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Toward Libraries of Biotinylated Chondroitin Sulfate Analogues: From Synthesis to In Vivo Studies

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Abstract: Chondroitin sulfate-E (CS-E) oligosaccharidic analogues (di to hexa) were prepared from lactose. In these compounds, the 2-acetamido group was replaced by a hydroxyl group. This modification speeded up the synthesis, and large oligosaccharides were constructed in a few steps from a lactose-originated block. The protecting groups used were as follows; Fmoc for hydroxyl groups to be glycosylated, allyl group for anomeric position protection, and trichoroacetimidate leaving groups were used to prepare up to octasaccharides. We took advantage of the presence of allyl group to develop a click biotinylation, through its transformation into a 3-azido-2-hydroxyl propyl group in two steps (epoxidation and sodium azide epoxide opening). The biotinylating agent was a water-soluble propargylated and biotinylated triethylene glycol (PEG). By using surface plasmon resonance (SPR), it was shown that the di-, tetra-, and hexasaccharides display a binding affinity and selectivity toward HSF/GSF and CXCL12 similar to that of CS-E. A parallel study confirmed their mimicry of natural compounds, based on the hexasaccharide interaction with Otx2, a homeodomain protein involved in brain maturation, thus validating our simplification approach to synthesize bioactive GAG.

Keywords: allyl groups · biotinylation · click chemistry · lactose · protecting groups · structure–activity relationships

Introduction

Chondroitin sulfates (CS) are important polysaccharides belonging to the family of glycosaminoglycans (GAGs). These linear and anionic polymeric chains are constituted by a repetition of O-sulfated disaccharidic units. The disaccharidic block contains a D-galactosamine 1→4-linked to a D-glucuronic acid and the disaccharides are 1→3-linked to form chains of various lengths (10 to 200 repeating units). The sulfate groups carried by the disaccharidic portions can be located on different positions and each sulfation pattern defines a subclass of CS.

The CS chains are linked to carrier proteins through a tetrasaccharidic moiety covalently bound to an L-serine residue to form structures called proteoglycans (PGs). Mainly found in the extracellular matrix of a wide variety of tissues but also on cell surfaces, the PGs present two degrees of structural heterogeneity. On one hand, each carrier protein could link several kinds of GAGs, additionally to CS. On the other hand, CS chains are non-regular polymers with variation on the sulfation pattern.

CS proteoglycans participate in important biological processes such as regulation of cells behaviors[1–3] (growth, proliferation, and migration), development and repair of the central nervous system (CNS),[4–10] or cellular recognition.[11–13] These critical biological activities seem to be governed by the finely tuned repartition of the negative charges borne by the sulfate and carboxylate groups.[14] The sulfation patterns expressed all along the CS chains would encode information decipherable by positively charged domains of protein receptors. However, very little is known about the role of the sulfation code and the structure–activity relationship was not well studied, due the the lack of pure and chemically defined large CS fragments.

The purification of homogeneous depolymerized products is not realistic beyond tetrasaccharides. The synthesis of large fragments is very complicated, because it implies galactosamine, and these oligosaccharides have been prepared

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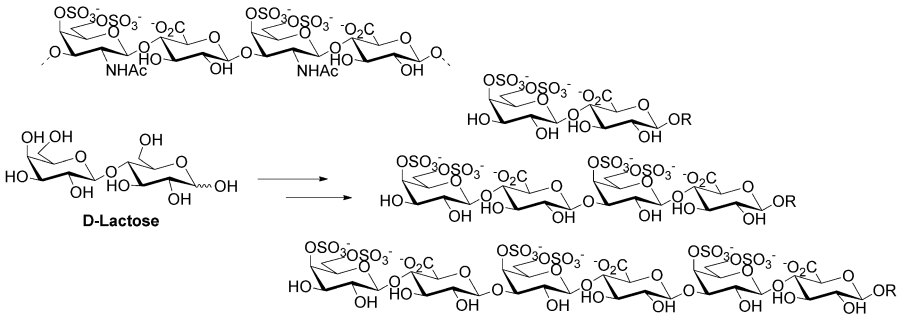


Figure 1. CS-E (top) and analogues (bottom).

after long sequences of reactions.[9,15–22] We propose here to prepare very close analogues of CS-E in a rapid manner (Figure 1) and to explore structure–activity relationships. As with the heparin series,[23] we only modified an uncharged residue: the 2-NHAc group was replaced by a 2-OH; the sulfation and charge patterns being completely preserved. Similar group exchanges were already successfully described for gangliosides and related compounds.[24] This slight modification has halved the chemical steps needed for CS-E synthesis.

To allow easy biological evaluation (i.e., protein binding), CS-E analogues were prepared in their biotinylated forms (di **3**, tetra **4**, and hexa **5**) and in their unconjugated forms (tetra **1** and hexa **2**) (Figure 1). Their synthesis can be easily and rapidly achieved from the abundant and cheap natural disaccharide lactose.

Result and Discussion

These chondroitin sulfate analogues were prepared by "oligomerization" of a key disaccharide lactose block (Figure 2). The synthetic plan is depicted on Scheme 1.

Benzylidene groups were used to protect positions 4′,6′ to be sulfated and benzoate was used for "permanent" and participating alcohol function protecting groups. A selectively cleavable group is needed on the 3′ position for chain elongation and an allyl group is used to block position 1 and also as a linker precursor in the biotinylation reaction.

Synthesis of disaccharidic blocks: The synthesis started from peracetylated allyl β-lactose **6**[25] accessible in a large scale (50 g) from D-lactose (Scheme 2). Compound **6** was deacetylated in Zemplen conditions, followed by the selective preparation of 4′,6′-O-benzylidene using benzaldehyde and zinc chloride. The remaining 6-primary position was then silylated to give **7** (65% yield from **6**).

