The Activation of Fibroblast Growth Factors by Heparin: Synthesis, Structure, and Biological Activity of Heparin-Like Oligosaccharides

José-Luis de Paz,[a] Jesús Angulo,[a] José-María Lassaletta,[a] Pedro M. Nieto,[a] Mariano Redondo-Horcajo,[b] Rosa M. Lozano,[b] Guillermo Giménez-Gallego,[b] and Manuel Martín-Lomas*[a]

*An effective strategy has been designed for the synthesis of oligosaccharides of different sizes structurally related to the regular region of heparin; this is illustrated by the preparation of hexasaccharide **1** and octasaccharide **2**. This synthetic strategy provides the oligosaccharide sequence containing a D-glucosamine unit at the nonreducing end that is not available either by enzymatic or chemical degradation of heparin. It may permit, after slight modifications, the preparation of oligosaccharide fragments with different charge distribution as well. NMR spectroscopy and molecular dynamics simulations have shown that the overall structure of **1** in solution is a stable right-hand helix with four residues per turn. Hexasaccharide **1** and, most likely, octasaccharide **2** are, therefore, chemically well-defined structural models of naturally occurring heparin-like oligosaccharides for use in binding and biological activity studies. Both compounds **1** and **2** induce the mitogenic activity of acid fibroblast growth factor (FGF1), with the half-maximum activating concentration of **2** being equivalent to that of heparin. Sedimentation equilibrium analysis with compound **2** suggests that heparin-induced FGF1 dimerization is not an absolute requirement for biological activity.*

KEYWORDS:

conformation analysis · growth factor activation · heparin · oligosaccharides · synthesis design

Introduction

Fibroblast growth factors (FGFs) constitute a family of signaling polypeptides involved in a wide variety of physiological processes. Disruption of their homeostasis is associated with numerous important diseases. FGF signaling at the molecular level is mediated by binding to specific cell-surface receptors (the FGFRs) and the biological activity of FGFs is tightly regulated by heparin-like glycosaminoglycans, either free or belonging to the cell plasma membrane.[1] These heparin-like glycosaminoglycans are predominantly formed by disaccharide repeating units of D-glucosamine (GlcN) and L-iduronic acid (IdoA), linked by α-(1 $\rightarrow$4) glycosidic linkages and typically containing sulfate groups located at positions 2 and 6 of the D-glucosamine unit and position 2 of the L-iduronic acid unit. But these polysaccharides also contain, to a lesser extent, D-glucuronic acid, *N*-acetylglucosamine, and unsubstituted glucosamine residues and show, in addition, considerable heterogeneity as far as the length of the saccharide chain and the degree of sulfation are concerned.[2] The structure of these saccharide chains has been widely studied but some features, which are mainly related to the conformation of the L-iduronic acid units, are still open questions.[3] The influence of saccharide length and charge distribution on FGF binding and the minimal structural requirements for the saccharide chain to regulate FGF biological activity are matters of current interest.[1h, 4] A number of studies seem to indicate that octasaccharides and longer heparin fragments bind and activate FGFs[4, 9] while controversial results have been reported on the stimulatory activity of hexasaccharides.[9, 10] Crystal structures of several FGFs,[5] FGF complexes with heparin fragments,[6] and FGF-2[7] (basic FGF) and FGF-1[8] (acidic FGF) bound to variants of the FGFR have been determined.

However, the role played by heparin-like oligosaccharides in the activation process is not well understood and several hypotheses have been launched to account for the existing experimental data.[10a] It seems to be widely accepted that the specific interaction of FGFs with heparin would give rise to FGF oligomerization and that these oligomers would then interact with the FGFR to induce the cellular response through FGFR

[a] *Prof. Dr. M. Martín-Lomas, Dr. J.-L. de Paz, J. Angulo, Dr. J.-M. Lassaletta, Dr. P. M. Nieto*
Grupo de Carbohidratos
Instituto de Investigaciones Químicas, CSIC
Isla de la Cartuja, Américo Vespucio s/n
41092 Seville (Spain)
Fax: (+34) 95-446-05-65
E-mail: manuel.martin-lomas@iiq.cartuja.csic.es

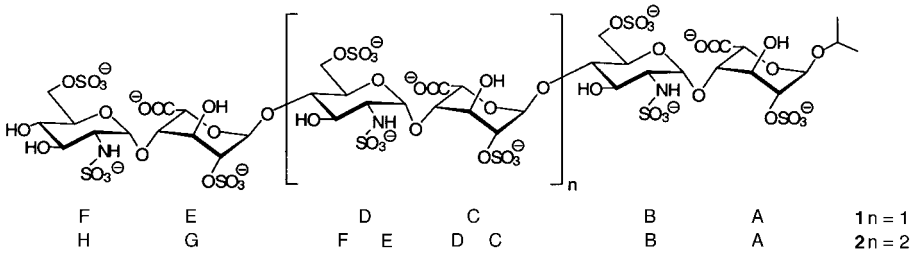
[b] *M. Redondo-Horcajo, Dr. R. M. Lozano, Prof. Dr. G. Giménez-Gallego*
Centro de Investigaciones Biológicas, CSIC
Velazquez 144, 28006 Madrid (Spain)

Supporting information for this article is available on the WWW under http://www.chembiochem.com or from the author.

dimerization.[11] However it is not clear whether this type of mechanism would generally apply to all members of the FGF family. The configuration of the heparin – FGF and heparin – FGF – FGFR complexes remains, therefore, a controversial issue.[12]

The above-mentioned structural, binding, and biological activity studies along with a number of others make evident the complexity involved in establishing the molecular basis of the FGF – heparin – FGFR interaction and in understanding the different functions of the FGF family. The heterogeneity of the heparin-like glycosaminoglycans makes it difficult to determine the binding sequences for the FGFs and FGFRs. Therefore, the development of effective and versatile synthetic strategies that permit the preparation of homogeneous oligosaccharides with defined sequence and charge distributions to perform reliable conformational, binding, and biological activity studies is highly desirable.

We now report a strategy for the synthesis of the regular region of these heparin-like oligosaccharides, the effectiveness of which is illustrated by the preparation of hexasaccharide **1** and octasaccharide **2** (Scheme 1). As a prerequisite to further investigation of the interaction of synthetic **1** and **2** with FGF-1—the crystal structure of binary and ternary complexes of FGF-1 with natural heparin fragments[6c] and with FGFR[8] have been recently reported—we have also examined the three-dimensional structure of synthetic **1** in solution and the effect of **1** and **2** on the stimulation of the mitogenic activity of FGF-1. In order to contribute to the understanding of FGF-1 activation, sedimentation equilibrium analyses with FGF-1 and the synthetic oligosaccharides have been performed as well.



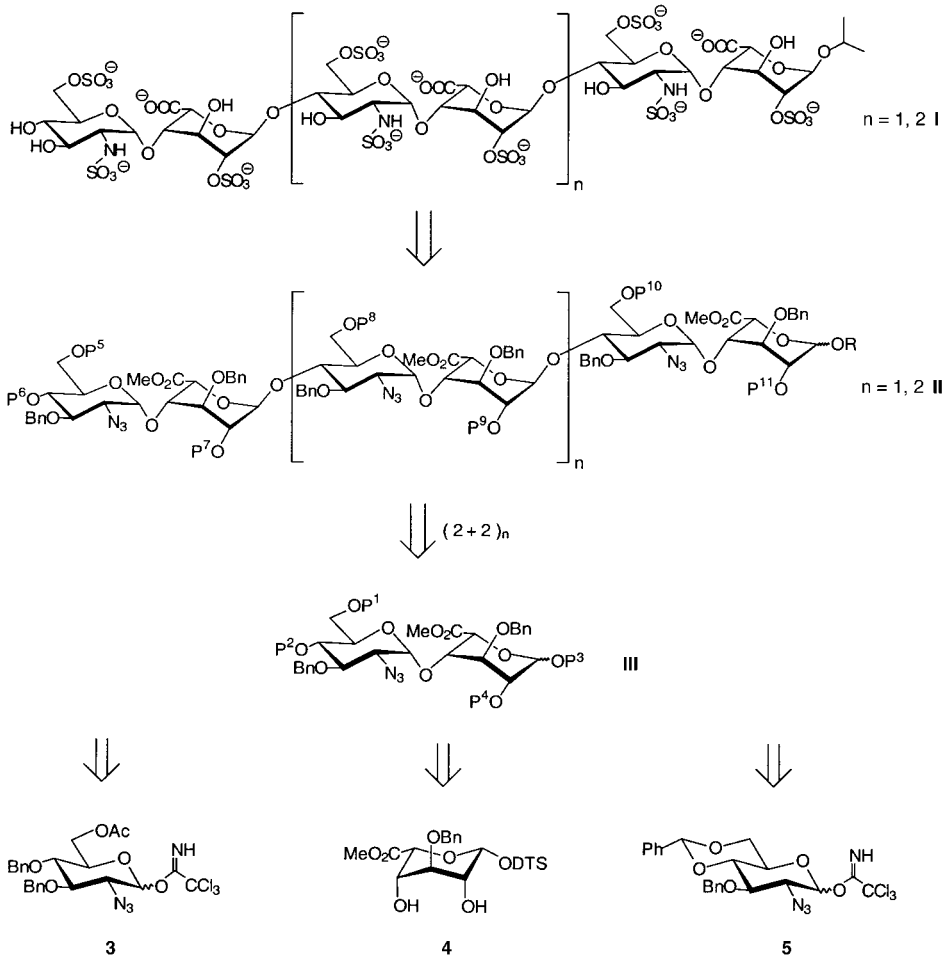
Scheme 1. *Structures of hexasaccharide **1** and octasaccharide **2**. For the sake of simplicity the L-iduronate units are all shown in the $^1C_4$ conformation.*

Results and Discussion

Synthesis

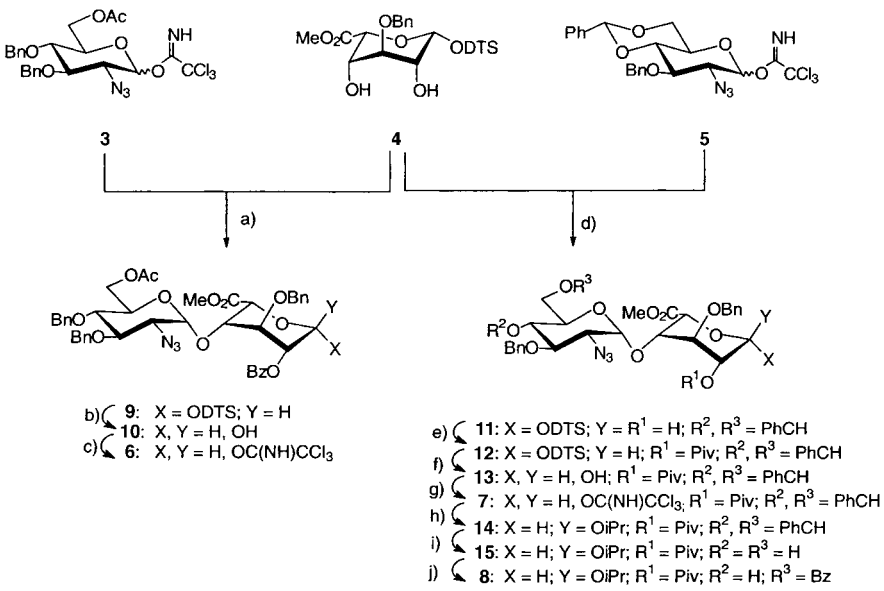
Oligosaccharides **1** and **2** contain L-iduronic acid residues at the reducing end and this sequence is not available either by enzymatic or chemical degradation of heparin. The synthesis of disaccharide sequences containing this structural motif has only recently been reported.[13] While this work was in progress, the synthesis of a tetra- and hexasaccharide containing the alternative sequence, with the L-iduronic acid unit at the nonreducing and the D-glucosamine residue at the reducing end, was reported along with the effect of these synthetic compounds on the inhibition of FGF-2 binding and proliferation induced by FGF-2 with cultured human aortic smooth muscle cells.[14] Previous studies on the synthesis and biological effect on FGF-2 of several pentasaccharide fragments containing L-iduronic or D-glucuronic acid residues and showing the above alternative sequence have also appeared.[15]

The synthesis of fragments of the regular region of heparin (**I**) here reported involves a convergent $n+2$ block approach, as indicated in Scheme 2. A careful protecting-group strategy was



Scheme 2. *General retrosynthetic strategy for the oligosaccharide fragments of the regular region of heparin. R, $P^1 - P^{11}$ = protecting groups, Bn = benzyl, Ph = phenyl, DTS = dimethylthexylsilyl (thexyl = 1,1,2-trimethylpropyl).*

designed to obtain the key disaccharide structures **III**, which may operate either as glycosyl donors or as glycosyl acceptors, thereby allowing the required stereochemistry of the glycosidic linkages to be achieved and the sulfate groups to be located at the desired positions at a later stage. The synthesis of structures **III** was envisaged (Scheme 2) to begin from the 2-azido-2-deoxy-D-glucopyranosyl trichloroacetimidates, **3** and **5**, and the iduronic acid derivative glycosyl acceptor **4**. These monosaccharide units have been prepared in a multigram scale: **5** from D-glucosamine hydrochloride by a diazo transfer reaction with triflic azide,[16] **3** from D-glucal,[17] and **4** from D-glucurono-6,3-lactone.[18]

