3D structure of a heparin mimetic analogue of a FGF-1 activator. A NMR and molecular modelling study†

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The motional behaviour of heparin oligosaccharides in solution is best described as a top rotor having two perpendicular rotation axes. This prevents an accurate extraction of interprotonic distances by NOESY/ROESY based methods. In this paper, we describe the solution structure of the hexasaccharide **1** calculated from high exactitude distance data obtained from *off-resonance* ROESY combined with a long MD simulation of 500 ns. In previous studies, we have found that two synthetic hexasaccharides having the sulphate groups directed towards one side of its central plane have an opposite biological activity, while **1** is unable to activate the FGF-1 signalling pathway, the other (**2**) is even more active than the regular region derived hexasaccharide (**3**) that mimics the natural active compound, heparin. From the structural analysis it was concluded that **1** has similar three-dimensional characteristics to **2** or **3** and therefore the differences in the activity should be due to the arrangement of the sulphate groups within the hexasaccharidic sequence.

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Introduction

The study of molecular recognition processes between carbohydrates and protein receptors has attracted considerable attention during the past few decades.^{1–3} Among these processes, the interactions of glycosaminoglycans (GAGs) and signalling proteins have been attracting the attention of many research groups due to their biological relevance and large number of cases. As a consequence of this, their structural details have been extensively studied.^{4–7} The versatility of the biosynthesis of these polysaccharides, which involves several enzymatic reactions in a non-template driven mode, yields a large number of possible substitution patterns that make GAGs highly dense information carriers. There are many examples where the specificity of the interaction between a GAG and a signalling protein relies on the substitution pattern.⁸ Among them, worth mentioning are the interactions with the fibroblast growth factor (FGF) family,⁹ chemokines,¹⁰ antithrombin-III (AT-III),¹¹ ECM¹² and plasma proteins.¹³

In the context of a wider research programme about the factors that regulate the activation of the FGF-FGFR signalling pathway by glycosaminoglycans, some hexa- and octasaccharides related to the structure of heparin and/or heparan

sulphate with diverse substitution patterns were prepared, tested, and their structures solved (Fig. 1).^{14–18}

The heparin regular region is made of repetitions of the D-GlcN-L-IdoA disaccharide, connected by $\alpha(1\text{--}4)$ linkages, with sulphate groups at positions 6 and 2 of glucosamine, and 2 of iduronate. In previous reports from our group, we have studied the activation of the mitogenic activity mediated by FGF-1 analysing the three-dimensional structure and dynamics of several hexa- and octasaccharides differing in their substitution pattern.^{14–18} The structure evaluation by high resolution NMR afforded similar overall shapes for the five substitution patterns: (a) two with sulphate groups on one side (**1**¹⁸ and **2**¹⁵), (b) one similar to the regular heparin pattern with sulphates on both sides (**3**)¹⁴ and (c) the other two with the absence of a type of sulphate group (either at position 6 primary hydroxyl of glucosamine residues, D-GlcN,6OH (**4**),¹⁶ or at position 2 of the iduronate rings, L-Ido2OH (**5**)).¹⁶ The activity results disclosed a strong influence of the sulphation pattern; while the regular heparin pattern (**3**) showed reasonable activity, the axially asymmetric substitution (**2**) had an improved activity (Fig. 1). On the other side, while the absence of sulphate groups in the glucosamine residues (**4**) weakened the activity; the removal of those at position 2 of the IdoA residues (**5**) removed completely the induction of activity.¹⁷

In a further work, the group prepared an alternative sequence, hexasaccharide **1**,¹⁸ based on crystallographic data and representing the most suitable minimum sequence for the interaction to yield activity according to the analysis by others and us.¹⁹ However, the biological results were negative

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Fig. 1 Structures (top) and schematic representation (bottom) of five hexasaccharides representing the heparin regular region (**3**), the same sequence with sulphate groups only on one side of the helix (**2**), an inactive sequence for FGF-1 induced mitogenic activity (**1**) and two hexasaccharides lacking one type of sulphate (**4** and **5**), both also inactive. The bottom panel represents the 3D structure of heparin (PDB code 1hpn²⁰) with the relative disposition of the sulphate groups (balls, up or down the molecular plane).

in terms of activity.¹⁸ Note that in compound **1** the spatial orientation of the negative charges is similar to **2**, with five sulfate groups displayed on one side of the helical structure. However, compound **1** contains the alternative sequence of constituent monosaccharides with an L-iduronic acid unit at

the non-reducing end and a D-glucosamine residue at the reducing terminal. As the original work did not include a detailed structural analysis, we have decided to obtain the structure of **1** with the highest possible resolution to analyse the potential reasons for the unexpected lack of activity.

