

Synthesis of Well-Defined Heparan Sulfate Proteoglycan Mimetics to Study Cell Surface Engineering and Drug Delivery

विद्या वाचस्पति की
उपाधि की अपेक्षाओं की आंशिक पूर्ति में प्रस्तुत शोध प्रबंधक

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Indian Institute of Science Education and Research, Pune

2024

***I would like to dedicate my thesis to my beloved Sister
Shikha***

CERTIFICATE

This is to certify that the work incorporated in this thesis entitled “***Synthesis of Well-Defined Heparan Sulfate Proteoglycan Mimics to Study Cell Surface Engineering and Drug Delivery***” submitted by Prashant Jain carried out by candidate at Indian Institute of Science Education and Research, Pune under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other university or institution.



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Engineering and Drug Delivery**

I declare that this written submission represents my idea in my own words and where other's ideas have been included; I have adequately cited and referenced the original sources. I declare that I have acknowledged collaborative work and discussions wherever such work has been included. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

The work reported in this thesis is the original work done by me under the guidance of

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*The woods are lovely, dark and deep,
But I have promises to keep,
And miles to go before I sleep,
And miles to go before I sleep.*

Saurabh Anand

Saurabh Anand

सौरभ आनंद

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Abbreviations

A

AT Antithrombin

B

BAIB Bis(acetoxy)iodobenzene

BMP-2 Bone Morphogenetic Protein 2

BM Basement Membrane

C

CAMs Cell Adhesion Molecules

CS Chondroitin Sulfate

CNS Central Nervous System

CSPG Chondroitin Sulfate Proteoglycans

CP (cp) Cis-platin

CSA Camphore Sulfonic Acid

Cy-5 Cyanine-5

CTCF Corrected Total Cell Fluorescence

D

DDQ 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone

DMAP 4-Dimethylaminopyridine

DMEM Dulbecco's Modified Eagle's Medium

DMF Dimethylformamide

DS Dermatan Sulfate

DIPEA N,N-Diisopropylethylamine

DBCO Dibenzocyclooctyne

DCC	Dicyclohexyl Carbodiimide
DiO	3,3'-Diocetadecyloxacarbocyanine Perchlorate
DOSY	Diffusion Ordered Spectroscopy
E	
ECM	Extracellular Matrix
Erk-1/2	Extracellular Signal-Regulated Protein Kinases 1 and 2
Ext1	Exostosin-1
EVs	Extracellular Vesicles
F	
FBS	Fetal Bovine Serum
FGF-1	Fibroblast Growth Factor - 1
FGF-2	Fibroblast Growth Factor - 2
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
Fmoc-Cl	Fluorenylmethyloxycarbonyl Chloride
G	
GAGs	Glycosaminoglycans
Gal	Galactose
GalN	Galactose Amine
GlcA	Glucuronic Acid
GFs	Growth Factors
GPI	Glycosylphosphatidylinositol
GDFs	Growth Differentiation Factor
H	
Hep	Heparin
HR-ESI-MS	High resolution Electrospray Ionisation Mass Spectrometry
HS	Heparan Sulfate

HSBPs	Heparan Sulfate Binding Proteins
HSPGs	Heparan Sulfate Proteoglycans
HIV	Human Immunodeficiency Viruses
HSV	Herpes Simplex Virus
HOBT	Hydroxybenzotriazole
HBTU	Hexafluorophosphate Benzotriazole Tetramethyl Uronium
HPLC	High-Performance Liquid Chromatography
HCMV	Human Cytomegalovirus
HPV	Human Papillomavirus
DENV	Dengue Virus
I	
IdoA	Iduronic Acid
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IR	Infra-Red
K	
KS	Keratan Sulfate
M	
MAPK	Mitogen-activated Protein Kinases
MS	Molecular Sieves
mESC	Mouse Embryonic Stem Cell
MsCl	Methanesulfonyl Chloride
MALDI-TOF-MS	Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry
N	
NAP-Br	2-(Bromomethyl)naphthalene
NHS	N-Hydroxysuccinimide

NIS	N-Iodosuccinimide
NMR	Nuclear Magnetic Resonance
NMP	N-Methylpyrrolidone
N-CAM	Neural Cell Adhesion Molecule
nM	Nano Molar
NRP1	Neuropilin-1

P

PBS	Phosphate Buffered Saline
PTSA	p-Toluenesulfonic Acid
py	Pyridine
PG	Proteoglycan
PM	Plasma Membrane
PEG	Polyethylene glycol

R

RT	Room Temperature
RMSD	Root Mean Square Deviation

T

TBDPS-Cl	tert-Butyldimethylsilyl Chloride
TEMPO	2,2,6,6-Tetramethyl-1-piperidinyloxy
THF	Tetrahydrofuran
TLC	Thin-layer Chromatography
TMSSPh	Phenyl Trimethylsilyl Sulphide
TGF- β	Transforming Growth Factor Beta
TFA	Trifluoroacetic acid
TMSOtf	Trimethylsilyl Trifluoromethanesulfonate
TIPS	Triisopropyl Silane

U

UV Ultra-violet

μM micromolar

V

VEGF Vascular Endothelial Growth Factor

VGIC Voltage Gated Ion Channel

V3 Third Ventricle

VZV Varicella-Zoster Virus

Abstract

Heparan sulfate proteoglycans (HSPGs) are essential components of the extracellular matrix and cell surfaces, playing pivotal roles in various biological processes. Composed of a core protein with covalently attached heparan sulfate chains, HSPGs are crucial for cell-cell interactions, signaling regulation, and the organization of the extracellular environment. The synthesis of proteoglycan mimetics involves sophisticated chemical and biochemical strategies aimed at replicating the intricate structures and functions of natural proteoglycans, such as HSPGs. Li et al. employ synthetic organic chemistry to design molecules that mimic the glycosaminoglycan (GAGs) chains (e.g., heparan sulfate (HS), chondroitin sulfate (CS)) and the core proteins to which they attach. These mimetics are engineered to exhibit specific biological activities, such as binding to growth factors, cytokines, or cell surface receptors, similar to natural HSPGs. Bioconjugation techniques are crucial for linking synthetic glycosaminoglycan chains to appropriate protein or peptide scaffolds, ensuring structural and functional mimicry. The synthesis of proteoglycan mimetics holds significant promise for applications in regenerative medicine, drug delivery systems, and biomaterial development, where precise control over biological interactions is essential for therapeutic efficacy (Sugahara et al., 2015).

Chapter 1 highlights that proteoglycans are vital components of the extracellular matrix and plasma membrane, featuring core proteins linked to (GAGs). These complexes store and deliver growth factors, thereby regulating cellular processes. Current research is focused on creating simplified proteoglycan mimics to influence biological activities, especially in areas such as stem cell research, neural plasticity, viral inhibition, cancer biology, and biomarker development.

Chapter 2 discusses the significance of synthesizing monodisperse, precise multivalent, fluorescently labeled peptide backbones using cholesterol as a template for creating proteoglycan mimetics (PGs). HSPGs play key roles in various biological functions due to their complex structures, which include varying uronic acids and sulfation patterns. This complexity necessitates the precise synthesis of proteoglycan mimetics to better understand their specific biological functions.

Chapter 3 explores how membrane-bound HSPGs act as receptors for growth factors and influence endocytosis. The study highlights how specific sulfation and uronic acid compositions in heparan sulfate impact cell dynamics and the internalization of HSPG mimetics. For example, 3-*O*-sulfated iduronic acid (PG@ID-2) facilitates faster endocytosis and affects cell migration and proliferation, underscoring the importance of sulfation patterns.

Chapter 4 explains the importance of developing stable membrane probes for studying cell dynamics. The microheterogeneity of heparan sulfate on fluorescent peptide backbone provides a new platform for probing cellular functions. Highly sulfated HS ligands with L-iduronic acid (PG@ID-6) termed as **NeoMProbe** shows prolonged presence on the plasma membrane, which is useful for mapping basement membranes and enhancing imaging techniques.

Chapter 5 highlights the critical role of the extracellular matrix, particularly HS, in regulating interactions with drugs and toxins. The structural diversity of HS influences how cisplatin binds to it, with a preference for L-iduronic acid. A proteoglycan mimic containing IdoA (P-I12) demonstrates selective toxicity in cancer cells, thereby enhancing drug delivery and suggesting potential applications in targeted therapies.

Chapter 6 discusses how voltage-gated ion channels are modulated by PG mimetics. The study reveals that the sulfation of heparan sulfate and the sialic acid linkages on proteoglycan mimetics affect glycocalyx remodeling and Kv2.1 channel behavior. High sulfation and flexible sialylation lead to prolonged cell membrane persistence and altered ion channel activity, offering insights for the development of therapeutic probes.

Publications

1. **Saurabh Anand***, Sandhya Mardhekar, Rakesh Raigawali, Nirmala Mohanta, Prashant Jain, Chethan D. Shanthamurthy, Boopathy Gnanaprakasam, and Raghavendra Kikkeri “Continuous-Flow Accelerated Sulfation of Heparan Sulfate Intermediates” *Organic Letters*, **2020**, 22, 9, 3402-3406. (* *equal contribution*)
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Chapter – 1

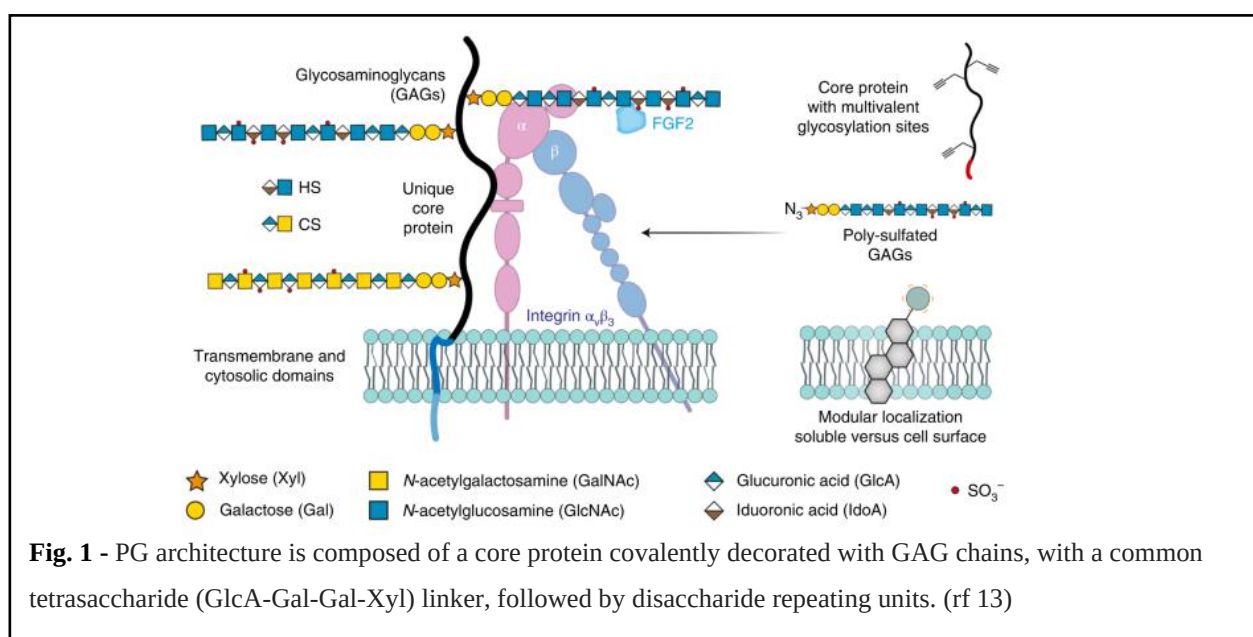
Introduction

Abstract

Proteoglycans are crucial components of the extracellular matrix (ECM) and plasma membrane, characterized by core proteins to which glycosaminoglycans (GAGs) are attached at serine or threonine residues *via* a tetrasaccharide linker. The biological functions of proteoglycans are mediated through the synergy between core proteins and GAGs. GAGs primarily act as reservoirs for growth factors (GFs), while the core proteins facilitate the formation of GAG-GF complexes that interact with GF receptors to regulate various cellular processes. As a result, there is increasing research interest in developing simplified proteoglycan mimics to modulate biological activities at the interface of the cell membrane and ECM. Our focus is on understanding mimics role in areas such as stem cell research, neural plasticity, and viral inhibition. Additionally, I will explore the significance of proteoglycan mimics in cancer biology, and their potential role as next-generation biomarkers.

1.1. Introduction

Proteoglycans are essential components of the ECM and plasma membrane, featuring core proteins to which GAGs are glycosylated at serine/threonine residues *via* a tetrasaccharide linker.¹ The biological activity of proteoglycan is harnessed by the combined actions of core proteins and GAGs, where GAGs primarily serve as a reservoir for GF and core protein articulate GAG-GF complexes to stimulate GF receptors to regulate cellular functions.^{2,3,4} Consequently, there is growing research interest in mimicking the structures of proteoglycans with simpler architectures to modulate biological activities at the junction of cell membrane and ECM.^{3,5,6,7,8} Proteoglycans at cellular, subcellular and intracellular components regulate the major cellular events by activating growth factor and morphogen signalling.^{5,9,10,11,12,13} (**Fig. 1**) whereas, proteoglycans localized on plasma membrane and extracellular serves as a receptor for viral and bacterial binding and infections. Hence, researcher emphasized great effort to understand the individual component of PG and identified key biologically active molecules.^{14,3,15,16,17} Structurally, proteoglycan composed of ‘core protein’ and ‘glycosaminoglycan’ synergies. Among them, GAGs accounts most established synergies, playing a pivotal role in PG biological activities.^{2,18,19} However, ‘core protein’ biological activity cannot be neglected, as they play critical role in increasing the life-time of the GAGs expression on plasma membrane and actively participate in the PG biological activities.^{20,9,21} I will mainly highlight the raft polymer and polypeptide based heparin sulfate PGs and its potential applications in stem cell, neurobiology and cancer research.



1.2. Heparan Sulfate Proteoglycans (HSPGs)

GAGs are negatively charged polymeric entities with distinct expression patterns across different tissue types. GAGs comprise four functional sulfated polysaccharides (HS, CS, DS and KS) differentiated by their hexamine-uronic acid compositions and non-sulfated hyaluronic acid polysaccharide.^{1,22} Among them, HSPGs have been identified as structurally highly diverse, resulting a wide range of biological activities. Approximately 17 proteoglycans have been identified to have one or more HS polysaccharide scaffolds. These HSPGs are ubiquitous in pericellular (basement membrane), cell surfaces, and intracellular domains in tissues.^{23,24,25,26,9,27,5}

In the basement membrane, the HSPGs include Perlecan, Agrin, and collagen XVIII proteoglycans (**Fig. 2**).^{1,5} These HSPGs are made up of 1-4 HS polysaccharide chains and are usually associated with laminins and collagen type IV polymeric networks. These basement membrane HSPGs enhance numerous cellular functions, including cell survival, division, migration, and proliferation, through their binding with a range of growth factors, such as VEGF, TGF- β , and FGF.^{28,29,30,31}

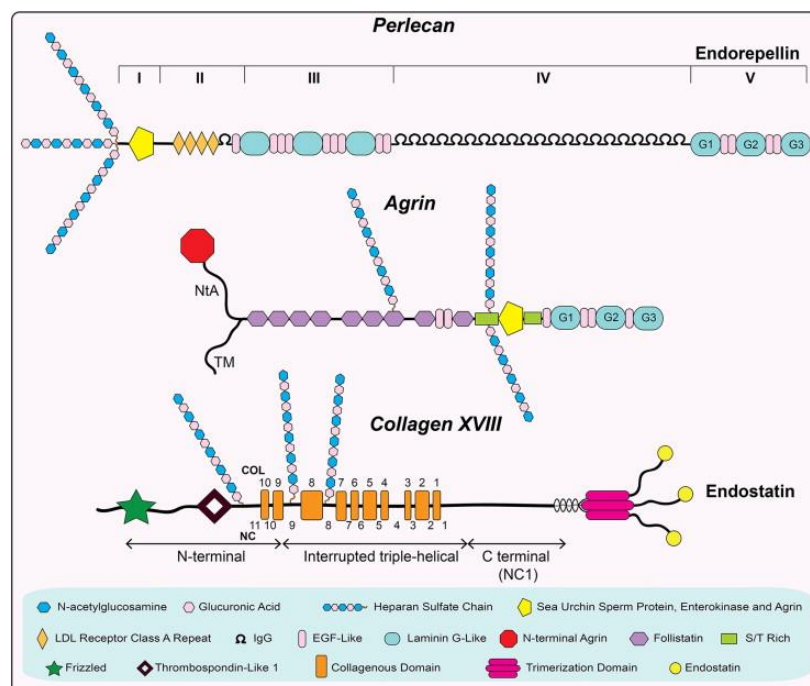
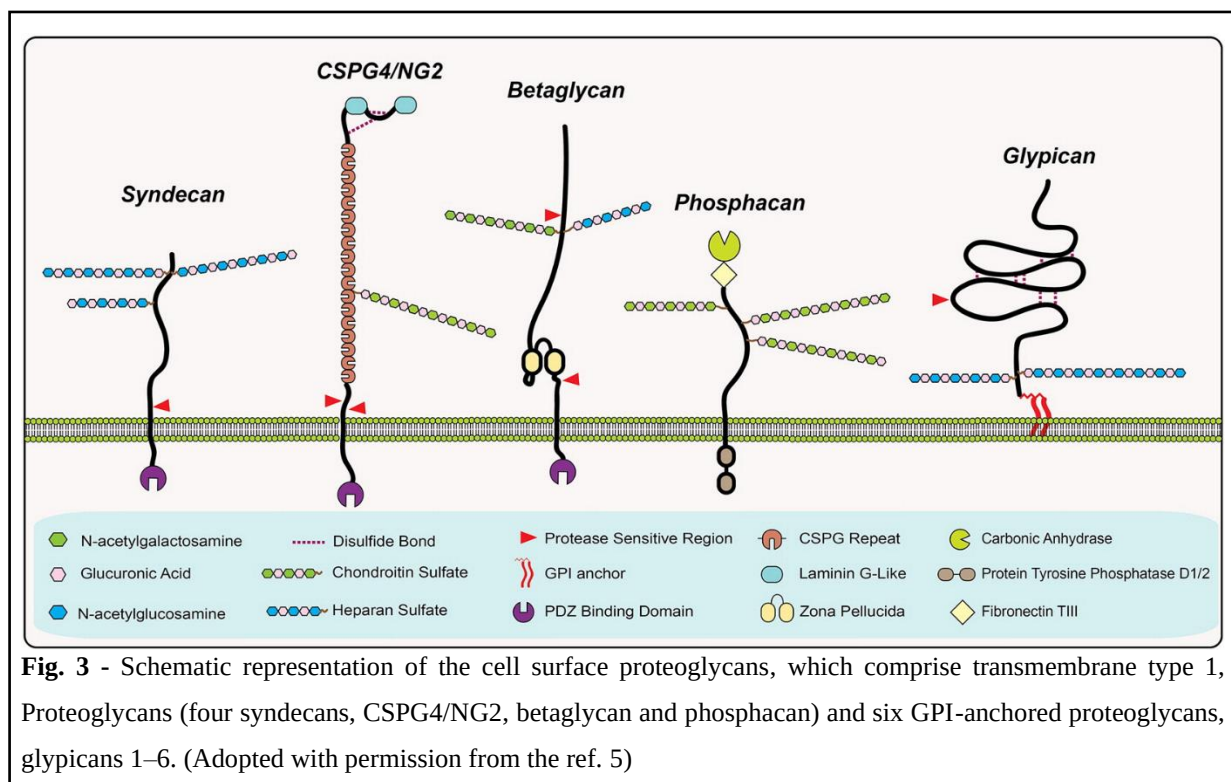


Fig. 2- Pericellular proteoglycans, which comprise perlecan agrin, and collagens XV III and XV. (Adopted with permission from the ref. 5)

Perlecan is located in both vascular and avascular tissues, interacting with a variety of protein ligands to regulate cell adhesion, thrombosis, and cell death. In contrast, Agrin, containing HS

both HS and CS GAGs sequences, is an extracellular proteoglycan highly expressed in nervous tissues, acting as a co-receptor for neurite activities.^{32,33,5,34} The HS in Agrin binds to FGF-2, thrombospondin, beta-amyloid peptide, N-CAM, and protein tyrosine phosphatase. Collagen XVIII is another HSPG containing a homotrimer of $\alpha 1$ chain of collagen protein. Collagen XVIII is a negative regulator of angiogenesis and directly implicates in the generation of adipose tissue and hyperlipidemia associated with visceral obesity and fatty liver.⁵

The transmembrane PGs consist of five HSPGs: syndecans, glypican, betaglycan, neuropilin-1, and CD44 (**Fig. 3**).⁵ Syndecans is one of the most frequently occurring cell surface HSPGs, found in almost all cells and tissues. There are four types of syndecans isomers in vertebrates (syn-1 to syn-4). Syndecans 1 to 3 are expressed in a tissue-specific fashion, whereas syndecan-4 is ubiquitously distributed. Their structure includes a variable ectodomain and a highly conserved cytoplasmic domain. The structural and functional diversity of syndecans is influenced by the GAGs compositions and structures, which vary with tissue type and species.^{5,35,36,37,30}



Syndecan-1 and syndecan-3 feature additional CS chains that enhance their structural diversity. Syndecans participate in various physiological and pathological processes, binding to numerous ligands from a range of protein families, including growth factors, cytokines, chemokines, fibrillin-1, fibronectin, collagen, tenascins, and vitronectin.^{38,39,40,41} Additionally, syndecans have a unique binding affinity for pathogens, such as HIV-1, HIV-2, herpes simplex

virus, dengue virus, and hepatitis C virus. Several bacterial pathogens, particularly *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Neisseria gonorrhoeae*, also bind to syndecans.^{42,43,44}

Glypicans represent another family of transmembrane proteoglycans, usually expressed with glycosyl phosphatidylinositol-linked (GPI-linked) proteoglycan chains. Literature reports indicate that six glypicans (GPC-1 to GPC-6) are found on the mammalian cell surface, mainly expressed during embryonic development. The lack of homology domains in glypican isoforms suggests that these HSPGs serve unique functions. Glypicans consist of 1 to 5 HS chains per proteoglycan, but GPC-5, produced by rhabdomyosarcoma cells, also features a CS domain. Glypicans regulate various growth and survival factors during cell differentiation and apoptosis. The abnormal expression of glypicans alters several signaling pathways, such as Wnt and Hedgehog, and promotes tumor progression. Anti-glypicans are considered the next generation of antibodies for cancer treatment.^{45,46,47,48,49,50}

β -glycan is a cell surface proteoglycan that functions as a co-receptor for TGF- β and is also named the TGF- β type III receptor. It is mainly involved in modulating reproduction and fetal growth. The ectodomain of beta-glycans is composed of 1-2 HS GAGs sequences and binds to all isoforms of TGF- β proteins, which include activins, inhibin, GDFs, and BMPs. Neuropilin-1 (NRP1), a transmembrane protein and co-receptor for various proteins, such as semaphorins and VEGFs, regulates endocytosis and transport to a number of cell surface receptors from the cytoplasm to the cell surface and vice versa. All of these aforementioned biological events by HSPGs highlight the important HS and core proteins of the PG.^{51,52,53,54,55,52,56}

In Summary, the HSPGs are extensively found on cell surfaces and within the pericellular space. The structures of HS on (PGs vary by tissue and serve to regulate different biological processes. For example, beta-glycan, with its distinct HS sequences, modulates the activity of TGF- β , which is not present in basement membrane HSPGs. Likewise, proteoglycans such as syndecans, glypicans, perlecan, and agrin likely share common HS domains that facilitate binding to various growth factors. Neuropilin-1, featuring 3-O-sulfated HS, is crucial for binding neurite growth factors. Consequently, understanding the subtle variations in HS structures is essential for deciphering and controlling specific biological functions.

1.3. Polymer Based HSPG Mimetics and its Applications

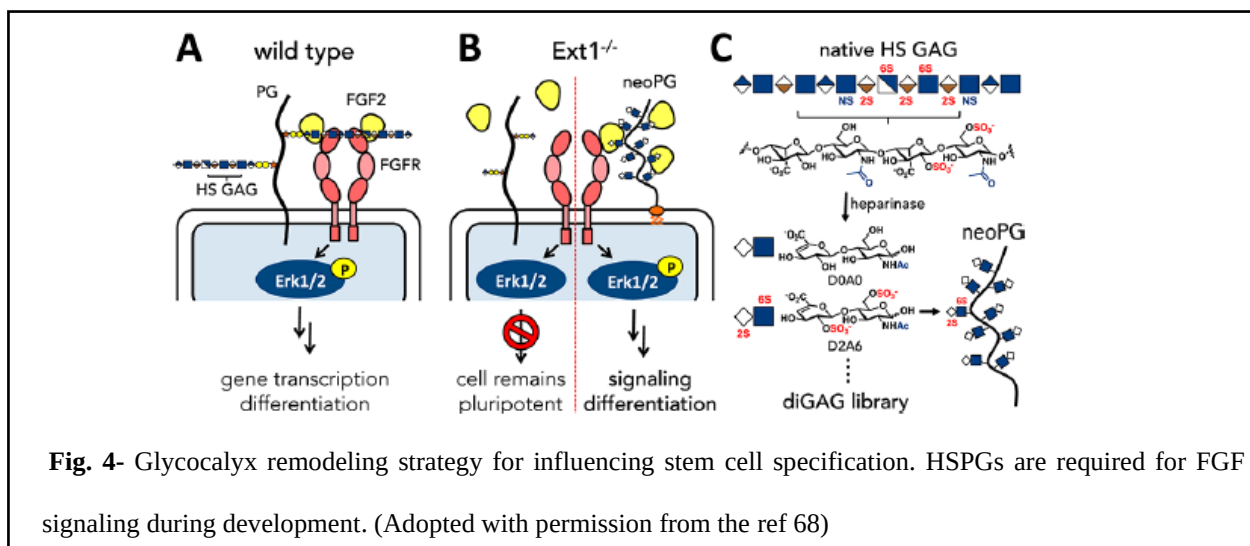
Polymer based HSPG are distinguished by the presence of carbohydrate groups HS as side chains in synthetic polymers, offering a more precise imitation of cell surface carbohydrates

like proteoglycans. These molecules play crucial roles in biological processes such as cell-cell interactions, adhesion, cancer metastasis, and pathogen infection, all mediated by protein-saccharide interactions. Given the inherently weak nature of protein-carbohydrate interactions, a multivalent system is necessary to significantly enhance these interactions.^{57, 58, 59, 60}

The focus on surface modification in glycopolymers development has enabled researchers to investigate the effects of sugar presentation and the physicochemical properties of these polymers over time. The first lactose-containing glycopolymer was synthesized in the 1980s, while in the Whitesides group explored sialic acid-containing glycopolymers and their multivalent interactions. These synthesized polymers exhibited various molecular weights and significant multivalent effects, rendering them valuable for diverse studies.^{61, 62}

1.3.1. Role of HSPG in Embryonic Stem Cells

As soluble multivalent ligands binding to cell-surface receptors and activating biological processes, Godula *et. al.* proposed a cell-surface engineering strategy targeting GAG-mediated growth factor signaling to influence stem cell differentiation. By remodeling the cell glycocalyx with synthetic glycopolymers, the researchers aimed to mediate biological processes with minimal membrane disruption. They utilized lipid-terminated chondroitin sulfate GAGs and other synthetic glycopolymers to enhance signaling pathways such as nerve growth factor and Erk1/2, promoting neural outgrowth and differentiation. Employing an enzyme to depolymerize HS, they incorporated HS disaccharides into poly(acrylamide) scaffolds (**Fig.4**)^{63,64,65,2,66,67} These scaffolds efficiently bound FGF-2 and promoted neural differentiation in mouse embryonic stem cells (mESCs). This method offers precise control over cell surface remodeling, enabling sustained differentiation signals and promising broad applications in stem cell biology and therapeutic differentiation.^{68,69,70,71}



1.3.2. Role of HSPG in Neural Plasticity

HSPGs play a crucial role in neural plasticity by interacting with key molecules such as NCAM and EGF, which are essential for synaptic formation and function. The specific sulfation patterns of HSPGs are critical in regulating these interactions, and alterations in HSPG expression or structure can lead to significant changes in CNS like ^{72,28,30,73,74}:

(i) *Neural Development*

HSPGs are intricately involved in neurogenesis, particularly in regions of the brain undergoing active mitosis. Glypican-1, among other glypicans, is expressed in the ventricular zone during central nervous system (CNS) development, whereas glypican-3 mutations are linked to Simpson-Golabi-Behmel syndrome, which entails neuroblastoma risks. Syndecan-3's expression peaks during glial differentiation and myelination. CSPGs like phosphacan, neurocan, and neuroglycan C regulate neural stem cell proliferation and neurogenesis, playing pivotal roles in embryonic CNS development and neural precursor proliferation through mechanisms such as FGF-2 signaling. ^{75,76,77,78,79,79,80}

(ii) *Neuronal Migration*

PGs, especially CSPGs, are essential in mediating the ECM signals that guide neuronal migration. In the CNS, CSPGs like RPTP β /phosphacan are found along radial glial fibres and in migrating neurons, facilitating adhesion molecule interactions and neuronal movement. CSPG expression inversely correlates with neural crest cell migration pathways, with specific CSPGs like versican inhibiting attachment and migration. Highly sulfated CS motifs enhance migration, underscoring the influence of GAG types and their sulfation on neural migration. ^{81,82,83,84,85}

(iii) *Neurite Outgrowth*

HSPGs and CSPGs are critical regulators of neurite outgrowth and axonal pathfinding. HSPGs support neurite extension by binding to growth-enhancing molecules and interacting with growth factors like FGF-2, influencing axonal targeting. CSPGs exhibit dual roles: while generally known for inhibiting axonal growth in glial scar regions, they also facilitate growth in other contexts. Specific CSPGs like phosphacan promote neurite outgrowth in certain neuronal populations, with their inhibitory or stimulatory roles being governed by their spatiotemporal expression and sulfation patterns. These interactions often converge on

pathways such as Rho/ROCK, which modulate cytoskeletal dynamics critical for neurite extension.^{86,87,88,89,90}

(iv) *CNS Plasticity*

HSPGs significantly contribute to CNS plasticity by interacting with molecules like NCAM and EGF, crucial for synaptic modulation and long-term potentiation (LTP). Alterations in HSPG sulfation patterns influence synaptic plasticity and cognitive functions. For instance, deficiencies in HSPG-modifying enzymes like Sulf-1 and Sulf-2 impair spatial learning and synaptic plasticity. CSPGs also modulate plasticity through perineuronal net (PNN) formation, which restricts synaptic flexibility at the end of the critical developmental period. Changes in CSPG sulfation can restore plasticity in the mature CNS, demonstrating their role in synaptic network stabilization and memory processes.^{85,81,91}

1.3.3. Role of HSPG in various Viral Entry

HS and HSPGs play crucial roles in the lifecycle of many viruses. These molecules serve as attachment receptors, mediators of entry, and facilitators of cell-to-cell spread. The interactions between viral glycoproteins and HS/HSPGs are vital for successful infection, influencing viral attachment, entry, and dissemination. Below, we discuss the role of HS and HSPGs in the engagement of various viruses (**Fig. 5**)^{92,93,94,95}

(i) *Herpesviruses*

Herpesviruses are a group of DNA viruses, divided into α -, β -, and γ -subfamilies, with varying host ranges and replication cycles. These viruses can enter latency after primary infection, leading to potential recurrent infections. HSPGs play critical roles in the lifecycle of each herpesvirus subfamily.

Herpes Simplex Virus Type-1 (HSV-1)

HSV-1 is a neurotrophic virus that infects cells using various glycoproteins, primarily gB and gC, which interact with HS on HSPGs. These interactions facilitate viral entry, where HS binding is crucial for the virus to "surf" to entry receptors. HSV-1 also uses HS to promote cell-to-cell fusion and spread, with 3-O-HS enhancing fusion, while heparan sulfate itself serves as a negative regulator.

Herpes Simplex Virus Type-2 (HSV-2)

HSV-2, another α -herpesvirus, primarily relies on gB for binding to HS, unlike HSV-1 which uses gC extensively. Differences in HS binding capacities and sensitivities to structural differences in glycoproteins lead to variations in engagement with HS. HSV-2 shows a greater dependence on gB for HS binding, and exhibits differences in sensitivity to heparan sulfate modifications and inhibitors compared to HSV-1.

Human Cytomegalovirus (HCMV)

HCMV, a β -herpesvirus, uses HS for initial attachment. The viral glycoproteins gB and a gM-gN complex mediate this interaction, which is essential for entry and spread. HCMV relies on HS for initial binding, and disrupting these interactions with cationic peptides or heparin significantly reduces infection rates *in vitro* and *in vivo*.

Varicella-Zoster Virus (VZV) and Human Herpesvirus 7 (HHV-7)

VZV and HHV-7 rely on HS for attachment and entry, VZV uses gB to bind HS, while HHV-7's primary target cells are CD4⁺ T lymphocytes, where HS serves as a co-receptor for infection.

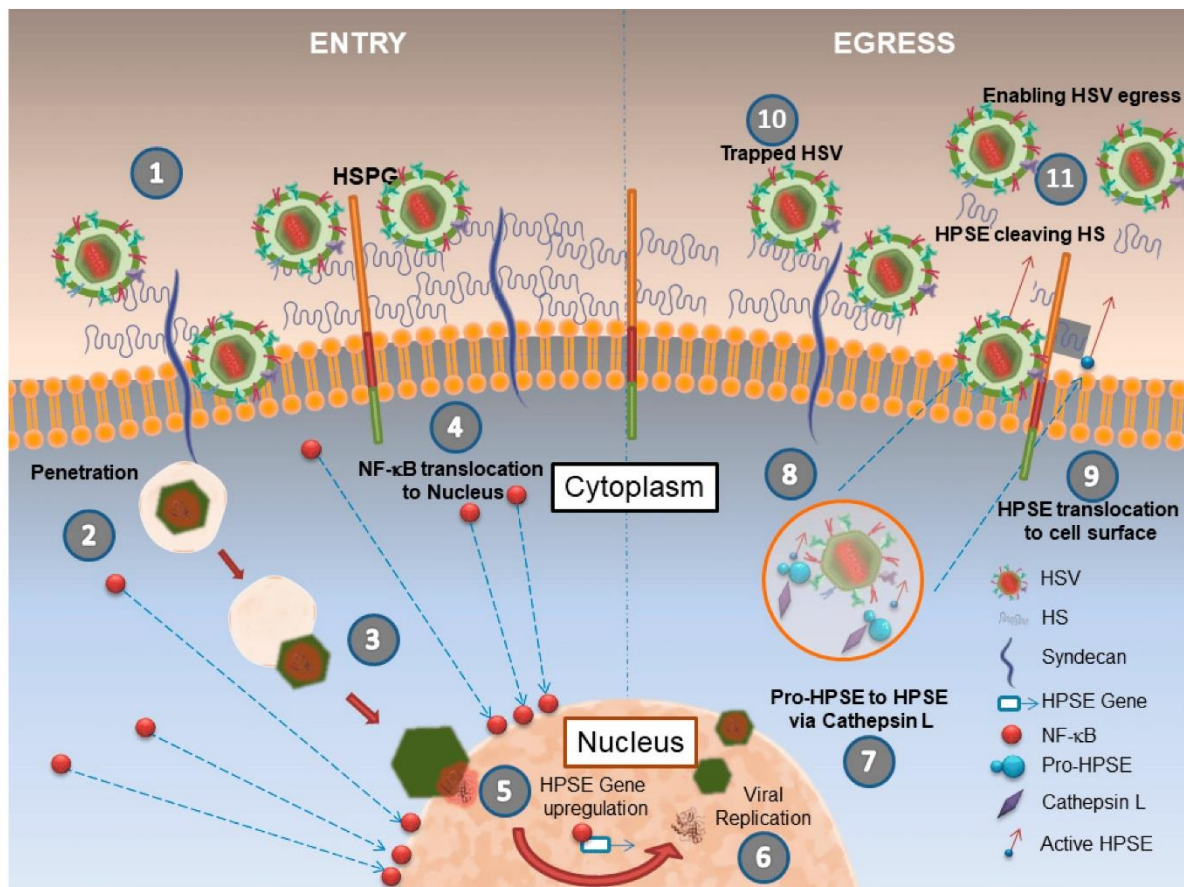


Fig. 5- Schematic representation of role of HSPG in HSV infection. **(1)** Virions attach to HSPGs including syndecans on a host cell. **(2)** Capsid penetration occurs at the plasma membrane (via membrane fusion or endocytosis). **(3)** The capsid is released into the cytoplasm, which reaches the nuclear membrane for docking and

release of viral DNA in the nucleus for replication. **(4)** Sensing viral invasion, NF- κ B translocates to the nucleus and **(5)** signals HPSE overexpression. **(6)** New capsids containing viral genomes mature and bud out of the nucleus. **(7–8)** Enveloped capsids with viral glycoproteins are exocytosed. The same vesicles are also suggested to carry Pro-HPSE, which is eventually activated by cathepsin-L. **(9)** The enzymatically active HPSE then translocates to the cell surface where it cleaves off heparan sulfate chains **(10)** to help release any surface bound virions. **(11)** removal of heparan sulfate enables unrestricted release of HSV-1 virions. (*Adopted with permission from the ref 95*)

(ii) Human Immunodeficiency Virus (HIV)

HIV relies on HS for initial attachment to host cells, particularly epithelial and CD4+ T cells. The viral envelope protein gp120 interacts with HS, facilitating the attachment and subsequent entry. HS on epithelial cells aids in the attachment of HIV, and the transcytosis through genital epithelia involves syndecans, a type of HSPG. HS is also involved in the formation of a complex with HIV gp120 and Toll-like receptors, activating immune pathways that disrupt cellular barriers and facilitate viral dissemination^{96,97,98}.

(iii) Human Papillomavirus (HPV)

HPV, a non-enveloped DNA virus, uses HS as a primary receptor for attachment to basal epithelial cells. This interaction is crucial for the virus's entry and subsequent infection of proliferating cells. The viral capsid protein L1 binds to HS, triggering conformational changes that facilitate entry. Inhibition of HS binding with heparin or heparinase reduces HPV infection rates, indicating the importance of HS in viral entry^{99,92}.

(iv) Hepatitis C Virus (HCV)

HCV engages HS for attachment and entry into hepatocytes. The viral E2 glycoprotein and apolipoprotein E (ApoE) are involved in binding to HS, which is essential for the infection process. Specific sulfation patterns of HS are critical for HCV binding, with N- and 6-O-sulfation being particularly important for effective viral attachment and entry^{92,101}.

(v) Human Respiratory Syncytial Virus (RSV)

RSV, an enveloped RNA virus, uses HS for binding through its G and F proteins, which mediate viral attachment and facilitate cell-to-cell spread. Blocking the interaction of RSV proteins with HS using heparin or heparinase reduces infection rates, highlighting the role of HS in RSV pathogenesis¹⁰².

(vi) Dengue Virus (DENV)

DENV, an enveloped RNA virus, uses HS for binding to specific cell types, including epithelial cells and fibroblasts. The viral protein NS1 interacts with HS, disrupting the endothelial glycocalyx layer and facilitating viral entry and spread. NS1-induced shedding of syndecans from the endothelial surface increases permeability, promoting inflammation and viral dissemination (**Fig. 6**)^{92,100,95}.

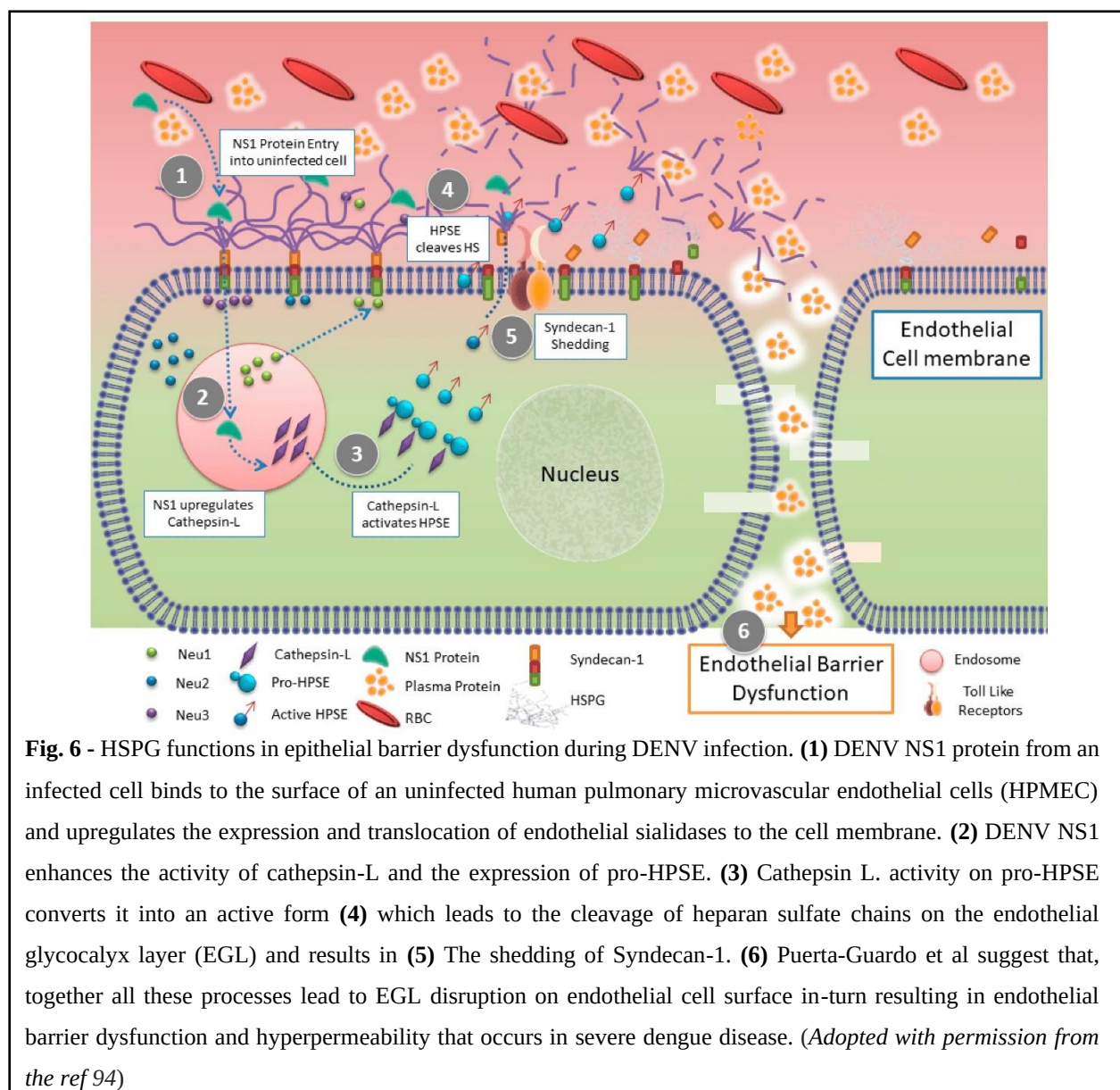


Fig. 6 - HSPG functions in epithelial barrier dysfunction during DENV infection. **(1)** DENV NS1 protein from an infected cell binds to the surface of an uninfected human pulmonary microvascular endothelial cells (HPMEC) and upregulates the expression and translocation of endothelial sialidases to the cell membrane. **(2)** DENV NS1 enhances the activity of cathepsin-L and the expression of pro-HPSE. **(3)** Cathepsin L. activity on pro-HPSE converts it into an active form **(4)** which leads to the cleavage of heparan sulfate chains on the endothelial glycocalyx layer (EGL) and results in **(5)** The shedding of Syndecan-1. **(6)** Puerta-Guardo et al suggest that, together all these processes lead to EGL disruption on endothelial cell surface in-turn resulting in endothelial barrier dysfunction and hyperpermeability that occurs in severe dengue disease. (Adopted with permission from the ref⁹⁴)

(vii) Ebola Virus (EBOV)

EBOV, a severe hemorrhagic virus, uses HS for attachment to host cells, particularly targeting the basolateral surfaces. HS-mediated attachment is crucial for viral entry and infection. The

virus shows a preference for basolateral attachment, and disruption of HS interactions at this site significantly reduces infection efficiency¹⁰³.

(viii) Coronaviruses

Coronaviruses, including HCoV-NL63, SARS-CoV, and SARS-CoV-2, use HS as a co-receptor for attachment and entry. The viral spike (S) protein interacts with HS to facilitate binding to the primary receptor, ACE2, and initiate infection. Recent studies suggest that SARS-CoV-2's spike protein binds to HS, and disrupting this interaction can reduce viral adhesion and entry, offering potential therapeutic targets for COVID-19 treatment^{92,104}.

In summary HS and HSPGs are essential for the lifecycle of many viruses, including herpesviruses, HIV, HPV, and coronaviruses. These molecules facilitate initial attachment, mediate viral entry, and aid in cell-to-cell spread. Targeting HS interactions offers a potential therapeutic approach to mitigate viral infections and their associated pathologies.

1.3.4. Role of HSPG in Cancer Biology

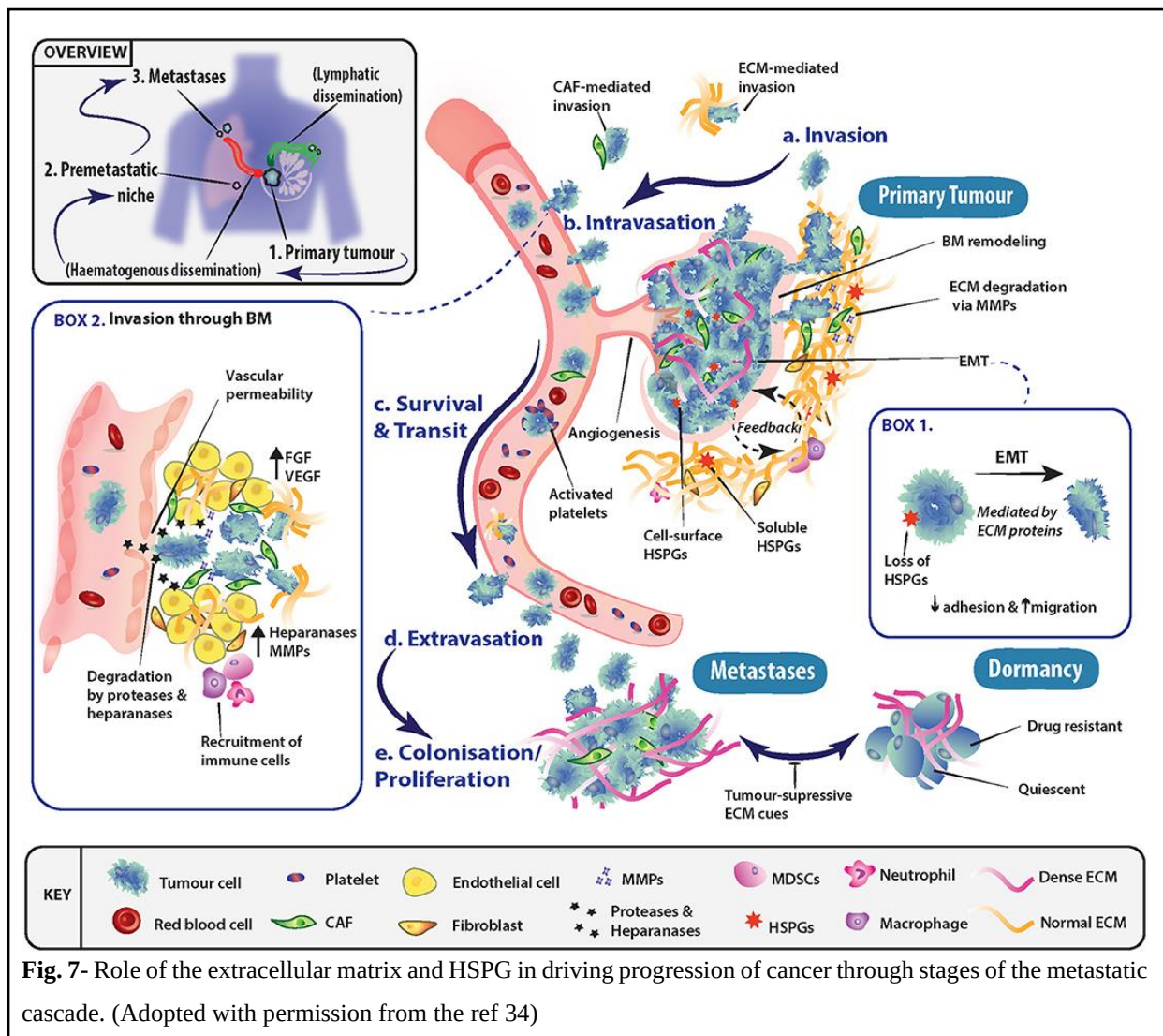
The provided paragraphs discuss the role of HS and HSPGs in cancer, focusing on various aspects such as cellular features, extracellular matrix remodeling, intercellular communication, and resistance to therapy (**Fig 7**)^{1,34}.

(i) Heparan Sulfate Changes in Cancer

Altered glycosylation, including changes in HS, is associated with cancer progression. HS modulates cellular events like proliferation and signaling through interactions with growth factors. Abnormal expression and activity of enzymes involved in HS biosynthesis contribute to cancer development. Moreover, heparinase, an enzyme that cleaves HS, influences tumor growth, metastasis, and resistance to therapy^{105,106,107}.

(ii) Role of HS and HSPG in Cancer Cellular Features and ECM Remodelling

HS and HSPGs affect cancer cell behavior by influencing processes like migration, invasion, and angiogenesis. They also regulate gene expression and signaling pathways, contributing to tumor progression and metastasis. Furthermore, alterations in HS levels and structure impact extracellular matrix stiffness and cell-matrix interactions, facilitating tumor invasion^{34,108,109}.



(iii) HS Roles in Cancer Intercellular Communication

HSPGs play a crucial role in cellular communication within the tumor microenvironment, including the secretion and uptake of extracellular vesicles (EVs). EVs, containing bioactive cargo, contribute to cancer progression, metastasis, and therapy resistance. HSPGs regulate EV biogenesis, secretion, and uptake, influencing various aspects of cancer development^{110,111,112}.

(iv) Impact of HS in Cancer Cell Resistance to Therapy

Altered expression of HSPGs and HPSE contributes to cancer cell resistance to chemotherapy and radiotherapy. HSPGs can modulate drug sensitivity and affect the tumor microenvironment, leading to therapy resistance and tumor recurrence. Additionally, heparinase influences EV secretion and cargo, promoting metastasis and therapeutic resistance^{113,114,15,115}.

Overall, HS and HSPGs play multifaceted roles in cancer progression, including cellular behavior, intercellular communication, and therapy resistance, making them potential targets for cancer treatment strategies.

1.4. Conclusion

HSPGs has diverse role across various biological processes, including their involvement in cellular functions, embryonic stem cell signaling, viral entry mechanisms, neural plasticity, and cancer biology. HSPGs found in pericellular and intracellular region, participate in cell survival, migration, and proliferation through interactions with growth factors and receptors. Whereas, transmembrane HSPGs like syndecans and glypicans play crucial roles in physiological and pathological processes, including viral infections and cancer progression. Recently, HSPG structures have been mimicked by using raft glycopolymers to study protein-carbohydrate interactions and cell-surface engineering. But in glycocalyx, HS ligands are not polyvalent in proteoglycan backbone. Hence, we need a better mimic to replicate their activity. In the following chapter, I will discuss a new class of proteoglycan mimics to study heparan sulfate mediated biological activities.

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Chapter 2

Synthesis of Heparan Sulfate (HS) Based Proteoglycan Mimetics

Abstract

HSPGs are complex molecules found on cell surfaces and in the ECM. They play critical roles in cellular communication and tissue organization by interacting with growth factors, cytokines, and other signaling molecules. Their ability to modulate ligand-receptor interactions regulates processes such as development, inflammation, and wound healing, highlighting their essential biological significance. Heparan sulfate is complex due to presence two types of uronic acid moieties, L-IdoA and D-GluA, with sulfate groups present on various *O*- and *N*-moieties, including *N*-sulfation, *N*-acetate, and free amine, contributing to microheterogeneity. To better understand which motif is important for each biological function on cell surface, we are reporting for the first time peptide based multivalent system which is monodisperse, precise multivalent, fluorescently labelled with cholesterol as a template for synthesizing PG mimetics.

2.1 Introduction

HSPGs are a class of proteoglycans that play a pivotal role in cellular processes and extracellular interactions. Structurally, HSPGs consist of a core protein to which several linear HS GAGs chains are covalently attached. HS and heparin are negatively charged biomolecules within the proteoglycan family, commonly found on cell surfaces and in the ECM. So far, around 17 HSPGs have been identified, including both cell membrane-bound and secreted types.¹ These proteins contain linear polysaccharides with repeating disaccharide units of glucosamine and uronic acid. In these units, glucosamine is α -linked to uronic acid, which can be *N*-acetylated or *N*-sulfated, with free amines being rare. Uronic acid composition varies, with glucuronic acid, β -linked to glucosamine, making up about 50% of HS disaccharide repeats, whereas iduronic acid, α -linked, is predominant in heparin.^{2,3}

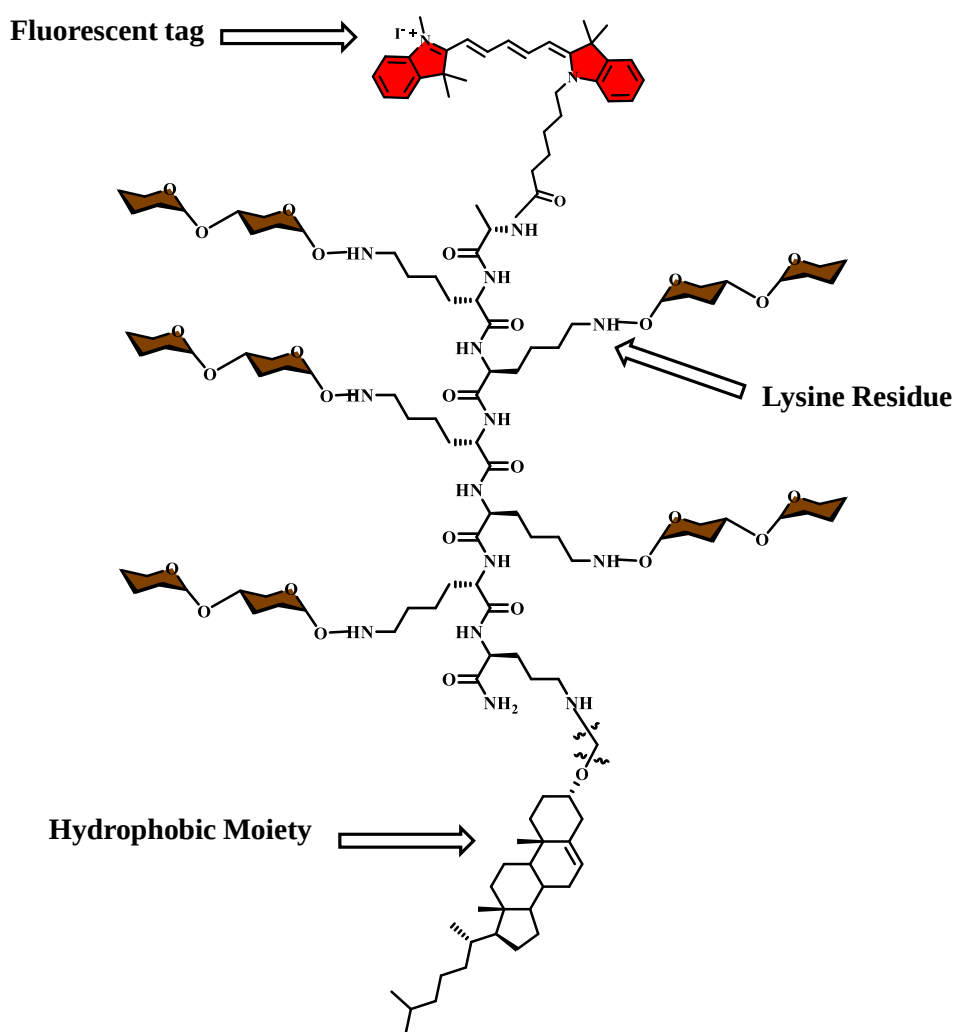
In addition to *N*-sulfation, these sugar moieties often have *O*-sulfate groups at specific positions, increasing HS microheterogeneity. While HS and heparin are sometimes used interchangeably, they are distinct. HS is synthesized by nearly all cells, whereas heparin is produced exclusively by connective tissue-type mast cells. They also differ in size and sulfation patterns, with heparin having a higher negative charge.⁴

These complex macromolecules are ubiquitously present on cell surfaces and within the extracellular matrix. HSPGs are crucial in modulating cell signaling pathways by binding to a variety of growth factors, cytokines, and morphogens, thereby influencing cellular proliferation, differentiation, and migration. They also facilitate the formation of extracellular matrix scaffolds, contributing to tissue structure and repair. Additionally, HSPGs act as co-receptors that enhance the specificity and sensitivity of receptor-ligand interactions, playing a significant role in developmental processes and in maintaining cellular homeostasis. Their ability to interact with a wide array of biological molecules underscores their importance in health and disease, including in cancer, where they can modulate tumor progression and metastasis.^{5,6}

Several research groups have synthesized well-defined HSPGs mimetics using different strategies to mimic the complex structure and function of HSPGs. Pioneer work is done by, Linhardt et al. developed chemoenzymatic approaches, leveraging bacterial glycosyltransferases and sulfotransferases to create structurally defined heparan sulfate oligosaccharides. Meanwhile, Rosenberg et al. and Bertozzi et al. focused on synthesizing HSPG mimetics through polymer-supported chemical synthesis, enabling precise control over

sulfation patterns and chain lengths.^{8,9} These diverse methodologies underline the complexity and versatility required to replicate the intricate nature of HSPGs.

Herein, we have developed a new modular system carrying amphiphilic group, fluorescent dye and a precise number of HS ligands ranging from disaccharide to hexasaccharide to study the HS mediated biological activity. The modular system is composed of three parts. (i) a classic hydrophobic cholesterol moiety, serving as an anchoring module for the mimics on the cell membrane; (ii) Cy5 dye serves as fluorescent probe to visualize cell membrane dynamics of the mimics; (iii) multivalent lysine residue was modified with azide functional to conjugate HS ligands. (Scheme 1)

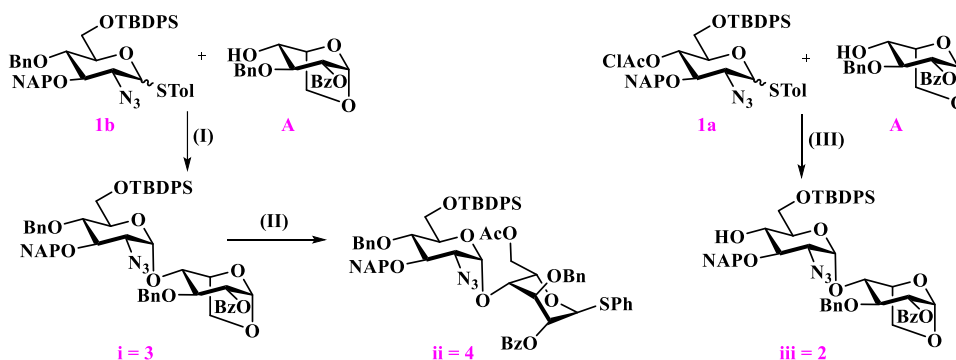


Scheme 1: Schematic representation of Proteoglycans (PGs) mimetics

2.2. Result and Discussion

2.2.1 Synthesis of NHAc and N-Sulfate Disaccharide Library

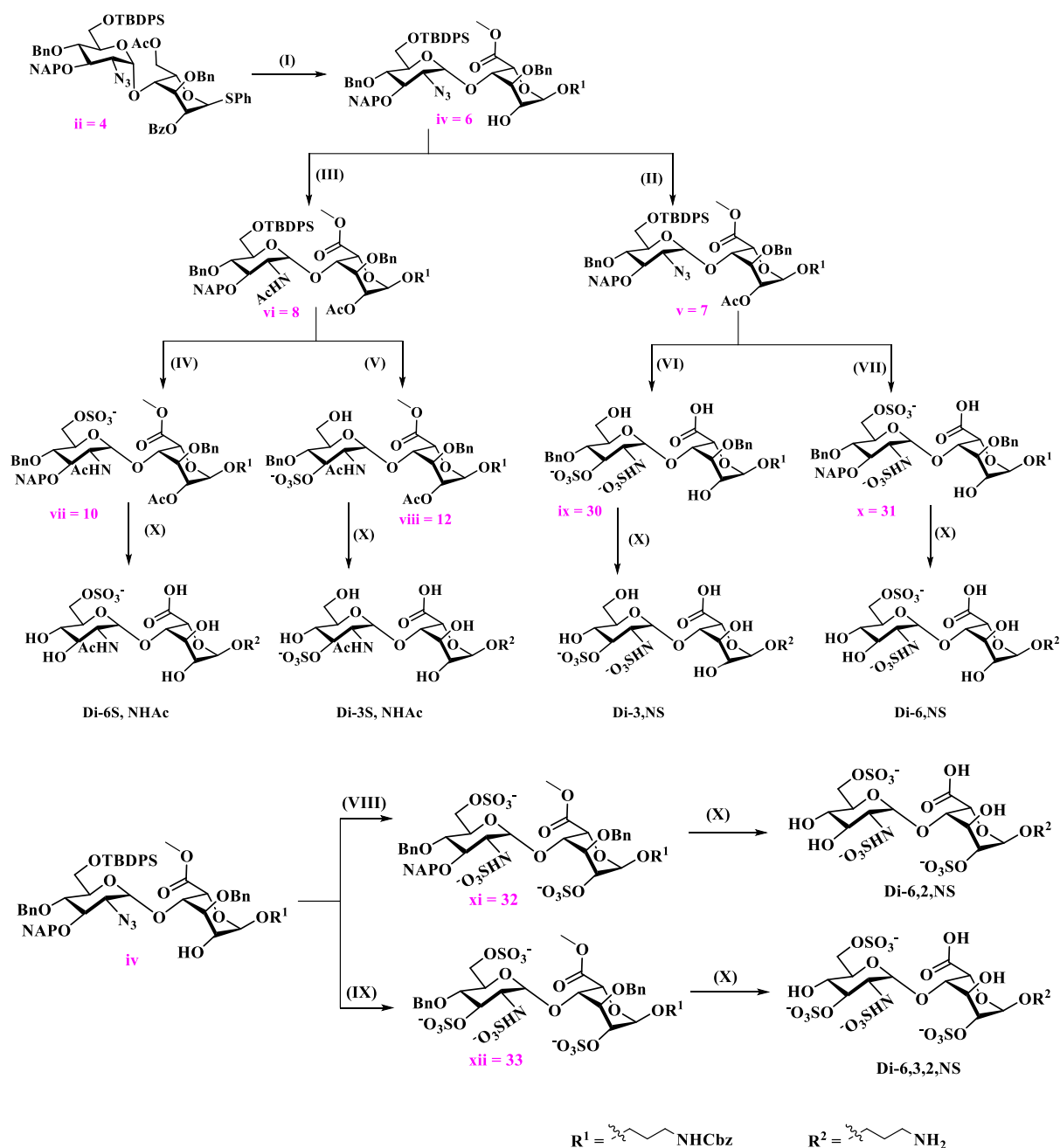
We achieved the synthesis of a regioselectively sulfate-modified HS-disaccharide analogue, featuring *N*-acetylation and *N*-sulfation, utilizing disaccharides **III**. The orthogonally protected HS disaccharide served as a key element, allowing for the precise installation of the sulfate group. The synthesis of the disaccharide involved the strategic use of glucosamine **1a**, **1b** and modified IdoA (**A**) building blocks from previously known procedure,^{10,11,12,13} following a well-established protocol. Employing a streamlined [1+1] glycosylation method, we successfully produced an α -1,4 linked disaccharides **i** and **iii** with an excellent yield. (scheme 2)



Scheme 2: (I) NIS, TMSOTf, 4 Å MS -78 °C to -20 °C, CH₂Cl₂, 30 min. (II) (a) Ac₂O, Cu(OTf)₂, rt, 12 h. (b) TMSSPh, ZnI₂, CH₂Cl₂, rt, 2 h. (III) (a) NIS, TMSOTf, 4 Å MS -78 °C to -20 °C, CH₂Cl₂, 30 min., (b) Thiourea, MeOH:Py, (1:1), 80 °C, 1 h.

Acetolysis was carried out on the reducing end iduronic acid (IdoA) of compound followed by the transformation of the resulting product into thiophenol donor **ii**, achieved by employing phenyl trimethylsulfide and ZnI₂.

Subsequently, glycosylation of the (3-hydroxypropyl)carbamate linker was performed using NIS and TMSOTf to obtain compound **iv**. The ensuing steps involved deacetylation followed by oxidation of the primary hydroxyl using TEMPO and methyl esterification. Following this, an acetolysis reaction was carried out on C-2 of IdoA to obtain compound **v** for the synthesis of the NS derivative. Additionally, the azide moiety of the glucosamine was converted into NHAc using Zn/CH₃COOH/Ac₂O, yielding compound **vi** for the synthesis of the NHAc derivative of the disaccharide.^{10,11,12,13}



Scheme 3: (I) (a) Benzyl (3-hydroxypropyl)carbamate, NIS, TfOH, 4 Å MS, -10 °C, CH₂Cl₂, 30 min. (b) NaOMe, CH₂Cl₂:MeOH (1:1), rt, 12 h. (c) TEMPO, CH₂Cl₂:MeOH (1:1), rt, 12 h. (d) LiOH·H₂O, H₂O:THF (1:1), rt, 2 h. (e) MeI, K₂CO₃, DMF, rt, 12 h. (II) (a) Ac₂O, DMAP, DCM:py (1:1), 0 °C, 12 h. (b) Zn, THF/AcOH/Ac₂O (3:2:1), rt, 12 h. (III) Ac₂O, DMAP, DCM:py (1:1), 0 °C, 12 h. (IV) (a) 70% HF.py, Py, 0 °C, 12 h. (b) SO₃·NEt₃, DMF, 80 °C, 3 days. (V) (a) DDQ, DCM:H₂O (18:1), rt, 2 h. (b) SO₃·NEt₃, DMF, 80 °C, 3 days. (c) 70% HF.py, Py, 0 °C, 12 h. (VI) (a) 70% HF.py, Py, 0 °C, 12 h. (b) SO₃·NEt₃, DMF, 80 °C, 3 days. (c) LiOH·H₂O, THF:H₂O (1:1), rt, 12 h. (d) PMe₃.THF, THF, 0.1M NaOH, rt, 12 h. (e) SO₃Py, MeOH, 0.1M NaOH, NEt₃, 0 °C, 36 h. (VII) (a) DDQ, DCM:H₂O (18:1), rt, 2 h. (b) SO₃·NEt₃, DMF, 80 °C, 3 days. (c) 70% HF.py, Py, 0 °C, 12 h. (d) LiOH·H₂O, THF:H₂O (1:1), rt, 12 h. (e) PMe₃.THF, THF, 0.1M NaOH, rt, 12 h. (f) SO₃Py, MeOH, 0.1M NaOH, NEt₃, 0 °C, 36 h. (VIII) (a) 70% HF.py, Py, 0 °C, 12 h. (b) SO₃·NEt₃, DMF, 80 °C, 3 days. (c) LiOH·H₂O, THF:H₂O (1:1), rt, 12 h. (d) PMe₃.THF, THF, 0.1M NaOH, rt, 12 h. (e) SO₃Py, MeOH, 0.1M NaOH, NEt₃, 0 °C, 36 h.

36 h. **(VII) (a)** DDQ, DCM:H₂O (18:1), rt, 2 h. **(b)** 70% HF.py, Py, 0 °C, 12 h. **(c)** SO₃·NEt₃, DMF, 80 °C, 3 days. **(d)** LiOH·H₂O, THF:H₂O (1:1), rt, 12 h. **(e)** PMe₃·THF, THF, 0.1M NaOH, rt, 12 h. **(f)** SO₃·Py, MeOH, 0.1M NaOH, NEt₃, 0 °C, 36 h. **(X)** Global Deprotection

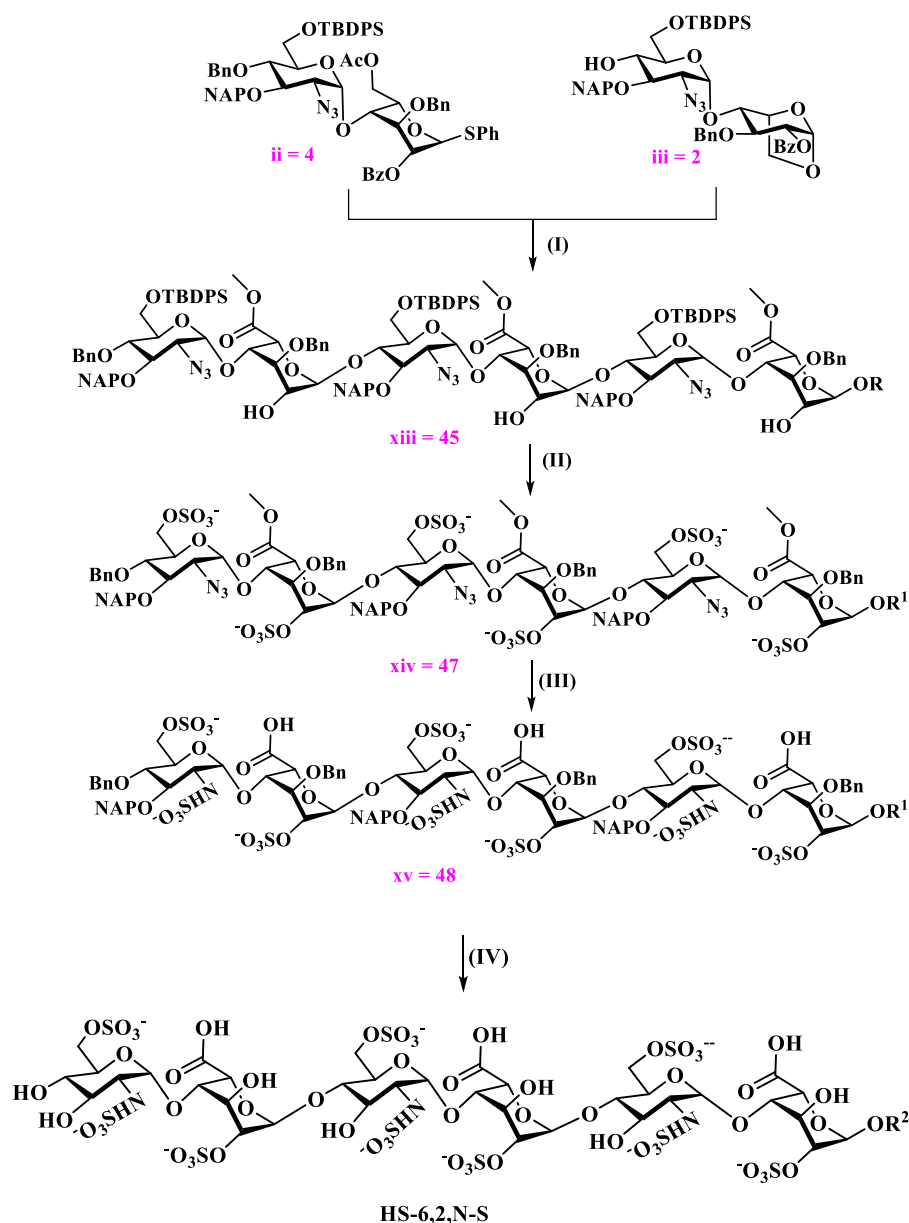
A divergent synthetic approach was employed to construct a comprehensive library of HS disaccharides. Specifically, the silyl protecting group TBDPS was selectively removed using 70% HF.py for the 6-*O*-sulfate derivative precursors (**vii**). Similarly, NAP deprotection done with DDQ yielded 3-*O*-sulfated precursors in a moderate yield followed by TBDPS deprotection (**viii**). Subsequently, introduction of sulfate groups on the backbone was facilitated by SO₃·NEt₃. For introducing *N*-sulfate on amine SO₃·Py. was used, after hydrolysis and reduction of azide using PMe₃·THF (**ix**, **x**, **xi**, **xii**).

Finally, a global deprotection process encompassing all sulfated derivatives, including non-sulfated analogues with NHAc and *N*-sulfate, yielded the desired HS-disaccharides with the amine linker at the reducing end. (*scheme 3*)

2.2.3 Synthesis of *N*- Sulfate derivative of Hexasaccharide

N-sulfated HS analogues with regioselective sulfate modifications were obtained from hexasaccharide precursors. Orthogonal protection of the hexasaccharide allowed for the selective installation of sulfate groups. The synthesis of the hexasaccharide was carried out using disaccharide building blocks **3** and **4**. Briefly, we adopted a [2+2+2] glycosylation approach to obtain the hexasaccharide in good yield. To synthesize hexasaccharide **14**, two glycosylation reactions were performed on compound **3** with **4**, utilizing NIS and trimethylsilyl trifluoromethanesulfonate, followed by attachment of a linker to the thiophenol hexasaccharide. The subsequent steps involved deacetylation, followed by that oxidation of the primary hydroxyl using TEMPO and methyl esterification.

Subsequently, the silyl protecting group TBDPS was selectively removed using 70% HF-pyridine, and the 6-*O*-sulfate derivative precursors were installed using SO₃·NMe₃. For introducing the *N*-sulfate on the amine, SO₃·Py was used after hydrolysis and reduction of the azide using PMe₃ in THF to yield compound **16**. Finally, a global deprotection process yielded the desired HS-hexasaccharide with the amine linker at the reducing end.

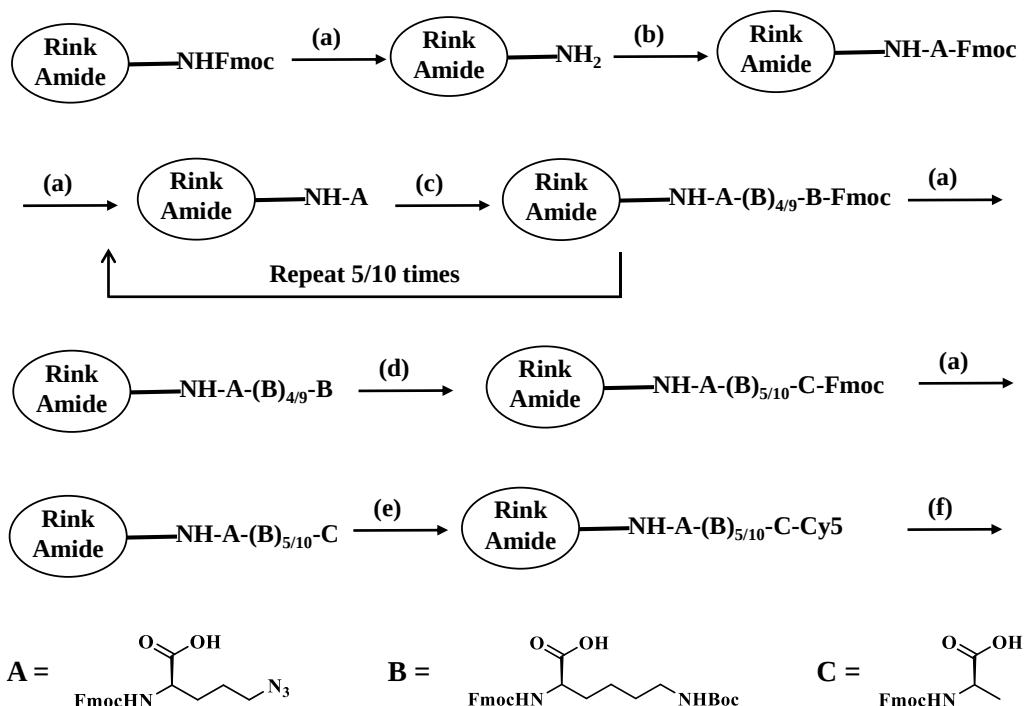


Scheme 3: (I) (a) NIS, TfOH, 4 Å MS, -10 °C, CH₂Cl₂, 30 min. (b) Ac₂O, Cu(OTf)₂, rt, 12 h. (c) TMSSPh, ZnI₂, CH₂Cl₂, rt, 2 h. (d) Benzyl (3-hydroxypropyl)carbamate, NIS, TMSOTf, 4 Å MS, -10 °C, CH₂Cl₂, 30 min. (e) NaOMe, CH₂Cl₂:MeOH (1:1), rt, 12 h. (f) TEMPO, CH₂Cl₂:MeOH (1:1), rt, 12 h. (g) LiOH.H₂O, H₂O:THF (1:1), rt, 2 h. (h) MeI, K₂CO₃, DMF, rt, 12 h. (II) (a) 70% HF.py, Py, 0 °C, 12 h. (b) SO₃NEt₃, DMF, 80 °C, 3 days. (III) (a) LiOH.H₂O, THF:H₂O (1:1), rt, 12 h. (b) PMe₃.THF, THF, 0.1M NaOH, rt, 12 h. (c) SO₃Py, MeOH, 0.1M NaOH, NEt₃, 0 °C, 36 h. (IV) Global Deprotection

2.2.4 Synthesis of Proteoglycan Mimics

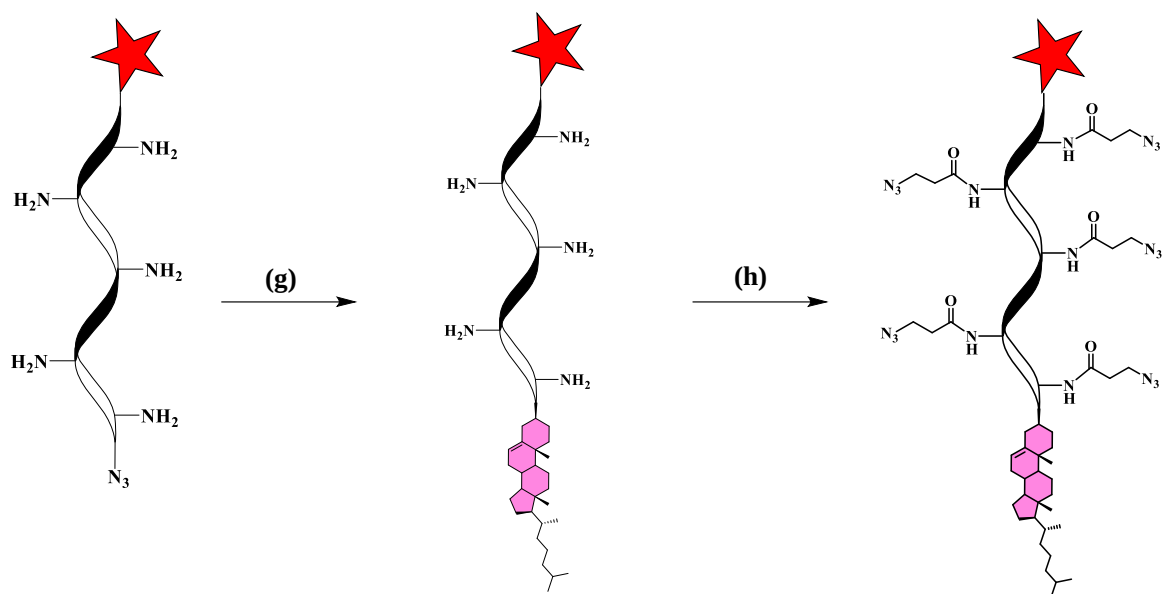
The synthesis of proteoglycan mimetics begins by modifying the side chain acid of Boc-Glu-(OtBu)-OH (**67**) into an azide (**71**), followed by the synthesis of Cy-5 with the acid moiety (**75**). A cholesterol-DBCO conjugated compound is then synthesized. Next, Fmoc chemistry and solid-phase peptide chemistry were used to synthesize peptides of varying lengths. First,

Fmoc-Glu-(N₃)-OH is conjugated to rink amide resin, followed by Fmoc deprotection and conjugation of Fmoc-Lys-(OtBu)-OH. This process is repeated to attach 5-10 lysine moieties to the resin, depending on the desired length (7- and 12-mers). Fmoc-Ala-OH is subsequently conjugated to the peptide, and Cy-5 dye is attached on solid phase. The peptide is then cleaved from the resin using a TFA, phenol, water, and TIPS cocktail.¹⁴



Scheme 4: (a) (i) 20 % Piperidine in DMF 3 times 10 min each (b) **A**, HOBT, HBTU, DIPEA, NMP, 1 h (c) **B**, HOBT, HBTU, DIPEA, NMP, 1 h (d) **C**, HOBT, HBTU, DIPEA, NMP, 1 h (e) **Cy-5**, HOBT, HBTU, DIPEA, NMP, 1 h (f) TFA:H₂O:Phenol:TIPS (18:1:1:0.5), 1 h

Subsequently, a click reaction with cholesterol-DBCO follows, converting lysine amine into an azide using an azide linker. Finally, a carbohydrate moiety with DBCO is conjugated to the peptide using click reaction to yield proteoglycan mimetics the completion of reaction was monitored using MALDI/TOF and HRMS-ESI-MS which is showing conjugation of 10 molecules to on 12 -mer peptide and 5 molecules on 7-mer peptide.¹⁴



Scheme 5: (g) Schematic representation of peptide template and PG mimetics (i) Cholesterol-DBCO, rt, 12 h (ii) Linker, rt, 12 – 16 h (h) Click reaction

2.3 Conclusion

We report here for the first time, a new class of peptide-based multivalent system by utilizing this we had synthesized proteoglycan mimetics of varying lengths and monodisperse in nature. These proteoglycan mimetics comprise four components: (i) cholesterol for hydrophobic insertion, (ii) a peptide backbone, (iii) a fluorescent tag, and (iv) a carbohydrate moiety, which can be easily modified. We have attached monosaccharides up to hexasaccharide of HS moieties and also conjugated sialic acid trisaccharide's, demonstrating the versatility of this peptide based multivalent template.

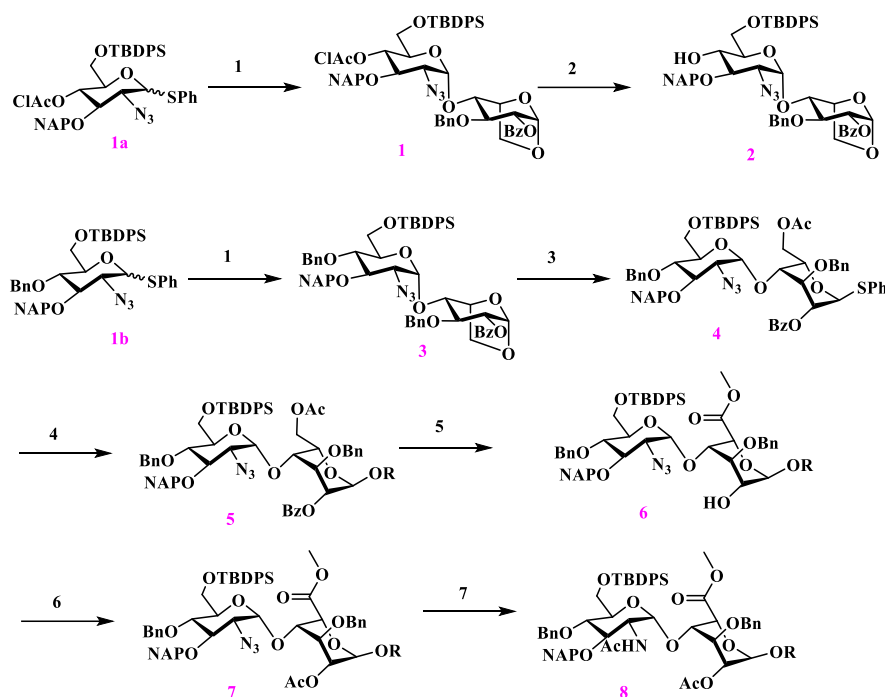
2.4 Experimental Section

2.4.1 General Information

All chemicals were utilized in their reagent-grade form, without further purification, unless specified otherwise. Analytical thin-layer chromatography (TLC) was executed on Merck silica gel 60 F254 plates, with compound visualization achieved through either UV irradiation or immersion in a CAM/ninhydrin solution followed by heating. Column chromatography employed a force flow of the specified solvent on Fluka Kieselgel 60 (230–400 mesh). The ^1H and ^{13}C NMR spectra for all compounds were recorded on Jeol 400 MHz and Bruker 600 MHz with a cryo probe. Residual solvent signals served as an internal reference (CDCl_3 δH , 7.26

ppm, δ C 77.3 ppm, CD₃OD δ H 3.31 ppm, δ C 49.0 ppm, and D₂O δ H 4.79 ppm). Chemical shifts (δ) are reported in ppm, and coupling constants (J) are expressed in Hz.

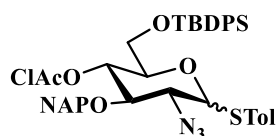
2.4.2 Synthesis of HS Disaccharide



Reagents & Yield: (1) NIS, TMSOTf, 68 % (2) Thiourea, 90 % (3) (i) Ac₂O, Cu(OTf)₂, 90 %, (ii) TMSSPh, ZnI₂, 87 % (4) Benzyl (3-hydroxypropyl)carbamate, NIS, TMSOTf, 85 % (5) (i) NaOMe, 90 %, (ii) TEMPO, BAIB, 87 %, (iii) LiOH.H₂O (iv) MeI, K₂CO₃, 80 %, (6) Ac₂O, 90 %, (7) Zn dust, THF:AcOH:Ac₂O (3:2:1), 82 %

4-Methylphenyl

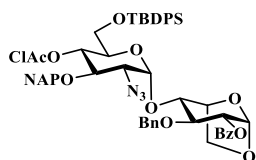
2-azido-4-*O*-chloroacetyl-6-*O*-*tert*-butyldiphenylsilyl-3-*O*-(2-naphthylmethyl)-2-deoxy-1-thio- α/β -D-glucopyranoside (1a)



To a solution of compound 3-*O*-Nap and benzylidene protected glucosamine building block (1.5 g, 2.78 mmol) in CH₂Cl₂ (5 mL) and MeOH (10 mL) was added pTSA (0.95 g, 5.56 mmol) and stirred for 6 h. Upon completion, the reaction mixture was quenched using Et₃N and extracted with ethyl acetate and brine. The organic layer was collected, dried over Na₂SO₄, filtered, concentrated and purified through flash silica gel column chromatography (ethyl acetate/ hexane= 1/1, v/v). Next, the obtained compound (1 g, 2.21 mmol) was dissolved in CH₂Cl₂ (10 mL) and stirred at ice cold temperature under N₂ atmosphere followed by the addition of imidazole (0.45 g,

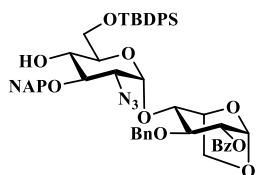
6.64 mmol) and DMAP (0.10 g, 0.88 mmol). Finally, TBDPS-Cl was added dropwise and reaction mixture was left for stirring. After 12 h, the reaction was quenched using MeOH and extracted with brine. The combined organic layer was collected, dried over Na₂SO₄, filtered, concentrated and purified through silica gel column chromatography (ethyl acetate/ hexane= 1/10, v/v). Further, the solution of 6-O-TBDPS compound (1 g, 1.45 mmol) in CH₂Cl₂ (8 mL) and pyridine (2 mL) was stirred at ice cold temperature for 15 minutes under N₂ atmosphere before the addition of chloroacetic anhydride (0.32 g, 1.85 mmol). Reaction completion was monitored by TLC (approx. 20 min). After the completion of reaction, the mixture was washed with 1 N HCl and brine. The organic layer was collected, dried over Na₂SO₄, filtered, concentrated and purified through silica gel column chromatography (ethyl acetate/ hexane= 1/20, v/v) to obtain compound **1a** in 95 % yield. ¹H NMR (400 MHz, Chloroform-d) (**α anomer**) δ 7.87 – 7.83 (m, 3H), 7.78 (s, 1H), 7.64 – 7.61 (m, 4H), 7.51 – 7.48 (m, 2H), 7.43 – 7.30 (m, 9H), 7.06 (d, *J* = 7.9 Hz, 2H), 5.59 (d, *J* = 5.4 Hz, 1H), 5.28 – 5.23 (m, 1H), 5.07 (d, *J* = 11.6 Hz, 1H), 4.80 (d, *J* = 11.6 Hz, 1H), 4.37 (ddd, *J* = 10.1, 4.2, 2.3 Hz, 1H), 4.03 (dd, *J* = 10.2, 5.4 Hz, 1H), 3.86 – 3.81 (m, 1H), 3.67 (qd, *J* = 11.7, 3.3 Hz, 2H), 3.53 – 3.44 (m, 2H), 2.33 (s, 3H), 1.02 (s, 9H). ¹³C NMR (101 MHz, Chloroform-d) δ 165.85, 138.06, 135.81, 135.67, 134.92, 134.89, 133.28, 133.12, 133.11, 132.96, 132.43, 130.01, 129.81, 129.72, 129.65, 128.47, 128.13, 127.79, 127.70, 127.17, 126.40, 126.31, 126.15, 87.46, 79.35, 75.46, 71.68, 71.56, 64.24, 62.35, 40.43, 26.77, 21.24, 19.28. (**β anomer**) ¹H NMR (400 MHz, Chloroform-d) δ 7.87- 7.85 (m, 3H), 7.78 – 7.74 (m, 3H), 7.70 (dd, *J* = 7.7, 1.4 Hz, 2H), 7.57 (d, *J* = 8.1 Hz, 2H), 7.54 – 7.51 (m, 2H), 7.47 – 7.41 (m, 7H), 7.09 (d, *J* = 8.0 Hz, 2H), 5.21 (t, *J* = 6.8 Hz, 1H), 5.03 (d, *J* = 11.5 Hz, 1H), 4.80 (d, *J* = 11.6 Hz, 1H), 4.43 (d, *J* = 10.0 Hz, 1H), 3.80 (dd, *J* = 11.6, 1.9 Hz, 1H), 3.68 – 3.64 (m, 1H), 3.58 (dd, *J* = 13.2, 5.4 Hz, 1H), 3.51 – 3.45 (m, 4H), 2.36 (s, 3H), 1.10 (s, 9H). ¹³C NMR (101 MHz, Chloroform-d) δ 165.67, 139.07, 135.79, 135.78, 134.83, 134.54, 133.25, 133.12, 132.99, 132.92, 130.01, 129.90, 129.81, 128.48, 128.08, 127.83, 127.78, 127.25, 126.51, 126.40, 126.33, 126.16, 86.05, 82.60, 78.65, 75.49, 70.67, 64.75, 62.20, 26.80, 21.29, 19.26. HR-ESI-MS (*m/z*): [M+Na]⁺ calcd for C₄₂H₄₄ClN₃O₅SSiNa, 788.2357; found, 788.2358.

1,6-Anhydro-[2-azido-4-O-chloroacetyl-6-O-*tert*-butyldiphenylsilyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl]-(1 $\rightarrow$ 4)-O-2-O-benzoyl-3-O-benzyl- β -L-idopyranose (1)



A solution of donor **1a** (1 g, 1.30 mmol) and acceptor (**A**) (0.41 g, 1.17 mmol) in CH₂Cl₂ (20 mL) was stirred under N₂ atmosphere in round bottom flask containing freshly dried 4 Å molecular sieves for 1 h. The mixture solution was cooled down to -78 °C followed by the addition of NIS (0.46 g, 2.08 mmol) and TMSOTf (71 µL, 0.39 mmol). The temperature was gradually increased to -20 °C and reaction mixture was left for stirring for another 15 minutes. After 15 minutes reaction completion was monitored by TLC and quenched using few drops of Et₃N. Molecular sieves were filtered using celite and organic layer was washed with aqueous Na₂S₂O₃ and brine. The collected organic layer was dried over Na₂SO₄, filtered, concentrated and purified through silica gel column chromatography (ethyl acetate/ hexane= 1/12, v/v) to obtain compound **1** in 65 % yield. ¹H NMR (400 MHz, Chloroform-d) δ 8.10 – 8.03 (m, 2H), 7.84 (m, 3H), 7.77 (d, J = 1.7 Hz, 1H), 7.63 (m, 4H), 7.63 – 7.56 (m, 1H), 7.53 – 7.44 (m, 4H), 7.47 – 7.34 (m, 9H), 7.27 (m, 2H), 7.28 – 7.20 (m, 3H), 5.55 (d, J = 1.8 Hz, 1H), 5.35 (d, J = 3.8 Hz, 1H), 5.18 – 5.06 (m, 2H), 5.05 (d, J = 11.5 Hz, 1H), 4.90 (d, J = 10.9 Hz, 1H), 4.80 (t, J = 11.3, 2H), 4.65 (t, J = 4.5 Hz, 1H), 4.19 (d, J = 7.6 Hz, 1H), 4.11 (t, J = 8.0 Hz, 1H), 4.10 – 3.95 (m, 2H), 3.79 – 3.69 (m, 2H), 3.66 (m, 1H), 3.64 – 3.56 (m, 1H), 3.57 (d, J = 2.2 Hz, 2H), 3.55 – 3.44 (m, 2H), 1.05 (s, 9H). ¹³C NMR (101 MHz, Chloroform-d) δ 165.84, 165.80, 137.92, 135.73, 135.68, 134.87, 133.55, 133.28, 133.14, 132.86, 132.74, 129.97, 129.47, 128.60, 128.52, 128.50, 128.07, 127.93, 127.89, 127.87, 127.82, 127.01, 126.45, 126.34, 125.96, 99.48, 99.37, 79.51, 78.94, 75.07, 74.96, 74.24, 71.75, 71.41, 65.77, 63.36, 62.64, 40.41, 26.82, 19.22. HR-ESI-MS (*m/z*): [M+Na]⁺ calcd for C₅₅H₅₆ClN₃O₁₁SiNa, 1020.3270; found, 1020.3271.

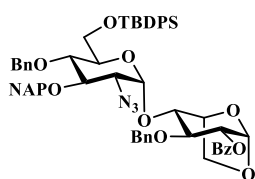
1,6-Anhydro-[2-azido-6-*O*-*tert*-butyldiphenylsilyl-3-*O*-(2-naphthylmethyl)-2-deoxy-α-D-glucopyranosyl]-(1→4)-*O*-2-*O*-benzoyl-3-*O*-benzyl-β-L-idopyranose (2**)**



To a solution of compound **1** (0.70 g, 0.7 mmol) in MeOH (5 mL) and pyridine (5 mL) was added thiourea (0.10 g, 1.4 mmol) and refluxed at 80 °C. After 1 h, volatiles were evaporated and residue was extracted with ethyl acetate, 1 N HCl and brine. The collected organic layer was dried over Na₂SO₄, filtered, concentrated and purified through silica gel column chromatography (ethyl acetate/ hexane= 1/8, v/v) to obtain compound **2** in 90 % yield. ¹H NMR (400 MHz, Chloroform-d) δ 8.07 – 8.04 (m, 2H), 7.89 – 7.83 (m, 5H), 7.69 – 7.65 (m, 52H), 7.59 – 7.54 (m, 2H), 7.52 – 7.46 (m, 3H), 7.46 – 7.42 (m, 4H), 7.42 – 7.36 (m, 5H), 7.26 – 7.19 (m, 4H), 5.53 (d, J = 1.8 Hz, 1H), 5.25 (d, J = 3.8 Hz, 1H), 5.11 (d, J = 11.4 Hz, 1H), 5.08 – 5.01 (m, 2H), 4.89 (d, J = 10.8 Hz, 1H), 4.75 (d, J = 10.8 Hz, 1H), 4.55 (t, J = 4.6 Hz, 1H),

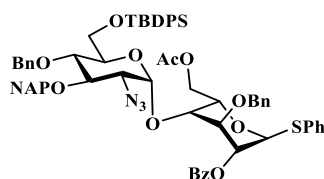
4.17 – 4.04 (m, 2H), 4.00 (d, $J = 4.3$ Hz, 1H), 3.94 – 3.80 (m, 4H), 3.72 (m, 2H), 3.63 – 3.57 (m, 1H), 3.39 – 3.34 (m, 1H), 2.66 (d, $J = 2.7$ Hz, 1H), 1.08 – 1.05 (m, 9H). ^{13}C NMR (101 MHz, Chloroform- d) δ 165.83, 137.98, 135.69, 135.68, 135.45, 133.51, 133.42, 133.22, 132.83, 132.71, 130.14, 130.10, 129.98, 129.48, 128.62, 128.57, 128.47, 128.09, 127.98, 127.96, 127.85, 127.83, 127.07, 126.35, 126.22, 125.98, 99.76, 99.33, 79.50, 79.44, 78.90, 75.33, 75.05, 74.32, 72.61, 71.89, 65.75, 64.36, 62.96, 26.93, 19.27. HR-ESI-MS (m/z): $[\text{M}+\text{Na}]^+$ calcd for $\text{C}_{53}\text{H}_{55}\text{N}_3\text{O}_{10}\text{SiNa}$, 944.3555; found, 944.3540.

1,6-Anhydro-[2-azido-4-*O*-benzyl-6-*O*-*tert*-butyldiphenylsilyl-3-*O*-(2 naphthylmethyl)-2-deoxy- α -D-glucopyranosyl]-(1 $\rightarrow$ 4)-*O*-2-*O*-benzoyl-3-*O*-benzyl- β -L- idopyranose (3)



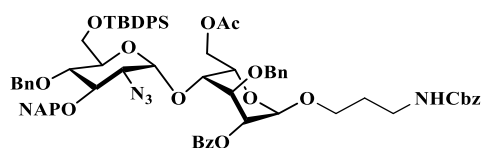
To a solution of compound **1b** donor (1g, 1.5 mmol) and acceptor **A** (0.41 g, 1.17 mmol) in CH_2Cl_2 (20 mL) was stirred under N_2 atmosphere in round bottom flask containing freshly dried 4 Å molecular sieves for 1 h. The mixture solution was cooled down to -78°C followed by the addition of NIS (0.46 g, 2.08 mmol) and TMSOTf (71 μL , 0.39 mmol). The temperature was gradually increased to -20°C and reaction mixture was left for stirring for another 15 minutes. After 15 minutes reaction completion was monitored by TLC and quenched using few drops of Et_3N . Molecular sieves were filtered using celite and organic layer was washed with aqueous $\text{Na}_2\text{S}_2\text{O}_3$ and brine. The collected organic layer was dried over Na_2SO_4 , filtered, concentrated and purified through silica gel column chromatography (ethyl acetate/ hexane= 1/12, v/v) to obtain compound **3** in 65 % yield. ^1H NMR (400 MHz, Chloroform- d) δ 8.08 – 8.04 (m, 2H), 7.83 (m, 4H), 7.70 – 7.67 (m, 4H), 7.62 – 7.54 (m, 2H), 7.51 – 7.42 (m, 7H), 7.42 – 7.35 (m, 5H), 7.31 – 7.27 (m, 3H), 7.24 – 7.18 (m, 3H), 7.17 – 7.13 (m, 2H), 5.52 (d, $J = 1.8$ Hz, 1H), 5.32 – 5.29 (m, 1H), 5.12 – 5.06 (m, 3H), 4.89 (t, $J = 10.4$ Hz, 2H), 4.76 (d, $J = 10.8$ Hz, 1H), 4.66 – 4.57 (m, 2H), 4.14 (d, $J = 7.7$ Hz, 1H), 4.10 – 3.99 (m, 3H), 3.88 (d, $J = 1.8$ Hz, 2H), 3.75 – 3.65 (m, 3H), 3.45 (dd, $J = 10.3, 3.8$ Hz, 1H), 1.07 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3) δ 165.8, 138.0, 137.5, 135.8, 135.7, 135.2, 133.4, 133.3, 133.2, 132.9, 129.9, 129.9, 129.8, 129.4, 128.6, 128.5, 128.4, 128.4, 128.1, 128.1, 128.0, 127.9, 127.8, 127.7, 127.0, 126.2, 126.1, 126.0, 99.7, 99.3, 80.0, 79.4, 78.9, 78.3, 77.4, 77.0, 76.7, 75.7, 75.5, 75.0, 74.2, 72.9, 65.7, 63.7, 62.6, 31.0, 26.9, 26.8, 25.3, 19.2. HR-ESI-MS (m/z): $[\text{M}+\text{Na}]^+$ calcd for $\text{C}_{60}\text{H}_{61}\text{N}_3\text{O}_{10}\text{SiNa}$, 1034.4024; found, 1034.4010.

Phenyl-[2-azido-4-*O*-benzyl-6-*O*-*tert*-butyldiphenylsilyl-3-*O*-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl]-(1 $\rightarrow$ 4)-*O*-2-*O*-benzoyl-3-*O*-benzyl-6-*O*-acetyl-1-thio- α -L-idopyranoside (4)



A solution of compound **3** (1 g, 0.98 mmol) in Ac₂O (10 mL) was stirred at ice cold temperature for 15 minutes before the addition of Cu(OTf)₂ (0.035 g, 0.098 mmol). After 16 h, the reaction mixture was concentrated under reduced pressure and the residue was extracted with ethyl acetate, NaHCO₃ and washed with brine. The combined organic layer was dried over Na₂SO₄, filtered, concentrated and preceded further without any purification. Next, the as obtained acetylated solution of compound (0.98 g, 0.88 mmol), ZnI₂ (0.59 g, 1.84 mmol) and phenyl trimethylsilyl sulphide (0.50 g, 2.72 mmol) in CH₂Cl₂ (15 mL) was stirred under N₂ atmosphere for 2 h. Upon completion, the reaction mixture was filtered through celite, evaporated and purified through silica gel column chromatography (ethyl acetate/ hexane= 1/5, v/v) to obtain compound **4** in 85 % yield. ¹H NMR (400 MHz, Chloroform-d) δ 8.20 – 8.16 (m, 2H), 7.81 – 7.74 (m, 3H), 7.70 (d, J = 1.5 Hz, 1H), 7.68 – 7.66 (m, 2H), 7.65 (d, J = 1.5 Hz, 1H), 7.58 – 7.55 (m, 3H), 7.51 – 7.46 (m, 4H), 7.43 – 7.37 (m, 5H), 7.36 – 7.27 (m, 14H), 7.19 – 7.16 (m, 2H), 5.62 (s, 1H), 5.40 (t, J = 2.5 Hz, 1H), 4.99 (d, J = 11.6 Hz, 1H), 4.90 - 4.88 (m, 1H), 4.82 – 4.68 (m, 3H), 4.63 (d, J = 3.7 Hz, 1H), 4.55 (d, J = 10.6 Hz, 1H), 4.41 (dd, J = 11.6, 8.1 Hz, 1H), 4.24 – 4.15 (m, 3H), 4.02 – 3.98 (m, 1H), 3.88 (d, J = 11.5, 1H), 3.81 – 3.65 (m, 4H), 3.36 (dd, J = 9.9, 3.7 Hz, 1H), 1.91 (s, 3H), 1.05 (s, 9H). ¹³C NMR (101 MHz, CHLOROFORM-D) δ 170.5, 165.9, 138.1, 137.4, 136.0, 135.7, 135.6, 135.2, 133.6, 133.4, 133.1, 133.1, 131.8, 130.0, 130.0, 129.8, 129.8, 129.0, 128.7, 128.6, 128.5, 128.3, 128.2, 128.2, 128.1, 127.9, 127.9, 127.8, 127.8, 127.7, 127.6, 127.1, 126.2, 126.2, 126.1, 99.1, 86.0, 80.9, 77.9, 77.5, 77.4, 77.2, 76.8, 75.4, 75.2, 75.1, 73.3, 72.9, 72.0, 69.8, 66.5, 64.2, 63.2, 62.3, 27.0, 20.8, 19.4. HR-ESI-MS (*m/z*): [M+Na]⁺ calcd for C₆₈H₆₉N₃O₁₁SSiNa, 1186.4320; found, 1186.4315.

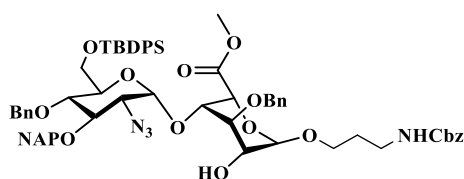
N-benzyloxycarbonyl-3-aminopropyl-*O*-[2-azido-4-*O*-benzyl-6-*O*-*tert*-butyldiphenylsilyl-3-*O*-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl]-(1 $\rightarrow$ 4)-*O*-2-*O*-benzoyl-3-*O*-benzyl-6-*O*-acetyl-1- α -L-idopyranoside (5)



A solution of donor **4** (0.90 g, 0.42 mmol) and linker benzyl (3-hydroxypropyl) carbamate (0.097 g, 0.46 mmol) in CH₂Cl₂ (15 mL) was stirred under N₂

atmosphere in round bottom flask containing freshly dried 4 Å molecular sieves for 1 h. Next, NIS (0.15 g, 0.67 mmol) and TfOH (7.4 µL, 0.084 mmol) was added at room temperature and reaction completion was monitored by TLC, quenched using few drops of NEt₃. Molecular sieves were filtered using celite and organic layer was washed with aqueous Na₂S₂O₃ and brine. The collected organic layer was dried over Na₂SO₄, filtered, concentrated and purified through silica gel column chromatography (ethyl acetate/ hexane= 1/3, v/v) to obtain compound **5** in 87 % yield. ¹H NMR (400 MHz, Chloroform-d) δ 8.17 – 8.09 (m, 2H), 7.84 – 7.72 (m, 3H), 7.69 – 7.63 (m, 4H), 7.58 (s, 1H), 7.50 – 7.45 (m, 2H), 7.43 – 7.38 (m, 2H), 7.37 – 7.27 (m, 20H), 7.20 – 7.14 (m, 2H), 5.64 (t, J = 6.0 Hz, 1H), 5.07 (d, J = 6.9 Hz, 3H), 4.94 (d, J = 1.4 Hz, 1H), 4.81 (dd, J = 11.5, 8.4 Hz, 2H), 4.67 (t, J = 11.3 Hz, 2H), 4.57 (d, J = 10.6 Hz, 1H), 4.43 – 4.21 (m, 4H), 4.07 (dd, J = 10.9, 4.1 Hz, 1H), 4.04 – 3.95 (m, 2H), 3.84 (dd, J = 10.4, 5.7 Hz, 2H), 3.78 – 3.65 (m, 3H), 3.58 – 3.40 (m, 3H), 3.30 – 3.18 (m, 2H), 1.92 (s, 3H), 1.85 (p, J = 6.9, 5.8 Hz, 2H), 1.04 (s, 9H). ¹³C NMR (101 MHz, CHLOROFORM-D) δ 170.6, 165.8, 156.7, 138.1, 137.6, 136.8, 136.0, 135.7, 135.2, 133.6, 133.4, 133.3, 133.1, 130.0, 129.9, 129.8, 128.7, 128.5, 128.4, 128.4, 128.2, 128.1, 128.1, 127.9, 127.8, 127.7, 127.7, 127.1, 126.2, 126.1, 98.8, 98.2, 80.8, 77.9, 77.4, 77.1, 76.8, 75.4, 75.2, 74.6, 73.1, 72.6, 72.0, 68.7, 67.1, 66.7, 65.3, 64.2, 63.4, 62.3, 39.8, 29.5, 26.9, 20.8, 19.4. HR-ESI-MS (*m/z*): [M+Na]⁺ calcd for C₇₃H₇₈N₄O₁₄SSiNa, 1285.5181; found, 1285.5174.

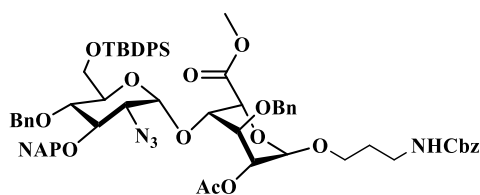
N-benzyloxycarbonyl-3-aminopropyl-O-[(methyl(2-azido-4-O-benzyl-6-O-*tert*-butyldiphenylsilyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1→4)-O-3-O-benzyl-1- α -L-idopyranosyluronate (6**)**



A solution of compound **5** (1g, 1 eq) in DCM:MeOH (1:1, 14mL), NaOMe(1.2 eq) was added and kept it for stirring for 12 h at room temperature followed by quenching with IR Na⁺ resin, filtered, evaporated and purified by silica gel column chromatography (ethyl acetate/ hexane= 1/2.5, v/v). After that compound was dissolved in DCM:H₂O (1:1) followed by addition by of TEMPO (0.2 eq) and BAIB (3 eq) and kept it stirring for 12 h at room temperature followed by extraction with saturated aqueous solution NH₄Cl. The organic layer was evaporated and purified by silica gel column chromatography. Then the compound was dissolved in THF:H₂O (1:1) followed by addition of lithium hydroxide (10 eq) and kept it on stirring for 2 h at room temperature followed by evaporation and dried over vacuum. After that reaction mixture was dissolved in dry DMF and potassium carbonate (3 eq), methyl iodide (9 eq) was added and kept it on stirring

for at room temperature. After 12 h extraction was done using 10 % HCl solution and organic layer was evaporated, purified using silica gel chromatography using ethyl acetate/hexane (2/3) to obtain compound **6** 84%. ^1H NMR (400 MHz, Chloroform-*d*) δ 7.84 – 7.68 (m, 6H), 7.68 – 7.61 (m, 2H), 7.50 – 7.27 (m, 19H), 7.26 – 7.13 (m, 5H), 5.56 (d, J = 6.0 Hz, 1H), 5.04 (s, 2H), 5.00 (d, J = 10.6 Hz, 3H), 4.93 – 4.88 (m, 2H), 4.80 (s, 1H), 4.76 (d, J = 11.4 Hz, 1H), 4.60 – 4.56 (m, 2H), 4.12 4.11 (m, 1H), 3.97 (dd, J = 11.6, 2.9 Hz, 1H), 3.93 – 3.80 (m, 5H), 3.71 - 3.67(m, 1H), 3.63 (s, 3H), 3.61 – 3.54 (m, 3H), 3.54 – 3.49 (m, 1H), 3.44 (d, J = 5.5 Hz, 1H), 1.90 – 1.77 (m, 2H), 1.08 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3) δ 169.8, 156.5, 138.3, 137.3, 136.6, 135.9, 135.6, 135.0, 133.4, 133.3, 133.1, 133.1, 129.8, 129.8, 128.7, 128.6, 128.4, 128.4, 128.3, 128.3, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 127.6, 127.2, 127.2, 127.1, 126.2, 126.2, 126.1, 126.0, 101.5, 94.9, 81.1, 77.6, 77.4, 77.3, 77.1, 76.7, 76.1, 74.8, 72.8, 71.9, 70.8, 70.7, 68.0, 67.0, 66.9, 66.6, 63.9, 62.1, 53.4, 52.4, 39.9, 29.5, 26.9, 19.4. HR-ESI-MS (m/z): $[\text{M}+\text{Na}]^+$ calcd for $\text{C}_{65}\text{H}_{72}\text{N}_4\text{O}_{13}\text{SiNa}$, 1167.4763; found, 1167.5478.

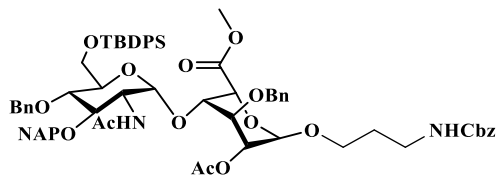
N-benzyloxycarbonyl-3-aminopropyl-O-[(methyl(2-azido-4-O-benzyl-6-O-*tert*-butyldiphenylsilyl)-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-2-O-acetyl-3-O-benzyl-1- α - L-idopyranosyluronate (7)



A solution of compound **6** (1g, 1 eq) in DCM:Py (1:1, 14mL), acetic anhydride (5 eq) and DMAP (0.2 eq) was added and kept it for stirring for 12 h at 0 °C followed by washing with 1N HCl 3 times and organic

layer was evaporated and purified by silica gel column chromatography (ethyl acetate/ hexane= 1/4, v/v) to obtain **7**, 86 %. ^1H NMR (400 MHz, Chloroform-*d*) δ 7.87 – 7.72 (m, 4H), 7.70 - 7.68 (m, 2H), 7.64 – 7.62 (m, 2H), 7.52 – 7.43 (m, 3H), 7.42 – 7.26 (m, 17H), 7.26 – 7.14 (m, 3H), 5.44 (t, J = 5.7 Hz, 1H), 5.05 (s, 2H), 5.02 – 4.91 (m, 3H), 4.90 (s, 1H), 4.87 – 4.80 (m, 2H), 4.79 – 4.71 (m, 2H), 4.65 (m, 2H), 4.02 – 3.93 (m, 3H), 3.91 – 3.81(m, 4H), 3.76 – 3.68 (m, 1H), 3.60 – 3.52 (m, 4H), 3.43 – 3.31 (m, 2H), 3.28 – 3.20 (m, 1H), 2.07 (s, 3H), 1.86 – 1.73 (m, 2H), 1.06 (s, 8H). ^{13}C NMR (101 MHz, CDCl_3) δ 170.1, 169.4, 156.5, 138.3, 137.3, 136.6, 135.8, 135.6, 135.1, 133.4, 133.3, 133.1, 133.1, 129.7, 128.5, 128.4, 128.4, 128.4, 128.3, 128.2, 128.0, 128.0, 128.0, 127.8, 127.7, 127.7, 127.6, 127.6, 127.5, 127.2, 127.1, 127.0, 126.1, 126.0, 126.0, 98.3, 97.3, 79.9, 77.9, 77.3, 77.0, 76.7, 75.5, 74.7, 72.6, 72.4, 72.0, 71.4, 68.0, 67.8, 67.6, 66.6, 63.6, 62.2, 52.2, 39.5, 29.4, 26.9, 26.9, 20.8, 20.7, 19.4. HR-ESI-MS (m/z): $[\text{M}+\text{Na}]^+$ calcd for $\text{C}_{67}\text{H}_{74}\text{N}_4\text{O}_{14}\text{SiNa}$, 1209.4868; found, 1209.4862.

