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BINDING OF HEPARIN TO ANTITHROMBIN III: A CHEMICAL PROOF OF THE CRITICAL ROLE PLAYED BY A 3-SULFATED 2-AMINO-2-DEOXY-D-GLUCOSE RESIDUE*

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ABSTRACT

Known methyl (prop-1-enyl 2,3-di-*O*-benzyl-α-D-glucopyranosid)uronate was first converted into methyl (prop-1-enyl 2,3-di-*O*-benzyl-4-*O*-levulinyl-α-D-glucopyranosid)uronate. Acid hydrolysis, followed by treatment with (bromomethylene)-dimethylammonium bromide, gave methyl (2,3-di-*O*-benzyl-4-*O*-levulinyl-α-D-glucopyranosyl bromide)uronate. Condensation of this bromide with 1,6-anhydro-2-azido-3-*O*-benzyl-2-deoxy-β-D-glucopyranose gave 1,6-anhydro-2-azido-3-*O*-benzyl-2-deoxy-4-*O*-(methyl 2,3-di-*O*-benzyl-4-*O*-levulinyl-β-D-glucopyranosyluronate)-β-D-glucopyranose. Acetolysis, followed by selective anomeric *O*-deacetylation and treatment with (bromomethylene)dimethylammonium bromide then gave 6-*O*-acetyl-2-azido-3-*O*-benzyl-2-deoxy-4-*O*-(methyl 2,3-di-*O*-benzyl-4-*O*-levulinyl-β-D-glucopyranosyluronate)-α-D-glucopyranosyl bromide. Condensation of this bromide with benzyl 6-O-acetyl-3-*O*-benzyl-2-benzyloxycarbonylamino-2-deoxy-4-*O*-(methyl 2-*O*-acetyl-3-*O*-benzyl-α-L-idopyranosyluronate)-α-D-glucopyranoside provided benzyl *O*-(methyl 2,3-di-*O*-benzyl-4-*O*-levulinyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(6-*O*-acetyl-2-azido-3-*O*-benzyl-2-deoxy-α-D-glucopyranosyl)-(1→4)-*O*-(methyl 2-*O*-acetyl-3-*O*-benzyl-α-L-idopyranosyluronate)-(1→4)-6-*O*-acetyl-3-*O*-benzyl-2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside. Removal of the levulinyl group followed by condensation with 6-*O*-acetyl-2-azido-3,4-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl bromide provided benzyl *O*-(6-*O*-acetyl-2-azido-3,4-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl)-(1→4)-*O*-(methyl 2,3-di-*O*-benzyl-β-D-glucopyranosyluronate)-(1→4)-*O*-(6-*O*-acetyl-2-azido-3-*O*-benzyl-2-deoxy-α-D-glucopyranosyl)-(1→4)-*O*-(methyl 2-*O*-acetyl-3-*O*-benzyl-α-L-idopyranosyluronate)-(1→4)-6-*O*-acetyl-3-*O*-benzyl-2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside in 78% yield. *O*-Deacetylation followed by re-esterification, *O*-sulfation, catalytic hydrogenolysis, saponification, and *N*-sulfation gave the nona-

*Dedicated to Professor Bengt Lindberg

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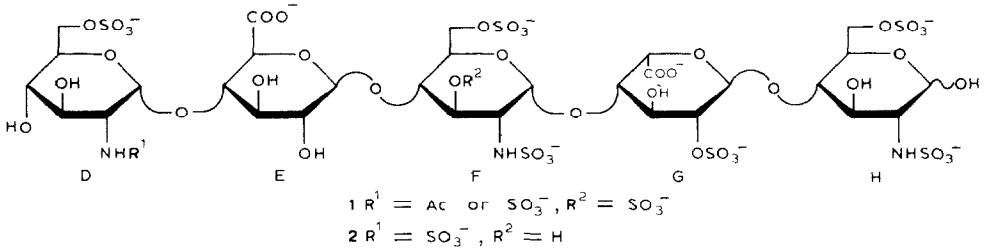


sodium salt of *O*-(2-deoxy-6-*O*-sulfo-2-sulfoamino-α-D-glucopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyluronic acid)-(1→4)-*O*-(2-deoxy-6-*O*-sulfo-2-sulfoamino-α-D-glucopyranosyl)-(1→4)-*O*-(2-*O*-sulfo-α-L-idopyranosyluronic acid)-(1→4)-2-deoxy-6-*O*-sulfo-2-sulfoamino-D-glucopyranose. This synthetic pentasaccharide neither binds to antithrombin III nor induces anti-factor Xa activity.

