EXPLORE THE INTERACTION OF PROTEIN AND SYNTHETIC HEPARAN SULFATE VIA A MODULAR APPROACH

by

CHENGLI ZONG

(Under the Direction of Geert-Jan Boons)

ABSTRACT

Although it is widely accepted that HS is an information-rich polymer, the oligosaccharide structures that mediates particular biological interactions has been defined in only a few cases. This problem stems from the structural complexity of HS, which in turn, arises from a complex biosynthetic pathway. Lack of structure-activity relationship for HS-binding proteins makes it difficult to understand the physiology and pathology of HS at a molecular level and greatly complicates harnessing its therapeutic potential. As a continuation of our modular synthesis of HS, we have employed an anomeric aminopentyl linker protected by a benzyloxycarbonyl group modified by a perfluorodecyl tag for our synthesis, which enables the purification of polar intermediates by fluorous solid phase extraction. It also facilitate the repeated glycosylations to drive reactions to completion.

Then, we have employed both bottom-up and top-down approach for the identification of HS ligand for lectins.

In the first approach, the sulfation pattern was diversified at a late stage during the synthesis via per-sulfation, selective sulfation, de-sulfation and enzymatic 3-O-sulfation. Same strategy was applied to three other tetrasaccharides generating a library of 50 compounds. Microarray study

with those compounds confirmed the essential role of 2-O-sulfate for FGF2 binding. Further, we found configuration of the uronic acid is also important. As compounds with 2-O-sulfate on glucuronic acid does not show any binding.

The microarray data also indicated: by increasing the number of sulfate, their affinity with most lectins increased accordingly, which might correlate with the biological function of highly sulfated HS. Selectivity was observed for tetrasaccharides with intermediate sulfation level. With longer sequences or a more diversified library, we might get a better picture for their binding preferences. On the other hand, we also explored the top down approach combining enzymatic digestion, affinity column separation, mass spectrometry assisted structure identification, chemical synthesis, binding study via SPR to identify HS ligand for ROBO1. A unique octasaccharide sequence with a $K_D$ value of 3.5 μM was identified.

INDEX WORDS: Glycosaminoglycans, heparan sulfate, uronic acids, modular synthesis, fluorous assisted synthesis, stereoselective synthesis, pre/post-glycosylation oxidation, ROBO1.

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# DEDICATION

I would like to dedicate my thesis to my parents Dehuai Zong and Cuiling Yang, and my wife Tiantian Sun, for their undying support, love and encouragement. Your confidence and belief in me and my abilities has given me immense motivation to succeed.

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LIST OF ABBREVIATIONS

Ac .....Acetyl

AcCl…….....Acetyl chloride

AcOH.....Acetic acid

$Ac_2O$.....Acetic anhydride

AgOTf.....Silver triflate

Ar .....Aryl

AT III.....Antithrombin

BAIB..... bis(acetoxy)iodobenzene

Bn .....Benzyl

BTSA.....*N, O*-bis(trimethylsilyl)acetamide

BuOH.....Butanol

Bz .....Benzoyl

C.....Carbon

Cbz .....Benzyloxycarbonyl

$CHCl_3$ .....Chloroform

$CH_3CN$.....Acetonitrile

CSA.....Camphorsulfonic acid

COSY.....Correlation spectroscopy

DBU.....1,8-Diazabicylco[5.4.0]undec-7-ene

DCC.....Dicyclohexyl carbodiimide

DCE.....1,2 Dichloroethane

DCM .....Dichloromethane/Methylene chloride

DMAP .....N, N-Dimethylaminopyridine

DMF.....N, Ndimethylamineformamide

ESI-MS .....Electrospray ionization mass spectrometry

EtSH .....Ethanethiol

EtOH..... Ethanol

$Et_2O$.....Diethyl ether

GlcA.....Glucuronic acid

GlcNAc .....N-Acetyl glucosamine

GlcNS .....N-sulfonato glucosamine

$H_2$.....Hydrogen

HF.....Hydrogen fluoride

HSQC..... Heteronuclear Single Quantum Coherence

$NaN_3$.....Sodium azide

NaOH.....Sodium hydroxide

NaOMe.....Sodium Methoxide

$Na_2S_2O_3$ .....Sodium thiosulfate

$NH_3$ .....Ammonia

Pd .....Palladium

Pd/C .....Palladium on carbon

$Pd(OH)_2$ .....Palladium hydroxide

PBS .....Phosphate buffered saline

PMe3 .....Trimethyl phosphine

ppm .....parts per million

TEMPO.....2,2,6,6-Tetramethylpiperidinyloxy

TFA.....Trifluoroacetic acid

TfOH.....Trifluoromethane sulfonic acid

TLC.....Thin layer chromatography

THF.....Tetrahydrofuran

TMS.....Trimethylsilyl

TMSOTf .....Trimethylsilyl trifluoromethanesulfonate

TNF-α.....Tumor necrosis factor alpha

TOCSY .....Total correlation spectroscopy

TsOH.....p-Toluenesulfonic acid

UV .....Ultraviolet

# CHAPTER 1

# INTRODUCTION AND LITERATURE OVERVIEW

## Glycosaminoglycan

Glycosaminoglycans (abbreviated as GAGs) are long unbranched polysaccharides consisting of a repeating disaccharide unit (except for keratan) initiated by a conserved tetrasaccharide (GlcA-β1,3-Gal-β1,3-Gal-β1,4Xyl-β) including chondroitin sulfate, dermantan sulfate (also known as chondroitin sulfate B), heparan sulfate, heparin and hyaluronic acid. GAG chains can be post-synthetically modified by sulphation and epimerization (GlcA conversion to IdoA), producing substantial heterogeneity.[1] Additionally, while approximately 2% of the genome encodes the requisite proteins needed for normal glycosylation, glycan structures themselves are not encoded. Therefore, glycans are an important form of post-translational modification and via an epigenetic mechanism can regulate gene expression.[2,3] The repeating unit consists of an amino sugar (N-acetylglucosamine or N-acetylgalactosamine) along with an uronic sugar (glucuronic acid or iduronic acid) or galactose (Figure 1.1). Many glycosaminoglycans form complexes known as proteoglycan consisting a core protein and one or more covalently attached glycosaminoglycan chains, and can be secreted，transmembrane or glycosylphosphatidylinositol (GPI)-anchored.[1]

A given proteoglycan presenting in different cell types, or during various pathological process, or in different individuals often exhibits differences in the number of glycosaminoglycan chains, their lengths, and the arrangement of sulfated residues along the chains.[4] These

characteristics, typical of all proteoglycans, create diversity that may facilitate the formation of binding sites of variable density and affinity for different ligands.[1]

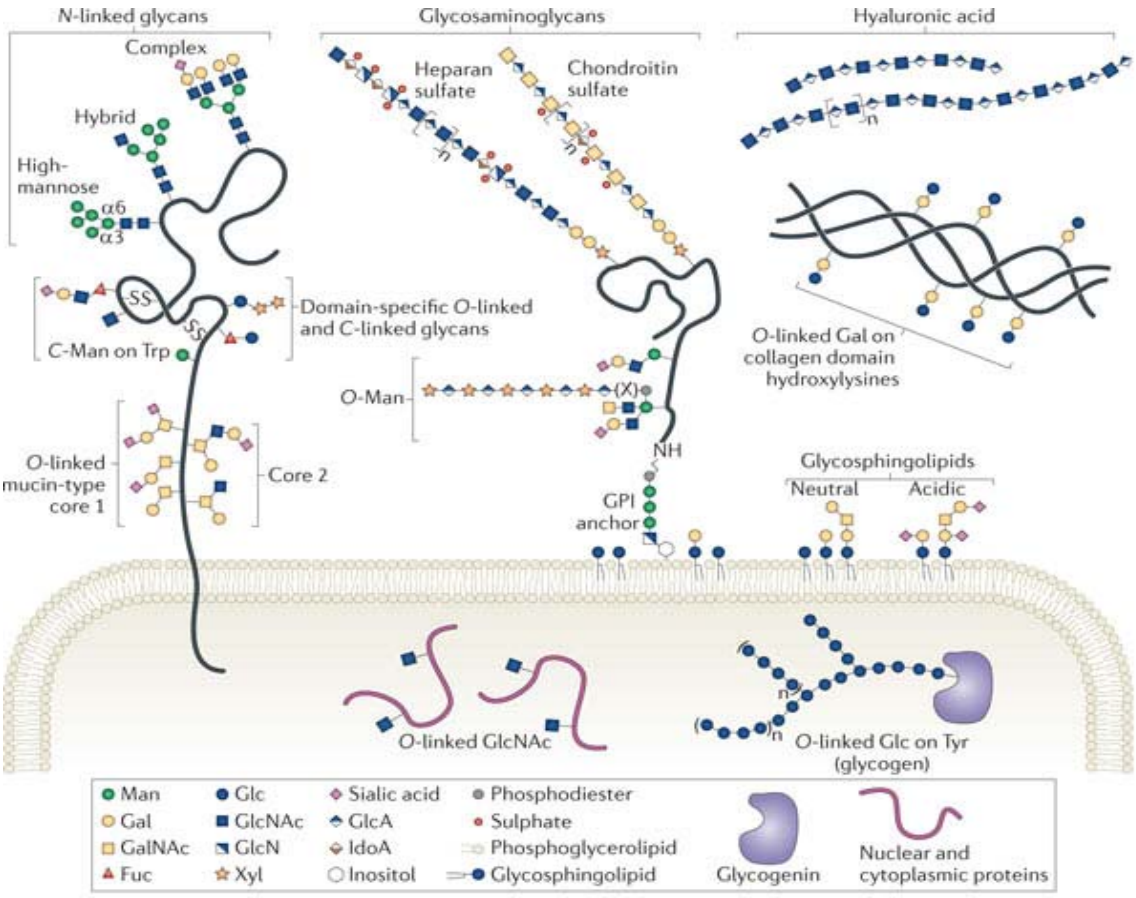



Figure 1.1 Major classes of vertebrate glycan structures. A symbol nomenclature of the various monosaccharides is described in the inset. Glc, glucose; Gal, galactose; Man, mannose; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; GlcA, glucuronic acid; IdoA, L-iduronic acid; Fuc. L-fucose; Xyl, xylose; Sia, silic acid; Ac, acetyl; P, phosphate; S, sulfate.

Unique among glycosaminoglycans, hyaluronic acid (HA), also referred to as hyaluronan or hyaluronate contains nonsulfated β-1, 3 linked D-glucuronic acid and D-N-acetylglucosamine disaccharide repeating unit that distributes widely throughout connective, epithelial and neural tissues, and is the only glycan that is not linked to a protein or lipid. Significant amount of HA is found in synovial fluid, vitreous humor, cartilage, blood vessels, skin and the umbilical cord. It

contributes significantly to cell proliferation, migration and may also be involved in the progression of some malignant tumors.[5] HA works by acting as a cushion and lubricant due to its outstanding ability to absorb water. In cartilage, changes in the composition of HA are related to osteoarthritis.[6] Thus it has been widely used as visco-supplementation in attempts to treat osteoarthritis of the knee via injecting it into the joint. However, clinical trials have proved hyaluronic acid is associated with insignificant benefit and an increased risk for serious adverse events such as inflammation. [7]

Now more studies have indicated HA gel injection can be useful for the treatment of ocular conditions such as upper eyelid retraction problem in thyroid eye disease [8] and lens regeneration. Over the last decade, in-depth studies have implied HA as an ideal carrier polymer for systemic drug delivery applications due to its biodegradable, non-toxic, non-immunogenic and non-inflammatory property.[9] It can achieve sustained drug release by months via gelation. Also, a relatively simple coupling method allows for modification of the sugar residues to get a variety of functional macrostructures that facilitates self-assembly and encapsulation of diverse drugs and gene payloads for targeted delivery. Of particular interesting to tumor selective delivery, the HA backbone is endowed with tumor targeting moieties that specifically recognizes CD44 – an integral membrane glycoprotein overexpressed on several tumors cell surfaces, including tumor initiating stem cells.[10]

Similar to HA, chondroitin sulfate (CS) is also an important component of cartilage and helps to maintain cartilage healthy by absorbing fluid into the connective tissue. It has been advocated, especially in the lay media, as a safe diet supplement for the management of symptoms of osteoarthritis. CS is a type of unbranched polysaccharide with variable length containing alternating D-glucuronic acid (GlcA) and N-acetyl-D-galactosamine (GalNAc). The GlcA residues can be epimerized to L-iduronic acid (IdoA); the resulting disaccharide is referred to as dermatan sulfate. In the most typical scenario, the C-4 and C-6 positions of the GalNAc with some chains having the C-2 position of glucuronic acid sulfated. They are usually covalently linked to proteins as part of a proteoglycan. As the most abundant glycosaminoglycan, CS is a major component of extracellular matrix and is essential for maintaining the structural integrity of different tissues.[11] Besides as a cushion in cartilage, CS can inhibit the expression of proteolytic enzymes that can cause cartilage breakdown,[12] and it can also provide building blocks to produce new or repair the damaged cartilage.[13] Loss of chondroitin sulfate in cartilage is related with osteoarthritis. The most common commercial available chondroitin sulfate comes from natural sources, such as shark and bovine cartilage. There is an only marginal improvement with the symptoms of osteoarthritis as being evidenced by several randomized clinical trials.[14, 15, 16] Thus CS has been used as a symptomatic slow-acting drug for the treatment of osteoarthritis. Recent

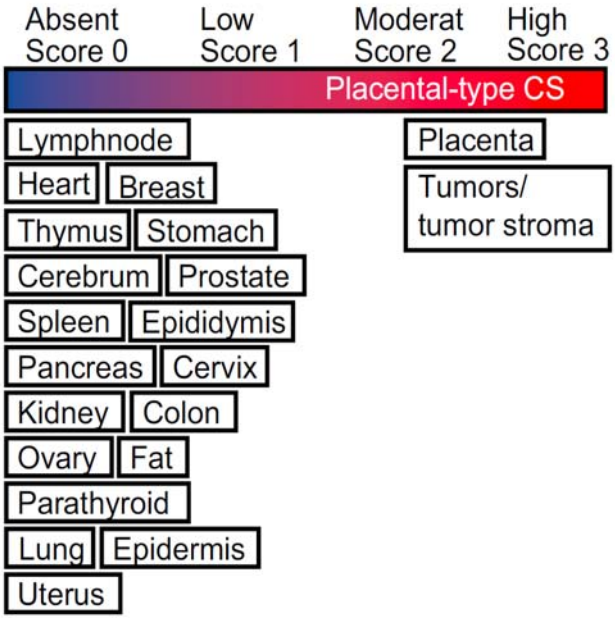



Figure 1.2 Schematic representation of pl-CS expression in the indicated tissue categories.

studies have identified a distinct type of chondroitin sulfate A (Figure 1.2) in aggressive cancer cell and placental syncytiotrophoblast cell surface which although incompletely resolved, might associate with the ability of trophoblasts to invade the uterine tissue and promote rapid cell proliferation and provide a new target for cancer treatment.[17]

Heparan sulfate chains are usually covalently linked to proteins to form proteoglycans (PGs) such as perlecan, agrin and collagen in the extracellular matrix, intracellular heparin PGs (serglycin); and cell-surface associated syndecans (SDCs) and glypicans (GPCs). Notably, whereas SDCs are transmembrane proteins, GPCs are associated with the plasma membrane *via* a GPI anchor. Topographical localization of HS chains on those two proteoglycans are also different: GPC HS chains are attached to the core protein close to the plasma membrane, whereas SDC HS chains are attached at more peripheral sites.[18]

As a more heavily and uniformly modified variant of HS, heparin is produced and stored in mast cells. These negatively charged polysaccharides are known to be necessary for storage of histamine, proteases and other inflammatory mediators within the granules.[19]

Syndecans 1-4 are components of cell plasma membrane. Each of them has a short cytoplasmic domain, an extracellular domain and a transmembrane domain with three to five HS or CS chains attached near the N-terminus. The molecular size of syndecan core proteins ranges from 22 to 45 kDa because of the distinct extracellular domains. Apart from participating cell-cell and cell-ECM interactions, syndecan is also involved in interactions inside of cells, such as cytoskeletal organization which responsible for cell adhesion and signal transduction.[6] Many cell types express more than one type of syndecans, and the expression levels may change during development and differentiation.[20] Mice lacking syndecan-1, -3 or -4 are all viable but exhibit

relatively mild defects, such as abnormal feeding behavior (syndecan-3) or delayed wound healing (syndecan-4).[21]

Glypicans are attached to the plasma membrane via a GPI anchor and carry three to four HS chains.[22] Glypican has six isoforms with molecular weight around 60 kDa and are all highly expressed during embryonic development. The glypican-3 deficient mice exhibit clinical features observed in Simpson-Golabi-Behmel syndrome patient such as perinatal death, abnormal kidney and lung development.[23]

Agrin, perlecan, and collagen XVIII are secreted ECM proteoglycans. Agrin, with molecular size 220 kDa, is most abundant in lung, kidney, and brain. It was found essential in the formation of neuromuscular junction by interacting with several receptors on the surface of skeletal muscle.[24, 25] While perlecan (~400 kDa) is the one of the largest extracellular matrix molecules and expressed in nearly all basement membrane and connective tissue. Recently, perlecan is also found in stromal space of various pathophysiological conditions. Perlecan is involved in early and late stages of embryogenesis, as well as in human diseases such as cancer and diabetes.[26] Deletion of perlecan[27] and agrin[28] lead to prenatal lethality due to severe developmental defects (perlecan) and disruption of neuromuscular junctions (agrin). Collagen XVIII is an essential component of epithelial and endothelial basement membrane and is also found in cartilage and fibrocartilage. Mice lacking collagen XVIII is viable but with developmental eye defects.[29]

**HS/heparin biosynthesis and structure**

Initiation of HS/heparin biosynthesis: HS/heparin chains are assembled on a core protein by enzymes in the Golgi compartment using nucleotide sugars imported from the cytoplasm. The protein and HS were linked by a tetrasaccharide sequence βGlcA1-3-β-Gal-1-3-β-Gal-1-4-β-Xyl-1-O-(Ser) at Ser residues of the respective core proteins (Figure 1.3). The GAG-attachment sites have the consensus peptide sequence Ser-Gly/Ala-X-Gly (where X stands for any amino acid) for GAG chain initiation.[30] The tetrasaccharides may be phosphorylated at C2 of the Xyl unit and sulfated at C4 and C6 of the two Gal units. It been shown that chondroitin or heparan sulfate polymerization does not occur when the Xyl residue of the linker region is phosphorylated.[31] Also, 4-O-sulfation of the second Gal residue in the linker region is associated with CS biosynthesis. Those studies seem to suggest phosphorylation and sulfation of the linker region may affect both HS and CS biosynthesis.[32] While others indicated the protein sequence proximate or distant from the GAG attachment site has a profound influence on the selectivity of CS or HS. [30]

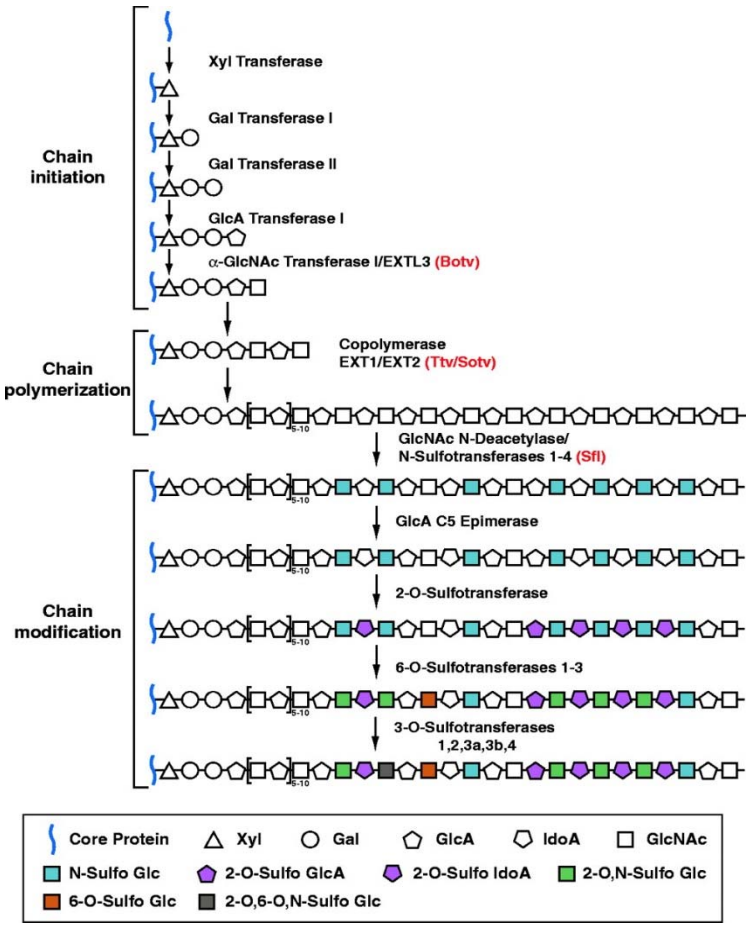


Figure 1.3 Heparan sulfate chain biosynthesis.

Polymerization of HS/heparin chains: after attaching a GlcNAc to the linker by exostosin-like-2/3 (EXTL-2/3), the chain is elongated by alternating addition of GlcA and GlcNAc catalyzed by polymerases (EXT-1/EXT-2 complex).[33] The nascent HS/heparin chains usually contain 20 to 100 disaccharide units.

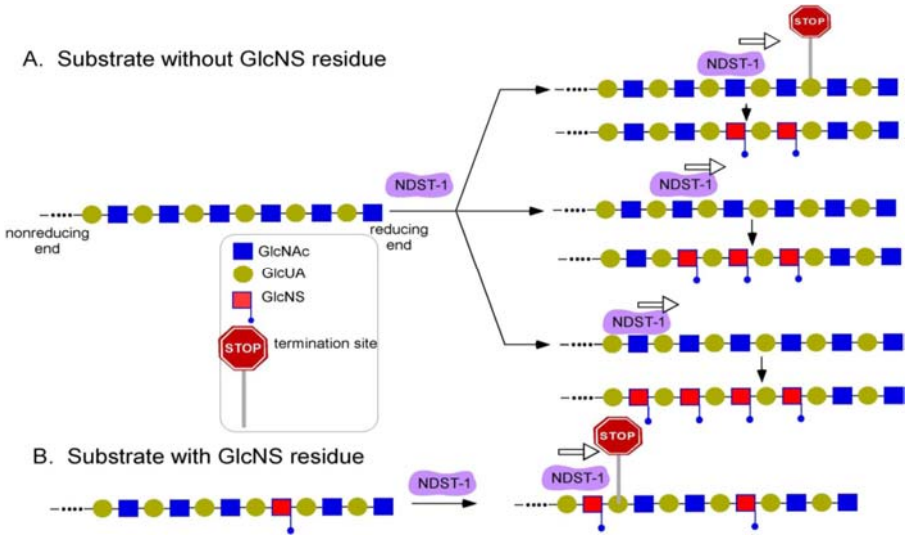



Figure 1.4 Mode of action of NDST-1. *Panel A* shows the scenario of the substrate without a GlcNS residue. *Panel B* shows the scenario of the substrate with a GlcNS residue.

Modification of HS/heparin chains: after the assembly of the chain, it undergoes a series of modification reactions involving N-deacetylation and *N*-sulfation of the GlcNAc, epimerization of GlcA to IdoA, and O-sulfation at various positions.[1] Modification of N-deacetylation/N-sulfation of GlcNAc residues into GlcNS was considered a prerequisite for all subsequent modifications and is catalyzed by N-deacetylase/N-sulfotransferase1-4 (Ndst 1-4). The N-deacetylase activity removes acetyl groups from GlcNAc residues, generating free amino groups, which are then sulfated through the N-sulfotransferase activity. Most cells express at least two Ndst isoforms, usually Ndst-1 and -2.[34] The latter enzyme was found as a predominance in mast cells, thus suggesting a role of Ndst-2 in the generation of NS domains. Transfection of HS-producing cells with NDST-2 led to the formation of an almost exclusively *N*-sulfated

polysaccharide, whereas overexpression of NDST-1 had little effect on the *N*-substitution pattern.[35] While NDST-1 is tentatively implicated in the formation of the NA/NS domains typically found in HS. It can initiate the *N*-sulfation at any internal GlcNAc residue, and the sulfation proceeds from nonreducing to reducing end. The *N*-sulfation stops at the residue that is five saccharide units away from the reducing end (Figure 1.4A). On the other hand, pre-existing GlcNS residues prevent the *N*-sulfation immediately located at the nonreducing end, leaving the gap between two GlcNS residues. The gap should be at least five saccharide units long (Figure 1.4B). [36]

*Ndsts* were considered as gateway enzymes since subsequent modifications by C5-epimerase, 2-*O*-sulfotransferase, 3-*O*-sulfotransferase, and 6-*O*-sulfotransferase are dictated by the location of *N*-sulfate groups. [36] However, heparan sulfate synthesized by mouse embryonic stem cells deficient in Ndst-1 and Ndst-2 completely lacks *N*-sulfation but still contains 6-*O*-sulfate groups, demonstrating that 6-*O*-sulfation can occur without prior *N*-sulfation.[37] Also, by incubating mature bovine kidney HS Ndst-2 in the presence of [$^{35}$S]PAPS, NS was inhibited by 2-O-sulfation while GlcNAc6S units were all modified by NS. These studies provide in vitro evidence showing that mediated modification is not necessarily just a gateway modification during HS biosynthesis.[38]

In HS biosynthesis, some GlcNAc residues escape N-deacetylation /N-sulfation and are not further modified. Thus, three types of domain structure are generated including N-acetylated regions (NA domains), 2-*O*, 6-*O*-sulfated and *N*-sulfated regions that contain both IdoA and GlcA residues (NS domains) and mixed regions of alternating N-sulfated and N-acetylated disaccharides (NA/NS domains). Some of the enzymes involved in this process are known to function as a complex, for instance, EXT-1/EXT-2, [39] EXT-2/Ndst-1 and Hsepi/Hs2st.[40]

Mice deficient in Ndst-1 are neonatally lethal due to lung defect;[41] Whereas Ndst-2-deficient mice are viable and fertile but have fewer connective-tissue-type mast cells; these cells have an altered morphology and contain severely reduced amounts of histamine and mast cell proteases.[42]

Glucuronyl C5-epimerase (Hsepi) catalyzes the reversible conversion of GlcA to IdoA that occurs in contiguous N-sulfated regions of HS/heparin chains as well as in regions composed of interspersed N-acetylated and N-sulfated disaccharide units. Substrate recognition requires N-sulfate at the non-reducing side of the HexA and will accept neither 2-O-sulfated nor 6-O-sulfated units.[43] The conversion involves two steps: the abstraction of the C5 proton, which results in the formation of a carbanion intermediate, followed by the addition of a proton to form either L-IdoA or D-GlA. It seems the production of IdoA residues in vivo is not directly correlated to the quantity of the enzyme, as the heterozygous embryos expressing 50% of the enzyme produced identical HS as in the wild-type.[43] Due to its flexible conformation, IdoA is critical for the interaction of HS with protein ligands. The Hsepi mutant mice resulting in a structurally altered HS/heparin lacking IdoA is neonatally lethal with renal agenesis, lung defects and skeletal malformations. Unexpectedly, major organ systems, including the brain, liver, gastrointestinal tract and heart appear normal. [44]

Following epimerization, 2-O-sulfotransferase (Hs2st) catalyzed the transfer of sulfate to the C2-position of IdoA adjacent to N-sulfated glucosamine within the nascent HS chain. *In vivo* study have confirmed the formation of complexes between C5-epimerase and Hs2st which not only locks the configuration of IdA but also contributes to the rapidity of heparan sulfate formation.[40] 2-O-sulfate transferred by Hs2st are needed to form signaling complexes between HS, FGF2, and FGFR1 that activate Erk1/2.[45] Deletion of Hs2st results in kidney and skeletal

malformations.[44] Although 2-O-sulfation occurs mostly on IdoA, 2-O-sulfated-GlcA residues are rare but can also be detected in HS/heparin chains.[46]

6-O-sulfotransferase (Hs6st) catalyzed 6-O-sulfation to both GlcNAc and GlcNS residues, with a preference for GlcNS residues. In mammals, at least three isoforms of Hs6st have been identified, and each of them has a distinct substrate preference. Many studies indicated that Hs6st-1 sulfates predominantly C6 of GlcNS residues in HexA-GlcNS unit, whereas Hs6st-2 and Hs6st-3 transfer sulfate preferentially to position 6 of GlcNS residues in IdoA2SGlcNS unit. Similar with other sulfotransferases, Hs6st transcripts are differentially expressed in different tissues as well as developmental stages. For example, compared with Hs6st-3 that is ubiquitously expressed, Hs6st-1and Hs6st-2 are highly expressed in mouse liver and brain respectively.[47] Therefore distinctive structures of HS produced in a spatiotemporally regulated manner can potentially control the function of heparin-binding proteins such as growth factors, morphogens, and extracellular proteins.

3-O-sulfation catalyzed by 3-O-sulfotransferase (Hs3st) is a relative rare modification within the biosynthesis system. In mammals, a family of seven isoforms have been discovered and constitutes the largest group of sulfotransferases involved in heparan sulfate formation.[48] Although rare, 3-O-sulfation is a biologically significant modification and is found initially essential for antithrombin (AT) binding and infection of type I herpes simplex virus. With the development of ultrasensitive instruments, more 3-O-sulfate related biological process will emerge. On the other hand, mice lacking Hs3st-1 only showed growth retardation, which suggests overlapping functions or compensatory mechanisms of the isoforms.[49] Interestingly, knockdown of Hs3st-7 results in a very distinct phenotype, including loss of cardiac ventricle contraction. These studies suggested that knockdown of Hs3st family members did not result in the same phenotype, indicating specific

Hs3st has distinct functions in vertebrates and lending *in vivo* evidence for the glyco-code hypothesis.[50]

Heparin and HS share similar biosynthetic pathway. But heparin has a higher level of IdoA, sulfation than HS, thus, the primary disaccharide unit in heparin is IdoA2S-GlcNS6S.

Regulation of HS structure: mounting evidence suggests specific sulfation patterns of HS can determine its affinity for a ligand. However, how the HS biosynthesis is regulated remains still largely unknown due to the lack of analytical methods for high-capacity, high-resolution sequence analysis of full-length HS chains. As a compromising alternative, many groups employ digested disaccharides for structural analysis. However, this method does not reveal the detailed sequence information of the oligosaccharide. Plus, certain regions of heparin/HS have been shown to be resistant to digestion down to disaccharides, biasing the composition results.[51,52]

Based on current biochemical studies, several mechanisms might contribute to the regulation: 1) the competition between HS synthesis and CS synthesis. 2) Variations in concentration of enzymes including sulfotransferases, heparanase and sulfatases under different biological conditions or in tissues, and the random binding to their substrates are possible reasons for HS structural variability. 3) the modification of HS/heparin chains is partly regulated by the substrate specificities of the enzymes involved in the process. For example, compared with single 2-O-sulfotransferase, there are three 6-O-sulfotransferases and seven 3-O-sulfotransferases.

**Similarities and differences between heparan sulfate and heparin.**

As shown in Figure 1.5, both heparan sulfate and heparin consist of alternating units of GlcN with GlcA or IdoA. Heparan sulfate contains a higher proportion of GlcA, whereas heparin contains more IdoA. Both molecules are sulfated (illustrated here by blue circles). O-sulfation may occur on position 2 of the uronic acid, 3 and 6 position of the amino-sugar, while the glucosamine

nitrogen may be sulfated, acetylated or, less frequently, unmodified, leading to 48 possible disaccharides. The diversity grows exponentially with the polymer length, leading to 2304 possible tetrasaccharides, 110,592 hexasaccharides and more than $5*10^6$ octasaccharides. [53,54] Furthermore, HS can adopt longer, more flexible chains in solution than heparin and exists as a proteoglycan component on cell surfaces and throughout tissue matrices. Whereas the closely related molecule heparin, which is co-released with histamine and covalently attached to serglycin, is synthesized by, and stored in mast cells. However, other than mast cells, some cells or tissues express heparin-like structures, for example, all leukocyte classes from four human donors[55] and liver cells[56]. The latter organ has a high level of 2-O-sulfate (40%). Such phenomenon might relate to their ability for immune response, hepatitis C virus binding[57], hepatic clearance of triglyceride-rich lipoproteins[58] and other HSPG-mediated endocytosis. In line with the studies above, conditional knockout mice deficient in 2-O-sulfotransferase, but not in 6-O-sulfotransferase, showed delayed clearance of lipoproteins in the liver.[59] Thus, it has become apparent that the designations heparin or HS are less clear-cut than this description implies, and that polysaccharides isolated from some organisms appear to be hybrid.[60]

It is possible, therefore, to consider heparin a tissue-specific form of HS with the conventional definitions of heparin and HS residing towards two opposite extremes. More detailed studies across many species and tissues will provide a more complete picture of the extent of sulfation and structural diversity throughout the animal kingdom.[54,60]

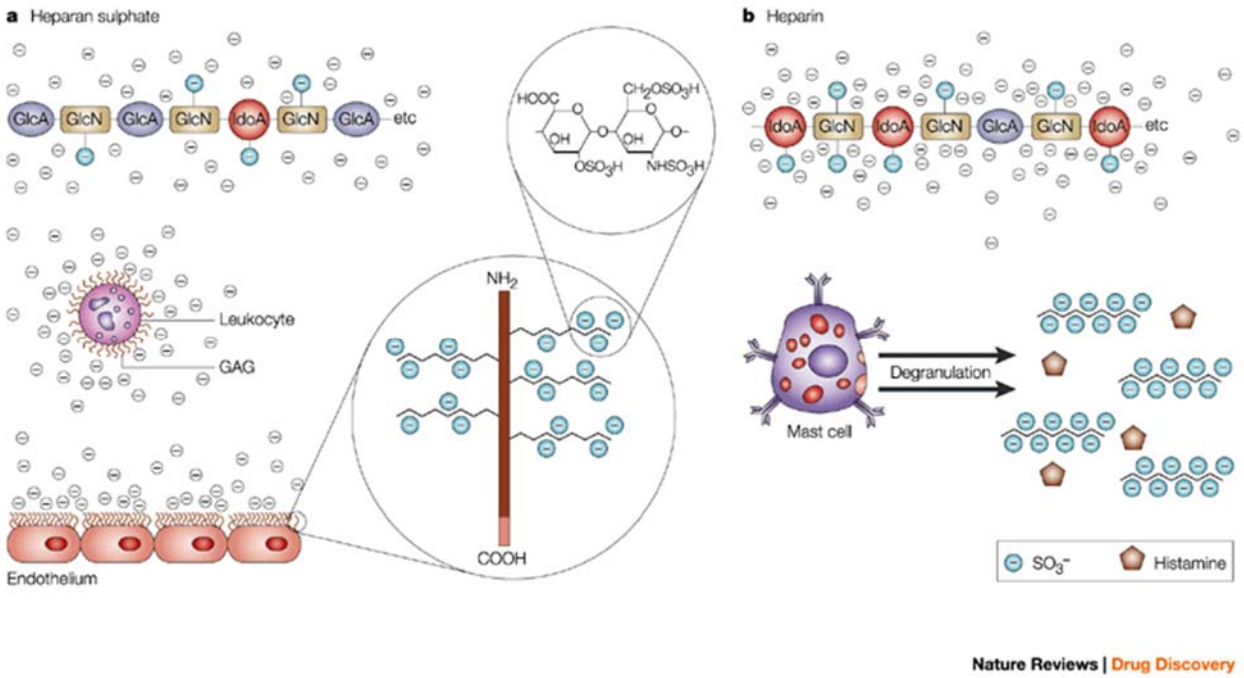



Figure 1.5 Similarities and differences between heparan sulphate and heparin.

**Interactions of HS and proteins**

HS contains multiple binding sites, thus, can oligomerize various signaling proteins. HS can also act as a storage reservoir and increase their local concentration, at the same time, prevent proteolysis. It can also facilitate the formation of morphogen gradients essential for cell specification during development and chemokine gradients during leukocyte recruitment and

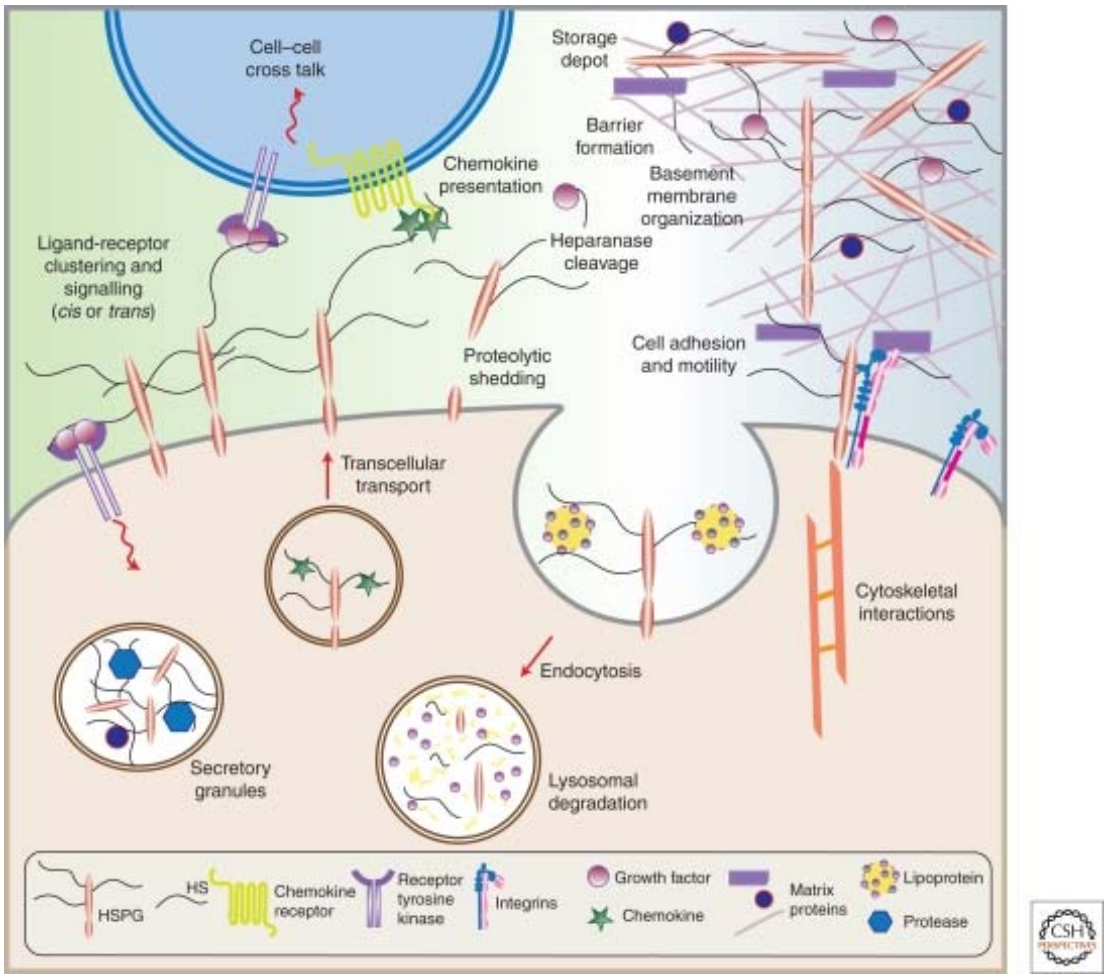



Figure 1.6 Multiple functions of HSPGs in cells and tissues.

homing.[61] As a major component of ECM, HSPGs contribute significantly to its integrity by binding and assembling ECM proteins, thereby playing important roles in cell–cell and cell–ECM interactions. Heparin can maintain proteases in an active state, and regulating various biological activities after secretion such as coagulation, host defense, and wound repair. These interactions play important roles in the control of physiological and pathological processes, such as morphogenesis, tissue repair, inflammation, vascularization and cancer metastasis (Figure 1.6).

Depending on the repertoire of other membrane proteins, perhaps most importantly integrin and lipoprotein receptors, cell-surface HSPGs may be located to various plasma membrane

domains. Moreover, the status of the HS biosynthesis, modification and degradation machineries set the stage for how cells bind to a wide variety of extracellular, polybasic ligands. Thus, an interesting and challenging study subject is the biological relevance of HS-ligand interactions.[18,21]

Binding of the negatively charged GAGs to positively charged amino acid residues such as lysine and arginine contributes to the binding of heparin/HS to proteins. The interaction with arginine sidechains is considered to be stronger. Other factors, for instance, the configuration of the uronic acid also affects the binding. Some of the data regarding the binding specificity were obtained by X-ray crystallography, in which small sugar fragments obtained by enzymatic digestion of heparin served as substitutes for the physiologically relevant heparin sulfate. Despite the high resolution in measuring atomic position, protein structural analysis by X-ray crystallography is subject to a number of limitations including structural packing in which conformational artefacts can be created, incomplete solvation, which contrasts the normal physiological conditions, and the fact that usually crystallography cannot be employed to study protein–polysaccharide complexes nor, in many cases, analyze glycosylated and structurally disordered proteins. Moreover, under physiological situations, the binding processes are usually dynamic while protein structures obtained from crystallography can be viewed as ‘frozen’ in a particular conformation.[62] Site-directed mutagenesis, although only provides binding formation about the protein, has been widely used as a key method to study heparan sulfate and protein interactions.

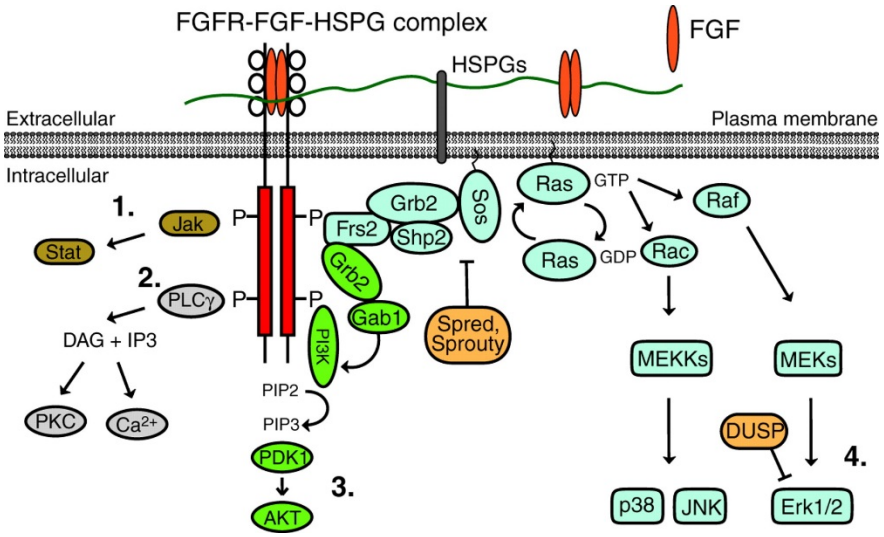



Figure 1.7 The FGF-FGFR signaling pathway

Fibroblast growth factors (FGFs) belong to a family of growth factors essential for embryonic development, wound healing, angiogenesis and various endocrine signaling pathways. HS has been proved to be a key cofactor during FGFs signaling. Binding of FGFs to their cognate cell surface FGF-receptors (FGFRs) requires HS chains as a template that bridges and stabilizes FGFs and FGFRs interaction (Figure 1.7).[63]

So far, 22 FGF members and four types of FGFRs (FGFR1-4) have been identified. FGFR contains three extracellular immunoglobulin (Ig)-like domains, an acidic, a transmembrane helix and two intracellular tyrosine kinase domains. Alternative RNA splicing resulting in two different Ig-like domain III (b and c) in FGFR 1-3, which is an essential determinant of ligand binding specificity.[64]

The interaction of FGF-FGFR-HS is essential in several signaling cascades implicated in many biological and pathological processes.The rasmitogen activated protein kinase (MAPK) pathway has been well studied and is associated with the mitogenic response that is responsible for cell migration and proliferation. [65] Recent studies on FGF1 and FGF2 have demonstrated that

the HS-dependent signaling relies on stabilization of the FGF-HS-FGFR ternary complex, and the overall level of sulfation is more important than the spatial distribution of the sulfation. [66]

Basic fibroblast growth factor (bFGF also called FGF2) was the first growth factor shown to depend on HS for interaction with its receptor.[67] Two crystal structures of FGF-HS-FGFR ternary complex have been published: symmetrical 2:2:2 complex involving two copies of each;[68] asymmetrical structure 2:2:1 complex with a single heparin chain that can bridge in between FGF2 and FGFR.[69] Later studies demonstrated both interaction modes are possible depending on the length and structure of the HS/heparin oligosaccharide. For example, heparin fragments larger than octasaccharide dimerize FGF2 on heparin, which leads to a rapid dimerization of FGFR1 on FGF2 (FGF2-FGFR-heparin 2:2:1); on the other hand, shorter heparin fragments form 1:1:1 complex (FGF2-Heparin-FGFR) and then the association of the 1:1:1 complexes to form symmetrical 2:2:2 complexes. [70]

Affinity chromatography was first employed and identified a dp 14 oligosaccharides with 2-O-sulfate that's responsible for FGF2 binding. Other oligosaccharides with a relatively low content of IdoA(2S) but a corresponding increase in 6-sulphate groups displayed a low or intermediate affinity for bFGF, emphasizing the significance of C-2 sulphation of IdoA residues.[71]

Structural and biochemical studies have shown that the chain length, sulfation pattern, and conformation of HS play critical roles in FGF binding and activity. Prof. Gaulier and coworkers reported NMR structural analysis using two-dimensional NOE spectroscopy (2D-NOESY) and transferred NOESY (trNOESY) on a non-6-O-sulfated synthetic tetrasaccharide (ANS-I2S-ANS-I2S-OPr) both in its free state and bound to FGF2. This tetrasaccharide comprises both the structural trisaccharide motif ANS-I2S-ANS that forms "kinks" in longer heparin chains induced by FGF binding[72] and the common binding motif I2S-ANS-I2S present in octasaccharides that

exhibited strong FGF2 binding.[73] These data suggest that tetrasaccharide could be the shortest HS that binds to FGF2. Furthermore, their study confirms that both the IdoA residues in TETRA adopt the chair $^{1}C_{4}$ conformation upon FGF2 binding to provide the best molecular fit in contrast to an analogous 6-O-sulfated tetrasaccharide motif observed in the FGF2-HS cocrystal structure where one of the IdoAs adopts skew-boat 2SO conformation. Thus, their study highlights the fact that the conformational plurality of IdoA can accommodate changes in the sulfation pattern to provide the necessary specificity for protein binding.[74]

HS binds to mammalian FGFs with different specificity arising from their variable N- and C-terminal regions. Prof. Huang and coworkers screened 48 HS-based disaccharides with FGF1 using isothermal titration calorimetry (ITC). In accordance with X-ray analysis of the cocrystal of FGF-1 and disaccharides, the interaction involves N-sulfonate of GlcN and the 2-O-sulfonate of IdoA. 3-O-sulfate contribute to the binding. While 6-O-sulfate does not improve the binding, it might involve in the oligomerization or interact with FGFR.[75] Controversially, by NMR study Prof. Guglier and coworkers showed that a tetrasaccharide of the form GlcNS,6X-I2S-GlcNS,6X-I2S-OPr (where X is OH or O-sulfate, and Pr is propyl) with at least one of the GlcNS,6X residues having a 6-O sulfate group was the minimum binding motif for FGF1.[76] Thus more in-depth study are required to define the specificity for the binding event.

This multiple components model stabilized by HS has been extended to many other signaling pathways, including Hedgehog (Hh),[77] bone morphogenic protein (BMP) and Wnt signaling[78]. However, in some cases, it seems even without heparan sulfate, the signaling protein can still interact with the according receptor to activate the signaling pathway, for example, FGFR related Erk activation.

In addition to growth factors, a large number of proteins with a broad range of functions are known to bind HS/heparin. Although it is known that sulfate, carboxyl groups and configuration of uronic acid in HS chains play key roles by interacting with basic residues on the proteins, little is understood about how these ionic interactions provide the necessary function.

Roundabout 1 (Robo1) is a transmembrane protein and mainly expressed in nervous systems and have been established to be the cognate receptor secreted axon guidance molecule (Slit). In addition to neuron-related functions such as axon branching and neuron migration, Slit-Robo interaction also regulates non-neuron-related functions, such as muscle precursor cell migration, leukocyte trafficking, development of lung, kidney, heart, and diaphragm, inflammation, tumor metastasis, angiogenesis and hematopoietic stem cell trafficking (Figure 1.8).[79]

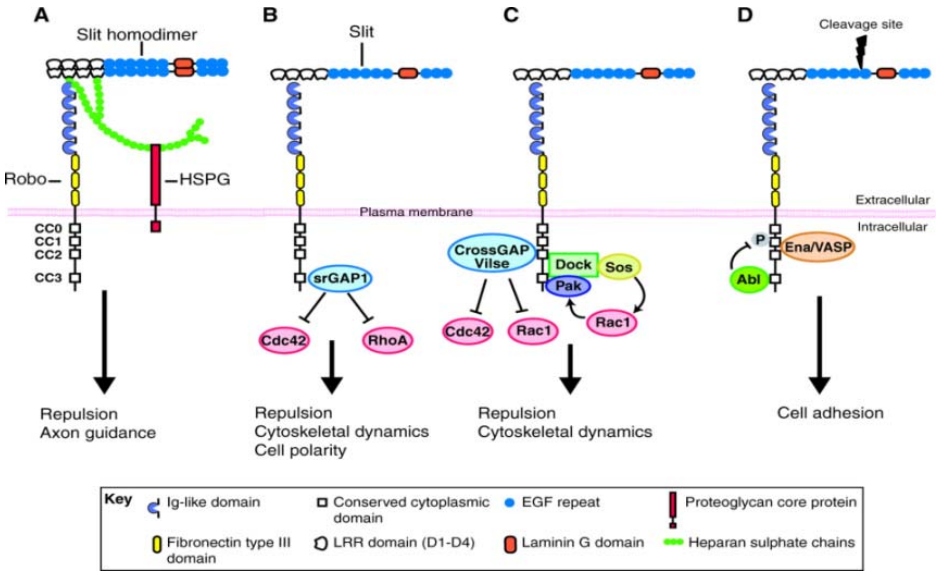



Figure 1.8 A schematic representation of Slit-Robo signaling

Ronca and coworkers have demonstrated Slit2 binds to HS on glypican-1 in brain tissues with high affinity.[80] *C. elegans* lacking HS-modifying enzymes exhibit distinct as well as overlapping axonal and cellular guidance defects in specific neuron classes which are related to

Robo function.[81] Along with other studies, it has been confirmed that HS can interact with Slit and Robo simultaneously, resulting in the formation of ternary Slit/Robo/HS signaling complexes on cell surface thereby facilitating the Slite-Robo signaling, reminiscent of the well-accepted FGF/FGFR/HS and VEGF/VEGFR/HS models.

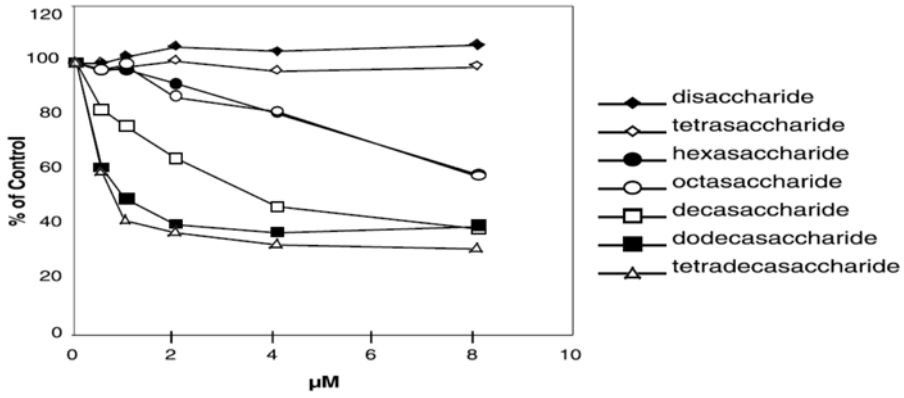



Figure 1.9 Inhibition of glypican-1 binding to Slit-2 protein in the presence of heparin

Heparin, as a highly sulfated form of HS that is abundantly available, was used as a substituent of HS to determine the structural features required for HS to interact with Slit2 and Slit3, and demonstrated that Slit shows a size dependent heparin/HS-specific high-affinity interaction (Figure 1.9).[80] Several research have shown that although all sulfate groups are necessary for the Robo1-heparin interaction, *N*-sulfo and 6-*O*-sulfo groups are essential for the Robo1 heparin binding (Figure 1.10).[80]

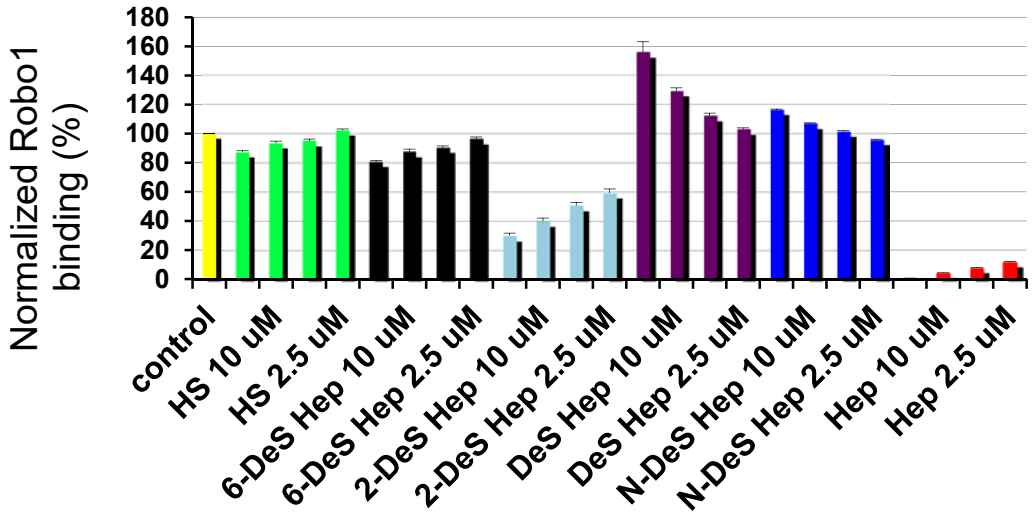



Figure 1.10 Bar graph of normalized Robo1 binding preference to surface immobilized heparin by competing with HS or different chemically modified heparin in solution.

**Heparan sulfate and diseases**

The interaction between HS and proteins is critical for many biological processes[79] including cell–cell and cell–matrix interactions, cell migration and proliferation, growth factor sequestration, chemokine and cytokine activation,[79] microbial recognition[79,82] and tissue morphogenesis during embryonic development.[84] Alteration in HS expression has been associated with disease.[85,86]

Heparin as an anticoagulant drug: after the discovery of heparin by McLean in 1916, Brinkhous and coworkers demonstrated the anticoagulant activity of heparin requires antithrombin III (AT III) as a plasma cofactor.[87] AT III is a serine protease inhibitor (serpin) and inhibits the activity of the coagulation factors Xa and thrombin (in the coagulation cascade) which are serine proteases. The serpins react with serine proteases to form inactive complexes which are then cleared from blood circulation. The binding of AT III to the coagulation factors is accelerated by binding of AT III to heparin. Initial interaction of a unique pentasaccharide within heparin with AT III is a weak interaction. Upon binding, AT III undergoes a conformational change which promotes additional interaction between heparin and AT III.88

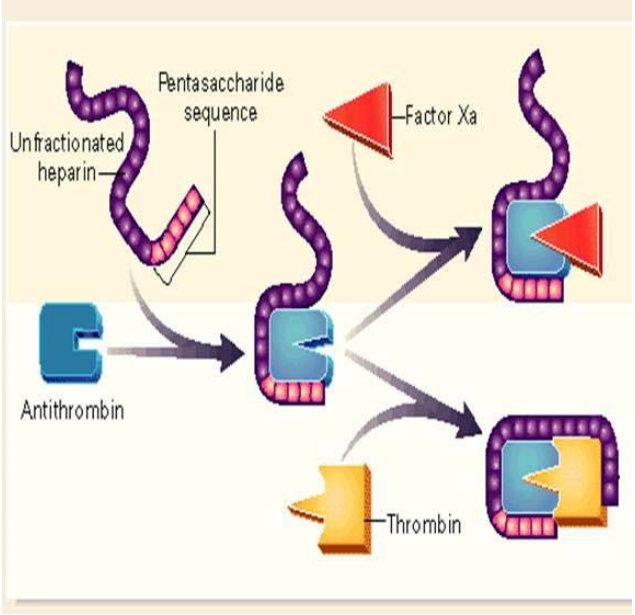


Figure 1.11 heparin mechanism of anticoagulant

This conformational change also leads to binding of a reactive site loop on AT III to the factor Xa and other proteases. The conformational altered AT III is covalently bound to the serine protease yielding a cleaved and conformationally altered AT III as an acyl enzyme intermediate.[89] As a result of the cleavage, the affinity between AT III and heparin decreased significantly, leading to the dissociation of the unaltered heparin from the complex, free to catalyze more AT III-serine protease reactions. While the pentasaccharide is sufficient to bind to AT III and inhibit factor Xa, a 16-18-mer containing the pentasaccharide is required to accelerate the reaction of AT III to thrombin (Figure 1.11). This can be explained by the fact that the polysaccharide binds to both AT III and thrombin, thus bringing both proteins in close proximity. While AT III binds strongly to

the pentasaccharide, the extended HS sequence binds non-specifically to thrombin. Thrombin diffuses along the polysaccharide from the nonreducing end toward AT III.[90]

Heparin can act as an anticoagulant, preventing the formation of clots and extension of existing clots within the blood. While heparin does not break down clots that have already formed (unlike tissue plasminogen activator), it allows the body's natural clot lysis mechanisms to work normally to break down clots that have formed.[91] Commercially available heparin obtained from porcine intestine is polydisperse and heterogeneous in sequence. One major disadvantage of heparin therapy is its low half-life of about one hour, thus it must be given frequently or as a continuous infusion. Another side effect caused by heparin is a severe immunoallergic reaction called heparin-induced thrombocytopenia (HIT, Figure 1.12).[92] Clinically administered heparin forms a molecular complex with platelet factor (PF4) and a strong IgG response to the heparin-PF4 complex results in platelet aggregation and eventual occurrence of HIT. Low-molecular-weight heparins (LMWHs) are obtained by chemical or enzymatic depolymerization of heparin and have lower mean average molecular weight. LMWHs, for instance, Enoxaparin, have replaced heparin owing to reduced hemorrhagic effects, improved efficacy and greater convenience of administration over heparin. [93,94]

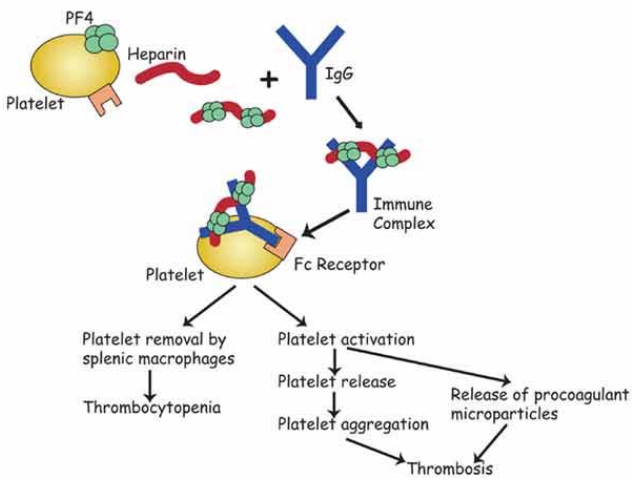



Figure 1.12 Heparin-induced thrombocytopenia

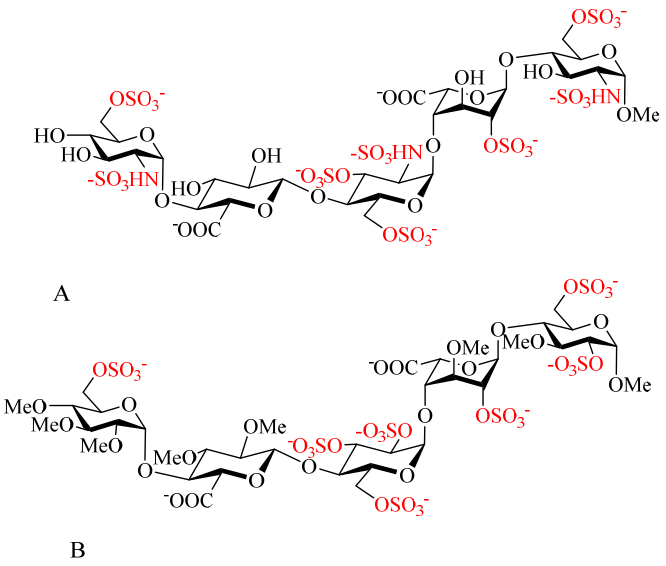



Figure 1. 13 Structure of A: Fondaparinux and B: Idraparinux

Subsequently, it was discovered that heparin binds to and potentiates the activity of AT through a unique pentasaccharide sequence.[95] A synthetic pentasaccharide under the brand of Fondaparinux (Figure 1.13) has been developed and is in the market for prevention and treatment of venous thrombosis. Fondaparinux mediates its effects indirectly through antithrombin III, but unlike heparin, it is selective for factor Xa. Another advantage of Fondaparinux over LMWH or unfractionated heparin is that the risk for heparin-induced thrombocytopenia (HIT) is substantially lower. Furthermore, as Fondaparinux has no affinity to PF-4, there have been case reports of it being used to patients with established HIT. However, its renal excretion precludes its use in patients with renal dysfunction.

Subsequently, Idraparinux, was synthesized in 25 steps and contains O-sulfates instead of N-sulfates and methylated or sulfated hydroxyl groups. Idraparinux has a longer half-life than fondaparinux and requires only one weekly administration. Both Idraparinux and Fondaparinux showed potent AT III-mediated anti-Xa inhibition and decreased nonspecific protein binding and reduced neutralization by PF4. [96,97]

Inflammation: HS is involved in multiple stages of the inflammatory response (Figur 1.14). During this process, leukocytes first attach to activated endothelial cells and then migrate into areas of damaged tissue. Binding of L-selectin expressed on leukocytes to endothelial HSPG initiates the attachment and rolling of leukocytes into blood vessels. Removal of cell-surface HS from aortic endothelial cells by bacterial heparinase digestion inhibited L-selectin-mediated binding of monocytes to the endothelial cells in vitro.[98] Further studies have shown that heparin and heparin-derived oligosaccharides have anti-inflammatory activity in vivo and function by blocking the action of L- and P-selectin, an effect that is independent of the anticoagulant activity of heparin.[99]

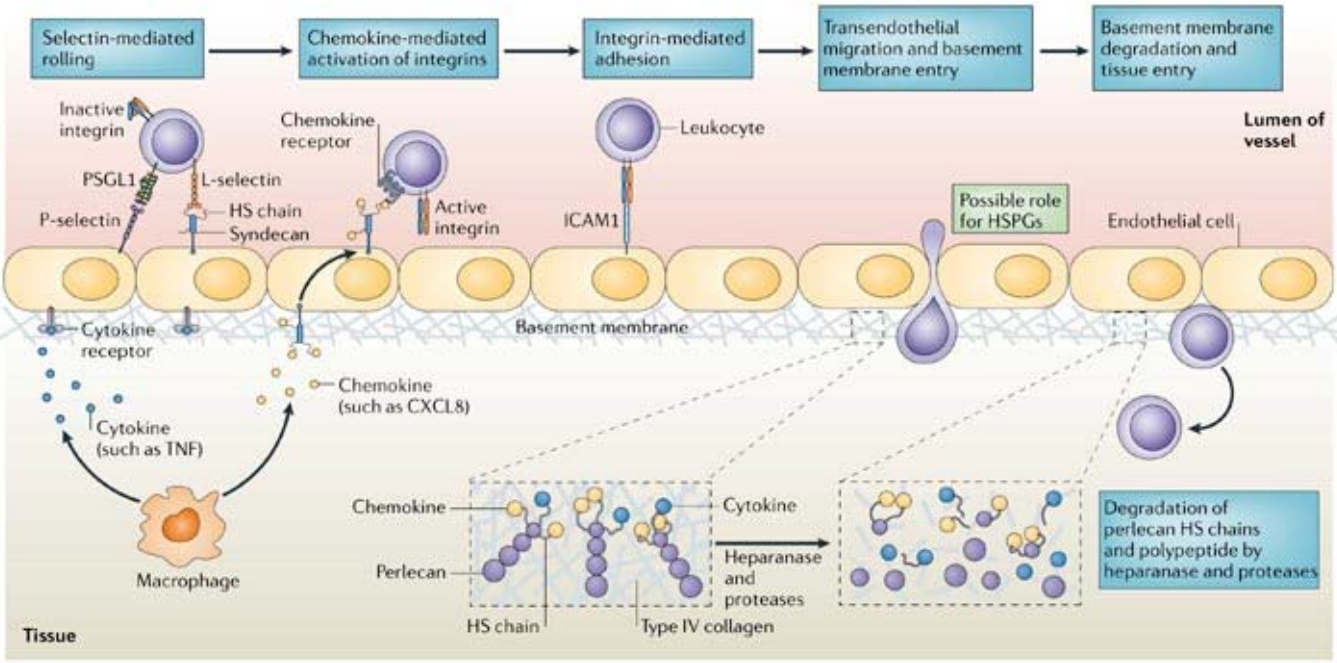



Figure 1.14 The importance of heparan sulfate at different stages during the entry of leukocytes into sites of inflammation.[100]

Modulation of chemokines by heparan sulfate: inflammatory chemokines are produced under stimuli and transported from the abluminal to the luminal side of endothelial cells, a process known as transcytosis. HS plays an important role in this process as evidenced by, for example, endothelial transcytosis of CXC- chemokine ligand 8 (CXCL8 or IL-8) depends on the heparin-

binding domain of the chemokine, indicating that the process is HS-dependent. It was also supported by showing that endothelial cells deficient in highly sulfated heparan sulfate are much less efficient at mediating chemokine (CXCL8) transcytosis. [101,102]

Chemokines bound to HS are presented to leukocytes, resulting in the activation of integrin and firm adhesion of rolling cells.[103] The interaction of chemokines with heparan sulfate can protect them from proteolysis and induce the oligomerization; oligomerized chemokines are thought to be more active than their monomeric counterparts as evidenced by, for example, RANTES with heparan sulfate binding site mutated can function as a dominant-negative inhibitor by forming non-functional heterodimers with wild-type RANTES.[104] It also helps the formation of a chemokine gradient of on the endothelial cell surface, which facilitates leukocytes following the gradients to enter the target tissue.[105]

Basement membrane heparan sulfate: after the activated leukocytes crossing the endothelial cell layer of the blood vessel, the subendothelial basement membrane represents an important physical obstruction for further migration. To pass the barrier constituted by perlecan, type XVIII collagen and agrin, a range of HS degradative enzymes are employed by leukocytes including various sulfatases, endo-β-glucuronidase heparanase.[106] During inflammation, heparanase helps leukocyte invasion and also regulates the response in several other ways. First, cytokines and chemokines anchored within the ECM or cell surfaces are liberated with the degradation of HS, which is crucial for chronic inflammation response. Then, enzymatically inactive proheparanase can bind to T cells and aid the adhesion of these cells to extracellular matrix components.[107] Further, heparanase can degrade HS expressed on the surface of migrating leukocytes, which might support cell invasion by preventing the interaction of the HS with basement membrane components.[108] The last, by releasing oligosaccharide fragments from HS,

heparanase can inhibit the inflammatory response via inhibiting TNF production by T cells and macrophages. [104,109]

Mounting evidence suggested that the composition of HS differs in various biological process that can modulate the interaction of HS with various chemokines and growth factors, and can further fine tune inflammation responses. For example, inactivation of Hs2st, results in enhanced neutrophil recruitment in several models of acute inflammation, which resulted in part from reduced rolling velocity underflow both *in vivo* and *in vitro* because of stronger binding of neutrophil L-selectin to mutant endothelial cells. Hs2st-deficient endothelial cells also displayed a striking increase in binding of IL-8 and macrophage inflammatory protein-2. The enhanced binding of these mediators of neutrophil recruitment resulted from a change in heparan sulfate structure caused by increased *N*-sulfation and 6-*O*-sulfation of glucosamine units in response to the decrease in 2-*O*-sulfation of uronic acid residues.[110]

Many other studies also indicated unique binding activities of heparan sulfate like molecules with various chemokines, HS degrading enzymes and growth factors involved in inflammatory responses.[111] Further exploration of HS related molecules as anti-inflammatory drugs depends heavily on obtaining pure HS oligosaccharide which is a major challenge due to its heterogeneity in nature and difficulties in synthesis.

