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Inter *vs.* intraglycosidic acetal linkages control sulfation pattern in semi-synthetic chondroitin sulfate

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ABSTRACT

Microbial-sourced unsulfated chondroitin could be converted into chondroitin sulfate (CS) polysaccharide by a multi-step strategy relying upon benzylidenation and acetylation reactions as key-steps for its regioselective protection. By conducting the two reactions *one-* or *two-pots*, CSs with different sulfation patterns could be obtained at the end of the semi-synthesis. In particular, a CS polysaccharide possessing sulfate groups randomly distributed between positions 4 and 6 of *N*-acetyl-galactosamine (GalNAc) units could be obtained through the *two-pots* route, whereas the *one-pot* pathway allowed an additional sulfation at position 3 of some glucuronic acid (GlcA) units. This difference was ascribed to the stabilization of a labile interglycosidic benzylidene acetal involving positions *O*-3 and *O*-6 of some GlcA and GalNAc, respectively, when the benzylidene-acetylation reactions were conducted in a *one-pot* fashion. Isolation and characterization of a polysaccharide intermediate showing interglycosidic acetal moieties was accomplished.

1. Introduction

Glycosaminoglycans (GAGs) are biomacromolecules ubiquitously distributed in extracellular matrices and at cell surfaces, with a high biological significance. Chondroitin sulfate (CS), the most abundant GAG of the human body, is involved in a myriad of physiological and pathological processes, including central nervous system development, signal transduction, morphogenesis, wound healing, viral and bacterial infections (Yamada & Sugahara, 2008). From a structural point of view, CS is a highly negatively charged polysaccharide, constituted of glucuronic acid (GlcA) and *N*-acetyl-galactosamine (GalNAc), linked together through alternating β-(1→3) and β-(1→4) glycosidic bonds. The resulting 4)-β-GlcA-(1→3)-β-GalNAc-(1→ repeating unit undergoes sulfation at various positions after *in vivo* polymerization, thus affording a polysaccharide chain with a variable sulfation pattern. Depending on the position of sulfate group substitutions, several disaccharide subunits could be identified along the backbone of natural CSs. The most common sulfation patterns are depicted in Fig. 1: the positions 4 and/or 6 of the GalNAc units are commonly sulfated, whereas positions 2 or 3 of the GlcA units are sulfated to a minor extent (Pomin, 2013).

CSs extracted from animal sources often possess a combination of different sulfation patterns. Recent studies suggested that CS may be capable of encoding functional information in a sequence-specific manner, mainly through the sequence of sulfate sites pattern on the saccharide backbone (Gama et al., 2006). This sequence seems to be strictly regulated *in vivo* and is tissue/age specific. It also depends on the physiological or pathological state of the organism. However, the details of this "sulfation code" have yet to be fully elucidated.

CSs isolated from vertebrate sources have some applications as therapeutics. In particular, the CS polysaccharide isolated from poultry bones as well as bovine and porcine nasal septa and tracheas – predominantly composed of A and C disaccharide subunits (CS-A,C) – is employed as a biomedical ingredient for the treatment of osteoarthritis and osteoarthrosis (Reginster, Heraud, Zegels, & Bruyere, 2007), in alternative to the conventional use of nonsteroidal anti-inflammatory drugs and analgesics, that can cause serious side effects. CS-A,C is also marketed as a nutraceutical to prevent lesions or degeneration of joint cartilages. Furthermore, it is a component of viscoelastic solutions used as surgical aids in cataract extraction and intraocular lens implantation procedures, as well as an ingredient for skin moisturizers and creams for the treatment of burns. Several other pharmaceutical applications have been recently proposed for CSs, in relation to different sulfation patterns (Yamada & Sugahara, 2008). In spite of the growing interest in pharmacological and nutraceutical applications of CSs, several problems still limit their availability: first, the low abundance of the

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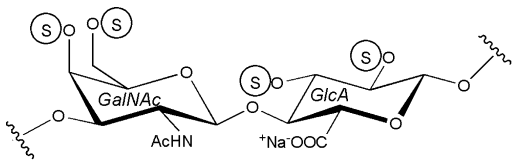


0: GalNAc-GlcA

A: GalNAc(4S)-GlcA
C: GalNAc(6S)-GlcA
D: GalNAc(6S)-GlcA(2S)
E: GalNAc(4,6S)-GlcA
K: GalNAc(4S)-GlcA(3S)
L: GalNAc(6S)-GlcA(3S)
M: GalNAc(4,6S)-GlcA(3S)
R: GalNAc-GlcA(2,3S)

Fig. 1. Disaccharide subunits found in natural CSs.

raw material and the laborious downstream purification, but also the strict regulations for animal-derived drugs. Furthermore, the high variability of the sulfation patterns in animal tissues requires a strict control of the sulfation level in CS products. It is worth noting that per-*O*-sulfated CS can induce a strong allergic-type response (Greinacher, Michels, Schäfer, Keifel, & Mueller-Eckhardt, 1992). Recently, its fraudulent addition as a contaminant in some heparin lots, caused a cluster of serious adverse events, including 149 deaths in US and Europe (Kishimoto et al., 2008).

The high biomedical interest in CS prompted many efforts in the fine manipulation of its structure by chemical and chemoenzymatic methods (Bedini & Parrilli, 2013; Scott & Panitch, 2013), mainly aimed to the modification of the sulfation pattern. In most cases natural polysaccharides are chemically sulfated without any control of the regioselectivity of the reaction (Fan et al., 2011; Möller et al., 2012; Jindal et al., 2013; Liu et al., 2014), nonetheless, for natural CSs some methods for the regioselective *O*-sulfation/de-*O*-sulfation were reported (Bedini & Parrilli, 2012). To this aim, we (Bedini et al., 2011, 2012) and others (Zoppetti & Oreste, 2004) developed protocols relying upon the regioselective protection of unsulfated chondroitin polysaccharide from microbial sources (Schiraldi, Cimini, & De Rosa, 2010; DeAngelis, 2012), followed by sulfation of the unprotected hydroxyls and final global deprotection. A plethora of protecting groups are commonly employed in synthetic carbohydrate chemistry (Wuts & Greene, 2007), nonetheless their application on polysaccharides is rather limited. Indeed, the regioselective installation of the protecting groups may involve well-defined hydroxyl position(s) in each repeating unit and, at the same time, the glycosidic linkages and any labile decoration on the polysaccharide backbone should survive unaltered during the protection–deprotection reactions. All the strategies developed for the chondroitin case relied upon the installation of a cyclic protecting group on the 4,6-diol of GalNAc units as first step. Either acetal-(benzylidene) or orthoester-type (Jacquinet, Lopin-Bon, & Vibert, 2009) cycles were installed (**1**/**3**, Scheme 1). After acetylation of the GlcA units, they could be both opened non regioselectively to afford intermediates **2**/**5** possessing either 4-*O*- or 6-*O*-acylated GalNAc units randomly distributed on the same polymer chain. Sulfation of the free alcohols and global deprotection finally afforded semi-synthetic CS polysaccharides. Very interestingly, the choice of the starting cyclic protecting group seemed to influence the sulfation pattern in the obtained CS product. Indeed, the benzylidene route afforded a CS-A,C polysaccharide with sulfate groups only at positions *O*-4 or *O*-6 of GalNAc units (Bedini et al., 2011), whereas the orthoester-based strategy furnished a CS polymer possessing additional sulfate groups at position *O*-3 of some GlcA units (Bedini et al., 2012), a feature quite uncommon in natural CSs (Sugahara & Yamada, 2000). Due to the high interest in producing CS polysaccharides from non-animal sources with several different but strictly controlled sulfation patterns, we decided to study and rationalize these results.

