Adaptable synthesis of chondroitin sulfate disaccharide subtypes preprogrammed for regiospecific *O*-sulfation

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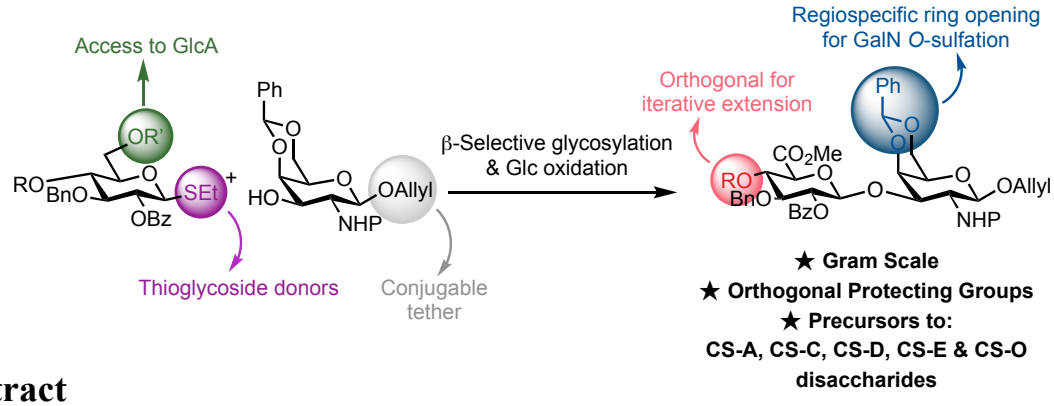
Graphical Abstract



Abstract

A divergent synthetic route to chondroitin sulfate (CS) disaccharide precursors, including rarer subtypes such as CS-D, has been developed. From common intermediates, a series of thioglycoside D-glucose donors and 4,6-*O*-benzylidene protected D-galactosamine acceptors are utilised in a robust glycosylation reaction, achieving β-selectivity and consistent yields (60-75%) on scales >2.0 g. A post-glycosylation oxidation to D-glucuronic acid and orthogonal protecting groups delivers access to CS-A, CS-C, CS-D, CS-E and CS-O precursor subtypes. Of further note is a 4-*O*-benzyl regioselective reductive ring opening of a 4,6-*O*-benzylidene protected disaccharide using $PhBCl_2$ and Et_3SiH to access a CS-D precursor, in 73% yield over two steps. Finally, synthesis of a 6-*O*-sulfated CS-C disaccharide containing a conjugable anomeric allyl tether is completed. These materials will provide a benchmark to further synthesise and study chondroitin sulfates.

Introduction

Chondroitin sulfate (CS) is a glycosaminoglycan composed of D-glucuronic acid (D-GlcA) and *N*-Acetyl-D-galactosamine (D-GalNAc), with repeating sequences of D-GlcA(1,3-β)-D-GalNAc-(1,4-β) disaccharides, typically in excess of 100 units.[1] During biosynthetic assembly, enzymatic modification of the repeating nascent disaccharide backbone gives rise to a number of different sulfation patterns, creating a multitude of CS subtypes and heterogenous structures.[2] Naturally occurring CS therefore contains an assortment of subtypes, and

proportions that also vary depending on the polysaccharide source. CS-A (D-GalNAc 4-*O*-sulfation) and CS-C (D-GalNAc 6-*O*-sulfation) are abundant compared to other subtypes such as CS-D (2'-*O*-D-GlcA-6-*O*-D-GalNAc sulfation) and CS-E (4,6-*O*-D-GalNAc sulfation).[3–5] CS interacts with growth factors, cytokines, chemokines and adhesion molecules,[6] regulating physiological processes including embryonic and brain development,[7,8] anti-inflammatory effects,[9] wound healing[10] and signalling.[11] Whilst it is established that CS sulfation patterns correlate to related biological function, the specificities of such molecular interactions is an underdeveloped field.[12] Access to homogenous, structurally defined CS oligosaccharides is therefore of utmost importance for the study of related CS-protein interactions, but is hampered by difficulties in obtaining significant amounts of structurally defined sequences from natural sources, particularly for rarer subtypes such as CS-D. Relatedly, synthetic approaches to heparan sulfate (HS) fragments have advanced more rapidly and proven extremely successful.[13–17]

