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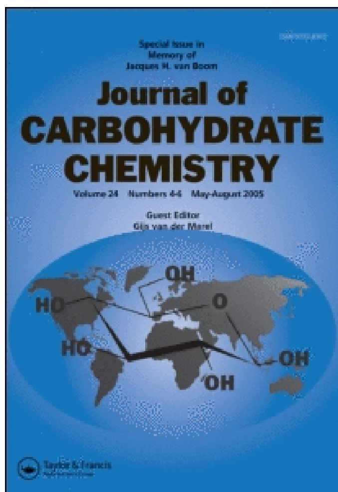

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Synthesis and Fibroblast Growth Factor Binding of Oligosaccharides Related to Heparin and Heparan Sulphate

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SYNTHESIS AND FIBROBLAST GROWTH FACTOR BINDING OF OLIGO-SACCHARIDES RELATED TO HEPARIN AND HEPARAN SULPHATE

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ABSTRACT

A series of six disaccharides, α-L-IdoA-(1→4)-α-D-GlcNAc-1→OMe, α-L-IdoA-(1→4)-α-D-GlcNSO$_3$-1→OMe, β-D-GlcA-(1→4)-α-D-GlcNAc-1→OMe, β-D-GlcA-(1→4)-α-D-GlcNSO$_3$-1→OMe, α-D-GlcNAc-(1→4)-β-D-GlcA-1→OMe, β-D-GlcNAc-(1→4)-β-D-GlcA-1→OMe, and two trisaccharide, β-D-GlcA-(1→4)-α-D-GlcNAc-(1→4)-β-D-GlcA-1→OMe, α-L-IdoA-(1→4)-α-D-GlcNSO$_3$-(1→4)-β-D-GlcA-1→OMe was prepared and screened for biological activity *in vitro*. The oligosaccharides were tested, together with a previously synthesized trisaccharide, α-L-IdoA-(1→4)-α-D-GlcNAc-(1→4)-β-D-GlcA-1→OMe, and three tetrasaccharides, α-L-IdoA-(1→4)-α-D-GlcNAc-(1→4)-β-D-GlcA-(1→3)-β-D-Gal-1→OMe, β-D-GlcA-(1→3)-β-D-Gal-(1→3)-β-D-Gal-(1→3)-2-PO$_3$-β-D-Xyl-1→OMe, β-D-GlcA-(1→3)-β-D-Gal-(1→3)-β-D-Gal-(1→3)-β-D-Xyl-1→OMe, for competitive binding to acidic and basic fibroblast growth factor in an assay using ^{125}I labelled heparin. It was found that the non-sulphated trisaccharides, α-L-IdoA-(1→4)-α-D-GlcNAc-(1→4)-β-D-GlcA-1→OMe and β-D-GlcA-(1→4)-α-D-GlcNAc-(1→4)-β-D-GlcA-1→OMe, and two of the disaccharides can bind to acidic as well as basic FGF.

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INTRODUCTION

Heparin is a well known antithrombotic drug.[1,2] Additionally, heparin has other biological effects such as anti-inflammatory[3] anti-metastatic[4] and angiogenic modulatory activity.[5] We are interested in studying how oligosaccharides from heparin/heparan sulphate modulate angiogenesis in order to obtain a basis for preparing potential drugs for use in treating coronary artery disease or neoplastic disease. Because angiogenesis is modulated by various growth factors we as well as others have studied the interaction between the heparin binding growth factors, acidic (FGF-1) and basic (FGF-2) fibroblast growth factor and oligosaccharides obtained by the depolymerization and fractionation of heparin and heparan sulphate.[4,6-15] By depolymerization and fractionation we obtained oligosaccharides of defined size and having a narrow range of sulphation.[6,16] Even those size homogenous, heparin/heparan sulphate derived oligosaccharides consist of many isomers with various degrees and position of sulphation. We have synthesized di-, tri- and tetrasaccharides of defined structure to determine the minimum saccharide structure of heparin/heparan sulphate which can interact with FGF. Previous reports only describe the interaction of sulphated heparin/heparan sulphate oligosaccharides with FGF. We therefore wanted to address the question of whether non-sulphated oligosaccharide sequences, which are abundant in heparan sulphate, would bind FGF. Here we report on the synthesis of eight di- and trisaccharides. We demonstrate that several of these saccharides (and some previously synthesized tri- and tetrasaccharides[17,18]) bind to both acidic and basic FGF. additional studies, reported separately,[19] demonstrate that some of these heparin/heparan sulphate derived saccharides are biologically active.

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Following compounds were prepared and assayed for FGF-binding:

α-L-IdoA-(1→4)-α-D-GlcNAc-1→OMe	1	Di-1
α-L-IdoA-(1→4)-α-D-GlcNSO$_3$-1→OMe	2	Di-2
β-D-GlcA-(1→4)-α-D-GlcNAc-1→OMe	3	Di-3
β-D-GlcA-(1→4)-α-D-GlcNSO$_3$-1→OMe	4	Di-4
α-D-GlcNAc-(1→4)-β-D-GlcA-1→OMe	5	Di-5

β-D-GlcNAc-(1→4)-β-D-GlcA-1→OMe	6	Di-6
β-D-GlcA-(1→4)-α-D-GlcNAc-(1→4)-β-D-GlcA-1→OMe	7	Tri-1
α-L-IdoA-(1→4)-α-D-GlcNSO$_3$-(1→4)-β-D-GlcA-1→OMe	8	Tri-2
α-L-IdoA-(1→4)-α-D-GlcNAc-(1→4)-β-D-GlcA-1→OMe		Tri-3
α-L-IdoA-(1→4)-α-D-GlcNAc-(1→4)-β-D-GlcA-(1→3)-β-D-Gal-1→OMe		Tetra-1
β-D-GlcA-(1→3)-β-D-Gal-(1→3)-β-D-Gal-(1→3)-β-D-Xyl-1→OMe		Tetra-2
β-D-GlcA-(1→3)-β-D-Gal-(1→3)-β-D-Gal-(1→3)-2-PO$_3$-β-Xyl-1→OMe		Tetra-3

