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Designing Allosteric Regulators of Thrombin. Exosite 2 Features Multiple Sub-Sites That Can Be Targeted By Sulfated Small Molecules for Inducing Inhibition

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Abstract

We recently designed a group of novel exosite 2-directed, sulfated, small, allosteric inhibitors of thrombin. To develop more potent inhibitors, monosulfated benzofuran tri- and tetrameric homologs of the parent designed dimers were synthesized in 7–8 steps and found to exhibit a wide range of potencies. Among these, trimer **9a** was found to be nearly 10-fold more potent than the first generation molecules. Michaelis-Menten studies indicated an allosteric mechanism of inhibition. Competitive studies using a hirudin peptide (exosite 1 ligand) and, unfractionated heparin, heparin octasaccharide and γ' -fibrinogen peptide (exosite 2 ligands), demonstrated exosite 2 recognition in a manner different from the parent dimers. Alanine scanning mutagenesis of 12 Arg/Lys residues of exosite 2 revealed a defect in **9a** potency for Arg233Ala thrombin only confirming the major difference in site of recognition between the two structurally related sulfated benzofurans. The results suggest that multiple avenues are available within exosite 2 for inducing thrombin inhibition.

Introduction

The coagulation cascade is set of sequential, and yet highly inter-woven, proteolytic reactions that operate efficiently to prevent excessive loss of blood and ingestion of microbes. It can also be triggered by an aberrant intravascular signal, which may lead to an internal clot that can be catastrophic. Several anticoagulants have been approved for treatment of such conditions including unfractionated heparin (UFH), low molecular weight heparins (LMWHs), warfarin, hirudin and its analogs, argatroban, dabigatran, rivaroxaban and apixaban. These agents either directly or indirectly target thrombin and/or factor Xa, two key enzymes of the coagulation cascade.^{1–3}

Thrombin is a trypsin-like serine protease that is formed rapidly upon initiation of coagulation and has been the primary target for development of novel anticoagulant

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Supplementary Information

General procedures for the synthesis of sulfated benzofurans and other intermediate compounds are described in Supplementary Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

therapy.^{2,4,5} Yet, plasma thrombin is an unusual protease that displays both pro- as well as anti-coagulant roles. Whereas it cleaves fibrinogen to stem the flow of blood, its specificity changes to the cleavage of protein C in the presence of thrombomodulin to induce blood patency.⁶⁻⁸ The characteristic thermodynamic feature of thrombin that achieves this maneuver is its conformational plasticity. Thrombin exhibits an ensemble of conformations that can rapidly interconvert, especially in the presence of appropriate ligands. In fact, nature appears to have engineered thrombin as a pivot to rapidly alter the flux down either the pro- or anti-coagulant pathways. Thus, regulating thrombin is critical and challenging.

A specific approach exploited by nature to regulate thrombin is allosteric modulation of its active site. Three distinct allosteric sites are known on thrombin including the sodium binding site and anion-binding exosites 1 and 2.⁸⁻¹⁰ Each of these are located approximately 10–30 Å away from the active site. Sodium binding converts thrombin from the ‘slow’ form to the ‘fast’ form, which has been inferred as a switch for transforming the anticoagulant flux to the procoagulant one,¹¹ although the physiologic significance of this has been questioned recently.¹² Exosites 1 and 2 are electropositive domains that engage several physiologic ligands including glycosaminoglycans (GAGs), thrombomodulin, fibrinogen, glycoprotein Iba and protease activated receptor-1.⁶⁻⁹ Both exosites 1 and 2 are energetically linked to the active site as demonstrated by altered rates of cleavage of substrates in the presence of different ligands. For example, exosite 1 ligand hirugen significantly increases the catalytic efficiency of thrombin for small chromogenic substrates,^{13,14} while exosite 2 ligand fragment 1.2 induces better recognition of thrombin’s active site by a small fluorophore.¹² In fact, the conformational plasticity of thrombin appears to present a series of states along the monotonous path between the zymogen-like and proteinase-like forms that can be stabilized by an allosteric ligand.¹² Thus, an appropriately designed ligand can select and stabilize a distinct thrombin state with its unique characteristics of substrate specificity and catalytic activity.

Nearly all allosteric regulators of thrombin discovered to date are polymeric molecules, i.e., proteins and sulfated polysaccharides. Some time ago, we reasoned that such interactions, especially of sulfated polysaccharides called GAGs, could serve as reasonable starting points for the design of medicinally relevant small molecules. Thus, sulfated low molecular weight lignins (LMWLs) were designed as oligomeric mimetics of sulfated GAGs and found to inhibit thrombin with nanomolar potency by utilizing exosite 2 (Figure 1).^{15,16} To transform the heterogeneous, sulfated LMWLs into homogeneous small molecules, we developed sulfated benzofuran monomers, which were found to retain exosite 2-mediated thrombin inhibition potential of the parent oligomers.¹⁷ Homologation of the monomers to sulfated benzofuran dimers increased the inhibitory potency 100–1000-fold and also displayed good human plasma anticoagulant effect.¹⁸ Further, the dimeric scaffold exhibited high selectivity for thrombin due to their recognition of a specific site in exosite 2.¹⁹

The medicinal potential of the sulfated benzofurans is high. The molecules are not only small, but also present a combination of anionic and hydrophobic forces, which afford interesting protein recognition and physicochemical properties.²⁰ Sulfated benzofuran dimers are highly water soluble, readily synthesized, and essentially nontoxic to mammalian cells.¹⁹ Their most interesting property – allosteric inhibition of thrombin – is different from all known clinically used thrombin inhibitors (competitive inhibition). Thus, to discover more potent allosteric sulfated benzofurans, we designed a library of higher analogs. Herein, we report on the synthesis, biological activity, mechanism of action and site of binding of sulfated benzofuran oligomers. A specific trimer (**9a**) was found to display much higher potency than the earlier generation inhibitors. Interestingly, **9a** recognized a site within exosite 2 that was different from that recognized by sulfated benzofuran dimers. The result suggests that multiple avenues within exosite 2 can be exploited to modulate the highly

dynamic conformational equilibria displayed by thrombin. This work puts forward a highly potent, small molecule as a direct allosteric inhibitor of thrombin and presents a new sub-site in exosite 2 for drug discovery.

Results

Rationale Behind the Study of Sulfated Benzofuran Trimers/Tetramer

Allosteric targeting of enzymes can afford major advantages of higher selectivity and tunable efficacy in comparison to the traditional orthosteric targeting. Higher selectivity arises from much less conserved allosteric sites as compared to catalytic sites, while tunable efficacy arises from the dependence of energetic coupling between the ligand and substrate binding sites on the structure of the ligand. Both of these properties are critical for regulating thrombin so as to maintain hemostatic balance. Our previous work with sulfated benzofuran dimers established that these unique small molecules have the dual property of high selectivity and efficacy expected of allosteric inhibitors.^{17–19} For example, a group of sulfated benzofuran dimers inhibited thrombin, but not the closely related factor Xa, and displayed variable efficacies in the range of 60 to 95%.¹⁹ The potency of inhibition was found to be 5–50 μ M, which was reasonable considering the molecular size and the exposed nature of Arg173, the residue involved in their recognition. Structure-activity relationship (SAR) studies revealed that a sulfate group at the 5-position, a hydrophobic ester at the 3-position and an alkyl ether at the 6-position were optimal for potency.¹⁸

Despite their reasonable potency, the sulfated benzofuran dimers are still ~200-fold less potent than sulfated LMWLs,¹⁶ which were the parent oligomers for the design of small molecules. Whereas sulfated LMWLs are heterogeneous and polydisperse structures with an average chain length of 5 – 13 monomers,¹⁶ sulfated benzofuran dimers contain only two monomers. Hence, we sought to assess whether homologation to a trimeric or tetrameric length would enhance the potency of inhibition with retention of exosite 2-dependent allosteric inhibition of thrombin. Thus, a focused library of ten sulfated benzofuran trimers and one tetramer was designed considering the previous SAR results. Two of the three benzofuran units were maintained essentially invariant as found in the parent sulfated benzofuran dimer.¹⁸ Variations were introduced at the available 3-, 5-, and 6-positions of the terminal benzofuran unit, which included hydrophilic to bulky hydrophobic groups at the 3-ester, hydrophobic groups at the 6-position, sulfate group at either 5- or 6-position, a carboxylic acid group at the 3-position, and an unsulfated 5-OH group (see Schemes 1 – 4).