Despite recent advances in the synthesis of CS oligosaccharides and related building blocks,[1,18–21] approaches are required that allow diversification to CS subtypes with varying sulfation patterns, chain lengths and conjugation capabilities. Herein, and as part of a wider program targeting approaches to other glycosaminoglycans,[22,23] we develop a reliable and versatile synthesis of CS D-GlcA-β-1,3-D-GalN building block disaccharides from a small library of D-Glc and D-GalN monosaccharides (*Figure 1*). Careful consideration of protecting groups was made, to enable: β-stereoselectivity using a participating C2-OBz protecting group, variation of sulfation site programming using regiospecific ring opening of D-GalN 4,6-*O*-benzylidene acetals, orthogonal D-GlcA-C4'-*O*-substituents for elongation potential and a reducing end anomeric allyl group as a conjugable handle or orthogonal group for alternative donor formation.

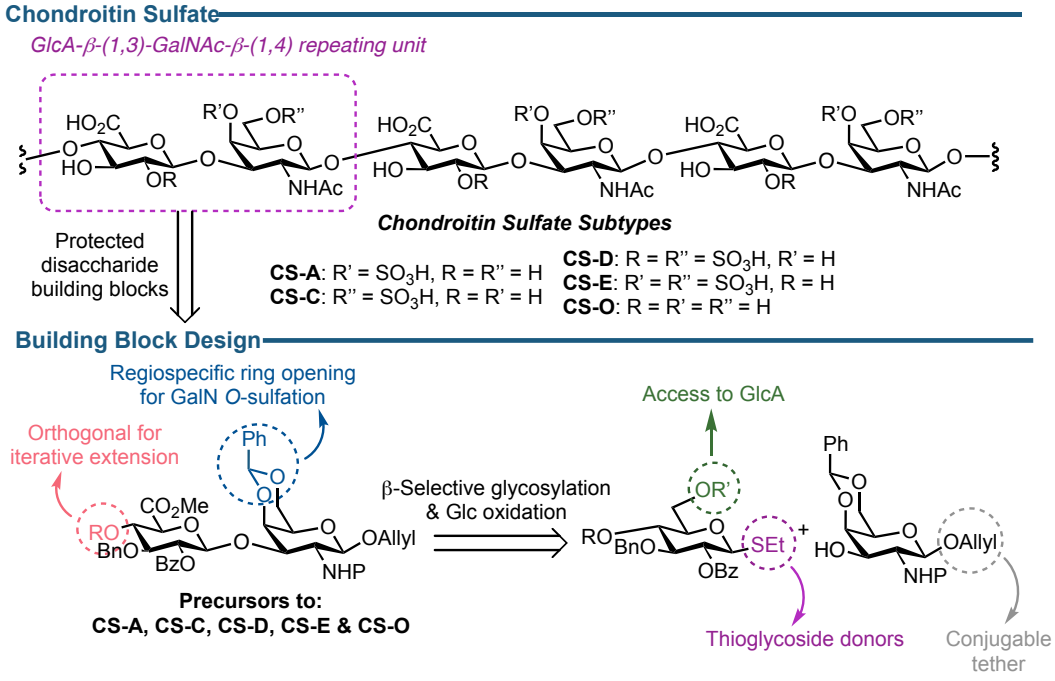


Figure 1: **Top:** Various subtypes of the glycosaminoglycan chondroitin sulfate (CS). The standard disaccharide repeat is shown inside the purple dotted box. **Bottom:** A retrosynthetic

