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Synthesis and conformational analysis of an analogue of the antithrombin-binding region of heparin: the role of the carboxylate function of α-L-idopyranuronate

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Abstract. An analogue of the antithrombin-III-binding region of heparin has been synthesized. This analogue [$GlcNSO_3$ (6-OSO_3)-GlcU-$GlcNSO_3$ (3,6-OSO_3)-*Xyl(2-OSO_3)*-$GlcNSO_3$ (6-OSO_3), *i.e.* compound **11**] lacks the carboxylate function of the α-L-iduronic acid moiety, which implies that the 2-*O*-sulphated α-L-idopyranuronate unit is replaced by 2-*O*-sulphated β-D-xylopyranose.
Compound **11** was prepared from fully protected pentasaccharide **10a**, which was synthesized from two disaccharides (**7** and **6b**) and a monosaccharide (**9**). Synthesis of disaccharide **6b** required preparation of a new, suitably protected xylopyranose-building block: 2-*O*-acetyl- or 2-*O*-benzoyl-3-*O*-benzyl-4-*O*-levulinoyl-α-D-xylopyranosyl fluoride **4a,b**.
High-field NMR spectroscopy of compound **11** afforded a complete set of interproton coupling data and Nuclear Overhauser Enhancement data, which have been used for conformational analysis. It appears that β-D-xylopyranose in compound **11** adopts exclusively the $^4C_1$ conformation, whereas it is known that α-L-idopyranuronate in the naturally occurring heparin fragment exhibits $^2S_0$ and $^1C_4$ conformations. Thus, the carboxylate function of α-L-idopyranuronate in the heparin fragment is an important conformational drive.
Since neither compound **11** nor another analogue containing L-idose (with similar conformational properties as the natural fragment) elicit AT-III-mediated activity, it is proposed that the carboxylate of idopyranuronate interacts with AT-III to bring about the active conformation of the protease inhibitor.

Introduction

Heparin consists of a mixture of sulphated heterogenous glycosaminoglycans, displaying anticoagulant properties. The anticoagulant action involves binding of heparin to antithrombin III (AT-III); the protease inhibitor thereby reacts more rapidly with proteases (*e.g.* thrombin, Xa, IXa etc.) of the blood coagulation cascade[1,2].
Heparin fragments have been isolated by affinity chromatography on immobilized AT-III and then analyzed. From these studies, it appeared that a unique pentasaccharide constitutes the minimal binding site for AT-III[3,4,11]; it is of particular interest that this pentasaccharide catalyzes AT-III-mediated inactivation of factor Xa but not of thrombin. The structure of this AT-III-binding site of heparin is shown in Fig. 1.
Much is known about the role of the sulphate substituents in activation of AT-III. It has been established that the 6-*O*-sulpate substituent[5] of glucosamine unit 2 and the 3-*O*[6,7] and *N*-sulphate substituents[8] of glucosamine unit 4 are essential for high affinity to AT-III and for accelerating inactivation of the coagulation factor Xa (anti-Xa activity). Furthermore, the 6-*O*-sulphate group[9] at glucosamine unit 6 has been found to contribute significantly during the acti-

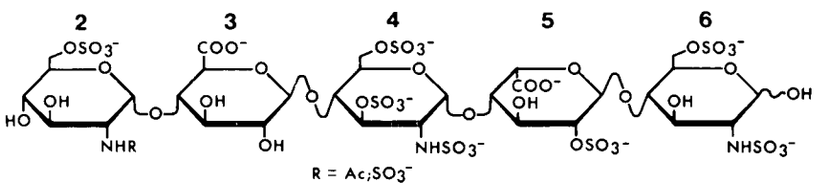


Fig. 1. Structure of the unique AT-III-binding fragment of heparin. This pentasaccharide (2-3-4-5-6) has virtually the same affinity for AT-III as the enzymatically obtained octasaccharide (1-2-3-4-5-6-7-8): IdU-GlcNAc (6-OSO_3)-GlcU-$GlcNSO_3$ (3,6-OSO_3)-IdU (2-OSO_3)-$GlcNSO_3$ (6-OSO_3)-IdU (2-OSO_3)-A-Man (6-OSO_3), see ref. 37.

vation of AT-III, while selective removal of the *N*-sulphate at glucosamine unit 6 reduces binding of high-affinity fragments to AT-III[8]. Since the tetrasaccharide comprising units 2-3-4-5 still exhibits significant anti-Xa activity[6], the sulphate groups of moiety 6 only increase the activity. On the other hand, the *N*-sulphate (or *N*-acetyl) group at glucosamine unit 2 and the 6-*O*-sulphate substituent at glucosamine 4 have been found to be non-essential[4,10,11]. However, the role of the two carboxylate functions of the pentasaccharide is as yet unknown, although it has been reported that AT-III-mediated anti-Xa activity of heparin decreases upon methylation of the uronic acid carboxylate functions[12]. One should be careful when inferring from this study that carboxylate functions participate in the activation process, since it was not established whether other vital substituents remained unaffected during the chemical modification.

Since well-defined heparin fragments and analogues are now accessible by total-synthesis[9,13,14,15], the role of the carboxylate functions in the heparin fragment can be studied with the help of synthetic model compounds. In this publication, we wish to report the synthesis and conformational analysis of an analogue of the AT-III-binding pentasaccharide which lacks the carboxylate function (*) at the L-iduronic acid moiety 5 (*i.e.* compound **11** in Scheme 1).

Results and discussion

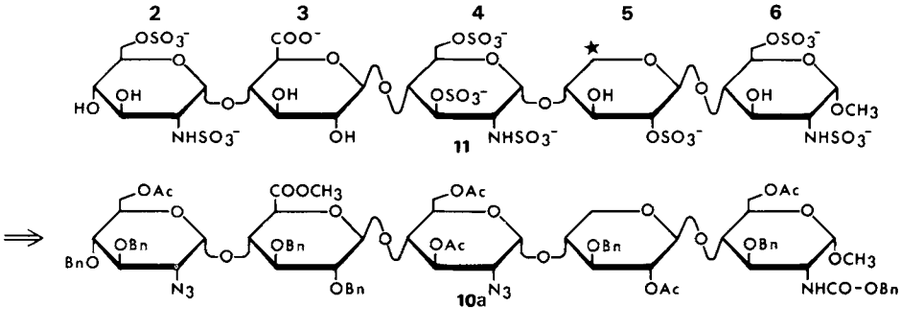
Synthesis

The synthesis of pentasaccharide **11** instead of the AT-III-binding heparin fragment involves the introduction of a 2-*O*-sulphated β-D-xylopyranose moiety at the position of 2-*O*-sulphated α-L-idopyranuronate. It is now well recognized[9,13,14,15] that synthesis of heparin-like oligosaccharides can be most conveniently performed using protected building blocks bearing acetyl (or benzoyl) protective groups at hydroxyl functions to be sulphated and benzyl protective groups for unsulphated hydroxyl groups. Consequently, pentasaccharide **11** can be prepared from the fully protected derivative **10a** (see Scheme 1). The azide functions of compound **10a** have been introduced at those protected glucosamine residues which require appropriate non-participating protective groups at C-2 to facilitate α-glycosidic bond formation.

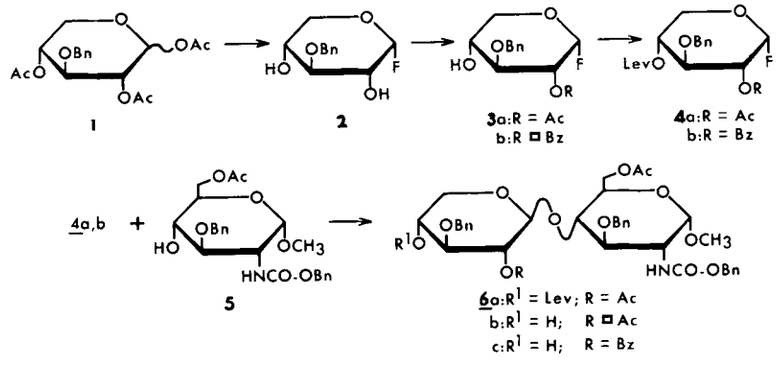
Assemblage of pentasaccharide **10a** requires coupling of the suitably protected β-D-xylopyranose-containing disaccharide **6b** (Scheme 2) with the known dissacharide **7**[14] (Scheme 3) to give the tetrasaccharide **8a**, which, in turn, can be coupled with monosaccharide **9** to afford the fully protected pentasaccharide **10a**. Ultimately, compound **10a** can be deprotected in a step-wise process and subsequently sulphated to give the desired compound **11** (Scheme 4).

