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Note

Synthesis of the basic disaccharide unit of heparin

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Heparin is a complex anionic polysaccharide used as a drug for its anticlotting and antithrombotic properties. The latter are mediated by the plasma protein antithrombin III (AT III). The binding of heparin to AT III involves a specific pentasaccharide sequence characterised by the presence of *N*-sulpho-3,6-di-*O*-sulpho-α-D-glucosamine, *N*-acetyl-6-*O*-sulpho-α-D-glucosamine, and β-D-glucuronic acid residues[1]. However the largest part of heparin molecules consists of the repetition of a disaccharide sequence made up of 2-*O*-sulpho-α-L-iduronic acid and *N*-sulpho-6-*O*-sulpho-α-D-glucosamine units. This sequence constitutes the so-called regular region of the polysaccharide[2]. As the prelude to the synthesis of longer homologous fragments, we report here the first synthesis of the regular-sequence disaccharide (**1**) and a new preparation of its 6-*O*-unsulphated counterpart[3] (**2**).

DISCUSSION

As usual for the synthesis of oligosaccharides of this type[4], the intermediates **4** and **9** are considered synthetic equivalents of **1** and **2**, respectively. Benzyl, 4-methoxybenzyl, and trityl ethers may be seen as latent hydroxyls, acetates (or, in the present case, hydroxyl at C-2 in **9**) as latent *O*-sulphates, and benzylamino as latent *N*-sulphate, while the carboxylate function is blocked either as a methyl (**4**) or a benzyl (**9**) ester. Because **3** was in stock from a previous synthesis, the key intermediate **4** was obtained from this known[5] disaccharide. The key reaction then, is the introduction of an alkyl protective group at position 4′ of **3**.

Etherification with a strong base and an alkyl halide is not applicable to **3** because of the risk of β-elimination. Compound **3** also failed to react with methyl iodide in *N*,*N*-dimethylformamide at room temperature or at 50°C in the presence

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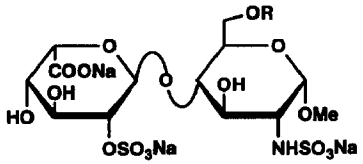

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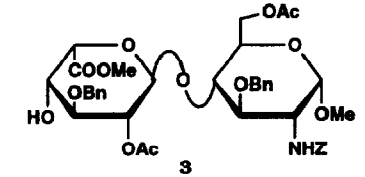
2 R = H



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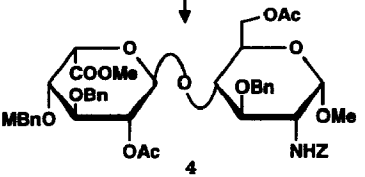

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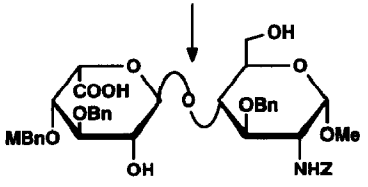

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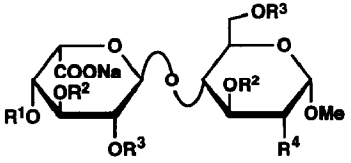
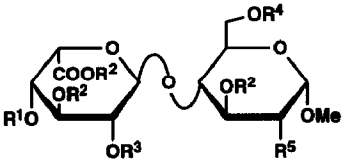


	R^1	R^2	R^3	R^4
6	MBn	Bn	SO_3Na	NHZ
7	H	H	SO_3Na	NH_2



	R^1	R^2	R^3	R^4	R^5
8	MBn	Bn	H	H	NHZ
9	MBn	Bn	H	Tr	NHZ
10	MBn	Bn	SO_3Na	Tr	NHZ
11	MBn	Bn	SO_3Na	H	NHZ
12	H	H	SO_3Na	H	NH_2

Z: benzyloxycarbonyl; MBn: *p*-methoxybenzyl.

of silver oxide (Ag_2O) or barium hydroxide. In contrast, using benzyl trichloroacetimidate[6] in a mixture of dichloromethane and cyclohexane, and triflic acid as catalyst, 4′-*O*-benzylation of **3** could be achieved, although in poor yield (30%), after 4 h. Finally, protection was best realised using *p*-methoxybenzyl trichloroace-

