Synthesis and Pharmacological Properties of a Close Analogue of an Antithrombotic Pentasaccharide (SR 90107A/ORG 31540)†

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The synthetic pentasaccharide (**1**) corresponding to the heparin sequence which binds to, and activates, antithrombin III (AT III) is a potent antithrombotic compound in several animal models of venous thrombosis. We describe here the preparation and the pharmacological properties of **34**, an analogue of oligosaccharide **1** with the latter's *N*-sulfates being replaced by sulfate esters and hydroxyl groups being methylated. These structural modifications allow a simpler and more efficient synthesis of such anionic oligosaccharides. Affinity for human AT III, anti-factor Xa activity, ability to inhibit thrombin generation, antithrombotic activity in a rat model of venous thrombosis, and elimination half-life in the rat have been determined for **1** and **34**. Surprisingly, introduction of *O*-sulfates in place of *N*-sulfates, and methylation of hydroxyl groups, contributes to reinforce the binding to AT III, resulting in an improved pharmacological profile.

Introduction

The activity of heparin and low molecular weight heparin, two widely used antithrombotic drugs, is essentially due to their ability to interact with antithrombin III (AT III), a serine protease inhibitor present in plasma in a latent form. This interaction mainly results in inhibition of the coagulation enzymes thrombin and factor Xa. Recent studies at the molecular level have shown that, upon heparin binding, AT III undergoes a conformational change which is sufficient to allow factor Xa inhibition (allostery mechanism), whereas thrombin inhibition further requires binding of both AT III and thrombin to the same heparin molecule (template mechanism).[1] This explains why very short heparin fragments can promote selective factor Xa inhibition without affecting thrombin. Indeed a unique pentasaccharide sequence, which represents the binding site of heparin to AT III, was shown to be a potent and selective anti-factor Xa compound.[2] We found that such selective factor Xa inhibitors also displayed antithrombotic properties in animal models of venous thrombosis,[3] and this prompted us to launch a research program aimed at the preparation of pure synthetic antithrombotic compounds based on AT III mediated factor Xa inhibition (for review, see ref 4). The lead compound in this series (**1**, SR 90107A/ORG 31540) is the exact copy of an AT III binding sequence present on heparin chains. Despite its complex structure which requires numerous synthesis steps,[5] this compound is currently undergoing clinical trials to prove the therapeutic potential of this class of pure indirect factor Xa inhibitors. To simplify the synthesis, various pentasaccharides in which *N*-sulfate groups have been replaced by *O*-sulfates and hydroxyl groups by *O*-alkyl groups have been prepared.[6−10] In this article, we report the synthesis and the biological properties of **34**, the exact copy of **1**. This work allows us to precisely assess how *O*-sulfation *vs N*-sulfation and alkylation of hydroxyl groups influence the pharmacological properties of such synthetic pentasaccharides.

Chemistry

The *N*,*O*-sulfated pentasaccharide (**1**, Scheme 1) used in this study was obtained as previously reported.[4,5] This compound is an exact copy of the heparin sequence required for AT III binding and therefore contains both glucuronic and iduronic acid units, and *N*-sulfated glucosamine. The strategy for the synthesis of such a complex oligosaccharide consists in the preparation of a fully protected molecule (**2**), the protective groups of which are then sequentially removed to introduce the desired substituents in the appropriate positions. Thus in **2**, *O*-acetyl substitutes for *O*-sulfate, *O*-benzyl for hydroxyl, methoxycarbonyl for carboxylate, azido and *N*-(benzyloxycarbonyl)amino for *N*-sulfate. For the preparation of **2**, glucuronic and iduronic acid units were elaborated from glucose, while glucosamine units were obtained either from glucose or from glucosamine itself.

Synthesis of 34. Since **34** does not contain *N*-sulfate groups but only sulfate esters, we used glucose as the precursor of all units. It should be underlined as well that the structures of **34** and **1** also differ by the presence of methyl ethers in place of hydroxyl groups. These apparently modest structural differences are of considerable interest from the chemical synthesis standpoint. On the one hand, the synthesis of **1** requires the use of three levels of protecting groups: permanent (removed at the very end of the synthesis to unmask the hydroxy functions), semipermanent (removed before introduction of the sulfate esters), and temporary (useful to temporarily protect a hydroxyl group during the elaboration of the pentasaccharidic backbone). So far benzyl ethers and acetyl esters have been used as permanent and semipermanent protecting groups, respectively, while several other protecting groups were temporarily employed.[4] On the other hand, for the

†Dedicated to Pr. Dr. Hans Paulsen on the occasion of his 75th anniversary.

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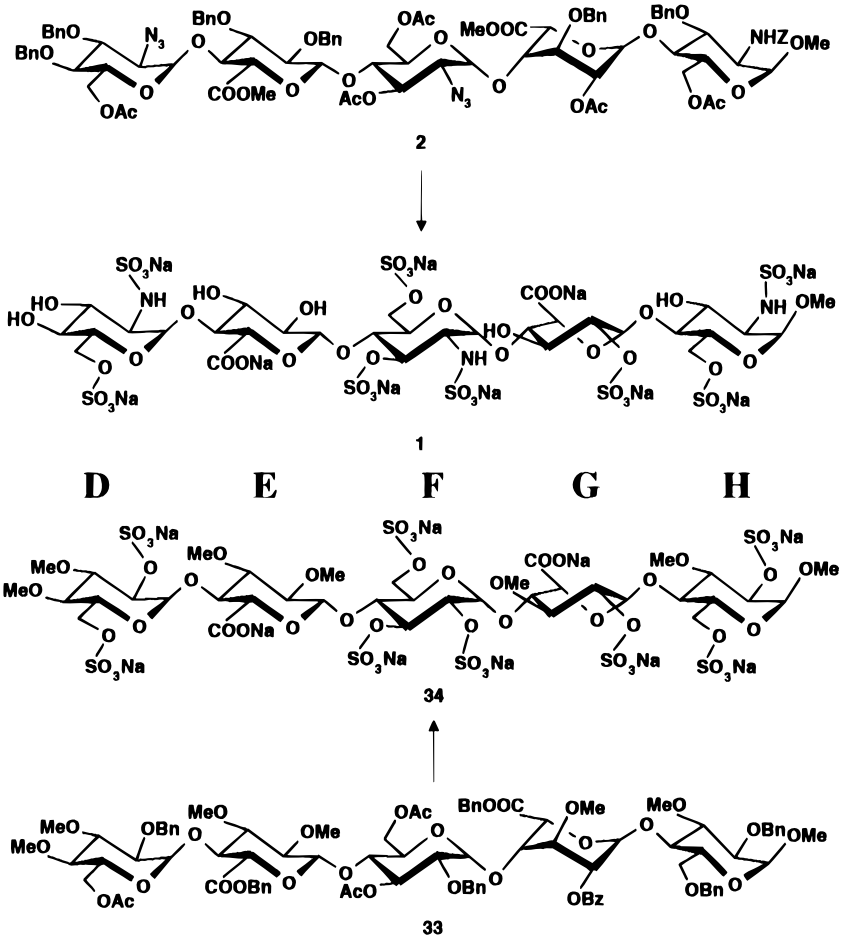
Scheme 1. Structures of **1** and **34** and Their Fully Protected Precursor Pentasaccharides **2** and **33**[a]



[a] D, E, F, G, H are used throughout the text to designate the corresponding monosaccharide units.