The protected D-xylopyranose monosaccharide, to be used in the synthesis of building block **6b**, has to fulfil the following requirements: *(i)* a permanent benzyl-blocking group for protection of the 3-*O* position; *(ii)* an acetyl (benzoyl) group at the 2-*O* position; *(iii)* a temporary blocking group for protection of 4-*O*; *(iv)* an activated anomeric centre at which the β-glycosidic bond has to be introduced. The synthesis of such a D-xylopyranose derivative can be conveniently accomplished by taking advantage of a D-xylopyranosyl fluoride derivative (Scheme 2).

Thus, 3-*O*-benzyl-α-D-xylopyranosyl fluoride **2** was prepared in 85% yield by treating 1,2,4-tri-*O*-acetyl-3-*O*-benzyl-D-xylopyranose **1**[16] with 70% hydrogen fluoride in pyridine[18], followed by saponification of acetyl esters with potassium carbonate in methanol. Compound **2** was then selectively acetylated at the 2-*O* position with 1-acetylimidazole in boiling dichloromethane for a week to give 50% of mono-acetylated **3a**[17]. Unfortunately, compound **3a**



Scheme 1. Compound **11** *is an analogue of the AT-III-binding heparin fragment in which the carboxylate function* (*) *of unit 5 is absent. Compound* **11** *can be prepared from fully protected pentasaccharide* **10a** *containing a β-D-xylopyranose moiety.*

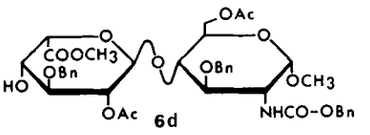


Scheme 2

was contaminated with *ca.* 10% of the 4-*O*-acetylated isomer. Levulinoylation of **3a** with levulinic* anhydride[19] in pyridine, in the presence of 4-(dimethylamino)pyridine, gave compound **4a** contaminated with the respective isomer. Attempts to remove the undesired isomer from **3a** or **4a** by chromatography on silica gel or by crystallization were unsuccessful. Tentatively, crude **4a** was coupled with aglycon **5** in dichloromethane and in the presence of boron trifluoride[20]. After work-up and chromatography, disaccharide **6a** was isolated in 60% yield. Disaccharide **6a** was still contaminated with some of the isomer **6a** (R^1 = Ac, R = Lev), displaying identical chromatographical behaviour. Fortunately, at this stage, the undesired isomer could be removed by crystallization to give pure **6a** in 40% yield. Finally, selective deprotection of the levulinoyl group with hydrazine in acetic acid/pyridine afforded disaccharide **6b** in 75% yield. The structure of **6b** was confirmed by proton–proton correlated ^{1}H-NMR spectroscopy, which clearly revealed acetylation of the 2'-hydroxyl function.

Subsequently, the synthesis of a suitably protected β-D--xylopyranose building block was improved by selective introduction of a benzoyl group instead of an acetyl group. Thus, compound **2** was treated with a slight excess of benzoyl chloride at −25°C to give, after purification by silica gel chromatography and crystallization, pure **3b** in 53% yield. Levulinoylation of **3b** gave **4b**, which, in turn, was coupled with **5** under the conditions described for the preparation of **6a** to give, after cleavage of the levulinoyl group, **6c** in 42% yield.

Synthesis of tetrasaccharide **8a** was executed by a coupling reaction of **6b** with glycon **7** (see Scheme 3). Initially, reaction conditions were elaborated using silver triflate as promoter. Similar conditions have been applied previously in the synthesis of the AT-III-binding pentasaccharide, in which coupling of glycon **7** with iduronic-acid-containing disaccharides (such as **6d**) afforded tetrasaccharides in 60–70% yield[9,13,14]. However, a silver triflate-promoted coupling of **7** with **6b** in the presence of 2,6-di-*tert*-butylpyridine as a non-nucleophilic base[14] gave, after column chromatography, tetrasaccharide **8a** in a poor yield of 13% (Table I, entry 1). Variation of the reaction conditions[21], or using different batches of silver triflate or base, did not increase the yield significantly (*e.g.* entries 2 and 3). Neither the application of mercury(II) bromide (entries 4 and 5) nor that of silver perchlorate (entries 6, 7 and 8) significantly improved the coupling reaction.

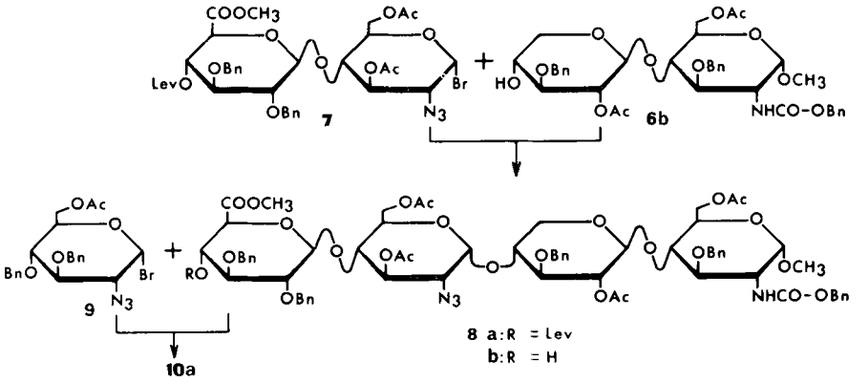


6d

We also investigated an alternative coupling procedure, as described by *Schmidt*[22] et al., in which aglycon **7** is functionalized at the anomeric centre with a trichloroacetimidate group instead of with bromide (entry 9). However, no formation of tetrasaccharide **8a** was observed.

Apparently, the 4'-hydroxyl group of disaccharide **6b** is much less reactive than that of the corresponding L-iduronic acid analogue **6d**. At first sight, this phenomenon is incomprehensible, since it is widely accepted that electron-withdrawing groups (such as the methoxycarbonyl group of **6d**) inactivate proximate hydroxyl functions[21]. However, the reactivity of a hydroxyl group also depends on the conformation of the saccharide bearing that hydroxyl group.

* Levulinic = 4-oxopentanoic.



Scheme 3

Table I Reaction conditions for coupling glycon **7** *with aglycon* **6b** *to give* **8a**.

Entry	Glycon 7 (eq)	Promoter (eq)	Acid scavenger[a]	Time	*T* (°C)	Yield (%)
1	1.5	$AgCF_3SO_3$ (2.5)	DTBP	16 hr	−20	13
2	1.5	$AgCF_3SO_3$ (2.5)	MS 10 A	16 hr	−30	20
3	2.5	$AgCF_3SO_3$ (2.5)	DTBP	16 hr	−66	–
4	1.5	$HgBr_2$ (1)	MS 4 A	5 days	20	20
5	1.5	$HgBr_2$ (1.5)	MS 4 A	10 days	40	–
6	2	$AgClO_4$ (0.5)	DTBP	6 days	20	19
7	1.5	$AgClO_4$ (1.5)	DTBP	1.5 hr	−10	20
8	1.5	$AgClO_4$ (2.5)	DTBP	8 hr	0	22
9	1.5[b]	TMS-triflate (0.1)	–	3 hr	−50	–

[a] DTBP = 2,6-di-*tert*-butylpyridine. [b] The α/β-trichloroacetimidate analogue of **7** (O−C=NH−CCl_3 instead of Br) was used.

Unravelling of the conformational details of **6b** and **6d**, by ^{1}H-NMR spectroscopy elicits different conformations of the D-xylopyranose and L-idopyranuronate ring, respectively. The vicinal coupling constants of the L-idopyranuronate ring of **6d** pertain to a $^1C_4$ conformation (see Table II), whereas the coupling constants of the D-xylopyranose ring of **6b** are typical of a $^4C_1$ conformation[23]. In view of these conformational differences, we assume that the 4'-hydroxyl group of **6d** is more reactive than that of **6b**. It is taken for granted that this conformational effect outweighs deactivation by the electron-withdrawing carboxylate ester. In this respect, it merits comment that similar phenomena have been observed for 4-hydroxyl groups of gluco- and mannopyranosyl aglycons, which are also much more reactive in $^1C_4$ pyranose conformations (*e.g.* 1,6-anhydropyranoses) than in $^4C_1$ conformations[21].

Table II Observed and calculated[b] interproton coupling constants for some β-D-xylopyranose and α-L-idopyranuronate moieties.

