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SYNTHESIS OF HEPARIN PARTIAL STRUCTURES AND THEIR BINDING ACTIVITIES TO PLATELETS

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Abstract: A synthetic pentasaccharide corresponding to the antithrombin III-binding region in heparin was also found to bind to human platelets. To identify the platelet-binding site in the pentasaccharide which is expected to be a novel sequence in heparin responsible for its platelet-binding, five partial structures of this particular pentasaccharide were synthesized. In a competitive assay using [^{3}H]-heparin, a trisaccharide, *O*-(2-deoxy-2-sulfamido-3,6-di-*O*-sulfo-α-D-glucopyranosyl)-(1→4)-*O*-(2-*O*-sulfo-α-L-idopyranosyluronic acid)-(1→4)-2-deoxy-2-sulfamido-6-*O*-sulfo-α-D-glucopyranose, was concluded to be a high-affinity site for heparin's binding to platelets.

Heparin, a heterogeneous sulfated polysaccharide known as an anticoagulant drug,[1] binds to human platelets and alters their functions,[2] that may be mediated by a binding interaction between specific structures in heparin and specific protein(s) on the platelet surface. We previously found that a disaccharide in heparin, *O*-(2-deoxy-2-sulfamido-6-*O*-sulfo-α-D-glucopyranosyl)-(1→4)-2-*O*-sulfo-α-L-idopyranosyluronic acid (abbreviated as GlcNS6S-IdoA2S), is a key unit responsible for binding to platelets.[3,4] Then, we further evaluated various heparin fragments and indicated the effect of the conformation of the inner sulfated iduronic acid on the binding activity.[5] Furthermore, we employed a modified binding assay system using a high concentration of [^{3}H]-labelled heparin to find high-affinity site(s) in heparin for platelets and elucidated that more than two units of GlcNS6S-IdoA2S are necessary to exhibit high-affinity, indicating the operation of a so-called clustering effect based on GlcNS6S-IdoA2S.[6] In addition, we found so far that the synthetic pentasaccharide[7] corresponding to the antithrombin III (AT III) binding region in heparin (**1**) possesses a distinct binding potency, although it does not contain GlcNS6S-IdoA2S, and the binding affinity of the pentasaccharide is still 3 orders of magnitude lower than naturally occurring heparins of higher molecular weight. It was also found that *O*-(2-*O*-sulfo-4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid)-(1→4)-*O*-(2-deoxy-2-acetamido-6-*O*-sulfo-α-D-glucopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyluronic acid)-(1→4)-2-deoxy-2-sulfamido-3,6-di-*O*-sulfo-α-D-glucopyranose (abbreviated as ΔHexA2S-GlcNAc6S-GlcA-GlcNS3S6S), similar to the non-reducing trisaccharide of **1**, was not responsible

