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Regioselective modifications of natural polysaccharides

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ABBREVIATIONS

$CH_3C(OCH_3)_3$ = Trimethyl orthoacetate

COSY = Correlation spectroscopy

CS = Chondroitin sulfate

CSA = (+)-camphor-10-sulfonic acid

DCC = Dicyclohexylcarbodiimide

DEA = Diethylamine

DeS = Dermatan sulfate

DIPEA = *N*,*N*-Diisopropylethylamine

DMA = *N*,*N*-Dimethylacetamide

DMF = *N*,*N*-Dimethylformamide

DMI = 1,3-Dimethyl-2-imidazolidinone

DMTr = Dimethoxytrityl

DMTMM = 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride

EDC = *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride

fCS = Fucosylated chondroitin sulfate

GalA = Galacturonic acid

GlcA = Glucuronic acid

GalNAc = *N*-Acetyl-galactosamine

GlcNAc = *N*-Acetyl-glucosamine

HA = Hyaluronic acid

HMBC = Heteronuclear multiple bond correlation

HOBt = 1-hydroxybenzotriazole

HP = Heparin

HS = Heparan sulfate

HSQC-DEPT = Heteronuclear Single Quantum Coherence-Distorsionless Enhancement by Polarization Transfer

HSQC-TOCSY = Heteronuclear Single Quantum Coherence-Total correlation spectroscopy

HyCl = Benzethonium chloride

KS = Keratan sulfate

NHS = *N*-hydroxysuccinimide

NIS = *N*-iodosuccinimide

NOESY = Nuclear Overhauser enhancement spectroscopy

MES = 4-morpholineethanesulfonic acid

MMTr = Monomethoxytrityl

$PhC(OCH_3)_3$ = Trimethyl orthobenzoate

PSS = Propylene glycol alginate sodium sulfate

pyBOP® = (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate

SLS = Static light scattering

TOCSY = Total correlation spectroscopy

TBA = Tetrabutylammonium

TBAF = Tetrabutylammonium fluoride

TBDMS = *tert*-Butyldimethylsilyl

TBDPS = *tert*-Butyldiphenylsilyl

TBTU = *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate

Thr = Threonine

TMSOTf = Trimethylsilyl trifluoromethanesulfonate

Tr = Triphenylmethyl

ABSTRACT

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Polysaccharides are polymeric carbohydrates, usually formed of repeating units (either mono-, or higher oligosaccharides) joined together by glycosidic bonds. Some of these macromolecules are characterized by high natural availability (starch, cellulose, glycogen and chitin among others) and they have also a great biological importance, since they can be a source of energy for animal species. Moreover, they are structural elements of cellular walls and identification sites of cellular surfaces. An important class of polysaccharides is that of glycosaminoglycans animal sourced biomacromolecules that play a pivotal role in several biological processes. CS is included into the family of sulfated GAGs and is involved in the treatment of osteoarthritis and osteoarthrosis. From the structural point of view it is composed of a disaccharide repeating unit containing GlcA and GalNAc linked together through β-(1→3) and β-(1→4) glycosidic bonds, and displaying different sulfation patterns after *in vivo* polymerization. Indeed, depending on the position of sulfate groups, different disaccharide subunits could be described (Figure I).

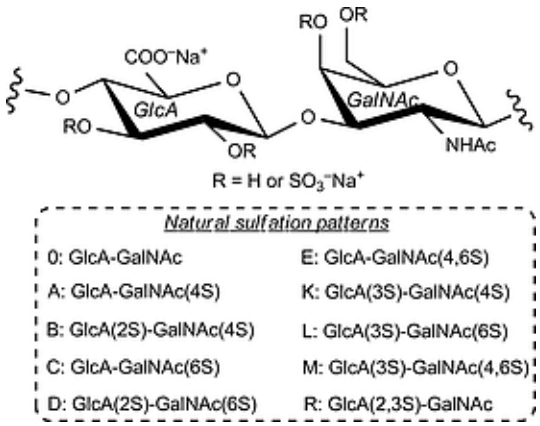

Figure I: Disaccharide subunits in natural CSs

Nonetheless, the low abundance of raw material, the labourious downstream purification and the growing application of this polysaccharide as a drug, led to development of a non-animal derived CS with a well-defined sulfation pattern, starting from *Escherichia coli* O5:K4:H4 sourced unsulfated chondroitin, through the optimization of a suitable sequenece of regioselective steps for its structural modification. This was based on the selective protection of *O*-4,6-GalNAc diol with a cyclic group (beznylidene), followed by acylation of *O*-2,3-GlcA diol on the polysaccharide backbone. By conducting benzylidenation and acetylation reactions *one*- or *two pots*, CSs with different sulfation patterns were obtained. In particular, sulfate groups randomly distributed either at position *O*-4 or at position *O*-6 of GalNAc units (CS-A,C) were obtained through the *two-pots* strategy, whereas the presence of additional sulfate groups was found at position *O*-3 of GlcA units when the protection reactions were conducted in *one-pot* fashion. This difference was ascribed to the formation of interglycosidic acetals during the insertion of benzylidene ring on *O*-4,6-GalNAc diol. These unusual acetals were rather acid-labile and could be not conserved after reaction work-up, thus, at the end of the

semi-synthetic strategy, a chondroitin polysaccharide bearing sulfate groups exclusively on GalNAc units was afforded. Differently, stabilization in alkaline environment of the labile interglycosidic acetals by the *two-pots* strategy and their following oxidative cleavage allowed the semi-synthesis of CS species possessing sulfate groups not only on GalNAc units but also at position *O*-3 of some GlcA ones. It is worth noting that the detailed understanding of the factors influencing finely tailored chemical modifications on microbial sourced chondroitin is rather valuable because it allows the preparation of biologically relevant CSs from non-animal sources and with different, but highly controlled sulfation patterns. Indeed, CS-A,C is employed for several biomedical applications, as well as CSs possessing GlcA units decorated at *O*-3 position with sulfate groups are interesting for their neurite outgrowth promotion in the central nervous system.

To GAGs family belongs also fCS. It is a glycosaminoglycan extracted from sea cucumbers (*Echinodermata*) and composed of a chondroitin sulfate backbone, substituted at position *O*-3 of GlcA units with heavily sulfated L-fucose side branches (Figure II).

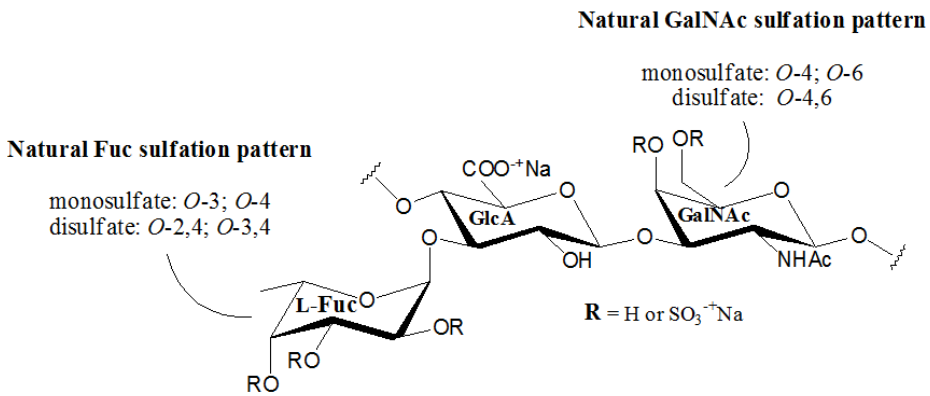

Figure II: Natural fucosylated chondroitin sulfate

fCS shows several biological properties, above all anticoagulant and antithrombotic activities that are tied to the branches of sulfated fucose on CS backbone. As heparin, fCS exerts these two activities by a serpin-dependent mechanism, in which thrombin inhibition is mediated by AT and HC-II. Importantly, and in contrast to heparin, fCS inhibits Xase factor and furthermore the Xa itself, through a serpin-independent mechanism too.

These peculiar properties position fCS to potentially substitute heparin as anticoagulant and antithrombotic agent; indeed, fCS is currently under investigation in clinical trials as a new antithrombotic drug. In order to overcome the serious downsides of using animal-sourced polysaccharides for therapeutic purposes, such as ethical problems, contamination risks and discrepancies in composition, a regioselective modification of a chondroitin polysaccharide, obtained by fed-batch fermentation of *E. coli* O5:K4:H4, was developed, with the final aim to produce a safer and highly controllable fCS-based drug candidate.

Derivatization started by esterification (either methylation or *n*-dodecylation) of carboxylic acid of GlcA subunits, to make chondroitin more soluble in aprotic solvents, then *O*-4,6 diol of GalNAc was protected by introduction of a benzylidene ring. The obtained derivatives were used as polysaccharide acceptors for glycosylation reactions, by coupling with suitable per-*O*-benzylated fucosyl donors under several conditions, trying to achieve a regiochemical and stereochemical control of glycosidic bond formation. Fucosylated products were further modified, obtaining at the end of semi-synthetic route fCS polysaccharides bearing persulfated Fuc branches.

In order to obtain different sulfation patterns on Fuc units, the semi-synthetic strategy was upgraded, with the synthesis of new suitably protected fucosyl donors, for achieving polysaccharides with a even

higher control of regio- and stereoselectivity of Fuc branching and sulfation pattern on the chondroitin backbone. Moreover, modification on polysaccharide backbone afforded a different glycosyl acceptor, useful to further enlarge the library of the semi-synthesized fucosylated chondroitin sulfate and chondroitin sulfates (fC and fCS, respectively) polysaccharides (Figure III) for future detailed structure-activity relationship investigations.

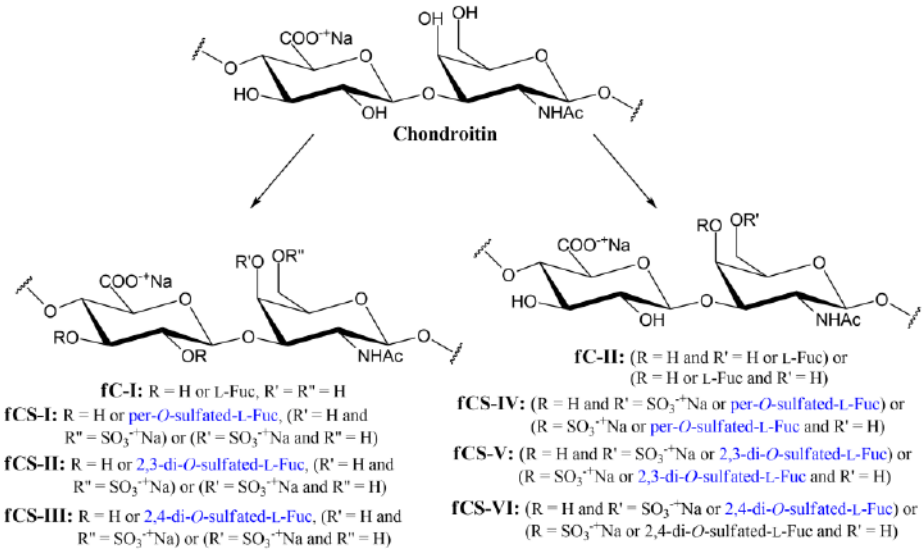

Figure III: Library of regioselectively semi-synthesized fC and fCS polysaccharides

They were preliminarily assayed for anticoagulant activity, displaying an AT-dependent activity against factor Xa in the same range of low molecular mass fCS species obtained by partial depolymerization of natural polysaccharides. For HC-II mediated factor IIa activity, data were very close to heparin for fCSs with Fuc branches on the GlcA units, regardless of their sulfation pattern, whereas two of the three

fCSs with Fuc branches on the GalNAc units, as well as unsulfated polysaccharides, displayed a much reduced anticoagulant activity.

Among biological properties of fCS polysaccharides, it is worth noting that the inhibition of P- and L-selectin interaction with sialyl Lewis(x), is stronger than the heparin one. Interestingly, oligosaccharides prepared by depolymerization of fCS from *Holoturia forskali* still maintained a high affinity for P- and L-selectins, but displaying a lower adverse effects than native polysaccharide. In order to evaluate the same inhibition activity of depolymerized fucosylated chondroitin sulfate (dfCS) from natural sources, a semi-synthetic fCS polysaccharide was submitted to β-eliminative depolymerization to give a oligosaccharide to be tested for its interaction with P- and L-selectins by STD-NMR techniques, displaying a slightly minor affinity with respect to that obtained from the natural one.

Chondroitin polysaccharide obtained from the fed-batch fermentation of *E.coli* O5:K4:H4 is, from a structural point of view, similar to the backbone of *Colwellia psychrerythraea* 34H capsular polysaccharide (CPS) displaying an unprecedented cryoprotectant function, and consisting of a tetrasaccharide repeating units composed of two aminosugars and two uronic acids, with one of the two latter bearing a L-threonine as substituent. In order to better understand the structure-cryoprotectant function relationship of this polysaccharide, microbial sourced chondroitin was coupled with L-threonine (Figure IV) under several conditions, producing a semi-synthetic derivative that displayed a ice recrystallization inhibition much lower than the *C. psychrerythraea* CPS. A combined NMR-molecular dynamic study of its 3D structure showed a rather far arrangement between the two polysaccharides, thus demonstrating that threonine decoration of biomacroolecules is not a sufficient element for gaining ice

ricrystallization inhibition in spite of several examples of Thr-rich (glycol)-proteins and polysaccharides with cryoprotectant activity in Nature.

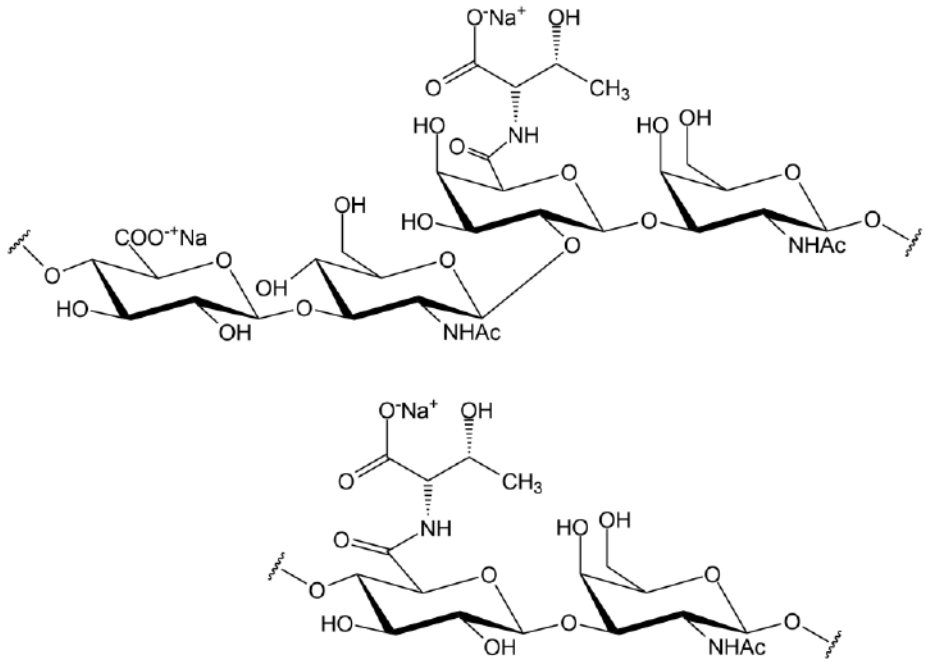

Figure IV: units of CPS from *C. psychrerythraea* 34H (top) and its semi-synthetic mimic obtained from microbial chondroitin (bottom)

Another polysaccharide that was subjected to regioselective modifications is alginate, that consists of 1→4-linked β-D-mannuronic acid (M) and its *C*-5 epimer α-L-guluronic acid (G) units. This natural copolymer is an important component of algae such kelp, and is also an exopolysaccharide of bacteria including *Pseudomonas aeruginosa*. Alginates are widely used in food, cosmetic and pharmaceutical industry. The sulfation of these polysaccharides exhibits compounds with carboxylic and sulfate groups close to each others as in heparin ones. Randomly sulfated alginates show anticoagulant activity, so regioselective modification of the polysaccharide backbone may help to

understand the relationship between structure and properties in alginate sulfates. Indeed, a semi-synthetic sulfated alginate derivative (propylene glycol alginate sodium sulfate, PSS), has been employed as anti-cardiovascular disease drug in China, without control of degree of sulfation. Due to incomplete solubility and highly heterogeneous structure of natural alginic acids the strategy to obtain a regioselectively sulfated alginate polysaccharide was applied to β-D-polymannuronic acid, that is the simplest polysaccharide possessing the most homogeneous structure of all alginic acids. It was protected at *O*-2,3 diol by either application of an orthoester or benzylidene ring and in the latter case, the polymannuronic acid was derivatized at carboxylic function too in order to enhance its solubility in aprotic solvent. At the end of the semi-synthetic route compounds with different sulfation pattern were obtained, but with unclear and probably not complete regioselectivity. Therefore, further optimization on semi-synthetic strategy is needed for the production of regioselectively sulfated alginates and for the evaluation of their structure-activity relationships.

CHAPTER 1

INTRODUCTION

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1.1 Carbohydrates and polysaccharides

Carbohydrates are by far the most abundant organic molecules found in Nature, and nearly all organisms synthesize and metabolize carbohydrates. (Gazarian et al., **2011**) They are the most information-rich class of biomacromolecules and with huge structural diversity compared to DNA or proteins. In monosaccharide structure, for example, the total number of isomer permutations for an hexamer with an "alphabet" of 20 monosaccharides achieves the astounding number of $1.44 \cdot 10^{15}$ structures, whereas from 20 aminoacids and 4 nucleotides, under the same conditions, only 20^6 and 4^6 structures could be obtained, respectively. (Gabius et al., **2000**) This characteristic explains the high complexity both in structural characterization (Figure 1) and in the synthesis of carbohydrates.

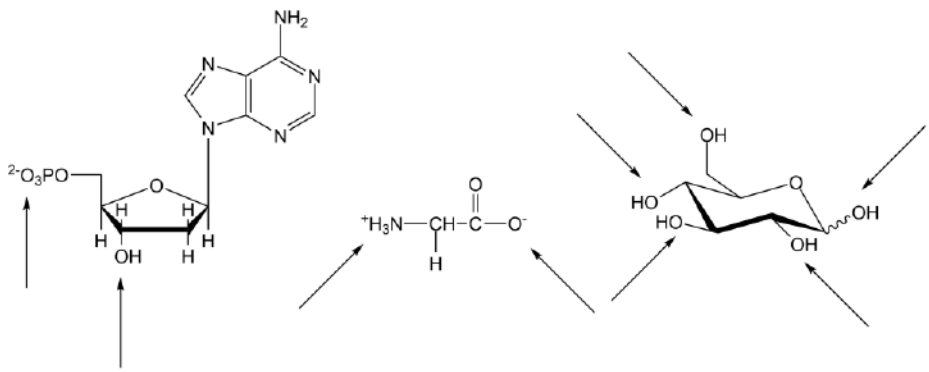


Figure 1: Comparison between the site of oligomerization explaining the structural diversity among nucleotides, aminoacids and monosaccharides

Main biological activities of glycans regard structural properties and specific interaction with other molecules, such as intrinsic and extrinsic glycan-binding proteins (GBPSs), of which the formers are typically involved in cell-cell interactions and the latters consist mainly of interactions with pathogens (Figure 2). (Varki et al., **2009***)*

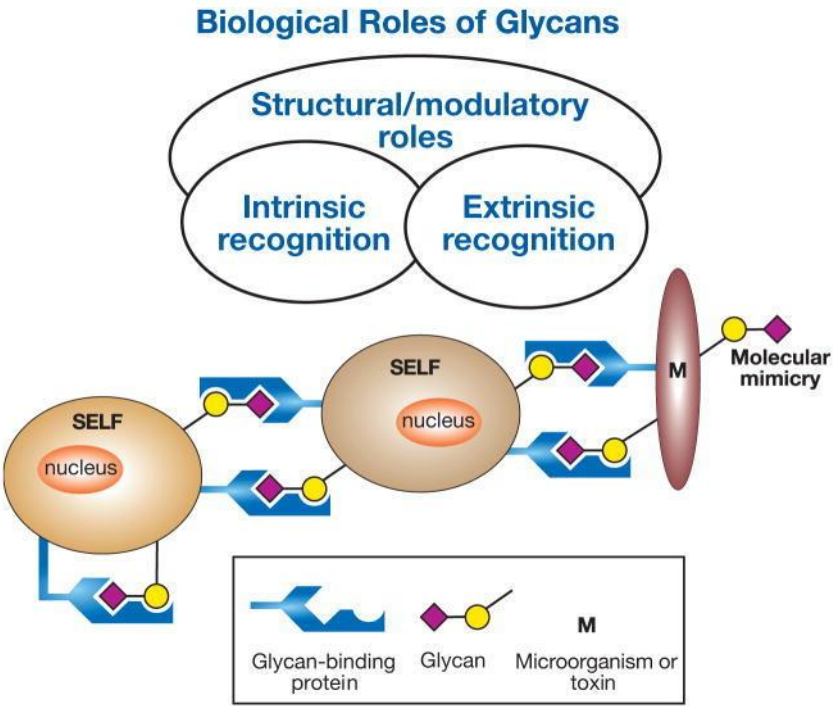


Figure 2: General classification of biological roles of glycans. (Gagneux et al., **1999**)

Among intrinsic GBPSs, it is worth mentioning the lectins, that are involved in a wide variety of cell-cell recognition, signaling and adhesion processes and in intracellular targeting of newly biosynthesized proteins. (Weiss et al., **1996**) The selectins are cell surface lectins able to recognize fucosylated, sialylated and sulfated ligands expressed on scaffold glycoproteins serving as functional

counter-receptors. They play a significant role in inflammation, immunological responses, and homing of lymphocytes and bone marrow stem cells, as well as in atherosclerosis, ischemia-reperfusion injury, inflammatory diseases, and metastatic spreading of some cancers. (Ley, **2001**)

Polysaccharides are polymeric carbohydrates, usually formed of repeating units (either mono-, or higher oligosaccharides) joined together by glycosidic bonds. These structures are often linear, but may contain various degrees of branching. Polysaccharides are often quite heterogeneous, containing slight modifications of the repeating unit. Depending on the structure, these macromolecules can have distinct properties from their monosaccharide building blocks. Polysaccharides are called homopolysaccharides when all monosaccharides in them are the same, on the other hand, when more than one type of monosaccharides is present, they are called heteropolysaccharides. Some of these macromolecules are characterized by high natural availability (starch, cellulose, glycogen, chitin, among others) and they also have a great biological importance, since they can be a source of energy for animal species. Moreover, they are structural elements of cellular walls and identification sites of cellular surfaces.

1.2 Sulfated polysaccharides and glycosaminoglycans

An important class of polysaccharides is that comprising the sulfated ones, that mainly occur in many seaweeds and animal species. The former class includes sulfated polysaccharides that can be classified depending on their structure, in sulfated fucans – polysaccharides with heterogeneous structures composed of L-fucose with different degrees of sulfation (Pomin et al., **2008**) -, carrageenans and agarans, that show

linear polysaccharide backbones composed almost exclusively of L- and/or D-galactose and 3,6-anhydro-D- or L-galactose units sulfated to a various extent (Campo et al., **2009**), and ulvans, that are usually constituted of 3-*O*-sulfated-L-rhamnose residues linked to xylose (either sulfated or not at its *O*-2 position) or to an uronic acid such as D-glucuronic or L-iduronic acid. (Lahaye et al., **2007**) Some of these sulfated polysaccharides have been found also in animals, especially sulfated fucans and galactans have been isolated from several marine invertebrates, showing a more regular structure than their analogues from seaweeds. (Pomin, **2009**) The most important animal sourced sulfated polysaccharides are GAGs, that are linked to variously composed proteoglycans in the extracellular matrix and, as other glycoconjugates, they play an important role in biological processes (Figure 3). (Esko et al., **2009**)

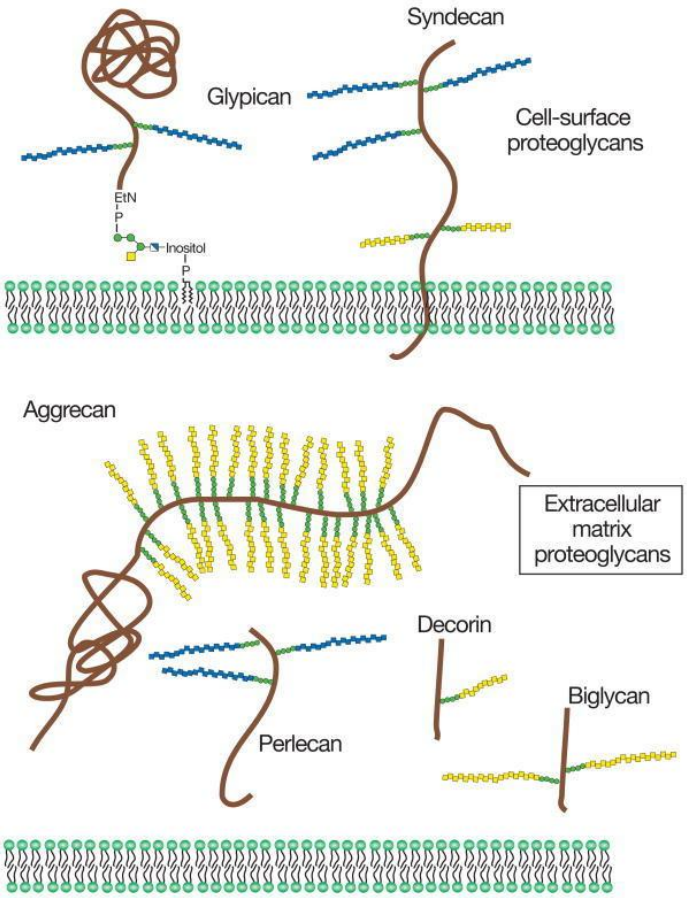


Figure 3: Some of the most important proteoglycans

Moreover, GAGs have been found on the surface of some pathogenic microorganisms, allowing them to escape hosts' immune response. (Raedts et al., **2011**)

From a structural point of view, GAGs are high densely charged negative heteropolysaccharides, consisting of a disaccharide repeating unit composed of a hexose (galactose) or an uronic acid (D-glucuronic or L-iduronic) acid and an aminosugar (glucosamine or galactosamine). (Mende et al., **2016**) They could be decorated with acetyl groups on the nitrogen atom of aminosugars or sulfated at hydroxyls and/or again on

nitrogen atom of aminosugars. GAGs include HP, HS, CS, fCS, DeS and KS, whereas hyaluronic acid is the only unsulfated one.

Sulfated polysaccharides and GAGs are known for their broad range of biological activities. (a) Pomin, **2015**. b) Ngo et al., **2013**. c) Jiao et al., **2011**. d) Campo et al., **2009**. e) Lahaye et al., **2007**. f) Toida et al., **2003**.) Some of them are already employed in therapeutic treatments (*e.g*. heparin and chondroitin sulfate for their activity as anticoagulant and for therapy of articular cartilage osteoarthritis, respectively), and a wide number of novel drugs, medical devices and nutraceuticals based on the bioactivities of sulfated polysaccharides are currently under development (a) Zhao et al. **2015**. b) Scott et al., **2013**. c) Patel, **2012**. d) Pomin et al., **2008**. e) Yamada et al., **2008**.)

Their biological functions are mainly related to specific cell-cell and cell-extracellular matrix interactions (a) Lim et al., **2015**. b) Esko et al., **2009**. c) Bishop et al., **2007**.) that are generally mediated by the formation of carbohydrates-protein complexes. Moreover, it has been suggested that the sulfation pattern sequence might be able to encode functional information to this aim. The sulfation code has been not unveiled yet, except for few cases, where its function in blood clotting control (van Boeckel et al., **1993**) and adhesion of pathogens (a) Martín et al., **2013**. b) Achur et al., **2003**.), guiding neuronal growth (a) Swarup et al., **2013**. b) Rogers et al., **2011**. c) Gama et al., **2006**.) or inducing cancer cell apoptosis (Poh et al., **2015**) have been elucidated.

1.3 Synthesis of carbohydrates

A plethora of protecting groups employed in organic chemistry are commonly used in synthetic carbohydrate chemistry, although their

application on polysaccharides is rather limited. They could be divided in two categories: permanent (they remain through all the synthesis and are removed at the end of it) and temporary protecting groups (that are removed during the synthesis to free reactive position of the substrate). In carbohydrate chemistry a large amount of protecting groups are usually needed, above all to pursue a regioselective protection. Their insertion and removal, as well as, their stability and orthogonality often recall the reaction conditions already developed for non carbohydrate molecules, but several protecting groups developed *ad hoc* for sugar species have been reported too. (Wuts et al., **2014**)

The regioselective protection may involve well-defined hydroxyl position(s) in each repeating unit avoiding, at the same time, any alteration of glycosidic linkages and any labile decoration on the polysaccharide backbone during the protection-deprotection reactions. From a regioselective point of view, modification of polysaccharides is completely different from monomeric molecules. Indeed, when the protection or deprotection reaction gives a non quantitative modification of small molecules, the desired derivative could be purified from other components of the mixture, whereas in polysaccharides any purification is impossible, due to the covalent bond between correctly and uncorrectly modified monosaccharide units. (Cumpstey, **2013**) Protecting groups are, without doubts, useful to protect reactive functions of the molecules, but they can also enhance or decrease the reactivity and participate in the reactions, thus affecting their kinetic, regiochemical and stereochemical aspects.

The most important protecting groups in the synthesis of carbohydrates are hydroxyls protecting groups. Moreover, carboxy- and amino-

protecting groups for uronic acid and aminosugars-containing polysaccharides, respectively, are highly important too.

1.3.1 Hydroxyl protecting groups

Due to the large number of hydroxyls on carbohydrates, this kind of protecting groups are the most widely employed. Ethers and esters are usually introduced to protect them, adopting very often benzyl ethers and acetate or benzoyl esters for a permanent protection, due to their stability and efficient introduction/removal methods. On the contrary, a wide number of temporary protecting groups has been reported in literature and new protection strategies are continuously developed. In order to obtain regioselectively protected polysaccharides, it could be considered that the order of reactivity of hydroxyls is tied to both their steric hindrance and to a primary OH > equatorial OH > axial OH scale, although the differentiation of secondary hydroxyls on the basis of their nucleophilicity is often difficult.

1.3.1.1 Ether-type protecting groups

The most common ether-type protecting groups are depicted in Figure 4 and consist of both ethereal (such as methyl, benzyl, trityl) and silyl ether functionalities, such as TBDMS and TBDPS ethers.

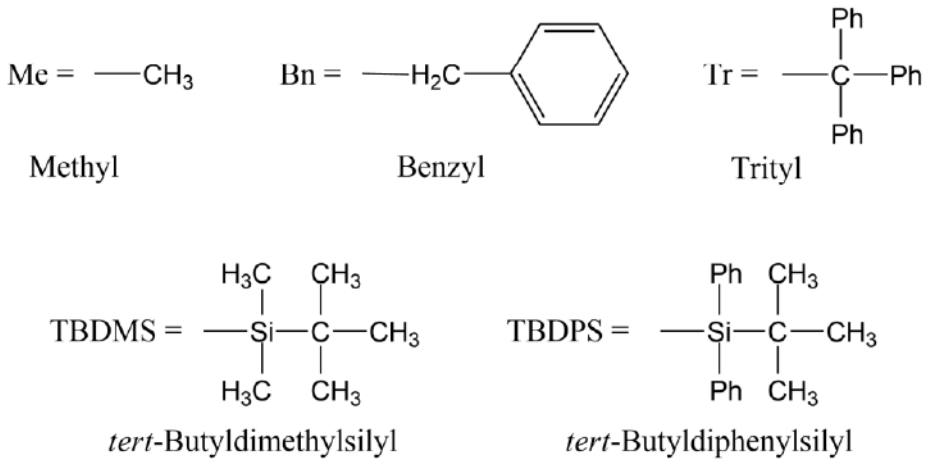


Figure 4: Commonly used ether and silyl ether protecting groups

These protecting groups have been applied not only in the synthesis of oligosaccharides and glycoconjugates, but also for the structural modification of polysaccharides. For example, the insertion of methyl and other alkyl ethers on cellulose was performed in the presence of alkyl halides under basic conditions investigating different solvents such as DMI/LiCl, (Takaragi et al., **1999**) DMA/LiCl (McCormick et al., **1987**), DMSO/LiCl mixtures (Petrus et al., **1995**) and SO_2/DEA/DMSO system (Isogai et al., **1986**) affording 2,3,6-tri-*O*-alkylated cellulose quantitatively.

Benzyl ethers are widely used as permanent protecting group for their well-known stability. Their insertion can be performed under strongly basic conditions as reported for benzylation of chitin employing sodium hydride (Umemura et al., **2012**) or sodium hydroxide as base (Somorin et al., **1979**), affording a perbenzylated or selectively *O*-6 protected polysaccharide, respectively. Benzyl ethers are usually removed under

hydrogenolytic, reductive (Na/ liquid ammonia) (Iseloh et al., **2002**) or oxidative conditions (Adinolfi et al., **1999**).

Trityl ethers regioselectively react with polysaccharides in a weakly basic environment preferentially at primary positions due to their bulkiness, as reported for cellulose, (Green, **1963**) chitin (Umemura et al., **2012**) and chitosan. (Holappa et al., **2005**) Trityl ethers can be easily cleaved by acid hydrolysis.

TBDMS and TBDPS are the most widely used silyl ethers and their insertion can be performed under less basic conditions than ethers. They are largely employed for derivatization of polysaccharides, especially to convert alcohol moieties into more lipophilic and often more reactive groups, as reported, for example, on cellulose, (a) Richter et al., **2003**. b) Groth et al., **2001**. c) Baumann et al., **2000**.) obtaining per-*O*-silylated derivatives. TBDMS groups were also regioseletively inserted on cellulose either at position *O*-6 or both at positions *O*-2 and *O*-6, in dependence of the employed reaction conditions. (a) Koschella et al., **1997**. b) Klemm et al., **1995**.) Removal of silyl ethers protecting groups can be easily achieved with fluoride ions (TBAF/THF).

1.3.1.2 Ester protecting groups

The most common ester protecting groups are showed in Figure 5. Carboxylate esters can be obtained using carboxylic acid as acylating agents in the presence of strong acid or nucleophile catalysts. Much more frequently, their activated derivatives, such as acid chlorides or anhydrides either under acid or basic conditions, are employed.

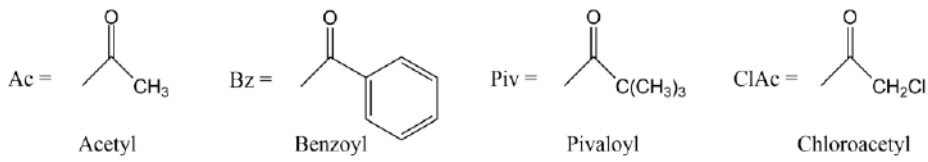


Figure 5: Commonly used ester protecting groups

When the reaction is conducted in basic conditions, a stoichometric amount of a base, such as pyridine or triethylamine has to be used. (Cumpstey, **2013**)

Ester protected polysaccharides were prepared on starch (Grote et al., **2005**) and cellulose (McCormick et al., **1987**) under basic conditions, obtaining in both cases an almost quantitative degree of substitution. Moreover, alginates were tested for hydroxyl protection by esterification too. (Pawar et al., **2011**) Regioselective esterification at the primary position of a polysaccharide was investigated mainly on cellulose, displaying a lower degree of substitution than ether protection. (Fox et al., **2011**)

1.3.1.3 Acetal and ketal protecting groups

Benzylidene and isopropylidene are example of acetal and ketal-type protecting groups, respectively. They are suitable for the contemporary protection of two hydroxyl groups. They are introduced under acid conditions in the presence of aldehyde/ketone or acetal/ketal reagents, respectively and can form five-membered rings on 1,2-*cis*-diol and/or six-membered ones on 1,3-*trans* diols. They can be removed by acid hydrolysis affording the free hydroxyls, but, in the case of a benzylidene ring, several other methods for their regioselective opening could be applied. (Ohlin et al., **2011**) Reductive opening of acetals

relies on a combination of a hydride reagent with a Lewis (or a proton) acid. In the case of benzylidene cycles installed at 4,6-positions of a sugar, the most common methods employed for opening to 6-*O*-benzyl protected derivatives use $NaCNBH_3$-HCl-THF, (a) Garegg et al., **1982**. b) Garegg et al., **1981**.) and $BH_3 \cdot NMe_3$-$AlCl_3$-THF mixtures. (Ek et al., **1983**) Instead, for the reductive opening to 4-*O*-benzyl derivatives, a $LiAlH_4$-$AlCl_3$ system in ethereal solvent is widely used, (Liptak et al., **1975**) although the employment of $BH_3 \cdot THF$ in THF with several different acids is a common method too. (a) Shie et al., **2009**. b) Daragics et al., **2009**. c) Tani et al., **2007**. d) Shie et al., **2005**. e) Wang et al., **2002**. f) Jiang et al., **1998**.) Benzylidene acetals can be also opened under oxidative, non regioselective conditions, obtaining, - for example, from, 4,6-rings – both, either a 4-*O*-benzoylated and a 6-*O*-protected derivative. (Adinolfi et al., **1999**)

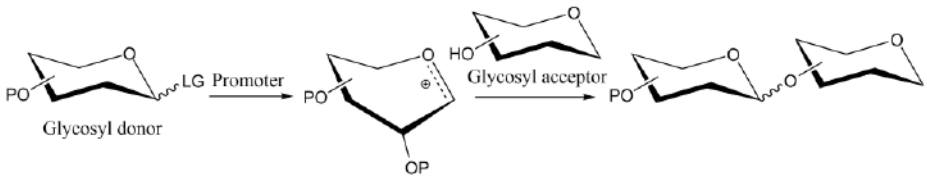
1.3.2 Carboxylic acid protecting groups

Natural polysaccharides can present carboxylic functions in their structure. The uronic acid species can be protected at *C*-6 position by formation of esters, reacting either as an electrophile or as a nucleophile. In the first case the activation could occur in strongly acid conditions, followed by the reaction with an alcohol to give the ester. Alternatively, the carboxylic acid can be transformed in a carboxylate ion and then, reacts with an alkylating agent to give the ester, as published for alginates (a) Pawar et al., **2013**. b) Pelletier et al., **2000**.) and pectins. (Pappas et al., **2004**)

1.3.3 Glycosidic bond formation

Some branched polysaccharides display several biological functions, including anti-tumour and immunostimulating activities. (Kurita et al., **2011**) Thus, introduction of branches on some specific linear polysaccharides could be useful for developing polysaccharide-based drugs. Recently, it has been developed a strategy for the regioselective glycosylation of polysaccharides, such as chitin-cellulose hybrids (Ishimaru et al., **2014**) and chitin polysaccharides (a) Kurita et al., **2003**. b) Kurita et al., **1998**.) with oxazoline or orthoester monosaccharide derivatives as branching reagents, but exclusively at *O*-6 primary position.

To this aim, the formation of new glycosydic bonds is achieved by glycosylation. This key reaction occurs between a *glycosyl donor* that acts as an electrophile, and a *glycosyl acceptor* that acts as a nuclophile. The former is fully protected with the presence of a leaving group (LG) at its anomeric position, whereas the latter presents all the positions protected but often only one free hydroxyl group (Scheme 1).



Scheme 1: Standard glycosylation reaction mechanism

The reaction needs the presence of a *promoter* (it is usually used in catalytic amount) to take place. It activates the glycosyl donor assisting the departure of the leaving group. Other additives such as molecular

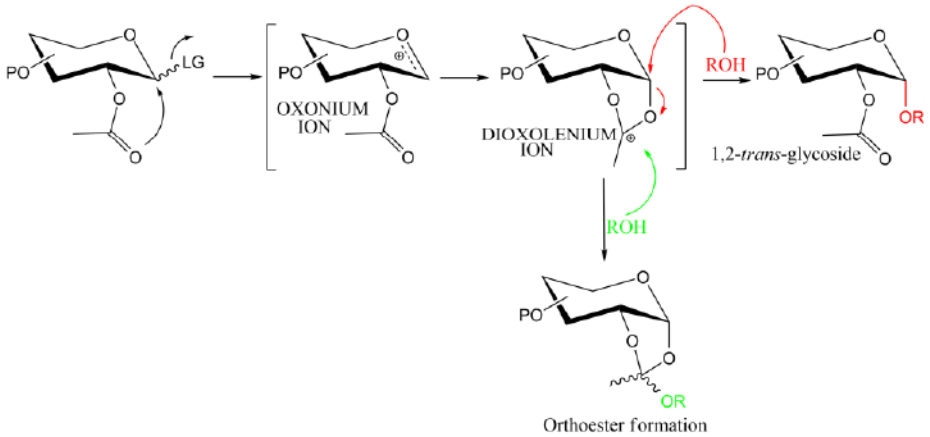
sieves or any base or acid that may act as scavengers could be also used depending on the specific conditions employed for the glycosylation. Protecting groups on the donor and acceptor backbones participate in the reaction and this feature is due to their electronic properties; thus, according to these, the *armed-disarmed* concept (Mootoo et al., **1988**) displayed that a glycosyl donor with ester-type protecting groups is electronically deactivated (disarmed) over the same glycosyl donor bearing ether-type protecting groups, that is electronically activated (armed). (Demchenko, **2008**) The difference in reactivity could be attributed to the electron-withdrawing effect of ester-type protecting groups that decrease the electron density and the nucleophilicity of leaving groups. Moreover, it has been discovered that glycosyl donors with a 2-*O*-benzyl-3,4,6-tri-*O*-acyl protecting group pattern are even more deactivated than the corresponding *disarmed* peracyl derivatives. (Kamat et al., **2005**)

This different behaviour could be attributed to the higher stabilization of the carbocationic intermediate, *via* the acyloxonium ion of the peracetylated donor, with respect to the oxocarbenium stabilization of 2-*O*-benzyl-3,4,6-tri-*O*-acyl one, (Demchenko, **2008**) taking into account of "*O*-2/*O*-5 cooperative effect", (Kamat et al., **2005**) although the nature of this latter has not been completely understood yet.

1.3.3.1 Stereochemistry of glycosidic bond formation

The *O*-glycosylation is a challenging aspect of synthetic carbohydrate chemistry for the necessity to form either α- or β-glycosydic bond, with a complete control of stereochemistry. In the presence of a participating protecting group at *C*-2 such as an acyl one, after the activation of a glycosyl donor and the subsequent exit of the leaving group, an

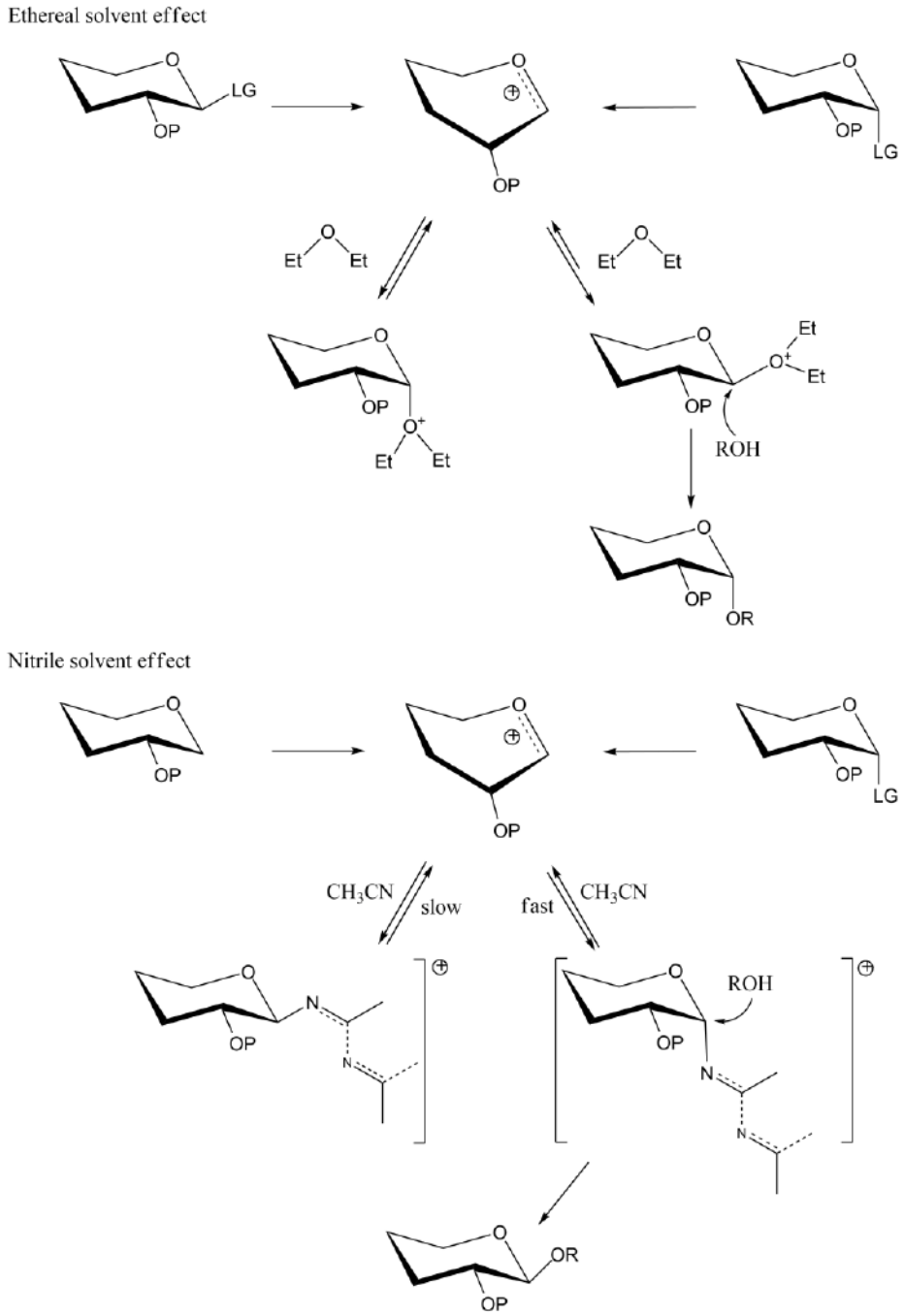
oxonium ion is formed. It reacts with the carboxylic oxygen of the acyl group, carrying out the anchimeric effect, and thus forming a dioxolenium ion. This latter is attacked by the glycosyl acceptor affording the 1,2-*trans*-glycosidic bond; thus, in the case of *gluco*-configured donor a β-anomer is formed, whereas in the case of a *manno*-configured one a α-anomer is obtained. A side reaction could occur employing a glycosyl donor with a participating protecting group at *C*-2, such as the formation of an orthoester that could be, anyway, a reversible process when strong Lewis and Brønsted are used as promoters (Scheme 2).



Scheme 2: Anchimeric effect in the control of stereochemistry of glycosidic bond formation

The control of stereoselectivity in a glycosylation reaction, can be also achieved by the solvent (solvent effect), in the case of anchimeric effect absence. Using polar solvents, the mechanism is a pure S_N1, with the formation of a solvated oxonium ion, that with the employment of nitrile-type solvents is solvated preferentially at α-face, forming the

kinetically controlled (for the reverse anomeric effect) α-nitrilium-nitrile complex, that affords a β-configured glycoside. With ether-type solvents the oxonium ion is solvated at β-face, forming the thermodynamically controlled β-ethereal complex, that affords a α-configured glycoside (Scheme 3).



Scheme 3: Solvent control on stereochemistry of glycosidic bond formation

1.3.3.2 Glycosyl donors and activation conditions

Glycosyl donors are classified depending on the leaving group linked to the anomeric position. The most commonly used are glycosyl halides, thioglycosides, trichloro- and *N*-phenyl-trifluoroacetimidates (Figure 6), although their number and activation methods are continuously in progress (a) Satoh et al., **2013**. b) Zhu et al., **2009**.)