Compound	Coupling constant (Hz)				
	$^3J_{1,2}$	$^3J_{2,3}$	$^3J_{3,4}$	$^3J_{4,5}$	$^3J_{4,5'}$
6b (Xyl p)	6.8	8.8	8.8	5.0	8.4
6d[a] (IdU p)	1.6	3.0	3.6	2.3	
11 (Xyl p)	7.8	9.3	8.8	4.9	9.7
β-Xyl p: $^1C_4$[b,c]	2.6	3.5	3.5	4.4	1.2
β-Xyl p: $^2S_0$[b,d]	4.9	9.6	5.0	1.3	4.4
β-Xyl p: $^4C_1$[b,c]	7.8	9.7	9.3	5.6	10.3

[a] Longe-range coupling constants are observed: $^4J_{1,3}$ 1.1 Hz; $^4J_{2,4}$ 1.3 Hz. [b] Calculated coupling constants according to the equations described by *Haasnoot* et al.[26,44]. [c] Assuming regular pyranose geometry. [d] Dihedral angles from ref. 30.

Notwithstanding the difficulties met during the preparation of tetrasaccharide **8a**, sufficient material was obtained to complete the synthesis. The levulinoyl group was selectively removed from **8a** using hydrazine hydrate in pyridine/acetic acid to give **8b** in 80% yield. Pentasaccharide **10a** was obtained in 60% yield by coupling **8b** with monosaccharide **9** in the presence of silver triflate and 2,6-di-*tert*-butylpyridine[14].

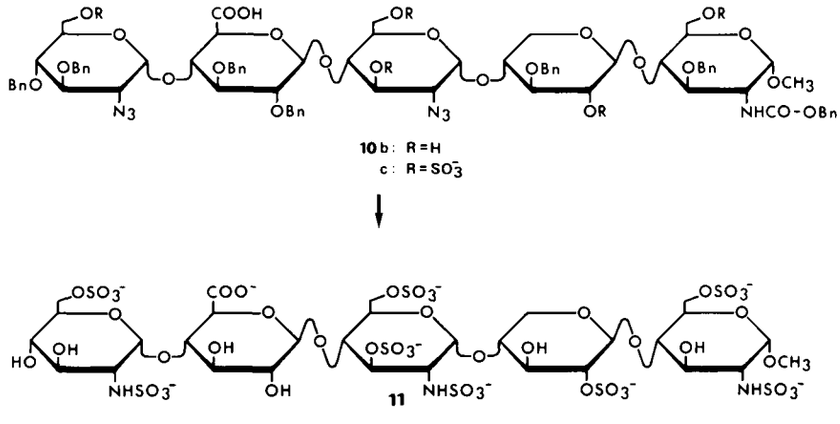
Further processing of compound **10a** (see Scheme 4) included quantitative saponification of acetyl groups and the methyl ester to give **10b**, followed by *O*-sulphation with sulphur trioxide/trimethylamine complex in DMF[13] to give, after chromatography, compound **10c** in 90% yield. The benzyl and azide groups of **10c** were simultaneously hydrogenolyzed in the presence of palladium on charcoal and then the deprotected compound was subjected to selective *N*-sulphation[13] with sulphur trioxide/trimethylamine complex in water at pH 9. The crude material thus obtained was desalted by gel-permeation chromatography and converted into the sodium salt to give pure pentasaccharide **11** in 56% yield. High-field ^{1}H-NMR (2D-COSY) revealed that no side-reactions had occurred during the last two steps, indicating that the relatively low yield in the last step has to be attributed to loss of material during gel-permeation chromatography or to the presence of residual salt in intermediate **10c**.

Biological activity and conformational analysis

Pure pentasaccharide **11** displayed an anti-Xa activity of only 0.1 U/mg in an amidolytic assay in plasma with chromogenic substrate S2222, whereas the synthetic AT-III-binding fragment (Fig. 1, R = SO_3^-) in this assay displays about 600 anti-Xa U/mg.

It is clear from this finding that the carboxylate function of L-iduronic acid plays an essential role in the activation process of AT-III. This sharp decrease in the biological activity may be ascribed to the different conformational behaviour of β-D-xylopyranose, with respect to α-L-idopyranuronate in the natural compound. The α-L-idopyranuronate part of the AT-III-binding fragment has been found to adopt both $^2S_0$ skew boat and $^1C_4$ chair conformations[24]; the $^2S_0$ form is favoured under low-ionic conditions, whereas the $^1C_4$ conformation predominates at high-ionic conditions (*e.g.* 3 M NaCl[25]). By contrast, 2-*O*-sulphated β-D-xylopyranose moiety in pentasaccharide **11** exclusively adopts the $^4C_1$ conformation. The occurrence of the $^4C_1$ conformation was unambiguously inferred from the high-field ^{1}H-NMR spectrum, of which all resonances could be assigned completely by means of a two-dimensional proton–proton correlation experiment (2D-COSY: see Fig. 2a). The six proton–spin system of β-D-xylopyranose was computer-simulated to provide accurate coupling constants, which correspond to the calculated coupling constants[26] for the $^4C_1$ conformation of β-D-xylopyranose (see Table 2).

It has to be noted that many β-D-xylopyranose derivatives do not only occur in the $^4C_1$ conformation. For instance, ^{1}H-NMR investigations revealed that esterified methyl β-D-xylopyranosides occur in $^4C_1 \rightleftarrows {}^1C_4$ equilibria[27,28,29]. Furthermore, methyl 2,3,4-tri-*O*-benzoyl-β-D-xylopyranoside, in a crystal, was found[30] to occur in a $^2S_0$ skew-boat conformation, although in solution again an equilibrium between $^4C_1$ and $^1C_4$ conformers is observed[27]. Thus, in principle, the β-D-xylopyranose unit may display the same conformations[31,32] as the α-L-idopyranuronate unit (*i.e.* $^1C_4$,



Scheme 4

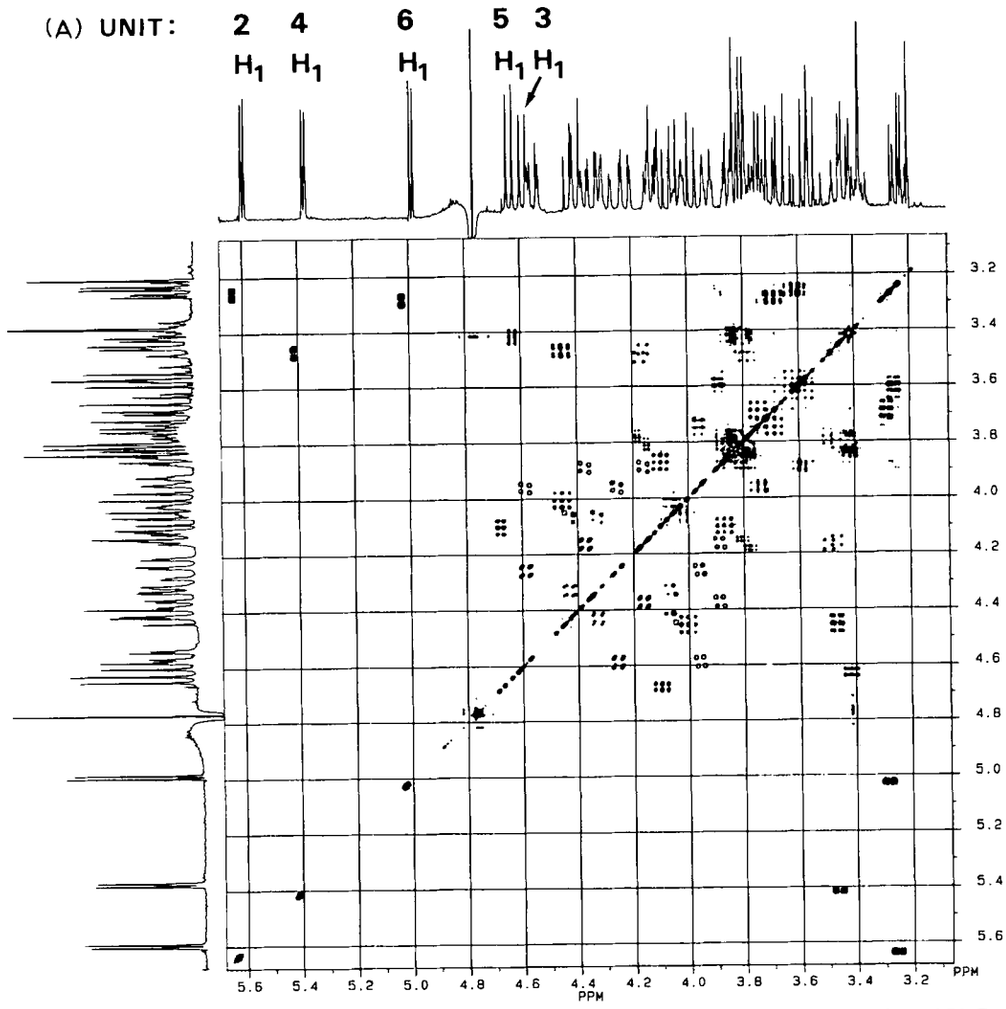


Fig. 2a. Contour plot of a proton–proton chemical shift correlated spectrum (COSY) of compound **11** *at 27°C. The normal 1D spectrum is plotted along the axes.*

