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Heparin: From the original "soup" to well-designed heparin mimetics

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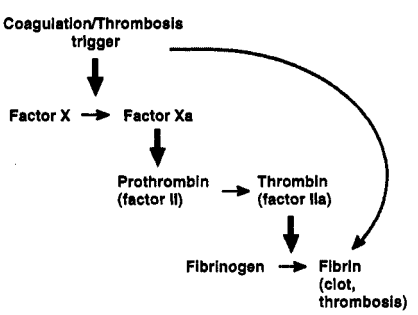
Abstract: Since its introduction in 1937 heparinotherapy has improved consistently. In this article we describe the rationale behind the different steps of the evolution that, starting from a very impure polysaccharide mixture, ended in the proposal for development of pure synthetic antithrombotic oligosaccharides.

INTRODUCTION

Cardiovascular diseases are a major cause of mortality and morbidity in developed countries. Among them, vascular thrombosis, the partial or total occlusion of blood vessels by a clot containing blood cells and fibrin, can affect arteries or veins. In the case of arteries it mainly results from platelet activation and leads to heart attack, angina or stroke, whereas venous thrombosis results in phlebitis and pulmonary emboli.

Since coagulation was shown to play a major role at the onset of venous thrombosis, heparin a powerful anticoagulant, has been used very early (1,2) to prevent and to cure this type of thrombosis, heparin is nowadays also used in the treatment of arterial thrombosis.

It was shown in 1973 that heparin requires antithrombin III (AT III) a 432 amino acid protein to express its anticoagulant activity. AT III, an essential regulator of blood coagulation, is a serine protease inhibitor present in plasma at about 2 μM concentration (3). In the presence of heparin the anticoagulant activity of AT III is considerably reinforced (4). The antithrombotic effect of heparin has been particularly assigned to the enhanced inhibition of two of the coagulation factors: factor Xa and thrombin (scheme 1).



Scheme 1. Blood coagulation results from a cascade of enzymatic reactions where the enzyme formed at one step produces the enzyme of the next step. When large amounts of Tissue Factor (the main trigger of coagulation) are poured into the circulation (surgery, cancer etc.) extensive coagulation and thrombosis may result, if the natural regulators of coagulation are overwhelmed. The antithrombotic activity of heparin is due to the enhancement of the inhibitory potency of AT III with respect to factor Xa and thrombin.

Heparin is a complex heterogeneous polysaccharide of the glycosaminoglycan family. Standard heparin preparations contain polysaccharide chains having a molecular weight in the range 3000-30.000 Da. These chains consist in sequences of disaccharides containing 1→4 linked uronic acid (α-L-iduronic or β-D-glucuronic) and α-D-glucosamine units. Due to the various sulfation patterns of these disaccharides (see for example figure 1) the structure of heparin is highly complex (5).

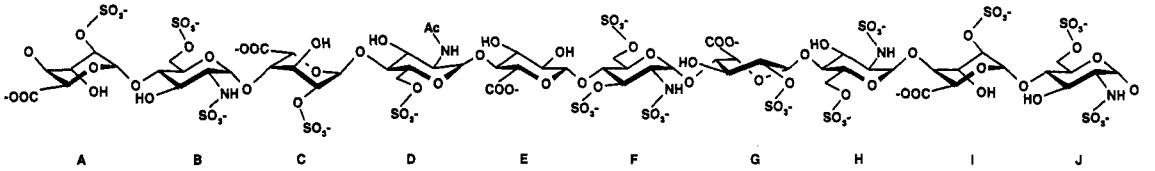


Figure 1. The structure of heparin

The highly complex polyanionic molecules that constitute a heparin preparation have been reported to interact with hundreds of proteins and biomolecules. Most of these interactions are non specific, they are merely assigned to the polyanionic character of the heavily sulfated chains. The intensity of these interactions is directly correlated to the charge and to the length of the heparin molecules.
The aim of the research outlined in this article is to improve heparinotherapy by trying, among the many interactions just mentioned, to favor those which are responsible for the antithrombotic effect of heparin, and to minimize the undesired ones.

