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SYNTHESIS OF AN ANTITHROMBIN BINDING HEPARIN-LIKE PENTASACCHARIDE LACKING 6-O SULPHATE AT ITS REDUCING END.

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Summary. The synthesis of a pentasaccharide of the structure of the antithrombin (AT-III) binding sequence, but lacking the 6-O sulphate group at the reducing end, is described and its α-Xa activity determined.

The last few years it has become evident that the important anticoagulant drug heparin, which is a mixture of sulphated glycosaminoglycans, contains a unique pentasaccharide subunit (Fig.I) capable of binding to antithrombin (AT-III)[1,2]. This pentasaccharide contributes 94% of the binding energy of the enzymatically obtained octasaccharide

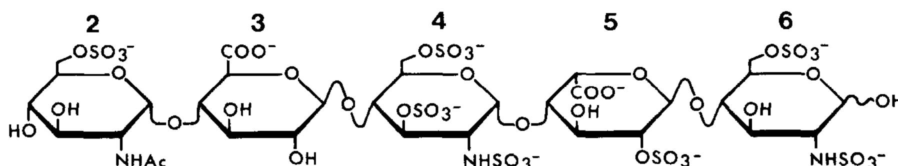
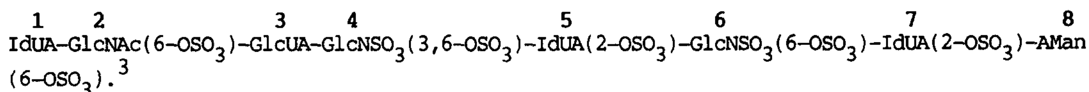


Fig. I

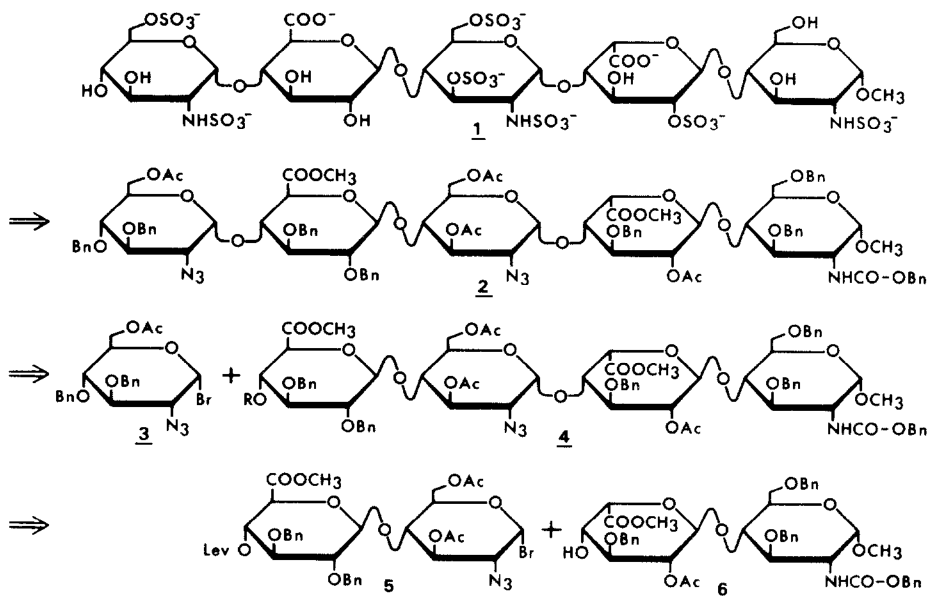

1 2 3 4 5 6 7 8
IdUA-GlcNAc(6-OSO_3)-GlcUA-GlcNSO_3(3,6-OSO_3)-IdUA(2-OSO_3)-GlcNSO_3(6-OSO_3)-IdUA(2-OSO_3)-AMan (6-OSO_3).[3]

Several studies have been undertaken to elucidate the role of the sulphate groups at nitrogen and oxygen. The most firm evidence of the influence of sulphate groups was obtained for the nitrogen sulphates. Thus, Lindahl showed that the acetyl group of unit **2** could be removed and replaced by a sulphate group without loss of antithrombin affinity[2]. On the other hand, the N-sulphate groups at units **4** and **6** were shown to be involved in interaction of the pentasaccharide with antithrombin.[3,4,5]

The role of some of the O-sulphate groups was also unambiguously determined. The 3-O-sulphate at unit **4**, which exclusively occurs in the binding pentasaccharide, proved to be essential for affinity. Sinaÿ and Petitou synthesized the pentasaccharide **2-6** (Fig.I) with sulphate at the nitrogen of unit **2**, and with[6] and without[7a] a 3-O sulphate group at unit **4**. The penta-saccharide with the 3-O sulphate group displayed an anti-Xa activity of about 600 U/mg in the