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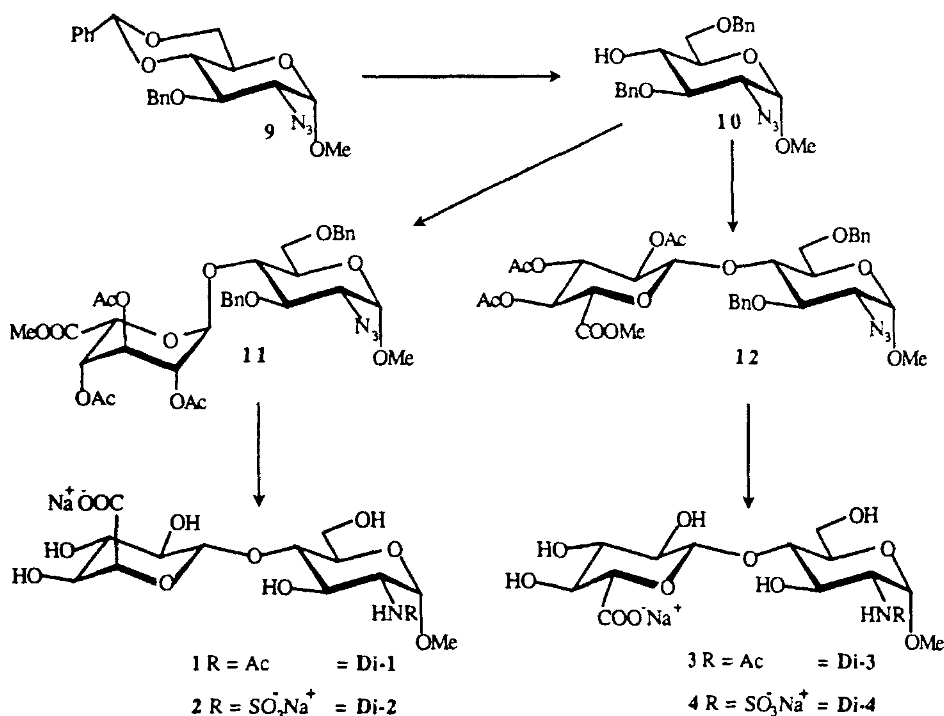
RESULTS AND DISCUSSION

Synthesis of heparin oligosaccharides.

Our aim was to synthesize di- and trisaccharides that are structural elements of heparin/heparan sulphate (or variants of the trisaccharide Tri-3) to find the smallest and most active FGF-binding structure. A large number of heparin related oligsaccharides have been prepared earlier.[20] Our synthetic route utilized intermediate building blocks previously reported.[17,18] The following steps were performed:

The methyl glycoside **9** was prepared from nitrate 2-azido-4,6-*O*-benzylidene-3-*O*-benzyl-2-deoxy-β-D-glucopyranoside[21] which was treated with methanol and tetraethylammonium bromide to give the α-methyl glycoside in 66% yield. The 4,6-*O*-benzylidene derivative **9** was then ring-opened by using sodium cyanoborohydride and HCl in diethyl ether[22] to give the OH-4 compound **10** in 88% yield. Glycosylation of **10** with methyl (2,3,4-tri-*O*-acetyl-β-L-idopyranosyl bromide)uronate[23] using silver triflate as promoter gave the disaccharide **11** in 71% yield. Deprotection of **11** by deacetylation, using 2 M sodium hydroxide in tetrahydrofuran, and hydrogenolysis over Pd-C gave a disaccharide which was either *N*-acetylated by treatment with acetic anhydride in water-methanol giving the disaccharide **1** in 76% yield or *N*-sulphated using triethylamine-sulfur trioxide complex giving compound **2** in 55% yield.

Compound **10** was glycosylated with methyl (2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide)uronate[24] using silver triflate as promoter and 2,6-di-*tert*-butyl-4-methyl pyridine (DTBMP) as acid acceptor, to give compound **12** in 65% yield. Deprotection and *N*-acetylation or *N*-sulphation of **12** was performed as described above to give compound **3** and **4** in 73% and 54% yield respectively.



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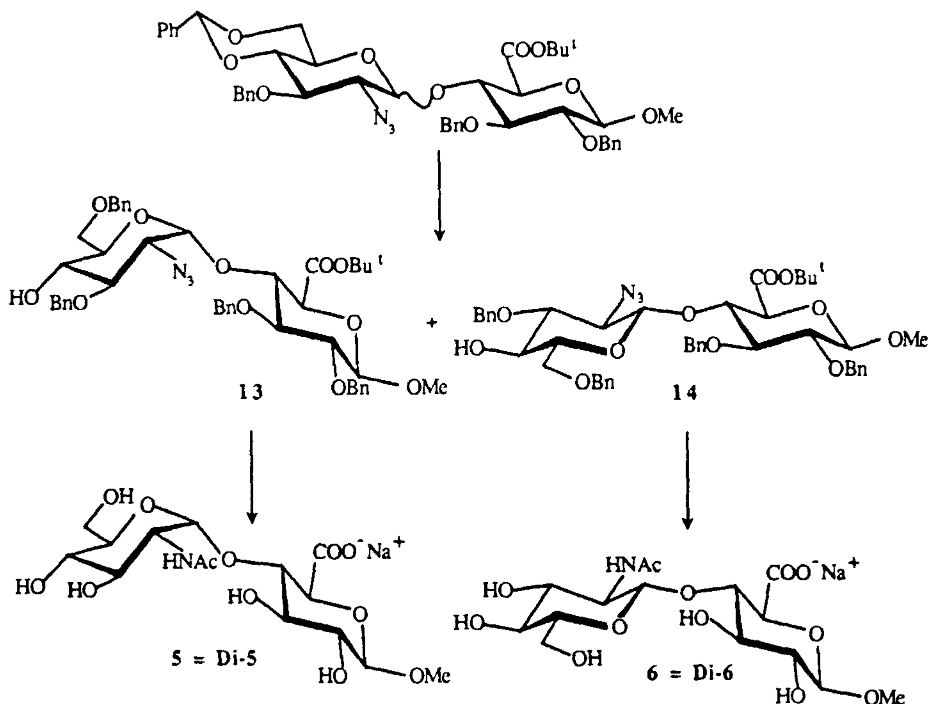
Tert-butyl (methyl 2,3-di-*O*-benzyl-β-D-glucopyranoside)uronate[16] was glycosylated with 2-azido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranosyl chloride[17] using silver triflate and DTBMP. The product, which was an inseparable α,β-mixture of disaccharides obtained in a total yield of 89%, was subjected to reductive opening of the 4,6-*O*-benzylidene acetal using sodium cyanoborohydride and HCl in diethyl ether to give the OH-4 disaccharides **13** and **14** which were separated by column chromatography. The desired α-glycoside **13** was obtained in 55% yield and the β-glycoside **14** was isolated in 25% yield. The disaccharides **13** and **14** were both deprotected by hydrolysis of the *tert*-butyl esters using

trifluoroacetic acid in dichloromethane,[25] followed by hydrogenolysis over Pd-C. Selective *N*-acetylations of the resulting amines by treatment with acetic anhydride in water-methanol gave compound **5** in 63% yield and compound **6** in 53% yield.



