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# A Chondroitin Sulfate Small Molecule that Stimulates Neuronal Growth

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Chondroitin sulfate glycosaminoglycans are sulfated polysaccharides implicated in cell division, neuronal development, and spinal cord injury.[1] While considerable attention has been focused on heparan sulfate glycosaminoglycans, much less is known about the chondroitin sulfate (CS) class. As with all glycosaminoglycans, the complexity and heterogeneity of CS have hampered efforts to understand its precise biological roles. For instance, CS has been shown to prevent the growth of axons; yet it is also found in developing, growth-permissive regions.[1c,2] Synthetic access to CS molecules of defined length and sulfation pattern, in combination with biological studies, should enable a systematic examination of structure−activity relationships. Toward this end, we report the synthesis and identification of a CS tetrasaccharide that promotes neuronal outgrowth. These studies represent the first biological investigations of synthetic CS molecules and define a tetrasaccharide as a minimal motif required for activity.

CS polysaccharides have been shown both to stimulate and to attenuate the growth of cultured neurons.[3,4] Notably, the molecules used in those studies were ∼200 saccharides in length, poorly defined, and heterogeneously sulfated, features that might account for the contradictory observations. Among the sulfation patterns implicated in the modulation of cell growth is the disulfated CS-E motif (Figure 1). To investigate the biological properties of CS-E and establish the minimal structural determinants for activity, we sought to synthesize di- and tetrasaccharides bearing this sulfation pattern.

We envisioned a convergent strategy to access various CS-E molecules from a single disaccharide building block, **4**. The sulfated 4- and 6-hydroxyls of D-galactosamine were masked with a *p*-methoxybenzylidene acetal. This group was chosen with future access to other sulfation motifs (e.g., CS-A, CS-C)[5] and extension to solid-phase methodologies in mind. In particular, oxidative removal using DDQ[6] or regioselective opening of the acetal ring[7] was anticipated to permit selective deprotection of either or both the 4- and 6-hydroxyls and circumvent the need for hydrogenolysis. The orthogonal *tert*-butyldimethylsilyl group was installed at the C-4 position on the nonreducing end of **4** to facilitate chain elongation. To achieve stereoselective formation of $\beta$-glycosidic linkages, we exploited well-precedented *N*-trichloroacetyl and benzoyl participating groups.[8] Finally, the anomeric hydroxyl of **4** was masked with an allyl group, which could be converted to activated glycosyl donors and offers a convenient means to conjugate CS to small molecules, proteins, or surfaces.

The synthesis of the target compounds is illustrated in Scheme 1. Monosaccharides **5** and **6** were generated from known *p*-tolyl-1-thio-$\beta$-D-glucopyranose[9] or 1,3,4,6-tetra-*O*-acetyl-2-azido-2-deoxy-D-galactopyranose,[10] respectively (Supporting Information). Coupling of **5** with **6** using the trichloroacetimidate procedure[11] afforded exclusively the $\beta$-linked disaccharide **4** in 74% yield. At this stage, activation of the disaccharide was envisioned to proceed through conversion of the C-1 allyl group to the lactol. However,

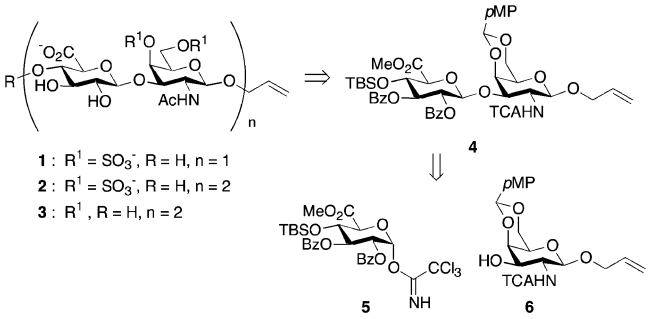


***Figure 1.*** Chondroitin sulfate molecules **1**−**3** and retrosynthetic analysis.

conventional methods such as $PdCl_2$, $Pd(PPh_3)_4$, $[Ir(COD)(PMePh_2)_2]PF_6$, and Wilkinson's catalyst did not yield the desired isomerization, presumably due to interference by the neighboring trichloroacetamide.[12] Fortunately, treatment with Grubbs' second-generation catalyst[13] in the presence of $H_2$ led to the desired outcome. Hydrolysis of the enol ether and conversion to trichloroacetimidate **7** proceeded smoothly under standard conditions.[14] Disaccharide **4** also provided ready access to glycosyl acceptor **8** via desilylation. Subsequent glycosylation of **7** and **8** delivered the desired tetrasaccharide with good stereoselectivity.

