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A Modular Approach to a Library of Semi-Synthetic Fucosylated Chondroitin Sulfate Polysaccharides with Different Sulfation and Fucosylation Patterns

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Abstract: Fucosylated chondroitin sulfate (fCS)—a glycosaminoglycan (GAG) found in sea cucumbers—has recently attracted much attention owing to its biological properties. In particular, a low molecular mass fCS polysaccharide has very recently been suggested as a strong candidate for the development of an antithrombotic drug that would be safer and more effective than heparin. To avoid the use of animal sourced drugs, here we present the chemical transformation of a microbial sourced unsulfated chondroitin polysaccharide into a small library of fucosylated (and sulfated) derivatives thereof. To this aim, a modular approach based on the different combination of only five reactions was employed, with an almost unprecedented polysaccharide branching by O-glycosylation as the key step. The library was differentiated for sulfation patterns and/or positions of the fucose branches, as confirmed by detailed 2D NMR spectroscopic analysis. These semi-synthetic polysaccharides will allow a wider and more accurate structure–activity relationship study with respect to those reported in literature to date.

Introduction

Fucosylated chondroitin sulfate (fCS) is a glycosaminoglycan (GAG) found up to now exclusively in sea cucumbers (*Echinoidea*, *Holothuroidea*). Its polysaccharide structure is usually constituted of a repeating unit with *N*-acetyl-D-galactosamine (GalNAc) and fucosylated D-glucuronic acid (GlcA) residues linked together through alternating β-1→3 and β-1→4 glycosidic bonds. The fucosylation decoration very often consists of a single L-fucose (Fuc) branch per repeating unit, linked at position *O*-3 of the GlcA units through an α-configured glycosidic bond,[1] but some slightly different structural features have been very recently suggested for fCS extracted from *Apostichopus japonicus*, *Actinopyga mauritania*, and *Ludwigothurea grisea*. In the former two cases, a random fucosylation at either the GlcA *O*-3 or GalNAc *O*-4/*O*-6 position has been proposed,[2] whereas in the latter fCS an α-Fuc-(1→3)-Fuc disaccharide branch was found.[3] Both the GlcA-GalNAc linear backbone and Fuc branches are sulfated to a various extent. Different varieties of sea cucumbers from the seawaters of different geographical zones produce a fCS polysaccharide with a different sulfation pattern at the Fuc and/or GalNAc sites.[4] The sulfation profiles found up to now are depicted in Figure 1. It is worth noting that in all fCSs studied up to now, the presence of two or even more different sulfation patterns on the Fuc branches of the same polysaccharide have been detected.

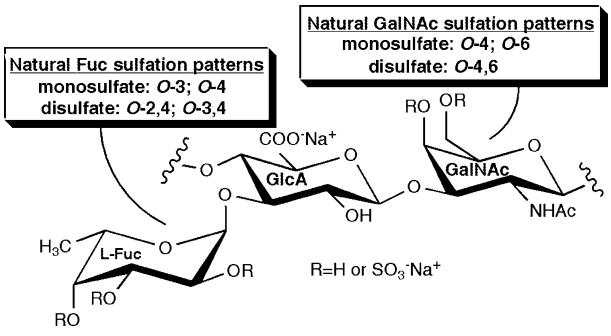

Figure 1. Sulfation patterns found in natural fCSs.

fCS shows interesting activity in biological events related to inflammation, angiogenesis, cellular growth, cancer metastasis, fibrosis, virus infection, hyperglycemia, atherosclerosis, and, above all, coagulation and thrombosis.[1] Interestingly, its anticoagulant and antithrombotic activity seems to be driven by both a serpin-dependent mechanism—mediated by antithrombin (AT) and heparin cofactor II (HC-II)[5]—and a serpin-inde-

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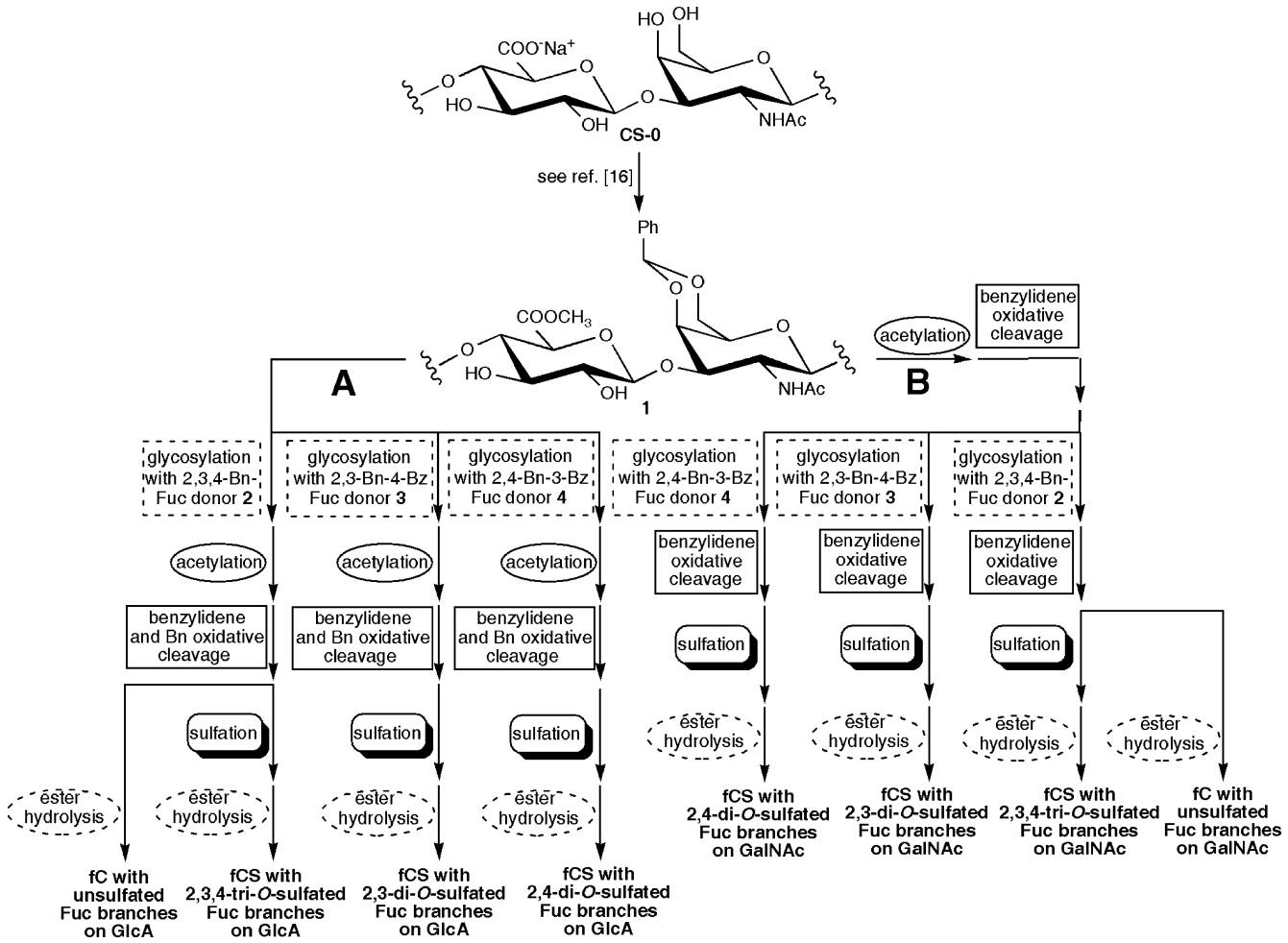
pendent one, which makes fCS active also on AT- and HC-II-free plasmas.[6] Furthermore, fCS seems to retain its activity also when it is orally administered.[7]