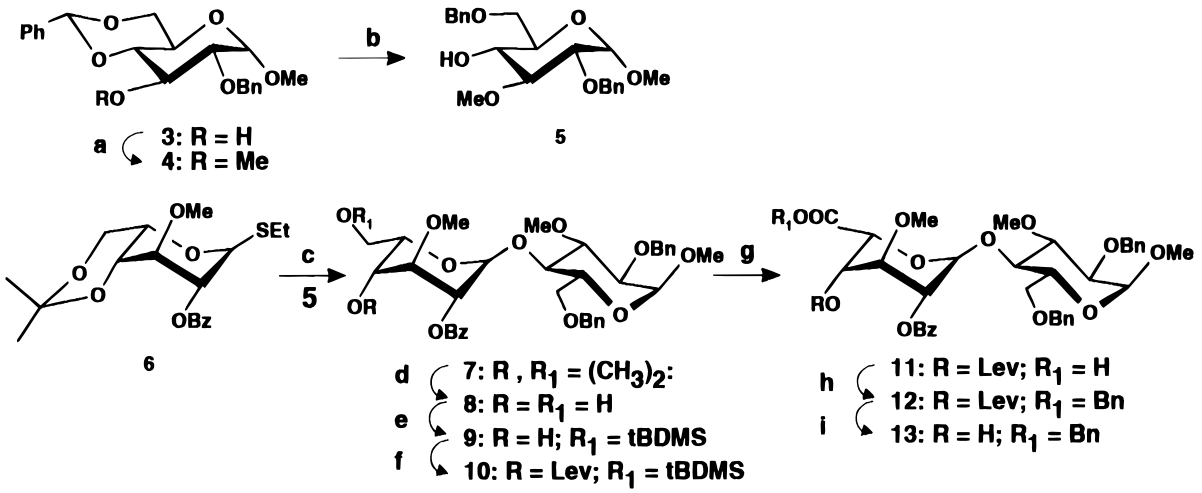
synthesis of **34**, hydroxyl groups do not need to be unmasked at the end of the synthesis (the methyl that protect them belong to the final structure), and further, there is no need to differentiate between *N*- and *O*-sulfates (only the latter are present in the final structure). As a consequence only two levels of protective groups are required, the temporary ones during the construction of the backbone and the semipermanent ones that will be replaced by sulfates in **34**. Thus the benzyl group (ether or ester) can serve, together with acetyl (or benzoyl) groups, to protect the positions to be sulfated or to block the carboxyl functions. This allows shorter synthetic routes and an easier deprotection−functionalization sequence at the end of the synthesis. The fully protected pentasaccharide **33** was chosen as the key precursor of **34**. Its elaboration represents the biggest part of the synthesis work.

The glycosyl acceptor **5** was obtained from **3**[11] after methylation and reductive opening of the benzylidene ring by the cyanoborohydride−tetrafluoroboric acid system (Scheme 2).[12] Condensation of **5** with the known[7] L-idose derivative **6** under henceforth classical thioglycoside activation[13,14] (*N*-iodosuccinimide, trifluoromethanesulfonic acid) led, as expected from the participation of the benzoyl at position 2, to the stereospecific formation of the desired disaccharide **7**, isolated in excellent yield (89%). The α-L configuration of the new interglycosidic bond was confirmed by the long-range coupling observed by 2D ^{1}H NMR between H-1 and H-3 of the idose ring in the $^1C_4$ conformation.

Hydrolysis of the isopropylidene group then delivered the 4,6-diol **8** which had to be oxidized into the corresponding uronic acid. This was achieved through temporary protection of the primary alcohol as a *tert*-butyldimethylsilyl ether, followed by levulinoylation of the secondary hydroxyl group and direct Jones' oxidation of the silyl ether **10**. Benzylation of the free acid **11** with benzyl bromide, in the presence of potassium hydrogen carbonate, in DMF, and removal of the levulinyl group, finally afforded the glycosyl acceptor **13** (64% from **7**).

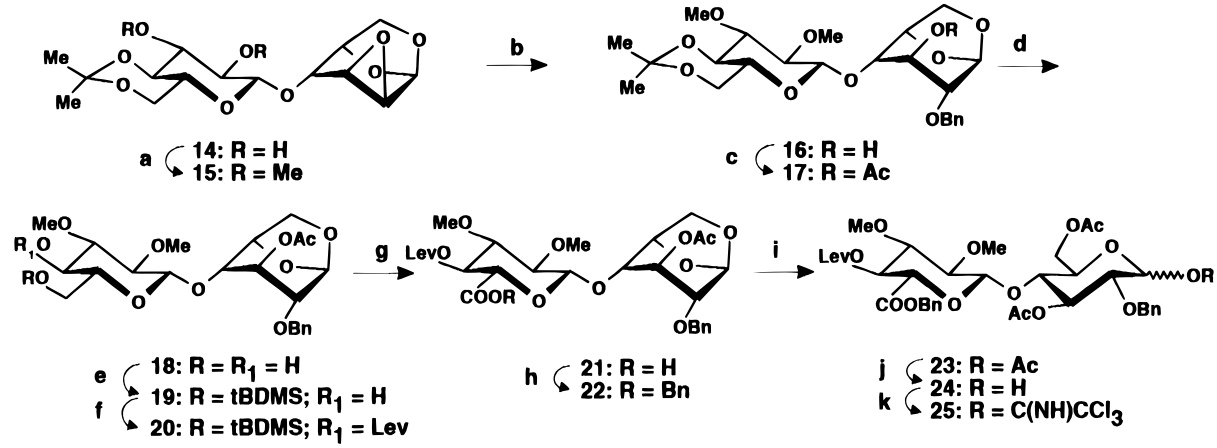
The glucuronic acid containing synthon **25** was prepared from **14** (Scheme 3).[15] Since **25** must be engaged in a glycosidation reaction with **13** to give an α-glycosidic bond, we used the nonparticipating benzyl group to protect the position 2 which later will be sulfated in the final pentasaccharide. After methylation of the secondary alcohols, *trans*-diaxial opening of the epoxide by sodium benzylate, followed by acetylation, gave *O*-(4,6-*O*-isopropylidene-2,3-di-*O*-methyl-*β*-D-glucopyranosyl)-(1→4)-3-*O*-acetyl-1,6-anhydro-2-*O*-benzyl-*β*-D-glucopyranose, **17**. The reaction sequence described above for the preparation of **12** from **7** was then applied to provide **22**. No intermediate purification was required, and pure **22** was isolated (46% from **14**) after a single column chromatography at the end of the sequence. Acetolysis of the 1,6-anhydro ring, using the acetic anhydride−trifluoroacetic acid system, followed by selective removal of the anomeric acetate by benzylamine in ether, gave **24** which was purified by column chro-

