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Chemical Approaches to Define the Structure-Activity Relationship of Heparin-like Glycosaminoglycans

Review

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Heparin, the drug of choice for the prevention and treatment of thromboembolic disorders, has been shown to interact with many proteins. Despite its widespread medical use, little is known about the precise sequences that interact with specific proteins. The minimum heparin binding sequence for FGF1 and FGF2 necessary to promote signaling was investigated. A characteristic pentasaccharide sequence, DEFGH, is required to accelerate the inhibition of thrombin and factor Xa in the blood-coagulation cascade. The first synthetic heparin pentasaccharide drug has been approved in Europe and the US and is sold under the trade name Arixtra. Other oligosaccharides with different composition are under clinical investigation. The enormous interest in the assembly of heparin oligosaccharides will stimulate the development of new synthetic approaches. Heparin-oligosaccharide-synthesis automation similar to that of DNA or peptide synthesis will play an important role.

Introduction

Proteoglycans are major components of the extracellular matrix that surround all mammalian cells. Different core proteins anchor glycosaminoglycan polysaccharides in the outside of the lipid bilayer. Heparin and heparan sulfate are the most complex glycosaminoglycans (GAGs), a family of molecules that also includes chondroitin sulfate, keratin sulfate, and dermatan sulfate. GAGs have important biological functions by binding to different growth factors, enzymes, morphogens, cell adhesion molecules, and cytokines [1–5].

Heparin, found only in mastocytes, has served as an anticoagulant in heart disease for more than 60 years [6, 7]. It has a molecular weight ranging from 5 to 40 kDa, with an average molecular weight of about 15 kDa. These linear, unbranched, highly sulfated polysaccharides are composed of disaccharide units consisting of a uronic acid 1,4-linked to a D-glucosamine unit (Figure 1). The uronic acid residues are more often L-iduronic acid (90%) than its C5 epimer D-glucuronic acid (10%). A prototypical heparin disaccharide contains three sulfate groups. These sulfate groups render heparin one of the most acidic macromolecules in nature. *O*-sulfation normally occurs at the 2 position of the uronic acids and the 3- and/or 6-position of the glucosamine. In addition, the glucosamine nitrogen may be sulfated, acetylated or, less frequently, may remain unmodified, thus resulting in 48 possible disaccharides that make up heparin [8].

Heparan sulfate is structurally related to heparin but is widely distributed in different cell types and tissues. With a molecular weight range of 5 to 50 kDa and an average molecular weight of 30 kDa, heparan sulfate chains are generally longer than those of heparin. Heparan sulfate is more heterogeneous than heparin, is richer in *N*-acetyl D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA) units, and contains less *O*-sulfates [9].

Much research on the structure, function, and biological activity of heparin and heparan sulfate has been carried out. A more detailed structure-activity relationship for heparin oligosaccharides has evolved in recent years. This more-detailed understanding of GAGs has been aided by the availability of synthetic, completely defined heparin oligosaccharides. Because many aspects of heparin chemistry [10–13] and biology [4, 14, 15] have been reviewed recently, after a brief general introduction, we will focus on studies using synthetic heparin oligosaccharides to elucidate the SAR of heparin and heparan sulfate.

Biosynthesis

To understand the way GAG sequences interact with particular proteins, it is helpful to consider the mechanism by which nature synthesizes different heparins. Much progress in understanding GAG biosynthesis has been made in recent years [16]. Heparin and heparan sulfate are assembled via a similar pathway. Chain initiation occurs in the Golgi apparatus. A tetrasaccharide unit that is attached to a core protein primes chain elongation. *N*-Acetyl D-glucosamine and glucuronic acid monosaccharide units are alternatingly incorporated from the nonreducing end of the nascent polymer with the corresponding sugar nucleotides. Glucuronic acid is attached by glucuronyltransferase followed by the addition of the first *N*-acetyl glucosamine residue. Two glycosyl transferases (EXT1 and EXT2) that form a hetero-oligomeric complex in the Golgi are responsible for the disaccharide addition [17]. As the polysaccharide chain forms, it undergoes a series of modification reactions catalyzed by at least four families of sulfotransferases and one epimerase [18]: *N*-deacetylation and *N*-sulfation is carried out by a *N*-deacetylase and a *N*-sulfotransferase. The transformation of D-glucuronic units to L-iduronic units is catalyzed by a C-5 epimerase. Different *O*-sulfotransferases are responsible for *O*-sulfation of the iduronic acid residues. The action of these enzymes results in tremendous heterogeneity within modified regions.

Structure of Heparin/Heparan Sulfate

Heparin does not fold into tertiary structures like proteins but exists primarily as a helical structure [19]. The sulfate and carboxyl groups of heparin promote specific interactions with biologically important proteins [20]. The pyranose rings of the monosaccharide residues within heparin oligosaccharides adopt the $^1C_4$

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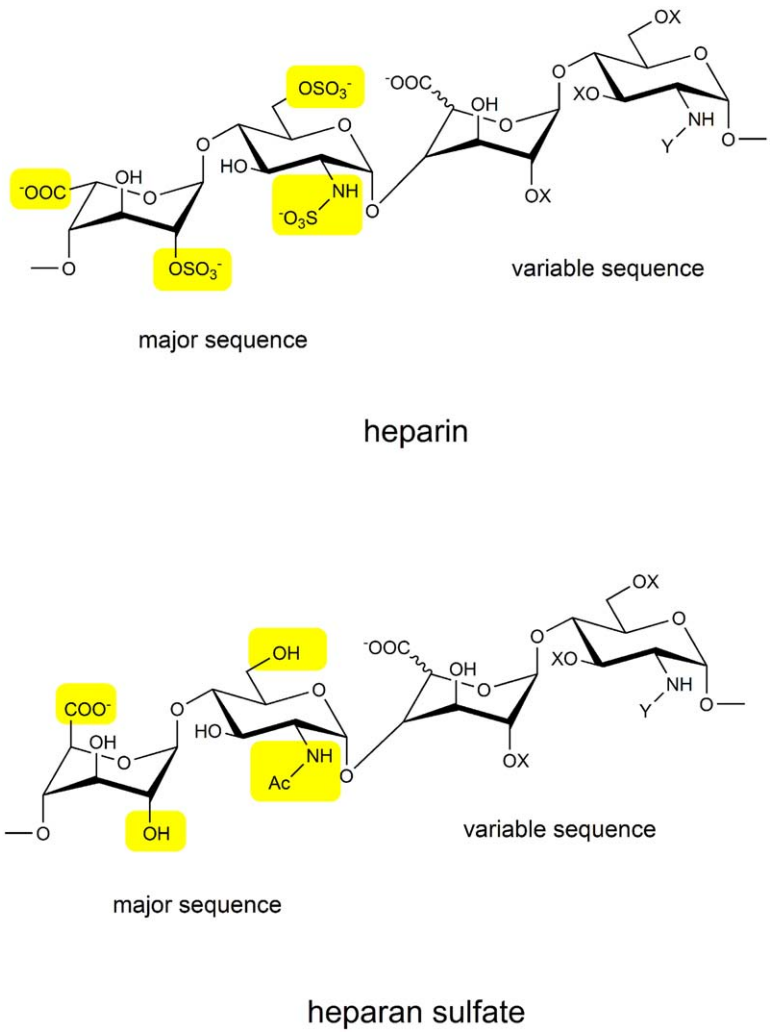


Figure 1. Major and Minor Disaccharide Repeating Units in Heparin and Heparan Sulfate

X = H or SO_3^-; Y = Ac, SO_3^-, or H.

and $^4C_1$ chair conformation as well as the $^2S_0$ skew-boat conformation [21–23]. NMR studies and X-ray structures show that the pyranose rings of glucuronic acid and glucosamine residues prefer the $^4C_1$ chair conformation whereby all nonhydrogen substituents, except the anomeric hydroxyl group of glucosamine, are equatorial [24, 25]. The iduronic acid pyranose is more flexible: bearing a sulfate group at C-2, the $^1C_4$ chair and the $^2S_0$ skew-boat conformation are preferred. Unsubstituted iduronic acid, however, resides predominantly in the $^1C_4$ form [24, 26]. The flexibility of the L-iduronic acid residues is important for specific heparin-protein interactions.

Different attractive forces are responsible for the heparin-protein interplay: ionic interactions are dominant as the negatively charged sulfate and carboxyl groups form ion pairs with positively charged amino acids on the protein [27]. Nonionic interactions such as hydrogen bonding as well as hydrophobic forces also contribute to the binding. The binding affinity of the interaction depends on the ability of the oligosaccharide sequence to provide optimal charge (orientation of the sulfates) and surface (van der Waals contact) complimentary with the protein. Crystal structure of highly sulfated GAG oligosaccharides have shown that ionic contacts are not sufficient to explain the optimal structural fit of a GAG oligosaccharide to the binding site of the protein, which influences the affinity of the interaction. From the conformation of the GAG, it can be envisioned that protein binding would induce local distortions that are manifested as changes in the glycosidic torsion angles. These conformation changes would enable an optimal structural fit in the terms of both ionic and van der Waals contacts between the oligosaccharide motif and the protein [28]. The complexity of GAG polymers has greatly complicated the evolution of detailed structure-activity relationships. However, it has been shown that defined length and sequences of GAGs are responsible for the binding to proteins and hence modulate the biological activity of particular proteins. A more detailed work on the structural insights into biological roles of GAGs and proteins has recently been reviewed [29].

Heparin Sequences of Biological Significance

Many aspects of heparin binding proteins have been reviewed [4], and the best studied interactions are briefly summarized below.

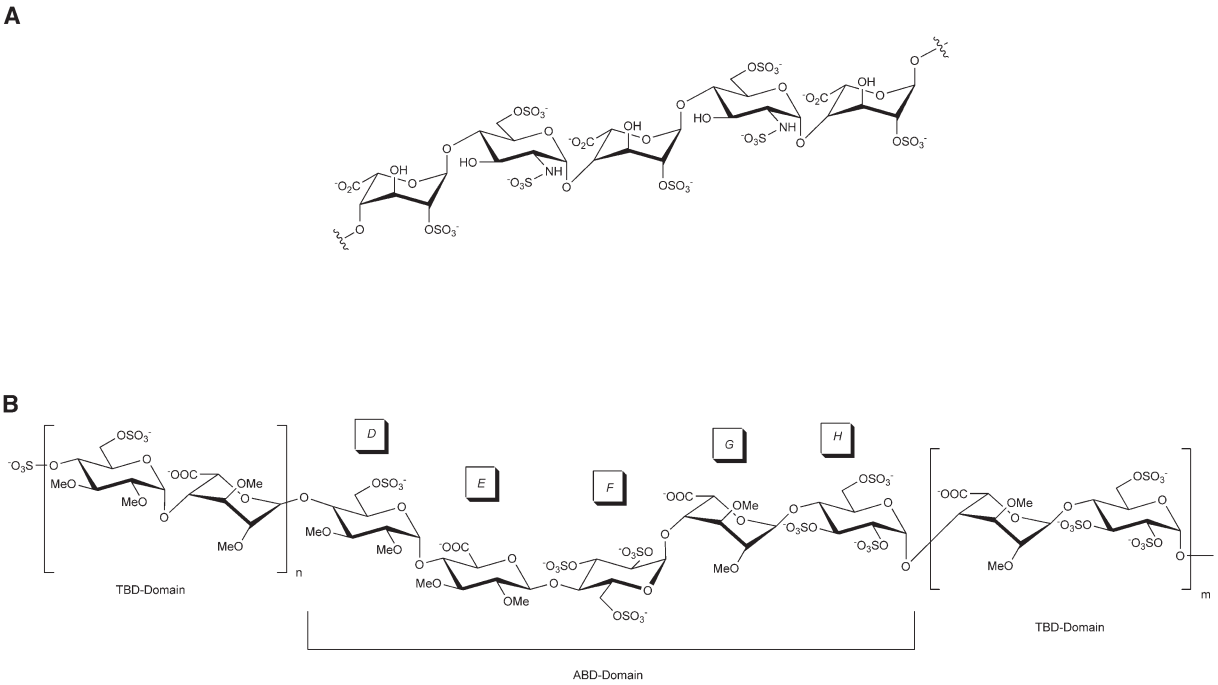


Figure 2. Heparin Oligosaccharides

(A) The FGF binding domain.

(B) The AT III binding domain: a heparin molecule contains an antithrombin binding domain (ABD) and thrombin binding domains (TBD).

Interactions With Growth Factors

Many growth factors, including the fibroblast growth factors (FGFs), bind to the extracellular matrix of target tissues by interacting with GAGs such as heparin and heparan sulfate. The mammalian FGFs belong to a protein family involved in cell proliferation, differentiation, and angiogenesis. Growth factors bind relatively tightly to GAGs [30–32].

Heparin interacts directly with the FGF2 receptor and mediates a high affinity FGF·FGFR complex [33]. One FGF molecule binds to four to five saccharide units in heparin (Figure 2A) [34, 35]. Heparan sulfate stabilizes the formation of FGF oligomers, an essential step to promote the oligomerization and activation of tyrosine kinase FGF receptor (FGFR). The FGFs interact with two distinct extracellular receptors and proteoglycan modified FGFR dimerization activates the FGF-mediated signal-transduction process.