TABLE I

^{1}H NMR data for the ring protons of L-iduronic acid and D-glucosamine residues in some segments of the heparin molecule

Sequence	Chemical shifts in ppm from internal TSP (coupling constants in Hz)											
	L-Iduronic acid					D-Glucosamine						
	H-1 ($J_{1,2}$) ($J_{1,3}$)	H-2 ($J_{2,3}$) ($J_{2,4}$)	H-3 ($J_{3,4}$)	H-4 ($J_{4,5}$)	H-5	H-1 ($J_{1,2}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-4 ($J_{4,5}$)	H-5 ($J_{5,6a}$) ($J_{5,6b}$)	H-6a ($J_{6a,6b}$)	H-6b
Sequence **1**, in heparin[8,12]	5.19 (3.95)	4.32 (7.54)	4.17 (3.56)	4.15 (3.13)	4.77	5.40 (3.66)	3.28 (9.98)	3.67 (9.09)	3.77 (9.23)	4.03 (2.15) (2.15)	4.41 (−11.23)	4.28
in AT III binding site[8,13]	5.18 (2.80)	4.29 (6.10)	4.17 (3.30)	4.16 (3.40)	4.73	5.03 (3.6)	3.30 (10.4)	3.64 (8.2)	3.78 (9.8)	3.93 (2.6) (3.2)	4.40 (−12)	4.33
Disaccharide **1**	5.20 (2.6) (0.6)	4.30 (3.8) (0.6)	4.08 (3.8)	4.01 (2.7)	4.78	5.08 (3.60)	3.32 (10.3)	3.73 (8.80)	3.79 (8.85)	4.04 (2.44) (4.87)	4.38 (−11.36)	4.35
Disaccharide **2**	5.18 (2.4) (0.9)	4.28 (3.8) (0.9)	4.09 (4.0)	4.01 (2.5)	4.80	5.09 (3.60)	3.31 (10.0)	3.72 (8.65)	3.76 (9.75)	3.81 (2.27) (4.37)	3.95 (−12.33)	3.90

timidate, prepared in the same way[6] as benzyl trichloroacetimidate, in diethyl ether with triflic acid as catalyst. Compound **4** was thus readily obtained in 30 min with an excellent yield (85%). Saponification of **4** with sodium hydroxide in methanol gave **5** (92%), which was *O*-sulphated[4] with sulphur trioxide–triethylamine complex in *N*,*N*-dimethylformamide at 50°C to give **6** (71%). Hydrogenolysis with Pd–C in a mixture of *tert*-butyl alcohol and water furnished **7**, which was then *N*-sulphated[4] with sulphur trioxide–pyridine complex in water at pH 9.5 to give **1**, the basic disaccharide of heparin (53%).

Protection of the acidic function of **5** with benzyl bromide in *N*,*N*-dimethylformamide in the presence of potassium hydrogencarbonate gave **8** (85%). Compound **8** was selectively 6-*O*-tritylated (95%), *O*-sulphated[4] using sulphur trioxide–triethylamine complex in *N*,*N*-dimethylformamide (90%), detritylated, and hydrogenolysed to give **12** (75%). Selective *N*-sulphation[4] of **12** using sulphur trioxide–pyridine in water at pH 9.5 gave **2** (66%).

Compounds **1** and **2** were purified on a MONO Q HR 10/10 column (Pharmacia) eluted with a sodium chloride gradient. After desalting on a Sephadex G25 column, **1** and **2** were freeze-dried to yield white fluffy material.

^{1}H NMR data for **1** and **2** are reported in Table I. Comparison with the values obtained for the same disaccharide sequence in heparin and in the synthetic heparin pentasaccharide fragment representing the binding site of heparin to antithrombin III (AT III binding site) suggests a difference in the conformational state of the iduronic acid residue.