Compound **13** was glycosylated with methyl (2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide)uronate using silver triflate and DTBMP to give the trisaccharide **15** in 50% yield. Compound **15** was deprotected by hydrolysis of the *tert*-butyl esters using trifluoroacetic acid in dichloromethane,[25] followed by deacetylation, using 2 M sodium hydroxide in tetrahydrofuran and hydrogenolysis over Pd-C. Selective *N*-acetylation of the resulting amine by treatment with acetic anhydride in water-methanol gave the trisaccharide **7** in 55% yield.

Methyl *O*-(methyl 2,3,4-tri-*O*-acetyl-α-L-idopyranosyluronate)-(1→4)-*O*-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl)-(1→4)-(*tert*-butyl 2,3 -di-*O*-benzyl-β-D-glucopyranosid)uronate[17] was deprotected as described for compound **7**. *N*-sulphation by treatment with triethylamine-sulfur-trioxide complex gave the trisaccharide **8** in 58% yield.

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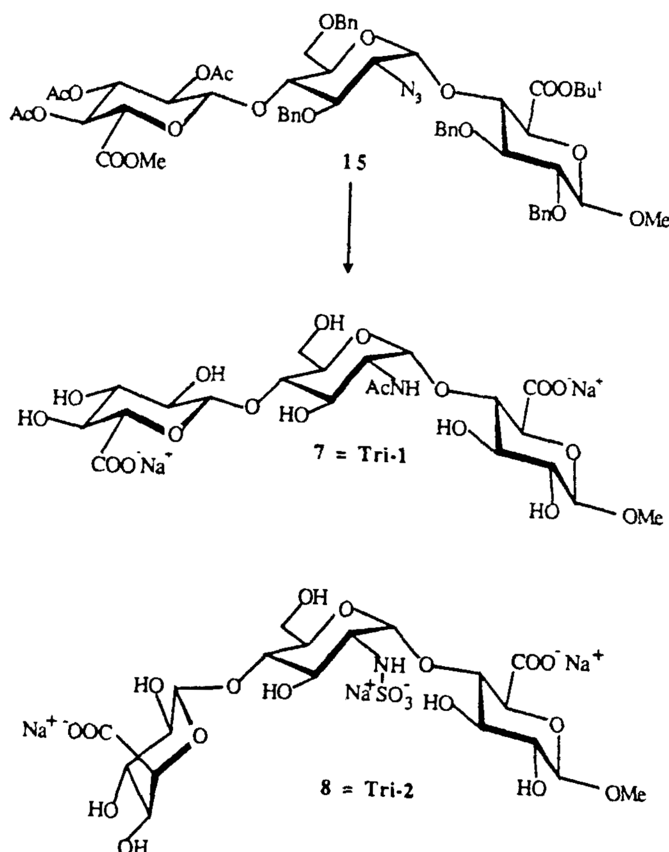

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FGF binding activity

The synthesized oligosaccharides (Table 1) were assayed for their ability to compete with iodinated heparin for binding FGF. Of the six disaccharides tested we found that Di-3 and Di-4 could compete with iodinated heparin for binding FGF. Di-6 (obtained as a side product during the synthesis of Di-5) contains a non heparin/heparan sulphate β-D-GlcNAc structure (as in hyaluronic acid). This disaccharide, and several disaccharides containing structure found in heparin/heparan sulphate do not bind FGF very well. Of the three trisaccharides tested both the two non-sulphated trisaccharides (Tri-1 and Tri-3) compete well with iodinated heparin binding to both FGF-1 (Figure 1) and FGF-2 (Figure 2). However, the *N*-sulphated trisaccharide (Tri-2) does not compete very well for binding to FGF. The tetrasaccharide (Tetra-1) which has an the extra monosaccharide β-D-Gal

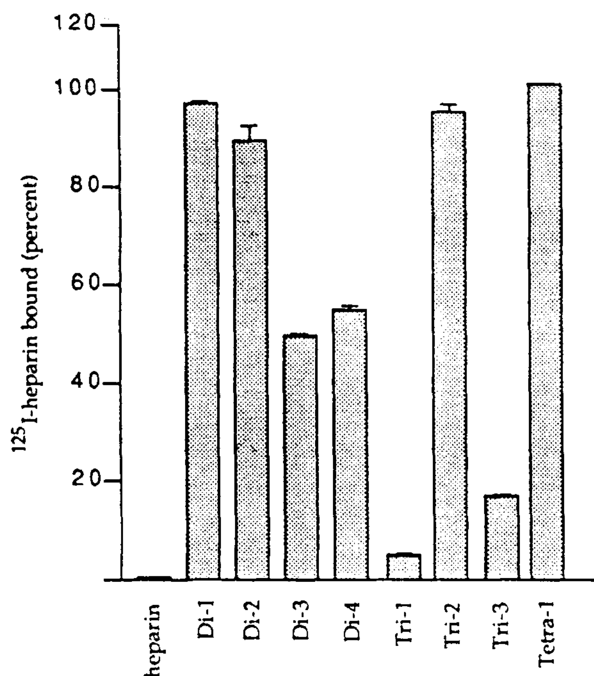

Figure 1. Oligosaccharide binding to acidic fibroblast growth factor (FGF-1). Iodinated heparin was incubated with FGF-1 in the presence of unlabelled heparin or synthetic oligosaccharides (40 μg/mL). The heparin-FGF-1complex was then immunoprecipitated with polyclonal antibody (against the carboxy terminus of FGF-1) and protein A Sepharose. Following washing with PBS, bound ^{125}I-heparin was counted. The data in this experiment is representative of two independent binding experiments.

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added to the reducing terminal of Tri-3 does not compete for binding, and two tetrasaccharides (Tetra-2 and Tetra-3) which correspond to the linkage region of heparin/heparan sulphate also do not compete for binding (Figure 2) (regardless of whether the phosphate group was present or not). With respect to FGF-1 (Figure 1), Tri-1 competes more efficiently than Tri-3 for binding, whereas for FGF-2 the difference between Tri-1 and Tri-3 is very small.