Scheme 2. Preparation of the L-iduronic Acid Containing Synthon **13**[a]



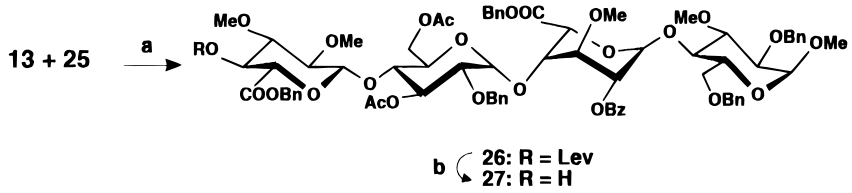
[a] Bn, benzyl; Bz, benzoyl; tBDMS, *tert*-butyldimethylsilyl. Reagents: (a) MeI, NaH, DMF; (b) HBF_4, $NaBH_3CN$, THF; (c) *N*-iodosuccinimide, TsOH, $PhCH_3$; (d) aqueous CF_3COOH, CH_2Cl_2; (e) tBDMSCl, DMAP, Et_3N, CH_2Cl_2; (f) Lev_2O, DMAP, Et_3N; (g) CrO_3/H_2SO_4, acetone; (h) BnBr, DMF, $KHCO_3$; (i) NH_2NH_2, AcOH/pyridine.

Scheme 3. Preparation of the D-Glucuronic Acid Containing Synthon **25**[a]



[a] Bn, benzyl; Bz, benzoyl; tBDMS, *tert*-butyldimethylsilyl. Reagents: (a) MeI, NaH, DMF; (b) BnONa, BnOH; (c) Ac_2O, DMAP, Et_3N, CH_2Cl_2; (d) aqueous CF_3COOH, CH_2Cl_2; (e) tBDMSCl, DMAP, Et_3N, CH_2Cl_2; (f) Lev_2O, DMAP, Et_3N; (g) CrO_3/H_2SO_4, acetone; (h) BnBr, DMF, $NaHCO_3$; (i) Ac_2O, CF_3COOH; (j) $PhCH_2NH_2$, Et_2O; (k) CCl_3CN, Cs_2CO_3, CH_2Cl_2.

Scheme 4[a]



[a] Reagents: (a) trimethylsilyl triflate, $PhCH_3$; (b) $PhCH_2NH_2$, Et_2O.

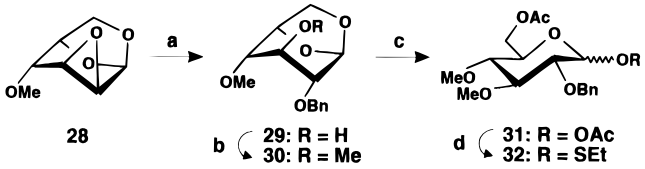
matography. Finally, reaction with trichloroacetonitrile in the presence of cesium carbonate yielded a 3:2 mixture of α- and β-imidates **25** (52% from **22**).

Reaction of equimolar amounts of **13** and **25** under the classical conditions for imidate coupling[16] delivered the corresponding α-coupled tetrasaccharide **26** isolated in 73% yield after column chromatography (Scheme 4). The α-configuration was proved by the small coupling constant (3.7 Hz) observed by ^1H NMR between H-1 and H-2 of the F unit. Removal of the levulinyl protection then gave the tetrasaccharide **27** (96%) ready to be glycosylated by **32**.

The choice of a benzyl ether at position 2 of the glycosyl donor **32** was, as for **25**, dictated by the need to obtain an α-linkage after coupling with **27**, and later to introduce a sulfate at this position. Opening of the epoxide ring of **28**[17] by sodium benzylate, followed by methylation, acetolysis, and treatment with ethanethiol in the presence of boron trifluoride, gave the thioglycoside donor **32**, as a mixture of α- and β-anomers (78% from **29**, Scheme 5).

Condensation of **32** and **27** using triflic acid and *N*-iodosuccinimide in a mixture of ether and dichloroethane finally afforded the pentasaccharide **33** (62%). The α-configuration was confirmed by ^1H NMR analysis (coupling constant between H-1 and H-2 of the D unit: 3.3 Hz). Final conversion of **33** into the target pentasaccharide **34** was carried out in a very efficient way

Scheme 5. Preparation of the Nonreducing End Unit[a]



[a] Bn, benzyl. Reagents: (a) BnONa, BnOH; (b) MeI, NaH, DMF; (c) Ac_2O, CF_3COOH; (d) EtSH, $BF_3{\cdot}Et_2O$, $PhCH_3$.

Table 1. *In Vitro* and *in Vivo* Properties of the Pentasaccharides **1** and **34**[a]

	1	**34**
K_D for AT-III (nM)	58 ± 3	7 ± 2
anti-Xa activity (units/mg)	850 ± 27	1040 ± 80
thrombin generation inhibition (IC_{50}, μM)	0.13 (0.10−0.17)	0.11 (0.09−0.17)
antithrombotic activity (ED_{50}, μg/kg)	81 (55−103)	20 (12−28)
elimination half-life ($t_{1/2}$, h)	0.7 ± 0.1	3.8 ± 1.5

[a] Values are means ± SD (n = 3) or 95% confidence interval.

as follows: hydrogenolysis of benzyl ethers and benzyl esters, saponification by sodium hydroxide in methanol, and sulfation using triethylamine−sulfur trioxide complex in dimethylformamide. Pure **34** was obtained directly (88% yield over the three steps) after gel permeation chromatography first in 0.2 M sodium chloride and then in water.

Biological Properties *in Vitro* and *in Vivo*

Affinity for AT III. Plasma AT III is the intended target of the synthetic pentasaccharides **1** and **34**. It was therefore of prime importance to assess the influence of the structural modifications described above on binding affinity. Interaction of the pentasaccharides with AT III induces a change in the intrinsic protein fluorescence which allows titration of the complex formed and determination of the affinity of the different ligands.[18] We used this method to determine the K_D values reported in Table 1. The substitution of *O*-sulfates for *N*-sulfates and the introduction of methyl groups resulted in an increase (8 fold) of the affinity for AT III.

Inhibition of Factor Xa. Binding of the pentasaccharides to AT III induces a conformational change which results in an accelerated inhibition of blood coagulation factor Xa by AT III.[2] The anti-factor Xa activity was determined, in buffer, by an amidolytic method adapted from Teien and Lie.[19] Activity of **1** and **34** paralleled their AT III affinity, and a good correlation was found between their anti-factor Xa activity and the natural log value of their K_d.

Inhibition of Thrombin Generation. An efficient venous antithrombotic agent must either inhibit thrombin or impair thrombin generation. The pentasaccharides are not thrombin inhibitors; it is therefore of prime importance to assess their ability to inhibit thrombin generation. As compared to factor Xa inhibition assay which is performed in buffer, thrombin generation inhibition was carried out in plasma. Thrombin is generated through proteolysis of prothrombin by factor Xa in the prothrombinase complex, the rate of thrombin generation is therefore largely dependent on available factor Xa,[20] and the same ranking as for factor Xa inhibition was therefore expected and indeed observed for **1** and **34** (Table 1).