The first critical step was the selective protection of the 3′ position of compound **7**. It could be achieved (72% yield)

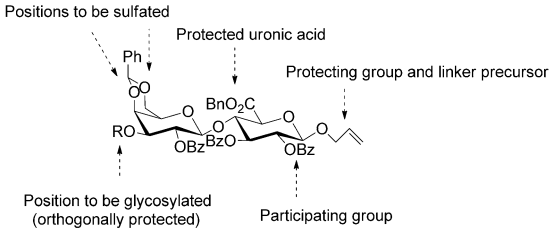
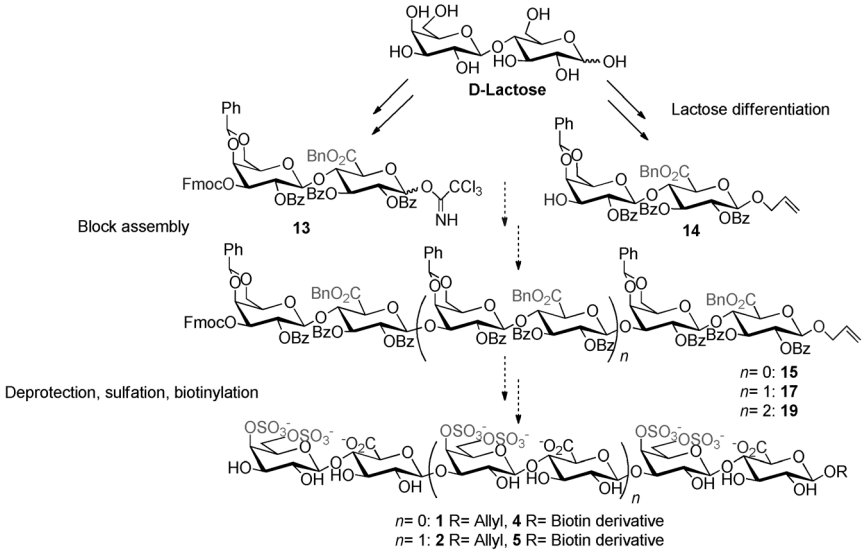


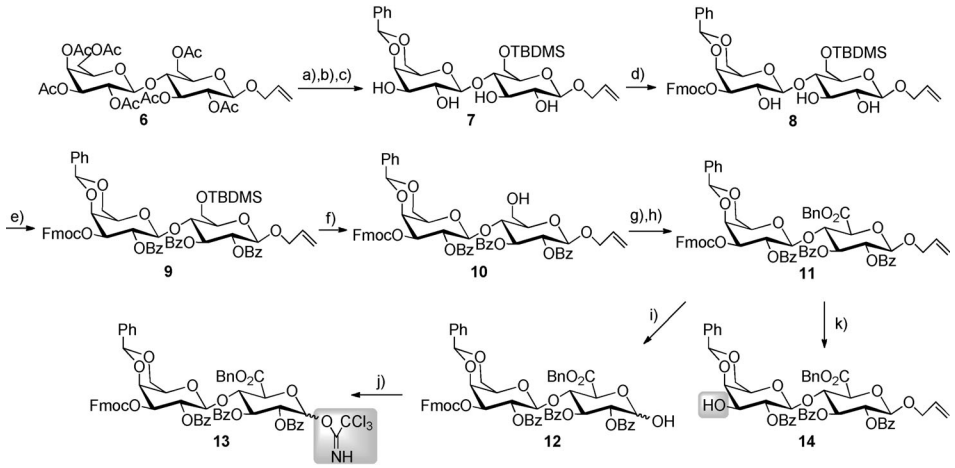
Figure 2. Common building block.

at room temperature by using FmocCl and sym-collidine to generate a hindered acylating agent in situ.[26] Benzoylation of **8** in mild conditions[27] (CH_2Cl_2/pyridine (3:1), low temperature) gave **9** (81% yield). The use of pure pyridine as solvent was found to be detrimental because of in situ partial removal of the Fmoc group and formation of 3′-*O*-benzoate.

The second critical step was the selective deprotection of the silyl group in the presence of *O*Fmoc; this was achieved using TMSOTf at low temperature[28] to produce compound **10** (84%). This probably occurred through the formation of



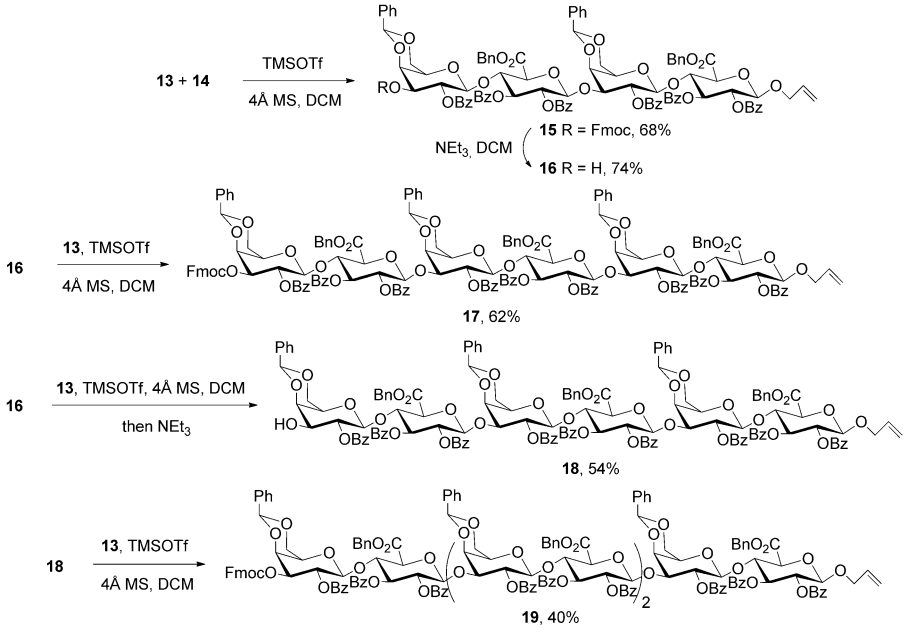
Scheme 1. Synthetic plan.



Scheme 2. Reagents and conditions: a) NaOMe, MeOH; b) PhCHO, $ZnCl_2$; c) TBDMSCl, pyridine, 65% (3 steps); d) FmocCl, sym-collidine, CH_2Cl_2, 72%; e) BzCl, CH_2Cl_2/pyridine, 0°C to RT, 81%; f) TMSOTf, CH_2Cl_2, −78°C, 84%; g) TEMPO, BAIB, CH_2Cl_2/water; h) BnBr, $NaHCO_3$, DMF, 82% in 2 steps; i) Pd(PPh_3)$_4$, $ZnCl_2$, Bu_3SnH, THF, RT, 89%; j) CCl_3CN, NaH, CH_2Cl_2, 0°C to RT, 74%; k) Et_3N, dichloromethane (DCM), RT, 90%.

a labile trimethylsilyl ether in position 6, which was readily hydrolyzed in the work up.[26] Oxidation using 2,2,6,6-tetramethylpiperidin *N*-oxide (TEMPO)/bis(acetoxy)iodobenzene (BAIB) followed by an esterification reaction of the crude carboxylic acid gave **11** (82% yield).

