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## Modified minimal-size fragments of heparan sulfate as inhibitors of endosulfatase-2 (Sulf-2)<sup>†</sup>

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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<sub>50</sub> 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).<sup>1a,b</sup> 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.<sup>2</sup> 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.<sup>3</sup> Sulf-2 expression is induced or upregulated in various cancers and its role has been identified as being pro-tumourigenic, 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.<sup>4a,b</sup> 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<sub>3</sub><sup>–</sup>) with the sulfamate motif (–OSO<sub>2</sub>NH<sub>2</sub>). This preliminary work utilised the smallest, most relevant unit of HS, α-GlcNS(6S) to template inhibitor design.<sup>5</sup> 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<sub>50</sub> 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.<sup>6</sup> 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.<sup>7</sup> The disaccharide, GlcNS(6Sulfamate)-IdoA(2S) only caused 20% Sulf-1 inhibition at 0.7 mM (IC<sub>50</sub> value not determined), and the trisaccharide and tetrasaccharide analogues were more potent with IC<sub>50</sub> values of 0.53 and 29.6 μM, respectively.

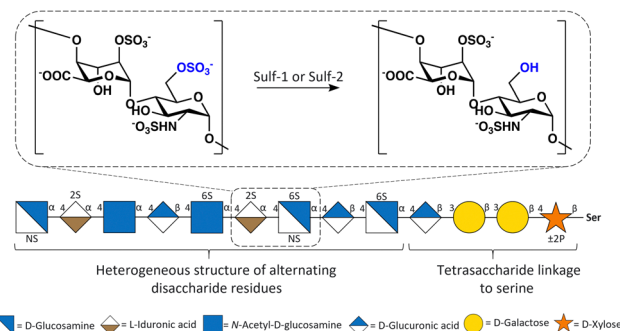


Fig. 1 Structure of HS highlighting the disaccharide residue, IdoA(2S)-GlcNS(6S), that Sulfs have a preference for.

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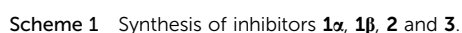
<sup>†</sup> Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d3cc02565a>

