Targeting the GPIbα Binding Site of Thrombin To Simultaneously Induce Dual Anticoagulant and Antiplatelet Effects

ARTICLE *in* JOURNAL OF MEDICINAL CHEMISTRY · MARCH 2014

Impact Factor: 5.45 · DOI: 10.1021/jm4020026 · Source: PubMed

7 AUTHORS, INCLUDING:

Jay N Thakkar
Virginia Commonwealth University
10 PUBLICATIONS **105** CITATIONS
SEE PROFILE

Bassem M Mohammed
Virginia Commonwealth University
13 PUBLICATIONS **19** CITATIONS
SEE PROFILE

Erika J Martin
Virginia Commonwealth University
53 PUBLICATIONS **532** CITATIONS
SEE PROFILE

Donald F Brophy
Virginia Commonwealth University
75 PUBLICATIONS **733** CITATIONS
SEE PROFILE

Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

Targeting the GPIbα Binding Site of Thrombin To Simultaneously Induce Dual Anticoagulant and Antiplatelet Effects

Akul Y. Mehta,[†] Jay N. Thakkar,[†] Bassem M. Mohammed,[‡,§] Erika J. Martin,[‡] Donald F. Brophy,[‡] Takao Kishimoto,[∥] and Umesh R. Desai*[,†]

[†]Department of Medicinal Chemistry and Institute for Structural Biology and Drug Discovery, Virginia Commonwealth University, Richmond, Virginia 23219, United States

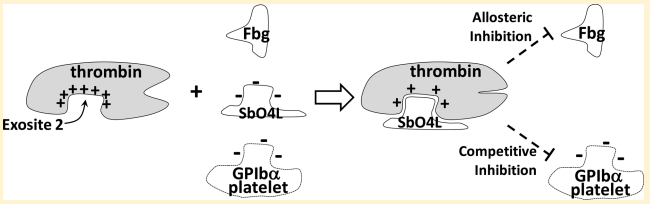
[‡]Department of Pharmacotherapy and Outcomes Science, Virginia Commonwealth University, Richmond, Virginia 23298, United States

[§]Department of Clinical Pharmacy, Faculty of Pharmacy, Cairo University, Cairo 11562, Egypt

[∥]Department of Biotechnology, Faculty of Engineering, Toyama Prefectural University, Imizu, 939-0398, Japan

Supporting Information

ABSTRACT: Exosite 2 of human thrombin contributes to two opposing pathways, the anticoagulant pathway and the platelet aggregation pathway. We reasoned that an exosite 2 directed allosteric thrombin inhibitor should simultaneously induce anticoagulant and antiplatelet effects. To assess this, we synthesized SbO4L based on the sulfated tyrosine-containing sequence of GPIbα. SbO4L was synthesized in three simple steps in high yield and found to be a highly selective, direct inhibitor of thrombin. Michelis−Menten kinetic studies indicated a noncompetitive mechanism of inhibition. Competitive inhibition studies suggested ideal competition with heparin and glycoprotein Ibα, as predicted. Studies with site-directed mutants of thrombin indicated that SbO4L binds to Arg233, Lys235, and Lys236 of exosite 2. SbO4L prevented thrombin-mediated platelet activation and aggregation as expected on the basis of competition with GPIbα. SbO4L presents a novel paradigm of simultaneous dual anticoagulant and antiplatelet effects achieved through the GPIbα binding site of thrombin.



INTRODUCTION

Thrombin, a key factor of the coagulation cascade, is a highly plastic enzyme.[1−6] Its catalytic activity and specificity can be modulated by a wide range of ligands such as fibrinogen, protease-activated receptors, glycoprotein Ibα (GPIbα), sulfated glycosaminoglycans (GAGs), thrombomodulin, hirudin, and many others.[3,5−12] These ligands usually engage one or both of the allosteric sites on thrombin called anion-binding exosites 1 and 2, which span a wide area on thrombin surface on either side of its active site. Although both allosteric sites are electropositive domains, exosite 2 prefers to bind to more negatively charged ligands, e.g., sulfated GAGs, than exosite 1.[13]

Allosterism is the origin of thrombin's highly specific interactions and regulatory functions. Whereas exosite 1 allosterism is the basis for a procoagulant signal in the form of fibrinogen cleavage,[14] it is also the reason for an anticoagulant signal in the form of thrombomodulin-initiated cleavage of protein C.[15] Likewise, exosite 1 is the basis for a highly specific inhibitory interaction in the form of hirudin.[12] In fact, nearly all exosite 1−ligand interactions utilize a unique combination of residues on thrombin that generate exquisite specificity. Interestingly, such specificity of interactions is not uniformly found for exosite 2 ligands. The classic example of such a nonspecific interaction is unfractionated heparin (UFH), a sulfated GAG (Figure 1).[11] Key reasons for the poor specificity resident in

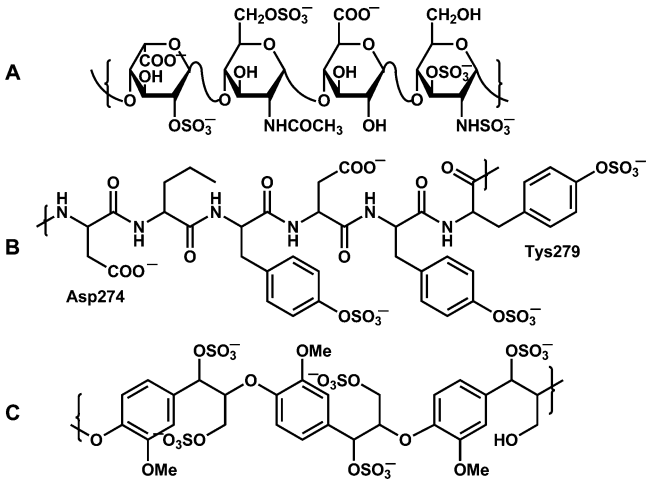


Figure 1. Representative structures of (A) unfractionated heparin (UFH), (B) GPIbα sequence Asp274-Leu275-Tys276-Asp277-Tys278-Tys279 that binds to exosite 2 of thrombin, and (C) sulfated β-O4 lignin (SbO4L).

Received: December 31, 2013
Published: March 17, 2014

exosite 2−sulfated GAG interactions include too many highly exposed electropositive residues and too few tightly bound water molecule(s) that can be displaced upon binding.[16]

Despite this lack of specificity with regard to sulfated GAGs, exosite 2 binds tightly and specifically to GPIbα, a platelet surface receptor. Exosite 2 therefore contributes to two opposing pathways. It binds to UFH and contributes to the anticoagulant pathway[17,18] while also interacting with GPIbα and contributing to the platelet activation pathway.[19−24]Interestingly, both pathways utilize sulfated ligands. Whereas a highly sulfated heparin chain is critical for the antithrombin-based anticoagulant response, sulfated tyrosines (Y_S) on GPIbα, e.g., $^{276}Y_S$, $^{278}Y_S$, and $^{279}Y_S$, are the basis for platelet activation and aggregation response (Figure 1). In fact, in the case of GPIbα, the $^{276}Y_SDY_SY_S^{279}$ sequence makes strong, direct interactions with exosite 2.[22−24] More importantly, both UFH and GPIbα essentially utilize an identical set of exosite 2 residues including Arg93, Arg101, Arg233, Lys235, Lys236, and Lys240.[11,22−24] Thus, nature appears to have developed exosite 2 as a mutually exclusive cue to engineer specificity of function.

We reasoned that this mutual exclusivity could be exploited in developing a dual anticoagulant and antiplatelet molecule, assuming that exosite 2 residues that contribute to the specificity of GPIbα could be targeted. Literature provides some precedent for the possibility of simultaneous dual outcomes. Thrombin-mediated platelet procoagulant activity can be blocked by UFH and low molecular weight (LMW) heparins.[21] Likewise, GPIbα appears to reduce the cleavage of fibrinogen by thrombin[25,26] and has been shown to inhibit thrombin-mediated activation of factor VIII via anionic peptide.[27] Thus, a highly sulfated ligand that specifically targets exosite 2 residues, which bind to GPIbα, may allosterically inhibit thrombin's procoagulant function while competitively inhibiting thrombin-mediated platelet activation and aggregation.

Recently, we designed a group of allosteric, direct thrombin inhibitors, which include sulfated LMW lignins and sulfated benzofurans.[28−31] These molecules were fairly potent (0.1−10 μM) and found to engage exosite 2 residues and induce allosteric inhibition. However, the chemoenzymatic preparation of sulfated LMW lignins resulted in high microheterogeneity because of the presence of various intermonomer linkages including β−O4, β−5, β−β, 5−5 and others.[28,32] This resulted in relatively poor selectivity of targeting serine proteases.[33] To improve upon this, we synthesized sulfated β-O4 lignin (SbO4L) as a mimic of the sulfated tyrosine sequence of GPIbα (Figure 1). SbO4L contains only one type of intermonomer linkage (β−O4), can be synthesized in only three simple steps, and exhibits high selectivity and potency (14 nM) for exosite 2 based allosteric thrombin inhibition while also preventing platelet activation arising from competition with sulfated tyrosines of GPIbα. This work presents the paradigm that potent and highly selective sulfated molecules could be developed to target the GPIbα binding site on thrombin and simultaneously induce anticoagulant and antiplatelet activities.