2. Material and methods

2.1. General methods

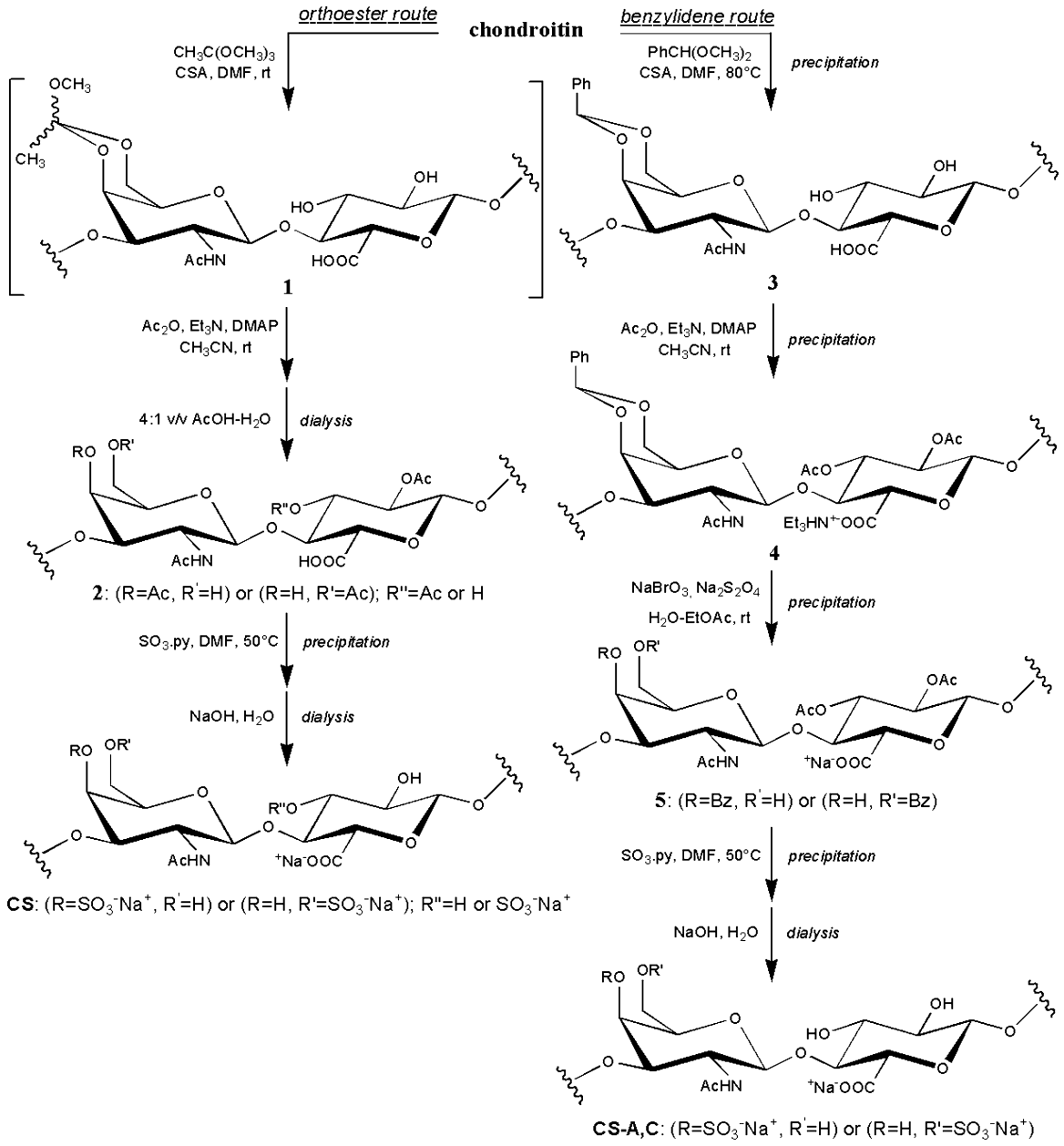
Commercial grade reagents and solvents were used without further purification. Unsulfated chondroitin sodium salt was obtained by fed-batch fermentation of *Escherichia coli* K4 followed by downstream purification processes performed as recently indicated (Cimini, Restaino, Catapano, De Rosa, & Schiraldi, 2010). Centrifugations were performed with an Eppendorf Centrifuge 5804 R instrument. Dialyses were conducted on Spectra/Por 10 kDa cut-off membranes. Freeze-dryings were performed with a 5Pascal Lio 5P 4 K freeze dryer. The term "pure water" refers to water purified by a Millipore Milli-Q Gradient system. NMR spectra were recorded on a Varian INOVA 500 (^{1}H NMR: 500 MHz, ^{13}C NMR: 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) 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 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 an unshifted Qsine function to both dimensions and data matrix was zero-filled by factor of 2 before Fourier transformation. TOCSY mixing time was set to 120 ms. 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 to 4096 × 2048 points reconstructing 1024 of these data points with forward linear prediction extrapolation (30 coefficients) and zero-filling those remaining.

2.2. Preparation of chondroitin 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, 11.10 mmol)

2.3. Semi-synthesis of CS via one-pot benzylidene-acetylation strategy

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 4 Å 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 2) and α,α-dimethoxytoluene (464 μL, 3.09 mmol), freshly dried over 4 Å molecular sieves, was added. A 0.21 M solution of (+)-camphor-10-sulfonic acid in freshly dried DMF (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 triethylamine (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) was obtained as a brown amorphous solid. It was then suspended in



Scheme 1. Orthoester and benzylidene routes for the semi-synthesis of CSs from unsulfated chondroitin.