Cancer is the second leading cause of death in the United States, exceeded only by heart disease. In 2015, there will be an estimated 1.5 million new cancer cases diagnosed and 0.5 million cancer deaths in the US according to the *United States Cancer Statistics*. The financial costs of cancer are overwhelming. The Agency for Healthcare research and Quality (AHRQ) estimates that the direct medical costs (total of all health care costs) for cancer in the US in 2011 were $88.7

billion. According to the National Institutes of Health, cancer cost the United States an estimated $263.8 billion in medical costs and lost productivity in 2010. [104]

Compared with normal cells (Figure 1.15) the damaged DNA of cancer cells is not repaired, and the cell goes on making new cells seizing a significant amount of the nutrients from the patient's body. Abnormal DNA can be inherited, but most often DNA damage is caused while the normal cell is reproducing or by something in the environment. Sometimes the cause of the DNA damage may be something obvious like cigarette smoking or sun exposure. But it's rare to know what exactly caused any one person's cancer.

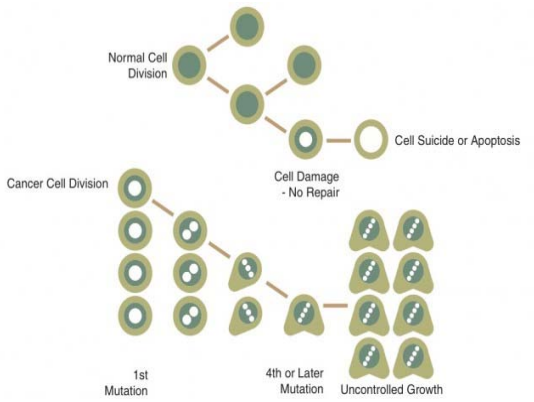



Figure 1.15 Normal cell versus cancer cell division

Cancer cells often travel via body's bloodstream or lymphatic system to other parts of the body to form new tumors which is named metastasis. 90% of cancer suffering and death is associated with metastasis. It is the single most significant challenge in the management of this disease.

The ability of HS to bind growth factors leads to numerous biological and pathological roles for HSPGs, including tumor angiogenesis, proliferation, and differentiation.[104] Individual HSPGs have distinctive roles in specific cancers (Table 1.1).

| Receptor | Cytokines | Growth factors |
|---|---|---|
| Unspecified HSPG | IL-5, IL-6, IL-8, IL-10, CXCL12/SDF-1, TNF-α, and PF-4 | FGF1, FGF2, FGF4, FGF7, FGF8, FGF10, and FGF18; HGF, PDGF, HBEGF, Neuregulin-1, VEGF, BMP-7, Noggin, Hh |
| SDC1–4 | CXCL12/SDF-1 | FGF2, HGF, VEGF, HBEGF, Hh, Midkine, Pleiotrophin, TGF-β, Wnt |
| GPC1–8 | | FGF1, FGF2, HGF, VEGF, BMP-7, Hh, Wnt, TGF-β, Midkine, IGF |
| Agrin | | FGF2 |
| Perlecan | | FGF2, FGF7 |
| TβRIII | | FGF2, TGF-β1, TGF-β2, TGF-β3, inhibin,BMP-7, BMP-2, BMP-4, GDF-5 |
| CD44 | MCP-1 | FGF2, VEGF, HBEGF, HGF |
| Nrp1–2 | | FGF2, FGF4, VEGF, PIGF, PDGFB, semaphorins, TGF-β |

Table 1. 1 HSPGs and their binding interaction. Abbreviations: BMP, bone morphogenetic protein; CXCL12, chemokine C-X-C motif ligand; FGF, fibroblast growth factor; GDF, growth and differentiation factor; HBEGF, heparin-binding epidermal growth factor-like growth factor; HGF, hepatocyte growth factor; Hh, Hedgehog; HS, heparan sulfate; TGF, transforming growth factor; IGF, insulin growth factor; IL, interleukin; MCP, monocyte chemoattractant protein; PDGF, platelet-derived growth factor; PF, platelet factor; PIGF, placental growth factor; SDF, stroma cell derived factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor. Heparin-binding growth factors are in bold font.

HSPGs are regulated and altered during pathological processes including tumor. Some of them, such as GPC1 and SDC2, are consistently upregulated, while others such as TβRIII, are downregulated in most cancers. Some HSPGs have conflicting roles in promoting or suppressing carcinogenesis, depending on tumor cell of origin, illustrating the diversity of biological functions for this outwardly similar family of signaling molecules.[112] The expression patterns might mirror those of ligands that require HSPGs to elicit their cellular responses.[113]

Heparan sulfate in cancer cell proliferation and angiogenesis: the overexpressed mitogenic growth factors including FGFs and EFGs exert selective pressure on individual tumor cells resulting in increased expression of HSPGs in certain cancers. For example, overexpression of GPC1 and SDC1 in breast cancer cell enhances the proliferative response to treatment with FGF, HBEFG and HGF. Knocking out SDC1 and GPC1 in myeloma and pancreatic cancer cells resulted in decreased proliferation, suggesting HSPGs can potentiate heparin-binding growth factor signaling even in the absence of exogenous ligand treatment.[114]

HS can also be shredded from the cell surface to influence cancer cell biology. For instance, cell surface anchored GPC3 and GPC1 promote tumor growth while the soluble forms of both HSPGs can inhibit carcinoma growth.[18,115] The proximity of HS chains on glypicans to the GPI anchor and cellular plasma membrane could facilitate the formation of growth factor signaling complexes, and help to explain the divergent roles of surface and soluble glypicans.

Since tumor growth is heavily dependent on vascularization and new blood supply, angiogenesis is important in tumor progression. Several angiogenic factors like vascular endothelial growth factor (VEGF), FGFs and hepatocyte growth factor (HGF), have been shown to interact with HSPGs.

Heparan sulfate in tumor metastasis: as mentioned above, HS plays a fundamental role during proliferation and angiogenesis, which are critical steps in the metastatic cascade. More evidence has also demonstrated HS can facilitate tumor metastasis via other mechanisms. For example, besides promoting proliferation, HS chains on SDC1 can bind matrix proteins to promote adhesion, maintain cell polarity, and reduce cell invasiveness.[116] Decreased expression of SDC1 in some tumors are thought to disrupt HS signaling functions to promote disease progression.[117] However, it remains unclear why expression of a given HSPG would affect one biology but not another in a particular tumor. To further complicate matters, increased adhesion does not uniformly suppress metastasis and can in fact promote extravasation of circulating tumor cells. For example, SDC2 and SDC4 promote adhesion to enhance invasion in lung and liver cancer.[118] Interestingly, glypicans do not appear to influence invasiveness, demonstrating specificity among HSPGs that is probably related to distinct HS structures.[119-121]

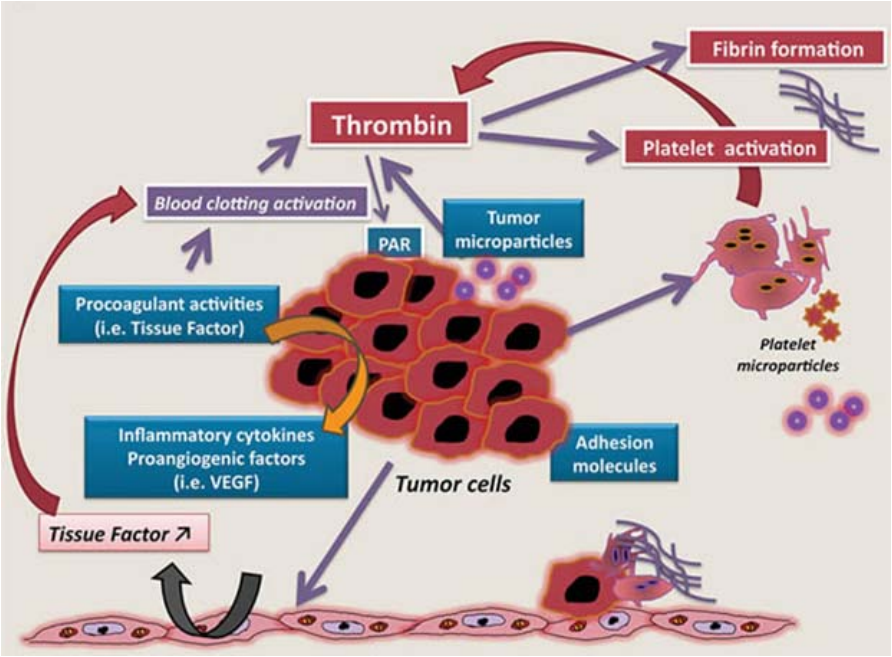



Figure 1.16 several hemostatic pathways are involved in the hemostatic system activation by tumor cells

HS chains constituting the basement membrane barrier can be destroyed by heparanase and sulfatases released from tumor cells to facilitate entry of tumor cells into blood circulation and further dissemination.[122]

Clinical application of heparin as antitumor agent: cancer-associated venous thrombosis (VTE) is linked with poor prognosis, and it is the second leading cause of death in cancer patients. Critical for the design of pharmacologic interventions for cancer-associated VTE is a better understanding mechanisms underlying the hypercoagulability. Tumor cell-specific clot-promoting properties play a prominent role, which may contribute to tumor growth and dissemination. [123] These include expression of tissue factor (TF) by tumor cells, production of circulating cell-derived microparticles and inflammatory cytokines by tumor and/or host cells, and direct adhesion of tumor cells to platelets, leukocytes, and endothelial cells (Figure 1.16). TF of tumor origin is a key molecule that initiates blood clotting and also supports tumor growth and metastasis via coagulation-independent mechanisms, such as up-regulation of VEGF and activation of PAR-2.[123]

Coagulation system activation, with the generation of thrombin, fibrin and activation of platelets, leukocytes, and endothelial cells, play crucial roles in the progression of cancer. [116] Within the circulatory system, fibrin and platelets guard tumor cells from immune elimination and promote their arrest at the endothelium, favoring the establishment of secondary tumors. Experimental studies in mice demonstrate the role of platelet glycoproteins GPIbα, GPVI, and P-selectin in supporting this process.[116] Moreover, platelets exert a protective role in the maintenance of tumor vascular integrity and may represent a target for the specific destabilization of tumor vessels.[124] Finally, circulating cell-derived microvesicles shed by tumor cells and platelets, which carry phospholipids and proteins that often originate from membrane lipid rafts of the parental cell, including functional transmembrane receptors such as TF,[125] are newly identified players in

maintaining cancer cell survival, invasiveness, and metastases. Some clinical trials have confirmed a close relationship between cancer and thrombosis, documented by the fact that while receiving chemotherapy cancer patients have an overall 7-fold increased risk of venous thromboembolism (VTE) compared to non-malignancy.[126]

Heparin as anticoagulant drugs, in this context, have been reported to improve survival in cancer patients, which might also relate to the ability of inhibition of cell–cell interaction by blocking cell adhesion molecules, the inhibition of extracellular matrix protease heparanase, and the inhibition of angiogenesis.[127]

In one study of a population registry, the 1-year survival of cancer patients diagnosed with VTE was one-third that of cancer patients without VTE (12% vs. 36%) matched for sex, age, tumor type, and duration of cancer.[128]

A randomized clinical trial enrolling 3200 patients evaluating the efficacy and safety of a prophylactic dose of the ultra-low-molecular-weight heparin semuloparin as an anticancer agent is reported by Agnelli and colleagues in 2011. The drug was administered subcutaneously once daily for a median duration of 3.5 months. Clinically, semuloparin significantly reduced the incidence of venous thromboembolism but had no significant effect on major bleeding or mortality. This study supported by other clinical trials strengthens the confidence in the estimated risk reduction for venous thromboembolism and the nonsignificant effect on major bleeding.[129]

According to the data, if 1000 patients with cancer were to use a prophylactic dose of low-molecular-weight heparin, over a period of 12 months death would be averted in approximately 30 patients, venous thromboembolism would be averted in 20, and 1 would have a major bleeding episode.

More clarity is required about which cancer patients would benefit most, the magnitude of this

survival benefit, and whether this benefit is appropriate for cancers that respond poorly to other therapies. For those patients truly looking for survival from their cancer will need to deal with some uncertainty about whether their type and stage of cancer are associated with the likely survival benefit of LMWH.

More recently, a number of preclinical and clinical studies have suggested that LMWHs may improve survival in cancer patients with mechanisms that are different from its antithrombotic effect but are linked to the ability of influencing directly the tumor biology. [130] However for other tumors, for instance, malignant gliomas, clinical trial was unable to detect neither a statistically significant reduction in VTE nor survival difference with LMWH compared with placebo.[131] Given the limitations of the available studies, the routine use of LMWH as primary anti-cancer therapy needs further clarification. As the molecular basis becomes better elucidated, heparin still has a great potential as an anticancer agent that can target both the malignant process and the resultant hypercoagulability.

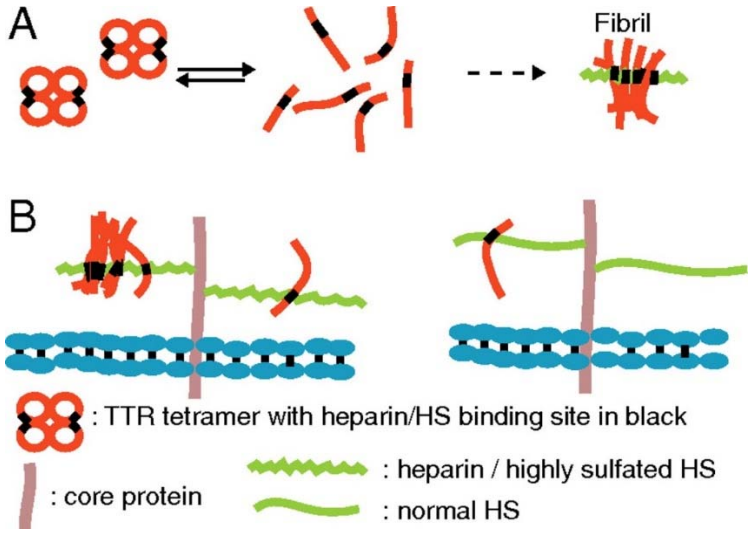



Figure 1.17 Illustration of the effect of heparin/HS on transthyretin (TTR) aggregation. (A) Tetramer destabilization (promoted by acidic condition) leads to formation of monomers on which the heparin/HS-binding domain is exposed. Mild acidic condition facilitates TTR binding to

heparin providing a scaffold to promote fibril formation. (B) A hypothetical mechanism of TTR amyloidosis in the cardiac tissues. Possible change (increase of sulfation degree) in HS structure in the aged heart (Left) in comparison to the normal HS structure in younger individuals (Right) may promote TTR–HS interactions, leading to the accumulation and deposition of TTR in the heart associated with aging.[132]

Amyloid diseases, including neurodegenerative conditions such as Parkinson's disease, Alzheimer's disease, and nonneuropathic localized amyloidosis such as type II diabetes, amyotrophic lateral sclerosis, prion diseases and AL amyloidosis are characterized by the formation of insoluble fibrillar aggregates of proteins in multiple organs and tissues (Figure 1.17).[133] There are more than 20 polypeptides known to refold, assemble in parallel and associate with HSPGs to form fibrils to form tissue deposits termed amyloid.[134] Many studies have demonstrated that HSPGs enhance fibrillization of the peptides and stabilize already formed aggregates against proteolytic degradation.[135] Others find that tau fibril uptake also occurs via HSPG binding, which can be blocked in cultured cells and primary neurons by heparin, chlorate, heparinase, and genetic knockdown of a key HSPG synthetic enzyme, *Ext1*.[136] The precise roles of HSPGs in amyloidosis remain unclear; however, a study with heparanase transgenic mice showed that shortened HS chains can prevent amyloid deposition due to high activity of heparanase.[137] Recently, HS has been identified as the first naturally occurring inhibitor of b-secretase (BACE1) that processes the amyloid precursor protein (APP). This inhibition is dependent on the structure and size of the HS chains, with emphasis on specific sulfation pattern. These discoveries have led to the investigation of HS analogs as potential therapeutic approaches to treat Alzheimer’s disease.[138]

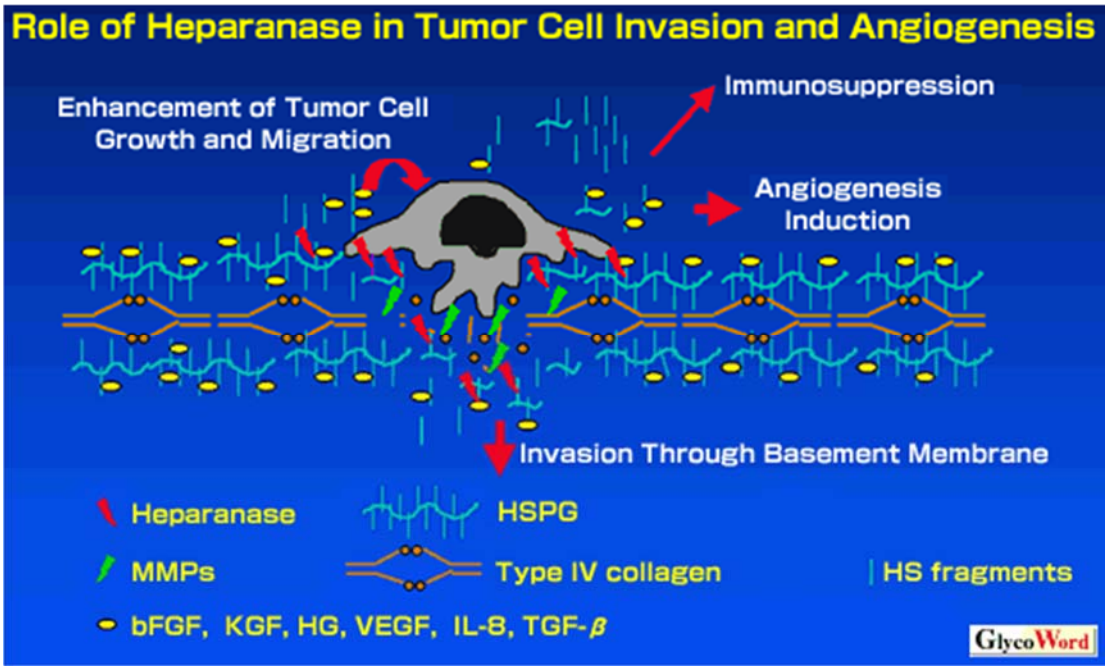



Figure 1.18 Role of heparanase in basement degradation by metastatic tumor cells

**Heparanase**

Heparanase is an endo-β-D-glucuronidase that catalyzes the hydrolytic cleavage of the β-1,4-glycosidic bond between an uronic acid and α-Dglucosamine in HS/heparin chains, yielding fragments of variable size. Previous studies indicate that a 2-O-sulfate group on a HexA residue located two monosaccharide units away from the cleavage site appears essential for the recognition.[139] However, the exact substrate structure remains unclear.

Heparanase is expressed as a 65 kDa precursor followed by proteolytic cleavage to form an activated enzyme composed of a 50 kDa subunit and non-covalently linked with an 8 kDa peptide. Heparanase exhibits maximal endoglycosidase activity between pH 5.0 and 6.0, and is inactivated at pH>8.0.[140]

Although heparanase is expressed at a low level in all tissues, it is an important regulator during the maintenance of the matrix integrity and balancing of soluble/insoluble molecules of the

ECM (Figure 1.18). Under pathological conditions, the level is often elevated.[141] Thus heparanase appears to be involved in embryonic implantation and development[142], wound repair[143], tissue remodeling[144], immune surveillance[145] and hair growth[146].

During tumor metastasis, heparanase is involved by playing several major roles: it promotes angiogenesis by clearing a path for new blood vessels to grow and releasing angiogenic factors, such as FGFs and VEGF;[147] it enables invasion of the tumor cells by digesting the ECM of the surrounding tissue;[148] it facilitates distribution tumor cells by allowing tumor cells to extravasate via digesting HS on the basement membrane of blood vessels.[149]

In addition to tumor metastasis, heparanase is found up-regulated in inflammation, wound healing, and diabetic nephropathy, effects being attributed to enhanced cell dissemination as a consequence of HS cleavage and remodeling of the ECM barrier. The involvement of heparanase in various pathological conditions indicates therapeutical applications of heparanase inhibitors. Several drugs have been studied and tested in clinical trials. Due to the high affinity of heparin for heparanase, it was shown to inhibit HS degradation by heparanase. On a general note, heparin has higher affinity for essentially all HS-binding proteins due to its high sulfation level; therefore, heparin can inhibit the interactions between growth factors and HS.[150] Other compounds such as PI88, which is a mixture of highly sulfated, monophosphorylated mannose oligosaccharides, derived from the extracellular phospholamban of the yeast Pichia (Hansenula) holstii, can also inhibit the activity of heparanase and has shown some benefit in delaying the time it takes for the hepatocellular carcinoma to reappear after surgery. [151]

In addition to the catalytic property of the enzyme, heparanase was noted to exert biological functions apparently independent of its enzymatic activity. For instance, adhesion of lymphoma

with T cells was enhanced by overexpression of non-enzymatic heparanase in the cells through activation of Akt signaling pathway.[152] [153]

**Heparan sulfate microarray**

By using only a minute amount of material, microarrays have been the subject of considerable interest over recent years, as a powerful tool in the post-genome era for exploring gene expression and molecular interactions on a large scale. Displaying glycans for the screen with protein probes provides valuable information for defining glycan-protein interactions. Glycan arrays have been applied to the assessment of adhesion of bacteria, viruses, and mammalian cells to glycans. [154]

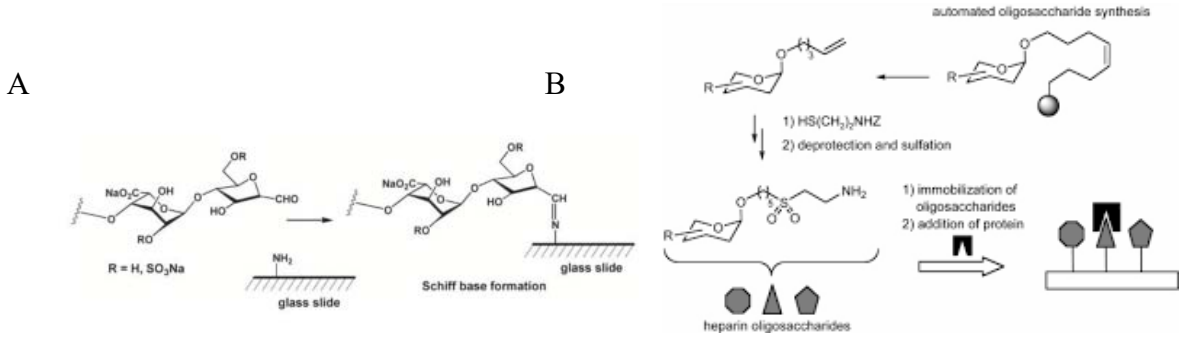



Figure 1.19 Immobilization of deaminated heparin oligosaccharides on amine-coated glass slides.

Before capable of making large heparin sulfate oligosaccharide, most groups utilized either enzymatically or chemically digested heparin as a substitute for heparin sulfate. The most successful method involves depolymerization of heparin with nitrous acid, which cleaves heparin chains at either N-unsubstituted or N-sulfated glucosamine residues, to produce oligosaccharides containing a 2, 5-anhydromannose unit at the reducing end (Figure 1.19A). The aldehyde group at position 1 of the 2, 5-anhydromannose unit is more reactive than aldehyde groups of reducing sugars since it is not in equilibrium with unreactive closed ring forms. The increased reactivity of the reducing end allows for the attachment of deaminated heparin fragments to surfaces by either formation of a Schiff base or via reductive amination. Using this method, Prof. Seeberger and

coworkers has demonstrated that as little as 63 ug/mL(~12 picomoles per spot) was sufficient to obtain a signal above background. [155] Later a novel linker strategy employing a double bond was developed. Strategic placement of the orthogonally protected amine linker was the key to the success of the array construction (Figure 1.19B). This method is compatible with the protecting group manipulations required for the synthesis of the highly sulfated oligosaccharides and can also be extended to an automated solid phase approach.[156]

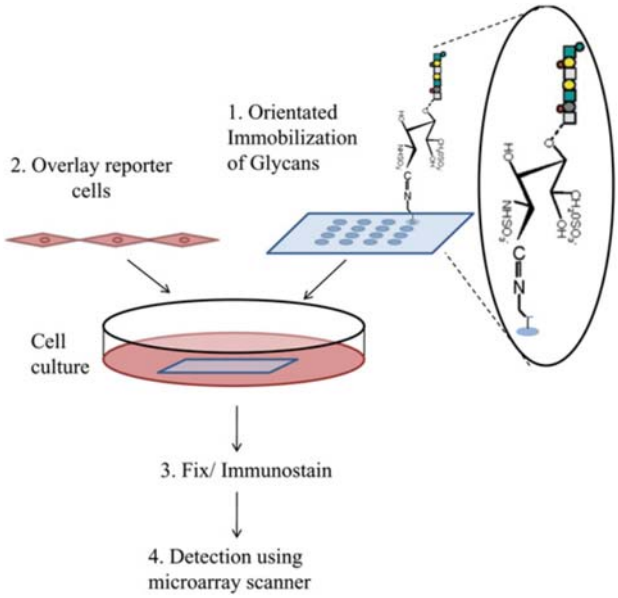



Figure 1.20 A Slide Array Method for Assaying Functional Responses of Cells to Immobilized Saccharides

Innovatively, Professor Turnbull has demonstrated that microarray can generate functional data on the consequences of cell-glycan interactions regarding cellular responses of living cells (such as activation of intracellular signaling pathways, Figure 1.20). Heparan sulfate-deficient Swiss 3T3 cells were overlaid onto an amino silane coated slide surface onto which heparin saccharides had been spotted and immobilized. The cells were transiently stimulated with FGF2 and immunofluorescence measured to assess downstream ERK1/2 phosphorylation. Activation of this signaling pathway response was restricted to cells exposed to heparin saccharides competent

to activate FGF2 signaling. Differential activation of the overlaid cells by different-sized heparin saccharides was demonstrated by quantitative measurement of fluorescent intensity. Such studies could directly address the structure-function relationships of HS saccharides at a functional level, and might be further exploited as a generic tool for glycan analysis.[157]

**Chemical synthesis of Heparan sulfate**

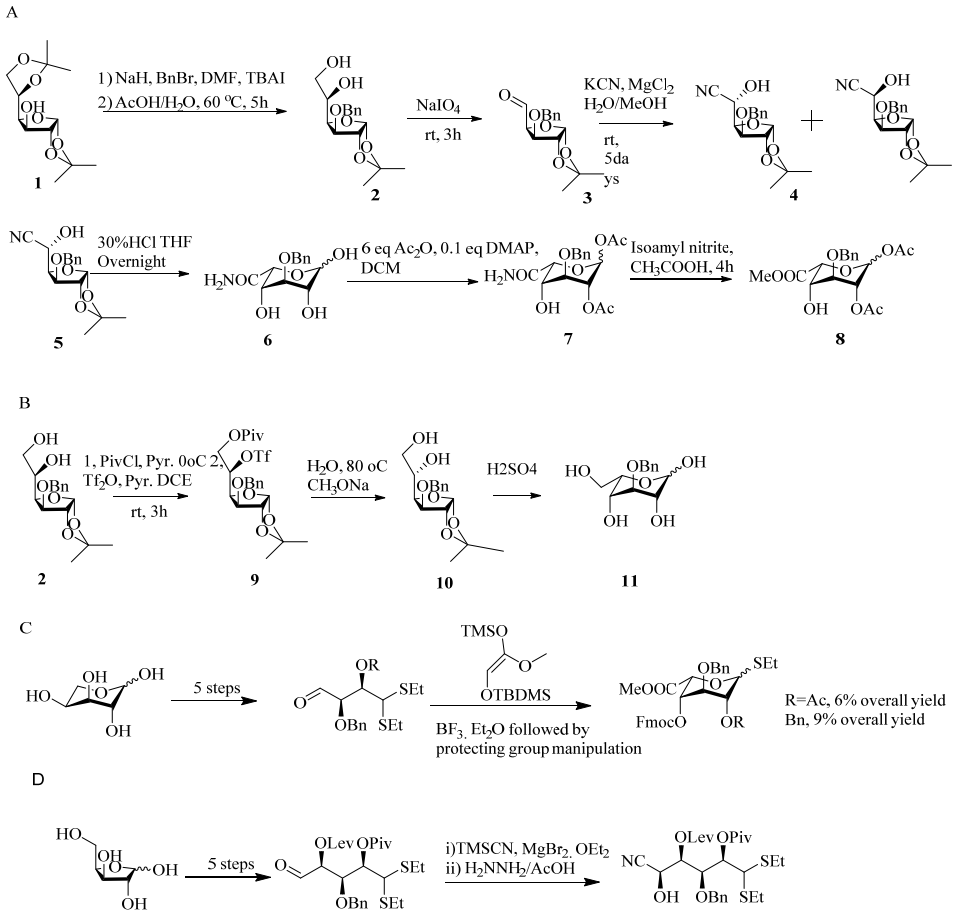
Although more than hundred HS-binding proteins are known, the precise HS sequences required for protein binding remain mostly undiscovered due to its complexity. Heparin is commonly employed experimentally as a proxy for HS due to its structural similarity with HS. Using heparin in its polysaccharide form, or as a means of generating oligosaccharides following enzymatic digestion, there have been many attempts to examine the structure–activity relationship with various signaling proteins.[158] However, the purification process of heparin aiming to achieve high anticoagulant potency favors chains with certain characteristics, leading to a more homogeneous mixture than most HS. Those sequences derived from heparin are representative of only a very limited subset of total possible sequences. Thus chemical synthesis provides an irreplaceable tool to generate structurally defined diversified heparin/HS structures.[159]

From the first synthesis in the mid-1980s impressive and innovative synthetic efforts have been performed to obtain HS fragments with well-defined structures. [160]

Challenges in Synthesis of Oligosaccharides of Heparin and HS: L-iduronic acid or the corresponding idopyranoside is not readily accessible from natural sources and quite expensive. Many efforts have been spent to access L-IdoA and its derivatives efficiently. Conventional methods include selective inversion of the configuration at C5-hydroxy, or inversion of the configuration at C-5 through the formation of an L-ido epoxide, oxidation of C5 to aldehyde

followed by stereoselective addition to form the desired configuration, and *de novo* synthesis (Scheme 1).

The biggest scale iduronic acid synthesis was performed by Prof. Gardiner and coworkers. Starting from commercially available 1, 2 : 5, 6-di-O-isopropylidene-α-D-glucofuranose, the L-Ido cyanohydrin 4 was prepared in four steps and 76% overall yield and 90% de via cyanohydrin reaction of aldehyde. It worth noticing that L-ido configuration diastereomer 4 was preferentially crystallized out from the reaction solution. Thus, no column was required. Then 4 was treated with acid to hydrolyze the cyanohydrin to amide bond followed by isoamyl nitrite-mediated amide bond cleavage to form iduronic ester 8.[161, 159] C-4 hydroxyl group must be protected orthogonally for the convenience of glycosylation. However, selective acetylation of intermediate **6** gives a mixture of several compounds including tri-acetylated, mono-acetylated, furan form monosaccharides beside the desired compound 7. Thus careful tuning of the condition is required.




Scheme 1. 1 Preparation of L-Iduronic Acid and L-Idose

An alternative method was reported as shown in Scheme 1.1B. The C5 was selectively protected with a leaving group such as a trifluorosulfonic ester, then nucleophilic displacement gives the inverted product. Although more steps are required to make the final building block, every step gives decent yield. Thus, this is the most prevalent method for idose synthesis in our lab.

*De novo* L-IdoA synthesis has also been reported by several groups. As an example, Seeberger and coworkers synthesized L-IdoA starting from L-arabinose, but the low selectivity in the Mukaiyama aldol reaction unifies a silyl enol ether and a thioacetal-containing aldehyde to give a low overall yield (6%).[162] Starting from D-xylose and switching the aldol reaction to

diastereoselective elongation of two monoprotected dialdehydes furnished the L- IdoA building block 11 in 24% overall yield.[60]

Despite the many routes developed toward the preparation of L-IdoA or L-idose, the synthesis of heparin/ HS oligosaccharides remains challenging as long as 8–12 synthetic steps are required for the preparation of a single monosaccharide building block.[163]

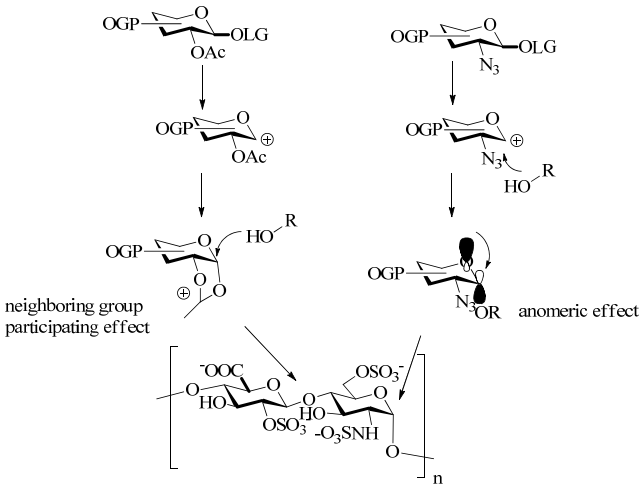
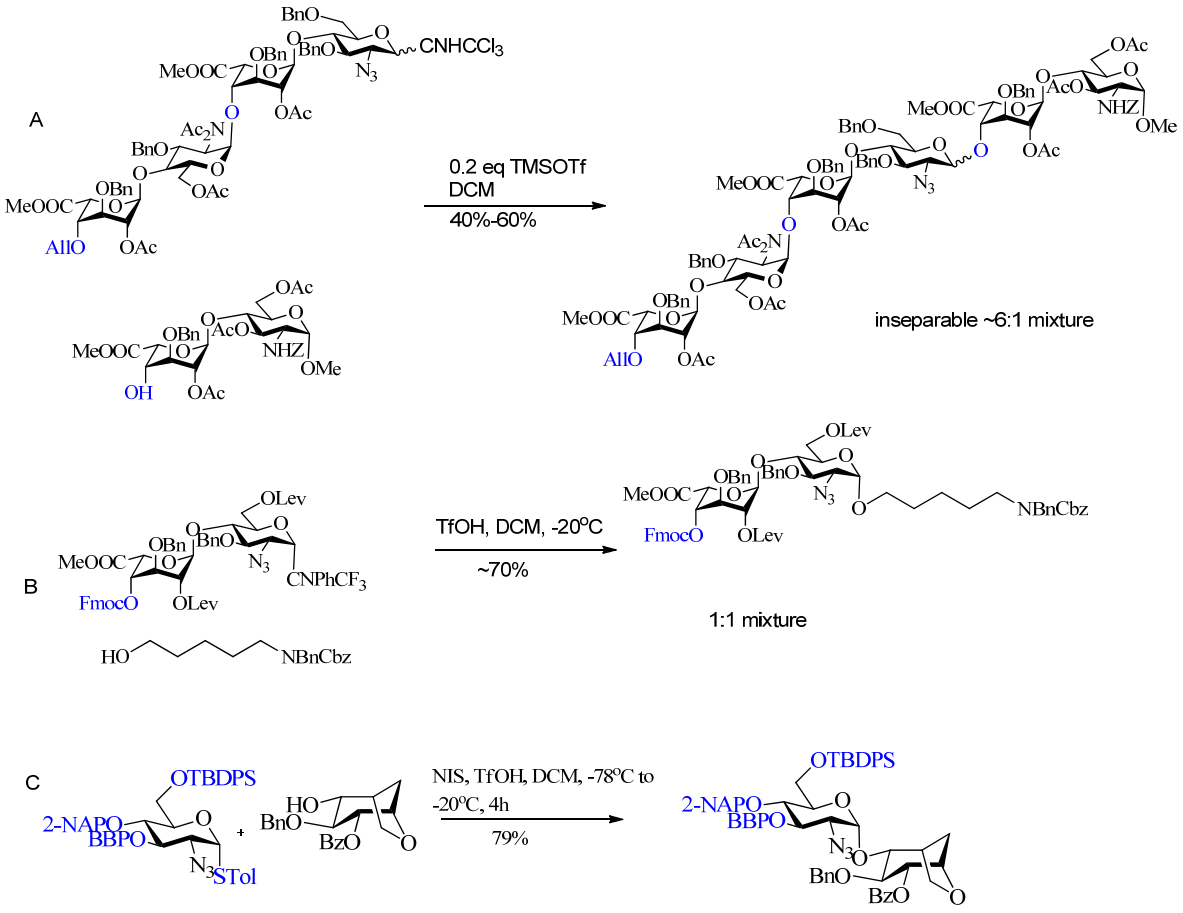



Figure 1.21 Stereochemical Control in Heparin synthesis

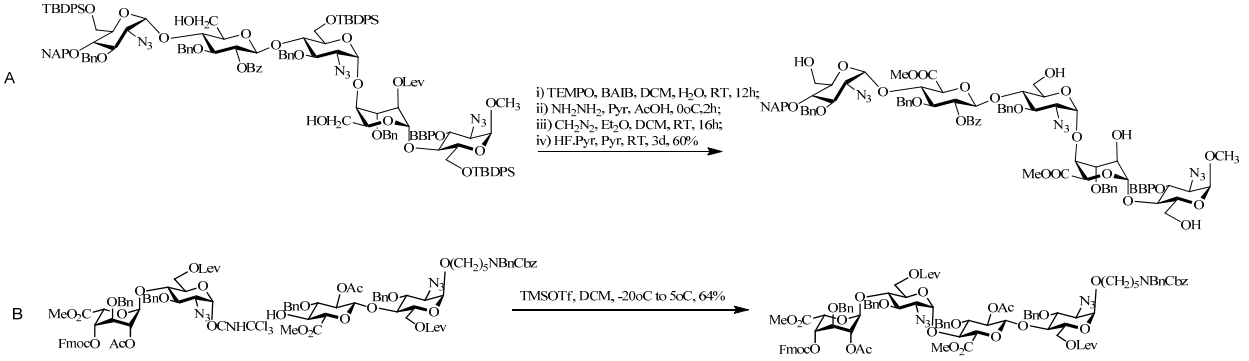
Despite the development of glycosylation technologies, stereochemical control remains nontrivial in the synthesis of heparin sulfate. The 1, 2-trans linkage of uronic acid and glucosamine can be controlled by a participating group on the C-2 position of uronic acid. However, lacking a directing group on C-2 position, the 1, 2 cis-alpha linkage between glucosamine and uronic acid can be more challenging. The most common approach is by using azido group as the amino protecting group taking advantage of the nonparticipating property during glycosylation favoring thermodynamically more stable alpha product.[164]




Scheme 1.2 the α selectivity can be affected by many factors

The C-6 protecting group has a significant influence on the stereoselectivity during glycosylation. For instance, with a C-6 benzyl either, an inseparable mixture was obtained (Figure 2A). Some has reported by locking the glucuronic acid component into the $^{1}C_{4}$ conformation α selectivity can be improved (Scheme 1.2C).[165] Recent research also indicates that by installing a bulky group on C-6 such as a *tert*-butyl diphenyl silane group or an ester which is a possible long-range participating group can also favor the alpha linkage (Scheme 2C).[166] However we found when compound XX with a C-6 ester reacted with a primary hydroxyl acceptor, the stereoselectivity was compromised probably due to the high reactivity of the acceptor making it capable of attacking from both sides (Figure 1.2B). Also, when it comes to large oligosaccharide

synthesis, caution should be given due to possible unexpected stereoselectivity.[167] Thus most strategies will construct this linkage on a disaccharide level.




Scheme 1. 3 Adjustment of the C-6 oxidation state can then be performed either before (A) or after (B) glycosylation

The Choice of Uronic Acid versus the un-oxidized Pyranoside: as glycosyl donors, uronic acids are typically less reactive than the corresponding un-oxidized pyranosides, which is caused by the electron-withdrawing effect of carboxylic ester that destabilizes the oxocarbenium ion like transition state during glycosylation. Therefore, it is common to employ a post-glycosylation oxidation approach in which an oligosaccharide is assembled followed by oxidation of the C-6 hydroxyl of glucosides or idosides to the corresponding carboxylic acid (Scheme 1.3). Early syntheses relied on the use of low efficient, toxic chromium reagents and high-yielding oxidation of long oligosaccharide become very challenging. This problem was subsequently overcome by using the TEMPO-mediated oxidations with NaOCl or iodobenzene diacetate as a co-oxidant to achieve high yields.[168]

Alternatively, due to the high reactivity as donors, unoxidized glycopyranosides can be used to prepare disaccharide intermediates as precursors for longer oligosaccharides. Adjustment of the oxidation state can be performed at the disaccharide level, thus avoiding a late-stage oxidation of the more valuable larger oligosaccharides.

Modular approaches for the chemical synthesis of HS oligosaccharides: Prof. Jian Liu and coworkers have employed an enzymatic approach for the synthesis of HS, yet because of the

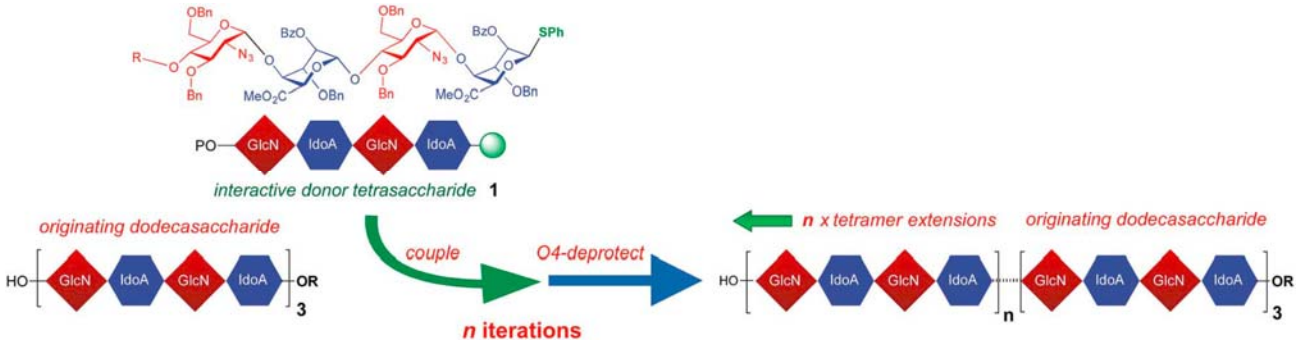


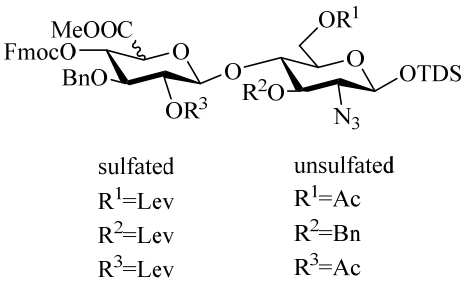

Figure 1.22 Strategy for iterative long heparin-like oligomer syntheses via tetrasaccharide building block homologation

enzyme specificity, only highly sulfated molecules can be obtained representing fragments more like heparin than heparan sulfate. Also large scale and efficient synthesis remains challenging due to the cost and availability of enzymes and PAPS.[169] Thus chemical synthesis remains irreplaceable. However as mentioned above, despite recent progress in HS synthesis, the chemical synthesis of long oligosaccharides remains a major challenge. Prof. Gardiner described a synthesis of the longest synthetic oligosaccharides of any type yet reported by iterative construction of a series of $[4]^n$-mer heparin-backbone oligosaccharides ranging from 16-mer through to the 40-mer in length (Figure 1.22).[170]

The structural diversity of HS is considered essential for its interaction with different signaling proteins. For instance, an octasaccharide sequence crucial for herpes simplex virus infection contains a rare 3-O-sulfation and different N-substitutions. While previous routes only

allows for the synthesis of Thus, a better modular approach is needed for the construction of more complexed HS fragments.

We have developed a modular approach for the chemical synthesis of HS oligosaccharides whereby a set of disaccharide building blocks, which resemble the different disaccharide motifs found in HS, can repeatedly be used for the assembly of a wide range of sulfated oligosaccharides (Figure 1.23). In this approach, levulinoyl esters (Lev) are employed for the protection of hydroxyls that need sulfation. In HS, the C-3 and C-6 of glucosamine and C-2 hydroxyls of uronic acids can be sulfated, and therefore depending on the sulfation pattern of a targeted disaccharide module, one or more of these positions are protected as Lev esters. In case for positions that do not need sulfation, acetyl esters are employed as a permanently protecting group, which is stable under the conditions used for the removal of Lev esters. An azido group is used as an amino-masking functionality because it does not perform neighboring group participation thereby allowing the introduction of alpha-glucosides. The C-4 hydroxyl, which is required for extension, is protected as a 9-fluorenylmethyl carbonate (Fmoc), and this protecting group can be removed with a hindered base such as $Et_3N$ without affecting the Lev ester, whereas the Lev group can be cleaved with hydrazine buffered with acetic acid and these conditions do not affect the Fmoc carbonate. The anomeric center of the modular disaccharides is protected as TDS glycosides, and this functionality can easily be removed by treatment with HF in pyridine without affecting the other protecting groups. The resulting lactol can then be converted into a leaving group for glycosylations with appropriate acceptors.




| sulfated | unsulfated |
|---|---|
| $R^1$=Lev | $R^1$=Ac |
| $R^2$=Lev | $R^2$=Bn |
| $R^3$=Lev | $R^3$=Ac |

Figure 1.23 Orthogonally protected disaccharide building blocks

Compared to conventional approaches, a modular synthetic strategy makes it possible to rapidly assemble libraries of HS oligosaccharides for structure-activity relationship studies.

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# CHAPTER 2

# FLUOROUS SUPPORTED MODULAR SYNTHESIS OF HEPARAN SULFATE OLIGOSACCHARIDES[1]

[1] Chengli Zong, Andre Venot, Omkar Dhamale, Geert-Jan Boons, *Organic letters*, **2013**, *2*, 342-345. Reprinted here with permission of the publisher.

**Abstract:**

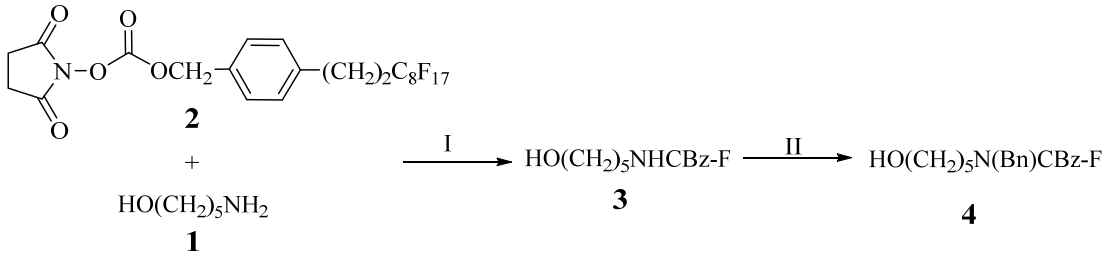
The modular synthesis of heparan sulfate fragments is greatly facilitated by employing an anomeric aminopentyl linker protected by a benzyloxycarbonyl group modified by a perfluorodecyl tag, which made it possible to purify highly polar intermediates by fluorous solid phase extraction. This tagging methodology made it also possible to perform repeated glycosylations to drive reactions to completion.

**Introduction**

Heparan sulfates (HS) are highly *N*- and *O*-sulfated polysaccharides involved in a number of important biological processes such as embryo development, inhibition of blood coagulation, organization of the extracellular matrix, angiogenesis, the presentation of enzymes and cytokines on cell surfaces, and as co-receptors for viral infections.[1-5]

In general, it is difficult to determine oligosaccharide sequences and sulfation patterns required for binding of HS binding proteins.[6-8] To address this difficulty, we have developed a modular approach for the chemical synthesis of HS oligosaccharides whereby a set of disaccharide building blocks, which resemble the different disaccharide motifs found in HS, can repeatedly be used for the assembly of a wide range of sulfated oligosaccharides.[9-13] In this approach, levulinoyl esters (Lev)[14] are employed for the protection of hydroxyls that need sulfation. In HS, the C-3 and C-6 of glucosamine and C-1 hydroxyls of uronic acids can be sulfated and therefore depending on the sulfation pattern of a targeted disaccharide module, one or more of these positions are protected as Lev esters. In case the C-2 position of a disaccharide module does not need sulfation, an acetyl ester is employed as a permanent protecting group, which is stable under the conditions used for the removal of Lev esters. An azido group is used as an amino-masking functionality because it does not perform neighboring group participation thereby allowing the introduction of α-

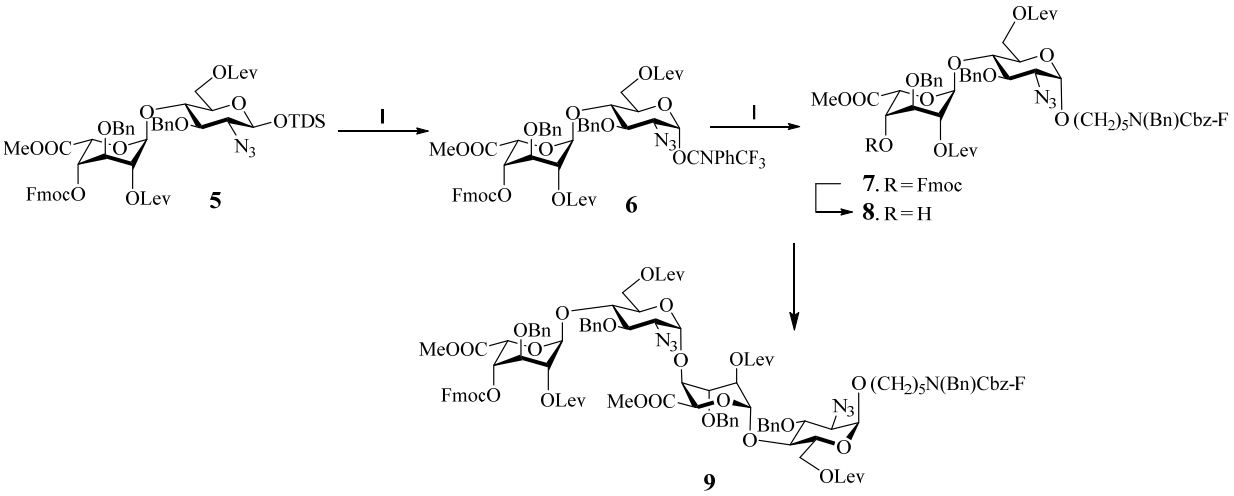
glucosides.[15] The C-4' hydroxyl, which is required for extension, is protected as 9-fluorenylmethyl carbonate (Fmoc), and this protecting group can be removed with a hindered base such as $Et_3N$ without affecting the Lev ester whereas the Lev group can be cleaved with hydrazine buffered with acetic acid and these conditions do not affect the Fmoc carbonate.[14] The anomeric center of the modular disaccharides is protected as TDS glycosides and this functionality can easily be removed by treatment with HF in pyridine without affecting the other protecting groups. The resulting lactol can then be converted into a leaving group for glycosylations with appropriate acceptors. Compared to conventional approaches,[9,16,17] a modular synthetic strategy makes it possible to rapidly assembly libraries of HS oligosaccharides for structure activity relationship studies.




Scheme 2.1 Preparation of fluorous tagged aminopentyl linker I) $NaHCO_3$, $H_2O$/THF = 4:1, rt, 1 h, 86%;II) i) $Ac_2O$, DMAP, THF, rt, 1 h; ii) NaH, BnBr, DMF, rt, 12 h; iii) MeONa, MeOH, rt, 2 h, 80% for three steps

Although modular oligosaccharide assembly is very attractive,[9-13,18] the endgame involving selective protecting group removal, *O*- and *N*-sulfation and global deprotection requires a relatively large number of steps providing polar compounds that are difficult to purify by conventional approaches thereby slowing down the preparation of libraries of HS oligosaccharides. Several platforms have been developed to speedup the process of oligosaccharide assembly.[19-24] We were attracted by light fluorous supported synthesis[25,26] because compounds tagged by a linear fluorous tag can easily be separated from non-fluorous material by

solid phase extraction using silica gel modified by fluorocarbons.[27-30] This generic procedure, which resembles more filtration than chromatography, depends primarily on the presence or absence of a fluorous tag, and not on polarity or other molecular features.




Scheme 2.2 Preparation of fluorous tagged tetrasaccharide I) i) HF in Pyridine, THF, rt, overnight; ii) $ClCNPhCF_3$, NaH, DCM, rt, 1 h, 70% for two steps; II) $HO(CH_2)_5N(Bn)CBz\text{-}F(4)$, TfOH, Tol/Dioxane = 1/3, rt, 15 min, 75%, a/b = 3/1; III) $Et_3N$/DCM (1/4), rt, 4 h, 100%; IV) TfOH, DCM, -20 ºC, 15 min, 72%

We envisaged that fluorous supported synthesis would speedup modular synthesis of HS oligosaccharides and would in particular be attractive for the final modifications of the fully assembled oligosaccharides as these procedures are high yielding but require large excesses of reagents and provide polar compounds that are difficult to purify by conventional approaches. Previously,[9] we employed an *N*-(benzyl)benzyloxycarbonyl aminopentanol linker for the modification of the reducing end of HS oligosaccharides, and thus linker **4** was selected, which contains a benzyloxycarbonyl protecting group modified by a perfluorodecyl tag. Linker **4** could easily be prepared by treatment of aminopentanol (**1**) with **2** in aqueous sodium bicarbonate to give, after purification by fluorous solid phase extraction, benzyloxycarbonyl protected **3** in a yield

of 86%. Selective *N*-benzylation of **3** to give **4** was accomplished by three-step procedure involving acetylation of the hydroxyl with acetic anhydride in pyridine followed by *N*-benzylation by treatment with benzyl bromide in the presence of NaH in DMF and then saponication of the acetyl ester using NaOMe in methanol.

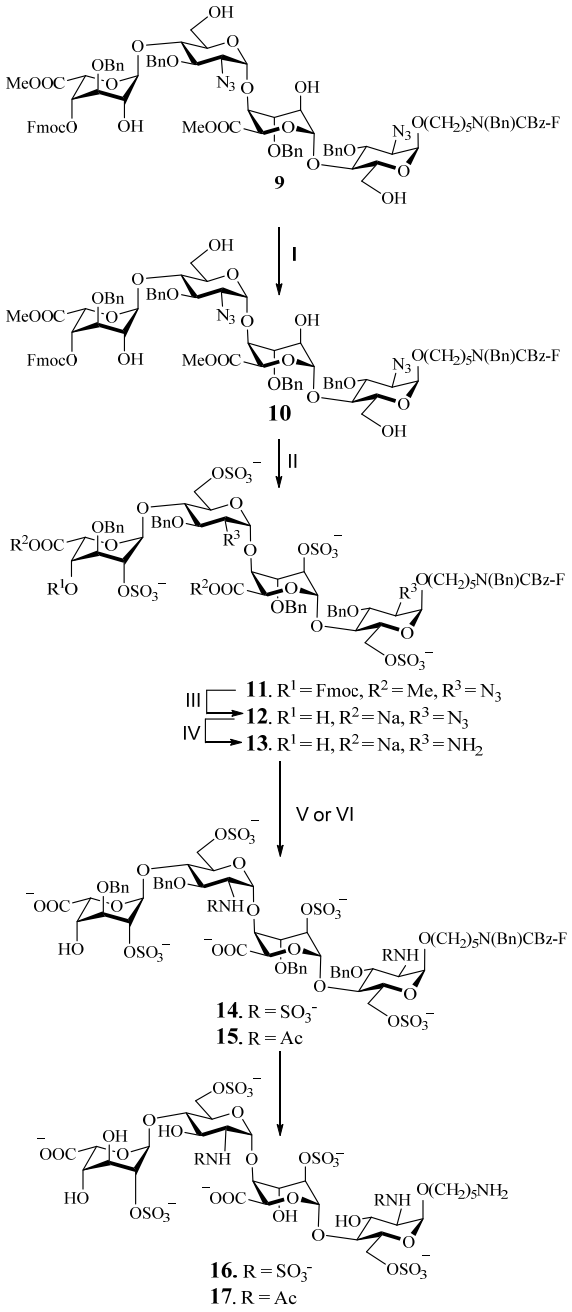
**Results and discussion**

Having at hand linker **4** modified with a perfluorodecyl tag, attention was focused on its installation into modular disaccharides by glycosylation. Thus, glycosyl donor **6** was prepared by removal of the anomeric TDS moiety of modular disaccharide **5**[9] with HF in pyridine to give a lactol (Scheme 2.2), which was converted into trifluoro-*N*-phenylacetimidate **6** by reaction with *N*-phenyltrifluoroacetimidoyl chloride in the presence of NaH in DCM.[31]

Previously, we observed that glycosylations of modular disaccharides such as **6** with a regular *N*-(benzyl)benzyloxycarbonyl aminopentanol led to mixtures of anomers that were difficult to separate by silica gel column chromatography. Therefore, an additional set of modular disaccharides, having a preinstalled linker, needed to be prepared.[9] A number of conditions were examined to improve the anomeric selectivity of the glycosylation of linker **4** with glycosyl donor **6**. A TfOH promoted glycosylation of **4** with **6** in DCM at -20ºC gave **7** as a mixture of anomers (α/β = 1/2, 70%), which surprisingly could readily be separated by traditional silica gel column chromatography. Increasing the temperature or the addition of thiophene[32] or DMF[33] did not have a notable effect on the anomeric ratio (α/β=1/1). The use of diethyl ether to improve the alpha anomeric selectivity[34] led to a low yield of product due to poor solubility of linker **4**. The use of a mixture of dioxane and toluene at ambient temperature[34] and TfOH as the promoter, gave compound **6** in a good yield of 75% as mainly the alpha anomer (α/β = 3/1) (Scheme 2.2). Linker

**3** was also examined for tagging modular disaccharides but the results were disappointing due poor solubility of this compound in commonly employed solvents for glycosylation.

Next, the Fmoc protecting group of **7** was removed by treatment with $Et_3N$ in DCM to give glycosyl acceptor **8** in a near quantitative yield after purification by fluorous solid phase extraction. A TfOH promoted coupling of glycosyl donor **6** with acceptor **8** led to the formation of tetrasaccharide **9** as exclusively the α-anomer (Scheme 2.2). As expected, fluorous solid phase extraction resulted in the removal of hydrolyzed acceptor and other non-fluorous by-products. The resulting compound **9** was, however, contaminated with glycosyl acceptor **8** due to an incomplete glycosylation. Therefore, the latter mixture was resubjected to the treatment with of glycosyl donor **6** (0.5 eq) and a catalytic amount of TfOH, which led to complete consumption of the remaining acceptor to provide, after fluorous solid phase extraction, pure tetrasaccharide **9** in a yield of 72%. Solid supported synthesis often exploits repeated reaction cycles to drive reactions to completion[19] and the results described here highlight that such an approach is possible for fluorous supported synthesis.




Scheme 2.3 Preparation of target tetrasaccharides I) AcOH.$NH_2NH_2$, DCM/MeOH = 1/1, rt, 2 h, 86%; II) Py.$SO_3$, DMF, rt, 5 h, 75%; III) LiOH, $H_2O_2$, THF, rt, 18 h, 82%; IV) 1M $PMe_3$, 0.1M NaOH, THF, rt, 5 h, 78%; V) Py.$SO_3$, $Et_3N$, 0.1M NaOH, MeOH, $CF_3CH_2OH$. 0 oC to rt, 18 h, 87%; VI) $Ac_2O$, MeOH, Et3N, 0 oC to rt, 1 h, 86%; VII) i) Pd/C, $H_2$, MeOH/H2O = 1:1, 0.1% AcOH, rt, 12h; ii) $Pd(OH)_2$/C,$H_2$, $H_2O$, 0.1% AcOH, rt, 12 h; 85% for **16**, 84% for **17**.`

The sulfate esters were installed following removal of the Lev esters from **9** with hydrazine acetate in a mixture of DCM and methanol, followed by sulfation of the hydroxyls of compound **10** using a large excess of pyridinium sulfur trioxide pyridine complex to provide compound **11** in high yield after purification by fluorous solid phase extraction (one reaction cycle). Next, the Fmoc and methyl esters of **11** were saponified by treatment of LiOH in a mixture of hydrogen peroxide and THF to give partially deprotected **12**. Purification by fluorous solid phase extraction was troublesome probably due to the formation of micelles. However, the addition of a small amount of 2,2,2-trifluoroethanol solved this problem and pure fluorous-tagged **12** could readily be obtained. The azido moiety of **12** was reduced with trimethyl phosphine in THF in the presence of NaOH[35,36] to give amine **13**, which was immediately sulfated with sulfur trioxide pyridine complex in the presence of triethylamine in methanol to afford, after fluorous solid phase extraction with 2,2,2-trifluoroethanol (to avoid micelle formation), *N*-sulfate **14** in a yield of 86%. As expected, the modified *N*-(benzyl)benzyloxycarbonyl tag was stable under the applied basic conditions. Alternatively, acetylation of the free amine of **13** with acetic anhydride in methanol provided acetamido derivative **15**. Finally, the benzyl ethers and benzyloxycarbamate of **14** and **15** were removed by a two-step procedure[37] involving hydrogenation over Pd/C in a mixture of MeOH/$H_2O$, which led to the removal of the spacer protecting groups, followed by hydrogenation over $Pd(OH)_2$ in $H_2O$ which resulted in the removal of the benzyl ethers to give HS oligosaccharides **16** and **17**, respectively. Addition of small amount of acetic acid was found to be necessary to speed up the hydrogenation. Prolonged hydrogenation in the absence of AcOH caused loss of sulfate groups. The $^1H$ NMR spectra of the sulfated oligosaccharides were fully assigned by 1D and 2D NMR spectroscopy. The α-anomeric configuration of 2-azido-glucosides was confirmed by the $J_{1,2}$ coupling constants and by the $^{13}C$ chemical shift of C-1 (~97 ppm). Furthermore, a downfield shift

of 0.5 ppm of H-6 was observed for *O*-sulfation of C-6 hydroxyls and 0.4 ppm of H-2 for *N*-sulfation.

**Conclusion**

The studies reported here highlight the appealing features of fluorous supported modular synthesis of HS oligosaccharides. During oligosaccharide assembly, the attraction of the technology is that two or more reaction cycles can easily be performed to drive reaction to completion and thus early installation of the fluorous tag is attractive.[38] Unlike solid supported synthesis, light fluorous technology does not require large excesses of reagents to drive the reactions to completion. The fluorous-tagged compounds could easily be analyzed by standard spectroscopic methods thereby providing control over the synthesis. Furthermore, the final modifications involving selective protecting group removal, *O*- and *N*-sulfation, and global deprotection were much faster because these reactions proceed with high efficiency. The resulting intermediates are normally difficult to purify by traditional chromatographic approaches. We observed, however that polar carbohydrates modified by a fluorous tag tend to aggregate complicating chemical transformations and solid phase extraction. This problem could easily be addressed by employing 2,2,2-trifluoroethanol as a cosolvent, which is more suitable than the use of $EtOC_4F_9$[39,40] or $PhCF_3$ to solve this problem.[41-43] Pohl and coworkers are developing a liquid handler to automate fluorous supported oligosaccharide synthesis[44] and it is to be expected that such a system will be very attractive for modular synthesis of HS oligosaccharides.

**Experimental Section**

**General procedures:** $^1H$ and $^{13}C$ (data from HSQC) NMR spectra were recorded on Varian Mercury 300 MHz, Varian INOVA 500 MHz or 600 MHz spectrometers. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS) as the internal standard.