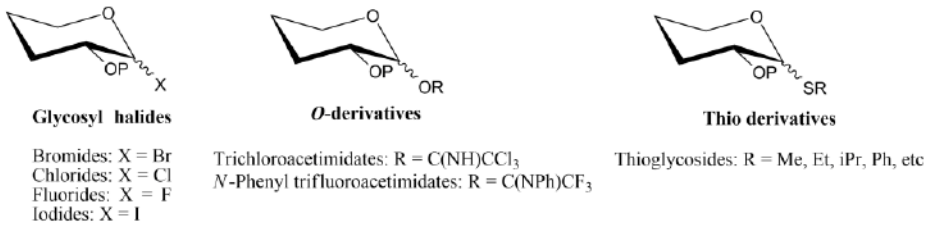


Figure 6: Typical glycosyl donors

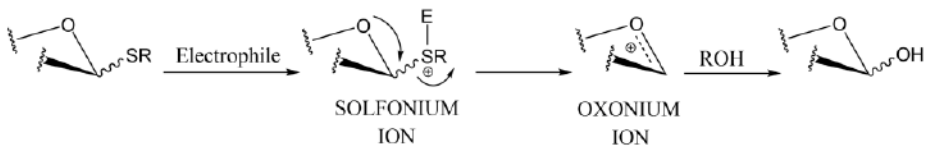
Glycosyl halides were the first donors described in glycosylations, (Michael, **1879**) with **glycosyl bromides** that are generally more reactive than **glycosyl chlorides**. Glycosylations employing them are performed in the presence of heavy metal salts as promoter in stoichometric amount (Koenigs-Knorr reaction). (a) Koenigs et al., **1901**. b) Wulff et al., **1974**. c) Igarashi, **1977**.) Up to now a wide variety of promoters to activate glycosyl halides have been developed such as $AgClO_4$ and AgOTf and combination thereof. (Sherman et al., **2001**) Besides heavy metals, Lewis acids such as $SnCl_4$, $BF_3{\cdot}OEt_2$ (Ogawa et al., **1976**) or $ZnCl_2$ (Higashi et al., **1990**) have been also introduced as promoters. **Glycosyl fluorides** are more stable than chloride and bromide ones and for long time they were not considered suitable glycosyl donors due to their unreactivity under Koenigs-Knorr conditions. Their use was lately introduced (Mukaiyama et al., **1981**)

employing over the years different kind of promoters for the activation, although the commonly used one is $BF_3 \cdot OEt_2$. (Toshima, **2000**) Despite **glycosyl iodides** had been already known (Fischer et al., **1910**), they were considered too unstable to be synthetically useful (Meloncelli et al., **2009**) and only recently it has been shown that they can offer some advantages with respect to glycosyl bromides and chlorides in terms of time, efficiency and selectivity (a) Ranade et al., **2013**. b) van Well et al., **2005**.) *O*-Glycosyl imidates donors (**trichloro-** and ***N*-phenyl-trifluoroacetimidates**) (Schmidt et al., **1980**) represent a very versatile method for glycosylation. This is ascribed to the catalytic amounts of promoter that are needed for their activation, differently from most glycosyl donors that, instead, require a stoichometric quantity.

Both trichloro- and *N*-phenyl-trifluoroacetimidate donors are relatively stable under basic or neutral conditions, but they easily react in the presence of catalytic amounts of a Brønsted or Lewis acid. For the first class of donor, typically TMSOTf (a) Grundler et al., **1984**. b) Schmidt et al., **1982**.) and $BF_3 \cdot OEt_2$ are involved in the activation and the imidate leaving group is expelled as trichloroacetamide after this step. Beside the above mentioned promoter, other several ones have been developed, including metal triflates [$Sm(OTf)_3$, (Adinolfi et al., M.; **2000**) $Sn(OTf)_2$, (a) Bartek et al., **1998**. b) Castro-Palomino et al., **1995**.) $Yb(OTf)_3$, (a) Adinolfi et al., **2001**. b) Adinolfi et al., **2002**.) $Cu(OTf)_2$ (Yamada et al., **2002**) and $Bi(OTf)_3$ (Adinolfi et al., **2006**)], acid molecular sieves (Adinolfi et al., **2003**) and I_2/Et_3SiH system. (Adinolfi et al., **2002**) Another technical development regarding the trichloroacetimidate donors is the *inverse procedure* (Schmidt et al., **1991**) for their activation, where the glycosyl donor is added to a mixture of a glycosyl acceptor and the promoter. According to this, it is

assumed that the highly reactive glycosyl trichloroacetimidates might react with the acceptor immediately after their activation with the promoter, because the last might be complexed in solution to heteroatoms of the acceptor. Glycosyl *N*-phenyl-trifluoroacetimidates can be activated under the same conditions of trichloroacetimidates, but they often require stronger conditions and both their higher stability (Yu et al., **2010**) and the less amount of byproducts than the latter could be ascribed to their enormous fame, in spite of their rather recent discovery. (Yu et al., **2001**)

The ease of synthesis and their stability make **thioglycosides** the most used donors in carbohydrates chemistry. Indeed, they display not only long shelf-lives, but they can be also decorated with several protecting groups, that can be effectively introduced and removed, leaving the thioglycoside function intact. Despite their stability, thioglycosides can be activated using mild thiophilic reagent (typically soft electrophiles) obtaining a sulfonium ion, that is a better leaving group than the SR moiety, and thus affords the oxonium ion. This latter can react with the glycosyl acceptor, then forming a glycosydic bond (Scheme 4).



Scheme 4: Thioglycoside activation

A very common activation method for thioglycosides relies on the employment of *N*-iodosuccinimide (NIS) as iodine-containing compound in the presence of triflic acid (TfOH) (Veeneman et al., **1990**) or with Lewis acids such as TMSOTf, $BF_3{\cdot}OEt_2$ or AgOTf.

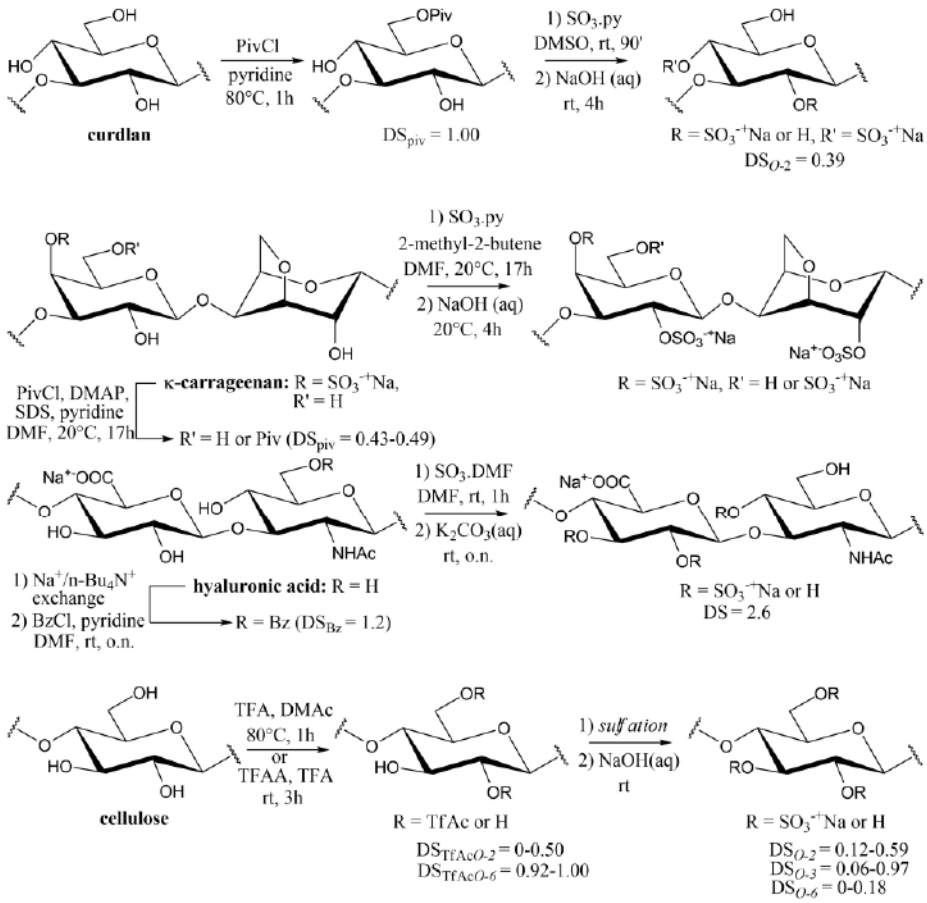
(Konradsson et al., **1990**) Even though the versatility of thioglycosides is very well documented, they can present some disadvantages, the most important one is the necessity of stoichiometric amounts of promoter that could lead to side reactions in the glycosylations.

1.3.4 Sulfation decoration of polysaccharides

The biological importance of sulfate-bearing polysaccharides has given a great impulse to the production of chemically or chemoenzymatically sulfated polysaccharides from natural unsulfated ones. Indeed, after the introduction of sulfate groups, they often acquire biological activities. Sulfation is usually performed under non regioselective conditions, thus achieving derivatives that present sulfate groups randomly distributed on the polysaccharide backbone. Moreover, under harsh conditions, persulfated polymers could be obtained but some of them can induce some very adverse side effects in humans. (a) Guerrini et al., **2008**. b) Kishimoto et al., **2008**.) The regioselective sulfation of polysaccharides has been reported both in enzymatic and chemical way, although the former method is limited by the low scale of reactions reported up to now and by the selective employment on GAGs, that are usually already sulfated. It can be, therefore, more advisable to develop also chemical methods for the regioselective introduction of sulfate groups into different polysaccharide backbones.

The already reported methods for the regioselective sulfation of polysaccharides rely on protection-sulfation-deprotection strategies, employing both esters and ethers as protecting groups. Bulky pivaloyl esters, for example, were inserted on the reactive primary hydroxyl of curdlan (Gao et al, **1997**) and carrageenan (de Araùjo et al., **2013**),

affording, after sulfation and global deprotection, in the former case polysaccharides composed of a 2,4-*O*-disulafted glucose repeating unit and in the latter 2,4-*O*-disulfated galactose and 2,4,6-*O*-trisulfated galactose together with 2-*O*-sulfated anhydrogalactose (Scheme 5).



Scheme 5: Three-steps (protection/sulfation/deprotection) strategies using ester protecting groups on polysaccharides.

Moreover, other ester groups, such as benzoyl and trifluoroacetyl, were used to regioselectively protect the primary *O*-6 hydroxyl of hyaluronic acid (Becher et al., **2010**) and cellulose (a) Zhang et al., **2013**. b) Baumann et al., **2000**. c) Klemm et al., **1995**.) Besides, also some bulky

ether groups, such as Tr, MMTr and DMTr were used as protecting groups in the regioselective sulfation of polysaccharides, showing their advantages to be removed orthogonally with respects to the other protecting groups. Indeed, primary alcohol protection with Tr, MMTrCl or DMTrCl was employed for the regioselective sulfation of chitosan (Nishimura et al., **1998**) and cellulose. (Heinze et al., **2000**)

The longest and up to now most sophisticated multi-step sequences for the regioselective sulfation of polysaccharides have been developed for the production of CS (a) Laezza et al., **2014**. b) Bedini et al., **2012**. c) Bedini et al., **2011**. d) Bianchi et al., **2012**. e) Zoppetti et al., **2004**.) and fCS (a) Laezza et al., **2016**. b) Laezza et al., **2015**) polysaccharides with different sulfation patterns. Some of them will be discussed in full details in Chapter II.

1.3.5 Conjugation of polysaccharides with amines

Uronic acids can react as electrophiles in the presence of amines to give amides. This reaction can be achieved using several coupling agents (as it will be discussed in more detail in Chapter IV), such as water-soluble EDC as published for alginates uronic acid modification. (Yang et al. **2011**) Moreover, the same modification could be achieved employing organic salts, that are suitable for organic solvent. (Vallée et al., **2009**)

1.4 Aim of the thesis

The aim of this thesis is the development of new synthetic organic chemistry strategies for the regioselective modifications of some polysaccharides. This includes:

- A semi-synthetic strategy for the obtainment of CS and fCS polysaccharides by regioselective sulfation and insertion of L-fucose branches on microbial sourced chondroitin polysaccharide (Chapter II)
- A preliminary study on interaction between selectins and one of the obtained fCS derivatives (Chapter III)
- The semi-synthesis of mimics of CPS from *Colwellia psychrerythraea* – a psychrophilic bacterium - by regioselective decoration of a microbial sourced chondroitin polysaccharide with L-threonine units and their antifreeze properties evaluation (Chapter IV)
- A study of regioselective sulfation of a polymannuronic acid alginate (Chapter V)

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CHAPTER 2

REGIOSELECTIVE SULFATION AND FOCUSYLATION OF MICROBIAL SOURCED CHONDROITIN POLYSACCHARIDE

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2.1 Introduction

Sulfation is a very important decoration. In fact GAGs could be divided into two families depending on the presence or the absence of sulfate group. HA is the only unsulfated glycosaminoglycan, whereas sulfated ones include HP, HS, CS, fCS, DeS and KS. (Bedini et al., **2016**)

CS is widely distributed in mammals and invertebrates, as well as in some bacteria (Bedini et al., **2016**) and is involved in different physiological and pathological processes. It is composed of a disaccharide repeating unit containing β-D-glucuronic acid and *N*-acetyl-galactosamine linked through β-(1→3) and β-(1→4) glycosydic bonds. After *in vivo* polymerization it affords a polysaccharide that can show different sulfation patterns. Based on positions of sulfate groups, several disaccharide subunits could be, indeed, described: the positions *O*-4 and/or *O*-6 on GalNAc residue are commonly sulfated, differently from positions *O*-2 or *O*-3 of the GlcA residue that are rarely sulfated. (Pomin, **2013**) (Figure 7)

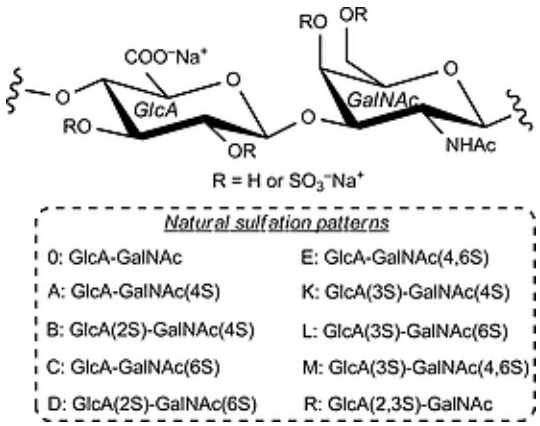


Figure 7: Disaccharide subunits in natural CSs

CS isolated from natural sources (Shi et al., **2014**) has applications in medicine for treatment of osteoarthritis and osteoarthrosis (Bishnoi et al., **2016**), but further potential employments have been proposed depending on different sulfation patterns. (a) Schiraldi et al., **2010**. b)Yamada et al., **2008**.) Nonetheless, the low abundance of raw material, the laborious downstream purification and the growing application of CS as drug led to development of chemical and chemoenzymatic (a) Bedini et al., **2013**. b) Scott et al., **2013**.) fine manipulation of its structure, mainly towards the modification of sulfation pattern. (Bedini et al., **2016**)

To GAGs family belongs also fCS. It is extracted from sea cucumbers (*Echinoidea, Holothureidea*) and presents a linear disaccharide backbone composed of GlcA and GalNAc units, linked together through β-(1→3) and β-(1→4) alternating glycosidic bonds. Depending on the different varieties of sea cucumbers from various seawaters, the disaccharide repeating unit could be characterized by the presence of

both differently distributed sulfate groups and differently sulfated L-fucose branches. (a) Myron et al., **2014**. b) Chen et al., **2011**.)

Single L-fucose branch per repeating unit is α-glycosidically linked at position *O*-3 of GlcA units, its presence is probably necessary to prevent the action of chondroitin sulfate-related enzymes, excreted from marine microorganisms. It has been also suggested that polysaccharides with high extent of sulfate groups are required in marine environments. (Myron et al., **2014**) Moreover, some slightly different structural features have been recently suggested for fCS extracted from *Apostichopus japonicus*, *Actinopyga mauritania*, and *Ludwigothurea grisea*. In the former two cases a random fucosylation at either GlcA *O*-3 or GalNAc *O*-4/*O*-6 position has been proposed, (a) Ustyuzhanina et al., **2016**. b) Yang et al., **2015**.), whereas in the latter fCS a α-Fuc-(1→3)-Fuc disaccharide branch was found. (Santos et al., **2015**) Several different sulfation patterns for both GlcA- and GalNAc-linked Fuc branches have been found (Figure 8).

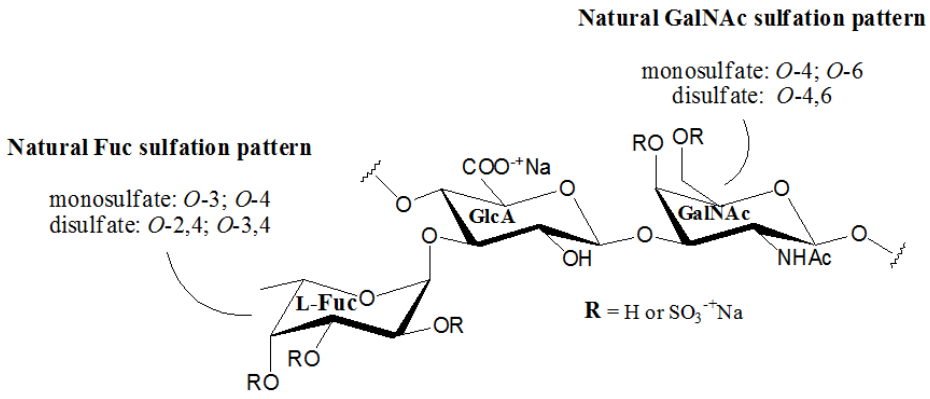


Figure 8: Sulfation patterns found in natural fCSs

fCS presents biological activities related to angiogenesis, atherosclerosis, cancer metastasis, cellular growth, hyperglycemia, fibrosis, viral infection, and, above all, coagulation and thrombosis. (Pomin, **2014**)

The anticoagulant and antithrombotic activities are strictly related to the presence of sulfated L-fucose branches, indeed, partially defucosylated and desulfated derivatives have no anticoagulant or antithrombotic activities. (a) Monteiro-Machado et al., **2015**. b) Mour o et al., **1998**. c) Mourao et al., **1996**.) Moreover, the highest anticoagulant activities have been found for 2,4-di-*O*-sulfated fucose-containing fCS, although the independence of the anticoagulant and antithrombotic ones from the different sulfation pattern of Fuc branches has been recently proposed. (a) Santos et al., **2015**. b) Myron et al., **2014**.) Anticoagulant and antithrombotic activities are mediated by AT and HCII (our o et al., **1996**) in a serpin-dependent mechanism, but, differently from heparin, fCS showed also a serpin-independent one, when it was tested on AT- and HC-II free plasmas, inhibiting, indeed the generation of factor Xa by the intrinsic tenase complex in a more potent way than thrombin formation in the prothrombinase complex. (Glauser et al., **2008**) Moreover, it seems to retain also the antithrombotic activity when it is orally uptaken. (Fonseca et al., **2006**)

The most used drug for anticoagulant treatment is unfractionated heparin, which long-term therapy displays several limitations, as well as a high tendency to pathogenic contamination due to heparin extraction from animal sources. (our o , **2015**) The introduction of ultra-low-molecular weight heparins (ULMWH) reduced these problems, but the development of a safer and more potent antithrombotic and anticoagulant drug is an engaging challenge.

(Mackman, **2008**) To this aim, the anticoagulant and antithrombotic activities showed by fCS have drawn attention, although the even more strict regulation for animal-derived drugs and the high variability of sulfation pattern in polysaccharide of animal origin require a severe control of its sulfation level. Indeed in 2008 a contamination of heparin lots by oversulfated chondroitin sulfate provoked 149 deaths (a) Kishimoto et al., **2008**. b) Guerrini et al., **2008**.) Moreover, natural fCS polysaccharides exhibit adverse effects, such as platelet aggregation, factor XII activation and bleeding, (Fonseca et al., **2010**) differently from low molecular weight fCS polysaccharides. For this reason, strategies to mild and regioselective depolymerization to obtain shorter fCS molecules without removal of sulfate groups and fucose branches (a) Li et al., **2016**. b) Liu et al., **2016**. c) Zhao et al., **2015**. d) Gao et al., **2015**. e) Wu et al., **2015**. f) Zhao et al., **2013**.) or total synthesis of di- and trisaccharide fragments of fCS were recently developed. (a) Vinnitskiy et al., **2017**. b) Ustyuzhanina et al., **2015**. c) Tamura et al., **2013**) Using the former strategy, it has been discovered that the minimum lenght that is responsible for the intrinsic tenase complex inhibition is a nonasaccharide. (Zhao et al., **2015**)

An alternative approach to non-animal sourced CS and fCS species is here proposed by accomplishing the chemical regioselective fucosylation and/or sulfation of a microbial-sourced chondroitin polysaccharide (a) Laezza et al., **2016**. b) Laezza et al., **2015**. c) Laezza et al., **2014**.)

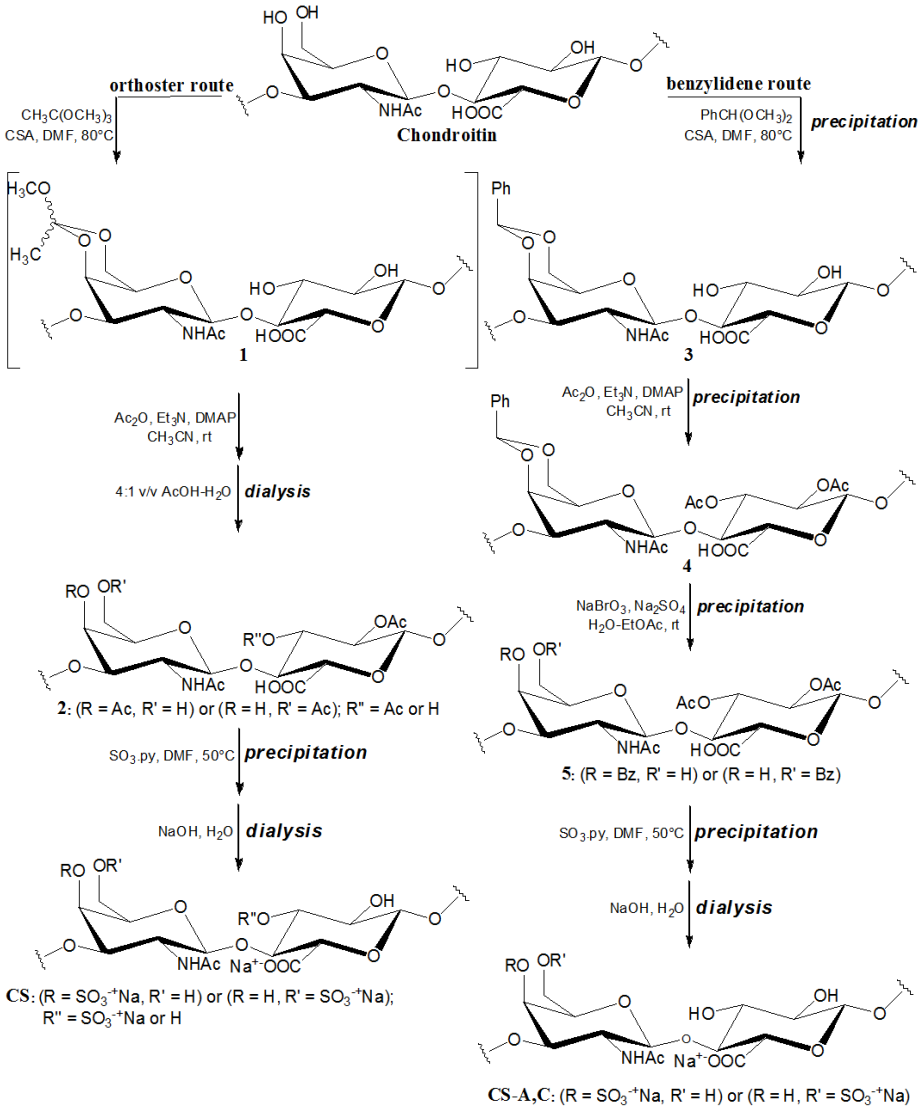
2.2 Results and discussion

2.2.1 Regioselective sulfation of microbial sourced chondroitin

An interesting approach to produce CS polysaccharides with a well-defined sulfation pattern from microbial sourced chondroitin (Cimini et al., **2010**) relies on regioselective protection of polysaccharide backbone, followed by sulfation of free hydroxyls and then global deprotection. The semi-syntheses reported up to now all started with protection of the GalNAc *O*-4,6 diol by a cyclic acetal (benzylidene) (Bedini et al., **2011**) or by an orthoester (Jacquinet et al., **2009**) (**1** and **3** respectively, Scheme 6) followed by acetylation of *O*-2,3 GlcA diol. The cycles were, successively, both opened under no regioselective conditions (Adinolfi et al., **1999**) obtaining derivatives **2** and **5** protected by acyl groups either at position *O*-4 or *O*-6 of GalNAc on disaccharide unit. Sulfation of free hydroxyls and global deprotection finally afforded semi-synthetic CS polisaccharides. The application of different cyclic protecting group seemed to influence the sulfation pattern, with the presence of sulfate groups at positions *O*-4 or *O*-6 of GalNAc units (CS-A,C) (Bedini et al., **2011**) in the case of a benzylidene-based strategy. Alternatively, diol protection by a orthoester ring afforded a CS polysaccharide with the uncommon feature (Sugahara et al., **2000**) of an additional sulfate group at position *O*-3 of some GlcA units. (Bedini et al., **2012**)

Here the two protection sequences were carefully investigated in order to explain the different output on CS sulfation pattern. The starting material used for the semi-synthesis of CS polysaccharides was obtained after a downstream purification (Cimini et al., **2010**) of a fed-

batch fermentation of *Escherichia coli* O5:K4:H4. Unsulfated chondroitin polysaccharide was, in this way, obtained with 89-94% purity grade free of any toxic lipopolysaccharide contaminant after NMR evaluation and capillary electrophoresis. (Restaino et al., **2009**) The presence of a sulfate group at position *O*-3 of some GlcA units obtained at the end of the orthoester route, was firstly hypothesized to be produced after a non-quantitative acetylation on derivative **1** (Scheme 6).



Scheme 6: Orthoester and benzylidene routes for the semi-synthesis of CSs from unsulfated chondroitin

Nonetheless, even by accomplishing the protection of free hydroxyls of **1** with acyl groups under several powerful conditions, the final CS polysaccharide still presented to a certain extent an additional sulfate group at *O*-3 of GlcA unit in every case.

Benzylidene and orthoester routes presented differences not only in the cyclic protecting group used for the *O*-4,6 diol on GalNAc units, but also in the work-up employed for the reactions. Indeed, in the benzylidene strategy the polysaccharide was isolated after each step by precipitation, on the contrary the first three steps of the orthoester route were conducted *one-pot* with only a final purification of the intermediate polysaccharide **2** by dialysis (Scheme 6). In order to reduce as many differences as possible between the two semi-synthetic strategies, the isolation of orthoester-protected intermediate **1** was pursued by precipitation. Unfortunately, all the efforts afforded a product showing very complex NMR spectra that was not possible to characterize in details, differently from benzylidene-protected polysaccharide **3**, that displayed benzylidene methine proton (δ_H = 5.53 ppm) and *N*-acetyl (δ_H = 1.76 ppm) signals with a 1:3 integral ratio in its ^{1}H-NMR spectrum (overlapped to ist HSQC-DEPT spectrum in Figure 9).

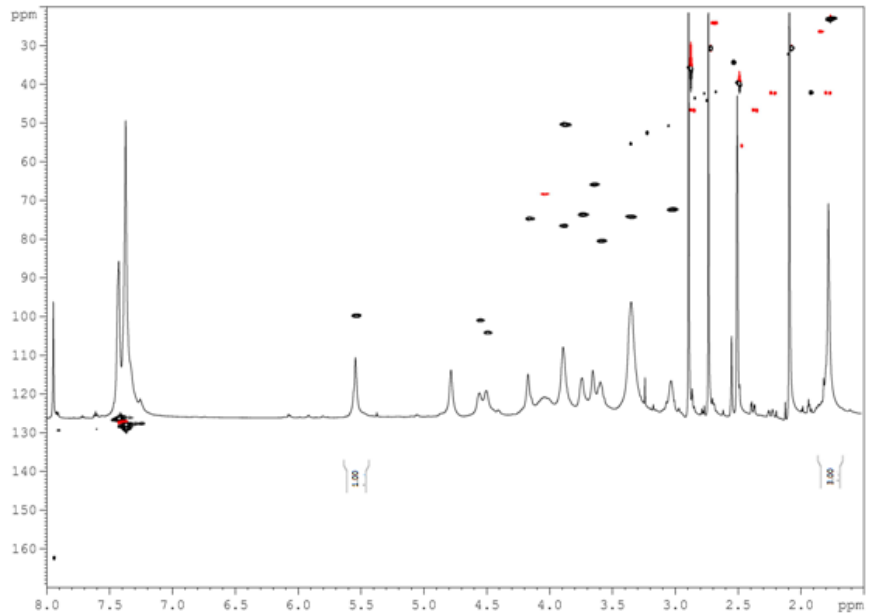


Figure 9: ^{1}H and ^{1}H-^{13}C HSQC-DEPT NMR spectra (600 MHz, DMSO-d_6, 298 K) of polysaccharide **3**

The higher lability of the orthoester moiety prevented the isolation of the polysaccharide intermediate **1**. Thus, it was necessary to get the benzylidene strategy as closest as possible to the orthoester one, instead of opposite. To this aim, benzylidenation and acetylation steps were conducted *one-pot* without the precipitation of the partially protected polysaccharide intermediate **3**. The benzylidene ring was then cleaved under oxidative conditions (Adinolfi et al., **1999**) and, by sulfation of free hydroxyls and then global deprotection, the semi-synthetic pathway was completed without any other difference respect to the original benzylidene route (Scheme 6), (Bedini et al., **2011**) affording in this case a CS polysaccharide possessing sulfate groups at positions *O*-4 or *O*-6 of GalNAc units and also at *O*-3 of some GlcA units, as through the orthoester route. This result was clearly showed in its

HSQC-DEPT spectrum (Figure 10) by the presence of two downfield-shifted carbinolic CH signals (δ_H = 4.75, δc = 77.2 ppm and δ_H = 4.36, δc = 81.9 ppm) – indicating (Sugahara et al., **1996**) a sulfation at *H*-4 of GalNAc and *H*-3 of GlcA units, respectively – and of one group of downfield-shifted CH_2 signals (δ_H = 4.17-4.22, δ_C = 66.9, 68.0 ppm) attribuible to sulfated *H*-6 of GalNAc units.

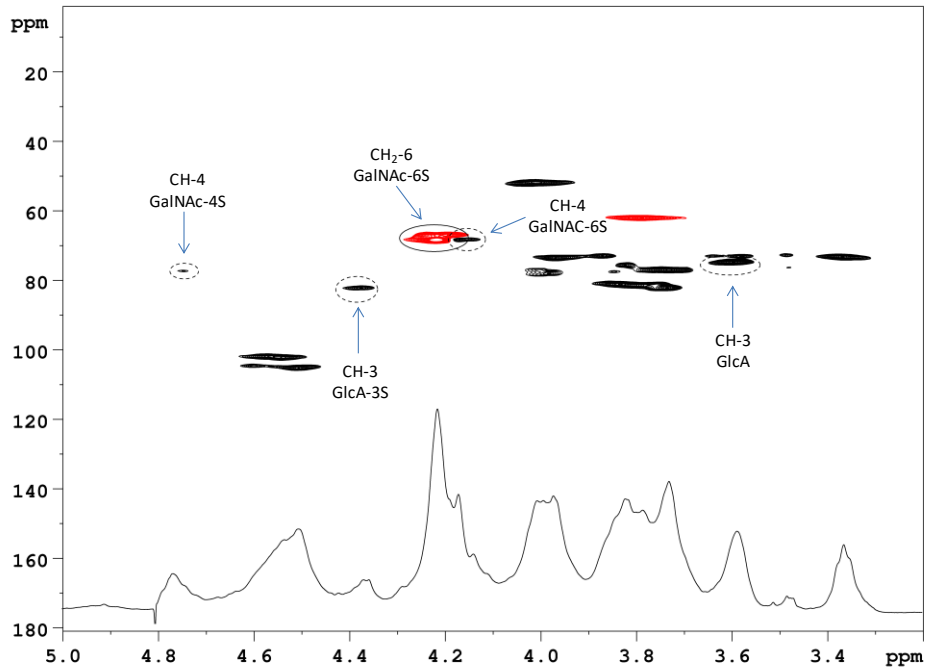
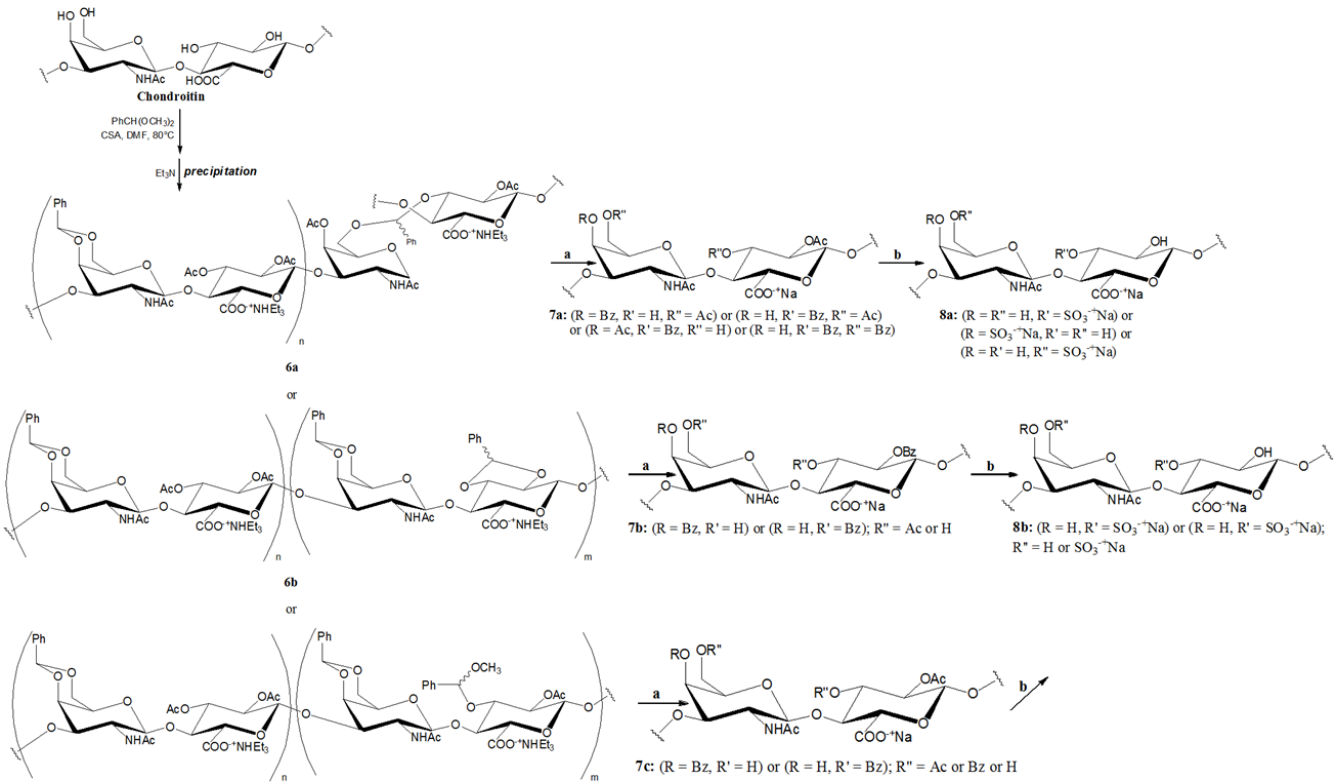


Figure 10: Zoom of ^1H and ^1H-^{13}C HSQC-DEPT NMR spectra (600 MHz, D_2O, 298 K) of semi-synthetic CS polysaccharide obtained through one-pot benzylidene (80°C)-acetylation route (the integral intensities of cross-peaks enclosed in the continous regions were used for the evaluation of the relative amount of sulfated and non-sulfated monose units: see Table 1).

This result was firstly ascribed to a non-quantitative acetylation at *O*-3 position of GlcA caused by the change of solvent from the *two-pot* (acetylation in CH_3CN) to the *one-pot* sequence (acetylation in DMF-CH_3CN or DMF only). To verify this hypothesis, purified intermediate **3** was protected by acetylation in DMF, instead of CH_3CN. By concluding the synthetic route as indicated in Scheme 6, the obtainment of a CS-A,C polysaccharide without any NMR-detectable GlcA-3S unit was afforded. Hence, a DMF effect operating during the acetylation step had to be discarded. Alternatively, the presence of some GlcA-3S units obtained after *one-pot* benzylidenation/acetylation strategy, could be attributed to the formation of unstable benzylidene acetals involving position *O*-3 of some GlcA units that could survive when no work-up was performed (Scheme 7). Instead, they might degrade after benzylidenation work-up by precipitation due to the presence of acid functional groups on the polysaccharide backbone (**3**, Scheme 6).



(a) Na_2S_2O4, $NaBrO_3$, H_2O-ethyl acetate, rt; (b) SO_3·py, DMF, 50°C; NaOH, H_2O, rt

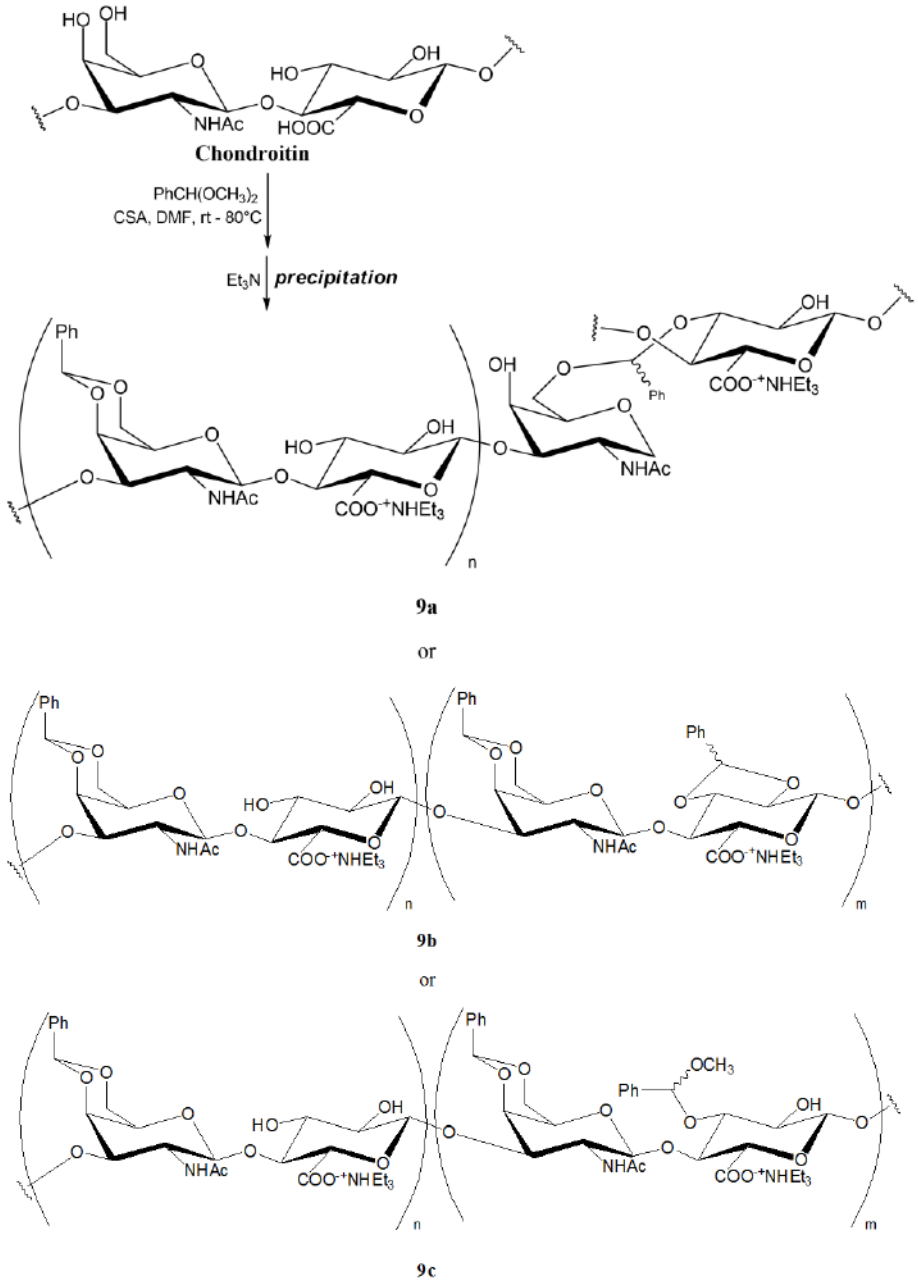
Scheme 7: Inter, *trans*-cyclic or acyclic acetal formation during one-pot benzylidene-acetylation route

Three different labile benzylidene moieties were hypothesized. An interglycosidic acetal involving *O*-3 and *O*-6 of some GalNAc and GlcA units, respectively, could have been formed in competition with a classical intraglycosidic 4,6-*O*-benzylidene, (**6a**, Scheme 7) as reported on some di- and trisaccharides (a) Manzo et al., **2000**. b) Sakairi et al., **1998**. c) Sakairi et al., **1993**. d) Jaramillo et al., **1988**. e) Alonso-Lopez et al., **1987**. f) Fanton et al., **1981**.) and on cyclodextrins. (Sakairi et al., **1996**) Differently, to the best of our knowledge, no examples has been reported on polysaccharides yet. Moreover, an interglycosidic benzylidene acetal has been demonstrated to be selectively cleaved under mild acid conditions in the presence of six-membered intraglycosydic 4,6-benzylidene cycles (Sakairi et al., **1998**), as observed also in our case, as only the latter kind of benzylidene was obtained by precipitation of derivative **3** in the *two-pot* benzylidene-acetylation strategy (Scheme 6). The interglycosidic benzylidene ring in **6a** could be also cleaved under oxidative conditions (Adinolfi et al., **1999**) together with the six-member one, in order to obtain a free hydroxyl group at position *O*-3 of some GlcA units (**7a**, Scheme 7), that after sulfation and global deprotection afforded CS-**8a**.

A second possibility for a labile benzylidene acetal including *O*-3 of some GlcA units was supposed to be a 2,3-*trans*-configured ring (**6b**), as described for gluco-configured hexoses. (a) Français et al., **2007**. b) Thiem et al., **1978**.) It might have been oxidatively cleaved as well, nevertheless the presence of some GlcA-3S in the final CS product might display that this cleavage proceeded with a marked regioselectivity, leaving principally a free hydroxyl group at position *O*-3 and a benzoate ester at *O*-2 (**7b**). Anyway, this hypothesis is in contrast with the oxidative opening of a 2,3-benzylidene ring on a

gluco-derivative under Hanessian-Hullar conditions that furnished an equimolar mixture of *O*-2 and *O*-3 alcohol products (Thiem et al., **1978**). A labile acyclic acetal was, also, hypothesized, (**6c**) as reported for secondary glucose hydroxyls on acetalation of dextrans. (a) Broaders et al., **2009**. b) Bachelder et al., **2008**.) It could be opened under oxidative conditions, by $NaBrO_3/Na_2S_2O_4$ treatment, affording a free alcohol at *O*-3 position of some GlcA units. Nonetheless the amount of acyclic acetal was observed to be approximately zero when acetalation of dextran was performed with $PhCH(OCH_3)_2$. (Cui et al., **2012**)

The hypothesis of these three labile acetals formation was tested by firstly neutralizing the carboxylic acid functions of GlcA and the residual CSA catalyst that usually co-precipitated with the polysaccharide. This was done by quenching the benzylidenation reaction of chondroitin with a stoichiometric excess of triethylamine (Et_3N) before precipitation work-up.



Scheme 8: Isolation of intermediate **9a/9b/9c** by precipitation from the reaction mixture and quenched with Et_3N.

The obtained product **9a/9b/9c** (Scheme 8) afforded at the end of the semi-synthetic steps, as expected, a polysaccharide displaying sulfate groups not only on GalNAc but also at position *O*-3 of some GlcA units. This confirmed that the stabilization in an alkaline environment of a labile acetal on some GlcA units controls the sulfation pattern of the final CS product.

In order to increase the production of the labile acetal, benzylidene conditions were slightly modified. The reaction was conducted under Ar atmosphere by employing freshly dried DMF and $PhCH(OCH_3)_2$ to avoid hydrolysis of the labile acetal by moisture. Moreover, the reaction was conducted at temperatures lower than 80°C, following the benzylidenation step by the standard strategy (Scheme 7), and evaluating the relative sulfation degree at *O*-3 position of GlcA units with respect to unsulfated GlcA ones in the final CS polysaccharides, by integration of HSQC-DEPT spectrum (Figure 11). (Guerrini et al., **2005**)

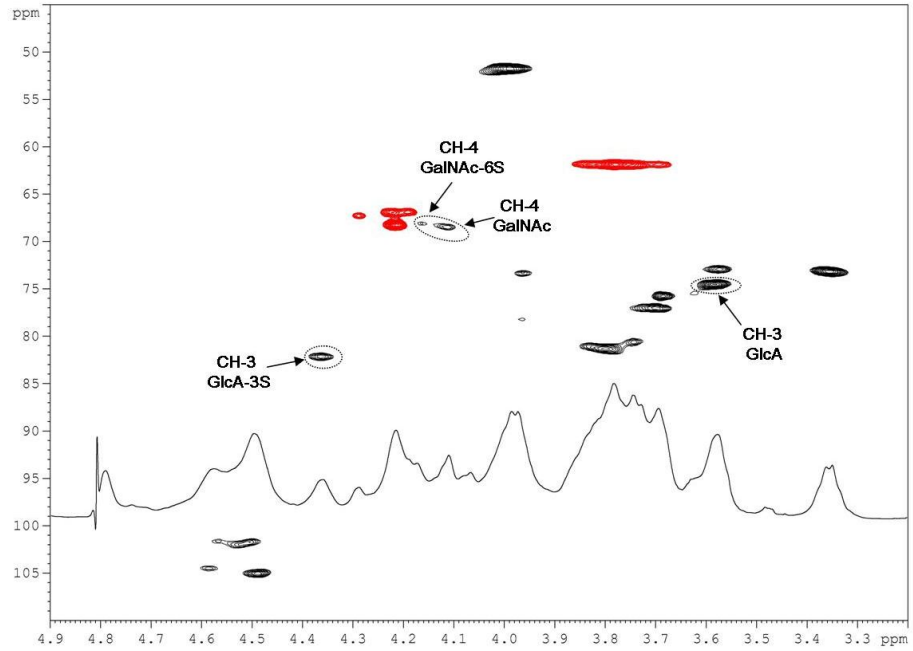


Figure 11: Zoom of ^{1}H and ^{1}H-^{13}C HSQC-DEPT NMR spectra (600 MHz, D_2O, 298 K) of CS obtained with the semi-synthetic strategy based on one-pot benzylidenation (rt)-acetylation (the integral intensities of cross-peaks enclosed in the dotted regions were used for the evaluation of the relative amount of sulfated and non-sulfated monose units: see Table 1).

