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Modified minimal-size fragments of heparan sulfate as inhibitors of endosulfatase-2 (Sulf-2)†

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Sulf-2 has been identified as a putative target for anticancer therapies. Here we report the design and synthesis of sulfated disaccharide inhibitors based on IdoA(2S)-GlcNS(6S). Trisulfated disaccharide inhibitor IdoA(2S)-GlcNS(6Sulfamate) demonstrated potent Sulf-2 inhibition. The IC_{50} value was determined to be 39.8 μM ± 18.3, which is comparable to a tetrasaccharide inhibitor of HSulf-1 reported in the literature. We propose that the disaccharide IdoA(2S)-GlcNS(6S) is the shortest fragment size required for effective inhibition of the Sulfs.

Endosulfatases (Sulf-1 and Sulf-2) are located in the extracellular matrix and are responsible for the selective desulfation of the sulfate group on the glucosamine 6-*O*-sulfate residues within heparan sulfate (HS) proteoglycans and have a strong substrate specificity for the [Glc/IdoA(2S)-GlcNS(6S)] trisulfated disaccharide (Fig. 1).[1a,b] The trisulfated disaccharide [Glc/IdoA(2S)-GlcNS(6S)] has a low abundance within HS, and therefore seemingly subtle modifications by Sulf activity result in major functional consequences.[2] This highlights the importance of Sulf activity and indicates how targeting the Sulfs could have significant downstream effects on HS-mediated processes. Sulf-2 inhibitors are putative anticancer therapeutics because the sulfs have been linked to the regulation of signalling pathways such as Wnt and FGF *via* the modulation of the 6-*O*-sulfation status of HS.[3] Sulf-2 expression is induced or upregulated in in various cancers and its role has been identified as being pro-tumourgenic, with Sulf-2 gene silencing or knock-out leading to decreased tumour formation. Therefore, Sulf-2 inhibition has been identified as a potential therapeutic target for many cancers.[4a,b] For this reason, the development of endosulfatase inhibitors has gained attention over the past decade.

Scheilwies *et al.* reported glucosamine-based small molecule inhibitors substituting the 6-*O*-sulfate ($-OSO_3^-$) with the sulfamate motif ($-OSO_2NH_2$). This preliminary work utilised the smallest, most relevant unit of HS, α-GlcNS(6S) to template inhibitor design.[5] The biochemical characterisation of this compound in a competition assay with fluorogenic substrate 4-methylumbelliferyl sulfate (4-MUS), revealed that the sulfamate inhibitor had an IC_{50} values of 95 μM against HSulf-1 and 130 μM against HSulf-2, and importantly was more selective for the Sulfs than other sulfatases investigated. In 2015, Miller *et al.* aimed to replicate the inhibitory activity of the glucosamine-6-sulfamate inhibitors and develop a structure activity relationship. All compounds synthesised were found to have minimal inhibition of Sulf-2 at 1 mM.[6] However, there were some discrepancies in assay protocol between the two papers that may explain the different inhibition potencies reported, so the question remains of whether **1** is a true inhibitor of Sulfs. Recently, Chiu *et al.* reported the design and synthesis of di-, tri- and tetra-saccharide fragments of HS with the sulfamate modification as inhibitors of Sulf-1.[7] The disaccharide, GlcNS(6Sulfamate)-IdoA(2S) only caused 20% Sulf-1 inhibition at 0.7 mM (IC_{50} value not determined), and the trisaccharide and tetrasaccharide analogues were more potent with IC_{50} values of 0.53 and 29.6 μM, respectively.

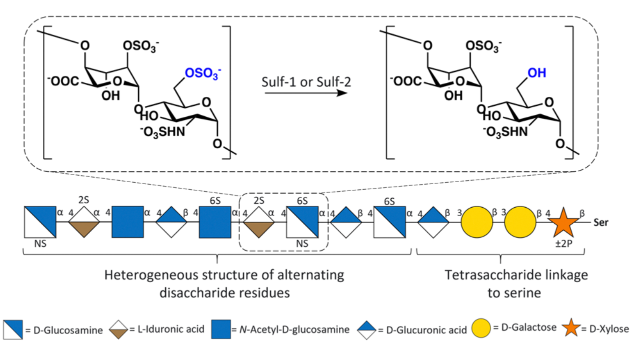

Fig. 1 Structure of HS highlighting the disaccharide residue, IdoA(2S)-GlcNS(6S), that Sulfs have a preference for.

