Chemical Contributions to Understanding Heparin Activity: Synthesis of Related Sulfated Oligosaccharides

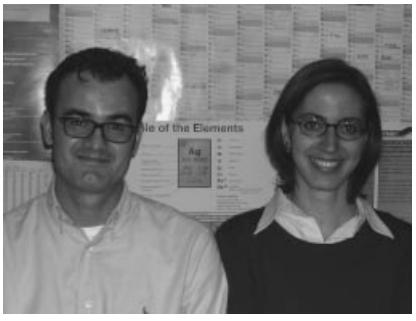
Laura Poletti[a] and Luigi Lay*[a]

Keywords: Carbohydrates / Oligosaccharides / Glycosylation / Heparin / Heparan sulfate

Heparin and heparan sulfate are complex polysaccharides that modulate several biological events through the recognition of a number of distinct proteins. Chemical synthesis is a powerful tool to identify the unique binding domains responsible for the specific interactions and to generate potent analogues of the natural sequences. The present review reports the synthetic efforts of the last decade that are focused on the preparation of tailored fragments of these two polymers and analogues thereof.

Contents

[a] Dipartimento di Chimica Organica e Industriale and Centro Interdisciplinare Studi bio-molecolari e applicazioni Industriali (CISI), Università degli Studi di Milano, and CNR (Istituto di Scienze e Tecnologie Molecolari),
Via G. Venezian 21, 20133 Milano, Italy
Fax: (internat.) + 39-02/50314061
E-mail: luigi.lay@unimi.it



Laura Poletti (right) was born in 1970 in Milan, Italy. She received her graduation in Chemistry at the Università degli Studi of Milan in 1995, under the supervision of Prof. Franco Cozzi. In 1999 she received her Ph.D. degree in Industrial Chemistry, from the same University, under the supervision of Prof. Giovanni Russo. During her graduate studies she worked on the synthesis of heparin-like oligosaccharides starting from strategically protected building blocks. In 2000 she moved to the Ronzoni Institute of Chemistry and Biochemistry, Milan, where she worked on a project dealing with the chemical modification of glycosaminoglycan polymers. In 2001 she went back to the University of Milan, where she is currently working as a postdoctoral fellow. Her research interest is focused on the synthesis of biologically relevant oligosaccharides and their analogues.

Luigi Lay (left) was born in Sassari (Italy) in 1965. He received his Ph.D. in Organic Chemistry at the University of Milan in 1994, under the supervision of Prof. Giovanni Russo. During 1995−1996 he was a postdoctoral fellow at the Institut für Organische Chemie, University of Konstanz (Germany) with Prof. R. R. Schmidt, where he was involved in an EU-funded project on the synthesis of glycoconjugates via new oligosaccharide building-blocks. In 1996 he joined the CNR (Consiglio Nazionale delle Ricerche, at the Centro di Studio sulle Sostanze Organiche e Naturali) in Milan as a researcher. In 2000 he became a senior researcher at the University of Milan (Department of Organic and Industrial Chemistry), in the group of Prof. Giovanni Russo. His research area is the synthesis of oligosaccharides and analogues, with a particular focus on partially sulfated oligosaccharide fragments related to heparin and heparan sulfate, and on oligosaccharides endowed with potential immunological acitivity.

MICROREVIEWS: This feature introduces the readers to the author's research through a concise overview of the selected topic. Reference to important work from others in the field is included.

 DOI: 10.1002/ejoc.200200721

1. Introduction

Heparan sulfate glycosaminoglycans (H/HS)[1,2] are members of the glycosaminoglycan (GAG) family, which are complex polysaccharides characterised by an alternating disaccharide unit of uronic acid (either L-iduronic or D-glucuronic acid) linked to a D-glucosamine unit (Figure 1). We use the term H/HS to imply both heparin (H) and heparan sulfate (HS).

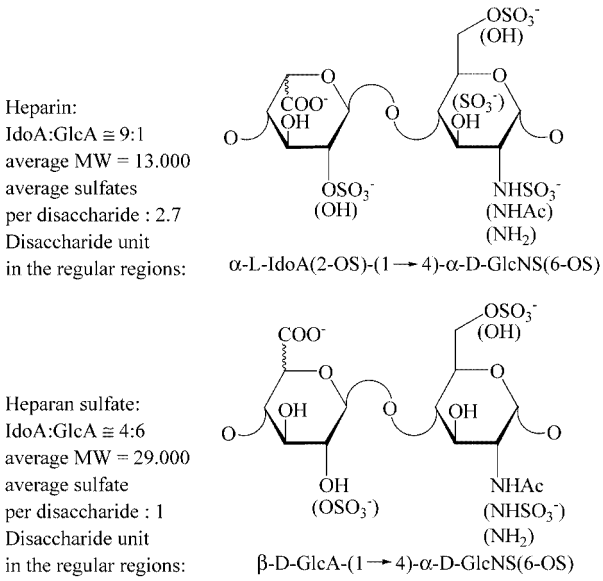


Figure 1. Disaccharide repeating units of heparin and heparan sulfate in the regular regions; possible modifications occurring in the heterogeneous domains are included in brackets

While HS is ubiquitous and usually found covalently attached to a core protein, H is almost exclusively synthesised and stored in connective-tissue mast cells and is released in the extracellular matrix free from the core protein.[3] H/HS are characterised by linear chains, comprised of 20−200 disaccharide repeating units, displaying a helical conformation.[4] Even though a clear-cut borderline between the two species hardly can be drawn, they differ in the sulfation pattern and in the ratio of D-GlcA/L-IdoA. HS is more heterogeneous than H, is richer in D-GlcNAc and D-GlcA units, and contains fewer *O*-sulfates. The structural complexity of H/HA arises from different modifications of individual disaccharides within the polymer, which give rise to over a million possible saccharide sequences. Usual modifications include the C-5 configuration at the uronic acid units (present as both L-IdoA or D-GlcA), *N*-acetylation or *N*-sulfation at the D-GlcN units, and 2-*O*-sulfation at the L-IdoA units (Figure 1). The occurrence of the so-called "rare" components (3-*O*-sulfate or *N*-unsubstituted D-GlcN, 2-*O*-sulfated D-GlcA units) adds further complexity to this polymer.

H/HS are present in the cell tissue/organ interface, where they have been shown to interact with more than a hundred proteins, often involving divalent cations,[5,6] that play crucial regulatory roles in normal physiological processes − such as embryogenesis, cell adhesion and viral invasion − as well as in pathophysiological conditions — including the processes of tumour onset, growth and metastasis.[7−9]

The high density of information contained in H/HS chains that is suggested by the wide range of H/HS-dependent processes relies both on the complexity of their structure and on their distinct ways of interaction with proteins. The most prominent type of interaction between H/HS and proteins is electrostatic in nature,[10] and occurs between the negatively charged groups of the polymer and the basic residues of the protein. However, a significant contribution is made by non-ionic interactions, namely hydrogen bonding and hydrophobic forces. In some cases, the H/HS−protein interaction seems to be influenced crucially by the flexibility of one or more L-IdoA units present in the binding site. Because of the strongly electron-withdrawing effect of the carboxyl group at C-5, the L-IdoA ring can adopt both $^1C_4$ and $^4C_1$ chair conformations, as well as a $^2S_0$ skew-boat conformation, depending on the nature of the adjacent residues (Figure 2).[11−14] The occurrence of a specific L-IdoA conformer can affect the shape of the carbohydrate helix in the linkage region, providing a sort of "signature" associated with the fit between a unique H/HS sequence and a given protein. The influence of this conformer has been assessed for the interaction of H/HS with some proteins, such as growth factors, blood coagulation factors (especially antithrombin III) and foot-and-mouth disease virus protein.[15−17]

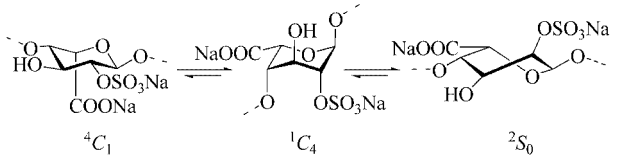


Figure 2. Conformational flexibility of the L-IdoA residues in H/HS

One could argue, therefore, that the nature of the interaction between H/HS and a given protein depends critically on the fine structure of distinct saccharide domains. Nevertheless, in only a few cases, as in the interaction with antithrombin, has the specific binding sequence been fully elucidated. It is widely accepted that the generation of the consensus sequence(s) towards the distinct proteins (usually 5−15 saccharides per single domain) occurs throughout the biosynthesis of the GAG, which starts with the formation of an initial D-GlcA-D-GlcNAc chain anchored to the core protein through a tetrasaccharide linkage region. During chain extension, distinct enzymes act in sequence, partially modifying the monosaccharide residues of the growing polymer, with each enzyme providing the substrate for the

following modification. In this way, incomplete *N*-deacetylation/*N*-sulfation, C-5 epimerization of D-GlcA, and sulfation occur at different positions, giving rise to extremely different and heterogeneous H/HS compositions. The central domains result as the most extensively sulfated regions (the so-called "regular regions") with three sulfate units per disaccharide repeating unit.[18]

The recognition of the consensus sequences towards distinct proteins is seriously hampered by the presence of the regular domains. In fact, most of the proteins, regardless of the specific epitope they recognise on the polysaccharide, easily bind H fragments derived from the central, highly sulfated regions through nonselective, ionic interactions, concealing the minimal binding sequence. On the other hand, the scarce availability of the HS polymer, which contains more-sparsely distributed sulfate units, limits the use of the more selective HS fragments in the binding studies. A further drawback is the difficulty in obtaining homogeneous H/HS oligosaccharide preparations from natural sources, which causes problems in characterization and detailed sequence definition.

The progress made in analytical methods and in H/HS chain sequencing,[19−21] as well as the crystallographic analysis of the complexes between H/HS oligosaccharides and some proteins, have added important elements to the understanding of this complex picture.[14,17,22−25] In this framework, synthetic oligosaccharides provided with tailored and well-defined sequences can greatly help in disclosing the minimal structural requirements for the interaction between H/HS domains and a cognate protein. On the other hand, the results of the binding studies provide new elements for the generation of novel synthetic sequences that can act as potent activating/inhibiting oligosaccharides. The early syntheses of H/HS fragments, which have been reviewed,[26] have focused on the preparation of the saccharide sequence interacting with antithrombin III and of some of its analogues. In this review, we present the results of synthetic efforts made during the last decade that are mainly devoted to the preparation of a new generation of H/HS analogues interacting with antithrombin III, as well as the elucidation of the basic interaction mechanisms between H/HS chains and other proteins, such as growth factors and platelet surface proteins.

2. Overview of Synthetic Approaches towards H/HS Fragments

Total synthesis of complex oligosaccharides is a multistep process requiring large amounts of monosaccharide blocks to be connected by stereoselective glycosylation reactions. Therefore, to gain insights into structure/activity relationships of H/HS fragments, efficient and versatile synthetic protocols are required for the preparation of oligomers of variable lengths. In most cases, this requirement translates into the synthesis of disaccharide building blocks composed of hexuronic acid and D-glucosamine units, with a protecting-group strategy that allows both selective *O*-sulfation and the assembly into longer structures by deblocking their reducing or nonreducing termini. In the syntheses of H/HS fragments, the bottleneck is an efficient gram-scale preparation of L-*ido* synthons, since L-idose or L-iduronic acid are not readily accessible from natural sources and their commercial costs are prohibitive. Therefore, since the early 1980s, several routes have been developed for the synthesis of L-*ido* building blocks, with most of them relying on manipulation of suitable D-*gluco* monomers.[27−35] Although some short and efficient strategies have been reported recently,[36−40] the rapid availability of significant amounts of L-*ido* building blocks still remains one of the major difficulties encountered in the synthesis of H/HS fragments.

Notwithstanding the method applied for the synthesis of L-*ido* sugars, a key issue for the synthesis of complex and polyfunctional molecules, like glycosaminoglycans, is the proper and careful choice of the protecting groups, which should meet the following requirements:

(a) The C-2 hydroxy group of the uronic acid units should bear participating protecting groups (e.g., acyl groups) to ensure the installation of 1,2-*trans* interglycosidic bonds, whereas the C-2 amino groups on D-GlcN residues should be masked or protected to allow the difficult introduction of 1,2-*cis*-linked D-glucosamine units. Typically, the nonparticipating azido group is used to suppress β-glucoside formation.

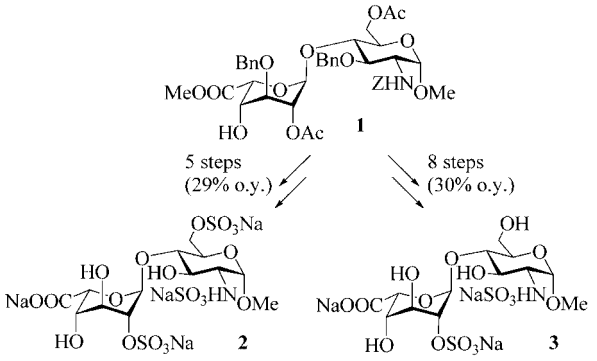
(b) The hydroxy groups destined to be *O*-sulfated in the final product and those that do not need sulfation should be differentiated by employing two orthogonal sets of temporary and permanent protecting groups, respectively.

(c) The protecting groups should be unaffected under the reaction conditions selected for the oxidation of the C-6 hydroxy groups in the generation of the uronic acids.

(d) The removal of the permanent protecting groups should be compatible with the presence of *O*- and *N*-sulfate functionalities.

A second essential point concerns the glycosylation strategies adopted in the synthesis of these types of oligosaccharides. As is detailed in the next section, the first synthetic approaches towards H fragments were developed for an efficient preparation of the pentasaccharide identified as the binding sequence for Antithrombin III. According to the state of the art in oligosaccharide synthesis at that time, the glycosylation reactions were performed using glycosyl bromides and orthoesters as donors. As new glycosylation methods were developed, more efficient glycosyl donors were employed in the synthesis of H/HS fragments, such as trichloroacetimidates, thioglycosides and vinyl glycosides, thus avoiding the use of heavy-metal salts as promoters.

The first synthesis of the regular sequence disaccharide of H was reported in 1994 by Petitou.[41] Disaccharide **2**, and its 6-*O*-unsulfated counterpart **3**, were obtained from protected **1** in five and eight steps, respectively (Scheme 1). As is shown below, small variants of this building block, with a similar array of protecting groups, have been used extensively in the synthesis of larger H/HS fragments and analogues thereof.



Scheme 1. Synthesis of the regular sequence disaccharides of heparin; Z = benzyloxycarbonyl

An approach to a complementary sequence has been introduced by our group with the synthesis of disaccharides **4** and **5** (Figure 3, a).[42] These compounds bear the D-glucosamine residue at the nonreducing end, and they allow a systematic approach to be made for the synthesis of new kinds of oligomers, such as $(GlcN\text{-}HexA)_n$, which have a reversed sequence with respect to those fragments obtained by chemical or enzymatic processing of the native polysaccharides.

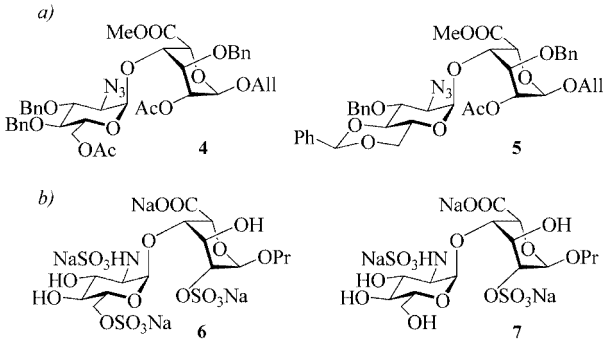
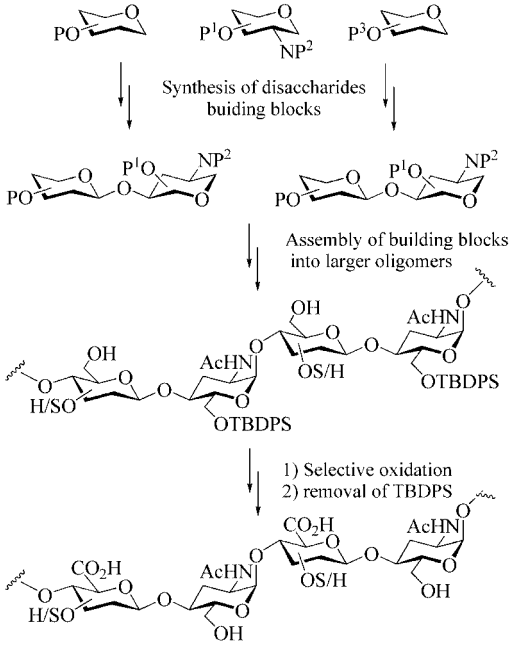


Figure 3. a) Structures of disaccharide building blocks **4** and **5** with "reversed" sequences; b) disaccharides **6** and **7**, the first two elements of this new series, were obtained from building block **4**

A second aim of our work was to determine the biological significance of the 6-*O*-sulfation of D-glucosamine in the interaction with fibroblast growth factors. Therefore, we designed a synthetic protocol suitable for obtaining all possible oligosaccharides in which the O-6 position of each D-glucosamine unit may, or may not, be sulfated. The pattern of orthogonal protecting groups in building blocks **4** and **5** allowed us to reach this goal, as is demonstrated by the synthesis of disaccharide **6** and its 6-*O*-unsulfated counterpart **7** from building block **4** (Figure 3, b), which are the first two members of this new series of oligosaccharides.

Boons' group reported a new modular approach for the synthesis of H/HS fragments, based on the concept that the enormous structural heterogeneity typical of these compounds can be reproduced by a proper assembly of nineteen disaccharide building blocks, resembling the naturally occurring disaccharide units. In a manner that is different from previous approaches, an important aspect of this strategy is the formation of uronic acids at a late stage of the synthesis, which improves the efficiency of the glycosylation steps with respect to the low glycosyl-donating properties of uronic acid derivatives. This effect was achieved by selective oxidation of the C-6 hydroxy groups of the glucosides and idosides with a catalytic amount of 2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO) and using sodium hypochlorite as co-oxidant,[43] which is a procedure that is compatible with the presence of sulfate groups. The synthetic strategy is illustrated schematically in Scheme 2 and is exemplified by the syntheses of disaccharides **8** and **9** and trisaccharide **10** (Figure 4).[44,45] First, the oligosaccharide backbone is assembled by combining different modular building blocks (Scheme 2), and thereafter all the protecting groups are removed, except the ones at C-6 of the D-glucosamine units. Next, the C-6 hydroxy groups of the glucosides (and/or idosides) are oxidised selectively to carboxylic acids and, finally, the primary hydroxy groups of D-glucosamine units are revealed to give the target fragment.



Scheme 2. A modular approach towards H/HS fragments; first, the oligosaccharide backbone is assembled by combining different building blocks, and thereafter all the protecting groups are removed, except the ones at C-6 of the D-glucosamine units; next, the C-6 hydroxy groups of the glucosides (and/or idosides) are oxidised selectively to carboxylic acids, and finally the primary hydroxy groups of D-glucosamine units are revealed to give the target fragment; TBDPS = *tert*-butyldiphenylsilyl

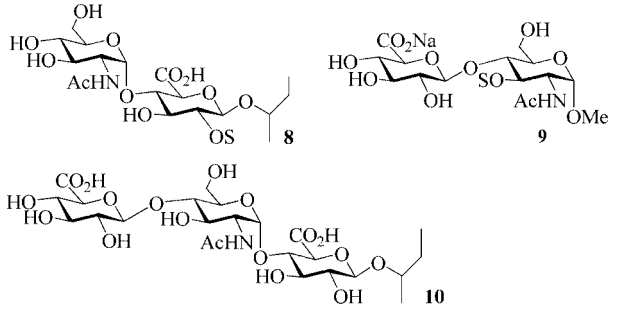
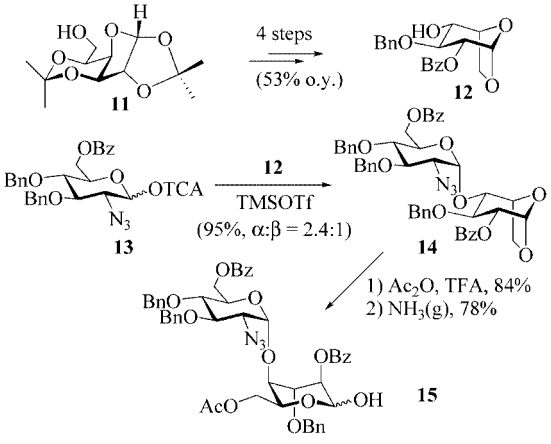


Figure 4. Structures of oligosaccharides **8**–**10**

Another essential feature for the successful application of this modular approach is the selection of a suitable set of protecting groups. The TBDPS group was used as a permanent protecting group of the C-6 position of D-glucosamine residues.