The most widespread and long-term used anticoagulant drug is unfractionated heparin, which unfortunately display several limitations such as risks of bleeding, platelet aggregation, and contamination with toxic agents, as well as a short half-life and a reduced activity on congenital or acquired antithrombin deficient patients.[8] The introduction of low- and ultra-low-molecular-mass heparins has alleviated these problems to some extent, nonetheless the development of even more effective and safer antithrombotic drugs is still a hot topic.[9] To this aim, the anticoagulant and antithrombotic properties displayed by fCS have attracted much attention, even if some drawbacks must be taken into account: the severe regulations for animal-derived drugs and the high variability of the sulfation patterns in polysaccharides extracted from animal tissues, which require strict control of the fCS sulfation level. Indeed, a too highly sulfated GAG such as per-*O*-sulfated chondroitin can induce a strong allergic-type response,[10] as demonstrated by hundreds of serious adverse events, including 149 deaths,[11] which occurred after the contamination of some heparin lots with per-*O*-sulfated chondroitin.[12] Furthermore, together with their anticoagulant activity, natural fCS polysaccharides exhibit some adverse effects, such as platelet aggregation, factor XII activation, and bleeding.[13] As low-molecular-mass fCS polysaccharides do not show these adverse effects, many efforts have recently been devoted to the development of mild and selective depolymerization methods aimed at shortening the fCS polymer chain without cleaving the sulfate groups as well as the labile Fuc branches.[2b,14] To avoid the use of animal sourced molecules, a total synthetic approach of some fCS di- and trisaccharide fragments has also been accomplished.[15] To the best of our knowledge, their anticoagulant activity has not yet been reported, nonetheless the obtainment of higher fCS oligosaccharides by selective depolymerization of the natural polysaccharide has very recently pointed to the fact that nonasaccharide is the minimum structural unit responsible for anticoagulant activity.[14b] An alternative approach to non-animal sourced fCS species has very recently been proposed by us, by accomplishing the chemical fucosylation of a microbial sourced chondroitin polysaccharide. Subsequent sulfation of the fucosylated polysaccharide afforded for the first time a semi-synthetic fCS, displaying per-*O*-sulfated Fuc branches randomly linked at GlcA and GalNAc units and with additional sulfate groups at either the *O*-4 or *O*-6 positions of GalNAc.[16] A preliminary anticoagulant assay revealed an activity profile similar to low-molecular-mass fCS polysaccharides obtained from natural sources, thus encouraging us to further work on this topic. With the aim of performing a wide structure–activity relationship (SAR) investigation of fCS polysaccharides that would be not restricted to the natural structures found up to now, we have now prepared a small library of six semi-synthetic fCSs with different linkage positions and/or sulfation patterns of the Fuc branches, and two unsulfated fucosylated chondroitins (fCs).

Results and Discussion

The production of unsulfated chondroitin (CS-0) from several microbial sources has recently been accomplished.[17] As with our recent works on the semi-synthesis of non-animal sourced chondroitin sulfate polysaccharides,[18] the CS-0 starting material for the preparation of the fCS and fC library was obtained by fed-batch fermentation of *Escherichia coli* K4, followed by microfiltration, protease treatment, diafiltration, and mild hydrolysis to eliminate both lipopolysaccharide (LPS) contaminants and unwanted fructosyl residues attached at position *C*-3 of some GlcA units.[19] After further purification steps using ethanol and repeated alcoholic precipitations, the unsulfated, defructosylated polysaccharide was obtained in 90% purity grade, as evaluated by NMR analysis and capillary electrophoresis.[20] The residual endotoxin content was evaluated by using the limulus amebocyte lysate (LAL) test and found to be lower than 0.1 EU mg^{-1}.[21]

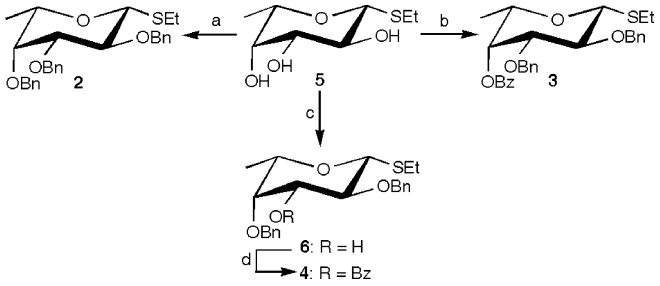
The transformation of CS-0 into eight different fC and fCS polysaccharides was planned through a modular approach (Scheme 1). After a preliminary esterification of GlcA carboxylic acid and protection of GalNAc 4,6-diol as benzylidene to convert CS-0 into polysaccharide **1**,[16] five reactions were applied in two different sequences to access fCSs and fCs with Fuc branches placed exclusively on either the GlcA or GalNAc units. Indeed, in synthetic sequence A, glycosylation of polysaccharide acceptor **1**, followed by i) capping of the unreacted hydroxyls by acetylation, ii) oxidative cleavage of benzyl (Bn) and benzylidene protecting groups,[22] iii) sulfation of the released hydroxyls, and iv) global deprotection by ester hydrolysis, can furnish polysaccharides with Fuc branches exclusively on the GlcA units. Alternatively (synthetic sequence B), by performing the acetylation and benzylidene ring oxidative cleavage before the glycosylation reaction and then the Bn cleavage, sulfation, and ester hydrolysis steps, fCSs and fCs with Fuc branches exclusively on the GalNAc units could be obtained. A second level of differentiation of the target polysaccharides concerned the sulfation pattern of the Fuc branches. This could be achieved by using a set of Fuc donors with a different patterns of protecting groups. Indeed, Bn groups were oxidatively cleaved before the sulfation step, whereas the benzoyl (Bz) ones were deprotected only at the end of the semi-syntheses. Thus, a per-*O*-benzylated Fuc donor could be used to obtain fCS polysaccharides with per-*O*-sulfated Fuc branches, whereas 2,3-di-*O*-Bn-4-*O*-Bz-Fuc and 2,4-di-*O*-Bn-3-*O*-Bz-Fuc donors could allow the access to fCSs with 2,3-di-*O*-sulfated or 2,4-di-*O*-sulfated Fuc branches, respectively. It is worth noting that the 2,4-di-*O*-sulfation pattern of Fuc branches was chosen because it is one of the most commonly found in natural fCSs[4] and it has also been identified to give the strongest anticoagulant activity.[4b,23] However, some results published during the development of this work pointed to the independence of the anticoagulant and antithrombotic activities from the different sulfation patterns of Fuc branches in natural fCSs.[3] The other two chosen Fuc sulfation patterns were selected because, to the best of our knowledge, they have been never found in natural fCSs, and therefore will us allow to enlarge



Scheme 1. Modular approach for the transformation of unsulfated chondroitin into a library of fCSs (see Scheme 2 for the structures of Fuc donors **2**, **3**, and **4**).

the SAR investigations and also serve as additional standards for structural characterization of fCSs from novel natural sources. A third level of differentiation could be also achieved among the target polysaccharides by avoiding the sulfation step in the semi-synthetic sequences, thus opening access to non-natural fCs.