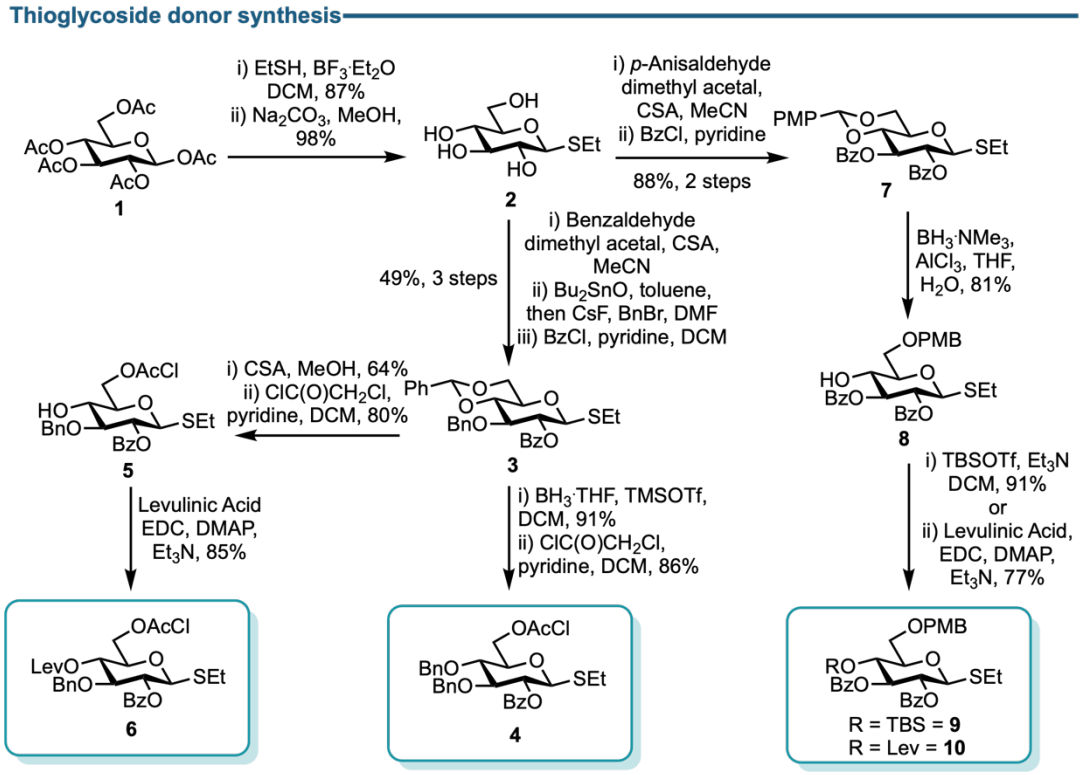
approach to install capability for regiodefined CS sulfation, conjugation and non-reducing end iterative extension into appropriate disaccharides from monosaccharide precursors.

Results and Discussion

Previous approaches to CS oligosaccharides and associated building blocks have utilised trichloroacetimidate (TCAI) D-GlcA donors, namely a pre-glycosylation oxidation strategy.[24,25] Whilst proven and undoubtedly useful, issues due to low reactivity of uronate donors and the possibility of donor-derived side product formation (e.g., orthoesters or *N*-trichloroacetamides) are notable considerations.[26] Here we instead opted to explore a post-glycosylation oxidation approach (D-Glc to D-GlcA) using thioglycoside donors (shelf-stable and generally synthesised in fewer steps than TCAI donors). Previously, Lei and co-workers established a post-glycosylation oxidation strategy to synthesise CS-E oligosaccharides using TCAI donors to access a key disaccharide for iterative synthesis.[27]