The hydroxy groups destined for *O*-sulfation in the final product were protected as levulinoyl esters, whereas an acetate or benzoate protecting group was introduced at the C-2 position to ensure the stereoselective formation of 1,2-*trans*-glycosidic linkages. Finally, benzyl ethers were used to protect the primary hydroxy groups that should remain unsulfated in the final product. The removal of all protecting groups turned out to be compatible with the presence of base- and acid-labile sulfate esters.

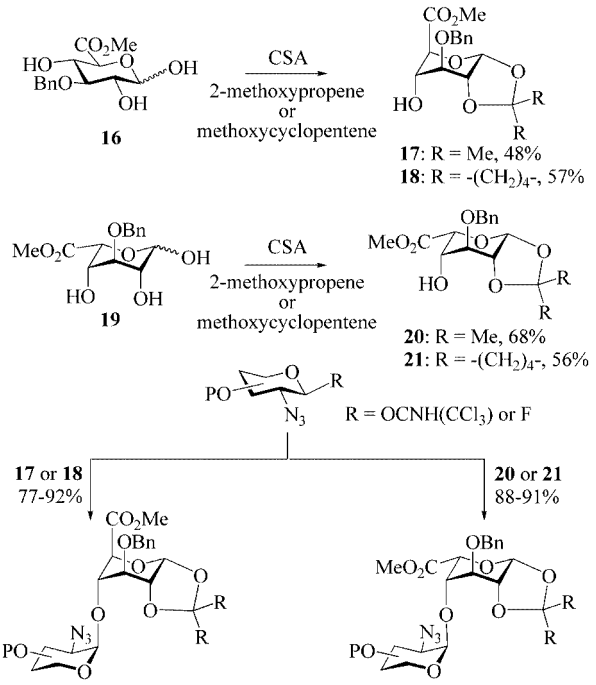
A different approach leading to disaccharide units of H/HS was described by Hung et al. and is summarised in Scheme 3.[46]



Scheme 3. Synthesis of disaccharide building block **15** from 1,2:3,5-di-*O*-isopropylidene-β-L-idofuranose **11**

1,6-Anhydro-β-L-idopyranosyl acceptor **12** was obtained in four steps from 1,2:3,5-di-*O*-isopropylidene-β-L-idofuranose (**11**)[47] and was glycosylated with the trichloroacetimidate of 2-azido-6-*O*-benzoyl-3,4-di-*O*-benzyl-2-deoxy-D-glucopyranose (**13**). Once isolated from its β-isomer, the α-linked disaccharide **14** was submitted to acetolysis of the 1,6-anhydro ring affording, after selective removal of the anomeric acetate, building block **15**, which was assembled into larger oligomers. The distribution of the protecting groups in **15** corresponds to the sulfation pattern of the regular region of H.

As mentioned above, despite many new achievements reported in this field, the stereocontrolled formation of α-D-glucosamine linkages during the assembly of H/HS backbones still remains a challenging task. Seeberger et al.[48] argued that the stereoselective installation of α-D-glucosamine linkages can be achieved by locking the $^1C_4$ conformation of the uronate acceptor. To test this hypothesis, conformationally constrained glucuronate and iduronate acceptors **17**, **18**, **20** and **21** were prepared from triols **16** and **19** by introduction of cyclic 1,2-acetals (Scheme 4). These



Scheme 4. Synthesis of α-linked disaccharides by glycosylation of conformationally $^1C_4$-constrained uronate acceptors

protecting groups ensure the presence of the $^1C_4$ conformation of the hexuronate monosaccharides.

The coupling reactions of different glucosaminyl donors with acceptors **17**, **18**, **20** and **21** invariably led to exclusive formation of the α-linked disaccharides. Taking advantage of these results, the authors synthesised a set of key building blocks (di- and trisaccharides) to be used in a highly convergent modular approach to H/HS oligosaccharides.[49] As a proof of concept, various tetrasaccharides, and even a hexasaccharide, were prepared by assembling these saccharide modules. The effect of different participating protecting groups on C-2 of the iduronic acid moieties also has been explored.

The linear backbone of H/HS oligosaccharides should make them ideal candidates for the application of solid-phase methodologies to their synthesis. Quite surprisingly, only one example has been reported in the literature.[50] The authors used a polyethylene glycol (PEG) supported solution synthesis[51,52] for the preparation of differently *O*-sulfated and *O*-methylated HS-related oligosaccharides of various lengths, up to dodecasaccharides.

Chemical synthesis of small subunits of H/HS also can be an auxiliary means to unravel details of the biosynthetic pathway of these polysaccharides, by affording helpful standards for the structural identification of natural "non-regular" fragments. Our group reported the synthesis of two disaccharides (Figure 5) containing 2-*O*- (**22**) and 3-*O*-sulfated (**23**) D-glucuronic acid residues.[53]

These compounds were submitted to reductive deamination and employed as standards to identify the precise location of the *O*-sulfate group in the D-glucuronic acid unit of disaccharides generated by degradation of HS isolated

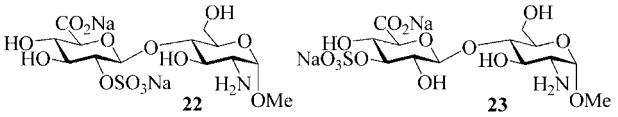


Figure 5. Structure of disaccharides **22** and **23**

from the human brain. The comparison by anion-exchange HPLC definitely revealed the occurrence of 2-*O*-sulfated D-glucuronic acid units in natural H/HS molecules.[54]

We conclude this section with a general comment on the expanding synthetic approaches towards H/HS fragments. Whilst the first pioneer syntheses followed mainly a target-oriented approach, the modular approaches proposed recently by Boons[44] and Seeberger[48] seem to be the most suitable ones for reproducing the enormous molecular diversity displayed by H/HS chains and to obtain new and more detailed insights into their recognition phenomena. In fact, the proper assembly of a number of stereoselectively constructed building blocks is an extremely promising approach for future developments, which are expected to culminate in the efficient production of complex, and even unprecedented, H/HS fragments. Moreover, these approaches could be applied to a solid-phase methodology. If so, production of libraries of biologically significant H/HS fragments are expected in the near future.

3. Synthesis of H/HS Fragments as Putative Binding Sequences towards Distinct Proteins

3.1 Interaction of H/HS with Blood Coagulation Factors

The first pharmacological use of H, which began in the 1930s, was as an antithrombotic drug in post-operative treatment. H/HS act as anticoagulants primarily through their interaction with antithrombin III (AT III), which enhances AT III mediated inhibition of blood coagulation factors, such as factor IIa (thrombin) and factor Xa, which belong to the serine protease family.

As was disclosed later, this activity is ascribed to a specific pentasaccharide sequence *DEFGH* (antithrombin

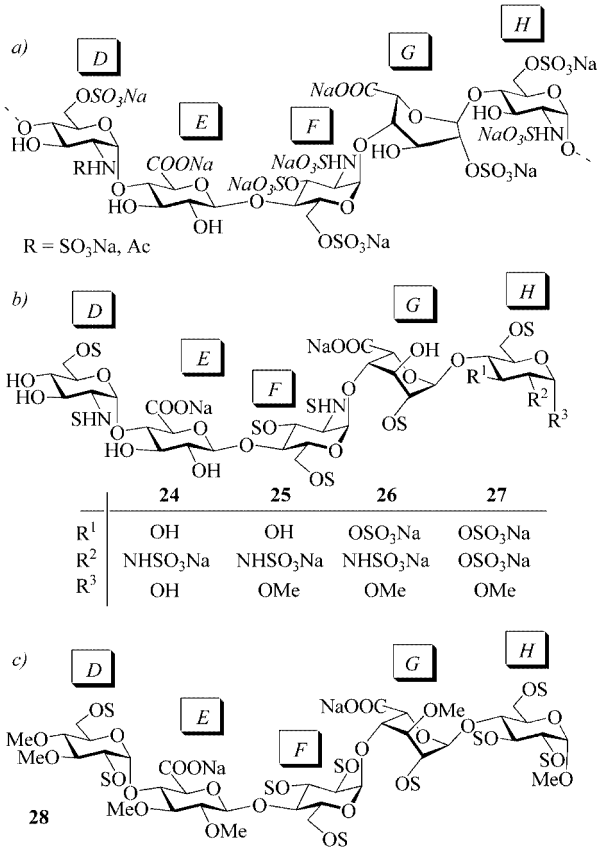


Figure 6. a) AT III binding domain (ABD) in H/HS; the charged groups mainly involved in the binding are reported in italics; unit *G* is represented in the unusual $^2S_0$ form; b) and c) some analogues of the natural ABD sequence

binding domain, ABD, Figure 6, a), which is present only in one-third of the H/HS chains, that displays a characteristic "rare" component, namely the 3-*O*-sulfated D-GlcN unit *F*.[55–58] An H/HS fragment containing the sole sequence *DEFGH* is able to interact with AT III, which accelerates the inactivation of factor Xa and other enzymes of the coagulation cascade.[15] Conversely, the AT III mediated inhibition of thrombin requires a minimum length of H/HS

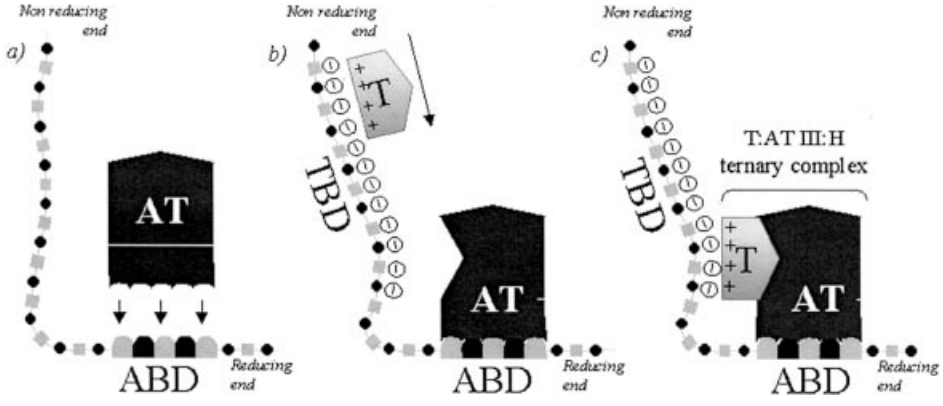


Figure 7. Heparin-mediated inhibition of thrombin by AT III; a) AT III binds to H through the interaction with the ABD pentasaccharide; b) binding to H causes a conformational change in AT III that enhances its affinity towards thrombin (T); thrombin is electrostatically attracted by the regular part of H and approaches AT III by sliding along the chain; c) AT III binds to thrombin forming a stable ternary complex T:AT III:H that inhibits the thrombin-induced coagulation cascade

chains of 14−20 saccharide units (see section 3.3), where the ABD is embedded into a long, regularly sulfated region.

Because of its relevant therapeutic implications, the mechanism for the formation of the H−AT III complex has been investigated widely. Initial binding of AT III to ABD (Figure 7, a) causes a conformational change in AT III that enhances its affinity towards thrombin (Figure 7, b).[59,60] In turn, thrombin is attracted electrostatically by the regular regions adjacent to the ABD (the so-called thrombin binding domain, TBD) and approaches AT III possibly by sliding along the chain through an unspecific, ionic interaction (Figure 7, b).[61,62] The subsequent binding of AT III with thrombin leads to the formation of a stable ternary complex T:AT III:H that inhibits the thrombin-induced coagulation cascade (Figure 7, c).

The different functional groups present on pentasaccharide *DEFGH* play distinct roles in this interaction process. Crystallographic studies have disclosed that mainly six charged groups are involved in the binding, as is shown in Figure 6 (a).[15] A number of conformational studies have been carried out on this sequence and on some synthetic analogues, such as **25** (Figure 6, b), with the aid of NMR spectroscopy[63,64] and force-field calculations.[65−67]

In particular, NMR spectroscopic data indicate the presence of the unusual $^2S_0$ skew-boat conformation as the major contributor in the conformational equilibrium of L-IdoA (unit *G*) in pentasaccharide **25**, especially when bound to AT III.[14,15,61] As is detailed below, activity studies performed on synthetic oligosaccharides revealed that, with respect to the $^1C_4$ and $^4C_1$ conformations, the $^2S_0$ conformation is able to interact more effectively with AT III and, thus, it represents the conformer responsible for the biological activity.[14,68]

3.2 Synthesis of Fragments with Exclusive Anti-Factor Xa Activity

Since its identification as the ABD, in the early 1980s, many synthetic approaches have been directed towards pentasaccharide *DEFGH*, especially the variant in which the unit *D* is *N*-sulfated (**24**, Figure 6, b).[26,69,70] However, the simultaneous generation of free amino groups and of the anomeric hydroxy group during the synthesis of **24** gave rise to undesired side reactions, such as partial oligomerization of the pentasaccharide. Therefore, the corresponding stable methylglycoside **25** (Figure 6, b) was synthesised.[71,72] The anti-Xa activity displayed by **25** was 700 U mg^{-1}, and it was used as the "reference pentasaccharide" in subsequent studies aimed at optimising the binding affinity for AT III through the synthesis of various potent analogues.

Various analogues were designed to better define the structure/activity relationships of "natural" pentasaccharide **25**. Their ability toward the activation of AT III was compared to that of the reference pentasaccharide, and provided new information on various aspects (sulfation pattern, selectivity, binding requirements) of the interaction between the pentasaccharide domain of H/HS and AT III. The results of these studies are summarised as follows:

(a) The binding sequence *DEFGH* bears two essential *O*-sulfate groups (units *D* and *F*), two essential *N*-sulfate groups (units *F* and *H*), and two essential carboxylate groups (hexuronic acids *E* and *G*) (Figure 6, a).

(b) The sulfation of the 3-hydroxy group of unit *H* improves the biological activity, whereas sulfation of other hydroxy groups leads to a drop in activity, up to the persulfated pentasaccharide which is totally inactive.

(c) The orientation of the key charged groups is important, since epimers show reduced activity.

(d) The type of charge is crucial. The replacement of essential sulfate or carboxylate groups with other charged groups (e.g., phosphates) leads to a substantial loss of activity.

(e) The presence of the pyranosidic rings of the hexuronic acid moieties is essential for specific recognition and activation of AT III, since the flexible open-chain analogues are almost inactive.

After screening the analogues of this class, compound **26** (Figure 6, b) was found to be the most active relative to the reference pentasaccharide (the measured anti-Xa activity for **26** was 1250 U mg^{-1}).

For more details on the syntheses of the analogues mentioned above, the reader is referred to ref.[26] and references cited therein.

Although they are attractive as lead compounds for drug development, the chemical complexity of glycosaminoglycan analogues, such as those described so far, renders their large-scale synthesis difficult. Therefore, in the early 1990s, the synthetic community began to devise the synthesis of simplified analogues of the "natural" pentasaccharide **25**.

A key breakthrough in this field was the design of "non"-glycosaminoglycan analogues, in which some *N*-sulfate groups are replaced by *O*-sulfate groups and some free hydroxy groups are replaced by *O*-alkyl (mostly *O*-methyl) ethers. A first insight in this direction was derived from the observation that pentasaccharide **27** (Figure 6, b), containing a D-glucose moiety instead of a D-glucosamine unit, was as active as analogue **26**.

Starting from compound **27**, the extent of chemical modification into putative pentasaccharide AT III ligands was gradually increased. First, several partially and fully *O*-methylated derivatives were synthesised[73] and they displayed very similar AT III affinity in comparison with the corresponding non-methylated pentasaccharides. Soon after this finding, various non-glycosaminoglycan analogues, where all the *N*-sulfate groups are replaced by *O*-sulfate groups and all free hydroxy groups are *O*-alkylated, were prepared and tested,[74] and these studies demonstrated that none of these structural modifications decreased the biological activity. On the contrary, fully *O*-methylated pentasaccharide **28**[75] (Figure 6, c) even exhibited a somewhat higher anti-Xa activity than the analogue **26** (1323 vs. 1250 U mg^{-1}). The synthesis of this type of analogue is greatly simplified with respect to that of the H/HS fragments:

(a) The *O*-methyl ethers are introduced at an early stage of the synthesis. Therefore, no further differentiation be-

tween two orthogonal sets of protecting groups is required and both acyl esters and benzyl ethers can be used to protect the hydroxy groups destined to be *O*-sulfated.

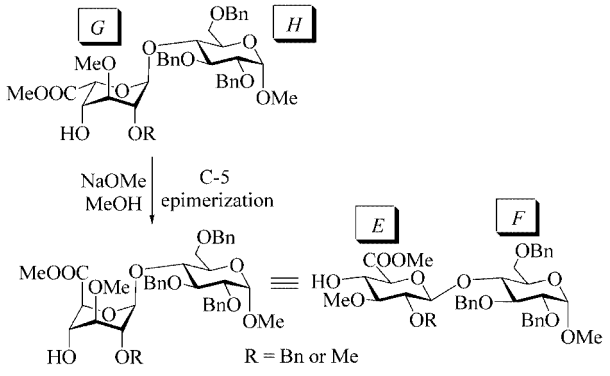
(b) No α-coupled D-glucosamines need to be introduced, which thus avoids elaborate synthetic routes for the preparation of azide-containing building blocks.

(c) Many more chemical modifications are feasible, which further simplifies the synthesis (e.g., carboxylates can be protected as benzyl esters and deprotected in a one-pot procedure during a hydrogenolysis step).

(d) A selective *N*-sulfation is not required at the end of the synthesis.

The surprisingly high anti-Xa activities of *O*-methylated non-glycosaminoglycan analogues suggests that neither the hydroxy groups nor the NH−sulfate groups of the pentasaccharide act as hydrogen bond donors in the interaction process with AT III.

A further shortening of the synthetic route towards these *O*-methylated non-glycosaminoglycan analogues turned out to be the synthesis of derivatives with a pseudo-alternating sequence, i.e. where the disaccharide fragments *EF* and *GH* have the same substitution pattern. The only remaining difference between the disaccharide moieties is the opposite configuration at C-5 of the hexuronic acid units. Therefore, a route was developed to transform a suitably protected disaccharide *GH* into its counterpart *EF* by base-catalysed epimerization (Scheme 5).



Scheme 5. Synthesis of derivatives with a pseudo-alternating sequence: a suitably protected disaccharide *GH* is transformed into its counterpart *EF* by base-catalysed epimerization

Various pentasaccharide analogues have been synthesised according to the strategy above [76,77] (Figure 8).