Heparin and heparan sulphate are very similar polysaccharides. One difference between them is the amount and substitution pattern of sulphate groups, with heparan sulphate having less sulphate groups than heparin[26]. Heparan sulphate also contains a higher ratio of glucuronic acid to iduronic acid compared to heparin.[26] In contrast to heparin, heparan sulphate also

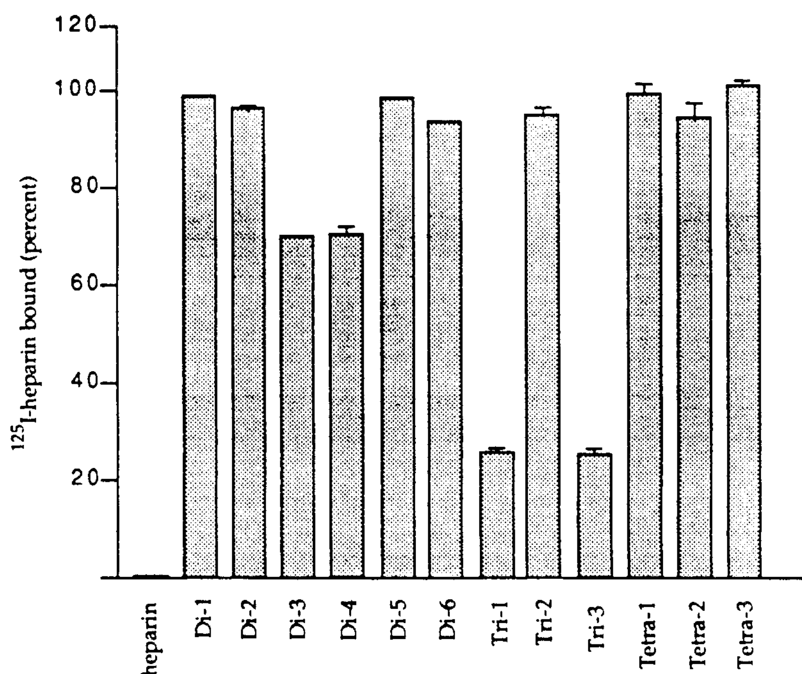

Figure 2. Oligosaccharide binding to basic fibroblast growth factor (FGF-2). Iodinated heparin was incubated with FGF-2 in the presence of unlabelled heparin or synthetic oligosaccharides (40 μg/mL). The heparin-FGF-2 complex was then immunoprecipitated with monoclonal antibody DG2 and protein A Sepharose. Following washing with PBS, bound ^{125}I-heparin was counted. The data in this experiment is representative of at least three independent binding experiments.

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contains non-sulphated oligosaccharide sequences. Such sequences generally are limited to disaccharides in heparin.[26,27] Heparan sulphate, found in the extra cellular matrix and on the cell surfaces is considered the major binding polysaccharide for FGF.[28,29]

The sulphated disaccharide (Di-4) and the non-sulphated saccharides (Di-3, Tri-1 and Tri-3) are to the best of our knowledge the smallest heparan sulphate/heparin derived saccharides shown to bind to FGF. The structure of the non reducing uronic acid of these saccharides does not affect the binding to FGF-2. This is similar to an observation by Maccarana *et al*[12] who found that for a heptasulphated pentasaccharide from heparin and an octasaccharide from heparan sulphate (which in their hands represented the smallest FGF-2 binding oligosaccharides) the non reducing terminal could

be either non-sulphated GlcA or IdoA. Mach *et al.*[13] have also shown that a hexasulphated tetrasaccharide derived from heparin can bind FGF-1, but not a disaccharide.

With respect to FGF-1, Tri-1 containing GlcA at the non-reducing terminal position, binds better than the Tri-3, which contains an IdoA at the same position. This suggests that there may be more specificity for binding to FGF-1 than to FGF-2 with respect to these non-sulphated trisaccharides. Surprisingly when we changed the substitution of the amino group on the glucosamine of Tri-3 from acetyl to sulphate to give Tri-2 the binding decreased considerably. It remains to be seen whether the corresponding sulphation of Tri-1 is more favourable for binding. Here we demonstrate that non-sulphated di- and trisaccharides are capable of binding FGF. This is important because it suggests that FGF may bind to the low sulphated heparan sulphate found in the extra cellular matrix and on the cell surface. It has been postulated that FGF binds to highly sulphated (heparin like) sequences in heparan sulphate.[10,30]

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Such highly sulphated sequences are however not very common in heparan sulphate (compared to low sulphated and non-sulphated sequences).[12] Our finding that non-sulphated trisaccharides as well as a non sulphated disaccharide binds to FGF constitutes an additional explanation of how FGF can bind to heparan sulphate *in vivo*. Previous studies demonstrate that heparin/heparan sulpate is required for FGF to bind to its high affinity receptor and to transmit a biological signal.[8,9] Separately we demonstrate that our non-sulphated trisaccharides are biologically active in these assays.[19]

EXPERIMENTAL

General Methods. Concentrations were performed under diminished pressure at < 40 °C (bath). Optical rotations were recorded for 0.5% solutions at room temperature (22-25 °C) using a Perkin-Elmer 241 polarimeter. NMR spectra were recorded either in $CDCl_3$ with Me_4Si as internal standard or in D_2O with sodium 4,4-dimethyl-4-silapentanoate-2,2',3,3'-d_4 as internal standard at 30 °C, using JEOL EX-400 and Varian 600 MHz instruments. All

^{1}H NMR assignments were based on 2D experiments. NMR spectra recorded for all new compounds, were in agreement with the postulated structures, and only selected data are reported. ^{1}H NMR shift values and coupling constants (values in parentheses) are often presented as tables, in which the sugar residues are given as GlcA, GlcN and IdoA. TLC was performed on Silica gel F254 (Merck) with detection by UV and /or by charring with H_2SO_4. Column chromatography was performed on Silica gel (Matrex Silica Si 60A, 35-70 µm, Amicon). Organic solutions were dried over magnesium sulphate. Molecular sieves were desiccated at 300 °C overnight. The purity of the target compounds was ascertained by NMR spectroscopy.