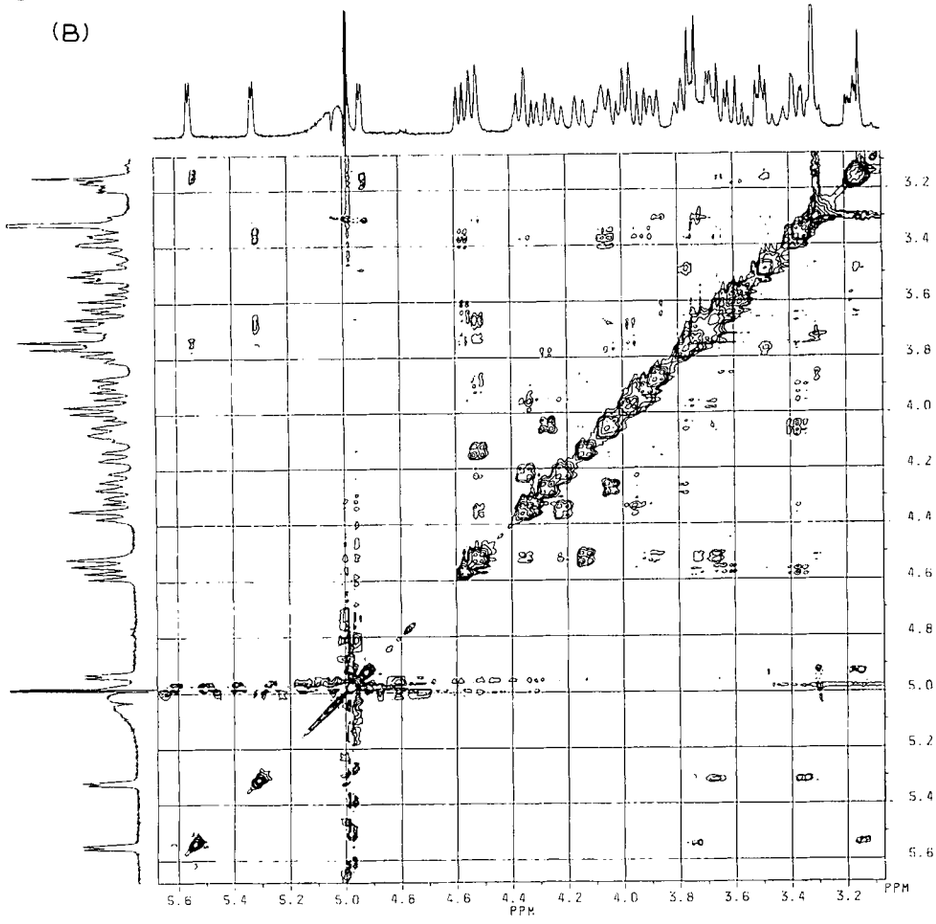


Fig. 2b. Contour plot (pure absorption mode) of a phase-sensitive NOESY spectrum of compound **11** *recorded at 3°C. The normal 1D spectrum recorded at the same temperature is plotted along th*

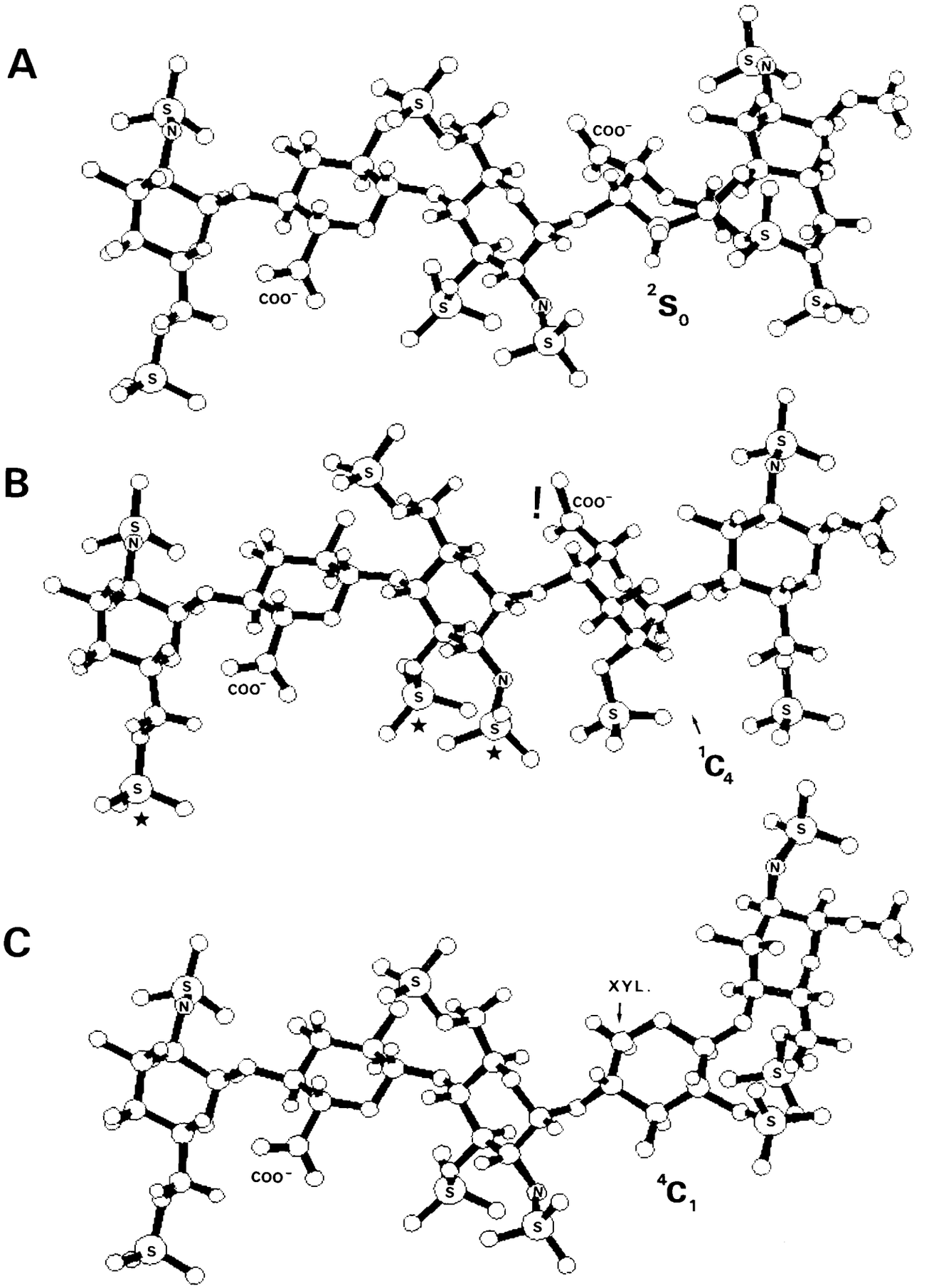


Fig. 3. *A. Molecular model of the AT-III-binding fragment of heparin (see Fig. 1; R = SO_3^-), displaying α-L-idopyranuronate in $^2S_0$ conformation.*

B. Molecular model of the AT-III-binding fragment of heparin, displaying α-L-idopyranuronate in $^1C_4$ conformation; it is proposed that this particular model is representative of the conformation of the pentasaccharide bound at AT-III. The essential sulphate substituents are located at the south side of the model (*), whereas the carboxylate group of iduronic acid is located at the north side (!).

C. Molecular model of the analogue **11**, *displaying β-D-xylose in $^4C_1$ conformation.*

$^4C_1$ and $^2S_0$ conformations). However, the ultimate population of conformations in solution is expected to differ considerably for these respective pyranoses, due to the fact that the carboxylate function of L-idopyranuronate most strongly affects the free-energy difference between individual conformations. Thus, 2-*O*-sulphated α-L-idopyranuronate tends to adopt the $^1C_4$ conformation[25,31], since in this form the bulky carboxylate group is disposed in an equatorial position and concomitantly the axial anomeric oxygen is stabilized by the anomeric effect. In addition, the $^1C_4$ conformation is stabilized[25] by the electron-withdrawing sulphate group at C-2. On the other hand, preponderance of the $^2S_0$ conformation in the AT-III-binding fragment[24,25] is a delicate compromise between the $^1C_4$ form, which, in this particular sequence, is destabilized by electronic repulsions among sulphate groups, and the $^4C_1$ form, which is disfavoured due to severe steric crowding of the axial carboxylate function.