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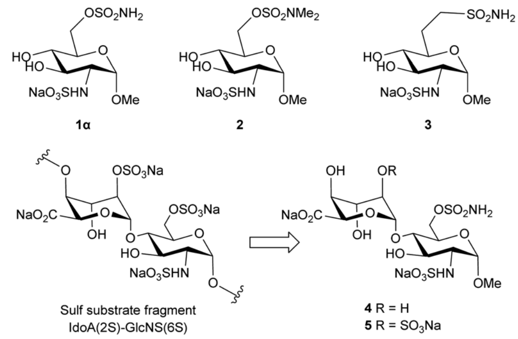


Fig. 2 Structure of putative disaccharide inhibitors.

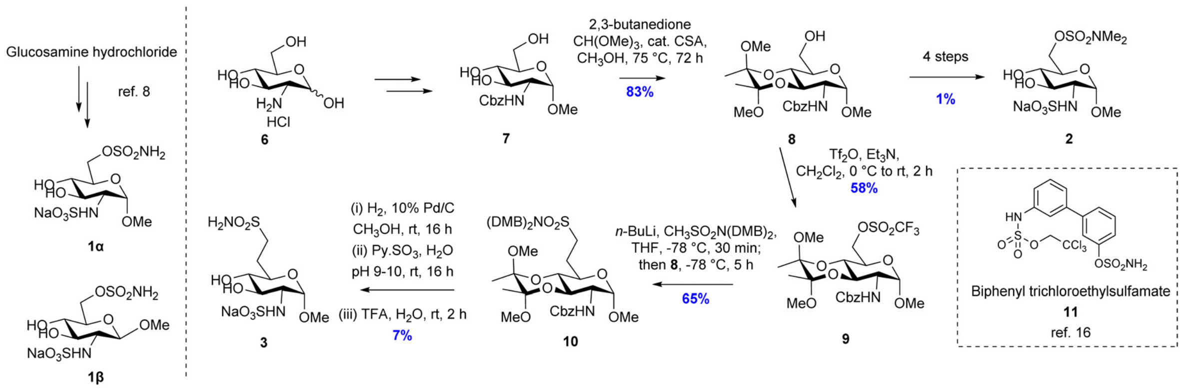
We propose that the IdoA(2S)-GlcNS(6Sulfamate) disaccharide will have superior potency as a minimal fragment Sulf inhibitor compared to the beforementioned reported disaccharide because the substrate specificity studies of the Sulfs point towards this disaccharide unit as being the most frequently desulfated among HS.[8] In this study, we report the re-evaluation of monosaccharide glucosamine-6-*O*-sulfamates as inhibitors of Sulf-2, and the synthesis of putative disaccharide inhibitors using the IdoA(2S)-GlcNS(6S) scaffold as a guide in order to determine whether this fragment size is the shortest effective moiety for HS inhibition.

Putative inhibitors **1** and **2** (Fig. 2) were synthesised according to literature protocols.[5,6] Additionally, a non-hydrolysable analogue **3**, bearing a methylene sulfonamide in the 6-position, was designed and prepared. The key step was the installation of the methylsulfonamide functional group which was achieved *via* nucleophilic substitution of an orthogonally-protected triflate **9**, which was synthesized from glucosamine hydrochloride according to Scheme 1. The 3- and 4-hydroxyls of intermediate **7**[6] were protected by reaction with 2,3-butanedione, trimethyl orthoformate and catalytic sulfonic acid in refluxing methanol, to give **8** as a single diastereomer in 83% yield.