The most thoroughly studied members of the fibroblast growth-factor family are FGF1 (acidic FGF) and FGF2 (basic FGF). The minimum heparin sequences required for FGF1 and FGF2 to promote assembly of active structures have been determined [36–40]. Short oligomers such as tri- and pentasaccharides are able to bind FGF2 [31, 37, 41]; however, longer oligomers are necessary for dimerization and activation of FGFs [42]. X-ray crystal structures of FGF1 [43] and FGF2 [31] complexed with heparin oligosaccharides helped identify functional groups crucial for signaling [19]. The binding region for FGF2 has been identified as a pentasaccharide sequence containing a single, essential, *O*-sulfate group at C2 of iduronic acid [41] and *N*-sulfated D-glucosamine. For efficient binding of FGF2, all uronic acid units must be present as L-iduronic acids [44, 45]. Although 6-*O*-sulfate groups are necessary for binding and activation of FGF1, sulfates are not required for binding to FGF2 [41, 46] but are thought to be essential for the mitogenic activity of FGF2 [47]. Crystal structures of FGF-heparin complexes further strengthen these observations [31, 43]. Although FGF-heparin interactions are dominated by interactions involving sulfate groups, nonsulfated oligosaccharides can also bind to the same site, as shown by the structure of a complex between FGF2 and a trisaccharide heparin-derivative [37]. For the FGF system, only one structure has been determined with sufficient resolution to allow for the direct identification of the structural details of the glycan [31].

Interactions With Antithrombin III

The most thoroughly studied heparin binding protein is the serine protease inhibitor antithrombin III (AT III) that interacts with thrombin and factor Xa in the blood-coagulation cascade [10]. The heparin-AT III interaction is responsible for the anticoagulant activity of heparin. A characteristic heparin pentasaccharide sequence, termed DEFGH, is necessary for binding to AT III, a process that has been studied in great detail [48, 49], including NMR spectroscopy [50] and X-ray crystallography [31, 51].

Crystallographic studies of an AT III/pentasaccharide complex have identified the important functional groups within the pentasaccharide sequence: four charged sulfate groups and two carboxyl groups are responsible for the binding to AT III [52]. The importance of the 3-*O*-sulfate group in unit F of the AT III binding site [53] was demonstrated by NMR. The 6-*O*-sulfate substituent of glucosamine D [54, 55], the *N*-sulfate group of unit F [56], and the carboxylate group of the iduronic acid residue G are also required for binding to AT III and affect anticoagulant activity. The 2-*O*-sulfate group on

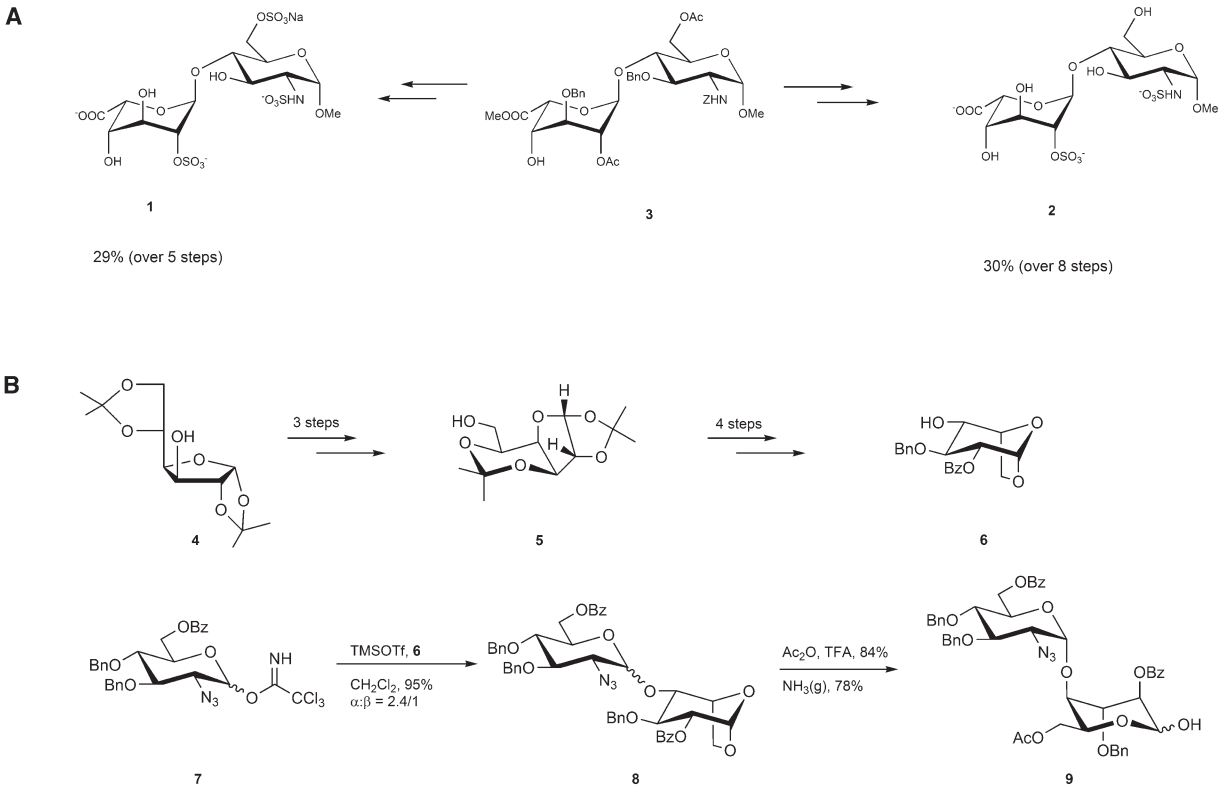


Figure 3. Modular Assembly Approaches

(A) Synthesis of the regular sequence disaccharides of heparin.

(B) Synthesis of the 1,6-anhydro-β-L-hexopyranose.

iduronic acid G and the 6-*O*-sulfate group of unit H are not essential for binding but contribute to the overall binding affinity [57].

Heparin's antithrombotic activity results from a ternary complex formed by heparin, AT III, and thrombin [58, 59]. The interaction of AT III with the pentasaccharide sequence DEFGH results in a conformational change of the protein [4, 60]. AT III-pentasaccharide binding directly accelerates the inhibition of factor Xa but not that of thrombin. The SAR of thrombin inhibition is much more complicated. In the case of thrombin, a conformational change is not sufficient to neutralize the enzyme. Although the pentasaccharide sequence is required for binding to AT III, a heparin chain containing 14–20 saccharide units is required to accelerate the AT III/thrombin interaction (Figure 2B). Heparin forms a bridge between AT III and thrombin in the ternary AT III/heparin/thrombin complex containing 6 to 8 sulfated monosaccharide units that do not interact with protein. Although the thrombin inhibition is a desirable effect of heparin, nonspecific interactions with other proteins (e.g., platelet factor 4) result in life-threatening side effects [61–63].

Chemokine Interactions

Chemokines, a family of over 40 structurally related glycoproteins that facilitate leukocyte migration, angiogenesis, breast cancer metastasis [64], and leukocyte degranulation, also interact with heparin. Platelet factor 4 (PF-4, CXC chemokine ligand 4), a basic tetramer of identical subunits, forms a very stable 1:1 complex with heparin (MW > 10,000) [65]. Therapeutically administered heparin binds to PF-4, thus resulting in heparin-induced thrombocytopenia (HIT), a dangerous, immunologically induced loss of platelets. Other chemokines that bind to heparin with varying affinity and specificity are the stromal-cell-derived factor-1α (SDF-1α), the monocyte chemoattractant protein-1 (MCP-1), and the macrophage inflammatory peptide-1 (MIP-1).

Annexin Interactions

Annexins comprise a family of over 30 calcium and phospholipids binding proteins. At least one of the annexins is expressed in nearly every eukaryotic cell type indicating the wide range of biological functions. The phospholipid/membrane binding properties of the annexins are responsible for their anti-inflammatory activity and exhibit anticoagulant and calcium-channel activity [66, 67]. In addition, annexins are involved in membrane fusion, exocytosis, and endocytosis [68].

Annexin V is one of the few proteins that bind heparin in a calcium-dependent manner [69] as verified by X-ray crystallography [70]. The interaction of annexin V with heparin involves an oligosaccharide sequence of 6–8 residues [69], and sulfate groups on heparin are important. Annexins IV, V, and VI were found to bind to heparin, heparan sulfate, and chondroitin sulfate [71].

Chemical Synthesis of Defined Heparin Oligosaccharides

Access to defined heparin oligosaccharides is of utmost importance to establish a detailed SAR- and cor-

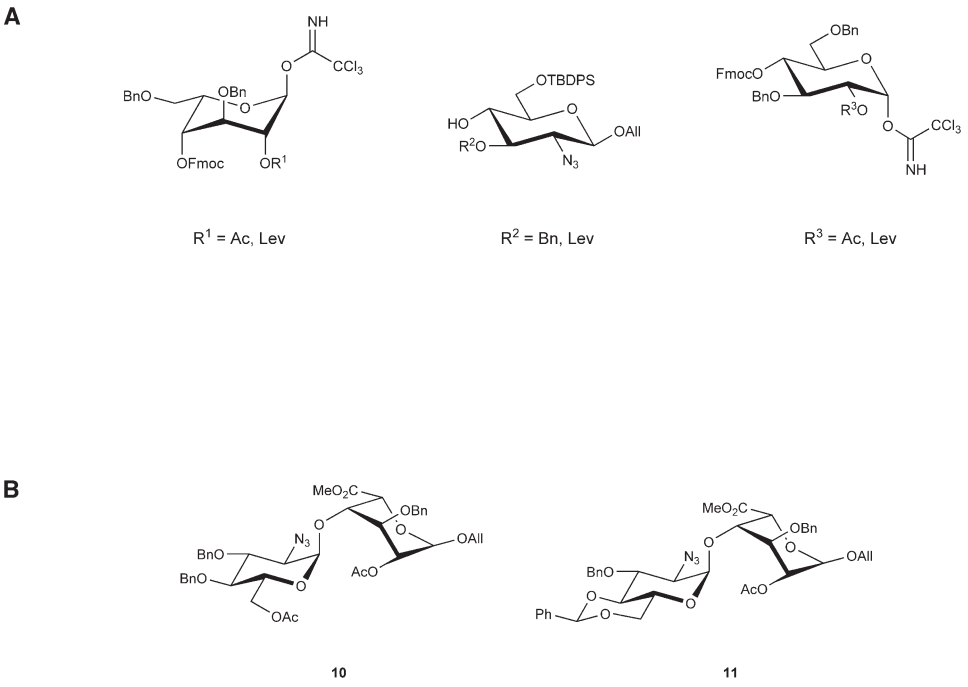


Figure 4. Buildings Blocks for the Assembly of Heparin Oligosaccharides

(A) Building blocks for the synthesis of 20 different heparan sulfate disaccharide units.

(B) Disaccharide moieties with reversed sequences.

relate-specific heparin sequences and sulfation patterns to protein binding and biological activity. The purification of oligosaccharides after enzymatic digestion of GAG chains has provided access to usable quantities of oligomers up to hexasaccharides [72–74]. Chemical synthesis, although challenging in the context of heparin oligosaccharides, has been used to procure defined sequences and analogs for biological studies and to define SAR [10–12]. In this section, we first briefly summarize the preparation of building blocks used during heparin oligosaccharide assembly followed by a review of the different general methods used to prepare oligosaccharides.

Synthesis of Building Blocks for Heparin Synthesis

The preparation of sufficient quantities of fully differentiated building blocks incorporating appropriate protecting groups is particularly important for the synthesis of complex oligosaccharides such as heparin. The placement of specific temporary protecting groups is required at the 4-hydroxyl group of each building block to allow for easy deprotection in anticipation of