INTRODUCTION

The pentasaccharide sequence **1** is required in heparin for binding to the plasma protein antithrombin III (AT III), thus eliciting the well known anticoagulant properties of the polysaccharide[1-4]. We[5] and others[6,7] have synthesised the pentasaccharide **1** ($R^1 = SO_3^-$) which binds to AT III with an affinity the same as that of high-affinity heparin species[8] and induces a high anti-factor Xa activity in plasma and antithrombotic activity *in vivo* in animal models[9].

A noticeable feature of this sequence is the occurrence of a 3-sulfated glucosamine residue F. This unique, typical amino sugar has been identified through the use of a 3-*O*-sulfatase from human urine[10]. In ^{13}C-n.m.r. analysis, the 3-*O*-sulfo group induces an upfield shift of the signals for the adjacent carbon atoms. Thus, a new "signal", originally detected in high-AT-III-affinity oligosaccharides[11], was later shown to be associated with the heparin antithrombin-III-binding sequence and, more precisely, assigned to C-2 of the 3-sulfated glucosamine unit F[2,12].

Because of its constant occurrence in high-affinity heparin species, the 3-*O*-sulfo group on residue F was assumed to be essential for binding to AT III[1-4,10-13]. This view has been supported by biosynthetic studies[14].

In order to establish unambiguously the importance of this unique sulfation in the binding of heparin to AT III, we have synthesised[15] the pentasaccharide **2** and now report the details of this work.

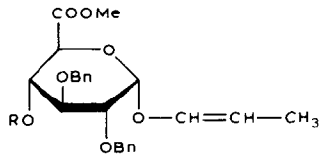
RESULTS AND DISCUSSION

The general strategy of this synthesis was based on benzyl ethers as permanent blocking groups, and the known[16] alcohol **12** was selected as a progenitor of the G–H sequence. For clarity, the route of synthesis to pentasaccharide **2** was as follows: **11** →**14** →**16** →**2** in which the alcohol **12** and the disaccharide glycosyl

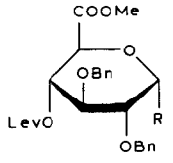
bromide **11** were the two key building-blocks of the potential target molecule **16**. The synthesis of **11** was thus undertaken.

Methyl (prop-1-enyl 2,3-di-*O*-benzyl-α-D-glucopyranosid)uronate[5] (**3**) was levulinated in 1,4-dioxane with levulinic (4-oxopentanoic) acid in the presence of dicyclohexylcarbodi-imide and 4-dimethylaminopyridine[17] to give the crystalline derivative **4** in 93% yield. Treatment of **4** with mercuric chloride and yellow mercuric oxide in acetone[18] gave the hemiacetal **5**. Mild bromination of **5** with (bromomethylene)dimethylammonium bromide[19] gave 83% of the bromide **6**. Condensation of **6** with the known[20] alcohol **7**, in dichloromethane at room temperature in the presence of silver carbonate for 5 days, gave the crystalline disaccharide derivative **8** in 53% yield. The ^{1}H-n.m.r. data for **8** showed that no downfield doublet indicative of a newly established α-glycosidic bond was present. Treatment of **8** with acetic anhydride–trifluoroacetic acid opened the anhydro ring and gave an α,β-mixture of the acetates **9** which was selectively *O*-deacetylated at C-1 with benzylamine in ether to give the disaccharide **10** in 85% yield. Mild bromination of **10** with (bromomethylene)dimethylammonium bromide gave the key bromide **11** in 48% yield.

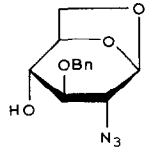
Condensation of **11** with **12** in dichloromethane at 0°, in the presence of silver triflate, molecular sieve (4 Å), and 2,4,6-trimethylpyridine, gave 42% of the amorphous tetrasaccharide derivative **13**. The ^{1}H-n.m.r. data (δ 5.21, d, $J_{1,2}$ 3.6 Hz, H-1 of unit F) demonstrated that the new glycosidic bond was α and confirmed that the terminal unit E (δ 4.45, d, $J_{1,2}$ 9.3 Hz, H-1) was β-bonded. Selective *O*-delevulinylation of **13** with hydrazine hydrate in pyridine–acetic acid[17] gave the alcohol **14**, which was condensed with the known[21] glycosyl bromide **15** in dichloromethane at −20°, in the presence of silver triflate and 2,4,6-trimethylpyridine, to give 78% of the amorphous pentasaccharide derivative **16**. The 300-MHz ^{1}H-n.m.r. data for **16** (δ 5.51, d, $J_{1,2}$ 3.7 Hz, H-1 of unit D) demonstrated that the new glycosidic bond was α.