Synthesis of Sulfated Benzofuran Trimers and a Tetramer

The synthesis of benzofurans **1a** – **1e** from catechol in one step followed by dimerization and functionalization (Scheme 1) to provide several sulfated derivatives has been described in detail in our earlier work.¹⁸ Briefly, mono-alkylation of **1a** – **1e** with three different alkyl halides primarily afforded **2a** – **2g** with 6-substitution. Intermediates **2a** – **2g** were protected and brominated to give reactive handles **4a** – **4g**. Coupling of allylic bromide **4a** with benzofurans **2a** or **2e** followed by deprotection resulted in dimers **6a** and **6e** in reasonably good overall yield (Scheme 1, 25 – 30 % overall isolated yield).

The synthesis of eight sulfated benzofuran trimers **9a** – **9g** and **13** – **14** was achieved by coupling reactive handles **4a** – **4g** with either **6a** or **6e** (Scheme 1). Unlike relatively easy coupling of two monomers **4a** and **2a**, the coupling of a dimer **6a** with **4a** – **4g** did not proceed well under normal conditions, perhaps because of enhanced steric hindrance. Microwave-based coupling afforded the intermediate trimers **7a** – **7g** (see Supplementary Material for structures and details) in 80 min with nearly 50% yield. These were then deprotected using potassium fluoride and sulfated using triethylamine-sulfur trioxide under

microwave conditions.²¹ The triethylammonium cation was exchanged for sodium using a weak cation exchange resin to yield monosulfated benzofuran trimers **9a** – **9g** (30 – 40% overall from **6a**). Likewise, coupling of **6e**, which contains an allylic group, with **4a** followed by deprotection gave trimer **11**, which was selectively hydrolyzed using catalytic amount of Pd(PPh₃)₄ in the presence of triethyl amine to give **12** in good yields.²² Both **11** and **12** were then chemically sulfated to their respective monosulfated derivatives **13** and **14** in good overall yields from dimeric starting point. It is interesting that sulfation of **12** proceeded smoothly despite the presence of carboxylic acid group, which has been known to disrupt the formation of the intermediate sulfo-ester.¹⁸

To assess the chain length dependence of thrombin inhibition, monosulfated benzofuran tetramer **17** was also synthesized (Scheme 2). Trimer intermediate **8a** was processed through base-catalyzed coupling, desilylation and sulfation to give **17** in an overall yield of 36%. Yet, inhibition results described below restricted our synthetic efforts to only one tetramer member. Finally, a ‘flipped’ sulfated benzofuran trimer **23** (Scheme 3) was synthesized to assess the specificity of recognition of parent trimers **9a** – **9g**. The ‘flipped’ trimer **23** contains a sulfate group at the 6-position, whereas trimers **9a** – **9g** and **13** – **14** (see Scheme 1) have a sulfate at the 5-position. Trimer **23** could be prepared by exploiting the 5-substituted regio isomer **18** formed in small amounts (~10 %) in the methylation of benzofuran **1a**. Coupling of **18** with **2a** followed by the established series of reactions described above resulted in the ‘flipped’ trimer **23** (Scheme 3).

Thrombin Inhibition Potential of Sulfated Benzofuran Trimers and Tetramer

The focused library of 11 potential inhibitors displays structural variations in the terminal benzofuran unit containing the 5-sulfate at the 6-position, which include –CH₃, –C₂H₅, and –CH(CH₃)₂ groups, and at the 3-position, which include –CH₃, –C₂H₅, –CH(CH₃)₂ and –CH₂CH₂OCH₃ groups (trimers **9a** – **9g**). Other structural variations include 3-allylic ester (**13**) or 3-carboxylic acid (**14**) in the benzofuran unit remote from the sulfated benzofuran unit and the ‘flipped’ benzofuran (**23**). In addition to tetramer **17**, trimer **12** was also included in the study because it displayed fairly good water solubility despite being devoid of any sulfate group.

Thrombin inhibition was studied using Spectrozyme TH hydrolysis assay, as described previously for dimers.¹⁶ Of the 11 molecules, nine molecules inhibited thrombin at pH 7.4 and 25 °C (Figure 2, Table 1). Trimer **9a** was found to be the most potent inhibitor with an *IC*₅₀ of 670 nM and an efficacy of approximately 80%. In comparison to the corresponding parent dimer,¹⁸ this represents a nine-fold increase in potency for the trimer with no difference in efficacy. Other inhibitors displayed potencies in the range of 2 to 165 μM, except for two molecules which were essentially inactive. Comparison of *IC*₅₀s of **9a** – **9d** shows an interesting relationship in terms of the nature of the 3-ester (Table 1). The potency decreases precipitously (>500-fold) as the substituent changes from ethyl/methyl (**9a/9b**) to *t*-butyl (**9c**) to methoxyethyl (**9d**) indicating stringent steric and/or polar requirements. This possibly suggests recognition of a rather constrained binding site by sulfated benzofuran trimers in the vicinity of the terminal 3-ester position. Likewise, the potency decreases 8.6- and 148-fold upon changing the 6-substituent from methyl (**9a**) to ethyl (**9f**) and to isopropyl (**9g**), respectively. This implies a significant size constraint on the opposite face of the same benzofuran unit too.

In contrast, the 3-allyl ester (**13**) present on the unit most away from the sulfated benzofuran unit introduces a modest 46-fold reduction in potency from **9a**. This potency reduces further only 2-fold upon hydrolysis of the allyl group (**13**) to a carboxylic acid (**14**) (Table 1) suggesting that the benzofuran monomer most away from the sulfated benzofuran unit is

binding to a site that is much less sterically and/or electronically constrained. This is in striking contrast to the complete lack of inhibition observed with the corresponding sulfated benzofuran dimer containing an equivalent carboxylic acid group.¹⁸ Elimination of the 5-sulfate from **14**, as in trimer **12**, eliminated thrombin inhibition potential suggesting the central significance of the lone sulfate group. The trimer scaffold was found to be optimal for activity as suggested by the loss of nearly 236-fold in potency with tetramer **17**. This is a bit surprising considering that sulfated LMWLs with chain lengths of 5 – 13 units inhibit thrombin with high potency (~30 nM).¹⁶ It is possible that this difference in activity arises from fundamental differences in the structure between sulfated LMWLs and sulfated benzofurans. Whereas the former contain dihydrobenzofuran units, the latter contain fully aromatic units. The dihydrobenzofuran units perhaps afford greater flexibility resulting in better engagement of the thrombin surface. The results suggest that the binding site on thrombin accommodates a few trimeric benzofurans, i.e., **9a** or **9b**, well and deviation from these structures is detrimental. This conclusion is also supported by results with the 6-sulfated regio isomer **23**, which displays an IC₅₀ as weak as the tetramer **17**.