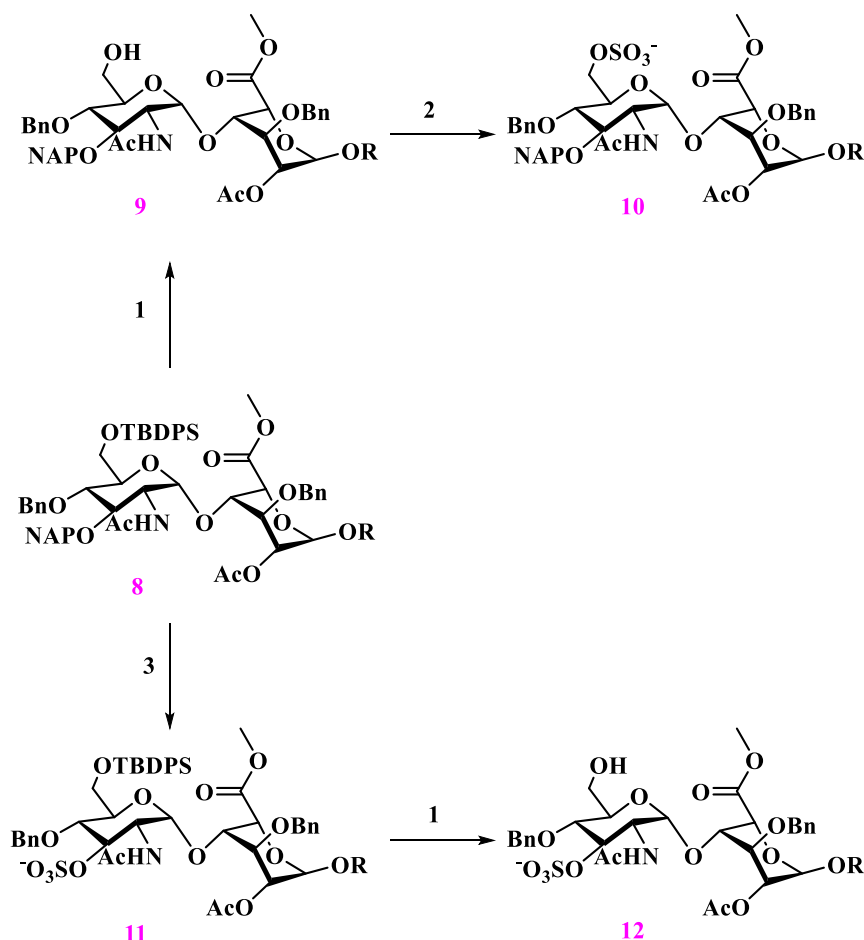
N-benzoyloxycarbonyl-3-aminopropyl-O-[(methyl(2-acetamido-4-O-benzyl-6-O-*tert*-butyldiphenylsilyl)-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-2-O-acetyl-3-O-benzyl-1- α - L-idopyranosyluronate (8)



A solution of compound **7** (1g, 1 eq) in THF:CH₃COOH:Ac₂O (3:2:1, 18mL), zinc dust (30 eq) was added and kept it for stirring for 12 h at room temperature followed by celite filtration and organic

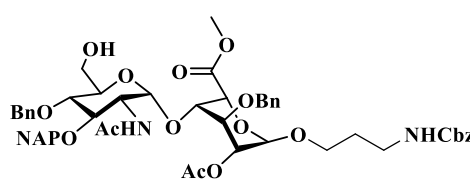
layer was evaporated. After that compound was re-dissolved in ethyl acetate and washed with 10 % sodium bicarbonate 3 times followed by evaporation of organic layer, purification by silica gel column chromatography (ethyl acetate/ hexane= 1/2, v/v) to obtain **8**, 81 %. ¹H NMR (400 MHz, Chloroform-d) δ 7.84 – 7.63 (m, 8H), 7.47 – 7.10 (m, 24H), 5.02 (s, 1H), 4.98 (d, J = 12.0 Hz, 1H), 4.92 (d, J = 11.2 Hz, 1H), 4.89 – 4.83 (m, 2H), 4.82 – 4.76 (m, 3H), 4.72 (d, J = 11.2 Hz, 1H), 4.48 (s, 1H), 4.37 (td, J = 10.1, 3.5 Hz, 1H), 4.05 (d, J = 2.9 Hz, 1H), 3.99 – 3.83 (m, 3H), 3.83 – 3.73 (m, 2H), 3.64 (s, 3H), 3.63 – 3.56 (m, 2H), 3.55 – 3.45 (m, 1H), 3.42 – 3.30 (m, 1H), 3.26 – 3.14 (m, 1H), 1.83 (s, 3H), 1.78 (m, 2H), 1.70 (s, 3H), 1.07 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 170.2, 169.5, 169.4, 156.5, 138.5, 136.9, 136.6, 135.9, 135.8, 135.6, 135.6, 133.4, 133.3, 133.1, 133.0, 129.8, 128.6, 128.5, 128.5, 128.4, 128.4, 128.3, 128.3, 128.2, 128.1, 128.1, 128.0, 127.9, 127.9, 127.8, 127.7, 127.7, 127.6, 127.6, 127.4, 127.4, 127.4, 126.8, 126.2, 126.2, 126.1, 98.2, 95.3, 80.5, 77.9, 77.5, 77.2, 76.9, 75.2, 74.8, 73.1, 72.4, 68.0, 67.7, 67.7, 66.6, 62.6, 52.4, 52.2, 39.3, 29.4, 27.0, 26.9, 26.9, 23.3, 20.7, 19.4. HR-ESI-MS (*m/z*): [M+Na]⁺ calcd for C₆₉H₇₈N₂O₁₅SiNa, 1225.5069; found, 1225.5059.

2.4.3 Orthogonal Deprotection & *O*-Sulfation of NHAc Disaccharide



Reagents & Yield: (1) 70 % HF.py, 90 – 92 %, (2) SO₃.NEt₃, 78 %, (3) (i) DDQ, 74 %, (ii) SO₃.NEt₃, 72 %

N-benzyloxycarbonyl-3-aminopropyl-O-[(methyl(2-acetamido-4-O-benzyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl))]- (1 $\rightarrow$ 4)-O-2-O-acetyl-3-O-benzyl-1- α -L-idopyranosyluronate (9)

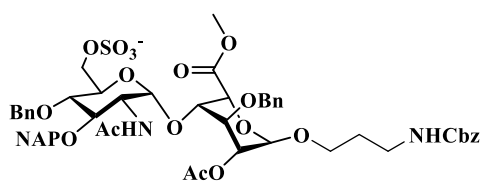


A solution of compound **8** (1g, 1 eq) in dry pyridine (5 mL) 70% HF.Py complex (5 eq) was added at 0 °C and kept it for stirring for 12 h. After that in reaction mixture ethyl acetate was added and washed with 1N

HCl 3 times followed by evaporation of organic layer, purification by silica gel column chromatography (ethyl acetate/ hexane= 2/3, v/v) to obtain **9**, 92 %. ¹H NMR (400 MHz, Chloroform-d) δ 7.84 – 7.67 (m, 4H), 7.49 – 7.42 (m, 2H), 7.40 – 7.26 (m, 12H), 7.26 – 7.16 (m, 4H), 5.91 – 5.80 (m, 1H), 5.46 (t, J = 5.8 Hz, 1H), 5.11 – 5.01 (m, 2H), 4.97 – 4.83 (m, 3H), 4.81 – 4.64 (m, 5H), 4.63 – 4.46 (m, 2H), 4.25 (td, J = 9.8, 3.6 Hz, 1H), 3.96 (q, J = 3.4, 2.9 Hz, 1H), 3.85 – 3.79 (m, 1H), 3.77 (s, 3H), 3.73 – 3.62 (m, 3H), 3.61 – 3.53 (m, 3H), 3.46 – 3.34 (m, 1H), 3.27 – 3.18 (m, 1H), 1.87 – 1.73 (m, 5H), 1.65 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 169.9, 169.5, 169.4, 156.5, 138.1, 136.8, 136.6, 135.7, 133.2, 133.0, 128.7, 128.6,

128.5, 128.5, 128.5, 128.5, 128.4, 128.3, 128.3, 128.2, 128.1, 128.1, 128.0, 128.0, 127.9, 127.8, 127.7, 127.7, 127.6, 127.5, 126.6, 126.3, 126.1, 126.1, 126.0, 98.3, 95.7, 80.1, 77.8, 77.4, 77.1, 76.8, 75.0, 74.8, 72.6, 72.2, 70.3, 69.7, 68.0, 67.9, 67.5, 66.7, 61.5, 52.4, 52.3, 39.5, 29.4, 23.3, 20.7. HR-ESI-MS (m/z): $[M+Na]^+$ calcd for $C_{53}H_{60}N_2O_{15}Na$, 987.3891; found, 987.3875.

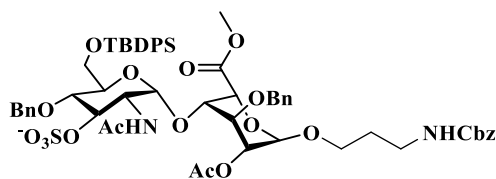
N-benzyloxycarbonyl-3-aminopropyl-O-[(methyl(2-acetamido-6-O-sulfonato-4-O-benzyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl))-(1 $\rightarrow$ 4)-O-2-O-acetyl-3-O-benzyl-1- α - L-idopyranosyluronate (10)



To a solution of starting material **9** (1 mmol, 1 eq) in DMF (2 mL) was added $SO_3 \cdot NEt_3$ complex (10 mmol per -OH, 10 eq) under N_2 atmosphere and stirred at 70 ° C for 3 days. Upon completion of reaction, the

reaction mixture was concentrated under reduced pressure and it was concentrated and purified using silica column chromatography (MeOH/DCM 1/10 v/v) to obtain product **10** 78 %. 1H NMR (400 MHz, Methanol- d_4) δ 7.86 – 7.78 (m, 2H), 7.78 – 7.71 (m, 1H), 7.52 – 7.45 (m, 2H), 7.44 – 7.26 (m, 16H), 5.08 (s, 2H), 4.99 – 4.96 (m, 3H), 4.94 – 4.93 (m, 1H), 4.85 – 4.72 (m, 3H), 4.42 – 4.33 (m, 2H), 4.29 (dd, J = 10.2, 3.3 Hz, 1H), 4.24 (s, 1H), 4.01 (s, 1H), 3.92 (d, J = 8.7 Hz, 1H), 3.89 – 3.83 (m, 1H), 3.82 (s, 3H), 3.78 – 3.67 (m, 2H), 3.57 (m, 1H), 3.34 – 3.18 (m, 1H), 2.01 (s, 3H), 1.91 (s, 3H), 1.82 (m, 2 H). ^{13}C NMR (101 MHz, MeOD) δ 171.8, 170.2, 170.0, 157.4, 138.4, 137.6, 137.0, 135.9, 133.4, 133.1, 128.3, 128.1, 128.1, 127.9, 127.9, 127.7, 127.6, 127.6, 127.5, 127.5, 127.3, 127.3, 126.0, 125.8, 125.6, 125.5, 98.6, 94.8, 80.4, 77.8, 74.9, 74.6, 72.0, 70.8, 70.4, 69.2, 68.1, 67.7, 66.4, 66.0, 52.7, 51.7, 48.6, 48.4, 48.1, 47.9, 47.7, 47.5, 47.3, 47.1, 37.9, 29.3, 21.8, 19.7. HR-ESI-MS (m/z): $[M+Na]^+$ calcd for $C_{53}H_{59}N_2O_{18}S^-$, 1043.3489; found, 1043.3485.

N-benzyloxycarbonyl-3-aminopropyl-O-[(methyl(2-acetamido-4-O-benzyl-6-O-tert-butylidiphenylsilyl-3-O-sulfonato-2-deoxy- α -D-glucopyranosyl))-(1 $\rightarrow$ 4)-O-2-O-acetyl-3-O-benzyl-1- α - L-idopyranosyluronate (11)

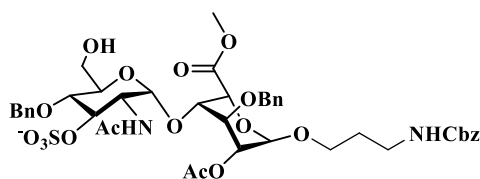


To a solution of starting material **8** (1 mmol, 1 eq) in CH_2Cl_2 (6 mL) and H_2O (340 μ L) was added DDQ (5 mmol, 5 eq) in 4 portions over the interval of 20 min.

After 1 h, the reaction mixture was quenched using $NaHCO_3$, extracted with CH_2Cl_2 and washed with brine. The collected organic layer was dried over Na_2SO_4 , filtered, concentrated and purified through silica gel column chromatography.

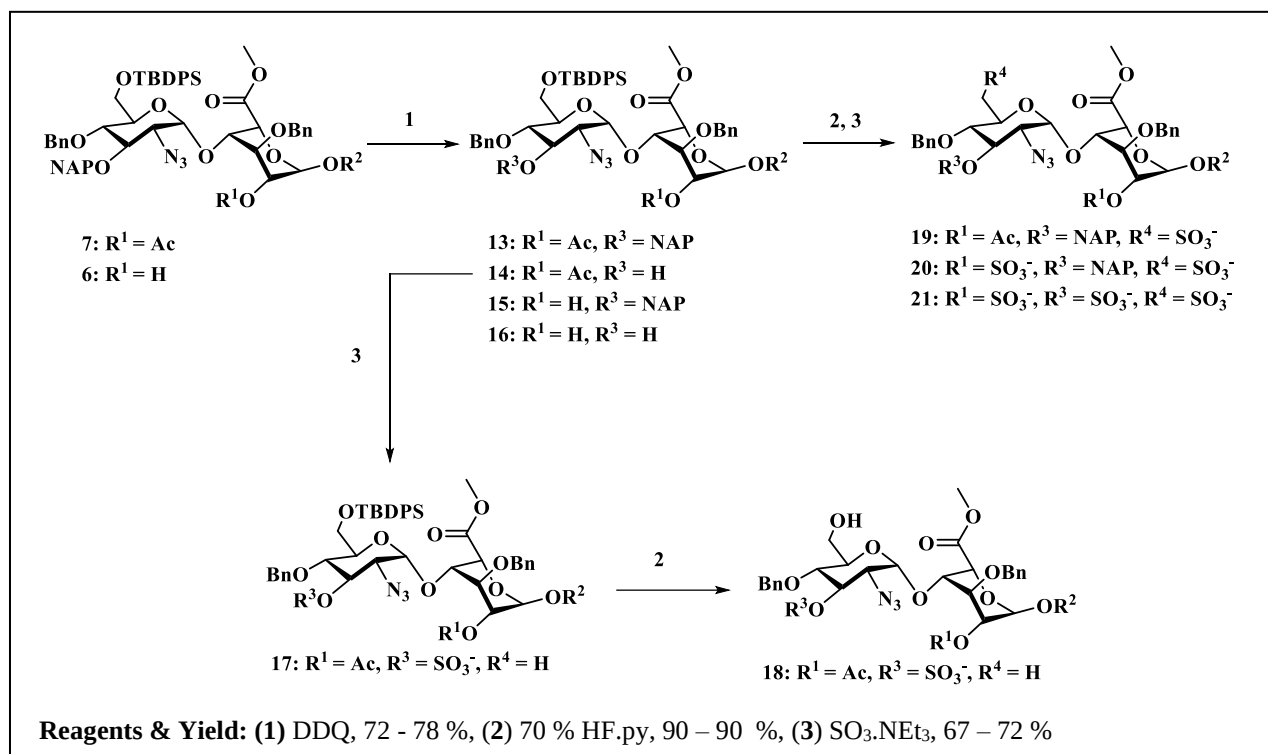
After that, compound was dissolved to 1 mmol in DMF (2 mL) was added SO₃.NEt₃ complex (10 mmol per -OH) under N₂ atmosphere and stirred at 70 °C for 3 days. Upon completion of reaction, the reaction mixture was concentrated under reduced pressure and it was concentrated and purified using silica column chromatography (MeOH/DCM 1/10 v/v) to obtain product **11** in 71 % yield. ¹H NMR (400 MHz, Methanol-d₄) δ 7.82 – 7.66 (m, 4H), 7.50 – 7.24 (m, 21H), 5.39 – 5.22 (m, 2H), 5.14 – 5.02 (m, 3H), 5.00 – 4.89 (m, 2H), 4.82 – 4.59 (m, 4H), 4.23 – 4.09 (m, 2H), 4.04 – 3.81 (m, 6H), 3.71 (s, 3H), 3.57 (dd, J = 10.3, 5.4 Hz, 1H), 3.27 (dq, J = 19.7, 6.7 Hz, 2H), 2.17 (s, 3H), 2.03 (s, 3H), 1.89 – 1.73 (m, 2H), 1.12 (s, 9H). ¹³C NMR (101 MHz, MeOD) δ 172.6, 170.6, 170.0, 157.3, 138.6, 137.6, 136.9, 135.6, 135.4, 133.3, 133.0, 129.6, 128.2, 128.1, 128.0, 128.0, 127.9, 127.7, 127.6, 127.5, 127.5, 127.5, 127.4, 127.2, 98.6, 96.4, 78.4, 76.1, 74.5, 73.0, 72.4, 72.2, 71.8, 68.3, 68.1, 66.5, 66.0, 62.7, 54.0, 53.8, 51.9, 48.5, 48.3, 48.1, 47.9, 47.7, 47.4, 47.2, 44.3, 38.0, 29.4, 29.3, 26.2, 22.0, 20.0, 18.8. HR-ESI-MS (*m/z*): [M]⁻ calcd for C₅₈H₆₉N₂O₁₈SSi⁻, 1141.4041; found, 1141.4019.

N-benzyloxycarbonyl-3-aminopropyl-O-[(methyl(2-acetamido-4-O-benzyl-3-O-sulfonato-2-deoxy- α -D-glucopyranosyl))-(1 $\rightarrow$ 4)-O-2-O-acetyl-3-O-benzyl-1- α - L-idopyranosyluronate (12**)**

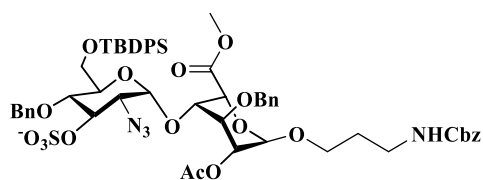


A solution of compound **11** (1g, 1 eq) in dry pyridine (5 mL) 70% HF.Py complex (5 eq) was added at 0 °C and kept it for stirring for 12 h. After that in reaction mixture was evaporated followed by purification by silica gel column chromatography (MeOH/DCM = 1/10, v/v) to obtain **12** in 85 % yield. ¹H NMR (400 MHz, Methanol-d₄) δ 7.55 – 7.44 (m, 2H), 7.41 – 7.25 (m, 13H), 5.28 – 5.15 (m, 2H), 5.07 (d, J = 2.0 Hz, 2H), 5.00 (d, J = 2.7 Hz, 1H), 4.91 (d, J = 3.3 Hz, 1H), 4.77 – 4.66 (m, 3H), 4.59 (d, J = 10.5 Hz, 1H), 4.15 (t, J = 3.6 Hz, 1H), 4.04 – 3.90 (m, 2H), 3.84 (s, 4H), 3.81 (d, J = 2.1 Hz, 2H), 3.79 – 3.71 (m, 1H), 3.66 (t, J = 9.2 Hz, 1H), 3.60 – 3.50 (m, 1H), 3.23 (m, 2H), 2.13 (s, 3H), 1.94 (s, 3H), 1.87 – 1.72 (m, 2H). ¹³C NMR (101 MHz, MeOD) δ 172.5, 170.7, 170.2, 157.4, 138.7, 137.8, 137.0, 128.4, 128.1, 127.8, 127.6, 127.5, 127.5, 127.5, 127.2, 98.7, 97.1, 77.8, 75.9, 74.5, 73.3, 72.9, 72.9, 72.2, 68.6, 68.3, 66.4, 66.0, 60.4, 53.9, 51.9, 48.6, 48.4, 48.2, 48.0, 47.8, 47.6, 47.3, 47.1, 37.9, 29.4, 29.3, 21.9, 21.8, 20.0. HR-ESI-MS (*m/z*): [M]⁻ calcd for C₄₂H₅₁N₂O₁₈S⁻, 903.2863; found, 903.2857.

2.4.4 Orthogonal Deprotection & *O*-Sulfation for NS Disaccharide



N-benzyloxycarbonyl-3-aminopropyl-*O*-[(methyl(2-azido-4-*O*-benzyl-6-*O*-*tert*-butyl diphenylsilyl-3-*O*-sulfonato-2-deoxy- α -D-glucopyranosyl))-(1 $\rightarrow$ 4)-*O*-2-*O*-acetyl-3-*O*-benzyl-1- α -L-idopyranosyluronate (17)



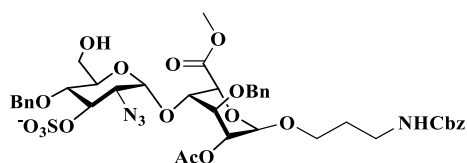
To a solution of starting material **7** (1 mmol, 1 eq) in CH₂Cl₂ (6 mL) and H₂O (340 μ L) was added DDQ (5 mmol, 1 eq) in 4 portions over the interval of 20 min.

After 1 h, the reaction mixture was quenched using

NaHCO₃, extracted with CH₂Cl₂ and washed with brine. The collected organic layer was dried over Na₂SO₄, filtered, concentrated and purified through silica gel column chromatography. After that, compound was dissolved to 1 mmol in DMF (2 mL) was added SO₃.NEt₃ complex (10 mmol per -OH) under N₂ atmosphere and stirred at 70 °C for 3 days. Upon completion of reaction, the reaction mixture was concentrated under reduced pressure and it was concentrated and purified using silica column chromatography (MeOH/DCM 1/10 v/v) to obtain product **21** 77 % in yield. ¹H NMR (400 MHz, Methanol-d₄) δ 7.79 – 7.73 (m, 4H), 7.47 – 7.28 (m, 4H), 5.35 (d, J = 10.7 Hz, 1H), 5.21 – 5.05 (m, 4H), 5.03 – 4.91 (m, 3H), 4.82 – 4.63 (m, 4H), 4.29 – 4.15 (m, 2H), 4.01 – 3.77 (m, 5H), 3.73 (s, 3H), 3.60 (m, 1H), 3.39 – 3.17 (m, 4H), 2.24 (s, 3H), 1.84 (q, J = 5.9 Hz, 2H). ¹³C NMR (101 MHz, MeOD) δ

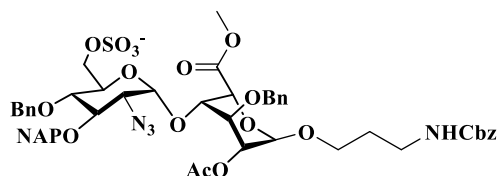
170.8, 170.3, 157.3, 138.5, 137.6, 136.9, 135.6, 135.4, 133.3, 133.1, 129.7, 128.3, 128.2, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 127.5, 127.3, 98.5, 96.9, 78.7, 76.4, 74.6, 72.6, 72.3, 71.2, 68.1, 68.0, 66.6, 66.1, 62.8, 62.4, 51.9, 48.5, 48.3, 48.1, 47.9, 47.7, 47.5, 47.3, 46.6, 38.1, 29.4, 29.3, 26.3, 19.9, 18.9. HR-ESI-MS (m/z): $[M]^-$ calcd for $C_{56}H_{65}N_4O_{17}SiS^-$, 1125.3840; found, 1125.3839.

N-benzyloxycarbonyl-3-aminopropyl-O-[(methyl(2-azido-4-O-benzyl-3-O-sulfonato-2-deoxy- α -D-glucopyranosyl))-(1 $\rightarrow$ 4)-O-2-O-acetyl-3-O-benzyl-1- α -L-idopyranosyluronate (18)



A solution of compound **7** (1g, 1 eq) in dry pyridine (5 mL) 70% HF.Py complex (5 eq) was added at 0 °C and kept it for stirring for 12 h. After that in reaction mixture was evaporated followed by purification by silica gel column chromatography (MeOH/DCM = 1/10, v/v) to obtain **18** in 85 % yield. 1H NMR (400 MHz, Methanol- d_4) δ 7.51 – 7.45 (m, 2H), 7.40 – 7.25 (m, 13H), 5.24 (d, J_z = 10.4 Hz, 1H), 5.09 – 5.04 (m, 4H), 4.90 (d, J = 3.3 Hz, 1H), 4.84 (d, J = 1.8 Hz, 1H), 4.76 – 4.68 (m, 2H), 4.58 (d, J = 10.5 Hz, 2H), 4.20 (t, J = 3.6 Hz, 1H), 4.06 (t, J = 4.0 Hz, 1H), 3.81 (s, 3H), 3.81 – 3.76 (m, 3H), 3.74 – 3.50 (m, 3H), 3.30 – 3.16 (m, 3H), 2.17 (s, 3H), 1.84 – 1.73 (m, 2H). ^{13}C NMR (101 MHz, MeOD) δ 171.0, 170.4, 157.4, 138.6, 137.7, 136.9, 128.4, 128.3, 128.2, 128.2, 128.1, 127.9, 127.8, 127.7, 127.7, 127.6, 127.5, 127.5, 127.3, 98.7, 97.8, 78.6, 76.2, 74.5, 72.9, 72.4, 72.4, 68.4, 68.1, 66.4, 66.1, 62.3, 60.3, 51.9, 48.6, 48.4, 48.2, 48.0, 47.7, 47.5, 47.3, 47.1, 38.0, 29.3, 19.9. HR-ESI-MS (m/z): $[M]^-$ calcd for $C_{40}H_{47}N_4O_{17}S^-$, 887.2668; found, 887.2657.

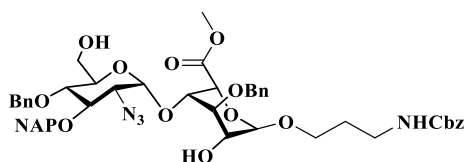
N-benzyloxycarbonyl-3-aminopropyl-O-[(methyl(2-azido-4-O-benzyl-6-O-sulfonato-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl))-(1 $\rightarrow$ 4)-O-2-O-acetyl-3-O-benzyl-1- α -L-idopyranosyluronate (19)



A solution of compound **7** (1mmol, 1 eq) in dry pyridine (5 mL) 70% HF.Py complex (5 eq) was added at 0 °C and kept it for stirring for 12 h. After that reaction mixture was evaporated followed by purification by silica gel column chromatography (ethyl acetate/ hexane= 1/10, v/v). After that, compound was dissolved to (1 mmol, 1 eq) in DMF (2 mL) was added $SO_3.NEt_3$ complex (10 mmol per -OH) under N_2 atmosphere and stirred at 70 °C for 3 days. Upon completion of reaction, the reaction mixture was concentrated under reduced pressure and it was concentrated

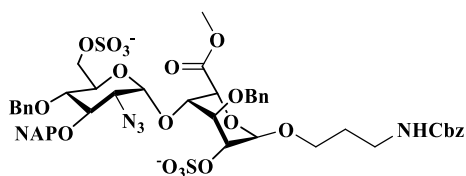
and purified using silica column chromatography (MeOH/DCM 1/10 v/v) to obtain product **18** in 81% yield. ¹H NMR (400 MHz, Methanol-d₄) δ 7.88 – 7.72 (m, 4H), 7.51 – 7.44 (m, 3H), 7.41 – 7.32 (m, 10H), 7.35 – 7.26 (m, 5H), 5.08 (s, 2H), 5.03 (d, J = 11.3 Hz, 1H), 5.01 – 4.93 (m, 3H), 4.95 – 4.86 (m, 2H), 4.82 (d, J = 10.8 Hz, 1H), 4.79 – 4.65 (m, 2H), 4.41 – 4.29 (m, 2H), 4.16 (t, J = 3.1 Hz, 1H), 3.98 (d, J = 3.2 Hz, 1H), 3.97 – 3.87 (m, 2H), 3.83 (m, 1H), 3.80 (s, 3H), 3.79 – 3.72 (m, 1H), 3.58 – 3.52 (m, 1H), 3.32 – 3.18 (m, 2H), 2.05 (s, 3H), 1.80 (m, 2H). ¹³C NMR (101 MHz, MeOD) δ 170.3, 170.1, 157.4, 138.3, 137.6, 135.5, 133.3, 133.1, 128.1, 128.1, 127.9, 127.9, 127.8, 127.7, 127.6, 127.6, 127.5, 127.5, 127.4, 127.3, 127.3, 126.2, 125.8, 125.6, 125.6, 98.6, 97.5, 79.4, 77.7, 74.5, 74.5, 72.8, 72.0, 70.5, 68.0, 67.6, 66.3, 66.0, 65.5, 63.2, 51.7, 48.5, 48.3, 48.1, 47.9, 47.7, 47.4, 47.2, 47.0, 38.0, 29.2, 19.6. HR-ESI-MS (*m/z*): [M][–] calcd for C₅₁H₅₅N₄O₁₇S[–], 1027.3288; found, 1027.3275.

N-benzyloxycarbonyl-3-aminopropyl-O-[(2-azido-4-O-benzyl-3-O-(2-naphthylmethyl)-2-deoxy-α-D-glucopyranosyl)]-(1→4)-O-3-O-benzyl-1-α- L-idopyranosyluronate (20a)



A solution of compound **6** (1g, 1 eq) in dry pyridine (5 mL) 70% HF.Py complex (5 eq) was added at 0 °C and kept it for stirring for 12 h. After that reaction mixture was evaporated followed by purification by silica gel column chromatography (ethyl acetate/ hexane= 1/10, v/v) to obtain **20a** in, 87 %. ¹H NMR (400 MHz, Chloroform-d) δ 7.84 – 7.70 (m, 4H), 7.48 – 7.41 (m, 3H), 7.39 – 7.26 (m, 11H), 7.25 (m, 3H), 5.57 (s, 1H), 5.05 (s, 1H), 5.01 (s, 1H), 4.97 (s, 2H), 4.87 – 4.82 (m, 2H), 4.69 – 4.59 (m, 2H), 4.50 (d, J = 12.3 Hz, 1H), 4.07 (s, 1H), 3.92 – 3.82 (m, 2H), 3.80 (s, 3H), 3.76 (d, J = 2.2 Hz, 1H), 3.74 – 3.64 (m, 3H), 3.64 – 3.57 (m, 2H), 3.54 – 3.52 (m, 2H), 3.49 – 3.43 (m, 2H), 3.30 – 3.20 (m, 1H), 1.90 – 1.77 (m, 2H). ¹³C NMR (101 MHz, CHLOROFORM-D) δ 170.1, 156.6, 137.9, 137.3, 136.6, 135.0, 133.4, 133.1, 128.8, 128.6, 128.5, 128.4, 128.3, 128.2, 128.1, 128.1, 128.0, 128.0, 127.8, 127.7, 127.1, 126.2, 126.1, 126.1, 101.7, 95.0, 81.1, 77.4, 77.3, 77.1, 76.8, 76.0, 74.8, 72.3, 71.4, 71.1, 68.1, 66.9, 66.8, 63.8, 61.3, 52.8, 40.0, 29.5. HR-ESI-MS (*m/z*): [M+Na]⁺ calcd for C₄₂H₄₈N₄NaO₁₃, 839.3116; found, 839.3110.

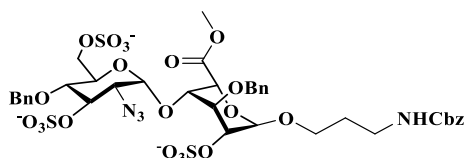
N-benzyloxycarbonyl-3-aminopropyl-O-[(methyl-(2-azido-4-O-benzyl-6-O-sulfonato-3-O-(2-naphthylmethyl)-2-deoxy-α-D-glucopyranosyl))]-(1→4)-O-2-O-sulfonato-3-O-benzyl-1-α- L-idopyranosyluronate (20)



The compound **20a** was dissolved to (1 mmol, 1eq) in DMF (2 mL) was added SO₃.NEt₃ complex (10 mmol per -OH) under N₂ atmosphere and stirred at 70 °C for 4 days. Upon completion of reaction, the reaction

mixture was concentrated under reduced pressure and it was concentrated and purified using silica column chromatography (MeOH/DCM 1.3/10 v/v) to obtain product **20** in 76% yield. ¹H NMR (400 MHz, Methanol-d₄) δ 7.91 – 7.73 (m, 4H), 7.54 – 7.19 (m, 18H), 5.22 (s, 1H), 5.15 – 4.98 (m, 5H), 4.80 (d, J = 10.9 Hz, 2H), 4.67 (d, J = 11.8 Hz, 2H), 4.52 (s, 1H), 4.37 – 4.25 (m, 3H), 4.14 (t, J = 2.8 Hz, 1H), 4.03 – 3.98 (m, 1H), 3.87 – 3.80 (m, 2H), 3.78 (s, 3H), 3.73 – 3.56 (m, 3H), 3.40 (dd, J = 10.3, 3.4 Hz, 1H), 3.33 – 3.17 (m, 2H), 1.89 – 1.77 (m, 2H). ¹³C NMR (101 MHz, MeOD) δ 170.8, 157.5, 138.3, 137.7, 136.9, 135.8, 133.3, 133.1, 128.1, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 127.6, 127.6, 127.5, 127.3, 127.2, 126.3, 125.8, 125.7, 125.6, 99.4, 96.4, 79.8, 77.6, 74.9, 74.5, 71.8, 71.3, 71.1, 71.0, 70.5, 67.1, 66.4, 66.1, 65.7, 63.5, 53.9, 51.9, 48.4, 48.2, 47.9, 47.7, 47.5, 47.3, 47.1, 38.1, 29.3, 29.2. HR-ESI-MS (*m/z*): [M]²⁻ calcd for C₄₂H₄₆N₄O₁₉S₂²⁻, 487.1104; found, 487.1098.

N-benzyloxycarbonyl-3-aminopropyl-O-[(methyl-(2-azido-4-O-benzyl-6-O-sulfonato-3-O-sulfonato-2-deoxy- α -D-glucopyranosyl))-(1 $\rightarrow$ 4)-O-2-O-sulfonato-3-O-benzyl-1- α -L-idopyranosyluronate (21**)**

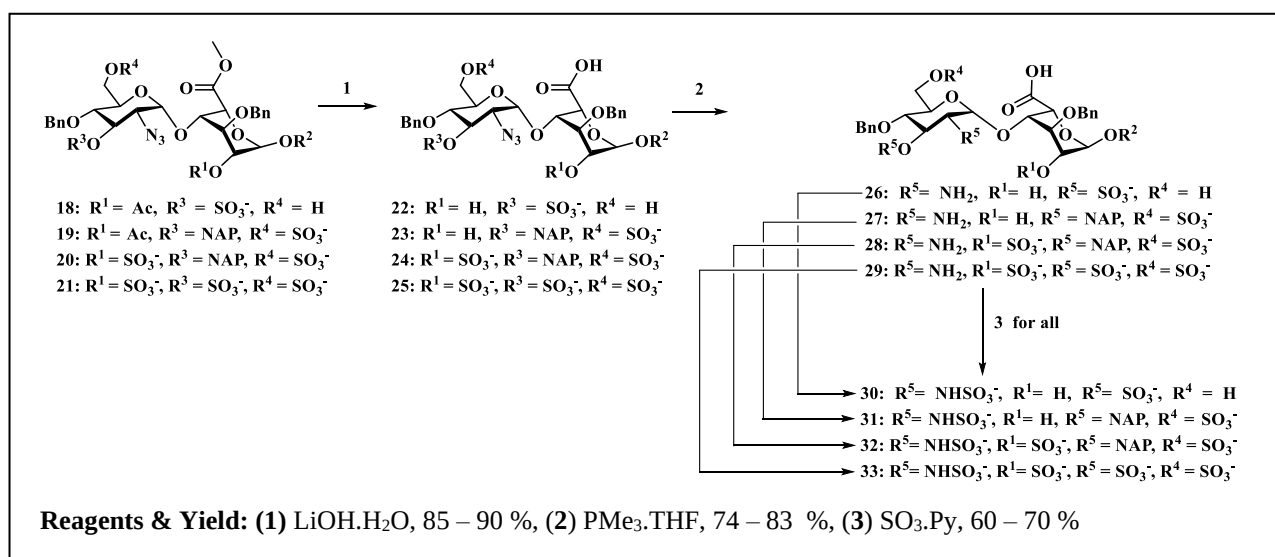


To a solution of starting material **6** (1 mmol, 1 eq) in CH₂Cl₂ (6 mL) and H₂O (340 μ L) was added DDQ (5 mmol) portion wise over the interval of 20 min. After 1

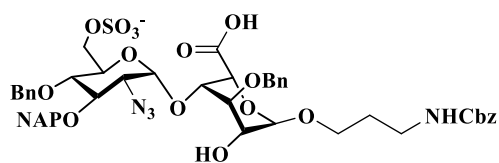
h, the reaction mixture was quenched using NaHCO₃, extracted with CH₂Cl₂ and washed with brine. The collected organic layer was dried over Na₂SO₄, filtered, concentrated and purified through silica gel column chromatography. After that, solution of product in dry pyridine (5 mL) 70% HF.Py complex (5 eq) was added at 0 °C and kept it for stirring for 12 h. After that in reaction mixture was evaporated followed by purification by silica gel column chromatography (ethyl acetate/hexane = 1/2, v/v). Then, compound was dissolved to 1 mmol in DMF (2 mL) was added SO₃.NEt₃ complex (10 mmol per -OH) under N₂ atmosphere and stirred at 70 °C for 4 days. Upon completion of reaction, the reaction mixture was concentrated under reduced pressure and it was concentrated and purified using silica column chromatography (MeOH/DCM 1.5/10 v/v) to obtain product **31** in 62% yield. ¹H NMR (400 MHz, Methanol-d₄) δ 7.58 – 7.20 (m, 15H), 5.17 (s, 1H), 5.06 (dd, J = 6.5, 2.8 Hz, 2H), 4.92 (d, J = 10.6 Hz, 2H), 4.78 (d, J = 10.8 Hz, 2H), 4.72 – 4.60 (m, 2H), 4.46 (s, 1H), 4.27 – 4.24

(m, 2H), 4.17 – 4.08 (m, 1H), 4.03 – 3.86 (m, 1H), 3.89 – 3.72 (m, 2H), 3.75 (s, 3H), 3.32 – 3.11 (m, 3H), 1.85 – 1.80 (m, 2H). ^{13}C NMR (101 MHz, MeOD) δ 170.4, 157.4, 138.6, 138.4, 137.8, 136.9, 128.8, 128.2, 128.1, 128.1, 128.0, 128.0, 127.9, 127.8, 127.8, 127.8, 127.7, 127.5, 127.5, 127.3, 99.4, 96.3, 77.7, 74.6, 71.8, 71.5, 70.2, 67.2, 66.0, 63.5, 53.9, 51.7, 48.6, 48.4, 48.1, 47.9, 47.7, 47.5, 47.3, 47.1, 44.4, 44.3, 29.2. HR-ESI-MS (m/z): $[\text{M}]^{2-}$ calcd for $\text{C}_{30}\text{H}_{37}\text{N}_4\text{O}_{20}\text{S}_3^{3-}$, 289.7060; found, 289.7047.

2.4.5 N-Sulfation of HS Disaccharide



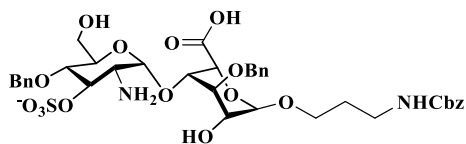
N-benzyloxycarbonyl-3-aminopropyl-O-[methyl((2-azido-4-O-benzyl-6-O-sulfonato-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl))-(1 $\rightarrow$ 4)-O-2-O-acetyl-3-O-benzyl-1- α -L-idopyranosyluronate (23)



A solution of compound **19** (0.6 g, 1 eq) in $\text{THF}:\text{H}_2\text{O}$ (1:1, 5 mL) $\text{LiOH} \cdot \text{H}_2\text{O}$ (20 eq) was added and kept it on stirring for room temperature. After 12 h reaction was quenched using IR H^+ resin and reaction mixture was evaporated and purified using reverse phase C-18 column chromatography using ($\text{MeOH}/\text{H}_2\text{O}$ 1/9 v/v). ^1H NMR (400 MHz, Methanol- d_4) δ 7.92 – 7.61 (m, 4H), 7.58 – 7.07 (m, 18H), 5.07 – 4.91 (m, 5H), 4.77 – 4.59 (m, 4H), 4.45 – 4.36 (m, 1H), 4.32 – 4.15 (m, 2H), 4.02 – 3.88 (m, 2H), 3.88 – 3.61 (m, 5H), 3.56 – 3.51 (m, 1H), 3.27 – 3.17 (m, 2H), 1.76 (m, 1H). ^{13}C NMR (101 MHz, MeOD) δ 157.4, 138.3, 137.9, 136.9, 133.3, 133.1, 128.1, 128.1, 127.9, 127.8, 127.7, 127.6, 127.6, 127.5, 127.3, 127.2, 126.1, 125.7, 125.6, 125.5, 101.3, 95.3, 80.8, 77.5, 75.1, 74.5, 71.6, 70.4, 66.1, 65.5,

63.9, 48.3, 48.1, 47.9, 47.7, 47.5, 47.2, 47.0, 38.4, 29.3. HR-ESI-MS (m/z): $[M]^-$ calcd for $C_{48}H_{51}N_4O_{16}S^-$, 971.3026; found, 971.3005.

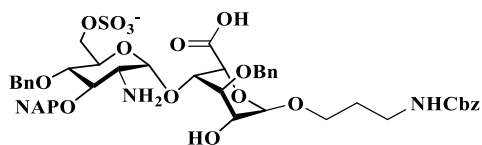
N-benzyloxycarbonyl-3-aminopropyl-O-[(2-azido-4-O-benzyl-3-O-sulfonato-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-3-O-benzyl-1- α -L-idopyranosyluronate (26)



A solution of compound **18** (0.6g, 1 eq) in THF:H₂O (1:1, 5 mL) LiOH.H₂O (20 eq) was added and kept it on stirring for room temperature. After 12 h reaction was

quenched using IR H⁺ resin and reaction fixture was evaporated and purified using reverse phase C-18 column chromatography using (MeOH/H₂O 1/9 v/v) to obtain **22**. A solution of **22** in THF (3 mL) 1M PMe₃.THF (8 eq) and 0.1M aqueous NaOH (10eq) was added and kept it on stirring for room temperature. After 12 h reaction was quenched using glacial acetic acid and reaction mixture solvent was evaporated and purified using reverse phase C-18 column chromatography using (MeOH/H₂O 2/3 v/v), followed by passing through DOWEX 50WX8 Na⁺ resin column to obtain **26** in 78 % yield. ¹H NMR (400 MHz, Methanol-d₄) δ 7.45 – 7.12 (m, 15H), 5.00 – 4.90 (m, 3H), 4.82 (s, 1H), 4.66 – 4.48 (m, 4H), 4.21 (s, 1H), 3.92 – 3.58 (m, 7H), 3.45 - 3.42 (3, 1H), 3.32 – 3.06 (m, 3H), 2.79 – 2.75 (m, 1H), 1.75 – 1.63 (m, 2H). ¹³C NMR (101 MHz, MeOD) δ 175.1, 157.3, 138.6, 138.2, 136.9, 128.1, 128.1, 128.0, 128.0, 127.9, 127.9, 127.7, 127.6, 127.6, 127.5, 127.3, 100.9, 95.5, 78.4, 74.4, 73.0, 72.5, 71.7, 71.1, 70.9, 67.9, 66.3, 66.1, 61.0, 55.8, 48.4, 48.2, 48.0, 47.8, 47.5, 47.3, 47.1, 38.7, 29.4. HR-ESI-MS (m/z): $[M]^-$ calcd for $C_{37}H_{45}N_4O_{16}S^-$, 805.2495; found, 805.2486.

N-benzyloxycarbonyl-3-aminopropyl-O-[(2-amino-4-O-benzyl-6-O-sulfonato-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-3-O-benzyl-1- α -L-idopyranosyluronate (27)

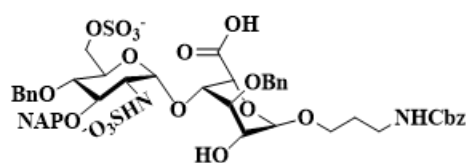


A solution of compound **23** (0.6g, 1 eq) in THF (3 mL) 1M PMe₃.THF (8 eq) and 0.1M aqueous NaOH (10eq) was added and kept it on stirring for room temperature.

After 12 h reaction was quenched using glacial acetic acid and reaction mixture solvent was evaporated and purified using reverse phase C-18 column chromatography using (MeOH/H₂O 2/3 v/v), followed by passing through DOWEX 50WX8 Na⁺ resin column to obtain compound **27** in 76 % yield. ¹H NMR (400 MHz, Methanol-d₄) δ 7.78 – 7.72 (m, 4H), 7.46 – 7.40 (m, 3H), 7.37 – 7.23 (m, 9H), 7.23 – 7.10 (m, 6H), 5.31 (d, J = 3.9 Hz, 1H), 5.12 (d, J = 11.5 Hz, 1H), 5.04 – 4.95 (m, 4H), 4.82 (s, 2H), 4.67 – 4.46 (m, 4H), 4.52 – 4.44 (m, 1H), 4.38 – 4.28

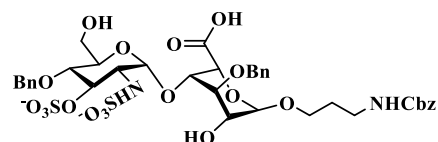
(m, 2H), 4.17 – 4.05 (m, 2H), 3.90 (s, 1H), 3.80 – 3.74 (m, 3H), 3.49 – 3.47 (m, 1H), 3.37 (dd, $J = 10.4, 3.7$ Hz, 0H), 3.28 – 3.15 (m, 2H), 1.76 – 1.72 (m, 2 H). ^{13}C NMR (101 MHz, MeOD) δ 157.4, 138.1, 136.9, 135.8, 133.4, 133.0, 128.1, 128.0, 127.9, 127.7, 127.6, 127.6, 127.5, 127.3, 127.2, 125.7, 125.5, 125.2, 101.1, 92.1, 77.8, 74.8, 74.4, 71.9, 71.3, 70.4, 66.3, 66.0, 65.4, 54.1, 48.3, 48.1, 47.8, 47.6, 47.4, 47.2, 47.0, 38.5, 29.3. HR-ESI-MS (m/z): $[\text{M}]^-$ calcd for $\text{C}_{48}\text{H}_{53}\text{N}_2\text{O}_{16}\text{S}^-$, 945.3141; found, 945.3128.

N-benzyloxycarbonyl-3-aminopropyl-O-[(2-sulfonatamido-4-O-benzyl-6-O-sulfonato-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-3-O-benzyl-1- α -L-idopyranosyluronate (31)



A solution of compound **27** (0.2g, 1 eq) in MeOH (3 mL) 0.1M NaOH (4 eq), triethylamine (8 eq), 10 eq of $\text{SO}_3\cdot\text{Py}$ was added at an interval of 1 h for 6 times by maintain 9-10 pH of reaction mixture and kept it on stirring for room temperature. The progress of reaction was monitored using TLC plate (ethyl acetate/pyridine/water/acetic acid 8/5/3/1 v/v/v/v). After completion, reaction mixture solvent was evaporated and purified using reverse phase C-18 column chromatography using (MeOH/ H_2O 2/3 v/v), followed by passing through DOWEX 50WX8 Na^+ resin column to obtain compound **31** in 64 % yield. ^1H NMR (400 MHz, Methanol- d_4) δ 7.86 – 7.74 (m, 4H), 7.60 – 7.58 (m, 1H), 7.43 – 7.36 (m, 4H), 7.32 – 7.24 (m, 9H), 7.22 – 7.19 (m, 3H), 5.38 (d, $J = 3.9$ Hz, 1H), 5.27 (d, $J = 11.3$ Hz, 1H), 5.03 (s, 2H), 4.93 (d, $J = 10.1$ Hz, 2H), 4.83 – 4.73 (m, 3H), 4.65 (q, $J = 11.6$ Hz, 2H), 4.43 – 4.23 (m, 1H), 4.25 (d, $J = 10.8$, 1H), 4.17 (d, $J = 6.7$ Hz, 2H), 3.94 – 3.86 (m, 2H), 3.84 – 3.73 (m, 2H), 3.73 – 3.65 (m, 1H), 3.63 – 3.48 (m, 2H), 3.29 – 3.15 (m, 2H), 1.82 – 1.74 (m, 2H). ^{13}C NMR (101 MHz, MeOD) δ 172.9, 157.4, 138.5, 138.2, 136.9, 136.7, 133.4, 133.0, 128.1, 128.0, 128.0, 127.8, 127.7, 127.7, 127.6, 127.5, 127.4, 127.3, 127.2, 127.1, 126.2, 126.2, 125.5, 125.3, 101.2, 96.5, 80.6, 77.3, 75.0, 74.6, 73.1, 71.8, 69.9, 66.7, 66.1, 65.9, 58.2, 48.3, 48.1, 47.9, 47.7, 47.5, 47.3, 47.0, 38.3, 29.3. HR-ESI-MS (m/z): $[\text{M}]^-$ calcd for $\text{C}_{48}\text{H}_{52}\text{N}_2\text{O}_{19}\text{S}^{2-}$, 512.1308; found, 512.1297.

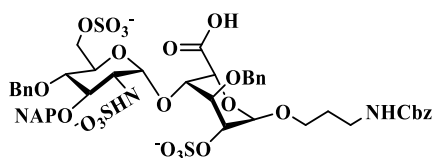
N-benzyloxycarbonyl-3-aminopropyl-O-[(2-sulfonatamido-4-O-benzyl-3-O-sulfonato-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-3-O-benzyl-1- α - L-idopyranosyluronate (30)



A solution of compound **26** (0.2g, 1 eq) in MeOH (3 mL) 0.1M NaOH (4 eq), triethylamine (8 eq), 10 eq of $\text{SO}_3\cdot\text{Py}$ was added at an interval of 1 h for 6 times by maintain 9-

10 pH of reaction mixture and kept it on stirring for room temperature. The progress of reaction was monitored using TLC plate (ethyl acetate/pyridine/water/acetic acid 8/5/3/1 v/v/v/v). After completion, reaction mixture solvent was evaporated and purified using reverse phase C-18 column chromatography using (MeOH/H₂O 2/3 v/v), followed by passing through DOWEX 50WX8 Na⁺ resin column to obtain compound **30** in 62 % yield. ¹H NMR (400 MHz, Methanol-d₄) δ 7.50 – 7.21 (m, 15H), 5.28 (d, J = 3.8 Hz, 1H), 5.04 (s, 2H), 4.96 (d, J = 11.0 Hz, 2H), 4.68 – 4.62 (m, 4H), 4.24 – 4.04 (m, 2H), 3.89 – 3.72 (m, 5H), 3.74 – 5.71 (m, 1H), 3.58 – 3.46 (m, 2H), 3.33 – 3.11 (m, 3H), 1.80 (p, J = 6.1 Hz, H). ¹³C NMR (151 MHz, MeOD) δ 174.0, 157.4, 138.6, 138.2, 136.9, 128.4, 128.1, 127.9, 127.9, 127.9, 127.6, 127.6, 127.5, 127.5, 127.3, 127.2, 101.0, 95.9, 78.3, 74.3, 73.1, 72.2, 71.7, 71.6, 71.2, 67.2, 66.1, 60.7, 58.4, 53.9, 48.1, 48.0, 47.8, 47.7, 47.5, 47.4, 47.3, 38.4, 29.3. HR-ESI-MS (*m/z*): [M]²⁻ calcd for C₃₇H₄₄N₂O₁₉S²⁻, 442.0885; found, 442.0876..

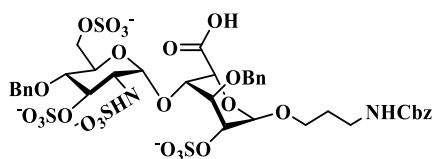
N-benzyloxycarbonyl-3-aminopropyl-O-(((2-sulfonatimido-4-O-benzyl-6-O-sulfonato-2-deoxy-α-D-glucopyranosyl)))-(1→4)-O-2-O-sulfonato-3-O-benzyl-1-α-L-idopyranosyl uronate (32)



A solution of compound **20** (0.2g, 1 eq) in THF:H₂O (1:1, 5 mL) LiOH.H₂O (20 eq) was added and kept it on stirring for room temperature. After 12 h reaction was quenched using IR H⁺ resin and reaction mixture was evaporated and purified using reverse phase C-18 column chromatography using (MeOH/H₂O 1/9 v/v). A solution of compound **46a** in THF (3 mL) 1M PMe₃.THF (8 eq) and 0.1M aqueous NaOH (10eq) was added and kept it on stirring for room temperature. After 12 h reaction was quenched using glacial acetic acid and reaction mixture solvent was evaporated and purified using reverse phase C-18 column chromatography using (MeOH/H₂O 2/3 v/v), followed by passing through DOWEX 50WX8 Na⁺ resin column. Then, a solution of compound **46b** in MeOH (3 mL) 0.1M NaOH (4 eq), triethylamine (8 eq), 10 eq of SO₃.Py was added at an interval of 1 h for 6 times by maintain 9-10 pH of reaction mixture and kept it on stirring for room temperature. The progress of reaction was monitored using TLC plate (ethyl acetate/pyridine/water/acetic acid 8/5/3/1 v/v/v/v). After completion, reaction mixture solvent was evaporated and purified using reverse phase C-18 column chromatography using (MeOH/H₂O 2/3 v/v), followed by passing through DOWEX 50WX8 Na⁺ resin column to obtain **32** in 58 % yield. ¹H NMR (400 MHz, Methanol-d₄) δ 7.94 – 7.74 (m, 4H), 7.64 (d, J = 8.4 Hz, 1H), 7.53 – 7.14 (m, 17H), 5.36 (d, J = 11.6 Hz, 2H), 5.03 (s, 3H), 4.61 – 4.54 (m, 5H), 4.45 – 4.34 (m, 3H), 4.47 – 4.07 (m, 3H), 3.91 – 3.72 (m, 2H), 3.70

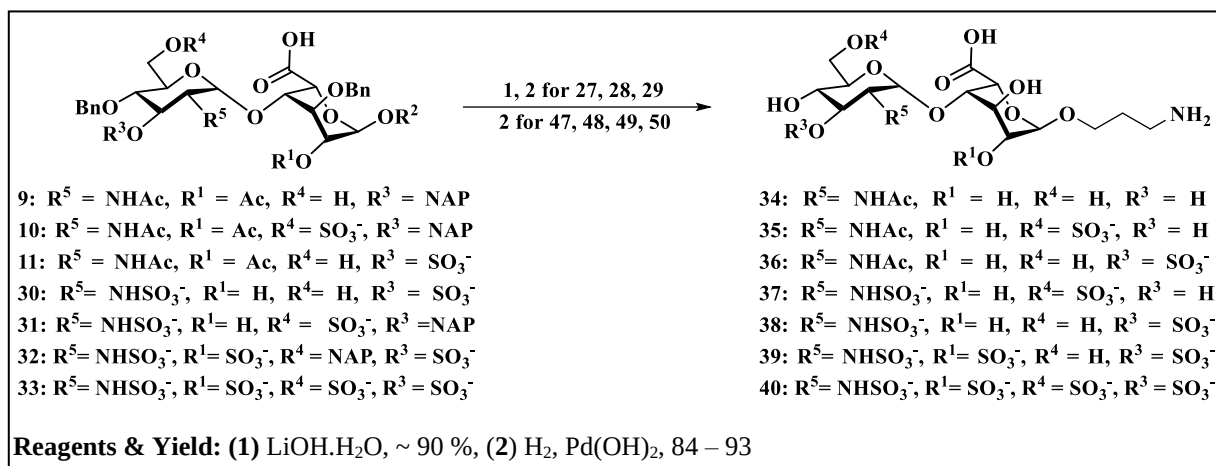
3.54(m, 4H), 3.24 – 3.15 (m, 2H), 1.80 – 1.74 (m, 2H). ^{13}C NMR (101 MHz, MeOD) δ 174.6, 157.4, 138.3, 138.2, 136.9, 133.4, 133.0, 128.3, 128.1, 128.0, 127.9, 127.8, 127.6, 127.6, 127.5, 127.4, 127.3, 127.3, 127.2, 126.3, 126.3, 125.5, 125.4, 98.8, 98.7, 80.5, 77.3, 75.0, 74.4, 73.8, 71.7, 70.8, 69.7, 67.2, 66.0, 65.8, 53.9, 48.4, 48.2, 48.0, 47.7, 47.5, 47.3, 47.1, 38.0, 29.3. HR-ESI-MS (m/z): $[\text{M}]^{3-}$ calcd for $\text{C}_{48}\text{H}_{51}\text{N}_2\text{O}_{22}\text{S}_3^{3-}$, 367.7371; found, 367.7359.

N-benzyloxycarbonyl-3-aminopropyl-O-(((2-sulfonatido-4-O-benzyl-6-O-sulfonato-3-O-sulfonato-2-deoxy- α -D-glucopyranosyl)))-(1 $\rightarrow$ 4)-O-2-O-sulfonato-3-O-benzyl-1- α -L-idopyranosyluronate (33)

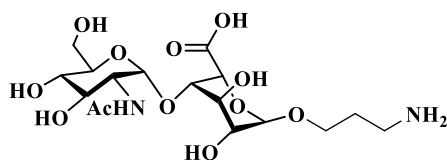


A solution of compound **21** (0.2g, 1eq) in THF:H₂O (1:1, 5 mL) LiOH.H₂O (20 eq) was added and kept it on stirring for room temperature. After 12 h reaction was quenched using IR H⁺ resin and reaction mixture was evaporated and purified using reverse phase C-18 column chromatography using (MeOH/H₂O 1/9 v/v). A solution of compound **25** in THF (3 mL) 1M PMe₃.THF (8 eq) and 0.1M aqueous NaOH (10eq) was added and kept it on stirring for room temperature. After 12 h reaction was quenched using glacial acetic acid and reaction mixture solvent was evaporated and purified using reverse phase C-18 column chromatography using (MeOH/H₂O 2/3 v/v), followed by passing through DOWEX 50WX8 Na⁺ resin column. Then, a solution of compound **29** in MeOH (3 mL) 0.1M NaOH (4 eq), triethylamine (8 eq), 10 eq of SO₃.Py was added at an interval of 1 h for 6 times by maintain 9-10 pH of reaction mixture and kept it on stirring for room temperature. The progress of reaction was monitored using TLC plate (ethyl acetate/pyridine/water/acetic acid 8/5/3/1 v/v/v/v). After completion, reaction mixture solvent was evaporated and purified using reverse phase C-18 column chromatography using (MeOH/H₂O 2/3 v/v), followed by passing through DOWEX 50WX8 Na⁺ resin column to obtain compound **33** in 55 % yield. ^1H NMR (400 MHz, Methanol-d₄) δ 7.55 (d, J = 7.4 Hz, 2H), 7.46 (d, J = 7.6 Hz, 2H), 7.43 – 7.26 (m, 11H), 5.34 (d, J = 15.4 Hz, 2H), 5.10 (s, 2H), 4.77 (d, J = 6.3 Hz, 2H), 4.71 – 4.60 (m, 3H), 4.57 (s, 1H), 4.49 (d, J = 10.9 Hz, 1H), 4.44 – 4.18 (m, 4H), 4.10 (d, J = 10.1 Hz, 1H), 3.93 – 3.77 (m, 2H), 3.72 – 3.61 (m, 2H), 3.31 – 3.17 (m, 2H), 1.89 – 1.80 (m, 2H). ^{13}C NMR (101 MHz, MeOD) δ 157.5, 138.4, 138.1, 136.9, 129.6, 129.5, 128.5, 128.1, 128.1, 127.9, 127.7, 127.5, 127.5, 127.4, 127.2, 98.6, 97.7, 78.1, 74.8, 73.4, 72.0, 71.7, 70.9, 69.5, 66.1, 65.9, 58.5, 48.4, 48.2, 47.9, 47.7, 47.5, 47.3, 47.1, 46.3, 38.1, 29.2. HR-ESI-MS (m/z): $[\text{M}]^{2-}$ calcd for $\text{C}_{37}\text{H}_{42}\text{N}_2\text{O}_{25}\text{S}_4^{4-}$, 260.5245; found, 260.5237.

2.4.6 Global Deprotection

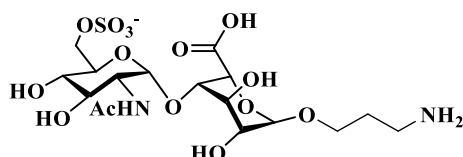


3-aminopropyl-O-[(2-acetamido- α -D-glucopyranosyl)-(1 $\rightarrow$ 4)-O- α -L-idopyranosiduronate (34)



A solution of compound **9** (60 mg, 1eq) in THF:H₂O (1:1, 5 mL) LiOH.H₂O (20 eq) was added and kept it on stirring for room temperature. After 12 h reaction was quenched using IR H⁺ resin and reaction mixture was evaporated and purified using reverse phase C-18 column chromatography using (MeOH/H₂O 1/9 v/v). The reaction mixture was dissolved in water and Pd(OH)₂ was added and stirred under H₂ atm. After 48 h, the mixture was filtered, concentrated and eluted through Bond Elute C-18 column using H₂O as eluent. The combined H₂O fraction were pooled and lyophilized to yield fully deprotected NAc-disaccharide **34**. ¹H NMR (400 MHz, Deuterium Oxide) δ 5.04 (d, J = 3.7 Hz, 1H), 4.79 (d, J = 2.6 Hz, 1H), 4.40 (d, J = 2.7 Hz, 1H), 3.95 (t, J = 3.1 Hz, 1H), 3.89 (t, J = 4.1 Hz, 1H), 3.81 (td, J = 9.9, 9.1, 2.9 Hz, 2H), 3.72 (s, 2H), 3.69 (dd, J = 2.9, 1.3 Hz, 1H), 3.66 – 3.60 (m, 2H), 3.58 – 3.55 (m, 1H), 3.39 (t, J = 9.3 Hz, 1H), 3.04 (td, J = 6.7, 1.9 Hz, 2H), 1.91 (s, 5H). ¹³C NMR (101 MHz, D₂O) δ 175.2, 174.4, 100.4, 94.4, 73.1, 71.9, 71.1, 69.6, 68.7, 68.4, 67.3, 66.4, 60.1, 53.5, 38.2, 26.4, 21.9. HR-ESI-MS (*m/z*): [M]⁻ calcd for C₁₇H₃₀N₂O₁₂, 454.1799; found, 454.1788.

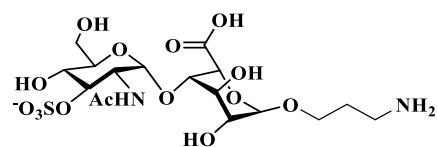
3-aminopropyl-O-[(2-acetamido-6-O-sulfonato- α -D-glucopyranosyl)-(1 $\rightarrow$ 4)-O- α -L-idopyranosiduronate (35)



A solution of compound **10** (72 mg, 1 eq) in THF:H₂O (1:1, 5 mL) LiOH.H₂O (20 eq) was added and kept it on stirring for room temperature. After 12 h reaction was

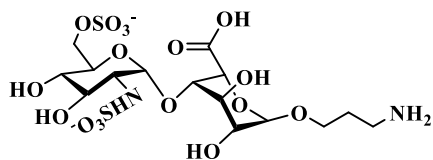
quenched using IR H⁺ resin and reaction fixture was evaporated and purified using reverse phase C-18 column chromatography using (MeOH/H₂O 1/9 v/v). The reaction mixture was dissolved in water and Pd(OH)₂ was added and stirred under H₂ atm. After 48 h, the mixture was filtered, concentrated and eluted through Bond Elute C-18 column using H₂O as eluent. The combined H₂O fraction were pooled and lyophilized to yield fully deprotected 6-O-sulfated NAc-disaccharide **35**. ¹H NMR (400 MHz, Deuterium Oxide) δ 5.06 (d, J = 3.7 Hz, 1H), 4.81 (d, J = 2.6 Hz, 1H), 4.44 (d, J = 2.6 Hz, 1H), 4.25 (dd, J = 11.1, 3.4 Hz, 1H), 4.12 (dd, J = 11.0, 2.1 Hz, 1H), 3.98 (t, J = 2.9 Hz, 1H), 3.92 (t, J = 4.0 Hz, 1H), 3.89 (d, J = 3.5 Hz, 1H), 3.86 (d, J = 3.6 Hz, 1H), 3.84 – 3.78 (m, 1H), 3.66 – 3.60 (m, 2H), 3.58 (dd, J = 4.7, 2.6 Hz, 1H), 3.48 (t, J = 9.6 Hz, 1H), 3.10 – 3.02 (m, 2H), 1.95 – 1.87 (m, 5H). ¹³C NMR (101 MHz, D₂O) δ 174.3, 100.4, 94.3, 72.9, 71.0, 70.1, 69.1, 68.5, 68.2, 67.0, 66.5, 53.3, 38.3, 26.3, 21.9, 18.1. HR-ESI-MS (*m/z*): [M][−] calcd for C₁₇H₂₉N₂O₁₅S[−], 533.1294; found, 533.1291.

3-aminopropyl-O-[(2-acetamido-3-O-sulfonato-α-D-glucopyranosyl)-(1→4)-O-α-L-idopyranosiduronate (36)



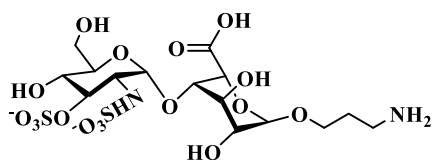
A solution of compound **12** (60 mg, 1 eq) in H₂O (2 mL) LiOH.H₂O (20 eq) was added and kept it on stirring for room temperature. After 12 h reaction was quenched using IR H⁺ resin and reaction fixture was evaporated and purified using reverse phase C-18 column chromatography using (MeOH/H₂O 1/9 v/v). The reaction mixture was dissolved in water and Pd(OH)₂ was added and stirred under H₂ atm. After 48 h, the mixture was filtered, concentrated and eluted through Bond Elute C-18 column using H₂O as eluent. The combined H₂O fraction were pooled and lyophilized to yield fully deprotected 3-O-sulfated NAc-disaccharide. ¹H NMR (400 MHz, Deuterium Oxide) δ 5.17 (d, J = 3.7 Hz, 1H), 4.91 (d, J = 2.7 Hz, 1H), 4.51 (d, J = 2.6 Hz, 1H), 4.44 (dd, J = 10.5, 9.0 Hz, 1H), 4.13 – 4.05 (m, 2H), 4.02 (t, J = 4.1 Hz, 1H), 3.95 – 3.89 (m, 1H), 3.89 – 3.82 (m, 3H), 3.73 (dt, J = 10.6, 5.6 Hz, 1H), 3.69 – 3.62 (m, 2H), 3.15 (td, J = 6.7, 1.4 Hz, 2H), 2.00 (s, 5H). ¹³C NMR (151 MHz, D₂O) δ 175.1, 174.3, 100.4, 94.9, 79.8, 73.7, 71.8, 68.7, 68.3, 68.2, 67.5, 66.4, 59.9, 52.1, 38.1, 26.3, 22.0. HR-ESI-MS (*m/z*): [M][−] calcd for C₁₇H₂₉N₂O₁₅S[−], 533.1294; found, 533.1262.

3-aminopropyl-O-[(2-sulfonatamido-6-O-sulfonato-α-D-glucopyranosyl)-(1→4)-O-α-L-idopyranosiduronate (37)



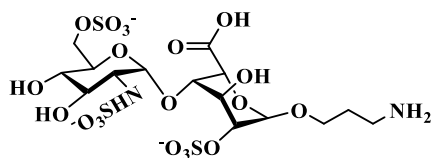
A solution of compound **30** in H₂O and Pd(OH)₂ was added and stirred under H₂ atm. After 48 h, the mixture was filtered, concentrated and eluted through Bond Elute C-18 column using H₂O as eluent. The combined H₂O fraction were pooled and lyophilized to yield fully deprotected NS-disaccharide. ¹H NMR (400 MHz, Deuterium Oxide) δ 5.34 (d, J = 3.7 Hz, 1H), 4.97 – 4.85 (m, 1H), 4.60 (d, J = 2.4 Hz, 1H), 4.34 (dd, J = 11.0, 3.1 Hz, 1H), 4.27 – 4.11 (m, 2H), 4.08 (t, J = 2.8 Hz, 1H), 3.97 – 3.83 (m, 2H), 3.78 – 3.68 (m, 2H), 3.66 – 3.54 (m, 2H), 3.25 (dd, J = 10.0, 3.6 Hz, 1H), 3.21 – 3.11 (m, 2H), 2.04 – 1.98 (m, 2H). ¹³C NMR (151 MHz, D₂O) δ 174.6, 100.4, 95.8, 74.2, 74.1, 71.1, 70.1, 69.1, 68.1, 68.0, 67.5, 67.5, 66.9, 66.8, 66.6, 66.5, 66.3, 57.6, 50.8, 43.2, 38.3, 26.2, 25.6. HR-ESI-MS (*m/z*): [M]²⁻ calcd for C₁₅H₂₆N₂O₁₇S₂²⁻, 285.0684; found, 285.0671.

3-aminopropyl-O-[(2-sulfonatamido-3-O-sulfonato- α -D-glucopyranosyl)-(1→4)-O- α -L-idopyranosiduronate (38)



A solution of compound **31** in H₂O and Pd(OH)₂ was added and stirred under H₂ atm. After 48 h, the mixture was filtered, concentrated and eluted through Bond Elute C-18 column using H₂O as eluent. The combined H₂O fraction were pooled and lyophilized to yield fully deprotected NS-disaccharide. ¹H NMR (600 MHz, Deuterium Oxide) δ 5.33 (d, J = 3.7 Hz, 1H), 4.93 (s, 1H), 4.66 (d, J = 2.5 Hz, 1H), 4.20 (t, J = 3.5 Hz, 1H), 4.07 (d, J = 2.6 Hz, 1H), 3.95 – 3.88 (m, 1H), 3.84 – 3.74 (3, 2H), 3.75 (d, J = 3.7 Hz, 1H), 3.74 – 3.65 (m, 2H), 3.63 – 3.60 (m, 1H), 3.51 (t, J = 9.6 Hz, 1H), 3.24 – 3.12 (m, 3H), 2.03 – 1.96 (m, 2H). ¹³C NMR (151 MHz, D₂O) δ 174.2, 100.5, 96.1, 74.1, 72.0, 71.2, 69.4, 67.8, 67.2, 66.6, 66.6, 60.0, 57.7, 55.4, 38.3, 26.2. HR-ESI-MS (*m/z*): [M]²⁻ calcd for C₁₅H₂₆N₂O₁₇S₂²⁻, 285.0684; found, 285.0673.

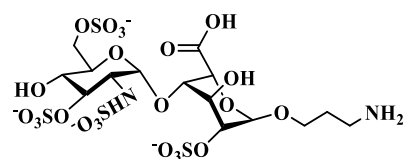
3-aminopropyl-O-[(2-sulfonatamido-6-O-sulfonato- α -D-glucopyranosyl)-(1→4)-O-2-O-sulfonato O- α -L-idopyranosiduronate (39)



A solution of compound **32** in H₂O and Pd(OH)₂ was added and stirred under H₂ atm. After 48 h, the mixture was filtered, concentrated and eluted through Bond Elute C-18 column using H₂O as eluent. The combined H₂O fraction were pooled and lyophilized to yield fully deprotected NS-disaccharide. ¹H NMR (400 MHz, Deuterium Oxide) δ 5.32 (d, J = 3.5 Hz, 1H), 5.08 (d, J = 2.6 Hz, 1H), 4.48 (d, J = 2.7 Hz, 1H), 4.28 (dd, J = 11.0, 2.8 Hz, 1H), 4.24

– 4.11 (m, 3H), 4.03 (t, $J = 3.1$ Hz, 1H), 3.91 – 3.84 (m, 2H), 3.67 – 3.60 (m, 1H), 3.59 – 3.46 (m, 2H), 3.19 (dd, $J = 10.2, 3.6$ Hz, 1H), 3.10 (t, $J = 6.5$ Hz, 2H), 1.97 – 1.90 (m, 2H). ^{13}C NMR (101 MHz, D_2O) δ 176.9, 101.2, 99.7, 78.4, 78.2, 73.5, 72.6, 71.7, 70.9, 70.8, 69.2, 69.0, 60.4, 40.9, 28.7. HR-ESI-MS (m/z): $[\text{M}]^{2-}$ calcd for $\text{C}_{16}\text{H}_{27}\text{N}_2\text{O}_{20}\text{S}_3^{3-}$, 216.3393; found, 216.3370.

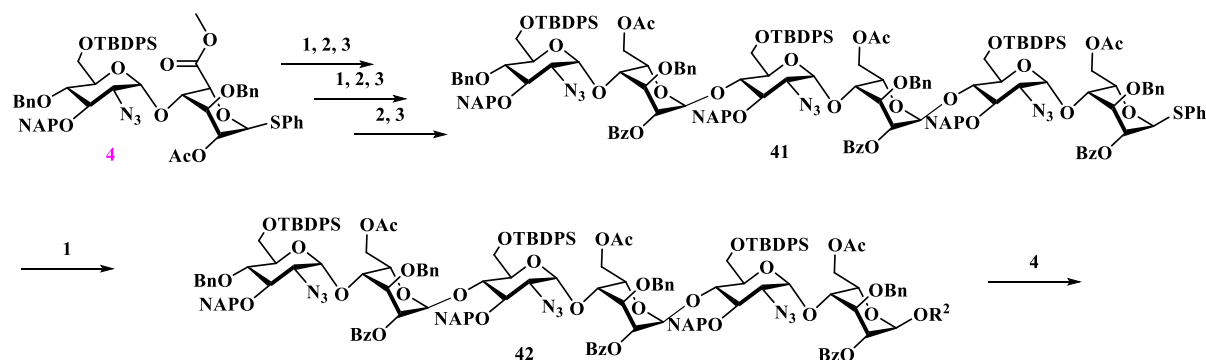
3-aminopropyl-O-[(2-sulfonatomido-6-O-sulfonato-3-O-sulfonato- α -D-glucopyranosyl)-(1 $\rightarrow$ 4)-O-2-O-sulfonato O- α -L-idopyranosiduronate (40)

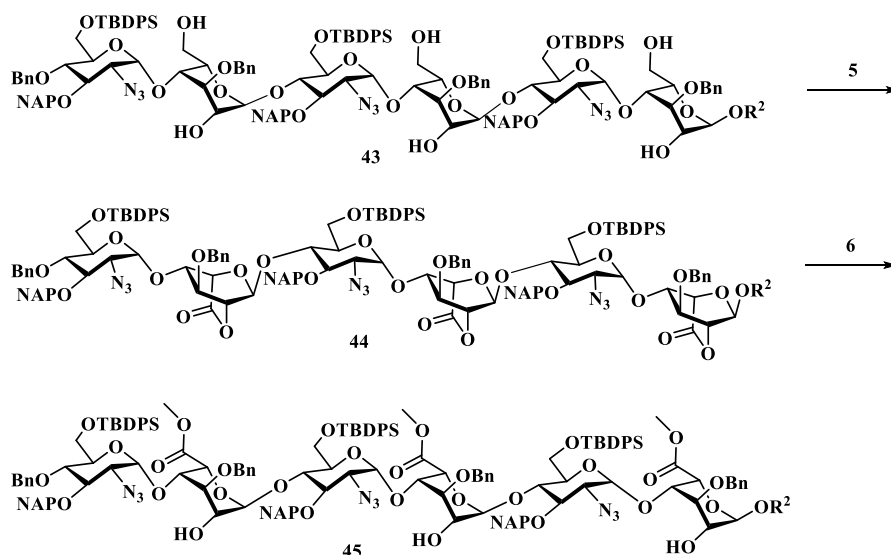


A solution of compound **33** in H_2O and $\text{Pd}(\text{OH})_2$ was added and stirred under H_2 atm. After 48 h, the mixture was filtered, concentrated and eluted through Bond Elute C-18 column

using H_2O as eluent. The combined H_2O fraction were pooled and lyophilized to yield fully deprotected NS-disaccharide. ^1H NMR (400 MHz, Deuterium Oxide) δ 5.34 (d, $J = 3.7$ Hz, 1H), 4.97 – 4.85 (m, 1H), 4.60 (d, $J = 2.4$ Hz, 1H), 4.34 (dd, $J = 11.0, 3.1$ Hz, 1H), 4.27 – 4.11 (m, 2H), 4.08 (t, $J = 2.8$ Hz, 1H), 3.97 – 3.83 (m, 2H), 3.78 – 3.68 (m, 2H), 3.66 – 3.54 (m, 2H), 3.25 (dd, $J = 10.0, 3.6$ Hz, 1H), 3.21 – 3.11 (m, 2H), 2.04 – 1.98 (m, 2H). ^{13}C NMR (151 MHz, D_2O) δ 174.6, 100.4, 95.8, 74.2, 74.1, 71.1, 70.1, 69.1, 68.1, 68.0, 67.5, 67.5, 66.9, 66.8, 66.6, 66.5, 66.3, 57.6, 50.8, 43.2, 38.3, 26.2, 25.6. HR-ESI-MS (m/z): $[\text{M}]^{2-}$ calcd for $\text{C}_{15}\text{H}_{26}\text{N}_2\text{O}_{23}\text{S}_4^{4-}$, 181.9919; found, 181.9901.

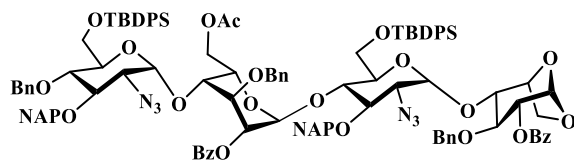
2.4.7 Synthesis of Hexasaccharide





Reagents & Yield: (1) NIS, TMSOTf, 75 – 80 %, (2) Cu(OTf)₂, Ac₂O, 85 – 92 % (3) TMSSPh, ZnI₂, 85 – 88 %, (4) NaOMe, 91 %, (5) TEMPO, BAIB, 56 %, (6) (a) LiOH.H₂O (b) MeI, K₂CO₃, 75 %

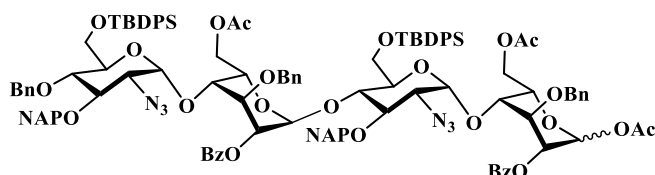
1,6-Anhydro-[(2-azido-4-O-benzyl-6-O-tert-butyldiphenylsilyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)-(1 $\rightarrow$ 4)-O-(2-O-benzoyl-3-O-benzyl-6-O-acetyl- α -L-idopyranosyl)-(1 $\rightarrow$ 4)-O-(2-azido-6-O-tert-butyldiphenylsilyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-2-O-benzoyl-3-O-benzyl- β -L-idopyranose (41a)



A solution of donor **4** (0.87 g, 0.74 mmol) and acceptor **2** (0.55 g, 0.60 mmol) in CH₂Cl₂ (15 mL) was stirred under N₂ atmosphere in round bottom flask containing freshly dried 4 Å molecular sieves for 2 h. The mixture solution was cooled down to -10 °C followed by the addition of NIS (0.26 g, 1.18 mmol) and TMSOTf (26 μ L, 0.148 mmol). After 15 minutes reaction completion was monitored by TLC and quenched using few drops of Et₃N. Molecular sieves were filtered using celite and organic layer was washed with aqueous Na₂S₂O₃ and brine. The collected organic layer was dried over Na₂SO₄, filtered, concentrated and purified through silica gel column chromatography (ethyl acetate/hexane= 1/6, v/v) to obtain compound **41a** in 95 % yield. ¹H NMR (400 MHz, Chloroform-d) δ 8.11 – 8.07 (m, 4H), 7.89 – 7.80 (m, 4H), 7.76 – 7.63 (m, 16H), 7.53 – 7.51 (m, 4H), 7.48 – 7.42 (m, 7H), 7.41 – 7.32 (m, 20H), 7.32 – 7.30 (m, 4H), 7.22 – 7.19 (m, 3H), 5.56 (d, J = 1.8 Hz, 1H), 5.41 (s, 1H), 5.27 (t, J = 2.5 Hz, 1H), 5.24 – 5.06 (m, 4H), 4.93 – 4.84 (m, 4H), 4.81 – 4.74 (m, 4H), 4.73 – 4.65 (m, 2H), 4.57 – 4.53 (m, 1H), 4.47 – 4.38 (m, 2H), 4.23 – 4.12 (m, 4H), 4.09 – 4.01 (m, 2H), 3.95 – 3.91 (m, 6H), 3.81 – 3.76 (m, 3 H), 3.72 – 3.57 (m, 4H), 3.43 (dd, J = 10.2, 3.9 Hz, 1H), 3.36 (dd, J = 10.1, 3.5 Hz, 1H), 1.46 (s, 3H), 1.07 (d, J = 10.9 Hz, 18H). ¹³C NMR (101 MHz, CHLOROFORM-D) δ 170.2, 165.8, 138.2, 138.0, 136.0, 135.9, 135.8, 135.7, 135.5, 135.3, 133.5, 133.5, 133.4, 133.3, 133.1,

133.1, 132.9, 130.2, 130.0, 129.9, 129.9, 129.8, 129.6, 129.5, 128.8, 128.6, 128.6, 128.6, 128.5, 128.5, 128.3, 128.2, 128.1, 128.0, 127.9, 127.9, 127.9, 127.8, 127.8, 127.7, 127.7, 127.7, 127.7, 127.6, 127.1, 127.0, 126.4, 126.2, 126.2, 126.1, 125.8, 125.8, 99.4, 99.3, 98.4, 97.4, 80.5, 79.3, 79.1, 78.5, 77.8, 77.5, 77.3, 77.1, 77.0, 76.8, 75.4, 75.2, 75.0, 74.2, 73.5, 73.4, 73.1, 73.0, 72.9, 68.9, 65.6, 65.4, 64.1, 62.5, 27.0, 26.9, 26.9, 20.8, 20.3, 19.5, 19.4. HR-ESI-MS (m/z): $[M+Na]^+$ calcd for $C_{115}H_{118}N_6O_{21}Si_2Na$, 1997.7787; found, 1997.7742.

Acetyl-O-[(2-azido-4-O-benzyl-6-O-*tert*-butyldiphenylsilyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)-(1 $\rightarrow$ 4)-O-(2-O-benzoyl-3-O-benzyl-6-O-acetyl- α -L-idopyranosyl)-(1 $\rightarrow$ 4)-O-(2-azido-6-O-*tert*-butyldiphenylsilyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-2-O-benzoyl-3-O-benzyl-6-O-acetyl- α/β -L-idopyranoside (41b)

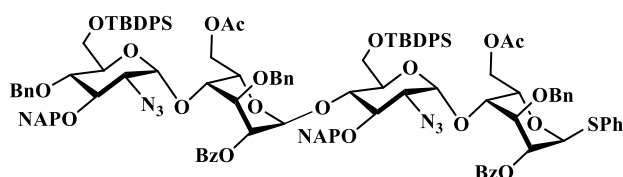


A solution of compound **41a** (1 g, 0.98 mmol) in Ac_2O (10 mL) was stirred at ice cold temperature for 15 minutes before the addition of $Cu(OTf)_2$ (0.035 g, 0.098

mmol). After 16 h, the reaction mixture was concentrated under reduced pressure and the residue was extracted with ethyl acetate, $NaHCO_3$ and washed with brine. The combined organic layer was dried over Na_2SO_4 , filtered, concentrated and purified through silica gel column chromatography (ethyl acetate/ hexane= 1/5, v/v) to obtain compound **41b** in 90 % yield. 1H NMR (400 MHz, Chloroform- d) δ 8.16 – 8.11 (m, 3H), 8.05 (d, J = 7.7 Hz, 3H), 7.86 – 7.77 (m, 4H), 7.64 (ddq, J = 22.1, 13.3, 7.6, 6.4 Hz, 21H), 7.56 – 7.29 (m, 50H), 7.28 – 7.14 (m, 17H), 6.24 (d, J = 2.3 Hz, 1H), 5.42 (s, 1H), 5.25 – 5.16 (m, 3H), 4.95 – 4.67 (m, 13H), 4.61 (dd, J = 7.2, 3.4 Hz, 2H), 4.41 (dq, J = 16.1, 6.1, 5.4 Hz, 5H), 4.30 – 4.10 (m, 8H), 4.09 – 4.01 (m, 3H), 3.93 – 3.63 (m, 18H), 3.52 (s, 1H), 3.35 – 3.30 (m, 3H), 2.12 (d, J = 3.9 Hz, 2H), 1.97 (s, 2H), 1.90 (s, 3H), 1.28 (s, 2H), 1.25 (s, 3H), 1.05 (s, 15H), 1.00 (s, 13H). ^{13}C NMR (101 MHz, Chloroform- d) δ 170.44, 170.31, 169.98, 169.97, 169.04, 168.91, 166.05, 165.76, 165.71, 138.18, 137.52, 137.51, 137.24, 135.90, 135.87, 135.64, 135.60, 135.49, 135.45, 135.23, 133.46, 133.40, 133.36, 133.34, 133.28, 133.25, 133.22, 133.17, 133.16, 133.02, 132.99, 132.74, 132.71, 129.90, 129.80, 129.76, 129.72, 129.65, 129.63, 129.50, 128.72, 128.69, 128.63, 128.60, 128.52, 128.47, 128.44, 128.36, 128.23, 128.18, 128.15, 128.13, 128.09, 128.05, 128.00, 127.98, 127.84, 127.76, 127.75, 127.71, 127.66, 127.63, 127.53, 127.43, 127.41, 127.37, 126.94, 126.10, 126.04, 126.00, 125.94, 125.88, 125.86, 125.69, 125.67, 125.61, 125.58, 98.52, 98.13, 97.91, 97.00, 96.98, 91.74, 90.56, 80.36, 79.11,

78.91, 77.64, 77.27, 75.31, 75.04, 73.97, 73.88, 73.83, 73.33, 73.29, 73.26, 72.93, 72.80, 72.68, 72.55, 72.46, 71.98, 68.63, 68.54, 67.80, 67.25, 64.65, 64.37, 64.29, 64.06, 62.48, 62.33, 62.26, 61.94, 26.87, 26.79, 21.01, 20.73, 20.66, 20.01, 19.95, 19.44, 19.34. HR-ESI-MS (m/z): $[M+Na]^+$ calcd for $C_{119}H_{124}N_6O_{24}Si_2Na$, 2100.8137; found, 2100.8131.

Phenyl-[(2-azido-4-*O*-benzyl-6-*O*-*tert*-butyldiphenylsilyl-3-*O*-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)-(1 $\rightarrow$ 4)-*O*-(2-*O*-benzoyl-3-*O*-benzyl-6-*O*-acetyl- α -L-idopyranosyl)-(1 $\rightarrow$ 4)-*O*-(2-azido-6-*O*-*tert*-butyldiphenylsilyl-3-*O*-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-*O*-2-*O*-benzoyl-3-*O*-benzyl-6-*O*-acetyl-1-thio- α -L-idopyranoside (41c**)**

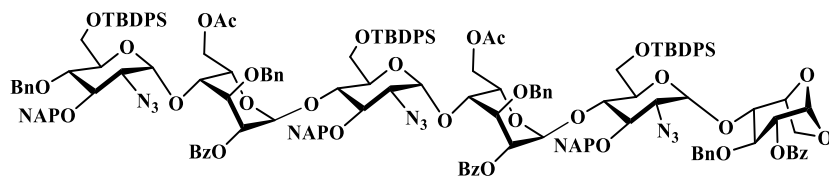


A solution of compound **41b** (0.98 g, 0.88 mmol), ZnI_2 (0.59 g, 1.84 mmol) and phenyl trimethylsilyl sulphide (0.50 g, 2.72 mmol) in CH_2Cl_2 (15 mL) was stirred under

N_2 atmosphere for 2 h. Upon completion, the reaction mixture was filtered through celite, evaporated and purified through silica gel column chromatography (ethyl acetate/ hexane= 1/5, v/v) to obtain compound **41c** in 85 % yield. 1H NMR (400 MHz, Chloroform- d) δ 8.14 – 8.12 (m, 2H), 8.05 – 8.01 (m, 2H), 7.83 – 7.75 (m, 1H), 7.67 – 7.56 (m, 16H), 7.49 – 7.43 (m, 8H), 7.41 – 7.37 (m, 7H), 7.36 – 7.33 (m, 5H), 7.32 – 7.26 (m, 18H), 7.25 – 7.21 (m, 4H), 7.19 – 7.11 (m, 6H), 5.63 (t, J = 1.4 Hz, 1H), 5.39 (s, 1H), 5.35 (s, 1H), 5.19 (t, J = 2.2 Hz, 1H), 4.96 – 4.83 (m, 3H), 4.82 – 4.75 (m, 2H), 4.78 – 4.65 (m, 4 H), 4.58 (d, J = 10.7 Hz, 1H), 4.48 (d, J = 3.9 Hz, 1H), 4.43 – 4.31 (m, 3H), 4.15 – 4.09 (m, 2H), 4.06 – 3.99 (m, 4H), 3.92 – 3.75 (m, 6H), 3.74 – 3.47 (m, 8H), 3.28 (dd, J = 10.2, 3.6 Hz, 2H), 1.22 (s, 3H), 1.03 (s, 3H), 0.98 (s, 18H). ^{13}C NMR (101 MHz, CHLOROFORM- D) δ 170.4, 170.1, 166.0, 165.8, 138.3, 137.6, 136.0, 136.0, 135.7, 135.7, 135.6, 135.5, 135.3, 133.5, 133.5, 133.4, 133.3, 133.3, 133.1, 133.1, 132.8, 131.8, 130.0, 129.9, 129.8, 129.7, 129.7, 129.7, 129.0, 128.8, 128.7, 128.6, 128.5, 128.5, 128.4, 128.3, 128.2, 128.2, 128.1, 127.9, 127.9, 127.8, 127.8, 127.8, 127.7, 127.7, 127.6, 127.5, 127.4, 127.0, 126.2, 126.1, 126.1, 126.0, 126.0, 125.8, 125.7, 98.3, 98.1, 97.0, 85.9, 80.4, 79.2, 77.7, 77.5, 77.3, 77.1, 76.8, 75.4, 75.1, 75.0, 74.3, 73.4, 73.0, 72.9, 72.9, 72.6, 72.5, 71.6, 69.9, 68.6, 66.4, 64.7, 64.5, 64.1, 63.0, 62.0, 26.9, 26.9, 20.7, 20.0. HR-ESI-MS (m/z): $[M+Na]^+$ calcd for $C_{123}H_{126}N_6O_{22}SSi_2Na$, 2149.8080; found, 2149.8072.

1,6-Anhydro-[(2-azido-4-*O*-benzyl-6-*O*-*tert*-butyldiphenylsilyl-3-*O*-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)-(1 $\rightarrow$ 4)-*O*-(2-*O*-benzoyl-3-*O*-benzyl-6-*O*-acetyl- α -L-idopyranosyl)-(1 $\rightarrow$ 4)-*O*-(2-azido-6-*O*-*tert*-butyldiphenylsilyl-3-*O*-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-*O*-(2-*O*-benzoyl-3-*O*-benzyl-6-*O*-acetyl- α -L-idopyranoside (41c**)**

idopyranosyl)-(1→4)-O-(2-azido-6-O-*tert*-butyldiphenylsilyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1→4)-O-2-O-benzoyl-3-O-benzyl- β -L-idopyranose (41d**)**

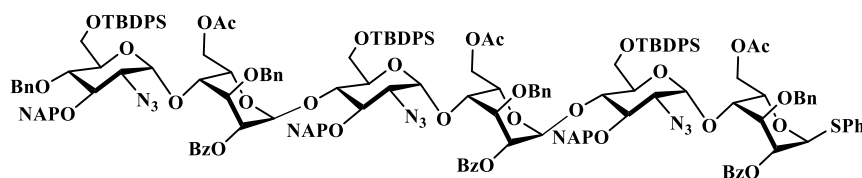


A solution of donor **41c** (0.87 g, 0.74 mmol) and acceptor **2** (0.55 g, 0.60 mmol) in CH₂Cl₂ (15 mL)

was stirred under N₂ atmosphere in round bottom flask containing freshly dried 4 Å molecular sieves for 2 h. The mixture solution was cooled down to -10 °C followed by the addition of NIS (0.26 g, 1.18 mmol) and TMSOTf (26 µL, 0.148 mmol). After 15 minutes reaction completion was monitored by TLC and quenched using few drops of Et₃N. Molecular sieves were filtered using celite and organic layer was washed with aqueous Na₂S₂O₃ and brine. The collected organic layer was dried over Na₂SO₄, filtered, concentrated and purified through silica gel column chromatography (ethyl acetate/ hexane= 1/6, v/v) to obtain compound **41d** in 82 % yield. ¹H NMR (400 MHz, Chloroform-d) δ 8.08 (d, J = 1.2 Hz, 1H), 8.06 - 8.04 (m, 2H), 8.05 - 8.03 (m, 2H), 7.84 - 7.75 (m, 3H), 7.72 - 7.65 (m, 9H), 7.65 - 7.7.60 (m, 11H), 7.59 - 7.54 (m, 3H), 7.50 - 7.43 (m, 6H), 7.43 - 7.36 (m, 9H), 7.36 - 7.29 (m, 23H), 7.29 - 7.27 (m, 3H), 7.24 - 7.19 (m, 11H), 7.16 - 7.13 (m, 4H), 5.52 (d, J = 1.8 Hz, 1H), 5.42 (s, 1H), 5.35 (d, J = 2.2 Hz, 1H), 5.22 (t, J = 2.9 Hz, 1H), 5.21 - 5.16 (m, 2H), 5.12 (d, J = 11.3 Hz, 1H), 5.05 (dd, J = 8.1, 1.8 Hz, 1H), 4.91 - 4.78 (m, 6H), 4.75 - 4.62 (m, 6H), 4.58 (d, J = 10.7 Hz, 1H), 4.49 - 4.36 (m, 2H), 4.40 - 4.28 (m, 3H), 4.19 - 4.08 (m, 5H), 4.05 - 4.00 (m, 3H), 3.96 - 3.87 (m, 5H), 3.87 - 3.80 (m, 3H), 3.77 - 3.66 (m, 4H), 3.65 - 3.59 (m, 3H), 3.57 - 3.47 (m, 4H), 3.40 (dd, J = 10.3, 3.8 Hz, 1H), 3.30 - 3.23 (m, 2H), 1.45 (s, 3H), 1.23 (s, 3H), 1.04 (d, J = 1.9 Hz, 18H), 0.98 (s, 9H). ¹³C NMR (101 MHz, CHLOROFORM-D) δ 170.1, 170.1, 165.9, 165.9, 165.8, 138.3, 138.0, 137.5, 137.5, 136.0, 136.0, 135.7, 135.7, 135.7, 135.5, 135.3, 133.5, 133.4, 133.4, 133.3, 133.3, 133.1, 133.1, 133.0, 132.9, 132.8, 130.0, 129.9, 129.9, 129.8, 129.8, 129.5, 128.9, 128.7, 128.6, 128.5, 128.5, 128.3, 128.2, 128.1, 128.0, 127.9, 127.9, 127.8, 127.8, 127.7, 127.5, 127.0, 126.5, 126.2, 126.1, 126.1, 126.1, 126.0, 125.9, 125.8, 99.4, 99.3, 98.3, 97.4, 97.1, 96.9, 79.3, 79.1, 78.9, 78.5, 77.7, 77.5, 77.4, 77.2, 77.0, 76.8, 75.4, 75.1, 75.1, 74.2, 73.7, 73.2, 72.9, 72.8, 64.4, 64.2, 64.1, 62.4, 62.2, 34.8, 27.0, 26.9, 26.8, 25.4, 20.3, 20.0, 19.5, 19.4. HR-ESI-MS (*m/z*): [M+Na]⁺ calcd for C₁₇₀H₁₇₅N₉O₃₂Si₃Na, 2961.1589; found, 2961.1571.

Phenyl-[(2-azido-4-O-benzyl-6-O-*tert*-butyldiphenylsilyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)-(1→4)-O-(2-O-benzoyl-3-O-benzyl-6-O-acetyl- α -L-idopyranosyl)-(1→4)-O-(2-azido-6-O-*tert*-butyldiphenylsilyl-3-O-(2-naphthylmethyl)-2-

deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-(2-O-benzoyl-3-O-benzyl-6-O-acetyl- α -L-idopyranosyl)-(1 $\rightarrow$ 4)-O-(2-azido-6-O-*tert*-butyldiphenylsilyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-2-O-benzoyl-3-O-benzyl-6-O-acetyl-1-thio- α -L-idopyranoside (**41**)

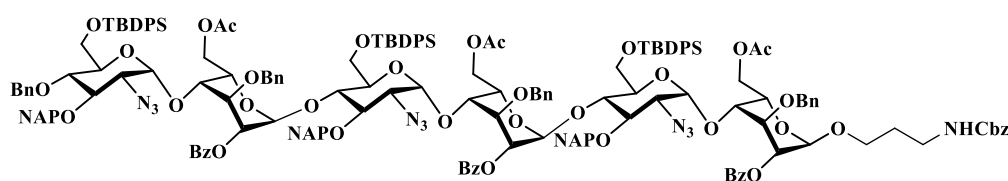


A solution of compound **41d** (1 g, 0.98 mmol) in Ac₂O (10 mL) was stirred at ice cold

temperature for 15 minutes before the addition of Cu(OTf)₂ (0.035 g, 0.098 mmol). After 16 h, the reaction mixture was concentrated under reduced pressure and the residue was extracted with ethyl acetate, NaHCO₃ and washed with brine. The combined organic layer was dried over Na₂SO₄, filtered, concentrated and purified through silica gel column chromatography (ethyl acetate/ hexane= 1/5, v/v) to obtain compound **41e**. Then a solution of compound **41e** (0.98 g, 0.88 mmol), ZnI₂ (0.59 g, 1.84 mmol) and phenyl trimethylsilyl sulphide (0.50 g, 2.72 mmol) in CH₂Cl₂ (15 mL) was stirred under N₂ atmosphere for 2 h. Upon completion, the reaction mixture was filtered through celite, evaporated and purified through silica gel column chromatography (ethyl acetate/ hexane= 1/5, v/v) to obtain compound **41** in 84 % yield. ¹H NMR (400 MHz, Chloroform-d) δ 8.12 – 8.09 (m, 2H), 8.02 – 7.99 (m, 4H), 7.81 – 7.73 (m, 3H), 7.66 – 7.63 (m, 3H), 7.63 – 7.58 (m, 11H), 7.58 – 7.52 (m, 10H), 7.49 (s, 1H), 7.46 – 7.42 (m, 6H), 7.39 – 7.36 (m, 6H), 7.36 – 7.30 (m, 15H), 7.29 – 7.25 (m, 22H), 7.22 – 7.20 (dd, J = 7.4, 3.5 Hz, 6H), 7.18 – 7.14 (m, 3H), 7.14 – 7.07 (m, 6H), 5.61 (s, 1H), 5.38 (s, 1H), 5.34 (d, J = 5.7 Hz, 2H), 5.13 (d, J = 20.1 Hz, 2H), 4.92 (d, J = 11.7 Hz, 1H), 4.85 – 4.81 (m, 2H), 4.77 (d, J = 11.3 Hz, 2H), 4.69 (s, 2H), 4.67 – 4.60 (m, 3H), 4.58 (d, J = 3.5 Hz, 2H), 4.54 (d, J = 10.7 Hz, 1H), 4.46 (d, J = 3.8 Hz, 1H), 4.40 (d, J = 6.4 Hz, 1H), 4.36 – 4.43 (m, 3H), 4.27 (d, J = 11.8 Hz, 1H), 4.23 (s, 1H), 4.15 (d, J = 9.5 Hz, 1H), 4.11 (d, J = 4.0 Hz, 1H), 4.07 – 4.01 (m, 3H), 4.02 – 3.95 (m, 5H), 3.90 – 3.87 (m, 1H), 3.85 – 3.73 (m, 6H), 3.68 (dd, J = 10.3, 5.2 Hz, 2H), 3.65 – 3.63 (m, 1H), 3.63 – 3.55 (m, 3H), 3.54 – 3.48 (m, 2H), 3.43 – 3.39 (m, 2H), 3.37 (s, 1H), 3.27 – 3.22 (m, 2H), 3.18 (dd, J = 10.3, 3.7 Hz, 1H), 1.84 (s, 3H), 1.23 (s, 3H), 1.17 (s, 3H), 1.00 (s, 9H), 0.98 (s, 9H), 0.94 (s, 9H). ¹³C NMR (101 MHz, CHLOROFORM-D) δ 170.4, 170.1, 170.0, 166.0, 165.9, 138.3, 137.6, 137.5, 137.4, 136.1, 136.0, 136.0, 135.8, 135.7, 135.7, 135.5, 135.5, 135.3, 133.6, 133.5, 133.5, 133.4, 133.3, 133.3, 133.3, 133.2, 133.2, 133.1, 133.1, 132.8, 132.8, 131.8, 130.0, 129.9, 129.9, 129.9, 129.8, 129.8, 129.7, 129.7, 129.7, 129.6, 129.0, 128.9, 128.7, 128.7, 128.6, 128.6, 128.5, 128.5, 128.5, 128.5, 128.4, 128.3, 128.3, 128.3, 128.2, 128.2, 128.1, 127.9, 127.9, 127.8, 127.8, 127.8, 127.7, 127.7, 127.7, 127.5, 127.5, 127.4, 127.0, 126.2, 126.2, 126.1, 126.0, 126.0, 125.9, 125.8, 125.8, 125.6, 98.2, 98.1, 97.0, 97.0, 96.9, 96.7, 85.9, 80.4, 79.2, 78.8, 77.7, 77.5, 77.5, 77.4, 77.2, 76.8, 75.4, 75.1, 74.3, 73.4, 73.3, 73.1, 72.9, 72.9, 72.8, 72.3, 72.2, 72.1, 71.9,

71.6, 69.9, 68.8, 68.4, 66.4, 64.5, 64.4, 64.1, 63.0, 62.4, 62.3, 62.1, 62.0, 28.5, 26.9, 26.9, 26.8, 24.8, 23.9, 20.7, 20.0, 20.0, 19.5.

N-benzyloxycarbonyl-3-aminopropyl-O-[(2-azido-4-O-benzyl-6-O-*tert*-butyldiphenylsilyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)-(1 $\rightarrow$ 4)-O-(2-O-benzoyl-3-O-benzyl-6-O-acetyl- α -L-idopyranosyl)-(1 $\rightarrow$ 4)-O-(2-azido-6-O-*tert*-butyldiphenylsilyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-(2-O-benzoyl-3-O-benzyl-6-O-acetyl- α -L-idopyranoside (42)

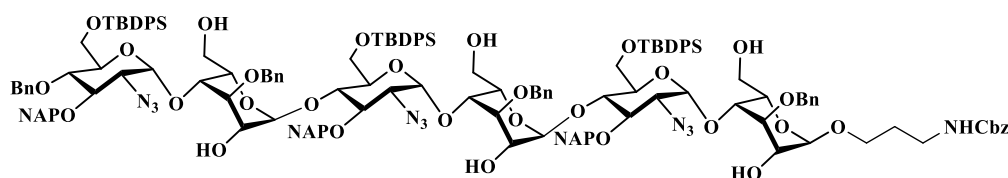


A solution of donor **41** (0.90 g, 0.42 mmol) and

linker benzyl (3-hydroxypropyl) carbamate (0.097 g, 0.46 mmol) in CH₂Cl₂ (15 mL) was stirred under N₂ atmosphere in round bottom flask containing freshly dried 4 Å molecular sieves for 2 h. Next, NIS (0.15 g, 0.67 mmol) and TfOH (7.4 μL, 0.084 mmol) was added at room temperature and reaction completion was monitored by TLC, quenched using few drops of Et₃N. Molecular sieves were filtered using celite and organic layer was washed with aqueous Na₂S₂O₃ and brine. The collected organic layer was dried over Na₂SO₄, filtered, concentrated and purified through silica gel column chromatography (ethyl acetate/ hexane= 1/3, v/v) to obtain compound **42** in 78 % yield. ¹H NMR (400 MHz, Chloroform-d) δ 8.12 – 8.10 (m, 2H), 8.05 – 7.99 (m, 3H), 7.83 – 7.74 (m, 3H), 7.68 – 7.60 (m, 12H), 7.60 – 7.54 (m, 7H), 7.51 (s, 1H), 7.49 – 7.45 (m, 3H), 7.42 – 7.36 (m, 9H), 7.36 – 7.31 (m, 20H), 7.30 – 7.27 (m, 15H), 7.25 – 7.20 (m, 7H), 7.19 – 7.10 (m, 9H), 5.66 (t, J = 5.5 Hz, 1H), 5.39 (d, J = 14.4 Hz, 2H), 5.16 (d, J = 20.4 Hz, 2H), 5.09 (s, 2H), 5.05 (s, 1H), 4.98 (s, 1H), 4.87 – 4.77 (m, 4H), 4.72 (d, J = 7.9 Hz, 2H), 4.68 (d, J = 3.0 Hz, 1H), 4.64 (d, J = 3.0 Hz, 1H), 4.60 (d, J = 3.5 Hz, 2H), 4.56 (d, J = 10.7 Hz, 1H), 4.43 – 4.24 (m, 7H), 4.18 – 4.04 (m, 4H), 4.05 – 3.93 (m, 6H), 3.92 – 3.65 (m, 11H), 3.63 – 3.52 (m, 6H), 3.44 (d, J = 7.1 Hz, 2H), 3.40 (s, 2H), 3.31 – 3.24 (m, 2H), 3.21 (dd, J = 10.3, 3.5 Hz, 2H), 1.89 – 1.80 (m, 5H), 1.21 (d, J = 14.5 Hz, 6H), 1.03 (s, 9H), 1.00 (s, 9H), 0.96 (s, 9H). ¹³C NMR (101 MHz, CHLOROFORM-D) δ 170.5, 170.1, 170.0, 165.9, 165.9, 165.8, 156.7, 138.3, 137.6, 137.5, 136.8, 136.1, 136.0, 136.0, 135.8, 135.7, 135.7, 135.7, 135.5, 135.3, 133.6, 133.5, 133.4, 133.3, 133.3, 133.3, 133.1, 132.8, 132.8, 130.0, 129.9, 129.9, 129.8, 129.8, 129.8, 129.7, 129.7, 129.7, 129.6, 129.3, 128.9, 128.8, 128.7, 128.7, 128.6, 128.6, 128.5, 128.5, 128.5, 128.5, 128.4, 128.4, 128.3, 128.3, 128.2, 128.2, 128.1, 128.1, 128.0, 127.9, 127.9, 127.8, 127.8, 127.8, 127.7, 127.7, 127.7, 127.7, 127.6, 127.5, 127.5, 127.4, 127.0, 126.2,

126.2, 126.1, 126.0, 126.0, 125.9, 125.8, 125.8, 125.7, 125.3, 98.2, 98.2, 97.9, 97.0, 96.9, 96.7, 80.4, 79.2, 78.8, 77.7, 77.5, 77.4, 77.2, 76.8, 75.4, 75.1, 73.9, 73.2, 73.1, 72.9, 72.7, 72.6, 72.2, 72.1, 71.8, 71.6, 68.8, 68.4, 67.2, 66.7, 65.1, 64.9, 64.5, 64.4, 64.1, 63.2, 62.4, 62.0, 39.8, 29.8, 29.5, 27.0, 27.0, 26.9, 26.9, 26.8, 20.7, 20.0, 19.5, 19.5, 19.4.

N-benzyloxycarbonyl-3-aminopropyl-O-[(2-azido-4-O-benzyl-6-O-*tert*-butyldiphenylsilyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)-(1 $\rightarrow$ 4)-O-(3-O-benzyl- α -L-idopyranosyl)-(1 $\rightarrow$ 4)-O-(2-azido-6-O-*tert*-butyldiphenylsilyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-(3-O-benzyl- α -L-idopyranosyl)-(1 $\rightarrow$ 4)-O-(2-azido-6-O-*tert*-butyldiphenylsilyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-3-O-benzyl- α -L-idopyranoside (43**)**

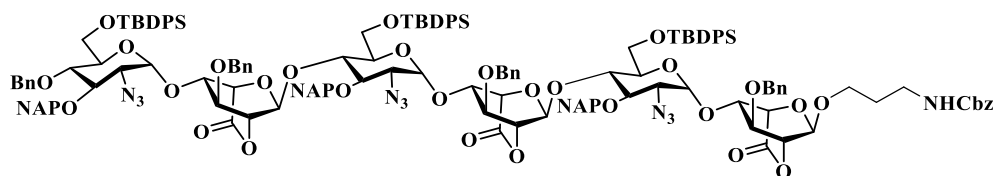


To a solution
of compound
42 (0.84 g,
0.37 mmol)

in CH₂Cl₂ (7 mL) and MeOH (7 mL) was added NaOMe (0.06g, 1.11 mmol) and stirred at room temperature. After 12 h, reaction mixture was quenched using Amberlite IR 120H⁺ resin, filtered, evaporated and purified through silica gel column chromatography (ethyl acetate/hexane= 1/2.5, v/v) to obtain compound **43** in 95 % yield. ¹H NMR (400 MHz, Chloroform-d) δ 7.87 – 7.83 (m, 2H), 7.83 – 7.77 (m, 7H), 7.77 – 7.72 (m, 6H), 7.71 – 7.70 (m, 1H), 7.69 (d, J = 1.3 Hz, 1H), 7.59 – 7.50 (m, 10H), 7.49 – 7.46 (m, 13H), 7.45 – 7.47 (d, J = 1.5 Hz, 4H), 7.42 – 7.37 (m, 13H), 7.36 – 7.32 (m, 6H), 7.30 – 7.28 (m, 7H), 7.20 – 7.11 (m, 2H), 6.99 – 6.92 (m, 1H), 5.72 (d, J = 7.5 Hz, 1H), 5.24 (d, J = 2.0 Hz, 1H), 5.16 (s, 1H), 5.09 – 5.02 (m, 4H), 5.01 – 4.94 (m, 2H), 4.91 – 4.83 (m, 3H), 4.81 (s, 1H), 4.77 -4.68 (m, 3H), 4.70 (s, 1H), 4.69 – 4.60 (m, 6H), 4.55 (d, J = 12.1 Hz, 1H), 4.31 – 4.26 (m, 2H), 4.20 (t, J = 6.8 Hz, 1H), 4.13 – 4.00 (m, 1H), 4.03 (t, J = 9.5 Hz, 1H), 3.91 (d, J = 6.0 Hz, 3H), 3.87 (s, 3H), 3.85 (s, 2H), 3.81 – 3.77 (m, J = 13.8 Hz, 5H), 3.74 – 3.70 (m, 3H), 3.67 (d, J = 5.0 Hz, 1H), 3.64 (d, J = 3.5 Hz, 1H), 3.61 (d, J = 4.3 Hz, 3H), 3.57 (s, 4H), 3.55 (s, 1H), 3.38 (d, J = 10.3 Hz, 2H), 3.29 – 3.16 (m, 3H), 2.81 – 2.69 (m, 2H), 1.90 – 1.81 (m, 2H), 1.11 (s, 9H), 1.08 (s, 9H), 1.06 (s, 9H). ¹³C NMR (101 MHz, CHLOROFORM-D) δ 156.8, 138.2, 137.6, 137.6, 137.5, 136.7, 136.2, 136.1, 135.9, 135.9, 135.9, 135.7, 135.7, 135.2, 135.2, 135.0, 133.6, 133.4, 133.2, 133.2, 132.9, 132.9, 132.7, 132.7, 130.0, 129.9, 129.9, 129.9, 129.8, 128.9, 128.8, 128.7, 128.7, 128.6, 128.6, 128.6, 128.5, 128.5, 128.4, 128.4, 128.2, 128.1, 128.0, 128.0, 127.9, 127.9, 127.8, 127.8, 127.8, 127.8, 127.7, 127.7, 127.6, 127.6, 127.2, 126.7, 126.6, 126.4, 126.3, 126.2, 126.2, 126.1,

126.0, 125.7, 125.7, 101.1, 100.2, 99.7, 94.9, 94.4, 94.0, 81.3, 80.1, 80.0, 77.9, 77.5, 77.4, 77.2, 76.8, 76.1, 75.6, 75.1, 73.8, 73.0, 72.9, 72.5, 72.4, 72.3, 72.1, 72.0, 70.4, 69.7, 69.3, 66.7, 66.3, 66.1, 64.7, 64.6, 64.1, 62.6, 62.0, 61.6, 61.5, 39.8, 36.7, 29.8, 27.0, 26.9, 26.9, 19.5.

N-benzyloxycarbonyl-3-aminopropyl-O-[(2-azido-4-O-benzyl-6-O-*tert*-butyldiphenylsilyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)-(1 $\rightarrow$ 4)-O-(3-O-benzyl- α -L-idopyranosidurono-6,2-lactone)-(1 $\rightarrow$ 4)-O-(2-azido-6-O-*tert*-butyldiphenylsilyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-(3-O-benzyl- α -L-idopyranosidurono-6,2-lactone)-(1 $\rightarrow$ 4)-O-(2-azido-6-O-*tert*-butyldiphenylsilyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-3-O-benzyl- α -L-idopyranosidurono-6,2-lactone (44**)**

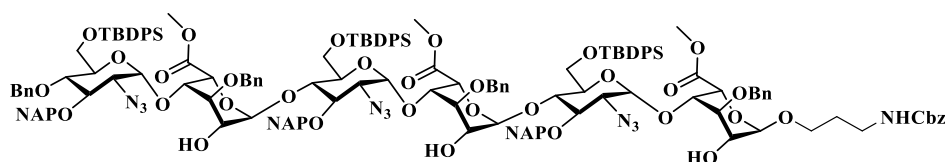


To a solution of compound **44** (0.67 g, 0.34 mmol) in

CH₂Cl₂ (5 mL) and H₂O (5 mL) was added TEMPO (0.01g, 0.07 mmol), BAIB (0.54 g, 1.70 mmol) and stirred at room temperature. After 16 h, the reaction mixture was extracted using saturated aqueous NH₄Cl. The collected organic layer was dried over Na₂SO₄, filtered, concentrated and purified through silica gel column chromatography (ethyl acetate/ hexane= 1/3, v/v) to obtain compound **44** in 55 % yield. ¹H NMR (400 MHz, Chloroform-d) δ 7.93 – 7.78 (m, 11H), 7.74 – 7.64 (m, 12H), 7.64 – 7.60 (m, 2H), 7.54 – 7.52 (m, 1H), 7.52 – 7.47 (m, 6 H), 7.46 (m, 3H), 7.45 – 7.43 (m, 4H), 7.42 – 7.39 (m, 14H), 7.38 (s, 3H), 7.37 – 7.33 (m, 12H), 7.32 – 7.29 (m, 9H), 7.19 (dd, J = 6.6, 3.0 Hz, 2H), 5.87 (t, J = 5.5 Hz, 1H), 5.51 (s, 1H), 5.45 (s, 1H), 5.26 (d, J = 12.4 Hz, 1H), 5.21 – 5.15 (m, 1H), 5.11 (d, J = 9.4 Hz, 2H), 5.05 (d, J = 19.9 Hz, 2H), 5.00 – 4.95 (m, 2H), 4.93 (d, J = 5.0 Hz, 1H), 4.88 – 4.81 (m, 2H), 4.80 – 4.72 (m, 2H), 4.70 – 4.64 (m, 2H), 4.61 (d, J = 12.0 Hz, 1H), 4.54 (s, 1H), 4.51 – 4.46 (m, 2H), 4.35 (d, J = 3.2 Hz, 1H), 4.31 – 4.27 (m, 1H), 4.26 – 4.21 (m, 3H), 4.20 – 4.16 (m, 2H), 4.03 – 3.93 (m, 4H), 3.90 – 3.83 (m, 3H), 3.83 – 3.77 (m, 4H), 3.77 – 3.70 (m, 4H), 3.67 – 3.63 (m, 3H), 3.62 – 3.54 (m, 4H), 3.62 – 3.55 (m, 4H), 3.46 – 3.28 (m, 3H), 2.87 – 2.79 (m, 1H), 1.94 – 1.85 (m, 2H), 1.10 (m, J = 5.5 Hz, 27H). ¹³C NMR (101 MHz, CHLOROFORM-D) δ 167.6, 167.4, 167.2, 156.7, 138.2, 137.1, 137.1, 136.9, 136.8, 136.4, 136.2, 136.1, 136.1, 136.0, 135.7, 135.7, 135.6, 135.3, 133.7, 133.4, 133.4, 133.4, 133.2, 133.1, 133.0, 132.9, 132.9, 132.8, 132.7, 130.1, 130.0, 129.8, 129.8, 128.9, 128.8, 128.7, 128.6, 128.6, 128.6, 128.5, 128.4, 128.4, 128.3, 128.3, 128.3, 128.2, 128.2, 128.2, 128.1, 128.1, 128.0, 127.9, 127.8, 127.8, 127.8, 127.7, 127.6,

127.3, 126.7, 126.6, 126.3, 126.2, 126.1, 126.1, 125.2, 124.9, 124.8, 100.0, 99.0, 96.9, 96.8, 81.1, 80.6, 80.3, 80.1, 78.3, 78.2, 78.1, 77.8, 77.5, 77.4, 77.2, 76.9, 76.2, 76.1, 75.9, 75.1, 74.4, 72.8, 72.6, 72.4, 72.3, 72.2, 71.9, 69.5, 69.2, 66.7, 63.4, 63.2, 40.0, 36.7, 29.6, 27.0, 24.8, 19.5, 19.4.