Antithrombotic Activity. Compounds **1** and **34** are aimed at the prevention of venous thrombosis. The Wessler model is the reference to assess the antithrombotic potency of the compounds in animal *in vivo*.[21] The well-known antithrombotic drugs heparin and low molecular weight heparin are highly efficient in this model. Here again we found (Table 1) a direct correlation between the affinity for AT III and the antithrombotic activity of each compound.

Pharmacokinetics. Renal excretion is the main route of elimination of the pentasaccharides.[22] As the rate of elimination is proportional to the concentration of free pentasaccharide in plasma, then one may expect this rate to be negatively correlated to the affinity for AT III.[22] Such a correlation was indeed confirmed, with **34** having a half-life considerably longer than that of **1** (Table 1).

Conclusion

Compared to the complex synthesis of **1**, the preparation of **34** is much simpler. Replacing *N*-sulfates by *O*-sulfates avoids the final *N*-sulfation step as well as the use of the hazardous azido group. Furthermore, methylation of the hydroxyl groups at the very beginning of the synthesis assures their protection and allows benzyl ethers and benzyl esters to serve, together with acetates, for the protection both of the hydroxyls to be sulfated and the carboxylates. This is of considerable interest since it offers many different possibilities for the preparation of compounds of this family. Regarding the biological properties of **34**, they are very similar to those of the reference compound **1**. Interestingly **34** displays a somewhat better affinity for AT III, which may explain its slightly higher potency in the pharmacological tests reported here and its slightly higher half-life. From a more fundamental point of view, since the interaction of AT III and the oligosaccharide is not altered by the present structural modifications, one may conclude that NH and OH groups are not hydrogen bond donors in the interaction with AT III. Further studies are necessary to assess the contribution to the binding of *O- vs N*-sulfates and methyl *vs* hydroxyl groups.

Experimental Section

All compounds were homogeneous by TLC analysis and had spectral properties consistent with their assigned structures. Melting points were determined in capillary tubes in a Mettler apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer Model 241 polarimeter at 22 ± 3 °C. Compound purity was checked by TLC on silica gel 60 F_{254} (E. Merck) with detection by charring with sulfuric acid. Unless otherwise stated, column chromatography were performed on silica gel 60, 40−63 or 63−200 μm (E. Merck). ^1H NMR spectra were recorded with Bruker AC 200, AM 250, AC 300, or AM500 instruments, for solution in $CDCl_3$ or D_2O. Before analysis in D_2O, samples were passed through a Chelex (Bio-Rad) ion exchange column and lyophilized three times from D_2O. Chemical shifts are relative to external TMS when the spectra are recorded in $CDCl_3$ and to external TSP when the spectra are recorded in D_2O. MS analyses were performed on a ZAB-2E instrument (Fisons). Elemental analyses were performed on a Fisons elemental analyzer.

Factor Xa (71 nkat per vial) and S-2222 substrate (Bz-Ile-Glu-Gly-Arg-pNA) were from Chromogenix (Mölndal, Sweden). Rabbit brain thromboplastin was from Sigma (Saint Quentin Fallavier, France). Platelet poor plasma (PPP) was obtained after centrifugation (15 min, 1500*g*) from blood collected from the ante-cubital vein of normal healthy volunteers using 3.8%

sodium citrate as anticoagulant (blood/citrate, 9:1, v/v). Human tissue factor (Innovin) was from Baxter-Dade (Miami, FL).

Methyl 2,6-Di-*O*-benzyl-3-*O*-methyl-α-D-glucopyranoside (5). MeI (1.6 mL, 25 mmol) was added, at 0 °C, to a solution of methyl 2-*O*-benzyl-4,6-*O*-benzylidene-α-D-glucopyranoside[11] (**3**, 6.3 g, 16.9 mmol) and NaH (0.6 g, 20 mmol) in DMF (60 mL). After 1 h, MeOH (5 mL) was introduced dropwise, and after 15 min the product was extracted with EtOAc. The solution was washed with aqueous 10% $Na_2S_2O_3$ and H_2O, dried (Na_2SO_4), and concentrated. Column chromatography gave methyl 2-*O*-benzyl-4,6-*O*-benzylidene-3-*O*-methyl-α-D-glucopyranoside (**4**, 8.44 g, 79%): ^{1}H NMR ($CDCl_3$) δ 7.24−7.51 (m, 10H, 2Ph), 5.51 (s, 1H, :C*H*Ph), 3.66 (s, 3H, OCH_3), 4.58 (d, 1H, J = 3.7 Hz, H-1), 3.65, 3.38 (2s, 6H, $2OCH_3$); TLC, R_f 0.60, 15:1 CH_2Cl_2/acetone.

HBF_4 (54% solution in Et_2O, 26 mL, 188 mmol) was added dropwise, with stirring, to a cooled (0 °C) solution of **4** (8 g, 21 mmol) and $NaBH_3CN$ (11.8 g, 188 mmol) in THF (110 mL). After 75 min the solution was filtered (Celite) and diluted with CH_2Cl_2, and NaOH was added until basic pH. The solution was then washed with brine and H_2O, dried (Na_2SO_4), and concentrated. Column chromatography (20:1 CH_2Cl_2/acetone) afforded **5** (5.7 g, 70%): ^{1}H NMR ($CDCl_3$) δ 7.36−7.24 (m, 10H, 2Ph), 3.66, 3.36 (2s, 6H, $2OCH_3$); TLC R_f 0.30, 18:1 CH_2Cl_2/acetone.

Methyl *O*-(2-*O*-Benzoyl-4,6-*O*-isopropylidene-3-*O*-methyl-α-L-idopyranosyl)-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-glucopyranoside (7). Triflic acid in toluene (0.15 M, 1.5 mL) was added under argon to a stirred, cold (−20 °C) solution of ethyl 2-*O*-benzoyl-4,6-*O*-isopropylidene-3-*O*-methyl-1-thio-α,β-L-idopyranoside[7] (**6**, 5.94 g, 15.5 mmol), **5** (6.03 g, 15.5 mmol), and *N*-iodosuccinimide (8.8 g, 39 mmol) in toluene (200 mL) containing finely grounded 4 Å molecular sieves. After 1 h solid $NaHCO_3$ (125 mg) was introduced, and 15 min later the solution was filtered, diluted with CH_2Cl_2, washed with H_2O, dried (Na_2SO_4), and evaporated. Column chromatography (30:1, then 28:1, then 26:1 CH_2Cl_2/acetone) gave **7** (11.18 g, 89%): ^{1}H NMR ($CDCl_3$) δ 8.10−7.21 (m, 15H, 3Ph), 5.06 (d, 1H, J = 3.1 Hz, H-1′), 4.55 (d, 1H, J = 3.7 Hz, H-1), 3.58, 3.44, 3.29 (3s, 3 OCH_3), 1.46, 1.47 (2s, 6H, :C(CH_3)$_2$). Anal. ($C_{18}H_{24}O_8$) C, H.