It can be understood that the 2-*O*-sulphated β-D-xylopyranose moiety in compound **11** adopts the $^4C_1$ conformation exclusively, since this carbohydrate lacks the bulky axial substituent at C-5 and its $^1C_4$ form is destabilized by repulsions among sulphate groups, as established for the AT-III-binding fragment.

Molecular modelling

In Fig. 3, molecular models are shown for the naturally occurring AT-III-binding pentasaccharide, having α-L-idopyranuronate either in $^2S_0$ (A) or $^1C_4$ (B) conformation, and for compound **11** (C), displaying β-D-xylopyranose in the $^4C_1$ conformation.

The molecular models A and B of the AT-III-binding fragment were constructed[25] starting from structures of synthetic trisaccharides, corresponding to the flexible part 4-5-6 (see Fig. 1), displaying α-L-idopyranuronate in $^2S_0$ or $^1C_4$ conformation, respectively.

The molecular models of these trisaccharides were designed previously, utilizing ^{1}H-NMR-spectroscopic and Nuclear-Overhauser-Enhancement (NOE) data[25]. These sub-structures were combined with three-dimensional structures of the rigid trisaccharide part 2-3-4 of the AT-III-binding fragment. These data have been recently published by *Ferro* et al.[33], who calculated conformations about the glycosidic bonds (φ and ψ) using force-field methods.

A molecular model of compound **11** (Fig. 3C) was constructed starting from the individual carbohydrate units in $^4C_1$ conformations, the atomic parameters of which were obtained from the X-ray or neutron-diffraction crystallographic structures. The exclusive occurrence of $^4C_1$ conformations is reflected by the vicinal proton–proton coupling constants of the individual carbohydrate units. Furthermore, the orientation about the C5–C6 bonds (dihedral angles ω) of the glucosamines can be estimated by determining the vicinal coupling constants between H-5 and H-6a,b; since both coupling constants are small, a gauche–gauche conformation (ω $-60°$) is preferred. The φ and ψ torsion angles, which define the conformation about the interglycosidic bonds, may be estimated from ^{1}H-NMR NOE data, while taking into account the *exo*-anomeric effect[34] and Van der Waals interactions.

Representative NOE data of compound **11** were obtained from 1- and 2-dimensional NOE experiments. Only weak negative NOEs were observed in NOE experiments performed at 300°K, using 360-MHz or 500-MHz NMR equipment. Fortunately, the NOEs become more intensive at lower temperature due to longer apparent correlation times of the pentasaccharide molecule. It is assumed that inter-proton distances derived from NOEs are representative for the solution conformation, since the individual saccharide units of compound **11** are rigid pyranoses in $^4C_1$ conformation and the α/β $1 \rightarrow 4$ linkages have only limited conformational space available. The interglycosidic distances between glyconic H-1 and aglyconic H-4 protons have been derived from NOEs observed at H-4, upon irradiation of the anomeric proton, or, in the case of NOESY experiments (see Fig. 2b), from cross-sections along anomeric proton frequencies. In both experiments, intraglycosidic NOEs are also observed at H-2 in the case of α-glycosidic bonds, and at H-3/H-5 in the case of β-glycosidic bonds; the known internuclear distances between anomeric proton and intraglycosidic protons H-2, H-3 or H-5 can be used as a reference for interglycosidic NOEs, since the NOE depends on the internuclear distance.

Molecular modelling has been performed interactively by applying the modelling programme CHEM-X. Thus, φ angles were varied between $\pm 40°$ and $\pm 80°$ as estimated by energy calculations of the *exo*-anomeric effect[34,35]. The ψ angles were varied up to an angle at which the distances between anomeric and aglyconic hydrogens correspond roughly with the NOE data. Interatomic distances of non-bonded atoms were not allowed to be shorter than the sum of their Van der Waals radii.

In Fig. 3, it can be seen that the three-dimensional features of compound **11** (Fig. 3C) differ significantly from those of the natural compound, displaying iduronic acid either in $^2S_0$ or $^1C_4$ conformation (Figs. 3A and 3B, respectively). In this respect, it should be recalled that, in our opinion, it is most likely that a heparin fragment, bound at AT-III, exposes the idopyranuronate ring in the $^1C_4$ conformation, as observed by NMR spectroscopy under high-ionic conditions[25]. In the corresponding molecular model (Fig. 3B), being a linear conformation, all essential sulphates are manifested at the south side of the pentasaccharide molecule, whereas the two non-essential groups are at the north side. In contrast, analogue **11** (Fig. 3C) displays a bent conformation, thus placing the sulphate groups of the right half of the pentasaccharide (*i.e.* residues 5–6) in a different position.

Evidently, the carboxylate function of iduronic acid in the AT-III-binding heparin fragment is a crucial conformational drive.

Interaction of a carboxylate function with AT-III

From the molecular model depicted in Fig. 3B, one might conclude that the carboxylate group of L-iduronic acid does not interact with AT-III, since the position of this group at the molecule is opposite to the side where the essential sulphates are attached. We have some evidence, however, that the carboxylate function of α-L-idopyranuronate interacts with AT-III to bring about activation of the protease inhibitor. This hypothesis is mainly endorsed by conformational analysis and pharmacological data of another analogue[36], which contains 2,6-di-*O*-sulphated α-L-idopyranose, instead of 2-*O*-sulphated α-L-idopyranuronate. The conformational properties of α-L-idopyranose in the latter analogue resemble those of idopyranuronate in the natural compound; at low ionic conditions, the ratio of $^1C_4$, $^2S_0$ and $^4C_1$ conformer population is about 1/2/1[36]. However, this analogue similarly does not elicit significant anti-Xa activity. Thus, replacement of the iduronic acid carboxylate group by a $CH_2OSO_3^-$ group nullifies activation of AT-III, despite a similar three-dimensional orientation of the saccharide backbone and the sulphate groups in the natural compound and the idopyranose analogue.

Apparently, the carboxylate group of iduronic acid, being placed opposite to the essential sulphates, interacts with a second binding site of AT-III. In this respect, it is interesting to note that selective hydrolysis[8] of the *N*-sulphate at unit 6, which is at the same side of and close to the

carboxylate group, leads to considerable reduction of AT-III activity; presumably, the remaining ammonium group neutralizes the required negative charge of the carboxylate function in its particular binding area.

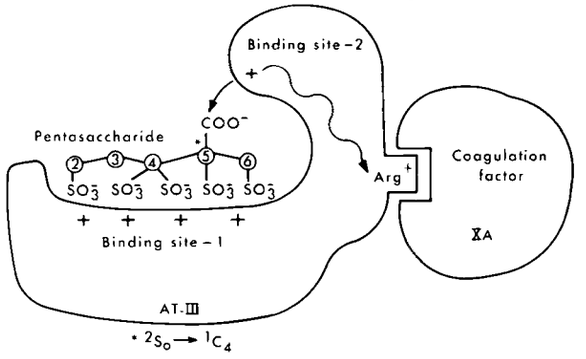


Fig. 4. Model for activation of AT-III by the high-affinity heparin pentasaccharide:
First, the three essential sulphate groups of moiety 2-3-4 bind to binding site −1 of AT-III, as do probably the 2-OSO_3^- of unit 5 and 6-OSO_3^- of unit 6. In the second step, the carboxylate group of unit 5 interacts with binding site −2 (as does probably the NSO_3^- of unit 6), causing the required conformational change in the AT-III protein.

In Fig. 4, a model is proposed for activation of AT-III upon binding of the heparin pentasaccharide. We assume that initially the crucial sulphates of trisaccharide moiety 2-3-4 interact[37] with positive charges at a first binding site of AT-III, whereupon iduronic acid adopts the $^1C_4$ conformation (Fig. 3B). Secondly, the carboxylate group of iduronic acid interacts with a second binding site at AT-III, causing a conformational change of AT-III to enhance the protease inactivating ability of the arginine residue. This model is in agreement with circular dichroism[40] and intrinsic fluorescence studies[41] as well as with kinetic studies[38,39], which reveal that binding of heparin to AT-III should be interpreted as a two-step mechanism[42,43].

Conclusion

The carboxylate function of α-L-idopyranuronate in the AT-III-binding fragment of heparin is essential for AT-III mediated anti-Xa activity. This functional group governs the conformational properties of the iduronate ring and thereby determines the overall shape of the pentasaccharide molecule. Furthermore, it is proposed that the carboxylate function interacts with AT-III during the activation process. This proposal is based on the fact that an analogue, containing L-idose[36] and displaying similar conformations as the natural fragment, does not exhibit AT-III mediated anti-Xa activity.