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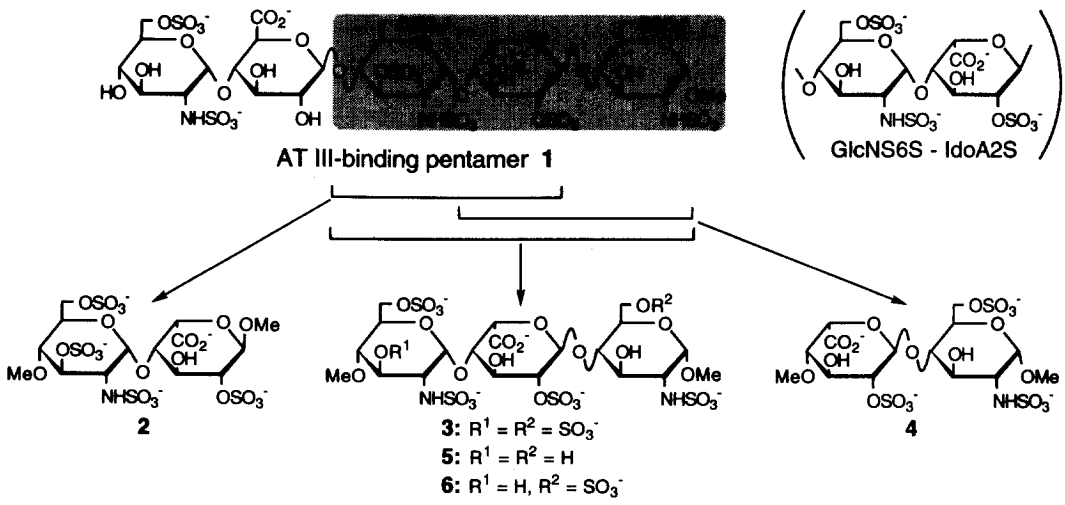
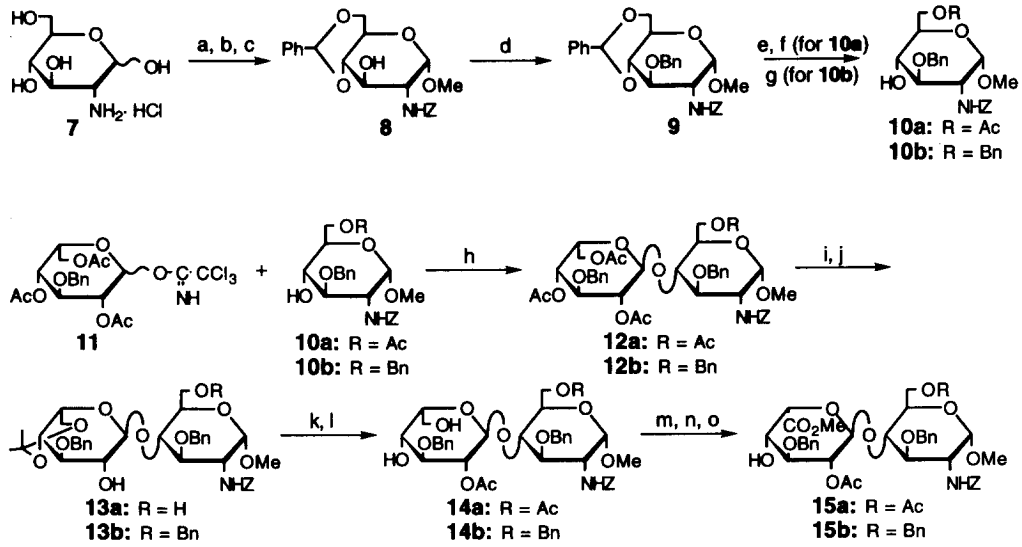
for the binding.[5] Therefore, we focused on the binding potency of the trisaccharide at the reducing end of **1** and prepared the trisaccharide **3**, its partially desulfated derivatives (**5** and **6**), and two disaccharides (**2**, **4**).



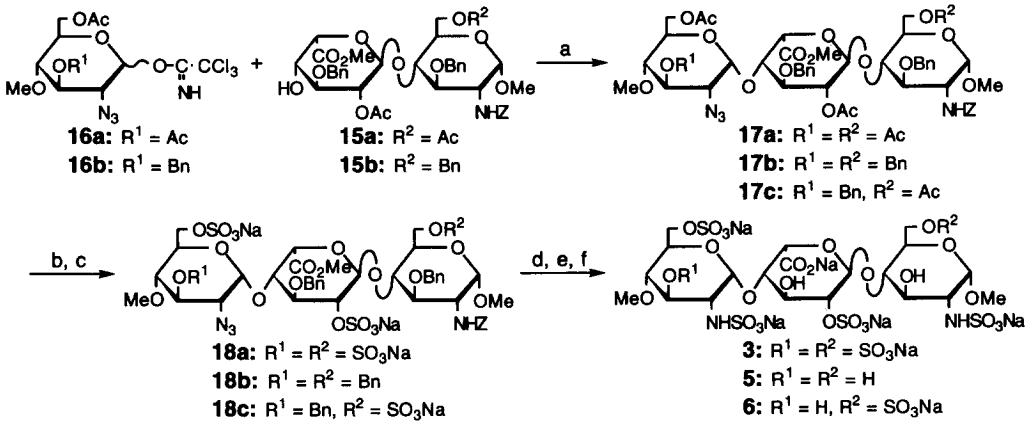
The synthesis of trisaccharide **3** was carried out as shown in Schemes 1 and 2. The glucosamine derivative **10a** as a reducing end building block for **3** was prepared in 6 steps from **7**. The coupling of **10a** with the L-idose derivative **11**[6,8] was performed using $Sn(OTf)_2$ at 0°C to give a β-linked disaccharide **12a** in a good yield. All acetyl protecting groups were removed, then the resulting 4'- and 6'-hydroxy groups of the idose residue were protected by isopropylidenation and the remaining 2'- and 6-hydroxy groups of **13a** were acetylated. After removing the isopropylidene group, the primary 6'-hydroxy group of idose residue was selectively oxidized to a carboxylic acid using 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO)[9], followed by $NaClO_2$ treatment.[10] The resulting carboxyl group was then esterified with (trimethylsilyl)diazomethane[11] to afford the disaccharide component **15a**.

