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Feasible Synthesis and Biological Properties of Six 'Non-Glycosamino' Glycan Analogues of the Antithrombin III Binding Heparin Pentasaccharide

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Abstract—In this paper, we report the synthesis of 'non-glycosamino' glycan analogues **5**–**10** of the antithrombin III binding pentasaccharide **1**. Pentasaccharides **5**–**10** feature a pseudo-alternating EFGH tetrasaccharide sequence, that is, the disaccharide fragments EF and GH have the same substitution pattern. In the synthetic strategy applied for the synthesis of pentasaccharides **5**–**10**, the properly protected EF disaccharide fragments **19** and **20** are obtained from their GH counterparts **17** and **18** by base-catalyzed epimerization. Series I, comprising pentasaccharides **5**–**7**, has an invariable EFGH tetrasaccharide containing 2-O-sulfate 3-O-methyl uronic acid moieties. Series II, on the other hand, contains pentasaccharides **8**–**10** and has an invariable EFGH tetrasaccharide containing 2,3-di-O-methyl uronic acid moieties. Coupling disaccharides **17** with **25** and **18** with **26** exclusively afforded the α-coupled tetrasaccharides **27** and **28**, respectively. Glycosylation of acceptor tetrasaccharides **29** and **30** with glucosyl donors **35**, **36** and **39** provided, after deprotection and sulfation, the title-compounds **5**–**10**. Biological data obtained with series I and II indicate that the *in vivo* half-life but not the intrinsic anti-Xa activity depends on the substitution pattern of the D-unit. In addition, the applicability of reversed UV capillary electrophoresis as an analytical tool to determine the purity of these 'non-glycosamino' glycans is demonstrated.

Introduction

Heparin is an anticoagulant drug composed of a mixture of glycosaminoglycans (GAGs) purified from biological sources such as intestinal mucosa. The antithrombotic action of heparin mainly results from interactions with the protease inhibitor antithrombin III (AT-III). The heparin/AT-III complex inactivates the blood coagulation factors factor Xa and thrombin.[1]

Apart from anticoagulant properties heparin exerts platelet inhibitory activity which has been associated with its tendency to induce bleeding.[2] Bleeding induction, which is an important risk factor in heparin anticoagulant therapy, has furthermore been related to the degree of sulfation of GAGs in general.[3] Both properties probably do not relate to the antithrombotic effect of heparin.[4]

It was anticipated that heparin fractions of a lower molecular weight might show higher specificity towards the inhibition of individual coagulation factors and a lower bleeding induction. Therefore, heparin was degraded to obtain low molecular weight heparins which differed in degree of sulfation and showed a decreased antithrombin activity.[5]

The shortest fragment in the heparin polymer with high affinity for AT-III was discovered to be pentasaccharide **1**[6,7] (Scheme I). This pentasaccharide was shown to accelerate the AT-III mediated inactivation of factor Xa (anti-Xa activity) but not that of thrombin. The antithrombotic activity of the synthetic counterpart of the pentasaccharide, i.e. **2**, was demonstrated in animal models of venous thrombosis.[8,9] Moreover, the pentasaccharide has no tendency to induce bleeding at therapeutic doses.[10]

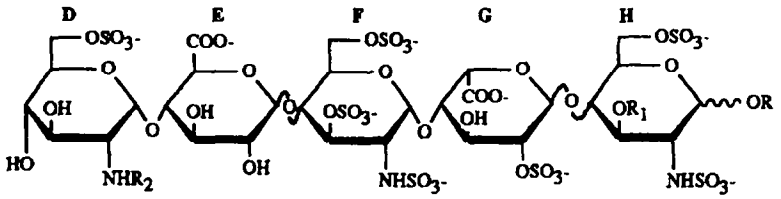
In order to get insight into structure–activity relationships of this new class of selective factor Xa inhibitors, a program towards the preparation of pentasaccharide analogues was started,[11–13] giving detailed information on the functional groups essential for the binding to and activation of AT-III.[14–16] These studies revealed that analogues containing two 3-O-sulfated glucosamine residues, i.e. **3**, display enhanced affinity for AT-III, resulting in a very potent antithrombotic compound with a prolonged period of action.[14,17]

Moreover, a molecular model of the interaction between AT-III and pentasaccharide **3** was constructed.[18] It is attractive to use these highly active, well-defined compounds as leads for drug development although their chemical complexity renders large-scale synthesis difficult. Hence, the synthesis of simplified analogues of the natural pentasaccharide has been pursued. Recently, the synthesis of the 'non-glycosamino' glycan analogue of compound **3** was reported.[19] This compound, i.e. **4** (Scheme II), features

O-sulfate instead of N-sulfate esters and O-alkyl ethers instead of hydroxyl groups. Indeed the synthesis of 'non-glycosamino' glycan analogue **4** is much easier than that of heparin fragments **2** and **3** because: (i) the synthetic strategy is more flexible in that both acyl esters and benzyl ethers can be used for the protection of hydroxyl groups to be sulfated; (ii) no amino sugars have to be introduced which require elaborate synthetic routes for the preparaton of azide containing building blocks; (iii) a strategy may be devised in which only benzyl protective groups are used, requiring only one deprotection step; (iv) at the end of the synthesis no selective N-sulfation has to be performed. To our surprise the alkylated, exclusively O-sulfated derivative **4** was even somewhat more active (Table 2) in comparison with the parent compound **3**, indicating that no 'key polar' hydroxyl groups are involved in the interaction with AT-III.[20]

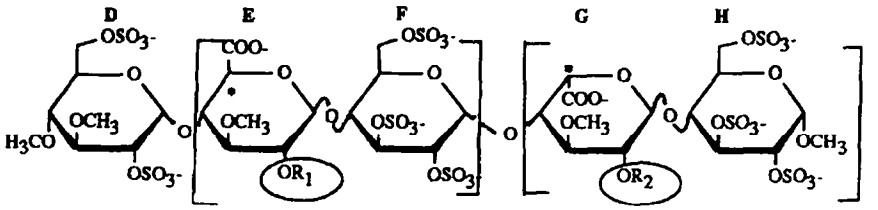
Having the simplified analogue **4** at our disposal we wondered whether even shorter synthetic routes could be devised towards these 'non-glycosamino' glycan analogues. In this respect, a particularly appealing prospect turned out to be the synthesis of analogues with a pseudo-alternating sequence (i.e. the substitution pattern of disaccharide moiety EF corresponds to that of GH) like pentasaccharide **6** in Scheme II. The only remaining difference between the disaccharide moieties EF and GH of compound **6** is that they have opposite configurations at C-5 of the uronic acid moieties E and G (Scheme II). Hence, a shorter synthetic route could be developed in which a properly protected disaccharide fragment GH is transformed into its counterpart EF by base-catalyzed epimerization[21] (Scheme III).

Taking compound **4** as an example, pentasaccharides displaying pseudo-symmetry can be obtained in two different ways: (i)introduction of a 2-O-sulfate at unit E (i.e. compound **6**, Scheme II); (ii) replacement of the 2-O-sulfate at unit G by an O-methyl group (i.e. compound **9**, Scheme II).



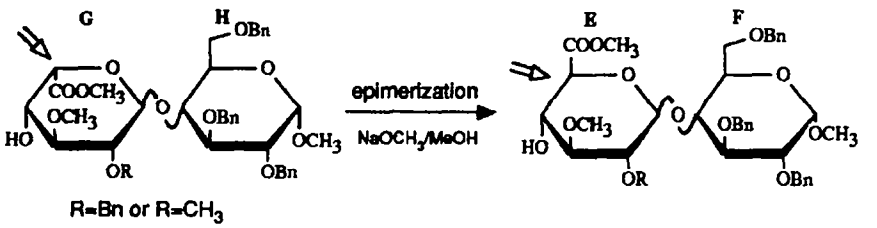
1: R=R$_1$=H, R$_2$=Ac or SO_3^-
2: R=αOCH$_3$, R$_1$=H, R$_2$=SO_3^-
3: R=αOCH$_3$, R$_1$=R$_2$=SO_3^-

Scheme I.



4: R$_1$=CH$_3$, R$_2$=SO_3^-
6: R$_1$=R$_2$=SO_3^-
9: R$_1$=R$_2$=CH$_3$

Scheme II.



Scheme III. Transformation of GH into EF by epimerization.

In this paper we report on the synthesis of two series, i.e. series I (compounds **5–7**) and series II (compounds **8–10**), of 'non-glycosamino' glycan pentasaccharides with a pseudo-alternating sequence (Scheme IV). Both series contain an invariable EFGH block, each of which is coupled with three different D-units varying in sulfation pattern. Series I contains 2-O-sulfate 3-O-methyl uronic acid moieties, i.e. E and G, whereas the series II contains 2,3-di-O-methyl uronic acid moieties (Scheme IV).

In addition we will present the specific anti-Xa activities and *in vivo* residence times in rat of these pentasaccharides.