3 R = H
4 R = Lev (levulinyl)



5 R = OH
6 R = Br



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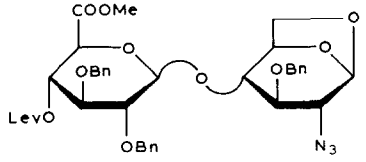

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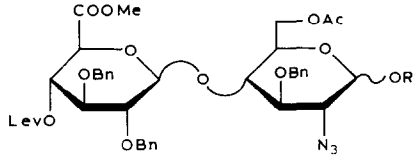


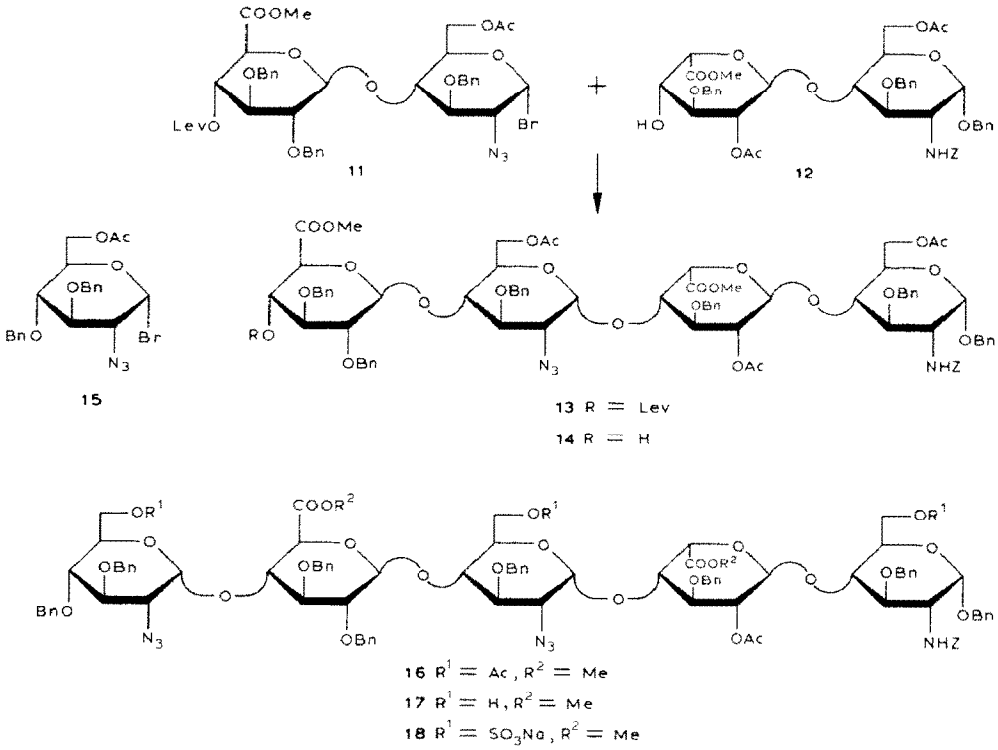
9 R = Ac
10 R = H

Conversion of the pentasaccharide **16** into **2** was accomplished along lines previously described[5b]. The 300-MHz ^{1}H-n.m.r. data for **2** are in full agreement with the structure. Noteworthy is the expected downfield displacement of ~ 0.7 p.p.m. of the signal of H-3 in unit F upon sulfation [δ 4.37 for the previously[5] synthesised pentasaccharide **1** ($R^2 = SO_3^-$) and δ 3.70 for **2**].

Antithrombin III-binding experiments[22] and anti-factor Xa activity measured either by a clotting assay or an amidolytic assay[15] showed that the synthetic pentasaccharide **2** neither binds to antithrombin III nor induces anti-factor Xa activity. This result points to a critical role for the 3-*O*-sulfo group in pentasaccharide DEFGH. These data clearly demonstrate that selective synthesis of various analogs of the active pentasaccharide DEFGH would result in a precise assessment of the sulfate groups that are essential for binding of heparin to antithrombin III.

EXPERIMENTAL

General methods. — Melting points were determined in capillary tubes with a Büchi apparatus and are uncorrected. Optical rotations were measured at 20–24° with a Perkin–Elmer Model 241 polarimeter. ^{1}H-N.m.r. spectra were recorded with a Bruker CXP 300 (300 MHz) instrument for solutions in $CDCl_3$ (internal Me_4Si) unless otherwise stated. The purity of products was determined by t.l.c. on Silica



Gel 60 F_{154} (Merck) with detection by charring with sulfuric acid. Column chromatography was performed on Silica Gel 60 (Merck, 63–200 μm) which was used without pretreatment. Elemental analyses were performed by the Service d'Analyse de l'Institut Choay (M. Zuber).