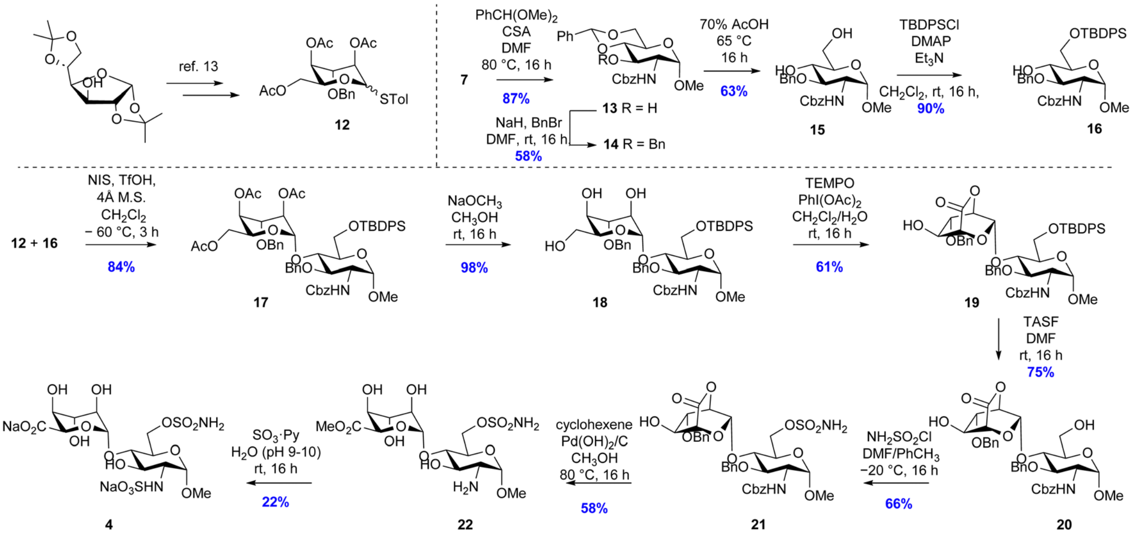
Triflation of **8** was achieved with triflic anhydride in the presence of Et_3N at 0 °C to give **9** in 58% yield. The nucleophile $LiCH_2SO_2(DMB)_2$ was generated *in situ* by deprotonation of methylsulfonamide $CH_3SO_2(DMB)_2$ with *n*-BuLi at −78 °C.[9] The methylene sulfonamide unit was then introduced by nucleophilic substitution of triflate **9** with $LiCH_2SO_2N(DMB)_2$ to give fully protected intermediate **10** in 65% yield. Finally, a three-reaction sequence was performed: (1) the carboxybenzyl group was deprotected in 46% yield by palladium-catalyzed hydrogenation; (2) addition of sulfur trioxide pyridine complex to the amine intermediate in water at pH 9–10 resulted in the sulfation of the amino group in 37% yield; (3) deprotection of the two *N*-2,4-dimethoxybenzyl and 3,4-bisacetal units was achieved using TFA in water in 52% yield. Following purification the putative non-hydrolysable inhibitor **3** was isolated as a sodium salt in 7% yield over three steps.

Two disaccharide inhibitors, **4** and **5**, were designed based on the trisulfated disaccharide fragment of HS identified by Sulf substrate specificity studies, incorporating the 6-*O*-sulfamate group, Fig. 2. Retrosynthetic analysis of inhibitor **4** identified key intermediates *p*-tolyl 2,4,6-tri-*O*-acetyl-3-*O*-benzyl-1-thio-L-idopyranoside **12** prepared from diacetone D-glucose, 6 steps, 22% yield[10] and glucosamine glycosyl acceptor **16**, which was synthesized according to Scheme 2. First, the 4-OH and 6-OH of intermediate **7** were protected using a benzylidene acetal, which formed **13** as a single diastereoisomer in 87% yield. Next, the 3-OH was protected using sodium hydride and benzyl bromide in DMF to obtain **14** in 58% yield. Benzylidene acetal **14** was hydrolysed to **15** using 70% acetic acid at 65 °C (63% yield) and finally, silyl ether formation using *tert*-butyldiphenylchlorosilane gave acceptor **16** in 90% yield.

