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# Design, synthesis, FGF-1 binding, and molecular modeling studies of conformationally flexible heparin mimetic disaccharides

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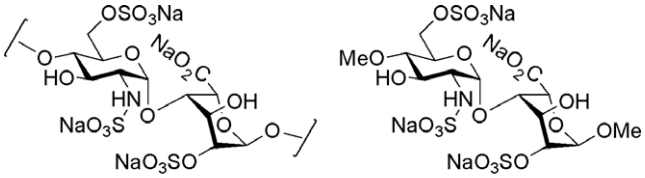
**Abstract**—Disaccharide mimetics of a heparin sequence that binds to fibroblast growth factors were prepared by coupling a D-galactose donor with a methyl β-D-gluco- or xylopyranoside acceptor. When fully sulfated, the glucose or xylose moieties exist in solution in equilibrium between the $^4C_1$ and $^1C_4$ conformers, as confirmed by $^1$H NMR spectroscopy, thus mimicking the conformationally flexible L-iduronic acid found in heparin. Docking calculations showed that the predicted locations of disaccharide sulfo groups in the binding site of FGF-1 are consistent with the positions observed for co-crystallized heparin-derived oligosaccharides. Predicted binding affinities are in accord with experimental $K_d$ values obtained from binding assays and are similar to the predicted values for a model heparin disaccharide.


The fibroblast growth factors (FGFs) are a family of structurally related proteins that play important roles in cell proliferation, differentiation, and migration, as well as disease processes such as tumor angiogenesis.[1,2] The most extensively studied members of the FGF family are FGF-1 and FGF-2 which function by binding to and dimerizing a family of signal-transducing FGF receptors (FGFRs), leading to receptor activation and cell signaling. This process is initiated through binding of heparan sulfate (HS) to the FGF and FGFR to form a ternary complex. The manner in which the components of the complex associate is not fully understood, and several models have been proposed.[3–5] Prevention of the formation of the HS:FGF:FGFR ternary complex via blocking the interaction of HS with the FGF may, therefore, form the basis for antiangiogenic therapies.[6–8]

Heparin and HS are glycosaminoglycans (GAGs) composed of repeating 1 → 4 linked disaccharide sequences of α-D-glucosamine (GlcN) and a uronic acid (β-D-glucuronic acid, GlcA, or α-L-iduronic acid, IdoA).[9] Short heparin/HS sequences (di- and trisaccharides[10] and tetrasaccharides[11]) bind to the FGFs, although more recent studies indicate that unsulfated di- and trisaccharides do not bind FGF,[12] and longer oligosaccharides are usually required for the promotion of dimerization and activation. X-ray crystal structures of heparin-derived oligosaccharides bound to FGFs alone[13,14] or in ternary complex with FGFR[15,16] have been determined. Analyses of these structures reveal that not all oligosaccharide sulfo and carboxyl groups bind to FGF, and that the disaccharide GlcN(2*S*,6*S*)-IdoA(2*S*) (**1**, Fig. 1) represents a minimal heparin/HS consensus sequence for FGF binding.[17] The 2-*O*-sulfo group of IdoA and the *N*-sulfo group of GlcN in **1** form the primary protein contacts and in the case of FGF-1,




1 2

**Figure 1.** Structure of the GlcN(2*S*,6*S*)-IdoA(2*S*) disaccharide sequence **1**, which represents a minimal consensus sequence for FGF:HS binding,[17] and a model disaccharide **2** considered in the theoretical calculations presented here.

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the GlcN 6-*O*-sulfo group also interacts favorably with the protein.[17] In these structures the GlcN residue of **1** adopts the normal $^4C_1$ chair conformation whilst the conformationally flexible[18] IdoA is found in the $^1C_4$ conformation when bound only to the protein[13,14] or in a skew-boat ($^2S_O$) conformation when part of a ternary complex.[15,16] Recent NMR studies indicate that FGF-1 can bind both conformations of IdoA in a bioactive hexasaccharide.[19]

A number of structurally simple heparin mimetics also bind to the FGFs in the HS binding site and inhibit FGF mitogenic activity, for example, sulfonated naphthalenes[20] and sulfated oligosaccharides[6,21] and their glycosides.[22,23] Taken together, these observations lead us to conclude that structural mimics of disaccharide **1** might be profitably explored as potential inhibitors of FGF-mediated angiogenesis.