N-benzyloxycarbonyl-3-aminopropyl-O-[(methyl(2-azido-4-O-benzyl-6-O-tert-butylidiphenylsilyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-(3-O-benzyl- α -L-idopyranosidurono-6,2-lactone)-(1 $\rightarrow$ 4)-O-(2-azido-6-O-tert-butylidiphenylsilyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-(3-O-benzyl- α -L-idopyranosidurono-6,2-lactone)-(1 $\rightarrow$ 4)-O-(2-azido-6-O-tert-butylidiphenylsilyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-3-O-benzyl- α -L-idopyranosyluronate (45)

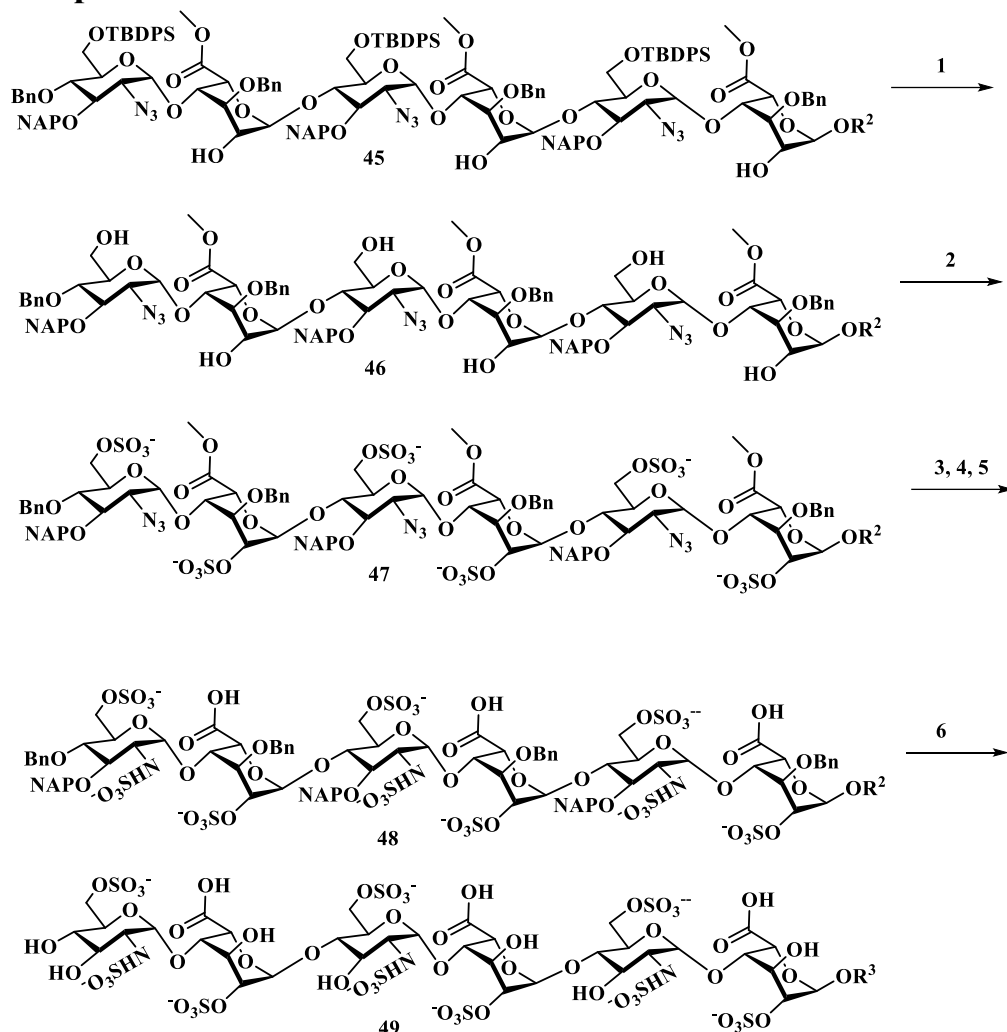


To a solution of compound **44** was dissolved in

DCM:H₂O (1:1) followed by addition by of TEMPO (0.2eq) and BAIB (3eq) and kept it stirring for 12 h at room temperature followed by extraction with saturated aqueous solution NH₄Cl. The organic layer was evaporated and purified by silica gel column chromatography. Then the compound was dissolved in THF:H₂O (1:1) followed by addition of lithium hydroxide (10eq) and kept it on stirring for 2 h at room temperature followed by evaporation and dried over vacuum. After that reaction mixture was dissolved in dry DMF and potassium carbonate (3eq), methyl iodide (9eq) was added and kept it on stirring for at room temperature. After 12 h extraction was done using 10 % HCl solution and organic layer was evaporated, purified using silica gel chromatography using ethyl acetate/hexane (2/3) to obtain compound **45** 78%. ¹H NMR (400 MHz, Chloroform-d) δ 7.88 – 6.97 (m, 76H), 5.55 (d, J = 5.9 Hz, 1H), 5.37 (d, J = 21.1 Hz, 2H), 5.09 – 4.93 (m, 6H), 4.93 – 4.76 (m, 5H), 4.77 – 4.52 (m, 10H), 4.14 – 4.04 (m, 3H), 4.02 – 3.60 (m, 19H), 3.63 – 3.34 (m, 13H), 3.35 – 3.19 (m, 3H), 2.67 (d, J = 6.7 Hz, 3H), 1.91 – 1.75 (m, 2H). ¹³C NMR (151 MHz, CDCl₃) δ 169.7, 168.7, 168.7, 156.5, 138.3, 137.2, 137.1, 136.6, 136.1, 136.0, 136.0, 136.0, 135.9, 135.8, 135.6, 135.1, 135.1, 133.4, 133.4, 133.3, 133.3, 133.2, 133.2, 133.1, 132.8, 132.8, 129.8, 129.8, 129.8, 128.8, 128.8, 128.8, 128.7, 128.6, 128.5, 128.4, 128.4, 128.4, 128.4, 128.3, 128.3, 128.2, 128.1, 128.0, 128.0, 127.9, 127.8, 127.8, 127.8, 127.7, 127.7, 127.6, 127.5, 127.5, 127.4, 127.2, 127.1, 126.5, 126.4, 126.2, 126.1, 126.0, 125.8, 125.7, 125.7, 125.6, 101.5, 100.6, 100.4, 95.0, 94.9, 94.3, 80.9, 79.5, 79.4, 77.5, 77.3, 77.1, 76.9, 76.0, 75.3, 75.1, 74.8, 73.7, 73.3, 73.1, 72.7, 72.7, 72.6, 72.6, 72.4, 72.1, 71.9,

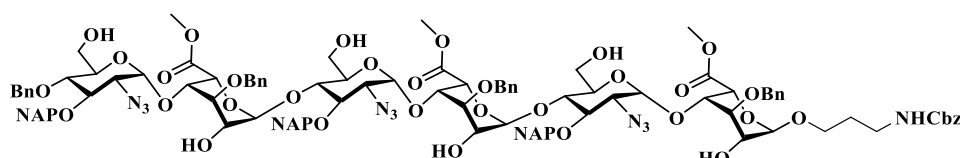
71.8, 71.0, 70.7, 70.5, 68.0, 67.2, 66.9, 66.8, 66.7, 66.5, 64.4, 64.2, 63.9, 62.4, 62.1, 61.7, 52.2, 51.0, 50.9, 39.9, 36.7, 27.0, 26.9, 26.9, 26.9, 19.5, 19.4, 19.4. HR-ESI-MS (m/z): $[M+Na]^+$ calcd for $C_{159}H_{174}N_{10}O_{33}Si_3Na$, 2858.1450; found, 2858.1437.

2.4.8 Deprotection & O & N- Sulfation



Reagents & Yield: (1) 70 % HF.Py, 90 %, (2) SO₃NEt₃, 71 %, (3) LiOH.H₂O, 88 %, (4) PMe₃.THF, 79 %, (5) SO₃.Py, 63 %, (6) H₂, Pd(OH)₂, 69 %

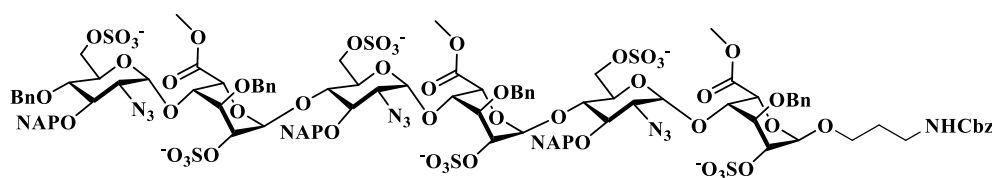
N-benzyloxycarbonyl-3-aminopropyl-O-[(methyl(2-azido-4-O-benzyl-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl))]- (1 $\rightarrow$ 4)-O-(3-O-benzyl- α -L-idopyranosiduronate)- (1 $\rightarrow$ 4)-O-[(methyl(2-azido-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl))]- (1 $\rightarrow$ 4)-O-(3-O-benzyl- α -L-idopyranosiduronate)- (1 $\rightarrow$ 4)-O-[(methyl(2-azido-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl))]- (1 $\rightarrow$ 4)-O-3-O-benzyl- α -L-idopyranosiduronate (46)



A solution of compound **45** (1g, 1 eq) in dry

pyridine (5 mL) 70% HF.Py complex (5 eq per TBDPS) was added at 0 °C and kept it for stirring for 12 h. After that in reaction mixture ethyl acetate was added and washed with 1N HCl 3 times followed by evaporation of organic layer, purification by silica gel column chromatography (ethyl acetate/ hexane= 2/3, v/v) to obtain **46**, 87 %. ¹H NMR (400 MHz, Chloroform-d) δ 7.84 – 7.55 (m, 10H), 7.48 – 7.17 (m, 36H), 5.61 – 5.53 (m, 1H), 5.32 – 5.16 (m, 2H), 5.05 (t, J = 12.1 Hz, 3H), 5.01 – 4.88 (m, 3H), 4.89 – 4.59 (m, 12H), 4.51 (d, J = 12.3 Hz, 1H), 4.07 – 3.83 (m, 9H), 3.79 (s, 8H), 3.73 – 3.65 (m, 3H), 3.67 – 3.38 (m, 14H), 3.36 – 3.17 (m, 3H), 2.98 – 2.86 (m, 4H), 1.91 – 1.78 (m, 2H). ¹³C NMR (101 MHz, CDCl₃) δ 171.2, 170.0, 169.3, 169.2, 156.5, 137.8, 137.2, 137.0, 136.6, 135.0, 135.0, 133.4, 133.0, 132.9, 128.8, 128.8, 128.6, 128.5, 128.5, 128.4, 128.3, 128.2, 128.2, 128.1, 128.0, 127.9, 127.7, 127.6, 126.9, 126.2, 126.1, 126.0, 125.9, 125.8, 125.5, 101.7, 101.7, 100.8, 100.7, 95.3, 95.3, 80.8, 77.4, 77.3, 77.1, 76.7, 75.8, 75.2, 74.7, 72.8, 72.5, 72.2, 71.9, 71.2, 68.0, 66.7, 63.7, 61.0, 60.4, 52.5, 51.6, 51.5, 39.9, 29.5, 21.1, 14.2. HR-ESI-MS (*m/z*): [M+Na]⁺ calcd for C₁₁₁H₁₂₀N₁₀O₃₃Na, 2120.8019; found, 2120.8011.

N-benzyloxycarbonyl-3-aminopropyl-O-[(methyl(2-azido-4-O-benzyl-6-O-sulfonato-3-O-(2-naphthylmethyl)-2-deoxy-α-D-glucopyranosyl))-(1→4)-O-(3-O-benzyl-2-O-sulfonato-α-L-idopyranosiduronate)-(1→4)-O-[(methyl(2-azido-6-O-sulfonato-3-O-(2-naphthylmethyl)-2-deoxy-α-D-glucopyranosyl))-(1→4)-O-(3-O-benzyl-2-O-sulfonato-α-L-idopyranosiduronate)-(1→4)-O-[(methyl(2-azido-6-O-sulfonato-3-O-(2-naphthylmethyl)-2-deoxy-α-D-glucopyranosyl))-(1→4)-O-3-O-benzyl-2-O-sulfonato-α-L-idopyranosiduronate (47)

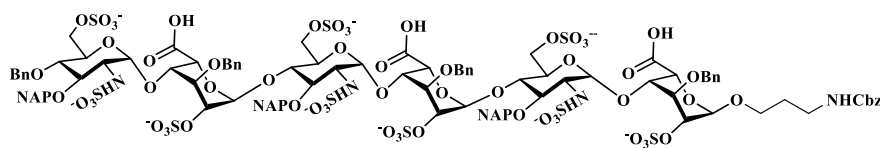


To a solution of starting material **46** (1 mmol) in

DMF (2 mL) was added SO₃.NEt₃ complex (10 mmol per -OH) under N₂ atmosphere and stirred at 70 °C for 5 days. Upon completion of reaction, the reaction mixture was concentrated under reduced pressure and it was concentrated and purified using silica column chromatography (MeOH/DCM 1/10 v/v) to obtain product **47** 68 %. ¹H NMR (600 MHz, Methanol-d₄) δ 7.94 – 6.78 (m, 44H), 5.46 – 4.90 (m, 10H), 4.81 – 4.53 (m, 16H), 4.41 – 3.52

(m, 31H), 3.51 – 3.38 (m, 2H), 3.33 – 3.17 (m, 3H), 2.98 – 2.63 (m, 3H), 1.81 – 1.77 (m, 2H). ^{13}C NMR (151 MHz, MeOD) δ 170.3, 170.1, 169.9, 157.4, 138.2, 138.0, 137.9, 137.7, 136.9, 135.5, 135.2, 133.4, 133.4, 133.3, 133.3, 133.1, 133.0, 133.0, 128.9, 128.9, 128.7, 128.5, 128.5, 128.4, 128.4, 128.4, 128.3, 128.2, 128.2, 128.1, 128.1, 128.0, 128.0, 127.9, 127.9, 127.8, 127.7, 127.7, 127.6, 127.6, 127.6, 127.5, 127.5, 127.5, 127.4, 127.3, 127.3, 127.3, 127.1, 127.0, 126.4, 126.3, 126.1, 126.0, 125.8, 125.7, 125.7, 125.7, 125.6, 125.5, 101.5, 100.7, 100.4, 96.2, 95.5, 95.4, 78.9, 77.2, 74.9, 74.6, 74.4, 74.3, 72.8, 72.6, 72.4, 70.7, 70.4, 68.3, 66.5, 66.1, 66.0, 65.7, 65.4, 64.0, 63.4, 53.5, 51.9, 51.8, 51.0, 50.9, 48.5, 48.3, 48.1, 48.0, 47.8, 47.7, 47.6, 47.4, 47.3, 38.1, 29.3. HR-ESI-MS (m/z): $[\text{M}/6]^{6-}$ calcd for $\text{C}_{111}\text{H}_{114}\text{N}_{10}\text{O}_{51}\text{S}_6$, 432.5837; found, 432.5829.

N-benzyloxycarbonyl-3-aminopropyl-O-[(2-sulfonatoimido-4-O-benzyl-6-O-sulfonato-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-(3-O-benzyl-2-O-sulfonato- α -L-idopyranosiduronate)-(1 $\rightarrow$ 4)-O-(2-sulfonatoimido-6-O-sulfonato-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-(3-O-benzyl-2-O-sulfonato- α -L-idopyranosiduronate)-(1 $\rightarrow$ 4)-O-(2-sulfonatoimido-6-O-sulfonato-3-O-(2-naphthylmethyl)-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-3-O-benzyl-2-O-sulfonato- α -L-idopyranosiduronate (48)

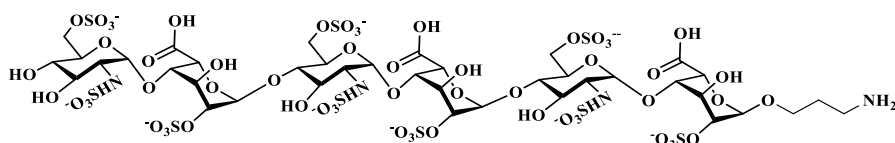


A solution of compound **47** (0.2 g, 1 eq) in THF:H₂O (1:1, 5

mL) LiOH.H₂O (20 eq) was added and kept it on stirring for room temperature. After 12 h reaction was quenched using IR H⁺ resin and reaction mixture was evaporated and purified using reverse phase C-18 column chromatography using (MeOH/H₂O 1/9 v/v). A solution of compound **48a** in THF (3 mL) 1M PMe₃.THF (8 eq) and 0.1M aqueous NaOH (10eq) was added and kept it on stirring for room temperature. After 12 h reaction was quenched using glacial acetic acid and reaction mixture solvent was evaporated and purified using reverse phase C-18 column chromatography using (MeOH/H₂O 2/3 v/v), followed by passing through DOWEX 50WX8 Na⁺ resin column. Then, a solution of compound **48b** in MeOH (3 mL) 0.1M NaOH (4 eq), triethylamine (8 eq), 10 eq of SO₃.Py was added at an interval of 1 h for 6 times by maintain 9-10 pH of reaction mixture and kept it on stirring for room temperature. The progress of reaction was monitored using TLC plate (ethyl acetate/pyridine/water/acetic acid 8/5/3/1 v/v/v/v). After completion, reaction mixture solvent was evaporated and purified using reverse phase C-18 column chromatography using (MeOH/H₂O 2/3 v/v), followed by passing through DOWEX 50WX8 Na⁺ resin column to obtain compound **48**. ^1H NMR (400 MHz,

Deuterium Oxide) δ 8.23 – 7.02 (m, 47H), 5.42 – 4.92 (m, 16H), 4.82 – 4.54 (m, 8H), 4.52 – 3.59 (m, 27H), 3.58 – 3.13 (m, 5H), 1.93 – 1.76 (m, 2H). ^{13}C NMR (151 MHz, MeOD) δ 174.8, 174.1, 172.4, 157.7, 138.7, 138.6, 138.5, 137.0, 136.7, 136.1, 133.4, 133.4, 133.2, 133.1, 133.0, 128.4, 128.4, 128.2, 128.1, 128.1, 128.0, 128.0, 127.9, 127.9, 127.9, 127.8, 127.8, 127.7, 127.7, 127.6, 127.6, 127.5, 127.4, 127.4, 127.4, 127.3, 127.3, 127.3, 127.2, 127.2, 127.1, 127.1, 127.0, 126.9, 126.5, 125.8, 125.8, 125.6, 125.4, 125.4, 125.3, 103.1, 102.0, 97.6, 96.9, 93.6, 92.7, 81.6, 80.6, 78.1, 77.7, 77.3, 76.2, 75.3, 75.1, 74.9, 74.8, 74.6, 74.4, 74.3, 73.2, 72.7, 70.5, 70.0, 69.7, 67.1, 67.0, 66.0, 65.9, 58.6, 53.8, 49.9, 48.5, 48.2, 48.1, 48.0, 47.8, 47.7, 47.5, 47.4, 47.2, 43.1, 42.1, 37.3, 29.5, 29.4, 29.3, 29.3, 21.4, 12.6, 12.5, 12.5, 10.5. HR-ESI-MS (m/z): $[\text{M}/9]^{9-}$ calcd for $\text{C}_{108}\text{H}_{111}\text{N}_4\text{O}_{60}\text{S}_9^{9-}$, 271.2333; found, 271.2321.

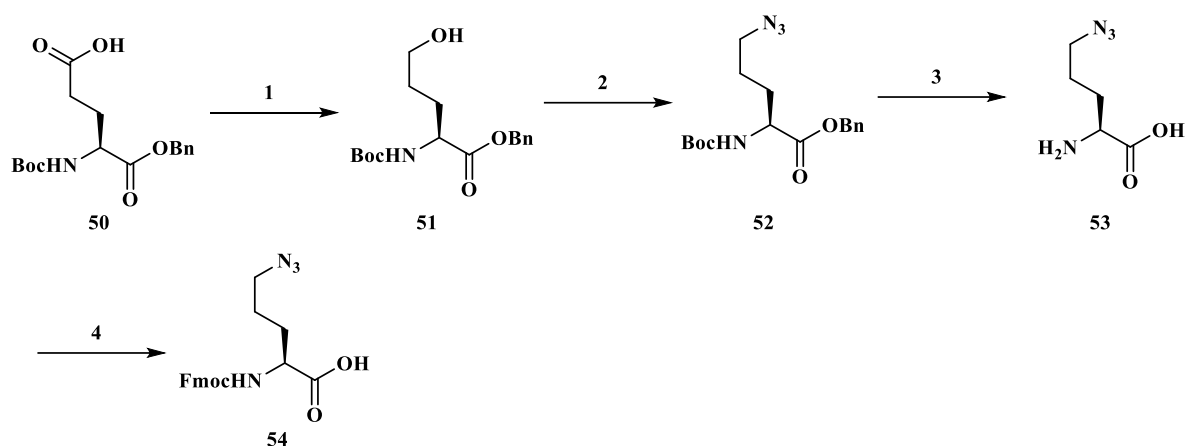
3-aminopropyl-O-[(2-sulfonatoimido-6-O-sulfonato-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-(2-O-sulfonato- α -L-idopyranosiduronate)-(1 $\rightarrow$ 4)-O-(2-sulfonatoimido-4-O-benzyl-6-O-sulfonato-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-(2-O-sulfonato- α -L-idopyranosiduronate)-(1 $\rightarrow$ 4)-O-(2-sulfonatoimido-6-O-sulfonato-2-deoxy- α -D-glucopyranosyl)]-(1 $\rightarrow$ 4)-O-2-O-sulfonato- α -L-idopyranosiduronate (49)



A solution of compound **48** in H_2O and $\text{Pd}(\text{OH})_2$ was

added and stirred under H_2 atm. After 48 h, the mixture was filtered, concentrated and eluted through Bond Elute C-18 column using H_2O as eluent. The combined H_2O fraction were pooled and lyophilized to yield fully deprotected NS-hexasaccharide. ^1H NMR (400 MHz, Deuterium Oxide) δ 5.35 (d, $J = 9.5$ Hz, 3H), 5.27 – 5.13 (m, 2H), 5.05 (s, 1H), 4.98 – 4.83 (m, 4H), 4.43 – 4.26 (m, 4H), 4.22 (d, $J = 13.9$ Hz, 4H), 4.14 – 4.09 (m, 3H), 3.95 – 3.90 (m, 1H), 3.82 (s, 3H), 3.80 – 3.67 (m, 5H), 3.65 – 3.52 (m, 4H), 3.29 3.20 (m, 3H), 3.20 – 3.03 (m, 3H), 2.06 – 1.94 (m, 2H). ^{13}C NMR (101 MHz, D_2O) δ 174.9, 174.8, 174.8, 102.2, 101.8, 97.6, 97.0, 95.2, 77.1, 76.7, 76.5, 76.2, 76.2, 76.0, 75.8, 73.2, 73.1, 72.8, 72.6, 71.1, 70.5, 69.8, 69.3, 69.2, 69.0, 68.6, 68.0, 67.7, 66.3, 66.0, 65.8, 57.9, 57.4, 54.0, 53.7, 37.6, 37.6, 26.7. HR-ESI-MS (m/z): $[\text{M}/9]^{9-}$ calcd for $\text{C}_{39}\text{H}_{57}\text{N}_4\text{O}_{58}\text{S}_9^{9-}$, 179.6917; found, 179.6895.

2.4.9 Synthesis of Amino Acid Building Block



Reagent and Yield: (1) (a) NHS, DCC (b) NaBH₄, 70 %, (2) (a) MsCl, NEt₃, 90 %, (b) NaN₃, 81 %, (3) (a) LiOH.H₂O, 91 %, (b) 40 % TFA in DCM, 94 %, (4) Fmoc-Cl, Na₂CO₃, 62 %.

Benzyl (S)-2-((tert-butoxycarbonyl)amino)-5-hydroxypentanoate (51)

The commercially available Boc-NH-Glu (COOH)-O tBu **50** (4.54 g, 15 mmol) was dissolved in 30 mL of dry THF. The resulting mixture was cooled to 0 °C using a combination of salt and ice, and 2.06 mL (15 mmol) of triethylamine was added. After a 5-minute interval, N-hydroxysuccinamide (2 equivalents) and N,N'-Dicyclohexylcarbodiimide (5 equivalents) were introduced and stirred for 12 hours. Subsequently, NaBH₄ (3 equivalents) was added to the reaction mixture and stirred for an additional 30 minutes. The progress of the reaction was monitored using TLC. Upon completion of the reaction (approximately 30 minutes), the THF solvent was evaporated under reduced pressure, and any excess NaBH₄ was neutralized using a 10% HCl solution. The resulting mixture was then subjected to extraction with ethyl acetate (3 × 50 mL). The organic layer was washed with 10% HCl solution (3 × 50 mL), 10% Na₂CO₃ (3 × 50 mL), brine solution (3 × 50 mL), and finally dried over anhydrous Na₂SO₄. The compound was purified using silica gel chromatography with ethyl acetate/hexane (1/3 v/v) to obtain compound **51**. ¹H NMR (400 MHz, Chloroform-d) δ 7.39 – 7.30 (m, 5H), 5.12 (s, 2H), 4.84 – 4.78 (m, 1H), 3.63 (t, J = 6.3 Hz, 2H), 3.59 – 3.50 (m, 1H), 2.52 – 2.42 (m, 2H), 1.95 – 1.87 (m, 1H), 1.83 – 1.75 (m, 1H), 1.43 (s, 9H). ¹³C NMR (101 MHz, CHLOROFORM-D) δ 173.6, 156.3, 135.8, 128.7, 128.4, 128.4, 79.8, 77.4, 77.1, 76.8, 66.6, 65.3, 52.3, 34.0, 31.0, 28.4, 26.3.

Benzyl (S)-5-azido-2-((tert-butoxycarbonyl)amino)pentanoate (52)

A solution of compound **51** comprising 1 equivalent in dry dichloromethane (DCM) underwent treatment with 2 equivalents of triethylamine at 0 °C. After 5 minutes of stirring, 3 equivalents

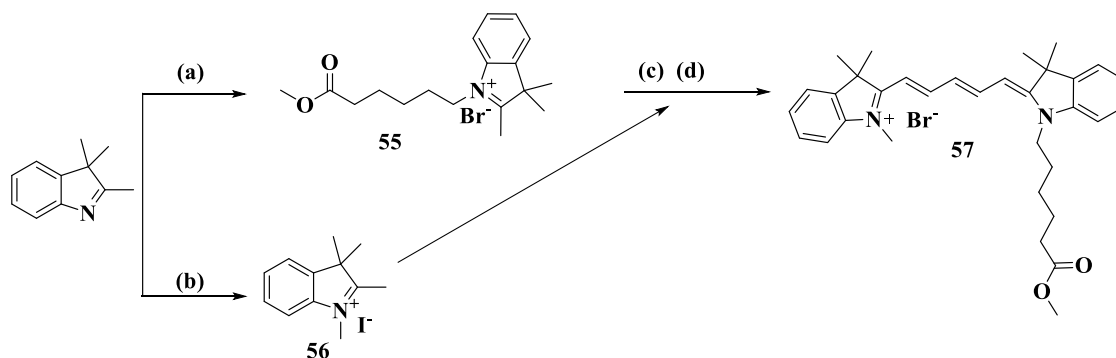
of methanesulfonyl chloride were introduced and the mixture was stirred for 45 minutes. Quenching of the reaction was achieved using 1N aqueous ammonium chloride (NH_4Cl), and the resultant mixture was subjected to thrice washing with NH_4Cl before undergoing drying over anhydrous sodium sulfate (Na_2SO_4). Subsequent purification involved silica gel chromatography utilizing an ethyl acetate/hexane solvent system (1:3 v/v). Post-purification, the compound was dissolved in dry dimethylformamide (DMF), and 2 equivalents of sodium azide (NaN_3) were incorporated, followed by stirring for 12 hours at 45 °C. To this resultant reaction mixture, 50 mL of ethyl acetate was added, followed by thrice washing with brine and final drying over Na_2SO_4 . The compound underwent another purification step using silica gel chromatography with an ethyl acetate/hexane solvent system (2:3 v/v) to obtain **52**. ^1H NMR (400 MHz, Chloroform-*d*) δ 7.41 – 7.28 (m, 5H), 5.13 (s, 2H), 4.62 (d, J = 8.1 Hz, 1H), 3.82 – 3.69 (m, 1H), 3.41 (qd, J = 12.3, 4.3 Hz, 2H), 2.45 (t, J = 7.4 Hz, 2H), 1.95 – 1.72 (m, 2H), 1.43 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3) δ 172.9, 155.3, 135.8, 128.6, 128.3, 128.3, 79.9, 77.4, 77.0, 76.7, 66.5, 54.9, 50.0, 30.8, 28.3, 27.4.

(S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-azidopentanoic acid (54)

Compound **52** (1 equivalent) was dissolved in a mixture of MeOH:THF:H₂O (1:1:1). Subsequently, 5 equivalents of LiOH.H₂O were added, and the mixture was stirred for 2 hours at room temperature. After evaporation, ethyl acetate was added, followed by three washes with 1N HCl. The resulting solution was dried over anhydrous Na_2SO_4 . Next, 40% TFA in DCM was added to the reaction mixture, and it was stirred for 2 hours at room temperature. After evaporation and vacuum drying, Compound **53** (1 equivalent) was dissolved in 20 mL of H₂O and cooled to 0 °C. To this solution, 10 mL of 10% Na_2CO_3 was added, followed by FmocCl (2 equivalents), and the reaction mixture was stirred for approximately 4 hours. The progress of the reaction was monitored by TLC. Upon completion, H₂O was evaporated under reduced pressure, and the resulting mixture was acidified with a 1N HCl solution. Extraction with ethyl acetate (3 × 50 mL) followed, and the combined organic layer was washed with a brine solution (3 × 50 mL). After drying over anhydrous Na_2SO_4 , the organic layer was evaporated under reduced pressure. The compound was then purified using silica gel chromatography with ethyl acetate/hexane (2/3 v/v) to obtain compound **54**. ^1H NMR (400 MHz, Chloroform-*d*) δ 7.76 (d, J = 7.5 Hz, 2H), 7.57 (dd, J = 18.3, 7.0 Hz, 2H), 7.40 (t, J = 7.5 Hz, 2H), 7.31 (t, J = 7.5 Hz, 2H), 5.38 (d, J = 8.1 Hz, 1H), 4.43 (t, J = 6.2 Hz, 2H), 4.22 (t, J = 6.6 Hz, 1H), 3.41 – 3.09 (m, 2H), 1.96 (s, 1H), 1.84 – 1.35 (m, 4H). ^{13}C NMR (101 MHz,

CHLOROFORM-D) δ 176.4, 156.2, 143.9, 143.7, 141.4, 127.9, 127.2, 127.2, 125.1, 125.1, 120.1, 120.1, 77.5, 77.1, 76.8, 67.2, 53.3, 50.9, 47.2, 29.8, 29.7, 24.9.

2.4.10 Synthesis of Cy-5 Dye



Reagent and Yield: (a) 6, 1,2- dichlorobenzene, 120 °C, 4 h (b) Methyl iodide, ACN, reflux, 7 h (c) Malonaldehyde bis(phenylimine)monohydrochloride, Acetic anhydride, reflux, 1 h (d) 7, Py., rt, 2 h

1-(6-methoxy-6-oxohexyl)-2,3,3-trimethyl-3H-indol-1-ium bromide (55)

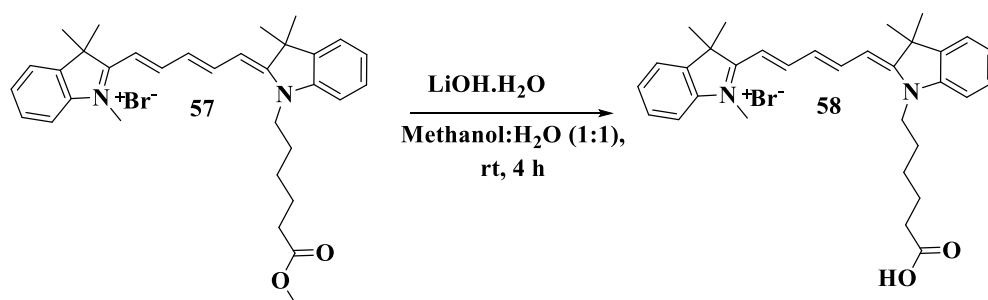
Commercially available 2,3,3-trimethyl-3H-indole (5 g, 31.4 mmol, 1 eq) and commercially available, methyl 6 – bromohexanoate (62.8 mmol, 2 eq) was dissolved in 1,2 – dichlorobenzene and it for stirring at 120 °C under nitrogen atmosphere for 4 h. After completion of reaction (~ 4 h), solvent was evaporated under reduce pressure at 52 °C. Reaction mixture was redissolved into minimum amount of cold chloroform (~5 mL), then cold acetone was added to precipitate the product. It was washed with cold acetone 3 times. After washing wine red paste of product was obtained. Yield 3.5 g (30 %). It was confirmed by HRMS. HR-ESI-MS (m/z): Calcd for $C_{18}H_{26}N_4O_2^+ [M]^+$ is 288.1958, found is 288.1956.

1,2,3,3-tetramethyl-3H-indol-1-ium iodide (56)

Commercially available 2,3,3-trimethyl-3H-indole (5 g, 31.4 mmol, 1 eq) and methyl iodide (62.8 mmol, 2 eq) was dissolved in ACN and it for stirring at 90 °C under nitrogen atmosphere for 7 h. After completion of reaction (~ 7 h), solvent was evaporated under reduce pressure. Reaction mixture was redissolved into minimum amount of cold chloroform (~5 mL), then cold acetone was added to precipitate the product. It was washed with cold acetone 3 times. After washing light maroon crystal of product was obtained. Yield 4 g (43 %). It was confirmed by HRMS. HR-ESI-MS (m/z): Calcd for $C_{12}H_{16}N^+ [M]^+$ is 174.1277, found is 174.1269.

2-((1E,3E)-5-((Z)-1-(6-methoxy-6-oxohexyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-3H-indol-1-ium iodide (57)

Compound **56** (2 g, 6.6 mmol, 1 eq) and commercially available Malonaldehyde bis(phenylimine)monohydrochloride (8 mmol, 1.2 eq) was dissolved in acetic anhydride (10 mL) and refluxed it for 1 h. After (~ 1 h), reaction mixture was cool down to rt. Then compound **55** (13.2 mmol, 2 eq) was dissolved in pyridine (15 mL) followed by dropwise addition to reaction mixture at rt and stirred it 2 h. After completion of reaction (~ 2 h), solvent was evaporated under reduced pressure. After that reaction mixture was redissolved in DCM (~ 10 mL) and organic layer was washed with 10 % HCl 3 times, 3 times with 10 % NaHCO₃, 3 times with brine and dried over Na₂SO₄. The crude compound was purified through column chromatography in MeOH/ DCM (8/100) solvent system to get pure compound **57**. Yield 825 mg (21 %). ¹H NMR (400 MHz, Chloroform-d) δ 8.15 (t, J = 13.0, 2H), 7.39 – 7.30 (m, 4H), 7.23 – 7.16 (m, 2H), 7.13 – 7.02 (m, 2H), 6.83 (t, J = 12.4 Hz, 1H), 6.31 (t, J = 13.6, 2H), 4.05 (t, J = 7.6 Hz, 2H), 3.69 (s, 3H), 3.63 (s, 3H), 2.33 (t, J = 7.3 Hz, 2H), 1.86 – 1.77 (m, 3H), 1.74 (d, J = 11.5 Hz, 12H), 1.71 – 1.64 (m, 3H), 1.55 – 1.45 (m, 2H). ¹³C NMR (100 MHz, Chloroform-d) δ 173.88, 172.89, 153.83, 142.74, 142.01, 141.35, 141.02, 128.61, 128.57, 126.44, 125.17, 125.10, 122.36, 122.21, 110.49, 110.42, 104.09, 103.66, 51.60, 49.44, 49.36, 44.25, 33.68, 32.49, 28.19, 28.01, 27.13, 26.38, 24.55. HR-ESI-MS (m/z): Calcd for C₃₃H₄₁N₂O₂⁺ [M]⁺ is 497.3163, found is 497.3161.

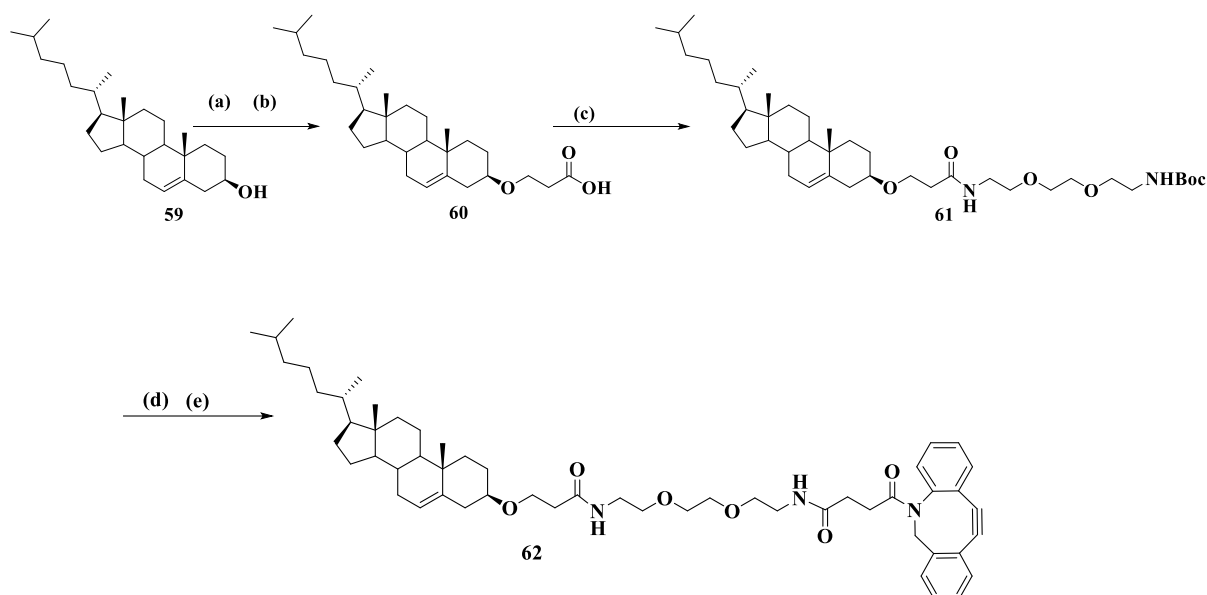


2-((1E,3E)-5-((Z)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-3H-indol-1-ium iodide (58)

Compound **57** (825 mg, 1.32 mmol, 1 eq) was dissolved in MeOH: H₂O (1:1) followed by addition of LiOH.H₂O (2.64 mmol, 2 eq) and stirred it at rt for 4 h. After completion of reaction (~ 4 h), organic solvent was evaporated under reduced pressure. Then the aqueous layer was washed with DCM 3 times (~ 30 mL) and later the organic layer was washed 10 % HCl 3 times (~ 50 mL), 3 times with brine and dried over Na₂SO₄. The crude compound was purified

through column chromatography in MeOH/ DCM (1/10) solvent system to get pure compound **58**. Yield 757 mg (94 %). ^1H NMR (400 MHz, Chloroform- d) δ 7.90 (t, J = 13.0, 2H), 7.41 – 7.27 (m, 4H), 7.21 – 7.13 (m, 2H), 7.07 (dd, J = 13.0, 7.9 Hz, 2H), 6.93 (t, J = 12.5 Hz, 1H), 6.54 (d, J = 13.6 Hz, 1H), 6.38 (d, J = 13.6 Hz, 1H), 4.01 (t, J = 7.7 Hz, 2H), 3.68 (s, 3H), 2.52 (t, J = 7.0 Hz, 2H), 1.78 (m, 5H), 1.67 (d, J = 3.4 Hz, 12H), 1.55 (m, 2H). ^{13}C NMR (101 MHz, Chloroform- d) δ 173.5, 172.8, 153.7, 153.7, 142.8, 142.0, 141.2, 141.0, 128.8, 128.7, 126.4, 125.2, 122.3, 122.2, 110.7, 110.6, 104.2, 103.7, 77.6, 77.3, 76.9, 50.3, 49.4, 49.3, 44.2, 34.7, 32.0, 31.9, 30.4, 29.7, 29.7, 28.1, 28.0, 26.8, 26.3, 24.5, 14.2. HR-ESI-MS (m/z): Calcd for $\text{C}_{32}\text{H}_{39}\text{N}_2\text{O}_2^+ [\text{M}]^+$ is 483.3006, found is 483.2998.

2.4.11 Synthesis of Cholesterol DBCO



Reagent and Yield: (a) tert-butyl acrylate, NaH, THF, rt, 16 h (b) 40 % TFA in DCM, rt, 2 h (c) tert-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate, DCC, DCM, 0 °C, 12 h (d) 40 % TFA in DCM, rt, 2 h (e) NHS – DBCO, NEt_3 , DCM, rt, 12 h.

3-(((3R,10S,13S,17S)-10,13-dimethyl-17-((S)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthrene-3-yl)oxy)propanoic acid (60)

Commercially available cholesterol (5 g, 12.9 mmol, 1 eq) was dissolved in 50 mL of THF, then NaH (181 mmol, 14 eq) was added in 4 portions. After that tert-butyl acrylate (3.9 mmol, 0.3 eq) was dissolved in 20 mL of THF and addition was done in dropwise manner. The progress of reaction was monitored by TLC. After completion of reaction (~ 16 h), reaction

was quenched by addition of 15 mL of MeOH and solvent was evaporated under reduce pressure. Then reaction mixture was redissolved in DCM and it's washed with 10 % HCl 3 times, 3 times with brine and dried over Na₂SO₄. The crude compound was purified through column chromatography in ethyl acetate / hexane (4/10) solvent system to get pure compound. Yield 1.1 g (55 %). After that, product (1.1 g, 2.2 mmol, 1 eq) was dissolved in 40 % TFA in DCM and stirred it for 2 h at rt. The progress of reaction was monitored by TLC. After completion of reaction (~ 2 h), solvent was evaporated under reduce pressure. Then reaction mixture was redissolved into DCM (~ 50mL) and its's washed with 10 % NaHCO₃ 3 times, 3 times with brine and dried over Na₂SO₄. The crude compound was purified through column chromatography in ethyl acetate / hexane (6/10) solvent system to get pure compound **60**. Yield 897 mg (90 %). ¹H NMR (400 MHz, Chloroform-d) δ 5.35 – 5.34 (m, 1H), 3.76 (t, J = 6.3, 2H), 3.20 (m, 1H), 2.62 (t, J = 6.3 Hz, 2H), 2.39 – 2.34 (m, 1H), 2.24 – 2.13 (m, 1H), 2.06 – 1.93 (m, 2H), 1.92 – 1.76 (m, 3H), 1.62 – 1.42 (m, 7H), 1.41 – 1.23 (m, 5H), 1.22 – 1.01 (m, 8H), 0.99 (s, 4H), 0.91 (d, J = 6.6 Hz, 3H), 0.86 (dd, J = 6.6, 1.8 Hz, 6H), 0.67 (s, 3H). ¹³C NMR (101 MHz, CHLOROFORM-D) δ 176.6, 140.7, 121.9, 79.7, 77.4, 77.1, 76.8, 63.1, 56.8, 56.2, 50.2, 42.4, 39.9, 39.6, 39.0, 37.2, 36.9, 36.3, 35.9, 35.3, 32.0, 32.0, 28.3, 28.3, 28.1, 24.4, 23.9, 22.9, 22.7, 21.1, 19.5, 18.8, 11.9, 0.1. HR-ESI-MS (m/z): Calcd for C₃₀H₅₀O₃ [M] is 458.3760, found is 458.3759.

N-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-3-(((3R,10S,13S,17S)-10,13-dimethyl-17-((S)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)propenamide (61)

Compound **77** (897 mg, 1.9 mmol, 1 eq) was dissolved in 10 mL of DCM. Then, DCC (2.9 mmol, 1.5 eq) and tert-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl) carbamate (2.9 mmol, 1.5 eq) was added at 0 oC under nitrogen atmosphere. The progress of reaction was monitored by TLC. After completion of reaction (~ 12 h), solvent was evaporated under reduced pressure. The crude compound was purified through column chromatography in ethyl acetate / hexane (8/10) solvent system to get pure compound **61**. Yield 1.1 mg (80 %). ¹H NMR (400 MHz, Chloroform-d) δ 6.85 (s, 1H), 5.29 (d, J = 5.1 Hz, 1H), 5.09 (t, J = 5.8 Hz, 1H), 3.68 (t, J = 5.9 Hz, 2H), 3.56 (s, 4H), 3.53 – 3.48 (m, 4H), 3.42 (q, J = 5.2 Hz, 2H), 3.27 (q, J = 5.4 Hz, 2H), 3.18 – 3.10 (m, 1H), 2.42 (t, J = 5.8 Hz, 2H), 2.33 – 2.28 (m, 1H), 2.17 – 2.10 (m, 1H), 1.98 – 1.72 (m, 5H), 1.51 – 1.42 (m, 5H), 1.39 (s, 12H), 1.34 – 1.24 (m, 3H), 1.23 – 1.19 (m, 2H), 1.12 – 1.05 (m, 4H), 1.04 – 0.97 (m, 3H), 0.95 (s, 3H), 0.87 (d, J = 6.5 Hz, 3H), 0.81 (dd, J = 6.6, 1.8 Hz, 6H), 0.63 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 172.0, 156.0, 140.5, 121.9, 79.4,

77.4, 77.1, 76.8, 70.2, 70.1, 69.8, 63.9, 56.7, 56.1, 50.1, 42.3, 40.3, 39.7, 39.5, 39.1, 39.0, 37.2, 37.1, 36.8, 36.2, 35.8, 31.9, 31.8, 28.4, 28.3, 28.2, 28.0, 24.3, 23.8, 22.8, 22.5, 21.0, 19.4, 18.7, 11.8. HR-ESI-MS (m/z): Calcd for C₄₁H₇₂N₂O₆ [M] is 688.5390, found is 688.5381.

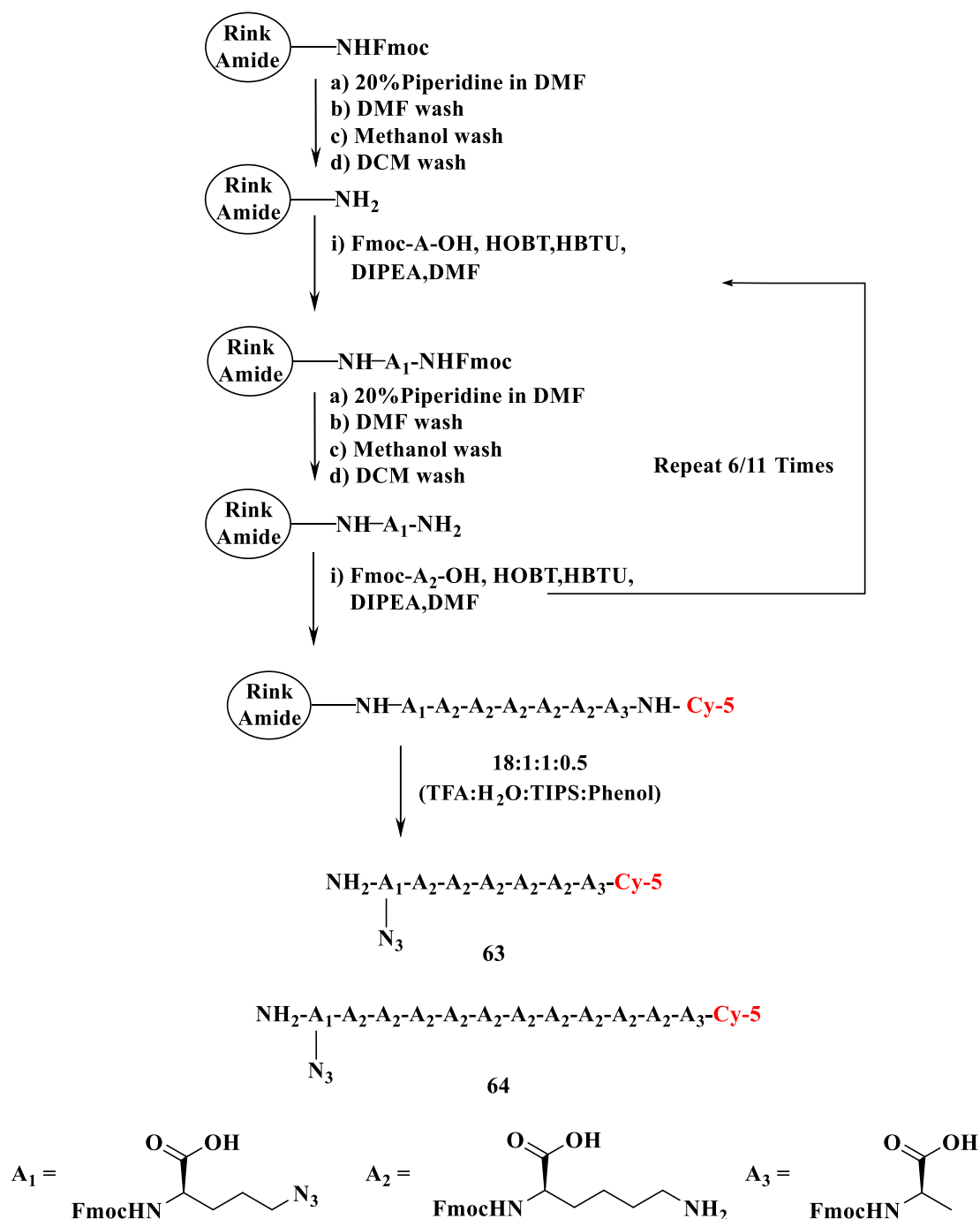
Synthesis of Compound 62:

After that Boc group was deprotected using 40 % TFA in DCM. The crude compound was purified by column chromatography ethyl acetate/ hexane (8/10). Yield 844 mg (90 %). Compound 6 (6 mg, 50.9 μ mol, 1eq) was dissolved in DCM followed by dropwise addition of NEt₃ (56 μ mol, 1.1 eq). After that NHS – DBCO (45.8 μ mol, 0.9 eq) was added and stirred for 12 h at rt. The progress of reaction was monitored using TLC. After completion of reaction (~12 h), solvent was evaporated under reduce pressure. The crude compound was purified through column chromatography in ethyl acetate/ hexane (1/2) solvent system to get pure compound **62**. Yield 8.1 mg (94 %). ¹H NMR (400 MHz, Chloroform-d) δ 7.66 (d, J = 7.3 Hz, 1H), 7.55 – 7.43 (m, 1H), 7.41 – 7.26 (m, 5H), 7.25 (d, J = 1.7 Hz, 1H), 6.81 (t, J = 5.6 Hz, 1H), 6.26 (t, J = 5.5 Hz, 1H), 5.35 – 5.29 (m, 1H), 5.14 (d, J = 13.9 Hz, 1H), 3.74 – 3.62 (m, 3H), 3.62 – 3.50 (m, 6H), 3.49 – 3.37 (m, 4H), 3.33 (dd, J = 5.7, 4.1 Hz, 2H), 3.20 – 3.12 (m, 1H), 2.84 – 2.77 (m, 1H), 2.48 – 2.38 (m, 3H), 2.36 – 2.30 (m, 1H), 2.19 – 2.14 (m, 1H), 2.07 – 1.75 (m, 8H), 1.63 – 1.28 (m, 9H), 1.25 (s, 3H), 1.19 – 0.97 (m, 6H), 0.97 (s, 3H), 0.91 (d, J = 6.5 Hz, 3H), 0.86 (dd, J = 6.6, 1.8 Hz, 6H), 0.66 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 171.2, 170.8, 161.4, 150.3, 147.1, 131.2, 128.4, 127.7, 127.2, 127.1, 126.7, 126.0, 124.5, 122.2, 121.4, 120.8, 117.3, 114.4, 113.6, 106.9, 76.4, 76.1, 75.8, 63.0, 55.7, 55.1, 54.5, 51.6, 49.1, 41.3, 38.7, 38.5, 38.1, 38.0, 36.1, 35.8, 35.2, 34.8, 30.9, 30.2, 29.2, 28.7, 27.2, 27.0, 24.3, 23.3, 22.8, 21.8, 21.5, 20.0, 18.4, 17.7, 10.8, 7.5, 6.5. Calcd for C₅₅H₇₇N₃O₆ [M] is 875.5812, found is 875.5801.

2.4.12 Synthesis of Peptide Backbone

A 5mer & 12mer peptide was manually synthesized via solid-phase peptide synthesis on rink amide resin at a 0.3 mmol scale, employing standard Fmoc conditions. The coupling reagents consisted of a combination of HBTU and HOBT, with DIPEA serving as the activator base. NMP was utilized as the coupling solvent, and 20% piperidine in DMF was applied for Fmoc group deprotection. To remove the peptides from the solid support, the resin underwent stirring for approximately 2 hours in a 10 mL cocktail containing trifluoroacetic acid (TFA)/triisopropylsilane/water/phenol (90:5:2.5:2.5). After filtration of the resin, the crude

peptides in the filtrate were evaporated under reduced pressure, and the residue was triturated with 20 mL of cold diethyl ether. The resulting precipitate was separated through centrifugation. The crude peptides were subsequently dissolved in 10 mL of MeOH and subjected to purification on a C-18 column using reversed-phase HPLC. A MeOH and water gradient system containing 0.1% TFA was employed for peptide purification. Confirmation of the purified peptides was accomplished through IR, NMR, and HR-ESI-MS.



7-mer Peptide Backbone (63)

^1H NMR (400 MHz, Acetonitrile- d_3) δ 8.21 (t, J = 13.1 Hz, 2H), 8.15 – 7.92 (m, 7H), 7.75 – 7.63 (m, 11H), 7.61 (m, 3H), 7.55 (m, 2H), 7.39 (t, J = 7.4 Hz, 4H), 6.68 (t, J = 12.4 Hz, 1H), 6.34 (dd, J = 13.8, 5.2 Hz, 2H), 4.36 (m, 8H), 4.14 (t, J = 7.5 Hz, 4H), 3.68 (s, 3H), 3.11 (m, 2H), 3.06 (m, 10H), 2.37 (t, J = 7.5 Hz, 2H), 2.11 (p, J = 2.5 Hz, 2H), 1.98 – 1.82 (m, 11H), 1.81 (s, 12H), 1.79 – 1.69 (m, 13H), 1.61 – 1.46 (m, 11H), 1.45 – 1.41 (m, 3H). ^{13}C NMR (151 MHz, Acetonitrile- d_3) δ 175.9, 175.1, 174.7, 174.0, 173.3, 161.7, 154.8, 154.7, 143.9, 143.2, 142.3, 142.2, 129.6, 129.4, 129.4, 126.0, 125.8, 125.4, 123.1, 123.0, 118.4, 118.3, 118.1, 116.9, 115.4, 111.7, 111.7, 104.0, 103.6, 56.2, 53.6, 51.6, 51.0, 50.3, 50.1, 50.0, 49.8, 49.7, 49.2, 49.1, 49.0, 49.0, 48.8, 48.7, 48.5, 48.4, 47.7, 45.8, 44.7, 43.2, 40.1, 35.9, 31.7, 31.2, 30.8, 30.7, 30.7, 29.6, 27.7, 27.6, 27.4, 27.0, 26.0, 25.8, 23.2, 23.2, 17.1. and HR-ESI-MS Calcd for $\text{C}_{70}\text{H}_{113}\text{N}_{18}\text{O}_8^+ [\text{M}]$ is 1334.5291, found is 1334.5280.

12-mer Peptide Backbone (64)

^1H NMR (600 MHz, Methanol- d_4) δ 8.13 (t, J = 11.3 Hz, 2H), 8.05 – 7.82 (m, 5H), 7.37 (dd, J = 7.6, 4.5 Hz, 2H), 7.30 (tt, J = 7.7, 1.9 Hz, 2H), 7.16 (dt, J = 14.6, 7.3 Hz, 4H), 6.55 – 6.46 (m, 1H), 6.16 (dd, J = 13.7, 4.0 Hz, 1H), 4.34 – 3.76 (m, 15H), 3.52 (s, 3H), 3.20 (dq, J = 4.4, 2.7, 2.2 Hz, 3H), 2.82 (h, J = 6.8 Hz, 23H), 2.23 (dt, J = 12.3, 6.1 Hz, 2H), 1.88 – 1.65 (m, 29H), 1.64 – 1.51 (m, 47H), 1.51 – 1.43 (m, 6H), 1.42 – 1.28 (m, 17H). ^{13}C NMR (151 MHz, MeOD) δ 176.4, 175.8, 175.7, 175.6, 175.2, 175.2, 175.1, 175.1, 175.0, 174.8, 174.6, 174.5, 174.1, 173.6, 173.4, 173.1, 161.3, 161.0, 158.0, 157.5, 157.2, 156.9, 154.2, 154.0, 142.8, 142.2, 141.2, 141.1, 128.6, 128.3, 125.2, 125.2, 124.9, 124.8, 122.0, 121.9, 119.6, 117.6, 117.5, 115.7, 115.7, 115.6, 114.3, 113.7, 111.8, 110.5, 110.5, 103.1, 102.8, 56.8, 56.7, 56.5, 56.3, 56.1, 55.4, 54.8, 54.7, 54.5, 53.8, 53.7, 53.4, 53.3, 53.1, 53.0, 52.8, 50.8, 50.7, 49.1, 49.1, 48.5, 48.1, 47.9, 47.8, 47.7, 47.5, 47.4, 47.2, 43.5, 39.2, 39.1, 38.9, 35.1, 30.2, 30.1, 29.9, 29.7, 29.5, 28.7, 27.5, 26.9, 26.7, 26.6, 26.4, 26.3, 25.4, 25.3, 25.1, 25.0, 23.1, 23.0, 22.9, 22.8, 22.7, 22.6, 22.3, 15.9. HR-ESI-MS Calcd for $\text{C}_{100}\text{H}_{173}\text{N}_{28}\text{O}_{13}^+ [\text{M}]^+$ is 1974.3731, found is 1974.3710.

2.4.13 Synthesis Protocol for Cholesterol Conjugation

General Procedure for Conjugation of Cholesterol-DBCO

A 7-mer peptide and 12-mer peptide (1 equivalent) was dissolved in 1 mL of MeOH, while cholesterol-DBCO (1 equivalent) was dissolved in 1 mL of DCM. The subsequent step involved the gradual addition of the cholesterol-DBCO solution into the peptide solution, with continuous stirring for 12 hours at room temperature. The progress of the reaction was tracked

through MALDI-TOF/TOF analysis. Upon completion of the reaction (approximately 12 hours), the solvent was evaporated under reduced pressure. The crude compound underwent purification via C-18 bond elute column chromatography in a MeOH/H₂O (6/10) solvent system, yielding the pure cholesterol-conjugated 7-mer peptide (**82** and 12-mer peptide (**83**) compound. Confirmation was accomplished through IR, NMR, and HR-ESI-MS analyses.

7-mer Peptide with Cholesterol (**65**)

¹H NMR (400 MHz, Methanol-d₄) δ 8.26 (t, *J* = 13.0, Hz, 2H), 8.00 – 7.80 (m, 1H), 7.72 – 7.54 (m, 4H), 7.51 – 7.49 (m, 2H), 7.46 – 7.34 (m, 4H), 7.34 – 7.20 (m, 6H), 6.62 (t, *J* = 12.4 Hz, 1H), 6.29 – 6.26 (d, *J* = 13.8 Hz, 2H), 5.35 (d, *J* = 4.9 Hz, 1H), 4.63 – 4.20 (m, 14H), 4.17 – 4.07 (m, 2H), 3.75 – 3.70 (m, 2H), 3.63 (s, 3H), 3.60 (d, *J* = 5.9 Hz, 3H), 3.56 – 3.48 (m, 4H), 3.38 – 3.35 (m, 3H), 3.19 – 3.13 (m, 1H), 2.98 – 2.89 (m, 10H), 2.44 – 2.27 (m, 7H), 2.17 – 1.81 (m, 17H), 1.73 (s, 12H), 1.71 – 1.62 (m, 15H), 1.57 – 1.42 (m, 19H), 1.40 – 1.29 (m, 10H), 1.21 – 1.03 (m, 8H), 1.00 (s, 3H), 0.94 (d, *J* = 6.5 Hz, 3H), 0.88 (dd, *J* = 6.6, 1.4 Hz, 6H), 0.72 (s, 3H). ¹³C NMR (151 MHz, Methanol-d₄) δ 174.8, 174.2, 173.1, 172.8, 161.7, 161.5, 154.3, 154.0, 142.8, 142.2, 141.2, 141.1, 140.5, 128.4, 125.2, 125.0, 124.8, 122.1, 121.9, 121.4, 117.8, 115.9, 110.5, 103.1, 102.7, 79.2, 69.9, 69.2, 63.7, 56.8, 56.2, 53.4, 50.3, 49.2, 49.1, 48.2, 48.0, 47.9, 47.7, 47.6, 47.5, 47.3, 47.2, 43.4, 42.1, 39.7, 39.3, 39.1, 38.8, 37.0, 36.6, 36.6, 36.0, 35.7, 35.0, 31.8, 31.6, 30.8, 30.1, 28.1, 27.9, 27.8, 26.9, 26.6, 26.4, 26.1, 25.1, 23.9, 23.5, 22.4, 21.8, 21.5, 20.8, 18.5, 17.8, 16.3, 16.0, 10.9. and HR-ESI-MS Calcd for C₁₂₅H₁₉₀N₂₁O₁₄⁺ [M] is 2209.4796, found is 2210.4816 (M+H).

12-mer Peptide with Cholesterol (**66**)

¹H NMR (600 MHz, Methanol-d₄) δ 8.31 – 8.19 (m, 2H), 7.94 (d, *J* = 77.2 Hz, 2H), 7.71 – 7.52 (m, 4H), 7.50 (dd, *J* = 7.6, 2.2 Hz, 3H), 7.45 – 7.36 (m, 5H), 7.33 – 7.25 (m, 6H), 6.63 (dd, *J* = 13.9, 11.1 Hz, 1H), 6.32 – 6.26 (m, 2H), 5.37 – 5.32 (m, 1H), 4.31 – 3.97 (m, 17H), 3.64 (s, 3H), 3.62 – 3.48 (m, 11H), 2.97 – 2.93 (m, 28H), 2.46 – 2.27 (m, 9H), 2.00 – 1.78 (m, 36H), 1.73 (d, *J* = 3.3 Hz, 17H), 1.62 – 1.67 (m, 28H), 1.63 – 1.36 (m, 48H), 1.29 (s, 3H), 1.21 – 1.01 (m, 10H), 1.00 (s, 3H), 0.94 (d, *J* = 6.5 Hz, 4H), 0.89 (d, *J* = 2.2 Hz, 4H), 0.88 (d, *J* = 2.2 Hz, 4H), 0.71 (s, 3H). ¹³C NMR (151 MHz, MeOD) δ 174.2, 174.2, 174.2, 173.1, 172.9, 162.0, 161.8, 161.5, 161.3, 154.3, 154.0, 142.8, 142.2, 141.2, 141.1, 140.5, 131.1, 129.8, 129.8, 129.1, 128.4, 127.1, 126.8, 125.2, 125.0, 124.8, 122.1, 121.9, 121.4, 120.3, 119.7, 117.8, 115.9, 113.9, 110.5, 103.1, 102.7, 79.3, 69.9, 69.9, 69.8, 69.2, 69.2, 65.1, 63.7, 62.7, 56.8, 56.2, 50.3, 49.2, 49.1, 48.5, 48.1, 47.9, 47.8, 47.6, 47.5, 47.4, 47.2, 43.4, 42.1, 39.7, 39.3, 39.1, 39.0, 39.0, 38.8,

37.0, 36.6, 36.6, 36.0, 35.7, 35.1, 33.6, 30.4, 30.3, 30.2, 29.6, 29.4, 29.4, 29.3, 29.2, 29.1, 29.0, 28.8, 28.0, 27.9, 27.7, 26.9, 26.7, 26.6, 26.4, 26.3, 25.1, 24.6, 23.9, 23.5, 22.8, 22.7, 22.7, 22.6, 22.3, 21.8, 21.6, 20.8, 18.5, 17.9, 13.1, 10.9. HR-ESI-MS Calcd for $C_{155}H_{252}N_{31}O_{19}^+$ $[M]^+$ is 2851.9700, found is 2852.9751 (M+H).

General Procedure for Conjugation of 3-Azidopropanoate Linker Conjugation

A 12-mer peptide, combined with cholesterol (1 equivalent), was dissolved in 1 mL of DMF. Subsequently, 40 μ L of DIPEA was added to the solution, followed by the gradual addition of a solution containing 2,5-dioxopyrrolidin-1-yl 3-azidopropanoate (10 equivalents per NH_2) in 1 mL of DMF. The resulting peptide solution was stirred for 12 hours at room temperature. The progression of the reaction was monitored through MALDI-TOF/TOF analysis. After the reaction's completion (approximately 12 hours), the solvent was evaporated under reduced pressure. The crude compound underwent purification through LH – 20 size exclusion column chromatography, utilizing MeOH as the solvent system. Additionally, it was passed through Amberlite® IR120, Na^+ form, to obtain the pure compound. Confirmation was done by IR, NMR, and HR-ESI-MS analyses.

7-mer Peptide with 3-Azidopropanoate Linker (67)

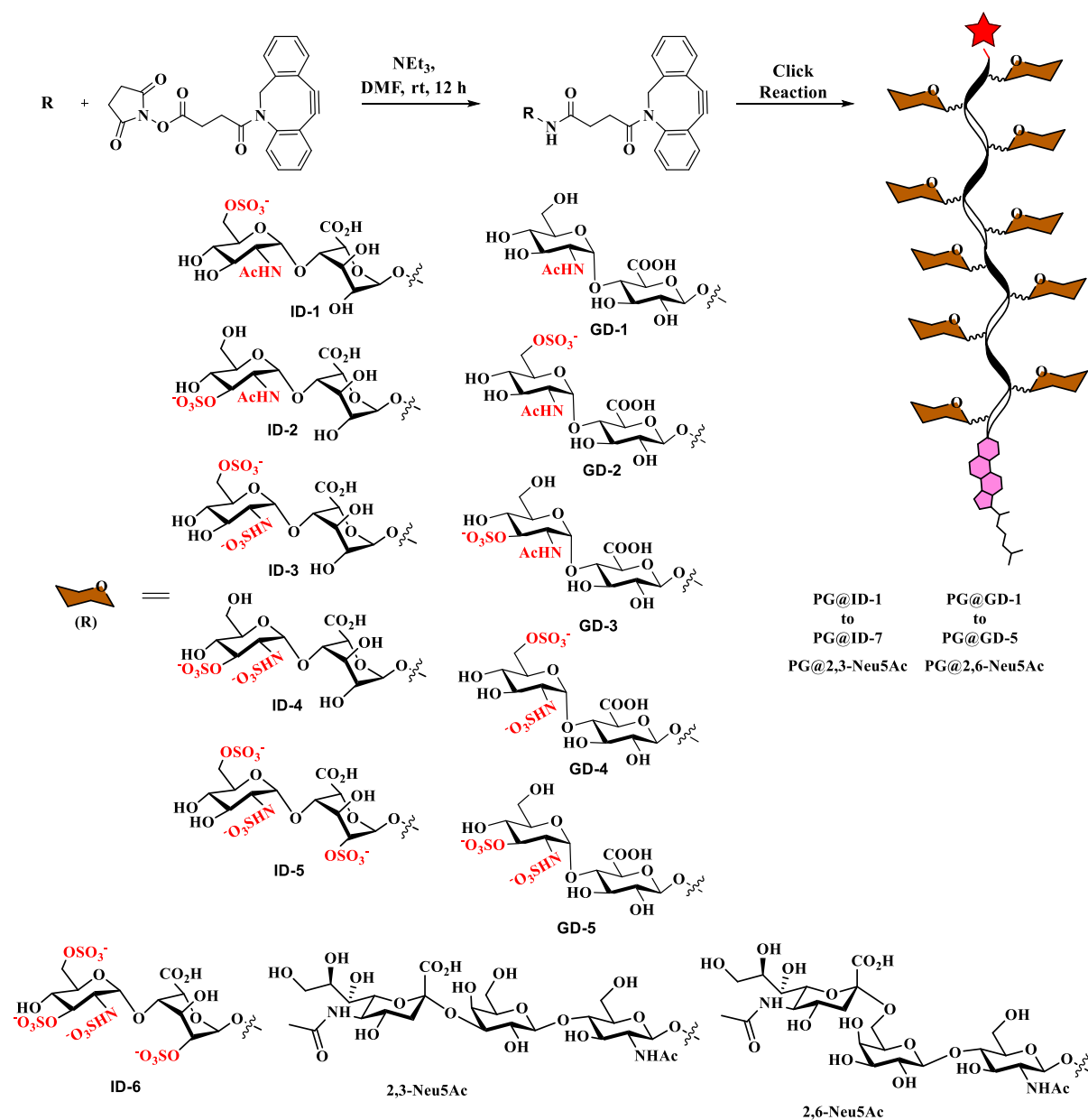
1H NMR (600 MHz, Methanol- d_4) δ 8.36 – 8.20 (m, 1H), 7.78 – 7.73 (m, 4H), 7.60 – 7.45 (m, 4H), 6.45 – 6.17 (m, 1H), 5.57 (d, J = 5.1, 1H), 4.40 – 4.26 (m, 8H), 4.11 – 3.96 (m, 8H), 3.93 – 3.89 (m, 7H), 3.86 – 3.82 (m, 9H), 3.81 – 3.76 (m, 12H), 3.44 – 3.38 (m, 8H), 3.12 – 3.05 (m, 14H), 2.94 (s, 10H), 2.89 – 2.75 (m, 10H), 2.70 – 2.56 (m, 19H), 2.50 – 2.46 (m, 2H), 2.41 – 2.18 (m, 6H), 2.14 – 2.05 (m, 10H), 1.97 – 1.93 (m, 10H), 1.80 – 1.72 (m, 10H), 1.70 – 1.57 (m, 16H), 1.52 – 1.50 (m, 8H), 1.42 – 1.23 (m, 9H), 1.18 – 1.14 (m, 3H), 1.11 – 1.09 (m, 6H), 0.32 (s, 3H). ^{13}C NMR (151 MHz, MeOD) δ 175.2, 174.2, 173.3, 171.8, 162.6, 143.3, 142.6, 141.7, 141.5, 141.1, 126.0, 125.8, 122.9, 122.7, 122.4, 111.3, 80.1, 78.4, 78.2, 78.0, 70.7, 70.4, 70.2, 65.8, 64.5, 63.6, 57.4, 56.8, 55.0, 54.8, 54.5, 50.8, 49.9, 49.4, 49.3, 49.1, 49.0, 48.9, 48.7, 48.6, 48.1, 44.3, 42.9, 40.4, 40.1, 39.7, 39.5, 37.7, 37.5, 37.4, 37.0, 36.7, 36.4, 35.9, 34.6, 32.5, 32.5, 32.3, 31.5, 30.6, 30.2, 30.1, 30.0, 29.9, 29.8, 29.6, 29.6, 28.8, 28.8, 28.6, 28.5, 28.1, 28.0, 26.0, 25.8, 25.4, 24.8, 24.3, 23.2, 23.1, 22.8, 21.6, 19.7, 19.1, 17.2, 14.3, 12.2, 1.4. and HR-ESI-MS Calcd for $C_{140}H_{205}N_{36}O_{19}^+$ $[M]^+$ is 2694.6176, found is 2694.6153.

12-mer Peptide with 3-Azidopropanoate Linker (68)

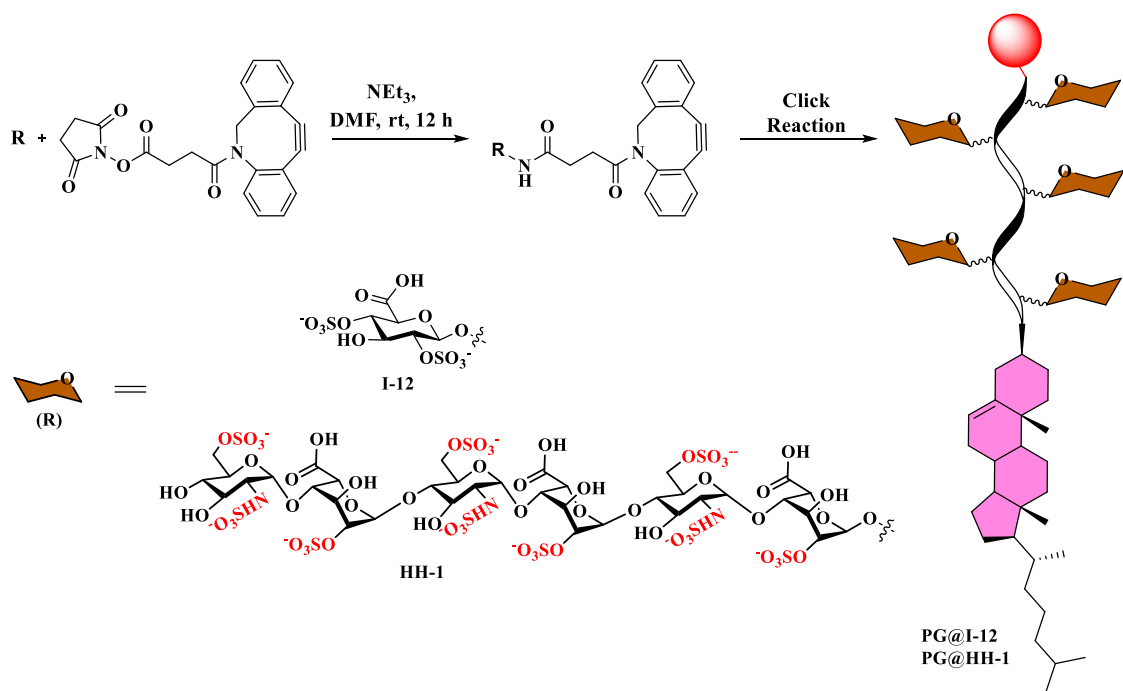
^1H NMR (600 MHz, Methanol- d_4) δ 7.71 (td, J = 14.7, 13.5, 8.4 Hz, 5H), 7.38 – 7.28 (m, 2H), 7.21 (d, J = 8.9 Hz, 1H), 7.14 (q, J = 7.4 Hz, 4H), 7.10 – 6.90 (m, 6H), 5.93 (dd, J = 21.7, 13.6 Hz, 1H), 5.05 (d, J = 5.0 Hz, 1H), 3.89 – 3.75 (m, 4H), 3.68 – 3.52 (m, 11H), 3.47 – 3.29 (m, 18H), 3.27 (dd, J = 11.4, 5.9 Hz, 28H), 2.99 – 2.79 (m, 24H), 2.62 – 2.47 (m, 7H), 2.21 – 2.12 (m, 23H), 1.69 – 1.48 (m, 25H), 1.45 (d, J = 3.6 Hz, 13H), 1.35 – 1.18 (m, 39H), 1.19 – 1.07 (m, 19H), 0.97 (s, 14H), 0.90 – 0.69 (m, 15H), 0.63 (d, J = 6.5 Hz, 4H), 0.57 (dd, J = 6.6, 2.6 Hz, 8H), 0.40 (s, 3H). ^{13}C NMR (151 MHz, MeOD) δ 175.8, 175.5, 175.4, 175.2, 174.5, 173.7, 173.2, 172.6, 171.0, 153.6, 153.4, 142.6, 142.0, 141.0, 140.8, 140.4, 131.2, 131.0, 129.4, 128.7, 127.9, 125.4, 125.1, 122.2, 122.1, 121.8, 110.8, 110.6, 103.1, 79.4, 77.7, 77.4, 77.2, 70.0, 69.8, 69.5, 65.2, 63.9, 63.0, 56.7, 56.1, 53.4, 51.5, 50.1, 49.3, 48.9, 48.7, 48.6, 48.4, 48.3, 48.1, 48.0, 47.4, 46.6, 44.0, 42.2, 39.7, 39.4, 39.3, 39.1, 38.9, 37.1, 36.9, 36.7, 36.1, 35.7, 35.3, 35.2, 34.0, 33.7, 32.2, 31.8, 31.8, 30.9, 29.6, 29.5, 29.5, 29.5, 29.3, 29.2, 29.1, 29.0, 28.9, 28.2, 28.1, 27.9, 27.6, 27.5, 27.3, 26.6, 25.4, 25.2, 24.8, 24.1, 23.7, 22.5, 22.5, 22.2, 21.0, 19.1, 18.4, 16.3, 13.7, 11.6. HR-ESI-MS Calcd for $\text{C}_{185}\text{H}_{282}\text{N}_{61}\text{O}_{29}^+$ [M] is 3822.2461, found is 3822.2245.

2.4.14 General Procedure for Conjugation of Sugar Molecules

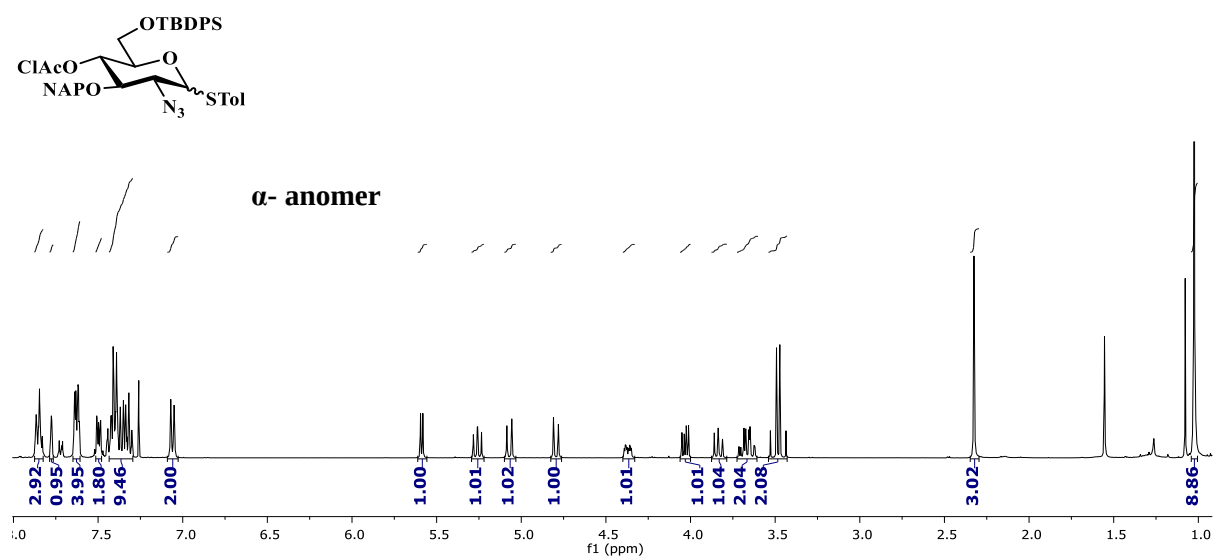
The sugar derivative was dissolved in 400 μL of dry DMF and stirred for 5 minutes. Next, NEt_3 (2 equivalents) was added, followed by dropwise addition of the solution of DBCO-NHS in 400 μL , while stirring at room temperature. After 12 hours, DMF was evaporated, and the crude product was purified using LH-20 size exclusion chromatography. Then, a compound with an azide linker (1 eq.) was dissolved in 500 μL of CHCl_3 and 500 μL of MeOH. Subsequently, a solution of sugar molecules (1.2 eq. per azide of peptide) in H_2O (1 mL) was added, followed by the addition of another 500 μL of MeOH. The resulting solution was stirred for 7 – 8 days at room temperature. The progress of the reaction was monitored by infrared spectroscopy (IR). After the completion of the reaction (approximately 7 - 8 days), the solvent was evaporated under reduced pressure.

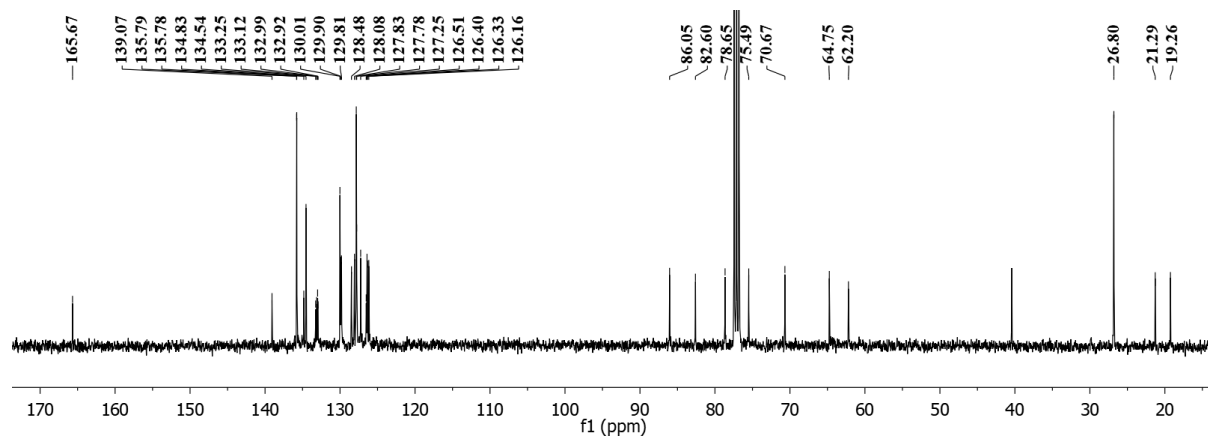
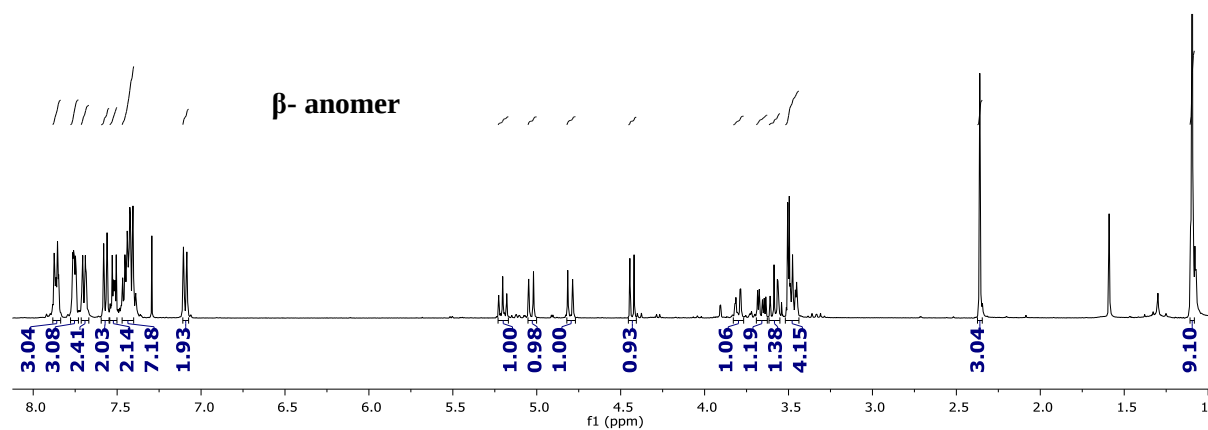
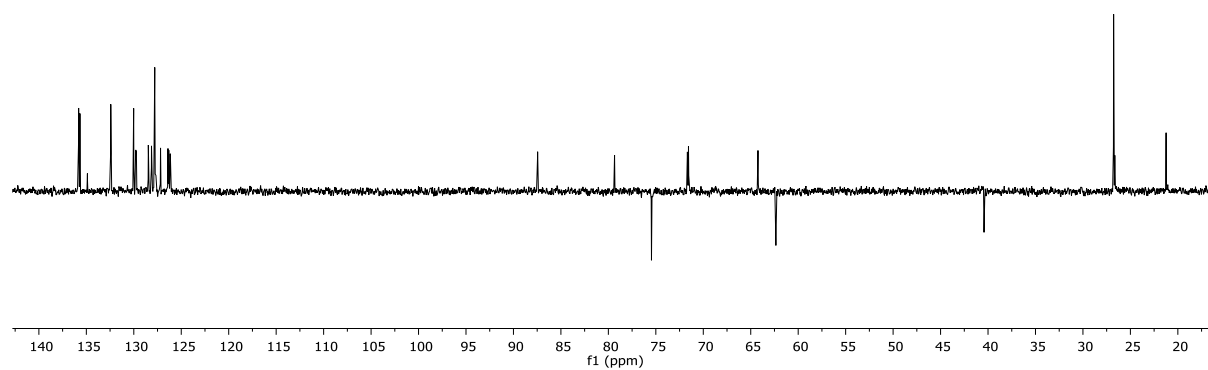
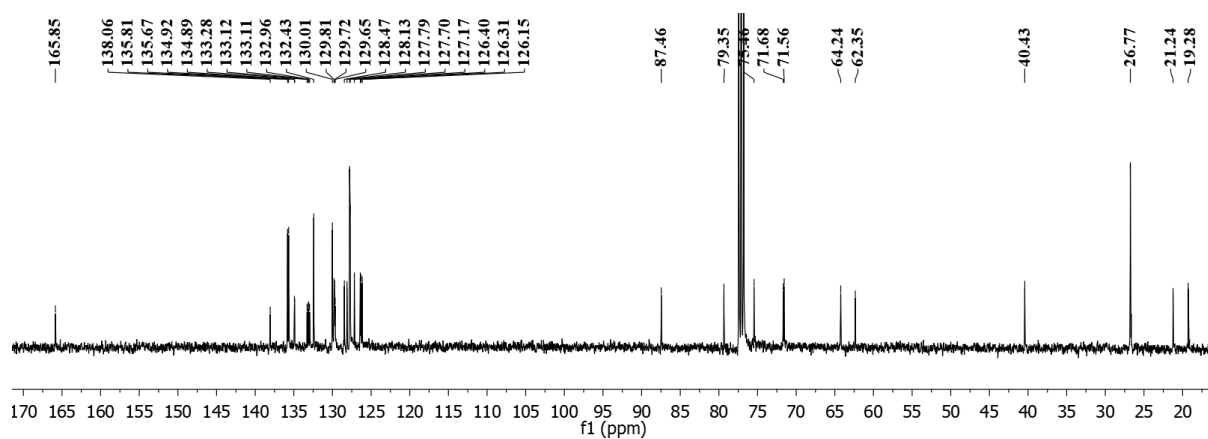


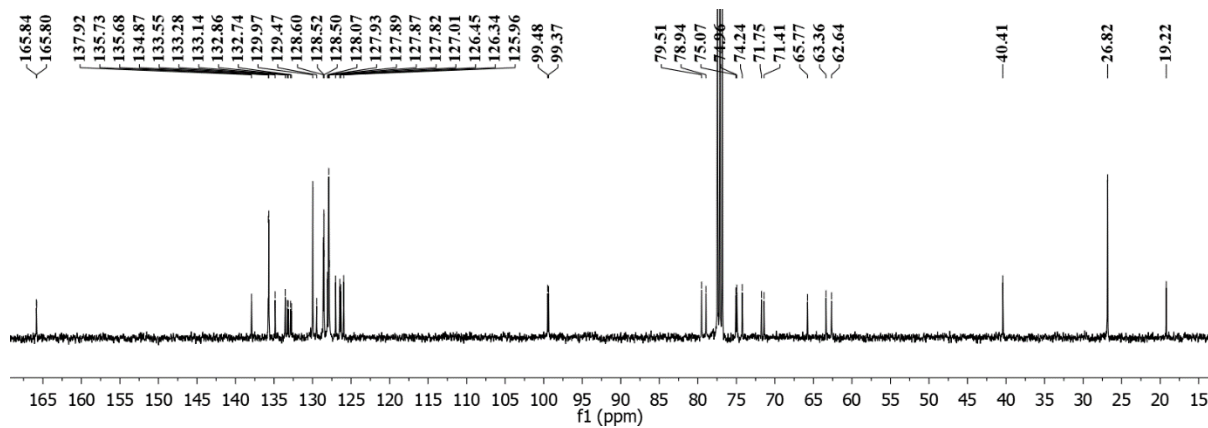
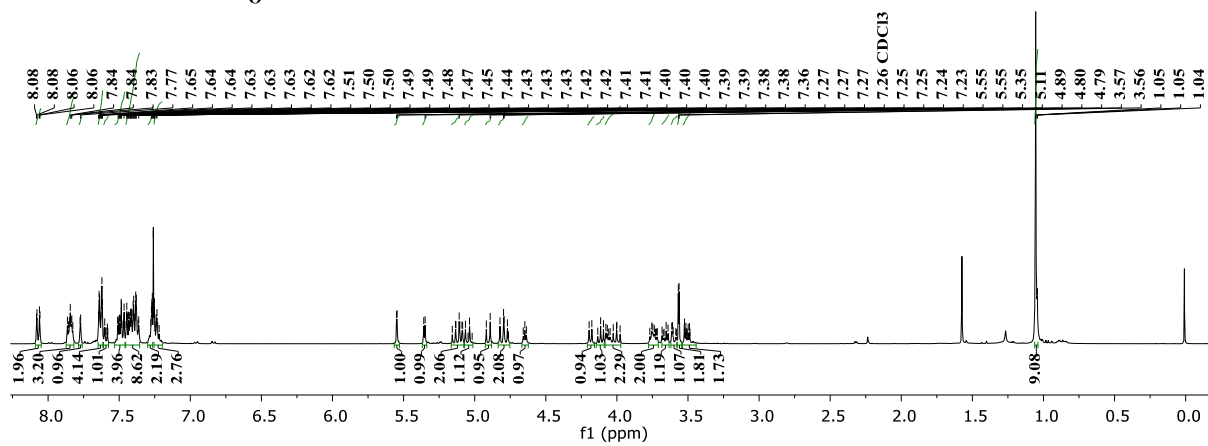
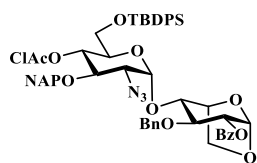
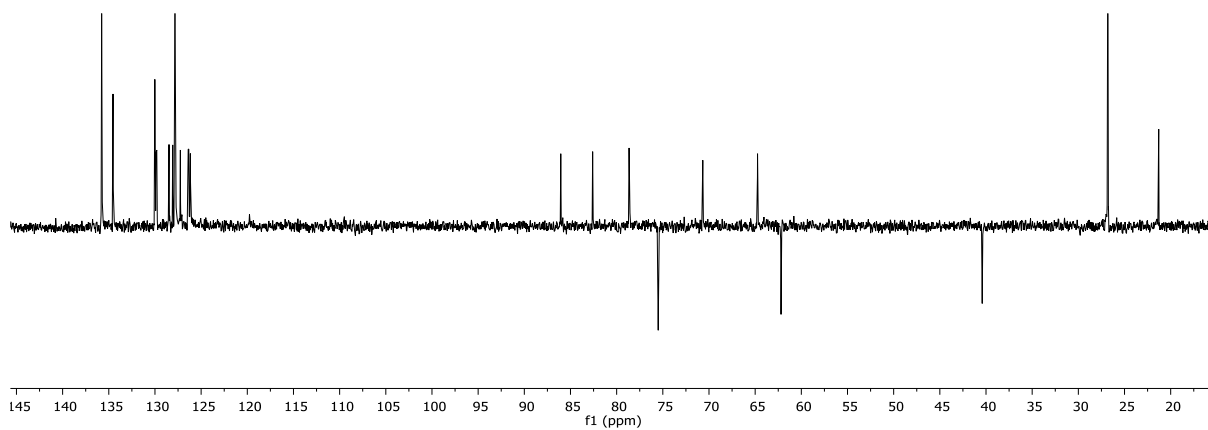
The crude product was then purified using a 10 kDa centrifuge filter, followed by a C-18 column with reversed-phase high-performance liquid chromatography (HPLC). A gradient system of MeOH and water was employed for compound purification. Confirmation of the product was achieved through IR, NMR, and HRMS-ESI.

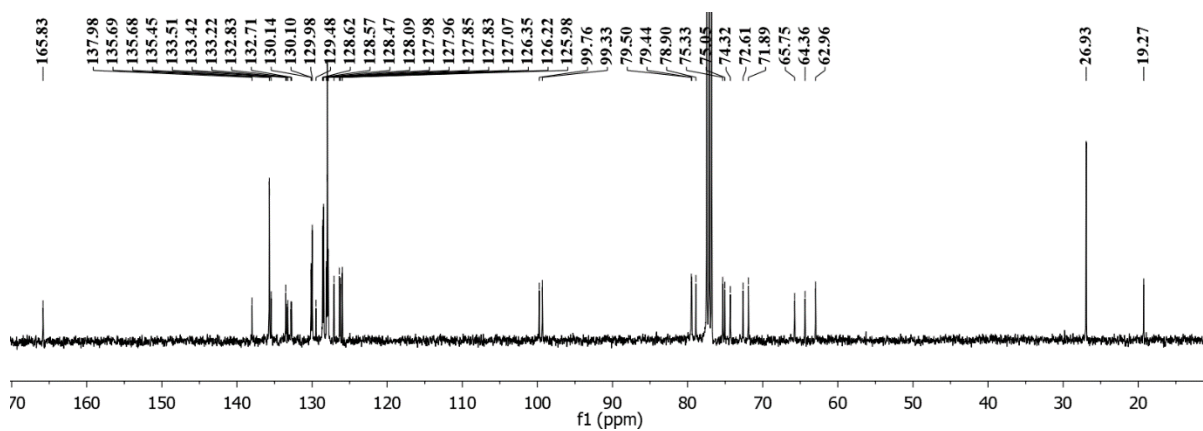
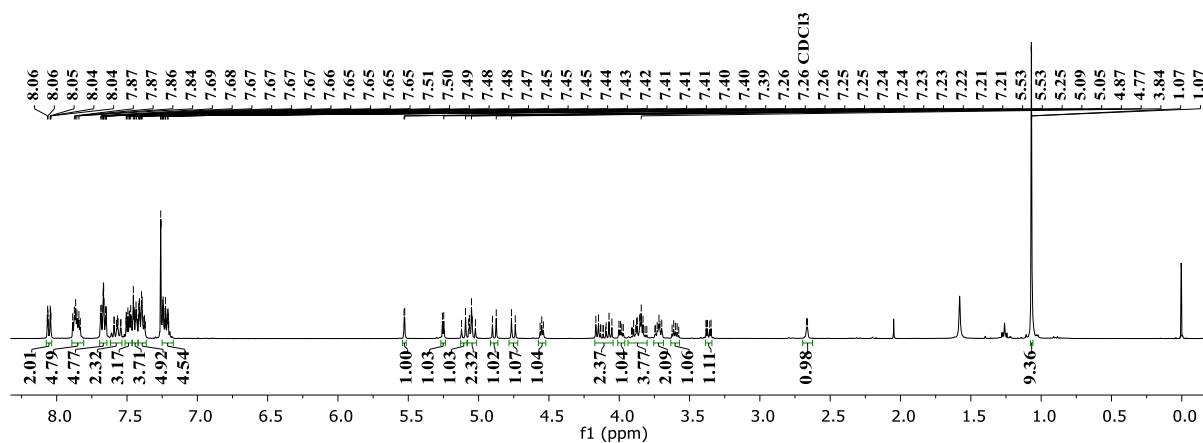
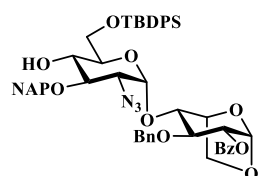
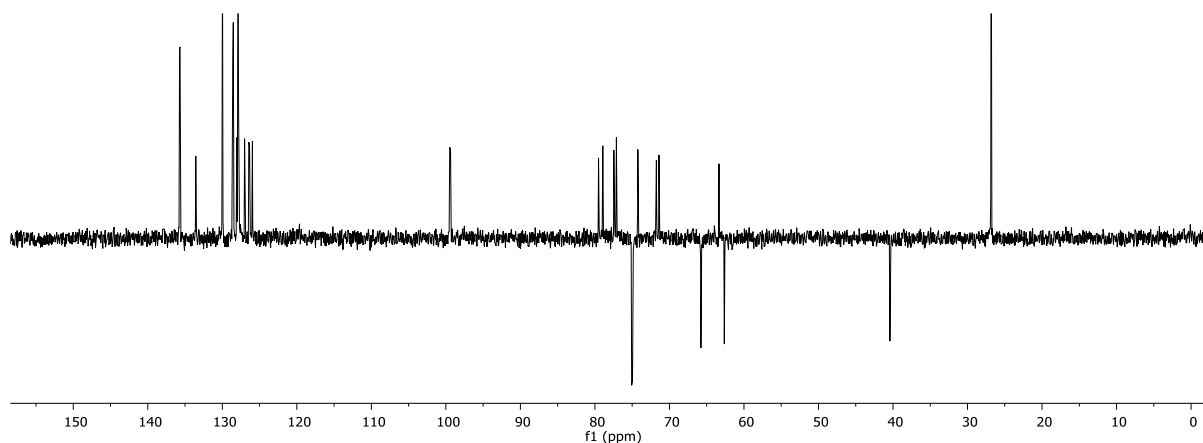


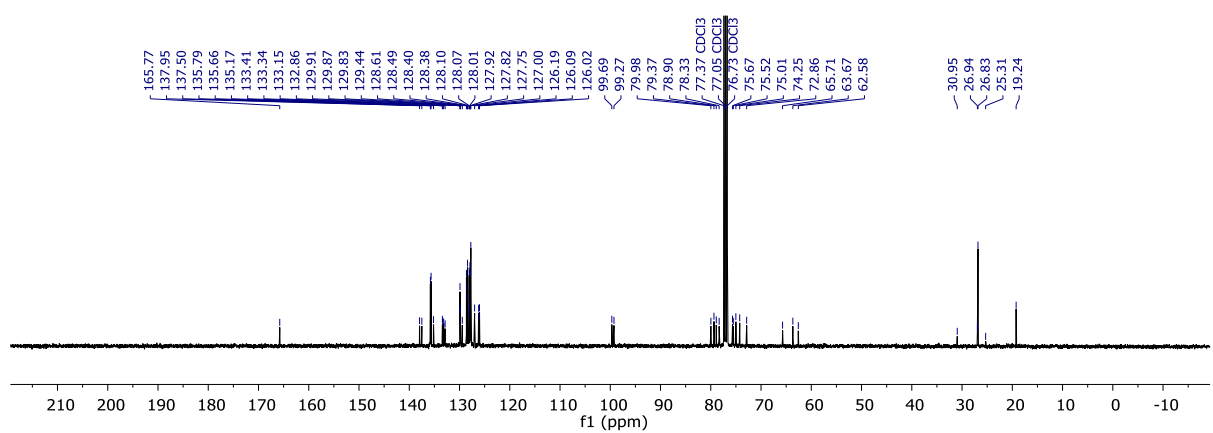
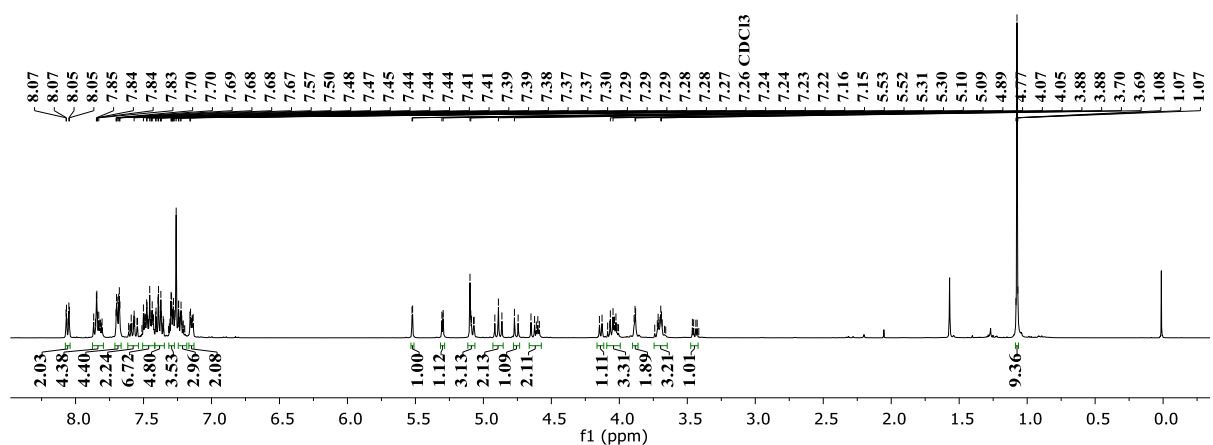
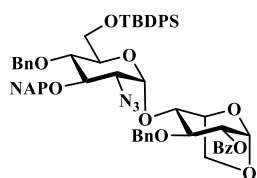
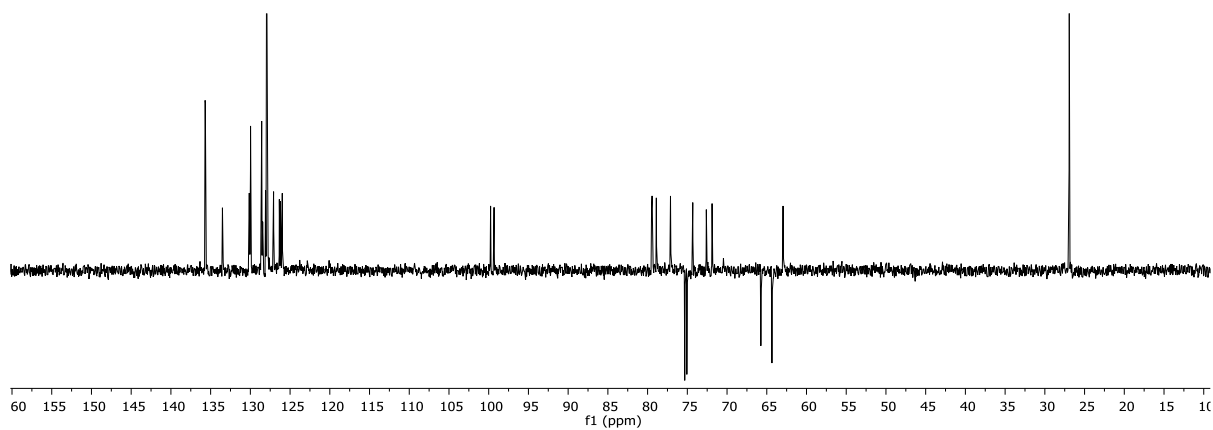
NMR and other Characterization Data

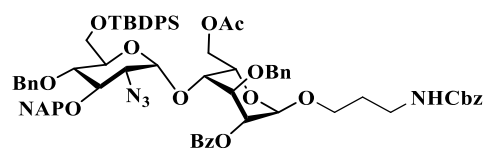
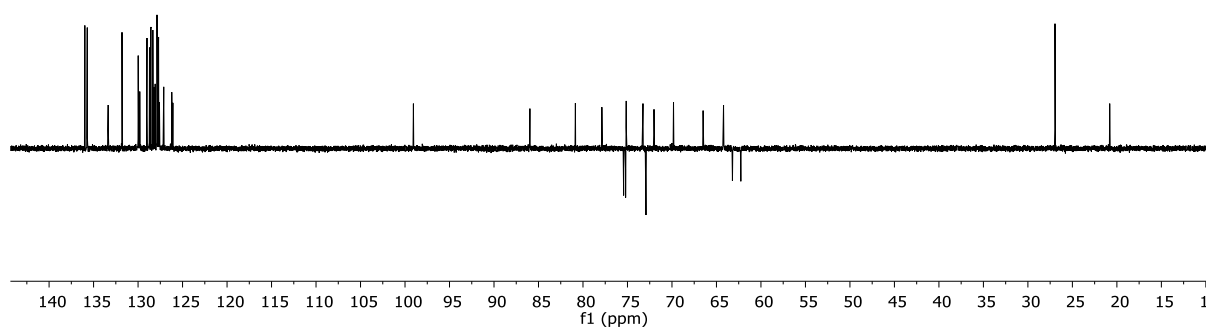
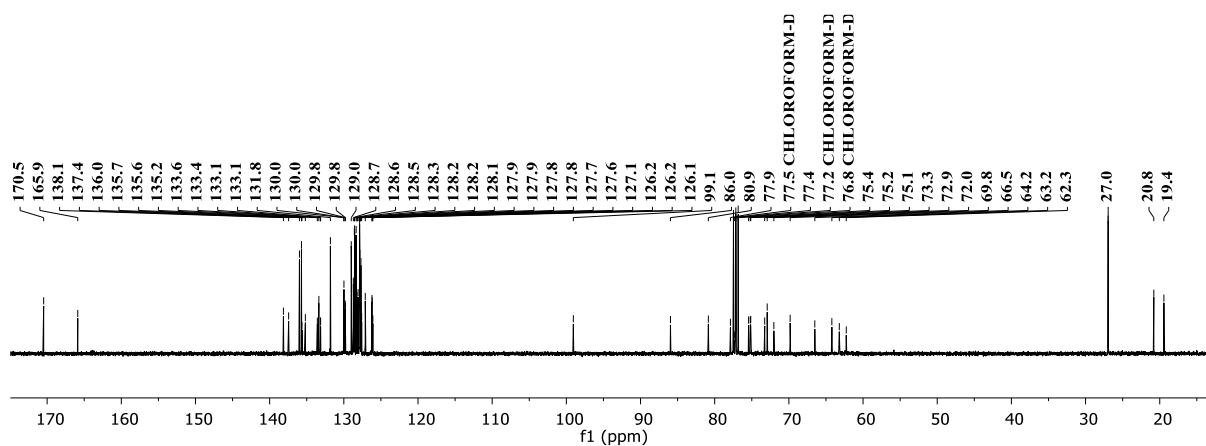
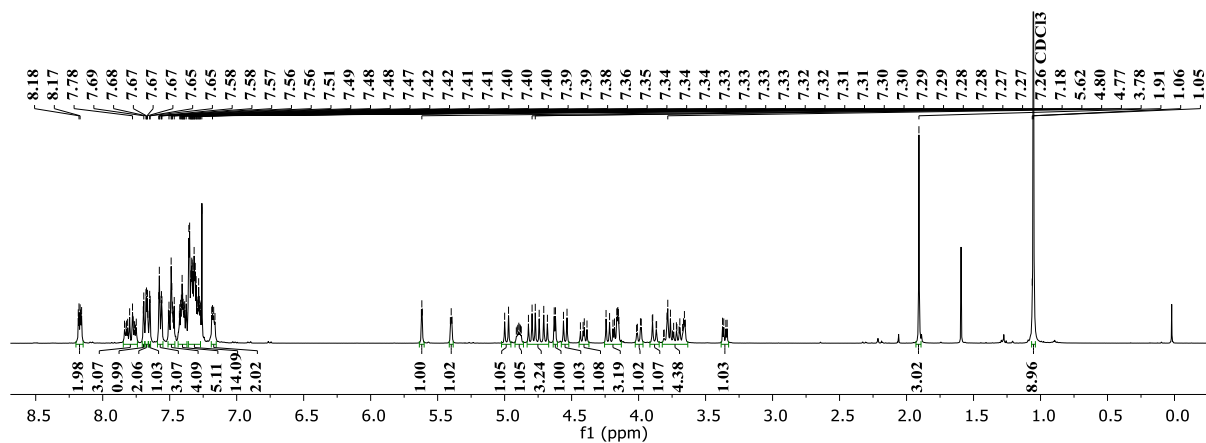
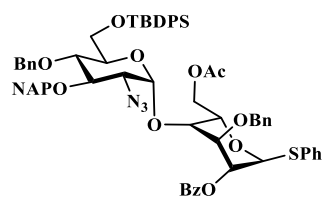


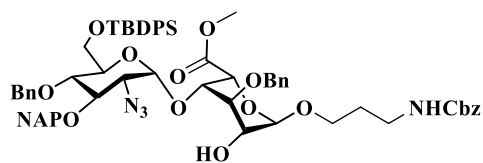
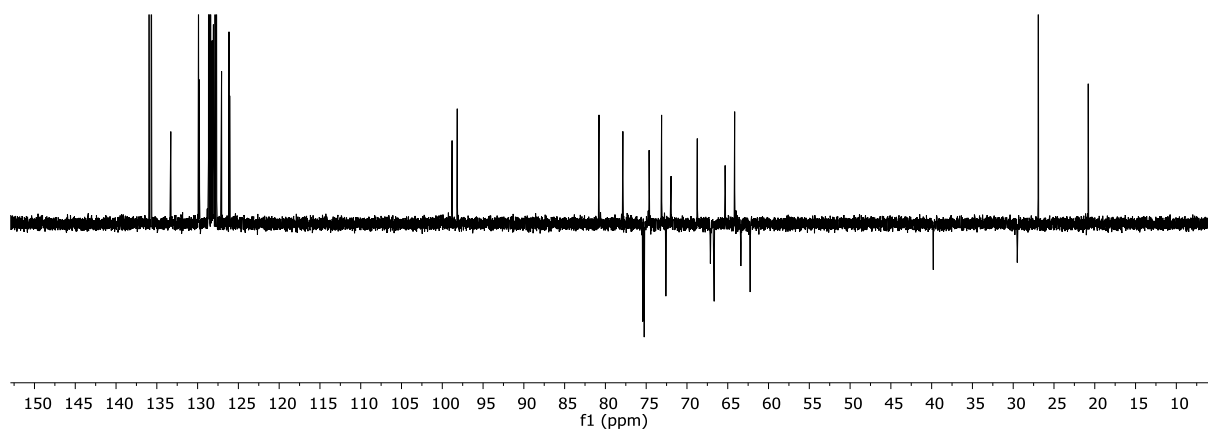
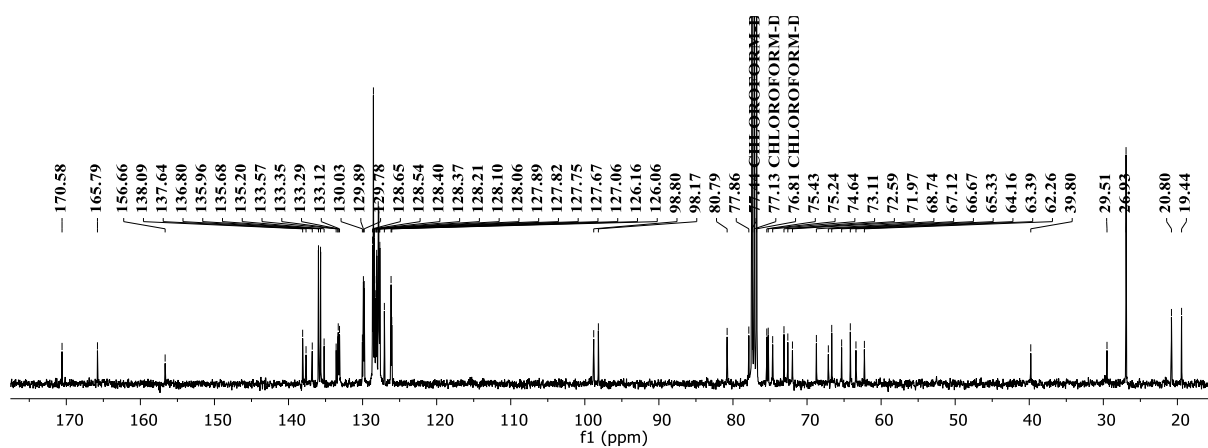
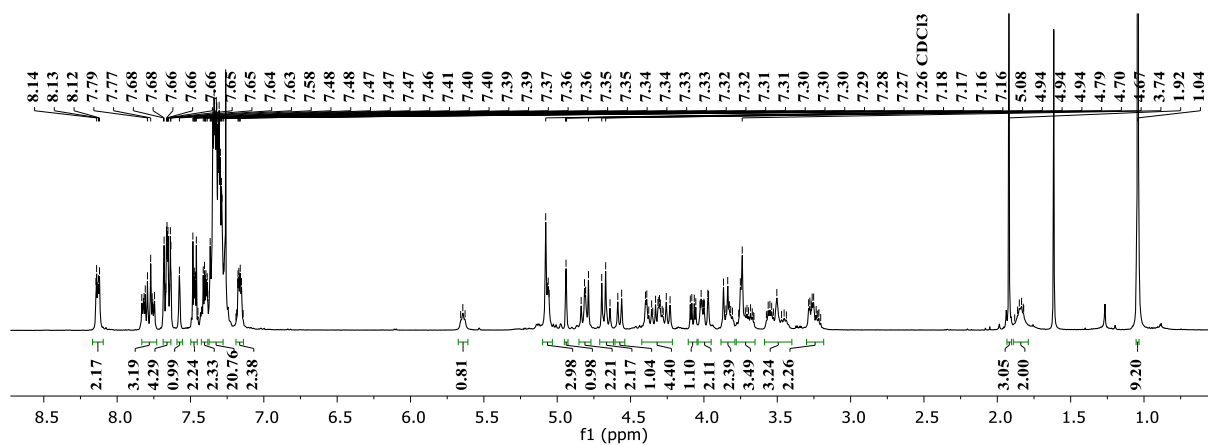