Compounds **29**−**31** and **32**−**34** belong to different series. Compounds **29**−**31** (series I) share the same *EFGH* block containing both *E* and *G* units that are 2-*O*-sulfated. Compounds **32**−**34** also have the same *EFGH* block, in which the *E* and *G* moieties are 2-*O*-methylated. Moreover, in each series the *EFGH* tetrasaccharide is coupled with three different *D* units varying in their sulfation/methylation patterns. The AT III binding affinities and anti-Xa activities of compounds **29**−**34** were evaluated. Despite the presence of a 2-*O*-methyl group instead of an important (albeit nonessential) 2-*O*-sulfate group in unit *G*, series II showed higher anti-Xa activities than series I. In particular, pentasaccharide **32**, containing the minimal number of *O*-sulfate

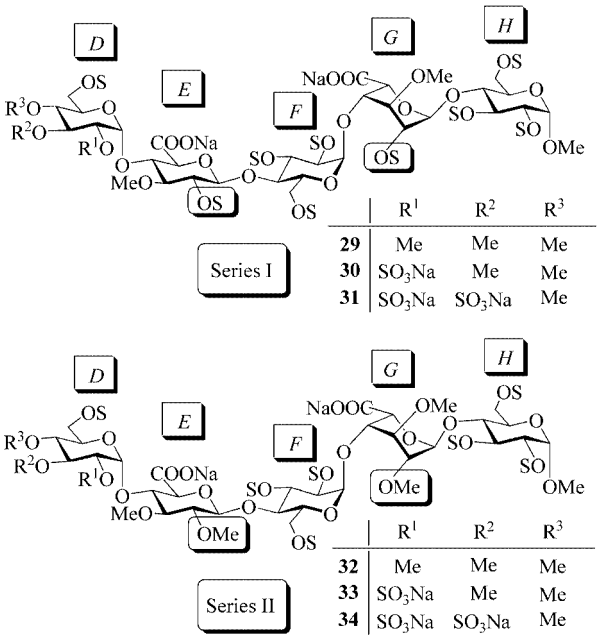
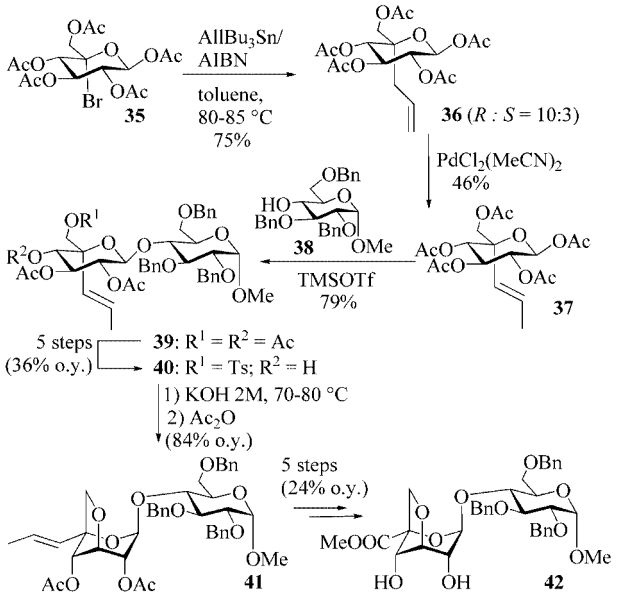


Figure 8. Structures of two series of pentasaccharides with pseudo-alternating sequences

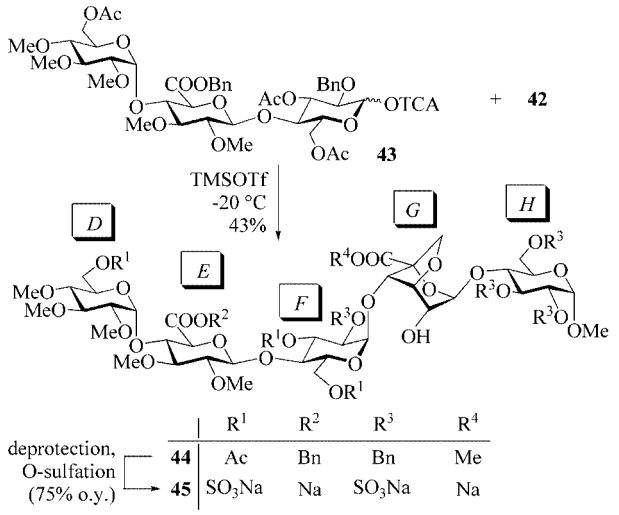
groups in the above series, exhibited the highest anti-Xa activity (1611 U mg^{-1}) and the longest in vivo residence time in rats ($T_{1/2}$ of elimination: 10.9 h) and it is the most potent analogue of **25** found so far. To explain these results, the authors claim that the loss of electrostatic interactions may be compensated by favourable hydrophobic or van der Waals interactions between the methylated pentasaccharide and the apolar surface amino acid residues of AT III.

To acquire as many clues as possible on the structure/activity relationship and to improve its anticoagulant and antithrombotic properties, so far more than fifty diverse analogues of pentasaccharide **25** have been synthesised and tested. The unique conformational properties of L-iduronic acid stimulated various studies aimed at determining to what extent, if any, they influence the molecular recognition between AT III and the ABD pentasaccharide. The main goal was to establish whether a precise conformation of L-iduronic acid is required or, alternatively, whether a conformational switch from one conformer to another might be necessary for the binding and activation of AT III. This question was cleverly answered by two different research groups. They deduced that more precise information on conformation/activity relationships of ABD pentasaccharide could be acquired by locking the three main conformations of the L-iduronic acid unit. From a synthetic point of view, this information resulted from the first synthesis of pentasaccharide **45**, where the $^1C_4$ conformation of the L-iduronic acid unit (*G*) is locked by a covalent bridge between the C-3 and C-5 atoms.[78] The approach towards pentasaccharide **45** is illustrated in Schemes 6 and 7.

The key disaccharide acceptor **42**, containing the conformationally constrained L-iduronate unit, was synthesised from bromide **35** through radical C-5 *C*-allylation, followed by olefin isomerization and coupling with alcohol **38**



Scheme 6. Synthesis of disaccharide intermediate **42**



Scheme 7. Synthesis of pentasaccharide **45** from intermediates **42** and **43**

(Scheme 6). Manipulation of the protecting groups gave the monotosylated derivative **40**, which, upon treatment with aqueous potassium hydroxide at 70−80 °C, was saponified and intramolecularly substituted to afford, after acetylation, the required 3′,6′-anhydro-bridged compound **41**. Finally, oxidative cleavage of the C-5′ propenyl group, followed by esterification and deacetylation, yielded **42**. Glycosylation of **42** with donor **43** (Scheme 7) furnished pentasaccharide **44** with good selectivity. Hydrogenolysis of the benzyl ethers and saponification, followed by *O*-sulfation of the remaining hydroxy groups, eventually gave pentasaccharide **45** containing the "pure" $^1C_4$ conformer of the L-iduronate residue. Since compound **45** showed very low activity of 65 U mg^{-1} in an anti-Xa assay, the authors concluded that the $^1C_4$ conformation of unit *G* was not the active one.

More recently, a systematic approach towards the understanding of the conformational effects into the AT III mediated anti-Xa activity of H/HS was developed by Petitou, Sinaÿ et al., who performed the total synthesis of three pentasaccharides in which the single L-iduronic residue is conformationally locked, either in the $^1C_4$, $^4C_1$ or $^2S_0$ form.[79,80] Pentasaccharide **32** was selected as the reference model for the biological activity. Conformational locking of L-iduronate was achieved as shown in Figure 9 (a).

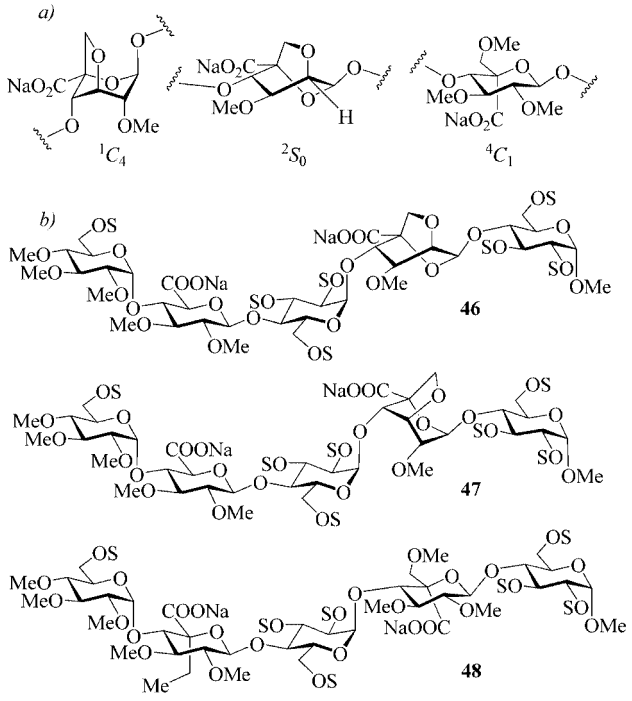
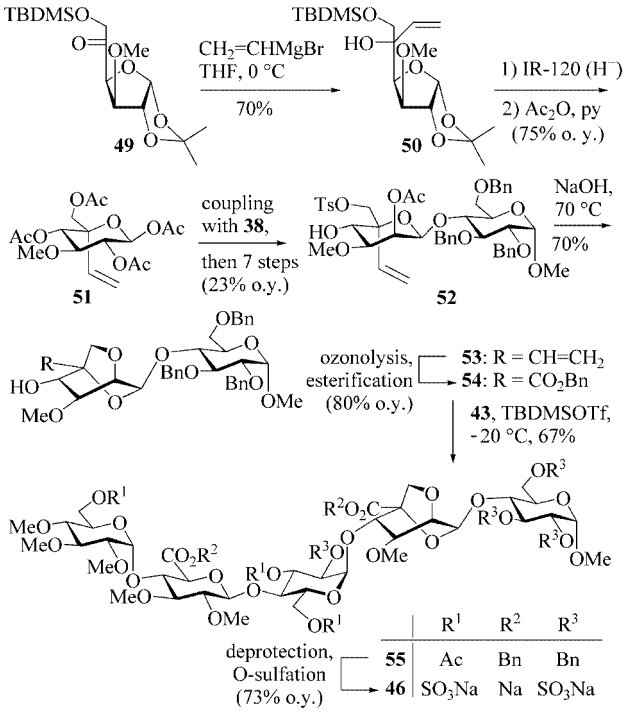


Figure 9. a) Locked $^1C_4$, $^2S_0$ and $^4C_1$ conformers of L-IdoA; b) structures of pentasaccharides **46**, **47** and **48** containing the locked $^2S_0$, $^1C_4$ and $^4C_1$ conformers, respectively, of L-IdoA

The $^1C_4$ and $^2S_0$ conformers were locked by covalently bridging the C-3/C-5 and C-2/C-5 ring atoms, respectively. The $^4C_1$ chair was frozen by introducing a methoxymethyl substituent at C-5. The authors were confident that such substitution would shift the iduronate ring towards the $^4C_1$ conformation, which is the preferred one adopted by non-constrained monosaccharides bearing a hydrocarbon chain instead of a hydrogen atom at C-5. This prediction turned out to be correct at the end of the synthesis. The introduction of conformationally constrained L-iduronate residues into pentasaccharide **32** led to mimics **46**, **47**, and **48** (Figure 9, b).[79,80] The synthesis of **46** is illustrated in Scheme 8.

In comparison with the previously described synthesis of **45**, the introduction of the *C*-vinyl appendage at C-5 into the intermediate **51** was achieved more efficiently through stereoselective addition of vinylmagnesium bromide to ketone **49** under chelation-controlled conditions. Further elaboration of **51**, including the coupling with **38** and the inversion of the configuration at C-2′ by Swern oxidation followed by reduction with $LiEt_3BH$, produced the monotosylated intermediate **52**, and then a two-atom bridge between C-2′ and C-5′ was installed to give **53**. Oxidative



Scheme 8. Synthesis of conformationally constrained pentasaccharide **46**

cleavage of the C−C double bond converted **53** into **54**, which was coupled with the imidate **43** to give protected pentasaccharide **55**, and eventually **46**.[79]

The same strategy was applied to the synthesis of mimic **47** by repeating the reaction sequence described above using the 3-*O*-allylated counterpart of ketone **49**. Usual chemical transformations and protecting-group manipulations eventually afforded pentasaccharide **47**.[80]

Both syntheses of **46** and **47** were based on key intermediates containing the structural element **A** (Figure 10) equipped with an equatorially oriented hydroxymethyl group and an axially oriented vinyl group.

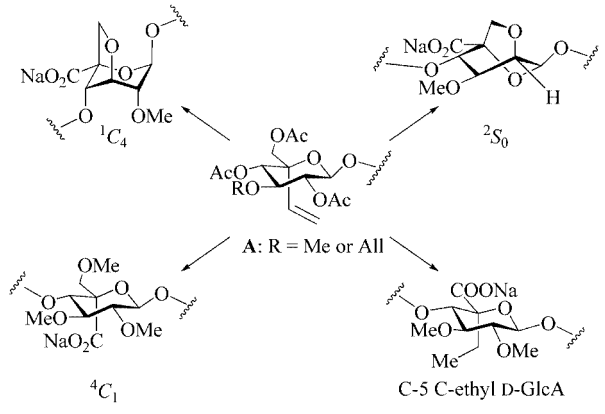
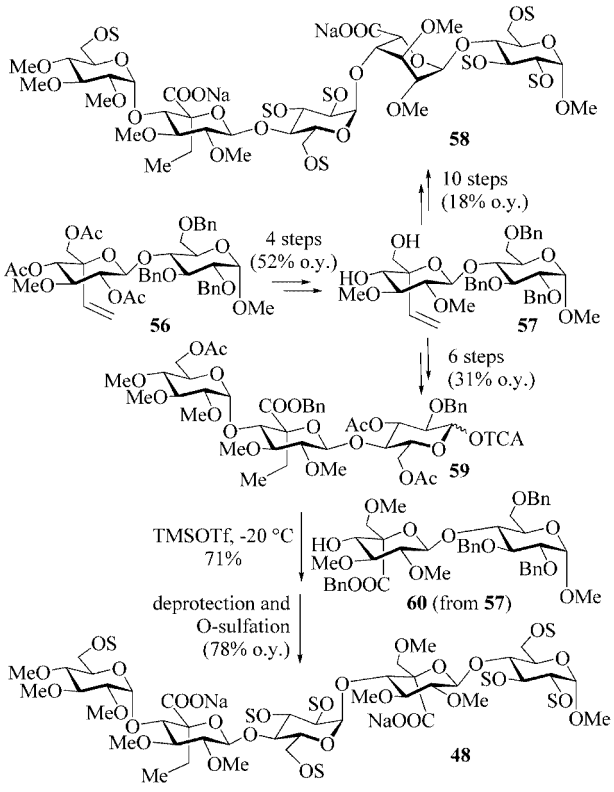


Figure 10. Element **A** as a precursor of conformationally constrained L-iduronate moieties

This compound is also a suitable precursor of the frozen $^4C_1$ conformer of L-iduronate in the synthesis of mimic **48**, through ozonolysis of the double bond followed by oxidation of the resulting aldehyde. In addition, an intermediate such as **A** can also be converted into a D-glucuronic acid derivative by hydrogenation of the double bond and oxidation of the primary alcohol (Figure 10). The versatility of intermediate **A** suggested a converging synthesis of mimic **48**, where both the hexuronic acid residues (units *E* and *G*) are obtained from the same precursor. However, the presence of the ethyl group at C-5 of D-glucuronic acid, in principle, could influence the interaction between the pentasaccharide and AT III. To rule out this hypothesis, pentasaccharide **58**, containing an ethyl group at C-5 of unit *E* and a flexible L-iduronate residue, was synthesised via disaccharide **57** obtained from **56** (Scheme 9) and its anti-Xa activity was evaluated. Finally, the synthesis of pentasaccharide **48** was accomplished (Scheme 9) by employing the same intermediate **57** utilised in the preparation of **58**.[80]



Scheme 9. Synthesis of conformationally constrained pentasaccharide **48** and its flexible counterpart, pentasaccharide **58**

The role of the L-iduronic acid conformation in the interaction of these H/HS mimetics with AT III was investigated by comparing their biological activities with that of the reference **32**. Pentasaccharide **46** (containing the $^2S_0$ conformer of unit *G*) showed high affinity for AT III, strongly inhibiting the blood coagulation factor Xa (1073 U mg^{-1}). In contrast, pentasaccharides **47** ($^1C_4$ form of unit *G*) and **48** ($^4C_1$ form of unit *G*) exhibited only low activities (43 and 115 U mg^{-1}, respectively). Finally, compound **58** (flexible unit *G*) was found to have anti-Xa activity very close to that of reference **32** (1345 U mg^{-1}), which thus indicates that the low activity of **48** results only from a conformational feature of unit *G*, and not from the presence of the ethyl

group at C-5 of unit *E*. In conclusion, this result was the first strong indication that the L-iduronic acid unit of ABD pentasaccharides (like **32**) adopts the $^2S_0$ conformation when bound to AT III and this unusual conformer governs the antithrombotic activity of H/HS.

These results had already been suggested by previous studies performed on pentasaccharide **61** (Figure 11), the counterpart of **25** containing a 3-deoxy-L-iduronic acid unit *G*.[81] ^{1}H NMR spectroscopic analysis of **61** showed that this structural modification shifted the conformational equilibrium towards the $^1C_4$ chair. This chair became the predominant conformer of unit *G* (65%), with the $^2S_0$ form also present, but only in 24% abundance. In contrast, the $^2S_0$ conformer contributes to the extent of 64% in **25**, which has a much higher affinity for AT III relative to **61**. The authors concluded that this result was a clear effect of the predominance of the $^2S_0$ conformer in the reference compound.

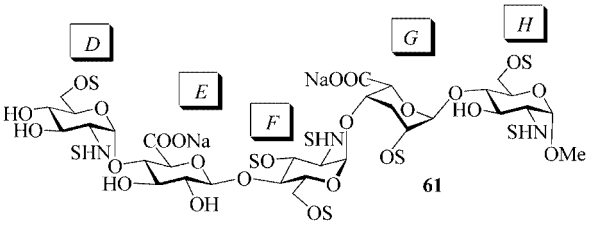
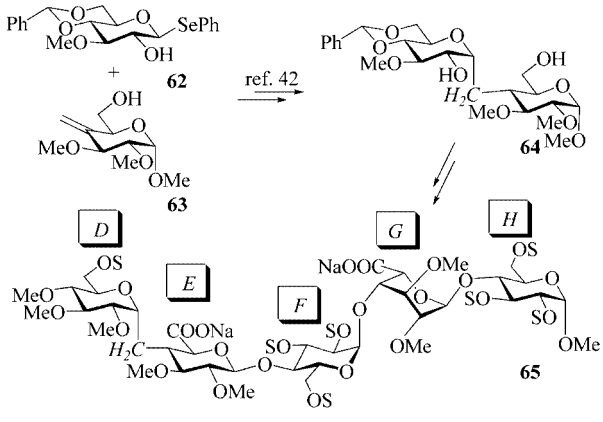


Figure 11. Structure of pentasaccharide **61** containing a 3-deoxy-L-iduronic acid unit *G*

Another kind of analogue of the ABD pentasaccharide was obtained by replacing the *O*-glycosidic bond between units *D* and *E* of the highly active mimic **32** with a *C*-glycosidic linkage.[82,83] The introduction of the *C*-interglycosidic bond to give disaccharide **64** (Scheme 10) was achieved through a methodology developed by the authors based on the radical coupling between selenophenyl glycoside **62** and *exo*-methylene derivative **63**.[84]



Scheme 10. Synthesis of pentasaccharide **65** containing a *C*-glycosidic linkage between the *D* and *E* units

Elongation of intermediate **64** eventually gave pentasaccharide **65**. The comparison between **65** and **32** did not show a significant difference in their biological properties, indicating that the substitution of an *O*-glycosidic bond by a *C*-glycosidic bond hardly affects the affinity for AT III and the anti-Xa activity.

3.3 Synthesis of Fragments with the Full Anticoagulant Properties of Heparin

Many chemical syntheses of various analogues of the pentasaccharide sequence *DEFGH* have been reported (see above), leading to highly potent artificial ABDs endowed with strong, but exclusive, anti-Xa activities. However, none of these compounds is able to inhibit thrombin. In contrast with the interaction of ABD with AT III, thrombin binding is only a matter of electrostatic interaction between the protein and the polyanionic chain of H/HS (Figure 7). Indeed, the interaction of the TBD with thrombin is about three orders of magnitude weaker than that of ABD with AT III. As detailed above, thrombin is inhibited through a template mechanism. The electrostatic attraction leads thrombin to the collision with H-bound AT III. Therefore, to constitute the thrombin:AT III:H ternary complex, a longer H chain is required, comprising between 14 and 20 saccharide units. Moreover, it has been reported that various side effects are associated with heparinotherapy, especially when employed in long-term treatments. The ability of H to interact with a multitude of different proteins may lead, for example, to heparin-induced thrombocytopaenia (HIT),[85] a side effect occurring in ca. 3% of H-treated patients, which derives from unwanted interactions with platelet factor 4 (PF4), and to haemorrhages.[86] Therefore, an ideal candidate for development of more-potent anticoagulant drugs should be devoid of these side effects.