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). It was suspended in DMF (2.1 mL), that was freshly dried over 4 Å 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) as a slightly yellow, woolly solid. ^{1}H-NMR (600 MHz, D_2O, 298 K) for CS obtained through a benzylidenation at 50–80 °C (Fig. 2 and Fig. A.3 in the Supplementary data): δ 4.78–4.74 (bs, H-4 GalNAc-4S), 4.60–4.46 (m, H-1 GalNAc-4S, H-1 GalNAc-6S, H-1 GlcA, H-1 GlcA-3S), 4.38–4.34 (bs, H-3 GlcA-3S), 4.28–4.11 (m, H-4 GalN-6S, H-6 GalNAc-6S), 4.04–3.92 (m, H-2 GalNAc-4S, H-2 GalNAc-6S, H-3 GalNAc-4S, H-4 GlcA-3S, H-5 GalNAc-6S), 3.89–3.67 (m, H-3 GalNAc-6S, H-4 GlcA, H-5 GalNAc-4S, H-5 GlcA, H-5 GlcA-3S, H-6 GalNAc-4S), 3.63–3.54 (bs, H-2 GlcA-3S, H-3 GlcA), 3.40–3.31 (bs, H-2 GlcA), 2.08–1.93 (bs, $COCH_3$). ^{1}H-NMR (600 MHz, D_2O, 298 K) for CS obtained through a benzylidenation at rt (Fig. A.4 in the Supplementary data): δ 4.60–4.44 (m, H-1 GalNAc, H-1 GalNAc-6S, H-1 GlcA, H-1 GlcA-3S), 4.38–4.34 (bs, H-3 GlcA-3S), 4.29–4.16 (m, H-4 GalN-6S, H-6 GalNAc-6S), 4.13–4.09 (bs, H-4 GalNAc), 4.03–3.93 (m, H-2 GalNAc, H-2 GalNAc-6S, H-4 GlcA-3S, H-5 GalNAc-6S), 3.88–3.65 (m, H-3 GalNAc, H-3 GalNAc-6S, H-4 GlcA, H-5 GalNAc, H-5 GlcA,

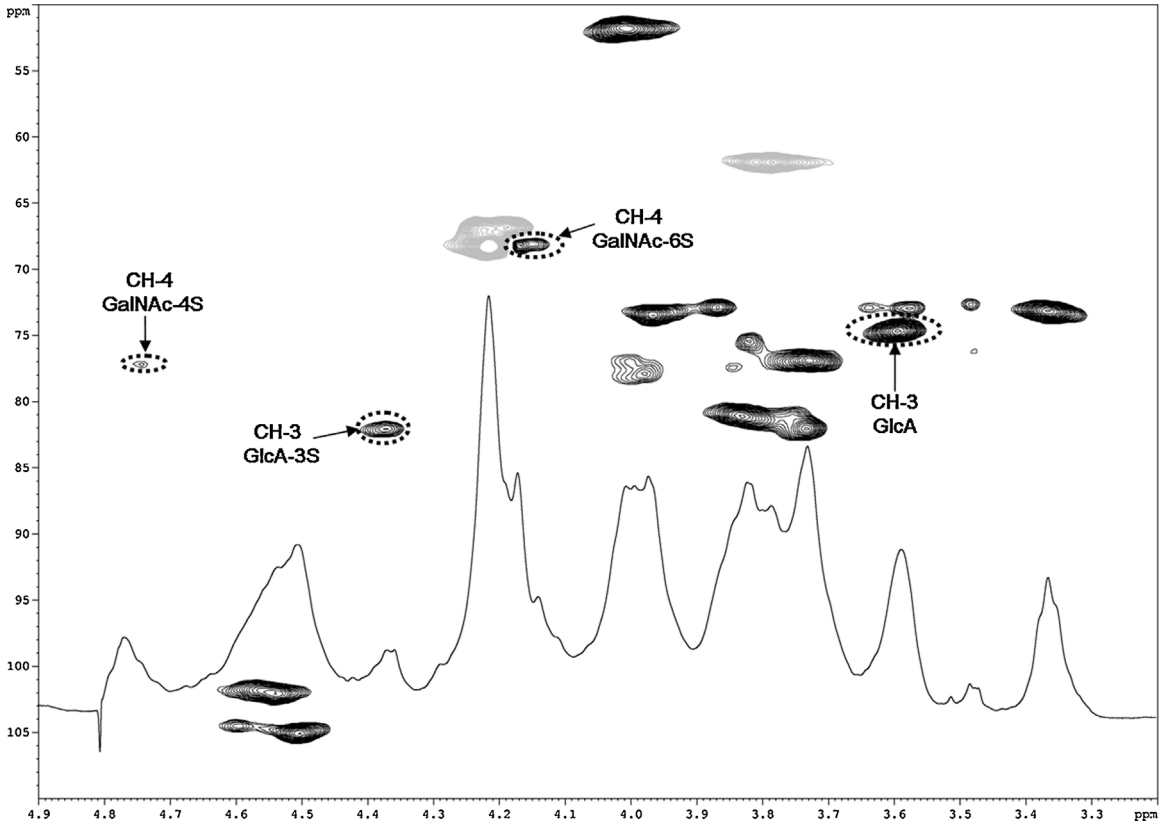


Fig. 2. Zoom of ^{1}H and HSQC-DEPT NMR spectra (600 MHz, D_2O, 298 K) of semi-synthetic CS polysaccharide obtained through *one-pot* benzylidene (80 °C)-acetylation route (the densities enclosed in the dotted regions were used for the evaluation of the relative amount of sulfated and non-sulfated monose units: see Table 2).

H-5 GlcA-3S, H-6 GalNAc), 3.62–3.53 (bs, H-2 GlcA-3S, H-3 GlcA), 3.39–3.31 (bs, H-2 GlcA), 2.11–1.92 (bs, $COCH_3$).

2.4. 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 4 Å 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 4 Å molecular sieves. A 0.21 M solution of (+)-camphor-10-sulfonic acid in freshly dried DMF (209 μL, 43.9 μmol) was then added and the mixture was stirred at rt overnight. Triethylamine (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) as a slightly yellow amorphous solid.

2.5. NMR degradation studies of **9a**

A solution of polysaccharide **9a** (6.1 mg) in D_2O (600 μL) was transferred to a NMR test tube and a first ^{1}H-NMR (500 MHz, 298 K) spectrum was measured. A 7% v/v solution of DCl or CD_3COOD (AcOD) in D_2O (30 μL) was then added. ^{1}H-NMR spectra were then measured at different time intervals.

3. Results and discussion

The presence of GlcA-3S units in CS obtained from the orthoester route was firstly hypothesized to be a direct consequence of a non-quantitative acetylation at position GlcA-*O*-3 on polysaccharide **1** (Scheme 1). Even by performing the acylation under several powerful conditions, the final CS polysaccharide contained GlcA-3S units to a certain extent in every case (Bedini et al., 2011).

