Carbohydrate Research, 73 (1979) 332–336

Note

The preparation of a heparin analogue from alginic acid

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(Received September 25th, 1978; accepted for publication, November 10th, 1978)

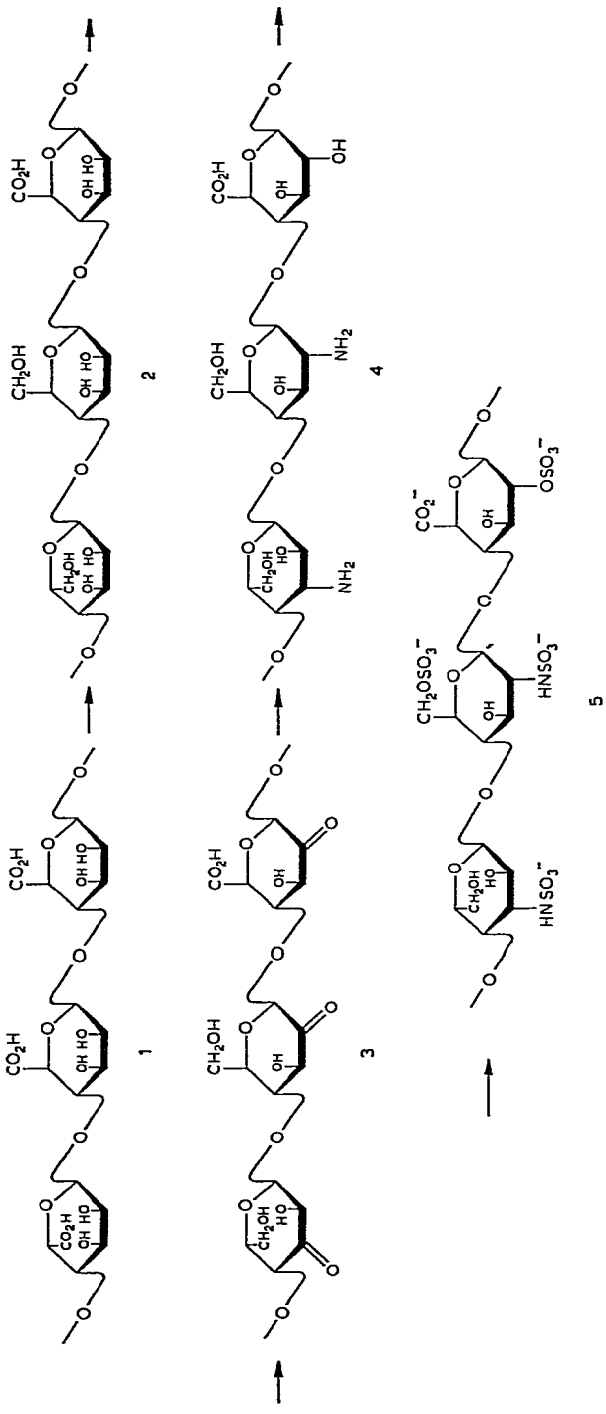
Alginic acid (**1**) contains (1 → 4)-linked β-D-mannopyranosyluronic and α-L-gulopyranosyluronic acid residues. This polysaccharide is a block polymer containing sequences of predominantly mixed structure, alternating with sequences built up solely of L-guluronic acid or D-mannuronic acid residues[1].

Heparin contains (1 → 4)-linked 2-amino-2-deoxy-α-D-glucopyranosyl residues, α-L-idopyranosyluronic acid residues, and, usually, smaller proportions of β-D-glucopyranosyluronic acid residues. The various sugar residues are incorporated to form a polymer of alternating hexosamine and uronic acid residues. All of the residues are partially *O*-sulphated; in addition, most of the 2-amino-2-deoxy-D-glucose residues are *N*-sulphated, the remainder being *N*-acetylated[2].

When methyl β-D-mannopyranoside is oxidised with aqueous bromine at pH 7, oxidation occurs[3] mainly at C-2. The resulting methyl β-D-*arabino*-hexopyranosidulose can be converted into methyl 2-amino-2-deoxy-β-D-glucopyranoside by reductive amination[4,5]. Similar treatment of carboxyl-reduced alginic acid should yield a polymer containing 2-amino-2-deoxy-β-D-glucopyranosyl residues. Further, when L-guluronic acid, the C-2 epimer of L-iduronic acid, with an axial hydroxyl group at C-2 in the $^4C_1$ conformation, is oxidised at C-2 and reduced, L-iduronic acid should be obtained. Thus, it should be possible to convert alginic acid into a polysaccharide containing the residues that are present in heparin.