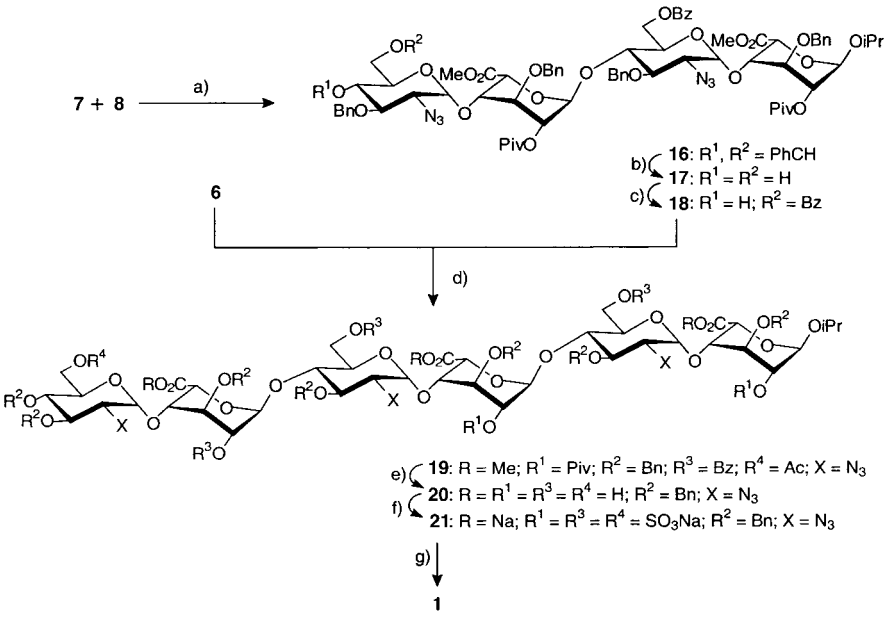


Scheme 3. *Synthesis of disaccharide building blocks. Reagents and conditions: a) 5% TMSOTf, CH_2Cl_2; BzCl, Pyr, 51%; b) $(HF)_x \cdot Pyr$, 88%; c) Cl_3CCN, K_2CO_3, 95%; d) 5% TMSOTf, CH_2Cl_2, 62% (35% of recovered **4**); e) PivCl, Pyr, DMAP (cat.), 91%; f) $(HF)_x \cdot Pyr$, 82%; g) Cl_3CCN, K_2CO_3, 99%; h) iPrOH, 2% TMSOTf, 70%; i) EtSH, PTSA (cat.), 90%; j) BzCN, Et_3N (cat.), MeCN, $-40\,^{\circ}C$, 95%. Bz = benzoyl, DMAP = 4-dimethylaminopyridine, Piv = pivaloyl, PTSA = p-toluenesulfonic acid, Pyr = pyridine, TMSOTf = trimethylsilyl trifluoromethanesulfonate.*

The disaccharides **6**, **7**, and **8** were prepared as building blocks for constructing, respectively, the nonreducing end, the inner regions, and the reducing end of oligosaccharides **1** and **2** as indicated in Scheme 3. Building block **6** was synthesized by regio- and stereoselective glycosylation of the iduronic acid derivative **4**[18] with trichloroacetimidate **3**[13] followed by benzoylation to give **9**, desilylation to afford **10**, and anomeric activation to yield finally **6** (43% overall yield). The regioselectivity observed in the glycosylation of **4** with **3**, as well as in the glycosylation of **4** with **5** (see below), most likely results as a consequence of the $^1C_4$ conformation of **4** ($^4J_{2,4} = 1.1$ Hz), favored by a strong OH4 → O2 hydrogen bond ($J_{2,OH2} = 0$, $J_{4,OH4} = 11.5$ Hz) that should decrease the nucleophilicity of OH2, as well as being due to the steric hindrance imposed on OH2 by the neighboring dimethylthexylsilyl group.

Building blocks **7** and **8** were prepared from the common precursor disaccharide **11**, which was synthesized in 63% yield by regio- and stereoselective glycosylation of **4**[18] with trichloroacetimidate **5**.[19] Pivaloylation of **11** gave **12**; this was then desilylated, to give **13**, and activated as trichloroacetimidate **7** in 74% overall yield. Compound **7**, which constituted the key building block for the construction of the inner regions of the target oligosaccharides, was also used for the synthesis of the reducing end disaccharide **8**. Glycosylation of **7** with isopropanol afforded the isopropyl glycoside **14** which was transformed into diol **15** and then finally regioselectively benzoylated to give **8** in 68% overall yield. The presence of the sterically demanding pivaloyl group along the synthetic pathway was crucial since it considerably decreased acyl migration in the desilylation step, avoided orthoester formation in the glycosylation step,[20] and contributed a better glycosyl donor performance than the corresponding 2-*O*-acetyl analogues.[21] Glycosylation of acceptor **8** with donor **7** afforded tetrasaccharide **16** in 79% yield.

The benzylidene protecting groups were removed from compound **16** to give **17**, which was then transformed into acceptor **18** by selective benzoylation in 93% yield (Scheme 4).



Scheme 4. *Assembly of hexasaccharide **19** and deprotection sequence to form **1**. Reagents and conditions: a) 3% TMSOTf, CH_2Cl_2, 79% (19% of recovered **8**); b) EtSH, PTSA (cat.), 75%; c) BzCN, Et_3N (cat.), MeCN, $-40\,^{\circ}C$, 93%; d) 3% TMSOTf, 58% (36% of recovered **18**); e) KOH, 74%; f) $SO_3 \cdot NMe_3$; Dowex 50WX4 (Na^+), 71%; g) 10% Pd/C, H_2; $SO_3 \cdot Pyr$, 87%.*

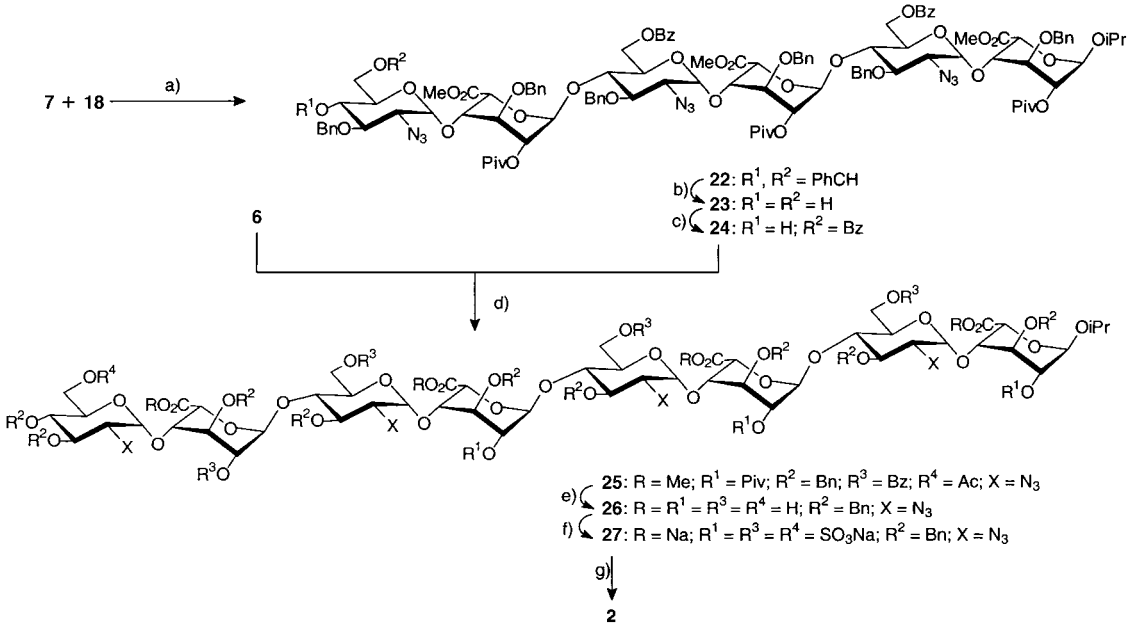
The coupling of **18** with donor **6** took place with only moderate yield (58%) to afford hexasaccharide **19**, although a substantial amount (39%) of valuable acceptor **18** could be recovered from the reaction mixture. Hexasaccharide **19** was then submitted to the deprotection/sulfation sequence. Acyl and methoxycarbonyl groups were removed by an aqueous/alcoholic solution of KOH and the resulting partially protected hexasaccharide **20** was sulfated and isolated as the sodium salt **21**. Hydrogenolytic cleavage of the benzyl groups and simultaneous reduction of the azido groups in **21** followed by selective N-sulfation yielded compound **1** (87% yield), which was purified by gel permeation chromatography according to the protocol previously reported for oligosaccharides of the irregular region of heparin.[22]

The effectiveness of this synthetic strategy for the preparation of heparin-like oligosaccharides with increasing size (>2 units) by repeating the sequence of reactions leading from **16** to **19** has been demonstrated by the synthesis of octasaccharide **2** (Scheme 5). Glycosylation of **18** with **7** gave hexasaccharide **22** in 52% yield. As previously a large quantity (40%) of unreacted **18** could be recovered from the reaction mixture. The benzylidene protecting groups were removed from compound **22** to give **23** (79% yield) and this was selectively benzoylated to yield **24** (90% yield). Glycosylation of **24** with **6** afforded **25** in 60% yield (and 40% of unreacted **24**). Octasaccharide **25** was then submitted to the deprotection/sulfation/purification sequence to give, through **26** (67% yield from **25**) and **27** (74% yield from **26**), finally the target compound **2** (60% yield from **27**). Hexasaccharide **1** and octasaccharide **2** were fully characterized by NMR spectroscopy and by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry of their complexes with the synthetic basic peptides $(Arg-Gly)_{10}$ and $(Arg-Gly)_{15}$, respectively, according to a procedure previously reported.[23]

The above synthetic strategy, which has been partially inspired by previous work on the antithrombin III binding heparin pentasaccharide,[24] provides oligosaccharide sequences of the regular region of heparin, the size of which can be varied, as exemplified by the synthesis of compounds **1** and **2**. Modifications of this strategy also allow the synthesis of oligosaccharides differing in the sulfation pattern. These synthetic compounds, as well as those reported by others[13–15] with different sequences and prepared by a different route, may be crucial in disclosing the structural and biological consequences of the diversity and complexity of the FGF family.

Structure

The structure of heparin-like oligosaccharides is a matter of current interest.[3] One of the most relevant topics in this regard is the conformation of the L-iduronate residues. NMR spectroscopy, X-ray fiber diffraction studies, and molecular modeling studies have indicated that these L-iduronate units may adopt $^1C_4$, $^4C_1$, and $^2S_0$ conformations within the glycosaminoglycan oligosaccharide chain.[3a] Heparin itself is a heterogeneous polysaccharide, and the NMR spectra of long oligosaccharide fragments of heparin suffer from polydispersity and signal overlapping that hamper the consideration of individual units in the chain. This problem has been previously approached working with a purified hexasaccharide fragment obtained by enzymatic depolimerization of heparin.[3b] This hexasaccharide presents, therefore, a monosaccharide sequence alternative to the sequence in hexasaccharide **1**, contains an unsaturated uronic acid residue at the nonreducing end, and, in addition, displays anomeric heterogeneity at the reducing end. Therefore, in order to obtain rigorous information on the three-dimensional



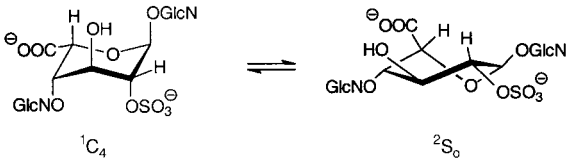
Scheme 5. *Assembly of octasaccharide **25** and deprotection sequence to form **2**. Reagents and conditions: a) 3% TMSOTf, CH_2Cl_2, 52% (40% of recovered **18**); b) EtSH, PTSA (cat.), 79%; c) BzCN, Et_3N (cat.), MeCN, $-40\,^{\circ}C$, 90%; d) 3% TMSOTf, 60% (33% of recovered **24**); e) KOH, 67%; f) $SO_3 \cdot NMe_3$; Dowex 50WX4 (Na^+), 74%; g) 10% Pd/C, H_2; $SO_3 \cdot Pyr$, 60%.*

Table 1. *Observed ^1H and ^{13}C chemical shifts (in ppm) and $^3J_{H,H}$ values (in Hz) in the NMR spectra of* **1**.

	H1; C1	$J_{1,2}$	H2; C2	$J_{2,3}$	H3; C3	$J_{3,4}$	H4; C4	$J_{4,5}$	H5; C5	H6; C6
IdoA (A)	5.22;	2.9	4.14;	–	4.16;	3.2	4.00;	2.6	4.50;	
	97.70		76.69		69.58		76.49		68.98	
GlcN (B)	5.30;		3.23;		3.66;		3.75;		3.96;	4.38(*R*), 4.21(*S*);
	97.39		58.62		70.23		76.44		69.78	66.93
IdoA (C)	5.24;	2.9	4.30;	4.5	4.17;	3.3	4.05;	2.7	4.82;	
	99.57		76.44		69.58		76.39		70.03	
GlcN (D)	5.37;		3.23;		3.62;		3.74;		3.99;	4.39(*R*), 4.22(*S*);
	97.31		58.62		70.23		76.44		69.78	66.93
IdoA (E)	5.18;	2.9	4.29;	5.6	4.16;	3.4	4.06;	2.8	4.79;	
	99.65		76.44		69.58		76.39		70.03	
GlcN (F)	5.40;		3.19;		3.59;		3.52;		3.95;	4.32(*R*), 4.17(*S*);
	97.15		58.62		71.53		69.98		70.58	67.08

structure of our synthetic oligosaccharides in solution, we undertook the conformation analysis of hexasaccharide **1**.

The ^1H and ^{13}C NMR spectra of **1** were fully assigned with conventional one- and two-dimensional spectroscopy (Table 1). The $^3J_{H,H}$ values, which have been previously used to determine the conformation of the L-iduronate residues,[3, 25] were obtained by recursive deconvolution in the frequency domain of DQF-COSY cross-peaks.[26] As expected the $^3J_{H,H}$ values obtained for all the D-glucosamine units indicated a single $^4C_1$ conformation. The $^3J_{H5,H6}$ and $^3J_{H5,H6'}$ values obtained and the detection of a short distance between H4 and H6 allowed the stereospecific assignment of these protons, which was in agreement with that reported based on chemical shift criteria[3b,c] and permitted definition of the ω angle as indicative of a major *gg* rotamer in all D-glucosamine residues. The $^3J_{H,H}$ values for the L-iduronate units (Table 1) could be accounted for by a fast equilibrium between $^1C_4$ and $^2S_0$ conformers where the $^1C_4$ populations for residues A, C, and E were 61, 55, and 62%, respectively, with no clear differences among the units, including the residue at the reducing end (Scheme 6). The potential border effects observed for natural samples are therefore minimized in the synthetic isopropyl glycoside **1**.



