Chemistry
A European
Journal

Chemistry Europe
European Chemical Societies Publishing

Accepted Article

Title: A Rapid and Mild Sulfation Strategy Reveals Conformational Preferences in Therapeutically Relevant Sulfated Xylooligosaccharides

Authors: Yen Vo, Brett D. Schwartz, Hideki Onagi, Jas S. Ward, Michael G. Gardiner, Martin G. Banwell, Keats Nelms, and Lara Malins

To be cited as: *Chem. Eur. J.* 10.1002/chem.202100527

Link to VoR: https://doi.org/10.1002/chem.202100527

WILEY-VCH

10.1002/chem.202100527

WILEY-VCH

A Rapid and Mild Sulfation Strategy Reveals Conformational Preferences in Therapeutically Relevant Sulfated Xylooligosaccharides

Yen Vo,[a] Brett D. Schwartz,[a]* Hideki Onagi,[a] Jas S. Ward,[a] Michael G. Gardiner,[a] Martin G. Banwell,[a] Keats Nelms[b] and Lara R. Malins [a]*

[a] Dr. Y. Vo, Dr. B. D. Schwartz, Dr. Hideki Onagi, Dr. J S. Ward, Dr. M. G. Gardiner, Prof. M. G. Banwell, Dr. L. R. Malins
Research School of Chemistry
Australian National University
Canberra, ACT 2601, Australia
E-mail: brett.schwartz@anu.edu.au; lara.malins@anu.edu.au
Twitter: @Malins_Lab
[b] Dr. K. Nelms
Beta Therapeutics Pty. Ltd.,
Level 6, 121 Marcus Clarke Street
Canberra, ACT 2601, Australia.

Supporting information for this article is given via a link at the end of the document.

Abstract: Although sulfated xylooligosaccharides are promising therapeutic leads for a multitude of afflictions, the structural complexity and heterogeneity of commercially deployed forms (e.g. Pentosan polysulfate **1**) complicates their path to further clinical development. We describe herein the preparation of the largest homogeneous persulfated xylooligomers prepared to date—comprising up to eight xylose residues—as standards for biological studies. Near quantitative sulfation was accomplished using a remarkably mild and operationally simple protocol which avoids the need for high temperatures and a large excess of the sulfating reagent. Moreover, the sulfated xylooligomer standards so obtained enabled definitive identification of a pyridinium contaminant in a sample of a commercially prepared Pentosan drug and provided significant insights into the conformational preferences of the constituent persulfated monosaccharide residues. As the spatial distribution of sulfates is a key determinant of the binding of sulfated oligosaccharides to endogenous targets, these findings have broad implications for the advancement of Pentosan-based treatments.

Introduction

Sulfation is a powerful regulator of bioactivity.[1] For example, glycosaminoglycans (GAGs), ubiquitous components of the extracellular matrix broadly involved in signaling and development, have complex and diverse carbohydrate sulfation patterns that have been linked to their specific biological functions.[2] The precise array of negatively charged sulfate groups serves to mediate a variety of binding events largely through targeted electrostatic interactions.[3] Accordingly, elucidation of the so-called GAG "sulfation code,"[3,4] including important work regarding 3-*O*-sulfation of heparan,[5] is crucial to unlocking the complexities of GAG biochemistry and has fueled considerable interest in sulfated GAG mimetics as useful therapeutic leads.[6]

A notable example is Pentosan polysulfate **1** (PPS, Figure 1A), a heparan sulfate mimic that is currently the only prescribed therapeutic for interstitial cystitis and is in clinical development

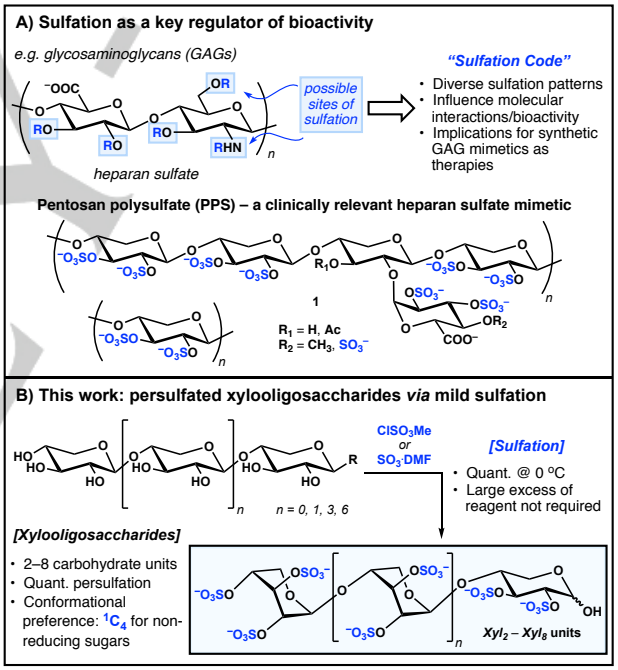


Figure 1. A) The importance of sulfation in glycosaminoglycans (GAGs) and therapeutic GAG mimetics; B) The persulfated xylooligosaccharides accessed in this work.

for the treatment of osteoarthritis. PPS is a complex, heterogeneous and semi-synthetic drug composed of sulfated, β-1→4-linked xylooligosaccharides of varying lengths. The structure is further complicated by intermittent C-2 substitution with sulfated 4-*O*-methyl-α-D-glucuronic acid units. Difficulties isolating the individual components and thereby establishing precise structure-activity relationships represent a major barrier to further clinical development of PPS-based treatments. Moreover, recent reports of retinal toxicity[7] associated with prolonged exposure to PPS warrant urgent examination of its precise composition. Prompted by the immense therapeutic potential of PPS and an on-going medicinal chemistry campaign

in our own laboratory, we sought to prepare homogeneous, low molecular weight sulfated xylooligomers lacking the glucuronic acid branch so as to facilitate detailed biological studies of unbranched oligosaccharides. It was envisioned that such a collection of standards (Figure 1B) would prove valuable in unravelling the Pentosan sulfation code.

Herein, we describe the realization of such objectives through the convergent synthesis of persulfated xylooligosaccharides incorporating up to eight xylose units (Xyl_2–Xyl_8-SO_3, Figure 1B) and so representing the largest single-species sulfated homologues reported to date.[8] This outcome was accelerated by optimization of a mild and quantitative sulfation protocol using methyl chlorosulfate, a largely overlooked reagent that also enabled herein the rapid sulfation of amino acids, steroids, and acid-sensitive substrates. Furthermore, as a result of rigorous NMR spectroscopic studies, insights have been gained into the conformational preferences of Pentosan-derived sulfated xylooligosaccharides. Such information on the spatial orientation of sulfate groups has important implications for computational drug design and the ability of sulfated xylooligosaccharides to engage biological targets for therapeutic benefit.