It is well known that heparin oligomers longer than a tetrasaccharide are anisotropic, exhibiting a hydrodynamic top rotor^{21,22} behaviour.^{23,24} Consequently, the NOEs depend, additionally to the distances, on the orientation of the interprotonic vector with respect to the molecular axis. As expected, all the six compounds exhibit such hydrodynamic anisotropic behaviour originating from the rigidity of the glycosidic linkages that may be reflecting the electrostatic repulsions of the negatively charged groups (*i.e.* sulphate and carboxylate). In previous reports, we have studied this issue by analysing the differences in perpendicular and parallel correlation times by means of the NOE/ROE quotient at different magnetic field strengths for all the substitution patterns **2–5**^{14–17} or by complete ¹³C relaxation analysis for **2** and **3**.²⁴

In an anisotropic molecule, the NOESY or ROESY based distances calculated using the isolated spin pair approximation with a single distance, and therefore a single correlation time, as a reference are not accurate. One of the approaches used to circumvent this inconvenience has been to estimate several reference distances selected by their orthogonal orientation with respect to the molecular vector. Thus, ranges for all the unknown distances were obtained.^{14–17} However, the accuracy needed to distinguish some of the characteristic features of heparin-derived oligosaccharides is lost.

In this work, we apply to hexasaccharide **1** the *off-resonance* ROESY methodology^{25,26} for the estimation of precise distances in anisotropic molecules independently of their relative orientation with respect to the molecular axis. Distances were calculated by obtaining simultaneously the correlation time and the distance for each pair of protons. This method relies on calculating several *off-resonance* ROESY relaxation rates by varying the tilted angle of the effective ROESY spin-lock field to achieve enough independent data as to extract the correlation time for each vector and from this to calculate the interprotonic distance.^{24,25}

Results and discussion

We have performed a combined NMR and molecular dynamics structural study of **1** to obtain an NMR derived structure as precise as possible that could shed some light on its unexpected lack of biological activity. The spectra of **1** were assigned by standard procedures, *i.e.* identifying the spin systems of each ring by scalar couplings and interconnecting them *via* interglycosidic NOEs.

In this work, we have used the *off-resonance* ROESY methodology to calculate experimental distances from dipolar relaxation measurements without a pre-assigned model of motion.²⁶ This procedure allows to extract simultaneously cross relaxation rates σ^{NOE} and σ^{ROE} and effective correlation

Table 1 σ^{NOE} and σ^{ROE} cross relaxation rates and correlation times of the intra and interresidual proton pairs calculated from *off-resonance* ROESY measurements (500 MHz, 298 K)

	σ^{NOE} (s^{-1})	σ^{ROE} (s^{-1})	τ_c (ns)
H1B–H4A	−0.09	0.61	0.50
H1B–H6A ^a	−0.03	0.17	0.51
H1C–H3B	−0.05	0.20	0.59
H1C–H4B	−0.07	0.40	0.53
H1D–H4C	−0.03	0.18	0.50
H1D–H6C	−0.07	0.41	0.51
H1D–H6'C	−0.05	0.16	0.75
H1F–H4E	−0.08	0.69	0.46
H1F–H6E	−0.13	0.83	0.50
H1F–H6'E	−0.04	0.12	0.85
H1B–H3B	−0.04	0.17	0.57
H4B–H5B	−0.11	0.54	0.57
H1C–H2C	−0.02	0.07	0.65
H2C–H4C	−0.01	0.07	0.54
H1D–H2D	−0.03	0.19	0.51
H1E–H2E	−0.01	0.06	0.62
H2E–H4E	−0.01	0.07	0.50
H4F–H5F	−0.05	0.47	0.45

^a Average values over the *proR* and *proS* measurements.