Scheme 6. *Conformational equilibrium of the L-iduronic acid (IdoA) residues. Calculated populations for the IdoA residues A, C, and E in hexasaccharide* ***1*** *are 61, 55, and 62%, respectively.*

Molecular dynamics simulations starting from structures containing all the L-iduronate residues in either the $^1C_4$ or the $^2S_0$ conformation were strongly dependent on the electrostatic treatment. Transitions between iduronate skew-boat conformations and, more rarely, between skew-boat and chair conformations were always found in the subnanosecond scale (see Supporting Information). The best results were obtained with $\varepsilon = 5r$ and taking as the main criterium the absence of conformations incompatible with the spectroscopic data. As previously reported,[3a] a $^2S_0 - {}^5S_1$ pseudorotational equilibrium found in the picosecond scale could not be experimentally substantiated. The simulations starting from different conformers were analysed individually as the system failed to reach a steady state regarding the iduronate conformational transitions. In all cases glycosidic linkage geometries of type *syn*-Ψ were found (Figure 1). This result is in agreement with the observed interresidue NOE contacts (H1′ – H3 and H1′ – H4 for GlcN – IdoA linkages and H1′ – H4 and H1′ – $H6_R$ for IdoA – GlcN linkages) as predicted by the adiabatic energy maps (not shown). The adiabatic energy maps corresponding to the GlcN – IdoA linkages showed some differences depending on the conformation of the L-iduronate residue being considered, but no attempt was made to justify the experimental data as anisotropy prevented the accurate evaluation of the interprotonic distances. A weak NOE effect was observed between GlN_iH3 and $IdoA_{i-1}H5$ which, according to the minimized models, could only take place for L-iduronate $^1C_4$ conformers; this confirms the $^3J_{H,H}$ analysis. The overall structure of **1** along the simulation was a stable right-hand helix with four residues per turn whatever the starting conformation of the L-iduronate units (Figure 2).[3a,b] This heparin-like structure qualitatively agrees with the interglycosidic NOE data. The ensemble of structures along the simulations could be satisfactorily superimposed with the hexasaccharide fragment of the central region of heparin (Figure 2).[3a]

Cross-relaxation rates, σ^{NOE}, were calculated at 500 and 400 MHz (Table 2) and the apparent correlation times were calculated from these values (Table 3). Some indications about the molecular tumbling of **1** could be obtained by comparison of the interprotonic distances H1 – H2 and H2 – H4 (2.4 and 2.5 Å, respectively) within the conformationally homogeneous D-glucosamine units with the σ^{NOE} values. The apparent correlation times for these vectors were considerably different (860 versus 1300 ps), which was a clear indication of an anisotropic tumbling of compound **1** giving rise to different correlation times for vectors with different orientations along the molecule axis (around 109°). This anisotropic tumbling did not allow a straightforward analysis of fast internal motions based on σ^{NOE} ratios, and ^{13}C relaxation data then have to be considered.[27] These fast motions have often been disregarded by the use of rigid top rotor models to account for homonuclear NMR data,

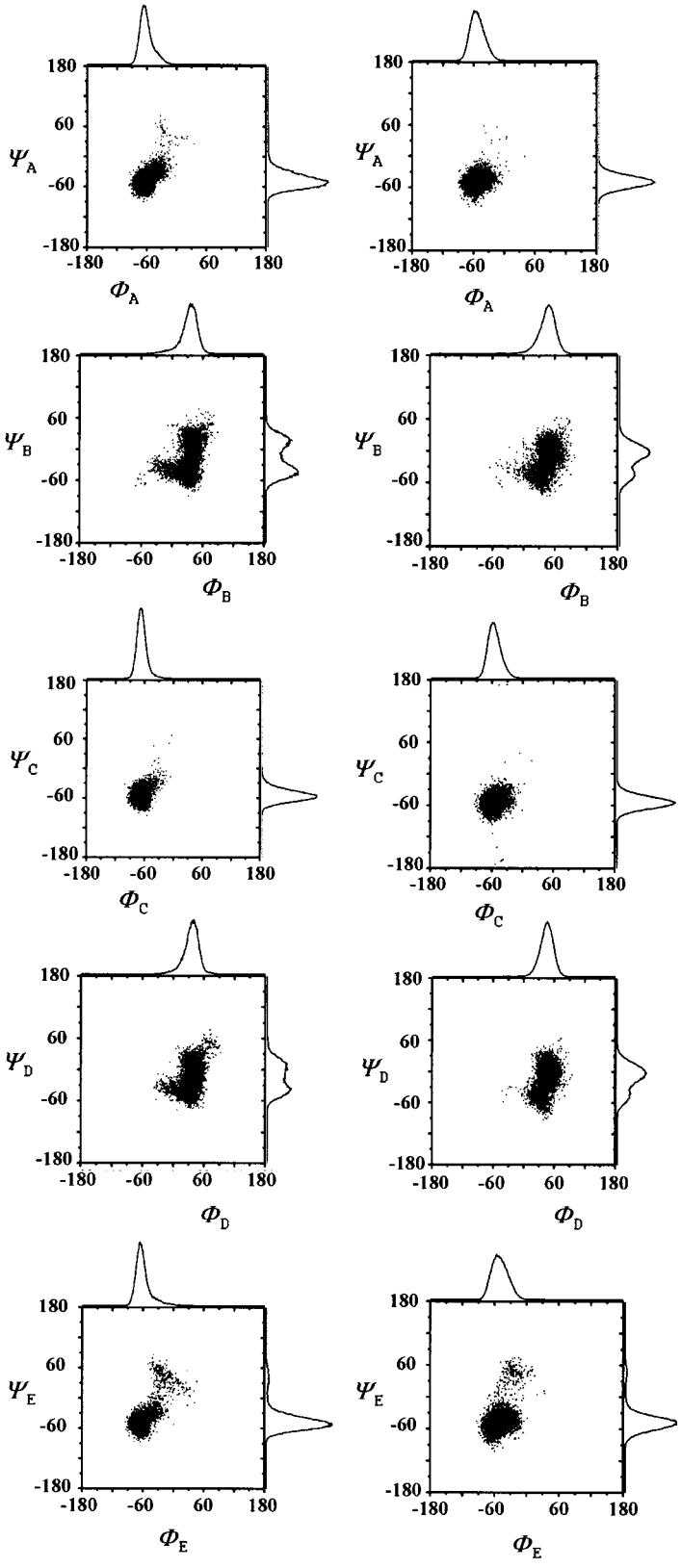


Figure 1. Φ/Ψ *distribution along the molecular dynamics simulation (4.5 ns) for each glycosidic linkage and starting structure of the hexasaccharide* ***1****. Starting structures with IdoA residues in the* $^1C_4$ *form are shown on the left and in the* $^2S_0$ *form on the right.*

with acceptable results in the calculation of the three-dimensional structure. The right-handed helix symmetry implicit in **1** could be used to isolate the effect of anisotropy on σ by assuming similarity of orientation of equivalent vectors regardless their position along the chain. Thus, differences in the apparent correlation times could be attributed to differential segmental motions. When grouped by symmetry, a clear trend on σ^{NOE} or apparent isotropic correlation times was found which indicated a slower motion for internal units than for external units in the oligosaccharide chain.

In conclusion, hexasaccharide **1**, and most probably octasaccharide **2**, possesses the structural elements of the regular region of heparin. The structure of the L-iduronate residues can be described as a fast equilibrium between $^1C_4$ and $^2S_0$ conformers and the position of these residues in the oligosaccharide chain does not seem to have appreciable influence in this conformational equilibrium. Only the external motions seemed experimentally to be faster for the terminal residues, thus indicating the existence of a border effect. Therefore hexasaccharide **1** and, most probably, octasaccharide **2** are chemically well-defined structural models of naturally occurring heparin-like oligosaccharides which may provide valuable information in the study of FGF binding and activation.

Biological activity

The mitogenic activity of FGF1 is strictly dependent on heparin.[28] Figure 3 shows the induction of the mitogenic activity of FGF1 by increasing concentrations of a commercial, relatively homogeneous heparin sample, hexasaccharide **1**, and octasaccharide **2**. As previously reported,[29] the activating effect of heparin reaches a maximum at concentrations of approximately 100 μg mL^{-1}, at which the maximum activating activity of octasaccharide **2** is also observed. Figure 3 also shows that the half-maximum activating concentration (a parameter directly related to the specific activity) of octasaccharide **2** and heparin are equivalent (6 – 10 μg mL^{-1}). The maximal activation level is, however, somewhat lower in the case of octasaccharide **2**. Hexasaccharide **1** does also activate FGF1 but considerably higher concentrations of **1** were needed to reach a maximal activation equivalent to that of heparin (Figure 3).These results are therefore in agreement with previous reports[4, 9] which stated that the octasaccharide is the minimal oligosaccharide size for significant biological activity.

Concerning the mechanism underlying FGF1 activation by heparin, the previous observation that FGF1 can be cross-linked in the presence of heparin has been taken as an indication that heparin-induced FGF1 oligomerization may be central for the FGFR dimerization required for intracellular signal transduction.[30] This heparin-mediated FGF1 dimerization has been observed in the crystalline structure of a FGF1 – heparin complex.[6c] On this basis, a model is generally accepted for the whole FGF family of proteins which implies that the formation of the heparin-induced FGF dimers is necessary as a previous step to FGFR dimerization. According to this model two FGF1 units previously cross-linked by heparin would bring together two FGFR units, thus promoting their reciprocal cross-phosphorylation.

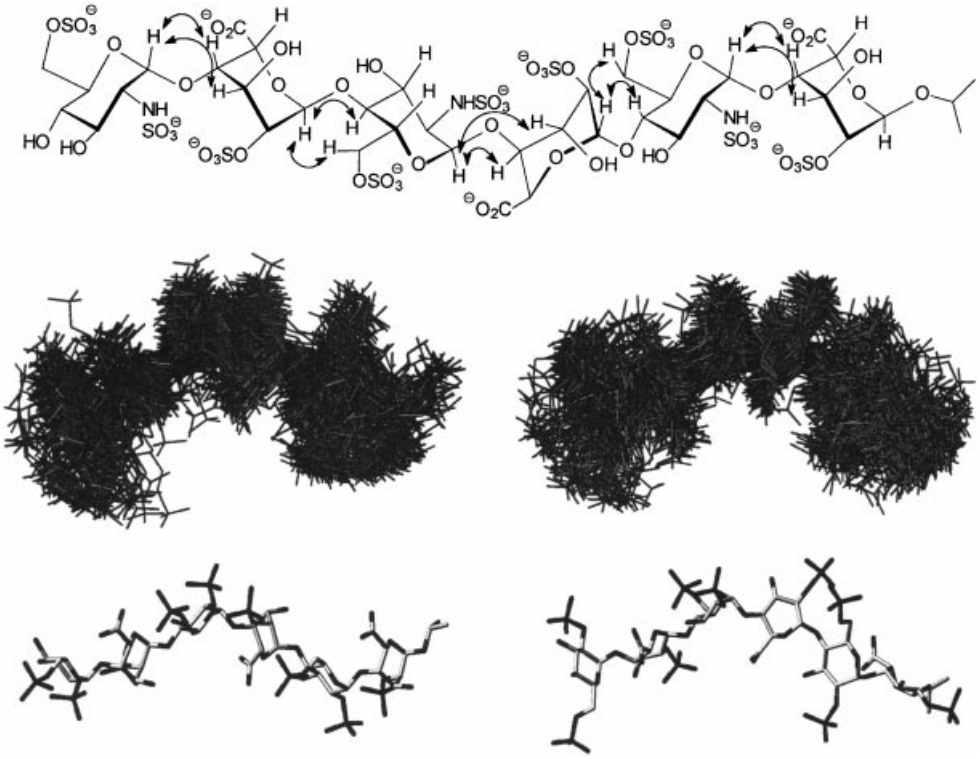


Figure 2. *Top: Representative interglycosidic NOE effects of **1**. Center: Superimposition of 100 structures taken along the molecular dynamics simulation for each starting structure (IdoA residues in the $^1C_4$ conformer (left) and in the $^2S_0$ (right)) of **1**. Bottom: Central hexasaccharide of the dodecasaccharide structure in the same orientation (left for $^1C_4$ and right for $^2S_0$ IdoA conformations).*

Table 2. *Cross-relaxation rates (σ^{NOE} in s^{-1}) from the spectrum of **1** at 500 and 400 MHz.*

		GlcN		IdoA		Interglycosidic	
		H1 – H2	H2 – H4	H4 – H5	H2 – H5	H1′ – H4	H1′ – H3
GlcN (F)	500 MHz	0.15	0.039				
	400 MHz	0.13	0.037				
(1 →4)	500 MHz					0.12	0.09
	400 MHz					0.10	0.07
IdoA (E)	500 MHz			0.19			
	400 MHz			0.15	0.042		
(1 →4)	500 MHz					0.12	
	400 MHz					0.09	
GlcN (D)	500 MHz	0.25	0.11				
	400 MHz	0.23	0.084				
(1 →4)	500 MHz					0.16	0.13
	400 MHz					0.15	0.11
IdoA (C)	500 MHz			0.22			
	400 MHz			0.15	0.05		
(1 →4)	500 MHz					0.13	
	400 MHz					0.12	
GlcN (B)	500 MHz	0.20	0.11				
	400 MHz	0.18	0.084				
(1 →4)	500 MHz					0.14	0.08
	400 MHz					0.13	0.07
IdoA (A)	500 MHz			0.11			
	400 MHz			0.099	0.034		

Table 3. *Calculated isotropic correlation times (τ_c in ns) for selected interglycosidic vectors in the hexasaccharide **1**.*