The reaction sequence is presented in **1**–**5**; the formulae give information on important chemical units, but should not be given any deeper significance. Part of the uronic acid residues in **1** were reduced[6] and the resulting polymer (**2**) was oxidised with aqueous bromine at pH 7, to give the modified alginic acid **3**. Reductive amination of **3** gave **4**, which was then partially sulphated, yielding the heparin analogue **5**.

As was evident from gel-filtration data, **5** was partially degraded during the reactions, and it also showed rather low, whole-blood anticoagulant activity (2.5 I.U./mg, compared with 110–150 I.U./mg for commercial heparin). On affinity chromato-


1
2
3
4
5

graphy on antithrombin III (AT III)–Sepharose[7], ~ 7% of the material of high molecular weight was adsorbed (**5a**) and could later be eluted by increasing the ionic strength of the buffer. The anticoagulant activity of **5a** was 35 units/mg in the whole-blood clotting assay, 91 units/mg in the thrombin-dependent assay, and 32 units/mg in the anti-factor X_a-dependent assay for heparin. The elemental analyses of **5a** and the fraction that did not bind to AT III (**5b**) revealed that **5a** contained a higher proportion of sulphur and nitrogen than did **5b**. In agreement with the elemental analyses, **5a** also contained considerably more D-glucopyranosylamine residues (14%) than **5b** (< 1%). Only traces of other amino-sugar residues could be detected. The neutral-sugar content of **5a** was 6% (glucose–galactose–mannose–gulose 1:1:1:2) and that of **5b** was 28% (glucose–galactose–mannose–gulose 1:0.1:2:3). Possibly because of non-specific degradations of the uronic acid residues in **5a** and **5b** during hydrolysis, ambiguous results were obtained in the quantitative g.l.c. analyses of these sugars. However, the presence of L-guluronic and D-mannuronic acid residues was observed in both **5a** and **5b**. It is noteworthy that neither **5a** nor **5b** contained L-iduronic acid. In the reductive amination reaction, some of the keto-glycosyl units are reduced to the parent sugars or their epimers, and as no L-iduronic acid was found, it seems very likely that oxidation does not occur on C-2 of the α-L-guluronic acid residues. This is possibly due to the fact that these units have the $^1C_4$ conformation, with HO-2 equatorial, in alginic acid[8]. As no appropriate reference materials are available for such polysaccharides as **5a** and **5b**, accurate quantifications of total uronic acid content by colour reactions are impossible.

Sulphation with sulphur trioxide–pyridine, occurs in the first place on amino groups[9]. Primary alcohol groups are more easily sulphated than secondary alcohols[10]. Consequently, it is reasonable to assume that, in **5a** and **5b**, all amino sugars are *N*-sulphated, and most of the primary hydroxyl groups and some of the secondary hydroxyl groups are *O*-sulphated.

The comparatively high content of 2-amino-2-deoxy-D-glucose in **5a** indicates that this sugar is essential for the activation of AT III.

Modifications of alginic acids having different proportions of L-guluronic and D-mannuronic acid residues and variations of the reaction conditions are in progress.

EXPERIMENTAL

General methods. — Concentrations were performed at reduced pressure at bath temperatures not exceeding 40°C. Optical rotations were determined with a Perkin–Elmer 141 polarimeter. G.l.c. was performed with a Packard model 427 instrument, fitted with a flame-ionisation detector. For quantitative g.l.c., an Autolab minigrator was used. Separations of aldononitriles were performed on glass columns (240 × 0.15 cm) containing 3% of NPGS on Varaport 30 (100–120 mesh) at 150 → 200°C (2°/min). G.l.c.–m.s. was performed with a Varian CH-7 gas chromatograph–mass spectrometer. P.c. was performed on Whatman No. 1 paper with ethyl acetate–acetic acid–water (3:1:1). Electrophoresis was performed on Whatman No. 1 paper with

sodium acetate buffer (pH 4.5) at 25°C. Detection was achieved with *p*-anisidine hydrochloride and silver nitrate–sodium hydroxide.

Conversion of alginic acid into the heparin analogue **5**. — (*a*) *Reduction of alginic acid*[6] (**1**). Sodium alginate, $[\alpha]_{578}$ −117° (Manugel DJ, Alginate Industries; 1 g, 5 mmol), was dissolved in water (100 ml), and the pH was adjusted to 4.75 with 0.1M HCl. 1-Ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride (2.2 g, 11.5 mmol) was added in small portions at room temperature to the stirred solution, and the pH was automatically maintained at 4.75 by the addition of 0.1M HCl (Methrom E 300 B pH-meter). After the last addition of the carbodi-imide hydrochloride, the solution was kept under continuous stirring at room temperature until no further change in pH was observed (~ 30 min). After the last addition of sodium borohydride, the reaction mixture was kept at room temperature for 2 h. The solution was acidified (HCl), and dialysed against distilled water. After freeze-drying, **2** was obtained (0.8 g). The uronic acid content was ~ 50%, as determined with carbazole[11].