Glycosyl Donor and Acceptor Building Block Synthesis

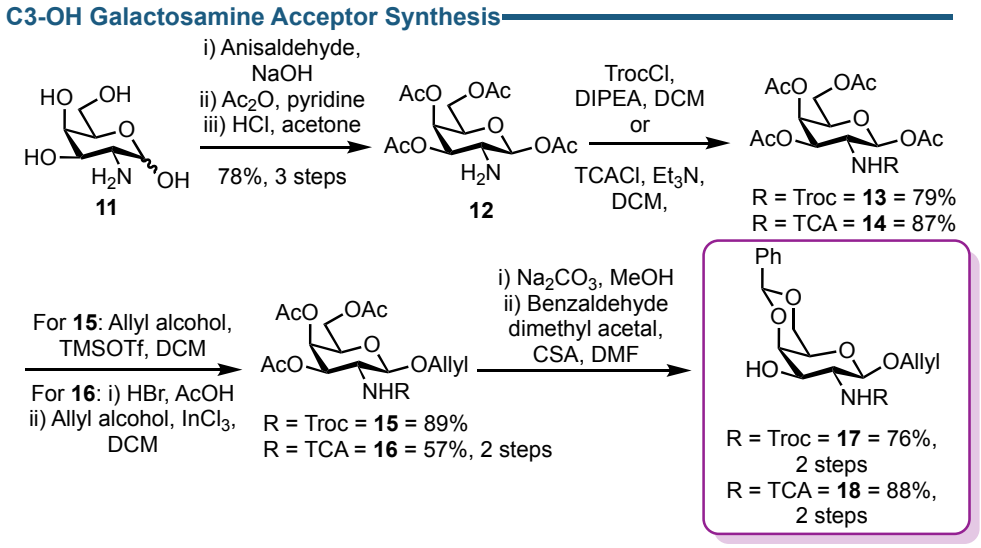
Synthesis towards thioglycoside donors commenced from commercial 1,2,3,4,6-penta-*O*-acetyl-β-D-glucose **1**, undergoing thioglycosylation with EtSH using $BF_3{\cdot}Et_2O$ as a Lewis acid and affording the β-thiol in 87% yield (***Scheme 1***). Deacetylation using Na_2CO_3 in MeOH afforded tetrol **2** in 98% yield and an appropriate material to diversify the route to the required building blocks. Benzylidene protection using benzaldehyde dimethyl acetal with CSA in MeCN was followed by regioselective C3-*O*-benzylation using $^{n}Bu_2SnO$ and CsF with benzyl bromide.[28] Subsequent C2-*O*-benzoylation using BzCl and pyridine afforded thioglycoside **3** in 49% yield over three steps. From this material two divergent routes were progressed. The first saw regioselective reductive ring opening using $BH_3{\cdot}THF$ and a catalytic amount of TMSOTf as Lewis acid to give a 6-OH,4-OBn protected material in 91% yield. COSY NMR correlation between a broad singlet at $\delta = 1.97$ ppm and H6 environments at $\delta = 3.93$ and 3.72 ppm indicated a C6-OH was present and the desired regiochemistry achieved. Reaction of this material with chloroacetyl chloride and pyridine afforded the desired donor **4** in 86% yield. Secondly, thioglycoside **3** was subjected to acidic benzylidene cleavage using CSA in MeOH at 40 °C, followed by regioselective C6-*O*-chloroacetylation at low temperature to afford alcohol **5** in 80% yield. Finally, C4-*O*-levulinoyl ester protection proceeded smoothly in 85% yield to generate glycosyl donor **6**, alternatively protected at C4, compared to **4**; the C4-*O*-Lev group enabling access to regioselective deprotection (versus C4 OBn in **4)** and access to a glycosyl acceptor form.



Scheme 1: Synthesis of thioglycoside donor panel starting from commercial material **1** and delivering orthogonally protected donors **4**, **6**, **9** and **10**.

Furthermore, from tetrol **2** *para*-methoxyphenyl benzylidene protection was completed, followed by C2,C3-*O*-benzoylation to afford thioglycoside **7** in 88% yield over two steps. Reductive ring opening of PMP-benzylidene **7** was explored, for the first time, employing $BH_3 \cdot NMe_3$ complex and $AlCl_3$. Initial yields in forming the desired C6-*O*-PMB regioisomer **8** were low (35-44%), noting amounts (22-30%) of returned **7** alongside an unwanted anomeric hydrolysis product formed during the 3 h reaction. By reducing the reaction time to 1 h (expedited by the addition of two equivalents of H_2O),[29] the isolated yield of **8** increased to 81% and was accessible on 10 g scale. Finally, C4-*O*-protection of **8** was completed using either TBDMSOTf or EDC/levulinic acid to afford orthogonally C4-protected donors **9** (91% yield) and **10** (77% yield), completing the panel.