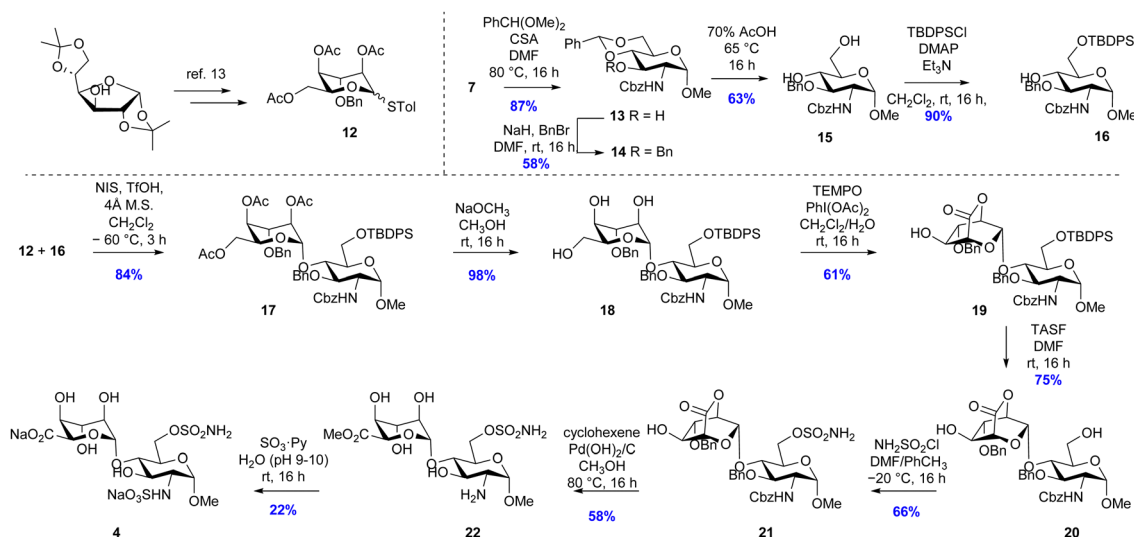




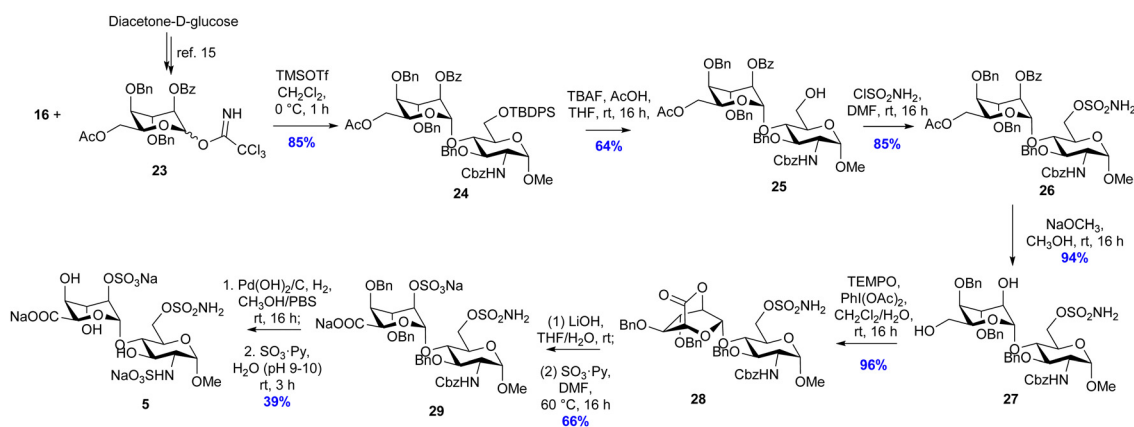
Triflation of **8** was achieved with triflic anhydride in the presence of Et<sub>3</sub>N at 0 °C to give **9** in 58% yield. The nucleophile LiCH<sub>2</sub>SO<sub>2</sub>(DMB)<sub>2</sub> was generated *in situ* by deprotonation of methylsulfonamide CH<sub>3</sub>SO<sub>2</sub>(DMB)<sub>2</sub> with *n*-BuLi at –78 °C.<sup>9</sup> The methylene sulfonamide unit was then introduced by nucleophilic substitution of triflate **9** with LiCH<sub>2</sub>SO<sub>2</sub>N(DMB)<sub>2</sub>.

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  $^1\text{H}$  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 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 debenzyla-tion 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)<sub>2</sub> in refluxing methanol.<sup>11</sup> 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<sup>®</sup> 50WX8 Na<sup>+</sup>-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* benzyla-tion of intermediate alcohol **19**. However, all attempts at benzyl protection of the ido 4-OH of **19** were unsuccessful and there-fore idose glycosyl donor **23** was synthesised according to Hu *et al.* (Scheme 3).<sup>12</sup> 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. Desilylation 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<sub>3</sub> in CH<sub>3</sub>OH 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<sup>®</sup> 50WX8 Na<sup>+</sup>-form column, **29** was isolated in 66% yield over two steps. Finally, the hydrogenolysis-labile protecting groups of **29** were cleaved by Pd(OH)<sub>2</sub>/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



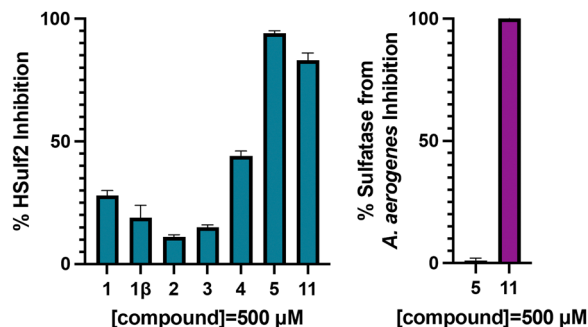


Fig. 3 (left) Sulf-2 and (right) sulfatase from *A. aerogenes* inhibition data for glucosamine-based inhibitors and biphenyl trichloroethylsulfamate **11**. Data represented as the mean  $\pm$  SD, ( $n = 2$ ). Inhibition values are reported as percentages of the uninhibited control values.

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  $\mu$ M) to compare inhibitory activity and the  $IC_{50}$  value was determined for the most potent inhibitor. Biphenyl trichloroethylsulfamate **11**<sup>13</sup> (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  $\mu$ M, and **1β**, **2** and **3** inhibited Sulf 2 by <15% at 500  $\mu$ M. As predicted, the extension of fragment size to the disaccharide scaffold led to an increased inhibition at 500  $\mu$ M. Disulfated disaccharide **4** inhibited Sulf-2 by 44% and trisulfated disaccharide **5** inhibited Sulf-2 almost completely (95%) at 500  $\mu$ M.

The inhibition of Sulf-2 by compound **11** was evaluated over a concentration range and the  $IC_{50}$  was found to be 39.8  $\mu$ M  $\pm$  17.6 (Fig. S1, ESI<sup>†</sup>). In comparison, the best biphenyl inhibitor reported by Reuillon *et al.*, compound **11**, was reported of having an  $IC_{50}$  value of 167  $\pm$  5  $\mu$ M 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  $\mu$ M, Fig. 3). Furthermore, at this single concentration compound **11** exhibited potent inhibition of sulfatase from *A. aerogenes* (100%) compared to compound **5** (1%  $\pm$  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.<sup>13</sup> 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 Sulf-2,<sup>14</sup> 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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