*Methyl (prop-1-enyl 2,3-di-*O*-benzyl-4-*O*-levulinyl-α-*D*-glucopyranosid)uronate* (**4**). — A solution of **3** (9.6 g) in 1,4-dioxane (100 mL) was stirred for 3 h in the presence of levulinic acid (5.2 g), dicyclohexylcarbodi-imide (9.3 g), and 4-dimethylaminopyridine (0.55 g), and then diluted with cold methyl acetate (200 mL). The precipitate was filtered-off, and the solution was washed with water, aqueous 10% $KHSO_4$, and water, dried (Na_2SO_4), and concentrated. The residue was eluted from a column of silica gel (500 g) with chloroform–ethyl acetate (15:1) to give **4** (11 g, 93%), m.p. 80–81° (from hexane–ethyl acetate), $[\alpha]_D$ +6° (*c* 1, chloroform). ^{1}H-N.m.r. data: δ 7.30 (m, 10 H, 2 Ph), 5.03 (dd, 1 H, $J_{3,4}$ 9, $J_{4,5}$ 10 Hz, H-4), 4.97 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 4.18 (d, 1 H, $J_{4,5}$ 10 Hz, H-5), 4.03 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9 Hz, H-3), 3.67 (s, 3 H, COOMe), 3.61 (dd, 1 H, $J_{1,2}$ 3.5, $J_{2,3}$ 9 Hz, H-2).

Anal. Calc. for $C_{29}H_{34}O_9$: C, 66.14; H, 6.50. Found: C, 65.83; H, 6.30.

*Methyl 2,3-di-*O*-benzyl-4-*O*-levulinyl-α-*D*-glucopyranuronate* (**5**). — A solution of mercuric chloride (16 g) in acetone–water (5:1, 100 mL) was added dropwise at room temperature to a stirred mixture of **4** (11 g), yellow mercuric oxide (12.76 g), and acetone–water (5:1, 300 mL). After 5 min, the mixture was filtered and concentrated, and a solution of the residue in chloroform was washed with saturated aqueous potassium iodide and water, dried (Na_2SO_4), and concentrated. The residue was crystallised from ethanol–water to give **5** (8.3 g, 81%), m.p. 99–101°, $[\alpha]_D$ +8° (*c* 1, chloroform). ^{1}H-N.m.r. data: δ 7.27 (m, 10 H, Ph), 5.26 (d after D_2O addition, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 5.09 (dd, 1 H, $J_{3,4}$ 8.5, $J_{4,5}$ 10 Hz, H-4), 4.44 (d, 1 H, $J_{4,5}$ 10 Hz, H-5), 3.98 (t, 1 H, $J_{2,3} = J_{3,4}$ = 8.5 Hz, H-3), 3.80 (d, 1 H, *J* 4 Hz, OH), 3.68 (dd, 1 H, $J_{1,2}$ 3.5, $J_{2,3}$ 8.5 Hz, H-2).

Anal. Calc. for $C_{26}H_{30}O_9$: C, 64.18; H, 6.21. Found: C, 64.09; H, 6.25.

*Methyl (2,3-di-*O*-benzyl-4-*O*-levulinyl-α-*D*-glucopyranosyl bromide)uronate* (**6**). — A solution of **5** (6 g) in dichloromethane (100 mL) was stirred for 12 h at room temperature in the presence of 2,4,6-trimethylpyridine (26.2 mL) and freshly prepared $[Me_2N=CHBr]^+Br^-$ (20 g), then diluted with dichloromethane (300 mL), washed with ice-cold water, dried (Na_2SO_4), and concentrated. The residue was eluted from a column of silica gel (60 g) with dichloromethane–ethyl acetate (10:1) to give **6** (5.67 g, 83.5%), $[\alpha]_D$ +130° (*c* 1, chloroform). ^{1}H-N.m.r. data: δ 7.30 (m, 10 H, 2 Ph), 6.30 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 5.09 (dd, 1 H, $J_{3,4}$ 9, $J_{4,5}$ 10 Hz, H-4), 4.43 (d, 1 H, $J_{4,5}$ 10 Hz, H-5), 4.0 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9 Hz, H-3), 3.67 (s, 3 H, COOMe), 3.56 (dd, 1 H, $J_{1,2}$ 3.5, $J_{2,3}$ 9 Hz, H-2).