To obtain the donor block, compound **11** was first deallylated by using Bu_3SnH and Pd(PPh_3)$_4$ and $ZnCl_2$ as catalysts.[29] The resulting hemiacetal **12** (89% yield) was converted into the anomeric trichloroacetimidate **13** by treatment with trichloroacetonitrile and sodium hydride. Finally, the acceptor block **14** was obtained after the cleavage of the Fmoc group on compound **11**.



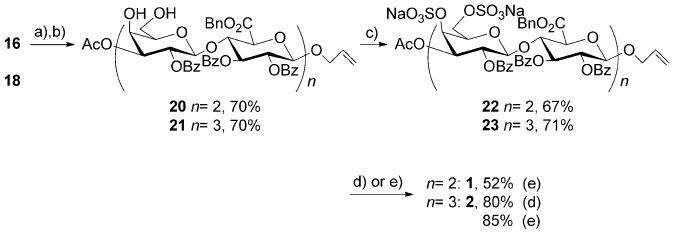
Scheme 3. Construction of oligosaccharides backbone through iterative glycosylation/deprotection sequences.

Oligosaccharides synthesis: With these building blocks in hand, iterative glycosylation reactions were undertaken by using TMSOTf as a promoter, in the presence of molecular sieves in dichloromethane at room temperature (Scheme 3). Rather high quantities of promoter (0.3 equiv) and "high" temperatures (20°C) were used to avoid the formation of the orthoester. After removing the Fmoc group, the oligomerisation process was continued up to the octasaccharide **19** (Scheme 3).

β Selectivity was ascertained in the NMR spectra, in particular ^{13}C NMR C1 signals between $\delta = 100$–102 ppm.[30] Lower chemical shifts were expected in the α configuration $\delta = 95$–97 ppm.[31] Yields were within the range of 60–70%, and comparable to published yields for similar reactions using disarmed uronate donors.

Final steps: sulfation and saponification: Unconjugated (allyl) CS analogues were prepared from tetra- and hexasaccharide (compounds **16** and **18**, respectively) by using the following sequence: a) benzylidene removal, b) sulfation of free OH, and c) saponification of ester groups. Although Fmoc is stable in benzylidene removal conditions, it is cleaved during the sulfation reaction. Consequently, to avoid the presence of an extra sulfate, it was replaced by an acetyl group. Benzylidene acetals were removed by using aqueous TFA, and the free hydroxyls were sulfated by using SO_3·Me_3N in DMF (Scheme 4). The large shifts in ^{1}H and ^{13}C signals in the NMR spectrum confirmed the positions of the sulfates (Table 1).

The last step is the saponification of ester groups by two different procedures. First, a two-step deprotection was used, involving a saponification under mild conditions with $LiOH/H_2O_2$ followed by treatment with aqueous NaOH[18]



Scheme 4. Final steps for non-biotinylated oligosaccharides. Reagents and conditions: a) Ac_2O, pyridine; b) TFA, water, THF/CH_2Cl_2, sonication; c) SO_3·NEt_3, DMF, 60 °C; d) LiOH, H_2O_2, THF/water, 0 °C to RT then NaOH (4 M), MeOH, 0 °C to RT; e) 4 M NaOH, MeOH, 0 °C to RT.

Table 1. Chemical shifts ^1H and ^{13}C of galactose protons before/after sulfation.

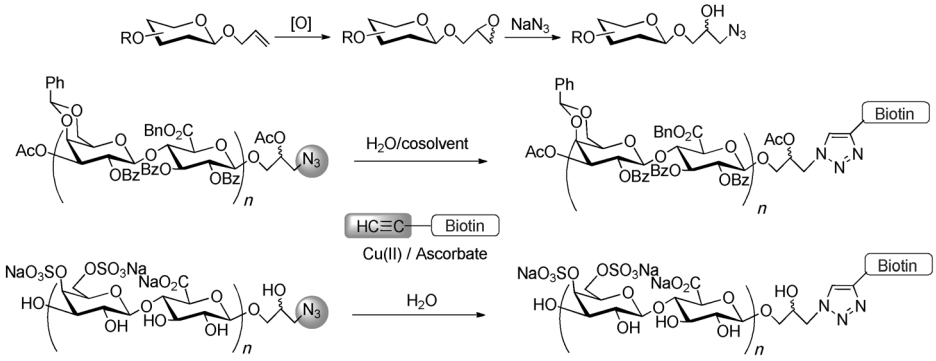
	$H4_{Gal}$	$H6_{Gal}$	$H5_{Gal}$	$C4_{Gal}$	$C6_{Gal}$	$C5_{Gal}$
20	4.02–3.71	3.30–3.03	3.30–3.03	69.30, 68.20	62.15, 61.81	74.68, 74.01
22	4.72, 4.70	3.81–3.64	3.89–3.64	77.27, 73.21	67.96	74.38, 74.28
21	4.02–3.65	3.29–3.00	3.29–3.00	68.25–68.16	62.10–61.83	74.68–74.03
23	4.74–4.65	4.20–3.59	3.91–3.59	77.26–72.94	68.23–67.89	74.31

(Scheme 5). These conditions are "traditionally" employed in GAG synthesis to avoid the β-elimination of the 4-*O*-glycosidic bond of the glucuronic ester moiety. Nevertheless, Martin-Lomas et al.[32] reported a direct saponification using KOH at room temperature in the case of heparin-like oligosaccharide synthesis. Thus, we decided to apply a similar approach for the deprotection of CS analogues and tetra- and hexasaccharide **22** and **23** were treated with aqueous NaOH in methanol to give the final compounds **1** and **2**. The NMR spectra are in accordance with the structure. Further structural confirmation was then undertaken by using mass spectrometry. MALDI or electrospray mass spectrometry of the sodium salt is generally deceptive due to poor volatility and concomitant extensive fragmentation. Recently, this has been improved with a new method from the group of Vasseur[33]; the principle is to use, instead of sodium salt, a large hydrophobic pyrenemethyl guanidinium counterion (pmg). Adducts between sulfated oligosaccharide and pmg are very stable and can be analyzed by MALDI MS (see the Supporting Information).