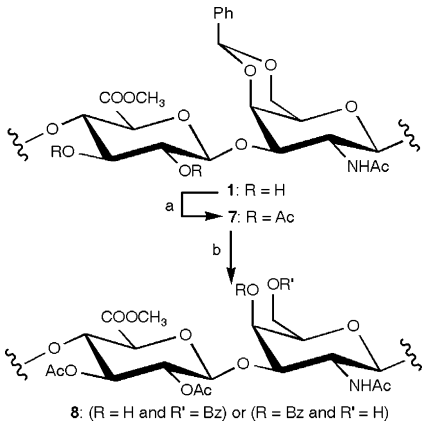
The key step of the semi-synthetic strategies depicted in Scheme 1 was the glycosylation reaction that connected the Fuc branches with the linear chondroitin backbone. Before performing it, Fuc donors and polysaccharide acceptors were prepared. For the donors, per-*O*-benzylated Fuc derivatives with both a *N*-phenyl-trifluoroacetimidate[24] and an ethyl thioglycoside[25] leaving group were shown to be efficient in the random fucosylation of a polysaccharide,[16] nonetheless thioglycosides were preferred owing to their longer shelf life and shorter sequence of steps for their preparation. Therefore, thioglycosides **2**, **3**, and **4** were selected as Fuc donors (Scheme 2). The former two were synthesized from ethyl 1-thio-β-L-fucopyranoside **5**[25] according to known procedures,[25,26] whereas **4** was unprecedented. It was thus obtained by regioselective protection of triol **5** in three steps (3-*O*-silylation, benzylation,



Scheme 2. Synthesis of Fuc donors **2**, **3**, and **4**. (a) See ref. [25]; (b) see ref. [26]; (c) i) TBDMSCl, imidazole, DMF, rt, 3 h, 61%; ii) BnBr, NaH, DMF, rt, 1 h, 93%; iii) TBAF, THF, rt, 2 h, 94%; (d) BzCl, 1:1 v/v pyridine/CH_2Cl_2, rt, 3 h, 80%.

and de-*O*-silylation)[27] to give **6** (53% overall yield), which was then benzoylated to afford **4** in 80% yield.

The polysaccharide acceptors for Fuc branching on GlcA or GalNAc were **1** and **8**, respectively. The former was already known[16] and served also for the synthesis of the latter in two steps (Scheme 3). First, the 2,3-diol on the GlcA units was pro-



Scheme 3. Synthesis of **8**. (a) Ac_2O, Et_3N, DMAP, CH_3CN, rt, overnight (DS = 2.00); (b) $NaBrO_3$, $Na_2S_2O_4$, H_2O/ethyl acetate, rt, overnight, 63% over two steps (DS = 0.97).

tected by acetylation with Ac_2O and Et_3N in CH_3CN in the presence of a catalytic amount of 4-dimethylaminopyridine (DMAP) to afford derivative **7** with a quantitative degree of substitution (DS = 2.00), as calculated by integration of the *H*-2 and *H*-3 carbinolic signals ($\delta = 4.65$ and 5.04 ppm, respectively) with respect to benzylidene methine one ($\delta = 5.44$ ppm) in the ^{1}H NMR spectrum (see also the Supporting Information). The benzylidene ring of derivative **7** was then cleaved under oxidative conditions ($NaBrO_3$ and $Na_2S_2O_3$ in a H_2O/ethyl acetate mixture)[21] to afford polysaccharide acceptor **8** in 63% yield (over two steps from **1**)[28] with either 4-*O*- or 6-*O*-benzoylated GalNAc units randomly distributed along the polymer chain. The almost quantitative DS (0.97) of the benzylidene cleavage reaction was evaluated by ^{1}H NMR integration of the *ortho*-H Bz aromatic signals ($\delta = 7.86$ and 7.95 ppm) with respect to the Ac ones ($\delta = 1.72$–1.98 ppm). The 4-*O*-benzoylated-/6-*O*-benzoylated-GalNAc ratio was estimated to be 1.3 by HSQC DEPT 2D NMR integration of the 4-*O*-Bz-GalNAc and 6-*O*-BzGalNAc 6-*O*-methylene signals ($\delta_{H/C} = 3.29/59.3$ and 4.21–4.34/62.9 ppm, respectively; see also the Supporting Information), assuming that the signals to be compared displayed similar $^1J_{CH}$ coupling constants and that a difference of around 5–8 Hz from the experimental set value did not cause a substantial variation in the integrated peak volumes.[29]

With fucosyl donors **2**–**4** and polysaccharide acceptors **1** and **8** in hand, the reaction conditions for glycosylations were set: *N*-iodosuccinimide (NIS)/TMSOTf was chosen as the typical thioglycoside activator system and 5:3 v/v CH_2Cl_2/DMF as solvent mixture, because DMF was shown to act as an α-stereodirecting modulator in glycosylations with 2-*O*-benzylated thioglycoside donors,[30] including the first 1,2-*cis*-glycosylation of a polysaccharide acceptor.[16] The reaction temperature was set at $-20\,^\circ$C to make the benzylidene acid cleavage rate slow enough to avoid Fuc branching also on the GalNAc units. The glycosylation of **1** with thioglycosides **2**–**4** was run under homogeneous conditions and gave derivatives **9i**–**iii**, respectively (Scheme 4). The complexity of their ^{1}H NMR spectra and the coprecipitation of part of the Fuc byproducts deriving from **2**–**4** with the polysaccharides impeded any DS evaluation of the fucosylation reactions. Therefore, **9i**–**iii** were directly subjected to further steps to transform them into target polysaccharides **fC-i** and **fCS-i**–**iii**. In particular, they were first treated with Ac_2O and Et_3N in CH_3CN in the presence of a catalytic amount of DMAP to protect the (possible) unreacted hydroxyls on the GlcA units (→**10i**–**iii**). Then, the oxidative cleavage of both benzylidene and Bn protecting groups with $NaBrO_3$ and $Na_2S_2O_3$ in a H_2O/ethyl acetate mixture[22] furnished derivatives **11i**–**iii** with two or three free hydroxyls on the Fuc branches as well as at either 4-*O*- or 6-*O*-position of the GalNAc units. Sulfation of the free alcohol moieties with the SO_3·pyridine complex in DMF at 50 °C and subsequent hydrolysis of Ac, Bz, and methyl ester groups under alkaline aqueous conditions afforded **fCS-i**–**iii**. By skipping the sulfation reaction on **11i**, unsulfated fucosylated chondroitin **fC-i** was obtained.

A slightly different sequence of steps allowed the transformation of polysaccharide acceptor **8** into **fC-ii** and **fCS-iv**–**vi**. Fucosylation of **8** with thioglycosides **2**–**4** was conducted under almost the same conditions indicated above for the glycosylation of polysaccharide **1**. Only the reaction temperature was changed, by increasing it to room temperature, because no acid-cleavable benzylidene groups had to be preserved. The obtained derivatives **12i**–**iii** were then subjected to Bn oxidative cleavage (→**13i**–**iii**) followed by sulfation of the deprotected hydroxyls on Fuc and final ester hydrolysis to afford **fCS-iv**–**vi**. By skipping the sulfation reaction on **13i**, **fC-ii** was obtained.

^{1}H NMR spectra of semi-synthetic **fC-i**,**ii** and **fCS-i**–**vi** polysaccharides (see Figure 2 and the Supporting Information) allowed the determination of their degree of fucosylation (DF) and the α/β stereochemical ratio of the Fuc glycosidic bonds. DF was evaluated as the ratio between the *H*-6 Fuc methyl ($\delta = 1.21$–1.35 ppm) and the acetyl signal ($\delta = 2.01$–2.10 ppm) integrations. No significant variations among the fC and fCS polysaccharides was observed, as the DF value ranged from 0.34 to 0.51 (Table 1). Interestingly, no differences could be detected between polysaccharides branched at the GlcA and GalNAc positions (**fC-i**, **fCS-i**–**iii** and **fC-ii**, **fCS-iv**–**vi**, respectively), al-