RESULTS

Synthesis and Characterization of SbO4L. We developed a three-step process to synthesize SbO4L, which included alkaline polymerization of ethyl 2-bromo-3-(4-hydroxy-3-methoxyphenyl)-3-oxopropanoate followed by sodium borohydride/methanol reduction and sulfation using triethylamine−sulfur trioxide complex (see Figure S1 in Supporting Information).[28,34−38]

The aqueous GPC−HPLC profile of SbO4L indicated the number (M_N), weight (M_W), and peak (M_P) average molecular weights of 9200, 12 300, and 9100, respectively, which implied that on an average the SbO4L chain is ∼23 residues long. In comparison, an average UFH chain is 50 residues long. The GPC−HPLC chromatograms also indicated absence of species with molecular weight less than 1000, suggesting an enriched preparation of longer oligomers that constituted composition greater than 95%. The polydispersity, i.e., the ratio of M_W to M_N, of SbO4L was found to be 1.336, which is similar to that of UFH used in the clinic.[39] The sulfation density calculated on the basis of the difference in M_W between SbO4L and its unsulfated precursor was ∼2 sulfate groups per monomer. Elemental analysis measurements indicated a composition of C 35.39, H 4.15, and S 11.54, which match a hypothetical SbO4L chain of 23 residues containing 27 sulfate groups and calculated composition of C 35.20, H 4.02, S 11.08. This indicates an average of 1.2 sulfate groups per monomer These measurements compare favorably with that of UFH and LMW heparins, which show an average of ∼1.6 anionic groups per monomer. Additional purity and consistency of SbO4L preparation studies were performed using reversed-phase ion pairing UPLC−MS[40] and in vitro thrombin inhibition tests on three independent batches of synthetic SbO4L. The three batches demonstrated essentially identical UPLC−MS fingerprints (see Figure S2A) and indistinguishable IC_{50} for thrombin inhibition (Figure S2B). Thus, overall, the elemental analysis measurements in combination with UPLC−MS fingerprints indicate the presence of uniform β−O4 linkage in SbO4L preparation in high purity (>95%) in comparison to sulfated LMW lignins studied earlier.[28]

SbO4L Is a Selective, Direct Inhibitor of Human Thrombin. The effect of SbO4L on the coagulation enzymes was studied using chromogenic substrate hydrolysis assays under optimal conditions for each enzyme, as described earlier.[28,29,33,40] The decrease in residual activity of each enzyme as SbO4L concentration was varied over 10^5-fold and was fitted using the dose−response eq 1 to calculate the IC_{50}, Hill slope, and maximal (Y_M) and minimal activity (Y_0) parameters (Figure 2, Table 1). SbO4L inhibited human α-thrombin with an IC_{50} of 0.17 μg/mL (∼14 nM), while factor Xa and factor XIa were inhibited at 90−500 μg/mL SbO4L (∼10−55 μM), suggesting highly selective inhibition. To test whether the high potency of direct inhibition could be affected by the indirect pathway, thrombin inhibition by SbO4L was studied in the presence of antithrombin. Except for a decrease in the efficacy of inhibition ($\Delta Y = Y_M - Y_0$) arising from the covalent inhibition induced by the serpin, the IC_{50} remained unaffected (Table 1 and Figure S3). This suggested that SbO4L does not interact with the heparin-binding site of antithrombin supporting selectivity of thrombin recognition. Likewise, activation of protein C by thrombin−thrombomodulin complex was inhibited by SbO4L, but the IC_{50} was ∼20-fold higher than that for direct thrombin inhibition (see Figure S4). The exact molecular reason for the 20-fold lower potency against thrombin−thrombomodulin complex is not clear. The glycoprotein thrombomodulin binds to exosite 1 of thrombin via its peptide chain while simultaneously binding to exosite 2 using its chondroitin sulfate chain. It is possible that the GAG chain of thrombomodulin competes with SbO4L. Alternatively, the SbO4L does not recognize thrombomodulin-bound conformationally altered thrombin. Inhibition of factor IXa was not noticeable at SbO4L concentrations as high as 81 μg/mL, while factor VIIa−recombinant tissue factor complex was inhibited at 9.4 μg/mL SbO4L. The results indicated that SbO4L inhibited

dx.doi.org/10.1021/jm4020026 | J. Med. Chem. 2014, 57, 3030−3039

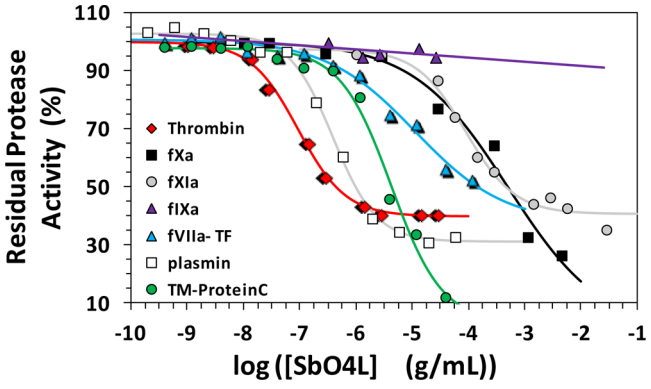


Figure 2. Direct inhibition of serine proteases by SbO4L. The inhibition of human α-thrombin, factors (f) Xa, fXIa, fIXa and fVIIa–tissue factor (TF) complex, plasmin, and thrombomodulin (TM)–protein C complex by SbO4L was studied using chromogenic substrate hydrolysis assays. Solid lines represent sigmoidal dose–response fits (eq 1) to obtain IC_{50}. Other serine proteases studied include cathepsin G, human leukocyte elastase, porcine pancreatic elastase, activated protein C, chymotrypsin, and trypsin at a concentration ~475-fold in excess over the IC_{50} of thrombin inhibition.

human thrombin ~20- to 2940-fold better than the closely related coagulation factors. It is important to note that molecules with structures comparable to SbO4L, such as sulfated GAGs (UFH and fondaparinux), do not inhibit these coagulation enzymes directly at concentrations as high as 100 μg/mL.[29] This implies that subtle differences exist between how the two types of molecules (SbO4L and sulfated GAGs) interact with thrombin.

To assess the selectivity against proteases outside the coagulation cascade, we screened cathepsin G, human leukocyte elastase, kallikrein, porcine pancreatic elastase, activated protein C, plasmin, chymotrypsin, and trypsin. These proteases have been known to bind UFH with varying affinities and have been studied earlier for inhibition with sulfated LMW lignins.[33] Except for plasmin, the activity of each protease remained unaffected in the presence of 81 μg/mL SbO4L (Table 1), suggesting a minimum of 475-fold selectivity. For plasmin, a dose–response study led to an IC_{50} of 0.38 μg/mL (Figure 2, Table 1), which is 2.2-fold higher than that against thrombin. Although plasmin does not contribute much to coagulation and platelet aggregation pathways, the observed high potency suggests that appropriate modifications in the SbO4L structure are necessary to better segregate thrombin and plasmin recognition. Yet the fortuitous plasmin targeting could also be of value because complex pathologies, such as disseminated intravascular coagulation, are known wherein simultaneous thrombin and plasmin inhibition is deemed to be important.[41] An important point to also note is that SbO4L is likely to bind other proteins present in human plasma, such as serum albumin, in the manner of heparin. Yet overall, SbO4L selectively inhibited human thrombin and plasmin among several structurally similar and relevant trypsin-like proteases.

SbO4L Is an Allosteric Inhibitor of Thrombin. To elucidate the mechanism of thrombin inhibition, substrate hydrolysis was studied in the presence of SbO4L. The initial rates of Spectrozyme TH cleavage at pH 7.4 in the presence of varying levels of SbO4L were hyperbolic, as expected on the basis of Michaelis–Menten kinetics (Figure 3). The profiles revealed that as the concentration of SbO4L increased from 12 ng/mL to 2.3 μg/mL, the maximal velocity of hydrolysis (V_{MAX}) decreased steadily and the $K_{M,app}$ remained essentially invariant (~1.5 μM). This implied that SbO4L does not affect small chromogenic substrate binding but brought about considerable changes in the active site that reduce the catalytic rate. Thus, SbO4L was found to be a noncompetitive, allosteric inhibitor of human thrombin in a manner similar to other sulfated LMW lignins studied earlier.[29]

Allostery Arises from Binding in Exosite 2 of Thrombin and Not in Exosite 1. To assess whether SbO4L engages one or both exosites of thrombin, competitive inhibition studies were performed using prototypical ligands, hirugen (exosite 1) and heparin (exosite 2). HirP, a hirudin-based dodecapeptide that binds avidly to exosite 1 (K_D = 28 nM),[42] did not affect the apparent IC_{50} of thrombin inhibition by SbO4L at concentrations nearly 3.1 times higher than the affinity (Figure 4A, Table 2). This implies that the SbO4L does not engage exosite 1 of thrombin. When the thrombin inhibition was studied in the presence of both SbO4L and UFH, the IC_{50} increased from