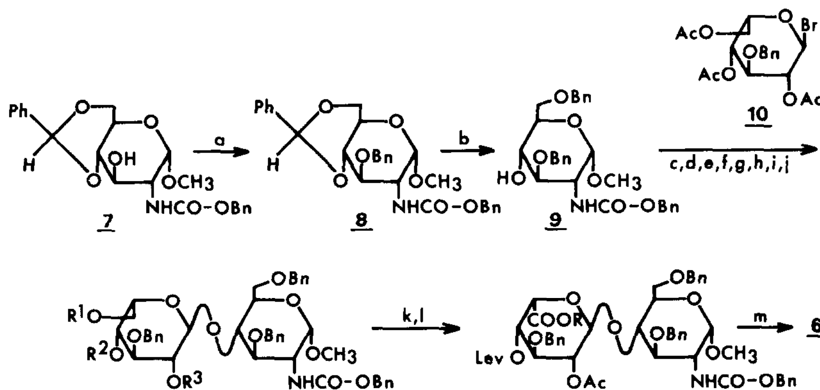
amidolytic assay in buffer with chromogenic substrate S2222, whereas the pentasaccharide lacking this sulphate group, elicited no anti-Xa activity.[7] Lindahl showed that removal of the 6-O sulphate group from unit **2** led to a virtually complete loss of affinity for antithrombin.[8] The 6-O sulphate group of unit **4** is assumed to be non-essential,[2,9b] whereas the role of the 2-O sulphate group of unit **5** and the 6-O sulphate group of unit **6** remains unclear up to now. As far as the sulphate groups at units **5** and **6** are concerned, Japanese workers compared the activity of porcine and cetacean heparin, the latter containing after nitrous acid degradation non-sulphated anhydromannose (unit **6**) and a larger portion of non-sulphated iduronic acid. Their observations suggest, that the 2-O sulphate of unit **5** is important for activity[9]. Further, they found that binding to antithrombin was significantly stronger for the porcine than for the cetacean high affinity octasaccharide, which they attributed to the absence of the 6-O sulphate group at the reducing end of cetacean octasaccharide.[9c] In contradiction with this, Atha et al.[3] concluded from binding studies that it is the N-sulphate and not the O-sulphate of unit **6** that is involved in the interaction with antithrombin.

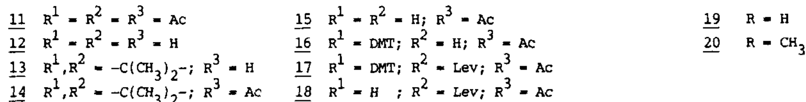
As part of an investigation on the synthesis of sulphated pentasaccharides of the heparin type[10], we embarked on the synthesis of pentasaccharide 1, which lacks the 6-O sulphate at unit **6**, in order to compare its biological activity with the pentasaccharide synthesized by Sinaÿ and Petitou et al.[6] and to determine the biological significance of the 6-O sulphate group of unit **6**. Pentasaccharide 1 was synthesized from the building blocks 3, 5 and 6 as depicted in the retrosynthetic Scheme I.[16]



Scheme I

Building block 6 was prepared from known methyl 4,6-O-benzylidene-2-(benzyloxycarbonyl)-amino-2-deoxy-α-D-glucopyranoside 7[11] (Scheme II), which was benzylated to 8 with benzyl-bromide in dioxane with KOH as base.





Reagents: a. BnBr/KOH/dioxane;73%; b. $NaCNBH_3$/THF/HCl;71%; c. $HgBr_2$,CH_2Cl_2,mol.sieves 4A;55%; d. KOtBu; e. $(CH_3)_2C(OCH_3)_2$/ DMF; f. Ac_2O/pyridine; g. HOAc; h. $C_6H_5C(C_6H_4OCH_3)_2Cl$/THF/pyridine; i. $(CH_3COCH_2CH_2CO)_2O$; j. HOAc(80%); k. CrO_3/acetone/ H_2SO_4;62%; l. CH_2N_2,100%; m. $N_2H_4.H_2O$; 100%.