The conformation of iduronic acid has been investigated in detail and it is now acknowledged that the iduronate ring is in an equilibrium involving the three conformers $^1C_4$, $^4C_1$, and $^2S_0$ (refs. 3, 7–9). This conformational equilibrium is reflected in ^{1}H NMR coupling constants[8,10], particularly, $J_{2,3}$ and $J_{3,4}$. Thus a strong participation of $^2S_0$ in the equilibrium induces a large $J_{3,4}$ (3.9–7.1 Hz) and an even larger $J_{2,3}$ (8.6–10.4 Hz), while a strong participation of $^4C_1$ involves large and similar $J_{2,3}$ and $J_{3,4}$ (7.9–10.1 Hz), and a strong participation of $^1C_4$ involves similar but small (1.5–4.4 Hz) coupling constants. For example, the well documented large contribution of $^2S_0$, for sequence **1** in heparin and in the AT III binding site, appears in the coupling constants reported in Table I ($J_{2,3} > J_{3,4}$). In contrast, we can conclude that in disaccharides **1** and **2**, the $^1C_4$ conformer is strongly predominant since we observe a small $J_{1,2}$ coupling and small and similar $J_{2,3}$ and $J_{3,4}$. The long range $J_{1,3}$ and $J_{2,4}$ couplings also support this conclusion.

With regard to the glucosamine units, comparison of the coupling constants between protons 5 and 6a, and 5 and 6b, in **1** and **2** indicates a weak contribution of the sulphate group to the rotamer equilibrium around the C-5–C-6 bond. An equilibrium between *gt* and *gg* conformers is generally observed[11].

EXPERIMENTAL

General.—^{1}H NMR spectra were recorded with Bruker AM 100 and AM 600 instruments for solutions in $CDCl_3$ or D_2O. Before analysis in D_2O, samples were passed through a Chelex (BioRad) ion-exchange column and lyophilized three times from D_2O. Melting points were determined in capillary tubes with a Mettler apparatus and are uncorrected. Optical rotations were measured with a Perkin–Elmer Model 141 polarimeter at 20°C. Compound purity was checked 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 (Merck 63–200 μm). Elemental analyses were carried out by the Service d'Analyses Sanofi, Gentilly.

Methyl 6-O-acetyl-3-O-benzyl-2-benzyloxycarbonylamino-2-deoxy-4-O-(methyl 2-O-acetyl-3-O-benzyl-4-O-p-methoxybenzyl-α-L-idopyranosyluronate)-α-D-glucopyranoside (**4**).—*p*-Methoxybenzyl trichloroacetimidate (0.36 g, 1.30 mmol) was added at 20°C under Ar to a solution of **3** (0.5 g, 0.64 mmol) in 1:5 CH_2Cl_2–ether (12 mL). Trifluoromethanesulphonic acid in ether (0.1 M, 0.1 mL) was added and the mixture was stirred for 30 min at 20°C. The reaction was stopped with 5% aq $NaHCO_3$ (0.5 mL). After filtration of the mixture and evaporation of the filtrate to dryness the crude residual syrup was purified silica gel chromatography (1:1 toluene-ether), yielding crystalline **4** (490 mg, 85%); mp 61°C; $[\alpha]_D^{20} + 94°$ (*c* 1, CH_2Cl_2). Anal. Calcd for $C_{48}H_{55}NO_{16}$: C, 63.92; H, 6.14; N, 1.55. Found: C, 63.99; H, 6.18; N, 1.48.

Methyl 3-O-benzyl-4-O-(3-O-benzyl-4-O-p-methoxybenzyl-α-L-idopyranosyluronic acid)-2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside (**5**).—To a solution of **4** (0.1 g, 0.11 mmol) in 5:1 CH_2Cl_2–MeOH (6 mL) 4 M aq NaOH (0.16 mL, 0.66 mmol) was added. After 2 h at 20°C the pH was adjusted to 2 by the addition of 6

N HCl. Dichloromethane was added, the organic phase was washed with water and dried ($MgSO_4$), and the solvent was evaporated to give **5** (80 mg, 92%) as a foam; mp 114°C; $[\alpha]_D^{20} - 33°$ (*c* 0.60, CH_2Cl_2). Anal. Calcd for $C_{43}H_{49}NO_{14} \cdot 0.5H_2O$: C, 63.53; H, 6.19; N, 1.72. Found: C, 63.42; H, 6.16; N, 1.68.