Sulfated benzofuran trimers display an impressive 26 – 91% range of efficacy of inhibition (Table 1). Such a broad range arising from this small library is striking. Interestingly, the efficacies for most potent inhibitors are also considerably different. It is difficult to understand the structural basis of the efficacy of inhibition without the crystal structure of the co-complex, however it is possible that the stringent interactions made by the benzofuran unit containing the 5-sulfate is the primary driver of the energetic coupling that induces inhibition. This hypothesis will have to be tested through detailed biophysical and/or computational studies.

A final aspect of thrombin inhibition induced by sulfated benzofuran trimers is the specificity of inhibition. When the molecules were screened against human factor Xa, which is a closely related coagulation enzyme, trimers **9a** – **15** did not display any significant inhibition at concentration as high as 365 μ M (see Table S1 in Supporting Information).

Kinetics of Thrombin Inhibition By Trimer **9a**

To identify the mechanism of thrombin inhibition induced by sulfated benzofuran trimers, the kinetics of substrate hydrolysis by thrombin was studied in the presence of **9a** under two conditions. Considering that the inhibitors being studied have unique structures and are likely to display allosteric properties in the manner of their parent designs,¹⁹ the inhibitors were incubated with thrombin either for 2 h at 37 °C or for overnight (16 h) at 25 °C. The initial rate of Spectrozyme TH amidolysis displayed the characteristic hyperbolic profile (Figure 3), as expected on the basis of Michaelis – Menten kinetics from which K_M and V_{MAX} were calculated. The K_M for Spectrozyme TH decreased from 9.5 μ M in the absence of **9a** to 3.1 μ M in the presence of 3.6 μ M **9a** at 25 °C (Table 2), a decrease of nearly 3.1-fold. A similar 4.1-fold decrease in K_M was observed at 37 °C in the presence of 9.9 μ M **9a**. Simultaneously the V_{MAX} of hydrolysis also decreased by 4.3- and 5.7-fold at 25 °C and 37 °C, respectively. This suggests that under both conditions the presence of **9a** affects the binding of the chromogenic substrate to the active site of the enzyme, while simultaneously reducing its forward rate constant. More importantly, the reduction in K_M and V_{MAX} at each temperature is essentially equivalent (either 0.86–1.2-fold per μ M at 25 °C or 0.4–0.6-fold per μ M at 37 °C). The simultaneous and equivalent decrease in K_M and V_{MAX} under both conditions indicates a primarily uncompetitive mechanism of thrombin inhibition by **9a**. This mechanism is interesting and different from the noncompetitive mechanism displayed by sulfated benzofuran dimers studied earlier.¹⁹ Thus, the trimers appear to be unexpectedly different from the dimers.

The uncompetitive mechanism implies that thrombin is inhibited with the formation of enzyme – substrate – inhibitor ternary complex. This suggests that the trimers may be inducing a change in the catalytic triad that disfavors catalysis even in the presence of substrate. This does not however, imply that the catalytic triad is made completely dysfunctional. Thrombin retains partial catalytic activity, which alludes to regulatory features of sulfated benzofuran trimers. Thus, a question arises whether thrombin can be completely inhibited by an active-site directed inhibitor, e.g., *D*-Phe-Pro-Arg-chloromethylketone (PPACK) following its inhibition by **9a**. Figure S10 (Supplementary Material) shows the thrombin activity profile of such a sequential inhibition by **9a** and PPACK. As expected, PPACK reduces thrombin's activity over and above that of **9a** and renders it totally inactive. Thus, if necessary, competitive and uncompetitive inhibitors can be simultaneously used to induce total inhibition.

Trimer **9a** Does Not Bind in Exosite 1 of Thrombin

To identify the site of interaction on thrombin, we measured the effect of a 12-residue hirugen analog, HirP, on the IC_{50} of **9a**. HirP binding to thrombin has been extensively studied earlier.^{16,23} It interacts with exosite 1 and enhances the k_{CAT} of substrate hydrolysis.¹⁶ The apparent IC_{50} of **9a** in the presence of HirP was measured in a manner similar to that used for direct inhibition studies. The presence of HirP did not significantly affect the inhibition profile of **9a** (Table 3, see Figure S1 in Supplementary Material). Using the logistic equation 1, the apparent IC_{50} s calculated in the presence of the three HirP concentrations tested were 0.9, 0.8 and 0.9 μ M indicating that **9a** does not appear to bind in the exosite 1 of thrombin.

Trimer **9a** Interacts With Exosite 2 of Thrombin

To identify whether sulfated benzofurans bind in exosite 2 of thrombin, we measured the apparent IC_{50} of **9a** in the presence of three different exosite 2 ligands including UFH, H8, and FibP. UFH, a heterogeneous polysaccharide with an average molecular weight of ~13000, is approximately 5-fold longer than octasaccharide H8, while FibP is a 20-amino acid peptide sequence derived from fibrinogen. All three ligands bind in exosite 2, but span slightly different amino acid residues and, therefore, could serve as good probes for localizing **9a** binding site. Whereas the crystal structure of H8 – thrombin complex indicates ionic interactions with Arg93, Arg101, Arg126, Arg165, and Lys240 residues,²⁴ the interaction of UFH with thrombin is considered to also involve Arg236.²⁵ FibP, on the other hand, interacts with H8 binding residues²⁶ and also Arg97, Arg173 and Arg175.²⁷

Figure 4A shows the dose-response profiles of **9a** inhibition of thrombin in the presence of UFH as a competitor. A characteristic shift in the profiles is observed as function of the concentration of UFH suggesting competition between the two ligands for thrombin binding. The apparent IC_{50} increases from 0.68 μ M in the absence of UFH to 5.6 μ M in the presence of 42.6 μ M UFH (Table 3). To evaluate whether this competition is essentially ideal, the predicted IC_{50} in the presence of UFH was calculated using Dixon – Webb equation 2 (K_{comp} = dissociation constant of the competitor) using the measured affinity of 6.8 μ M for UFH – thrombin interaction in the laboratory.²⁴ The measured and Dixon – Webb predicted IC_{50} s compare favorably at each concentration of UFH (Figure 4B) indicating that **9a** and UFH compete with each other essentially ideally for binding in exosite 2 of thrombin.

$$IC_{50,predicted}=IC_{50,[comp]=0} \times \left(1+\frac{[comp]_0}{K_{comp}}\right) \quad (\text{Eq. 2})$$

To test whether **9a** competes with H8, similar studies were performed in the presence of 1 to 20 μM H8 (K_{H8} was found to be 2.7 μM). Figure 4C shows the dose-response curves and Table 3 lists the calculated apparent IC_{50} values. The potency increases to 1.8 μM at 20 μM H8, which represents a small increase that does not compare well with the predicted increase based on Dixon – Webb analysis (Figure 4D). This suggests that H8 does not compete with **9a**, although a marginal overlap in interactions between the two cannot be excluded.

To assess whether **9a** engages thrombin residues that interact with FibP, competition studies were performed in the presence of 0.2 to 6.5 μM FibP ($K_{\text{FibP}} = 0.6 \mu\text{M}$).²⁷ No shift in the dose-response profile was observed in the presence of this competitor and the apparent IC_{50} did not increase suggesting no competition between the two ligands (Table 3 and see Figure S2).