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Methyl 2-Azido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-α-D-gluco-pyranoside (9). Tetraethylammonium bromide (1.4 g, 6.8 mmol) was added to a stirred solution of 2-azido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranosylnitrate[17] (1.0 g, 2.3 mmol), collidine (1.50 mL, 11.3 mmol) and methanol (0.60 mL, 15 mmol) in CH_2Cl_2 (50 mL). The mixture was stirred at room temperature for 48 h and was then washed with $NaHCO_3$ (aq), water, dried, filtered and concentrated. The residue was purified by column chromatography (toluene-ethyl acetate, 8:1) to give **9** (0.60 g, 1.5 mmol, 65%) isolated as a solid, having $[\alpha]_{578}$ +23° (*c* 0.5, $CHCl_3$). R_f 0.66 (petroleum ether-ethyl acetate 4:1). NMR data ($CDCl_3$): ^{13}C, δ 55.4 (MeO), 62.6, 63.2, 68.9, 75.0, 76.7, 82.8 (C-2,3,4,5,6 and CH_2Ph), 99.4 (C-1) and 101.5 (PhCH); ^{1}H, δ 3.44 (dd, $J_{1,2}$ 3.7, $J_{2,3}$ 9.5 Hz, H-2), 3.71 (t, $J_{3,4}$ = $J_{4,5}$ 9.3 Hz H-4), 3.77 (t, $J_{5,6a}$ = $J_{6a,6b}$ 10.0 Hz, H-6a), 3.86 (m, H-5), 4.06 (t, H-3), 4.29 (dd, $J_{5,6b}$ 4.6 Hz, H-6b), 4.78 (d, H-1).

Anal. Calcd for $C_{21}H_{23}N_3O_5$: C, 63.4; H, 5.9; N, 10.6. Found: C, 63.1; H, 6.0; N, 10.4.

Methyl 2-Azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside (10). Diethyl ether saturated with HCl was added, at room temperature, to a stirred mixture of **9** (0.46 g, 1.2 mmol), $NaCNBH_3$ (0.44 g, 7.0 mmol) and 3A molular sieves in THF (20 mL) until the mixture was acidic (as determined with indicator paper). The mixture was stirred for 10 min and was then diluted with CH_2Cl_2 and filtered. The solution was washed with $NaHCO_3$ (aq), water, dried, filtered and concentrated. The residue was purified by column chromatography (toluene-ethyl acetate 8:1) to give **10** (0.62 g, 1.6 mmol, 88%) isolated as a syrup, having $[\alpha]_{578}$ +62° (*c* 0.5, $CHCl_3$), R_f 0.41

(petroleum ether-ethyl acetate 4:1). NMR data ($CDCl_3$): ^{13}C, δ 55.3 (MeO), 63.1, 69.8, 70.0, 72.1, 73.7, 75.1, 80.0 (C-2,3,4,5,6 and 2 CH_2Ph), 98.8 (C-1); ^1H, δ 3.40 (s MeO), 3.34 (dd, $J_{1,2}$ 3.7, $J_{2,3}$ 10.0 Hz, H-2), 3.65-3.72 (H-4, H-5, H-6), 3.79 (t, $J_{3,4}$ 8.6 H-3), 4.76 (d, H-1).

Methyl *O*-(methyl 2,3,4-tri-*O*-acetyl-α-L-idopyranosyluronate)-(1→4)-O-2-azido-3,6-di-O-benzyl-2-deoxy-α-D-glucopyranoside (11). A mixture of **10** (0.28 g, 0.70 mmol), freshly prepared methyl (2,3,4-tri-O-acetyl-β-L-idopyranosyl bromide)uronate[23] (0.39 g, 1.1 mmol), 4A molecular sieves in CH_2Cl_2 (2 mL) was stirred under N_2 at room temperature for 10 min and was then cooled to -15 °C. Silver triflate (0.35 g, 1.4 mmol) was added and the mixture was stirred at this temperature for 1 hour. Triethylamine (0.8 mL) was added and the mixture was filtered through Celite, concentrated and purified by column chromatography (toluene-ethyl acetate 4:1) to give **11** (0.35 g, 0.50 mmol, 71%) isolated as a syrup, having $[\alpha]_{578}$ +5° (*c* 0.5, $CHCl_3$), R_f 0.61 (toluene-ethyl acetate 1:1). NMR data ($CDCl_3$): ^{13}C, δ 20.6, 20.8 21.0 (Me acetyl), 52.1, 55.4 (2 MeO), 97.0 (C'-1), 98.5 (C-1), 137.6, 137.9 (aromatic C, BnO), 168.0 (C'-6), 169.0, 169.4, 169.6 (C=O acetyl). ^1H NMR data are shown in the following table.

	H-1	H-2	H-3	H-4	H-5
GlcN	4.81 (3.7)	3.49 (10.1)	3.80 (9.5)	4.05 (9.5)	3.71
IdoA	5.31 (3.1)	4.78 (5.2)	5.15 (4.7)	5.02 (3.7)	4.88

Methyl *O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-(1→4)-*O*-2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside (12). A mixture of **10** (0.14 g, 0.30 mmol), methyl (2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide)uronate[24] (0.20 g, 0.48 mmol), 4A molecular sieves and 2,6-di-*tert*-butyl-4-methylpyridine (DTBMP) (35 mg, 0.17 mmol) in CH_2Cl_2 (2 mL) was stirred under N_2 at room temperature for 10 min and was then cooled to -15 °C. Silver triflate (0.15 g, 0.58 mmol) was added and the mixture was stirred at this temperature for 1 hour. Triethylamine (0.4 mL) was added and the temperature was allowed to reach room temperature. The mixture was then filtered through Celite, concentrated, and purified by column chromatography (toluene-ethyl acetate 4:1) to give **12** (0.15 g, 0.22 mmol, 65%) isolated as a syrup, having $[\alpha]_{578}$ +60° (*c* 0.5,

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$CHCl_3$), R_f 0.58 (toluene-ethyl acetate 1:1). NMR data ($CDCl_3$): ^{13}C, δ 20.5, 20.5, 20.6 (Me acetyl), 52.7 (MeO), 98.8 (C-1), 100.0 (C'-1), 137.5, 138.4 (aromatic C, BnO), 166.8 (C'-6), 168.9, 169.3, 170.0 (C=O, acetyl). ^1H NMR data are shown in the following table.