times τ_c by measuring a linear combination of NOE and ROE effects controlled by the spin lock offset (see details in the Experimental section). In order to quantify the distances, arrays of several series of *off-resonance* ROESY experiments at several mixing times were recorded at different tilted angles (6, 10, 20 and 30 kHz). All growth curves for each proton and each tilted angle were linearly fitted. Then, calculating the growth rate of several series of *off-resonance* ROESY experiments corresponding to different spin lock offsets, the values of σ^{NOE} , σ^{ROE} and τ_c can be independently obtained for each proton pair (Table 1) without the use of an internal reference distance.²⁶

We have performed intensive molecular dynamics calculations of **1**, in order to get a deeper insight into its structural properties. We have run the simulation using AMBER 12²⁷ with the latest GLYCAM parameters²⁸ and partial charges,²⁹ which represent a consistent parameterization to model glycosaminoglycans, and it has been employed with that aim.³⁰ After the appropriate equilibration steps we have run a long simulation (500 ns) in explicit water (TIP3P),³¹ periodic boundary conditions (PBC)³² using the particle mesh Ewald method (PME).³³ Results were consistent with stable ¹C₄ puckering with scarce transitions to ²S₀, and a narrow *syn-Φ* and *syn-ψ* glycosidic linkage distribution (Fig. S1†).

From the molecular dynamics data we calculated the theoretical interprotonic distances and compared them with the experimental ones obtained from the *off-resonance* ROESY experiments (Table 2). The agreement between the experimental and theoretical distances was in general good for all the interglycosidic proton pairs. The experimental distances fit well with the NOE calculated distances using both r^{-3} or r^{-6} averages, as the usage of one or another average depends on the relationship of the global and internal motions. This observation indicates that either this is an intermediate

Table 2 Theoretical and experimental distances calculated from MD simulations or from 2D *off-resonance* ROESY experiments

Proton pair	Theoretical distances, MD ^a (Å)		Experimental distances (Å)
	$\langle r^{-3} \rangle^{-1/3}$	$\langle r^{-6} \rangle^{-1/6}$	
H1B–H4A	2.3	2.3	2.3
H1B–H6A ^c	3.7	3.3	2.8
H1C–H3B	2.5	2.4	2.8
H1C–H4B	2.4	2.4	2.4
H1D–H4C	2.3	2.3	2.8
H1D–H6 <i>proR</i> ^d	2.9	2.7	2.4
H1D–H6 <i>proS</i> ^d	3.3	3.0	3.0
H1E–H3D	2.3	2.3	—
H1E–H4D	2.5	2.5	—
H1F–H4E	2.3	2.3	2.2
H1F–H6 <i>proR</i>	3.2	2.9	2.2
H1F–H6 <i>proS</i>	4.0	3.6	3.2
H1B–H2B	2.6	2.6	—
H1B–H3B	4.2	4.2	2.9
H2B–H5B	3.9	3.9	3.0 ^c
H4B–H5B	2.4	2.4	2.3
H1D–H2D	2.6	2.6	2.8
H1D–H3D	4.2	4.2	—
H2D–H5D	4.0	3.9	3.3 ^c
H4D–H5D	2.4	2.4	—
H1F–H2F	2.6	2.5	—
H1F–H3F	4.2	4.2	4.0 ^c
H2F–H5F	4.0	4.0	—
H4F–H5F	2.4	2.4	2.3

^a Distances calculated as $\langle r^{-3} \rangle^{-1/3}$ and $\langle r^{-6} \rangle^{-1/6}$ from 500 ns of MD simulation. ^b Distances derived from ¹H-NMR *off-resonance* 2D-ROESY experiments. ^c Theoretical distances obtained from r^{-3} and r^{-6} average, respectively, over the *proR* and *proS* values. ^d Values assigned to each diastereotopic hydrogen (*proR* and *proS*) under the approximation that the most populated conformer around the GlcN (C) ω torsion is *gg* (in agreement to MD results, see below).

hydrodynamic behaviour of the molecule that fits to both models of motion or that the distances do not fluctuate significantly (Table 2).

One of the key issues of the iduronate ring is the balance between the populations of the conformers in equilibrium depending on the position along the chain, their substitution, or the charge distribution of neighbour residues. This is reflected in the values of the intra-ring three-bond coupling constants and in the H2–H5 distance, that in some cases give observable NOE peaks.^{4,18} We have performed several analyses of the populations of conformers of the L-IdoA residues assuming a mixture of two conformations, ¹C₄ and ²S₀, in fast equilibrium in the chemical shift time scale (Table 3).