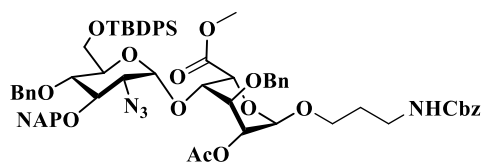
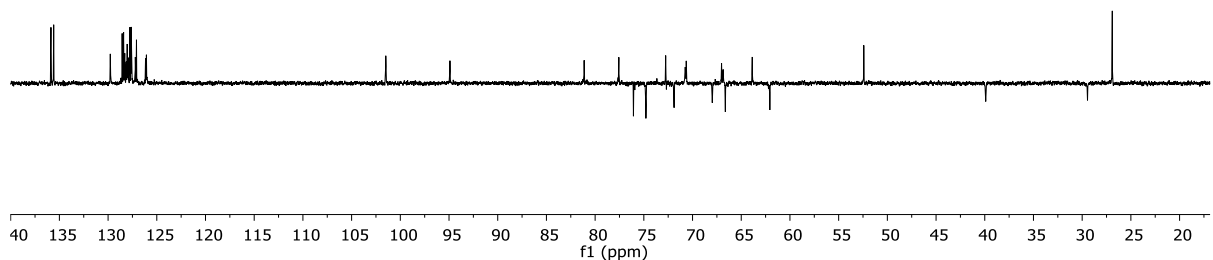
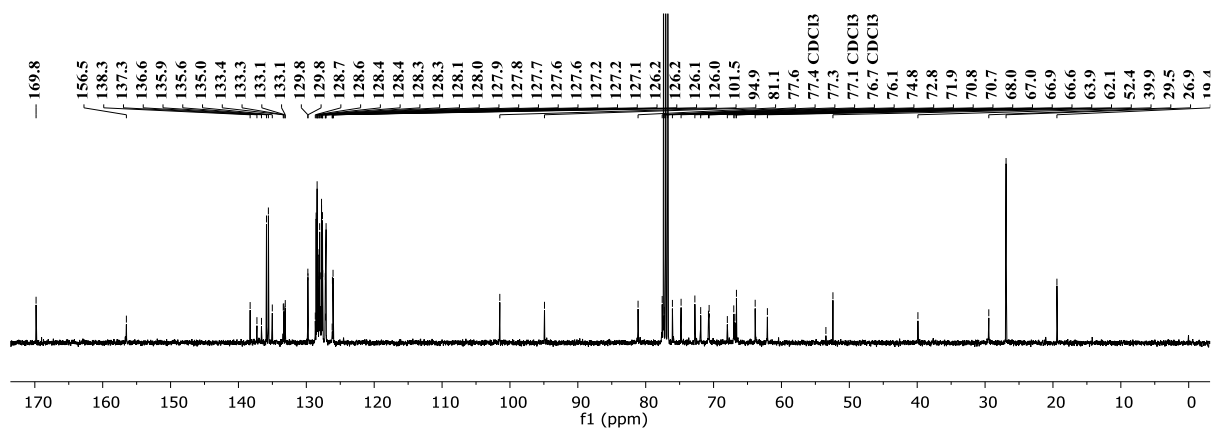
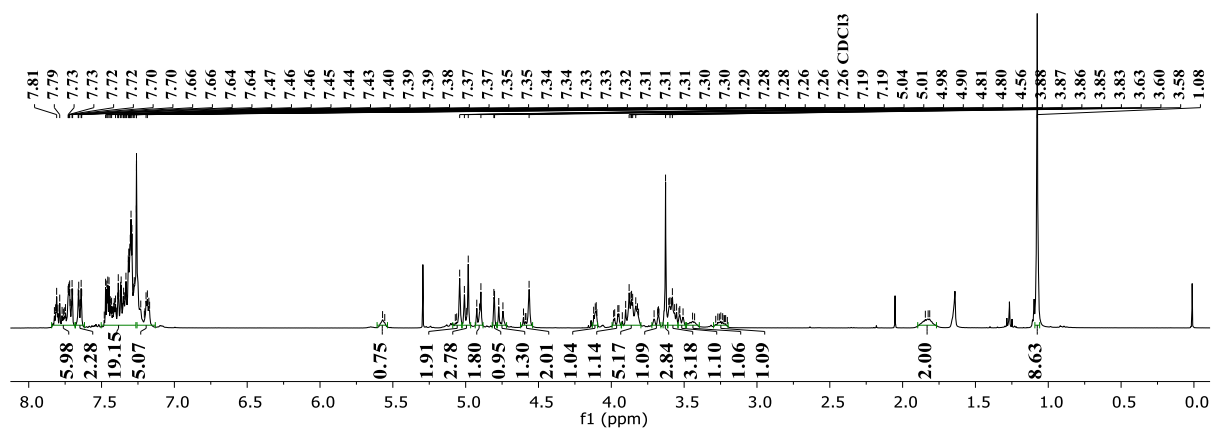


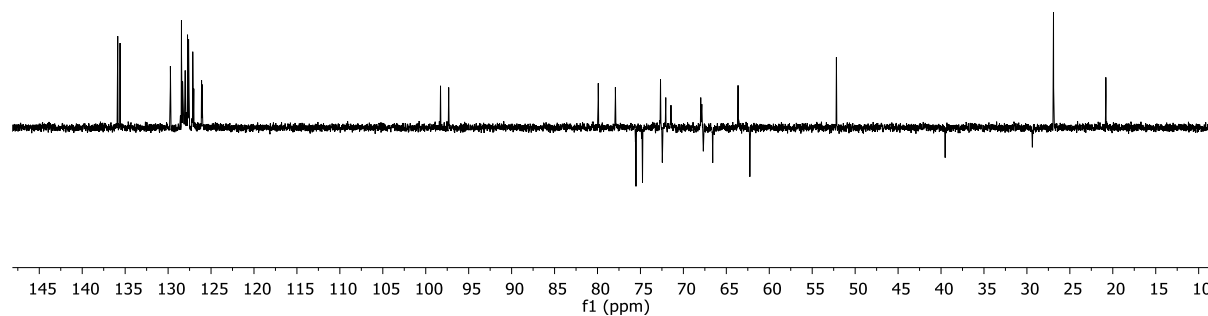
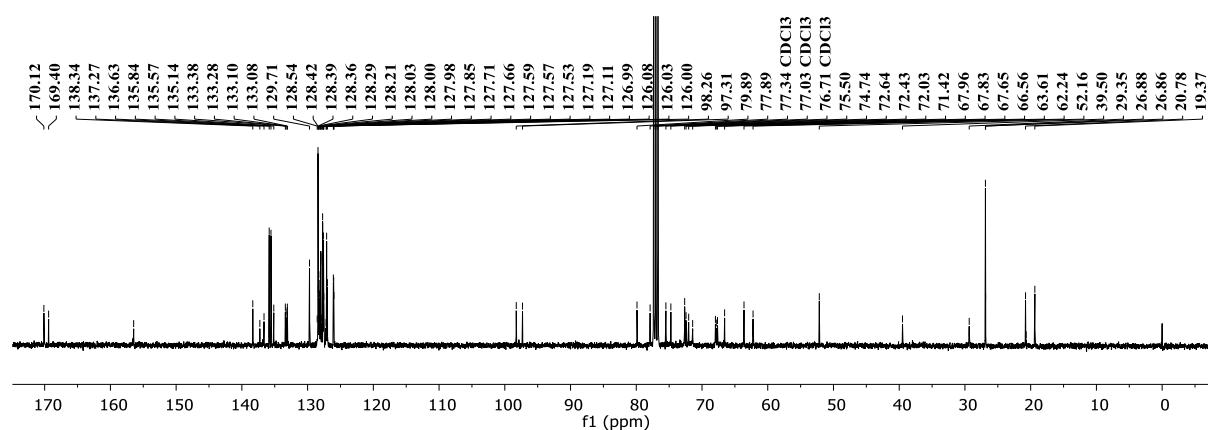


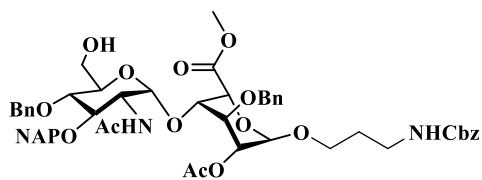
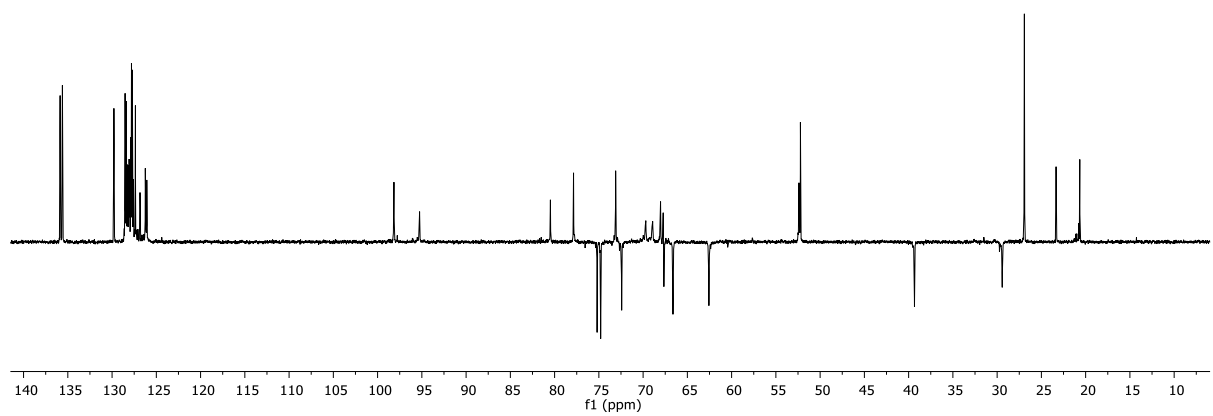
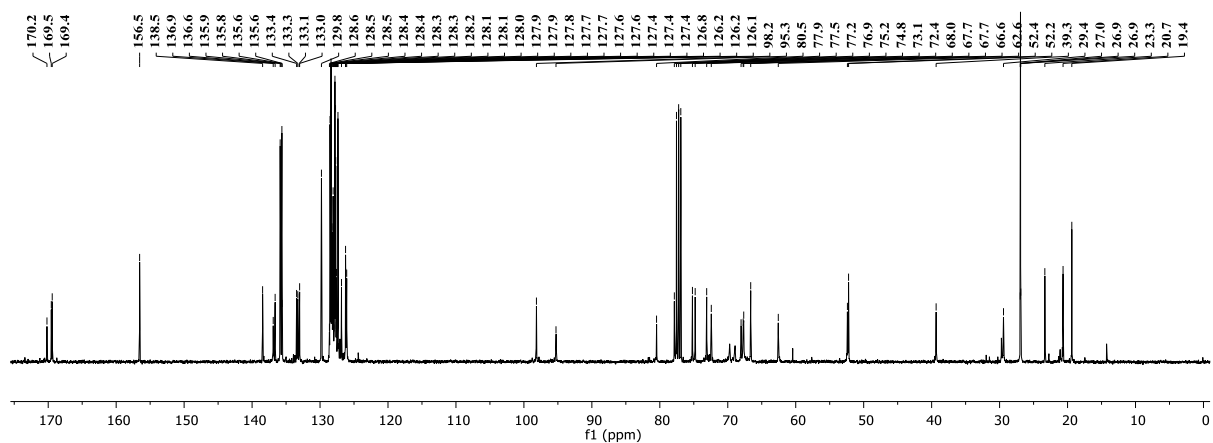
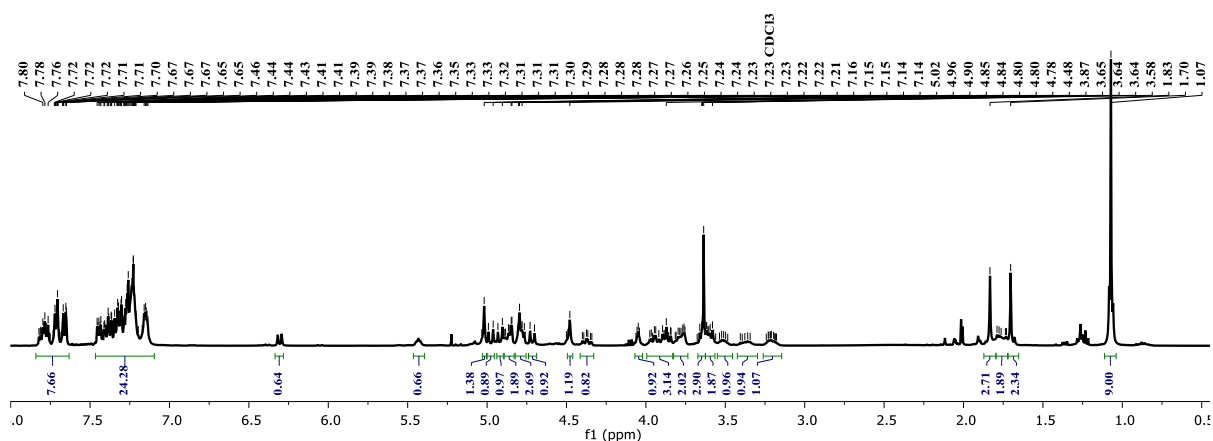


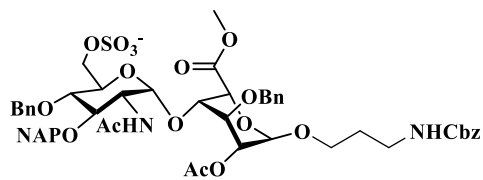


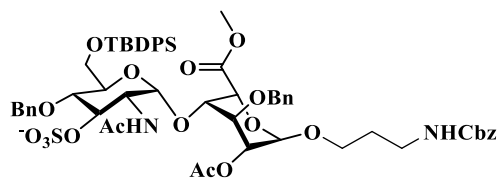
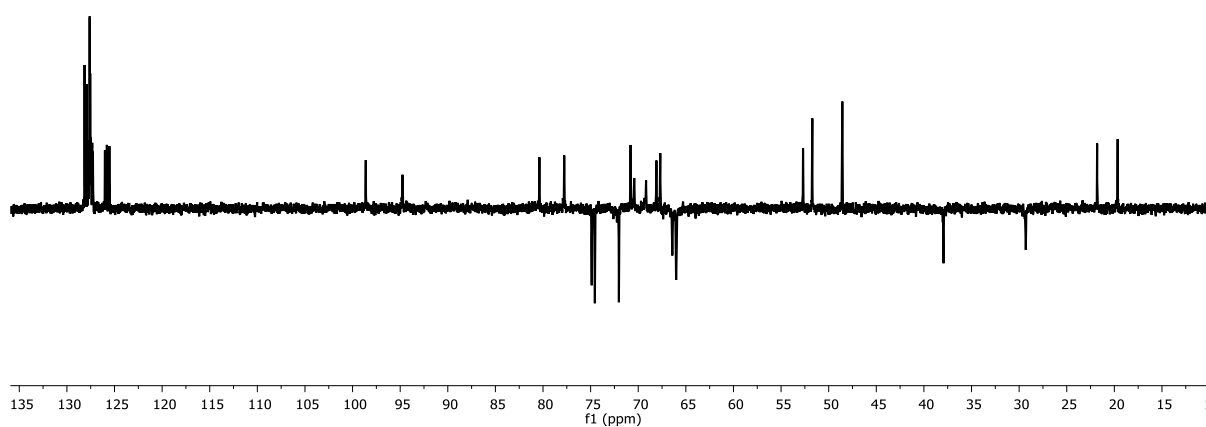
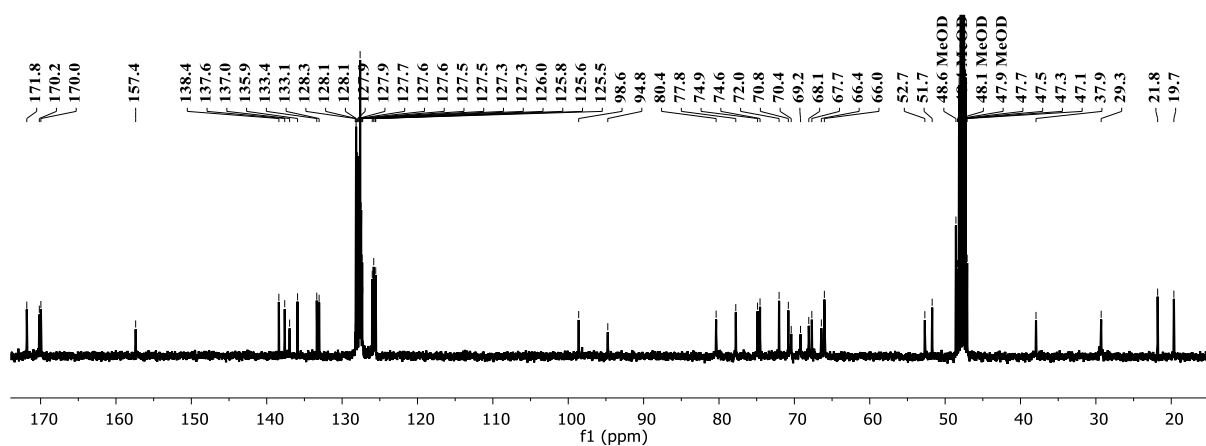
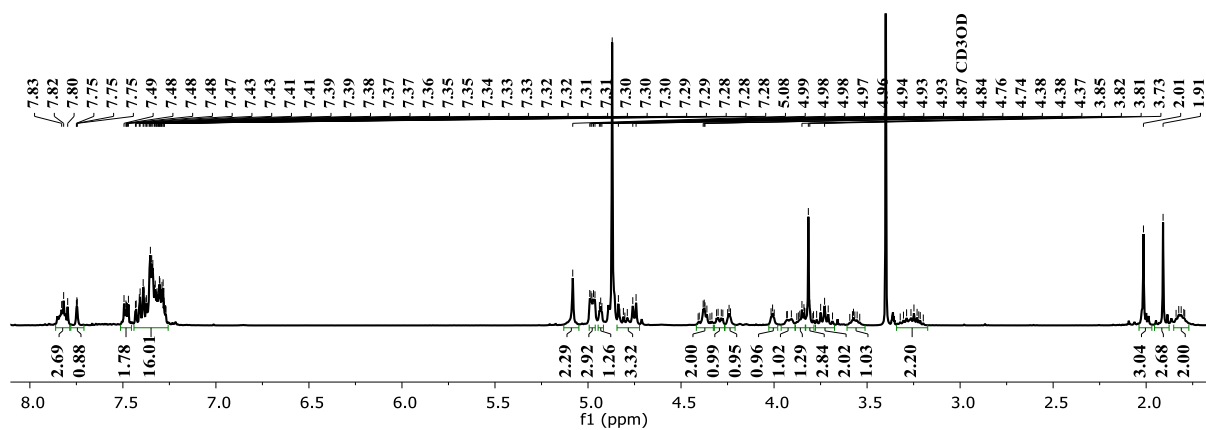


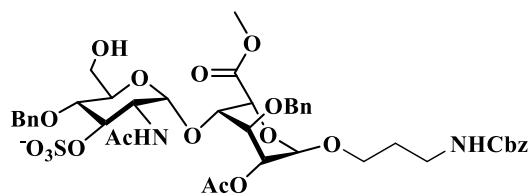
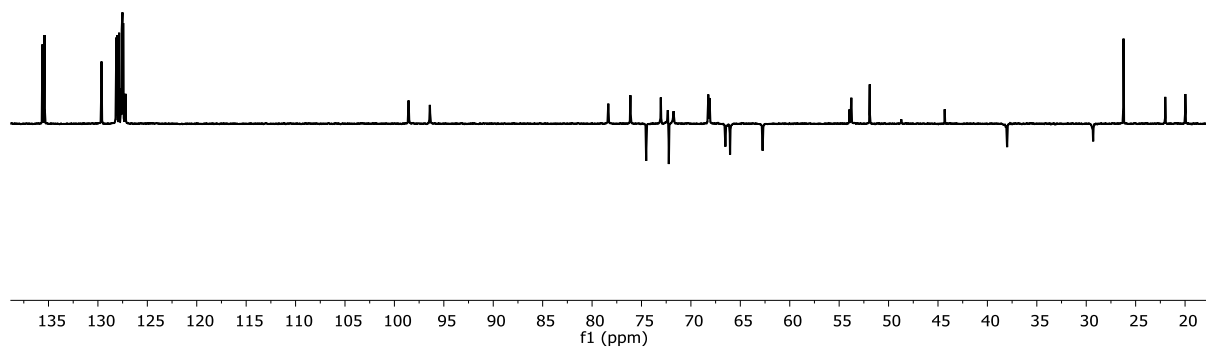
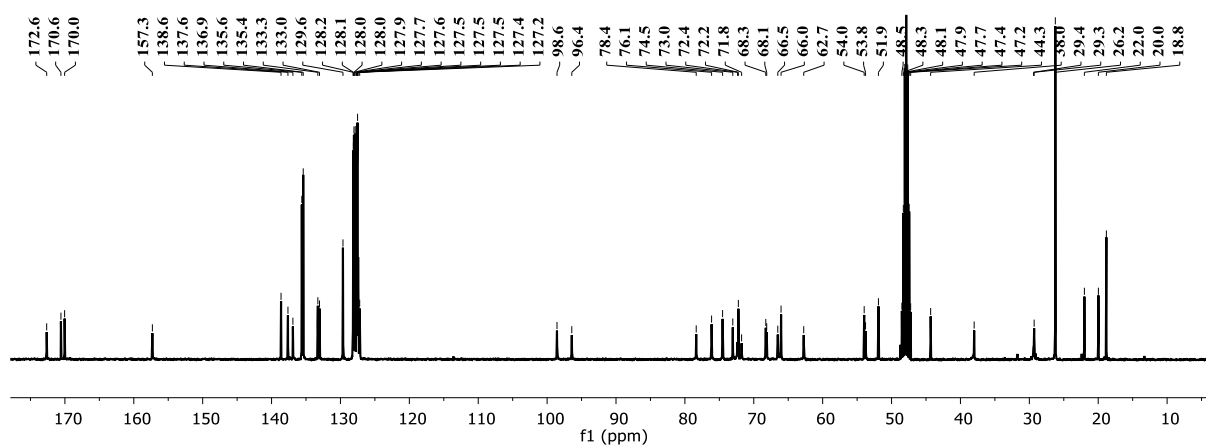
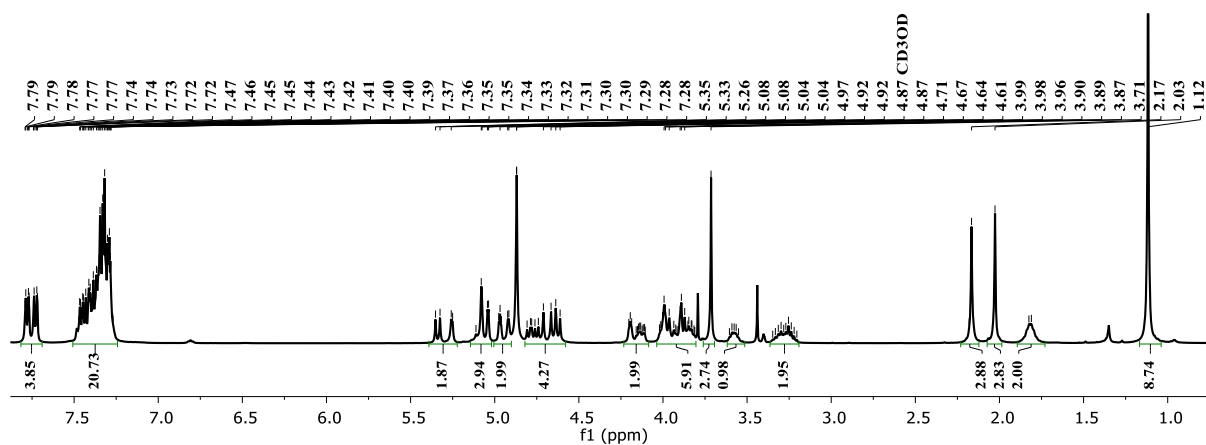


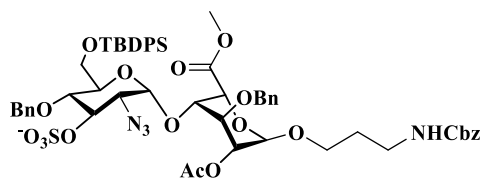


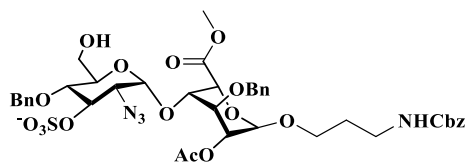


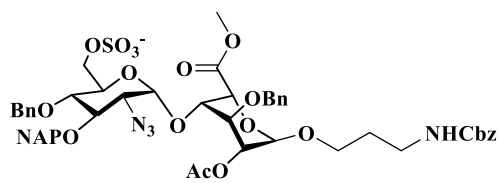
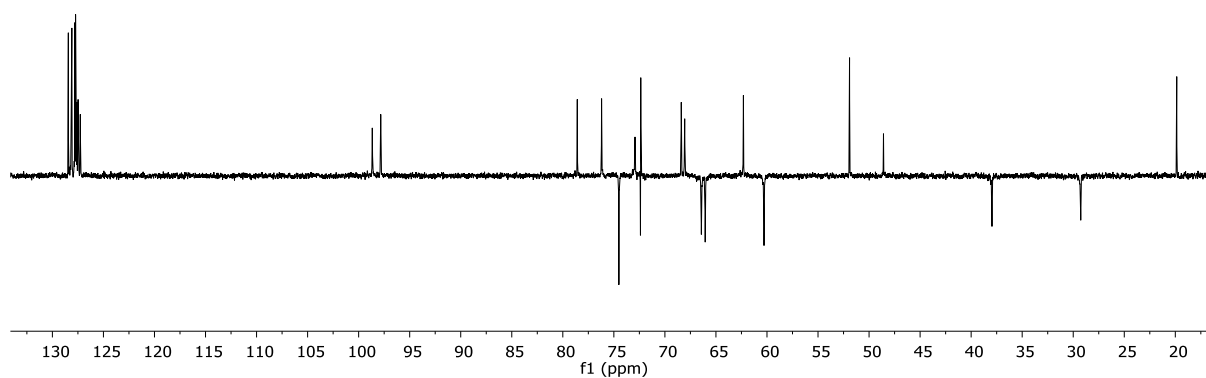
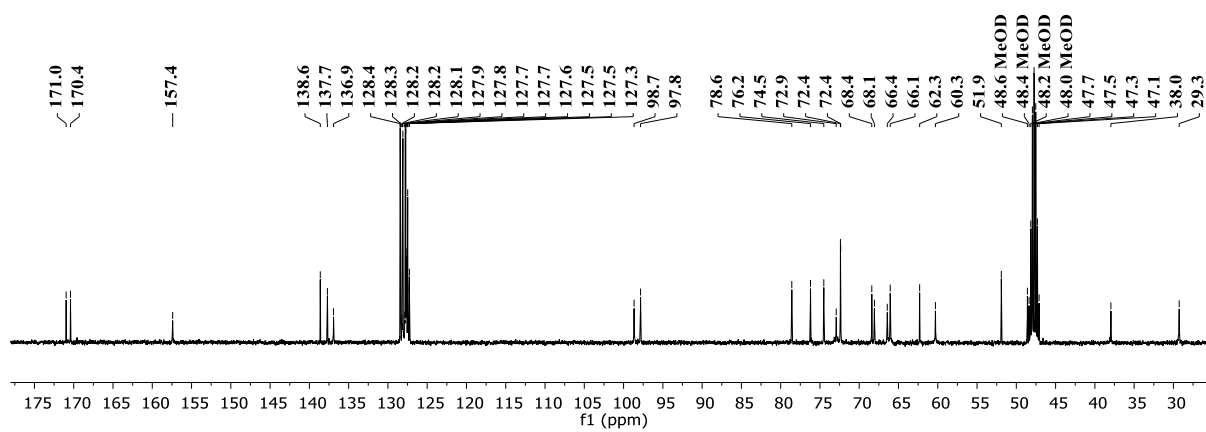
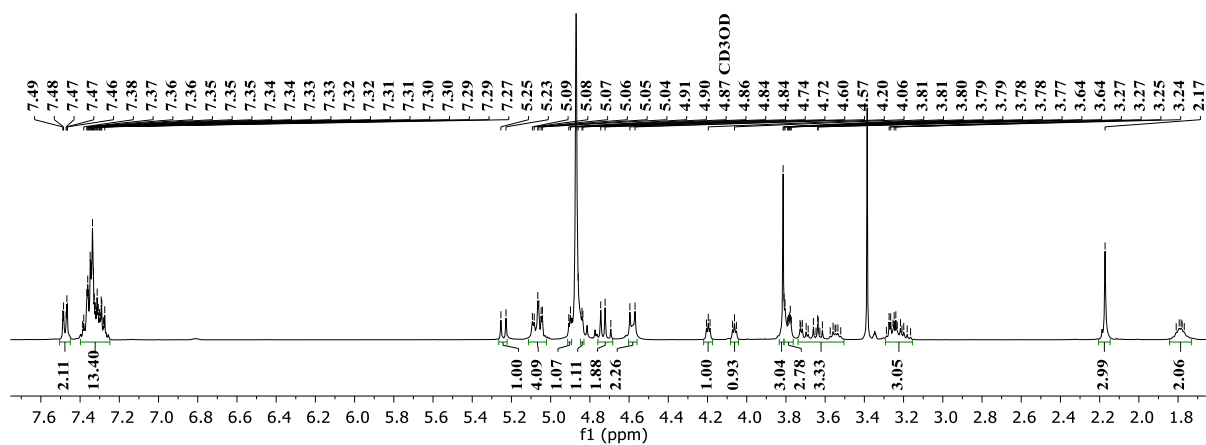


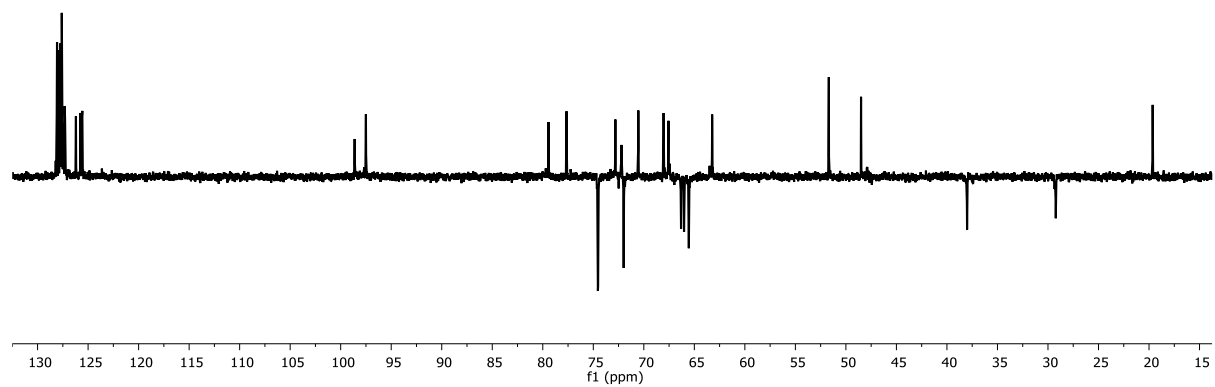
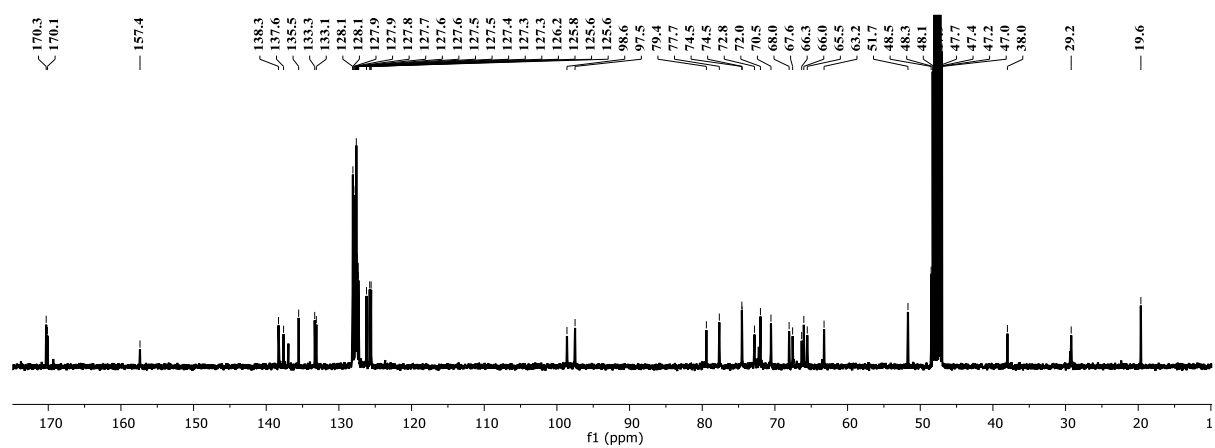


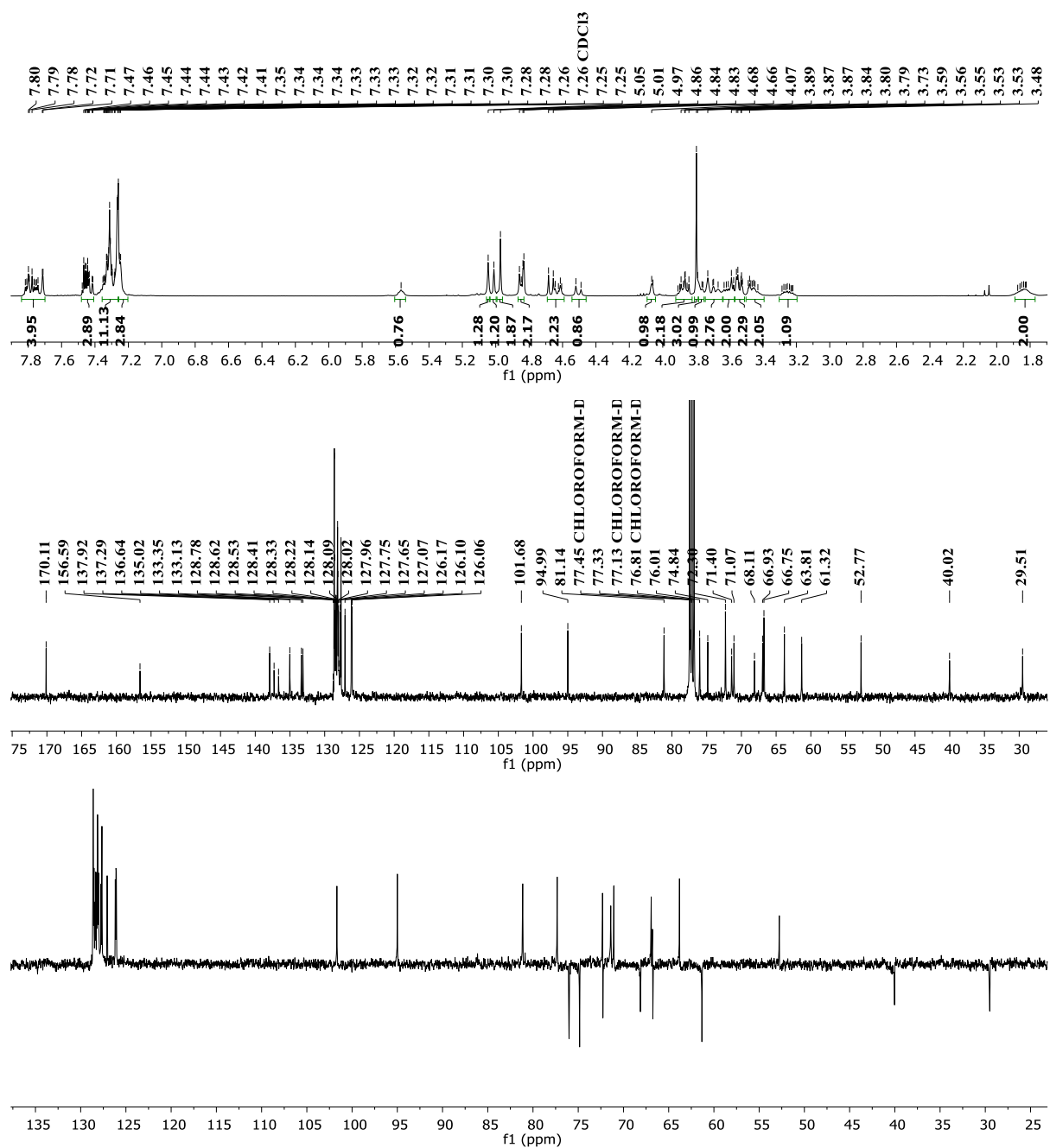


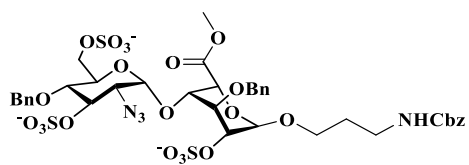
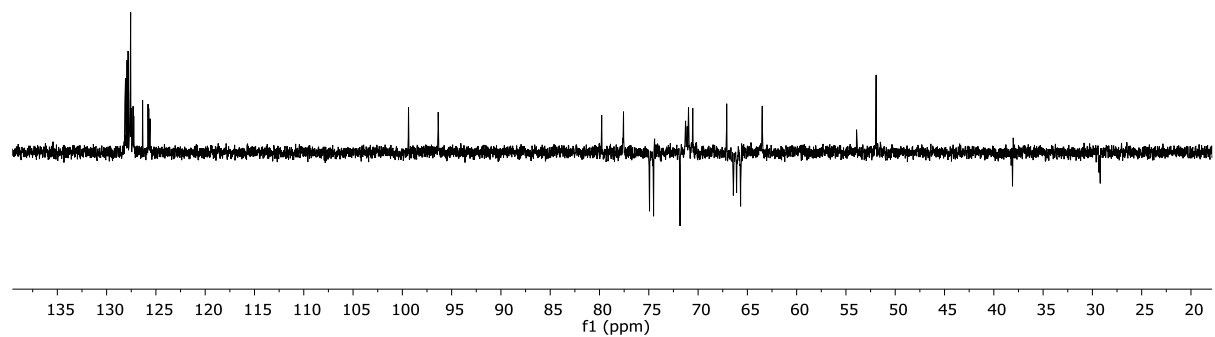
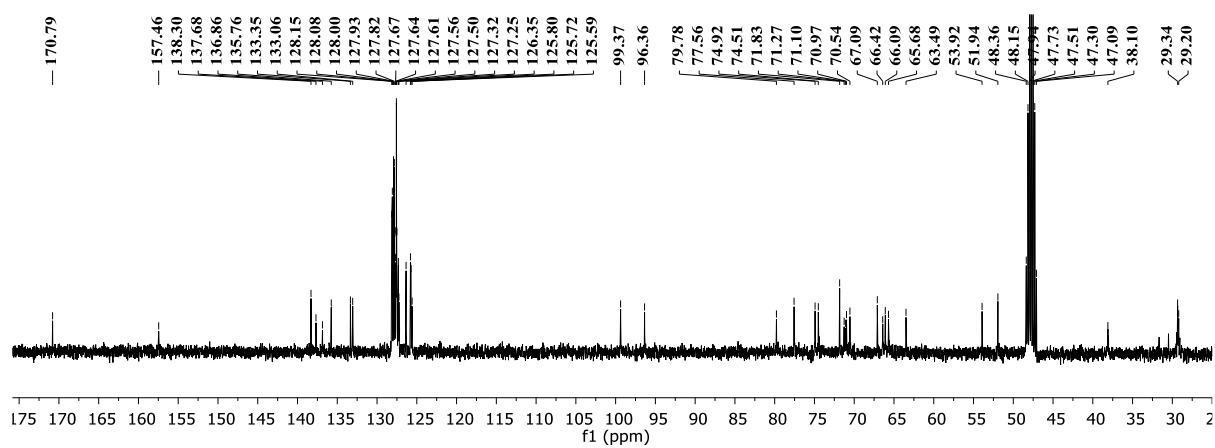
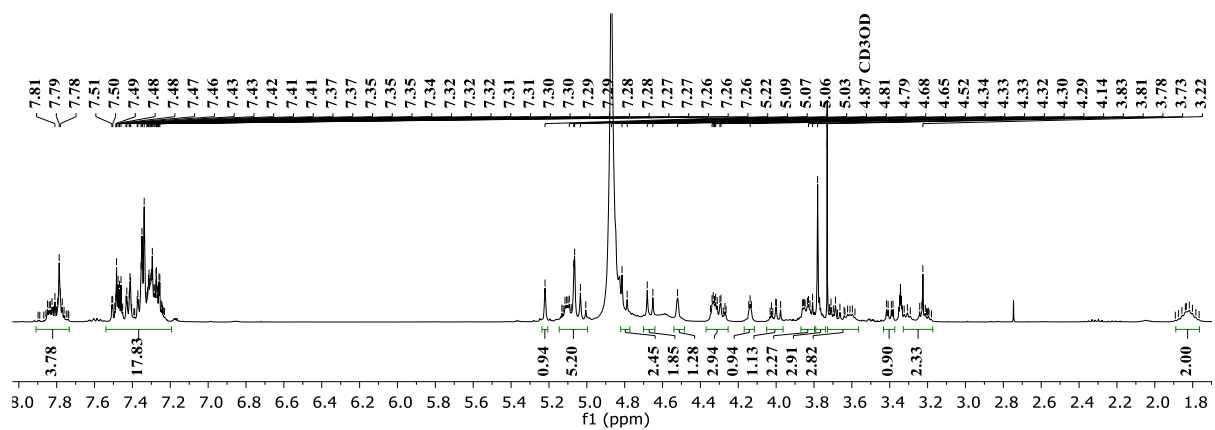


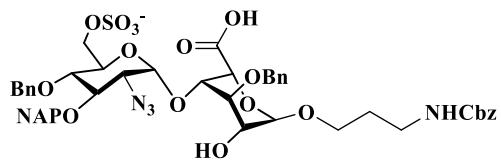
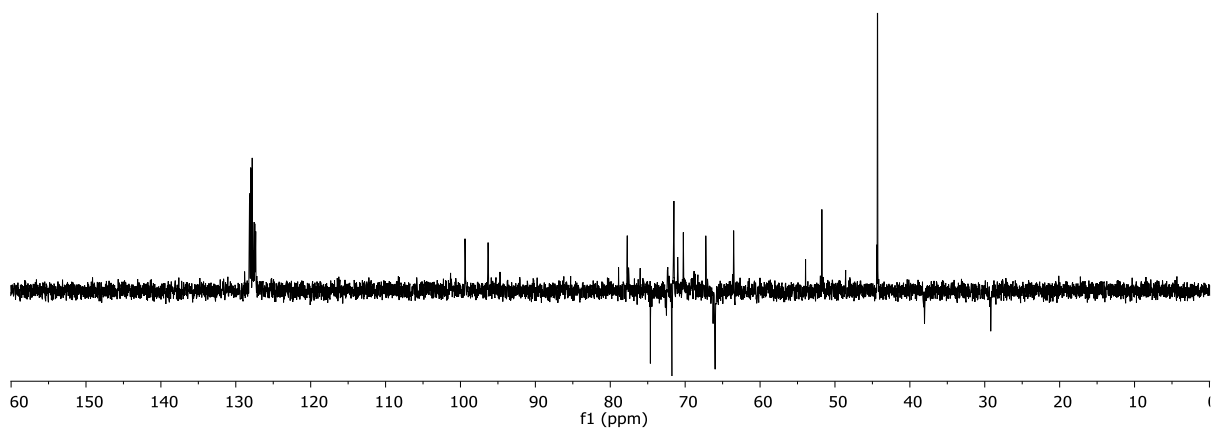
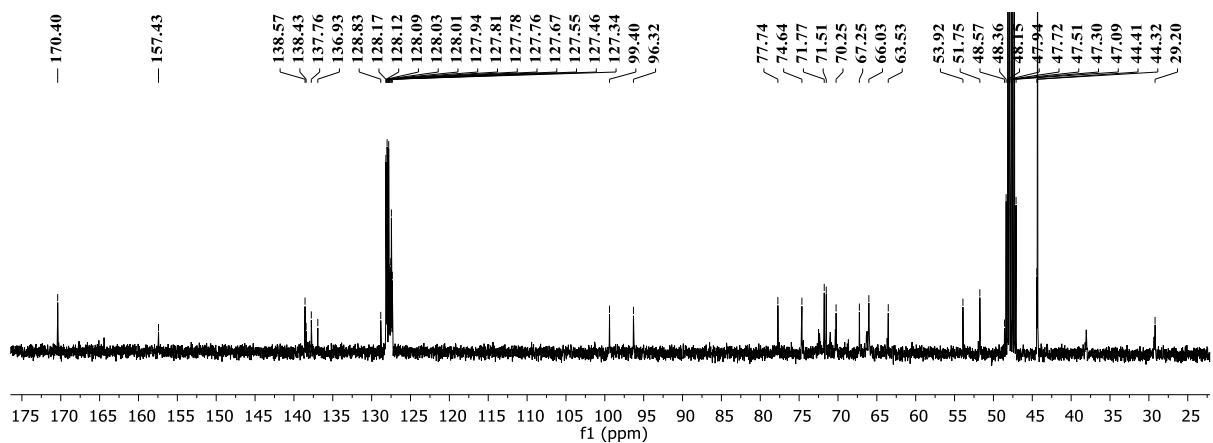
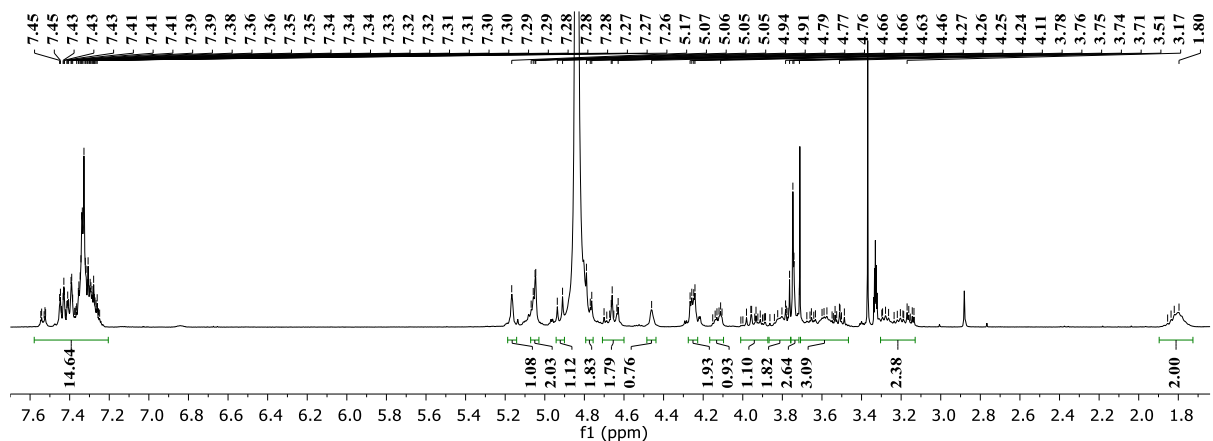


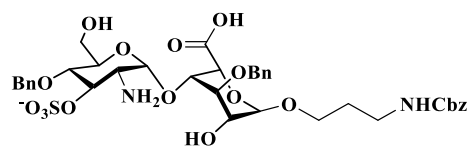
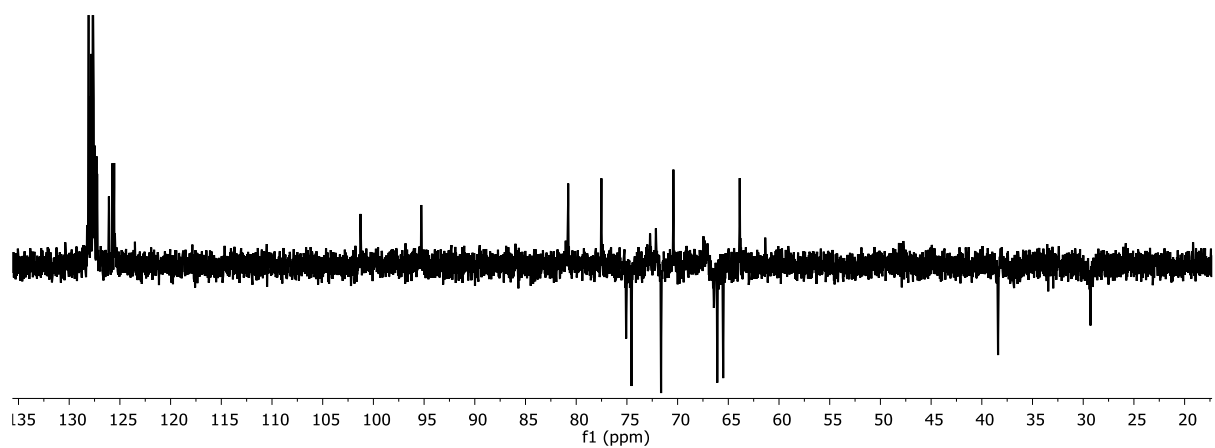
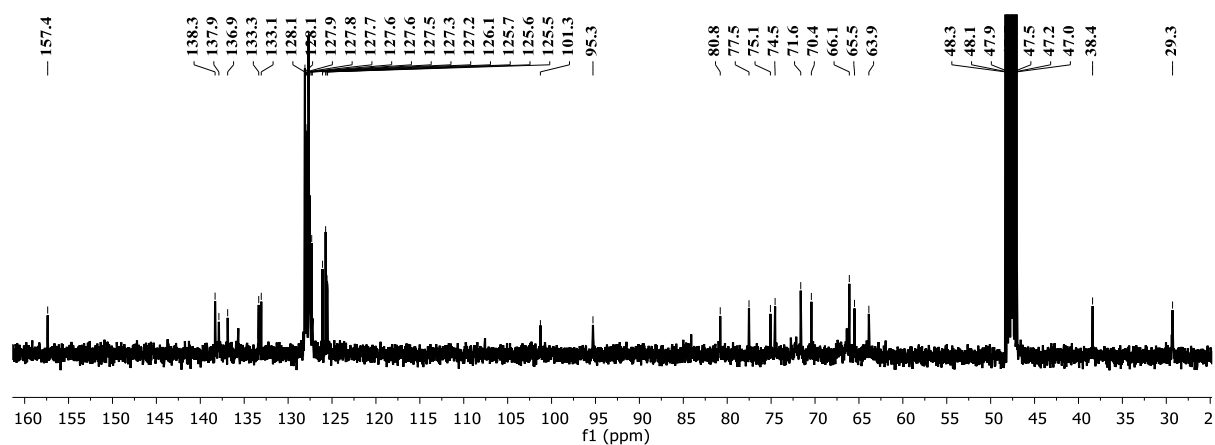
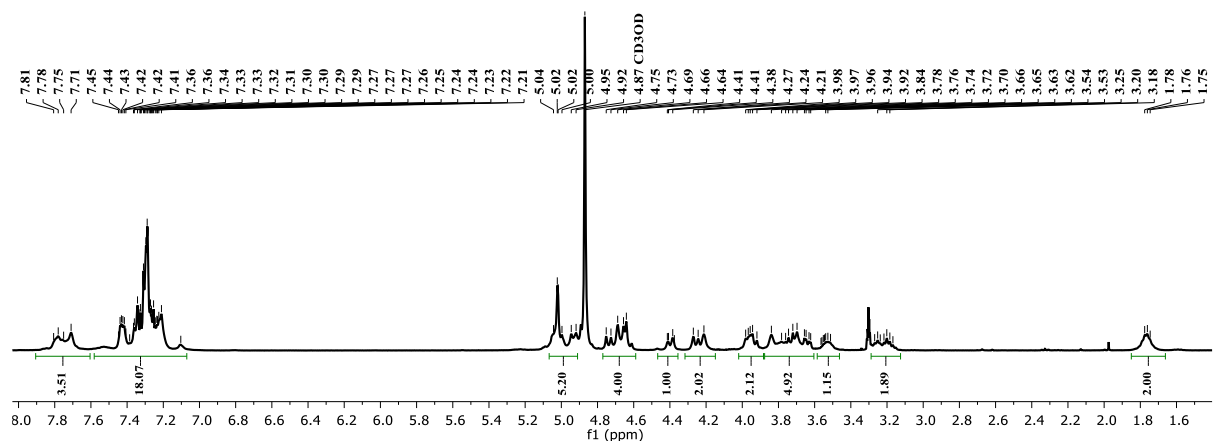


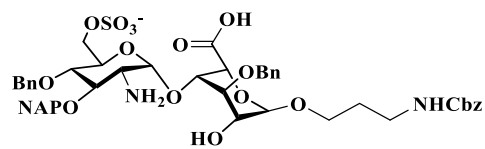
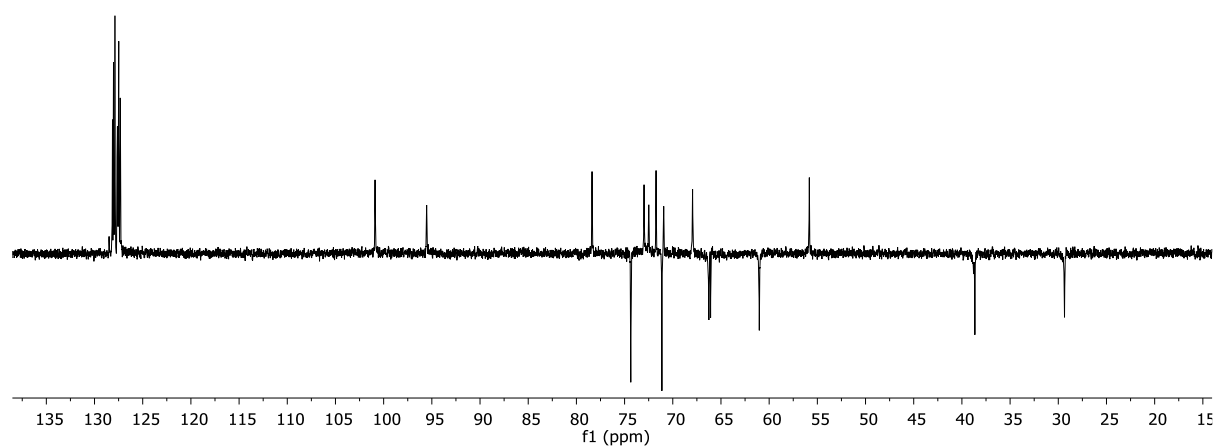
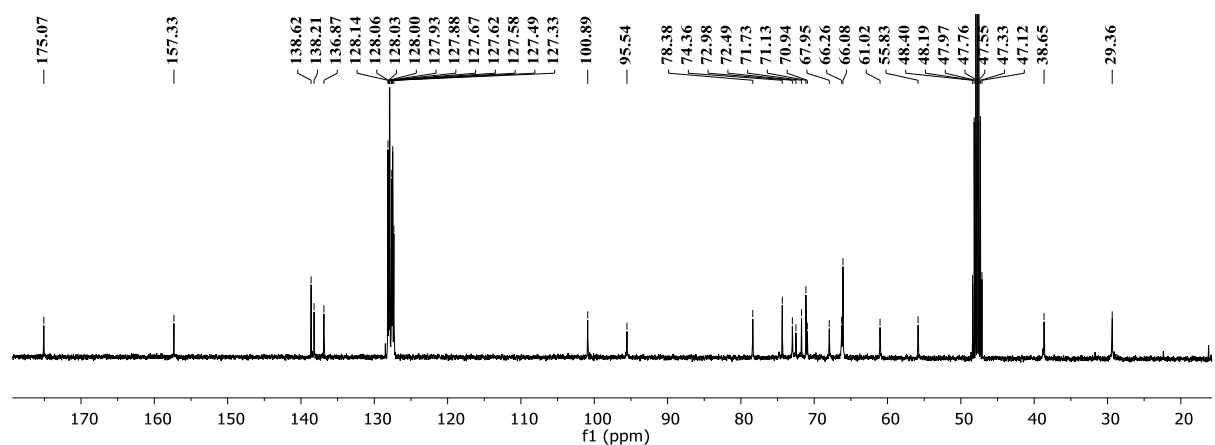
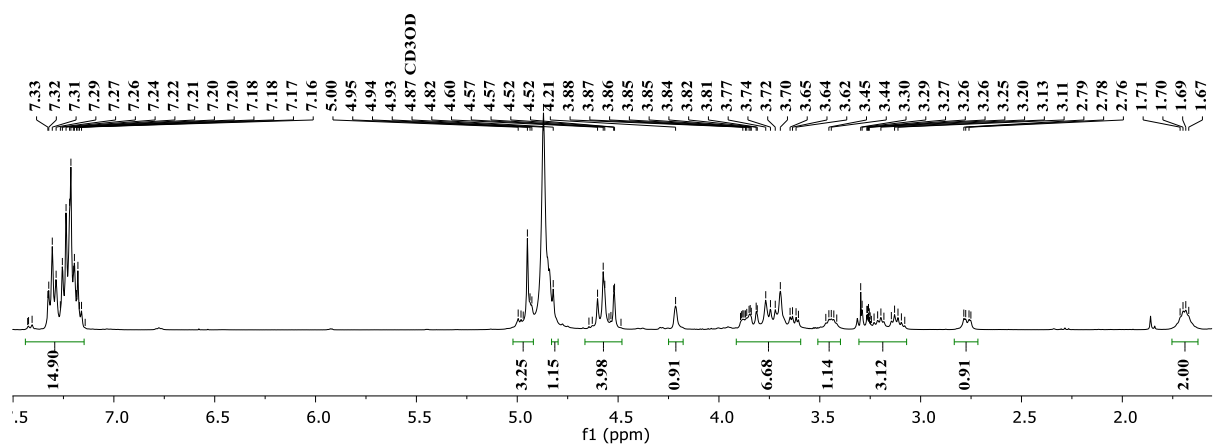


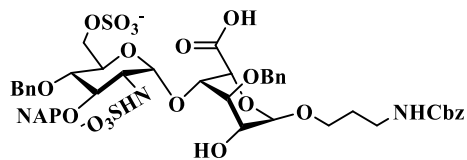
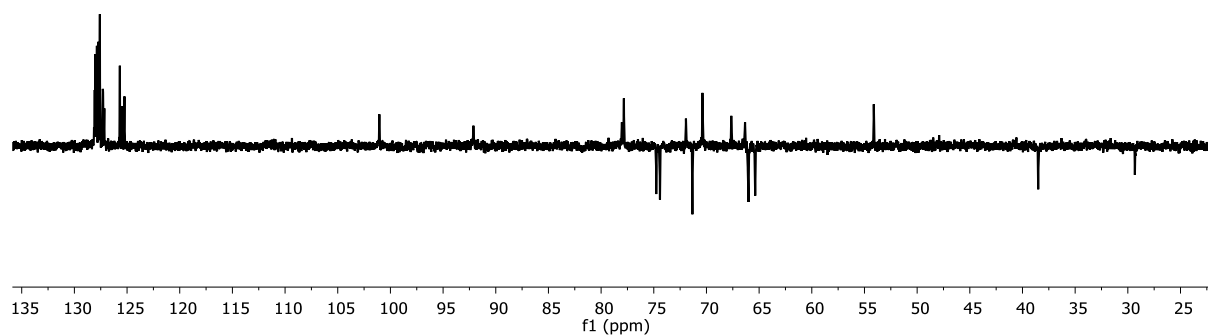
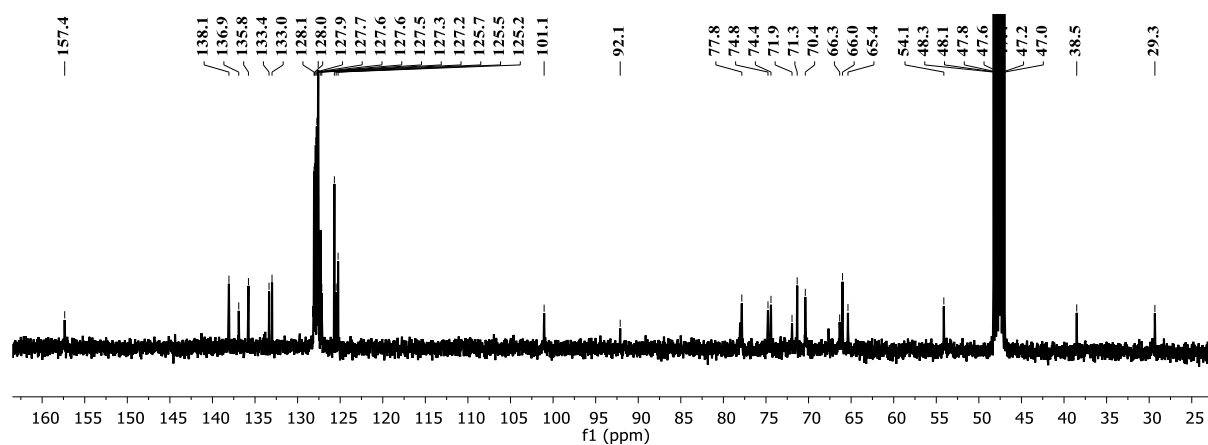
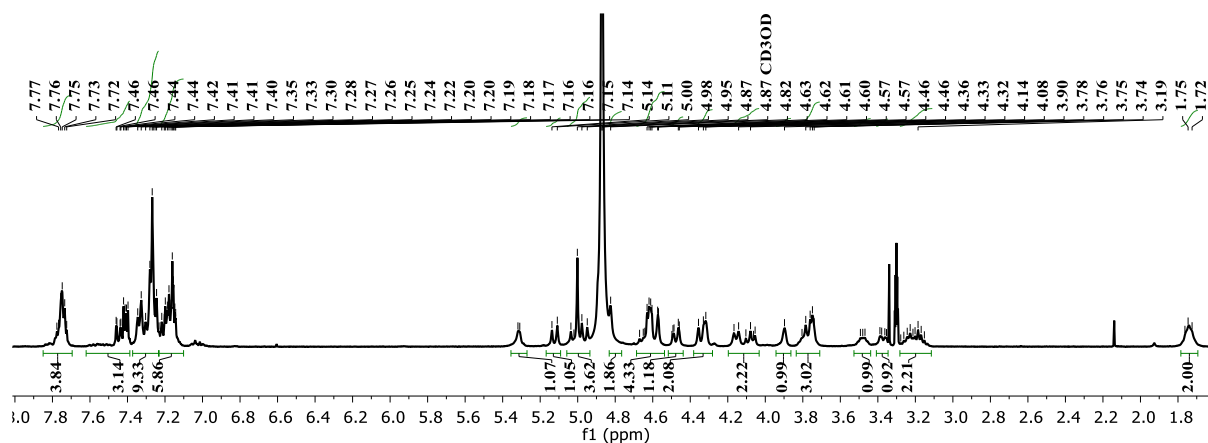


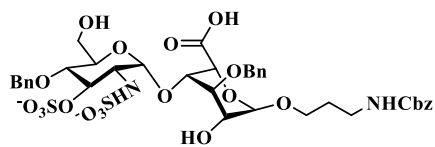
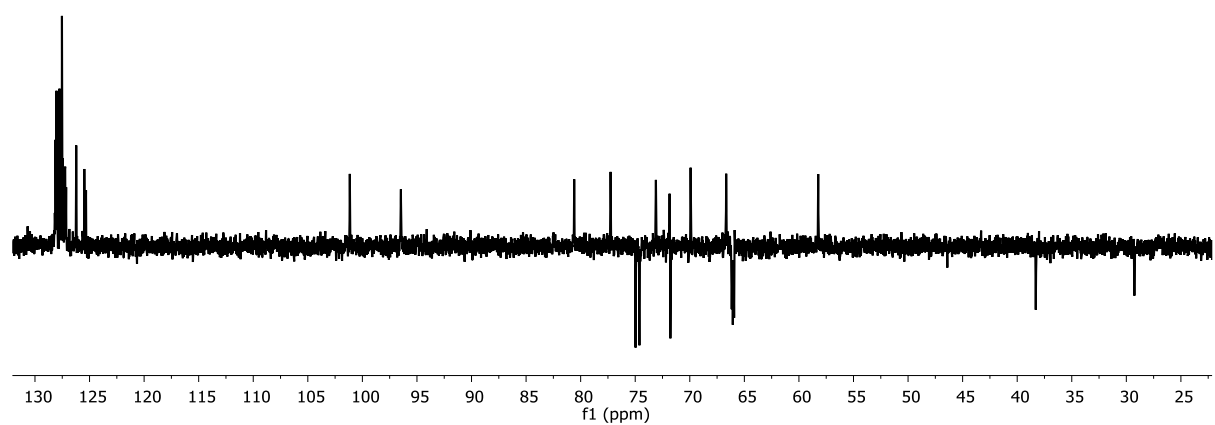
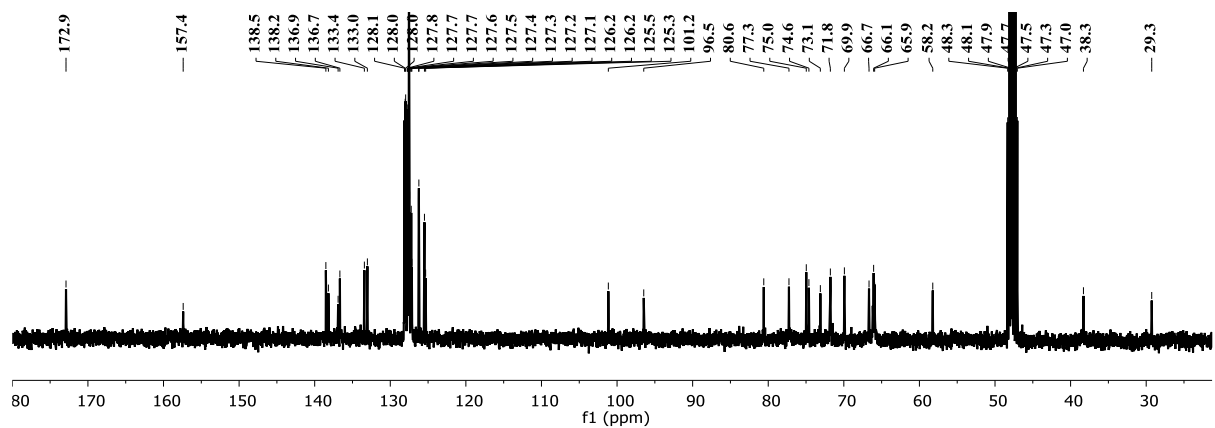
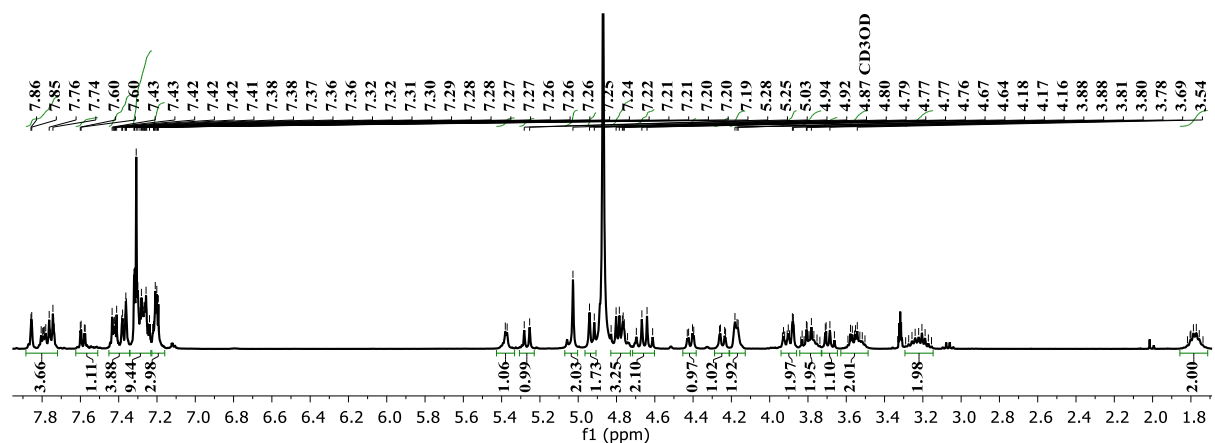


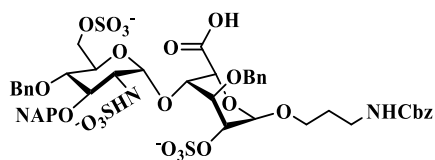
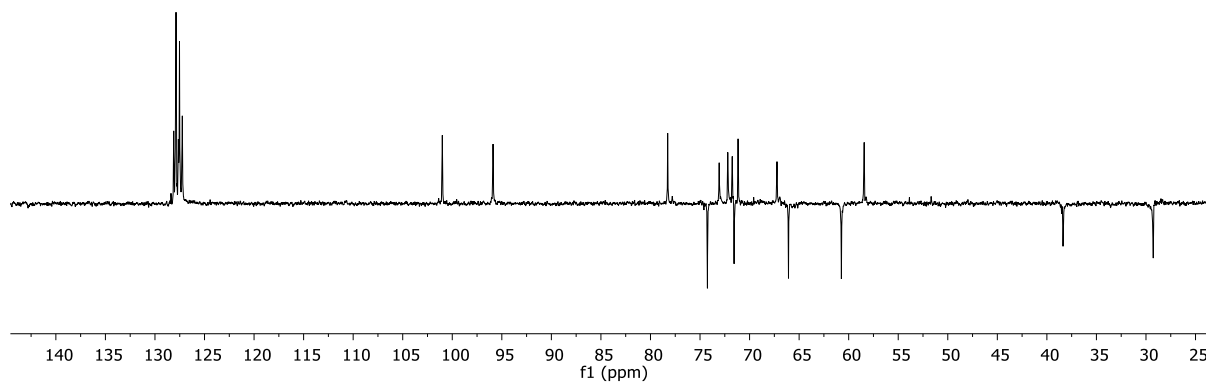
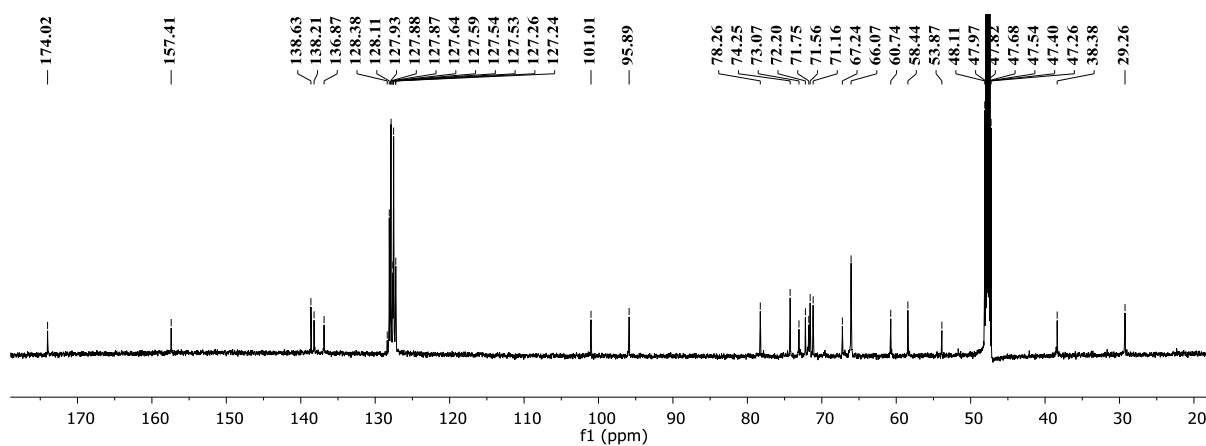
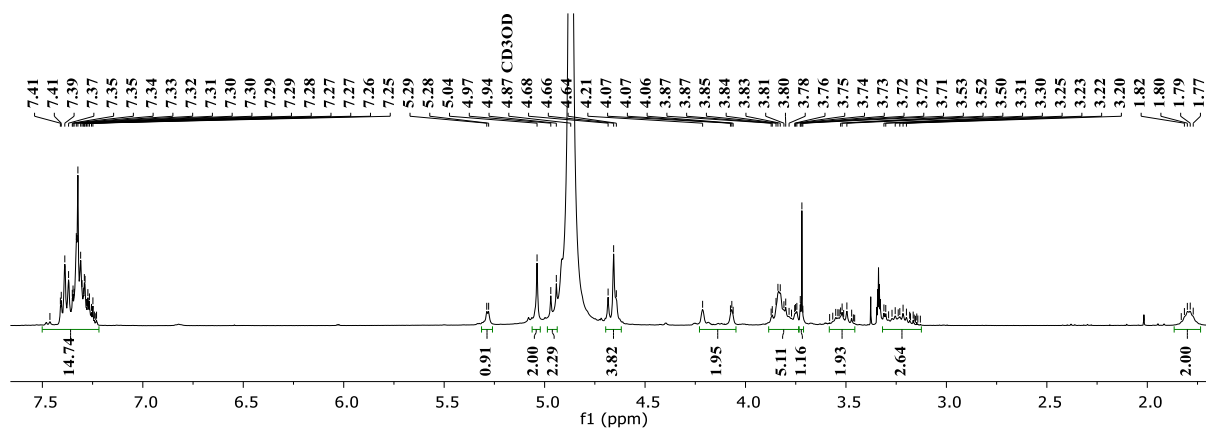


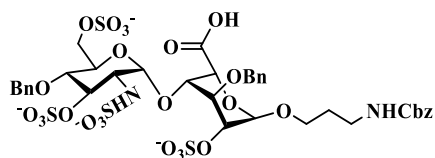
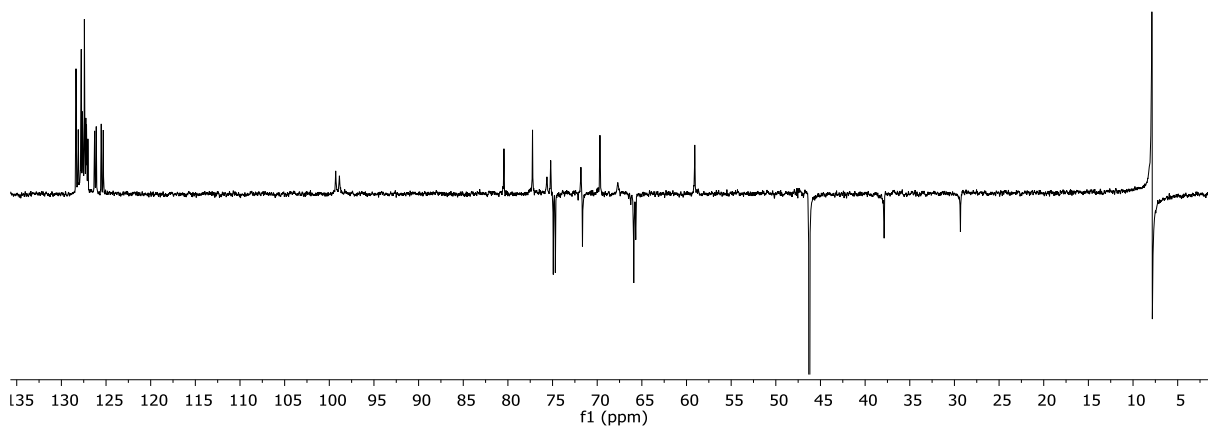
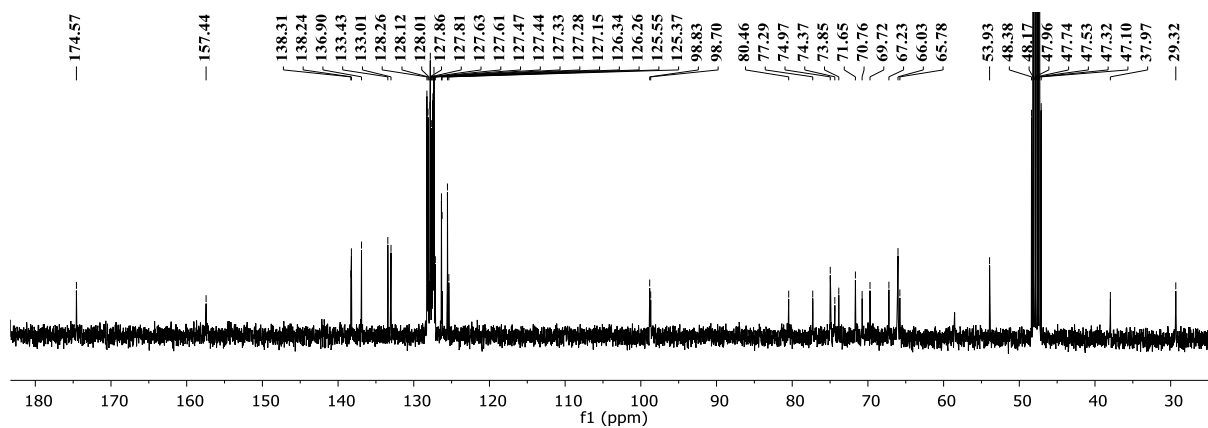
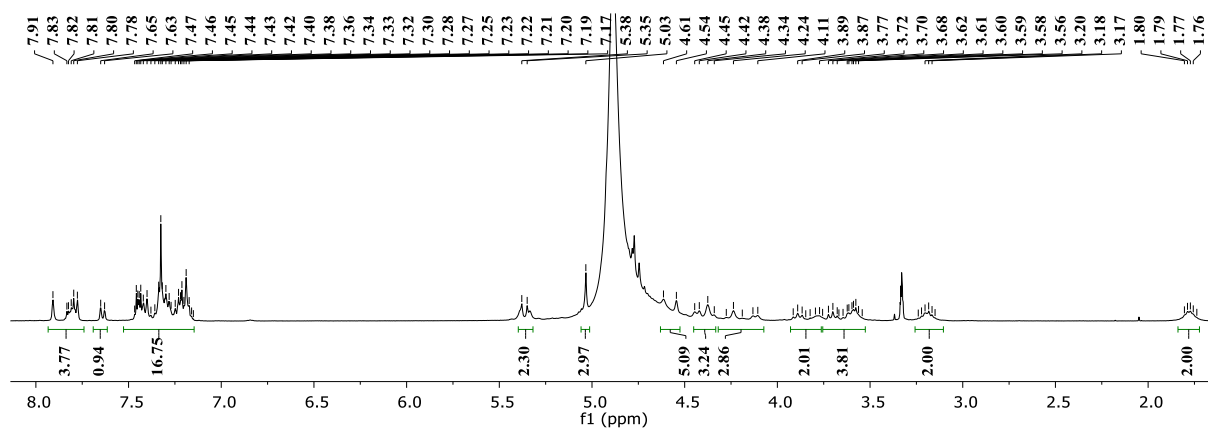


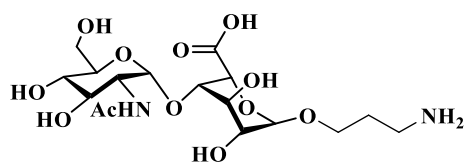
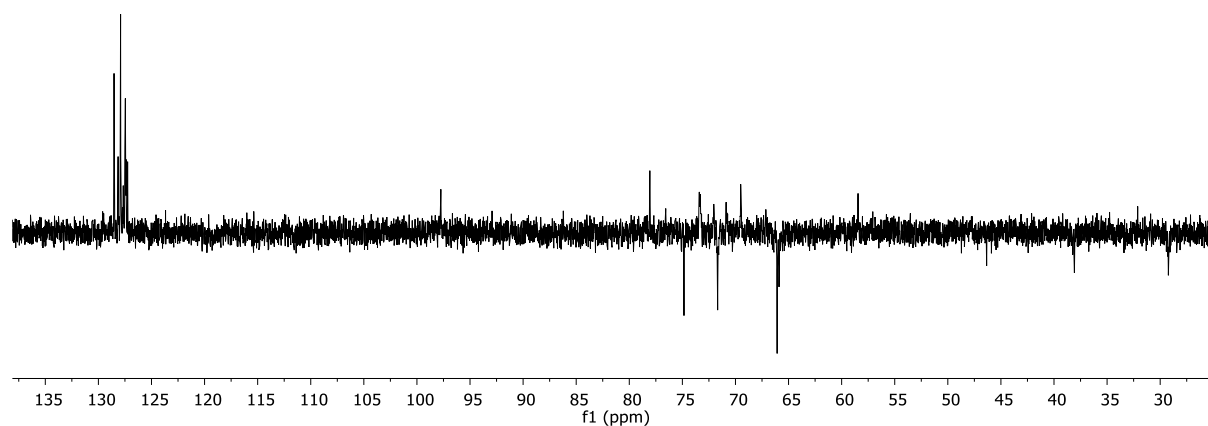
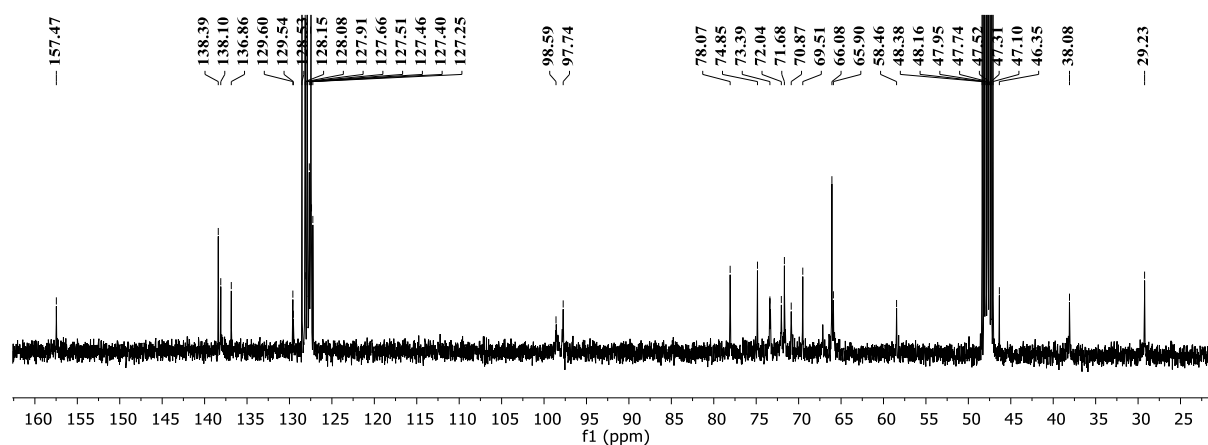
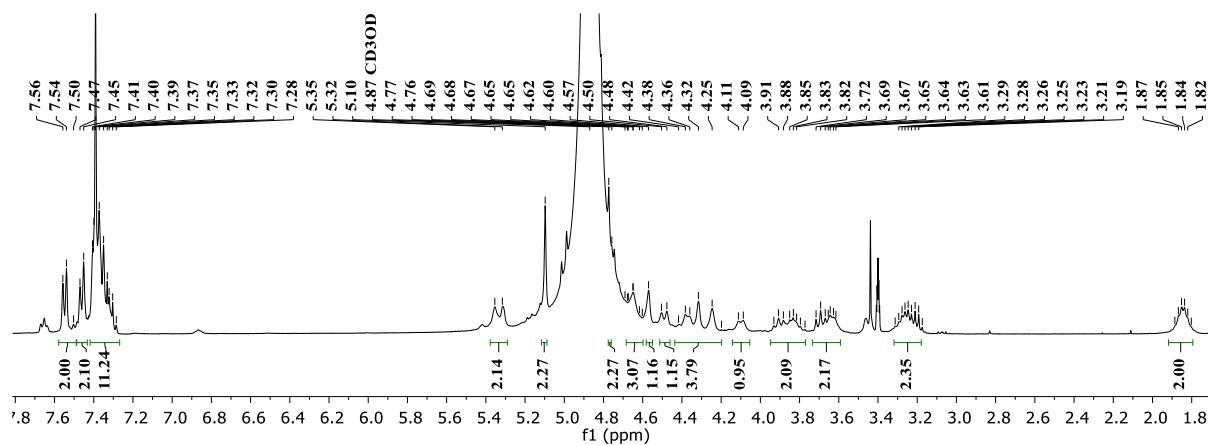


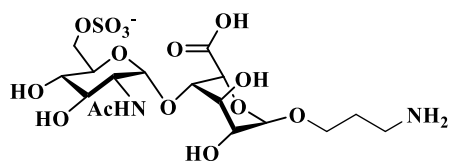
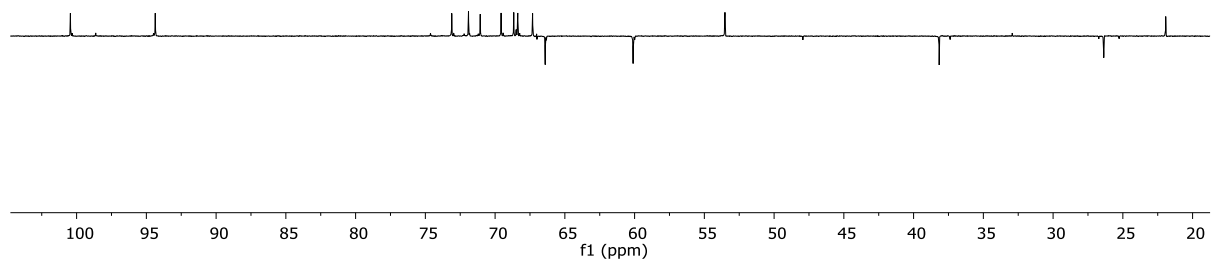
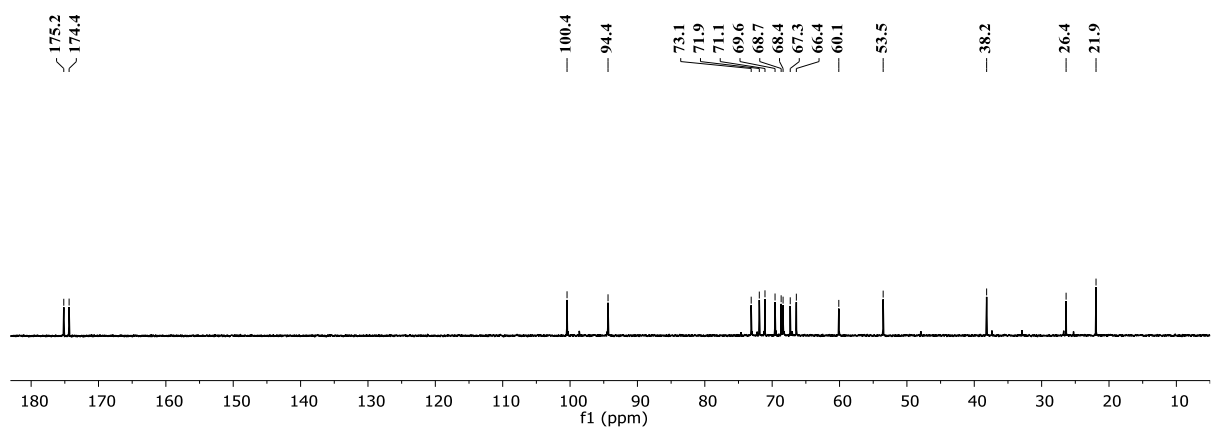
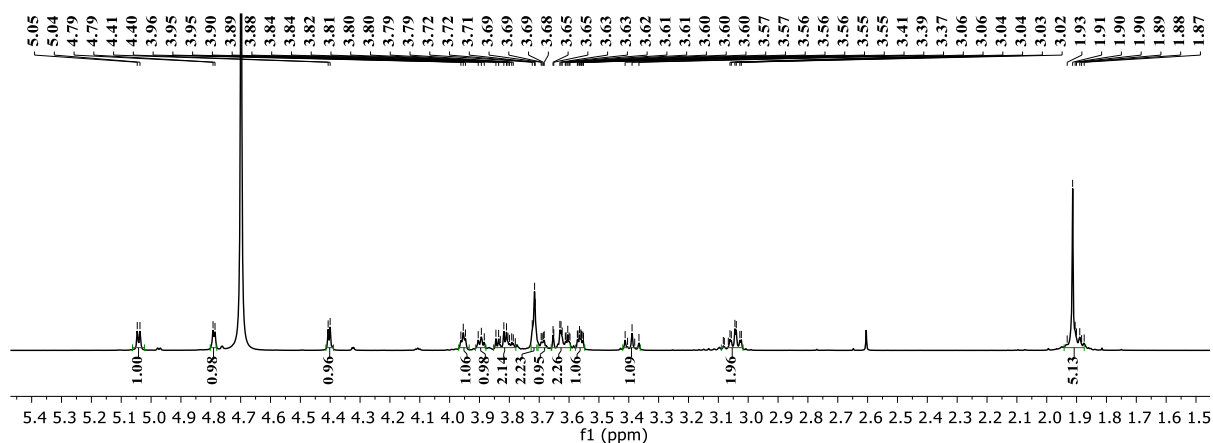


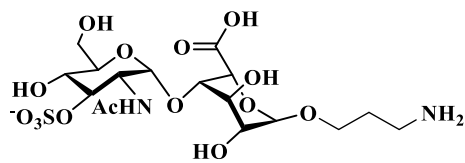
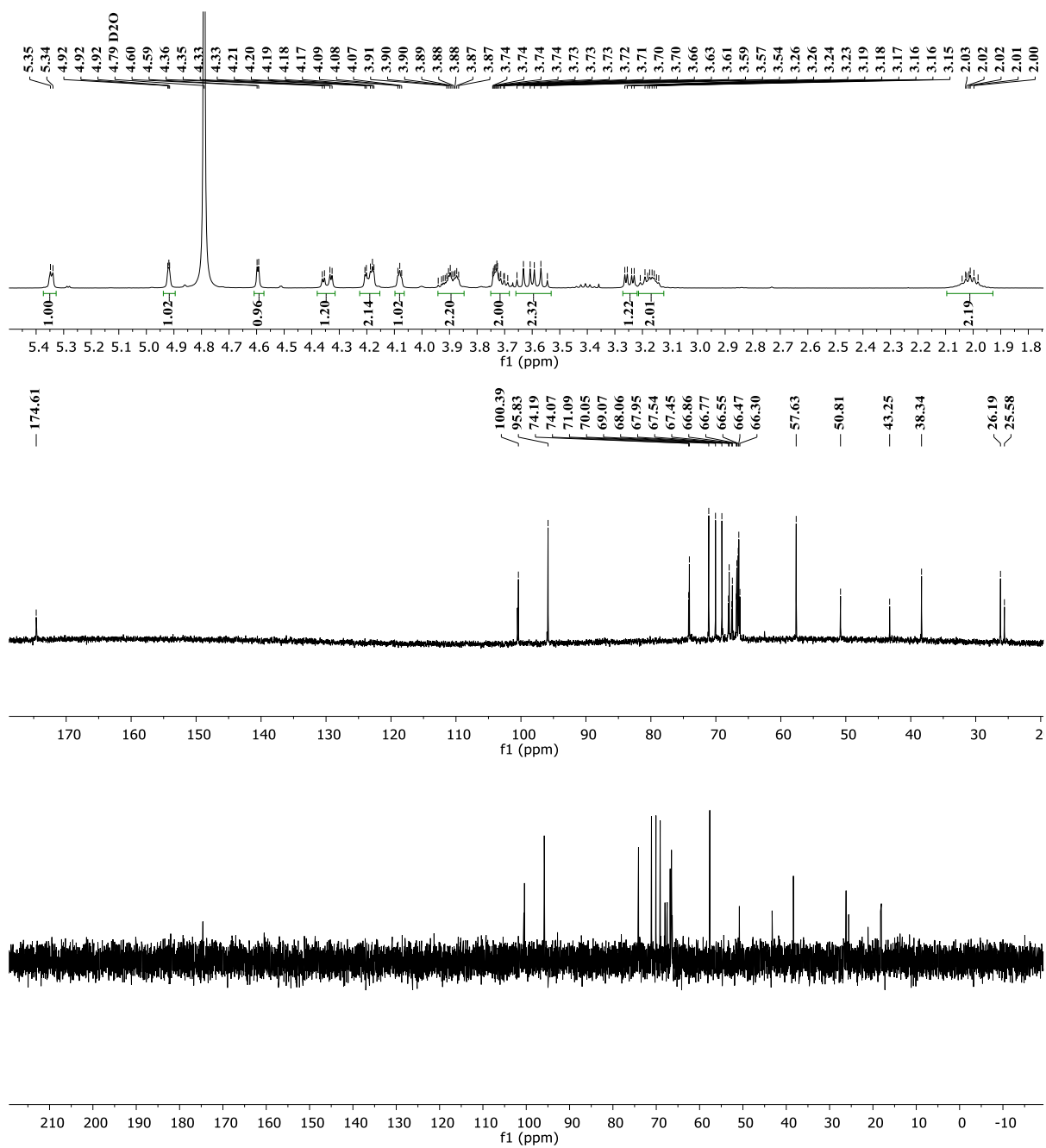


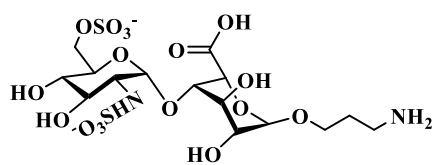
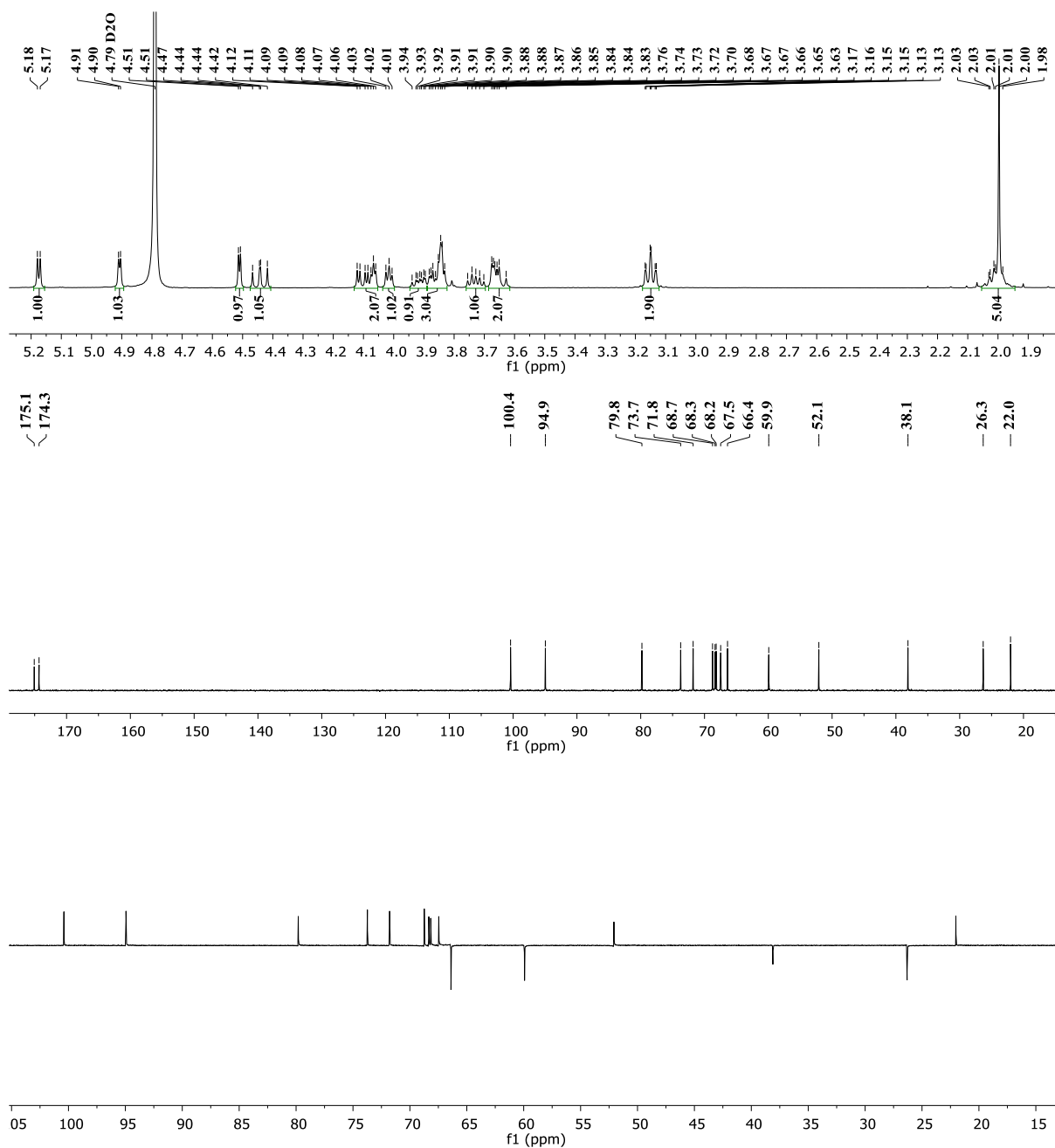


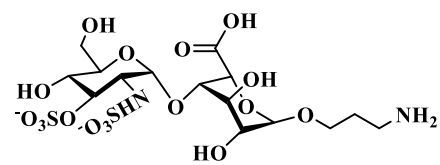
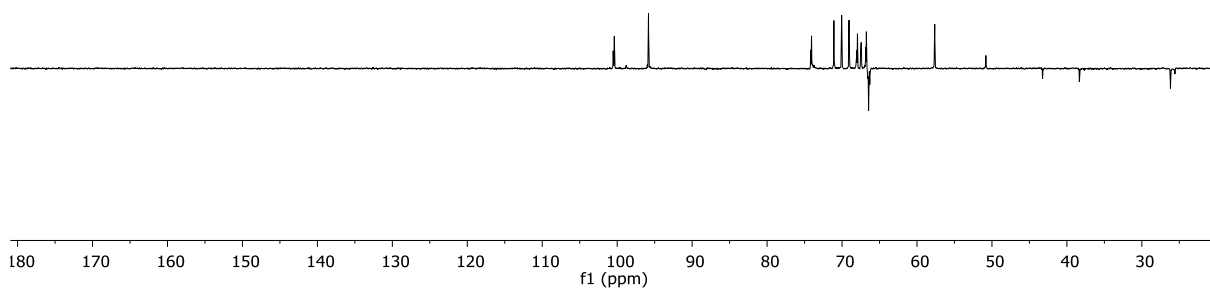
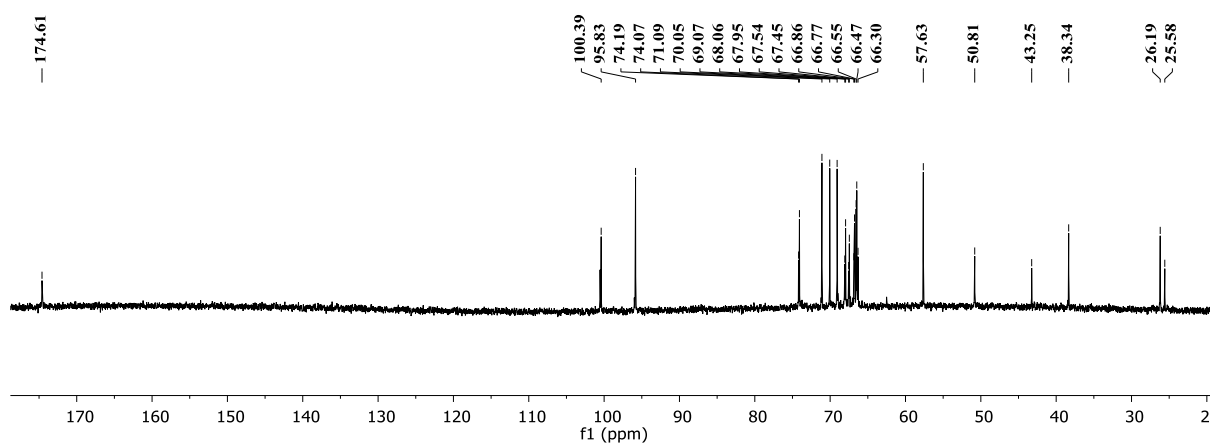
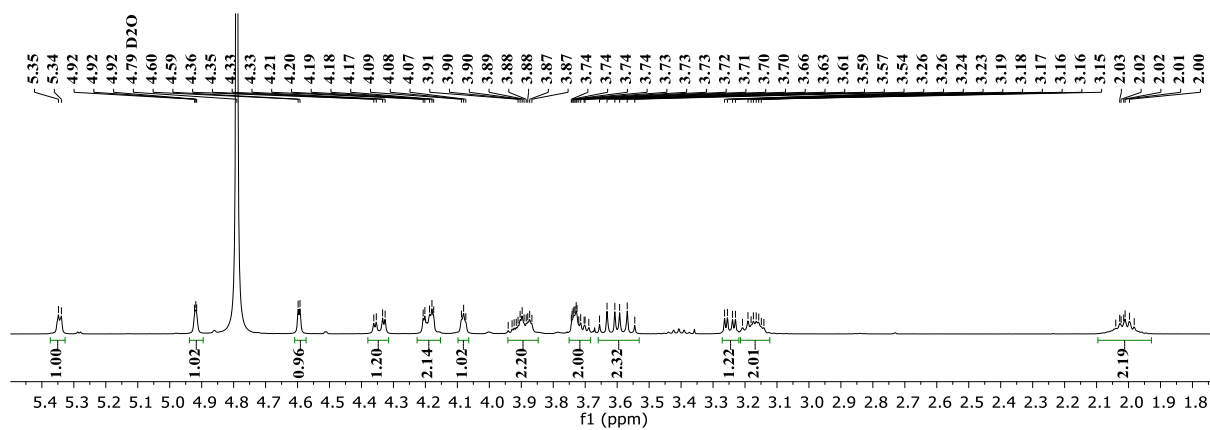


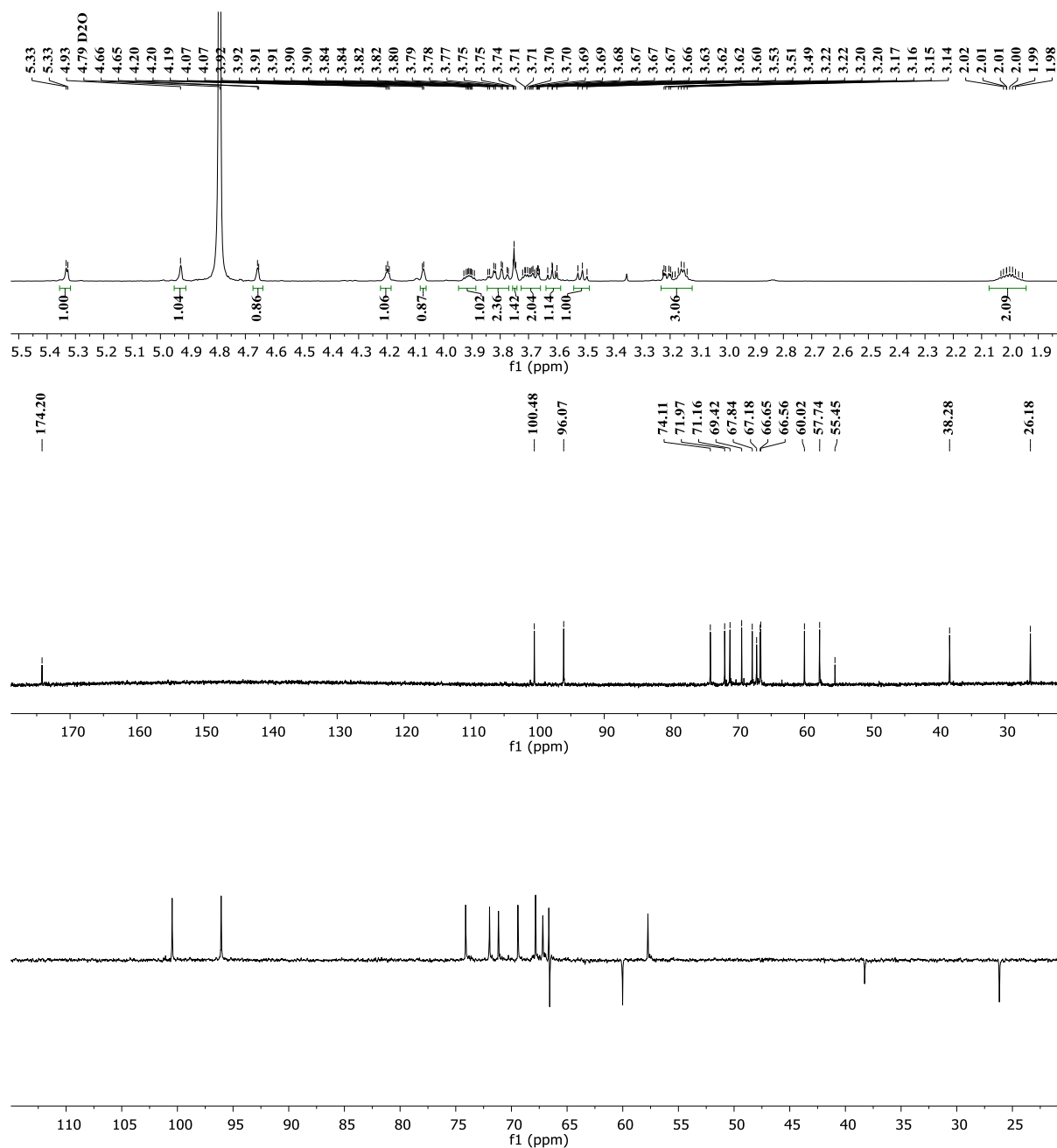


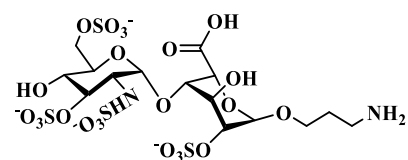
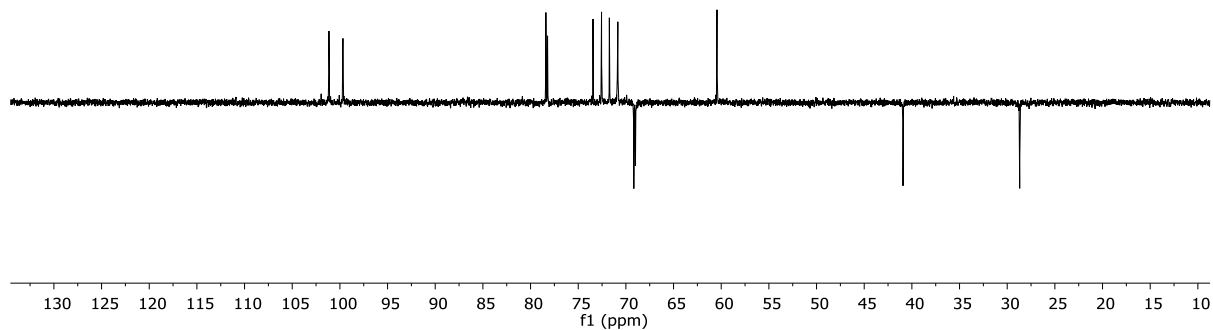
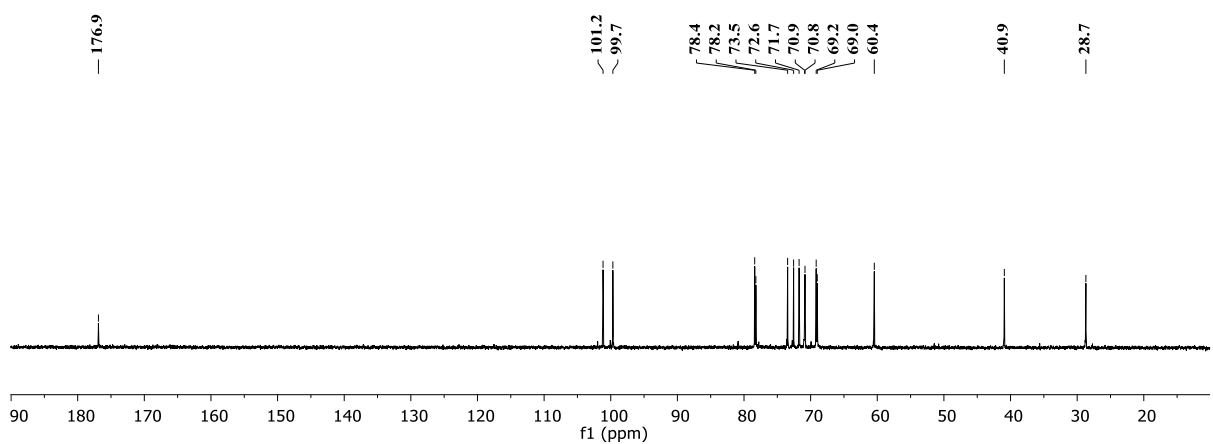
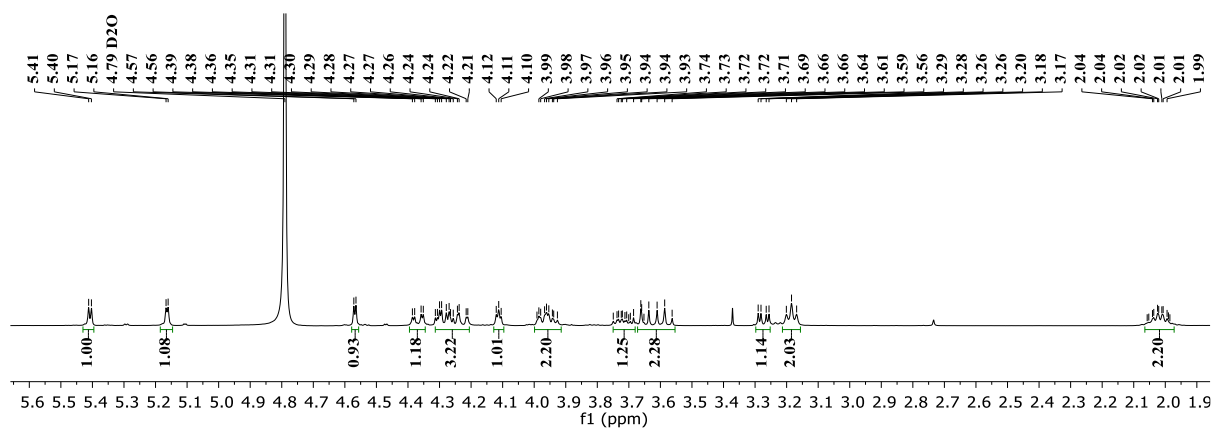


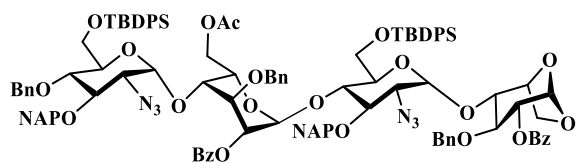
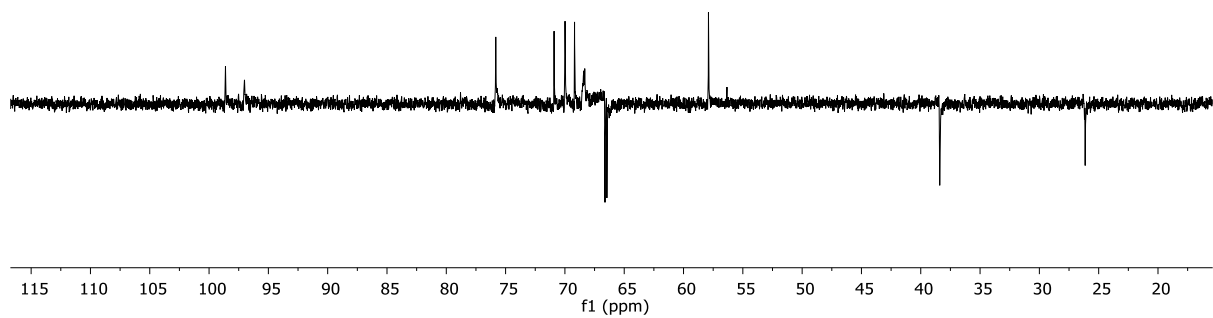
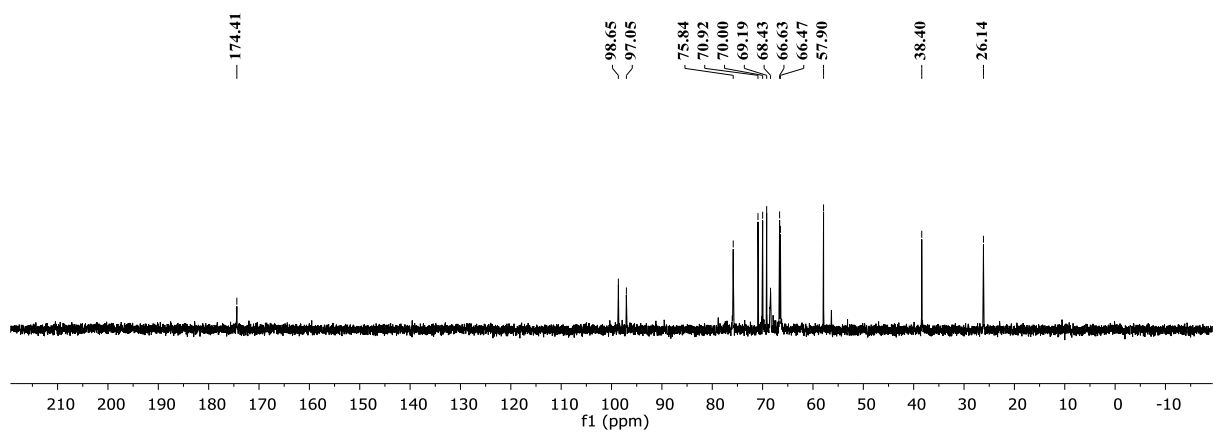
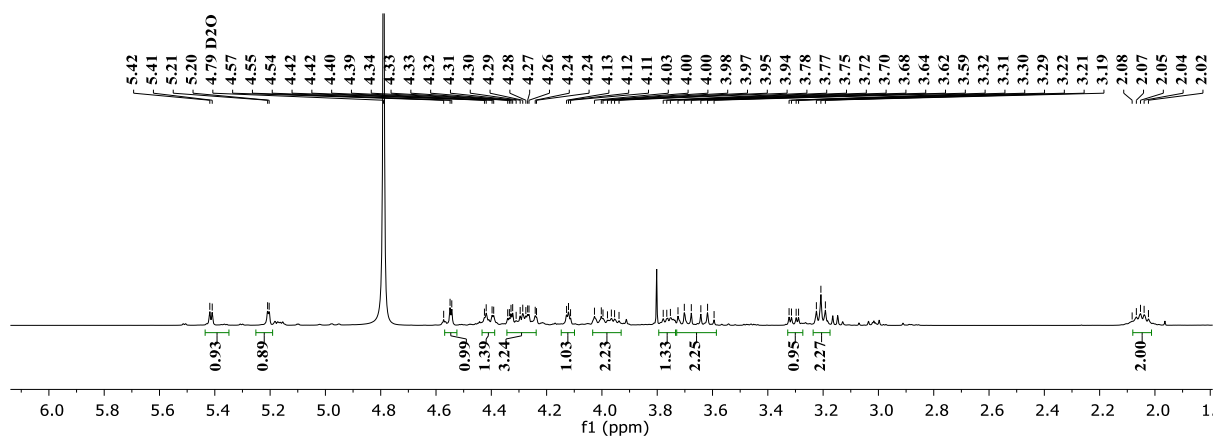