By comparing the first three steps of the orthoester and benzylidene routes, one could notice differences not only in the cyclic protecting group used for GalNAc units but also in the work-up employed for the reactions. Indeed, in the benzylidene route the polysaccharide was isolated after each step by precipitation, whereas the first three steps of the orthoester route were conducted *one-pot* with only a final purification of the intermediate polysaccharide by dialysis (Scheme 1). In order to eliminate as many differences as possible between the two routes, the isolation of the orthoester-protected intermediate **1** was pursued by precipitation. Unfortunately, all attempts afforded a product displaying very complex NMR spectra that could not be characterized in details, in contrast with benzylidene-protected polysaccharide **3**, that clearly showed 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 (Fig. A.1: see the Supplementary data). The difficulty in isolating a pure orthoester-protected polysaccharide intermediate was associated with the higher lability of the orthoester moiety with respect to the benzylidene ring. Thus, we decided to try to get the benzylidene route closer to the orthoester one, and not *vice versa*. To this aim, the precipitation work-up after the first reaction was deleted and the benzylidenation and acetylation steps were conducted *one-pot*, followed by precipitation of the fully-protected polysaccharide intermediate. The synthetic pathway was then completed by conducting oxidative benzylidene ring cleavage, sulfation and global deprotection without any other difference with respect to the original benzylidene route (Scheme 1) (Bedini et al., 2011). A CS polysaccharide possessing not only sulfate groups at positions 4 or 6 of GalNAc units but also at position 3 of some GlcA

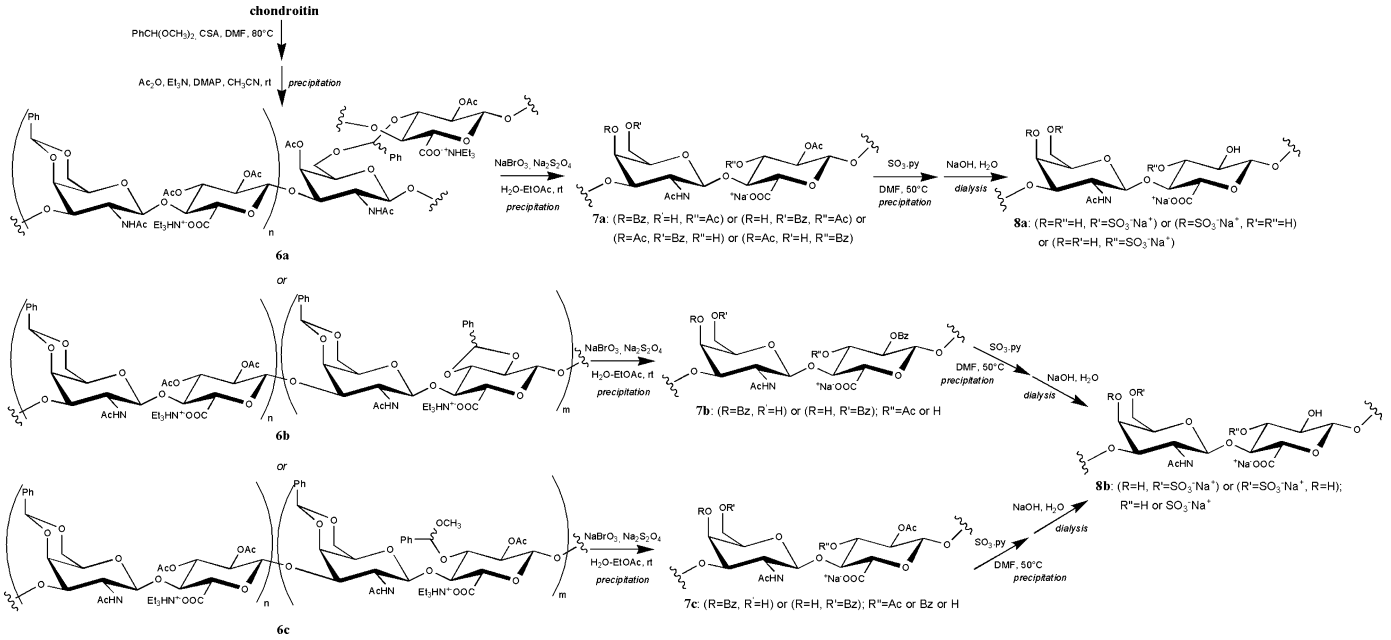
units was obtained in this case, as through the orthoester route. This was clearly showed in its HSQC-DEPT spectrum (Fig. 2) by the presence of two downfield-shifted carbinolic CH signals (δ_H = 4.75, δ_C = 77.2 ppm and δ_H = 4.36, δ_C = 81.9 ppm) – indicating (Sugahara et al., 1996; Kinoshita et al., 1997; Kumar Shetty et al., 2009; Bedini et al., 2012) 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) attributable to sulfated H-6 of GalNAc units. This result was firstly hypothesized to be caused by a non-quantitative acetylation at GlcA-*O*-3 position reputedly associated with the change of solvent from the *two-pots* (acetylation in CH_3CN) to the *one-pot* sequence (acetylation in DMF–CH_3CN or DMF only). To test this hypothesis, purified benzylidene-protected intermediate **3** was acetylated in DMF, instead of CH_3CN. By concluding the synthetic route, the obtainment of a CS-A,C polysaccharide without any NMR-detectable GlcA-3S unit was restored (Fig. A.2: see the Supplementary data). Hence, a DMF effect operating during the acetylation step had to be discarded.

An alternative hypothesis for explaining the presence of some GlcA-3S units in the semi-synthetic CS from the *one-pot* benzylidenation/acetylation route, was the formation of unstable benzylidene acetals involving position 3 of some GlcA units. These could survive when no work-up was performed after the benzylidenation reaction and the following acetylation step under basic conditions was conducted *one-pot* (Scheme 2), whereas they should degrade after benzylidenation work-up by precipitation because of acid functional groups on the polysaccharide backbone (**3**, Scheme 1). Three different labile benzylidene moieties could be hypothesized. Firstly, an interglycosidic acetal involving *O*-3 and *O*-6 of some GalNAc and GlcA units, respectively, could be formed in competition with a classical intraglycosidic 4,6-*O*-benzylidene (**6a**, Scheme 2). The formation of interglycosidic acetals and ketals have been reported in literature on some di- and trisaccharides (Fanton et al., 1981; Alonso-Lopez et al., 1987; Jaramillo, Fernandez-Mayoralas, & Martin-Lomas, 1988; Sakairi & Kuzuhara, 1993; Sakairi et al., 1998; Manzo, Barone, & Parrilli, 2000) as well as on cyclodextrins (Sakairi, Nishi, Tokura, & Kuzuhara, 1996), whereas, to the best of our knowledge, no examples exist on polysaccharides. Furthermore, an interglycosidic benzylidene acetal has been demonstrated to undergo selective cleavage under mild acid conditions in the presence of six-membered 4,6-benzylidene cycles (Sakairi et al., 1998), in agreement with the observation that only the latter kind of benzylidene motif was obtained by precipitation of the acid polysaccharide **3** in the *two-pots* benzylidene-acetylation route (Scheme 1). The interglycosidic benzylidene ring in **6a** could be also oxidatively cleaved, together with the six-membered one, by treatment with $NaBrO_3$ and $Na_2S_2O_4$ (Adinolfi, Barone, Guariniello, & Iadonisi, 1999) to liberate a free alcohol at position *O*-3 of some GlcA units (**7a**, Scheme 2), that could be then sulfated to afford CS-**8a**.