Table 1. Sulfated β-O4 Lignin-Based Selective Inhibition of Thrombin among Many Serine Proteases

serine protease	log[IC_{50} (g/mL)][a,d]	IC_{50} (μg/mL)[d]	Y_M [a,d]	Y_0 [a,d]	HS [a,d]
activated protein C	*b*	>81	*b*	*b*	*b*
cathepsin G	*b*	>81	*b*	*b*	*b*
chymotrypsin	*b*	>81	*b*	*b*	*b*
human neutrophil elastase	*b*	>81	*b*	*b*	*b*
porcine pancreatic elastase	*b*	>81	*b*	*b*	*b*
factor VIIa–tissue factor[c]	~ −5.0 [c]	>11	100 ± 2	~38	~0.5
factor IXa	*b*	>81	*b*	*b*	*b*
factor Xa	−3.3 ± 0.6	500 ± 300	100 ± 4	~1	0.5 ± 0.2
factor XIa	−4.1 ± 0.1	89 ± 9	98 ± 3	41 ± 2	1.0 ± 0.2
kallikrein	*b*	>81	*b*	*b*	*b*
plasmin	−6.4 ± 0.1	0.38 ± 0.04	103 ± 1	31 ± 2	1.1 ± 0.1
thrombin	−6.8 ± 0.1	0.17 ± 0.01	99 ± 1	29 ± 1	1.6 ± 0.1
thrombin with antithrombin[e]	−6.7 ± 0.1	0.20 ± 0.01	64 ± 1	6 ± 1	1.9 ± 0.4
thrombin/recombinant thrombomodulin–protein C	−5.4 ± 0.1	4.20 ± 0.08	98 ± 2	3 ± 5	1.0 ± 0.1
trypsin	*b*	>81	*b*	*b*	*b*

[a]The IC_{50}, HS, Y_M, Y_0 values were obtained following nonlinear regression analysis of direct inhibition of the protease. [b]Not applicable. Essentially no inhibition was observed at high concentration noted in the IC_{50} column. [c]Value estimated because of relatively weak inhibition. [d]Errors represent ±SD based on two or three independent experiments. [e]In the presence of 200 nM AT.

dx.doi.org/10.1021/jm4020026 | J. Med. Chem. 2014, 57, 3030–3039

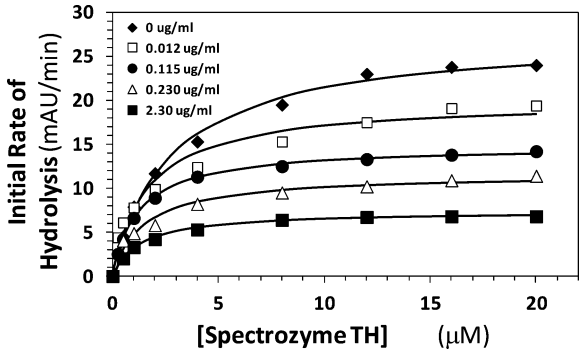


Figure 3. Michaelis–Menten kinetics of Spectrozyme TH hydrolysis by thrombin in the presence of SbO4L. The initial rate of hydrolysis at various substrate concentrations was measured in a pH 7.4 buffer. The concentrations of SbO4L were 0 (◆), 0.012 (□), 0.115 (●), 0.23 (△), and 2.3 μg/mL (■). Solid lines represent nonlinear fits to the data using the standard Michaelis–Menten equation.

0.2 to 1.25 μg/mL (Figure 4B, Table 2), which paralleled that predicted by the Dixon–Webb relationship for ideal competitive behavior (Figure S5). This implied that SbO4L competes with UFH for binding to thrombin, which suggests interaction with residues in or near exosite 2.

SbO4L Competes with GPIbα. To further understand SbO4L allostery, we decided to utilize recombinant GPIbα as a competitor.[22−24,43,44] Although GPIbα inhibits thrombin-mediated fibrinogen to fibrin conversion, it does not affect thrombin's hydrolysis of small peptides.[26] This afforded a simple experiment based on thrombin inhibition to test competition between SbO4L and GPIbα. As the concentration of GPIbα increased from 0 to 458 nM, the IC_{50} of thrombin inhibition by SbO4L increased from 0.17 to 1.15 μg/mL

Table 2. Inhibition of Human Plasma α-Thrombin by SbO4L in the Presence of Exosite 1 (HirP) and Exosite 2 (UFH and GPIbα) Ligands

[HirP], nM	IC_{50} (μg/mL)[a]	Y_M	Y_0	HS
0	0.15 ± 0.01	102 ± 14	24 ± 1	1.2 ± 0.1
8.6	0.17 ± 0.02	103 ± 2	23 ± 1	1.2 ± 0.1
86	0.14 ± 0.02	102 ± 3	17 ± 2	1.2 ± 0.2
[UFH], μM	IC_{50} (μg/mL)[a]	Y_M	Y_0	HS
0.2	0.22 ± 0.01	99 ± 1	26 ± 1	1.6 ± 0.1
2.0	0.34 ± 0.02	98 ± 1	26 ± 1	1.8 ± 0.1
20	0.66 ± 0.05	96 ± 1	24 ± 1	1.6 ± 0.2
100	1.25 ± 0.14	100 ± 1	28 ± 2	0.9 ± 0.1
[GPIbα], nM	IC_{50} (μg/mL)[a]	Y_M	Y_0	HS
0	0.23 ± 0.01	101 ± 2	18 ± 1	1.5 ± 0.1
229	0.69 ± 0.05	99 ± 1	22 ± 1	1.5 ± 0.1
458	1.15 ± 0.05	101 ± 2	28 ± 1	1.6 ± 0.1

[a]The IC_{50}, HS, Y_M, Y_O values were obtained following nonlinear regression analysis of direct inhibition of human plasma α-thrombin. Errors represent ±SD based on two independent experiments.

(Figure 4C), which paralleled the behavior predicted on the basis of ideal competition (see Figure S6). Thus, SbO4L competes with GPIbα for binding to human thrombin. Considering that SbO4L binds to thrombin in exosite 2, the above two results suggested that some or all of the residues involved in UFH and GPIbα binding are likely to be important for SbO4L too.

SbO4L Binds to Arg233, Lys235, and Lys236 of Exosite 2. To pinpoint the exosite 2 residues that interact with SbO4L, we studied 11 recombinant thrombins containing substitution of basic residues with alanine. These single-site or multisite thrombin mutants have been studied earlier and

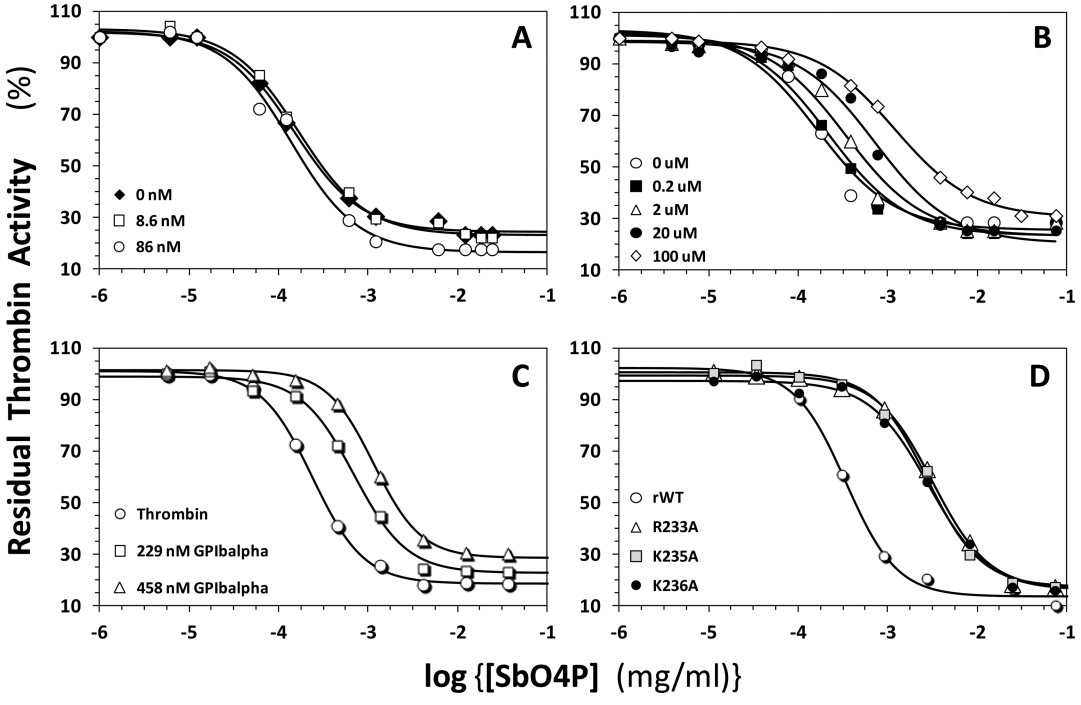


Figure 4. Effect of a hirudin-based peptide HirP (A), UFH (B), and GPIbα (C) on the inhibition of human α-thrombin by SbO4L and of alanine replacement of selected exosite 2 residues on the inhibition profile of SbO4L (D). The inhibition human plasma or recombinant thrombin by SbO4L was studied through Spectrozyme TH hydrolysis assays at pH 7.4 in the presence of fixed concentrations of competitors (as shown). Solid lines represent fits by the dose–response eq 1 to obtain the apparent IC_{50}.

dx.doi.org/10.1021/jm4020026 | J. Med. Chem. 2014, 57, 3030−3039

possess a fully functional catalytic machinery.[45,46] Alanine replacement at Arg126, Arg165, Lys169, Arg173, Arg175, or Lys240 showed minimal change (0.9- to 2.1-fold) in SbO4L inhibitory potential compared to their wild-type recombinant reference. Likewise, a triple mutant containing Arg93,101, 107Ala did not affect the IC_{50}. However, single-site alanine substitutions at Arg233, Lys235, and Lys236 positions were found to have 8- to 9-fold increase in IC_{50} compared to that for the wild-type thrombin (Figure 4D). This indicated that each of these residues is important for SbO4L-mediated inhibition of thrombin. Interestingly, these residues are also known to be crucial for interaction with heparin[11] and GPIbα.[21−24]