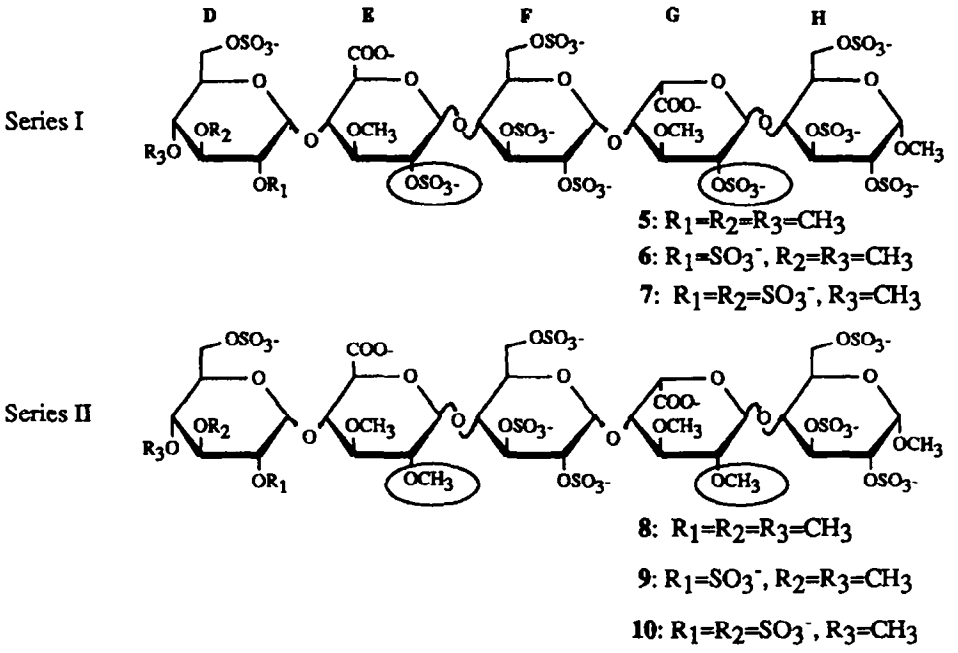
Results and Discussion

Synthesis of disaccharides 17 and 18

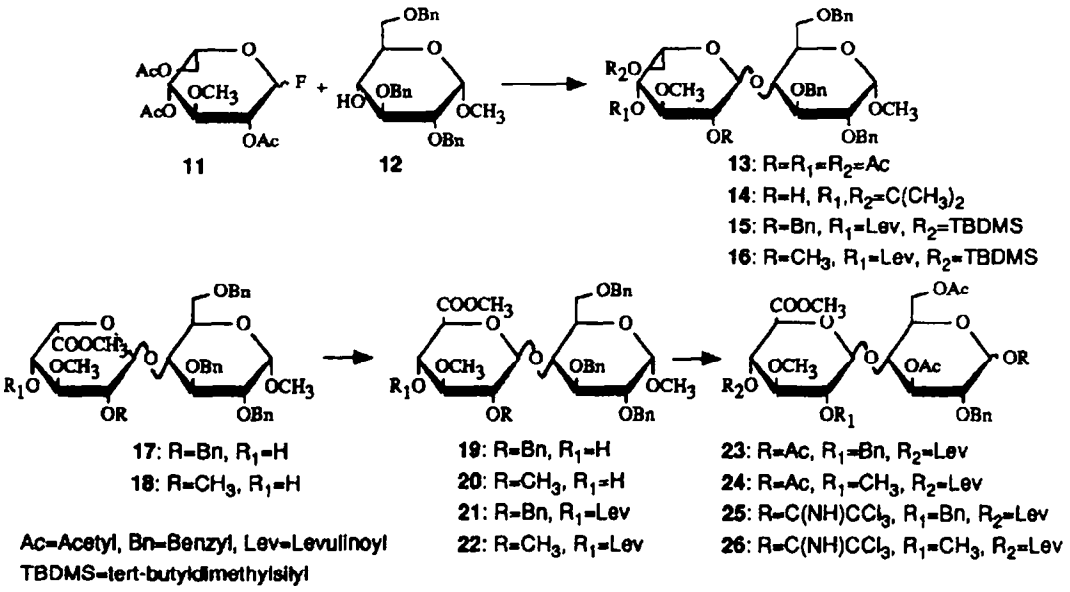
The key intermediates in the synthesis of series I and II are the suitably protected iduronic acid building blocks **17** and **18**, which can be transformed into their glucuronic acid counterparts (Scheme III) by epimerization. Since the required epimerization is carried out under basic conditions we had to protect **17** and **18** exclusively with alkyl groups. Fortunately, this does not give rise to synthetic difficulties in this 'non-glycosamino' glycan series of pentasaccharides, as we select ether or ester protecting groups for all hydroxyl groups to be sulfated. Glycosylation of acceptor **12**[22] with idopyranosyl fluoride **11**[23] in the presence of $BF_3 \cdot OEt_2$ and molecular sieves 4 Å in dichloromethane at 0 °C gave the disaccharide fragment **13** in 90 % yield (Scheme V). Saponification of the ester functions of compound **13** afforded compound **14**. According to well-established reaction sequences[24] compound **14** could be converted into compound **15** having a 2'-O-benzyl group. Jones oxidation of **15** (the 6'-O-TBDMS protective group is removed *in situ* under the applied conditions), followed by methyl-esterification and removal of the 4'-O-Lev protective group afforded the key intermediate **17**. Compound **18** was prepared in an analogous manner starting from **14**.

Synthesis of disaccharides 19 and 20

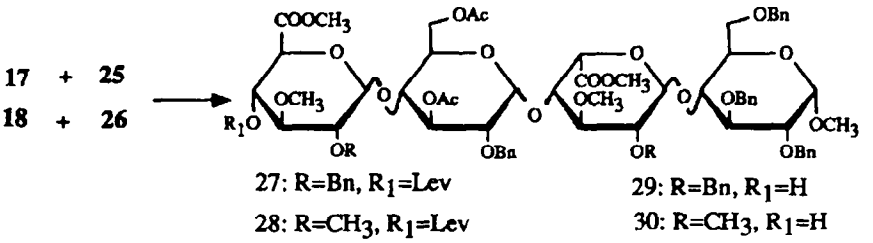
One of the crucial steps in our synthetic strategy involves epimerization of L-iduronic acid methylester **17** to give its D-glucuronic acid counterpart **19**. In this respect it is interesting to note that attempts to obtain L-iduronic acid derivative **17** by epimerization of D-glucuronic acid methylester **19** failed. Apparently the glucuronic acid configuration is lower in energy than the corresponding iduronic acid. Hence, treatment of **17** with sodium methoxide in methanol under reflux accomplished epimerization to afford **19**. Despite the strictly anhydrous conditions, partial hydrolysis of the methyl ester could not be avoided during the epimerization reaction. This decreases the yield of the reaction to a certain extent, since only esterified iduronic acid is prone to epimerization. Thus, after neutralization of the reaction mixture with Dowex H^+, the free carboxylate group was esterified with methyl iodide in *N*,*N*-dimethylformamide in the presence of $KHCO_3$ to give a mixture of **19** and starting material **17** (80 % yield, ratio 10:1). Application of benzyl esters in combination with sodium benzylate as base did not improve the isolated yield of the desired glucuronic acid derivative. Disaccharide fragment **20** was prepared in an analogous way starting from **18**.



Scheme IV. Series I and II.



Scheme V. Preparation of disaccharide fragments.



Scheme VI.

Synthesis of disaccharides 25 and 26

Another crucial step involves the acetolysis of the α-methoxy disaccharide **21**. Prior to acetolysis the 4'-OH has to be temporarily protected with a levulinoyl ester in order to ensure selective deblocking in the tetrasaccharide stage. Thus, treatment of **19** with levulinic acid and DCC afforded disaccharide **21** in a yield of 95 % (Scheme V). We now turned our attention towards the functionalization of the anomeric centre of **21** by acetolysis. Application of a mixture of trifluoroacetic acid/acetic anhydride/acetic acid (3.5/25/1, v/v/v) for the acetolysis required a long reaction time of 2 days[21] and afforded, after subsequent saponification of the anomeric acetate with piperidine and preparation of the imidate with cesium carbonate–trichloroacetonitrile[25] disaccharide imidate **25** in a yield of 75 % over three steps. However, acetolysis of **21** by the action of 2 % sulfuric acid in acetic anhydride[26] at –20 °C for 15 min, followed by anomeric deacetylation and imidate formation gave **25** in a comparable yield. The applied acetolysis conditions not only led to removal of the α-methoxy group but also of the 3- and 6-O-benzyl group. Fortunately, the 2-O-benzyl ether essential for the subsequent α-glycosidation remained unaffected. Disaccharide imidate **26** was prepared in an analogous manner starting from **20**.

Synthesis of tetrasaccharides 29 and 30

The assembly of the tetrasaccharide glycosyl-acceptors **29** and **30** is outlined in Scheme VI. Glycosylation of acceptor disaccharide **17** with disaccharide imidate **25** in the presence of the promoter trimethylsilyl triflate and molecular sieves 4 Å in dichloromethane at –15 °C afforded tetrasaccharide **27** in a yield of 75 %. It is of interest to note that, despite the application of an anomeric mixture[27,28] of imidates **25**, exclusively the α-coupled tetrasaccharide was isolated. This phenomenon should be explained by a high energy barrier for the transition state leading to the β-coupled product presumably caused by steric hindrance between donor and acceptor.[29] Selective removal of the levulinoyl function of **27** afforded acceptor tetrasaccharide **29**. Tetrasaccharide **30** was prepared in an analogous way starting from disaccharides **18** and **26**.

Preparation of imidates 35, 36 and 39

For the assemblage of the fully protected pentasaccharides **40–45** three different glucosyl

imidates had to be prepared. The synthesis of the appropriate glucosyl imidates **35**, **36** and **39** is outlined in Scheme VII. For the preparation of the glucosyl imidates **35** and **36**, 1,6-anhydro glucose units **31** and **32**[11] were subsequently methylated, subjected to acetolysis with trifluoroacetic acid, to afford **33** and **34**, treated with piperidine and with trichloroacetonitrile in the presence of cesium carbonate to give imidates **35** and **36** in an overall yield of 81 % and 87 %, respectively. Methylation of compound **37**, followed by acetolysis with sulfuric acid gave compound **38**. Subsequent saponification of the anomeric acetate and treatment with trichloroacetonitrile in the presence of cesium carbonate afforded imidate **39** in 60 % overall yield.

Construction of pentasaccharides 5–7

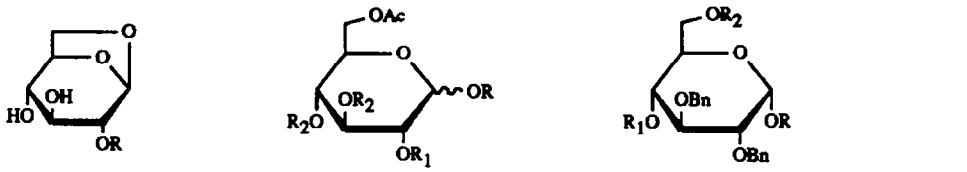
Coupling of glucosyl trichloroacetimidate **39** with acceptor tetrasaccharide **29** in the presence of trimethylsilyl trifluoromethylsulfonate and powdered molecular sieves 4 Å at −15 °C in dichloromethane afforded the α-coupled pentasaccharide **42** in a yield of 64 %, together with about 10 % of the β-coupled product. The fully protected pentasaccharide **42** was then saponified using lithium hydroperoxide[30] in order to avoid a β-elimination reaction leading to a Δ-4,5 uronic acid derivative. Subsequent hydrogenolysis and O-sulfation using triethylamine–sulfur trioxide complex, afforded sulfated pentasaccharide **7** in 75 % overall yield. Preparation of sulfated pentasaccharides **5** and **6** was accomplished analogously using glucosyl imidates **35** and **36**, respectively.

Construction of pentasaccharides 8–10

Coupling of glucosyl trichloroacetimidate **39** with acceptor tetrasaccharide **30**, as described for the coupling of **39** with **29** afforded the α-coupled pentasaccharide **45** in 75 % yield, together with some β-coupled product (not quantified). Deprotection and sulfation of fully protected pentasaccharide was performed as for pentasaccharide **42**, to give sulfated pentasaccharide **10** in about 80 % yield. Preparation of sulfated pentasaccharides **8** and **9** was accomplished analogously using glucosyl imidates **35** and **36**, respectively.