Click biotinylation: Biotinylation is a convenient way to reproducibly immobilize hydrophilic compounds on surfaces. Biotin binds to streptavidin with a very high affinity ($10^{15}\,M^{-1}$). We have, in the past, used biotin and biotin sulfone for the immblization of oligosaccharides onto microplate chips and beads.[34] Unfortunately, this method involves a carboxylic function at the end of the linker carried by the carbohydrate moiety, which is not compatible with benzoates and benzyl uronic esters. Jacquinet et al.[35,36] recently described the biotinylation of sulfated and unsulfated CS oligosaccharides by using a $NHZ{-}CH_2{-}CH_2$ linker.

Here, we wanted to use directly the allyl group and its double bond to introduce a biotin moiety. A first possible strategy is to add cysteamine on the allyl and then to use an amide-coupling reaction group. This well-known photo-induced addition of thiols onto double bounds has been used in sugar research.[37] Another strategy is the ozonolysis of the double bound to give a reactive aldehyde and to use it for reductive amination.[38,39] These methods are not fully convenient because they use large amounts of reagents and/or have limited yields due to solubility and low reactivity problems. We now propose an alternative approach based on the Huisgen[40] reaction. The use of the conditions described by the groups of Sharpless[41] and Meldal[42] (copper-catalyzed click chemistry) allows one to perform the reaction in various solvents using an equimolar ratio of reagents. The triazole linker is stable and not protonated until pH < 2.[43] Click chemistry has already been used in glycochemistry to prepare conjugates, arrays,[44] cyclodextrine analogues,[45] and multivalent lectin ligands.[46] This reaction was used successfully to conjugate a CS-D (from natural sources) to PEG or BSA with a good yield.[47] Some examples of "click biotinylation" have been reported in the case of *S*-Sialosides[48] and CD22 ligands.[49]

We chose to insert the azide function on the carbohydrate moiety and the alkyne group on the biotin derivative. Thus, the critical step is the clean introduction of an azide on the allyl chain. Nifantiev et al.[50] described the conversion of an allyl group into a 2-azido ethyl on methyl urinate-bearing oligosaccharides. Despite good overall yields (64–85%), this sequence is tedious (7 steps), and the uronate function is not compatible with the required reduction step of an aldehyde function. Consequently, we decided to prepare the azido compounds through an electrophilic epoxide, which could be obtained by the oxidation of the allylic double bond (Scheme 5). The synthesis only involves two steps, which are compatible with the uronic ester.



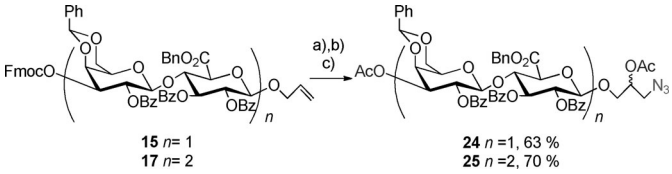
Scheme 5. Allyl to azido propyl conversion and alternatives for biotinylation.

Two strategies were envisioned for the coupling: biotinylation before or after the de-

protection/sulfation sequence (Scheme 5) by using a copper(II)/ascorbate catalytic system.

The reaction of NaN_3/NH_4Cl[51] on glycidyl epoxide is regioselective and only gives 3-azido 2-hydroxy propyl. In contrast, the epoxidation of the allyl glycoside by using peracid usually gives a 1:1 mixture of diastereomers. The presence of two epimeric OH should not be critical for biological activity as long as this position is not sulfated.

Compounds **15** and **17** were treated with *meta*-chloroperbenzoic acid (*m*-CPBA) in dichloromethane at RT to give the intermediate epoxides (Scheme 6). The crude compounds were reacted with NaN_3/NH_4Cl in THF/ethanol/water (2:2:1) to give the 3-azido 2-hydroxy propyl derivative. The acetylation in the presence of catalytic DBU in pyridine removed the Fmoc group (not fully compatible with the epoxide opening) and protected two alcohol functions that were not to be sulfated. The disaccharide **24** and tetrasaccharide **25** were obtained in 63 and 70% yields, respectively. The NMR signal of acetate was used to confirm the presence of a 1:1 mixture of the two epimers.



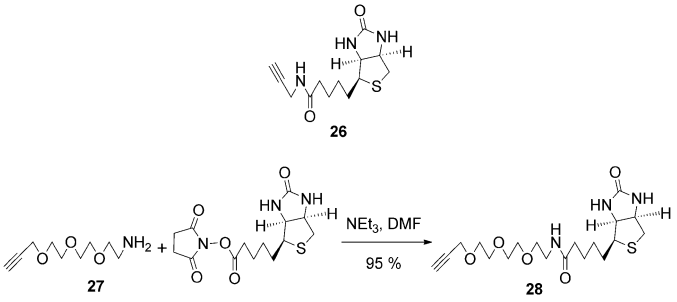
15 *n*= 1
17 *n*= 2

24 *n* =1, 63 %
25 *n* =2, 70 %

Scheme 6. Reagents and conditions: a) *m*-CPBA, CH_2Cl_2; b) NaN_3, NH_4Cl, THF/EtOH/water, heated to reflux; c) DBU (cat.), Ac_2O, pyridine, 63% after 3 steps.

Two biotin units were envisioned; the simplest compound used is the known biotin N-propargyl amide **26**;[52] the use of which was disappointing in preliminary experiments because it is poorly soluble in organic solvent and in water, and gave a poor yield in the coupling reaction. Compound **28**, a more versatile PEGylated version of **26**, was preferred: one important feature is that **28** is water soluble. This compound was obtained from the alkyne-amine **27**[53] and biotin-NHS (Scheme 7).

The protected disaccharide **24** and tetrasaccharide **25** were then converted into the biotin-conjugated compounds **29** and **30** in good yields. An acetal cleavage/sulfatation sequence gave the protected biotinylated compounds **31** and **32** (Scheme 8).