NMR data is presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, dd = doublet of doublet, m = multiplet and/or multiple resonances), coupling constant in Hertz (Hz), integration. All NMR signals were assigned on the basis of $^{1}H$ NMR, COSY and HSQC experiments. Mass spectra were recorded on an ABISciex 5800 MALDI-TOF-TOF or Shimadzu LCMS-IT-TOF mass spectrometer. The matrix used was 2, 5-dihydroxy-benzoic acid (DHB) and Ultamark 1621 as the standard. TLC-analysis was performed on Silica gel 60 F254 (EMD Chemicals inc.) with detection by UV-absorption (254nm) when applicable, and by spraying with a solution of $(NH_4)_6Mo_7O_{24}.H_2O$ (25 g/L) in 5% sulfuric acid in ethanol followed by charring. $CH_2Cl_2$ was freshly distilled from calcium hydride under nitrogen prior to use. Acid washed molecular sieves (4Å) were flame activated *in vacuo*. All reactions were carried out under an argon atmosphere.

**General procedure for F-SPE (Fluorous Solid Phase Extraction):** F-SPE cartridge (FluoroFlash® SPE Cartridges, 5 grams, 10 cc tube) was purchased from Fluorous Technologies. Inc. Other than specified, the crude mixture was loaded on the F-SPE cartridge using 90% DMF in water (5 mL). 20% water in methanol (20 mL) was then used to elute all of non-fluorous compounds while the fluorous molecules were retained on the cartridge. Further elution with 100% methanol or THF (20 mL) was then used to get the fluorous molecules from the cartridge.

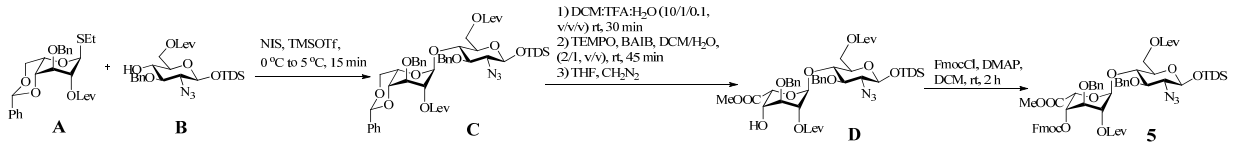
***N*-1H,1H,2H,2H-Perfluorodecyl-benzyloxycarbonyl-5-Aminopentanol (3)**

Compound **1** (1.0 g, 1.44 mmol), **2** (170.0 mg, 1.73 mmol) and $NaHCO_3$ (363.0 mg, 4.32 mmol) in a mixture of water and THF (4/1, v/v, 50 mL) was stirred for 1 h at ambient temperature. The reaction mixture was concentrated under reduced pressure and the residue was purified by F-SPE as described in general procedure to provide **3** as white amorphous solid (861.9 mg, 86.2 %). $^{1}H$ NMR (300 MHz, $CDCl_3$) δ 7.43 – 7.12 (m, 4H, C*H* Ar), 5.07 (s, 2H, C$H_2$Cbz), 4.75 (s, 1H,

O*H*), 3.64 (t, *J* = 6.6 Hz, 2H, C*H*$_2$CH$_2$C$_8$F$_{17}$), 3.21 (q, *J* = 6.6 Hz, 2H, CH$_2$C*H*$_2$C$_8$F$_{17}$), 3.00 – 2.80 (m, 2H, OC*H*$_2$), 2.36 (tt, *J* = 17.5, 8.1 Hz, 2H, NC*H*$_2$ Linker), 1.71 – 1.13 (m, 6H, (C*H*$_2$)$_3$ Linker). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 128.8, 128.7, 66.5, 62.9, 41.2, 32.1, 30.0, 26.4, 22.8. HR MALDI-TOF MS: m/z: calcd for $C_{23}H_{22}F_{17}NO_3$ [M+Na]$^+$: 706.1226; found: 706.1234.

***N*-(benzyl)-1H,1H,2H,2H-Perfluorodecyl-benzyloxycarbonyl-5-Aminopentanol (4)**

Ac$_2$O (168.9 mg, 1.7 mmol) and a catalytic amount of DMAP were added to a solution of **3** (1.0 g, 1.4 mmol) in THF (20 mL). The reaction mixture was stirred for 1 h and then diluted with DCM (20 mL), washed with brine (20 mL) and the organic layer was dried (MgSO$_4$), filtered, concentrated under reduced pressure and the resulting oil left *in vacuo* for 2 h. The crude product was dissolved DMF (20 mL), cooled (0 ºC) and NaH (60%) (99.6 mg, 2.5 mmol) was added. After stirring for 10 min BnBr (236.3 $\mu$L, 2.0 mmol) was added. After stirring for an additional 12 h the reaction was then diluted by MeOH (20 mL). MeONa was used to adjust the pH to 9 and stirring was continued for another 2 h followed by the addition of AcOH to adjust pH to 7. The mixture was concentrated under reduced pressure and the residue was purified by F-SPE to afford **4** as white amorphous solid (853.4 mg, 80%): $^1$H NMR (300 MHz, CDCl$_3$) δ 7.42 – 7.10 (m, 9H, CH Ar), 5.30 – 5.05 (m, 2H, C*H*$_2$Cbz), 4.49 (d, *J* = 4.1 Hz, 2H, NC*H*$_2$Bn), 3.71 – 3.50 (m, 2H, OCH$_2$), 3.37 – 3.13 (m, 2H, NC*H*$_2$ Linker), 2.91 (m, *J* = 7.2, 2.7 Hz, 2H, C*H*$_2$CH$_2$C$_8$F$_{17}$), 2.36 (m, *J* = 17.9 Hz and 10.8 Hz, 2H, CH$_2$C*H*$_2$C$_8$F$_{17}$), 1.66 – 1.20 (m, 6H, (CH$_2$)$_3$ Linker). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 128.4, 128.2, 67.0, 62.8, 50.5, 47.0, 33.1, 32.3, 27.7, 26.3, 23.0. HR MALDI-TOF MS: m/z: calcd for $C_{38}H_{28}F_{17}NO_3$ [M+Na]$^+$: 796.1695; found: 796.1690.



Scheme 2.4 Preparation of disaccharide building block

**Dimethylthexylsilyl *O*-(2-*O*-levulinoyl-3-*O*-benzyl-4,6-*O*-benzylidene-α-L-idopyranosyl)-(1→4) -*O*-2-azido-3-*O*-benzyl-6-*O*-levulinoyl-2-deoxy-β-D-glucopyranoside (C)**

Compound **A** (3.00 g, 6.75 mmol) and **B** (3.02 g, 5.63 mmol) were combined in a flask (50 mL), coevaporated with toluene (3 × 5 mL), and dissolved in DCM (30 mL). Powdered freshly activated 4 Å molecular sieves (3 g) were added, and the mixture was stirred for 30 min at ambient temperature and then cooled to 0 ºC. NIS (1.90 g, 8.45 mmol) and TMSOTf (109 µL, 563 µmol) were added to the mixture, and stirring was continued until TLC indicated disappearance of the glycosyl donor (~15 min). The reaction mixture was allowed to warm to +5 ºC and then quenched by the addition of pyridine. The mixture was filtered, the filtrate was concentrated *in vacuo*. The residue was chromatographed over silica gel using a gradient of hexanes and EtOAc to give disaccharide **C** (4.55 g, 83.0%). $^1$H NMR (500 MHz, CDCl3): *δ* 7.44-7.25 (m, 15H, CH Aromatic), 5.30 (s,1H, C*H* benzylidene), 4.98-4.97 (m, 1H, H2B), 4.92 (bs, 1H, H1B), 4.82 (d, 1H, *J* = 11.0 Hz, C*H*HBn), 4.74 (d, 1H, *J* = 11.5 Hz, CH*H*Bn), 4.63 (d, 1H, *J* = 11.5 Hz, C*H*HBn), 4.55-4.51 (m, 3H, H1A, H6aA, CH*H*Bn), 4.07 (dd, 1H, *J* = 5.5 Hz, *J* = 12.0 Hz, H6bA), 3.89 (bs, 1H, H5B), 3.84-3.79 (m, 3H, H4A, H4B, H6aB), 3.68 (bt, 1H, *J* = 3.0 Hz, H3B), 3.49-3.45 (m, 1H, H5A), 3.38-3.34 (m, 1H, H2A), 3.23 (t, 1H, *J* = 9.0 Hz, H3A), 3.18 (dd, 1H, *J* = 1.5 Hz, *J* = 12.5 Hz, H6bB), 2.78-2.68 (m, 2H, C*H*2 Lev), 2.66- 2.60 (m, 2H, C*H*2 Lev), 2.58-2.51 (m, 4H, 2 x C*H*2 Lev), 2.17 (s, 3H, C*H*3 Lev), 2.01 (s, 3H, C*H*3 Lev), 1.67-1.54 [m, 1H, C*H*(CH3)2], 0.90-0.88 [4s, 12H, C(C*H*3)2 and CH(C*H*3)2], 0.19-0.17 [2s, 6H, Si (CH3)2]. 13C NMR (75.5 MHz, CDCl3): δ 206.7, 206.3 172.1, 171.9, 138.1, 138.0, 137.6, 128.9, 128.3, 128.2, 128.0, 127.9, 127.9, 127.6, 127.4, 126.6, 100.4, 97.8, 96.9, 81.2, 77.4, 77.0, 76.5, 75.0, 74.9, 73.9, 73.4, 73.4, 72.1, 69.0, 69.0, 67.0, 62.5, 60.2, 37.9, 37.7, 33.9, 29.7, 29.6, 28.1, 27.9, 24.7, 19.9, 19.8, 18.4, 18.3,-2.1, -3.2. HR MALDI-TOF MS: m/z: calcd for $C_{51}H_{67}N_3O_{14}Si$ $[M+Na]^+$: 996.4290, found 996.4295.

**Dimethylthexylsilyl *O*-(methyl-2-*O*-levulinoyl-3-*O*-benzyl-α-L-idopyranosyluronate)-(1→4)-*O* -2-azido-3-*O*- benzyl-6-*O*-levulinoyl-2-deoxy-β-D-glucopyranoside (D)**

A solution of compound C (4.55 g, 4.67 mmol) in a mixture of DCM:TFA:$H_2O$ (10/1/0.1, v/v/v, 30 mL) was stirred at ambient temperature for 30 min. The reaction mixture was concentrated *in vacuo*, and the residue was coevaporated with toluene. To a vigorously stirred solution of the obtained diol in a mixture of DCM:$H_2O$ (2/1, v/v, 20 mL) was added (2,2,6,6-Tetramethylpiperidin-1-yl)-oxyl (175.9 mg, 1.13 mmol) and (diacetoxyiodo)-benzene (4.5 g, 14.08 mmol). Stirring was continued (~45 min) until TLC indicated complete conversion of the starting material to a spot of lower $R_f$ (DCM/MeOH, 10/1, v/v). The reaction mixture was quenched by the addition of aqueous $Na_2S_2O_3$ (10%, 20 mL). The mixture was extracted with EtOAc (2 × 20 mL), and the combined aqueous layers were back-extracted with EtOAc (10 mL). The combined organic layers were dried (MgSO4), filtered, and concentrated *in vacuo*. The oily residue was dissolved in THF (0.1 M) and treated with an excess of freshly prepared ethereal solution of diazomethane until the reaction mixture remained yellow. The excess diazomethane was quenched by the addition of AcOH until the mixture became colorless. The mixture was concentrated *in vacuo* and the residue was coevaporated with toluene(3 × 10 mL), and the residue was purified by silica gel column chromatography to yield compound **D** (3.0 g, 70.7% for three steps). 1H NMR (500 MHz, CDCl3): δ 7.44-7.25 (m, 10H, CH Aromatic), 4.99 (s, 1H, H2B), 4.91 (bs, 2H, H1B, H5B), 4.73 (d, 1H, *J* = 11.5 Hz, C*H*HBn), 4.66 (bs, 2H, CH*H*Bn, C*H*HBn), 4.61 (d, 1H, *J* = 11.5 Hz, CH*H*Bn), 4.49 (d, 1H, *J* = 7.5 Hz, H1A), 4.46 (dd, 1H, *J* = 2.0 Hz, *J* = 12.5 Hz, H6aA), 4.11 (dd, 1H, *J* = 5.0 Hz, *J* = 12.5 Hz, H6bA), 3.97 (bd, 1H, *J* = 9.0 Hz, H4B), 3.85 (t, 1H, *J* = 9.5Hz, H4A), 3.73 (t, 1H, *J* = 2.5 Hz, H3B), 3.47 (s, 3H, CO2C*H*3), 3.45-3.44 (m, 1H, H5A), 3.34-3.30 (m, 1H, H2A), 3.22 (t, 1H, *J* = 9.0 Hz, H3A), 2.80- 2.73 [m, 4H, 2 x C*H*2 Lev], 2.61-

2.58 [m, 4H, 2 x C*H*2 Lev], 2.19 (s, 3H, C*H*3 Lev), 2.17 (s, 3H, C*H*3 Lev), 1.67-1.63 [m, 1H, C*H*(CH3)2], 0.90-0.88 [4s, 12H, C(C*H*3)2, CH(C*H*3)2], 0.19-0.17 [2s, 6H, Si(C*H*3)2]. 13C NMR (75.5 MHz, CDCl3): δ 206.5, 206.3, 172.1, 171.3, 169.4, 138.0, 137.3, 128.4, 128.3, 128.1, 128.1, 128.0, 127.9, 127.5, 127.3, 97.7, 97.0, 81.0, 77.4, 77.0, 76.6, 74.7, 74.5, 74.3, 72.3, 68.9, 68.4, 67.7, 67.3, 62.5, 51.9, 37.8, 37.8, 33.9, 33.7, 31.9, 30.1, 29.8, 29.7, 29.3, 27.9, 24.8, 19.9, 19.8, 18.4, 18.3, 14.1, -2.1, -3.2. HR MALDI-TOF MS: m/z: calcd for $C_{45}H_{63}N_3O_{15}Si$ $[M+Na]^+$: 936.3926, found 936.3956.

**Dimethylthexylsilyl *O*-(methyl-2-*O*-levulinoyl-3-*O*-benzyl-α-L-idopyranosyluronate)-(1→4)-*O* -2-azido-3-*O*- benzyl-6-*O*-levulinoyl-2-deoxy-β-D-glucopyranoside (D)**

To a solution of compound **C** (3.0 g, 3.29 mmol) in DCM at 0 ºC was added FmocCl (8.49 g, 32.9 mmol) and DMAP (40.3 mg, 0.33 mmol). The reaction mixture was brought to room temperature, and stirring was continued until TLC indicated complete consumption of the starting material (~2 h). After quenching the reaction with MeOH (5 mL), the mixture was diluted with DCM (50 mL) and washed with saturated aqueous sodium bicarbonate (2 × 50 mL) and brine (50 mL). The organic phase was dried (MgSO4), filtered, and concentrated in vacuo. The residue was chromatographed over silica gel using a gradient of hexanes and EtOAc to give compound **5** (2.9 g, 78.8%).

***N*-(Benzyl)-1H,1H,2H,2H-Perfluorodecyl-benzyloxycarbonyl-5-aminopentanol-*O*-(methyl-2-*O* -levulinoyl-3-*O*-benzyl-4-fluorenylmethoxycarbonyl-α-L-idopyranosyluronate)-(1→4)-*O*-2-azido-3-*O*-benzyl-6-*O*-levulinoyl-2-deoxy-β-D-glucopyranoside (7)**

A mixture of compound **5** (100 mg, 88.1 μmol), 30% HF in pyridine (0.75 mL) and THF (1.5 mL) was stirred at rt for 18 h, and then diluted with DCM (50 mL), washed with water (50 mL), saturated aqueous sodium bicarbonate (50 mL), and brine (50 mL). The organic phase was

dried ($MgSO_4$), filtered, and concentrated under reduced pressure and the resulting oil was left *in vacuo* for 2 h. NaH (60%) (3.5 mg, 88.1 μmol) was added to a mixture of the crude material and $CF_3CNPhCl$ (53.0 $\mu$L, 264.3 μmol) in DCM. After 1 h, the mixture was dried and purified by silica gel to give compound **6** which was used directly for glycosylation. Compounds **6** (27.0 mg, 23.3 μmol) and **4** (22.8 mg, 28.0 μmol) were coevaporated with toluene (3×3 mL), and then dissolved in a mixture of toluene and dioxane (1/3, v/v, 800 $\mu L$). Freshly activated powdered 4 Å molecular sieves were added, and the mixture was stirred for 30 min at ambient temperature. After the addition of TfOH (0.93 $\mu L$, 10 μmol), the reaction mixture was stirred for 15 min at room temperature and then quenched with pyridine (5 $\mu$L). The mixture was filtered, the filtrate was concentrated under reduced pressure and the residue was purified by silica gel (Tol/THF, 100/1~10/1) to give **7** (alpha) as white foam (22.5 mg, 56 %). $^1$H NMR (600 MHz, $CDCl_3$) δ 7.80 – 7.08 (m, 27H, C*H* Ar), 5.22 – 5.11 (m, 2H, C$H_2$Cbz), 5.05 (d, $J$ = 12.4 Hz, 1H, H-1$^B$), 5.00 – 4.80 (m, 4H, H-1$^A$, H-2$^B$, H-4$^B$, H-5$^B$), 4.76 – 4.58 (m, 4H, 2×C$H_2$Bn), 4.55 – 4.43 (m, 4H, H-6a$^A$, NC$H_2$Bn, C*H*HFmoc), 4.42 – 4.34 (m, 1H, CH*H*Fmoc), 4.23 – 4.15 (m, 2H， H-6b$^A$, C*H*Fmoc), 3.96 – 3.83 (m, 2H, H-4$^A$, H-3$^B$), 3.80 (t, $J$ = 9.7 Hz, 1H, H-3$^A$), 3.65 (m, 1H, OC*H*H Linker), 3.45 (s, 3H, $CO_2CH_3$), 3.33 (dd, $J$ = 10.3, 3.7 Hz, 1H, H-2$^A$), 3.30 (m, 2H, NC$H_2$ Linker), 2.90 (m, 2H, C$H_2CH_2C_8F_{17}$), 2.86 – 2.53 (m, 8H, ($CH_2$)$_2$ Lev), 2.43 – 2.30 (m, 2H, $CH_2CH_2C_8F_{17}$), 2.18 (s, 3H, C$H_3$Lev), 2.06 (s, 3H, C$H_3$Lev), 1.68 – 1.52 (m, 4H, ($CH_2$)$_2$ Linker), 1.34 (m, C$H_2$ Linker). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 128.9, 128.4, 128.3, 127.9, 127.8, 127.3, 127.2, 127.1, 126.3, 126, 125.9, 124.9, 123.3, 121.0, 120.0, 118.4, 97.5, 97.1, 81.0, 78.2, 74.7, 74.5, 74.2, 73.9, 73.8, 72.9, 72.8, 71.9, 71.8, 71.3, 70.1, 68.9, 68.2, 67.8, 67.3, 67.2, 66.8, 66.7, 66.3, 65.8, 65.1, 63.4, 62.4, 53.7, 53.1, 52.1, 51.4, 51.1, 50.4, 49.3, 47.1, 46.6, 46.2, 37.7, 32.9, 29.8, 29.6, 29.5, 28.2, 27.8,

27.6, 26.1, 23.2. HR MALDI-TOF MS: m/z: calcd for $C_{82}H_{81}F_{17}N_7O_{19}$ $[M+Na]^+$: 1771.5121; found: 1771.5123.

***N*-(Benzyl)-1H,1H,2H,2H-Perfluorodecyl-benzyloxycarbonyl-5-aminopentanol-*O*-(methyl-2-*O* -levulinoyl-3-*O*-benzyl-α-L-idopyranosyluronate)-(1→4)-*O*-2-azido-3-*O*-benzyl-6-*O*-levulinoyl-2-deoxy-β-D-glucopyranoside (8)**

Compound **7** (179.0 mg, 102.9 μmol) and $Et_3N$ in DCM (1/4, v/v, 5 mL) was stirred for 4 h. The mixture was concentrated under reduced pressure and the resulting residue purified by F-SPE to give **8** as white foam (156.9 mg, 100 %). $^1$H NMR (600 MHz, $CDCl_3$) δ 7.50 – 6.99 (m, 19H, C*H* Ar), 5.15 (d, *J* = 23.5 Hz, 2H, C$H_2$Cbz), 5.00 (d, *J* = 10.6 Hz, 1H, H-1$^B$), 4.91 (m, 2H, H-2$^B$, H-5$^B$), 4.86 (broad s, 1H, H-1$^A$), 4.73 (d, *J* = 11.0 Hz, 2H, C$H_2$Bn), 4.69 – 4.59 (m, 2H, C$H_2$Bn), 4.49 (s, 2H, NC$H_2$Bn), 4.44 – 4.40 (m, 1H, H-6a$^A$), 4.29 – 4.17 (m, 1H, H-6b$^A$), 3.97 (t, *J* = 10.9 Hz, 1H, H-4$^B$), 3.92 – 3.79 (m, 3H, H-3$^A$, H-4$^A$, H-5$^A$), 3.74 (s, 1H, H-3$^B$), 3.65 (m, 1H, OC*H*H Linker), 3.52 – 3.16 (m, 7H, H-2$^A$, OC$H_3$, NC$H_2$ Linker, OC*H*H Linker, ), 2.90 (m, 2H, C$H_2$C$H_2$C$_8$F$_{17}$), 2.76 – 2.50 (m, 8H, (C$H_2$)$_4$Lev), 2.30 (m, 2H, CH$_2$C$H_2$C$_8$F$_{17}$), 2.17 (m, 6H, 2×-C$H_3$), 1.75 – 1.47 (m, 4H, (C$H_2$)$_2$ Linker), 1.45 – 1.18 (m, 2H, C$H_2$ Linker). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 128.4, 128.3, 127.9, 127.8, 97.7, 97.6, 81.0, 78.3, 76.5, 74.7, 74.6, 74.5, 74.5, 73.0, 72.3, 70.4, 69.9, 68.7, 68.3, 68.2, 67.7, 67.4, 66.8, 66.4, 63.3, 62.4, 51.9, 50.3, 47.0, 46.2, 37.8, 36.0, 32.8, 29.9, 29.6, 27.9, 27.8, 26.0, 23.2. HR MALDI-TOF MS: m/z: calcd for $C_{67}H_{71}F_{17}N_4O_{17}$ $[M+Na]^+$: 1549.4441; found: 1549.4445.

***N*-(Benzyl)-1H,1H,2H,2H-Perfluorodecyl-benzyloxycarbonyl-5-aminopentanol-*O*-[(methyl-2- *O*-levulinoyl-3-*O*-benzyl-4-*O*-fluorenylmethoxycarbonyl-α-L-idopyranosyluronate)-(1→4)-*O*-(2-deoxy-2-azido-3-*O*-benzyl-6-*O*-levulinoyl-β-D-glucopyranoside)-(1→4)-*O*-**

**(methyl-2-*O*-levulinoyl-3-*O*-benzyl-α-L-idopyranosyluronate)]-(1→4)-*O*-2-azido-3-*O*-benzyl-6-*O*-levulinoyl-2-deoxy-β-D-glucopyranoside (9)**

Compound **6** (27.0 mg, 23.3 μmol) and **8** (30.0 mg, 19.7 μmol) were coevaporated with toluene (3×3 mL), and then dissolved in DCM (0.4 mL). Freshly activated powdered 4 Å molecular sieves were added, and the mixture was stirred for 30 min at ambient temperature. After the addition of TfOH (2.1 $\mu$L, 23.3 μmol), the reaction mixture was stirred for 15 min and then quenched with pyridine (5 $\mu$L). The mixture was filtered and the filtrate was concentrated under reduced pressure. The residue was subjected to F-SPE and the resulting product and unreacted acceptor was re-subjected to the glycosylation conditions above using donor **6** (13.5 mg, 12.7 μmol) and TfOH (1.00 $\mu$L, 11.8 μmol). The resulting product was purified by F-SPE to afford **9** as white foam (34.5 mg, 72%). $^{1}$H NMR (500 MHz, $CDCl_3$) δ 7.83 – 7.08 (m, 37H, C*H* Ar), 5.21 (d, *J* = 3.7 Hz, 1H, H-1$^{B}$), 5.15 (d, *J* = 17.0 Hz, 2H, C*H*$_2$Cbz), 5.06 (dt, *J* = 8.7, 2.6 Hz, 2H, H-1$^{C}$, H-1$^{D}$), 4.98 – 4.93 (m, 2H, H-4$^{D}$, H-2$^{B}$), 4.91 (t, *J* = 4.1 Hz, 1H, H-2$^{D}$), 4.87 (d, *J* = 3.3 Hz, 1H, H-5$^{D}$), 4.85 – 4.79 (m, 2H, C*H*HBn, H-5$^{B}$), 4.79 – 4.57 (m, 9H, C*H*$_2$Bn), 4.53 – 4.42 (m, 4H, NC*H*$_2$Bn, H-6a$^{C}$, C*H*HFmoc), 4.39 (dd, *J* = 10.5, 7.2 Hz, 1H, CH*H*Fmoc), 4.30 – 4.10 (m, 4H, C*H*Fmoc, H-6$^{A}$, H-6b$^{C}$), 4.02 (t, *J* = 4.5 Hz, 1H, H-4$^{B}$), 3.98 – 3.77 (m, 7H, H-3$^{A}$, H-4$^{A}$, H-3$^{B}$, H-4$^{B}$, H-4$^{C}$, H-5$^{C}$, H-3$^{D}$), 3.65 – 3.61 (t, *J* = 6.0 Hz, 2H, H-3$^{C}$, OC*H*H Linker), 3.51 – 3.41 (m, 8H, OC*H*H Linker, H-4$^{A}$, 2×C*H*$_3$), 3.34 – 3.12 (m, 4H, H-2$^{A}$, H-2$^{C}$, NC*H*$_2$ Linker), 2.90 (m, 2H, C*H*$_2$CH$_2$C$_8$F$_{17}$), 2.86 – 2.45 (m, 16H, (C*H*$_2$)$_8$Lev), 2.42 – 2.31 (m, 2H, CH$_2$C*H*$_2$C$_8$F$_{17}$), 2.17 (s, 6H, 2×C*H*$_3$Lev), 1.64 – 1.53 (m, 4H, (C*H*$_2$)$_2$ Linker), 1.30 (m, 2H, C*H*$_2$ Linker). $^{13}$C NMR (126 MHz, $CDCl_3$) 128.6, 128.1, 127.7, 126.9, 125.4, 120.5, 98.3, 97.9, 97.5, 97.0, 78.3, 75.5, 75.0, 74.5, 73.9, 73.6, 72.4, 72.1, 71.6, 70.4, 69.8, 69.6, 69.5, 69.2, 68.4, 67.4, 67.1, 63.5, 62.5, 62.3, 59.8, 52.3,

52.0, 50.6, 47.3, 46.8, 46.5, 38.1, 37.9, 33.1, 31.5, 31.0, 29.9, 29.1, 28.2, 28.0, 27.8, 26.4, 23.4. HR MALDI-TOF MS: m/z: calcd for $C_{119}H_{124}F_{17}N_7O_{33}$ $[M+Na]^+$: 2524.7866; found: 2524.7859.

**_N_-(Benzyl)-1H,1H,2H,2H-Perfluorodecyl-benzyloxycarbonyl-5-aminopentanol-_O_-[(methyl-3- _O_-benzyl-4-_O_-fluorenylmethoxycarbonyl-α-L-idopyranosyluronate)-(1→4)-_O_-(2-deoxy-2-azido-3-_O_-benzyl-β-D-glucopyranoside)-(1→4)-_O_-(methyl-3-_O_-benzyl-α-L-idopyranosyluronate)]-(1→4)-_O_-2-deoxy-2-azido-3-_O_-benzyl-β-D-glucopyranoside (10)**

Hydrazine acetate (92.0 mg, 1.0 mmol) was added to a solution of **9** (125.0 mg, 50.0 μmol) in DCM and MeOH (1/1, v/v, 5 mL). The mixture was stirred for 2 h and then diluted with DCM (20 mL), washed water (20 mL) and brine (20 mL). The organic layer was dried ($MgSO_4$), filtered and the filtrate concentrated under reduced pressure. The residue was purified by F-SPE to afford **10** as white foam (90.0 mg, 85.7 %). $^1$H NMR (600 MHz, $CDCl_3$) δ 7.75 – 7.11 (m, 37, C*H* Ar), 5.35 (s, 1H, H-$1^D$), 5.30 – 5.23 (m, 1H, H-$1^B$), 5.18 – 5.11 (m, 2H, C$H_2$Cbz), 4.97 (m, 2H, H-$1^C$, H-$4^D$), 4.87 (m, 2H, H-$1^A$, H-$5^B$), 4.83 – 4.57 (m, 8H, 4×C$H_2$Bn), 4.49 (m, 2H, NC$H_2$Bn), 4.42 – 4.36 (m, 2H, C$H_2$Fmoc), 4.30 – 4.11 (t, $J$ = 7.6 Hz, 1H, C*H*Fmoc), 4.09 – 4.04 (m, 1H, H-$5^C$), 3.96 – 3.74 (m, 11H, H-$3^A$, H-$4^A$, H-$5^A$, H-$6^A$, H-$2^B$, H-$3^B$, H-$4^C$, H-$6^C$, H-$3^D$), 3.70 – 3.59 (m, 3H, H-$3^C$, H-$2^D$, C*H*H Linker), 3.55 – 3.43 (m, 5H, H-$2^C$, CH*H* Linker, $CO_2CH_3$), 3.38 (s, 3H, $CO_2CH_3$), 3.32 (m, 1H, H-$2^A$), 3.28 – 3.18 (m, 2H, NC$H_2$ Linker), 2.90 (m, 2H, C$H_2CH_2C_8F_{17}$), 2.42 – 2.25 (m, 2H, $CH_2CH_2C_8F_{17}$), 1.73 – 1.49 (m, 4H, $(CH_2)_2$ Linker), 1.26 (s, 2H, C$H_2$ Linker). $^{13}$C NMR (151 MHz, $CDCL_3$) δ128.4, 128.3, 128.1, 128.0, 127.9, 127.5, 127.4, 124.9, 120, 101.0, 100.6, 97.6, 95.7, 79.2, 78.3, 75.8, 75.5, 75.3, 75.0, 74, 73.9, 73.2, 73.0, 72.8, 72.4, 72.3, 72.2, 70.4, 69.9, 68.4, 68.3, 68.1, 67.6, 66.8, 63.9, 63.4, 61.2, 61.1, 53.6, 53.5, 52.3, 52.0, 50.8, 50.6, 50.4, 46.5, 46.2, 32.8, 28.9, 27.2, 26.1, 23.0. HR MALDI-TOF MS: m/z: calcd for $C_{99}H_{100}F_{17}N_7O_{25}$ $[M+Na]^+$: 2232.6395; found: 2232.6401.

***N*-(Benzyl)-1H,1H,2H,2H-Perfluorodecyl-benzyloxycarbonyl-5-aminopentanol-*O*-[(methyl-2- *O*-sulfonate-3-*O*-benzyl-4-*O*-fluorenylmethoxycarbonyl-α-L-idopyranosyluronate)-(1→4)-*O*-(2-deoxy-2-azido-3-*O*-benzyl-6-*O*-sulfonate-β-D-glucopyranoside)-(1→4)-*O*-(2-*O*-sulfonate-3-*O*-benzyl-α-L-idopyranosyluronate)]-(1→4)-*O*-2-deoxy-2-azido-3-*O*-benzyl-6-*O*-sulfonate-β-D-glucopyranoside (11)**

Sulfur trioxide pyridine complex (272.0 mg, 1.7 mmol) was added to a solution of **10** (90.0 mg, 42.7 μmol) in DMF (3 mL). The mixture was stirred at ambient temperature for 5 h and then saturated $NaHCO_3$ (300 $\mu$L) was added and stirring was continued for 30 min. The mixture was directly loaded on a F-SPE cartridge which was eluted with 80% methanol/$H_2O$ and then methanol to give **11** as white foam (80.0 mg, 74.7 %). $^1$H NMR (500 MHz, $CDCl_3$) δ 7.97 – 6.96 (m, 37H, C*H* Ar), 5.53 – 5.38 (m, 2H, H-1$^B$, H-1$^D$), 5.22 – 5.08 (m, 3H, H-1$^C$, C*H*$_2$Bn), 5.03 – 4.95 (m, 2H, H-2$^B$, H-2$^D$), 4.95 – 4.75 (m, 4H, H-1$^A$, H-5$^D$, C*H*$_2$Bn), 4.76 – 4.47 (m, 10H, H-5$^B$, H-4$^D$, NC*H*$_2$Cbz, C*H*$_2$Fmoc, 2×C*H*$_2$Bn), 4.47 – 4.29 (m, 7H, H-6$^A$, H-3$^B$, H-6$^C$, H-3$^D$, C*H*Fmoc), 4.29 – 4.19 (m, 4H, H-4$^A$, H-4$^B$, H-4$^C$, H-5$^C$), 4.08 – 3.89 (m, 4H, H-4$^A$, H-4$^B$, H-4$^C$, H-5$^C$), 3.79 – 3.70 (m, 2H, H-5$^A$, C*H*H Linker), 3.65 (m, 2H, H-3$^A$, H-3$^C$), 3.51 – 3.16 (m, 11H, H-2$^A$, H-2$^C$, CH*H* Linker, NC*H*$_2$ Linker, 2×$CO_2$C*H*$_3$), 2.92 (broad m, 2H, C*H*$_2$$CH_2C_8F_{17}$), 2.60 – 2.38 (m, 2H, $CH_2$C*H*$_2$$C_8F_{17}$), 1.71 – 1.47 (m, 4H, (C*H*$_2$)$_2$ Linker), 1.33 (m, 2H, C*H*$_2$ Linker). $^{13}$C NMR (126 MHz, $CDCl_3$) 129.0, 128.4, 128.3, 128.1, 128.0, 127.9, 127.5, 125.2, 119.8, 119.7, 98.0, 97.9, 95.0, 81.2, 78.6, 74.8, 74.5, 74.3, 73.9, 72.7, 72.5, 72.4, 71.2, 70.4, 70.3, 70.1, 70.0, 69.9, 69.8, 69.5, 68.8, 67.7, 67.2, 67.1, 67.0, 66.2, 65.9, 65.7, 64.4, 63.8, 53.6, 52.9, 52.7, 51.8, 51.7, 51.6, 50.3, 49.9, 49.2, 48.5, 48.0, 46.7, 45.0, 32.4, 29.0, 27.5, 25.8, 23.2. HR ESI-TOF MS: m/z: calcd for $C_{99}H_{96}F_{17}N_7Na_4O_{37}S_4$ [M-4$Na^+$+$H^+$]: 808.8178; found: 808.8173.

***N*-(Benzyl)-1H,1H,2H,2H-Perfluorodecyl-benzyloxycarbonyl-5-aminopentanol-*O*-[(2-*O*-sulfon ate-3-*O*-benzyl-α-L-idopyranosyluronate)-(1→4)-*O*-(2-deoxy-2-azido-3-*O*-benzyl-6-*O*-sulfonate-β-D-glucopyranoside)-(1→4)-*O*-(2-*O*-sulfonate-3-*O*-benzyl-α-L-idopyranosyluronate)]-(1→4)-*O*-2-deoxy-2-azido-3-*O*-benzyl-6-*O*-sulfonate-β-D-glucopyranoside (12)**

A premixed solution of 30% $H_2O_2$ in water (500 $\mu$L) and 1 M LiOH (2.2 mL) was added to a solution of **11** (80.0 mg, 31.8 μmol) in THF (2.2 mL). The reaction mixture was stirred at ambient temperature for 18 h, concentrated under reduced pressure (bath temperature 20 ºC) and loaded on F-SPE column using a mixture of DMF, $H_2O$ and $CF_3CH_2OH$ (9/1/0.1, v/v/v, 3 mL). The column was eluted with 40% MeOH/$H_2O$ (10 mL) and MeOH (20 mL). Appropriate fractions were concentrated under reduced pressure (bath temperature 20 ºC), and the residue was passed through a column of Biorad 50 × 8 $Na^+$ resin (0.6 × 5 cm) using $CH_3OH$ as eluent, providing **12** as white foam (60.0 mg, 82.0 %). $^1$H NMR (600 MHz, $CD_3OD$) δ 7.78 – 6.95 (m, 29H, C*H* Ar), 5.40 – 5.21 (m, 2H, H-1$^B$, H-1$^D$), 5.11 – 4.98 (m, 3H, H-1$^C$, C*H*$_2$Cbz), 4.94 – 4.85 (m, 4H, H-2$^B$, H-2$^D$, C*H*$_2$Bn), 4.77 (m, 2H, H-1$^A$, C*H*HBn), 4.66 – 4.36 (m, 10H, H-4$^B$, H-5$^B$, H-5$^D$, NC*H*$_2$Bn, 5H of C*H*HBn), 4.33 – 4.29 (m, 2H, H-6$^C{}_a$, C*H*HBn), 4.29 – 4.14 (m, 4H, H-6$^A$, H-4$^D$, CH*H*Bn), 4.13 – 4.03(m, 1H, H-6$^C{}_b$), 4.02 (s, 1H, H-3$^B$), 3.97 (t, *J* = 9.3 Hz, 1H, H-4$^C$), 3.94 – 3.79 (m, 5H, H-5$^A$, H-3$^C$, H-4$^C$, H-5$^C$, H-3$^D$), 3.78 – 3.53 (m, 3H, H-3$^A$, H-4$^A$, OC*H*H Linker), 3.52 – 3.45 (m, 1H, H-3$^C$), 3.35 – 3.25 (m, 1H, OCH*H* Linker), 3.27 – 3.17 (m, 3H, H-2$^A$, NC*H*$_2$ Linker), 3.14 (m, 1H, H-2$^C$), 2.81 (broad s, 2H, C*H*$_2$CH$_2$C$_8$F$_{17}$), 2.35 (s, 2H, CH$_2$C*H*$_2$C$_8$F$_{17}$), 1.60 – 1.40 (m, 4H, (C*H*$_2$)$_2$ Linker), 1.26 – 1.20 (m, 2H, C*H*$_2$ Linker). $^{13}$C NMR (151 MHz, $CD_3OD$) δ 128.4, 127.9, 127.7, 97.6, 94.4, 82.9, 78.9, 76.0, 75.5, 75.4, 74.6, 74.5, 72.8, 72.7, 72.4, 72.1, 71.8, 71.7, 71.6, 71.0, 70.3, 69.7, 69.5, 69.4, 68.7, 68.6, 68.1, 67.4, 67.1, 66.8, 66.7, 66.3, 66.2, 64.4, 63.7, 63.6,

60.5, 52.8, 52.2, 51.8, 51.6, 51.4, 50.9, 50.2, 50.1, 49.6, 48.9, 48.8, 48.4, 48.0, 47.9, 47.3, 46.9, 46.1, 45.6, 44.9, 44.8, 44.2, 43.6, 43.4, 42.9, 35.6, 32.3, 28.8, 27.1, 25.8, 23.2. HR ESI-TOF MS: m/z: calcd for $C_{82}H_{80}F_{17}N_7Na_6O_{35}S_4$ [M-6$Na^+$+3$H^+$]: 725.4514; found: 725.4512.

**_N_-(Benzyl)-1H,1H,2H,2H-Perfluorodecyl-benzyloxycarbonyl-5-aminopentanol-_O_-[(2-_O_-sulfon ate-3-_O_-benzyl-α-L-idopyranosyluronate)-(1→4)-_O_-(2-deoxy-2-amino-3-_O_-benzyl-6-_O_-sulfonate- β -D-glucopyranoside)-(1→4)-_O_-(2-_O_-sulfonate-3-_O_-benzyl-α-L-idopyranosyluronate)]-(1→4)-_O_-2-deoxy-2-amino-3-_O_-benzyl-6-_O_-sulfonate-β-D-glucopyranoside Hexasodium salt (13).**

A mixture of **13** (60.0 mg, 26.0 μmol), 1 M solution of $PMe_3$ in THF (1.3 mL, 1.3 mmol), 0.1 M NaOH (2.6 mL, 259.6 μmol) in THF (3.0 mL) was stirred at ambient temperature for 5 h and then concentrated under reduced pressure (bath temperature 20 °C) after the pH was adjusted to 8.5 by the addition of AcOH. The residue was purified by F-SPE to provide **13** as white foam (45.0 mg, 78.0 %). $^1$H NMR (600 MHz, $CD_3OD$) δ 7.47 – 7.00 (m, 29H, C*H* Ar), 5.84 (s, 1H, H-$1^B$), 5.55 (s, 1H, H-$1^D$), 5.22 – 5.02 (m, 4H, 3H of C*H*$_2$Bn, H-$2^D$), 5.00 – 4.92 (m, 4H, 3H of C*H*HBn, H-$1^A$), 4.84 (s, 1H, H-$2^B$), 4.73 (d, *J* = 11.0 Hz, 1H, C*H*HBn), 4.69 – 4.56 (m, 4H, CH*H*Bn, H-$1^C$, H-$5^B$, H-$5^D$), 4.56 – 4.46 (m, 3H, NC*H*$_2$Bn, C*H*HBn), 4.45 – 4.27 (m, 3H, H-$4^D$, CH*H*Bn, H-$6^{C}_a$), 4.23 – 4.12 (m, 4H, H-$6^A$, H-$6^{C}_b$, H-$3^D$), 4.09 – 4.02 (m, 1H, H-$3^A$), 3.98 – 3.91 (m, 2H, H-$4^B$, H-$5^C$), 3.95 (m, 2H, NC*H*$_2$ Linker), 3.84 (broad m, 1H, H-$5^A$), 3.78 (broad s, 1H, H-$3^B$), 3.66 – 3.59 (m, 1H, C*H*H Linker), 3.52 – 3.37 (m, 1H, H-$3^C$), 3.29 – 2.89 (m, 1H, CH*H* Linker), 2.92 (m, 2H, C*H*$_2$CH$_2$C$_8$F$_{17}$), 2.82 (t, *J* = 10.9 Hz, 1H, H-$2^A$), 2.41 (m, 3H, H-$2^C$, CH$_2$C*H*$_2$C$_8$F$_{17}$), 1.71 – 1.47 (m, 4H, (C*H*$_2$)$_2$ Linker), 1.33 (m, 2H, C*H*$_2$ Linker). $^{13}$C NMR (151 MHz, $CD_3OD$) δ 129.1, 128.3, 128.1, 128.0, 127.7, 127.1, 127.0, 126.7, 126.5, 103.0, 98.5, 97.2, 95.6, 94.6, 82.3, 80.6, 77.9, 76.7, 75.3, 75.1, 74.0, 73.3, 72.7, 71.2, 71, 70.5, 70.3, 69.7, 69.3, 69,

68.6, 67.9, 67.8, 67.6, 67.4, 67.2, 67.1, 67.0, 66.8, 66.7, 66.5, 65.6, 56.1, 55.6, 54.9, 51.2, 50.2, 49.6, 49.5, 48.9, 48.4, 47.8, 46.4, 46.1, 45.5, 44.8, 32.1, 29.2, 28.7, 27.3, 27.2, 25.6, 22.9. HR ESI-TOF MS: m/z: calcd for $C_{82}H_{84}F_{17}N_3Na_6O_{35}S_4$ [M-6$Na^+$+3$H^+$]: 708.1244; found: 708.1242.

**5-aminopentanol-*O*-[(2-*O*-sulfonate-α-L-idopyranosyluronate)-(1→4)-*O*-(2-deoxy-2-sulfoamino-6-*O*-sulfonate-β-D-glucopyranoside)-(1→4)-*O*-(2-*O*-sulfonate-α-L-idopyranosyluronate)]-(1→4)-*O*-2-deoxy-2-sulfoamino-6-*O*-sulfonate-β-D-glucopyranoside Octasodium salt (15)**

Sulfur trioxide pyridine complex (337.9 mg, 2.0 mmol) was added to **13** (24.0 mg, 10.6 μmol) in in a mixture of $CH_3OH$ (2 mL), trifluoroethanol (0.2 mL), triethylamine (0.3 mL) and 0.1 M NaOH (424 $\mu$L, 42.4 μmol) at 0 °C. After stirring for 18 h, the reaction mixture was concentrated under reduced pressure (bath temperature 20 °C). The residue was purified by F-SPE as described for the purification of compound **12** to afford **14,** which was used directly for hydrogenolysis. Pd/C (10%, 16 mg) was added to a solution of **14** (16.2 mg, 6.58 μmol) in $CH_3OH$, $H_2O$, $CH_3CO_2H$ (1/1/0.02, v/v/v, 4 mL). The mixture was stirred for 12 h under an atmosphere of hydrogen and then filtered through a PTFE syringe filter (0.2 mm, 13 mm), washed with a mixture of $CH_3OH$ and $H_2O$ (1/1, v/v, 2 mL), and the solvents were concentrated under reduced pressure. The residue was dissolved in distilled water, $CH_3CO_2H$ (1/0.01, v/v, 4 mL), and $Pd(OH)_2$ on carbon (Degussa type, 20%, 16 mg) was added. The mixture was stirred for 12 h under an atmosphere of hydrogen and then filtered through a PTFE syringe filter. The residue was washed with $H_2O$ (2 mL) and after freezing drying the filtrate, the residue was dissolved in $H_2O$ and passed through a column of Biorad 50 × 8 $Na^+$ resin (0.6 × 2.5 cm). The appropriate fractions were freeze dried to provide **15** as white foam (8.0 mg, 85.0 %). $^1$H NMR (500 MHz, $D_2O$) δ 5.31 (s, 1H, H-$1^B$), 5.16 – 5.02 (m, 3H, H-$1^A$, H-$1^C$, H-$1^D$), 4.78 – 4.60 (m, 2H, H-$2^B$, H-$2^D$), 4.30 – 4.15 (m, 8H,

H-6$^{A}$, H-6$^{C}$, H-5$^{D}$), 4.07 (t, *J* = 4.0 Hz, 1H, H-4$^{B}$), 4.04 – 3.84 (m, 5H, H-3$^{A}$, H-4$^{A}$, H-5$^{A}$, H-3$^{B}$, H-5$^{C}$, H-3$^{D}$), 3.70 – 3.52 (m, 6H, H-3$^{A}$, H-3$^{C}$, H-4$^{C}$, H-4$^{D}$, C*H*H Linker), 3.45 (m, 1H, CH*H* Linker), 3.17 (dd, *J* = 10.6, 2.4 Hz, 1H, H-2$^{C}$), 2.96 (m, 3H, H-2$^{A}$, NC*H*$_2$ Linker), 1.66 – 1.52 (m, 4H, (CH$_2$)$_2$ Linker), 1.40 – 1.32 (m, 2H, CH$_2$ Linker). $^{13}$C NMR (126 MHz, D$_2$O) δ 101.6, 99.1, 97.0, 96.4, 81.8, 77.0, 76.8, 76.7, 76.3, 75.9, 74.1, 72.8, 69.6, 69.5, 69.4, 69.3, 69.1, 68.9, 68.8, 67.9, 67.1, 66.6, 66.4, 60.1, 57.9, 52.1, 51.0, 49.9, 48.6, 47.0, 39.5, 28.0, 25.1, 22.5. HR ESI-TOF MS: m/z: calcd for $C_{29}H_{43}N_3Na_8O_{39}S_6$ [M-7Na$^+$+4NH$^+$+H$^+$]: 672.5575; found: 672.5572.

**_N_-(Benzyl)-1H,1H,2H,2H-Perfluorodecyl-benzyloxycarbonyl-5-aminopentanol-_O_-[(2-_O_-sulfonate-3-_O_-benzyl-α-L-idopyranosyluronate)-(1→4)-_O_-(2-deoxy-2-acetamido-3-_O_-benzyl-6-_O_-sulfonate-β-D-glucopyranoside)-(1→4)-_O_-(2-_O_-sulfonate-3-_O_-benzyl-α-L-idopyranosyluronate)]-(1→4)-_O_-2-deoxy-2-acetamido-3-_O_-benzyl-6-_O_-sulfonate-β-D-glucopyranoside Hexasodium salt (16)**

Acetic anhydride (15.0 μL, 160.0 μmol) was added to a mixture of **13** (18.0 mg, 8.0 μmol) in CH$_3$OH (2 mL) and Et$_3$N (22.5 μL, 160.0 μmol) at 0 °C. After stirring for 1 h at ambient temperature, the mixture was coevaporated with toluene under reduced pressure (bath temperature 20 °C) and the residue was purified by F-SPE as described for the preparation of compound **12** to provide **13** as white foam (24.0 mg, 87.0%). $^1$H NMR (500 MHz, CD$_3$OD) δ 7.52 – 6.90 (m, 29H, C*H* Ar), 5.84 (broad s, 1H, H-1$^{B}$), 5.63 (s, H-1$^{D}$), 5.24 – 5.09 (m, 2H, CH$_2$Bn), 5.04 (d, *J* = 11.5 Hz, 1H, C*H*HBn), 4.96 – 4.71 (m, 7H, H-2$^{B}$, H-1$^{C}$, H-5$^{D}$, 2×C*H*$_2$Bn), 4.67 – 4.49 (m, 8H, H-1$^{A}$, H-5$^{B}$, H-5$^{C}$, H-4$^{D}$, NC*H*$_2$Bn, C*H*$_2$Bn), 4.41 (broad s, *J* = 12.0 Hz, 2H, C*H*HBn, H-6$^{C}_{a}$), 4.39 – 4.32 (m, 2H, H-6$^{A}_{a}$, H-3$^{D}$), 4.31 – 4.21 (m, 3H, H-2$^{C}$, H-6$^{A}_{b}$, H-6$^{C}_{b}$), 4.18 (t, *J* = 9.9 Hz, 1H, H-4$^{C}$), 4.12 – 4.07 (m, 2H, H-4$^{A}$, H-4$^{B}$), 4.01 (t, *J* = 9.9 Hz, 1H, H-3$^{C}$), 3.98 – 3.93 (m, 1H, H-2$^{A}$), 3.89 (broad s, 1H, H-5$^{A}$), 3.82 – 3.53 (m, 3H, H-3$^{A}$, H-3$^{B}$, OC*H*H Linker), 3.37 – 3.17 (m, 3H, OCH*H*

Linker, NC$H_2$ Linker), 2.96 – 2.92 (m, 2H, C$H_2$CH$_2$C$_8$F$_{17}$), 2.48 (s, 2H, CH$_2$C$H_2$C$_8$F$_{17}$), 2.08 (s, 3H, COC$H_3$), 1.69 – 1.42 (m, 7H, (C$H_2$)$_2$ Linker, COC$H_3$), 1.43 – 1.26 (m, 2H, C$H_2$ Linker). $^{13}$C NMR (126 MHz, CD$_3$OD) δ 128.0, 127.9, 97.3, 97.1, 95.7, 94.2, 78.8, 75.9, 75.8, 75.2, 75.1, 73.8, 73.7, 73.0, 72.8, 72.1, 72.0, 71.2, 71.1, 70.8, 70.3, 70.1, 69.6, 69.4, 68.9, 67.8, 67.7, 67, 65.9, 53.4, 53.1, 50.4, 50.3, 49.9, 49.2, 48.7, 48.2, 47.4, 47.2, 47.0, 45.8, 38.1, 32.5, 29.0, 27.5, 25.9, 23.3, 22.5, 21.6, 21.3. HR ESI-TOF MS: m/z: calcd for $C_{86}H_{88}F_{17}N_3Na_6O_{37}S_4$ [M-6Na$^+$+3H$^+$]: 736.1314; found: 736.1312.

**5-aminopentanol-*O*-[(2-*O*-sulfonate-6-*O*-sulfonate-α-L-idopyranosyluronate)-(1→4)-*O*-(2-deoxy-2-acetamido-6-*O*-sulfonate-β-D-glucopyranoside)-(1→4)-*O*-(2-*O*-sulfonate-α-L-idopyranosyluronate)]-(1→4)-*O*-2-deoxy-2-acetamido-6-*O*-sulfonate-β-D-glucopyranoside Hexasodium salt (17)**

Compound **16** (17.0 mg, 7.4 μmol) was deprotected using a similar procedure as described for the preparation of **15** to afford **17** (8.2 mg, 84.0%). $^1$H NMR (500 MHz, D$_2$O) δ 5.12 (broad s, 1H, H-1$^B$), 5.05 – 5.03 (m, 2H, H-1$^C$, H-1$^D$), 4.82 – 4.79 (m, 2H, H-2$^B$, H-2$^D$), 4.75 (m, 1H, H-1$^A$), 4.31 – 4.26 (m, 1H, H-6$^A$$_a$), 4.22 – 4.11 (m, 7H, H-6$^A$$_b$, H-4$^B$, H-5$^B$, H-4$^D$, H-5$^D$, H-6$^C$), 4.01 – 3.87 (m, 5H, H-5$^A$, H-3$^B$, H-2$^C$, H-5$^C$, H-3$^D$), 3.84 (dd, $J$ = 10.6, 3.6 Hz, 1H, H-2$^A$), 3.76 (t, $J$ = 10.6 Hz, 1H, H-3$^A$), 3.70 – 3.60 (m, 3H, H-4$^A$, H-3$^C$, OC$H$H Linker), 3.38 – 3.43 (m, 1H, OCH$H$ Linker), 2.91 – 2.88 (t, $J$ = 3.0 Hz, 2H, NC$H_2$ Linker), 1.95 (s, 3H, C$H_3$CO), 1.92 (s, 3H, C$H_3$CO), 1.60 – 1.56 (m, 4H, (C$H_2$)$_2$ Linker), 1.37 (m, 2H, C$H_2$ Linker). $^{13}$C NMR (126 MHz, D$_2$O) δ 100.9, 99.1, 96.5, 93.7, 77.8, 76.7, 74.2, 73.1, 72.8, 71.3, 70.1, 69.9, 69.1, 69.0, 68.5, 68.0, 67.9, 67.1, 66.4, 64.6, 54.0, 53.4, 39.6, 38.5, 28.2, 26.4, 22.6, 22.3, 21.0. HR ESI-TOF MS: m/z: calcd for $C_{33}H_{49}N_3Na_6O_{35}S_4$ [M-4Na$^+$+2H$^+$]: 611.5491; found: 611.5493.

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# CHAPTER 3

# A HEPARAN SULFATE MICROARRAY UNCOVERS NOVEL ASPECTS OF HEPARAN SULFATE-PROTEIN BINDING[2]

[2] Chengli Zong, Wenyuan Xiao, Xiuru Li, Weigang Lu, Margaretha Wolfert, Josettte Wilkes, Lianchun Wang, Tracy Handel, Geert-Jan Boons. To be submitted to Nature Chemistry.

**Abstract**

Although it is widely accepted that HS is an information-rich polymer, the oligosaccharide structures that mediates particular biological interactions has been defined in only a few cases. This problem stems from the structural complexity of HS, which in turn, arises from a complex biosynthetic pathway. Lack of structure-activity relationship for HS-binding proteins makes it difficult to understand the physiology and pathology of HS at a molecular level and greatly complicates harnessing its therapeutic potential. Based on a modular approach for the synthesis of HS, we diversified the sulfation pattern at a late stage during the synthesis via per-sulfation, selective sulfation, de-sulfation and enzymatic catalyzed 3-O-sulfation. Same strategy was applied to three other tetrasaccharides generating a library of 50 compounds. Microarray study confirmed the essential role of 2-O-sulfate on IdoA for FGF2 binding. Microarray data with other lectins indicated: by increasing the number of sulfate, their affinity increased accordingly, which might correlate with the biological function of highly sulfated HS as a reservoir. Selectivity was observed for tetrasaccharides with intermediate sulfation level. With longer sequences or a more diversified library, we might get a better picture for their binding preferences.

**Introduction**

Heparan sulfates (HS) are naturally occurring polydisperse linear polysaccharides that are heavily *O*- and *N*-sulfated.[1-6] They reside on the cell surface and in the extracellular matrix (ECM) of virtually all mammalian tissue types where they interact with numerous signaling proteins, growth factors, and ECM components.[7] The interaction between GAGs and proteins is critical for many biological processes including cell–cell and cell–matrix interactions, cell migration and proliferation, growth factor sequestration, chemokine and cytokine activation,[8] microbial recognition[9,10] and tissue morphogenesis during embryonic development.[11] Alteration in HS

expression has been associated with disease,[12,13] and for example, significant changes in the composition of proteoglycans occur in the stroma surrounding tumors, which appear to support tumor growth and invasion. Many pathogens including bacteria, viruses and parasites attack host cells by binding to HS, which is often a decisive factor for infection.[14-17]

HS chains are assembled in the Golgi on core proteins by the initial formation of linear polymer composed of alternating *N*-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) moieites.[18] Subsequently, it undergoes a series of processing reactions involving *N*-deacetylation, *N*-sulfation, epimerization of GlcA to IdoA, and *O*-sulfation to generate relatively short segments of modified sugars interspersed by regions of unmodified sugars. The considerable structural complexity of HS arises from incomplete sulfation and epimerization leading to at least twenty different disaccharide moieties. Analyses of HS isolated from different mammalian tissues indicate the existence of tissue-specific compositions. Furthermore, immuno-histochemical analyses using antibodies that recognize specific HS-epitopes have uncovered unique patterns of HS-motifs within tissues.[19,20] These observations support a model in which HS structural diversity is not random, but regulated in a context dependent manner, which makes it possible to recruit specific binding partners leading to specific physiological of pathological processes.

Although it is widely accepted that HS is an information-rich polymer, the oligosaccharide structures that mediates particular biological interactions has been defined in only a few cases. This problem stems from the structural complexity of HS, which in turn, arises from a complex biosynthetic pathway. Lack of structure-activity relationship for HS-binding proteins makes it difficult to understand the physiology and pathology of HS at a molecular level and greatly complicates harnessing its therapeutic potential.

Glycoarrays are emerging as a key glycomics technology because they have a distinct advantage that only minute amounts of precious oligosaccharides are required to obtain fast, quantitative, systematic identification and characterization of carbohydrate-protein interactions.[21-24] The development of HS oligosaccharide arrays has, however been stymied by the difficulties of preparing large collections of these compounds, and previously reported HS arrays contained only small numbers of compounds that could not provide structure-activity relationships.[25-29]

We describe here the preparation of an unprecedented library of 50 HS-oligosaccharides that was employed for the development a microarray, which in turn was used to define ligand requirements of a number of HS binding proteins including growth factors and chemokines. These studies uncovered that different HS-binding proteins exhibit different structure-binding relationships. Furthermore, it was found that the HS microarray results can guide the design of compounds that can interfere in processes such as cell division implicating their applications as therapeutic agents. Collectively, our studies support a notion that changes in cell surface HS composition can modulate protein function.

**Results and Discussion**

Attention was focused on the preparation of a diverse library of HS-tetrasaccharides. It was the expectation that the structural diversity of such compounds is not too large to be accessible with an appropriate synthetic approach. Furthermore, it is known that relatively short HS oligosaccharides can bind with high affinity to HS-binding proteins,[30,31] and therefore, it was the expectation that a sufficiently large collection of HS-tetrasaccharides should provide important means to determine structure activity relationships and may lead to the identification of potent inhibitors of cellular activity.

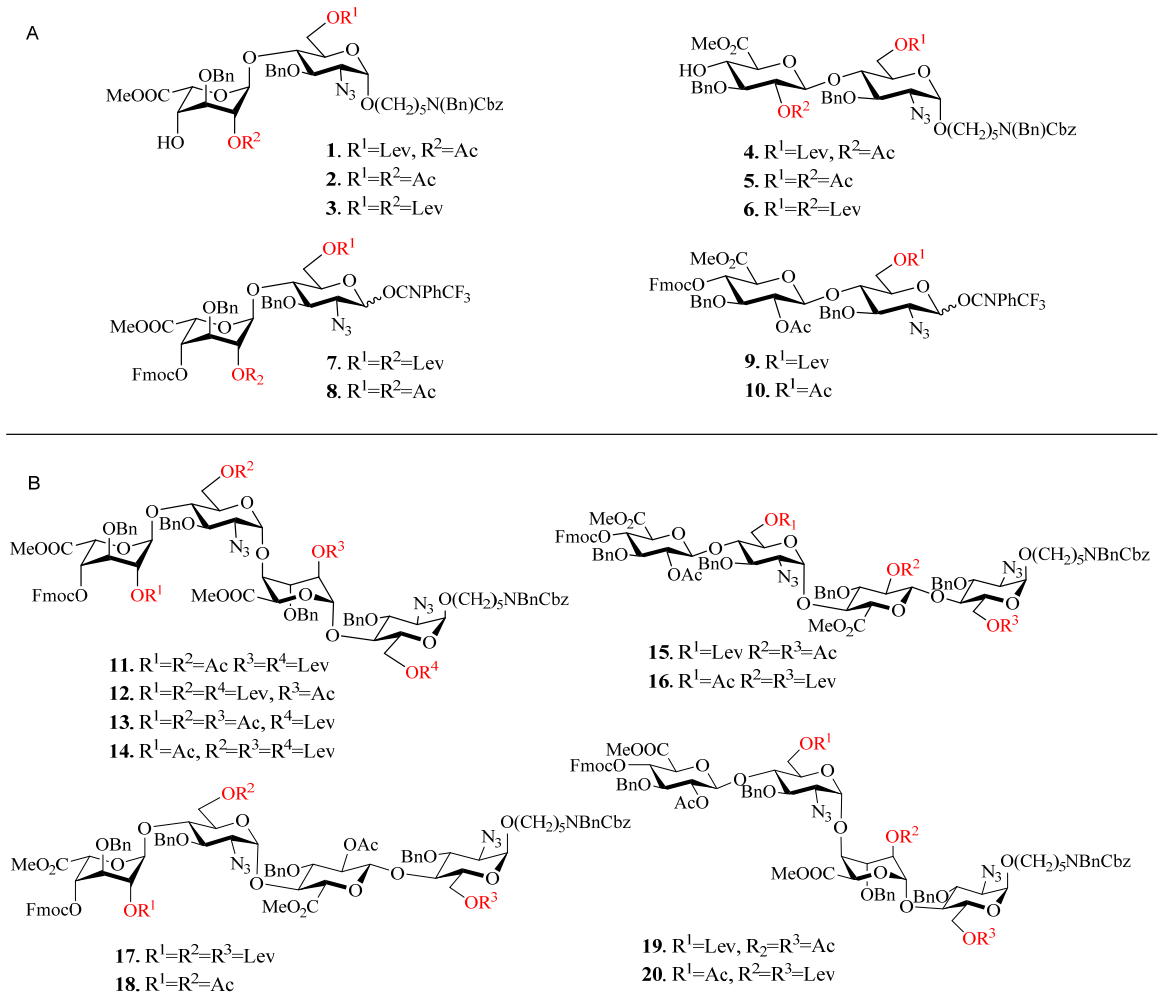
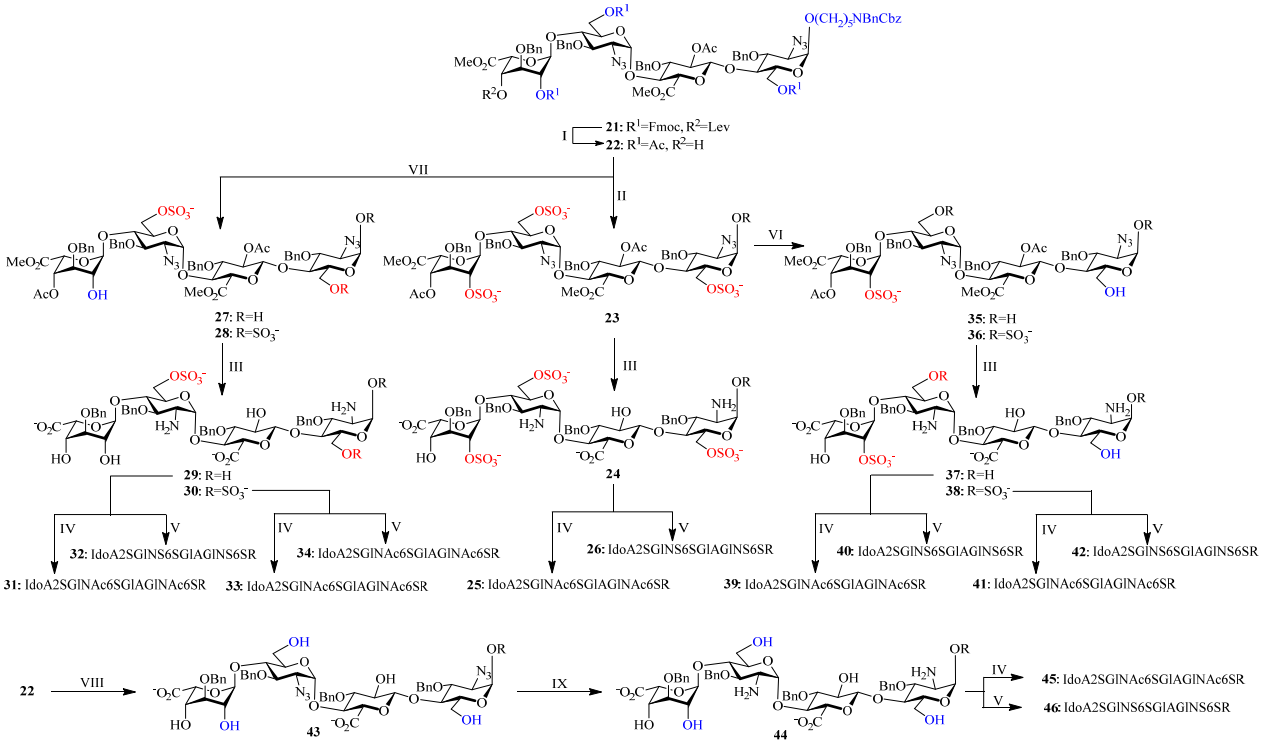



Figure 3.1 (A) modular disaccharide building blocks. (B) assembled tetrasaccharide

We envisaged that selective chemical and enzymatic manipulations of tetrasaccharides prepared by a modular approach[32,33] could provide a large number of compounds for array development. The attraction of such a strategy is that one complex oligosaccharide, which requires a large number of chemical steps for its preparation, can be employed for the synthesis of several differently sulfates derivatives.