Interestingly the iduronate rings of **1** have a rich and varied behaviour. In the NOESY experiments, the non-reducing end iduronate (F) does not show a NOE cross-peak between H2 and H5, which is characteristic of the participation of the ²S₀ conformation in the equilibrium. On the other hand, the iduronates B and D display some features corresponding to the expected participation in the equilibrium of the conformer ²S₀, as is the medium–weak intensity NOE cross peak between the protons H2 and H5. The NOESY experiments show that the proportion of ²S₀ decreases along the chain being larger in B than in D, and not observing the exclusive NOE peak in the

Table 3 Population-weighted H2–H5 distance (in Å) of each iduronate ring compared with the experimental one determined from 2D-NOESY experiments. The 1C_4 and 2S_0 puckers populations previously calculated by iterative fitting of the experimentally derived IdoA ${}^3J_{HH}$ and two different sets of canonical ${}^3J_{HH}$ values (Hricovíni 2006 and 2011)^{34,35} have been used. The canonical H2–H5 distance for both the 1C_4 and 2S_0 conformers, 4 Å and 2.4 Å, respectively, have been taken from the NMR resolved structure of heparin with PDB code 1hpn

	IdoA2S (B)	IdoA2OH (D)	IdoA2S (F)
Hricovíni 2006 ³⁴	2.8	2.8	3.1
Hricovíni 2011 ³⁵	2.8	2.9	3.1
Experimental	3.0	3.3	Undetected

case of the last iduronate, F, with the lowest population among the three. We compared the theoretical H2–H5 distances from the output of the MD simulation with the experimental ones based on *off-resonance* ROESY measurements (Table 3). A decrease of the equilibrium population of the 2S_0 conformation can be detected along the chain towards the non-reducing end.

A more precise method to determine the conformer population in this case is by using three bonds interprotonic coupling constants. In this case, we have used two different sets of canonical values for the 1C_4 and 2S_0 conformers of an IdoA residue (corresponding to an α -D-IdoA2S-OH monosaccharide³⁴ and an α -D-GlcNS,6S-(1-4)- α -D-IdoA2S-OMe disaccharide³⁵) (Table 4). Then, we have calculated the conformer populations assuming a mixture of two conformations for the three iduronate rings. When we calculated the conformer populations using the coupling constant analysis we found that they follow the same tendency (see Table S2 in ESI,[†] entry H2–C2–C3–H3: 6.0–5.5–4.4 Hz for B, D and F iduronate respectively). Interestingly, when ring F is subjected to this analysis some appreciable population of 2S_0 (17–19%) is obtained, suggesting that the energetic barrier is still low enough to reach the equilibrium. In this case the exclusive NOE for this conformation cannot be detected as the population of the conformation is too low.

When we analysed the conformer populations along the MD simulations using the puckering values we observed scarce conformational transitions from 1C_4 to 2S_0 (Fig. S1[†]). Although the frequency of the transitions was not as high as to explain the coupling constant analysis, we detected some transitions, which qualitatively correspond to our experimental observations where the population of skew boat decreases from B to F. While the behaviour of ring D can be explained by the absence of the sulphate group in the iduronate, we

Table 4 Populations of the skew-boat 2S_0 pucker of the L-IdoA rings calculated by multi-parametric fitting of the experimental and theoretical proton–proton vicinal coupling constants

Canonical ${}^3J_{HH}$	IdoA2S (B)	χ_{red}^2	IdoA2OH (D)	χ_{red}^2	IdoA2S (F)	χ_{red}^2
Hricovíni 2006 ³⁴	40%	0.20	33%	0.41	17%	0.09
Hricovíni 2007 ³⁵	37%	0.27	32%	0.44	19%	0.37

propose that the case F can be due to the situation on the chain at the non-reducing end.

An additional characteristic of the conformation of IdoA containing GAGs is the rigidity of its backbone in contrast with the large conformational equilibrium of the iduronate residues. It has been proposed that the electrostatic repulsions between charged groups are responsible for this behaviour. The distributions of the values of glycosidic linkage dihedral angles Φ and Ψ along the MD simulation are shown in Fig. S2.[†] The results are consistent with its habitual behaviour, where the linkages GlcN–Ido are more rigid than the Ido–GlcN and the Ψ angle covers a wider range than Φ (Fig. 2). The effect of the central iduronate glycosidic linkages on the stiffness of the backbone is smaller than for the other linkages reflecting the absence of the sulphate group at position 2 of the iduronate ring.

