Pergamon

Bioorganic & Medicinal Chemistry 6 (1998) 1509–1516

BIOORGANIC & MEDICINAL CHEMISTRY

Introducing a *C*-Interglycosidic Bond in a Biologically Active Pentasaccharide Hardly Affects its Biological Properties

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Received 18 December 1997; accepted 4 March 1998

Abstract—We describe here the synthesis and the biological activity of a '*C*-pentasaccharide', a new analogue of the antithrombin III (AT III) binding region of heparin containing a methylene bridge in place of an interglycosidic oxygen atom. The affinity for AT III and the anti-factor Xa activity of this compound have been compared with that of the corresponding selected '*O*-pentasaccharide'. Such a structural modification slightly decreased the affinity of this compound for AT III as well as its anti-factor Xa activity (880 ± 40 anti-Xa units versus 1180 ± 30 anti-Xa units for the C-pentasaccharide and the *O*-pentasaccharide, respectively). This compound therefore represents the first example of a new class of anti-factor Xa pentasaccharides containing a *C*-interglycosidic bond.

Introduction

Recent studies have demonstrated that oligosaccharides are involved in a number of biological processes[1] and more and more constitute potential active substances for drug development.[2] One problem in this context is the susceptibility of native structure to glycosidases. Several structural modifications have thus been developed to increase the resistance of oligosaccharides to enzymatic hydrolysis: introduction of *S*-interglycosidic bonds,[3] alkylation[4] and acylation[5] of noncritical hydroxyl functions. Various methods have also been reported for the introduction of *C*-interglycosidic bonds, resulting in *C*-disaccharides.[6] A critical feature is that the biological activity must be present and if possible increased in the analogue thus obtained, which requires the active conformation being preserved. The underlying basic question is indeed to evaluate to what extend the replacement of the oxygen atom by a methylene group—which eradicates the *exo* anomeric effect—affects the conformational properties of the molecule[7] resulting in enhanced or impaired biological activity. To document this problem, the comparison of biologically active compounds only differing by the presence of '*C*' versus '*O*' interglycosidic bonds is therefore highly desirable. Among the very restricted number of oligosaccharides clinically investigated is the pentasaccharide that reproduces the exact sequence required in heparin for binding and activation of antithrombin III (AT III).[8] Several analogues of this compound have been obtained which are not susceptible to enzymatic degradation.[9] However, having such a series of biologically active pentasaccharides at our disposal, we decided to investigate how a *C*-disaccharide bond in one of them would influence the interaction with the target protein 'receptor' antithrombin III.[10]

Key words: Antithrombotics; heparin analogue; oligosaccharide; *C*-pentasaccharide; antithrombin III.
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Results and Discussion

Total synthesis

We selected **14** (Fig. 1) as the target pentasaccharide because its structure was well adapted to the type of chemistry we had previously developed for the synthesis

0968-0896/98/$—see front matter
PII: S0968-0896(98)00094-7

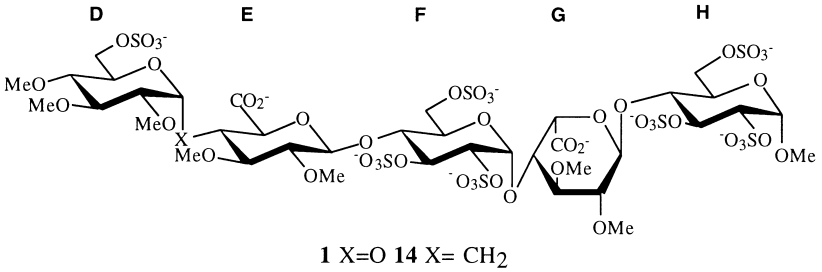


Figure 1. Structure of the synthetic *O*- and *C*-pentasaccharides.

of the *C*-disaccharide building block precursor of the DE part of the molecule.[11] The preparation of the corresponding '*O*-pentasaccharide' **1** has been described, and its biological properties thoroughly investigated.[12]

The precursor of the unit F is the alcohol **5**, which is synthesized according to Scheme 1. The epoxide **2**[13] was opened in a trans diaxial manner with sodium benzylate to give **3** which, after *O*-acetylation to **4**, was hydrolysed to provide the crystalline compound **5**.

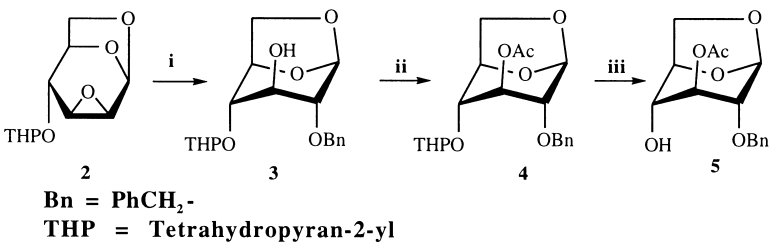
Compound **5** was then glycosylated with the previously described[10,11] derivative **6** to give the trisaccharide **7** (Scheme 2). The reaction was carried out in acetonitrile to take advantage of the 'nitrilium effect'.[14] We thus obtained in excellent yield (88%) a mixture containing **7** and its α-anomer **8** (β:α ratio 5.5:1).

Acetolysis of the 1,6-anhydro ring[15] (Scheme 3) quantitatively gave the expected α,β-acetates **9** (α:β 3:1) which after selective removal of the anomeric *O*-acetyl group by hydrazine acetate in DMF[16] provided the hemiacetal **10** (90%, α:β 1:1). The latter was converted into the imidate **11** using trichloroacetonitrile and DBU[17] in dichloromethane (94%, α:β 5:1). This trisaccharide **11** was then coupled to the alcohol **12**[18] to give the fully protected pentasaccharide **13** in 80% yield from **11** (two equivalents of **12** were used, and we did not try to recover the excess). This previously reported 3 + 2 strategy of coupling[19] allowed us to take advantage of the stereoselective α-coupling observed during glycosylation at position 4 of L-iduronic acid derivatives; only the α-anomer was formed during the reaction. Conversion of **13** into **14** was achieved through hydrogenation, saponification, and sulfation (65% overall yield for the three steps).

