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IN VITRO EVALUATION OF SYNTHETIC HEPARIN-LIKE CONJUGATES COMPRISING DIFFERENT THROMBIN BINDING DOMAINS

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Abstract: The syntheses of several heparin-like glycoconjugates (i.e., **16a–f**) containing identical AT III binding domains (ABD) and spacers but different thrombin binding domains (TBDs) are described.[1] Biological activities of conjugates **16a–f** indicate that the thrombin inhibitory activity is mainly determined by the charge density of the TBD moiety.

The sulphated glycosaminoglycan heparin[2] binds with high affinity to the plasma protein antithrombin III (AT III), thereby accelerating its inhibitory activity towards factor Xa and thrombin, two serine proteases involved in blood coagulation. The shortest fragment in the heparin polymer with high affinity for AT III was discovered to be a pentasaccharide.[3,4] This pentasaccharide was shown to exclusively accelerate the AT III mediated inactivation of factor Xa but not that of thrombin. A program towards the synthesis of analogues of the native pentasaccharide afforded potent alkylated (nonglycosaminoglycan) derivatives being relatively easy to prepare.[5–7]

The next challenge was to extend the concept of AT III-mediated inhibition of factor Xa by the pentasaccharide towards synthetically feasible derivatives displaying both anti-factor Xa and anti-thrombin activity.

On the basis of a model[8] of the heparine/AT III/thrombin ternary complex (see Fig. 1) van Boeckel et al.[9a,b] synthesized glycoconjugates displaying both αXa and αIIa activity. These glycoconjugates (e.g., compounds **16d–f**) comprise an AT III binding domain (ABD) linked via a linear, neutral spacer of about 50 atoms in length to negatively charged thrombin binding domains (TBD's). In order to get a deeper insight into the specificity of the molecular interaction between thrombin and different TBD's the biological properties of the synthetic conjugates **16a–c**, comprising dermatan sulphate- and heparin-like TBD's (i.e., TBD **I–III**, see Fig. 2) are evaluated and compared with earlier described conjugates **16d–f**.[9a,b]

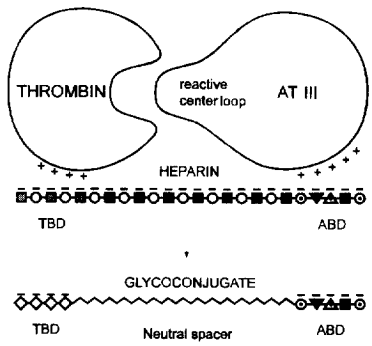

Figure 1. Schematic representation of the ternary complex of thrombin and AT III with heparin and glycoconjugates **16a–f**.

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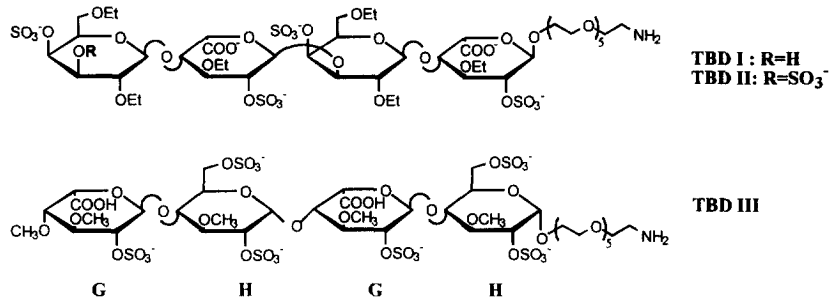
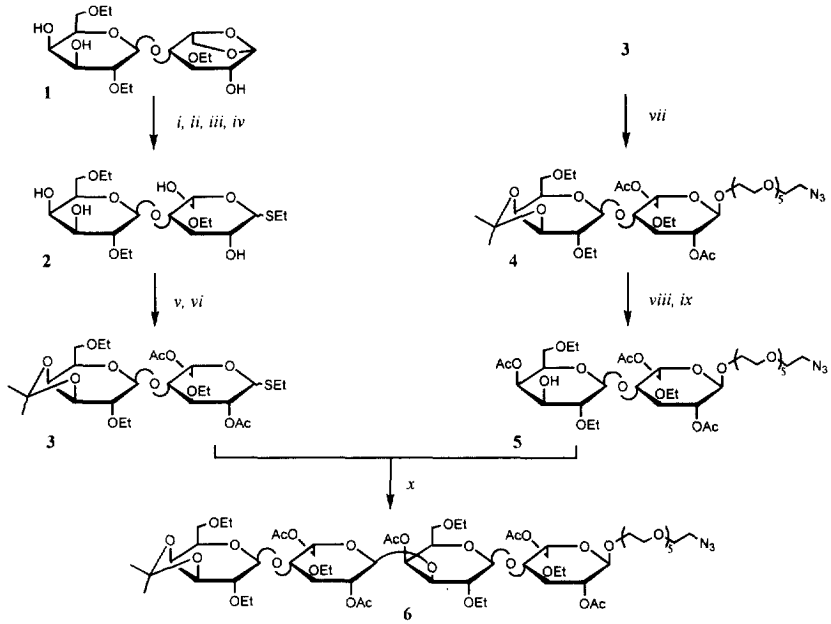