On these premises, obtaining an antithrombotic molecule with the full anticoagulant properties of H (i.e., one displaying dual anti-Xa and anti-thrombin activity and at the same time being free of undesired interactions with blood and vessel components) is a highly attractive goal. The mechanistic considerations above suggest that an oligosaccharide molecule able to mimic the full anticoagulant properties of H should contain both an ABD and a suitable TBD, to give a sequence ranging from 14 to 20 saccharide residues.[87] Such an oligosaccharide structure could be obtained by connecting ABD and TBD through a spacer (saccharidic or not) that is long enough to accommodate both AT III and thrombin in a ternary complex. Three main questions arise for the design of this type of compound: (a) What is the correct location of the TBD along the oligosaccharide chain to induce thrombin inhibition? In principle, there are two possible ways to elongate the ABD with a TBD (either at the reducing or nonreducing end, see Figure 12, a, b). (b) What is the nature of the spacer (linear, flexible or rigid; charged or neutral) required for the connection between ABD and TBD? (c) What are the minimal structural requirements of the TBD (as those of the ABD have been previously established)?

To address the first issue, a three-dimensional model of the ternary complex was constructed[61] to predict the correct relative orientation of ABD and TBD along the H chain. The analysis of this model strongly suggested that the TBD should be attached through the linker at the non-

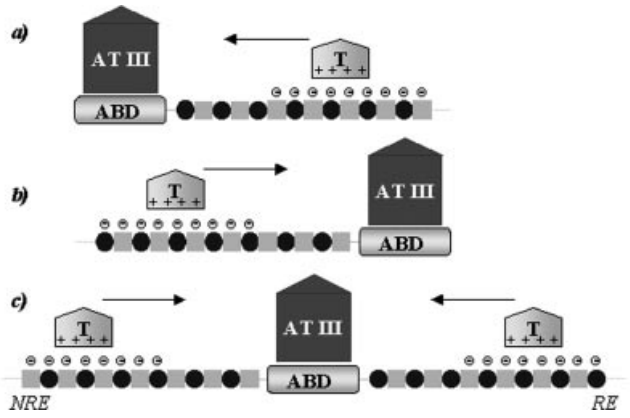


Figure 12. Possible relative arrangements of the TBD and ABD; NRE: nonreducing end; RE: reducing end

reducing end of the ABD (Figure 12, b). The definitive experimental proof of this assumption came only recently,[88] and is described below. Additionally, the same model revealed that the saccharidic portion, acting as a linker between the TBD and the ABD, does not participate in any interaction with the two proteins. Therefore, a first series of compounds were synthesised that contain a linear, flexible and neutral molecular spacer connected at the nonreducing end of an ABD and bearing a TBD at the opposite side. In all elements of this series, a neutral oligoethylene glycol type of spacer was utilised that comprises approximately 50 atoms (corresponding to a chain of about 8−10 monosaccharide units). Various high-affinity mimics of pentasaccharide **25** were included as ABDs, whereas a systematic investigation was carried out on various types of putative TBDs.

The general strategy consists of four key steps (Figure 13):

(a) A suitably protected glycosyl donor, containing a first part of the spacer (12 atoms, terminating with a protected or masked amino group) attached to its nonreducing end (4-OH), is condensed with a tetrasaccharide acceptor to give, after unmasking the free amino group, the tethered ABD pentasaccharide (Figure 13, a).

(b) The linker is elongated by attaching the bifunctional molecule **66** through the free amino group, giving rise to a longer spacer terminating with a thioacetyl group (Figure 13, b).

(c) A second portion of the spacer, containing a terminal amino group, must be introduced into the selected TBD (Figure 13, c).

(d) Finally, the equipped TBD and ABD moieties are condensed in two steps through the thiophilic linker sulfosuccinimidyl 4-(iodoacetylamino)benzoate (sulfo-SIAB) (Figure 13 d).

First, a number of differently sulfated saccharidic TBDs were explored. Because of the nonspecific nature of the interaction between thrombin and H, in principle any negatively charged oligosaccharide could act as a TBD, provided

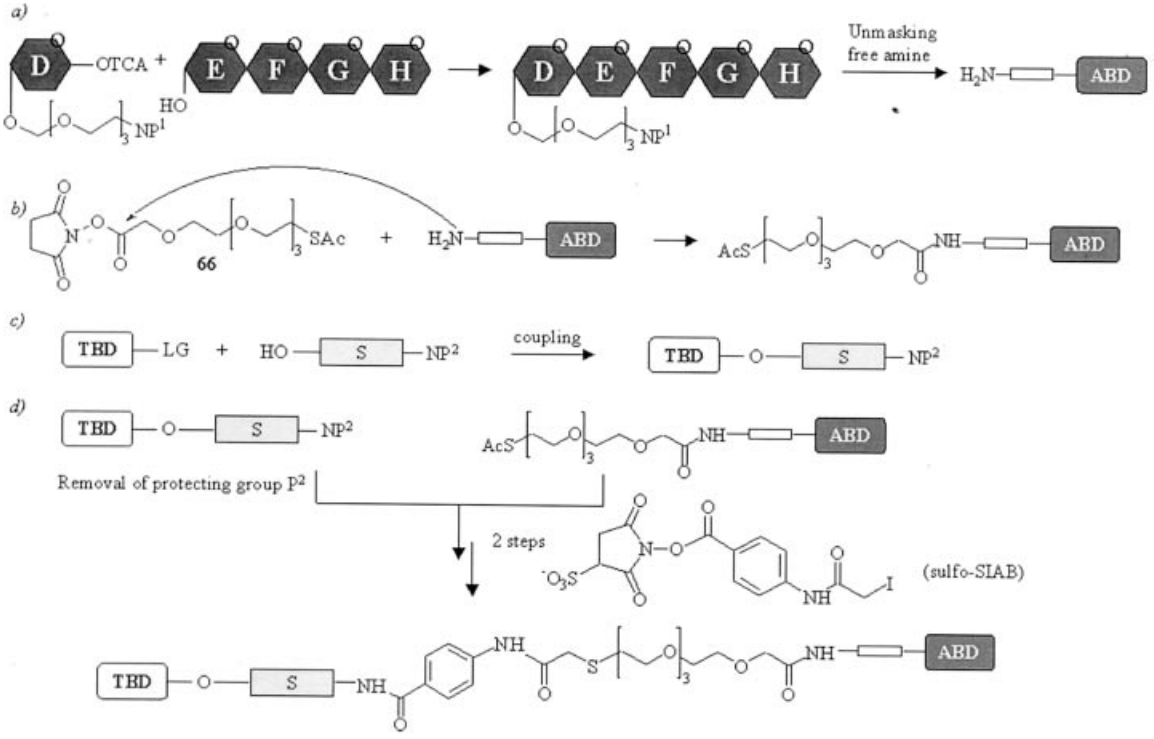
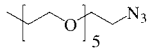
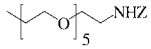
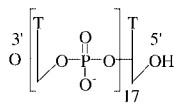
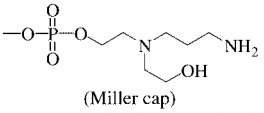


Figure 13. Synthesis of heparin-like conjugates with dual antithrombotic activity; a) glycosyl donor *D*, containing a first part of the spacer attached to its nonreducing end, is condensed with tetrasaccharide acceptor *EFGH* to give the ABD pentasaccharide; the protecting group at the nitrogen atom is then removed; b) the spacer is elongated by attaching bifunctional molecule **66**, giving rise to a longer arm terminating with a thioacetyl group; c) a second portion of the spacer (S, see Table 1), containing a terminal protected NH_2 functionality, is connected to the selected TBD (see Table 1); d) the equipped TBD and ABD moieties are condensed in two steps through the thiophilic linker sulfo-SIAB

Table 1. Conjugates **67**−**85** containing a flexible spacer bridging the ABD and the TBD and their relative anti-thrombin activities (see Figure 13)

Entry	Compd.	TBD (number of sulfates/phosphates)		Spacer length (atoms)	S	ABD	Anti-IIa activity ($U \cdot mg^{-1}$)
1	**67**	sulfated sugars	cellobiose (7-S)	53		**32**	10
2	**68**		maltotriose (10-S)	53		**32**	64
3	**69**		maltopentaose (16-S)	56		**32**	330
4	**70**		maltopentaose (7-S)	54		**32**	14
5	**71**		maltopentaose (10-S)	18		**32**	1
6	**72**		maltopentaose (10-S)	32		**32**	15
7	**73**		maltopentaose (10-S)	46		**32**	20
8	**74**		maltopentaose (10-S)	59		**32**	120
9	**75**		ABD **32** (7-S)	53		**32**	14
10	**76**		ABD **34** (9-S)	53		**34**	36
11	**77**		ABD **31** (11-S)	53		**31**	160
12	**78**		DS tetrasaccharide (4-S)	53		**32**	2
13	**79**		DS tetrasaccharide (5-S)	53		**32**	10
14	**80**		H tetrasaccharide	53		**32**	10
15	**81**	phosphorylated sugars	cellobiose (7-P)	53		**32**	22
16	**82**		cellobiose (7-P)	53		**32**	5
17	**83**		maltotriose (10-P)	53		**32**	167
18	**84**	octadecathymidilate		45	 (Miller cap)	**31**	5
19	**85**	NAPAP (see Figure 14)		2	–	**31**	0.35 μm[a]

[a] The IC_{50} value is reported.

it fulfils the minimal requirements for binding the protein. Therefore, short, persulfated oligosaccharides, such as cellobiose, maltotriose and maltopentaose were examined[87,89] (Table 1, Entries 1−3) using pentasaccharide **32** as the ABD. As expected, the three conjugates **67**−**69** showed very similar anti-Xa activities, this property being dependent exclusively on the structure of the ABD, and growing anti-thrombin activity from **67** (10 U mg^{-1}) to **69** (330 U mg^{-1}). These preliminary results show that the rigid and charged H chain between TBD and ABD is not an essential requirement for thrombin inhibition. In addition, through the synthesis of conjugate **70** (Entry 4) containing a heptasulfated maltopentaosyl TBD, it was demonstrated that the highest activity of conjugate **69** is due to the number of sulfate groups rather than the number of monosaccharide units: compound **70** showed almost the same anti-thrombin activity as **67** (14 U mg^{-1}).

The effect of the spacer length was also examined (Table 1, Entries 5−8). As anticipated, the minimal distance between the TBD and the ABD to have a substantial anti-thrombin activity is around 50 atoms, corresponding to 8−10 saccharidic units. The preparation of symmetric conjugates containing the same domain, that are able to bind both AT III on one end and thrombin on the opposite terminus, is an attractive task. Therefore, the ABD pentasaccharides **32** (7 sulfates), **34** (9 sulfates) and **31** (11 sulfates) were tethered to give conjugates **75**−**77**, respectively (Entries 9−11). Once more, it was observed that the anti-thrombin activity increases with the number of sulfate groups, from **75** to **77**. A further demonstration of the aspecific nature of the interaction between H and thrombin was achieved with the synthesis and biological evaluation of three new conjugates, namely compounds **78**−**80**, that include pentasaccharide **32** as the ABD (Table 1, Entries 12−14).[90] Whereas the TBDs in compounds **78** and **79** are tetrasaccharides structurally related to dermatan sulfate (DS), a member of the GAG family containing galactose instead of hexosamine units, the TBD in **80** is a tetrasaccharide very close to the "regular region" of H, with 2,6-di-*O*-sulfo-D-glucose in place of D-glucosamine. Their anti-thrombin activity was compared with that of conjugates **67** and **68**. Compounds **79** and **80** showed the same activity of **67**, much lower than **68**, whereas **78** was essentially inactive. This observation is a confirmation that the negative charge density, rather than its distribution or the carbohydrate structure, is the predominant factor in thrombin inhibitory activity.

The effect of the type of charge was also explored by synthesising conjugates **81**−**83** containing perphosphorylated TBDs tethered to the high-affinity ABD **32** (Entries 15−17). Perphosphorylated cellobiose (14 negative charges) and maltotriose (20 negative charges) were included as TBDs into conjugates **81** and **83**, whereas cellobiose containing seven isopropyl phosphate groups (7 negative charges) was used in **82**.[91] The obtained TBDs were linked at the nonreducing end of the ABD using the technique described above and the thrombin inhibition of the conjugates was compared with that of **67** and **68**. As expected, the doubled charge density of **81** (with respect to **67**) and **83** (with respect to **68**) induced a doubling of their anti-thrombin activity. On the contrary, conjugate **82** showed very low activity, probably because of steric hindrance of the isopropyl groups hampering an appropriate interaction with thrombin. These results clearly indicate that the struc-

ture/activity relationships of the TBD and the ABD are very different. In contrast to the ABD−AT III interaction, the type of charge is not a crucial factor, provided that a sufficiently high charge density is reached. Furthermore, whereas in the ABD−AT III interaction the introduction of alkyl groups led to a strong enhancement of the binding affinity (see *O*-methylated analogues of pentasaccharide **25**), the presence of the isopropyl groups in conjugate **82** caused a drop in its biological activity.

Two examples of non-carbohydrate TBDs were also reported. In the first one, an oligonucleotide, the readily accessible octadecathymidylate **84**, was used as a TBD (Entry 18).[92] Compound **84** was prepared using an automated DNA synthesizer and contained at its 3′-position an 2-[(3-aminopropyl)(2-hydroxyethyl)amino]ethyl phosphate moiety (the "Miller cap")[93] as an arm for the connection, through sulfo-SIAB, to the spacer-endowed ABD (pentasaccharide **31** was used in this case). The obtained conjugate **84** displayed only a very low anti-thrombin activity (5 U mg^{-1}), probably because of the modest charge density of the oligonucleotide.

In another experiment, a derivative of [*N*-(2-naphthylsulfonyl)glycyl-(D)-{4-[amino(imino)methyl]phenyl}alanyl]piperidine(NAPAP) was conjugated as a TBD to the ABD pentasaccharide **31**, giving compound **85** (Figure 14 and Entry 19 in Table 1).[94] NAPAP is known as a potent inhibitor of thrombin that acts directly on its active site. Because of this unique feature, the authors hoped that conjugate **85** could exhibit dual anti-Xa and anti-thrombin activity through a different mechanism, where the binding and inhibition of the two blood coagulation factors are two independent events (in other words, the inhibition of thrombin is not mediated by activated AT III). Actually, conjugate **85** elicited both high anti-thrombin and AT III-mediated anti-Xa activity. It is noteworthy that experiments in vivo showed that there is a kind of synergy between the TBD (NAPAP) and the ABD, since compound **85** is a stronger inhibitor than a combination of the free pentasaccharide **31** and NAPAP.

All glycoconjugates falling in the class described so far, sharing a flexible polyethylene glycol type of spacer bridging the AT III and thrombin binding domains, elicited full anticoagulant H activity. However, they also possess some typical drawbacks of H, such as neutralization by PF4. It

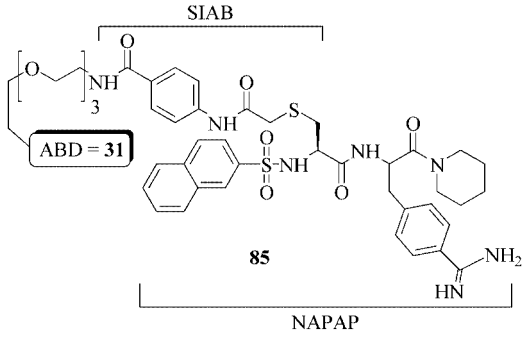


Figure 14. Structure of non-carbohydrate TBD conjugate **85**

has been established that the interaction of H and PF4 depends on two parameters: neutralization is enhanced upon increasing the size and the charge density of the sulfated oligosaccharide. In addition, the flexibility of the sugar chain also has been suggested as a possible cause of this undesired interaction. As already mentioned, the absence of side effects is a prerequisite for an antithrombotic molecule candidate for drug development. Therefore, a second class of synthetic ABD−TBD conjugates was developed containing rigid saccharidic spacers.[62] In a first approach towards these molecules, sulfated oligosaccharides were proposed composing of a continuum of ABDs, ranging from 10 to 20 sugar units.[95] Such a sequence was designed on the basis of the following considerations:

(a) Since the interaction of H and thrombin is merely electrostatic, a negatively charged ABD could also serve as a TBD, and this feature should simplify the synthetic route.

(b) The problem of the relative position of the two domains could be circumvented, since a continuum of ABDs would necessarily display their appropriate relative orientations (Figure 12, c).

Moreover, another point must be taken into account in designing these types of molecules. As mentioned above, the AT III−ABD binding affinity is much stronger than that of thrombin−TBD. Therefore, a continuum of ABDs would bind AT III with high affinity and prevent the efficient interaction of the saccharidic chain with thrombin. To achieve the desired dual anti-Xa and anti-thrombin activities, a fine-tuning of the two distinct binding events is required, i.e., the affinities of the complexes AT III−ABD and thrombin−TBD must have the same order of magnitude. This requirement could be accomplished by selecting ABDs with reduced affinity for AT III, i.e., those falling in the same micromolar range reported for thrombin and H. The authors envisaged that these ABDs with relatively low affinity for AT III could be obtained by removing some of the essential structural features of high-affinity compounds, leading to a simplified synthetic strategy. Taking the high-affinity pentasaccharide **32** as the reference compound, they argued that hexasaccharides composed of a single repeated disaccharide unit should display a lower, but still significant, affinity for AT III, possibly in the micromolar range. In particular, hexasaccharides **86**−**88** (Figure 15) were selected as targets: compound **86** to assess the effect of a third trisulfated D-glucose unit added at the nonreducing end of the reference pentasaccharide, and symmetric compounds **87** and **88**, containing only D-glucuronic and L-iduronic acid, respectively, to test the influence of the introduction of only one type of hexuronic acid residue. A great advantage in the synthesis of all three hexasaccharides is the use of a converging strategy where disaccharide building blocks were prepared first and then repeatedly condensed with each other by the imidate glycosylation procedure.[96] This approach turned out to be very convenient, especially in view of the preparation of larger homologous fragments. After deprotection and sulfation, the affinity of compounds **86**−**88** for AT III was determined and compared with that of the reference pentasaccharide **32**. Whereas hexasacchar-

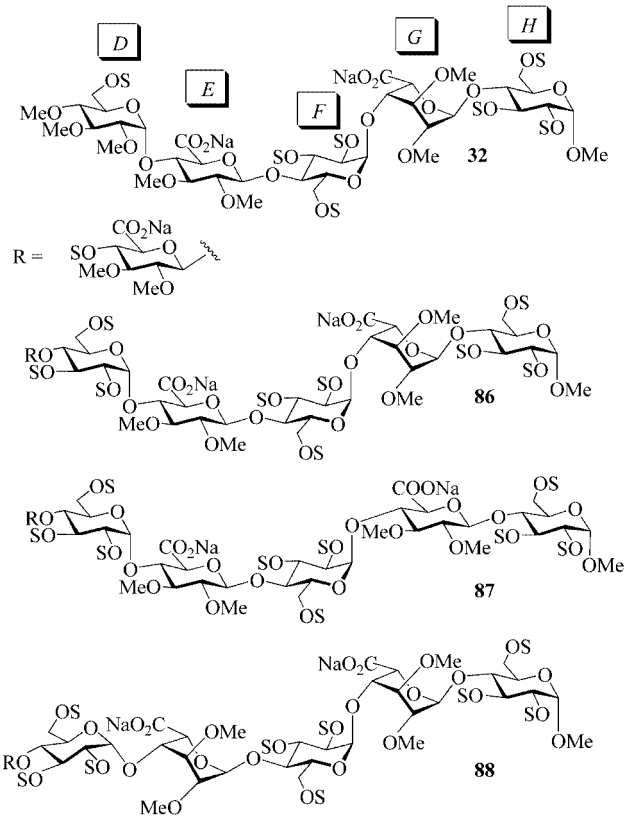
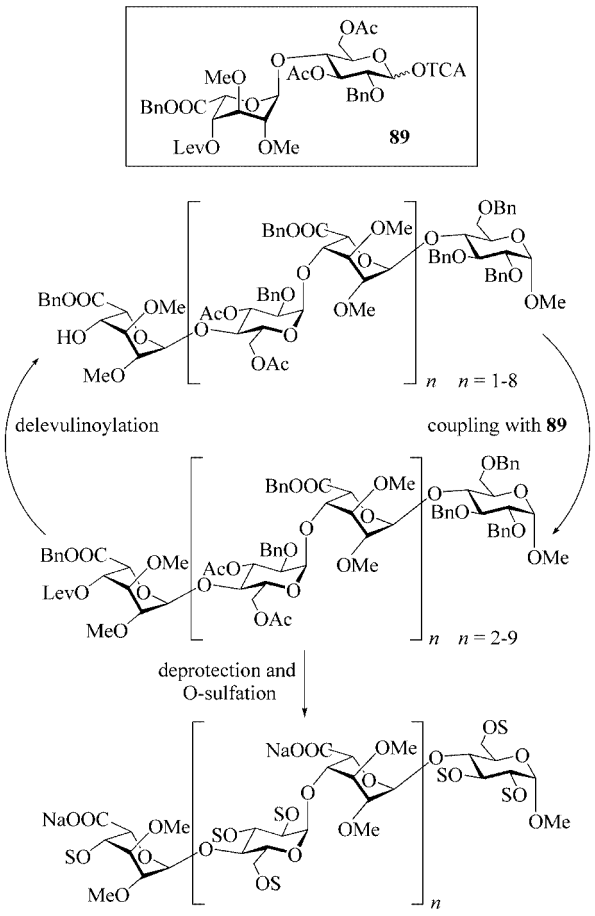


Figure 15. Structures of hexasaccharides **86**−**88**



90: $n = 4$; **91**: $n = 5$; **92**: $n = 6$; **93**: $n = 7$; **94**: $n = 8$; **95**: $n = 9$

Scheme 11. Iterative synthesis of oligomers **90**−**95** from donor **89**

ide **86** still preserved a high affinity for AT III (demonstrating that the addition of a third trisulfated D-glucose unit has only a slight effect on the recognition of AT III), compound **87**, containing only D-glucuronic acid, showed a dramatic loss of affinity.

