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ARTICLE TYPE

Efficient chemical synthesis of heparin-like octa-, deca- and dodecasaccharides and inhibition of FGF2- and $VEGF_{165}$-mediated endothelial cell functions

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A concise chemical synthesis of a series of structurally-defined heparin-like oligosaccharides is described. This work provides an efficient entry to octa-, deca-, and dodecasaccharides, including the first synthesis of $(GlcNS6S\text{-}IdoA2S)_5$ and $(GlcNS6S\text{-}IdoA2S)_6$. Evaluation of the in vitro activity of these species against FGF2- and $VEGF_{165}$-dependent endothelial cell proliferation and migration establishes that octa- and decasaccharides are more potent in targeting FGF2-induced effects, where cell migration is affected more significantly than proliferation. These structure-activity relationships exemplify the significance of 6-*O*-sulfation in regulating the activity of angiogenic growth factors.

Heparin and heparan sulphate (H/HS) are highly-charged, ubiquitous, naturally-occurring glycosaminoglycans (GAGs) which are involved in regulating a wide range of biologically important cellular signalling events that control a variety of biological functions, including angiogenesis.[1] Amongst these, angiogenic signalling pathways that control angiogenesis are regulated by pro-angiogenic and anti-angiogenic cytokines, many of which depend on H/HS for their biological activity.

Fibroblast Growth Factor 2 (FGF2) and Vascular Endothelial Growth Factor 165 ($VEGF_{165}$) are potent pro-angiogenic cytokines which require HS to bind and activate their respective receptors.[2a,b] We have previously demonstrated the relevance of the H/HS-cytokine axis to human cancer,[2c-h] through investigation of size fractionated heparin-like oligosaccharides as putative competitive inhibitors of H/HS function *in vitro*[2a,i] and *in vivo*,[2j] demonstrating the potency of octa- and deca-saccharides.

There is considerable interest in developing synthetic, structurally-defined H/HS sequences as tools to further probe these angiogenic signalling pathways and for other structural interaction studies. Efficient synthetic routes, as well as access to a diversity of functionality, are essential to provide such agents to interrogate a range of biological targets and also with relation to potentially developing new anti-angiogenic therapies.[3]

A number of reports concerning the construction of various H/HS architectures are known and address variation of sequence length and sulfation pattern.[4] The majority of these target the $(IS)_n$ repeating sequence,[5] with disaccharide-based strategies typically introducing the S-I anomeric linkage or employing iditol-based rather than iduronate donors. Noteworthy also are recent approaches utilising chemoenzymatic methodologies[6] and efforts towards sequences containing mixed (GS/IS) oligomers.[7]

Herein we report the first example of the total synthesis of structurally defined $(SI)_5$ deca- and $(SI)_6$ dodecasaccharides **27** and **28** (Figure 1) and *in vitro* evaluation of their ability to modulate FGF2- and $VEGF_{165}$-dependent endothelial cell functions.

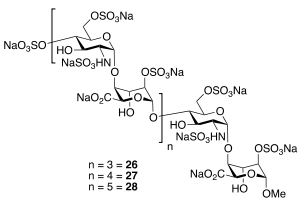

Figure 1. Synthetic heparin-like $[GlcNS6S\text{-}IdoA2S]_n$ oligosaccharides **26**-**28**.

The synthesis of these novel deca- and dodecassaccharides compliments the synthesis of the alternative dodecasaccharide sequence $(IS)_6$ reported by the Bonnaffé group[1d] and an $(SI)_4$ octasaccharide, similar to **26**, reported by Martin-Lomas' group.[4d] Furthermore, our optimized approach provides rapid iterative access to multi-hundred mg quantities of octasaccharide **15,** scalability which is pivotal to further elongations up to and including novel dodecasaccharide **19**. The work was underpinned by developing a reliable $2+(2)_n$ disaccharide iteration strategy for oligosaccharide chain elongation using stable thioglycoside iduronate donors, illustrated generically in Figure 2.