	H-1	H-2	H-3	H-4	H-5
GlcN	4.76 (3.9)	3.36 (10.2)	3.80 (9.6)	3.99 (9.3)	3.62
GlcA	4.51 (7.8)	4.93 (9.3)	4.99 (9.3)	5.17 (9.8)	3.67

Methyl *O*-(α-L-idopyranosyluronic acid)-(1→4)-*O*-2-acetamido-2-deoxy-α-D-glucopyranose sodium salt (1). Compound **11** (75 mg, 0.11 mmol) was dissolved in THF (4 mL) and cooled to +4 °C. Cold aq NaOH (1 mL, 2M) was added dropwise and the mixture was stirred at +8 °C until TLC indicated complete reaction. The mixture was neutralized with Dowex H^+ and concentrated. The residue was dissolved in 1:1 water-EtOH (10 mL) and hydrogenolyzed over Pd-C for 24 h. After filtering through Celite and concentrating, the residue was dissolved in 4:1 water-MeOH (4 mL) and the pH of the solution was adjusted to 7.5 with satd. $NaHCO_3$ (aq). Acetic anhydride (40 μL) was added in small portions and the mixture was stirred for 2 h at room temperature. The mixture was concentrated and the residue was dissolved in water and passed through a column of Dowex Na^+. The eluate was concentrated and purified on a column of P2 Biogel, using water (containing 1% 1-butanol) as eluent, to give **1** (36 mg, 83 μmol, 76%) isolated as a solid, having $[\alpha]_{578}$ +28° (*c* 0.5, H_2O), R_f 0.54 (ethyl acetate-ethanol-acetic acid-water 4:3:2:2). NMR data (D_2O): ^{13}C, δ 24.7 (Me *N*-acetyl), 56.5 (MeO), 58.0, 62.8, 72.5, 73.5, 74.0, 74.3, 74.4, 75.3, 81.0 (ring C), 100.6 (C-1), 104.1 (C'-1), 177.3, 178.7 (C'-6 and C=O, *N*-acetyl). ^1H NMR data are shown in the following table.

	H-1	H-2	H-3	H-4	H-5
GlcN	4.78 (3.9)	3.96 (10.5)	3.80 (8.6)	3.75	ND
IdoA	4.83 (5.9)	3.50 (8.3)	3.68 (6.9)	3.88 (4.4)	4.54

Methyl *O*-(α-L-idopyranosyluronic acid)-(1→4)-*O*-2-deoxy-2-sulfamido-α-D-glucopyranose disodium salt (2). Compound **11** (75 mg, 0.11 mmol) was treated with NaOH in THF and then hydrogenolyzed over Pd-C

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for 24 h as described for the preparation of 1. The residue from the hydrogenolysis was dissolved in water (5 mL) and the pH was adjusted to 9 with NaOH (2 M). Triethylamine-sulfur trioxide (0.18 g, 1.1 mmol) was added in small portions during 1 h and the mixture was then stirred for 2 h at room temperature. The mixture was concentrated and the residue was dissolved in water and passed through a column of Dowex Na^+. The eluate was concentrated and purified on a column of P2 Biogel, using water (containing 1% 1-butanol) as eluent, to give **2** (28 mg, 60 μmol, 55%) isolated as a solid, having $[\alpha]_{578}$ +33° (*c* 0.5, H_2O), R_f 0.31 (ethyl acetate-ethanol-acetic acid-water 4:3:2:2). NMR data (D_2O): ^{13}C, δ 57.7 (MeO), 58.1, 62.9, 73.7, 74.1, 74.4, 74.4, 75.3, 75.5, 80.9 (ring C), 102.4 (C-1), 104.0 (C'-1), 178.7 (C'-6). ^1H NMR data are shown in the following table.

	H-1	H-2	H-3	H-4	H-5
GlcN	4.78 (3.7)	3.61 (10.5)	3.63 (8.9)	3.78	3.91
IdoA	4.81 (5.6)	3.49 (8.1)	3.67 (7.1)	3.87 (4.5)	4.54

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Methyl *O*-(β-D-glucopyranosyluronic acid)-(1→4)-*O*-2-acetamido-2-deoxy-α-D-glucopyranose sodium salt (3). Compound **12** (30 mg, 43 μmol) was treated as described for the preparation of **1** to give **3** (14 mg, 32 μmol, 73%) isolated as a solid, having $[\alpha]_{578}$ +36° (*c* 0.5, $CHCl_3$), R_f 0.55 (ethyl acetate-ethanol-acetic acid-water 4:3:2:2). NMR data (D_2O): ^{13}C, δ 24.8 (Me *N*-acetyl), 56.2 (OMe), 58.1, 62.9, 72.5, 73.2, 74.6, 75.9, 78.3, 78.6, 82.2 (ring C), 100.6 (C-1), 105.2 (C'-1), 177.3 (C=O, *N*-acetyl), 178.4 (C'-6). ^1H NMR data are shown in the following table.

	H-1	H-2	H-3	H-4	H-5
GlcN	4.78 (3.3)	3.92 (10.8)	3.84 (8.0)	3.70	ND
GlcA	4.54 (7.8)	3.36 (9.6)	3.53 (8.4)	ND	ND

Methyl *O*-(β-D-glucopyranosyluronic acid)-(1→4)-*O*-2-deoxy-2-sulfamido-α-D-glucopyranose disodium salt (4). Compound **12** (30 mg, 43 μmol) was treated as described for the preparation of **2** to give **4** (11 mg, 23 μmol, 54%) isolated as a solid, having $[\alpha]_{578}$ +27° (*c* 0.5, H_2O), R_f 0.49 (ethyl acetate-ethanol-acetic acid-water 4:3:2:2). NMR data (D_2O): ^{13}C, δ 58.3 (OMe),

60.3, 62.9, 72.6, 73.1, 74.6, 75.9, 78.3, 78.3, 81.8 (ring C), 101.1 (C-1), 105.0 (C'-1), 178.3 (C'-6). ^{1}H NMR data are shown in the following table.