Relatedly, a scalable route towards *N*-Troc and *N*-trichloracetyl (TCA) protected D-GalN acceptors **17** and **18** was developed starting from commercial D-GalN **11**. The free amine was temporarily masked using *p*-anisaldehyde under basic conditions (***Scheme 2***). This was followed by global acetylation and subsequent acidic hydrolysis of the aldimine protecting group using HCl in refluxing acetone to afford amine **12** in 78% yield over three steps. This method of temporary amino protection was high yielding, β-selective and chromatography free. It should though be noted that initial large-scale attempts (> 5.0 g) resulted in imine hydrolysis during acetylation, reducing the yields markedly and affording the undesired *N*-acyl by-product. To overcome this, rigorous drying under high vacuum for 8 h was necessary, but enabled synthesis of **12** to be reproducibly scaled up to >40.0 g.

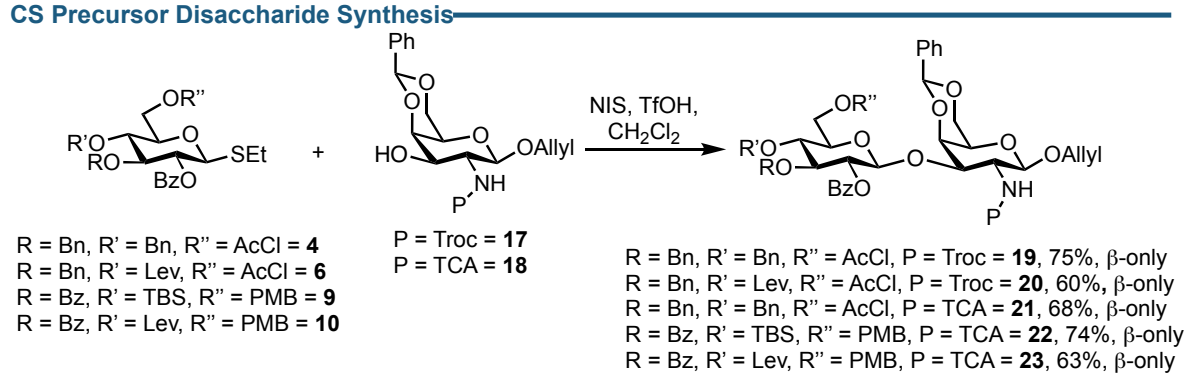


Scheme 2: Synthesis of D-galactosamine C3-OH acceptors with variant *N*-protecting groups, *N*-Troc **17** and N-TCA **18**.

From amine **12** both *N*-Troc and *N*-TCA protected acceptors were synthesised. *N*-Troc protection of **12** was completed on scales up to 15.0 g in 84% yield. Subsequent protection of the anomeric position with a conjugable aglycon (OAllyl) was completed using allyl alcohol and TMSOTf to afford β-**15** in 89% yield and a 19:1 β:α ratio, with the α-anomer separable *via* column chromatography. Deacetylation of **15** using Na_2CO_3 in MeOH was followed by 4,6-*O*-benzylidene acetal protection to deliver acceptor **17** on multigram scale and in 76% yield over two steps. The desired β-anomeric stereochemistry within **17** was confirmed using ^{1}H NMR and an observed equatorial-axial coupling constant of 8.2 Hz for H1 ($^3J_{H1\text{-}H2}$). For the synthesis of *N*-TCA acceptor **18**, a similar series of reactions were completed (***Scheme 2***), noting however that anomeric allylation of *N*-TCA derivative **14** was achieved *via* the glycosyl bromide, using allyl alcohol and indium chloride to selectively synthesize β-**16** in 57% yield. The required deacetylation and benzylidene protection steps afforded acceptor **18** in 88% yield over two steps.