SbO4L Inhibits Clot Formation in Human Plasma. A physiologic implication of inhibition of thrombin-mediated fibrin formation is delayed clotting in cell-free systems such as human plasma. Prothrombin (PT) and activated partial thromboplastin times (APTT) are traditional tests used to assess anticoagulation potential of new molecules in human plasma. Figure 5 shows the variation in plasma PT and APTT

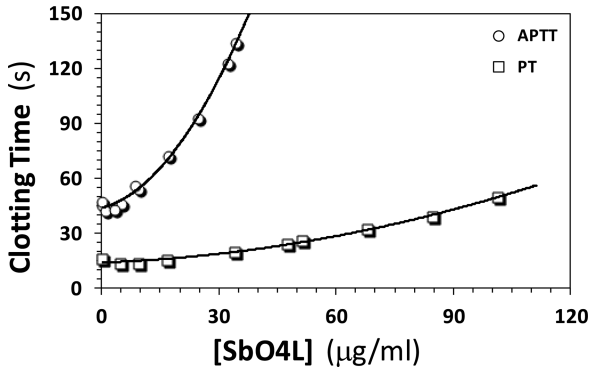


Figure 5. Prolongation of clotting time as a function of SbO4L concentration in either the prothrombin time (PT) or the activated partial thromboplastin time (APTT) assay. The solid lines are trend lines and not exponential fits. Errors in clotting time measurement were in the range of symbol size and have been omitted.

in the presence of SbO4L. A significant concentration-dependent prolongation of clotting times was observed suggesting good anticoagulation potential. A 2-fold increase in PT required 68 μg/mL, corresponding to 7.5 μM, which is significantly lower than that needed for a generic LMW heparin (142 μg/mL, 31.6 μM) and a clinically used LMW heparin (enoxaparin, 339 μg/mL or 75 μM).[47] Likewise, a 2-fold increase in APTT required 20 μg/mL (2.2 μM) SbO4L, which compares favorably with a concentration of 5.9 μg/mL (1.3 μM) for generic LMW heparin and 5.4 μg/mL (1.2 μM) for enoxaparin.[47] These results show that SbO4L is an effective anticoagulant in plasma and nearly as potent as LMW heparins. These initial anticoagulation results may also imply the possibility of SbO4L possessing side effects common to heparins, i.e., bleeding. Yet a homogeneous SbO4L analogue may demonstrate much reduced side effects.

SbO4L Prevents Thrombin-Mediated Platelet Aggregation and Activation. We reasoned that if SbO4L competes with GPIbα for binding to thrombin, it should reduce platelet activation as well as platelet aggregation induced by thrombin. Platelet aggregation is typically monitored by following the increase in light transmittance of platelet rich plasma (PRP) following the addition of a platelet agonist.[20] However, this strategy does not work well with α-thrombin because of the simultaneous formation of fibrin mesh in blood. For this reason, we used γ-thrombin, which is not effective in cleaving fibrinogen to fibrin, while exhibiting good ability to initiate platelet aggregation.[20] In the presence of 515 μg/mL SbO4L, 76 nM γ-thrombin did not induce any platelet aggregation in contrast to rapid aggregation observed in the absence of SbO4L (Figure 6A). This antiplatelet effect was maintained when the SbO4L concentration was reduced ∼5-fold to 105 μg/mL but was essentially lost at 54 μg/mL (Figure 6B).

Thrombin-mediated platelet aggregation arises due to thrombin binding to GPIbα, which transduces platelet activation signal.[20] Thus, competition between SbO4L and GPIbα should reduce the signal transduction. A molecular event characteristic of platelet activation is the release of ATP following degranulation, which serves to amplify the activation signal further in a feedback manner.[48] Thus, we reasoned that the presence of SbO4L should also reduce ATP release in a dose-dependent manner. ATP secretion by dense granules of platelets following activation by α-thrombin (1 U) was monitored using the well-established luminescence method in the presence of luciferase/luciferin.[48,49] As the concentration of SbO4L was increased from 0 to 248 μg/mL, the levels of ATP secreted decreased gradually by ∼85% (Figure 6C).

It is interesting that the ATP secretion assay shows a fine concentration dependence (Figure 6B) in contrast to the aggregation assay, which shows a more "on or off" effect (Figure 6C). Whereas the luminescence-based ATP secretion assay is a highly sensitive molecular assay that measures small changes in ATP levels, the transmittance-based aggregation assay relies more on physical forces between activated platelets, which depend on the colligative nature of aggregation. This results in the apparent nonuniform dose-dependence between the two assays. Overall, both platelet aggregation and ATP release studies support the expectation that SbO4L competes with platelet GPIbα for binding to thrombin.

■ DISCUSSION

All current clinically used direct thrombin inhibitors (DTIs) target the active site. While highly successful, DTIs still suffer from over-anticoagulation, in many cases resulting in enhanced bleeding risk.[50−53] We sought to explore a new paradigm of allosteric, direct thrombin inhibition, which we predicted would be easier to achieve for thrombin because of its highly plastic nature.[1−6] In principle, allosteric inhibition promises better regulatory advantages than simply direct, active site inhibition. Whereas potency (or dose) is the only parameter that can be used for regulating inhibition induced by active site inhibitors, potency (IC_{50}) and maximal efficacy (ΔY) are two independent parameters available for regulating inhibition induced by allosteric inhibitors. Our work on sulfated benzofurans shows that allosteric thrombin inhibition is tunable because it was possible to achieve variable levels of maximal efficacy in a structure-dependent manner.[30,31] SbO4L also appears to exhibit this feature (ΔY = 70%, Table 1), although studies with additional SbO4L analogues are necessary to ascertain this principle. Such tunability can be expected to play a major role in avoiding over-anticoagulation. Thus, SbO4L offers a new paradigm with regard to mechanism of anticoagulation, which is fundamentally different from all clinically used anticoagulants.

The mechanism by which SbO4L inhibits thrombin was deduced by a combination of Michaelis−Menten kinetics, competitive inhibition, and mutagenesis studies. SbO4L binds in exosite 2 and interacts with Arg233, Lys235, and Lys236,

dx.doi.org/10.1021/jm4020026 | J. Med. Chem. 2014, 57, 3030−3039

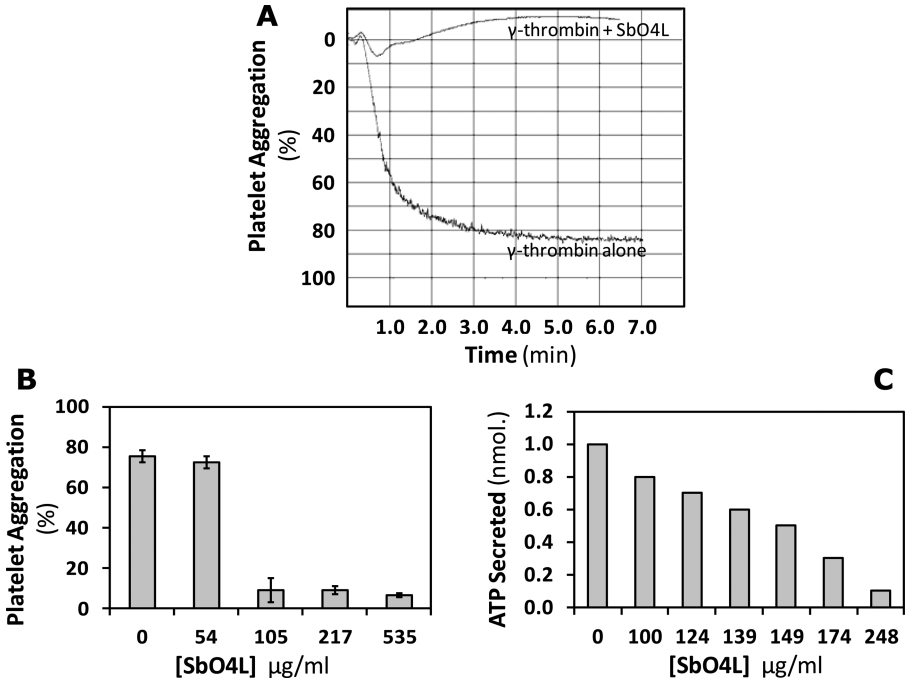


Figure 6. (A) Representative traces of human platelet rich plasma (PRP) aggregation induced by 76 nM γ-thrombin in the presence or absence of 217 μg/mL SbO4L. Aggregation was monitored by following time dependence of transmittance at 620 nm following addition of γ-thrombin. (B) Variation in level of aggregation as a function of the concentration of SbO4L. (C) Reduction in the level of ATP released by platelets in the presence of varying levels of SbO4L.

which are critical for interaction with sulfated tyrosines of GPIbα.[21−24] We find that this exosite 2 subdomain is energetically coupled in a negative manner to thrombin's catalytic triad, resulting in allosteric inhibition. This energetic coupling was alluded to earlier in GPIbα studies,[25,27,31,54] but SbO4L demonstrates that the plasticity of thrombin can be exploited in a synergistic manner to develop dual mechanism (anticoagulant and antiplatelet) modulators. Although at present SbO4L is not attractive as a clinically relevant molecule, it elegantly presents the concept of targeting the GPIbα binding site for developing a novel category of allosteric cum competitive anticoagulants.

