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DESIGN AND SYNTHESIS OF A NOVEL SYNTHETIC NAPAP-PENTASACCHARIDE CONJUGATE DISPLAYING A DUAL ANTITHROMBOTIC ACTION

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Abstract: The synthesis of a novel antithrombotic consisting of a heparin pentasaccharide conjugated to the active site inhibitor *N*-(2-naphtalenesulfonyl)-glycyl-(D)-4-aminophenyl-alanyl-piperidine (NAPAP) (i.e. compound **I**) is reported. This conjugate shows a unique pharmacological profile both in vitro and in vivo having direct anti-thrombin and ATIII-mediated anti-Xa activity. Furthermore, conjugate **I** has a prolonged in vivo half-life compared to NAPAP (1.5 h vs 9 min.).

Introduction

The serine proteases thrombin and factor Xa play a pivotal role in thrombosis and haemostasis. Factor Xa catalyzes the conversion of prothrombin into thrombin, whereas thrombin is involved in activation of blood platelets and the cleavage of fibrinogen into clottable fibrin. Therefore factor Xa and thrombin have become highly interesting pharmaceutical targets for the treatment of thrombotic disorders.

The sulfated glycosaminoglycan heparin has been a subject of research for many years.[1] Heparin enhances the inhibitory potency of antithrombin III (ATIII) towards both thrombin and factor Xa. A unique and well defined pentasaccharide (PS) domain[2] in heparin is responsible for the activation of ATIII. This PS domain is sufficient to promote the inhibition of factor Xa, while the activity of thrombin remains unaffected. For effective thrombin inhibition[3] heparin sequences are required which contain in addition to the PS domain ten to twelve consecutive saccharide units in order to form a ternary complex together with ATIII and thrombin. Based on this mechanism of ternary complex formation a variety of heparin mimics[4] were designed comprised of an *O*-sulfated/*O*-methylated PS-analog[5] (see Figure 1) tethered via a polethylene glycol spacer to a negatively charged thrombin binding domain. These heparin mimics proved to be effective ATIII-mediated inhibitors of factor Xa and thrombin in vitro.

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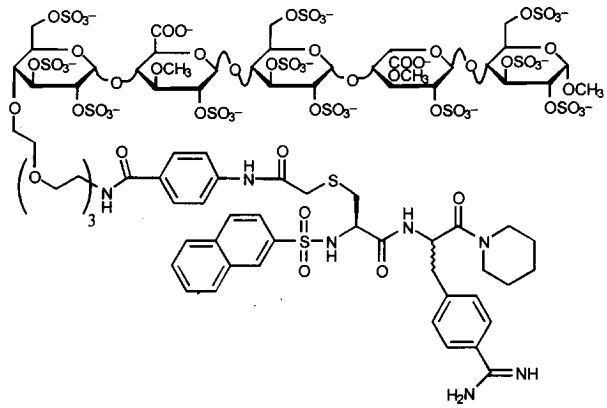
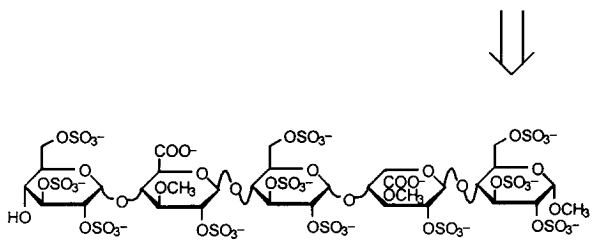
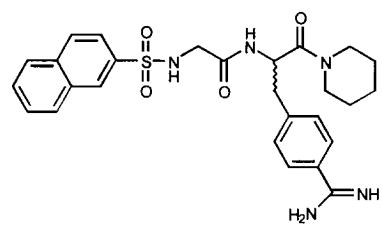


NAPAP-PS dual inhibitor **I**



Heparin PS-analog

ATIII-mediated anti-Xa activity: 1160 U mg^{-1}



NAPAP

anti-thrombin activity: IC_{50} = 0.57 μM

Figure 1. Structures of NAPAP, PS, and NAPAP-PS conjugate.