Methyl *O*-(Benzyl 2-*O*-benzoyl-3-*O*-methyl-α-L-idopyranosyluronate)-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-glucopyranoside (13). Aqueous CF_3COOH (70%, 22 mL) was added to a solution of **7** (9.9 g, 14 mmol) in CH_2Cl_2 (500 mL). After 40 min at room temperature the solution was diluted with CH_2Cl_2, washed with cold saturated aqueous $NaHCO_3$ and H_2O, and dried (Na_2SO_4). Concentration yielded crude methyl *O*-(2-*O*-benzoyl-3-*O*-methyl-α-L-idopyranosyl)-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-glucopyranoside (**8**, 9.39 g): ^{1}H NMR ($CDCl_3$) δ 8.0−7.15 (m, 15H, 3Ph), 5.04 (1H, J = 2 Hz, H-1′), 4.57 (d, 1H, J = 3.5 Hz, H-1), 3.67, 3.49, 3.34 (3s, 3 OCH_3), 2.60 (2H, OH).

The above crude **8** (9 g) dissolved in CH_2Cl_2 (36 mL) was heated at 50 °C for 4 h with Et_3N (3 mL, 22 mmol), 4-(dimethylamino)pyridine (210 mg, 1.74 mmol), and *tert*-butyldimethylsilyl chloride (1.09 g, 20.3 mmol). Levulinic anhydride (5g, 22.5 mmol), Et_3N (3.2 mL, 22.5 mmol), and 4-(dimethylamino)-pyridine (400 mg, 3.26 mmol) were then added. After 4 h the mixture was diluted with CH_2Cl_2, successively washed with 5% aqueous $KHSO_4$, H_2O, saturated aqueous $NaHCO_3$, and H_2O, dried (Na_2SO_4), and concentrated to yield methyl *O*-(2-*O*-benzoyl-4-*O*-levulinyl-3-*O*-methyl-6-*O*-(*tert*-butyldimethylsilyl)-α-L-idopyranosyl)-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-glucopyranoside (**10**, 14.3 g): TLC R_f 0.76, 1:1 cyclohexane/EtOAc.

A solution of CrO_3 (3.55 g, 35.5 mmol) in aqueous H_2SO_4 (3.5 M, 14.5 mL) was slowly added to a cooled (0 °C) solution of the above crude **10** in acetone (700 mL). After 3 h CH_2Cl_2 was introduced, the mixture was poured into iced H_2O, stirred vigorously, washed with H_2O until neutral, and dried. Concentration gave the acid methyl *O*-(2-*O*-benzoyl-4-*O*-levulinyl-3-*O*-methyl-α-L-idopyranosyluronic acid)-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-glucopyranoside (**11**, 12.46 g): TLC R_f 0.34, 15:1 CH_2Cl_2/MeOH.

A solution of the above crude **11** in DMF (100 mL) was treated, at room temperature, with BnBr (16 mL, 130.5 mmol) and $KHCO_3$ (6.7 g, 67 mmol) overnight. MeOH (35 mL) was added, and the product was extracted with Et_2O, washed with H_2O, dried, and concentrated. Column chromatography (1:1 cyclohexane/EtOAc) then gave methyl *O*-(benzyl 2-*O*-benzoyl-4-*O*-levulinyl-3-*O*-methyl-α-L-idopyranosyluronate)-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-glucopyranoside (**12**, 11.6 g): TLC R_f 0.54, 1:1 cyclohexane/EtOAc.

A solution of hydrazine hydrate (1 M in 3:2 pyridine/AcOH, 67 mL) was added to a cooled (0 °C) solution of the above **12** in pyridine (67 mL). After 30 min at 0 °C, concentration followed by column chromatography (14:1 CH_2Cl_2/EtOAc) yielded **13** (6.58 g, 64% from **7**): ^{1}H NMR ($CDCl_3$) δ 8.00−7.15 (m, 20H, 4Ph), 5.15 (d, 1H, H-1′), 4.57 (d, 1H, H-1), 3.48, 3.47, 3.32 (3s, 3 OCH_3). Anal. ($C_{43}H_{48}O_{13}$) C, H.

***O*-(Benzyl 4-*O*-levulinyl-2,3-di-*O*-methyl-β-D-glucopyranosyluronate)-(1→4)-3-*O*-acetyl-1,6-anhydro-2-*O*-benzyl-β-D-glucopyranose (22).** *O*-(4,6-*O*-Isopropylidene-β-D-glucopyranosyl)-(1→4)-1,6:2,3-dianhydro-β-D-mannopyranose[15] (**14**, 22 g, 63.5 mmol) was methylated as described for the preparation of **4** to give *O*-(4,6-*O*-isopropylidene-2,3-di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-1,6:2,3-dianhydro-β-D-mannopyranose (**15**, 21.7 g): TLC R_f 0.67, 1:1 toluene/acetone.

Freshly prepared sodium benzylate (1 M in benzyl alcohol, 315 mL) was slowly added to the above crude **15** (21.7 g). The mixture was heated during 4 h at 80 °C and neutralized with Dowex 50 H^+, and *O*-(4,6-*O*-isopropylidene-2,3-di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-1,6-anhydro-2-*O*-benzyl-β-D-glucopyranose was obtained (**16**, 23 g) after filtration on a silica gel pad (5:1 toluene/acetone): TLC R_f 0.21, 4:1 toluene/acetone.

Et_3N (44 mL, 313 mmol), 4-(dimethylamino)pyridine (1.28 g, 10.5 mmol), and Ac_2O (25 mL, 261 mmol) were added to a solution of **16** (23 g, 52 mmol) in CH_2Cl_2 (390 mL). After 30 min the mixture was successively washed with 5% aqueous $KHSO_4$, H_2O, saturated aqueous $NaHCO_3$, and H_2O and dried (Na_2SO_4). Concentration gave *O*-(4,6-*O*-isopropylidene-2,3-di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-3-*O*-acetyl-1,6-anhydro-2-*O*-benzyl-β-D-glucopyranose (**17**, 23.4 g) used as such in the next step: TLC R_f 0.37, 4:1 toluene/acetone.

The isopropylidene group of **17** was removed as described for the preparation of **8** to give *O*-(2,3-di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-3-*O*-acetyl-1,6-anhydro-2-*O*-benzyl-β-D-glucopyranose (**18**, 21.6 g): TLC R_f 0.51, 2:3 toluene/acetone.

This compound was then silylated and levulinylated as described for the preparation of **10** to give crude *O*-(4-*O*-levulinyl-2,3-di-*O*-methyl-6-*O*-(*tert*-butyldimethylsilyl)-β-D-glucopyranosyl)-(1→4)-3-*O*-acetyl-1,6-anhydro-2-*O*-benzyl-β-D-glucopyranose (**20**, 54.2 g): TLC R_f 0.52, 2:1 toluene/acetone.

Oxidation of the crude residue obtained in the preceding step, as described for the preparation of **11**, gave the crude acid *O*-(4-*O*-levulinyl-2,3-di-*O*-methyl-β-D-glucopyranosyluronic acid)-(1→4)-3-*O*-acetyl-1,6-anhydro-2-*O*-benzyl-β-D-glucopyranose (**21**, 37.9 g): TLC R_f 0.32, 12:2:0.6:1 EtOAc/pyridine/AcOH/H_2O.