Allosteric inhibition promises higher selectivity because allosteric sites are expected to be much less conserved in comparison to catalytic sites.[55] This expectation is certainly borne out by SbO4L, which does not inhibit any related coagulation factor including factors IXa, XIa, Xa, VIIa−tissue factor, or kallikrein (Table 1). Yet it inhibits plasmin with fairly high potency. Sulfated LMW lignins, molecules related to SbO4L, also allosterically inhibit plasmin.[41] This implies that SbO4L most probably binds plasmin in an exosite 2-like electropositive site. In fact, human plasmin contains 11 Arg/Lys residues that can possibly interact with multiple sulfate groups of SbO4L.[56] Of these, Arg779 is positioned identically to Arg233 of thrombin,[11] which is recognized by SbO4L. Yet significant differences between the two binding sites are also evident from the crystal structures. Thus, it should be possible to rationally design an analogue of SbO4L, possibly containing fewer sulfate groups, that displays even higher levels of thrombin specificity. Such an analogue could be a small molecule, e.g., a small, homogeneous SbO4L sequence, which would be more attractive from the clinical perspective.

It is interesting that although both heparin and SbO4L are highly sulfated, the former does not induce exosite 2 mediated thrombin inhibition, but the latter does. Perhaps there are subtle differences in the biophysical forces contributing to the interaction of the two highly sulfated ligands. One difference is obvious. SbO4L contains multiple aromatic rings, while heparin contains none. GPIbα also relies on its sulfated tyrosine to effectively interact with Arg/Lys residues of exosite 2.[20−24,43,44] More importantly, GPIbα induces partial thrombin inhibition in the absence of antithrombin.[25,26] Thus, SbO4L is a true mimetic of GPIbα. The atomistic reason why SbO4L interacts with thrombin so well could be the occurrence of cation−π or π−π interactions in SbO4L−thrombin complex, in addition to sulfate−Arg/Lys interactions. Such additional hydrophobic interactions have also explained the affinity for hirudin to exosite 1.[57] Both cation−π and π−π interactions are highly directional[58] and generate high selectivity of binding.

Finally, SbO4L is likely to provide a foundation for developing novel small antithrombotic agents. We have previously demonstrated this possibility through the design of sulfated benzofuran monomers,[59] dimers,[30] and trimers[31] from oligomeric sulfated lignins. Thus, an appropriate small monomeric, dimeric, or trimeric molecule based on the SbO4L structure can be expected to exhibit simultaneous anticoagulant and antiplatelet effect.

EXPERIMENTAL PROCEDURES

Proteins and Chemicals. Human plasma proteases, thrombin, γ-thrombin, and factors Xa, IXa, XIa, and VIIa, recombinant tissue factor, activated protein C, human protein C, and human plasma antithrombin were from Haematologic Technologies (Essex Junction, VT). Cathepsin G, chymotrypsin, kallikrein, trypsin, pancreatic porcine elastase, and recombinant human thrombomodulin were obtained from Sigma Aldrich (St. Louis, MO). Recombinant glycoprotein Ibα (GPIbα) was obtained from R&D Systems (Minneapolis, MN). Human leukocyte elastase was from Elastin Products Company (Owensville, MO). Chromogenic substrates

dx.doi.org/10.1021/jm4020026 | J. Med. Chem. 2014, 57, 3030−3039

Spectrozyme TH, Spectrozyme FXa, Spectrozyme FIXa, Spectrozyme VIIa, Spectrozyme PCa, Spectrozyme PL, and Spectrozyme CTY were obtained from Sekisui Diagnostics (Stamford, CT). Substrates *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA, *N*-(methoxysuccinyl)-Ala-Ala-Pro-Val-*p*NA, and *N*-succinyl-Ala-Ala-Ala-*p*NA were obtained from Sigma Aldrich (St. Louis, MO), and S-2266, S-2222, and S-2366 were obtained from Diapharma (West Chester, OH). Stock solutions of proteins were prepared in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% polyethylene glycol (PEG) 8000 (thrombin and factor XIa) or 20 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM EDTA and 0.1% PEG8000 (factor Xa). Factor VIIa stock solutions of proteins were prepared in 25 mM HEPES buffer, pH 7.4, containing 100 mM NaCl and 5 mM $CaCl_2$, while factor IXa stock solutions were prepared in 100 mM HEPES buffer, pH 8.0, containing 100 mM NaCl and 10 mM $CaCl_2$. Porcine UFH (MW, 15 000) was purchased from Sigma (St. Louis, MO). HirP, a Tyr63-sulfated hirudin-(54−65) peptide labeled with 5-(carboxy)fluorescein, i.e., [5F]Hir[54−65](SO_3^-), was a gift from Dr. Paul Bock (Vanderbilt University).[42] Monomer ethyl 2-bromo-3-(4-hydroxy-3-methoxyphenyl)-3-oxopropanoate was synthesized using a protocol developed earlier.[34] All other chemicals were analytical reagent grade from either Sigma Chemicals (St. Louis, MO) or Fisher (Pittsburgh, PA) and used without further purification.

Synthesis of SbO4L. The synthesis of SbO4L was derived on the basis of our earlier reported polymerization and sulfation strategies.[34−38] Briefly, anhydrous K_2CO_3 (0.58 g) was added to a stirring solution of ethyl 2-bromo-3-(4-hydroxy-3-methoxyphenyl)-3-oxopropanoate (0.97 g) in anhydrous DMF (5 mL) under nitrogen. After 24 h the reaction mixture was poured onto ice−water mixture (120 mL) and the pH adjusted to ∼2.5 with 2 M HCl. The precipitated polymer was filtered, washed with water, and lyophilized to remove moisture. The solid (600 mg) was suspended in methanol (10 mL) at 50 °C followed by careful addition of $NaBH_4$ (683 mg) while maintaining 50 °C. The mixture gradually turned into a clear solution, which was stirred for 24 h. The solution was then neutralized with acetic acid and poured into 0.5 M HCl (200 mL) to precipitate the reduced polymer, which was isolated by centrifugation, washed with water, and lyophilized. Low molecular weight chains of the polymer were removed by dissolution of the mixture in 1,4-dioxane (5 mL) and preferential precipitation of high molecular weight chains in diethyl ether (100 mL). The precipitate was filtered and dried using high vacuum. The reduced polymer (50 mg) so obtained was made to react with triethylamine−sulfur trioxide complex (200 mg) in anhydrous DMF (5 mL) at 65 °C for 24 h.[28,29,37,38] Following reaction, 30% (w/v) aqueous sodium acetate (35 mL) was added and the mixture stirred overnight. The solution was then poured into ice-cold ethanol (100 mL) to precipitate crude SbO4L, which was filtered and washed twice with ice-cold ethanol (10 mL each). The recovered solid was desalted using FloatALyzer G2 (Spectrum Labs) dialysis tubes (MWCO, 0.1−0.5 kDa). Lyophilization of the dialyzed solution gave SbO4L. Elemental analysis of SbO4L was carried out by Atlantic Mircolabs (Norcross, GA). The elemental composition was found to be C 35.39, H 4.15, and S 11.54. The calculated composition of a hypothetical SbO4L chain of 23 residues containing 27 sulfate groups and 31 water molecules would be C 35.20, H 4.02, S 11.08 corresponding to $C_{229}H_{311}O_{204}S_{27}Na_{27}$. In combination with GPC−HPLC and UPLC−MS (below), the elemental analysis results indicated a SbO4L purity of >95%.

Molecular Weights of SbO4L. The number-, weight-, and peak-average molecular weights (M_W, M_N, and M_P, respectively) of SbO4L were measured as described earlier.[28] Briefly, gel permeation chromatography (GPC) of SbO4L on a Asahipak GS-320 HQ (7.5 mm i.d. × 300 mm) column (Showa Denko K. K. Shodex, New York, NY) using 0.1 N NaOH mobile phase flowing at 0.7 mL/min gave a characteristic broad elution profile (detection at 254 nm). Polystyrene standards of different molecular weights and ferulic acid were used for calibration purposes. The relationship between logarithm of the molecular weight and the elution volume (V) of the standards was found to be linear with a correlation coefficient of 0.99. The SbO4L chromatogram was sliced into 1000 time periods providing 1000 average molecular weights with their corresponding absorbances. These slices were used to calculate M_W, M_N, and M_P values.[28]

Reversed-Phase Ion Pairing (RPIP) Ultraperformance Liquid Chromatography (UPLC) and Mass Spectrometry (MS). The reproducibility of three independent batches of SbO4L preparations was compared using RPIP−UPLC−MS, which has been used previously to characterize sulfated mixtures possessing high molecular weights.[40] Briefly, a Waters Acquity H-class UPLC system equipped with a PDA detector and a tandem quadrupole mass spectrometer was used. A 1.7 μm BEH C18 column (2.1 mm × 150 mm) was used for fingerprinting, which required a 5 μL injection of SbO4L (400 μg/mL) at 30 ± 2 °C and a solvent system composed of 10% v/v acetonitrile−water (solvent A) and 75% v/v acetonitrile−water (solvent B). Both solvents contained *n*-hexylamine (25 mM) as an ion-pairing agent along with acid modifier formic acid (0.1%). Resolution of SbO4L was achieved using a linear gradient from 0% to 100% solvent B in 40 min. Absorbance was monitored from 230 to 400 nm, and the eluent was directly injected into the mass spectrometer. ESI-MS detection conditions utilized positive ion mode with capillary voltage of 4 kV, cone voltage of 20 V, desolvation temperature of 350 °C, and nitrogen gas flow rate of 650 L/h. Mass scans were collected in the range of 500−2048 amu within 0.6 s to obtain the UPLC−MS fingerprints.