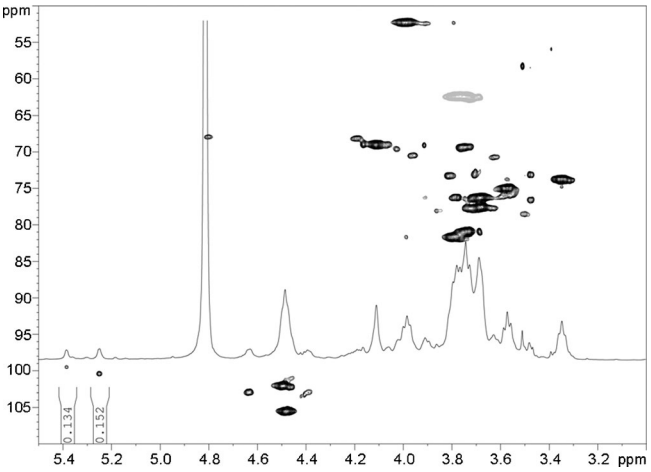
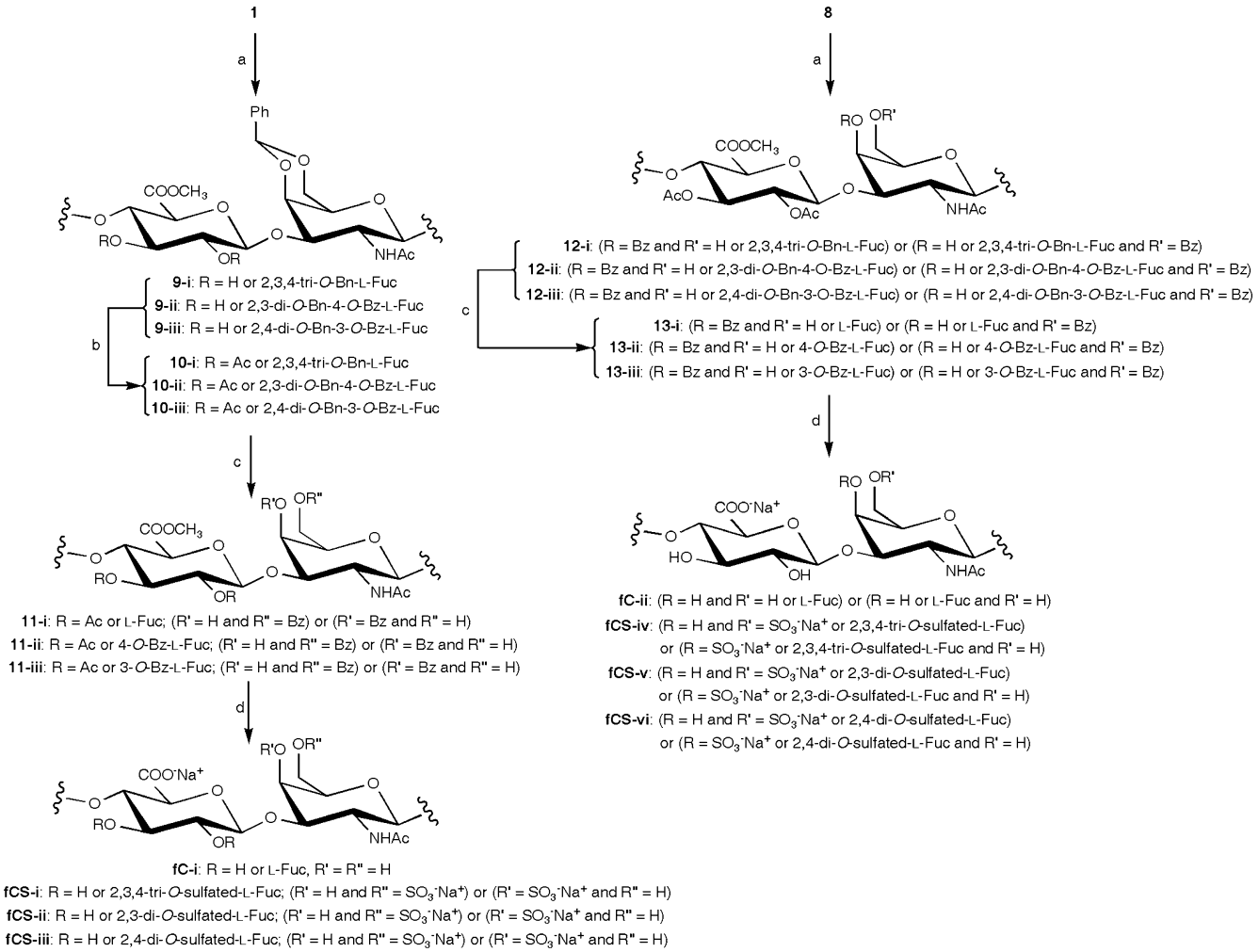


Figure 2. Magnified region of ^{1}H and HSQC DEPT NMR spectra of **fC-i** (600 MHz, D_2O, 298 K).



Scheme 4. Synthesis of **fSC-i–vi** and **fC-i,ii**. (a) **2** (→**9-i/12-i**) or **3** (→**9-ii/12-ii**) or **4** (→**9-iii/12-iii**), NIS, TMSOTf, 5:3 v/v CH_2Cl_2/DMF, AW-300 4 Å-MS, −20 °C (for **1**) or rt (for **8**), 4 h; (b) Ac_2O, Et_3N, DMAP, CH_3CN, rt, overnight; (c) $NaBrO_3$, $Na_2S_2O_4$, H_2O/ethyl acetate, rt, overnight; (d) i) SO_3·pyridine, DMF, 50 °C, overnight (skipped for **fC-i,ii**: see text); ii) NaOH, H_2O, rt, 6 h.

Table 1. Yield and structural data of **fC-i,ii** and **fCS-i–vi**.

	Yield[a] [%]	DF[b]	α/β ratio[c]	Branching site ratio[d]	Degree of sulfation[e]	M_w[f] [kDa]	M_w/M_n[g]
fC-i	33	0.34	5.2	0.9[h]	–	7.3	1.35
fC-ii	39	0.38	3.5	0.9[i]	–	6.9	1.35
fCS-i	41	0.51	3.6	0.9[h]	GalNAc *O*-4 = 0.32; GalNAc *O*-6 = 0.63	10.9	1.50
fCS-ii	41	0.43	5.5[j]	0.6[h,j]	GalNAc *O*-4 = 0.31; GalNAc *O*-6 = 0.62 GlcA *O*-2 = 0.06: GlcA *O*-3 = 0.07	9.4	1.49
fCS-iii	17	0.44	2.8	1.1[h]	GalNAc *O*-4 = 0.28; GalNAc *O*-6 = 0.56 GlcA *O*-2 = 0.13: GlcA *O*-3 = 0.19	8.3	1.58
fCS-iv	47	0.45	4.3	1.3[i]	GalNAc *O*-4 = 0.25; GalNAc *O*-6 = 0.33	9.4	1.56
fCS-v	38	0.43	4.0	2.1[i]	GalNAc *O*-4 = 0.25; GalNAc *O*-6 = 0.36	8.7	1.45
fCS-vi	40	0.50	1.4	2.4[i]	GalNAc *O*-4 = 0.14; GalNAc *O*-6 = 0.39	9.9	1.44

[a] Overall mass yield determined with respect to starting glycosyl acceptor (**1** or **8**). [b] Determined by ^{1}H NMR integration of Fuc methyl and GalNAc acetyl signals. [c] Estimated by ^{1}H NMR integration according to Eq. (1), except where indicated differently. [d] Estimated by ^{1}H NMR integration, except where indicated differently. [e] Estimated by HSQC DEPT integration (see Figures 4 and 5 and the Supporting Information). [f] M_w = weight-averaged molecular mass. [g] M_n = number-averaged molecular mass. [h] [α-Fuc-(1→3)-GlcA]/[α-Fuc(1→2)-GlcA] branching ratio. [i] [α-Fuc-(1→6)-GalNAc]/[α-Fuc(1→4)-GalNAc] branching ratio. [j] Determined by HSQC DEPT integration, owing to signals overlapping.

though fucosylation reactions were conducted at different temperatures (−20 °C and room temperature, respectively). The α/β ratio of Fuc branching could not be determined by integration of the α- and β-Fuc anomeric signals, because the latter could be not unambiguously detected in NMR spectra owing to low intensity and overlap with the signals of the α-

Fuc[31] or GlcA and GalNAc units. Therefore, an indirect estimation of the α/β ratio was done by evaluating the β-Fuc branches amount as the difference between the *H*-6 and *H*-1 α-Fuc signal integrations—as the former counts for both α- and β-linked Fuc units—according to Equation (1):

$$\alpha/\beta = \frac{I(\mathrm{CH}-1\alpha\mathrm{Fuc})}{\frac{I(\mathrm{CH}-6\mathrm{Fuc})}{3} - I(\mathrm{CH}-1\alpha\mathrm{Fuc})} \quad (1)$$

The α/β ratio was found to be rather variable, but with the α-stereochemistry clearly predominant in all cases, as expected from the α-stereodirecting effect of DMF in fucosylations.[16,30] It is worth noting that no evident increase in α/β ratio could be observed for polysaccharides deriving from fucosylations conducted with donors **3** and **4** (to give **fCS-ii–iii** and **fCS-v–vi**, respectively), in spite of the Bz groups at position *O*-4 and *O*-3, respectively, which are believed to enhance the α-stereoselectivity of fucosylation by remote participation.[32] Conversely, the lowest α/β ratios were observed for **fCS-iii** and **fCS-vi**, both deriving from fucosylations with 3-*O*-benzoylated donor **4**.