Taken together, these results indicate that **9a** binds in exosite 2, but its interaction is different from that identified for sulfated benzofuran dimers.¹⁹ Whereas the dimers compete fully with FibP and partially with UFH, the trimers compete well with UFH, show a very weak, if at all, competition with H8 and do not compete with FibP. Thus, despite an obvious similarity in structure between the dimers and trimers, their interaction at a molecular level with thrombin is dramatically different.

Sulfated Benzofuran Trimer **9a** Interacts with a Specific Arginine in Exosite 2

To further narrow down the site of binding, we studied the inhibition of 12 recombinant thrombins (a wild-type and other mutants) containing single replacement of Arg and Lys residues of exosite 2 to Ala. These recombinant thrombins were expressed in baby hamster kidney cells and have been studied earlier by the Rezaie laboratory.^{28,29} Each mutant was screened for direct inhibition by **9a** using S2238, which is a relatively more sensitive thrombin substrate. The recombinant wild-type thrombin displayed an IC_{50} of 0.26 μM , which is nearly 2.5-fold lower than that for human plasma α -thrombin. Such differences are not uncommon and may arise due to variations in the glycosylation pattern between recombinant and plasma proteins. Despite this difference in potency, we proceeded with the study of **9a** inhibition of thrombin mutants containing alanine replacement for nearly all positively charged residues of exosite 2. These residues are known to interact with UFH, H8 and FibP (see above). In addition, Lys169Ala, Arg233Ala and Arg235Ala thrombins were also studied.

The dose-response profiles were a bit challenging because the recombinant proteins tended to display a slightly higher Hill slope than that for human plasma thrombin. A higher HS requires careful selection of inhibitor concentrations to define the dose-response profiles adequately, which introduce an experimental challenge due to limited availability of recombinant mutants. Yet, at least 9 – 10 data points were used to define the dose-response profiles. Figure 5A shows the profiles for the Arg233Ala and wild-type thrombins and the measured IC_{50} s for all thrombins studied are listed in Table 4. The potencies for all recombinant thrombins ranged from 0.19 μM to 0.43 μM , except for the Arg233Ala mutant that showed an IC_{50} of 0.98 μM (Table 4). Figure 5B shows a comparison of the change in IC_{50} for each mutant from that of the wild-type. The Arg233Ala thrombin is the only enzyme with a significantly higher defect in **9a** recognition (3.8-fold loss in potency) as compared to mutants that display at best a 1.5-fold defect. This indicates that Arg233 of exosite 2 is the primary residue of contact for **9a**.

Plausible Binding Mode of Trimer **9a** in Exosite 2

To gain some insight into plausible mode of exosite 2 recognition by sulfated benzofuran trimer **9a**, we utilized genetic algorithm-based docking and scoring program GOLD, which

had been used successfully earlier for a sulfated benzofuran dimer.¹⁹ Trimer **9a** was docked into exosite 2 near Arg233. Following an exhaustive search, GOLD identified a small group of favorable binding modes that display an ionic interaction between the guanidine of Arg233 and 5-*O*-sulfate of trimer **9a** (Figure 6). Four of the top eight binding poses displayed similar orientation (RMSD < 2.5 Å). The preferred binding mode shows that the benzofuran ring orients in the shallow groove of exosite 2 such that the 3-ester side chain (Me- or Et-) can interact well with the narrow hydrophobic channel formed by Phe232 and other backbone atoms (Arg233, Leu234, Lys235 and Lys236). The size of this channel most probably cannot accommodate larger groups, thus explaining the observed loss of inhibitory activity in **9c** and **9d** (>300-fold loss, Table 1). Interestingly, the trimer **14** containing a carboxylic acid group at 3-position of a terminal benzofuran inhibits thrombin, while the corresponding dimer was found to be completely inactive in our earlier work.¹⁸ This model of trimer **9a** binding in exosite 2 provides a plausible explanation as the terminal benzofuran containing the carboxylic acid side chains is found to be placed in a much less constrained region with significant exposure to solvent. This also explains why trimer **13** with an allyl ester inhibits with an essentially similar potency as trimer **14** with a carboxylic acid group (Table 1). Support for this model also comes in the form of tetramer **17**, which binds with a 225-fold weaker affinity. Although **9a** binding model shows room for an extra benzofuran ring at the terminus opposite to the 5-*O*-sulfate group, the inter-monomer bond will have to undergo considerable bending while encountering the ridge formed by Glu97, Asp100, Arg101, Lys169, Arg175, Ile176, Thr177 and Asp178 residues, which reduces its affinity.

Yet, the **9a** model should be interpreted with caution. The bond between Arg233 and 5-*O*-sulfate is primarily electrostatic and not strong. This results in the weaker defect noted upon mutation of Arg233 (~3-fold). A stronger bond, such as a linear hydrogen-bond, would have engineered a much more powerful defect, as observed with Arg173Ala mutation on the affinity of sulfated benzofuran dimer.¹⁹ A significant portion of **9a** binding energy is likely to arise from hydrophobic forces, which are driven by entropically favorable displacement of water molecules.³⁰ Considering that **9a** is only the best molecule in a small library, considerable structural modifications may need to be introduced for engineering a more specific inhibitor.

Trimer **9a** Prolongs Human Plasma Clotting Time

To assess the anticoagulant potential of **9a**, we performed two human plasma clotting assays that are routinely used to screen new molecules in vitro. These are PT and APTT assays that attempt to measure the effect of an anticoagulant on the extrinsic and intrinsic flux of the coagulation cascade, respectively. Trimer **9a** displayed the characteristic increase in clotting times confirming its anticoagulant potential (Figure 7). The anticoagulant effect is better in APTT conditions than in PT conditions. The concentrations of **9a** required to double the PT and APTT were calculated as described earlier for sulfated benzofuran dimers and other compounds.¹⁸ A 2-fold increase in APTT and PT required 139 μM and 568 μM **9a**. These results also suggest that the sulfated benzofuran trimers are approximately 10-fold more potent in human plasma than the dimers.¹⁸ This follows the improvement in IC₅₀ of trimers as compared to the dimers and validates the rationale behind homology. Yet, trimer **9a** is still 10 times less potent in plasma anticoagulation than the parent polymeric sulfated LMWLs.¹⁵ This implies that further modifications on the trimeric scaffold, and not the tetrameric scaffold, would be necessary to reach the potency of the longer scaffold.

Discussion

This work shows that monosulfated benzofuran trimers can modulate thrombin's catalytic activity with high potency and variable efficacy by interacting with the anion-binding

exosite 2. A specific trimer **9a** was at least 9-fold more potent than the corresponding dimer in both direct enzyme inhibition assay and human plasma clotting assay.¹⁸ These results are in line with the expectation derived from the activity of sulfated LMWLs.¹⁵ The polymeric sulfated LMWLs were designed to be heterogeneous and polydisperse so as to mimic the nature of the natural anticoagulant, heparin, but also suffer from the limitations arising from the presence of millions of sequences. The sulfated benzofuran trimers are synthetically prepared, homogeneous and well-characterized. Trimer **9a** has good anticoagulant potential; however, much work is needed to assess its wider pharmacological activity before establishing its firm clinical relevance. This would include anticoagulation studies in human blood, effect on thrombin's macromolecular targets including protein C, glycoprotein Iba, and protease activated receptor-1.^{6-8,11} At the present time, trimer **9a** represents an exciting lead toward the discovery of the first clinically relevant, allosteric regulator of human plasma thrombin.