HEPARIN IN CLINICS

The first reports on the clinical use of heparin date back to the late thirties (1,2). Due to the bad quality of the drug then used, tolerance problems were encountered. To improve the situation, and also to reduce the bleeding tendency, it was suggested to lower the dose of anticoagulant injected. Thus in 1971, results of a clinical trial were published, showing that low doses of heparin were well tolerated while the antithrombotic activity was preserved (6). To explain this result it was proposed that inhibition of factor Xa is the key feature of the antithrombotic action of heparin. Since each step of the coagulation cascade is an amplification, much less heparin is required to inhibit a certain amount of factor Xa that to inhibit the thrombin it would generate (7). Soon after it was discovered that the molecular weight of heparin chains strongly influenced factor Xa and thrombin inhibition. Thus whereas high molecular weight heparin chains promote factor Xa and thrombin inhibition to the same extent, only factor Xa is inhibited by low molecular weight heparin chains in the presence of AT III (8). This observation has lead to the Low Molecular Weight Heparin class of drugs. The latter are obtained by partial depolymerisation of heparin, they preferentially inhibit factor Xa but still retain some anti-factor IIa (thrombin) inhibitory properties. They display the same antithrombotic potency as heparin and are endowed with better pharmacokinetics due to weaker interactions with blood and vessel components (9).

STRUCTURE OF ANTI-FACTOR Xa OLIGOSACCHARIDES OBTAINED FROM HEPARIN

The quest for the shorter heparin fragments able to activate AT III towards factor Xa inhibition was favored by the discovery, again in 1976, that standard heparin preparations could be fractionated according to affinity for AT III. It was found that two thirds of the components in a standard heparin preparation were devoid of affinity for the protein and were inactive in anticoagulant tests. On the contrary the compounds which bound to AT III did show a strong anticoagulant effect (8,10,11). Using affinity chromatography and gel filtration it was possible, starting from mixtures of partially depolymerised heparin preparations, to obtain oligosaccharides which showed high affinity for AT III and high anti-factor Xa activity (figure 2). The structure of these compounds could be definitively established (12, 13) after the discovery of a unique 3-*O*-sulfo substituent in the antithrombin III binding sequence of heparin (15). From all these studies it was concluded that the actual antithrombin III binding sequence in heparin was a pentasaccharide (16, 17).

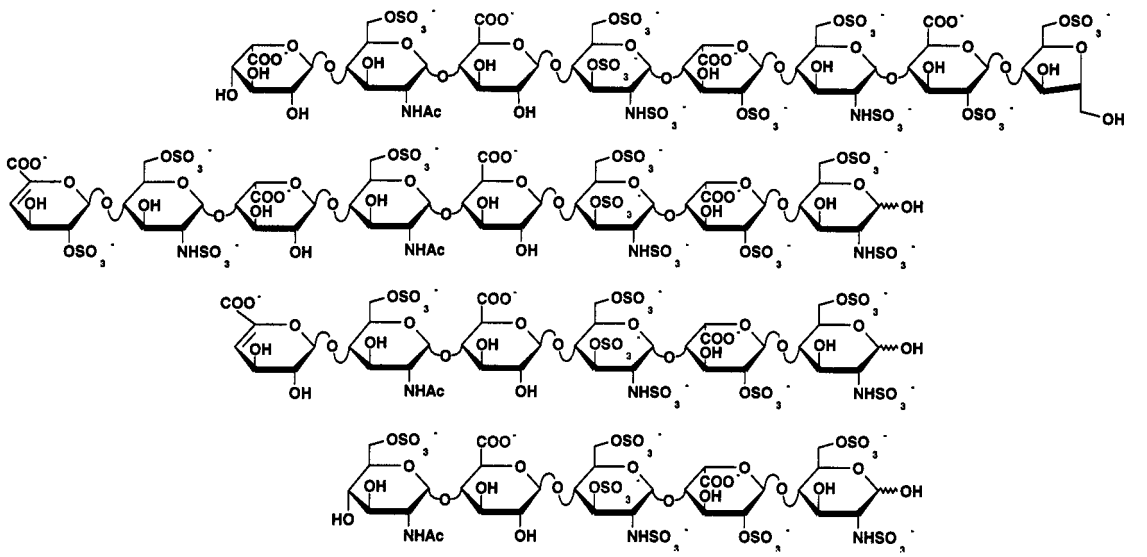
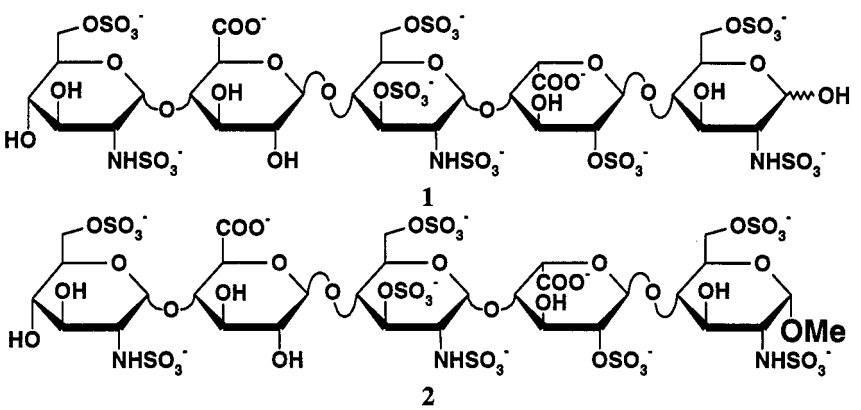