Anal. Calc. for $C_{26}H_{29}BrO_8$; C, 56.82; H, 5.31. Found: C, 56.98; H, 5.21.

*1,6-Anhydro-2-azido-3-*O*-benzyl-2-deoxy-4-*O*-(methyl 2,3-di-*O*-benzyl-4-*O*-levulinyl-β-*D*-glucopyranosyluronate)-β-*D*-glucopyranose* (**8**). — A solution of freshly prepared **6** (5.7 g) and **7**[20] (4.7 g) in anhydrous dichloromethane (40 mL) was stirred for 5 days at room temperature under dry argon in the presence of acti-

vated, powdered molecular sieve (4 Å, 5 g) and silver carbonate (4.3 g). The mixture was then diluted with dichloromethane (100 mL), filtered, washed with water, dried (Na_2SO_4), and concentrated. The residue was eluted from a column of silica gel (200 g) with hexane–ethyl acetate (1:1) to give **8** (4.1 g, 53%), m.p. 92–94° (from hexane–ethyl acetate), $[\alpha]_D$ +5° (*c* 1, chloroform). ^{1}H-N.m.r. data: δ 7.30 (m, 15 H, 3 Ph), 5.47 (broad s, 1 H, H-1), 3.66 (s, 3 H, COOMe), 3.19 (broad s, 1 H, H-2).

Anal. Calc. for $C_{39}H_{43}N_3O_{12}$: C, 62.80; H, 5.81; N, 5.63. Found: C, 62.62; H, 5.59; N, 5.52.

1,6-Di-O-acetyl-2-azido-3-O-benzyl-2-deoxy-4-O-(methyl 2,3-di-O-benzyl-4-O-levulinyl-β-D-glucopyranosyluronate)-D-glucopyranose (**9**). — A solution of **8** (4.5 g) in acetic anhydride (42 mL) and trifluoroacetic acid (12 mL) was stirred for 12 h at room temperature and then concentrated. The residue was eluted from a column of silica gel (200 g) with hexane–ethyl acetate (6:5) to give amorphous **9** (2.7 g, 52%). ^{1}H-N.m.r. data: δ 7.25 (m, 15 H, 3 Ph), 6.13 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1α), 3.50 (s, 3 H, COOMe).

Anal. Calc. for $C_{43}H_{49}N_3O_{15}$: C, 60.91; H, 5.82; N, 4.95. Found: C, 60.62; H, 5.69; N, 4.83.

6-O-Acetyl-2-azido-3-O-benzyl-2-deoxy-4-O-(methyl 2,3-di-O-benzyl-4-O-levulinyl-β-D-glucopyranosyluronate)-D-glucopyranose (**10**). — A solution of **9** (2.46 g) in ether (120 mL) and benzylamine (12 mL) was stirred for 6 h at room temperature, diluted with ether (700 mL), washed with cold M HCl and water, and concentrated. The residue was eluted from column of silica gel (180 g) with chloroform–ethyl acetate (2:1) to give amorphous **10** (2.047 g, 84.5%). ^{1}H-n.m.r. data: δ 7.20 (m, 15 H, Ph), 5.15 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1α), 3.50 (s, 3 H, COOMe).

Anal. Calc. for $C_{41}H_{47}N_3O_{14}$: C, 61.10; H, 5.88; N, 5.21. Found: C, 60.92; H, 5.98; N, 5.27.

6-O-Acetyl-2-azido-3-O-benzyl-2-deoxy-4-O-(methyl 2,3-di-O-benzyl-4-O-levulinyl-β-D-glucopyranosyluronate)-α-D-glucopyranosyl bromide (**11**). — A solution of **10** (162 mg) in dichloromethane (5 mL) was stirred for 18 h at 0° in the presence of 2,4,6-trimethylpyridine (0.32 mL) and freshly prepared $[Me_2N{=}CHBr]^+Br^-$ (300 mg), then diluted with dichloromethane (15 mL), washed with ice-cold water, dried (Na_2SO_4), and concentrated. The residue was eluted from a column of silica gel (4 g) with dichloromethane–ethyl acetate (5:1) to give unstable **11** (83 mg, 47.5%) which was immediately used for the next reaction.