Experimental

General procedures

Pyridine was dried by heating with CaH_2 under reflux and then distilled. DMF was stirred with CaH_2 at room temperature and distilled at reduced pressure. Methanol was heated with magnesium and then distilled. Dichloromethane, chloroform, 1,2-dichloroethane and toluene were distilled from P_2O_5. Nitromethane was dried with $CaCl_2$. Pyridine, 1,2-dichloroethane and DMF were stored over molecular sieves 4 Å, methanol over molecular sieves 3 Å, toluene over sodium-wire and dichloromethane over alumina. Melting points are corrected and optical rotations were recorded at ambient temperature using a Perkin-Elmer 241 polarimeter. TLC analysis was performed on Merck-Fertigplatten (Kieselgel 60 F 254, 5 × 10 cm). Compounds were visualized by spraying with sulphuric acid/ethanol (1/9, v/v).
Proton NMR spectra were recorded at 360 HMz on a Bruker AM-360 spectrometer at 27°C; chemical shifts are given in ppm (δ) relative to TMS as internal reference. Suppression of the residual solvent peak (HDO) was accomplished by presaturation. Proton–proton correlation spectra (COSY) were recorded using the standard (90°-t_1-90°/45°-Acq.) pulse sequence and magnitude mode processing.
Two-dimensional NOE spectra (NOESY) were recorded at 3°C in phase-sensitive mode using a (90°-t_1-90°-τ-90°-Acq.) sequence and the Time-Proportional Phase Increment (TPPI) method[45]. A mixing time of 0.3 sec was chosen, which was randomly varied (± 10%) during the experiment to suppress scalar coupling correlations. The spectrum was processed to pure absorption mode using Gaussian and sine-square window functions in f_2 and f_1, respectively. One-dimensional NOE-difference spectra were obtained by irradiation of resonances at a decoupler setting 40 dB below 0.2 Watt for 0.8 sec. Averaging took place in blocks of 16 scans. On- and off-resonance free-induction decays (FIDs) were subtracted and the resulting difference spectrum was processed using exponential window functions.
Computer simulations of the proton spectra were carried out using a LAOCOON-type computer programme (PANIC.84) running on the Aspect-3000 computer of the Bruker AM-360 spectrometer. Final parameters were obtained by iterative calculation.
Coupling constants of the molecular models of xylopyranose, as collected in Table II, were calculated using the computer programme CAGPLUS[44] (written in PASCAL) running on an Aspect 2000 computer.
Molecular modelling was performed on a SIGMA 5688 graphics terminal by means of the computer programme CHEM-X (Chemical Design Ltd., Oxford, U.K.) implemented on a VAX-780 computer.

3-O-Benzyl-α-D-xylopyranosyl fluoride (**2**)

A solution of 1,2,4-tri-*O*-acetyl-3-*O*-benzyl-D-xylopyranose (**1**) (4 g, 11 mmole) in dry dichloromethane (16 ml) was stirred at 0°C in a polyethylene vessel. To this solution was added (under nitrogen) 10 ml of a solution of 70% HF in pyridine (Merck). After 1 h, the mixture was poured into ice-water (300 ml), containing $NaHCO_3$ (45 g). After extraction with dichloromethane and evaporation of the solvent, pure 2,4-di-*O*-acetyl-3-*O*-benzyl-α-D-xylopyranosyl fluoride could be obtained directly by crystallization from isopropanol (yield 55%). On the other hand, after chromatography on silica gel (toluene/ethyl acetate, 93/7, v/v), the yield could be increased to 91% (3.26 g). M.p. 48°C; $[\alpha]_D^{20}$ +40.4° (*c* 1, $CHCl_3$); R_f 0.63 (toluene, ethyl acetate, 8/2, v/v).
Saponification of the latter compound (3.26 g, 10 mmole) was performed in dry methanol (70 ml) at −20°C in the presence of K_2CO_3 (830 mg). After stirring for 2 h, the reaction mixture was filtered, evaporated and the residue dissolved in dichloromethane (150 ml) and washed with brine. Evaporation of the organic layer gave crystalline **2** (1.86 g, 77%). M.p. 136°C (decompose); R_f 0.28 (dichloromethane/acetone, 95/5, v/v); $[\alpha]_D^{20}$ +48.2 (*c* 1, CH_3OH). ^1H NMR (CD_3OD): 5.65 (d, 1H, $J_{1,2}$ 3.2 Hz, $J_{1,F}$ 54 Hz).

Methyl 4-O-(2-O-acetyl-3-O-benzyl-4-O-levulinoyl-β-D-xylopyranosyl)-6-O-acetyl-3-O-benzyl-2-(benzyloxycarbonylamino)-2-deoxy-α-D-glucopyranoside (**6a**)

First, crude **4a** was prepared from compound **2**. Thus, compound **2** (1.8 g, 7.4 mmole) was dissolved in 1,2-dichloroethane (80 ml) containing 1-acetylimidazole (992 mg, 9.0 mmole). The reaction mixture was stirred for 7 days at 40°C, then washed with $NaHCO_3$ (saturated) and chromatographed on silica gel (toluene/ethyl acetate, 8/2 v/v). Crude compound **3a** (1.05 g, 3.7 mmole) was dissolved in pyridine (4 ml). Levulinoic anhydride in ether (21 ml, 0.25 M) was added, together with a catalytic amount of 4-(dimethylamino)pyridine, whereupon the mixture was stirred for 5 h at 0°C, after which time some water (1 ml) was added. The reaction mixture was diluted with dichloromethane (100 ml) and washed with a saturated solution of $NaHCO_3$ and brine. The organic layer was evaporated and the residue chromatographed on a small column of silica gel (10 g). Elution with dichloro-

methane/acetone (98/2, v/v) afforded crude **4a** (1.27 g, 90%). R_f 0.58 (dichloromethane/acetone, 95/5, v/v).
Crude compound **4a** (900 mg, 2.36 mmole) and aglycon **5** (1.08 g, 2.36 mmole) were dissolved in dry dichloromethane (30 ml). Molecular sieves (4 Å, 2.5 g) were added and the mixture stirred under nitrogen at −25°C. To this mixture was added dropwise boron trifluoride etherate (0.5 ml) in dry dichloromethane (5 ml). After 18 h at −25°C, TLC analysis (dichloromethane/acetone, 9/1, v/v) revealed complete disappearance of fluoride **4a** (R_f 0.82) and formation of the coupling product **6a** (R_f 0.40), together with some aglycon **5** (R_f 0.44). The reaction mixture was then washed with $NaHCO_3$ solution and brine. After evaporation of the organic solvent, the crude residue was dissolved in a mixture of pyridine (18 ml) and acetic anhydride (6 ml), containing a catalytic amount of 4-(dimethylamino)pyridine. After 1 h at room temperature, TLC analysis showed acetylation of the remaining aglycon **5** to be complete. After evaporation of the solvents and co-evaporation with toluene (3 × 50 ml), the crude mixture was chromatographed on silica gel (60 g). Elution with dichloromethane/acetone (95/5 → 90/10, v/v), evaporation of the appropriate fractions and crystallization from acetone/hexane afforded pure compound **6a** (775 mg, 40%). M.p. 157–159°C; R_f 0.40 (dichloromethane/acetone, 9/1, v/v); $[\alpha]_D^{20}$ +44.8° (*c* 1, $CHCl_3$).
^{1}H NMR ($CDCl_3$). Unit 5: 4.48 (d, 1H, $J_{1,2}$ 6.8 Hz, H-1); 4.99 (dd, 1H, $J_{2,3}$ 8.8 Hz, H-2); 3.63 (t, 1H, $J_{3,4}$ 8.8 Hz, H-3); 4.92 (ddd, 1H, $J_{4,5\,eq}$ 5.0 Hz, H-4); 4.02 (dd, 1H, J_{gem} 12.0 Hz, H-5_{eq}); 3.10 (dd, 1H, H-5_{ax}).
Unit 6: 4.64 (d, 1H, $J_{1,2}$ 3.7 Hz); 3.95 (dt, 1H, $J_{2,3}$ 10.5 Hz, $J_{NH,2}$ 10.5 Hz, H-2); 3.55 (dd, 1H, $J_{3,4}$ 8.4 Hz, H-3); 3.7–3.8 (c, 2H, H-4, H-5); 4.39 (dd, 1H, $J_{5,6a}$ 2 Hz, J_{gem} 12.0 Hz, H-6a); 4.15 (dd, 1H, $J_{5,6b}$ 4.5 Hz, H-6b); 3.32 (s, 3H, α-OCH_3).