Figure 2.

As it is known that heparin and dermatan sulphate interact with thrombin, we expected at first sight that conjugates **16a–c** would display enhanced antithrombin activity relative to randomly sulphated oligosaccharides.

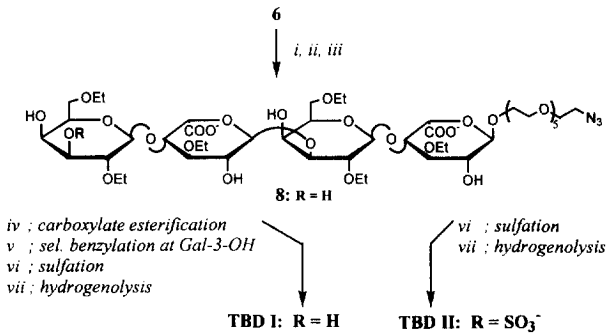
Synthesis of dermatan tetrasaccharide derivatives TBD I and II (Schemes 1 and 2)

The synthesis of the fully protected precursor **6** of the dermatan sulphate-like TBDs **I** and **II** is outlined in Scheme 1. The synthesis of **6** requires the preparation of two disaccharides, **3** and **5**, both of which have been derived from compound 1.[10] Disaccharide **5** is equipped with an α oriented spacer at the reducing end of the molecule and contains an azido functionality, which is stable during all protecting group manipulations.



Scheme 1: *(i)* Ac_2O, pyridine, 30 min, rt (100%); *(ii)* HOAc/Ac_2O/TFA 1/25/3.5 v/v/v, 15 h, rt (86%); *(iii)* Ethanethiol, $BF_3.Et_2O$, toluene, 1.5 h, rt (88%); *(iv)* KOtBu, CH_3OH, dioxane, 1 h, rt (100%); *(v)* 2,2-dimethoxypropane, *p*TosOH (91%); *(vi)* Ac_2O, pyridine, 20 h, rt (80%); *(vii)* 1-azido-17-hydroxy-3,6,9,12,15-pentaoxaheptadecane,[11] NIS, TfOH, toluene, MS 4 Å, -20 °C (80%); *(viii)* 70% HOAc, 50 °C, 2 h (94%); *(ix)* trimethyl orthoacetate, then 90% HOAc (99%); *(x)* NIS, TfOH, toluene, MS 4 Å, -20 °C (85%).

Hydrogenolysis of this azido group in the final step of the synthesis will afford a primary amine suitable for conjugation of the TBD with the ABD. First, compound **1** was converted into **2** in an excellent yield by successively acetylation of the free hydroxyl groups, ring opening of the 1,6-anhydro functionality, mercaptolysis of the anomeric acetate and saponification of the remaining acetate esters. Subsequent introduction of the 3',4'-*O*-isopropylidene protective group and acetylation afforded compound **3**. This intermediate could be used as glycosyl donor as well as starting building block for the the synthesis of glycosyl acceptor **5**. NIS-promoted coupling of donor **3** with 1-azido-17-hydroxy-3,6,9,12,15-pentaoxaheptadecane[11] was performed giving 1-azidohexaethyleneglycol glycoside **4** in 80% yield. Acid catalysed hydrolysis of the isopropylidene group of **4** was followed by conversion of the diol into the 3',4'-cyclic methyl orthoester. Regioselective ring opening with acetic acid afforded **5** in almost quantitative yield. Coupling of acceptor **5** with donor **3** under thioglycoside activation led to the formation of the desired tetrasaccharide **6**.