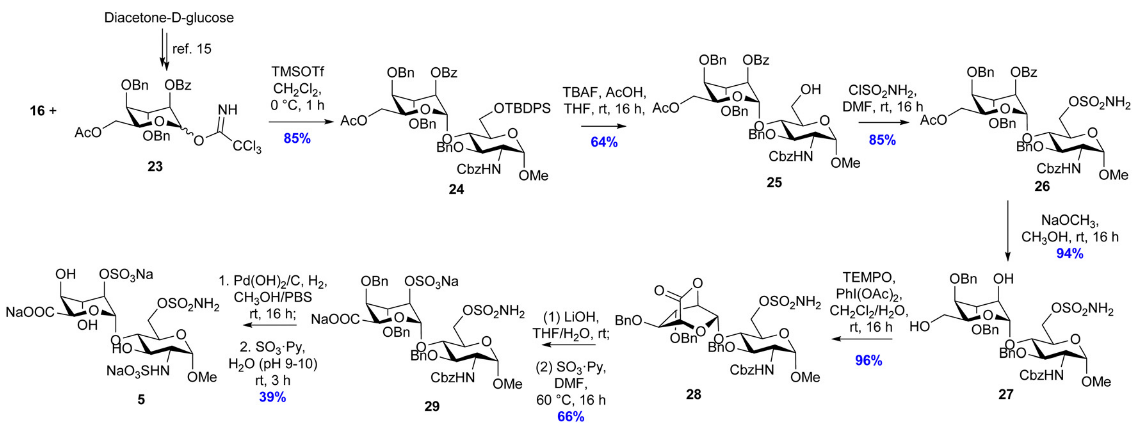
The glycosylation reaction between **12** and **16** was achieved using the NIS/TfOH reagent system to activate the thioglycoside donor to give the desired disaccharide intermediate **17** isolated in 84% yield as a single (alpha) anomer (Scheme 3). Anomeric stereochemistry was assigned by ^1H NMR spectroscopy. Next, the acetate esters were removed under Zemplén conditions to give triol **18** in 98% yield. The primary alcohol was oxidised using catalytic TEMPO and stoichiometric PIDA which produced lactone **19** in 61% yield. Desilylation of compound **19** initially proved challenging due to the instability of the lactone with nucleophiles causing low yields under TBAF deprotection conditions. Even when buffered with acetic acid, only a low yield (45%) of **20** was isolated. The optimised conditions used tris(dimethylamino)sulfonium difluorotrimethylsilicate (TASF) that gave an isolated yield of 75%. Next, regioselective sulfamoylation of **20** was achieved under



Scheme 1 Synthesis of inhibitors **1α**, **1β**, **2** and **3**.



Scheme 2 Synthesis of inhibitor **4**.



Scheme 3 Synthesis of inhibitor **5**.

conditions reported by Miller *et al.*, to give sulfamate **21** in 66% yield. Multiple conditions were trialled for the global debenzylation and deprotection of the amino-Cbz group, and the optimal conditions were found to be catalytic transfer hydrogenation using cyclohexene as the hydrogen donor with 20% $Pd(OH)_2$ in refluxing methanol.[11] Under these conditions, methyl ester **22** was isolated in 58% yield. Finally, the primary amine was sulfated using sulfur trioxide–pyridine complex in basic aqueous medium. Purification by ion-paired reverse-phase HPLC using 2 M triethylammonium bicarbonate and acetonitrile gradient, followed by elution through a Dowex® 50WX8 Na^+-form column, gave **4** in 22% isolated yield.

It was originally envisioned that the synthesis of putative inhibitor **5** could diverge from the synthesis of **4**, *via* benzylation of intermediate alcohol **19**. However, all attempts at benzyl protection of the ido 4-OH of **19** were unsuccessful and therefore idose glycosyl donor **23** was synthesised according to Hu *et al.* (Scheme 3).[12] With the alternative ido-glycosyl donor in hand, the glycosylation reaction between **23** and glycosyl acceptor **16** was activated using TMSOTf and proceeded effectively to afford the desired disaccharide **24** in 85% yield. Desilyation of the 6-*O*-TBDPS group of **24** using TBAF buffered in acetic acid proceeded to afford **25** in 64% yield. Subsequent sulfamoylation of the primary alcohol to **26** was achieved in 74% yield by altering the previous conditions to use 2 equivalents of sulfamoyl chloride at 0 °C. Subsequently, the base-labile protecting groups of compound **26** were removed by catalytic $NaOCH_3$ in CH_3OH to produce diol **27** in 94% yield. The resulting diol **27** was then subjected to oxidation with the TEMPO/PIDA reagent system to afford lactone **28** in 58% yield. **28** was immediately hydrolysed in basic aqueous medium, and the resulting 2-OH moiety was treated with sulfur trioxide–pyridine complex under microwave irradiation. After elution through a Dowex® 50WX8 Na^+-form column, **29** was isolated in 66% yield over two steps. Finally, the hydrogenolysis-labile protecting groups of **29** were cleaved by $Pd(OH)_2/C$ catalysed hydrogenation in methanol and aqueous phosphate buffered saline (20 mM, pH 7.4), to give a primary amine intermediate, which was successively subjected to sulfur trioxide pyridine