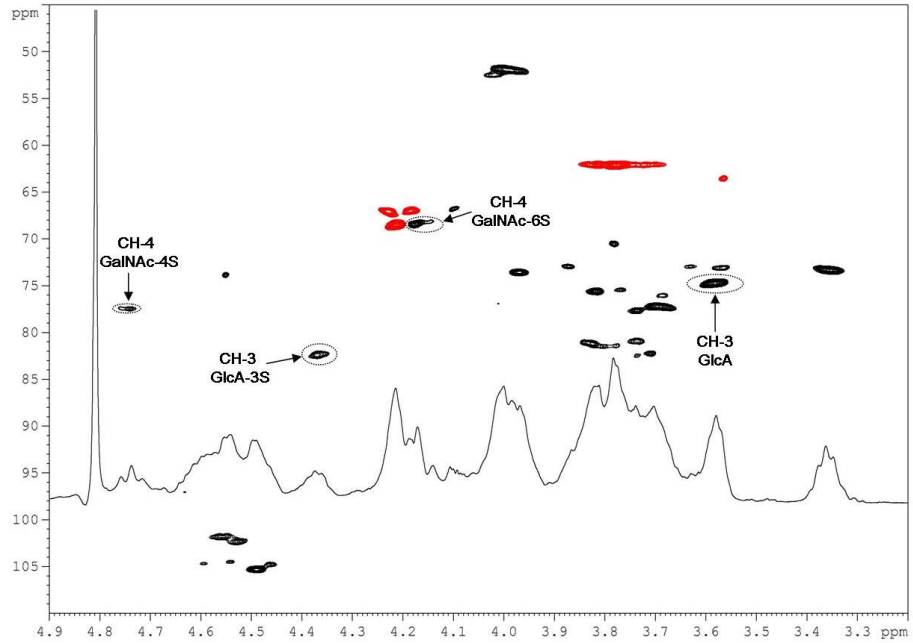


Figure 12: Zoom of ^{1}H and ^{1}H-^{13}C HSQC-DEPT NMR spectra (600 MHz, D_2O, 298 K) of CS obtained with the semi-synthetic strategy based on one-pot orthoesterification (rt)-acetylation (the integral intensities of cross-peaks enclosed in the dotted regions were used for the evaluation of the relative amount of sulfated and non-sulfated monose units: see Table 1).

Table 1: Amount of sulfated and non-sulfated monose units in semi-synthetic CSs depending on reaction conditions for GalNAc 4,6-diol protection

Entry	T [°C][a]	GlcA-3S[b]/GlcA	GalNAc[c]/GalNAc-4 or 6S	GalNAc[c]-4S/GalNAc-6S
1	80	0.29	~0[d]	0.34
2	50	0.27	~0[d]	0.46
3	RT	0.50	2.04	~0[e]
4[f]	RT	0.50	~0[d]	0.59

[a]Referred to benzylidenation reaction conducted one-pot with acetylation, except where differently indicated
[b]Ratio evaluated by integration of *CH*-3 signal of GlcA-3S (δ_H = 4.36, δ_C = 81.9 ppm), and GlcA (δ_H = 3.58, δ_C = 74.4 ppm) units in the HSQC-DEPT spectrum (600 MHz, D_2O, 298 K) (see Figure 10)
[c]Ratio evaluated by integration of *CH*-4 signal of GalNAc (δ_H = 4.12, δ_C = 68.4 ppm), GalNAc-4S (δ_H = 4.75, δ_C = 77.2 ppm), and GalNAc-6S (δ_H = 4.17, δ_C = 68.1 ppm) units in the HSQC-DEPT spectrum (600 MHz, D_2O, 298 K) (see Figure 10)
[d]*CH*-4 signal of unsulfated GalNAc units non detectable in the HSQC-DEPT spectrum (see Figure 10)
[e]*CH*-4 signal of GalNAc-4S not detectable in HSQC-DEPT spectrum (see Figure 10)
[f]Orthoesterification-acetylation semi-synthetic protocol (Bedini et al., **2012**)

By conducting the benzylidenation at 50°C, no increase of GlcA-3S was detected, whereas at rt the ratio GlcA-3S/GlcA increased, giving the same value observed for the *one-pot* orthoesterification-acetylation strategy (Figure 12 and Table 1). (Bedini et al., **2012**) Nonetheless, by lowering the benzylidenation reaction at rt, the amount of unsulfated GalNAc increased, because of a non-quantitative benzylidenation of GalNAc *O*-4,6 diol at rt. The resulting free hydroxyls were then acetylated and deprotected only in the final step, thus avoiding sulfation.

In order to understand which kind of labile benzylidene was responsible for GlcA-3S units in the CSs, it was necessary a 2D-NMR characterization of polysaccharide intermediate **9a/9b/9c**, isolated by

precipitation work-up after the benzylidenation reaction conducted at rt and quenched with Et_3N. The intermediate gave rather clear 2D-NMR spectra in a 4 mM NaOD solution in D_2O, even if the labile acetals formed could be underestimated (Figures 13 and 14).

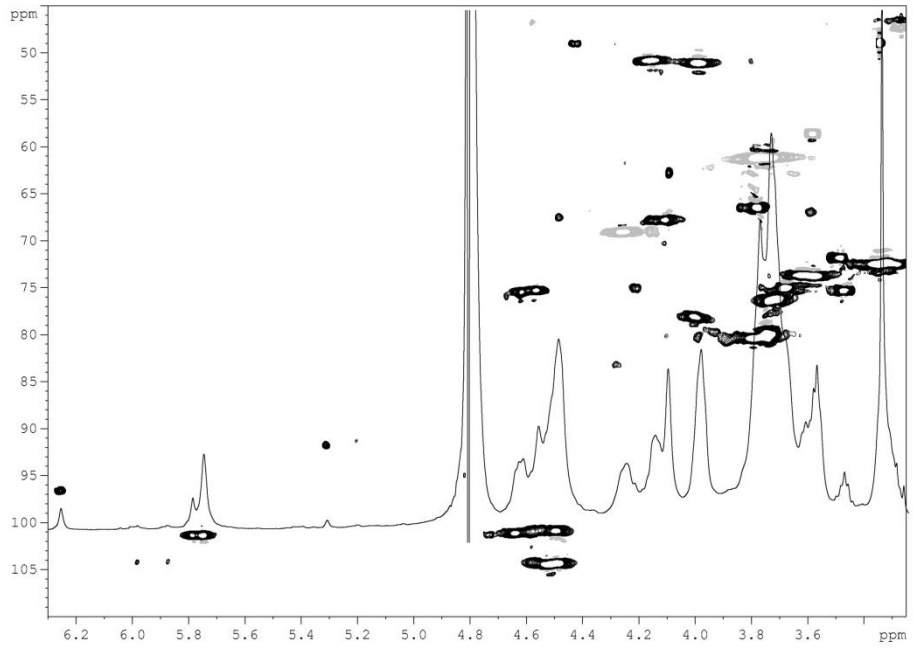


Figure 13: Zoom of ^{1}H and ^{1}H-^{13}C HSQC-DEPT NMR spectra (600 MHz, 4mM NaOD in D_2O, 298 K) of polysaccharide **9a**

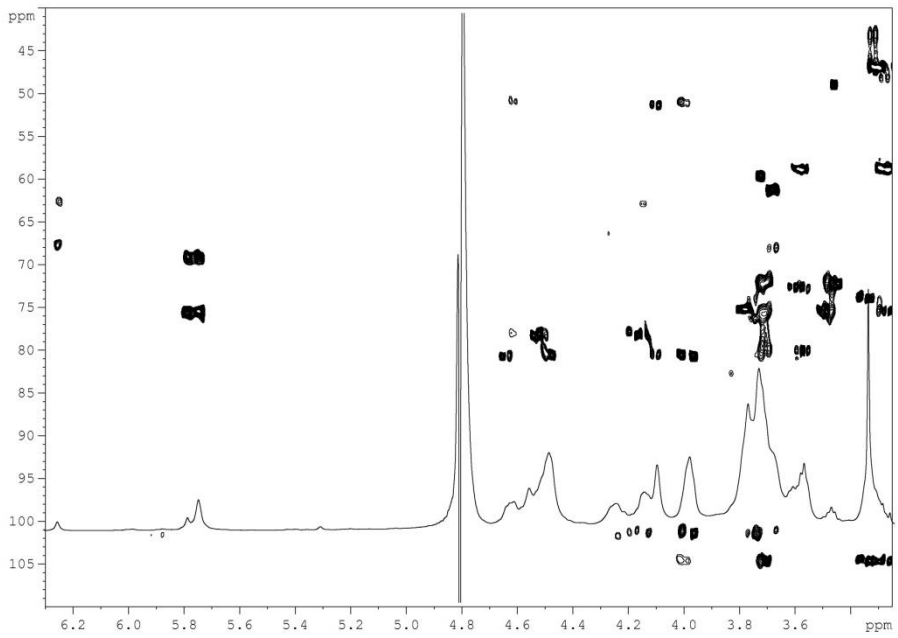


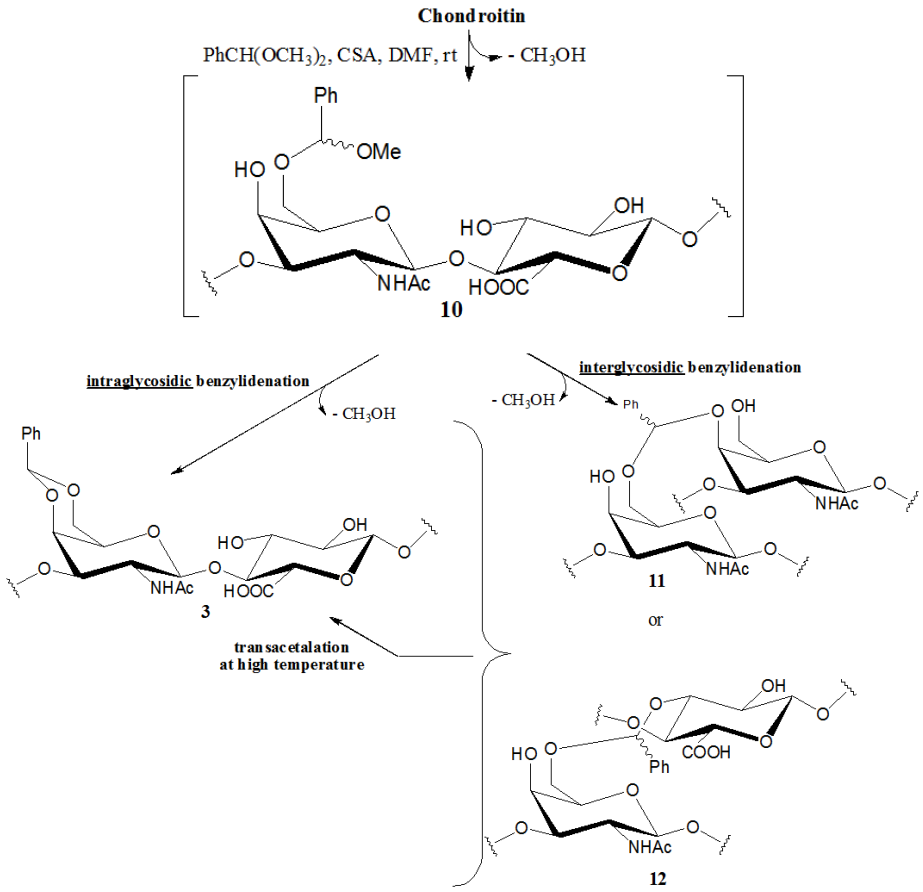
Figure 14: Zoom of ^{1}H, and ^{1}H-^{13}C HMBC NMR spectra (600 MHz, 4mM NaOD in D_2O, 298 K) of polysaccharide **9a**

The HSQC-DEPT spectrum displayed three different benzylidene methine signals, where the most intense one (δ_H = 5.78, δc = 101.6 ppm) correlated with two signals in the HMBC spectrum. The first one at δ_C = 69.5 ppm, was an antiphase signal in the HSQC-DEPT spectrum respect to the other integral intensities of cross-peaks and easily attributable to the methylene group at position *O*-6 of GalNAc (δ_H = 4.25, δ_C = 69.5 ppm). The second one could be ascribed to the *CH* at position *O*-4 of GalNAc (δ_H = 4.58, δ_C = 75.7 ppm) by HSQC-DEPT, COSY and TOCSY analysis, thus allowing the assignment of the most intense benzylidene methine signal to the 4,6-*O*-benzylidene ring on GalNAc. Also the most ^{1}H-downfield shifted benzylidene methine signal showed in the HSQC-DEPT spectrum (δ_H = 6.26, δ_C = 36.9 ppm) correlated with two densities in the H B C spectrum. These were at δ_C

= 67.3 and δ_C = 62.7 ppm, corresponding, respectively, to the signal of *CH* at position *O*-4 of GalNAc units in the HSQC-DEPT spectrum (δ_H = 4.49, δ_C = 67.3 ppm) and to a signal in antiphase with respect to the other integral intensities of cross-peaks and attributable to GalNAc methylene group (δ_H = 3.95, δ_C = 62.0 ppm). These signals could be assigned to an interglycosidic 4,6-*O*-acetal connecting two GalNAc units of the same or two different polysaccharide chains, because the absence of any shift of carbon signals (Mucci et al., **2000**) reminiscent of the NMR behaviour of CH_2 and CH groups involved in acyclic acetals in disaccharide derivatives. (a) Lipták et al., **2002**. b) Barili et al., **1997**.) Nevertheless, the presence of both or only one kind of connection has not been investigated, yet. The third most ^{1}H-upfield shifted benzylidene methine signal (δ_H = 5.32, δ_C = 91.4 ppm) suggested the formation of an interglycosidic benzylidene acetal between GalNAc-*O*-6 and GlcA-*O*-3 positions (**9a**, Scheme 8) in analogy with the interglycosidic 4,6-*O*-benzylidene associated to the most ^{1}H-downfield benzylidene methine proton (δ_H = 6.26 ppm). This was hypotesized by the correlation of benzylidene methine signal with two integral intensities of cross-peaks in the H BC spectrum at δ_C = 62.7 and δ_C = 75.1 ppm, respectively (Figure 14). The former corresponded in the HSQC-DEPT spectrum to a signal (δ_H = 3.65 ppm) in antiphase with respect to the other integral intensities of cross-peaks and thus again attributable to a GalNAc methylene group. Even if it was no possible to assign unambiguously the latter cross-peak in the HSQC-DEPT spectrum, due to overlapping with integral intensities of cross-peaks related to the major underivatized and six-membered benzylidene benzylidene protected disaccharide units, its carbon

chemical shift value (δ_C = 75.1 ppm) was rather similar to the *CH* at position *O*-3 of an underivatized GlcA unit (δ_H = 3.60, δ_C = 73.7 ppm). The *O*-6 GalNAc–*O*-3 GlcA connection could involve adjacent monose along the polysaccharide chain or far way ones in the same chain or in the two different ones. It is worth noting that the analysis of these 2D spectra allowed to discard the alternative hypotheses for the obtainment of CS polysaccharides sulfated at position *O*-3 of GlcA units (**9b** and **9c**, Scheme 8). Indeed, literature data showed rather upfield shifted values (δc ~ 50-53 ppm) for the methoxy group of acyclic benzylidene moieties on sugars (a) Fascione et al., **2010**. b) Mulard et al., **1994**.), thus making the hypothesis of acyclic acetal formation at position *O*-3 of some GlcA (**9c**, Scheme 8) inadmissible. A *trans*-2,3-benzylidene moiety one (**9b**) could be discarded as well, because the integral intensities of cross-peaks at δ_C = 62.0 and 62.7 ppm in the HMBC spectrum were compatible with neither *C*-2 nor *C*-3 of *trans*-configured benzylidene protected GlcA units. (Thiem et al., **1978**) In conclusion, NMR data obtained from HSQC-DEPT and HMBC spectra were compatible only with the formation of interglycosidic benzylidenes as labile acetal motifs. Connections between position *O*-6 of some GalNAc units and position *O*-4 of other GalNAc as well as *O*-3 of GlcA ones were proposed. The former allowed the sulfation of *O*-4 or *O*-6 of GalNAc, whereas the latter connection accounted for the sulfation at *O*-3 of GlcA units in the CSs obtained at the end of the *one-pot* benzylidene-acetylation route.

To summarize, a proposed mechanism for the formation of interglycosidic acetals in competition with a classical six-membered intraglycosidic one during the benzylidenation reaction is displayed in Scheme 9.



Scheme 9: Proposed mechanism for competitive formation of intra- vs. interglycosidic benzylidene acetals on chondroitin.

α,α-Dimethoxytoluene is firstly attacked by the primary, most reactive hydroxyl of GalNAc units, to give acyclic acetal **10**, that can be then intramolecularly transacetalated by hydroxyl at position 4 either of the same GalNAc unit, to obtain a six-membered intraglycosidic benzylidene ring **3**, or of another GalNAc along the same or another polymeric strand as well as by *O*H-3 of GlcA to give interglycosidic acetal moieties (**11** and **12**, respectively). The higher lability of the latters allowed their conversion into the former by another

intramolecular transacetalation, when the benzylidenation reaction was conducted under thermodynamic control conditions. Indeed, at high temperatures (50-80°C) a lower GlcA-3S/GlcA ratio was detected at the end of the semi-synthesis with a benzylidenation conducted at rt.

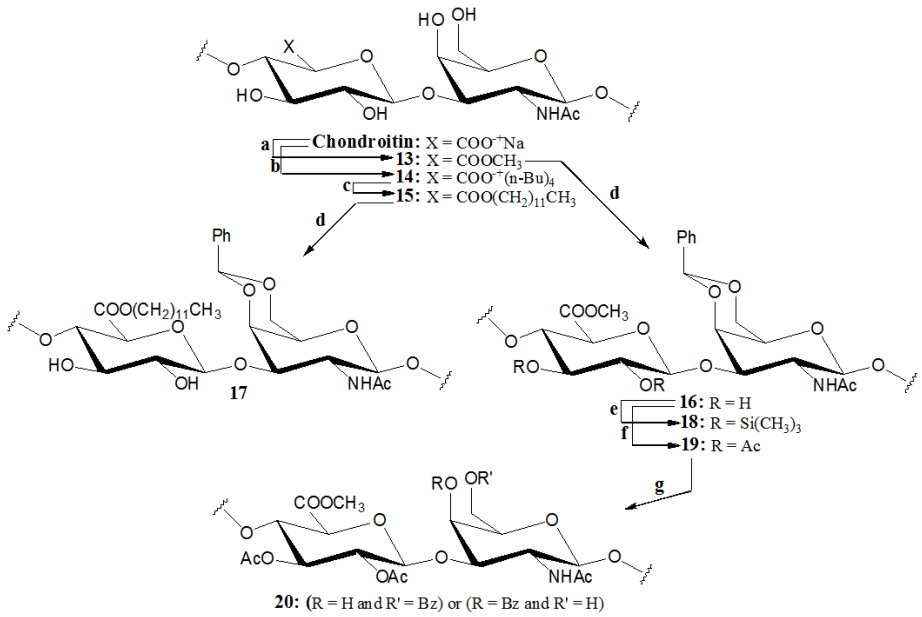
2.2.2 Semi-synthesis of fucosylated chondroitin sulfate

The key step for the obtainment of fCS polysaccharides from microbial sourced chondroitin was the insertion of Fuc branches on the linear polymer backbone of chondroitin by glycosylation reaction.

The only chemical *O*-glycosylation of polysaccharides reported by far regarded, to the best of our knowledge, the coupling between suitably protected polysaccharide derivatives with glycosyl orthoester or glycosyl oxazoline donors under rather harsh conditions to give products displaying mono- or disaccharide branches 1,2-*trans*-linked exclusively to the *O*-6 position of polysaccharide backbone. (a) Ishimaru et al., **2014**. b) Kurita et al., **2011**. c) Kurita et al., **2003**. d) Kurita et al., **1998**.) This route could not be useful for chondroitin fucosylation to give fCS, because α-Fuc branches are usually 1-2-*cis*-linked to a secondary position (GlcA-*O*-3) of the chondroitin backbone (Figure 8). Although recently enzymatic (a) Lezyk et al., **2016**. b) Benesova et al., **2013**. c) Sakurama et al., **2012**. d) Serna et al., **2011**. e) Cobucci-Ponzano et al., **2009**. f) Wada et al., **2008**. g) Cobucci-Ponzano et al., **2008**. h) Farkas et al., **2000**.) and chemical methods (a) Daly et al., **2013**. b) Fujiwara et al., **2011**. c) Adinolfi et al., *Synlett* **2004**. d) P rion et al., **2003**. e) Vermeer et al., **2001**. f) Schmid et al., **1998**.) for fucosylation of mono- and oligosaccharide derivatives have been published, glycosylation reactions involving deoxyhexosyl donors

are generally known to be rather challenging, due to the lower electron-withdrawing effect because of the absence of one (or more) hydroxyls. (Comegna et al., **2007**) This feature could lower the yield of the glycosylation, especially with poorly reactive glycosyl acceptors as polysaccharide derivatives, because the leaving group on deoxyglycosyl donor could release too early the anomeric position.

The semi-synthetic route started with the esterification of chondroitin (obtained from *E. coli* fermentation as described in paragraph 2.2.1) in order to afford polysaccharide derivatives soluble in aprotic solvents. Quantitative methylation was accomplished by stirring twice a methanolic suspension of the polysaccharide in the presence of acetyl chloride at rt overnight (Scheme 10). It was not possible to evaluate the DS of **13** by comparison of integration values of acetyl and methoxy signals (δ_H = 2.02 and 3.85 ppm) in ^{1}H-NMR spectrum due to the overlapping of OCH_3 with some carbinolic signals. However, this was circumvented by HSQC-DEPT integration, that gave an almost quantitative DS (0.92). Alternatively, chondroitin derivatization at carboxylic function with a long alkyl chain (*n*-dodecyl) was accomplished on tetrabutylammonium salt **14** (Valoti et al., **2012**) under conditions optimized for alginate esterification. (Pawar et al., **2013**) Thus, a suspension of **14** in DMF was treated with *n*-dodecyl iodide in the presence of TBAF at 80°C to give chondroitin ester **15** with a DS = 0.96.



Scheme 10: (a) 1: Dowex 50 WX8 (H^+ form), 2: CH_3COOCl, MeOH, rt, twice, 73%, DS = 0.92; (b) see ref. Valoti et al., **2012**; (c) $C_{12}H_{25}I$, TBAF, D F, 80°C, DS = 0.96; (d) α,α-dimethoxytoluene, CSA, DMF, 80°C, 89% (DS = 0.95) for **16**, 97% over two steps from **14** (DS = 0.96) for **17**; (e) $(CH_3)_3SiCl$, $[(CH_3)_3Si]_2NH$, pyridine, 50°C, 88-93% (DS =1.03-2); (f) Ac_2O, Et_3N, DMAP, CH_3CN, rt, overnight, DS = 2; (g) $NaBrO_3$, $Na_2S_2O_4$, H_2O-ethyl acetate, rt, overnight, 63% over two steps (DS = 0.97).

Both derivatives **13** and **15** were then regioselectively protected at the *O*-4,6 diol of GalNAc units with a benzylidene ring by treating them with α,α-dimethoxytoluene in DMF at 80°C in the presence of CSA as catalyst. HSQC-DEPT 2D-NMR integration of integral intensities of cross-peaks related to acetyl and benzylidene methine displayed a DS = 0.95 and 0.96 for **16** and **17**, respectively. It was not possible to determine the DS for derivatives **16** and **17** by comparison of acetyl (δ_H = 1.77 and 1.73 ppm for **16** and **17**, respectively) and benzylidene methine signals (δ_H = 5.51 and 5.48 ppm for **16** and **17**, respectively) in

^{1}H-NMR spectrum due to overlapping of the formers with CSA signals as a contaminant. Methyl ester polysaccharide **14,** differently from derivative **15** (solubility ~ 10 mg/mL)**,** was completely insoluble in CH_2Cl_2-etheral solvent mixtures, that are usually employed for α-stereoselective glycosylations. For this reason, *O*-2,3 diol of GlcA units on derivative **14** was trimethylsilylated. Under the same conditions already reported for trimethylsilylation of chitosan derivatives ($(CH_3)_3SiCl_2$, $[(CH_3)_3Si]_2Na$ in pyridine at rt) (Kurita et al., **1998**), no derivatization was observed and polysaccharide **16** was collected unreacted. By performing the reaction at 50°C overnight, followed by precipitation with diisopropyl ether, polysaccharide **18** was obtained with a quantitative DS (2.0), but not in pure form. Indeed, traces of pyridine were still present and this might have been not compatible with the acid-catalyzed fucosylation reaction. As purification of **18** by further precipitation or azeotropic distillation failed, it was alternatively precipitated from its crude reaction mixture with water, but obtaining in this case a lower DS (1.03) as expected due to TMS ether lability in aqueous mixtures.

In order to enhance the number of different fCS and fC polysaccharides of the target semi-synthetic library, the semi-synthetic route was designed to gain products with Fuc branches linked on either GlcA or GalNAc units. The formers could be obtained with fucosylation of acceptors **16**, **17** and **18** as key step, whereas for gaining the latters, polysaccharide acceptor **20** was prepared from **16**.

Firstly, the *O*-2,3 diol on GlcA units was acetylated with Ac_2O and Et_3N in the presence of a catalytic amount of DMAP affording the quantitatively substituted (DS = 2) polysaccharide **19**. The DS was evaluated by comparison between integration of *H*-2 and *H*-3 carbinolic

signal (δ = 4.65 and 5.04 ppm, respectively) and benzylidene methine one (δ = 5.44 ppm) in the ^{1}H-NMR spectrum (Figure 15).

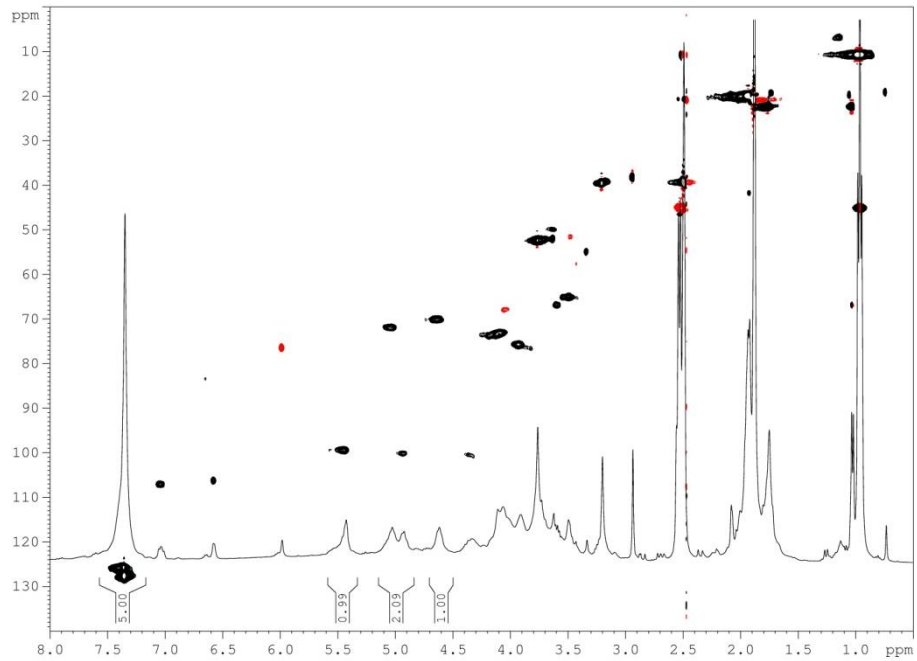


Figure 15: ^{1}H and ^{1}H-^{13}C HSQC-DEPT spectra of polysaccharide **19** (400 MHz, DMSO-d_6, 298 K)

Benzylidene ring was then non regioselectively cleaved under oxidative conditions (Adinolfi et al., **1999**) affording acceptor **20** in 63% yield (over two steps from **16**) with a quantitative DS (0.97) with either 4-*O*- or 6-*O*-benzoylated GalNAc units randomly distributed along the polymer chain. The DS was evaluated by ^{1}H-NMR integration of the *H*-ortho Bz aromatic signals (δ_H = 7.86 and 7.95 ppm) with respect to the Ac ones (δ_H = 1.72-1.98 ppm).

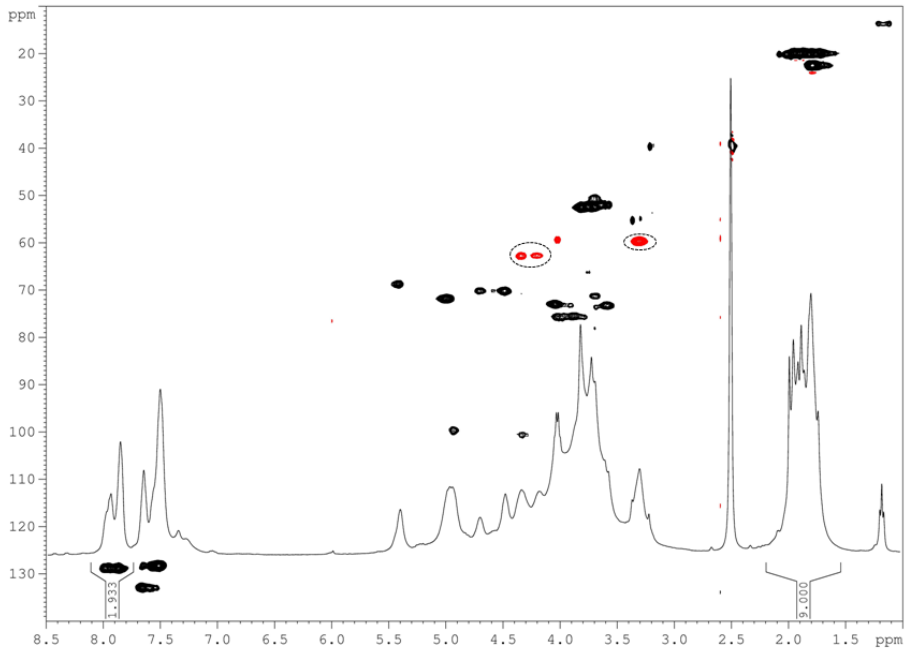
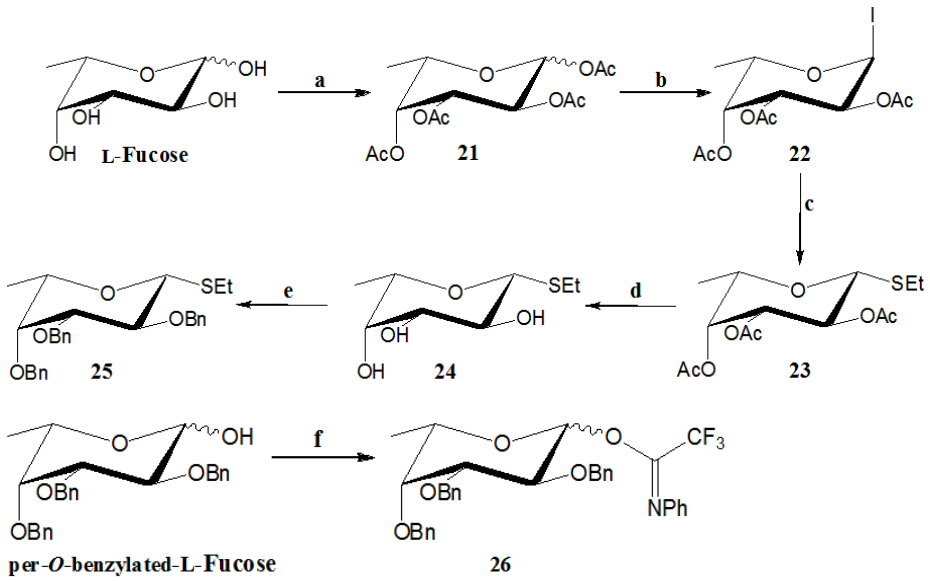


Figure 16: ^{1}H and ^{1}H-^{13}C HSQC-DEPT spectra of polysaccharide **20** (400 MHz, DMSO-d_6, 298 K). Integral intensities of cross-peaks enclosed in dotted circles were integrated for 4-Bz-GalNAc *vs.* 6-Bz-GalNAc evaluation

The ratio 4-*O*-/6-*O*-benzoylated-GalNAc was evaluated to be 1.3 by HSQC-DEPT 2D-NMR integration of the 4-Bz-GalNAc and 6-Bz-GalNAc 6-*O*-methylene signals ($\delta_{H/C}$ = 3.29/59.3 and 4.21-4.34/62.9 ppm, respectively) (Figure 16). Fuc branches were conjugated to chondroitin polysaccharide backbone, by glycosylation of suitable glycosyl donors as per-*O*-benzylated fucosyl thioglycoside **25** (Loon, **1985**) and *N*-phenyl-trifluoroacetimidate **26** (Scheme 11) (Adinolfi et al., **2004**) that were selected for the presence of a non-partecipating ether-type protecting group at position *O*-2, as required for a 1,2-*cis*-stereoselectivity in the glycosylation reaction.

Starting from commercially available L-fucose, through a synthetic strategy involving a peracetylation, a iodination at anomeric position and a thioalkilation in the presence of thiourea ($(NH_2)_2CS$), EtI and Et_3N, derivative **23** was obtained in 53% yield. Successively, acyl groups were removed employing typical Zemplén conditions and free hydroxyls were, in turn, protected by benzyl groups in the presence of BnBr and NaH in DMF, affording glycosyl donor **25** in 75% yield. Alternatively, glycosyl donor **26** was obtained in 57% yield, by treating per-*O*-benzylated-L-fucose with trifluoroacetimidoyl chloride ($CF_3(NPh)Cl$) in basic conditions.

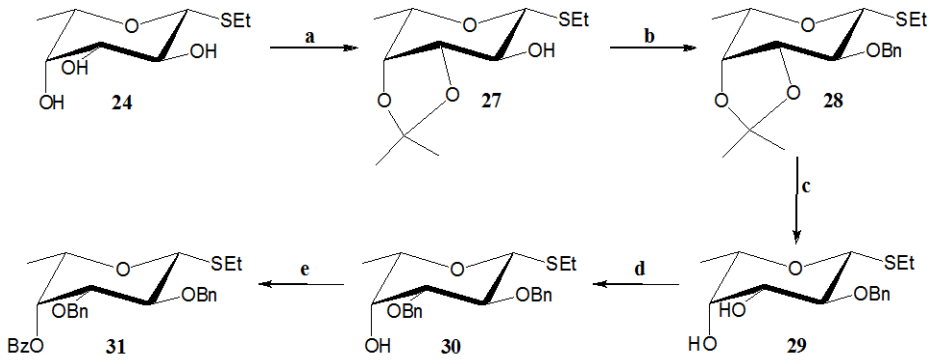
Per-*O*-benzylated fucosyl thioglycoside, as well as the trifluoroacetimidate donor were used for the obtainment of fCS polysaccharides with per-*O*-sulfated Fuc branches.



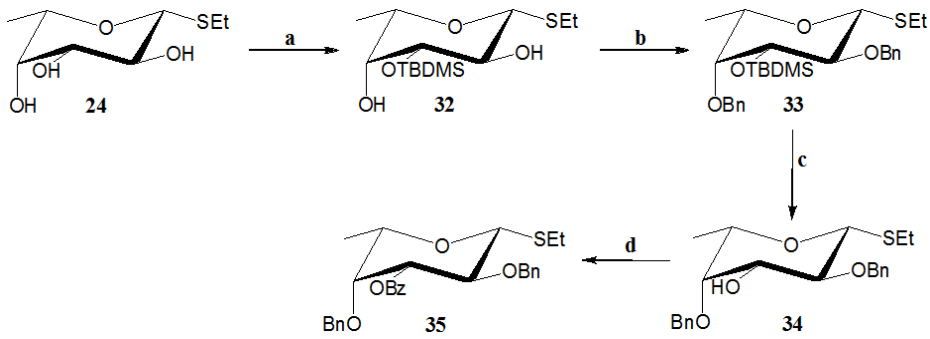
Scheme 11: (a) Ac_2O, pyridine, rt, quant.; (b) I_2, Et_3SiH, CH_2Cl_2, 40°C; (c) $(NH_2)_2CS$, CH_3CN, 60°C; EtI, Et_3N, rt, 53% (over two steps from **21**); (d) Na, MeOH, rt, 83%; (e) BnBr, NaH, DMF, rt, 75%; (f) $CF_3(NPh)Cl$, NaH, CH_2Cl_2, 0°C, 57%

Although these two glycosyl donors were shown to be efficient in the fucosylation of a polysaccharide (see below), the thioglycoside was preferred due to its longer shelf life. Therefore, in order to obtain a different sulfation pattern on Fuc branches, different protecting groups were applied on thioglycoside donors and 2,3-di-*O*-Bn-4-*O*-Bz-Fuc glycosyl donors **31** (Scheme 12) and 2,4-di-*O*-Bn-3-*O*-Bz-Fuc **35** (Scheme 13). The former was obtained by regioselective protection of triol **24** (3-*O*-silylation, benzylation and de-*O*-silylation) (Comegna et al., **2008**) to give **34** (68% overall yield), which was then benzoylated to afford **35** in 73% yield. The latter was synthesized from fucopyranoside **24** too, by protection of *O*-3,4-diol and *O*-2 position by isopropylidene ring and benzyl group, respectively. After isopropylidene opening under acid conditions, affording **29** in 98%

yield, free hydroxyls were benzylated and then benzoylated, obtaining fucosyl donor **35** in 73% yield. (a) Fujiwara et al., **2011**. b) Loon, **1985**.) These were selected as suitable Fuc donors to allow the access to fCSs with 2,3-di-*O*-sulfated or 2,4-di-*O*-sulfated Fuc branches, respectively. This latter enable was chosen as target because it is one of the most commonly found sulfation pattern in natural fCS polysaccharides and also identified to give the strongest anticoagulant activity, even though results suggesting their independence from the different sulfation pattern of Fuc branches were recently published. (Santos et al., **2015**)



Scheme 12: (a) $(CH_3)_2C(OCH_3)_2$, CSA, rt, 98%; (b) BnBr, NaH, DMF, rt, 90%; (c) 4:1 v/v TFA/H_2O, rt, 98%; (d) Bu_2SnO, C_6H_6/MeOH, 60°C; BnBr, Bu_4NI, 60°C, 95%; (e) BzCl, pyridine, CH_2Cl_2, rt, 82%



Scheme 13: (a) TBDMSCl, ImH, DMF, rt, 77%; (b) BnBr, NaH, DMF, rt, 93%; (c) TBAF, THF, MeOH, rt, 95%; (d) BzCl, pyridine, CH_2Cl_2, rt, 73%

To the best of our knowledge, the other two sulfation patterns on Fuc branches (2,3-di-*O*-sulfation and 2,3,4-tri-*O*-sulfation) have never been found in natural polysaccharides, and therefore, this semi-synthetic work will allow to enlarge the structure-activity relationship investigations and could furnish additional standards for the structural characterization of fCSs from new natural sources.

In fucosylations α-stereoselectivity usually relies on the presence of a non-partecipating group at *O*-2 position (Nigudkar et al., **2015**) of a glycosyl donor, even if competitive β-glycosylations (Vermeer et al., **2001**) have been found in literature. Novel glycosyl donors for this purpose were synthesized. (Abronina et al., **2015**) The influence of solvent is also fundamental (Nigudkar et al., **2015**), since the use of an ethereal or nitrilic solvent, rather than CH_2Cl_2, can direct the stereoselective formation of an α- or β-glycosydic bond, respectively. Recently, α-glycosylation procedure (fucosylation included) with thioglycoside donors was developed in the presence of DMF, that displayed a behaviour similar to an ethereral solvent. (Lu et al., **2011**). Therefore, fucosylation reactions of dodecyl ester polysaccharide

acceptor **17** were conducted under homogeneous conditions in 5:3 v/v CH_2Cl_2-DMF or in 3:1 v/v THF-CH_2Cl_2 (Table 2 for details, entries I, II and III). Methyl ester **16** was coupled exclusively in 5:3 v/v CH_2Cl_2-DMF (entries IV, V), because the shorter alkyl chain impeded its solubility in THF-CH_2Cl_2 mixtures, whereas silylated polysaccharide **18** was glycosylated in a ternary mixture of solvents (1:1:1 v/v/v CH_2Cl_2-DME-DMF) ensuring homogenous conditions too (entry VI). Also for coupling between fucosyl donors **25**, **31**, **35** and polysaccharide acceptors **16** and **20** (Table 2 for details, entries VII-XII), glycosylations were conducted under homogeneous conditions choosing NIS-TMSOTf as activator system and 5:3 v/v CH_2Cl_2-DMF as solvent mixture. Only the temperature was decreased to -20°C in some of the reactions with acceptor **16**, because acid-cleavable benzylidene groups could be cleaved by TMSOTf at rt (Table 2 for details, entries VII, VIII, IX).

The glycosylated polysaccharide derivatives were obtained by precipitation from the reaction mixture with suitable solvents. In the case of the coupling reaction between polysaccharide **17** and fucosyl donor **26** under conditions of entry II, the acceptor was recovered unreacted, probably due to quenching of TMSOTf used as catalyst by residual TBAF as an impurity coming from esterification reaction. In the other cases, the co-precipitation of part of Fuc byproducts with the polysaccharides complicated the ^{1}H-NMR spectra, not allowing a correct evaluation of fucosylation degree (DF).

Table 2: Glycosylation reactions of chondroitin acceptors **16-18** and **20** with fucosyl donors **25**, **26**, **31** and **35.**

Entry	Acceptor	Donor	Solvent	Product
I[a]	**17**	**25**	3:1 v/v $THF-CH_2Cl_2$	**36-I**
II[b]	**17**	**26**	3:1 v/v $THF-CH_2Cl_2$	**36-II**
III[c]	**17**	**26**	5:3 v/v CH_2Cl_2-DMF	**36-III**
IV[a]	**16**	**25**	5:3 v/v CH_2Cl_2-DMF	**37-IV**
V[b]	**16**	**26**	5:3 v/v CH_2Cl_2-DMF	**37-V**
VI[a]	**18**	**25**	1:1:1 v/v CH_2Cl_2-DME-DMF	**37-VI**
VII[d]	**16**	**25**	5:3 v/v CH_2Cl_2-DMF	**38-VII**
VIII[d]	**16**	**31**	5:3 v/v CH_2Cl_2-DMF	**38-VIII**
IX[d]	**16**	**35**	5:3 v/v CH_2Cl_2-DMF	**38-IX**
X[a]	**20**	**25**	5:3 v/v CH_2Cl_2-DMF	**39-X**
XI[a]	**20**	**31**	5:3 v/v CH_2Cl_2-DMF	**39-XI**
XII[a]	**20**	**35**	5:3 v/v CH_2Cl_2-DMF	**39-XII**

[a]Reaction conditions: acceptor, donor (5 eq), NIS (5.5 eq), TMSOTf (5.5 eq), AW-300 4Å MS, rt, 4h

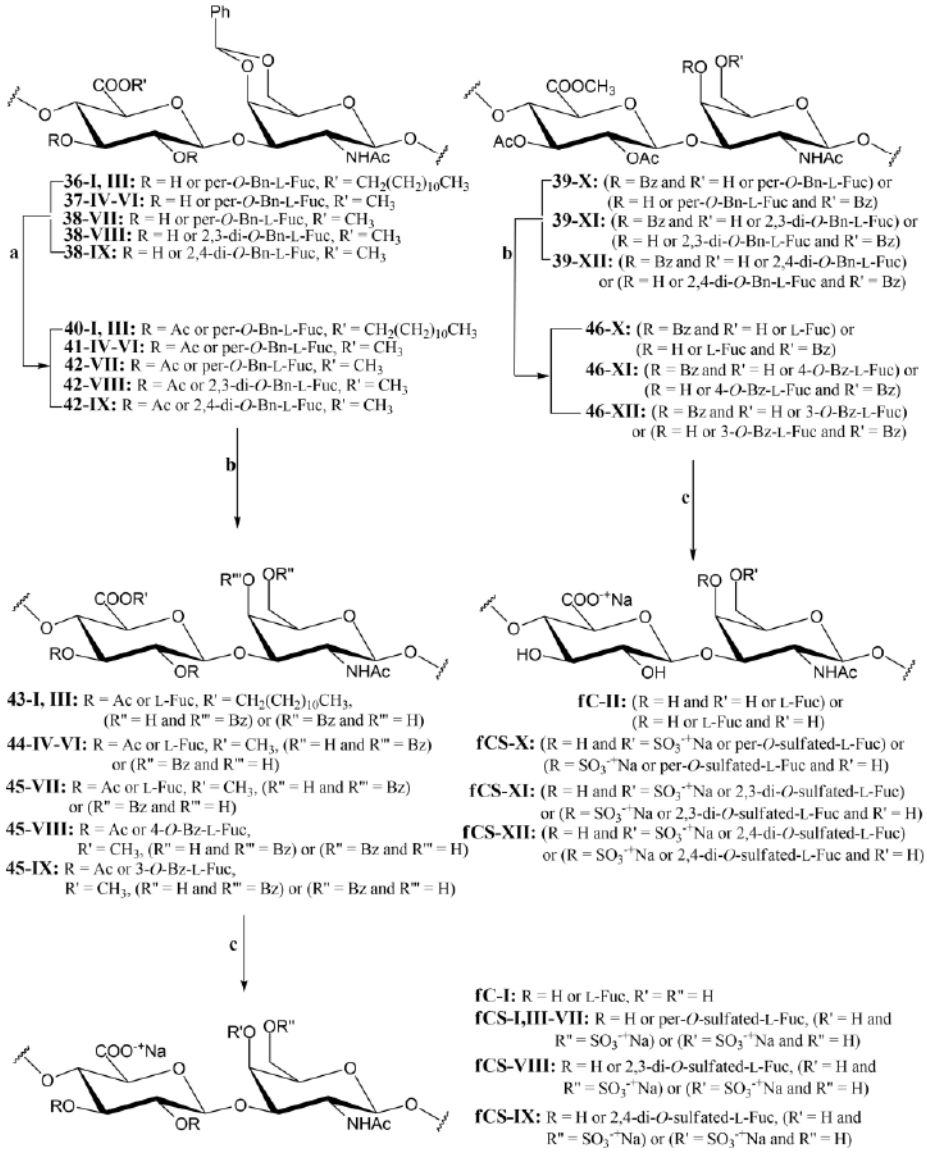
[b]Reaction conditions: acceptor, donor (5 eq), TMSOTf (0.1 eq), AW-300 4Å MS, rt, 4h (inverse procedure)

[c]Reaction conditions: acceptor, donor (5 eq), TMSOTf (0.5 eq), AW-300 4Å MS, rt, 4h (inverse procedure)

[d]Reaction conditions: acceptor, donor (5 eq), NIS (5.5 eq), TMSOTf (5.5 eq), AW-300 4Å MS, -20°C, 4h

Therefore glycosylation derivatives **36-39** were further modified in order to transform them into fCSs and investigate their structural features, without trying to purify them at this stage.

The eight derivatives **36-38** were acetylated, to protect free hydroxyl groups that did not couple during the fucosylations, under homogeneous conditions with Ac_2O in the presence of Et_3N and 4-(dimethylamino)pyridine (DMAP) in CH_3CN (Scheme 14). Benzyl groups were removed to have free hydroxyls - to be then sulfated - under oxidative conditions with $NaBrO_3$ and $Na_2S_2O_4$ in a H_2O-ethyl acetate two phase mixture. (Adinolfi et al., **1999**) Under these conditions benzylidene ring on *O*-4,6 diol was non regioselectively opened, as well as Bn ethers were removed to give derivatives **43-I,III**, **44-IV-VI**, **45-VII-IX**, possessing Fuc branches on GlcA units and **46-X-XII** possessing Fuc branches with some free hydroxyls and one more either at position *O*-4 or *O*-6 of GalNAc units randomly distributed on the polymer chain. (Bedini et al., **2011**)



Scheme 14: (a) Ac_2O, Et_3N, DMAP, CH_3CN, rt; (b) $Na_2S_2O_4$, $NaBrO_3$, H_2O-ethyl acetate, rt; (c) SO_3·py, DMF, 50°C; NaOH, H_2O, rt.