In contrast, compound **88**, with only L-iduronic acid, exhibited the ideal binding properties, since its affinity for AT III (K_d = 0.35 ± 0.01 μM) was very close to that reported for H and thrombin (1 μM). Moreover, **88** also displayed a significant anti-Xa activity.

Having identified hexasaccharide **88** as a suitable ABD, the challenge of synthesising larger homologous fragments was faced by iterating the strategy employed to prepare **88** (Scheme 11).[97] Thus, glycosyl donor **89** was coupled with the new glycosyl acceptor derived from cleavage of the levulinoyl group of the oligosaccharide obtained at the preceding step. This stepwise addition of one disaccharide unit was reiterated until a series of fragments, ranging from deca- to eicosamer, was obtained. Each single oligomer was deprotected and sulfated, providing oligosaccharides **90**−**95**.

As expected, all the fragments showed very similar anti-Xa activities. However, the 6-, 10-, 12-, and 14-mers, **88**, **90**−**92**, respectively, were inactive in the thrombin inhibition assay (because they were too short), whereas fragments containing from 16 to 20 saccharide units elicited anti-thrombin activity in a size-dependent manner (the eicosamer **95** was half as potent as standard H).

An important outcome of this study was to establish that H fragments shorter than 15−16 saccharide units are unable to display dual anti-Xa and anti-thrombin activity. However, compounds **93**−**95** cross-reacted with PF4 and they activated platelets in the presence of plasma from HIT patients. Therefore, new classes of related molecules, possessing two distinct binding domains, were explored.

A first improvement was achieved with the synthesis of hexadecasaccharide **96**,[98] which was synthesised according to strategy A (Figure 16), leading to molecules composed of three distinct domains. The D-glucose derivative (unit *D*) at the nonreducing end was embedded into the selected molecular spacer and added during the glycosylation step with the *EFGH* portion of the ABD. Next, a suitably protected sugar chain was attached to the nonreducing end of the molecular spacer to act, after deprotection and *O*-sulfation, as a TBD. The assembly of compound **96** was achieved by using a permethylated polymaltose chain as a neutral molecular spacer and persulfated maltotriose as the TBD. Moreover, the AT III binding domain of **96** was closely related to pentasaccharide **32**, except for the 6-OMe groups in units *F* and *H* in place of sulfate groups. The analysis of the biological properties of compound **96** allowed important information to be gathered. First, the replacement of

the flexible polyethylene glycol spacer (used in conjugates **67**−**85**) with a rigid polymethylated polyglucose spacer enhanced the anti-thrombin activity, as expected. Moreover, thanks to the lower charge density and to the rigidity of the spacer, the neutralization by PF4 of **96** was considerably reduced, by a factor of 20 in comparison with standard H. In two subsequent papers, Petitou et al. reported the synthesis of new oligosaccharides composed of an odd number of saccharide units. First, the preparation of pentadeca- (**98a**), heptadeca- (**98b**) and nonadecasaccharides (**98c**) was described[88,99] according to strategy B of Figure 16. According to this approach, the final molecule contains only two distinct blocks: an ABD pentasaccharide and a homologous sugar chain including both the TBD and the molecular spacer. The two blocks are assembled in two steps. First, a portion of the sugar chain terminating with unit *D* at the reducing end is condensed with *EFGH* tetrasaccharide, completing the AT III binding domain. Next, the TBD/spacer chain is elongated up to the desired length. Compounds **98a**−**c** contained pentasaccharide **32** as the ABD, elongated at its nonreducing terminus by oligomers of 3-*O*-methyl-2,6-di-*O*-sulfo-D-glucose with alternating α- and β-(1→4) linkages. This sequence was chosen to mimic the charge density of the regular region of H (it bears two negative charges per saccharide unit), but at the same time it allowed a great simplification of the chemistry. Additionally, the definitive experimental proof of the correct orientation of the AT III and thrombin binding domains was obtained only by a direct comparison between H mimetics having the same ABD and TBD, but differing for their relative arrangements. Therefore, also octadecasaccharide **99**, containing the TBD linked at the reducing end of the ABD, was synthesised by applying the same strategy described for compounds **98a**−**c**.

The affinity for AT III and anti-Xa activity were very similar for compounds **98a**−**c** and **99**. Moreover, **98a**−**c** inhibited thrombin in a size-dependent manner (**98c** was as potent as the most active fraction isolated from standard H), but they were also neutralised by PF4. In contrast, mimic **99** was shown to be a very poor inhibitor of thrombin, confirming that the TBD must be located at the nonreducing end of the ABD to obtain efficient inhibition of both thrombin and factor Xa. This work allowed also the definitive establishment that the minimal H sequence to observe both thrombin and factor Xa inhibition must include fifteen saccharide units (see **98a**). However, since pentadecasaccharide **98a**, which is the smallest fragment able to display the full anticoagulant properties of H, still interacts with PF4, the only remaining possibility to avoid this side effect is to reduce the charge of the molecule, the second parameter governing nonspecific interactions of H. Therefore, heptadecasaccharide **97** (Figure 16) was synthesised,[100] according to strategy A, with the following features: (a) the usual high-affinity pentasaccharide **32** was included as the ABD; (b) the ABD was first elongated by a neutral hexasaccharide comprising alternating α- and β-(1→4)-linked 2,3,6-tri-*O*-methyl-D-glucose residues; (c) finally, a TBD comprising six units of 3-*O*-methyl-2,6-di-*O*-sulfo-D-glucose with alternating α- and β-(1→4) interglycosidic bonds was anchored at the nonreducing end of this molecular spacer. The biological activity of **97** was compared with that of compound **98b** to assess the effect of the diminished charge density. Saccharide **97** was shown to be an extremely potent anticoagulant molecule, inhibiting both factor Xa and thrombin. Most importantly, compound **97** was not neutralised by PF4, even when added at a very large concentration (100 μg mL^{-1}). However, the synthesis of molecules like **97** still requires a great number of elaborate steps, hampering an efficient scale-up for further drug development. Therefore, the possibility of uniformly decreasing the charge density along the chain was investigated by using a lowly sulfated sequence composed of a repetition of α-linked 2,3-di-*O*-methyl-6-*O*-sulfo-D-glucose residues as the TBD. This approach led to the preparation of three compounds, hexadecasaccharide **100a**, octadecasaccharide **100b**, and eicosasaccharide **100c** (Figure 16).[101] In the TBD of compounds **100a**−**c**, the single sulfate group was located at the primary position of each D-glucose unit, because it is easier to differentiate. The biological activities of compounds **100a**−**c** were determined: as expected, only small differences in the affinity for AT III and in the inhibition of factor Xa were observed for **100a**−**c**. All three oligomers are anticoagulants that are less potent than standard H, but they showed a size-dependent thrombin inhibition profile. Even more importantly, none of the synthetic compounds was neutralised by PF4.

3.4 Synthesis of Fragments Involved in the Interaction with Fibroblast Growth Factors

Another class of biologically relevant H/HS ligands in the extracellular matrix are the fibroblast growth factors (FGFs),[102] a family of potent growth-promoting and cell-differentiating proteins[103] comprising 21 members, the most studied of which are acidic FGF (FGF1) and basic FGF (FGF2). Their low-affinity binding with H/HS is thought to sequester them in the extracellular matrix, creating a reservoir of inactive FGFs that are released in an active form when required.[104,105] More importantly, H/HS also modulate the binding of FGFs with their high-affinity receptors (the tyrosine kinase membrane receptors, FGFRs) in the presence of divalent cations,[1] through a mechanism whose elucidation is still under debate.[106]

Experimental data suggest that FGF1 and FGF2 exhibit different modes of activation and structural requirements for the binding site on the H/HS chain.[107,108] In this area of research, two main issues are controversial:

(a) The binding stoichiometry among H/HS chain, FGF and FGFR. It seems widely accepted that FGFR activation requires its dimerization, and this process is conceivably induced by H-mediated cross-linking of FGFs.[109,110] Two distinct kinds of FGF oligomers have been hypothesised: a *trans*, sandwich-type dimer, with the H chain positioned at the interface between two protomers (Figure 17, a);[16,23,24,111,112] a *cis* oligomer, where two proteins are positioned side-by-side along two contiguous domains on the H/HS chain (Figure 17, b).[113,114] The two modes of associ-

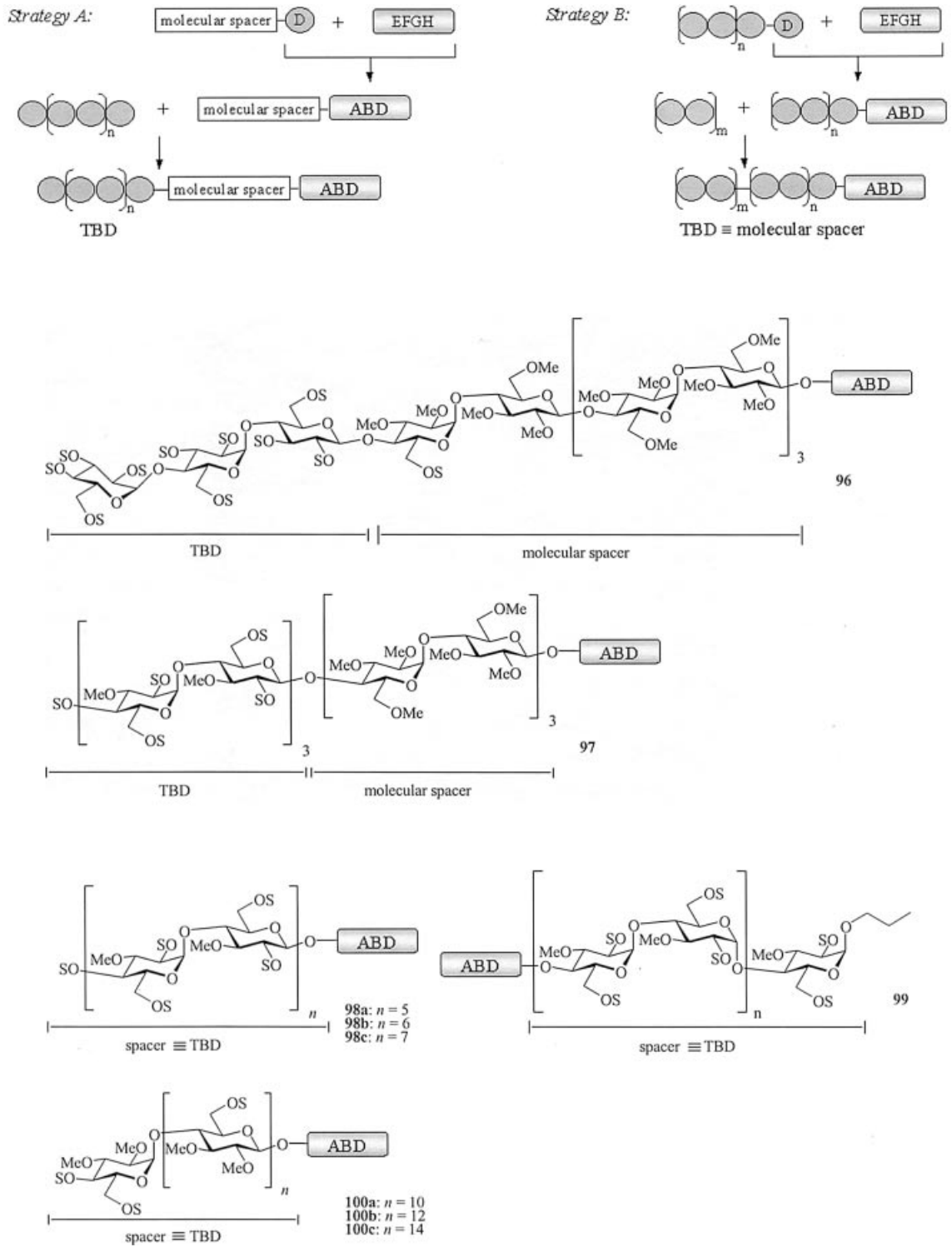


Figure 16. Strategy A leads to molecules composed of three distinct domains, whereas strategy B leads to molecules containing only two distinct domains (see text for further details); a: molecules synthesised according to strategy A; b: molecules synthesised according to strategy B

ation may depend on the length of the fragment, which is a major determinant for the biological activity.[115] However, the abundant experimental data have not yet provided convincing evidence favouring either of the proposed models.

(b) The minimal binding domain, i.e. the minimal sulfation pattern and the epitope length. It has been discovered that the specific binding motif towards FGF1 implicates the trisaccharide IdoA(2-OS)-GlcNS(6-OS)-IdoA(2-OS), which is abundant in the regular regions, included in a longer fragment.[116] However, determining the sulfation patterns remains an open issue. Ornitz et al. reported contradictory data that suggested that even the non-sulfated trisaccharide

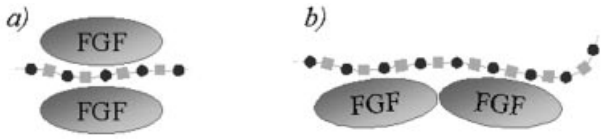


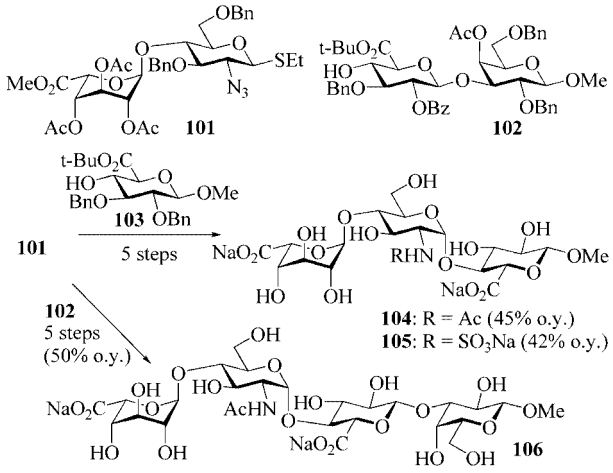
Figure 17. Distinct modes of H/HS-induced FGF oligomerization; a) two FGF molecules are positioned *trans* with respect to the H/HS chain, without contact between the two protomers; this kind of dimer engages short H/HS domains, which are involved in the binding of two sets of sulfate/carboxylate groups from opposite sides of the helix; b) two FGF molecules are positioned *cis* with respect to the H/HS chain; this dimer requires longer H/HS domains, but engages the sulfate/carboxylate groups only from one side of the helix; some amino acids of the two protomers are involved in protein−protein contacts

GlcA-GlcNAc-GlcA-OMe is able to bind FGF1,[117] while Casu et al. showed that partially sulfated fragments, as short as a tetrasaccharide, interact with FGF1.[118] The structural requirements of FGF2 are relatively simpler to assess. It is well established that while 2-OS units are necessary for the binding, 6-OS units are not, but they are required for the biological activity that is displayed only by long fragments (deca/dodecasaccharides).[119−122] In 1993, Lindahl et al. reported the pentasaccharide HexA-GlcNS-HexA-GlcNS-IdoA(2-OS) as the minimal sequence that is able to bind FGF2.[123,124] While the identity of the HexA units still remained uncertain, the IdoA(2-OS) unit at the reducing end of the domain was believed to be crucial for the interaction. Other authors suggested that, in order to exhibit an efficient binding towards FGF2, all the uronic acid units of this sequence must be present as L-IdoA.[125,126]

The binding studies reported so far have relied almost exclusively on H/HS fragments derived from the regular regions of the polymer. On the other hand, as has been already mentioned, the availability of differently sulfated fragments greatly aids in disclosing the specific sulfation patterns of the epitopes. Very recently the biosynthetic generation of oligosaccharide libraries for the identification of protein-binding motifs has been reported,[127] while only a moderate number of chemical syntheses of differently sequenced oligosaccharides as putative ligands towards FGFs have appeared in the literature. The first fragments were synthesised by Westman et al., who reported a series of sulfated and non-sulfated di-, tri- and tetrasaccharides, with sequences that are widely occurring in HS domains, with the aim of testing their binding abilities towards FGFs. The synthesis started from disaccharide intermediates **101** and **102** (Scheme 12).[128]

Disaccharide **101** was coupled with both acceptor **103** and alcohol **102** providing, after removal of the protecting groups and selective acetylation/*N*-sulfation, final compounds **104**−**106**.[129] According to a similar block procedure, disaccharides **107**−**110**, **111α** and **111β** and trisaccharide **112** were synthesised (Figure 18).

The biological tests performed on these fragments revealed, surprisingly, that both trisaccharides **104** and **112** were able to bind FGF2 in a competitive manner with iodinated H, and additionally they showed an even higher affinity in binding to FGF1. Also disaccharides **109** and **110** can compete with H in the binding with FGF2, although less efficiently. These results suggest that non-sulfated HS domains also are able to interact, to a certain extent, with these proteins.[113,117]



Scheme 12. Synthesis of non-sulfated tri- and tetrasaccharides **104**−**106**

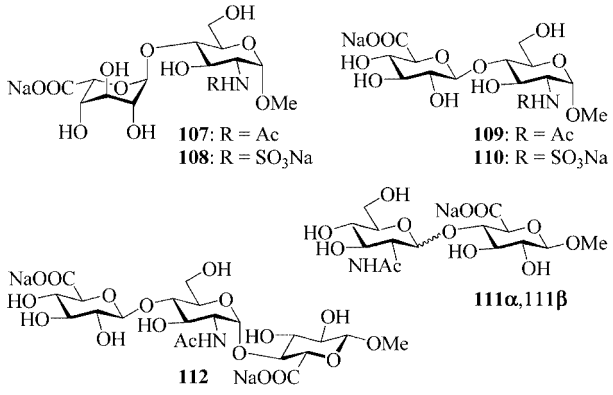


Figure 18. Structures of disaccharides **107**−**111** and trisaccharide **112**

After these preliminary results were reported, the efforts of other research groups have been directed mainly at the development of synthetic approaches suitable for the preparation of entire families of H/HS fragments, whose members differ in some structural feature (e.g., in the configuration of the uronic acid or in the sulfation pattern). In this way, comparisons of the binding ability and/or the biological activity displayed by the different members of each series have allowed the elucidation of some structural requirements for the binding motifs.