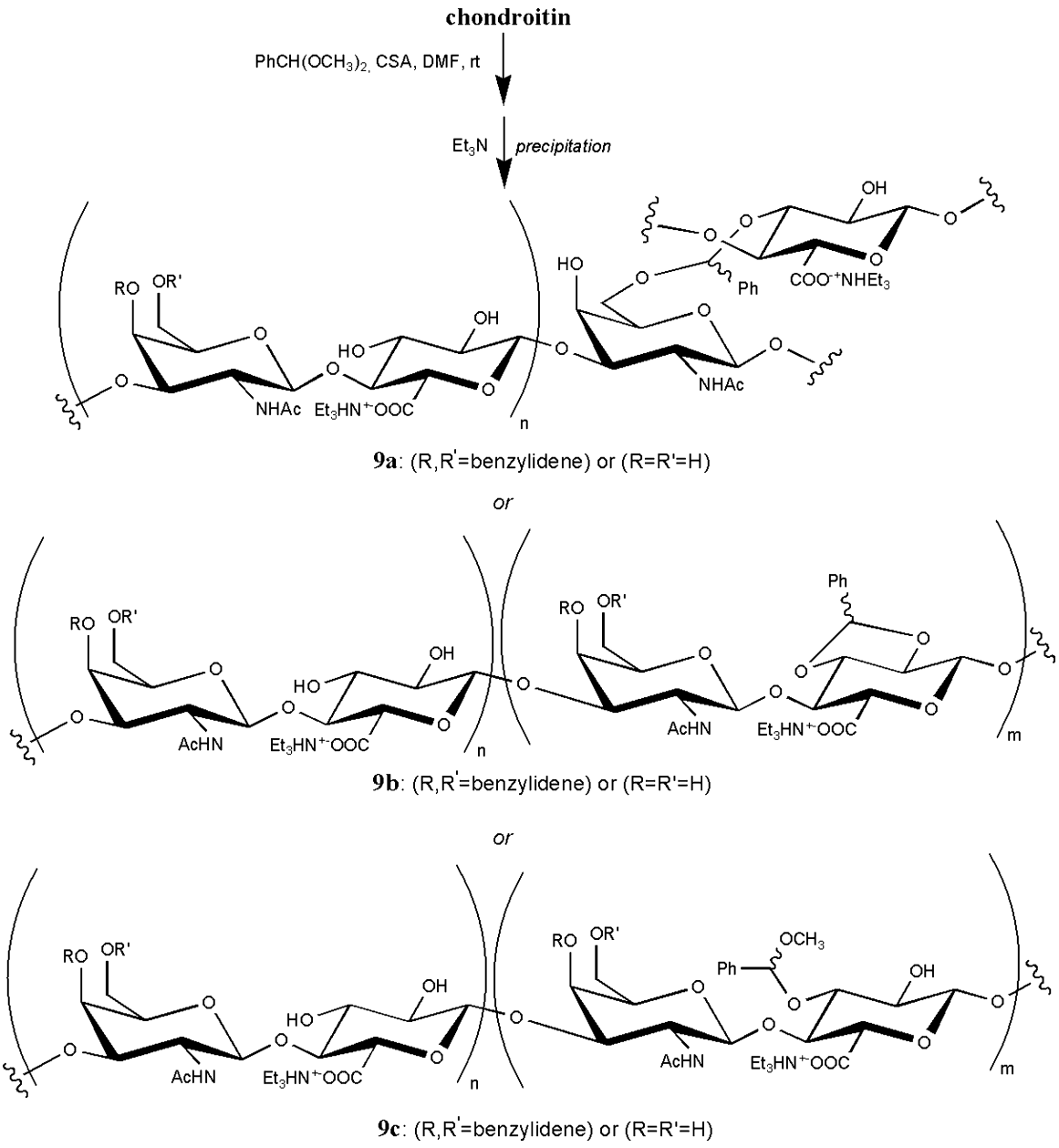
A second hypothesis for a labile benzylidene acetal involving position 3 of some GlcA units could be a 2,3-*trans*-configured ring (**6b**), that has been described for *gluco*-configured hexoses (Thiem & Elvers, 1978; Français, Urban, & Beau, 2007). The *trans*-benzylidene moiety at 2,3-diol of some GlcA units in **6b** could be oxidatively cleaved by $NaBrO_3/Na_2S_2O_4$ treatment as well, nonetheless the observed sulfate groups positioning at *O*-3 of some GlcA units in the final CS product would need to hypothesize that this cleavage proceeded with a marked regioselectivity, leaving predominantly a free alcohol at position *O*-3 and a benzoate ester at *O*-2 (polysaccharide **7b**). Actually, this hypothesis contrasts with the oxidative opening of a 2,3-benzylidene ring on a *gluco*-derivative under Hanessian–Hullar conditions which furnished an equimolar mixture of *O*-2- and *O*-3 alcohol products (Thiem & Elvers, 1978), therefore we less relied on it.

Another labile benzylidene moiety could be an acyclic acetal (**6c**), that again should liberate a free alcohol at position 3 of some GlcA units by $NaBrO_3/Na_2S_2O_4$ treatment (**7c**). The formation of transient acyclic acetals on glucose secondary hydroxyls has been recently observed on acetalation of dextrans (Bachelder, Beaudette, Broaders, Dashe, & Fréchet, 2008; Broaders, Cohen, Beaudette, Bachelder, & Frechet, 2009), nonetheless their amount was approximately zero when acetalation was conducted with $PhCH(OCH_3)_2$ (Cui et al., 2012).

We tested the hypothesis of a labile acetal formation (interglycosidic, *trans*-cyclic or acyclic), by firstly quenching the benzylidenation reaction of chondroitin with a stoichiometric excess of triethylamine before precipitation, in order to neutralize GlcA carboxylic acid functions and residual (+)-camphor-10-sulfonic acid (CSA) catalyst that usually co-precipitated with the



Scheme 2. Interglycosidic, *trans*-cyclic or acyclic acetal formation during *one-pot* benzylidene-acetylation route.



Scheme 3. Isolation of benzylidene intermediate **9a**/**9b**/**9c** by precipitation from the reaction mixture conducted at rt and quenched with triethylamine.

polysaccharide. The obtained product **9a**/**9b**/**9c** (Scheme 3) went through the subsequent steps of the standard benzylidene route, affording at the end of the semi-synthesis, as expected, a polysaccharide possessing sulfate groups not only on GalNAc but also at position 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.