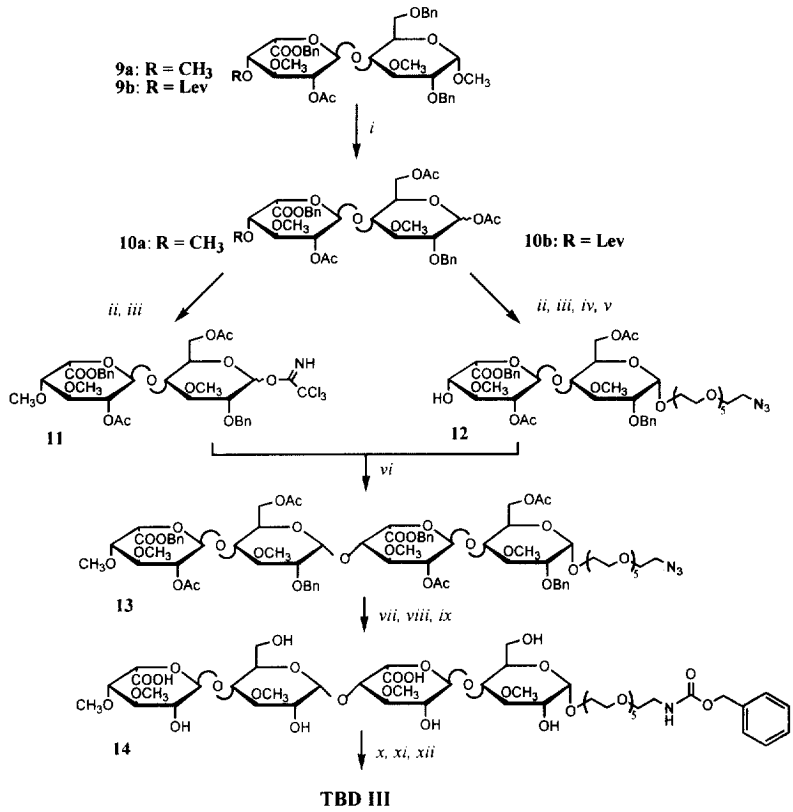


Scheme 2: *(i)* KOtBu, methanol, dioxane, 1 h, rt (96%); *(ii)* TEMPO, 2,2,6,6,-tetramethylpiperidin-1-oxyl, $NaHCO_3$, KBr, tetrabutylammonium chloride, NaOCl, 1 h, 0 °C (70%); *(iii)* 70% HOAc, 5 h, 35 °C (66%); *(iv)* BnBr, $KHCO_3$, DMF (83%); *(v)* $Bu_2Sn(OCH_3)_2$, BnBr, CsF, DMF, 4 h, rt (100%); *(vi)* $Et_3N.SO_3$-complex, DMF, 16 h, 55 °C (92%); *(vii)* Pd-C, H_2,H_2O (76%).

Then compound **6** was converted into **8** in three steps. Saponification of the acetyl esters followed by selective TEMPO oxidation[12] of the primary hydroxyls and hydrolysis of the isopropylidene group provided tetrasaccharide **8**. To obtain TBD **I** the uronic acids of compound **8** were first protected as benzyl esters. Regioselective 3-*O*-benzylation of the nonreducing end unit was effected by reaction of the stannylidene complex[13] with benzyl bromide. *O*-sulphation of the hydroxyl groups followed by reduction of the azido group and simultaneous hydrogenolysis of the benzyl esters and benzyl ether provided TBD **I** in 58% overall yield from **8.** Similarly, *O*-sulphation of compound **8** followed by hydrogenolysis afforded TBD **II**.