Benzylidene cleavage was confirmed by the splitting and downfield shift of aromatic signals (δ_H = 7.32 ppm for the benzylidene and δ_H = 7.50, 7.65, 7.94 ppm for the resulting benzoate, respectively) in the ^{1}H-

NMR spectra of polysaccharide derivatives **43-I,III**, **44-IV-VI**, **45-VII-IX**. After sulfation of the free hydroxyls with SO_3·pyridine in DMF at 50°C, followed by global deprotection under alkaline hydrolytic conditions **fCS-I, III-XII** were obtained. By skipping the sulfation reaction on **45-VII**, unsulfated fucosylated chondroitin **fC-I** could be also obtained.

The polysaccharides obtained from glycosylation reactions with glycosyl acceptor **20**, were subjected to Bn ether oxidative cleavage (**39-X-XII→46-X-XII**, Scheme 14), followed by sulfation of the deprotected hydroxyls on Fuc and final ester hydrolysis to afford **fCS X-XII**. By skipping the sulfation reaction on **46-I**, **fC-II** was obtained. All the polysaccharides were purified by dialysis, filtration on a C-18 silica gel cartridge and size exclusion chromatography in order to eliminate all the low molecular weight byproducts accumulated during the semi-synthetic route.

The DF of semi-synthetic **fC-(I,II)** and **fCS-(I-XII)** polysaccharides was evaluated through their ^{1}H-NMR spectra, by ratio between Fuc *H*-6 methyl (δ_H = 1.21-1.35 ppm) and GalNAc acetyl signals (2.0-2.10 ppm) integrations. No significant variations among fC and fCS polysaccharides was observed, as the DF value ranged from 0.14 to 1.15 (Table 3, for details). Interestingly, no differences was detected between polysaccharides branched at GlcA and GalNAc positions (**fC-I**, **fCS-I-IV,VII-IX** and **fC-II**, **fCS-X-XII**, respectively), although fucosylation reactions were conducted at different temperatures.

fCS-IV, fCS-V, obtained from methyl ester acceptor **16** had a degree of fucosylation of 0.77 and 1.15 respectively, whereas **fCS-VII** deriving from silylated polysaccharide acceptor **18** showed a DF of 0.14. The degree of fucosylation of fCSs derived from acceptor **17**, instead, had

to be calculated in a different way, due to the presence of residual *n*-dodecyl chain, also after reiteration of the hydrolysis reaction with LiOOH (Lucas et al., **1990**), on polysaccharide backbone of **fCS-I** and **fCS-III**, as showed by a signal at δ_H = 0.88 ppm in their ^{1}H-NMR spectra attributable to methyl group of the chain. Since their β-(ω-1) methylene signals overlap with those of the Fuc methyls, the DF of **fCS-I** and **fCS-III** had to be calculated by applying the following equation:

$$DF = \frac{I\,(CH_3\,Fuc) - \frac{20}{3}\,I\,(CH_3\,dodecyl)}{I\,(CH_3\,NHAc)} \quad (Eq.1)$$

fCS-I and **fCS-III** DF values were similar to those of **fCS-IV** and **fCS-V** ones, nonetheless the former polysaccharides had to be discarded for structure-activity relationship investigations due to the incomplete replacement of the free carboxylic acid moiety on GlcA units.

Table 3: Yield and structural data of **fC-I,II** and **fCS-I-XII**

	Yield[a]	DF[b]	α/β ratio[c]	Branching sites ratio[d]
fC-I	33%	0.34	5.2	0.9[e]
fC-II	39%	0.38	3.5	0.9[f]
fCS-I	56%	0.60	n.d.[i]	n.d.[i]
fCS-III	58%	0.87	n.d.[i]	n.d.[i]
fCS-IV	98%	0.77	2.9	40% : 22% : 38% [g,h]
fCS-V	76%	1.15	2.6	33% : 24% : 43% [g,h]
fCS-VI	89%	0.14	n.d.[i]	n.d.[i]
fCS-VII	41%	0.51	3.6	0.9[e]
fCS-VIII	41%	0.43	5.5	0.6[e,g]
fCS-IX	17%	0.44	2.8	1.1[e]
fCS-X	47%	0.45	4.3	1.3[f]
fCS-XI	38%	0.43	4.0	2.1[f]
fCS-XII	40%	0.50	1.4	2.4[f]

[a]Mass yield determined with respect to starting glycosyl acceptor (**16-18, 20**) over five steps.
[b]Determined by ^{1}H-NMR integration of Fuc methyl and GalNAc acetyl signals.
[c]Estimated by ^{1}H-NMR integration according to Eq.2
[d]Estimated by ^{1}H-NMR integration, except where differently indicated.
[e][α-Fuc-(1→3)-GlcA]/[α-Fuc-(1→2)-GlcA] branching ratio.
[f][α-Fuc-(1→6)-GalNAc]/[α-Fuc-(1→4)-GalNAc] branching ratio.
[g]Determined by HSQC-DEPT integration, due to signals overlapping
[h][α-Fuc-(1→3)-GlcA]/[α-Fuc-(1→2)-GlcA]/[α-Fuc-(1→6)- or α-Fuc-(1→4)-GalNAc] branching percent ratio.
[i]Not determined

The α/β stereochemical ratio of Fuc branching was not evaluated by integration of α- and β-Fuc anomeric signals, because the latter could be unambiguously detected in neither ^{1}H-NMR nor HSQC-DEPT spectra due to low intensity and overlapping with signal of α-Fuc (a) Hua et al., **2004**. b) Mulloy et al., **2000**.) or GlcA and GalNAc units. Therefore, an indirect estimation of α/β ratio was done by evaluating β-Fuc branches amount as a difference of *H*-6 and *H*-1α Fuc signal

integrations – as the former counts for both α- and β-linked Fuc units – according to the following equation:

$$\alpha/\beta = \frac{I\ (CH\_1\alpha\_Fuc)}{\frac{I\ (CH\_6Fuc)}{3} - I(CH\_1\alpha\_Fuc)} \quad (Eq.2)$$

The α/β ratio was found to be rather variable, but with α-stereochemistry clearly predominant in all cases, as expected from the DMF effect described above. (Lu et al., **2011**) It is worth noting that no evident increase in α/β ratio was observed for fucosylation reactions with partially benzoylated Fuc donors that gave **fCS-VIII-IX** and **fCS-XI-XII**, in spite of Bz groups at position *O*-4 and *O*-3, that are believed to enhance the α-stereoselectivity of fucosylation by remote participation. (Gerbst et al., **2001**) Differently, the lowest α/β ratio was observed for **fCS-XII**, deriving from fucosylation with 3-*O*-benzoylated donor **35**.

In order to investigate the regio- and stereochemistry of Fuc branches as well as their sulfation pattern, semi-synthetic **fCS-IV** and **fCS-V** were subjected to a detailed 2D-NMR (COSY, TOCSY, NOESY, HSQC-DEPT and HSQC-TOCSY) analysis first, obtaining for them very similar 2D-NMR spectra.

HSQC-DEPT spectrum of **fCS-IV** (Figure 17 and Table 4) displayed three integral intensities of cross-peaks ($\delta_{H/C}$ = 5.64/98.1, 5.50/98.4, and 5.39/96.7 ppm, respectively) in the typical region for anomeric *CH* of a α-linked sulfated Fuc unit. Thus, three different α-linked Fuc branches (indicated as α-Fuc^{I}, α-Fuc^{II} and α-Fuc^{III}, respectively) was hypothesized to be present on the chondroitin backbone. Moreover, their sulfation pattern was evaluated by COSY and TOCSY spectra

(Figures 18 and 19) allowing the assignment of the chemical shift for *H*-2, *H*-3 and *H*-4 signals of a α-linked Fuc branches with values very similar between each other (δ_H = 4.51-4.53, 4.51-4.61 and 4.87-4.94 ppm for *H*-2, *H*-3 and *H*-4, respectively).

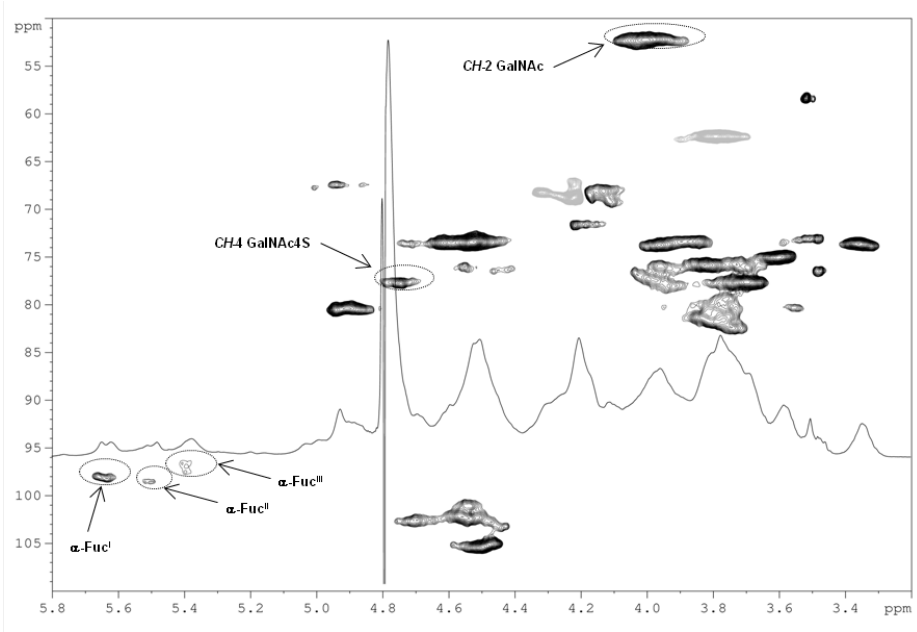


Figure 17: Zoom of ^{1}H and ^{1}H-^{13}C HSQC-DEPT NMR spectra of **fCS-IV** (600 MHz, D_2O, 298 K). Integral intensities of cross-peaks enclosed in the dotted circles of HSQC-DEPT spectrum were integrated for Fuc branching site regio- and stereochemistry as well as GalNAc-4,6-sulfation assessment

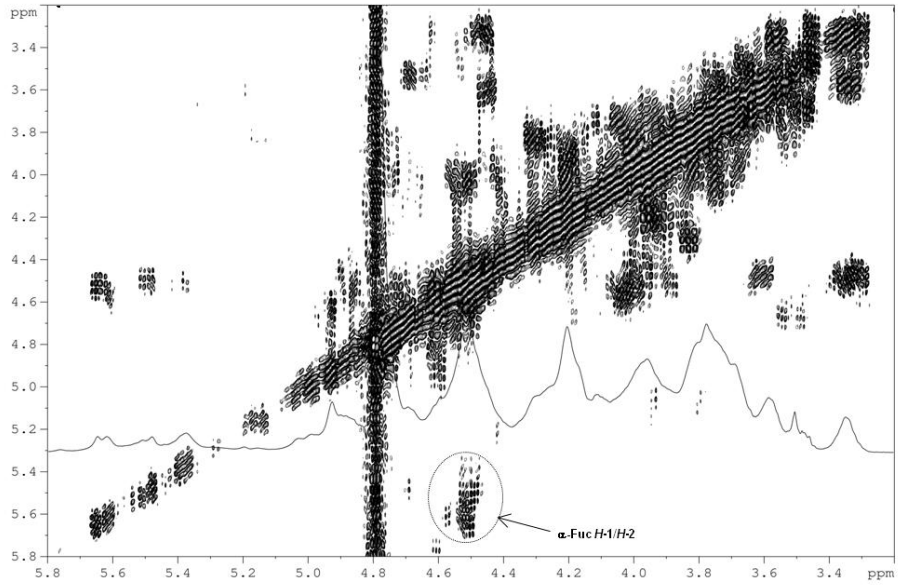


Figure 18: Zoom of ^{1}H and COSY NMR spectra of **fCS-IV** (600 MHz, D_2O, 298 K)

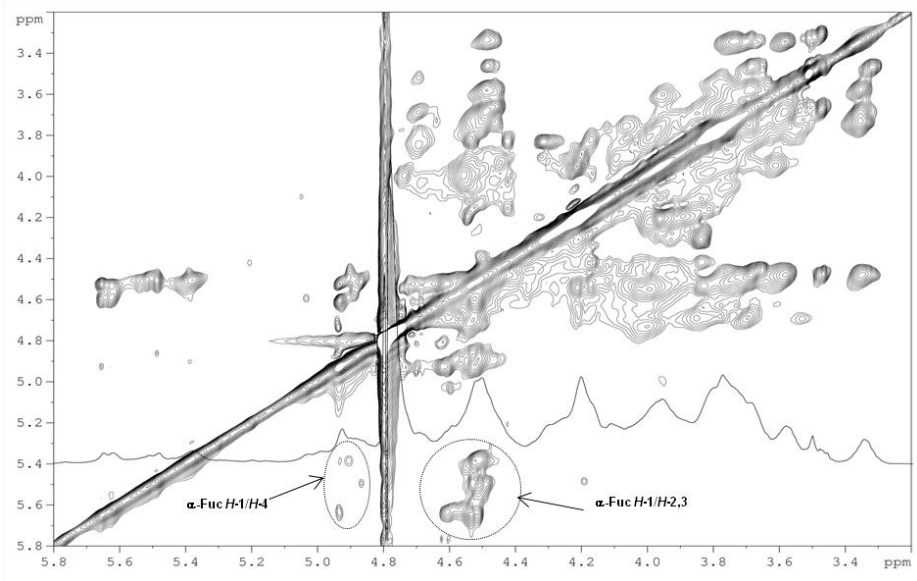


Figure 19: Zoom of ^{1}H and TOCSY NMR spectra of **fCS-IV** (600 MHz, D_2O, 298 K)

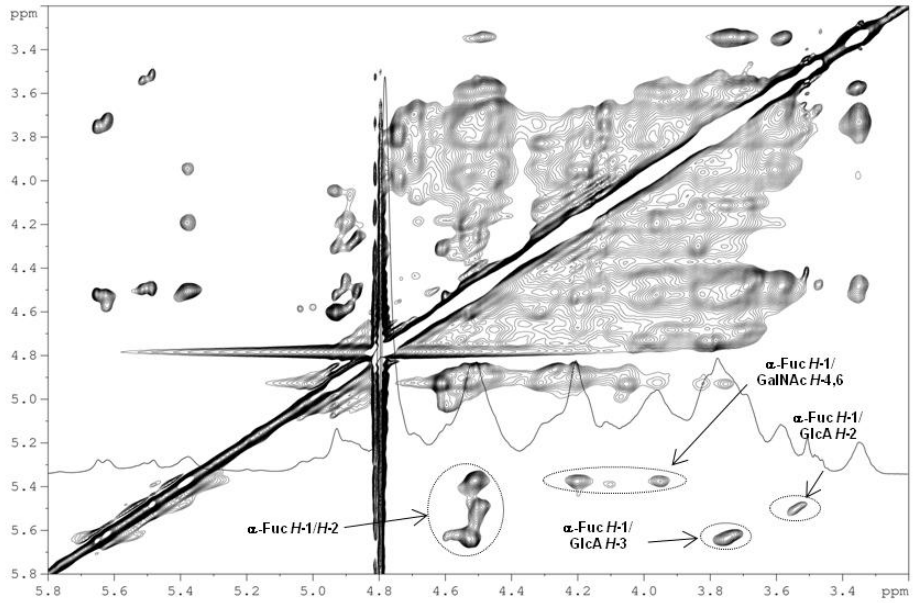


Figure 20: Zoom of ^{1}H and NOESY NMR spectra of of **fCS-IV** (600 MHz, D_2O, 298 K)

Their downfield shifts suggested a quantitative cleavage of benzyl protecting groups on Fuc branches under oxidative conditions that gave a 2,3,4-triol (**41-IV→44-IV, Scheme 14), that was then quantitatively sulfated in the subsequent step. The same sulfation pattern and stereochemistry of linkage for Fuc^{I}, Fuc^{II} and Fuc^{III} hypothesized that they are differentiated for a distinct branching site on the polysaccharide backbone. This was investigated by means of NOESY spectrum (Figure 20) that revealed a correlation between Fuc^{I} signal (δ_H = 5.64 ppm) with a integral intensity of cross-peak at δ_H = 3.73 ppm, attributable to *H*-3 of some GlcA units by means of the other 2D-NMR spectra and literature data. (Panagos et al., **2014**. b) Chen et al., **2011**.)**

In a similar way, Fuc^{II} signal (δ_H = 5.50 ppm) was assigned to the anomeric *CH* of a Fuc branch linked at *O*-2 ($\delta_{H/C}$ = 3.54/80.3 ppm) of

some GlcA units. The presence of both fucosylated GlcA *O*-2 and *O*-3 positions was also confirmed by the correlation between Fuc methyl integral intensity of cross-peak ($\delta_{H/C}$ = 1.31-1.33/17.2 ppm) and Fuc-*CH*-5 signals ($\delta_{H/C}$ = 4.94/67.4 and 4.22/67.7 ppm) in COSY, TOCSY and HSQC-TOCSY spectra. Indeed, a marked difference between the chemical shift of *H*-5 atom of *O*-2 and *O*-3-linked α-Fuc units has been recently highlighted. (Panagos et al., **2014**) Two inter-residue correlation for Fuc^{III} anomeric signal (δ_H = 5.39 ppm) were showed by NOESY spectrum (Figure 20): a major one at δ_H = 4.21 ppm and a minor one at δ_H = 3.95 ppm. By HSQC-DEPT spectrum, the former was associated to *O*-6 (δ_C = 67.4 ppm) of some GalNAc units. Since the DS for benzylidenation reaction of acceptor **13** was nearly quantitative (Scheme 10), a partial acidic cleavage of the benzylidene ring mediated by TMSOTf during the fucosylation step may explain this finding. The minor NOESY correlation from Fuc^{III} anomeric signal was not unambiguously assigned, nonetheless the existence of a benzylidene cleavage process during glycosylation suggested that *O*-4 position of some GalNAc units was fucosylated. Relative integration of Fuc^{I}, Fuc^{II}, Fuc^{III} HSQC-DEPT integral intensities of cross-peaks returned an estimate of relative Fuc branching degree at positions GlcA-*O*-3, GlcA-*O*-2 and GalNAc-(*O*-4,6), respectively (Table 3).

The presence in the HSQC-DEPT spectrum (Figure 17) of signals of both sulfated ($\delta_{H/C}$ = 4.75/77.6 ppm) and non-sulfated ($\delta_{H/C}$ = 4.08-4.14/67.9-68.9 ppm) *CH* at position *O*-4 as well as sulfated ($\delta_{H/C}$ = 4.21-4.32/68.2-68.7 ppm) and non-sulfated ($\delta_{H/C}$ = 3.79/62.4 ppm) *CH* at position *O*-6 of GalNAc units. (a) Bedini et al., **2011**. b) Mucci et al., **2000**.) suggested a typical A,C sulfation pattern on chondroitin backbone. This is in agreement with the known non-regioselective

oxidative opening of the benzylidene ring to give a free hydroxyl at either 4-*O*- or 6-*O*-position of GalNAc units (Bedini et al., **2011**) before sulfation step. By HSQC-DEPT relative integration of GalNAc *CH*-4 signal ($\delta_{H/C}$ = 4.75/77.6 ppm) with respect to the *CH*-2 signal of whichever GalNAc unit ($\delta_{H/C}$ = 4.02/52.2ppm) a 26% of sulfation degree at position *O*-4 was estimated, whereas evaluation of *O*-6 sulfation degree by integration of sulfated GalNAc *CH*-6 signal ($\delta_{H/C}$ = 4.21-4.32/68.2-68.7 ppm) was complicated by overlapping with other ones in the HSQC-DEPT spectrum.

Table 4: ^{1}H (plain) and ^{13}C (italic) chemical shift attribution of **fCS-IV** (600 MHz, D_2O, 298 K)

Residue[a]	**1**	**2**	**3**	**4**	**5**	**6**	**Other signals**
α-Fuc2,3,4S^{I}-(1→3)-GlcA	5.64 *98.1*	4.53 *73.6*	4.61 *73.4*	4.94 *80.3*	4.94 *67.4*	1.33 *17.2*	---
α-Fuc2,3,4S^{II}-(1→2)-GlcA	5.50 *98.4*	4.51 *73.6*		4.87 *80.3*	4.22 *67.7*	1.31 *17.2*	---
α-Fuc2,3,4S^{III}-(1→4 or 6)-GalNAc	5.39 *96.7*			4.91 *80.3*	n.d.[b]	n.d.[b]	---
GalNAc6S	4.55 *102.2*	4.02 *52.2*	3.82 *81.4*	4.04-4.14 *67.9-68.9*	3.84-3.96 *73.3-73.6*	4.21 - 4.32 *68.2 - 68.7*	Ac 1.96-2.05 *23.9*
GalNAc4S			3.59 *76.9*	4.75 *77.6*	3.81 *75.8*	3.79 *62.4*	
α-Fuc2,3,4S^{III}-(1→6)-*GalNAc*	n.d.[b]		n.d.[b]	n.d.[b]	3.84-3.96 *73.3-73.6*	4.15 *67.4*	
GlcA	4.48 *105.6*	3.33 *73.6*	3.53 *74.9*	3.73 *81.3*	3.71 *77.5*	---	---
α-Fuc2,3,4S^{I}-(1→3)-*GlcA*		3.57 *73.1*	3.73 *82.3*	n.d.[b]		---	---
α-Fuc2,3,4S^{II}-(1→2)-*GlcA*	4.70 *102.4*	3.54 *80.3*	3.69 *76.1*	3.85 *81.4*		---	---

[Chemical shift expressed in δ relative to internal acetone (^{1}H: $(CH_3)_2CO$ at δ = 2.22 ppm; ^{13}C: $(CH_3)_2CO$ at δ = 30.9 ppm)]

[a]Indicated by italic characters.

[b]Not determiable.

A 2D-NMR analysis was conducted also on **fC-I**, **fC-II** and **fCS-VII-XII** in order to investigate the regiochemistry of the Fuc branches, and limited to the latter polysaccharides, the sulfate groups distribution. HSQC-DEPT spectrum of **fC-I** (Figure 21) showed at highest chemical shift values the presence of two integral intensities of cross-peaks ($\delta_{H/C}$ = 5.38/99.5 and 5.25/100.4 ppm), that were assigned to the anomeric *CH* of the two α-linked Fuc units. The cross-peakes in NOESY spectrum related to these signals showed a correlation of the more downfield shifted signal (δ_H = 5.38 ppm) with a integral intensity of cross-peak at δ_H = 3.70 ppm, attributable to GlcA *H*-3 by means of the other 2D-NMR spectra and literature data. (Panagos et al., **2014**) In the same way, the other *H*-1 α-Fuc signal (δ_H = 5.25 ppm) was assigned to a branching unit linked at *O*-2 ($\delta_{H/C}$ = 3.50/78.6 ppm) of some GlcA units. The assignment of Fuc branches site was repeated for **fC-II** and **fCS-VII-XII** applying the same approach. By the integration of the anomeric α-Fuc signal in the ^{1}H-NMR spectra was displayed that polysaccharides obtained by glycosylation of acceptor **16** at -20°C (**fC-I** and **fCS-VII-IX**) had no preference for GlcA *O*-2 or *O*-3 position, with the branching site ratio close to 1 in all cases (Table 3). Differently, for the polysaccharides obtained from acceptor **20** (**fC-II** and **fCS-X-XII**) fucosylation was slightly preferred at *O*-6 with respect to *O*-4 of GalNAc (6-OH/4-OH ratio of 1:3), but limited to **fCS-XI,XII**.

2D-NMR spectra analysis confirmed that reactions conducted on glycosyl acceptor **15** at -20°C gave derivatives possessing Fuc branches only at positions *O*-2 and *O*-3, avoiding benzylidene cleavage, differently from **fCS-IV** that was obtained from the same acceptor, by

conducting fucosylation at rt. Indeed, this reaction gave derivative bearing Fuc branch also on GalNAc units.

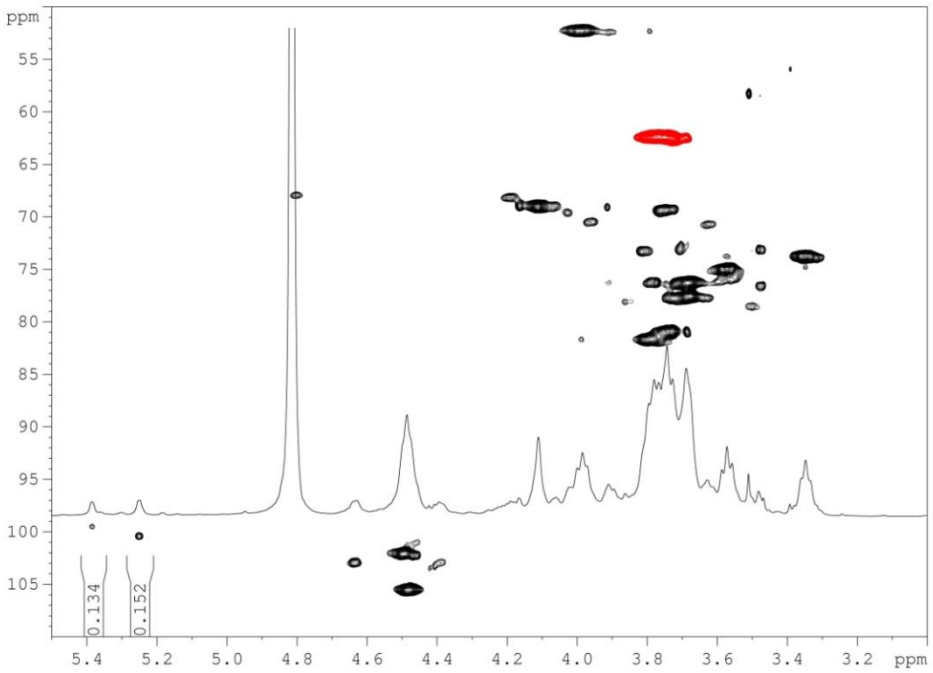


Figure 21: Zoom of ^{1}H and ^{1}H-^{13}C HSQC-DEPT NMR spectra of **fC-I** (600 MHz, D_2O, 298K)

In the case of fCS polysaccharides, NMR confirmed the Fuc sulfation patterns expected from the position of orthogonally cleavable (Bn) and permanent (Bz) protecting groups on fucosyl donors **25**, **31** and **35** used in glycosylations. For example, the cross-peaks related to anomeric α-Fuc signals in the COSY, TOCSY and NOESY spectra confirmed the difference in the sulfation pattern of Fuc units α-linked to *O-4* or *O-6* atom of GalNAc residues in **fCS-X-XII**. Indeed, fucosylation reaction with per-*O*-benzylated thioglycoside **25** afforded **fCS-X** with α-Fuc

branches displaying *H*-2, *H*-3 and *H*-4 signals all downfield shifted for *O*-sulfation (δ_H = 4.52, 4.70-4.74 and 4.95 ppm, respectively), whereas only *H*-2 and *H*-3 signals showed a similar shift (δ_H = 4.53, 4.67-4.69 ppm, respectively; δ_H = 4.22 for *H*-4) in **fCS-XI** (deriving from 2,3-di-*O*-benzylated donor **31**, as well as *H*-2 and *H*-4 ones in **fCS-XII** (δ_H = 4.41, 4.69 ppm, respectively; δ_H = 4.07-4.20 ppm for *H*-3), that was obtained by glycosylation with 2,4-di-*O*-benzylated thioglycoside **35** (Figure 22). The same NMR analysis was applied to confirm the differences in α-Fuc sulfation pattern of **fCS-VII-IX**.

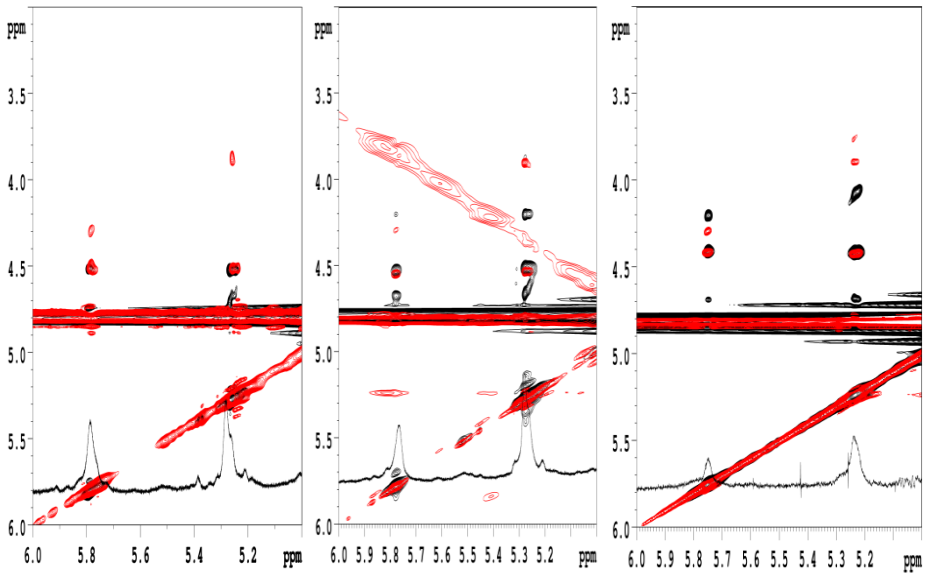


Figure 22: Zoom of TOCSY (black) and NOESY (red) spectra of **fCS-X** (left), **fCS-XI** (middle), **fCS-XII** (right) (600 MHz, D_2O, 298 K)

2-D NMR analysis also allowed to define the sulfation pattern in residue other than Fuc branches, as already discussed for **fCS-IV**. For example, the HSQC-DEPT spectrum of **fCS-X** (Figure 23) displayed signals of both sulfated ($\delta_{H/C}$ = 4.22/68.8 pppm) and non-sulfated

(fucosylated or not: $\delta_{H/C}$ = 3.90/69.4 or 3.71-3.85/62.5 ppm, respectively) *CH*$_2$ at postion 6, as well sulfated ($\delta_{H/C}$ = 4.70/76.1 and 4.74/77.7 ppm) and non-sulfated (fucosylated or not: $\delta_{H/C}$ = 4.29/73.8 or 4.17/68.8 ppm, respectively) *CH* at position 4 of GalNAc units (a) Bedini et al., **2011**. b) Mucci et al., **2000**.) This was in agreement with the non-regioselective distribution of free hydroxyls at either position 4 or 6 of GalNAc on acceptor **20** together with the their non quantitative fucosylation before the sulfation reaction, as already discussed above (DF = 0.45, Table 3). An estimation of sulfation degree at position *O*-4 and *O*-6 was possible by HSQC-DEPT integral intensities of cross-peaks integration. Due to partial overlapping of the integral intensities of cross-peaks related to *CH*$_2$ at position 6 of GalNAc6S and *CH* at position 4 of unsulfated and non-fucosylated GalNAc, the integrations were made with respect to the *CH*-2 integral intensity of cross-peak of whichever GalNAc unit ($\delta_{H/C}$ = 4.01/52.2 ppm). In particular, the relative integration of GalNAc4S *CH*-4 integral intensities of cross-peaks with the *CH*-2 one gave a 0.25 degree of sulfation position *O*-4. A 0.33 value was estimated for position *O*-6, by integrating the integral intensities of cross-peaks related to unsulfated GalNAc units (6-*O*-fucosylated or not) with respect to the *CH*-2 one and then applying the following equation:

$$O\text{-6 degree of sulfation} = 1 - \frac{I[(CH_2\_6\ GalNAc-(6-OH)]+ [(CH_2\_6\ GalNAc-(6-Fuc)]}{2*I(CH\_2\ GalNAc)]} \quad (Eq.3)$$

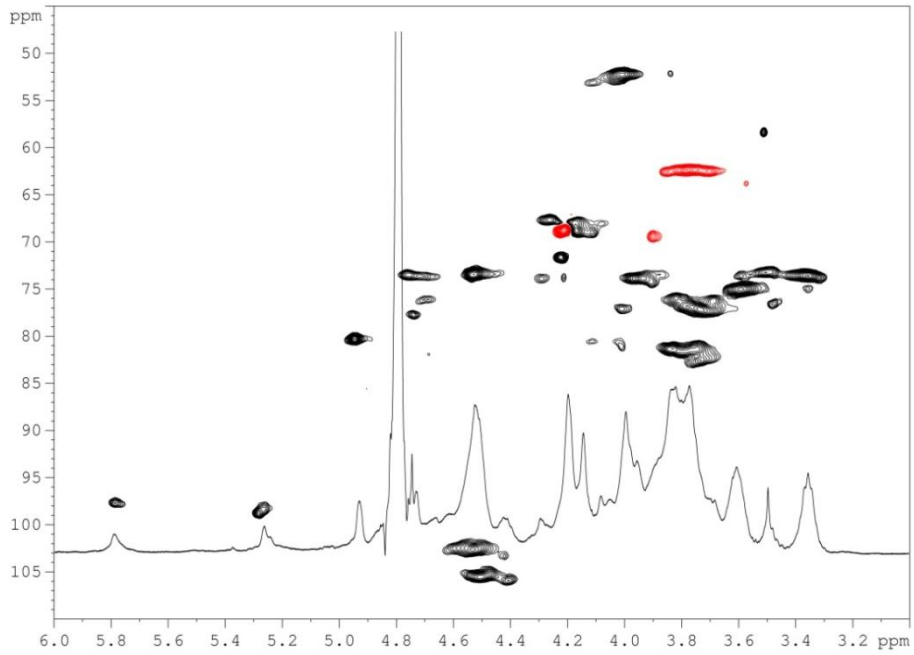


Figure 23: Zoom of ^{1}H and ^{1}H-^{13}C HSQC-DEPT spectrum (600 MHz, D_2O, 298 K) of **fCS-X**

In the case of **fCS-VIII** and **fCS-IX** polysaccharides, after a careful investigation of the 2D-NMR spectra, a small amount of sulfate groups also on GlcA units was detected, as demonstrated by the presence of characteristic signals for *O*-2 and *O*-3 sulfation at $\delta_{H/C}$ = 4.11/80.5 (Gargiulo et al., **2009**) and 4.35/82.5 (Bedini et al., **2012**) ppm, respectively. This was ascribed to a non-quantitative acetylation of GlcA hydroxyls (**38-VII-IX → 42-VII-IX**) that left some free alcohol moieties ready to be sulfated at the end of the semi-synthetic route (Scheme 14). As for GalNAc units, also in the GlcA case sulfation degrees at *O*-2 and *O*-3 positions could be estimated by integration of these integral intensities of cross-peaks with respect to the GalNAc *CH*-2 one in the HSQC-DEPT spectrum (Figure 24).

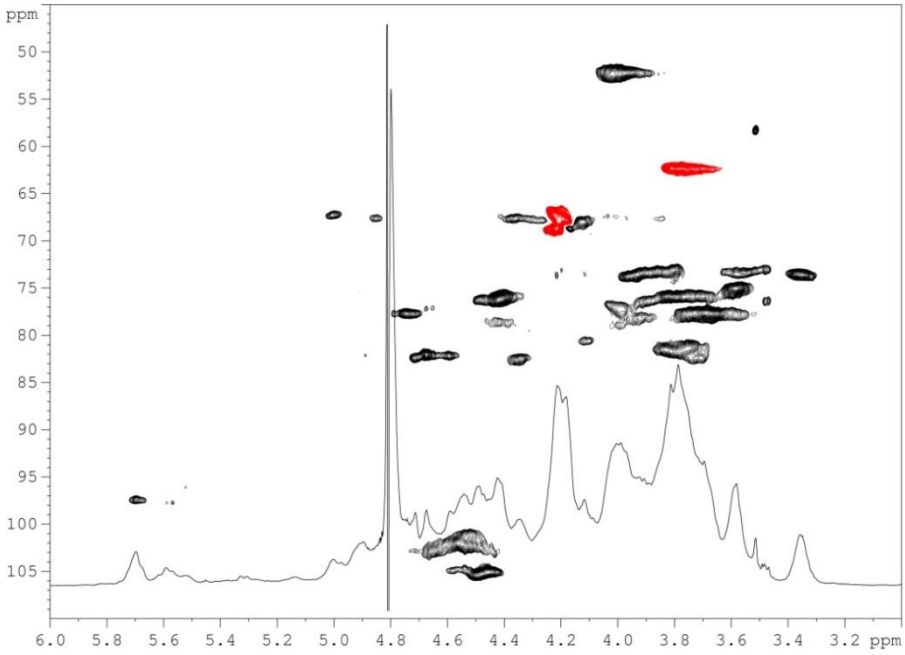


Figure 24: Zoom of ^{1}H and ^{1}H-^{13}C HSQC-DEPT spectrum (600 MHz, D_2O, 298 K) of **fCS-XI**

Comprehensive tables of NMR assignments for the structural characterization of the all obtained fC and fCS polysaccharides are characterised in the following pages.

Table 5: ^{1}H (plain) and ^{13}C (italic) chemical shift attribution of **fC-I** (D_2O, 600 MHz, 298 K)

Residue[a]	**1**	**2**	**3**	**4**	**5**	**6**	**Other signals**
α-Fuc- (1→2)-GlcA	5.25 *100.4*	3.75 *69.4*	3.71 *73.0*	3.63 *70.7*	4.19 *68.1*	1.19 *16.6*	---
α-Fuc- (1→3)-GlcA	5.38 *99.5*		3.81 *73.3*	3.97 *70.4*	4.80 *68.0*	1.22 *16.6*	---
GalNAc	4.49 *102.0*	4.00 *52.2*	3.79 *81.6*	4.11 *69.0*	3.68 *76.2*	3.69- 3.78 *62.4*	Ac: 2.01 *23.8*
GalNAc[b]	4.39 *103.0*	3.92, 4.00 *52.2*	3.81, 3.86 *78.1,* *81.6*	4.03, 4.11 *69.0-* *69.4*	n.d.[c]		
GlcA	4.48 *105.5*	3.34 *73.7*	3.57 *74.9*	3.73 *81.0*	3.68 *77.7*	---	---
α-Fuc- (1→2)-*GlcA*	4.63 *102.9*	3.50 *78.6*	3.78 *76.2*	n.d.[c]	n.d.[c]	---	---
α-Fuc- (1→3)-*GlcA*	4.48 *105.5*	3.54 *75.3*	3.70 *80.9*	n.d.[c]	n.d.[c]	---	---

[Chemical shift expressed in δ relative to internal acetone (^{1}H: $(CH_3)_2CO$ at δ = 2.22 ppm; ^{13}C $(CH_3)_2CO$ at δ = 2.22 ppm]

[a]Indicated by italic character

[b]GalNAc unit whose chemical shifts are shifted, probably due to proximal fucosylation

[c]Not determinable

Table 6: ^{1}H (plain) and ^{13}C (italic) chemical shift attribution of **fC-II** (D_2O, 600 MHz, 298 K)

Residue[a]	**1**	**2**	**3**	**4**	**5**	**6**	**Other signals**
α-Fuc-(1→4)-GalNAc	5.37 *100.9*	3.75 *69.4*	3.91 *70.8*	3.82 *73.0*	4.07-4.18 *68.3*	1.21 *16.6*	---
α-Fuc-(1→6)-GalNAc	4.92 *99.9*		3.85 *70.8*				---
GalNAc	4.49 *102.1*	3.99 *52.2*	3.79 81.6	4.11-4.16 *68.8-69.0*	3.68 *76.3*	3.69-3.78 *62.4*	Ac: 2.01 23.8
α-Fuc-(1→4)-*GalNAc*		4.13 *52.6*	3.93 *81.5*	4.20 *76.7*	n.d.[c]		
α-Fuc-(1→6)-*GalNAc*		n.d.[c]	3.98 *81.7*	4.11-4.16 *68.8-69.0*	3.89 *74.1*	3.71,3.86 *67.2*	
GlcA	4.48 *105.5*	3.34 *73.7*	3.57 *74.9*	3.74 *80.9*	3.68 *77.6*	---	---
G*lcA*[b]	4.40 *105.5*	3.48 *73.1*	3.64 *76.2*	n.d.[c]	n.d.[c]	---	---

[Chemical shift expressed in δ relative to internal acetone (^{1}H: $(CH_3)_2CO$ at δ = 2.22 ppm; ^{13}C $(CH_3)_2CO$ at δ = 2.22 ppm]

[a]Indicated by italic character

[b]GlcA unit whose chemical shifts are shifted, probably due to proximal fucosylation

[c]Not determinable

Table 7: ^{1}H (plain) and ^{13}C (italic) chemical shift attribution of **fCS-VII** (D_2O, 600 MHz, 298 K)

Residue[a]	**1**	**2**	**3**	**4**	**5**	**6**	**Other signals**
α-Fuc2,3,4S-(1→2)-GlcA	5.51 *98.4*	4.52 *73.5*		4.88 *80.4*	4.27 67.5	1.29 *17.1*	---
α-Fuc2,3,4S-(1→3)-GlcA	5.64 *98.0*	4.54 *73.5*	4.62 *73.5*	4.94 *80.4*	4.94 *67.5*	1.33 *17.1*	---
GalNAc6S	4.55 *102.2*	3.86-4.01 *52.2*	3.84 *81.2*	4.10 *69.4*	3.96 *73.8*	4.21 *68.7*	Ac: 2.01, 2.10 *23.8*
GalNAc4S			4.01 *76.9*	4.74 77.5	3.82 75.8	3.78 *62.4*	
GlcA	4.50 *105.4*	3.36 *73.5*	3.59 *74.9*	3.74 *82.4*	3.74 *77.7*	---	---
α-Fuc2,3,4S-(1→2)-*GlcA*	4.69 *102.6*	3.54 *80.1*	3.76 *75.7*	3.86 n.d.[b]	3.78 *77.7*	---	---
α-Fuc2,3,4S-(1→3)-*GlcA*	4.50 *105.4*	3.59 *73.2*	3.75 *79.6*	n.d.[b]	n.d.[b]	---	---

[Chemical shift expressed in δ relative to internal acetone (^{1}H: $(CH_3)_2CO$ at δ = 2.22 ppm; ^{13}C $(CH_3)_2CO$ at δ = 2.22 ppm]

[a]Indicated by italic character

[b]Not determinable

Table 8: ^{1}H (plain) and ^{13}C (italic) chemical shift attribution of **fCS-VIII** (D_2O, 600 MHz, 298 K)

Residue[a]	**1**	**2**	**3**	**4**	**5**	**6**	**Other signals**
α-*Fuc2,3S*-(1→2)-GlcA	5.48, 5.51 *98.7*	4.51 *73.6*	4.46 *76.2*	4.12 *71.6*	4.21 *67.9*	1.23 *16.6*	---
α-*Fuc2,3S*-(1→3)-GlcA	5.21, 5.26 *98.8*	4.43 *73.0*	4.43 *76.2*		4.21 *71.4*		---
GalNAc6S	4.43-4.55 *102.0-103.3*	3.83 - 4.01 *52.2*	3.83 *81.4*	4.10 *69.3*	3.89,[b] 3.96 *73.4,*[b] *73.8*	4.21 *67.3,*[b] *68.8*	Ac: 2.02, 2.09 *23.8*
GalNAc4S			4.00 *77.1*	4.74 *77.7*	3.81 *75.8*	3.72-3.81 *62.3*	
GlcA	4.49 *105.5*	3.35 *73.6*	3.59 *75.0*	3.74 *82.8*	3.68 *77.6*	---	---
α-Fuc2,3S-(1→2)-*GlcA*	4.69 *102.7*	3.54 *80.3*	3.78 *77.0*	n.d.[c]	n.d.[c]	---	---
α-Fuc2,3S-(1→3)-*GlcA*	4.49 *105.5*	3.57 *73.5*	3.80 *80.3*	n.d.[c]	n.d.[c]	---	---
GlcA3S	4.58 *104.8*	3.54 *73.4*	4.35 *82.5*	3.91 *77.9*	3.68 *77.6*	---	---
GlcA2S	4.68 *102.7*	4.11 *80.5*	3.79 *76.1*	3.92 *79.3*		---	---

[Chemical shift expressed in δ relative to internal acetone (^{1}H: $(CH_3)_2CO$ at δ = 2.22 ppm; ^{13}C $(CH_3)_2CO$ at δ = 2.22 ppm]

[a]Indicated by italic character

[b]Signals related to GalNAc linked to GlcA3S or GlcA2S unit

[c]Not determinable

Table 9: ^{1}H (plain) and ^{13}C (italic) chemical shift attribution of **fCS-IX** (D_2O, 600 MHz, 298 K)

Residue[a]	**1**	**2**	**3**	**4**	**5**	**6**	**Other signals**
α-*Fuc2,4S*-(1→2)-GlcA	5.58 *97.8*	4.41 *76.2*	4.16 *68.7*	4.67 *82.1*	4.37 *67.6*	1.26 *16.8*	---
α-*Fuc2,4S*-(1→3)-GlcA	5.70 *97.5*	4.41, 4.48 *76.2*	4.12, 4.19 *68.0*	4.71 *82.5*	4.85, 5.00 *67.2, 67.6*	1.31, 1.35 *17.1*	---
GalNAc6S	4.43-4.55 *102.0-103.2*	3.94-4.02 *52.2*	3.82 *81.3*	n.d.[c]	3.81,[b] 3.96 *73.1,*[b] *73.8*	4.18-4.22 *67.3,*[b] *67.8,*[b] *68.7*	Ac: 2.01, 2.07 *23.8*
GalNAc4S			4.00 *77.1*	4.74 *77.5*	3.82 *75.8*	3.69-3.81 *62.4*	
GlcA	4.50 *105.4*	3.36 *73.5*	3.58 *75.0*	3.73 *81.2*	3.69 *77.7*	---	---
α-Fuc2,4S-(1→2)-*GlcA*	4.65 *102.8*	3.57 *77.7*	3.76 *75.9*	3.86 *n.d.*[c]	n.d.[c]	---	---
α-Fuc2,4S-(1→3)-*GlcA*	4.50 *105.4*	3.58 *73.5*	3.76 *81.3*	n.d.[c]	n.d.[c]	---	---
GlcA3S	4.56 *104.8*	3.53 *73.4*	4.35 *82.5*	3.92 *78.1*	3.69 *77.7*	---	---
GlcA2S	4.66 *102.8*	4.11 *80.5*	3.79 *75.9*	3.88 *n.d.*[c]		---	---

[Chemical shift expressed in δ relative to internal acetone (^{1}H: $(CH_3)_2CO$ at δ = 2.22 ppm; ^{13}C $(CH_3)_2CO$ at δ = 2.22 ppm]

[a]Indicated by italic character

[b]Signals related to GalNAc linked to GlcA3S or GlcA2S unit

[c]Not determinable

Table 10: ^{1}H (plain) and ^{13}C (italic) chemical shift attribution of **fCS-X** (D_2O, 600 MHz, 298 K)

Residue[a]	**1**	**2**	**3**	**4**	**5**	**6**	**Other signals**
α-Fuc2,3,4S-(1→4)-GalNAc	5.79 *97.7*	4.52 *73.5*	4.74 *73.6*	4.95 *80.2*	4.15 *68.8*	1.24 *16.5*	---
α-Fuc2,3,4S-(1→6)-GalNAc	5.28 *98.7*		4.70 *73.6*		4.22 *71.7*	1.29 *17.2*	---
GalNAc6S	4.50-4.57 *102.6*	4.01 *52.2*	3.83 *81.4*	4.17 *68.8*	3.98 *73.9*	4.22 *68.8*	Ac: 2.01 *23.8*
α-Fuc2,3,4S-(1→4)-*GalNAc6S*			3.78 *81.4*	4.29 *73.8*	n.d.[c]		
GalNAc4S			4.01 *77.1*	4.74 *77.7*	n.d.[c]	3.71-3.85 *62.5*	
α-Fuc2,3,4S-(1→6)-*GalNAc4S*			n.d.[c]	4.70 *76.1*	n.d.[c]	3.90 *69.4*	
GlcA	4.50 *105.5*	3.36 *73.7*	3.59 *75.0*	3.75 *81.4*	3.81 *76.2*	---	---
GlcA[b]	4.41 *105.9*	3.51, 3.57 *73.5*	3.78 *76.8*	n.d.[c]	n.d.[c]	---	---

[Chemical shift expressed in δ relative to internal acetone (^{1}H: $(CH_3)_2CO$ at δ = 2.22 ppm; ^{13}C $(CH_3)_2CO$ at δ = 2.22 ppm]

[a]Indicated by italic character

[b]GlcA unit whose chemical shifts are shifted, probably due to proximal fucosylation

[c]Not determinable

Table 11: ^{1}H (plain) and ^{13}C (italic) chemical shift attribution of **fCS-XI** (D_2O, 600 MHz, 298 K)

Residue[a]	**1**	**2**	**3**	**4**	**5**	**6**	**Other signals**
α-Fuc2,3S-(1→4)-GalNAc	5.79 *97.9*	4.53 *73.3*	4.69 *76.1*	4.22 *71.7*	4.08 *68.0*	1.24 *16.6*	---
α-Fuc2,3S-(1→6)-GalNAc	5.27 *98.7*		4.67 *76.1*		4.17 *68.8*		---
GalNAc6S	4.50-4.56 *102.6*	4.01 *52.2*	3.83 *81.4*	4.17 *67.9*	3.97 *73.9*	4.21 *68.8*	Ac: 2.01 *23.8*
α-Fuc2,3S-(1→4)-*GalNAc6S*			n.d.[c]	4.28 *74.1*	n.d.[c]		
GalNAc4S			4.00 *77.1*	4.74 *77.7*	3.82 *76.0*	3.71-3.84 *62.5*	
α-Fuc2,3S-(1→6)-*GalNAc4S*			n.d.[c]		n.d.[c]	3.90 *69.7*	
GlcA	4.51 *105.5*	3.37 *73.7*	3.61 *75.0*	3.72 *82.5*	3.71 *77.5*	---	---
GlcA[b]	4.42 *105.8*	3.51, 3.57 *73.5*	3.78 *76.8*	n.d.[c]	n.d.[c]	---	---

[Chemical shift expressed in δ relative to internal acetone (^{1}H: $(CH_3)_2CO$ at δ = 2.22 ppm; ^{13}C $(CH_3)_2CO$ at δ = 2.22 ppm]

[a]Indicated by italic character

[b]GlcA unit whose chemical shifts are shifted, probably due to proximal fucosylation

[c]Not determinable

Table 12: ^{1}H (plain) and ^{13}C (italic) chemical shift attribution of **fCS-XII** (D_2O, 600 MHz, 298 K)

Residue[a]	**1**	**2**	**3**	**4**	**5**	**6**	**Other signals**
α-Fuc2,4S-(1→4)-GalNAc	5.75 *97.6*	4.41 *76.3*	4.20 *67.5*	4.69 *82.0*	4.07 *67.9*	1.28 *16.8*	---
α-Fuc2,4S-(1→6)-GalNAc	5.23 *98.4*		4.07 *67.9*		4.22 *71.5*		---
GalNAc6S	4.51-4.59 *103.0*	4.01 *52.4*	3.83 *81.7*	4.16 *68.4*	3.97 *74.0*	4.21 *68.8*	Ac: 2.01 *23.8*
α-Fuc2,4S-(1→4)-*GalNAc6S*			3.79 *81.7*	4.29 *73.8*	n.d.[c]		
GalNAc4S			4.01 *77.4*	4.74 *77.7*	3.89 *73.6*	3.70-3.79 *62.4*	
α-Fuc2,4S-(1→6)-*GalNAc4S*			n.d.[c]		n.d.[c]	3.75, 3.88 *67.2*	
GlcA	4.54 *105.7*	3.38 *73.5*	3.63 *75.0*	3.78 *81.7*	3.87 *76.0*	---	---
GlcA[b]	4.44 *106.1*	3.52, 3.62 *73.0, 73.2*	3.83 *76.1*	n.d.[c]	n.d.[c]	---	---

[Chemical shift expressed in δ relative to internal acetone (^{1}H: $(CH_3)_2CO$ at δ = 2.22 ppm; ^{13}C $(CH_3)_2CO$ at δ = 2.22 ppm]

[a]Indicated by italic character

[b]GlcA unit whose chemical shifts are shifted, probably due to proximal fucosylation

[c]Not determinable

2.2.3 Molecular weight evaluation and anticoagulant activity of semi-synthetic fucosylated chondroitin sulfate

High-performance size exclusion chromatography combined with a triple detector array (HP-SEC-TDA) (a) La Gatta et al., **2010**. b) Bertini et al., **2005**.) allowed the evaluation of weight-averaged molecular mass (M_w), and then the polydispersity (M_w/M_n) for the semi-synthetic **fC-I-II**, **fCS-IV,VII-XII** (Table 13). It is an index used as a measure of a broadness of a molecular weight distribution of a polymer and it was lower with respect to the natural one (M_w ~ 55-65 kDa) and the starting chondroitin (M_w = 23.7 kDa for **fCS-IV**) and (M_w = 38 kDa for **fC-I,II** and **fCS-VII-XII**), presumably due to polysaccharide chain shortening during acid-mediated reactions (glycosylation and sulfation). Nonetheless, polydispersity was only marginally increased .