Glycosidic linkage	Vector	τ_c
GlcN (F)/IdoA (E)	H1′(F) – H4(E)	0.78
GlcN (F)/IdoA (E)	H1′(F) – H3(E)	0.69
GlcN (D)/IdoA (C)	H1′(D) – H4(C)	1.21
GlcN (D)/IdoA (C)	H1′(D) – H3(C)	0.81
GlcN (B)/IdoA (A)	H1′(B) – H4(A)	1.13
GlcN (B)/IdoA (A)	H1′(B) – H3(A)	0.88
IdoA (E)/GlcN (D)	H1′(E) – H4(D)	0.66
IdoA (C)/GlcN (B)	H1′(C) – H4(B)	0.83

Our results of sedimentation equilibrium analyses with heparin and octasaccharide **2** are shown in Figure 4. The profile of FGF1 solutions at the ionic strength of the mitogenic assays[31] shifted from that corresponding to a molecular species of

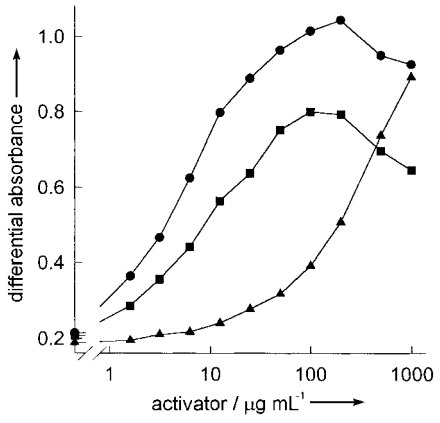


Figure 3. *Effect of increasing concentrations of heparin (●), hexasaccharide **1** (▲), and octasaccharide **2** (■) on the mitogenic activity of FGF1.*

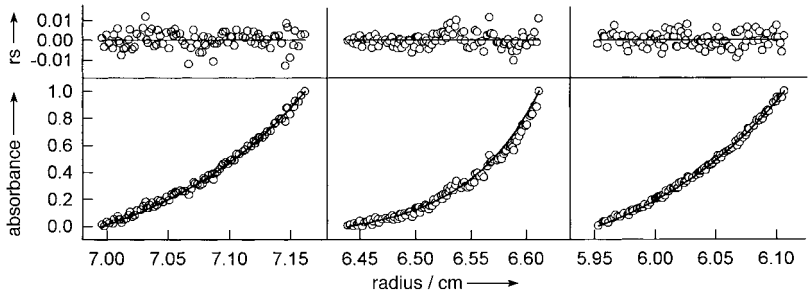


*Figure 4. Effect of heparin and octasaccharide **2** on the sedimentation equilibrium of FGF1. Left, FGF1 without activators; center, FGF1 in the presence of 100 $\mu g\,mL^{-1}$ of heparin; right, FGF1 in the presence of 100 $\mu g\,mL^{-1}$ of octasaccharide **2**. Absorbance was normalized to the minimum and maximum values of the plotted gradient. Absorbance maxima and minima were, respectively, 0.017 and 0.245, 0.032 and 0.167, and 0.161 and 0.502. Absorbance was measured at 280 nm in the experiment represented on the left and at 236 nm in those represented in the center and on the right. The estimated M_r values were 13920, 30800, and 14270, respectively.*

approximately 14 kD to that corresponding to a molecular species of about 30 kD when mitogenesis-activating concentrations of heparin were added to the FGF1 solution. However, the profile of FGF1 solutions in the presence of activating concentrations of octasaccharide **2** corresponded to a molecular species of approximately 17 kD. These results suggest that heparin-induced FGF1 dimerization in the culture medium surrounding the cells is not an absolute requirement for FGF1-induced mitogenesis signaling. In other words, FGF1 dimerization previous to FGF1 – FGFR binding does not seem to be an absolute requirement for biological activity.

Experimental Section

General procedures: Thin layer chromatography (TLC) analyses were performed on silica gel 60 F_{254} precoated on aluminum plates (Merck) and the compounds were detected by staining with sulfuric acid/ethanol (1:9) or with anisaldehyde solution (anisaldehyde (25 mL) with sulphuric acid (25 mL), ethanol (450 mL), and acetic acid (1 mL)) followed by heating at over 200 °C. Column chromatography was carried out on silica gel 60 (0.2 – 0.5 mm, 0.2 – 0.063 mm, or 0.040 – 0.015 mm; Merck). Optical rotations were determined with a Perkin-Elmer 341 polarimeter. ^1H and ^{13}C NMR spectra were acquired on Bruker DPX-300, DRX-400, and DRX-500 spectrometers and chemical shifts are given in ppm relative to tetramethylsilane as an internal reference or relative to D_2O. Elemental analyses were performed with a Leco CHNS-932 apparatus, after drying analytical samples over phosphorous pentoxide for 24 h. Mass spectra (fast atom bombardment; FAB MS) were carried out by the Mass Spectrometry Service, Facultad Química, Seville, with a Kratos MS-80 RFA spectrometer. Gel filtration chromatography (Sephadex LH-20 and G-25; Pharmacia) and ion-exchange chromatography (Dowex 50WX4 Na^+; Fluka) were used in order to achieve purification of the final products. Maldi-tof mass spectra were recorded with a MALDI-TOF GSG System spectrometer. Samples of the intermediate products were dissolved in AcOEt or MeOH at mM concentrations and 2,5-dihydroxybenzoic acid was used as the matrix. The Maldi-tof mass spectra of compounds **1** and **2** were achieved by detecting the ionic complexes formed with $(Arg\text{-}Gly)_{10}$ and $(Arg\text{-}Gly)_{15}$ as basic peptides[23] (synthesised by the Peptides Synthesis Service, Facultad Química, Barcelona). In this case, 3-hydroxypicolinic acid was used as the matrix, the concentration of sample was 25 g L^{-1} in H_2O/CH_3CN (1:1), and α-D-fucose (approximately 10% (w/w)) was always added to the solution.

NMR Measurements: Correlation NMR measurements were performed on Bruker DRX-500 and DRX-400 instruments with acetone as an external reference in a 2 mM sample (pH 7.0). DQF-COSY,[32] TOCSY,[33] NOESY,[34] and HMQC[35] experiments were performed using *z*-gradient pulse program sequences when possible. In all two-dimensional experiments TPPI[36] detection of F1 was used. The mixing times of NOESY experiments ranged from 200 – 600 ms. Data were transformed into phase-sensitive mode after weighting with shifted square sine-bell functions. In the NMR data given for the tetra-, hexa-, and octasaccharides below, the letters correspond to the saccharides, as labeled in Scheme 1.

Cross-relaxation rates, σ^{NOE}, were calculated at two field strengths (500 and 400 MHz) from NOESY experiments at several mixing times (200, 300, 350, 400, 500, and 600 ms), with assumption of the isolated spin-pairs approximation at short mixing times by extrapolation to time zero of the ratio $V_{cross\text{-}peak}/V_{diagonal}$. The apparent correlation times were calculated with the spectral density function of an isotropic rigid molecule, from the ratio $\sigma_{500}/\sigma_{400}$.

Molecular modeling: Molecular modeling has been carried out with the AMBER 5.0[37] program and the GLYCAM[38] parameter set for carbohydrates, including sulphate- and sulphamate-specific parameters.[39] The initial structures were constructed from dodecasaccharide structures taken from the Brookhaven Data Bank by Mulloy (File: 1hpn.pdb). Typical molecular dynamics simulations were performed with an integration step of 1.5 fseg at 297 K with a termal bath coupling of 0.5 ps and the hydrogen-bond lengths were kept constant with the SHAKE algorithm. After an equilibration period of 15 ps, the data were registered for 9 ns.

Biological Assays: Heparin – sepharose was obtained from Pharmacia, nitrocellulose filters from Millipore, culture plates from Costar, ITS + culture supplement from Collaborative Research Inc., Na – heparin (average molecular weight: 3 kD) from Sigma, L-glutamine, Ham's F-12 medium, and Dulbecco's modified Eagle's medium (DMEM) from Flow. Distilled water filtered through a Milli-Q (Millipore) water purifier fitted with an Organex column (Millipore) was used in all solutions.

Mitogenic activity was measured as previously described.[40] Cells were counted by measuring the total amount of crystal violet retained by cell nuclei by differential absorption (620 minus 690 nm).[40, 41] For assaying the activation of FGF1 by heparin, hexasaccharide **1**, and octasaccharide **2**, a mitogenic unit of the protein (320 pg mL^{-1}) was added to each well of the assay plate.[42]. A 139 residue form of FGF1 was used.[43]. The protein was synthesized and purified as previously reported with expresion vector pMG47.[41]

Sedimentation equilibrium experiments were performed with an Optima XL-A analytical ultracentrifuge (Beckman Instruments Inc.) equipped with a UV/Vis optical scanner with 12 mm six-channel centerpieces of eppon – charcoal at 20° and 25000 rpm. Radial absorbance scans were taken at sedimentation equilibrium. For determination of the absorbance base line of each sample, the solution was afterwards depleted of protein in solution by centrifugation at 40000 rpm. Analyzed solutions were always 6 µM FGF1 in 10 mM sodium phosphate (pH 7.2) with 80 mM NaCl. Fitting of the experimental data to the expression describing the radial concentration distribution of an ideal solute on sedimentation equilibrium

was carried out by nonlinear least-squares procedures with the programs XLAEQ and EQAASSOC (supplied by Beckman Instruments Inc.). The weight averages of the complexes were calculated using the partial specific volumes for the protein (0.735 $cm^3 g^{-1}$)[43] and heparin (0.51 $cm^3 g^{-1}$).[44].

Starting Materials: 6-*O*-Acetyl-2-azido-3,4-di-*O*-benzyl-2-deoxy-α,β-D-glucopyranosyl trichloroacetimidate (**3**)[17] and methyl (dimethylthexylsilyl-3-*O*-benzyl-β-L-idopyranosyl)uronate (**4**)[18] were prepared according to literature procedures. 2-Azido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-α,β-D-glucopyranosyl trichloroacetimidate (**5**) was prepared from D-glucosamine hydrochloride with diazo transfer from triflic azide[16] as the key transformation.

Disaccharide 9: TMSOTf (39 μL, 0.22 mmol) was added to a cooled (0 °C) solution of **4** (1.904 g, 4.32 mmol) in dry CH_2Cl_2 (100 mL) under an argon atmosphere. While the reaction was stirred, a solution of **3** (2.480 g, 4.32 mmol) in dry CH_2Cl_2 (25 mL) was added dropwise. After 30 min the mixture was neutralized with saturated aqueous $NaHCO_3$ solution, and then CH_2Cl_2 (400 mL) was added at room temperature (RT). The suspension was washed with H_2O (250 mL). The organic layer was dried ($MgSO_4$) and concentrated in vacuo, and the residue was separated by flash column chromatography (eluent: toluene/AcOEt (12:1)) to obtain unreacted acceptor (363 mg, 19%) and fractions containing the desired disaccharide; these were combined, concentrated, and dissolved in Pyr (15 mL). Benzoyl chloride (4.1 mL, 35 mmol) was added and the solution was stirred at RT. After 24 h, the mixture was diluted with CH_2Cl_2 (400 mL), washed with H_2O (300 mL), dried ($MgSO_4$), and concentrated in vacuo. The residue was purified by flash column chromatography (toluene/AcOEt (12:1)) to yield **9** (2.115 g, 51%). $[\alpha]_D^{RT} = +18.0^\circ$ ($c = 1$, $CHCl_3$); TLC: (toluene/AcOEt (12:1)) $R_f = 0.26$; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 8.11 - 7.22$ (m, 20H, Ph), 5.17 (br s, 1H, H1), 5.07 (br s, 1H, H2), 4.84 – 4.74 (2 × d, 2H, $J = 11.7$ Hz, CH_2Ph), 4.72 (d, 1H, $J_{1',2'} = 3.4$ Hz, H1′), 4.67 – 4.49 (2 × d, 2H, $J = 10.7$ Hz, CH_2Ph), 4.48 (br s, 1H, H5), 4.38 (dd, 1H, $J_{5',6'a} = 1.8$, $J_{6'a,6'b} = 12.4$ Hz, H6′(A)), 4.30 (dd, 1H, $J_{5',6'b} = 2.3$ Hz, H6′(B)), 4.23 (m, 1H, H3), 4.07 (m, 1H, H5′), 4.01 (br s, 1H, H4), 3.96 – 3.87 (2 × d, 2H, $J = 10.7$ Hz, CH_2Ph), 3.75 (s, 3H, COOCH_3), 3.53 – 3.43 (m, 2H, H3′ and H4′), 3.12 (dd, 1H, $J_{2,3} = 10.1$ Hz, H2′), 1.99 (s, 3H, OCOCH_3), 1.56 (m, 1H, CH(CH$_3$)$_2$), 0.79 – 0.75 (4 × s, 12H, C(CH_3)$_2$ and CH(CH_3)$_2$), 0.25 – 0.13 (2 × s, 6H, Si(CH_3)$_2$); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 170.59$, 168.62 and 166.58 (C=O), 137.86 – 127.74 (Ph), 99.65 (C1′), 93.97 (C1), 79.86, 77.35, 75.65, 75.02, 74.88, 74.60, 73.45, 73.03, 70.09, 68.71, 63.72, 62.29, 52.21 (COOCH_3), 33.98 (*C*H(CH$_3$)$_2$), 24.77 (*C*(CH$_3$)$_2$), 20.88 (OCOCH_3), 20.17 – 18.36 (C(*C*H$_3$)$_2$ and CH(*C*H$_3$)$_2$), −1.99 and −3.36 (Si(*C*H$_3$)$_2$); FAB MS: $m/z = 976$ [M+Na$^+$]; elemental analysis (%): calcd for $C_{51}H_{63}N_3O_{13}Si$: C 64.20, H 6.66, N 4.40; found: C 64.47, H 6.64, N 4.24.