Results and Discussion

Since uniform persulfation of carbohydrates can be challenging, often requiring elevated temperatures and a large excess of the sulfating agent,[9] conditions leading to the targeted persulfated polysaccharides required careful consideration. Prompted by reports[10] on the successful exhaustive sulfation of xylan with SO_3•pyridine to prepare PPS, we first examined this method. Specifically, xylose **2**, methyl-β-xyloside **3** and xylobiose **4** were each subjected to reaction with SO_3•pyridine at elevated temperature (Figure 2A). Unfortunately, under these conditions, clean persulfation of compounds **2**–**4** was compromised by the covalent addition of pyridine to the reducing ends of these systems and concomitant cleavage of the β-1→4 linkage of xylobiose. The addition of pyridine to the reducing terminus of xylans has recently been observed by others,[11] and our NMR analysis of a commercial sample of a marketed Pentosan drug supports the presence of pyridinium contaminants stemming from the sulfation process (see Figure 6 and pages 56 and 239 –244 of the Supporting Information for further discussion).

On the basis of the foregoing, our attention turned to the use of SO_3•DMF **6**, noted for its high reactivity as a sulfating agent, including in the production of xylan sulfates from plant isolates.[10a] Accordingly, xylobiose **4** was treated with this reagent at –15 °C and after two hours the α- and β-anomeric forms of xylobiose hexasulfate **7** were obtained together with pentasulfate **8**, a reducing sugar, resulting from hydrolysis of the initially formed anomeric sulfate. Interestingly, in the ^{1}H NMR spectrum of the crude persulfated material, the C-1 (anomeric) hydrogen associated with anomer β-**7** (5.80 ppm) appeared as a singlet ($^3J_{H1\text{-}H2}$ = 0-1 Hz) (Figure 2B), as did the C-1` hydrogens of both compounds C-1α **7** and C-1β **7**. The conformational equilibria associated with sulfated monosaccharides have been assessed by Wessel,[12] Nifantiev[13] and Nishida[14] through careful analysis of ^{1}H NMR coupling constants. These studies revealed that a shift from the typical $^4C_1$ to the "inverted" or "all-axial" $^1C_4$ conformation of β-D-substituted xylopyranosides occurs with increasing degrees of sulfation (e.g. sulfation at either *O*-2 and *O*-3 (**11**), or at *O*-2, *O*-3 and *O*-4 (**12**) (Figure 2B) as evidenced by small $^3J_{H1\text{-}H2}$ coupling constants. Moreover, Nishida postulated that the characteristic 2,3-di-*O*-sulfate pattern in the β-1→4-xyloglycan structure of Pentosan PS **1** may reflect a $^1C_4$ conformational preference among the constituent xylose units.[14] However, the complexity of the ^{1}H NMR spectral data displayed by Pentosan PS mixtures limits the "readable" coupling constant data and so this proposal remains to be verified. Accordingly, elucidating the conformational preferences of individual Pentosan-type persulfated xylooligosaccharides emerged as a second aim of our work, with the targeted Xyl_2-SO_3 **8**, Xyl_3-SO_3 **13**, Xyl_5-SO_3 **14** and Xyl_8-SO_3 **15** systems expected to serve as highly informative reference standards through which the structure of Pentosan PS itself could be better understood (Figure 5, *vide infra*).

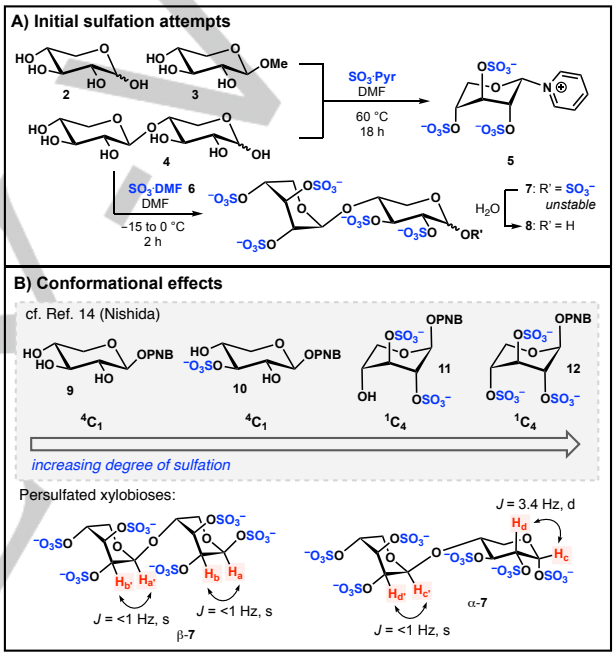


Figure 2. A) Initial sulfation attempts using SO_3•pyridine and SO_3•DMF; B) Conformational effects of increasing sulfation and observed coupling constants for persulfated xylobiose **7**. Note: sulfate counterions are omitted throughout for clarity.

In situ sulfation approach using methyl chlorosulfate

Although SO_3•DMF **6** readily afforded persulfated **7**, before embarking on the synthesis of more complex polysulfated oligosaccharides, we sought to revisit viable approaches to exhaustive sulfation. A variety of SO_3•amine complexes[15] are commonly[16] exploited for this purpose. However, the majority of these require the application of elevated temperatures (e.g. >70 °C) and large excesses of the reagent (5-10 equiv. per OH group) to ensure high degrees of sulfation,[9] prompting the exploration of microwave-assisted[17] and acid-catalyzed approaches,[18] as well as the judicious use of co-solvents.[8b,19] The reactivity of the SO_3 complex can be roughly correlated to the Lewis basicity of the amine, with stronger bases forming more stable and thus less reactive complexes.[15] Given the weak basicity of DMF, the efficacy of SO_3•DMF at low temperatures in our initial study is unsurprising. However, as

SO_3•DMF is typically prepared by treating DMF with sulfur trioxide,[20] we sought to identify an operationally simpler protocol. A seemingly neglected entry in a patent published in 1951[21] on the sulfation of leuco dyes led us to explore this process for the *in situ* production of SO_3•DMF. Accordingly, dropwise addition of methyl chlorosulfate **16**[22,23] to ice-cold DMF resulted in an exothermic reaction with notable gas evolution due to the production of chloromethane.[24] Crystals were obtained from the reaction mixture after 48 h at 4 °C and a single-crystal X-ray analysis provided the first confirmation of the structure of SO_3•DMF (Figure 3).