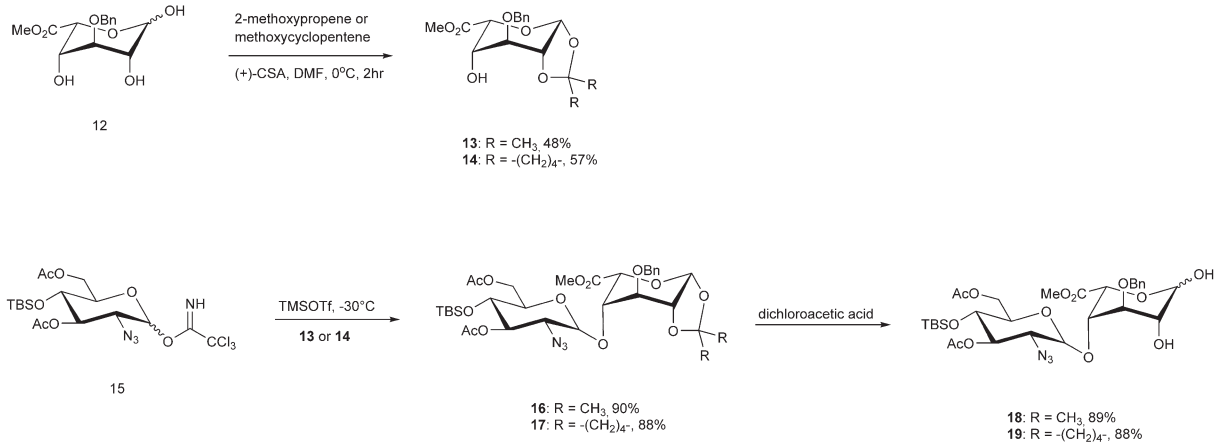


Figure 5. Assembly of a Disaccharide with a Locked Iduronic Acid Acceptor

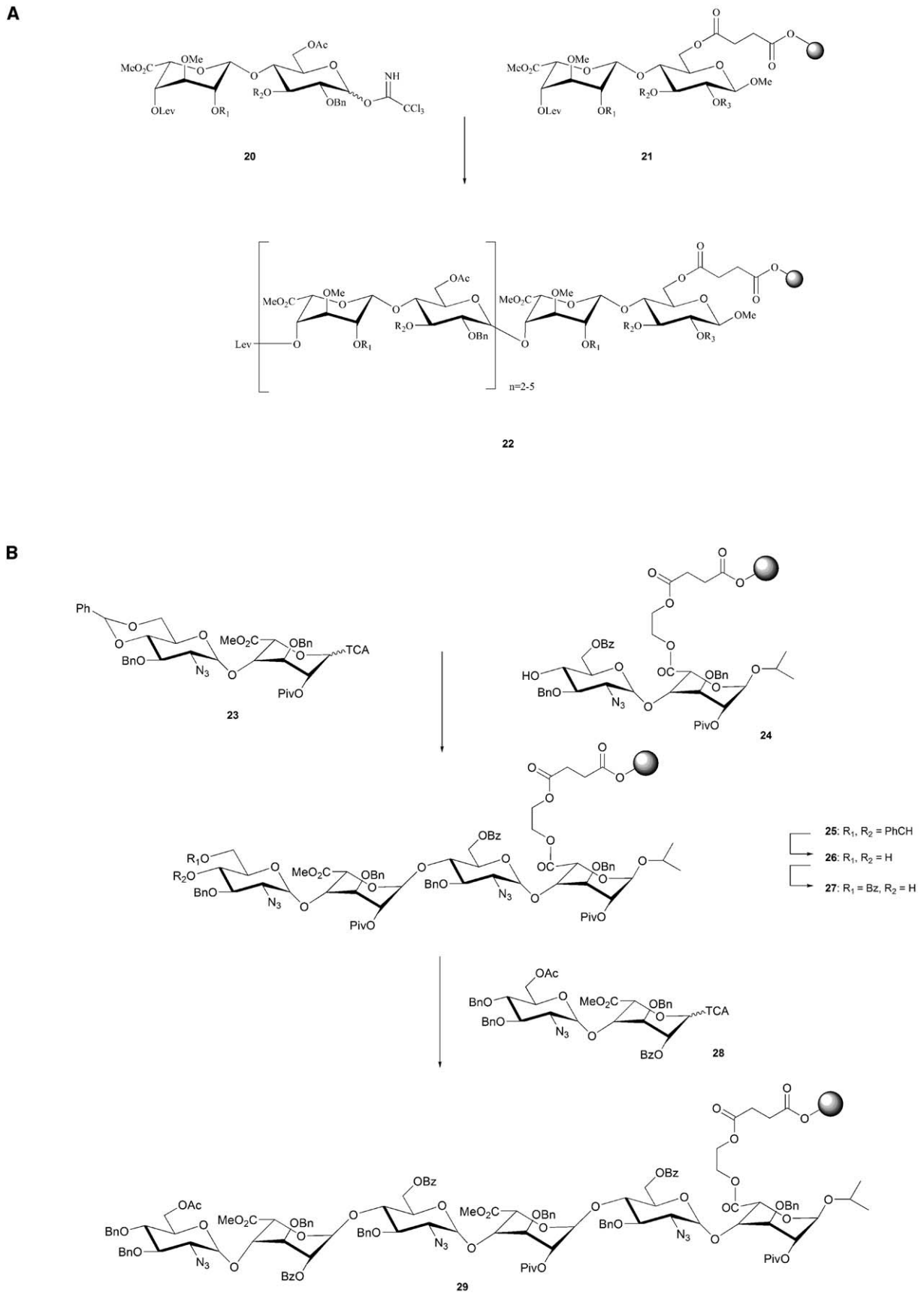
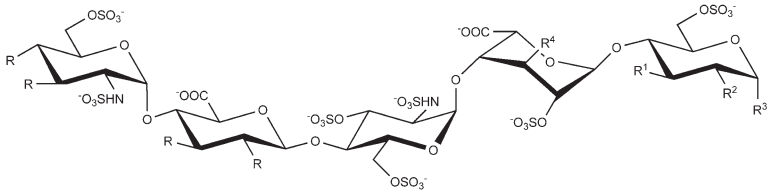


Figure 6. Synthesis of Polymer Bound Oligosaccharides



30-36

Figure 7. Different Pentasaccharides with Xa Affinity

	R	R^1	R^2	R^3	R^4	Anti-Xa activity
30	OH	OH	$NHSO_3^-$	OH	OH	700 units/mg
31	OH	OH	$NHSO_3^-$	OMe	OH	700 units/mg
32	OH	OSO_3^-	$NHSO_3^-$	OMe	OH	1270 units/mg
33	OH	OSO_3^-	OSO_3^-	OMe	OH	1300 units/mg
34	OH	OSO_3^-	OSO_3^-	OMe	OMe	1110 units/mg
35	OMe	OSO_3^-	$NHSO_3^-$	OMe	OH	1288 units/mg
36	OMe	OSO_3^-	OSO_3^-	OMe	OMe	1323 units/mg

chain elongation. Here, levulinoyl esters [75, 76], Fmoc carbonates [77], silyl ethers [75, 78, 79], and PMB ethers [80–82] have found use. The hydroxyl groups to be *O*-sulfated at the end and those that should remain free have to be protected differentially. Commonly, acetate esters serve to mark the hydroxyl groups to be sulfated, whereas benzyl ethers mask hydroxyl groups that will not be modified. The amine group of D-glucosamine requires the placement of different protecting groups. Azides have been commonly used in this function [79, 82–84].

The choice of anomeric leaving groups presents another important consideration in oligosaccharide assembly. Increasingly efficient glycosylation reactions have been developed, and glycosyl trichloroacetimidate and thioglycoside building blocks have replaced methods requiring the use of heavy metal activators. Commonly used, commercially available, and inexpensive starting materials for the synthesis of differentially protected glucosamine and glucuronic acid building blocks are D-glucosamine and diacetone glucose.

Because L-iduronic acid is not accessible from natural sources, many strategies to obtain this monosaccharide by manipulation of D-glucuronic acid derivatives have been developed [85–95]. Recently, a host of methods starting from D-glucuronolactone, D-diacetone glucose, and D-glucuronic acid glycals as starting materials have been reported [8, 96–105]. Nevertheless, the synthesis of iduronic acid building blocks remains a major challenge en route to the desired heparin oligosaccharides.

Initially, total synthesis efforts targeted a single heparin oligosaccharide and yielded many valuable insights. In recent years, modular approaches aiming at the synthesis of a broad range of heparin/heparan sulfate oligosaccharide fragments have been developed.

Modular Synthesis of Heparin Oligosaccharides

The first modular assembly approach targeted the common heparin disaccharide repeating unit **1** and its 6-*O*-unsulfated counterpart **2** (Figure 3A) [80]. Disaccharide **1** was prepared in five steps from disaccharide **3** (29% overall yield) [85], whereas the assembly of **2** required eight steps (30% yield).

Another modular approach relied on the late stage formation of uronic acids by selective oxidation of the C-6 hydroxyl groups of glucose and idose residues. Thus, problems such as epimerization of the C-5 position of uronic acids and poor coupling efficiency of uronic acid-based glycosylating agents were circumvented [79]. It was calculated that 20 disaccharide building blocks would be required to assemble all possible linear oligosaccharide chains.

Another synthesis to disaccharide subunits starts from diacetone glucose **4** that was converted via 1,2:3,5-di-*O*-isopropylidene-β-L-idofuranose **5** to the desired 1,6-anhydro-β-L-hexopyranose **6** (Figure 3B) [107, 108]. Union of **6** and **7** upon TMSOTf activation provided disaccharide **8**. The α-isomer was isolated and acetylated, and the 1,6-anhydro-β-L-idopyranosyl ring was opened. Removal of the anomeric acetate afforded the desired lactol **9**, ready for the next glycosylation.

Six different monosaccharide building blocks that can be combined to form the 20 disaccharides found in heparan sulfate were prepared (Figure 4A) [106]. Four different protecting groups (Lev, Fmoc, TBDPS, and All) were employed in these syntheses. The Fmoc group

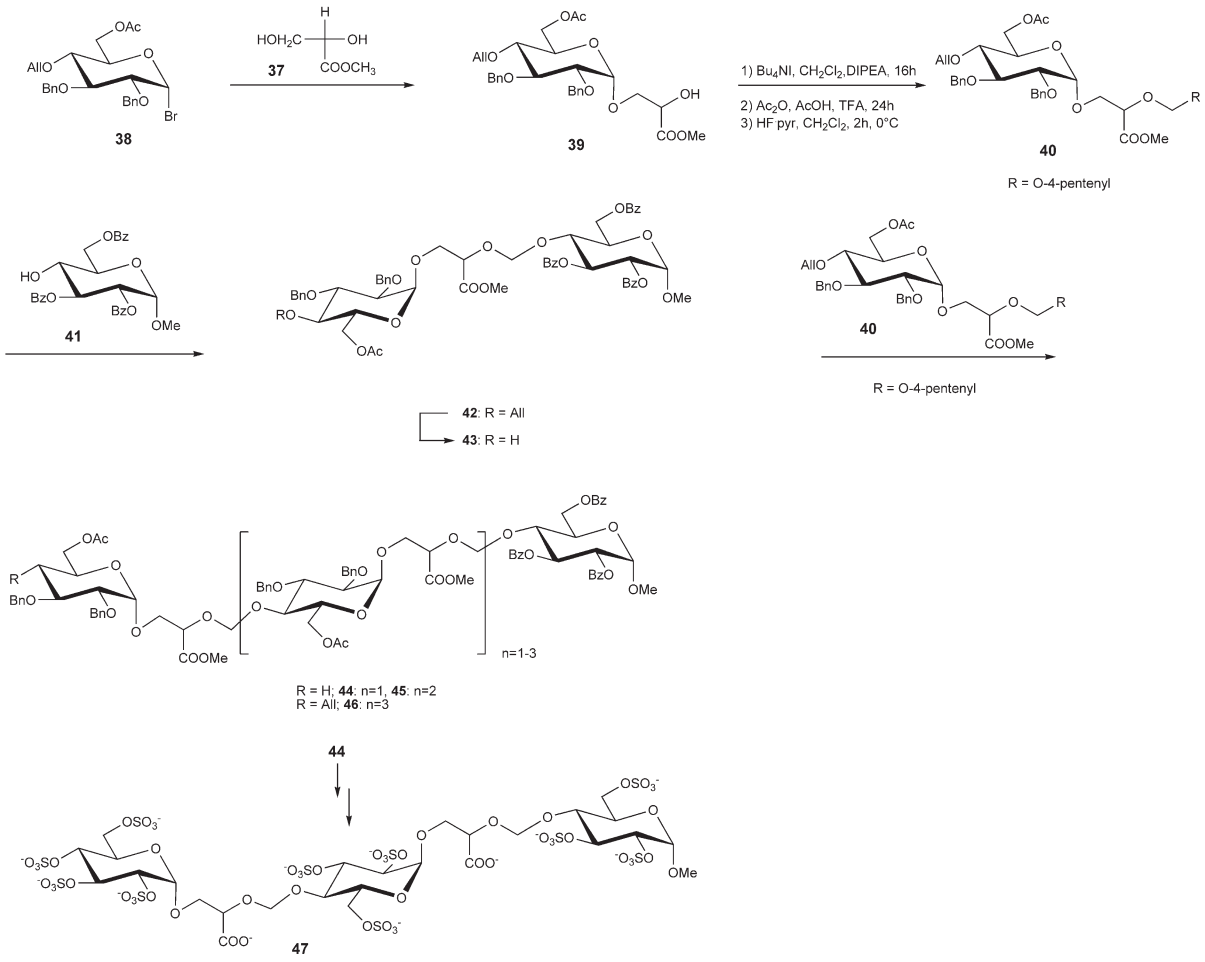


Figure 8. Preparation of Heparin Analogs

was chosen to mark the C-4′ hydroxyls for chain elongation, whereas the anomeric center of the disaccharide was protected in form of an allyl glycoside. Benzyl ethers masked primary hydroxyls that were subsequently oxidized to carboxylic acids and secondary hydroxyls that remain unsulfated. Levulinoyl esters were utilized to protect the C-2′ position to ensure stereoselective formation of 1,2-*trans*-glycosidic linkages. Finally, the C-6 position of glucosamine was protected as TBDPS ether.

Two disaccharides (**10** and **11**) containing glucosamines at the nonreducing end allowed for a systematic approach to prepare inverse sequence (glucosamine-iduronic acid) oligosaccharides in contrast to fragments obtained by chemical or enzymatic processing of polysaccharides (Figure 4B) [109].

The selective installation of the α-glucosamine glycosidic linkage found in this disaccharide by coupling glucosamine with an iduronic acid is the most challenging task in the synthesis of heparin oligosaccharides. A new approach for stereochemical control of glycosylation reactions by controlling the conformation of the nucleophile was developed [110]. Introduction of a cyclic protecting group (e.g., isopropylidene or cyclopentylidene) as conformational lock for the monosaccharide nucleophile **12** ensured the exclusive formation of the desired α-linked disaccharides (**18** and **19**) (Figure 5) [104].

Preparation of disaccharide building blocks by regio- and stereoselective glycosylation of an iduronic acid ester diol with a 2-azido-2-deoxy-D-glucopyranosyl trichloroacetimidate was also reported [111]. Four different series of hexa- and octasaccharides were synthesized with this disaccharide unit.