The synthesis of heparin/HS oligosaccharides is challenging,[24] and syntheses of 'non-regular' disaccharides, that is, with GlcN at the non-reducing end, have only been reported in the past decade. Despite recent progress,[25–27] a major hurdle in this area continues to be synthetic access to IdoA. Simple disaccharides, therefore, were sought that could be readily synthesized while mimicking the essential features of **1**, namely the α-(1 → 4) linkage between the two monosaccharide units, the spatial orientation of the two key sulfo groups [GlcN(2*S*) and IdoA(2*S*)], and the conformational flexibility of the IdoA residue. It was considered that suitable disaccharides might be synthesized by glycosylation of an IdoA mimic with a differentially 2-*O*-protected glycosyl donor.

The D-thiogalactosides **3** and **4** were chosen as glycosyl donors (Fig. 2) because of the ease with which the 2-OH can be differentially protected. It was assumed that *N*-sulfo groups could be substituted for by *O*-sulfo, as previously demonstrated with analogues of the AT III-binding heparin pentasaccharide.[28] It was also hypothesized that D-galactose (Gal) in the place of GlcN at the non-reducing end would not significantly reduce FGF binding. The β-configuration and the non-participating 2-*O*-benzyl group were expected to favor the stereoselective formation of the desired α-(1 → 4) linkage. Like the naturally occurring GlcN, the Gal residue should adopt a $^4C_1$ conformation in solution. Importantly, the protecting group strategy also permits the other hydroxyl groups of this residue to remain either unprotected or derivatized as non-polar methyl ethers[28] which allows for the testing of small hydrophobic groups in FGF ligand binding. Glycosyl donor **4** allows for sulfonation at *O*-6 enabling examination of the effects of the 6-*O*-sulfo group that is present in **1**.

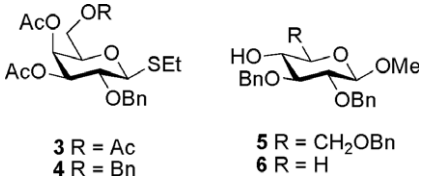



**Figure 2.** Structures of glycosyl donors (**3** and **4**) and acceptors (**5** and **6**) used in this study.

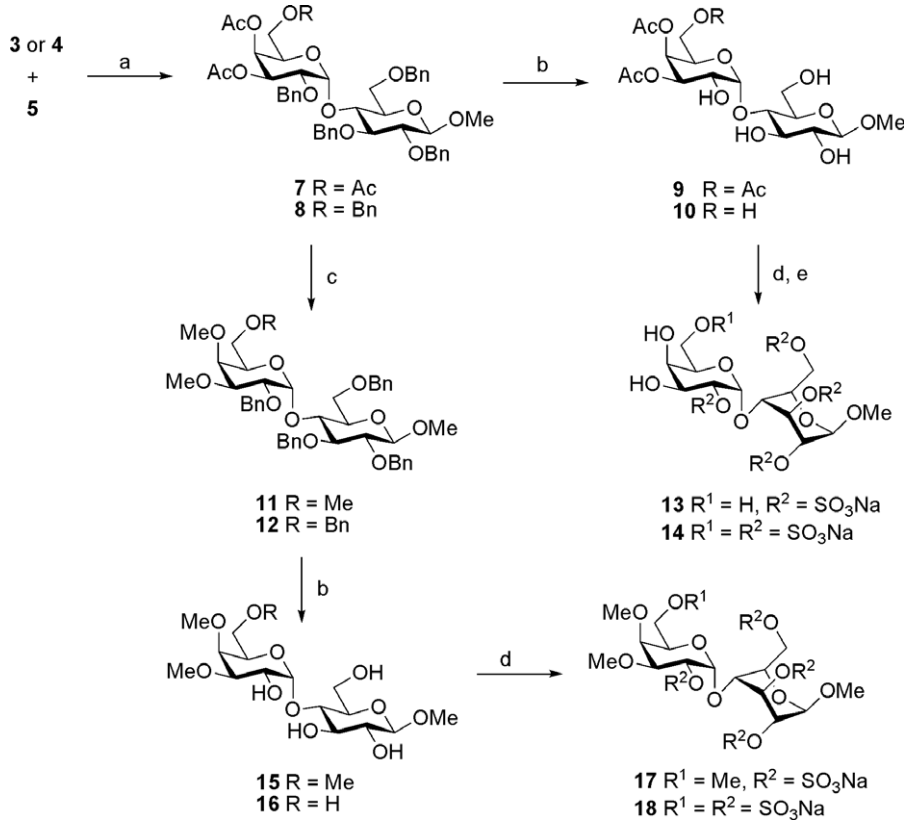
The selection criteria for IdoA mimics as glycosyl acceptors included ease of synthesis and ability to adopt the $^1C_4$ conformation in solution, thus presenting a suitably orientated 2′-*O*-sulfo group for binding to FGF. Highly sulfated β-D-gluco- and -xylopyranosides are known to exist in a conformational equilibrium between the two chair conformers in favor of the $^1C_4$.[29,30] The tribenzyl β-D-glucoside **5** and dibenzyl β-D-xyloside **6** were thus selected as glycosyl acceptors because, once deprotected and sulfonated, the glucose or xylose ring should exhibit the desired conformational flexibility. It was hypothesized that the additional sulfates in the ring beyond that required at *O*-2′ would not unduly interfere with binding.