Figure 2. Structure of heparin oligosaccharides having affinity for AT III. The two octasaccharides were isolated from heparin after partial nitrous acid (1st line) and heparinase (2nd line) degradation followed by affinity chromatography and gel filtration. The overlapping hexasaccharide sequence (3rd line) contains the actual antithrombin III binding sequence, the bottom line pentasaccharide (12-17).

FIRST GENERATION SYNTHETIC PENTASACCHARIDES

Having identified the pentasaccharide sequence responsible for binding and activation of AT III we embarked on the synthesis of such oligosaccharides. The first target, pentasaccharide **1**, was obtained by total chemical synthesis (18, 19) and shown to possess affinity for AT III and high anti-factor Xa activity (20). This compound was also active in animal models of venous thrombosis (21). In a second step, to facilitate the synthesis and to avoid the reaction of the hemiacetal with proteins amino group, the free reducing end of this compound was blocked under the form of a methyl glycoside (22, 23). The compound **2** thus obtained has been prepared at large scale and is currently undergoing clinical trials to demonstrate the efficiency of such selective anti-factor Xa inhibitors in the prevention and the treatment of venous thrombosis.



1

2

To investigate the structure activity relationships of the interaction of pentasaccharide **2** and AT III we have synthesized numerous analogues of **2**. Shorter fragments were prepared and were practically inactive. Numerous compounds missing sulfate groups at well defined positions were also obtained and their study allowed us to definitively prove the critical role of some of them while others were found to

only reinforce the interaction with the protein, as shown figure 3 (22, 23). The spatial distribution of the critical groups shown figure 3 suggests the presence of two distinct binding sites, at the south, and at the north of the molecule. We found that introduction of an extra sulfate group on the second binding site (at position 3 of the reducing end glucosamine unit) resulted in a large increase of affinity for the protein and in anti factor Xa activity (24).

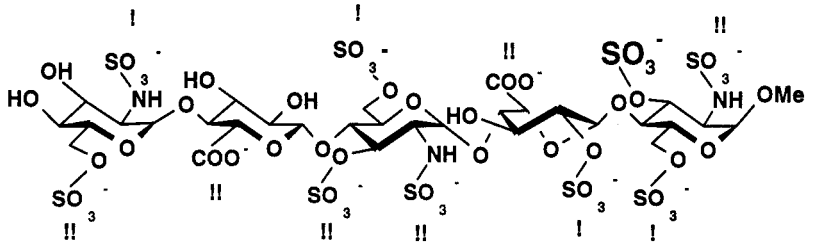


Figure 3. The role of the sulfate groups borne by the pentasaccharide sequence. The functional groups that play a critical role (four sulfates and the two carboxyls) are noted !!; those that only contribute to reinforce the interaction are indicated !. The nature of the two uronic acids is also important, a large drop in activity is observed when either of them is replaced by its epimer. Introduction of an extra sulfate group at position 3 of the reducing end glucosamine unit results in a large increase in affinity and anti-factor Xa activity (24).

SECOND GENERATION SYNTHETIC PENTASACCHARIDES

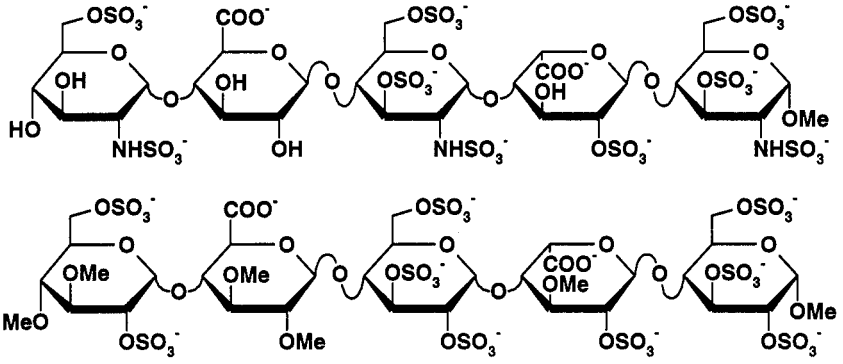


Figure 4. Replacement of *N*-sulfo by *O*-sulfo and OH by OMe simplifies the synthesis and results in compounds having similar biological properties (Kd 16 nM *vs* 22 nM, and 1323 *vs* 1250 anti-Xa units/mg).