Trichloroacetimidate 6: Cl_3CCN (593 μL, 5.9 mmol) and K_2CO_3 (55 mg, 0.39 mmol) were added to a solution of **10** (320 mg, 0.39 mmol) in dry CH_2Cl_2 (4 mL). After stirring at RT for 4 h, the mixture was filtered off and concentrated in vacuo, and the residue was purified by chromatography over a short silica gel column (hexane/AcOEt (2:1)) to yield **6** (357 mg, 95%) as a α/β mixture. TLC: (hexane/AcOEt (2:1)) $R_f = 0.53$ and 0.38 (β and α); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 8.67$ (s, 0.6H, N*H*(β)), 8.64 (s, 0.4H, N*H*(α)), 8.13 – 7.10 (m, 20H, Ph), 6.55 (br s, 0.6H, H1(β)), 6.29 (d, 0.4H, $J_{1,2} = 1.8$ Hz, H1(α)), 5.43 (m, 0.4H, H2(α)), 5.33 (br s, 0.6H, H2(β)), 5.00 (br s, 0.4H, H5(α)), 4.93 – 4.88 (m, 1H, CH_2Ph), 4.79 – 4.65 (m, 3.6H, H5(β), H1′, and CH_2Ph), 4.52 – 4.46 (m, 1H, CH_2Ph), 4.38 – 4.35 (m, 1.4H, H6′(A) and H3(α)), 4.25 – 4.23 (m, 1.6H, H6′(B) and H3(β)), 4.15 (br s, 0.4H, H4(α)), 4.03 – 3.88 (m, 3.6H, H5′, H4(β), and CH_2Ph), 3.79 – 3.78 (2 × s, 3H, COOCH_3(α and β)), 3.51 – 3.41 (m, 2H, H3′ and H4′), 3.23 – 3.19 (m, 1H, H2′), 1.99 (s, 3H, OCOCH_3(α, β)); elemental analysis (%): calcd for $C_{45}H_{45}N_4O_{13}Cl_3$: C 56.52, H 4.74, N 5.86; found: C 56.17, H 4.98, N 5.79.

Disaccharide 11: TMSOTf (50 μL of a 0.26 M solution in dry CH_2Cl_2) was added to a cooled (0 °C) solution of **4** (114 mg, 259 μmol) in dry CH_2Cl_2 (4 mL) under an argon atmosphere. While the reaction was stirred, a solution of **5** (82 mg, 155 μmol) in dry CH_2Cl_2 (1.5 mL) was then added dropwise. After 30 min the mixture was neutralized with saturated aqueous $NaHCO_3$ solution and then CH_2Cl_2 (50 mL) was added at RT. The suspension was washed with H_2O (50 mL). The organic layer was dried ($MgSO_4$) and concentrated in vacuo, and the residue was purified by flash column chromatography (toluene/AcOEt (30:1)) to yield **11** (78 mg, 62%) and unreacted acceptor (40 mg, 35%). $[\alpha]_D^{RT} = 4.3^\circ$ ($c = 1$, $CHCl_3$); TLC: (hexane/AcOEt (4:1)) $R_f = 0.30$, (toluene/AcOEt (15:1)) $R_f = 0.32$; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 7.43 - 7.24$ (m, 15H, Ph), 5.51 (s, 1H, PhC*H*O), 5.02 (br s, 1H, H1), 4.90 (d, 1H, $J_{1',2'} = 3.8$ Hz, H1′), 4.87 – 4.73 (2 × d, 2H, $J = 10.7$ Hz, CH_2Ph), 4.64 – 4.61 (2 × d, 2H, $J = 11.8$ Hz, CH_2Ph), 4.55 (br s, 1H, H5), 4.25 (dd, 1H, $J_{5',6'a} = 2.5$, $J_{6'a,6'b} = 8.9$ Hz, H6′(A)), 4.06 (br s, 1H, H4), 3.97 – 3.92 (m, 2H, H3 and H3′), 3.79 (s, 3H, COOCH_3), 3.69 – 3.62 (m, 4H, H2, H4′, H5′, and H6′(B)), 3.53 (dd, 1H, $J_{2,3} = 9.8$ Hz, H2′), 3.03 (d, 1H, $J_{2,OH} = 9.9$ Hz, O*H*), 1.66 (m, 1H, C*H*(CH$_3$)$_2$), 0.90 – 0.88 (4 × s, 12H, C(CH_3)$_2$ and CH(CH_3)$_2$), 0.24 – 0.19 (2 × s, 6H, Si(CH_3)$_2$); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 168.97$ (C=O), 137.74 – 126.05 (Ph), 101.56 (PhCHO), 95.87 (C1′), 94.89 (C1), 82.16, 77.19, 75.25 (CH$_2$Ph), 73.72, 72.70 (CH$_2$Ph), 71.52 (C4), 68.46, 63.25, 63.06, 52.48 (COOCH_3), 34.04 (*C*H(CH$_3$)$_2$), 25.14 (*C*(CH$_3$)$_2$), 20.39 – 18.46 (C(CH_3)$_2$ and CH(CH_3)$_2$), −1.88 and 3.22 (Si(CH$_3$)$_2$); FAB MS: $m/z = 828$ [M+Na$^+$]; elemental analysis (%): calcd for $C_{42}H_{55}N_3O_{11}Si$: C 62.59, H 6.88, N 5.21; found: C 62.61, H 6.74, N 5.36.

Trichloroacetimidate 7: Cl_3CCN (0.85 mL, 8.5 mmol) and K_2CO_3 (86 mg, 0.63 mmol) were added to a solution of **13** (425 mg, 0.57 mmol) in dry CH_2Cl_2 (5 mL). After stirring at RT for 6 h, the mixture was then filtered off and concentrated in vacuo, and the residue was purified by chromatography over a short silica gel column (hexane/AcOEt (3:1)) to yield **7** (500 mg, 99%) as a α/β mixture. TLC: (hexane/AcOEt (3:1)) $R_f = 0.48$ and 0.28 (β and α); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 8.69 - 8.64$ (2 × s, 1H, N*H*(α and β)), 7.45 – 7.24 (m, 15H, Ph), 6.40 (d, $J_{1,2} = 2.1$ Hz, H1(β)), 6.32 (d, $J_{1,2} = 2.6$ Hz, H1(α)), 5.52 – 5.50 (2 × s, 1H, PhC*H*O (α and β)), 5.22 (dd, $J_{2,3} = 5.2$ Hz, H2(α)), 5.17 (dd, H2(β)), 4.95 – 4.62 (m, 6H), 4.30 – 3.61 (m, 7H), 3.74 (s, 3H, COOCH_3(α and β)), 3.37 (m, 1H, H2′(α and β)), 1.24 – 1.22 (2 × s, 9H, OCOC(CH_3)$_3$); FAB MS: $m/z = 913$ [M+Na$^+$]; elemental analysis (%): calcd for $C_{41}H_{45}N_4O_{12}Cl_3$: C 55.20, H 5.08, N 6.28; found: C 55.01, H 5.04, N 6.32.

Disaccharide 8: BzCN (42 mg) and a catalytic amount of Et_3N (drops) were added to a cooled (−40 °C) solution of **15** (205 mg, 0.29 mmol) in dry CH_3CN (2 mL). After 1 h, MeOH was added, and the mixture was then warmed to RT and stirred for 15 min. The solvent was then removed in vacuo, and the residue was dissolved in MeOH and concentrated twice more. The purification was carried out by flash column chromatography (hexane/AcOEt (4:1)) to afford **8** (223 mg, 95%). $[\alpha]_D^{RT} = +7.5^\circ$ ($c = 1$, $CHCl_3$); TLC: (hexane/AcOEt (4:1)) $R_f = 0.24$; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 8.01 - 7.23$ (m, 15H, Ph), 5.19 (d, 1H, $J_{1,2} = 4.5$ Hz, H1), 5.06 (d, 1H, $J_{1',2'} = 3.6$ Hz, H1′), 4.93 (dd, 1H, H2), 4.85 (s, 2H, CH_2Ph), 4.79 – 4.76 (m, 3H, CH_2Ph, H5, and H6′(A)), 4.69 (d, 1H, $J = 11.2$ Hz, CH_2Ph), 4.40 (dd, 1H, $J_{6'a,6'b} = 12.4$, $J_{5',6'b} = 2.1$ Hz, H6′(B)), 4.17 (dd, 1H, H4), 3.97 – 3.92 (m, 3H, H3, H5′, and C*H*(CH$_3$)$_2$), 3.78 (s, 3H, COOCH_3), 3.74 (dd, 1H, H3′), 3.50 (ddd, 1H, H4′), 3.20 (dd, 1H, $J_{2,3} = 10.2$ Hz, H2′), 2.94 (d, 1H, $J_{4,OH} = 3.8$ Hz, O*H*), 1.22 (s, 9H, OCOC(CH_3)$_3$), 1.20 – 1.15 (2 × d, 6H, $J = 6.2$ Hz, CH(CH_3)$_2$); FAB MS: $m/z = 828$ [M+Na$^+$]; elemental analysis (%): calcd for $C_{42}H_{51}N_3O_{13} \cdot {}^1/_2H_2O$: C 61.90, H 6.43, N 5.16; found: C 61.68, H 6.44, N 5.06.

Tetrasaccharide 16: TMSOTf (140 μL of a 0.11 M solution in dry CH_2Cl_2) was added to a cooled solution (0 °C) of **8** (421 mg, 0.52 mmol) and **7** (606 mg, 0.68 mmol) in dry CH_2Cl_2 (7 mL) under

an argon atmosphere. After 75 min, saturated aqueous $NaHCO_3$ solution and CH_2Cl_2 (250 mL) were added and the mixture was washed with H_2O (200 mL). The organic layer was dried ($MgSO_4$) and concentrated in vacuo, and the residue was purified by flash column chromatography (hexane/AcOEt (4:1) and toluene/ AcOEt (9:1)) to yield **16** (633 mg, 79%) and unreacted acceptor (80 mg, 19%). $[\alpha]_D^{RT} = -0.8^\circ$ ($c = 1.55$, $CHCl_3$); TLC: (hexane/AcOEt (4:1)) $R_f = 0.19$; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 8.05 - 7.14$ (m, 30H, Ph), 5.51 (s, 1H, PhC*H*O), 5.40 (d, 1H, $J_{1,2} = 5.1$ Hz, H1(C)), 5.22 (d, 1H, $J_{1,2} = 4.8$ Hz, H1(A)), 5.03 (d, 1H, $J_{1,2} = 3.6$ Hz, H1(B)), 5.00 (dd, 1H, H2(C)), 4.97 (d, 1H, $J_{1,2} = 3.8$ Hz, H1(D)), 4.94 – 4.90 (m, 2H, H2(A) and CH_2Ph), 4.84 (d, 1H, $J = 11.0$ Hz, CH_2Ph), 4.79 – 4.63 (m, 8H, H5(A), H6(B), and CH_2Ph), 4.59 (d, 1H, $J_{4,5} = 4.7$ Hz, H5(C)), 4.46 (dd, 1H, H6′(B)), 4.19 (dd, 1H, $J_{5,6} = 4.7$, $J_{6,6'} = 10.0$ Hz, H6(D)), 4.14 (dd, 1H, H4(A)), 4.06 – 4.02 (m, 3H, H4(B and C) and H5(B or D)), 3.97 – 3.92 (m, 3H, H3(A and C) and C*H*$(CH_3)_2$), 3.86 – 3.81 (m, 2H, H3(B or D) and H5(B or D)), 3.75 (m, 1H, H3(B or D)), 3.66 – 3.62 (m, 2H, H4(D) and H6′(D)), 3.77 and 3.34 (2 × s, 6H, COOC*H*$_3$(A and C)), 3.34 – 3.28 (m, 2H, H2(B and D)), 1.24 – 1.14 (m, 24H, OCOC(C*H*$_3$)$_3$ and CH(C*H*$_3$)$_2$); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta =$ 177.29, 177.22, 170.21, 169.16 and 165.98 (C=O), 137.71 – 126.07 (Ph), 101.50 (PhCHO), 99.43 (C1 d), 97.90 (C1 b), 97.70 (C1 c), 97.20 (C1 a), 82.44, 78.27, 76.48, 76.28, 75.86, 75.32, 74.85, 74.80, 73.94, 73.89, 73.24, 71.26, 70.94, 70.88, 70.78, 70.40, 69.95, 68.49 (C6 d), 63.31, 63.06, 62.86, 62.25, 52.25 and 51.75 ($COOCH_3$), 38.83, 38.17, 27.19 and 27.13 ($OCO(CH_3)_3$), 23.35 and 21.79 ($CH(CH_3)_2$); MALDI-TOF MS: $m/z =$ 1557 [M+Na$^+$], 1573 [M+K$^+$]; elemental analysis (%): calcd for $C_{81}H_{94}N_6O_{24}$: C 63.35, H 6.17, N 5.47; found: C 63.55, H 6.35, N 5.20.