Benzylidenation conditions were then subjected to slight modifications in order to enhance the formation of the labile acetal. The reaction was conducted under argon atmosphere and by employing freshly dried DMF and $PhCH(OCH_3)_2$ in order to avoid hydrolysis of the labile acetal by moisture. Furthermore, the reaction was performed at temperatures lower than 80 °C. Every semi-synthesis under the modified benzylidenation conditions was then completed with the standard following steps (Scheme 2) and the relative amount of GlcA-3S units with respect to unsulfated GlcA ones in the obtained CS polysaccharides was evaluated by integration of HSQC-DEPT spectrum (Guerrini, Naggi, Guglieri, Santarsiero, & Torri, 2005) (Fig. 2 and Figs. A.3–A.5: see the Supplementary data). By lowering the reaction temperature to 50 °C no increase of GlcA-3S units was detected (Table 1, entries 1 and 2), whereas by conducting the reaction at rt GlcA-3S/GlcA ratio increased (entry 3). Interestingly, the same ratio was obtained for *one-pot* benzylidenation–acetylation and orthoesterification–acetylation (Bedini et al., 2012) protocols when protection reactions were conducted at rt in both cases (entries 3 and 4). Nonetheless, by conducting the benzylidenation reaction at rt, a relevant amount of unsulfated GalNAc units was detected. This could be ascribed to a non-quantitative benzylidenation of GalNAc 4,6-diol at rt. The free hydroxyls still present on GalNAc units after benzylidenation at rt were then protected as acetyls and therefore could be not liberated and sulfated in the two following steps.

In order to understand which kind of labile benzylidene was responsible of the GlcA-3S units in the CSs obtained *via* the *one-pot* benzylidene-acetylation route, a 2D-NMR spectroscopic analysis of polysaccharide **6a**/**6b**/**6c** may be necessary. Unfortunately, its NMR

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-4or6S	GalNAc-4S[c]/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 (see Section 2 for details), 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 ppm; δ_C = 74.4 ppm) units in the HSQC-DEPT spectrum (600 MHz, D_2O, 298 K) (see Fig. 2 and Figs. A.3–A.5 in the Supplementary data).
[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, respectively, in the HSQC-DEPT spectrum (600 MHz, D_2O, 298 K) (see Fig. 2 and Figs. A.3–A.5 in the Supplementary data).
[d] CH-4 signal of unsulfated GalNAc units not detectable in the HSQC-DEPT spectrum (see Fig. 2 and S3–S5 in the Supporting information).
[e] CH-4 signal of GalNAc-4S units not detectable in the HSQC-DEPT spectrum (see Fig. A.4 in the Supplementary data).
[f] Orthoesterification–acetylation semi-synthetic protocol (Bedini et al., 2012).

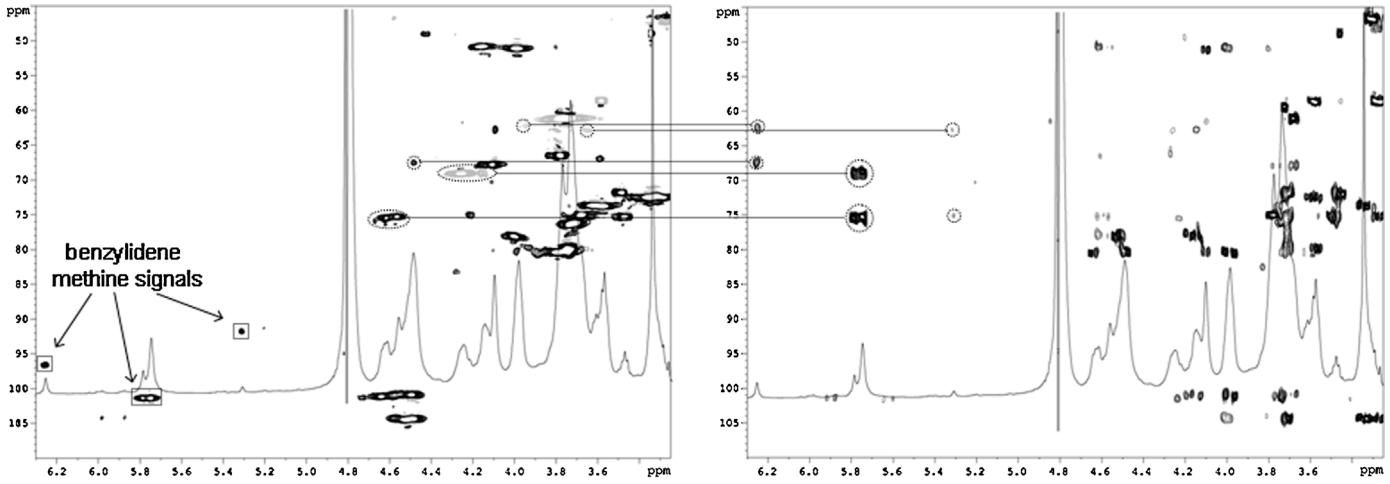


Fig. 3. Zoom of ^{1}H, HSQC-DEPT (left) and HMBC (right) NMR spectra (600 MHz, 4 mM NaOD in D_2O, 298 K) of polysaccharide **9a**.

spectra in DMSO-d_6 were too complicate to address this aim. Conversely, polysaccharide intermediate **9a**/**9b**/**9c**, that was isolated by precipitation from the benzylidene reaction mixture conducted at rt and quenched with triethylamine (Scheme 3), gave clearer 2D-NMR spectra in a 4 mM NaOD solution in D_2O, even if the formation of labile acetals could be underestimated.