With the fully protected di- and tetrasaccharides in hand, we embarked on the final deprotection−sulfation steps. Radical-mediated conversion of the *N*-trichloroacetyl group to *N*-acetyl[8] followed by oxidative cleavage of the *p*-methoxybenzylidene acetal[6] afforded **9** and **10**. Despite an earlier report suggesting that simultaneous sulfation of the C-4 and C-6 hydroxyls might be challenging,[7a] treatment of **9** and **10** with $SO_3$·trimethylamine complex in DMF delivered the sulfated compounds in 67% and 93% yields, respectively. The target CS-E di- and tetrasaccharides **1** and **2** were successfully obtained after silyl deprotection and saponification utilizing NaOH or sequential LiOOH−NaOH treatment[15] to minimize $\beta$-elimination at the C-4 position. Deprotection of **9** under similar conditions furnished the unsulfated tetrasaccharide **3**.

To explore the ability of **1**−**3** to modulate neuronal growth, primary hippocampal neurons were cultured on poly-DL-ornithine-coated coverslips with or without each compound. After 48 h, the neurons were fixed, immunostained with anti-tau antibodies, and examined by confocal fluorescence microscopy. Sulfated tetrasaccharide **2** exhibited striking effects on neuronal morphology and growth (Figure 2). The number of neurites emanating from the cell body was enhanced, and the growth of the major extension was dramatically stimulated by 39.3 ± 3.6% relative to the poly-DL-ornithine control. In contrast, sulfated disaccharide **1** and unsulfated tetrasaccharide **3** had no significant effect on neuronal outgrowth. These results indicate that a tetrasaccharide represents a minimum structural motif and that sulfation is critical for CS activity.

The observed effects of **2** support previous studies implicating the CS-E motif in the growth and development of neurons. For example, CS-E is found on the protein appican, an isoform of the


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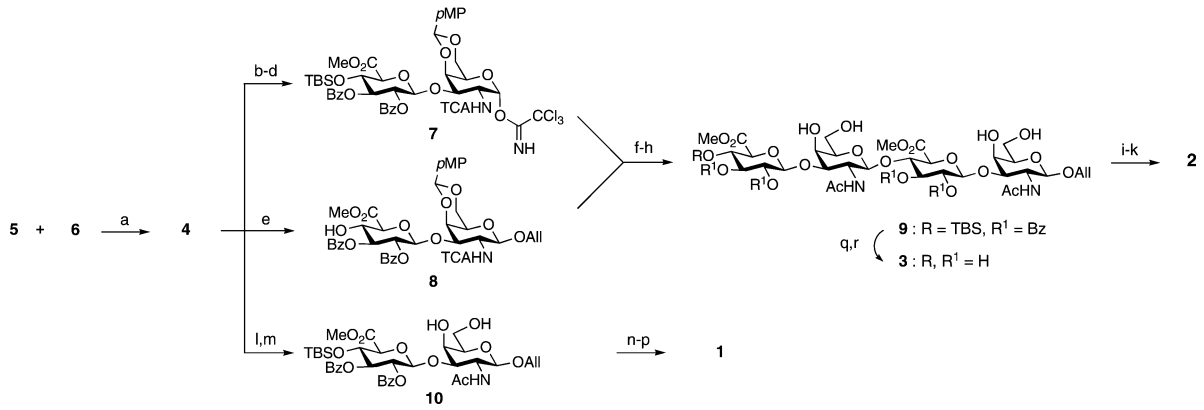
***Scheme 1.*** Synthesis of **1**−**3**[a]




