Bioorganic Chemistry

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A Microbiological–Chemical Strategy to Produce Chondroitin Sulfate A,C**

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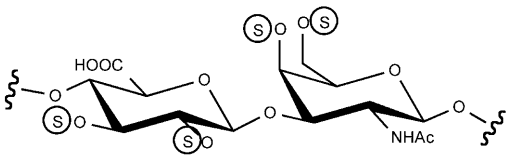
Chondroitin sulfate (CS) is a glycosaminoglycan found in both vertebrates and invertebrates. It is ubiquitously distributed in connective tissue extracellular matrices and on cell surfaces as CS-proteoglycan, in which the CS linear polysaccharide chain is covalently attached to a core protein. CS polysaccharide consists of a 4)-β-GlcA-(1→3)-β-GalNAc-(1→ disaccharide repeating unit, with a variable sulfation pattern (GlcA = glucuronic acid, GalNAc = *N*-acetylgalactosamine). The position 4 or 6 (or both) of the GalNAc units is commonly sulfated, while position 2 or 3 of GlcA units is sulfated to a minor extent (Scheme 1). The sulfation pattern is tightly regulated in vivo; it is tissue- and age-specific and is considered able to encode functional information.[1]

Osteoarthritis is the most common musculoskeletal disease, affecting about 10% of the world's population aged 60 and older.[2] Conventional osteoarthritis treatment consists of nonsteroidal anti-inflammatory drugs and analgesics, but many of these drugs can cause serious side effects. Therefore, CS polysaccharide predominantly composed of A and C disaccharide subunits (CS-A,C) has recently substituted such drugs for the therapy of tibiofibular osteoarthritis of the knee and in articular cartilage osteoarthritis.[3] For these pharmacological applications, CS-A,C is obtained by extraction from bovine, porcine, and shark cartilages. Nonetheless, the low abundance of the raw material and the laborious downstream purification limit the availability of CS-A,C, in spite of the growing interest in expanding the application of CS to pharmacological fields other than osteoarthritis treatment. Furthermore, the ever-stricter regulations for animal-derived drugs has led to a renewed search for synthetic replacements. In the case of CS-A,C this demand is even more pressing, owing to the high variability of sulfation patterns in animal tissues. It is worth noting that per-*O*-sulfated CS, which has been fraudulently added as a contaminant in some heparin lots, induced a strong allergic-type response, causing severe symptoms and, in some cases, patient death.[4]



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

Scheme 1. Typical disaccharide subunits found in natural CSs.

In spite of the urgent demand for synthetic CS, several reports appeared in the literature in the last decade about the synthesis of small CS oligosaccharides[5] and glycomimetic polymers,[6] whereas a synthetic access to CS-A,C polysaccharide is still lacking. We describe herein how this goal is reached through an innovative microbiological–chemical approach setting a preparative synthesis for actual industrial scale-up.

Some bacteria that produce glycosaminoglycan-like capsular polysaccharides have been isolated.[7] Among them, *Escherichia coli* O5:K4:H4 biosynthesizes a capsular polysaccharide having a chondroitin backbone with the additional presence of a β-fructose residue linked at the *O*-3 position of the GlcA units.[8] Thus, the fermentation broth of *E. coli* O5K4:H4 under a fed-batch process, with subsequent microfiltration, protease treatment, diafiltration of the harvested broth, and mild hydrolysis,[9] afforded the de-fructosylated polysaccharide free of any toxic lipopolysaccharide contaminant. At a gram scale, the described protocol furnished chondroitin with 89–94% purity grade, as evaluated by NMR spectroscopy and capillary electrophoresis.[9,10] Its weight-averaged molecular weight and polydispersity were 45.0 kDa and 1.40, respectively, as evaluated by high-performance size-exclusion chromatography combined with a triple detector array (HP-SEC-TDA).[11]

The key feature for the chemical conversion of chondroitin into CS-A,C is to insert a single sulfate group on GalNAc units either at position 4 or 6, avoiding the double sulfation (E subunit) as well as sulfation on GlcA units (D, K, L and M