(*b*) *Oxidation*[3] *of* **2**. The partially carboxyl-reduced alginic acid (**2**, 200 mg) was dissolved in water (25 ml), and an aqueous solution of bromine (12 ml, 0.1M) was added at room temperature to the stirred solution. The pH was kept at 7 by automatic addition of NaOH (0.1M). When the oxidant was consumed (6 h), the pH was adjusted to 6.5, and the solution containing **3** was concentrated to about one-third of its volume.

(*c*) *Reductive amination*[4,5] *of* **3**. To the stirred, concentrated solution of **3** (200 mg) were added ammonium acetate (2 g) and sodium cyanoborohydride (2 g) at room temperature. The pH was adjusted to 6.0 and the solution stirred at 40°C for 2 days. In order to reduce unreacted carbonyl groups, the pH was adjusted to 4.0 and the reaction mixture was allowed to stand at room temperature for an additional 2 h. The polysaccharide (**4**) was precipitated by addition of ethanol, collected on a glass filter, washed with ethanol (2 litres), and dried at room temperature; yield of **4**, 150 mg (Found: C, 35.4; H, 4.9; N, 1.25%).

(*d*) *Sulphation*[12] *of* **4**. Compound **4** (150 mg) was treated with glacial acetic acid (2 h, room temperature). After filtration, **4** was suspended in *N*,*N*-dimethylformamide (5 ml). The stirred suspension was cooled to 0°C and sulphur trioxide–pyridine complex (1.5 g, Fluka) was added. The solution was allowed to reach room temperature gradually and was then stirred for an additional 2 h. Pyridine (3 ml) was added and the polysaccharide **5** was precipitated with acetone–methanol (9:1). The precipitate was dissolved in water and the pH was adjusted to 7.0 with M sodium hydroxide. Reprecipitation with acetone–methanol (9:1) yielded the sodium salt of **5** (150 mg) (Found: C, 23.8; H, 2.8; N, 0.5; O, 48.2; S, 12.5; Na, 11.2%).

Fractionation of **5** *on antithrombin III–Sepharose*. — The modified alginic acid (**5**, 100 mg) was subjected to affinity chromatography on a column of AT III–Sepharose[7], equilibrated in a buffer (0.05M Tris and 0.15M sodium chloride) at pH 7.4. About 7% of the material (**5a**) was bound to the AT-III gel, and the rest (**5b**) was eluted with the equilibration buffer volume. The retained material (**5a**) was then desorbed with 0.05M Tris buffer and 0.15M sodium chloride at pH 7.4. The fractions **5a** and **5b** were desalted on Sephadex G-25 and lyophilised. Fraction **5a** (7 mg) had $[\alpha]_{578}$

+7.1° (Found: C, 21.2; H, 3.4; N, 2.4; O, 58.7; S, 7.5; Na, 6.8%). The material of high molecular weight from **5b** (eluted with the void volume on G-25; 10 mg) had $[\alpha]_{578}$ —74° (Found: C, 25.5; H, 3.8; N, 0.9; O, 60.1; S, 4.9; Na, 4.8%).

Samples of **5a** and **5b** (1 mg of each) were hydrolysed (HCl, 6M) for 24 h at 110°C, and the products were analysed on an automatic amino-acid analyser[13]; authentic samples of 2-amino-2-deoxy-D-glucose and -D-mannose were used as standards. The hydrolysate of **5a** contained 15% of 2-amino-2-deoxy-D-glucose, but that from **5b** contained less than 1% of this sugar. Only very small amounts of 2-amino-2-deoxy-D-mannose could be detected in the hydrolysates from **5a** and **5b**. The 2-amino-2-deoxy-D-glucose contents of **5a** (15%) and **5b** (1.2%) were also measured by the deamination procedure devised by Lagunoff and Warren[14].

The total uronic acid content in **5a** and **5b** was comparable as determined by the carbazole method[11], but an accurate quantification was impossible, as no reference material was available. G.l.c.[15], p.c., and electrophoresis of hydrolysates of **5a** and **5b** revealed the presence of guluronic acid, but no iduronic acid and only traces of mannuronic acid[16].

The neutral-sugar content in nitrous acid-degraded[17] and hydrolysed **5a** and **5b** was measured by the aldononitrile method[18] with *myo*-inositol as the internal standard; **5a** contained glucose (2%), mannose (7%), gulose (3%), and galactose (2%), and **5b** contained glucose (6%), mannose (9%), gulose (9%), and galactose (traces).

ACKNOWLEDGMENTS

The authors thank Professor Ulf Lindahl for valuable discussions and help with some of the analyses, and Professor Olof Theander for valuable discussions. The work was financially supported by KABI AB, Stockholm.

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