Analysis of the title compounds

The identity and homogeneity of the title compounds were each ascertained by ^{1}H NMR spectroscopy and FAB-mass spectrometry. In addition we demonstrated the applicability of reversed UV capillary electrophoresis (CE) as an analytical tool to determine the purity of the 'non-glycosamino' glycans. Recently, CE was reported as a sensitive and high-resolution method for the determination of disaccharide

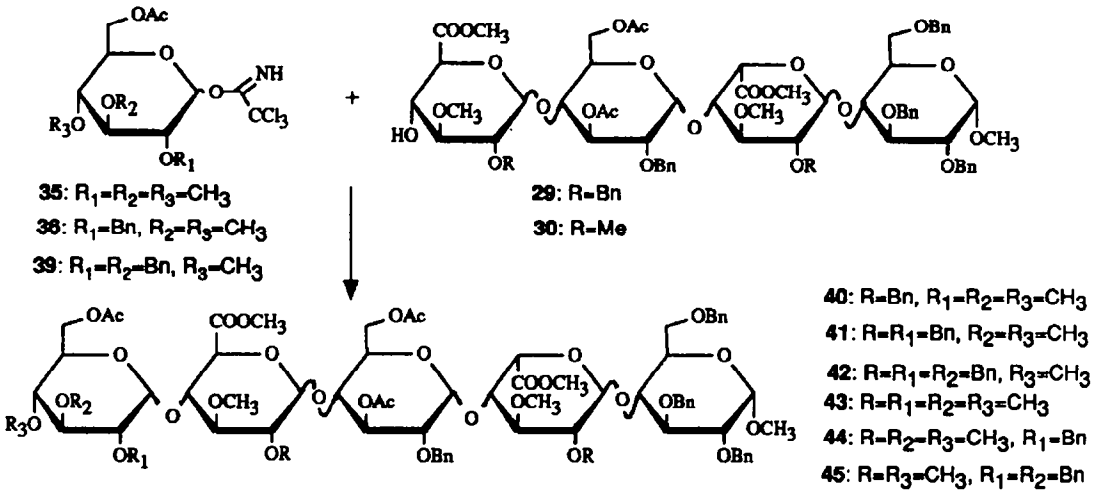


31: R=H
32: R=Bn

33: R=Ac, R_1=R_2=CH_3
34: R=Ac, R_1=Bn, R_2=CH_3
35: R=C(NH)CCl_3, R_1=R_2=CH_3
36: R=C(NH)CCl_3, R_1=Bn, R_2=CH_3

37: R=CH_3, R_1=H, R_2=Bn
38: R=Ac, R_1=CH_3, R_2=Ac
39: R=C(NH)CCl_3, R_1=Me, R_2=Ac

Scheme VII.



35: R_1=R_2=R_3=CH_3
36: R_1=Bn, R_2=R_3=CH_3
39: R_1=R_2=Bn, R_3=CH_3

29: R=Bn
30: R=Me

40: R=Bn, R_1=R_2=R_3=CH_3
41: R=R_1=Bn, R_2=R_3=CH_3
42: R=R_1=R_2=Bn, R_3=CH_3
43: R=R_1=R_2=R_3=CH_3
44: R=R_2=R_3=CH_3, R_1=Bn
45: R=R_3=CH_3, R_1=R_2=Bn

Scheme VIII.

composition of several proteaglycans.[31,32] The suitability of NMR spectroscopy and CE/(in)direct UV detection for the qualitative and quantitative analysis of low molecular weight (derivatives of) glycosaminoglycans has been exemplified previously.[33]

Since NMR spectroscopy and CE/indirect UV detection are totally independent methods, the combination of both procedures enables a reliable analysis. The use of indirect UV detection allows a quantitative interpretation of the data since a comparable detector response is obtained for all types of pentasaccharides. The electropherograms obtained for the title compounds are compiled in Figure 1, whereas the results are listed in Table 1. The title compounds all displayed a purity exceeding 95 %.

Biological activities: anti-Xa activities, AT-III binding affinities

The anti-Xa activities and AT-III binding affinities are collected in Table 2. The pentasaccharides **5–7** (series I) and **8–10** (series II) display comparable anti-Xa activities and binding affinities. However, the anti-Xa activities of series II are higher than those obtained for series I. This is quite remarkable, since in this series a critical sulfate ester, i.e. iduronic acid 2-O-sulfate, has been replaced by a methyl ether. Compound **8**, in particular, containing not less than seven methyl ethers, displays the very high anti-Xa activity of 1611 units/mg. However, note that the binding affinity (Kd) for AT-III is only slightly enhanced in comparison with compound **3**. One could speculate[34] that hydrophobic or van der Waals interactions between the methylated pentasaccharide and the apolar surface amino acid residues of AT-III effectively compensate the loss of electrostatic interactions relative to the glycosaminoglycan compound. Additionally, the enhanced lipophilic character of the alkylated compound leads to a reduced free energy of desolvation and this may also contribute to the enhanced anti-Xa activity.

Biological activities: in vivo *residence times in rat*

The time courses of the *in vivo* anti-Xa activity in the rat were evaluated for the title pentasaccharides. Figure 2 shows the time courses of anti-Xa activities of the

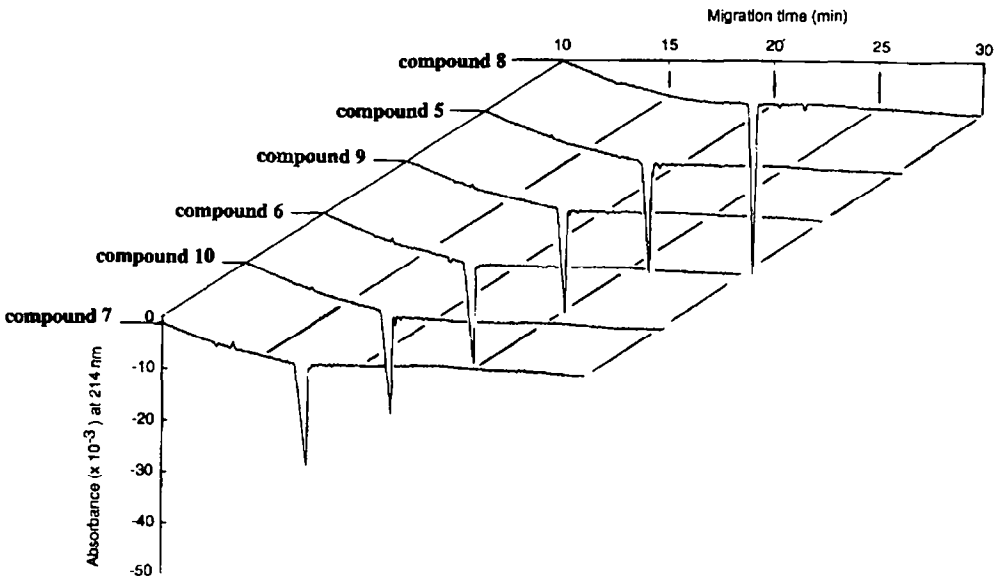


Figure 1. Electropherograms of the title compounds.

Table 1. Analysis of the purity of pentasaccharides **5–10** by capillary electrophoresis using indirect UV detection. The purity is measured as the percentage of the total peak area

pentasaccharide	*migration time (min)*	*purity (%)*
Series I		
5	17.99	≥95
6	17.22	≥95
7	16.80	≥95
Series II		
8	19.23	≥95
9	17.90	≥95
10	17.11	≥95

Table 2. Anti-Xa activities, binding affinities and elimination half-lives in rat of pentasaccharides

Pentasaccharide	*Anti-Xa activity (U/mg)*	*Binding affinity (Kd) (nM)*	*T½ of elimination (h)*
2	700	754	0.7
3	1250	25	7.3
4	1323	17	10.6
Series I			
5	1217	36	5.1
6	1159	53	2.2
7	1184	75	1.6
Series II			
8	1611	13	10.9
9	1318	36	7.8
10	1404	30	5.7

tested pentasaccharides after intravenous injection in rats, illustrating the obtainable range of elimination rates. However, it was recognized that pentasaccharides with the highest number of sulfate groups, i.e. pentasaccharides **7** and **10**, tend to have a shorter residence time than is expected from their K_d value.

Heparin is known to be subjected to multiple routes of elimination within the body,[35–40] which have been implicated in its non-linear pharmacokinetic profile.[41] With low molecular weight heparins, prolonged *in vivo* anti-Xa activity has been observed corresponding with much lower affinities for endothelial cells.[42]

Synthetic pentasaccharides, derived from the AT-III binding pentasaccharide **1**, have been found to exhibit a much more straightforward pharmacokinetic profile. In general, *in vivo* residence times of the majority of these pentasaccharides have been shown to be highly predictable from their binding affinities towards AT-III.[43] The half-life is determined by the ratio bound/free pentasaccharide, depending on the dissociation constant (K_d) of the pentasaccharide–AT-III complex, because freely circulating pentasaccharides are rapidly cleared by the kidneys. However, pentasaccharide **7** displays a shorter residence time than is expected from its K_d value,[44] suggesting an additional route of clearance. In order to explore such alternative routes for the title compounds, time courses of anti-Xa activity were also determined in nephrectomized rats. It was expected that the residence times of exclusively renally cleared pentasaccharides will be increased to a level equal or above that of circulating AT-III.[45] In contrast, an unexpected rapid clearance was obtained not only for pentasaccharide **7** but also for pentasaccharide **10**. These experiments indicate additional mechanisms of clearance for pentasaccharides with a heavily sulfated D-unit, **7** and **10**, because the expected increase in half-life was not observed in the experiments with nephrectomized rats.

The present paper demonstrates the versatile usefulness of the 'epimerization concept' for the preparation of pseudo-symmetrical 'non-glycosamino' glycan analogues of the AT-III binding pentasaccharide. The epimerization concept simplifies the synthesis of the title compounds, because the EF disaccharide block is obtained from the GH block. Additionally, we

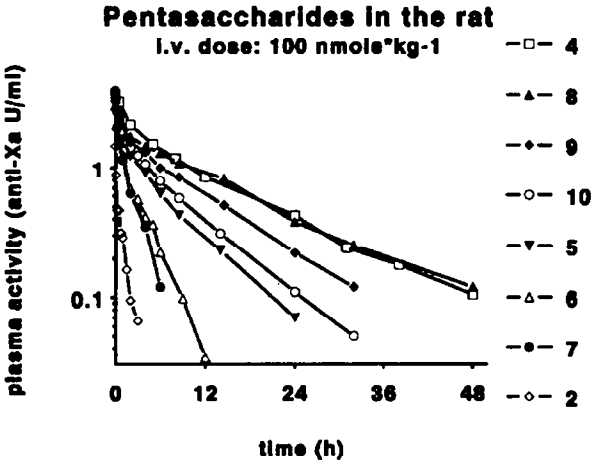


Figure 2. Residence times of the title compounds in rat.

demonstrated the applicability of reversed UV capillary electrophoresis (CE) as an analytical tool to determine the purity of 'non-glycosamino' glycans. In series II, having the crucial iduronic acid 2-O-sulfate ester replaced by a methyl ether, we obtained a very potent derivative, i.e. compound **8**, having 1611 anti-Xa units/mg and displaying a long *in vivo* residence time in rat. Moreover, the *in vivo* residence time appears to relate to minor chemical modifications in the D-unit. Compounds with a heavily sulfated D-unit, i.e. **7** and **10**, are not exclusively cleared by the kidneys.