Methyl 4-O-(2-O-acetyl-3-O-benzyl-β-D-xylopyranosyl)-6-O-acetyl-3-O-benzyl-2-(benzyloxycarbonylamino)-2-deoxy-α-D-glucopyranoside (**6b**)

To a solution of **6a** (730 mg, 0.9 mmole) in pyridine (10 ml) was added 10 ml of pyridine/acetic acid/hydrazine hydrate (6/4/0.5 v/v). The mixture was then stirred for 6 min at room temperature. Dichloromethane (100 ml) was added and the mixture washed with water, $NaHCO_3$ solution and brine. The organic layer was evaporated and applied to a column of silica gel (15 g). The product was eluted from the column with dichloromethane/acetone (95/5 → 90/10, v/v) to give **6b**, which crystallized from dichloromethane/ether (499 mg, 75%). M.p. 154–156.5°C; R_f 0.32 (dichloromethane/acetone, 9/1, v/v); $[\alpha]_D^{20}$ +54.4° (*c* 0.72, $CHCl_3$).
^{1}H NMR ($CDCl_3$). Unit 5: 4.42 (d, 1H, $J_{1,2}$ 6.8 Hz, H-1); 4.95 (dd, 1H, $J_{2,3}$ 8.8 Hz, H-2); 3.43 (t, 1H, $J_{3,4}$ 8.8 Hz, H-3); 3.7–3.8 (c, H-4); 3.97 (dd, 1H, $J_{4,5\,eq}$ 5.0 Hz, J_{gem} 12.0 Hz, H-5_{eq}); 3.10 (dd, 1H, $J_{4,5\,ax}$ 8.4 Hz, H-5_{ax})
Unit 6: 4.64 (d, 1H, $J_{1,2}$ 3.7 Hz); 3.31 (s, 3H, α-OCH_3).

2-O-Benzoyl-3-O-benzyl-α-D-xylopyranosyl fluoride (**3b**)

A solution of benzoyl chloride (653 mg, 4.6 mmole) in 2.7 ml anhydrous pyridine was added dropwise at −25°C to a solution of compound **2** (880 mg, 3.6 mmole) in pyridine (100 ml) and stirred for 3 h. The mixture was diluted with dichloromethane and washed with saturated aqueous Na_2CO_3 and brine. The organic layer was then dried ($MgSO_4$) and concentrated to dryness. The residue was eluted from a column of silica gel (50 g, dichloromethane/acetone; 98/2 → 93/7, v/v), after which the product was crystallized from hexane/acetone to give compound **3b** (660 mg, 53%). M.p. 91–93°C; R_f 0.5 (dichloromethane/acetone, 95/5, v/v); $[\alpha]_D^{20}$ +104.8° (*c* 0.17, CH_2Cl_2).
^{1}H NMR ($CDCl_3$): 5.73 (dd, 1H, $J_{1,2}$ 2.8 Hz, $J_{1,F}$ 53.7 Hz, H-1); 5.07 (ddd, 1H, $J_{2,3}$ 9.6 Hz, $J_{2,F}$ 4.4 Hz, H-2).

2-O-Benzoyl-3-O-benzyl-4-O-levulinoyl-α-D-xylopyranosyl fluoride (**4b**)

A solution of levulinoic anhydride (1038 mg, 4.85 mmole) in anhydrous ether (19.4 ml) was added dropwise at 0°C to a solution of compound **3b** (960 mg, 2.77 mmole) in pyridine (4 ml). After 24 h at 0°C, water (15 ml) was added to the reaction mixture which was stirred for 15 min at room temperature. The reaction mixture was then diluted with dichloromethane, washed with aqueous saturated $NaHCO_3$ and brine and the organic layer was dried ($MgSO_4$) and evaporated to dryness. The residue was eluted from a silica gel column (50 g, dichloromethane/acetone, 97/3; v/v) after which the product was crystallized from ether/hexane to give compound **4b** (1.13 g, 92%). M.p. 86–88°C; R_f 0.60 (dichloromethane/acetone, 95/5, v/v); $[\alpha]_D^{20}$ +147.3° (*c* 0.22, CH_2Cl_2).
^{1}H NMR ($CDCl_3$): 5.75 (dd, 1H, $J_{1,2}$ 2.6 Hz, $J_{1,F}$ 54.0 Hz, H-1); 5.09 (ddd, 1H, $J_{2,3}$ 10.0 Hz, $J_{2,F}$ 4.0 Hz, H-2); 4.11 (dd, 1H, $J_{3,4}$ 10.0 Hz, H-3); 5.03 (ddd, 1H, H-4); 3.96 (dd, 1H, $J_{4,5\,eq}$ 6.0 Hz, J_{gem} 11.8 Hz, H-5_{eq}); 3.69 (dd, 1H, $J_{4,5\,ax}$ 11.8 Hz, H-5_{ax}).

Methyl 4-O-(2-O-benzoyl-3-O-benzyl-β-D-xylopyranosyl)-3-O-benzyl-2-(benzyloxycarbonylamino)-2-deoxy-α-D-glucopyranoside (**6c**)

A mixture of compound **4b** (154.5 mg, 0.35 mmole), compound **5** (160.7 mg, 0.35 mmole) and molecular sieves (4 Å, 400 mg) in dichloromethane (5 ml) was stirred at −25°C and a solution of BF_3 etherate (0.7 mmole) in dichloromethane (0.7 ml) was added dropwise to the reaction mixture. After 36 h, the reaction mixture was diluted with dichloromethane, washed with saturated aqueous $NaHCO_3$ and brine and the organic layer dried ($MgSO_4$) and evaporated to dryness. The residue was purified by chromatography on silica gel (10 g, dichloromethane/acetone, 95/5, v/v) which afforded the required disaccharide (147 mg, 48%). R_f 0.44 (dichloromethane/acetone, 9/1, v/v). The levulinoyl protective group was removed under similar conditions as described for the preparation of 2′-*O*-acetyl-derivative **6b**. The crude product was purified by silica gel chromatography (dichloromethane/acetone, 95/5 → 9/1, v/v) to give compound **6c** (110 mg, 88%). R_f 0.28 (dichloromethane/acetone, 9/1, v/v); $[\alpha]_D^{20}$ +40.0° (*c* 0.39, CH_2Cl_2).
^{1}H NMR ($CDCl_3$). Unit 5: 4.58 (d, 1H, $J_{1,2}$ 6.9 Hz, H-1); 5.25 (dd, 1H, $J_{2,3}$ 8.9 Hz, H-2); 3.98 (dd, 1H, $J_{4,5\,eq}$ 5.0 Hz, J_{gem} 12.0 Hz, H-5_{eq}); 3.17 (dd, 1H, $J_{4,5\,ax}$ 9.0 Hz, H-5_{ax}).
Unit 6: 3.60 (d, 1H, $J_{1,2}$ 3.8 Hz, H-1); 3.24 (s, 3H, −OCH_3).

Tetrasaccharide **8b**

A mixture of compound **6b** (440 mg, 0.60 mmole), activated molecular sieves (4 Å, 4.0 g), 2,6-di-*tert*-butyl-pyridine (190 mg, 1 mmole) and $AgClO_4$ (308 mg, 1.48 mmole) in dichloromethane (12 ml) was stirred at 0°C. At this temperature, a solution of compound **7** (680 mg, 0.84 mmole) in dichloromethane (8 ml) was added dropwise to the reaction mixture. After 24 h at 0°C, the mixture was diluted with dichloromethane, washed with aqueous $NaHCO_3$ and brine and the organic layer dried ($MgSO_4$) and evaporated to dryness. The crude product was purified by chromatography on silica gel (10 g) (dichloromethane/acetone, 97/3 → 93/7, v/v) to give compound **8a** (22%). The levulinoyl group was removed from **8a** by treatment with hydrazine acetate in pyridine as described for the preparation of **6b**. After purification on silica gel (dichloromethane/acetone, 95/5 → 9/1. v/v), compound **8b** (144 mg, 80%) was isolated. R_f 0.70 (dichloromethane/acetone, 9/1, v/v); $[\alpha]_D^{20}$ +57.4° (*c* 0.34, CH_2Cl_2).
^{1}H NMR ($CDCl_3$). Unit 3: 4.31 (d, 1H, $J_{1,2}$ 7.9 Hz, H-1); 3.79 (s, 3H, −$COOCH_3$).
Unit 4: 5.14 (d, 1H, $J_{1,2}$ 3.6 Hz, H-1); 5.43 (dd, 1H, $J_{2,3}$ 10.5 Hz, $J_{3,4}$ 9.0 Hz, H-3).
Unit 5: 4.43 (d, 1H, $J_{1,2}$ 7.5 Hz, H-1); 4.98 (dd, 1H, $J_{2,3}$ 9.0 Hz, H-2); 3.64 (t, 1H, $J_{3,4}$ 8.2 Hz, H-3); 3.22 (dd, 1H, $J_{4,5}$ 9.0 Hz, J_{gem} 12.0 Hz, H-5_{ax}).
Unit 6: 4.65 (d, 1H, $J_{1,2}$ 3.7 Hz, H-1); 3.31 (s, 3H, −OCH_3).