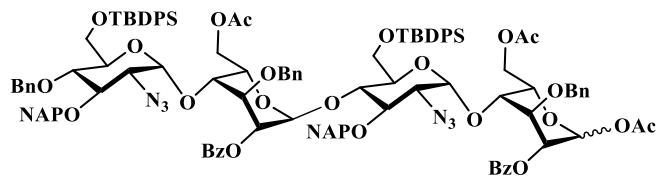


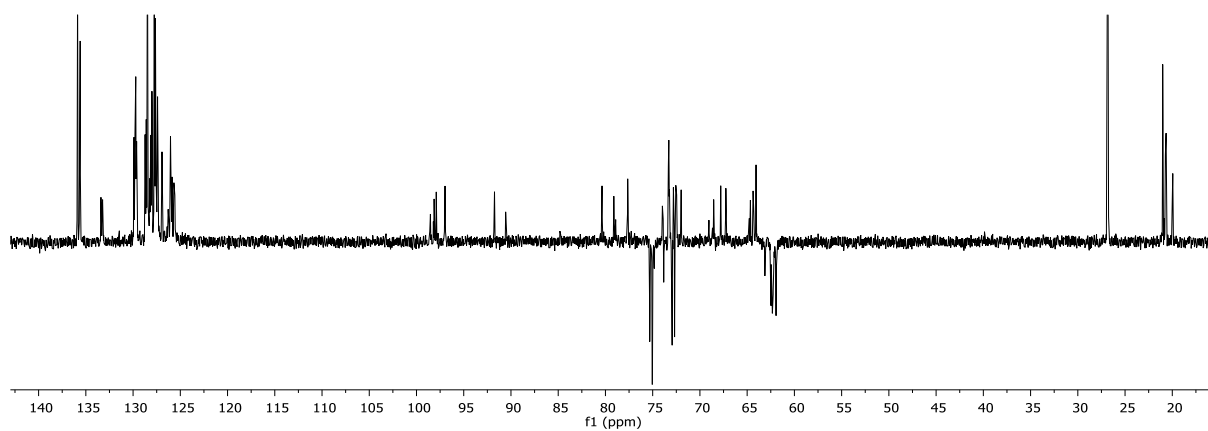


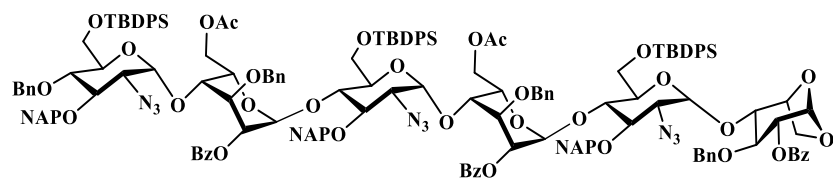
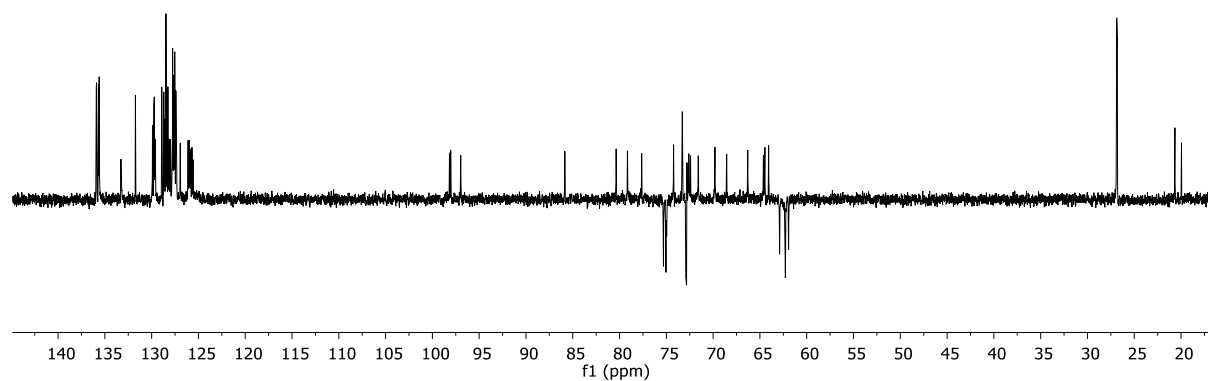
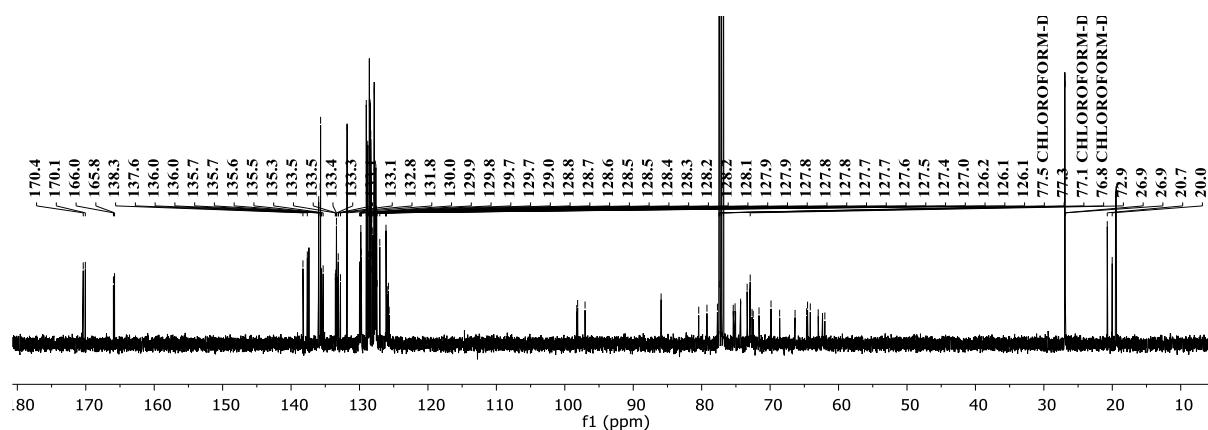
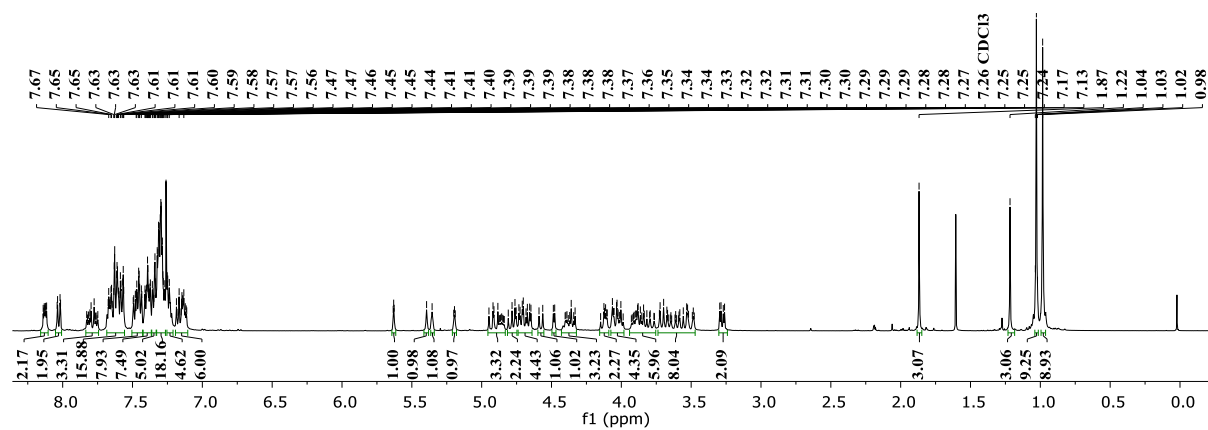


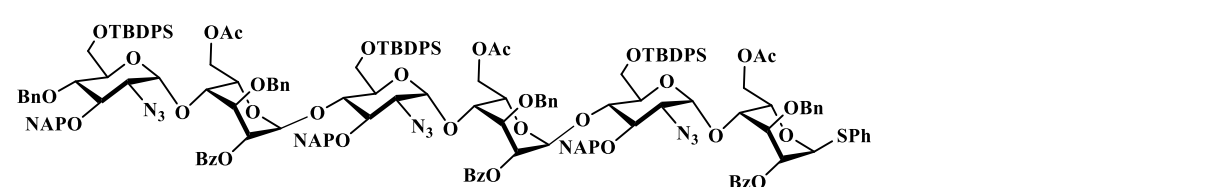
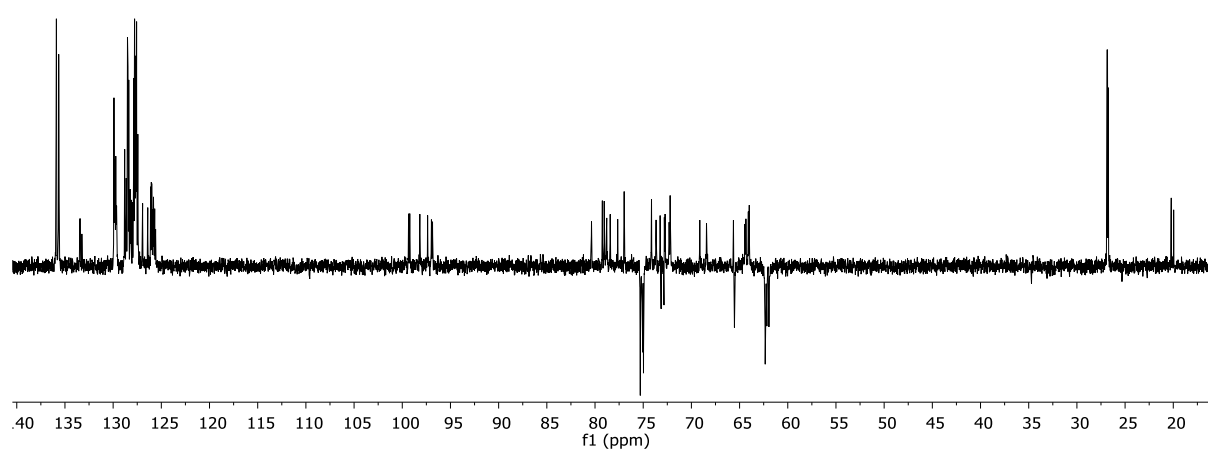
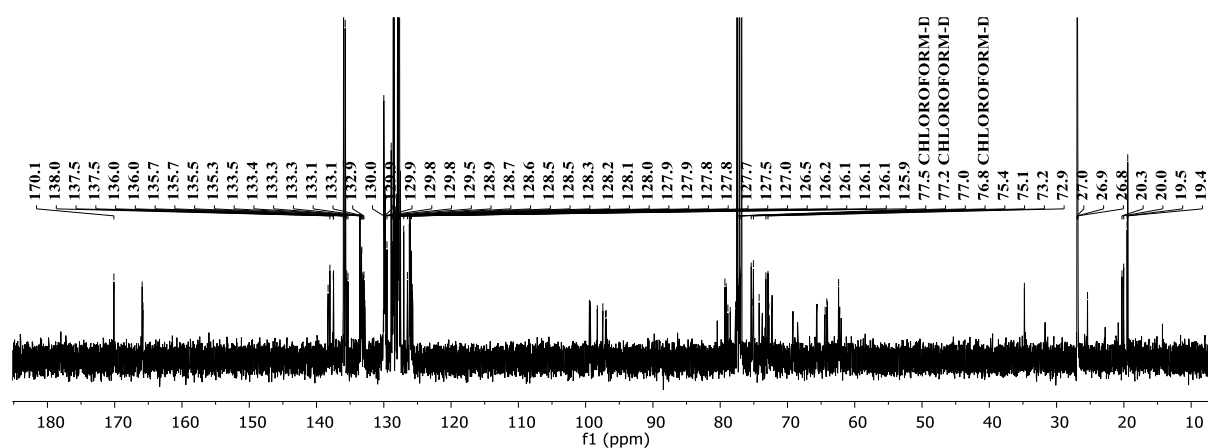
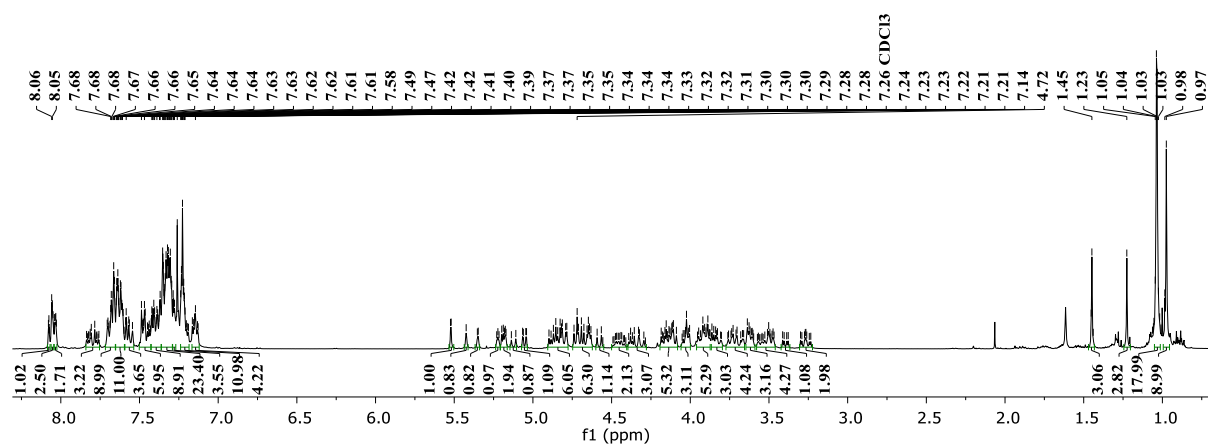


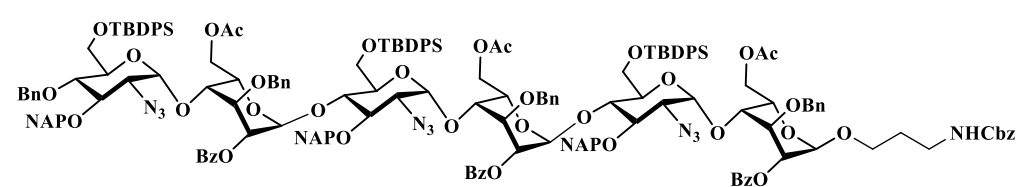
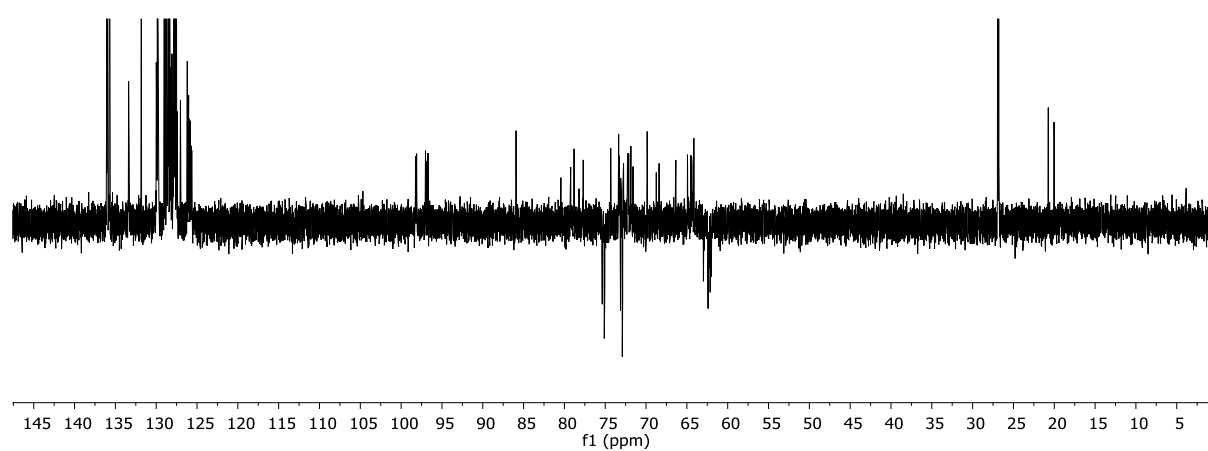
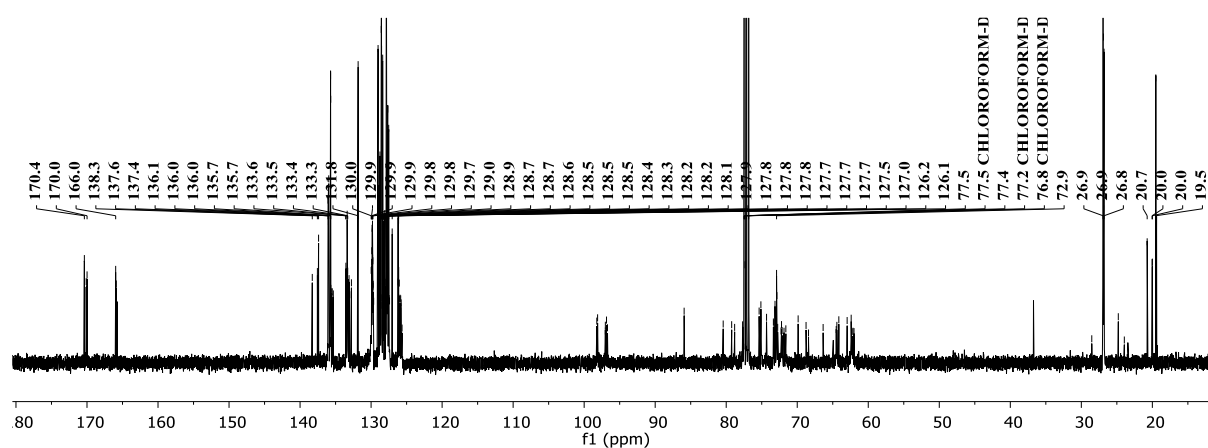
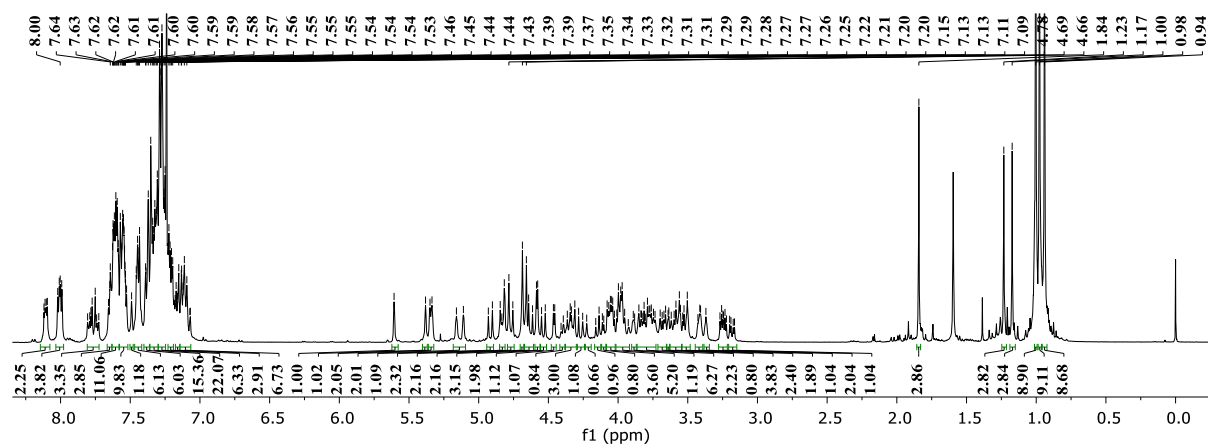


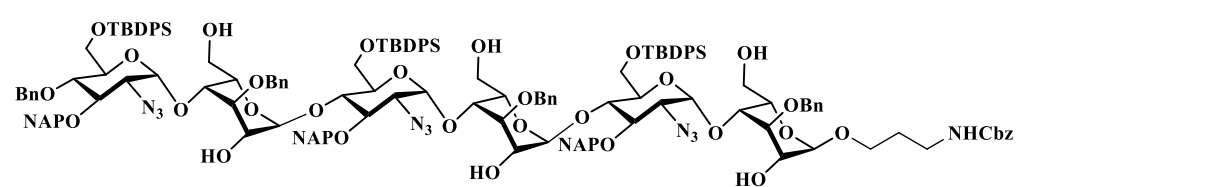
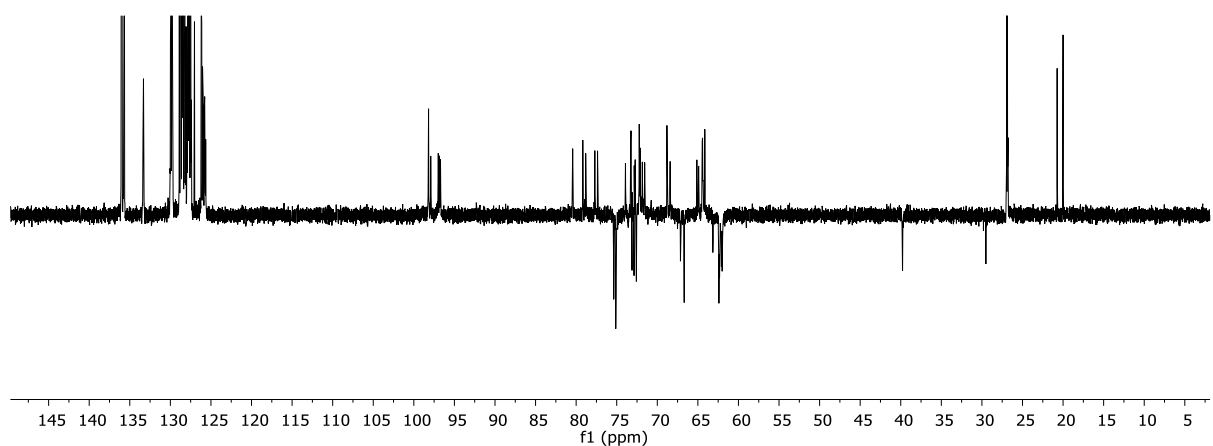
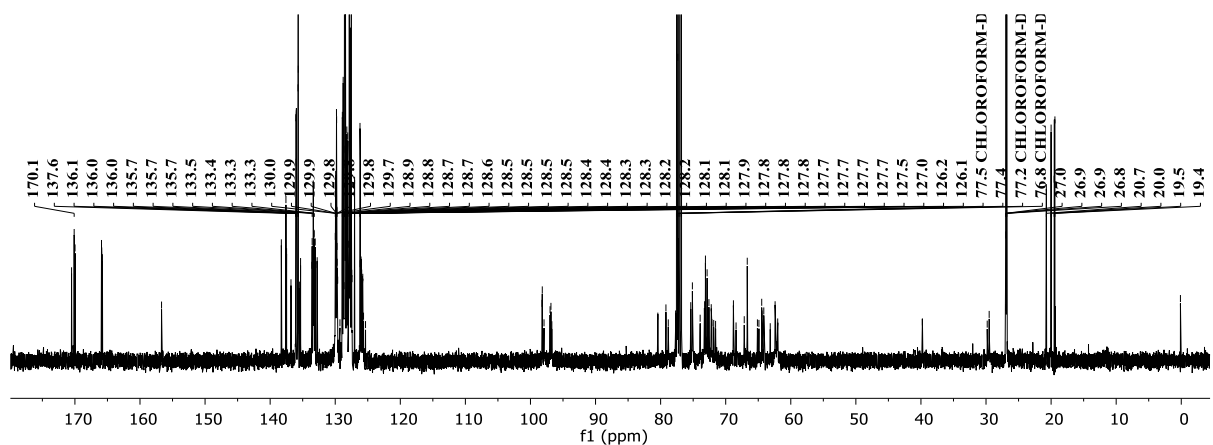
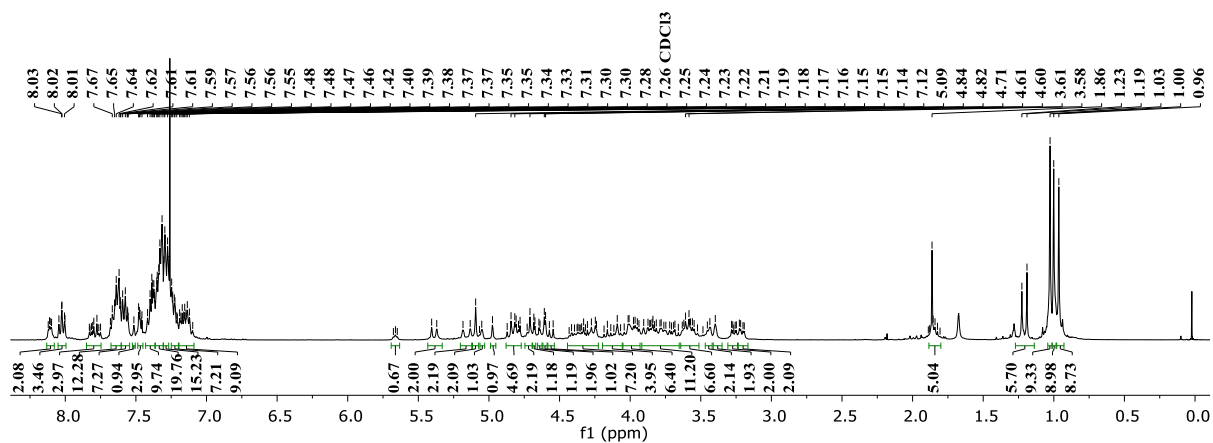


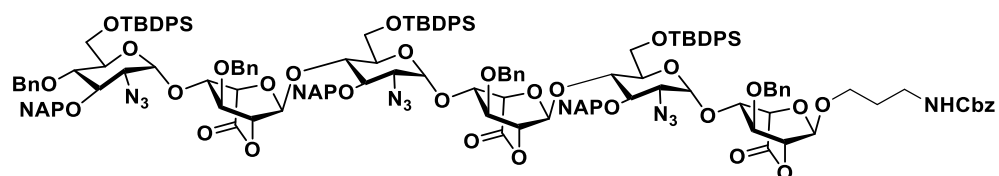
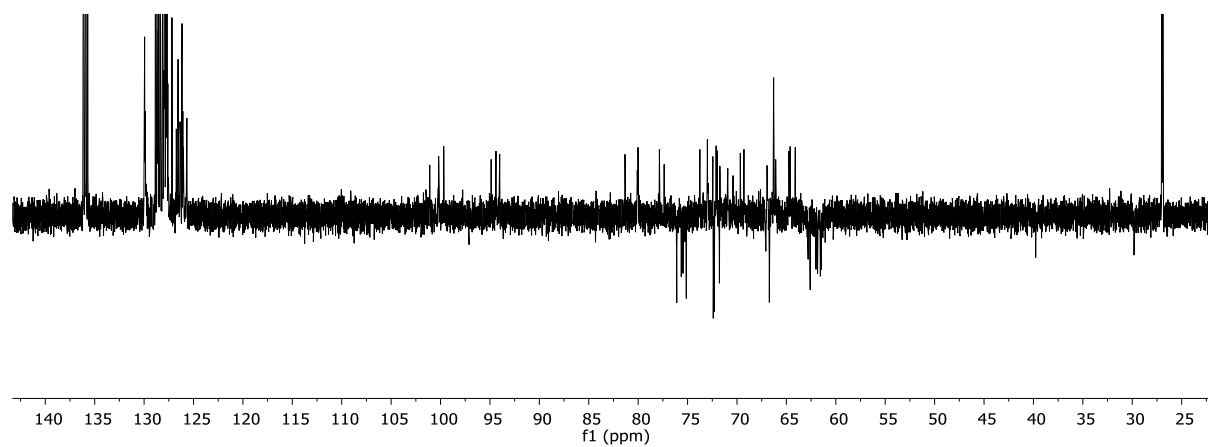
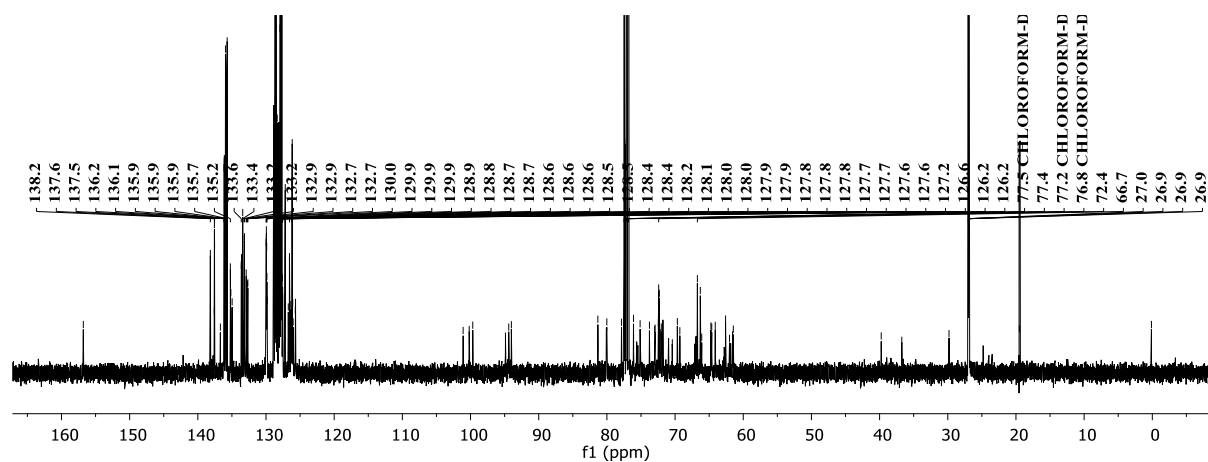
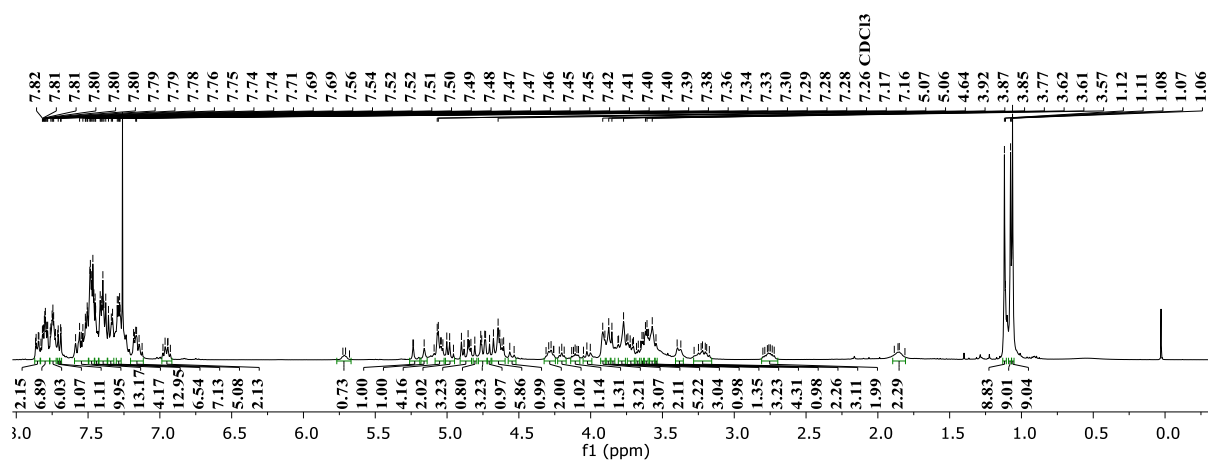


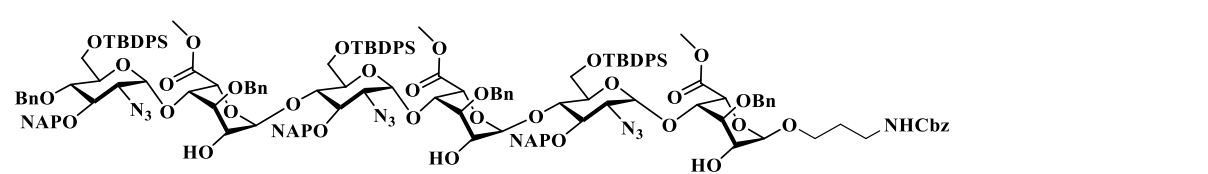
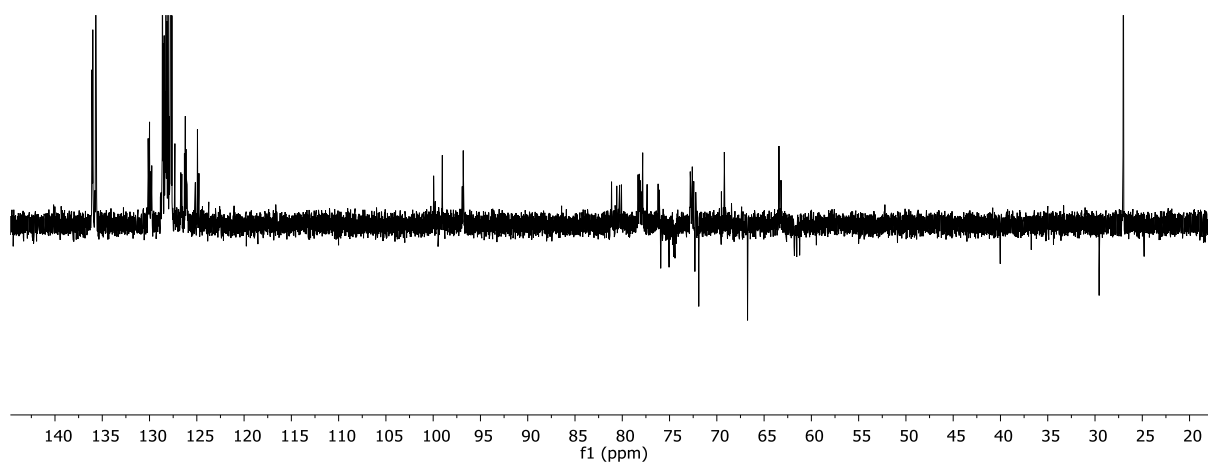
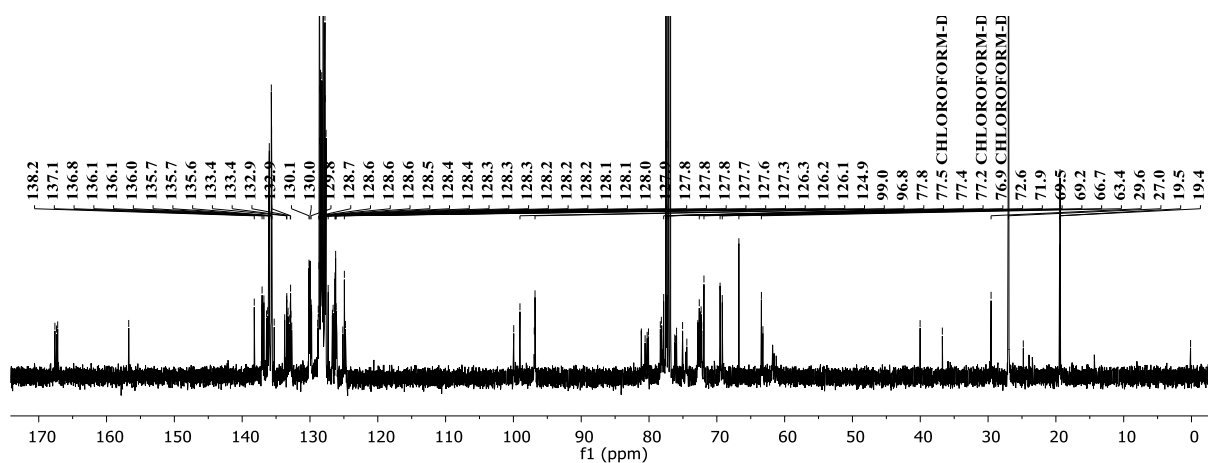
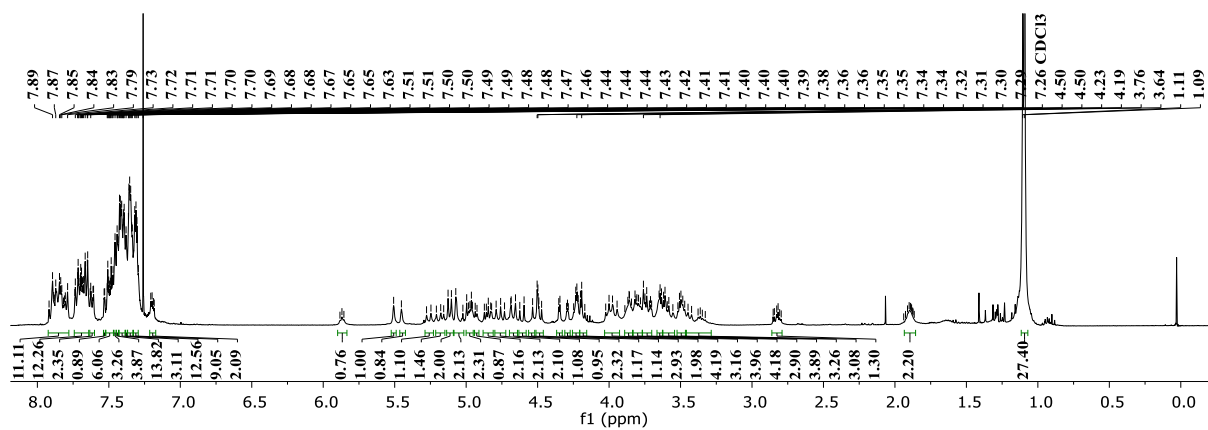


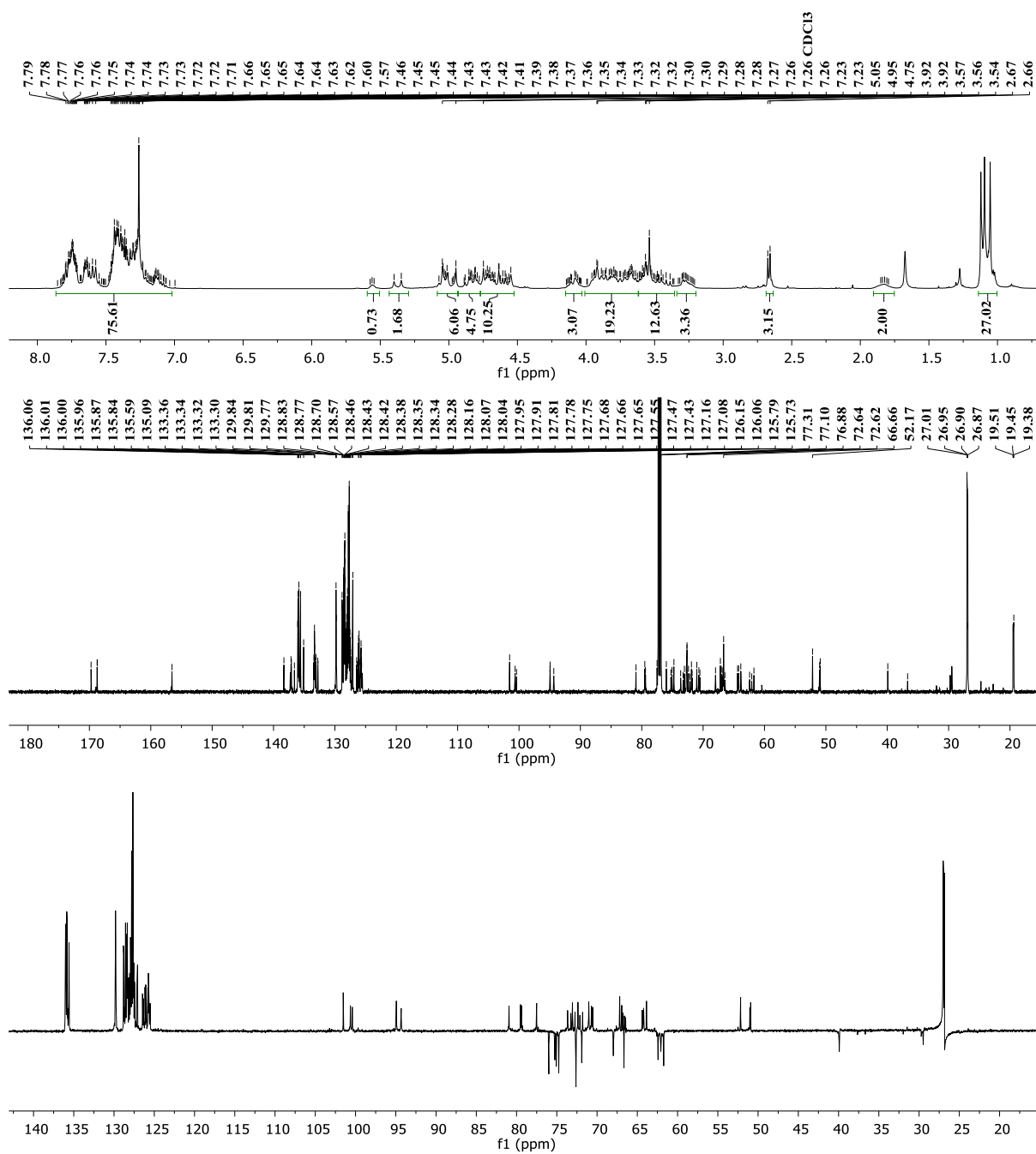


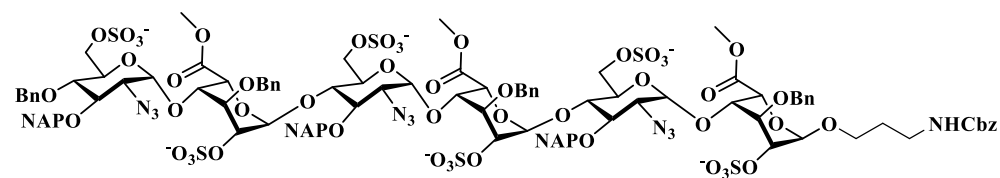
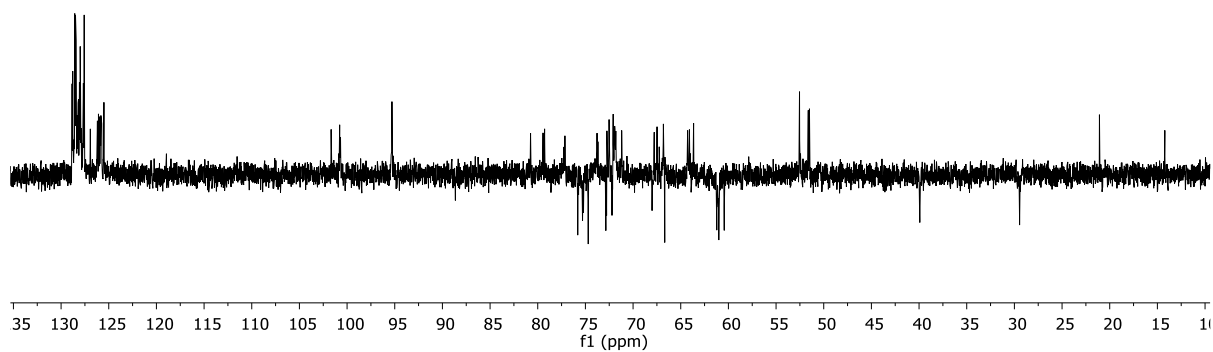
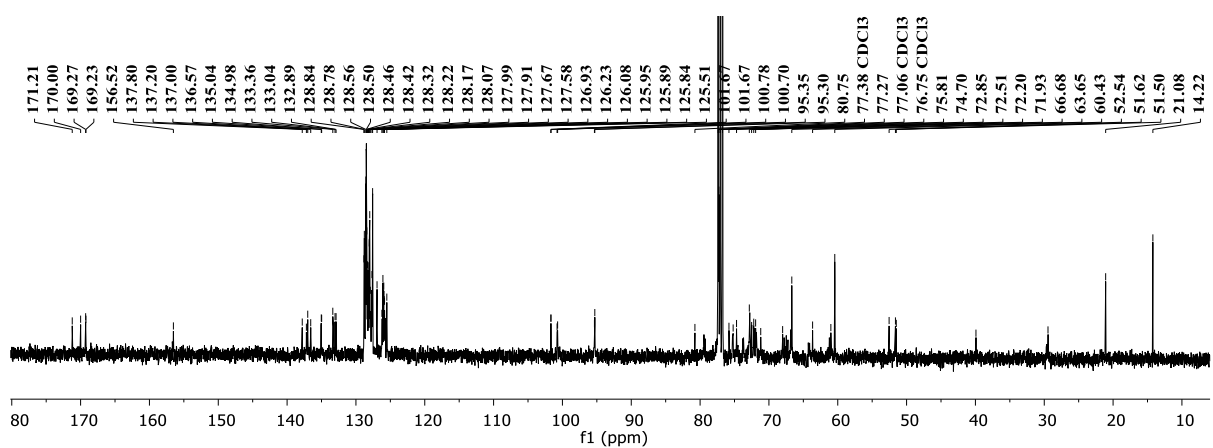
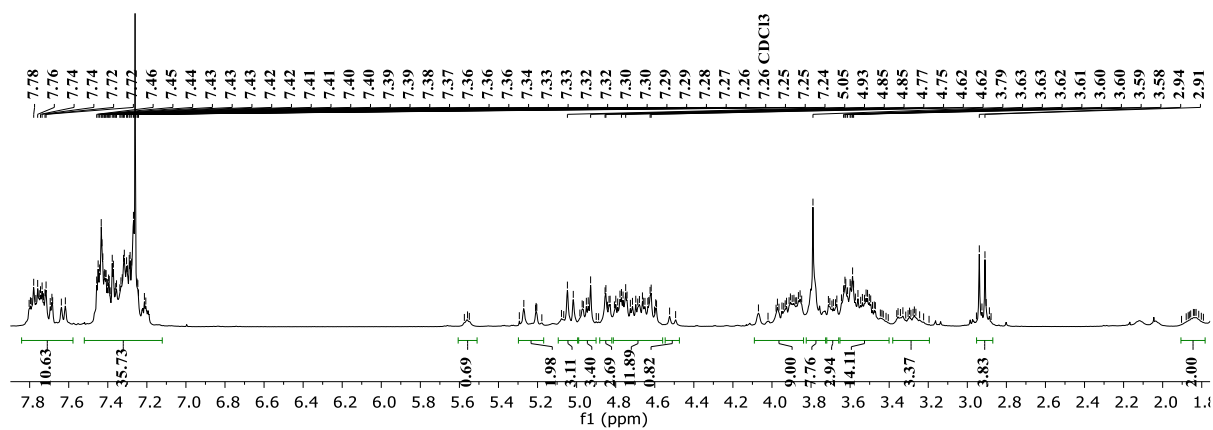


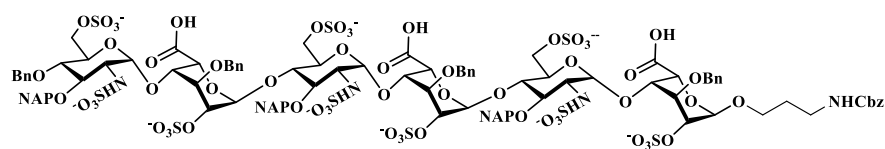


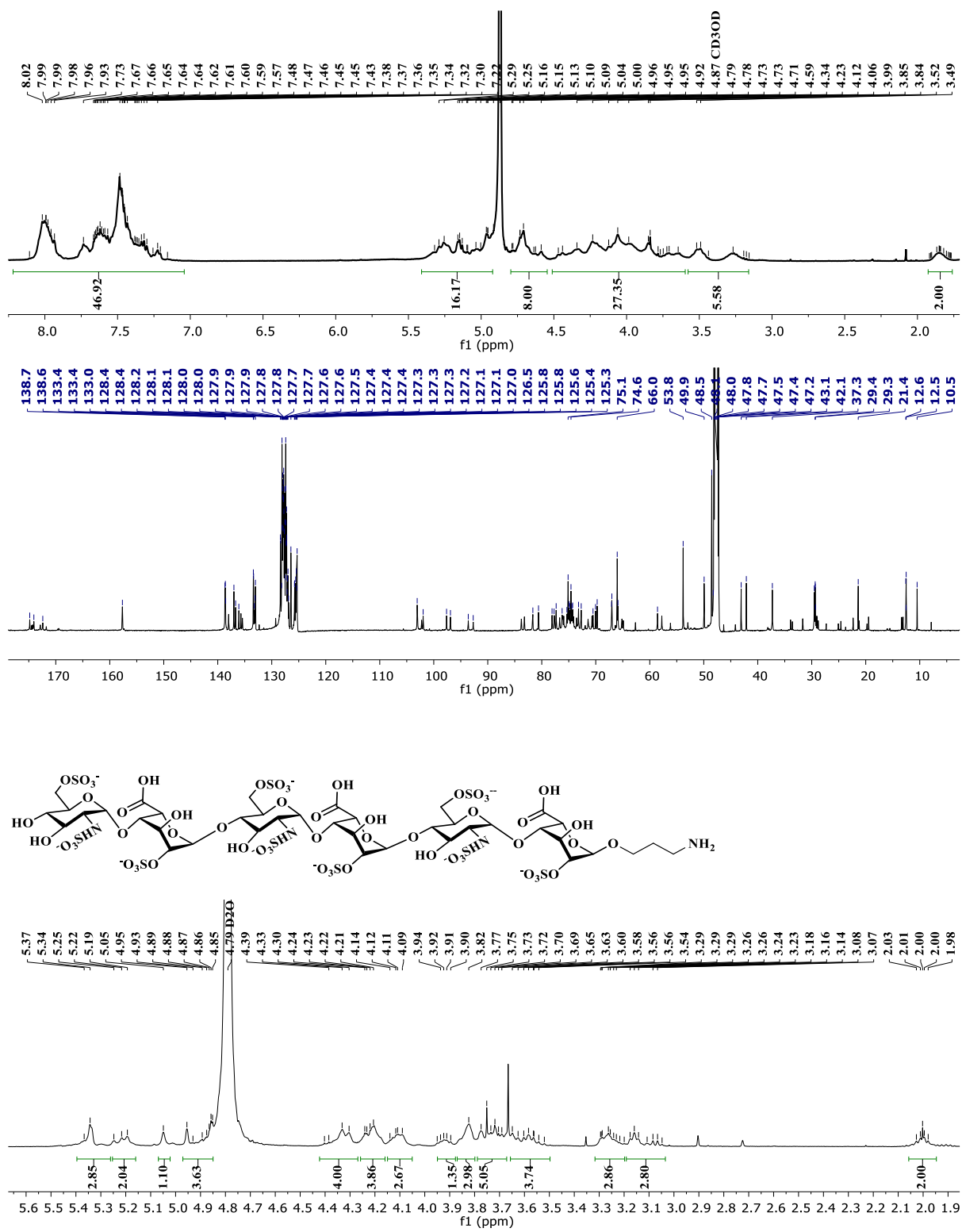


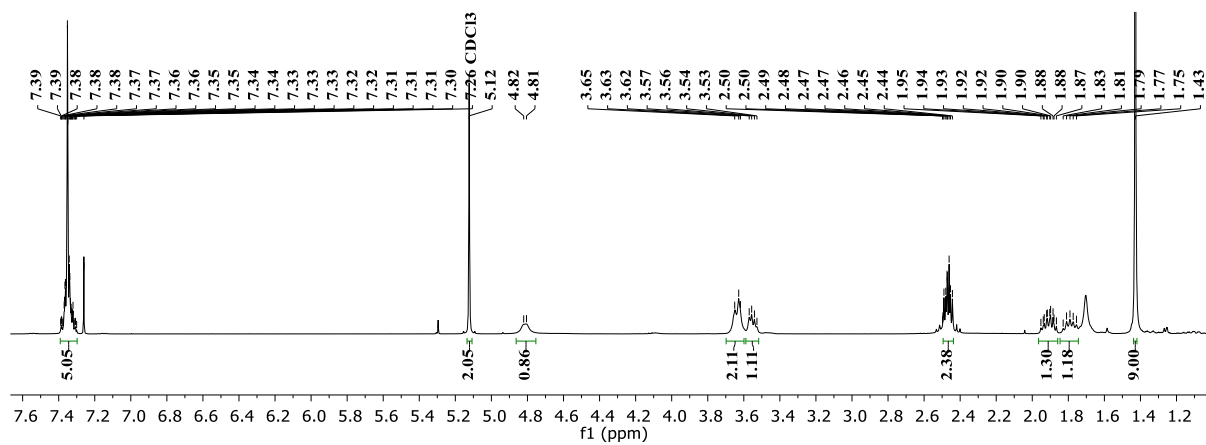
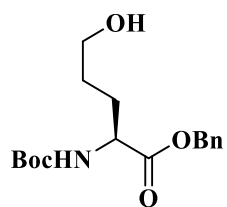
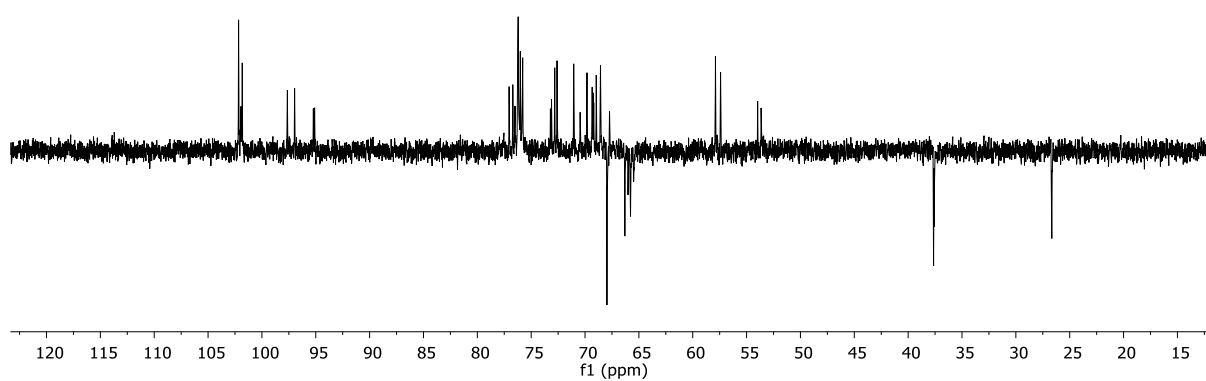
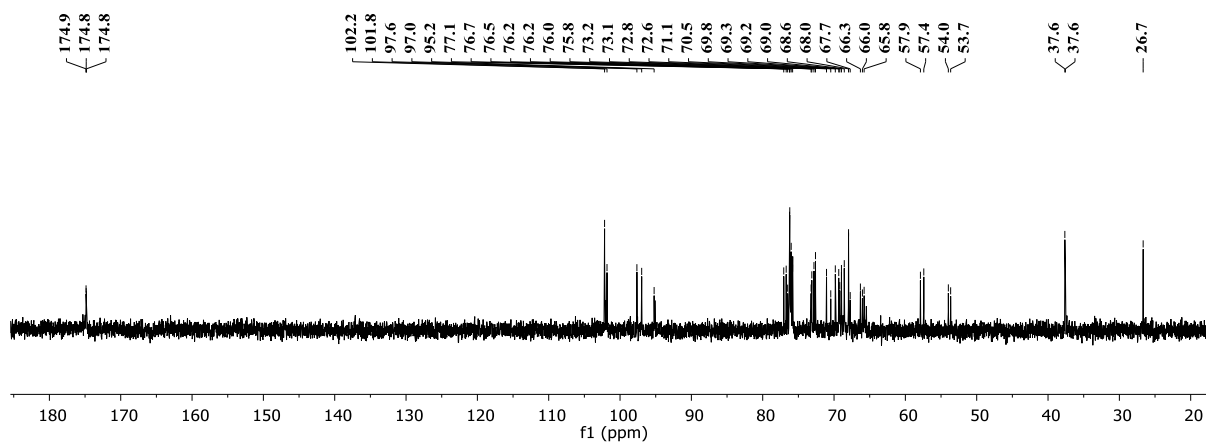


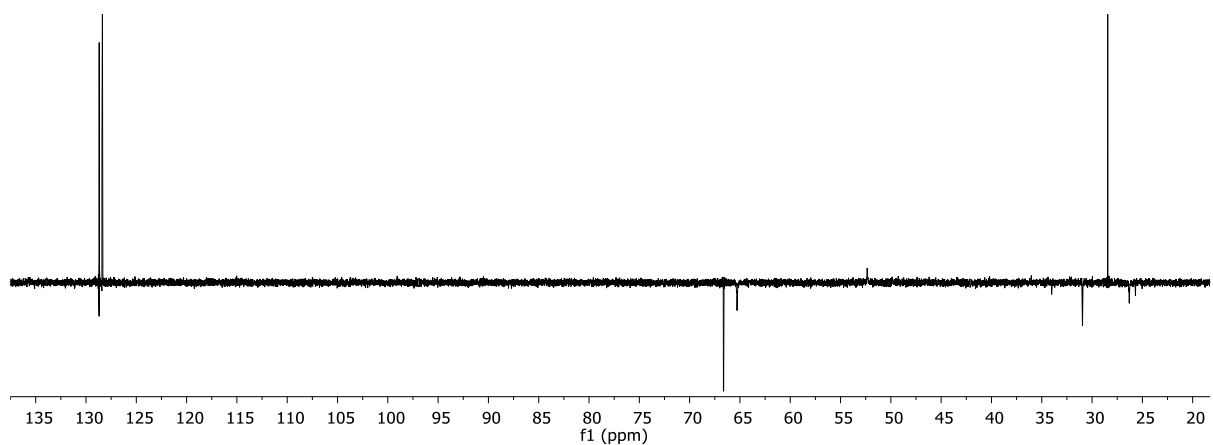
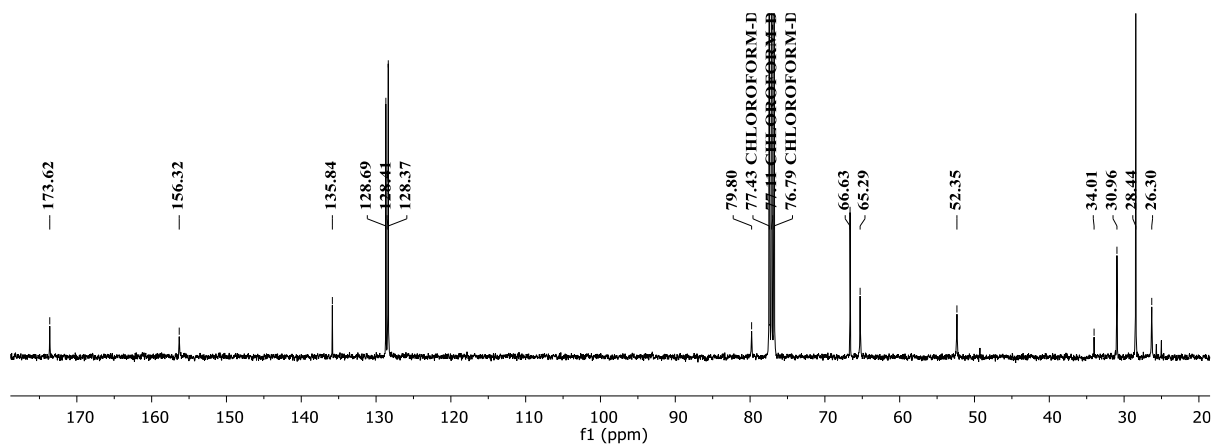


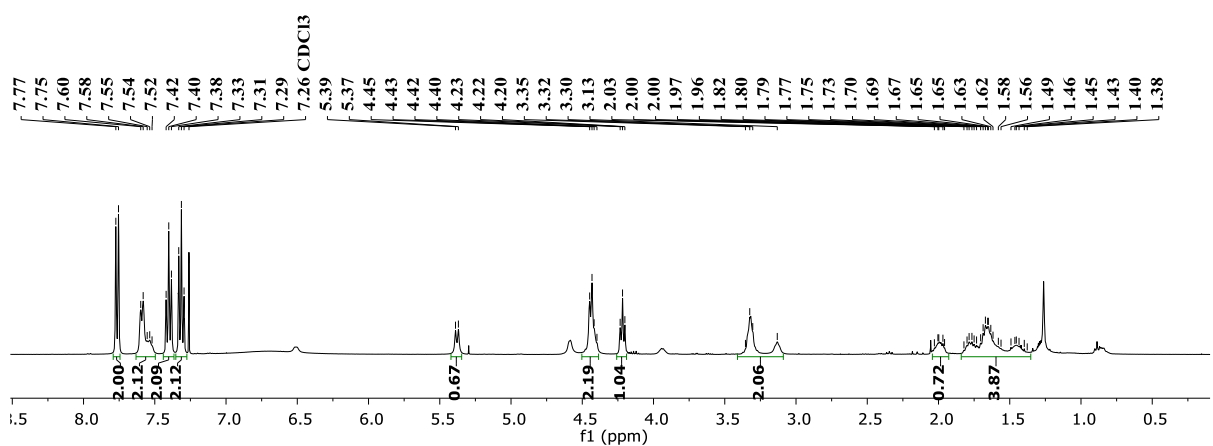
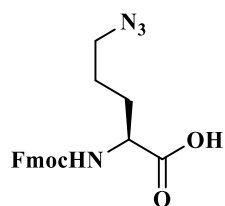
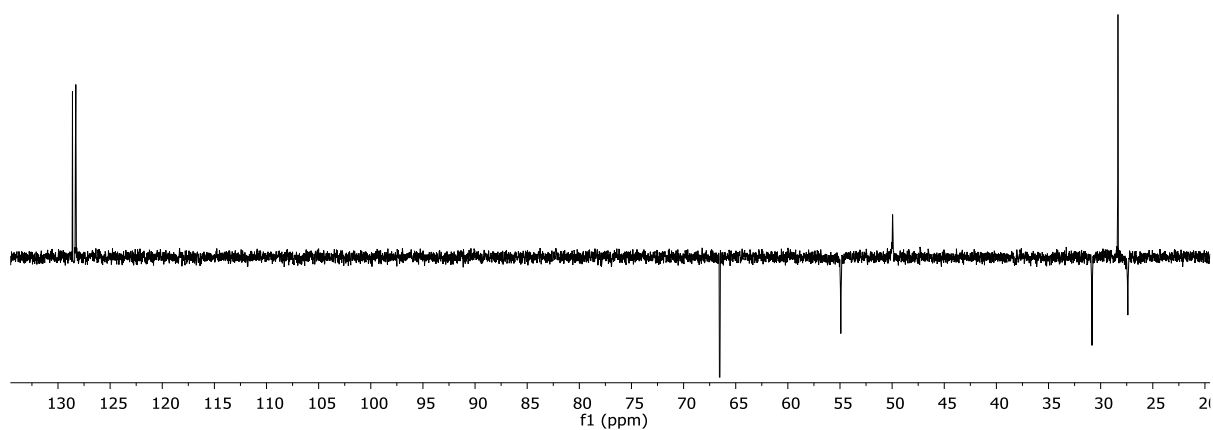
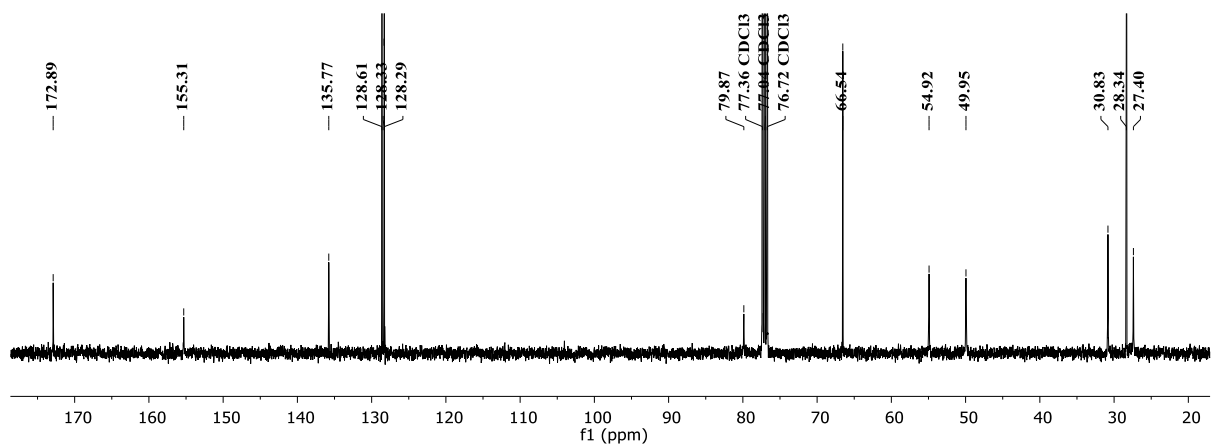


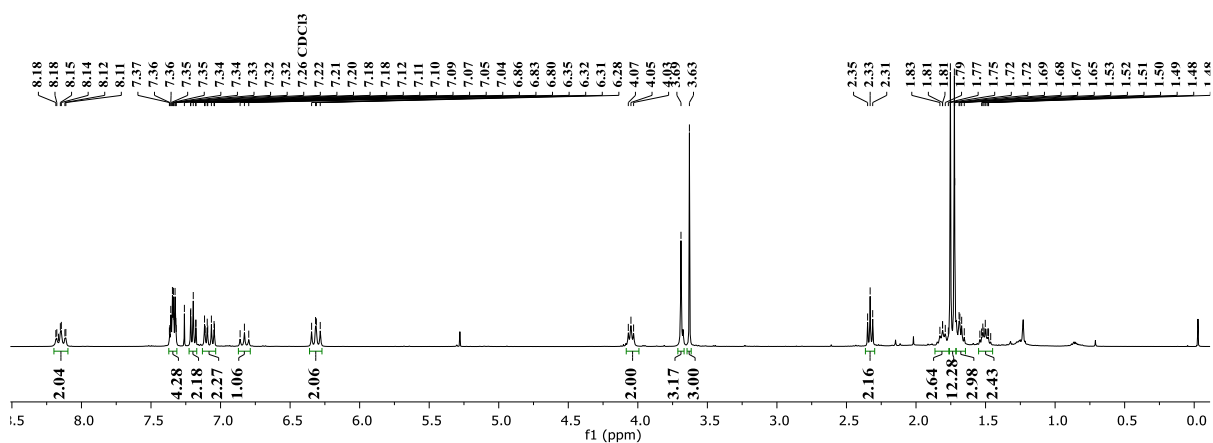
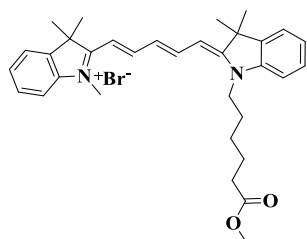
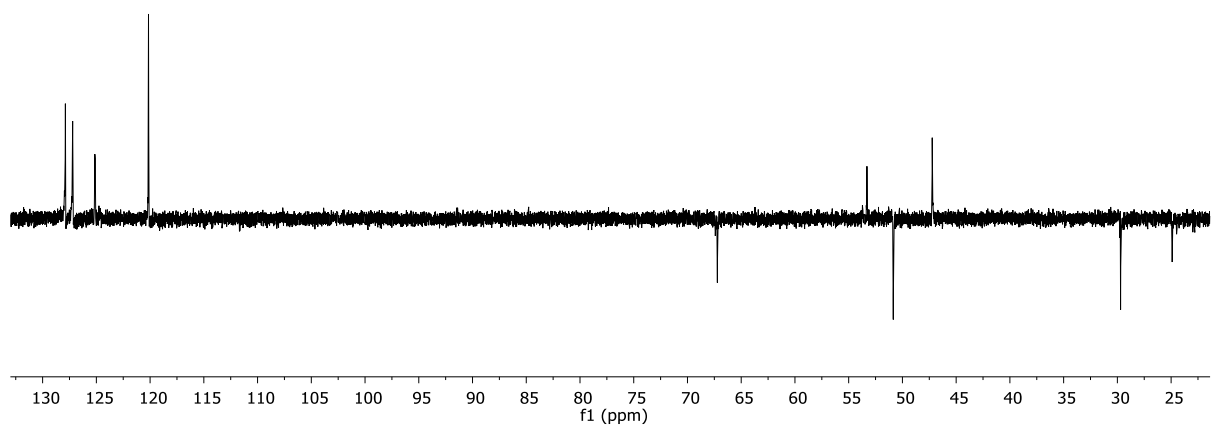
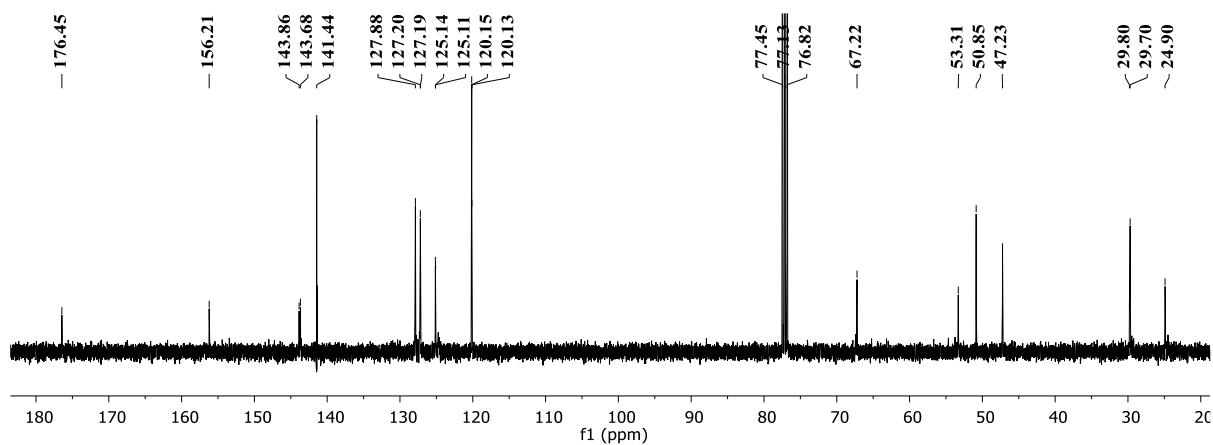


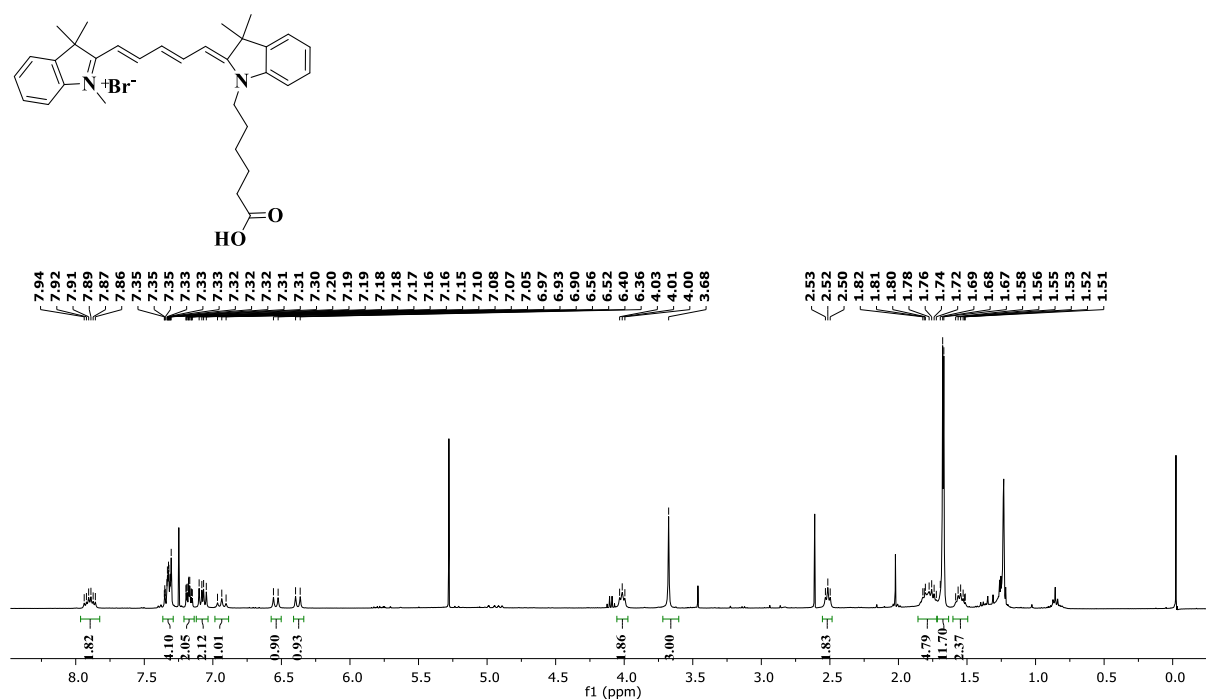
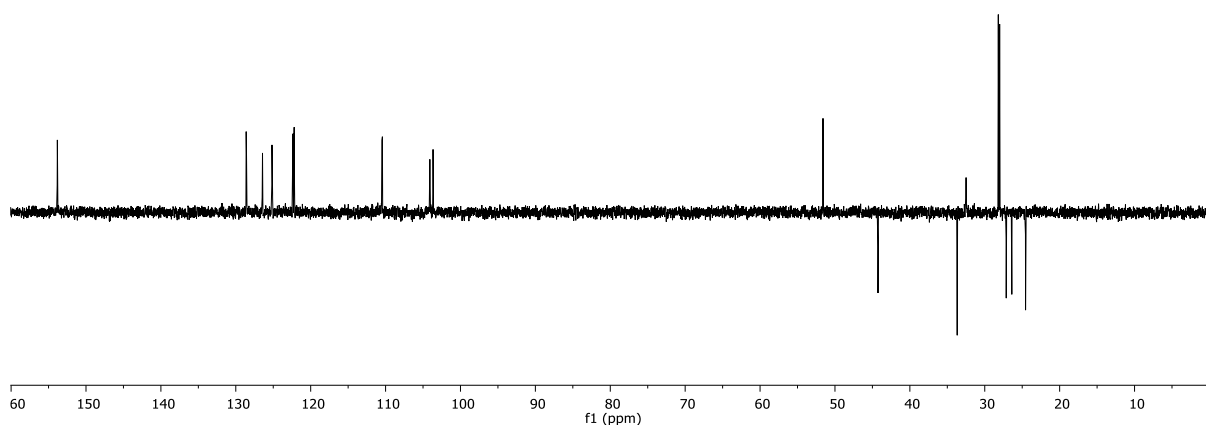
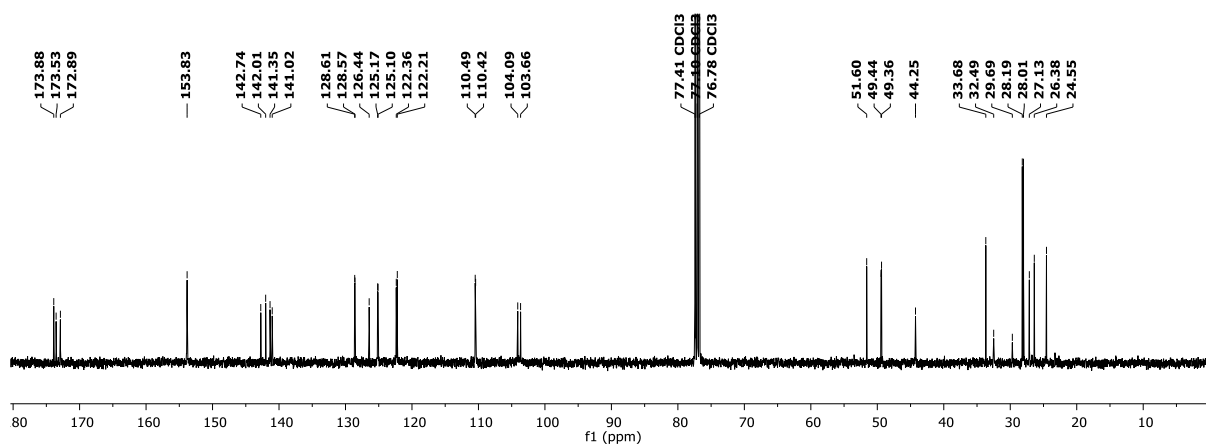


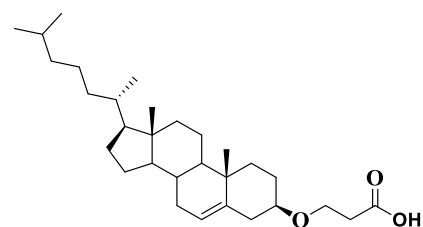
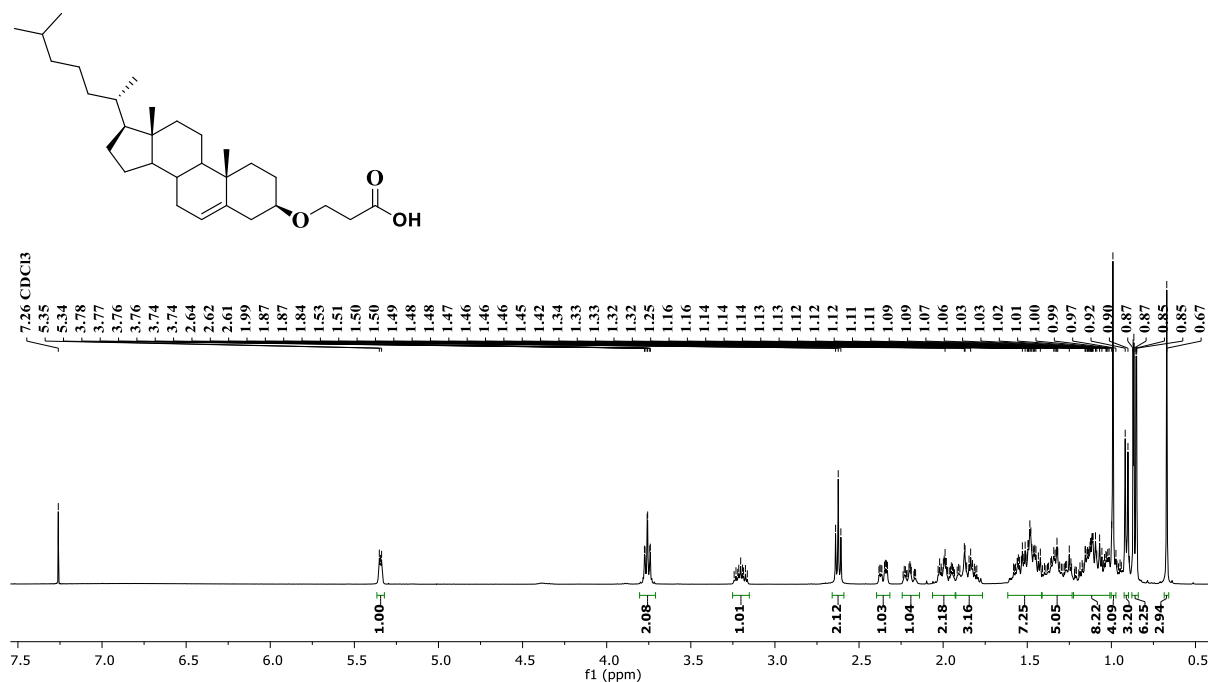
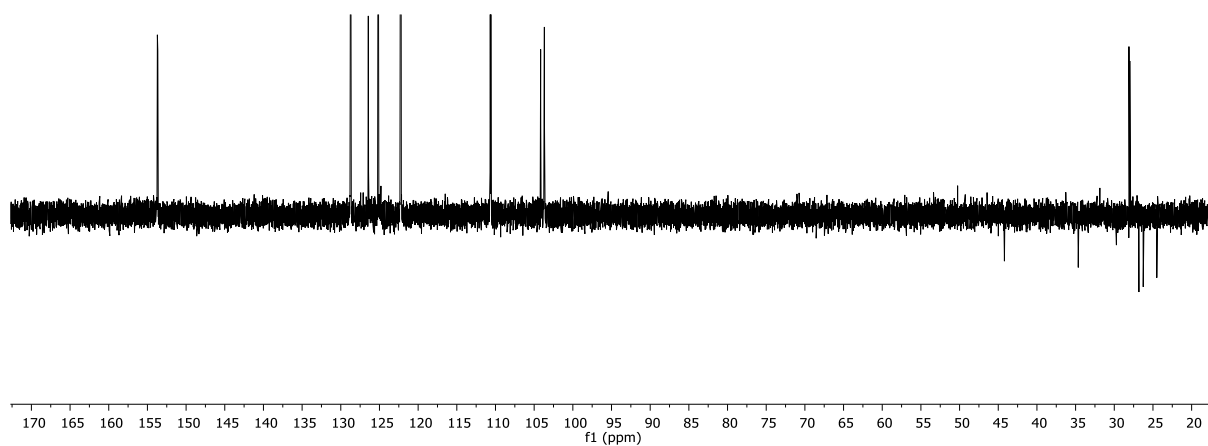
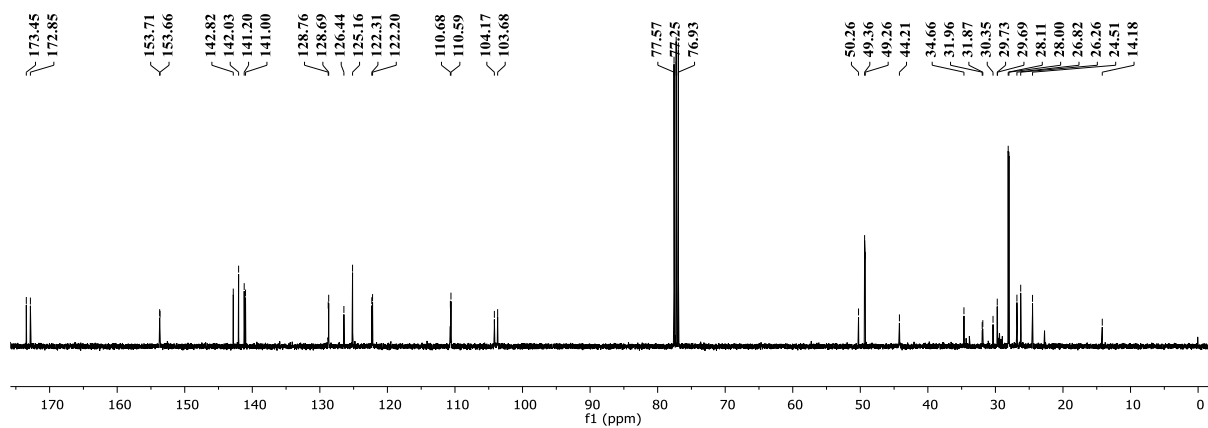


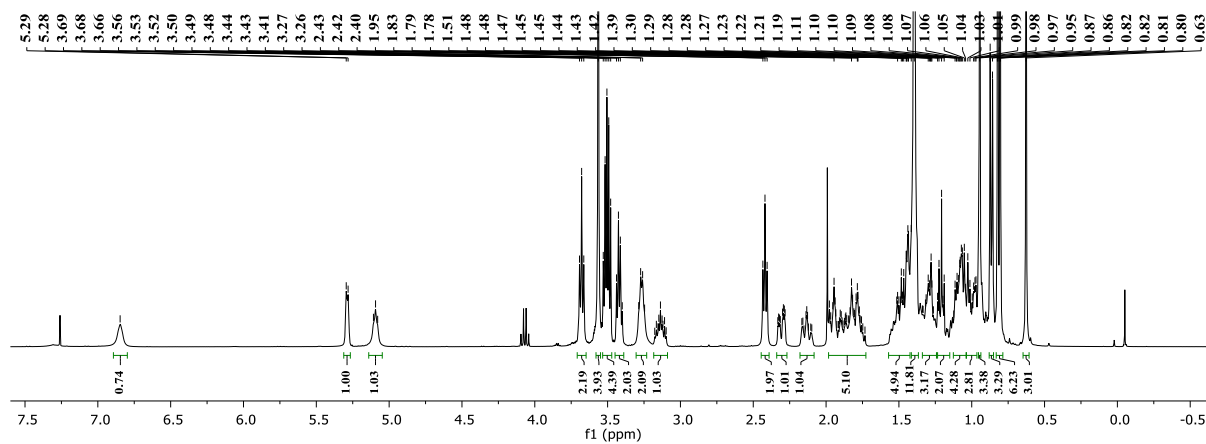
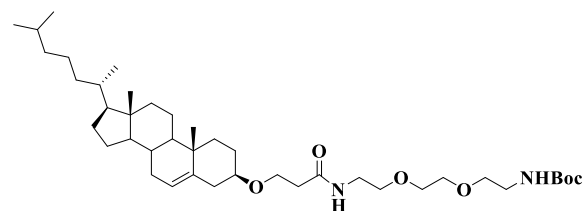
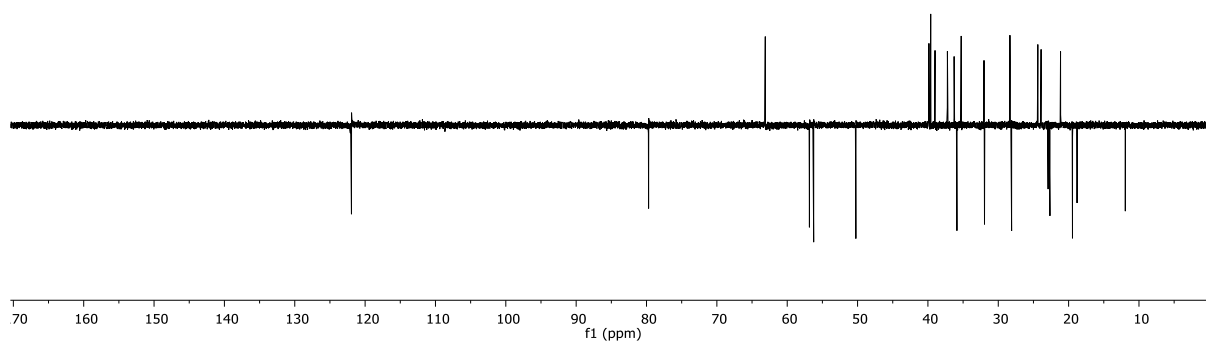
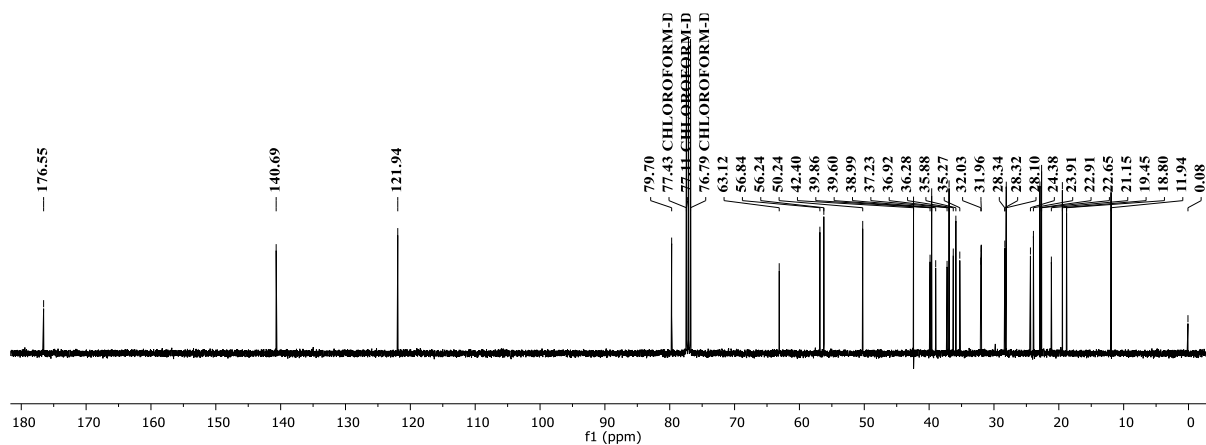


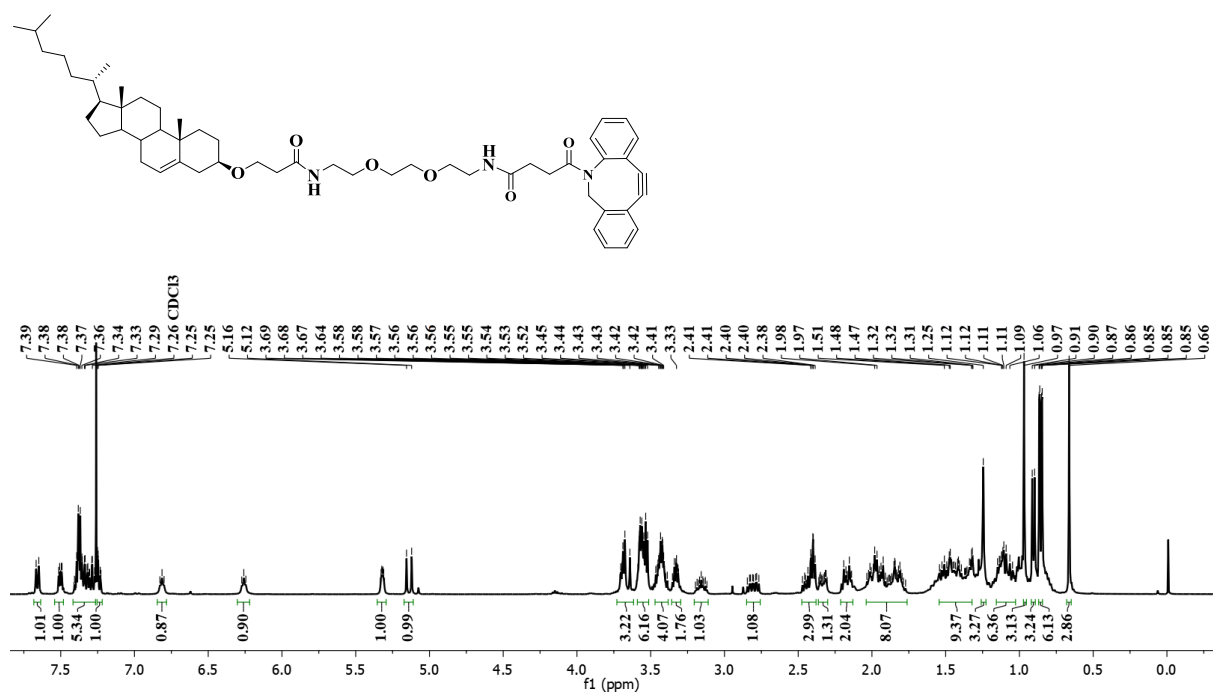
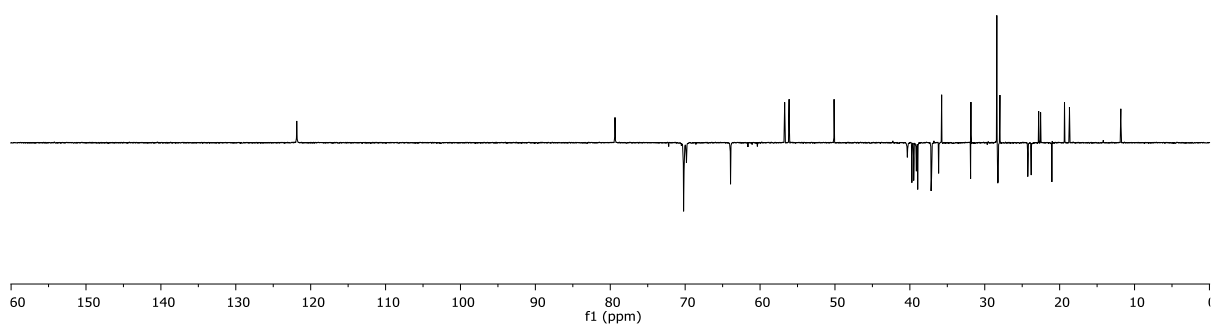
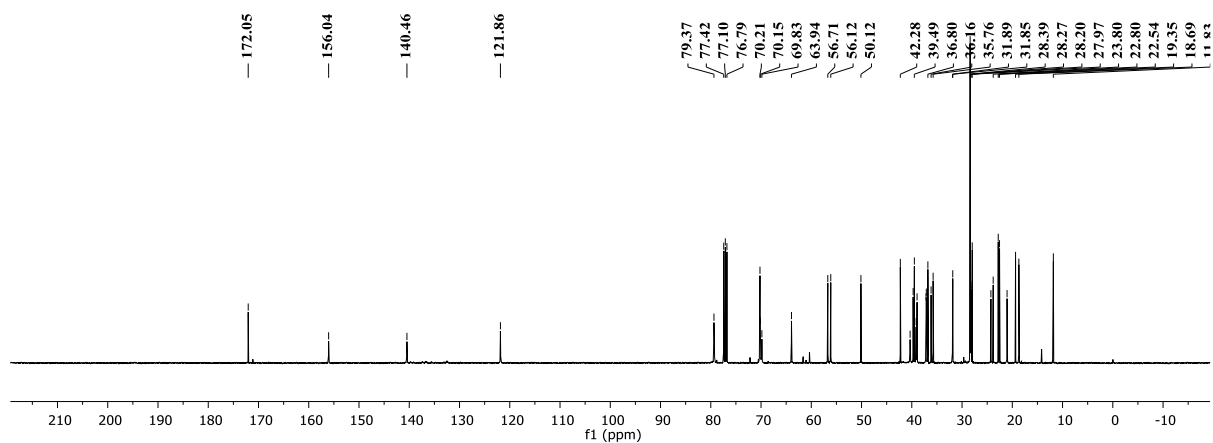


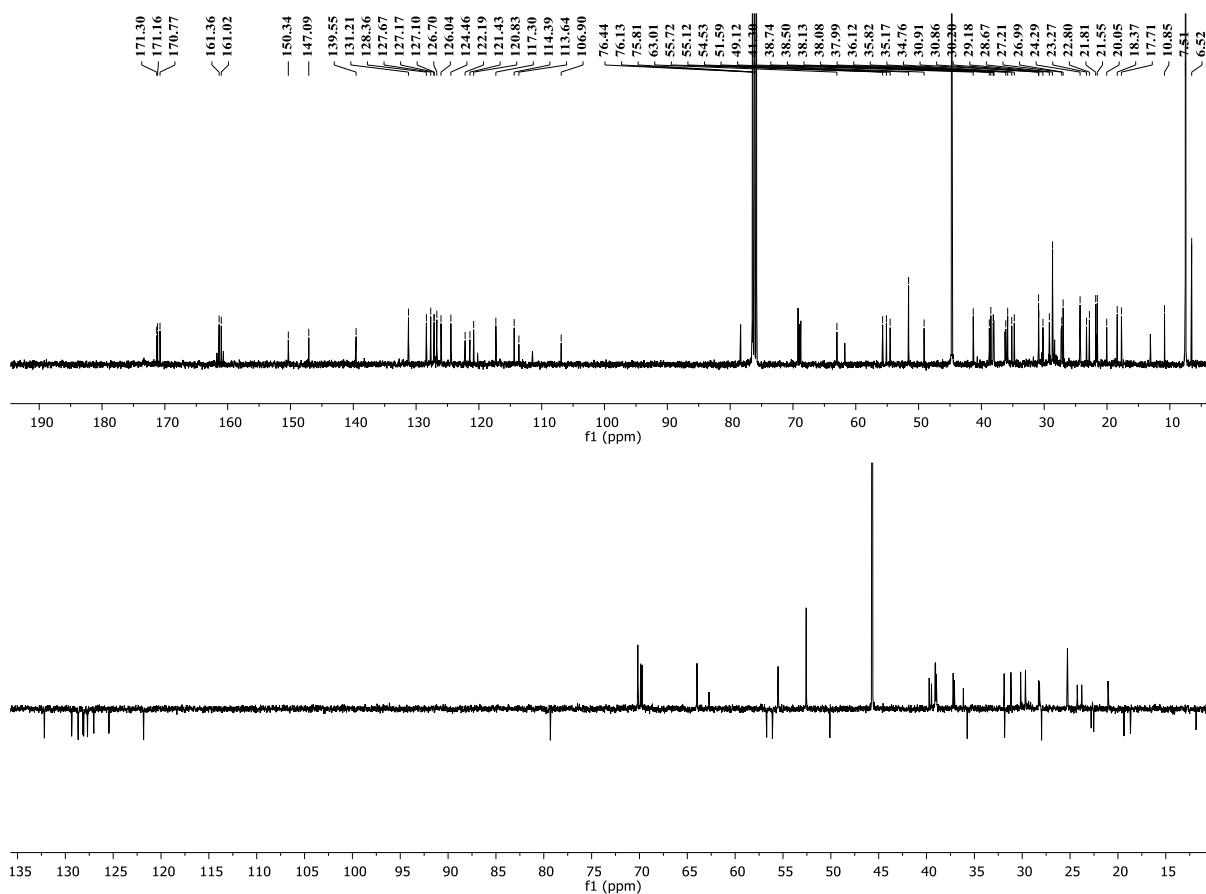




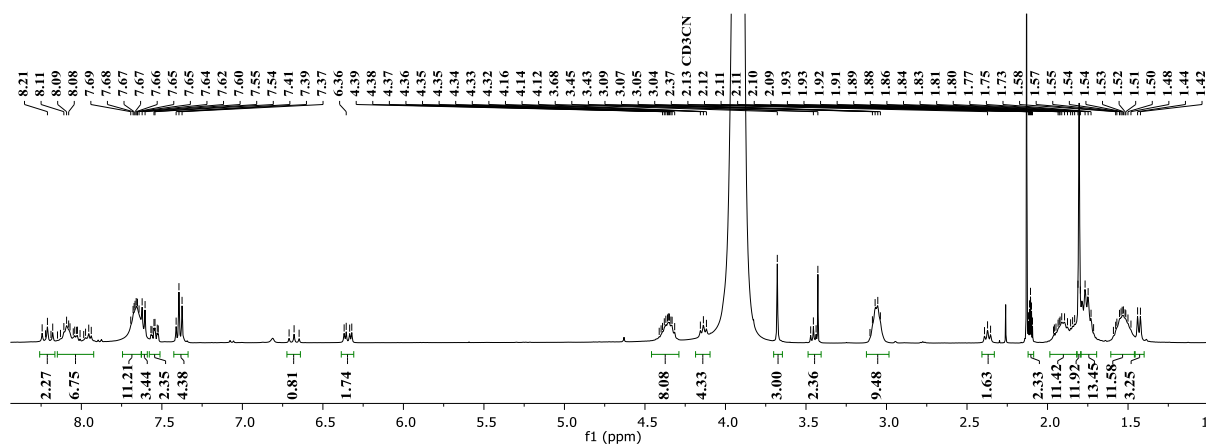


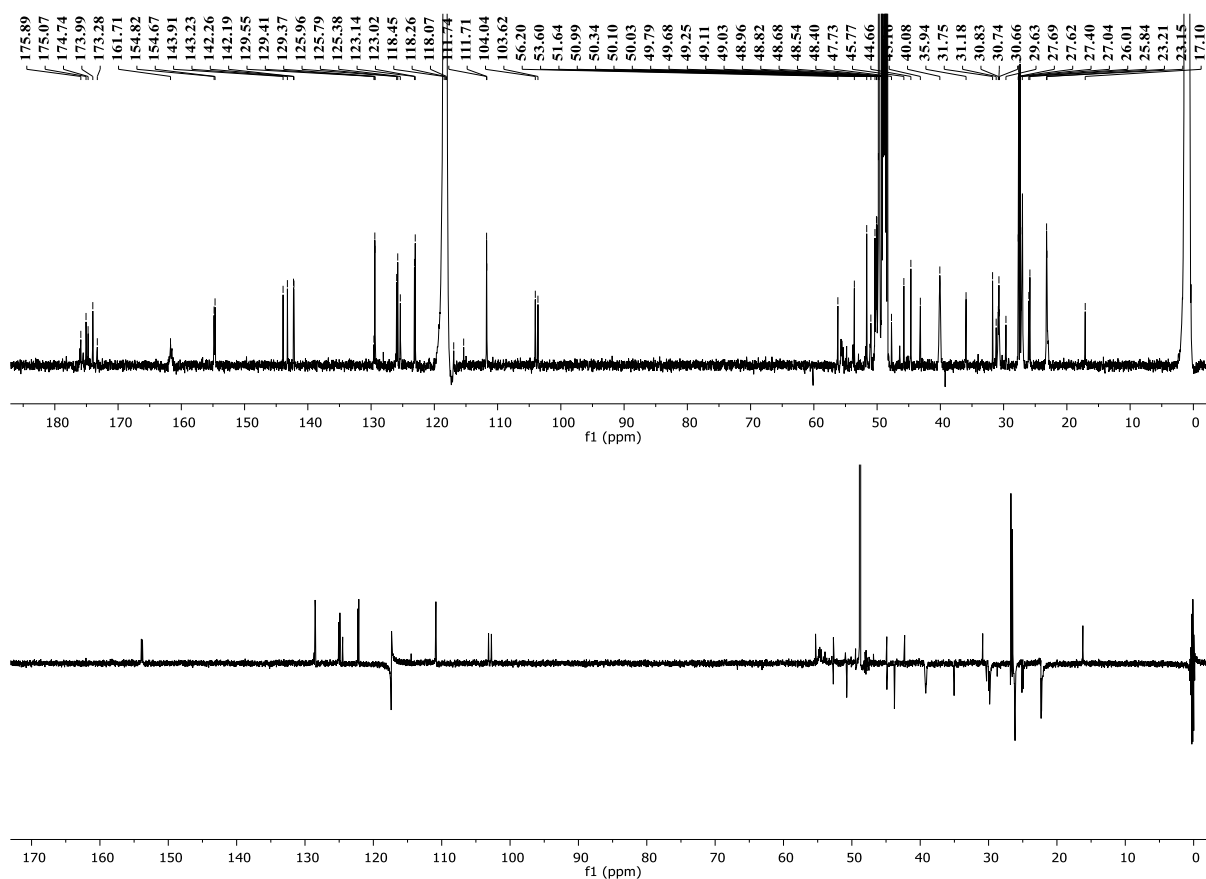




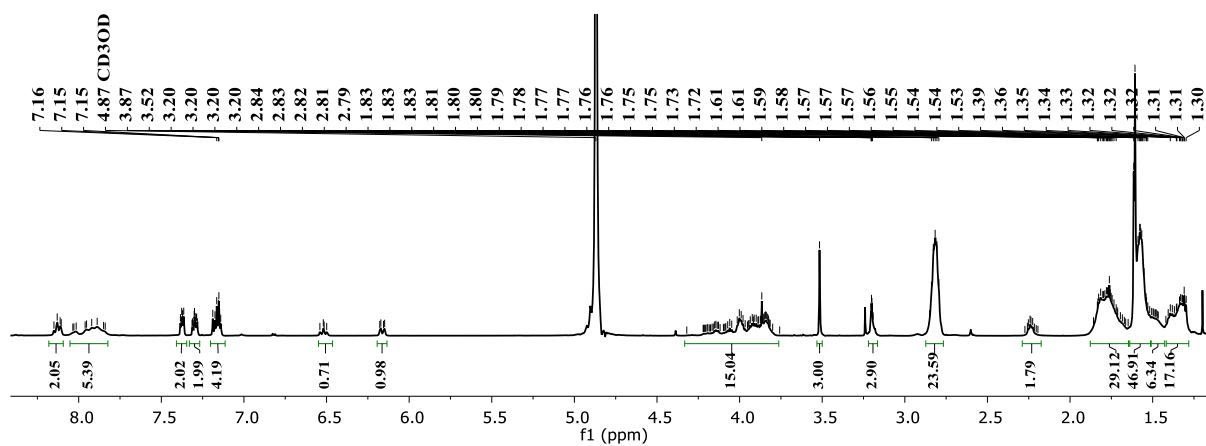


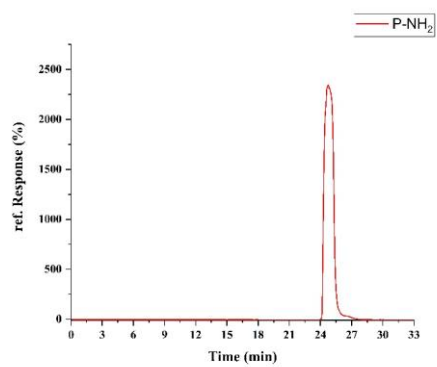
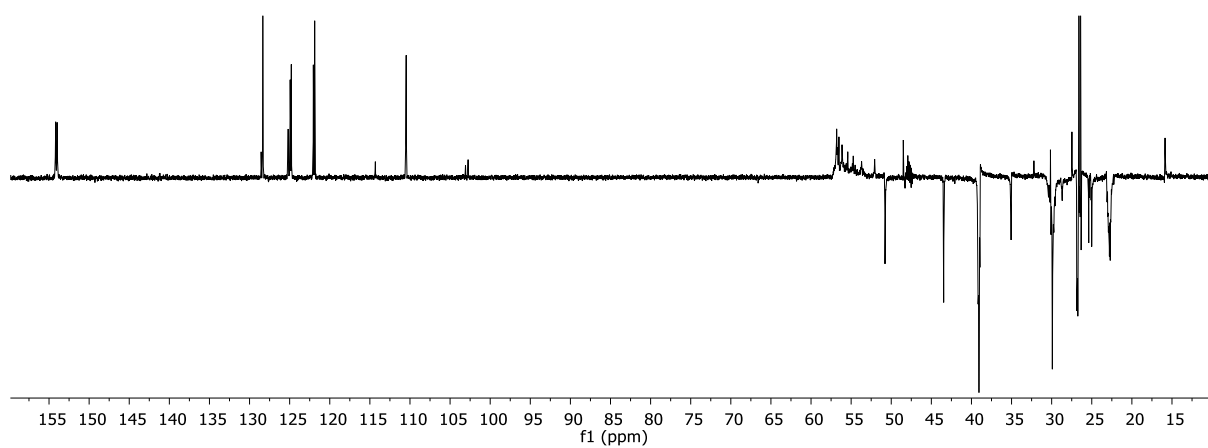
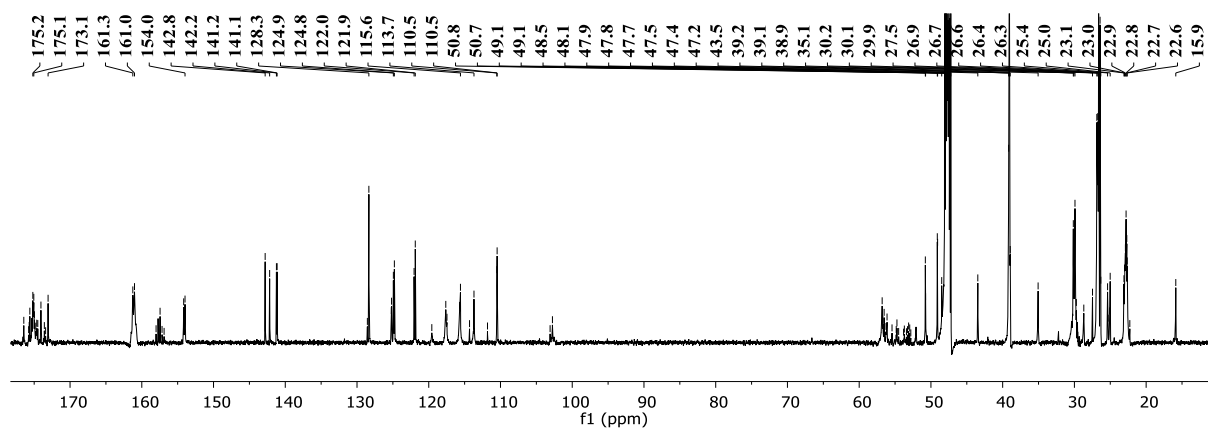
7-mer Peptide NMR