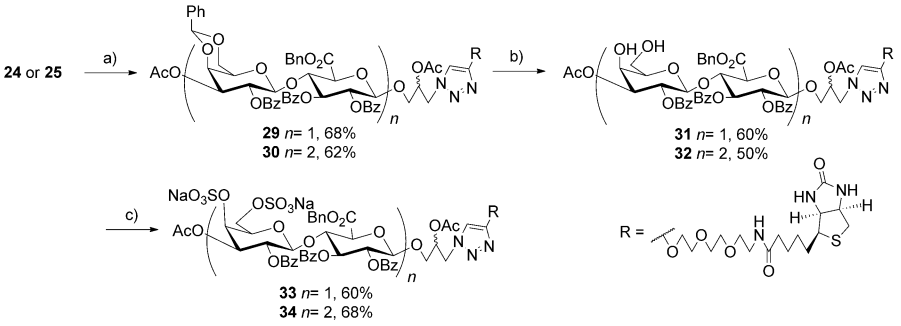
26

27

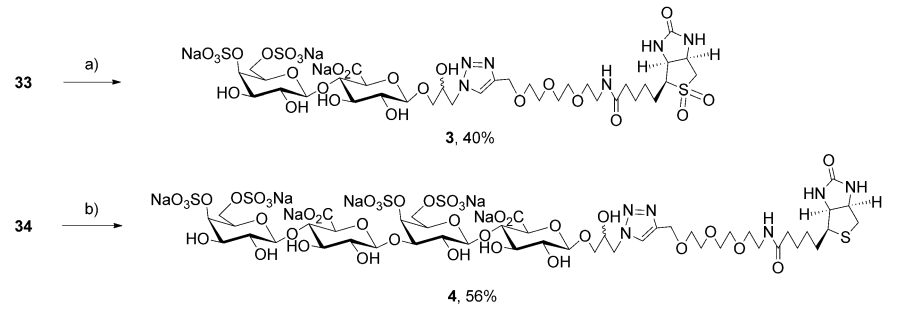
28

Scheme 7. Clickable-biotin derivatives.

The disaccharide **33** was deprotected following the two-step mild saponification ($LiOH/H_2O_2$) to give compound **3** (Scheme 9). Analysis by using mass spectrometry showed that the sulfide function of the biotin moiety was oxidized into a sulfone group. This side reaction is not detrimental; biotin sulfone is known to be strongly recognized by avidin.[54] Although biotin sulfone is compatible with biological tests, we choose to saponify the other compounds using the direct procedure to avoid the possible uncomplete oxidation of the biotin leading to a mixture of sulfoxide and sulfone. Thus, the protected biotinylated tetrasaccharide **34**



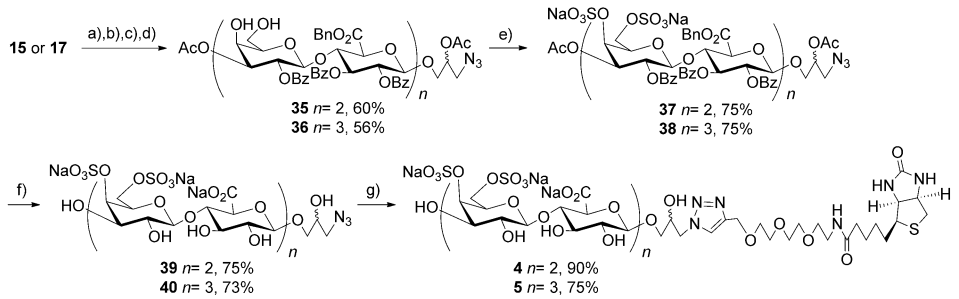
Scheme 8. Reagents and conditions: a) **28**, $Cu(OAc)_2$/ascorbate, CH_2Cl_2/water; b) TFA, water, THF/CH_2Cl_2, sonication; c) $Me_3N{\cdot}SO_3$, DMF, 60 °C.



Scheme 9. Reagents and conditions: a) LiOH, H_2O_2, THF/water, 0 °C to RT then NaOH (4 M), MeOH, 0 °C to RT; b) NaOH (4 M), MeOH, 0 °C to RT.

was treated with NaOH (4 M) in MeOH to give the final CS tetrasaccharidic analogue **4** (Scheme 9).

The yields are not as good as those in the allyl series, mostly because of the increased polarities and purification difficulties due to the presence of the biotin. An alternative was explored, that is, to delay the introduction of the biotin (Scheme 10). The functionalization with the azide group directly followed by benzylidene acetals cleavage was carried out in good overall yields (60%). After sulfation/saponification, the deprotected oligosaccharides were coupled to the biotin derivative **28** in water. Very good yields were obtained for this last "click biotinylation"(90% for **4**, 75% for **5**).



Scheme 10. Reagents and conditions: a) *m*-CPBA, CH_2Cl_2; b) NaN_3, NH_4Cl, THF/EtOH/water, heated to reflux; c) DBU (cat.), Ac_2O, pyridine; d) TFA, water, THF/CH_2Cl_2; e) $Me_3N{\cdot}SO_3$, DMF, 60 °C ; f) NaOH, MeOH, 0 °C to RT; g) compound **28**, $CuSO_4$/ascorbate, water.

Biological Evaluation

Analysis of chemokine-oligosaccharide interaction by SPR: To investigate the activities of these CS-E-like oligosaccharides, surface plasmon resonance (SPR)-based binding and competition assays were performed with three model proteins, namely HGF/SF, CXCL12α and CXCL12γ. Hepatocyte growth factor/scatter factor (HGF/SF) is a multifunctional plasminogen-related protein that induces growth, motility, and differentiation of epithelial, endothelial, and neural cells through activation of its tyrosine kinase receptor MET.[55] HGF/SF also interacts with HS and CS/DS chains[56] and it has been demonstrated that efficient activation of the MET receptor requires HGF/SF engagement with GAGs to form a signalling ternary complex. The minimal size of the oligosaccharide that binds HGF/SF with high affinity is a tetrasaccharide from heparan sulfate but a hexasaccharide from dermatan sulfate.[57] CXCL12 (or SDF for stromal cell-derived factor) is also a GAG binding protein that occurs in different isoforms. It belongs to the chemokine family that promotes directional kinesis of cells through the establishment of concentration gradients that are believed to be maintained within tissues through GAGs binding.[58] CXCL12α exclusively binds to HS, whereas CXCL12γ, a CXCL12 isoform characterized by a long C-terminal and highly basic extension, interacts both with HS and CS/DS. Altogether, HGF/SF, CXCL12α and CXCL12γ represent interesting models to investigate the activity of the CS analogues prepared here, regarding both their nature (CS versus HS) and their degree of polymerisation (di- to hexasaccharide).