The HSQC-DEPT spectrum (Fig. 3, left) revealed the presence of three different benzylidene methine signals (δ_H = 6.26, δ_C = 96.9 ppm; δ_H = 5.78, δ_C = 101.6 ppm; δ_H = 5.32, δ_C = 91.4 ppm). The most intense one (δ_H = 5.78, δ_C = 101.6 ppm) correlated with two signals in the HMBC spectrum (Fig. 3, right), the first one at δ_C = 69.5 ppm, corresponding to a signal in the HSQC-DEPT spectrum in antiphase with the respect to the other densities and thus easily attributable to the methylene group at position *O*-6 of GalNAc units (δ_H = 4.25, δ_C = 69.5 ppm). The second one could be assigned to the CH at position *O*-4 of GalNAc units (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 moiety of GalNAc. The most ^{1}H-downfield shifted benzylidene methine signal (δ_H = 6.26, δ_C = 96.9 ppm) in the HSQC-DEPT spectrum correlated with two densities in the HMBC spectrum at δ_C = 67.3 and δ_C = 62.7 ppm, respectively, the former corresponding to a signal at δ_H = 4.49 ppm in the HSQC-DEPT spectrum attributable to a CH at position *O*-4 of GalNAc units, and the latter to a signal in antiphase with the respect to the other densities and thus again attributable to GalNAc methylene group (δ_H = 3.95, δ_C = 62.0 ppm). These 4,6-GalNAc carbon signals having no shifts with respect to the same signals in underivatized chondroitin disaccharide unit (Mucci, Schenetti, & Volpi, 2000) reminded the NMR behaviour of CH_2 and CH groups involved in acyclic acetals in disaccharide derivatives (Barili, Catelani, D'Andrea, De Rensis, & Falcini, 1997; Lipták, Jánossy, Borbás, & Szejtlic, 2002). Therefore, these signals could be conceivably associated with an interglycosidic 4,6-*O*-benzylidene acetal connecting two GalNAc units of the same polysaccharide chain or two different ones. The third, most ^{1}H-upfield shifted benzylidene methine signal (δ_H = 5.32, δ_C = 91.4 ppm) (Fig. 3, left) correlated with two densities in the HMBC spectrum at δ_C = 62.7 and δ_C = 75.1 ppm, respectively (Fig. 3, right). The former corresponded in the HSQC-DEPT spectrum to a signal (δ_H = 3.65 ppm) in antiphase with respect to the other densities and thus again attributable to a GalNAc methylene group. Even if it was not possible to assign unambiguously the latter cross-peak in the HSQC-DEPT spectrum, due to overlapping with densities related to the major underivatized and six-membered benzylidene protected disaccharide units, its carbon chemical shift value (δ_C = 75.1 ppm) was rather similar to the CH at position 3 of an underivatized GlcA unit (δ_H = 3.60, δ_C = 73.7 ppm). This suggested the formation of an interglycosidic benzylidene acetal between GalNAc-*O*-6 at GlcA-*O*-3 positions (**9a**, Scheme 3), in analogy with the interglycosidic 4,6-*O*-benzylidene associated to the most ^{1}H-downfield benzylidene methine proton (δ_H = 6.26 ppm). The GalNAc-*O*-6-GlcA-*O*-3 connection could involve adjacent monose units along the polysaccharide chain or far away ones in the same chain or in two different ones. It is worth noting that the analysis of these HSQC-DEPT and HMBC spectra allowed to discard the alternative hypotheses for the obtainment of GlcA-3S containing CS polysaccharides (Scheme 3). Indeed, literature data showed rather upfield shifted values (δ_C ~ 50–53 ppm) for the methoxy group of acyclic benzylidene moieties on sugars (Mulard, Kováč, & Glaudemans, 1994; Fascione & Turnbull, 2010). This made the presence of acyclic acetal at position *O*-3 of some GlcA (**9c**, Scheme 3) units inadmissible in our sample. The hypothesis of

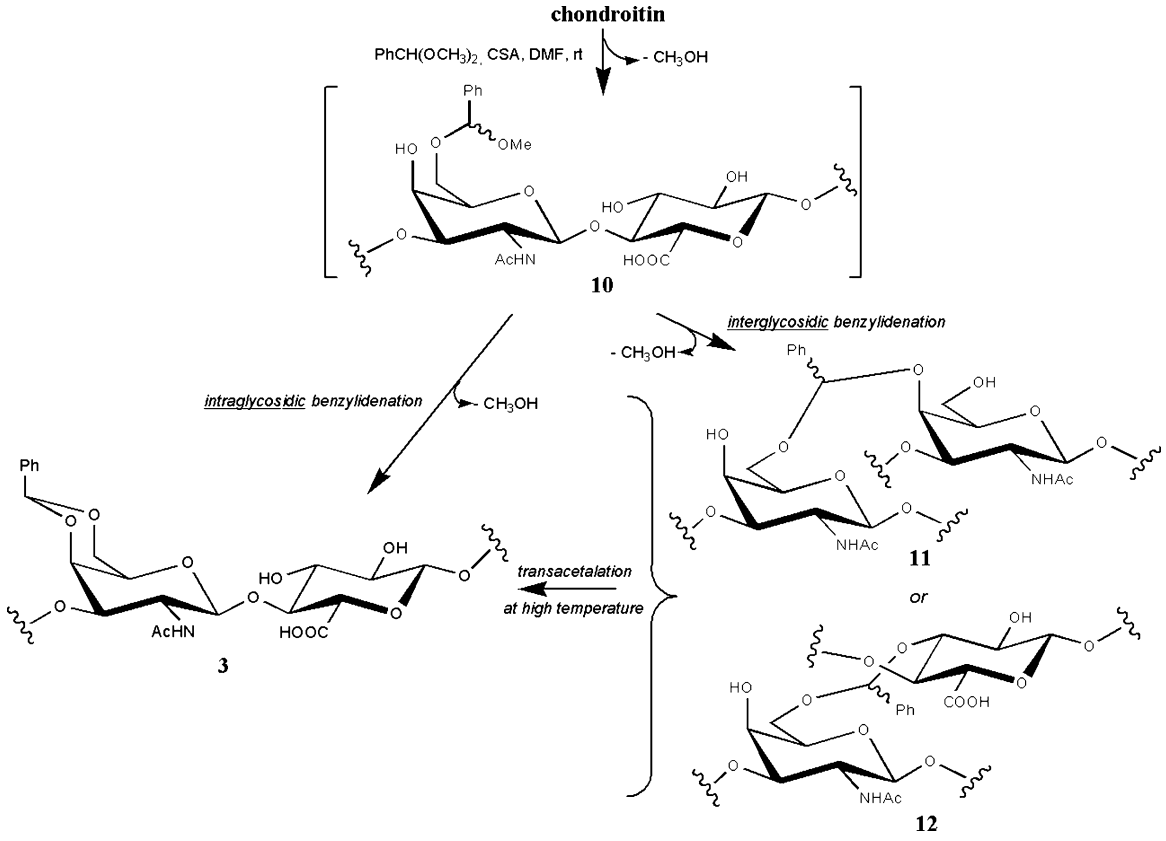
Table 2
Interglycosidic *vs.* intraglycosidic benzylidene hydrolysis on polysaccharide **9a**.

Time[a] [min]	$DS_{intra,DCl}$[b]	$DS_{inter,DCl}$[c]	Time[d] [min]	$DS_{intra,AcOD}$[b]	$DS_{inter,AcOD}$[c]
0	0.55	0.28	0	0.55	0.28
10	0.25	0	60	0.54	0.13
20	0.20	0	120	0.47	0.08
45	0.13	0	300	0.35	0.04
220	0	0	420	0.29	0
			1320	0.23	0

[a] Time after DCl addition to D_2O solution of **9a**.
[b] Degree of substitution of the polysaccharide with intraglycosidic six-membered benzylidene acetal evaluated by integrating the related methine proton signal (δ_H = 5.78 ppm) with respect to the *N*-acetyl one (δ_H = 2.01 ppm) (see Figs. A.6 and A.7 in the Supplementary data).
[c] Degree of substitution of the polysaccharide with interglycosidic benzylidene acetal evaluated by integrating the related methine proton signal (δ_H = 6.26 ppm) with respect to the *N*-acetyl one (δ_H = 2.01 ppm) (see Figs. A.6 and A.7 in the Supplementary data).
[d] Time after AcOD addition to D_2O solution of **9a**.

a *trans*-2,3-benzylidene moiety (**9b**) could be discarded as well, because the ^{13}C chemical shift of the HMBC cross peaks signals at δ_C = 62.0 and 62.7 ppm were compatible with neither *C*-2 nor *C*-3 of GlcA units protected with a *trans*-configured benzylidene (Thiem & Elvers, 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 interglycosidic linkage allowed the sulfation of no positions other than *O*-4 or *O*-6 of GalNAc units at the end of the semi-synthesis, whereas the latter connection could explain the formation of GlcA-3S units in the CSs obtained at the end of the *one-pot* benzylidene-acetylation route.