Solid-Phase Synthesis of Heparin Oligosaccharides

Ideally, defined heparin sequences could be prepared by automated solid-phase synthesis with methods analogous to those that already exist for the assembly of oligonucleotides and peptides. Although automated solid-phase synthesis of oligosaccharides is now possible [112], only a few polymer-supported syntheses of heparin oligosaccharides have been disclosed [113–115]. Soluble polyethylene glycol (PEG) polymers gave encouraging results for the synthesis of fully protected oligosaccharides **22** that were obtained by coupling of disaccharide **20** with polymer-bound iduronic acid **21** (Figure 6A). Each elongation cycle incorporated levulinoate cleavage, coupling, and capping.

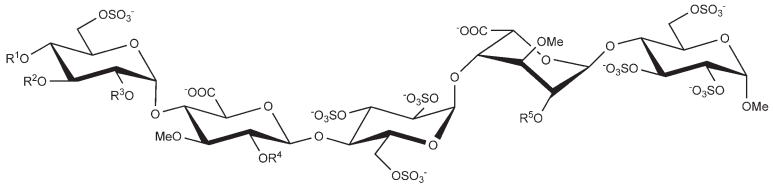


Figure 9. Structures of Pentasaccharides with Pseudoalternating Sequence

	R^1	R^2	R^3	R^4	R^5	Anti-Xa activity
48	Me	Me	Me	SO_3^-	SO_3^-	1217 units/mg
49	Me	Me	SO_3^-	SO_3^-	SO_3^-	1159 units/mg
50	Me	SO_3^-	SO_3^-	SO_3^-	SO_3^-	1184 units/mg
51	Me	Me	Me	Me	Me	1611 units/mg
52	Me	Me	SO_3^-	Me	Me	1318 units/mg
53	Me	SO_3^-	SO_3^-	Me	Me	1404 units/mg

In a second approach, disaccharide **23** was coupled to polyethylene glycol monomethyl ether (MPEG) bound disaccharide **24** to furnish MPEG bound tetrasaccharide **25**. Subsequent reaction of **27** with disaccharide **28** afforded MPEG bound hexasaccharide **29** that was cleaved at the end from the solid support (Figure 6B).

This section summarized different approaches for the modular synthesis of oligosaccharides. Future effort will focus on the synthesis of larger quantities of heparin and heparan sulfate with an automated oligosaccharide synthesizer to produce libraries of biologically significant heparin and heparan oligosaccharides.

Synthesis of Heparin Oligosaccharides with Anti-Factor Xa Activity

The anticoagulant activity of pentasaccharide DEFGH (Figure 2B) prompted synthetic efforts aimed at the procurement of this structure as well as a host of related sequences (Figure 7). The first synthetic heparin oligosaccharide **30** with a high affinity for AT III was synthesized in 1986 [116, 117].

α-methyl-glycoside **31**, displaying the same biological properties as **30**, was prepared to simplify the chemical synthesis [118, 119]. The synthesis of analogs of **30** has been reviewed in detail [10]. Pentasaccharides **32–36** were synthesized to establish a detailed structure-activity relationship. Replacement of the reducing end glucosamine by a glucose residue indicated that *O*-sulfates are effective substitutes for *N*-sulfates [119]. Introduction of an extra 3-*O*-sulfate group at the reducing end of pentasaccharide **32** increased factor Xa affinity [120]. Partial and complete *O*-methylation (**31–36**) did not significantly alter AT III affinity [121, 122].

To assess the role of iduronic acid in the pentasaccharide with Xa affinity we replaced this unit with a glyceric acid oxymethylene linker to give **37** (Figure 8) [123]. These fragments are readily accessible and offer the advantage that no α/β mixture is formed during glycosylation. Coupling of **37** with glycosyl bromide **38**, followed by epimerization, gave **39**. Union of **40** and **41** furnished trisaccharide **42**. After deallylation, **43** was coupled with **40** to afford pentasaccharide **44**. The repetition of deprotection and coupling steps afforded heptasaccharide **45** and nonasaccharide **46**.

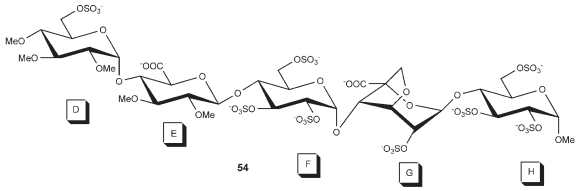
Analogs **48–53** closely resemble heparin oligosaccharides and their function but are simpler to synthesize. These so-called "nonglycosamino" glycans contain only *O*-sulfate esters and *O*-alkyl ethers [75]. All hydroxyl groups are permanently capped as methyl ethers, thus eliminating the need to discriminate between nonsulfated and sulfated hydroxyl groups. Many analogs with pseudo-alternating sequences have been synthesized [124, 125].

Pentasaccharides **48–50** contain a common tetrasaccharide composed of 2-*O*-sulfate and 3-*O*-methyl uronic acid moieties, whereas pentasaccharides **51–53** contain an invariable tetrasaccharide composed of 2,3-di-*O*-methyl uronic acid moieties (Figure 9). Pentasaccharide **51**, containing not less than seven methyl ethers, displays the highest anti-Xa activity (1611 units/mg) and is the most potent analog of **31** identified to date.

Octasulfated pentasaccharide **54** containing an L-iduronic acid unit in a fixed $^1C_4$ conformation was synthesized to determine whether the $^1C_4$ or the $^2S_0$ conformation of L-iduronic acid is necessary for binding and activation of AT III (Figure 10A) [126]. Pentasaccharide **54** exhibited very low anti-Xa activity, indicating that either the $^2S_0$ conformation of L-iduronic acid or a flexible L-iduronic acid residue, or the presence of both conformers, is essential to bind and activate AT III.

Further studies involving different pentasaccharides

A



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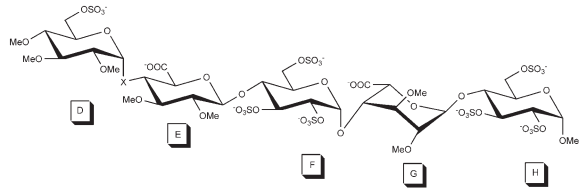
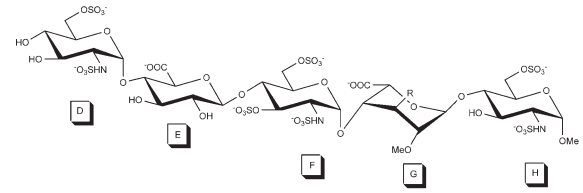
B



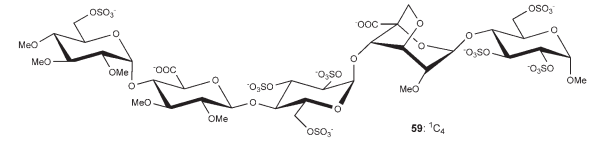
55: X = CH_2
56: X = O

C

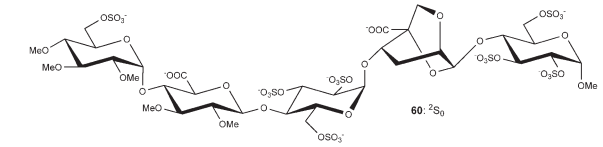


57: R = H
58: R = OH

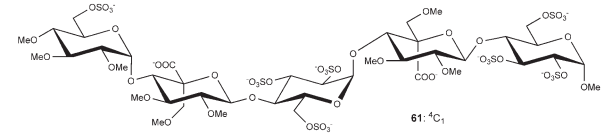
D



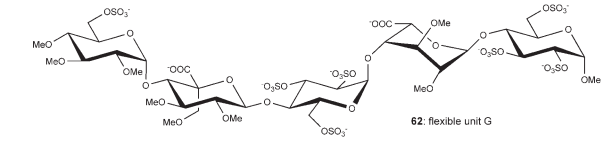
59: $^1C_4$



60: $^2S_0$

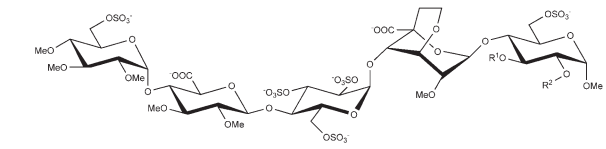


61: $^4C_1$



62: flexible unit G

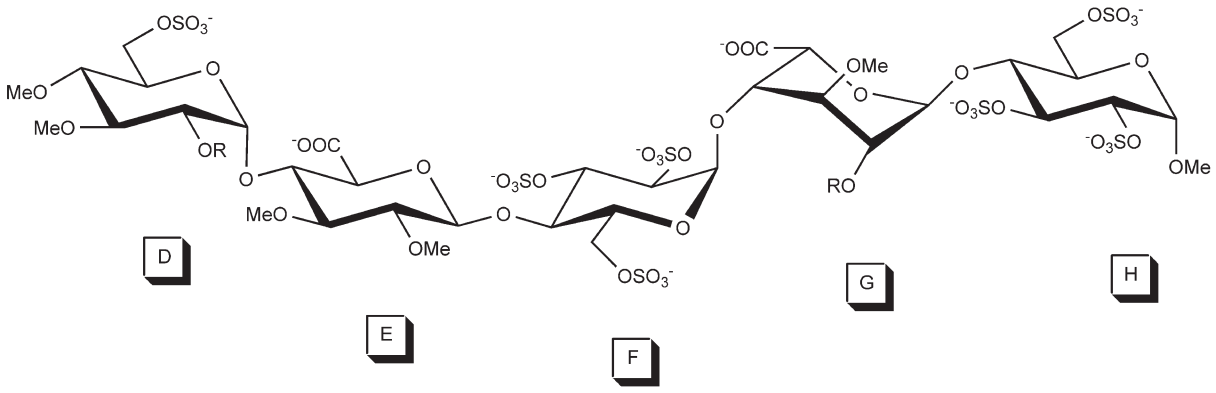
E



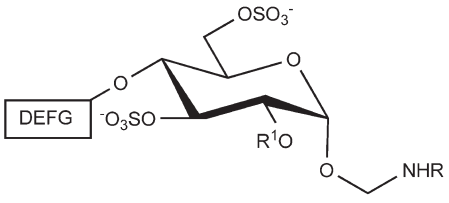
	R^1	R^2	Anti-Xa activity
Ref (63)	SO_3^-	SO_3^-	1217 units/mg
64	Me	Me	1159 units/mg
65	SO_3^-	Me	1184 units/mg
66	Me	SO_3^-	1611 units/mg

Figure 10. Different Heparin Oligosaccharides

(A) Bridged heparin analog.
(B) Pentasaccharide with a *C*-interglycosidic bond.
(C) Pentasaccharide **64**, deoxygenated at C-3 of unit G.
(D) Pentasaccharides containing iduronic acid unit G locked in the $^2S_0$, $^1C_4$, and $^4C_1$ conformation.
(E) Pentasaccharides with two carbon atoms in the bridge.

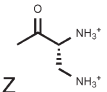


51: R = Me
67: R= SO_3^-



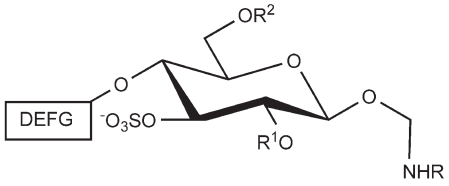
68: $R^1 = SO_3^-$, $R = H_2^+$

69: R^1 = Me, $R = H_2^+$

70: R^1 = Me, R = 

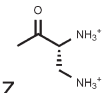
71: R^1 = Me, R = Z

72: R^1 = Me, R = 



73: $R^1 = SO_3^-$, $R^2 = SO_3^-$, $R = H_2^+$

74: R^1,R^2 = Me, $R = H_2^+$

75: R^1,R^2 = Me, R = 

76: R^1,R^2 = Me, R = Z

77: R^1,R^2 = Me, R = 

Figure 11. Pentasaccharides Containing Charged Amino Groups at the Reducing End

indicated that a significant shift of the conformational equilibrium from $^1C_4$ toward the $^2S_0$ conformation was observed when the L-iduronic acid residue is adjacent to a 3-*O*-sulfated glucosamine (unit F) [23]. The additional sulfate on unit F is the key structural element responsible for binding to AT III. Pentasaccharides with an extra sulfate group introduced in unit H show higher affinity for AT III, possibly because of the increased presence of the $^2S_0$ conformation [119].

Replacement of an *O*-glycoside by a *C*-glycosidic bond in anti-factor Xa pentasaccharides slightly decreased the affinities of **55** (880 units/mg versus 1180 units/mg) for AT III and anti-factor Xa (Figure 10B) [127, 128]. A C-3-deoxygenated L-iduronic acid residue was incorporated as unit G to increase the $^1C_4$-content of iduronic acid (Figure 10C) [129].