To analyze the protein binding capacities of the di-, tetra- and hexasaccharides we first adopted a relevant system in which the oligosaccharides were coupled, owing to their biotin group, to a solid phase (streptavidin-activated sensorchip), thus mimicking the presentation of CSPG at the cell surface, whereas the proteins of interest were injected in the fluid phase. Injection of a range of concentration of HGF/SF (up to 5.5 nM), a known CS binding protein[59], over the CS-E-like di-, tetra- or hexasaccharide surfaces, gave a series of sensorgrams (Figure 3a–c), which show that the hexasaccharide **5** displays a strong HGF/SF-binding capacity, whereas the tetra- or disaccharide are only poorly active or completely inactive. To investigate whether these materials are specific CS mimetics (rather than the polyanions of more general GAG mimetics) we made use of the CXCL12 chemokine, of which two isoforms CXCL12α and CXCL12γ bind to heparan sulfate, whereas the CXCL12γ only recognizes CS.[60] As shown in Figure 3d–i, we found that CXCL12α did not bind to any part of the oligosaccharide, whereas CXCL12γ strongly interacted with the hexasaccharide **5**, and albeit to a much lesser extent, with the tetrasaccharide **4**.

Next, to investigate the binding of these oligosaccharides to protein in solution, a competition assay was designed in which CXCL12γ was co-incubated with the non biotinylated CS-E-like tetra- or hexasaccharide or (for comparison) with heparin-derived hexasaccharide and injected over a chondroitine sulfate-functionalized sensorchip. Similar to the direct binding assay, competition was observed for both the heparin and the CS hexasaccharides, but not for the tetrasaccharide (Figure 4) . Interestingly, this experiment showed that the hexasaccharide **2** is almost as active as the heparin derived oligosaccharide.

Analysis of Otx2-oligosaccharide interaction: Homeoproteins define a family of transcription factors with important developmental and physiological properties. It was shown recently that in addition to regulating transcription in a cell autonomous way, these transcription factors also have a non-cell autonomous signalling activity.[61] A particularly interesting case is provided by the homeoprotein Otx2 that is found in parvalbumin-expressing GABAergic interneurons (PV-cells) in the visual cortex. The presence of Otx2 in PV-

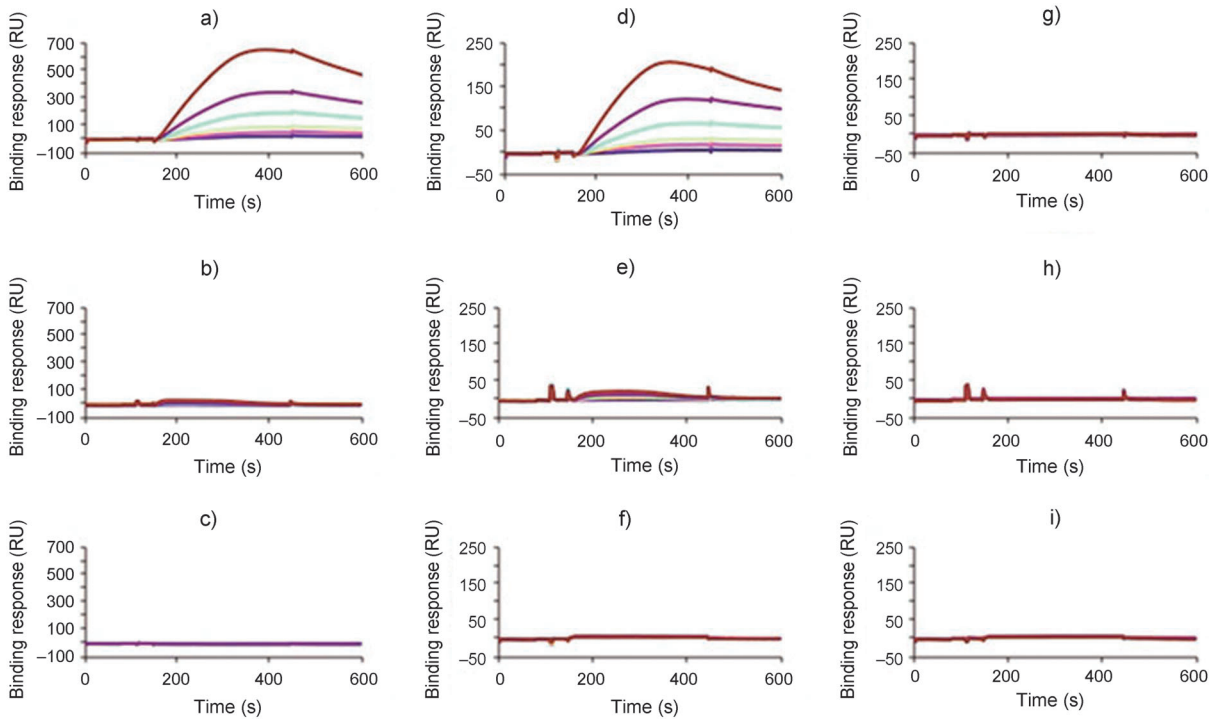


Figure 3. SPR analysis of HGF/SF and CXCL12 binding to di-, tetra-, and hexasaccharides. Overlay of sensorgram showing binding of HGF/SF (a,b,c), CXCL12γ (d,e,f) or CXCL12α (g,h,i) to hexa- (a,d,g), tetra- (b,e,h) or di- (c,f,i) saccharides. In each panel, the protein concentrations used were (from top to bottom) 5.5–3.7–2.5–1.6–1.1, and 0.73 nM for HGF/SF, 13.3–8.9–5.9–3.9–2.6, and 1.7 nM for CXCL12γ and 150–100–66.7–44.4–29.6, and 19.7 nM for CXCL12α.

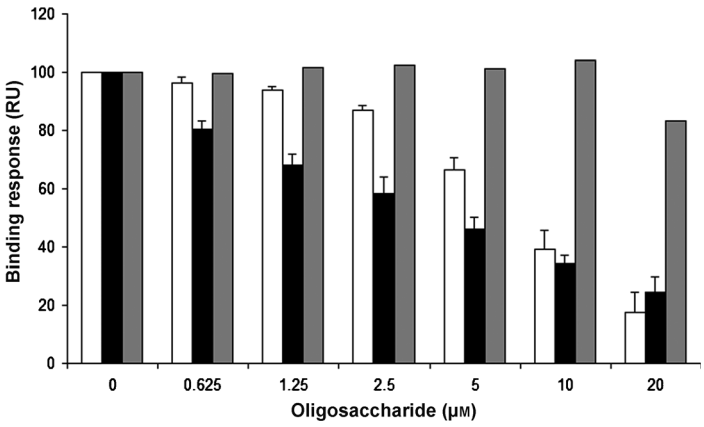


Figure 4. Binding of soluble heparin and CS-E analogues to CXCL12γ. Oligosaccharides: hexasaccharide **2** (white), heparin hexasaccharide (black) or tetrasaccharide **1** (grey) from 0 to 20 μM were incubated with CXCL12γ (10 nM) and injected over a chondroitin sulfate activated surface. The binding responses (mean of two experiments for the hexasaccharide) were recorded and plotted against the oligosaccharide concentration.

cells does not reflect its synthesis by this cell type but its import from an extracellular source.[10] Otx2 accumulates in PV-cells starting at day 20 (P20) of the mouse postnatal life and continuing throughout life. This accumulation opens at P20 and closes at P40 a period of physiological plasticity, called the critical period (CP) for binocular vision. This means that closing one eye during this period leads to a strong decrease of its visual acuity. This does not happen if the eye is closed before P20 or after P40.