Crude methyl 3-O-benzyl-4-O-(3-O-benzyl-4-O-p-methoxybenzyl-2-O-sulpho-α-L-idopyranosyluronic acid)-2-benzyloxycarbonylamino-2-deoxy-6-O-sulpho-α-D-glucopyranoside, trisodium salt (**6**).—To a solution of **5** (145 mg, 0.184 mmol) in dry DMF (3 mL) under Ar, SO_3–Et_3N complex (460 mg, 2.5 mmol) was added and the mixture was stirred at 50°C for 48 h. Saturated aq $NaHCO_3$ was added (5 mL) and stirring was continued for 3 h at 20°C, then the mixture was evaporated to dryness. Compound **6** was extracted from the salts with MeOH. Filtration and evaporation to dryness gave a white foam (148 mg, 71%), used for the next step without purification.

Crude methyl 2-amino-2-deoxy-6-O-sulpho-4-O-(2-O-sulpho-α-L-idopyranosyluronic acid)-α-D-glucopyranoside, disodium salt (**7**).—A solution of **6** (86 mg, 0.085 mmol) in 3:2 *tert*-butyl alcohol–water (3 mL) was ultrafiltered (0.22 μm), then 10% Pd–C (50 mg) was added under N_2, and H_2 was bubbled through the stirred solution for 20 h at 20°C. After filtration and evaporation to dryness, **7** was obtained as a colourless syrup (61 mg). Complete removal of benzyl groups was checked by recording UV and ^1H NMR spectra. The product was used for the next step without further purification.

Methyl 2-deoxy-6-O-sulpho-2-sulphoamino-4-O-(2-O-sulpho-α-L-idopyranosyluronic acid)-α-D-glucopyranoside, tetrasodium salt (**1**).—A solution of **7** (61 mg) in water (5 mL) was maintained at pH 9.5 by the addition of 2 M NaOH with a pH stat. Sulphur trioxide–pyridine complex (28 mg, 0.17 mmol) was added, and after 2 h of stirring at 20°C the same amount of SO_3–pyridine complex was again added. After 2 h at 20°C the mixture was layered on top of a Sephadex G25F column, which was eluted with 0.2 M aq NaCl. After pooling the fractions and concentration, the product was desalted on a Sephadex G25F column. Evaporation to dryness afforded **1** (white foam, 50 mg), which was purified on Mono Q ion-exchange resin (elution with a linear 0.12 to 1.2 M NaCl gradient), desalted on Sephadex G25F, and freeze-dried, yielding pure (as judged by ^1H NMR) **1** (29 mg, 53% from **6**); $[\alpha]_D^{20} + 36°$ (*c* 0.3, H_2O). ^1H NMR (600 MHz, D_2O): See Table I.

Methyl 3-O-benzyl-4-O-(benzyl 3-O-benzyl-4-O-p-methoxybenzyl-α-L-idopyranosyluronate)-2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside (**8**).—Potassium hydrogencarbonate (86 mg) and benzyl bromide (75 μL, 0.63 mmol) were added under Ar to a solution of **5** (230 mg, 0.286 mmol) in dry DMF (12 mL). After 15 h of stirring at 20°C water was introduced and the product was extracted with CH_2Cl_2. The organic layer was washed with water, dried ($MgSO_4$), filtered, and evaporated to dryness. Product **8** was obtained (218 mg, 85%) after crystallisation (EtOAc–hexane); mp 87°C; $[\alpha]_D^{20} - 10°$ (*c* 0.8, CH_2Cl_2). Anal. Calcd for $C_{50}H_{55}NO_{14} \cdot 0.5H_2O$. C, 66.50; H, 6.14; N, 1.55. Found: C, 66.85; H, 6.21; N, 1.51.