A novel aspect of trimer **9a** is that it inhibits thrombin with an efficacy of approximately 80%, and not 100%, which alludes to the possibility of allosteric regulation. It has been proposed that regulators, and not inhibitors, of coagulation, are likely to exhibit reduced bleeding complications. A regulator of coagulation will ensure a balance between pro-coagulation and anti-coagulation states, while an inhibitor may tip the balance to anti-coagulation, especially with 100% inhibition efficacy. Thus, sulfated benzofuran trimers exhibit considerable promise as safer anticoagulants.

An interesting finding of this work is that **9a** binds in a site different from that of the sulfated benzofuran dimers (see Figure 6). Whereas **9a** recognizes Arg233 in exosite 2, the corresponding sulfated dimer recognizes Arg173.¹⁹ Arg233 is also important for recognition of sulfated LMWLs, although two additional residues were found to be more important.³¹ Replacement of Arg233 for Ala reduced the potency of sulfated LMWLs by ~2.5-fold, which is similar to the defect found for **9a**. Thus, sulfated benzofuran trimers appear to mimic certain oligomeric sequences of sulfated LMWLs. Although both Arg233 and Arg173 are located in exosite 2, they are approximately 20 – 25 Å apart. This suggests two different modes of binding for the two structurally related allosteric inhibitors. Thus, there are two distinct sub-sites within exosite 2 that bind to the sulfated benzofuran scaffold and modulate the conformation of the catalytic site so as to attenuate its activity. This allosteric phenomenon most probably arises from the highly plastic nature of thrombin that allows selective stabilization of a conformational state driven by ligand binding.^{10,12} The observation that molecules as small as sulfated benzofuran dimers and trimers are able to function as allosteric regulators is exciting.

An interesting corollary of the two different binding modes for sulfated benzofuran dimer and its higher oligomer is that the two inhibitors could generate synergistic effects. However, the combination experiment did not display any synergism (not shown). We hypothesize that the lack of synergism between the two binding sites probably arises from the binding of trimer first to thrombin, which induces a conformational change in exosite 2 as well as the active site that disfavors binding of the dimer to an altered exosite 2. This result alludes to the highly pliable nature of allosteric regulation of thrombin, as also reported recently for other ligands.³²

Despite the lack of synergistic effects, the two different binding sites presents two independent avenues as well as one co-dependent opportunity for further rational drug design. The two independent avenues would entail structure-based design of analogs of the dimer and trimer scaffolds so as to enhance potency and specificity as well as tailor efficacy. The co-dependent opportunity would entail designing a larger, new molecule that ties the two oligomers so as to significantly enhance affinity and specificity. This approach would

mimic the fragment-based drug design strategy, wherein the two fragments would be the trimer and the dimer. Although interesting, the strategy is not necessarily straightforward and guaranteed to work. An example of the challenge is our results with tetramer **17**, which was found to be much less active. Yet, the nanomolar potency of longer oligomers present in sulfated low molecular weight lignins^{15,16} alludes to the feasibility of the approach.

Overall, the homogenous sulfated benzofuran trimer scaffold is the first scaffold that affords exosite 2 driven allosteric regulators of thrombin with sub-micromolar potency. These molecules may yield true regulators of thrombin considering that they display variable efficacy of inhibition. Further studies with macromolecular substrates of thrombin are needed to better assess allosteric regulation.

Experimental Procedures

Chemicals, Reagents, Proteins and Analytical Chemistry

Reagents, chemicals and solvents were purchased either from Sigma-Aldrich (Milwaukee, WI) or Fisher (Pittsburgh, PA) and were used as received. Reagents and chemicals were handled under inert nitrogen atmosphere using syringe techniques for liquid and dried spatula for solids. Glassware was oven dried for 12 h before use. Anhydrous sodium sulfate was used for drying organic extracts following work-up. Reactions were monitored by analytical TLC on UNIPATE™ silica gel GHLF 250 μm pre-coated plates (ANALTECH, Newark, DE) with UV detection (254 nm). Column chromatography was performed using Teledyne ISCO (Lincoln, NE) Comb flash RF system and disposable normal silica cartridges of 30–50 μ particle size, 230–400 mesh size and 60 Å pore size. Sodium exchange chromatography was performed using SP Sephadex C-25 sodium cation exchange resin from GE Healthcare Life Sciences (Piscataway, NJ). Sulfation and coupling reactions under microwave conditions were performed using a CEM-Discover synthesizer (Matthews, NC). The purity of sulfated benzofuran trimers and intermediates along their synthesis was assessed using Shimadzu VP HPLC system. The purity of compounds synthesized in this work was assessed by reversed-phase HPLC and ascertained to be >95% before proceeding for subsequent reaction or biological analysis.

¹H and ¹³C NMR spectra were recorded on either 300 or 400 MHz (Varian Mercury or Bruker Ultrashield™ Plus) in appropriate deuterated solvents including CDCl₃, DMSO-*d*₆ or D₂O. NMR signals are reported in ppm with the internal solvent signals as standards. The NMR data is reported as chemical shift (ppm) followed by the splitting pattern (s = singlet, d = doublet, t = triplet, m = multiplet), coupling constant (Hz), and integration. Mass spectrometry was performed on using Micromass ZMD4000 single quadrupole mass spectrometer with ESI ionization probe operating in negative ion mode (Waters Corp., Milford, MA). The samples were dissolved in acetonitrile-containing formic acid (5% v/v) and infused at 10 μL/min. The source block temperature and the probe temperature were typically held at 100 and 120 °C, respectively, while corona and cone voltages were selected through manual optimization. The desolvation nitrogen flow was 500 L/hour. The MS profiles were acquired in the mass range from 110 to 1000 daltons at 400 amu/sec.

Human plasma α-thrombin was from Haematologic Technologies (Essex Junction, VT). Stock solutions of α-thrombin were prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl. Chromogenic substrates Spectrozyme TH (H-*D*-cyclohexylalanyl-alanyl-arginine *para*-nitroanilide diacetate) was purchased from American Diagnostica (Greenwich, CT) and S2238 (H-*D*-Phe-Pip-Arg-*p*-nitroanilide) was purchased from ANASPEC (Fremont, CA). UFH and PPACK were purchased from Sigma (St. Louis, MO). Heparin octasaccharide (H8) was purchased from V-Labs (Covington, LA). Tyr63-sulfated hirudin-(54–65) (HirP) was a generous gift from Dr. Paul Bock. A peptide

corresponding to the 20 amino acids at the carboxyl terminal of γ' -fibrinogen chain (VRPEHPAETE-Y(PO₃)-DSL-Y(PO₃)-PEDDL) (FibP) was obtained from Dr. David Farrell. All other chemicals were analytical reagent grade from either Sigma Chemicals (St. Louis, MO) or Fisher (Pittsburgh, PA) and used as such.

Recombinant Thrombin Mutants

Recombinant wild-type and mutant thrombins were prepared in Dr. Rezaie's laboratory, as described earlier.^{28,29} Briefly, Arg93Ala, Arg97Ala, Arg101Ala, Arg126Ala, Arg165Ala, Lys169Ala, Arg173Ala, Arg175Ala, Arg233Ala, Lys235Ala, Lys236Ala and Lys240Ala thrombins were prepared in prothrombin-1 form by PCR mutagenesis and expression in baby hamster kidney cells (BHK) using the pNUT-PL2 expression/purification vector system. The mutants were purified to homogeneity by immunoaffinity chromatography using the Ca²⁺-dependent monoclonal antibody, HPC4, and activated to thrombin. The active-site concentrations of thrombin mutants were determined by an amidolytic activity assay and stoichiometric titrations with antithrombin. These concentrations were within 90–100% of those expected on the basis of their absorbance at 280 nm.