In two precedent studies[62,63] it was shown that Otx2 infused directly inside the visual cortex is primarily internalized by PV-cells. This specific recognition is mediated by the recognition of Otx2 by glycosaminoglycans (GAGs) present within the perineuronal nets (PNNs) enwrapping the PV-cells. The preferential transfer of endogenous Otx2 into PV-cells is lost upon injection of chondroitinase ABC, suggesting that chondroitin sulfate (CS) participates in the recognition of Otx2. Within the Otx2 sequence was identified a short domain that has the structure of a GAG-binding domain (RKQRRERTTFTRAQL) and participates in PNN recognition. By using isothermal calorimetry, it was demonstrated that among the CS family, disulfated CS (CS-D and CS-E) have the highest affinities for the GAG-binding domain of Otx2.[57]

This is why we have investigated if Otx2 recognizes the CS-E-like hexasaccharide. To that end, we used the gel shift assay, an assay allowing one to test whether a protein binds to a biotinylated DNA probe in the presence of competing GAGs. In the present case, the biotinylated version of the hexasaccharide (**5**) was used as a probe. As illustrated in Figure 5a, the incubation of the hexasaccharide **5** with Otx2 led to a shift in its migration. Blotting the membrane with an antibody against Otx2 showed that the shift is really due to the interaction of the hexasaccharide with Otx2 (Fig-

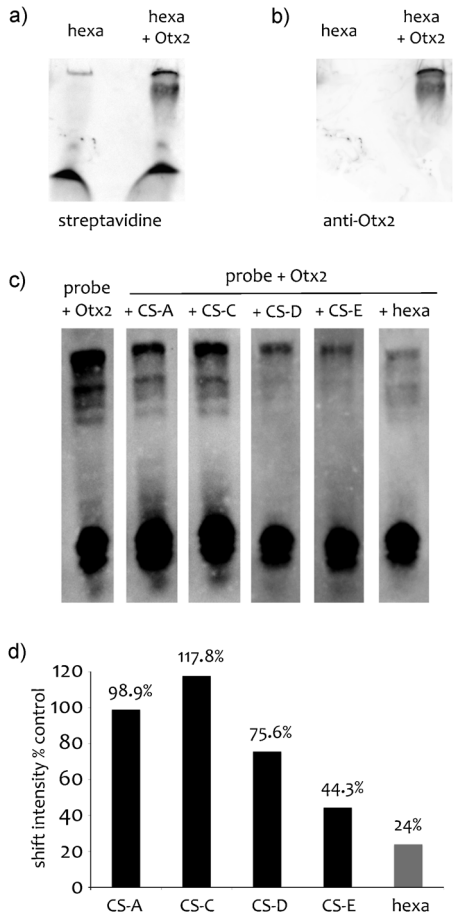


Figure 5. Otx2 binds to hexasaccharides: a,b) Gel shift assay showing that Otx2 binds to hexasaccharide **5**. Biotinylated hexasaccharide is revealed by streptavidin-HRP (a) and Otx2 protein by anti-Otx2 antibody (b). c) Gel shift assay using a biotinylated DNA probe showing the competition ability for Otx2 binding of commercial chondroitin sulfates and of the hexasaccharide **2**. d) Quantification of the DNA shift intensity, compared to control (probe + Otx2).

ure 5b). This demonstrates that Otx2 binds the CS-E-like hexasaccharide.

The GAG-binding domain of Otx2 corresponds to the first helix of its DNA-binding domain (homeodomain) plus the RK doublet upstream of the helix. We thus formed the hypothesis that a CS recognized by Otx2 should antagonize its binding to specific promoter sequences. Using the gel shift assay with an IRBP1 DNA probe that binds specifically to Otx2, we tested the competing activities of different commercial sugars. At 4 pmol, CS-E almost entirely displaced the transcription factor from its cognate binding site (Figure 5c–d). CS-D was slightly less active and CS-A and CS-C were devoid of competing activity. Strikingly, the synthetic hexasaccharide **2** presented the highest competing activity, suggesting that cell surface and DNA recognition involve similar domains of Otx2, in particular the first helix of the homeodomain.

As already mentioned, the preferential internalization of endogenous Otx2 by PV-cells is mediated by its specific binding to complex sugars present at the surface of the cells. Accordingly, Otx2 internalization is antagonized by the infusion of the GAG-binding domain and severely decreased following the injection of chondroitinase ABC into the visual cortex .[63] This led us to investigate if the hexasaccharide **2** could modify the amount of endogenous Otx2 internalized by PV-cells following its infusion into the adult mouse visual cortex for three days. As illustrated in Figure 6a and b and quantified in Figure 6e, a marked decrease in the number of Otx2-positive cells was observed in cortical supragranular layers of infused hemispheres, compared to the untreated hemispheres (at 3 days: $(77.3\pm13.9)\%$ of control; at 7 days: $(53.1\pm0.8)\%$ of control). Thus, Otx2 localization in the visual cortex was disrupted by the presence of **2**. In addition, blocking Otx2 transfer induced a significant loss of the staining for WFA, a lectin that binds with high affinity to the PNNs (Figure 6c–e, at 3 days: $(87\pm10.9)\%$; at 7 days: $(72.2\pm1.3)\%$). Indeed, the persistent accumulation of Otx2 is needed to maintain the PNNs around the PV-cells in the visual cortex .[62] PV expression was not modified significantly (at 3 days: $(100.3\pm6.5)\%$; at 7 days: $(107.3\pm11.5)\%$), demonstrating an absence of cell death (Figure 6c–e).

