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BIOLOGICAL PROPERTIES OF SYNTHETIC GLYCOCONJUGATE MIMICS OF HEPARIN COMPRISING DIFFERENT MOLECULAR SPACERS

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Abstract: The in vitro antithrombotic activity of synthetic glycoconjugates **I** and **II**, comprising a flexible polyethylene glycol type and a rigid polyglucose type spacer, respectively, are compared to heparin.

Keywords: Antithrombotics; Carbohydrates; Glycosidation.

Introduction: The sulphated glycosaminoglycan heparin[1] binds with high affinity to the protease inhibitor antithrombin III (AT III), thereby increasing the inhibitory potency of AT III relative to the coagulation factors Xa and thrombin (IIa). The smallest active part of heparin consists of an unique, well-defined pentasaccharide (PS) which brings about a selective inhibition[2] of factor Xa. For the effective inhibition of thrombin[3] heparin fragments require apart from the presence of the unique PS domain also a chain length of about 16-18 sugar units in length. Molecular modelling studies[4] revealed that heparin mimics may be obtained when a sulphated thrombin binding saccharide is tethered to the nonreducing end of the AT III binding pentasaccharide (Antithrombin Binding Domain; ABD) through a neutral molecular spacer. Furthermore it has been proposed[5] that any sulphated oligosaccharide may serve as a Thrombin Binding Domain (TBD) since this domain is involved in a nonspecific interaction with thrombin. In the meantime synthetically more accessible O-sulphated/O-methylated PS analogues have been obtained.[6] Various glycoconjugates with the above described structural features were synthesized[7] and tested for their ability to inhibit both factor Xa and thrombin. Initially, compounds having an easily accessible polyethylene glycol type spacer were prepared. For example, compound **I**[8] (Fig. 1) represents the first class of glycoconjugates displaying both ATIII mediated anti-Xa and anti-thrombin activity.

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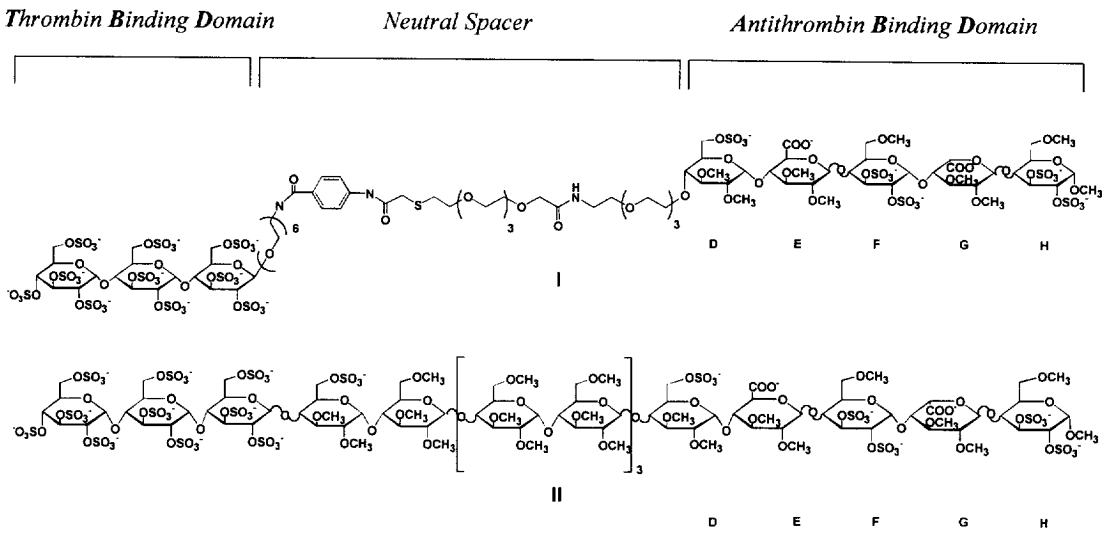


Figure 1

This particular glycoconjugate comprises a high affinity PS-analogue, which is connected via a linear polyethylene glycol spacer to a persulphated maltotrioside moiety. In search for acceleration of the inhibitory effect on thrombin it was found that the length of the spacer as well as the number and/or nature[9] of negatively charged groups on the TBD of the glycoconjugates are crucial.