The possibility to modulate the biological parameters of the products and their relative ease of preparation makes the 'non-glycosamino' glycan analogues of the AT-III binding pentasaccharide attractive candidates for pharmaceutical development.

Experimental

General methods

TLC and HPTLC were performed on Kieselgel 60 F254 (Merck) with detection by charring with sulfuric acid or sulfuric acid–molybdate–phosphoric acid. Column chromatography was performed on Kieselgel 60 (70–230 mesh, Merck). NMR spectra (internal Me_4Si or D_2O) were recorded on a Bruker WM 360 spectrometer

equipped with an ASPECT 3000 computer (^{1}H 360 MHz). FAB-mass spectra were recorded with a Finnigan MAT 90 mass spectrometer equipped with a WATV Cs ion gun (Wagner Analysen Technik Vertriebs GmbH, Worpswede, F.R.G.). The gun produced a beam of fast Cs ions (beam current ~2 μA at 22 kV). Thioglycerol or triethanolamine was used as the matrix.

Coupling of donor 11 with acceptor 12 to give disaccharide 13

A mixture of idopyranosyl fluoride **11** (31.9 g, 99 mmol) and acceptor **12** (48.7 g, 105 mmol) in dichloromethane (1 L) was stirred for 1 h in the presence of molecular sieves 4 Å (22 g). The mixture was cooled to −10 °C and a solution of borontrifluoride etherate (9.5 mL) in dichloromethane (100 mL) was added dropwise in 1 h. Thereafter the mixture was stirred for 1 h at 0 °C, filtered and subsequently washed with aqueous $NaHCO_3$ and brine, dried ($MgSO_4$), filtered and evaporated to dryness. The residual oil was purified by silica gel chromatography (1500 g, toluene/ethyl acetate, 4/1) to afford disaccharide **13** in 60 % yield (45 g). R_f 0.22 (toluene/ethyl acetate, 4/1). NMR data ($CDCl_3$): ^{1}H, δ ring G (non-reducing end): 1.86, 2.01, 2.08 (s, 3H, acetates), 4.98 (bs, 1H, H-1), 4.80 (c, 1H, H-2), 3.46 (c, 1H, H-3), 4.68 (c, 1H, H-4), 4.61 (c, 1H, H-5), 3.49 (s, 3H, OCH_3); ring H (reducing end): 4.58 (d, 1H, J = 4 Hz, H-1), 3.60 (dd, 1H, J = 4 Hz and 9 Hz, H-2), 3.85 (t, 1H, J = 9 Hz, H-3), 4.12 (c, 1H, H-4), 3.82 (c, 3H, H-5, H-6a, H-6b), 3.37 (s, 3H, OCH_3), 4.52, 4.65, 4.90 (3 CH_2Ph), 7.10–7.38 (15H, Ph).

Preparation of disaccharide 17

Compound **13** (46 g, 59.4 mmol) was dissolved in a mixture of methanol and dioxane (700 mL, 1:1, v/v). After the addition of a catalytic amount (0.5 g) of potassium *tert*-butoxide the mixture was stirred for 4 h, neutralized by the addition of Dowex-H$^+$ anion exchange resin, filtered and concentrated to dryness (R_f 0-45, dichloromethane/methanol, 9/1). The residual oil was dissolved in a mixture of DMF and 2,2-dimethoxypropane (200 mL, 3:1, v/v) and acidified to pH 4.5 with *p*-toluenesulfonic acid and stirred for 1 h, diluted with ethyl acetate, subsequently washed with aqueous $NaHCO_3$ and water, dried ($MgSO_4$), filtered and concentrated to give the crude acetonide **14** (R_f 0.30, toluene/ethyl acetate, 7/3). Crude **14** was coevaporated with DMF (50 mL), dissolved in DMF (300 mL) and treated with NaH (70 mmol) and benzyl bromide (13 g). After 1 h methanol was added to the reaction mixture and stirring was continued for 15 min. The mixture was diluted with ethyl acetate (600 mL), subsequently washed with aqueous $NaHCO_3$ and water, dried ($MgSO_4$), filtered and concentrated to give the crude 2'-O-benzyl derivative (R_f 0.35, dichloromethane/ethyl acetate, 9/1). The latter was treated with aqueous acetic acid (80 %, 150 mL) at 60 °C for 1 h, and thrice coevaporated with toluene (250 mL) to give, after silica gel chromatography (toluene/ethyl acetate, 1/1–1/4) the 4',6'-di-OH derivative (R_f 0.60, toluene/ethyl acetate, 1/4). A solution of the latter in pyridine (200 mL) was treated with *tert*-butyldimethylsilyl chloride (9.5 g) for 16 h, concentrated to a small volume, diluted with ethyl acetate, subsequently washed with dilute acid (0.3 M HCl) and water, dried ($MgSO_4$), filtered and concentrated to give the crude 6'-O-TBDMS derivative (R_f 0.61, hexane/ethyl acetate, 6/1). To a solution of the crude 6'-O-TBDMS derivative (42 g) in dioxane (350 mL), levulinic acid (11.6 g), dicyclohexylcarbodiimide (20.6 g) and 4-*N*,*N*-dimethylaminopyridine (1 g) were added. After 2 h, the mixture was diluted with with cold ether (500 mL), filtered, washed with aqueous $NaHSO_4$ and water, dried ($MgSO_4$), filtered and concentrated to give **15** (R_f 0.41, hexane/ethyl acetate, 3/2). To a stirred solution of **15** (27.4 g, 29 mmol) in acetone (350 mL) at 0 °C a solution of chromium trioxide (15.7 g) in a water/sulfuric acid (96 mL, 5:1, v/v) was added dropwise in 20 min. The mixture was stirred for 3 h at rt when methanol (33 mL) was added to the mixture at 0 °C. After 30 min sodium acetate (30 g), water (350 mL) and ethyl acetate (600 mL) were added. The organic layer was washed with water (100 mL), dried ($MgSO_4$), filtered and evaporated to dryness to give the crude iduronic acid derivative. To a solution of the crude iduronic acid derivative in DMF (150 mL) molecular sieves 4 Å (10 g) and potassium hydrogen carbonate (10 g) were added. After 15 min methyl iodide (10 mL) was added, the mixture was stirred for 3 h, filtered, diluted with ethyl acetate and subsequently washed with aqueous sodium thiosulfate and water, dried ($MgSO_4$), filtered, and concentrated to dryness. Silica gel chromatography of the residual oil (toluene/ethyl acetate, 3/2–1/1) afforded the fully protected iduronic acid methylester (84 %). To a solution of the latter (21.4 g, 25 mmol) in pyridine (120 mL), acetic acid (154 mL) and hydrazine hydrate (17.6 mL) were added. After 6.5 min the mixture was poured into water (500 mL) and extracted with ethyl acetate (2 × 00 mL). The combined organic layers were subsequently washed with dilute acid (0.3 M HCl, 2 × 250 mL) and water, dried ($MgSO_4$), filtered and concentrated to give, after silica gel chromatography (hexane/ethyl acetate, 1/1–2/3), disaccharide **17** (80 %, 15.2 g). R_f 0.45 (hexane/ethyl acetate, 2/3). NMR data ($CDCl_3$): ^{1}H, δ ring G (non-reducing end): 5.20 (bs, 1H, H-1), 3.45 (c, 1H, H-2), 3.54 (c, 1H, H-3), 3.93 (c, 1H, H-4), 4.86 (d, 1H, J = 2 Hz, H-5), 3.39 (s, 3H, OCH_3), 3.46 (s, 3H, $C(O)CH_3$), 3.41 (d, 1H, OH); ring H: 4.54 (d, 1H, J = 3.8 Hz, H-1), 3.56 (dd, 1H, J = 3.8 Hz and 9 Hz, H-2), 3.83 (t, 1H, J = 9 Hz, H-3), 3.90 (t, 1H, J = 9 Hz, H-4), 3.74 (dt, 1H, J = 3 Hz and 9 Hz, H-5), 3.35 (s, 3H, OCH_3), 4.42, 4.55, 4.63, 4.87 (4 CH_2Ph).

Compound **18** (R_f 0.42, hexane/ethyl acetate, 2/3) was prepared according to the procedure described for **17** with the difference that methyl iodide instead of benzyl bromide was used for the alkylation of **14**.

Epimerization of 17 to give 19

To a solution of compound **17** (5 g, 6.58 mmol) in methanol (75 mL), a solution of sodium methoxide in

methanol (1 M, 33 mL) was added. The mixture was refluxed for 16 h, cooled to rt, neutralized with Dowex-H^+ anion exhange resin, filtered and concentrated. The residual oil was dissolved in DMF (50 mL), molecular sieves 4 Å (2.3 g), potassium hydrogen carbonate (2.3 mL) and methyl iodide (2.3 mL) were added. After 3 h, the mixture was filtered, diluted with ethyl acetate (125 mL), subsequently washed with aqueous sodium thiosulfate and water, dried ($MgSO_4$), filtered and evaporated to dryness to give, after silica gel chromatography (hexane/ether 1/1–1/9), the glucuronic acid derivative **19** (73 %, 3.65 g). R_f 0.72 (hexane/ethyl acetate, 3/7). NMR data ($CDCl_3$): ^1H, δ ring E (non-reducing end): 4.37 (d, 1H, J = 8 Hz, H-1), 2.96 (t, 1H, J =9 Hz, H-3), 3.18 (dd, 1H, J = 8 Hz and 9 Hz, H-2), 3.81 (d, 1H, J = 9 Hz, H-5), 2.90 (d, 1H, J = 12 Hz,OH); ring F: 4.57 (d, 1H, J = 3.8 Hz, H-1), 3.86 (t, 1H, J = 9 Hz, H-3), 3.94 (t, 1H, J = 9 Hz, H-4).

Compound **20** was prepared in an analogous way starting from **18**.