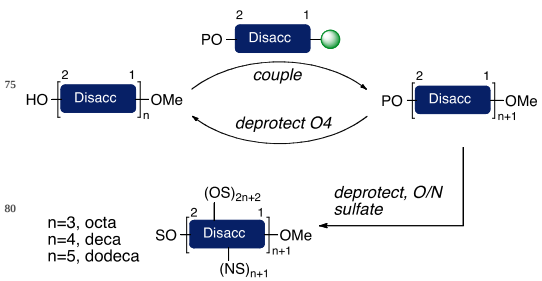

Figure 2. Iterative homologation from disaccharide level through to 8-, 10- and 12-mers, followed by deprotections/sulfations to access heparin-like oligosaccharides.

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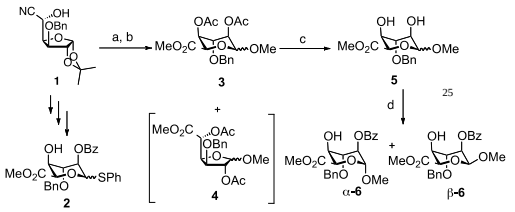
This efficient synthesis utilizes only two disaccharide building blocks, **9** and **10**, proceeding with very effective control over introduction of the pivotal α-1,4, I-S linking stereochemistry with yields reliably averaging 75% for each successive round of (2-step) homologation.

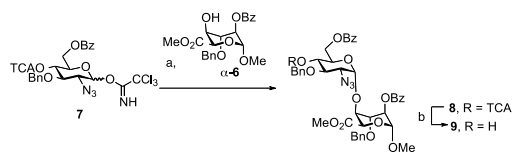
To provide the requisite reducing-end cap monosaccharide **6** required to prepare the key initial disaccharide **9**, we further exploited our diastereomerically pure cyanohydrin derivative **1**.[8] We have previously shown the conversion of **1** into thioglycoside iduronates of type **2** (Scheme 1) and their use as effective glycosyl acceptors to access H/HS disaccharide building blocks (including donor **10**)**.** Whilst that elaboration relied on an initial conversion of **1** *via* intermediary L-iduronamide derivatives, here we report that alternative Pinner type conditions convert **1** directly into methyl glycosides of the iduronate methyl ester in 77% yield. Whilst this afforded the expected mixture of pyranoside and furanoside diols, subsequent acetylation furnished **3** and **4** in high yield (93%) and allowed ready separation of these pyranoside and furanoside isomers, thus facilitating provision of **5** through deacetylation of **3** in 89% yield (Scheme 1).



Scheme 1. L-iduronate methyl ester acceptors. (a) AcCl, MeOH, 77% (b) Ac_2O, Pyridine, DCM, 53% for **3**, 40% for **4** (c) NaOMe, MeOH, 89% (d) $^nBu_2SnO$, MeOH then BzCl, dioxane, 70% (44% for **α-6**, 26% for **β-6**).

Following regioselective C-2 acylation of **5** using stannane-acetal chemistry,[4g] chromatographic separation afforded **α-6** and **β-6** in 70% overall yield. This route provides a new and scalable entry (13.1 g of **5** prepared) into iduronate acceptors of this type,[9] utilizing simple hydrolysis and acylation processes and is available in only four steps from cyanohydrin **1** (which we have shown to be available on Kg scale) and only eight steps (34% overall yield) from commercially available diacetone-D-glucose.

Glycosylation of α-**6** was then effected using glucosamine-derived trichloroacetimidate donor **7**[8a,c] under standard conditions, giving novel disaccharide **8** in 78% yield (Scheme 2). The α-selectivity of this glycosylation was confirmed as >95% by ^{1}H NMR analysis (GlcN $J_{1,2}$ = 3.7 Hz for **8**).