Scheme 3.1 Diversification of sulfation patterns at late stage during the synthesis (I) i) $Et_3N$, MeOH/DCM; ii) $Ac_2O$, Pyr., DMAP; iii) $NH_2NH_2AcOH$, DCM/MeOH, RT, 2h, 77%; (II) $Py.SO_3$ excess, DMF, 2 h, 81%; (III) i) LiOH, $H_2O_2$, THF, 4 h, then 4M NaOH, MeOH, 12 h; ii) $PMe_3$, THF, MeOH, 0.1M NaOH, 1 h (19, 70%; 22, 67%; 23, 54%; 26, 78%; 27, 75%); (IV) i) $Ac_2O$, MeOH, $Et_3N$, 30 min; ii) Pd/C, $H_2$, MeOH, $H_2O$, 4 h, iii) $Pd(OH)_2$, $H_2$, $H_2O$, 14 h (1, 85%; 3, 77%; 5, 72%; 7, 65%; 11, 82%); (V) i) $Py.SO_3$, MeOH, $Et_3N$, 0.1M NaOH, 12 h; ii) Pd/C, $H_2$, MeOH, $H_2O$, 4 h; iii) $Pd(OH)_2$, $H_2$, $H_2O$, 14 h (2, 67%; 4, 69%; 6, 77%; 8, 68%; 12, 71%); (VI) BTSA,

Pyr., 60ºC, 2 h (20, 50%; 21, 27%); (VII) Py.$SO_3$, controlled addition, DMF (24, 48%; 25, 35%); (VIII) $PMe_3$, THF, MeOH, 0.1M NaOH, 1 h, 84%; (IX) LiOH, $H_2O_2$, THF, 4 h, then 4M NaOH, MeOH, 12 h, 87%.

First tetrasaccharides **11**-**20** were prepared by parallel combinatorial glycosylations of glycosyl donors **7**-**6** with glycosyl acceptors **3**-**9** to give in each case only the α-anomeric product in good yield (Figure 3.1). The resulting compounds differ in the composition of iduronic and glucoronic acids and the presence of Lev esters at different positions. The latter functionality could selectively be cleaved by hydrazine acetate allowing the installation of sulfate esters. Reduction of the azides gave amines that could then be converted into an acetamido or sulfamide derivatives. The benzyl ethers and benzyloxycarbamates of the resulting compounds could be removed by a two-step procedure involving hydrogenation over Pd/C in a mixture of *t*-butanol/$H_2O$ which led to the removal of the spacer protecting groups followed by hydrogenation over $Pd(OH)_2$ which led to the removal of the benzyl ethers to give the desired HS tetrasaccharide.

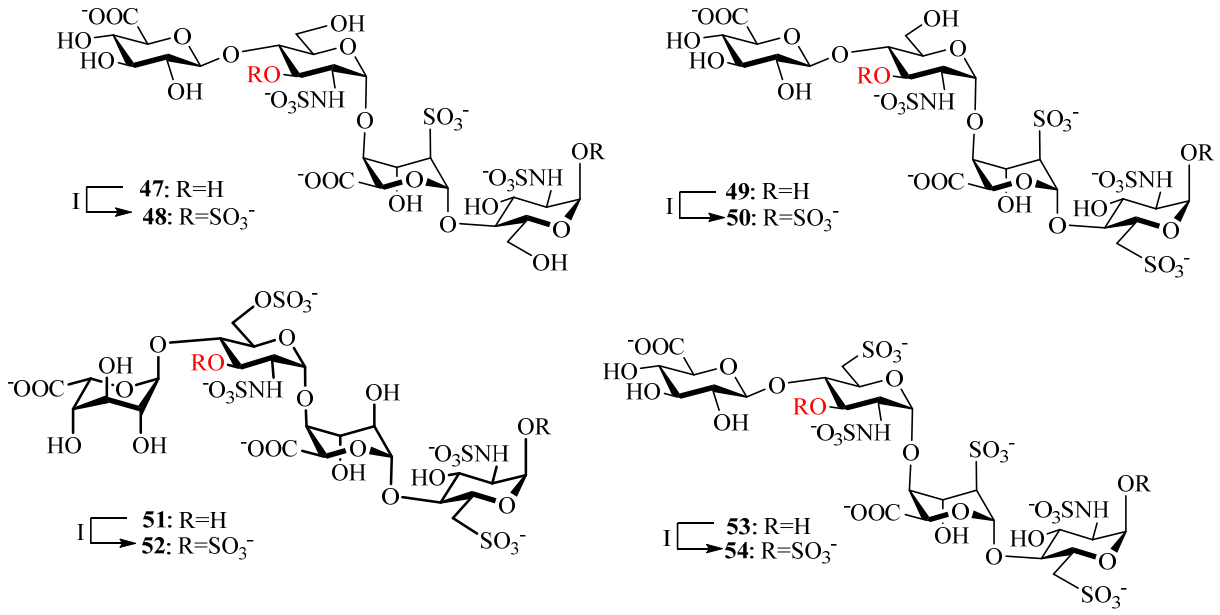
We envisaged that selective manipulations of hydroxyls and amines of the tetrasaccharides would provide easy entry into additional HS-oligosaccharides. As an example of such an approach, we describe in detail the conversion of **21** into twelve differently HS-oligosaccharides (compounds **25**, **26**, **31**-**33**, **39**-**42**, **45** and **46**). Thus, the Lev esters of **21** were cleaved by treatment with hydrazine acetate in a mixture of methanol and dichloromethane followed by sulfation of the resulting hydroxyls of **22** with pyridinium sulfur trioxide to give compound **23** in a yield of 81%. Next, the esters of **23** were hydrolyzed by first treating the compound with LiOH in a mixture of hydrogen peroxide and THF and then sodium hydroxide in methanol. The azido moiety was reduced with trimethyl phosphine in THF in the presence of NaOH[34,35] to give amine **24** in a yield of 70%, which was sulfated with a large excess of pyridinium sulfur trioxide in the presence of

triethylamine or acetylated with acetic anhydride. The benzyl ethers and benzyloxycarbamate were removed via a two-step hydrogenation over Pd/C and $Pd(OH)_2/C$ to give HS oligosaccharides **25** and **26**. Alternatively, treatment of **22** with 4 eq. of pyridinium sulfur trioxide in DMF for 30 min at 0ºC resulted in selective sulfation of primary hydroxyls to give a mixture of mono-sulfate **27** (48%) and bis-sulfate **28** (35%), which could readily be separated by column chromatography using Iatrobeads. Alternatively, sulfur trioxide trimethylamine, which is a less reactive sulfating reagent, could be used at elevated temperature to give the same compounds. Thus, it is evident that primary hydroxyls are much more reactive towards sulfation than secondary ones, and furthermore the hydroxyl at the distal $GlcN_3$ moiety from the linker is significantly more reactive than a similar hydroxyl at proximal $GlcN_3$ residue. By adding a sulfate, signal of the according ring proton downshifted by 0.4 ppm, and $^{13}C$ by 4ppm. Site of the sulfation is confirmed by NMR spectroscopy including $^1H$, TOCSY (Total Correlation Spectroscopy) and $^1H$-$^{13}C$ HSQC (Heteronuc lear single quantum coherence spectroscopy). Saponification of the esters, reduction of the azides to amines following by *N*-sulfation or *N*-acetylation and finally hydrogenation, gave the HS oligosaccharides **31**-**34**. After the hydrolysis of esters, the intermediates are best separated by C18 chromatography.

It was found that selective removal of sulfate esters offers an opportunity to prepare additional derivatives. Thus, **23** was converted into the pyridinium salt and treatment with *N*, *O*-bis(trimethylsilyl) acetamide (BTSA) in pyridine at 60ºC for 2 h resulted only in the removal of primary sulfate esters to give derivatives **35** (50%) and **36** (27%).[36,37] Thus, primary sulfate esters can be selectively hydrolyzed in the presence of secondary ones, and furthermore the primary sulfate at the proximal $GlcN_3$ moiety is more susceptible to treatment with BTSA than the one the distal $GlcN_3$ residue. Standard *N*-modification and deprotection procedures gave entry into

derivatives **39-42**. Finally, it is also possible to only modify the amines, which gave entry into compounds **45** and **46**.

Encouraged by these results three additional tetrasaccharides (**13**, **15** and **20**) having different backbone compositions were subjected to similar manipulation to give an addition **19** sulfated oligosaccharides. It was found that the proximal glucosamine moiety of a tetrasaccharide having a GlAGlN-IdoAGlN backbone could be selectively sulfated to give additional compounds.




Scheme 3.2 3OST1 mediated 3-O-sulfation (I) 3-O-sulfotransferase 1, PAPS, MES buffer, $MnCl_2$, $MgCl_2$.

Next, we explored whether the library of 44 compounds could be further expanded by employing sulfotransferases.[38,39] Specifically, there was a lack of compounds in the library modified by 3-O-sulfates, which is a relatively rare modification vital for anticoagulant activity, cellular entry of viruses, and associated with embryonic development and cancer.[40] Seven different 3-OST isoforms have been identified, each of which can sulfate distinct glucosamine residues of

HS. The substrate specificity of 3-OST1 has been studied in detail and this enzyme has a relatively broad substrate specificity requiring N-sulfonate and iduronyl residue with/without 2-O-sulfate at the reducing side of the target residue.[41] Tetrasaccharides (**47**, **49**, **51**, **53**) were selected as putative substrates of recombinant 3-OST1 and as expected incubation in the presence of PAPS gave derivatives (**48**, **50**, **52**, **54**) as shown in Scheme 3.2. Since tetrasaccharide is not the optimum substrate for 3OST1, the enzymatic reaction usually gives a mixture of the product and some of the precursor, which can be easily separated by a weak anion exchange chromatography such as a DEAE (diethylaminoethyl) column.

The synthetic HS oligosaccharides are modified with an aminopentyl spacer, and thus a microarray could readily be constructed by piezoelectric non-contact printing on *N*-hydroxysuccinimide (NHS)-activated glass slides.[42] All samples were printed as six replicates (1 mM in pH 9, 50 mM sodium phosphate buffer), and after an incubation  screening experiments.

First, we examined the HS ligand requirements of fibroblast growth factors 2 (FGF2), which can mediate diverse functions such as morphogenesis, maintaining tissue homeostasis, and regulating metabolic processes by binding and dimerizing FGF receptors (FGFRs) in a heparan sulfate (HS) cofactor assisted manner.[43]  Dysfunction of FGF2 signaling contributes to many human diseases, such metabolic disorders including chronic kidney disease and insulin resistance as well as many acquired forms of cancers, and thus interfering in FGF2 signaling offers many opportunities for drug discovery. Binding studies using chemically modified heparin and small numbers of synthetic compounds has provided some information about the ligand requirements of FGF2, and thus it offers an appropriate starting point to validate the array technology.

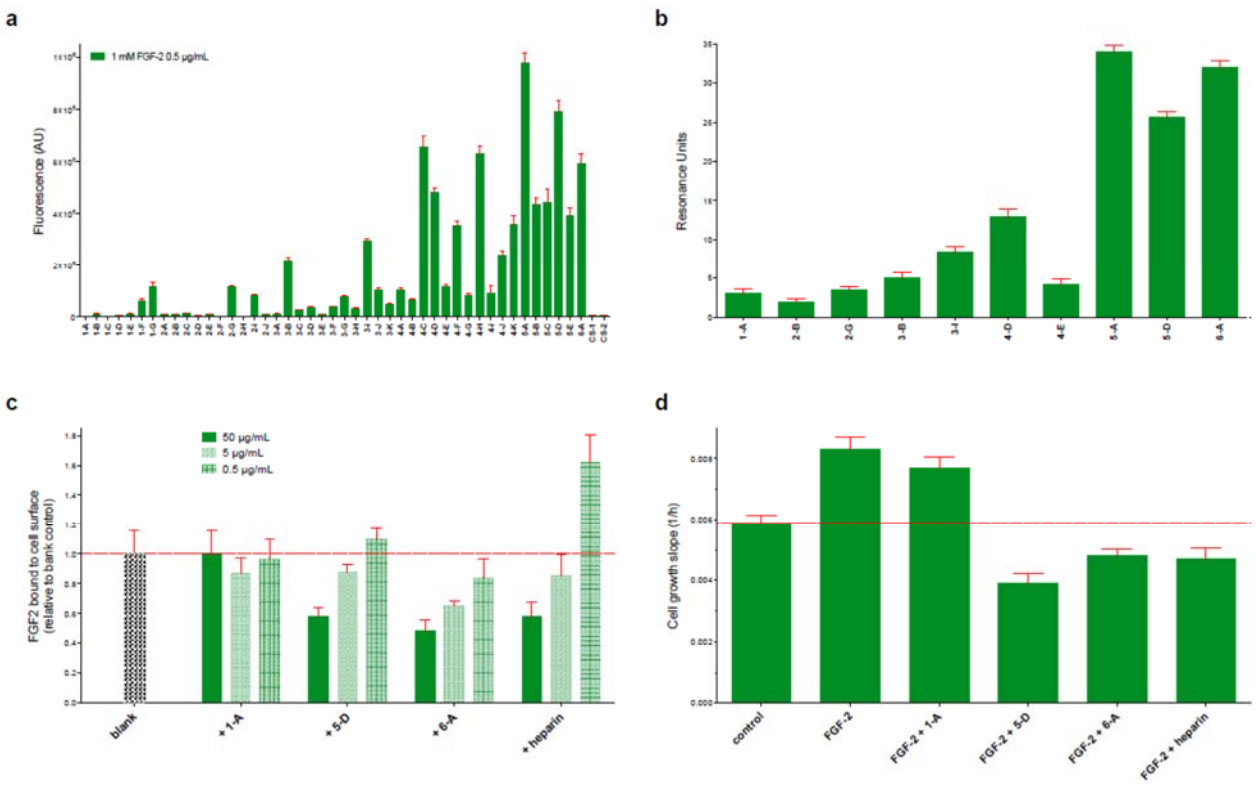



Figure 3.2 Tetrasaccharide inhibited FGF2 induced cell proliferation. (A) Microarray results of synthetic heparin sulfate tetrasaccharide library with 0.5ug/mL FGF2. (B) SPR consolidation of the binding with 100 um tetrasaccharide. As high binders in the array, at 50 ug/mL compound **5-D** and **6-A** inhibited the binding of FGF2 on the mouse endothelium cell surface (C) and FGF2 induced cell proliferation (D).

Thus, sub-arrays were incubated with FGF2 at different concentrations in PBS for 1 h followed by washing and exposure to an anti-FGF2 antibody and an anti-mouse antibody labeled with Alexafluor532. To analyze the data, the compounds were organized according to the number of sulfates present and Ido *vs.* GlcA contend (Figure 3.2 A). It is apparent that FGF2 recognizes all highly sulfated compounds, and thus it seems that it tolerates sulfate esters at all positions. The compound having intermediate levels of sulfation (3 and 4 sulfates) exhibited clear structure

activity relationships. As 3-*O*-sulfate (**4-C**, *vs.* **3-B**, and **5-A** *vs.* **4-D**) and 6-*O*-sulfates (**4-D**, *vs.* **3-B**, **5-A**, *vs.* **4-C**) appear less critical elements for binding and removal of such a function had no or a marginal impact on the responsiveness. The largest increases in binding were observed when 2-*O*-sulfates (**3-I** *vs*. **2-H**, **4-F** *vs*. **3-H**, **4-D** *vs*. **3-C**, **5-C** *vs*. **4-E**, and **5-B** *vs*. **4-G**) and *N*-sulfates (**4-D** *vs*. **3-D**, **5-E** *vs*. **3-F**, **5-D** *vs*. **3-K**) were present. The latter observation was further supported by comparing the responsiveness of compounds **3-B** and **3-C**, which are isomers having a sulfate ester at C-2 or C-6 of the proximal disaccharide moiety, and only the compound **3-B** which has a 2-O-sulfate exhibited binding. Similar trends were observed for other compounds such as **4-D** and **4-E**, which also differ in the position of sulfate esters. These results agree with previous studies that have indicated that 2-*O*- and N-sulfates are important for FGF2 binding.[44-46]

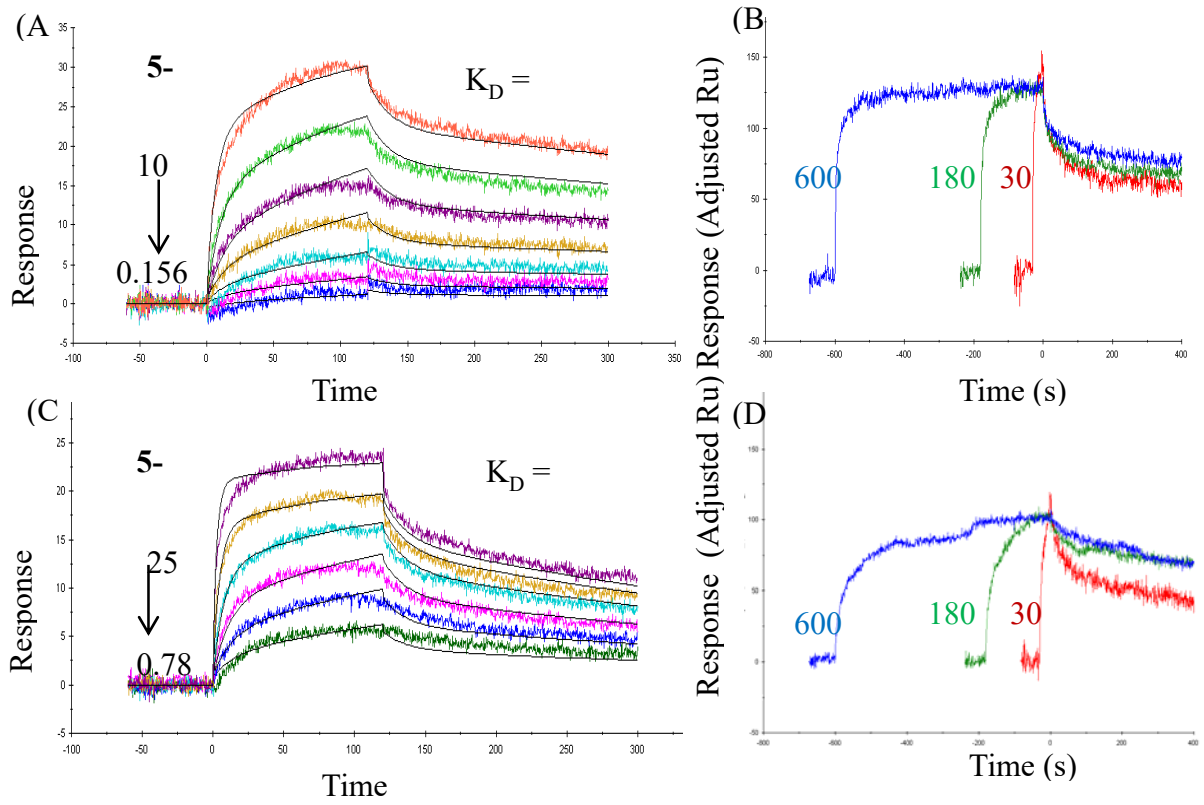


Figure 3. 3 SPR sensorgrams of **5-A** and **5-D** with $FGF_2$. The $FGF_2$ was immobilized in a CM5 chip by amine coupling chemistry, and the binding was analyzed by injecting the respective HS at serial two-fold dilutions starting at 10 and 25 μM for **5-A** and **5-D**, respectively. (B) and (D) are contact time experiments using FGF-2 and compounds **5-A**, **5-D** respectively, to confirm a two-state binding model. Adjusted binding curves for the (C) FGF2-**5-A** (10 μM) and (D) FGF2-**5-D** (10 μM) for exposure times of 30 s (*red*), 180 s (*green*), and 600 s (*blue*) are shown. The analyte injection end point of each time point was set to the same value on the *y* axis (100) and *x* axis (0) to indicate the point at which the comparison of the various dissociation phases began. A significant difference was observed between 30 *versus* 600 s.

To validate the array data, binding studies were performed by surface plasmon resonance (SPR). Thus, FGF-2 was immobilized on NHS-activated groups of a CM-5 sensor chip surface and titration experiments were performed with the synthetic compounds **1-A**, **2-B**, **2-G**, **3-B**, **3-I**, **4-D**, **4-E**, **5-A**, **5-D**, and **6-A** which showed no, weak, moderate, and strong binding to FGF-2 in the microarray experiments, respectively. Gratifyingly, the SPR response units for the various compounds correlated well with the microarray responses (Figure 3.2B). The titration curves for **5-A** and **5-D** fitted well to a two-state binding mode (Figure 3.3 A and C) and $K_D$ values of 0.41 and 1.2 μM, respectively were determined. The two-state binding model for compounds **5-A** and **5-D** was confirmed by a contact time experiment in which the dissociation phase of binding sensorgrams is analyzed as a function of time (Figure 3.3 B and D) and as expected longer contact times resulted in slower rates of dissociation. A model in which weak binding is followed by a tight binding event can rationalize these observations.

The tetrasaccharides **5-D** and **6-A**, which exhibit high affinity and compound **1-A**, which has low affinity for FGF2 were further evaluated in cellular assays. First, we examined whether

the synthetic compounds and heparin could inhibit FGF2 binding to endothelial cells. As can be seen in Figure 3.2 C, the compounds **5-D** and **6-A** inhibited FGF2 binding in a dose dependent manner whereas compound **1-A** did not exhibit any activity. Heparin inhibited FGF2 binding at low concentrations whereas at high concentration addition recruitment of FGF2 was observed. These observations are in agreement with literature data, and probably at low concentration heparin can induce the formation of FGF-FGFR dimer complexes and can therefore recruit additional FGF to the cell surface.[47,48] Importantly, the high affinity synthetic compounds **5-D** and **6-A** could inhibit FGF induced proliferation of endothelial cells as shown in Figure 3.2 D,, whereas compound **1-A** failed to inhibit FGF-2 mediated cellular activity. Interestingly, the synthetic compounds exhibited similar activity compared to heparin.

Next, we examined whether different HS-binding proteins exhibit different preferences for the HS-oligosaccharides. For this purpose, recombinant IP10, Rantes, IL8, MCP-1, MCP-3, MCP-4 modified by HA[49] and SLIT2 modified with a C-terminal 6-His tag were exposed to the microarray and detection was accomplished by anti-HA followed by anti-rabbit conjugated with AlexaFluor532 and CF™ dye conjugated mouse anti-6X His tag antibodies, respectively. Although in general a higher level of sulfation led to increases in binding (Figure 3.4), distinct differences in binding patterns were observed for the different chemokines.

Although MCP1 exhibits a lower affinity for HS than MCP3, probably due to different binding sequence and binding mode, the structure activity relationship for MCP1, 3 and 4 is similar. These proteins do not appear to have a preference for IdoA or GlA, N-sulfate, 6-O-sulfate or 2-O-sulfate, and a higher level of sulfation led to higher responses. An MCP-1 mutant in which the basic amino acids Arg-18 and Lys-19 were replaced with nonpolar Ala[50] did not show binding

for the HS oligosaccharides. The MCP3 mutants K18A/K19A[51] also exhibited a significant decrease in oligosaccharide binding, which validates the microarray results.

IP10 and RANTES showed a preference for 6-O-sulfates over 2-O-sulfates, and for example binding of compound **4-E**, bearing a 6-O-sulfate at the non-reducing glucosamine, was substantially stronger than for **4-D** which is an isomeric compound having a sulfate ester at the C-

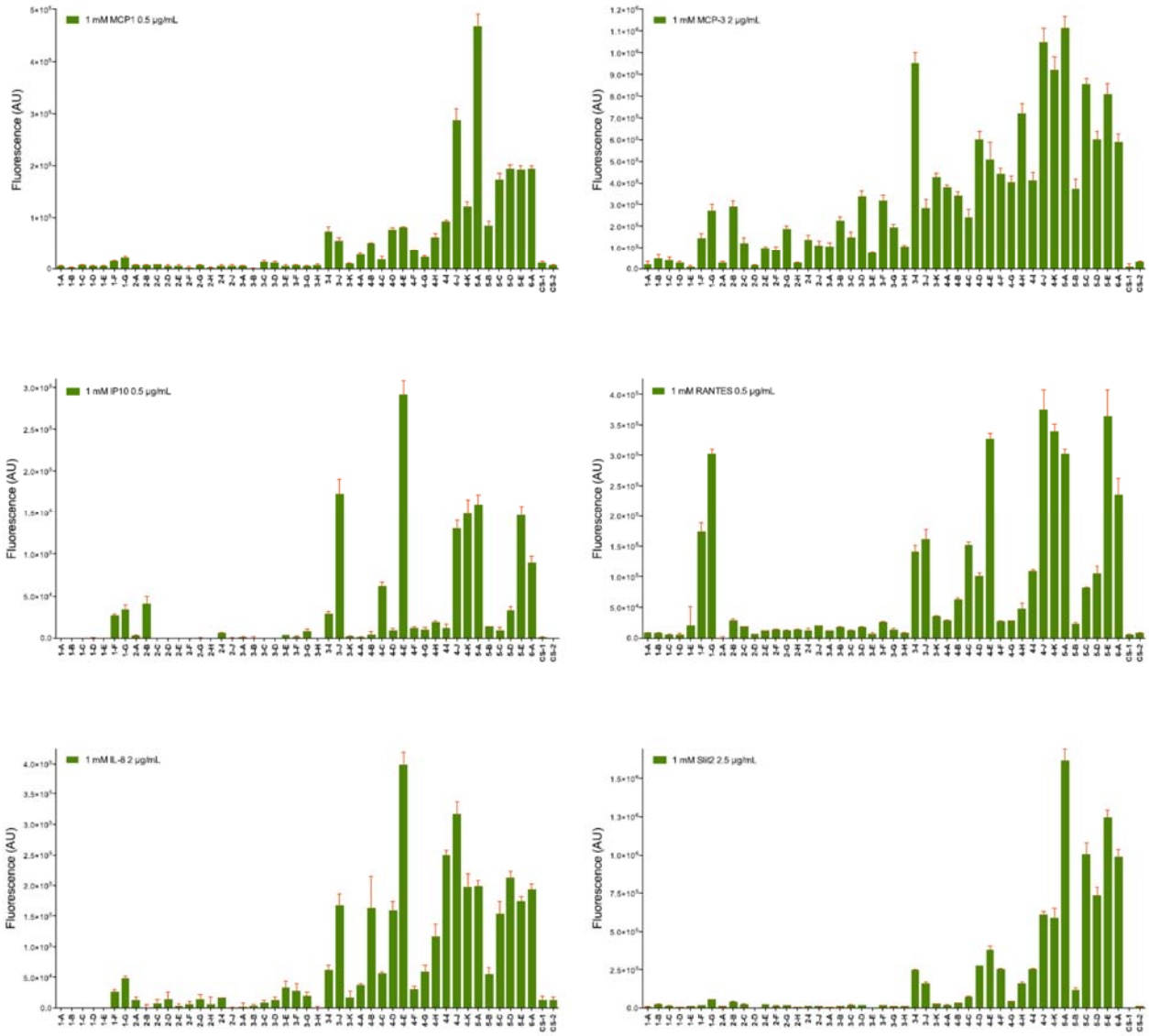



Figure 3.4 Microarry screening of other lectins

2 position of the distal iduronic acid moiety. This trend was, however, different for tetrasaccharide having a different backbone, and for example compound **4-I** and **4-H**. Adding 2-*O*-sulfate(s) on

compound **4-I** to form compounds **5-D** and **6-A** does not significantly affect binding (same is true for compounds **4-G** and **5-C**). Both proteins appear not to tolerate a glucuronic acid at the reducing glucosamine moiety (compound **4-G**, **4-B**, **4-A**, and **4-F**). Compared to IP10 and RANTES, IL8 has a broader substrate preference and more compounds exhibit high affinity (**4-I**, **5-C**). Furthermore, Slit2 bound to HS with a similar pattern as IL8 with a slight preference for higher sulfated oligosaccharides

The interaction of HS with chemokines results in the formation of protein gradients that regulate cellular processes such as the migration of cells into inflamed tissues, tissue repair and development. It is known that endothelial cells of different tissues express different chemokine profiles.[52] Furthermore, it has been observed these that different endothelial issues can express unique patterns of HS-motifs. Our studies show that different chemokines exhibit different ligand requirements for their HS oligosaccharides. Thus, it is likely that HS and chemokine expressions operate synergistically to regulate these processes.

**Conclusions**

Given the enormous structural diversity in GAGs and in HS in particular, it is tempting to speculate that this diversity may encode information and perhaps may even constitute an HS code. Specifically, one could envision that specific combinations of HS-modification patterns may provide a cell-type and/or region-specific "address" that dictates which specific signaling pathways, or combinations thereof, are activated. This model provides an intriguing mechanistic basis for the concept that many receptor/ligand systems may act in a combinatorial manner, for example, to guide cellular and axonal migration. Although the various proteins examined in this study did not recognize specific compounds, clear differences were seen in the structure-binding data and support a notion that different HS-binding proteins recognize different patterns of sulfate

thereby providing opportunities to control protein binding by controlling HS-biosynthesis. The technology described here to prepare relatively large collections of HS oligosaccharides combined with progress in sequencing of HS, and cellular activity measurements will uncover the possible existence of a HS code.

**Experimental section**

General procedures: $^{1}H$ and $^{13}C$ (data from HSQC) NMR spectra were recorded on Varian Mercury 300 MHz, Varian INOVA 500 MHz, 600 MHz, Georgia Research Alliance (GRA)- University of Georgia 800 MHz spectrometers. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS) as the internal standard. NMR data is presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, dd = doublet of doublet, m = multiplet and/or multiple resonances), integration, coupling constant in Hertz (Hz). All NMR signals were assigned on the basis of $^{1}H$ NMR, COSY and HSQC experiments. Mass spectra were recorded on an ABISciex 5800 MALDi-TOF-TOF or Shimadzu LCMS-IT-TOF mass spectrometer. The matrix was used was 2, 5-dihydroxy-benzoic acid (DHB) and Ultamark 1621 as the standard. TLC-analysis performed on Silica gel 60 F254 (EMD Chemicals inc.) with detection by UV-absorption (254nm) when applicable, and by spraying with a solution of (NH4)6Mo7O24.H2O (25 g/L) in 5% sulfuric acid in ethanol followed by charring. $CH_2Cl_2$ was freshly distilled from calcium hydride under nitrogen prior to use. Acid washed molecular sieves (4Å) were flame activated *in vacuo*. All moisture sensitive reactions were carried out under an argon atmosphere.

| compound number in array | compound number in schemes | structures | compound number in array | compound number in schemes | structures |
|---|---|---|---|---|---|
| 1-A | 19 | GlAGlNAc-GlA2SGlNAc | 3-H | 32 | IdoAGlNS6SGlAGlNS |
| 1-B | 15 | GlAGlNAc-IdoA2SGlNAc | 3-I | 33 | IdoA2SGlNAc6SGlAGlNAc6S |
| 1-C | 26 | GlAGlNAc-IdoAGlNAc6S | 3-J | 60 | IdoAGlNS-IdoAGlNS66s |
| 1-D | 35 | IdoA2SGlNAc-GlAGlNAc | 3-K | 11 | IdoAGlNac6SIdoA2SGlNAc6S |
| 1-E | 30 | IdoAGlNAc6S-GlAGlNAc | 4-A | 18 | GlAGlNSGlA2SGlNS6S |
| 1-F | 47 | IdoAGlNAc-IdoAGlNAc6S | 4-B | 12 | GlAGlNS6SGlAGlNS6S |
| 1-G | 48 | IdoAGlNAc6s-IdoAGlNAc | 4-C | 51 | GlAGlNS3SIdoA2SGlNS |
| 2-A | 55 | GlAGLNS6SGlAGlNAc | 4-D | 23 | GlAGlNSIdoA2SGlNS6S |
| 2-B | 17 | GlAGlNAc-GlA2SGlNAc6S | 4-E | 8 | GlAGlNS6SIdoAGlNS6S |
| 2-C | 14 | GlAGlNAc6S-GlAGlNAc6S | 4-F | 36 | IdoA2SGlNS6SGlAGlNS |
| 2-D | 22 | GlAGlNAc-IdoA2SGlNS | 4-G | 4 | IdoAGlNS6SGlAGlNS6S |
| 2-E | 24 | GlAGlNAc-IdoA2SGlNAc6S | 4-H | 57 | IdoAGlNS-IdoA2SGlNS6S |
| 2-F | 10 | GlAGlNAc6S-IdoAGlNAc6S | 4-I | 13 | IdoAGlNS6SIdoAGlNS6S |
| 2-G | 34 | IdoA2SGlNAc6S-GlAGlNAc | 4-J | 59 | IdoA2SGlNS6S-IdoAGlNAc6S |
| 2-H | 9 | IdoAGlNAc6S-GlAGlNAc6S | 4-K | 39 | IdoA2SGlNAc6SIdoA2SGlNAc6S |
| 2-I | 56 | IdoAGlNS-IdoAGlNS | 5-A | 52 | GlAGlNS3SIdoA2SGlNS6S |
| 2-J | 7 | IdoAGlNAc6S-IdoAGlNAc6S | 5-B | 3 | IdoA2SGlNS6SGlAGlNS6S |
| 3-A | 20 | GlAGlNS-GlA2SGlNS | 5-C | 31 | IdoA2SGlNS6SGlAGlNS6S |
| 3-B | 16 | GlAGlNSIdoA2SGlNS | 5-D | 38 | IdoAGLNS6SIdoA2sGlNS6S |
| 3-C | 27 | GlAGlNSIdoAGlNS6S | 5-E | 50 | IdoAGlNS3S6SIdoAGlNS6S |
| 3-D | 25 | GlAGlNAcIdoA2SGlNS6S | 6-A | 40 | IdoA2SGlNS6SIdoA2SGlNS6S |
| 3-E | 58 | GlAGlNS6S-IdoAGlNS | CS-1 | 53 | GlAGalNAcGlAGalNAc4S |
| 3-F | 5 | GlAGlNAc6SIdoA2SGlNAc6S | CS-2 | 46 | GlAGlNS6SGlAGlNAc |
| 3-G | 37 | IdoA2SGlNS-GlAGlNS | | | |

Table 3.1 compound number and structures

**General Procedure for Preparation of Tetrasaccharide.** Disaccharide donor (1.2 equiv) and acceptor (1.0 equiv) were coevaporated with toluene (3 × 3 mL), and then dissolved in anhydrous DCM to maintain a concentration of 0.05 M. Freshly activated powdered 4 Å molecular sieves were added, and the mixture was stirred for 30 min at ambient temperature and then cooled to -20 °C. After adding TfOH (1 equiv), the reaction mixture was stirred for 1 hour and then quenched by the addition of pyridine (5 μL). The mixture was filtered, the filtrate was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography using a gradient of toluene and EtOAc(from 60/40 to 40/60, v/v) to give pure tetrasaccharide.

**General Procedure for Cleavage of Lev Esters.** Hydrazine acetate (5 equiv per Lev group) was added to a solution of the starting material in a mixture of DCM and MeOH (1/1, v/v, 0.02 M). Stirring was continued until TLC (toluene/EtOAc, 1/1, v/v) indicated disappearance of starting material (∼2 h). The reaction mixture was diluted with DCM (30 mL), washed with water (3 × 25 mL) and brine (25 mL), dried ($MgSO_4$), and filtered. The filtrate was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography using a gradient of hexanes or toluene and EtOAc (from 60/40 to 40/60, v/v) to give pure product.

**General Procedure for O-Sulfation.** Sulfur trioxide pyridine complex (10 equiv per OH) was added to a solution of the starting material in DMF (0.02 M). The mixture was stirred at ambient temperature for 2-4 h until TLC ($CHCl_3$/$CH_3OH$, 90/10, v/v) indicated completion of the reaction. After the addition of triethylamine and $CH_3OH$ (1/1, v/v, 1mL), stirring was continued for 30 min. The mixture was concentrated under reduced pressure, and the residue was applied to a column of Iatrobeads (1.5 g), which was eluted with a gradient of $CH_3OH$ in $CHCl_3$ (from 96/4 to 88/12, v/v). Fractions containing product were concentrated under reduced pressure and the residue was passed

through a column of Biorad 50 × 8$Na^+$ resin (0.6 × 5 cm) using $CH_3OH$ as eluent, to give pure product.

**General procedure for selective 6-O-sulfation:** Sulfur trioxide pyridine complex (2 equiv per OH) was added to a solution of starting material in DMF (0.002 M). The mixture was stirred for 30 minutes at 0 °C and then 30 minutes at room temperature until TLC ($CHCl_3$/$CH_3OH$, 90/10, v/v) indicated completion of the reaction. After the addition of triethylamine and $CH_3OH$ (1/1, v/v, 1mL), stirring was continued for 30 min. The mixture was concentrated under reduced pressure, and the residue was applied to a column of Iatrobeads (1.5 g), which was eluted with a gradient of $CH_3OH$ in $CHCl_3$ (from 96/4 to 88/12, v/v). Fractions containing product were concentrated under reduced pressure and the residue was passed through a column of Biorad 50 × 8$Na^+$ resin (0.6 × 5 cm) using $CH_3OH$ as eluent, providing product.

**General procedure for selective de-6-O-sulfation: S**tarting material was converted from sodium salt form to pyridinium form by passing the solution of starting material in methanol through ion-exchange resin (Amberlite IR-120, H+ form, 0.5 ml) with 0.2 mL pyridine in the receiving flask. The solution was concentrated under reduced pressure before adding *N*, *O*-bis(trimethylsilyl)acetamide (40 equiv per sulfate) to a mixture of starting material in anhydrous pyridine. The reaction mixture was stirred at 70 ºC for 2h until TLC ($CHCl_3$/$CH_3OH$, 90/10, v/v) indicated completion of the reaction. Then the solvent was evaporated under reduced pressure and co-evaporation with methanol and water for three times (1/1, v/v, 2 mL). The residue was applied to a C18 column, which was eluted with a gradient of water and methanol (from 90/10 to 5/95, v/v). Fractions containing product were concentrated under reduced pressure and the residue was passed through a column of Biorad 50 × 8$Na^+$ resin (0.6 × 5 cm) using $CH_3OH$ as eluent, to give pure product.

**General procedure for 3-OST1 mediated 3-O-sulfation**: 0.1mg 3-OST1 (0.85 mg/mL) was added to a solution of 0.1 mg tetrasaccharide, PAPS (40 μM final concentration) in 0.5 mL pH 7 buffer containing 50 mM 2-(*N*-morpholino)ethanesulfonic acid, 10 mM $MnCl_2$, 5mM $MgCl_2$, and 1% triton X-100. The reaction mixture was incubated at 37 ºC for 7h. Then 0.1 M NaOH was added until pH=9. The crude reaction mixture was directly loaded on to a DEAE column (0.6 × 2.5 cm) which was eluted with a gradient of ammonium bicarbonate solution (from 0.1M to 1.5M). Fractions containing product were concentrated under reduced pressure and the residue was passed through a column of Biorad 50 × $8Na^+$ resin (0.6 × 5 cm) using $CH_3OH$ as eluent, to give pure product.

**General Procedure for Saponification of Methyl Esters and De-O-acetylation**. A premixed solution of 30% solution of $H_2O_2$ in water (100 equiv per $CO_2Me$) and 1M LiOH (50 equiv per $CO_2Me$) were added to a solution of the starting material in THF (0.02 M). The reaction mixture was stirred at room temperature for 8 h. Then a 4M solution of NaOH was added until pH=14. The reaction mixture was left stirring for 18 h at room temperature. In the case that the reaction had not gone to completion, stirring was continued at 35 °C for an additional 12 h. The reaction mixture was then brought to pH 8-8.5 by addition of AcOH, and concentrated under reduced pressure. The residue was vortexed with water and applied to a C-18 column, which was eluted with a gradient of $H_2O$ and $CH_3OH$ (from 90/10 to 70/30, v/v). ). Fractions containing product were concentrated under reduced pressure and the residue was passed through a column of Biorad 50 × $8Na^+$ resin (0.6 × 5 cm) using $CH_3OH$ as eluent, to give pure product.

**General Procedure for Reduction of Azide Group.** 1M solution of $PMe_3$ in THF (8 equiv per azide group) was added to the solution of 0.1M NaOH (10 equiv per azido group) and the starting material in THF (1.0 mL for 0.013 mmol). The reaction mixture was stirred at room temperature

for 1 h until TLC ($CHCl_3/CH_3OH/H_2O$=70/30/5, v/v/v, or C-18 plates with $H_2O/CH_3OH$=40/60, v/v) indicated completion of the reaction. The presence of amino groups was confirmed using ninhydrin as visualizing agent. The pH was then adjusted to 8.5 by careful addition of AcOH, and the mixture was concentrated under reduced pressure. The residue was vortexed with water and applied to a C-18 silica gel column, which was eluted with a gradient of $H_2O$ and $CH_3OH$ (from 90/10 to 40/60, v/v). The appropriate fractions were concentrated under reduced pressure, and the residue was passed through a column Biorad 50 × $8Na^+$ resin (0.6 × 5 cm) using $CH_3OH$ as eluent, to give pure product.

**General Procedure for N-Acetylation.** Acetic anhydride (10 equiv per $NH_2$) was added to a solution of the starting material in a mixture of anhydrous $CH_3OH$ (0.02 M) and $Et_3N$ (20 equiv per $NH_2$). The progress of the reaction was monitored by TLC (silica gel, $CHCl_3/CH_3OH/H_2O$, 60/30/3, v/v/v; or C-18 silica gel, $H_2O/CH_3OH$, 40/60, v/v). After stirring for 1 h at room temperature, the mixture was coevaporated with toluene in vacuo and the residue passed through a short column of Biorad 50 × $8Na^+$ resin (0.6 × 5 cm) using a mixture of $CH_3OH$ and $H_2O$ (90/10, v/v) as eluent, and appropriate fractions were concentrated in vacuo. The residue was vortexed with water and applied to a C-18 column, which was eluted with a stepwise gradient of $H_2O$ and $CH_3OH$ (from 90/10 to 40/60, v/v). The appropriate fractions were concentrated under reduced pressure to give *N*-acetylated product.

**General Procedure for N-Sulfation.** $SO_3$·Py (20 equiv per $NH_2$) was added to the solution of starting material in a mixture of $CH_3OH$ (1 mL for 0.006 mmol) and triethylamine (0.3 mL). 0.1 M NaOH was used to adjust the pH to 11. The progress of the reaction was monitored by TLC (silica gel TLC, EtOAc/pyridine/water/$CH_3CO_2H$, 8/5/3/1, v/v/v/v). After stirring for an additional 12 h, the reaction mixture was coevaporated with water and the residue passed through a short

column of Biorad 50 × 8$Na^+$ resin (0.6 × 5 cm) with $CH_3OH$ and $H_2O$ (90/10, v/v) as eluent. Appropriate fractions were concentrated under reduced pressure, and the residue was vortexed with water and applied to C-18 silica gel column, which was then eluted with a gradient of $H_2O$ and $CH_3OH$ (from 90/10 to 40/60, v/v). The appropriate fractions were concentrated under reduced pressure to give *N*-sulfated product.

**General Procedure for Selective N-Sulfation and N-Acetylation.** $SO_3$·Py (5 equiv per NH2) was added to the starting material in $CH_3OH$ (1 mL for 0.006 mmol) in a mixture of triethylamine (0.3 mL) and 0.1 M NaOH (2 equiv per NH2) at 0 °C. The progress of the reaction was monitored by TLC (silica gel TLC, EtOAc/pyridine/water/$CH_3CO_2H$, 8/5/3/1, v/v/v/v). Two additional portions of $SO_3$·Py (5 equiv per $NH_2$) were added at 0 °C after 1 and 2 h. After stirring for additional 4 h, the reaction mixture was coevaporated with water the residue passed through a short column of Biorad 50 × 8Na+ resin (0.6 × 5 cm) using $CH_3OH$ and $H_2O$ (90/10, v/v) as eluent. Appropriate fractions were concentrated under reduced pressure, and the residue was vortexed with water and applied to C-18 silica gel column, which was then eluted with a gradient of $H_2O$ and $CH_3OH$ (from 90/10 to 40/60, v/v). The appropriate fractions were concentrated under reduced pressure to provide *N*-sulfated product. Then the product was N-acetylated according to general procedure for N-acetylation to give the product.

**General Procedure for Global Debenzylation.** Pd/C (10%, 1.5 times the weight of starting material) was added to a solution of the starting material in *tert*-butanol and $H_2O$ (1/1, v/v, 1 mL for 5 mg). The mixture was placed under an atmosphere of hydrogen, and the progress of the reaction was monitored by TLC (silica gel EtOAc/pyridine/water/acetic acid, 3/5/3/1, v/v/v/v). The mixture was filtered through a PTFE syringe filter (0.2 mm, 13 mm) when TLC indicated the disappearance of the starting material. The residue was washed with a mixture of *tert*-butanol and

$H_2O$ (1/1, v/v, 2 mL), and the solvents were concentrated under reduced pressure. Then palladium hydroxide on carbon (Degussa type, 20%, 1.5 times the weight of starting material) was added to the solution of the residue in distilled water (1 mL for 5 mg). The reaction mixture was placed under an atmosphere of hydrogen, and stirred for 12 h until TLC (EtOAc/pyridine/water/acetic acid, 4/5/3/1, v/v/v/v) indicated the completion of the reaction. The mixture was filtered through a PTFE syringe filter, and the residue was washed with $H_2O$ (2 mL). The filtrate was freeze dried, the residue was passed through a short column of Biorad 50 × 8$Na^+$ resin (0.6 × 2.5 cm) using $H_2O$ as the eluent, and the appropriate fractions were freeze dried to give the final product.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(methyl-2-*O*-levulinoyl-3-*O*-benzyl-4-*O*-fluorenylmethyloxycarbonyl-α-L-idopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-2-deoxy-6-levulinoyl-α-D-glucopyranoside)-(1→4)-*O*-(methyl-2-*O*-acetyl-3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-2-azido-3-*O*-benzy-6-*O*-levulinoyl-2-deoxy-α-D-glucopyranoside (21)**

Donor **7** (128.6 mg, 0.125 mmol) and acceptor **4** (100.0 mg, 0.105 mmol) was coupled according to the general procedure for glycosylation to give tetrasaccharide **21** (118.9 mg, 58.0%);1H NMR (500 MHz, $CDCl_3$) δ 7.49 – 7.03 (m, 30H, C*H* Aromatic), 5.37 (d, J = 3.6 Hz, 1H, $H1^D$), 5.18 (d, J = 16.6 Hz, 2H, CH*H* of Bn), 5.05 – 4.55 (m, 14H, $H1^A$, $H1^B$, $H2^B$, $H1^C$, $H2^D$, $H4^D$, $H5^D$, 8H of Bn), 4.49 (s, 2H, C$H_2$ of Bn), 4.45 – 4.08 (m, 4H, $H6^A$, $H6^C$), 4.00 – 3.20 (m, 23H, $H2^A$, $H3^A$, $H4^A$, $H5^A$, $H3^B$, $H4^B$, $H5^B$, $H2^C$, $H3^C$, $H4^C$, $H5^C$, $H3^D$, $H4^D$, NC$H_2$ and OC$H_2$ of linker, 2*COC$H_3$), 3.00 – 2.39 (m, 12H, 6*C$H_2$ of Lev), 2.29 – 1.93 (m, 12H, 4*C$H_3$), 1.60 – 1.50 (m, 4H, 2*C$H_2$ linker), 1.41 – 1.13 (m, 2H, C$H_2$ linker). $^{13}$C NMR (126 MHz, cdcl3) δ 128.4, 127.9, 127.8, 101.3, 98, 97.6, 97.5, 82.2, 78.5, 78.3, 75.6, 75.6, 75.6, 75.6, 75, 75, 74.9, 74.8, 74.8, 74.8, 74.8, 74.3, 73.3, 73.3, 73.2, 73.2, 69.9, 68.9, 68.7, 68.6, 68.2, 68.1, 67.4, 67.2, 63.7, 63.1, 62.4, 62.1, 62.1, 56.2,

52.8, 52.5, 50.6, 47.3, 46.5, 38.2, 38.1, 38.1, 30, 30, 29.9, 29.2, 28, 28, 23.4, 21, 20.9. MALDI-HRMS: m/z calcd for C106H117N7NaO32 $[M+Na^+]^+$: 2022.7641; found, 2202.7663.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(methyl-3-*O*-benzyl-4-*O*-fluorenylmethyloxycarbonyl-α-L-idopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(methyl-2-*O*-acetyl-3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-2-azido-3-*O*-benzy-2-deoxy-α-D-glucopyranoside (22)**

$Et_3N$ (0.5 mL) was added to a mixture of compound **21** (82.0 mg, 0.040 mmol) in MeOH/DCM (1/1, v/v, 5 mL). The reaction mixture was stirred at room temperature for 1h. Then solvent was removed under reduced pressure and the crude was dissolved in a mixture of pyridine and acetic anhydride (10/1, v/v, 5 mL). The reaction mixture was stirred for 1h and quenched by 0.5 mL MeOH. After evaporation under reduced pressure, the crude was subjected to cleavage of Lev esters according to general procedure to give tetrasaccharide **22** (47.8 mg, 77% for three steps); $^1$H NMR (500 MHz, $CDCl_3$) δ 7.54 – 6.99 (m, 30H, C*H* Aromatic), 5.38 (d, *J* = 3.8 Hz, 1H, $H1^C$), 5.33 (d, *J* = 4.4 Hz, 1H, $H1^D$), 5.17 (d, *J* = 18.0, 2H, C$H_2$Cbz), 5.13 (s, 1H, $H4^D$), 5.12 (t, *J* = 8.5 Hz, 1H, $H2^B$), 5.07 (d, *J* = 11.0 Hz, 1H, C*H*HBn), 4.84 – 4.81 (m, 2H, $H5^D$, C*H*HBn), 4.80 – 4.79 (m, 2H, $H1^A$, $H1^B$), 4.74 – 4.59 (m, 4H, 2*C$H_2$Bn), 4.50 (d, *J* = 10.0 Hz, 2H, NC$H_2$Bn), 4.22 (t, *J* = 8.6 Hz, 1H, $H4^B$), 4.00 (d, *J* = 8.9 Hz, 1H, $H5^B$), 3.94 – 3.68 (m, 10H, $H3^A$, $H4^A$, $H3^B$, $H6^A$, $H3^C$, $H4^C$, $H6^C$, $H3^D$), 3.68 – 3.55 (m, 3H, $H5^A$, $H5^C$, $H2^D$, OC*H*H Linker), 3.51 (s, 3H, C*H*3OOC), 3.47 (s, 3H, C$H_3$OOC), 3.40– 3.28 (m, 1H, OCH*H* Linker), 3.28 (dd, *J* = 10.2, 3.8 Hz, 1H, $H2^C$), 3.23 – 3.18 (m, 3H, $H2^A$, C$H_2$N Linker), 2.00 (s, 3H, C$H_3$CO), 1.99 (s, 3H, C$H_3$CO), 1.73 – 1.01 (m, 6H, 3*C$H_2$ Linker). $^{13}$C NMR (126 MHz, $CDCl_3$) δ 128.44, 128.18, 127.65, 101.08, 100.89, 98.09, 97.86, 82.4, 78.45, 77.81, 76.3, 76.09, 75.44, 75.25, 75.07, 75.03, 74.87, 74.86, 73.91, 73.55, 72.36, 71.14, 70.31, 69.86, 68.73, 68.49, 67.37, 63.66, 63.18, 61.17, 59.75, 53.42, 52.76, 52.28,

50.59, 46.34, 31.48, 29.89, 28.83, 22.9, 20.92, 14.23 MALDI-HRMS: m/z calcd for C78H91N7NaO25 $[M+Na^+]^+$: 1548.5962; found 1548.5989.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(methyl-2-*O*-sulfonate-3-*O*-benzyl-4-*O*-acetyl-α-L-idopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(methyl-2-*O*-acetyl-3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-2-azido-3-*O*-benzy-6-*O*-sulfonate-2-deoxy-α-D-glucopyranoside (23)**

Compound **21** (45.0 mg, 0.030mmol) was dissolved in anhydrous DMF (0.8 mL) and O-sulfated according to general procedure for complete O-sulfation to give **23** (44.0 mg, 81%); $^1$H NMR (500 MHz, $CD_3OD$) δ 7.63 – 6.99 (m, 30H, C*H* Aromatic), 5.43 (s, 1H, $H1^D$), 5.37 (d, *J* = 3.4 Hz, 1H, $H1^C$), 5.16 (d, *J* = 18.4 Hz, 2H, C*H*$_2$Bn), 5.11 – 5.04 (m, 2H, $H1^B$, CH*H*Bn ), 5.00 – 4.88 (m, 4H, $H2^B$, $H2^D$, $H5^D$, CH*H*Bn), 4.82 – 4.65 (m, 4H, $H1^A$, 3H of C*H*$_2$Bn), 4.59 (t, *J* = 4.5 Hz, 1H, $H4^D$), 4.51 (s, 1H, NC*H*$_2$Bn), 4.47 – 4.28 (m, 3H, $H6^C$, $H6a^A$), 4.28 – 4.17 (m, 2H, $H5^B$, $H6b^A$), 4.17 – 3.97 (m, 4H, $H3^B$, $H4^B$, $H4^C$, $H3^D$), 3.92 (s, 1H, $H4^A$), 3.81 (s, 1H, $H3^A$), 3.76 – 3.56 (m, 10H, $H5^A$, $H3^C$, $H5^C$, 2*$CH^3$, OC*H*H Llinker), 3.45 (dd, *J* = 10.5, 3.4 Hz, 1H, $H2^C$), 3.40 – 3.20 (m, 3H, $H2^A$, NC*H*$_2$ Linker), 2.02 (s, 3H, C*H*$_3$CO), 1.98 (s, 3H, C*H*$_3$CO), 1.65 – 1.22 (m, 6H, 3×$CH_2$ Linker). $^{13}$C NMR (126 MHz, $CD_3OD$) δ 128.76, 128.75, 128.36, 128.28, 128.16, 127.92, 127.29, 127.25, 127.24, 100.67, 97.99, 97.79, 82.15, 78.62, 77.79, 77.71, 76.99, 75.09, 75.08, 75.07, 74.89, 74.88, 74.70, 74.69,74.53, 73.49, 72.36, 72.36, 71.97, 71.93, 70.88, 69.84, 69.37, 67.96, 67.31, 65.73, 65.56, 65.55, 65.25, 65.23, 64.37, 62.74, 53.92, 53.51, 52.42, 51.57, 50.36, 48.2, 48.00, 47.56, 47.37, 28.85, 27.49, 22.72, 19.91, 19.54. ESI-MS: m/z calcd for C78H88N7Na2O34S3, $[M-Na^+]^-$: 1808.4335; found 1808.4340.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(2-*O*-sulfonate-3-*O*-benzyl-α-L-idopyranosyluronate)-(1→4)-*O*-(2-amino-3-*O*-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-2-amino-3-*O*-benzy-6-*O*-sulfonate-2-deoxy-α-D-glucopyranoside (24)**

Compound **23** (60.0 mg, 0.033 mmol) was dissolved in THF (5 mL) and subjected to the saponification, de-*O*-acetylation, and azide reduction according to the general procedure to provide **24** as a sodium salt (38.7mg, 70% for three steps); $^{1}$H NMR (600 MHz, $CD_3OD$) δ 7.60 – 7.02 (m, 30H, C*H* Aromatic), 5.51 (d, *J* = 2.8 Hz, 1H, $H1^{C}$), 5.37 (s, 1H, $H1^{D}$), 5.32 (m, 1H, C*H*HBn), 5.16 (d, *J* = 20.6 Hz, C$H_2$Bn), 4.97 (m, 3H, $H1^{A}$, C$H_2$Bn), 4.87 (s, 1H, $H1^{B}$), 4.74 – 4.69 (m, 1H, C*H*HBn), 4.61 – 4.47 (m, 6H, $H2^{D}$, $H5^{D}$, C$H_2$Bn, NC$H_2$Bn), 4.40 – 4.32 (m, 3H, $H6a^{C}$, $H6^{A}$), 4.26 – 4.13 (m, 4H, $H5^{A}$, $H4^{B}$, $H4^{C}$, $H6b^{C}$), 4.04 (m, 2H, $H5^{B}$, $H5^{A}$), 3.98 – 3.78 (m, 7H, $H3^{A}$, $H4^{A}$, $H3^{B}$, $H3^{C}$, $H5^{C}$, $H3^{D}$, $H4^{D}$), 3.78 – 3.67 (m, 1H, OC*H*H linker), 3.61 (t, *J* = 8.4 Hz, 1H, $H2^{B}$), 3.53 – 3.36 (m, 1H, OC*H*H linker), 3.36 – 3.17 (m, 3H, $H2^{A}$, C$H_2$N linker), 3.09 (dd, *J* = 7.2, 2.8 Hz, 1H, $H2^{C}$), 1.73 – 1.19 (m, 6H, 3×C$H_2$ linker). $^{13}$C NMR (151 MHz, $CD_3OD$) δ 129.1, 128.74, 127.99, 127.97, 127.89, 102.4, 97.11, 94.69, 92.11, 82.99, 76.31, 75.61, 75.47, 75.12, 74.93, 74.73, 73.96, 73.86, 73.66, 73.59, 72.73, 72.04, 71.99, 71.94, 71.16, 70.66, 69.53, 68.66, 68.11, 67.18, 66.98, 64.94, 64.86, 64.78, 52.92, 52.29, 51.13, 50.01, 48.48, 47.89, 46.9, 46.77, 27.15, 22.93 ESI-MS: m/z calcd for $C72H82N3Na3O32S3$ $[M-2Na]^{2-}$: 832.6874; found 832.6881.

**5-aminopentyl-*O*-(2-*O*-sulfonate-α-L-idopyranosyluronate)-(1→4)-*O*-(2-acetamino-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(β-D-glucopyranosyluronate)-(1→4)-*O*-2-acetamino-6-*O*-sulfonate-2-deoxy-α-D-glucopyranoside (25)**

Compound **24** (18.0 mg, 0.0102 mmol) was dissolved methanol (3 mL) and subjected to *N*-acetylation and global debenzylation according to the general procedures to give **25** (10.7 mg,

85%); $^{1}$H NMR (500 MHz, $D_2O$) δ 5.41 (d, *J* = 3.8 Hz, 1H, $H1^{C}$), 5.19 (s, 1H, $H1^{D}$), 4.91 (d, *J* = 2.9 Hz, $H1^{A}$), 4.82 (s,1H, $H5^{D}$), 4.61 (d, *J* = 7.9 Hz, 1H, $H1^{B}$), 4.48 – 4.48 (m, 5H, $H6^{A}$, $H6^{C}$, $H2^{D}$), 4.12 (t, 1H, $H3^{D}$), 4.08 (m, 1H, $H5^{A}$), 4.03 – 4.00 (m, 2H, $H5^{C}$, $H4^{D}$), 3.98 – 3.69 (m, 10H, $H2^{A}$, $H3^{A}$, $H4^{A}$, $H3^{B}$, $H4^{B}$, $H5^{B}$, $H2^{C}$, $H3^{C}$, $H4^{C}$, OC*H*H Linker), 3.60 – 3.52 (m, 1H, OCH*H* Linker), 3.38 (t, *J* = 8.0 Hz, 1H, $H2^{B}$), 3.04 (t, *J* = 7.4 Hz, 2H, C$H_2$N Linker), 2.07 (s, 3H, $CH_3$), 2.06 (s, 3H, $CH_3$), 1.92 – 1.64 (m, 4H, 2×C$H_2$ Linker), 1.64 – 1.29 (m, 2H, $CH_2$ Linker). $^{13}$C NMR (126 MHz, d2o) δ 102.2, 99.2, 97.1, 96.7, 79.4, 76.6, 76.5, 76.5, 74.4, 73.8, 70.0, 69.5, 69.4, 69.4, 69.2, 69.1, 68.9, 68.3, 68.2, 67.0, 66.4, 53.6, 39.6, 28.2, 26.6, 22.5, 22.0. ESI-MS: m/z calcd for: $C33H50N3Na3O32S3$ $[M-2Na^{+}]^{2-}$: 582.5622; found 582.5631.

**5-aminopentyl-*O*-(2-*O*-sulfonate-α-L-idopyranosyluronate)-(1→4)-*O*-(6-sulfamino-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(β-D-glucopyranosyluronate)-(1→4)-*O*-2-sulfamino-6-*O*-sulfonate-2-deoxy-α-D-glucopyranoside (26)**

Compound **24** (10.0 mg, 0.006 mmol) was dissolved in methanol (2 mL) and subjected to *N*-sulfation and global debenzylation according to the general procedures to give **26** (5.2 mg, 67%); $^{1}$H NMR (600 MHz, $D_2O$) δ 5.64 (d, *J* = 3.8 Hz, 1H, $H1^{C}$), 5.19 (s, 1H, $H1^{D}$), 5.16 (d, *J* = 3.6 Hz, 1H, $H1^{A}$), 4.85 (s, 1H, $H5^{D}$), 4.62 – 4.22 (m, 5H, $H6^{A}$, $H6^{C}$, $H2^{D}$), 4.12 (t, *J* = 3.0 Hz, 1H, $H3^{D}$), 4.07 – 3.93 (m, 3H, $H5^{A}$, $H5^{C}$, $H4^{D}$), 3.93 – 3.56 (m, 8H, $H3^{A}$, $H4^{A}$, $H3^{B}$, $H4^{B}$, $H5^{B}$, $H3^{C}$, $H4^{C}$, OC*H*H linker), 3.60 – 3.52 (m, 1H, OCH*H* linker), 3.40 (t, *J* = 8.0 Hz, 1H, $H2^{B}$), 3.30 (m, 2H, $H2^{A}$, $H2^{C}$), 3.03 (t, *J* = 7.4 Hz, 2H, C$H_2$N linker), 1.91 – 1.42 (m, 4H, 2×C$H_2$ linker), 1.64 – 1.29 (m, 2H, C$H_2$ linker). $^{13}$C NMR (151 MHz, $D_2O$) δ 101.6, 98.8, 97.2, 96.9, 78.7, 76.7, 76.3, 76.2, 75.9, 73.9, 72.8, 69.6, 69.3, 69.3, 68.9, 68.8, 68.7, 68.4, 68.1, 68.1, 66.7, 66.2, 57.9, 57.3, 55.4, 48.7, 39.3, 28.2, 27.8, 22.3. ESI-MS: m/z calcd for: $C29H44N3Na5O36S5$ $[M-2Na^{+}]^{2-}$: 642.4904; found 642.4911.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(methyl-3-*O*-benzyl-4-*O*-acetyl-α-L-idopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-6-sulfate-2-deoxy-α-D-**

**glucopyranoside)-(1→4)-*O*-(methyl-2-*O*-acetyl-3-*O*-benzyl-6-sulfate-β-D-glucopyranosyluronate)-(1→4)-*O*-2-azido-3-*O*-benzy-2-deoxy-α-D-glucopyranoside (27)**

Compound **22** (16.7 mg, 0.104 mmol) was dissolved in DMF (10 mL) treated according to general procedure of selective-*O*-sulfation to gave **27** (10.0 mg, 48%); $^{1}$H NMR (500 MHz, $CD_3OD$) δ 7.38 – 7.05 (m, 30H, C*H* Aromatic), 5.40 (d, *J* = 3.9 Hz, 1H, $H1^{C}$), 5.17 – 4.96 (m, 6H, $H1^{D}$, 5H of C$H_2$Bn), 4.74 (s, 2H, C$H_2$Cbz), 4.70 – 4.61 (m, 2H, $H1^{A}$, $H1^{B}$), 4.61 – 4.44 (m, 6H, $H5^{D}$, NC$H_2$Bn, 3H of C$H_2$Bn), 4.42 – 4.26 (dd, *J* = 11.2, 4.4 Hz, 2H, $H6^{C}$), 4.21 – 3.51 (m, 23H, $H2^{A}$, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H6^{A}$, $H2^{B}$, $H3^{B}$, $H4^{B}$, $H5^{B}$, $H2^{C}$, $H3^{C}$, $H4^{C}$, $H5^{C}$, $H2^{D}$, $H3^{D}$, $H4^{D}$, 2×$CH_3$, OC*H*H linker), 3.35 – 3.20 (m, 3H, OC*H*H linker, C$H_2$N linker), 1.80 (s, 3H, C$H_3$CO), 1.78 (s, 3H, C$H_3$CO), 1.65 – 1.20 (m, 6H, 3×C$H_2$ linker). $^{13}$C NMR (126 MHz, $CD_3OD$) δ 128.1, 128.1, 128.0, 127.9, 127.8, 127.3, 127.1, 103.8, 100.7, 97.4, 96.9, 85.3, 80.4, 79.6, 78.3, 77.5, 77.4, 76.1, 75.2, 74.9, 74.3, 73.9, 73.8, 73.7, 72.5, 71.5, 70.7, 70.2, 70.1, 69.8, 67.6, 66.9, 66.4, 60.8, 53.5, 52.8, 50.4, 48.8, 48.4, 48.1, 21.8. ESI-MS: m/z calcd for: $C_{78}H_{90}N_7O_{28}S$ $[M\text{-}Na^{+}]^{-}$: 1604.5560; found 1604.5575.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(methyl-3-*O*-benzyl-4-*O*-acetyl-α-L-idopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-6-sulfate-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(methyl-2-*O*-acetyl-3-*O*-benzyl-6-sulfate-β-D-glucopyranosyluronate)-(1→4)-*O*-2-azido-3-*O*-benzy-6-sulfate-2-deoxy-α-D-glucopyranoside (28)**

Compound **22** (29.0 mg, 0.019 mmol) was dissolved in DMF (10 mL) treated according to general procedure of selective-*O*-sulfation to gave tetrasaccharide **28** (11.5 mg, 35%); $^{1}$H NMR (500 MHz, $CD_3OD$) δ 7.47 – 6.96 (m, 30H, C*H* Aromatic), 5.33 (d, *J* = 3.7 Hz, 1H, $H1^{C}$), 5.26 (d, *J* = 2.5 Hz, 1H, $H1^{D}$), 5.16 (d, *J* = 19.8 Hz, 2H, C$H_2$Cbz), 5.10 – 4.96 (m, 5H, $H1^{B}$, $H2^{B}$, $H4^{D}$, C$H_2$Bn), 4.84

(s, 1H, H5$^{D}$), 4.78 (d, *J* = 11.1 Hz, 2H, H1$^{A}$, C*H*HBn), 4.75 – 4.65 (m, 3H, 3H of C*H*$_2$Bn), 4.66 (d, *J* = 10.6 Hz, 1H, C*H*HBn), 4.64 (d, *J* = 11.1 Hz, C*H*HBn), 4.62 (s, 2H, NC*H*$_2$Bn), 4.45 – 4.37 (m, 2H, H6a$^{A}$, H6a$^{C}$), 4.35 – 4.26 (m, 3H, H6b$^{A}$, H5$^{B}$, H6b$^{C}$), 4.10 (t, *J* = 9.1 Hz, 1H, H4$^{B}$), 4.03 (t, *J* = 9.1 Hz, 1H, H3$^{B}$), 3.94 (t, *J* = 8.9 Hz, 2H, H4$^{A}$, H4$^{C}$), 4.81 – 4.78 (m, 1H, H3$^{A}$), 3.77 (t, *J* = 3.3 Hz, 1H, H2$^{D}$), 3.73 – 3.68 (m, 4H, H5$^{A}$, H3$^{C}$, H5$^{C}$, OC*H*H Linker), 3.58 (s, 3H, C*H*$_3$), 3.48 (dd, *J* = 10.5, 3.0 Hz, 1H, H2$^{C}$), 3.42 (s, 3H, C*H*$_3$), 3.40 – 3.34 (m, 1H, OCH*H* Linker), 3.25 (broad s, 1H, H2$^{A}$), 1.91 (s, 3H, C*H*$_3$CO), 1.85 (s, 3H, C*H*$_3$CO), 1.62 – 1.06 (m, 6H, 3*C*H*$_2$ Linker). $^{13}$C NMR (126 MHz, CD$_3$OD) δ 128.5, 128.4, 128.4, 127.8, 127.4, 127.2, 100.9, 100.6, 98.2, 97.7, 82.0, 78.6, 77.7, 77.4, 75.2, 75.0, 74.7, 74.6, 73.4, 72.6, 70.5, 69.0, 67.9, 67.4, 67.3, 67.2, 65.3, 63.9, 62.6, 53.3, 52.5, 52.2, 51.4, 50.2, 49.1, 47.9, 46.7, 46.3, 29.4, 27.2, 22.9, 19.6, 19.2. ESI-MS: m/z calcd for: C78H89N7O31S2 [M-2Na$^{+}$]$^{2-}$: 589.2269; found 589.2275.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(2-*O*-sulfonate-3-*O*-benzyl-α-L-idopyranosyluronate)-(1→4)-*O*-(2-amino-3-*O*-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-2-amino-3-*O*-benzy-2-deoxy-α-D-glucopyranoside (29)**

Compound **27** (28.0 mg, 0.017 mmol) was dissolved in THF (0.9 mL) and subjected to saponification, de-*O*-acetylation and azide reduction according to the general procedure to give **29** as sodium salt (20.2 mg, 78% for three steps). $^{1}$H NMR (600 MHz, CD$_3$OD) δ 7.56 – 7.04 (m, 30H, C*H* Aromatic), 5.43 (d, *J* = 3.2 Hz, 1H, H1$^{C}$), 5.24 – 4.96 (m, 6H, H1$^{D}$, 5H of C*H*$_2$Bn), 4.76 – 4.42 (m, 10H, 3 H of C*H*$_2$Bn, C*H*$_2$Cbz, NC*H*$_2$Bn, H1$^{A}$, H1$^{B}$, H5$^{D}$), 4.42 – 4.28 (m, 2H, H6$^{C}$), 4.10 (m, 2H, H5$^{C}$, H5$^{D}$), 4.05 – 3.45 (m, 14H, H3$^{A}$, H4$^{A}$, H5$^{A}$, H6$^{A}$, H2$^{B}$, H3$^{B}$, H4$^{B}$, H5$^{B}$, H3$^{C}$, H4$^{C}$, H2$^{D}$, H3$^{D}$, H4$^{D}$, OC*H*H linker), 3.77 (m, 1H, OC*H*H linker), 2.60 (m, 4H, H2$^{A}$, H2$^{C}$, NC*H*$_2$ Linker), 1.17 – 1.60 (m, 6H, 3×C*H*$_2$ Linker). $^{13}$C NMR (151 MHz, CD$_3$OD) δ 128.9, 128.1, 127.6,

127.4, 103.5, 100.3, 98.3, 96.9, 84.4, 81.1, 80.6, 79.3, 77.7, 75.0, 74.9, 74.8, 74.4, 74.3, 73.9, 72.3, 72.0, 71.4, 70.9, 70.6, 69.9, 69.9, 69.8, 67.6, 67.2, 67.0, 66.9, 66.0, 59.9, 54.7, 51.5, 49.9, 49.5, 48.6, 48.3, 48.1, 47.8, 46.1, 29.3, 28.7, 28.7, 27.5. ESI-MS: m/z calcd for: C72H84N3Na2O26S $[M\text{-}Na^+]^-$ : 1484.4865; found 1484.4869.