Glycosylation Methodology Development for CS Disaccharide Synthesis

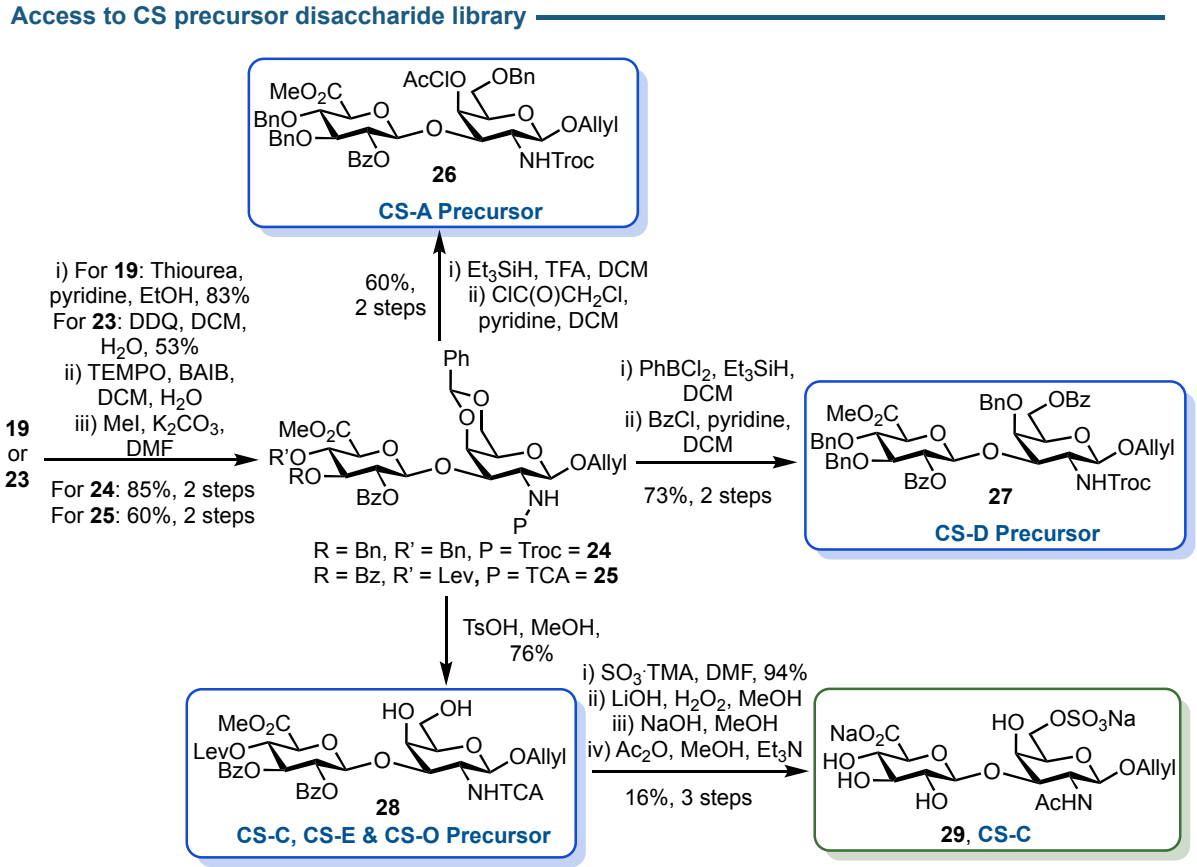
We next explored glycosylation capability of our donor panel (compounds **4**, **6**, **9** and **10**) with D-GalN acceptors **17** and **18**. Glycosylation of thioglycoside donor **4** using *N*-Troc acceptor **17** and an NIS/TfOH promotor system afforded disaccharide **19** in 75% yield (***Scheme 3***). The reaction proceeded smoothly, and isolated yields were consistent on >2.0 g scale. The desired β-(1,3) glycosidic linkage was confirmed through a large $J_{H1'\text{-}H2'}$ coupling constant of 8.0 Hz (^{1}H δ = 4.83 ppm). A H1'-C3 HMBC correlation further confirmed the desired linkage had formed. The glycosylation conditions were successfully replicated with C4-*O*-Lev donor **6**, generating disaccharide **20** in 60% yield. Switching to *N*-TCA acceptor **18** and donor **4** furnished disaccharide **21** in 68% yield and glycosylation of **18** with donor **9** delivered disaccharide **22** in 74% yield. Finally, use of C4-*O*-Lev protected donor **10** afforded disaccharide **23** in a slightly lower 63% yield. From these results the reactivity of an *N*-Troc D-GalN acceptor was established as comparable to an *N*-TCA derivative.