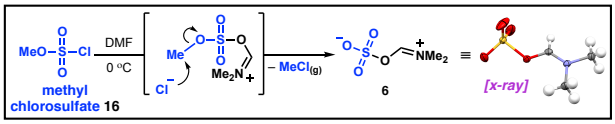
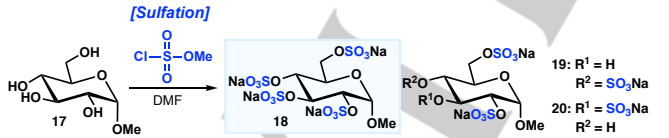


Figure 3. Plausible mechanism of SO_3•DMF formation from methyl chlorosulfate in DMF.

To test the feasibility and efficiency of a one-pot sulfation protocol with SO_3•DMF generated *in situ* from methyl chlorosulfate and DMF, methyl-α-glucoside **17** was chosen as a model substrate and subjected to various conditions (Table 1). In particular, addition of near stoichiometric amounts of methyl chlorosulfate (1.1 equiv. per OH group) at 0 °C for 1 h afforded 47% of the tetrasulfate **18**, with the remaining material comprised of trisulfates **19** and **20**. Increasing the number of equivalents of $ClSO_3Me$ per OH residue to 1.5 afforded 93% of product **18** after 1 h at 0 °C. Performing the reaction at room temperature led to exhaustive persulfation, as did increasing the equivalents of $ClSO_3Me$ to 2.0 per OH group at 0 °C. Drawbacks of this process include the production of sodium methyl sulfate and dimethylamine generated through exposure of the reagent to adventitious moisture and quenching of the reaction mixture with sodium bicarbonate. Nevertheless, dimethylamine bound as the conjugate base can be readily removed by ion-exchange and sodium methyl sulfate can be removed by either flash chromatography or size-exclusion chromatography, especially during desalting (see Supporting Information for details).

Table 1. Optimization of sulfation conditions.



Entry	Equiv. $ClSO_3Me$ per OH group	*t* (min)	Reaction temp.	Conversion (%)[a] 18	19	20
1	1.1	60	0 °C	47	21	32
2	1.5	60	0 °C	93	3	4
3	1.5	10	21 °C	99	<0.5	<0.5
4	2.0	60	0 °C	99	<0.5	<0.5

[a]Determined by ^{1}H NMR analysis; [b]Starting temperature before addition of $ClSO_3Me$

With optimal sulfation and purification procedures established, a small suite of substrates (Figure 4), including amino acids (Ser, **21** and Tyr, **22**), a disaccharide (**23**), and steroids (**24** and **25**) was subjected to these conditions. No erosion of optical purity was observed for Fmoc-L-**21** (see Supporting Information, pages 16 and 57), and even acid-sensitive substrates (e.g. the precursor to **26**) could be sulfated in high yield using a slightly modified protocol involving pre-treatment of the starting material with DIEA. The successful sulfation of the bioisosteric cubane motif[25] to afford product **27** was confirmed by single-crystal X-ray analysis.

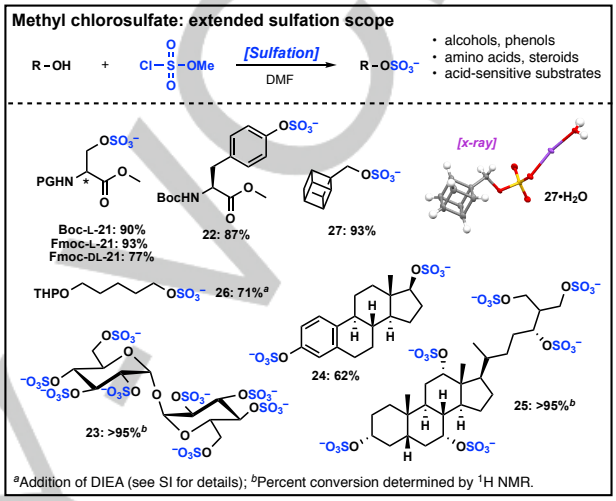


Figure 4. Scope of sulfation with methyl chlorosulfate. Note: sulfate counterions are omitted throughout for clarity.

Preparation of pentasulfo-xylobiose (Xyl_2-SO_3) and heptasulfo-xylotriose (Xyl_3-SO_3) standards

Having established an efficient sulfation protocol, we next turned our attention to the synthesis of the target oligosaccharide reference standards. Syntheses of Xyl_2-SO_3 **8** and Xyl_3-SO_3 **13** were greatly simplified by starting with an inexpensive, commercially available xylooligomeric mixture (Xyl_2 to Xyl_5) derived from corncobs.[26] Exhaustive acetylation of this mixture[8a] followed by flash chromatography provided substantial amounts of the xylobiose and xylotriose peresters **28** and **29**, respectively (see Figure 5 and Supporting Information for details).

With these suitable building blocks in hand, a protecting group strategy was devised to overcome product instability resulting from sulfation at C-1, as observed in our initial studies (see Figure 2A). It was envisioned that global sulfation of oligosaccharides bearing anomeric *O*-benzyl (*path a*) or *S*-tolyl (*path b*) groups could be followed by selective deprotection to deliver the target reducing sugars (Figure 5A). Following *path a* in the first instance, anomeric deacetylation followed by Schmidt glycosylation with benzyl alcohol provided compound **31** after global deacetylation (Figure 5A). Treatment of **31** with $ClSO_3Me$ (–30 to > 0 °C) in DMF in an operationally simple sulfation process afforded the β-benzylated pentasulfo-species **32** that was cleanly hydrogenolyzed to provide target **8**.