The Gal-Glc disaccharides were prepared as shown in Scheme 1. The acceptor alcohol **5**[31] was glycosylated at −20 °C with thioglycoside donor **3**,[32] using *N*-iodosuccinimide/triflic acid (NIS/TfOH) as promoter, to give a 4:1 α/β mixture of anomers from which pure disaccharide **7** was obtained by flash chromatography in satisfactory yield (61%).[33] Hydrogenolysis of the benzyl ethers ($H_2$/Pd(OH)$_2$ on C) proceeded in good yield (91%) and subsequent sulfonation (sulfur trioxide trimethylamine complex) and deacetylation (1 M NaOH) gave the desired tetrasulfate **13**, which was purified by size exclusion chromatography (Bio-Gel P-2). The purity (⩾96%) was determined by capillary electrophoresis (CE)[34] and NMR spectroscopy. The trimethylated derivative **11** was prepared by quantitative deacetylation and methylation (NaOMe/MeOH and NaH/MeI) of **7**. Hydrogenolysis of the benzyl ether protecting groups ($H_2$/Pd on C) gave tetrol **16** in good yield (79%), and subsequent sulfonation afforded trimethylated tetrasulfate **17**. The corresponding pentasulfates **14** and **18** were prepared analogously starting from glycosyl donor **4**.[35]

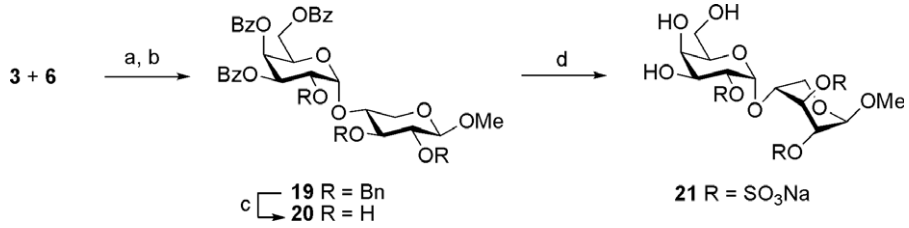
For the synthesis of the xylose analogue of **13** (Scheme 2), glycosylation of xylose acceptor **6**[36] with donor **3** gave an inseparable mixture of anomers (α/β = 5:1) that was converted into the corresponding mixture of tribenzoates from which the pure α-anomer **19** was obtained by flash chromatography. Hydrogenolysis to give triol **20**, followed by sulfonation/debenzoylation, afforded the trisulfate **21** in moderate yield (24%, three steps from **19**).

Purification of the sulfated disaccharides was challenging and the final products were obtained in low yields (3–24%), in part, due to contamination by inorganic salts and the presence of undersulfated species which were difficult to separate chromatographically. The magnitude of the vicinal coupling constants observed in the $^1$H NMR spectra indicates that the Glc rings of **13**, **14**, **17**, and **18**, and the D-xylose ring of **21**, exist in a chair–chair equilibrium in favor of the $^1C_4$ chair conformation (Table 1), as anticipated.[29,30]

The binding affinities of the sulfated disaccharides **13**, **14**, **17**, **18**, and **21** for FGF-1, measured using a surface




**Scheme 1.** Reagents and conditions: (a) NIS, TfOH, $CH_2Cl_2$, −20 °C; (b) $H_2$, $Pd(OH)_2$ or Pd/C; (c) i—NaOMe, MeOH, ii—NaH, MeI; (d) $SO_3{\cdot}Me_3N$, DMF, 60 °C; (e) 1 M NaOH.




**Scheme 2.** Reagents and conditions: (a) NIS, TfOH, $CH_2Cl_2$, −20 °C; (b) i—NaOMe, MeOH; ii—BzCl, Py; iii—chromatography; (c) $H_2$, $Pd(OH)_2$, $CHCl_3$; (d) i—$SO_3{\cdot}Me_3N$, DMF, 60 °C; ii—1 M NaOH.