However, the degree of sulphation of the glycoconjugates is limited with respect to unwanted side-effects in anticoagulant therapies. Thus, Greinacher[10] demonstrated that highly sulphated heparin fractions in particular induce the immunological type of heparin-associated-thrombocytopenia (HAT). HAT is associated with lifethreathening thrombo-embolic complications and has an incidence of 2-3% in heparin-treated patients. The antigen in HAT consists of multimolecular complexes of heparin or other sulphated oligosaccharides with platelet proteins i.e. mainly platelet factor 4 (PF4). The interaction between sulphated oligosaccharides and PF4 was shown to enhance upon increasing charge density and flexibility of the sulphated oligosaccharide. For this reason we also turned our attention to synthetic ABD-TBD conjugates containing non-sulphated rigid spacers. In addition we were eager to compare the thrombin inhibitory properties[11] of conjugates with flexible spacers (i.e. compound **I**) with their rigid counterparts, while taking into account that the distance between ABD and TBD moieties corresponds to our model of the ternary (AT III-heparin-thrombin) complex.[4] Thus, glycoconjugate **II** was designed; its fully methylated polymaltose chain was selected as molecular spacer resembling the backbone of heparin.

Synthesis of the spacer molecule 1

A retrosynthetic analysis of glycoconjugate **II**, as shown in Figure 2, was inspired by our broad experience with the chemical synthesis of glycosaminoglycans and derivatives thereof.[6] Accordingly, two C-O

disconnections are made in the target molecule to provide the three key intermediates **1**, **2** and **3**, the latter two of which are accessible via published procedures.[12,13] The methylated saccharide spacer **1**, including the nonreducing end glucose derivative (D-unit) of the PS, can be prepared by a repeating cycle of chemical steps starting from the known glucose and maltose derivatives, **4**[14] and **5**[15], respectively (see Scheme 1). The elongation cycle to the nonasaccharide **8c** comprises the following five (i.e. step *i-v*; see Scheme 1) consecutive steps. First, N-iodosuccinimide promoted coupling in the presence of triflic acid[16] of acceptor **4** with donor **5** gave the desired 1,4-trans glycoside in very high yield.