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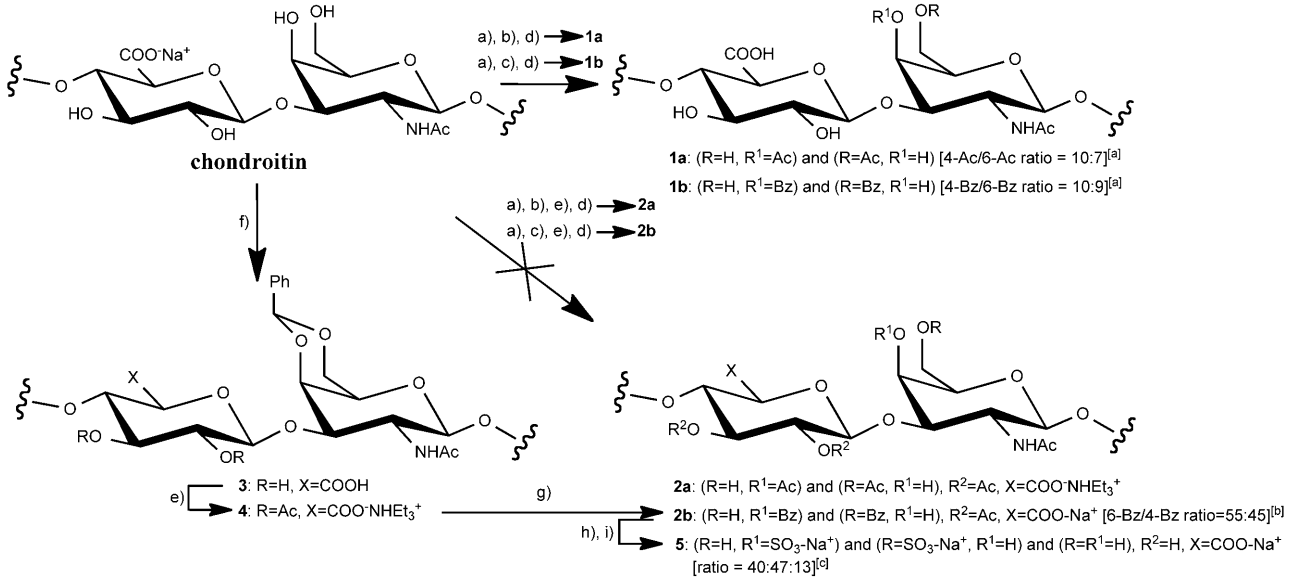
subunits). A recently reported strategy that consists of producing CS-A and CS-C polysaccharides separately and then mixing them[12] is unsatisfactory, because it does not allow both A and C disaccharide subunits to be incorporated into the same polysaccharide chain, as required for the desired product. To guarantee the presence of sulfate groups either at the 4 or 6 positions of GalNAc units, we pursued a strategy entailing the selective protection of the 4,6-GalNAc diol with a cyclic group, followed by acylation of 2,3-GlcA diol, and then cycle opening under reaction conditions leading to random deprotection of a single hydroxy group per disaccharide subunit, which could be then sulfated.