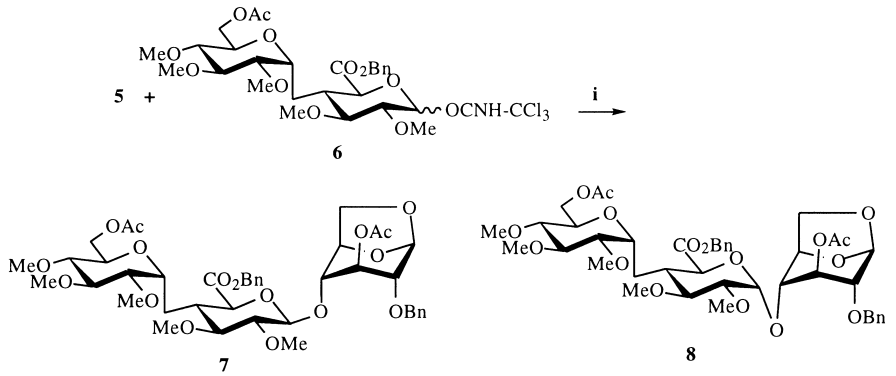
Biological properties in vitro

Affinity for antithrombin III. AT III is the plasma 'receptor' of the synthetic pentasaccharides. It is therefore of prime importance to assess the influence of the structural modifications described above on the binding affinity for AT III. Interaction of the pentasaccharides with AT III induces a change in fluorescence of AT III which allows titration and determination of the affinity of the different ligands.[20] We used this method to determine the affinities of **1** and **14** for AT III (Table 1). The substitution of a *O*-glycosidic bond by a *C*-glycosidic bond resulted in a slight decrease in the affinity for AT III ($Kd = 1.9 \pm 0.1$ versus 2.8 ± 0.2 nM for **1** and **14**, respectively).

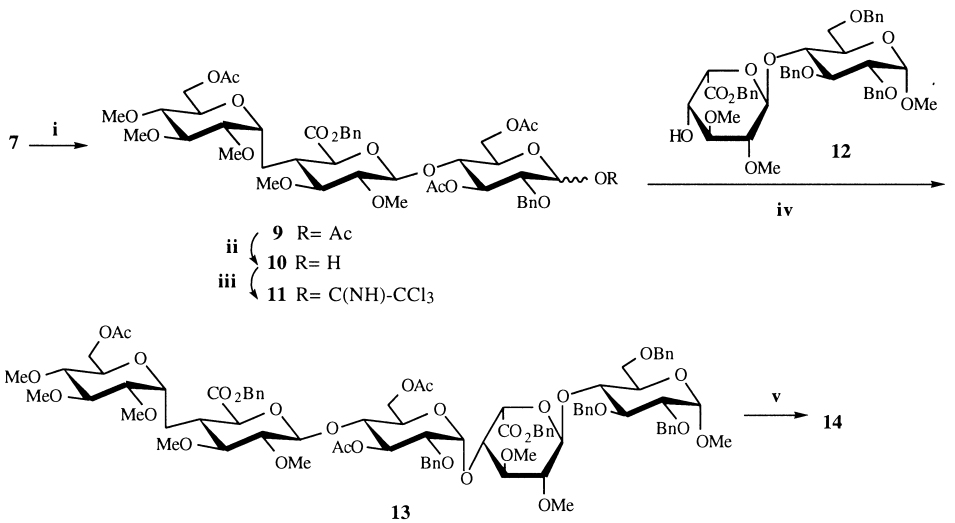
Inhibition of factor Xa. Binding of the pentasaccharides to AT III induces a conformational change of the protein[20] which results in an accelerated inhibition of blood coagulation factor Xa by AT III. The anti-factor Xa activity was determined by an amidolytic method adapted from Teien and Lie.[21] Since the amount of AT III-pentasaccharide complex depends on the affinity of the oligosaccharide for AT III, the anti-Xa activity of **1** and **14** followed the same ranking as for their affinity for AT III (Table 1).



Scheme 1. Synthesis of the alcohol **5**. Reagents and conditions: (i) BnONa, BnOH, 110 °C, 64%; (ii) Ac_2O, Et_3N, DMAP, CH_2Cl_2, 20 °C; (iii) PTSA, MeOH, 20 °C, 87% form **3**.



Scheme 2. Synthesis of the *C*-trisaccharide **8**. Reagent and conditions: TMSOTf, CH_3CN, −37 °C, 88% **7**:**8** (5.5:1).



Scheme 3. Synthesis of the *C*-pentasaccharide **14**. Reagents and conditions: (i) H_2SO_4, AcOH, Ac_2O), −20 °C, 100%; (ii) NH_3NH_2OAc, DMF, 90%; (iii) CCl_3CN, DBU, CH_2Cl_2, 94%; (iv) TMSOTf, CH_2Cl_2, −20 °C, 79%; (v) 1. H_2, Pd/C, CH_2Cl_2, MeOH, 99%, 2. NaOH, H_2O, MeOH, 0 °C, 77%, 3. SO_3. Et_3N, DMF, 55 °C, 85%.

Conclusion

We conclude from the results of factor Xa inhibition that substitution of an *O*-glycosidic by a *C*-glycosidic bond does not change the ability to induce the conformational change in AT III resulting in active site loop exposure and trapping of the target serine protease factor Xa. Since we have recently shown[22] that the DEF trisaccharide part of the pentasaccharide molecules is responsible for the recognition of AT III, the active conformation is preserved in the *C*-pentasaccharide. The lower affinity of the '*C*-glycoside' may be due to the increased flexibility of the *C*-pentasaccharide leading to a higher cost in conformational entropy when it binds to its receptor protein.