Benzyl O-(methyl 2,3-di-O-benzyl-4-O-levulinyl-β-D-glucopyranosyluronate)-(1→4)-O-(6-O-acetyl-2-azido-3-O-benzyl-2-deoxy-α-D-glucopyranosyl)-(1→4)-O-(methyl 2-O-acetyl-3-O-benzyl-α-L-idopyranosyluronate)-(1→4)-6-O-acetyl-3-O-benzyl-2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside (**13**). — A solution of freshly prepared **11** (360 mg) and benzyl 6-*O*-acetyl-3-*O*-benzyl-2-benzyloxycarbonylamino-2-deoxy-4-*O*-(methyl 2-*O*-acetyl-3-*O*-benzyl-α-L-idopyranosyluronate)-α-D-glucopyranoside[16] (**12**, 710 mg) in anhydrous dichloromethane (10 mL) was stirred for 15 min at −20° in the presence of activated, powdered molecular sieve (4

Å, 200 mg). 2,4,6-Trimethylpyridine (0.07 mL) and silver triflate (117 mg) were added. The mixture was stirred in the dark for 2 h at −20°, and then a second addition of 2,4,6-trimethylpyridine (0.07 mL) and silver triflate (117 mg) was made. The mixture was stirred for 12 h at 0°, when a third addition of 2,4,6-trimethylpyridine (0.07 mL) and silver triflate (117 mg) was made. After 34 h at 0°, the mixture was diluted with dichloromethane (60 mL), filtered, washed with aqueous 10% $KHSO_4$, dried (Na_2SO_4), and concentrated. The residue was eluted from a column of silica gel (15 g) with dichloromethane–ethyl acetate (4.5:1) to give **13** (287 mg, 42.5%) as a colourless glass, $[\alpha]_D$ +45.5° (*c* 1.8, chloroform). ^{1}H-N.m.r. data: unit E, δ 4.45 (d, 1 H, $J_{1,2}$ 9.3 Hz, H-1), 3.50 (s, 3 H, COOMe), 3.67 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3), 5.15 (t, 1 H, $J_{3,4}$ = $J_{4,5}$ = 9.5 Hz, H-4); unit F, δ 5.21 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1), 3.24 (dd, 1 H, $J_{1,2}$ 3.6, $J_{2,3}$ 10 Hz, H-2); unit G, δ 3.39 (s, 3 H, COOMe).

Anal. Calc. for $C_{87}H_{96}N_4O_{28}\cdot H_2O$: C, 62.80; H, 5.93; N, 3.36. Found: C, 62.69; H, 5.81; N, 3.29.

Further elution gave **12** (390 mg, 55%).

Benzyl O-*(methyl 2,3-di*-O-*benzyl*-β-D-*glucopyranosyluronate)*-*(1→4)*-O-*(6*-O-*acetyl-2-azido-3*-O-*benzyl-2-deoxy*-α-D-*glucopyranosyl)*-*(1→4)*-O-*(methyl 2*-O-*acetyl-3*-O-*benzyl*-α-L-*idopyranosyluronate)*-*(1→4)*-*6*-O-*acetyl-3*-O-*benzyl-2-benzyloxycarbonylamino-2-deoxy*-α-D-*glucopyranoside* (**14**). — M Hydrazine hydrate in pyridine–acetic acid (3:2, 0.9 mL) was added to a solution of **13** (272 mg) in pyridine (0.9 mL). The mixture was stirred for 5 min at room temperature, diluted with dichloromethane (20 mL), washed with aqueous 10% $KHSO_4$, aqueous saturated $NaHCO_3$ and water, dried (Na_2SO_4), and concentrated. The residue was eluted from a column of silica gel (10 g) with ethyl acetate–hexane (1:1) to give **14** (219 mg, 86%), $[\alpha]_D$ +51.5° (*c* 1, chloroform). ^{1}H-N.m.r. data: unit E, δ 3.52 (s, 3 H, COOMe), 2.77 (broad s, 1 H, OH); unit F, δ 5.20 (d, 1 H, $J_{1,2}$ 3.7 Hz, H-1), 3.27 (dd, 1 H, $J_{1,2}$ 3.7, $J_{2,3}$ 10 Hz, H-2); unit G, δ 3.37 (s, 3 H, COOMe); 7.30 (m, 35 H, Ph), 2.10, 2.06, and 1.98 (3 s, 9 H, 3 Ac).

Anal. Calc. for $C_{82}H_{90}N_4O_{26}$: C, 63.65; H, 5.86; N, 3.62. Found: C, 63.47; H, 5.82; N, 3.62.