First, an orthoester cycle (orthoacetate or orthobenzoate) was installed on 4,6-GalNAc diol[5d] by treatment of chondroitin with trimethyl orthoacetate (or orthobenzoate). A successive one-pot treatment with aqueous acetic acid gave a polysaccharide with **1a** or **1b** motifs, in which either 4-*O*- or 6-*O*-acylated GalNAc units ($\delta_H = 5.28$ and 4.18 ppm in **1a**, respectively; $\delta_H = 5.54$ and 4.44 ppm in **1b**, respectively: see Table S1 in Supporting Information) were present, thus attesting the non-regioselective cleavage of the orthoester cycle (Scheme 2). Nonetheless, the insertion of a per-*O*-acetylation step prior to orthoester opening failed to give **2a**/**b**: underacetylation at *O*-3 of GlcA units was always detected, even under forced acylation conditions. A 4,6-benzylidene ring was chosen as an alternative GalNAc protection, because its cleavage under several oxidative conditions is reported to proceed with no or very low regioselectivity.[13] Thus, chondroitin was treated with α,α-dimethoxytoluene to give **3**. Exhaustive benzylidenation of all GalNAc 4,6-diols of the polysaccharide was demonstrated by the 1:3 ratio obtained for benzylidene methine proton ($\delta_H = 1.76$ ppm) and acetyl ($\delta_H = 5.53$ ppm) signals in the ^{1}H NMR spectrum (Table S1 and Figure S4 in Supporting Information). Polysaccharide **3** was then uneventfully per-*O*-acetylated to **4**, as indicated by downfield shifts of the GlcA H-2 and H-3 signals in the ^{1}H NMR spectrum at $\delta_H = 4.65$ and 4.86 ppm, respectively (Table S1 in Supporting Information). Under oxidative conditions employing $NaBrO_3/Na_2S_2O_4$ in an ethyl acetate/water mixture,[13b] **2b** was obtained, for which 2D NMR spectroscopy demonstrated the presence of either 4-*O*- ($\delta_H = 5.40$ ppm) or 6-*O*-benzoylated ($\delta_H = 4.19$, 4.36 ppm) GalNAc units. A residual amount of oxidation-resistant benzylidene cycle could be detected by the presence of a benzylidene methine signal ($\delta_H = 5.48$ ppm, $\delta_C = 99.6$ ppm) in the HSQC-DEPT spectrum (Table S1 and Figure S3 in Supporting Information). Sulfation of **2b** with SO_3·py in DMF and subsequent alkaline deacylation furnished chondroitin sulfate **5** (CS-**5**) in 61% global yield[14] from chondroitin on a multigram scale. *E. coli* O5:K4:H4 O-antigen presence was detectable by neither ^{1}H NMR spectroscopy (Figure 1) nor chemical composition analysis. CS-**5** was subjected to a detailed 2D NMR spectroscopic characterization. In particular, the HSQC-DEPT spectrum (Figure 1) revealed the presence of two signals in antiphase with respect to the other densities, attributable to sulfated ($\delta_H = 4.11$ ppm, $\delta_C = 67.3$ ppm) and non-sulfated ($\delta_H = 3.68$ ppm, $\delta_C = 60.9$ ppm) carbinolic proton at position 6 of GalNAc units, respectively. COSY and TOCSY allowed the identification of GalNAc and GlcA proton sequence from H-1 to H-4 and H-5 respectively, revealing the concurrent presence of sulfated ($\delta_H = 4.64$ ppm, $\delta_C = 76.3$ ppm) and non-sulfated ($\delta_H =$



Scheme 2. Transformation of chondroitin into CS-A,C: a) Dowex 50 WX8 (H^+ form), room temperature, 5 min; b) trimethyl orthoacetate, CSA, DMF, room temperature, 5 h; c) trimethyl orthobenzoate, CSA, DMF, room temperature, 5 h; d) 80% AcOH, room temperature, 1.5 h; e) Ac_2O, Et_3N, DMAP, CH_3CN, room temperature, 24 h; f) α,α-dimethoxytoluene, CSA, DMF, 80°C, 22 h; g) $NaBrO_3$, $Na_2S_2O_4$, 7:3 v/v H_2O-ethyl acetate, room temperature, 24 h; h) SO_3·py, DMF, 50°C, 22 h; i) NaOH, H_2O, room temperature, 6 h. [a] Determined by integration of signals in ^{1}H NMR spectra. [b] Determined by integration of signals in HSQC-DEPT NMR spectra. [c] Determined by enzymatic digestion and subsequent HPLC analysis (see below). CSA = camphorsulfonic acid, DMAP = 4-(dimethylamino)pyridine, DMF = *N*,*N*-dimethylformamide, py = pyridine.

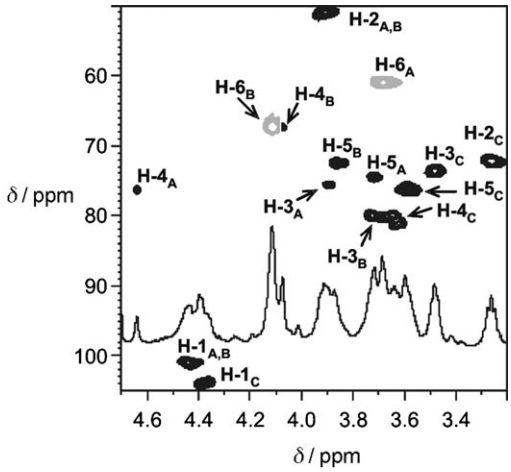


Figure 1. ^{1}H and HSQC-DEPT NMR spectra (zoom) of CS-**5** (A = GalNAc4S, B = GalNAc6S, C = GlcA).