	H-1	H-2	H-3	H-4	H-5
GlcN	5.05 (3.9)	3.29 (10.4)	3.71 (8.9)	3.78	3.94
GlcA	4.56 (7.8)	3.39 (9.3)	3.54	ND	ND

Methyl *O*-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl)-(1→4)-*O*-(*tert*-butyl 2,3-di-*O*-benzyl-β-D-glucopyranoside)uronate (13) and Methyl *O*-(2-azido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(*tert*-butyl 2,3-di-*O*-benzyl-β-D-glucopyranoside)uronate (14). A mixture of 2-azido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranosylchloride[17] (1.00 g, 2.24 mmol), *tert*-butyl (methyl 2,3-di-*O*-benzyl-β-D-glucopyranoside)uronate[17] (1.08 g, 2.42 mmol), 4A molecular sieves and DTBMP (920 mg, 4.48 mmol) in dry diethyl ether (10 mL) was stirred under N_2 at room temperature for 10 min and was then cooled to -15 °C. Silver triflate (1.38 g, 5.39 mmol) was added and the mixture was stirred at room temperature for 1 hour. The mixture was diluted with CH_2Cl_2 (20 mL) and cooled to 0 °C. The solution was washed with sodium thiosulphate 10% (aq) and water, dried and concentrated. The residue was purified by column chromatography (petroleum ether-ethyl acetate 6:1) to give methyl *O*-(2-azido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-α,β-D-glucopyranosyl)-(1→4)-*O*-(*tert*-butyl 2,3-di-*O*-benzyl-β-D-glucopyranoside)uronate (1.62 g, 1.99 mmol, 89%) isolated as a solid, having R_f 0.54 (petroleum ether-ethyl acetate 4:1). NMR data ($CDCl_3$): ^{13}C δ 97.8 (C'-1, α), 101.4 (C'-1, β), 104.9 (C-1); ^{1}H, δ 4.38 (d, $J_{1,2}$ 7.6 Hz H-1), 4.56 (d, $J_{1,2}$ 8.0 Hz H'-1β), 5.56 (d, $J_{1,2}$ 3.9 Hz H'-1α).

The α,β-mixture (0.65 g, 0.80 mmol) was treated with diethyl ether saturated with HCl and $NaCNBH_3$ as described for the preparation of **10**, and purified by column chromatography (petroleum ether-ethyl acetate 6:1). Compound **13** (0.36 g, 0.44 mmol, 55%) was isolated as an amorphous solid having $[\alpha]_{578}$ +28° (*c* 0.5, $CHCl_3$), R_f 0.43 (petroleum ether-ethyl acetate 4:1). NMR data ($CDCl_3$): ^{13}C, δ 28.8 (Me *tert*-butyl), 57.0 (MeO), 97.2 (C'-1), 104.7 (C-1), 167.3 (C-6); ^{1}H NMR data are shown in the following table.

	H-1	H-2	H-3	H-4	H-5
GlcA	4.39 (7.3)	3.47 (8.8)	3.72 (8.8)	4.18 (9.3)	3.79
GlcN	5.58 (3.9)	3.21 (10.3)	3.72 (9.3)	3.77 (9.8)	3.60

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Compound **14** (0.16 g, 0.20 mmol, 25%) was isolated as an amorphous solid having $[\alpha]_{578}$ -6° (*c* 0.5, $CHCl_3$), R_f 0.39 (petroleum ether-ethyl acetate 4:1). NMR data ($CDCl_3$): ^{13}C, δ 27.9 (Me *tert*-butyl), 57.2 (MeO), 101.1 (C'-1), 104.7 (C-1), 167.7 (C'-6); ^{1}H NMR data are shown in the following table.

	H-1	H-2	H-3	H-4	H-5
GlcA	4.34 (7.8)	3.39 (8.8)	3.58 (9.3)	4.15 (8.8)	3.82
GlcN	4.50 (7.8)	3.29 (8.8)	3.19 (9.3)	3.65 (8.8)	3.35

Methyl *O*-(2-acetamido-2-deoxy-α-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyluronic acid sodium salt (5). Compound **13** (75 mg, 92 μmol) was dissolved in a solution of CF_3CO_2H in CH_2Cl_2 (20%) and stirred at room temperature for 1 h, The mixture was diluted with CH_2Cl_2 and washed with $NaHCO_3$ (aq) and water, dried and concentrated. The residue was dissolved in 1:1 water-ethanol (10 mL) and hydrogenolyzed over Pd-C for 24 h. After filtering through Celite and concentrating the residue was *N*-acetylated and purified as described for **1** to give compound **5** (25 mg, 58 μmol, 63%) isolated as a solid having $[\alpha]_{578}$ +74° (*c* 0.5, H_2O), R_f 0.51 (ethyl acetate-ethanol-acetic acid-water 4:3:2:2). NMR data (D_2O): ^{13}C, δ 24.7 (Me *N*-acetyl), 56.5 (MeO), 60.0, 62.9, 72.5, 73.6, 74.7, 76.2, 78.6, 79.5, 79.7, (ring C), 99.6 (C'-1), 106.0 (C-1), 177.3 (C-6), 177.9 (C=O, acetyl). ^{1}H NMR data are shown in the following table.

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	H-1	H-2	H-3	H-4	H-5
GlcA	4.37 (8.1)	3.30 (9.0)	3.68 (8.8)	3.77	ND
GlcN	5.40 (3.7)	3.88 (10.3)	3.72 (8.5)	3.48	ND

Methyl *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-β-D-glucopyranosyluronic acid sodium salt (6). Compound 14 (70 mg, 86 μmol) was treated as described for the preparation of **5** to give **6** (20 mg, 46 μmol, 53%) isolated as a solid having $[\alpha]_{578}$ +1° (*c* 0.5, H_2O), R_f 0.51 (ethyl acetate-ethanol-acetic acid-water 4:3:2:2). NMR data (D_2O) ^{13}C, δ 25.2 (Me *N*-acetyl), 58.2 (MeO), 60.1, 63.3, 72.5, 75.5, 76.6, 76.7, 78.7, 79.5, 82.8, 82.9 (ring C), 103.6 (C'-1), 106.2 (C-1), 177.2 (C-6), 177.7 (C=O *N*-acetyl). ^{1}H NMR data are shown in the following table.

	H-1	H-2	H-3	H-4	H-5
GlcA	4.37 (8.1)	3.33 (9.5)	3.59 (8.8)	3.76 (9.5)	3.92
GlcN	4.55 (8.8)	3.69 (8.1)	3.53 (9.5)	3.44	3.77

Methyl *O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl)-(1→4)-*O*-(*tert*-butyl 2,3-di-*O*-benzyl-β-D-glucopyranoside)uronate (15). A mixture of 13 (0.12 g, 0.12 mmol), methyl (2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide)uronate[24] (75 mg, 0.18 mmol), 4A molecular sieves and DTBMP (15 mg, 72 μmol) in CH_2Cl_2 (2 mL) and silver triflate (0.25 g, 1.0 mmol) was treated as described for the preparation of compound **11** to give **15** (70 mg, 62 μmol, 50%) isolated as an amorphous solid, having $[\alpha]_{578}$ +28° (*c* 0.5, $CHCl_3$), R_f 0.68 (toluene-ethyl acetate 1:1). NMR data ($CDCl_3$): ^{13}C, δ 20.5, 20.6, 20.7 (Me acetyl), 28.1 (Me *tert*-butyl), 52.7, 57.1 (MeO) 96.7 (C'-1), 99.8 (C''-1), 104.9 (C-1), 166.9, 167.5 (C''-6, C-6), 168.8, 169.3, 170.5 (C=O, acetyl). ^1H NMR data are shown in the following table.