Pentasaccharide **10a**

A mixture of aglycon **8b** (190 mg, 0.14 mmole), silver triflate (215 mg, 0.84 mmole), activated molecular sieves (4 Å, 450 mg) and 2,6-di-*tert*-butylpyridine (0.190 mg, 1 mmole) in dichloromethane (3 ml) was stirred at −55°C under nitrogen. Glycopyranosyl bromide **9** (199 mg, 0.41 mmole), dissolved in dichloromethane (2 ml), was added dropwise over 1 h. The reaction mixture was stirred for 2 h at −55°C, then diluted with 20 ml dichloromethane and filtered over Hyflo. The organic solution was washed with $NaHCO_3$ solution and brine, dried ($MgSO_4$) and evaporated to dryness. The crude reaction product was purified by chromatography on silica gel (25 g) (dichloromethane/acetone, 97/3 → 93/7, v/v) to give pure pentamer **10a** (150 mg, 60%). R_f 0.65 (dichloromethane/acetone, 93/7, v/v); $[\alpha]_D^{20}$ +73.7° (*c* 0.3, CH_2Cl_2).

^{1}H NMR ($CDCl_3$). Unit 2: 5.50 (d, 1H, $J_{1,2}$ 3.6 Hz, H-1); 3.27 (dd, 1H, $J_{2,3}$ 10.4 Hz, H-2).
Unit 3: 4.32 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1); 3.43 (dd, 1H, $J_{2,3}$ 9.2 Hz, H-2); 3.85 (d, 1H, $J_{4,5}$ 10.0 Hz, H-4); 3.75 (s, 3H, $COOCH_3$).
Unit 4: 5.12 (d, 1H, $J_{1,2}$ 3.6 Hz, H-1); 3.32 (dd, 1H, $J_{2,3}$ 10.5 Hz, H-2); 5.41 (dd, 1H, $J_{3,4}$ 9.0 Hz, H-3).
Unit 5: 4.42 (d, 1H, $J_{1,2}$ 7.2 Hz, H-1); 4.97 (dd, 1H, $J_{2,3}$ 9.0 Hz, H-2).
Unit 6: 4.62 (d, 1H, $J_{1,2}$ 3.7 Hz, H-1); 3.31 (s, 3H, α-OCH_3).
^{13}C NMR ($CDCl_3$), unit 2: 97.56 (C-1); unit 3: 103.24 (C-1); unit 4: 98.72 (C-1); unit 5: 101.17 (C-1); unit 6: 98.72 (C-1).

Pentasaccharide **10c**

To a mixture of chloroform (4.5 ml), methanol (27 ml) and aqueous sodium hydroxide solution (4.5 ml, 4N) was added dropwise at room temperature a solution of compound **10a** (145 mg, 0.082 mmole) in chloroform (4.5 ml). The reaction mixture was stirred for 16 h and acidified at 0°C to pH 1.5 with aqueous hydrochloric acid (6N). The reaction mixture was extracted with dichloromethane (5 × 30 ml) and the combined organic layers were washed with ice-water (25 ml), dried ($MgSO_4$) and evaporated to dryness to give compound **10b** (134 mg, quantitative yield). R_f 0.6 (dichloromethane/methanol; 9/1, v/v).
A solution of compound **10b** (134 mg, 0.082 mmole) in *N,N*-dimethylformamide (30 ml) was stirred for 16 h at 50°C in the presence of sulphur trioxide/trimethylamine complex (420 mg, 3.0 mmole). The mixture was cooled and $NaHCO_3$ (700 mg, 8.3 mmole) in water (1 ml) was added, whereupon the mixture was stirred for a further 20 min at room temperature. After evaporation to dryness, the residue was eluted from a column of silica gel (5 g, dichloromethane/methanol, 95/5 → 8/2, v/v) to yield a pure fraction of compound **10c** and a fraction, which was contaminated with lower sulphated products. This impure material was dissolved in *N,N*-dimethylformamide (20 ml) and stirred in the presence of sulphur trioxide/trimethylamine complex (420 mg, 3.0 mmole) for 16 h at 50°C. The reaction mixture was cooled and worked up as described above. The crude material was eluted from a column of silica gel (5 g, dichloromethane/methanol, 95/5 → 8/2, v/v) to give a pure second batch of pentamer **10c** (total yield 158 mg, 90%). R_f 0.13 (ethyl acetate/pyridine/acetic acid/water, 11/7/1.6/4, v/v).

Pentasaccharide **11**

A solution of compound **10c** (137 mg, 0.065 mmole) in a mixture of water (5 ml) and methanol (15 ml) was kept under hydrogen in the presence of 10% Pd/C (95 mg) for 20 h. It was then filtered under nitrogen and concentrated. The residue was dissolved in water (20 ml) and hydrogenated again for two days in the presence of fresh 10% Pd/C (95 mg). The suspension was filtered and concentrated.
The crude product, dissolved in water (10 ml), was stirred for two days at room temperature in the presence of sulphur trioxide/trimethylamine complex (100 mg, 0.72 mmole) and Na_2CO_3 (100 mg, 0.94 mmole). A second and a third portion of sulphur trioxide/trimethylamine complex and Na_2CO_3 were added on the third and the fifth day, respectively. After a further two days, the reaction mixture was neutralized to pH 8, concentrated to 5 ml, applied on a column of Sephadex G 10 and eluted with water. The isolated product was then eluted from a column of Dowex 50 WX (Na^+ form) with water to give pure compound **11** (61 mg, 56%). $[\alpha]_D^{20}$ + 36.2 (*c* 0.95, H_2O).
^{1}H NMR (D_2O). Unit 2: 5.60 (d, 1H, $J_{1,2}$ 3.9 Hz, H-1); 3.22 (dd, 1H, $J_{2,3}$ 10.0 Hz, H-2); 3.58 (dd, 1H, $J_{3,4}$ 9.5 Hz, H-3); 3.55 (dd, 1H, $J_{4,5}$ 9.0 Hz, H-4); 3.86 (c, 1H, $J_{5,6a}$, $J_{5,6b}$ 2–3 Hz, H-5); 4.12 (dd, 1H, J_{gem} 11.0 Hz, H-6a); 4.34 (dd, 1H, H-6b).
Unit 3: 4.60 (d, 1H, $J_{1,2}$ 7.8 Hz, H-1); 3.38 (c, 1H, H-2); 3.81 (c, 1H, H-3); 3.75 (c, 2H, H-4, H-5).
Unit 4: 5.38 (d, 1H, $J_{1,2}$ 3.8 Hz, H-1); 3.43 (dd, 1H, $J_{2,3}$ 10.5 Hz, H-2); 4.42 (dd, 1H, $J_{3,4}$ 8.5 Hz, H-3); 3.98 (dd, 1H, $J_{4,5}$ 9.0 Hz, H-4); 4.04 (c, 1H, $J_{5,6a}$, $J_{5,6b}$ 1.5–3.5 Hz, H-5); 4.30 (dd, 1H, J_{gem} 11.0 Hz, H-6a); 4.40 (dd, 1H, H-6b).
Unit 5: 4.64 (d, 1H, $J_{1,2}$ 7.80 Hz, H-1); 4.06 (dd, 1H, $J_{2,3}$ 9.26 Hz, H-2); 3.84 (dd, 1H, $J_{3,4}$ 8.79 Hz, H-3); 3.75 (ddd, 1H, $J_{4,5\,eq}$ 4.90 Hz, $J_{4,5\,ax}$ 9.71 Hz, H-4); 4.13 (dd, 1H, J_{gem} 11.6 Hz, H-5_{eq}); 3.45 (dd, 1H, H-5_{ax}).
Unit 6: 5.00 (d, 1H, $J_{1,2}$ 3.9 Hz, H-1); 3.26 (dd, 1H, $J_{2,3}$ 10.5 Hz, H-2); 3.66 (dd, 1H, $J_{3,4}$ 9.5 Hz, H-3); 3.72 (dd, 1H, $J_{4,5}$ 9 Hz, H-4); 3.93 (c, 1H, $J_{5,6a}$, $J_{5,6b}$ 2–3 Hz, H-5); 4.23 (dd, 1H, J_{gem} 11.0 Hz, H-6a); 4.56 (dd, 1H, H-6b).

References and Notes

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