The 2-azido sugar derivative **16a**, which was prepared according to our method with a slight modification,[6] was coupled with the disaccharide component **15a** using t-butyldimethylsilyl triflate (TBDMSOTf) at -20°C to give selectively an α-linked trisaccharide **17a**.[6,12] After removing the acetyl groups with sodium methoxide, the resulting hydroxy groups were *O*-sulfated using sulfur trioxide-pyridine complex at room temperature. The methyl ester was hydrolyzed and the catalytic reduction was performed using Pd-C to remove the benzyl and benzyloxycarbonyl groups and to reduce the azide group. Finally, *N*-sulfation was done at the 2''- and 2-amino groups to give the desired trisaccharide **3**. Partially desulfated analogues of **3**, trisaccharides **5** and **6**, were prepared with a similar procedure using azido derivative **16b** and disaccharide **15a** (to **6**) or **15b** (to **5**). Compounds **3**, **5** and **6** were purified by chromatography on Sepahdex G-25, and formation of them were confirmed by ^1H NMR and ESI-MS.[13-15]



a: ZCl, Na_2CO_3 /H_2O; b: HCl /MeOH; c: $PhCH(OMe)_2$, CSA /THF, 59% (3 steps); d: BnBr, BaO, $Ba(OH)_2 \cdot 8H_2O$ /DMF, 78%; e: 60% AcOH; f: Ac_2O, pyridine /CH_2Cl_2, 75% (2 steps); g: $NaBH_3CN$, HCl /THF, 61%; h: $Sn(OTf)_2$ /$(CH_2Cl)_2$, **12a** 75%, **12b** 83%; i: 0.05 M NaOMe; j: $(CH_3)_2C(OCH_3)_2$, CSA /acetone, **13a** 77%, **13b** 76% (2 steps); k: Ac_2O, DMAP, pyridine /CH_2Cl_2; l: 90% AcOH, **14a** 99%, **14b** 85% (2 steps); m: TEMPO, NaClO, KBr; n: $NaClO_2$, NaH_2PO_4 /H_2O, t-BuOH; o: $TMSCHN_2$, **15a** 86%, **15b** 75% (3 steps).