A 2D NMR (COSY, TOCSY, NOESY, HSQC DEPT, HSQC TOCSY) analysis of the semi-synthetic polysaccharides was then conducted to investigate the regiochemistry of the Fuc branches and, limited to fCS products, the sulfate group distribution. The HSQC DEPT spectrum of **fC-i** (Figure 2) showed at the highest ^{1}H chemical shift values the presence of two densities ($\delta_{H/C}$ = 5.38/99.5 and 5.25/100.4 ppm), which could be associated with the anomeric CH of two α-linked Fuc units. The analysis of the cross peaks related to these signals in the NOESY spectrum showed a correlation of the more downfield shifted signal (δ_H = 5.38 ppm) with a density at δ_H = 3.70 ppm, attributable to GlcA *H*-3 by means of the other 2D NMR spectra and literature data.[16,33] Similarly, the other *H*-1 α-Fuc signal (δ_H = 5.25 ppm) could be assigned to a branching unit linked at *O*-2 ($\delta_{H/C}$ = 3.50/78.6 ppm) of some GlcA units. The same approach for the assignment of Fuc branching sites was repeated for **fC-ii** as well as for all the semi-synthesized fCSs (see Figure 3 and the Supporting Information). Then, the integration of the anomeric α-Fuc signals in the ^{1}H NMR spectra allowed an evaluation of the branching site regiochemistry. Polysaccharides obtained by fucosylation of acceptor **1** (**fC-i** and **fCS-i–iii**) showed no preference for the GlcA *O*-2 or *O*-3 position, with the branching site ratio close to 1 in all cases (Table 1). A slight preference for fucosylation at *O*-6 with respect to *O*-4 of GalNAc units was observed for polysaccharides obtained by glycosylation of acceptor **8** (possessing a 6-OH/4-OH ratio of 1.3: see above), but limited only to **fCS-v,vi**.

In the case of fCS polysaccharides, NMR analysis also allowed us to confirm the Fuc sulfation patterns expected from the position of orthogonally cleavable (Bn) and permanent (Bz) protecting groups on fucosyl donors **2**, **3**, and **4** used in the glycosylations. For example, the cross peaks related to anomeric α-Fuc signals in the COSY, TOCSY, and NOESY spectra confirmed the differences in the sulfation pattern of Fuc units α-linked to the *O*-4 or *O*-6 atoms of GalNAc residues in **fCS-iv–vi**. Indeed, α-Fuc branches of **fCS-iv** (obtained from fucosylation reaction with perbenzylated thioglycoside **2**) displayed *H*-2, *H*-3, and *H*-4 signals all downfield shifted for *O*-sulfation (δ = 4.52, 4.70–4.74, and 4.95 ppm, respectively), whereas only the *H*-2 and *H*-3 signals showed a similar shift (δ = 4.53, 4.67–4.69 ppm, respectively; δ = 4.22 for *H*-4) in **fCS-v** (deriving from 2,3-di-*O*-benzylated donor **3**), as well as *H*-2 and *H*-4 ones in **fCS-vi** (δ = 4.41, 4.69 ppm, respectively; δ = 4.07–4.20 for *H*-3), which was obtained by glycosylation with 2,4-di-*O*-benzylated thioglycoside **4** (see Figure 3). The same NMR analysis was applied to confirm the differences in the α-Fuc sulfation patterns of **fCS-i–iii** (see the Supporting Information). 2D NMR analysis also allowed us to define the sulfation pattern in residues other than the Fuc branches. For example, the HSQC DEPT spectrum of **fCS-iv** (Figure 4) displayed signals for both sulfated ($\delta_{H/C}$ =

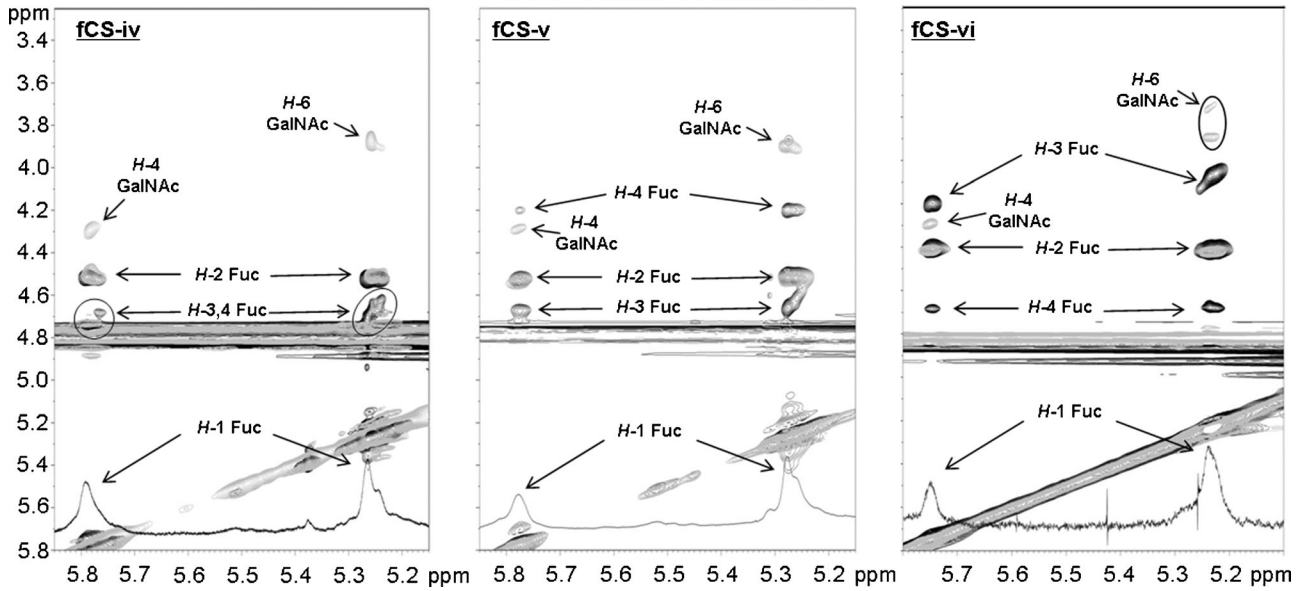


Figure 3. Magnified region of TOCSY (black) and NOESY (grey) NMR spectra (600 MHz, D_2O, 298 K) of fCS polysaccharides derived from acceptor **8**.