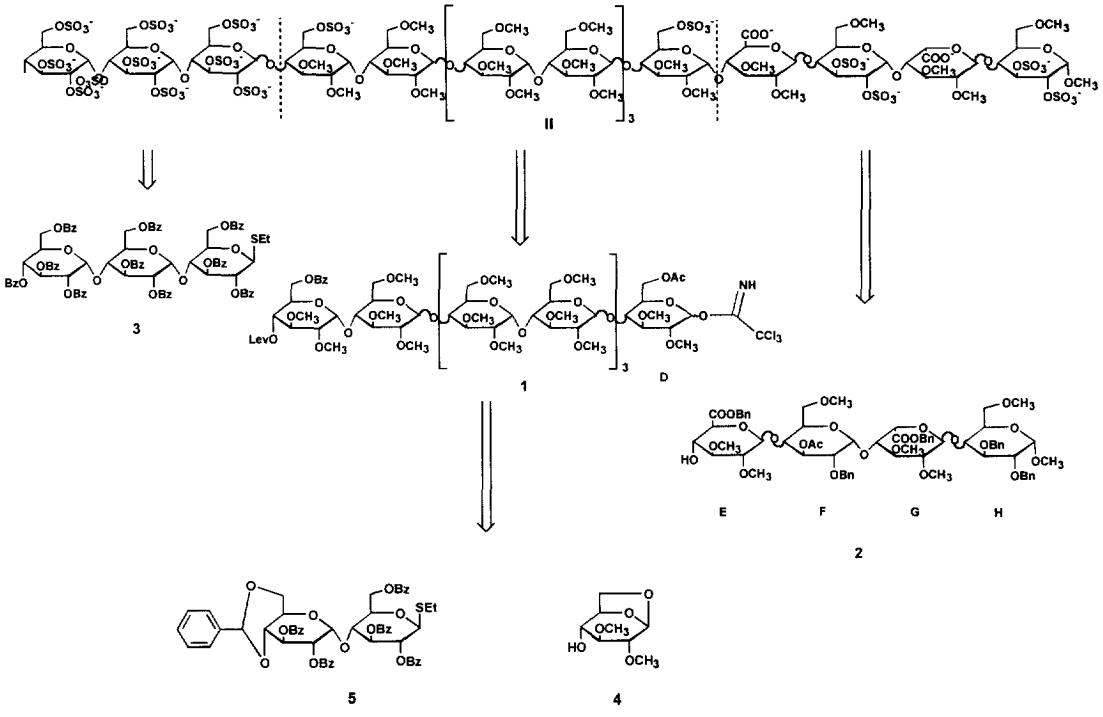


Fig. 2: Retrosynthetic analysis of glycoconjugate **II**

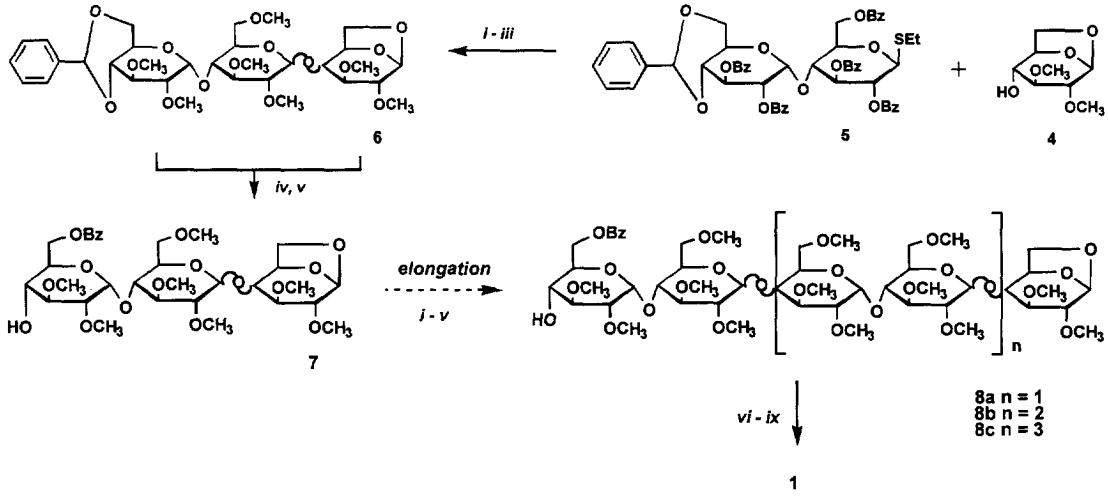
Secondly, saponification followed by methylation using standard conditions gave the fully protected trisaccharide **6** in an excellent overall yield. Acid treatment of **6** resulted in a 4,6-diol system, of which the primary hydroxyl was selectively benzoylated with 1-benzoyloxy-1-H-benzotriazole[17] to give **7** in 78% yield

After reiteration of the above described elongation steps (i.e. steps *i-v*) commencing with the coupling of acceptor **7** with **5** the partly protected pentasaccharide **8a** was obtained. The third cycle of elongation steps gave the intermediate heptasaccharide **8b** and a final repetition of the elongation steps delivered nonasaccharide **8c**, which subsequently was protected with the temporary levulinoyl group. Acetolysis of the 1,6 anhydro moiety,[18] followed by selective anomeric saponification with morpholine and treatment with

trichloroacetonitrile in the presence of cesium carbonate furnished the target nonasaccharide imidate **1** in an excellent overall yield.