In this paper we now present a novel antithrombotic, which consists of a PS-analog conjugated to a derivative of *N*-(2-naphtalenesulfonyl)-glycyl-(D)-4-aminophenyl-alanyl-piperidine[6] (NAPAP) (i.e. compound **I**, see Figure 1). NAPAP is a potent representative (EC_{50} = 0.75 μM) of the low molecular weight thrombin inhibitors acting directly on thrombin's active site. The NAPAP-PS conjugate **I** is expected to elicit new pharmacological properties as it may stimulate the ATIII-mediated anti-Xa activity on the one hand (see Figure 2, **A**), while on the other hand it may inhibit thrombin directly (see Figure 2, **B**). Since earlier pharmacokinetic studies[7] clearly demonstrated that the half-life of a heparin pentasaccharide is governed by its affinity for ATIII, this novel dual inhibitor should possess a prolonged antithrombin activity compared to NAPAP. Moreover, this approach opens the way to the preparation of NAPAP-PS conjugates having taylor-made half-lives.[8] Furthermore, this conjugate has a higher solubility in aqueous media than NAPAP making the former more suitable for parenteral administration.[9]

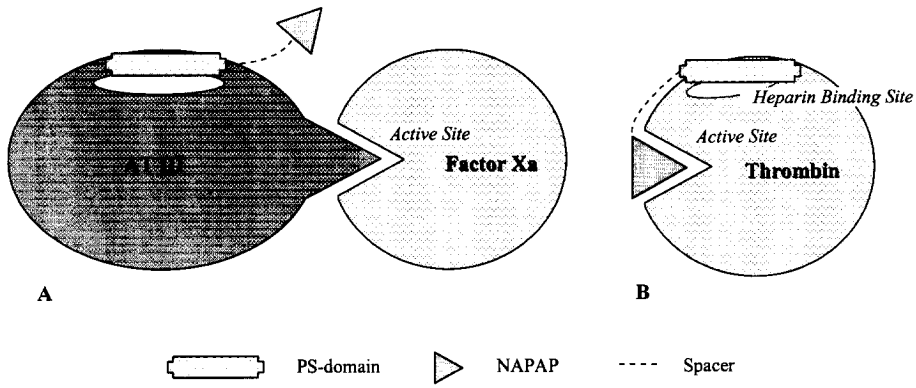
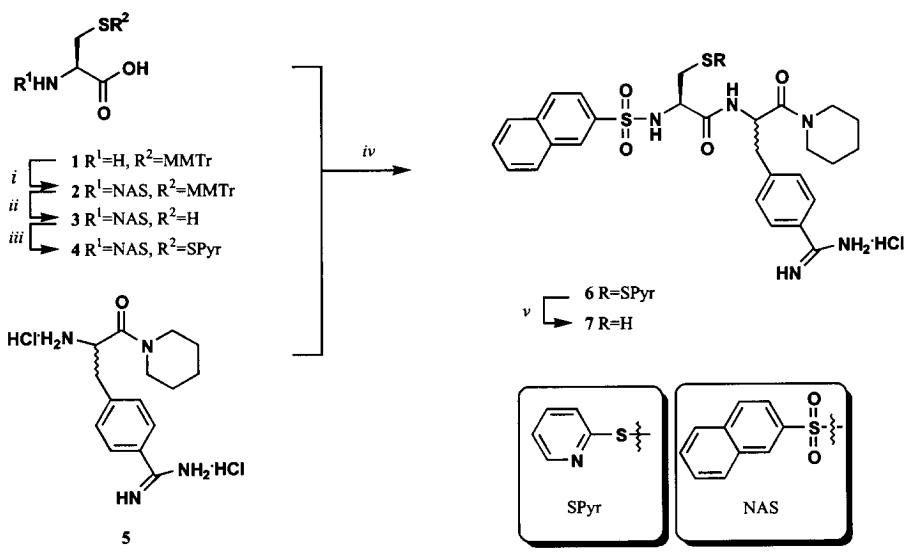