Scheme 3: CS precursor disaccharide synthesis using variously protected donors and acceptors and NIS/TfOH glycosylation conditions. Following column chromatography of the crude reactions only β-anomer was isolated for compounds **19-22**.

Post Glycosylation Oxidation to Access CS Precursor Disaccharides

With access to a robust glycosylation procedure in place, we sought to complete post-glycosylation oxidation of the disaccharide D-Glc component (to D-GlcA) and from there access the required protecting group patterns for CS-A/C/D/E/O. Accordingly, we removed the 6'-*O*-protecting groups from within disaccharides **19** and **23** (thiourea for AcCl cleavage in **19** and DDQ for oxidative PMB removal in **23**). From here oxidative conditions using a biphasic TEMPO/BAIB system were applied to deliver common disaccharides **24** and **25** in 85% and 60% yields respectively (***Scheme 4***). The lower yield for disaccharide **25** was attributed to a sensitivity of the *N*-TCA protecting group to excess K_2CO_3 in the carboxylate methylation step.



Scheme 4: Towards a variably protected series of CS-disaccharide precursors and exemplar synthesis of a CS-C disaccharide. Inset image of

From disaccharides **24** and **25** we completed access to five CS precursors. Firstly, to access a protecting group pattern towards CS-A (D-GalNAc-4-*O*-sulfation pattern), reductive ring opening of **24** was completed to provide a C6-*O*-benzyl moiety in product **26**. Disaccharide **24** was subjected to Et_3SiH and TFA in CH_2Cl_2 at 0 °C and the crude product from this step was then subject to 6-*O*-chloroacetylation, which proceeded smoothly to afford CS-A precursor **26** in 60% yield over two steps and programmed with orthogonal 4-*O*-protecting group for sulfation. Towards a CS-D disaccharide (D-GalNAc-6-*O*- and D-GlcA-2-*O*-sulfation) we required alternate reductive ring opening conditions. Sakagami and Hamana previously identified a combination of $PhBCl_2$ and Et_3SiH for regioselective ring opening of benzylidene acetals to 4-OBn/6-OH products.[30] Reactions were generally performed in CH_2Cl_2 at −78 °C with an excess of Et_3SiH and $PhBCl_2$. However, in CH_2Cl_2, when an excess of Et_3SiH is used, the silane reduces $PhBCl_2$ to PhBHCl, which can hydroborate alkenes, presenting a potential problem for our anomeric *O*-allyl moiety. To avoid this, use of only 1.1 equivalents of Et_3SiH in CH_2Cl_2 at low temperatures has been reported.[31] Upon adopting these conditions for disaccharide **24** for 20 minutes all starting material was consumed and there was no notable side product formation by TLC analysis. Subsequent C6-*O*-benzoylation of the crude material delivered disaccharide **27** in 73% over two steps. NMR analysis confirmed orthogonal 4-*O*-/6-*O*-protecting group patterns in both **26** and **27** (illustrated for **27** in ***Figure 2***).