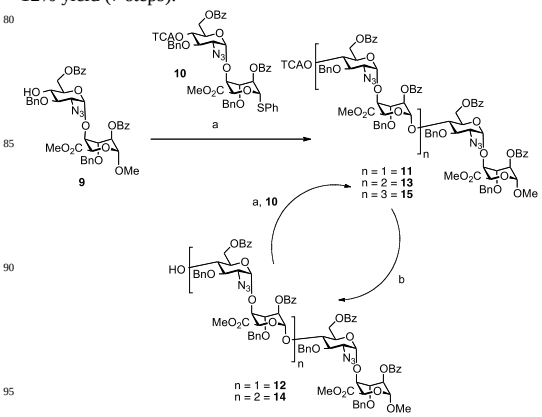


Scheme 2. Disaccharide acceptor synthesis (a) TMSOTf, DCM, 78% (b) MeOH, Pyridine, 95%, TCA = $C(O)CCl_3$.

Facile removal of the 4-*O*-TCA group from **8** using mildly basic conditions provided acceptor **9** in 95% yield. This novel disaccharide then served as the pivotal reducing terminal for iteration towards longer oligosaccharide sequences.

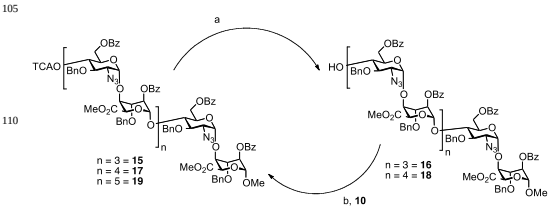
With effective access to **9**, our iterative $2+(2)_n$ process constituted removal of the glucosamine-4-*O*-TCA from each new intermediate oligosaccharide followed by coupling with disaccharide donor unit **10** in each iterative cycle so that the synthesis only needed to address the introduction of I-S linkages.

Hence, coupling of **9** with **10** furnished tetrasaccharide **11** in 66% yield (Scheme 3) and removal of the 4-*O*-TCA protecting group from **11** then gave **12** in excellent yield (91%), ready for further elongation. Continuation of this iterative glycosylation sequence was then successfully applied through two further cycles, homologating tetrasaccharide **12** into octasaccharide **15** (Scheme 3) with good yields and selectivity for each glycosylation step and consistently over 85% yield for 4-*O*-TCA deprotection. Our multi-gram access to the disaccharide building blocks, combined with this efficient homologation sequence, meant this methodology was effective for batch synthesis of >800mg quantities of octasaccharide **15**. This provides an impressive 5 step route from disaccharide **9** to protected octasaccharide **15** in 19% overall yield and compares well to previous work delivering the closest related octasaccharide in 12% yield (7 steps).[4d]



Scheme 3. Iteration to protected heparin-like octasaccharide. (a) NIS, AgOTf, DCM; **11** (66%), **13** (57%), **15** (64%) (b) MeOH, Pyridine; **12** (91%), **14** (86%).

Octasaccharide **15** was then further elaborated to afford novel deca and dodecasaccharides **17** and **19** using the same iteration process (Scheme 4), with acceptor octasaccharide **16** elaborated into the novel dodecasaccharide **19** in 40% overall yield.



Scheme 4. Iteration to longer heparin-like-oligosaccharides. (a) MeOH, Pyridine; **16** (89%), **18** (91%) (b) NIS, AgOTf, DCM; **17** (57%), **19** (79%).

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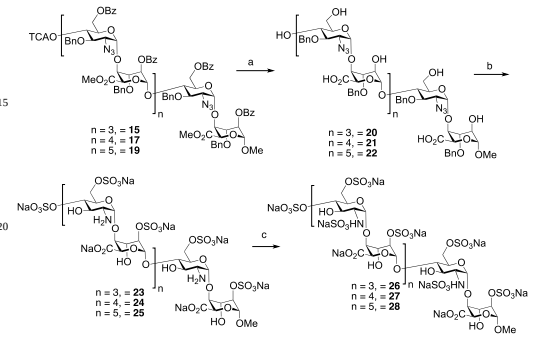
This oligosaccharide iteration proved extremely reliable and each round of glycosylation/deprotection could be completed in under 24 h. This demonstrates an efficient capability to more readily access a range of heparin-like oligosaccharides on a scale not accessible by other means and with the potential for incorporation of disaccharides with specific sulphation patterns.