Tetrasaccharide 18: BzCN (44 mg) and a catalytic amount of Et_3N (drops)were added to a cooled (−40 °C) solution of **17** (441 mg, 0.31 mmol) in dry CH_3CN (5 mL). After 30 min, MeOH was added, and the mixture was warmed to RT and stirred for 15 min. The solvent was then removed in vacuo, and the residue was dissolved in MeOH and concentrated twice more. The purification was carried out by flash column chromatography (hexane/AcOEt (4:1)) to afford **18** (441 mg, 93%). $[\alpha]_D^{RT} = +14.3^\circ$ ($c = 0.58$, $CHCl_3$); TLC: (hexane/AcOEt (2:1)) $R_f =$ 0.38; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 8.05 - 7.23$ (m, 30H, Ph), 5.39 (d, 1H, $J_{1,2} = 5.0$ Hz, H1(C)), 5.23 (d, 1H, $J_{1,2} = 5.0$ Hz, H1(A)), 5.04 (d, 1H, $J_{1,2} = 3.5$ Hz, H1(B)), 5.02 (dd, 1H, H2(C)), 4.99 (d, 1H, $J_{1,2} = 3.5$ Hz, H1(D)), 4.94 (d, 1H, $J = 10.5$ Hz, CH_2Ph), 4.91 (dd, 1H, H2(A)), 4.81 – 4.65 (m, 10H, C*H*$_2$Ph, H6(B and D) and H5(A)), 4.61 (d, 1H, $J_{4,5} = 4.7$ Hz, H5(C)), 4.47 (br d, 1H, $J_{6,6'} = 12.0$ Hz, H6′(B or D)), 4.29 (br d, 1H, $J_{6,6'} =$ 12.0 Hz, H6′(B or D)), 4.16 (dd, 1H, H4(A)), 4.10 – 4.07 (m, 3H, H4(C), H5(B), and H4(B)), 3.97 – 3.92 (m, 3H, H3(A), H3(C), and C*H*$(CH_3)_2$), 3.84 (m, 1H, H5(D)), 3.77 (m, 4H, COOC*H*$_3$ and H3(B)), 3.64 (dd, 1H, H3(D)), 3.49 (ddd, 1H, H4(D)), 3.43 (s, 3H, COOC*H*$_3$(A or C)), 3.32 (dd, 1H, $J_{2,3} = 10.5$ Hz, H2(B)), 3.20 (dd, 1H, $J_{2,3} = 10.5$ Hz, H2(D)), 3.03 (d, 1H, $J_{4,OH} = 4.0$ Hz, O*H*), 1.20 – 1.15 (m, 24H, OCOC(C*H*$_3$)$_3$ and CH(C*H*$_3$)$_2$); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 177.26$, 177.24, 170.23, 169.30, 167.34 and 166.14 (C=O), 137.76 – 126.81 (Ph), 98.82 (C1 d), 97.83 (C1 b), 97.73 (C1 c), 97.19 (C1 a), 79.02, 78.08, 76.34, 76.29, 75.24, 74.99, 74.33, 73.81, 73.63, 73.30, 71.35, 71.28, 71.13, 71.04, 70.97, 70.53, 70.48, 69.93, 63.01 – 62.54 (C2 b, C2 d, C6 b, and C6 d), 52.24 and 51.86 ($COOCH_3$), 38.83, 27.19 and 27.13 ($OCO(CH_3)_3$), 23.36 and 21.80 ($CH(CH_3)_2$); MALDI-TOF MS: $m/z = 1573$ [M+Na$^+$], 1589 [M+K$^+$]; elemental analysis (%): calcd for $C_{81}H_{94}N_6O_{25} \cdot H_2O$: C 61.98, H 6.16, N 5.35; found: C 61.74, H 6.31, N 5.13.

Hexasaccharide 19: TMSOTf (50 μL of a 0.035 M solution in dry CH_2Cl_2) was added to a solution of **18** (70 mg, 45 μmol) and **6** (56 mg, 59 μmol) in dry CH_2Cl_2 (0.5 mL) at RT under an argon atmosphere. After 30 min, saturated aqueous $NaHCO_3$ solution and CH_2Cl_2 (50 mL) were added and the mixture was washed with H_2O (25 mL). The organic layer was dried ($MgSO_4$) and concentrated in vacuo and the residue was purified by flash column chromatography (toluene/AcOEt (8:1)) to yield **19** (62 mg, 58%) and unreacted acceptor (25 mg, 36%). $[\alpha]_D^{RT} = +13.5^\circ$ ($c = 1$, $CHCl_3$); TLC: (toluene/AcOEt (8:1)) $R_f = 0.19$; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 8.05 - 7.18$ (m, 50H, Ph), 5.56 (d, 1H, $J_{1,2} = 4.4$ Hz, H1(E)), 5.41 (d, 1H, $J_{1,2} = 5.8$ Hz, H1(C)), 5.24 (d, 1H, $J_{1,2} = 5.0$ Hz, H1(A)), 5.15 (dd, 1H, H2(E)), 5.04 (d, 1H, $J_{1,2} =$ 3.5 Hz, H1(B)), 5.00 (dd, 1H, H2(C)), 4.95 (d, 1H, $J_{1,2} = 3.5$ Hz, H1(D)), 4.92 – 4.88 (m, 4H, H1(F), C*H*$_2$Ph, and H2(A)), 4.79 – 4.47 (m, 15H, C*H*$_2$Ph, H5(A and E), H6(B and D), and H6′(B)), 4.44 (d, 1H, $J = 5.2$ Hz, H5(C)), 4.35 – 4.33 (m, 2H, H6′(D) and C*H*$_2$Ph), 4.28 – 4.23 (m, 2H, H6(F) and C*H*$_2$Ph), 4.17 – 4.12 (m, 3H, H3(E), H4(A), and H6′(F)), 4.04 – 3.85 (m, 10H, H3(A and C), H4(B – E), H5(B, D, and F), and C*H*$(CH_3)_2$), 3.76 – 3.73 (m, 4H, H3(B) and COOC*H*$_3$), 3.63 (dd, 1H, H3(D)), 3.56 (dd, 1H, H3(F)), 3.47 – 3.44 (m, 4H, H4(F) and COOC*H*$_3$), 3.32 – 3.28 (m, 5H, H2(B and D) and COOC*H*$_3$), 3.19 (dd, 1H, $J_{1,2} = 3.4$, $J_{2,3} = 10.2$ Hz, H2(F)), 1.95 (s, 3H, OCOC*H*$_3$), 1.20 – 1.16 (m, 24H, OCOC(C*H*$_3$)$_3$ and CH(C*H*$_3$)$_2$); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 177.23$, 177.12, 170.43, 170.24, 169.24, 169.14, 165.97, 165.92 and 165.15 (C=O), 137.75 – 127.22 (Ph), 99.00 (C1 f), 98.29 (C1 e), 98.03 (C1 d), 97.80 (C1 b), 97.49 (C1 c), 97.18 (C1 a), 80.02, 78.41, 77.96, 77.40, 76.43, 76.19, 75.50, 75.33, 75.26, 75.15, 75.12, 74.96, 74.75, 74.06, 73.86, 73.84, 73.73, 73.32, 71.40, 71.28, 71.23, 71.09, 70.82, 70.63, 70.29, 70.04, 69.95, 63.52 – 62.03 (C2′ b, C2′ d, C2′ f, C6′ b, C6′ d, and C6′ f), 52.23, 51.88 and 51.62 ($COOCH_3$), 27.19 and 27.07 ($OCO(CH_3)_3$), 23.34 and 21.80 ($CH(CH_3)_2$), 20.76 ($OCOCH_3$); FAB MS: $m/z = 2368$ [M+Na$^+$]; elemental analysis (%): calcd for $C_{124}H_{137}N_9O_{37}$: C 63.50, H 5.89, N 5.37; found: C 63.42, H 6.19, N 5.08.

Hexasaccharide 20: A 2 N solution of KOH (0.7 mL) was added to a solution of **19** (35 mg, 15 μmol) in CH_2Cl_2 (0.6 mL), MeOH (3.3 mL), and H_2O (0.3 mL). After 24 h, the mixture was acidified to pH 2 – 3 with a 2 N solution of HCl and was then diluted with CH_2Cl_2 (30 mL) and H_2O (15 mL). The organic phase was washed with H_2O until neutral, dried ($MgSO_4$), and concentrated. The residue was eluted from a Sephadex LH-20 chromatography column with MeOH/CH_2Cl_2 (1:1) to afford **20** (20 mg, 74%). TLC: (CH_2Cl_2/MeOH (8:1)) $R_f = 0.34$; ^{1}H NMR (500 MHz, CD_3COCD_3): $\delta = 7.47 - 7.17$ (m, 35H, Ph), 5.35 – 5.07 (m, 6H, H1(A – F)), 1.22 – 1.18 (2d, 6H, $J = 6.2$ Hz, CH(C*H*$_3$)$_2$); MALDI-TOF MS: $m/z = 1802.7$ [M+Na$^+$], 1818.5 [M+K$^+$].

Hexasaccharide 21: A solution of **20** (20 mg, 11 μmol) in dry *N*,*N*-dimethylformamide (DMF; 1.5 mL) was stirred for 2 days at 55 °C under an argon atmosphere in the presence of a sulphur trioxide – trimethylamine complex (47 mg, 5 equivalents for each hydroxy group). (This reactive complex had been previously washed with H_2O, EtOH, and CH_2Cl_2 and dried because this kind of sulphating material usually contains a lot of acid.) The mixture was cooled, MeOH (1 mL) and CH_2Cl_2 (1 mL) were added, and the solution was layered on the top of a Sephadex LH-20 chromatography column which was eluted with MeOH/CH_2Cl_2 (1:1). The fractions, which contained sulphated hexasaccharide, were pooled and evaporated to dryness. The residue was converted into the sodium salt by elution from a column of Dowex 50WX4-Na$^+$ with MeOH/H_2O (2:1) to give **21** (20 mg, 71%). TLC: (AcOEt/Pyr/H_2O/AcOH (8:5:3:1)) $R_f = 0.33$; ^{1}H NMR (500 MHz, D_2O): $\delta = 7.63 - 7.30$ (m, 35H, Ph), 5.39, 5.38, 5.34 (m, 3H, H1(A, C, and E)), 5.21, 5.18, 5.04 (m, 3H, H1(B, D, and F)), 3.56 – 3.48 (m, 3H, H2(B, D, and F)), 1.33 – 1.32 (2d, 6H, $J = 6.2$ Hz, CH(C*H*$_3$)$_2$).

Dodecasodium salt of hexasaccharide 1: A solution of **21** (22 mg, 8.9 μmol) in MeOH/H_2O (1.5 mL, 9:1) was hydrogenated in the presence of 10% Pd/C. After 24 h, the suspension was filtered and concentrated to give the desired product which was homogeneous by TLC analysis with AcOEt/Pyr/H_2O/AcOH (4:5:3:1) as the eluent ($R_f = 0.21$). No aromatic signal was detected by NMR spectroscopy. This compound was directly used for the N sulfation.

The hydrogenated hexasaccharide (16 mg) was dissolved in H_2O (1 mL) and the pH value of the solution was adjusted to 9.5 with a 1 N solution of NaOH. A pyridine–sulphur trioxide complex (21 mg, 5 equivalents for each amine group) was added in three portions over 1 h and the pH value was maintained at 9.5 by subsequent addition of a 1 N solution of NaOH. Second, third, and fourth additions of the pyridine–sulphur trioxide complex were made after stirring for 2, 4, and 6 h, respectively. After 24 h, the mixture was neutralized with a 0.1 N solution of HCl and then subjected to chromatography over a Sephadex G-25 column with a 0.9% solution of NaCl. The appropriate fractions were pooled and passed through a column of Dowex 50WX4-Na^+ with a 0.5 M solution of NaCl as the eluent and then through a column of Sephadex G-25 with H_2O/EtOH (9:1) as the eluent. The fractions which contained the final hexasaccharide were lyophilized to give **1** (15 mg, 87%). Before NMR spectroscopic studies, it was useful to make a last elution over a column of Dowex 50WX4-Na^+ in order to avoid the formation of calcium salts instead of sodium salts and to get a better resolution in the spectra. TLC: (AcOEt/Pyr/H_2O/AcOH (2:5:3:1)) $R_f = 0.43$; $[\alpha]_D^{RT} = +22.7^\circ$ ($c = 0.67$, H_2O); $[\alpha]_{Hg}^{RT}$ (546 nm) $= +26.7^\circ$ ($c = 0.67$, H_2O); ^{1}H NMR (500 MHz, D_2O, 10 °C): $\delta = 3.98$ (m, 1H, C*H*(CH_3)$_2$), 1.15 (2 × d, 6H, $J = 6.2$ Hz, CH(C*H*$_3$)$_2$); ^{13}C NMR (125 MHz, D_2O, 27 °C): $\delta = 72.03$ (*C*H(CH_3)$_2$); MALDI-TOF MS: calcd for [*M*($C_{39}H_{65}O_{58}N_3S_9$)+(Arg–Gly)$_{10}$+H]$^+$: 3943.0; found: 3943.9. (The complete NMR spectrum assignment of the sugar protons and carbons appears in Table 1.)

Hexasaccharide 22: TMSOTf (50 μL of a 0.04 M solution in dry CH_2Cl_2) was added to a solution of **18** (82 mg, 53 μmol) and **7** (57 mg, 64 μmol) in dry CH_2Cl_2 (700 μL) under an argon atmosphere. After 2 h, saturated aqueous $NaHCO_3$ solution and CH_2Cl_2 (100 mL) were added and the mixture was washed with H_2O (75 mL). The organic layer was dried ($MgSO_4$) and concentrated in vacuo, and the residue was purified by flash column chromatography (toluene/AcOEt (8:1)) to yield **22** (62 mg, 52%) and unreacted acceptor (33 mg, 40%). $[\alpha]_D^{RT} = +3.4^\circ$ ($c = 1.33$, $CHCl_3$); TLC: (toluene/AcOEt (8:1)) $R_f = 0.30$; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 8.05$–7.21 (m, 45H, Ph), 5.50 (s, 1H, PhC*H*O), 5.49 (d, 1H, $J_{1,2} = 6.1$ Hz, H1(E)), 5.42 (d, 1H, $J_{1,2} = 5.4$ Hz, H1(C)), 5.25 (d, 1H, $J_{1,2} = 5.0$ Hz, H1(A)), 5.05–5.02 (m, 2H, H2(E) and H1(B)), 5.00–4.97 (m, 3H, H2(C) and H1(D and F)), 4.95–4.90 (m, 2H, H2(A) and C*H*$_2$Ph), 4.84–4.60 (m, 14H, C*H*$_2$Ph, H6(B and D), and H5(A)), 4.54 (d, 1H, $J = 4.9$ Hz, H5(C or E)), 4.50–4.47 (m, 2H, H6′(B or D) and H5(C or E)), 4.39 (dd, 1H, H6(B or D)), 4.18–4.14 (m, 2H, H4(A) and H6(F)), 4.09–3.74 (m, 13H, H3(A, C, and E), H4(B–E), H5(B, D, and F), H3(B, D, or F) (2H), and C*H*(CH_3)$_2$), 3.67–3.57 (m, 3H, H3(B, D, or F) (1H), H4(F), and H6′(F)), 3.77, 3.38, and 3.30 (3 × s, 9H, COOC*H*$_3$), 3.34–3.28 (m, 3H, H2(B, D, and F)), 1.20–1.14 (m, 33H, OCOC(C*H*$_3$)$_3$ and CH(C*H*$_3$)$_2$); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 177.27$, 177.22, 177.12, 170.30, 169.54, 169.19, 166.01, 165.92, (C=O), 137.76–126.07 (Ph), 101.49 (PhCHO), 99.46 (C1(F)), 98.13 (C1(D)), 97.83 (C1(B)), 97.53 (C1(C)), 97.45 (C1(E)), 97.20 (C1(A)), 82.43, 78.33, 77.93, 76.64, 76.49, 75.83, 75.45, 75.18, 74.83, 74.79, 74.46, 74.06, 73.74, 73.39, 71.69, 71.64, 71.32, 71.24, 71.09, 71.03, 70.66, 69.99, 69.95, 68.49, 63.33, 63.02, 62.89, 62.83, 62.33, 61.82, 52.30, 51.88 and 51.72 (COOCH_3), 27.22, 27.19 and 27.14 (OCO(CH_3)$_3$), 23.38 and 21.83 (CH(CH_3)$_2$); MALDI-TOF MS: $m/z = 2303$ [*M*+Na^+]; 2319 [*M*+K^+]; FAB MS: $m/z = 2303$ [*M*+Na^+].