Acetolysis of 21 to give 23

Compound **19** (3.65 g, 4.80 mmol) was treated with levulinic acid and dicyclohexylcarbodiimide, as described for the preparation of **15**, to give **21** in a yield of 90 % (R_f 0.57, hexane/ether, 2/3). To a solution of compound **21** (3.70 g, 4.32 mmol) in acetic anhydride (25 mL) at –20 °C a mixture of concentrated sulfuric acid in acetic anhydride (25 mL, 2 % sulfuric acid, v/v) of the same temperature was added. After stirring for 15 min at –20 °C sodium acetate (1 g) was added, the mixture was brought to ambient temperature, concentrated to a small volume, diluted with ethyl acetate and subsequently washed with aqueous $NaHCO_3$ and water, dried ($MgSO_4$), filtered and evaporated to dryness to give, after silica gel chromatography (toluene/ethyl acetate, 3/2–2/3), disaccharide **23** in a yield of 85 % (R_f 0.34, toluene/ethyl acetate, 2/3). NMR data ($CDCl_3$): ^1H, δ ring E (non-reducing end): 4.21 (d, 1H, J = 8 Hz, H-1), 3.04 (dd, 1H, J = 8 Hz and 9 Hz, H-2), 3.26 (t, 1H, J = 9 Hz, H-3), 4.96 (dd, 1H, J = 9 Hz and 10 Hz, H-4), 3.79 (d, 1H, J = 10 Hz, H-5), 3.47 (s, 3H, OCH_3), 3.71 (s, 3H, $C(O)OCH_3$), 4.74 (CH_2Ph); ring F: 6.32 (d, 1H, J = 4 Hz, H-1), 3.55 (dd, 1H, J = 4 Hz and 9 Hz, H-2), 5.41 (t, 1H, J = 9 Hz, H-3), 3.61 (c, 1H, H-4), 3.98 (m, 1H, H-5), 4.29 (c, 1H, H-6a), 4.53 (c, 1H, H-6b), 5.13 (CH_2Ph), 7.2–7.4 (10H, Ph).

Compound **24** was prepared in an analogous way starting from **22**.

Preparation of imidate 25

To a solution of disaccharide **23** (3.07 g, 3.67 mmol) in THF (60 mL), piperidine (5.5 mL) was added. After 16 h the mixture was diluted with ether (60 mL) and subsequently washed with dilute acid (0.3 M HCl, 2 × 25 mL), water and aqueous $NaHCO_3$, dried ($MgSO_4$), filtered and evaporated to dryness to give an oil (R_f 0.3, toluene/ethyl acetate, 2/3). The latter was dissolved in a mixture of dichloromethane and trichloroacetonitrile (16 mL, 15/1, v/v) and cesium carbonate (150 mg) was added. After 45 min the mixture was filtered, concentrated and applied to silica gel chromatography (toluene/ethyl acetate 3/2–2/3) to give imidate **25** in 85 % yield over two steps (2.78 g). R_f 0.45 (toluene/ethyl acetate, 2/3). NMR data ($CDCl_3$): ^1H, δ ring E (non-reducing end): 4.34 (d, 1H, J = 8 Hz, H-1), 3.06 (dd, 1H, J = 8 Hz and 9 Hz, H-2), 3.24 (t, 1H, J = 9 Hz, H-3), 4.94 (dd, 1H, J = 9 Hz and 10 Hz, H-4); ring F: 6.54 (d, 1H, J = 4 Hz, H-1), 5.54 (t, 1H, J = 9 Hz, H-3), 8.62 (s, 1H, NH).

Compound **26** was prepared in an analogous way starting from **24**.

Preparation of tetrasaccharide 29

To a solution of aglycon **17** (676 mg, 0.89 mmol) and imidate **25** (795 mg, 0.89 mmol) in dichloromethane (23 mL) molelcular sieves 4 Å (785 mg) were added. After stirring for 30 min the mixture was cooled to –20 °C and 0.3 mL of a stock solution of trimethylsilyl trifluoromethanesulfonate (0.26 mL) in dichloromethane (3 mL) was added. After 15 min additional imidate **25** (120 mg) and trimethylsilyl trifluoromethanesulfonate (0.1 mL stock solution) were added to the reaction mixture. After 15 min the reaction mixture was filtered, diluted with dichloromethane (25 mL) and subsequently washed with aqueous $NaHCO_3$, brine and water, dried ($MgSO_4$), filtered and concentrated. The residual oil was applied to a column of Sephadex LH20 (120 × 2 cm^2) eluted with dichloromethane/methanol 2/1 to give tetrasaccharide **27** as an oil in 89 % yield (R_f 0.46, toluene/ethyl acetate, 1/1). Treatment of **27** with hydrazine acetate, as described for the preparation of **17**, afforded, after silica gel chromatography (toluene/ether, 2/1–1/3) the tetrasaccharide acceptor **29** in 86 % yield. R_f 0.36 (toluene/ether, 1/1). NMR data ($CDCl_3$): ^1H, δ ring E (non-reducing end): 4.26 (d, 1H, J = 8 Hz, H-1), 3.14 (c, 2H, H-2, H-3), 3.71 (c, 1H, H-4), 3.76 (d, 1H, J = 9 Hz, H-5); ring F: 5.20 (d, 1H, J = 3 Hz, H-1), 3.47 (c, 1H, H-2), 5.45 (t, 1H, J = 9 Hz, H-3), 3.62 (t, 1H, J = 9 Hz, H-4), 4.08 (m, 1H, H-5), 4.17 (dd, 1H, J = 5 Hz and 12 Hz, H-6a), 4.29 (dd, 1H, J = 3 Hz and 12 Hz, H-6b); ring G: 5.32 (d, 1H, J = 7 Hz, H-L), 3.20 (dd, 1H, J = 7 Hz and 9 Hz, H-2), 3.75 (t, 1H, J = 9 Hz, H-3), 4.52 (d, 1H, J = 6 Hz, H-5); ring H: 4.54 (d, 1H, J = 4 Hz, H-1), 3.47 (dd, 1H, J = 4 Hz and 9 Hz, H-2), 3.83 (c, 2H, H-3, H-4), 3.57 (c, 1H, H-5).

Compound **30** was prepared in an analogous way starting from acceptor **18** and imidate **26**.

1,6-Di-O-acetyl-2,3,4-tri-O-methyl-D-glucopyranose (33)

To a stirred mixture of **31** (162 mg, 1 mmol) and NaH (84 mg) in DMF (5 mL) was added methyl iodide (3.5 mmol) dropwise at 0 °C. The mixture was stirred for 30 min at 20 °C, then diluted with CH_2Cl_2 (25 mL), washed with brine (15 mL), dried ($MgSO_4$), and concentrated. To a solution of the crude mixture in

acetic anhydride (5 mL) was added dropwise at 0 °C trifluoroacetic acid (0.2 mL). After 3 h at room temperature the mixture was coevaporated with toluene (3 × 25 mL) to afford **9** as an oil (300 mg, 100 %), R_f 0.62 (4/1 toluene/EtOH). NMR data ($CDCl_3$): ^1H, δ 2.07, 2.16 (s, 3H, acetates), 3.15 (dd, 1H, J = 8.5 Hz and 11 Hz, H-4), 3.28 (dd, 1H, J = 3 Hz and 9.8 Hz, H-2), 3.44–3.66 (c, 10H, 3 × OCH_3, H-3), 3.78 (dt, 1H, H-5), 4.26 (c, 2H, H-6a, H-6b), 6.26 (d, 1H, J = 3 Hz, H-1).

1,6-Di-O-acetyl-2-benzyl-3,4-di-O-methyl-D-glucopyranose (34)

Treatment of **32** (250 mg, 1 mmol), as described for compound **33**, with NaH (60 mg) and methyl iodide (2.4 mmol), subsequent acetolysis with acetic anhydride/trifluoroacetic acid (5.2 mL, 25/1) afforded **34** (380 mg, 100 %), R_f 0.52 (4/1 toluene/EtOAc). NMR data ($CDCl_3$): ^1H, δ 2.04, 2.14 (s, 3H, acetates), 3.12 (dd, 1H, J = 9 Hz and 10.5 Hz, H-4), 3.42–3.66 (c, 8H, 2 × OCH_3, H-2, H-3), 3.79 (dt, 1H, H-5), 4.22 (c, 2H, H-6a, H-6b), 4.66 (AB, 2H, PhCH_2), 6.24 (d, 1H, J = 3.4 Hz, H-1), 7.28–7.41 (5H, Ph).

1,6-Di-O-acetyl-2,3-di-O-benzyl-4-O-methyl-D-glucopyranose (38)

Treatment of **37** (460 mg, 1 mmol) with NaH (30 mg) and methyl iodide (1.2 mmol), as described for compound **33**, afforded the corresponding 4-O-methyl derivative in a quantitative yield. To a stirred solution of the latter in acetic anhydride (5 mL) at –20 °C was added a cold mixture (–20 °C) of H_2SO_4 in acetic anhydride (2 % v/v, 5 mL). After 15 min excess sodium acetate was added, the mixture was diluted with EtOAc (40 mL), successively washed with aqueous $NaHCO_3$ (2 × 25 mL), water (25 mL), the organic layer was dried ($MgSO_4$), and concentrated. Column chromatography (9/1 toluene/EtOAc) of the residue gave **38** (320 mg, 70 %), R_f 0.55 (4/1 toluene/EtOAc). NMR data ($CDCl_3$): ^1H, δ 2.08, 2.18 (s, 3H, acetates), 3.25 (dd, 1H, J = 9.0 Hz and 10.0 Hz, H-4), 3.54 (s, 3H, OCH_3), 3.62 (dd, 1H, J = 3.5 Hz and 9.0 Hz, H-2), 3.82–3.88 (c, 2H, H-3, H-5), 4.25–4.28 (c, 2H, H-6a, H-6b), 4.61–4.95 (2 × AB, 4H, 2 × CH_2Ph), 6.29 (d, 1H, J = 3.5 Hz, H-1), 7.20–7.40 (c, 10H, 2 × Ph).

6-O-Acetyl-2,3,4-tri-O-methyl-D-glucopyranose trichloroacetimidate (35)

A mixture of **33** (300 mg, 1 mmol) and piperidine (0.72 mL) in THF (10 mL) was kept at room temperature for 16 h, diluted with CH_2Cl_2 (25 mL), washed with aqueous HCl (0.3 M, 15 mL), water (15 mL), dried ($MgSO_4$) and concentrated to give the corresponding compound with a free reducing end. The latter crude compound (248 mg, 0.94 mmol) was dissolved in CH_2Cl_2 (8 mL), trichloroacetonitrile (0.5 mL) and cesium carbonate (630 mg) were added and the mixture was stirred for 1 h, diluted with EtOAc (25 mL), filtered, washed with aqueous $NaHCO_3$, dried ($MgSO_4$), and concentrated. Column chromatography (4/1 toluene/EtOAc) of the residue afforded **35** (330 mg, 81 %), R_f 0.60 (1/1 toluene/EtOAc). NMR data ($CDCl_3$): ^1H, δ 2.08 (s, 3H, acetate), 3.18 (dd, 1H, J = 8.5 Hz and 10 Hz, H-4), 3.38 (dd, 1H, J = 3.4 Hz and 9.8 Hz, H-2), 3.45–3.61 (c, 10H, 3 × OCH_3, H-3), 3.93 (m, 1H, H-5), 4.23–4.4.36 (c, 2H, H-6a, H-6b), 6.47 (d, 1H, J = 3.4 Hz, H-1), 8.65 (s, 1H, NH).