Scheme II

Reductive ring opening according to the method of Garegg et al.[12] provided 6-O-benzyl derivative 9 in high yield. Koenigs-Knorr coupling of 9 with idopyranosyl bromide 10[10] gave disaccharide 11 in 55% yield. Cleavage of the acetyl groups was achieved with potassium t-butylate in a mixture of dioxane/methanol. The crude product 12 was successively treated with dimethoxypropane/pTSOH to protect the 4',6'-hydroxyl groups (13) and with acetic anhydride/pyridine to protect the remaining hydroxyl group (14). Without purification the isopropylidene group of 14 was removed with 80% acetic acid to give 15 and the primary hydroxyl group was selectively protected by treatment with dimethoxytritylchloride in THF/pyridine. The 4'-OH group of 16 was masked with a levulinoyl protective group by treatment with levulinic acid anhydride in pyridine with dimethylaminopyridine as catalyst (17), after which the dimethoxytrityl group was cleaved with 80% acetic acid (18). After purification by silica chromatography, 18 was obtained in an overall yield of 27% from 11. Oxidation of 18 was performed by Jones oxidation[13], and the acid 19 was directly esterified with diazomethane to give iduronic acid derivative 20 in 62% yield. The levulinoyl group was quantitatively removed by treatment with hydrazine hydrate in pyridine/acetic acid to give building block 6.[14] Disaccharide 6 was coupled with bromide 5[10] to tetrasaccharide 4 (R = Lev) in dichloromethane at -20°C using silver triflate as promoter and 2,6-di-t-butylpyridine[15] as acid scavenger. After silica chromatography (toluene-acetone 9:1) 47% of pure tetrasaccharide was obtained, from which the levulinoyl group was cleaved with hydrazine hydrate, to afford 4

(R = H)[14], which in turn was coupled with known 3[10] at -60° with silver triflate as promoter and collidine as acid scavenger. After silica chromatography (toluene-acetone 95:5) 78% of pentasaccharide 2[14] was obtained. Saponification of the acetyl groups and the methylesters ($CHCl_3$-CH_3OH-H_2O/8.5 M NaOH), O-sulphation ($Me_3N.SO_3$/DMF, 50°, 24 h), hydrogenolysis (H_2, Pd/C, CH_3OH/H_2O, 5 days) and N-sulphation ($Me_3N.SO_3/H_2O$, r.t., pH 9.5) then gave 1. Crude 1 was desalted on Sephadex G-10 and fractionated on DEAE-Sephadex to afford pure 1 in a yield of 30% from 2. The ^{1}H-NMR spectrum of 1 (360 MHz, D_2O) showed δ 5.62 (d, $J_{1,2}$ 4 Hz, H-1''''), 5.45(d, $J_{1,2}$ 3.5 Hz, H-1''), 5.17 (br d, $J_{1,2}$ 3.5 Hz, H-1'), 5.02 (d, $J_{1,2}$ 4 Hz, H-1), 4,62 (d, $J_{1,2}$ 8 Hz, H-1''') and the ^{13}C-NMR (90 MHz, D_2O) showed δ 101.31 (C-1'''), 99.60 (C-1'), 98.45 (C-1), 97,73 (C-1''''), 96.46 (C-1'').

The synthetic pentasaccharide 1 when tested for its α-Xa activity in the amidolytic assay displayed an activity of 150 U/mg, which is about 25% of the activity of the natural pentasaccharide synthesized by Sinaÿ and Petitou et al. This indicates that the 6-O sulphate group at unit 6, although not indispensable, plays a critical role in the binding with antithrombin.

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14. ^{1}H-NMR (360 MHz, $CDCl_3$) compound 6: δ 5.13 (br., H-1'), 4.69 (d, J 4 Hz, H-1), 3.39 (s, $COOCH_3$), 3.34 (s, OCH_3); compound 4 (R = H): δ 5.37 (dd, J 9 Hz, H-3'''), 5.30 (d, J 3,5 Hz, H-1'), 4.98 (br.s, H-1''), 4.70 (d, J 3.5 Hz, H-1), 4.33 (d, J 7.5 Hz, H-1'''), 3.78, 3.51 (s, $COOCH_3$); 3.32 (s, OCH_3); 3.20 (dd, J 3.5, H-2''); compound 2 (200 MHz, $CDCl_3$) δ 5.49 (d, J 3 Hz, H-1''''), 5.36 (dd, J 9 Hz, H-3''), 5.29 (d, J 3.5 Hz, H-1'), 4.91 (d, J 3.5 Hz, H-1''), 3.73, 3.49 (s, $COOCH_3$), 3.32 (s, OCH_3), 3.19 (dd, J 3.5, H-2'').
15. See note 29 of ref. 10.
16. Abbreviations: Bn=benzyl, Ph=phenyl, Ac=acetyl, Lev=levulinoyl, DMT=dimethoxytrityl.

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