Esterification of **21**, as described for the synthesis of **12**, followed by column chromatography (5:1 toluene/acetone), afforded pure *O*-(benzyl 4-*O*-levulinyl-2,3-di-*O*-methyl-β-D-glucopyranosyluronate)-(1→4)-3-*O*-acetyl-1,6-anhydro-2-*O*-benzyl- β-D-glucopyranose (**22**, 20 g, 46% from **14**): ^{1}H NMR ($CDCl_3$) δ 7.29−7.25 (m, 10H, 2Ph), 5.40 (d, 1H, J = 1 Hz, H-1), 4.69 (d, 1H, J = 7 Hz, H-1′), 3.57, 3.50 (2s, 2 OCH_3), 2.3−2.6 (m, 4H, levulinyl CH_2CH_2), 2.12, 2.03 (2s, 6H, 1Ac and levulinyl CH_3); TLC R_f 0.45, 2:1 toluene/acetone.

***O*-(Benzyl 4-*O*-levulinyl-2,3-di-*O*-methyl-β-D-glucopyranosyluronate)-(1→4)-3,6-di-*O*-acetyl-2-*O*-benzyl-α,β-D-glucopyranosyl Trichloroacetimidate (25).** CF_3COOH (10 mL, 130 mmol) was added to a solution of the above **22** (8 g, 11.7 mmol) in Ac_2O (112 mL, 1.17 mol). After 1.5 h the solution was concentrated. Toluene (10 mL) was evaporated twice from the residue to eliminate traces of Ac_2O. Crude *O*-(benzyl 4-*O*-levulinyl-2,3-di-*O*-methyl-β-D-glucopyranosyluronate)-(1→4)-1,3,6-tri-*O*-acetyl-2-*O*-benzyl-α,β-D-glucopyranose (**23**, 9.55 g) was obtained: TLC R_f 0.36, 3:1 toluene/acetone.

Benzylamine (49 mL, 453 mmol) was added to a solution of the above crude **23** (9.55 g) in Et_2O (400 mL). After 6 h at room temperature the solution was washed with 1 M aqueous HCl and H_2O, dried, and concentrated. Column chromatography (2:1 toluene/acetone) afforded pure *O*-(benzyl 4-*O*-levulinyl-2,3-di-*O*-methyl-β-D-glucopyranosyluronate)-(1→4)-3,6-di-*O*-acetyl-2-*O*-benzyl-α,β-D-glucopyranose (**24**, 6.35 g, 73% from **22**): TLC R_f 0.43, 2:1 toluene/acetone. Anal. ($C_{37}H_{46}O_{16}$) C, H.

Trichloroacetonitrile (4.5 mL, 85 mmol) and cesium carbonate (5 g, 15.3 mmol) were added under argon to a solution of **24** (6.33 g, 8.48 mmol) in CH_2Cl_2 (105 mL). After 1.5 h the solution was filtered and concentrated. Column chromatography of the residue (6:1 toluene/acetone) afforded a mixture (α/β 62:38) of the *O*-(benzyl 4-*O*-levulinyl-2,3-di-*O*-methyl-β-D-glucopyranosyluronate)-(1→4)-3,6-di-*O*-acetyl-2-*O*-benzyl-α,β-D-glucopyranosyl trichloroacetimidates (**25**, 5.4 g, 71%): TLC R_f 0.34 and 0.43, 3:1 toluene/acetone; ^{1}H NMR ($CDCl_3$) δ 8.70−8.60 (2s, 1H, =NH of α- and β-anomers), 7.17−7.35 (m, 10H, 2Ph), 6.44 (d, J = 3.5 Hz, 0.62H, H-1α), 5.83 (d, J = 7.5 Hz, 0.38H, H-1β), 3.48, 3.50 (2s, 6H, 2OMe), 2.20−2.60 (m, 4H, levulinyl CH_2CH_2), 2.11, 2.06, 2.05, 1.93, 1.85, 1.57 (6s, Ac and levulinyl CH_3).

Methyl *O*-(Benzyl 2,3-di-*O*-methyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(3,6-di-*O*-acetyl-2-*O*-benzyl-α-D-glucopyranosyl)-(1→4)-*O*-(benzyl 2-*O*-benzoyl-3-*O*-methyl-α-L-idopyranosyluronate)-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-glucopyranoside (27). Trimethylsilyl triflate (40 μL) was added under argon to a stirred, cooled (−20 °C) solution of the above imidates **25** (1.81 g, 2 mmol) and **13** (1.57 g, 2 mmol) in toluene (50 mL) containing 4 Å molecular sieves (3.3 g). After 30 min, solid $NaHCO_3$ (0.1 g) was introduced, and stirring was prolonged overnight. The solution was filtered, washed with H_2O, dried, and concentrated. Column chromatography (3:2 cyclohexane/EtOAc) provided pure methyl *O*-(benzyl 2,3-di-*O*-methyl-4-*O*-levulinyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(3,6-di-*O*-acetyl-2-*O*-benzyl-α-D-glucopyranosyl)-(1→4)-*O*-(benzyl 2-*O*-benzoyl-3-*O*-methyl-α-L-idopyranosyluronate)-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-glucopyranoside (**26**, 2.24 g, 73%): ^{1}H NMR ($CDCl_3$) δ 7.10−8.04 (m, 30H, 6Ph), 5.26 (d, 1H, J = 2.6 Hz, H-1 G unit), 4.95 (d, 1H, J = 3.7 Hz, H-1 F unit), 4.51 (d, 1H, J = 3.5 Hz, H-1 H unit), 4.20 (d, 1H, J = 7.9 Hz, H-1 E unit), 3.47, 3.42, 3.32, 3.28, 3.25 (5s, 15H, 5OMe), 2.26−2.58 (m, 4H, levulinyl CH_2CH_2), 2.12, 2.07, 1.92 (3s, 9H, 2Ac and levulinyl CH_3). Anal. ($C_{80}H_{22}O_{28}$) C, H.

The levulinyl group of **26** (2.21 g, 1.47 mmol) was selectively removed as described for the preparation of **13** to give pure (TLC) methyl *O*-(benzyl 2,3-di-*O*-methyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(3,6-di-*O*-acetyl-2-*O*-benzyl-α-D-glucopyranosyl)-(1→4)-*O*-(benzyl 2-*O*-benzoyl-3-*O*-methyl-α-L-idopyranosyluronate)-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-glucopyranoside (**27**, 1.98 g, 96%) after column chromatography (1:1 cyclohexane/EtOAc): TLC R_f 0.37, 1:1 cyclohexane/EtOAc; ^{1}H NMR ($CDCl_3$) δ 7.18−8.04 (m, 30H, 6Ph), 5.27 (d, 1H, J = 3.1 Hz, H-1 G unit), 4.98 (d, 1H, J = 3.7 Hz, H-1 F unit), 4.53 (d, 1H, J = 3.5 Hz, H-1 H unit), 4.21 (d, 1H, J = 7.7 Hz, H-1 E unit), 3.58, 3.42, 3.32, 3.29, 3.28 (5s, 5OMe), 2.06, 1.89 (2s, 2Ac).