Synthesis of Sulfated Benzofuran Trimers 9a – 9g

To the solution of **6a** (0.3 mmol) and **4a – 4g** (0.4 mmol) in ethyl acetate (3 mL) was added cesium carbonate (0.3 mmoles) in anhydrous DMF (0.5 mL) in a microwave tube (10 mL). The reaction mixture was exposed to microwaves at 90 °C for 80 min, quenched with methylene chloride, and extracted with brine. The organic layer was dried over magnesium sulfate, concentrated, purified using flash chromatography on silica gel (20–60 % ethyl acetate in hexanes) resulting in **7a – 7g** (40–60 % yield). The purity of these products was assessed by reversed phase HPLC and found to be greater than 95% (see Supplementary Material for details on spectral characterization). A solution of **7a – 7g** (0.3 mmol) in DMF (3 mL) was then reacted with KF (4 mmoles) and acetic acid (10 μ L) at RT for 5 h. The reaction was quenched by methylene chloride and extracted with brine solution. The aqueous layer was extracted with a fresh batch of methylene chloride. The organic layer was combined, dried over magnesium sulfate and concentrated in vacuum. The solid so obtained was precipitated using methanol to obtain products **8a – 8g** in ~90% yield. These were characterized by spectroscopic/spectrometric techniques (see Supplementary Material) and sulfated using trimethylamine-sulfur trioxide complex, as described earlier.²¹ In this reaction, to a solution of **8a – 8g** (50 mg) in acetonitrile:DMF (4:1, 0.45 mL) in a microwave tube was added triethylamine (10 equiv) and trimethylamine-sulfur trioxide complex (15 equiv). The reaction mixture was exposed to microwaves (50 W) for 40 min at 100 °C followed by vacuum concentration to remove all solvent. The solid so obtained was directly loaded on a Sephadex C-25 cation exchange resin and eluted with water. Fractions containing the sulfated product were lyophilized to obtain a solid residue, which was further purified on flash chromatography using 0–20% methanol in methylene chloride. For cation exchange, approximately 100 mg sulfated sample was loaded onto 10 g of the cation exchanger in 10 $\times$ 460 mm column and eluted with deionized, distilled water at 0.5 mL/min. The purity of these compounds was found to be >95% by reversed phase HPLC. Characterization of the sulfated trimers **9a – 9g** was performed using ¹H and ¹³C NMR spectroscopy and ESI-MS (see Supplementary Material).

Synthesis of Sulfated Benzofuran Trimers 13 and 14

The synthesis of these began with alkylation reaction using cesium carbonate, as described above for trimers **9a – 9g**. Thus, **6a** (0.3 mmol) was reacted with **4a** (0.4 mmol) in ethyl acetate (4 mL) and anhydrous DMF (1 mL) in the presence of cesium carbonate (0.3 mmoles), followed by work and purification (see Supplementary Material for details) to

obtain **10** in 60 % yield. Desilylation of **10** (0.3 mmol) in DMF (3 mL) with KF (4 mmoles) and acetic acid (10 μ L) gave **11** (see Supplementary Material). To the solution of **11** (0.19 mmol) in methylene chloride (1.5 mL) was added Pd(PPh₃)₄ (0.009 mmol) and PPh₃ (0.018 mmol), and the mixture was stirred for 10 min in ice bath. To this mixture was then added pyrrolidine (0.21 mmol) and stirred for 30 min. The reaction was quenched, extracted with brine, dried, and concentrated in vacuum to obtain a solid, which was purified by flash chromatography to yield **12**. Trimers **11** and **12** were sulfated to **13** and **14**, respectively, under microwave conditions with trimethylamine-sulfur trioxide complex, as described above, purified, and characterized (see Supplementary Material for details).

Synthesis of Sulfated Benzofuran Tetramer **17** and ‘Flipped’ Sulfated Benzofuran Trimer **23**

The synthesis of these compounds using intermediates **2a**, **6a**, **7a** and **18** involved standard reaction conditions established in the synthesis of other sulfated benzofuran trimers. These reactions included Cs₂CO₃-based alkylation, KF-acetic acid-based desilylation, and MeN:SO₃-based microwave sulfation. Each of these have been described above as well as in Supplementary Material, which also provides the spectral characterization data.

Inhibition of Thrombin Using Chromogenic Substrate Hydrolysis Assay

Thrombin activity in the presence of sulfated benzofuran inhibitors was studied using a chromogenic substrate hydrolysis assay in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM CaCl₂ and 0.1 % PEG8000 in PEG20000-coated acrylic cuvettes. Spectrozyme TH was used as substrate and the residual thrombin activity was quantified, as reported earlier.¹⁶ Briefly, a solution of 10 μ L of an inhibitor to give 1–900 μ M final concentration was diluted with 970 μ L of the buffer and 5 μ L of thrombin (5 nM final concentration). Following incubation for 10 min, 15 μ L of 2 mM Spectrozyme TH was added (30 μ M final concentration) and the initial rate of hydrolysis from the linear increase in absorbance at 405 nm as a function of time was rapidly measured. Logistic equation 1 was used to fit the dose-dependence of residual thrombin activity to obtain IC₅₀. In this equation, Y is the ratio of residual thrombin activity in the presence of the inhibitor to that in its absence; Y_M and Y₀ are the maximum and minimum possible values of the residual percentage of thrombin activity, respectively; and IC₅₀ is the concentration of inhibitors that results in 50% inhibition of enzyme activity. Sigmaplot 8.0 (SPSS, Inc. Chicago, IL) was used to perform non-linear curve fitting in which Y_M, Y₀, HS (Hill Slope), and IC₅₀ were allowed to float.

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

Michaelis-Menten Kinetics of Substrate Hydrolysis by Thrombin

The initial rate of Spectrozyme TH hydrolysis by 5 nM thrombin was monitored from the linear increase in absorbance at 405 nm corresponding to less than 10% consumption of the substrate. The initial rate was measured as a function of various concentrations of the substrate (2 – 80 μ M) in the presence of fixed concentration of sulfated benzofuran trimer, which was incubated with thrombin either overnight at 25°C or for 2 h at 37°C in 20 mM Tris-HCl buffer, pH 7.4 containing 100 mM NaCl, 2.5 mM CaCl₂ and 0.1 % PEG8000. The data was fitted using the standard Michaelis-Menten equation to determine K_M and V_{MAX}.

Competitive Binding Studies with Exosite 1 or Exosite 2 Ligands

Thrombin inhibition by **9a** was studied in the presence of either exosite 1 competitor HirP or exosite 2 competitors including UFH, H8 and FibP in a manner similar to that described for

direct thrombin inhibition. Briefly, a solution of **9a** (0.045–22.5 μ M) and thrombin (5–10 nM) was incubated overnight at 25 °C with either HirP (0–15 nM), UFH (0–42.6 μ M), H8 (0–20 μ M) or FibP (0.2–6.5 μ M) in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM CaCl₂ and 0.1 % PEG 8000. Following overnight incubation, the residual thrombin activity was measured through Spectrozyme TH hydrolysis assay. The dose-dependence of the fractional residual thrombin activity at each concentration of **9a** was fitted using equation 1 to obtain the apparent IC₅₀, HS, Y₀ and Y_M.