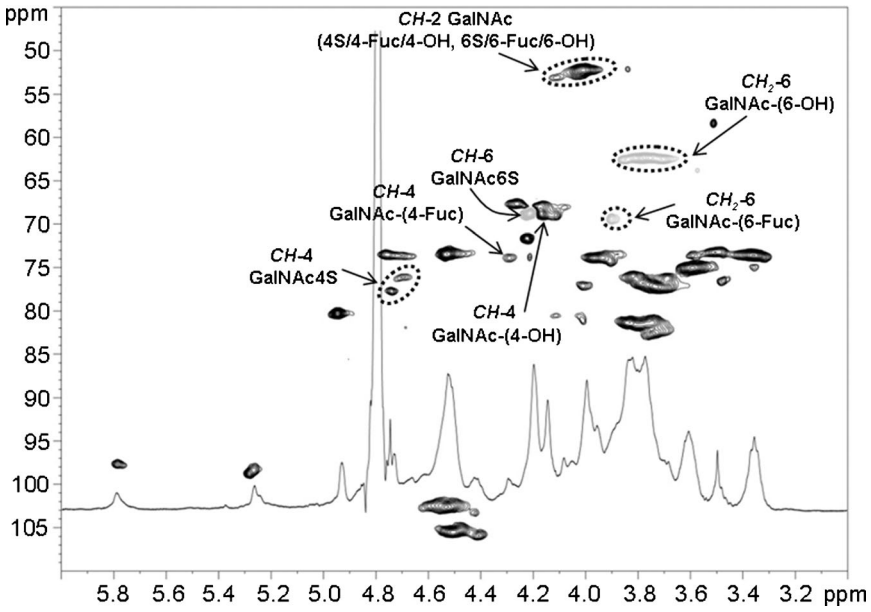


Figure 4. Magnified region of ^{1}H and HSQC DEPT NMR spectra (600 MHz, D_2O, 298 K) of **fCS-iv** (densities enclosed in the dotted circles were integrated for GalNAc *O*-4 and *O*-6 sulfation degree estimation).

4.22/68.8 ppm) 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 position 6, as well as 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 the GalNAc units[18e,34] (see also the Supporting Information). This was in agreement with the non-regioselective distribution of free hydroxyls at either position 4 or 6 of the GalNAc units on polysaccharide acceptor **8** together with their non-quantitative fucosylation (**8**→**12-i**, Scheme 4) before the sulfation step (**13-i**→**fCS-iv**), as already discussed above (DF = 0.45, Table 1). An estimation of sulfation degree at positions *O*-4 and *O*-6 was possible by HSQC DEPT density integration. Owing to the partial overlap of the densities related to the 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 density of GalNAc unit ($\delta_{H/C}=4.01/52.2$ ppm). In particular, the relative integration of GalNAc4S *CH*-4 densities with respect to the *CH*-2 one gave a 0.25 degree of sulfation at position *O*-4. A value of 0.33 was estimated for position *O*-6, by integrating the densities related to unsulfated GalNAc units (6-*O*-fucosylated or not) with respect to the *CH*-2 one and then applying Equation (2):

O-6 degree of sulfation :

$$1-\frac{I[(CH_2-6GalNAc-(6-OH)]+I[CH_2-6GalNAc(6-Fuc)]}{2\times I(CH-2GalNAc)} \quad (2)$$

A similar approach was repeated for the other fCS polysaccharides of the semi-synthetic library. Data for 4- and 6-*O*-sulfation at GalNAc sites are reported in Table 1. It is worth noting that fCS polysaccharides with Fuc branches on GlcA units (**fCS-i–iii**) display a cumulative value of sulfation degree on GalNAc units that is very close to 1. This is indicative of an A,C sulfation pattern—as expected from the non-regioselective oxidative opening of the benzylidene ring before the sulfation step[18e] (Scheme 4, **10-i–iii**→**11-i–iii**)—with a very low amount of unsulfated GalNAc units along the polysaccharide chain. Lower cumulative values for sulfation degree on the GalNAc units were obtained for **fCS-iv–vi** (Table 1). Indeed, in these cases, sulfate groups could be inserted only on those GalNAc residues that were not fucosylated in the glycosylation step, as ascertained by values close to 1 for the sum of DF and GalNAc *O*-6 and *O*-4 sulfation degrees.[35]

A careful investigation of the 2D NMR spectra of the semi-synthetic library allowed us to detect a small amount of sulfate groups also on the GlcA units in the case of **fCS-ii** and **fCS-iii** polysaccharides, as demonstrated by the presence of characteristic signals for *O*-2 and *O*-3 sulfation at $\delta_{H/C}=4.11/80.5$[29a] and 4.35/82.5 ppm,[18d] respectively. This could be ascribed to the non-quantitative protection of GlcA hydroxyls in the acetylation step (**9-ii,iii**→**10-ii,iii**) that left some free alcohol moieties ready to be sulfated at the end of the semi-synthesis (Scheme 4). Similarly to the GalNAc case, the GlcA sulfation degrees at *O*-2 and *O*-3 positions (Table 1) could be estimated by integration of these densities with respect to the GalNAc *CH*-2 one in the HSQC DEPT spectrum (Figure 5 and the Supporting Information). A comprehensive list of NMR assignment data for all the semi-synthesized polysaccharides is reported in the Supporting Information. These could be useful as standard data for researchers dealing with the structural characterization of novel natural fCSs, which could show a Fuc sulfation pattern and/or branching site different from those already reported in the literature.

The structural characterization of the semi-synthetic fC and fCS polysaccharides was completed with the determination of

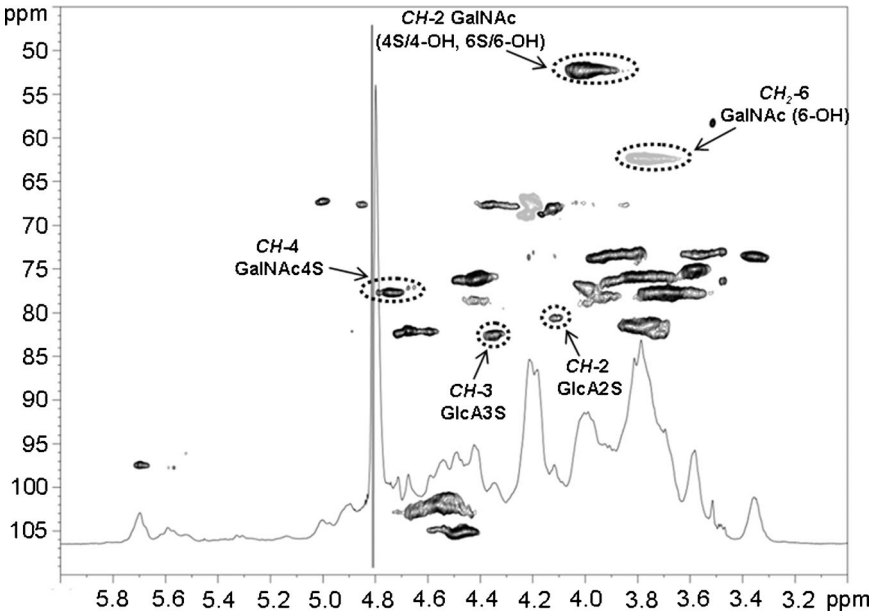


Figure 5. Magnified region of ^{1}H and HSQC DEPT NMR spectra (600 MHz, D_2O, 298 K) of **fCS-iii** (densities enclosed in the dotted circles were integrated for GalNAc *O*-4 and *O*-6 as well as GlcA *O*-2 and *O*-3 sulfation degree estimation).

their molecular mass by high-performance size exclusion chromatography combined with a triple detector array (HP-SEC-TDA).[36] All polysaccharides showed a weight-averaged molecular mass (M_w) that was much lower (7–10 kDa, Table 1) with respect to natural fCSs ($M_w \approx 55$–65 kDa).[14d] This is presumably due to acid-mediated reactions (carboxylic acid esterification[16] and glycosylation) in the semi-synthetic sequences, which shorten the polysaccharide chain of the starting microbial chondroitin ($M_w = 38$ kDa), although the polydispersity (M_w/M_n) was found to be only poorly increased (1.35–1.58 for the semi-synthetic polysaccharides; 1.34 for starting chondroitin). The fC species showed slightly lower M_w values with respect to fCS polysaccharides, owing to the absence of sulfate groups on the polymer chain. Interestingly, fCS derivatives with a M_w of 8–12 kDa are known to retain the anticoagulant activity of the longer, native polysaccharides, while minimizing the undesired effects, such as platelet aggregation and bleeding exhibited by the latter.[14]

Two very preliminary assays of the anticoagulant activity of the semi-synthetic polysaccharides were made by evaluating their AT-dependent activity against factor Xa and HC-II-mediated anti-factor IIa activity. Approximately 600–1000-fold lower values with respect to heparin were found for anti-Xa activity (Table 2), as already reported for low-molecular-mass fCS species obtained by partial depolymerization of natural polysaccharides.[5,14d,33] A behavior similar to natural species was found also for the reduction of HC-II mediated factor IIa activity.[14d] Indeed, data were very close to heparin for fCSs with Fuc branches on the GlcA units (**fCS-i**–**iii**), regardless of their sulfation pattern (Figure 6), whereas two of the three fCSs with Fuc branches on the GalNAc units (**fCS-v**–**vi**), as well as unsulfated polysaccharides (**fC-i**–**ii**), displayed a much reduced anticoagulant activity. These preliminary data encourage a much more detailed screening of the anticoagulant and antithrombotic effects of the semi-synthesized polysaccharides. This work is currently underway, and the results will be published in due course.