Table 13: Molecular weight and polydispersity of **fC-I-II** and **fCS-I-XII**

	M_w[a] (kDa)	M_w/M_n[b]
fC-I	7.3	1.35
fC-II	6.9	1.35
fCS-I	n.d.[c]	n.d.[c]
fCS-III	n.d.[c]	n.d.[c]
fCS-IV	8.5	1.35
fCS-V	n.d.[c]	n.d.[c]
fCS-VI	n.d.[c]	n.d.[c]
fCS-VII	10.9	1.50
fCS-VIII	9.4	1.49
fCS-IX	8.3	1.58
fCS-X	9.4	1.56
fCS-XI	8.7	1.45
fCS-XII	9.9	1.44

[a]Weight-averaged molecular mass
[b]Number-averaged molecular mass
[c]Not determined

Interestingly, M_w values between 7 and 10 kDa are indicated in the literature to be in an optimal range to retain anticoagulant activity (at least 6-8 repeating units are required), while minimizing undesirable effects – such as platelet aggregation, bleeding, hypotension – exhibited by natural high molecular weight fCSs. (a) Wu et al., **2015**. b) Zhao et al., **2013**. c) Wu et al., **2010**. d) Sheehan et al., **2006**.)

Semi-synthetic **fC-I-II**, as well as, **fCS-IV, VII-XII** were preliminarly assayed for anticoagulant activity. AT-dependent activity against factor Xa was 600-1000-fold lower than heparin (Table 14), as already reported for low molecular mass fCS species obtained by partial depolymerisation of natural polysaccharides. (a) Wu et al., **2015**. b) Panagos et al., **2014**. c) Mourão et al., **1996**.) The Xa activities (0.1-0.38 IU/mg) were more than 20-fold higher than CS from shark (0.017

IU/mg), thus confirming that the anticoagulant activity could be mostly attributed to Fuc branching units. Surprisingly, **fC-I-II**, carrying unsulfated Fuc branches, displayed a factor Xa activity similar to fCS polysaccharides (0.20 and 0.25 IU/mg for **fC-I** and **fC-II**, respectively) and for **fCS-IV**.

Table 14: AT-dependent activity against factor Xa

	Activity [IU/mg]
fC-I	0.20
fC-II	0.25
fCS-IV	0.38
fCS-VII	0.18
fCS-VIII	0.27
fCS-IX	n.d[a]
fCS-X	0.26
fCS-XI	0.33
fCS-XII	0.23
Heparin	198

[a]Not determined

A behaviour similar to natural fCS species was found also for the reduction of HC-II mediated factor IIa activity (Wu et al., **2015**) of **fCS-VII-XII**. Indeed, data were very close to heparin for fCSs with Fuc branches on the GlcA units (**fCS-VII-IX**), regardless of their sulfation pattern (Figure 25), whereas two of the three fCS with Fuc branches on the GalNAc units (**fCS-X-XII**), as well as unsulfated polysaccharides (**fC-I–II)**, displayed a much reduced anticoaugulant activity

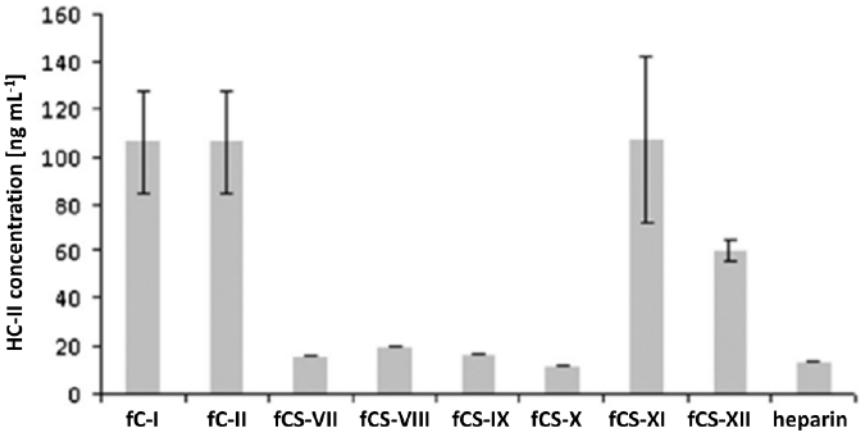


Figure 25: HC-II-mediated anti factor IIa activity

However, many more biological investigations are necessary in order to better characterize the anticoagulant and antithrombotic activities of these semi-synthetic polysaccharides, and they are scheduled for the near future.

2.3 Conclusions

The formation of unusual interglycosidic acetals involving positions *O*-6 of some GalNAc and *O*-4 of another GalNAc or *O*-3 of GlcA units (along the same polymeric chain or across two strands) of microbial sourced chondroitin during the preliminary protection for its regioselective sulfation was described. The lability under acid conditions of these interglycosidic acetals did not preserve them after reaction work-up. Thus, at the end of the semi-synthetic strategy CS-A,C possessing sulfate groups exclusively on GalNAc units was achieved. Conversely, the stabilization in an alkaline environment of

the labile interglycosidic acetals during benzylidenation work-up afforded at the end of the semi-synthesis CS species possessing sulfate groups not only on GalNAc units but also at position *O*-3 of some GlcA units, as already observed for an orthoester-based protection strategy. In the latter case, a transient interglycosidic orthoester involving position *O*-3 of some GlcA units and position *O*-6 of some GalNAc could be hypothesized, in analogy to the interglycosidic acetal for the one-pot benzylidenation-acetylation route. It is worth noting that the detailed understanding of the factors influencing finely tailored chemical modifications on microbial sourced chondroitin is rather valuable because it allows the preparation of biologically relevant CSs from non-animal sources with different, but highly controlled sulfation patterns. In particular, CS-A,C is a biomedical ingredient for several applications (Yamada et al., **2008**), and GlcA-3S-containing CSs – usually isolated from squid and king crab cartilages as well as from squid liver integuments (a) Kumar Shetty et al., **2009**. b) Kinoshita et al., **1997**. c) Sugahara et al., **1996**.) – are also interesting biomacromolecules because they promote neurite outgrowth in the central nervous system. (Mikami et al., **2006**)

Furthermore, the production of a library of twelve fCS polysaccharides with different Fuc sulfation pattern and/or branch position, and two fCs with different Fuc linkage site, was obtained from microbial sourced chondroitin too, through a semi-synthetic strategy based on the modular use of five different reactions: glycosylation, acetylation, selective cleavage of orthogonal protecting groups (Bn and benzylidene), sulfation and global deprotection. The key step of the synthetic strategy was the glycosylation with differently protected Fuc donors of suitably protected chondroitin acceptors. To the best of our knowledge, this is

the first example of the obtainment of a library of chemically modified polysaccharides by using a *O*-glycosylation as the key step, a reaction that is very well known in the field of oligosaccharide synthesis (Zhu et al., **2009**) but is almost unexplored for polysaccharides, especially in the case of branching at the secondary, less reactive hydroxyl positions. The structures of the semi-synthetic fCS and fC polysaccharides were fully characterized by 2D NMR spectroscopy, confirming in all cases the fucosylation and sulfation patterns expected from the designed reaction sequences. A single, well-defined sulfation pattern was found for Fuc branches in all the semi-synthesized fCS polysaccharides, contrary to the natural ones studied up to now, which display two or even more different sulfation patterns within the same polymer chain. The obtained NMR data could be useful for structural characterization purposes, as they can be employed as standards for comparison with data obtained from novel natural fCSs showing a Fuc sulfation pattern and/or branching site different from the known ones. More importantly, this library of semi-synthetic polysaccharides will allow a wider and more accurate structure-activity relationship study of fCSs with respect to those performed up to now, that have been limited to only the natural, heterogeneous structures found in sea cucumbers. Furthermore, by HP-SEC-TDA analysis, a lower molecular mass was found for all the obtained polysaccharides with respect to the natural ones. This is encouraging for avoiding the adverse effects displayed by the latter, while at the same retaining the anticoagulant activity. To confirm this, a careful screening of the biological activities of these semi-synthetic fCS and fC polysaccharides has been launched. The results will drive further chemical efforts towards the obtainment of fCS polysaccharides with an even more well-defined structure in terms of Fuc site

branching. In particular, the development of further regioselective protecting steps for the differentiation of the hydroxyl groups in GlcA 2,3- or GalNAc 4,6-diols will be pursued, as well as the polysaccharide branching with more complex glycosyl donors such as Fuc disaccharide ones. The final aim will be the full development of an effective, safe, non-animal sourced antithrombotic drug candidate.

2.4 Experimental section

2.4.1General methods

Reagents and solvents were used without further purification, except where differently indicated. Unsulfated chondroitin sodium salt was obtained at the laboratories of Prof. Chiara Schiraldi group (Department of Experimental Medicine, Università della Campania Luigi Vanvitelli) by fed-batch fermentation of *Escherichia coli K4* followed by down-stream purification processes performed as recently indicated. (Cimini et al., **2010**) Centrifugations were performed with an Eppendorf Centrifuge 5804 R instrument. Dialyses were conducted on Spectra/Por 10 kDa cut-off membranes at 4°C. Freeze-dryings were performed with a 5Pascal Lio 5P4 K freeze dryer. The term "pure water" refers to water purified by a i llipore i lli-Q Gradient system. NMR spectra were recorded on a Bruker DRX-400 (^{1}H: 400 MHz, ^{13}C: 100 MHz), on a Varian INOVA 500 (^{1}H: 500 MHz, ^{13}C: 125 MHz) instrument or on a Bruker DRX-600 (^{1}H: 600 MHz, ^{13}C: 150 MHz) instrument equipped with a cryo probe, in D_2O (acetone as internal standard, ^{1}H: $(CH_3)_2CO$ at δ = 2.22 ppm; ^{13}C: $(CH_3)_2CO$ at δ = 31.5 ppm), $CDCl_3$ (^{1}H: $CHCl_3$ at δ = 7.26 ppm; ^{13}C: $CDCl_3$ at δ = 77.0 ppm)

or DMSO-d_6 (^{1}H: CHD_2SOCD_3 at δ = 2.49 ppm; ^{13}C: CD_3SOCD_3 at δ = 39.5 ppm). Double quantum-filtered phase-sensitive COSY, phase sensitive NOESY and TOCSY experiments were performed using spectral widths of either 6000 Hz in both dimensions, using data sets of 4096 × 256 points. Quadrature indirect dimensions were achieved through States-TPPI method; spectra were processed applying a cosine square function to both dimensions and data matrix was zero-filled by factor of 2 before Fourier transformation. TOCSY and NOESY mixing times were set to 120 ms and 200 ms, respectively. HSQC-DEPT experiments were measured in the ^{1}H-detected mode via single quantum coherence with proton decoupling in the ^{13}C domain, using data sets of 2048 × 256 points and typically 32 increments. For HMBC spectrum, data set consisted of 2048 × 256 points and long range constant was set to 8 Hz. In these heteronuclear experiments the data matrix was extended to4096 × 2048 points reconstructing 1024 of these data points with forward linear prediction extrapolation (30 coefficients) and zero-filling those remaining. As for HSQC-TOCSY, data sets of 2048 × 256 points were used, with 100 increments, mixing time was set to 100 ms and spectra were transformed as indicated for homonuclear spectra, data matrix were doubled and linear prediction applied the new points by using 30 coefficients, Qsine functions were used as window function and values selected depended by the best resolution obtained.

Size-exclusion chromatographies were performed on a Bio-Gel P2 column (0.75 x 67.5 cm, Bio-Rad) using 50 mM ammonium acetate as a buffer at a flow rate of 0.2 mL/min. The column eluate was monitored continuously with a Knauer K-2310 refractive index refractometer.

A ViscotekTM instrument (Malvern) was used to determine molecular mass data.

2.4.2 Synthetic procedure for regioselective sulfation of chondroitin

Preparation of chondroitin acid form from chondroitin sodium salt: Chondroitin sodium salt (4.600 g, 11.47 mmol repeating unit) was dissolved in pure water (140 mL) and passed through a short Dowex 50 WX8 column (H^+ form, 20–50 mesh, approx. 450 cm^3). Elution with pure water was continued until pH of the eluate was neutral. Freeze-drying of the collected eluate gave chondroitin (4.208 g, 97%).

One-pot **benzylidenation-acetylation on chondroitin (6a):** Chondroitin (117.1 mg, 0.309 mmol repeating unit) was manually chopped and then suspended under Ar atmosphere in DMF (3.1 mL), that was freshly dried over 4Å molecular sieves. The mixture was vigorously stirred at 80°C for 3 h. If needed, the temperature of the mixture was then changed (rt, 50°C or 80°C: see Table 1) and α,α-dimethoxytoluene (464 μL, 3.09 mmol), freshly dried over 4Å molecular sieves, was added. A 0.21 M solution of CSA in freshly dried D F (371 μL,77.9 μmol) was then added and the mixture was stirred at 80°C (or 50°C or rt) overnight. The mixture was then treated at rt with Et_3N (861 μL, 6.18 mmol). After few minutes, CH_3CN (5.6 mL) and then acetic anhydride (2.0 mL, 21.6 mmol) and 4-(dimethylamino)pyridine (15.1 mg, 0.124 mmol) were added. After overnight stirring, the obtained brown solution was treated with

diisopropyl ether (11.7 mL). A white solid was obtained. It was isolated by centrifugation at 4°C (3000 rpm, 10 min). After overnight desiccation under vacuum, polysaccharide **6a** (141.4 mg, 87%) was obtained as a brown amorphous solid.

Oxidative opening of benzylidene ring (7a): Derivative **6a** (122.2 mg, 0.234 mmol) was suspended in ethyl acetate (2.6 mL) and then treated with a 0.27 M solution of $NaBrO_3$ in pure water (2.6 mL, 0.702 mmol). A 0.24 M solution of $Na_2S_2O_4$ in pure water (2.4 mL, 0.576 mmol) was added portionwise over a period of 10 min. After overnight stirring at rt under visible light irradiation, centrifugation of the mixture at 4°C (3000 rpm, 10 min) allowed the isolation of a gummy yellowish precipitate that was desiccated under vacuum overnight to give **7a** (123.7 mg, 98%).

Sulfation and global deprotection (8a): Derivative **7a** (144.9 mg, 0.269 mmol) was suspended in DMF (2.1 mL), that was freshly dried over 4Å molecular sieves and then treated with a 1.17 M solution of pyridine–sulfur trioxide complex in freshly dried DMF (4.6 mL, 5.382 mmol). After 1 h stirring at 50°C, the suspension turned into a clear solution. Stirring at 50°C was continued overnight, after that a cold, saturated NaCl solution in acetone (18.0 mL) was added. The obtained yellowish precipitate was collected by centrifugation at 4°C (3000 rpm, 10 min) and then dissolved in pure water (11.7 mL). The solution was stirred at 50°C for 1 h, then cooled to rt and treated with a 15% w/v NaOH solution to adjust pH to 13. The solution was stirred for 6 h at rt and then 4 M HCl was added until neutralization. Dialysis and

subsequent freeze-drying yielded the CS polysaccharide (104.1 mg, 69%) as a slightly yellow, woolly solid.

Isolation of polysaccharide 9a: Chondroitin (62.4 mg, 0.165 mmol repeating unit) was manually chopped and then suspended under Ar atmosphere in DMF (3.1 mL), that was freshly dried over 4Å molecular sieves. The mixture was vigorously stirred at 80°C for 3 h, then cooled to rt and treated with α,α-dimethoxytoluene (248 μL, 1.65 mmol), that was freshly dried over 4Å molecular sieves. A 0.21 M solution of CSA in freshly dried D F (209 μL, 43.9 μmol) was then added and the mixture was stirred at rt overnight. Et_3N (460 μL, 3.30 mmol) and, after few minutes, diisopropyl ether (8.0 mL) were then added. The obtained precipitate was collected by centrifugation at 4°C (3000 rpm,10 min) and dried overnight under vacuum to yield polysaccharide **9a** (78.9 mg, 81%) as a slightly yellow amorphous solid.

2.4.3 Synthetic procedure for fucosylated chondroitin sulfate polysaccharides

Chondroitin methyl ester (13): Chondroitin sodium salt from *E. coli* O5:K4:H4 fed-batch fermentation (2.183 g, 5.445 mmol repeating unit) was dissolved in pure water (140 mL) and passed through a short Dowex 50 WX8 column (H^+ form, 20-50 mesh, approx. 450 cm^3). Elution with pure water was continued until pH of the eluate was neutral. Freeze-drying of the collected eluate gave chondroitin (2.010 g, 5.303 mmol), that was manually chopped and then suspended under Ar atmosphere in dry methanol (250 mL). After overnight stirring at rt, a 0.58 M methanolic solution of acetyl chloride (124 mL, 72.4 mmol)

was added *via* cannula and stirring was continued overnight. The reaction was then quenched by neutralization with 1M aqueous $NaHCO_3$. The mixture was concentrated by rotoevaporation and then freeze-dried. The protocol was repeated to give, after dialysis and freeze-drying, chondroitin methyl ester **13** (1.567 g, 73%, DS = 0.92) as a white waxy solid. NMR data (400 MHz, D_2O) are reported below (Table 15)

Table 15: ^{1}H (plain) and ^{13}C (italic) chemical shift attribution of derivative **13**

Residue	1	2	3	4	5	6	Other signals
GlcA	4.60 *105.1*	3.39 *72.4*	3.68 *75.4*	3.86 *80.4*	4.10 *74.1*	---	(methyl ester) 3.85 *54.0*
GalNAc	4.50 *101.6*	3.98 *51.5*	4.09 *80.3*	4.07 *68.3*	3.66 *75.3*	3.77 *61.6*	Ac 2.02 *23.2*

[Chemical shift expressed in δ relative to internal acetone (^{1}H: $(CH_3)_2CO$ at δ = 2.22 ppm; ^{13}C: $(CH_3)_2CO$ at δ = 30.9 ppm)]

Chondroitin *n*-dodecyl ester (15): A suspension of TBA salt of chondroitin **14** (Valoti et al., **2012**) (0.962 g, 1.551 mmol repeating unit) was suspended in DMF (32 mL) that was freshly dried over 4Å MS. TBAF (3.59 mL, 12.40 mmol) was added to give a clear solution that was then treated with *n*-dodecyl iodide (6.3 mL, 25.5 mmol). The solution was stirred at 80°C overnight and then treated with diisopropyl ether (130 mL) to give a gummy precipitate, that was collected by centrifugation and then dried under vacuum overnight to afford an approximately 30:70 w/w mixture of **15** and TBAI/TBAF (2.456 g, DS = 1). NMR data (400 MHz, DMSO-d_6) are reported below (Table 16)

Table 16: ^{1}H (plain) and ^{13}C (italic) chemical shift attribution of derivative **15**

Residue	1	2	3	4	5	6	Other signals
GlcA	4.45 *103.9*	3.08 *72.3*	3.31 *74.3*	3.52 *80.0*	3.74 *73.3*	---	(dodecyl ester) α: 4.01, 4.14 *64.5* β: 1.56 *27.2* γ-(ω-1): 1.23-1.26 *28.8* ω: 0.93 *13.1*
GalNAc	4.38 *101.0*	3.78 *50.9*	3.67 *78.9*	3.69 *66.9*	3.40 *75.0*	3.47 *60.1*	Ac 1.76 *21.2*

[Chemical shift expressed in δ relative to isotopic impurity of DMSO-d_6 (^{1}H: CHD_2SOCD_3 at δ = 2.49 ppm) in D S O-d_6 (^{13}C: CD_3SOCD_3 at δ = 39.5 ppm)]

Chondroitin methyl ester 4,6-benzylidene (16): Chondroitin methyl ester **13** (0.598 g, 1.519 mmol repeating unit) was suspended in DMF (30 mL), that was freshly dried over 4Å MS, and stirred at 80°C for 2 hours. α,α-Dimethoxytoluene (2.3 mL, 15.2 mmol) freshly dried over 4Å MS and then CSA (88.3 mg, 0.380 mmol) were added. The mixture was stirred at 80°C overnight, after that a clear, yellowish solution was obtained. It was cooled to rt and treated with diisopropyl ether (72 mL). The obtained white precipitate was collected by centrifugation and then dried under vacuum overnight. Derivative **16** was obtained as a yellowish amorphous solid, as a 74% w/w mixture with CSA and DMF (0.878 g, 89%, DS = 0.99). NMR data (400 MHz, DMSO-d_6) are reported below (Table 17)

Table 17: ^{1}H (plain) and ^{13}C (italic) chemical shift attribution of derivative **16**

Residue	1	2	3	4	5	6	Other signals
GlcA	4.50 *103.4*	3.04 *72.1*	3.39 *73.6*	3.63 *79.4*	3.85 *76.5*	---	(benzylidene ring) CH: 5.51 *99.5* Ph: 7.38 *126.1* (methyl ester) 3.71 *52.0*
GalNAc	4.54 *100.1*	3.86 *50.2*	3.85 *73.3*	4.16 *74.4*	3.61 *65.4*	4.03 *68.2*	Ac 1.77 *22.7*

[Chemical shift expressed in δ relative to isotopic impurity of D SO-d_6 (^{1}H: CHD_2SOCD_3 at δ = 2.49 ppm) in D S O-d_6 (^{13}C: CD_3SOCD_3 at δ = 39.5 ppm)]

Chondroitin n-dodecyl ester 4,6-benzylidene (17): Chondroitin *n*-dodecyl ester **15** (0.237 g approx. 30% w/w pure) was dissolved in DMF (12 mL), that was freshly dried over 4Å MS, and stirred at 80°C for 2 hours. α,α-Dimethoxytoluene (0.65 mL, 4.34 mmol) freshly dried over 4Å MS and then a 0.2 M solution of CSA in freshly dried DMF (0.52 mL, 0.104 mmol) were added. The solution was stirred at 80°C overnight. It was then cooled to rt and treated with water (40 mL) to give a white precipitate that was collected by centrifugation and then dried under vacuum overnight. A 86% w/w mixture of derivative **17** with TBAF and DMF (97.4 mg, 97% over two steps from **14**, DS = 0.96) was obtained as a white amorphous solid. NMR data (400 MHz, DMSO-d_6) are reported below (Table 18)

Table 18: ^{1}H (plain) and ^{13}C (italic) chemical shift attribution of derivative **17**

Residue	1	2	3	4	5	6	Other signals
GlcA	4.49 *104.4*	3.03 *72.5*	3.37 *73.9*	3.65 *79.3*	3.83 *73.5*	---	(benzylidene ring) CH: 5.48 *99.9* Ph: 7.37 *127.4* (dodecyl ester) α: 4.01, 4.15 *64.6* β: 1.56 *27.4* γ-(ω-1): 1.23-1.26 *28.7* ω: 0.93 *13.1*
GalNAc	4.58 *100.4*	3.81 *50.7*	3.83 *76.9*	4.14 *74.7*	3.58 *65.8*	4.03 *68.3*	Ac 1.73 *22.7*

[Chemical shift expressed in δ relative to isotopic impurity of D SO-d_6 (^{1}H: CHD_2SOCD_3 at δ = 2.49 ppm) in D S O-d_6 (^{13}C: CD_3SOCD_3 at δ = 39.5 ppm)]

Chondroitin methyl ester 4,6-benzylidene 2,3-*bis*-trimethylsilylether (18): Polysaccharide **16** (63.2 mg, 74% w/w pure) was suspended in pyridine (4 mL). $[(CH_3)_3Si]_2NH$ (274 µL, 1.31 mmol) and $(CH_3)_3SiCl$ (166 µL, 1.31 mmol) were then added. After overnight stirring at 50°C, a clear solution was obtained. It was cooled to rt and treated with diisopropyl ether (16 mL) to give a yellowish precipitate, that was collected by centrifugation and then dried under vacuum overnight. A 52:48 w/w mixture of derivative **18** and pyridine (103 mg,

88%, DS = 2) was obtained. Alternatively, after overnight stirring at 50°C, the crude reaction mixture was treated with water (16 mL) to afford, after centrifugation and overnight drying, pure polysaccharide **18** (50.3 mg, 93%, DS = 1.03) as a white precipitate.

Chondroitin derivative (19): Polysaccharide **16** (202 mg, 0.419 mmol) was treated with CH_3CN (4.4 mL) and then with Et_3N (1.2 mL), Ac_2O (2.8 mL) and DMAP (18.8 mg, 0.168 mmol). After overnight stirring at rt, a clear solution was obtained. Diisopropyl ether (15 mL) was then added to give a yellowish precipitate that was collected by centrifugation and then dried under vacuum overnight to afford derivative **19** (227 mg, DS = 2) contaminated mostly by Et_3N and DMAP. NMR data (400 MHz, DMSO-d_6) are reported below (Table 19)

Table 19: ^{1}H (plain) and ^{13}C (italic) chemical shift attribution of derivative **19**

Residue	1	2	3	4	5	6	Other signals
GlcA	4.93 *100.8*	4.65 *70.0*	5.04 *71.7*	3.91 *75.5*	4.10 *73.2*	---	NAc 1.76 *22.3* OAc 1.93 *19.9*
GalNAc	4.34 *99.9*	3.63 *49.9*	3.82 *76.5*	4.18 *73.7*	3.54 *65.1*	4.04 *67.7*	(Benzylidene ring) CH: 5.44 *99.2* Ph: 7.35 *127.4* (Methyl ester) 3.77 *52.5*

[Chemical shift expressed in δ relative to isotopic impurity of D SO-d_6 (^{1}H: CHD_2SOCD_3 at δ = 2.49 ppm) in D S O-d_6 (^{13}C: CD_3SOCD_3 at δ = 39.5 ppm)]

Chondroitin derivative (20): Derivative **19** (96.5 mg) was suspended in EtOAc (1.9 mL) and then treated with a 0.27 M solution of $NaBrO_3$ in pure water (1.9 mL). A 0.24 M solution of $Na_2S_2O_4$ in pure water (1.9 mL) was added portionwise over a period of 10 min. The mixture was vigorously stirred at rt overnight under visible light irradiation. The yellowish solid was collected by centrifugation and then dried under vacuum to give pure polysaccharide **20** (66.9 mg, 63% over two steps from **16**, DS = 0.97). NMR data (400 MHz, DMSO-d_6) are reported below (Table 20)

Table 20: ^{1}H (plain) and ^{13}C (italic) chemical shift attribution of derivative **20**

Residue	1	2	3	4	5	6	Other signals
GlcA (4-Bz)	4.93 *99.6*	4.68 *70.2*	4.99 *71.8*	3.80 *75.7*	4.04 *72.9*	---	NAc 1.79 *22.4*
GlcA (6-Bz)		4.48 *70.0*		3.88 *75.5*		---	OAc 1.72-1.93 *19.8-20.2*
GalNAc (4-Bz)	4.32 *100.6*	3.69 *51.9*	4.02 *75.5*	5.41 *68.8*	3.61 *73.2*	3.29 *59.3*	(Benzoyl ester) Ph: 7.95, 7.86, 7.66, 7.52 *128.8, 128.8, 132.8, 128.3* (Methyl ester) 3.77 *52.5*
GalNAc (6-Bz)				3.90 *73.2*	3.69 *73.2*	4.21, 4.34 *62.9*	

[Chemical shift expressed in δ relative to isotopic impurity of D SO-d_6 (^{1}H: CHD_2SOCD_3 at δ = 2.49 ppm) in D S O-d_6 (^{13}C: CD_3SOCD_3 at δ = 39.5 ppm)]

1,2,3,4-tetra-*O*-acetyl-L-fucopyranoside (21): Commercially available L-fucose (2.55 g, 15.53 mmmol) was suspended in Ac_2O (6.10 mL) and pyridine (6.10 mL). After overnight stirring at rt, the obtained solution was diluted with CH_2Cl_2 (100 mL) and washed with HCl 0.5 M (100 mL). The organic phase was collected, dried over anhydrous Na_2SO_4, filtered and concentrated obtaining 1,2,3,4-tetra-*O*-acetyl-L-fucopyranoside (5.16 g, quant.) ^{1}H NMR (600 MHz, $CDCl_3$, mixture of anomers α/β: 35/65): δ α-form = 6.24 (d, $J_{1,2}$ = 3.1 Hz, 1H,

H-1), 5.23-5.29 (m, 3H, H-2, H-3, H-4), 4.27 (q, $J_{5,6}$ = 6.4 Hz, 1H, H-5) 2.18 (s, 3H, OCOCH_3), 2.15 (s, 3H, OCOCH_3), 2.02 (s, 3H, OCOCH_3), 2.00 (s, 3H, OCOCH_3), 1.16 (d, $J_{6,5}$ = 6.4 Hz, 3H, H-6).
δ β-form = 5.68 (d, $J_{1,2}$ = 8.3 Hz, 1H, H-1), 5.23-5.29 (m, 3H, H-2, H-3, H-4), 5.08 (dd, $J_{3,4}$ = 3.4 Hz, $J_{3,2}$ = 10.35 Hz, 1H, H-3), 3.95 (q, $J_{5,6}$ = 6.4 Hz, 1H, H-5), 2.20 (s, 3H, OCOCH_3), 2.12 (s, 3H, OCOCH_3), 2.04 (s, 3H, OCOCH_3), 1.99 (s, 3H, OCOCH_3), 1.23 (d, $J_{6,5}$ = 6.0 Hz, 3H, H-6).

2,3,4-tri-*O*-acetyl-1-iodo-*α*-L-fucopyranoside (22): Compound **21** (6.27 g, 18.87 mmol) was dissolved in CH_2Cl_2 (38.2 mL), treated with I_2 (7.82 g, 30.80 mmol), Et_3SiH (4.3 mL, 26.92 mmol) and stirred for 3 h at 50°C at reflux. The mixture was then diluted with CH_2Cl_2 (150 mL) and washed with a 1:1 v/v solution of $NaHCO_3/Na_2S_2O_3$. The organic phase was collected, dried over anhydrous Na_2SO_4, filtered and concentrated obtaining **22** as an oil, for the presence of siloxanes as contaminants. The crude product was used in the next step without purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.10 (d, $J_{1,2}$ = 3.9 Hz, 1H, H-1), 5.34 (d, $J_{4,3}$ = 2.4 Hz, 1H, H-4), 5.29 (dd, $J_{3,4}$ = 3.2 Hz, $J_{3,2}$ = 10.2 Hz, 1H, H-3), 4.44 (m 1H, H-2), 4.28 (q, $J_{5,6}$ = 6.4 Hz, 1H, H-5), 2.17 (s, OCOCH_3, 3H) , 2.11 (s, OCOCH_3, 3H) ,2.00 (s, OCOCH_3, 3H), 1.23 (d, $J_{6,5}$ = 6.4 Hz, 3H, H-6).

Ethyl 2,3,4-tri-*O*-acetyl-*β*-L-fucopyranosyl thioglycoside (23): A suspension of crude **22** in dry CH_3CN (20.0 mL) was treated with $(NH_2)_2CS$ (2.18 g, 28.70 mmol) and stirred for 30 min at 60°C. The mixture was cooled to rt, then EtI (3.81 mL, 47.39 mmol) and Et_3N (10.6 mL, 76.05 mmol) were added. After stirring for 2 h at rt, the

yellowish solution was diluted with CH_2Cl_2 (100 mL) and washed with 0.1 M HCl (100 mL). The organic phase was collected, dried over anhydrous Na_2SO_4, filtered and concentrated. The obtained residue was purified by column chromatography (4:1 to 2:1 v/v hexane-ethyl acetate) to afford pure ethyl 2,3,4-tri-*O*-acetyl-β-L-fucopyranosyl thioglycoside (3.37 g, 53% over two steps from **21**). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.28 (d, $J_{4,3}$ = 3.2 Hz, 1H, H-4), 5.23 (t, $J_{2,1}$ = $J_{2,3}$ = 10.0 Hz, 1H, H-2), 5.05 (dd, $J_{3,4}$ = 3.2 Hz, $J_{3,2}$ = 10.0 Hz, 1H, H-3), 4.46 (d, $J_{1,2}$ = 9.9 Hz, 1H, H-1), 3.83 (q, $J_{5,6}$ = 6.3 Hz, 1H, H-5), 2.74 (m, SC*H*$_2$CH$_3$, 2H), 2.22 (s, OCOC*H*$_3$, 3H), 2.07 (s, OCOC*H*$_3$, 3H), 1.99 (s, OCOC*H*$_3$, 3H), 1.29 (t, J_{vic} = 7.4 Hz, SCH$_2$C*H*$_3$, 3H), 1.23 (d, $J_{6,5}$ = 6.4 Hz, 3H, H-6)

Ethyl *β*-L-fucopyranosyl thioglycoside (24): Compound **23** (3.34 g, 9.99 mmol) was suspended in dry MeOH (22.0 mL) and treated with a 2.35 M solution of Na in dry MeOH (3.15 mL, 7.40 mmol). The yellow solution was stirred for 2 h at rt, neutralized with Amberlyst®, filtered and concentrated, obtaining pure **24** (1.97 g, 83%) ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.30 (d, $J_{1,2}$ = 9.4 Hz, 1H, H-1), 3.80 (s, 1H, H-4) 3.68 (q, $J_{5,6}$ = 6.5 Hz, 1H, H-5), 3.62 (m, 2H, H-2, H-3), 2.75 (m, SC*H*$_2$CH$_3$, 2H), 1.35 (d, $J_{6,5}$ = 6.0 Hz, 3H, H-6), 1.32 (t, J_{vic} = 7.2 Hz, SCH$_2$C*H*$_3$, 3H)

Ethyl 2,3,4-tri-*O*-benzyl-*β*-L-fucopyranosyl thioglycoside (25): A solution of **24** (1.97 g, 8.26 mmol) in DMF that was freshly dried over activated 4Å molecular sieves (16.0 mL), was cooled to 0°C and treated with BnBr (4.04 mL, 34.08 mmol) and then NaH (60% dispersion in mineral oil, 817.9 mg, 34.08 mmol). After 10 min stirring at 0°C, the

temperature was raised to rt and the mixture was stirred for 1 hour further. The reaction was then quenched by careful addition of water (50 mL) at 0°C and in small aliquots, and then of CH_2Cl_2 (100 mL). The organic layer was collected, dried over anhydrous Na_2SO_4, filtered and concentrated to give a residue that was subjected to column chromatography (9:1 to 5:1 v/v hexane-ethyl acetate) to afford pure ethyl 2,3,4-tri-*O*-benzyl-*β*-L-fucopyranosyl thioglycoside (3.15 g, 75%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.41-7.22 (m, 15H, H-Ar), 5.01 (d, J_{gem} = 11.8 Hz, 1H, OC*H*HPh), 4.92 (d, J_{gem} = 10.2 Hz, 1H, OC*H*HPh), 4.82 (J_{gem} = 10.2 Hz, 1H, OC*H*HPh), 4.78 (J_{gem} = 10.2 Hz, 1H, OC*H*HPh), 4.76 (J_{gem} = 11.8 Hz, 1H, OC*H*HPh) ,4.71 (J_{gem} = 11.8 Hz, 1H, OC*H*HPh), 4.41 (d, $J_{1,2}$ = 9.6 Hz, 1H, H-1), 3.85 (t, $J_{2,1} = J_{2,3}$ = 9.4 Hz, 1H, H-2), 3.64 (d, $J_{4,3}$= 2.1 Hz, 1H, H-4), 3.58 (dd, $J_{3,4}$ = 2.7, $J_{3,2}$ = 9.2 Hz, 1H, H-3), 3.50 (q, $J_{5,6}$ = 6.4 Hz, 1H, H-5), 2.76 (m, SC*H*$_2$CH$_3$, 2H), 1.33 (t, J_{vic} = 7.4 Hz, SCH$_2$C*H*$_3$, 3H), 1.25 (d, $J_{6,5}$ 6.4 Hz, 3H, H-6).

2,3,4-tri-*O*-benzyl-L-fucopyranosyl-*N*-phenyl-trifluoroacetimidate (26): A solution of commercially available 2,3,4-tri -*O*-benzyl-L-fucopyranose (3.13 g, 7.20 mmol) in dry CH_2Cl_2 (43 mL) was cooled to 0°C and treated with CF_3(NPh)Cl (1.618 mL, 12.98 mmol) and then NaH (60% dispersion in mineral oil, 345.8 mg, 14.42 mmol). After 3 h stirring at 0°C, the mixture was purified through column chromatography (toluene 99.9%, Et_3N 0.1%) affording pure compound **26** (2.49 g, 57%). ^{1}H-NMR data in agreement with literature (Adinolfi et al., **2004**)

Ethyl 3,4-*O*-isopropylidene-*β*-L-fucopyranosyl thioglycoside (27): Compound **24** (1.87 g, 7.84 mmol) was treated with $(CH_3)_2C(OCH_3)_2$ (9.95 mL, 80.88 mmol) and with CSA (83.5 mg, 0.36 mmol). After 2h stirring at rt, the solution was diluted with CH_2Cl_2 (75.0 mL) and then washed with 1M $NaHCO_3$ (75.0 mL). The organic phase was collected, dried over anhydrous Na_2SO_4, filtered and concentrated to afford pure compound **27** (1.91 g, 98%) ^{1}H NMR (600 MHz, $CDCl_3$): 4.20 (d, $J_{1,2}$ = 10.2 Hz, 1H, H-1), 4.05 (dd, $J_{4,5}$ = 2.2 Hz, $J_{4,3}$ = 5.5 Hz, 1H, H-4), 4.03 (t, $J_{3,4}$ = 5.5 Hz, $J_{3,2}$ = 6.8 Hz, 1H, H-3), 3.87 (dq, $J_{5,4}$ = 2.2 Hz, $J_{5,6}$ = 6.6 Hz, 1H, H-5), 3.54 (q, $J_{2,3}$ = 6.8 Hz, $J_{2,1}$ = 10.2 Hz, 1H, H-2), 2.74 (m, SC*H*$_2$CH$_3$, 2H), 1.53 (s, CC*H*$_3$, 3H), 1.40 (d, $J_{6,5}$ = 6.4 Hz, 3H, H-6), 1.36 (s, CC*H*$_3$, 3H), 1.31 (t, J_{vic} = 7.4 Hz, 3H, SCH$_2$C*H*$_3$)

Ethyl 2-*O*-benzyl-3,4-*O*-isopropylidene-*β*-L-fucopyranosyl thioglycoside (28): A solution of **27** (2.15 g, 8.66 mmol) in freshly dried *N*,*N*-dimethylformamide over activated 4Å molecular sieves (17.5 mL) was cooled to 0°C and treated with BnBr (1.23 mL, 10.39 mmol) and then NaH (60% dispersion in mineral oil, 249.4 mg, 10.39 mmol). After 10 min stirring at 0°C, the temperature was raised to rt and the mixture was stirred for 1 hour further. The reaction was then quenched by careful addition of water (20 mL) at 0°C and in small aliquots, and then of CH_2Cl_2 (100 mL). The organic layer was collected, dried over anhydrous Na_2SO_4, filtered and concentrated to give a residue that was subjected to column chromatography (9:1 to 7:1 v/v cyclohexane-ethyl acetate) to afford pure ethyl 2-*O*-benzyl-3,4-*O*-isopropylidene-*β*-L-fucopyranosyl thioglycoside (2.65 g, 90%). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.43-7.26 (m, 5H, H-Ar), 4.84 (d, J_{gem} = 11.4 Hz, 1H, OC*H*HPh), 4.76 (d, J_{gem} = 11.3 Hz, 1H, OC*H*HPh), 4.38 (d, $J_{1,2}$ = 9.8

Hz, 1H, H-1), 4.19 (t, $J_{3,4}$ = 5.6 Hz, $J_{3,2}$ = 6.7 Hz, 1H, H-3), 4.05 (dd, $J_{4,5}$ = 2.2 Hz, $J_{4,3}$ = 5.6 Hz, 1H, H-4), 3.81 (dq, $J_{5,4}$ = 2.2 Hz, $J_{5,6}$ = 6.6 Hz, 1H, H-5), 3.43 (q, $J_{2,3}$ = 6.7 Hz, $J_{2,1}$ = 9.8 Hz, 1H, H-2), 2.70 (m, SCH_2CH_3, 2H), 1.45 (s, CCH_3, 3H), 1.38 (d, $J_{6,5}$ = 6.5 Hz, 3H, H-6), 1.36 (s, CCH_3, 3H), 1.29 (J_{vic} = 7.43 Hz, SCH_2CH_3, 3H)

Ethyl 2-*O*-benzyl-*β*-L-fucopyranosyl thioglycoside (29): Compound **28** (2.64 g, 7.80 mmol) was suspended in a 4:1 v/v TFA/H_2O solution and stirred for 1 h at rt. The solution was diluted with H_2O (200 mL) and washed with CH_2Cl_2 (200 mL). The organic phase was washed with 1M $NaHCO_3$ (200 mL), collected, dried over anhydrous Na_2SO_4, filtered and concentrated affording pure ethyl 2-*O*-benzyl-*β*-L-fucopyranosyl thioglycoside (2.28 g, 98%). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.43-7.30 (m, 5H, H-Ar), 4.97 (d, J_{gem} = 11.0 Hz, 1H, OC*H*HPh), 4.68 (d, J_{gem} = 11.1 Hz, 1H, OC*H*HPh), 4.40 (d, $J_{1,2}$ = 9.6 Hz, 1H, H-1), 3.75 (d, $J_{4,3}$ = 3.3 Hz, 1H, H-4), 3.64-3.60 (m, 2H, H-3, H-5), 3.45 (t, $J_{2,1}$ = $J_{2,3}$ = 9.3 Hz, 1H, H-2), 2.77 (m, SCH_2CH_3, 2H), 1.35-1.29 (m, SCH_2CH_3, 6H, H-6)

Ethyl 2,3-di-*O*-benzyl-1-*β*-L-fucopyranosyl thioglycoside (30): Bu_2SnO (1.83g, 7.35 mmol) was added to compound **29** (2.42 g, 8.11 the mixture was suspended in a 10:1 v/v C_6H_6/MeOH solution (35.2 mL) and stirre 60°C. After 3 h the solution was cooled to rt, concentrated and then, under Ar atmosphere Bu_4NBr (2.26 g, 6.12 mmol), dry toluene (17.9 mL) and BnBr (738 µL, 61.2 mmol) were added. After overnight stirring at 65°C, the solution was cooled to rt, concentrated and the residue was purified through column chromatography (6:1 to 1:4 v/v hexane-ethyl acetate) affording pure

compound **30** (2.15 g, 95%). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.41-7.28 (m, 10H, H-Ar), 4.88 (d, J_{gem} = 10.2 Hz, 1H, OC*H*HPh), 4.76 (d, J_{gem} = 10.2 Hz, 1H, OC*H*HPh), 4.72-4.70 (m, 2H, 2 OC*H*HPh), 4.39 (d, $J_{1,2}$ = 9.7 Hz, 1H, H-1), 3.82 (s, 1H, H-4), 3.62 (t, $J_{2,1} = J_{2,3}$ = 9.6 Hz, 1H, H-2), 3.56-3.52 (m, 2H, H-3, H-5), 2.81-2.68 (m, SC*H*$_2$CH$_3$, 2H), 1.34 (d, $J_{6,5}$ = 6.4 Hz, 3H, H-6), 1.31 (t, J_{vic} = 7.4 Hz, 3H, SCH$_2$C*H*$_3$).