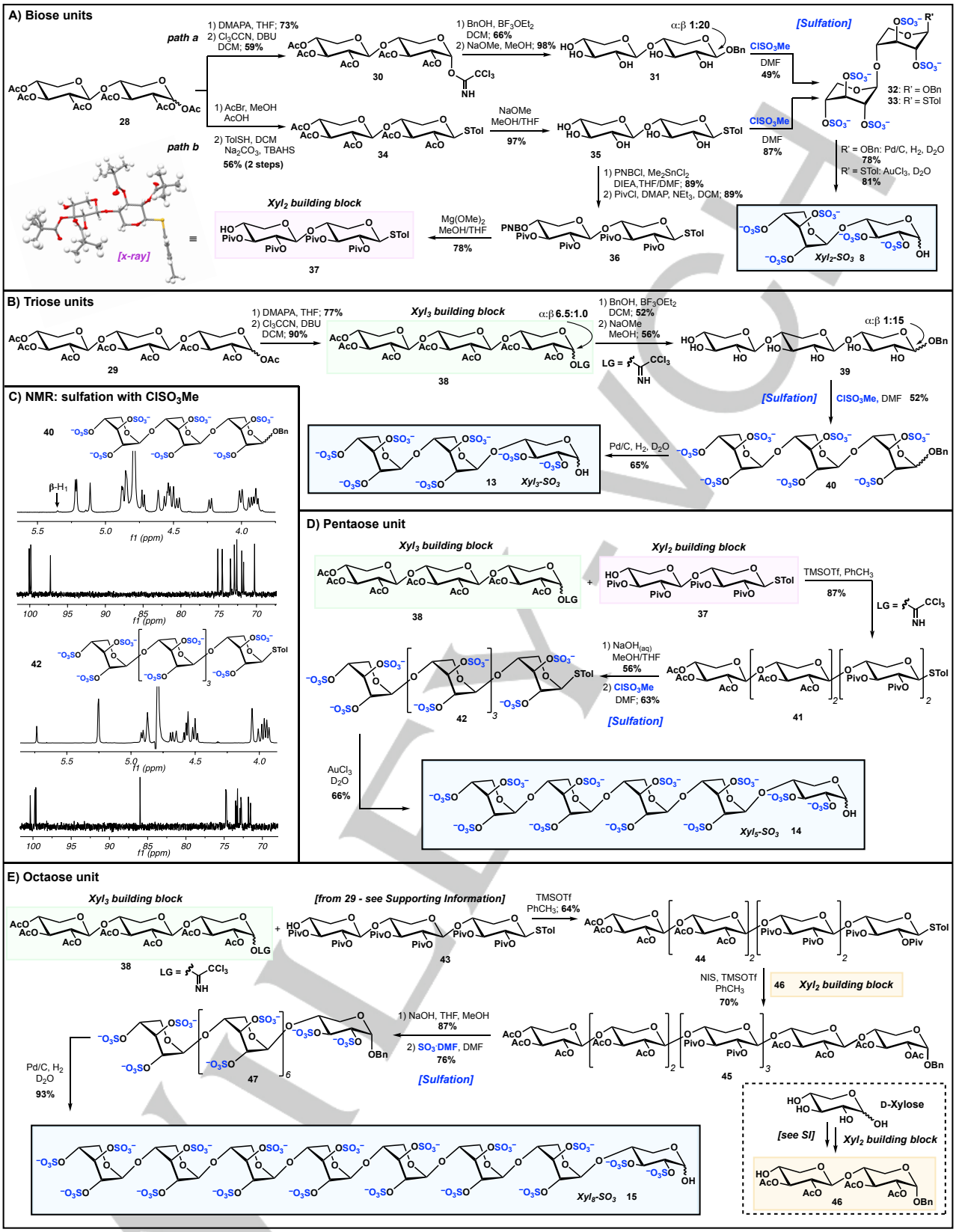


Figure 5. A) Synthesis of sulfated xylooligosaccharide standards; A) xylobiose units; B) xylotriose units; C) ^{1}H and ^{13}C NMR spectra of sulfated derivatives; D) xylopentaose unit; E) xylooctaose unit. Note: sulfate counterions are omitted throughout for clarity.

Access to the β-thiotolyl-xylobioside **35** was accomplished through the intermediate glycosyl bromide (*path b*). Saponification of the resulting penta-acetate **34** gave polyol **35** that was itself smoothly persulfated with methyl chlorosulfate in DMF to give **33**. The anomeric hydroxyl was installed through hydrolysis of the glycosidic C–S bond using Au(III) chloride,[27] forming the reducing sugar **8**. The *p*-toluene thiol-derived by-products produced in this final step, though initially troublesome, were removed using extraction, trituration and size-exclusion chromatography techniques. The spectral data derived from product **8** obtained by either route were identical, in all respects, and confirmed the near quantitative conversion in the key sulfation step, thus validating the utility of our *in situ* sulfation approach. The presence of the persulfated product was supported by mass spectrometry (ESI$^-$, see Supporting Information for details) and the homogeneity of the product as determined by NMR spectroscopic analysis, is indicative of a high degree of sulfation. Having established this proof of concept, access to the heptasulfo-xylotriose Xyl_3-SO_3 homologue **13** was readily achieved from peracetate **29** using a *O*-benzyl protecting group strategy and *in situ* sulfation with methyl chlorosulfate in DMF (Figure 5B; see Figure 5C for representative NMR spectral data for the key sulfation step).

Preparation of undecasulfo-xylopentaose (Xyl_5-SO_3)

Syntheses of homologues of xylose higher than xylotriose have rarely been pursued.[28,29,30] We envisaged that the five-unit β-1→4-xylooligomer could be accessed through union of the Xyl_2 acceptor **37** and the Xyl_3 donor **38** (Figure 5D). Conversion of tolyl-1-thio-β-xylobioside (**35**) to the acceptor **37** was achieved via a selective protection of the C-4'-OH as the *p*-nitrobenzoyl ester followed by pivaloyl protection of the remaining hydroxyl groups to give polyester **36** (Figure 5A). Selective deprotection of the C-4'-*p*-PNB group using $Mg(OMe)_2$ afforded target **37**, the structure of which was confirmed by single-crystal X-ray analysis. Glycosylation of compound **37** with the Xyl_3 donor **38** under Schmidt conditions then provided the fully protected xylopentaose **41** (Figure 5D). Analysis of the ^{1}H NMR spectrum of compound **41** revealed that the five H-1/H-2 hydrogens at the anomeric centers displayed coupling constants in the range of 5.9 to 9.3 Hz, consistent with their di-axial dispositions and so confirming β-1→4 linkages throughout the length of the xylopentaose scaffold. Compound **41** was deacylated under standard conditions and the ensuing polyol sulfated *in situ* using $ClSO_3Me$/DMF. Under such conditions the undecasulfo-derivative **42** was obtained, and after treatment with Au(III) chloride, the desired reducing sugar **14** was isolated in high purity and with a high degree of sulfation (see Figure 5C for select NMR data).