Synthesis of the heparin tetrasaccharide derivative TBD III (Scheme 3)

The key-intermediates in the synthesis of TBD **III** are the suitable protected building blocks **11** and **12** obtained from disaccharides **9a** and **9b**.[14] For the peparation of imidate **11** compound **9a** was first subjected to acetolysis with a mixture of acetic anhydride and sulfuric acid to afford **10a**. Selective removal of the anomeric



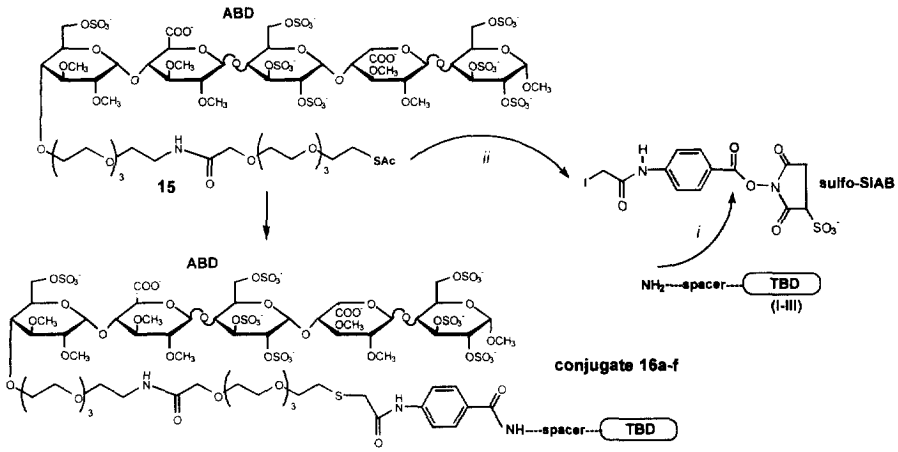
Scheme 3: *(i)* Ac_2O, H_2SO_4, -20 °C, 15 min (45-62%); *(ii)* Piperidine, THF, rt, 48 h (84%); *(iii)* CH_2Cl_2, CCl_3CN, Cs_2CO_3, 1 h (74%); *(iv)* 1-azido-17-hydroxy-3,6,9,12,15-pentaoxaheptadecane,[11] CH_2Cl_2, MS 4 Å, TMSOTf, 0 °C (74% α/β); *(v)* N_2H_4.HOAc, pyridine, 0 °C, 10 min (36% pure α); *(vi)* TMSOTf, MS 4 Å, CH_2Cl_2,-20 °C (64%); *(vii)* Pd-C, H_2, *t*BuOH/H_2O (100%); *(viii)* 0.4 N NaOH/CH_3OH 3/1 (v/v) 3 h, 20 °C (86%); *(ix)* Benzyl chloroformate, $NaHCO_3$, 20 h, 20 °C (92%); *(x)* $Et_3N.SO_3$-complex, DMF, 16 h, 55 °C (100%); *(xi)* 0.2 N HCl, 20 h, 4 °C (84%); *(xii)* Pd-C, H_2, H_2O (90%).

acetate by the action of piperidine in tetrahydrofuran was followed by treatment of the hemiacetal with trichloroacetonitrile in the presence of cesium carbonate to give glycosyl donor **11** in an overall yield of 38%. Glycosyl acceptor **12** was synthesized by a five-step procedure starting from **9b**. Acetolysis, anomeric saponification, and preparation of the imidate was performed as described for compound **11**. The coupling reaction with 1-azido-17-hydroxy-3,6,9,12,15-pentaoxaheptadecane was performed using TMSOTf as promotor to give a mixture of α/β coupled spacer in a 2/1 ratio. After selective removal of the levulinoyl group the mixture was purified by silicagel chromatography to afford the α anomer **12** in an overall yield of only 18%. Coupling of donor **11** with acceptor **12** in the presence of TMSOTf afforded tetrasaccharide **13** in 64% yield. After deprotection of **13** by hydrogenolysis and base treatment the primary amino group of the spacer was protected with a benzyloxycarbonyl function to avoid *N*-sulfation in the next step. As earlier experienced [9a] *O*-sulphation of compound **14** afforded a mixture of two products, indicating that the amide group in the spacer moiety was

partly sulphated. Fortunately, the latter N-sulphate could selectively be removed by treatment with 0.2 N HCl at 4 ^{0}C. Final hydrogenolysis of the benzyloxycarbonyl group afforded TBD **III** in high yield.