Ethyl 2,3-di-*O*-benzyl-4-*O*-benzoyl-*β*-L-fucopyranosyl thioglycoside (31): Compound **30** (2.13 g, 5.48 mmol) was suspended in dry CH_2Cl_2 (24.0 mL) and treated with BzCl (958 µL, 8.25 mmol) and pyridine (6.0 mL). After 2h stirring at rt, the solution was diluted with CH_2Cl_2 (50.0 mL) and washed with 1.0 M HCl (50.0 mL). The organic phase was collected, dried over anhydrous Na_2SO_4, filtered and concentrated affording a residue that was purified through column chromatography (12:1 to 6:1 v/v hexane-ethyl acetate), to afford a pure compound **31** (2.21 g, 82%). ^{1}H NMR (600 MHz, $CDCl_3$): δ=8.14-7.24 (m, 15H, H-Ar), 5.63 (d, $J_{4,3}$ = 2.9 Hz, 1H, H-4), 4.85-4.81 (m, 2H, 2 OC*H*HPh) 4.77 (d, J_{gem} = 10.26 Hz, 1H, OC*H*HPh), 4.57 (d, J_{gem} = 11.7 Hz, 1H, OC*H*HPh), 4.50 (d, $J_{1,2}$ = 9.70 Hz, 1H, H-1), 3.77 (q, $J_{5,6}$ = 6.40 Hz, 1H, H-5), 3.73 (dd, $J_{3,2}$ = 9.17 Hz, $J_{3,4}$ = 3.21 Hz, 1H, H-3), 3.69 (t, $J_{2,1} = J_{2,3}$ = 9.7 Hz, 1H, H-2), 2.81 (m, SC*H*$_2$CH$_3$, 2H), 1.35 (t, J_{vic} = 7.4 Hz, 3H, SCH$_2$C*H*$_3$), 1.29 (d, $J_{6,5}$ = 6.4 Hz, 3H, H-6)

Ethyl 3-*O*-*tert*-butyldimethylsilyl-*β*-L-fucopyranosyl thioglycoside (32): A solution of compound **24** (1.95 g, 8.18 mmol) in DMF (8.0 mL) was treated with ImH (1.59 g, 23.45 mmol) and TBDMSCl (1.93 g, 12.85 mmol) after cooling to 0°C. After 20 min stirring, the temperature was raised to rt and the mixture was stirred for 2 h further.

The solution was diluted with AcOEt (100 mL) and washed with H_2O (100 mL). The organic phase was collected, dried over anhydrous Na_2SO_4, filtered and concentrated to give a residue that was subjected to column chromatography (10:1 to 6:1 v/v hexane-ethyl acetate) affording pure **32** (2.03 g, 77%) ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.24 (d, $J_{1,2}$ = 9.0 Hz, 1H, H-1), 3.64-3.54 (m, 4H, H-2, H-3, H-4, H-5), 2.81 (m, SC*H*$_2$CH$_3$, 2H), 1.36 (d, $J_{6,5}$ = 6.4 Hz, 3H, H-6), 1.30 (t, J_{vic} = 7.4 Hz, 3H, SCH$_2$C*H*$_3$), 0.92 (s, 9H, SiC(C*H*$_3$)$_3$), 0.16 (s, 3H, SiC*H*$_3$), 0.13 (s, 3H, SiC*H*$_3$).

Ethyl 2,4-di-*O*-benzyl-3-*O*-*tert*-butyldimethylsilyl-*β*-L-fucopyranosyl thioglycoside (33): A solution of **32** (1.80 g, 5.58 mmol) in DMF that was freshly dried over activated 4Å molecular sieves (16.5 mL) was cooled to 0°C and treated with BnBr (1.66 mL, 13.39 mmol) and then NaH (60% dispersion in mineral oil, 321.2 mg, 13.39 mmol). After 10 min stirring at 0°C, the temperature was raised to rt and the mixture was stirred for 1 hour further. The reaction was then quenched by careful addition of water (50 mL) at 0°C and in small aliquots, and then of CH_2Cl_2 (100 mL). The organic layer was collected, dried over anhydrous Na_2SO_4, filtered and concentrated to give a residue that was subjected to column chromatography (12:1 to 8:1 v/v hexane-ethyl acetate) to afford pure ethyl 2,4-di-*O*-benzyl-3-*O*-*tert*-butyldimethylsilyl-*β*-L-fucopyranosyl thioglycoside (2.60 g, 93%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.44-7.29 (m, 10H, H-Ar), 5.10 (d, J_{gem} = 11.4 Hz, 1H, OC*H*HPh), 4.88 (d, J_{gem} = 10.2 Hz, 1H, OC*H*HPh), 4.74 (d, J_{gem} = 10.2 Hz, 1H, OC*H*HPh), 4.63 (d, J_{gem} = 11.4 Hz, 1H, OC*H*HPh), 4.40 (d, $J_{1,2}$ = 9.3 Hz, 1H, H-1), 3.76 (dd, $J_{3,2}$ = 9.0 Hz, $J_{3,4}$ = 2.8 Hz, 1H, H-3), 3.73 (t, $J_{2,1} = J_{2,3}$ = 9.3 Hz, 1H, H-2), 3.55 (q, $J_{5,6}$ =

6.4 Hz, 1H, H-5), 3.46 (d, $J_{4,3}$ = 3.0 Hz, 1H, H-4), 2.81 (m, SCH_2CH$_3$, 2H), 1.29 (t, J_{vic} = 7.5 Hz, 3H, SCH$_2$CH_3), 1.24 (d, $J_{6,5}$ = 6.2 Hz, 3H, H-6), 0.97 (s, 9H, SiC(CH_3)$_3$), 0.13 (s, 3H, SiCH_3), 0.08 (s, 3H, SiCH_3).

Ethyl 2,4-di-*O*-benzyl-*β*-L-fucopyranosyl thioglycoside (34): Compound **33** (2.57 g, 5.11 mmol) was suspended in THF (37.0 mL) and treated with 1.0 M solution of TBAF in THF (12.8 mL). The yellowish solution was stirred for 2h at rt and the reaction was quenched by concentration at the rotoevaporator, and obtaining a crude mixture that was purified trough column chromatography (12:1 to 8:1 v/v hexane-ethyl acetate). Pure **34** was obtained (1.89 g, 95%). ^{1}H NMR (400 MHz, CDCl$_3$): δ = 7.42-7.29 (m, 10H, H-Ar), 4.95 (d, J_{gem} = 10.6 Hz, 1H, OC*H*HPh), 4.78-4.62 (m 3H, 3 OC*H*HPh), 4.37 (d, $J_{1,2}$ = 9.6 Hz, 1H, H-1), 3.68 (dd, $J_{3,2}$ = 9.3 Hz, $J_{3,4}$ = 3.1 Hz, 1H, H-3), 3.53 (t, $J_{2,1}$ = $J_{2,3}$ = 9.7 Hz, 1H, H-2), 3.50 (d, $J_{4,3}$ = 3.0 Hz, 1H, H-4), 3.47 (q, $J_{5,6}$ = 6.4 Hz, 1H, H-5), 2.81 (m, SCH_2CH$_3$, 2H), 1.35 (t, J_{vic} = 7.4 Hz, 3H, SCH$_2$CH_3), 1.29 (d, $J_{6,5}$ = 6.4 Hz, 3H, H-6).

Ethyl 2,4-di-*O*-benzyl-3-*O*-benzoyl-*β*-L-fucopyranosyl thioglycoside (35): Compound **34** (1.89 g, 4.86 mmol) was dissolved in dry CH_2Cl_2 (10.0 mL) and treated with BzCl (849 μL, 7.30 mmol) obtaining a yellow solution that was stirred at rt. After 30 min a white precipitate was formed. It was dissolved by adding more CH_2Cl_2 (10.0 mL). The reaction was then quenched by addition of MeOH, diluted with CH_2Cl_2 (100 mL) and washed with 0.1 M HCl (100 mL). The organic phase was collected, dried over anhydrous Na_2SO_4, filtered and concentrated to give a residue that was subjected to column chromatography (12:1 to 6:1 v/v hexane-ethyl acetate) to afford pure ethyl 2,4-di-*O*-benzyl-3-*O*-

benzoyl-*β*-L-fucopyranosyl thioglycoside (1.76 g, 73%). ^{1}H NMR (400 MHz, $CDCl_3$): δ=8.04-7.18 (m, 15H, H-Ar), 5.25 (dd, $J_{3,4}$ = 3.1 Hz $J_{3,2}$ = 9.7 Hz, 1H, H-3), 4.90 (d, J_{gem} = 10.6 Hz, 1H, OC*H*HPh), 4.72 (d, J_{gem} = 11.7 Hz, 1H, OC*H*HPh), 4.69 (d, J_{gem} = 11.7 Hz, 1H, OC*H*HPh), 4.59 (d, J_{gem} = 10.6 Hz, 1H, OC*H*HPh), 4.55 (d, $J_{1,2}$ = 9.6 Hz, 1H, H-1), 4.01 (t, $J_{2,1}$ = $J_{2,3}$ = 9.7 Hz, 1H, H-2), 3.89 (d, $J_{4,3}$ = 3.1 Hz, 1H, H-4), 3.74 (q, $J_{5,6}$ = 6.4 Hz, 1H, H-5), 2.81 (m, SC*H*$_2$CH$_3$, 2H), 1.35 (t, J_{vic} = 7.4 Hz, 3H, SCH$_2$C*H*$_3$), 1.29 (d, $J_{6,5}$ = 6.4 Hz, 3H, H-6)

Glycosylation with ethyl 2,3,4-tri-*O*-benzyl-*β*-L-fucopyranosyl thioglycoside 25. A mixture of polysaccharide **16** (or **17** or **18** or **20**) (48.4 mg, 0.101 mmol) and fucosyl thioglycoside **25** (241 mg, 0.505 mmol) was co-evaporated three times with dry toluene (2.5 mL). AW-300 4Å molecular sieves and then CH_2Cl_2 (3.4 mL) and DMF (2.5 mL) (or THF, or in addition with DME in the case of **17** and **18,** respectively), that were freshly dried over 4Å molecular sieves, were added to the mixture under argon atmosphere. NIS (125 mg, 0.550 mmol) and then TMSOTf (99 μL, 0.550 mmol) were added. The mixture was stirred at rt (or at -20°C) under argon atmosphere for 4 hours, then the molecular sieves were eliminated by decantation. A few drops of triethylamine and then diisopropyl ether (30 mL) were added and the mixture was stored at -28°C overnight. The obtained yellowish precipitate was collected by centrifugation and then dried under vacuum overnight to afford derivative **37-IV** (94.6 mg) or **38-VII** (at rt and -20°C, respectively from **16)**, **36-I**, **37-VI** (from **17** and **18,** respectively) and **39-X** (from acceptor **20**).

Glycosylation with 2,3,4-tri-*O*-benzyl-L-fucopyranosyl *N*-phenyl-trifluoroacetimidate 26. Polysaccharide **16** (or **17**) (98.1 mg, 0.204 mmol) and fucosyl donor **26** (617 mg, 1.02 mmol) were co-evaporated three times with dry toluene (5.0 mL) in two separated round-bottomed flasks. AW-300 4Å molecular sieves and then DMF (6.0 mL) (or THF), that was freshly dried over 4Å molecular sieves, were added to the polysaccharide, whereas 4Å molecular sieves and then CH_2Cl_2 (10.0 mL), that was freshly dried over 4Å molecular sieves, were added to the donor. The mixture containing the polysaccharide was then treated with a 0.49M solution of TMSOTf in CH_2Cl_2 (42 μL, 20.6 μmol), and then the mixture containing the donor was immediately added *via* cannula under argon atmosphere. The mixture was stirred at rt for 4 hours, then the molecular sieves were eliminated by decantation. A few drops of triethylamine and then diisopropyl ether (30 mL) were added and the mixture was stored at -28°C overnight. The obtained white precipitate was collected by centrifugation and then dried under vacuum overnight to afford derivative **37-V** (153.9 mg, from acceptor **16**) or **36-II,III** (from acceptor **17**).

Glycosylation with ethyl 2,3-di-*O*-benzyl-4-*O*-benzyl-1-*β*-L-fucopyranosyl thioglycoside 31. A mixture of polysaccharide **16** (or **20**) (61.9 mg, 0.124 mmol) and fucosyl thioglycoside **31** (317.0 mg, 0.643 mmol) was co-evaporated three times with dry toluene (2.5 mL). AW-300 4Å molecular sieves and then CH_2Cl_2 (6.28 mL) and DMF (3.77 mL), that were freshly dried over 4Å molecular sieves, were added to the mixture under argon atmosphere. NIS (158.1 mg, 0.707 mmol) and then TMSOTf (34.8 μL, 0.193 mmol) were added. The mixture was stirred at -20°C (or at rt in the case of polysaccharide **20**)

under argon atmosphere for 4 hours, then the molecular sieves were eliminated by decantation. A few drops of triethylamine and then diisopropyl ether (30 mL) were added and the mixture was stored at -28°C overnight. The obtained yellowish precipitate was collected by centrifugation and then dried under vacuum overnight to afford derivative **38-VIII** (72.9 mg) (or **39-XI** in the case of polysaccharide acceptor **20**).

Glycosylation with ethyl 2,4-di-*O*-benzyl-3-*O*-benzoyl-*β*-L-fucopyranosyl thioglycoside 35. A mixture of polysaccharide **16** (or **20**) (68.5 mg, 0.138 mmol) and fucosyl thioglycoside **35** (351 mg, 0.712 mmol) was co-evaporated three times with dry toluene (2.5 mL). AW-300 4Å molecular sieves and then CH_2Cl_2 (6.95 mL) and DMF (4.17 mL), that were freshly dried over 4Å molecular sieves, were added to the mixture under argon atmosphere. NIS (176.3 mg, 0.783 mmol) and then TMSOTf (38.5 μL, 0.214 mmol) were added. The mixture was stirred at -20°C (or rt in the case of polysaccharide **20**) under argon atmosphere for 4 hours, then the molecular sieves were eliminated by decantation. A few drops of triethylamine and then diisopropyl ether (30 mL) were added and the mixture was stored at -28°C overnight. The obtained yellowish precipitate was collected by centrifugation and then dried under vacuum overnight to afford derivative **38-IX** (52.1 mg) (or **39-XII** in the case of polysaccharide acceptor **20**).

Acetylation. Derivative **37-IV** (or **36-I**, and **38-VII**) (88.4 mg) was suspended in CH_3CN (2.0 mL) and then Ac_2O (650 μL), Et_3N (275 μL) and DMAP (4.8 mg) were added. After overnight stirring at rt, a clear

solution was obtained. In the case of methyl ester derivatives (**37-IV→41-IV**), diisopropyl ether (15 mL) was added to give a white precipitate that was collected by centrifugation and then dried under vacuum overnight to afford derivative **41-IV** (or **42-VIII**) (72.5 mg). In the case of dodecyl ester derivatives (**36-I→40-I**), no polysaccharide precipitation could be obtained with any solvent. Therefore, the crude mixture was concentrated to give a residue that was co-evaporated several times with toluene. A brownish oil (381 mg) containing polysaccharide **40-I** was obtained. It was not further purified, but used in the following synthetic step as it was. The same stategy was applied for acetylation of **37-V-VI** (**37-V-VI →41-V-VI**), **36-III** (**36-III→40-III**) and **38-IX-X** (**38-IX-X →42-IX-X**)

Oxidative cleavage of benzyl and benzylidene protecting groups. A suspension of polysaccharide **41-IV** (67.7 mg) in ethyl acetate (800 μL) was treated with a 0.27 M solution of $NaBrO_3$ in pure water (800 μL). A 0.24 M solution of $Na_2S_2O_4$ in pure water (750 μL) was added portionwise over a period of 10 min. The triphasic mixture was vigorously stirred at rt overnight under visible light irradiation. The yellowish solid was then collected by centrifugation to afford derivative **44-IV** (36.8 mg). The same strategy was applied for derivatives **40I,III**, **41-V-VI** and **42-VII-IX** that afforded **43-I,III**, **44-V-VI** and 45-**VII-I**, respectively.

Sulfation and alkaline deprotection. Derivative **44-IV** (33.3 mg) was suspended in DMF (570 μL), that was freshly dried over 4Å molecular sieves and then treated with a 1.17 M solution of pyridine–sulfur trioxide complex in freshly dried DMF (840 μL). After 2h stirring at

50°C the suspension turned into a clear solution. Stirring at 50°C was continued overnight. A saturated NaCl solution in acetone (5 mL) was added. The obtained yellowish precipitate was collected by centrifugation and then dissolved in pure water (7.0 mL). The solution was treated with a 15% w/v NaOH solution to adjust pH to 13, stirred for 6 h at rt and then 1.0 M HCl was added until neutralization. Dialysis and subsequent freeze-drying yielded a slightly yellow solid, that was further purified by filtration through a Sep-pak® C-18 cartridge and then by size-exclusion chromatography. Freeze-drying of the fractions afforded **fCS-IV** (35.2 mg) as a white waxy solid. The same strategy was applied to afford **fCS-I,III-XII**.

Alkaline deprotection to fC-I-II. Derivative **46-I** (or **45-I**) (80.4 mg) was suspended in pure water (6.0 mL) obtaining a yellowish suspension that was treated with a 15% w/v NaOH solution to adjust pH to 13. The mixture was stirred for 6 h at rt and then 1.0 M HCl was added until neutralization. The reaction was repeated twice. Dialysis and subsequent freeze-drying yielded a slightly yellow solid, that was further purified by filtration through a Sep-pak® C-18 cartridge and then by size-exclusion chromatography. Freeze-drying of the fractions afforded **fC-I** (31.4 mg) (or **fC-II**) as a white waxy solid.

2.4.4Determination of molecular mass

Hydrodynamic characterization of fC and fCS samples was performed by using the SEC-TDA equipment by Viscotek (Malvern, Italy). The chromatographic system consists of two modules: (i) a GPCmax VE 2001 integrated system composed of a specific pump for gel

permeation chromatography, an in-line solvent degasser, and an autosampler; and (ii) a TDA305 module (triple detector array) that includes a column oven and a triple detector. The latter is the key element of the GPC-Viscotek as it is equipped with a refractive index (RI) detector, a four-bridge viscosimeter (VIS), and a light scattering (LS) detector. The latter consists of a right-angle light scattering (RALS) detector and a low-angle light scattering (LALS) detector that performed measurements of the scattered light at 7° with respect to the incident beam with an optimal signal-to-noise ratio. The OmniSEC software program was used for the acquisition and analysis of the Viscotek data. Two in series TSK-GEL GMPWXL columns (Tosoh Bioscience, Cat. No. 8–08025, hydroxylated polymethacrylate base material, 100–1000 Å pore size, 13 μm mean particle size, 7.8×30.0 cm), preceded by a TSK-GEL guard column GMPWXL (Tosoh Bioscience, Cat. No. 08033, 12 μm mean particle size, 6.0×4.0 cm), were used. The samples were analyzed at concentrations ranging from 0.3 to 4 g L^{-1} to have a column load for each analysis (injection volume×sample concentration×intrinsic viscosity) of approximately 0.2 dL and, at the same time, appreciable LALS and VIS signals when analyzing low-molecular-mass species (see equations below). An isocratic elution with 0.1 M $NaNO_3$ aqueous solution (pH 7.0) at a flow rate of 0.6 mL min^{-1} was carried out. Analyses were performed at 40 °C with a running time of 60 min. The molecular mass and size distribution, polydispersity, hydrodynamic radius, and intrinsic viscosity were derived. For the detected signals, the following equations apply: RI signal=$K_1 \times dn/dc \times C$; VIS signal=$K_2 \times [\eta] \times C$; LALS signal=$K_3 \times M_w \times (dn/dc)^2 \times C$; where $[\eta]$ is the intrinsic viscosity (dL g^{-1}); C is the mass concentration (mg mL^{-1}); dn/dc is the refractive index

increment (mL g^{-1}); and K_1, K_2, and K_3 are instrumental constants. (Hartmann et al., **2004**)

By solving the equations above, the system allowed the simultaneous determination of sample concentration, molecular mass, and intrinsic viscosity. (La Gatta et al., **2010**)

2.4.5 Anticoagulant activity

AT-dependent antifactor Xa activity was evaluated by a kinetic colorimetric method by using a commercial kit (Stachrom Heparin, Stago). Heparin was used as the standard for building a calibration curve (r^2 = 0.976) with 0.1 to 0.5 $IUmg^{-1}$ active solutions. The sample (100 mL) was added to purified bovine AT (100 mL) diluted in Tris EDTA buffer (pH 8.4), and the mixture was incubated with factor Xa (200 mL) for 2 min at 37 °C. Then, factor Xa chromogenic substrate (200 mL) was added. The absorbance was read at 405 nm against a blank and was inversely proportional to the concentration of the polysaccharides. The anti HC-IIa assay was performed by using a sandwich enzyme immunoassay (USCN, Life Science Inc.) according to the manufacturer's protocol (http://www.nlbiochemex.com/e90284ga.html).

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CHAPTER 3

DEPOLYMERIZATION OF SEMI-SYNTHETIC FUCOSYLATED CHONDROITIN SULFATE AND INTERACTION WITH SELECTINS

CHAPTER 3

DEPOLYMERIZATION OF SEMI-SYNTHETIC FUCOSYLATED CHONDROITIN SULFATE AND INTERACTION WITH SELECTINS

3.1 Introduction

The biological activities of GAGs require to specify their composition in order to identify which is responsible for any of their distinct properties. (Toida et al., **2009**) For example, a pentasaccharide sequence of heparin, responsible for binding with antithrombin, was discovered after studies on heparin oligosaccharides. (Munoz et al., **2004**) Moreover, low molecular weight heparins (LMWHs) have been used to replace the unfractionated one as anticoagulant and antithrombotic drugs due to their reduced side effects. (a) Hirsh et al., **2004**. b) Linhardt **2003**. c) Green et al., **1994**. d) Matsch et al., **1990**. e) Monreal et al., **1990**.) Among glycosaminoglycans, fucosylated chondroitin sulfate is considered as a potential substitute of heparin for its anticoagulant and antithrombotic properties acting with both a serpin-dependent mechanism and a serpin-independent one, which could make fCS active also on cases for which heparin fails. (a) Wu et al., **2015**. b) Pomin **2014**. c) Glauser et al., **2008**) However, fCS could also cause undesirable effects, such as platelet aggregation, factor XII activation and bleeding. (Li et al., **2016**) Depolymerized fucosylated chondroitin sulfate still display high antithrombotic activity

but with less side effects, similarly to the case of unfractionated heparin (UFH) and LMWHs (a) Buyue et al., **2009**. b) Kitazato et al., **2003**. b) Kenji et al., **1996**.)

Different methods of GAGs depolymerization, characterized by specific advantages and limitations, have been developed up to now. (Panagos et al., **2016**) Enzymatic depolymerization is the most widespread method, nonetheless its high cost and lack of action on highly sulfated GAGs limit its use. (Vieira et al., **1991**) The employment of ultrasound represents, to the best of our knowledge, the only mechanical strategy to obtain depolymerized hyaluronic acid (Miyazaki et al., **2001**) or LMWH oligosaccharides. (Achour et al., **2013**)

Acid-catalyzed hydrolysis is, instead, a chemical approach for polysaccharide depolymerization. In theory, this strategy of depolymerization could be suitable for large-scale preparation of low molecular fCSs, nonetheless it can easily lead to loss of fucose branches or to desulfation, thus influencing the anticoagulant and the antithrombotic activity of the depolymerized fCS. (Li et al., **2016**)

Free-radical depolymerization of GAGs using a Fenton-type reaction is, among the chemical methods, the most widely used one (a) Li et al., **2016**. b) Liu et al., **2016**. c) Panagos et al., **2014**. d) Panagos et al., **2012**. Wu et al., **2010**.) because it guarantees reproducibility, constant composition and control of the extent of depolymerization. (Petit et al., **2006**)

Since GAGs are characterized by the presence of uronic acid units, they could be also submitted to β-elimination of the substituent at *O*-4 position, thus breaking the 1→4 glycosidic bond and forming $\Delta^{4,5}$

unsaturated uronic acid residues at non-reducing end of the resulting fragments. (Gao et al., **2015**)

In order to evaluate the anticoagulant and the antithrombotic activity of fucosylated chondroitin sulfate depolymerized by radical reaction, native fCS polysaccharides have been recently extracted from *Cucumaria frondosa*, *Thelenota ananas,* (Liu et al., **2016**) *Isostichopus badionotus* (Li et al., **2016**) and *Holoturia forskali* (Panagos et al., **2014**) and submitted to copper (II) Fenton-type depolymerization as described by Wu in 2010, or with suitable modifications thereof. The results displayed different activities between depolymerized fCS from *H. forskali* and the other sea cucumbers.

Since heparin is involved in inhibition of P- and L-selectin binding to sialyl Lewis(x) carbohydrate determinant, reducing metastasis and inflammation, the fCS polysaccharides extracted from *L. grisea* (Borsig et al., **2007**) and from *H. forskali* were studied for this action as well. (Panagos et al., **2014**) Natural fCS displayed more potent inhibition than heparin in the P- and L-selectin-sialyl Lewis(x) interaction due to the presence of fucose branches, that resemble motifs present in sialyl Lewis(x) ligand. (Borsig et al., **2007**) No inhibition of E-selectin was observed, whereas defucosylated polysaccharide abolished this effect both *in vitro* and *in vivo*, as described for the above-mentioned anticoagulant and antithrombotic activities. (a) Pacheco et al., **2000**. b) our o et al., **1996**.) Interestingly, fCS oligosaccharides prepared from *H. forskali* by a Fenton-type depolymerization maintained a high affinity for P- and L-selectins, but displaying lower adverse effects than native fCS.

In order to evaluate the same inhibition activity of depolymerized fucosylated chondroitin sulfate from natural sources, it was planned to

submit semi-synthetic fCS polysaccharide (**fCS-XII**, see Chapter II), showing a sulfation pattern different from natural ones to β-eliminative depolymerization (Gao et al., **2015**) to give a oligosaccharide to be tested for its interaction with P- and L-selectins by "ligand-based" NMR techniques such as Saturation Transfer Difference (STD). (a) Marchetti et al., **2016**. b) Lepre et al., **2004**. c) Mayer et al., **2001**.) This one is the widespread method for detection and characterization of transient receptor-ligand interactions in solution, due to its efficiency to determine the binding epitope of ligands, detecting the region in closer contact to the receptor protein.

A STD spectrum is obtained by the difference between on- and off-resonance experiments (Figure 26). (Angulo et al., **2011**)

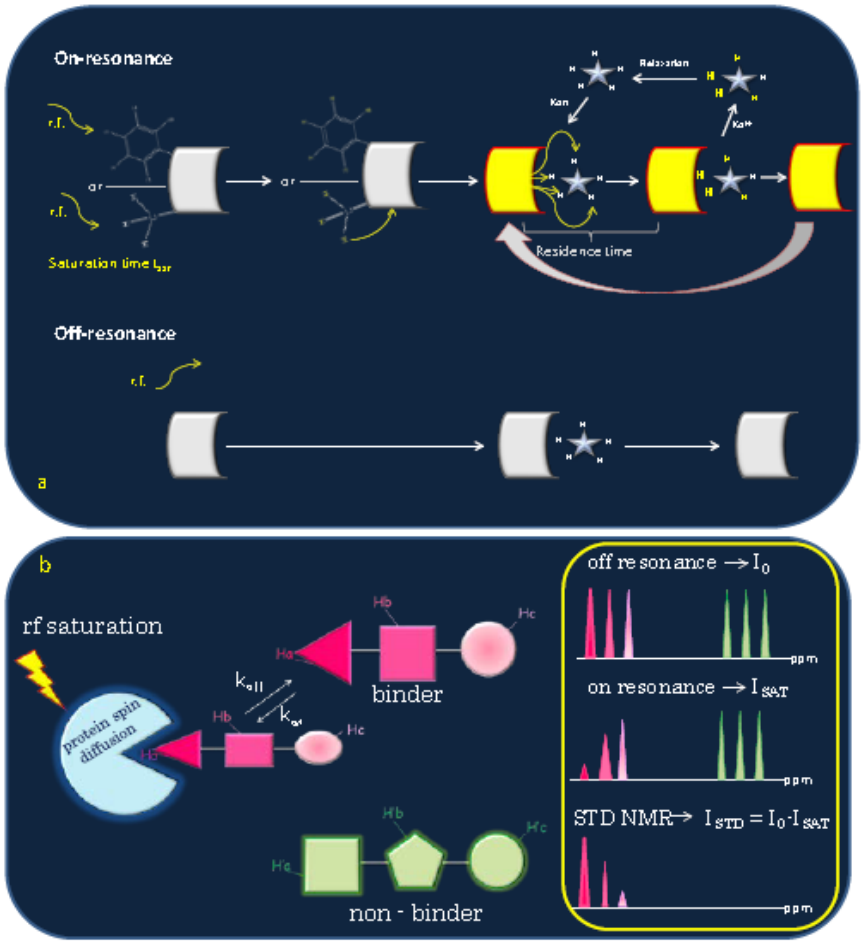


Figure 26: Schematic representation of STD NMR spectroscopy

In the former, selective RF (radio frequency)-pulse train is set for a given time (saturation time) to a region of the spectrum with only resonances from protein (-4 ppm), whereas the latter is a reference spectrum, where saturation rf is applied in an off resonance region from both receptor and ligand.

Once saturated, the receptor transfers the saturation to ligand through the ^{1}H-^{1}H cross relaxation pathway, obtaining a difference (STD) spectrum that allows to determine the existence of interactions in

solution and the binding epitope of ligands, detecting the regions in closer contact with the receptor

3.2 Results and discussion

The presence on fCS backbone of uronic acid units led to choose a β-elimination (Gao et al., **2015**) as a suitable depolymerization method. Indeed, commercial LMWHs have been obtained by controlled chemical or enzymatic β-eliminative depolymerization. (a) Gao et al., **2015**. b) Hirsh et al., **1992**.) To this purpose, semi-synthetic **fCS-XII** was firstly subjected to an ion exchange in the presence of benzethonium chloride, in order to change the Na^+ counterion at carboxylate and sulfate groups. The obtained derivative **47** was modified at its carboxylic functions through a benzylation reaction with benzyl chloride (BnCl) at 35°C, in order to obtain a derivative more soluble in aprotic solvent, and that could give more easily a depolymerization at the subsequent step. (Gao et al.,**2015**) Ester **48** was obtained in 36% yield with the DS = 0.39 (Figure 27) as evaluated by comparison of integration between signals δ_H = 5.38 and 5.21 ppm region (*H*-1 protons and CH_2 atoms) and the CH_3 ones (δ = 1.23) of fucose branches and then applying the following equation:

$$DS = \frac{I\ (CH\_1\ \alpha\_Fuc + CH_2\ Bn) - \frac{I\ (CH_3\ \alpha\_Fuc)}{3}}{CH_3\ GalNAc} \quad (Eq.4)$$

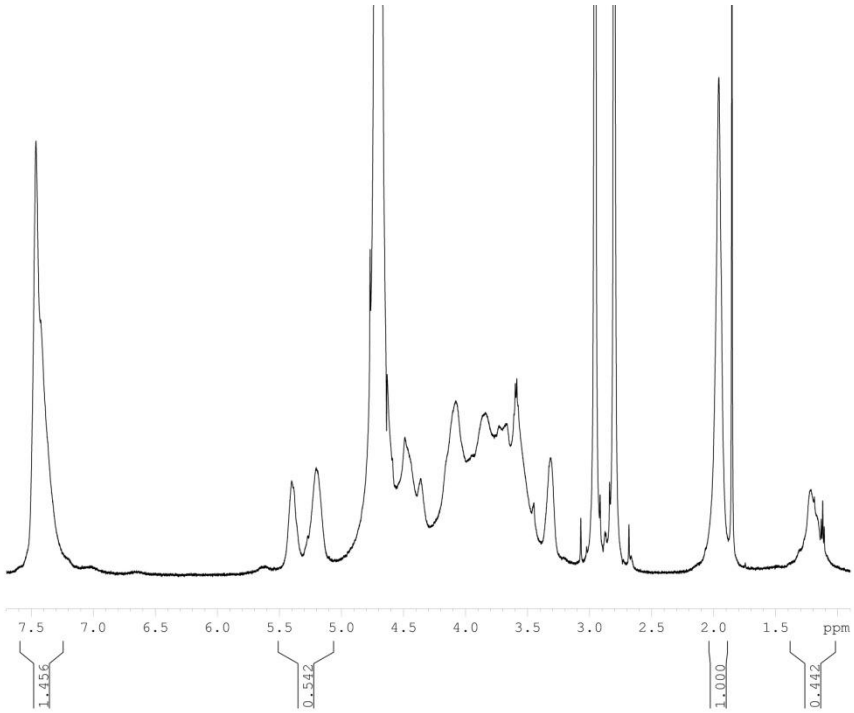
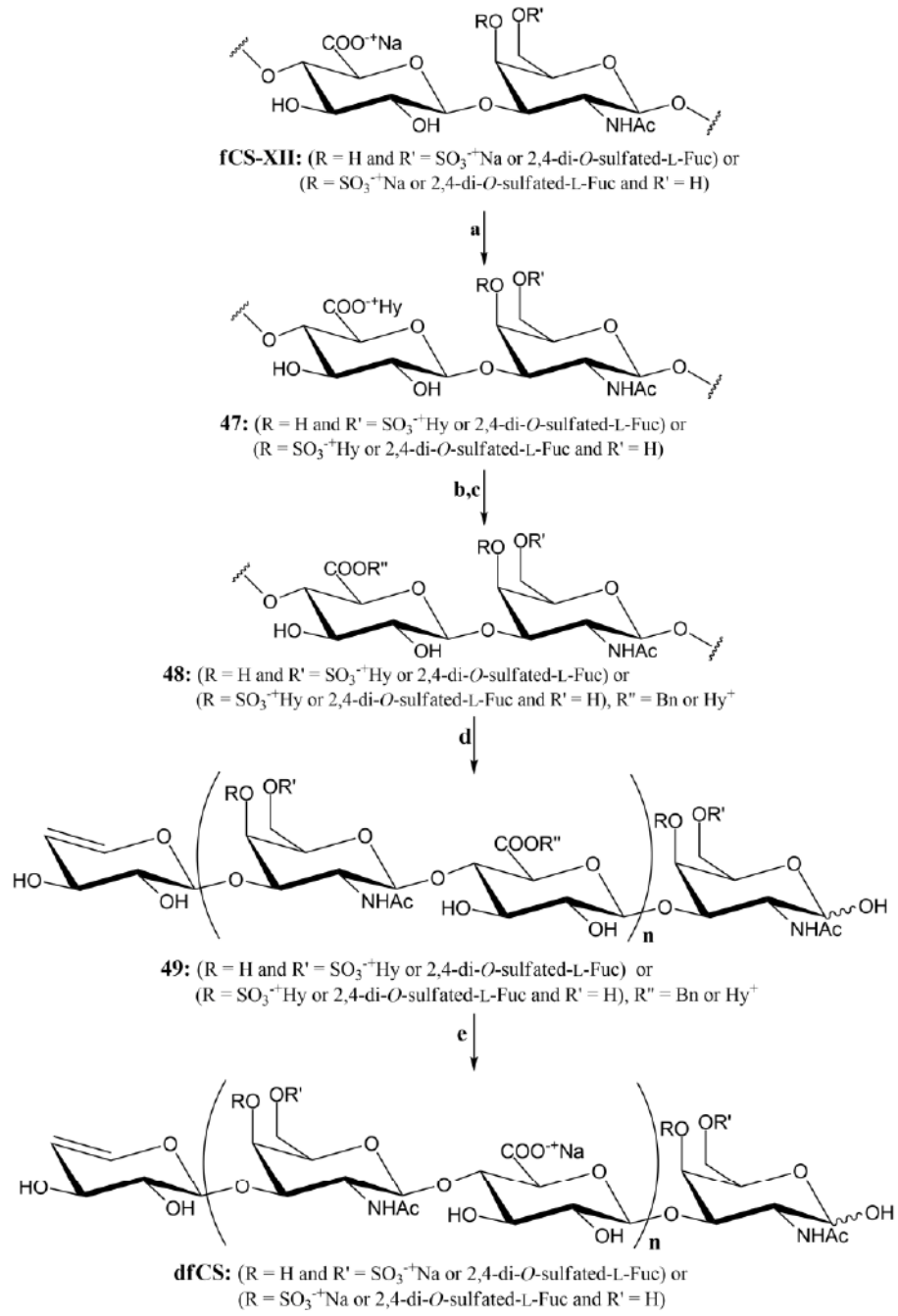


Figure 27: ^{1}H spectrum of **48** (600 MHz, D_2O, 298 K)

After a second transalification, the polysaccharide derivative was dissolved in DMF and treated with a 0.04 M solution of EtONa in ethanol for 30 minutes at rt and then subjected to global alkaline deprotection to remove benzyl ester groups and afford **dfCS** (Scheme 15).



Scheme 15: (a) HyCl, H_2O, rt, quant.; (b) BnCl, DMF, 35°C, overnight, 36%, $DS_{benzylation}$ = 0.39; (c) HyCl, H_2O, rt; (d) 0.04 M EtONa in EtOH, DMF, rt, DS = 0.23; (e) NaOH, H_2O, rt, 19% after three steps, DS = 0.24

After a dialysis and size-exclusion chromatography pure **dfCS** was obtained with a 19% yield and a DS = 0.24 that was evaluated comparing the integration of *H*-4 of unsaturated GlcA (δ_H = 5.86 ppm) and acetyl of GalNAc (δ_H = 2.05-1.99 ppm).

In order to investigate the structure of the depolymerized derivatives, a detailed 2D-NMR (HSQC-DEPT and HSQC-TOCSY) analysis was conducted. HSQC-DEPT spectrum of **dfCS** (Figure 28) displayed two cross peaks ($\delta_{H/C}$ = 5.22/99.2 and 5.70/99.1 ppm, respectively) in the typical region for anomeric CH of a α-linked sulfated Fuc units. Thus, two different α-linked Fuc branches linked either at *O*-6 or at *O*-4 of GalNAc units (see also Chapter II) were still present after the depolymerization route, as well as the sulfate groups on fucose (δ_H = 4.44 and 4.68 ppm, for *H*-2 and *H*-4, respectively) and on GalNAc units ($\delta_{H/C}$ = 4.74/78.9 and 4.21/69.8 ppm for sulfation at *CH*-4 and *CH*-6, respectively). Moreover, HSQC-DEPT spectrum displayed three integral intensities of cross-peaks in the alkene region ($\delta_{H/C}$ = 6.02/108.7, 5.96/108.7 and 5.86/109.6 ppm, respectively), that might be attributed to three different unsaturated GlcA units probably accounting for GlcA units at non reducing terminal linked to 4-*O*-, 6-*O*- or non-fucosylated GalNAc units (Table 21).

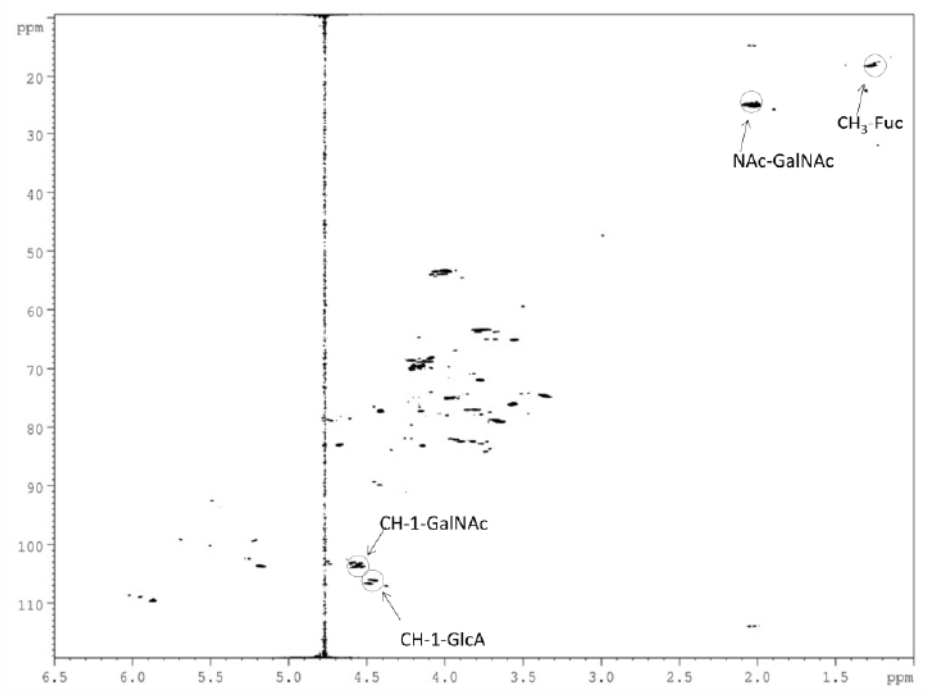


Figure 28: Zoom of ^{1}H-^{13}C HSQC-DEPT spectrum of **dfCS** (800 MHZ, D_2O, 298 K)

Table 21: ^{1}H (plain) and ^{13}C (italic) chemical shift attribution of **dfCS** (800 MHz, D_2O, 298 K)

Residue[a]	**1**	**2**	**3**	**4**	**5**	**6**	**Other signals**
GlcA	4.48 *106.5*	3.36 *74.6*	3.58 *76.1*	3.73 *84.0*	3.65 *79.1*	---	---
GalNAc4S	4.59-4.51 *103.9-103.7*	4.05-3.97 *54.0-53.1*	4.01 *77.9*	4.73 *78.8*	3.87 *74.3*	3.70 - 3.80 *63.4*	Ac 2.05-1.99 *25.0*
αFuc2,4S-(1→6)-*GalNAc4S*			n.d.[b]		n.d.[b]	3.72 - 3.55 *65.0*	Ac 2.05-1.99 *25.0*
GalNac6S			3.82 *82.4*	4.16 *69.7*	3.89 *75.0*	4.21 *69.8*	Ac 2.05-1.99 *25.0*
αFuc2,4S-(1→4)-*GalNAc6S*			3.76 *82.8*	n.d.[b]	3.98 *75.2*	4.20 *69.8*	Ac 2.05-1.99 *25.0*
αFuc2,4S-(1→6)-GalNAc	5.23 *99.2*	4.44 *77.2*	4.13 *68.8*	4.68 *83.1*	4.22 *68.6*	1.27 *18.2*	---
αFuc2,4S-(1→4)-GalNAc	5.70 *99.1*	4.44 *77.2*	n.d.[b]	n.d.[b]	4.16 *68.9*	1.26 *18.1*	---
ΔGlcA^{I}	n.d.[b]	n.d.[b]	n.d.[b]	6.02 *108.7*	---	---	---
ΔGlcA^{II}	n.d.[b]	n.d.[b]	n.d.[b]	5.95 *109.0*	---	---	---
ΔGlcA^{III}	5.18 103.7	4.09 68.2	3.77 72.0	5.86 *109.6*	---	---	---

Chemical shift expressed in δ.
[a] Indicated by italic characters.
[b] Not determinable

The depolymerised fCS derivative was subjected to STD experiments in collaboration with Dr. Dusan Uhrin research group of University of Edinburgh, in order to evaluate its interaction with L- and P-selectins. A preliminary analysis was performed on NMR data obtained from dfCS demonstrated the contact with L-selectin. By comparison of STD spectrum with the ^{1}H-NMR reference spectrum (Figure 29) it was possible to suggest the ligand protons that are in the proximity of the protein.

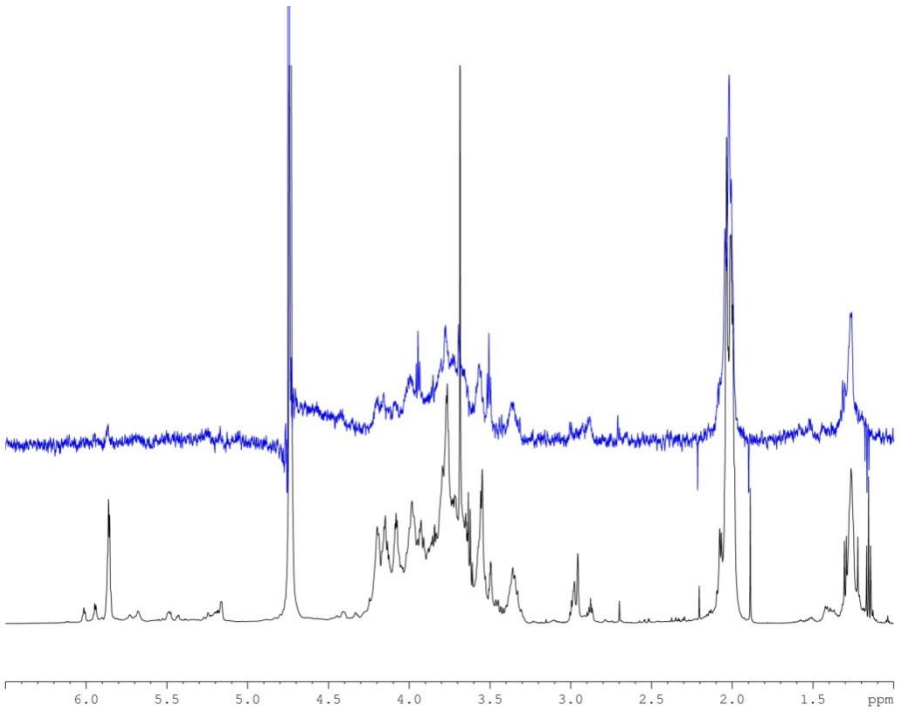


Figure 29: Reference ^{1}H-NMR spectrum (bottom) and STD 1D NMR spectrum (top) of mixture L-selectin-dfCS.

It can be seen that larger STD signals observed belong to CH_3 of Fuc branches and for Ac of GalNAc, that was present also in the L-selectin spectrum, indicating small distances between those ligand protons and the receptor surface in the bound state. The data obtained by comparing

the relative percentages of saturation received by the different ligand moieties showed that signal enhancements belonging to GlcA units were also present, although these protons received lower transfer of magnetization from the protein and thus their individual contributions were weaker (see epitope group mapping Figure 30).

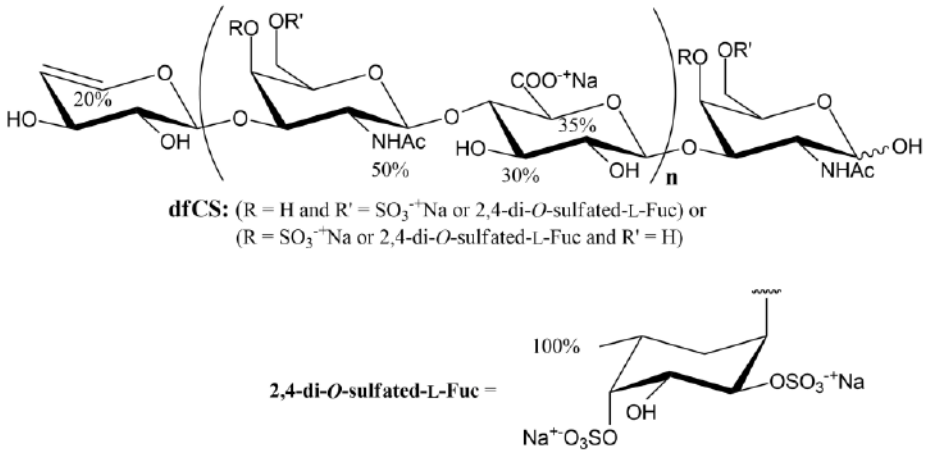


Figure 30: Chemical structure and epitope binding of dfCS to L-selectin.