[a] Conditions: (a) TMSOTf, $CH_2Cl_2$, −40 → −15 °C, 74%. (b) $RuCl_2$(=CHPh)($PCy_3$)L, L = 1,3-dimesityl-4,5-dihydroimidazolylidene (20 mol %), $CH_2Cl_2$, $H_2$, 77%. (c) $I_2$, $H_2O$, pyr/THF, 81%. (d) DBU, $CCl_3CN$, $CH_2Cl_2$, 90%. (e) HF·pyr, pyr/THF, 0 °C, 85%. (f) TMSOTf, $CH_2Cl_2$, −15 °C, 31%. (g) TBTH, AIBN, DMA/benzene, 25 → 80 °C, 85%. (h) DDQ, $H_2O/CH_2Cl_2$, 75%. (i) $SO_3$·$Me_3N$, DMF, 50 °C, 67%. (j) HF·pyr, pyr/THF/$H_2O$, 0 °C. (k) LiOH, $H_2O_2$, THF/$H_2O$, then NaOH, MeOH/$H_2O$, 25% over three steps. (l) TBTH, AIBN, benzene, 25 → 80 °C, 85%. (m) DDQ, $H_2O/CH_2Cl_2$, 62%. (n) $SO_3$·$Me_3N$, DMF, 50 °C, 93%. (o) HF·pyr, pyr/THF/$H_2O$, 0 °C. (p) NaOH, MeOH/$H_2O$, 55% over two steps. (q) HF·pyr, pyr/THF/$H_2O$, 0 °C. (r) LiOH, $H_2O_2$, THF/$H_2O$, then NaOH, MeOH/$H_2O$, 52% over three steps.

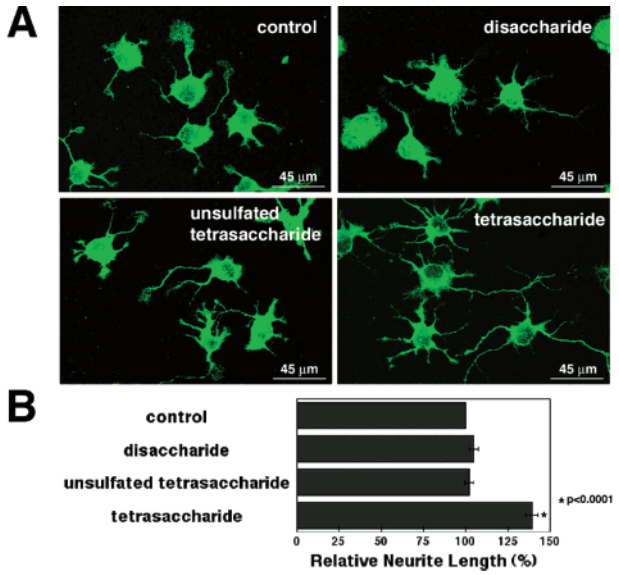



***Figure 2.*** CS-E tetrasaccharide **2** stimulates the outgrowth of hippocampal neurons. (A) Immunofluorescence images of neurons 48 h after treatment with the indicated compound. (B) Statistical analysis of neurite length. See Supporting Information for conditions.

amyloid precursor protein that exhibits neurotrophic activity.[16] Moreover, polysaccharides enriched in the CS-E motif have been shown to promote the outgrowth of neurons.[4] Our studies provide the first, direct investigations into the structure−activity relationships of CS using homogeneous, synthetic molecules. The findings indicate that the CS-E sulfation motif is likely an important structural determinant for CS activity in vivo, endowing CS polysaccharides with the ability to induce neuronal growth.

In summary, we have identified a CS tetrasaccharide that stimulates the growth and differentiation of neurons. The ability of CS small molecules to recapitulate the activity of larger polysaccharides should provide new chemical approaches to understand and manipulate neuronal growth and regeneration. Future investigations will explore the role of other sulfation patterns and the targets of CS-E in vivo.

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**Note Added after ASAP Posting.** After this paper was posted ASAP on 06/02/2004, a correction was made to structure **10** in Scheme 1. The corrected version was posted 06/04/2004.

**Supporting Information Available:** Syntheses and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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