Fig. 2 Φ and Ψ glycosidic dihedral angles frequency distribution curves for each linkage. A bin size of 10° has been used.

Fig. 3 Superimposition of 20 conformers randomly taken from the MD simulation. The backbone of the two central residues (IdoA(D) and GlcN(C)) has been considered for the superimposition, and thus, the increase of flexibility from the center to the ending residues of the polysaccharide chain is clearly observed. The hydrogen atoms and the sulfate groups have been removed for clarity.

The diffusional anisotropy is the quotient between the parallel and perpendicular correlation times and the most precise method to calculate it is by a complete relaxation analysis using ^{13}C T_1 , T_2 and heteronuclear NOE, avoiding the influence of the internal correlation times that decrease these ratios. However, this time consuming method could be replaced by the relationship between the correlation times of two orthogonal interprotonic vectors of similar distances aligned with respect to the molecular axis. We have calculated the ratios of the apparent correlation times, obtained from the *off-resonance* ROESY, for two vectors: H1–H2 and H2–H4 of glucosamine ($\tau_{\perp}/\tau_{\parallel} = 1.2$ for ring D and 1.24 for ring E) which are orthogonal between them, and the vector H1–H2 is nearly parallel to the molecular axis of the hexasaccharide **1**. Therefore, this quantity stands for the anisotropy factor. Finally, we compare the anisotropy factor obtained from the quotients between the experimental correlation times for **1**, with those previously obtained for **2** and **3** ($\tau_{\text{CH1-H2}}/\tau_{\text{CH2-H4}} = 1.22$, 1.50 and 1.46 for **1**, **2** and **3**, respectively).¹⁵ Therefore, it can be concluded that **1**, although slightly less anisotropic than **2** or **3**, still exhibits this characteristic behaviour.

In general the structural characteristics of **1** are similar to those observed for **2** and **3**: linear overall shape, rotational anisotropy, backbone rigidity and iduronate conformational equilibrium. The superimposition of 20 frames taken along the molecular dynamics simulation is shown in Fig. 3.

Experimental

NMR

A NMR sample was prepared by dissolving 2.5 mg of **1** in 600 μL of D_2O 100%, pH* was adjusted to 7.0. 2D experiments were recorded using the following versions with z -gradients when possible: gs-DQF-COSY,³⁶ TOCSY³⁷ using 80 ms of spin lock, NOESY,^{38,39} ROESY,⁴⁰ HSQC using gradient selected with sensitivity enhanced versions,⁴¹ and coupled HSQC.

Estimation of ^1H – ^1H distances. In order to estimate the longitudinal and transversal cross relaxation rates, σ^{NOE} and σ^{ROE} , compensated *off-resonance* ROESY^{25,42} experiments have

been acquired at 500 MHz using different mixing times (100, 200, 300, 400 and 500 ms) and several radiofrequency offsets (6, 10, 20 and 30 kHz) for the spin-lock. The spin locking field was 7.14 kHz and the experiments were carried out at 298 K.

Molecular dynamics

Input preparation. The initial coordinates were based on the NMR-resolved dodecasaccharide structure of natural heparin (PDB code 1hpn²⁰). The topology and coordinates files were built with the *tLEAP* module of AMBER 11 package.⁴³ The system was neutralized with sodium ions and then immersed in a TIP3P³¹ water box, giving rise to a molecular system of 4526 atoms. The GLYCAM_06 h parameters²⁸ were used to model the sugar moiety, including the sulfate and sulfamate groups,⁴⁴ and the AMBER ff12SB parameters²⁹ for the water molecules and calcium ions. The partial charges of GLYCAM06²⁸ were employed for the sugar moiety, adjusting the partial charge on the O- and N-atoms bound to the SO_3 groups according to the GLYCAM procedure for charge development. For the *O*-isopropyl group, partial charges were derived from the molecular electrostatics potential (MEP) using the RESP method with a constraint of 0.01, for consistency with the procedure employed in GLYCAM06 force field²⁸ development. The HF/6-31G* level of theory was used for both the structure optimization and the MEP calculation. Detailing the procedure employed, a methyl-*O*-isopropyl and a D-Glc-OMe were built and charge constraints imposed as follows: the total charge of both molecular models is set to 0, the methyl group in the D-Glc-OMe must have a charge of +0.194 whereas the charge of the oxygen involved in the glycosidic linkage is set to –0.194, and both methyl groups are set to be equivalent and to be removed during the last step of charge derivation, and, thus, being both compounds merged to form a D-Glc-*O*-isopropyl. Additionally, the partial charges on aliphatic hydrogens and on the *O*-isopropyl group were constrained to 0 and –0.194, respectively, in agreement with the GLYCAM procedure. The standard error and the relative root mean square error were, respectively, 0.005 and 0.188. It is noticeable that a similar protocol has been successfully applied for the development of parameters for different sugar derived compounds.^{45,46} The quantum mechanical calculations and the RESP procedure were carried out with ante-R.E.D 2.0 and R.E.D IV of the R.E.D web server.⁴⁷