Table 1. Biological properties of the pentasaccharides **1** and **14** in vitro

Compd	Affinity for AT III (*K*d in nM)	Anti-factor Xa activity (U/mg)
1 (*O*-glycoside)	1.9 ± 0.1	1180 ± 30
14 (*C*-glycoside)	2.8 ± 0.2	880 ± 40

Values are arithmetic means ± SD ($n = 6$).

Experimental

General

All solvents and reagents were of the best commercially available grade or were purified and dried according to standard procedures. Reactions were monitored by TLC

on silica gel 60 F_{254} (Merck) with detection by charring with H_2SO_4. Column chromatography was performed on silica gel 60 (E. Merck 63–200 μm). NMR spectra were recorded with Bruker AM 100, AC 250, AM 400 or AM 500 instruments for solution in $CDCl_3$ (internal Me_4Si) unless otherwise stated. Protons H-4ha and H-4hb correspond respectively to the low field and high field hydrogen of the methylene linkage. MS analyses were performed on Nermag R10-10 instrument using chemical ionization (NH_3) and detection of positive ions. Melting points were determined in capillary tubes with a Büchi 510 apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter at 23 ± 3 °C. Elemental analyses were carried out at the Service Central d'Analyses (C.N.R.S., Vernaison, France).

1,6-Anhydro-2-*O*-benzyl-4-*O*-(tetrahydropyran-2-yl)-β-D-glucopyranose (3). A mixture of 1,6:2,3-dianhydro-4-*O*-(tetrahydropyran-2-yl)-β-D-mannopyranose **2** (6.16 g, 27.05 mmol) and a solution of sodium benzylate in benzyl alcohol (1 M, 135 mL) was heated at 110 °C for 0.5 h. The cooled mixture was neutralized (Dowex 50 H^+) filtered, and concentrated. The residue was purified by flash column chromatography (CH_2Cl_2/AcOEt 10:1 to 2:1) to give **3** as a white solid. (6.09 g, 64%). Isomer 1: $[\alpha]_D$ −69° (*c* 1.00; CH_2Cl_2); ^1H NMR: δ 7.42–7.25 (m, 5 H, Ph), 5.42 (s, 1 H, H-1), 4.52 (d, 1 H, *J* = 5 Hz, H-5), 3.42 (dd, 1 H, *J* = 5,6 Hz, H-4), 3.26 (d, 1 H, *J* = 5.9 Hz, H-2). Isomer 2: melting point 121 °C; $[\alpha]_D$ +29° (*c* 0.97; CH_2Cl_2); ^1H NMR δ 7.39–7.28 (m, 5H, Ph), 5.44 (s, 1H, H-1), 3.55 (dd, 1 H, *J* = 1.3 Hz and 5.6 Hz, H-4), 3.26 (d, 1 H, *J* = 4.5 Hz, H-2). MS, FAB, positive mode: *m*/*z* thioglycerol + NaCl, 359 $(M+Na)^+$; thioglycerol + KF, 375 $(M+K)^+$; Anal. calcd for $C_{18}H_{24}O_6$ (336.37): C, 64.27 ; H, 7.19. Found: C, 64.28 ; H, 7.34.

3-*O*-Acetyl-1,6-anhydro-2-*O*-benzyl-4-*O*-(tetrahydropyran-2-yl)-β-D-glucopyranose (4). A solution of **3** (2 g, 6 mmol), triethylamine (5 mL, 35.6 mmol), 4-dimethylaminopyridine (0.15 g, 1.2 mmol) and acetic anhydride (2.8 mL, 29.8 mmol) in dichloromethane (20 mL) was stirred overnight at room temperature, diluted with dichloromethane, washed with 10% aq $KHSO_4$, water then satd aq $NaHCO_3$ and water, dried ($MgSO_4$) and concentrated. The compound was used as such in the next step.

3-*O*-Acetyl-1,6-anhydro-2-*O*-benzyl-β-D-glucopyranose (5). A solution of crude **4** in a 0.25 M solution of *p*-toluenesulfonic acid in methanol (24 mL) was stirred for 1 h at room temperature, diluted with dichloromethane, washed with water, dried ($MgSO_4$), and concentrated. The residue was chromatographed on silica gel (cyclohexane/AcOEt 1:1) to give **5** (1.53 g, 87% two steps). $[\alpha]_D$ −82° (*c* 1.00; CH_2Cl_2), mp 79 °C; ^1H NMR: δ 7.36–7.26 (m, 5H, Ph), 5.36 (br. s, 1H, H-1), 4.93 (br.s, 1H, H-3), 4.10 (dd, 1H, *J* = 7.5Hz, H-6), 3.76 (dd, 1H, *J* = 5.7 Hz, 7.5 Hz, H-6′), 3.58 (br. m, 1H, H-4), 3.26 (br. m, 1H, H-2), 2.08 (s, 3H, Ac); MS, FAB, positive mode: *m*/*z* thioglycerol + NaCl, 317 $(M+Na)^+$; thioglycerol + KF, 333 $(M+K)^+$; Anal. calcd for $C_{15}H_{18}O_6$ (294.29): C, 61.21 ; H, 6.16. Found : C, 61.33 ; H, 6.23.