From the studies mentioned above we deduced that the hydroxyl and amino groups of the monosaccharide units were probably not involved in the interaction with AT III. For obvious reasons it would be a tremendous advantage from the synthesis standpoint to only deal with *O*-sulfo groups instead of *O*-sulfo and *N*-sulfo. Replacement of OH groups by OMe would considerably simplify protecting groups manipulations since OMe could be introduced at the very beginning of the process, and no orthogonal protective group strategy is required. As expected the compounds obtained (figure 4) displayed similar biological properties as their hydroxylated and *N*-sulfated counterparts (25-29). Further symmetrisation of the molecule in this series of second generation pentasaccharides allowed us to devise shorter synthetic routes toward this kind of pentasaccharides (30).

SYNTHETIC THROMBIN INHIBITORS

As indicated earlier in this article a very short specific pentasaccharide sequence is responsible for the binding of heparin to AT III. On binding to this sequence AT III undergoes a conformational change which allows the inhibitory protein to be recognized by factor Xa which is then inhibited. Thrombin inhibition occurs through a more complicated mechanism where the heparin molecule serves as a template to assemble the thrombin-AT III complex (31). The conformational change of AT III, which accompanies high affinity binding to AT III, is not by itself required for thrombin inhibition since this latter can also be promoted, although in a less efficient way, by heparin chains devoid of AT III binding pentasaccharide sequence (32).

Having at our disposal various types of pure factor Xa inhibitors, we decided to investigate whether the type of chemistry we have developed would be useful to produce thrombin inhibitors. It results from the mechanism of thrombin inhibition just mentioned that to this end we need to produce larger molecules, containing a pentasaccharide AT III binding domain (ABD) and a thrombin binding domain (TBD). While we had much information concerning the ABD, not so much was known concerning the TBD. It has been assumed that thrombin interaction with heparin does not require a very precise arrangement of sulfate groups, but merely results from electrostatic attraction between the positively charged protein and the negatively charged heparin chain. Recent publications indicated however that a tetrasaccharide sequence was required for optimal thrombin binding (33), and it has been known for some time that the fragments of heparin required for thrombin inhibition, including the pentasaccharide sequence must contain at least from 14 to 20 monosaccharide units (34-37). Another open question however was the relative positions of the ABD and the TBD, should the TBD be at the reducing end or at the non-reducing end of the ABD pentasaccharide?

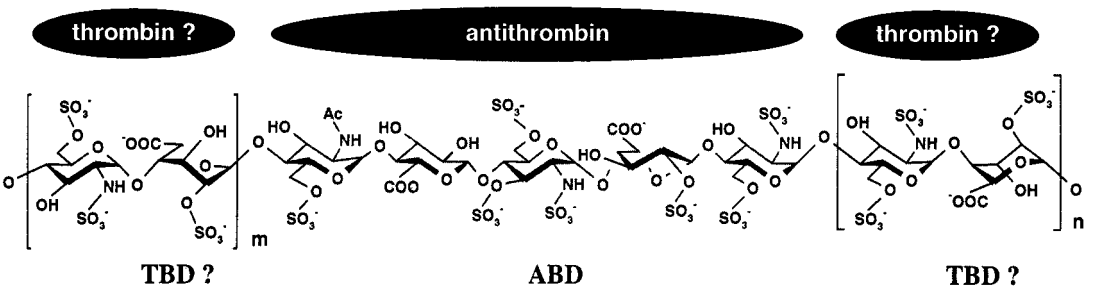


Figure 5. In a heparin molecule the AT III binding domain (ABD) is prolonged at both ends by potential binding sites for thrombin (TBD). Only one (unknown at the start of this project) of the ABD→TBD and TBD←ABD arrangements is efficient for thrombin inhibition.

Taking into account, on the one hand, the asymmetric distribution of interacting sulfates and carboxylates on the pentasaccharide sequence and, on the other hand, the position of the amino acid residues involved at the protein side, it was possible using the three dimensional structure of AT III, to dock the pentasaccharide on the surface of the protein (38), then to deduce, from the position of the loop containing the inhibitory site of AT III, that thrombin should approach the heparin-AT III complex coming from the non-reducing end of the pentasaccharide sequence. Furthermore, molecular modeling of the ternary complex between heparin, AT III and thrombin indicated that the minimum size of the molecule required to assemble such a complex was a pentadecasaccharide. It also confirmed that part of the structure was used to bridge the ABD and the TBD (39).