Hexasaccharide 24: BzCN (100 μL of a 0.46 M solution in dry CH_3CN) and a catalytic amount of Et_3N (drops) was added to a cooled (−40 °C) solution of **23** (92 mg, 42 μmol) in dry CH_3CN (1.5 mL). After 30 min, MeOH was added and the mixture warmed up to RT and stirred for 15 min. The solvent was then removed in vacuo and the residue dissolved in MeOH and concentrated twice more. The purification was carried out by flash column chromatography (hexane/AcOEt (2:1)) to afford **24** (86 mg, 90%). $[\alpha]_D^{RT} = +15.8^\circ$ ($c = 1$, $CHCl_3$); TLC: (hexane/AcOEt (2:1)) $R_f = 0.52$; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 8.07$–7.23 (m, 45H, Ph), 5.51 (d, 1H, $J_{1,2} = 6.2$ Hz, H1(E)), 5.42 (d, 1H, $J_{1,2} = 5.6$ Hz, H1(C)), 5.26 (d, 1H, $J_{1,2} = 4.9$ Hz, H1(A)), 5.06–5.01 (m, 5H, H2(C and E) and H1(B, D, and F)), 4.96–4.91 (m, 2H, H2(A) and C*H*$_2$Ph), 4.86–4.62 (m, 15H, C*H*$_2$Ph, H6(B, D, and F), and H5(A)), 4.56 (d, 1H, $J = 5.0$ Hz, H5(C or E)), 4.50 (m, 2H, H5(C or E) and H6(B or D)), 4.41 (dd, 1H, H6(B or D)), 4.28 (dd, 1H, H6(F)), 4.16 (dd, 1H, H4(A)), 4.13–4.05 (m, 5H, H5(B or D) and H4(B–E)), 3.97–3.95 (m, 5H, H5(B or D), H3(A, C, and E), and C*H*(CH_3)$_2$), 3.84 (m, 1H, H5(F)), 3.77 (m, 1H, H3(B or D)), 3.61 (m, 2H, H3(B or D) and H3(F)), 3.50 (ddd, 1H, H4(F)), 3.77, 3.39, and 3.37 (3 × s, 9H, COOC*H*$_3$), 3.34 (m, 2H, H2(B and D)), 3.20 (dd, 1H, $J_{1,2} = 3.6$, $J_{2,3} = 10.0$ Hz, H2(F)), 3.05 (d, 1H, $J_{4,OH} = 4.0$ Hz, O*H*), 1.25–1.15 (m, 33H, OCOC(C*H*$_3$)$_3$ and CH(C*H*$_3$)$_2$); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 177.27$, 177.24, 177.12, 170.30, 169.53, 169.30, 167.36, 166.04, 166.01, (C=O), 137.75–127.17 (Ph), 98.76 (C1(F)), 98.06 (C1(D)), 97.82 (C1(B)), 97.58 (C1(C)), 97.45 (C1(E)), 97.19 (C1(A)), 78.99, 78.15, 77.89, 76.59, 76.50, 76.40, 75.41, 75.37, 75.25, 75.21, 74.59, 74.23, 74.05, 73.96, 73.79, 73.73, 73.39, 71.70, 71.67, 71.33, 71.18, 71.11, 70.67, 70.43, 69.97, 69.95, 63.01, 62.88, 62.84, 62.66, 62.33, 62.04, (C2(B, D, and F) and C6(B, D, and F)), 52.29, 51.87 and 51.81 (COOCH_3), 27.22, 27.19 and 27.14 (OCO(CH_3)$_3$), 23.37 and 21.82 (CH(CH_3)$_2$); FAB MS: $m/z = 2320$ [*M*+Na^+].

Octasaccharide 25: TMSOTf (50 μL of a 0.03 M solution in dry CH_2Cl_2) at RT under an argon atmosphere was added to a solution of **24** (86 mg, 37 μmol) and **6** (47 mg, 49 μmol) in dry CH_2Cl_2 (0.5 mL). After 2 h, saturated aqueous $NaHCO_3$ solution and CH_2Cl_2 (75 mL) were added and the mixture was washed with H_2O (50 mL). The organic layer was dried ($MgSO_4$) and concentrated in vacuo and the residue was purified by flash column chromatography (toluene/AcOEt (8:1) and hexane/AcOEt (3:1)) to yield **25** (70 mg, 60%) and unreacted acceptor (28 mg, 33%). $[\alpha]_D^{RT} = +14.5^\circ$ ($c = 1$, $CHCl_3$); TLC: (toluene/AcOEt (8:1)) $R_f = 0.35$; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 8.06$–7.15 (m, 65H, Ph), 5.57 (d, 1H, $J = 4.7$ Hz, H1(G)), 5.52 (d, 1H, $J = 6.3$ Hz, H1(C)), 5.43 (d, 1H, $J = 6.1$ Hz, H1(E)), 5.26 (d, 1H, $J = 5.1$ Hz, H1(A)), 5.17 (dd, 1H, H2(G)), 5.06–4.91 (m, 7H, H1(B, D, F, and H) and H2(A, C, and E)), 4.86–4.23 (m, 18H, C*H*$_2$Ph), 4.73–4.48 (m, 7H, H5(A, C, and G), H6(B, D, and F), and H6′(B)), 4.39–4.25 (m, 4H, H5(E), H6′(D and F), and H6(H)), 4.17–4.14 (m, 3H, H4(A), H3(G), and H6′(H)), 4.08–4.03 (m, 6H, H4(B–D, F, and G), and H5(B)), 3.97–3.85 (m, 7H, H3(A, C, and E), H4(E), and H5(D, F, and H)), 3.95 (m, 1H, C*H*(CH_3)$_2$), 3.78, 3.44, 3.36, 3.23 (4 × s, 12H, COOC*H*$_3$), 3.77 (m, 1H, H3(B)), 3.60–3.56 (m, 3H, H3(D, F, and H)), 3.48 (m, 1H, H4(H)), 3.33–3.32 (m, 3H, H2(B, D, and F)), 3.21 (d, 1H, $J_{1,2} = 3.5$, $J_{2,3} = 10.2$ Hz, H2(H)), 1.95 (s, 3H, OCOC*H*$_3$), 1.21–1.14 (m, 33H, OCOC(C*H*$_3$)$_3$ and CH(C*H*$_3$)$_2$); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 177.27$, 177.14, 177.11, 170.48, 170.30, 169.56, 169.27, 169.19, 166.01, 165.94 and 165.18 (C=O), 137.76–127.15 (Ph), 99.00 (C1(H)), 98.27 (C1(G)), 98.07 (C1(D and F)), 97.81 (C1(B)), 97.41 and 97.34 (C1(C and E)), 97.20 (C1(A)), 80.03, 78.41, 78.04, 77.88, 76.71, 76.51, 76.33, 75.87, 75.61, 75.44, 75.40, 75.35, 75.22, 75.02, 74.98, 74.71, 74.12, 73.98, 73.91, 73.71, 73.39, 71.83, 71.77, 71.65, 71.41, 71.32, 71.13, 70.95, 70.69, 70.41, 70.05, 69.97, 63.50–61.84 (C2(B, D, F, and H) and C6(B, D, F, and H)), 52.29, 51.91 and 51.58 (4 × COOCH_3), 27.22 and 27.14 (3 × OCO(CH_3)$_3$), 23.36 and 21.82 (CH(CH_3)$_2$), 20.769 (OCOCH_3); MALDI-TOF MS: $m/z = 3112$ [*M*+Na^+], 3128 [*M*+K^+]; elemental analysis (%): calcd for $C_{163}H_{180}N_{12}O_{49}$: C 63.33, H 5.87, N 5.44; found: C 63.12, H 6.03, N 5.33.

Octasaccharide 26: A 2 N solution of KOH (1.2 mL) was added to a solution of **25** (60 mg, 19 μmol) in CH_2Cl_2 (1 mL), MeOH (4 mL), and H_2O (0.5 mL). During the reaction period it was necessary to add more MeOH (4 mL) in order to homogenize the solution. After 48 h, the mixture was acidified to pH 2–3 with a 2 N solution of HCl and was then diluted with CH_2Cl_2 (100 mL) and H_2O (50 mL). The organic phase was washed with H_2O until neutral, dried ($MgSO_4$), and

concentrated. The residue was eluted with MeOH/CH_2Cl_2 (1:1) from a column of Sephadex LH-20 to afford **26** (30 mg, 67%). TLC: (CH_2Cl_2/MeOH (6:1)) $R_f = 0.56$; ^{1}H NMR (500 MHz, CD_3OD): $\delta = 7.38 - 7.18$ (m, 45H, Ph), 5.29 – 5.02 (m, 8H, H1(A – H)), 1.24 – 1.21 (m, 6H, CH(CH_3)$_2$); MALDI-TOF MS: calcd for [M+Na$^+$]: 2347.4; found: 2346.0; calcd. for [M+K$^+$]: 2363.5; found: 2365.5.

Octasaccharide 27: A solution of **26** (25 mg, 11 μmol) in dry DMF (1.5 mL) was stirred for 3 days at 55 °C under an argon atmosphere in the presence of a sulphur trioxide – trimethylamine complex (60 mg, 5 equivalents for each hydroxy group). (This reactive complex had been previously washed with H_2O, EtOH, and CH_2Cl_2 and dried because this kind of sulphating material usually contains a lot of acid.) The mixture was cooled, MeOH (1 mL) and CH_2Cl_2 (1 mL) were added, and the solution was layered on the top of a column of Sephadex LH-20, which was eluted with MeOH/CH_2Cl_2 (1:1). The fractions that contained the sulphated octasaccharide were pooled and evaporated to dryness. The residue was converted into the sodium salt by elution from a column of Dowex 50WX4 (Na$^+$) with MeOH/H_2O (2:1) to give **27** (26 mg, 74%). TLC: (AcOEt/Pyr/H_2O/AcOH (8:5:3:1)) $R_f = 0.37$; ^{1}H NMR (500 MHz, D_2O): $\delta = 7.60 - 7.26$ (m, 45H, Ph), 5.36 – 5.30 (m, 4H, H1(A, C, E, and G)), 5.20 – 4.98 (m, 4H, H1(B, D, F, and H)), 3.51 – 3.48 (m, 4H, H2(B, D, F, and H)), 1.29 – 1.28 (m, 6H, CH(CH_3)$_2$).

Hexadecasodium salt of octasaccharide 2: A solution of **27** (24 mg, 7.4 μmol) in MeOH/H_2O (1.5 mL, 9:1) was hydrogenated in the presence of 10% Pd/C. After 36 h, the suspension was filtered and concentrated to give the hydrogenated octasaccharide, which was homogeneous by TLC analysis with AcOEt/Pyr/H_2O/AcOH (3:5:3:1) as the eluent ($R_f = 0.31$). No aromatic signal was detected by NMR spectroscopy. This compound was directly used for the N sulphation.

The hydrogenated octasaccharide was dissolved in H_2O (1 mL) and the pH value of the solution was adjusted to 9.5 with a 1 N solution of NaOH. A pyridine – sulphur trioxide complex (24 mg, 5 equivalents for each amine group) was added in three portions over the course of 1 h and the pH value was maintained at 9.5 by subsequent addition of a 1 N solution of NaOH. Second, third, and fourth portions of the pyridine – sulphur trioxide complex were added after stirring for 2, 4, and 6 h, respectively. After 24 h, the mixture was neutralized to pH 7.2 with a 0.1 N solution of HCl and then eluted from a chromatography column of Sephadex G-25 with a 0.9% solution of NaCl. The appropriate fractions were pooled and passed through a column of Dowex 50WX4 (Na$^+$) with a 0.5 M solution of ClNa as the eluent and then through a column of Sephadex G-25 with H_2O/EtOH (9:1) as the eluent. The fractions which contained the final octasaccharide were lyophilized to give **2** (12 mg, 60%). Before NMR studies were performed, it was useful to make a last elution through a column of Dowex 50WX4 (Na$^+$) in order to avoid the formation of calcium salts instead of sodium salts and to get a better resolution in the spectra. $[\alpha]_D^{RT} = +21.0°$ ($c = 0.67$, H_2O); TLC: (AcOEt/Pyr/H_2O/AcOH (1:5:3:1)) $R_f = 0.40$; ^{1}H NMR (500 MHz, D_2O) $\delta = 5.38$ (m, 3H, H1(D, F, and H)), 5.33 (m, 1H, H1(B)), 5.19 (m, 4H, H1(A, C, E, and G)), 4.79 (m, 3H, H5(C, E, and G)), 4.50 (m, 1H, H5(A)), 4.40 – 4.20 (m, 6H, H6(B, D, and F)), 4.33 and 4.17 (m, 2H, H6(H)), 4.30 (m, 3H, H2(C, E, and G)), 4.20 – 4.11 (m, 5H, H2(A), and H3(A, C, E, and G)), 4.11 – 4.02 (m, 3H, H4(C, E, and G)), 4.05 – 3.93 (m, 3H, H5(B, D, and F)), 4.03 (m, 1H, H4(A)), 3.99 (m, 1H, CH(CH$_3$)$_2$), 3.96 (m, 1H, H5(H)), 3.78 – 3.58 (m, 6H, H-3(B, D, and F) and H4(B, D, and F)), 3.61 (m, 1H, H3(H)), 3.53 (m, 1H, H4(H)), 3.24 (m, 3H, H2(B, D, and F)), 3.20 (m, 1H, H2(H)), 1.16 (m, 6H, CH(CH_3)$_2$); MALDI-TOF MS: calcd for [M($C_{51}H_{84}O_{77}N_4S_{12}$)+(Arg-Gly)$_{15}$+H]$^+$: 5586.7; found: 5587.2.