Inhibition of Recombinant Thrombins

Direct inhibition of recombinant wild-type and mutant thrombins by **9a** was measured through the chromogenic substrate hydrolysis assay described above, except for the use of a more sensitive substrate, S2238, in these experiments. Solutions of **9a** (0.5 – 150 μ L) of 90 μ M were diluted with the assay buffer in PEG20000-coated acrylic cuvette following by addition of recombinant thrombins (1.5–7.5 nM final concentration) and overnight incubation at 25 °C. S2238 (20 μ L of 2 mM stock) was added to measure the residual enzyme activity from the initial rate of increase in absorbance at 405 nm. Logistic equation 1 was used to fit the dose-response profile to obtain the apparent IC₅₀, HS, Y₀ and Y_M.

Human Plasma Clotting Assays

Prothrombin (PT) and activated partial thromboplastin (APTT) times were measured using a BBL Fibrometer (Becton-Dickinson, Sparks, MD) in a standard 1-stage recalcification assay. For the APTT assay, 90 μ L citrated human plasma, 10 μ L inhibitor (or vehicle for blank test), and 100 μ L of pre-warmed 0.2% ellagic acid were mixed and incubated for 4 minutes. Clotting was initiated by adding 100 μ L of 25 mM CaCl₂ (37 °C) and time necessary for the plasma to clot recorded. For PT assays, 10 μ L sample of the inhibitor (or vehicle for blank test), and 90 μ L of citrated human plasma were mixed and incubated for 30 s at 37 °C followed by addition of 200 μ L pre-warmed thromboplastin (reconstituted as per direction provided by manufacturer). Each clotting assay was performed in duplicate. The data were fit to a quadratic trend line, which was used to determine the concentration of the inhibitor necessary to double the clotting time, 2 $\times$ APTT or 2 $\times$ PT.

Molecular Modeling of Sulfated Benzofuran Trimer **9a** Binding to Thrombin

The crystal structure of thrombin (PDB code 3uwj, resolution 1.5 Å) was obtained from the RCSB protein data bank and prepared in Sybyl-X v. 2.0 (www.tripos.com/sybyl). Cocrystallized ligands and water molecules were removed using the “prepare protein” module. The missing side chains of Lys236 and Lys240 were engineered *in silico* by implementing the Lovell rotamer library³³ in Sybyl-X. His91 and His230, which were close to Arg233, were set to the epsilon protonated state to maximize hydrogen bonding and avoid steric clashes. Hydrogen atoms were added and the protein minimized for 1000 steps with a gradient of 0.05 kcal/molÅ using the Powell method.³⁴ Trimer **9a** was docked using GOLD v. 5.1³⁵ utilizing all residues within 22 Å of this basic residue. An experiment consisting of 100 GA runs was employed to ensure sufficient conformational and spatial sampling. A hydrogen bond constraint was used to encourage interaction between Arg233 and the sulfate group of trimer **9a**. Docked poses were scored using GOLD score, from which the top 10 poses were selected for detailed analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

APTT	activated partial thromboplastin time
BHK	baby hamster kidney
FibP	gamma prime fibrinogen peptide
GAG	Glycosaminoglycan
H8	heparin octasaccharide
HS	Hill slope
LMWH	low molecular weight heparin
LMWL	low molecular weight lignin
PEG	Polyethylene glycol
PT	prothrombin time
SAR	structure-activity relationship
UFH	unfractionated heparin

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Figure 1.

Rationale for the study of monosulfated benzofuran trimers. Sulfated low molecular weight lignins were designed to mimic the interactions of sulfated glycosaminoglycans and found to directly and allosterically inhibit human α -thrombin.^{15,16} The heterogeneity of these macromolecules was eliminated in the design of sulfated benzofuran monomers, which also inhibited thrombin.¹⁷ The potency of inhibition increased by orders of magnitude following homologation to the dimeric scaffold.^{18,19} This led to the rationale that the trimeric and tetrameric scaffold would be more potent and allosteric inhibitors. R represents different functional groups.

Figure 2.

Direct inhibition of human α -thrombin by designed sulfated benzofuran trimers and a tetramer. The inhibition of thrombin was measured spectrophotometrically through hydrolysis of Spectrozyme TH 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. Solid lines represent fits to the data using equation 1 to obtain IC_{50} , HS , Y_M , and Y_O , as described in 'Experimental Procedures'.

Figure 3. Michaelis-Menten kinetics of Spectrozyme TH hydrolysis by human α -thrombin in presence of trimer **9a**. The initial rate of hydrolysis at various inhibitor concentrations ($\blacklozenge = 0 \mu\text{M}$; $\triangle = 0.36 \mu\text{M}$; $\bullet = 0.63 \mu\text{M}$; $\square = 1.8 \mu\text{M}$; $\circ = 3.6 \mu\text{M}$) was measured spectrophotometrically in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM CaCl_2 and 0.1 % PEG8000 following overnight incubation at 25 °C. Solid lines represent non-linear regressional fits to the data by the standard Michaelis-Menten equation to yield K_M and V_{MAX} .

Figure 4.

Competition by exosite 2 ligands and Dixon-Webb analysis of the competitive effects. Competitive effect of UFH (A) and H8 (C) on the inhibition of human α -thrombin by **9a** in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM CaCl₂ and 0.1 % PEG8000 at 25 °C. Solid lines represent fits using the logistic equation 1 to obtain the apparent IC_{50} as described in 'Experimental Procedures'. Comparisons of the predicted and experimentally measured IC_{50} values in the presence of UFH and H8 at various competitor concentrations are shown in B and D, respectively. Open bars represent the measured values, whereas closed bars are those predicted using Dixon-Webb equation 2.

Figure 5. Identification of residues that contribute to the potency of sulfated benzofuran trimer **9a** using recombinant site-directed mutants of human thrombin. Thrombin mutants were expressed in the Rezaie laboratory and their inhibition by **9a** studied in a manner identical to the plasma α -thrombin. Data shown in A is the dose-response profile for recombinant wild-type and R233A thrombins. Solid lines in A represent sigmoidal dose-response fits using equation 1. The ratio of IC₅₀ values of the recombinant mutant to that of recombinant wild-type thrombin is plotted in B. See text for details.

Figure 6.

Proposed mode of binding of sulfated benzofuran trimer **9a** in exosite 2 of thrombin. Utilizing a 100 GA runs, GOLD-based docking of **9a** led to four nearly equivalent poses of the top eight solutions. Trimer **9a** (stick representation in yellow) was found to bind to Arg233 in a region distant from that of a sulfated benzofuran dimer (sticks, cyan), which engages Arg173.¹⁹ Thrombin surface is shown in light grey with positively charged amino acids of exosite 2 in blue.

Figure 7.

Prolongation of human plasma clotting time as a function of concentration of sulfated benzofuran trimer **9a** in either APTT assay (○) or PT assay (□). Solid lines are trend lines (and not non-linear regressional fits). APTT and PT assays were performed in duplicate, as described in the experimental section.

Scheme 1. Synthesis of sulfated benzofuran trimers **9a – 9g** and **13 – 14**.

Scheme 2.
Synthesis of sulfated benzofuran tetramer **17**.

Scheme 3.
Synthesis of 'flipped' sulfated benzofuran trimer **23**.