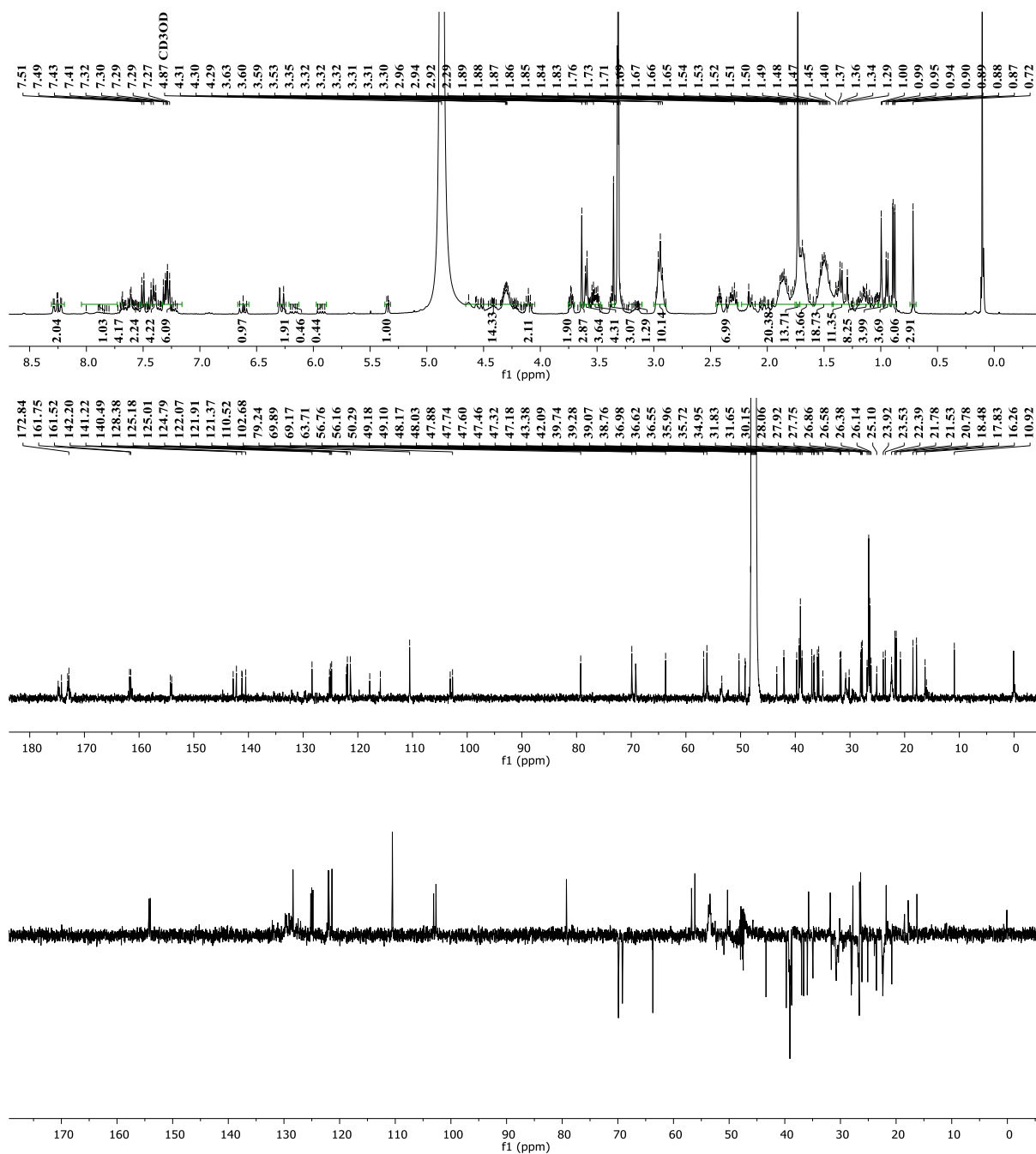


12-mer Peptide NMR

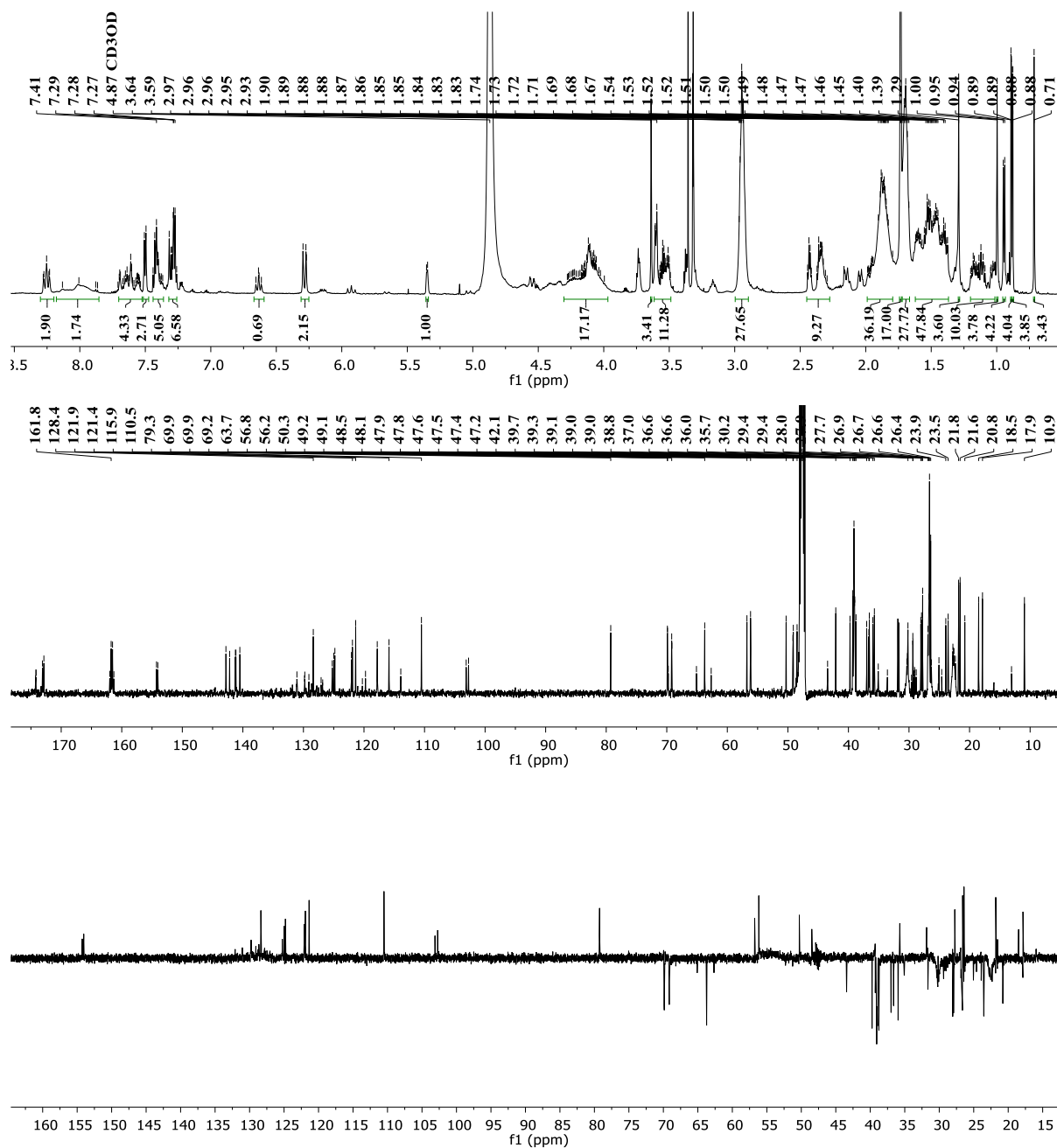




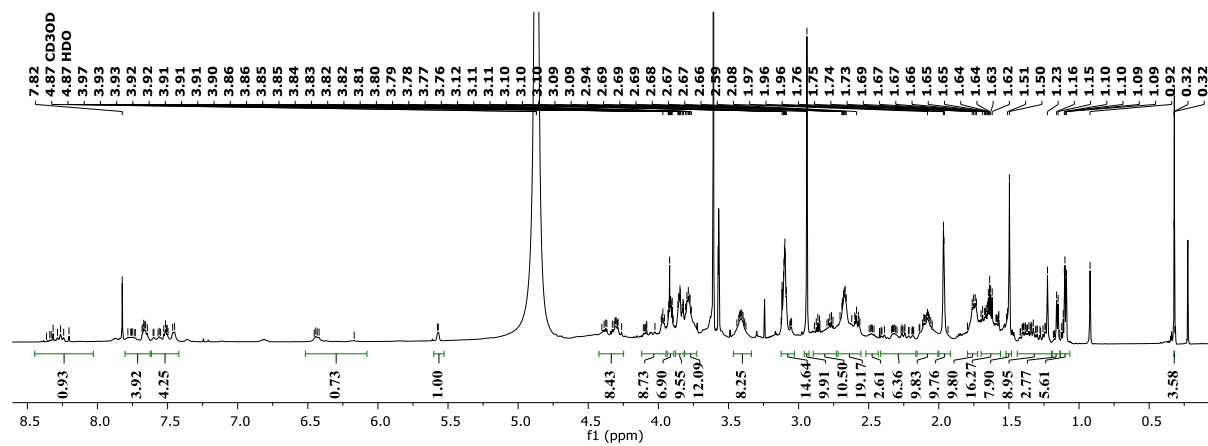
7-mer Peptide with Cholesterol

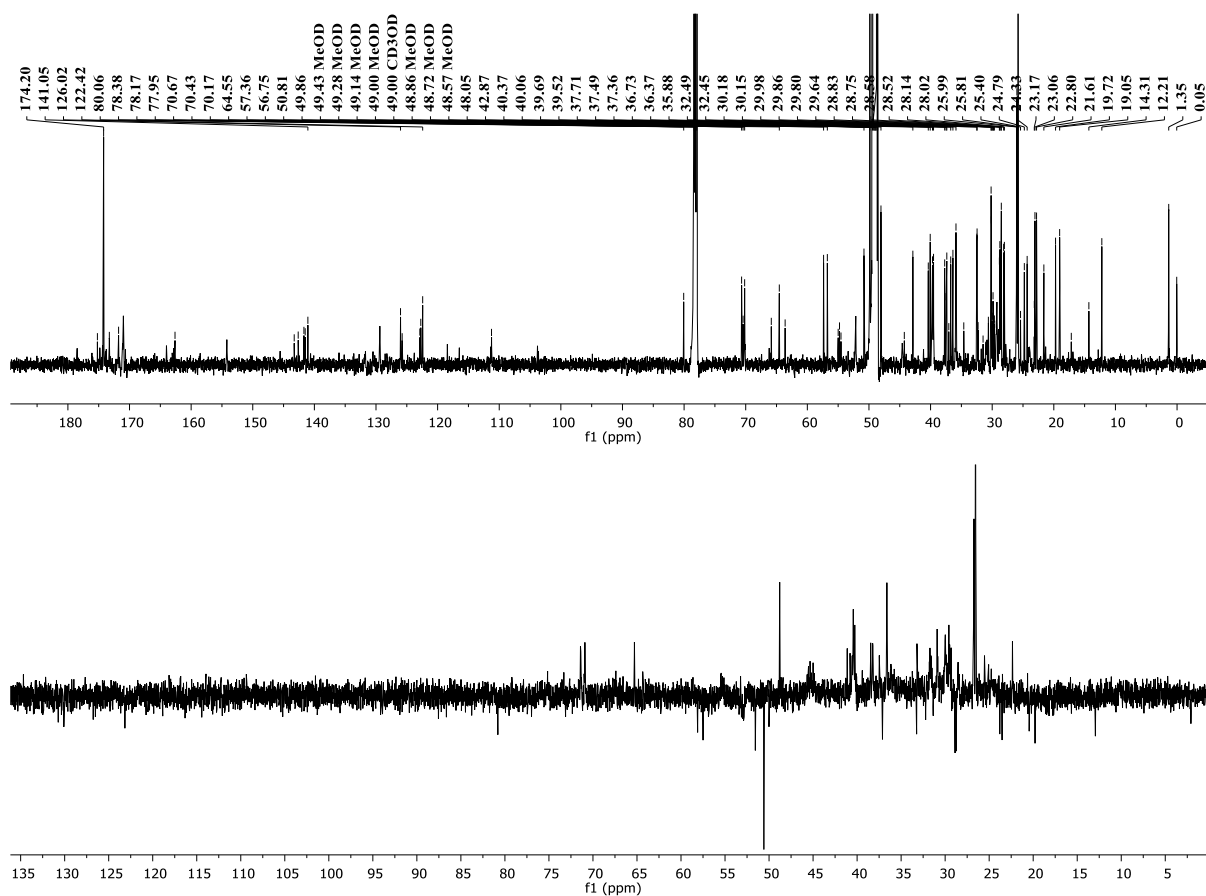


12-mer Peptide with Cholesterol

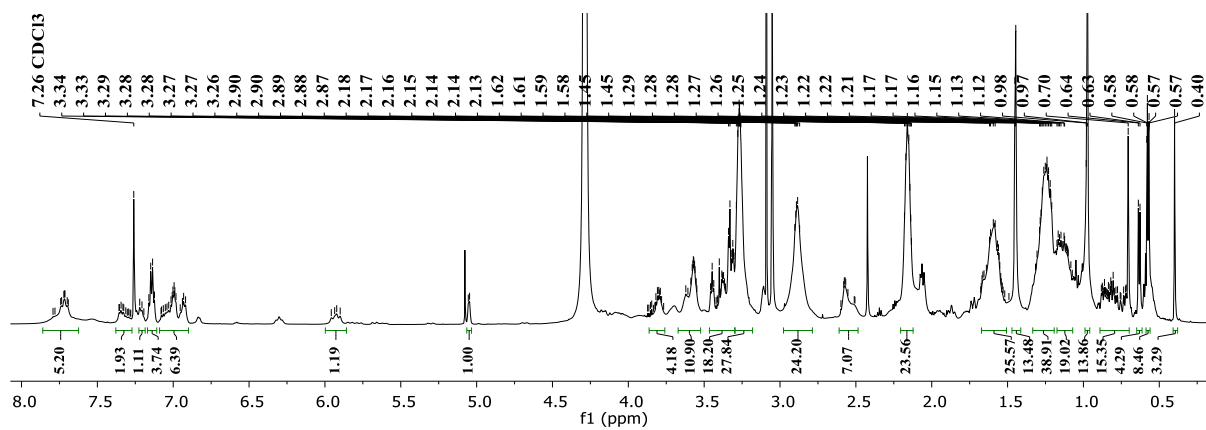


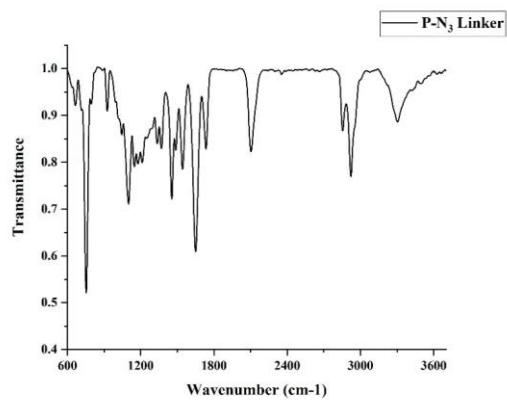
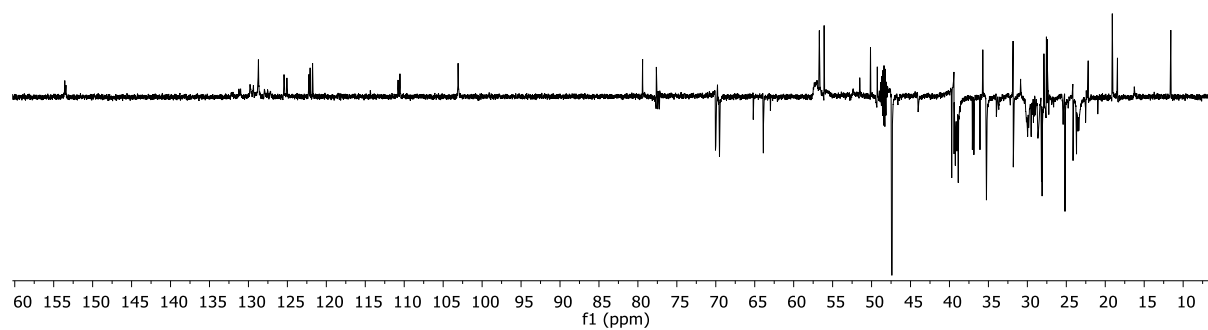
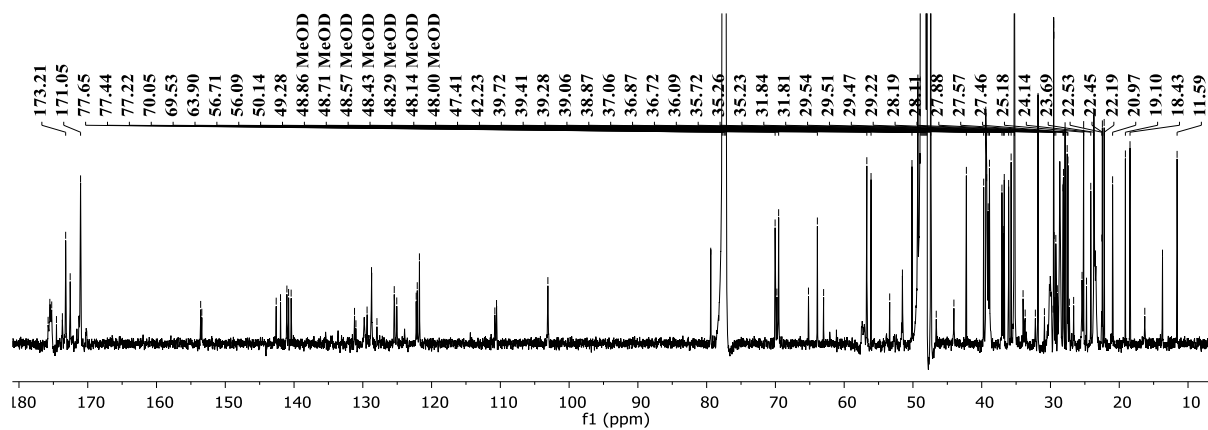
7-mer Peptide with 3-Azidopropanoate Linker



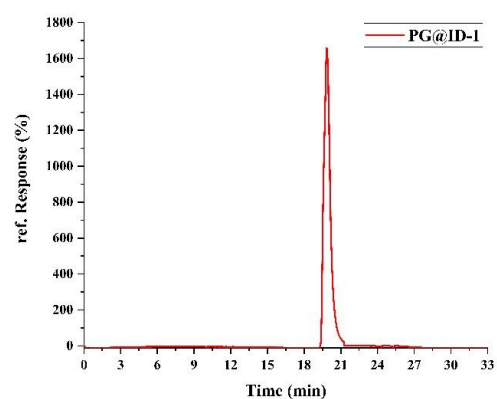
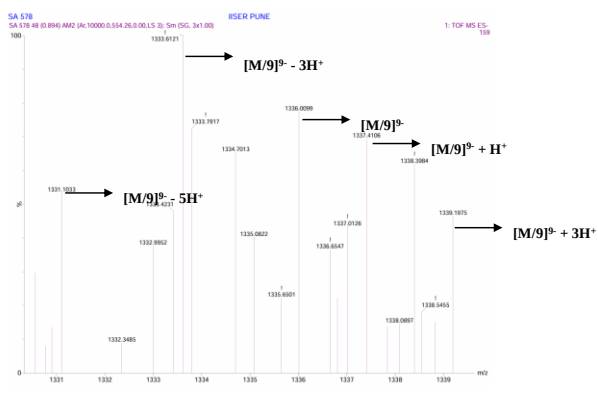
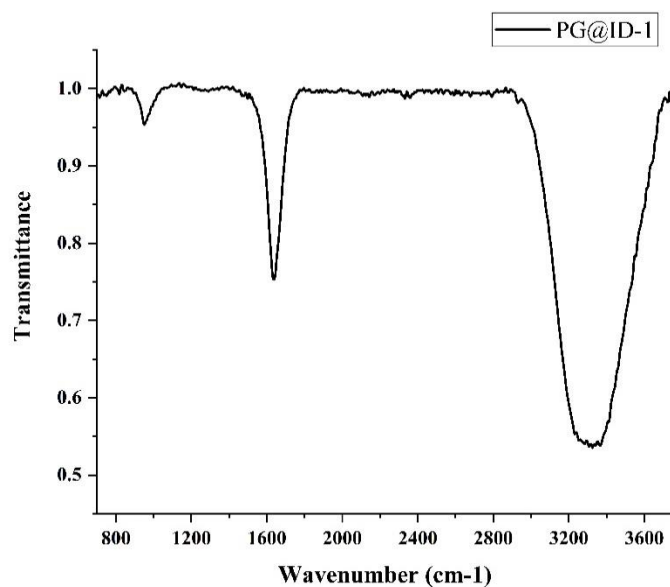
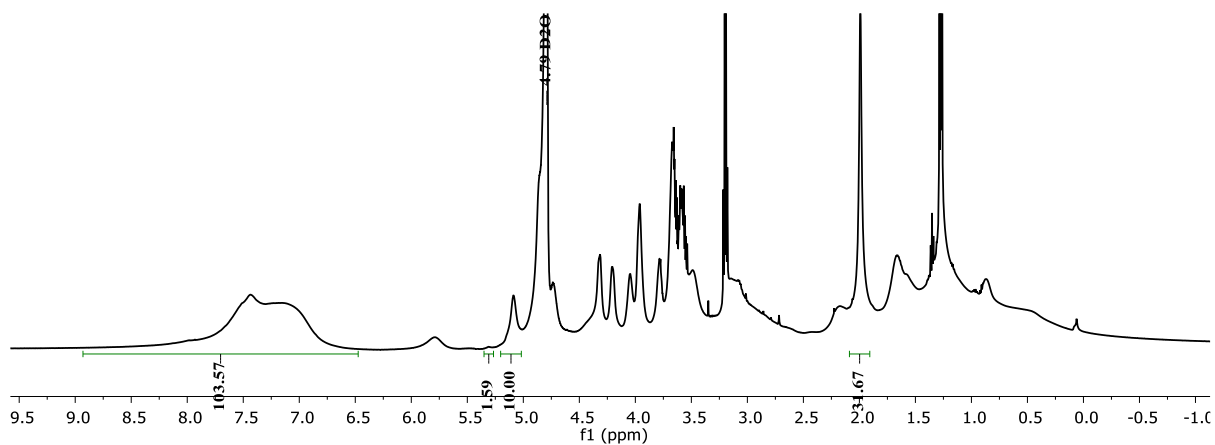


12-mer Peptide with 3-Azidopropanoate Linker

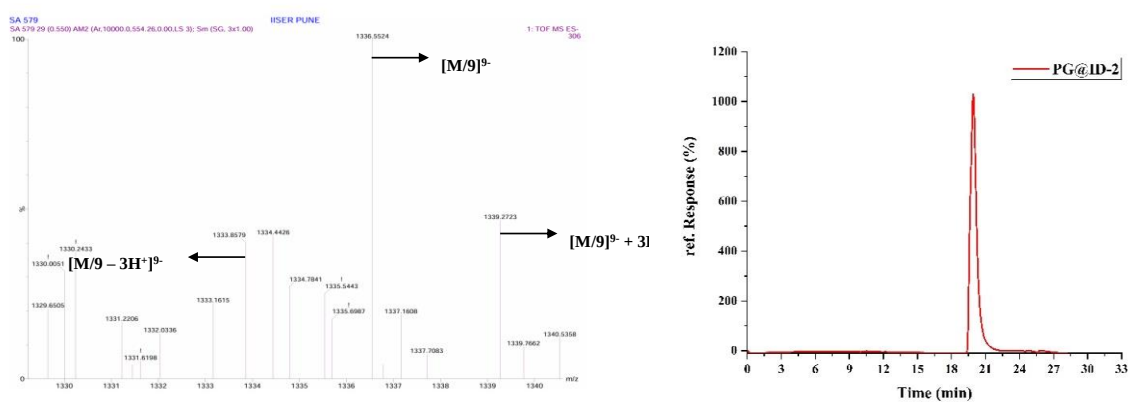
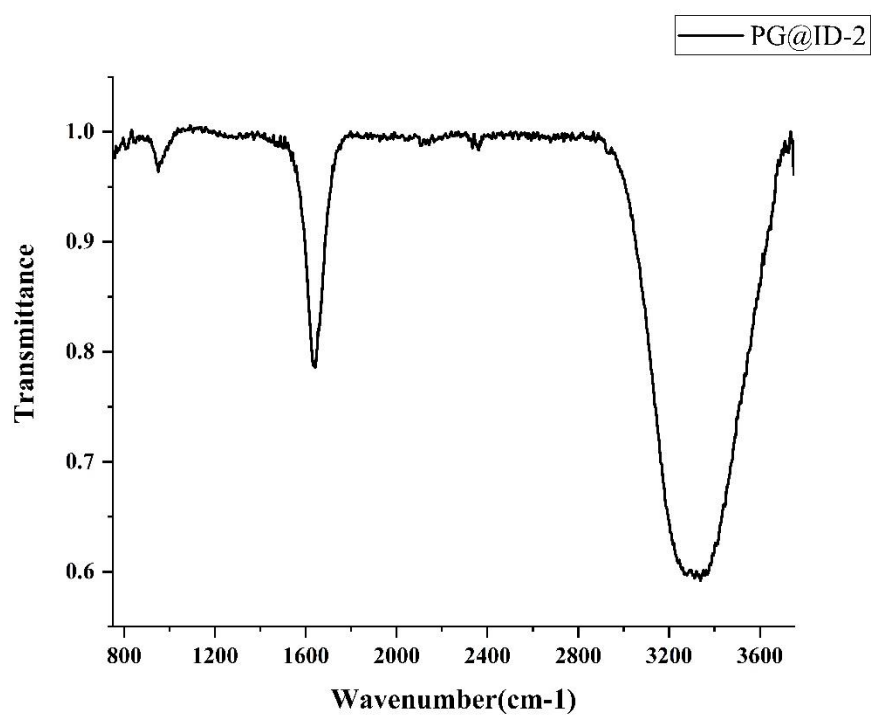
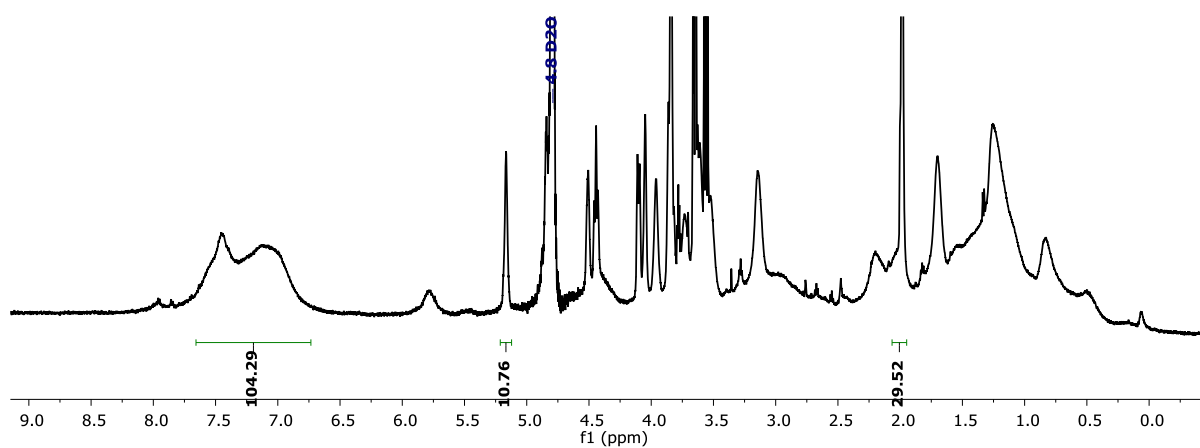




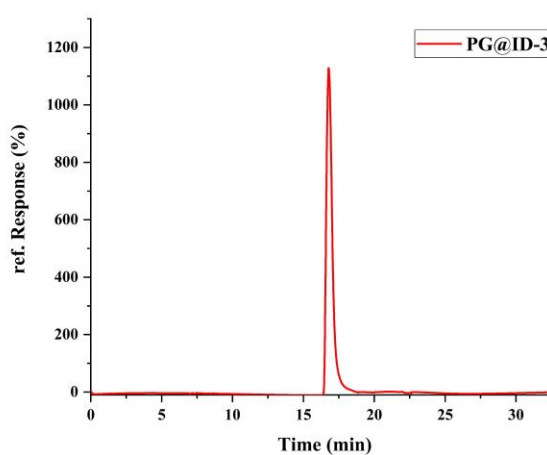
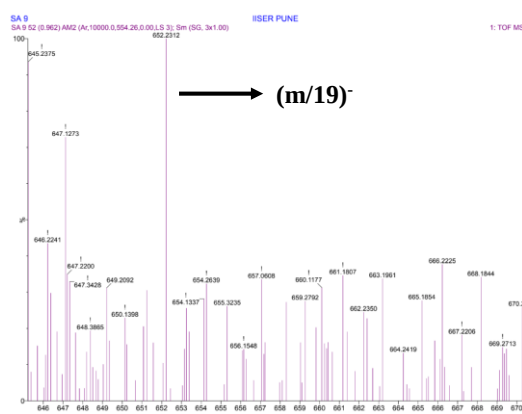
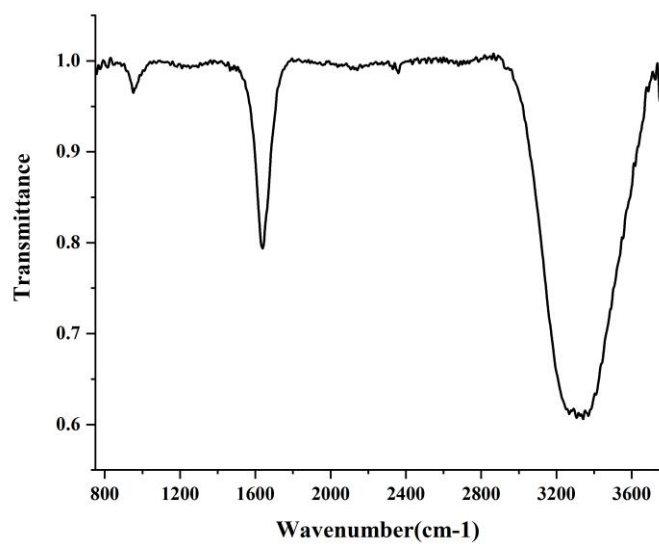
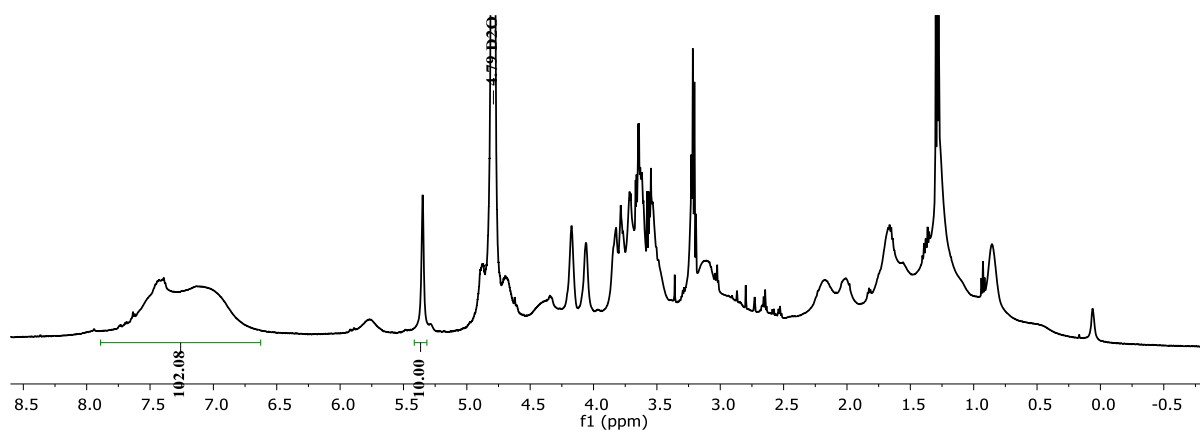
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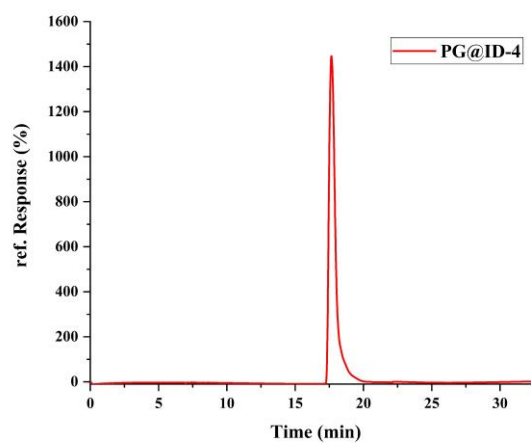
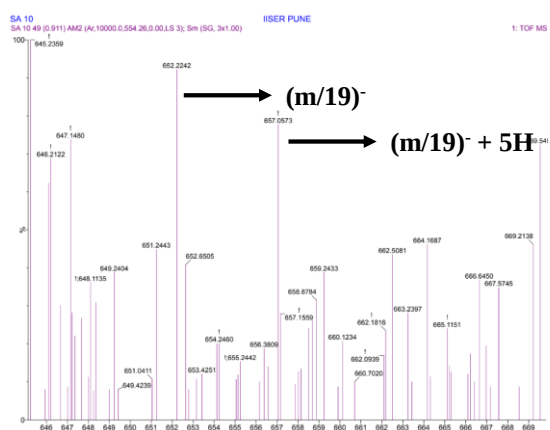
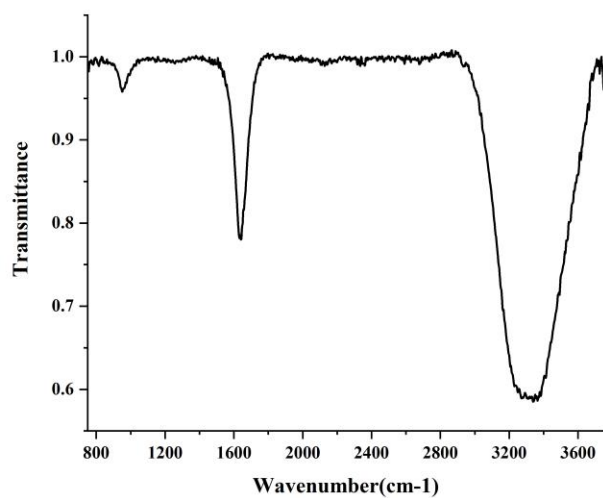
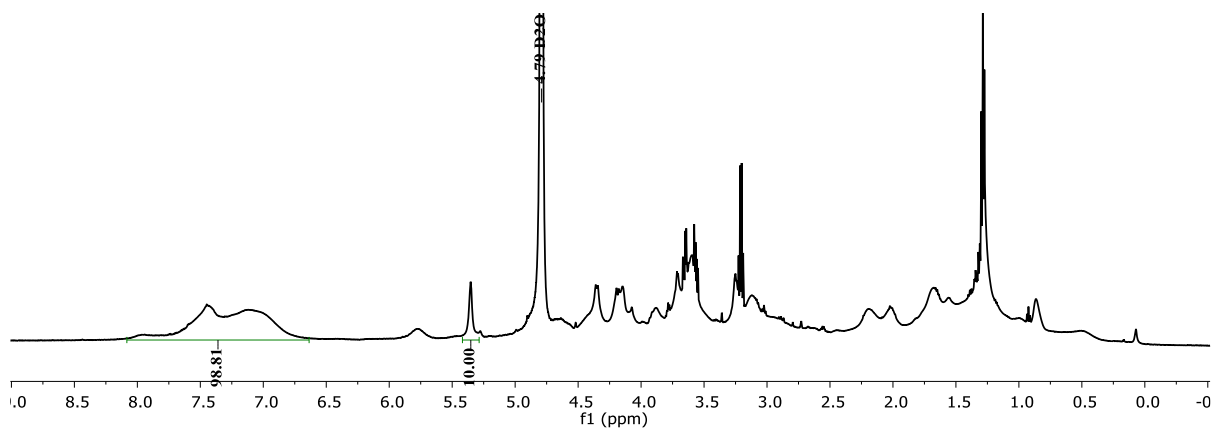
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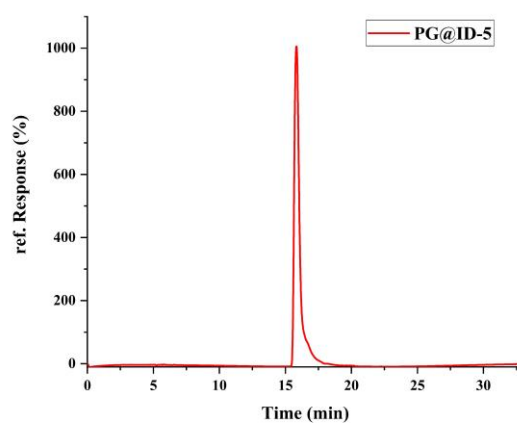
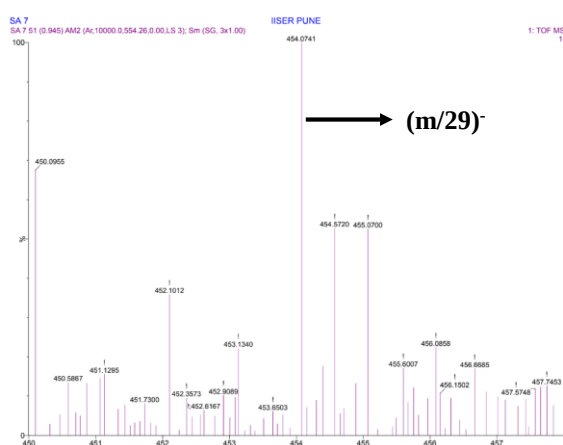
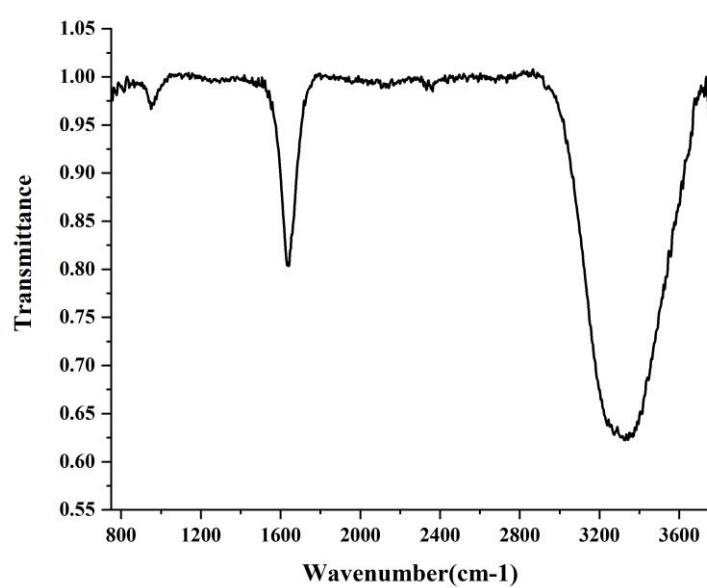
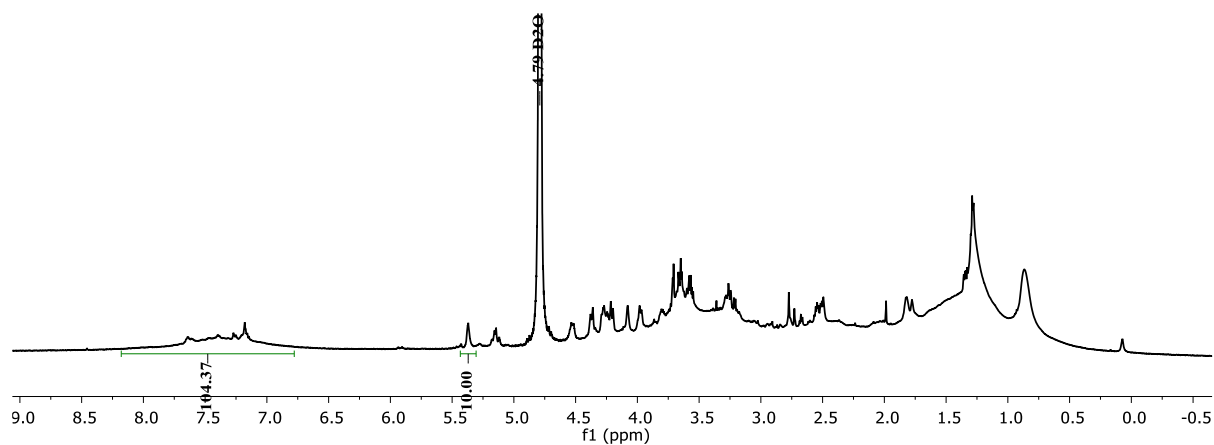
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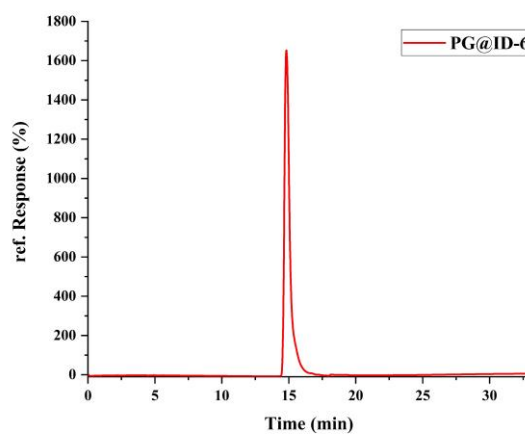
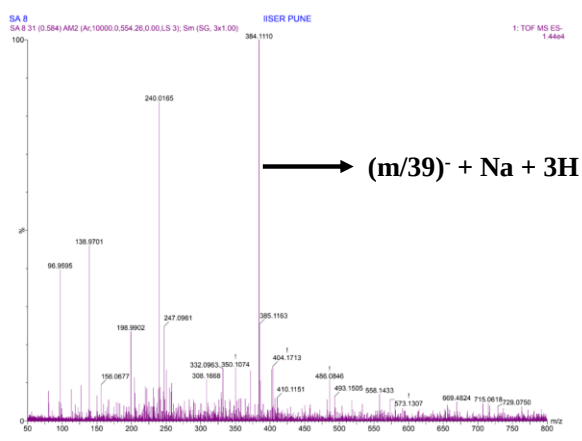
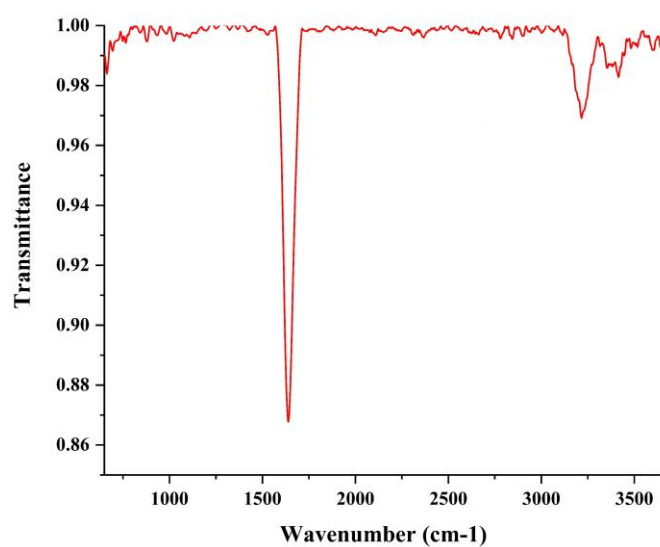
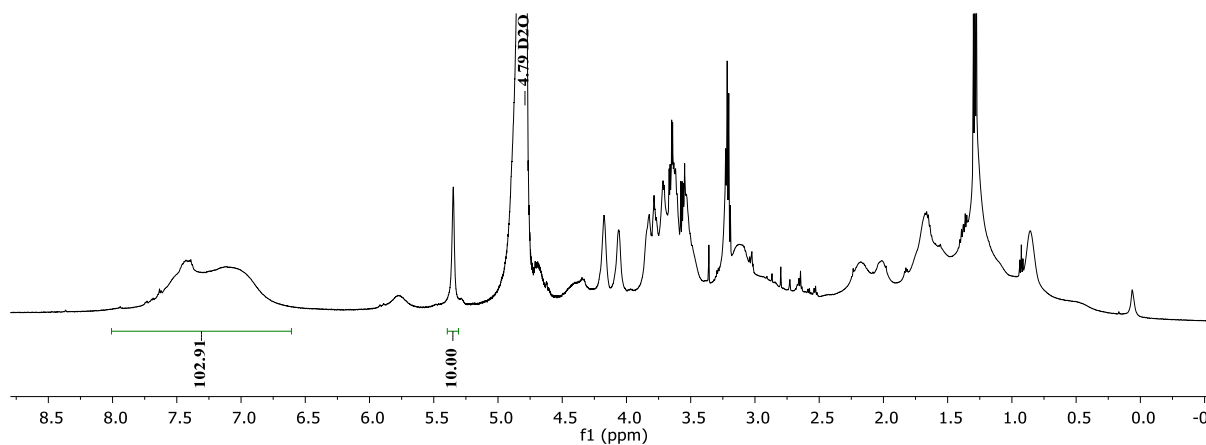
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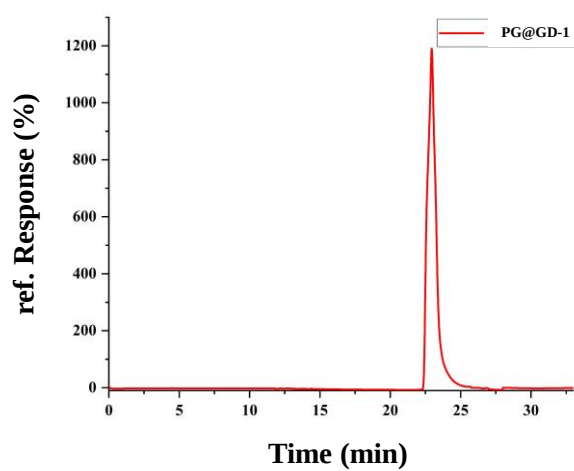
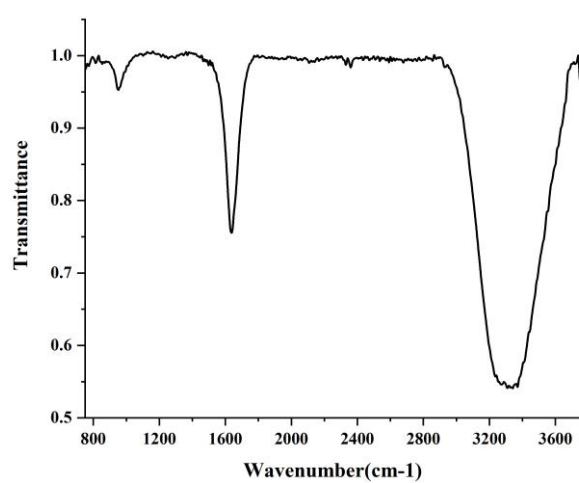
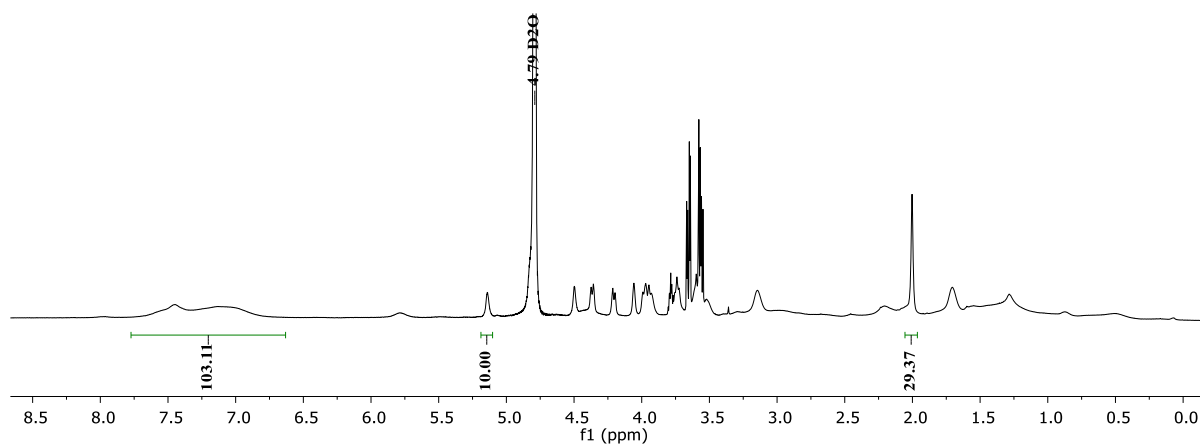
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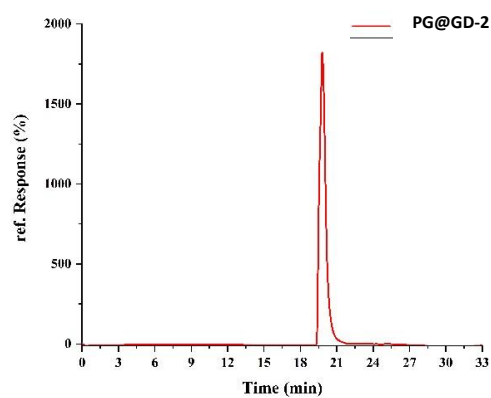
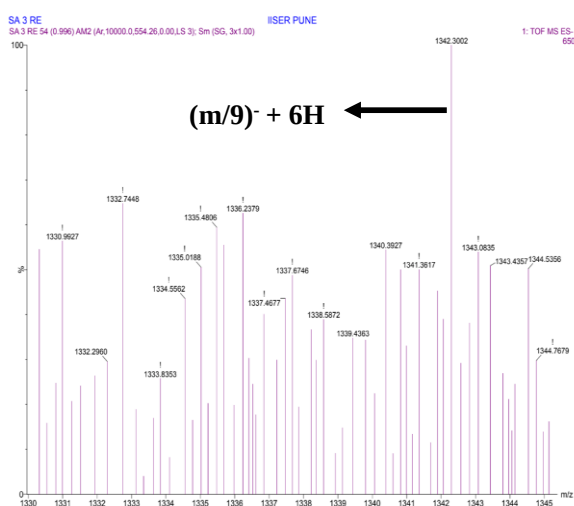
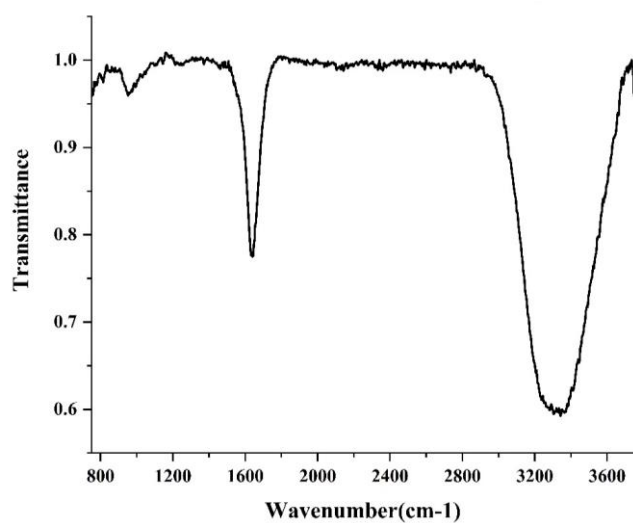
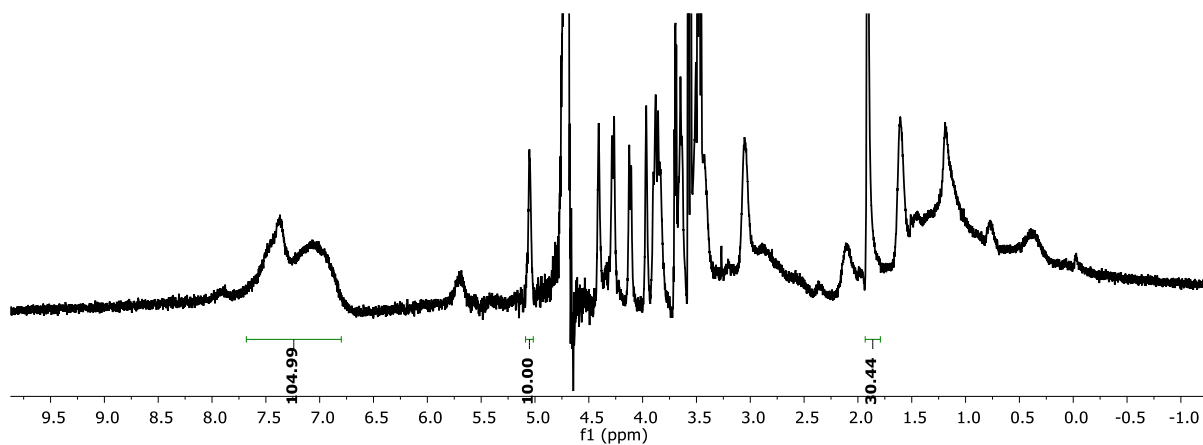
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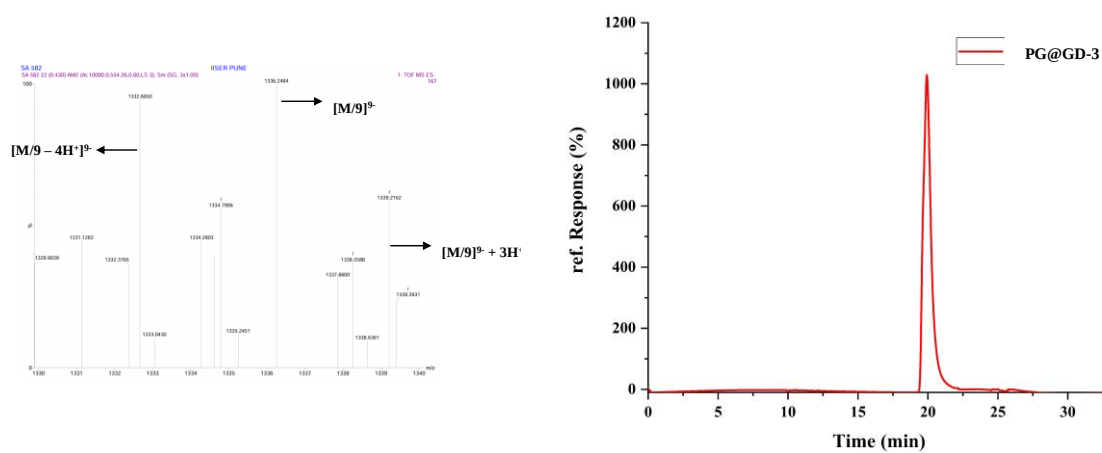
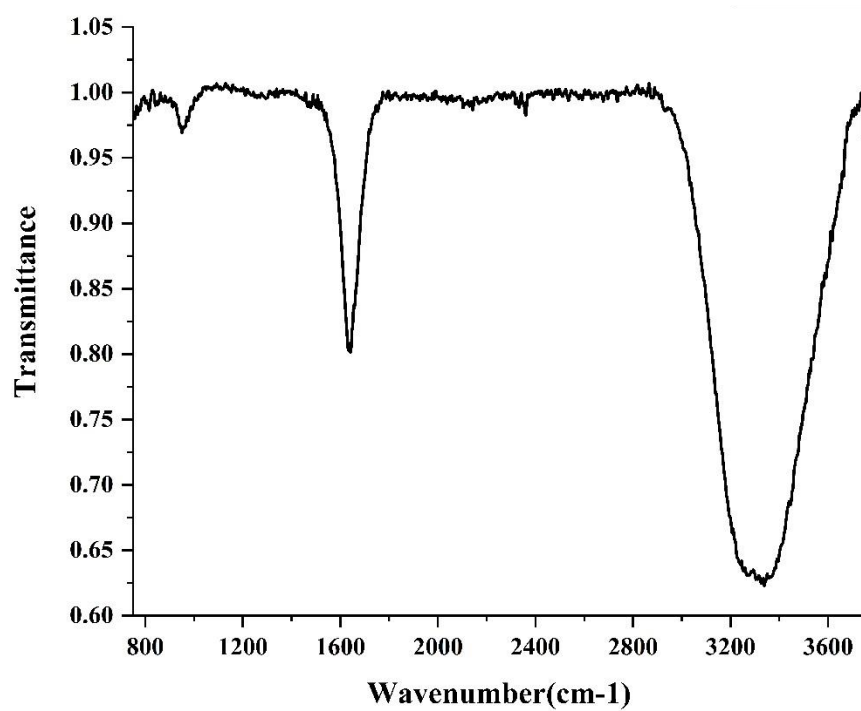
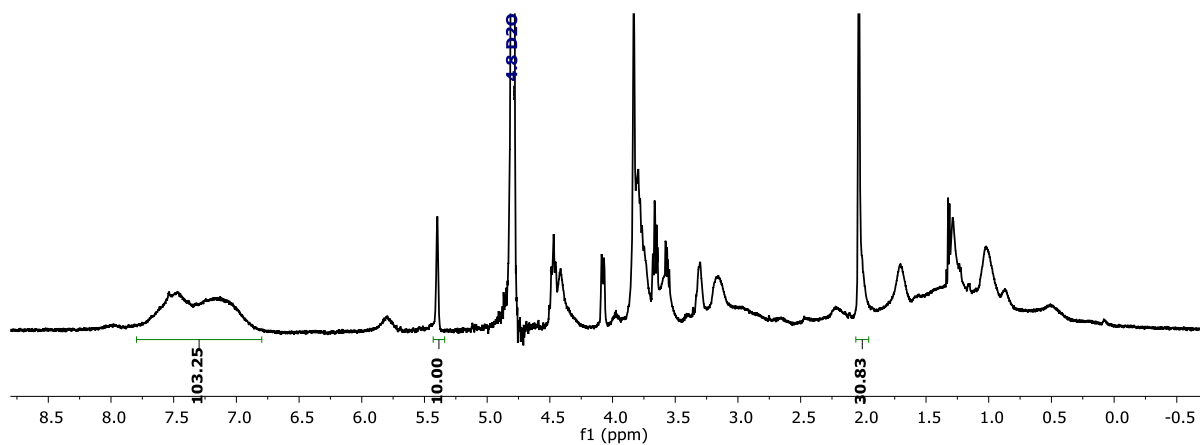
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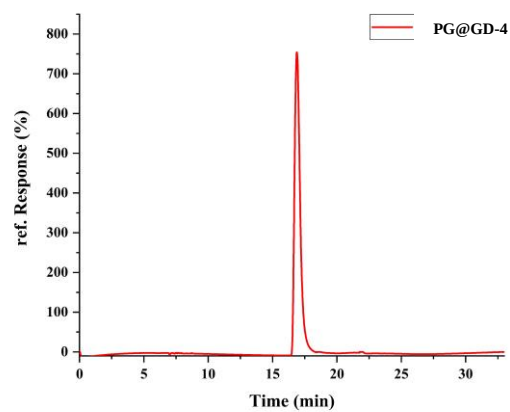
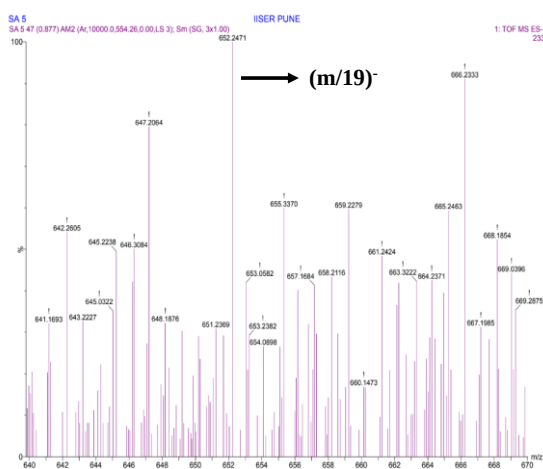
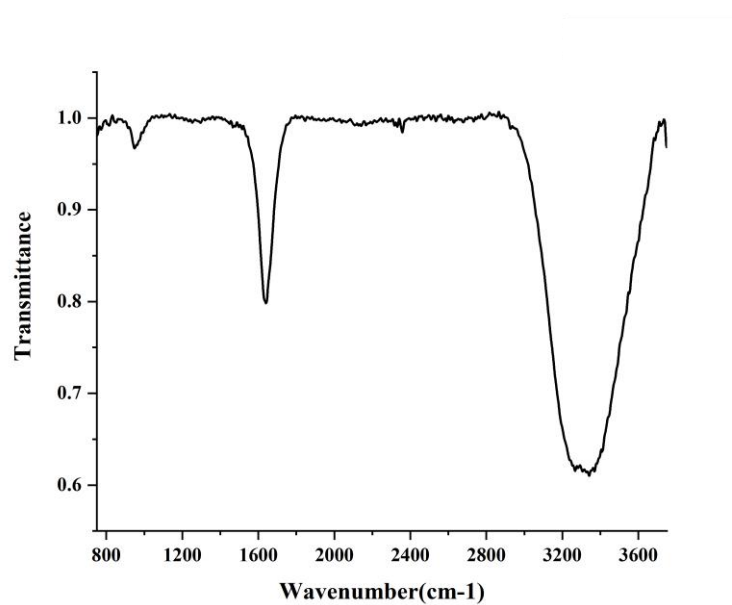
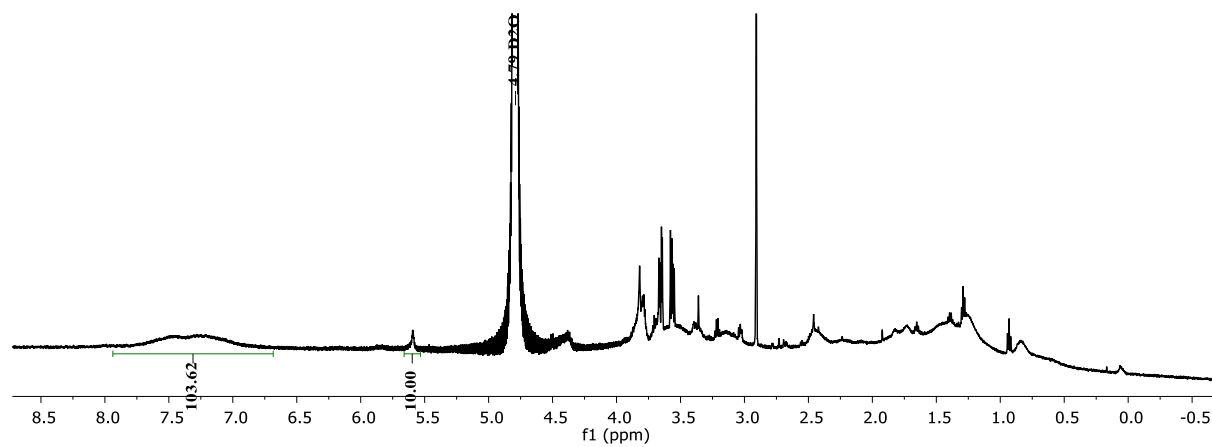
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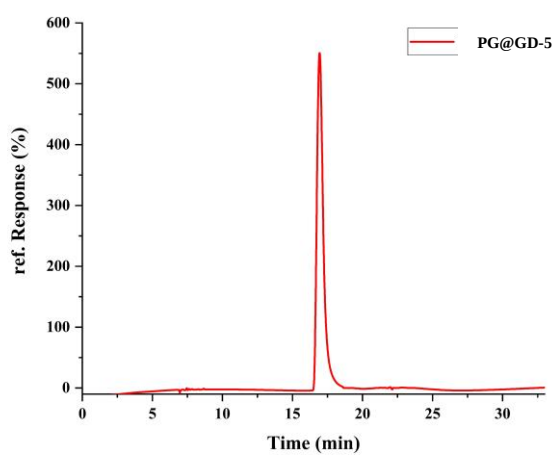
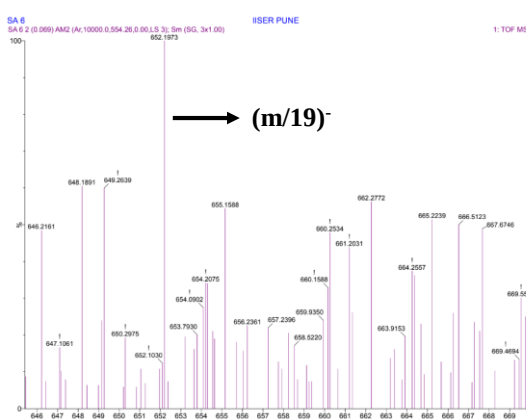
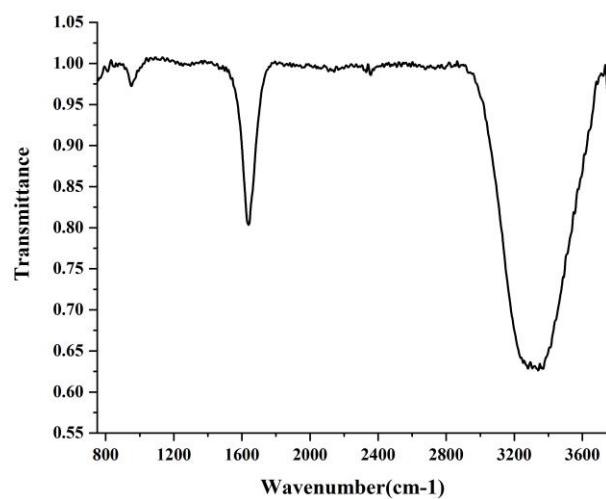
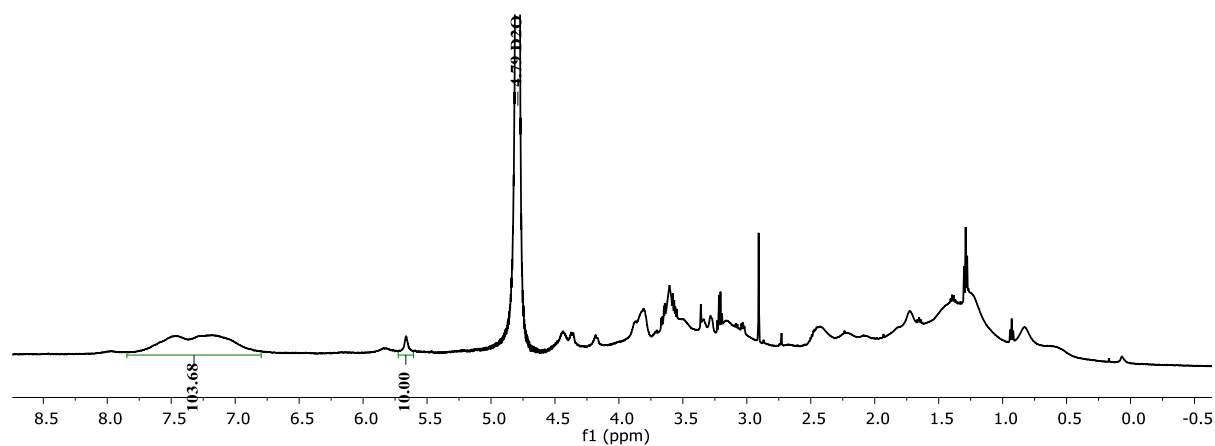
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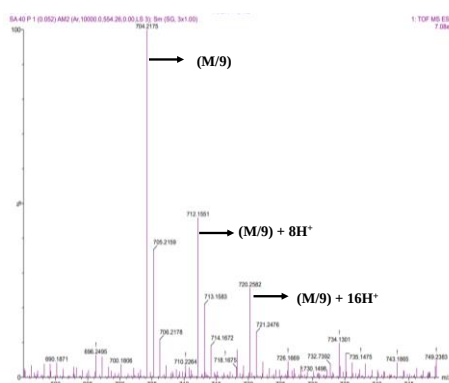
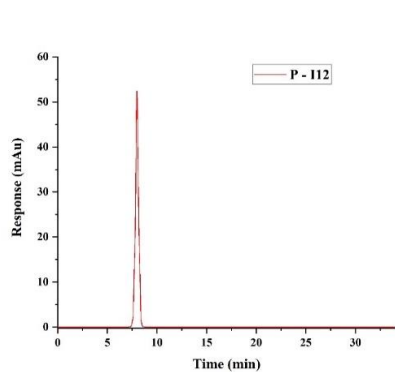
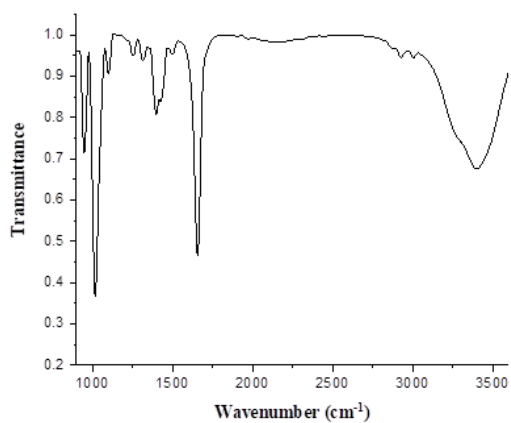
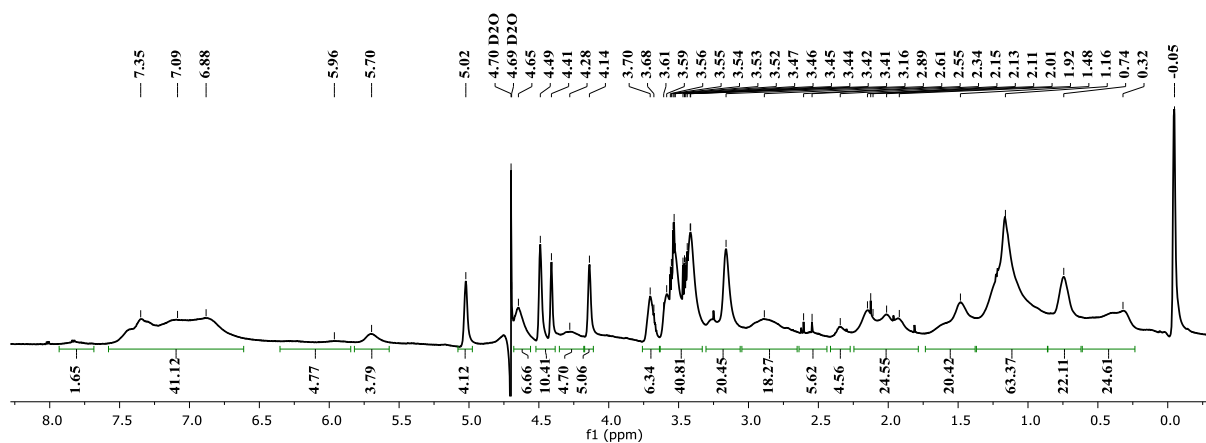
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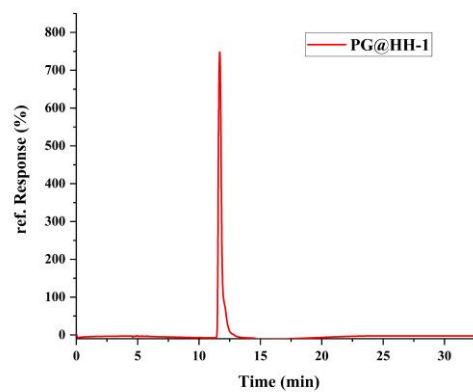
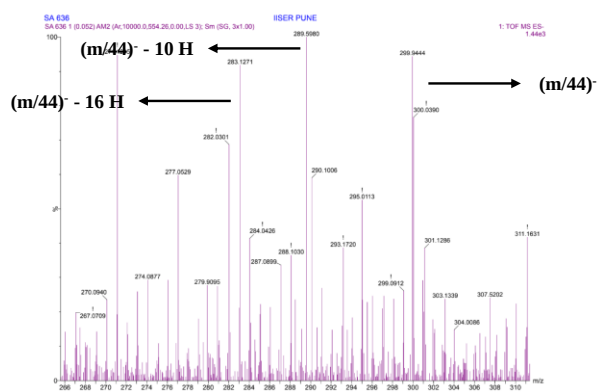
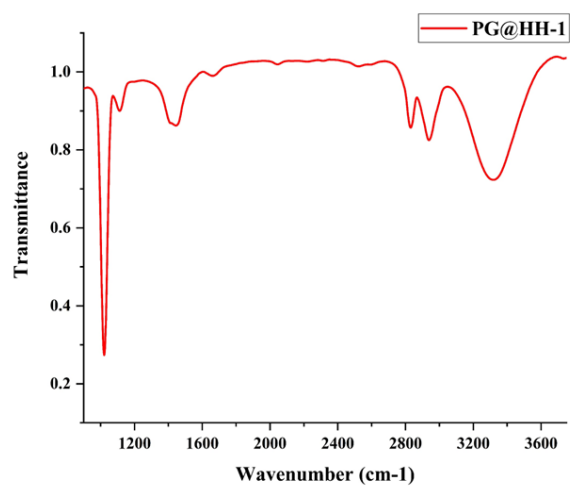
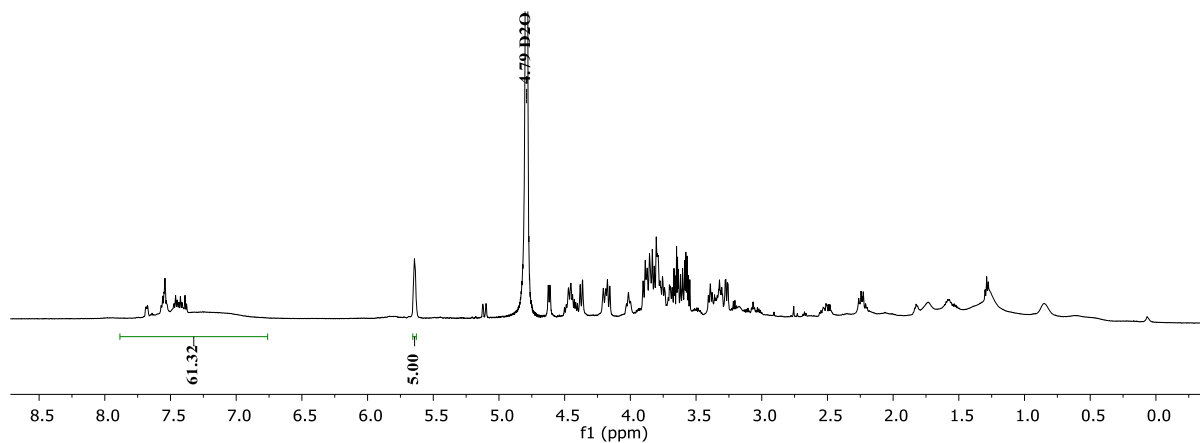
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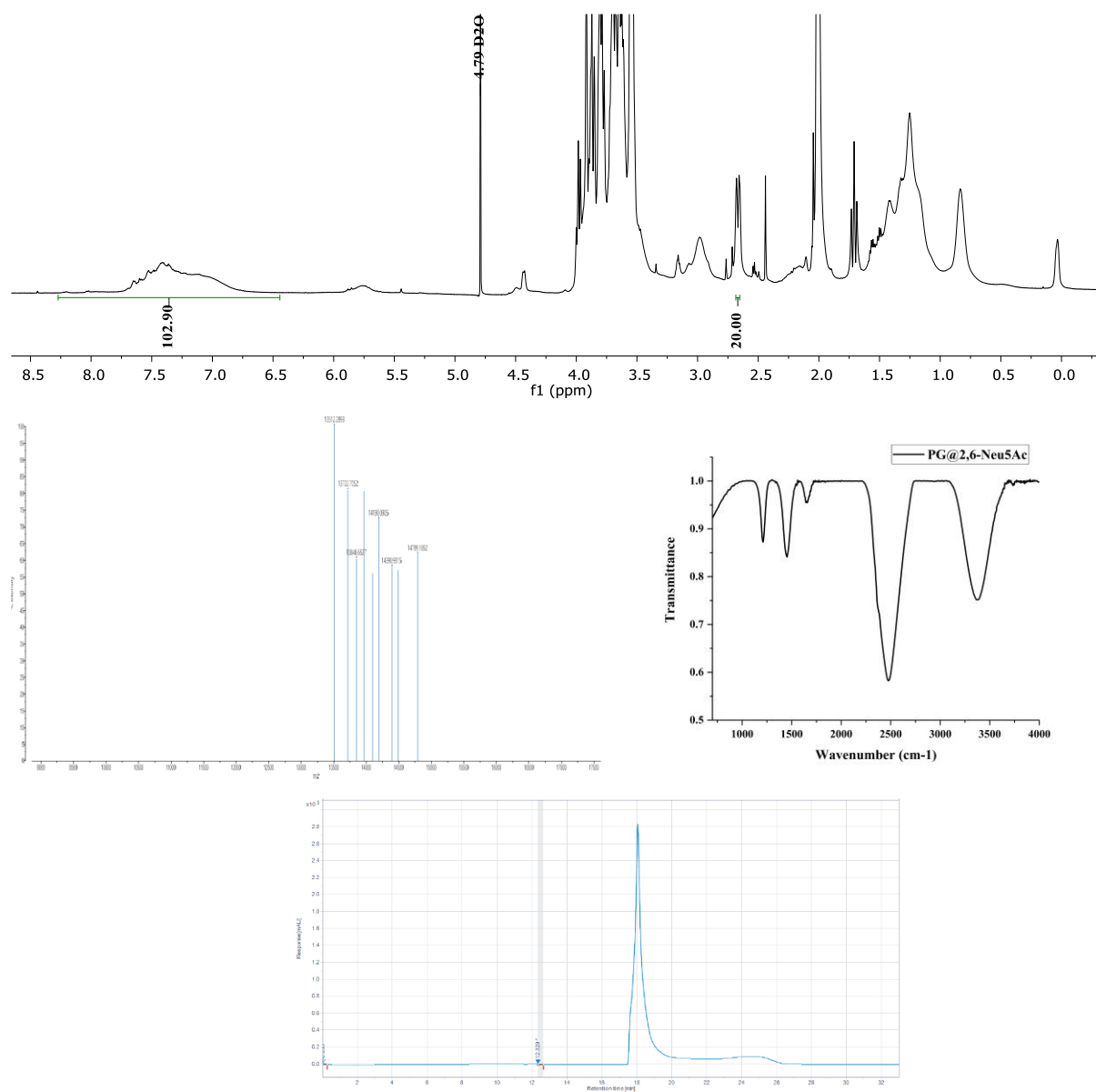
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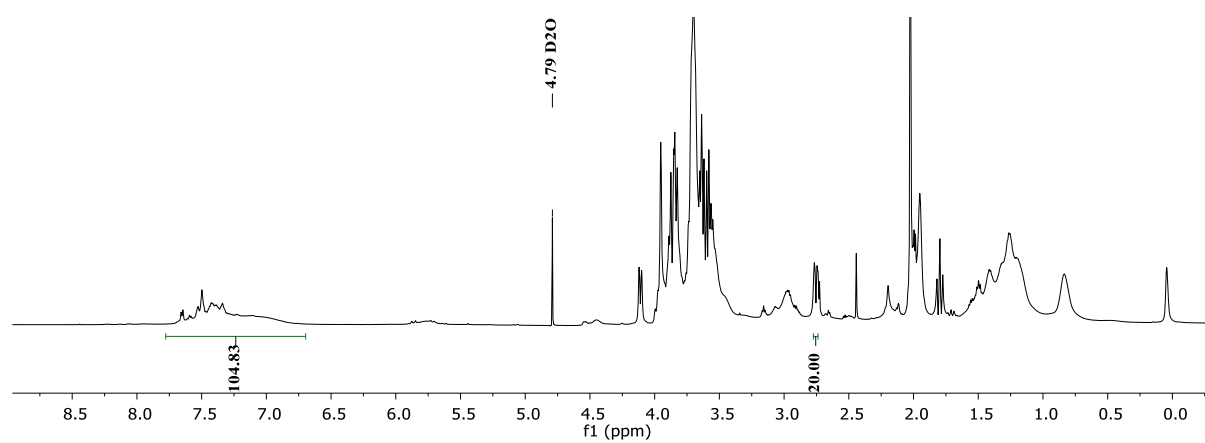
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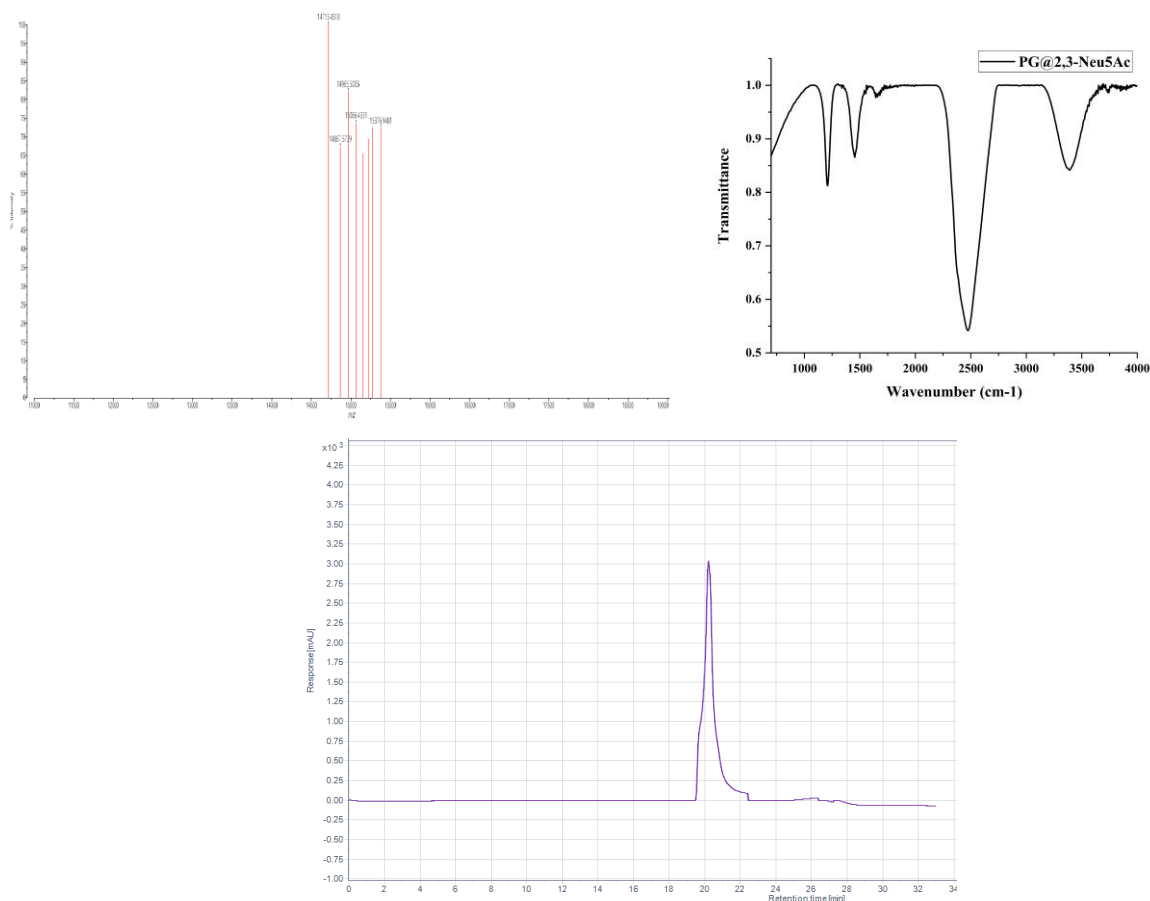


PG-1 (PG@2,6-Neu5Ac)



PG-2 (PG@2,3-Neu5Ac)





2.6 Reference

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Chapter 3
Sulfation Code of Heparan Sulfate
Proteoglycan Mimetics Modulating Cell
Membrane Dynamics

Abstract

Membrane-bound HSPGs serve as receptors/co-receptors for growth factors and cargo delivery. However, key question unraveled include the structural heterogeneity of the HS glycan moiety, altering glycocalyx remodeling and endocytosis of proteoglycans. Herein, we have discovered that specific sulfation and uronic acid composition of HS significantly alter cell-membrane dynamics and internalization mechanism of HSPG mimetics. Time-dependent confocal imaging studies of structurally well-defined HSPG mimetics showed that 3-*O*-sulfated iduronic acid (**PG@ID-2**) display fasted endocytosis over 6-*O*-sulfated uronic acid counterpart (**PG@ID-1**, **PG@GD-1** or **PG@GD-2**). Molecular dynamics simulation and unsupervised machine learning have revealed that **PG@ID-2** display a different set of physico-chemical properties (PCP) compared to their 6-*O*-sulfate counterparts and induce a more rigid structure enabling faster diffusion through the cell membrane than other analogues. Such disparity also influenced **PG@ID-2**/FGF-2-mediated fibroblast cell migration and cell proliferation in wound healing assay. Overall, these findings shed light on unseen aspects of sulfation patterns on proteoglycans, modulating biological processes.

3.1 Introduction

Decoding the structure-function relationship of biomacromolecules poses formidable challenges due to their diverse roles, each functionality influencing or autonomously governing biological processes.^{1,2,3} Thus, unravelling the individual or collective actions of each function is crucial for a more comprehensive understanding of biomacromolecules. Amongst those, PGs stand out as distinctive protein/polysaccharide conjugated biomacromolecules that function as receptors or co-receptors for various proteins and cargo delivery vehicles.^{4,5,6} Hence, understanding the parameters influencing proteoglycan functionality is important for development of drug-delivery and glycocalyx remodeling probes.^{7,8}

Proteoglycans are a protein-glycosaminoglycan conjugates ubiquitously found at the interface of cell-matrix.⁹ Based on their glycosaminoglycan composition they are classified as HSPGs or CSPGs.¹⁰ HSPGs are attractive targets for glycocalyx remodelling and cargo delivery, as they are expressed on the cell membrane and are directly involved in binding to various morphogens and growth factors through their structural diversity of HS, and participate in spectrum of biological processes.^{11,12}

HS is an anionic polymer composed of disaccharides repeating units of glucosamine (GlcN) and uronic acid sugar units. The structural diversity of HS originates from the sulfation patterns, particularly where GlcN is sulfated at the 6-O and 3-O positions, whereas the uronic acids (L-iduronic acid or D-glucuronic acid) are sulfated at the 2-O position. Additionally, the amine of GlcN can be *N*-sulfated, *N*-acetylated, or *N*-unsubstituted. Overall, a simple disaccharide of HS can be assembled into 48 different possible structures.^{13,14,15,16,17,18,19,20,21}

The conventional approach to synthesize proteoglycan mimetics involves a polymer or peptide-based lipid-glycoconjugates.^{22,23,24} Such mimetics could remodel the glycocalyx to facilitate growth factor-mediated proliferation, differentiation of stem cells, promote neurite outgrowth, and exhibit anticoagulant and antiviral activities.^{25,26,27,28,29,30,31,32,33} Furthermore, PG mimetics have been shown to regulate innate immune systems and inhibit Wnt activity.³⁴ While these studies have yielded ground-breaking results, some challenges impeded a systematic investigation of their biological functions, for example due to structurally compromised HS ligands like Δ 4,5-unsaturated uronic acid or fragmented heparinoids, as well as the polydispersity of the proteoglycan backbone.^{35,36}

To overcome these limitations, here we report the synthesis of 12-mer lysine-based proteoglycan mimetics carrying Cy-5 fluorescent and cholesterol linker at the C- and N-

terminal of the peptide. The lysine residue was converted into azide, and well-defined HS-disaccharide ligands were conjugated via copper-free click reaction. We used *N*-acetate heparinoids carrying 6-*O* and 3-*O*-sulfation and also changed the uronic acid composition to examine the synergistic effect on glycocalyx remodelling and endocytosis processes on cancer and normal cells.

3.2 Results and Discussion

3.2.1 Synthesis of Proteoglycans Mimetics

To examine sulfation pattern dependent proteoglycan activity, we functionalized disaccharide heparinoids with a peptide-based PGs scaffold, also carrying Cy-5 fluorescent tag for imaging studies.

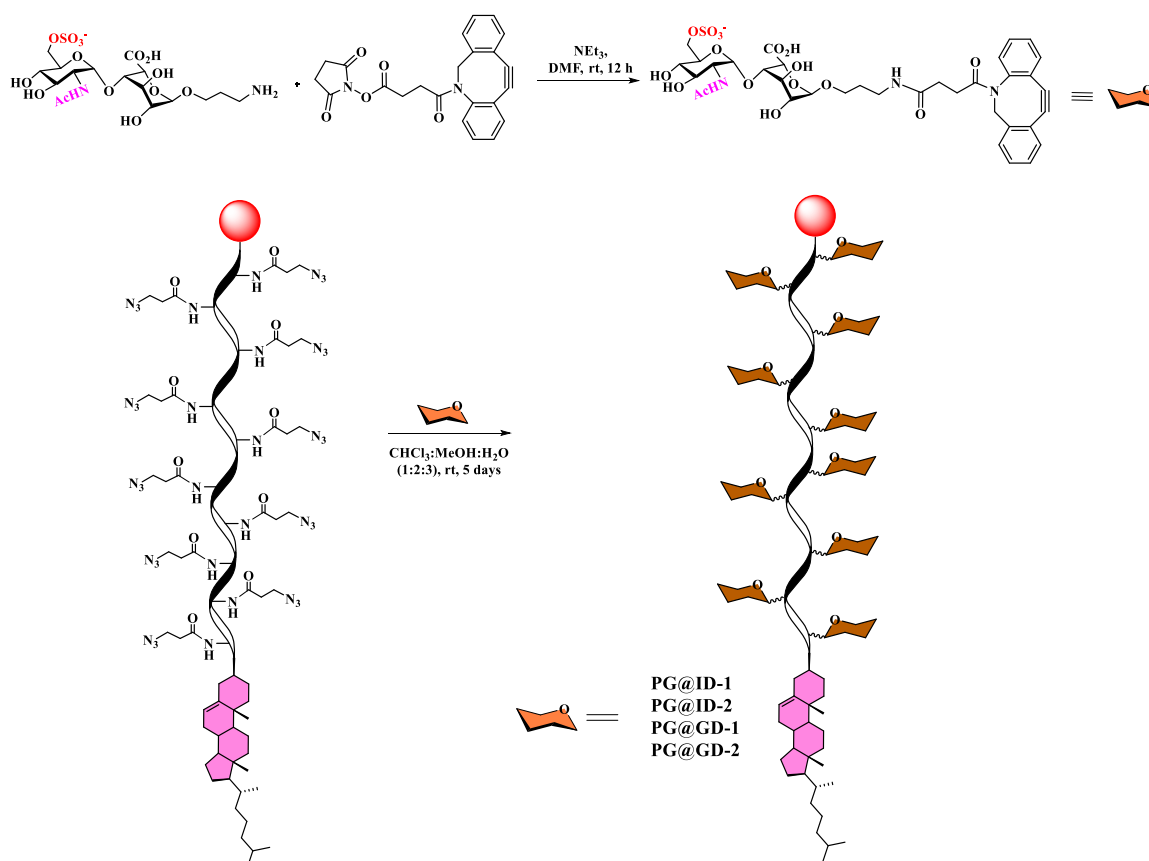


Figure 1. Synthesis of PGs mimetics by conjugation of NAc series of HS molecules to the 12-mer peptide

The above shown molecules are from chapter 2 and it's utilized to study cell membrane modulation.

3.2.2 *In vitro* Studies

3.2.2.1 Cell Surface Decoration Studies

Next, live cell imaging studies were conducted to evaluate glycocalyx remodeling and uptake, using MDA-MB-468 as a prototype (also examined NIH-3T3 cells and SK-BR-3 cells. Cells were grown on eight-well glass chamber slides and allowed to grow until reaching 70-80% confluence. Proteoglycan mimetics (2 μ M) were introduced, and live images were recorded at various time intervals (0.5, 1.5, 2.5, and 3.5 hours), revealing that all four proteoglycan mimetics were incorporated into the plasma membrane within minutes (**Fig. 2 – 3**), though at varying rates of cell membrane incorporation and internalization.

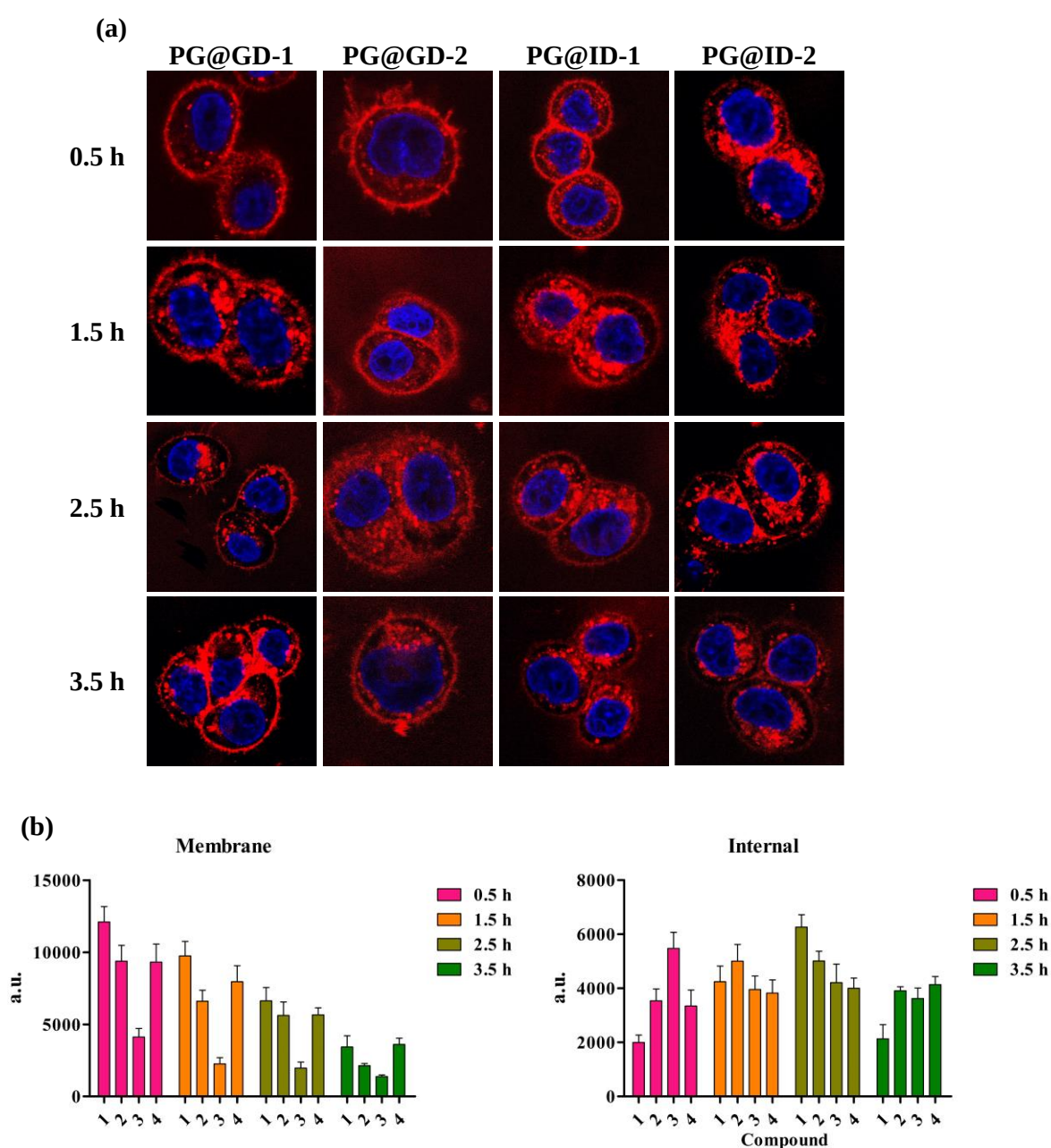


Figure 2. (a) Time dependent confocal imaging of proteoglycan mimetics in MDA-MB-468 cells after 30 mins of incubation, **(b)** Average surface fluorescence quantification of proteoglycan mimetics in MDA-MB-468 cells at different time interval on the cell membrane and inside the cell n = 50.

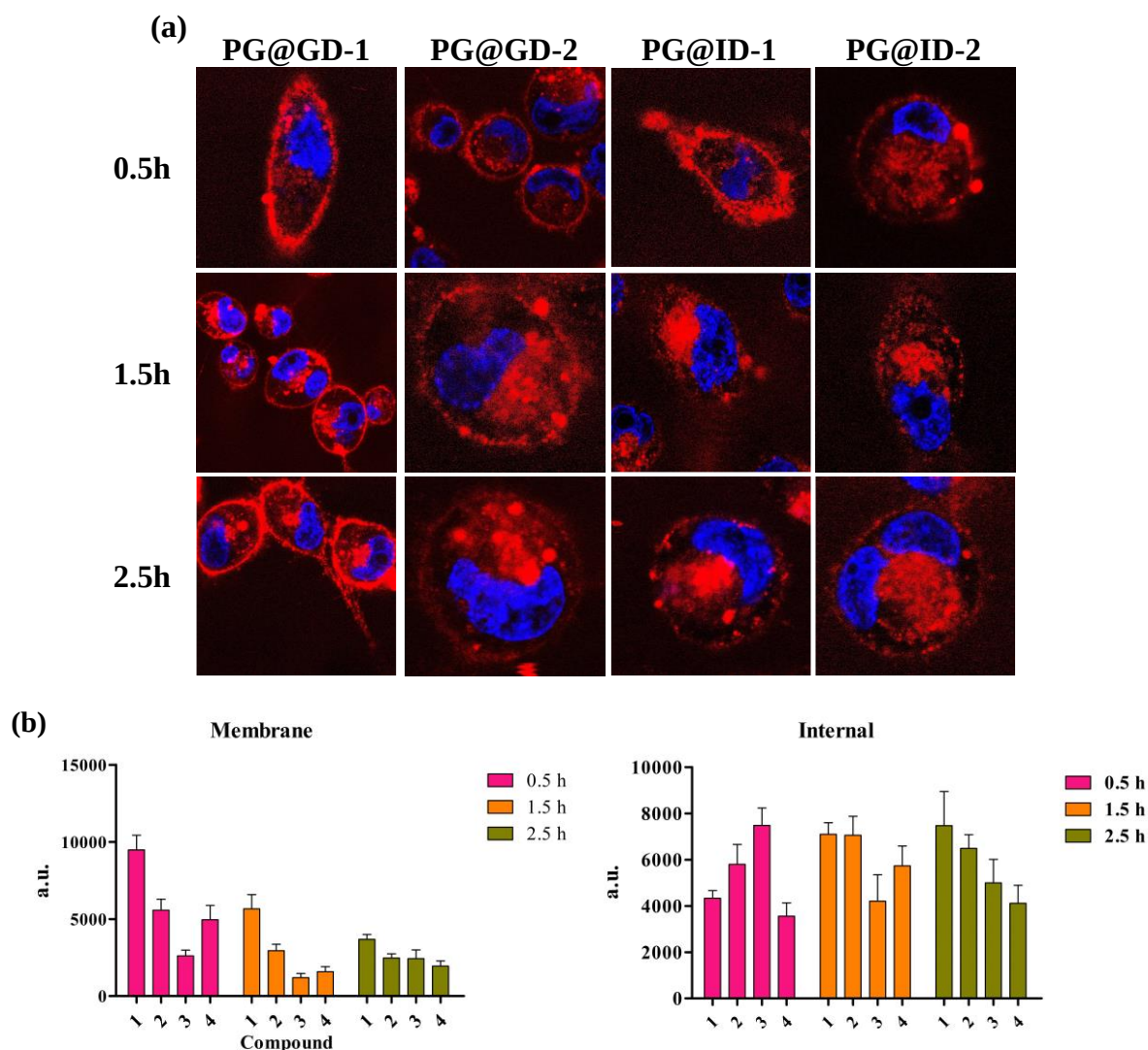
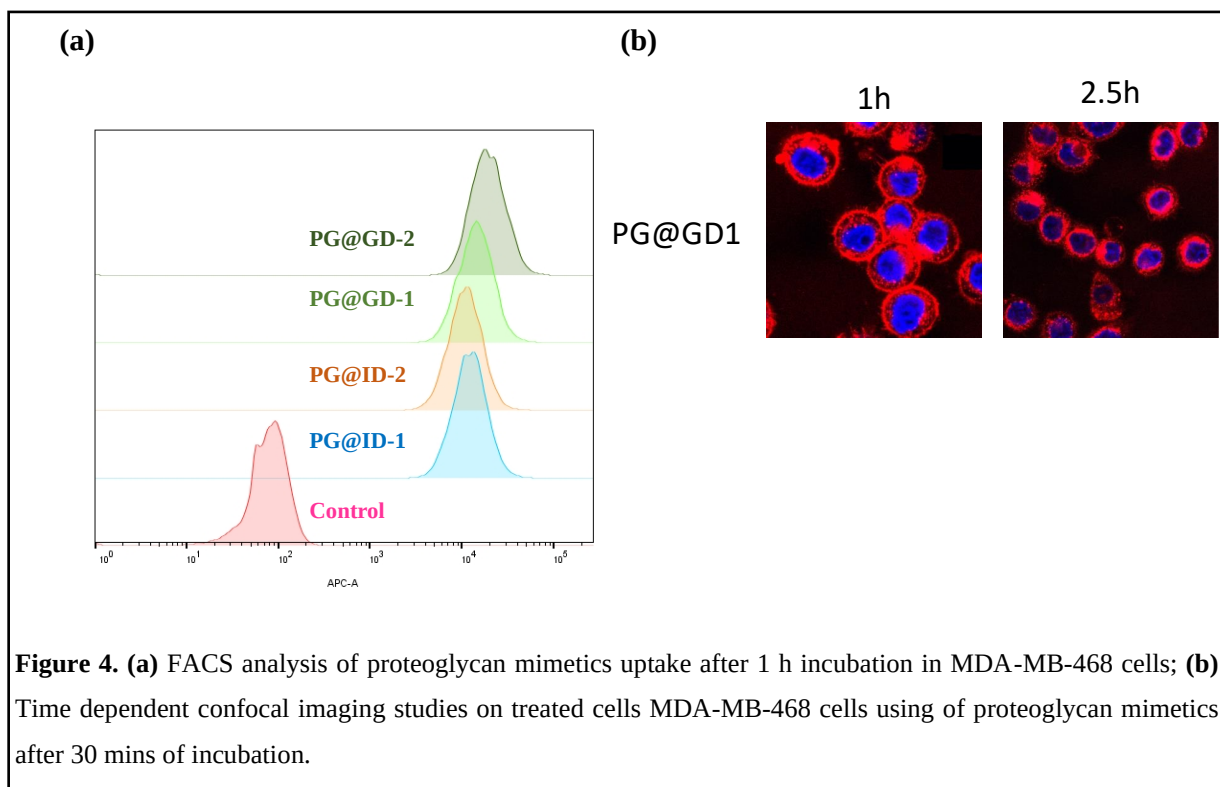


Figure 3. (a) Time dependent confocal imaging of proteoglycan mimetics in NIH3T3 cells after 30 mins of incubation, **(b)** Average surface fluorescence quantification of proteoglycan mimetics in NIH3T3 cells at different time interval on the cell membrane and inside the cell n = 50.

PG@GD-1 exhibited robust incorporation into the cell plasma membrane compared to the other three mimetics. Conversely, PG@ID-2 displayed the least incorporation after 0.5 h, with the amount nearly half of the fluorescent intensity of PG@ID-1 and PG@GD-2. However, after 1 h of incubation, all four molecules commenced internalization, and the majority of the molecules were internalized after 3.5 h in MDA-MB-468 2.5 h in NIH-3T3 (SK-BR-3 in **Fig. 9**). Subsequently, flow cytometry was employed for quantifying the amount of fluorescent

HSPG mimetics in cells. After 1 h, a similar amount of mimetics (90 – 95%) were incorporated to the cells (**Fig. 4a**).



These results suggest that 3-*O*-sulfation in PG@ID-2 possesses such unique features that alter their molecular representation on the cell membrane and internalization processes.

The glycocalyx is a negatively charged surface, and electrostatic repulsion between proteoglycan mimetics and cell-surface glycans might interfere with the uptake mechanism.¹² To better understand the rapid endocytosis of PG@ID-2, we performed imaging studies with heparinase and periodide-treated MDA-MB-468 cells. PG@GD-1 showed similar uptake trend compared to untreated, revealing that anionic repulsion is least influencing the uptake process (**Fig. 4b**).

3.2.2.2 Mechanistic Studies

Further uptake mechanism was investigated, MDA-MB-468 cells were incubated with NaN_3 for 30 minutes to deplete ATP, followed by treatment with PG@GD-1 (2 μM) for 2 hours. There was no decrease in cellular uptake, suggesting the internalization process is passive and does not require energy, for example does not involve ATP-dependent membrane receptors/channels (**Fig. 5**).

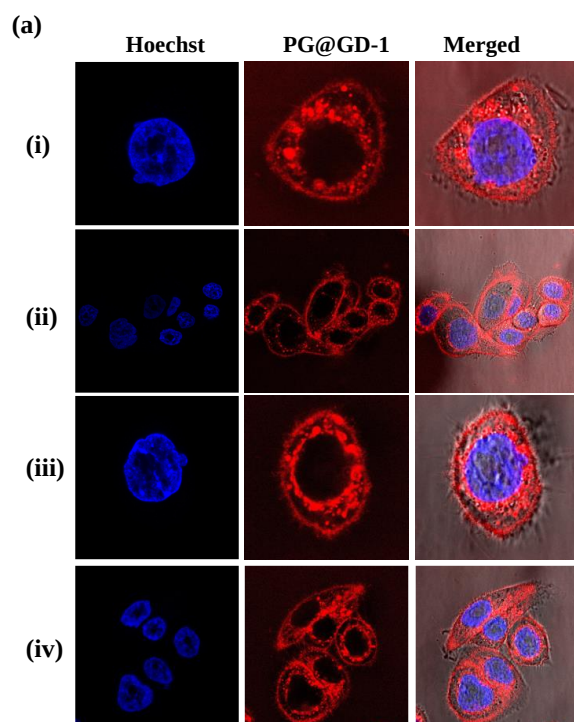


Figure 5. Confocal imaging of mechanistic studies with PG@GD-1 (i) Energy dependent pathway studies, (ii) Dynamin dependent pathway studies, (iii) Methyl- β -cyclodextrin studies, (iv) Chlorpromazine studies

Similarly, cells treated with various endocytosis inhibitors as dynasore hydrate (an inhibitor for the dynamin pathway), methyl- β -cyclodextrin (m- β -CD; an inhibitor of caveolae-mediated endocytosis), or chlorpromazine (inhibitors of clathrin-mediated endocytosis) also did not inhibit the internalization process. Together, these data support internalization of PG mimetic through a passive process, as would occur by lipid raft mediated uptake.¹³

3.2.3 Theoretical Studies

To provide structural insights into the penetration ability of these proteoglycan mimetics, in silico analysis was performed. A set of molecular dynamics (MD)-derived descriptors characterizing the physico-chemical properties of the compounds was selected to discover the properties of the unbound molecules that are putatively linked to the ability of the compounds to penetrate biological membranes (Table 1). The inclusion of descriptors of the binding sites located on the biological membranes should also be considered, as these would likely enhance the information gained from computational studies of the unbound HSPG-containing compounds. Nevertheless, it can be assumed that the characteristics of the analyzed molecules are likely to affect the ability to penetrate biological membranes, as the flexibility or ability of the compounds to adopt a favorable conformation prior to penetrating biological membranes

would likely be limited by the individual physico-chemical properties of the compounds *in vitro*.

Using the CPPTRAJ module of AMBER 20, a set of physico-chemical properties over the entire MD trajectory was calculated (Table 2). A significant difference in value ranges can be seen, not only between each of the HSPGs, but also between individual HS side chains within the compounds, suggesting these differences may relate to membrane penetration propensities. To assess the correlation between the descriptors and uncover potential relationships between them, pairwise Spearman correlation was calculated after standardizing the data set. Removing the data on the main chain descriptors allowed to distinguish the patterns more clearly. Most likely, this is due to the fact that the differences between compounds stem mainly from the different heparinoids attached to the PG backbone, which do not affect the behaviour of the main chain. Therefore, subsequent analyses and visualizations were conducted only using the descriptors relating to the HS ligands.

The correlation coefficients for the descriptors are summarized in experimental section. Groups of descriptors form clusters of strong positive and negative correlation. Expectedly, for all compounds a cluster of positive correlation can be observed for the SASA (Solvent-Accessible Surface Area (*shown in table 2*)) of the HSPGs, the overall volume of each compound, and the number of H-bonds between the heparinoids and the solvent identified in each MD trajectory snapshot. Particularly noteworthy are the differences in the correlation patterns observed between the compounds. While PG@GD-1 and PG@ID-1 show a similarity in correlation patterns, PG@GD-2 is characterized by a much more sparse correlation heatmap.

Apart from the aforementioned solvent-related correlation cluster, the EED (end-to-end distance (*shown in table 2*)) of PG@GD-1 and PG@ID-1 showed a predominantly positive correlation with the RMSD (root mean square derivation) and R_{gyr} (radius gyration) of the respective HSPGs, implying a high flexibility of the HS ligand. Conversely, the weak negative correlation between the EED and RMSD of heparinoids of PG@GD-2 implies its more rigid structure, wherein the heparinoids are more extended compared to the other compounds, yet the RMSD is low, implying a relatively stable extended structure. Interestingly, PG@GD-2 shows a more complex and simultaneously stronger pattern of correlation for Angle and SASA than PG@GD-1 and PG@ID-1, hinting at a distinct behavior of the HS ligands throughout the MD simulation.

Compared to compounds PG@GD-1, PG@ID-1, and PG@GD-2, a different pattern of correlation can be observed for PG@ID-2 (**Fig. 6**). The complex signal involving both strong positive and negative correlations between many descriptors implies a greater interdependence of the descriptors. In order to capture the complexity of dependence of the descriptors on each other principal component analysis (PCA) was conducted on the set of the collected descriptors. The calculated PCs were analyzed in terms of the cumulative explained variance. To preserve the most important information, we reduced the dimension of the dataset from the initial 67 descriptors to a total of 3 PCs, which explained 37.3% of variance of the heparinoids-descriptor data subset. The obtained PCs constituted a new dimension, with each PC being linearly independent from the others, therefore representing a unique combination of the original descriptors. The detailed analysis of the PCA loadings, shown in (**fig 11**), enabled to untangle the physico-chemical meaning of each of the PCs.

The first PC (PC1) corresponds to a compact structure observed in the MD trajectory of the compounds. High values of PC1 are positively correlated with lower EED of the heparinoids and a lower volume of a compound, alongside greater solvent accessibility of the centrally-located GAG side chains and more frequent H-bonds formed between the heparinoids and the main chain and solvent. PC2 describes a more extended and spread-out shape of the compounds. The main positive correlation with PC2 can be seen with larger EED and R_{gyr} values, indicating extended GAG side chains. A high RMSD value for most heparinoids is negatively correlated with PC2, indicating a less flexible behavior of the attached chains. Likely due to their extended nature, the HS ligands are characterized by less H-bonds established with the main chain. Interestingly, PC3 seems to correspond to a mix between the two opposite forms of the compounds captured by PC1 and PC2. Higher values of PC3 are correlated with more extended terminal side chains for GAGs 8, 9, and 10, characterized by larger R_{gyr} values. At the same time, the central heparinoids and those attached to the opposing end are more compact, with less solvent-accessible surface area. All of the heparinoids are also characterized by a bent structure as seen in high positive and negative correlation for all of the angle descriptors. Importantly, the heparinoids are characterized by more H-bond interactions among each other, which is different from other conformations.

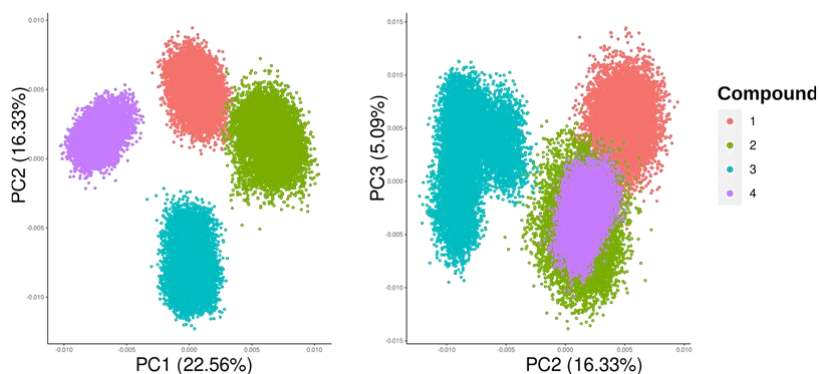


Figure 6. The data corresponding to each compound per MD trajectory snapshot plotted in the 2D representation of the new PCA dimensionality. Each data point is colored according to the compound it represents

Logistic regression was used to evaluate the extent to which the PCs can explain the ability of the compounds to penetrate the biological membrane, encoded as a binary variable with 0 corresponding to lower penetration ability and 1 corresponding to higher membrane penetration ability. The independent variables of the linear regression model were the three PCs obtained from PCA, with the ability of the compounds to penetrate the biological membrane as response variable. The results of this analysis are detailed in Table 3. All PCs are identified as statistically significant in explaining the binary response variable ($p < \alpha = 0.05$). As a result, the PCA-reduced dimension correctly reproduces the physico-chemical properties of the molecules and the PCs can be used to further explain the observed differences between the compounds. The best separation of compounds can be observed in the PC1/PC2 space, however the similarity of PG@GD-1, PG@ID-1, and PG@GD-2 is seen for both the PC1/PC2 space and even more so in the PC2/PC3 space (**Fig. 6**).

The observed differences in PG@GD-1, PG@ID-1, and PG@GD-2 are to be best explained by PC1, however PG@ID-2 is separated from the compounds in PC2. This corresponds to a generally more extended and rigid structure of PG@ID-2 compared to the remaining compounds, and a much more compact structure of PG@GD-2. To summarize, we find that more extended conformation of the PG@ID-2 together with its higher rigidity could serve as physico-chemical basis for a different behavior of this compound in the penetration experiment.

3.2.4 Microarray and Wound Healing Studies

To assess whether such disparity in membrane dynamics influences growth factor mediated cell migration and cell proliferation, we first conducted glycan microarray of HS ligands with the FGF-1 and FGF-2 growth factors. While both FGF-1 and FGF-2 bound the natural heparan

sulphate (HS), FGF-2 had three-times stronger binding to HS than FGF-1, and only FGF-2 could also bind all four HS mimetic glycans at high binding preference (**Fig. 7, section 3.4.3**).

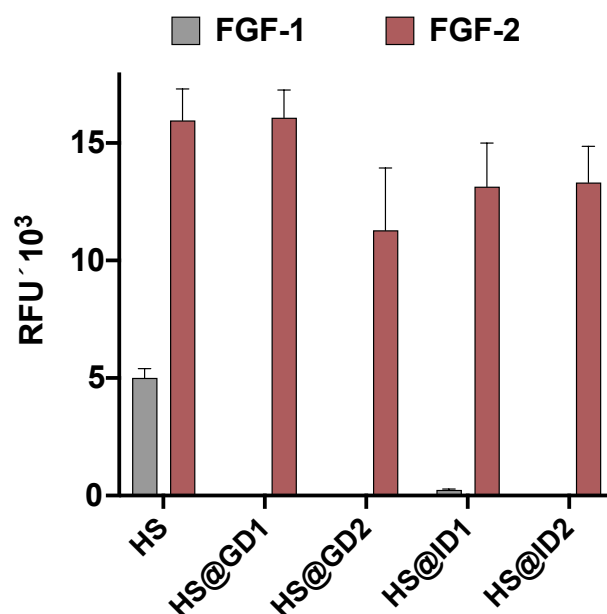


Figure 7. Microarray analysis of heparinoids with human FGF-2 for that 100 μ M of PGs mimetics were used.

This differential binding preference of the HS-mimetics by FGF-2 but not with FGF-1 could provide important research and theranostic tool. Follow up studies with FGF-2 in a cell migration assay³⁷ on NIH-3T3 cells, PG@ID-2 showed significant wound healing effect compared to the other three PG mimetics (**Fig. 8**), supporting the notion that rigid structures of PG@ID-2 can effectively clustered the FGF-2/FGFR receptors than plasma membrane assembly of HSPG and induce cell proliferation.

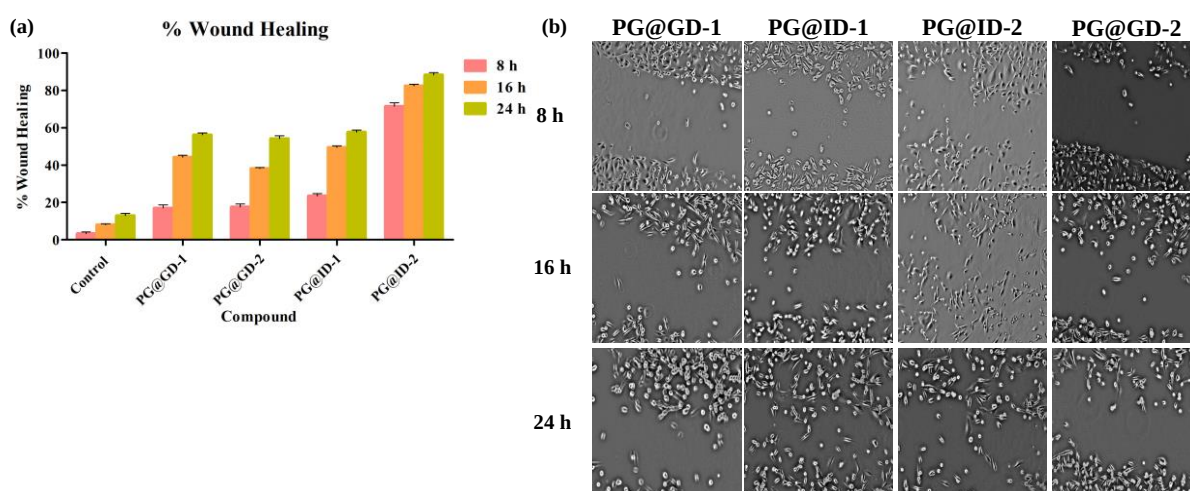


Figure 8. (a) Quantification of wound healing studies at different time intervals. (b) Bright field imaging of wound healing studies in NIH-3T3 cells in the presence of FGF-2 and PGs mimetic.

3.3 Conclusions

We have provided a detailed analysis of the structural diversity of HS in modulating plasma membrane dynamics and the internalization of HSPG mimetics carrying well-defined heparinoids. Using live-cell imaging studies, molecular dynamics and machine learning approaches, we have shown that 3-*O* sulfation in iduronic acid-based HS disaccharide induces rigidity in the system, allowing for faster penetration of the cell membrane compared to the 6-*O*-sulfated counterparts. Microarray studies have confirmed that although N-acetate-based heparinoids display similar FGF-2 binding preferences, disparities in cell membrane dynamics and internalization processes significantly modulate their growth factor-mediated cellular responses. These results show that HS behavior as an individual component differs from that of HSPGs. Further detailed investigation with other HS disaccharide ligands or longer oligosaccharides can shed light on efficient strategies to enhance the cellular delivery of cargoes and differentially affect growth factors.

3.4 Experimental Section

3.4.1 *In vitro* Studies

Experiment was performed on MDA-MB-468, SK-BR-3, and NIH3T3 cells were cultured at 37 °C in a 5% CO₂ atmosphere in DMEM media supplemented with 10% fetal bovine serum (FBS) and 0.1% streptomycin.

3.4.1.1 Cell Decoration Studies

A total of 2×10^4 cells were seeded onto 8-well confocal imaging plates and incubated at 37 °C overnight. Subsequently, the cells were treated with **PG@ID-1**, **PG@ID-2**, **PG@GD-1**, and **PG@GD-2** (2 μM) for 30 minutes. Following the treatment, the cells underwent three washes with PBS, and the nuclei were stained with Hoechst 33342. After the staining process, the media was added, and fluorescent images were captured using a Leica SP8 microscope. (*excitation* = 633 nm and *emission* = 650 – 720 nm)

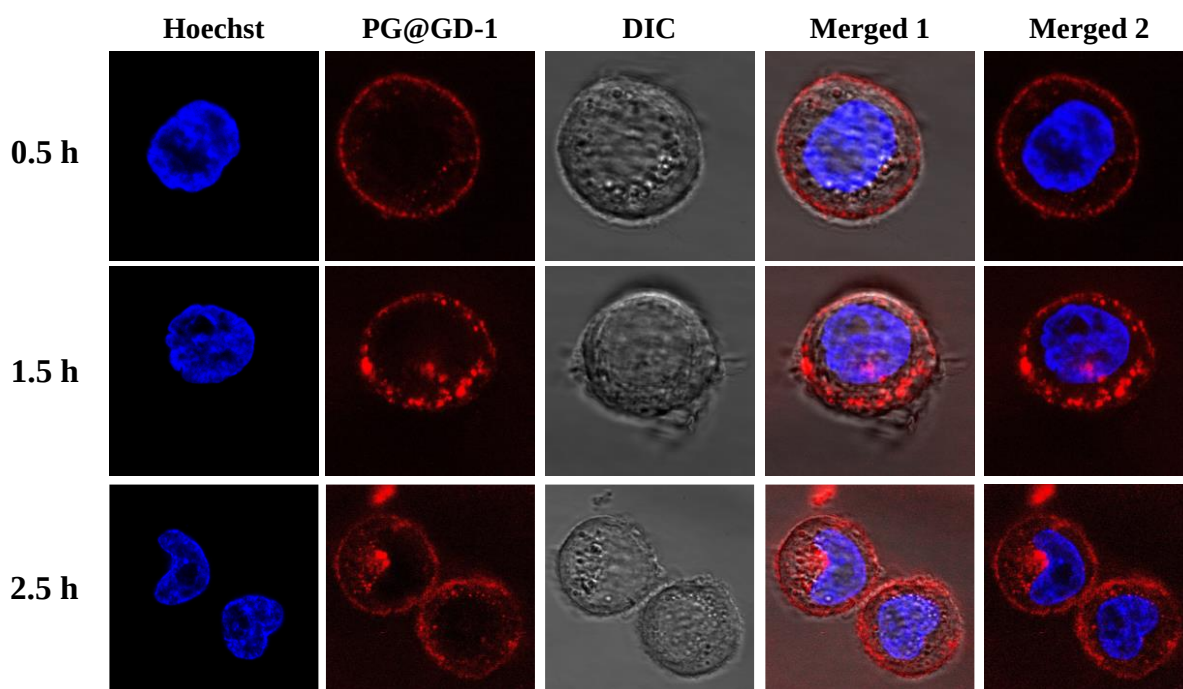


Figure 9. Compounds cell surface activity in SK-BR-3 cell line at 2 μ M concentration after 1h.