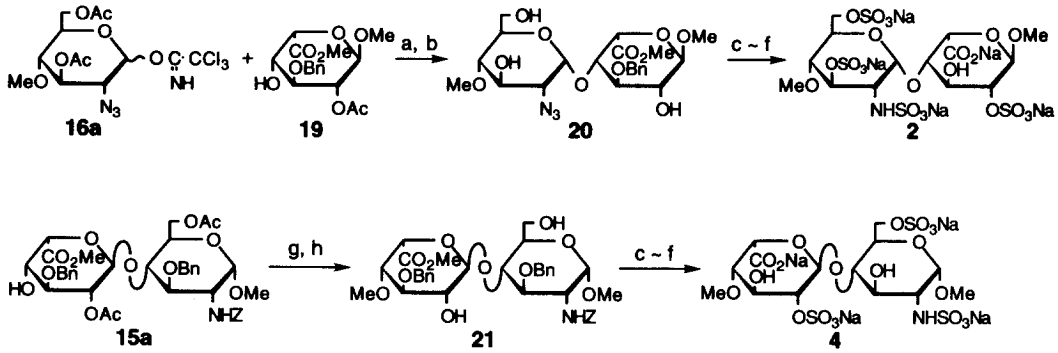
Scheme 1



a: TBDMSOTf, MS4A /toluene, **17a** 76%, **17b** 89%, **17c** 88%; b: 0.05 M NaOMe, **a** 82%, **b** 89%, **c** 89%; c: SO_3·pyridine complex /DMF; d: NaOH /MeOH; e: 10% Pd-C, H_2; f: SO_3·pyridine complex /H_2O, pH=9.5, **3** 50%, **5** 69%, **6** 77% (4 steps).

Scheme 2

The preparation of the disaccharides **2** and **4** were carried out similarly (Scheme 3). The azido derivative **16a** was coupled with the L-iduronic acid derivative **19**.[6] The resulting disaccharide was then treated with sodium methoxide to remove acetyl groups to give **20**. *O*-Sulfation, saponification, catalytic reduction, and *N*-sulfation were sequentially performed to afford **2**. For the synthesis of **4**, the 4'-hydroxy group of the disaccharide **15a** was methylated using methyl iodide and Ag_2O, where the yield of the desired 4'-methyl ether was not satisfactory owing to partial elimination of the acetyl groups and methylation of the resulting hydroxy groups. Then a series of de-protection and sulfation were performed in a similar manner to give **4**. Compounds **2** and **4** were purified by chromatography on Sepahdex G-25, and formation of them were confirmed by ^1H NMR and ESI-MS.[16,17]



a: TBDMSOTf, MS4A /toluene, 79%; b: 0.1 M NaOMe, 54%; c: SO_3·pyridine complex /DMF; d: NaOH /MeOH; e: 10% Pd-C, H_2; f: SO_3·pyridine complex /H_2O, pH=9.5, **2** 57%, **4** 68% (each 4 steps); g: MeI, Ag_2O /CH_3CN; h: 0.05 M NaOMe, 23% (2 steps, recovery of **15a**, 37%).

Scheme 3

The platelet-binding activities of the synthetic compounds **2-6** were evaluated by a competitive binding assay using high concentration of [^3H]-labelled heparin according to our previous method[6] to find high-affinity site(s) in heparin for platelets. Their activities were compared with those of a commercial heparin (average molecular weight 17500, from porcine intestine, Nacalai Tesque, Kyoto, Japan) and AT III-binding pentasaccharide **1** (Fig. 1). The disaccharide **2** possesses a structure similar to that of GlcNS6S-IdoA2S, except for the presence of the additional sulfate group at the 3'-position. Another disaccharide **4** corresponds to the 'reversed sequence' of GlcNS6S-IdoA2S, where the numbers of carboxyl and sulfate groups are identical to those of GlcNS6S-IdoA2S. Both of the disaccharides did not show a distinct binding activity. Together with our previous results using the oligomer-model compounds containing two or three units of GlcNS6S-IdoA2S,[6] it became evident that only one unit of any disaccharide can not form a high-affinity site for the binding to platelets. On the contrary, the trisaccharide **3** exhibited a distinct binding activity, although the potency was lower than that of high molecular weight commercial heparin or pentasaccharide **1**. Since the relating tetrasaccharide, ΔHexA2S-GlcNAc6S-GlcA-GlcNS3S6S, was found to possess no binding activity,[5] the GlcA-GlcNS3S6S sequence may not contribute to the binding. Therefore, the difference between **1** and **3** may be due to the cooperative and non-

specific binding effect conferred by the additional GlcNS6S-GlcA in **1**. The other trisaccharides **5** and **6**, analogues of **3**, did not show any binding activity in the present assay system. From these results, we conclude that the trisaccharide sequence, *O*-(2-deoxy-2-sulfamido-3,6-di-*O*-sulfo-α-D-glucopyranosyl)-(1→4)-*O*-(2-*O*-sulfo-α-L-idopyranosyluronic acid)-(1→4)-2-deoxy-2-sulfamido-6-*O*-sulfo-α-D-glucopyranose (abbreviated as GlcNS3S6S-IdoA2S-GlcNS6S), forms a platelet-binding high-affinity site in heparin.