**Table 1.** Experimental data for synthesized disaccharides

| Disaccharide | $J_{1,2}$ | $J_{2,3}$ | $K_d$ (μM)[b] | $\Delta G$(obs) | Gscore ($^1C_4$) | Gscore ($^4C_1$) |
|---|---|---|---|---|---|---|
| **13** | 2.8 | <4.0 | 77.5 ± 2.5 | −5.60 | −4.18 | −2.72 |
| **14** | 3.6 | 3.2 | 21.8 ± 0.6 | −6.36 | −4.43 | −4.94 |
| **17** | 2.2 | 1.1 | 233 ± 23 | −4.95 | −4.22 | −3.58 |
| **18** | 3.6 | 2.8 | 47.9 ± 1.3 | −5.89 | −4.70 | −4.06 |
| **21** | <2.8 | n.o.[a] | 1400 ± 170 | −3.89 | −4.13 | −3.97 |

Selected vicinal coupling constants $J$ (Hz) for the reducing end β-D-glucopyranosyl or β-D-xylopyranosyl moiety of sulfated disaccharides (data extracted from $^1$H NMR spectra determined in $D_2O$ at 400 MHz.)

Dissociation constants ($K_d$) and corresponding $\Delta G$ values measured for sulfated disaccharides binding to FGF-1 and calculated XPGlide Gscores for disaccharides docked with FGF-1.

[a] Not observed.

[b] All values are means and standard deviation of at least two independent measurements.

plasmon resonance solution affinity assay,[21] are shown in Table 1. The affinities are in the μM range and compare well with reported binding affinities for FGF-1 of the synthetic heparin tetrasaccharides GlcN(2*S*,6*S*)-IdoA(2*S*)-GlcN(2*S*,6*S*)-IdoA(2*S*)-OPr ($IC_{50}$ = 0.24 μM) and GlcN(2*S*)-IdoA(2*S*)-GlcN(2*S*,6*S*)-IdoA(2*S*)-OPr

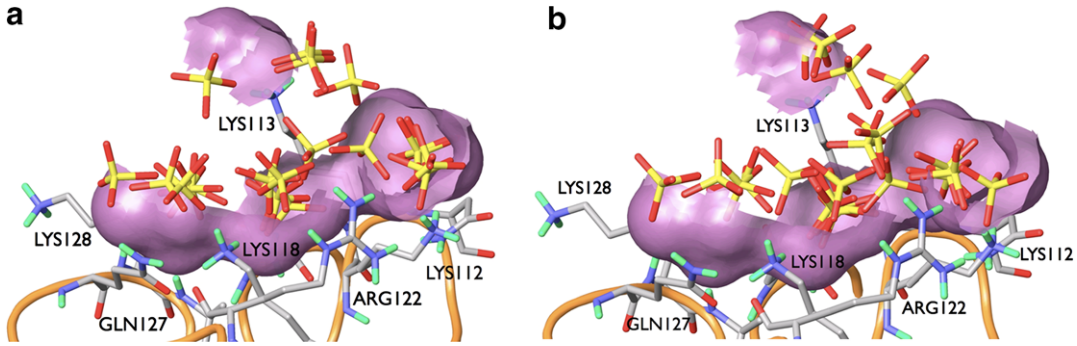



**Figure 3.** Sulfate groups of the relaxed, FGF-1-docked poses of the synthesized disaccharide ligands. (a) The disaccharides with Glc in the $^{1}C_{4}$ conformation. (b) The disaccharides with Glc in the $^{4}C_{1}$ conformation. The molecular surface of the sulfate and carboxylate groups of the cocrystallized hexasaccharide ligand that are involved in hydrogen bonding interactions with the protein is shown, and the backbone α-carbon atoms of FGF-1 are represented by the orange tube. Carbon, nitrogen, sulfur, oxygen, and hydrogen atoms are shown in gray, blue, yellow, red and green, respectively.

($IC_{50}$ = 290 μM).[11] The extra sulfo group of **14** resulted in a small (3.5-fold) improvement in affinity over **13**, while capping the free hydroxyl groups as methyl ethers (i.e., **17** and **18**) resulted in decreased affinity compared with their non-methylated counterparts. Disaccharide **21** bound poorly to FGF-1 suggesting that the 6′-*O*-sulfo group may be important in binding, perhaps by mimicking the carboxylate in IdoA. These results suggest that it may be possible to tailor the specificity of binding to different HS-binding growth factors by altering the substituents around the two key 2-*O*-sulfo groups.