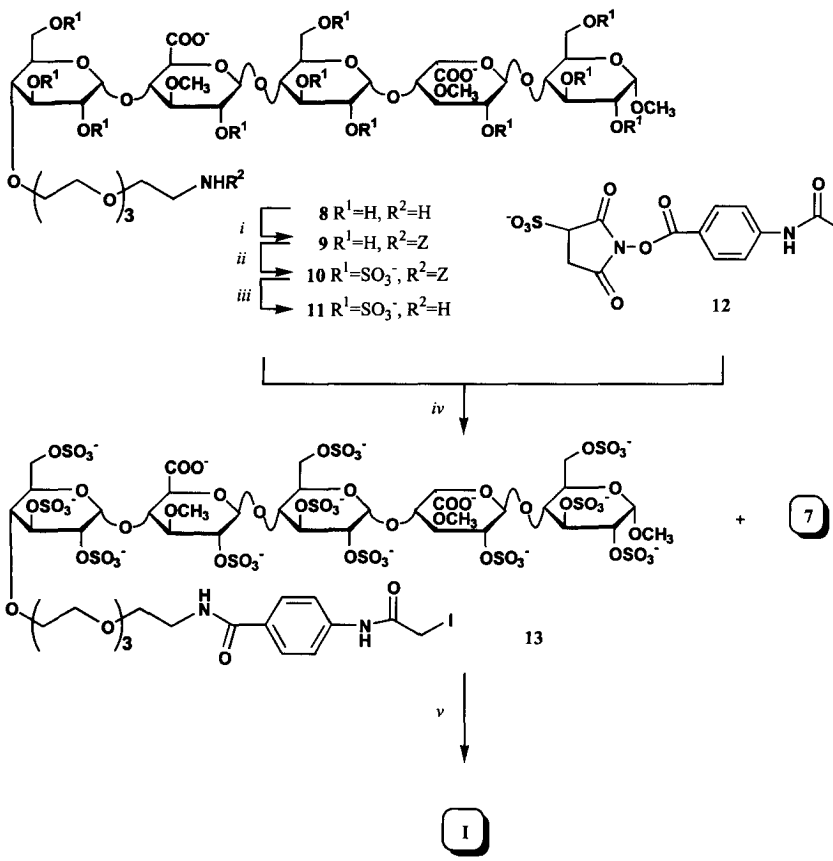
Figure 2. Schematic picture showing the action of a NAPAP-PS dual inhibitor. The PS-domain stimulates (**A**) the inhibitory potency of ATIII towards factor Xa by binding to ATIII, whereas the NAPAP part inhibits thrombin directly (**B**) by interaction with its active site.

Results and Discussion

The construction of conjugate **I** required suitably derivatized NAPAP and PS building blocks. Earlier investigations[9] indicated that derivatization at the α-carbon of the glycine moiety of NAPAP did not influence its inhibitory potency against thrombin. Therefore, we synthesized NAPAP derivative **7** (see Scheme 1), the thiol-function of which allowed conjugation with thiophilic groups like maleimides and bromo- or iodoacetamides.[10] Synthesis of compound **7** commences with sulfonylation of *S*-[4-monomethoxytrityl]-(L)-cysteine[11] (**1**) with 2-naphtalenesulfonyl chloride (NAS-Cl) under Schotten-Baumann conditions.



Scheme 1: (i) NAS-Cl, dioxane/10% Na_2CO_3 (1/1, v/v), 1 h, 76%; (ii) TFA/triisopropylsilane/CH_2Cl_2 (1/1/18, v/v/v), 20 min; (iii) Aldrithiol™, isopropanol/2 N AcOH (1/1, v/v), 1 h, 55% (2 steps); (iv) EDCI, HOBt, *N*-ethylmorpholine, DMF, 16 h, 70%; (v) Bu_3P, MeOH, 1 h.



Scheme 2: (i) Z-OSu, DMF/H_2O (1/4), *N*-ethylmorpholine, 15 min, 91%; (ii) a. $(C_2H_5)_3N{\cdot}SO_3$, DMF, 55 °C, 16 h, b. 0.2 N HCl, 4 °C, 16 h; (iii) H_2, Pd/C, *tert*-BuOH, H_2O, 3 h, 60% (3 steps); (iv) 0.1 M Na_2HPO_4 (pH 7.5), 3 h; (v) DMF, MeOH/0.1 M Na_2HPO_4 (pH 7.0) (1/4/5, v/v/v), 3 h, 52% (2 steps).

Detritylation[11] of the resulting sulfonamide **2** gave the free thiol **3**, which was subsequently treated with an excess of dithiodipyridine (Aldrithiol™) to yield thiopyridyl protected **4**. Condensation of **4** with the known[9b] (D/L)-4-amidinophenylalanyl piperidine dihydrochloride (**5**, H-(D/L)-Adf-PiP) using the well established peptide coupling agent 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) in the presence of 1-hydroxybenzotriazole (HOBt) and *N*-ethylmorpholine as a base afforded compound **6** as a mixture of diastereoisomers in an overall yield of 29% (from **1**). Compound **6** was reduced using a slight excess (1.1 equiv) of tri-*n*-butylphosphine in methanol to give the free thiol **7**. In order to gain access to the NAPAP-PS conjugate **I** iodoacetyl containing PS **13** had to be synthesized, which could be obtained from known[4d] **8** in five consecutive steps (see Scheme 2). Protection of the amine in **8** with *N*-(benzyloxycarbonyloxy)-succinimide (Z-OSu) yielded **9**. Sulfation of the free hydroxyls in **9** with triethylamine/sulfur trioxide complex and subsequent treatment with 0.2 N HCl (to remove the *N*-SO_3^- group formed during sulfation) gave **10**. Reductive cleavage of