General conjugation method of ABD with TBD (Scheme 4)

The final ABD-TBD conjugation was accomplished according to Scheme 4. The ABD derivative we selected (i.e., compound **15**) consists of a pentasaccharide (Org 34006) hooked to a poly(ethyleneglycol)-type spacer at its non-reducing end. The same ABD derivative was also used for the preparation of the earlier described[9a] conjugates **16d–f**.



Scheme 4: *(i, ii)* one-pot reaction: sulfo-SIAB, 0.1 M NaH_2PO_4 buffer pH 7.5, 0.05 M NH_2OH, argon atmosphere (60-90%).

The amino group of the TBD derivatives (TBD **I–III**) was first reacted with sulfosuccinimidyl (4-iodoacetylamino) benzoate (sulfo-SIAB) in sodium dihydrogen phosphate buffer. Subsequent addition of hydroxylamine and ABD derivative **15** under argon atmosphere furnished, after Sephadex G-50 chromatography, the conjugates **16a–c** in 60–90% yield.

Table 1. AT-III-mediated anti-IIa activities of glycoconjugates **16a–f**

Conjugate	ABD	Sp. L.[a]	TBD	anti II a activity
16a	Org 34006	53	dermatan tetra $4SO_3^-$	2 U/mg
16b	Org 34006	53	dermatan tetra $5SO_3^-$	10 U/mg
16c	Org 34006	53	heparin tetra $6SO_3^-$	10 U/mg
16d	Org 34006	53	cellobiose $7SO_3^-$ [9a]	10 U/mg
16e	Org 34006	53	cellobiose $7PO_3^{2-}$ [9b]	26 U/mg
16f	Org 34006	53	maltotriose $10SO_3^-$ [9a]	65 U/mg

[a] Spacer length in number of atoms

The identity of conjugates **16a–c** was established by NMR[15] and/or MALDI mass spectroscopy.[16] Conjugates **16a–f** were tested[17] in vitro for AT-III mediated anti-thrombin activity (see Table 1). Conjugates **16b** and **16c** displayed the same antithrombin activity compared to conjugate **16d** with the persulphated cellobiose TBD. Conjugate **16a**, with only four sulphate groups on the TBD, showed only a low antithrombin activity.

From these results it can be concluded that the thrombin inhibitory activity of the conjugates is mainly determined by the charge density of the TBD moiety, whereas the carbohydrate structure of the TBD and its charge distribution has hardly any effect.

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15. ^{1}H NMR, 400 MHz, D_2O: conjugate **16a**: ABD-unit 1 (reducing end): δ 5.19 (d, 1H, $J_{1,2}$ = 3.8Hz, H-1). ABD-unit 2: 5.21 (br.s, 1H, H-1). ABD-unit 3: 5.44 (d, 1H, $J_{1,2}$ = 3.8 Hz, H-1). ABD-unit 4: 4.69; ABD-unit 5 (non reducing end): 5.49 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1). TBD-unit 1 (reducing end): 5.11 (br.s, 1H, H-1); TBD-unit 2 and 4: 4.62 (d, 1H, $J_{1,2}$ = 7.9Hz, H-1). TBD-unit 3: 5.20 (d, 1H, $J_{1,2}$ = 5.0Hz, H-1). Conjugate **16b**: TBD-unit 1: 5.06 (br. s, 1H, H-1). TBD-unit 2: 4.46 (d, 1H, $J_{1,2}$ = 5.2 Hz). TBD-unit 3: 5.16 (br.s, 1H, H-1). TBD-unit 4: 4.62. Conjugate **16c**: TBD-unit 1: 5.22 (d, 1H, $J_{1,2}$ = 2.3 Hz, H-1). TBD-unit 2 and 4: 5.12. TBD-unit 3: 5.38 (d, 1H, $J_{1,2}$ = 2Hz, H-1).
16. Maldi mass spectra of conjugates **16a** and **16c**; $(Arg\text{-}Gly)_{15}$ was used as basic peptide for formation of ionic complexes. Calc. for conjugate **16a** ($C_{112}H_{189}O_{99}N_3S_{12}$): 3547.5; found: 6766.4-3217.6 = 3548.8. Calc. for conjugate **16c** ($C_{105}H_{175}O_{105}N_3S_{14}$): 3607.4; found: 6829.8-3217.6 = 3612.2
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