The fully protected octa-, deca- and dodecasaccharides **15**, **17** and **19** were then elaborated into the target species *via* a four-step deprotection and *N*/*O*-sulfation sequence (Scheme 5).



Scheme 5. Deprotection and sulfation of octa-, deca- and dodecasaccharides. (a) LiOH, THF/MeOH/H_2O; **20** (89%), **21** (90%), **22** (68%). (b) Py.SO_3 complex, pyridine or SO_3.NMe_3, DMF, μW then H_2, $Pd(OH)_2$/C, MeOH/THF/H_2O; **23** (75%, 2 steps), **24** (87%, 2 steps), **25** (71%, 2 steps). (c) Py.SO_3 complex, $NaHCO_3$, H_2O; **26** (78%), **27** (73%), **28** (82%).

Firstly, ester saponification released the free carboxylic acids **20**-**22** and subsequent exhaustive *O*-sulfation was then effected using either Py.SO_3 complex in pyridine at 50°C (for **20**) or by using $Me_3N.SO_3$ under microwave conditions[10] (for **21** and **22**). Utilisation of microwave irradiation for this step saw a significant reduction in reaction time (1.5 h *vs* 18h) and better overall yields. *O*-sulfation was followed by hydrogenation to remove the benzyl protecting groups and reduce the azides to furnish **23**-**25** in good yields over the two steps. A final step *N*-sulfation of the glucosamine NH_2 residues was effected using Py.SO_3 complex in H_2O to provide 8-, 10- and 12-mer heparin-like oligosaccharides **26**-**28**.

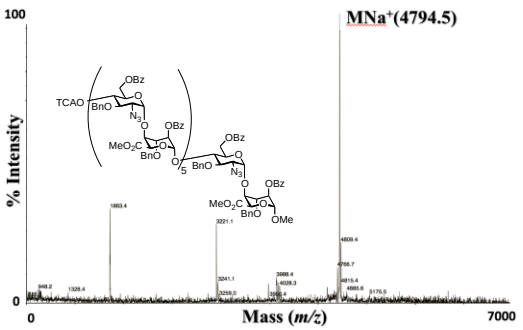


Figure 3. MALDI MS of dodecasaccharide **19** (MNa^+ shown).

Protected and partially/fully-deprotected oligosaccharides of this nature present analytical challenges. In this series, high field NMR (800MHz) of the fully protected octa-, deca- and dodecasaccharides (**15**, **17**, and **19**), provided assignment of the diagnostic anomeric signals and constituent disaccharide repeating units, whilst MS analysis using MALDI techniques, proved very reliable (see Figure 3 for analysis of dodecamer **19**).

Optimum analysis of oligosaccharides **23**-**28** required negative mode ESI-MS on samples that had undergone a carboxylic and sulfonic acid counter-ion salt switch (from Na^+ to NH_4^+) prior to analysis. This produced significantly less complicated spectra compared to those seen with the common Na^+ counterion.[11]

Characterization of final oligosaccharide length and homogeneity was supported by 800MHz NMR analyses and PAGE analysis for synthetic compounds **26**-**28** (Figure 4), compared to heparin digest oligosaccharides of known length (Iduron). Thus, PAGE runs comparing octa-, deca- and dodecasaccharides from biological digests with synthetic **26**-**28** and showed good correlations.