***O*-(6-*O*-Acetyl-2,3,4-tri-*O*-methyl-α-D-glucopyranosylmethyl)-(1→4)-C-(benzyl 4-deoxy-2,3-di-*O*-methyl-β-D-glucopyranosyluronate)-(1→4)-3-*O*-acetyl-1,6-anhydro-2-*O*-benzyl-β-D-glucopyranose (7) and *O*-(6-*O*-Acetyl-2,3,4-tri-*O*-methyl-α-D-glucopyranosylmethyl)-(1→4)-*C*-(benzyl 4-deoxy-2,3-di-*O*-methyl-α-D-glucopyranosyluronate)-(1→4)-3-*O*-acetyl-1,6-anhydro-2-*O*-benzyl-β-D-glucopyranose (8).** A 0.05 M solution of TMSOTf in CH_3CN (250 μL) was slowly added to a cooled solution of **6** (179 mg, 255 mmol) and **5** (84 mg, 0.285 mmol) in acetonitrile under argon at −37 °C. After 1.5 h solid $KHCO_3$ (200 mg) was introduced and the temperature was allowed to rise to room temperature. After filtration and concentration, column chromatography of the residue (CH_2Cl_2:EtOAc:acetone, 20:1:2) gave **7** (156 mg, 74%) and **8** (30 mg, 14%). For **7**: $[\alpha]_D$ −32° (*c* 1.0, $CHCl_3$); ^1H NMR (400 MHz) δ 7.37–7.24 (m, 10H, H arom), 5.40 (s, 1H, H-1), 5.27 (s, 1H, H-3), 5.14 (s, 2H, COOCH_2Ph), 4.76 and 4.51 (d, 1H, *J* = 12.2 Hz, OCH_2Ph), 4.68 (d, 1H, $J_{5,6b}$ = 5.5 Hz, H-5), 4.55 (m, 1H, $J_{1',2'}$ = 7.3 Hz, H-1′), 4.39 (ddd, 1H, $J_{4'ha,1''}$ = 11.7 Hz, $J_{1'',2''}$ = 5.2 Hz, $J_{4'hb,1''}$ = 2.8 Hz, H-1″), 4.25–4.17 (m, 2H, H-6a″, H-6b″), 3.96 (d, 1H, $J_{6a,6b}$ = 7.3 Hz, H-6a), 3.89 (d, 1H, $J_{4',5'}$ = 10.7 Hz, H-5′), 3.75 (dd, 1H, H-6b), 3.66 (s, 1H, H-4), 3.59, 3.57, 3.52, 3.49 and 3.39 (s, 3H, OCH_3), 3.53–3.45 (m, 1H, H-5′), 3.26–3.16 (m, 5H, H-2, H-2′, H-3′, H-2″, H-3″), 3.01 (dd, 1H, $J_{4'',5''}$ = 9.5 Hz, $J_{3'',4''}$ = 8.0 Hz, H-4″), 2.25–2.17 (m, 1H, H-4′), 2.06 and 2.05 (s, 3H, OCOCH_3), 1.75 (ddd, 1H, $J_{4',4'ha}$ = 2.3 Hz, H-4′ha), 1.56 (ddd, 1H, $J_{4'ha,4'hb}$ = 14.5 Hz, $J_{4',4'hb}$ = 8.0 Hz, H-4′hb); ^{13}C NMR (100.58 MHz) δ 170.88, 169.30 and 168.53 (C=O), 137.71 and 135.02 (C arom), 128.47–127.58 (C arom), 102.67 (C-1′), 100.64 (C-1), 84.53, 83.31, 82.75, 81.23, 79.58, 76.01, 75.82, 73.59, 73.21, 69.78 and 69.33 (C-2, C-3, C-4, C-5, C-2′, C-3′, C-5′, C-2″, C-3″, C-4″, C-5″), 71.01 (C-1′, 70.96 (OCH$_2$Ph), 67.11 (COOCH$_2$Ph), 64.82 (C-6), 63.41 (C-6″), 60.29, 60.20, 60.16, 58.84 and 58.46, (OCH$_3$), 38.40 (C-4′), 23.66 (C methylene), 21.03 and 20.79 (COCH$_3$). MS, *m*/*z* 850 $(M+NH_4)^+$; Anal. calcd for $C_{42}H_{56}O_{17}$ (832.894): C 60.57, H 6.78, found: C 60.72, H 7.03.

For **8**: $[\alpha]_D$ +21° (*c* 1.0, $CHCl_3$); ^1H NMR (400 MHz) δ 7.40–7.25 (m, 10H, arom), 5.44 (s, 1H, H-1), 5.27 (d, 1H, $J_{1',2'}$ = 3.5 Hz, H-1′), 5.19 and 5.13 (d, 1H, *J* = 12.5 Hz, COOCH_2Ph), 5.04 (m, 1H, H-3), 4.79 and 4.61 (d, 1H, *J* = 12.0 Hz, OCH_2Ph), 4.69 (d, 1H, $J_{5,6b}$ = 5.5 Hz, H-5), 4.44–4.38 (m, 1H, H-1″), 4.34 (d,

1H, $J_{4',5'}$ = 9.5 Hz, H-5′), 4.26–4.18 (m, 2H, H-6a″, H-6b″), 3.91 (d, 1H, $J_{6a,6b}$ = 7.5 Hz, H-6a), 3.68 (dd, 1H, H-6b), 3.57, 3.50, 3.48, 3.46 and 3.42 (s, 3H, OCH_3), 3.57–3.51 (m, 2H, H-4, H-3′), 3.47–3.42 (m, 1H, H-5″), 3.54 (dd, 1H, $J_{2',3'}$ = 8.5 Hz, H-2′), 3.27 (dd, 1H, $J_{2'',3''}$ = 9.0 Hz, $J_{1'',2''}$ = 5.5 Hz, H-2″), 3.25 (s, 1H, H-2), 3.19 (t, 1H, $J_{3'',4''}$ = 8.8 Hz, H-3″), 3.02 (dd, 1H, $J_{4'',5''}$ = 9.8 Hz, H-4″), 2.27–2.19 (m, 1H, H-4′), 2.07 and 2.06 (s, 3H, OCOCH_3), 1.81–1.67 (m, 2H, H-4′ha, H-4′hb); ^{13}C NMR (62.896 MHz) δ 170.97, 170.10 and 169.66 (C=O), 137.68 and 135.14 (C arom), 128.56–127.69 (C arom), 100.40 (C-1), 97.38 (C-1′), 83.17, 81.40, 81.34, 80.79, 79.72, 75.86, 74.86, 74.33, 73.12, 71.65, 69.74 and 69.35 (C-2, C-3, C-4, C-5, C-2′, C-3′, C-5′, C-1″, C-2″, C-3″, C-4″, C-5″), 71.35 (OCH_2Ph), 67.15 (COOCH_2Ph), 65.18 (C-6), 63.53 (C-6″), 60.49, 60.32, 59.48, 58.54 and 58.33 (OCH_3), 37.79 (C-4′), 24.67 (C methylene), 21.10 and 20.83 (COCH_3). MS, *m*/*z* 850 $(M+NH_4)^+$