Molecular dynamics simulations. The molecular dynamics simulations have been run on a 4-node AMD Opteron Interlagos cluster (2.3 GHz, 16 cores per node).

MD simulations were carried out with AMBER 12.²⁷ The equilibration protocol consisted of an initial minimization of the water box (20 000 steps), followed by a minimization of the whole system (10 000 steps); finally, the system was heated (40 000 steps) at a constant volume until 300 K using a time constant for the heat bath coupling of 1 ps. The production dynamics have been carried out at a constant temperature of 300 K, by applying the Langevin thermostat⁴⁸ with a collision frequency of 5 ps^{-1} , and at a constant pressure (1 bar),

applying periodic boundary conditions (PBC)³² and using the particle mesh Ewald method^{33,49} (PME) to account for the long range electrostatic effect (the grid spacing was approximately 1 Å). The SHAKE algorithm^{50,51} was also employed, thus, allowing a 2 fs time step, and non-bonded interactions were cutoff at 8.0 Å and updated every 25 steps.

The equilibration protocol and production dynamics have been performed with the *sander.MPI* and *pmemd.MPI* modules of AMBER 12,²⁷ respectively. One MD simulation of 500 ns has been performed; saving the trajectory coordinates each picosecond. The analysis of the MD trajectory was performed using the *ptraj* module of AMBER 12.²⁷ The Cremer–Pople coordinates were calculated with a script from R. J. Woods' group (see the Acknowledgements section).

Conclusions

We have analysed the 3D structure of the hexasaccharide **1** which is inactive as an inductor of the FGF1–FGFR signalling pathway.¹⁸ We have found that the structure of the hexasaccharide **1** has the same overall features as the previously studied heparin-like oligosaccharides, a rigid linear structure due to the geometry of the glycosidic linkages driven by the electrostatic repulsions between the charges of the sulphate groups.^{14–17} In particular, it is very close to the structure of **2** that has remarkable mitogenic activity mediated by FGF-1 and shares many of its structural characteristics with the non-symmetrical distribution of the sulphate groups on both sides of the molecule.¹⁷

Although, differences have been found in the populations of the conformational equilibrium with the previously analysed compounds with the same length but with different sulphation patterns and sequences. The populations of the ²S₀ conformer are lower than in **2**, for residues B and D, and even undetectable in terms of NOE for the terminal iduronate E. However, this is not likely to be the cause of the inactivity as the differences in energy that can be inferred from differences in populations are not large enough to justify differences on binding. Furthermore, it has been reported that the conformational equilibrium remains even when the hexasaccharide **2** is within the complex with FGF-1 as a result of interactions with flexible side chains *via* electrostatic charges.^{52,53}

The solution structure of the heparin was described by Mulloy *et al.* as an extended helix with four residues per turn²⁰ that leads to clustering three consecutive sulphate groups directed towards the same side, relative to the central chain, while the subsequent cluster is directed towards the opposite side. The distribution of sulphate group charges in **1** can be considered to be analogous to that of compound **2** but in the reversed directionality (from the reducing to the non-reducing end; see Fig. 1). We are now exploring a potential explanation for the lack of activity of **1** based on the previous results for the FGF-1 and FGF-2 specific interactions, together with the X-ray structures of the ternary complexes with the extracellular domains of the receptor FGFR.

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