We originally hypothesized that GlcNS6S-IdoA2S was a key platelet binding disaccharide based on the fact that heparinase I digestion of heparins cleaves this disaccharide and also significantly reduces the platelet-binding activity of the resulting fragments. This hypothesis was then confirmed by binding tests of synthetic model compounds,[4,6]: GlcNS6S-IdoA2S forms a low affinity site, and the clustering effect based on multi GlcNS6S-IdoA2S units increased the affinity drastically as described above. Yamada et al. recently reported the formation of oligosaccharides containing not inner but reducing *N*- and 3,6-*O*-trisulfated glucosamine by partial digestion of commercial heparin with heparinase I.[18] Thus, GlcNS3S6S-IdoA2S may even be a preferential target to GlcNS6S-IdoA2S for the cleavage by this enzyme. If true, these observations continue to support our data implicating GlcNS3S6S-IdoA2S-GlcNS6S as an important platelet-binding domain structure.

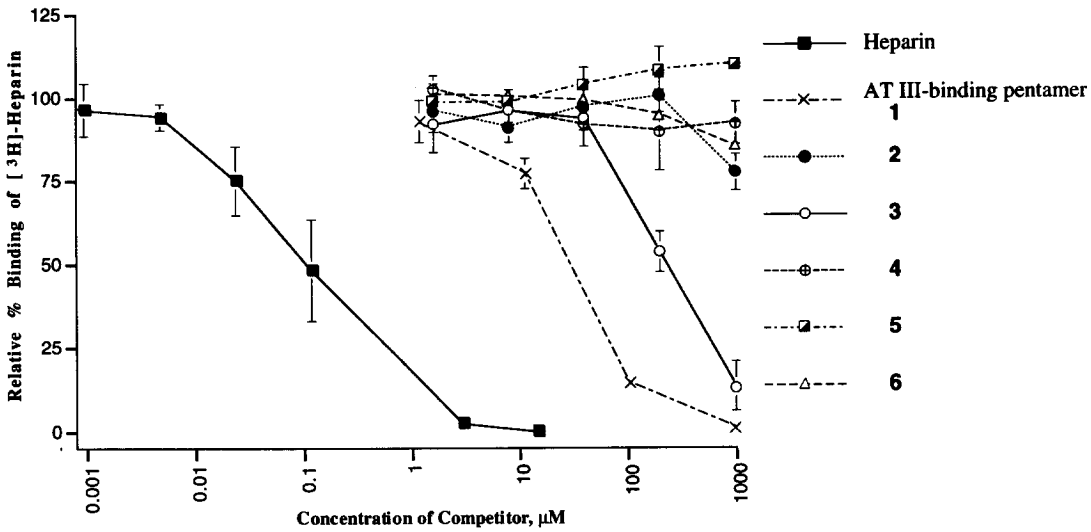


Fig. 1 Binding competitive activity of AT III-binding pentamer **1**, our synthetic compounds **2~6** and commercial heparin