A modular approach mainly has been applied for this purpose and various building blocks have been designed to allow the preparation of the following oligosaccharide series:

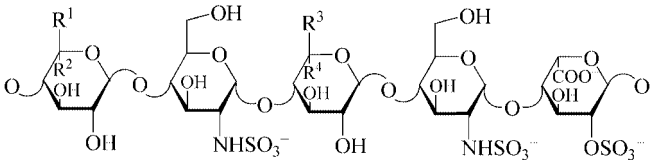
(a) Oligosaccharides differing in the configuration of the HexA units (L-IdoA/D-GlcA).

(b) Oligosaccharides differing in the sulfation pattern.

(c) Oligosaccharides, belonging to the H regular regions, differing only in fragment length.

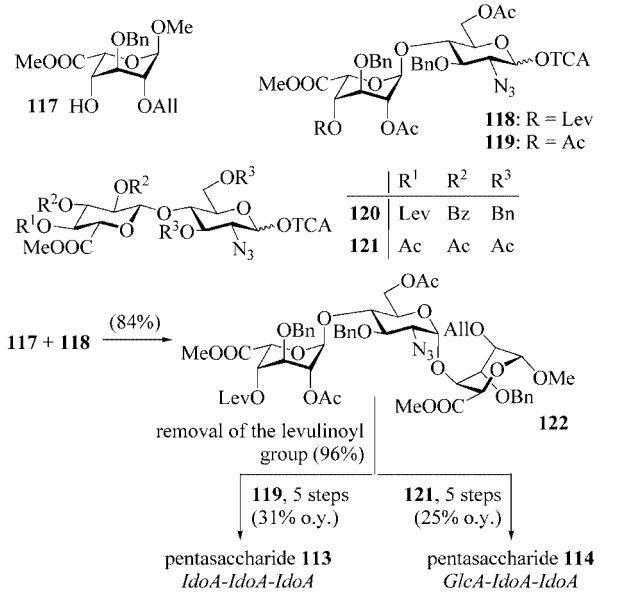
A family of type (a) was synthesised by Sinaÿ et al. Shortly after identifying the sequence HexA-GlcNS-HexA-GlcNS-IdoA(2-OS) as a putative binding site towards FGF2,[123,124] they began a project aimed at elucidating the exact nature of the HexA units by performing the total syntheses of the four possible pentasaccharide isomers **113**−**116** (Table 2).[130,131]

Table 2. Structures of pentasaccharides **113**−**116**



	R^1	R^2	R^3	R^4	
113	H	COO^-	H	COO^-	IdoA-IdoA-IdoA
114	COO^-	H	H	COO^-	GlcA-IdoA-IdoA
115	H	COO^-	COO^-	H	IdoA-GlcA-IdoA
116	COO^-	H	COO^-	H	GlcA-GlcA-IdoA

The synthesis was performed using the five building blocks **117**−**121** (Scheme 13). In the protecting group strategy, the allyl group was used to mask the unique hydroxy group that would be sulfated in the final products and the levulinoyl ester was chosen to block position 4 of the HexA units to allow the elongation from the nonreducing end of the chain. Thus, acceptor **117** was coupled with donor **118**, providing trisaccharide intermediate **122**. After removal of its levulinoyl group, **122** was engaged in the condensation with trichloroacetimidate donors **119** and **121** yielding, after deprotection and 2-*O*-sulfation of the reducing L-IdoA unit, target compounds **113** and **114**. Likewise, pentasaccharides **115** and **116** were obtained by applying the same procedure to acceptor **117** and donor **120**.



Scheme 13. Synthesis of pentasaccharides **113**−**114**

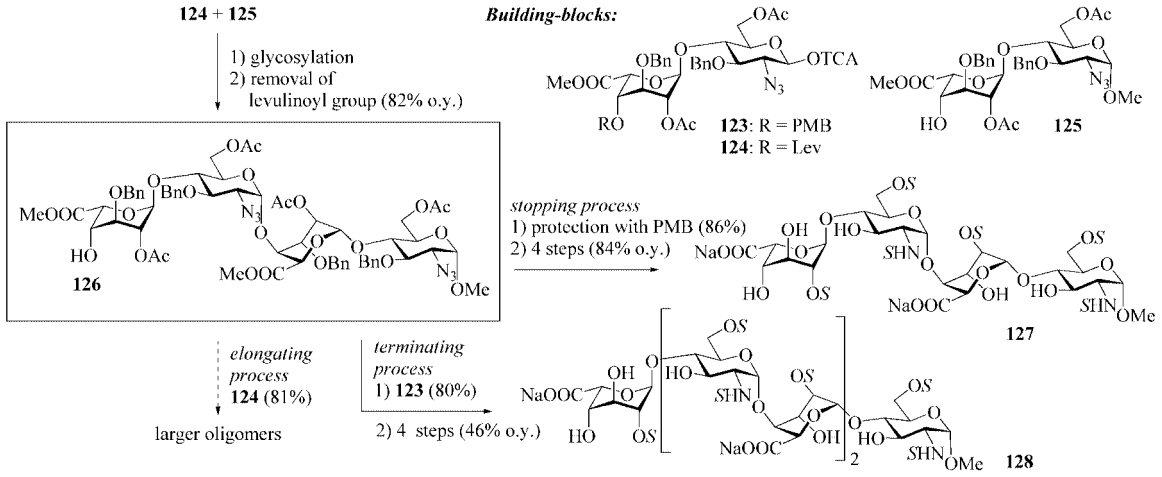
Conformational analysis of the pentasaccharides revealed that the L-IdoA conformation depends strictly on the position it occupies in the pentasaccharide. Thus, while the nonreducing end L-IdoA moiety (when present) is mainly in the $^4C_1$ form, the $^1C_4$ conformer predominates in the central unit, with 25% of the $^2S_0$ form. Finally, the L-IdoA unit at the reducing end is present almost exclusively as the $^2S_0$ conformer. Biacore® analysis[100,132] and in vivo tests performed on these compounds showed that they are able to antagonize iodinated H−FGF2 binding and to inhibit proliferation of FGF2-induced human aortic smooth-muscle cells (HASMC), with pentasaccharide **113** being the most potent. These results confirmed previous findings suggesting that the L-IdoA unit is the uronic acid mainly involved in the interaction between FGF2 and HS.[125,126]

Soon after this report appeared, the same group investigated a synthetic methodology that was still based on the assembly of building blocks and applied it to the preparation of tetra- and larger oligosaccharides corresponding to the regular regions of H [oligosaccharides of type (c)]. Disaccharides **123**−**125** were chosen as the capping block, the elongating block and the seeding block of the synthesis, respectively (Scheme 14).[133] After the preparation of key intermediate **126**, derived from disaccharides **124** and **125**, in principle three options were possible: (a) a stopping step, reprotecting the 4-OH group of the nonreducing L-IdoA unit with a PMB group and applying the deprotection/sulfation sequence leading to tetrasaccharide **127**; (b) a terminating step, coupling **126** with donor **123**, eventually yielding hexasaccharide **128**; (c) a coupling with donor **124** for further elongation of the chain. Binding and inhibition tests performed on compounds **127** and **128** revealed that the hexasaccharide efficiently antagonised iodinated H−FGF2 binding to HASMC and it was able to inhibit FGF-induced proliferation of HASMC, with H as the reference compound. Tetrasaccharide **127**, although still active, exhibited a markedly weaker efficiency. Once more, the length of the fragment appeared to be the determinant for the efficiency of the biological activity.

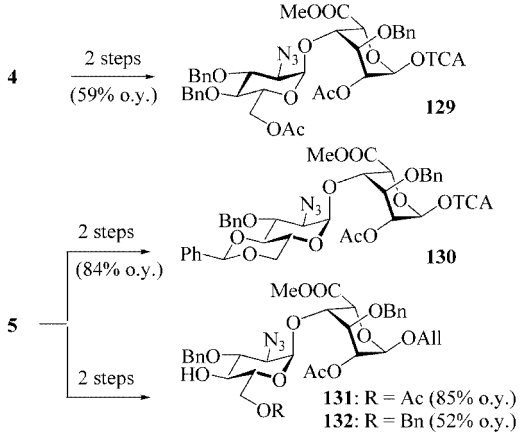
Our group was the first to devise two disaccharide building blocks (compounds **4** and **5**, Figure 3) for the preparation of a family of type (b), with the aim of ascertaining the role of the 6-*O* sulfate group of the D-GlcNS unit in the binding with FGFs.[42]

The first two members of the series, disaccharides **6** and **7** (Figure 3), were first prepared to validate the synthetic procedure. These two H-like fragments, although unable to bind FGFs, could be helpful standards for conformational analysis. They have also been reported as the minimal H motif recognised by platelets.[134] Building block **4** was then manipulated to afford donor **129**, while disaccharide **5** provided donor **130** and acceptors **131** and **132** (Scheme 15).[135]

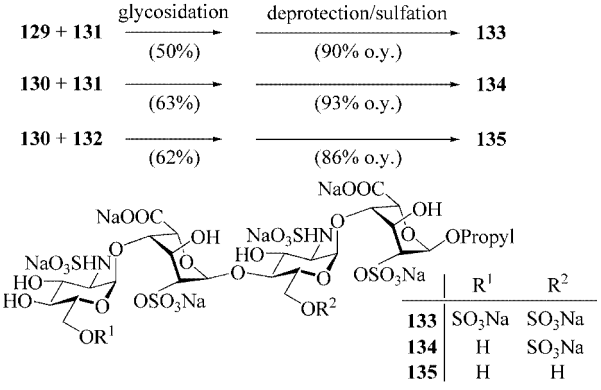
Coupling between glycosyl acceptor **131** and donors **129** and **130** gave fully protected tetrasaccharides that, after sulfation and removal of the protecting groups, yielded target oligosaccharides **133** and **134**, respectively (Scheme 16). Likewise, donor **130** was coupled with acceptor **132** to



Scheme 14. Synthesis of oligosaccharides of the regular region of heparin



Scheme 15. Synthesis of donors **129** and **130** and acceptors **131** and **132** from building blocks **4** and **5**



Scheme 16. Preparation of tetrasaccharides **133**−**135** from intermediates **129**−**132**

eventually provide compound **135**.[135] The binding activity of these H-like fragments towards FGF1 was tested by Biacore® analysis, NMR spectroscopy binding experiments and MALDI-TOF mass spectrometry of the oligosaccharide−protein complexes.[118] Interestingly, both compounds **133** and **134** contain the recently identified HS binding sequence IdoA(2-OS)-GlcNS(6-OS)-IdoA(2-OS).[116] Moreover, whereas the strong interaction of **133** primarly involves the GlcNS(6-OS)-IdoA(2-OS) disaccharide moiety at its nonreducing end, all the residues appeared to be involved in the weaker complex of compound **134**. Together, these results suggest that H/HS-induced dimerisation of FGF1 requires only one 6-OS unit per tetrasaccharide. Actually, compound **135** revealed negligible activity, while tetrasaccharides **133** and **134** interacted significantly with FGF1. MALDI mass spectra revealed that both compounds **133** and **134** induce oligomerisation of this protein, forming 2:1 FGF1−tetrasaccharide complexes.

Larger oligosaccharides containing the same sequence and belonging to fully sulfated H domains were synthesised by the group of Martín-Lomas, who applied the same building block strategy following an $n+2$ convergent block approach. The synthesis started from disaccharide precursors **136**−**138**, available from D-glucosamine and D-glucurono-6,3-lactone (Figure 19).[136] These compounds represent, respectively, the nonreducing end, the inner disaccharide and the reducing end of the final oligosaccharides. The assembly of these three building blocks and the following deprotection/sulfation procedure provided hexasaccharide **139** and octasaccharide **140**. Conformational analysis of hexasaccharide **139** revealed that it displays a helical conformation and that its L-IdoA units exist in fast equilibrium between $^1C_4$ (slightly preferred) and $^2S_0$ conformers, regardless of their position in the chain. Interestingly, these results, when compared to those reported by Sinaÿ et al. on pentasaccharides **113**−**116** (Table 2), suggest that the sequence of H fragments (L-IdoA-D-GlcN vs. D-GlcN-L-IdoA) can strongly affect the conformational equilibrium of the L-IdoA units. Also, the binding of Ca^{2+} cations to this hexasaccharide was investigated, since the interaction of H with several proteins, including FGFs and their receptors, is mediated by this cation.[1,6,109,137] The results indicate that specific Ca^{2+} binding exists in the H chain without affecting its overall helical structure, yet it affects the flexibility of the oligosaccharide backbone.[138] The specific binding site was revealed by further studies performed on disaccharide model **141**, which is readily available from **136**, that

showed that the Ca^{2+} coordinates the COO^-, 2-OSO_3^- and $HNSO_3^-$ groups of the interglycosidic and iduronate ring oxygen atoms and an apical water molecule.[139] Compounds **139** and **140** were then tested for their activation properties towards FGF1.[140] While octasaccharide **140** gave results equivalent to those of H in activating the mytogenic signal, hexasaccharide **139** was less efficient. Quite surprisingly, sedimentation experiments showed that the active form of FGF1 is a monomer, suggesting that FGF1 dimerisation is not a prerequisite for FGF1-induced signaling.

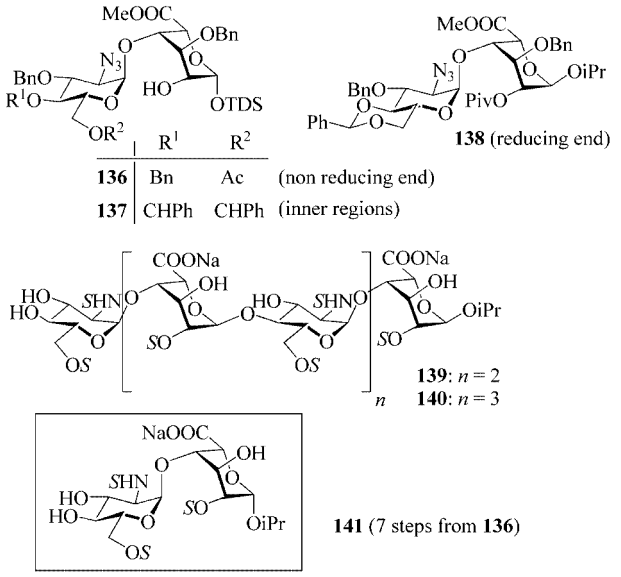


Figure 19. Synthesis of oligosaccharides **152**−**154** from building blocks **149**−**151**; *i*Pr = isopropyl; TDS = thexyl dimethylsilyl; Piv = pivaloyl

To validate this hypothesis, partially sulfated fragments **142** and **143** were synthesised from disaccharide precursor **137** (Scheme 17).[141] These two fragments were designed to display the charged groups only on one side of their helical structure and, in principle, they should give only a monomeric complex with FGF1. Preliminary results indicated that hexasaccharide **142** displays significant biological activity and, thus, demonstrates that even a 1:1 complex between FGF1 and an H fragment can induce mitogenesis.

3.5 Synthesis of Fragments Involved in the Interaction with Platelet Cells

As mentioned above, there are a number of undesired side effects related to the administration of H as an anticoagulant. Among them, H-mediated platelet aggregation causes an increasing of the binding of fibrinogen to platelets, with paradoxical, pro-thrombotic effects as a consequence. This change of the platelet function is due to the binding of H to platelet-surface integrin $\alpha_{IIb}\beta_3$.[142] This binding is saturable, specific and reversible, as determined by inhibition studies,[143] and bears many of the characteristics of an integrin-dependent process. Moreover, it has been shown that the pro-thrombotic effect is independent of the interaction of H with platelet-secreted H-binding proteins, such as fibronectin, thrombospondin or PF4. To develop more efficient and safer H-related anticoagulant drugs, the molecular basis of these interactions must be better defined. During the last decade, Suda et al. pursued a program in this direction.[144] Preliminary studies performed on partially depolymerised H suggested that the interaction with platelets is molecular weight dependent. The binding is mainly ionic in nature and it seems not to involve a specific sequence on the H chain, even if pentasaccharide *DEFGH* is able to interact with platelets. More importantly, the same studies suggested that the disaccharides GlcNS(6-OS)-IdoA(2-OS) and GlcNS-IdoA(2-OS) appear to be crucial for the binding to the platelet surface.[134] The authors observed that the binding potency is related to the number and frequency of these fragments along the H chain, and claimed the existence of a clustering effect on binding. The first prediction was confirmed through the chemical synthesis of the model disaccharide **144** (Figure 20),[145] which showed decreased platelet-binding affinity when compared to commercial H, but exhibited higher affinity than compound **145**, which contains the same charge density and is obtained from H digestion with heparinase I. This feature

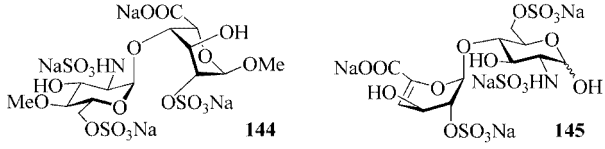
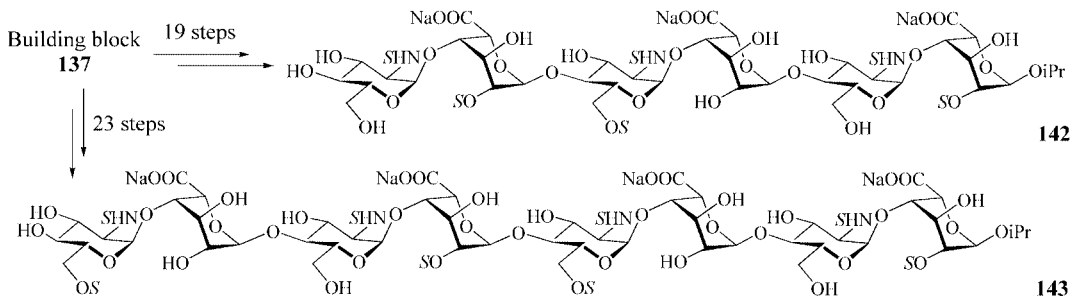


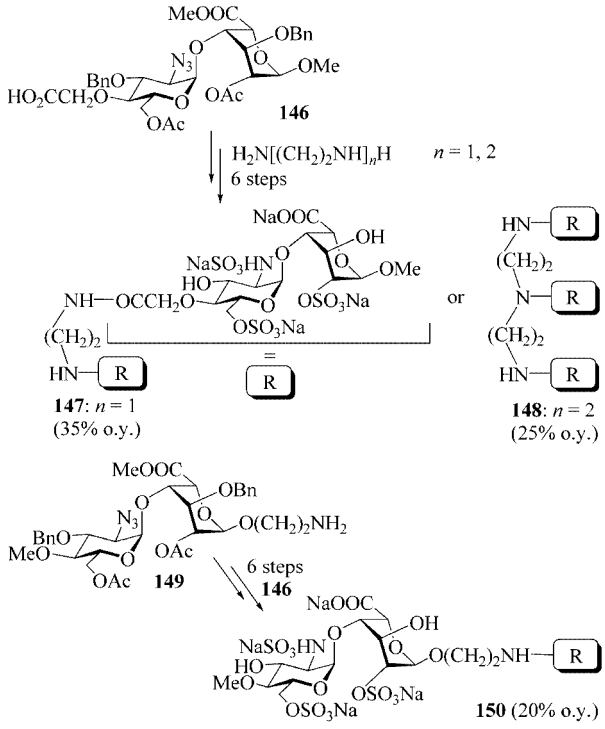
Figure 20. Structures of disaccharides **144** and **145**



Scheme 17. Synthesis and structure of oligosaccharides **142** and **143**

is a clear indication that the unique disaccharide sequence of **144** is involved in specific H-platelet interactions.