6-O-Acetyl-2-benzyl-3,4-di-O-methyl-D-glucopyranose trichloroacetimidate (36)

Treatment of **34** (380 mg, 1 mmol) with piperidine and trichloroacetonitrile/cesium carbonate, as described for compound **33**, afforded **36** (420 mg, 87 %), R_f 0.73 (3/2 toluene/EtOAc). NMR data ($CDCl_3$): ^1H, δ 2.11 (s, 3H, acetate), 3.16 (dd, 1H, J = 8.5 Hz and 10.5 Hz, H-4), 3.36 (dd, 1H, J = 3.5 Hz and 8.5 Hz, H-2), 3.54, 3.67 (s, 3H, OCH_3), 3.64 (t, 1H, J = 8.5 Hz, H-3), 4.04 (m, 1H, H-5), 4.38 (c, 2H, H-6a, H-6b), 4.73 (AB, 2H, CH_2Ph), 6.39 (d, 1H, J = 3.5 Hz, H-1), 7.11–7.40 (5H, Ph), 8.70 (s, 1H, NH).

6-O-Acetyl-2,3-di-O-benzyl-4-O-methyl-D-glucopyranose trichloroacetimidate (39)

Treatment of **38** (320 mg, 0.7 mmol), as described for compound **33**, with piperidine and trichloroacetonitrile/cesium carbonate gave **39** (338 mg, 86 %), R_f 0.55 (7/3 toluene/EtOAc). NMR data ($CDCl_3$), ^1H, δ 2.09 (s, 3H, acetate), 3.29 (dd, 1H, J = 9.0 Hz and 10.0 Hz, H-4), 3.55 (s, 3H, OCH_3), 3.70 (dd, 1H, J = 9.0 Hz and 4.0 Hz, H-2), 3.95 (c, 2H, H-3, H-5), 4.26–4.38 (c, 2H, H-6a, H-6b), 4.70–4.98 (c, 4H, CH_2Ph), 6.43 (d, 1H, J = 4.0 Hz, H-1), 7.20–7.40 (10H, Ph), 8.70 (s, 1H, NH).

*Coupling of **35** with **30** to give **43***

A mixture of **35** (49 mg, 0.12 mmol) and **30** (129 mg, 0.10 mmol) in CH_2Cl_2 (3.5 mL) was stirred for 1 h in the presence of activated molecular sieves 4 Å (112 mg). At a temperature of –20 °C 0.3 mL of a stock solution of trimethylsilyl trifluoromethanesulfonate (25 μL) in CH_2Cl_2 (3 mL) was added dropwise to the reaction mixture. After 15 min extra **35** (0.06 mmol) and stock solution (0.2 mL) of trimethylsilyl trifluoromethanesulfonate in CH_2Cl_2 were added. After 15 min the mixture was diluted with CH_2Cl_2 (20 mL), filtered, washed with aqueous $NaHCO_3$ and brine (10 mL), the organic layer was dried ($MgSO_4$) and concentrated. The residue was purified by colomn chromatography (1/1 → 2/3 heptane/EtOAc) to give **43** (92 mg, 60 %), R_f 0.37 (2/3 heptane/EtOAc). NMR data ($CDCl_3$): ^1H, δ 2.06, 2.09 (s, 3H, acetate), 3.46, 3.48, 3.50, 3.53, 3.55 (7 × OCH_3), 4.53–5.00 (10H, CH_2Ph), 7.20–7.40 (25H, Ph), ring D: 5.49 (d, 1H, J = 3 Hz, H-1), 3.14 (dd, 1H, J = 3 Hz and 7 Hz), 3.38 (c, 1H, H-3), 3.07 (dd, 1H, J = 6 Hz and 7 Hz, H-4), 3.38 (c, 1H, H-5), 4.23 (c, 2H, H-6a, H-6b), ring E: 4.34 (d, 1H, J = 6.0

Hz, H-1), 3.03 (dd, 1H, J = 6 Hz and 7 Hz, H-2), 3.38 (c, 1H, H-3), 3.96 (dd, 1H, J = 6 Hz and 7 Hz, H-4), 3.83 (d, 1H, J = 7 Hz, H-5), 3.61 (s, 3H, $C(O)CH_3$), ring F: 5.12 (d, 1H, J = 3.0 Hz, H-1), 3.41 (dd, 1H, J = 3 Hz and 7 Hz, H-2), 3.72 (c, 2H, H-3, H-4), 3.97 (c, 1H, H-5), 4.35 (dd, 1H, J = 3 and 9 Hz, H-6a), 4.47 (dd, 1H, J = 2 and 9 Hz, H-6b), ring G: 5.29 (d, 1H, J = 5.0 Hz, H-1), 2.97 (dd, 1H, J = 5 Hz and 6 Hz, H-2), 3.67 (t, 1H, J = 6 Hz, H-3), 3.82 (c, 1H, H-4), 4.46 (d, 1H, J = 6 Hz, H-5), 3.63 (s, 3H, $C(O)CH_3$), ring H: 4.59 (d, 1H, J = 3.0 Hz, H-1), 3.51 (c, 1H, H-2), 3.87 (t, 1H, J = 7.0 Hz, H-3), 3.39 (s, 3H, OCH_3).

Coupling of 36 with 30 to give 44

Coupling imidate **36** with **30** (0.10 mmol) according to the same procedure as described for **43** furnished fully protected pentasaccharide **44** (98 mg, 61 %), R_f 0.18 (3/7 hexane/ether). NMR data ($CDCl_3$): ^1H, δ 2.07, 2.09 (s, 3H, acetates), 3.46, 3.48, 3.50 (18H, 6 × OCH_3), 4.51-4.99 (c, 12H, CH_2Ph), 7.19–7.40 (c, 30H, Ph), ring D: 5.41 (d, 1H, J = 3 Hz, H-1), 3.36 (c, 1H, H-2), 3.07 (dd, 1H, J = 6 and 7 Hz, H-3), 3.46 (t, 1H, J = 7 Hz, H-4), 4.19 (dd, 1H, J = 2 Hz and 9 Hz, H-6a), 4.25 (dd, 1H, J = 3 Hz and 9 Hz, H-6b), ring E: 4.33 (d, 1H, J = 6 Hz, H-1), 3.03 (dd, 1H, J = 6 Hz and 7 Hz, H-2), 3.34 (t, 1H, J = 7 Hz, H-3), 3.83 (d, 1H, J = 6 Hz, H-5), 3.51 (s, 3H, $C(O)CH_3$), ring F: 5.11 (d, 1H, J = 3 Hz, H-1), 3.72 (c, 2H, H-3, H-4), 3.97 (c, 1H, H-5), ring G: 5.29 (d, 1H, J = 5 Hz, H-1), 2.97 (dd, 1H, J = 5 and 6 Hz, H-2), 3.68 (c, 1H, H-3), 3.82 (t, 1H, J = 5 Hz, H-4), 4.45 (d, 1H, J = 5 Hz, H-5), 3.65 (s, 3H, $C(O)CH_3$), ring H: 4.57 (d, 1H, J = 3 Hz, H-1), 3.47 (c, 1H, H-2), 3.83 (t, 1H, J7 Hz, H-3), 3.37 (s, 3H, OCH_3).

Glycosylation of 30 with 39 to afford 45

Compound **45** was prepared according to the procedure described for **43** by coupling imidate **39** with **30** (0.10 mmol). Fully protected pentasaccharide **45** could be isolated in 75 % (122 mg), R_f 0.27 (1/4 heptane/ether). NMR data ($CDCl_3$): ^1H, δ 1.98, 2.07, 2.09 (s, 3H, acetates), 3.47, 3.49, 3.50, 3.52 (5 × OCH_3), 4.56–4.91 (c, 12H, CH_2Ph), 7.18–7.40 (c, 30H, Ph), ring D: 5.42 (d, 1H, J = 3 Hz, H-1), 3.46 (c, 1H, H-2), 3.18 (dd, 1H, J = 6 and 7 Hz, H-4), 3.42 (c, 1H, H-5), ring E: 4.14 (d, 1H, J = 6 Hz, H-1), 2.99 (dd, 1H, J = 6 Hz and 7 Hz, H-2), 3.32 (t, 1H, J = 7 Hz, H-3), 3.89 (t, 1H, J = 7 Hz, H-4), 3.83 (d, 1H, J = 7 Hz, H-5), 3.62 (s, 3H, $C(O)CH_3$), ring F: 5.18 (d, 1H, J = 3 Hz, H-1), 3.42 (dd, 1H, J = 3 Hz and 7 Hz, H-2), 5.28 (t, 1H, J = 7 Hz, H-3), 3.58 (t, 1H, J = 7 Hz, H-4), 4.08 (c, 1H, H-5), 4.49 (dd, 1H, J = 2 Hz and 9 Hz, H-6a), ring G: 5.37 (d, 1H, J = 5 Hz), 2.94 (dd, 1H, J = 5 Hz and 6 Hz, H-2), 3.64 (t, 1H, J = 6 Hz, H-3), 3.83 (c, 1H, H-4), 4.41 (d, 1H, J = 5 Hz, H-5), 3.72 (s, 3H, $C(O)CH_3$), ring H: 4.57 (d, 1H, J = 3 Hz, H-1), 3.50 (c, 1H, H-2), 3.38 (c, 1H, H-3), 3.38 (s, 3H, OCH_3).

Coupling of 35 with 29 to yield 40

Glycosylation of **29** (0.10 mmol) with imidate **35** according to the procedure as described for **43** afforded fully protected pentasaccharide **40** (98 mg, 60 %), R_f 0.53 (4/1 toluene/acetone). NMR data ($CDCl_3$): ^1H, δ 2.07, 2.09 (s, 3H, acetates), 3.46, 3.47, 3.49, 3.51 (5 × OCH_3), 4.52–5.00 (c, 12H, 6 × CH_2Ph), 7.18–7.40 (c, 30H, Ph), ring D: 5.45 (d, 1H, J = 3 Hz, H-1), ring E: 4.23 (d, 1H, J = 6 Hz, H-1), ring F: 5.18 (d, 1H, J = 3.5 Hz, H-1), ring G: 5.38 (d, 1H, J = 7 Hz, H-1), 4.57 (d, 1H, J = 3 Hz, H-1).