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13. Spectral data for compound **3**: ^{1}H NMR (600 MHz, D_2O), δ 5.40 (1H, d, J = 3.3 Hz), 5.07 (1H, s), 4.90 (1H, d, J = 3.3 Hz), 4.64-4.61 (1H, m, superimposed with HDO signal), 4.27 (1H, dd, J = 4.6 Hz, J = 11.2 Hz), 4.24 (1H, m), 4.23 (1H, m), 4.20 (1H, m), 4.20 (1H, s), 4.09 (1H, d, J = 10.2 Hz), 4.05 (2H, m), 3.89 (1H, d, J = 9.1 Hz), 3.84 (1H, m), 3.64 (1H, m), 3.54 (1H, t, J = 9.6 Hz), 3.47 (3H, s), 3.37 (1H, t, J = 9.2 Hz), 3.30 (3H, s), 3.29 (1H, dd, J = 3.6 Hz), 3.16 (1H, dd, J = 3.6 Hz, J = 10.7 Hz); ESI-MS (negative) *m/z* 510.99 [(M-7Na+5H)$^{2-}$].
14. Spectral data for compound **5**: ^{1}H NMR (600 MHz, D_2O), δ 5.23 (1H, d, J = 3.6 Hz), 5.09 (1H, s), 4.91 (1H, d, J = 3.3 Hz), 4.66 (1H, m), 4.20-4.18 (2H, m), 4.09 (1H, m), 4.08 (1H, m), 3.95 (1H, s), 3.86 (1H, d, J = 10.7 Hz), 3.80 (1H, dd, J = 2.2 Hz, J = 12.1 Hz), 3.76 (1H, dd, J = 4.4 Hz, J = 12.4 Hz), 3.64 (1H, m), 3.60 (2H, m), 3.54 (1H, t, J = 9.5 Hz), 3.46 (3H, s), 3.29 (3H, s), 3.24 (1H, t, J = 9.8 Hz), 3.14 (1H, dd, J = 3.6 Hz, J = 10.6 Hz), 3.14 (1H, dd, J = 3.3 Hz, J = 10.2 Hz); ESI-MS (negative) *m/z* 431.02 [(M-5Na+3H)$^{2-}$].
15. Spectral data for compound **6**: ^{1}H NMR (600 MHz, D_2O), δ 5.27 (1H, d, J = 3.6 Hz), 5.11 (1H, s), 4.91 (1H, d, J = 3.6 Hz), 4.65 (1H, m, superimposed with HDO signal), 4.25 (1H, dd, J = 1.7 Hz, J = 11.3 Hz), 4.23 (1H, d, J = 5.5 Hz), 4.20 (1H, m), 4.18 (1H, m), 4.09 (1H, d, J = 4.9 Hz), 4.08 (1H, s), 3.97 (1H, s), 3.88-3.84 (2H, m), 3.62 (1H, m), 3.60 (1H, t, J = 9.9 Hz), 3.54 (1H, t, J = 9.9 Hz), 3.46 (3H, s), 3.30 (3H, s), 3.24 (1H, t, J = 9.5 Hz), 3.16 (1H, dd, J = 3.6 Hz, J = 10.4 Hz), 3.14 (1H, dd, J = 3.6 Hz, J = 10.4 Hz); ESI-MS (negative) *m/z* 471.00 [(M-6Na+4H)$^{2-}$].
16. Spectral data for compound **2**: ^{1}H NMR (600 MHz, D_2O), δ 5.34 (1H, d, J = 3.6 Hz), 4.84 (1H, d, J = 3.0 Hz), 4.34 (1H, m), 4.26 (1H, t, J = 9.8 Hz), 4.21 (1H, dd, J = 1.9 Hz, J = 11.3 Hz), 4.10 (1H, s), 4.08 (1H, dd, J = 3.6 Hz, J = 10.7 Hz), 4.07 (1H, m), 3.98 (1H, t, J = 3.5 Hz), 3.91 (1H, d, J = 9.6 Hz), 3.47 (3H, s), 3.36 (1H, t, J = 9.5 Hz), 3.31 (3H, s), 3.29 (1H, dd, J = 3.3 Hz, J = 10.7 Hz); ESI-MS (negative) *m/z* 701.91 [(M-5Na+4H)-].
17. Spectral data for compound **4**: ^{1}H NMR (600 MHz, D_2O), δ 5.03 (1H, s), 4.90 (1H, d, J = 3.6 Hz), 4.66-4.61 (1H, m, superimposed with HDO signal), 4.23 (1H, dd, J = 2.2 Hz, J = 11.3 Hz), 4.17 (1H, m), 4.15 (1H, s), 4.15 (1H, dd, J = 5.4 Hz, J = 11.7 Hz), 3.87 (1H, m), 3.59 (1H, t, J = 9.5 Hz), 3.57 (1H, s), 3.55 (1H, t, J = 9.5 Hz), 3.31 (3H, s), 3.30 (3H, s), 3.16 (1H, dd, J = 3.6 Hz, J = 9.9 Hz); ESI-MS (negative) *m/z* 622.04 [(M-4Na+3H)-].
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