The presumed clustering effect was next examined with the synthesis of compounds **147**, **148** and **150** (Scheme 18).[146] Compounds **147** and **148** contain two and three units of the key disaccharide, respectively, connected in a tail-to-tail orientation, whereas in **150** the same two units are connected head-to-tail. Disaccharide **146** was the key precursor of **147** and **148**. Two units of **146** were coupled with ethylenediamine, eventually providing **147**. Likewise, three units of **146** were coupled with diethylenetriamine to afford **148**. Finally, compound **150** was prepared from disaccharide **149** equipped with an ethanolamine spacer. The coupling of **149** with **146**, followed by sulfation and deprotection, gave the head-to-tail oligomer.



Scheme 18. Synthesis of conjugates **147**, **148** and **150**

The platelet-binding activities of compounds **147**, **148** and **150** were compared with those of commercial H and synthetic disaccharide **144**. All the oligomers showed a substantial binding affinity for platelets, although weaker than H. Interestingly, compound **148** exhibited a higher binding activity than **147**, which, thus, indicates the role of the clustering effect. Moreover, the relative arrangement of the two disaccharide units has a great influence on the binding properties, since compound **150** is significantly more active than **147**.

In the approach described above, the synthesis of each individual oligomer required first the preparation of the suitably protected disaccharide, followed by the assembly step and finally by sulfation and deprotection to give the target compounds. In view of the preparation of more complex oligomeric compounds, a new and more flexible strategy was highly desirable for assembling "pre"-formed sulfated and deprotected oligosaccharides. With this purpose in mind, an efficient method was developed for the assembly of functionalised oligosaccharides by reductive amination with diverse linkers containing aromatic amino groups[147] and it was applied to the synthesis of compounds **154**, **155** and **157**.[148] The first compound prepared was the sulfated trisaccharide **151** (Scheme 19), which contains an additional D-glucose moiety at the reducing end of disaccharide **144**.

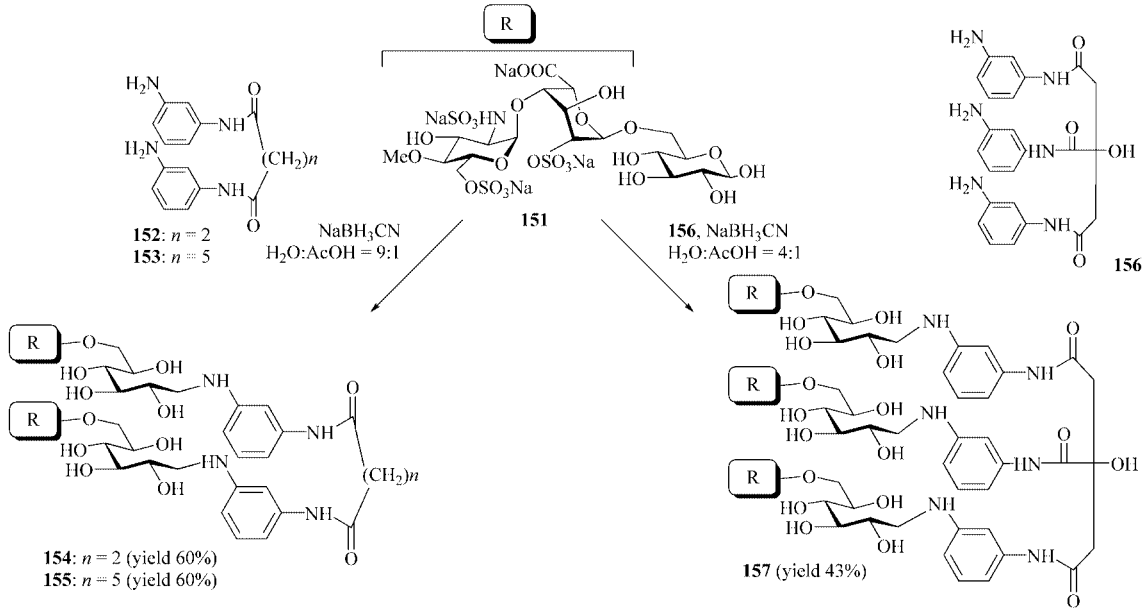
This trisaccharide provides the aldehyde function for the reductive amination step, and it also acts as the hydrophilic spacer in the conjugated molecule. The conjugation of **151** with the amino linkers was accomplished as illustrated in Scheme 19. Compound **151** was incubated with divalent linkers **152** and **153** at 37 °C (pH = 3) in the presence of $NaBH_3CN$ to afford dimers **154** and **155**, respectively. Likewise, the incubation of **151** with the trivalent linker **156** generated the trimeric compound **157**. The platelet-binding activities of **151**, **154**, **155** and **157** were assessed in a competitive binding assay in comparison with commercial H. An enhancement in the binding affinity was observed with respect to the number of GlcNS(6-OS)-IdoA(2-OS) residues. As a matter of fact, trisaccharide **151** was almost inactive, whereas the trimeric conjugate **157** showed the highest activity, which, thus, confirms that a multivalent effect is in operation.

As mentioned above, synthetic pentasaccharide **25** (the ABD in H) binds efficiently to platelets, although it does not contain the key fragment GlcNS(6-OS)-IdoA(2-OS). This finding stimulated the synthesis of various fragments of this pentasaccharide aimed at identifying putative novel sequences responsible for the platelet-binding activity. Previous studies had shown that a tetrasaccharide containing the nonreducing portion *DEF* of **25** was unable to bind platelets. Therefore, the synthesis was focused on the reducing end *FGH* trisaccharide **159**, on its partially desulfated derivatives **160** and **161**, and on its disaccharidic components **158** (*FG*) and **162** (*GH*, Figure 21).[149]

To obtain the desired sulfation pattern in the final compounds, specific protection was required at position 6 of unit *H* and position 3 of unit *F*. Among the synthesised fragments, only trisaccharide **159** exhibited a distinct binding activity towards platelets in a competitive assay using commercial H and pentasaccharide **25** as reference compounds. This finding led to the conclusion that the sequence *FGH* of **25** is a high-affinity domain for platelets, and suggests that of this fragment, combined with a clustering effect of GlcNS(6-OS)-IdoA(2-OS), is responsible for the binding of H to platelets.

3.6 Synthesis of Fragments Involved in the Interaction with Lipoprotein Lipase

Besides the anticoagulant and antithrombotic activity, heparin also exerts a lipolytic activity through its interaction with lipoprotein lipase (LpL). This enzyme catalyses



Scheme 19. Synthesis of conjugates **154**, **155** and **157**

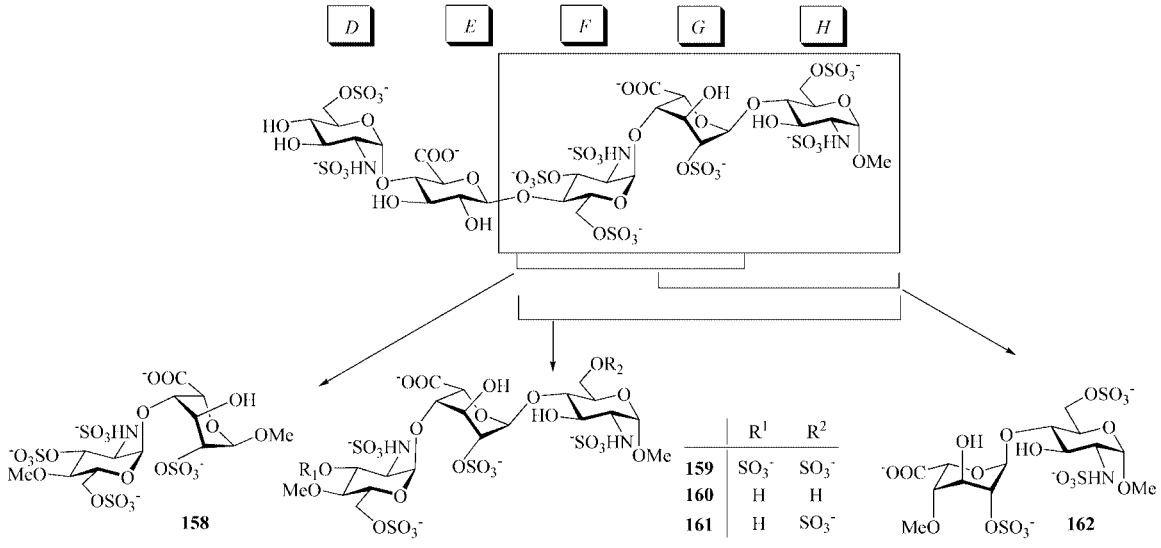
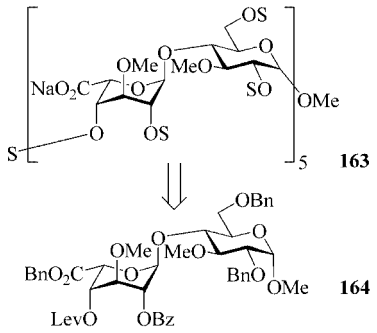


Figure 21. Structures of di- and trisaccharides structurally related to the *FGH* portion of pentasaccharide **25**

the hydrolysis of triacylglycerols in circulating lipoproteins. It is located in the vascular endothelium, noncovalently bound to cell-membrane HS, and it is displaced and released in circulation by H. H is not only able to release LpL, but also epathic lipase, causing an increase in cholesterol levels as a side effect in H-treated patients.[150] The lipase-releasing activity is correlated to the molecular weight and the charge density of the polysaccharide chain,[151,152] and a regularly sulfated H/HS decasaccharide sequence has been reported as the shortest fragment able to bind LpL strongly.[153] Exploiting the synthetic methodologies developed in the preparation of H analogues with antithrombotic activity, Petitou et al. synthesised decasaccharide **163** (Scheme 20) to investigate whether a well-defined synthetic mimic could release this enzyme from its endothelium compartment,[154] since it maintains the charge density and the correct conformation of the natural decasaccharide fragment.

Its synthesis was performed starting from disaccharide **164**,[74] through an $n+2$ block strategy and applying standard deprotection/sulfation protocols. Decasaccharide **163** is able to release lipase activity, although with less efficiency than H. The synthesis of the H mimic **163** provided a suitable approach to study the H−lipase interaction through



Scheme 20. Retrosynthesis of an H mimic, decasaccharide **163**

well-defined fragments, and allowed elucidation of the structural features (length, number and position of the sulfates) for the lipase-releasing activity of H.

4. Summary and Outlook

H/HS are biologically relevant biopolymers, able to interact with a multitude of proteins, with a degree of specificity ranging from merely ionic to extremely specific in nature. Actually, the only case of a highly specific interaction that is clearly defined, and which is the most deeply studied so far, is with blood coagulation factors, but the importance of the recognition of other proteins is strongly emerging. The chemical synthesis of H/HS oligomers plays a dual crucial role. First, it is an important tool in revealing the sites in chains of natural H/HS that are responsible for the recognition of proteins. In addition, only synthesis allows for the specific modifications to be made that are required to modulate the biological activity of the distinct domains. The arsenal of synthetic methodologies in carbohydrate chemistry has increased significantly in recent decades and it enables the preparation of new biologically active H/HS fragments, but the high degree of functionalization, coupled with the heterogeneity of these polymers, continues to make H/HS fragments challenging synthetic targets. However, the enormous interest raised by the multifaceted biological function of these polymers is stimulating the development of new and versatile synthetic approaches towards these complex molecules. Preliminary reports on some innovative methodologies are described in this review, and their successful application to the synthesis of tailored sulfated fragments is expected in the near future.

Abbreviations: GlcN = D-glucosamine, HexA = unspecified hexuronic acid, GlcA = D-glucuronic acid, IdoA = L-iduronic acid, S = $SO_3^-Na^+$, TCA = $CNHCCl_3$ (trichloroacetimidate), Ac = acetyl, All = allyl, Bn = benzyl, Bz = benzoyl, Lev = levulinoyl.

Acknowledgments

We gratefully acknowledge CNR (Istituto di Scienze e Tecnologie Molecolari), CISI and MIUR for financial support. We also thank Prof. G. Russo and Dr. S. Vignando for a critical revision of the manuscript.

[1] B. Casu, U. Lindahl, *Adv. Carbohydr. Chem. Biochem.* **2001**, *57*, 159−206 and references therein.
[2] J. D. Esko, S. B. Selleck, *Annu. Rev. Biochem.* **2002**, *71*, 435−471 and references therein.
[3] J. D. Esko, U. Lindahl, *J. Clin. Invest.* **2001**, *108*, 169−173 and references therein.
[4] B. Mulloy, M. J. Forster, C. Jones, D. B. Davies, *Biochem. J.* **1993**, *293*, 849−858 and references therein.
[5] M. Kan, F. Wang, F. Xu, J. W. Craab, J. Hon, W. L. McKeehan, *Science* **1993**, *259*, 1918−1921.
[6] I. Capila, M. J. Hernaiz, Y. D. Mo, T. R. Mealy, B. Campos, J. R. Dedman, R. J. Linahrdt, *Structure* **2001**, *9*, 57−64.
[7] H. E. Conrad, *Heparin-binding proteins*, Academic, San Diego, USA, **1998**.
[8] I. M. Bernfield, M. Gotte, P. W. Park, O. Reizes, M. L. Fitzgerald, J. Lincecum, M. Zaco, *Annu. Rev. Biochem.* **1999**, *68*, 729−777.
[9] Capila, R. J. Linhardt, *Angew. Chem. Int. Ed.* **2002**, *41*, 390−412.
[10] R. E. Hileman, J. R. Fromm, J. M. Weiler, R. J. Linhardt, *BioEssays* **1998**, *20*, 156−167.
[11] D. A. Rees, E. R. Morris, J. F. Stoddart, E. S. Stevens, *Nature* **1985**, *317*, 480.
[12] B. Casu, J. Choay, D. R. Ferro, G. Gatti, J.-C. Jacquinet, M. Petitou, A. Provasoli, M. Ragazzi, P. Sinaÿ, G. Torri, *Nature* **1986**, *322*, 215−216.
[13] B. Mulloy, M. J. Forster, *Glycobiology* **2000**, *10*, 1147−1156.
[14] M. Hricovíni, M. Guerrini, A. Bisio, G. Torri, M. Petitou, B. Casu, *Biochem. J.* **2001**, *359*, 265−272.
[15] L. Jin, J. P. Abrahams, R. Skinner, M. Petitou, R. N. Pike, N. W. Carrel, *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 14683−14688.
[16] A. DiGabriele, I. Lax, D. I. Chen, C. M. Svahn, M. Jaye, J. Schlessinger, W. A. Hendrickson, *Nature* **1998**, *393*, 812−817.
[17] E. E. Fry, S. M. Lea, T. Jackson, J. W. Newman, F. M. Ellard, W. E. Blakemore, R. Abu-Gazaleh, A. Samuel, A. M. King, D. I. Stuart, *EMBO J.* **1999**, *18*, 543−554.
[18] U. Lindahl, M. Kusche-Gullberg, L. Kjellén, *J. Biol. Chem.* **1998**, *273*, 24979−24982 and references therein.
[19] R. R. Vivès, D. A. Pye, M. Salmivirta, J. J. Hopwood, U. Lindahl, J. T. Gallagher, *Biochem. J.* **1999**, *339*, 767−773.
[20] J. E. Turnbull, J. J. Hopwood, J. T. Gallagher, *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 2698−2703.
[21] G. Venkataraman, Z. Shriver, R. Raman, R. Sasisekharan, *Science* **1999**, *286*, 537−542.
[22] S. Faham, R. E. Hileman, J. R. Fromm, R. J. Linhardt, D. C. Rees, *Science* **1996**, *271*, 1116−1120.
[23] M. T. Huhtala, O. T. Pentikäinen, M. S. Johnson, *Structure* **1999**, *7*, 699−709.
[24] L. Pellegrini, D. F. Burke, F. von Delft, B. Mulloy, T. L. Blundell, *Nature* **2000**, *407*, 1029−1034.
[25] J. Schlessinger, A. L. Plotnikov, O. A. Ibrahimi, A. V. Eliseenkova, B. K. Yeh, A. Yayon, R. J. Linhardt, M. Mohammadi, *Mol. Cell* **2000**, *6*, 743−750.
[26] C. A. A. van Boeckel, M. Petitou, *Angew. Chem.* **1993**, *105*, 1741−1761; *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 1671−1690.
[27] I. Macher, K. Dax, E. Wanek, H. Weidmann, *Carbohydr. Res.* **1980**, *80*, 45−51.
[28] J.-C. Jacquinet, M. Petitou, P. Duchaussoy, I. Lederman, J. Choay, G. Torri, P. Sinaÿ, *Carbohydr. Res.* **1984**, *130*, 183−193.
[29] N. Baggett, A. K. Samra, *Carbohydr. Res.* **1984**, *127*, 149−153.
[30] C. A. A. van Boeckel, T. Beetz, J. N. Vos, A. J. M. de Jong, S. F. van Aelst, R. H. van den Bosch, J. M. R. Mertens, F. A. van der Vlugt, *J. Carbohydr. Chem.* **1985**, *4*, 293−321.
[31] T. Chiba, P. Sinaÿ, *Carbohydr. Res.* **1986**, *151*, 379−389.
[32] D. Medakovic, *Carbohydr. Res.* **1994**, *253*, 299−300.
[33] I. R. Vlahov, R. J. Linhardt, *Tetrahedron Lett.* **1995**, *36*, 8379−8382.
[34] H. G. Bazin, R. J. Kerns, R. J. Linhardt, *Tetrahedron Lett.* **1997**, *38*, 923−926.