Ethyl 6-*O*-Acetyl-2-*O*-benzyl-3,4-di-*O*-methyl-1-thio-α,β-D-glucopyranoside (32). The epoxide ring of 1,6:2,3-dianhydro-4-*O*-methyl-β-D-mannopyranose[17] (**28**, 6.39 g, 40.4 mmol) was opened as descibed for the preparation of **16** to give 1,6-anhydro-2-*O*-benzyl-4-*O*-methyl-β-D-glucopyranose (**28**, 10.51 g, 97%) after column chromatography (5:1, then 4:1, 3:1, 1:2, 1:3 hexane/EtOAc): ^{1}H NMR ($CDCl_3$) δ 7.26−7.34 (m, 5H, Ph), 5.43 (m, 1H, H-1), 3.47 (1s, 3H, OMe).

Compound **28** (3.57 g, 13.4 mmol) was methylated as described for the preparation of **4** to give 1,6-anhydro-2-*O*-benzyl-3,4-di-*O*-methyl-β-D-glucopyranose (**29**, 3.45 g, 89%) after column chromatography (2:1 hexane/EtOAc): ^{1}H NMR ($CDCl_3$) δ 7.26−7.38 (m, 5H, Ph), 5.42 (m, 1H, H-1), 3.47, 3.31 (2s, 6H, 2OMe).

Acetolysis of the anhydro ring of **30** (3.2 g, 11.44 mmol) was performed as described for the preparation of **23** to give crude 1,6-di-*O*-acetyl-2-*O*-benzyl-3,4-di-*O*-methyl-β-D-glucopyranose (**31**, 4.4 g): ^{1}H NMR ($CDCl_3$) δ 7.33 (m, 5H, Ph), 6.23 (d, J = 2.9 Hz, H-1α), 5.55 (d, J = 7 Hz, H-1β), 3.67, 3.54 (2s, 6H, 2OMe) 2.14, 2.08, 2.05 (3s, 6H, OAc).

Thioethanol (0.2 mL, 2.6 mmol) was added to a solution of **31** (0.5 g, 1.3 mmol) in toluene (25 mL) followed by $BF_3{\cdot}Et_2O$ complex (1.3 mL, 1.3 mmol, dropwise addition). After 1.5 h aqueous saturated $NaHCO_3$ (15 mL) was introduced, and the solution was diluted with CH_2Cl_2, washed with H_2O and brine, dried (Na_2SO_4), and concentrated. Column chromatography of the residue (5:1, then 4:1, 3:1 hexane/EtOAc) afforded **32** (0.46 g, 91% from 30) as a colorless syrup: ^{1}H NMR ($CDCl_3$) δ 7.30−7.42 (m, 5H, Ph), 5.30 (d, J = 5.1 Hz, H-1α), 4.79 (d, J = 8.1 Hz, H-1β), 3.66, 3.64, 3.53, 3.52 (4s, 6H, OMe), 2.42−2.69 (m, 2H, SCH_2CH_3), 2.08 (s, OAc), 1.15−1.38 (m, 3H, SCH_2CH_3); TLC R_f 0.37 1:1 hexane/EtOAc. Anal. ($C_{19}H_{28}O_6S$) C, H, S.

Methyl *O*-(6-*O*-Acetyl-2-*O*-benzyl-3,4-di-*O*-methyl-α-D-glucopyranosyl)-(1→4)-*O*-(benzyl 2,3-di-*O*-methyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(3,6-di-*O*-acetyl-2-*O*-benzyl-α-D-glucopyranosyl)-(1→4)-*O*-(benzyl 2-*O*-benzoyl-3-*O*-methyl-α-L-idopyranosyluronate)-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-glucopyranoside (33). Triflic acid (3.4 μL, 0.038 mmol) was added under argon to a stirred, cold (−20 °C) solution of **32** (0.15 g, 0.384 mmol), **27** (0.45 g, 0.32 mmol), and *N*-iodosuccinimide (0.087 g, 0.384 mmol) in 2:1 1,2-dichloroethane/Et_2O (21 mL) containing 4 Å molecular sieves (0.6 g). After 30 min solid $NaHCO_3$ (10 mg) was added, and 15 min later the solution was filtered, diluted with CH_2Cl_2, washed with 10% aqueous $Na_2S_2O_3$ and H_2O, dried (Na_2SO_4), and evaporated. Column chromatography (16:1 CH_2Cl_2/acetone) gave pure (TLC, R_f 0.5, 1:1 cyclohexane EtOAc) **33** (0.38 g, 62%): ^{1}H NMR ($CDCl_3$) δ 7.18−8.04 (m, 35H, 7Ph), 5.44 (d, 1H, J = 3.3 Hz, H-1 D unit), 5.25 (d, 1H, J = 2.2 Hz, H-1 G unit), 4.95 (d, 1H, J = 3.5 Hz, H-1 F unit), 4.52 (d, 1H, J = 3.3 Hz, H-1 H unit), 4.15 (d, 1H, J = 8.1 Hz, H-1 E unit), 3.56, 3.49, 3.43, 3.41, 3.32, 3.28 (7s, 7OMe), 2.09, 2.06, 1.87 (3s, 9H, 3Ac).

Methyl *O*-(3,4-Di-*O*-methyl-2,6-di-*O*-sulfo-α-D-glucopyranosyl)-(1→4)-*O*-(2,3-di-*O*-methyl-β-D-glucopyranosyluronic acid)-(1→4)-*O*-(2,3,6-tri-*O*-sulfo-α-D-glucopyranosyl)-(1→4)-*O*-(3-*O*-methyl-2-*O*-sulfo-α-L-idopyranosyluronic acid)-(1→4)-3-*O*-methyl-2,6-di-*O*-sulfo-α-D-glucopyranoside, Decasodium Salt (34). A solution of **33** (0.25 g, 0.144 mmol) in DMF (11 mL) was stirred during 36 h under a weak stream of H_2 in the presence of 10% Pd/C catalyst (0.25 g). After filtration the solution was concentrated to give methyl *O*-(6-*O*-acetyl-3,4-di-*O*-methyl-α-D-glucopyranosyl)-(1→4)-*O*-(2,3-di-*O*-methyl-β-D-glucopyranosyluronic acid)-(1→4)-*O*-(3,6-di-*O*-acetyl-α-D-glucopyranosyl)-(1→4)-*O*-(2-*O*-benzoyl-3-*O*-methyl-α-L-idopyranosyluronic acid)-(1→4)-3-*O*-methyl-α-D-glucopyranoside (0.170 g): TLC R_f 0.87, 5:5:1:3 EtOAc/pyridine/AcOH/H_2O.