This work was supported by DGES (Grant No.: PB96 – 0820). J.L.de P. and J.A. thank the Spanish Ministry of Education and the Fundacion Ramón Areces, respectively, for fellowships.

[1] a) J. Taipale, J. Keski-Oja, *FASEB J.* **1997**, *11*, 51 – 59; b) H. E. Conrad, *Heparin-binding Proteins*, Academic Press, San Diego, **1998**; c) I. J. Mason, *Cell* **1994**, *78*, 547 – 552; d) R. E. Friesel, T. Maciag, *FASEB J.* **1995**, *9*, 919 – 925; e) J. Slavin, *Cell Biol. Int.* **1995**, *19*, 431 – 444; f) Z. Galzie, A. R. Kinsella, J. A. Smith, *Biochem. Cell Biol.* **1997**, *75*, 669 – 685; g) W. L. McKeehan, F. Wang, M. Kan, *Prog. Nucleic Acid Res. Mol. Biol.* **1998**, *59*, 135 – 176; h) S. Faham, R. J. Linhardt, D. C. Rees, *Curr. Opin. Struct. Biol.* **1998**, *8*, 578 – 586.

[2] a) U. Lindahl, *Heparin*, Boca Raton, Florida, **1989**; b) S. E. Stringer, J. T. Gallagher, *Int. J. Biochem. Cell Biol.* **1997**, *29*, 709 – 714; c) B. Casu, *Adv. Carbohydr. Chem. Biochem.* **1985**, *43*, 51 – 134.

[3] a) B. Mulloy, M. J. Foster, C. Jones, D. B. Davis, *Biochem. J.* **1993**, *298*, 849 – 858; b) D. Mikhailov, K. H. Mayo, I. R. Vlahov, T. Toida, A. Pervin, R. L. Linhardt, *Biochem. J.* **1996**, *318*, 93 – 102; c) D. Mikhailov, R. L. Linhardt, K. H. Mayo, *Biochem. J.* **1997**, *328*, 51 – 61; d) G. Torri, B. Casu, G. Gatti, M. Petitou, J. Choay, J. C. Jacquinet, P. Sinaÿ, *Biochem. Biophys. Res. Commun.* **1985**, *128*, 134 – 140; e) B. Casu, J. Choay, D. R. Ferro, G. Gatti, J.-C. Jacquinet, M. Petitou, A. Provasoli, M. Ragazzi, P. Sinay, G. Torri, *Nature* **1986**, *322*, 215 – 216.

[4] A. Walker, J. E. Turnbull, J. T. Gallagher, *J. Biol. Chem.* **1994**, *269*, 931 – 935.

[5] a) G. Szebenyi, J. F. Fallon, *Int. Rev. Cytol.* **1999**, *185*, 45 – 106; b) A. Romero, A. Pineda-Lucena, G. Giménez-Gallego, *Eur. J. Biochem.* **1996**, *241*, 453 – 461.

[6] a) D. Ornitz, A. Herr, M. Nilsson, J. Westman, C. Svahn, G. Waskman, *Science* **1995**, *268*, 432 – 436; b) S. Faham, R. Hileman, J. Fromm, R. Linhardt, D. Rees, *Science* **1996**, *271*, 1116 – 1120; c) A. D. DiGabriele, I. Lax, D. I. Chen, C. M. Svahn, M. Jaye, J. Schlessinger, W. A. Hendrickson, *Nature* **1998**, *393*, 812 – 817.

[7] A. N. Plotnikov, J. Schlessinger, S. R. Hubbard, M. Mohammadi, *Cell* **1999**, *98*, 641 – 650.

[8] a) D. J. Stauber, A. D. DiGabriele, W. A. Hendrickson, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 49 – 54; b) J. Schlessinger, A. N. Plotnikov, O. A. Ibrahimi, A. Eliseenkova, B. K. Yeh, A. Yayon, R. J. Lindhardt, M. Muhammadi, *Mol. Cell* **2000**, *6*, 743 – 750; c) L. Pellegrini, D. Barke, F. Von Dolft, B. Mulloy, T. I. Blundell, *Nature* **2000**, *407*, 1029 – 1034.

[9] a) D. M. Ornitz, A. Yayon, J. G. Flanagan, C. M. Svahn, E. Levi, P. Leder, *Mol. Cell Biol.* **1992**, *12*, 240 – 247; b) H. Wang, T. Toda, Y. S. Kim, I. Capila, R. Y. Hileman, M. Bernfield, R. J. Linhardt, *Biochem. Biophys. Res. Commun.* **1997**, *235*, 369 – 373; c) M. Ishihara, D. J. Tyrell, G. B. Stauber, S. Brown, L. S. Cousens, R. J. Stack, *J. Biol. Chem.* **1993**, *268*, 4675 – 4683; d) D. Avizer, E. Levy, M. Safran, C. Svahn, E. Buddecke, A. Schmidt, G. David, I. Vlodavsky, A. Yayon, *J. Biol. Chem.* **1994**, *269*, 114 – 121.

[10] a) F. Y. Zhou, M. Kan, R. T, Owens, W. L. McKeehan, J. A. Thompson, R. J. Linhardt, M. Höök, *Eur. J. Cell Biol.* **1997**, *73*, 71 – 80; b) A. G. Gambarini, C. A. Miyamoto, G. A. Lima, H. B. Nader, C. P. Dietrich, *Mol. Cell Biochem.* **1993**, *124*, 121 – 129.

[11] T. Spivak-Kroizman, M. A. Lehman, I. Dikic, J. E. Ladbury, D. Pinchasi, J. Huang, M. Jaye, G. Crumley, J. Schlesinger, I. Lax, *Cell* **1994**, *79*, 1015 – 1024.

[12] a) G. Wasman, A. B. Herr, *Nat. Struct. Biol.* **1998**, *5*, 527 – 530; b) G. Venkataraman, R. Raman, V. Sasisekharan, R. Sasisekharan, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 3658 – 3663.

[13] B. La Ferla, L. Lay, M. Guerini, L. Poletti, L. Panza, G. Russo, *Tetrahedron* **1999**, *55*, 9867 – 9880.

[14] C. Tabeur, J.-M. Mallet, F. Bono, J.-M. Herbert, M. Petitou, P. Sinaÿ, *Bioorg. Med. Chem.* **1999**, *7*, 2003 – 2012.

[15] a) J. Kovensky, P. Duchaussoy, M. Petitou, P. Sinaÿ, *Tetrahedron: Asymmetry* **1996**, *7*, 3119 – 3128; b) J. Kovensky, P. Duchaussoy, F. Bono, M. Salmivirta, P. Sizun, J.-M. Herbert, M. Petitou, P. Sinaÿ, *Biorg. Med. Chem.* **1999**, *7*, 1567 – 1580.

[16] A. Vasella, C. Witzig, J. L. Chiara, M. Martín-Lomas, *Helv. Chim. Acta* **1991**, *74*, 2073 – 2077.

[17] D. Tailler, J. C. Jaquinet, A. M. Noirot, J. M. Beau, *J. Chem. Soc. Perkin Trans. 1* **1992**, 3163 – 3164.

[18] R. Ojeda, J. L. de Paz, M. Martín-Lomas, J. M. Lassaletta, *Synlett* **1999**, *8*, 1316 – 1318.

[19] W. Kinzi, R. R. Schmidt, *Liebigs Ann. Chem*. **1985**, 1537 – 1545.
[20] H. Kunz, A. Harreus, *Liebigs Ann. Chem*. **1982**, 41 – 53.
[21] J.-L. de Paz, PhD Thesis, University of Seville, **2000**.
[22] H. Lucas, J. E. M. Basten, T. G. Van Dinther, D. G. Mauleman, S. F. Van Aelst, C. A. A. Van Boeckel, *Tetrahedron* **1990**, *46*, 8207 – 8228.
[23] P. Juhasz, K. Biemann, *Carbohydr. Res*. **1995**, *270*, 131 – 147.
[24] J. C. Jacquinet, M. Petitou, P.Duchausoy, I. Lederman, J. Choay, G. Torri, P. Sinaÿ, *Carbohydr. Res*. **1984**, *130*, 221 – 241; b) P. Sinaÿ, J. C. Jacquinet, M. Petitou, P. Duchaussoy, I. Lederman, J. Choay, G. Torri, *Carbohydr. Res*. **1984**, *132*, C5 – C9; c) M. Petitou, J. C. Jaquinet, P. Duchaussoy, I. Lederman, J. Choay, G. Torri, P. Sinaÿ, *Carbohydr. Res*. **1986**, *147*, 221 – 236; d) C. A. A. Van Boekel, T. Beetz, J. N. Vos, A. J. M. de Jong, S. F. Van Aelst, R. H. van den Bosc, J. M. R. Mertens, F. A. Vander Vlugt, *J. Carbohydr. Chem*. **1985**, *4*, 293 – 231; e) M. Petitou, G. Jurand, M. Derrien, J. Choay, *Biorg. Med. Chem. Lett*. **1991**, *1*, 95 – 98; f) M. Petitou, P. Duchaussoy, I. Lederman, J. Choay, P. Sinaÿ, *Carbohydr. Res*. **1988**, *179*, 163 – 172; g) C. A. A. Van Boeckel, M. Petitou, *Angew. Chem.* **1993**, *105*, 1741 – 1760, *Angew. Chem. Int. Ed. Engl*. **1993**, *32*, 1671 – 1690; h) I. Ichikawa, R. Monden, H. Kuzuhara, *Tetrahedron Lett*. **1986**, *27*, 611 – 614.
[25] a) D. Ferro, A. Provasoli, M. Ragazzi, B. Casu, G. Torri, V. Bossennec, B. Perly, P. Sinaÿ, M. Petitou, J. Choay, *Carbohydr. Res*. **1990**, *195*, 157 – 167; b) D. Ferro, A. Provasoli, M. Ragazzi, G. Torri, B. Casu, G. Gatti, J. C. Jaquinet, P. Sinaÿ, M. Petitou, J. Choay, *J. Am. Chem. Soc*. **1986**, *108*, 6774 – 6778.
[26] D. Jeannerat, G. Bodenhausen, *J. Magn. Reson*. **1999**, *141*, 133 – 140.
[27] M. Hrikovini, G. Torri, *Carbohydr. Res*. **1995**, *268*, 159 – 175.
[28] G. Giménez-Gallego, G. Con, V. B. Hatcher, K. A. Thomas, *Biochem. Biophys. Res. Commun.* **1986**, *135*, 541 – 548.
[29] R. M. Lozano, G. Rivas, G. Giménez-Gallego, *Eur. J. Biochem*. **1997**, *248*, 30 – 36.
[30] A. Ullrich, J. Schlessinger, *Cell* **1990**, *61*, 203 – 212.
[31] R. M. Lozano, M. Jimenez, J. Santoro, M. Rico, G. Gimenez-Gallego, *J. Mol. Biol*. **1998**, *281*, 899 – 915.
[32] A. L. Davis, E. D. Laue, J. Keeler, D. Moskau, J. Lohman, *J. Magn. Reson.* **1991**, *93*, 54 – 62.
[33] A. Bax, D. Davies, *J. Magn. Reson.* **1985**, *65*, 355 – 366.
[34] J. Jeener, B. H. Meier, P. Bachmann, R. R. Ernst, *J. Chem. Phys*. **1979**, *71*, 4546 – 4553.
[35] W. Willker, D. Leibfritz, R. Kerssebaum, W. Bermel, *J. Magn. Reson.* **1993**, *31*, 287 – 292.
[36] D. Marion, K. Wutrich, *Biochem. Biophys. Res. Commun.* **1983**, *113*, 286 – 292.
[37] D. A. Case, D. A. Pearlman, J. W. Cadwell, T. E. Cheatham III, W. S. Ross, C. L. Simmerling, T. A. Darden, K. M. Merz, R. V. Stanton, A. L. Cheng, J. J. Vincent, M. Crowley, D. M. Ferguson, R. J. Radmer, G. L. Seibel, U. C. Singh, P. K. Weiner, P. A. Kollman, AMBER 5, University of California, San Francisco, **1997**.
[38] R. J. Woods, R. A. Dwek, C. J. Edge, B. Freiser-Reed, *J. Phys. Chem*. **1995**, *99*, 3832 – 3846.
[39] C. Altona, C. J. M. Huige, *J. Comput. Chem.* **1995**, *16*, 56 – 79.
[40] S. Ortega, J. L. García, M. Zazo, J. Varela, I. Muñoz-Willery, P. Cuevas, G. Giménez-Gallego, *Bio/Technology* **1992**, *10*, 795 – 798.
[41] M. Zazo, R. M. Lozano, S. Ortega, J. Varela, R. Díaz-Orejas, J. M. Ramirez, G. Giménez-Gallego, *Gene* **1992**, *113*, 231 – 238.
[42] A. Pineda-Lucena, M. A. Jiménez, R. M. Lozano, J. L. Nieto, J. Santoro, M. Rico, G. Giménez-Gallego, *J. Mol. Biol*. **1996**, *264*, 162 – 178.
[43] A. Pineda-Lucena, M. A. Jiménez, J. L. Nieto, J. Santoro, M. Rico, G. Giménez-Gallego, *J. Mol. Biol*. **1994**, *242*, 81 – 98.
[44] H. Mach, D. B. Volkin, C. J. Burke, C. R. Middaugh, R. J. Lindhardt, J. R. Fromm, D. Longanathan, L. Mattson, *Biochemistry* **1993**, *32*, 5480 – 5489.

Received: December 18, 2000
Revised version: April 12, 2001 [F 171]