**5-aminopentyl-*O*-(2-*O*-sulfonate-α-L-idopyranosyluronate)-(1→4)-*O*-(2-acetamino-6-*O*-sulfonate-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(β-D-glucopyranosyluronate)-(1→4)-*O*-2-acetamino-3-deoxy-α-D-glucopyranoside (31)**

Compound **29** (15.0 mg, 0.009 mmol) was dissolved in MeOH (3 mL) and subjected to *N*-acetylation and global debenzylation according to the general procedures to give tetrasaccharide **31** (6.0 mg, 65% for two steps). $^1$H NMR (500 MHz, $CD_3OD$) δ 5.44 (d, *J* = 3.7 Hz, 1H, $H1^A$), 4.92 (m, 2H, $H1^C$, $H1^D$), 4.60 (dd, *J* = 23.0, 5.8 Hz, 2H), 4.63 (d, *J* = 3.7 Hz, 1H, $H5^D$), 4.60 (d, *J* = 5.0 Hz, 1H, $H1^B$), 4.40 (dd, *J* = 11.0, 1.8 Hz, 1H, $H6a^C$), 4.22 (dd, *J* = 10.9, 1.3 Hz, 1H, $H6b^C$), 4.05 (d, *J* = 8.5 Hz, 1H, $H5^C$), 4.07 – 3.54 (3.97 – 3.76 (m, 11H, $H2^A$, $H3^A$, $H4^A$, $H5^A$, $H6^A$, $H2^B$, $H3^B$, $H4^B$, $H5^B$, $H2^C$, $H3^C$, $H4^C$, $H2^D$, $H3^D$, $H4^D$, OC*H*H Linker), 3.62 (m, 1H, $H2^D$), 3.51 (m, 1H, OCH*H* Linker), 3.03 (t, *J* = 7.4 Hz, 2H, NC$H_2$ Linker), 2.07 (s, 3H, C$H_3$), 2.06 (s, 3H, C$H_3$), 1.75 – 1.35 (m, 6H, 3×C$H_2$ Linker); $^{13}$C NMR (126 MHz, $CD_3OD$) δ 101.6, 98.9, 97.2, 96.9, 78.7, 76.7, 76.3, 76.2, 76.0, 75.9, 73.9, 72.8, 69.6, 69.3, 69.3, 68.9, 68.8, 68.7, 68.4, 68.1, 66.8, 66.2, 57.9, 57.3, 55.4, 48.8, 39.3, 28.2, 27.8, 26.3. ESI-MS: m/z calcd for: C33H52N3Na2O26S $[M\text{-}Na^+]^-$: 984.2361; found 984.2379.

**5-aminopentyl-*O*-(2-*O*-sulfonate-α-L-idopyranosyluronate)-(1→4)-*O*-(2-sulfamino-6-*O*-sulfonate-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(β-D-glucopyranosyluronate)-(1→4)-*O*-2-sulfamino-3-deoxy-α-D-glucopyranoside (32)**

Compound **29** (11.0 mg, 0.009 mmol) was dissolved in methanol (2 mL) and subjected to N-sulfation and global debenzylation according to the general procedures to give tetrasaccharide **32** (5.0 mg, 68% for two steps). $^1$H NMR (600 MHz, $CD_3OD$) δ 5.66 (d, *J* = 3.7 Hz, 1H, $H1^A$), 5.17 (d, *J* = 3.6 Hz, 1H, $H1^C$), 4.91 (d, *J* = 4.5 Hz, 1H, $H1^D$), 4.84 (s, 1H，$H5^D$), 4.62 (m, 2H, $H1^B$ , $H5^D$), 4.39 (dd, *J* = 11.1, 2.0 Hz, 1H, $H6a^C$ ), 4.21 (dd, *J* = 11.0, 1.8 Hz, 1H, $H6a^C$), 4.01 (d, *J* = 10.1 Hz, 1H, $H5^C$), 3.97 – 3.76 (m, 11H, $H4^A$, $H5^A$, $H6^A$, $H3^B$, $H4^B$, $H5^B$, $H4^C$, $H3^D$, $H4^D$, OC*H*H Linker), 3.57 – 3.55 (m, 2H, $H3^A$, $H3^C$), 3.54 (dd, *J* = 7.0, 4.6 Hz, 1H, $H2^D$ ), 3.49 (m, 1H, OC*H*H Linker), 3.35 (t, *J* = 7.4 Hz, 1H, $H2^B$), 3.27 (dd, *J* =10.5, 3.8 Hz, 1H, $H2^A$ ), 3.22 (dd, *J* = 10.3, 3.6 Hz, 1H, $H2^C$), 2.98 (t, *J* = 7.4 Hz, 2H, NC$H_2$ Linker), 1.75 – 1.35 (m, 6H, 3×C$H_2$ Linker); $^{13}$C NMR (151 MHz, $CD_3OD$) δ 100.1, 95.7, 95.4, 95.3, 77.3, 75.8, 74.9, 71.4, 70.3, 69.6, 69.4, 69.4, 69.2, 69.1, 68.9, 68.3, 67.8, 67.4, 66.4, 64.5, 58.7, 56.5, 56.1, 37.9, 26.5, 25.01. ESI-MS: m/z calcd for: $C_{29}H_{46}N_3Na_3O_{30}S_3$ $[M\text{-}Na^+]^-$: 1081.1027; found 1081.1036.

The synthesis of compound **30**, **33** and **34** have been reported.[3]

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(methyl-2-*O*-sulfonate-3-*O*-benzyl-4-*O*-acetyl-α-L-idopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(methyl-2-*O*-acetyl-3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-2-azido-3-*O*-benzy-2-deoxy-α-D-glucopyranoside (35)**

Compound **23** (65 mg, 0.036 mmol) was treated according to general procedure of de-*O*-sulfation to gave tetrasaccharide **35** (28.0 mg, 50%); $^1$H NMR (500 MHz, $CD_3OD$) δ 7.70 – 6.92 (m, 30H, C*H* Aromatic), 5.43 (d, *J* = 3.5 Hz, 1H, $H1^C$), 5.41 (s, 1H, $H1^D$), 5.17 (d, *J* = 19.6 Hz, 2H, C$H_2$Bn), 5.10 – 4.98 (m, 2H, $H2^B$, C$H_2$Bn), 5.96 – 4.68 (m, 8H, $H1^A$, $H1^B$, $H4^D$, $H5^D$, 2*C$H_2$Bn), 4.61 – 4.39 (m, 5H, $H2^D$, C$H_2$Bn, NC$H_2$Bn), 4.17 – 4.09 (m, 3H, $H4^B$, $H5^B$, $H2^D$), 4.05 – 3.72 (m, 7H,

[3] Arungundram, S. *et al.* Modular synthesis of heparan sulfate oligosaccharides for structure-activity relationship studies. *J. Am. Chem. Soc.* **131**, 17394-17405 (2009).

H4$^{A}$, H6$^{A}$, H3$^{B}$, H4$^{C}$, H6$^{C}$), 3.71 – 3.55 (m, 9H, H3$^{A}$, H3$^{C}$, H5$^{C}$, 2*C$H_3$), 3.53 – 3.42 (m, 3H, H5$^{A}$, H2$^{C}$, OC*H*H linker), 3.40 – 3.18 (m, 3H, H2$^{A}$, NC$H_2$ Linker), 2.01 (s, 3H, C$H_3$CO), 2.00 (s, 3H, C$H_3$CO), 1.69 – 1.24 (m, 6H, 3*C$H_2$ Linker).$^{13}$C NMR (126 MHz, $CD_3OD$) δ 128.6, 128.2, 128.1, 127.7, 100.9, 98.3, 97.9, 97.8, 82.4, 78.5, 77.9, 74.9, 74.8, 74.4, 74.3, 73.6, 73.5, 73.1, 72.5, 72.3, 72.0, 71.8, 69.5, 67.9, 67.3, 65.7, 64.1, 63.1, 59.8, 59.5, 52.3, 51.4, 51.3, 50.4, 49.3, 48.2, 47.6, 47.2, 47.0, 46.6, 29.0, 27.4, 23.5, 19.8, 18.6. ESI-MS: m/z calcd for: $C_{78}H_{90}N_7O_{28}S$ [M-$Na^+$]$^-$ : 1604.5560; found 1604.5571.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(methyl-2-*O*-sulfonate-3-*O*-benzyl-4-*O*-acetyl-α-L-idopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(methyl-2-*O*-acetyl-3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-2-azido-3-*O*-benzy-2-deoxy-α-D-glucopyranoside (36)**

Compound **23** (65 mg, 0.036 mmol) was treated according to general procedure for de-*O*-sulfation to gave tetrasaccharide **36** (16 mg, 27%); $^1$H NMR (500 MHz, $CD_3OD$) δ7.56 – 7.01 (m, 30H, C*H* Aromatic), 5.43 (s, 1H, H1$^{D}$), 5.35 (d, *J* = 3.6 Hz, 1H, H1$^{C}$), 5.15 (d, *J* = 19.6 Hz, 2H, C$H_2$Cbz), 5.09 – 4.86 (m, 5H, H2$^{B}$, H4$^{D}$, H5$^{D}$, C$H_2$Bn), 4.90 – 4.75 (m, 3H, H1$^{A}$, H1$^{B}$, C*H*HBn), 4.72 and 4.67 (each d, *J* = 7.2 Hz, 2H, C*H*HBn), 4.59 (d, *J* = 7.2 Hz, 1H, C*H*HBn), 4.55 (s, 1H, H2$^{D}$), 4.51 (m, 3H, C*H*HBn, C$H_2$N linker), 4.41 (d, *J* = 8.2 Hz, 1H, C*H*HBn), 4.28 (dd, *J* = 36.0, 12.0 Hz, 2H, H6$^{C}$), 4.17 – 4.10 (m, 1H, H4$^{B}$, H5$^{B}$), 4.04 (t, *J* = 9.5 Hz, 1H, H4$^{C}$), 3.95 (t, *J* = 8.7 Hz, 1H, H3$^{B}$), 3.91 – 3.72 (m, 3H, H4$^{A}$, H6$^{A}$), 3.71 – 3.67 (m, 3H, H5$^{A}$, H3$^{C}$, H5$^{C}$, OC*H*H linker), 3.65 – 3.50 (m, 1H, H3$^{A}$), 3.47 (dd, *J* = 10.4, 3.5 Hz, 1H, H2$^{C}$), 3.41 – 3.32 (m, 1H, OC*H*H linker), 3.23 (m, 1H, H2$^{A}$), 1.99 (s, 3H, C$H_3$CO), 1.97 (s, 3H, C$H_3$CO), 1.68 – 1.21 (m, 6H, 3*C$H_2$ linker).$^{13}$C NMR (126 MHz, $CD_3OD$) δ 129.3, 128.6, 128.1, 127.9, 127.4, 127.1, 100.9, 97.9, 97.8, 82.1, 78.4, 77.8, 76.9, 74.9, 74.8, 74.3, 72.3, 72.2, 72.1, 71.8, 70.9, 69.7, 67.9, 67.3, 65.9, 65.6, 65.54, 64.1, 63.0,

59.8, 52.6, 51.5, 50.4, 49.3, 49.2, 48.2, 47.0, 46.6, 29.6, 27.5, 23.4, 19.8. ESI-MS: m/z calcd for: $C_{78}H_{89}N_7O_{31}S_2$ $[M-2Na^+]^{2-}$: 841.7527; found 841.7529.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(2-*O*-sulfonate-3-*O*-benzyl-α-L-idopyranosyluronate)-(1→4)-*O*-(2-amino-3-*O*-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-2-amino-3-*O*-benzy-2-deoxy-α-D-glucopyranoside (37)**

Compound **35** (35.0 mg, 0.022 mmol) was dissolved in THF (0.9 mL) and subjected to saponification, de-*O*-acetylation and azide reduction according to the general procedure to give **37** as sodium salt (21.7 mg, 67% for three steps). $^1$H NMR (600 MHz, $CD_3OD$) δ 7.83 – 6.96 (m, 30H, C*H* Aromatic), 5.54 (s, 2H, $H1^C$, $H1^D$), 5.26 – 5.06 (m, 5H, 5H of C*H*$_2$Bn), 4.96 (m, 4H, $H1^A$, $H5^D$, C*H*$_2$Bn), 4.72 – 4.68 (m, 2H, C*H*$_2$Bn), 4.65 (m, 1H, $H1^B$), 4.59 (m, 1H, $H2^D$), 4.58 – 4.41 (m, H, C*H*$_2$Bn, C*H*$_2$N linker), 4.11 (s, 1H, $H3^D$), 4.08 – 3.77 (m, 9H, $H3^A$,$H4^A$, $H6^A$, $H5^B$, $H4^C$, $H6^C$, $H4^D$), 3.73 – 3.59 (m, 3H, $H5^A$, $H3^B$, OC*H*H linker), 3.43 – 3.40 (m, 1H, OC*H*H linker), 3.22 (m, 1H, $H2^B$), 3.09 (s, 1H, $H2^C$), 1.77 – 1.08 (m, 6H, 3*C*H*$_2$ linker). $^{13}$C NMR (151 MHz, $CD_3OD$) δ 129.1, 128.8, 128.1, 127.5, 102.9, 95.1, 76.3, 74.9, 74.8, 73.4, 72.6, 72.1, 71.3, 71.2, 69.0, 67.9, 67.8, 67.7, 67.1, 67.1, 66.2, 59.5, 59.4, 53.2, 53.1, 51.8, 51.1, 50.1, 49.2, 46.2, 29.5, 28.7, 27.1. ESI-MS: m/z calcd for: $C_{72}H_{84}N_3Na_2O_{26}S$ $[M-Na^+]^-$: 1484.4865; found 1484.4865.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(2-*O*-sulfonate-3-*O*-benzyl-α-L-idopyranosyluronate)-(1→4)-*O*-(2-amino-3-*O*-benzyl-6-*O*-sulfonate-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-2-amino-3-*O*-benzy-2-deoxy-α-D-glucopyranoside (38)**

Compound **36** (40.0 mg, 0.024 mmol) was dissolved in THF (0.9 mL) and subjected to saponification, de-*O*-acetylation and azide reduction according to the general procedure to give **38**

as sodium salt (20.1 mg, 54% for three steps). $^{1}$H NMR (500 MHz, $CD_3OD$) δ 7.54 – 7.07 (m, 30H, C*H* Aromatic), 5.36 (d, *J* = 4.0 Hz, 1H, $H1^{C}$), 5.28 (s, 1H, $H1^{D}$), 5.24 – 4.98 (m, 5H, 5H of C*H*$_2$Bn), 4.81 (d, *J* = 1.5 Hz, 1H, $H5^{D}$), 4.80 – 4.72 (broad m, 1H, $H1^{A}$), 4.75 (d, *J* = 11.5 Hz, C*H*HBn), 4.69 (d, *J* = 11.5 Hz, CH*H*Bn), 4.64 – 4.53 (m, 4H, $H1^{B}$, $H2^{D}$, $CH_2$Bn), 4.50 (s, 2H, NC*H*$_2$Bn), 4.37 (d, *J* = 11.7 Hz, 1H, C*H*HBn), 4.31 (s, 1H, $H6^{C}$), 4.20 (d, *J* = 9.7 Hz, 1H, $H5^{C}$), 4.12 (s, 1H, $H3^{D}$), 4.08 – 4.11 (m, 3H, $H6a^{A}$, $4^{B}$, $H3^{C}$), 3.96 (t, *J* = 2.6 Hz, 1H, $H4^{D}$), 3.90 – 3.81 (m, 5H, H3A, $H5^{A}$, $H6b^{A}$, $H5^{B}$, $H3^{C}$), 3.70 – 3.53 (m, 4H, $H4^{A}$, $H2^{B}$, $H3^{B}$, OC*H*H Llinker), 3.38 – 3.32 (m, 1H, OCH*H* Linker), 3.30– 3.25 (m, 2H, NC*H*$_2$ Linker), 3.15 (d, *J* = 7.0 Hz, 1H, $H2^{A}$), 3.05 (dd, *J* = 10.3, 3.7 Hz, 1H, $H2^{C}$), 1.66 – 1.22 (m, 6H, 3*$CH_2$ Liner). $^{13}$C NMR (126 MHz, $cd_3od$) δ 129.3, 128.6, 128.5, 128.3, 128.2, 128.0, 127.9, 126.9, 126.3, 104.1, 98.3, 97.9, 97.3, 85.6, 78.3, 78.1, 76.2, 76.0, 75.5, 75.2, 74.9, 74.5, 71.8, 71.7, 71.0, 70.4, 69.0, 67.8, 67.6, 67.4, 67.0, 63.9, 62.9, 60.3, 60.2, 60.2, 50.2, 48.3, 46.8, 28.7, 27.8, 27.1. ESI-MS: m/z calcd for: C72H83N3Na2O29S2 $[M-2Na^{+}]^{2-}$ : 781.7180; found 781.7189.

**5-aminopentyl-*O*-(2-*O*-sulfonate-α-L-idopyranosyluronate)-(1→4)-*O*-(2-acetamino-6-*O*-sulfonate-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(β-D-glucopyranosyluronate)-(1→4)-*O*-2-acetamino-3-deoxy-α-D-glucopyranoside (39)**

Compound **37** (12.0 mg, 0.008 mmol) was dissolved in methanol (2.5 mL) and subjected to *N*-acetylation and global debenzylation according to the general procedures to give **39** (8.7 mg, 77% for two steps). $^{1}$H NMR (600 MHz, $D_2O$) δ 5.62 (d, *J* = 3.6 Hz, 1H, $H1^{C}$), 5.19 (s, 1H, $H1^{D}$), 5.15 (d, *J* = 3.4 Hz, 1H, $H1^{A}$), 4.87 (m, 1H, $H5^{D}$), 4.64 (d, *J* = 7.9 Hz, 1H, $H1^{B}$), 4.39 – 4.28 (m, 2H, $H6a^{C}$, $H2^{D}$), 4.23 (d, *J* = 10.9 Hz, 1H, $H6b^{C}$), 4.11 (t, *J* = 3.6 Hz, 1H, $H3^{D}$), 4.03 – 3.96 (m, 2H, $H4^{D}$, $H5^{C}$), 3.96 – 3.91 (m, 1H, $H6a^{A}$), 3.90 – 3.66 (m, 10H, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H6b^{A}$, $H3^{B}$, $H4^{B}$, $H5^{B}$, $H3^{C}$, $H4^{C}$, OC*H*H Linker), 3.55 (m, 1H, OCH*H* Linker), 3.41 (t, *J* = 8.6 Hz, 1H, $H2^{B}$), 3.34

– 3.26 (m, 2H, H2$^{A}$, H2$^{C}$), 3.04 (t, *J* = 7.4 Hz, 2H, NC*H*$_2$ Linker), 1.79 – 1.43 (m, 6H, 3×C*H*$_2$ Linker); $^{13}$C NMR (151 MHz, d$_2$o) δ 100.9, 98.7, 97.1, 96.8, 78.3, 76.5, 76.4, 76.3, 76.1, 75.9, 74.1, 72.7, 70.3, 69.5, 69.4, 68.9, 68.8, 68.7, 68.6, 67.8, 66.2, 66.1, 66.1, 60.1, 57.7, 57.6, 39.3, 27.7, 27.5, 26.5, 22.4, 19.7. ESI-MS: m/z calcd for: C33H52N3Na2O26S [M-Na$^+$]$^-$: 984.2361; found 984.2375.

**5-aminopentyl-*O*-(2-*O*-sulfonate-α-L-idopyranosyluronate)-(1→4)-*O*-(2-sulfamino-6-*O*-sulfonate-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(β-D-glucopyranosyluronate)-(1→4)-*O*-2-sulfamino-3-deoxy-α-D-glucopyranoside (40)**

Compound **37** (15.0 mg, 0.010 mmol) was dissolved in methanol (3 mL) and subjected to N-sulfation and global debenzylation according to the general procedures to give **40** (6.9 mg, 69% for two steps). $^1$H NMR (600 MHz, D$_2$O) δ 5.60 (d, *J* = 3.6 Hz, 1H, H1$^{C}$), 5.16 (m, 2H, H1$^{A}$, H1$^{D}$), 4.85 (m, 1H, H5$^{D}$), 4.61 (d, *J* = 7.9 Hz, 1H, H1$^{B}$), 4.30 (s, 1H, H2$^{D}$), 4.08 (s, 1H, H3$^{D}$), 4.01 – 3.84 (m, 2H, H4$^{D}$, H5$^{C}$), 3.84 – 3.62 (m, 10H, H3$^{A}$, H4$^{A}$, H5$^{A}$, H6$^{A}$, H3$^{B}$, H4$^{B}$, H5$^{B}$, H3$^{C}$, H4$^{C}$, H6$^{C}$, OC*H*H Linker), 3.57 – 3.52 (m, 1H, OCH*H* linker), 3.40 (t, *J* = 8.6 Hz, 1H, H2$^{B}$), 3.34 – 3.23 (m, 1H, H2$^{A}$, H2$^{C}$), 3.03 (t, *J* = 7.3 Hz, 2H, NC*H*$_2$ Linker), 1.88 – 1.46 (m, 6H, 3×C*H*$_2$ Linker). $^{13}$C NMR (151 MHz, D$_2$O) δ 101.1, 99.0, 97.2, 96.7, 78.3, 77.2, 76.4, 76.2, 75.9, 73.6, 72.8, 70.6, 70.3, 69.5, 69.5, 68.9, 68.7, 68.2, 67.8, 67.7, 60.1, 60.1, 59.8, 59.6, 58.0, 57.7, 39.2, 39.2, 27.6, 26.5, 22.3.. ESI-MS: m/z calcd for: C33H52N3Na2O26S [M-Na$^+$]$^-$ : 984.2361; found 984.2375.

**5-aminopentyl-*O*-(2-*O*-sulfonate-α-L-idopyranosyluronate)-(1→4)-*O*-(2-acetamino-6-*O*-sulfonate-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(β-D-glucopyranosyluronate)-(1→4)-*O*-2-amino-3-deoxy-α-D-glucopyranoside (41)**

Compound **38** (7.0 mg, 0.004 mmol) was dissolved methanol (1.5 mL) and subjected to *N*-acetylation and global debenzylation according to the general procedures to give tetrasaccharide

**41** (3.6 mg, 72% for two steps). $^{1}$H NMR (500 MHz, $D_2O$) δ 5.41 (d, *J* = 3.8 Hz, 1H, $H1^{C}$), 5.20 (s, 1H, $H1^{D}$), 4.90 (d, *J* = 3.4 Hz, 1H, $H1^{A}$), 4.85 (s, 1H, $H5^{D}$), 4.61 (d, *J* = 7.9 Hz, 1H, $H1^{B}$), 4.36 and 4.27 (dd, *J* = 20.0, 18.4 Hz, 2H, $H6a^{C}$), 4.32 (t, *J* = 2.0 Hz, $H2^{D}$), 4.11 (t, *J* = 3.8 Hz, 1H, $H3^{D}$), 4.10 – 3.67 (m, 10H, $H2^{A}$, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H6b^{A}$, $H3^{B}$, $H4^{B}$, $H5^{B}$, $H2^{C}$, $H3^{C}$, $H4^{C}$, OC*H*H Linker), 3.51 (m, 1H, OCH*H* Linker), 3.38 (t, *J* = 8.6 Hz, 1H, $H2^{B}$), 3.06 – 2.99 (m, 2H, $H2^{A}$, $H2^{C}$), 3.08 – 2.99 (t, *J* = 7.4 Hz, 2H, C$H_2$N Linker), 2.06 (d, *J* = 2.9 Hz, 3H, COC$H_3$), 1.94 (s, 3H, COC$H_3$), 1.78 – 1.38 (m, 6H, 3×C$H_2$ Linker). $^{13}$C NMR (126 MHz, d2o) δ 101.6, 99.2, 96.9, 96.6, 79.1, 76.6, 76.5, 76.3, 74.6, 73.8, 70.8, 69.6, 69.5, 69.4, 69.3, 68.8, 68.0, 66.7, 66.4, 61.2, 60.4, 53.8, 39.6, 28.3, 26.7, 23.4, 22.6, 22.1, 20.4. ESI-MS: m/z calcd for: C33H51N3Na2O29S2 $[M\text{-}2Na^{+}]^{2-}$: 531.5928; found 531.5933.

**5-aminopentyl-*O*-(2-*O*-sulfonate-α-L-idopyranosyluronate)-(1→4)-*O*-(2-sulfamino-6-*O*-sulfonate-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(β-D-glucopyranosyluronate)-(1→4)-*O*-2-sulfamino-3-deoxy-α-D-glucopyranoside (42)**

Compound **38** (15.0 mg, 0.008 mmol) was subjected to N-sulfation and global debenzylation according to the general procedures to give tetrasaccharide **42** (8.7 mg, 77% for two steps). $^{1}$H NMR (600 MHz, $D_2O$) δ 5.62 (d, *J* = 3.6 Hz, 1H, $H1^{C}$), 5.19 (s, 1H, $H1^{D}$), 5.15 (d, *J* = 3.4 Hz, 1H, $H1^{A}$), 4.87 (m, 1H, $H5^{D}$), 4.64 (d, *J* = 7.9 Hz, 1H, $H1^{B}$), 4.39 – 4.28 (m, 2H, $H6a^{C}$, $H2^{D}$), 4.23 (d, *J* = 10.9 Hz, 1H, $H6b^{C}$), 4.11 (t, *J* = 3.6 Hz, 1H, $H3^{D}$), 4.03 – 3.96 (m, 2H, $H4^{D}$, $H5^{C}$), 3.96 – 3.91 (m, 1H, $H6a^{A}$), 3.90 – 3.66 (m, 10H, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H6b^{A}$, $H3^{B}$, $H4^{B}$, $H5^{B}$, $H3^{C}$, $H4^{C}$, OC*H*H Linker), 3.55 (m, 1H, OCH*H* Linker), 3.41 (t, *J* = 8.6 Hz, 1H, $H2^{B}$), 3.34 – 3.26 (m, 2H, $H2^{A}$, $H2^{C}$), 3.04 (t, *J* = 7.4 Hz, 2H, NC$H_2$ Linker), 1.79 – 1.43 (m, 6H, 3×C$H_2$ Linker); $^{13}$C NMR (151 MHz, $D_2O$) δ 100.9, 98.7, 97.1, 96.8, 78.3, 76.5, 76.4, 76.3, 76.1, 75.9, 74.1, 72.7, 70.3, 69.5, 69.4,

68.9, 68.8, 68.7, 68.6, 67.8, 66.2, 66.1, 66.1, 60.1, 57.7, 57.6, 39.3, 27.7, 27.5, 26.5, 22.4, 19.7. ESI-MS: m/z calcd for: $C_{29}H_{45}N_3Na_4O_{33}S_4$ $[M-2Na^+]^{2-}$: 591.5204; found 591.5210.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(3-*O*-benzyl-α-L-idopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-2-azido-3-*O*-benzy-2-deoxy-α-D-glucopyranoside (43)**

Compound **22** (25.0 mg, 0.019 mmol) was dissolved in THF (0.9 mL) and subjected to saponification and de-*O*-acetylation according to the general procedure to give **43** (20.5mg, 87%); $^1$H NMR (600 MHz, $CD_3OD$) δ 7.51 – 6.91 (m, 29H, C*H* Aromatics), 5.51 (d, *J* = 3.0 Hz, 1H, $H1^C$), 5.10 -5.20 (m, 4H, 3H of C$H_2$ Bn, $H1^D$), 4.88 – 4.49 (m, 8H, $H1^A$, $H1^B$, $H5^D$, 5H of C$H_2$ Bn), 4.44 (s, 2H, C$H_2$ of Cbz), 4.34 (d, *J* = 11.2 Hz, 1H, C*H* of Bn), 4.05 – 3.52 (m, 17H, $H3^A$, $H4^A$, $H5^A$, $H6^A$, $H3^B$, $H4^B$, $H5^B$, $H3^C$, $H4^C$, $H5^C$, $H6^C$, $H2^D$, $H3^D$, $H4^D$, OC*H*H linker), 3.50 (t, *J* = 8.6 Hz, 1H, $H2^B$), 3.42 – 3.10 (m, 5H, $H2^A$, $H2^C$, OCH*H* linker, NC$H_2$ linker), 1.70 – 0.93 (m, 6H, C$H_2$ linker). $^{13}$C NMR (151 MHz, $CD_3OD$) δ 129.0, 128.4, 127.9, 127.8, 127.7, 125.4, 103.3, 99.9, 97.8, 97.7, 79.6, 84.7, 77.9, 77.3, 75.2, 75.0, 74.9, 74.6, 74.1, 72.2, 72.1, 71.5, 68.9, 67.9, 67.4, 67.0, 63.2, 59.9, 59.7, 50.0, 46.3, 29.3, 28.5, 23.1. ESI-MS: m/z calcd for: $C_{72}H_{81}N_7NaO_{23}$ $[M-Na^+]^-$: 1434.5287; found 1434.5297.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(3-*O*-benzyl-α-L-idopyranosyluronate)-(1→4)-*O*-(2-amino-3-*O*-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-2-amino-3-*O*-benzy-2-deoxy-α-D-glucopyranoside (44)**

Compound **43** (18.0 mg, 0.013 mmol) was dissolved in THF (3 mL) and subjected to azide reduction according to the general procedure to give **44** (14.3 mg, 84%); $^1$H NMR (500 MHz, $CD_3OD$) δ 7.62 – 6.96 (m, 29H, C*H* Aromatics), 5.34 (d, *J* = 3.7 Hz, 1H, $H1^C$), 5.17 – 5.04 (m, 5H, C*H* of $CH_2$ Bn), 5.00 – 4.45 (m, 10H, $H1^A$, $H1^B$, $H1^D$, C*H* of $CH_2$ Bn), 4.38 (s, 1H, $H5^D$), 4.07

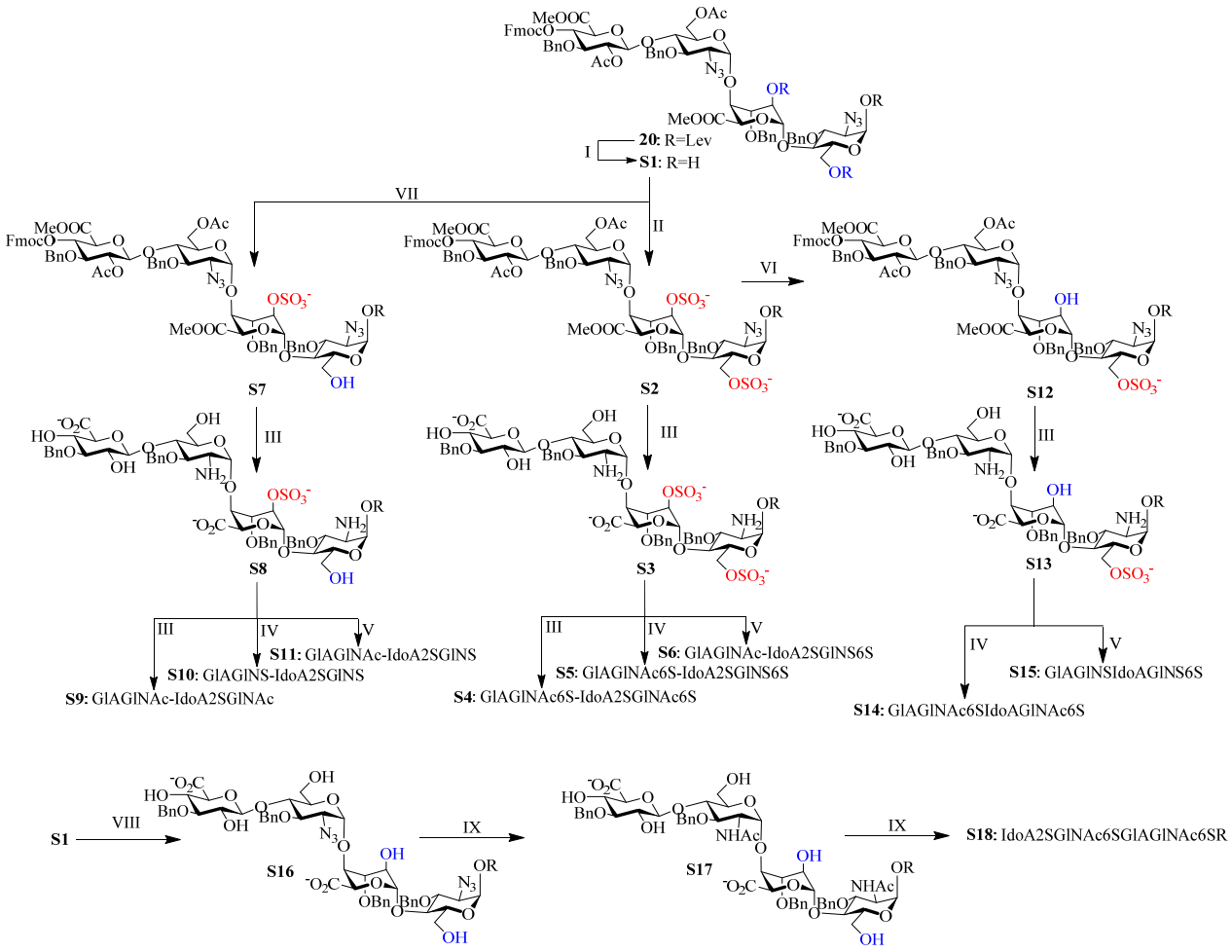
– 3.37 (m, 27H, $H3^A$, $H4^A$, $H5^A$, $H6^{A,}$ $H2^B$, $H3^B$, $H4^B$, $H5^B$, $H3^C$, $H4^C$, $H5^C$, $H6^C$, $H2^D$, $H3^D$, $H4^D$, OC$H_2$ linker), 3.30 – 3.10 (m, 2H, NC$H_2$ linker), 2.60 – 2.43 (m, 2H, $H2^A$, $H2^C$), 1.61 – 1.11 (m, 6H, C$H_2$ linker). $^{13}$C NMR (126 MHz, $CD_3OD$) δ 134.0, 128.0, 127.8,127.6, 126.5, 103.6, 99.7, 98.4, 98.1, 86.6, 80.7,76.8, 75.3, 74.6, 74.5, 74.3, 73.6, 73.0, 72.7, 71.6, 66.9, 66.2, 60.9, 60.2, 55.3, 50.0, 46.3, 29.8, 27.0, 23.8. ESI-MS: m/z calcd for: C72H81N7NaO23 $[M\text{-}Na^+]^-$: 1382.5477; found 1382.5489.

**5-aminopentyl-*O*-(α-L-idopyranosyluronate)-(1→4)-*O*-(2-acetamino-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(β-D-glucopyranosyluronate)-(1→4)-*O*-2-acetamino-3-deoxy-α-D-glucopyranoside (45)**

Compound **44** (6 mg, 0.004 mmol) was dissolved in methanol (1.2 mL) and subjected to N-acetylation and global debenzylation according to the general procedures to give tetrasaccharide **45** (3.1 mg, 82% for two steps). $^1$H NMR (600 MHz, $D_2O$) δ 5.23 (d, $J$ = 3.8 Hz, 1H, $H1^C$), 4.72 (d, $J$ = 2.5 Hz, 1H, $H1^A$), 4.62 (s, 1H, $H1^D$), 4.39 (d, $J$ = 7.7 Hz, 1H, $H1^B$), 4.37 (d, $J$ = 4.7 Hz, 1H, $H5^D$), 3.82 – 3.46 (m, 19H, $H2^A$, $H3^A$, $H4^A$, $H5^A$, $H6^A$, $H4^B$, $H5^B$, $H2^C$, $H3^C$, $H4^C$, $H5^C$, $H6^C$, $H2^D$, $H3^{D,}$ $H4^D$, OC$H$H linker), 3.35 – 3.31 (m, 2H, $H3^B$, OCH$H$ linker), 3.19 (t, $J$ = 7.6 Hz, 1H, $H2^B$), 2.84 (t, $J$ = 6.7 Hz, 2H, NC$H_2$ linker), 1.89 (S, 3H, COC$H_3$), 1.87 (s, 3H, COC$H_3$), 1.58 – 1.22 (m, 6H, C$H_2$ linker). $^{13}$C NMR (151 MHz, $D_2O$) δ 101.9, 99.9, 96.7, 96.3, 79.2, 75.8, 74.6, 74.1, 72.3, 71.4, 71.0, 69.1, 69.0, 67.7, 59.7, 59.4, 53.4, 39.2, 27.9, 26.4, 22.2, 21.7. ESI-MS: m/z calcd for: C33H53N3NaO23 $[M\text{-}Na^+]^-$ : 882.2973; found 882.2980.

**5-aminopentyl-*O*-(α-L-idopyranosyluronate)-(1→4)-*O*-(2-sulfamino-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(β-D-glucopyranosyluronate)-(1→4)-*O*-2-sulfamino-3-deoxy-α-D-glucopyranoside (46)**

Compound **44** (7 mg, 0.005 mmol) was dissolved in MeOH (1 mL) and subjected to *N*-sulfation and global debenzylation according to the general procedures to give tetrasaccharide **46** (3.5 mg, 71% for two steps).$^{1}$H NMR (500 MHz, $D_2O$) δ 5.50 (d, $J$ = 3.8 Hz, 1H, $H1^{C}$), 5.03 (d, $J$ = 3.6 Hz, 1H, $H1^{A}$), 4.69 (s, 1H, $H1^{D}$), 4.47 (d, $J$ = 7.9 Hz, 1H, $H1^{B}$), 4.41 (d, $J$ = 4.8 Hz, 1H, $H5^{D}$), 3.87 – 3.30 (m, 18H, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H6^{A}$, $H3^{B,}$ $H4^{B}$, $H5^{B}$, $H3^{C}$, $H4^{C}$, $H5^{C}$, $H6^{C}$, $H2^{D}$, $H3^{D}$, $H4^{D}$, OC$H_2$ linker), 3.26 (dd, $J$ = 9.4, 8.0 Hz, 1H, $H2^{B}$), 3.15 (m, 2H, $H2^{A}$, $H2^{C}$), 2.92 – 2.86 (t, $J$ = 6.4 Hz, 2H, NC$H_2$ linker), 1.63 – 1.26 (m, 6H, C$H_2$ linker). $^{13}$C NMR (126 MHz, $D_2O$) δ 101.6, 101.0, 97.1, 96.8, 76.3, 73.2, 72.7, 71.6, 71.5, 71.3, 71.1, 62.6, 62.5, 62.4, 59.7, 57.7, 39.5, 26.9, 27.5, 22.6. ESI-MS: m/z calcd for: $C_{29}H_{47}N_3Na_2O_{27}S_2$ $[M\text{-}2Na^{+}]^{2-}$: 489.5823; found 489.5839.




Scheme 3.3 Diversifation of GlAGlNIdoAGlN (I) $NH_2NH_2AcOH$, DCM, MeOH, 81% (I) $Py.SO_3$ excess, DMF, RT, 75%; (II) i) LiOH, $H_2O_2$, THF, 4h, RT. then 4M NaOH, MeOH, RT, 12h; ii) $PMe_3$, THF, MeOH, 0.1M NaOH (**S3**, 60%; **S13**, 72%, **S8**, 79%); III) i) $Ac_2O$, MeOH, $Et_3N$, 74%; ii) Pd/C, $H_2$, MeOH, $H_2O$ 4h, iii) $Pd(OH)_2$, $H_2$, $H_2O$, 14h (**S4**, 75%; **S9**, 76%; **S14**, 64%; **S18**, 86%); IV) i)$Py.SO_3$ excess, MeOH, $ET_3N$, 0.1M NaOH; ii) Pd/C, $H_2$, MeOH, $H_2O$ 4h; iii) $Pd(OH)_2$, $H_2$, $H_2O$, 14h (**S6**, 67%; **S11**, 63%; **S15**, 57%); (V) i) $Py.SO_3$ controlled addition, MeOH, $Et_3N$, 0.1M NaOH; ii) $Ac_2O$, MeOH, $Et_3N$; iii) Pd/C, $H_2$, MeOH, $H_2O$ 4h; iv) $Pd(OH)_2$, $H_2$, $H_2O$,

14h (**S5**, 63%; **S10**, 52%); (VI) BTSA excess, Py, 60oC, 4-5h, 60%; (VII) Py.$SO_3$, controlled addition, DMF 30%; (VIII) LiOH, $H_2O_2$, THF, 4h, RT, then 4M NaOH, MeOH, RT, 12h, 83%.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-O-(methyl-2-O-acetyl-3-O-benzyl-4-O-(9-fluorenylmethoxycarbonyl)-β-D-glucopyranosyluronate)-(1→4)-O-(2-azido-3-O-benzyl-2-deoxy-6-O-acetyl-α-D-glucopyranoside)-(1→4)-O-(methyl-2-O-levulinate-3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-azido-3-O-benzy-6-O-levulinate-2-deoxy-α-D-glucopyranoside (20)**

Donor **10** (270.0 mg, 0.263 mmol) and acceptor **3** (420.0 mg, 0.144 mmol) was coupled according to the general procedure for glycosylation to give tetrasaccharide **20** (394.0 mg, 77.0%). $^1$H NMR (500 MHz, cdcl$_3$) δ 7.78 – 6.95 (m, 38H, CH Aromatics), 5.22 (d, *J* = 3.6 Hz, 1H, H1B), 5.20 – 4.97 (m, 6H, H1C, H2D, H4D, 3CH of CH2Ph), 4.92 (s, 1H, H2B), 4.84 – 4.58 (m, 8H, H1A, H1D, H5B, 5H of CH2Ph), 4.57 – 4.11 (m, 8H, H6A, H6C, 4H of CH2Ph), 4.02 – 3.56 (m, 12H, H3A, H4A, H5A, H3B, H4B, H2C, H3C, H4C, H5C, H3D, H5D, OCHH linker), 3.54 (s, 3H, CO2CH3), 3.44 (s, 3H, CO2CH3), 3.40 – 3.12 (m, 5H, H2A, H2C, OCHH linker, NCH2 linker), 2.85 – 2.42 (m, 8H, CH2 of Lev), 2.15 (s, 3H, COCH3), 2.11 (s, 3H, COCH3), 2.05 (s, 3H, COCH3), 1.95 (s, 3H, COCH3), 1.63 – 1.06 (m, 6H, 3CH2 linker). $^{13}$C NMR (126 MHz, $CDCl_3$) δ 128.2, 128.1, 127.3, 125.5, 120.5, 101.1, 98.2, 97.5, 97.4, 80.4, 79.8, 78.4, 76.0, 75.4, 73.2, 72.8, 72.7, 72.3, 70.1, 69.9, 69.6, 69.2, 67.41, 63.2, 63.0, 62.7, 61.7, 52.9, 52.8, 50.6, 47.9, 37.9, 29.9, 29.3, 29.2, 26.4, 22.3, 21.0, 20.3. MALDI-MS: m/z calcd for C99H105N7NaO31 $[M+Na^+]^+$: 1910.6753; found, 1910.6768.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-O-(methyl-2-O-acetyl-3-O-benzyl-4-O-(9-fluorenylmethoxycarbonyl)-β-D-glucopyranosyluronate)-(1→4)-O-(2-azido-3-O-benzyl-2-**

**deoxy-6-O-acetyl-α-D-glucopyranoside)-(1→4)-O-(methyl-3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-2-azido-3-O-benzy-2-deoxy-α-D-glucopyranoside (S1)**

Compound **19** (394 mg, 0.202 mmol) was dissolved in MeOH/DCM (1/1, v/v, 10 mL) and subject to removal of levulinate esters according to the general procedure to give compound **S1** (288 mg, 81%).$^{1}$H NMR (500 MHz, $CDCl_3$) δ 7.83 – 7.01 (m, 38H, C*H* Aromatics), 5.23 (s, 1H, $H1^B$), 5.15 – 4.97 (m, 5H, $H2^D$, $H4^D$, 3*C*H* of Bn), 4.97 – 4.90 (m, 1H, $H1^C$), 4.83 – 4.74 (m, 1H, $H1^A$), 4.75 – 4.23 (m, 14H, $H5^B$, $H1^D$, $H6^C{}_a$, 11C*H* of Bn), 4.18 – 4.08 (m, 2H, $H6^C{}_b$, C*H* of Fmoc), 3.98 (s, 1H, $H4^B$), 3.87 (d, *J* = 9.8 Hz, 1H, $H5^D$), 3.84 – 3.49 (m, 12H, $H3^A$, $H4^A$, $H5^A$, $H6^A$, $H2^B$, $H3^B$, $H3^C$, H4C, $H5^C$, $H3^D$, OC*H*H linker), 3.49 – 3.33 (m, 8H, $H2^C$, OCH*H* linker, 2*COOC$H_3$), 3.33 – 3.12 (m, 3H, $H2^A$, NC$H_2$ linker), 2.02 (s, 3H, COC$H_3$), 1.91 (s, 3H, COC$H_3$), 1.60 – 1.40 (m, 4H, 2*C$H_2$ linker), 1.39 – 1.13 (m, 2H, C$H_2$ linker). $^{13}$C NMR (126 MHz, $CDCl_3$) δ 128.3, 127.8, 126.7, 124.9, 120.1, 101.1, 97.5, 97.2, 94.2, 79.3, 78.2, 77.8, 74.7, 74.6, 74, 74, 72.9, 72.7, 72.4, 72.2, 70.4, 69.6, 69.1, 68.6, 68.1, 68, 67.2, 66.2, 63.9, 62.9, 62.2, 52.7, 52.3, 50.3, 46.9, 46.2, 30.5, 24.8, 21.8, 20.8, 18.9. MALDI-MS: m/z calcd for $C93H101N7NaO27$ $[M+Na^+]^+$: 1770.6643; found, 1770.6690.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-O-(methyl-2-O-acetyl-3-O-benzyl-4-O-(9-fluorenylmethoxycarbonyl)-β-D-glucopyranosyluronate)-(1→4)-O-(2-azido-3-O-benzyl-2-deoxy-6-O-acetyl-α-D-glucopyranoside)-(1→4)-O-(methyl-2-O-sulfonate-3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-azido-3-O-benzy-6-O-sulfonate-2-deoxy-α-D-glucopyranoside (S2)**

Tetrasaccharide **S1** (100 mg, 0.057 mmol) was dissolved in anhydrous DMF (3.0 mL) and O-sulfated according to the general procedure for O-sulfation providing **S2** as sodium salt (83 mg, 75%). $^{1}$H NMR (500 MHz, $CCl_3D$) *δ* 8.12 – 6.89 (m, 38H, C*H* Aromatics), 5.65 (s, 1H, $H1^B$), 5.16

(d, *J* = 12.9 Hz, 2H, CH2 of Bn), 5. 12 – 5.03 (m, 3H, $H1^{C}$, $H2^{D}$, $H4^{D}$), 4.90 – 4.70 (m, 5H, $H1^{A}$, $H2^{B}$, 3*C*H* of Bn), 4.69 – 4.11 (m, 15H, $H6^{A}$a, $H2^{B}$, $H3^{B}$, $H6^{C}$, $H1^{D}$, 6*C*H* of Bn, C*H* and $CH_2$ of Fmoc), 4.01 – 3.95(m, 2H, $H6^{A}$b, $H5^{D}$), 3.73 – 3.04 (m, 21H, $H2^{A}$, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H4^{B}$, $H2^{C}$, $H3^{C}$, $H4^{C}$, $H5^{C}$, $H3^{D}$, O$CH_2$ linker, N$CH_2$ linker, 2*COO$CH_3$), 2.00 (s, 3H, CO$CH_3$), 1.96 (s, 3H, CO$CH_3$), 1.64 – 1.34 (m, 4H, 2*$CH_2$ linker), 1.27 – 1.23 (m, 2H, $CH_2$ linker). $^{13}$C NMR (126 MHz, $CDCl_3$) δ 120.1, 124.9, 126.7, 128.3, 127.8, 97.2, 67.2, 94.2, 72.9, 74.6, 68.1, 74.7, 72.2, 97.5, 68.6, 74.0, 101.1, 74.0, 50.3, 70.4, 66.2, 62.2, 46.9, 72.7, 69.6, 72.4, 77.8, 79.3, 78.2, 68.0, 69.1, 52.7, 63.9, 52.3, 62.9, 46.2, 20.8, 18.9, 30.5, 24.8, 21.8. $MAL^{D}I$-MS: m/z calcd for C93H99N7Na3O33S2 $[M+Na^+]^+$: 1974.5418; found, 1974.5430.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-O-(3-O-benzyl-β-D-glucopyranosyluronate)-(1→4)-O-(2-amino-3-O-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(2-O-sulfonate-3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-amino-3-O-benzy-6-O-sulfonate-2-deoxy-α-D-glucopyranoside (S3)**

Tetrasaccharde **S2** (40 mg, 0.020 mmol) was dissolved in THF (1.4 mL) and subjected to saponification, de-O-acetylation and azide reduction according to the general procedure to give **S3** as sodium salt (15 mg, 60% for three steps). $^1$H NMR (500 MHz, $CD_3OD$) δ 7.62 – 7.00 (m, 30H, C*H* Aromatics), 5.44 (s, 1H, $H1^{B}$), 5.25 – 5.07 (m, 4H, $H1^{C}$, 3CH of Bn), 5.00 (s, 1H, $H5^{B}$), 4.99 – 4.74 (m, 4H, 4C*H* of Bn), 4.74 – 4.47 (m, 5H, $H1^{A}$, $H2^{B}$, $H1^{D}$, 4C*H* of Bn), 4.40 – 4.22 (m, 5H, $H6^{A}$, $H3^{B}$, $H4^{B}$, C*H* of Bn), 4.03 – 3.75 (m, 7H, $H4^{A}$, $H5^{A}$, $H3^{C}$, $H4^{C}$, $H6^{C}$, $H5^{D}$), 3.74 – 3.09 (m, 9H, $H3^{A}$, $H5^{C}$, $H2^{D}$, $H3^{D}$, $H4^{D}$, O$CH_2$ linker, N$CH_2$ linker), 2.99 (d, *J* = 9.9 Hz, 1H, $H2^{C}$), 2.60 – 2.41 (m, 1H, $H2^{A}$), 1.63 – 1.37 (m, 4H, 2*$CH_2$ linker), 1.28 – 1.24 (m, 2H, $CH_2$ linker). $^{13}$C NMR (126 MHz, $CD_3OD$) δ 128.8, 128, 127.7, 127.6, 127.4, 126.8, 102.7, 98.3, 98, 94, 84.1, 81.1, 75.6, 75.4, 75.1, 74.6, 74.3, 74.2, 73.6, 72.1, 71.9, 71.8, 70.5, 70.3, 70.2, 69.4, 68, 67.3, 67, 66.5, 59.8,

54.8, 50.1, 46.6, 28.6, 27.3, 23.1. MAlDI-MS: m/z calcd for: $C_{72}H_{83}N_3Na_5O_{29}S_2$ $[M\text{-}Na^+]^-$: 1632.4042; found 1632.4060.

**5-Aminopentyl-O-(β-D-Glucopyranosyluronate)-(1→4)-(2-deoxy-2-N-acetamino-6-O-sulfonate-α-D-glucopyranoside)-(1→4)-O-(α-L-idopyranosyluronate-(1→4)-O-2-deoxy-2-N-acetamino-2-deoxy-6-O-sulfonate-α-D-glucopyranoside Hexasodium Salt (S4)**

Tetrasaccharide **S3** (7.0 mg, 0.004 mmol) was subjected to N-acetylation and global debenzylation according to the general procedures to give tetrasaccharide **S4** (3.6 mg, 75% for two steps). $^1$H NMR (500 MHz, $D_2O$) δ 5.24 (d, *J* = 2.3 Hz, 1H, $H1^B$), 5.16 (d, *J* = 3.6 Hz, 1H, $H1^C$), 4.89 (d, *J* = 3.6 Hz, 1H, $H1^A$), 4.80 (s, 1H, $H5^B$), 4.54 (d, *J* = 7.9 Hz, 1H, $H1^D$), 4.46 – 4.39 (m, 1H, $H1a^A$), 4.35 – 4.26 (m, 3H, $H6b^A$, $H2^B$), 4.23 (t, *J* = 2.4 Hz, 1H, $H3^B$), 4.12 – 3.71 (m, 13H, $H2^A$, $H3^A$, $H4^A$, $H5^A$, $H4^B$, $H2^C$, $H3^C$, $H4^C$, $H5^C$, $H6^C$, $H5^D$, OC*H*H linker), 3.60 – 3.51 (m, 3H, $H3^D$, $H4^D$, OCH*H* linker), 3.41 (t, *J* = 6.4 Hz, 1H, $H2^D$), 3.04 (t, *J* = 7.6 Hz, 2H, NC$H_2$ linker), 2.10 (s, 3H, COC$H_3$), 2.06 (s, 3H, COC$H_3$), 1.80 – 1.70 (m, 4H, 2*C$H_2$ linker), 1.60 - 1.50 (m, 2H, C$H_2$ linker). $^{13}$C NMR (126 MHz, $D_2O$) δ102.3, 99.3, 96.7, 93.9, 78.1, 75.9, 75.3, 74.8, 73.0, 71.9, 70.9, 69.9, 69.1, 68.6, 68.0, 67.4, 65.5, 59.7, 54.0 53.3, 39.7, 28.2, 26.6, 22.5. MALDI-MS: m/z calcd for: $C_{33}H_{51}N_3Na_2O_{29}S_2^{2-}$ $[M\text{-}2Na^+]^{2-}$: 531.5928; found 531.6931.

**5-Aminopentyl-O-(β-D-Glucopyranosyluronate)-(1→4)-(2-deoxy-2-acetamino-α-D-glucopyranoside)-(1→4)-O-(2-sulfate-α-L-idopyranosyluronate-(1→4)-O-2-deoxy-2-sulfoamino-2-deoxy-6-O-sulfonate-α-D-glucopyranoside Hexasodium Salt (S5)**

Tetrasaccharide **S3** (5.5 mg, 0.004 mmol) was subjected to selective N-sulfation, N-acetylation and global debenzylation according to the general procedures to give tetrasaccharide **S5** (1.8 mg, 38% for two steps). 1H NMR (500 MHz, $D_2O$) δ 5.09 (s, 1H, $H1^B$), 5.04 – 4.98 (m, 2H, $H1^A$, $H1^C$), 4.70 (d, J = 2.4 Hz, 1H, $H5^B$), 4.39 (d, J = 7.8 Hz, 1H, $H1^D$), 4.27 (dd, J = 11.3, 2.1 Hz, 1H, $H6^A{}_a$),

4.19 – 4.06 (m, 3H, $H6^{A}{}_{b}$, $H2^{B}$, $H3^{B}$), 3.97 – 3.35 (m, 16H, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H4^{B}$, $H2^{C}$, $H3^{C}$, $H4^{C}$, $H5^{C}$, $H6^{C}$, $H3^{D}$, $H4^{D}$, $H5^{D}$, $H6^{D}$, OC$H_2$ linker), 3.30 – 3.23 (m, 1H, $H2^{D}$), 3.20 – 3.12 (m, 1H, $H2^{A}$), 2.91 (t, J = 7.4 Hz, 2H, NC$H_2$ linker), 1.95 (s, 3H, NHCOC$H_3$), 1.69 – 1.29 (m, 6H, 3*C$H_2$ linker). $^{13}$C NMR (126 MHz, $D_2O$) δ 99.3, 97.1, 94.0, 102.4, 78.1, 76.0, 75.5, 74.7, 73.0, 71.9, 71.7, 70.8, 70.0, 69.9, 68.5, 68.3, 67.5, 65.4, 68.9, 59.7, 58.1, 53.3, 39.7, 28.1, 26.2, 22.6, 22.5. ESI-MS: m/z calcd for: C31H48N3Na3O31S3 $[M\text{-}2Na^{+}]^{2-}$: 561.5569; found 561.5576.

**5-Aminopentyl-O-(β-D-Glucopyranosyluronate)-(1→4)-(2-deoxy-2-N-sulfoamino-α-D-glucopyranoside)-(1→4)-O-(α-L-idopyranosyluronate)-(1→4)-O-2-deoxy-2-N-sulfoamino-2-deoxy-6-O-sulfonate-α-D-glucopyranoside Hexasodium Salt (S6)**

Tetrasaccharide **S3** (8.0 mg, 0.004 mmol) was subjected to N-sulfation and global debenzylation according to the general procedures to give tetrasaccharide **S6** (3.7 mg, 67% for two steps). $^{1}$H NMR (800 MHz, $D_2O$) δ 5.39 (d, *J* = 3.6 Hz, 1H, $H1^{C}$), 5.23 (d, *J* = 3.1 Hz, 1H, $H1^{B}$), 5.11 (d, *J* = 3.7 Hz, 1H, $H1^{A}$), 4.71 (d, *J* = 2.8 Hz, 1H, $H5^{B}$), 4.50 (d, *J* = 7.9 Hz, 1H, $H1^{D}$), 4.35 (dd, *J* = 11.3, 2.1 Hz, 1H, $H6^{A}{}_{a}$), 4.31 – 4.26 (m, 2H, $H6b^{A}$, $H2^{B}$), 4.18 (dd, *J* = 5.9, 3.7 Hz, 1H, $H3^{B}$), 4.07 (t, *J* = 3.3 Hz, 1H, $H4^{B}$), 4.01 (m, 1H, $H5^{A}$), 3.93 – 3.82 (m, 3H, $H5^{C}$, $H6^{C}$), 3.76 – 3.66 (m, 8H, $H3^{A}$, $H4^{A}$, $H3^{C}$, $H4^{C}$, $H2^{D}$, $H4^{D}$, $H5^{D}$, OC*H*H linker), 3.58 – 3.47 (m, 3H, $H3^{D}$, $H4^{D}$, OCH*H* linker), 3.37 (t, *J* = 8.5 Hz, 1H, $H2^{D}$), 3.25 (m, 2H, $H2^{A}$, $H2^{C}$), 3.01 (t, *J* = 7.4 Hz, 2H, NC$H_2$ linker), 1.74 – 1.60 (m, 4H, 2*C$H_2$ linker),1.52 - 1.44 (m, 2H, C$H_2$ linker). $^{13}$C NMR (201 MHz, $D_2O$) δ 101.6, 98.4, 96.5, 95.9, 76.8, 75.3, 74.9, 74.6, 73.7, 72.3, 71.3, 70.1, 69.1, 68.9, 68.6, 68.2, 67.5, 67.5, 66.7, 66.6, 58.2, 57.2, 55.2, 28.2, 26.6, 22.5. MALDI-MS: m/z calcd for: C29H45N3Na4O33S42 $[M\text{-}2Na^{+}]^{2-}$: 591.5210; found 591.5216.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-O-(methyl-2-O-acetyl-3-O-benzyl-4-O-(9-fluorenylmethoxycarbonyl)-β-D-glucopyranosyluronate)-(1→4)-O-(2-azido-3-O-benzyl-2-**

**deoxy-6-O-acetyl-α-D-glucopyranoside)-(1→4)-O-(methyl-3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-azido-3-O-benzy-6-sulfonate-2-deoxy-α-D-glucopyranoside (S7)**

Tetrasaccharide **S1** (45.0 mg, 0.025mmol) was subjected to selective O-sulfation according to the general procedures to give tetrasaccharide **S7** (14.5 mg, 30%).$^{1}$H NMR (500 MHz, $CD_3OD$) δ 8.09 – 7.15 (m, 38H, C*H* Aromatics), 5.28 (d, *J* = 4.3 Hz, 1H, $H1^{B}$), 5.22 – 5.06 (m, 4H, $H1^{C}$, 3C*H* of Bn), 4.96 (t, *J* = 7.9 Hz, 1H, $H2^{D}$), 4.93 – 4.80 (m, 4H, $H1^{A}$, $H4^{D}$, C$H_2$ of Bn), 4.78 – 4.68 (m, 3H, $H5^{B}$,$H1^{D}$, C*H* of Bn), 4.68 – 4.56 (m, 4H, 4C*H* of Bn), 4.54 – 4.39 (m, 6H, $H6^{C}{}_{a}$, C$H_2$ of Fmoc, 3C*H* of Bn), 4.36 – 4.26 (m, 2H, $H6^{A}$), 4.23 (t, *J* = 6.6 Hz, 1H, C*H* of Fmoc), 4.17 – 4.13 (m, 2H, $H6^{C}{}_{b}$, $H5^{D}$), 3.99 – 3.63 (m, 11H, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H2^{B}$, $H3^{B}$, $H4^{B}$, $H3^{C}$, $H4^{C}$, $H5^{C}$, $H3^{D}$, OC*H*H linker), 3.50 (s, 3H, COOC$H_3$), 3.45 (s, 3H, COOC$H_3$), 3.40 – 3.34 (m, 3H, $H2^{A}$, $H2^{C}$, OCH*H* linker), 3.27 (t, *J* = 7.1 Hz, 2H, NC$H_2$ linker), 2.01 (s, 3H, COC$H_3$), 1.97 (s, 3H, COC$H_3$), 1.70 – 1.50 (m, 4H, 2*C$H_2$ linker), 1.40 – 1.30 (m, 2H, C$H_2$ linker). $^{13}$C NMR (126 MHz, $CD_3OD$) δ 128.9, 127.7, 127.5, 125.3, 124.7, 119.6, 100.3, 100.1, 97.3, 96.2, 79.1, 78.2, 78, 77.6, 76.5, 76.2, 75.3, 74.5, 74.3, 73.5, 73.1, 72.5, 72.1, 70.2, 70, 69.8, 69.4, 67.6, 67, 66.1, 62.9, 62.1, 51.9, 51.5, 50.1, 46.5, 46.2, 29.8, 26.1, 23.1, 20.5, 19.3. MALDI-MS: m/z calcd for: $C_{93}H_{100}N_7O_{30}S$ [M-$Na^+$]$^-$: 1826.6241; found: 1826.6252.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-O-(3-O-benzyl-β-D-glucopyranosyluronate)-(1→4)-O-(2-amino-3-O-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-amino-3-O-benzy-6-O-sulfate-2-deoxy-α-D-glucopyranoside (S8)**

Tetrasaccharde **S7** (33 mg, 0.018 mmol) was dissolved in THF (1.2 mL) and subjected to saponification, de-O-acetylation and azide reduction according to the general procedure to give **S8**

as sodium salt (17.5 mg, 79% for three steps). $^{1}$H NMR (500 MHz, $CD_3OD$) δ 7.50 – 6.96 (m, 30H, C*H* Aromatics), 5.18 – 5.01 (m, 4H, $H1^B$, 3*C*H* of Bn), 4.92 – 4.79 (s, 5H, $H1^C$, $H5^B$, 3*C*H* of Bn), 4.64 – 4.28 (m, 8H, $H1^A$, $H1^D$, 6*C*H* of Bn), 4.20 (s, 1H, $H3^B$), 4.19 – 4.15 (m, 1H, $H6^A{}_a$), 4.09 (d, $J$ = 10.3 Hz, 1H, $H6^A{}_b$), 3.91– 3.60 (m, 9H, $H4^A$, $H5^A$, $H3^C$, $H4^C$, $H5^C$, $H6^C$), 3.58 – 3.06 (m, 8H, $H3^A$, $H2^D$, $H3^D$, $H5^D$, OC$H_2$ linker, NC$H_2$ linker), 2.76 – 2.67 (m, 1H, $H2^C$), 2.45 – 2.37 (m, 1H, $H2^A$), 1.50 – 1.35 (m, 4H, 2*C$H_2$ linker), 1.25 – 0.95 (m, 2H, C$H_2$ linker). $^{13}$C NMR (126 MHz, $CD_3OD$) δ 130.7, 128.2, 128, 127.8, 127.7, 102.8, 101.3, 98.4, 95.4, 84, 82.8, 81, 76.1, 75.8, 75.3, 75.1, 74.9, 74.7, 74.1, 73.2, 72.2, 71.8, 71.3, 70.7, 69.2, 68.9, 67.2, 66.5, 66.2, 65.2, 59.8, 54.9, 54.8, 50, 46.2, 28.7, 27.2, 23.0. MALDI-MS: m/z calcd for: $C72H84N3Na2O26S$ $[M\text{-}Na^+]^-$ 1484.4865; found: 1484.4869.