All in all, these results demonstrate that the hexasaccharide **2** can be used as an inhibitor of Otx2 in vitro binding to gene promoters and of its in vivo internalization. Given that Otx2 internalization beyond critical period closure is necessary to maintain a non-plastic state, it will be interesting to test if, as is the case for the GAG-binding domain,[57] the CS-E hexasaccharide analogue can be used to reactivate plasticity in the adult.

Conclusion

The efficient synthetic approach described here allowed us to obtain CS-E oligosaccharides analogues from the cheap and abundant lactose disaccharide. The easy access to highly differentiated lactoside blocks lead to the rapid construction of structurally complex oligosaccharides (up to octasaccharide) in good yields and stereoselectivity. The building block **17** has proved to be very versatile and has enabled us to directly use the allyl-protected oligosaccharides for the coupling reaction with biotin. This biotinylation was based on the copper-catalyzed Huisgen cycloaddition and was successfully applied at two different stages: on final sulfated oligosaccharides or on protected intermediates. This attractive "click biotinylation" method can be extended to other allylated substrates.

The preliminary biological investigations showed good activities of the designed analogues with two different GAG protein receptors. Moreover, the specific interaction with HGF/SF (regarding size dependency) and CXCL12γ versus CXCL12α (regarding HS/CS binding) demonstrated the ability of the analogues to mimic CS fragments, which validates the proof of concept. The biological properties of CS analogues will be further explored through studies with CS-specific proteins. It is worth noting that the CS disaccharide

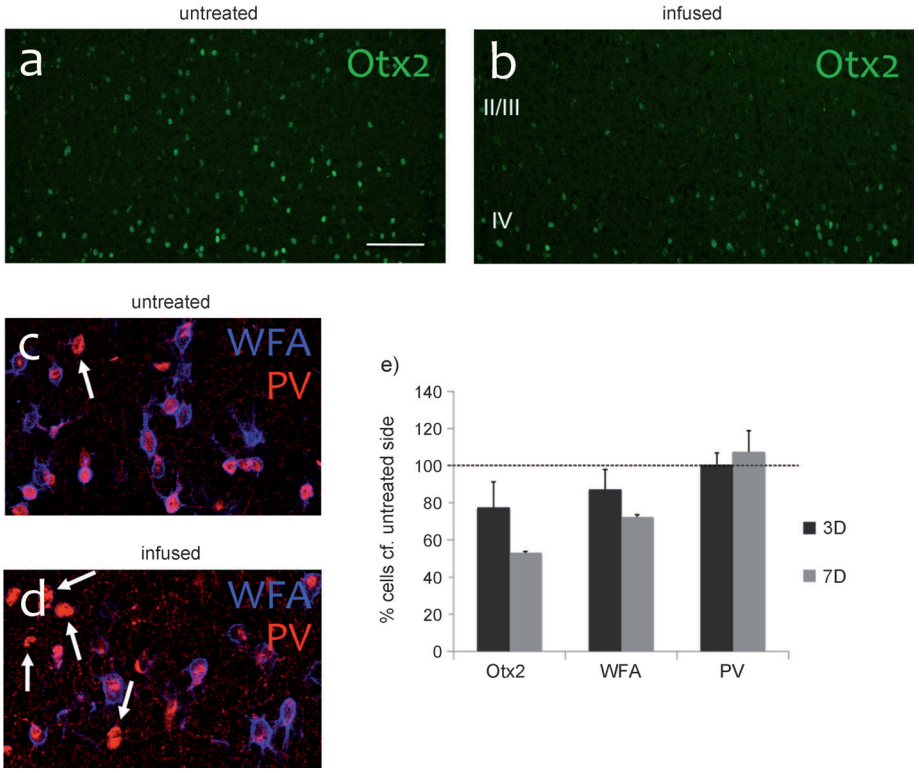


Figure 6. Otx2 binding to hexasaccharide interferes with Otx2 transfer in vivo: a,b) Hexasaccharide **2** infused for 3 days in adult V1 decreases the number of Otx2-stained cells. c,d) Hexasaccharide **2** infusion decreases the number of WFA-stained cells, but not that of PV-positive cells. Arrows indicate PV-cells that are not surrounded by WFA when staining. e) Percentage of Otx2, WFA, and PV-positive cells in the supragranular layers of V1b (infused compared to contralateral, untreated side), 3 days or 7 days post infusion. Scale bar = 100 μm.

unit E has been associated with the formation of osteosarcoma cell line tumor nodules in the liver and with metastasis of Lewis lung carcinoma cells, presumably through binding to a number of growth factors including VEGF, FGF, and HGF/SF.[63] There is also strong evidence that over-expression or the HGF/SF-MET system contributes to growth, invasion, and metastasis in a large variety of tumors.[64] Therefore interfering with the HGF/SF-MET interactions, with the CS-E analogues might be of interest in such pathologies. It is also worth noting that amongst the many interleukins that bind to heparin, some of them have been shown to interact with chondroitin sulfate, including interleukins-4,[65] 7,[66] and 8.[67] The analogues described in this study could thus be of interest in the context of the immune response.

The CS-E-like hexasaccharide binds to the homeoprotein Otx2. Our in vivo experiments showed that its infusion into the adult visual cortex can block the transfer of Otx2 into PV-cells, suggesting that it competes with endogenous GAGs involved in Otx2 recognition. The permanent transfer and capture of Otx2 by PV-cells is necessary to maintain a consolidated non-plastic state of the adult visual cortex.[56,57] The hexasaccharide **2** could then be used to open windows of plasticity in the adult cortex. Other homeoproteins that can be transferred between cells possess a GAG-binding domain, raising the intriguing possibility of a sugar code for homeoprotein recognition and transfer. It is of note that the hexasaccharide competes with specific DNA probes for Otx2 binding. This means that the same domain, encompassing the 1st helix of the homeodomain, is involved in DNA and CS-E recognition. It is thus possible to envisage the use of synthetic GAGs composing the "sugar code" to antagonize the transcriptional activity of distinct homeoproteins.

The success of the nitrogen-to-oxygen replacement approach and the preliminary interesting biological results reported here have opened the way toward the preparation of other CS analogues (of a different series) but also other GAG analogues, such as dermatan sulfate (DS) or keratane sulfate (KS). These compounds could be extensively used for various applications such as structure–activity studies, understanding of complex biological processes, or medicinal chemistry.

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