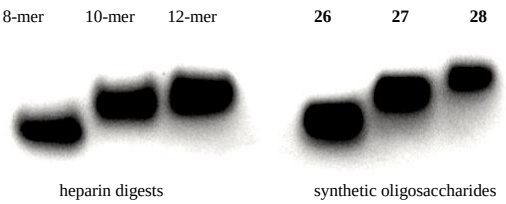


Figure 4. Azure A stained PAGE analysis of **26** (8-mer), **27** (10-mer) and **28** (12-mer) *vs* heparin digest 8-, 10- and 12-mer comparisons (4 μg loading).†

We previously reported that FGF2- and $VEGF_{165}$-mediated signalling pathways and endothelial cell functions are inhibited by a series of lower-sulphated synthetic $(S0I2)_n$ ($n \leq 6$) HS oligosaccharides.[2a] The most potent inhibition was achieved with longer oligosaccharide sequences and *N*-sulfation of glucosamine residues was essential for activity.

We thus used FGF2- and $VEGF_{165}$-dependent endothelial cell proliferation and migration *in vitro* assays to evaluate whether introducing per-6-*O*-sulfation into $(SI)_n$ sequences altered the potential to inhibit FGF2- and $VEGF_{165}$-dependent endothelial cell functions. (Fig. 5 and 6).

The proliferation results show that oligosaccharides **26** and **27** inhibit FGF2, whilst dodecasaccharide **28** supports the activity of FGF2 (Fig. 5); findings that are in keeping with our previous *in vivo* study of size-fractionated 6-*O*-sulfated heparin oligosaccharides.[2j] Moreover, this contrasts dramatically with the effect of our previously-reported synthetic $[GlcNS\text{-}IdoA2S]_6$-OMe dodecasaccharide, where FGF2-mediated cell proliferation was inhibited by 85%. Access to the new synthetic 6-*O*-sulfated dodecasaccharide **28** thus enables proof of a key structure-function switch in which **28** supports FGF-mediated proliferation, whilst its direct 6-*O*-desulfated synthetic analogue is very substantively inhibitory.

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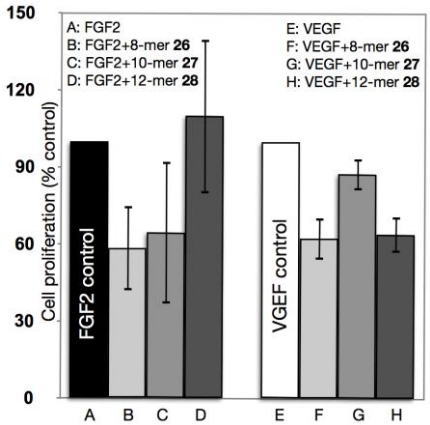

Figure 5. ***In vitro*** **endothelial cell proliferation data for 26-28**. Oligosaccharides **26-28** affect FGF2- and $VEGF_{165}$-induced human umbilical vein endothelial cell (HUVEC) proliferation. HUVECs were maintained in endothelial cell growth media without supplements containing 1% fetal bovine serum (FBS) for six hours before adding FGF2 and $VEGF_{165}$ at 5 ng/ml and 2.5 ng/ml concentration, respectively. HUVECs were cultured with the growth factors in the presence or absence of oligosaccharides (50 μg/ml) for 96 hours. Cell proliferation was evaluated using sulforhodamine B assay. FGF2- and $VEGF_{165}$-induced HUVEC proliferation in the absence of oligosaccharides is expressed as 100%. Results are shown as mean ± SEM.

All three oligosaccharides **26-28** inhibited FGF2-mediated endothelial cell migration by 45-70 % (Figure 6). However, our previously-reported synthetic $[GlcNS\text{-}IdoA2S]_6$-OMe dodecasaccharide completely inhibited FGF2-mediated cell migration. This also provides another significant advancement in proof of the very different effects of sulfation within such synthetic oligosaccharides.