Condensation of 36 with 29 to furnish 41

Coupling of imidate **36** with acceptor **29** (0.10 mmol) analogous to the procedure described for **43** afforded pentasaccharide **41** (106 mg, 62 %), R_f 0.50 (93/7 CH_2Cl_2/acetone). NMR data ($CDCl_3$): ^1H, δ 1.95, 2.04, 2.09 (s, 3H, acetate), 4.40–4.93 (14H, CH_2Ph), 7.18–7.42 (35H, Ph), ring D: 5.36 (d, 1H, J = 3.0 Hz, H-1), 3.36 (c, 1H, H-2), 3.46 (c, 1H, H-3), 3.06 (dd, 1H, J = 6 Hz and 7 Hz, H-4), 3.37 (c, 1H, H-5), 4.23 (c, 2H, H-6a, H-6b), ring E: 4.22 (c, 1H, H-1), 3.28 (dd, 1H, J = 6 Hz and 7 Hz, H-2), 3.37 (c, 1H, H-3), 3.90 (c, 1H, H-4), 3.84 (c, 1H, H-5), 3.64 (s, 3H, $C(O)CH_3$), ring F: 5.18 (d, 1H, J = 3.0 Hz, H-1), 3.46 (c, 1H, H-2), 5.32 (t, 1H, J = 7.0 Hz, H-3), 3.59 (t, 1H, J = 7.0 Hz, H-4), 4.08 (c, 1H, H-5), 4.22 (c, 1H, H-6a), 4.37 (dd, 1H, J = 2.0 Hz and 9.0 Hz, H-6b), ring G: 5.38 (d, 1H, J = 5.0 Hz, H-1), 3.20 (dd, 1H, J = 5.0 Hz and 6.0 Hz, H-2), 3.72 (t, 1H, J = 6.0 Hz, H-3), 3.82 (c, 1H, H-4), 4.45 (d, 1H, J = 4.0 Hz, H-5), 3.72 (s, 3H, $C(O)CH_3$), ring H: 4.54 (d, 1H, J = 3.4 Hz, H-1), 3.46 (c, 1H, H-2), 3.80 (c, 2H, H-3, H-4), 3.90 (c, 1H, H-5), 3.60 (c, 2H, H-6a, H-6b), 3.35 (s, 3H, OCH_3).

Glycosylation of 29 with 39 to obtain 42

Application of the procedure described for **43** on the coupling of imidate **39** with tetrasaccharide **29** (0.10 mmol) gave pentasaccharide **42** (107 mg, 60 %). R_f 0.60 (7/3 toluene/EtOAc). NMR data ($CDCl_3$): ^1H, δ 1.94, 1.96, 2.09 (s, 3H, acetate), 3.41, 3.45, 3.51 (3 × OCH_3), 4.36–5.24 (c, 16H, CH_2Ph), 7.12–7.41 (c, 40H, Ph), ring D: 5.46 (d, 1H, J = 3 Hz, H-1), 3.47 (c, 1H, H-2), 3.74 (t, 1H, J = 7 Hz, H-3), 3.20 (c, 1H, H-4), 3.52 (c, 1H, H-5), ring E: 4.56 (d, 1H, J = 5 Hz, H-1), 3.20 (c, 1H, H-2), 3.38 (t, 1H, J = 7 Hz, H-3), 3.99 (dd, 1H, J = 6 Hz and 7 Hz, H-4), 3.88 (d, 1H, J = 6 Hz, H-5), 3.76 (s, 3H, $C(O)CH_3$), ring F: 5.22 (d, 1H, J = 3 Hz), 3.47 (c, 1H, H-2), 5.42 (t, 1H, J = 7 Hz, H-3), 3.60 (t, 1H, J = 7 Hz, H-4), 4.04 (m, 1H, H-5), 4.14 (dd, 1H, J = 4 and 8 Hz, H-6a), 4.28 (c, 1H, H-6b), ring G: 5.32 (d, 1H, J = 5 Hz, H-1), 3.20 (c, 1H, H-2), 3.74 (e, 1H, H-3), 3.84 (c, 1H, H-4), 4.48 (d, 1H, J = 5 Hz, H-5), 3.81 (s, 3H, $C(O)CH_3$), ring H: 4.55 (d, 1H, J = 3 Hz, H-1), 3.45 (c, 1H, H-2), 3.82 (c, 2H, H-3, H-4), 3.58 (c, 1H, H-5), 3.60 (c, 2H, H-6a, H-6b), 3.34 (s, 3H, OCH_3).

General deprotection/sulfation methods

Saponification. To a solution of the fully protected pentamer (50 μmol) in THF/30 % H_2O_2(9 mL, 2/1) was added dropwise at –5 °C aqueous LiOH (1.25 M, 1.3 mL). After 16 h at 0 °C methanol (6 mL) and

aqueous NaOH (4 M, 1.7 mL) were added to the mixture. After 6 h at room temperature the mixture was acidified to pH 2 with HCl (2 M), diluted with EtOAc, and the organic layer was extracted with aqueous Na_2SO_3 (pH = 2.5), dried ($MgSO_4$) and evaporated. The residue was purified over silica gel (RP 18, CH_3CN/H_2O, 65/35–7/3) to give the saponified pentamer (40 μmol, 80 %).

Hydrogenolysis. A solution of the saponified pentamer (40 μmol) in *t*-butanol/water (15 mL, 2/1) was stirred under hydrogen atmosphere in the presence of 10 % Pd/C (75 % w/w based on the pentamer) for 16 h, filtered and the filtrate was concentrated. The fully deprotected pentamer (40 μmol, 100 %) was directly used for the sulfation.

Sulfation. The fully deprotected pentamer (40 μmol) was dissolved in DMF. Under nitrogen atmosphere, triethylamine sulfur trioxide complex was added (5 eq. for each hydroxyl group) and the mixture was stirred for 16 h at 50 °C. The mixture was cooled to 0 °C and aqueous $NaHCO_3$ was added (5 eq. per eq. triethylamine sulfur trioxide complex). The mixture was concentrated to a small volume and applied on a Sephadex G-25 column eluted with water/acetonitrile (4/1, v/v). The appropriate fractions were pooled, concentrated to a small volume, applied on a Dowex 50 XW4-Na^+ ion-exchange column in water and the eluate was lyophilized to provide the sulfated pentamer as a white fluffy solid (39 μmol, 97 %).

Compound 5. $[\alpha]_D$ +46.7 ° (*c* 0.55, H_2O); NMR data (D_2O): ^1H, δ 3.58, 3.59, 3.61, 3.63, 3.65 (5 × OCH_3), ring D: 5.48 (d, 1H, *J* = 3 Hz, H-1), 3.33 (dd, 1H, *J* = 3 and 7 Hz, H-2), 3.56 (t, 1H, *J* = 7 Hz, H-3), 3.36 (t, 1H, *J* = 7 Hz, H-4), 3.89 (dt, 1H, *J* = 2 Hz and 7 Hz, H-5), ring E: 4.67 (d, 1H, *J* = 5 Hz, H-1), 4.28 (c, 1H, H-2), 3.63 (c, 1H, H-2), 3.95 (t, 1H, *J* = 7 Hz, H-4), 3.76 (d, 1H, *J* = 7 Hz, H-5), ring F: 5.50 (d, 1H, *J* = 3 Hz, H-1), 4.35 (c, 1H, H-2), 4.60 (c, 1H, H-3), 3.93 (t, 1H, *J* = 7 Hz, H-4), 4.13 (c, 1H, H-6a), ring G: 5.16 (d, 1H, *J* = 4 Hz, H-1), 4.42 (dd, 1H, *J* = 4 and 6.5 Hz, H-2), 3.81 (dd, 1H, *J* = 4.5 Hz and 6.5 Hz, H-3), 4.28 (c, 1H, H-4), 4.97 (d, 1H, *J* = 5 Hz, H-5), ring H: 5.14 (d, 1H, *J* = 3 Hz, H-1), 4.35 (c, 1H, H-2), 4.60 (c, 1H, H-3), 4.02 (c, 2H, H-4, H-5), 4.32 (c, 1H, H-6a), 4.50 (c, 1H, H-6b), 3.46 (s, 3H, OCH_3). FAB-mass spectrum: m/z 1879 $(M - Na)^-$, 1857 $(M - 2Na + H)^-$, 1835 (M – 3Na + 2H), 1903 $(M + H)^+$, 1881 $(M - Na + 2H)^+$.

Compound 6. $[\alpha]_D$ +39.8 ° (*c* 1.05, H_2O); NMR data (D_2O) ^1H, δ 3.61, 3.65, 3.69 (4 × OCH_3), ring D: 5.53 (d, 1H, *J* = 3.6 Hz, H-1), 4.20 (dd, 1H, *J* = 3.6 Hz and 10 Hz, H-2), 3.67 (c, 1H, H-3), 3.41 (t, 1H, *J* = 9.6 Hz, H-4), ring E: 4.75 (d, 1H, *J* = 8 Hz, H-1), 4.26 (c, 1H, H-2) 3.63 (c, 1H, H-3), 3.94 (c, 1H, H-4), 3.80 (d, 1H, *J* = 7 Hz, H-5), ring F: 5.48 (d, 1H, *J* = 4 Hz, H-1), 4.35 (c, 1H, H-2), 4.59 (c, 1H, H-3), 3.92 (c, 1H, H-4), ring G: 5.16 (d, 1H, *J* = 5 Hz, H-1), 4.41 (dd, 1H, *J* = 5 Hz and 9 Hz, H-2), 3.81 (dd, 1H, *J* = 4 Hz and 9 Hz, H-3), 4.96 (d, 1H, *J* = 3.6 Hz, H-5), ring H: 5.14 (d, 1H, *J* = 3.8 Hz, H-1), 4.59 (c, 1H, H-3), 4.02 (c, 2H, H-4, H-5), 3.46 (s, 3H, OCH_3). FAB-mass spectrum: m/z 1967 $(M - Na)^-$, 1945 $(M - 2Na + H)^-$, 1923 $(M - 3Na + 2H)^-$, 2013 $(M + Na)^+$, 1991 $(M + H)^+$.