3.4.1.2 Mechanistic Studies

To explore the cellular internalization mechanism, MDA-MB 468 cells were cultured overnight in an 8-well chamber plate (2×10^4 cells per well). They were then subjected to a specific inhibitor for 30 minutes, followed by a 30-minute treatment with **PG@GD-1** and **PG@ID-2** at 37 °C. For the investigation of energy-dependent, dynamin-mediated, clathrin-mediated, and caveolae-mediated processes, cells were exposed to NaN₃ (50 mM), dynasore hydrate (50 μ M), chlorpromazine (25 μ M), and methylated- β -cyclodextrin (10 mM) for 30 minutes, respectively. Following the 30-minute treatment with **PG@GD-1** and **PG@ID-2**, cells underwent a thorough wash to remove unbound materials and were subsequently stained with Hoechst 33342 before proceeding to imaging.

3.4.1.3 Heparinase and per-iodide Treated Cell Decoration Studies

To investigate the glycocalyx anionic interaction modulating the activity, we first treated the cell with heparinase (0.03 IU/ml) for 1h at 37 °C, followed by sodium periodate (1 mM) for 30 min at 4 °C respectively. Next cells incubated with **PG@GD-1** and **PG@ID-2** at 2 μ M concentration. Our results showed no such significant difference in the activity as compared to untreated cells, indicating the glycocalyx anions-HSPGs interaction is least influential.

3.4.2 Molecular Dynamics Simulations

All-atom molecular dynamics (MD) simulations of the studied compounds were performed in AMBER20.³⁸ Compounds **PG@GD-1**, **PG@ID-1**, **PG@ID-2**, and **PG@GD-2** were built in Leap and optimized in Gaussian 09 using B3LYP (6-31G*) level of theory.³⁹ These molecules were parameterized combining ff19SB,⁴⁰ GAFF⁴¹ and the GLYCAM06j⁴² force field parameters. The partial atomic charges compatible with the AMBER were obtained by RESP procedure.⁴³ A truncated octahedron TIP3P periodic box of 12 Å water layer from the box's border to the solute was used to solvate the molecule. The charge was neutralized with Na⁺ counterions. Two steps of energy minimization were performed: first 500 steepest descent and then 1000 conjugate gradient cycles with harmonic restraints of 100 kcal/mol/Å² on the solute, followed by 3000 steepest descent and 3000 conjugate gradient cycles without restraints. Then, the system was heated up to 300 K for 10 ps with harmonic restraints of 100 kcal/mol/Å² on solute and equilibrated for 100 ps at 300 K and 10⁵ Pa in isothermal isobaric ensemble (NTP). Afterward, MD simulations were carried out in the same NTP for 100 ns with SHAKE algorithm for the covalent bonds containing hydrogen atoms. The 2fs time integration step, 8 Å cutoff for non-bonded interactions and the Particle Mesh Ewald method to treat electrostatics were used.

Descriptors of the studied compounds. Based on the obtained MD trajectories, a set of physico-chemical properties (“descriptors”) was analyzed with CPPTRAJ module of AMBER 20(Table 1).⁴⁴

Table 1. MD-derived descriptors of the main chain and GAG side chain fragments of the analyzed compounds used in the study.

No.	Descriptor	Symbol [units]	Physical meaning
1-10	Angle formed between the main chain and the GAG side chain of the compound	Angle [°]	The angle between the GAG side chains and the main chain of each compound characterizes the rigidity of the compound. The angles are calculated using the <i>vectormath</i> command in <i>cpptraj</i> . The calculation involves two vectors: one that runs along the axis connecting the centers of mass from the first to the last residue of the main chain, and another for each GAG, which runs along the axis connecting the centers of mass from the first to the last residue of the GAG side chains.

11-21	End-to-end distance	EED [Å]	The overall extension of the main chain and GAG side chains, defined as the distance between the center of mass of atoms of the first residue of a given fragment (main chain, GAG side chain) and the center of mass of the last residue of the same fragment, calculated using the <i>distance</i> command in <i>cpptraj</i> .
22-33	Root Mean Square Deviation	RMSD [Å]	The RMSD of the main chain and the GAG side chains with the reference to their starting position characterizes flexibility of the compound. It is calculated using the <i>rms</i> command in <i>cpptraj</i> .
34-45	Solvent-Accessible Surface Area	SASA [Å ²]	The surface area accessible to the solvent provides information about the solubility of the compound. It is calculated using the <i>surf</i> command in <i>cpptraj</i> .
46	Volume	volume [Å ³]	It is calculated using in-house R scripts with the use of <i>OptimalDesign</i> library.
47-58	Radius of gyration	Rgyr [Å]	Radius of gyration characterizes the shape of the compound. It is calculated using the <i>radgyr</i> command in <i>cpptraj</i> .
59-66	Number of formed H-bonds	MCGAG GAGMC MCMC GAGGAG MCWAT WATMC GAGWAT WATGAG	The number of H-bonds formed between the compound fragments acting either as H-bond donor or acceptor as well as the number of H-bonds formed between the individual fragments (main chain (MC), GAG side chains (GAG)) and the solvent (WAT) characterize intramolecular and compound-solvent interactions. It is calculated using the <i>hbond</i> command <i>cpptraj</i> .

Principal component and correlation analysis. To detect groups of highly correlated descriptors, Spearman correlation coefficients were computed for the descriptor dataset and plotted as a heatmap. The descriptor dataset was standardized prior to performing analysis. Principal component analysis (PCA) was used as a feature extraction approach to reduce the dimensionality of the original dataset and to uncover patterns in the descriptor dataset that could later be linked to the target variable, i.e. the ability of compounds to penetrate the biological membrane. PCA of the descriptor dataset was carried out in R.⁴⁵ PCA, an

unsupervised machine learning technique, transforms a set of related variables into a new set of uncorrelated variables called Principal Components (PCs), which are linear combinations of the original variables that capture the core physico-chemical information and reduce the noise present in the dataset. Since the PCs are derived without considering the target variable, they solely reflect the physico-chemical characteristics of the HSPG mimics as simulated in the MD study. Spearman correlation was used to assess the relationship between each individual descriptor and the target variable. The values of each descriptor measured during the MD simulation were correlated with the penetrating propensity values for each HSPG individually to investigate which descriptors can be assumed to be most strongly linked with the ability to penetrate biological membranes.

Linear regression analysis. Logistic regression analysis was carried out in R (*stats* base package) with the purpose of determining the strength of the link between the computed PCs and the ability of HSPGs to penetrate the biological membranes. Statistical significance of the regression model was conducted using the Wald-test, with a cutoff for significance $\alpha = 0.05$; p-values of the test for the independent variables (PCs) below the α level were considered statistically significant.

Visualization. Visualization was carried out in R⁴⁶ using the ggplot⁴⁷ and gplots libraries.⁴⁸

Table 2. Summary of the MD-derived descriptors.

Descriptor		PG@GD-1				PG@ID-1				PG@ID-2				PG@GD-2			
		min	mean	sd	max	min	mean	sd	max	min	mean	sd	max	min	mean	sd	max
1	angle1	12.6	114.4	26.6	179.5	24.9	113.2	35.0	179.2	1.8	116.5	30.7	179.3	0.6	54.6	39.7	177.6
2	angle2	0.5	89.0	41.4	179.6	0.6	94.3	32.9	177.1	41.0	132.2	27.2	179.8	42.2	121.0	23.3	179.7
3	angle3	1.6	95.5	30.4	179.1	12.8	108.6	30.3	179.4	0.4	92.8	36.5	177.3	7.5	104.2	27.7	178.3
4	angle4	25.7	146.9	20.9	179.4	3.3	97.5	37.5	179.2	55.7	129.3	27.7	179.7	30.1	114.5	22.4	179.1
5	angle5	3.6	83.4	17.1	154.1	1.0	89.4	34.5	178.9	9.0	124.7	30.3	179.9	48.9	102.4	22.8	169.0
6	angle6	5.0	72.7	28.0	155.3	2.7	90.3	31.1	175.7	1.7	100.3	40.2	178.5	5.5	125.0	25.3	179.1
7	angle7	1.8	82.8	25.8	151.6	8.1	101.4	23.0	173.5	1.4	85.0	37.6	176.4	0.5	62.8	23.4	146.9
8	angle8	1.1	50.9	22.0	131.3	9.8	100.8	29.8	178.3	4.1	87.3	19.5	142.9	0.4	59.4	16.3	123.3
9	angle9	0.4	62.2	31.5	179.4	0.5	31.5	25.2	141.9	2.5	87.0	35.8	154.4	2.5	82.5	30.7	179.4

10	angle10	8.6	135.1	32.1	179.8	94.3	158.3	11.8	179.5	0.7	90.5	34.6	171.2	0.2	59.9	35.3	158.2
11	eed_mc	17.9	20.6	0.9	24.3	21.2	23.9	1.1	29.8	17.1	26.4	2.3	30.7	23.5	28.2	0.9	30.5
12	eed1	6.7	8.5	0.4	9.8	6.3	7.8	0.5	9.1	6.5	8.0	0.7	9.2	7.5	9.4	0.2	9.9
13	eed2	6.7	8.5	0.4	9.7	6.4	7.8	0.5	9.1	7.6	8.6	0.2	9.2	7.7	9.4	0.2	9.9
14	eed3	6.6	8.6	0.3	9.5	6.3	7.9	0.5	9.0	7.5	8.6	0.2	9.5	8.8	9.5	0.2	9.9
15	eed4	7.1	8.6	0.4	9.7	6.3	7.8	0.5	9.2	6.6	8.2	0.6	9.5	7.4	9.4	0.2	9.9
16	eed5	7.2	8.6	0.4	9.7	6.4	8.2	0.5	9.7	7.7	8.6	0.2	9.5	8.7	9.4	0.2	9.9
17	eed6	7.4	8.7	0.4	9.7	6.4	7.9	0.4	9.1	6.8	8.6	0.2	9.3	8.1	9.5	0.1	9.9
18	eed7	6.8	8.5	0.4	9.7	6.4	7.9	0.4	9.0	7.2	8.6	0.2	9.2	8.0	9.4	0.2	9.9
19	eed8	7.2	8.6	0.4	9.8	6.7	8.5	0.4	9.8	7.4	8.6	0.2	9.4	8.0	9.5	0.2	9.9
20	eed9	6.4	8.5	0.4	9.7	5.6	8.4	0.5	9.7	7.4	8.7	0.3	9.5	8.1	9.4	0.2	10.0
21	eed10	7.0	8.5	0.4	9.7	6.5	8.1	0.4	9.0	6.8	8.6	0.2	9.4	7.5	9.4	0.2	9.9
22	rmsd_mc	0.5	2.2	0.2	2.7	0.7	2.7	0.4	3.9	0.7	2.0	0.7	4.2	0.6	2.0	0.2	2.9
24	rmsd1	0.3	1.2	0.2	2.1	0.5	1.8	0.3	3.0	0.2	1.7	0.7	3.1	0.4	1.4	0.3	2.7
25	rmsd2	0.3	1.3	0.3	2.3	0.4	1.3	0.3	2.4	0.3	1.1	0.3	2.6	0.2	1.1	0.3	2.6
26	rmsd3	0.3	1.2	0.2	2.2	0.4	1.3	0.3	2.3	0.4	2.2	0.4	3.1	0.2	1.1	0.2	1.9
27	rmsd4	0.3	1.2	0.3	2.2	0.3	1.5	0.3	3.0	0.2	2.1	0.9	3.2	0.3	1.1	0.3	2.1
28	rmsd5	0.3	1.4	0.2	2.4	0.3	1.6	0.3	2.7	0.3	2.3	0.3	3.0	0.3	1.0	0.2	1.7
29	rmsd6	0.4	1.5	0.3	2.3	0.3	1.3	0.3	2.4	0.3	1.1	0.3	2.8	0.3	1.0	0.2	1.8
30	rmsd7	0.3	1.4	0.2	2.4	0.3	1.5	0.4	2.7	0.3	1.1	0.2	2.7	0.3	1.2	0.2	2.3
31	rmsd8	0.3	1.2	0.2	2.8	0.6	1.9	0.3	3.0	0.4	2.3	0.3	3.0	0.2	1.1	0.2	2.2
32	rmsd9	0.4	1.4	0.4	3.0	0.6	2.2	0.3	3.0	0.3	1.3	0.3	2.7	0.3	1.1	0.3	2.4
33	rmsd10	0.4	1.5	0.3	2.4	0.6	1.6	0.3	2.5	0.3	1.1	0.3	2.6	0.3	1.1	0.3	3.0
34	sasa_mc	58.3	170.1	39.6	379.9	94.7	218.4	67.4	542.4	124.1	250.5	57.0	541.5	86.4	236.8	56.9	539.3
36	sasa1	333.3	533.7	51.6	625.1	248.3	535.4	69.7	628.2	291.2	502.5	55.4	602.9	202.1	438.8	89.3	616.0
37	sasa2	379.1	516.0	59.9	630.5	290.8	503.1	68.5	621.8	312.1	459.3	44.7	603.2	324.7	478.2	52.7	609.7
38	sasa3	178.4	356.0	74.4	602.1	244.5	477.4	74.7	631.3	282.7	480.6	57.2	601.0	237.3	398.9	51.8	562.9
39	sasa4	308.7	477.2	57.9	634.9	251.6	516.0	67.5	634.7	258.1	414.5	68.6	598.4	220.6	418.5	85.6	605.3
40	sasa5	175.8	394.4	62.7	602.9	322.5	543.3	55.8	630.1	136.2	369.0	91.8	604.8	110.5	323.7	55.5	525.6

41	sasa6	252.6	451.8	75.5	617.7	325.9	510.5	59.6	622.1	277.3	512.5	56.1	606.7	316.7	492.0	48.0	613.1
42	sasa7	270.2	483.3	79.5	626.8	241.9	496.1	58.1	615.4	266.4	476.0	61.8	596.8	253.1	465.5	74.0	613.6
43	sasa8	236.4	482.9	59.8	613.1	321.8	528.0	60.0	632.9	196.6	404.3	66.5	604.0	261.3	441.4	68.0	612.3
44	sasa9	310.2	539.9	56.2	629.4	374.5	514.4	35.3	626.0	92.2	373.3	95.9	606.2	294.9	466.0	62.8	607.1
45	sasa10	211.1	482.7	75.3	635.5	85.8	294.6	56.1	557.8	211.8	439.9	80.6	598.7	215.0	477.2	85.6	619.3
46	volume	39741.5	66083.6	12526.9	131767.3	40870.3	67786.7	11651.0	128609.5	36398.9	55694.7	9750.0	95856.6	35135.8	58202.0	12271.1	119354.8
48	rgyr_mc	6.4	7.2	0.3	8.3	7.8	8.5	0.2	9.6	7.5	8.8	0.4	9.9	8.1	9.2	0.2	9.9
50	rgyr1	3.8	4.2	0.1	4.6	3.6	4.1	0.1	4.5	3.4	3.8	0.2	4.2	3.9	4.2	0.0	4.4
51	rgyr2	3.8	4.2	0.1	4.5	3.5	4.1	0.1	4.5	3.8	4.0	0.1	4.2	3.9	4.2	0.0	4.3
52	rgyr3	3.8	4.2	0.1	4.5	3.7	4.1	0.1	4.5	3.8	4.0	0.1	4.3	4.0	4.2	0.0	4.3
53	rgyr4	3.7	4.2	0.1	4.6	3.4	4.1	0.1	4.5	3.7	4.0	0.1	4.3	4.0	4.2	0.0	4.4
54	rgyr5	3.8	4.3	0.1	4.5	3.6	4.2	0.1	4.6	3.8	4.0	0.1	4.3	4.0	4.2	0.0	4.3
55	rgyr6	3.8	4.3	0.1	4.6	3.6	4.1	0.1	4.5	3.5	4.0	0.1	4.2	3.9	4.2	0.0	4.3
56	rgyr7	3.8	4.2	0.1	4.5	3.7	4.1	0.1	4.5	3.7	4.0	0.1	4.2	4.0	4.2	0.0	4.3
57	rgyr8	3.8	4.2	0.1	4.6	3.7	4.3	0.1	4.7	3.8	4.0	0.1	4.3	4.0	4.2	0.0	4.3
58	rgyr9	3.7	4.2	0.1	4.6	3.7	4.3	0.1	4.6	3.7	4.0	0.1	4.3	4.0	4.2	0.0	4.3
59	rgyr10	3.8	4.2	0.1	4.5	3.7	4.2	0.1	4.5	3.5	4.0	0.1	4.2	3.7	4.2	0.1	4.3
61	MCGAG	0	2	1	6	0	1	1	6	0	1	1	6	0	1	1	6
62	GAGMC	0	0	1	5	0	1	1	5	0	1	1	5	0	0	1	4
63	MCMC	0	0	1	6	0	0	1	3	0	0	0	2	0	0	0	2
64	GAGGAG	5	16	3	31	2	12	4	30	7	21	4	41	9	24	4	39
65	MCWAT	0	3	2	11	0	4	2	11	0	4	2	12	0	5	2	13
66	WATMC	0	5	2	11	0	6	2	14	0	6	2	14	0	6	2	14
67	GAGWAT	14	35	6	57	16	38	6	61	12	30	6	53	8	28	5	53
68	WATGAG	150	190	9	228	152	188	10	228	146	181	9	218	151	182	9	216

Table 3. Statistical analysis of the logistic regression model using PCs as independent variables. The statistical significance of the PCs was assessed by the Wald test. Statistical significance is indicated by a bold font.

	Estimate	Std. Error	z value	Prob. ($> z $)
(Intercept)	-23.0	9.2	-2.5	0.0123
PC1	-2.4	1.0	-2.5	0.0135
PC2	-14.6	5.8	-2.5	0.0117
PC3	6.7	2.7	2.5	0.0133

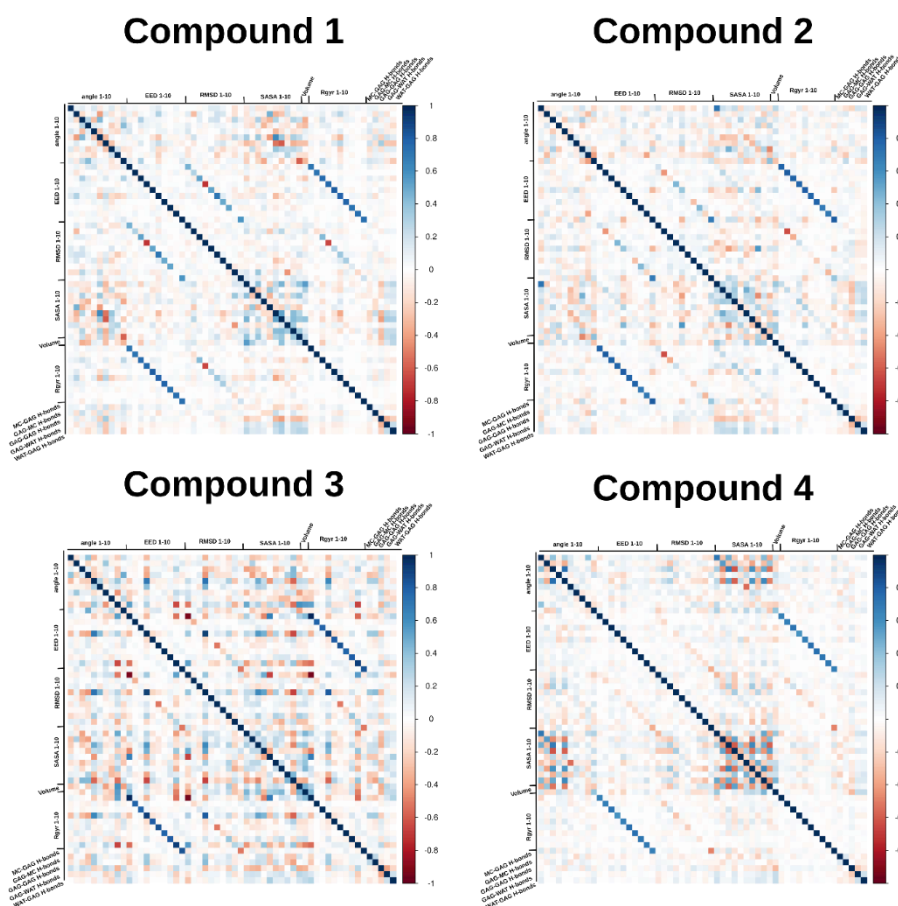


Figure 10. Heatmap of Spearman correlation coefficients between the MD-derived descriptors calculated per compound. A strong positive correlation is shown in blue colors, a strong negative correlation by red colors, while no correlation corresponds to a white color. (Note: Comp 1: **PG@GD-1**; Comp 2: **PG@ID-1**; Comp 3: **PG@ID-2** and Comp 4: **PG@GD-2**).

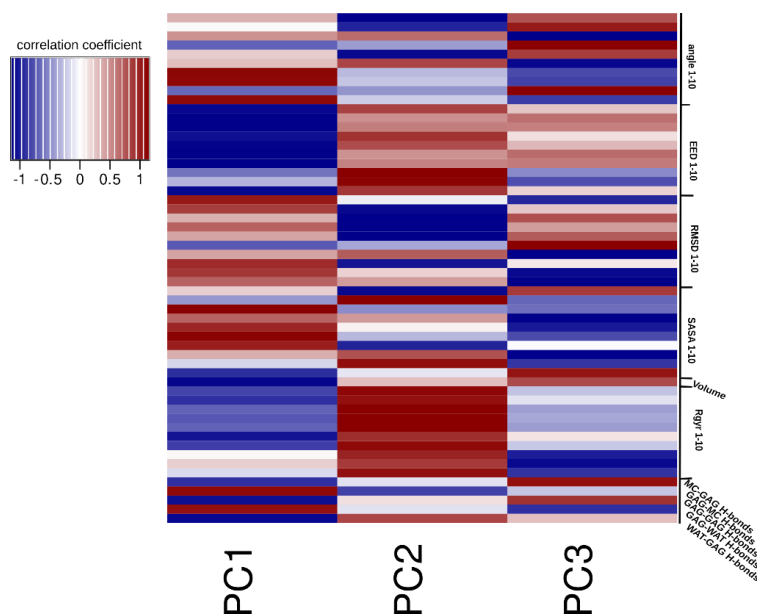


Figure 11. Heatmap showing the contribution of MD-derived descriptors to the obtained PCs.

3.4.3 Glycan Microarray

Materials. PBSx10 was purchased from Hy-labs, ethanolamine from Fisher, ovalbumin grade from Sigma-Aldrich, human FGF-1 (acidic), human FGF-2 (basic recombinant), biotinylated-rabbit-anti-human FGF-1 acidic and biotinylated-rabbit-anti-human-FGF-2 polyclonal antibodies from Peprotech, Chrom Pure human IgG whole molecule and Cy3-sreptavidin from Jackson Immuno Research. AlexaFlour-555-Hydraside from Invitrogen.

HS Microarray Fabrication. Glycans microarrays were fabricated as previously described {28745644; 22549775}. Arrays (Version VRH2) were fabricated with Nano Print LM-60 Microarray Printer (Arrayit) on epoxide-derivatized slides (PolyAn 2D) with 16 sub-array blocks per slide, each sub-array containing 380 spots arranged in 20×19 matrix. Glycoconjugates were distributed into 384-well source plates using 4 replicate wells per sample and 8 µl per well. Each glycoconjugate was diluted into 100 µM in an optimized printing buffer (300 mM phosphate buffer, pH 8.4). To monitor printing, ChromPure human IgG whole molecule (at 20 ng/µl in PBS pH 7.4, 10% glycerol) and AlexaFlour-555-Hydraside (at 2 ng/µl in 178 mM phosphate buffer, pH 5.5) were used for each sub-array. The arrays were printed with four 946MP3 pins (5 µm tip, 0.25 µl sample channel, ~100 µm spot diameter; Arrayit). The humidity level in the arraying chamber was maintained at ~70% during printing. Printed slides were left on arrayer deck over-night, allowing humidity to drop to ambient levels (40–45%). Next, slides were packed, vacuum-sealed and stored at room temperature (RT) until used.

HS Glycan Microarray Binding Assay. Slides were developed and analyzed as previously described {22549775} with some modifications. Slides were rehydrated with dH₂O and incubated for 30 min in a staining dish with 50°C pre-warmed ethanolamine (0.05 M) in Tris-HCl (0.1 M, pH 9.0), then washed with 50°C pre-warmed dH₂O. Slides were centrifuged at 200×g for five min then fitted with ProPlate™ Multi-Array 16-well slide module (Grace Biolab) to divide into the sub-arrays (blocks). Slides were washed with PBST (PBS, 0.1% Tween 20), aspirated and blocked with 200 µl/sub-array of blocking buffer (PBS pH 7.3, 1% w/v ovalbumin) for 1 hour at RT with gentle shaking. Next, the blocking solution was aspirated and 10 ng/µl in 100 µl/ sub-array of primary detection diluted in blocking buffer, were incubated with gentle shaking for 2 hours at RT.

Binding was detected with biotinylated-anti-FGF-1 or biotinylated-anti-FGF-2 (1 ng/µl, 100 µl/sub-array) diluted in PBS for 1 hour at RT with gentle shaking, followed by Cy-3-sterptavidin (1.5 ng/µl) detection diluted in PBS at 200 µl/sub-array, for 1 hour at RT with gentle shaking. Slides were washed 4 times with PBST, then with PBS (without Tween-20) for 10 min followed by removal from ProPlate™ Multi-Array slide module and immediately dipping in a staining dish with dH₂O for 10 min with shaking. Slide then were centrifuged at 200×g for 5 minutes and the dry slides immediately scanned.

Array Slide Processing. Processed slides were scanned and analyzed as described {28745644; 22549775} at 10 µm resolution with a Genepix 4000B microarray scanner (Molecular Devices) using 350 gain. Image analysis was carried out with Genepix Pro 7.0 analysis software (Molecular Devices). Spots were defined as circular features with a variable radius as determined by the Genepix scanning software. Local background subtraction was performed. RFU from each spot was calculated.

3.4.4 Wound Healing Assay

To investigate the wound healing study, we initially placed two well-defined silicon inserts, creating a cell-free gap, in a 24-well plate. Subsequently, NIH3T3 (2×10⁴) cells were cultured in a two-well silicon chamber overnight. Following this incubation period, the silicon inserts were removed and washed with PBS. Next, **PG@ID-1**, **PG@ID-2**, **PG@GD-1** and **PG@GD-2** were added with the **FGF-2 (50 ng/ml)** growth factor, and the mixture was incubated for 30 minutes. Afterward, the samples were washed with PBS and left for further incubation. Bright field images of the wound were captured at 8-hour, 16-hour, and 24-hour intervals to assess the percentage of wound healing.

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Chapter 4
NeoMProbe: A New Class of
Fluorescent Cellular and Tissue
Membrane Probe

Abstract

The development of a long-lasting cell and tissues membrane probes are in high demand for advancing our understanding of membrane dynamics during differentiation and disease conditions. Herein, we report that the microheterogeneity of heparan sulfate (HS) on fluorescent neo-proteoglycans backbone offer a new platform for designing membrane probes. Confocal live-cell imaging studies of cancer and normal cell lines using a panel of Cy5 fluorescently tagged neo-proteoglycans confirmed that highly sulfated HS ligand with L-iduronic acid composition (**PG@ID-6**), induce prolong expression on plasma membrane (PM) compared to low-sulfated and uronic acid counterparts. Mono- and multi-photon microscopic imaging of tissue sections with **NeoMProbe (PG@ID-6)** allowed mapping basement membrane (BM) and demonstrated staining efficacy equivalent to antibodies against BM components. Finally, *in vivo* whole-body imaging of **NeoMProbe** and subsequent tissue section imaging confirmed versatile and efficient membrane mapping by the probe.

4.1 Introduction

Plasma membrane (PM) and basement membrane (BM) are constituent of cells and tissues, playing a central tenant in a broad spectrum of physiological and pathological processes.¹ Visualizing these membranes using biomarkers offer a comprehensive overview of the local conditions of tissues and cells, including health and activity.² In recent years, there has been a significant expansion in the design of fluorescence markers aimed at targeting the PM to elucidate cellular events.³ Despite this progress, critical challenges in PM imaging remain, particularly in distinguishing the PM of various cell types at the disease conditions and maintaining marker longevity to explore PM dynamics.^{3a} Chromophores such as chromone, Nile red, BODIPY, perylene, and fluorescein isothiocyanate, have been modified with zwitterion and amphiphilic groups, to developed for PM staining.⁴ However, these probes internalized within short time interval, allowing poor information on PM dynamics.⁵ On the other hand, the basement membrane (BM) visualization using synthetic probe is extremely challenging, as the environment is more complex and heterogeneous, resulting in non-specific staining. Hence, fluorescent tagged antibodies directed at BM components have been extensively used.⁶ Overall, a new model is required to design synthetic membrane probes that are effective not only at the cellular level but also for *ex vivo* and *in vivo* membrane mapping. Herein, we report the design of a novel class of membrane probes that exploit the function of proteoglycan to promote membrane anchoring and image.

Proteoglycans (PG) are the combination of proteins and glycosaminoglycans (GAGs), where the protein ectodomains anchored highly-sulfated GAGs including heparan sulfate (HS) and ubiquitously expressed on cell membrane and extra-cellular matrix.⁷ Consequently, HS based neo-proteoglycan (neoPG) mimics are attractive targets for glycocalyx remodelling and membrane probe development.⁸ Pioneering advent in glycopolymers and glycopeptide-based neoPG have demonstrated their PM retention for longer time scale, ensuring the active growth factors signaling.⁹ A thorough understanding of the key elements controlling the membrane dynamics of neoPG could inspire new strategies for effective glycocalyx remodelling and novel membrane probe development. To this end, we synthesized a panel of Cy5-tagged neoPG to study the structural heterogeneity of HS altering the membrane persistence and differentiating the cell types at tissue slices. We employed a single, multi-photon imaging techniques and whole-body imaging studies to show the compatibility of these membrane probes.

4.2 Result and Discussion

Synthesis of structurally well-defined neoPG mimetics were initiated by solid-phase peptide synthesis and conjugation of HS disaccharide ligands, and cholesterol moiety was carried out by copper-free click reaction. A library of HS disaccharides was synthesized by modifying the previously document-ed protocols.¹⁰ In the HS disaccharide library, ID-1 to -6 represent L-iduronic acid domain HS ligands, while compounds GD-1 to -5 correspond to D-glucuronic acid-based HS ligands (**Fig. 1**). Compounds ID-1 to -2 and GD-1 to -3 are composed of either 6-O or 3-O-sulfated or non-sulfated N-acetate glucosamine derivatives. For comparison, we synthesized highly sulfated N-sulfated ID-3 to -6 and GD-4 to -5 ligands. These HS ligands were conjugated on 12-mer peptide back-bone carrying cholesterol and Cy5-fluorescent moiety. Purity, monodispersity of the neoPG mimetics were characterized by NMR, mass spectroscopy and HPLC techniques. The photo-physical properties of PGs mimetics were investigated in water at room temperature. All complexes showed characteristic peaks of Cy-5 with 5 - 6 nm bathochromic shift compared to the Cy-5 as such, indicating the glycoconjugation (**Table 1 & 2**).

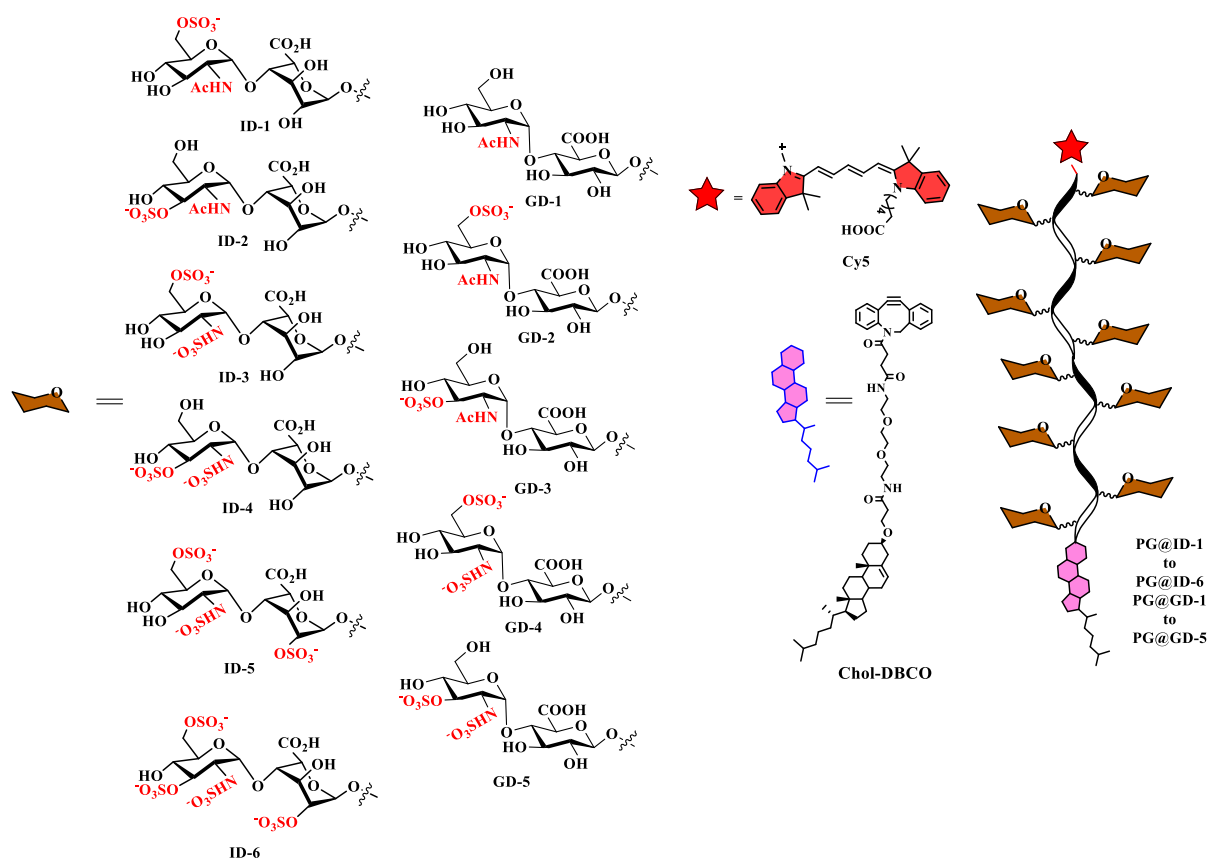


Figure 1. Proteoglycans (PGs) mimetics compositions and schematic representation. (Further **PG@ID-6** will be termed as **NeoMProbe**)

We next examined the PM dynamics of PGs mimetics using cancer cells MDA-MB-468 and normal cells NIH3T3 cells, as they represent different compositions of anionic glycans in the glycocalyx and such disparity may induce anionic repulsion with PGs and alter the assemble on PM.¹¹ A solution of PG mimetics (2 μ M) was added to the cells and washed after 30 min incubation and confocal images of all PG mimetics displayed clear decoration on the PM and some analogs showed internalization. To our surprise PG@ID-2 showed internalization within 30 mins (**Fig. 2a**), whereas remaining PG mimetics displayed intense fluorescence on PM. Among uronic acid compositions, L-iduronic acid based HSPG mimetics showed significant PM fluorescence compared to D-glucuronic acid counterparts. Notably, heavily sulfated L-iduronic acid-based PGs (PG@ID-5 and PG@ID-6) exhibited intense PM fluorescent compared to non-fluorescent or low sulfated HSPGs (**Fig. 2c**).

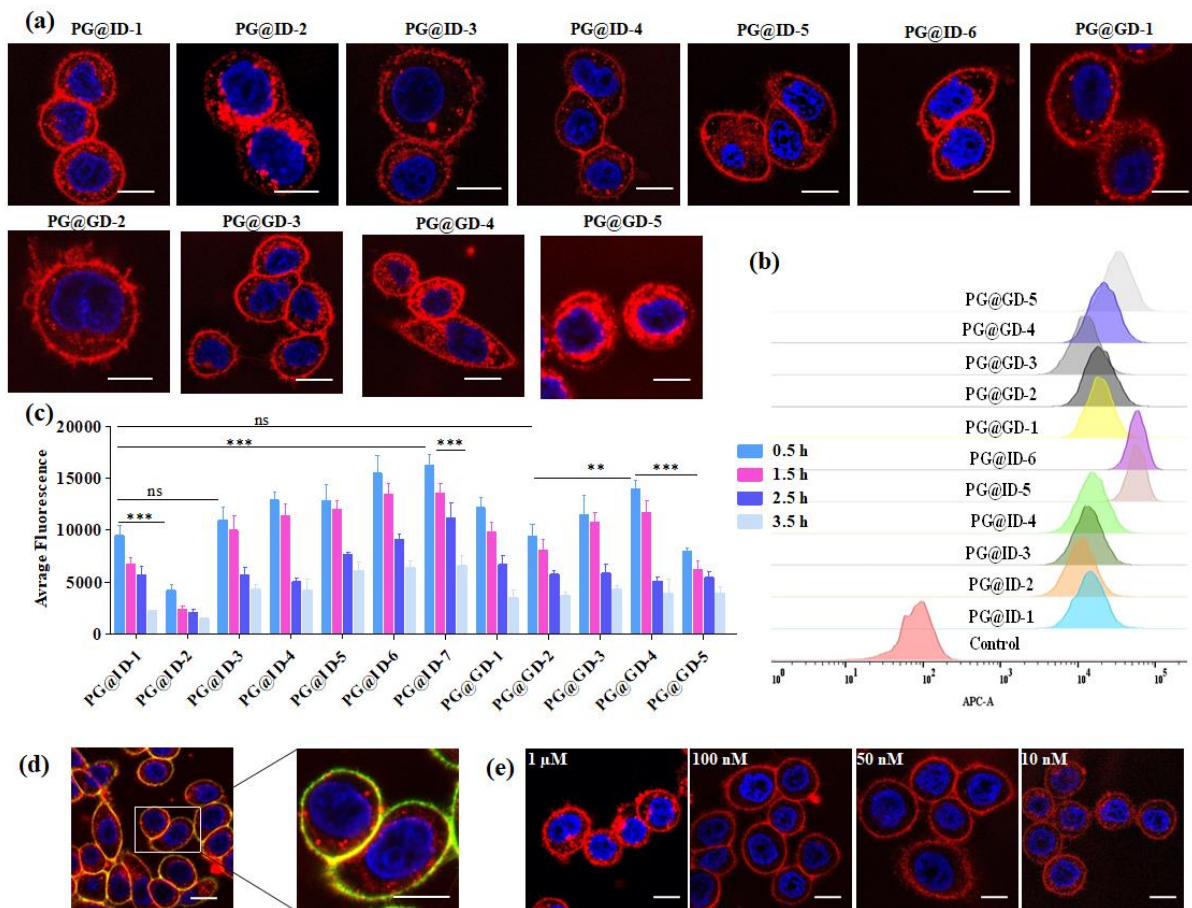


Figure 2. (a) Confocal imaging of proteoglycan mimetics in MDA-MB468 cells after 30 mins of incubation; (b) FACS analysis of proteoglycan mimetics uptake after 1 h incubation in MDA-MB 468 cells; (c) Average surface fluorescence quantification of proteoglycan mimetics in MDA-MB 468 cells at different time interval n = 50, Asterisk indicates statistically significant differences (**p < 0.001; ***p < 0.0001; ns, not significant). Statistical analysis is performed using a two-tailed Student's t-test; (d) Colocalization of **PG@ID-6** with CD44 Monoclonal

antibody-FITC cell membrane marker in MDA-MB 468 cell line after 30 mins; (e) Confocal images of **PG@ID-6** at different concentration (scale bar 10 μ m).

These results clearly illustrated that microheterogeneity of HS fine-tune the PM dynamics of neoPG mimetics. FACS measurement after 1 h further reiterates structural heterogeneity of HS modulating the PM expression level (**Fig. 2b**). Next, we determined PM dynamics of PGs mimetics by live imaging studies at different time intervals. All compounds undergo 50-90% endocytosis after 3.5 h incubation in MDA-MB-468. **Among them, PG@ID-6, named as NeoMProbe** showed slow internalization process compared to other analogs and also maximum expression (**Fig. 2c, 6 & 7**).

Unlike cancer cells, NIH-3T3 normal cells showed faster internalization process (**Fig. 8 and 9**), illustrating the glycocalyx composition significantly alter the PM dynamics of PGs mimetics. Our data also suggested that the endocytosis process of **NeoMProbe** is neither clatherin nor caveolin-dependent (**Fig. 14**). Further, heparinase and periodate treated cancer cells, significant alter the PM dynamics of **NeoMProbe** (**Fig. 15**), suggesting the anionic composition of glycocalyx-HS ligands of **NeoMProbe** interaction alters PM dynamics. Similar to commercial PM makers,¹² **NeoMProbe** stained PM of cancer cells up to 10 nM concentration, indicating the potential commercial usage of the probe (**Fig. 2e & 15 – 17**). Finally, FITC-conjugated anti-CD44 and E-cadherin co-staining experiment confirmed membrane assembly of **NeoMProbe** (**Fig. 2d & 13**).

The efficacy of the PM probe at the cellular level inspired us to extend our testing to tissue membrane using **NeoMProbe**. Accordingly, we prepared 1-mm thick tissue sections from mouse liver, kidney, heart, lung, and brain, and stained them with **NeoMProbe** for 1 hour and washed before imaging (**Fig. 16 – 19, Fig. 3 & 4**).

Previous studies have indicated that lipophilic PM markers such as DiI, DiD, and PKH26 often suffer from poor water solubility, resulting in uneven labelling of tissue membranes.¹¹ In contrast, the zwitterion-based probes such as MemBright series have successfully stained liver hepatocyte membranes and primary neuronal cultures.¹³ However, probing BM and PM in tissues and animals necessitates a specific modification for appropriate distribution across tissue membranes. We hypothesized that the neoPG backbone could modify the properties of chromophores and enhance BM/PM staining in tissues and animals. To our surprise, the kidney slices exhibited exceptional staining of BM in both the tubular and glomerulus regions (**Fig. 3a-c**). In the glomerulus region, **NeoMProbe** distinctively marked the Bowman's capsule and

glomeruli, differentiating them from Bowman's space (**Fig. 3c**). With-in the Bowman's capsule, the **NeoMProbe** notably stained the BM closely associated to epithelial cell membrane. In the glomerular region, the double immunostaining with FITC anti-E-cadherin and **NeoMProbe** displayed staining of BM between the podocytes and endothelial cell lines (**Fig. 3e**). In the tubular region, **NeoMProbe** provided strong staining of the BM of distal and proximal tubules, which was further confirmed by E-cadherin co-staining (**Fig. 3d**). Upon closer inspection at multi-photon 3D-images, the staining was primarily observed on the BM close to proximal tubular epithelial cell membrane and brush border. These results demonstrate that the proteoglycan backbone significantly modulate fluorescent staining property at the tissue level. Moreover, these results are comparable to the kidney section staining of BM with immunostaining of $\alpha 3$ subunit of collagen VI in the literature.¹⁴ Overall, **NeoMProbe** serves as a potential marker for kidney BM, with future prospective applications in the study of anti-glomerular basement membrane diseases. Similarly, liver tissue staining with **NeoMProbe** exhibited preferential staining of sinusoidal walls within the sinusoidal lumen and didn't stained PM of hepatocyte cell line, suggesting its potential of differentiating membrane in a heterogeneous liver system (**Fig. 3f-h**). Since the endothelial cell membrane and blood vessel of the sinusoidal microvesicle are closely associated, **NeoMProbe** behaves similar to cell maskTM and VE-cadherin antibodies staining the BM of sinusoidal microvesicles (**Fig. 3i**).¹⁵ In heart sections, **NeoMProbe** effectively stained the BM of cardiac muscle fibers, allowing differentiation of branching and striation junctions (**Fig. 3l**).¹⁶ Additionally, **NeoMProbe** labelled the epithelial BM of pneumocyte type I and type II cells in the alveolar regions of the lung sections (**Fig. 3l & 3k**).¹⁷

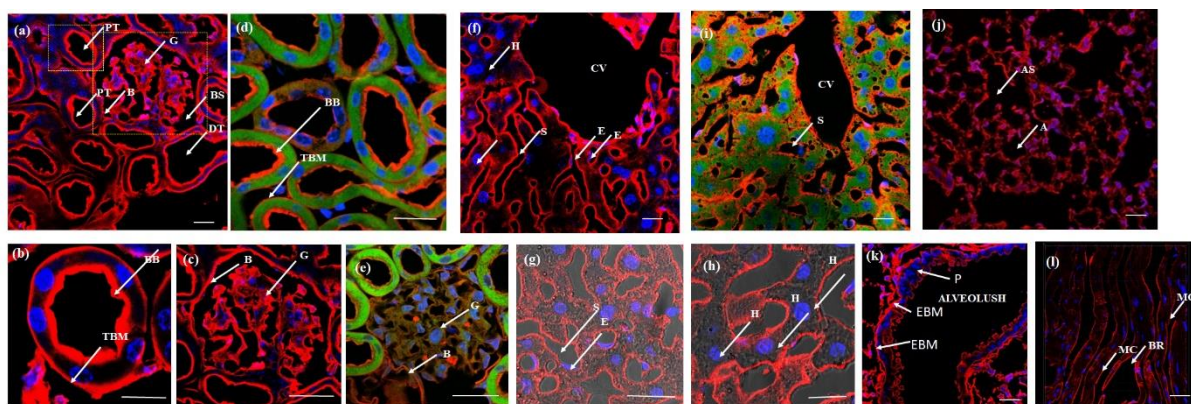


Figure 3. Tissue slides, 1- mm thick, were incubated with **NeoMProbe** and DAPI: (a) Kidney sections displayed staining of the BM of distal (DT) and proximal tubules (PT), glomerulus (G), and Bowman's capsule region. The yellow box corresponds to the zoomed region. (b)-(c) Zoomed kidney section displaying BM and brush border

staining of **NeoMProbe** (brush border (BB) and tubular basement membrane (TBM) bowman's space (BS)); (d)-(e) Double immunofluorescent images kidney slices with **NeoMProbe** (red) and FITC anti-E-cadherin (green); (f)-(h) Liver sections exhibited selective staining at the sinusoidal (S) and central vein (CV) membranes over the hepatocyte cell membrane (H) sinusoidal endothelial cells (E); (i) Double immunofluorescent images of liver slices with **NeoMProbe** (red) and FITC anti-E-cadherin (green); (j) Lung section showed marking of the BM of alveolar sacs (AS), alveoli (A) region (k) Zoomed region of alveolus, **NeoMProbe** showed staining the epithelial basement membrane (EBM) close to the pneumocyte type 1 and type II cells (P); (l) Heart sections demonstrated staining of **NeoMProbe** at the BM of close to the muscle endothelial cells (MC) and also highlight the branching (BR) region (scale bar 100 μ m).

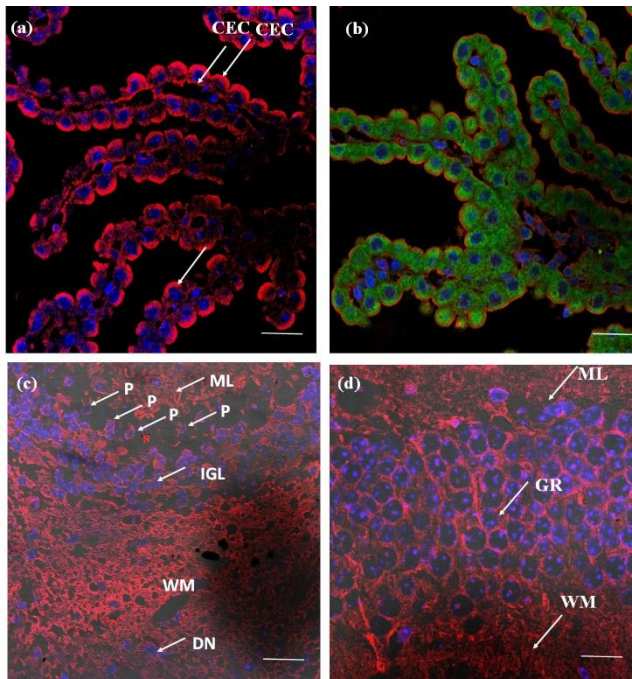


Figure 4: Brain slides, 1 mm thick, were incubated with PG@ID-6 and DAPI: (a) 100 X confocal images of 3rd ventricle region showed selective staining the cell membrane of cuboidal epithelial cells (CEC) in the choroid plexus; (b) Immunofluorescent double labelling analysis of 3rd ventricle with **NeoMProbe** and FITC anti-E-cadherin monoclonal antibody; (c) Cerebellum and (d) Hippocampal region showed differentiation among white matter (WM) granular region (GR), Purkinje layer (P), inner granular layer (IGL) and molecular layer (ML). Hippocampal region showed staining of neurite axon networks of different layers (scale bar 100 μ m).

Next, we carried out immunofluorescent analysis on sections of the brain, focusing on the cerebellum, hippocampal and third ventricle regions (**Fig. 4**). In the third ventricle section, we observed notable BM staining of the choroid plexus epithelial cell wall, distinguishing it from the stromal capillary. When immunofluorescent double labelling analyses were conducted using FITC anti-E-cadherin and **NeoMProbe**, E-cadherin was observed in a PM layer beneath the **NeoMProbe**, showing no co-localization (**Fig. 4a & b**). This clearly demonstrates that the BM and PM of epithelial cells in the third ventricle are differentiated. In cerebellar regions, **NeoMProbe** exhibited more prominent staining networks in the white matter and molecular layer compared to the granular region. It is hypothesized that **NeoMProbe** marked the myelinated climbing fibres and mossy fibres, which are extensively networked in these areas (**Fig. 4c**). In the Purkinje region, **NeoMProbe** stained the outer layer of the Purkinje cells but did not stain the axons or dendrites emanating from them. Further efforts with confocal and

multiphoton imaging did not result in better images, indicating the need for more detailed investigation. Similar trend was also observed in the hippocampal region, warranting further investigation of brain sections (**Fig. 4d**).

Inspired by the successful *ex vivo* imaging studies, we subsequently conducted *in vivo* live imaging to assess the compatibility of the probe with animals and explore its real-time applications for membrane probing. To this end, we performed BALB/c mice live imaging studies. **NeoMProbe** was injected intraperitoneal, and whole-body images were collected at different time intervals (Figure 5a & 5b). As anticipated, **NeoMProbe** demonstrated slow uptake. Within 15 minutes to 1 hour, the majority of the probe was localized in the lower part of the body, particularly in the kidneys, liver, intestines, and gastrointestinal tract (Figure 5a). After 2 hours, the entire body appeared stained with the probe, including the brain, and the staining persisted for nearly 5 and slowly cleared for next 24 hours, clearly showcasing its potential as a biomarker for whole-body imaging. When we examined tissue sections of the liver, kidney, and brain after 3 h post-injection, kidney slices showed persistent **NeoMProbe** presence in the tubular and glomerular regions (Figure 5c-e). However, their biodistribution and sequestration is quite different from the Cy-5 probe as such,¹⁸ indicating the neoPG backbone modulate Cy-5 *in vivo* activity. Unlike *ex vivo* staining, **NeoMProbe** exhibited wide biodistribution.

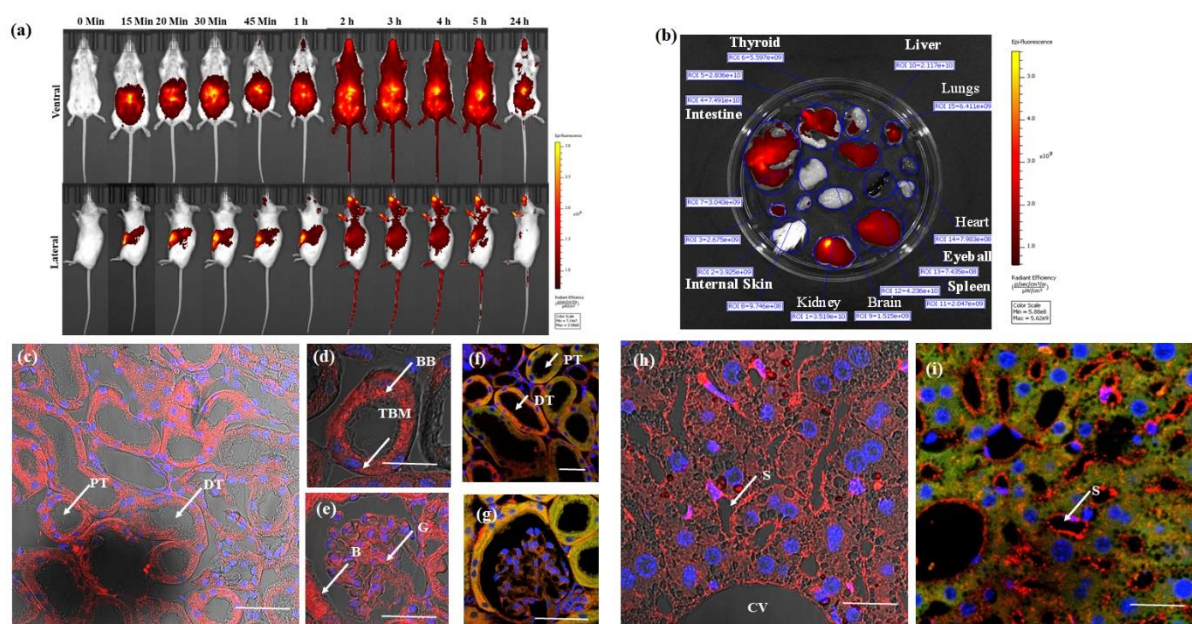


Figure 5. (a) Live animal imaging at different time interval after injecting 2 μ M of **NeoMProbe** (b) photograph of major organs collected after 3 h of intraperitoneal injection of the **NeoMProbe**. (c)-(e) mono-photon images of kidney slices after 3 h of post injection of **NeoMProbe**; (f)-(g) Double immunofluorescent staining (anti E-

cadherin (green) and **NeoMProbe** (red) of kidney slices; (h) Liver section processed after 2 h of intraperitoneal injection of **NeoMProbe**; (i) Double immunofluorescent staining of liver slices (scale bar 100 μ m).

The double immunofluorescent staining with anti E-cadherin confirmed that the **NeoMProbe** was not only staining the BM, but also on PM and other cellular compartments (**Figure 5f-g**). Similarly, liver sections also showed significant persistence on the sinusoidal membrane as well as hepatocyte cellular region (**Figure 5h-i**). However, to our surprise, we did not observe any fluorescence in the brain sections, indicating that the molecules have very poor permeability to brain tissue. It is expected that further modification of the multivalency and oligosaccharide chain length on **NeoMProbe** will increase BM specificity *in vivo*, which is currently under investigation.

4.3 Conclusion

Here, we introduced an innovative method for producing membrane probes that is compatible across cellular, tissue, and animal levels. Unlike amphiphilic and zwitterion-based fluorescent probe, these probes are designed by using proteoglycan mimetic backbone and these probes were synthesized by using peptides and heparan sulfate ligands. Confocal imaging studies of Cy-5 neoPG mimetics demonstrated that the cell membrane persistence depends on the sulfation patterns. Our results indicated that proteoglycan mimetics carrying *N*-sulfated and L-iduronic acid-based HS ligands (**PG@ID-6** or **NeoMProbe**) exhibited greater cell membrane persistence compared to those with *N*-acetylated and D-glucuronic acid derivatives. Surprisingly, 3-*O*-sulfated *N*-acetylated-based neoPG showed rapid internalization compared to other sulfation patterns. Inspired by the cellular studies, we conducted *ex vivo* imaging of **NeoMProbe**, which displayed BM staining of tissues. Notably BM in the tubular and granular region of kidney sections was selectively mapped over cell membrane decoration, while, in liver section, **NeoMProbe** stained sinusoidal over hepatocyte region. In the heart and lung section, BM on muscle cells and pneumocyte type I/II cells were stained. These results align with the BM component antibody staining. *In vivo* live imaging, followed by tissue section imaging, underscore the potential of these probes as membrane biomarkers. The system's flexible architecture permits the use of membrane probes to explore various fluorescent probes, with Cy-5 being interchangeable with any other fluorescent label. This simple exchange of fluorescent or carbohydrate epitopes will greatly streamline the process of generating collections of **NeoMProbes** and glyocalyx remodeling system.

4.4 Experimental Section

4.4.1 Physical Characterization

Standard compound used for calculation of quantum yield is Atto 655.

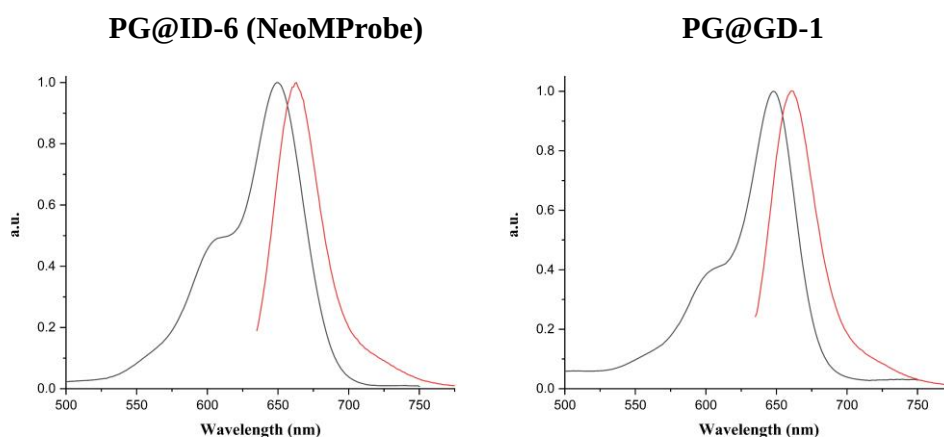


Table S1: Absorbance, emission and quantum yield of L-Iduronic acid-based molecules

S.No.	Compound	Absorbance (nm)	Emission (nm)	Quantum Yield
1.	PG@ID-1	648	660	22.8
2.	PG@ID-2	649	661	21.9
3.	PG@ID-3	649	662	22.3
4.	PG@ID-4	650	663	22.8
5.	PG@ID-5	648	662	22.2
6.	PG@ID-6	650	665	22.8

Table S2: Absorbance, emission and quantum yield of D-Glucuronic acid-based molecules

S.No.	Compound	Absorbance (nm)	Emission (nm)	Quantum Yield
1.	PG@GD-1	649	658	21.8
2.	PG@GD-2	648	660	22.0
3.	PG@GD-3	649	662	22.3
4.	PG@GD-4	650	664	22.1
5.	PG@GD-5	651	663	22.5

4.4.2 Cell Lines

MDA-MB-468 and NIH3T3 cells were cultured at 37 °C in a 5% CO₂ atmosphere in DMEM media supplemented with 10% fetal bovine serum (FBS) and 0.1% streptomycin.

4.4.3 Confocal Imaging Study

A total of 2×10^4 cells were seeded onto 8-well confocal imaging plates and incubated at 37 °C overnight. Subsequently, the cells were treated with **PG@ID-1**, **PG@ID-2**, **PG@ID-3**,

PG@ID-4, PG@ID-5, PG@ID-6, PG@GD-1, PG@GD-2, PG@GD-3, PG@GD-4 and **PG@GD-5** (2 μ M) for 30 minutes and for **PG@ID-6 (NeoMProbe)** we use different concentration from 10nM, 50 nM, 100 nM, 500 nM, 1 μ M & 2 μ M. Following the treatment, the cells underwent three washes with PBS, and the nuclei were stained with Hoechst 33342. After the staining process, the media was added, and fluorescent images were captured using a Leica SP8 (*excitation 633 nm and emission = 650 – 720 nm*) and Zeiss Multiphoton (*excitation = 485 nm and emission = 650 – 700 nm*) microscope.

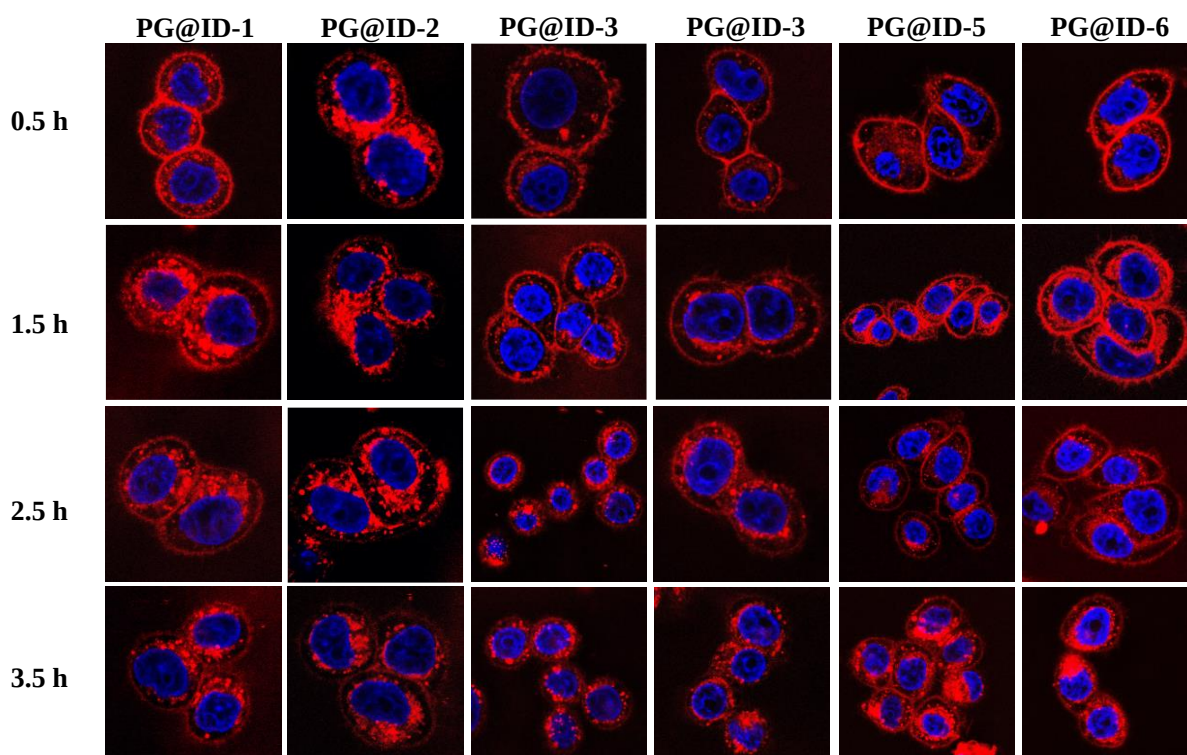


Fig. 6: Time dependent confocal images of MDA-MB-468 treated with 2 μ M of PG@ID's library after 30 min of incubation

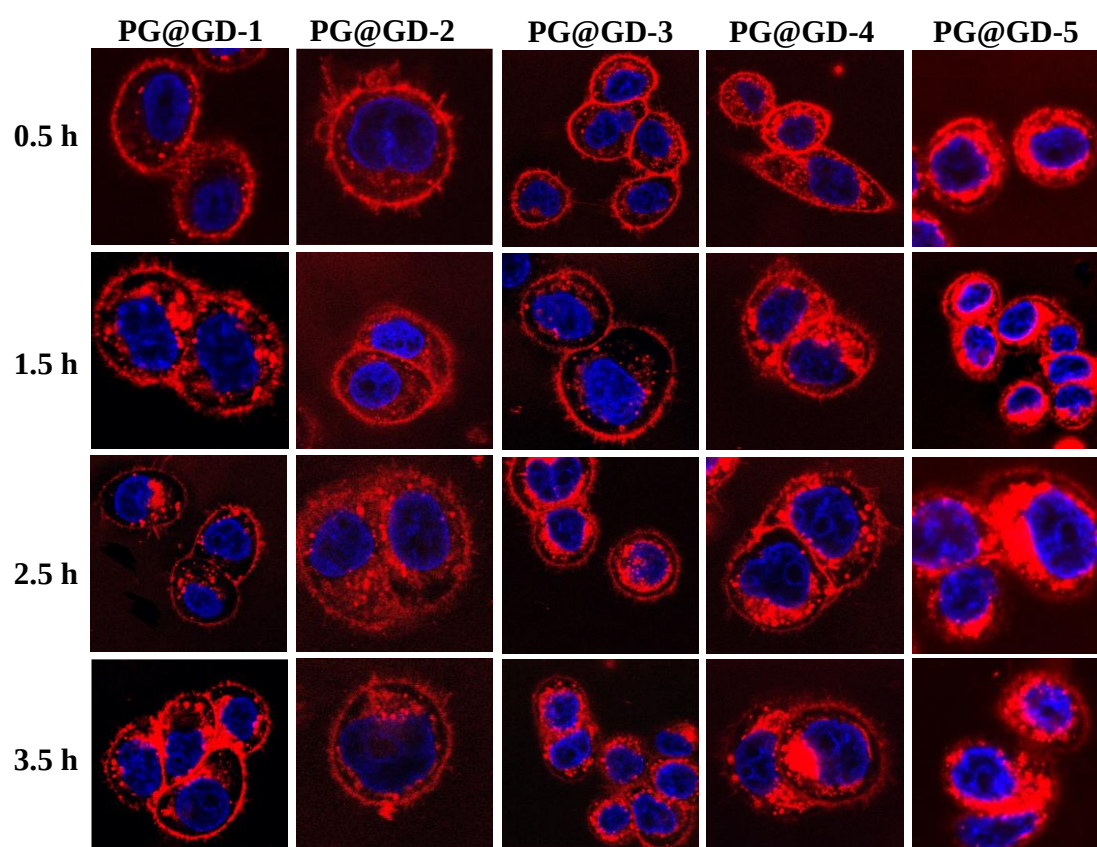


Fig. 7: Time dependent confocal images of MDA-MB-468 using 2 μ M of PG@GD's library after 30 min of incubation

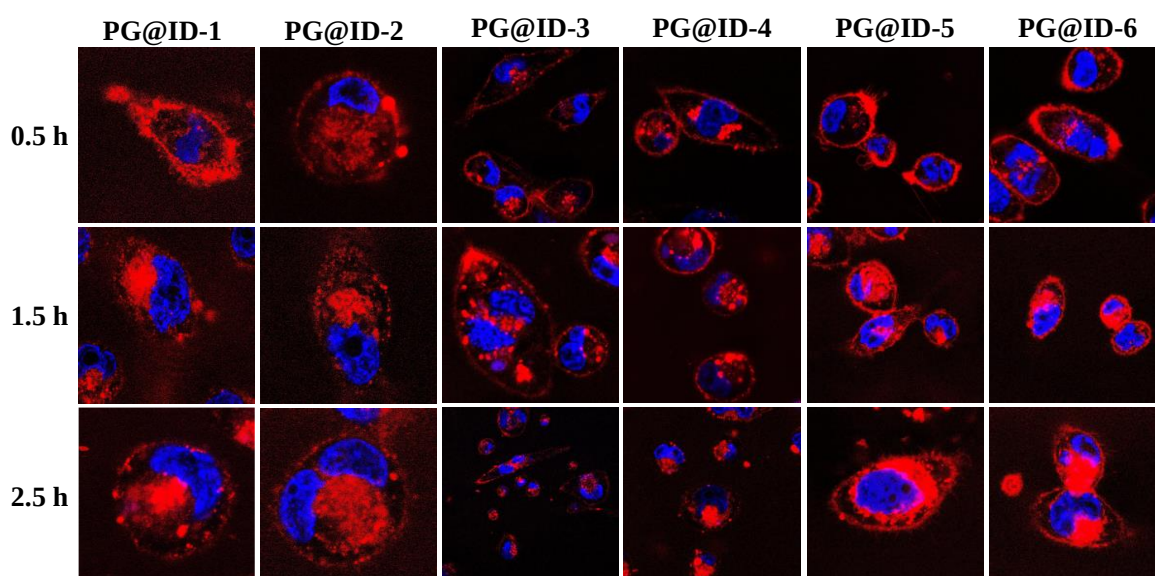


Fig. 8: Time dependent confocal images of NIH3T3 treated with 2 μ M of PG@ID's library after 30 min of incubation

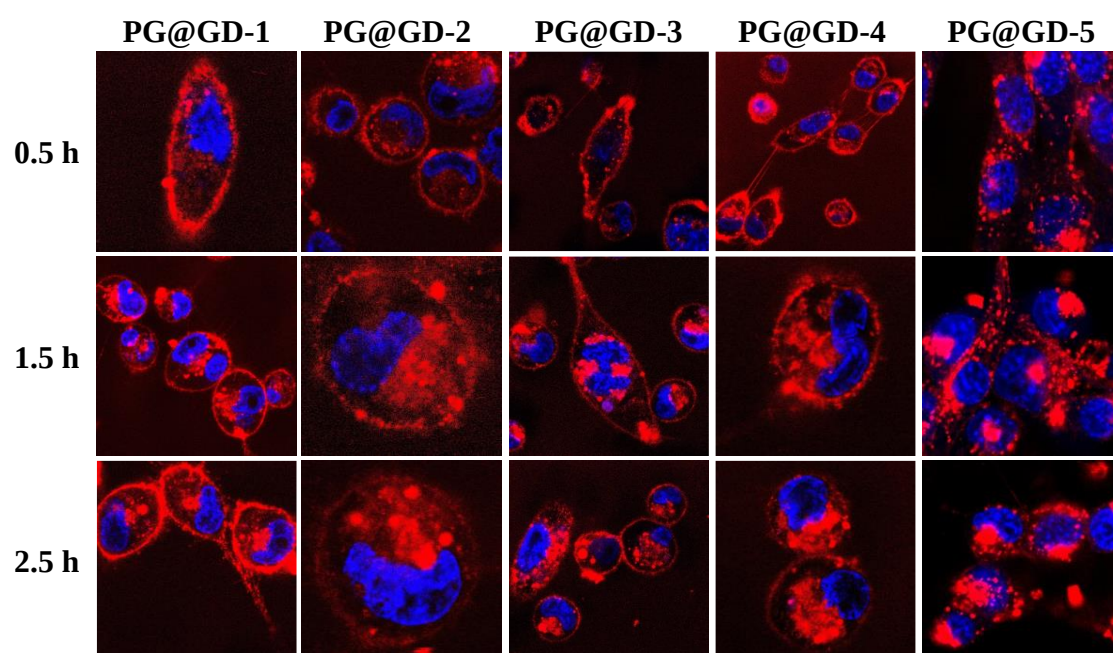


Fig. 9: Time dependent confocal images of NIH3T3 treated with 2 μ M of PG@GD's library after 30 min of incubation

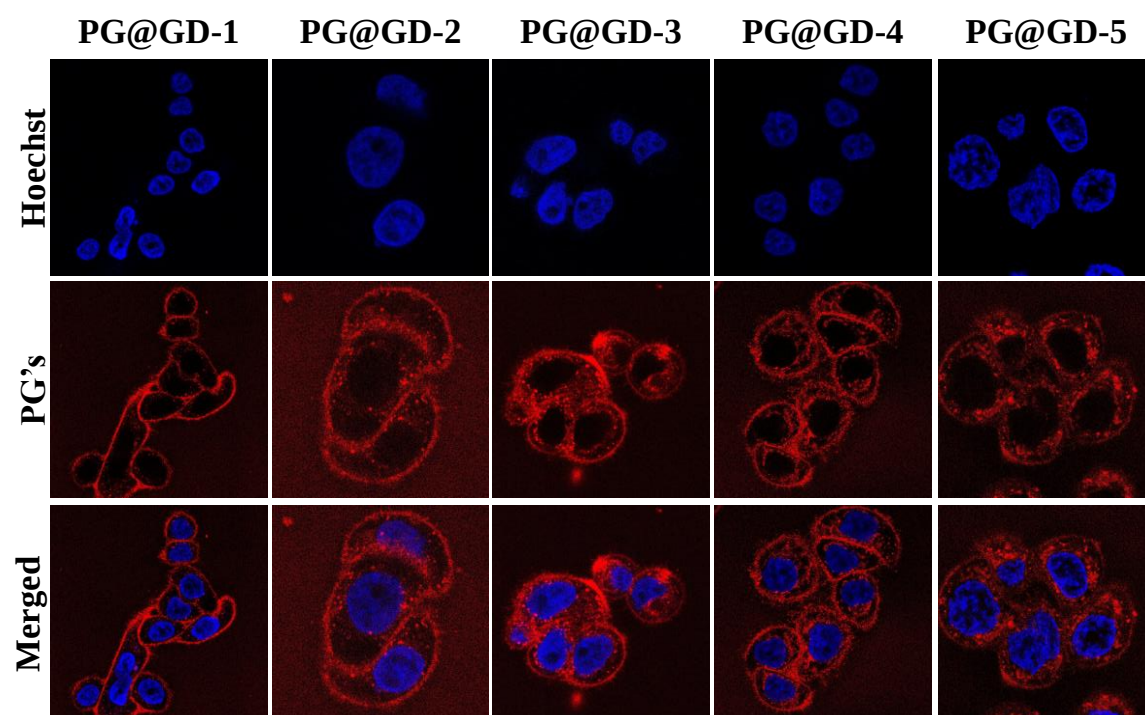


Fig. 10: Confocal images of MDA-MB-468 treated with 500 nM of PG@GD's library after 30 min of incubation

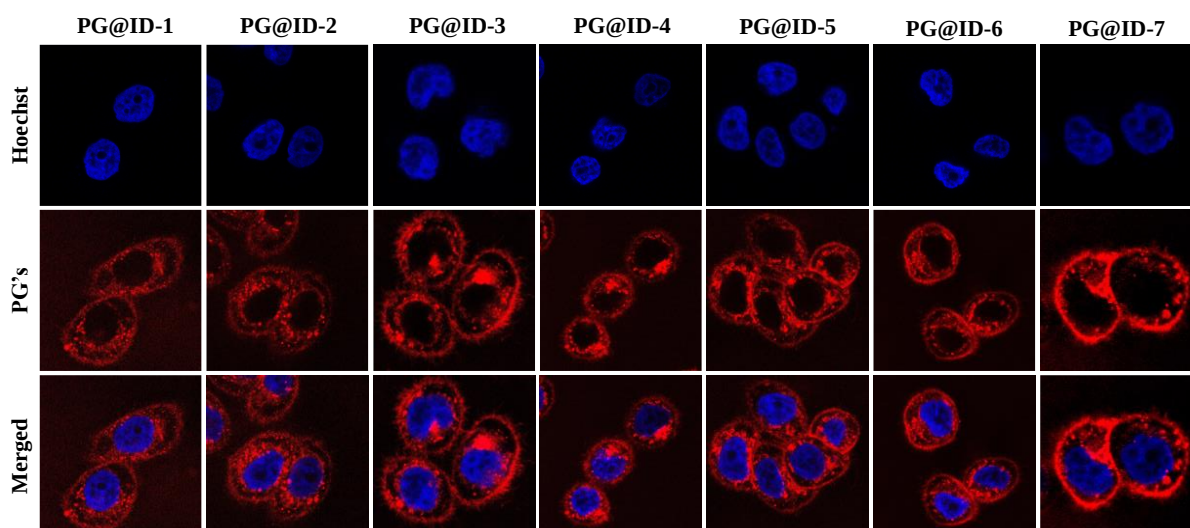


Fig. 11: Confocal images of MDA-MB-468 treated with 500 nM of PG@GD's library after 30min of incubation

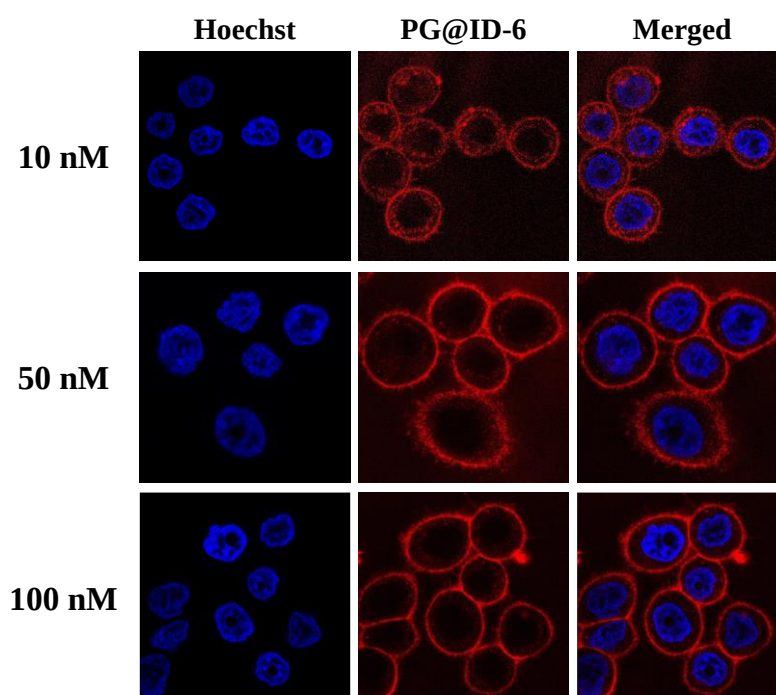


Fig. 12: Confocal images of MDA-MB-468 treated with PG@ID- 6 (**NeoMProbe**) from 10 nM to 100 nM concentration after 30 min of incubation

4.4.4 Co-Localization Assay in Cellular Level

To study the cellular co-localization of **NeoMProbe** (PG@ID-6), we stained MDA-MB 468 cells grown overnight in an 8-well plate (1×10^4 cells per well). The cells were treated with 2 μ M **NeoMProbe**, followed by treatment with different markers: (a) DiO, (b) FITC-CD44, and

(c) FITC-E-Cadherin antibody for 30 minutes at 37 °C. After that, the nuclei were stained with Hoechst for 10 minutes, and washing with PBS 2 times, and live cell imaging was performed.

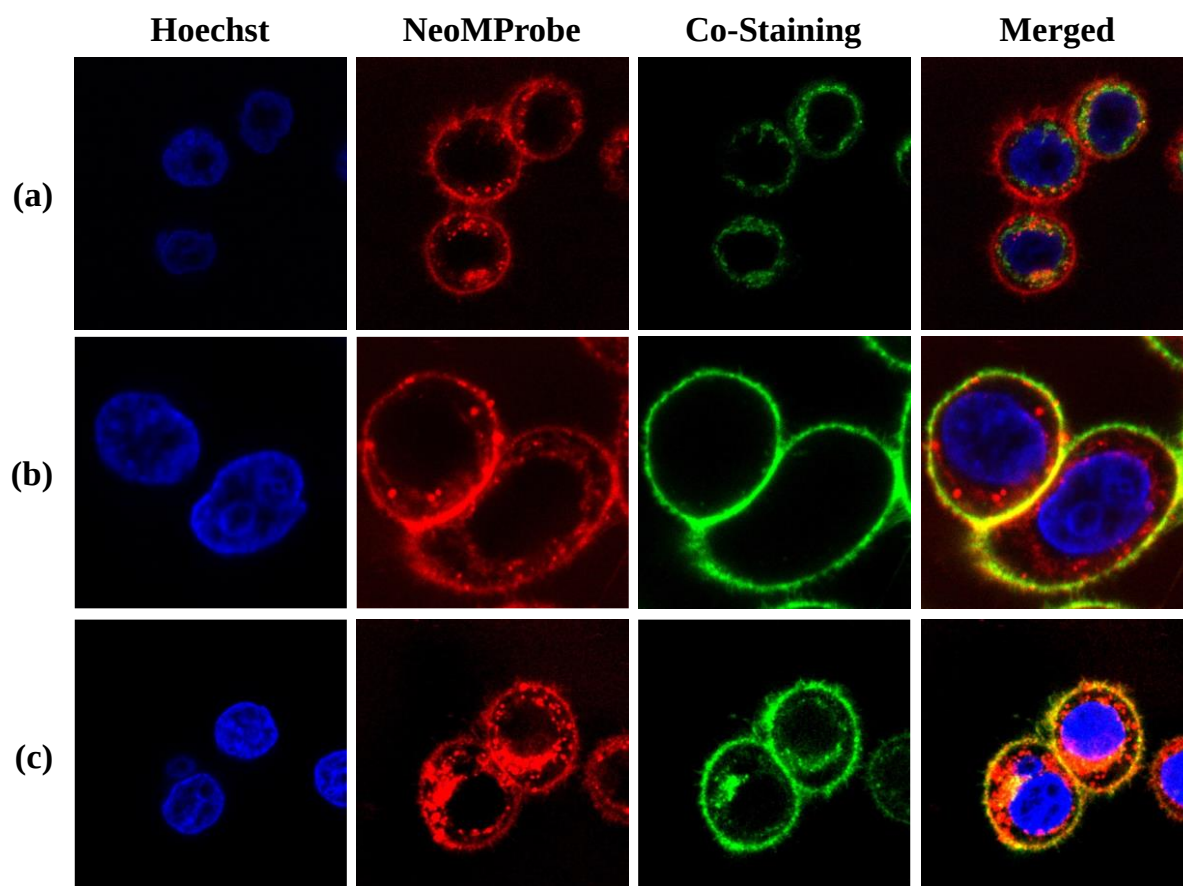


Fig. 13: Co-staining confocal imaging of MDA-MB-468 treated with **NeoMProbe** (PG@ID- 6) and (a) DiO (b) FITC-CD44 antibody and (c) FITC-E-cadherin antibody

4.4.5 Cellular Uptake Mechanism

To explore the cellular internalization mechanism, MDA-MB 468 cells were cultured overnight in an 8-well chamber plate (2×10^4 cells per well). They were then subjected to a specific inhibitor for 30 minutes, followed by a 30-minute treatment with **PG@ID-6 (NeoMProbe)** at 37 °C. For the investigation of energy-dependent, dynamin-mediated, clathrin-mediated, and caveolae-mediated processes, cells were exposed to NaN₃ (50 mM), dynasore hydrate (50 μM), chlorpromazine (25 μM), and methylated-β-cyclodextrin (10 mM) for 30 minutes, respectively. Following the 30-minute treatment with **PG@ID-6 (NeoMProbe)**, cells underwent a thorough wash to remove unbound materials and were subsequently stained with Hoechst 33342 before proceeding to imaging.

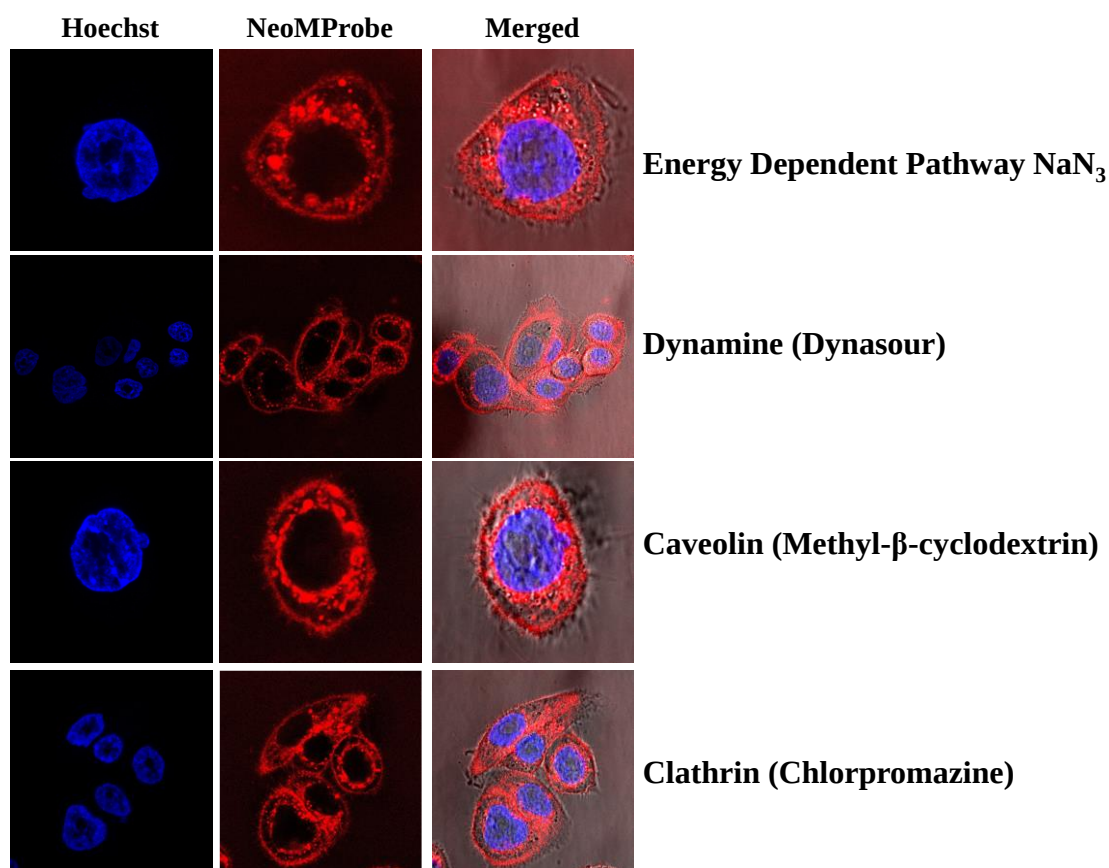


Fig. 14: Confocal imaging studies of uptake mechanism using **NeoMProbe** on MDA-MB-468 cell line

4.4.6 Heparinase and Per-Iodide Treated Assay

To investigate the glycocalyx anionic interaction modulating the activity, we first treated the cell with heparinase (0.03 IU/ml) for 1h at 37 °C, followed by sodium periodate (1 mM) for 30 min at 4 °C respectively. Next cells incubated with **PG@ID-6 (NeoMProbe)** at 2 μM concentration. Our results showed no such significant difference in the activity as compared to untreated cells, indicating the glycocalyx anions-HSPGs interaction is least influential.

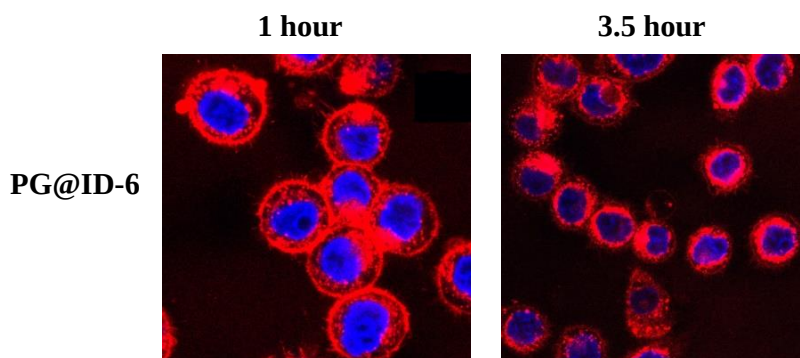


Fig. 15: Confocal imaging of **NeoMProbe** uptake assay after Heparinase and Per-iodide treatment using MDA-MB-468 cell line

4.4.7 FACS Analysis

Cells (5×10^5 cells per well) were seeded into a 24-well plate and incubated overnight. Subsequently, the cells were subjected to treatment with PG's mimetics for 1 hour. Followed by washing 3 times and re-suspended in FACS buffer for analysis.

4.4.8 Tissue Section Images

4.4.8.1 *ex vivo* Tissue Section Images

To illustrate the potential of the PG's mimetics as a tissue membrane staining, we stained the mouse tissue sections. For dewaxing of paraffin-embedded mouse tissue sections: Slides were initially heated at 60 °C for 15 minutes, then placed into glass slide holders and subjected to dewaxing as follows: Twice in 100% xylene wash for 5 minutes each, followed by immersion in 100% isopropanol with brief agitation (10–20 seconds) for 3 minutes, and finally twice in H₂O with brief agitation. For rehydration, the glass side were immersed in PBS for 30 minutes. Subsequently, tissue sections were treated with a 2 μ M and 500 nM of **PG@ID-6** for 1 hour at rt, followed by PBS washing for 3 minutes. Finally, sections were treated with Hoechst for 20 minutes at rt, followed by PBS washing 2 times for 3 minutes, and then subjected to confocal imaging.

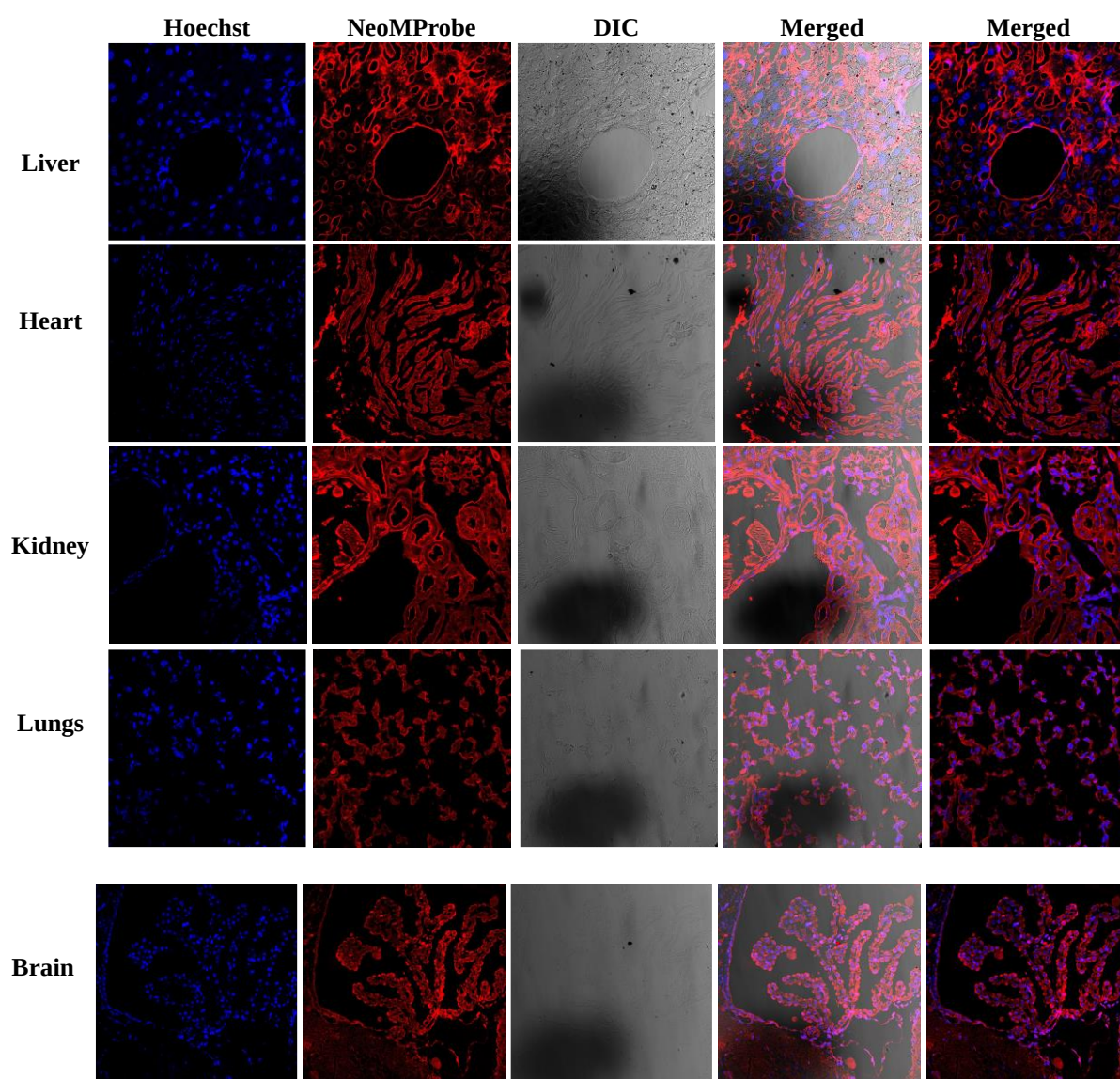
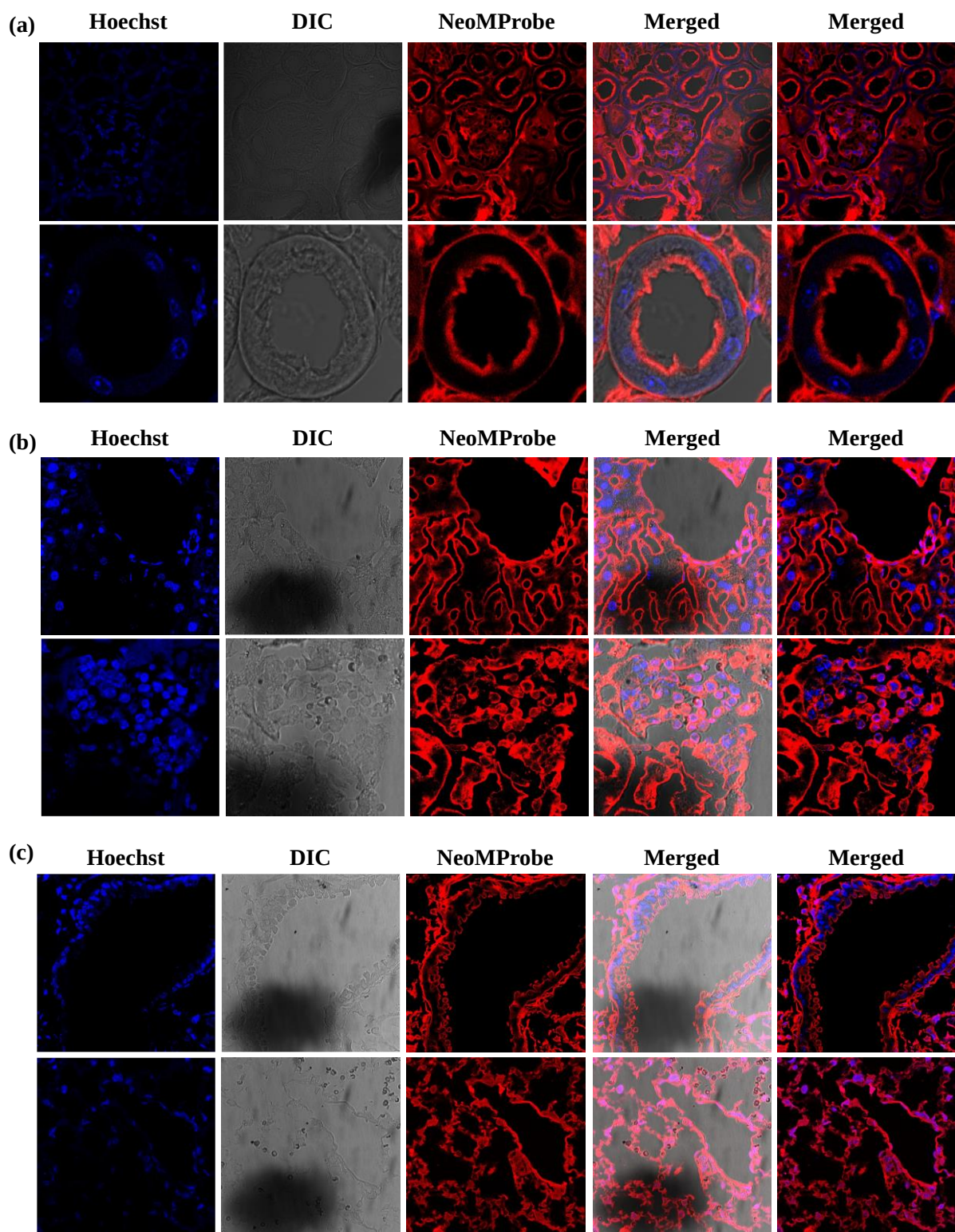


Fig. 16: Confocal images of tissue sections using **NeoMProbe@500 nM** concentration after 1 h of treatment



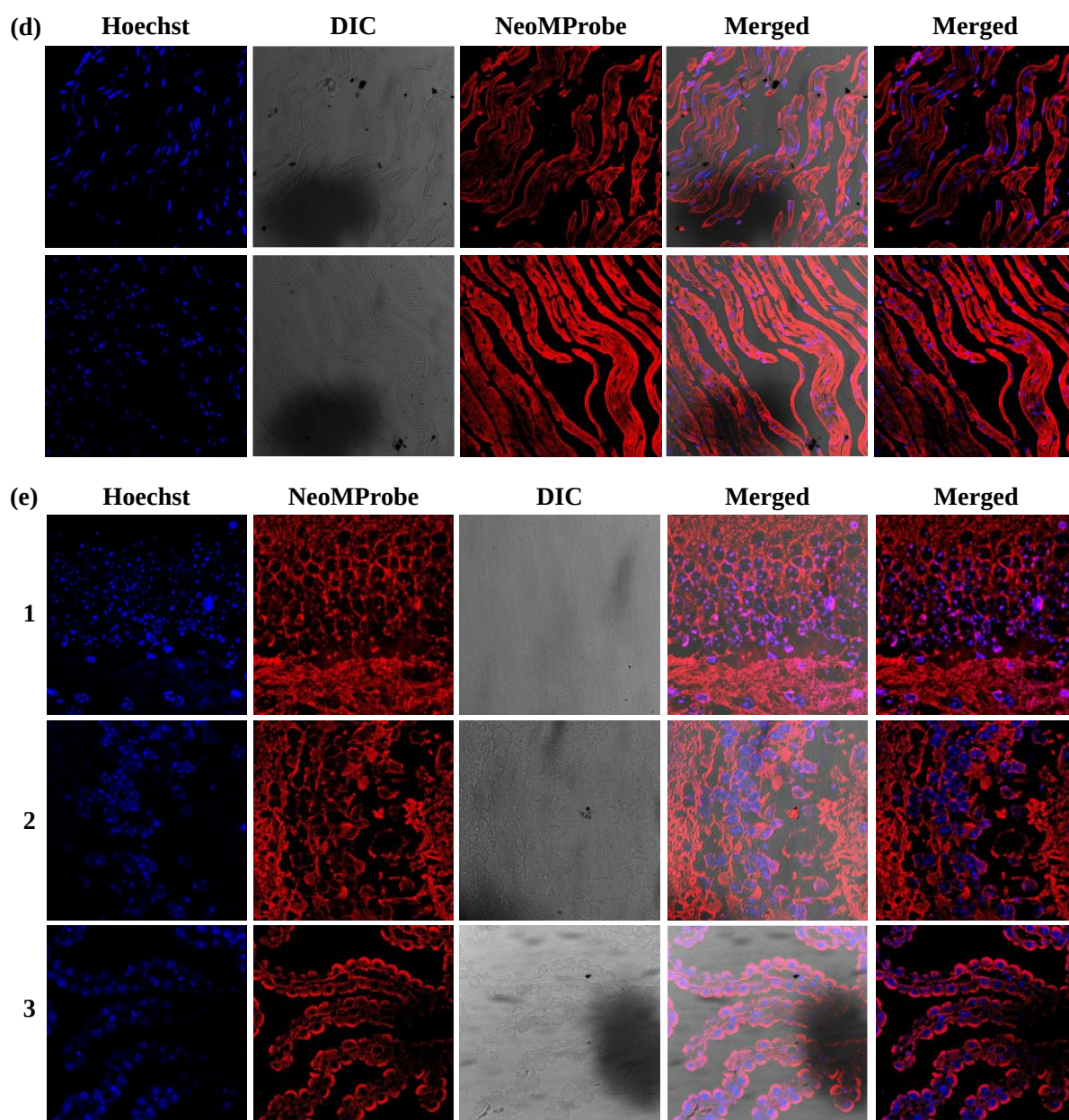


Fig. 17: Confocal imaging of tissue sections using **NeoMProbe@2 μ M** concentration after 1 h of treatment **(a)** Kidney Section **(b)** Liver Section **(c)** Lungs Section **(d)** Heart Section **(e)** Brain section **(1.** Hippocampus region **2.** Cerebellum region **3.** Third ventricle region (V3))

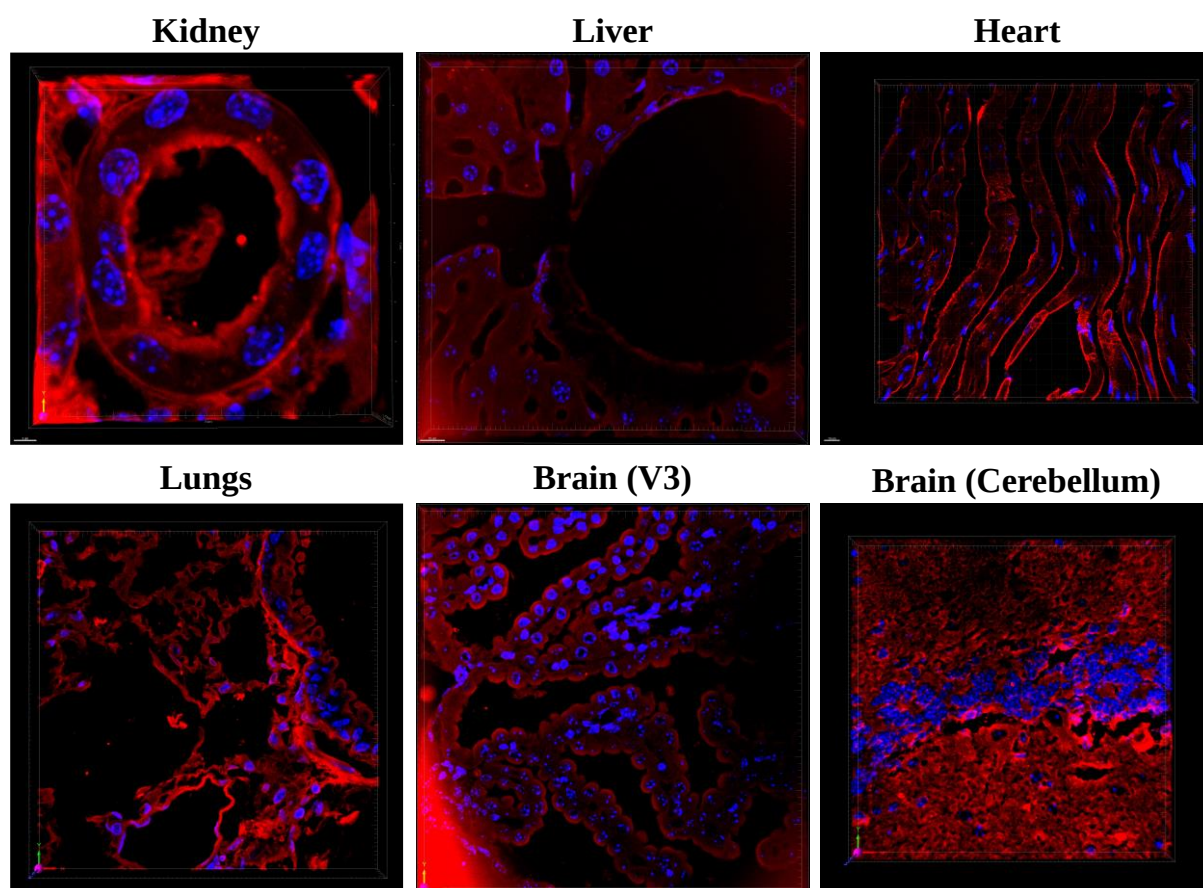


Fig. 18: Z stack confocal imaging of different tissue sections using **NeoMProbe@2** μ M concentration

4.4.8.2 *ex vivo* Tissue Section Co-Staining

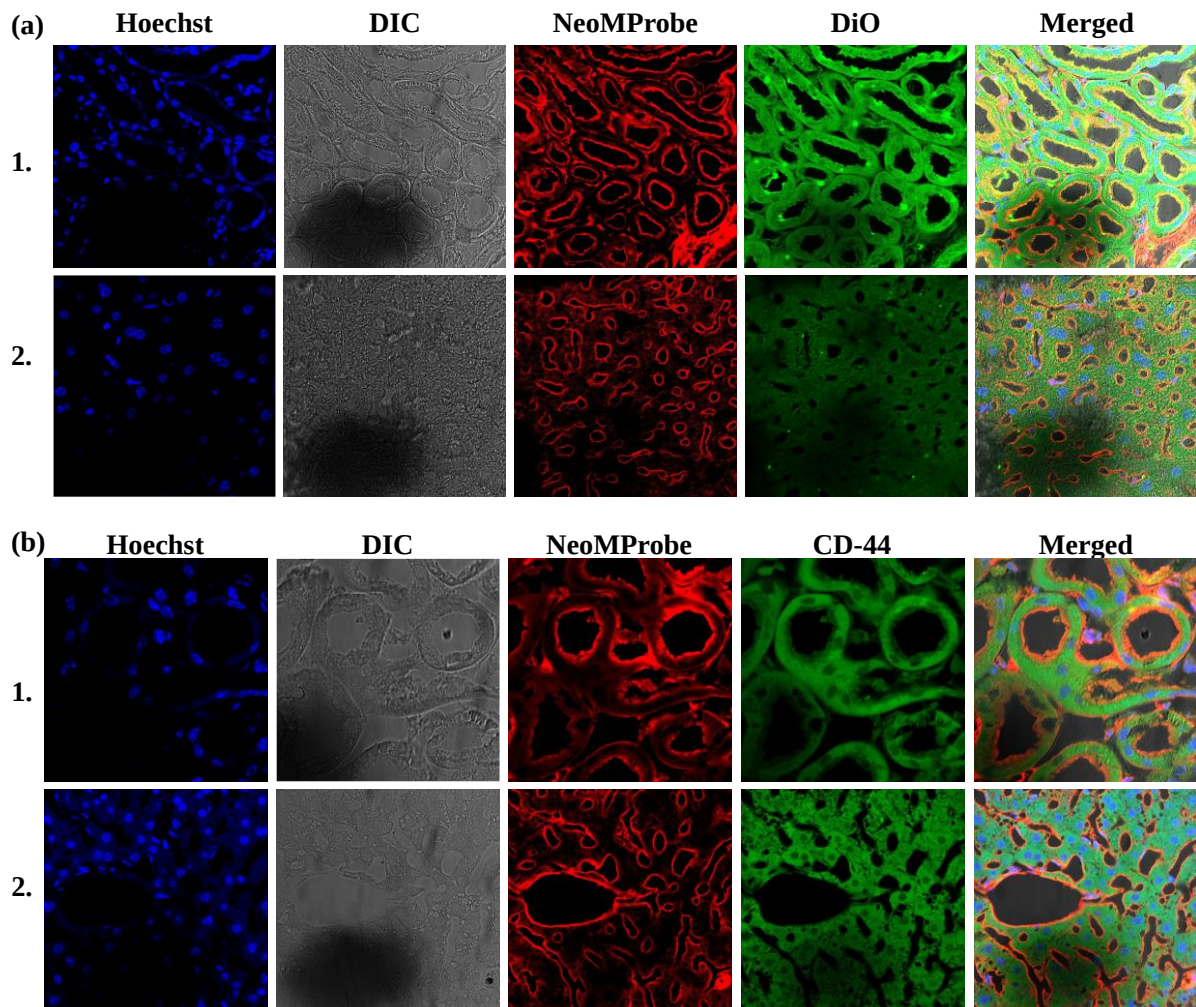
To illustrate the potential of the PG's mimetics as a tissue membrane staining, we stained the mouse tissue sections. For dewaxing of paraffin-embedded mouse tissue sections: Slides were initially heated at 60 °C for 15 minutes, then placed into glass slide holders and subjected to dewaxing as follows: Twice in 100% xylene wash for 5 minutes each, followed by immersion in 100% isopropanol with brief agitation (10–20 seconds) for 3 minutes, and finally twice in H₂O with brief agitation. For rehydration, the glass side were immersed in PBS for 30 minutes:

(a) Subsequently, tissue sections were treated with a 2 μ M of **PG@ID-6** and DiO ((3,3' - dioctadecyloxycarbocyanine, perchlorate) for 30 minutes at rt, followed by PBS washing for 3 minutes. Finally, sections were treated with Hoechst for 20 minutes at rt, followed by PBS washing 2 times for 3 minutes, and then subjected to confocal imaging.

(b) Subsequently, it was treated with 0.1 % trypsin for 1 h followed by washing with PBS for 5 min 2 times. After that permeabilization was done using mixture of PBS and 0.25 % of triton

X100 followed by washing with PBS for 5 min 2 times. Then, using 1% CD-44 anti-body in PBS staining was done for 2 h at rt followed by washing with PBS for 5 min 2 times. Finally, tissue sections were treated with a 2 μ M of **PG@ID-6** and Hoechst for 30 minutes at rt, followed by PBS washing for 3 minutes 2 times, and then subjected to confocal imaging.

(c) Subsequently, it was treated with 0.1 % trypsin for 1 h followed by washing with PBS for 5 min 2 times. After that permeabilization was done using mixture of PBS, gelatin 1:1 and 0.25 % of triton X100 followed by washing with PBS for 5 min 2 times. Then, using 1% E-cadherin anti-body in PBS staining was done for 3 h at rt followed by washing with PBS for 5 min 2 times. Finally, tissue sections were treated with a 2 μ M of **PG@ID-6** and Hoechst for 30 minutes at rt, followed by PBS washing for 3 minutes 2 times, and then subjected to confocal imaging.



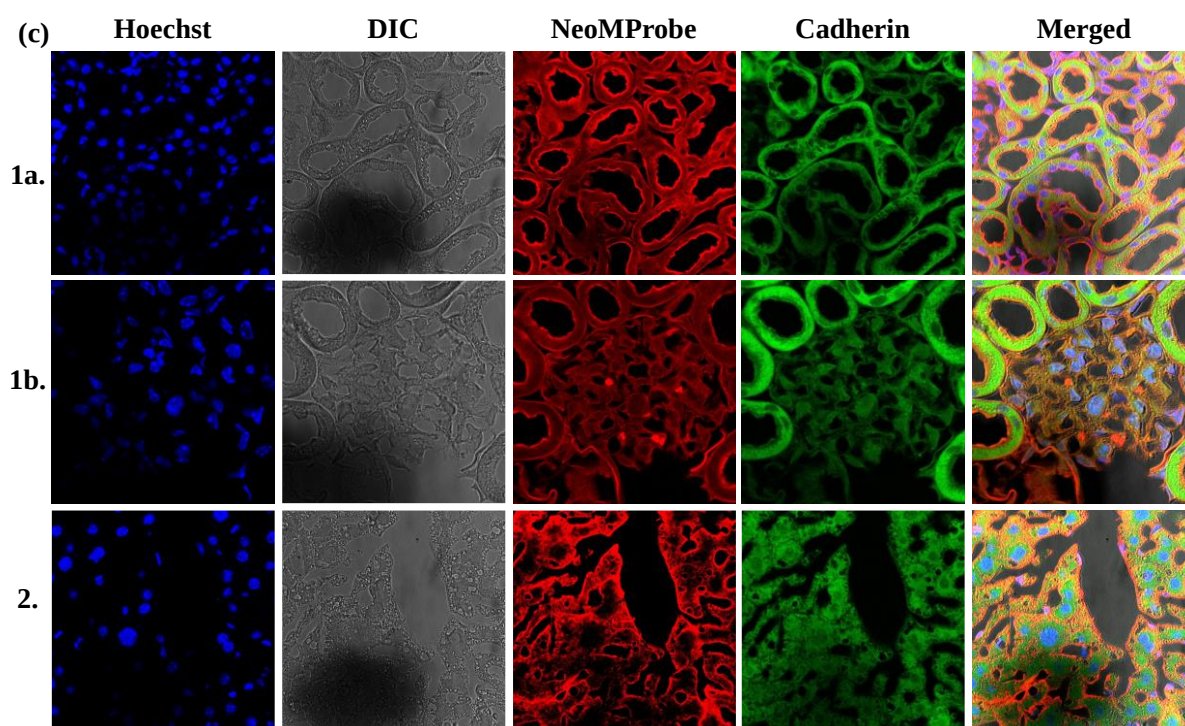


Fig. 19: Co-staining confocal imaging of kidney (**1**) and liver (**2**) using (**a**) DiO (**b**) CD-44 (**c**) E-Cadherin

4.4.9 Animal Studies

4.4.9.1 *in vivo* Studies

BALB/c 6-8 weeks old were taken from the National Facility for Gene Function in Health and Disease of IISER, Pune. Prior to experiments the mice were maintained in an animal house, with the proper amount of food and water. All experiments were performed in accordance with the relevant guidelines and regulations of the Institutional Animal Ethical Committee, set up by CPCSEA, Govt. of India. The mice ($n = 3$) were anesthetized by ketamine. After that **PG@ID-6 (NeoMProbe)** 1.8 mg/Kg were injected intraperitoneal. Then *in vivo* imaging was done using Quantum GX2 Micro-CT system (*excitation* = 640 nm and *emission* = 680 nm). First, before injection image was taken marked at 0 h, after injection at different time interval imaging was done upto 24 h. One mouse was sacrificed after 3 h. Subsequently, major organs including kidney and liver were collected and fixed with 4% paraformaldehyde. Organs were embedded in paraffin and 1 mm sections were cut using a microtome. The tissue sections were fixed on a poly- L -lysine-coated glass plates.

4.4.9.2 Post Injection *in vivo* Tissue Section

To illustrate the potential of the PG's mimetics as a *in vivo* tissue membrane stanning, we stained the mouse tissue sections. For dewaxing of paraffin-embedded mouse tissue sections: Slides were initially heated at 60 °C for 15 minutes, then placed into glass slide holders and subjected to dewaxing as follows: Twice in 100% xylene wash for 5 minutes each, followed by immersion in 100% isopropanol with brief agitation (10–20 seconds) for 3 minutes, and finally twice in H₂O with brief agitation. For rehydration, the glass side were immersed in PBS for 30 minutes. Subsequently, tissue was treated with Hoechst for 20 minutes at rt, followed by PBS washing 2 times for 3 minutes, and then subjected to confocal imaging.