Due to the overlapping of signals of different units other experiments are in progress to better understand the ligand structure that binds the protein. In the case of P-selectin the same experiment was carried out and the analysis was performed on the obtained NMR data by comparison of STD spectrum with its corresponding reference (Figure 31).

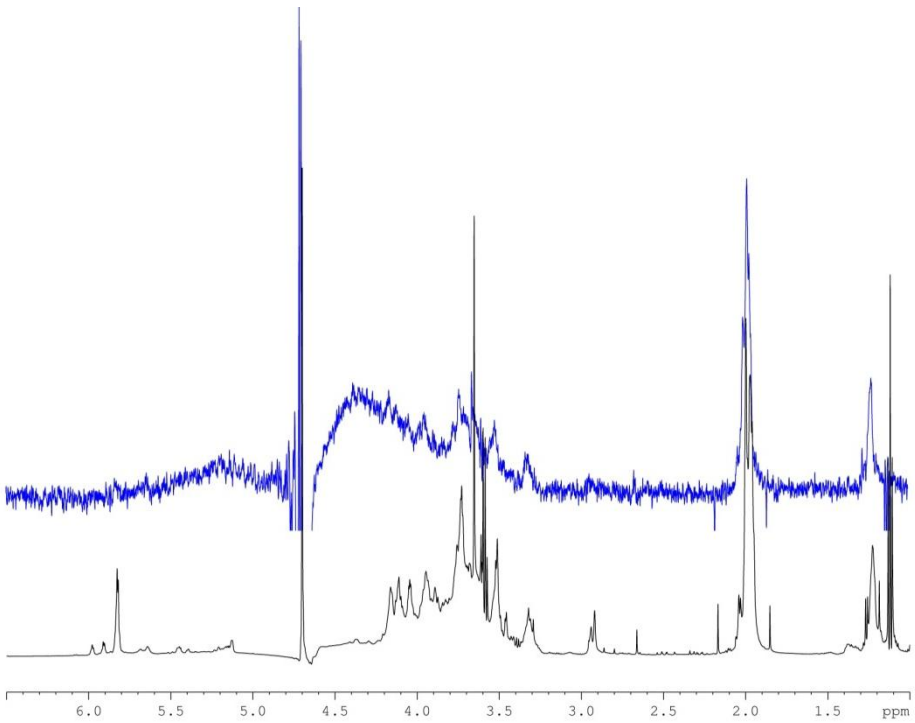


Figure 31: Reference ^{1}H-NMR spectrum (bottom) and STD 1D NMR spectrum (top) of mixture P-selectin-dfCS.

Larger STD signals were observed in this case for C*H*$_3$ of Fuc branches and for *H*-2 of GlcA, indicating small distances between those ligand protons and the receptor surface in the bound state. The data obtained by comparing the relative percentages of saturation received by the different ligand moieties showed that signal enhancements belonging to Ac of GalNAc, that was present also in the P-selectin spectrum, and to *H*-3 GlcA units were also present, with these protons received high transfer of magnetization from the protein and thus their individual contributions were strong. (see epitope group mapping Figure 32).

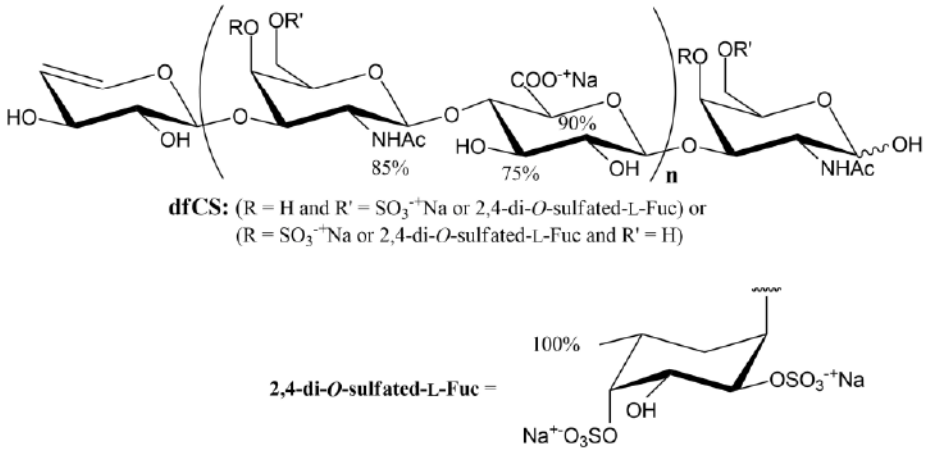


Figure 32: Chemical structure and epitope binding of dfCS to P-selectin.

Due to the overlapping of signals of different units, the integrals and then percentages are only approximate. Indeed, other experiments are in progress to better understand the ligand structure that binds the protein.

3.3 Conclusions

In this chapter, a preliminary strategy for non-degradative β-eliminative depolymerization on semi-synthetic fucosylated chondroitin sulfate has been reported. Although STD-NMR studies revealed the binding of oligosaccharide backbone of dfCS, even if with a slightly smaller affinity relative to the that obtained for oligosaccharides prepared from the native polysaccharide, (data not shown, personal communication Dusan Uhrin). High heterogeneity of the semi-synthetic derivative did not allow an exact evaluation of which parts of the ligand are more involved in the interaction process. Thus, in order to better understand the relationship between structure and L- and P-selectins interactions, it

will be necessary to obtain a less heterogeneous semi-synthetic fCS polysaccharide and, in case, also to design a new depolymerization method on fCS.

3.4 Experimental section

3.4.1 General methods

Commercial grade reagents and solvents were used without further purification. Centrifugations were performed with an Eppendorf Centrifuge 5804 R instrument.

Dialyses were conducted on Spectra/Por 1.0 kDa cut-off membranes at 4°C. Size-exclusion chromatography was performed on a Bio-Gel P2 column (0.75 x 67.5 cm, Bio-Rad) using 50 mM ammonium acetate as a buffer at a flow rate of 0.2 mL/min. The column eluate was monitored continuously with a Knauer K-2310 refractive index refractometer.

Freeze-dryings were performed with a 5Pascal Lio 5P4 K freeze dryer. The term "pure water" refers to water purified by a illipore illi-Q Gradient system.

NMR spectra were recorded on a Bruker AVANCE III-800 (^{1}H: 800 MHz, ^{13}C: 200 MHz) or on a Bruker AVANCE III-600 (^{1}H: 600 MHz, ^{13}C: 150 MHz) instruments equipped with a TCI cryo probe, in D_2O (acetone as internal standard, ^{1}H: $(CH_3)_2CO$ at δ = 2.22 ppm; ^{13}C: $(CH_3)_2CO$ at δ = 31.5 ppm). HSQC-DEPT and HSQC-TOCSY experiments were measured with ^{1}H-detected mode via single quantum coherence with ^{13}C adiabatic decoupling of carbon-proton couplings using data sets of 2048 × 1024 points and typically 8 and 24 scans, for HSQC-DEPT and HSQC-TOCSY, respectively. Mixing time for HSQC-TOCSY was set to 40 ms. Quadrature indirect dimensions were

achieved through Echo-Antiecho method; spectra were processed applying a cosine square function to both dimensions and data matrix was zero-filled by factor of 2 before Fourier transformation. Data matrix was doubled and linear prediction applied the new points by using 32 coefficients; cosine square functions were used as window functions and the phase shift selected depended by the best resolution obtained.

3.4.2 Synthetic procedure

Transalification: fCS-XII (68.3 mg, 0.079 mmol) was dissolved in pure water (2.72 mL) and treated with a 0.16 M aqueous solution of benzethonium chloride (2.72 mL, 0.454 mmol). A white precipitate was outright formed, collected by centrifugation and then dessicated under vacuum affording compound **47** (255.0 mg, quant.) with the presence of benzethonium chloride as contaminant. This reaction was repeated under the same conditions on derivative **48**

Esterification: Compound **47** (254.6 mg, 0.105 mmol) was dissolved in D F (3.40 mL) and treated with BnCl (432.0 μL, 3.75 mmol). After overnight stirring at 35°C, the mixture was cooled to rt and treated with a 3 aqueous solution of sodium acetate (850 μL) and ethanol (7.66 mL). The obtained white precipitate was collected by centrifugation and dessicated under vacuum affording compound **48** (79.8 mg, 36%).

Depolymerization: Derivative **48** (86.3 mg, 0.041 mmol) was suspended in DMF (1.0 mL) and treated with a 0.04 M solution (1.0 mL, 0.04 mmol) of sodium ethoxide in ethanol. After 30 minutes

stirring at rt, the mixture was treated with a 1.21M suspension of NaOAc in ethanol (3 mL) and the obtained precipitate was collected by centrifugation and dessicated under vacuum affording derivative **49** (1.321 g)

Global deprotection: Compound **49** was dissolved in pure water (3.0 mL), treated with a 15% w/v NaOH solution and stirred at rt for 30 minutes. After neutralization with 1.0M solution of HCl, the mixture was treated with ethanol and the obtained precipitate was collected by centrifugation and dessicated under vacuum obtaining a white solid (4.7 mg). The supernatant, after dialysis, and subsequent freeze-drying, yielded a white solid, that was further purified by size-exclusion chromatography. Freeze-drying of the collected fractions afforded **dfCS** (7.6 mg, 19% after three steps)

3.4.3 STD experiments

The samples were dissolved in 99.9% D_2O (540 µl) containing deuterated 10 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 7.2) to which a stock solution of EDTA (20 µl) and TSP was added. The stock solution was prepared by dissolving 4 mg of EDTA and 9 mg of TSP in 200 µl of the phosphate buffer. The pH was adjusted to 7.2 by adding few drops of a concentrated solution of NaOH in D_2O.

STD NMR experiments on the mixtures were performed with protein/ligand molar ratios varying from 1:50 to 1:100 using the BRUKER stddiffesgp.3 pulse sequence with water suppression via a double-pulsed field gradient spin-echo (2 ms sinc pulses) and the following parameters: 8, 4 and 1.36 seconds relaxation, presaturation

and acquisition times, respectively. The on- and off-resonance irradiation at -4 ppm and 30 ppm. The irradiation was achieved by 78 repetitions of 50 ms Gaussian pulses applied at $\gamma B_1/2\pi$ = 180 Hz. 16 scans were accumulated per one of 128 averaging cycles using one-scan alternate accumulation into on- and off-resonance spectra. The overall spectrometer was 10.6 hours per experiment.

3.5 Bibliography

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CHAPTER 4

SYNTHESIS, CONFORMATIONAL STUDY AND ANTIFREEZE ACTIVITY OF THREONINE DECORATED MICROBIAL SOURCED CHONDROITIN

CHAPTER 4

SYNTHESIS, CONFORMATIONAL STUDY AND ANTIFREEZE ACTIVITY OF THREONINE DECORATED MICROBIAL SOURCED CHONDROITIN

4.1 Introduction

In the last two decades many works were published reporting chemical derivatizations of carboxylic function of CS to afford different derivatives. (a) Bedini et al., **2016**. b) Zhao et al., **2015**. c) Scott et al., **2013**. d) Weyers et al., **2013**. e) Bedini et al., **2012**. f) Schiller et al., **2010**.) Ester modified CS polysaccharide were reported, for example, in the attachment of three different non-stereoidal anti-inflammatory drugs through a PEG-spacer, that was studied as prodrug systems (Peng et al., **2006**) or carboxylic acid esterification of GlA units in different native fCSs has very recently been accomplished with several alkyl bromides. (Wu et al., **2015**)

Moreover carboxy group derivatives have also been used to cross-link CS polysaccharide chain into networks that are able to adsorb large amount of water and have found several biomedical application in the field of tissue engineering and regenerative medicine. (Bedini et al., **2016**)

Among the derivatization reactions, carboxyl group modification with amines or hydrazides is very common. Indeed CS polysaccharide has

been coupled through amide linkages with several small biomolecules, such as tyramine (a) Ni et al., **2015**. b) Jin et al., **2013**.), histamine (Yu et al., **2014**), biotin (Altgärde et al., **2013**), and cholesterol (Yu et al., **2013**) among others. Moreover, biomacromolecules, such as tissue proteins and hyaluronic acid, were coupled too. (a) Yan et al., **2013**. b) Yu et al., **2013**. c) Daamen et al., **2003**. d) Kuipers et al., **2000**.) Nonetheless, to the best of our knowledge no systematic study of how the activation conditions of the carboxylic acid of chondroitin or CS influence the degree of substitution has been done yet. Furthermore, a very variable DS for amidation of such polysaccharides has been obtained, with values ranging from 0.01 to 0.88. (Bedini et al., **2016**)

Colwellia psychrerythraea 34H is a psychrophilic Gram-negative bacterium found in Arctic marine sediments (Ewert et al., **2013**) that produces extracellular polysaccharides (a) Methé et al., **2005**. b) Huston et al., **2004**.) with cryoprotectant function and apparent ice-affinity (a) Ewert et al., **2011**. b) Marx et al., **2009**.) The adaptation of bacteria to low temperature can be regulated by several different mechanism, such as the production of cold-active enzymes (Huston et al., **2004**), as well as, antifreeze proteins (AFPs), antifreeze glycoproteins (AFGPs) and cryoprotectants. (Deming, **2009**) It has been found that C. *psychrerythraea* 34H antifreeze activity is driven by an original, alternative mechanism due to the production of polysaccharides with ice recrystallization inhibition activity. (a) Casillo et al., **2017**. b) Carillo et al., **2015**.) One of them, a capsular polysaccharide (CPS), is characterized by a GAG-like linear tetrasaccharide repeating unit composed of alternating uronic acids and *N*-acetyl-hexosamines (GlcA, GalA, GalNAc and GlcNAc), with a Thr decoration on GalA to complete the structure (Figure 33). The CPS was

suggested to confer the cryoprotectant activity to the bacterium. (Carillo et al., **2015**)

Anti-freeze molecule applications can range from cell, tissue, organ and frozen food cryopreservatives as well as cryosurgical aids. The design of recrystallization inhibitors that mimic the structure of antifreeze natural products is a challenging field in synthetic organic and polymer chemistry. Both total synthetic small molecules and macromolecules were obtained and then tested for this activity. (a) Balcerzak et al., **2014**. b) Gibson, **2010**.) The GAG-like structure of the CPS from *C. psychrerythraea* 34H suggested to regioselectively decorate unsulfated chondroitin polysaccharide with a Thr on GlcA units and then assay its antifreeze activity. To this aim a semi-synthetic approach was applied (Laezza et al., **2017**) taking advantage of the high availability of pure unsulfated chondroitin from the fed-batch fermentation of *Escherichia coli* K4 followed by a suitable downstream purification. (Cimini et al., **2010**) This polysaccharide was employed, as previously reported in Chapter II, as starting material for the obtainment of non-animal sourced CS and fucosylated CS polysaccharides through tailored semi-synthetic strategy (a) Laezza et al., **2016**. b) Laezza et al., **2015**. c) Laezza et al., **2014**. d) Bedini et al., **2012**. e) Bedini et al., **2011**.) In this case, it was employed to study the different conditions of carboxylic acid activation in order to achieve the highest possible DS of Thr decoration of the chondroitin polysaccharide backbone and to evaluate the relationship between structure and anti-freeze activity.

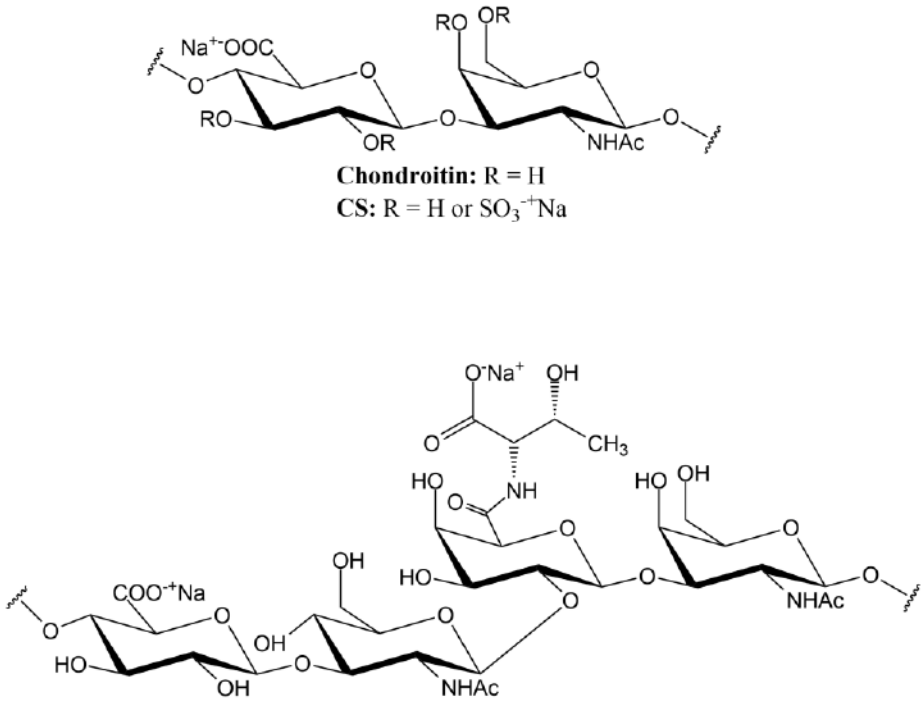


Figure 33: Repeating units of CS, unsulfated chondroitin (top) and CPS from *C. psychrerythraea* 34H (bottom)

4.2 Results and discussion

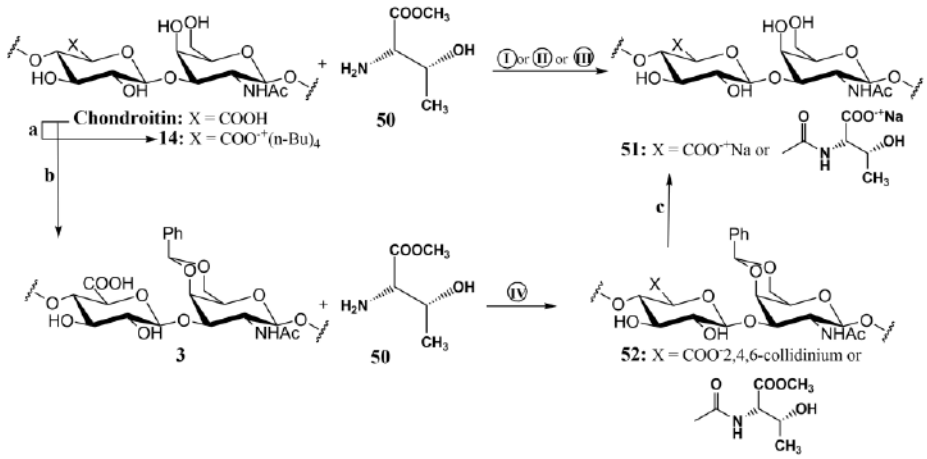
4.2.1 Threonine decoration of chondroitin polysaccharide

Amide bond formation relies on the activation of carboxylic function. By limiting to the most common ones, carboxylic acid activators can be classified according to their structure into phosponium (Coste, **2002**) and uronium/guanidinium salts (Bienert et al., **2002**) (that were recently applied in order to reduce side reactions of amidation), carbodiimides (a) Williams et al., **1981**. b) Mikolajczyk et al., **1981**.) and triazines (a) Borke et al., **2015**. b) D'Este et al., **2014**.)

By looking for example of amidation reactions of GAGs in literature it was found that hyaluronic acid was coupled with amines after a GlcA

carboxylic function activation not only with EDC/NHS, but also with DMTMM (a) D'Este et al., **2014**. b) Gemma et al., **2007**.) or other triazine species, displaying DSs far from a quantitative value. (Borke et al., **2015**) Moreover, amidation of carboxylic function of GlcA units in CS was performed with EDC, or more rarely DCC in the presence or not of a protic additive such as HOBt or NHS. (Bedini et al., **2016**) Our first attempt to decorate microbial sourced chondroitin polysaccharide employed threonine methyl ester **50** (Devedjiev et al., **2008**) in the presence of EDC and NHS in MES buffered water (pH = 6) (Scheme 16). After overnight reaction at rt, ester cleavage was conducted one-pot by adding sodium hydroxide. Dialysis and subsequent freeze-drying furnished polysaccharide **51-I** in 86% mass yield (Table 22), that was subjected to ^{1}H NMR analysis for evaluation of Thr amide DS. By integration of the CH_3 signal of Thr moiety (δ_H = 1.20 ppm) with respect to acetyl one (δ_H = 2.01 ppm), a 0.14 value was obtained (Figure 34). In order to understand the structure-function relationship between Thr decoration and antifreeze activity, it was necessary to enhance the DS of coupling reaction. Since amidation in the presence of phosponium and uronium/guanidinium salts were usually performed in DMF, chondroitin was firstly converted into its TBA salt using aqueous TBAOH according to a known procedure (Valoti et al., **2012**), in order to obtain a more soluble polysaccharide in such solvent. A clear solution of chondroitin-TBA salt in DMF was then treated at rt with a phosphonium salt activator such as pyBOP® in the presence of HOBt and DIPEA as a base. The amide coupling was alternatively conducted with an uronium salt activator such as TBTU and 2,4,6-collidine as base. Successive *one-pot* cleavage of Thr methyl ester, dialysis, and freeze-drying, afforded polysaccharides, for which the

integration of their ^{1}H-NMR signals allowed DS evaluation. In pyBOP®/HOBt case (**51-II**, Scheme 16) only a slightly increase of DS (0.19) with respect to EDC/NHS reaction was observed, whereas a much significant enhancement was obtained for polysaccharide **51-III** (DS = 0.43), nonetheless the coupling reaction was still very far from affording a quantitative Thr decoration.



Scheme 16: (a) see ref. Valoti et al., **2012**; (b) α,α-dimethoxytoluene, CSA, DMF, 80°C, overnight, quant., DS = 1; (I) 1: EDC, NHS, MES, H_2O, rt, overnight, 2: NaOH, H_2O, rt, 86%, DS = 0.14; (II) 1: pyBOP®, HOBt, DIPEA, DMF, rt, overnight, 2: NaOH, H_2O, rt; 69%, DS = 0.19; (III) 1: TBTU, 2-4-6-collidine, DMF, rt, overnight, 2: NaOH, H_2O, rt, 76%, DS = 0.43; (IV) TBTU, 2-4-6-collidine, DMF, rt, overnight, (c) 1: $NaBrO_3$, $Na_2S_2O_4$, H_2O-ethyl acetate, rt, overnight, 2: NaOH, H_2O, rt, 51%, DS = 1

Table 22: Yield and DS of threonine decorated chondroitin polysaccharides

Polysaccharide	Mass yield	DS
51-I	86%	0.14
51-II	69%	0.19
51-III	76%	0.43
51-IV	51%	1.00

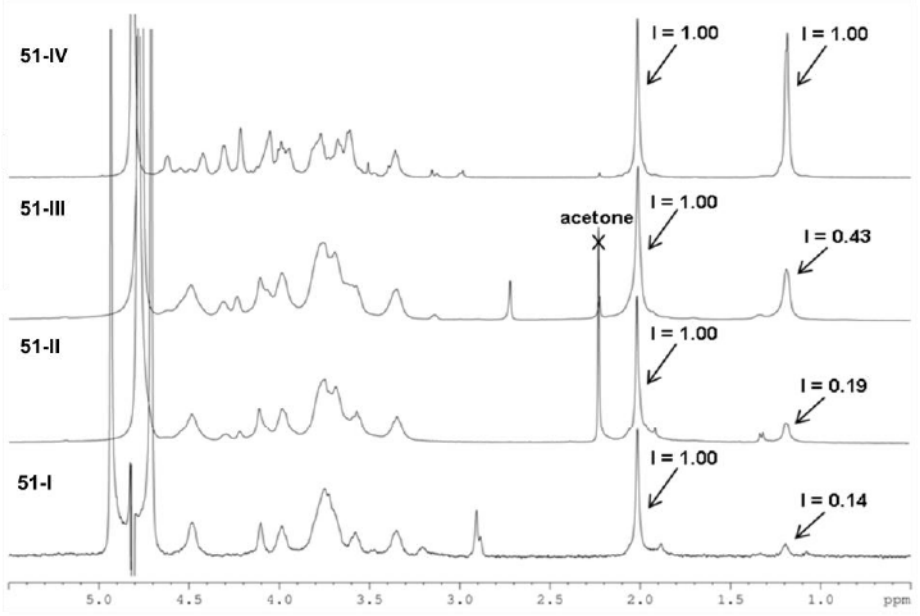


Figure 34: ^{1}H-NMR spectra (600 MHz, D_2O, 298 K) of polysaccharides **51-I-IV**(I = Integral area)

It was hypothesized that low DS values could be due to a strong network of hydrogen bonds between carboxylic functions and hydroxyl groups of chondroitin, that should be rather effective especially in an aprotic solvent such as DMF. This could impede the activation of carboxylic acids in every GlcA unit of the polysaccharide chain. To minimize this effect, the protection of some hydroxyl groups of

chondroitin was planned by employing a benzylidene ring that was installed at 4,6-diol of GalNAc units by reaction with α,α-dimethoxytoluene in the presence of CSA as acid catalyst, as already reported in Chapter II. (Bedini et al., **2011**) This chondroitin derivative was then subjected to amide coupling with **50** under the activation condition that performed best on the unprotected polysaccharide (TBTU/2,4,6-collidine). The derivative **52** was then isolated by precipitation after addition of diisopropyl ether, as suitable solvent, to the reaction mixture. Its ^{1}H-NMR spectrum could not give an exact DS evaluation, as methyl signal of linked Thr amide moiety (δ_H = 1.09 ppm) partially overlapped with the same kind of signal from free Thr amine **50** (δ_H = 1.02 ppm), that co-precipitated with **52**. A global deprotection of **52** was performed in two steps, by firstly treating it with $NaBrO_3$ and $Na_2S_2O_4$ in H_2O-ethyl acetate mixture to cleave oxidatively the benzylidene ring (Adinolfi et al., **1999**) affording a polysaccharide with Bz esters at position either *O*-4 or *O*-6 of GalNAc units (Bedini et al., **2011**), that were then hydrolysed under alkaline aqueous conditions together with Thr methyl ester to give polysaccharide **51-IV**. To our delight, integration of its ^{1}H-NMR spectrum showed a quantitative decoration (DS = 1.00) of GlcA with Thr amides (Figure 34). Attribution of chemical shift values of polysaccharide **51-IV** (Table 23) was possible by analysis of ^{13}C and two-dimensional NMR spectra (COSY, TOCSY, NOESY, HSQC-DEPT) and their comparison with the data reported in the literature for unsulfated chondroitin. (Mucci et al., **2000**) In particular, the integral intensity of cross-peak at $\delta_{H/C}$ 4.21/61.0 ppm in the HSQC-DEPT spectrum (Figure 35) could be associated to the *CH* group at position α of Thr units linked through an amide bond to GlcA carboxylic moieties.

Table 23: ^{1}H (plain) and ^{13}C NMR (italic) chemical shift attribution of **51-IV**

Residue	**1**	**2**	**3**	**4**	**5**	**6**	**Other Signals**
GlcA	4.62 *105.0*	3.35 *72.9*	3.62 *74.2*	3.94 *77.1*	4.05 *68.7*	-- *170.3*	--
GalNAc	4.42 *100.0*	3.99 *51.6*	3.80 *81.6*	4.07 *74.5*	3.60 *75.6*	3.76-3.67 *61.7*	NH*C*OCH$_3$: *175.7* NHCOC*H*$_3$: 2.01, *23.1*
	α	β	γ				
Thr	4.21 *61.7*	4.30 *68.4*	1.20 *19.9*				COOH: *176.9*

NMR experiments conducted in D_2O (600 MHz, 298 K).
Chemical shifts expressed in δ relative to internal acetone [^{1}H: $(CH_3)_2CO$ at δ = 2.22 ppm; ^{13}C: $(CH_3)_2CO$ at δ = 30.9 ppm].

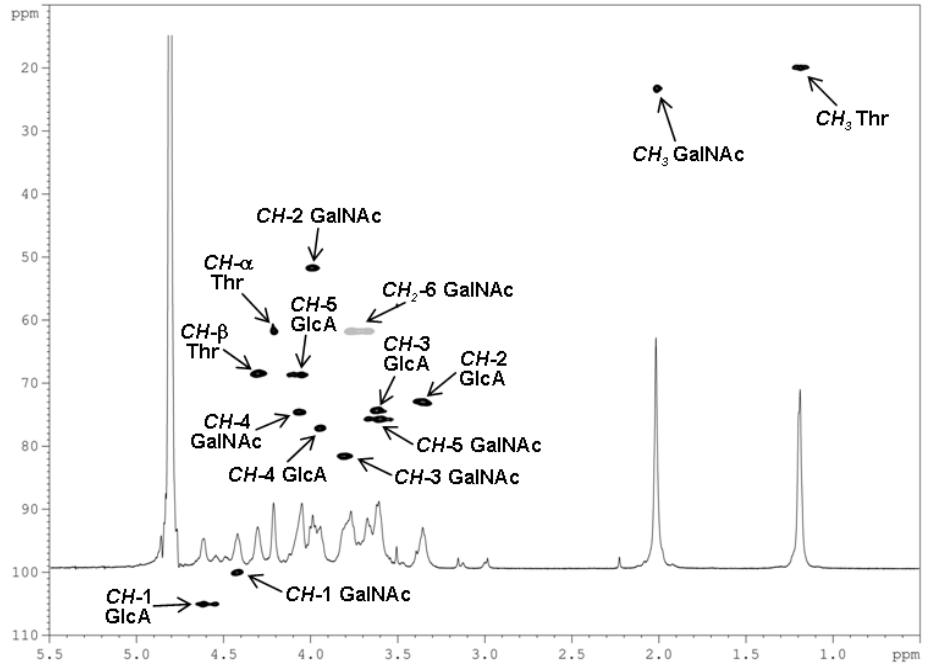


Figure 35: ^{1}H and ^{1}H-^{13}C HSQC-DEPT NMR spectra (600 MHz, D_2O, 298 K) of polysaccharide **51-IV**

4.2.2 Conformational study

With the aim of determining the three-dimensional arrangement of **51-IV**, an in-depth analysis of their NMR spectra has been performed. The NOE connectivities in the NOESY spectra between 1,3-diaxial protons [*H*-1/*H*-3/*H*-5 and *H*-2/*H*-4 for GlcA(Thr) and *H*-1/*H*-3/*H*-5 for GalNAc], unambiguously indicated that both sugar moieties assume the classical $^{4}C_1$ chair conformation. NOESY spectra also showed interesting inter-residue NOEs that are diagnostic for the determination of the spatial evolution of the polysaccharide. Particularly, strong NOEs between *H*-1 of GlcA(Thr) and *H*-3 of GalNAc, and between *H*-1 of GalNAc and *H*-4 of GlcA(Thr) indicate the relative spatial

orientation of the sugars. The lack of long-range NOEs suggests that the overall structure of the polysaccharide is fairly linear. This structural information was employed to construct a simplified model of the semi-synthetic chondroitin polysaccharide [made up by six repetitions of the GlcA(Thr)-GalNAc disaccharide subunit] through NMR-restrained simulated annealing (SA) calculations (this work was made in collaboration with the group of Prof. Antonio Randazzo at the Department of Pharmacy of the University of Naples Federico II). An ensemble of 400 isoenergetic structures was calculated featuring for all the considered distances a maximum violation of 0.12 Å. Subsequently, different conformations of this ensemble were clustered considering the position of the sugar ring atoms belonging to the central 4 disaccharide subunits with a root-mean-squared difference (rmsd) value of 1.5 Å. Clustering of the obtained 400 conformations resulted in 38 different conformational families among which cluster 3 represented the 25% of the total ensemble demonstrating a good convergence of our calculations toward a well-defined structure. For this cluster, the representative conformation was considered (i.e. the one closest to the centroid of the cluster) for further 310 K molecular dynamics (MD) simulations aimed at inspecting the thermodynamic stability in explicit solvent. From these calculations (NMR-restrained SA and MD simulations), it was clear that the structure adopts a fairly linear conformation which is stable over time as demonstrated by plotting of the rmsd values during the last 10 ns of the production run. This conformation is stabilized by the formation of a series of inter-residue H-bond interactions, which are pretty conserved along the polysaccharide structure (Figure 36). Interestingly, the calculated conformation does recall neither the “zigzag” arrangement of the *C.*

psychrerythraea Thr-decorated CPS (Carillo et al., **2015**) nor the helical conformation very recently detected for the exopolysaccharide (EPS) secreted by the same bacteria and displaying alanine (Ala) amide groups on GalA units. (Casillo et al., **2017**) Interestingly, the presence of a pseudo-helical conformation in EPS allowed entrapping water molecules in discrete clefts, where no tetrahedral arrangement was detected thereby disfavouring the formation of ice crystals. From these considerations, it might be suggested that the linear structure of the semi-synthetic Thr-decorated chondroitin polysaccharide **51-IV** might not be conducive to an antifreeze property.

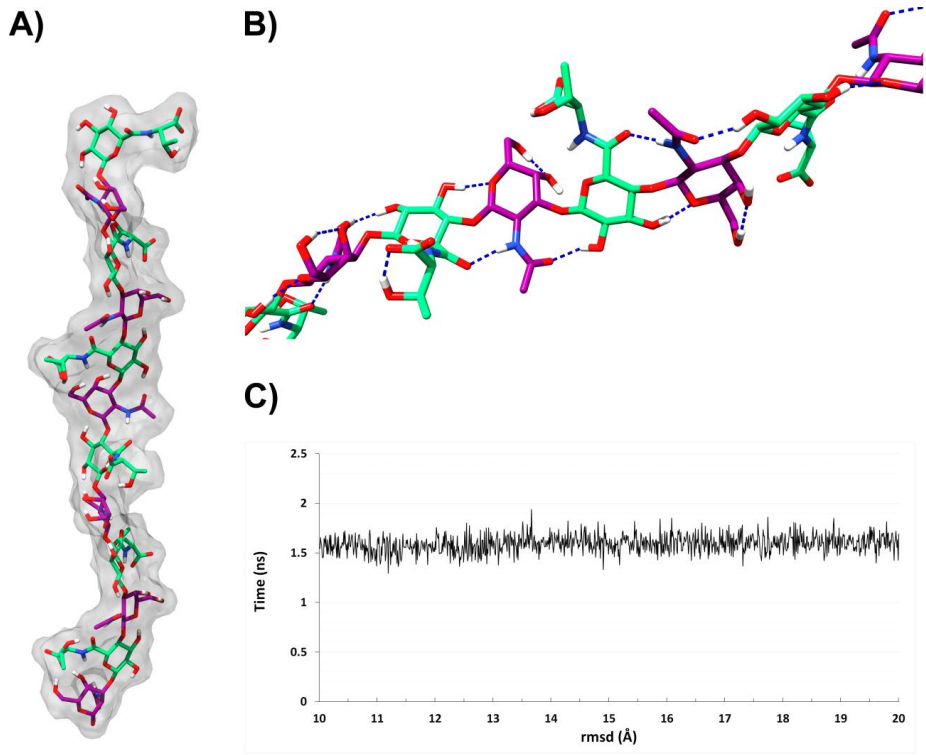


Figure 36: Front (A) and zoomed (B) view of the representative structure of the inspected polysaccharide as calculated by restrained SA calculations. GlcA(Thr) and GalNAc are represented as green and purple sticks. H-bonds are represented as dashed blue lines. C) rmsd fluctuations of the studied of the polysaccharide heavy atoms, along the last 10 ns of the MD simulation with respect to the conformation reported in (A)

4.2.3 Antifreeze activity and molecular weight determination

In order to assess the cryoprotective effect of the Thr decorated polysaccharides, ice recrystallization inhibition (IRI) activity was measured. Antifreeze proteins, antifreeze glycoproteins and several synthetic polymers have been shown to be potent ice recrystallization

inhibitors. (a) Congdon *et al*., **2013**. b) Gibson, **2010**) IRI has been linked to survival in frozen environments and can be applied to cellular cryopreservation. (Deller et al., **2014**) In order to test the IRI activity, a modified 'splat' assay was used. (Knight et al., **1988**) Briefly, 10 μL droplets of the polysaccharide in PBS was dropped down a tube onto a cooled (∼−70 °C) glass cover slip, and then transferred to a microscope set at −6 °C. After 30 min, the mean largest grain size (MLGS) was measured and reported relative to a PBS control. It is important to note that in this assay, MLGS values below ~ 20% indicate zero growth (as the initial crystals cannot have zero size). The IRI activity of the polysaccharides is reported in Figure 37 alongside a negative control (PEG) and another polysaccharide, dextran (M_w ~ 40 kDa). Compared to PEG, the polysaccharides do have some moderate IRI activity, reducing the grain size by up to 40 % compared to PBS alone. The activity seems similar to Dextran, which is only a very weak IRI; however it should be noted that IRI activity has been observed in nearly all systems to increase with molecular weight of the species tested here. It was determined by means of the SLS (Schmitz, **1990**) in collaboration with the group of Prof. Luigi Paduano at the Department of Chemical Sciences of the University of Naples Federico II and the species that were 35.1±1.7 kDa, 22.4±1.1 kDa and 16.9±0.8 kDa, respectively for **51-II-IV**. Interestingly, there is a trend that as the threonine content increases, the IRI activity is reduced. There have been several examples of Thr- and/or Ala-rich (glyco)proteins and polysaccharides acting as antifreeze agents in Nature (a) Carillo et al., **2015**. b) Lin et al., **2011**. c) Graether et al., **2000**) It is clear that Thr decorations alone is insufficient for introduce potent IRI activity, and is

the observed activity is slightly less than the EPS from *C. psychrerythraea*.

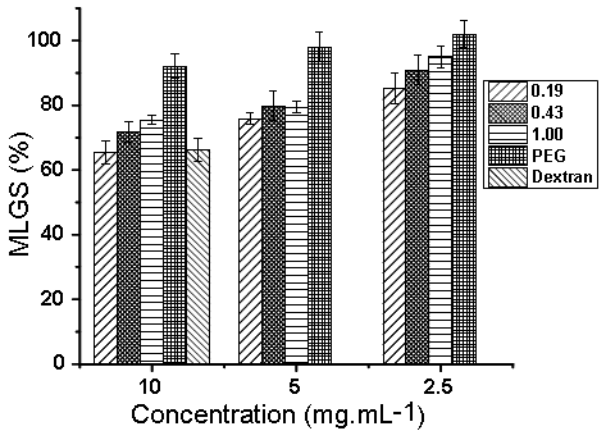


Figure 37: IRI activity of the chondroitin polysaccharide **51-II-IV** (0.19, 0.43 and 1.00 indicate the DSs of Thr amidation), PEG (negative control) and dextran (positive control). Mean largest grain size is expressed as a percentage of PBS buffer, and small MLGS values indicate increased IRI activity

4.3 Conclusions

A semi-synthetic mimic of the antifreeze CPS from *C. psychrerythraea* was obtained after decoration of carboxylic function of microbial sourced chondroitin with Thr amides. To this aim, several amide coupling conditions were screened in order to obtain a quantitative DS for Thr decoration. The lower DSs (0.14-0.43) were obtained by performing the reaction on underivatized chondroitin, whichever the kind of carboxylic acid activator (carbodiimide-, phosphonium- or uronium-type) was employed, whereas a quantitative DS was displayed by performing the reaction on a partially protected polysaccharide derivative. This result was ascribed to a weakening of the hydrogen

bond network acting in DMF – the solvent used for the amidation reaction – after masking some hydroxyls of the polysaccharide with protecting groups.

The three-dimensional arrangement of the Thr-decorated polysaccharide was then investigated by NOESY NMR and SA and MD simulations, determining a stable, fairly linear conformation. This was rather different from the "zigzag" arrangement determined for the CPS from *C. psychrerythraea*. (Carillo et al., **2015**) This difference explained the much lower ice recrystallization inhibition activity measured for the semi-synthetic polysaccharide with respect to the natural one. Indeed, the absence of discrete clefts in the linear structure of the former does not allow the entrapment of water molecules in a non tetrahedral arrangement in the first hydration shell, that disfavors the formation of ice crystals. Therefore, this work demonstrates that in spite of several examples of Thr- and/or Ala-rich (glyco)proteins and polysaccharides acting as antifreeze agents in Nature (a) Carillo et al., **2015**. b) Lin et al., **2011**. c) Graether et al., **2000**.), the presence of a high number of Thr decorations on the biomacromolecule is not a sufficient element for gaining ice recrystallization inhibition activity. However, it is worth noting that also the presence of a polysaccharide structure with discrete clefts such as in a pseudo-helical conformation has been recently demonstrated to be not a sufficient condition alone for IRI activity. Indeed, a very recently discovered Thr/Ala free CPS, still isolated from *C. psychrerythraea* and displaying a helical structure, does not show any antifreeze activity. (Casillo et al., **2017**)

This finding will help future investigations towards the development of (semi-)synthetic polymer mimics of natural antifreeze agents to be applied in the cryopreservation field.

4.4 Experimental section

4.4.1.General methods

Commercial grade reagents and solvents were used without further purification, except where differently indicated. The term "pure water" refers to water purified by a Millipore Milli-Q Gradient system. Centrifugations were performed with an Eppendorf Centrifuge 5804R instrument at 4°C (4600 g, 10 min). Dialyses were conducted on Spectra/Por 3.5 kDa cut-off membranes at 4°C. Size-exclusion chromatographies were performed on a Bio-Gel P2 column (0.75 x 67.5 cm, Bio-Rad) using 50 mM ammonium bicarbonate as a buffer at a flow rate of 0.2 mL/min. The column eluates were monitored continuously with a Knauer K-2310 refractive index refractometer. Freeze-dryings were performed with a 5Pascal Lio 5P 4K freeze dryer. NMR spectra were recorded on a Bruker DRX-600 (^{1}H: 600 MHz, ^{13}C: 150 MHz) instrument equipped with a cryo probe, in D_2O (acetone as internal standard, ^{1}H: $(CH_3)_2CO$ at δ 2.22 ppm; ^{13}C:$(CH_3)_2CO$ at δ 31.5 ppm) or DMSO-d_6 (^{1}H: CHD_2SOCD_3 at δ 2.49 ppm; ^{13}C: CD_3SOCD_3 at δ 39.5 ppm). Gradient-selected COSY, phase sensitive NOESY, and TOCSY experiments were performed using spectral widths of 6000 Hz in both dimensions, using data sets of 4096 × 256 points. Quadrature indirect dimensions were achieved through States-TPPI method; spectra were processed applying a cosine square function to both dimensions and data matrix was zero-filled by factor of 2 before Fourier transformation. TOCSY and NOESY mixing times were set to 120 and 200 ms, respectively. HSQC-DEPT experiments were measured in the ^{1}H-detected mode via single quantum coherence with

proton decoupling in the ^{13}C domain, using data sets of 2048 × 256 points and typically 32 increments.

Phosphate-buffered saline (PBS) solution was prepared using preformulated tablets (Sigma-Aldrich) in 200 mL of Milli-Q water (>18.2 Ω mean resistivity) to give [NaCl] = 0.138 , [KCl] = 0.0027 M, and pH 7.4.

SLS measurements were performed with a home-made instrument composed by a Photocor compact goniometer, a SMD 6000 Laser Quantum 50 mW light source operating at 5325 Å, a photomultiplier (PMT-120-OP/B) and a correlator (Flex02-01D) from Correlator.com.

4.4.2 Synthetic procedure

Preparation of polysaccharide 51-I: Chondroitin sodium salt (22.0 mg, 54.9 μmol) was dissolved in pure water (1.0 mL) and passed through a short Dowex 50 WX8 column (H^+ form, 20-50 mesh, approx. 5 cm^3). Elution with pure water was continued until pH of the eluate was neutral. Freeze-drying of the collected eluate gave chondroitin (20.0 mg, 52.8 μmol), that was suspended in pure water (4.0 mL) and treated with EDC (22.7 mg, 0.146 mmol) and NHS (8.5 mg, 73.9 μmol). After 40 min stirring at rt, ES (40.0 mg, 0.204 mmol) and then Thr methyl ester **50** (35.7 mg, 0.264 mmol) were added and stirring was continued overnight. Few drops of 1M NaOH solution were then added to adjust pH to 12. After 6 hours stirring at rt, the solution was then neutralized with 1M HCl. Dialysis and subsequent freeze-drying afforded polysaccharide **51-I** (17.2 mg, 86%) as a white waxy solid.

Preparation of polysaccharides 51-II and 51-III: Chondroitin TBA salt **14** (31.8 mg, 51.3 μmol) – obtained from chondroitin sodium salt according to a known procedure (Valoti et al., **2012**) – was suspended in DMF (2.1 mL) that was freshly dried over activated 4Å molecular sieves. The mixture was heated to 80°C and stirred for 3 hours, after that a clear solution was obtained. After cooling to rt, in the case of obtainment of **51-II** the solution was treated with 0.27M solutions of pyBOP® and HOBt in freshly dried D F (376 μL each, 0.102 mmol), then with a 0.48M solution of **50** in freshly dried D F (300 μL, 0.144 mmol), and finally with DIPEA (88.8 μL, 0.510 mmol) that was freshly dried over activated 4Å molecular sieves. Alternatively, in the case of obtainment of **51-III**, the solution was treated with a 0.44 M solution of TBTU in freshly dried D F (229 μL, 0.101 mmol), then with a 0.48 M solution of **50** in freshly dried D F (300 μL, 0.144 mmol), and finally with 2,4,6-collidine (66.5 μL, 0.503 mmol) that was freshly dried over activated 4Å molecular sieves. Both solutions were stirred at rt overnight and then treated with few drops of 1M NaOH aqueous solution. After further 6 hours stirring, the solutions were neutralized by adding a 1M HCl aqueous solution and then dialyzed and freeze-dried. Since the ^{1}H-NMR spectrum of both products clearly displayed the signals of residual TBA ions, a further purification was made by dissolving the polysaccharides in pure water (3.0 mL) and then passing the obtained solutions through a short Dowex 50 WX8 column (H^+ form, 20-50 mesh, approx. 5 cm^3). Elution with pure water was continued until pH of the eluates was neutral. The obtained solutions were then treated with 1M NaOH to adjust the pH to 12 and then neutralized by adding 1M HCl. Dialysis and subsequent freeze-drying

of the afforded polysaccharides **51-II** (22.0 mg, 69%) and **51-III** (24.2 mg, 76%), respectively, as white waxy solids.

Preparation of polysaccharide 3: Chondroitin in free acid form (56.3 mg, 0.148 mmol) – obtained from chondroitin sodium salt as indicated above for the preparation of **51-I** – was suspended in DMF (2.8 mL) that was freshly dried over activated 4Å molecular sieves. The mixture was heated to 80°C and stirred for 3 hours, after that a clear solution was obtained. After cooling to rt, it was treated with α,α-dimethoxytoluene (222 μL, 1.48 mmol), that was freshly dried over activated 4Å molecular sieves, and then with a 0.21M solution of CSA in freshly dried D F (178 μL, 37.4 μmol). After overnight stirring at 80°C, a yellowish solution was obtained. It was cooled to rt and treated firstly with Et_3N (0.4 mL) and then with diisopropyl ether (10 mL). The mixture was cooled to -30°C for 2 hours. A white flocculant solid was obtained. It was isolated by centrifugation and then desiccated under vacuum overnight to afford **3** (89.0 mg, quant.) as a yellowish amorphous solid.

Preparation of polysaccharide 52: A suspension of **3** (27.2 mg, 58.2 μmol) in freshly dried D F (2.2 mL) was heated at 80°C. After two hours stirring, a clear solution was obtained. It was cooled to rt and then treated with a 0.44M solution of TBTU in freshly dried DMF (323 μL, 0.142 mmol), then with a 0.22 solution of **50** in freshly dried D F (790 μL, 0.174 mmol), and finally with 2,4,6-collidine (93.8 μL, 0.709 mmol), that was freshly dried over activated 4Å molecular sieves. After overnight stirring at rt, the solution was treated with diisopropyl ether (7.0 mL). A white precipitate was obtained. It was

collected by centrifugation and then desiccated under vacuum overnight to give **52** (34.8 mg).