4.07 ppm, $\delta_C = 67.2$ ppm) carbinolic proton at position 4 of GalNAc units. The very close resemblance between the ^{1}H NMR spectra of CS-**5** and two lots of pharmacological CS (Figure 2)[15] corroborated the CS-A,C structure for CS-**5**, which was further confirmed by enzymatic assay.[16] Indeed, HPLC chromatograms of the *Proteus vulgaris* chondroitinase

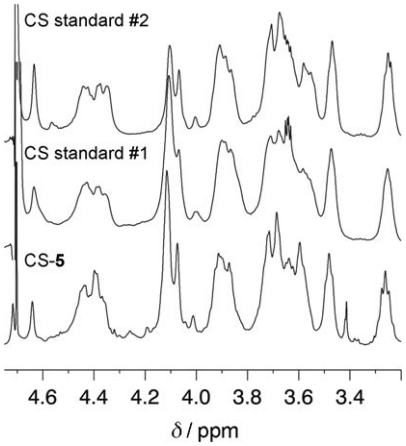
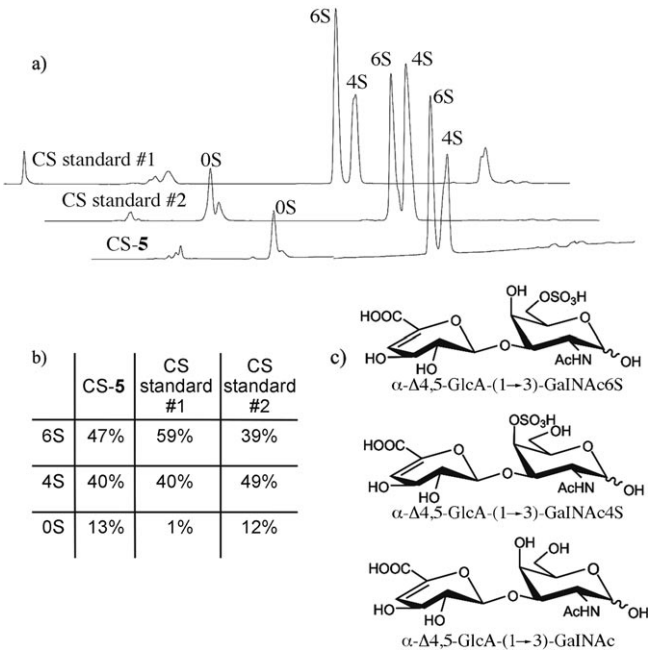


Figure 2. Comparison of ^{1}H NMR spectra (zoom) of CS-**5** and two lots of pharmacological CS.

ABC digest of CS-**5** and of the two CS standard lots showed a close resemblance between them, displaying three peaks corresponding to α-Δ4,5-GlcA-(1→3)-GalNAc4S, α-Δ4,5-GlcA-(1→3)-GalNAc6S, and α-Δ4,5-GlcA-(1→3)-GalNAc disaccharides (Figure 3). It is worth noting that the presence of a minor amount of unsulfated disaccharide subunits in CS-**5** is due to GalNAc units in **2b** maintaining a residual, oxidation-resistant benzylidene cycle that was hydrolytically cleaved to 4,6-diol during sulfation workup.

The molecular size of CS-**5** was evaluated by HP-SEC-TDA. The weight-averaged molecular weight and polydispersity were 17.3 kDa and 1.56, respectively. The acid conditions employed in some synthetic steps (carboxylate



	CS-**5**	CS standard #1	CS standard #2
6S	47%	59%	39%
4S	40%	40%	49%
0S	13%	1%	12%

Figure 3. HPLC of ABC lyase digests of CS-**5** and two lots of pharmacological CS: a) chromatograms (peak identification was conducted by co-injection of pure standards), b) peak integration, and c) products of enzymatic digestion.

neutralization with Dowex 50 WX8 H^+, orthoesterification and sulfation workup; see the Supporting Information for experimental details) conceivably caused the decrease in the weight-averaged molecular weight from starting chondroitin (45.0 kDa) to CS-**5**. It is worth noting that the CS-**5** weight-averaged molecular weight is very close to that of pharmacological CS (16.8 kDa), which has been shown to be more bioavailable and thus more efficacious in osteoarthritis treatment than naturally occurring high-molecular-weight CS.[17] The toxicity in vivo of CS-**5** was preliminarily tested on murine models. Alterations were not found, even at the highest dose (32.8 mg kg^{-1} day^{-1} for 4 weeks).

In conclusion, for the first time the transformation of chondroitin from a microbial source into CS possessing A and C disaccharide subunits in the same polysaccharide chain has been reported. The high global yield, the low price of the used reagents, and, above all, the very close resemblance between synthesized and pharmacological CS-A,C strongly propose this product as a valuable candidate for the replacement of CS animal-sourced drugs, thus avoiding concern about interspecies viral contamination.

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