Benzyl O-*(6*-O-*acetyl-2-azido-3,4-di*-O-*benzyl-2-deoxy*-α-D-*glucopyranosyl)*-*(1→4)*-O-*(methyl 2,3-di*-O-*benzyl*-β-D-*glucopyranosyluronate)*-*(1→4)*-O-*(6*-O-*acetyl-2-azido-3*-O-*benzyl-2-deoxy*-α-D-*glucopyranosyl)*-*(1→4)*-O-*(methyl 2*-O-*acetyl-3*-O-*benzyl*-α-L-*idopyranosyluronate)*-*(1→4)*-*6*-O-*acetyl-3*-O-*benzyl-2-benzyloxycarbonylamino-2-deoxy*-α-D-*glucopyranoside* (**16**). — A solution of freshly prepared 6-*O*-acetyl-2-azido-3,4-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl bromide[21] (**15**, 350 mg) and **14** (208 mg) in anhydrous dichloromethane (8 mL) was stirred for 30 min at room temperature in the presence of activated, powdered molecular sieve (4 Å, 100 mg). Silver triflate (201 mg) and 2,4,6-trimethylpyridine (114 μL) were added, and the mixture was stirred for 1 h in the dark at −20°, diluted with dichloromethane (50 mL), filtered, washed with aqueous 10% $KHSO_4$, dried (Na_2SO_4), and concentrated. The residue was eluted from a column of silica gel (10 g) with hexane–

ethyl acetate (4:3) to give **16** (203 mg, 77.5%), $[\alpha]_D$ +57° (*c* 1, chloroform). ^{1}H-N.m.r. data: unit D, δ 5.51 (d, 1 H, $J_{1,2}$ 3.7 Hz, H-1), 3.28 (dd, 1 H, $J_{1,2}$ 3.7, $J_{2,3}$ 10.7 Hz, H-2); unit E, 3.53 (s, 3 H, COOMe); unit F, δ 5.20 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 3.25 (dd, 1 H, $J_{1,2}$ 3.5, $J_{2,3}$ 10.5 Hz, H-2); unit G, δ 3.35 (s, 3 H, COOMe); 7.30 (m, 45 H, 9 Ph), 2.01, 2.06, and 2.09 (3 s, 12 H, 4 Ac).

Anal. Calc. for $C_{104}H_{113}N_7O_{31}$: C, 63.82; H, 5.76; N, 5.01. Found: C, 63.58; H, 5.76; N, 4.96.

Benzyl O-*(2-azido-3,4-di-*O*-benzyl-2-deoxy-α-*D*-glucopyranosyl)-(1→4)-*O*-(methyl 2,3-di-*O*-benzyl-β-*D*-glucopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-2-deoxy-α-*D*-glucopyranosyl)-(1→4)-*O*-(methyl 3-*O*-benzyl-α-*L*-idopyranosyluronate)-(1→4)-3-*O*-benzyl-2-benzyloxycarbonylamino-2-deoxy-α-*D*-glucopyranoside* (**17**). — 5M Sodium hydroxide (2.5 mL) was added at room temperature to a solution of **16** (193 mg) in chloroform (5 mL), methanol (18 mL), and water (2.5 mL). After 7 h, the mixture was diluted with chloroform (50 mL) and acidified with 6 M HCl (4 mL). The organic layer was washed with water, treated with ethereal diazomethane, and concentrated. The residue was eluted from a column of silica gel (7 g) with chloroform–methanol (40:1) to give amorphous **17** (96 mg, 55%), $[\alpha]_D$ +45° (*c* 1, chloroform). ^{1}H-N.m.r. data (CD_3OD): unit D, δ 5.50 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1); unit E, δ 3.61 (s, 1 H, COOMe); unit F, δ 5.11 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1); unit G, δ 5.24 (d, 1 H, $J_{1,2}$ 3.7 Hz, H-1), 3.09 (s, 3 H, COOMe); 7.30 (m, 45 H, 9 Ph).

Anal. Calc. for $C_{96}H_{105}N_7O_{27}$: C, 64.45; H, 5.91; N, 5.48. Found: C, 64.55; H, 5.94; N, 5.33.