Decrease of the nonbonding interactions between C-3 and other axial substituents shifts the conformational equilibrium to render unit G predominantly in a $^1C_4$ conformation (65%). The $^2S_0$ conformer that pre-

Table 1. Different Oligosaccharides to Study Thrombin Binding

Nr	TBD	Spacer Length	ABD	Anti-Factor Xa Activity (units/mg)	Antithrombin Activity (units/mg)
78	cellobiose (7-S)	53	**51**	740	10
79	maltotriose (10-S)	53	**51**	490	64
80	maltopentaose (16-S)	56	**51**	280	330
81	TBD=ABD (7-S)	53	**51**	770	14
82	TBD=ABD (9-S)	53	**53**	640	36
83	TBD=ABD (11-S)	53	**50**	280	160
84	maltopentaose (10-S)	18	**51**	540	1
85	maltopentaose (10-S)	32	**51**	700	15
86	maltopentaose (10-S)	46	**51**	420	20
87	maltopentaose (10-S)	59	**51**	630	120
88	maltotriose (16-S)	56	**51**	41	280
89	DS tetra (4-S)	53	**51**		2
90	DS tetra (5-S)	53	**51**		10
91	heparin tetra (6-S)	53	**51**		10
92	cellobiose (7-P)	53	**51**	500	22
93	cellobiose (7-IP)	53	**51**	690	5
94	maltotriose (10-P)		**51**	1000	167
36	T_{18}-Oligonucleotide		**51**	173	5
51		—		1611	—
Heparin	oligosaccharide	±50		160	160

dominates in **58** (64%) is now considerably less abundant (24%). Because pentasaccharide **58** has a much higher affinity for AT III than **57**, the conformation of unit G highly influences AT III affinity. Conformationally locked pentasaccharides were prepared to further address the influence of uronic acid conformation on AT III binding. The L-iduronic units were locked in the $^1C_4$ (**59**) or $^2S_0$(**60**) conformation by covalently bridging the C-2/C-5 and C-3/C-5 ring atoms or in the $^4C_1$(**61**) conformation by introducing a methoxymethyl substituent at C-5 (Figure 10D) [101, 130].

Pentasaccharide **60** that contains an $^2S_0$ iduronic acid binds to AT III and can inhibit factor Xa (1073 units/mg). In contrast, the pentasaccharides **59** (43 units/mg) and **61** (115 units/mg), having unit G locked in the $^1C_4$ or $^4C_1$ conformation, respectively, exhibit little inhibitory activity. The anti-Xa activity of compound **62** that contains a flexible G unit is similar to reference pentasaccharide **51**.

Insertion of one additional bridging carbon atom in pentasaccharide **63** (Figure 10E) resulted in an iduronic acid that failed to adopt the ideal $^2S_0$ conformation as indicated by ^{1}H-NMR-coupling constants [78]. The anti-factor Xa activity (1198 units/mg) of pentasaccharide **63** is similar to that of pentasaccharide **36** (1323 units/mg), and its biological activity is slightly better than that of **60** (1073 units/mg) containing only one carbon atom to bridge C-2 and C-5.

Three pentasaccharides, **64–66**, containing the $^2S_0$-locked iduronic acid as well as various sulfate or methyl groups on the reducing end were synthesized to determine whether the absence of the noncritical sulfate groups in unit H influences the biological activity [131]. The 3-*O*-sulfate in unit H increases the affinity for AT III and shifts the equilibrium toward the $^2S_0$ conformation. The 6-*O*-sulfate group in H interacts with the 2-*O*-sulfate group on unit G resulting in enhanced biological activity, whereas the removal of one of these sulfate groups results in lower activity.

The crystal structure of the AT III-pentasaccharide (**67**) complex illustrates that all carboxylate groups are interacting with the positively charged Lys and Arg residues of AT III [52]. The carboxylate groups of Glu113 and Asp117 are separated from the α-*O*-methyl group and the anomeric center. Therefore, the exchange of the α-*O*-methyl group in **51** by an α-*C*-glycosidic ethylamine tether should result in an additional binding interaction with Glu113 and Asp117 in AT III. Pentasaccharides **68–77** having one or two positively charged amino groups at the reducing end were prepared (Figure 11) [132]. The R^1 sulfate of **68** as well as the R^1 and R^2 sulfates in **73** were replaced by methyl groups in **69** and **74** to minimize unwanted intramolecular salt bridges between the terminal amino group and the sulfate groups. Pentasaccharides **70** and **75** contained additional positively charged amino substituents on the ethylamino tether.

All pentasaccharides were less active than **51**. The amino groups in **71** and **76** are not interacting favorably with the negatively charged target amino acids Glu113 and Asp117. Based on the lower activity of **74** (77 units/mg) compared to **69** (21 units/mg), it can be concluded that the 6-*O*-sulfate may form an intramolecular salt bridge with the amine. Incorporation of two positively charged groups (**70** and **75**) results in decreased activity (18 units/mg) and further suggests that the amino acid residues are not available for additional binding interactions.

Heparin Oligosaccharides with Full Anticoagulant Properties

All pentasaccharides described above exhibit strong anti-factor Xa activity but fail to inhibit thrombin. Longer heparin chains (14–20 saccharides) are required for antithrombin activity. Long, sulfated oligosaccharides can be obtained by connecting two sequences such as ABD and TBD through a spacer. Several aspects need to be considered in defining the nature of the spacer: (1) the type of the spacer (charged or neutral; linear, flexible, or rigid), (2) the direction of chain

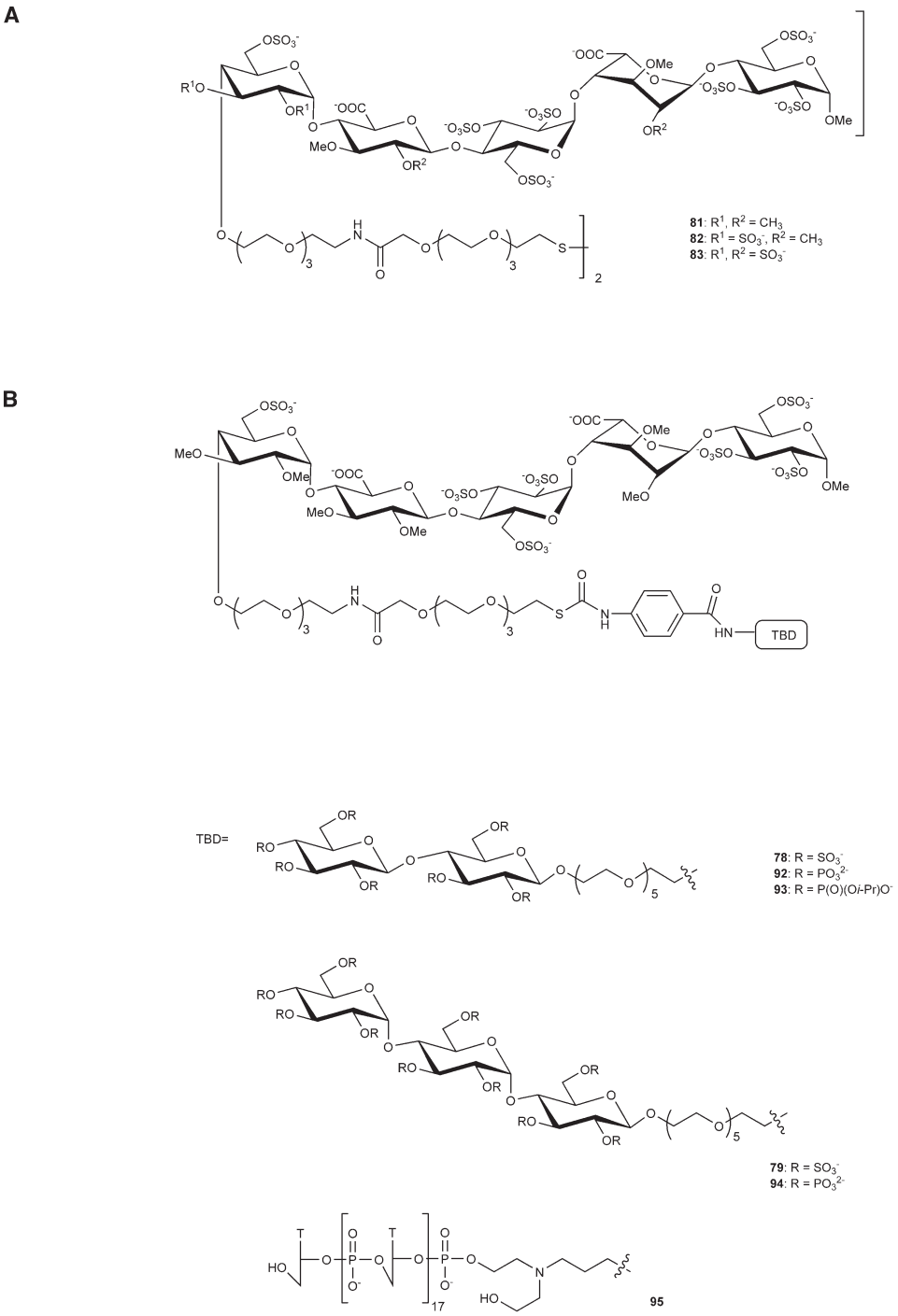


Figure 12. Heparin Oligosaccharides with Full Anticoagulant Activity

(A) Glycoconjugate derived through dimerization.

(B) Alternative TBDs for thrombin inhibition.

elongation (from the reducing end or nonreducing end), and (3) the structural requirements of the thrombin binding domain of heparin.

With these considerations in mind, a model of a heparin/AT III/thrombin complex containing different glycoconjugates in the AT III binding domain ABD, a linear spacer (50 atoms in length corresponding to an oligosaccharide of 12–18 units), and a persulfated maltotrioside representing the thrombin binding domain TBD was prepared [133]. The model suggests that TBD should be attached via the linker at the nonreducing end of ABD. Different, short, persulfated oligosaccharides **78–91** were synthesized and attached to ABD (**51**) to study the binding of TBD to thrombin. Compounds

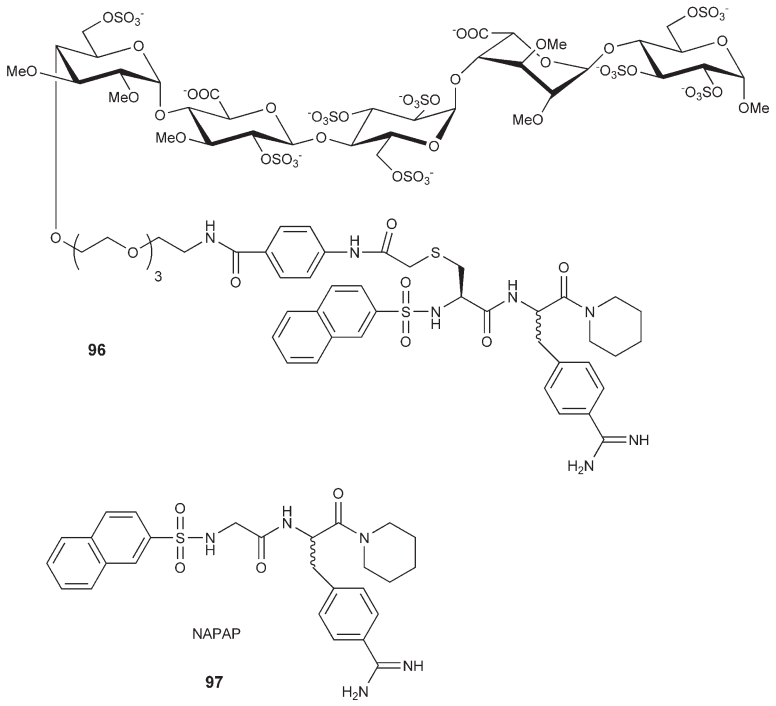


Figure 13. Example for a Noncarbohydrate TBD

78–80 display both good AT III-mediated anti-factor Xa and antithrombin activity and indicate that an increase in charge of the TBD unit results in higher antithrombin activity (Table 1).

The synthesis of symmetric conjugates that are able to bind AT III on one end and thrombin on the other end was also of interest. Therefore, pentasaccharides **50**, **51**, and **53** containing seven, nine, and eleven sulfates were dimerized to produce glycoconjugates **81–83** (Figure 12A).

Based on these findings, it was concluded that antithrombin activity increases with the number of sulfate groups. The number of sulfates in the thrombin binding domain appeared to be more important than the number of carbohydrates. The influence of the length of the spacer was investigated with conjugates **84–87**. With an 18 atom spacer, only anti-factor Xa activity was observed, whereas the incorporation of a 32 atom spacer induced some antithrombin activity. An increase in spacer length to 46 or 59 atoms resulted in a sharp increase in antithrombin activity. Conjugate **88** was synthesized to establish if reduction of the AT III affinity of the pentasaccharide in a conjugate affects both the anti-factor Xa and antithrombin activity. Conjugate **88** is similar to **80** but contains a pentasaccharide with a 50-fold-lower activity for AT III. Surprisingly, the antithrombin activity of **88** is almost identical to that of **80** but exhibits a 7-fold-lower anti-factor Xa activity. An explanation for this phenomenon could be that in case of conjugate **88**, the assembly of the ternary complex occurs through the expected binary AT III-**88** complex. This leads to an anti-factor Xa and antithrombin activity and a binary thrombin-**88** complex that leads only to an AT III-mediated antithrombin activity. The formation of the latter binary complex occurs more easily relative to the thrombin-heparin binary complex because the pentasaccharide domain of conjugate **88** has a lower activity for AT III, whereas its thrombin binding domain should interact more strongly with thrombin.

Heparin-like conjugates **89–91** that contain identical ABD but different TBD domains were prepared [134]. Conjugate **89** contains a TBD domain with four sulfate groups and exhibited low antithrombin activity. Conjugates **90** and **91** showed an antithrombin activity similar to **78**. The charge density of the TBD moiety determines the antithrombin inhibitory activity, whereas the carbohydrate structure of TBD has no effect.

Perphosporylated cellobiosyl saccharides **92** and **93** with seven and 14 negative charges, respectively, as well as maltotrioside **94** carrying 20 negative charges were synthesized to probe the effect of the charged group on high affinity ABD domains (Figure 12B). Incorporation of lipophilic groups into the TBD domain was expected to enhance the interaction with thrombin. The introduction of a phosphate ester in TBD increased its affinity for thrombin, whereas the replacement of one sulfate group by a phosphate ester in the ABD decreased its affinity of ABD for AT III.