In $VEGF_{165}$-mediated cell proliferation and migration assays (Figures 5 and 6) the activities of the 8-mer (**26)** and 12-mer (**28**) were almost identical whereas the 10-mer (**27**), whilst having little effect on proliferation (Fig 2a), was significantly more effective, inhibiting cell migration by 70%. Notably, this is comparable in effect to the inhibition of VEGF-mediated migration by our synthetic 6-*O*-desulfated $[GlcNS\text{-}IdoA2S]_6$-OMe dodecasaccharide.[2a] This provides an interesting contrast between the relationship of oligosaccharide length and sulfation levels in inhibiting VEGF-mediated processes.

Overall, these results, when compared with our prior biological inhibition data for the lesser-sulphated synthetic analogue series, indicate that the number and specific positions of sulphate residues in HS-related oligosaccharides have a significant role in affecting different FGF2- and $VEGF_{165}$-mediated processes. The lower inhibitory activity of the fully-6-*O*-sulphated synthetic series **26-28** (compared with the de-6-*O*-sulphated series) against FGF2- and $VEGF_{165}$-mediated endothelial cell functions, particularly exemplified by the very different effects of the dodecasaccharides on FGF2-mediated proliferation and migration, could be due to the closer structural analogy to native HS S-domains, where such sequences are involved in the activation of growth factors and growth factor receptors on endothelial cells and are detected in tumour endothelium.[2f-h,12]

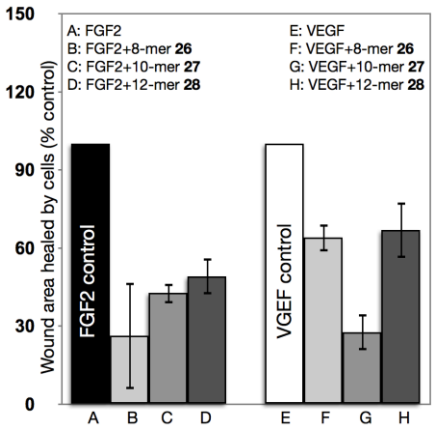

Figure 6. Inhibition of FGF2- and $VEGF_{165}$-induced HUVEC migration. HUVECs were seeded to form confluent monolayers that were maintained in endothelial cell growth media without supplements containing 2% FBS for 24 hours. Following serum-starvation monolayers were wounded and FGF2 or $VEGF_{165}$ with or without oligosaccharides (50 μg/ml) were added at 5 ng/ml and 2.5 ng/ml concentration, respectively, for 24 hours. The images of unpopulated areas were analysed using MetaMorph image analysis software by measuring unpopulated area at 0 and 24 hours. Cell advancement area was derived for each treatment. The control treatment with FGF2 or $VEGF_{165}$ alone is presented as 100%. Results are expressed as mean ± SEM

Conclusions

In summary we have demonstrated an efficient $2+(2)_n$ iduronate donor-disaccharide-based synthesis of heparin-like oligosaccharides, delivering the first examples of deca- and dodecasaccharides with the (SI) repeat unit. The protected octasaccharide precursor can be prepared on up to multi-hundred-mg scales and demonstrates a robust entry to access essential, structurally-defined $[GlcNS6S\text{-}IdoA2S]_n$ oligosaccharides. Evaluation of these compounds in *in vitro* endothelial cell-based assays has enabled us to highlight the critical role that the glucosamine-6-*O*-sulphate residue plays in the regulation of cytokine activity by HS and provides important structure-activity information, which will prove insightful in the future design and development of new anti-angiogenic synthetic HS agents.

Notes and References

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Author contributions

†Iduronate development, oligosaccharide synthesis and wrote manuscript (GJM), §iduronate development (SUH), ¶biological evaluations (EA, CC, GR), overall project planning, supervision and writing (JMG and GJ).

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† Electronic Supplementary Information (ESI) available: [^{1}H, ^{13}C, HMQC/HSQC, COSY data for new compounds, mass spectral data and synthetic procedures for saccharides, PAGE analysis and biological assays]. See DOI: 10.1039/b000000x/

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