SYNTHESIS AND BIOCHEMICAL STUDIES ON SULFATED MONOMERS OF LOW MOLECULAR WEIGHT LIGNINS

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SYNTHESIS AND BIOCHEMICAL STUDIES ON SULFATED MONOMERS OF LOW MOLECULAR WEIGHT LIGNINS

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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Anticoagulants are used as the first line therapy for management and prevention of thrombotic disorders. Thrombin and factor Xa have been the prime targets for regulation of the coagulation cascade.

In this work, a small library of 17 benzofuran derivatives were synthesized and screened against thrombin and factor Xa. The derivatives that displayed inhibitory potential were docked on the exosite-II of factor Xa using a docking protocol that was developed in our research group. These compounds were based on the β-5 structural unit found in the oligomer ‘CDSO3’, which was prepared in our lab and was found to inhibit both thrombin and factor Xa by an exosite-II mediated allosteric disruption of the catalytic triad.
The results revealed that these β-5 like derivatives are inhibitory against thrombin and factor Xa, although their potency is weak. Thrombin and factor Xa appear to recognize different structural features suggesting a significant selectivity in recognition. Furthermore, a slight preference for the benzofuran scaffold was observed with factor Xa. Probing the mechanism of inhibition using Michaelis-Menten kinetics reveal that these compounds display uncompetitive inhibition of these proteases and the mechanism of inhibition is allosteric.

Docking of these compounds on factor Xa were done using GOLD (Genetic algorithm for ligand docking) and the results, explain the observed inhibition profile. The computed docked poses also give an idea of the residues on the exosite-II of factor Xa critical for inhibition.

The molecules studied here are radically different in terms of structure and mechanism of inhibition from any other ligand described in literature. This represents an opportunity to discover novel molecules with a possibly different pharmacological and toxicological profile.
Chapter 1 Introduction

1.1 The cell based model of coagulation

Blood coagulation is a self defense system preventing excess loss of blood and infiltration of microbes following injury. The initial model of the coagulation cascade was proposed in 1960\textsuperscript{1, 2} and recently a cell based model was proposed. The cell based coagulation cascade is proposed to occur by three steps – initiation, amplification and propagation\textsuperscript{3-5} (Figure 1).

Cell surfaces localize the activation of coagulant factors, so that systemic activation of the factors is prevented. Exposure of the transmembrane tissue factor (TF) is how coagulation is initiated \textit{in vivo} in response to a vascular injury\textsuperscript{6}. The exposed TF binds to factor VIIa to form the extrinsic tenase complex, which activates factor X and factor IX\textsuperscript{7}. Activated factor X (fXa) activates factor V (fVa) and converts sufficient amounts of prothrombin to thrombin to amplify the cascade and move the site of action from TF bearing cells to platelet surfaces\textsuperscript{8}. On the platelet surface fIXa binds to fVIIla to form the intrinsic tenase complex which activates fX, which binds to fVla and forms the prothrombinase complex. This complex efficiently converts prothrombin to thrombin. Thrombin converts fibrinogen to fibrin monomers and activates fXIIIa which cross links the fibrin monomers and stabilizes the clot\textsuperscript{9}.
Figure 1 The coagulation cascade. The endogenous regulators of the cascade include antithrombin (AT), tissue factor pathway inhibitor (TFPI) and activated protein C (APC). HMWK: high molecular weight kininogen, TM: thrombomodulin, TM:IIa: thrombomodulin-thrombin complex, TF: tissue factor, PL: phospholipid, F: fibrin, Fm: fibrin monomers. (Figure was adapted from Henry, B. L.; Desai, U. R. Recent research developments in the direct inhibition of coagulation proteinases--inhibitors of the initiation phase. Cardiovasc. Hematol. Agents Med. Chem. 2008, 6, 323-336).
1.2 Regulation of coagulation

Several endogenous proteins regulate the procoagulant response and prevent hyper–coagulation by inhibiting the cascade at multiple points.

TFPI has a role in modulating the formation of the extrinsic tenase complex. First TFPI binds to fXa forming TFPI/fXa complex. This complex binds to fVIIa in the TF/VIIa complex thereby regulating its catalytic activity. Also TFPI potentiates the activity of heparins.