**5-Aminopentyl-O-(β-D-Glucopyranosyluronate)-(1→4)-(2-deoxy-2-N-acetamino-α-D-glucopyranoside)-(1→4)-O-(α-L-idopyranosyluronate)-(1→4)-O-2-deoxy-2-N-acetamino-2-deoxy-6-sulfate-α-D-glucopyranoside Hexasodium Salt (S9)**

Tetrasaccharide **S8** (6.0 mg, 0.007 mmol) was subjected N-acetylation and global debenzylation according to the general procedures to give tetrasaccharide **S9** (2.4 mg, 64% for three steps). $^{1}$H NMR (500 MHz, $D_2O$) δ 5.19 (d, $J$ = 3.8 Hz, 1H, $H1^C$), 4.99 (d, $J$ = 3.4 Hz, $H1^B$), 4.91 (d, $J$ = 3.7 Hz, 1H, $H1^A$), 4.73 (d, $J$ = 2.9 Hz, 1H, $H5^B$), 4.54 (d, $J$ = 7.9 Hz, 1H, $H1^D$), 4.38 (dd, $J$ = 11.3, 2.2 Hz, 1H, $H6^A{}_a$), 4.29 (dd, $J$ = 11.3, 5.7 Hz, 1H, $H6^A{}_b$), 4.15-4.05(m, 2H, $H5^A$, $H4^B$), 4.00-3.67 (m, 14H, $H2^A$, $H3^A$, $H4^A$, $H2^B$, $H3^B$, $H3^C$, $H4^C$, $H5^C$, $H6^C$, $H5^D$, OC*H*H linker), 3.60 – 3.50 (m, 3H, $H3^D$, $H4^D$, OCH*H* linker), 3.42 (t, $J$ = 8.2 Hz, 1H, $H2^D$), 3.03 (t, $J$ = 7.6 Hz, 2H, NC$H_2$ linker), 2.04(s, 3H, C$H_3$CO), 2.02(s, 3H, C$H_3$CO), 1.77 – 1.63 (m, 4H, 2*C$H_2$ linker), 1.48 (m, 2H, C$H_2$ linker). $^{13}$C NMR (126 MHz, $D_2O$) 102.4, 102.3, 97.9, 94.4, 78.6, 76.0, 75.3, 74.4, 73.0, 72.0, 71.0,

70.0, 69.9, 69.5, 69.1, 68.2, 67.2, 59.7, 53.7, 39.7, 28.2, 25.6, 22.2, 21.4. ESI-MS: m/z calcd for: $C33H52N3Na2O26S$ $[M\text{-}Na^{+}]^{-}$: 984.2361; found 984.2371.

**5-Aminopentyl-O-[(β-D-Glucopyranosyluronate)-(1→4)-(2-deoxy-2-N-sulfoamino-α-D-glucopyranoside)-(1→4)-O-(α-L-idopyranosyluronate-(1→4)-O-2-deoxy-2-N-sulfoamino-2-deoxy-6-O-sulfonate-α-D-glucopyranoside Hexasodium Salt (S10)**

Tetrasaccharide **S8** (8.0 mg, 0.004 mmol) was subjected to N-sulfation and global debenzylation according to the general procedures to give tetrasaccharide S10 (3.7 mg, 67% for two steps). $^{1}$H NMR (800 MHz, $D_2O$) δ 5.39 (d, *J* = 3.6 Hz, 1H, $H1^{C}$), 5.23 (d, *J* = 3.1 Hz, 1H, $H1^{B}$), 5.11 (d, *J* = 3.7 Hz, 1H, $H1^{A}$), 4.71 (d, *J* = 2.8 Hz, 1H, $H5^{B}$), 4.50 (d, *J* = 7.9 Hz, 1H, $H1^{D}$), 4.35 (dd, *J* = 11.3, 2.1 Hz, 1H, $H6a^{A}$), 4.31 – 4.26 (m, 2H, $H6b^{A}$, $H2^{B}$), 4.18 (dd, *J* = 5.9, 3.7 Hz, 1H, $H3^{B}$), 4.07 (t, *J* = 3.3 Hz, 1H, $H4^{B}$), 4.01 (m, 1H, $H5^{A}$), 3.93 – 3.82 (m, 3H, $H5^{C}$, $H6^{C}$), 3.76 – 3.66 (m, 8H, $H3^{A}$, $H4^{A}$, $H3^{C}$, $H4^{C}$, $H2^{D}$, $H4^{D}$, $H5^{D}$, OC*H*H linker), 3.58 – 3.47 (m, 3H, $H3^{D}$, $H4^{D}$, OCH*H* linker), 3.37 (t, *J* = 8.5 Hz, 1H, $H2^{D}$), 3.25 (m, 2H, $H2^{A}$, $H2^{C}$), 3.01 (t, *J* = 7.4 Hz, 2H, NC$H_2$ linker), 1.74 – 1.60 (m, 4H, 2*C$H_2$ linker),1.52 - 1.44 (m, 2H, C$H_2$ linker). $^{13}$C NMR (201 MHz, $D_2O$) δ 101.6, 98.4, 96.5, 95.9, 76.8, 75.3, 74.9, 74.6, 73.7, 72.3, 71.3, 70.1, 69.1, 68.9, 68.6, 68.2, 67.5, 67.5, 66.7, 66.6, 58.2, 57.2, 55.2, 28.2, 26.6, 22.5. MALDI-MS: m/z calcd for: $C29H45N3Na4O33S42$ $[M\text{-}2Na^{+}]^{2-}$: 591.5210; found 591.5216.

**5-Aminopentyl-O-(β-D-Glucopyranosyluronate)-(1→4)-(2-deoxy-2-sulfamino-α-D-glucopyranoside)-(1→4)-O-(α-L-idopyranosyluronate)-(1→4)-O-2-deoxy-2-sulfamino-6-sulfate-2-deoxy-α-D-glucopyranoside Hexasodium Salt (S11)**

Tetrasaccharide **S8** (12.0 mg, 0.008 mmol) was subjected to N-sulfation and global debenzylation according to the general procedures to give tetrasaccharide **S11** (5.0 mg, 57% for two steps). $^{1}$H NMR (500 MHz, $D_2O$) δ 5.24 (d, *J* = 3.6 Hz, 1H, $H1^{C}$), 5.03 (d, *J* = 3.5 Hz, 1H, $H1^{A}$), 4.90 (s, 1H,

H1$^{B}$), 4.40 (d, *J* = 7.8 Hz, 1H, H5$^{B}$), 4.20 (d, *J* = 11.4 Hz, 1H, H6$^{A}{}_{a}$), 4.13 (dd, *J* = 11.2, 5.5 Hz, 1H, H6$^{A}{}_{b}$), 4.01 (s, 1H, H3$^{B}$), 3.95-3.90 (m, 2H, H5$^{A}$, H4$^{B}$), 3.79 – 3.55 (m, 7H, H3$^{A}$, H4$^{A}$, H3$^{C}$, H4$^{C}$, H6$^{C}$, H5$^{D}$, OCH*H* linker ), 3.49 – 3.37 (m, 3H, H3$^{D}$, H4$^{D}$, OC*H*H linker),3.28(t, *J* = 8.2 Hz, 1H, H2$^{D}$), 3.20 – 3.11 (m, 2H, H2$^{A}$, H2$^{C}$), 2.90 (t, *J* = 7.5 Hz, 2H, NC*H*$_2$ linker), 1.70-1.50 (m, 4H, 2*C*H*$_2$ linker), 1.45-1.30 (m, 2H, C*H*$_2$ linker). $^{13}$C NMR (126 MHz, D$_2$O) 102.2, 102.0, 97.3, 95.6, 78.8, 75.2, 74.9, 73.0, 72.0, 70.9, 70.0, 69.1, 68.9, 68.4, 68.3, 67.3, 61.3, 57.8, 55.3, 39.7, 28.0, 26.8, 22.7. ESI-MS: m/z calcd for: C29H46N3Na3O30S32 [M-2Na$^{+}$]$^{2-}$: 540.5516; found 540.5519.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-O-(methyl-2-O-acetyl-3-O-benzyl-4-O-(9-fluorenylmethoxycarbonyl)-β-D-glucopyranosyluronate)-(1→4)-O-(2-azido-3-O-benzyl-2-deoxy-6-O-acetyl-α-L-idopyranosyluronate)-(1→4)-O-(methyl-2-sulfonate-3-O-benzyl-α-L-S2)-(1→4)-O-(2-azido-3-O-benzy-2-deoxy-α-D-glucopyranoside (S12)**

Tetrasaccharide **S2** (55.0 mg, 0.004 mmol) was subjected to selective O-desulfation according to the general procedures to give tetrasaccharide **S12** (30.6 mg, 60%).$^{1}$H NMR (500 MHz, CD$_3$OD) δ 7.90 – 7.07 (m, 38H, C*H* Aromatics), 5.37 (d, *J* = 3.3 Hz, 1H, H1$^{B}$), 5.21 – 5.09 (m, 3H, H1$^{C}$, C*H*$_2$Ph), 5.05 (t, *J* = 11.4 Hz, 1H, C*H*HPh), 4.95 – 4.75 (m, 7H, H1$^{A}$, H5$^{B}$, H1$^{D}$, H2$^{D}$, H4$^{D}$, C*H*$_2$Ph), 4.74 – 4.40 (m, 10H, H2$^{B}$, H6$^{C}{}_{a}$, 8*H* of C*H*$_2$Ph), 4.33 (s, 1H, H3$^{B}$), 4.24 (t, *J* = 6.6 Hz, 1H, C*H* Fmoc), 4.18 (d, *J* = 9.9 Hz, 1H, H5$^{D}$), 4.11 (m, 1H, H6$^{C}{}_{b}$), 3.98 – 3.61 (m, 11H, H3$^{A}$, H4$^{A}$, H5$^{A}$, H6$^{A}$, H4$^{B}$, H3$^{C}$, H4$^{C}$, H5$^{C}$, H3$^{D}$, OC*H*H linker), 3.45 – 3.19 (m, 11H, H2$^{A}$, H2$^{C}$, OCH*H* linker, 2*COOC*H*$_3$, NC*H*$_2$ linker), 2.06 (s, 3H, COC*H*$_3$), 1.93 (s, 3H, COC*H*$_3$), 1.71 – 1.51 (m, 4H, 2*C*H*$_2$ linker), 1.50 – 1.35 (m, 2H, C*H*$_2$ linker). $^{13}$C NMR (126 MHz, CD$_3$OD) δ127.9, 127.6, 127.2, 126.9, 125.4, 124.7, 119.4, 100.1, 98.5, 97.7, 95.1, 82.5, 79.4, 78.2, 77.9, 74.9, 74.4, 73.7, 72.7, 72.3, 71.9, 71.8, 70.8, 70.1, 70.0, 69.9, 69.4, 67.5, 67.3, 67.0, 63.3, 62.9, 62.2, 60.4, 51.2, 50.1,

48.1, 46.5, 46.4, 28.9, 28.0, 23.1, 19.7, 19.2. MALDI-MS: m/z calcd for: C93H100N7O30S [M-$Na^+$]$^-$: 1826.6241; found: 1826.6256.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-O-(3-O-benzyl-β-D-glucopyranosyluronate)-(1→4)-O-(2-amino-3-O-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(2-O-sulfonate-3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-amino-3-O-benzy-2-deoxy-α-D-glucopyranoside (S13)**

Tetrasaccharde **S12** (30 mg, 0.020 mmol) was dissolved in THF (1.4 mL) and subjected to saponification, de-O-acetylation and azide reduction according to the general procedure to give **S13** as sodium salt (17.5 mg, 72% for three steps). $^1$H NMR (500 MHz, Methanol-$d_4$) δ 7.58 – 7.07 (m, 30H, C*H* Aromatics), 5.40 (s, 1H, H1$^B$), 5.27 (d, *J* = 3.6 Hz, 1H, H1$^C$), 5.17 (d, *J* = 13.8 Hz, 2H, C$H_2$ of Bn), 5.09 (d, *J* = 10.7 Hz, 1H, C*H* of Bn), 4.95 - 4.84 (m, 5H, H1$^A$, H5$^B$, 4*C*H* of Bn), 4.79 (d, *J* = 11.3 Hz, 1H, C*H* of Bn), 4.72 – 4.60 (m, 3H, H2$^B$, H1$^D$, C*H* of Bn), 4.51 (s, 2H, C$H_2$ of Bn), 4.45 (d, *J* = 11.7 Hz, 1H, C*H* of Bn), 4.30 (s, 1H, H3$^B$), 4.26 (s, 1H, H4$^B$), 4.03 – 3.8 (m, 8H, H3$^A$, H4$^A$, H5$^A$, H6$^A$, H6$^C$, H5$^D$), 3.72 – 3.30 (m, 10H, H2$^C$, H3$^C$, H4$^C$, H5$^C$, H2$^D$, H3$^D$, H4$^D$, OC$H_2$ linker), 3.20 – 3.30 (m, 2H, NC$H_2$ linker), 3.15 – 3.20 (m, 1H, H2$^A$), 1.65 – 1.55 (m, 4H, 2*C$H_2$ linker), 1.40 -1.30 (m, 2H, C$H_2$ linker). $^{13}$C NMR (126 MHz, $CD_3OD$) δ 128.8, 128.2, 127.9, 127.8, 126.8, 125.7, 102.5, 99, 97.9, 93.5, 84.2, 79.8, 78.4, 75.2, 74.7, 74.3, 72.3, 72, 71.6, 71.5, 70.2, 70, 68.7, 67.4, 67, 64.3, 63.1, 60.4, 60.1, 50.1, 46.7, 30.3, 27.3, 23.1 MALDI-MS: m/z calcd for: C72H84N3Na2O26S [M-$Na^+$]$^-$:1484.4865; found: 1484.4872.

**5-Aminopentyl-O-(β-D-Glucopyranosyluronate)-(1→4)-(2-deoxy-2-acetamino-α-D-glucopyranoside)-(1→4)-O-(2-sulfate-α-D-idopyranosyluronate)-(1→4)-O-2-deoxy-2-acetamino-2-deoxy-α-D-glucopyranoside Hexasodium Salt (S14)**

Tetrasaccharide **S13** (15.0 mg, 0.004 mmol) was subjected to N-acetylation and global debenzylation according to the general procedures to give tetrasaccharide **S14** (7.6 mg, 76% for two steps). $^{1}$H NMR (500 MHz, $D_2O$) δ 5.22 (s, 1H, $H1^B$), 5.13 (d, $J$ = 3.6 Hz, 1H, $H1^C$), 4.90 (d, $J$ = 3.5 Hz, 1H, $H1^A$), 4.87 (s, 1H, $H5^B$), 4.53 (d, $J$ = 7.8 Hz, 1H, $H1^D$), 4.29 (dd, $J$ = 3.2, 1.8 Hz, 1H, $H2^B$), 4.23 (t, $J$ = 3.2 Hz, 1H, $H3^B$), 4.09 (s, 1H, $H4^B$), 4.02 (dd, $J$ = 10.6, 3.5 Hz, 1H, $H2^A$), 4.09 – 3.68 (m, 13H, $H3^A$, $H4^A$, $H5^A$, $H6^A$, $H2^C$, $H3^C$, $H4^C$, $H5^C$, $H6^C$, $H5^D$, OC*H*H linker), 3.60 – 3.48 (m, 3H, $H3^D$, $H4^D$, OCH*H* linker), 3.40 (t, $J$ = 7.6 Hz, 1H, $H2^D$), 3.03 (t, $J$ = 7.6 Hz, 2H, NC$H_2$ linker), 2.10 (s, 3H, COC$H_3$), 2.06 (s, 3H, COC$H_3$), 1.70 – 1.50 (m, 4H, 2*C$H_2$ linker), 1.55 – 1.43 (m, 2H, C$H_2$ linker).$^{13}$C NMR (126 MHz, $D_2O$) δ 102.3, 99.1, 96.7, 93.9, 78.1, 76.0, 75.3, 74.7, 73.1, 72.0, 71.7, 71.1, 68.3, 67.9, 65.2, 60.1, 54.3, 53.3, 39.6, 28.3, 25.7, 23.3, 22.6. ESI-MS: m/z calcd for: $C_{33}H_{52}N_3Na_2O_{26}S$ $[M\text{-}Na^+]^-$: 984.2361; found 984.2369.

**5-Aminopentyl-O-(β-D-Glucopyranosyluronate)-(1→4)-(2-deoxy-2-sulfamino-α-D-glucopyranoside)-(1→4)-O-(2-sulfate-α-L-idopyranosyluronate)-(1→4)-O-2-deoxy-2-sulfamino-2-deoxy-α-D-glucopyranoside Hexasodium Salt (S15)**

Tetrasaccharide **S13** (10.0 mg, 0.006 mmol) was subjected to N-sulfation and global debenzylation according to the general procedures to give tetrasaccharide **S15** (7.6 mg, 63% for two steps). $^{1}$H NMR (500 MHz, $D_2O$) δ 5.22 (s, 1H, $H1^B$), 5.13 (d, $J$ = 3.6 Hz, 1H, $H1^C$), 4.90 (d, $J$ = 3.5 Hz, 1H, $H1^A$), 4.87 (s, 1H, $H5^B$), 4.53 (d, $J$ = 7.8 Hz, 1H, $H1^D$), 4.29 (dd, $J$ = 3.2, 1.8 Hz, 1H, $H2^B$), 4.23 (t, $J$ = 3.2 Hz, 1H, $H3^B$), 4.09 (s, 1H, $H4^B$), 4.02 (dd, $J$ = 10.6, 3.5 Hz, 1H, $H2^A$), 4.09 – 3.68 (m, 13H, $H3^A$, $H4^A$, $H5^A$, $H6^A$, $H2^C$, $H3^C$, $H4^C$, $H5^C$, $H6^C$, $H5^D$, OC*H*H linker), 3.60 – 3.48 (m, 3H, $H3^D$, $H4^D$, OCH*H* linker), 3.40 (t, $J$ = 7.6 Hz, 1H, $H2^D$), 3.03 (t, $J$ = 7.6 Hz, 2H, NC$H_2$ linker), 2.10 (s, 3H, COC$H_3$), 2.06 (s, 3H, COC$H_3$), 1.70 – 1.50 (m, 4H, 2*C$H_2$ linker), 1.55 – 1.43 (m, 2H, C$H_2$ linker).$^{13}$C NMR (126 MHz, $D_2O$) δ 102.3, 99.1, 96.7, 93.9, 78.1, 76.0, 75.3, 74.7, 73.1,

72.0, 71.7, 71.1, 68.3, 67.9, 65.2, 60.1, 54.3, 53.3, 39.6, 28.3, 25.7, 23.3, 22.6. ESI-MS: m/z calcd for: C29H46N3Na3O30S32 $[M\text{-}Na^{+}]^{-}$: 540.5516; found 540.5520.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-O-(3-O-benzyl-β-D-glucopyranosyluronate)-(1→4)-O-(2-azido-3-O-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-azido-3-O-benzy-2-deoxy-α-D-glucopyranoside (S16)**

Tetrasaccharde **S1** (19 mg, 0.011 mmol) was dissolved in THF (1.0 mL) and subjected to saponification, and de-O-acetylation according to the general procedure to give **S16** as sodium salt (13.0 mg, 83% for three steps). $^{1}$H NMR (500 MHz, $CD_3OD$) δ 7.50 – 7.17 (m, 30H, C*H* Aromatics), 5.20 – 5.11 (m, 4H, $H1^{C}$, 3H of C$H_2$ Bn), 5.08 – 4.99 (m, 2H, $H1^{B}$, C*H* of C$H_2$ Bn), 4.88 – 4.86 (m, 2H, $H5^{B}$, C*H* of C$H_2$ Bn), 4.78 – 4.64 (m, 3H, $H1^{A}$, 2*C*H* of C$H_2$ Bn), 4.62 – 4.50 (m, 4H, $H1^{D}$, 3*C*H* of C$H_2$ Bn), 4.33 (t, *J* = 2.8 Hz, 1H, $H4^{B}$), 3.69 – 3.50 (m, 15H, $H2^{B}$, $H3^{B}$, $H3^{C}$, $H4^{A}$, $H4^{C}$, $H5^{A}$, $H5^{C}$, $H5^{D}$, $H6^{A}$, $H6^{C}$, OC*H*H linker), 3.49 – 3.20 (m, 7H, $H3^{A}$, $H2^{D}$, $H3^{D}$, $H4^{D}$, OCH*H* linker, NC$H_2$ linker), 2.78 (dd, *J* = 10.0, 3.5 Hz, 1H, $H2^{C}$), 2.56 (dd, *J* = 9.9, 3.6 Hz, 1H, $H2^{A}$), 1.53 – 1.43 (m, 4H, OC$H_2$ linker), 1.31 – 1.14 (m, 2H, OC$H_2$ linker). $^{13}$C NMR (126 MHz, $CD_3OD$) δ 128.5, 128.4, 128.1, 127.9, 127.7, 103.1, 101.4, 98.8, 96.3, 84.4, 82.2, 81.5, 75.7, 75.5, 75.4, 75, 74.9, 74.9, 74.5, 74.4, 72.6, 72.2, 71.9, 71.7, 70.2, 68.6, 67.5, 67.3, 61, 60.4, 60.3, 55.4, 55.3, 50.4, 48.1, 46.8, 29, 27.5. MALDI-MS: m/z calcd for: C72H81N7NaO23 $[M\text{-}Na^{+}]^{-}$: 1434.5287; found: 1434.5292.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-O-(3-O-benzyl-β-D-glucopyranosyluronate)-(1→4)-O-(2-acetadmido-3-O-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-acetadmido-3-O-benzy-2-deoxy-α-D-glucopyranoside (S17)**

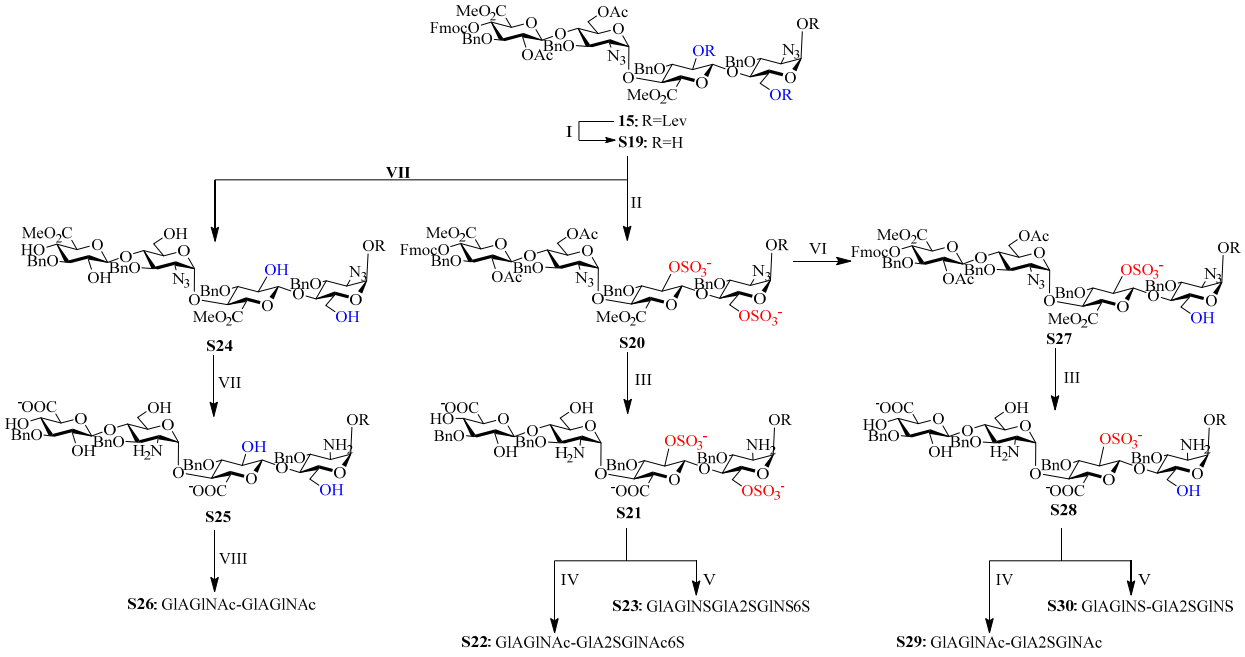
Tetrasaccharde **S16** (13.0 mg, 0.009 mmol) was dissolved in THF (1.0 mL) and subjected to azide reduction and N acetylation according to the general procedure to give **S17** as sodium salt (10.2 mg, 77% for two steps). $^{1}$H NMR (500 MHz, $CD_3OD$) δ 7.54 – 7.14 (m, 30H, CH Aromatics), 5.25 – 5.07 (m, 3H, $H1^B$, 2*H* of Bn), 5.02 (d, *J* = 11.7 Hz, 1H, H of C*H*$_2$Ph), 4.96 (d, *J* = 11.4 Hz, 1H, $H5^B$), 4.95 – 4.75 (m, 3H, $H1^C$, 2*H* of C*H*$_2$Ph), 4.75 – 4.43 (m, 8H, $H1^A$, $H1^D$, 6*H* of C*H*$_2$Ph), 4.22 – 3.17 (m, 23H, $H2^A$, $H3^A$, $H4^A$, $H5^A$, $H6^A$, $H2^B$, $H3^B$, $H4^B$, $H2^C$,$H3^C$, $H4^C$, $H5^C$, $H6^C$, $H2^D$, $H3^D$, $H4^D$, $H5^D$, OC*H*$_2$ linker, NC*H*$_2$ linker), 1.83 (s, 3H, C*H*$_3$CO), 1.77 – 1.71 (m, 3H, C*H*$_3$CO), 1.64 – 1.41 (m, 4H, 2*C*H*$_2$ linker), 1.31 – 1.14 (m, 2H, OC*H*$_2$ linker). $^{13}$C NMR (126 MHz, $CD_3OD$) δ 128.1, 128.0, 127.9, 127.8, 127.7, 127.2, 103.2, 101.4, 96.0, 84.3, 79.3, 76.9, 75.6, 75.3, 74.8, 74.4, 73.8, 73.7, 72.1, 72.0, 71.7, 68.0, 67.7, 67.6, 67.3, 67.1, 60.8, 59.9, 53.4, 52.8, 50.3, 48.1, 46.6, 28.9, 27.5, 23.3, 21.6, 20.5. ESI-MS: m/z calcd for: $C_{76}H_{89}N_3NaO_{25}$ $[M\text{-}Na^+]^-$ 1466.5688; found: 1466.5695.

**5-Aminopentyl-O-(β-D-Glucopyranosyluronate)-(1→4)-(2-deoxy-2-acetamino-α-D-glucopyranoside)-(1→4)-O-(α-L-idopyranosyluronate)-(1→4)-O-2-deoxy-2-acetamino-2-deoxy-α-D-glucopyranoside Hexasodium Salt (S18)**

Tetrasaccharide **S17** (10.2 mg, 0.007 mmol) was subjected to global debenzylation according to the general procedures to give tetrasaccharide **S18** (5.4 mg, 86%). $^{1}$H NMR (500 MHz, $D_2O$) δ 5.06 (d, *J* = 3.8 Hz, 1H, $H1^C$), 4.84 (d, 1H, *J* = 3.4 Hz, $H1^B$), 4.77 (d, *J* = 3.6 Hz, 1H, $H1^A$), 4.57 (d, *J* = 2.3 Hz, 1H, $H5^B$), 4.39 (d, 1H, *J* = 7.9 Hz, 1H, $H1^D$), 3.95 (t, *J* = 3.4 Hz, 1H, $H4^B$), 3.86 – 3.51 (m, 15H, $H2^A$, $H2^B$, $H2^C$, $H3^A$, $H3^B$, $H3^C$, $H4^A$, $H4^C$, $H5^A$, $H5^C$, $H5^D$, $H6^A$, $H6^C$, OC*H*H linker), 3.46 – 3.33 (m, 3H, $H3^D$, $H4^D$, OCH*H* linker), 3.25 (d, 1H, *J* = 7.9 Hz, 1H, $H2^D$), 2.95 – 2.84 (m, 2H, NC*H*$_2$ linker), 1.94 – 1.86 (m, 6H, 2*COC*H*$_3$), 1.65 – 1.47 (m, 4H, 2*C*H*$_2$ liker), 1.39 – 1.27 (m, 2H, C*H*$_2$ linker).$^{13}$C NMR (126 MHz, $D_2O$) δ 102.5, 101.8, 96.7, 94.5, 78.3, 75.9, 75.5,

74.7, 73.1, 72.0, 71.0, 70.9, 70.3, 70.1, 69.8, 68.0, 60.0, 54.1, 53.7, 39.6, 28.2, 22.1, 26.8, 22.5.

ESI-MS: m/z calcd for: $C_{33}H_{53}N_3NaO_{23}$ $[M\text{-}Na^+]^-$: 882.2968; found 882.2979.




Scheme 3.4 Diversifation of GlAGlNGlAGlN (I)$NH_2NH_2$AcOH, DCM/MeOH, RT, 2h, 83%; (II) Py.$SO_3$ excess, DMF, RT, 93%; (III) i) LiOH, $H_2O_2$, THF, 4h, then 4M NaOH, MeOH, 12h; ii) $PMe_3$, THF, MeOH, 0.1M NaOH (**S20**, 88%; **S28**, 81%); (IV) i) $Ac_2O$, MeOH, $Et_3N$, 30 min; ii) Pd/C, $H_2$, MeOH, $H_2O$, 4h; ii) $Pd(OH)_2$/C, $H_2$, $H_2O$, 14h (**S22**, 78%, **S26**, 75%; **S29**, 69%; ); (V) i) Py.$SO_3$, MeOH, $Et_3N$, 0.1M NaOH, 12h; ii) Pd/C, $H_2$, MeOH, $H_2O$, 4h; ii) $Pd(OH)_2$/C, $H_2$, $H_2O$, 14h (**S23**, 71%; **S30**, 82%); (VI) BTSA, Pyr., 60 ºC, 2h, 78%; (VII) LiOH, $H_2O_2$, THF, 4 h, then 4M NaOH, MeOH, 12 h, 67%; (VIII) $PMe_3$, THF, MeOH, 0.1M NaOH, 92%.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(methyl-2-*O*-acetyl-3-*O*-benzyl-4-*O*-acetyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-6-*O*-acetyl-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(methyl-2-*O*-levulinate-3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzy-6-*O*-levulinate-2-deoxy-α-D-glucopyranoside (15)**

Disaccharide donor **10** (160.0 mg, 0.173 mmol) and acceptor **6** (156.0 mg, 0.144 mmol) was coupled according to the general procedure for glycosylation to give tetrasaccharide **15** (118.9 mg, 59.0%). $^{1}$H NMR (500 MHz, $CDCl_3$) δ 7.35 – 6.86 (m, 38H, C*H* Aromatics), 5.45 (s, 1H, $H1^{C}$), 5.16 – 4.90 (m, 5H, 3H of C$H_2$Ph, $H2^{D}$, $H4^{D}$), 4.97 – 4.72 (m, 1H, $H2^{A}$), 4.71 – 4.23 (m, 8H, $H1^{B}$, $H1^{D}$, $H6^{A}$a, 5H of CH2Ph), 4.13 – 3.03 (m, 22H, $H2^{A}$, $H3^{A}$, $H5^{A}$, $H6^{A}$b, $H2^{B}$, $H3^{B}$, $H4^{B}$, $H5^{B}$, $H2^{C}$, $H3^{C}$, $H4^{C}$, $H5^{C}$, $H6^{C}$, $H3^{D}$, $H5^{D}$, OC$H_2$ and NC$H_2$ linker), 2.02 (s, 3H, COC$H_3$), 1.99 (s, 3H, COC$H_3$), 1.89 (s, 3H, COC$H_3$), 1.66 – 1.33 (m, 4H, 2*C$H_2$ linker), 1.36 – 1.07 (m, 2H, C$H_2$ linker). $^{13}$C NMR (500 MHz, $CDCl_3$) δ 129.9, 128, 127.8, 127.6, 127.4, 125.9, 101.1, 100.9, 97.5, 97, 81.8, 79.5, 77.9, 77.7, 75.5, 75.1, 74.2, 74.1, 74, 73.8, 73, 72.8, 72.2, 70.7, 69.2, 68.9, 67.7, 66.8, 62.6, 62.3, 61.1, 50, 47.7, 45.8, 38.8, 31.2, 29.4, 28.5, 27.2, 25.8, 23.9, 21.2, 20.4. ESI-MS: m/z calcd for $C89H93N7NaO27$ $[M+Na^{+}]^{+}$: 1910.6753; found, 1910.6770.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(methyl-2-*O*-acetyl-3-*O*-benzyl-4-*O*-acetyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-6-*O*-acetyl-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(methyl-3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzy-2-deoxy-α-D-glucopyranoside (S19)**

Tetrasaccharide **15** (394.0 mg, 0.202mmol) was subjected to Cleavage of Lev Esters according to general procedure to give **S19** (188.0 mg, 81%). $^{1}$H NMR (500 MHz, $CDCl_3$) δ 7.35 – 6.86 (m, 38H, C*H* Aromatics), 5.45 (s, 1H, $H1^{C}$), 5.16 – 4.90 (m, 5H, 3H of C$H_2$Ph, $H2^{D}$, $H4^{D}$), 4.97 – 4.72 (m, 1H, $H2^{A}$), 4.71 – 4.23 (m, 8H, $H1^{B}$, $H1^{D}$, $H6^{A}$a, 5H of CH2Ph), 4.13 – 3.03 (m, 22H, $H2^{A}$, $H3^{A}$, $H5^{A}$, $H6^{A}$b, $H2^{B}$, $H3^{B}$, $H4^{B}$, $H5^{B}$, $H2^{C}$, $H3^{C}$, $H4^{C}$, $H5^{C}$, $H6^{C}$, $H3^{D}$, $H5^{D}$, OC$H_2$ and NC$H_2$ linker), 2.02 (s, 3H, COC$H_3$), 1.99 (s, 3H, COC$H_3$), 1.89 (s, 3H, COC$H_3$), 1.66 – 1.33 (m, 4H, 2*C$H_2$ linker), 1.36 – 1.07 (m, 2H, C$H_2$ linker). $^{13}$C NMR (500 MHz, $CDCl_3$) δ 128.2, 127.9, 127.8, 103.3, 100.8, 97.6, 96.9, 83.8, 79.6, 78.6, 77.7, 75.2, 74.8, 74.6, 74.3, 74.1, 73.1, 72.3, 71,

70.9, 69, 67.3, 67.2, 67.2, 63.2, 62.7, 61.5, 61.2, 61.1, 52.6, 52.5, 50.3, 45.9, 28.8, 27.3, 23.2, 21.7, 21.6, 20.8, 20.5, 20.4. ESI-MS: m/z calcd for C89H93N7NaO27 $[M+Na^+]^+$: 1714.6017; found, 1714.6032.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(methyl-2-*O*-acetyl-3-*O*-benzyl-4-*O*-acetyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-6-*O*-acetyl-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(methyl-2-*O*-sulfate-3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzy-6-*O*-sulfonate-2-deoxy-α-D-glucopyranoside (S20)**

Tetrasaccharide S19 (120.0 mg, 0.069mmol) was subjected to O-sulfation according to general procedure to give **S20** (125.0 mg, 93%); $^1$H NMR (500 MHz, $CD_3OD$) δ 7.96 – 6.81 (m, 38H, C*H* Ar), 5.46 (d, *J* = 3.6 Hz, 1H, $H1^C$), 5.31 (d, *J* = 10.1 Hz, 1H, H of C$H_2$Ph), 5.23 – 5.03 (m, 5H, $H1^B$, $H4^D$, 3*C*H* of C$H_2$Ph), 4.98 (t, *J* = 9.4 Hz, 1H, $H2^D$), 4.87 – 4.37 (m, 15H, $H1^A$, $H6^A$a, $H2^B$, $H1^D$, 8*CH of $CH_2$Ph, C*H* and C$H_2$ of Fmoc), 4.20 – 3.85 (m, 12H, $H3^A$, $H4^A$, $H5^A$, $H6^A{}_b$, $H5^B$, $H3^C$, $H4^C$, $H5^C$, $H6^C$, $H3^D$, $H5^D$), 3.76 – 3.07 (m, 14H, $H2^A$, $H4^B$, $H5^B$, $H2^C$, 2*COOC$H_3$, OC$H_2$ linker, NC$H_2$ linker), 2.06 (s, 3H, COC$H_3$), 1.95 (s, 3H, COC$H_3$), 1. 60 – 1.40 (m, 4H, 2*C$H_2$ linker), 1.42 – 1.15 (m, 2H, C$H_2$ linker). $^{13C}$ NMR (126 MHz, $CD_3OD$) δ 148.7, 128.5, 128, 127.7, 127.5, 124.1, 101, 100.5, 96.2, 82.8, 80, 79.6, 77.8, 77.3, 74.7, 74.4, 74.4, 74.3, 74.3, 73.6, 72.6, 72.4, 71.3, 69.1, 67.1, 65.7, 65.5, 62.8, 61.8, 61.6, 51.7, 51.6, 50.1, 48.4, 47.9, 47, 46.5, 46.4, 19.5, 19.3 ESI-MS: m/z calcd for C91H97N7O32S22 $[M-Na^+]^-$: 931.7816; found, 931.7829.

**N-($^B$enzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(2-*O*-sulfate-3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzy-6-*O*-sulfonate-2-deoxy-α-D-glucopyranoside (S21)**

Tetrasaccharide **S20** (107.0 mg, 0.062 mmol) was subjected to the saponification, de-*O*-acetylation and azide reduction according to the general procedure to provide tetrasaccharide **S21** as a sodium salt (65.0 mg, 88% for three steps); $^{1}$H NMR (500 MHz, $CD_3OD$) δ 7.76 – 7.01 (m, 30H, C*H* Ar), 5.27 (d, *J* = 3.6 Hz, 1H, $H1^{C}$), 5.24 – 5.07 (m, 5H, $H1^{B}$, 4H of CH2Ph), 5.02 (d, *J* = 11.0 Hz, 1H, CH of CH2Ph), 4.94 – 4.47 (m, 10H, $H1^{A}$, $H1^{D}$, $H2^{B}$, 7H of CH2Ph), 4.40 – 4.30 (m, 1H, $H5^{B}$), 4.20 – 3.13 (m, 21H, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H6^{A}$, $H3^{B}$, $H4^{B}$, $H2^{C}$, $H3^{C}$, $H4^{C}$, $H5^{C}$, $H6^{C}$, $H2^{D}$, $H3^{D}$, $H4^{D}$, $H5^{D}$, OC*H*$_2$ linker, NC*H*$_2$ linker), 2.75 – 2.60 (m, 2H, $H2^{A}$, $H2^{C}$), 1.68 – 1.41 (m, 4H, 2*$CH_2$ linker), 1.30 – 1.20 (m, 2H, C*H*$_2$ linker). $^{13C}$ NMR (126 MHz, $CD_3OD$) δ 129.1, 128.5, 127.9, 127.8, 127.7, 127.5, 102.7, 100.5, 98.3, 97.3, 84.1, 81.1, 80.7, 80.6, 79.8, 78.6, 75.6, 75.5, 75.3, 74.3, 74.2, 74.1, 74, 73.7, 73, 72.7, 72.1, 69.9, 67.5, 67.4, 67.0, 65.7, 65.6, 61.6, 61, 59.9, 58.8, 58.2, 55.3, 54.2, 52.5, 51.8, 51.2, 50.8, 50.3, 50.1, 49.6, 49.5, 49.1, 49, 48.4, 47.9, 47.7, 47.3, 46.9, 46.4, 45.5, 45.4, 27.4, 23.0. ESI-MS: m/z calcd for $C72H79N7Na2O29S2$ $[M-2Na^{+}]^{2-}$: 807.7085; found, 807.7090.

**5-aminopentyl O-(β-D-glucopyranosyluronate)-(1→4)-*O*-(2-acetamino-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(2-*O*-sulfate-β-D-glucopyranosyluronate)-(1→4)-*O*-(2-acetamino-6-*O*-sulfonate-2-deoxy-α-D-glucopyranoside (S22)**

Tetrasaccharide **S21** (33.0 mg, 0.020 mmol) was subjected to the N-acetylation and global hydrogenation to provide tetrasaccharide **S22** as a sodium salt (16.4 mg, 88% for two steps); $^{1}$H NMR (500 MHz, $D_2O$) δ 5.43 (d, *J* = 3.8 Hz, 1H, $H1^{C}$), 4.90 (d, *J* = 2.8 Hz, 1H, $H1^{A}$), 4.8(d, *J* = 7.9 Hz 1H, $H1^{B}$), 4.58 (dd, *J* = 11.2, 3.1 Hz, 1H, $H6^{A}{}_{a}$), 4.53 (d, *J* = 7.9 Hz, 1H, $H1^{D}$), 4.28 (dd, *J* = 11.2, 2.1 Hz, 1H, $H6^{A}{}_{b}$), 4.13 (t, *J* = 7.8 Hz, 1H, $H2^{B}$), 4.02 (t, *J* = 9.9 Hz, 1H, $H3^{B}$), 3.97 – 3.68 (m, 14H, $H2^{A}$, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H4^{B}$, $H5^{B}$, $H2^{C}$, $H3^{C}$, $H4^{C}$, $H5^{C}$, $H6^{C}$, $H5^{D}$, OC*H*H linker), 3.58 – 3.50 (m, 3H, $H3^{D}$, $H4^{D}$, OCH*H* linker), 3.41 (t, *J* = 6.4 Hz, 1H, $H2^{D}$), 3.04 (t, *J* = 7.6 Hz,

2H, NC$H_2$ linker), 2.08 (s, 3H, COC$H_3$), 2.04 (s, 3H, COC$H_3$), 1.78 – 1.60 (m, 4H, 2*C$H_2$ linker), 1.53 – 1.43 (m, 2H, C$H_2$ linker).$^{13}$C NMR (126 MHz, $D_2O$) δ 102.5, 100.0, 97.0, 96.7, 80.5, 78.5, 76.2, 75.9, 75.4, 75.3, 73.1, 72.0, 70.9, 69.4, 68.7, 68.1, 66.3, 66.2, 59.6, 53.5, 39.8, 28.4, 26.7, 22.7, 22.2 ESI-MS: m/z calcd for C33H51N3Na2O29S2 $[M-2Na^+]^{2-}$: 531.5928; found, 531.5936.

**5-aminopentyl-O-(β-**D**-glucopyranosyluronate)-(1→4)-*O*-(2-sulfamino-2-deoxy-α-**D**-glucopyranoside)-(1→4)-*O*-(2-*O*-sulfate-β-**D**-glucopyranosyluronate)-(1→4)-*O*-(2-sulfamino-6-*O*-sulfonate-2-deoxy-α-**D**-glucopyranoside (S23)**

Tetrasaccharide **S21** (30.0 mg, 0.018 mmol) was subjected to the N-sulfation and global hydrogenation to provide tetrasaccharide **S23** as a sodium salt (15.0 mg, 71% for two steps); $^1$H NMR (500 MHz, $D_2O$) δ 5.43 (d, *J* = 3.8 Hz, 1H, $H1^C$), 4.90 (d, *J* = 2.8 Hz, 1H, $H1^A$), 4.8(d, *J* = 7.9 Hz 1H, $H1^B$), 4.58 (dd, *J* = 11.2, 3.1 Hz, 1H, $H6^A{}_a$), 4.53 (d, *J* = 7.9 Hz, 1H, $H1^D$), 4.28 (dd, *J* = 11.2, 2.1 Hz, 1H, $H6^A{}_b$), 4.13 (t, *J* = 7.8 Hz, 1H, $H2^B$), 4.02 (t, *J* = 9.9 Hz, 1H, $H3^B$), 3.97 – 3.68 (m, 14H, $H2^A$, $H3^A$, $H4^A$, $H5^A$, $H4^B$, $H5^B$, $H2^C$, $H3^C$, $H4^C$, $H5^C$, $H6^C$, $H5^D$, OC*H*H linker), 3.58 – 3.50 (m, 3H, $H3^D$, $H4^D$, OCH*H* linker), 3.41 (t, *J* = 6.4 Hz, 1H, $H2^D$), 3.04 (t, *J* = 7.6 Hz, 2H, NC$H_2$ linker), 2.08 (s, 3H, COC$H_3$), 2.04 (s, 3H, COC$H_3$), 1.78 – 1.60 (m, 4H, 2*C$H_2$ linker), 1.53 – 1.43 (m, 2H, C$H_2$ linker).$^{13}$C NMR (126 MHz, $D_2O$) δ 102.5, 100.0, 97.0, 96.7, 80.5, 78.5, 76.2, 75.9, 75.4, 75.3, 73.1, 72.0, 70.9, 69.4, 68.7, 68.1, 66.3, 66.2, 59.6, 53.5, 39.8, 28.4, 26.7, 22.7, 22.2 ESI-MS: m/z calcd for C29H45N3Na4O33S4 $[M-2Na+]^{2-}$: 591.5210; found, 591.5216.

**N-(**B**enzyl)-benzyloxycarbonyl-5-aminopentyl-O-(3-*O*-benzyl-β-**D**-glucopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-2-deoxy-α-**D**-glucopyranoside)-(1→4)-*O*-(3-*O*-benzyl-β-**D**-glucopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzy-2-deoxy-α-**D**-glucopyranoside (S24)**

Tetrasaccharide **S19** (53.0 mg, 0.034mmol) was subjected to saponification and de-*O*-acetylation according to general procedure to give **S24** (29.0 mg, 67%); $^1$H NMR (500 MHz, $CD_3OD$) δ 7.53

– 7.09 (m, 30H), 5.55 (d, $J$ = 3.8 Hz, 1H, $H1^{C}$), 5.23 – 5.06 (m, 5H, 5H of C$H_2$Ph), 4.96 – 4.46 (m, 10H, $H1^{A}$, $H1^{B}$, $H1^{D}$, 7C$H$ of C$H_2$Ph), 4.12 – 3.14 (m, 37H, $H2^{A}$, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H6^{A}$, $H2^{B}$, $H3^{B}$, $H4^{B}$, $H5^{B}$, $H2^{C}$, $H3^{C}$, $H4^{C}$, $H5^{C}$, $H6^{C}$, $H2^{D}$, $H3^{D}$, $H4^{D}$, $H5^{D}$, OC$H_2$ linker, NC$H_2$ linker), 1.76 – 1.44 (m, 4H, 2*C$H_2$ linker), 1.43 – 1.20 (m, 2H, C$H_2$ linker). $^{13C}$ NMR (126 MHz, $CD_3OD$) δ 129.4, 127.9, 127.8, 127.7, 125.9, 125.2, 104.3, 103.1, 97.8, 97.5, 86.6, 84.3,84.2, 84.0, 77.9, 76.2, 75.8, 75.6, 75.4, 75.3, 75.0, 74.8, 74.7, 74.6, 74.3, 73.6, 71.8, 67.6, 67.2, 63.1, 59.8, 59.7, 50.3, 48.3, 48.0, 31.4, 27.6, 25.1. ESI-MS: m/z calcd for C68H73N7NaO23 $[M\text{-}Na^{+}]^{-}$: 1378.4661; found, 1378.4673.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(2-amino-3-*O*-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(2-*O*-sulfate-3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(2-amino-3-*O*-benzy-6-*O*-sulfonate-2-deoxy-α-D-glucopyranoside (S25)**

Tetrasaccharide **S24** (29.0 mg, 0.019 mmol) was subjected to azide reduction according to the general procedure to provide tetrasaccharide **S25** as a sodium salt (24.5 mg, 92%); $^{11}$H NMR (500 MHz, $CD_3OD$) δ 7.67 – 6.94 (m, 30H, CH Ar), 5.32 (d, $J$ = 3.8 Hz, 1H, $H1^{C}$), 5.25 – 5.12 (m, 4H, CH of C$H_2$Ph), 5.07 – 4.49 (m, 19H, $H1^{A}$, $H1^{B}$, $H1^{D}$, $^{C}$H of CH2Ph), 4.31 – 3.15 (m, 34H, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H6^{A}$, $H2^{B}$, $H3^{B}$, $H4^{B}$, $H5^{B}$, $H3^{C}$, $H4^{C}$, $H5^{C}$, $H6^{C}$, $H2^{D}$, $H3^{D}$, $H4^{D}$, $H5^{D}$), 2.71 – 2.54 (m, 2H, $H2^{A}$, $H2^{C}$), 1.59 – 1.50 (m, 4H, CH2 linker), 1.20 – 1.30 (m, 2H, CH2 linker). $^{13C}$ NMR (126 MHz, $CD_3OD$) δ 130.0, 129.0, 128.8, 128.3, 128.0, 127.7, 125.6, 103.1, 102.0, 98.0, 97.5, 84.5, 84.3, 80.8, 78.7, 78.1, 77.9, 77.6, 76.1, 75.8, 75.5, 75.2, 75.1, 74.7, 74.6, 74.5, 74.4, 74.3, 73.3, 72.7, 72.4, 72.3, 71.6, 67.5, 67.3, 67.2, 63.0, 59.9, 59.8, 52.0, 51.5, 50.3, 49.2, 48.7, 48.6, 48.1, 47.2, 46.6, 28.9, 27.5, 23.2. ESI-MS: m/z calcd for C68H77N3NaO23 $[M\text{-}Na^{+}]^{-}$: 1326.4851; found, 1326.4851.

**5-aminopentyl-O-(β-D-glucopyranosyluronate)-(1→4)-*O*-(2-acetamino-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(β-D-glucopyranosyluronae)-(1→4)-*O*-(2-acetamino-2-deoxy-α-D-glucopyranoside (S26)**

Tetrasaccharide **S25** (25.0 mg, 0.018 mmol) was subjected to the N-acetylation and global hydrogenation to provide tetrasaccharide **S26** as a sodium salt (13.0 mg, 78% for two steps); $^{1}$H NMR (500 MHz, $D_2O$) δ 5.28 (d, *J* = 3.4 Hz, 1H, $H1^{C}$), 4.77 (d, *J* = 2.7 Hz, 1H, $H1^{A}$), 4.43 and 4.39 (each d, *J* = 7.9 Hz, 2H, $H1^{B}$, $H1^{D}$), 3.82 – 3.10 (m, 21H, $H2^{A}$, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H6^{A}$, $H2^{B}$, $H3^{B}$, $H4^{B}$, $H5^{B}$, $H2^{C}$, $H3^{C}$, $H4^{C}$, $H5^{C}$, $H6^{C}$, $H2^{D}$, $H3^{D}$, $H4^{D}$, $H5^{D}$, OCH2 linker), 2.87 (t, *J* = 8.0 Hz, 2H, NC$H_2$ linker), 1.92 (d, *J* = 6.7 Hz, 6H, 2*COC$H_3$), 1.54 – 1.45 (m, 4H, 2*C$H_2$ linker), 1.42 – 1.20 (m, 2H, C$H_2$ linker). $^{13C}$ NMR (126 MHz, $D_2O$) δ 102.5, 102.2, 96.9, 96.5, 79.3, 78.8, 76.7, 76.6, 76.0, 75.3, 73.7, 73.2, 71.9, 70.8, 69.3, 68.0, 60.1, 59.8, 53.7, 53.6, 39.6, 28.2, 26.7, 23.1, 22.5, 22.0. ESI-MS: m/z calcd for C33H53N3NaO23 $[M\text{-}Na^{+}]^{-}$: 882.2973; found, 882.2980.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(methyl-2-*O*-acetyl-3-*O*-benzyl-4-*O*-acetyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-6-*O*-acetyl-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(methyl-2-*O*-sulfate-3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzy-2-deoxy-α-D-glucopyranoside (S27)**

Tetrasaccharide **S20** (110.0 mg, 0.059mmol) was subjected to de-*O*-sulfation according to general procedure to give **S27** (63.0 mg, 65%); $^{1}$H NMR (500 MHz, $CD_3OD$) δ 7.54 – 7.00 (m, 30H, C*H* Ar), 5.36 (s, 1H, $H1^{C}$), 5.14 (d, *J* = 10.4 Hz, 1H, CH of C$H_2$Ph), 5.11 – 5.00 (m, 4H, 4H of C$H_2$Ph), 4.95 (t, *J* = 9.8 Hz, 1H, $H2^{B}$), 4.92 – 4.30 (m, 44H, $H1^{A}$, $H1^{B}$, $H2^{B}$, $H6^{C}$a, $H1^{D}$, $H2^{D}$, $H5^{D}$, 7CH of C$H_2$Ph), 4.11 – 3.00 (m, 26H, $H2^{A}$, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H6^{A}$, $H3^{B}$, $H4^{B}$, $H5^{B}$, $H2^{C}$, $H3^{C}$, $H4^{C}$, $H5^{C}$, $H6^{C}$b, $H3^{D}$, $H4^{D}$, 2*COOC$H_3$, OC$H_2$ linker, NC$H_2$ linker), 2.00 – 1.69 (m, 9H, 3*COC$H_3$), 1.50 – 1.40 (m, 4H, 2*C$H_2$ linker), 1.30 – 1.20 (m, 2H, C$H_2$ linker). $^{13C}$ NMR (126 MHz, $CD_3OD$) δ

127.9, 127.8, 127.4, 127.1, 126.6, 101.6, 100.6, 97.7, 96.2, 82.5, 79.5, 78.1, 77.8, 77.3, 74.6, 74.3, 73.8, 73.7, 72.6, 72.2, 71.2, 71.1, 69.2, 66.9, 62.7, 61.6, 61.5, 59.6, 52.9, 52.6, 51.7, 51.4, 51.2, 50.5, 50, 49.8, 49.6, 48.9, 48.1, 47.7, 47.6, 46.7, 46.2, 45.7, 29.2, 29.1, 26.9, 23.0, 21.6, 21.5, 20.4, 20.3, 19.3, 19.2, 19.0, 18.8, 18.7, 17.1. ESI-MS: m/z calcd for C80H92N7O29S [M-$Na^+$]$^-$ 1646.5666; found, 1646.5675.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-O-(3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(2-*O*-sulfate-3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzy-6-*O*-sulfonate-2-deoxy-α-D-glucopyranoside (S28)**

Tetrasaccharide **S27** (63.0 mg, 0.062 mmol) was subjected to the saponification and de-*O*-acetylation according to the general procedure to provide tetrasaccharide **S28** as a sodium salt (47.6 mg, 81% for two steps); $^1$H NMR (500 MHz, $CD_3OD$) δ 7.69 – 7.08 (m, 30H, CH Ar), 5.59 (d, *J* = 3.9 Hz, 1H, $H1^C$), 5.38 (d, *J* = 9.6 Hz, 1H, CH of C$H_2$Ph), 5.25 (d, *J* = 11.5 Hz, 1H, CH of CH2Ph), 5.17 (d, *J* = 21.9 Hz, 2H, C$H_2$Ph), 5.08 (d, *J* = 11.0 Hz, 1H, CH of C$H_2$Ph), 4.98 – 4.40 (m, 15H, $H1^A$, $H1^B$, $H2^B$, $H1^D$, 7*CH of CH2Ph), 4.32 – 3.01 (m, 24H, $H2^A$, $H3^A$, $H4^A$, $H5^A$, $H6^A$, $H3^B$, $H4^B$, $H5^B$, $H2^C$, $H3^C$, $H4^C$, $H5^C$, $H6^C$, $H2^D$, $H3^D$, $H4^D$, $H5^D$, $H6^D$, OC$H_2$ linker, NC$H_2$ linker), 1.67 – 1.44 (m, 4H, 2*C$H_2$ linker), 1.40 – 1.30 (m, 2H, C$H_2$ linker). $^{13C}$ NMR (126 MHz, $CD_3OD$) δ 130.0, 129.0, 128.8, 128.3, 128.0, 127.7, 125.6, 103.1, 102.0, 98.0, 97.5, 84.5, 84.3, 80.8, 78.7, 78.1, 77.9, 77.6, 76.1, 75.8, 75.5, 75.2, 75.1, 74.7, 74.6, 74.5, 74.4, 74.3, 73.3, 72.7, 72.4, 72.3, 71.6, 67.5, 67.3, 67.2, 63.0, 59.9, 59.8, 52.0, 51.5, 50.3, 49.2, 48.7, 48.6, 48.1, 47.2, 46.6, 28.9, 27.5, 23.2. ESI-MS: m/z calcd for C72H80N7Na2O26S [M-Na+]$^-$: 1536.4675; found, 1536.4679.

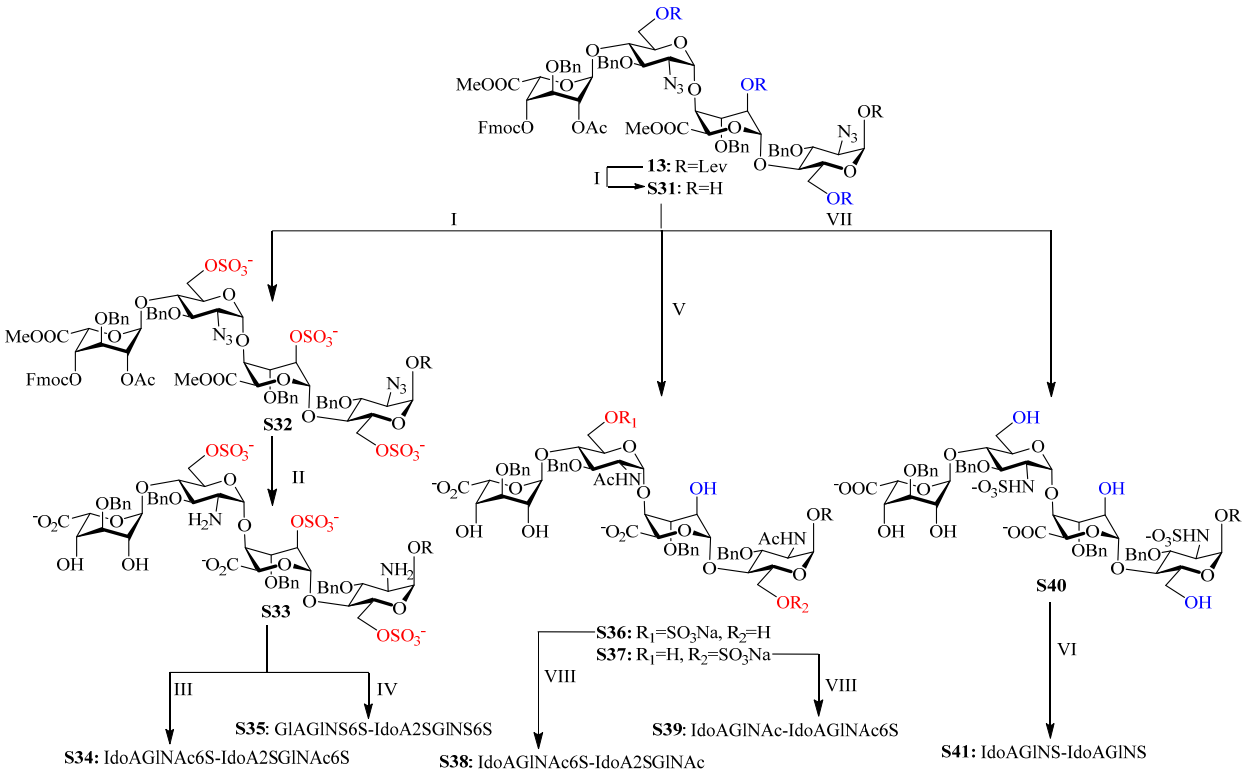
**5-aminopentyl-O-(β-D-glucopyranosyluronate)-(1→4)-*O*-(2-acetamino-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(2-*O*-sulfate-β-D-glucopyranosyluronate)-(1→4)-*O*-2-acetamino-2-deoxy-α-D-glucopyranoside (S29)**

Tetrasaccharide **S28** (15.0 mg, 0.009 mmol) was subjected to the N-acetylation and global hydrogenation to provide tetrasaccharide **S29** as a sodium salt (6.6 mg, 69% for two steps); $^{1}$H NMR (500 MHz, $D_2O$) δ 5.43 (d, *J* = 3.8 Hz, 1H, $H1^{C}$), 4.90 (d, *J* = 2.8 Hz, 1H, $H1^{A}$), 4.8(d, *J* = 7.9 Hz 1H, $H1^{B}$), 4.58 (dd, *J* = 11.2, 3.1 Hz, 1H, $H6^{A}{}_{a}$), 4.53 (d, *J* = 7.9 Hz, 1H, $H1^{D}$), 4.28 (dd, *J* = 11.2, 2.1 Hz, 1H, $H6^{A}{}_{b}$), 4.13 (t, *J* = 7.8 Hz, 1H, $H2^{B}$), 4.02 (t, *J* = 9.9 Hz, 1H, $H3^{B}$), 3.97 – 3.68 (m, 14H, $H2^{A}$, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H4^{B}$, $H5^{B}$, $H2^{C}$, $H3^{C}$, $H4^{C}$, $H5^{C}$, $H6^{C}$, $H5^{D}$, OC*H*H linker), 3.58 – 3.50 (m, 3H, $H3^{D}$, $H4^{D}$, OCH*H* linker), 3.41 (t, *J* = 6.4 Hz, 1H, $H2^{D}$), 3.04 (t, *J* = 7.6 Hz, 2H, $NCH_2$ linker), 2.08 (s, 3H, COC$H_3$), 2.04 (s, 3H, COC$H_3$), 1.78 – 1.60 (m, 4H, 2*C$H_2$ linker), 1.53 – 1.43 (m, 2H, C$H_2$ linker). $^{3}$C NMR (126 MHz, $D_2O$) δ 102.5, 100.0, 97.0, 96.7, 80.5, 78.5, 76.2, 75.9, 75.4, 75.3, 73.1, 72.0, 70.9, 69.4, 68.7, 68.1, 66.3, 66.2, 59.6, 53.5, 39.8, 28.4, 26.7, 22.7, 22.2. ESI-MS: m/z calcd for C33H52N3Na2O26S $[M\text{-}2Na^{+}]^{2-}$: 984.2361; found, 984.2379.

**5-aminopentyl-O-(β-D-glucopyranosyluronate)-(1→4)-*O*-(2-sulfamino-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(2-*O*-sulfate-β-D-glucopyranosyluronate)-(1→4)-*O*-(2-sulfamino-2-deoxy-α-D-glucopyranoside (S30)**

Tetrasaccharide **S28** (17.0 mg, 0.011 mmol) was subjected to the N-sulfation and global hydrogenation to provide tetrasaccharide **S30** as a sodium salt (9.8 mg, 82% for two steps); $^{1}$H NMR (500 MHz, $D_2O$) δ 5.69 (d, *J* = 3.7 Hz, 1H, $H1^{C}$), 5.17 (d, *J* = 3.6 Hz, 1H, $H1^{A}$), 4.73 (d, *J* = 7.8 Hz, 1H, $H1^{B}$), 4.54 (d, *J* = 7.9 Hz, 1H, $H1^{D}$), 4.15 (dd, *J* = 7.8 Hz, 1H, $H2^{B}$), 4.05 (t, *J* = 6.3 Hz, 1H, $H3^{B}$), 3.99 – 3.64 (m, 14H, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H6^{A}$, $H4^{B}$, $H5^{B}$, $H3^{C}$, $H4^{C}$, $H5^{C}$, $H6^{C}$, $H5^{D}$, OCH*H* linker), 3.61 – 3.52 (m, 3H, $H3^{D}$, $H4^{D}$, OC*H*H linker), 3.45 – 3.37 (t, *J* = 6.4 Hz, 1H, $H2^{D}$),

3.28 (m, 2H, H2$^{A}$, H2$^{C}$), 3.05 (t, *J* = 7.4 Hz, 2H, NC*H*$_2$ linker), 1.79 – 1.63 (m, 4H, 2*C*H*$_2$ linker), 1.58 – 1.45 (m, 2H, C*H*$_2$ linker). $^{13}$C NMR (126 MHz, D$_2$O) δ 103.0, 101.2, 97.9, 97.6, 82.5, 80.4, 80.0, 78.4, 77.2, 76.2, 75.9, 75.7, 73.5, 71.9, 70.9, 70.8, 70.1, 67.6, 60.1, 57.9, 39.7, 27.8, 26.9, 22.5. ESI-MS: m/z calcd for C29H46N3Na3O30S3 [M-2Na$^{+}$]$^{2-}$: 540.5516; found, 540.5529.