***O*-(6-*O*-Acetyl-2,3,4-tri-*O*-methyl-α-D-glucopyranosyl-methyl)-(1→4)-*C*-(benzyl 4-deoxy-2,3-di-*O*-methyl-β-D-glucopyranosyluronate)-(1→4)-1,3,6-tri-*O*-acetyl-2-*O*-benzyl-α,β-D-glucopyranose (9).** A 5% solution of concd H_2SO_4 in acetic acid (80 μL) was added to a solution of **7** (120 mg, 0.144 mmol) in acetic anhydride (12 mL) at −20 °C. After 15 min, CH_2Cl_2 (50 mL) and saturated aq $NaHCO_3$ (40 mL) were added. The aqueous phase was extracted three times with CH_2Cl_2, the organic layers were combined, dried ($MgSO_4$) and concentrated. Flash chromatography (cyclohexane:EtOAc:acetone, 3:1:1) gave **9** (134 mg, 100%; α,β 3:1 mixture). ^{1}H NMR (400 MHz) δ: α anomer: 7.41–7.23 (m, 10H, arom), 6.30 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1), 5.38 (t, 1H, $J_{2,3}$ = 9.7 Hz, $J_{3,4}$ = 9.7 Hz, H-3), 5.16 and 5.11 (d, 1H, J = 12.0 Hz, COOCH_2Ph), 4.62 and 4.49 (d, 1H, J = 12.5 Hz, OCH_2Ph), 4.53–4.47 (m, 1H, H-6a), 4.35 (ddd, 1H, $J_{4'ha,1''}$ = 11.5 Hz, $J_{1'',2''}$ = 5.5 Hz, $J_{4'hb,1''}$ = 2.8 Hz, H-1″), 4.24 (dd, 1H, $J_{6a,6b}$ = 12.8 Hz, $J_{5,6b}$ = 4.3 Hz, H-6b), 4.22–4.19 (m, 2H, H-6a″, H-6b″), 4.13 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1′), 3.98 (ddd, 1H, $J_{4,5}$ = 10.0 Hz, $J_{5,6a}$ = 2.0 Hz, H-5), 3.70 (d, 1H, $J_{4',5'}$ = 11.0 Hz, H-5′), 3.65 (t, 1H, H-4), 3.55 (dd, 1H, H-2), 3.54, 3.51, 3.50, 3.49 and 3.39 (s, 3H, OCH_3), 3.43–3.39 (m, 1H, H-5″), 3.21 (dd, 1H, $J_{2'',3''}$ = 9.0 Hz, H-2″), 3.13 (t, 1H, $J_{3'',4''}$ = 9.0 Hz, H-3″), 3.08 (dd, 1H, $J_{2',3'}$ = 9.5 Hz, $J_{3',4'}$ = 9.5 Hz, H-3′), 3.01–2.96 (m, 2H, H-2′, H-4″), 2.16, 2.09, 2.07 and 1.92 (s, 3H, OCOCH_3), 2.15–2.08 (m, 1H, H-4′), 1.65 (ddd, 1H, $J_{4',4'ha}$ = 2.0 Hz, H-4′ha), 1.49 (ddd, $J_{4'ha,4'hb}$ = 15.5 Hz, $J_{4',4'hb}$ = 8.0 Hz, H-4′hb). Selected data for β anomer: 5.62 (d, 1H, $J_{1,2}$ = 8.3 Hz, H-1), 5.22 (t, 1H, $J_{2,3}$ = 9.4 Hz, $J_{3,4}$ = 9.4 Hz, H-3), 4.68 and 4.60 (d, 1H, J = 12.0 Hz, OCH_2Ph), 4.12 (d, 1H, $J_{1',2'}$ = 7.8 Hz, H-1′), 3.74 (m, 1H, H-5); ^{13}C NMR (100.58 MHz) δ α anomer: 170.92, 170.30, 170.14, 169.17 and 168.45 (C=O), 137.35 and 134.74 (C arom), 128.66–127.68 (C arom), 103.65 (C-1′), 89.09 (C-1), 85.23, 83.89, 82.72, 81.11, 79.54, 76.00, 75.98, 75.45, 70.96, 70.88, 70.70 and 69.89 (C-2, C-3, C-4, C-5, C-1′, C-2′, C-3′, C-5′, C-2″, C-3″, C-4″, C-5″), 72.52 (OCH_2Ph), 67.30 (COOCH_2Ph), 63.33 (C-6″), 61.69 (C-6), 60.38, 60.27, 60.21, 59.32 and 58.54 (OCH_3), 38.69 (C-4′), 23.79 (C methylene), 20.97, 20.81 and 20.74 (OCOCH_3). Selected data for β anomer: 103.53 (C-1′), 93.62 (C-1). MS, *m*/*z* 952 $(M+NH_4)^+$; Anal. calcd for $C_{46}H_{62}O_{20}$ (934.984): C 59.09, H 6.68, found: C 59.04, H 6.83.