Preparation of heptadecasulfo-xylooctaose (Xyl_8-SO_3)

A Xyl_3 + Xyl_3 + Xyl_2 building-block approach was devised to allow rapid access to the Xyl_8 system (Figure 5E). The latent[31] thioglycoside acceptor **43**,[32] which acts as a linchpin for accessing the Xyl_8 system, was prepared in a fashion analogous to congener **37** from peracetylated xylotriose **29** (see Supporting Information for details). Reaction of compound **43** with the Xyl_3 trichloroacetimidate **38** employing Schmidt glycosylation conditions provided the hexasaccharide **44**. Activation of this with *N*-iodosuccinimide and trimethylsilyl trifluoromethanesulfonate in toluene followed by addition of the benzyl-α-xylobioside acceptor **46**[33] afforded the protected Xyl_8 **45** in 70% yield. Saponification of the associated acetate and pivalate groups provided the corresponding polyol that was sulfated directly with SO_3•DMF, owing to the availability of the reagent in the laboratory, to give product **47**. To complete the synthesis, hydrogenolysis of an aqueous solution of compound **47** under standard conditions afforded the heptadecasulfo-Xyl_8 reducing sugar **15**, ^{1}H and ^{13}C NMR spectral analysis of which provided data consistent with those reported for sulfated, non-glucuronic acid branched pentosans.[11a,b,34]

Sulfation of pyridinium xylotriose and a pentosan dosing study

With the ultimate goal of deconvoluting the complex structure-activity relationships of individual components of Pentosan PS-based therapies, and given the troubling identification of pyridinium artefacts in commercial Pentosan treatments, we next turned our attention to the synthesis of a persulfated pyridinium standard (Figure 6). Prior NMR studies have established a covalent linkage of the pyridinium motif to the reducing end of sulfated xylans,[11a,b,d] resulting from global sulfation of xylan using SO_3•pyridine complex. Nevertheless, a discrete sulfated xylooligosaccharide standard bearing an anomeric pyridinium moiety has not been reported. Given that minor impurities such as these may be implicated in the emerging retinal toxicity associated with long-term PPS exposure,[7] we envisaged that such a synthetic standard could facilitate detailed biological assays and safety studies.

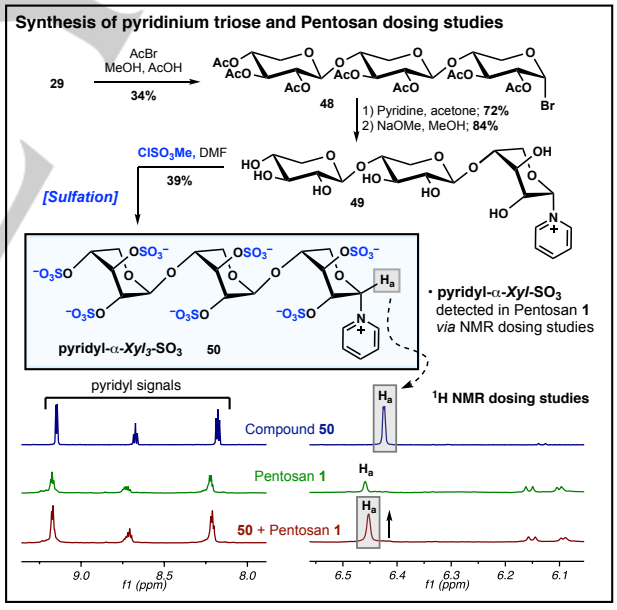


Figure 6 Synthesis of persulfated pyridinium xylotriose **50** and confirmation of pyridinium artefacts in a commercial sample of a Pentosan drug. Note: sulfate counterions are omitted throughout for clarity.

Accordingly, we carried out the synthesis of sulfated pyridinium triose **50** from peracetate **29**. Conversion of the latter into the α-bromide **48**, followed by reaction of this with pyridine[11c] and global deacetylation readily afforded the anomeric pyridinium species **49**. Sulfation of this last compound with $ClSO_3Me$ in DMF afforded the persulfated α-pyridinium compound **50** that was subject to full characterization. Dosing studies, undertaken

using NMR spectroscopic methods and in which a commercial sample of a Pentosan-based drug was treated with an authentic sample of sulfated standard **50**, confirmed the presence of an α-pyridinium contaminant in Pentosan PS, as indicated by an increase in intensity of the resonance attributed to the anomeric H-1 proton (see H_a in Figure 6 and Supporting Information for further details). As such, using compound **50** as a reference standard provides a valuable means for identifying impurities present in samples of Pentosan PS currently used in the clinic.

Conformational analysis of sulfated xylooligosaccharides in D_2O

The number and distribution of sulfate groups in glycosaminoglycans (GAGs) is an important determinant of biological activity that follows, *inter alia*, from the impact of such residues on molecular conformation. Such matters have attracted significant attention, particularly as they pertain to heparin and heparan sulfate.[35] The conformational impacts of sulfate groups in sugars, which have been largely elucidated through ^{1}H-^{1}H coupling constant analyses of ^{1}H NMR spectra,[12-14,35b,36] reveal intriguing conformational consequences arising from sulfation. Using high-field, 2D NMR spectroscopic techniques, it was possible to fully assign the ^{1}H and ^{13}C resonances observed for the sulfated xylobiose and xylotriose standards, these serving as robust models for their higher homologues (see Supporting Information for full assignments).

For example, the 700 MHz ^{1}H NMR spectrum of α-Xyl$_3$-SO$_3$ **13** (Figure 7) revealed that the *non-reducing* xylose units exist preferentially in the ^{1}C$_4$ conformation as indicated by a series of resonances with small to negligible couplings arising from the ring hydrogens [with the exception of geminal couplings between the xylose methylene hydrogens (e.g. H_g, H_h, Figure 7)]. For example, a coupling of 1.2 Hz was observed between H-1' (H_d) and H-2' (H_e) while H-1'' (H_i) appeared as an apparent singlet. The signal attributed to H-2' (H_e) was observed as an apparent triplet and exhibited a coupling with H-3' (H_f) of 2.4 Hz. Sufficient dispersion of signals allowed for detection of the anticipated W-couplings between H-5$_a$' (H_h) and H-3' (H_f) (1.7 Hz, app. dt) and between H-5$_a$'' (H_n) and H-3''(H_k) (1.6 Hz, app. dt) (Figure 7).