Molecular docking calculations were performed using the extra precision mode[37] (XP) of the *Glide* program[38–40] to examine the FGF-1 binding modes of model (**2**) and synthesized (**13**, **14**, **17**, **18**, and **21**) disaccharides. The protein structure used for this study was of FGF-1 co-crystallized with a heparin-derived hexasaccharide ligand (PDB accession code 2AXM). The disaccharides were constructed with the Glc moiety in both the $^{4}C_{1}$ and $^{1}C_{4}$ conformers, with ring flipping disallowed during the conformer generation stage of docking. After docking, the best docked pose (i.e., lowest Gscore) for each ligand was relaxed in the presence of the frozen protein to remove any unfavorable intra-ligand contacts.

The calculated Gscores are indicative of modest binding affinity and similar in magnitude to those observed (see Table 1) and importantly, the binding modes of the disaccharides mimic the co-crystallized hexasaccharide ligand by positioning their sulfate groups in similar sites. This is illustrated in Figure 3 where the positions of the disaccharide sulfates are shown relative to the negatively charged functional groups of the hexasaccharide (sulfate and carboxylate) involved in hydrogen bonding interactions with the protein. In all cases the bound disaccharide conformation involves placement of at least one sulfate group in a region also occupied by a charged group from the hexasaccharide. While it is not possible for a disaccharide to present all sulfate groups to the protein in the preferred locations, a consequence of the reduced size and flexibility of the disaccharide compared to the hexasaccharide, the preference for negatively charged functional groups in specific locations within the FGF-1 binding site is clear, as noted previously.[41,42]

FGF-1 docking of the model disaccharide **2** yielded a best Gscore of −4.01 kcal/mol, which is similar to those obtained for the synthesized disaccharides (Table 1). The bound conformation of **2** corresponding to this Gscore is shown in Figure 4a. Three of the four charged groups interact in formal hydrogen bonding interactions with the protein with only the 6-*O*-sulfo group not participating in hydrogen bonding. If, however, the geometry of **2** and the surrounding protein residues are relaxed

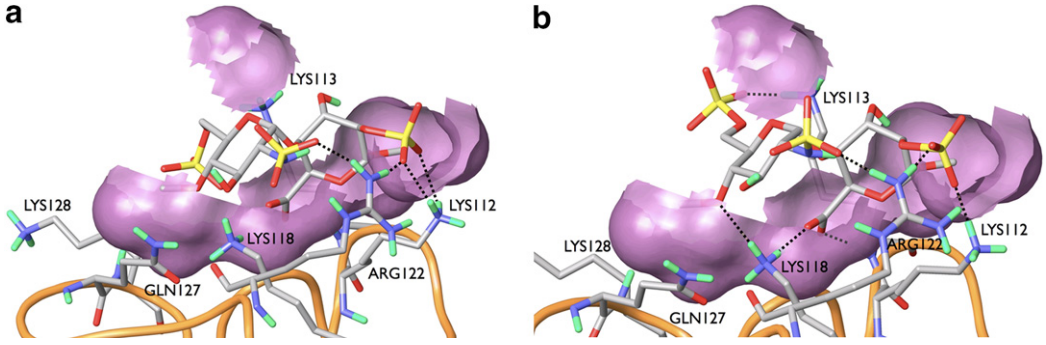



**Figure 4.** Conformations of the model disaccharide **2** bound to FGF-1. (a) The XPGlide docked pose after relaxation of the ligand. (b) The bound conformation after relaxation of both the ligand and nearby protein residues. The molecular surface and color scheme are the same as in Figure 3. Hydrogen bonding interactions between FGF-1 and **2** are depicted by dotted lines.

together, then all of the disaccharide's charged functional groups are able to participate in hydrogen bonding interactions with the protein. This is shown in Figure 4b and illustrates how an induced fit might occur in the heparin binding site of FGF-1 upon ligand binding. Together these results indicate that the most active disaccharides synthesized here have predicted binding affinities and binding modes similar to the heparin-derived disaccharide they were designed to mimic.

In conclusion, simple disaccharide mimetics of a heparin sequence that bind to FGF-1 were prepared to mimic the conformational flexibility of IdoA. Docking calculations showed that the predicted locations of disaccharide sulfo groups in the binding site of FGF-1 are consistent with the positions observed for co-crystallized heparin-derived oligosaccharides. Predicted binding affinities are in accord with experimental $K_d$ values obtained from binding assays and are similar to the predicted values for a model heparin disaccharide. These results may aid in the design of potential inhibitors of FGF-mediated angiogenesis.

## Acknowledgments

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## Supplementary data

General experimental procedures and details of the synthesis, purification, and characterization of disaccharides **13**, **14**, **17**, **18**, and **21**, and of the computational methods used in this study. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.10.071.

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