Assemblage of the target glycoconjugate II

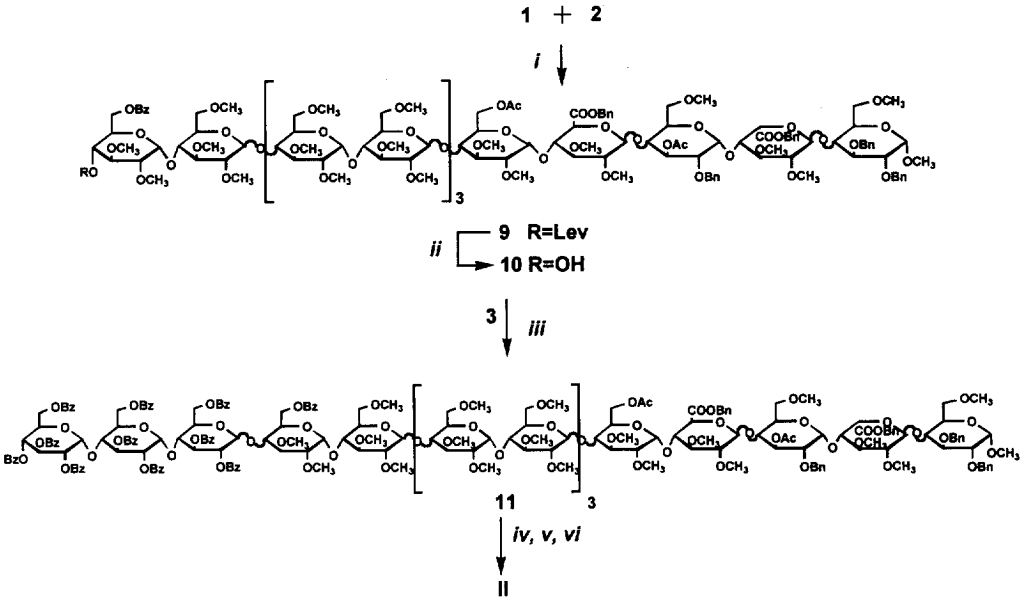
The assemblage of glycoconjugate **II** is depicted in Scheme 2 and starts with the coupling of imidate **1** with the partly protected tetrasaccharide **2** in the presence of TMSOTf[19] in dichloromethane. After workup and purification by size exclusion chromatography (Sephadex LH60) the 13- mer **9** was obtained as a mixture of anomers (α:β=85:15) in 63% yield. The anomeric mixture was further processed to the 16-mer **11** at which state the α/β mixture could be separated.



Scheme 1: (i) NIS, TfOH, MS4Å, toluene, -20 °C (83%); (ii) KOtBu, MeOH, dioxane, 4 h at rt; (iii) CH_3I, NaH, DMF, 5 h at rt (94% in 2 steps); (iv) 80% AcOH, 50 °C, 6 h (86%); (v) 1-Benzoyloxy-1H-benzotriazole, $(C_2H_5)_3N$, CH_2Cl_2, 15 h at rt (91%);(vi) Levulinic acid, 1-(3-Dimethylaminopropyl)-3-ethyl-carbodiimide.HCl, dioxane, DMAP, 3 h rt (93%); (vii) Ac_2O, AcOH, TFA, 4 h at rt (99%); (viii) Morpholine, toluene, 35 °C, 48 h (85%); (ix) Cs_2CO_3, $NCCCl_3$, CH_2Cl_2, 3 h at rt (90%).