Scheme 3.5 Diversifation of IdoAGlNIdoAGlN (I) Py.SO$_3$, DMF, RT, 76%; (II) i) LiOH, H$_2$O$_2$, THF, 4h, RT, then 4M NaOH, MeOH, RT, 12h; ii) PMe$_3$, THF, MeOH, 0.1M NaOH, 81%; (III) i) Py.SO$_3$, MeOH, Et$_3$N, 0.1M NaOH; ii) Pd/C, H$_2$, MeOH, H$_2$O; iii) Pd(OH)$_2$/C, H$_2$, H$_2$O, 58%; (IV) i) Ac$_2$O, MeOH, Et$_3$N; ii) Pd/C, H$_2$, MeOH, H$_2$O; iii) Pd(OH)$_2$/C, H$_2$, H$_2$O (**S34**, 79%; **S38**, 86%); (V) i) LiOH, H$_2$O$_2$, THF, 4h, RT, then 4M NaOH, MeOH, RT, 12h; ii) PMe$_3$, THF, MeOH, 0.1M NaOH; iii) Py.SO$_3$, MeOH, Et$_3$N, 0.1M NaOH, 73%; (VI) i) Pd/C, H$_2$, MeOH, H$_2$O; ii) Pd(OH)$_2$/C, H$_2$, H$_2$O, 86%; (VII) i) Py.SO$_3$, DMF, RT; ii) LiOH, H$_2$O$_2$, THF, 4h, RT, then 4M

NaOH, MeOH, RT, 12h; iii) PMe3, THF, MeOH, 0.1M NaOH, 81%; iv) $Ac_2O$, MeOH, $Et_3N$ (48, 6%; 49, 14%); (VIII) i) Pd/C, $H_2$, MeOH, $H_2O$; ii) $Pd(OH)_2$/C, $H_2$, $H_2O$ (**S35**, 81%; **S39**, 80%)

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-O-[(3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-azido-3-O-benzyl-6-O-sulfate-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(2-O-sulfate-3-O-benzyl-α-L-idopyranosyluronate)]-(1→4)-O-(2-azido-3-O-benzy-6-O-sulfonate-2-deoxy-α-D-glucopyranoside (S32)**

Tetrasaccharide **S31** (200 mg, 0.117 mmol) in anhydrous DMF (0.8 mL) was subjected to O-sulfation and saponification according to general procedure give **S32** (155.0 mg, 76%); $^1$H NMR (600 MHz, $CD_3OD$) δ 7.51 – 6.93 (m, 30H, CH Aromatics), 5.33 (s, 1H, $H1^B$), 5.12 – 5.02 (m, 3H, C*H*$_2$Ph, $H1^D$), 4.98 (d, *J* = 3.3 Hz, 1H, $H1^C$), 4.91 (d, *J* = 10.9 Hz, 1H, C*H* of C*H*$_2$Ph), 4.85 – 4.27 (m, 15H, 11H of C*H*$_2$Ph, $H1^A$, $H2^B$, $H5^B$, $H5^D$), 4.26 – 3.06 (m, 49H, $H2^A$, $H3^A$, $H4^A$, $H5^A$, $H6^A$, $H3^B$, $H4^B$, $H2^C$, $H3^C$, $H4^C$, $H5^C$, $H6^C$, $H2^D$, $H3^D$, $H4^D$, OC*H*$_2$ linker, NC*H*$_2$ linker), 1.64 – 1.13 (m, 6H, 3*C*H*$_2$ linker).128.6, 128.0, 127.9, 127.7, 127.5, 127.2, 100.7, 98, 97.4, 95.8, 78.6, 78.2, 76.0, 74.8, 73.9, 73.4, 72.8, 72.4, 72.0, 71.9, 71.4, 71.1, 70.8, 70.7, 69.9, 69.8, 69.7, 69.6, 69.3, 69.1, 67.5, 67, 67, 67, 66.2, 65.7, 65.6, 63.6, 63.5, 52.6, 51.6, 50.4, 50, 49.6, 48.9, 47.8, 46.8, 46.2, 28.7, 27.2, 22.9. ESI-MS: m/z calcd for C72H78N7Na3O32S3 $[M-2Na^+]^{2-}$: 858.6779; found, 858.6791.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-O-[(3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-amino-3-O-benzyl-6-O-sulfate-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(2-O-sulfate3-O-benzyl-α-L-idopyranosyluronate)]-(1→4)-O-(2-amino-3-O-benzy-6-O-sulfonate-2-deoxy-α-D-glucopyranoside (S33)**

Tetrasaccharide **S32** (61.5 mg, 0.036 mmol) was subjected to azide reduction according to general procedure to provide tetrasaccharide **S33** as a sodium salt (48.3 mg, 81% for three steps); $^1$H NMR

(600 MHz, Methanol-$d_4$) δ 7.41 – 6.80 (m, 30H, C*H* Aromatics), 5.29 (d, *J* = 3.3 Hz, 1H, $H1^B$), 5.19 – 4.99 (m, 5H, $H1^C$, $H1^D$, 3H of C$H_2$Ph), 4.92 (d, *J* = 12.0 Hz, 1H, C*H* of C$H_2$Ph), 4.84 – 3.01 (m, 96H, $H1^A$, $H3^A$, $H4^A$, $H5^A$, $H6^A$, $H2^B$, $H3^B$, $H4^B$, $H5^B$, $H2^C$, $H3^C$, $H4^C$, $H5^C$, $H6^C$, $H2^D$, $H3^D$, $H4^D$, $H5^D$, OC$H_2$ linker, NC$H_2$ linker, 10C*H* of C$H_2$Ph), 2.80 – 2.61 (m, 1H, $H2^A$), 1.59 – 1.03 (m, 6H, 3*C$H_2$ linker). $^{13}$C NMR (151 MHz, $CD_3OD$) δ 129.0, 127.9, 127.1, 126.3, 99.5, 98.0, 96.2, 90.9, 80.4, 79.0, 76.6, 74.8, 74.6, 74.5, 74.3, 74.2, 74.0, 73.5, 73.0, 72.2, 72.0, 71.9, 71.5, 71.3, 71.2, 71, 70.6, 70.5, 70.0, 69.8, 69.6, 69.5, 69.2, 68.9, 68.6, 68.3, 67.7, 67.6, 67.3, 67, 65.6, 65.5, 64.7, 64.4, 53.8, 53.6, 28.6, 27.2, 22.9. ESI-MS: m/z calcd for $C72H82N3Na3O32S3$ $[M-2Na]^{2-}$ 832.6874; found, 832.6879.

**5-aminopentyl-O-[(α-L-idopyranosyluronate)-(1→4)-O-(2-actylamino-6-O-sulfate-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(2-sulfate-α-L-idopyranosyluronate)]-(1→4)-O-(2-acetylamno-6-O-sulfonate-2-deoxy-α-D-glucopyranoside (S34)**

Tetrasaccharide **S33** (11.0 mg, 0.007 mmol) was subjected to N-acetylation and global debenzylation according to the general procedures to give tetrasaccharide **S34** (6.0 mg, 79% for two steps); $^1$H NMR (500 MHz, $CD_4OD$) δ 5.25 (s, 1H, $H1^B$), 5.16 (d, *J* = 3.6 Hz, 1H, $H1^C$), 4.98 – 4.66 (m, 4H, $H1^A$, $H1^D$, $H5^B$, $H5^D$), 4.50 – 3.44 (m, 7H, $H2^A$, $H3^A$, $H4^A$, $H5^A$, $H6^A$, $H2^B$, $H3^B$, $H4^B$, $H2^C$, $H3^C$, $H4^C$, $H5^C$, $H6^C$, $H2^D$, $H3^D$, $H4^D$, OC$H_2$ linker, NC$H_2$ linker), 2.09 (s, 3H, COC$H_3$), 2.05 (d, *J* = 8.6 Hz, 3H, COC$H_3$), 1.91 – 1.41 (m, 6H, 3*C$H_2$ linker). $^{13C}$ NMR (126 MHz, $CD_3OD$) δ 100.5, 98.0, 92.8, 95.4, 76.8, 76.1, 70.6, 70.4, 69.5, 69.4, 68.8, 68.5, 68.0, 67.7, 66.9, 66.6, 66.1, 65.0, 52.6, 52.1, 26.8, 23.9, 21.1, 20.7, 21.2. ESI-MS: m/z calcd for $C33H50N3Na3O32S3$ $[M-2Na^+]^{2-}$: 582.5622; found, 582.5635.

**5-aminopentyl-O-[(α-L-idopyranosyluronate)-(1→4)-O-(2-sulfamino-6-O-sulfate-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(2-sulfate-α-L-idopyranosyluronate)]-(1→4)-O-(2-sulfamino-6-O-sulfonate-2-deoxy-α-D-glucopyranoside (S35)**

Tetrasaccharide **S33** (24.0 mg, 0.014 mmol) was subjected to N-sulfation and global debenzylation according to the general procedures to give tetrasaccharide **S35** (8.0 mg, 58% for two steps); $^{1}$H NMR (500 MHz, $D_2O$) δ 5.32 (d, *J* = 3.4 Hz, 1H, $H1^{C}$), 5.15 (d, *J* = 3.1 Hz, 1H, $H1^{B}$), 5.03 (d, *J* = 3.6 Hz, 1H, $H1^{A}$), 4.78 (d, *J* = 4.5 Hz, 1H, $H1^{D}$), 4.73 – 4.48 (m, 2H, $H5^{B}$, $H5^{D}$), 4.29 – 3.38 (m, 18H, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H6^{A}$, $H2^{B}$, $H3^{B}$, $H4^{B}$, $H3^{C}$, $H4^{C}$, $H5^{C}$, $H6^{C}$, $H2^{D}$, $H3^{D}$, $H4^{D}$, O*C*$H_2$ linker), 3.20 – 3.15(m, 2H, $H2^{A}$, $H2^{C}$), 2.92 (t, *J* = 7.4 Hz, 2H, N*C*$H_2$ linker), 1.71 – 1.28 (m, 6H, 3*C*$H_2$ linker). $^{13C}$ NMR (126 MHz, $D_2O$) δ 101.5, 98.8, 97.0, 96.4, 81.7, 77.2, 76.2, 75.8, 71.8, 71.0, 70.9, 70.7, 69.7, 69.6, 69.5, 69.1, 68.9, 68.7, 68.0, 67.9, 66.7, 66.1, 57.8, 39.6, 28.0, 26.1, 22.5. ESI-MS: m/z calcd for C29H44N3Na5O36S5 $[M-2Na^{+}]^{2-}$:642.4904; found, 642.4912.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-O-[(3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-acetylamino-3-O-benzyl-6-O-sulfate-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(3-O-benzyl-α-L-idopyranosyluronate)]-(1→4)-O-(2-acetylamino-3-O-benzy-2-deoxy-α-D-glucopyranoside (S36)**

Tetrasaccharide **S31** (40.5 mg, 0.023mmol) was subjected to selective O-sulfation, saponification, de-acetylation, azide reduction and N-acetylation according to general procedure to provide tetrasaccharide **S36** as a sodium salt (2.0 mg, 6% for five steps); $^{1}$H NMR (500 MHz, $CD_3OD$) δ 7.46 – 6.92 (m, 30H, C*H* Aromatics), 5.30 – 5.05 (m, 4H, $H1^{A}$, $H1^{C}$, C$H_2$Ph), 4.85 – 4.29 (m, 14H, $H5^{B}$, $H5^{D}$, $H6^{B}$, 10H of C$H_2$Ph), 4.25 – 3.10 (m, 20H, $H2^{A}$, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H2^{B}$, $H3^{B}$, $H4^{B}$, $H2^{C}$, $H3^{C}$, $H4^{C}$, $H5^{C}$, $H6^{C}$, $H2^{B}$, $H3^{B}$, $H4^{B}$, O*C*$H_2$ linker, N*C*$H_2$ linker), 2.00 – 1.06 (m, 12H, 2*COC$H_3$, 6H of C$H_2$ linker).$^{13C}$ NMR (126 MHz, $CD_3OD$) δ 129.0, 127.7, 127.0, 101.4, 100.3, 97.1, 95.1,

79.0, 75.0, 74.6, 74.3, 73.9, 73.7, 72.0, 71.7, 71.6, 71.2, 71.0, 70.6, 70.1, 70.0, 68.3, 67.4, 67.0, 66.3, 60.7, 53.0, 50.1, 46.9, 29.3, 28.2, 24.6, 21.5, 21.4. ESI-MS: m/z calcd for C72H82N3Na3O32S3 $[M-2Na^+]^{2-}$: 832.6874; found, 832.6882.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-O-[(3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-acetylamino-3-O-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(3-O-benzyl-α-L-idopyranosyluronate)]-(1→4)-O-(2-acetylamino-3-O-benzy-6-O-sulfonate-2-deoxy-α-D-glucopyranoside (S37)**

Tetrasaccharide **S31** (40.5 mg, 0.023mmol) was subjected to selective O-sulfation, saponification, de-acetylation, azide reduction and N-acetylation according to general procedure to provide tetrasaccharide **S37** as a sodium salt (5 mg, 14% for five steps); $^1$H NMR (500 MHz, $CD_3OD$) δ 7.45 – 7.03 (m, 30H, C*H* Aromatics), 5.24 – 5.07 (m, 4H, $H1^A$, $H1^C$, C$H_2$Ph), 4.97 (d, *J* = 12.0 Hz, 1H, C*H* of C$H_2$PH), 4.90 – 4.40 (m, 11H, $H5^B$, $H5^D$, 9*H* of C$H_2$Ph), 4.85– 3.06 (m, 37H, $H2^A$, $H3^A$, $H4^A$, $H5^A$, $H6^A$, $H2^B$, $H3^B$, $H4^B$, $H2^C$, $H3^C$, $H4^C$, $H5^C$, $H2^D$, $H3^D$, $H4^D$, OC$H_2$ linker, NC$H_2$ linker), 1.80 (s, 3H, COOC$H_3$), 1.72 – 1.61 (m, 3H, COOC$H_3$), 1.61 – 0.94 (m, 6H, 3C$H_2$ linker). $^{13C}$ NMR (126 MHz, $CD_3OD$) δ 127.8, 127.5, 127.4, 127.2, 103.0, 98.4, 97.3, 96.1, 79.9, 79.4, 76.5, 74.2, 73.3, 72.7, 72.3, 71.7, 71.5, 70.6, 70.5, 70.2, 68.5, 67.2, 67.1,67.0, 66.9, 66.3, 61.1, 52.8, 50.1, 47.2, 27.9, 27.7, 22.7, 22.2, 21.1. ESI-MS: m/z calcd for C76H88N3Na2O28S $[M-Na^+]^-$: 1568.5076; found, 1568.5079.

**5-aminopentyl-O-[(α-L-idopyranosyluronate)-(1→4)-O-(2-acetylamino-6-O-sulfate-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(α-L-idopyranosyluronate)]-(1→4)-O-(2-acetylamino-2-deoxy-α-D-glucopyranoside (S38)**

Tetrasaccharide **S36** (2.0 mg, 0.003mmol) was subjected to global deprotection according to general procedure to provide tetrasaccharide **S38** as a sodium salt (1.0 mg, 80%); $^1$H NMR (500

MHz, $D_2O$) δ 5.03 (d, *J* = 3.7 Hz, 1H, $H1^C$), 4.84 (d, *J* = 3.4 Hz, 1H, $H1^B$), 4.75 (d, *J* = 3.4 Hz, 1H, $H1^A$), 4.65 (*J* = 3.4 Hz, 1H, $H1^D$), 4.57 (d, *J* = 2.8 Hz, 1H, $H5^B$), 4.39 (d, *J* = 4.8 Hz, 1H, $H5^D$), 4.23 (dd, *J* = 11.4, 2.2 Hz, 1H, $H6^A{}_a$), 4.16 – 4.07 (m, 1H, $H6^A{}_b$), 3.95 – 3.48 (m, 20H, $H2^A$, $H3^A$, $H4^A$, $H5^A$, $H3^B$, $H4^B$, $H2^C$, $H3^C$, $H4^C$, $H5^C$, $H6^C$, $H2^D$, $H3^D$, $H4^D$, OC*H*H linker), 3.44 – 3.31 (m, 2H, $H2^B$, OCH*H* linker), 2.87 (t, *J* = 7.6 Hz, 2H, NC$H_2$ linker), 1.90 (s, 3H, NHCOC$H_3$), 1.89 (s, 3H, NHCOC$H_3$), 1.63 – 1.25 (m, 6H, 3*$CH_2$ linker). $^{13C}$ NMR (126 MHz, $D_2O$) δ 102.1, 101.0, 96.7, 94.2, 78.7, 77.2, 74.1, 72.5, 71.6, 71.5, 71.1, 69.6, 69.1, 67.9, 66.9, 59.5, 53.6, 39.5, 27.8, 26.4, 21.9, 22.3.ESI-MS: m/z calcd for C33H52N3Na2O26S [M-$Na^+$]$^-$: 984.2361; found, 984.2365.

**5-aminopentyl-O-[(α-L-idopyranosyluronate)-(1→4)-O-(2-acetylamino-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(α-L-idopyranosyluronate)]-(1→4)-O-(2-acetylamino-6-O-sulfate-2-deoxy-α-D-glucopyranoside (S39)**

Tetrasaccharide **S37** (5.0 mg, 0.003mmol) was subjected to global deprotection according to general procedure to provide tetrasaccharide **S39** as a sodium salt (2.5 mg, 81%); $^1$H NMR (500 MHz, $D_2O$) δ 5.03 (d, *J* = 3.7 Hz, 1H, $H1^C$), 4.84 (d, *J* = 3.4 Hz, 1H, $H1^B$), 4.75 (d, *J* = 3.4 Hz, 1H, $H1^A$), 4.65 (*J* = 3.4 Hz, 1H, $H1^D$), 4.57 (d, *J* = 2.8 Hz, 1H, $H5^B$), 4.39 (d, *J* = 4.8 Hz, 1H, $H5^D$), 4.23 (dd, *J* = 11.4, 2.2 Hz, 1H, $H6^A{}_a$), 4.16 – 4.07 (m, 1H, $H6^A{}_b$), 3.95 – 3.48 (m, 20H, $H2^A$, $H3^A$, $H4^A$, $H5^A$, $H3^B$, $H4^B$, $H2^C$, $H3^C$, $H4^C$, $H5^C$, $H6^C$, $H2^D$, $H3^D$, $H4^D$, OC*H*H linker), 3.44 – 3.31 (m, 2H, $H2^B$, OCH*H* linker), 2.87 (t, *J* = 7.6 Hz, 2H, NC$H_2$ linker), 1.90 (s, 3H, NHCOC$H_3$), 1.89 (s, 3H, NHCOC$H_3$), 1.63 – 1.25 (m, 6H, 3*C$H_2$ linker). $^{13}$C NMR (126 MHz, $D_2O$) δ 102.1, 101.0, 96.7, 94.2, 78.7, 77.2, 74.1, 72.5, 71.6, 71.5, 71.1, 69.6, 69.1, 67.9, 66.9, 59.5, 53.6, 39.5, 27.8, 26.4, 21.9, 22.3.ESI-MS: m/z calcd for C33H52N3Na2O26S [M-$Na^+$]$^-$: 984.2361; found, 984.2369.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-O-[(3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-sulfamino-3-O-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(3-O-benzyl-α-L-idopyranosyluronate)]-(1→4)-O-(2-sulfamino-3-O-benzy-2-deoxy-α-D-glucopyranoside (S40)**

Tetrasaccharide **S31** (20.5 mg, 0.012 mmol) was subjected to saponification, de-acetylation, azide reductionand N-sulfation according to general procedure to provide tetrasaccharide **S40** as a sodium salt (13.0 mg, 73% for three steps); $^{1}$H NMR (d600 MHz, $CD_3OD$) δ 7.52 – 7.02 (m, 30H, C*H* Aromatics), 5.40 (d, *J* = 3.4 Hz, 1H, $H1^{C}$), 5.20 – 5.10 (m, 5H, $H1^{A}$, $H1^{B}$, $H1^{D}$, C$H_2$Ph), 5.02 (d, *J* = 10.6 Hz, 1H, C*H* of C$H_2$Ph), 4.90 – 4.45 (m, 11H, $H5^{B}$, $H5^{D}$, 9H of C$H_2$Ph), 4.33 (t, *J* = 2.3 Hz, 1H, $H4^{B}$), 4.16 – 3.13 (m, 31H, $H2^{A}$, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H6^{A}$, $H2^{B}$, $H3^{B}$, $H2^{C}$, $H3^{C}$, $H4^{C}$, $H5^{C}$, $H6^{C}$, $H2^{D}$, $H3^{D}$, $H4^{D}$, OC$H_2$ linker, NC$H_2$ linker), 1.73 – 1.10 (m, 6H, 3*C$H_2$ linker). $^{13}$C NMR (151 MHz, $CD_3OD$) δ 131.2, 127.6, 127.4, 122.3, 101.2, 97.8, 97.7, 95.5, 78.3, 76.1, 75.3, 74.6, 74.3, 72.5, 72.5, 72.0, 71.3, 70.2, 70.0, 69.7, 69.5, 69.1, 68.2, 67.9, 67.3, 66.5, 60.7, 57.7, 57.6, 49.4, 47.7, 46.2, 29.7, 28.0, 23.0. ESI-MS: m/z calcd for C72H83N3Na2O29S2 $[M-2Na^{+}]^{2-}$: 783.7180; found, 783.7189.

**5-aminopentyl-O-[(α-L-idopyranosyluronate)-(1→4)-O-(2-sulfamino-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(α-L-idopyranosyluronate)]-(1→4)-O-(2-sulfamino-2-deoxy-α-D-glucopyranoside (S41)**

Tetrasaccharide **S40** (13.0 mg, 0.008mmol) was subjected to global deprotection according to general procedure to provide tetrasaccharide **S41** as a sodium salt (7.0 mg, 86%); $^{1}$H NMR (600 MHz, $D_2O$) δ 5.23 (d, *J* = 3.6 Hz, 1H, $H1^{C}$), 4.99 (d, *J* = 3.7 Hz, 1H, $H1^{A}$), 4.85 (d, *J* = 3.1 Hz, 1H, $H1^{B}$), 4.60 (d, *J* = 3.2 Hz, 1H, $H1^{D}$), 4.56 (d, *J* = 2.6 Hz, 1H, $H5^{B}$), 4.37 (d, *J* = 4.9 Hz, 1H, $H5^{D}$), 3.98 – 3.85 (m, 2H, $H4^{B}$, $H3^{B}$), 3.76 – 3.15 (m, 21H, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H6^{A}$, $H2^{B}$, $H3^{C}$, $H4^{C}$,

H5$^{C}$, H6$^{C}$, H2$^{D}$, H3$^{D}$, H4$^{D}$, OC$H_2$ linker), 3.13 – 3.07 (m, 2H, H2$^{A}$, H2$^{C}$), 2.87 (t, $J$ = 7.5 Hz, 2H, NC$H_2$ linker), 1.67 – 1.22 (m, 6H, 3*C$H_2$ linker). $^{13C}$ NMR (151 MHz, $D_2O$) δ 101.2, 100.9, 96.8, 95.2, 79.8, 77.2, 76.1, 72.5, 71.4, 70.9, 70.8, 69.5, 69.3, 69.2, 68.5, 67.6, 60.2, 59.5, 57.8, 39.2, 27.9, 26.2, 20.9. ESI-MS: m/z calcd for C29H47N3Na2O27S2 [M-2Na$^{+}$]$^{2-}$: 489.5823; found, 489.5829.

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# CHAPTER 4

# A COMBINATORIAL APPROACH FOR THE IDENTIFICATION OF A HIGH AFFINITY HS OCTASACCHARIDE FOR ROBO1[4]

[4] Chengli Zong, Rongrong Huang, Yulun Chiu, Wenyuan Xiao, Xiuru Li, Weigang Lu, Joshua S. Sharp, Eduard Condac, Lianchun Wang, Mayumi Ishahara, Shuo Wang, Annapoorani Ramiah, Kelley W. Moremen, Muchena John Kailemia**,** I. Jonathan Amster, Geert-Jan Boons. To be submitted to Nature Chemistry.

**Abstract**

Identifying protein binding HS remains a challenge due to its complexity as well as the lack of a technology for structural elucidation. Here we have combined enzymatic digestion, affinity column separation, mass spectrometry assisted structure elucidation, chemical synthesis, binding study via SPR to identify a high affinity HS octasaccharide for ROBO1, which has a mixed pattern of N-substitutions. Beside azide, Cbz and benzyl protected amino groups, trichloroacetyl is installed in post glycosylation fashion that can be removed under basic condition followed by N-acetylation and deprotection to give the octasaccharide. High affinity between the synthetic octasccharide and Robo1 is validated by surface plasmon resonance (SPR). Collectively, our study has uncovered a high affinity octasaccharide ligand for Robo1 for the first time, and such strategy have the potential of becoming a general route of identifying other HS sequences involved in many biological and pathological processes.

**Introduction**

Heparan sulfates (HS) are naturally occurring polydisperse linear polysaccharides that are heavily *O*- and *N*-sulfated.[1-6] They are expressed on the cell surface, in the extracellular matrix (ECM) and basement membrane (BM) of virtually all mammalian tissue types where they interact with numerous signaling proteins and ECM components.[7] The interaction between GAGs and proteins is critical for many biological processes including cell–cell and cell–matrix interactions, cell migration and proliferation, growth factor sequestration, chemokine and cytokine activation,[8] microbial recognition[9,10] and tissue morphogenesis during embryonic development.[11] Alteration in HS expression has been associated with disease,[12,13] and for example, significant changes in the composition of proteoglycans occur in the stroma surrounding tumors, which appear to support

tumor growth and invasion. Many pathogens including bacteria, viruses and parasites attack host cells by binding to HS, which is often a decisive factor for infection.[14-16]

Recent studies have revealed the essential role of heparan sulfate during Slite-Robo interaction,[17] which, besides directing axon branching and neuron migration, also regulates non-neuron-related functions, such as muscle precursor cell migration, leukocyte trafficking, development of lung, kidney, heart, and diaphragm, inflammation, tumor metastasis, angiogenesis and hematopoietic stem cell trafficking. While the crystallographic studies[18] and structure-based mutagenesis identified Robo residues involved in heparin binding, the structural specificity of heparin/HS required for their binding to Robo was not clear. To address this issue, surface plasmon resonance (SPR) analysis is carried out by Wang *et al*[19] to study heparin binding to human Robo1 IG1-2, where SPR competition studies between heparin and several chemically modified heparins (with certain sulfation groups being removed) suggests that N-sulfo and 6-O-sulfo groups are essential in heparin-Robo1 interactions. Further exploration of the specific structure was impeded by the heterogeneity of HS and lack of ex vivo tools.

The considerable structural complexity of HS stems from incomplete sulfation and epimerization leading to at least twenty different disaccharide moieties. The overexpression of various sulfatases under certain pathological and physiological conditions that can take off sulfate, adds further complexity.[20] As a more heavily and uniformly modified variant of HS, heparin is commonly used in biochemical assays as protein binding ligand for functional studies due to its high abundance, commercial availability and similar structural property but less complexity compared to HS. However the diverse domain sequences provided by the sparse modification of HS are irreplaceable and essential for examination of structural specificity of HS-protein interactions.[21] Due to its low abundance, ultrasensitive methods such as mass spectrometry are

usually required for HS analysis. It becomes increasingly clear that efficient, accurate structural analysis requires orthogonal analytical approaches to help decipher the information encoded in the oligosaccharide sequences.[21]

Here, we have employed hydrophilic interaction chromatography-high resolution mass spectrometry (HILIC-HRMS) platform to analyze the compositional differences among three octaccharide libraries obtained by enzymatic partial polymerization of heparan sulfate and affinity purification by Robo1 and GFP immobilized resin. The composition of a Robo1 specific binding octasaccharide is identified and the structure is elucidated by sequential permethylation, desulfation and pertrideuteroacetylation followed by on-line separation and structural analysis. By replacing the labile and strongly polar sulfate groups with much more stable and hydrophobic trideuteroacetyl groups, the oligosaccharides can be well separated by reverse-phase capillary HPLC and fragmented by MS/MS without losing information regarding the sites of modification. The structural complexity of HS oligosaccharides increases at an exponential rate as oligosaccharides get larger, manual interpretation of MS/MS spectra and identification of isomeric sequences can be very challenging. Therefore, an in-house developed program, GAG-ID, is also used to facilitate data analysis.[21] The structure of an octasaccharide is proposed with a mixed patterns of N-substitutions. Thus, following our modular approach for the synthesis of HS, beside azide, Cbz and benzyl protected amino groups, trichloroacetyl is installed in post glycosylation fashion which is removed later during the synthesis under basic condition followed by N-acetylation and deprotection to give the octasaccharide. High affinity between the synthetic octasccharide and Robo1 is validated by surface plasmon resonance (SPR). Collectively, our study

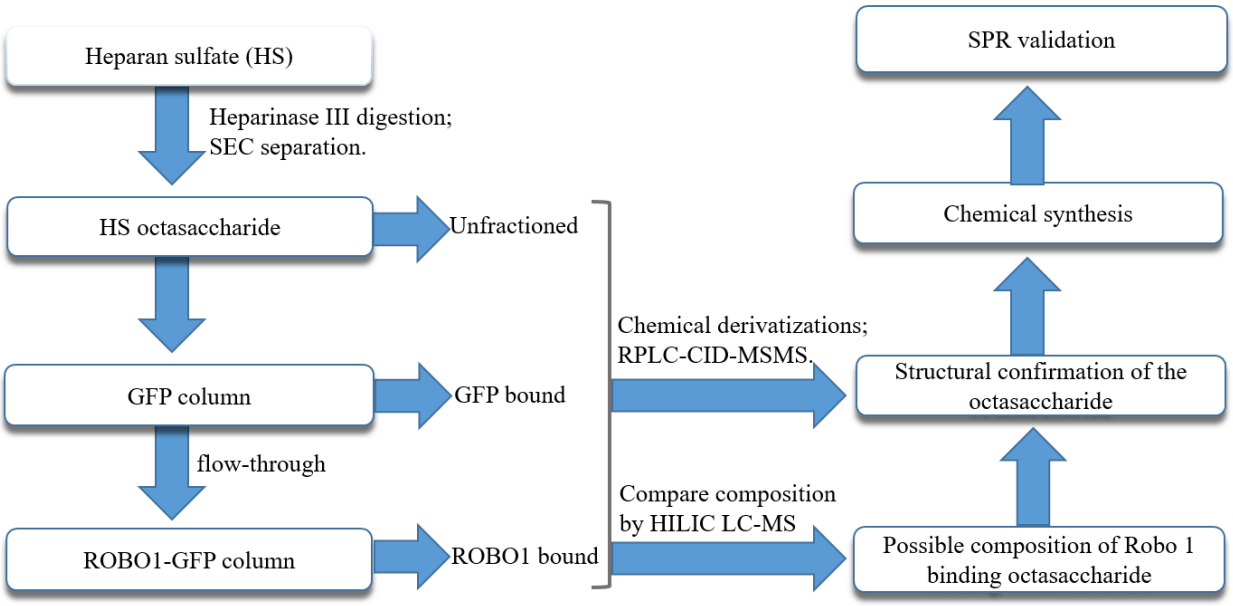



Figure 4.1 Schematic representation of the identification of a high affinity Robo1 binding HS octasaccharide.

has uncovered a high affinity octasaccharide ligand for Robo1 for the first time, and such strategy have the potential of becoming a general route to identify other HS sequences involved in many biological and pathological processes.

**Results and discussion**

Affinity purification of Robo1-bound HS octasaccharides

Green fluorescent protein (GFP) is widely used biochemically as a reporter and is known to bind HS. With Robo1-GFP in hand, HS octasaccharides derived from partial enzymatic digestion by heparanase III were passed through a GFP affinity column in low-salt buffer to

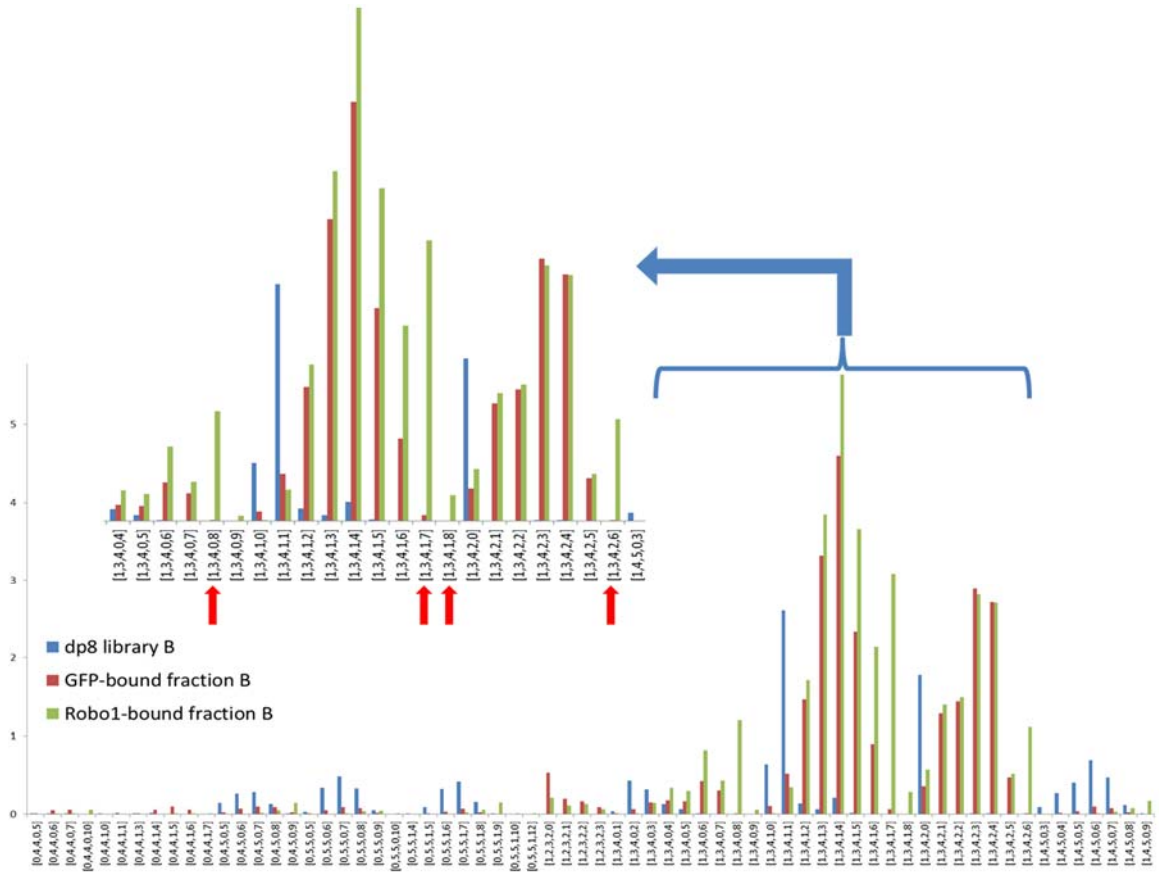


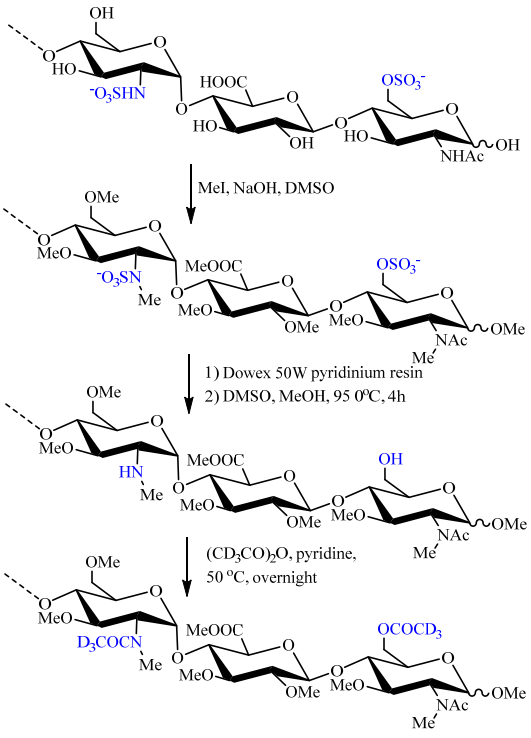

Figure 4.2 Composition analysis of octasaccharide library, GFP-bound fraction and Robo1-bound fraction. Each composition is given as follows: [ΔHexA, HexA, GlcN, Ac, $SO_3$]. Abundances are relative to the overall amount of HS detected in that sample, and are not directly comparable between samples. *Inset:* An enlarged view of the region with the most abundant compositions; the four compositions only observed significantly in the Robo1-bound fraction are indicated by red arrows, with the largest amount of specific binding being detected for the composition [1,3,4,1,7].

capture HS sequences that bound to GFP. Octasaccharides that did not bind the GFP column were then applied to a Robo1-GFP affinity column, where Robo1-bound fractions were eluted using a

high-salt buffer. By comparing compositions of intact octasaccharide library, Robo1-bound fractions and GFP-bound fractions, HS octasaccharides detected exclusively or with substantially higher abundance in Robo1-bound fractions would be considered as potential specific Robo1-bound ligands.

Composition analysis by HILIC-MS

While chemical derivatization strategy was useful for detailed structural sequencing, the sensitivity was sacrificed by performing multiple derivatization steps. Therefore, HILIC-MS analysis of underivatized HS dp8 fractions was first performed to obtain composition information prior to chemical derivatizations, which can also narrow down the possible sequences existed within the sample of interest [22]. The software package GlycReSoft was used to process deconvoluted HILIC-MS data as described previously [23]. Four dp8 compositions, [1,3,4,0,8], [1,3,4,1,7], [1,3,4,1,8] and [1,3,4,2,6] were observed in the Robo1-bound fraction but not the GFP-bound fraction (Figure 4.2), indicating the binding specificity of these structures with Robo1. The most abundant composition found to bind specifically to Robo1 was [1,3,4,1,7], which was barely identified in either the intact dp8 library or the GFP-bound fraction, but was one of the most abundant compositions in the Robo1-bound fraction.




| STRUCUTRE | DB_MZ | Composition |
|---|---|---|
| HexA2S-GlcNAc6S-HexA-GlcNAc-ol | 1057.469 | [0,2,2,1,4] |
| d-HexA2S-GlcNS6S-HexA-GlcNAc6S-ol<br>d-HexA2S-GlcNS6S-HexA2S-GlcNAc-ol<br>d-HexA2S-GlcNAc6S-HexA2S-GlcNS-ol | 1059.476 | [1,1,2,1,4] |
| d-HexA2S-GlcNS6S-HexA-GlcNAc-ol<br>d-HexA2S-GlcNAc6S-HexA-GlcNS-ol | 1028.462 | [1,1,2,1,3] |
| d-HexA-GlcNS3S-HexA2S-GlcNAc-ol<br>d-HexA2S-GlcNS3S-HexA-GlcNAc-ol | 1014.446 | [1,1,2,1,3] |
| d-HexA-GlcNS6S-HexA-GlcNS-ol<br>d-HexA2S-GlcNS-HexA-GlcNS-ol<br>d-HexA-GlcNS-HexA2S-GlcNS-ol | 1000.467 | [1,1,2,0,3] |
| d-HexA-GlcNAc6S-HexA-GlcNS-ol<br>d-HexA-GlcNS-HexA-GlcNAc6S-ol<br>d-HexA-GlcNS-HexA2S-GlcNAc-ol<br>d-HexA2S-GlcNS-HexA-GlcNAc-ol | 997.448 | [1,1,2,1,2] |
| d-HexA-GlcNAc6S-HexA-GlcNAc-ol | 994.429 | [1,1,2,2,1] |
| d-HexA-GlcNS3S-HexA-GlcNS-ol | 986.451 | [1,1,2,0,3] |
| d-HexA-GlcNS3S-HexA-GlcNAc-ol | 983.433 | [1,1,2,1,2] |
| d-HexA-GlcNS-HexA-GlcNS-ol | 969.453 | [1,1,2,0,2] |
| d-HexA-GlcNS-HexA-GlcNAc-ol<br>d-HexA-GlcNAc-HexA-GlcNS-ol | 966.434 | [1,1,2,1,1] |
| d-HexA-GlcNAc-HexA-GlcNAc-ol | 963.416 | [1,1,2,2,0] |
| d-HexA-GlcNAc6S-HexA-GlcN-ol<br>d-HexA2S-GlcNAc-HexA-GlcN-ol | 958.468 | [1,1,2,1,1] |

Table 4.1 Chemical derivatization and Identified tetrasaccharides sequences from the derivatized HS octasaccharides in the Robo1-bound fraction sample.

Chemical derivatization of Robo1-bound HS octasaccharides

The Robo1-bound fraction of the dp8 library was sequentially and quantitatively derivatized by permethylation, desulfation, and peracetylation as previously described [24], and the derivatized Robo1-bound oligosaccharides were analyzed by reverse phase data-dependent LC-MS/MS. No sequences could be identified with confidence in the theoretical dp8 or dp6 databases. Confident identifications of sequences of sulfation and acetylation were obtained when the data were searched against a comprehensive dp4 database and these identifications were verified manually, indicating the existence of several HS tetrasaccharide sequences. Previous studies using oligosaccharide standards have shown that β–elimination at permethylated uronic acid residues during the permethylation step under basic conditions, generating shorter sequences as the by-products [25]. The extent of β–elimination depends on length, derivatization conditions and possibly

sequence. No permethylation conditions have been identified that can prevent such β–elimination without sacrificing permethylation efficiency. Nevertheless, the generated tetrasacchrides represent partial sequences of the original octasaccharides, and therefore are still useful for structural characterization.

Automatic sequencing results of the Robo1-bound fraction also gave twenty four manually verified tetrasaccharide sequences as top hits from the GAG-ID program spread across thirteen compositions. The identified partial sequences and compositions as listed in Table 4.1.

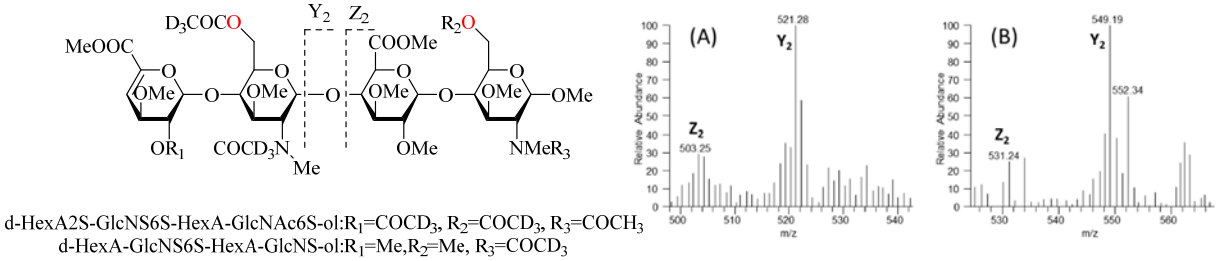


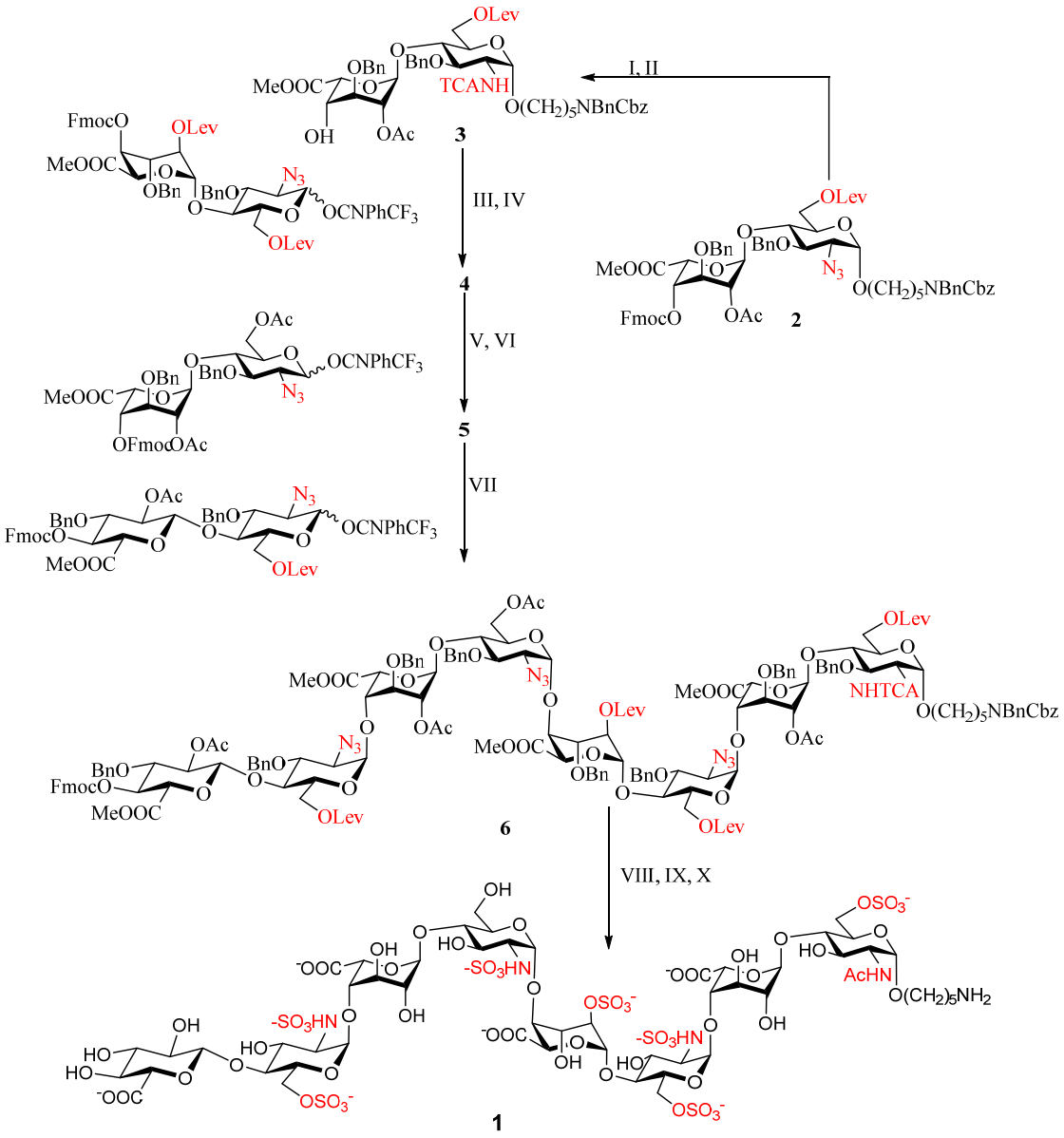

Figure 4.3 Zoom-in MS/MS spectra for parent ion $[M+Na]^+$ with m/z of 1000.467 for dp4 [1,1,2,0,3] (A) and m/z of 1059.476 for dp4 [1,1,2,1,4] (B). The observed $Z_2/Y_2$ ratio was approximately 0.3 for both spectrums, indicating an internal IdoA instead of GlcA.

Although we are not able to directly sequence the complete HS octasaccharides, the identified tetrasaccharide sequences are still useful for structural characterization. Therefore based on the HILIC-MS analysis, we targeted our partial sequences towards combinations that would yield the [1,3,4,1,7] **(**Figure 4.2) composition as candidate sequences to synthesize and test for binding. For the Robo1-bound fraction, the combination of dp4 compositions [1,1,2,1,4] and [1,1,2,0,3] could comprise dp8 composition [1,3,4,1,7]. These partial sequences provide information to narrow down the possible sequences from hundreds to tens.

The tetrasaccharide sequences d-HexA2S-GlcNS6S-HexA-GlcNAc6S-ol and d-HexA-GlcNS6S-HexA-GlcNS-ol were chosen from dp4 composition [1,1,2,1,4] and [1,1,2,0,3] due to their high ion intensity in the LC-MS/MS spectra. The internal HexA residue for the two tetrasaccharides were both manually assigned as IdoA due to the observed $Z_2/Y_2$ ratio of approximately 0.3 (Figure 4.3). According to previous results from tetrasaccharides identified from HS dp4, $Z_2/Y_2$ ratios of ~0.3 of tetrasaccharides with free reducing ends are indicative of IdoA, while $Z_2/Y_2$ ratios of ~0.9 of tetrasaccharides with free reducing ends are indicative of GlcA. Due to unsaturation at the non-reducing end that occurs upon lyase cleavage and β-elimination, no information regarding the epimerization of the non-reducing or the central uronic acid can be determined based on MS/MS data, as that residue is converted to a dHexA upon lyase cleavage or β-elimination. We know that IdoA is more likely to have 2-O-sulfation than GlcA, so the internal HexA2S residue is assigned as probably IdoA2S. With that, we propose a potential candidate as a Robo1-bound HS dp8 sequence: d-HexA-GlcNS6S-IdoA-GlcNS-IdoA2S-GlcNS6S-IdoA-GlcNAc6S (**1**). As heparinase III favors cleavage at GlcA more than IdoA, the non-reducing end dHexA was assigned as probably GlcA for synthesis. The octasaccharide features unique structures for HS by more 6-O-sulfate than 2-O-sulfate, and more iduronic acid than glucuronic acid.

Chemical synthesis of the octasaccharide GlA-GlcNS6S-IdoA-GlcNS-IdoA2S-GlcNS6S-IdoA-GlcNAc6S

Following our modular approach for the synthesis of HS, the octasaccharide can be assembled by four different disaccharide building blocks and an aminopentyl group will be




Scheme 4.1 Octasaccharide Synthesis (I) i) Zn, AcOH, DCM; ii) TCACl, THF, 70% for two steps; (II) $Et_3N$, DCM, 82%; (III) TfOH, DCM, -30°C, 54%; (IV) $Et_3N$, DCM, 79%; (V)TfOH, DCM, -30°C, 56%; (VI) $Et_3N$, DCM, 74%; (VII) TfOH, DCM, -30°C, 53%; (VIII) i) $NH_2NH_2$ AcOH, DCM/MeOH; ii) $SO_3$Pyr., DMF iii) $H_2O_2$, LiOH, NaOH, THF, 48h; iv) $Ac_2O$, $Et_3N$, MeOH, 26% for four steps; (IX) i) $PMe_3$, THF, NaOH aq; ii) $SO_3$Pyr., THF, $Et_3N$, NaOH, 72h, installed on the reducing end for the purpose of future derivation. To differentiate N-substitutions,

tricholoroacetyl group (TCA) will be added to the existing orthogonal protecting groups to mask the acetylated amine. TCA has been widely used for chondroitin sulfate synthesis due to 1) neighboring participating effect to give 1, 2-trans glyosidic bond; 2) multiple available method for deprotection and 3) good performance during glycosylation. Here, with the α-glyosidic bond between glucosamine and the linker, TCA will be installed following a post glycosylation manner. Then the azide will reduced and tricholoracetylated. At the final stage of the synthesis, N-TCA can be transformed to N-acetate without affecting azide, benzyl and Cbz protected amine.

With disaccharide bulding bocks in hand, the azide of fully protected compound 2 can be reduced to amine by zinc and acetic acid in dicholomethane followed by the treatment of TCACl in THF with sodium bicarbonate as a base. The resulting disaccharide can be transformed to acceptor by removal of the Fmoc carbonate with a mild base such as triethyl amine and then, coupled with a disaccharide donor with 1 eq. triflic acid to form tetrasaccharide in a yield of 54% as only the α-anomer. Then, following similar procedures, three additional glycosylations will be repeated to afford the fully protected octasaccharide (Scheme 4.1) with good yield. A critical step is the transformation of TCA to acetyl group. Zinc/copper in acetic acid to reduce TCA to acetyl group was commonly used during chondroitin sulfate synthesis. However for large oligosaccharide, such procedure usually gives low yield probably due to glycosidic bond cleavage. Other reducing method, such as hydrogenation can lead to incomplete transformation. Thus, we decided to first selectively remove all the Lev esters by hydrazine acetate, followed by O-sulfation and saponification which removed the TCA group simultaneously. The resulting octasaccharide was treated with acetic anhydride and triethyl amine in methanol followed by azide reduction by Staudinger reaction using $PMe_3$, N-sulfation and final deprotection to give the final compound **1** (Scheme 4.1). It should be noted that N-sulfation takes much longer time than for tetrasaccharide

synthesis, which is probably caused by hindered amine positions in the helix conformation formed by long oligosaccharide. The final compound was purified by P2 chromatography and characterized by one and two dimentional NMR experiment together with high resolution ESI-MS.

To validate the affinity, binding studies were performed by surface plasmon resonance (SPR). Thus, Robo1 was immobilized on a CM-5 sensor chip surface with NHS-activated carboxylic acid and titration experiments were performed with the synthetic octasaccharide. The titration curves fitted well to a two-state binding mode (Figure 4.5) and a $K_D$ value of 5.8 μM was determined, which is comparable with porcine intestinal heparin (16 kDa, two-stage binding mode, $K_D$ value 1.4 μM). The two-state binding model for the octsaccharide was confirmed by a contact time experiment in which the dissociation phase of binding sensorgrams is analyzed as a function of time and as expected longer contact times resulted in slower rates of dissociation. A model in which weak binding is followed by a tight binding event can rationalize these observations.

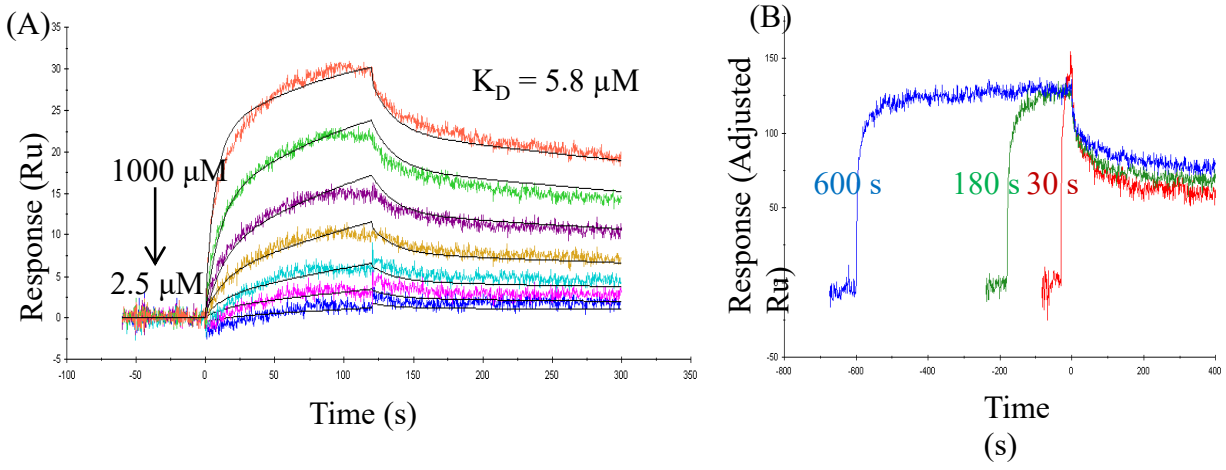



Figure 4.5 SPR study of ROBO1 with octasaccharide **1** (A) SPR sensorgrams of Robo1-octasaccharide interaction. XX mM Robo IG1-2 was immobilized on a CM-5 sensor chip surface via amine coupling with NHS-activated carboxylic acid and titration experiments were performed

with the synthetic compounds with various concentrations of octasaccharide (from top to bottom): 1000, 80, 40, 20 and 10, 2.5 uM, respectively. The black curves are the fitting curves using models from BIAevaluate 4.0.1 (B) contact time experiments using Robo1 and compounds **1**, to confirm a two-state binding model.

To further determine which part of the octasaccharide contributes more to the binding, we have also synthesized all three tetrasaccharide fragments (synthesis is in experimental section). SPR study indicated low binding affinity for each of them demonstrating all sugar units contribute to the binding. Further, the affinity of two other tetrasaccharide that differs by only one 2-O-sulfate (IdoA-GlcNS6S-IdoA-GlcNS6S and IdoA-GlcNS6S-IdoA2S-GlcNS6S) with Robo1 was also evaluated by SPR. Many studies have indicated the interaction between HS and protein for instance FGFs, is in direct correlation with the level of sulfate. However in the case Robo1, we have found, by adding a 2-O-sulfate the affinity has dropped significantly, indicating more contribution of 6-O-sulfates to the high affinity. Our initial NMR titration study has also confirmed the trend, however 2-O-sulfate seems important in inducing the protein conformational change.

**Conclusion**

By interacting with a variety of signaling molecules, HS can mediate a variety of physiological and pathological process. However, due to complexity of HS and sequencing method limitations, identifying the ligand requirements is extremely difficult. Rapid progresses in separation techniques and mass spectrometry (MS) have led to an increasing use of these methodologies in HS structural studies. Many of these studies involves disaccharide compositional analysis that uses only minute amount of sample and has short analysis times, however this method does not reveal the detailed sequence information of the oligosaccharide. Plus, certain regions of heparin/HS have been shown to be resistant to digestion down to

disaccharides, biasing the composition results. There are many other issues related with HS structural analysis such as sulfate loss during tandem mass spectroscopy and low reteintaion on reversed phase chromatography which is the most common chromatography used HPLC. To overcome these problems, better sequencing methods are desperately required. Thus by affinity purification of partially degraded HS fragments such as octasaccharides, the detailed structure is preserved with a much higher chance. Then, the library of octasaccharides is sequentially modified by permethylation, desulfation and pertrideuteroacetylation to remove fragile sulfates and increase the hydrophobicity while the detailed structural information is still preserved. By MS/MS analysis, a unique octasaccharide was unveiled, and synthesized via a modular approach. In HS synthesis, differentiating N-sulfate, N-acetate and amine is still a challenging task. Here we have shown TCA can be employed in a post glycosylation modification as a temporary amino protecting group. It has shown good performance during glycosylation and can be readily removed under basic condition. Of course, several steps can be improved in this procedure, for instance a better methylation method that can prevent β elimination. We can imagine by using our method the structural requirements for many heparan sulfate binding proteins will be disclosed in the near future, which in turn will help us to understand how HS regulate so many biological processes and harness many new therapeutic applications!

**Experimental section**

**General Procedure for glycosylation.** Disaccharide donor (1.2 equiv) and acceptor (1.0 equiv) were coevaporated with toluene (3 × 3 mL), and then dissolved in anhydrous DCM to maintain a concentration of 0.05 M. Freshly activated powdered 4 Å molecular sieves were added, and the mixture was stirred for 30 min at ambient temperature and then cooled to -20 °C. After adding TfOH (1 equiv), the reaction mixture was stirred for 1 hour and then quenched by the addition of

pyridine (5 μL). The mixture was filtered, the filtrate was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography using a gradient of toluene and EtOAc(from 60/40 to 40/60, v/v) to give pure tetrasaccharide.

**General Procedure for Fmoc removal.** The mixture of fully protected oligosaccharide in $Et_3N$ and DCM (20/80, v/v) was stirred for 2 hours until TLC indicated the completion. Then the mixture was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography using a gradient of hexanes and EtOAc (from 60/40 to 40/60, v/v) to give the according acceptor.

**General Procedure for Cleavage of Lev Esters.** Hydrazine acetate (5 equiv per Lev group) was added to a solution of the starting material in a mixture of DCM and MeOH (1/1, v/v, 0.02 M). Stirring was continued until TLC (toluene/EtOAc, 1/1, v/v) indicated disappearance of starting material (∼2 h). The reaction mixture was diluted with DCM (30 mL), washed with water (3 × 25 mL) and brine (25 mL), dried ($MgSO_4$), and filtered. The filtrate was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography using a gradient of hexanes or toluene and EtOAc (from 60/40 to 40/60, v/v) to give pure product.

**General Procedure for O-Sulfation.** Sulfur trioxide pyridine complex (10 equiv per OH) was added to a solution of the starting material in DMF (0.02 M). The mixture was stirred at ambient temperature for 2-4 h until TLC ($CHCl_3$/$CH_3OH$, 90/10, v/v) indicated completion of the reaction. After the addition of triethylamine and $CH_3OH$ (1/1, v/v, 1mL), stirring was continued for 30 min. The mixture was concentrated under reduced pressure, and the residue was applied to a column of Iatrobeads (1.5 g), which was eluted with a gradient of $CH_3OH$ in $CHCl_3$ (from 96/4 to 88/12, v/v). Fractions containing product were concentrated under reduced pressure and the residue was passed

through a column of Biorad 50 × 8$Na^+$ resin (0.6 × 5 cm) using $CH_3OH$ as eluent, to give pure product.

**General Procedure for Saponification of Methyl Esters and De-O-acetylation**. A premixed solution of 30% solution of $H_2O_2$ in water (100 equiv per $CO_2Me$) and 1M LiOH (50 equiv per $CO_2Me$) were added to a solution of the starting material in THF (0.02 M). The reaction mixture was stirred at room temperature for 8 h. Then a 4M solution of NaOH was added until pH=14. The reaction mixture was left stirring for 18 h at room temperature. In the case that the reaction had not gone to completion, stirring was continued at 35 °C for an additional 12 h. The reaction mixture was then brought to pH 8-8.5 by addition of AcOH, and concentrated under reduced pressure. The residue was vortexed with water and applied to a C-18 column, which was eluted with a gradient of $H_2O$ and $CH_3OH$ (from 90/10 to 70/30, v/v). ). Fractions containing product were concentrated under reduced pressure and the residue was passed through a column of Biorad 50 × 8$Na^+$ resin (0.6 × 5 cm) using $CH_3OH$ as eluent, to give pure product.

**General Procedure for Reduction of Azide Group.** 1M solution of $PMe_3$ in THF (8 equiv per azide group) was added to the solution of 0.1M NaOH (10 equiv per azido group) and the starting material in THF (1.0 mL for 0.013 mmol). The reaction mixture was stirred at room temperature for 1 h until TLC ($CHCl_3/CH_3OH/H_2O$=70/30/5, v/v/v, or C-18 plates with $H_2O/CH_3OH$=40/60, v/v) indicated completion of the reaction. The presence of amino groups was confirmed using ninhydrin as visualizing agent. The pH was then adjusted to 8.5 by careful addition of AcOH, and the mixture was concentrated under reduced pressure. The residue was vortexed with water and applied to a C-18 silica gel column, which was eluted with a gradient of $H_2O$ and $CH_3OH$ (from 90/10 to 40/60, v/v). The appropriate fractions were concentrated under reduced pressure, and the

residue was passed through a column Biorad 50 × 8$Na^+$ resin (0.6 × 5 cm) using $CH_3OH$ as eluent, to give pure product.

**General Procedure for N-Acetylation.** Acetic anhydride (10 equiv per $NH_2$) was added to a solution of the starting material in a mixture of anhydrous $CH_3OH$ (0.02 M) and $Et_3N$ (20 equiv per $NH_2$). The progress of the reaction was monitored by TLC (silica gel, $CHCl_3/CH_3OH/H_2O$, 60/30/3, v/v/v; or C-18 silica gel, $H_2O/CH_3OH$, 40/60, v/v). After stirring for 1 h at room temperature, the mixture was coevaporated with toluene in vacuo and the residue passed through a short column of Biorad 50 × 8$Na^+$ resin (0.6 × 5 cm) using a mixture of $CH_3OH$ and $H_2O$ (90/10, v/v) as eluent, and appropriate fractions were concentrated in vacuo. The residue was vortexed with water and applied to a C-18 column, which was eluted with a stepwise gradient of $H_2O$ and $CH_3OH$ (from 90/10 to 40/60, v/v). The appropriate fractions were concentrated under reduced pressure to give *N*-acetylated product.

**General Procedure for N-Sulfation.** $SO_3$·Py (20 equiv per $NH_2$) was added to the solution of starting material in a mixture of $CH_3OH$ (1 mL for 0.006 mmol) and triethylamine (0.3 mL). 0.1 M NaOH was used to adjust the pH to 11. The progress of the reaction was monitored by TLC (silica gel TLC, EtOAc/pyridine/water/$CH_3CO_2H$, 8/5/3/1, v/v/v/v). After stirring for an additional 12 h, the reaction mixture was coevaporated with water and the residue passed through a short column of Biorad 50 × 8$Na^+$ resin (0.6 × 5 cm) with $CH_3OH$ and $H_2O$ (90/10, v/v) as eluent. Appropriate fractions were concentrated under reduced pressure, and the residue was vortexed with water and applied to C-18 silica gel column, which was then eluted with a gradient of $H_2O$ and $CH_3OH$ (from 90/10 to 40/60, v/v). The appropriate fractions were concentrated under reduced pressure to give *N*-sulfated product.