Crude methyl 3-O-benzyl-4-O-(benzyl 3-O-benzyl-4-O-p-methoxybenzyl-α-L-idopyranosyluronate)-2-benzyloxycarbonylamino-2-deoxy-6-O-triphenylmethyl-α-D-glucopyranoside (**9**).—Triethylamine (0.5 mL, 3.55 mmol) was added under Ar to a solution of **8** (64 mg, 0.071 mmol) in 1:3 CH_2Cl_2–toluene (4 mL). The solution was heated to 110°C and triphenylmethyl chloride (120 mg, 0.42 mmol) was added in thirds at 2 h intervals. The reaction was stopped by the addition of MeOH (0.5 mL), and after 15 min of stirring at 20°C water was added. The mixture was diluted with CH_2Cl_2 and the organic layer was washed with brine, dried ($MgSO_4$), filtered, and evaporated to dryness to give a syrup, which was purified on silica gel (4:1 hexane–acetone). Compound **9** (77 mg, 95%) was obtained as a yellowish foam and used for the next step without further purification.

Crude methyl 3-O-benzyl-4-O-(benzyl 3-O-benzyl-4-O-p-methoxybenzyl-2-O-sulpho-α-L-idopyranosyluronate)-2-benzyloxycarbonylamino-2-deoxy-6-O-triphenylmethyl-α-D-glucopyranoside, sodium salt (**10**).—To a solution of **9** (123 mg, 0.108 mmol) in dry DMF (2 mL) under Ar, SO_3–Et_3N complex (98 mg, 0.54 mmol) was added, and the mixture was stirred at 55°C for 3 h. Saturated aq $NaHCO_3$ was added, and after 2 h of stirring at 20°C the solution was evaporated to dryness. The white solid residue was dissolved in CH_2Cl_2 and the organic layer was washed with water, dried, filtered, and evaporated to dryness. Compound **10** (120 mg, 90%) was obtained as a yellowish foam and used directly for the next step.

Methyl 3-O-benzyl-4-O-(benzyl 3-O-benzyl-4-O-p-methoxybenzyl-2-O-sulpho-α-L-idopyranosyluronate)-2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside, sodium salt (**11**).—An aq 70% solution of perchloric acid (73 μL) was added to a cooled (0°C) solution of **10** (120 mg, 0.096 mmol) in CH_2Cl_2 (12 mL). The mixture was stirred for 5 min, then washed with water. The organic layer was dried ($MgSO_4$) and evaporated to dryness. The syrup obtained was purified on silica gel (20:1 CH_2Cl_2–MeOH) to give pure (as judged by ^1H NMR) **11** (76 mg, 78%); $[\alpha]_D^{20}$ + 20° (*c* 0.95; MeOH).

Crude methyl 2-amino-2-deoxy-4-O-(2-O-sulpho-α-L-idopyranosyluronic acid)-α-D-glucopyranoside, monosodium salt (**12**).—To a solution of **11** (76 mg, 0.075 mmol) in a mixture of CH_2Cl_2 (2 mL), *tert*-butyl alcohol (5 mL), and water (1 mL), 10% Pd–C (30 mg) was added under N_2. Hydrogen was bubbled through the mixture with stirring for 15 h. Removal of the catalyst by filtration and evaporation to dryness gave **12** (34.0 mg, 97%) which was used in the next step without purification.

Methyl 2-deoxy-2-sulphoamino-4-O-(2-O-sulpho-α-L-idopyranosyluronic acid)-α-D-glucopyranoside, trisodium salt (**2**).—A solution of **12** (76 mg, 0.075 mmol) in water (5 mL) was maintained at pH 9.5 by controlled addition of 2 M NaOH via a pH stat. Sulphur trioxide–pyridine complex (24 mg, 0.30 mmol) was added at 20°C in two portions, the second after a h interval. After 2 h the mixture was layered on top of a Sephadex G 25 F chromatography column, made up in and eluted with 0.2 M NaCl. Fractions were pooled and the product was desalted on Sephadex G 25 F, then purified on a Mono Q ion-exchange column eluted with a linear (0.12 to 0.8

M) NaCl gradient. Compound **2** (30 mg, 66% from **9**) was obtained after desalting on Sephadex G 25 and freeze-drying; $[\alpha]_D^{20} + 35°$ (*c* 0.2, H_2O). ^1H NMR (600 MHz, D_2O): See Table I.

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