Based on the observation that oligonucleotides can associate with the heparin binding site [135], conjugate **95** (Figure 12B) was prepared in which part of the spacer and the TBD domain were replaced by an oligonucleotide. The low anti-factor Xa activity (173 units/mg) and antithrombin activity (5 units/mg) of **95** illustrated the existence of a weak interaction of oligonucleotides with the TBD domain. The interaction of the ATB domain with AT III requires more than just the interaction of the TBD domain with thrombin.

N-(2-Naphthalenesulfonyl)-glycyl-(D)-4-aminophenylalanyl-piperidine (NAPAP) derivate **96** served as an-

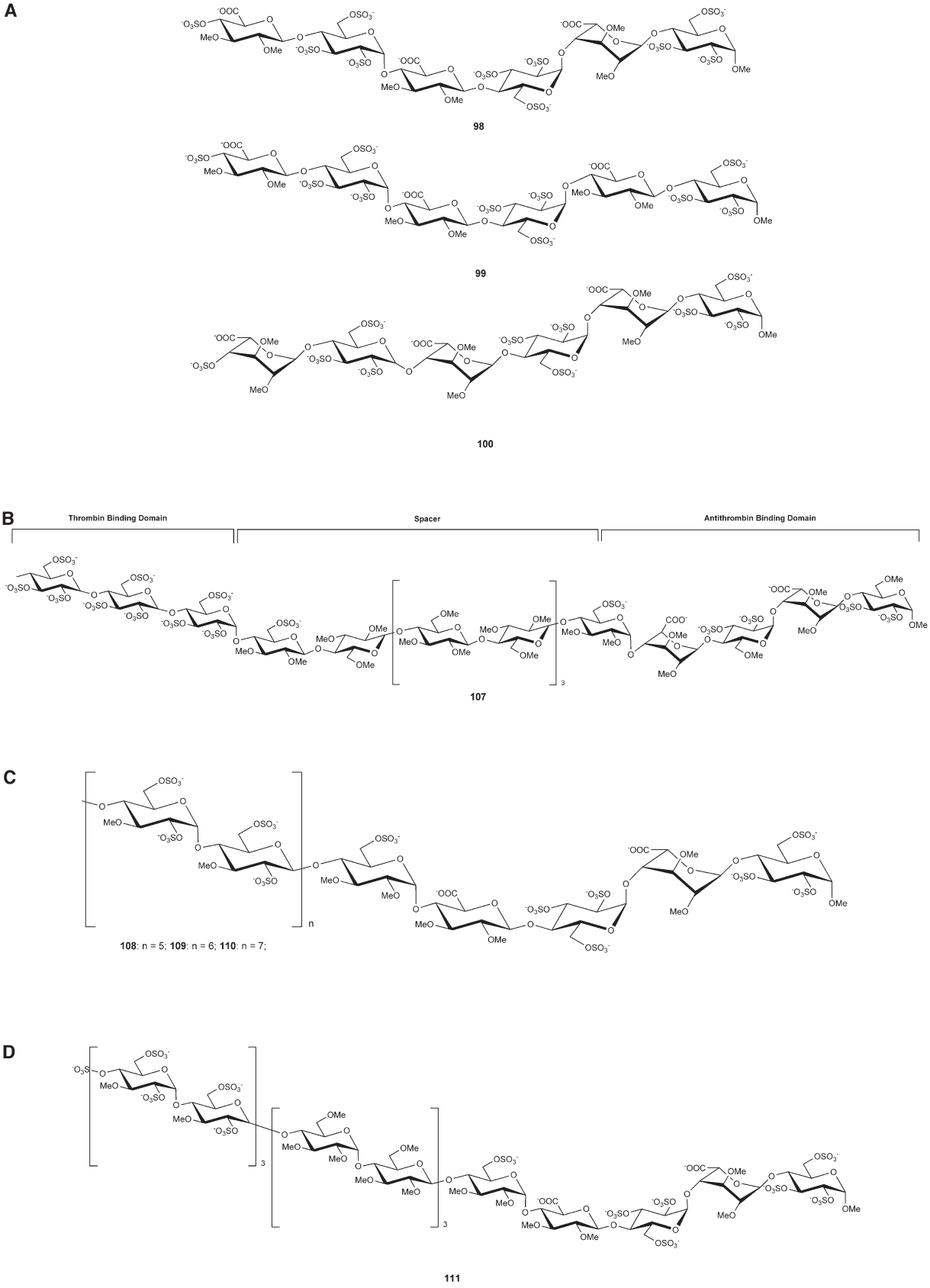


Figure 14. Heparin Oligosaccharides with Different Spacers

(A) Carbohydrates with full anticoagulant properties for heparin.

(B) Oligosaccharide with a permethylated polymaltose spacer.

(C) Retrosynthetic way for the synthesis of glycoconjugates with a rigid spacer.

(D) Heptadecasaccharide, not neutralized by PF4.

Table 2. Heparin Oligosaccharides to Study Full Anticoagulant Properties

Nr	Size	Anti-Factor Xa Activity (units/mg)	Antithrombin Activity IC_{50} (ng/mL)
101	10-mer	325	> 10,000
102	12-mer	405	> 10,000
103	14-mer	360	> 10,000
104	16-mer	310	130
105	18-mer	360	23
106	20-mer	290	6.7
Heparin	-	**170**	**3**
108	15-mer	370	41
109	17-mer	270	5.3
110	19-mer	290	1.7
111	17-mer	270	9.3
112	16-mer	350	490
113	18-mer	260	360
114	20-mer	210	88

other noncarbohydrate TBD (Figure 13) [136]. NAPAP **97** itself binds directly to the active site of thrombin (EC_{50} = 0.75 μM). The NAPAP-conjugate **96** was designed to stimulate AT III-mediated anti-factor Xa activity and to inhibit thrombin. Indeed, conjugate **96** exhibited antithrombin activity (IC_{50} = 0.35 μM) and anti-factor Xa activity (885 units/mg) and confirmed that the NAPAP conjugate is a better inhibitor than the combination of the free pentasaccharide and NAPAP.

All approaches discussed thus far incorporated a flexible spacer. Another class of oligosaccharides containing a rigid spacer and a decreased charge density was synthesized. ABD domains with at least six negative charges also served as a TBD because the interaction of thrombin with heparin results from an electrostatic attraction that depends on the density of negative charges. It should be noted that AT III-ABD binding affinity is much higher than thrombin-TBD binding affinity. A sequence of repeating ABD's would increase the affinity for AT III, but prevent thrombin binding. Oligosaccharides with anti-factor Xa and anti-thrombin affinity require an affinity of AT III-ABD and thrombin-TBD in the same order of magnitude. Based on the high AT III affinity of pentasaccharide **49**, hexasaccharides consisting of a repeating disaccharide unit should have a reduced but still significant affinity for AT III. The highly symmetrical antithrombin binding domain, obtained from a single disaccharide, results in a much-simplified synthesis of

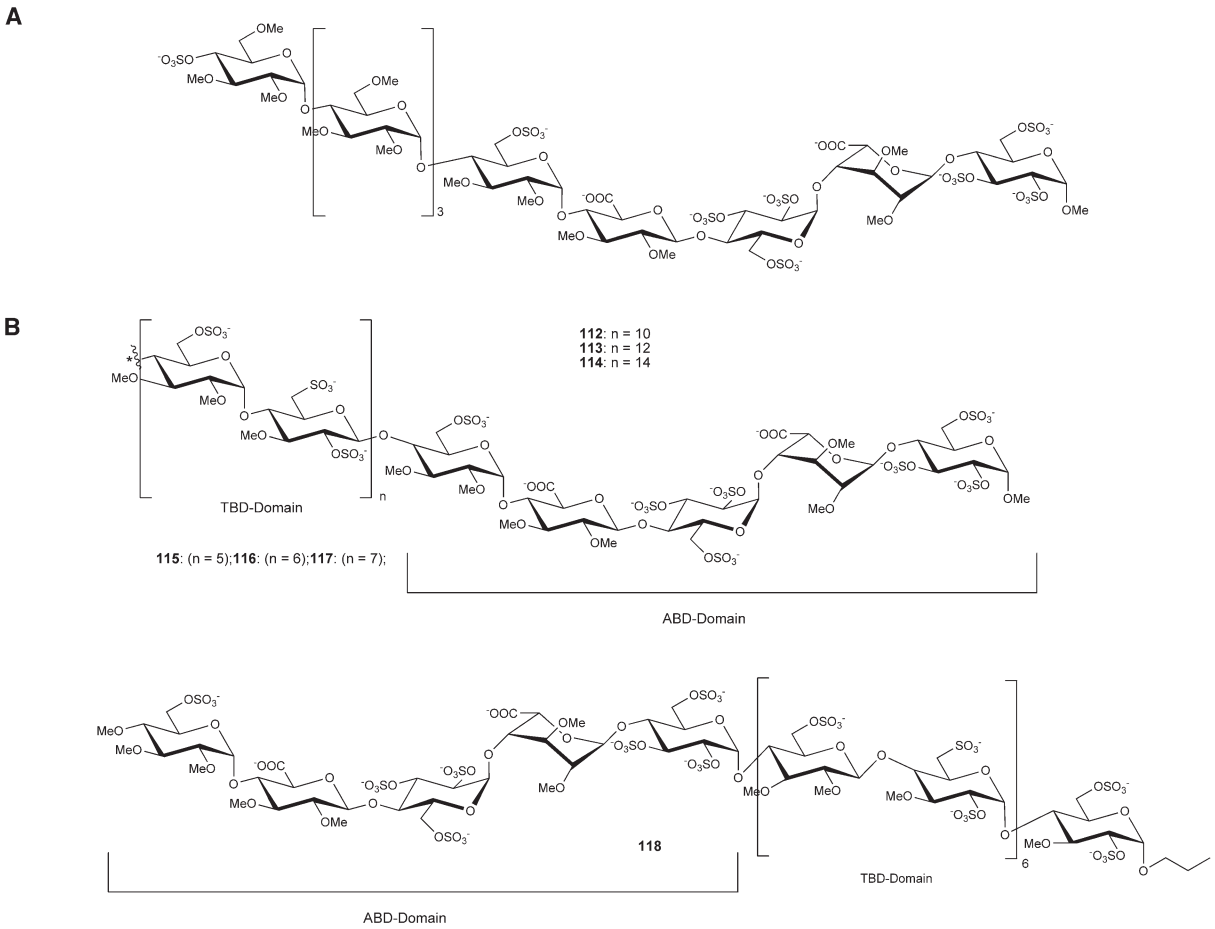


Figure 15. Different Heparin Mimetics

(A) Heparin mimetic with low sulfated sequence.

(B) Heparin mimetics to proof the location of the TBD domain.

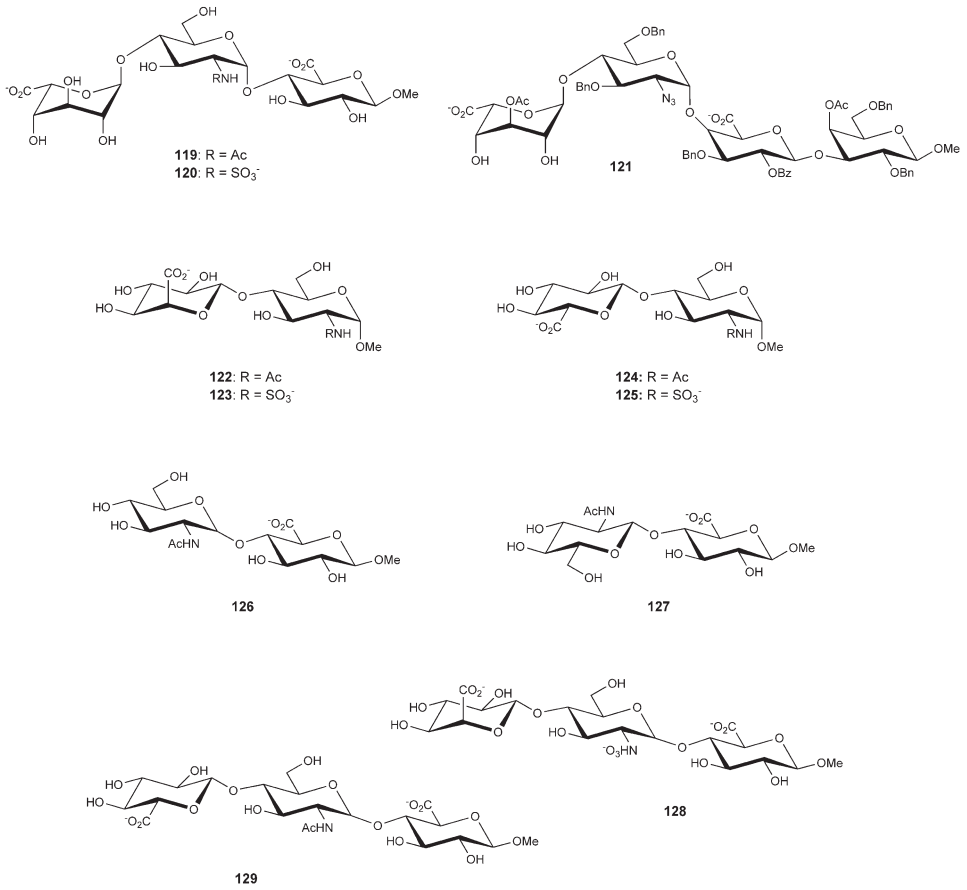


Figure 16. Oligosaccharides Involved in FGF Interaction

glycoconjugates for drug development. The first carbohydrates exhibiting full heparin anticoagulant properties were synthesized in 1998 [137, 138].