Aqueous NaOH (5 M, 2.9 mL) was added to a solution of the above crude compound in MeOH (26 mL). After 40 min Dowex 50 H^+ was introduced until neutral pH. The solution was concentrated, and the residue was layered on top of a Sephadex G 25 column (1.6 × 100 cm) eluted with H_2O. Concentration of the pooled fractions gave methyl *O*-(3,4-di-*O*-methyl-α-D-glucopyranosyl)-(1→4)-*O*-(2,3-di-*O*-methyl-β-D-glucopyranosyluronic acid)-(1→4)-*O*-(α-D-glucopyranosyl)-(1→4)-*O*-(3-*O*-methyl-α-L-idopyranosyluronic acid)-(1→4)-3-*O*-methyl-α-D-glucopyranoside (0.132 g): TLC R_f 0.14, 5:5:1:3 EtOAc/pyridine/AcOH/H_2O.

Et_3N/SO_3 complex (1.05 g, 5.82 mmol) was added to a solution of the above compound (0.132 g) in DMF (6 mL), and the solution was heated at 50 °C for 20 h. $NaHCO_3$ (1.8 g dissolved in H_2O) was then introduced, and the solution was layered on top of a Sephadex G-25 column (1.6 × 100 cm) equilibrated in 0.2 M NaCl. The fractions were pooled, concentrated, and dessalted on the same gel filtration column, equilibrated in H_2O. Lyophilization then gave **34** (0.23 g, 88% from **33**): LSIMS (liquid secondary ion mass spectrometry), negative mode, m/z 1791 $(M - Na)^-$, 1769 $(M - 2Na + H)^-$; ^{1}H NMR (D_2O) δ 5.53 (d, 1H, J = 3.7 Hz, H-1 D unit), 5.45 (d, 1H, J = 3.7 Hz, H-1 F unit), 5.12 (d, 1H, J = 2.6 Hz, H-1 G unit), 5.05 (d, 1H, J = 3.5 Hz, H-1 H unit), 4.65 (d, 1H, J = 7.7 Hz, H-1 E unit), 3.60, 3.59, 3.55, 3.54, 3.49, 3.42 (s, OMe).

34 was >95% pure (chiral detection) by HPLC on a Carbopac PA100 column eluted with a linear 1.1−2.0 M NaCl gradient.

Binding Constants. Fluorescence measurements were performed as described by Atha *et al.*[18] using a Perkin-Elmer LS-50 type spectrofluorimeter; excitation $\lambda = 280$ nm, emission $\lambda = 338$ nm; equipped with a thermostated sample compartment, at 37 °C, under continuous stirring. Oligosaccharides were added into the cuvette containing 2 mL of Tris-HCl buffer, 0.01 M, pH 7.0, 0.15 M NaCl, and 5−60 nM human AT-III (purified according to Mc Kay[23]). The ratio and the concentrations of AT III−oligosaccharide complexes were calculated considering a 1:1 reaction stoichiometry, and dissociation constants (K_D) were determined by Scatchard analysis using a specific application of RS/1 computer program (BBN Software Product Corp., Cambridge, MA). The results are mean ± SEM, $n = 3$.

Anti-factor Xa Activity. Anti-factor Xa activity was determined by an amidolytic method (modification of the procedure of Teien and Lie[19]): factor Xa (7.5 nkat/mL in 20 mM Tris/maleate buffer, pH 7.4, NaCl 150 mM; 100 μL) was incubated during 2 min with AT III (0.5 unit/mL in 20 mM Tris/maleate buffer, pH 7.4, NaCl 150 mM; 100 μL) at 37 °C in the presence of the oligosaccharides (at various concentrations in 20 mM Tris/maleate buffer, pH 7.4, NaCl 150 mM; 100 μL). To measure residual factor Xa, S-2222 substrate (Bz-Ile-Glu-Gly-Arg-pNA; 1 mM in 50 mM Tris/HCl buffer, pH 8.4, NaCl 175 mM, EDTA 2 7.5 mM; 100 μL) was added. The reaction was stopped 2 min later by addition of 50% aqueous acetic acid (100 μL), and the absorbance at 405 nm was read. The percentage of inhibition was then calculated [inhibition % = 100 × (A_{405} of buffer control − A_{405} of sample)/A_{405} of buffer control], and the activity of the compounds was determined by comparison with a calibrated standard, using Excel 4.0 Software (Microsoft, Redmond USA). The results are mean ± SEM, $n = 3$.

Thrombin Generation Test. The thrombin generation methodology was adapted from Hemker *et al.*[20] who used a poor substrate of thrombin (S-2222, Bz-Ile-Glu-Gly-Arg-pNA) to allow easier monitoring of the reaction. Under the conditions of the assay, the very low concentration of factor Xa, as compared to thrombin, could not be detected and does not interfere with the determination of thrombin. Briefly, in a plastic disposable semimicrocuvette were successively added, at 37 °C, rabbit brain thromboplastins (50 μL, *i.e.* the amount necessary to obtain a coagulation time of 90 s in the Quick assay, see ref 20), S-2222 substrate (1.6 mM, in 50 mM Tris-HCl buffer, pH 7.35, NaCl 0.15 M; 50 μL), the compound to be tested (in 50 mM Tris-HCl buffer, pH 7.35, NaCl 0.15 M; 50 μL), and $CaCl_2$ (0.25 M in H_2O; 50 μL). Thrombin generation was initiated by the addition of defibrinated platelet poor plasma (100 μL). The absorbance at 405 nm was plotted *vs* time during 20 min. The area under the curve and the IC_{50} were determined, using the four-parameter logistic model, with a confidence interval of 95%. The adjustment was obtained by nonlinear regression using the Levenberg−Marquard algorithm in RS/1 software (BBN Software Product Corp., Cambridge, MA). The results are mean, and 95% confidence interval, $n = 3$.

Antithrombotic Activity. Thrombus formation by a combination of stasis and hypercoagulability was induced as described by Vogel *et al.*[21] Male Sprague−Dawley rats (250−300 g; Iffa Credo, France) were anesthetized with sodium pentobarbitone (30 mg/kg, ip). The vena cava was exposed and dissected free from surrounding tissue. Two loose sutures were prepared 0.7 cm apart on the inferior vena cava, and all collateral veins were ligated. The various pentasaccharides were administered iv in the dorsal penile vein 5 min before thrombosis induction. Human tissue factor (1 ng/kg) was then injected iv, in the dorsal penile vein, during 30 s, and 10 s after the end of the injection, stasis was established by tightening the two sutures. Stasis was maintained for 20 min, and the thrombus formed was removed, rinsed, dried overnight at 60 °C, and weighed. The results are expressed as ED_{50} (50% reduction in thrombus weight) mean and 95% confidence interval, $n = 6$.

Pharmacokinetics. Pharmacokinetics were performed in rats after iv bolus injection of the compounds. The jugular vein was canulated under general anesthesia (sodium pentobarbitone 30 mg/kg, iv), and the animals were allowed to recover from the surgery overnight. The pentasaccharides were injected *via* the catheter at a dose of 100 nmol/kg. Blood was collected in a 0.129 M citrate solution (1/10; v/v). Concentrations of pentasaccharides in plasma were determined by measuring the anti-factor Xa activity as described above. Each pentasaccharide was used as its own reference. Elimination half-lives were calculated by the slope of the terminal phase using a computational method (Siphar/PC, version 4.0). The results are mean ± SEM, $n = 4$.

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