***O*-(6-*O*-Acetyl-2,3,4-tri-*O*-methyl-α-D-glucopyranosyl-methyl)-(1→4)-*C*-(benzyl 4-deoxy-2,3-di-*O*-methyl-β-D-glucopyranosyluronate)-(1→4)-3,6-di-*O*-acetyl-2-*O*-benzyl-α,β-D-glucopyranose (10).** Freshly prepared hydrazinium acetate (33 mg, 0.358 mmol) was added to a solution of **9** (134 mg, 0.144 mmol) in DMF (10 mL). After 30 min at room temperature, saturated aqueous sodium chloride (40 mL) was introduced and the product was extracted by ethyl acetate. The organic solution was dried ($MgSO_4$) and concentrated. Flash chromatography of the residue (cyclohexane:ethyl acetate:acetone, 5:2:2) afforded **10** (116 mg, 90%; α: β 1:1). ^{1}H NMR (400 MHz) δ α anomer: 7.39–7.27 (m, 10H, H arom), 5.42 (t, 1H, $J_{2,3}$ = 9.5 Hz, $J_{3,4}$ = 9.5 Hz, H-3), 5.22 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1), 5.17 and 5.12 (d, 1H, J = 12.5 Hz, COOCH_2Ph), 4.62 (s, 2H, OCH_2Ph), 4.54 (dd, 1H, $J_{6a,6b}$ = 12.0 Hz, $J_{5,6a}$ = 2.0 Hz, H-6a), 4.36 (ddd, 1H, $J_{4'ha,1''}$ = 11.0 Hz, $J_{1'',2''}$ = 5.5 Hz, $J_{4'hb,1''}$ = 2.8 Hz, H-1″), 4.28–4.12 (m, 4H, H-5, H-6b, H-6a″, H-6b″), 4.14 (d, 1H, $J_{1',2'}$ = 7.8 Hz, H-1′), 3.72 (d, 1H, $J_{4',5'}$ = 11.0 Hz, H-5′), 3.65–3.57 (m, 1H, H-4), 3.55, 3.51, 3.50, 3.49 and 3.39 (s, 3H, OCH_3), 3.52–3.47 (m, 1H, H-2), 3.44–3.37 (m, 1H, H-5″), 3.22 (dd, 1H, $J_{2'',3''}$ = 9.0 Hz, H-2″), 3.14 (t, 1H, $J_{3'',4''}$ = 9.0 Hz, H-3″), 3.09 (t, 1H, $J_{2',3'}$ = 9.5 Hz, $J_{3',4'}$ 9.5 Hz, H-3′), 3.02–2.96 (m, 2H, H-2′, H-4″), 2.16–2.07 (m, 1H, H-4′), 2.09 (s, 6H, OCOCH_3), 2.08 (s, 3H, OCOCH_3), 1.70–1.61 (m, 1H, H-4′ha), 1.54–1.57 (m, 1H, $J_{4'ha,4'hb}$ = 15.5 Hz, H-4′hb). Selected data for β anomer: 5.16 and 5.11 (d, 1H, J = 12.5 Hz, COOCH_2Ph), 4.84 and 5.66 (d, 1H, J = 12.0 Hz, OCH_2Ph), 4.78 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1), 3.71 (d, 1H, $J_{4',5'}$ = 11.0 Hz, H-5′), 3.31 (dd, 1H, $J_{2,3}$ = 9.5 Hz, H-2); ^{13}C NMR (100.58 MHz) δ mixture of anomers: 170.93, 170.47, 170.43, 170.18, 168.44 and 168.39 (C=O), 138.04, 137.41, 134.71 and 134.68 (C quart arom), 128.54–127.47 (C arom), 103.50 and 103.45 (C-1′ α and β), 97.10 (C-1 β), 90.53 (C-1 α), 85.05, 85.00, 83.76, 82.63, 81.00, 79.70, 79.49, 76.33, 76.21, 75.88, 75.82, 73.02, 72.86, 70.87, 70.81, 69.78 and 68.46, (C-2, C-3, C-4, C-5, C-2′, C-3′, C-5′, C-1″, C-2″, C-3″, C-4″, C-5″), 73.60, 72.51 and 67.17 (OCH_2Ph, COOCH_2Ph α and β), 63.30 (C-6′ α and β), 62.18 and 62.04 (C-6 α and β), 60.22, 60.18, 60.11, 59.09 and 58.40 (OCH_3), 38.60 and 38.55 (C-4′ α and β), 23.69 (C

methylene α and β), 20.77, 20.75, 20.72 and 20.63 ($OCOCH_3$). MS, m/z 910 $(M+NH_4)^+$; Anal. calcd. for $C_{44}H_{60}O_{19}$ (892.947): C 59.18, H 6.77, found: C 59.15, H 6.73.