[35] M. Dromowicz, P. Köll, *Carbohydr. Res.* **1998**, *308*, 169−171.
[36] H. Hinou, H. Kurosawa, K. Matsuoka, D. Terunuma, H. Kuzuhara, *Tetrahedron Lett.* **1999**, *40*, 1501−1504.
[37] M. Adinolfi, G. Barone, F. DeLorenzo, A. Iadonisi, *Synlett* **1999**, 336−338.
[38] R. Ojeda, J. L. de Paz, M. Martin-Lomas, J. M. Lassaletta, *Synlett* **1999**, 1316−1318.
[39] A. Lubineau, O. Gavard, J. Alais, D. Bonnaffé, *Tetrahedron Lett.* **2000**, *41*, 307−311.
[40] P. Schell, H. A. Orgueira, S. Roehrig, P. H. Seeberger, *Tetrahedron Lett.* **2001**, *42*, 3811−3814.
[41] G. Jaurand, C. Tabeur, M. Petitou, *Carbohydr. Res.* **1994**, *255*, 295−301.
[42] B. La Ferla, L. Lay, M. Guerrini, L. Poletti, L. Panza, G. Russo, *Tetrahedron* **1999**, *55*, 9867−9880.
[43] N. J. Davis, S. L. Flitsch, *J. Chem. Soc., Perkin Trans. 1* **1994**, 359−368.
[44] M. Haller, G.-J. Boons, *J. Chem. Soc., Perkin Trans. 1* **2001**, 814−822.
[45] M. Haller, G.-J. Boons, *Eur. J. Org. Chem.* **2002**, 2033−2038.
[46] S.-C. Hung, S. R. Thopate, F.-C. Chi, S.-W. Chang, J.-C. Lee, C.-C. Wang, Y.-S. Wen, *J. Am. Chem. Soc.* **2001**, *123*, 3153−3154.
[47] S.-C. Hung, R. Puranik, F.-C. Chi, *Tetrahedron Lett.* **2000**, *41*, 77−80.
[48] H. A. Orgueira, A. Bartolozzi, P. Schell, P. H. Seeberger, *Angew. Chem. Int. Ed.* **2002**, *41*, 2128−2131.
[49] H. A. Orgueira, A. Bartolozzi, P. Schell, R. E. J. N. Litjens, E. R. Palmacci, P. H. Seeberger, *Chem. Eur. J.* **2003**, *9*, 140−169.
[50] C. M. Dreef-Tramp, H. A. M. Willems, P. Westerduin, P. van Veelen, C. A. A. van Boeckel, *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1175−1180.
[51] S. P. Douglas, D. M. Withfield, J. J. Krepinsky, *J. Am. Chem. Soc.* **1991**, *113*, 5095−5097.
[52] D. J. Gravert, K. D. Janda, *Chem. Rev.* **1997**, *97*, 489−509.
[53] L. Cipolla, F. Nicotra, L. Lay, U. Lindahl, L. Panza, G. Russo, *Glycoconj. J.* **1996**, *13*, 995−1003.
[54] N. Razi, J. Kreuger, L. Lay, G. Russo, L. Panza, B. Lindahl, U. Lindahl, *Glycobiology* **1995**, *5*, 807−811.
[55] J. Choay, J. C. Lormeau, M. Petitou, *Ann. Pharm. Fr.* **1981**, *39*, 37−44.
[56] B. Casu, P. Oreste, G. Torri, G. Zoppetti, J. Choay, J. C. Lormeau, M. Petitou, P. Sinaÿ, *Biochem. J.* **1981**, *197*, 599−609.
[57] L. Thunberg, G. Bäckström, U. Lindahl, *Carbohydr. Res.* **1982**, *100*, 393−410.
[58] U. Lindahl, L. Thunberg, G. Bäckström, J. Riesenfeld, K. Nordling, I. Björk, *J. Biol. Chem.* **1984**, *259*, 12368−12376.
[59] R. W. Carrel, D. L: Evans, P. E. Stein, *Nature* **1991**, *353*, 576−578.
[60] P. G. Gettings, B. Fan, B. C. Crews, I. V. Turko, S. T. Olson, V. J. Streusand, *Biochemistry* **1993**, *32*, 8385−8389.
[61] C. A. A. van Boeckel, P. D. J. Grootenhuis, A. Visser, *Nat. Struct. Biol.* **1994**, *1*, 423−425.
[62] M. Petitou, J.-P. Hérault, A. Bernat, P.-A. Driguez, P. Duchaussoy, J.-C. Lormeau, J.-M. Herbert, *Nature* **1999**, *398*, 417−422.
[63] G. Torri, B. Casu, G. Gatti, M. Petitou, J. Choay, J.-C. Jacquinet, P. Sinaÿ, *Biochem. Biophys. Res. Commun.* **1985**, *128*, 134−140.
[64] D. R. 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.
[65] D. R. Ferro, A. Provasoli, M. Ragazzi, G. Torri, B. Casu, G. Gatti, J.-C. Jacquinet, P. Sinaÿ, M. Petitou, J. Choay, *J. Am. Chem. Soc.* **1986**, *108*, 6773−6778.
[66] M. Ragazzi, D. R. Ferro, B. Perly, P. Sinaÿ, M. Petitou, J. Choay, *Carbohydr. Res.* **1990**, *195*, 169−185.
[67] P. D. J. Grootenhuis, C. A. A. van Boeckel, *J. Am. Chem. Soc.* **1991**, *113*, 2743−2747.
[68] U. R. Desai, M. Petitou, I. Bjork, S. T. Olson, *J. Biol. Chem.* **1998**, *273*, 7478−7487.
[69] P. Sinaÿ, J.-C. Jacquinet, M. Petitou, P. Duchaussoy, I. Lederman, J. Choay, G. Torri, *Carbohydr. Res.* **1984**, *132*, C5−C9.
[70] M. Petitou, P. Duchaussoy, I. Lederman, J. Choay, P. Sinaÿ, J.-C. Jacquinet, G. Torri, *Carbohydr. Res.* **1986**, *147*, 221−236.
[71] M. Petitou, P. Duchaussoy, I. Lederman, J. Choay, J.-C. Jacquinet, P. Sinaÿ, G. Torri, *Carbohydr. Res.* **1987**, *167*, 67−75.
[72] M. Petitou, G. Jaurand, M. Derrien, J. Choay, *Bioorg. Med. Chem. Lett.* **1991**, *1*, 95−98.
[73] G. Jaurand, J. E. M. Basten, I. Lederman, C. A. A. van Boeckel, *Bioorg. Med. Chem. Lett.* **1992**, *2*, 901−904.
[74] M. Petitou, P. Duchaussoy, G. Jaurand, F. Gouvernec, I. Lederman, J.-M. Strassel, T. Bârzu, B. Crépon, J.-P. Hérault, J.-C. Lormeau, A. Bernat, J.-M. Herbert, *J. Med. Chem.* **1997**, *40*, 1600−1607.
[75] J. E. M. Basten, G. Jaurand, B. Olde-Hauter, P. Duchaussoy, M. Petitou, C. A. A. van Boeckel, *Bioorg. Med. Chem. Lett.* **1992**, *2*, 905−908.
[76] H. Lucas, J. E. M. Bosten, P. Konradsson, C. A. A. van Boeckel, *Angew. Chem.* **1993**, *105*, 462−464; *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 434−436.
[77] P. Westerduin, C. A. A. van Boeckel, J. E. M. Basten, M. A. Broekhoven, H. Lucas, A. Rood, H. van der Heijden, R. G. M. van Amsterdam, T. G. van Dinther, D. G. Meuleman, A. Visser, G. M. T. Vogel, J. B. L. Damm, G. T. Overklift, *Bioorg. Med. Chem.* **1994**, *2*, 1267−1280.
[78] N. Sakairi, J. E. M. Basten, G. A. van der Marel, C. A. A. van Boeckel, J. H. van Boom, *Chem. Eur. J.* **1996**, *2*, 1007−1013.
[79] S. K. Das, J.-M. Mallet, J. Esnault, P.-A. Driguez, P. Duchaussoy, P. Sizun, J.-P. Hérault, J.-M. Herbert, M. Petitou, P. Sinaÿ, *Angew. Chem.* **2001**, *113*, 1723−1726; *Angew. Chem. Int. Ed.* **2001**, *40*, 1670−1673.
[80] S. K. Das, J.-M. Mallet, J. Esnault, P.-A. Driguez, P. Duchaussoy, P. Sizun, J.-P. Hérault, J.-M. Herbert, M. Petitou, P. Sinaÿ, *Chem. Eur. J.* **2001**, *7*, 4821−4834.
[81] P.-S. Lei, P. Duchaussoy, P. Sizun, J.-M. Mallet, M. Petitou, P. Sinaÿ, *Bioorg. Med. Chem.* **1998**, *6*, 1337−1346.
[82] A. Helmboldt, M. Petitou, J.-M. Mallet, J.-P. Hérault, J.-C. Lormeau, P.-A. Driguez, J.-M. Herbert, P. Sinaÿ, *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1507−1510.
[83] M. Petitou, J.-P. Hérault, J.-C. Lormeau, A. Helmboldt, J.-M. Mallet, P. Sinaÿ, J.-M. Herbert, *Bioorg. Med. Chem.* **1998**, *6*, 1509−1516.
[84] Y. C. Xin, J.-M. Mallet, P. Sinaÿ, *J. Chem. Soc., Chem. Commun.* **1993**, 864−865.
[85] T. E. Warkentin, B. H. Chong, A. Greinacher, *Thromb. Hemost.* **1998**, *79*, 1−7.
[86] D. P. Thomas, *Thromb. Hemost.* **1997**, *78*, 1422−1425.
[87] P. D. J. Grootenhuis, P. Westerduin, D. Meuleman, M. Petitou, C. A. A. van Boeckel, *Nature Struct. Biol.* **1995**, *2*, 736−739.
[88] M. Petitou, A. Imberty, P. Duchaussoy, P.-A. Driguez, M.-L. Ceccato, F. Gouvernec, P. Sizun, J.-P. Hérault, S. Pérez, J.-M. Herbert, *Chem. Eur. J.* **2001**, *7*, 858−873.
[89] P. Westerduin, J. E. M. Basten, M. A. Broekhoven, V. de Kimpe, W. H. A. Kuijpers, C. A. A. van Boeckel, *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 331−333.
[90] J. E. M. Basten, C. M. Dreef-Tromp, B. de Wijs, C. A. A. van Boeckel, *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1201−1206.
[91] R. C. Buijsman, J. E. M. Basten, C. M. Dreef-Tromp, G. A. van der Marel, C. A. A. van Boeckel, J. H. Van Boom, *Bioorg. Med. Chem.* **1999**, *7*, 1881−1890.
[92] R. C. Buijsman, W. H. A. Kuijpers, J. E. M. Basten, E. Kuyl-Yeheskiely, G. A. van der Marel, C. A. A. van Boeckel, J. H. van Boom, *Chem. Eur. J.* **1996**, *2*, 1572−1577.
[93] J. Thaden, P. S. Miller, *Bioconjug. Chem.* **1993**, *4*, 395−401.
[94] R. C. Buijsman, J. E. M. Basten, T. G. van Dinther, G. A. van der Marel, C. A. A. van Boeckel, J. H. van Boom, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2013−2018.
[95] M. Petitou, P. Duchaussoy, P.-A. Driguez, G. Jaurand, J.-P. Hérault, J.-C. Lormeau, C. A. A. van Boeckel, J.-M. Herbert, *Angew. Chem. Int. Ed.* **1998**, *37*, 3009−3014.
[96] P. Duchaussoy, G. Jaurand, P.-A. Driguez, I. Lederman, F.

Gouvernec, J.-M. Strassel, P. Sizun, M. Petitou, J.-M. Herbert, *Carbohydr. Res.* **1999**, *317*, 63−84.
[97] P. Duchaussoy, G. Jaurand, P.-A. Driguez, I. Lederman, F. Gouvernec, J.-M. Strassel, P. Sizun, M. Petitou, J.-M. Herbert, *Carbohydr. Res.* **1999**, *317*, 85−99.
[98] C. M. Dreef-Tromp, J. E. M. Basten, M. A. Broekhoven, T. G. van Dinther, M. Petitou, C. A. A. van Boeckel, *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2081−2086.
[99] M. Petitou, P. Duchaussoy, P.-A. Driguez, J.-P. Hérault, J.-C. Lormeau, J.-M. Herbert, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1155−1160.
[100] M. Petitou, P.-A. Driguez, P. Duchaussoy, J.-P. Hérault, J.-C. Lormeau, J.-M. Herbert, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1161−1166.
[101] P.-A. Driguez, I. Lederman, J.-M. Strassel, J.-M. Herbert, M. Petitou, *J. Org. Chem.* **1999**, *64*, 9512−9520.
[102] W. H. Burgess, T. Maciag, *Annu. Rev. Biochem.* **1989**, *58*, 575−606.
[103] R. Ishiai-Michaeli, A. Eldor, I. Vlodavsky, *Cell Regul.* **1990**, *1*, 833−842.
[104] J. Folkman, M. Klagsbrum, J. Sasse, M. Wadzinski, D. Ingber, I. Vlodavsky, *Am. J. Pathol.* **1988**, *130*, 393−400.
[105] I. Vlodavsky, Z. Fuks, R. Ishiai-Michaeli, P. Bashkin, E. Levi, G. Korner, R. Bar-Shavit, M. Klagsbrum, *J. Cell. Biochem.* **1991**, *45*, 167−176.
[106] S. Faham, R. J. Linhardt, D. C. Rees, *Curr. Opin. Struct. Biol.* **1998**, *8*, 578−586.
[107] G. Venkataraman, Z. Shriver, J. C. Davies, R. Sasisekharan, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 1892−1897.
[108] L. Pellegrini, *Curr. Opin. Struct. Biol.* **2001**, *11*, 629−634 and references therein.
[109] T. Spivak-Kroizman, M. A. Lehman, I. Dikic, J. E. Ladbury, D. Pinchasi, J. Huang, M. Jaye, G. Crumley, J. Schlessinger, I. Lax, *Cell* **1994**, *79*, 1015−1024.
[110] C.-P. Kwan, G. Venkataraman, Z. Shriver, R. Raman, D. Liu, Y. Qi, L. Varticovski, R. Sasisekharan, *J. Biol. Chem.* **2001**, *276*, 23421−23429.
[111] D. J. Stauber, A. DiGabriele, W. A. Hendrickson, *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 49−54.
[112] J. Schlessinger, A. L. Plotnikov, O. A. Ibrahimi, A. V. Eliseenkova, B. K. Yeh, A. Yayon, R. J. Linhardt, M. Mohammadi, *Mol. Cell* **2000**, *6*, 743−750.
[113] A. B. Herr, D. M. Ornitz, R. Sasisekharan, G. Venkataraman, G. Waskman, *J. Biol. Chem.* **1997**, *272*, 16382−16389.
[114] F. J. Moy, M. Safran, A. P. Seddon, D. Kitchen, P. Böhlen, D. Aviezer, A. Yayon, R. Powers, *Biochemistry* **1997**, *36*, 4782−4791.
[115] H. Mach, D. B. Volkin, C. J. Burke, C. R. Middaugh, R. J. Linhardt, J. R. Fromm, D. Loganathan, *Biochemistry* **1993**, *32*, 5480−5489.
[116] J. Kreuger, M. Salmivirta, L. Sturiale, G. Giménez-Gallego, U. Lindahl, *J. Biol. Chem.* **2001**, *276*, 30744−30752.
[117] D. M. Ornitz, A. B. Herr, M. Nilsson, J. Westman, C.-M. Svahn, G. Waskman, *Science* **1995**, *268*, 432−436.
[118] M. Guerrini, T. Agulles, A. Bisio, M. Hricovíni, L. Lay, A. Naggi, L. Poletti, L. Sturiale, G. Torri, B. Casu, *Biochem. Biophys. Res. Commun.* **2002**, *292*, 222−230.
[119] H. Habuchi, S. Suzuki, T. Saito, T. Tamura, T. Harada, K. Yoshida, K. Kimata, *Biochem. J.* **1992**, *285*, 805−813.
[120] M. Ishihara, P. N. Shaklee, Z. Yang, W. Liang, Z. Wei, R. J. Stak, K. Holme, *Glycobiology* **1994**, *4*, 451−458.
[121] D. A. Pye, R. R. Vives, J. E. Turnbull, P. Hyde, J. T. Gallagher, *J. Biol. Chem.* **1998**, *273*, 22936−122942.
[122] L. Lundin, H. Larsson, J. Kreuger, U. Lindahl, M. Salmivirta, L. Claesson-Welsh, *J. Biol. Chem.* **2000**, *275*, 24653−24660.
[123] M. Maccarana, B. Casu, U. Lindahl, *J. Biol. Chem.* **1993**, *268*, 23898−23905.
[124] S. Guimond, M. Maccarana, B. B. Olwin, U. Lindahl, A. C. Rapraeger, *J. Biol. Chem.* **1993**, *268*, 23906−23914.
[125] J. E. Turnbull, D. E. Fernig, Y. Ke, M. C. Wilkinson, J. T. Gallagher, *J. Biol. Chem.* **1992**, *267*, 10337−10341.
[126] A. Walker, J. E. Turnbull, J. T. Gallagher, *J. Biol. Chem.* **1994**, *269*, 931−935.
[127] P. Jemth, J. Kreuger, M. Kusche-Gullberg, L. Sturiale, G. Giménez-Gallego, U. Lindahl, *J. Biol. Chem.* **2002**, *277*, 30567−30573.
[128] M. Nilsson, C.-M. Svahn, J. Westmann, *Carbohydr. Res.* **1993**, *246*, 161−172.
[129] J. Westmann, M. Nilsson, D. M. Ornitz, C.-M. Svahn, *J. Carbohydr. Chem.* **1995**, *14*, 95−113.
[130] J. Kovensky, P. Duchaussoy, M. Petitou, P. Sinaÿ, *Tetrahedron: Asymmetry* **1996**, *7*, 3119−3128.
[131] J. Kovensky, P. Duchaussoy, F. Bono, M. Salmivirta, P. Sizun, J.-M. Herbert, M. Petitou, P. Sinaÿ, *Bioorg. Med. Chem.* **1999**, *7*, 1567−1580.
[132] U. Jönnson, L. Flägerstam, B. Johnnson, R. Karlsson, K. Lundh, S. Lofas, B. Persson, H. Roos, I. Ronnberg, *Biotechniques* **1991**, *11*, 620−627.
[133] C. Tabeur, J.-M. Mallet, F. Bono, J.-M. Herbert, M. Petitou, P. Sinaÿ, *Bioorg. Med. Chem.* **1999**, *7*, 2003−2012.
[134] Y. Suda, D. Marques, J. C. Kermode, S. Kusumoto, M. Sobel, *Thromb. Res.* **1993**, *69*, 501−508.
[135] L. Poletti, M. Fleischer, C. Vogel, M. Guerrini, G. Torri, L. Lay, *Eur. J. Org. Chem.* **2001**, 2727−2734.
[136] J.-L. de Paz, J. Angulo, J.-M. Lassaletta, P. M. Nieto, M. Redondo-Horcajo, R. M. Lozano, G. Giménez-Gallego, M. Martín-Lomas, *ChemBiochem.* **2001**, *2*, 673−685.
[137] M. Kan, F. Wang, F. Xu, J. W. Craab, J. Hon, W. L. McKeehan, *Science* **1993**, *259*, 1918−1921.
[138] J. Angulo, J.-L. de Paz, P. M. Nieto, M. Martín-Lomas, *Isr. J. Chem.* **2000**, *40*, 289−299.
[139] F. Chevalier, J. Angulo, R. Lucas, P. M. Nieto, M. Martín-Lomas, *Eur. J. Org. Chem.* **2002**, 2367−2376.
[140] S. Ortega, J. L. García, M. Zazo, J. Varala, I. Muñoz-Willery, P. Cuevas, G. Giménez-Gallego, *Bio/Technology* **1992**, *10*, 795−798.
[141] R. Ojeda, J. Angulo, P. M. Nieto, M. Martín-Lomas, *Can. J. Chem.* **2002**, *80*, 917−936.
[142] M. Sobel, W. R. Fish, N. Toma, S. Luo, K. Bird, K. Mori, S. Kusumoto, S. D. Blystone, Y. Suda, *J. Vasc. Surg.* **2001**, *33*, 587−594.
[143] M. Sobel, B. Adelman, *Thromb. Res.* **1988**, *50*, 815−826.
[144] S. Koshida, *Trends in Glycoscience and Glycotechnology* **2001**, *13*, 209−213.
[145] Y. Suda, K. Bird, T. Shiyama, S. Koshida, D. Marques, K. Fukase, M. Sobel, S. Kusumoto, *Tetrahedron Lett.* **1996**, *37*, 1053−1056.
[146] S. Koshida, Y. Suda, Y. Fukui, J. Ormsby, M. Sobel, S. Kusumoto, *Tetrahedron Lett.* **1999**, *40*, 5725−5728.
[147] S. Koshida, Y. Suda, A. Arano, M. Sobel, S. Kusumoto, *Tetrahedron Lett.* **2001**, *42*, 1293−1296.
[148] S. Koshida, Y. Suda, M. Sobel, S. Kusumoto, *Tetrahedron Lett.* **2001**, *42*, 1289−1292.
[149] S. Koshida, Y. Suda, M. Sobel, J. Ormsby, S. Kusumoto, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 3127−3132.
[150] M. Monreal, E. Lafoz, A. Urrutia, J. Roncales, R. Galimany, C. Biosca, A. Corominas, *Haemostasis* **1995**, *25*, 283−287.
[151] Z. M. Merchant, E. E. Erbe, W. P. Eddy, D. Patel, R. J. Linhardt, *Atherosclerosis* **1986**, *62*, 151−158.
[152] B. Casu, E. A. Johnson, M. Mantovani, B. Mulloy, P. Oreste, R. Pescador, G. Prino, G. Torri, G. Zoppetti, *Arzneim.-Forsch./Drug Res.* **1983**, *33*, 135−142.
[153] N. Parthasarathy, I. J. Goldberg, P. Sivaram, B. Mulloy, D. M. Flory, W. D. Wagner, *J. Biol. Chem.* **1994**, *269*, 22391−22396.
[154] M. Petitou, P. Duchaussoy, A. Bernat, P. Hoffmann, J.-M. Herbert, *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2067−2070.

Received December 28, 2002