Thus treatment of **9** with hydrazine in a mixture of pyridine and acetic acid gave the oligosaccharide **10** with an unprotected 4-hydroxyl group. The latter hydroxyl group was coupled with thioethyl maltotriose **3** by the action of N-iodosuccinimide/triflic acid to give the desired 16-mer **11** in 62% yield, after purification by size exclusion (Sephadex LH60) and silica gel chromatography. Indeed, ^{1}H NMR (600 MHz) spectroscopy of **11** revealed a homogeneous product devoid of anomeric mixtures. The fully protected saccharide **11** was then deprotected in two steps. First, hydrogenolysis of the benzyl protecting groups was easily effected in quantitative yield by palladium on activated charcoal in ethyl acetate, followed by saponification of all esters with sodium hydroxide. Subsequent treatment with triethylamine-sulphur trioxide complex afforded after size exclusion chromatography (Sephadex G-25) the target glycoconjugate **II** in an excellent yield. The

identity and purity of **II**[20] was fully ascertained by ^{1}H NMR spectroscopy, ESI mass spectroscopy, HPLC analysis (anion exchange) and reversed UV capillary electrophoresis.[20]



Scheme 2 *(i) TMSOTf, MS 4Å, CH_2Cl_2, 0 °C, 15 min (63%; α/β = 85/15); (ii) $H_2NNH_2.H_2O$, AcOH, pyridine, 6 min. at rt (99%); (iii) NIS/TfOH MS 4Å, toluene, 15 min. at rt (62% pure α); (iv) H_2, Pd/C, rt (98%); (v) 0.5N NaOH, MeOH, 20 h at rt (89%); (vi) $(C_2H_5)_3N.SO_3$ complex, DMF, 55 °C, 16 h, Sephadex G-25 (93%).*

Biological activities

The ATIII mediated protease inhibitory activities of the glycoconjugates have been tested in vitro[22] (see Table 1). The replacement of the flexible polyethylene glycol spacer (i.e of compound **I**) by a rigid methylated polyglucose spacer (i.e. of compound **II**) increases the anti-IIa activity about tenfold.[11] On the other hand the difference in anti-Xa activities between the compounds **I** and **II** is small and mainly the result of their difference in molecular weight. The neutralisation of the anti-IIa activity by PF4 was determined by measuring the remaining anti-IIa activity after incubation with various concentrations of PF4 .

Compound	anti-Xa[1] (U/mg)	anti-IIa[2] (U/mg) pH = 8.4	pH= 7.4	PF4 neutralisation[3] (anti-IIa)
heparin	158	158	158	1
I	738	136	38	0.4
II	458	1878	1173	0.05

Table 1

[1] The inhibitory activity of compound **I** and **II** on factor Xa was tested in human plasma against reference natural pentasaccharide.

[2] The anti-IIa activity was assessed in a buffer system (pH = 8.4) against standard heparin.

[3] The PF4 neutralisation expressed as a factor compared to heparin at similar concentrations PF4 and compound.

The extent of neutralisation of the anti-IIa activity by PF4 is decreased by a factor of twenty when highly sulphated heparin is compared to glycoconjugate **II**, while it also confirmed that flexible derivatives interact more easily with PF4 than their rigid analogues.

The results presented in this paper clearly demonstrate that connection of the ABD with TBD in a glyconjugate mimic of heparin, with a rigid spacer instead of a flexible spacer, results in a substantial increase in anti-thrombin activity, whereas the undesired interaction with PF4 is diminished .

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20. $[\alpha]^{20}_{D}$= +67.6 (c=1, water); Mass (ESI): molecular weight: 4370.6 (H^+-form) $C_{128}H_{222}O_{113}S_{16}$ (calc. 4370.14); ^{1}H-NMR (600 MHz, D_2O); anomeric protons (δ, ppm): reducing end: unit 1-6: 5.17 ; 5.03; 5.41; 4.42; 5.49; 4.66; units 7, 9 and 11: 5.67: units 8, 10, and 12: 4.46; unit 13-16: 5.61; 4.94; 5.59; 5.69.
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