Direct Inhibition of Coagulation Proteases. Inhibition of thrombin and factors Xa, IXa, XIa, and VIIa by SbO4L was measured using chromogenic substrate hydrolysis assays, as described previously.[29,45] For these assays, a 10 μL aqueous solution of SbO4L was diluted with 930 μL of an appropriate buffer in a PEG20000-coated polystyrene cuvette. Plasmin and recombinant tissue factor−factor VIIa inhibition was studied in a 96-well microplate format, as described below in the section on heparin-binding serine proteases, except for measuring the activity over the full range of SbO4L concentrations. The buffers used in these experiments include 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% PEG8000 for thrombin, factor Xa, and factor XIa; 100 mM HEPES buffer, pH 8, containing 100 mM NaCl and 10 mM $CaCl_2$ for factor IXa; 25 mM HEPES buffer, pH 7.4, containing 100 mM NaCl and 5 mM $CaCl_2$ for factor VIIa and factor VIIa−tissue factor complex; and 50 mM Tris-HCl, 150 mM NaCl, and 0.1% PEG8000, pH 7.4, for plasmin at 37 °C. This was followed by addition of a 10 μL solution of the protease (stock concentrations used: 600 nM thrombin, 108 nM factor Xa, 152 nM factor XIa, 22.5 μM factor IXa, 4 μM factor VIIa) and incubation for 10 min. In the case of fVIIa−tissue factor complex, 5 μL of 266 nM factor VIIa and 5 μL of 800 nM recombinant tissue factor were mixed to prepare the complex. Following incubation, a 50 μL solution of chromogenic substrate was rapidly added and the residual enzyme activity was measured from the initial rate of increase in A_{405}. The chromogenic substrates used were Spectrozyme TH (H-D-hexahydrotyrosol-Ala-Arg-*p*-nitroanilide), Spectrozyme FXa (methoxycarbonyl-D-cyclohexylglycyl-Gyl-Arg-*p*-nitroanilide), Spectrozyme FIXa (D-Leu-Phe-Gly-Arg-*p*-nitroanilide), and Spectrozyme FVIIa (methanesulphonyl-D-cyclohexylalanyl-butyl-Arg-*p*-nitroanilide). Inhibition of factor XIa was studied using Spectrozyme FXa. Relative residual proteinase activity at each concentration was calculated using the activity measured under otherwise identical conditions, except for the absence of SbO4L. The IC_{50} was calculated from the regressional analysis using logistic eq 1.

$$Y = Y_0 + \frac{Y_M - Y_0}{1 + 10^{\{(\log[SbO4L]_0 - \log IC_{50})HS\}}} \quad (1)$$

In this equation Y is the fractional residual protease activity, i.e., the ratio of protease activity in the presence of inhibitor to that in its absence; Y_M and Y_0 are the maximum and minimum fractional residual activities, respectively; IC_{50} is the concentration of the inhibitor that results in 50% inhibition of enzyme activity; and HS is the Hill slope.

Direct Inhibition of Other Heparin-Binding Serine Proteases. The inhibition of other heparin-binding serine proteases by SbO4L was studied using the substrate hydrolysis assay (above) in a 96-well microplate format adapted from previous literature.[33] For these assays, 5 μL of SbO4L (1.62 mg/mL) was diluted with 85 μL of

dx.doi.org/10.1021/jm4020026 | J. Med. Chem. 2014, 57, 3030−3039

an appropriate buffer at either room temperature or 37 °C followed by addition of 5 μL of the protease solution and incubation for 5 min. The activity of the protease was assessed by addition of 5 μL of chromogenic substrate and measuring the initial rate of increase in A_{405} (<10% substrate consumption). The optimal assay conditions for the heparin-binding serine proteases were deduced from the literature[29,33] and included 50 mM Tris-HCl, 50 mM NaCl, pH 8.0, at room temperature for HLE and cathepsin G; 20 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, and 0.1% PEG8000, pH 7.5, at 37 °C for kallikrein and PPE; 50 mM Tris-HCl, 125 mM NaCl, and 10 mM $CaCl_2$, pH 8.0, at 37 °C for APC; and 50 mM Tris-HCl, 150 mM NaCl, and 0.1% PEG8000, pH 7.4, at 37 °C for trypsin and chymotrypsin. The enzyme concentrations used in these experiments were 200 nM cathepsin G, 1 μg/mL HLE, 0.005 U/mL kallikrein, 0.0125 U/mL PPE, 7 nM APC, 0.5 μg/mL chymotrypsin, and 0.145 μg/mL trypsin. Chromogenic substrates used were 1 mM *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA (cathepsin G), 100 μM *N*-(methoxysuccinyl)-Ala-Ala-Pro-Val-*p*NA (HLE), 50 μM H-D-Val-Leu-Arg-*p*NA (S-2266) (kallikrein), 100 μM *N*-succinyl-Ala-Ala-Ala-*p*NA (PPE), 200 μM *H*-D-(γ-carbobenzoxyl)-Lys-Pro-Arg-*p*NA (Spectrozyme PCa) (APC), 160 μM *N*-benzoyl-Ile-Glu-(-OR)-Gly-Arg-*p*NA (S-2222) (trypsin), and 250 μM H-D-Ala-Pro-Phe-*p*NA (Spectrozyme CTY) (chymotrypsin). Each assay was performed two or three times from which mean and standard deviations were calculated.

Inhibition of Thrombin in the Presence of Antithrombin. A 96-well microplate format was used to measure the IC_{50} of SbO4L inhibition of thrombin in the presence and absence of antithrombin adapted from previous reports.[28,29,60] In this format, a 5 μL aqueous solution of SbO4L was diluted with 180 μL of 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% PEG8000 at 37 °C. This was followed by addition of 5 μL of thrombin to give nearly 6 nM final enzyme concentration and 5 μL of antithrombin to give 200 nM serpin in each well. The mixture was incubated at 37 °C for 10 min followed by addition of 5 μL of 5 mM Spectrozyme TH. The residual enzyme activity was determined from the initial rate of increase in A_{405}. The apparent IC_{50} was calculated using logistic eq 1.

Inhibition of Thrombin−Thrombomodulin Mediated Activation of Protein C. The protocol for measuring the activation of protein C by thrombin−thrombomodulin (TH−TM) complex essentially followed the literature.[61] Briefly, 5 μL of SbO4L at concentrations ranging from 2.3 ng/mL to 2.3 mg/mL was added to 70 μL of 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl and 1 mM $CaCl_2$ at 37 °C, followed by addition of 5 μL of thrombin (240 nM) and 5 μL of TM (400 nM). The mixture was incubated for 10 min and 5 μL of protein C (10 μM) added, and the mixture was incubated at 37 °C for another 10 min. After this period, further generation of activated protein C (APC) was quenched by the addition of 5 μL of 10 mM EDTA and 16 μM argatroban (~100K_i). The APC generated was quantified from the initial rate of hydrolysis of 400 μM S-2366. The percent activation of protein C was calculated from the initial rate in the presence and absence of SbO4L, and the apparent IC_{50} was calculated using eq 1.

Michaelis−Menten Kinetics. The initial rate of Spectrozyme TH hydrolysis by 3 nM thrombin was monitored from the linear increase in absorbance at 405 nm corresponding to less than 10% consumption of the substrate.[29] The initial rate was measured as a function of substrate concentrations (0.6−20 μM) in the presence of a fixed concentration of SbO4L (0−2.3 μg/mL) in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% PEG8000 at 25 °C. Standard Michaelis−Menten equation was used to calculate K_M and V_{MAX}.

Competitive Binding Studies with Exosite 1 and 2 Ligands. SbO4L-dependent thrombin inhibition studies in the presence of competitors were performed as described earlier.[29] SbO4L (0−23 μg/mL) was incubated with either HirP (0−86 nM) or porcine UFH (0−100 μM) and thrombin (5 nM) in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% PEG 8000 at 25 °C in PEG20000-coated polystyrene cuvettes for 10 min. For competition with GPIbα, SbO4L (0−1 mg/mL) was incubated with recombinant GPIbα (0−458nM) in 10 mM HEPES buffer with 10 mM Tris-HCl, pH 7.8 containing 100 mM NaCl and 0.1% PEG 8000 in 96-well plate for 10 min. Following incubation, Spectrozyme TH (100 μM) was added and the initial rate of 405 nm absorbance change was measured. The dose dependence of the fractional residual protease activity at each concentration of the competitor was fitted by eq 1 to calculate the $IC_{50,app}$ of inhibition. The MW of porcine UFH was assumed to be 15 000, while its affinity was measured earlier in the laboratory (15.6 ± 3.1 μM).[29]

To quantify the degree of competition of SbO4L with its competitors, the Dixon−Webb relationship was used to calculate the apparent $IC_{50,app}$ of the inhibitor in the presence of a competitor with known affinity ($K_{competitor}$).[29,45] The equation is given as

$$IC_{50,app} = IC_{50,obs}\left[1 + \frac{[competitor]}{K_{competitor}}\right] \quad \text{(Eq. 2)}$$

Inhibition of Recombinant Thrombin Mutants. The IC_{50} of SbO4L against recombinant mutants of thrombin was measured using the chromogenic hydrolysis assay in a 96-well format. In general, SbO4L (0−2 mg/mL) was incubated with individual mutants of thrombin (6 nM) in a 20 mM Tris-HCl buffer containing 100 mM NaCl, 2.5 mM $CaCl_2$, 0.1% PEG 8000 at a pH 7.4 for 10 min. Following this, substrate Spectrozyme TH (100 μM) was added to the mixture and the initial rate of increase in absorbance at 405 nm was measured. The rate of initial hydrolysis was used to calculate residual thrombin activity, which was used to calculate the IC_{50} from eq 1.

Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT). Clotting times were determined in standard one-stage recalcification assays with a BBL Fibrosystem fibrometer (Becton-Dickinson, Sparles, MD), as reported previously.[47] For PT assays, 10 μL of SbO4L (or a reference agent) was mixed with 90 μL of citrated human plasma, incubated for 30 s at 37 °C followed by addition of 200 μL of prewarmed thromboplastin. For APTT assays, 10 μL of SbO4L solution was mixed with 90 μL of citrated human plasma and 100 μL 0.2% ellagic acid. Clotting was initiated by adding 100 μL of 25 mM $CaCl_2$. Each experiment was performed twice, and the averaged data were plotted. A quadratic trend line was used to calculate the concentration of SbO4L that doubles the clotting time (2 × APTT or 2 × PT).

Platelet Aggregation and ATP Release Assays. The effects of SbO4L on γ-thrombin-induced platelet aggregation and α-thrombin-induced platelet ATP secretion were performed as follows. Whole human blood was collected in sodium citrate from healthy volunteers and used to prepare platelet-rich plasma (PRP) by centrifuging at 70*g* for 10 min and platelet-poor plasma (PPP) by centrifuging at 900*g* for 10 min. The platelet count in the PRP was adjusted to 220 000 platelets/μL using the PPP. Studies were performed using the Chronolog model 700 Optical Lumi-Aggregometer (Chrono-Log Corporation, Havertown, PA). For the platelet aggregation assay, 250 μL of PRP was incubated with SbO4L (0−535 μg/mL) for 3 min at 37 °C under stirring conditions. Aggregation was initiated by using either 152 or 76 nM γ-thrombin. Aggregation was monitored using the optical settings of the aggregometer for at least 6 min following the addition of the agonist. For the ATP secretion assay, the manufacturer's protocol was used with a slight modification to test SbO4L.[48,49] Briefly, 450 μL of PRP was incubated with 50 μL of CHRONO-LUME reagent containing luciferase enzyme and 0−5 μL of SbO4L (0−248 μg/mL final concentration) for 3 min at 37 °C. Following this, 1 unit of CHRONO-PAR thrombin (α-thrombin) was added into the solution and the luminescence was monitored for at least 6 min. An ATP standard of 2 nmol was run separately under similar conditions to standardize the luminescence and obtain quantitative results of the amount of ATP released in each run.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures S1−S6 (cited in the main text). This material is available free of charge via the Internet at http://pubs.acs.org.

dx.doi.org/10.1021/jm4020026 | J. Med. Chem. 2014, 57, 3030−3039

AUTHOR INFORMATION

Corresponding Author

*Phone: 804-828-7328. Fax: 804-827-3664. E-mail: urdesai@vcu.edu.

Author Contributions

A.Y.M. synthesized and characterized SbO4L. A.Y.M. and J.N.T. performed enzyme inhibition, mechanism, and plasma studies and contributed to manuscript preparation. A.Y.M., B.M.M., and E.J.M. did platelet experiments. D.F.B. supervised platelet experiments. T.K. provided an authentic sample of β-O4 lignin. U.R.D. designed and supervised the study, intrepreted the results, and wrote the paper.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the Grants HL090586 and HL107152 from the National Institutes of Health. We thank Dr. Paul E. Bock of Vanderbilt University for HirP and Dr. Alireza R. Rezaie of St. Louis University for the recombinant thrombin mutants.

ABBREVIATIONS USED

GAG, glycosaminoglycan; GPC, gel permeation chromatography; GPIbα, glycoprotein Ibα; HirP, a Tyr63-sulfated hirudin-(54−65) peptide labeled with 5-(carboxy)fluorescein; LMW, low molecular weight; M_N, number average molecular weight; M_P, peak average molecular weight; M_W, weight average molecular weight; PEG, polyethylene glycol; PRP, platelet rich plasma; SbO4L, sulfated β-O4 lignin; UFH, unfractionated heparin

REFERENCES

(1) Di Cera, E.; Page, M. J.; Bah, A.; Bush-Pelc, L. A.; Garvey, L. C. Thrombin allostery. *Phys. Chem. Chem. Phys.* **2007**, *9*, 1291−1306.

(2) Kamath, P.; Huntington, J. A.; Krishnaswamy, S. Ligand binding shuttles thrombin along a continuum of zymogen- and proteinase-like states. *J. Biol. Chem.* **2010**, *285*, 28651−28658.

(3) Huntington, J. A. Thrombin plasticity. *Biochim. Biophys. Acta* **2012**, *1824*, 246−252.

(4) Gasper, P. M.; Fuglestad, B.; Komives, E. A.; Markwick, P. R.; McCammon, J. A. Allosteric networks in thrombin distinguish procoagulant vs. anticoagulant activities. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 21216−21222.

(5) Lechtenberg, B. C.; Freund, S. M.; Huntington, J. A. An ensemble view of thrombin allostery. *Biol. Chem.* **2012**, *393*, 889−898.

(6) Malovichko, M. V.; Sabo, T. M.; Maurer, M. C. Ligand binding to anion-binding exosites regulates conformational properties of thrombin. *J. Biol. Chem.* **2013**, *288*, 8667−8678.

(7) Esmon, C. T. The roles of protein C and thrombomodulin in the regulation of blood coagulation. *J. Biol. Chem.* **1989**, *264*, 4743−4746.

(8) Davie, E. W.; Fujikawa, K.; Kisiel, W. The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry* **1991**, *30*, 10363−10370.

(9) De Candia, E.; Hall, S. W.; Rutella, S.; Landolfi, R.; Andrews, R. K.; De Cristofaro, R. Binding of thrombin to glycoprotein Ib accelerates the hydrolysis of PAR-1 on intact platelets. *J. Biol. Chem.* **2001**, *276*, 4692−4698.

(10) Lane, D. A.; Philippou, H.; Huntington, J. A. Directing thrombin. *Blood* **2005**, *106*, 2605−2612.

(11) Carter, W. J.; Cama, E.; Huntington, J. A. Crystal structure of thrombin bound to heparin. *J. Biol. Chem.* **2005**, *280*, 2745−2749.

(12) Rydel, T. J.; Tulinsky, A.; Bode, W.; Huber, R. Refined structure of the hirudin−thrombin complex. *J. Mol. Biol.* **1991**, *221*, 583−601.

(13) Huntington, J. A. Molecular recognition mechanisms of thrombin. *J. Thromb. Haemostasis* **2005**, *3*, 1861−1872.

(14) Pechik, I.; Madrazo, J.; Mosesson, M. W.; Hernandez, I.; Gilliland, G. L.; Medved, L. Crystal structure of the complex between thrombin and the central "e" region of fibrin. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 2718−2723.

(15) Hall, S. W.; Nagashima, M.; Zhao, L.; Morser, J.; Leung, L. L. Thrombin interacts with thrombomodulin, protein C, and thrombin-activatable fibrinolysis inhibitor via specific and distinct domains. *J. Biol. Chem.* **1999**, *274*, 25510−25516.

(16) Mosier, P. D.; Krishnasamy, C.; Kellogg, G. E.; Desai, U. R. On the specificity of heparin/heparan sulfate binding to proteins. Anion-binding sites on antithrombin and thrombin are fundamentally different. *PLoS One* **2012**, *7*, e48632.

(17) Dementiev, A.; Petitou, M.; Herbert, J. M.; Gettins, P. G. The ternary complex of antithrombin−anhydrothrombin−heparin reveals the basis of inhibitor specificity. *Nat. Struct. Mol. Biol.* **2004**, *11*, 863−867.

(18) Olson, S. T.; Björk, I. Predominant contribution of surface approximation to the mechanism of heparin acceleration of the antithrombin−thrombin reaction. Elucidation from salt concentration effects. *J. Biol. Chem.* **1991**, *266*, 6353−6364.

(19) Dormann, D.; Clemetson, K. J.; Kehrel, B. E. The GpIb thrombin-binding site is essential for thrombin-induced platelet procoagulant activity. *Blood* **2000**, *96*, 2469−2478.

(20) Soslau, G.; Class, R.; Morgan, D. A.; Foster, C.; Lord, S. T.; Marchese, P.; Ruggeri, Z. M. Unique pathway of thrombin-induced platelet aggregation mediated by glycoprotein Ib. *J. Biol. Chem.* **2001**, *276*, 21173−21183.

(21) De Cristofaro, R.; De Candia, E.; Landolfi, R.; Rutella, S.; Hall, S. W. Structural and functional mapping of the thrombin domain involved in the binding to the platelet glycoprotein Ib. *Biochemistry* **2001**, *40*, 13268−13273.

(22) Celikel, R.; McClintock, R. A.; Roberts, J. R.; Mendolicchio, G. L.; Ware, J.; Varughese, K. I.; Ruggeri, Z. M. Modulation of alpha-thrombin function by distinct interactions with platelet glycoprotein Ibalpha. *Science* **2003**, *301*, 218−221.

(23) Dumas, J. J.; Kumar, R.; Seehra, J.; Somers, W. S.; Mosyak, L. Crystal structure of the GpIbalpha−thrombin complex essential for platelet aggregation. *Science* **2003**, *301*, 222−226.

(24) Lechtenberg, B. C.; Freund, S. M.; Huntington, J. A. GpIbα interacts exclusively with exosite II of thrombin. *J. Mol. Biol.* **2014**, *426*, 881−893.