	H-1	H-2	H-3	H-4	H-5
GlcA	4.38 (8.0)	3.46 (8.6)	3.75 (9.5)	4.12 (9.0)	3.18
GlcN	5.62 (3.9)	3.22 (10.5)	3.64 (9.5)	3.97 (9.5)	3.62
GlcA	4.53 (8.1)	4.86 (9.3)	4.95 (9.5)	5.13 (9.8)	3.65

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Methyl *O*-(β-D-glucopyranosyluronic acid)-(1→4)-*O*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-(1→4)-*O*-β-D-glucopyranosyluronic acid disodium salt (7). Compound **15** (70 mg, 62 μmol) was deprotected in three steps. Hydrolysis of the *tert*-butyl ester was performed with CF_3CO_2H in CH_2Cl_2 (20%) according to the method described for compound **5**. Subsequent treatment with cold aq NaOH (1 mL, 2M) in THF followed by hydrogenolysis over Pd-C in water: EtOH (1:1) gave the deprotected trisaccharide. Finally, the amino group was acetylated by satd. $NaHCO_3$ (aq) and acetic anhydride as described for compound **1** to give **7** (20 mg, 34 μmol, 55%) isolated as a solid, having $[\alpha]_{578}$ +34° (*c* 0.5, H_2O), R_f 0.35 (ethyl acetate-ethanol-acetic acid-water 4:3:2:2). NMR data (D_2O): ^{13}C, δ 24.8 (Me *N*-acetyl), 56.1 (MeO), 60.0, 62.2, 72.0, 73.3, 73.4, 74.6, 75.7, 76.2, 78.0, 78.9, 79.5, 79.7, 81.3 (ring C), 99.5 (C'-1), 105.2 (C''-1), 106.0 (C-1), 177.1, 177.9 (C-6, C''-6), 178.4 (C=O, acetyl); ^1H NMR data are shown in the following table.

	H-1	H-2	H-3	H-4	H-5
GlcA	4.37 (8.1)	3.30 (8.9)	3.70 (9.0)	3.72 (8.7)	ND
GlcN	5.39 (3.8)	3.94 (10.5)	3.82 (8.4)	ND	ND
GlcA	4.52 (8.0)	3.38 (9.7)	3.52	ND	ND

Methyl *O*-(α-L-idopyranosyluronic acid)-(1→4)-*O*-(2-sulfamido-2-deoxy-α-D-glucopyranosyl)-(1→4)-*O*-β-D-glucopyranosyluronic acid trisodium salt (8). Methyl *O*-(methyl 2,3,4-tri-*O*acetyl-α-L-idopyranosyl-uronate)-(1→4)-*O*-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl)-(1→4)-*O*-(*tert*-butyl 2,3-di-*O*-benzyl-β-D-glucopyranosid)uronate[17] (75 mg, 66 μmol) was deprotected in three steps as described for compound **7**. The obtained amino compound was treated with triethylamine-sulfur trioxide as described for compound **2** to give **8** (25 mg, 38 μmol, 58%) isolated as a solid, having $[\alpha]_{578}$ +25°(*c* 0.5, H_2O), R_f 0.18 (ethyl acetate-ethanol-acetic acid-water 4:3:2:2). NMR data (D_2O): ^{13}C, δ 57.3 (MeO), 60.1, 62.1, 71.7, 74.2, 74.3, 74.4, 74.5, 75.4, 76.0, 79.1, 79.2, 79.3, 79.6 (ring C), 98.6 (C'-1), 104.3 (C-1), 106.0 (C''-1), 177.7, 178.8 (C-6, C''-6); ^1H, δ ^1H NMR data are shown in the following table.

	H-1	H-2	H-3	H-4	H-5
GlcA	4.42 (8.3)	3.32 (9.3)	3.75	3.83	ND
GlcN	5.65 (3.9)	3.31 (10.5)	3.92 (8.7)	ND	ND
IdoA	4.82 (5.9)	3.54 (8.3)	3.70 (6.9)	3.89 (4.4)	4.57

Heparin iodination. Fluorescinated heparin (fl-heparin; 2-3 fluorescines /heparin molecules was a gift from C. Parish, Canberra, Australia). 30 μg (5 μL) fl-heparin was mixed with 14 μL 140 mM NaCl; 1.5 μL 0.2 M borate, pH 8.0; 5 μL ^{125}I (100 μCi/μL, Amersham) in a 1.5 mL microcentrifuge tube. The above mixture was then transferred to a glass test tube containing 5.2 μg 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Sigma) which was dissolved in $CHCl_3$, diluted, and dried in a glass tube. The reaction was incubated for 30 min on ice and mixed by hand every 5 min. The reaction was stopped by dilution into 250 μL PBS. The sample was applied to a NAP-5 column (Pharmacia) and eluted in PBS. 250 μL fractions were collected. Fractions 2 and 3, containing labelled heparin were pooled and stored at -70 °C. Specific activity was estimated to be greater than 7000 cpm/ng.

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Binding assays. Glycosaminoglycan binding to FGF-1 and FGF-2 was determined by incubating 4 nM FGF with 150,000 cpm iodinated heparin and competitor polysaccharide (40 μg/mL). The complex was immuno-precipitated with a 1:250 dilution of either monoclonal antibody DG2 (DuPont/Merk, for FGF-2) or a 1:500 dilution of an anti peptide polyclonal antibody (Merk, for FGF-1) with 30 μL of a 2x slurry of protein A Sepharose (Sigma) (diluted 1:4 with Sepharose CL6B). Total volume of the binding reaction was brought to 250 μL with DMEM/0.1% BSA. The immunoprecipitated complex was washed twice with 750 μL ice cold PBS and counted in a gamma counter (Beckman).

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