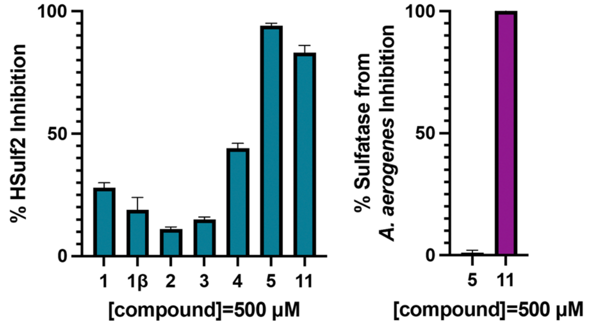


Fig. 3 (left) Sulf-2 and (right) sulfatase from *A. aerogenes* inhibition data for glucosamine-based inhibitors and biphenyl trichloroethylsulfamate **11**. Data represented as the mean ± SD, (n = 2). Inhibition values are reported as percentages of the uninhibited control values.

complex in basic aqueous medium to afford final compound **5** in 39% over two steps as the tri-sodium salt.

The inhibition of HSulf-2 with inhibitors **1**, **2**, **3**, **4** and **5** was determined using a competition assay with purified, recombinant HSulf-2 and a fluorogenic substrate 4-methylumbelliferyl sulfate (4-MUS) (Fig. 3). HSulf-2 shows pro-tumoral behaviour and therefore is a prime target for the design of inhibitors. Compounds were tested at a single concentration (500 μM) to compare inhibitory activity and the IC_{50} value was determined for the most potent inhibitor. Biphenyl trichloroethylsulfamate **11**[13] (Scheme 4), was included as a benchmark Sulf-2 inhibitor. Inhibition of sulfatase from *Aerobacter aerogenes*, a bacterial sulfatase was used to assess selectivity of the compounds.

In the monosaccharide series, parent glucosamine-6-*O*-sulfamate **1** was found to display weak inhibition of 28% at 500 μM, and **1β**, **2** and **3** inhibited Sulf 2 by <15% at 500 μM. As predicted, the extension of fragment size to the disaccharide scaffold led to an increased inhibition at 500 μM. Disulfated disaccharide **4** inhibited Sulf-2 by 44% and trisulfated disaccharide **5** inhibited Sulf-2 almost completely (95%) at 500 μM.

The inhibition of Sulf-2 by compound **11** was evaluated over a concentration range and the IC_{50} was found to be 39.8 μM ± 17.6 (Fig. S1, ESI†). In comparison, the best biphenyl inhibitor reported by Reuillon *et al.*, compound **11**, was reported of having an IC_{50} value of 167 ± 5 μM against Sulf-2. In the present study, compound **11** was used as a benchmark compound and it was found to be less potent than compound **11** (80% *vs.* 95% inhibition of Sulf-2 at 500 μM, Fig. 3). Furthermore, at this single concentration compound **11** exhibited potent inhibition of sulfatase from *A. aerogenes* (100%) compared to compound **5** (1% ± 1). This shows that compound **5** is more potent and more selective than the previous best inhibitor of Sulf-2 reported in the literature.

A small library of saccharide-based endosulfatase inhibitors was prepared incorporating a 6-sulfamate group in place of the glucosamine 6-*O*-sulfate. The presented study supports previous findings that the replacement of the glucosamine-6-*O*-sulfate with the 6-sulfamate group leads to effective inhibition of HSulf-2 activity. The putative inhibitors were evaluated in a competition assay with recombinant HSulf-2 and a fluorogenic substrate 4-methylumbelliferyl sulfate (4-MUS). Trisulfated **5** was found to be superior to the other inhibitors investigated and is more potent against Sulf-2 and more selective for Sulf-2 *vs.* other sulfatases than a biphenyl trichloroethylsulfamate inhibitor reported in the literature.[13] We propose that compound **5**, and consequently the disaccharide IdoA(2S)-GlcNS(6S), may represent the minimal-size fragment of HS required for effective inhibition of the endosulfatases. The disaccharide IdoA2S-GlcNS(6S) is not a substrate of the Sulfs,[14] however this fragment-size does efficiently bind to the active site (evidenced by inhibition in the 4-MUS assay), making it a good scaffold for inhibitor design. While inhibitor **5** displays effective inhibition, the fate of **5** in the presence of Sulf-2 remains unknown: whether the C(6)O–S bond is hydrolyzed or **5** simply binds to the active site and functions as a competitive inhibitor requires further investigation.

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Conflicts of interest

There are no conflicts to declare.

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