(25) Li, C. Q.; Vindigni, A.; Sadler, J. E.; Wardell, M. R. Platelet glycoprotein Ib alpha binds to thrombin anion-binding exosite II inducing allosteric changes in the activity of thrombin. *J. Biol. Chem.* **2001**, *276*, 6161−6168.

(26) Jandrot-Perrus, M.; Clemetson, K. J.; Huisse, M. G.; Guillin, M. C. Thrombin interaction with platelet glycoprotein Ib: effect of glycocalicin on thrombin specificity. *Blood* **1992**, *80*, 2781−2786.

(27) De Cristofaro, R.; De Filippis, V. Interaction of the 268−282 region of glycoprotein Ibalpha with the heparin-binding site of thrombin inhibits the enzyme activation of factor VIII. *Biochem. J.* **2003**, *373*, 593−601.

(28) Monien, B. H.; Henry, B. L.; Raghuraman, A.; Hindle, M.; Desai, U. R. Novel chemo-enzymatic oligomers of cinnamic acids as direct and indirect inhibitors of coagulation proteinases. *Bioorg. Med. Chem.* **2006**, *14*, 7988−7998.

(29) Henry, B. L.; Monien, B. H.; Bock, P. E.; Desai, U. R. A novel allosteric pathway of thrombin inhibition: exosite II mediated potent inhibition of thrombin by chemo-enzymatic, sulfated dehydropolymers of 4-hydroxycinnamic acids. *J. Biol. Chem.* **2007**, *282*, 31891−31899.

(30) Sidhu, P. S.; Liang, A.; Mehta, A. Y.; Abdel Aziz, M. H.; Zhou, Q.; Desai, U. R. Rational design of potent, small, synthetic allosteric inhibitors of thrombin. *J. Med. Chem.* **2011**, *54*, 5522−5531.

(31) Sidhu, P. S.; Abdel Aziz, M. H.; Sarkar, A.; Mehta, A. Y.; Zhou, Q.; Desai, U. R. Designing allosteric regulators of thrombin. Exosite 2 features multiple subsites that can be targeted by sulfated small molecules for inducing inhibition. *J. Med. Chem.* **2013**, *56*, 5059−5070.

(32) Vanholme, R.; Morreel, K.; Ralph, J.; Boerjan, W. Lignin engineering. *Curr. Opin. Plant Biol.* **2008**, *11*, 278−285.
(33) Henry, B. L.; Thakkar, J. N.; Liang, A.; Desai, U. R. Sulfated, low molecular weight lignins inhibit a select group of heparin-binding serine proteases. *Biochem. Biophys. Res. Commun.* **2012**, *417*, 382−386.
(34) Kishimoto, T.; Uraki, Y.; Ubukata, M. Synthesis of bromoacetophenone derivatives as starting monomers for beta-O-4 type artificial lignin polymers. *J. Wood Chem. Technol.* **2008**, *28*, 97−105.
(35) Kishimoto, T.; Uraki, Y.; Ubukata, M. Chemical synthesis of beta-O-4 type artificial lignin. *Org. Biomol. Chem.* **2006**, *4*, 1343−1347.
(36) Kishimoto, T.; Uraki, Y.; Ubukata, M. Synthesis of beta-O-4-type artificial lignin polymers and their analysis by NMR spectroscopy. *Org. Biomol. Chem.* **2008**, *6*, 2982−2987.
(37) Al-Horani, R. A.; Desai, U. R. Chemical sulfation of small molecules-advances and challenges. *Tetrahedron* **2010**, *66*, 2907−2918.
(38) Raghuraman, A.; Riaz, M.; Hindle, M.; Desai, U. R. Rapid and efficient microwave-assisted synthesis of highly sulfated organic scaffolds. *Tetrahedron Lett.* **2007**, *48*, 6754−6758.
(39) Zhang, F.; Yang, B.; Ly, M.; Solakyildirim, K.; Xiao, Z.; Wang, Z.; Beaudet, J. M.; Torelli, A. Y.; Dordick, J. S.; Linhardt, R. J. Structural characterization of heparins from different commercial sources. *Anal. Bioanal. Chem.* **2011**, *401*, 2793−2803.
(40) Al-Horani, R. A.; Ponnusamy, P.; Mehta, A. Y.; Gailani, D.; Desai, U. R. Sulfated pentagalloylglucoside is a potent, allosteric, and selective inhibitor of factor XIa. *J. Med. Chem.* **2013**, *56*, 867−878.
(41) Henry, B. L.; Abdel Aziz, M.; Zhou, Q.; Desai, U. R. Sulfated, low-molecular-weight lignins are potent inhibitors of plasmin, in addition to thrombin and factor Xa: novel opportunity for controlling complex pathologies. *Thromb. Haemostasis* **2010**, *103*, 507−515.
(42) Verhamme, I. M.; Olson, S. T.; Tollefsen, D. M.; Bock, P. E. Binding of exosite ligands to human thrombin. Re-evaluation of allosteric linkage between thrombin exosites I and II. *J. Biol. Chem.* **2002**, *277*, 6788−6798.
(43) Ruggeri, Z. M.; Zarpellon, A.; Roberts, J. R.; Mc Clintock, R. A.; Jing, H.; Mendolicchio, G. L. Unravelling the mechanism and significance of thrombin binding to platelet glycoprotein Ib. *Thromb. Haemostasis* **2010**, *104*, 894−902.
(44) Kobe, B.; Guncar, G.; Buchholz, R.; Huber, T.; Maco, B. The many faces of platelet glycoprotein Ibalpha−thrombin interaction. *Curr. Protein Pept. Sci.* **2009**, *10*, 551−558.
(45) Abdel Aziz, M. H.; Mosier, P. D.; Desai, U. R. Identification of the site of binding of sulfated, low molecular weight lignins on thrombin. *Biochem. Biophys. Res. Commun.* **2011**, *413*, 348−352.
(46) Yang, L.; Rezaie, A. R. Calcium-binding sites of the thrombin−thrombomodulin−protein C complex: possible implications for the effect of platelet factor 4 on the activation of vitamin K-dependent coagulation factors. *Thromb. Haemostasis* **2007**, *97*, 899−906.
(47) Henry, B. L.; Thakkar, J. N.; Martin, E. J.; Brophy, D. F.; Desai, U. R. Characterization of the plasma and blood anticoagulant potential of structurally and mechanistically novel oligomers of 4-hydroxycinnamic acids. *Blood Coagulation Fibrinolysis* **2009**, *20*, 27−34.
(48) Cattaneo, M. Light transmission aggregometry and ATP release for the diagnostic assessment of platelet function. *Semin. Thromb. Hemostasis* **2009**, *35*, 158−167.
(49) Soslau, G.; Parker, J. The bioluminescent detection of platelet released ATP: collagen-induced release and potential errors. *Thromb. Res.* **1992**, *66*, 15−21.
(50) Bauer, K. A. New anticoagulants. *Hematol. Am. Soc. Hematol. Educ. Program* **2006**, 450−456.
(51) Yeh, R. W.; Baron, S. J.; Healy, J. L.; Pomerantsev, E.; McNulty, I. A.; Cruz-Gonzalez, I.; Jang, I. K. Anticoagulation with the direct thrombin inhibitor argatroban in patients presenting with acute coronary syndromes. *Catheterization Cardiovasc. Interventions* **2009**, *74*, 359−364.
(52) Ganetsky, M.; Babu, K. M.; Salhanick, S. D.; Brown, R. S.; Boyer, E. W. Dabigatran: Review of pharmacology and management of bleeding complications of this novel oral anticoagulant. *J. Med. Toxicol.* **2011**, *7*, 281−287.
(53) Majeed, A.; Schulman, S. Bleeding and antidotes in new oral anticoagulants. *Best Pract. Res., Clin. Haematol.* **2013**, *26*, 191−202.
(54) Sabo, T. M.; Maurer, M. C. Biophysical investigation of GpIbalpha binding to thrombin anion binding exosite II. *Biochemistry* **2009**, *48*, 7110−7122.
(55) Hedstrom, L. Serine protease mechanism and specificity. *Chem. Rev.* **2002**, *102*, 4501−4524.
(56) Wang, X.; Lin, X.; Loy, J. A.; Tang, J.; Zhang, X. C. Crystal structure of the catalytic domain of human plasmin complexed with streptokinase. *Science* **1998**, *281*, 1662−1665.
(57) Myles, T.; Le Bonniec, B. F.; Betz, A.; Stone, S. R. Electrostatic steering and ionic tethering in the formation of thrombin-hirudin complexes: the role of the thrombin anion-binding exosite-I. *Biochemistry* **2001**, *40*, 4972−4979.
(58) Salonen, L. M.; Ellermann, M.; Diederich, F. Aromatic rings in chemical and biological recognition: energetics and structures. *Angew. Chem., Int. Ed.* **2011**, *50*, 4808−4842.
(59) Verghese, J.; Liang, A.; Sidhu, P. P.; Hindle, M.; Zhou, Q.; Desai, U. R. First steps in the direction of synthetic, allosteric, direct inhibitors of thrombin and factor Xa. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 4126−4129.
(60) Henry, B. L.; Connell, J.; Liang, A.; Krishnasamy, C.; Desai, U. R. Interaction of antithrombin with sulfated, low molecular weight lignins: opportunities for potent, selective modulation of antithrombin function. *J. Biol. Chem.* **2009**, *284*, 20897−20908.
(61) Slungaard, A.; Key, N. S. Platelet factor 4 stimulates thrombomodulin protein C-activating cofactor activity. A structure−function analysis. *J. Biol. Chem.* **1994**, *269*, 25549−25556.