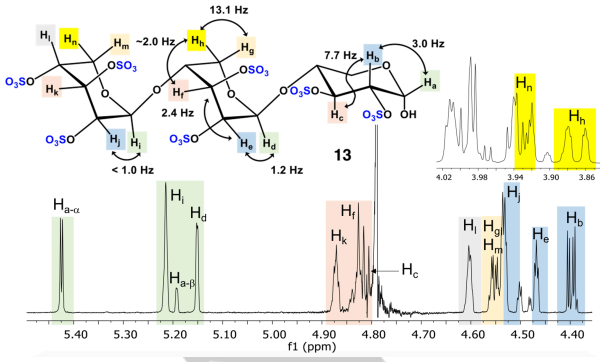


Figure 7. Selected region of the ^{1}H NMR spectrum of compound **13**. Note: negative charges and counterions on sulfates omitted for clarity.

Furthermore, the sulfated, β-configured precursors (**32**, **33**, **40**, and **42**) all exhibited apparent singlets for the anomeric hydrogens throughout the oligomer ($J_{H1\text{-}H2}$ = < 1 Hz) (see Table 2 for a summary of all anomeric H1-H2 couplings of sulfated xylooligomers prepared in this study) as well as small vicinal couplings (< 2 Hz) for discernable resonances due to the remaining ring protons. The all axial orientations of the sulfate groups implied by such a series of couplings likely serves to minimize electrostatic repulsion that would otherwise be associated with negatively charged sulfates occupying neighboring equatorial positions in a ^{4}C$_1$ conformation.[37] Such results also align with observations made by others for monosaccharide systems[12-14] and provide strong evidence in support of Nishida's hypothesis that the β-1→4-xyloglycan structure of Pentosan PS is dominated by a ^{1}C$_4$ conformational preference.

The situation is distinct for the third pyranose ring, namely the *reducing end* of α-Xyl$_3$-SO$_3$ **13**. A coupling constant between H-1 (H_a, Figure 7) and H-2 (H_b) of $J_{H1\text{-}H2}$ = 3.0 Hz is in accord with an all equatorial arrangement of the sulfate residues in an α-configured ^{4}C$_1$ system that is presumably dictated by the anomeric effect (cf. α-xylobiose **4** in the Supporting Information; $J_{H1\text{-}H2}$ = 3.7 Hz). Coupling constants between H-2 and H-3 (7.7 Hz, H_b-H_c), and H-4 and H-5$_a$ (7.4 Hz) also support an equilibrium favoring the ^{4}C$_1$ conformer. Such findings are consistent with observations made for the analogous, persulfated methyl-α-xyloside.[12] In the homologous α-Xyl$_5$ **14** and α-Xyl$_8$ **15** systems, the reducing end anomeric H-1-H-2 couplings were also found to be consistently ~3.0 Hz (see Table 2).

Table 2. Selected coupling constants for xylooligosaccharide derivatives.

Compound	Residue	$J_{H\text{-}1/H\text{-}2}$ (Hz)	Compound	Residue	$J_{H\text{-}1/H\text{-}2}$ (Hz)
α-xylobiose **4**	1	3.7	Bn-β-Xyl$_3$-SO$_3$ **40**	1	<1.0
β-xylobiose **4**	1	7.9		2–3	<1.0
	2	7.8	Xyl$_3$-SO$_3$ **13**	1	3.0
Bn-β-Xyl$_2$-SO$_3$ **32**	1	<1.0		2–3	<1.0
	2	<1.0	STol-β-Xyl$_5$-SO$_3$ **42**	1	<1.0
STol-β-Xyl$_2$-SO$_3$ **33**	1	<1.0		2–5	<1.0
	2	<1.0	α-Xyl$_5$-SO$_3$ **14**	1	3.0
α-Xyl$_2$-SO$_3$ **8**	1	2.9		2–5	<1.0
	2	<1.0	Bn-α-Xyl$_8$-SO$_3$ **47**	1	3.5
β-Xyl$_2$-SO$_3$ **8**	1	3		2	1.0
	2	<1.0		3–8	<1.0
pyridyl-α-Xyl$_3$-SO$_3$ **50**	1	<1.0	α-Xyl$_8$-SO$_3$ **15**	1	2.9
	2–3	<1.7		3–8	<1.0

Interestingly, the minor, H-1 β-anomer of Xyl$_3$ **13** exhibited a coupling between H-1 and H-2 of 3.0 Hz, while couplings of 3.0 Hz between H-4 and either H-5$_a$ or H-5$_b$ were also observed. These data imply an equilibrium favoring the "inverted" ^{1}C$_4$ conformer for the *reducing end* of the minor C-1-β anomer. Collectively, these findings provide considerable insight into the spatial display of sulfate groups in persulfated xylooligosaccharides and are likely to have important implications for biological and computational studies on the binding interactions of these molecules.

Conclusion

The present study describes an efficient synthetic route to the largest single-species sulfated xylooligomers reported to date. As key constituents of the therapeutically-relevant sulfated xylooligosaccharide drug Pentosan PS, these synthetic standards were targeted as valuable tools for deconvoluting the composition of the complex, heterogeneous nature of this therapeutically deployed material. Towards this objective, near-quantitative sulfation of xylooligomers was accomplished through the use of methyl chlorosulfate in DMF as a mild and operationally simple approach to the *in situ* generation of SO_3•DMF. This sulfation protocol was extended to a variety of aliphatic and aromatic alcohols, encompassing amino acids, steroids and acid-sensitive substrates. Its use in the preparation of a novel sulfated and pyridinium-ion containing xylotriose facilitated the identification of anomeric α-pyridinium contaminants in a commercial source of Pentosan PS **1**—a finding which may have important implications for enhancing the safety profile of treatments employing this material. Finally, detailed conformational analyses of certain sulfated xylooligosaccharides revealed a preference for the $^1C_4$ conformer in all associated non-reducing residues. Given the importance of sulfate arrays in GAG binding interactions, this structural information is expected to provide a means for the development of PPS-type drugs with improved therapeutic profiles. Studies probing the biological activity of the discrete Pentosan "standards" described above are currently underway in our laboratories.

Experimental Section

See supporting information for experimental details.

Acknowledgements

We are grateful to Beta Therapeutics Pty. Ltd. for providing funding for this work. We thank Dr. Tristan Reekie (ANU) for providing a sample of cubylmethanol. We would also like to acknowledge Ms. Anitha Jeyasingham and Mr. Joseph Boileau (ANU) for assistance with mass spectrometry, Dr. Chris Blake (ANU) for support with NMR spectroscopy, Mr. Daniel Bartkus and Dr. Nicholas Kanizaj (ANU) for technical assistance, and Mr. Christopher Fitzgerald and Assoc. Prof. Malcolm McLeod for useful discussions.