Three hexasaccharides that contain one additional trisulfated glucose unit at the nonreducing end (**98**), a D-glucuronic acid (**99**), or an L-iduronic acid as the only uronic acid in the compound (**100**) were synthesized (Figure 14A). The introduction of the additional trisulfated glucose in **98** results in binding to AT III. The binding affinity of **99** that contains only D-glucuronic acids decreased dramatically. Oligomer **100**, which contains only L-iduronic acids, displayed ideal binding properties with an affinity for AT III (K_d = 0.35 μM) similar to the heparin-thrombin binding (K_d = 1 μM). Anti-factor Xa activity of **100** was reported at 325 units/mg.

Based on these findings, larger heparin fragments related to **100** were synthesized [139]. All compounds (**101–106**) show similar anti-factor Xa activity. Smaller oligosaccharides (hexa-, octa-, deca-, dodeca-, and tetradecamer) do not inhibit thrombin, whereas for larger oligomers (hexadeca- [**104**], octadeca- [**105**], and eicosasaccharide [**106**]) activity increases with size. The eicosamer is half as potent as standard heparin (Table 2).

Exchange of the polyethylene glycol spacer to a rigid permethylated polymaltose spacer (**107**) increased the antithrombin activity 10-fold, whereas anti-factor Xa activity remained similar to **80** (Figure 14B). Because of the lower charge density and the rigidity of the spacer, neutralization of **107** by PF4 was reduced by a factor of 20 when compared to standard heparin [140].

Glycoconjugates **108–110** possessing a specific ABD domain and a TBD domain that is not recognized by AT III were synthesized to create oligomers with a charge density similar to that of heparin (Figure 14C) [141]. Preparation of these structures relied on elongation of the ABD domain at the nonreducing end by the addition of 3-*O*-methyl-2,6-di-*O*-sulfo-D-glucose oligomers with alternating α, and β (1→4) linkages.

Anti-factor Xa activity and affinity for AT III were similar for all compounds, whereby thrombin inhibition increased with growing chain length. Nonadecamer **110** was as potent as the most active fraction isolated from standard heparin. Heptadecasaccharide **111**, which contained even less charge density, exhibited the same anticoagulant properties but could not be neutralized

Table 3. Pentasaccharides Involved in FGF Interaction

Nr	
130	Ido-GlcNS-Ido-GlcNS-Ido
131	Ido-GlcNS-Glc-GlcNS-Ido
132	Glc-GlcNS-Ido-GlcNS-Ido
133	Glc-GlcNS-Glc-GlcNS-Ido

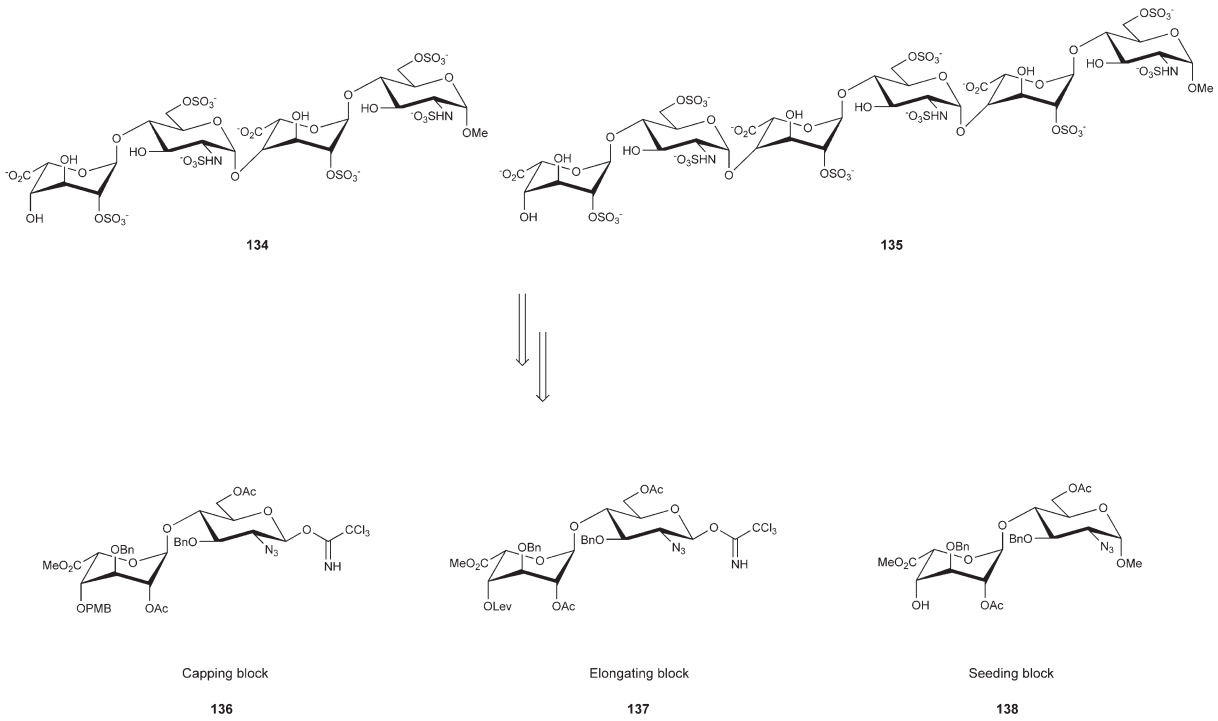


Figure 17. Strategy for the Synthesis of Larger Oligosaccharides

by PF4 even at very high concentrations (100 μg/ml) (Figure 14D) (Table 2) [142].

A new family of heparin mimetics (**112–114**) combined a pentasaccharide ABD domain with a TBD domain composed of a low sulfated sequence of repeating 2,3-di-*O*-methyl-6-*O*-sodium sulfonato-α-D-glucosyl units (Figure 15A) [143]. Only small differences in the AT III affinity and in the inhibition of Xa were observed. These conjugates are less potent than heparin, but none are neutralized by PF4, indicating that this group of molecules may constitute alternatives to standard heparin (Table 2).

Until 2001, there was no experimental proof that the thrombin binding domain in heparin is located at the nonreducing end of the antithrombin binding domain and that the factor Xa inhibition is not affected by elongation of the antithrombin binding pentasaccharide sequence. Different heparin mimetics were used to investigate this hypothesis (Figure 15B) [76]. The *N*-sulfated glucosamine units of heparin were replaced by *O*-sulfated glucose, and *O*-methyl groups were incorporated in place of hydroxyl groups to simplify the synthesis. In the TBD domain, 2,6-di-*O*-sulfonato-β-D-glucose substituted 2-*O*-sulfonato-α-L-iduronic acid to maintain the number of charges per saccharide unit as in heparin.

All analogs (**115–118**) exhibited the same activity for anti-factor Xa and affinity for AT III. Again, inhibition of thrombin was increasing with chain length. Oligosaccharide **118** does not inhibit thrombin in the presence of AT III. These results demonstrate that the TBD must be located at the nonreducing end of ABD to inhibit thrombin.

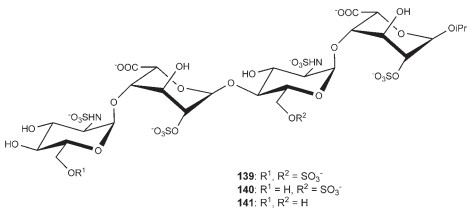
Synthesis of Heparin Oligosaccharides Involved in FGF Interaction

After the minimum heparin binding sequence for FGF1 and FGF2 had been determined (HexA-GlcNS-HexA-GlcNS-IdoA-2OS), different syntheses to access these heparin fragments were developed. The oligosaccharides differ in configuration of the HexA units, sulfation pattern, and length.

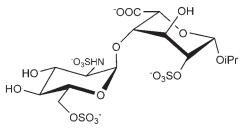
Tri (**119** and **120**)- and tetrasaccharide (**121**) derivatives were synthesized with a modular strategy (Figure 16) [144]. This modular strategy also yielded disaccharides **122–127** and trisaccharides **128** and **129** [145]. Disaccharide **124** and **125** bind to FGF2 less tightly than heparin. The two trisaccharides **128** and **129** bind to FGF2 and show higher affinity for FGF1.

Four different pentasaccharides **130–133** were prepared to elucidate the exact nature of the hexuronic acid unit (Table 3) [84, 146]. All pentasaccharides inhibited FGF2 binding to heparin or heparan sulfate and the proliferation of FGF-induced human aortic smooth-muscle cells (HASMC). Pentasaccharide **130** is most effective, whereas **131–133** showed only weak potency. From these observations, it was concluded that iduronic acid is mainly responsible for the interaction between heparin and FGF2. The conformation of the different iduronic acid residues was analyzed by NMR. The iduronic acid at the nonreducing end is predominantly present in the $^4C_1$ form. The iduronic acid in the center prefers the $^1C_4$ form, and the reducing end iduronic acid adopts the $^2S_0$ conformation.

Tetrasaccharide and hexasaccharide methyl glycoside **134** and **135** were prepared from three different



139: R^1, $R^2 = SO_3^-$
140: $R^1 = H$, $R^2 = SO_3^-$
141: R^1, $R^2 = H$



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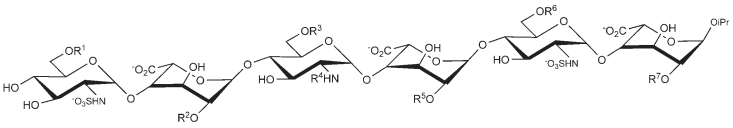
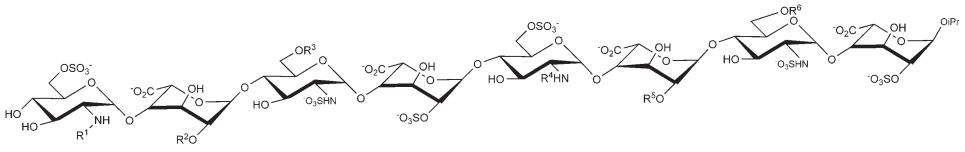


142, 145, 147, 148



143, 146

	R^1	R^2	R^3	R^4	R^5	R^6	R^7
142	SO_3^-	SO_3^-	SO_3^-	SO_3^-	SO_3^-	SO_3^-	SO_3^-
143	SO_3^-	SO_3^-	SO_3^-	SO_3^-	SO_3^-	SO_3^-	
145	H	SO_3^-	SO_3^-	Ac	H	H	SO_3^-
146	Ac	H	H	Ac	H	H	
147	SO_3^-	H	SO_3^-	SO_3^-	H	SO_3^-	H
148	H	SO_3^-	H	SO_3^-	SO_3^-	H	SO_3^-

Figure 18. Oligosaccharides with Different Sulfation Pattern

disaccharides: seeding disaccharide **138**, elongation disaccharide **137**, and capping disaccharide **136** (Figure 17) [81]. Within each disaccharide, iduronic acid was placed at the nonreducing end and glucosamine at the reducing end. Hexasaccharide **135** antagonized iodinated heparin-FGF2 binding and inhibited FGF2-induced proliferation of human aortic smooth-muscle cells (HASMC), whereas tetrasaccharide **134** showed only weak efficacy.

Three tetrasaccharides **139–141** containing the sequence GlcN-IdoA and different sulfation patterns at C-6 of the glucosamines were prepared (Figure 18) [147]. The synthesis relied on the coupling of two versatile disaccharide building blocks with orthogonal protecting groups [109]. The same strategy followed by a convergent n + 2 block approach allowed the preparation of longer oligosaccharides, which are not available by enzymatic or chemical degradation of heparin with a glucosamine unit at the nonreducing end [148–150].

Hexa- **142** and octasaccharide **143** contain the structural motif of the major region of heparin and were tested for activation of FGF1. Octasaccharide **143** activates the mytogenic signal like heparin, whereas hexasaccharide **142** was less efficient. From sedimentation experiments, it has been concluded that the active form of FGF1 is a monomer, assuming that the dimerization of FGF1 is not necessary for FGF1-induced signaling. Hexasaccharide **142** prefers a helical conformation, and the iduronic acid units exist in a fast equilibrium between the $^2S_0$ and the $^1C_4$ form with a slight preference for the latter. Comparison of the conformation of **142** with that of pentasaccharides **130–133** reveals that the sequence of the oligosaccharides (IdoA-GlcN versus GlcN-IdoA) can affect the conformation of the iduronic acid units.