Benzyl O-*(2-azido-3,4-di-*O*-benzyl-2-deoxy-6-*O*-sulfo-α-*D*-glucopyranosyl)-(1→4)-*O*-(methyl 2,3-di-*O*-benzyl-β-*D*-glucopyranosyluronate)-(1→4)-*O*-(2-azido-3-*O*-benzyl-2-deoxy-6-*O*-sulfo-α-*D*-glucopyranosyl)-(1→4)-*O*-(methyl 3-*O*-benzyl-2-*O*-sulfo-α-*L*-idopyranosyluronate)-(1→4)-3-*O*-benzyl-2-benzyloxycarbonylamino-2-deoxy-6-*O*-sulfo-α-*D*-glucopyranoside tetrasodium salt* (**18**). — A solution of **17** (88 mg) in *N*,*N*-dimethylformamide (1.5 mL) was stirred for 12 h at 50° in the presence of sulfur trioxide–trimethylamine complex (77 mg). The mixture was then cooled, chloroform (0.75 mL) and methanol (0.75 mL) were added, and the mixture was eluted from a column (2 × 20 cm) of Sephadex LH-20 equilibrated with chloroform–methanol (1:1), using the same solvent. The product was eluted from a column of silica gel (6 g) with ethyl acetate–pyridine–acetic acid–water (8:4:1:2) to yield a pure fraction, a solution of which in methanol (2 mL) was eluted from a column of Sephadex SP-25 (Na^+ form) with methanol–water (9:1) to afford **18** (97 mg, 89%), $[\alpha]_D$ +36° (*c* 1, methanol). ^{1}H-N.m.r. data (CD_3OD): unit D, δ 5.38 (d, 1 H, $J_{1,2}$ 3.7 Hz, H-1); unit E, δ 3.59 (s, 3 H, COOMe); unit G, δ 2.87 (s, 3 H, COOMe); 7.30 (m, 45 H, 9 Ph). This derivative was used immediately for the next reaction and was not submitted for elemental analysis.

*O-(2-Deoxy-6-*O*-sulfo-2-sulfoamino-α-*D*-glucopyranosyl)-(1→4)-*O*-(β-*D*-glucopyranosyluronic acid)-(1→4)-*O*-(2-deoxy-6-*O*-sulfo-2-sulfoamino-α-*D*-glucopyranosyl)-(1→4)-*O*-(2-*O*-sulfo-α-*L*-idopyranosyluronic acid)-(1→4)-2-deoxy-6-*

O-sulfo-2-sulfoamino-D-glucopyranose nonasodium salt (**21**). — A solution of **18** (55 mg) in methanol–water (9:1, 5 mL) was hydrogenated in the presence of 5% Pd-C (50 mg) for 8 days. The suspension was filtered and concentrated, and a solution of the residue in water (4.5 mL) was saponified for 3 h with 5M NaOH (1 mL). The pH was then adjusted to 9.5. Sulfur trioxide–trimethylamine complex (108 mg) was added, the pH being maintained at 9.5 by automatic addition of 0.5M NaOH. The mixture was filtered after 12 h and then eluted from a column (1.8 × 40 cm) of Sephadex G-50 equilibrated with water, using the same solvent. The pentasaccharide fractions were combined and concentrated. The residue was eluted from a column (1.25 × 45 cm) of Sephadex G-25 with 0.2M sodium chloride. The pentasaccharide fractions were eluted from a column (1.6 × 15 cm) of Dowex AGl-X2 resin equilibrated with 0.5M sodium chloride, using a gradient of sodium chloride (0.5 → 3M). The pentasaccharide fractions were combined and desalted on a column (1.6 × 40 cm) of Sephadex G-25. The pure fractions were combined and lyophilised to give **19** as an amorphous, white powder (10 mg, 30%), $[\alpha]_D$ +40° (*c* 1, water). ^{1}H-N.m.r. data (D_2O, 35°, internal TSP): unit D, δ 5.64 (d, 1 H, $J_{1,2}$ 3.7 Hz, H-1); unit E, δ 4.62 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 3.40 (dd, $J_{1,2}$ 8, $J_{2,3}$ 9 Hz, H-2), 3.87 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9 Hz, H-3); unit F, δ 5.45 (d, 1 H, $J_{1,2}$ 3.7 Hz, H-1), 3.25 (H-2), 3.70 (H-3); unit G, δ 5.24 (d, 1 H, $J_{1,2}$ 2.8 Hz, H-1), 4.33 (dd, 1 H, $J_{1,2}$ 2.8, $J_{2,3}$ 6.1 Hz, H-2), 4.11 (t, 1 H, $J_{3,4} = J_{4,5}$ = 3.3 Hz, H-4), 4.81 (d, 1 H, $J_{4,5}$ 3.3 Hz, H-5); unit H, δ 5.44 (d, 1 H, $J_{1,2}$ 3.7 Hz, H-1α), 4.70 (d, 1 H, $J_{1,2}$ 8.3 Hz, H-1β). No destructive elemental analysis was performed on this precious substance, but the ^{1}H-n.m.r. spectrum demonstrated >95% purity.

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