According to these informations it should be possible to obtain thrombin inhibitors by linking to the non reducing end of an AT III binding pentasaccharide a sulfated oligosaccharide able to attract thrombin. This can be achieved using two sulfated carbohydrate entities prolonged by spacers bearing suitable functional groups. The two species can then be linked to each other using a coupling reagent, as shown figure 7 (40).

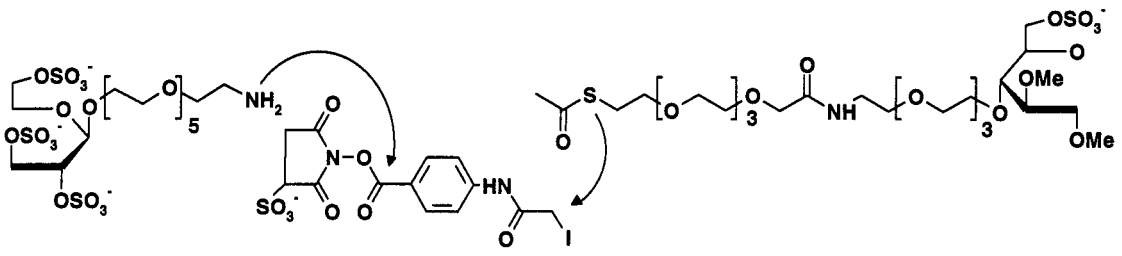


Figure 7. An example of coupling reaction to obtain a conjugate.

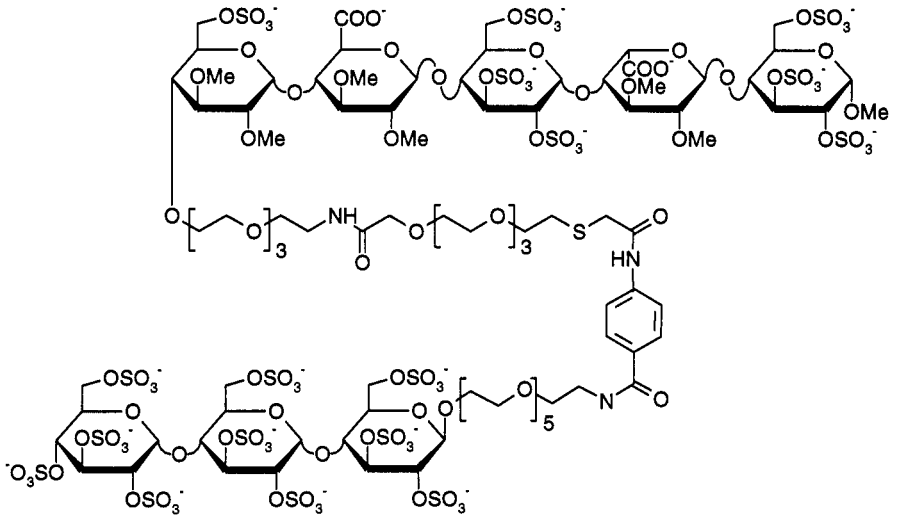


Figure 8. A conjugate between an AT III binding pentasaccharide and decasulfated maltotriose (40).

In order to assess the influence of the relative position of the ABD and TBD, we have synthesized two bisconjugates using the same spacer. The first one was obtained by connecting two molecules of the same AT III binding pentasaccharide through their non-reducing end. This compound displayed anti-factor Xa and thrombin inhibition in the presence of AT III. The second bisconjugate, resulted from the connection of two pentasaccharide molecules through their reducing end. It only displayed anti-factor Xa activity. These results confirm the deductions of the molecular modeling concerning the relative orientation of the ABD and the TBD.

Using different AT III binding sequence, different thrombin binding domains and by varying the length of the spacer it is possible to finely adjust the biological profile of the conjugate as shown Table 1.

Table 1. Biological properties of various conjugates obtained using the same pentasaccharide AT III binding sequence and various thrombin binding domains.

TBD	anti-Xa activity unit/mg	anti-IIa activity unit/mg
maltotriose	490	64
cellobiose	740	10
maltopentaose	280	330
TBD = ABD	770	14
heparin	160	160

CONCLUSION

The chemical synthesis approach has been very fruitful both from the standpoint of the understanding of the mode of action of heparin and of the production of potential active substances for new drugs. Thus it is clear now that the interaction of the complex polysaccharide and AT III depends on the presence of a very precise array of sulfate and carboxylate groups that induce a conformational change necessary and sufficient to inhibit blood coagulation factor Xa.

Most importantly, we can obtain in a pure state all the heparin mimetics desired. These synthetic products constitute active principles for new drugs the biological profile of which can be adapted at will to reproduce some of the many activities of heparin. Such compounds also constitute invaluable tools for the pharmacologists to better understand the mode of action of heparin itself.

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