Keywords: carbohydrates • conformation analysis • pentosan • persulfation • xylooligosaccharides

[1] Steroids: (a) J. W. Mueller, L. C. Gilligan, J. Idkowiak, W. Arlt, P. A. Foster, *Endocr. Rev.* **2015**, *36*, 526-563; Tyr Sulfation: (b) Y.-S. Yang, C.-C. Wang, B.-H. Chen, Y.-H. Hou, K.-S. Hung, Y.-C. Mao, *Molecules* **2015**, *20*, 2138-2164; (c) M. J. Stone, R. J. Payne, *Acc. Chem. Res.* **2015**, *48*, 2251-2261.

[2] D. Soares da Costa, R. L. Reis, I. Pashkuleva, *Annu. Rev. Biomed. Eng.* **2017**, *19*, 1-26.

[3] (a) C. I. Gama, S. E. Tully, N. Sotogaku, P. M. Clark, M. Rawat, N. Vaidehi, W. A. Goddard, A. Nishi, L. C. Hsieh-Wilson, *Nat. Chem. Biol.* **2006**, *2*, 467-473; Also see for example, a review on heparan sulfate-protein interactions: (b) D. Xu, J. D. Esko, *Annu. Rev. Biochem.* **2014**, *83*, 129-157.

[4] H. Habuchi, O. Habuchi, K. Kimata, *Glycoconj. J.* **2004**, *21*, 47-52.

[5] (a) P. Chopra, A. Joshi, J. Wu, W. Lu, T. Yadavalli, M.A. Wolfert, D. Shukla, J. Zaia, G-J. Boons, *Proc. Natl. Acad. Sci. U.S.A.* **2021**, *118* (3) e2012935118; (b) B. E. Thacker, D. Xua, R. Lawrence, J. D. Esko, *Matrix Biol.* **2014**, *35*, 60-72.

[6] (a) B. Ernst, J. L. Magnani, *Nat. Rev. Drug Discov.* **2009**, *8*, 661-677; (b) R. A. Scott, A. Panitch, *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* **2013**, *5*, 388-398.

[7] (a) W. A. Pearce, R. Chen, N. Jain, *Ophthalmology* **2018**, *125*, 1793-1802; (b) A. M. Hanif, R. Shah, J. Yan, J. S. Varghese, S. A. Patel, B. E. Cribbs, G. O'Keefe, A. M. Hendrick, J. G. Shantha, G. B. Hubbard, P. S. Patel, P. Rao, S. Yeh, N. Jain, *Ophthalmology* **2019**, *126*, 1464-1466; (c) M. J. Wingelaar, J. J. Raevis, K. A. Conlin, K. E. Stepien, *Urology* **2020**, *141*, e41-e42.

[8] (a) For partially sulfated methyl-β-xylobioside and methyl-β-xylotrioside see: B. Abad-Romero, K. Mereiter, H. Sixta, A. Hofinger, P. Kosma, *Carbohydr. Res.* **2009**, *344*, 21-28; (b) Persulfated D-xylobiose and methyl-β-D-xylobioside have recently been reported: C. H. O. Meara, L. A. Coupland, F. Kordbacheh, B. J. C. Quah, C.-W. Chang, D. A. Simon Davis, A. Bezos, A. M. Browne, C. Freeman, D. J. Hammill, P. Chopra, G. Pipa, P. D. Madge, E. Gallant, C. Segovis, A. F. Dulhunty, L. F. Arnolda, I. Mitchell, L. M. Khachigian, R. W. Stephens, M. von Itzstein, C. R. Parish, *Nat. Commun.* **2020**, *11*, 6408.

[9] R. A. Al-Horani, U. R. Desai, *Tetrahedron* **2010**, *66*, 2907-2918.

[10] (a) S. Daus, K. Petzold-Welcke, M. Kötteritzsch, A. Baumgaertel, U. S. Schubert, T. Heinze, *Macromol. Mater. Eng.* **2011**, *296,* 551-561; (b) K. Tihlarik, E. Lattova, *Chem. Pap.* **1991**, *45,* 547-552.

[11] (a) L. Gabriel, W. Günther, F. Pielenz, T. Heinze, *Macromol. Chem. Phys.* **2020**, *221,* 1900327; (b) A. Alekseeva, R. Raman, G. Eisele, T. Clark, A. Fisher, S. Lee, X. Jiang, G. Torri, R. Sasisekharan, S. Bertini, *Carbohydr. Polym.* **2020**, *234,* 115913; (c) R. Sagar, S. Rudić, D. P. Gamblin, E. M. Scanlan, T. D. Vaden, B. Odell, T. D. W. Claridge, J. P. Simons, B. G. Davis, *Chem. Sci.* **2012**, *3,* 2307-2313; (d) L. Ahrgren, A. N. de Belder, T. Mälson, *Carbohydr. Polym.* **1991**, *16,* 211-214.

[12] K. C. Probst, H. P. Wessel, *J. Carbohydr. Chem.* **2001**, *20,* 549-560.

[13] A. G. Gerbst, V. B. Krylov, N. E. Nifantiev, *Pure Appl. Chem.* **2019**, *91,* 1223-1229.

[14] T. Nagatsuka, H. Uzawa, Y. Nishida, *Chem. Commun.* **2009**, *27,* 4109-4111.

[15] E. E. Gilbert, *Chem. Rev.* **1962**, *62,* 549-589.

[16] Common reagents include: SO_3•pyridine, SO_3•NMe_3, SO_3•NBu_3: (a) D. M. Gill, L. Male, A. M. Jones, *Chem. Commun.* **2019**, *55*, 4319–4322; (b) J. A. Alshehri, A. M. Benedetti, A. M. Jones, *Sci. Rep.* **2020**, *10*, 16559.

[17] (a) A. Raghuraman, M. Riaz, M. Hindle, U. R. Desai, *Tetrahedron Lett.* **2007**, *48*, 6754-6758; (b) S. Maza, J. L. de Paz, P. M. Nieto, *Tetrahedron Lett.* **2011**, *52*, 441-443.

[18] V. B. Krylov, N. E. Ustyuzhanina, A. A. Grachev, N. E. Nifantiev, *Tetrahedron Lett.* **2008**, *49*, 5877-5879.