Table 1Inhibition of human α -thrombin by sulfated benzofurans.^a

	$\log IC_{50}$	IC_{50} (μ M)	HS	ΔY^a (%)
9a	-6.17 ± 0.03	0.67 ± 0.04^b	1.5 ± 0.2	79 ± 9
9b	-5.69 ± 0.13	2.0 ± 0.6	1.0^c	39 ± 2
9c	-3.78 ± 0.07	165 ± 25	2.1 ± 0.5	$\sim 94^d$
9d	—	>500	NA ^e	NA
9f	-5.23 ± 0.11	5.8 ± 1.5	2.3 ± 1.2	43 ± 3
9g	$\sim -4.0^d$	$\sim 99^d$	UD ^f	$\sim 26^d$
12	—	>500	NA	NA
13	-4.51 ± 0.06	31 ± 4	3.5 ± 1.2	53 ± 4
14	-4.18 ± 0.17	66 ± 25	1.0^c	49 ± 5
17	-3.80 ± 0.15	158 ± 55	1.0^c	62 ± 16
23	-3.81 ± 0.13	154 ± 45	1.2 ± 0.3	$\sim 91^d$

^aLogistic equation 1 was used to fit the dose-dependence of the residual thrombin activity to obtain $\log IC_{50}$, HS, Y_0 , and Y_M values for inhibition in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM CaCl₂, and 0.1% PEG8000 at 25 °C (see 'Experimental Procedures'). $\Delta Y = Y_M - Y_0$.

^bErrors represent ± 1 S. E.

^cHS was fixed to 1.0 to define the inhibition parameters better.

^dApproximate value because either HS, Y_M or Y_0 was not defined well.

^eNot applicable.

^fUndefined.

Table 2Michaelis-Menten kinetics of Spectrozyme TH hydrolysis by α -thrombin in the presence of the trimer 9a.^a

[9a] (μ M)	Overnight incubation at 25°C		2 hr incubation at 37°C	
	K_M (μ M)	V_{MAX} (mAU/min)	K_M (μ M)	V_{MAX} (mAU/min)
0	9.5 ± 1.8^b	81 ± 5	7.7 ± 2.0	86 ± 7
0.36	11.1 ± 2.5	80 ± 6	— ^c	—
0.63	7.7 ± 1.3	69 ± 3	6.7 ± 2.3	72 ± 8
1.53	—	—	2.2 ± 0.8	30 ± 2
1.80	3.2 ± 0.5	35 ± 1	—	—
3.60	3.1 ± 0.60	19 ± 1	2.7 ± 0.6	25 ± 1
9.90	—	—	1.9 ± 0.6	15 ± 0.8

^a K_M and V_{MAX} were measured by monitoring the initial rate of thrombin hydrolysis of Spectrozyme TH (2 – 80 μ M) from the linear increase in absorbance at 405 nm in the presence of fixed concentration of 9a that was incubated either overnight at 25°C or for two hours at 37°C in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM CaCl₂ and 0.1 % PEG8000. The data was fitted by the standard Michaelis – Menten equation to obtain K_M and V_{MAX} , as described in ‘Experimental Procedures’.

^b Error represents ± 1 S.E.

^c Not measured

Table 3Inhibition of α -thrombin by trimer 9a in presence of exosite 1 or exosite 2 ligands.^a

	$\log IC_{50}$	IC_{50} (μ M)	HS	ΔY^a (%)
0 μ M	-6.17 ± 0.03	0.67 ± 0.04^b	1.5 ± 0.2	79 ± 9
[Hirugen peptide]				
3 nM	-6.07 ± 0.03	0.9 ± 0.1	2.5 ± 0.5	44 ± 2
5 nM	-6.10 ± 0.03	0.8 ± 0.1	2.4 ± 0.3	46 ± 2
15 nM	-6.04 ± 0.03	0.9 ± 0.1	3.0 ± 0.7	43 ± 1
[Heparin]				
4.2 μ M	-6.00 ± 0.04	1.0 ± 0.1	2.2 ± 0.4	71 ± 7
12.8 μ M	-5.68 ± 0.03	2.1 ± 0.1	1.8 ± 0.2	80 ± 11
25.0 μ M	-5.42 ± 0.02	3.8 ± 0.2	1.7 ± 0.2	81 ± 10
42.6 μ M	-5.26 ± 0.06	5.6 ± 0.8	1.2 ± 0.2	$\sim 90^c$
[Octasaccharide H8]				
1.0 μ M	-5.87 ± 0.02	1.3 ± 0.1	2.3 ± 0.3	58 ± 3
3.0 μ M	-5.87 ± 0.04	1.4 ± 0.1	2.6 ± 0.6	54 ± 4
10 μ M	-5.78 ± 0.02	1.6 ± 0.1	2.8 ± 0.4	50 ± 2
20 μ M	-5.75 ± 0.03	1.8 ± 0.1	2.6 ± 0.5	49 ± 2
[γ' -Fibrinogen peptide]				
0.2 μ M	-5.94 ± 0.02	1.2 ± 0.1	2.4 ± 0.3	73 ± 8
0.65 μ M	-5.95 ± 0.03	1.1 ± 0.1	2.1 ± 0.3	71 ± 8
2.0 μ M	-5.92 ± 0.02	1.2 ± 0.1	3.3 ± 0.6	67 ± 4
6.5 μ M	-5.88 ± 0.06	1.3 ± 0.2	2.1 ± 0.6	65 ± 7

^aInhibition studies were performed in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM CaCl₂ and 0.1 % PEG 8000 in PEG20000-coated acrylic cuvettes. S-2366 was used as thrombin substrate and trimer 9a was incubated overnight with thrombin at 25 °C. Logistic equation 1 was used to fit the dose-dependence of the residual thrombin activity to obtain $\log IC_{50}$, HS, Y_0 , and Y_M . $\Delta Y = Y_M - Y_0$. See 'Experimental Procedures' for details.

^bRepresents $1 \pm$ S.E.

^cApproximate value because Y_0 was not well defined.

Table 4Inhibition of recombinant wild-type and mutant thrombins by trimer 9a.^a

Thrombin	log IC ₅₀	HS	IC ₅₀ (μM)	ΔY ^a (%)
rWT	-6.58 ± 0.02	3.4 ± 0.5 ^b	0.26 ± 0.01	49 ± 2
R93A	-6.73 ± 0.04	1.8 ± 0.3	0.19 ± 0.02	66 ± 6
R97A	-6.44 ± 0.04	2.6 ± 0.5	0.37 ± 0.03	47 ± 3
R101A	-6.41 ± 0.07	2.1 ± 0.6	0.39 ± 0.06	55 ± 6
R126A	-6.55 ± 0.07	1.8 ± 0.5	0.29 ± 0.05	65 ± 11
R165A	-6.59 ± 0.11	1.1 ± 0.3	0.26 ± 0.07	61 ± 8
K169A	-6.48 ± 0.03	2.1 ± 0.2	0.33 ± 0.02	59 ± 3
R173A	-6.59 ± 0.04	2.4 ± 0.7	0.26 ± 0.03	62 ± 8
R175A	-6.363 ± 0.04	5.2 ± 2.1	0.43 ± 0.04	37 ± 3
R233A	-6.01 ± 0.04	3.1 ± 0.6	0.98 ± 0.10	44 ± 2
K235A	-6.57 ± 0.02	2.0 ± 0.1	0.27 ± 0.01	50 ± 1
K236A	-6.40 ± 0.01	3.0 ± 0.3	0.40 ± 0.04	52 ± 1
K240A	-6.57 ± 0.04	2.3 ± 0.4	0.27 ± 0.03	49 ± 3

^aInhibition studies were performed in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM CaCl₂ and 0.1 % PEG 8000 in PEG20000-coated acrylic cuvettes. S-2366 was used as thrombin substrate and trimer 9a was incubated overnight with the enzyme at 25 °C. Logistic equation 1 was used to fit the dose-dependence of the residual thrombin activity to obtain log IC₅₀, HS, Y₀, and Y_M. ΔY = Y_M - Y₀. See 'Experimental Procedures' for details.

^bRepresents 1 ± S.E.