**General Procedure for Global Debenzylation.** Pd/C (10%, 1.5 times the weight of starting material) was added to a solution of the starting material in *tert*-butanol and $H_2O$ (1/1, v/v, 1 mL for 5 mg). The mixture was placed under an atmosphere of hydrogen, and the progress of the reaction was monitored by TLC (silica gel EtOAc/pyridine/water/acetic acid, 3/5/3/1, v/v/v/v). The mixture was filtered through a PTFE syringe filter (0.2 mm, 13 mm) when TLC indicated the disappearance of the starting material. The residue was washed with a mixture of *tert*-butanol and $H_2O$ (1/1, v/v, 2 mL), and the solvents were concentrated under reduced pressure. Then palladium hydroxide on carbon (Degussa type, 20%, 1.5 times the weight of starting material) was added to the solution of the residue in distilled water (1 mL for 5 mg). The reaction mixture was placed under an atmosphere of hydrogen, and stirred for 12 h until TLC (EtOAc/pyridine/water/acetic acid, 4/5/3/1, v/v/v/v) indicated the completion of the reaction. The mixture was filtered through a PTFE syringe filter, and the residue was washed with $H_2O$ (2 mL). The filtrate was freeze dried, the residue was passed through a short column of Biorad 50 × 8$Na^+$ resin (0.6 × 2.5 cm) using $H_2O$ as the eluent, and the appropriate fractions were freeze dried to give the final product.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(methyl-2-*O*-acetyl-3-*O*-benzyl-4-*O*-fluorenylmethoxycarbonyl-β-L-idopyranosyluronate)-(1→4)-*O*-2-azido-3-*O*-benzyl-6-*O*-levulinate-2-deoxy-α-D-glucopyranoside (2)**

The starting disaccharide was made following protocols reported in our JACS paper. $^1$H NMR (600 MHz, $CDCl_3$) δ 7.80 – 7.02 (m, 28H, C*H* Ar), 5.17 (d, *J* = 19.8 Hz, 2H, C$H_2$Ph), 5.10 (s, 1H, $H1^B$), 4.99 – 4.93 (m, 2H, $H4^B$, $H5^B$), 4.89 (d, *J* = 2.8 Hz, 1H, $H2^B$), 4.88 – 4.78 (m, 1H, $H1^A$), 4.79 – 4.59 (m, 4H, 4H of C$H_2$Ph), 4.56 – 4.45 (m, 3H, $H6_a{}^A$, C$H_2$Ph), 4.47 – 4.30 (m, 2H, $CH_2$ of Fmoc), 4.20 - 4.12 (m, 2H, $H6b^A$, CH of Fmoc), 3.97 – 3.15 (m, 9H, H2A, $H3^A$, $H4^A$, $H5^A$, $H3^B$, OC$H_2$ linker, NC$H_2$ linker), 2.89 – 2.51 (m, 4H, 2*C$H_2$ Lev), 2.17 (s, 3H, COC$H_3$), 2.03 (s, 3H,

COC*H*$_3$), 2.00 (s, 3H, COC*H*$_3$), 1.79 – 1.46 (m, 4H, 2*C*H*$_2$ linker), 1.41 – 1.22 (m, 2H, C*H*$_2$ linker). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 128.3, 127.9, 127.8, 127.4, 124.9, 120.0, 97.5, 97.3, 78.2, 74.5, 74.4, 72.8, 72.7, 71.3, 70.1, 69.0, 68.2, 67.2, 67.1, 66.7, 63.4, 62.2, 52.1, 50.4, 46.6, 46.2, 37.8, 29.7, 28.1, 29.0, 28.6, 23.3, 20.9. MALDI-MS: m/z calcd for C69H74N4NaO18, 1269.4896; found, 1269.4879[M+$Na^+$]$^+$.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(methyl-2-*O*-acetyl-3-*O*-benzyl-4-*O*-fluorenylmethoxycarbonyl-β-L-idopyranosyluronate)-(1→4)-*O*-2-azido-3-*O*-benzyl-6-*O*-levulinate-2-deoxy-trichloro acetylamino-α-D-glucopyranoside (3)**

A mixture of disaccharide **2** (530 mg, 0.425 mmol), zinc powder (1.10 g, 1.70 mmol) and acetic acid (49 uL, 0.85 mmol) in 5 mL THF was stirred for 3 hours at room temperature. After completion, the mixture was filtered and the filtrate was concentrated under reduced pressure. Then, trichloroacetyl chloride (72 uL, 0.638 mmol) and NaHCO3 (71 mg, 0.85 mmol) was added to the crude mixture dissolved in 5 mL anhydrous THF. The reaction was finished within 30 minutes followed by the addition of $Et_3N$. Then the mixture was stirred for 1 hour and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography using a gradient of hexanes and EtOAc(from 60/40 to 40/60, v/v) to give pure disaccharide **3** (339 mg, 70% for two steps). $^1$H NMR (800 MHz, $CDCl_3$) δ 7.54 – 7.01 (m, 24H, C*H* Ar), 5.25 – 5.09 (m, 2H, C*H*$_2$Ph), 5.02 (s, 1H, H1$^B$), 4.93 (s, 1H, H3$^B$), 4.85 (s, 1H, H5$^B$), 4.82 – 4.69 (m, 3H, H1$^A$, C*H*$_2$Ph), 4.63 (d, *J* = 11.7 Hz, 1H, C*H*HPh), 4.59 – 4.41 (m, 4H, H6a$^A$, 3H of C*H*$_2$Ph), 4.30 – 4.16 (m, 2H, H2$^A$, H6$_b$$^A$), 4.03 – 3.13 (m, 17H, H3$^A$, H4$^A$, H5$^A$, H4$^B$, OC*H*$_2$ linker, NC*H*$_2$ linker), 2.86 – 2.48 (m, 4H, 2*C*H*$_2$ of Lev), 2.19 (s, 3H, COC*H*$_3$), 2.09 (s, 3H, COC*H*$_3$), 2.04 (s, 2H, COC*H*$_3$), 1.74 – 1.44 (m, 4H, C*H*$_2$ linker), 1.41 –1.15 (m, 2H, C*H*$_2$ linker). $^{13}$C NMR (201 MHz, $CDCl_3$) δ 128.2, 128.1, 127.8, 127.3, 97.9, 96.7, 96.6, 78.8, 78.7, 74.5, 74.4, 74.3, 72.3,

72.2, 69.3, 68.4, 68.2, 68.1, 67.6, 67.3, 67.2, 62.4, 54.6, 52.1, 50.4, 46.9, 46.2, 37.8, 29.9, 28.9, 28.7, 28.0, 27.5, 21.1, 21.0, 28.6, 23.5. MALDI-MS: m/z calcd for C56H65Cl3N2O17Na $[M+Na^+]^+$: 1165.3247; found, 1165.3270.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(methyl-2-*O*-levulinate-3-*O*-benzyl-4-*O*-fluorenylmethoxycarbonyl-β-L-idopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-6-*O*-levulinate-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(methyl-2-*O*-acetyl-3-*O*-benzyl-β-L-idopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzy-6-*O*-acetyl-2-deoxy-trichloro acetylamino-α-D-glucopyranoside (4)**

Disaccharide donor (287.0 mg, 0.247 mmol) was coupled with acceptor **3** (180.0 mg, 0.158 mmol) according to the general procedure for glycosylation to give tetrasaccharide **4** (188.0 mg, 54.0%).$^1$H NMR (600 MHz, $CDCl_3$) δ 7.83 – 7.01 (m, 38H, C*H* Ar), 5.23 – 5.09 (m, 3H, $H1^B$, C$H_2$Ph), 5.05 (d, *J* = 3.1 Hz, 1H, $H1^D$), 4.94 (t, *J* = 3.7 Hz, 1H, $H4^D$), 4.92 – 4.86 (m, 2H, $H4^D$, $H2^B$), 4.84 (d, *J* = 3.3 Hz, 1H, $H5^B$), 4.82 (t, *J* = 3.4 Hz, 1H, $H2^D$), 4.81 – 4.63 (m, 5H, $H1^A$, $H5^D$, 3H of C$H_2$Ph), 4.61 – 4.32(m, 6H, $H6a^A$, C*H*2 of Fmoc, 3H of C*H*2Ph), 4.25 – 4.05 (m, 5H, $H2^A$, $H6b^A$, $H6^C$, C*H* of Fmoc), 4.02 – 3.11 (m, 7H, $H3^A$, $H4^A$, $H5^A$, $H3^B$, $H4^B$, $H2^C$, $H3^C$, $H4^C$, $H5^C$, $H3^D$, OC$H_2$ linker, NC$H_2$ linker), 2.90 – 2.34 (m, 4H, 2*C$H_2$ of Lev), 2.17 (s, 3H, COC$H_3$), 2.15 (s, 3H, COC$H_3$), 2.03 (s, 6H, 2*COC$H_3$), 1.66 – 1.14 (m, 6H, 3*C$H_2$ of linker). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 130.6, 129.6, 127.8, 124.9, 118.7, 98.0, 97.1, 97.0, 96.6, 78.4, 78.1, 74.9, 74.8, 74.5, 73.8, 73.4, 73.3, 72.3, 70.1, 70.0, 69.5, 69.1, 68.9, 68.0, 67.8, 67.2, 63.3, 62.1, 60.3, 54.5, 52.0, 50.4, 47.9, 47.0, 46.0, 37.6, 30.1, 29.8, 29.7, 28.7, 27.8, 23.5, 20.8. MALDI-MS: m/z calcd for C108H118Cl3N5NaO33 $[M+Na^+]^+$: 2140.6672; found, 2140.6690.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(methyl-2-*O*-acetyl-3-*O*-benzyl-4-*O*-fluorenylmethoxycarbonyl-β-L-idopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-6-*O*-acetyl-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(methyl-2-*O*-levulinate-3-*O*-benzyl-β-L-idopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzy-6-*O*-levulinate-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(methyl-2-*O*-acetyl-3-*O*-benzyl-β-L-idopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzy-6-*O*-levulinate-2-deoxy-trichloroacetylamino-α-D-glucopyranoside (5)**

The Fmoc of tetrasaccharide **4** (188 mg, 0.098 mmol) was removed according to general procedure and then coupled with disaccharide donor to afford hexasaccharide **5** (105 mg, 56%). $^{1}$H NMR (600 MHz, $CDCl_3$) δ 7.80 – 7.02 (m, 48H, C*H* Aromatic), 5.24 - 5.18 (m, 3H, $H1^B$, $H1^D$, $H1^F$), 5.18 – 5.10 (m, 2H, C$H_2$ Bn), 5.04(d, $J$ = 3.9 Hz, $H1^E$), 4.94 – 4.53 (m, 21H, $H1^A$, $H2^B$, $H5^B$, $H1^C$, $H2^D$, $H5^D$, $H2^F$, $H5^F$, 6*C$H_2$ Bn), 4.53 – 4.31 (m, 7H, $H6^A$a, $H6^C$a, $H6^E$a, C$H_2$ Bn, C$H_2$ Fmoc), 4.19 (m, 5H, $H2^A$, $H6^A$b, $H6^C$b, $H6^E$b, C*H* Fmoc), 4.02 – 3.58 (m, 15H, $H3^A$, $H4^A$, $H5^A$, $H3^B$, $H4^B$, $H3^C$, $H4^C$, $H5^C$, $H3^D$, $H4^D$, $H3^E$, $H4^E$, $H5^E$, $H3^F$, OC*H*H linker), 3.73 – 3.47 (m, 9H, 3*COOC$H_3$), 3.40 – 3.15 (m, 5H, H2C, H2E, OCH*H* linker, NC$H_2$ linker), 2.88 – 2.25 (m, 12H, C$H_2$ lev), 2.25 – 1.85 (m, 18H, 6*C$H_3$), 1.60 – 1.40 (m, 4H, C$H_2$ linker), 1.35 – 1.16 (m, 2H, C$H_2$ linker).$^{13}$C NMR (151 MHz, $CDCl_3$) δ 128.3, 127.9, 127.6, 125.4, 124.9, 120.0, 97.7, 97.4, 97.2, 96.5, 78.0, 74.9, 74.6, 74.2, 73.5, 72.2, 71.5, 69.8, 69.7, 69.6, 69.4, 69.0, 68.3, 67.9, 67.1, 63.0, 62.0, 54.3, 52.0, 50.3, 46.5, 37.9, 29.6, 29.5, 28.5, 27.6, 23.3, 20.7. MALDI-MS: m/z calcd for C139H153Cl3N8NaO45 $[M+Na^+]^+$: 2781.8893; found, 2781.8907.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-*O*-(methyl-2-*O*-acetyl-3-*O*-benzyl-4-*O*-fluorenylmethoxycarbonyl-β-D-idopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-6-*O*-acetyl-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*--(methyl-2-*O*-acetyl-3-*O*-benzyl-4-*O*-fluorenylmethoxycarbonyl-β-L-idopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-6-*O*-acetyl-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(methyl-2-*O*-levulinate-3-*O*-benzyl-β-L-idopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzy-6-*O*-levulinate-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(methyl-2-*O*-acetyl-3-*O*-benzyl-β-L-idopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzy-6-*O*-levulinate-2-deoxy-trichloroacetylamino-α-D-glucopyranoside (6)**

The Fmoc of hexasaccharide **5** (70 mg, 0.028 mmol) was removed and then coupled with disaccharide donor (100 mg, 0.090 mmol) according to general procedure to afford octasaccharide **6** (50 mg, 53%). $^{1}$H NMR (600 MHz, $CDCl_3$) δ 7.84 – 7.07 (m, 57H, C*H* Aromatics), 5.34 (d, *J* = 5.4 Hz, 1H, $H1^{H}$), 5.28 – 4.57(m, 33H, $H1^{A}$, $H1^{B}$, $H2^{B}$, $H5^{B}$, $H1^{C}$, $H1^{D}$, $H2^{D}$, $H5^{D}$, $H1^{E}$, $H1^{F}$, $H2^{F}$, $H5^{F}$, $H1^{G}$, $H2^{H}$, $H5^{H}$, 18H of C$H_2$Ph), 4.56 – 4.11 (m, 17H, CH and $CH_2$ of Fmoc, $H2^{A}$, $H6^{A}$, $H4^{B}$, $H6^{C}$, $H4^{D}$, $H6^{E}$, $H4^{F}$, $H6^{G}$, $H4^{H}$, $H5^{H}$), 4.07 – 3.07 (m, 35H, $H3^{A}$, $H4^{A}$, $H5^{A,}$ $H3^{B}$, $H2^{C}$, $H3^{C}$, $H4^{C}$, $H5^{C}$, $H3^{D}$, $H2^{E}$, $H3^{E}$, $H4^{E}$, $H5^{E}$, $H3^{F}$, $H2^{G}$, $H3^{G}$, $H4^{G}$, $H5^{G}$, $H3^{H}$, 4*COOC$H_3$, NC$H_2$, OC$H_2$ of linker), 2.97 – 2.29 (m, 16H, 8*C$H_2$ of Lev), 2.26 – 1.85 (m, 27H, 9*COC$H_3$), 1.74 – 0.90 (m, 6H, 3*C$H_2$ linker). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 131, 130.7, 130.1, 128.2, 128.1, 128, 127.6, 126.3, 126.1, 125.8, 125.6, 125.3, 125.1, 120, 100.5, 98, 97.9, 97.8, 97.2, 97.1, 96.4, 79.2, 77.6, 75.4, 75.3, 75.2, 75, 74.7, 74.6, 74.1, 73.5, 72.8, 72.4, 72.3, 71, 70.3, 70.2, 70.2, 70.1, 69.6, 69.3, 69.3, 69.2, 68, 67.9, 67.1, 62.6, 61.9, 61.6, 61.5, 54.3, 54.2, 52.4, 52, 51.8, 50.3, 50.2, 46.8, 46.4, 46.1, 37.8, 37.7, 37.5, 33.4, 31.3, 31, 29.6, 29.4, 29.3, 28.5, 28.2, 27.7, 27.6, 27.1, 27, 23.2, 23.1, 22.3,

22.2, 20.5. MALDI-MS: m/z calcd for C173H192Cl3N11NaO58 $[M+Na^+]^+$: 3479.1376; found, 3479.1395.

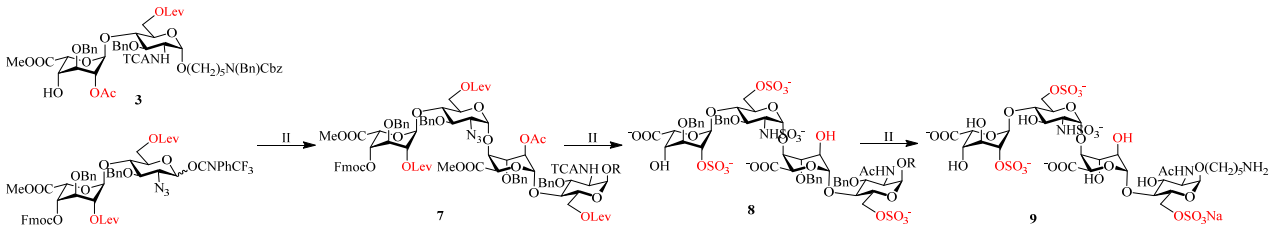
**5-aminopentyl-*O*-(β-D-idopyranosyluronate)-(1→4)-*O*-(2-sulfonate-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(β-L-idopyranosyluronate)-(1→4)-*O*-(2-sulfonate-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(2-*O*-sulfonate-β-L-idopyranosyluronate)-(1→4)-*O*-(2-sulfonate-6-*O*-sulfonate-2-deoxy-α-D-glucopyranoside)-(1→4)-*O*-(β-L-idopyranosyluronate)-(1→4)-*O*-(2-sulfonate-6-*O*-sulfonate-2-deoxy-acetylamino-α-D-glucopyranoside (1)**

Hydrazine acetate (80 mg, 0.087 mmol) was added to a solution of the octasaccharide **6** (50 mg, 0.014 mmol) in a mixture of DCM and MeOH (1/1, v/v, 2 mL). Stirring was continued until TLC (toluene/EtOAc, 1/1, v/v) indicated disappearance of starting material (~2 h). The reaction mixture was diluted with DCM (30 mL), washed with water (3 × 25 mL) and brine (25 mL), dried ($MgSO_4$), and filtered. The filtrate was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography using a gradient of hexanes or toluene and EtOAc (from 60/40 to 40/60, v/v) to give pure product. Sulfur trioxide pyridine complex (33 mg, 0.209 mmol) was added to a solution of the product in DMF (2 mL). The mixture was stirred at ambient temperature for 2-4 h until TLC ($CHCl_3$/$CH_3OH$, 90/10, v/v) indicated completion of the reaction. After the addition of triethylamine and $CH_3OH$ (1/1, v/v, 1mL), stirring was continued for 30 min. The mixture was concentrated under reduced pressure, and the residue was dissolved in THF (2 mL). A premixed solution of 30% solution of $H_2O_2$ in water (370 uL) and 1M LiOH (1.6 mL) were added to the mixture and the resulting mixture was stirred at room temperature for 8 h. Then a 4M solution of NaOH was added until pH=14. The reaction mixture was left stirring for 18 h at room temperature. Then, pH of the reaction mixture was then brought to 8-8.5 by addition of AcOH, and concentrated under reduced pressure. The residue was vortexed with water and applied to a C-

18 column, which was eluted with water followed by methanol. The methanol fraction was concentrated under reduced pressure and the residue was subjected with acetic anhydride (5 uL, 0.0195 mmol) in anhydrous $CH_3OH$ (1 mL) and $Et_3N$ (9 uL). The progress of the reaction was monitored by TLC (silica gel, $CHCl_3/CH_3OH/H_2O$, 60/30/3, v/v/v; or C-18 silica gel, $H_2O/CH_3OH$, 40/60, v/v). After stirring for 1 h at room temperature, the mixture was coevaporated with toluene in vacuo and the residue passed through a short column of Biorad 50 × $8Na^+$ resin (0.6 × 5 cm) using a mixture of $CH_3OH$ and $H_2O$ (90/10, v/v) as eluent, and appropriate fractions were concentrated in vacuo. The residue was vortexed with water and applied to a C-18 column, which was first flushed with water followed by methanol. The methanol fraction was concentrated under reduced pressure to give *N*-acetylated product (11 mg, 26% for four steps).

1M solution of $PMe_3$ in THF (100 uL) was added to the solution of 0.1M NaOH (300 uL) and the starting material in THF (0.5 mL). The reaction mixture was stirred at room temperature for 1 h until TLC ($CHCl_3/CH_3OH/H_2O$=70/30/5, v/v/v, or C-18 plates with $H_2O/CH_3OH$=40/60, v/v) indicated completion of the reaction. The presence of amino groups was confirmed using ninhydrin as visualizing agent. The pH was then adjusted to 8.5 by careful addition of AcOH, and the mixture was concentrated under reduced pressure. The residue was vortexed with water and applied to a C-18 silica gel column, which was first flushed with water followed by methanol. The methanol fraction was concentrated under reduced pressure to give azide reduced product. Then $SO_3$·Py (70 mg, 0.44 mmol) was added to the solution of starting material in a mixture of $CH_3OH$ (0.5 mL) and triethylamine (0.3 mL). 0.1 M NaOH was used to adjust the pH to 11. The progress of the reaction was monitored by TLC (silica gel TLC, EtOAc/pyridine/water/$CH_3CO_2H$, 8/5/3/1, v/v/v/v). After stirring for an additional 12 h, the reaction mixture was coevaporated with water and the residue passed through a short column of Biorad 50 × $8Na^+$ resin (0.6 × 5 cm) with $CH_3OH$

and $H_2O$ (90/10, v/v) as eluent. Appropriate fractions were concentrated under reduced pressure, and the residue was vortexed with water and applied to C-18 silica gel column, which was then eluted with a gradient of $H_2O$ and $CH_3OH$ (from 90/10 to 40/60, v/v). The appropriate fractions were concentrated under reduced pressure to give *N*-sulfated product (4.5 mg, 50%). Then $Pd(OH)_2/C$ (10%, 1.5 ) was added to a solution of the starting material in pH 7.0 PBS buffer (2 mL). The mixture was placed under an atmosphere of hydrogen, and the progress of the reaction was monitored by TLC (silica gel EtOAc/pyridine/water/acetic acid, 3/5/3/1, v/v/v/v). After 48 hours TLC indicated completion of the reaction. The mixture was filtered through a PTFE syringe filter, and the residue was washed with $H_2O$ (2 mL). The filtrate was freeze dried, the residue was passed through a short column of Biorad 50 × $8Na^+$ resin (0.6 × 2.5 cm) using $H_2O$ as the eluent, and the appropriate fractions were freeze dried to give the final product **1** (2.1 mg, 68%).$^1$H NMR (600 MHz, $D_2O$) δ 5.25 - 5.22 (each d, each *J* = 3.6 Hz, 3H, $H1^C$, $H1^E$, $H1^G$), 5.06 (d, *J* = 2.9 Hz, 1H, $H1^D$), 4.86 and 4.78 (each d, *J* = 3.1 Hz, 2H, $H1^B$, $H1^F$), 4.72 (d, *J* = 3.6 Hz, 1H, $H1^A$), 4.67 (s, 2H, $H5^B$, $H5^F$), 4.53 (d, *J* = 3.1 Hz, 1H, $H5^D$), 4.44 (d, *J* = 7.9 Hz, 1H, $H1^H$), 4.35 – 4.05 (m, 8H, $H2^D$, $H3^D$, $H6^A$, $H6^C$, $H6^G$), 4.25 - 3.82 (m, 9H, $H5^A$, $H3^B$, $H4^B$, $H5^C$, $H4^D$, $H5^E$, $H4^F$, $H5^G$), 3.78 (dd, *J* = 10.5, 3.6 Hz, 1H, $H2^A$), 3.75 – 3.45 (m, 12H, $H4^A$, $H2^B$, $H3^C$, $H4^C$, $H3^E$,$H4^E$, $H6^E$, $H2^F$,$H3^G$, $H4^G$, OC*H*H Linker), 3.43 – 3.31 (m, 3H, $H3^H$, $H4^H$, OCH*H* Linker), 3.19 (t, *J* = 8.5Hz, 1H, $H2^H$), 3.11 (m, 3H, $H2^C$, $H2^E$, $H2^G$), 2.85 (t, *J* = 7.6 Hz, 2H, NC$H_2$ Linker), 1.88 (s, 3H, C$H_3$CONH), 1.58 – 1.41 (m, 4H, 2*C$H_2$ Linker), 1.35 – 1.25 (m, 2H, C$H_2$ Linker). $^{13}$C NMR (201 MHz, $D_2O$) δ 101.7, 101.5, 99.1, 96.5, 95.1, 78.7, 76.5, 75.8, 75.7, 75.0, 74.1, 72.9, 71.8, 70.9, 69.5, 69.4, 69.2, 69.1, 68.7, 67.9, 67.0, 66.3, 65.8, 59.7, 58.0, 57.8, 57.4, 53.6, 39.4, 28.0, 26.3, 22.3, 21.8. ESI-MS: m/z calcd for $C_{55}H_{90}N_5O_{63}S_7$ $[M-11Na^++10H^+]^-$: 2052.2043; found, 2053.7543.



Scheme 4.2 synthesis of compound 9 I) TMSOTf, DCM, -30ºC, 51%; (II) i) $NH_2NH_2$.AcOH, DCM, MeOH, RT; ii) Py.$SO_3$, DMF, RT, 2h; iii) LiOH, $H_2O_2$, THF, 4h, then 4M NaOH, MeOH, 12h; iv) $PMe_3$, THF, MeOH, 0.1M NaOH, 1h; v) Py.$SO_3$, MeOH, $Et_3N$, 0.1M NaOH, 12h, 46% for six steps; (III) i) Pd/C, $H_2$, MeOH, $H_2O$, 4h; ii) $Pd(OH)_2$/C, $H_2$, $H_2O$, 14h, 80%.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl O-[(methyl-2-O-levulinoyl-3-O-benzyl-4-O-fluorenylmethyloxycarbonyl-α-L-idopyranosyluronate)-(1→4)-O-(2-azido-3-O-benzyl-6-O-levulinoyl-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(methyl-2-O-acetyl-3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-tricholoacetylamino-3-O-benzy-6-O-levulinoyl-2-deoxy-α-D-glucopyranoside (7)**

Glycosylation of donor (123 mg, 0.106mmol) and acceptor **3** (350 mg, 0.088 mmol) was performed according to general procedure to give tetrasaccharide **7** (94 mg, 51%). $^1$H NMR (500 MHz, Chloroform-*d*) δ 7.75 – 7.18 (m, 38H, C*H* Aromatic), 5.22 (d, *J* = 3.3 Hz, 1H, $H1^B$), 5.18 (d, *J* = 22.1 Hz, 2H, C$H_2$Cbz), 5.07 (d, *J* = 2.7 Hz, 1H, $H1^D$), 4.96 (t, *J* = 4.0 Hz, 1H, $H4^D$), 4.92 (t, *J* = 3.9 Hz, 1H, $H2^B$), 4.90 (d, *J* = 3.5 Hz, 1H, $H1^C$), 4.86 (d, *J* = 3.3 Hz, 1H, $H5^D$), 4.85 (d, *J* = 3.3 Hz, 1H, $H2^D$), 4.80 – 4.77 (m, 3H, $H1^A$, $H5^B$, C*H*HBn), 4.75 – 4.69 (m, 5H, C*H*HBn, 2* C$H_2$Bn), 4.58 (d, *J* = 10.7 Hz, 2H, C$H_2$Bn), 4.55 – 4.44 (m, 5H, C$H_2$Bn, C$H_2$Fmoc, $H6^A$), 4.39 (dd, *J* = 10.6, 7.2 Hz, 1H, C*H*HBn), 4.22 – 4.15 (m, 4H, C*H*Fmoc, $H6^C$, $H2^A$), 3.99 (t, *J* = 4.4 Hz, 1H, $H4^B$), 3.97 – 3.85 (m, 4H, $H4^C$, $H3^B$, $H4^A$, $H3^D$), 3.82 – 3.81 (m, 2H, $H5^A$, $H5^C$), 3.79 – 3.57 (m, 3H, $H3^A$, $H3^C$, OC*H*H linker), 3.45 (s, 6H, 2* C$H_3$), 3.41 – 3.16 (m, 4H, $H2^C$, OC*H*H linker, NC$H_2$ Linker), 2.94 – 2.35 (m, 12H, 6* C$H_2$Lev), 2.19 (s, 1H, C$H_3$), 2.17 (s, 1H, C$H_3$), 2.05 (s, 2*

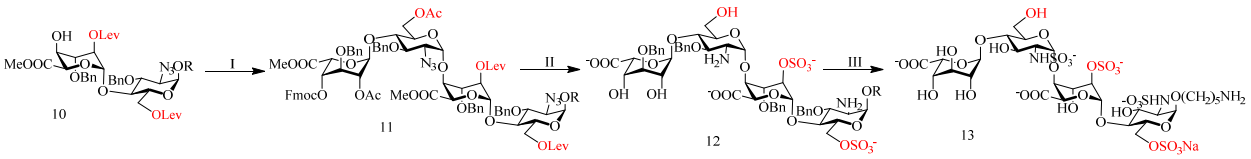
C$H_3$), 1.82 – 1.29 (m, 6H, 3*C$H_2$ linker). $^{13}$C NMR (126 MHz, cdcl$_3$) δ 128.2, 128.1, 127.6, 125.0, 120.1, 98.1, 97.3, 97.2, 96.5, 78.4, 78.1, 75.1, 74.6, 74.5, 73.9, 73.5, 73.3, 73.3, 71.4, 70.1, 69.5, 69.1, 68.9, 68.1, 67.2, 67.2, 63.3, 62.2, 62.1, 61.9, 54.5, 53.2, 51.9, 50.7, 50.4, 46.9, 46.6, 46.2, 37.9, 37.6, 37.5, 30.9, 29.7, 29.5, 28.8, 28.0, 27.8, 27.4, 23.4, 20.8. MALDI-MS: m/z calcd for C99H103Cl3N5NaO31 [M+Na$^+$]$^+$: 1985.5600; found, 1985.5612.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-O-[(2-O-sulfate-3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-azide-3-O-benzyl-6-O-sulfate-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-tricholoacetylamino-3-O-benzy-6-O-fate-2-deoxy-α-D-glucopyranoside (8)**

Tetrasaccharide **7** (94.0 mg, 0.042 mmol) was subjected to de-levulinoylation, sulfation, de-O-acetylation, saponification, N-acetylation according to the general procedure to provide tetrasaccharide **8** as a sodium salt (37.4 mg, 46% for five steps); $^1$H NMR (500 MHz, CD$_3$OD) *δ* 7.49 – 6.95 (m, 30H, C*H* Aromatics), 5.65 (s, 1H, H1$^D$), 5.26 (s, 1H, H1$^B$), 5.20 – 4.84 (m, 19H, H1$^A$, H5B, H1$^C$, H2$^D$, H5$^D$, 14C*H* of C$H_2$Ph), 4.36 – 3.11 (m, 21H, H2$^A$, H3$^A$, H4$^A$, H5$^A$, H6$^A$, H2$^B$, H3$^B$, H4$^B$, H2$^C$, H3$^C$, H4$^C$, H5$^C$, H6$^C$, H3$^D$, H4$^D$, OC*H*2 linker, NC$H_2$ linker), 1.75 – 1.41 (m, 7H, NHCOCH$_3$, 2*C$H_2$ linker), 1.43 – 1.14 (m, 2H, C$H_2$ linker). $^{13}$C NMR (126 MHz, CD$_3$OD) δ 127.8, 127.7, 127.3, 101.3, 98.2, 95.4, 94.5, 75.4, 75.7, 74.7, 74.5, 73.4, 72.3, 71.8, 71.5, 71.4, 70.3, 68.4, 66.8, 67.5, 67.2, 67.0, 66.5, 66.2, 64.4, 53.0, 50.1, 48.0, 28.6, 27.1, 22.0, 20.3. ESI-MS: m/z calcd for C70H74N5Na4O33S3 [M-Na+]-: 1700.3024; found, 1700.3032.

**5-aminopentyl O-[(2-O-sulfate-α-L-idopyranosyluronate)-(1→4)-O-(2-sulfamino-6-O-sulfate-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(α-L-idopyranosyluronate)-(1→4)-O-(2-acetylamino-6-O-fate-2-deoxy-α-D-glucopyranoside (9)**

Tetrasaccharide **8** (37.4 mg, 0.005 mmol) was subjected azide reduction, N-sulfation, global debenzylation according to the general procedure to provide tetrasaccharide **9** as a sodium salt (6.6 mg, 25% for three steps); $^{1}$H NMR (500 MHz, $D_2O$) $\delta$ 5.24 (d, $J$ = 3.7 Hz, 1H, $H1^{C}$), 5.02 (s, 1H, $H1^{D}$), 4.87 (d, $J$ = 3.0 Hz, 1H, $H1^{B}$), 4.74 (d, $J$ = 3.6 Hz, 1H, $H1^{A}$), 4.69 (d, $J$ = 2.4 Hz, 1H, $H5^{D}$), 4.57 (d, $J$ = 2.6 Hz, 1H, $H5^{B}$), 4.34 – 4.05 (m, 5H, $H6^{A}$, $H6^{C}$, $H2^{D}$), 4.05 – 3.89 (m, 4H, $H5^{A}$, $H4^{B}$, $H3^{B}$, $H3^{D}$), 3.91 – 3.83 (m, 2H, $H5^{C}$, $H4^{D}$), 3.81 (dd, $J$ = 10.6, 3.6 Hz, 1H, $H2^{A}$), 3.76 – 3.68 (m, 1H, $H3^{A}$), 3.67 – 3.48 (m, 6H, $H4^{C}$, $H4^{A}$, $H3^{C}$, $H3^{B}$, $H2^{B}$, OC*H*H linker), 3.38 (dt, $J$ = 10.5, 5.9 Hz, 1H, OC*H*H linker), 3.12 (dd, $J$ = 10.4, 3.7 Hz, 1H, $H2^{C}$), 2.87 (t, $J$ = 7.6 Hz, 2H), 1.67 – 1.22 (m, 6H, 3*C$H_2$ linker). $^{13}$C NMR (126 MHz, $D_2O$) δ 101.8, 98.9, 96.5, 95.1, 78.8, 76.4, 74.6, 74.1, 69.4, 69.2, 69.1, 68.8, 67.9, 66.9, 66.5, 57.8, 53.7, 39.5, 28.3, 26.8, 22.5, 21.9. ESI-MS: m/z calcd for $C_{31}H_{47}N_3Na_4O_{34}S_4$ $[M-2Na^{+}]^{2-}$: 614.5213; found, 614.5223.



Scheme 4.3 (I) donor, TMSOTf, DCM, -30ºC, 31%; (II) i) $NH_2NH_2$.AcOH, DCM, MeOH, RT; ii) Py.$SO_3$, DMF, RT, 2h; iii) LiOH, $H_2O_2$, THF, 4h, then 4M NaOH, MeOH, 12h; iv) $PMe_3$, THF, MeOH, 0.1M NaOH, 1h; v) Py.$SO_3$, MeOH, Et3N, 0.1M NaOH, 12h, 42% for six steps; (III) i) Pd/C, $H_2$, MeOH, $H_2O$, 4h; ii) $Pd(OH)_2$/C, $H_2$, $H_2O$, 14h, 56%.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl O-[(methyl-2-O-acetyl-3-O-benzyl-4-O-fluorenylmethyloxycarbonyl-α-L-idopyranosyluronate)-(1→4)-O-(2-azido-3-O-benzyl-6-O-**

**acetyl-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(methyl-2-O-levulinoyl-3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-azido-3-O-benzy-6-O-levulinoyl-2-deoxy-α-D-glucopyranoside (11)**

Glycosylation of donor (140 mg, 0.133 mmol) and acceptor **10** (350 mg, 0.324 mmol) was performed according to general procedure to give tetrasaccharide **11** (80 mg, 31%) $^{1}$H NMR (800 MHz, $CDCl_3$) δ 7.83 – 7.08 (m, 38H, C*H* Aromatics), 5.37 (d, *J* = 3.3 Hz, 1H, $H1^{B}$), 5.33 (s, 1H, $H1^{D}$), 5.23 – 5.15 (m, 4H, $H1^{A}$, $H1^{C}$, C$H_2$ of Bn), 5.05 – 4.30 (m, 19H, $H6^{A}$, $H2^{B}$, $H5^{B}$, $H2^{D}$, $H4^{D}$, $H5^{D}$, C*H* and C$H_2$ of Fmoc, 5*C$H_2$ of Bn), 4.29 – 3.68 (m, 10H, $H3^{A}$, $H4^{A}$, $H5^{A}$, $H3^{B}$, $H4^{B}$, $H3^{C}$, $H4^{C}$, $H5^{C}$, $H3^{D}$, OC*H*H linker), 3.64 – 3.17 (m, 15H, $H2^{A}$, $H2^{C}$, OCH*H* linker, N$H_2$ linker, 2*COOC$H_3$), 2.60 – 2.20 (m, 8H, C$H_2$ of Lev), 2.19 – 1.77 (m,12H, 4*COC$H_3$), 1.74 – 1.07 (m, 6H, 3*C$H_2$ linker). $^{13}$C NMR (201 MHz, $CDCl_3$) δ 128.1, 128.0, 127.3, 124.8, 120.1, 97.6, 92.1, 91.9, 78.2, 77.7, 75.1, 75.0, 74.7, 73.9, 73.1, 72.2, 71.5, 70.6, 70.1, 69.3, 68.4, 67.7, 67.5, 67.2, 66.9, 64.1, 63.8, 62.9, 62.1, 62.0, 52.2, 50.5, 46.5, 46.3, 37.9, 29.7, 28.9, 27.9, 23.3, 21.0, 20.9, 20.8, 20.7. MALDI-MS: m/z calcd for C99H105N7NaO31 $[M+Na^+]^+$: 1910.6753; found, 1910.6759.

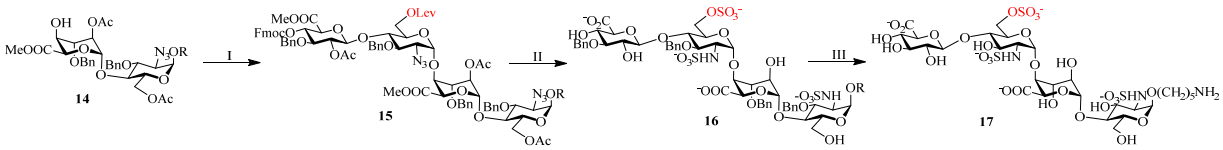
**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl O-[(3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-amino-3-O-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(2-sulfonate-3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-amino-3-O-benzy-6-O-sulfonate-2-deoxy-α-D-glucopyranoside (12)**

Tetrasaccharide **11** (80.0 mg, 0.042 mmol) was subjected to the de-levulinoylation, sulfation, de-O-acetylation, saponification and azide reduction according to the general procedure to provide tetrasaccharide **12** as a sodium salt (22.0 mg, 42% for five steps); $^{1}$H NMR (600 MHz, $CD_3OD$) δ 7.50 – 6.95 (m, 30H, C*H* Aromatics), 5.52 (s, 1H, $H1^{B}$), 5.26 – 5.07 (m,3, $H1^{D}$, C$H_2$ of Bn), 5.06

– 4.53 (m, 12H, H1$^{A}$, H2$^{B}$, H5$^{B}$, H1$^{C}$, 7H of C$H_2$Ph), 4.53 – 4.46 (m, 3H, H5$^{D}$, C$H_2$ of Cbz), 4.40 – 4.13 (m, 7H, H5$^{A}$, H6$^{A}$, H3$^{B}$, H4$^{B}$, H5$^{C}$, C*H* of Bn), 4.05 – 3.45 (m, 9H, H4$^{A}$, H6$^{A}$, H3$^{C}$, H4$^{C}$, H2$^{D}$,H3$^{D}$, H4$^{D}$, OC*H*H linker), 3.40 – 3.15 (m, 4H, H3$^{A}$, OCH*H* linker, NC$H_2$ linker), 2.86 – 2.78 (m, 1H, H2$^{C}$), 2.46 (s, 2H, H2$^{A}$), 1.60 – 1.20 (m, 6H, C$H_2$ linker). $^{13}$C NMR (151 MHz, $CD_3OD$) δ 128.8, 127.8, 127.7, 98.4, 97.8, 97.6, 96.7, 81.1, 79.1, 78.8, 76.2, 75.4, 75.2, 74.1, 72.8, 72.2, 72.1, 71.9, 70.5, 70.4, 70.3, 69.7,69.4, 67.9, 67.4, 67.1, 66.6, 61.4, 55.0, 54.9, 51.4, 46.4, 30.1, 27.4, 24.3 ESI-MS: m/z calcd for C68H75N3Na2O29S2 [M-2Na$^{+}$]$^{2-}$: 753.6867; found, 753.6867.

**5-aminopentyl-O-[(α-L-idopyranosyluronate)-(1→4)-O-(2-sulfamino-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(2-sulfonate-α-L-idopyranosyluronate)-(1→4)-O-(2-sulfamino-6-O-sulfonate-2-deoxy-α-D-glucopyranoside (13)**

Tetrasaccharide **12** (22.0 mg, 0.042 mmol) was subjected to N-sulfation and global debenzylation according to the general procedure to provide tetrasaccharide **13** as a sodium salt (9.5 mg, 56% for two steps);$^{1}$H NMR (600 MHz, Deuterium Oxide) δ 5.26 (d, *J* = 3.7 Hz, 1H, H1$^{C}$), 5.10 (d, *J* = 2.6 Hz, 1H, H1$^{A}$), 4.98 (d, *J* = 3.8 Hz, 1H, H1$^{A}$), 4.63 (s, 1H, H1$^{D}$), 4.58 (d, *J* = 2.7 Hz, 1H, H5$^{B}$), 4.38 (d, *J* = 5.0 Hz, 1H, H5$^{D}$), 4.23 (dd, *J* = 11.3, 2.2 Hz, 1H, H6$^{A}$a), 4.19 – 4.02 (m, 3H, H2$^{B}$, H3$^{B}$, H6Ab), 3.94 (t, *J* = 3.3 Hz, 1H, H4$^{B}$), 3.88 (t, *J* = 7.6 Hz, 1H, H5$^{A}$), 3.79 – 3.46 (m, 11H, H3$^{A}$, H4$^{A}$, H3$^{B}$, H3$^{C}$, H4$^{C}$, H5$^{C}$, H6$^{C}$, H3$^{D}$, H4$^{D}$, OCH*H* linker), 3.44 – 3.40 (m, 1H, OC*H*H linker), 3.34 – 3.31 (t, *J* = 9.9 Hz, 1H, H2$^{D}$), 3.15 – 3.08 (m, 2H, H2$^{A}$, H2$^{C}$), 2.88 (t, *J* = 7.4 Hz, 2H, NC$H_2$ linker), 1.65 – 1.23 (m, 6H, 3*C$H_2$ linker). $^{13}$C NMR (151 MHz, $d_2o$) δ 98.8, 98.2, 96.4, 96.6, 77.3, 75.7, 72.3, 71.6, 71.5, 71.2, 70.8, 69.6, 69.4, 69.0, 68.9, 68.6, 67.8, 67.1, 59.4, 57.8, 39.4, 26.8, 27.9, 22.3. ESI-MS: m/z calcd for C68H75N3Na2O29S2 [M-2Na$^{+}$]$^{2-}$: 591.5210; found, 591.5210.



Scheme 4.4 synthesis of compound 17 (I) donor, TMSOTf, DCM, -30ºC, 44%; (II) i) $NH_2NH_2$.AcOH, DCM, MeOH, RT; ii) Py.$SO_3$, DMF, RT, 2h; iii) LiOH, $H_2O_2$, THF, 4h, then 4M NaOH, MeOH, 12h; iv) $PMe_3$, THF, MeOH, 0.1M NaOH, 1h; v) Py.$SO_3$, MeOH, $Et_3N$, 0.1M NaOH, 12h, 27% for six steps; (III) i) Pd/C, $H_2$, MeOH, $H_2O$, 4h; ii) $Pd(OH)_2$/C, $H_2$, $H_2O$, 14h, 88%.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl O-[(methyl-2-O-acetyl-3-O-benzyl-4-O-fluorenylmethyloxycarbonyl-β-D-glucopyranosyluronate)-(1→4)-O-(2-azido-3-O-benzyl-6-O-levulinoyl-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(methyl-2-O-acetyl-3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-azido-3-O-benzy-6-O-acetyl-2-deoxy-α-D-glucopyranoside (15)**

Glycosylation of donor (150mg, 0.135 mmol) and acceptor **14** (262 mg, 0.270 mmol) was performed according to general procedure to give tetrasaccharide **15** (131.0 mg, 42%) $^1$H NMR (600 MHz, $CDCl_3$) δ 7.83 – 7.05 (m, 38H, C*H* Aromatics), 5.26 (d, *J* = 4.2 Hz, 1H, $H1^B$), 5.22 (d, *J* = 11.2 Hz, 1H, C*H*HPh), 5.18 – 5.15 (d, *J* = 15.0 Hz, 2H, C$H_2$Ph), 5.12 – 5.08 (m, 2H, $H4^D$, $H5^D$), 4.96 – 4.88 (m, 2H, H1C, $H2^B$), 4.86 – 4.77 (m, 2H, $H1^A$, CH*H*Ph), 4.77 – 4.52 (m, 9H, $H5^B$, $H1^D$, $H2^D$, 7H of C$H_2$Ph), 4.49 (d, *J* = 12.3 Hz, 2H, C$H_2$Ph), 4.45 – 4.25 (m, 4H, $H6^A{}_a$, $H5^D$, C$H_2$ of Fmoc,), 4.22 – 4.15 (m, 4H, $H6^A{}_b$, $H6^C$, C*H* of Fmoc), 4.03 – 3.13 (m, 22H, $H2^A$, $H3^A$, $H4^A$, $H5^A$, $H3^B$, $H4^B$, $H2^C$, $H3^C$, $H4^C$, $H5^C$, $H3^D$, NC$H_2$ linker, OC$H_2$ linker), 3.05 – 2.36 (m, 4H, 2*$CH_2$ of Lev), 2.21 (s, 3H, COC$H_3$), 2.10 (s, 3H, COC$H_3$), 2.2 (s, 3H, COC$H_3$), 1.96 (s, 3H, COC$H_3$), 1.71 – 0.98 (m, 6H, 3*C$H_2$ linker). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 132.0, 128.3, 127.8, 127.6, 125.1, 120.0, 100.7, 98.1, 97.8, 97.5, 79.4, 77.6, 76.7, 75.4, 75.2, 74.9, 74.8, 74.6, 74.5,

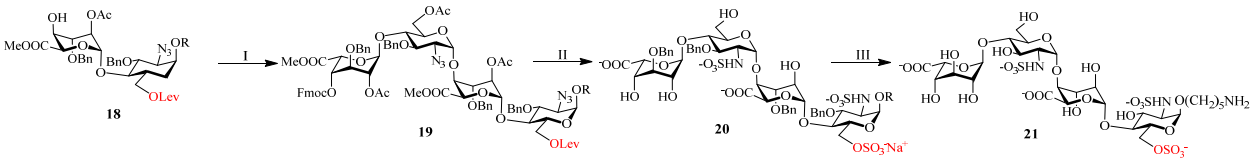
72.7, 72.6, 70.3, 70.1, 69.6, 69.1, 69.0, 68.8, 68.3, 66.8, 62.8, 62.5, 61.7, 60.6, 52.5, 50.3, 46.9, 46.7, 38.0, 31.5, 29.9, 28.3, 28.1, 23.3, 21.8, 21.1.15,111MALDI-MS: m/z calcd for C96H101N7NaO30 $[M+Na^+]^+$:1854.6491; found, 1854.6501.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl O-[(3-O-benzyl-β-D-glucopyranosyluronate)-(1→4)-O-(2-sulfamino-3-O-benzyl-6-O-sulfate-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-2-sulfamino-3-O-benzy-2-deoxy-α-D-glucopyranoside (16)**

Tetrasaccharide **15** (131.0 mg, 0.042 mmol) was subjected to the de-levulinoylation, sulfation, de-O-acetylation, saponification, azide reduction and sulfation according to the general procedure to provide tetrasaccharide **16** as a sodium salt (30.0 mg, 27% for five steps); $^1$H NMR (500 MHz, $CD_3OD$) δ 7.68 – 6.98 (m, 30H, C*H* Aromatic), 5.33 (d, *J* = 3.5 Hz, 1H, $H1^C$), 5.19-5.11 (m, 4H, $H1^A$, $H1^B$, C$H_2$Cbz), 5.08 (d, *J* = 11.4 Hz, 1H, C*H*HBn), 4.95 – 4.89 (m, 2H, C$H_2$Bn), 4.88-4.74 (m, 7H, $H1^D$, $H5^B$, 2*C$H_2$Bn, C*H*HBn), 4.69 (d, *J* = 2.1 Hz, 2H, C$H_2$Bn), 4.54-4.48 (m, 3H, $H6a^C$, C$H_2$Bn), 4.31 – 4.21 (m, 2H, $H6b^C$, $H4^B$), 4.13 (d, *J* = 9.8 Hz, 1H, $H5^D$), 4.08 – 3.98 (m, 2H, $H3^B$, $H4^D$), 3.92 (s, 1H, $H2^B$), 3.87 – 3.71 (m, 4H, $H6^A$, $H3^C$, $H3^D$), 3.69 (d, *J* = 9.7 Hz, 1H, $H4^A$), 3.66 – 3.49 (m, 5H, $H5^A$, $H3^A$, $H4^C$, $H5^C$, OC*H*H linker), 3.47 – 3.33 (m, 4H, $H2^A$, $H2^C$, $H2^D$, OC*H*H linker), 3.23 (m, 2H, NC$H_2$ Linker), 1.67-1.2 (m, 6H, 3*C$H_2$ linker). $^{13}$C NMR (126 MHz, $CD_3OD$) δ 128.9, 128.6, 127.9, 127.6, 127.6, 127.5, 127.2, 127.0, 101.7, 101.3, 97.6, 95.4, 84.3, 79.9, 77.5, 76.3, 76.0, 75.9, 75.3, 74.3, 74.3, 74.2, 73.4, 72.2, 71.9, 71.8, 71.6, 69.8, 69.3, 67.6, 67.1, 66.9, 65.5, 65.4, 60.7, 58.2, 50.14, 47.1, 46.2, 28.8, 27.3. ESI-MS: m/z calcd for C68H74N3Na3O32S3 $[M-Na^+]^-$: 804.6561; found, 804.6572.

**5-aminopentyl O-[(β-D-glucopyranosyluronate)-(1→4)-O-(2-sulfamino-6-O-sulfate-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(α-L-idopyranosyluronate)-(1→4)-O-2-sulfamino-2-deoxy-α-D-glucopyranoside (17)**

Tetrasaccharide **16** (30.0 mg, 0.018mmol) was subjected to global debenzylation according to the general procedure to provide tetrasaccharide **17** as a sodium salt (18.0 mg, 88%); $^{1}$H NMR (500 MHz, $D_2O$) δ 5.27 (d, *J* = 4.0 Hz, 1H, $H1^{C}$), 5.02 (d, *J* = 3.6 Hz, 1H, $H1^{A}$), 4.87 (d, *J* = 3.1 Hz, 1H, $H1^{B}$), 4.61 (d, *J* = 2.4 Hz, 1H, $H5^{B}$), 4.47 (d, *J* = 7.9 Hz, 1H, $H1^{D}$), 4.34 (dd, *J* = 11.2, 2.3 Hz, 1H, $H6a^{C}$), 4.08 (dd, *J* = 11.2, 2.0 Hz, 1H, $H6b^{C}$), 4.03 – 3.90 (m, 3H, $H5^{D}$, $H5^{C}$, $H4^{B}$), 3.78 – 3.51 (m, 10H, $H6^{A}$, $H4^{D}$, $H5^{A}$, $H4^{A}$, $H4^{C}$, $H3^{B}$, $H3^{C}$, $H2^{B}$, OC*H*H linker), 3.49 – 3.34 (m, 3H, $H3^{D}$, $H3^{A}$, OC*H*H linker), 3.23 (t, *J* = 8.5 Hz, 1H, $H2^{D}$), 3.15 (m, 2H, $H2^{A}$, $H2^{C}$), 2.90 (q, *J* = 7.7 Hz, 2H, NC$H_2$ Linker), 1.69 – 1.29 (m, 6H, 3*C$H_2$ linker). $^{13}$C NMR (500 MHz, $D_2O$) δ 101.9, 101.3, 97.0, 95.1, 77.9, 77.2, 75.8, 75.7, 75.1, 74.8, 73.0, 71.9, 70.7, 69.5, 69.2, 68.8, 68.6, 67.8, 67.8, 65.9, 65.8, 60.3, 57.8, 39.5, 26.4, 15.3, 10.1. ESI-MS: m/z calcd for $C29H46N3Na3O30S3$ [M-$2Na^{+}$]$^{2-}$: 540.5516; found, 540.5523.




Scheme 4.5 synthesis of compound 21 (I) donor, TMSOTf, DCM, -30ºC, 41%; (II) i) $NH_2NH_2$.AcOH, DCM, MeOH, RT; ii) Py.$SO_3$, DMF, RT, 2h; iii) LiOH, $H_2O_2$, THF, 4h, then 4M NaOH, MeOH, 12h; iv) $PMe_3$, THF, MeOH, 0.1M NaOH, 1h; v) Py.$SO_3$, MeOH, $Et_3N$, 0.1M NaOH, 12h, 42% for six steps; (III) i) Pd/C, $H_2$, MeOH, $H_2O$, 4h; ii) $Pd(OH)_2$/C, $H_2$, $H_2O$, 14h, 56%.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl-O-[(methyl-2-O-acetyl-3-O-benzyl-4-O-fluorenylmethyloxycarbonyl-α-L-idopyranosyluronate)-(1→4)-O-(2-azido-3-O-benzyl-6-acetyl-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(methyl-2-O-acetyl-3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-azido-3-O-benzy-6-O-levulinoyl-2-deoxy-α-D-glucopyranoside (19)**

Glycosylation of donor (106 mg, 0.101 mmol) and acceptor **18** (94.1 mg, 0.092 mmol) was performed according to general procedure to give tetrasaccharide **19** (75 mg, 44%).$^{1}$H NMR (500 MHz, $CDCl_3$) δ 7.84 – 7.02 (m, 38H, C*H* Aromatics), 5.26 – 5.12 (m, 4H, $H1^B$, $H2^D$, C$H_2$ of Bn), 5.04 – 4.61 (m, 13H, $H1^A$, $H2^B$, $H5^B$, $H1^C$, $H2^D$, $H4^D$, $H5^D$, 3*C$H_2$ of Bn), 4.55 – 4.33 (m, 6H, $H6^A$a, 2*$CH_2$ of Bn, C*H*H of Fmoc), 4.25 – 4.15 (m, 5H, $H6^A$b, $H6^C$, C*H* of Fmoc, CH*H* of Fmoc), 4.03 – 3.60 (m, 10H, $H3^A$, $H4^A$, $H5^A$, $H3^B$, $H4^B$, $H3^C$, $H4^C$, $H5^C$, $H3^D$, OC*H*H linker), 3.52 – 3.335 (m, 7H, OCH*H* linker, COOC$H_3$), 3.37 – 3.19 (m, 4H, $H2^A$, $H2^C$, NC$H_2$ linker), 2.83 – 2.52 (m, 4H, C$H_2$ linker), 2.22 – 1.81 (m, 12H, 4*C$H_3$CO), 1.75 – 1.50 (m, 6H, 3*C$H_2$ linker). $^{13}$C NMR (126 MHz, $CDCl_3$) δ 128.5, 128.3, 128.2, 127.9, 124.7, 120.3, 98.1, 97.7, 97.6, 97.2, 78.1, 78.0, 74.7, 74.5, 73.9, 73.4, 73.1, 71.6, 70.2, 69.5, 68.6, 68.2, 68.1, 67.2, 63.2, 62.2, 62.1, 52.0, 50.3, 46.5, 46.4, 37.7, 30.0, 29.4, 28.2, 27.8, 23.2, 20.7. MALDI-MS: m/z calcd for C167H188Cl3N11O57 $[M+Na^+]^+$: 3364.1216; found, 3364.1230.

**N-(Benzyl)-benzyloxycarbonyl-5-aminopentyl O-[(3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-sulfamino-3-O-benzyl-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(-3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-O-(2-sulfamino-3-O-benzy-6-O-sulfate-2-deoxy-α-D-glucopyranoside (20)**

Tetrasaccharide **19** (75.0 mg, 0.040 mmol) was subjected to the de-levulinoylation, sulfation, de-O-acetylation, saponification, azide reduction and N-sulfation according to the general procedure

to provide tetrasaccharide **20** as a sodium salt (21.0 mg, 32% for five steps); $^{1}$H NMR (500 MHz, $CD_3OD$) δ 7.64 – 6.69 (m, 30H, C*H* Aromatic), 5.40 (d, *J* = 3.4 Hz, 1H, $H1^{C}$), 5.22 (s, 1H, $H1^{B}$), 5.21 – 5.06 (m, 4H, $H1^{D}$, C*H*$_2$Cbz, $H1^{D}$), 5.02 (d, *J* = 10.6 Hz, 1H, C*H*HBn), 4.96 (s, 1H, $H5^{B}$), 4.89 (d, *J* = 11.1 Hz, 1H, C*H*HBn), 4.76 (d, *J* = 10.7 Hz, 1H, C*H*HBn), 4.74 – 4.55 (m, 5H, C*H*HBn, 2*C*H$_2$Bn), 4.50 (s, 3H, C*H*$_2$Bn, $H5^{D}$), 4.33 (s, 1H, $H4^{B}$), 4.26 (s, 1H, $H6a^{A}$), 4.20 – 4.10 (m, 3H, $H6b^{A}$, $H5^{C}$, $H3^{B}$), 4.09 – 4.01 (m, 2H, $H4^{D}$, $H2^{B}$), 3.99 (t, *J* = 9.8 Hz, 1H, $H3^{C}$), 3.94 – 3.79 (m, 3H, $H4^{C}$, $H4^{A}$, $H6_a^{C}$), 3.80 – 3.66 (m, 3H, $H5^{A}$, $H2^{D}$, $H6_b^{C}$), 3.65 – 3.46 (m, 4H, OC*H*H linker, $H3^{D}$, $H3^{A}$, $H2^{C}$), 3.44 – 3.32 (m, 2H, OC*H*H linker, $H2^{A}$), 3.21 ( m, 2H, NC*H*$_2$ Linker), 1.62 – 1.11 (m, 6H, 3*C*H$_2$ linker). $^{13}$C NMR (126 MHz, $CD_3OD$) δ 129.1, 127.5, 101.7, 97.6, 97.4, 95.9, 78.4, 77.0, 76.9, 75.9, 75.3, 74.8, 74.7, 72.9, 72.5, 72.3, 72.2, 71.8, 70.6, 70.5, 69.5, 69.4, 68.6, 67.8, 67.3, 66.3, 65.7, 61.2, 58.3, 57.4, 50.1, 46.7, 46.5, 27.3. ESI-MS: m/z calcd for $C119H143N5O65S7$ $[M-7Na^{+}+5H^{+}]^{2-}$: 2905.6094; found, 1452.8060.

**5-aminopentyl-O-[(α-L-idopyranosyluronate)-(1→4)-O-(2-sulfamino-2-deoxy-α-D-glucopyranoside)-(1→4)-O-(α-L-idopyranosyluronate)-(1→4)-O-(2-sulfamino-3-O-benzy-6-O-sulfate-2-deoxy-α-D-glucopyranoside (21)**

Tetrasaccharide **20** (12.0 mg, 0.007 mmol) was subjected to global debenzylation according to the general procedure to provide tetrasaccharide **21** as a sodium salt (4.0 mg, 50% for five steps); $^{1}$H NMR (800 MHz, $D_2O$) *δ* 5.27 -5.22 (each d, *J* = 3.6 Hz, 3H, $H1^{C}$, $H1^{E}$, $H1^{G}$), 5.06 (d, *J* = 2.9 Hz, 1H, $H1^{D}$), 4.86 (d, *J* = 3.1 Hz, 1H, $H1^{B}$), 4.78 (d, *J* = 3.0 Hz, 1H, $H1^{F}$), 4.59 (d, *J* = 2.6 Hz, 1H, $H1^{A}$), 4.55 – 4.51 (m, 3H, $H5^{B}$, $H5^{D}$, $H5^{F}$), 4.44 (d, *J* = 7.9 Hz, 1H, $H1^{A}$), 4.35 – 4.20 (m, 3H, $H6_a^{A}$, $H6_a^{c}$, $H6_a^{G}$), 4.20 – 4.05 (m, 5H, $H6_b^{A}$, $H6_b^{c}$, $H6_b^{G}$, $H2^{D}$, $H3^{D}$), 4.00 – 3.81 (m, 7H, $H3^{B}$, $H3^{F}$, $H4^{D}$, $H5^{A}$, $H5^{C}$, $H5^{E}$, $H5^{G}$), 3.80 (dd, *J* = 10.5, 3.6 Hz, 1H, $H2^{A}$), 3.79 – 3.45 (m, 15H, $H2^{A}$, $H3^{C}$, $H3^{E}$, $H3^{G}$, $H4^{A}$, $H4^{B}$, $H4^{C}$, $H4^{E}$, $H4^{F}$, $H4^{G}$, $H5^{H}$, $H6^{E}$, $H4^{C}$, OC*H*H linker), 3.42 – 3.35 (m,

3H, $H2^H$, $H3^H$, $H4^H$, OCH*H* linker), 3.21 (t, 1H, *J* = 8.3 Hz, $H2^H$), 3.15 - 3.10 (m, 3H, $H2^C$, $H2^E$, $H2^G$), 2.87 (t, *J* = 7.4 Hz, 2H, NC$H_2$ Linker), 1.64 – 1.28 (m, 6H, 3*C$H_2$ linker). $^{13}$C NMR (201 MHz, $d_2o$) δ 101.8, 100.9, 96.9, 95.3, 78.5, 77.1, 74.5, 72.4, 71.5, 71.0, 70.8, 69.8, 69.6, 68.9, 68.8, 68.7, 68.0, 68.0, 67.9, 67.0, 59.5, 57.7, 39.4, 27.9, 26.3, 22.3. ESI-MS: m/z calcd for C55H89N5O63S7 $[M-7Na^{+}+5H^{+}]^{2-}$: 2051.1970; found, 1025.5971.

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# Chapter 5

# CONCLUSION

Although it is widely accepted that HS is an information-rich polymer, the oligosaccharide structures that mediates particular biological interactions has been defined in only a few cases. This problem stems from the structural complexity of HS, which in turn, arises from a complex biosynthetic pathway. Lack of structure-activity relationship for HS-binding proteins makes it difficult to understand the physiology and pathology of HS at a molecular level and greatly complicates harnessing its therapeutic potential.

In our research, we have employed two different approaches for the identification of HS ligand with various lectins.

The bottom up approach requires a large number of structurally well-defined heparan sulfate. Previously, We have developed a modular approach for the chemical synthesis of HS oligosaccharides whereby a set of disaccharide building blocks, which resemble the different disaccharide motifs found in HS, can repeatedly be used for the assembly of a wide range of sulfated oligosaccharides. At the final stage, the azides will be reduced followed by either sulfation or acetylation generting two targets. Compared to conventional approaches, a modular synthetic strategy makes it possible to rapidly assemble libraries of HS oligosaccharides for structure-activity relationship studies.

In the current study, we have employed an anomeric aminopentyl linker protected by a benzyloxycarbonyl group modified by a perfluorodecyl tag, which enables fast purification of

highly polar intermediates by fluorous solid phase extraction. It also facilitates the repeated glycosylations to drive reactions to completion.

Then, we have successfully diversified the sulfation pattern at a late stage during the synthesis. For instance, beside per-sulfation, sulfate can be selectively installed on the more reactive primary hydroxyl groups in the presence of secondary hydroxyl groups under diluted condition. If both primary OH is available, a mixture of all three sulfation patterns can be obtained. To avoid per-sulfated side product, a much less reactive reagent: sulfurtrioxide trimethylamine can be employed at elevated temperature generating negligible side product demonstrating its superiority for selective sulfation.

On the other hand, the oligosaccharide can be transformed to its pyridinium salt form followed by treatment of BTSA and stirred at 50$^{o}$C in pyridine to selectively remove primary sulfate.

We also explored selective de-2-O-sulfation by lyophilizing the tetrasaccharide with 0.5 M NaOH, which however gave unknown side products and does not affect our study since the desired compound can be obtained via selective 6-O-sulfation.

Besides methods mentioned above, we also employed 3-OST1 to generate 3-O-sulfated tetrasaccharides, which might be important for some biological events. However, the transformation could not come to completion suggesting longer sequences might serve as better substrates. The product can be separated out from the mixture via anion exchange resin with a gradient of ammonium bicarbonate.

With those method in hand, we have applied same strategy with three other tetrasaccharides generating a library of 50 compounds and screened their affinity with various lectins via microarray. The binding of FGF2 and heparin has been well studied suggesting the essential role

of 2-O-sulfate, which has been confirmed by our study. We also discovered importance of the uronic acid configuration. As tetrasaccharide with 2-O-sulfate on glucuronic acid did not show any affinity.

Microarray study with other lectins suggested that by increasing the number of sulfate, the affinity increased accordingly, which is in accordance with the biological roles of highly sulfated heparan sulfate. Although rare and usually present on the chain reducing end, they can function as a reservoir of lectins without discriminating their subtypes, which might help to form lectin gradient and preventing proteolysis. After heparanase cleavage, those lectins will be released and might bind to their low sulfated-real ligand to form functional complex. Although the trend was not consistent, we indeed observe selectivity with intermediate level of sulfated tetrasaccharides. With sequences longer than tetrasaccharide or a more diversified library, we might get a better picture for their binding preferences.

On the other hand, we also explored the top down approach combining enzymatic digestion, affinity column separation, mass spectrometry assisted structure identification, chemical synthesis, binding study via SPR to identify a high affinity ROBO1 binding HS octasaccharide with a Kd value of 3.5 μM.

Heparan sulfate related study is challenging due to its complexity as well as a lack of tool for sequencing. Yet, our study together with many others, seem to indicate that the length, the type and level of sulfation, configuration of uronic acid are all important. Mostly likely, for some proteins, the sulfation level is more important than a specific type of sulfate, for instance, IP10, IL8 and other inflammatory factors that usually can form a gradient of those factors. While for others, for instance antithrombin III have a specific code involving 3-O-sulfate. There are seven subtypes of 3-OST in mammals and constitute the largest group of sulfotransferases involved in

heparan sulfate formation. Each of them seems to prefer a particular type of sequence. Nature will not waste its valuable energy on something un-useful. Thus, there must be many other proteins that can specifically recognize a 3-O-sulfate containing sequence. Currently, with the development of our method, we are at a good stage to synthesize a large number of 3-O-sulfated oligosaccharides for microarray based screening.