Because the interaction of heparin with several proteins, including FGFs and their receptors, is mediated by Ca^{2+} [151–155], further investigations on **142** have been performed. Electrostatic interactions of heparin with ions occur via charged saccharide groups. Calcium^{2+}-heparin interactions in which the cation binds preferentially to the carboxylate groups of the iduronic acid units have been observed. Modification of the carboxylate groups by conversion into methyl esters or by protonation resulted in the loss of binding capability. The lack of the sulfamido group of the glucosamine units

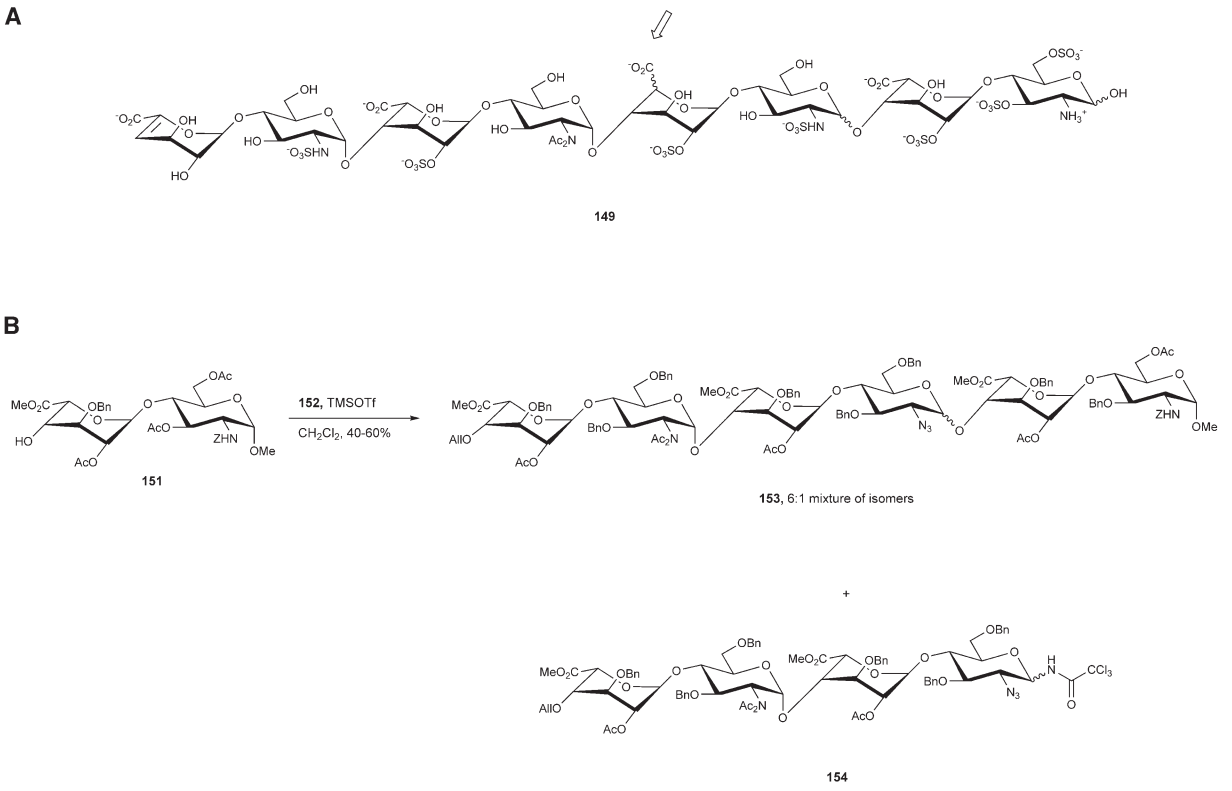


Figure 19. Heparin Oligosaccharides Recognized by Herpes Simplex Virus

(A) Target structure binding to HSV-1 with unknown stereochemistry at the indicated center.

(B) Synthesis of hexasaccharide **153**.

results in the loss of Ca^{2+} binding to heparin. Combination of size and charge is responsible for site-specific Ca^{2+} binding, whereas other ions (e.g., Na^{+} and Mg^{2+}) bind more specifically.

Binding of Ca^{2+} to **142** was found to be specific [156, 157]. The ion influences the backbone flexibility and rigidifies the glycosidic linkage, and the conformational equilibrium of the iduronic acid unit may be shifted to the $^{1}C_{4}$ conformation. NMR-spectroscopical studies of disaccharide unit **144** confirmed that the $^{1}C_{4}$ conformation showed also here the highest affinity for Ca^{2+}. Furthermore, the ion coordinates the carboxylate and 2-*O*-sulfate groups of the iduronic acid units as well as the *N*-sulfate moiety of glucosamine with the glycosidic and iduronate ring oxygen atoms. Calcium-heparin interaction studies were extended to hexasaccharides **147** and **148** [158]. NMR studies in concert with molecular modeling revealed that the 6-*O*-sulfate group of glucosamine is necessary for the interaction of heparin with Ca^{2+}, whereas the sulfate group at C-2 of iduronic acid is not required. Hexasaccharide **145** and octasaccharide **146** were prepared with the convergent n + 2 block approach to test whether the active form of FGF is a monomer [149]. Exhibiting charged groups only on one side of the helical structure, a monomeric complex with FGF1, should be formed exclusively. Conformational analyses showed that the absence of the 2-*O*-sulfate groups in C and G, the 6-*O*-sulfate group in B and F, and the *N*-sulfate group in D and H do not change the conformation of the iduronic acid rings. Hexasaccharide **145** was found to be biologically active and demonstrated that a 1:1 complex between a heparin fragment and FGF1 can induce mitogenesis.

The closely related hexasaccharides **147** and **148** have the same sequence as **142** and **145**, but their charge distribution has been designed to gain structural and biological information to study the importance of sulfate groups glucosamine C-6 and iduronic acid C-2 [150].

The oligosaccharides contain the basic structural features of heparin, but different charge distribution and orientation results in a different biological behavior. The two oligosaccharides **142** and **143** contain the trisaccharide motif (IdoA-2OS-GlcN-6OS-IdoA-2OS) required for high affinity binding to FGF1, whereas oligosaccharides **145–148** were missing this motif. Oligosaccharides lacking this internal trisaccharide motif can stimulate FGF1 more efficiently than those with the regular heparin structure [159]. Hexasaccharide **145** containing sulfate groups only on one side of the helical structure can activate FGF1 as effectively as octasaccharide **143**. These results underscore the importance of charge distribution in the activation process of FGF1 and suggest that the FGF dimerization is not absolutely required for biological activity.

Synthesis of Heparin Oligosaccharides Recognized by Herpes Simplex Virus

Heparan sulfate serves as adhesion receptor for bacteria, parasites, and viruses. Its negative charges can be

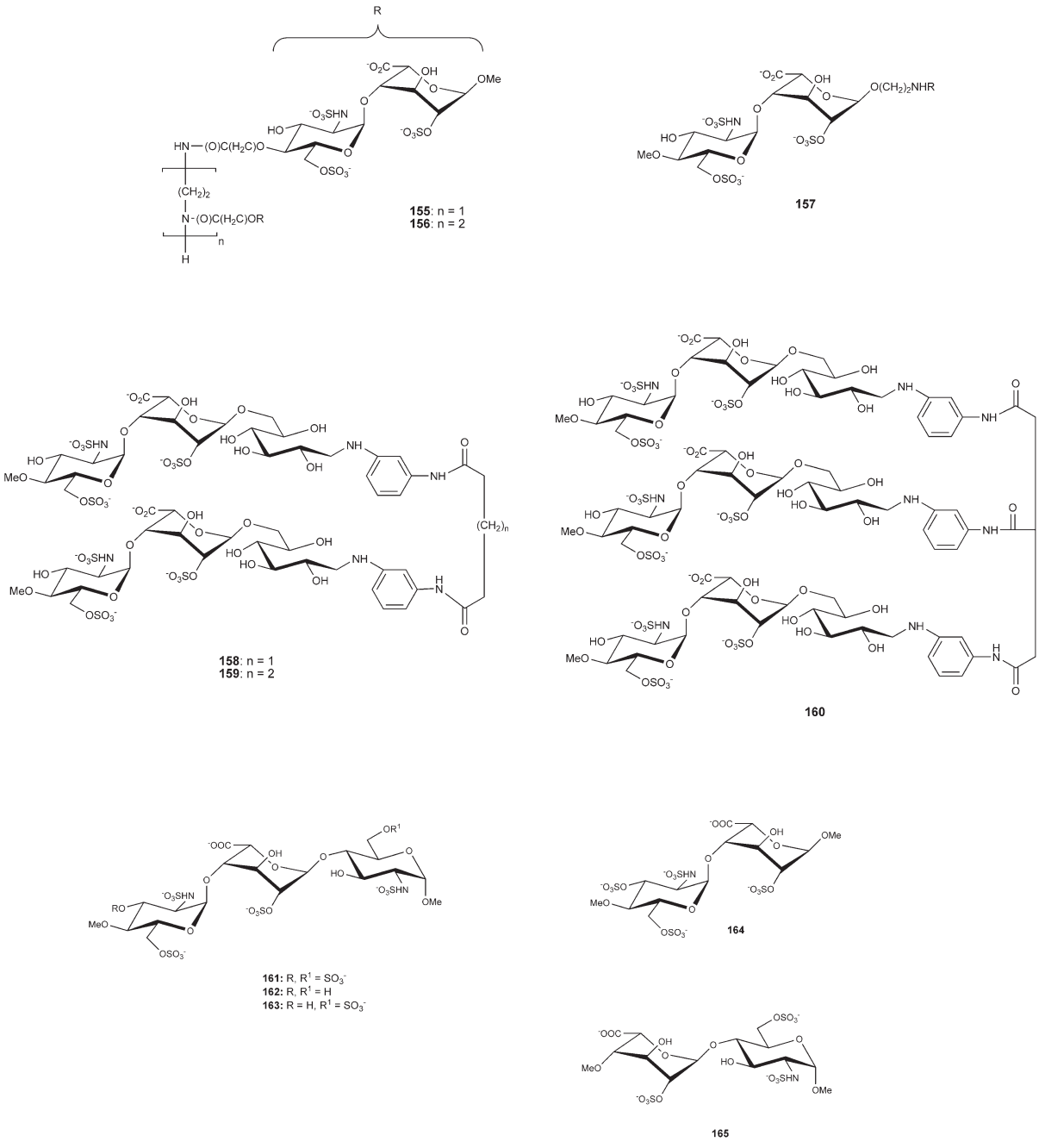


Figure 20. Compounds **155–165** to Investigate Platelet Binding Activities

recognized by viral proteins [160]. Herpes simplex viruses (HSVs) are members of the neurotropic subgroup of the herpes virus family. Infection with herpes simplex virus 1 (HSV-1) and herpes simplex virus 2 (HSV-2) are most common in humans. HSV-1 binds to cells by interaction of envelope glycoproteins gB and gC with cell-surface heparan sulfate. A third viral glycoprotein, gD, is interacting with one of multiple specific receptors, which results in a viral entry of the virion envelope with a cell membrane. This fusion requires the concerted action of the three glycoproteins and is triggered by the binding of gD to its cognate receptors. A heparan sulfate octasaccharide (**149**) that binds to HSV-1 gD was identified (Figure 19A) [161–163].

Based on this information, a fully protected *N*-differentiated heparin oligosaccharide was prepared in efforts to establish a structure-activity relationship [164]. Because of the limitations of the analytical methods, the nature of one of the uronic acid residues was not known. Therefore, initially, an iduronic acid residue was selected in this position. The protecting-group strategy was adapted from previous heparin syntheses: *O*-sulfonates were masked as *O*-acetates, hydroxyl groups as *O*-benzyl ethers, carboxylic acids as methyl esters, and

N-sulfonates as azides. In addition, the free amine was protected as benzylcarbamate and the *N*-diacetate as *N*-acetate.

Two disaccharides, **150** and **151**, and tetrasaccharide **152** were readily prepared. Coupling of **151** and **152** afforded hexasaccharide **153** as an α/β mixture (6:1) and the rearranged tetrasaccharide **154** (Figure 19B). The selectivity is decreased by the 2-*N*-CBz glucosamine residue that could force the iduronic acid unit out of the favorable $^1C_4$ conformation. This finding underscores that the selectivity of glycosylation reactions is greatly influenced by the formation of the nucleophile. This fact has to be taken into consideration for further syntheses of large heparin-like glycosaminoglycans.

Synthesis of Heparin Oligosaccharides Interacting with Platelets

Heparin binds to platelets and can cause activation and aggregation. The platelet binding site for heparin has to be determined to understand how heparin is altering platelet function. Given the heterogeneous nature of heparin, this is a challenging task. A disaccharide unit (GlcNS[6-OS]-IdoA[2-OS]) in heparin was found to be the key for the binding interaction [165]. The disaccharide cannot be obtained by enzymatic or chemical degradation. Therefore, a series of oligomers **155–160** containing this disaccharide were prepared, and their platelet binding activities determined (Figure 20) [166, 167].

Compound **156** and **160**, which contain three units of GlcNS(6-OS)-IdoA(2-OS), bound stronger than their counterparts **155**, **158**, and **159**, which contain only two key disaccharides. These findings underscore the role of the GlcNS(6-OS)-IdoA(2-OS) clustering effect for binding. The binding potency is not influenced by the distance between the GlcNS(6-OS)-IdoA(2-OS) units. Head-to-tail dimer **157** has a higher binding activity than the tail-to-tail dimer **155**. The arrangement of the two units of GlcNS(6-OS)-IdoA(2-OS) has an influence on the activity.

Pentasaccharide **31**, which does not contain the key disaccharide GlcNS(6-OS)-IdoA(2-OS), binds to platelets. Fragments of **31** were synthesized to determine which part of **31** is responsible for the platelet binding. The tetrasaccharide that contains the nonreducing fragment *DEF* of **31** does not bind platelets. More detailed studies were focused on the reducing end *FGH* trisaccharide **161** and on its partially desulfated derivatives **162** and **163** as well as on disaccharide derivatives **164** and **165** (Figure 20). Only trisaccharide **161** bound comparably to **156** and **160**. Based on these results, it has been suggested that the sequence GlcNS(3-OS)(6-OS)-IdoA(2-OS)-GlcNS(6-OS) is a high-affinity binding site for platelets.

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