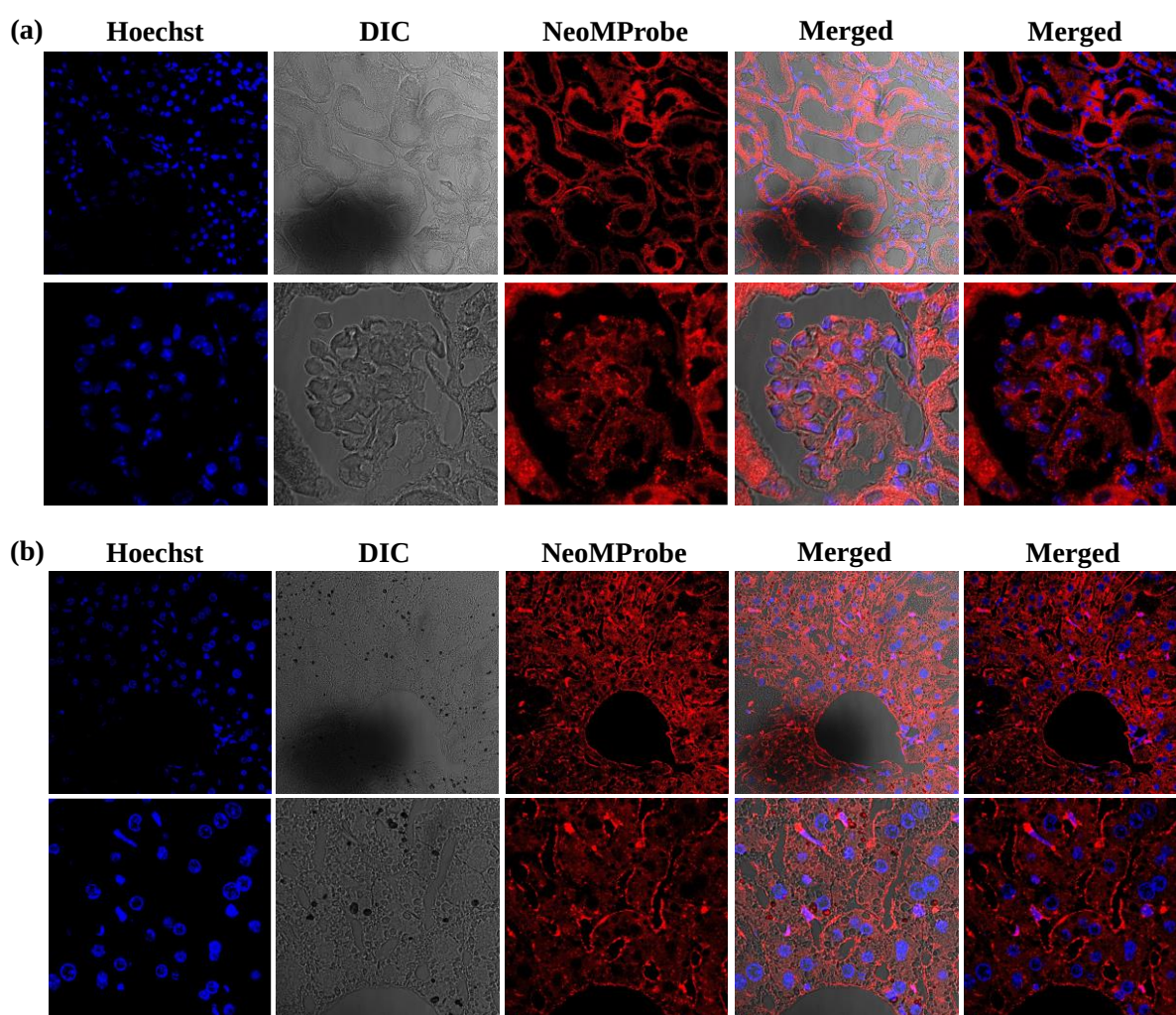


Fig. 20: Confocal imaging of tissue sections using **NeoMProbe** post injection **(a)** Kidney Section **(b)** Liver Section

4.4.9.3 Post Injection *in vivo* Tissue Section Co-Staining

To illustrate the potential of the PG's mimetics as a tissue membrane staining, we stained the mouse tissue sections. For dewaxing of paraffin-embedded mouse tissue sections: Slides were initially heated at 60 °C for 15 minutes, then placed into glass slide holders and subjected to dewaxing as follows: Twice in 100% xylene wash for 5 minutes each, followed by immersion in 100% isopropanol with brief agitation (10–20 seconds) for 3 minutes, and finally twice in H₂O with brief agitation. For rehydration, the glass side were immersed in PBS for 30 minutes. Subsequently, it was treated with 0.1 % trypsin for 1 h followed by washing with PBS for 5 min 2 times. After that permeabilization was done using mixture of PBS, gelatin 1:1 and 0.25 % of triton X100 followed by washing with PBS for 5 min 2 times. Then, using 1% E-cadherin anti-body in PBS staining was done for 3 h at rt followed by washing with PBS for 5 min 2 times. Finally, tissue sections were treated with a 2 µM of **PG@ID-6 (NeoMProbe)** and Hoechst for 30 minutes at rt, followed by PBS washing for 3 minutes 2 times, and then subjected to confocal imaging.

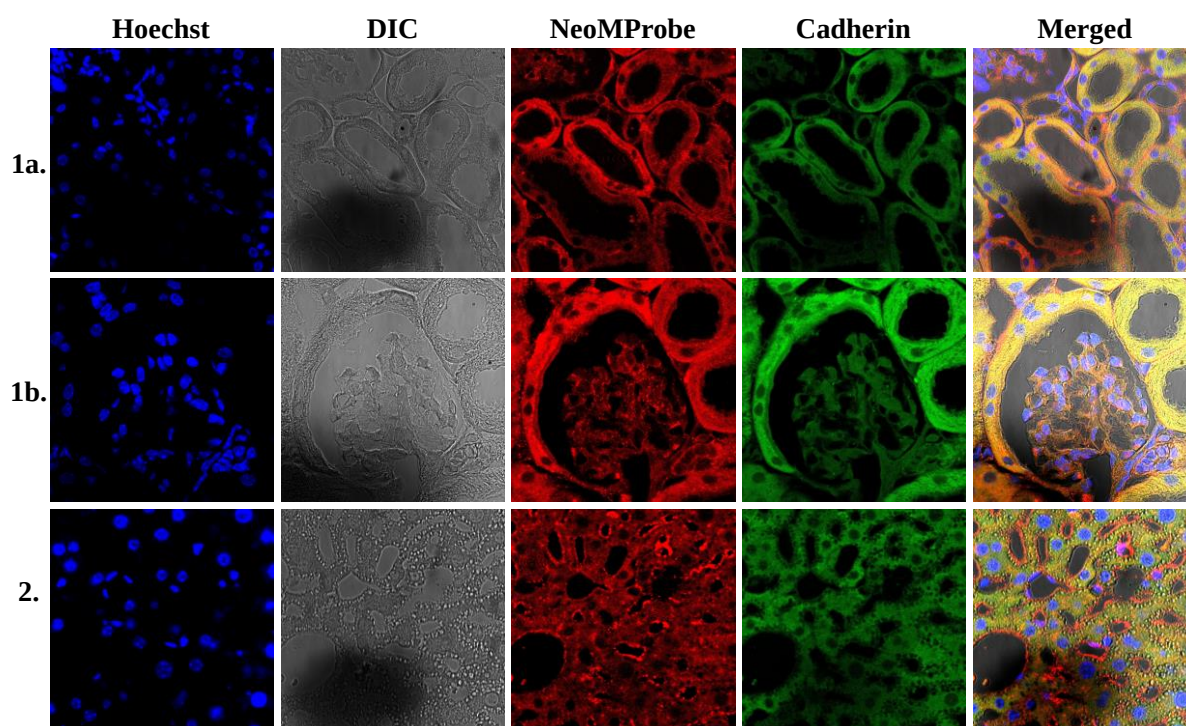


Fig. 21: Co-staining confocal imaging of kidney (**1a and 1b**) and liver (**2**) using E-Cadherin

4.5 Reference

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Chapter 5

Molecular Recognition and Proteoglycan Mimetics Arrangement: Modulating Cisplatin Toxicity

Abstract

The mammalian cell surface utilizes the extracellular matrix (ECM) and proteoglycans to capture and regulate various molecules, including drugs and toxic metal ions. Understanding the structure of the ECM, particularly heparan sulfate (HS), is crucial for developing effective therapeutic strategies. HS exhibits significant structural diversity due to varying degrees of sulfation and acetylation on its disaccharide units. Here, we have shown that cisplatin (CP) preferentially binds to sulfated uronic acids, especially L-iduronic acid (IdoA), over D-glucuronic acid (GlcA). IdoA demonstrated faster and more favourable binding. A proteoglycan mimic containing IdoA (P-I12) was synthesized and shown to modulate CP binding and cytotoxicity differently in cancer cells than normal cells, suggesting potential applications in targeted drug delivery. P-I12 enhances CP toxicity in cancer cells by facilitating its endocytosis and release in lysosomes. These findings indicate distinct modes of cell-surface engineering in normal and cancer cells, with these differences significantly impacting CP-mediated toxicity.

5.1 Introduction

The mammalian cell surface is equipped with an intricate defense mechanism mediated by the extracellular matrix (ECM) and proteoglycans, which enables the capture and regulation of various biomolecules such as proteins, drugs, neurotransmitters, and toxic metal ions.^{1,2,3,4,5} The ECM, consisting primarily of fibrous proteins and glycosaminoglycans (GAGs) like heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), and hyaluronic acid (HA),⁶ plays a pivotal role in orchestrating these biological processes.

Heparan sulfate (HS) is particularly noteworthy due to its structural complexity, characterized by variations in sulfation, acetylation, chain length, and uronic acid composition. Recent investigations underscore the significance of specific sulfation patterns and uronic acid configurations in governing drug binding interactions, underscoring the importance of decoding HS oligosaccharide-drug interactions for a comprehensive understanding of drug resistance mechanisms.^{7,8,9,10,11,12,13}

Studies by Farrell *et al.* have demonstrated that sulfated glucosamine and uronic acid residues within HS bind polynuclear platinum complexes (PCC), thereby modulating HS activity^{14,15,16} and influencing various biological phenomena such as viral entry inhibition and anti-angiogenic effects.^{16,17,18,19,20} However, the precise recognition mechanisms underlying the interaction between sulfated uronic acid residues and drug molecules, particularly cisplatin (CP), remain inadequately explored.

This study elucidates that O-sulfate and carboxylic acid residues on uronic acid sugars play a regulatory role in the binding of cisplatin. Furthermore, when sulfated-L-IdoA (a specific form of uronic acid) is conjugated to proteoglycan mimics and expressed on cell surfaces, it elicits distinct patterns of cell surface modification in cancerous and normal cells. These variations in molecular recognition and cell surface engineering collectively impact the cytotoxicity of cisplatin. Additionally, in cancer cells, proteoglycan mimics are internalized through a lysosomal pathway, suggesting a potential mechanism for drug release.

This research illuminates the intricate interplay between ECM components, drug molecules, and cellular responses, providing valuable insights into potential avenues for modulating drug activity and overcoming drug resistance in cancer therapy.

5.2 Result and Discussion

With the aim the to explore the potential of cisplatin's cytotoxicity modulated by uronic acid compositions i.e L-Iduronic Acid (I12) and D-Glucuronic Acid (G12) on the cell surface (**Fig 1**). We performed DOSY NMR studies²¹ for identifying the binding of these molecules with cisplatin and further cellular studies was performed.

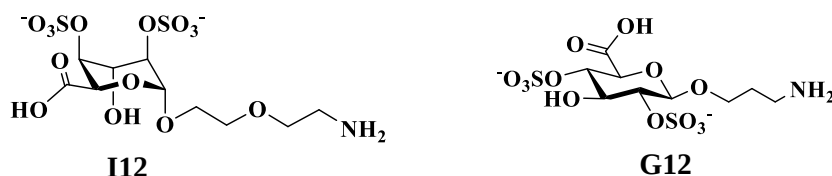
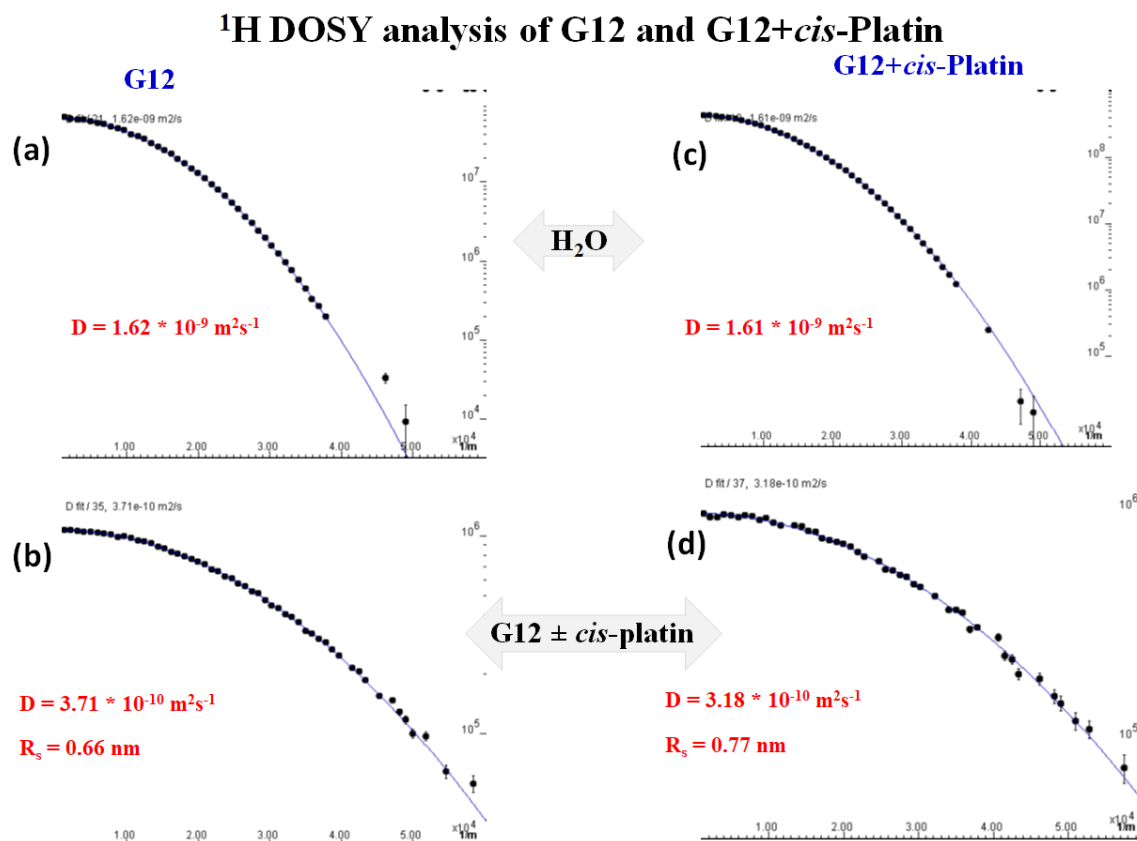


Figure 1. Structure of I12 and G12

5.2.1 NMR Studies

In this study, **G12** and **I12** were used as representative uronic acid ligands to investigate their binding to CP using NMR spectroscopy. More specifically, diffusion order spectroscopy (DOSY) and chemical shift measurement were employed to understand the complex formation.²¹ This technique is based on measuring the diffusion coefficient of the complex, which is inversely proportional to the molecular hydrodynamic radius. As CP does not have any detectable protons or carbons, the change in the rate of diffusion after the addition of CP was studied for the resonances of G12 and I12 ligands. Then DOSY spectra was obtained for G12 and I12 molecules in both conditions are qualitatively compared in **Fig. 1 and 2**. The diffusion data of G12, with and without CP, was analyzed quantitatively and compared with the diffusion rates of the residual protons of solvent D₂O. The diffusion rate (D) for water was observed to be $1.62 \times 10^{-9} \text{ m}^2\text{s}^{-1}$, and for G12, it was $37.1 \times 10^{-9} \text{ m}^2\text{s}^{-1}$ (**Fig. 1**). Interestingly, after the addition of CP, the rate of diffusion for water remained the same, but the diffusion of G12 resonance was observed to be slower ($31.8 \times 10^{-9} \text{ m}^2\text{s}^{-1}$), providing clear evidence for the interaction between G12 and CP. Using the value of D, the Stocks radius (R_s) or hydrodynamic radius for G12 was calculated to be 0.66 nm and 0.77 nm for G12+CP. The increase in R_s indicates an increased hydrodynamic radius of G12 due to interaction with CP. A similar trend was observed for I12, the diffusion rate (D) for water in I12 was observed $9.73 \times 10^{-10} \text{ m}^2\text{s}^{-1}$, and it was $2.23 \times 10^{-10} \text{ m}^2\text{s}^{-1}$ for the I12. Similarly, water rate of diffusion didn't change after addition of cisplatin but for I12 diffusion rate

decreased to $2.04 \times 10^{-10} \text{ m}^2\text{s}^{-1}$ which gives a shred of clear evidence for the interaction between I12 and cisplatin. The R_s is 1.1 nm and for I12+CP is 1.12 nm (**Fig. 2**), indicating that both ligands bind to CP.



Comparison of Rate of Diffusion

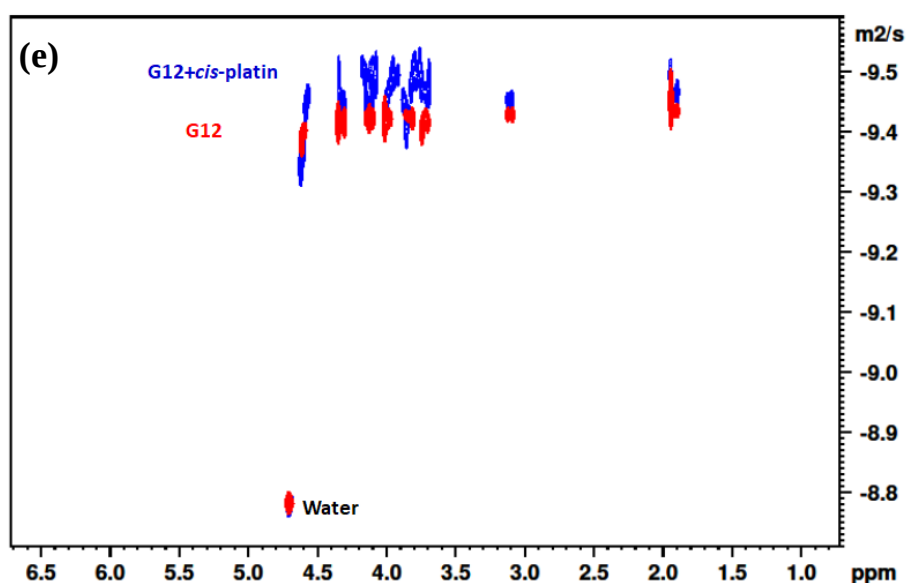


Figure 1: The quantitative analysis of diffusion data of G12 before and after the addition of cisplatin, the plot of rate of diffusion for (a) residual water in G12 (b) a randomly selected resonance of G12 (c) residual water after the addition of cisplatin and (d) G12+cisplatin (e) Comparison rate of diffusion

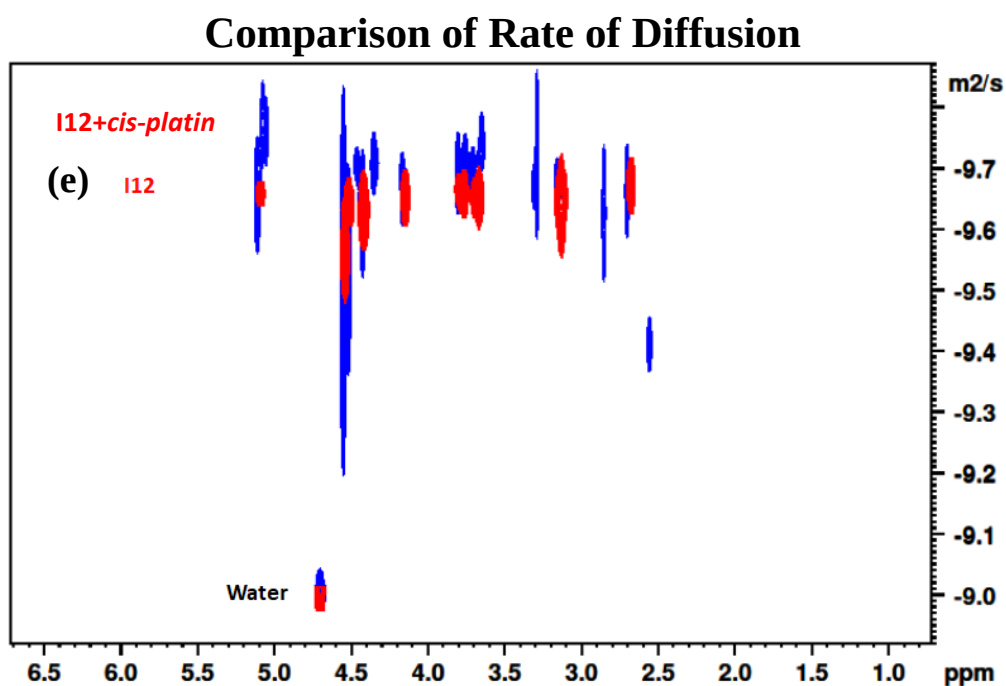
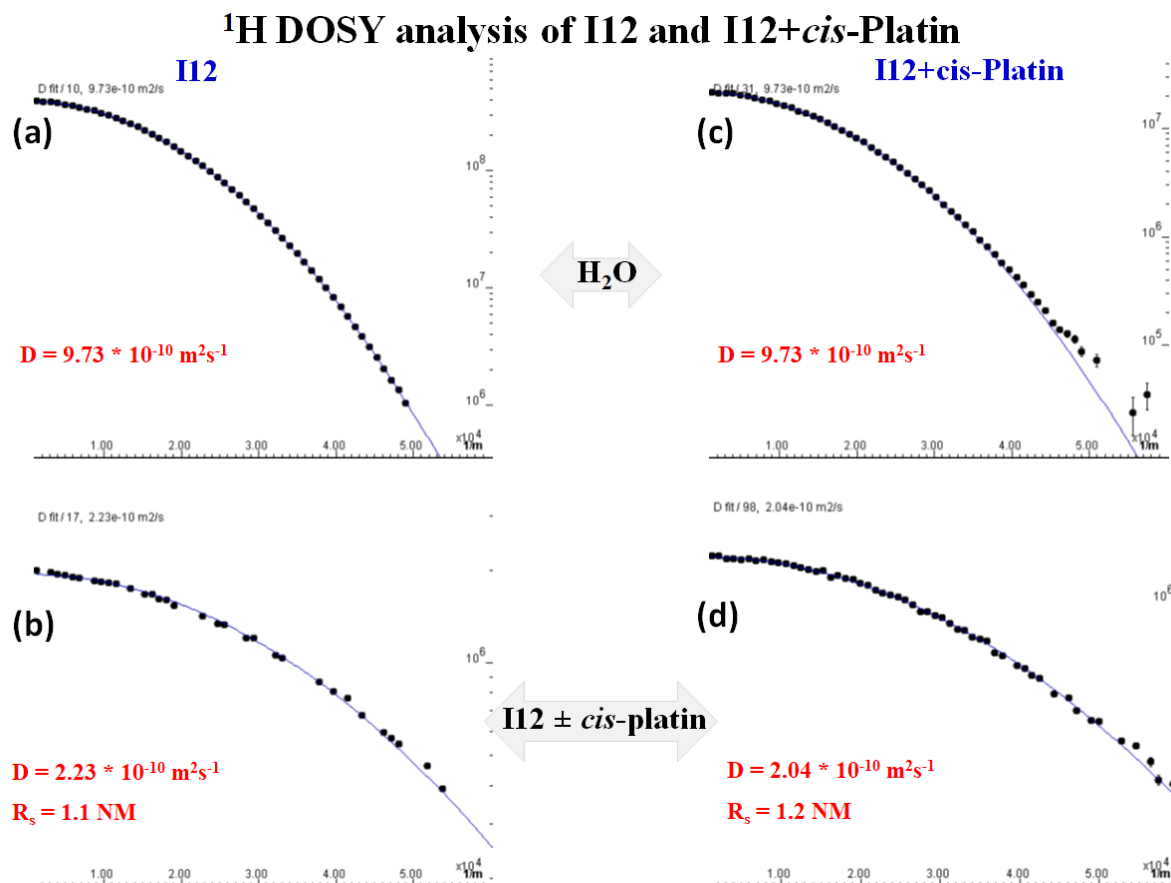


Figure 2: The quantitative analysis of diffusion data of I12 before and after the addition of cisplatin, the plot of rate of diffusion for (a) residual water in I12 (b) a randomly selected resonance of I12 (c) residual water after the addition of cisplatin and (d) I12+cisplatin (e) Comparison rate of diffusion

Next, the binding mode was tested by measuring the complex's chemical shift and spin-lattice relaxation time constant (T_1). For this, a ^1H - ^{13}C HSQC (heteronuclear single quantum correlation) experiment was performed on **G12** and **I12** with and without the addition of CP. In G12, after ^{13}C chemical shifts. Whereas C4 was the second site, and C3 was the third site on the backbone with a substantial change in their chemical shifts. In contrast, I12 showed that C5 and C2 had the maximum effect of interaction with CP. This trend continued in T_1 relaxation studies. Overall, the carboxylic acid at C5 and sulfate at C4 in G12 and C2 in I12 are the interaction sites in these molecules (**Fig. 3a**). It is noteworthy that the complex formation rate with I12-CP is 0.172h^{-1} and for G12-CP is 0.06h^{-1} which is much faster (**Fig. 3b**). Therefore, I12 was chosen for further biological studies due to its favourable binding properties with CP. Overall, these findings provide insights into the binding mode of CP with G12 and I12 and lay the foundation to develop a novel drug-resistance mechanism.

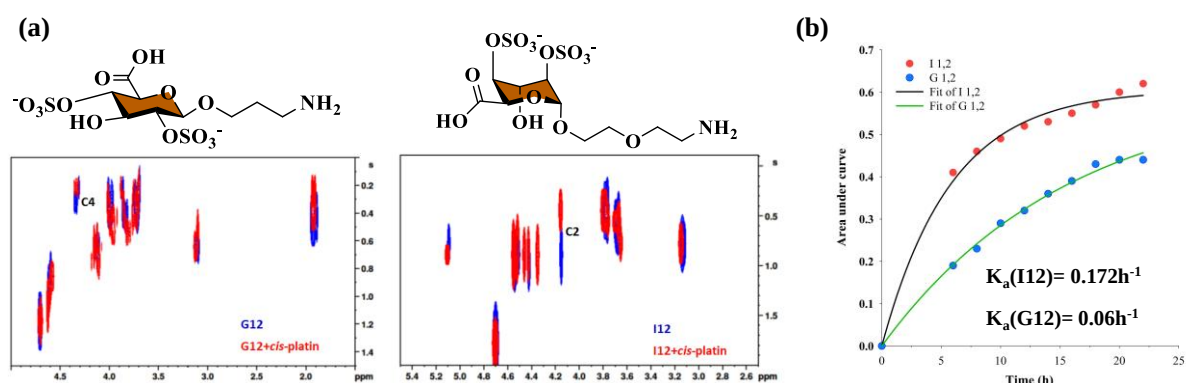


Figure 3: (a) The stacked 400 MHz ^1H NMR spectra for T_1 studies acquired using an inversion recovery pulse sequence. The particular colour for identification purposes highlights the colour-coded contours and the compounds (b) rate of formation of complex

5.2.2 Synthesis of P-I12

Next to investigate, whether the ability of I12-CP modulate the drug resistance mechanism, a short peptide-based proteoglycan mimic carrying an I12 residue was synthesized. This was achieved by synthesizing lysine based-short peptide using a solid phase synthesis, which was then functionalized with cholesterol and Cy-5 fluorescent tag at C- and N-terminal of the peptide respectively **Fig. 4**. The peptide was purified by reverse phase semi preparative HPLC

and characterized by ^1H -NMR and MALDI-TOF (synthesis shown in chapter 2). Next, the amine residue of the lysine side chain was converted into azide group. Finally, the I12 ligand was functionalized onto the peptide using strained promoted click reaction. The purity of the peptide was characterized by HPLC and HRMS. The mass spectral data of P-I12 (m/z 704.2017; $[\text{M}]^+/9$ and 712.1551 $[\text{M}^+/9 + 8\text{H}^+]$) confirm the proteoglycan mimic structure. Further, UV-visible and fluorescent spectra of the complex reiterate the presence of Cy-5 probe in the peptide backbone.

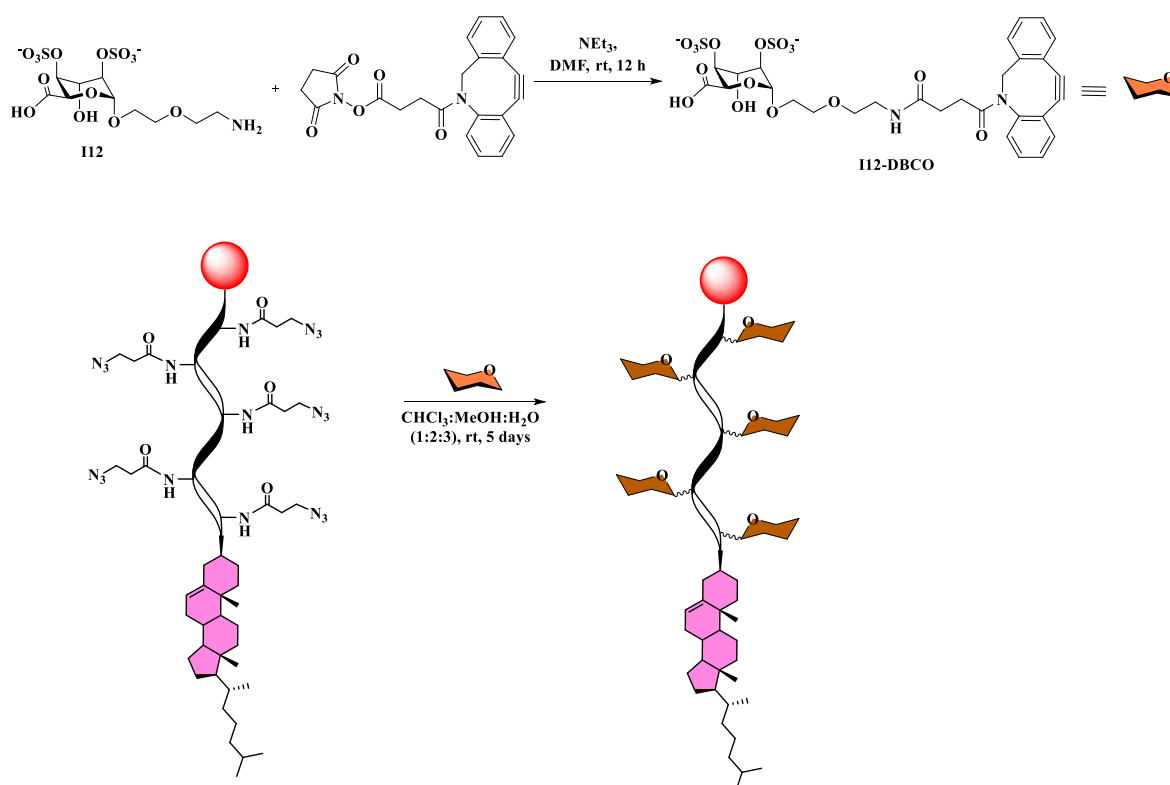


Figure 4. Synthesis scheme proteoglycans mimetics of P-I12

5.2.3 *In vitro* Studies

5.2.3.1 Cell Surface Decoration Studies

To explore the potential biological application of P-I12, we conducted an investigation into its glycocalyx engineering using a normal and cancer cell (NIH-3T3 and MDA-MB-468). Initially, we incubated the cells with different concentrations of P-I12 (1-10 μM) (**Fig. 5**).

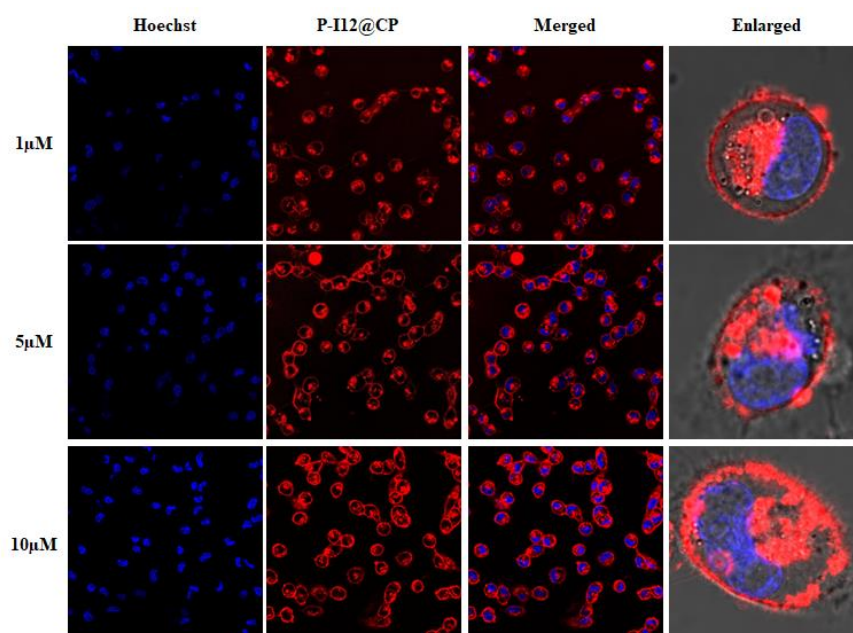


Figure 5 Concentration dependent confocal imaging studies with P-I12 using MDA-MB-468

For further studies we found that 2 μ M was adequate for all imaging studies, and we studied time dependent studies with MDA-MB-468 and NIH3T3 (**Fig. 6a & 6b**). Followed by immediate exposure and imaging of P-I12, we observed that a significant portion of the glycopeptide populated on the cell surface, while some of them underwent endocytosis.

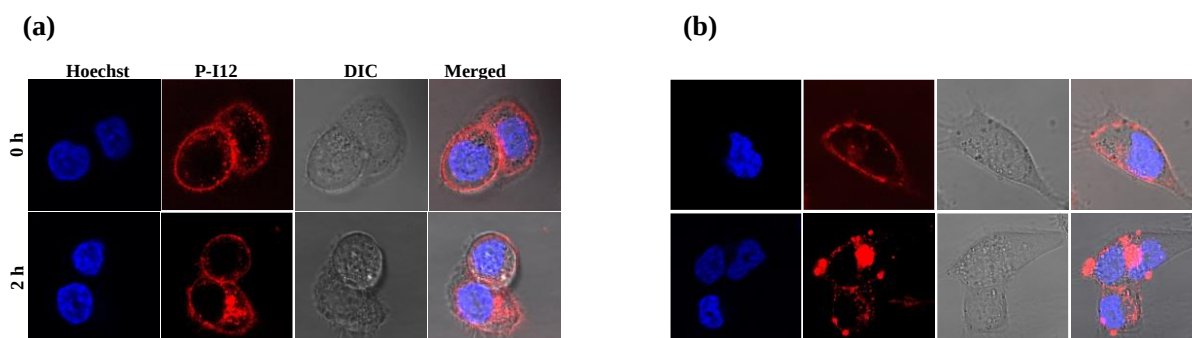


Figure 6. (a) Time dependent confocal imaging studies with P-I12 using MDA-MB-468 (b) Time dependent confocal imaging studies with P-I12 using NIH3T3

Upon quantification of the amount of molecules expressed on the cell-surface vs those internalize, a noteworthy distinction emerged in normal cell and cancer cells. In case NIH-3T3, we observed 1:2 ratio of glycopeptide on cell-surface compared to those internalize. In contrast, MDA-MB-468 cells displayed 1.5:1 ratio of bio-distribution. After 2 h of incubation, MDA-MB-468 showed 2:3 ratio of distribution as compared to NIH-3T3, which showed an approximately 1:4 ratio of biodistribution (**Fig. 7**). After 16 h incubation, the majority peptide found inside the cell lines.

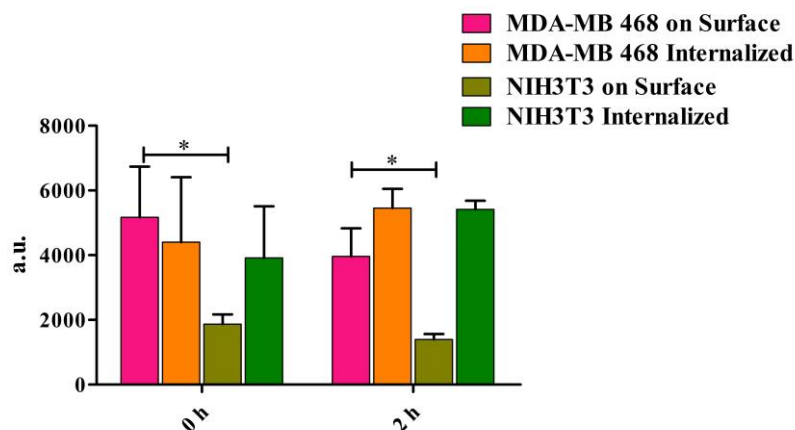


Figure 7. Cell surface quantification at different time interval using P-I12

It's uptake mechanism studied with different inhibitors (**Fig. 7b**) did not inhibit the internalisation process. Together, these data support internalisation of PG mimetic through a passive process, as would occur by lipid raft mediated uptake.

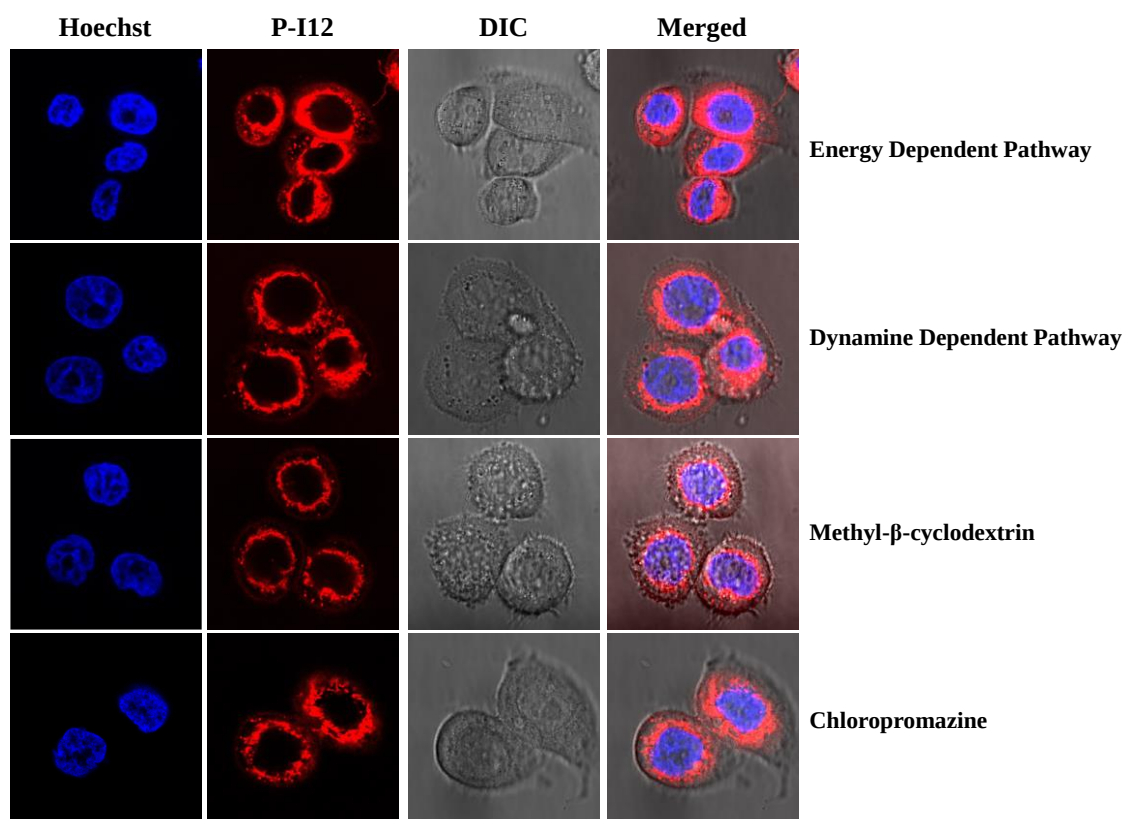


Figure 8. Uptake Mechanism studies with P-I12 using MDA-MB-468

We hypothesized that such disparity of P-I12 biodistribution may be attributed to the nature glycocalyx of normal and cancer cells. Given that the glycocalyx of normal cell is denser than that of cancer cells, P-I12 may readily self-assemble on the surface of cancer cells. Conversely,

the thick glycocalyx layer encapsulate the P-I12, leading to endocytosis and allow less pronounce cell-surface engineering. Given these findings, we anticipated that there might be differences in the cytotoxicity of cis-platin (CP) between cancer and normal cells when the cell surface was engineered with the P-I12 peptide.

5.2.3.2 Cell Viability Studies

To investigate this further, we performed the MTT toxicity assay after 48 h on different triple negative aggressive cancer cells (MDA-MB-231, MCF-7 MDA-MB-468)^{22,23,24,25} and normal cells (NIH-3T3). Cells were incubated with different concentration of P-I12 peptide and treating with CP drug at the concentration nearby IC₅₀ value for each cell line. As a control, we also treated cells with CP alone. At low concentration of P-I12 (10-30 μ M), we didn't observe any significant protection of the cells. However, at 50 μ M of P-I12, we observed substantial toxicity induced by CP in cancer compared to normal cells **Fig. 9**.

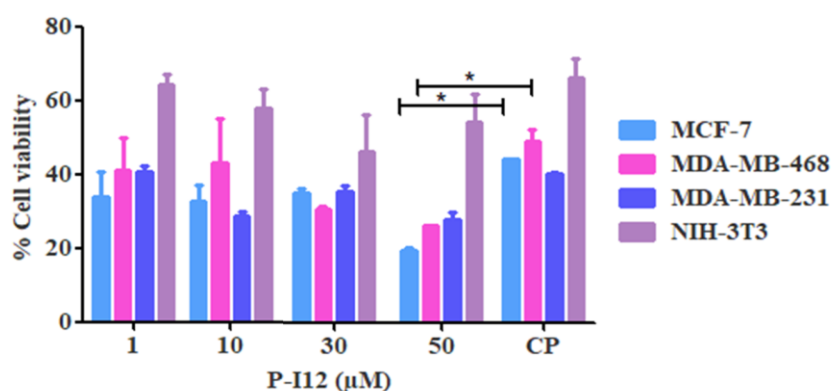


Figure 9. Cell viability for different cancer cells and normal cell in presence of cisplatin alone (CP concentration for NIH-3T3 25 μ M, for rest of cells 15 μ M) and in presence of mixture of P-I12 and cisplatin

This disparity might be due to the differences in the cell-surface engineering of P-I12 in cancer and normal cells. Since P-I12 was able to decorate on cell-surface of cancer cells for more than 2 h, it could bind to CP drug, internalize it and release the drug, leading to toxicity. Conversely, in normal cells, the amount of P-I12 expression on cell surface is lower, potentially resulting in a less complexation between I12 and CP ligand, and thus reduced toxicity. To confirm the CP toxicity mediated by P-I12, we additionally treated MDA-MB-468 with heparinase and assessed cell viability **Fig. 10a**. All these findings corroborated the pivotal role of P-I12 in inducing toxicity. The amount of CP inside the cells was also quantified using ICP-MS. All these findings corroborated the pivotal role of P-I12 in inducing toxicity. **Fig. 10b**.

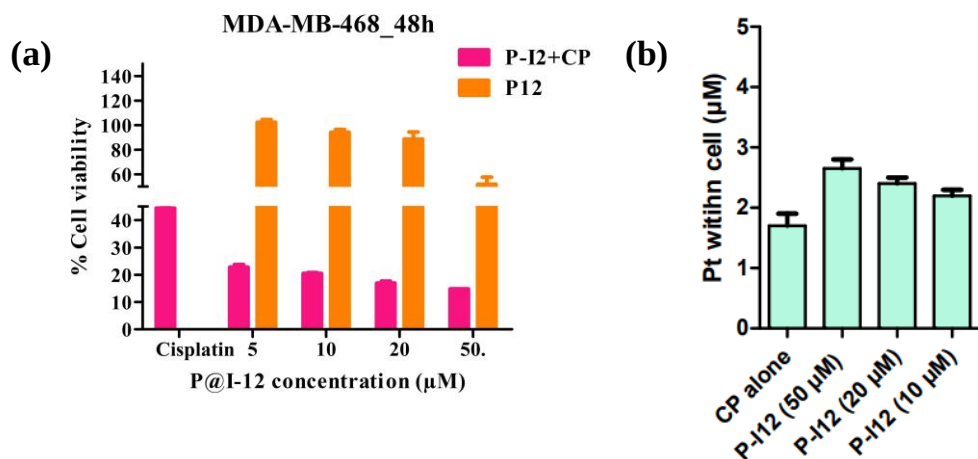


Figure 10. (a) Cell viability assay with heparinase treated MDA-MB-468 cancer cells (b) ICP-MS data for Platinum concentration within heparinase treated MDA-MB468 cancer cells

5.2.3.3 Intracellular Trafficking Studies

Finally, to uncover the mechanism of endocytosis in cancer cells, we performed co-localization assays of P-I12 using early, late endosome and lysosome markers at different time intervals. We observed that most of the peptide was sequestered in either early or late endosome in the first 6 h **Fig. 11a & 11b**. After that the peptide was repopulated from late endosomal to lysosomal region, and this trend continued between 12 to 48 h. After 48 h, P-I12 was found majorly sequestered in lysosomal region.

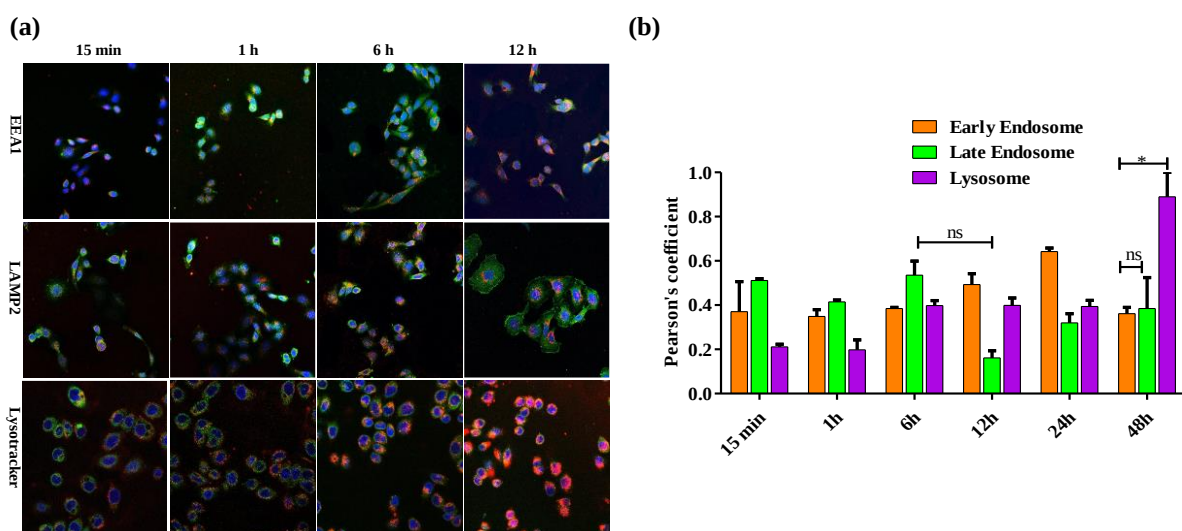


Figure 11. (a) Intracellular trafficking studies, (b) Pearson co-efficient in different compartment at different time interval.

Since the pH of the lysosomal region is around 4.4-5.0 and sulfate group responsible for I12-CP complexation was found sensitive to acidic pH, we hypothesize that P-I12@CP in acidic environment regulates CP release from the peptide, inducing slow and pronounced toxicity.

5.3 Conclusion

In summary, our research uncovered that the uronic acid residue of heparan sulfate (HS) plays a pivotal role in two distinct modes of conjugation with cisplatin (CP). The sulfated L-iduronic acid ligand binds to CP via the 2-O-sulfate and carboxylic acid residue, whereas D-glucuronic acid utilizes 4-O-sulfate and C5-carboxylic acid-mediated interaction. Notably, L-iduronic acid demonstrates a faster complexation rate compared to D-glucuronic acid. Additionally, proteoglycan mimics (P-I12) exhibit significant disparities in their ability to influence cell-surface engineering and endocytosis in both cancerous and normal cells. This variation profoundly affects the toxicity of the CP drug. Considering the limited presence of L-iduronic acid units on P-I12 (5 units per peptide), we anticipate a substantial improvement in the cancer cell-specific drug release activity by enhancing multivalency on the peptide backbone.

5.4 Experimental Procedure

5.4.1 NMR Studies

Uronic acid compounds (**G12** and **I12**) (1 mM) were mixed with cisplatin (1 mM) in D₂O solvent and used as such for all NMR studies.

5.4.2 Diffusion Ordered Spectroscopy (DOSY)

The DOSY in NMR has appeared as an effective tool in studying non-covalent intermolecular interactions between two or more molecules in solution. The diffusion coefficient is inversely proportional to the molecular hydrodynamic radius (molecular size). Since the diffusion coefficient depends on the size and intermolecular interactions, it results in the addition in size and reduction in diffusion rates. The NMR spectroscopy can be utilized in translational diffusion measurement using a pulsed-field gradient (PFG). The PFG produces a variation in the linear magnetic field across the NMR sample, which leads to the variation in the precession frequencies for a particular signal as it depends on the effective magnetic field. The signal evolution in the presence of PFG is refocused by another PFG of the same duration and strength after the magnetization inversion by 180° radio frequency pulse. This refocusing is only

effective in the absence of diffusion in the sample, and the translational diffusion results in a magnetization decay as δ .

$$I = I_0 e^{-D(\gamma\delta G)^2 (\Delta\delta/3)}$$

Where I represent the intensity observed after the refocusing gradient, I_0 is the intensity without gradient, D stands for the diffusion coefficient, γ is the gyromagnetic ratio, δ is the gradient duration of strength G , and Δ is the delay between both the gradients.

With the knowledge of diffusion constants (D), the formula weights (FW) of a molecule or system can be derived from the relationship-

$$\log D \propto \log \text{FW}$$

The estimated formula weight effectively approximates the number of interacting molecules and their ratio. The spin-echo diffusion method with PFG³ has been broadly applied in investigating intermolecular HB, ion-pairing, aggregation, π - π stacking in supramolecular chemistry.⁴ and the transition metal catalysts.⁵ The DOSY has been used in determining the formula weights of polymers and the structures of organometallic complexes in solution.⁶ The DOSY technique is also useful in examining the absence or presence of intermolecular interactions or molecular aggregation.

The free molecules have a particular diffusion rate, and it is observed slower if they interact with other molecules as the hydrodynamic radius increases. This technique has proven effective in examining the protein-ligand interaction. where the diffusion coefficient of a small ligand changes drastically if bound to a huge protein molecule. Due to the faster decay in the diffusion filter, small free molecules with fast diffusion that are free can be removed from the spectrum, and the molecules that bind to a large biomolecule remain in the spectrum because of their significantly slower diffusion.

Similar to the relaxation's studies, the intermolecular interactions are monitored as a change in the molecular size (hydrodynamic radius) after interaction between two or more molecules. There is a substantial observable change for the smaller molecules compared to the large molecule. This is because the sum of the hydrodynamic ratio is observed after the interaction, which changes nominal for the larger molecules as the size of the ligand or small molecules is comparatively less. In our system, the smaller molecule cisplatin does not have any detectable protons or carbons; hence the change in the rate of diffusion after the addition of cisplatin is studied for the resonances of glucose derivatives G12 and I12.

For diffusion studies, the pseudo-2D ¹H DOSY experiments with and without the addition of cisplatin were carried out for both the studied glucose derivatives G12 and I12. All the physical parameters (before and after adding cisplatin) were kept identical for comparison purposes.

5.4.3 Detection of Conformational Changes with Cisplatin

Detecting the of conformational or structural changes in addition to the ligand could be direct evidence of the interaction between the studied molecule and the ligand. Such interactions could be evident in many ways by NMR spectroscopy, such as a change in chemical shift, line broadening or appearance of additional resonances in the resulting spectra.

On adding the cisplatin to the molecules G12 and I12 we could not see any substantial changes immediately in the chemical shifts of both the molecules either in ^1H or ^{13}C NMR spectra. But on acquiring the spectra approximately after 6 hrs, we evident the appearance of additional resonances in the ^1H and ^{13}C spectra of both the molecules G12 and I12. The change in resonances is evident almost for all the resonances of ^1H spectra of the glucose ring's proton, but the substantial difference could only be seen for the specific resonances. The 1D ^1H NMR spectra of G12 with and without cisplatin are compared in *fig. 7*.

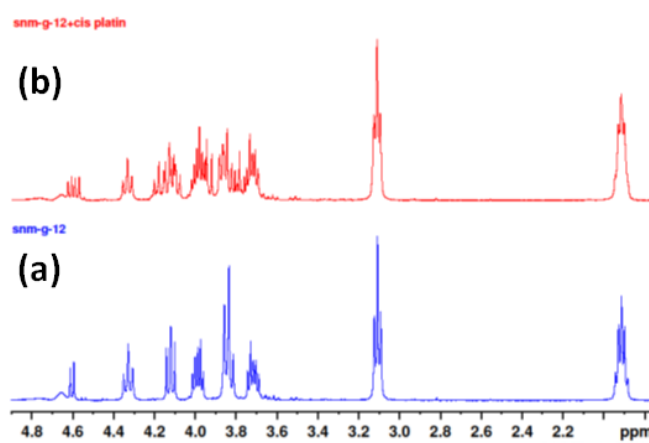


Figure 7: 1D ^1H 400 MHz NMR spectrum of (a) molecule G12 (b) the molecules G12+*cisplatin*. The appearance of additional resonances for most of the signals can be evident by comparing both the spectrum, which is more prominent for the protons of the ring, ranging from 3.6-4.6 ppm. This signal splitting is due to the interaction between G12 and *cisplatin*.

The appearance of additional signals for some ^{13}C of the ring is also obtained. To visualize these signals, the multiplicity edited by 135° pulse in ^1H - ^{13}C HSQC (heteronuclear single quantum correlation) experiment on G12 and I12 with and without the addition of *cisplatin* were carried out and given in *fig. 8*. The multiplicity editing is advantageous in distinguishing the CH_2 resonances from the CH and CH_3 in the HSQC spectrum. The additional CH signals in ^1H - ^{13}C HSQC spectrum of G12 after the addition of cisplatin were obtained in the ^1H and

^{13}C dimension, which can be visualized in the expanded spectrum given in *fig. 9*. The appearance of these additional resonances in the ^1H and ^{13}C dimensions is concluded due to the interaction between G12 and cisplatin.

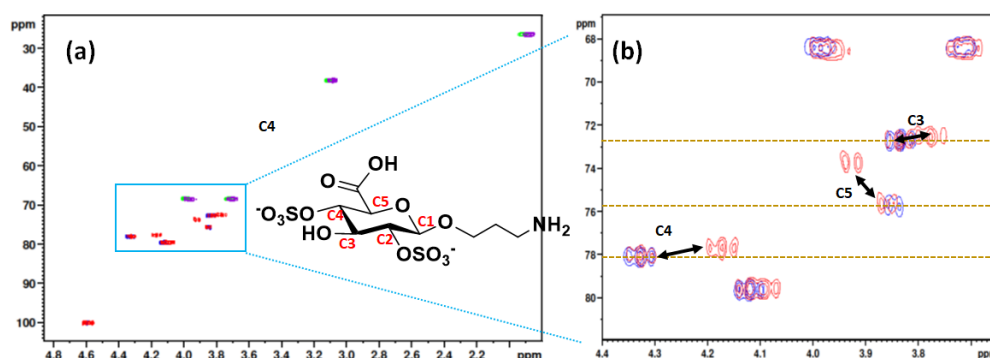


Figure 8: The stacked plot of multiplicity edited ^1H - ^{13}C HSQC (heteronuclear single quantum correlation) experiment by 135° pulse on G12 and G12+cisplatin. Blue and green contours belong to G12, and the red and purple contours to the G12+cisplatin.

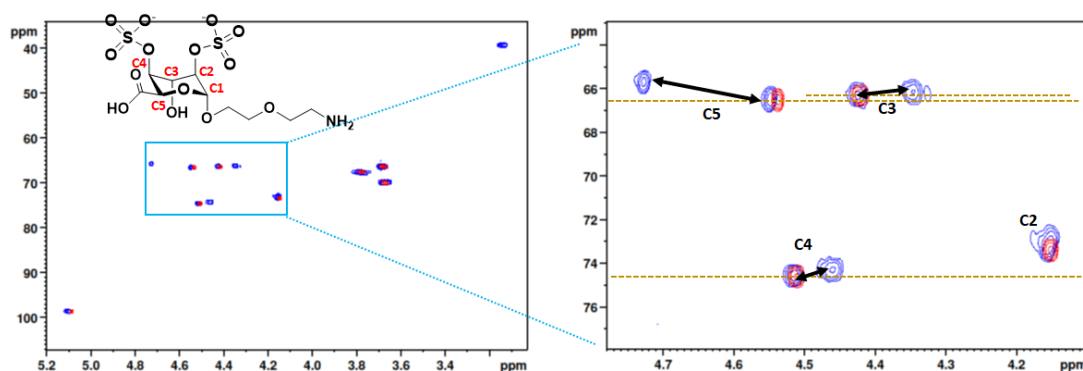


Figure 9: The stacked plot of multiplicity edited ^1H - ^{13}C HSQC (heteronuclear single quantum correlation) experiment by 135° pulse on I12 and I12+cisplatin. Blue and green contours belong to I12, and the red and purple contours to the I12+cisplatin.

Interestingly, the displacement in the newly appearing peaks is observed only for a few resonances, which is useful in identifying the most affected sites on G12 by the interaction of cisplatin. The extent of the displacement can be correlated to the effect of the interaction by cisplatin on the particular site.

The molecules G12 and I12 exist in the most stable conformer, which is probably the chair form, and after the addition of cisplatin, it shifts to the two different conformers, which may be the chair, boat, or two different kinds of twisted or any combination.

The ^1H and ^{13}C spectra also reveal information about the most affected sites by the interaction of cisplatin in the glucose molecules. As discussed, almost all ^1H resonances of the glucose ring are influenced by interaction with the cisplatin, but this change is selective for the specific

sites of the backbone carbons. Few ^{13}C obtained were almost unaffected and appeared at the same chemical shift as in the free molecule; on the other hand, few ^{13}C resonances were obtained splitted and shifted in the ^{13}C dimension with different extents of shifting. The extent of displacement in the ^{13}C dimension could be used to classify the most and least affected sites on interaction with cisplatin.

The G12 and I12 were fully assigned before the interaction studies using advanced 2D NMR techniques such as COSY, ^1H - ^{13}C HSQC, ^1H - ^{13}C HMBC etc., as per the requirement. In molecule G12, after interaction with cisplatin, carbon C5, which is substituted with carboxylic acid, shows the maximum shift in both the ^1H and ^{13}C chemical shifts; however, C4 is the second, and the carbon C3 is the third cite on the backbone with a substantial change in their chemical shifts. This change in ^1H and ^{13}C resonances is due to the conformational change in the glucose derivatives by the interaction of cisplatin. Similar to G12, the carboxylic substituted C5 of I12 shows the maximum shift in both ^1H and ^{13}C chemical shifts, but the ^1H and ^{13}C shifts are more for C3 than C4.

Based on the above observations, it is clear that the binding site is the sulfate group on the glucose backbone; additionally, the carbonyl group at position C5 also interacts with cisplatin through hydrogen bonding. As the mode of binding in I12 is different where the sulfate groups at position C4 and C2 interact with the cisplatin by replacing its two chlorides, position C3 is most affected compared to C4. On the other hand, in G12 the cisplatin interacts with sulfate at C4, and such an effect is not prominent for position C3. The different trend in the extent of the shift in the resonances of C3 and C4 is discarding the possibility of the carbonyl group as the only interacting site with cisplatin; hence the main interaction sites are the sulfate and carboxylic groups.

5.4.4 Concentration Dependent Binding Studies

A quantitative NMR investigation of L-IdoA/D-GlcA with cisplatin was conducted to determine the optimal stoichiometry of the complex. In this regard, L-IdoA/D-GlcA was mixed with cisplatin (CP) in D_2O and left in the NMR tube for one day, after which NMR measurements were taken. *fig 10a and 10b* illustrates that there is no significant alteration in the NMR spectra after forming a 1:1 complex, as compared to a 1:2, 1:5, or 1:10 ratio of the complex. This suggests that the 1:1 complex is deemed ideal for further studies on complexation.

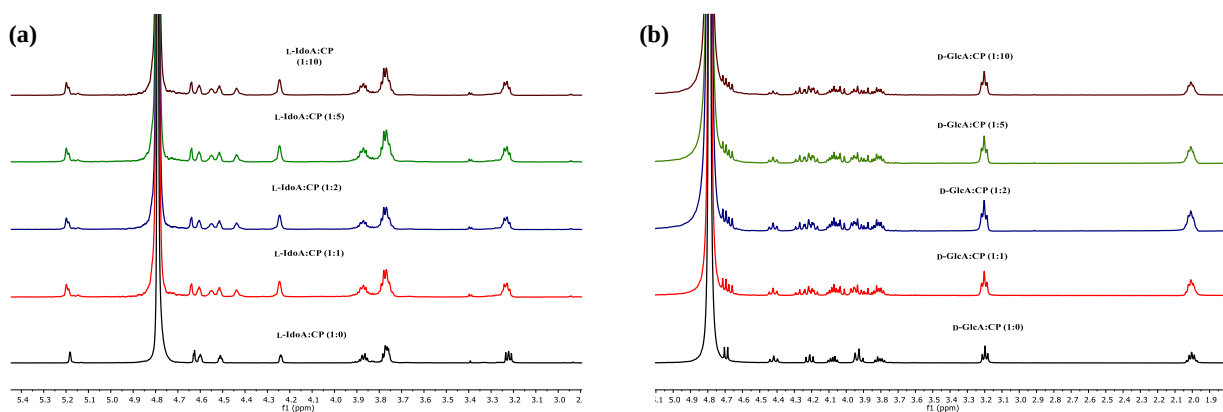


Figure 10. (a) Quantitative NMR studies of L-IdoA with cisplatin, (b) Quantitative NMR studies of D-GlcA with cisplatin.

5.4.5 Time Dependent Binding Studies

Time-dependent NMR studies were conducted with the L-IdoA/D-GlcA and cisplatin (CP) complex **Fig. 13a & 13b** disclosed the emergence of new peaks at 3.45 and 4.45 ppm, simultaneously with the reduction of peaks at 4.43, 4.52, and 4.55 ppm during the conformation of the L-IdoA-CP complex. A similar trend was also noted in the formation of the D-GlcA-CP complex.

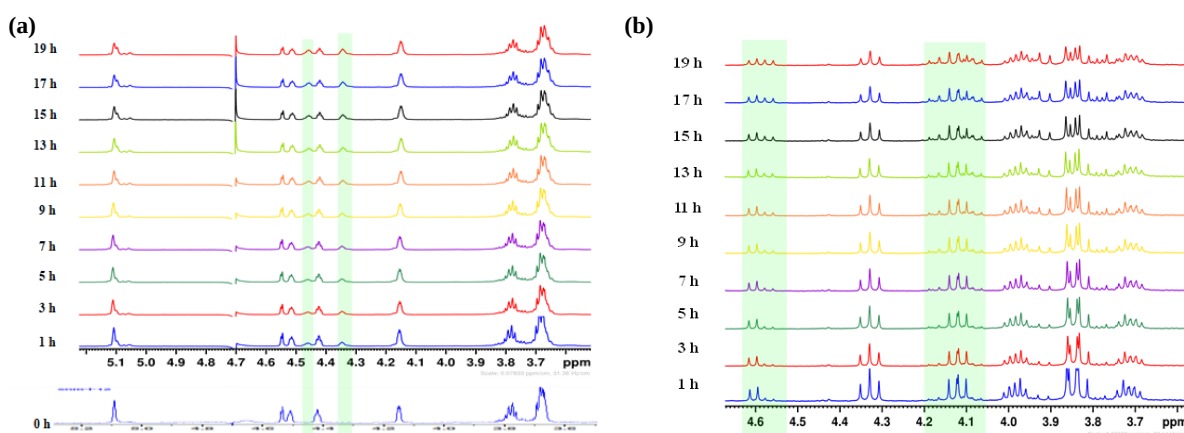


Figure 13. (a) Time dependent complex formation between L-IdoA and CP (1:1 mixture), (b) Time dependent complex formation between D-GlcA and CP (1:1 mixture).

5.4.6 pH Dependent Binding Studies

An investigation into the drug release mechanism was conducted at 1:1 concentration of the complex under different pH conditions. **Fig. 14** revealed that the complex is nearly or entirely released at pH 4.5. Conversely, at pH 7 and pH 6 no significant changes were observed in the

spectra compared to the complex. Furthermore, at pH 4.5, the NMR spectra clearly indicate that the dissociation of the complex is a rapid mechanism.

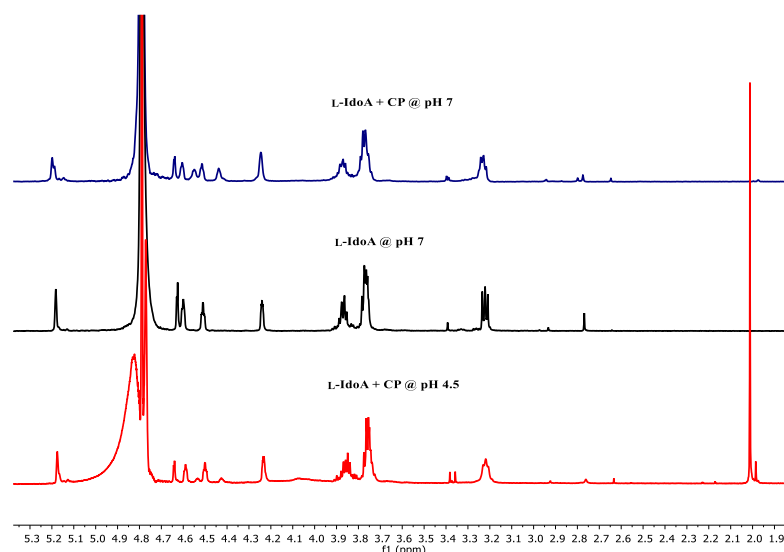


Figure 14. NMR analysis of L-IdoA-CP complex at different pH.

5.4.7 Rate of Complex Formation

To determine the rate of complex formation of G12 and I12, we performed kinetic studies of ligand-CP at different time intervals (0 to 48 h) and the changes in the NMR peak was evaluated. Based on the integration value of C2 and C4 of **I12** and **G12**.

5.4.8 Cell Surface Decoration Studies Protocol

To perform cell surface engineering studies 2×10^4 cells were plated in 8 well microscopy plates and incubated overnight. Next day different concentrations of P-I12 were added and cells were incubated for different time intervals. On completion of incubation, cells washed with PBS and taken for imaging

5.4.9 Intracellular Trafficking Studies

To study cellular co-localization of IdA-paprtide conjugated cisplatin complex MDA-MB 468 cells were grown on cover glass (10^4 cells per well) overnight. Cells treated with 10 μ M P-I12@CP for different time intervals (15 min to 24 h), followed by treatment with markers of different organelles. For lysosome labelling cells treated with lyso green for 1 h at 37 $^{\circ}$ C followed by staining with Hoechst for 10 min and proceeded further for fixation with 4% paraformaldehyde and mounting of glass slides. For early and late endosome co-localization cells first fixed using 4% paraformaldehyde then permeabilized with 0.1% triton X100 for 10

min followed by blocking with 2% BSA for 45 min at RT. Then cells labelled with primary EEA1 and LAMP2 antibody overnight at 4 °C followed by washing with PBS and treatment with secondary antibody at 1:2000 dilution for 45 min at RT. Coverslips washed and mounted on glass slides for imaging.

5.4.10 Mechanistic Studies

To examine the endocytosis mechanism, we initially assessed its energy-dependent pathway. MDA-MB-468 cells were incubated with NaN₃ for 30 minutes to deplete ATP, followed by treatment with P-I12 for 2 hours. No reduction in cellular uptake of MGDs was observed, confirming that the endocytic pathway is independent of energy. To investigate the involvement of dynamin-dependent uptake, dynasore hydrate was introduced. However, no blockade of uptake was observed, indicating that internalization is not dependent on clathrinor caveolae-mediated pathways. Similarly, the addition of methyl- β -cyclodextrin (m- β -CD, an inhibitor of caveolae-mediated endocytosis) and chlorpromazine (an inhibitor of clathrinmediated endocytosis) did not exhibit any blockade, suggesting that the pathway is unconventional, possibly involving cell-surface engineering and the phagocytosis pathway.

5.4.10.1 Cell Viability Studies on Non-Treated Cell

MTT assay was used to check cell proliferation. Cells (5×10^3 cells per well) were plated in 96 well plate in DMEM medium and incubated overnight followed by treatment with different concentration of P-I12 and fix concentration of cisplatin (Concentration nearby IC50 concentration) and further incubation for 48 h. Afterward 10 μ l of MTT (5 mg/ml) reagent was added to each well and incubated plates further for 4h at 37 °C. Purple precipitate formed was dissolved by adding 100 μ l of DMSO and plate was read at 570 nm.

5.4.10.2 Cell Viability Studies on Treated Cell

To comprehend the cisplatin delivery mediated by P-I12, a cell viability assay was conducted on heparinase-treated MDA-MB-468 cell lines. Initially, cancer cells were cultured on a 96-well plate and treated with heparinase enzyme (100 ng/mL) (Sigma Aldrich, cat no. SAE0116) for 1 hour following the literature protocol.⁷ Subsequently, the cells were treated with different concentrations of peptides alongside cisplatin and peptides alone, all at an optimal concentration of CP (3 μ M), which induced 50% cell viability. Figure S18 illustrates that with an increasing concentration of the peptide, cisplatin-mediated toxicity also escalated.

5.4.11 ICP-MS Studies

To further confirm cisplatin-mediated toxicity, ICP-MS was performed to measure the platinum concentration inside the cells. For this purpose, MDA-MB 468 cell first treated with heparinase enzyme followed by treatment with peptide alongside cisplatin and cisplatin alone for 48h. Cell pellets were collected digested with nitric acid, and subjected to ICP-MS. The observed concentration of CP inside the cells ranged between 2.5-2.8 μM with P-I12-treated cells, while the concentration of CP in the absence of P-I12 was 1.5-2 μM . This indicates that the amount of the compound is doubled in the presence of P-I12.

5.4.12 Procedure for Conjugation of Sugar Molecules

Procedure for conjugation of I-12 to peptide was written in chapter 2 experimental section 2.4.12 to 2.4.14.

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Chapter 6
Allosteric Modulation of the Voltage
Gated Potassium Channel by
Proteoglycan Mimetics

Abstract

Voltage-gated ion channels (VGICs) are allosterically modulated by glycosaminoglycan proteoglycans and sialic acid glycans. However, the structural diversity and heterogeneity of these biomolecules hinders precise delineation of the underlying structure-activity relationships. Herein, we present the first evidence that the sulfation of heparan sulfate (HS) and the glycosidic linkages of sialic acid glycan on a glycopeptide backbone influence glycocalyx remodeling and alter the gating of the Kv2.1 channel. Utilizing a panel of amphiphilic glycopeptides comprising HS disaccharides and sialic acid trisaccharide glycans, we observed that sulfation of HS and flexible $\alpha(2-6)$ sialylation result in prolonged persistence of glycopeptides on the cell membrane compared to non-sulfated HS and $\alpha(2-3)$ sialylation respective. This variation in glycocalyx composition was associated with a noticeable difference in the effects of these compounds on the activation and inactivation properties of the voltage-gated Kv2.1 channel with our strongest membrane associating compound demonstrating the most potent channel-activation propensity. Our findings demonstrate that negative charges on these glycopeptides play a critical role in their membrane association propensities and endow them with VGIC activation properties. These results set the stage for the development of glycocalyx probes as next-generation ion channel modulators with therapeutic potential.

6.1. Introduction

Voltage-gated ion channels (VGICs) are transmembrane receptors found ubiquitously on cardiac, muscle, and nerve cells, where they orchestrate various physiological processes, including electrical signaling, hormone secretion, and ion homeostasis.¹ Alterations in the activity of these channels leads to homeostatic plasticity, which ultimately results in neurological disorders such as epilepsy, autism, and Alzheimer's disease and cardiac arrest.² Unlike ligand-gated ion channels,³ VGICs are regulated by alterations in membrane potential instead of ligand binding.⁴ Nevertheless, some voltage-independent extrinsic and intrinsic factors also modulate VGIC activity. For example, VGICs are intrinsically regulated by post-translation modifications such as phosphorylation and glycosylation. Additionally, several external factors such as toxins and small molecules bind to VGICs and modulate their activity.⁵ Furthermore, the functioning of VGICs is known to be affected by changes in intracellular and extracellular ionic concentrations that are in turn intrinsically governed by the composition of the glycocalyx especially with respect to sialic acid and glycosaminoglycan carbohydrate compositions.⁶ Consequently, a thorough understanding of the specificity of carbohydrate ligands in VGIC activity could engender novel strategies for drug development.

The glycocalyx is composed of sialic acid which is the terminal carbohydrate in all glycoproteins and glycolipids,⁷ and glycosaminoglycans that are polysaccharides ubiquitously expressed as proteoglycans on the cell membrane and in the extracellular matrix.⁸ One of striking features of these two carbohydrate structures is that they are anionic in nature making them potential ligands for metal ions. Indeed, the carboxylic acid residue of sialic acid and sulfate groups of glycosaminoglycans bind to various metal ions, including heavy metals and alkaline metal ions and modulate the ionic concentration at the extracellular and intercellular level.⁹

Previous studies have shown that defective biosynthesis of sialic acid glycans and glycosaminoglycans, particularly heparan sulfate and chondroitin sulfate, alter membrane fluidity and allosterically modulate the activation kinetics and current amplitude of VGICs, and also alter coupling between pH and VGIC activation.⁶ For example, the HS proteoglycan glypican-1 has been demonstrated to modulate the biophysical properties of voltage-gated K⁺ channels in the cardiac tissue, and therefore, has an important role in cardiac disease pathophysiology.¹⁰ Moreover, calcium chelation by chondroitin 4-*O* and 6-*O*-sulfated causes alterations in the gating properties of voltage-gated calcium ion channels,¹¹ and sialylated

glycans play profound roles in regulating neuronal excitability by modulating the gating of all types of VGICs (Na^+ , K^+ , and Ca^{2+} channels).¹² Taken together, these studies implicate negatively charged cell surface glycans as physiologically important VGIC regulators. Detailed molecular levels understanding of this phenomenon, however, is hindered by the highly heterogeneous chemical composition of HS and sialic acid glycans.^{7,8} Therefore, to better understand the structure-activity relationship between these glycans and VGICs, the synthesis of well-defined proteoglycan mimics and sialic acid-based glycopeptides structures is crucial.

Herein, we introduce amphiphilic fluorescent glycopeptides carrying HS and sialic acid glycans to investigate glycocalyx remodeling and its subsequent effects on VGIC activity (**Fig. 1**). As a prototype, chemically synthesized HS disaccharides and sialic acid trisaccharide's ligands were functionalized onto a 12-mer peptide backbone, bearing a Cy-5 fluorescent tag and cholesterol moiety at the C- and N-terminus, respectively. Live-cell imaging and electrophysiological studies provided insights into how sulfation, glycosidic linkages, and the cell-surface retention of these glycopeptides modulate Kv2.1 channel activation and deactivation.

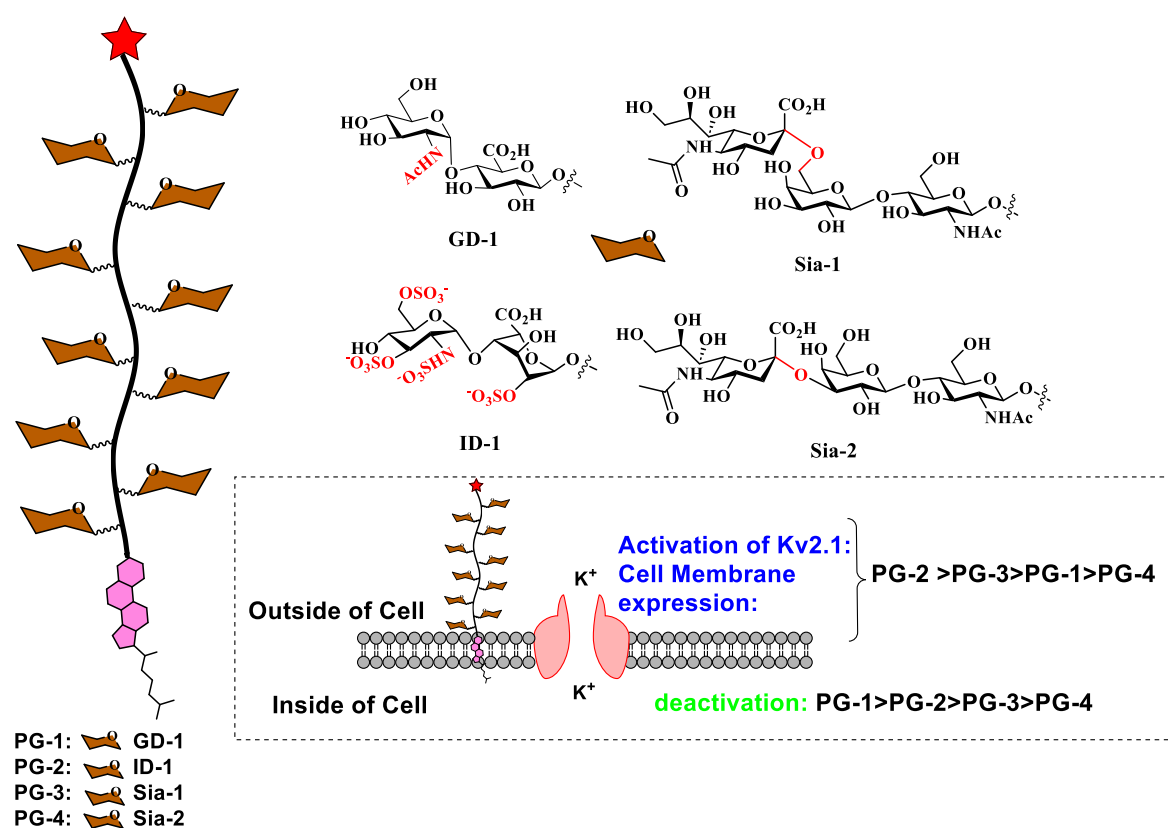
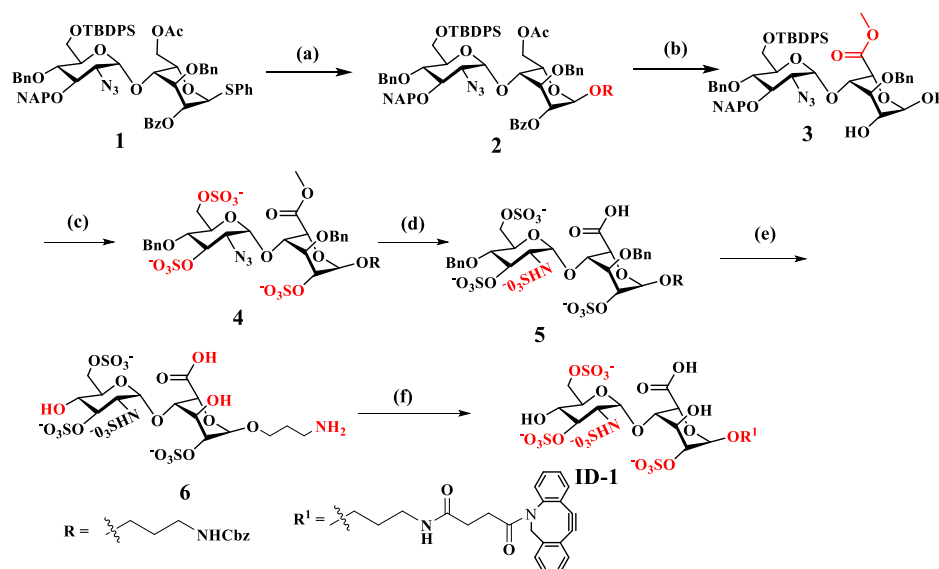


Fig. 1. Schematic representation of proteoglycans synthesized in this study and their voltage-gated ion channel modulation activity profiles.

6.2 Result and Discussion

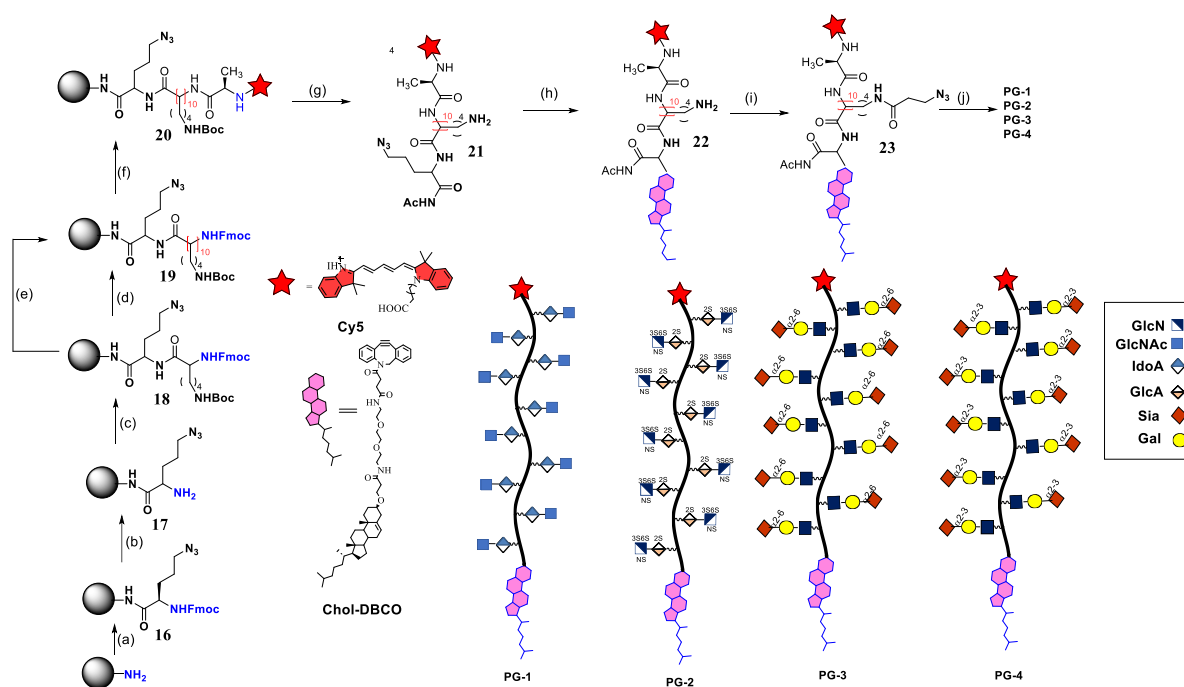
The glycocalyx is composed of highly heterogeneous populations of heparan sulphate and sialic acid glycans. To achieve a well-defined cell surface engineering probe, we synthesized structurally homogeneous versions of these glycans. The uronic acid-based heparan sulphate ligand (**G1** & **I1**) and the sialic acid glycans (**S1** and **S2**) were synthesized using established literature procedures.¹³ Briefly, glucosamine (**A1** & **A2**) and uronic acid building blocks (**B1** & **B2**) were synthesized as outlined in prior literature^{13d} and glycosylated using *N*-iodosuccinimide (NIS) in combination with the trimethylsilyl trifluoromethanesulfonate (TMSOTf) promoter. Subsequent deacetylation and oxidation with 2,2,6,6-tetramethyl-1-piperidinyloxy free radical (TEMPO) and [bis(acetoxy)iodo]benzene (BAIB), followed by methyl esterification, yielded **2** or **6** disaccharide precursors. TBDPS and 2-NAP deprotection of **2** using hydrogen fluoride, pyridine complex (HF.py) and 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), *O*-sulfation in the presence of sulfur trioxide triethylamine complex (SO₃.NEt₃) in DMF yielded **3**, followed by azide to amine conversion was done in the presence of zinc dust, acetic acid and N-sulfation in the presence of sulfur trioxide. Pyridine complex (SO₃.Py) yielded precursor **4** and global deprotection yielded I1 ligand (**Scheme 1**).



Scheme 1. Synthesis of HS disaccharide. **Reagents & Yield:** (a) Benzyl (3-hydroxypropyl)carbamate, NIS, TMSOTf, 85 % (b) (i) NaOMe, 90 %, (ii) TEMPO, BAIB, 87 %, (iii) LiOH.H₂O (d) MeI, K₂CO₃, 80 %, (c) (i) DDQ, 72 %, (ii) 70 % HF.py, 90 %, (iii) SO₃.NEt₃, 67 % (d) (i) LiOH.H₂O, 85 %, (ii) PMe₃.THF, 74 %, (iii) SO₃.Py, 55 % (e) H₂, Pd(OH)₂, 84 %; (f) DBCO-active ester, DMF/H₂O.

Lithium hydroxide-based hydrolysis and debezoylation of 6, followed by hydrogenolysis yielded G1 ligand. Sialic acid glycans (S1 and S2) were synthesized by glycosylating sialic acid dibutyl phosphate donor (C) with galactose acceptor (8 & 12), in the presence of NIS/TfOH promotor yielded $\alpha(2-6)$ and $\alpha(2-3)$ sialic acid disaccharides (9 & 13), which was glycosylated to N-troc protected glucosamine building block, yielded trisaccharide precursor (10 & 14). In order to obtain better trisaccharide yield, we first deprotected to N-Troc, followed by N-acetylation and standard global deprotection yielded S1 and S2 trisaccharide ligands respectively. Finally, the di and trisaccharide ligands (I1, G1, S1 and S2) were conjugated to DBCO linker to yield respective sugar precursor for glycopeptide conjugation.

Next, the glycopeptide backbone was synthesized from Rink amide resin using Fmoc-protected amino acid and coupling reagents, HOBt and HBTU respectively (**Scheme 2**).



Scheme 3. Synthesis of proteoglycan mimetics. (a) Fmoc-Glu(N₃)-OH, HOBt, HBTu, DIPEA (b) 30 % pyridine in DMF (g) Fmoc-Lys(OtBu)-OH, HOBt, HBTu, DIPEA (c) 30 % pyridine in DMF (i) Fmoc-Lys(OtBu)-OH, HOBt, HBTu, DIPEA (d) (i) Fmoc-Ala-OH, HOBt, HBTu, DIPEA (ii) Cy-5, HOBt, HBTu, DIPEA (e) TFA:H₂O:Phenol:TIPS (18:1:0.5:0.5) (f) (i) Chol-DBCO, 90 % (g) Azidopropanoate-NHS, NEt₃, 86 % (h) GD-1-DBCO, H₂O:MeOH, 90 %

First, 12-mer peptide, carrying ten lysine residues positioned between 5-azido-pentanoic acid and L-alanine at the N- and C-terminals of the peptide was synthesized and Cy-5 fluorescent probe was conjugated at N-terminal. After cleaving the peptide from the resin, it was subjected to copper-free click reaction with cholesterol linker (Chol-DBCO) 14 to yield the peptide

backbone 22, which was purified by HPLC and characterized by NMR and MALDI-TOF. The amine-end of the lysine residue was conjugated to an azido linker and clicked to the DBCO-sugars to obtain the desired glycopeptides (PG-1 to PG-4). The peptides were purified using HPLC and characterized through NMR, HRMS, UV, and fluorescent spectroscopy.

The HS (PG-1 & PG-2) and sialic acid (PG-3 and PG-4) glycopeptides were tested for their cell membrane persistence before investigating their VGIC activity. These experiments were performed on MDA-MB-468 cells and NIH3T3 cells that possess different glycocalyx compositions (Fig 2 & S3).¹⁵ Cells were seeded on eight-well glass chamber slides and allowed to grow until reaching 70-80% confluence. Fluorescent glycopeptides (2 μ M) were introduced, and live images were recorded after 30 min and 90 min, respectively (**Figure 2a**).

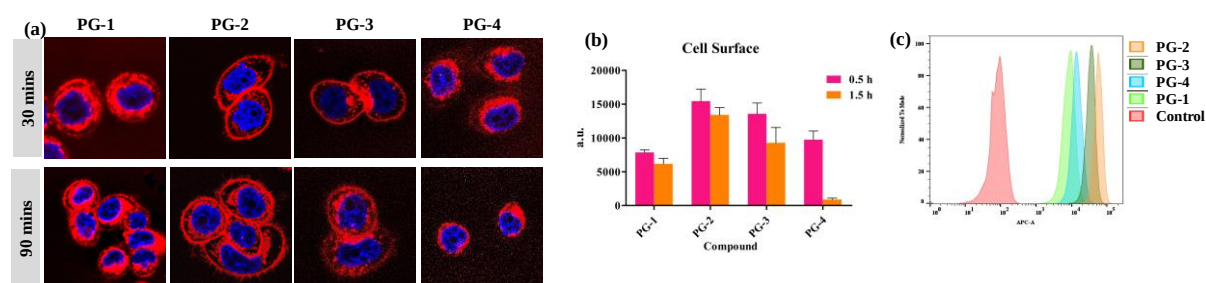


Figure 2. (a) Confocal imaging of glycopeptides uptake by MDA-MB-468 cell lines at different time interval; (b) Average fluorescence quantification of PG mimics on cell membrane at different time interval $n = 50$ cells; (c) FACS analysis of glycopeptides uptake after 1 h incubation.

All four proteoglycan mimetics were incorporated into the plasma membrane within minutes, although at varying rates of incorporation and internalization (**Figure 2a & b**). PG-2 was imbibed into the cell plasma membrane much more efficiently as compared to the other mimetics whereas PG-4 and PG-1 showed the least incorporation after 30 min. After 1.5 h, PG-4 was fully internalized with the cells, whereas PG-2 was retained in the plasma membrane demonstrating its high affinity for the plasma membrane. These studies demonstrated that the cell membrane affinities of these compounds were primarily dependent on their sugar scaffolds rather than their peptide backbones. It is proposed that two crucial structural attributes play a significant role in glycocalyx remodeling: the high sulfation of PG-2, which may induce electrostatic repulsion with the glycocalyx composition 16 on cells and regulate longer-term persistence compared to non-sulfated HS glycopeptide (PG-1); and (b) the rotational freedom within $\alpha(2-3)$ versus $\alpha(2-6)$ glycosidic linkages, 17 which imposes rigidity or flexibility on the peptide backbone, thereby altering cell membrane expression.

Overall, imaging studies affirmed that the composition of sulfation and the glycosidic linkages significantly modulate the expression of glycopeptides on cell membranes, which may influence the ion concentration on the membrane and can modulate VGIC activity.

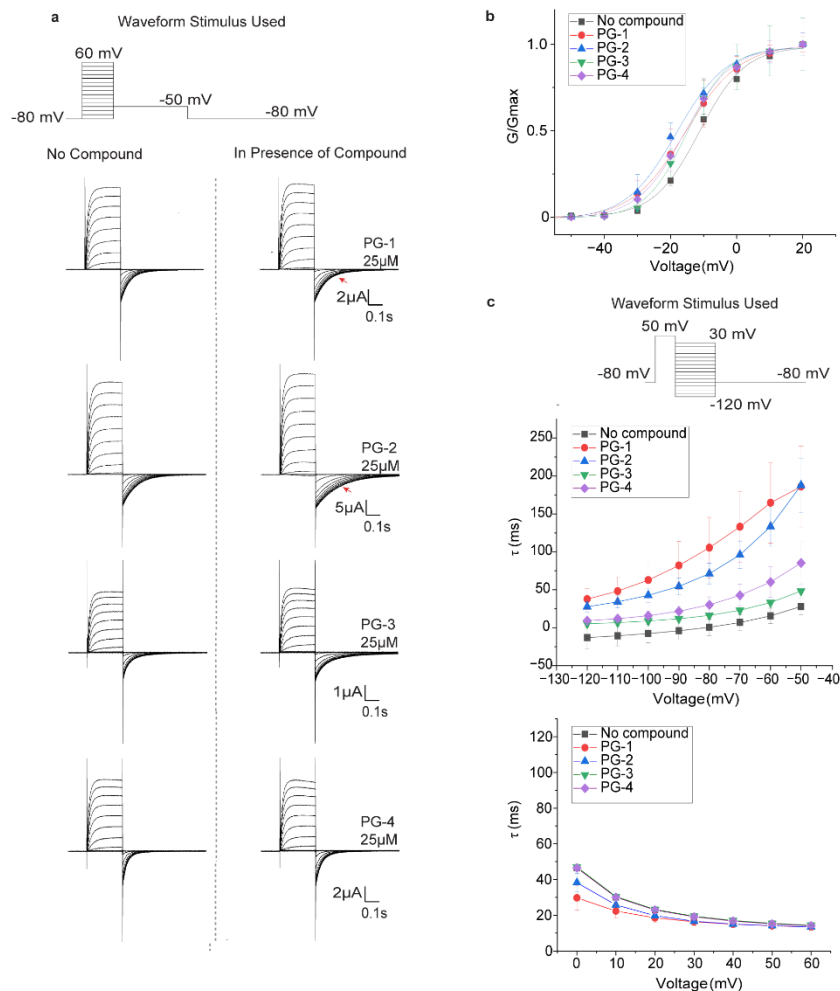


Figure 3. Representative traces, G–V relations, and activation/deactivation kinetics of Kv2.1 channels with PG series of compounds, a) Representative traces of ionic currents obtained in activation studies before applying (left) and in presence of (right) PG-1, PG-2, PG-3, and PG-4, b) G–V relations for Kv2.1 before and after application of 25 μ M of PG-1, PG-2, PG-3, and PG-4. Data points indicate mean $\pm$ s.e., c) Deactivation (top) and activation kinetics (bottom) of Kv2.1 before and after treatment with 25 μ M of the compounds. τ represents time constants from single exponential fits to channel deactivation (top) and activation (bottom). Data points are mean $\pm$ s.e.

To characterize the effects of these compounds on the activity of VGICs, we evaluated their effects on the voltage-gated potassium channel, Kv2.1, by employing the two-electrode voltage clamp electrophysiology technique. Experiments were performed on Kv2.1 expressed heterologously on the surface of *Xenopus laevis* oocytes. The effects of glycopeptides (PG-1

to PG-4, 25 μ M) on Kv2.1 activation were characterized by performing three step-voltage protocols, wherein the holding potential was maintained at -80 mV, and depolarization pulses were administered in 10 mV increments followed by a deactivation pulse at -50 mV to yield tail currents. These experiments demonstrated a leftward shift of the G–V relationship of the channel in presence of these compounds (a characteristic feature of VGIC activators) with PG-2 exhibiting the highest level of channel activation ($\Delta V_{0.5} = -7.0$), as compared to PG-3 ($\Delta V_{0.5} = -4.4$), PG-1 ($\Delta V_{0.5} = -4.1$), and PG-4 ($\Delta V_{0.5} = -3.7$) (**Figure 3b**, **Table 2**). A notable characteristic of PG-1 and PG-2 is their ability to slow down channel closure, as indicated by the deceleration of Kv2.1 tail currents in their presence (as shown by arrows in **Figure 3a**).

To study the slowing down of tail currents more rigorously, we performed deactivation studies using a voltage protocol wherein we pulsed to 50 mV followed by hyperpolarizing to different tail voltages in 10 mV steps (voltage protocol depicted in **Figure 3c**). These studies clearly demonstrated that all four compounds slow down channel closure with PG-1 and PG-2 demonstrating the most profound effects (slowing down channel closure by ~ 7 -fold at -50 mV; **Figure 3c** top; **Table 1**).

Our results establish that these glycopeptides are channel activators that act by stabilizing the open state of the channel and slowing down channel closure, and that sulfated HS glycans possess higher activity than their sialic acid counterparts. Fascinatingly, we find that our most potent Kv2.1 activator, PG-2, also demonstrated the strongest incorporation into the cell plasma membrane (**Figure 1b & c**). This observation supports the hypothesis that the Kv2.1-activation effects of these compounds are membrane-mediated. As such, these findings demonstrate that sulfated HS based glycopeptides have tremendous potential as lead molecules for targeting VGICs associated with neuromuscular and neurological disorders.

τ (ms)	No Compound	PG-1	PG-2	PG-3	PG-4
Activ. at 10 mV	30.1 $\pm$ 0.9	29.7 $\pm$ 6.8	38.2 $\pm$ 5.2	46.5 $\pm$ 1.4	47.0 $\pm$ 1.8
Deactiv. at -50 mV	28.1 $\pm$ 10.7	185.9 $\pm$ 53.4	187.6 $\pm$ 35.4	118.2 $\pm$ 36.5	48.4 $\pm$ 2.9

Table 1. Effect of PG series of compounds on Kv2.1. τ values indicate the time constant of channel opening or closing at selected voltages.

Condition (Normalised to 20mV)	$V_{0.5}$	$\Delta V_{0.5}$	z
No compound (n=20)	-11.3 $\pm$ 0.6	--	3.8 $\pm$ 0.3
PG-1 (n=3)	-15.4 $\pm$ 0.5	-4.1 $\pm$ 0.8	3.2 $\pm$ 0.2

PG-2 (n=3)	-18.3 ± 0.7	-7.0 ± 0.9	3.4 ± 0.3
PG-3 (n=3)	-15.7 ± 0.4	-4.4 ± 0.7	3.6 ± 0.2
PG-4 (n=3)	-15.0 ± 0.5	-3.7 ± 0.8	4.2 ± 0.3

Table 2. $V_{0.5}$, $\Delta V_{0.5}$, and z values of channel activation obtained upon application of PG compounds on Kv2.1 channels. $V_{0.5}$ values represent the voltage at which half of all channels are open, z values represent the charge of the ions moving across the membranes.

6.3 Conclusion

In summary, we have successfully demonstrated how varying the carbohydrate structures on the amphiphilic glycopeptide backbone can modulate the activation and inactivation of the voltage-gated Kv2.1 channel. As a prototype, we synthesized fluorescent amphiphilic glycopeptides conjugated with HS disaccharide and sialic acid trisaccharide glycans and studied their expression on the cell surface. Our findings indicate that native sulfated HS disaccharide (PG-2) and sialic acid $\alpha(2-6)$ glycopeptides are more likely to be expressed on the cell membrane compared to non-sulfated and $\alpha(2-3)$ glycopeptides. Electrophysiological studies using *Xenopus laevis* oocytes showed that PG-2 has strong tendency to activate Kv2.1 channel, while heparan sulfate glycopeptides (PG-1 and PG-2) profoundly altered channel closure kinetics compared to sialic acid composed glycopeptides. Given the complexity in sulfation patterns and polysaccharide nature of HS on the cell membrane, HS oligosaccharides composed proteoglycan mimics is anticipated to enhance electrophysiological activity. We are currently expanding the glycopeptide platform with these HS ligands to observe strong VGIC activation and deactivation process with therapeutic applications.

6.4 Experimental Section

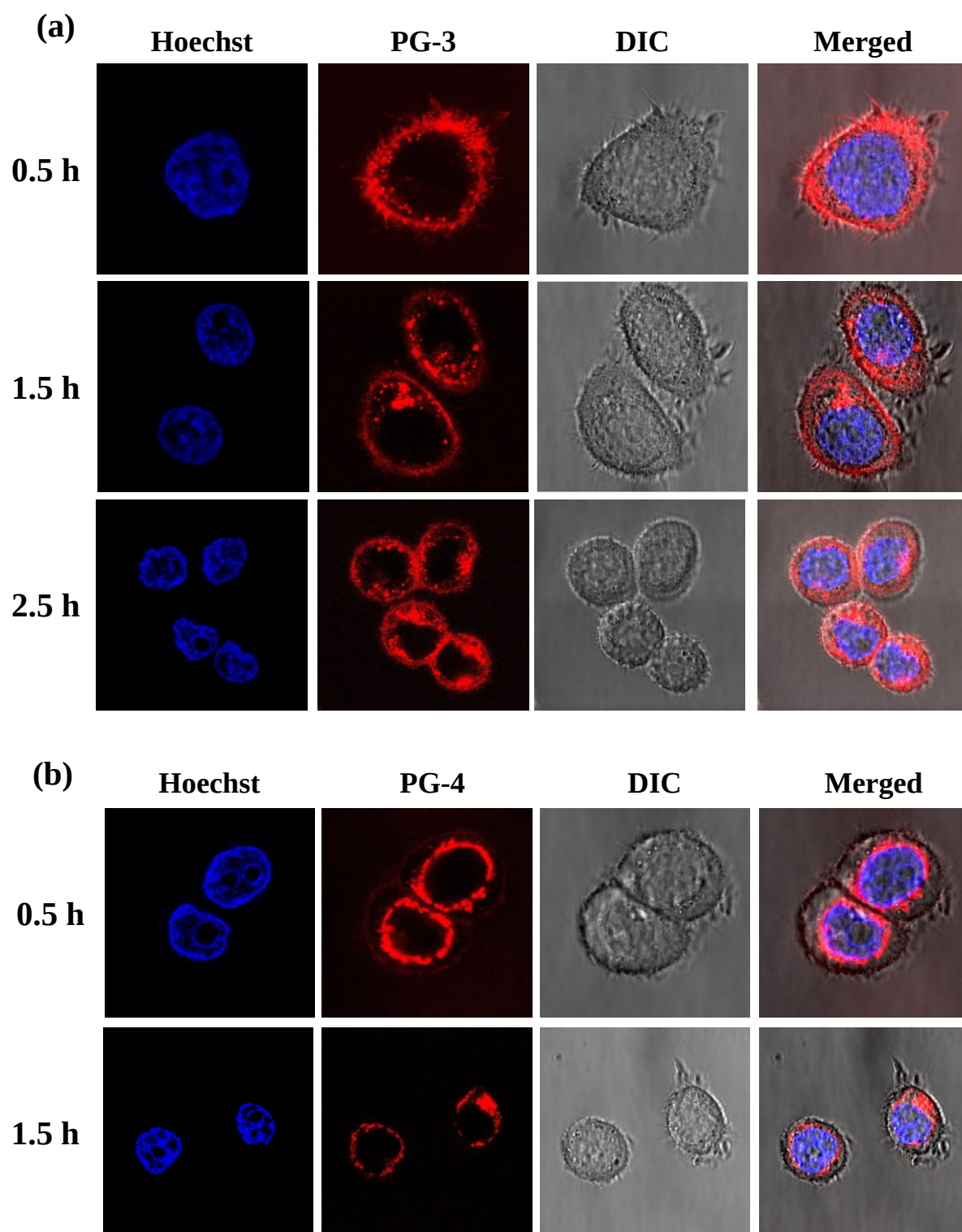
6.4.1 Cell Lines

MDA-MB-468 and NIH3T3 cells were cultured at 37 °C in a 5% CO₂ atmosphere in DMEM media supplemented with 10% fetal bovine serum (FBS) and 0.1% streptomycin.

6.4.2 Confocal Imaging Study

A total of 2×10^4 cells were seeded onto 8-well confocal imaging plates and incubated at 37 °C overnight. Subsequently, the cells were treated with **PG-1 to PG-4** (2 μ M) for 30 minutes. We use 2 μ M concentration. Following the treatment, the cells underwent three washes with PBS, and the nuclei were stained with Hoechst 33342. After the staining process, the media

was added, and fluorescent images were captured using a Leica SP8 (*Absorption* = 633 nm and *Emission* = 650 – 720 nm).



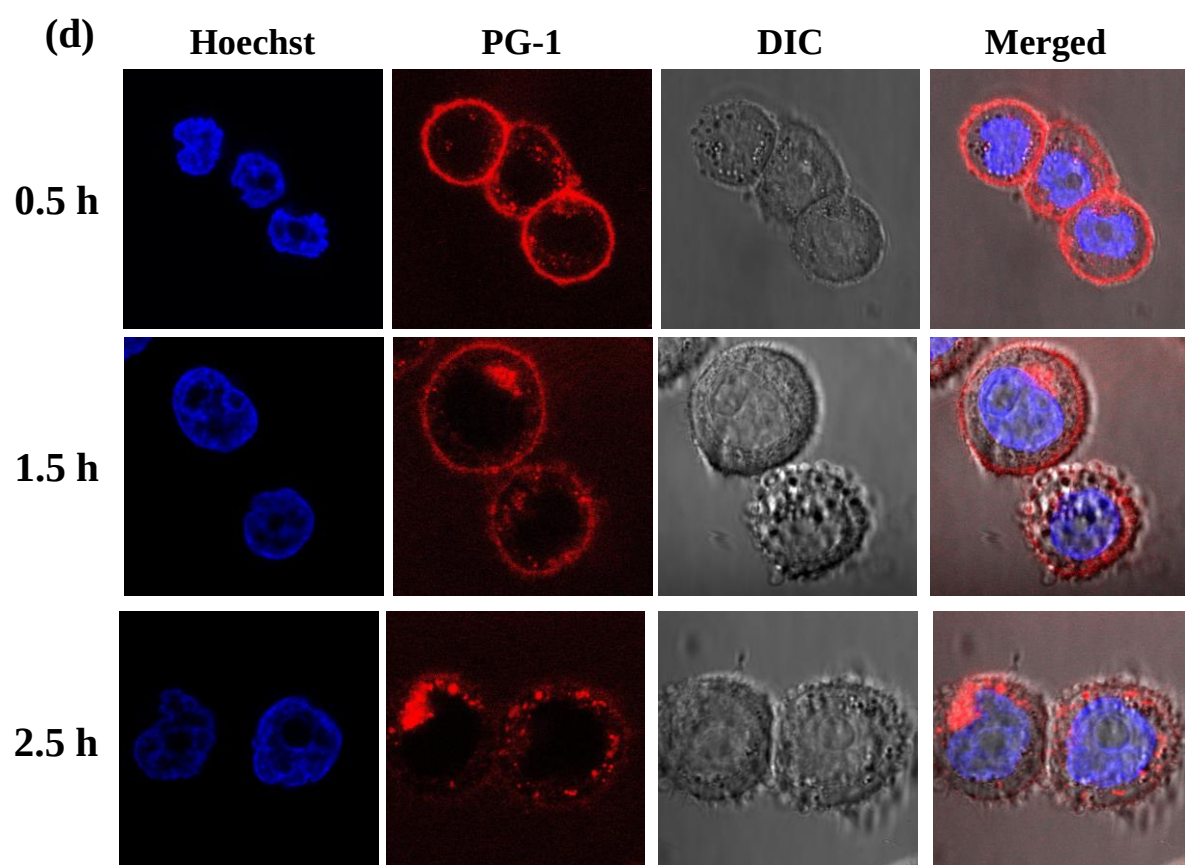
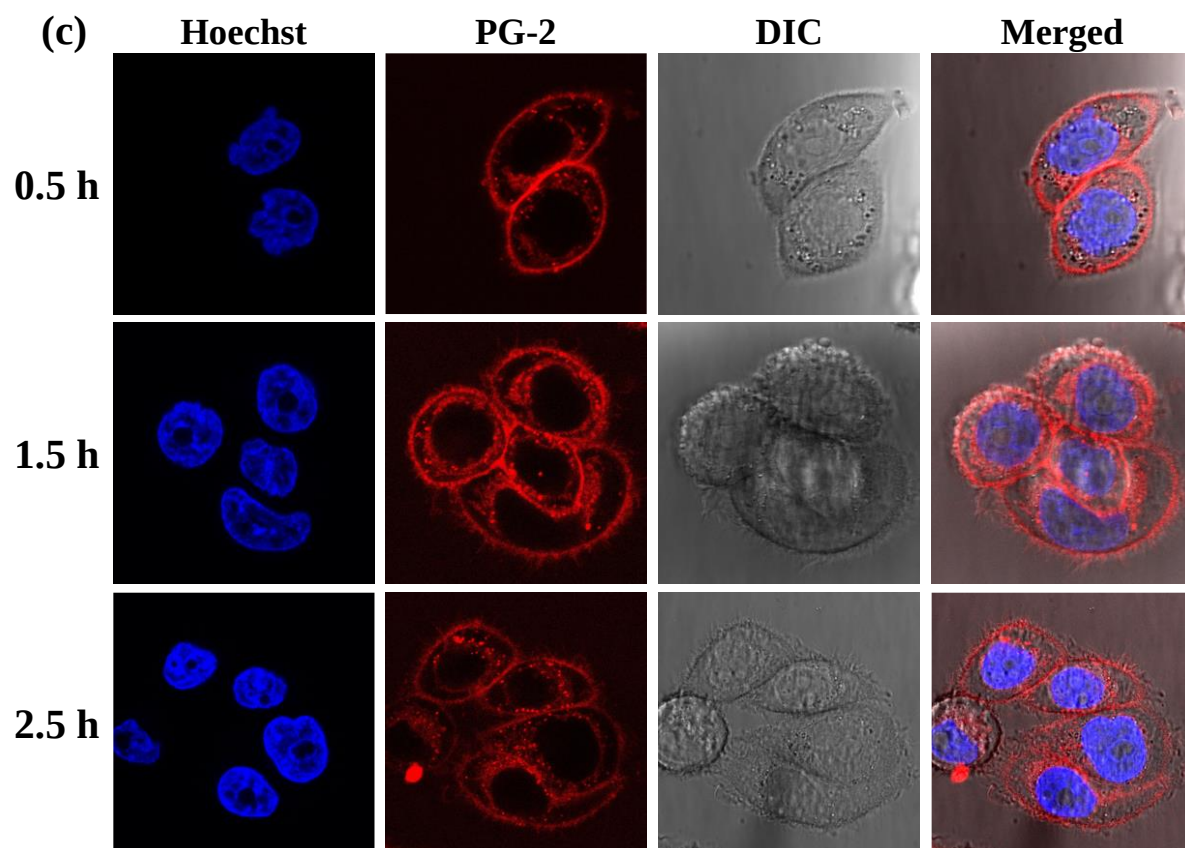


Figure 4. Confocal imaging of glycopeptides uptake by MDA-MB-468 cell lines at different time interval (a) PG-4, (b) PG-3, (c) PG-2, and (d) PG-1

6.4.3 FACS Analysis

Cells (5×10^5 cells per well) were seeded into a 24-well plate and incubated overnight. Subsequently, the cells were subjected to treatment with PG's mimetics for 1 hour. Followed by washing 3 times and re-suspended in FACS buffer for analysis.

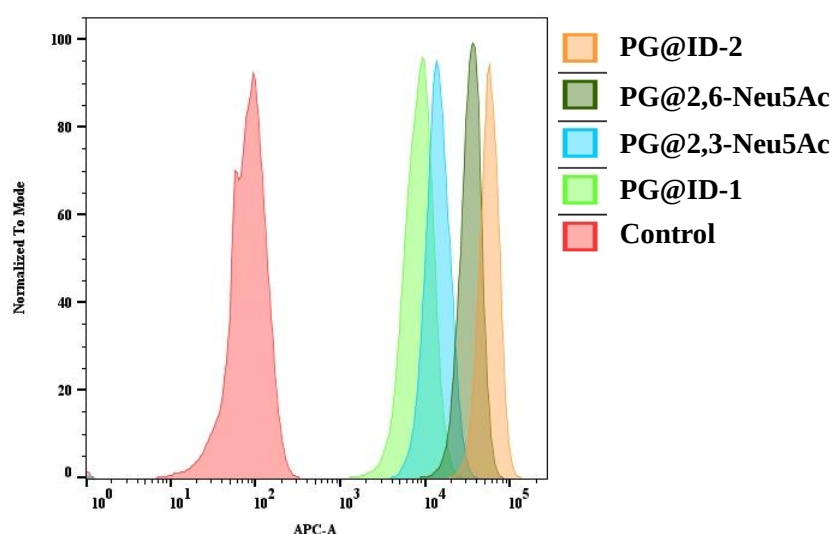


Figure 5. FACS analysis of proteoglycan mimetics uptake after 1 h incubation in MDA-MB 468 cells.

6.4.4 Voltage Clamp

Whole cell currents from *Xenopus* oocytes expressing Kv2.1 were recorded using an OC-725 oocyte clamp (Warner Instruments, Hamden, CT). All data was filtered at 1 kHz (eight pole Bessel) and digitized at 10 kHz. Micropipettes were filled with 3 M KCl. The extracellular solutions contained KCl (50 mM), NaCl (50 mM), MgCl₂ (1 mM), CaCl₂ (0.3 mM), and HEPES (20 mM) at pH 7.4, and the effect of each compound was monitored by three step-voltage protocols before and after the application of each compound. The voltage protocol to monitor activation consisted of depolarizing from a holding potential of -80 mV to $+60$ mV, with 10 mV increments per sweep. The voltage protocol for studying deactivation kinetics on the other hand consisted of an initial depolarization to $+50$ mV from a holding potential of -80

mV, followed by brief pulses ranging from –120 mV to +30 mV, to monitor kinetics of channel closure at various voltages, while the inactivation protocol consisted of long depolarizations of four seconds at +30 mV from a holding potential of +80 mV, with 10 mV increments per sweep. This was followed by a short 250 ms period at holding voltage, after which conductivity during a brief test pulse at +50 mV was measured. All experiments were performed at room temperature (~24°C), and all compounds were applied and tested in the absence of light.

6.4.5 Data Analysis

G–V relationships were obtained from currents elicited upon depolarization. The conductance at each voltage was calculated according to the equation $G = I/V - V_{rev}$, where G represents conductance, I represents the ionic current through the potassium channel in microamperes, V represents the voltage at which the current is elicited, and V_{rev} represents the reversal potential. The conductance is plotted against respective voltages and fit to the data using a single Boltzmann function, according to the following equation:

$$G/G_{max} = \left(1 + \exp^{-zF(V-V_{1/2})/RT}\right)^{-1}$$

where z is the valence, F is the Faraday constant, R is the gas constant, and T is the temperature. Mean time constants (τ) for activation and deactivation kinetics were obtained by fitting a single exponential equation to ionic currents of activation and tail currents respectively, and plotted against voltage.

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Conclusions and Future Prospects

It is becoming increasingly clear that innovative methods are required to study glycan interactions within the broader context of the cellular glycocalyx. Gadula and colleagues reported a lipid-functionalized glycopolymer-based approach for glycocalyx remodeling. They introduced heparan sulfate ligands onto these glycopolymers to remodel the plasma membrane of stem cells. This remodeling demonstrated how specific sulfation patterns of HS regulate growth factor-mediated stem cell differentiation. Mechanistic studies further identified the involvement of FGF2-HS in regulating MAPK pathways, which ultimately induce pluripotency and differentiation in mouse stem cells. A recent study by the same group highlighted how the uronic acid composition of heparan sulfate proteoglycan (HSPG) mimetics modulates viral infection and Wnt pathway activation. Alternatively, Hsieh-Wilson and her colleagues utilized liposomal delivery of lipidated chondroitin sulfate GAGs to the surfaces of cortical neurons, where they stimulated neurotrophin signaling and promoted neural outgrowths. Overall, cell surface engineering with synthetic mimetics has shown promising approach to understanding the roles of individual HS components in biological activity.

Inspired by these findings in the literature, we developed a novel platform to mimic HSPGs. We employed amphiphilic fluorescent glycopeptides for cell surface engineering and explored their potential biological applications. We synthesized a panel of HS proteoglycan mimetics, comprising disaccharides to hexasaccharide with a diverse range of sulfation patterns and uronic acid compositions. We reported that the degree of sulfation and uronic acid composition significantly influence the cell surface engineering of proteoglycan mimetics. We identified NeoMProbe as a potential probe for the plasma membrane and basement membrane. Furthermore, these molecules have proven to be a potential LYTAC model for the lysosomal degradation of extracellular proteins and cis-platin delivery. Finally, cell surface engineering with NeoMProbe was found to significantly modulate the activation and deactivation of voltage-gated ion channels. Overall, we have demonstrated the molecular-level effects of these glycomaterials on various biological roles of the glycocalyx. Despite considerable progress, advancing these molecules to the *in vivo* level remains a challenging task. We believe that future improvements in bioconjugation techniques and the synthesis of higher HS oligosaccharide-based proteoglycan mimetics will provide a more intricate understanding of glycocalyx GAGomics.