[*O*-(6-*O*-Acetyl-2,3,4-tri-*O*-methyl-α-D-glucopyranosylmethyl)-(1→4)-*C*-(benzyl 4-deoxy-2,3-di-*O*-methyl-β-D-glucopyranosyluronate)-(1→4)-3,6-di-*O*-acetyl-2-*O*-benzyl-α,β-D-glucopyranosyl] trichloroacetimidate (11). Trichloroacetonitrile (0.65 mL, 6.5 mmol) and DBU (15 μL, 0.1 mmol) were added to a solution of **10** (117 mg, 0.131 mmol) in CH_2Cl_2 (10 mL). After 20 min at room temperature, the solution was concentrated. Flash chromatography (cyclohexane:ethyl acetate, 13:10 + 1% Et_3N) gave **11** (128 mg, 94%; α:β : 5:1). ^1H NMR (400 MHz, C_6D_6) δ α anomer: 8.51 (s, 1H, N-H), 7.45–7.05 (m, 10H, arom), 6.73 (d, 1H, $J_{1,2}=3.5$ Hz, H-1), 6.06 (t, 1H, $J_{2,3}=9.7$ Hz, $J_{3,4}$ 9.7 Hz, H-3), 5.12 and 5.01 (d, 1H, $J=12.0$ Hz, $COOCH_2Ph$), 4.79–4.74 (m, 1H, H-6a), 4.61 (dd, 1H, $J_{6a'',6b''}=11.7$ Hz, $J_{5'',6a''}=2.0$ Hz, H-6a″), 4.54–4.49 (m, 3H, H-5, H-6b, OCH_2Ph), 4.48 (ddd, 1H, $J_{4'ha,1''}=11.6$ Hz, $J_{1'',2''}=5.5$ Hz, $J_{4'hb,1''}=2.5$ Hz, H-1″), 4.37 (dd, 1H, $J_{5'',6b''}=5.5$ Hz, H-6b″), 4.34 (2d, 2H, $J=12.0$ Hz, OCH_2Ph, $J_{1',2'}=7.5$ Hz, H-1′), 3.84 (t, 1H, $J_{4,5}=9.7$ Hz, H-4), 3.79 (d, 1H, $J_{4',5'}=11.0$ Hz, H-5′), 3.67 (ddd, 1H, $J_{4'',5''}=9.5$ Hz, H-5″), 3.59 (dd, 1H, H-2), 3.50, 3.39, 3.33, 3.32 and 3.15 (s, 3H, OCH_3), 3.29 (t, 1H, $J_{2'',3''}=9.5$ Hz, $J_{3'',4''}=9.5$ Hz, H-3″), 3.22 (dd, 1H, H-2″), 3.09–2.97 (m, 3H, H-2′, H-3′, H-4″), 2.45–2.36 (m, 1H, H-4′), 2.05, 1.94 and 1.71 (s, 3H, $OCOCH_3$), 1.77 (ddd, 1H, $J_{4',4'ha}=1.8$ Hz, H-4′ha), 1.52 (ddd, 1H, $J_{4'ha,4'hb}=15.5$ Hz, $J_{4',4'hb}=8.0$ Hz, H-4′hb). Selected data for β anomer: 8.62 (s, 1H, N-H), 6.04 (d, 1H, $J_{1,2}=7.5$ Hz, H-1), 5.58 (t, 1H, $J_{2,3}=9.0$ Hz, H-3), 4.87 and 4.67 (d, 1H, $J=12.0$ Hz, OCH_2Ph); ^{13}C NMR (100.58 MHz, C_6D_6) δ α anomer: 170.99, 170.44, 170.10 and 169.53 (C=O), 163.41 (CCl_3), 161.93 (C=NH), 138.73 and 135.98 (C arom), 129.73–128.14 (C arom), 104.32 (C-1′), 94.38 (C-1), 86.05, 84.86, 84.25, 82.48, 81.04, 77.47, 77.15, 77.11, 77.05, 72.60, 72.17 and 71.13 (C-2, C-3, C-4, C-5, C-2′, C-3′, C-5′, C-1″, C-2″, C-3″, C-4″, C-5″), 73.18 (OCH_2Ph), 68.10 ($COOCH_2Ph$), 64.30 and 62.87 (C-6, C-6″), 60.80, 60.75, 60.71, 59.66 and 58.73 (OCH_3), 39.91 (C-4′), 24.40 (C methylene), 21.45, 21.16 and 20.84 ($OCOCH_3$). MS, m/z 1055 $(M+NH_4)^+$, 892 $(M\text{-}OCNHCCl_3+NH_3)^+$.

Methyl *C*-(6-*O*-acetyl-2,3,4-tri-*O*-methyl-α-D-glucopyranosylmethyl)-(1→4)-*O*-(benzyl 4-deoxy-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 2,3-di-*O*-methyl-α-L-idopyranosyl uronate)-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranoside (13). A 0.05 M solution of TMSOTf in CH_2Cl_2 (0.35 mL) was slowly added under argon to a stirred solution of **11** (77 mg, 0.074 mmol) and **12** (118 mg, 0.155 mmol) in CH_2Cl_2, containing 4 Å molecular sieves at −20 °C. After 15 min solid $KHCO_3$ (500 mg) was introduced and the temperature was allowed to rise to room temperature. After filtration and concentration, column chromatography of the residue (CH_2Cl_2:EtOAc:acetone, 20:1:2) followed by gel filtration through a Sephadex LH-20 column (CH_2Cl_2/MeOH) gave **13** (96 mg, 79%). $[\alpha]_D$ +15° (*c* 0.24, $CHCl_3$); ^1H NMR (500 MHz): 7.38–7.22 (m, 30H, H arom), 5.40 (t, 1H, $J_{2'',3''}=9.7$ Hz, $J_{3'',4''}=9.7$ Hz, H-3″), 5.31 (d, 1H, $J_{1',2'}=6.8$ Hz, H-1′), 5.26 and 4.97 (d, 1H, $J_{gem}=12.2$ Hz, OCH_2Ph), 5.20 (d, 1H, $J_{1'',2''}=3.5$ Hz, H-1″), 5.14 and 5.10 (d, 1H, $J_{gem}=12.2$ Hz, OCH_2Ph), 4.89 and 4.77 (d, 1H, $J_{gem}=11.0$ Hz, OCH_2Ph), 4.75 and 4.58 (d, 1H, $J_{gem}=12.4$ Hz, OCH_2Ph), 4.69 and 4.53 (d, 1H, $J_{gem}=12.3$ Hz, OCH_2Ph), 4.64 and 4.51 (d, 1H, $J_{gem}=12.1$ Hz, OCH_2Ph), 4.56 (d, 1H, $J_{1,2}=3.6$ Hz, H-1), 4.46 (d, 1H, $J_{4',5'}=6.2$ Hz, H-5′), 4.43 (dd, 1H, $J_{6a'',6b''}=12.0$ Hz, $J_{5'',6a''}=1.7$ Hz, H-6″a), 4.36 (ddd, 1H, $J_{4'''ha,1'''}=11.4$ Hz, $J_{1''',2'''}=5.5$ Hz, $J_{4'''hb,1'''}=2.6$ Hz, H-1‴), 4.25–4.18 (m, 3H, H-6b″,H-6a‴, H-6b‴), 4.06–4.02 (m, 1H, H-5″), 4.06 (d, 1H, $J_{1''',2'''}=7.9$ Hz, H-1‴), 3.85 (dd, 1H, $J_{3',4'}=8.5$ Hz, H-4′), 3.84–3.78 (m, 2H, H-3, H-4), 3.76–3.65 (m, 4H, H-5, H-6a, H-6b, H-3′), 3.67 (d, 1H, $J_{4''',5'''}=10.0$ Hz, H-5‴), 3.55 (t, 1H, $J_{4'',5''}=9.7$ Hz, H-4″), 3.54, 3.48, 3.47, 3.46, 3.45, 3.39, 3.36 and 3.18 (s, 3H, OCH_3), 3.48–3.37 (m, 3H, H-2, H-2″, H-5‴), 3.21 (dd, 1H, $J_{2''',3'''}=8.7$ Hz, H-2‴), 3.12 (t, 1H, $J_{3''',4'''}=8.7$ Hz, H-3‴), 3.02 (dd, 1H, $J_{3''',4'''}=10.3$ Hz, $J_{2''',3'''}$ 8.3 Hz, H-3‴), 2.99 (t, 1H, $J_{4''',5'''}=8.7$ Hz, H-4‴), 2.93 (dd, 1H, $J_{2',3'}=8.1$ Hz, H-2′), 2.85 (dd, 1H, H-2‴), 2.12–2.07 (m, 1H, H-4‴), 2.09, 1.95 and 1.90 (s, 3H, $OCOCH_3$), 1.64 (ddd, 1H, $J_{4'''ha,4'''hb}=15.3$ Hz, $J_{4''',4ha'''}=1.8$ Hz, H-4‴ha), 1.57 (ddd, 1H, $J_{4''',4hb'''}=8.1$ Hz, H-4‴hb). MS, m/z 1650 $(M+NH_4)^+$; Anal. calcd for $C_{87}H_{108}O_{30}$ (1633.792): C 63.96, H 6.66, Found: C 63.65, H 6.52.