the Z-group in **10** and condensation of the corresponding amine **11** with the thiophilic *sulfo*-SIAB™ linker **12** gave the required PS **13**. Compound **13** was reacted with a two-fold excess of an epimeric mixture of NAPAP derivative **7**, which afforded the NAPAP-PS conjugate **I** as a mixture of diastereoisomers in an overall yield of 28% (based on **8**). The homogeneity and the identity of compound **I** was firmly established by mass spectrometry and NMR-spectroscopy.[12] Conjugate **I** indeed displayed a unique antithrombotic profile (in vitro) in that it shows both high anti-thrombin (IC_{50} = 0.35 μM) and ATIII-mediated anti-Xa activity (885 U mg^{-1}).[13] We were anxious to find the pharmacological implications of this unprecedented profile in an *in vivo* model. Measurement of the antithrombotic activity in an aorta-flow model[14] revealed that conjugate **I** is a stronger inhibitor than a combination of the free pentasaccharide and (±)-NAPAP. In addition, preliminary pharmacokinetic studies indicated that the elimination half-life of conjugate **I** is prolonged significantly compared to NAPAP (~1.5 h vs 9 min in rat)[5c], thus confirming that the half-life of this novel class of antithrombotics is determined by the interaction of the PS with ATIII.

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8. Various pentasaccharide analogs have been prepared (see ref 5b), which display half-lives between 1 to 11 hrs (in rat). Since there is a direct correlation between the binding affinity of a pentasaccharide domain with ATIII and its half-life in circulation (see ref 7), various conjugates could be prepared with different PS analogs, thus tuning the half-lives of these conjugates.

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12. ^{1}H NMR (D_2O, 600 MHz, 300 K, HH-COSY): δ (HOD = 4.76) 3.60, 3.53, 3.43 (3 x s, 9H, $CH_3O_{E,G,H}$); ***ring D***: 5.53 (m, 1H, H1), 4.15 (m, 1H, H2), 4.58 (m, 1H, H3), 3.56 (m, 1H, H4), 3.92 (m, 1H, H5), 4.26, 4.13 (2 x m, 2H, H6, H6'); ***ring E***: 4.70 (d, 1H, H1, $J_{1,2}$ = 8.1 Hz,), 4.21 (m, 1H, H2), 3.62 (m, 1H, H3), 3.92 (m, 1H, H4), 3.74 (m, 1H, H5); ***ring F***: 5.39 (d, 1H, H1, $J_{1,2}$ = 3.8 Hz), 4.22 (m, 1H, H2), 4.56 (m, 1H, H3), 3.83 (t, 1H, H4, $J_{3,4} = J_{4,5}$ = 9.8 Hz), 4.12 (m, 1H, H5); ***ring G***: 5.15 (bs, 1H, H1), 4.35 (m, 1H, H2), 3.76 (m, 1H, H3), 4.21 (m, 1H, H4), 4.80 (m, 1H, H5); ***ring H:*** 5.10 (d, 1H, H1, $J_{1,2}$ = 3.6 Hz), 4.31 (m, 1H, H2), 4.54 (m, 1H, H3), 4.21 (m, 1H, H4); ***spacer***: 7.51, 7.53, 7.13, 7.12 (4 x d, 4H, H_{arom} SIAB), 3.73 (m, 2H, $CH_2CH_2NH_2$), 3.66 (m, 12H, OCH_2 TEG), 3.31 (m,, 2H, CH_2NH_2); ***peptide***: 8.27, 8.22 (2 x s, 1H, H_{arom} NAS), 7.98-7.60 (m, 6H, H_{arom} NAS), 7.71, 7.64, 7.46, 7.44 (4 x d, 4H, H_{arom} Adf), 4.60, 4.45 (2 x t, 1H, αCH Adf, $J_{\alpha CH,\beta CH}$ = 6.6 Hz), 4.00, 3.97 (2 x m, 1H, αCH Cys), 3.10-2.85 (m, 4H, CH_2N piperidine), 2.82-2.70 (m, 3H, βCH_2 Cys, βCH Adf), 2.61 (m, 1H, βCH' Adf), 1.55-1.15 (m, 6H, CH_2 piperidine); ES-MS: $[M\text{-}H]^-$ 2680.6.

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