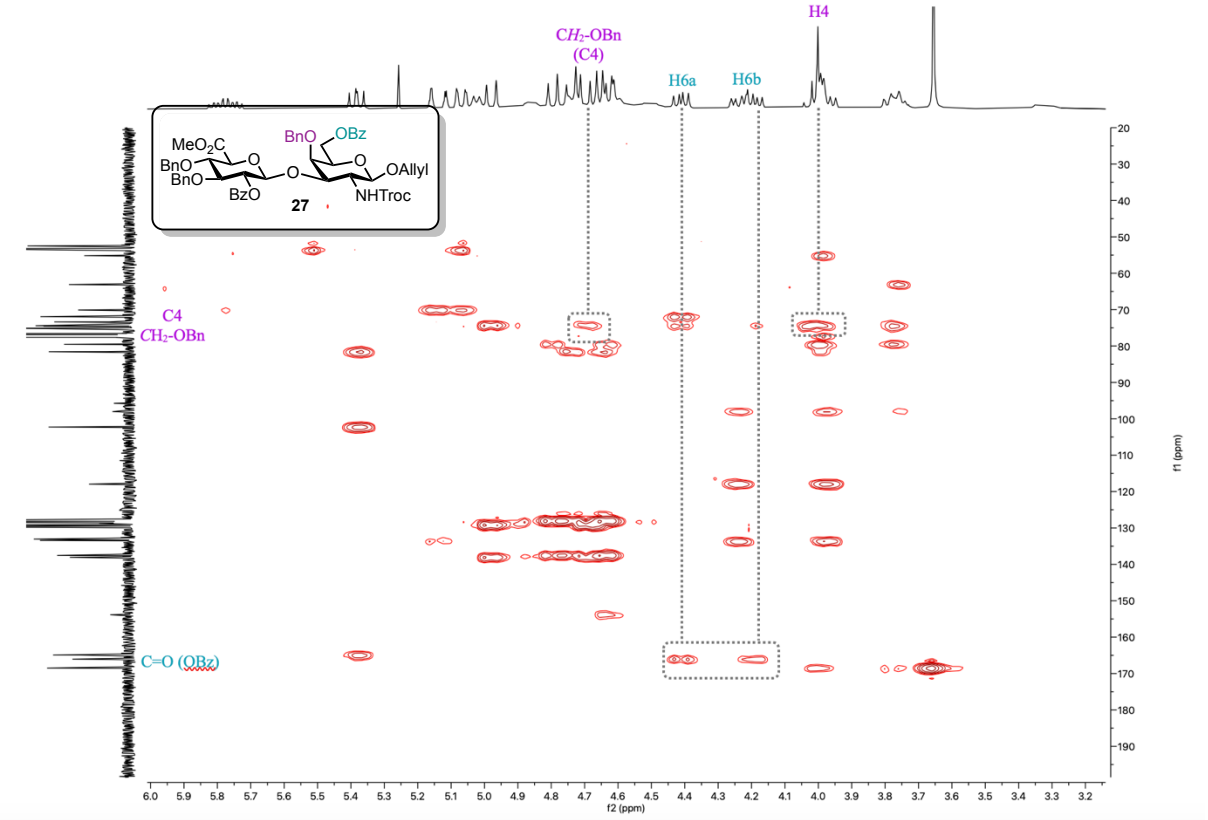


Figure 2: HMBC NMR (400 MHz) for disaccharide **27**, highlighting the result of regioselective ring opening using $PhBCl_2$ and Et_3SiH.

Lastly, acidic cleavage of the benzylidene acetal within disaccharide **25** afforded **28** as a precursor to CS-C, CS-E and CS-O. To exemplify the utility of our approach we completed a formal synthesis of a CS-C disaccharide (***Scheme 4***, green box). 6-*O*-Sulfation of disaccharide **28** using 2.5 equivalents of $SO_3{\cdot}NMe_3$ was achieved in 94% yield. Deprotection of all esters and the *N*-TCA group was completed using standard conditions, followed by a final *N*-acetylation to deliver regiospecifically sulfated disaccharide **29** in 19% yield over three steps.

Conclusion

An efficient approach to the synthesis of CS disaccharide precursors programmed towards different natural sulfation patterns has been established. This divergent strategy enables access to protected CS-A, CS-C, CS-D, CS-E and CS-O derivatives from common disaccharide precursors. Optimisation of a central glycosylation reaction using shelf-stable thioglycoside D-Glc donors enables reproducible multigram scale synthesis with β-stereoselectivity and ultimate access to D-GlcA(1,3-β)-D-GalN systems incorporating multiple orthogonal protecting groups. An exemplar synthesis of a free CS-C disaccharide is completed, containing an anomeric *O*-allyl handle, demonstrating capability for conjugation, for example using alkene click chemistry. Overall, this methodology offers an important contribution to provide the building blocks required for iterative CS oligosaccharide synthesis, for example using a [2+2] glycosylation approach and pre-programmed for regiospecific *O*-sulfation. The synthesis of longer, bespoke CS sequences for biological application is currently underway and will be reported in due course.

Acknowledgements

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