A higher lability of interglycosidic benzylidene acetals with respect to classical six-membered ones on oligosaccharides has been already reported in literature (Sakairi & Kuzuhara, 1993; Sakairi et al., 1998). A comparison between inter- and intraglycosidic benzylidene stability in **9a** was done by monitoring the progress of acetals cleavage under acid conditions by ^1H-NMR. The study was focused on the more intense interglycosidic benzylidene methine signal, corresponding to an acetal connection between an oxygen atom at position 6 of GalNAc and *O*-4 of another GalNAc unit. It was conducted by comparing the integration ratios of the signal of the interglycosidic and intraglycosidic benzylidene methine proton (δ_H = 6.26 and δ_H = 5.78 ppm, respectively) with respect to the *N*-acetyl signal (δ_H = 2.01 ppm), before and after the addition of an acid to the D_2O solution of polysaccharide **9a**. The higher lability of the interglycosidic benzylidene was ascertained by the NMR degradation study, showing that it was quantitatively cleaved 10 min after DCl addition (final DCl concentration = 0.33% v/v: see Section 2 for details), whereas at the same time approximately 50% of the six-membered benzylidene substitution was removed ($DS_{intra,t=0}$ = 0.55, $DS_{intra,t=10\ min,DCl}$ = 0.25) (Table 2, Fig. A.6: see the Supplementary data). By repeating the degradation experiment with a weaker acid such as AcOD, the difference



Scheme 4. Proposed mechanism for competitive formation of intra- *vs.* interglycosidic benzylidene acetals on chondroitin.

in stability between the interglycosidic and the intraglycosidic benzylidene acetal was even clearer. Indeed, 1 h after AcOD addition (final AcOD concentration = 0.33% v/v: see Section 2 for details) more than 50% of the interglycosidic benzylidene was cleaved ($DS_{inter,t=0}$ = 0.28, $DS_{inter,t=60\ min,AcOD}$ = 0.13), whereas at the same time less than 2% of the six-membered ring was removed ($DS_{intra,t=0}$ = 0.55, $DS_{intra,t=60\ min,AcOD}$ = 0.54). Interglycosidic acetal cleavage was completed 6 h after AcOD addition, whereas more than 40% of the 4,6-benzylidene cycle survived 22 h after AcOD addition ($DS_{intra,t=1320\ min,AcOD}$ = 0.23) (Table 2, Fig. A.7: see the Supplementary data). A similar behaviour could be observed by comparing the six-membered benzylidene acetal and the minor interglycosidic one, nonetheless a quantitative comparison of the degradation rates was not possible in this case, due to the low abundance of the minor interglycosidic benzylidene methine proton signal (δ_H = 5.32 ppm) in **9a**.

A proposed mechanism for the formation of interglycosidic acetals in competition with a classical six-membered intraglycosidic one during the benzylidenation reaction is depicted in Scheme 4. Conceivably, α,α-dimethoxytoluene is firstly attacked by the primary hydroxyl at position 6 of GalNAc units to give an acyclic acetal (**10**), that can be then subjected to a second intramolecular transacetalation by the hydroxyl at position 4 of the same GalNAc unit to close the six-membered intraglycosidic benzylidene ring (**3**), or by *OH*-4 of another GalNAc along the same or another polymeric strand as well as by *OH*-3 of GlcA to give interglycosidic acetal moieties (**11** and **12**, respectively). The higher lability of the latters allows their conversion into the former by another intramolecular transacetalation, when the benzylidenation reaction is conducted under thermodynamic control conditions. Indeed, at high reaction temperatures (50–80 °C) a lower GlcA-3S/GlcA ratio was detected at the end of the semi-synthesis, when compared with the CS product obtained starting the semi-synthesis with a benzylidenation conducted at rt (Table 2). The marked difference in stability between inter- and intraglycosidic benzylidene rings could be exploited in the future for developing more and more precisely tailored strategies towards semi-synthetic CS species. In particular, the possibility to place a labile acetal moiety at position *O*-3 of some GlcA units will be exploited in the future for the development of a semi-synthetic strategy to convert microbial-sourced unsulfated chondroitin into fucosylated CSs. These are CS polysaccharides isolated from sea cucumbers (Echinodermata) possessing a sulfated fucose branch at position *O*-3 of GlcA units as additional structural moiety (Chen et al., 2011). To our knowledge, a semi-synthetic access to fucosylated CSs have been not gained yet, whether it would be very useful because these polysaccharides have been shown to possess very interesting and unique anticoagulant and antithrombotic activities (Pomin, 2014). Work is currently in progress in our laboratory to this aim and the results will be reported in due course.

Finally, it is worth noting that no examination of the *in vitro* or *in vivo* biological activities of the reported semi-synthetic CSs has been made in the current study. Because some chemically sulfated derivatives, such as oversulfated CS, are known to show toxicity to humans (Greinacher et al., 1992) caution is advised before the *in vivo* use of the polysaccharides described herein.

4. Conclusions

In this paper we reported the results of our study aimed to understand in full details the factors influencing the sulfation pattern in the semi-synthesis of CS polysaccharides from microbial sourced unsulfated chondroitin. We proposed the formation of interglycosidic acetals during the protection as benzylidene of the GalNAc-4,6-diol on the starting unsulfated chondroitin. These unusual acetals involve positions *O*-6 of some GalNAc units and *O*-4 of another GalNAc or *O*-3 of GlcA along the same polymeric chain or across two strands. These interglycosidic acetals were rather acid-labile and could not be conserved after reaction work-up. Thus, the following steps of the semi-synthetic strategy (acetylation, oxidative cleavage of the 4,6-*O*-benzylidene ring, sulfation and global deprotection) afforded semi-synthetic CS-A,C possessing sulfate groups exclusively on GalNAc units. Conversely, the stabilization in an alkaline environment of the labile interglycosidic acetals during benzylidenation work-up 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 units, as already observed for a semi-synthetic strategy employing an orthoester-based protection. 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. Nonetheless, the attempts to stabilize and characterize this labile interglycosidic orthoester moiety had no success (data not shown).

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. In particular, CS-A,C is a biomedical ingredient for several applications (Yamada & Sugahara, 2008), and GlcA-3S-containing CSs – usually isolated from squid and king crab cartilages as well as from squid liver integuments (Sugahara et al., 1996; Kinoshita et al., 1997; Kumar Shetty et al., 2009) – are also interesting biomacromolecules because they promote neurite outgrowth in the central nervous system (Mikami & Sugahara, 2006). Furthermore, the control of the factors governing the inter- *vs.* intraglycosidic selectivity of benzylidenation in chondroitin will be also useful for the fine chemical manipulation of other biologically relevant polysaccharides.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.05.085.

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