Table 2. AT-dependent activity against factor Xa.

	fC-i	fC-ii	fCS-i	fCS-ii	fCS-iii
Activity [IU mg^{-1}]	0.20	0.25	0.18	0.27	n.d.[a]
	fCS-iv	**fCS-v**	**fCS-vi**	**heparin**	
Activity [IU mg^{-1}]	0.26	0.33	0.23	198	

[a] Not determined.

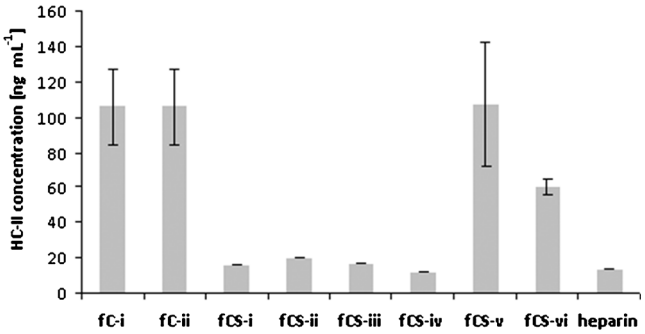


Figure 6. HC-II-mediated anti-factor IIa activity.

Conclusion

A library of six fCS polysaccharides with different Fuc sulfation patterns and/or branch positions, and two fCs with different Fuc linkage sites, was produced from microbial sourced chondroitin through semi-synthetic strategies based on the modu-

lar use of five different reactions: glycosylation, acetylation, selective cleavage of orthogonal protecting groups (Bn and benzylidene), sulfation, and global deprotection. 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[37] but is almost unexplored for polysaccharides,[38] 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 SAR 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. 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, which will be published in due course, 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- and 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.

Experimental Section

General methods

Commercial grade reagents and solvents were used without further purification, except where indicated differently. 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 with Spectra/Por 3.5 kDa cut-off membranes at 4 °C. Analytical thin layer chromatographies (TLCs) were performed on aluminum plates precoated with Merck Silica Gel 60 F254 as the adsorbent. The plates were developed with 10% H_2SO_4 ethanolic solution and then heating to 130 °C. Flash column chromatographies were performed with Kieselgel 60 (63–200 mesh). Size-exclusion chromatographies were performed with a Bio-Gel P2 column (0.75× 67.5 cm, Bio-Rad) by using 50 mM ammonium bicarbonate as a buffer at a flow rate of 0.2 mL min^{-1}. The column eluates were monitored continuously with a Knauer K-2310 refractive index refractometer. Freeze-dryings were performed with a 5 Pascal Lio 5P 4 K freeze-dryer. Optical rotations were measured with a JASCO P-1010 polarimeter. Elemental analyses were performed with a Carlo Erba 1108 instrument. Positive MALDI-MS spectra were recorded with a Applied Biosystem Voyager DE-PRO MALDI-TOF mass spectrometer in positive mode: compounds were dissolved in CH_3CN at a concentration of 0.1 mg mL^{-1} and one microliter of these solutions was mixed with one microliter of a 20 mg mL^{-1} solution of 2,5-dihydroxybenzoic acid in 7:3 v/v CH_3CN/H_2O. NMR spectra were recorded with a Bruker DRX-400 (^{1}H: 400 MHz, ^{13}C: 100 MHz) instrument or with 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 $\delta = 2.22$ ppm; ^{13}C: $(CH_3)_2CO$ at $\delta =$ 31.5 ppm) or [D_6]DMSO (^{1}H: CHD_2SOCD_3 at $\delta = 2.49$ ppm; ^{13}C: CD_3SOCD_3 at $\delta = 39.5$ ppm) or $CDCl_3$ (^{1}H: $CHCl_3$ at $\delta = 7.26$ ppm; ^{13}C: $CDCl_3$ at $\delta = 77.0$ ppm). Gradient-selected COSY, phase-sensitive NOESY, and TOCSY experiments were performed by using spectral widths of 6000 Hz in both dimensions, and by using data sets of 4096×256 points. Quadrature indirect dimensions were achieved through the States-TPPI (time proportional phase incrementation) method; spectra were processed by applying an unshifted Qsine function to both dimensions and the data matrix was zero-filled by a 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 by single quantum coherence with proton decoupling in the ^{13}C domain, by using data sets of 2048×256 points and typically 32 increments. As for HSQC TOCSY, data sets of 2048×256 points were used, with 100 increments, the 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 functions, and the values selected depended by the best resolution obtained. A Viscotek™ instrument (Malvern) was used to determine molecular mass data.

Ethyl 3-*O*-benzoyl-2,4-di-*O*-benzyl-1-thio-β-L-fucopyranoside (4)