[19] M. von Itzstein, C.-W. Chang, Patent WO 2019113646A1. *Sulfation method*, **2019**.

[20] SO_3•DMF is commercially available but is only intermittently available in Australia. The reagent can be freshly prepared according to literature protocols: (a) M. L. Wolfrom, T. M. Shen Han, *J. Am. Chem. Soc.* **1959**, *81*, 1764-1766; (b) W. L. Garbrecht, *J. Org. Chem.* **1959**, *24*, 368-372; (c) D. W. Clayton, J. A. Farrington, G. W. Kenner, J. M. Turner, *Chem. Soc.* **1957**, 1398-1407.

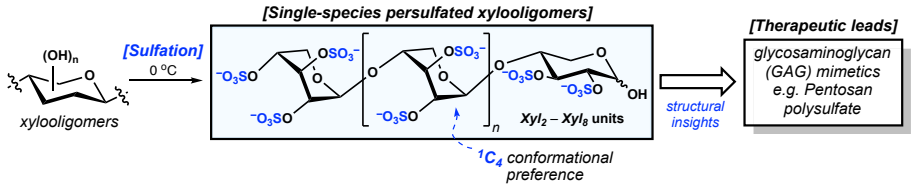
[21] S. Coffey, D. A. W. Fairweather, D. E. Hathaway, F. H. Slinger, U. S. Patent 2,563,819, **1951**; Chem. Abstracts, **1951**, 45, 9881

[22] Despite its ease of preparation, very few uses of methyl chlorosulfate have been reported. See for example: (a) M. S. Heller, D. P. Lorah, C. P. Cox, *J. Chem. Eng. Data* **1983**, *28*, 134-137; (b) R. J. Cremlyn, L. Wu, *Phosphorus Sulfur Relat. Elem.* **1988**, *39*, 165-171. (c) C. Chatgilialoglu, D. Griller, S. Rossini, *J. Org. Chem.* **1989**, *54*, 2734-

2737; (d) S. Silva, C. D. Maycock, *Tetrahedron Lett.* **2018**, *59*, 1233-1238.
[23] W. W. Binkley, E. F. Degering, *J. Am. Chem. Soc.* **1938**, *60*, 2810-2811.
[24] Volatiles captured in a balloon were then sparged into $CDCl_3$. Analysis of the resulting solution by ^1H NMR revealed a single resonance appearing at 3.01 ppm, consistent with that reported for chloromethane: M. J. Lacey, C. G. Macdonald, A. Pross, J. S. Shannon, S. Sternhell, *Aust. J. Chem.* **1970**, *23*, 1421-1429.
[25] T. A. Reekie, C. M. Williams, L. M. Rendina, M. Kassiou, *J. Med. Chem.* **2019**, *62*, 1078-1095.
[26] XOS-95P, Shandong Longlive Bio-technology Co., Ltd., Qingdao, China.
[27] A. M. Vibhute, A. Dhaka, V. Athiyaratha, K. M. Sureshan, *Chem. Sci.* **2016**, *7*, 4259-4263.
[28] K. Takeo, Y. Ohguchi, R. Hasegawa, S. Kitamura, *Carbohydr. Res.* **1995**, *278*, 301-313.
[29] M. J. Pedersen, R. Madsen, M. H. Clausen, *Chem. Commun.* **2018**, *54*, 952-955.
[30] B. Abad-Romero, D. Haltrich, A. Potthast, T. Rosenau, H. Sixta, P. Kosma, *Macromol. Symp.* **2006**, *232*, 93-97.
[31] R. Roy, F. O. Andersson, M. Letellier, *Tetrahedron Lett.* **1992**, *33*, 6053-6056.
[32] Prepared from peracetylated xylotriose **29** by employing a sequence analogous to that used to prepare xylobiose acceptor **37** - See Supporting Information.
[33] Prepared from D-xylose – see Supporting Information.
[34] L. De Ferra, A. Naggi, M. Zenoni, B. Pinto, Patent WO 2014114723 A1: Method for the qualification of preparations of pentosan polysulfate, raw materials and production processes thereof, **2014**.
[35] (a) A. Canales, J. Angulo, R. Ojeda, M. Bruix, R. Fayos, R. Lozano, G. Giménez-Gallego, M. Martín-Lomas, P. M. Nieto, J. Jiménez-Barbero, *J. Am. Chem. Soc.* **2005**, *127*, 5778-5779; (b) P.-H. Hsieh, D. F. Thieker, M. Guerrini, R. J. Woods, J. Liu, *Sci. Rep.* **2016**, *6*, 29602. (c) N. S. Gandhi, R. L. Mancera, *Chem. Biol. Drug Des.* **2008**, *72*, 455-482.
[36] (a) A. G. Gerbst, V. B. Krylov, D. A. Argunov, A. S. Solovev, A. S. Dmitrenok, A. S. Shashkov, N. E. Nifantiev, *Carbohydr. Res.* **2016**, *436*, 20-24; (b) H. P. Wessel, *J. Carbohydr. Chem.* **1992**, *11*, 1039-1052.
[37] Wessel and Bartsch postulated that β-methyl tetrasulfoglucopyranoside exists preferentially in a $^1C_4$ conformation but that replacement of the negatively charged sulfates with sulfamoyl groups (as neutral sulfate mimics) leads to a favoring of the $^4C_1$ conformation: (a) H. P. Wessel, S. Bartsch, *Carbohydr. Res.* **1995**, *274*, 1-9; Later computational studies suggest that β-methyl tetrasulfoglucopyranoside exists as an equilibrium of $^1C_4$ and skew-boat conformers: (b) A. G. Gerbst, V. B. Krylov, D. A. Argunov, A. S. Dmitrenok, N. E. Nifantiev, *ACS Omega* **2019**, *4*, 1139–1143.

10.1002/chem.202100527

Entry for the Table of Contents



Preparation of the largest homogeneous persulfated xylooligomers reported to date is facilitated by a rapid and quantitative one-pot sulfation protocol based on the *in situ* generation of SO_3•DMF. The synthesized oligosaccharide standards afforded unique structural insights into persulfated xylooligomers, including a $^1C_4$ conformational preference for all associated non-reducing residues. Application of the method to the synthesis of a sulfated pyridyl-α-xylotriose also enabled identification of a pyridinium contaminant in a commercial sample of pentosan polysulfate, a widely-employed sulfated xylooligosaccharide-based therapy.

Institute and/or researcher Twitter usernames: @Malins_Lab