Methyl *C*-(6-*O*-sulfo-2,3,4-tri-*O*-methyl-α-D-glucopyranosylmethyl)-(1→4)-*O*-(4-deoxy-2,3-di-*O*-methyl-β-D-glucopyranosyluronic acid)-(1→4)-*O*-(2,3,6-tri-*O*-sulfo-α-D-glucopyranosyl)-(1→4)-*O*-(2,3-di-*O*-methyl-α-L-idopyranosyluronic acid)-(1→4)-2,3,6-tri-*O*-sulfo-α-D-glucopyranoside (14). Hydrogenolysis of benzyl ethers and benzyl esters. A solution of **13** (70 mg, 0.043 mmol), in CH_2Cl_2:MeOH (1:1, 2 mL), was stirred for 5 h at room temperature under hydrogen atmosphere (5 bar) in the presence of 10% Pd/C (40 mg). After filtration and concentration, the residue (46.3 mg, 99%) was used in the next step.

Saponification of the esters. NaOH (4 M aq) was added (0.5 M final concentration) at 0 °C to a solution of the residue in MeOH (2 mL). After 2 h at 0 °C water was introduced, followed by Dowex 50 H^+ resin, until pH 1–2. After filtration and concentration, the residue was passed through a Sephadex G-25 column (1.6×115 cm)

eluted with water to give pure fully deprotected compound (33 mg, 77%). At this stage, complete removal of protective groups was checked by high field ^{1}H NMR.

Sulfation. Et_3N–sulfur trioxide complex (5 mmol/mmol of hydroxyl function) was added to a solution of the deprotected compound (29.8 mg, 0.030 mmol) in DMF (3 mL). After one day at 55 °C with protection from light, the solution was layered on top of a Sephadex G-25 column (1.6×115 cm) eluted with 0.2 M NaCl. The fractions containing the product were concentrated, and desalted using the same column, equilibrated in water. Lyophilization provided **14** (45.2 mg, 85%). $[\alpha]_D$ +45° (*c* 0.99, H_2O). ^{1}H NMR (500 MHz, D_2O) δ 5.41 (d, 1H, $J_{1,2}$ = 3.7 Hz, H-1 F-unit), 5.15 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1 H-unit), 5.07 (d, 1H, $J_{1,2}$ = 2.8 Hz, H-1 G-unit), 4.60 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1 E-unit), 4.43 (m, 1H, $J_{1,2}$ = 3.7 Hz, H-1 D-unit), 3.63, 3.60, 3.57, 3.56, 3.52, 3.47, 3.46, (s, 24H, 8 OMe), 2.00 (H-4, E-unit), 1.84 (ddd, Hha CHaHb), 1.58 (ddd, Hhb CH_haH_hb). MS: *m*/*z* 840 $[(M\text{-}2Na)^2\text{-}/2]$.

Affinity for AT III. Fluorescence measurements were performed using a Perkin–Elmer LS-50 type spectrofluorimeter (excitation λ = 280 nm, emission λ = 338 nm) equipped with a thermostated sample compartment, at 37 °C, under continuous stirring. Oligosaccharides were added into the sample containing 2 mL of 0.01 M Tris–HCl buffer, pH 7.5, 0.15 M NaCl and 5–60 nM human AT-III. 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 the RS/1 computer program (BBN Software Product Corporation, Cambridge, MA, USA).

Anti-factor Xa activity. Human factor Xa (2.4 nkat/mL) was incubated for 2 min with human AT III (0.17 U/mL) at 37 °C in the presence of various concentrations of oligosaccharides in Tris–maleate 20 mM buffer pH 7.4, NaCl 150 mM. To measure the residual factor Xa, S-2222 substrate (dissolved in 50 mM Tris–HCl buffer, pH 8.4, NaCl 175 mM, EDTA 2 7.5 mM) was added (0.25 mM final). The reaction was stopped 2 min later by addition of 50% aqueous acetic acid and the absorbance at 405 nm was read. The percentage of inhibition was then calculated [inhibition % = 100 ((OD blank-OD sample)/OD blank], and the activity of the compounds determined by comparison with a calibrated standard, using Excel 4.0 Software (Microsoft, Redmond, USA).

References and Notes

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