A solution of triol **5**[25] (1.966 g, 9.452 mmol) in DMF (16 mL) was cooled to 0 °C and treated with imidazole (1.608 g, 23.63 mmol) and *tert*-butyldimethylsilyl chloride (TBDMSCl; 1.952 g, 12.95 mmol). After 10 min of stirring at 0 °C, the temperature was raised to room temperature and stirring was continued for an additional 3 h. The solution was then diluted with ethyl acetate (100 mL) and washed with water (100 mL). The organic phase was collected, dried over anhydrous Na_2SO_4, filtered, and concentrated. The obtained residue was purified by column chromatography (10:1 to 6:1 v/v *n*-hexane/ethyl acetate) to afford pure ethyl 3-*O*-*tert*-butyldimethylsilyl-1-thio-β-L-fucopyranoside (1.844 g, 61%), which showed ^{1}H and ^{13}C NMR data fully in line with those already reported for its D-enantiomer.[27] A solution of the product (1.796 g, 5.580 mmol) in DMF (16 mL) was cooled to 0 °C and treated with benzyl bromide (1.66 mL, 13.4 mmol) and then NaH (60% dispersion in mineral oil, 321 mg, 13.4 mmol). After 10 min of stirring at 0 °C, the temperature was raised to room temperature and stirring was continued for another 1 h. 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 *n*-hexane/ethyl acetate) to afford pure ethyl 2,4-di-*O*-benzyl-3-*O*-*tert*-butyldimethylsilyl-1-thio-β-L-fucopyranoside (2.605 g, 93%), which showed ^{1}H and ^{13}C NMR data fully in line with those already reported for its D-enantiomer.[27] The product (2.570 g, 5.120 mmol) was then dissolved in THF (37 mL) and treated with a 1.0 M solution of tetra-*n*-butylammonium fluoride (TBAF) in THF (12.8 mL). After 2 h of stirring at room temperature, the solution was concentrated and the obtained residue was subjected to column chromatography (10:1 to 6:1 v/v *n*-hexane/ethyl acetate) to afford pure ethyl 2,4-di-*O*-benzyl-1-thio-β-L-fucopyranoside **6** (1.894 g, 94%), which showed ^{1}H and ^{13}C NMR data fully in line with those already reported for its D-enantiomer.[27] A solution of **6** (840 mg, 2.17 mmol) in 1:1 v/v CH_2Cl_2/pyridine (18.7 mL) was treated with benzoyl chloride (377 μL, 3.25 mmol). After 3 h of stirring at room temperature, the reaction was quenched by adding CH_3OH (2.0 mL). The solution was diluted with CH_2Cl_2 (40 mL) and washed with 0.1 M HCl (40 mL). The organic layer was 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 *n*-hexane/ethyl acetate) to afford pure **4** (857 mg, 80%) as a white powder. R_f = 0.6 (3:1 v/v hexane/ethyl acetate); $[\alpha]_D^{25} = -89.9$ (c = 1.0 in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.04–7.18 (m, 15H, H-Ar), 5.25 (dd, J = 9.7, 3.1 Hz, 1H, H-3), 4.90 (d, J = 10.6 Hz, 1H, OC*H*HPh), 4.72 (d, J = 11.7 Hz, 1H, OC*H*HPh), 4.69 (d, J = 11.7 Hz, 1H, OC*H*HPh), 4.59 (d, J = 10.6 Hz, 1H, OC*H*HPh), 4.55 (d, J = 9.6 Hz, 1H, H-1), 4.01 (t, J = 9.7 Hz, 1H, H-2), 3.89 (d, J = 3.1 Hz, 1H, H-4), 3.74 (quartet, J = 6.4 Hz, 1H, H-5), 2.81 (m, 2H, SCH_2CH$_3$), 1.35 (t, J = 7.4 Hz, 3H, SCH$_2$CH_3), 1.29 ppm (d, J = 6.4 Hz, 3H, H-6); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 165.9 (CO), 137.9–127.6 (C-Ar), 85.0 (C-1), 78.0, 77.3, 76.4, 75.5, 75.2, 74.3 (C-2, C-3, C-4, C-5, 2OCH$_2$Ph), 24.9 (SCH$_2$CH$_3$), 16.9 (C-6), 14.9 ppm (SCH$_2$CH$_3$); MALDI-TOF MS for $C_{29}H_{32}O_5S$ (m/z): M_r (calcd): 492.20; M_r (found): 514.13 $(M+Na)^+$; elemental analysis calcd (%) for $C_{29}H_{32}O_5S$: C 70.70, H 6.55, S 6.51; found: C 70.45, H 6.68, S 6.41.

Chondroitin derivative (7)

Polysaccharide **1**[16] (202 mg, 0.419 mmol) was treated with CH_3CN (4.4 mL) and then with Et_3N (1.2 mL), acetic anhydride (2.8 mL), and DMAP (18.8 mg, 0.168 mmol). After overnight stirring at room temperature, 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 **7** (227 mg), which was contaminated mostly by Et_3N and DMAP.

Chondroitin derivative (8)

Derivative **7** (96.5 mg) was suspended in ethyl acetate (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 room temperature overnight under visible-light irradiation. The yellowish solid was collected by centrifugation and then dried under vacuum overnight to give pure polysaccharide **8** (66.9 mg, 63% over two steps from **1**, $DS_{acetylation}$ = 2.0, $DS_{debenzylidenation}$ = 0.97).

Example of glycosylation reaction

A mixture of polysaccharide acceptor **1** (51.5 mg, 0.107 mmol) and fucosyl donor **2** (256 mg, 0.535 mmol) was coevaporated three times with dry toluene (2.5 mL each). AW-300 4 Å molecular sieves and then DMF (3.2 mL) and CH_2Cl_2 (5.2 mL), which were freshly dried over 4 Å molecular sieves, were added to the mixture under an argon atmosphere. The mixture was stirred at −20 °C (or at room temperature for **8**) for 10 min, and then treated with NIS (180 mg, 0.799 mmol) and a 0.45 M solution of TMSOTf in freshly dried CH_2Cl_2 (460 μL, 0.207 mmol). After 4 h of stirring under an argon atmosphere at −20 °C (or room temperature for **8**), the molecular sieves were removed by decantation. A few drops of triethylamine and then 1:1 v/v hexane/ethyl acetate (40 mL) were added, and the mixture stored at −28 °C overnight. The obtained yellowish precipitate was collected by centrifugation and then dried under vacuum overnight to afford a mixture containing derivative **9i** (99.5 mg).

Example of acetylation reaction

A suspension of derivative **9i** (96.1 mg) in CH_3CN (2.1 mL) was treated with triethylamine (160 μL), acetic anhydride (710 μL), and finally with DMAP (4.8 mg). After overnight stirring at room temperature, the resulting brownish solution was concentrated to give a residue that was coevaporated several times with toluene. A brownish oil (165 mg) was obtained. It was not further purified, but used in the following synthetic step as it was.

Example of cleavage of benzyl (and benzylidene) protecting groups

Derivative **10i** (163 mg) was treated with ethyl acetate (6.7 mL) and the resulting suspension was treated with a 0.27 M solution of $NaBrO_3$ in pure water (6.7 mL). A 0.24 M solution of $Na_2S_2O_4$ in pure water (6.3 mL) was added portionwise over a period of 10 min. The triphasic mixture was vigorously stirred at room temperature overnight under visible-light irradiation. The yellowish solid was then collected by centrifugation. It was dried under vacuum overnight to afford derivative **11i** (37.5 mg).

Example of sulfation and deprotection reactions

A suspension of derivative **11i** (26.9 mg) in DMF (1.7 mL), which was freshly dried over 4 Å molecular sieves, was treated with a 1.17 M solution of pyridine/sulfur trioxide complex in freshly dried DMF (0.6 mL). After overnight stirring at 50 °C, the obtained clear solution was cooled to room temperature and then treated with a saturated NaCl solution in acetone (20 mL). The obtained yellowish precipitate was collected by centrifugation and then dissolved in pure water (8.0 mL). A 15% w/v NaOH solution was then added to adjust the pH to 13. The solution was stirred at room temperature for 6 h, then it was neutralized by treatment with 1 M HCl. Dialysis and subsequent freeze-drying yielded a slightly yellow solid, which 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-i** (14.5 mg) as a white waxy solid.

Determination 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 re-

fractive 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.[39] By solving the equations above, the system allowed the simultaneous determination of sample concentration, molecular mass, and intrinsic viscosity.[36a]

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 IU mg^{-1} active solutions. The sample (100 μL) was added to purified bovine AT (100 μL) diluted in Tris EDTA buffer (pH 8.4), and the mixture was incubated with factor Xa (200 μL) for 2 min at 37 °C. Then, factor Xa chromogenic substrate (200 μL) 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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Keywords: carbohydrates · glycosylation · polysaccharides · semi-synthesis · sulfation

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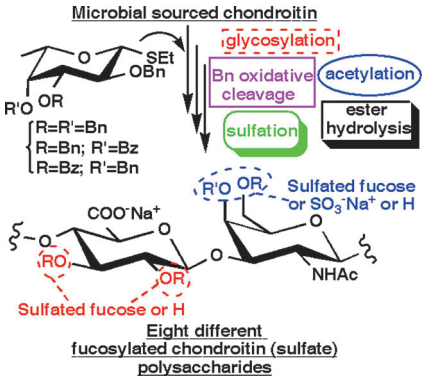
FULL PAPER

A small library of fucosylated chondroitin sulfate polysaccharides was obtained from microbial sourced unsulfated chondroitin by a semi-synthetic modular approach based on the different combination of only five reactions. An almost unprecedented polysaccharide branching by *O*-glycosylation was the key step. Eight polysaccharides with different fucose and/or sulfation patterns were obtained and carefully characterized by 2D NMR spectroscopy.



Polysaccharide Chemistry

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A Modular Approach to a Library of Semi-Synthetic Fucosylated Chondroitin Sulfate Polysaccharides with Different Sulfation and Fucosylation Patterns