Compound 7. $[\alpha]_D$ +41.6 ° (*c* 1, H_2O); NMR data (D_2O): ^1H, δ 3.61, 3.69 (3 × OCH_3), ring D: 5.61 (d, 1H, *J* = 3 Hz, H-1), 4.26 (dd, 1H, *J* = 3 Hz and 7 Hz, H-2), 4.62 (c, 1H, H-3), 3.53 (t, 1H, *J* = 7 Hz, H-4), 3.99 (c, 1H, H-5), ring E: 4.76 (d, 1H, *J* = 6 Hz, H-1), 4.28 (c, 1H, H-2), 3.70 (t, 1H, *J* = 7 Hz, H-3), 3.97 (t, 1H, *J* = 7 Hz, H-4), 3.81 (d, 1H, *J* = 7 Hz, H-5), ring F: 5.48 (d, 1H, *J* = 3 Hz, H-1), 4.36 (c, 1H, H-2), 4.64 (c, 1H, H-3), 3.93 (t, 1H, *J* = 7 Hz, H-4), ring G: 4.86 (d, 1H, *J* = 4 Hz, H-1), 4.26 (c, 1H, H-2), 3.84 (c, 1H, H-3), 4.42 (c, 1H, H-4), 5.23 (d, 1H, *J* = 3.5 Hz, H-5), ring H: 5.16 (d, 1H, *J* = 3 Hz, H-1), 4.37 (c, 1H, H-2), 4.63 (c, 1H, H-3), 4.04 (c, 2H, H-4, H-5), 4.38 (c, 1H, H-6a), 4.48 (c, 1H, H-6b), 3.47 (s, 3H, OCH_3). FAB-mass spectrum: m/z 2055 $(M - Na)^-$, 2033 $(M - 2Na + H)^-$.

Compound 8. $[\alpha]_D$ +55 ° (*c* 1, H_2O); NMR data (D_2O): ^1H, δ 3.53, 3.56, 3.58, 3.62, 3.63, 3.65 (7 × OCH_3), ring D: 5.47 (d, 1H, *J* = 3 Hz, H-1), 3.32 (dd, 1H, *J* = 3 Hz and 7 Hz, H-2), 3.55 (t, 1H, *J* = 7 Hz, H-3), 3.34 (t, 1H, *J* = 7 Hz, H-4), 3.88 (m, 1H, H-5), 4.13 (dd, 1H, *J* = 2 Hz and 8 Hz, H-6a), ring E: 4.68 (d, 1H, *J* = 6 Hz, H-1), 3.21 (dd, 1H, *J* = 6 Hz and 7 Hz, H-2), 3.55 (c, 1H, H-3), 3.89 (t, 1H, *J* = 7 Hz, H-4), 3.73 (d, 1H, *J* = 7 Hz, H-5), ring F: 5.42 (d, 1H, *J* = 3 Hz, H-1),4.35 (dd, 1H, *J* = 3 Hz and 7 Hz, H-2), 4.55 (t, 1H, *J* = 7 Hz, H-3), 3.98 (t, 1H, *J* = 7 Hz, H-4), 4.21 (c, 1H, H-5), ring G: 5.14 (b, 1H, H-1), ring H: 5.16 (d, 1H, *J* = 3 Hz, H-1), 4.36 (dd, 1H, *J* = 3 Hz and 7 Hz, H-2), 4.67 (t, 1H, *J* = 7 Hz, H-3), 3.97 (t, 1H, *J* = 7 Hz, H-4), 4.09 (c, 1H, H-5), 3.47 (s, 3H, OCH_3). FAB-mass spectrum: m/z 1705 $(M - Na + 2H)^+$, 1703 $(M - Na)^-$.

Compound 9. $[\alpha]_D$ +53 ° (*c* 1, H_2O); NMR data (D_2O): ^1H, δ 3.56, 3.59, 3.62, 3.66, 3.67, 3.68 (6 × OCH_3), ring D: 5.57 (d, 1H, *J* = 3 Hz, H-1), 4.22 (dd, 1H, *J* = 3 Hz and 7 Hz, H-2), 3.71 (t, 1H, *J* = 7 Hz, H-3), 3.43 (t, 1H, *J* = 7 Hz, H-4), 3.96 (c, 1H, H-5), 4.18 (dd, 1H, *J* = 2 Hz and 8 Hz, H-6a), ring E: 4.71 (d, 1H, *J* = 7 Hz, H-1), 3.27 (t, 1H, *J* = 7 Hz, H-2), 3.57 (t, 1H, *J* = 7 Hz, H-3), 3.92 (t, 1H, *J* = 7 Hz, H-4), 3.79 (d, 1H, *J* = 7 Hz, H-5), ring F: 5.45 (d, 1H, *J* = 3 Hz, H-1), 4.32 (c, 1H, H-2), ring G: 5.16 (b, 1H, H-1), ring H: 5.19 (d, 1H, *J* = 3 Hz, H-1), 4.39 (dd, 1H, *J* = 3 Hz and 6 Hz, H-2), 4.69 (t, 1H, *J* = 6 Hz, H-3), 3.49 (s, 3H, OCH_3). FAB-mass spectrum: m/z 1791 $(M - Na)^-$, 1769 $(M - 2Na + H)^-$, 1747 $(M - 3Na + 2H)^-$.

Compound 10. $[\alpha]_D$ +47.5 ° (*c* 1, H_20); NMR data (D_2O) ^1H, δ 3.53, 3.56, 3.59, 3.64, 3.65 (5 × OCH_3), ring D: 5.60 (d, 1H, *J* = 3 Hz, H-1), 4.24 (dd, 1H, *J* = 3 Hz and 7 Hz, H-2), 4.59 (t, 1H, *J* = 7 Hz, H-3), 3.51 (t, 1H, *J* = 7 Hz, H-4), 3.97 (c, 1H, H-5), 4.17 (dd, 1H, *J* = 1.5 Hz and 8 Hz, H-6a), ring E: 4.66 (d, 1H, *J* = 6 Hz, H-1), 3.25 (dd, 1H, *J* = 6 Hz and 7 Hz, H-2), 3.59 (t, 1H, *J* = 7 Hz, H-3), 3.91 (t, 1H, *J* = 7 Hz, H-4), 3.77 (d, 1H, *J* = 7 Hz, H-5), ring F: 5.42 (d, 1H, *J* = 3 Hz, H-1), 4.32 (dd, 1H, *J* = 3 and 7 Hz, H-2), 4.54 (t, 1H, *J* = 7 Hz, H-3), 3.98 (t, 1H, *J* = 7 Hz, H-4), 4.22 (c, 1H, H-5), ring G:

4.79 (b, 1H, H-1), 4.21 (c, 1H, H-2), 3.79 (c, 1H, H-3), 3.51 (c, 1H, H-4), 5.09 (d, 1H, J = 5 Hz, H-5), ring H: 5.17 (d, 1H, J = 3 Hz, H-1), 4.37 (dd, 1H, J = 3 Hz and 7 Hz, H-2), 4.67 (t, 1H, J = 7 Hz, H-3), 3.97 (t, 1H, J = 7 Hz, H-4), 4.10 (m, 1H, H-5), 4.29 (c, 1H, H-6a), 4.41 (c, 1H, H-6b), 3.47 (s, 3H, OCH_3). FAB-mass spectrum: m/z 1879 $(M - Na)^-$, 1857 $(M - 2Na + H)^-$, 1835 $(M - 3Na + 2H)^-$.

Capillary electrophoresis. The synthetic pentasaccharides were each dissolved in milli Q water (Millipore, Milford, MA, U.S.A.) to a concentration of 1 mg/mL and separated by high performance CE essentially as described earlier.[33] However, in our CE experiments 5 mM sulfosalicylic acid (pH 3.0) was used as the electrophoresis buffer at a thermostat temperature of 25 °C, applying a potential of 5 kV (87.7 V/cm) across the capillary. Electropherograms were recorded using indirect UV detection by quenching of the UV signal at 214 nm. The injection time was 2 s. A highly purified preparation of the reference pentasaccharide **3** was used as a reference compound in the CE experiments. The purity of this preparation was determined to be at least 99.5 % (mol/mol) by $^{13}C/^{1}H$ NMR spectroscopy as described.

Measurements of anti-Xa activity. The anti-Xa activity of the pentasaccharides was determined amidolytically in an *in vitro* assay, according to Teien and Lie.[46] Pentasaccharides were dissolved in human plasma diluted four-fold with Tris–HCl buffer, pH 8.4 containing 50 mM Tris, 100 mM NaCl, and 7.5 mM Na_2EDTA. On microtiter plates at least 6 pentasaccharide concentrations were tested in duplicate (final concentration range of 0.02–0.2 U/mL). Of each sample 50 µL was supplemented with 50 µL bovine factor Xa (1.0 U/mL). After incubation for 2 min, 100 µL of the chromogenic substrate S-2222 (0.5 mM; Kabi Vitrum, Stockholm, Sweden, Bz-Ile-Glu-Gly-Arg-pNA) was added and the optical density at 405 nm was determined after 2 and 22 min. The differences in optical densities (ΔODs) were calculated and the anti-Xa activities were read from a calibration curve obtained with 12 different concentrations of compound **2** (natural pentasaccharide). The anti-Xa activity of compound **2** was determined before, using the Fourth International Standard of Heparin for calibration.

For measurement of the anti-Xa activity in rat plasma, the following modifications were applied: all plasma samples were initially diluted four-fold with Tris–HCl buffer pH 8.4 and optionally further to a desired concentration range with four-fold diluted control rat plasma to ensure a constant AT-III level. Each diluted plasma sample was measured in duplicate in three different concentrations. Each sample was supplemented with 50 µL human AT-III (0.25 U/mL), dissolved in the Tris–HCl buffer to ensure complete detection of both AT-III bound and unbound pentasaccharide. The anti-Xa activity was read from a calibration curve which was made from the tested pentasaccharide itself dissolved in diluted rat plasma.

Blood collection. Male Wistar Hsd/Cpb:Wu rats (250–300 g body weight) were obtained from Harlan, Zeist, The Netherlands. One day prior to administration of the pentasaccharides, the rats were anaesthetized by ip injection of methohexital-sodium (40 mg/mL, 200 µL/100 g body weight; Brietal®, Eli Lilly, Amsterdam, The Netherlands). The right jugular vein was cannulated with a siliconized PE-50 (Clay Addams, Parsiappany, U.S.A.) cannula which had been filled with saline containing 25 % (v/v) citrated plasma. The cannula was led subcutaneously to the neck and exteriorized.

Pentasaccharides were injected as 100 µM saline solutions. Under light ether anaesthesia, a volume of 100 µL/100 g body weight was administered iv via the penis vein, corresponding to a dose of 100 nmol/kg body weight. Blood samples to a maximum of 500 µL were taken at preset time points from the cannula in plastic syringes containing 10 % (v/v) sodium citrate (38 g/L ultra pure water) and centrifuged at 125,000 N/kg for 1 min at room temperature. The supernatants were stored at –20 °C until use.

Data analysis. The pharmacokinetic data were analyzed from the plasma anti-Xa levels versus time curves by means of a two-compartment model. A sum of two exponential terms, $C = Ae^{-at} + Be^{-bt}$, was fitted to the plasma concentration data using the computerized iterative procedure of MW/PHARM (Mediware, Groningen, the Netherlands). The model is based on the Simplex-method in which Ae^{-at} represents the distribution and Be^{-bt} the elimination phase. Subsequently, the elimination half lives were calculatated using weighing factors which kept the happy mean between the relative and absolute error independent of the concentration.

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