Preparation of polysaccharide 51-IV: Derivative **52** (29.9 mg) was suspended in ethyl acetate (560 µL) and treated with a 0.27 solution of $NaBrO_3$ in pure water (560 µL, 0.151 mmol), and a 0.24 solution of $Na_2S_2O_4$ in pure water (535 µL, 0.128 mmol). The mixture was vigorously stirred at rt overnight under visible light irradiation, after that it was centrifugated. The supernatant was diluted with water (25 mL) and ethyl acetate (25 mL). The aqueous phase was collected, concentrated, dialyzed and freeze-dried. The obtained white powder (11.0 mg) was dissolved in pure water (5.0 mL) and treated with 1M HCl to adjust pH to 2. The solution was stirred at 50°C for 1 hour, then 1M NaOH was added to adjust pH to 12. Further stirring was conducted at rt for 6 hours. The solution was then neutralized by adding 1M HCl, and dialyzed. Freeze-drying afforded a white solid that was further purified by filtration through a Sep-pak C-18 cartridge to give pure **51-IV** (7.5 mg, 51%) as a white waxy solid.

4.4.3 Molecular weight determination

All measurements were performed at (25.00 ± 0.05) °C with temperature controlled through the use of a thermostat bath. Stock solutions of pure chondroitin and of the derivatives **2b**, **2c**, **2d** were prepared at 4.9 10^{-3} g mL^{-1}, 1.12 10^{-3} g mL^{-1}, 3.4 10^{-3} g mL^{-1} , 1.24 10^{-3} g mL^{-1} respectively. Deionized water filtered (0.22µm) was used in all cases. Molecular weight for each compounds was determinate twice.

The mass-average molecular weight, M_w, from the below equation was obtained: (Schmitz, **1990**)

$$\frac{k_{ls}c}{R_\theta} = \left[\frac{1}{M_w} + 2Bc\right]\left[1 + \frac{R_g^2}{3}q^2\right] \quad (Eq.5)$$

where c is the sample mass concentration, B the second virial coefficient, $k_{ls} = 4\pi^2 n_o^2 (dn/dc)^2 / \left(N_A \lambda^4\right)$ where n_0 = 1.33 is the refractive index of water, dn/dc = 0.185 is the refractive index increment (Paduano et al., **1992**), N_A is the Avogadro's number, λ is the laser wavelength in vacuum, R_θ is the excess Rayleigh ratio at 90°. The values of R_θ were obtained from $R_\theta = \left(I_s - I_{s,0}\right) / I_{s,R}\left(n_0^2 / n_R^2\right) R_{\theta,R}$ where I_s is the scattered intensity of the solution, $I_{s,0}$ is the scattered intensity of water, $I_{s,R}$ is the scattering intensity of toluene (the standard) and, n_R = 1.496 and $R_{\theta,R}$ = 2.85 10^{-5} cm^{-1} are the refractive index and the Rayleigh ratio of toluene respectively (Kaye et al., **1973**), $q = (4\pi n / \lambda) sin(\theta / 2)$ and R_g is the radius of gyration.

4.4.4 Conformational study

A simplified model of the Thr-decorated chondroitin polysaccharide **51-IV** with six repetitions was constructed through the carbohydrate builder within the Glycam web server (Woods Group GLYCAM Web; Complex Carbohydrate Research Center, University of Georgia, Athens, GA, 2005–2014; http://www.glycam.com) while the Thr residue attached to the GlcA unit was constructed employing the builder module in the Maestro package of the Schroedinger Suite 2014. Restrained simulated annealing (SA) calculations were performed on

polysaccharide using the AMBER 14.0 package (Case et al., **2014**) with sugars described by the latest GLYCAM06 force field (GLYCAM_06j-1). (Kirschner et al., **2008**) Parameters for Thr residue were retrieved from the ff14sb force field within the AMBER 14.0 package as well as missing bond parameters. For annealing simulations, the General Born solvation (igb = 2) with monovalent salt concentration corresponding to 0.1 M was used. The complex was heated to 600 K in the first 5 ps, cooled to 100 K for the next 13 ps, and then cooled to 0 K for the last 2 ps. The temperature of the system was maintained with a varying time constant: 0.4 ps during heating, 4 ps during cooling to 100 K, 1 ps for the final cooling stage, and then reduced from 0.1–0.05 for the last picosecond. The force constants for NOE constraints were increased from 3 to 30 kcal mol^{-1} $Å^{-2}$ during the first 5 ps and then maintained constant for the rest of the simulation. These force constants were applied in the form of a parabolic, flat-well energy term. The upper distance bounds were retrieved by NOE cross-peak volume integrations performed with the iNMR (www.inmr.net), using the NOESY experiment collected at mixing time of 100 ms. The NOE volumes were then converted to distance restraints after they were calibrated using the known fixed distance (*H*-6a/*H*-6b of GalNAc). An unrestrained energy minimization step completed the SA run. This SA/energy minimization procedure was repeated 400 times. SA simulations were then analysed by clustering the resulting polysaccharide **51-IV** conformations through the average linkage method and a cluster member cutoff of 1.5 Å root-mean-squared difference (rmsd) calculated on the sugars rings atoms belonging to the central four dimers. This clustering allowed selecting 38 different conformational clusters for which the most populated one had a

frequency of occurrence of 110/400 conformations. Moreover, conformations of this latter cluster feature the lowest overall potential energy and NMR restraint violations. Thus, the representative structure (i.e., the closest to the centroid of the cluster) of this cluster was considered for subsequent molecular dynamics (MD) simulations. After charge neutralization by the addition of 6 Na^+ ions, the complex was solvated with 9381 water molecules in a truncated octahedral box of pre-equilibrated TIP3P water. (Jorgensen et al., **1983**) Several equilibration steps were performed comprising minimization of the solvent molecules with the polysaccharide fixed, minimization of the whole system, and slow heating to 300 K with weak positional restraints on M1 atoms under constant-volume conditions. The following 20 ns production runs were applied in the NPT ensemble. The particle mesh Ewald method (a) Essmann et al., **1995**. b) Darden et al., **1993**.) was used to evaluate the electrostatic interactions with a direct space sum cutoff of 10 Å. With the bond lengths involving hydrogen atoms kept fixed with the SHAKE algorithm, a time step of 2 fs was employed. (van Gunsteren et al., **1977**) Related conformational substates populated during the MD simulation were analyzed with the A B E S's PT A module. (Shao et al., **2007**) To investigate the thermodynamic parameters of the water molecules around the polysaccharide, the GIST program included in cpptraj module in AMBER 14 was used (Roe et al., **2013**). We set the GIST grid size to 0.75 $Å^3$, and set the GIST analysis region to cover the whole polysaccharide structure. The box sizes were 90 × 90 × 90 $Å^3$. Illustrations of the structures were generated using Chimera. (Pettersen et al., **2004**)

4.4.5 Antifreeze activity

Ice recrystallisation inhibition was measured using a modified splay assay (Knight et al., **1988**). A 10 μL sample of polysaccharide or polymer dissolved in PBS buffer (pH 7.4) was dropped 1.40 m onto a chilled glass coverslip sat on a piece of polished aluminium placed on dry ice. Upon hitting the chilled glass coverslip, a wafer with diameter of approximately 10 mm and thickness 10 μm was formed instantaneously. The glass coverslip was transferred onto the Linkam cryostage and held at −8 °C under N_2 for 30 minutes. Photographs were obtained using an Olympus CX 41 microscope with a UIS-2 20×/0.45/∞/0–2/FN22 lens and crossed polarizers (Olympus Ltd, Southend on sea, UK), equipped with a Canon DSLR 500D digital camera. Images were taken of the initial wafer (to ensure that a polycrystalline sample had been obtained) and after 30 minutes. Image processing was conducted using Image J, which is freely available. In brief, ten of the largest ice crystals in the field of view were measured and the single largest length in any axis recorded. This was repeated for at least three wafers and the average (mean) value was calculated to find the largest grain dimension along any axis. The average of this value from three individual wafers was calculated to give the mean largest grain size (MLGS). This average value was then compared to that of a PBS buffer negative control providing a way of quantifying the amount of IRI activity. This testing method ensures that positive results are only reported if all ice crystals are inhibited, as opposed to a average per wafer, which would smooth out the presence of rouge ice crystal growth.

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CHAPTER 5

STUDY OF REGIOSELECTIVE SULFATION OF POLYMANNURONIC ACID POLYSACCHARIDE

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5.1 Introduction

Alginate is a particular type of non-branched polysaccharide extracted from cell wall of brown seaweeds (*Phaeophyceae*), and an exopolysaccharide of bacteria including *Pseudomonas aeruginosa*. It is characterized by a linear structure consisting of 1,4-linked β-D-mannuronic acid (M) and its *C*-5 epimer α-L-guluronic acid (G) (Figure 38).

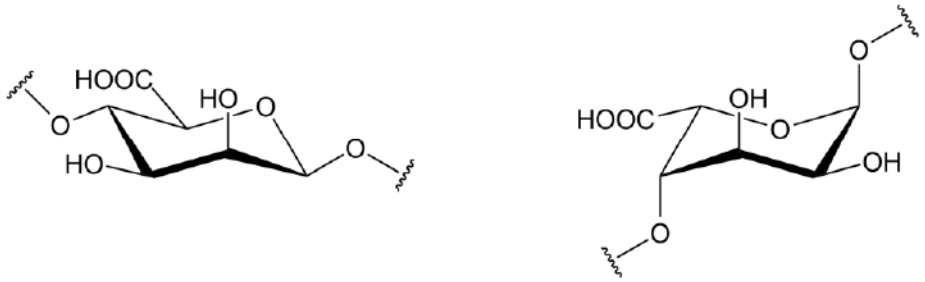


Figure 38: 1→4 ß-D-mannuronic acid (left) and 1→4 α-L-guluronic acid (right) monosaccharide units

These units are organized in a block-wise manner with their distribution, as well as, the M/G ratio that could be different, depending on the species of brown algae. (a) Cong et al., **2014**. b) Arlov et al., **2014**. c) Pawar et al., **2012**. d) Lee et al., **2012**. e) Draget et al., **1997**.) Sodium alginate was extensively employed in food, cosmetics (Chunmei et al., **2009**) and its property to strongly interact with

divalent cation (although this action seems to be carried out only by G-blocks) (George et al., **2006**) led to calcium cross-linked hydrogels, that have been used for pharmaceutical purposes (a) Lee et al., **2012**. b) Ghidoni et al., **2008**. c) Zimmermann et al., **2007**.) such as wound dressing materials for the treatment of acute or chronic wounds. (Boateng et al., **2008**) Moreover, the alginates display a crucial role in the progression of cystic fibrosis wherein bacterial biofilm formed from them are secreted by *P. aeruginosa.* (Ramsey et al., **2005**) Monosaccharide composition could strongly influence the geometry of the alginate chain (Smidsrød et al., **1990**) and any of their potential applications. The presence of free hydroxyls and carboxylic groups along the backbone makes alginates ideal candidates for chemical functionalization. (Yang et al., **2011**) This is usually used to enhance existing properties (improvement of ionic strength by additional covalent cross-linking, increase hydrophobicity of the backbone, improve biodegradation) or to introduce completely new properties otherwise non existing in the unmodified polysaccharide (afford anticoagulant properties, provide chemical/biochemical anchors to interact with cell surfaces or bestow temperature dependent characteristics such as lower critical solution temperature). (Pawar et al., **2012**) The free hydroxyl groups of alginate can be chemically sulfated in a random way (Fan et al., **2011**) affording polysaccharide with a similar behaviour to sulfated GAGs such as HS and HP (a) Arlov et al., **2014**. b) Freeman et al., **2008**.) Alginate sulfate, among the several biological functions of sulfated polysaccharides, may exhibit anticoagulant activity through the inhibition of intrinsic pathway of clotting cascade, that is directly related to the degree of sulfation (a) Fan et al., **2011**. b) Huang et al., **2003**.), anti-angiogenic and anti-

tumour activity. (Cong et al., **2014**) It is known that most biological functions in sulfated polysaccharides are related to their sulfation pattern and sequence. (a) Gama, et al., **2006**. b) Tadahisa et al., **2006**.) To the best of our knowledge, until now only methods for a random sulfation of alginates have been reported yet, but not for regioselective ones. This is a gap that should be urgently filled in. Indeed, a semi-synthetic sulfated alginate derivative PSS has been employed as anti-cardiovascular disease drug for nearly three decades in China, with no control of sulfate groups placement on the polysaccharide, in spite of the influence of DS on sulfated alginate activity (Arlov et al., **2014**) and of the reported cases of bleeding and anaphylaxis associated to PSS. (Xue et al., **2016**) Moreover, low molecular weight PSS derivatives (FPS) were recently evaluated for anticoagulant and antithrombotic activities displaying a good correlation between the molecular weight and the biological activities. (Xin et al., **2016**)

Due to incomplete solubility and highly heterogeneous structure of both G- and M-rich natural alginic acids the strategy to obtain a regioselectively sulfated polysaccharide was applied to the structurally simplest alginic acid, that is β-polymannuronic acid. This polysaccharide consists of a →4)-β-D-ManA-(1→ backbone (Figure 39), with a more homogeneous structure and a lower quantity of α-L-GulA units usually below 10-15% than all the other alginic acids.

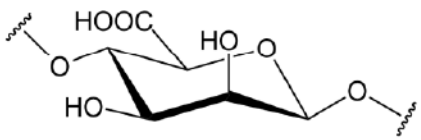


Figure 39: Polymannuronic acid repeating unit

This last property confers a higher solubility to this polysaccharide with respect to all the other alginic acids.

5.2 Results and discussion

Strategies for alginate modifications depend on three important parameters, such as solubility, reactivity and characterization.

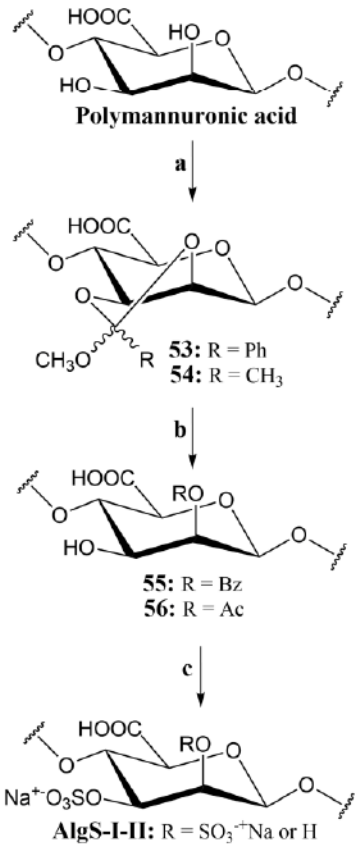
As regards solubility, alginates may be dissolved in aqueous, organic or mixed aqueous-organic media at a variable extent that could influence derivative substitution pattern. Furthermore, alginates can be chemically modified at secondary hydroxyl positions *O*-2 and *O*-3 (a) Arlov et al., **2014**. b) Cong et al., **2014**. c) Fan et al., **2011**.) or at the carboxylic acid group at *C*-6 position. (a) Pawar et al., **2012**. b) Yang et al., **2011**. c) Vallée et al., **2009**.)

Selective modification of either *C*-2 or *C*-3 hydroxyl group is challenging due to their minor reactivity differences and, moreover, it may be controlled in terms of selective modification of M or G residues. The comprehension of the substitution pattern needs several alginate samples with a different range of M/G ratios. Moreover, a lack of commercial availability of alginates with controlled G/M sequences may impede complete structural characterization of derivatives because of the complex nature of the polysaccharide backbone.

The starting material for the regioselective sulfation strategy was commercially available polymannuronic acid that presented 85% of M residues. It was protected at diol *O*-2,3 by either application of an orthoester or benzylidene ring. (Bedini et al., **2012**)

In the orthoester case, protection was repeated twice (showing no increase of degree of substitution), by stirring overnight at rt a DMF

suspension of polysaccharide in the presence of $PhC(OCH_3)_3$ or $CH_3C(OCH_3)_3$ and CSA as catalyst, followed by *one-pot* ring opening to liberate the equatorial position *O*-3, as one could hypothesize from the regiochemistry of orthoester opening on monosaccharide *cis*-diols (Mukhopadhyay et al., **2003**) and **55** and **56** were obtained in 68% and 57% yield, respectively. It was not possible to evaluate neither the DS nor the regioselectivity due to the presence of impurities that gave signals overlapped to the polysaccharide ones in the NMR spectra. Thus, both derivatives **55** and **56** were further treated with SO_3·pyridine in DMF at 50°C, to sulfate the free hydroxyl at position *O*-3 of polysaccharide backbone, followed by global alkaline deprotection in order to remove acyl protecting groups at position *O*-2 of polymannuronic acid (Scheme 17).



Scheme 17: (a) $PhC(OCH_3)_3$ or $CH_3C(OCH_3)_3$, DMF, CSA, rt; (b) H_2O, rt, 68% for **55**, 57%, for **56**; (c) 1: SO_3·py, DMF, 50°C, 2: NaOH, H_2O, rt, 98%, 75%, for **AlgS-I** and **AlgS-II**, respectively.

AlgS-I and **AlgS-II** were obtained in 98% and 75% yield from the intermediate partially protected polysaccharides, respectively and their regiochemistry of sulfation was preliminary evaluated by ^{13}C-NMR analysis, obtaining for them very similar spectra, that were compared with the ^{13}C-NMR spectrum of starting polymannuronic acid (Figure 40).

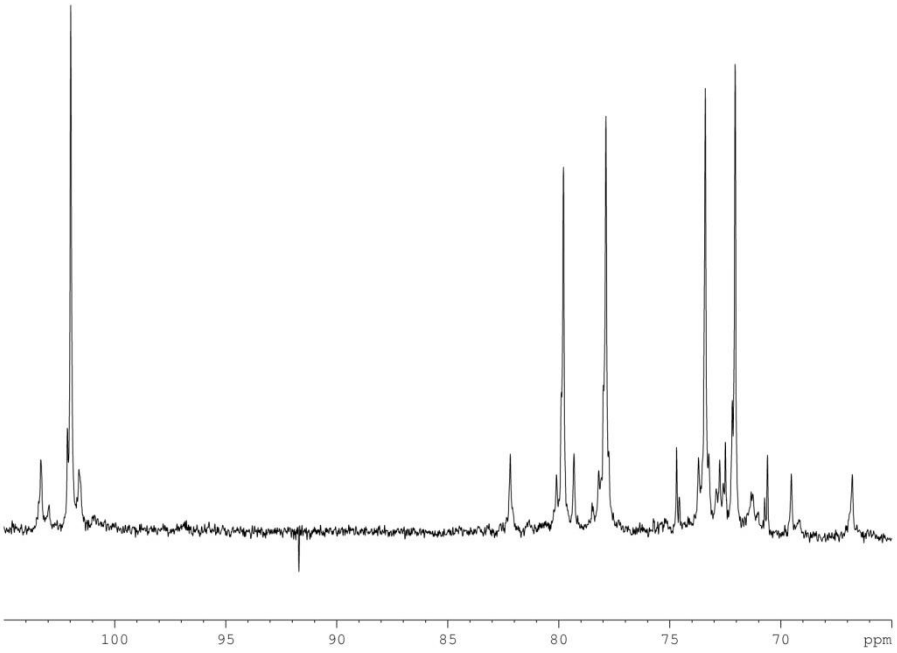


Figure 40: Zoom of ^{13}C-NMR spectrum of unprotected polymannuronic acid (400 MHz, D_2O, 298 K)

The latter displayed four signals at δ_C = 72.1, 73.4 , 77.9 and 79.8 ppm, that were ascribed to *C*-2, *C*-3, *C*-5 and *C*-4, respectively. Instead, the ^{13}C-NMR spectrum of **AlgS-I** (Figure 41), displayed two downshifted signals (δ_C = 76.6 and 78.9 ppm), that were hypothesized to belong to the downshift resonances of *C*-2 and *C*-3 of ManA units due to sulfation step in the semi-synthetic route. (Cong et al., **2014**)

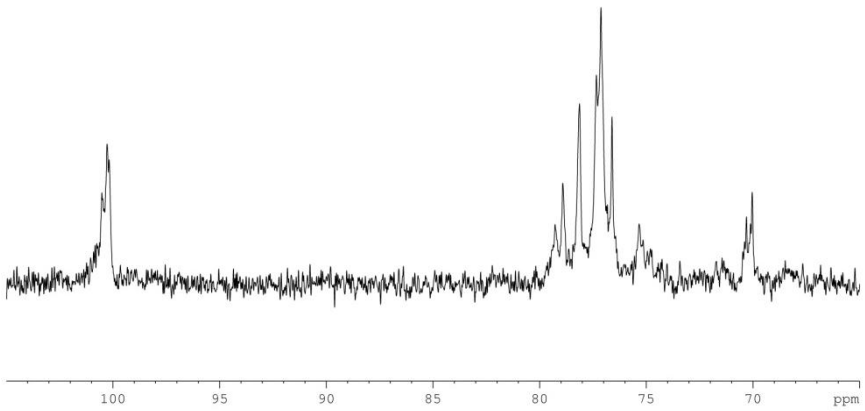


Figure 41: Zoom of ^{13}C-NMR spectrum of **AlgS-I** (400 MHz, D_2O, 298 K)

After sulfation, the carbon substituted by the sulfate ester groups shifted to a lower field, meanwhile the adjacent carbons generally shifted to slightly higher fields, (Grasdalen, **1983**) as proved by the shift of *C*-4 signal of polymannuronic acid polysaccharide from δ_C = 79.8 ppm to δ_C = 77.1 ppm, that might indicate the sulfation of the adjacent *C*-3. or eover, the signal at δ_C = 70.0 ppm could be ascribed to the shift of non sulfated *C*-2, adjacent to sulfated *C*-3. The just mentioned results suggested that the protection by orthoester ring was not quantitative because sulfate groups were found not exclusively at *O*-3 position as predictable. Therefore, it was necessary to change the semi-synthetic strategy by choosing a benzylidene ring as cyclic protecting group, in order to obtain a regioselectively sulfated polysaccharide.

For the purpose of obtaining a more soluble derivative in aprotic solvent polymannuronic acid was firstly derivatized at its carboxylic function. Carboxyl-modified alginate esters were obtained by treating a

tetrabutylammonium alginate salt DMF solution with suitably synthesized *n*-alkyl iodides in the presence of TBAF at 80°C. (Pawar et al., **2013**) Due to the presence of TBAF and *n*-alkyl iodides as contaminants, and the overlapping of their α-methylene signals with those related to TBA alginate salt ones, it was not possible to evaluate correctly the DS by ^{1}H-NMR. However, the semi-synthetic route was carried on because it was not strictly necessary to gain a quantitative esterification for obtaining an alginate derivative that was soluble in aprotic solvents.

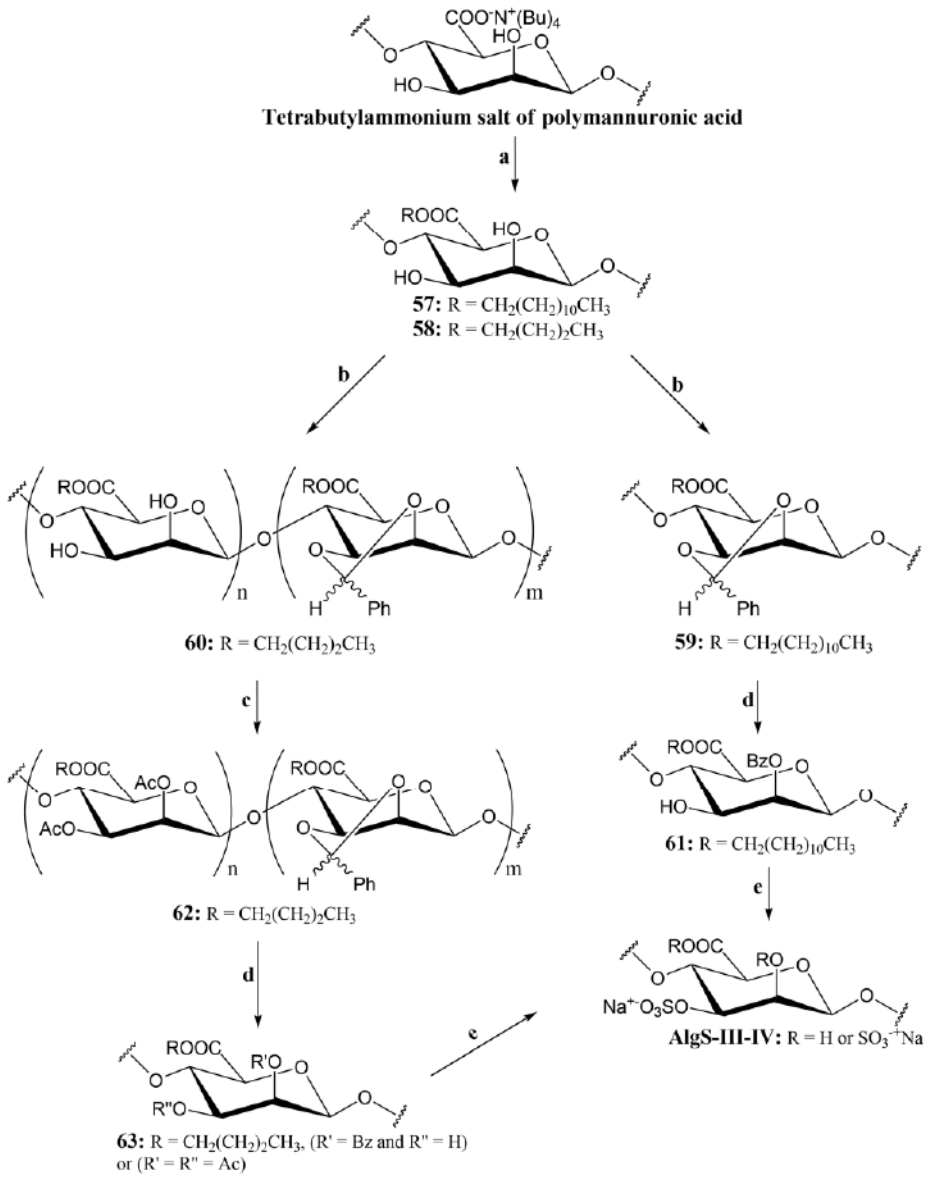
Derivatives **57** and **58** carrying a *n*-dodecyl or a *n*-butyl ester moiety, respectively were protected at *O*-2,3 diol with a benzylidene ring, by treating them with α,α-dimethoxytoluene in DMF at 80°C in the presence of CSA as catalyst.

^{1}H-NMR integration of signals related to anomeric protons and benzylidene methine displayed a DS = 0.99 for **59** and 0.71 and for **60**, respectively.

Benzylidene ring was, then, regioselectively cleaved under oxidative conditions (Adinolfi et al., **1999**) for derivative **59**, affording polysaccharide **61** in 58% yield with a DS = 1, and supposedly giving 2-*O*-benzoylated ManA units. The DS was evaluated by ^{1}H-NMR integration of the *H*-ortho Bz aromatic signals (δ_H = 7.86 and 7.95 ppm) with repect to the carbinolic and anomeric ones (δ_H = 5.33-3.67 ppm). On the contrary, for derivative **60** the free hydroxyls that did not couple during the benzylidene step were acetylated with Ac_2O and Et_3N in the presence of a catalytic amount of DMAP affording **62** in 68% with a DS = 0.65. This was evaluated by comparison between integration of polysaccharide carbinolic and anomeric signals (δ = 5.56-4.17 ppm) and Ac one (δ = 2.08 ppm) in the ^{1}H-NMR spectrum. This

latter compound was submitted to oxidative opening of benzylidene ring with $NaBrO_3$ and $Na_2S_2O_4$ in a H_2O-ethyl acetate two phase mixture (Adinolfi et al., **1999**) affording **63**. Benzylidene cleavage was confirmed in this case by the splitting and downfield shift of aromatic signals (δ_H = 7.32 ppm for the benzylidene and δ_H = 7.50, 7.65, 7.94 ppm for the resulting benzoate).

Sulfation of free hydroxyls at 3-*O*-position of ManA units with SO_3·py complex in DMF at 50°C, followed by an alkaline hydrolysis (that was repeated three times only for *n*-dodecyl ester derivative) of Ac, Bz and ester groups afforded **AlgS-III** and **AlgS-IV** (Scheme 18).



Scheme 18: (a) $C_{12}H_{25}I$ or C_4H_9I, TBAF, DMF, 80°C; (b) α,α-dimethoxytoluene, CSA, DMF, 80°C, (45%, 44%, $DS_{benzylidenation}$ = 0.99, 0.71 for **59** and **60**, respectively); (c) Ac_2O, Et_3N, DMAP, CH_3CN, rt, overnight, 59%, DS = 0.65 for **62**; (d) $NaBrO_3$, $Na_2S_2O_4$, H_2O-ethyl acetate, rt, overnight, 58%, 71%, DS = 1 for **61** and **63** respectively), (e) 1: SO_3·py, DMF, 50°C, 2: NaOH, H_2O, rt., 27% for **AlgS-III**

The sulfation pattern was evaluated by ^{13}C-NMR analysis, obtaining for both of them very similar spectra (Figure 42).

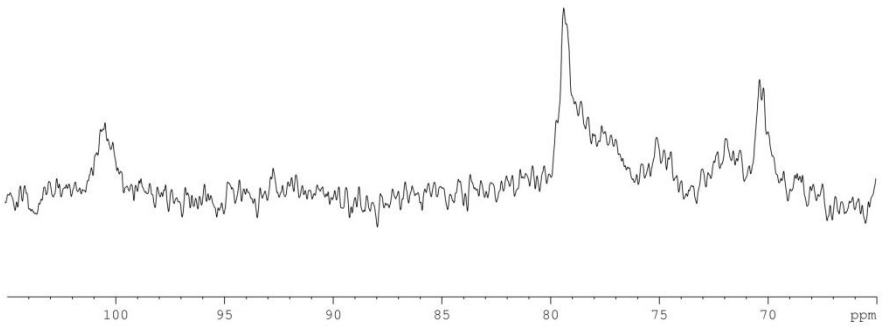


Figure 42: Zoom of ^{13}C-NMR spectrum of **AlgS-III** (400 MHz, D_2O, 298 K)

The ^{13}C-N spectrum showed peaks at δ_C = 75.0, 71.9 and 77.4 ppm that were hypothesized to belong to sulfated, non-sulfated *C*-3 and *C*-4 respectively (Arlov et al., **2014**). or eover, the signal at δ_C = 70.3 ppm could be ascribed to shifted *C*-2 for the presence of sulfated *C*-3, giving a preliminary hypothesis of a regioselective sulfation of polymannuronic acid. Although one can find discordant data found in literature about the sulfation of alginates (a) Arlov et al., **2014**. b) Cong et al., **2014**. c) Quanca et al., **2013**.), the obtained results encouraged us to pursue the employment of benzylidene group in the protection step of the semi-synthetic route towards regioselectively sulfated alginates that need to be optimized yet.

5.3 Conclusions

The study of regioselective sulfation of a commercially available polymannuronic acid polysaccharide, produced compounds with different sulfation patterns. The protection of *O*-2,3-diol on polysaccharide backbone, by an orthoester ring produced polymannuronic acid with sulfate groups either at position *O*-2 or at positions *O*-2 and *O*-3. Key reaction for a regioselective derivatization might be, then, a quantitative protection of *O*-2,3-diol on polysaccharide backbone by a suitable non-labile cyclic protecting group, that was preliminarily tested, in this study, by using a benzylidene ring protection. Further optimization on such protecting group will be applied in order to obtain a suitable regioselectively sulfated algnate to be fully charachterized and studied in details, also evaluating the relationship between biological function and structure.

5.4 Experimental section

5.4.1 General methods

Commercial grade reagents and solvents were used without further purification, except where differently indicated. The term "pure water" refers to water purified by a Millipore Milli-Q Gradient system. Centrifugations were performed with an Eppendorf Centrifuge 5804R instrument at 4°C (4600 g, 10 min). Dialyses were conducted on Spectra/Por 3.5 kDa cut-off membranes at 4°C. Freeze-dryings were performed with a 5Pascal Lio 5P 4K freeze dryer. NMR spectra were recorded on a Bruker DRX-400 (^{1}H: 400 MHz, ^{13}C: 100 MHz) instrument or Bruker DRX-600 (^{1}H: 600 MHz, ^{13}C: 150 MHz)

instrument equipped with a cryo probe, in D_2O (acetone as internal standard, ^1H: $(CH_3)_2CO$ at δ = 2.22 ppm; ^{13}C: $(CH_3)_2CO$ at δ = 31.5 ppm) or DMSO-d_6 (^1H: CHD_2SOCD_3 at δ = 2.49 ppm; ^{13}C: CD_3SOCD_3 at δ = 39.5 ppm).

5.4.2 Synthetic procedure

Orthoesterification and one-pot orthoester opening: Commercially available polymannuronic acid (85.0 mg, 0.44 mmol) was suspended in DMF (4.12 mL) that was freshly dried over activated 4Å molecular sieves. The mixture was treated with $PhC(OCH_3)_3$ (725 μL, 4.22 mmol), CSA (140 mg, 0.60 mmol) and stirred overnight at rt. The yellowish solution was treated with H_2O (15 mL) and a white precipitate was obtained. The mixture was stirred for 2 h at rt, dialyzed then freeze-dried, obtaining **55** as white solid (88.4 mg, 68%). Alternatively this reaction was conducted on commercially available polymannuronic acid using $CH_3C(OCH_3)_3$ (1.59mL, 12.6 mmol) as protecting group, obtaining **56** as white solid (84.7 mg, 57%).

Sulfation and global deprotection: Derivative **55 (**40.0 mg, 0.1337 mmol) was suspended in DMF (5.80 mL) that was freshly dried over activated 4 Å molecular sieves and treated with 1.10 M solution of SO_3.py in freshly dried DMF (2.44 mL, 2.674 mmol). After overnight stirring at 50°C the mixture was cooled to rt and treated with NaCl saturated acetone (8.0 mL), then to -30°C for 2 hours. A white flocculant solid was obtained. It was isolated by centrifugation, dissolved in H_2O (5 mL) and 1.0 M NaOH was added to adjust pH to 12. Further stirring was conducted at rt for 6 hours. The solution was

then neutralized by adding 1.0M HCl, and dialyzed. Freeze-drying afforded **AlgS-I** (32.6 mg, 98%) as a white solid. The reaction was repeated in the same conditions on compound **56** to afford **AlgS-II**.

***n*-Dodecyl iodide:** Sodium iodide (2.38 g, 15.88 mmol) was dissolved in acetone (26 mL), treated with $C_{12}H_{25}Br$ (1.91 mL, 7.95 mmol) and stirred at 60°C for 3 h at reflux. The mixture was cooled to rt, then diluted in *n*-hexane (100 mL) and washed with H_2O. The organic phase was collected, dried over anhydrous Na_2SO_4, filtered and concentrated affording a yellowish oil (2.28 g 48%).

***n*-Butyl iodide:** 1-Butanol (3.70 mL, 40.4 mmol) was treated with HI (9.0 mL, 119.7 mmol) and stirred at reflux for 4 h at 120°C. After cooling the mixture to 0°C, it was washed with cold H_2O. The organic phase was collected, dried over anhydrous Na_2SO_4, filtered, affording then an oil (3.14 g, 42%) that was directly stored at -28°C.

Tetrabutylammonium salt of polymannuronic acid: Commercially available polymannuronic acid (176.5 mg, 0.919 mmol) was suspended in H_2O (1.71 mL) and tetrabutylammonium hydroxide (40% w/w in MeOH) was added to adjust pH to 10. The mixture was freeze-dried, obtaining polysaccharide as a brown solid (217.6 mg, 55%)

***n*-Dodecyl ester of polymannuronic TBA salt:** Tetrabutylammonium salt of polymannuronic acid (193.4 mg, 0.446 mmol) was suspended in DMF (12.80 mL) that was freshly dried over activated 4 Å molecular sieves, obtaining a brownish suspension that was treated with 1.0 M TBAF solution in THF (1.03 mL, 1.03 mmol), in order to obtain a

better solubility. In the end *n*-dodecyl iodide (1.82 mL, 7.38 mmol) was added and after overnight stirring at 80°C, the mixture, that appared as a suspension, was cooled to rt and treated with diisopropyl ether (28 mL). A brown flocculant solid was obtained. It was isolated by centrifugation and then desiccated under vacuum overnight to afford **57** (222.4 mg) with TBAF and DMF as contaminant. The reaction was repeated twice.

***n*-Butyl ester of polymannuronic TBA salt:** Tetrabutylammonium salt of polymannuronic acid (57.7 mg, 0.133 mmol) was suspended in freshly dried DMF (3.80 mL) over activated 4Å molecular sieves, obtaining a brownish suspension that was treated with 1.0 M TBAF solution in THF (634.4 μL, 0.6344 mmol) in order to obtain a higher solubility. In the end, *n*-butyl iodide (249.0 μL, 2.20 mmol) was added and after overnight stirring at 80°C, the mixture was cooled to rt and treated with a diisopropyl ether/AcOEt (1:1 v/v) solution (32 mL). A white precipitate was obtained, collected by centrifugation and desiccated under vacuum overnight, affording derivative **58** with TBAF and DMF in large amount as contaminant. The compound was dissolved in DMF and treated with AcOEt (20 mL) obtaining a precipitate that was collected by centrifugation and desiccated under vacuum overnight, affording purified derivative **58** (33.0 mg, 100%)

Benzylidenation: A suspension of **57** (128.2 mg, 0.336 mmol) (or **58**) in freshly dried DMF over activated 4Å molecular sieves (8.50 mL) was heated at 80°C for 3 hours, obtaining a brownish suspension. The mixture was cooled to rt and treated with α,α-dimethoxytoluene (504.3 μL, 3.36 mmol), that was freshly dried over activated 4Å molecular

sieves, and then with a 0.21 M solution of CSA in freshly dried DMF (404.2 μL, 84.0 μmol). After overnight stirring to 80°C, the obtained brownish solution was cooled to rt and treated with H_2O (28.8 mL), (either AcOEt or a 1:1 v/v solution of *n*-hexane/AcOEt in the case of *n*-dodecyl ester and *n*-butyl ester derivatives, respectively) then the formed precipitate was collected by centrifugation and desiccated under vacuum overnight to afford **59** (or **60)** (71.8 mg, 45%)

Acetylation: Derivative **60** (18.8 mg, 75.7 μmol) was treated with Et_3N (209.0 μL) and suspended in CH_3CN (116.0 μL). Ac_2O (497.0 μL) and DMAP (3.36 mg) were added and after overnight stirring at rt, the mixture was treated with a 1:1 v/v solution of Hexane/EtOAc. The obtained brownish precipitate was collected by centrifugation and dessicated under vacuum overnight to afford compound **62** (14.8 mg, 59%).

Oxidative opening: Derivative **59** (62.7 mg, 0.140 mmol) was suspended in ethyl acetate (1.54 mL) and treated with a 0.27 M solution of $NaBrO_3$ in pure water (1.54 mL, 0.411 mmol), and a 0.24 M solution of $Na_2S_2O_4$ in pure water (1.46 mL, 0.345 mmol). The mixture was vigorously stirred at rt overnight under visible light irradiation, after that it was centrifugated and the precipitate was desiccated under vacuum affording **61** (12.1 mg). The supernatant was diluted with water (25 mL) and washed with ethyl acetate (25 mL). The organic layer was collected, dried with anhydrous Na_2SO_4, filtered and concentrated and added to the precipitate, affording a total amount of derivative of 37.4 mg (58%). The reaction was conducted in the same way on compound **62** to afford **63**

Sulfation and global alkaline deprotection: Derivative **61** (30.6 mg, 0.066 mmol) (or **63**) was suspended in DMF (524.6 μL), that was freshly dried over 4Å molecular sieves and then treated with a 1.17 M solution of SO_3·py in freshly dried DMF (1.13 mL). After overnight stirring at 50°C the suspension turned into a clear solution and it was cooled to rt, diluted with pure water (7.0 mL) and treated with a 1.0 M $NaHCO_3$ solution to adjust pH to 7. The obtained yellowish precipitate was collected by centrifugation and then dissolved in pure water (20.0 mL). The solution was treated with a 15% w/v NaOH solution to adjust pH to 13. The solution was stirred for 6 h at rt and then 1.0 M HCl was added until neutralization. The reaction was repeated twice and dialysis and subsequent freeze-drying yielded **AlgS-III** (or **AlgS-IV**) as a white solid (7.4 mg, 27%).

5.5 Bibliography

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APPENDIX

PhD COURSE ACTIVITY SUMMARY

PhD Course Activity Summary

Candidate: **Antonio Laezza**

Supervisor: **Dr. Emiliano Bedini**

Assessor: **Dr. Flavio Cermola**

1) **Attended Courses:**

- Zorn, R. "Neutron Scattering", 27-30/10/2015; 3 CFU
- Duilio, A. "Produzione ricombinante di proteine naturali e mutanti", 20-24/07/2015; 3 CFU
- ont esarchio, D. "Sintesi, struttura ed applicazioni di oligonucleotidi naturali e modificati", from 04-13/02/2015; 3 CFU
- Naviglio, D. "Tecniche estrattive solido-liquido impiegate nella preparazione del campione per l'analisi chimica e nella produzione di estratti per usi industriali", 09-17/12/2014; 3 CFU
- Parrilli, . "Glicoscienza", 17-24/07/2014; 3 CFU
- Pucci, P. "Corso Avanzato di Spettrometria di Massa", from 30/06/2014 to 4/07/2014; 4 CFU

2) **Attended Seminars:**

Title	Speaker	Date	Place
Chemical biology and medicinal chemistry of glycosphingolipid	**Prof. Herman Overkleeft**	**19/12/16**	**University of Naples Federico II**
Basics of detergents formulations and challenge	**Dr. Giulia Bianchetti**	**16/03/2016**	**University of Naples Federico II**

Innovative approaches for polysaccharide-based vaccines	**Prof. Francesco Berti**	**20/11/2015**	**University of Naples Federico II**
The bacterial cell wall by liquid state and DNP solid state NMR	**Prof. Jean-Pierre Simorre**	**29/06/2015**	**University of Naples Federico II**
Sugar engineering: Building new therapeutics with glycosyltransferase enzymes	**Prof. Paul De Angelis**	**03/06/2015**	**CNR**
Pharmaceutical companies: External manufacturing and quality assurance	**Prof. Domenico Demasi**	**25/05/2015**	**University of Naples Federico II**
Lectins from bacteria and fungi: therapeutical targets and research tools	**Prof. Anne Imberty**	**15/05/2015**	**University of Naples Federico II**
Matrix Metalloproteinases: Inter-domain flexibility and recognition of substrates	**Prof. Marco Fragai**	**08/05/2015**	**University of Naples Federico II**
European Large Facilities: Neutron and Synchrotron sources	**Prof. Serge Pérez**	**06/05/2015**	**University of Naples Federico II**

New chiral catalysts derived from Fe(II) and Bi(III) for asymmetric synthesis	**Prof. Thierry Ollevier**	**15/12/2014**	**University of Naples Federico II**
Le fitotossine: un'avventura luga 40 anni	**Prof. Antonio Evidente**	**10/12/2014**	**University of Naples Federico II**
Rediscovering the levan polysaccharide	**Prof. Ebru Toksoy Öner**	**28/10/2014**	**University of Naples Federico II**
Incontro con la Peroni. Azienda leader birraia	**Dr. Luigi Serino**	**12/05/2014**	**University of Naples Federico II**

3) Attended Integration Exams (for candidates not graduated in Chemical Science):

Title	Professor	Date
/	/	/
/	/	/

4) Visiting periods in Institutions different from University of Naples "Federico II":

Host Institution	Country	Start Date	End Date
University of Edinburgh	**United Kingdom**	**01/05/2016**	**31/07/2016**

5) Publications (include submitted and in preparation):

- **Laezza, A.**; Brodaczewska, N.; Uhrin, D.; Iadonisi, A.; Bedini, E. *"Depolymerization of semi-synthetic fucosylated chondroitin sulfate and interaction with selectins"*, in preparation

- **Laezza, A.**; Casillo, A.; Cosconati, A.; Biggs, C.I.; Fabozzi, A.; Paduano, L.; Iadonisi, A.; Novellino, E.; Gibson, M.I.; Randazzo, A.; Corsaro, M.M.; Bedini E. *"Decoration of chondroitin polysaccharide with threonine: synthesis, conformational study and antifreeze activity*", **2017**, submitted

- Bedini, E.; **Laezza, A.**; Parrilli, M.; Iadonisi, A. "*Chemical methods for the selective sulfation and desulfation of polysaccharides*", **2017**, submitted

- **Laezza, A.**; Iadonisi, A.; Pirozzi, A.V.A.; Diana, P.; De Rosa, M.; Schiraldi, C.; Parrilli, .; Bedini, E. "*A modular approach to a library of semi-synthetic fucosylated chondroitin sulfate polysaccharides with different sulfation and fucosylation pattern*",*Chem. Eur. J.* **2016**, *50*, 18215-18226

- Bedini, E.; **Laezza, A.**; Iadonisi, A. "*Chemical derivatization of sulfated glycosaminoglycans*", *Eur. J. Org. Chem.* **2016**, 18, 3018-3042

- **Laezza, A.**; Iadonisi, A.; De Castro, C.; De Rosa, M.; Schiraldi, C.; Parrilli, M.; Bedini, E. *"Chemical fucosylation of a polysaccharide: a semi-synthetic access to fucosylated chondroitin sulfate*", *Biomacromolecules* **2015**, *16*, 2237-2245

- **Laezza, A.**; De Castro, C.; Parrilli, .; Bedini, E. "*Inter vs. intraglycosidic acetal linkages control sulfation pattern in semi-synthetic chondroitin sulfate*", *Carbohydr. Polym.* **2014**, *112*, 546-55

6) **Attended congresses/workshops/summer schools/contribution:**

- BMMC VII - 7th Baltic Meeting on Microbial Carbohydrates; 25-29/09/2016, Guestrow (Germany). **Oral communication:** A semi-synthetic strategy to fucosylated chondroitin sulfate from microbial sourced chondroitin (Laezza, A.; Iadonisi, A.; De Castro, C.; De Rosa, M.; Schiraldi, C.; Parrilli, M.; Bedini, E.)

- Challenge in Organic Synthesis: Efficient Processes for Novel Applications; 11-12/12/2015, Naples (Italy)

- 4th EPNOE International Polysaccharide Conference; 19-22/10/2015, Warsaw (Poland). **Oral communication:** A semi-synthetic strategy to fucosylated chondroitin polysaccharides from microbial sourced chondroitin (Laezza, A.; Iadonisi, A.; De Castro, C.; De Rosa, M.; Schiraldi, C.; Parrilli, M.; Bedini, E.)

- Physics and Chemistry in Polysaccharide Science: From Molecules to Materials; 18/10/2015, Warsaw (Poland)

- COST Action BM1003 Final Meeting: Microbial cell surface determines of virulence, antibiotic resistance and inflammation in different pathologies; 13-14/10/2014, SS. Marcellino e Festo, University of Naples Federico II

- XIV Convegno-Scuola sulla Chimica dei Carboidrati; 22-25/06/2014, Pontignano (Italy). **Poster communication:** A semi-synthetic approach to fucosylated chondroitin sulfate from microbial derived chondroitin (Laezza, A.; Marzaioli, A.M.; De Castro, C.; Parrilli, M.; Bedini, E.)

- COST Traning School BM1003: Microbial cell surface determinants of virulence, as targets for new therapeutics in Cystic Fibrosis; 03-05/06/2014; University of Naples Federico II, Complesso di Monte Sant'Angelo.