Activated protein C (APC); in the protein C pathway, thrombin binds to thrombomodulin, a receptor in the endothelium. On binding, thrombin activates protein C which inactivates fVa and fVIIa.

Antithrombin (AT) regulates thrombin, fIXa, fXa and also TF/VIIa complex. Heparin induces a massive 300 – 4000 fold increase in the rate of inhibition of the target proteases by AT.

1.3 Thrombin and Factor Xa

Thrombin and factor Xa are the most important proteases in the coagulation cascade. Both the pharmaceutical industry and academia are actively involved in the design and development of molecules which target either one of these two proteases. The X-ray crystal structure of these proteases provides a useful starting point for rational design of their inhibitors.
Figure 2A  **The topology of Thrombin.** All the important domains are highlighted.

(Figure was made in SYBYL 8.1 from the protein structure 1XMN)

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Figure 2B  **The topology of fXa.** (Figure was made in SYBYL 8.1 from the protein structure 1HCG)
1.3.1 The structure of Thrombin

Thrombin is a serine protease which is similar to trypsin and chymotrypsin. It has 2 polypeptide chains; A (36 residues) and B (259 residues) linked by disulfide bridges (Figure 2A). Physiologically it cleaves fibrinogen to fibrinopeptides A and B which are cross linked by fXIIIa to form clot. A large number of structures of thrombin have been deposited in the protein data bank. The enzyme is organized around 2 adjacent β-barrels in chain B and the active site residues Ser^{195}, His^{57} and Asp^{102} (chymotrypsinogen numbering) is located between them. The active site is surrounded by the 60 and the γ-loops that restrict accessibility to the active site nucleophile. The nomenclature given by Schechter and Berger has been used in literature. Here substrate residues are termed P (for peptide) and the residues of the interacting proteases are called S (for subsite). Substrate residues that extend toward the N terminus of the scissile bond are numbered P2, P3… and substrate residues which extend toward the C terminus are termed P2’, P3’ …(Figure 3)

Figure 3 Schechter and Berger nomenclature for naming subsites (S) on a protease and positions (P) on a polypeptide substrate.

\[
\text{N-terminus} \quad \text{Cleavage point} \quad \text{C-terminus}
\]

\[
\begin{align*}
\text{---P}_3\text{---P}_2\text{---P}_1\text{---P}_1'\text{---P}_2'\text{---P}_3'\text{---} & \quad \text{substrate peptide} \\
\text{---S}_3\text{---S}_2\text{---S}_1\text{---S}_1'\text{---S}_2'\text{---S}_3'\text{---} & \quad \text{enzyme subsites}
\end{align*}
\]
The subsites and interacting domains present in thrombin are

The **S1 site** has Asp^{189}, which is present in the bottom that recognizes substrates/inhibitors with a basic P1 group. Also the Ser^{195}, His^{57}, Asp^{102} catalytic triad and an oxyanion hole are present. The active site is buried inside a canyon due to the presence of 60 and γ-insertion loops that are not present in chymotrypsinogen.

The **S2 site** has the residues Tyr^{60}A and Trp^{60}D that create the S2 pocket which recognizes lipophillic P2 groups. Proline binds better in the S2 pocket.

The **S3 site** is created by the side chains of Leu^{99}, Ile^{174} and Trp^{215}. It interacts with the hydrophobic group P3 and is more acidic when compared to other serine proteases due to a Glu at 192.

The **S4 site** aliphatic residues show a preference for the S4 pocket.

The **S1’, S2’ and S3’ sites** recognize hydrophobic residues P1’, P2’, P3’.

**60 and the γ-insertion loop;** the active site is buried in a narrow cleft made of the 60 and the γ-insertion loop. The residues which make the 60 loop are more hydrophobic, whereas the γ-insertion loop is hydrophilic and flexible. These both loops play a major role in substrate specificity.

Two basic domains are present approximately 10-20Å away from the active site on opposite ends. The first one located to the east of the active site is **Exosite-I**, which consists of residues Lys^{21}, Arg^{62}, Arg^{68}, Arg^{70}, Tyr^{71}, Arg^{73}, Lys^{106} and Lys^{107}. This site is involved in the binding of fibrinogen, fibrin and coagulation factors V, VIII and XIII and interacts with residues which are toward the N terminus of the substrate. The positive
field created by this site provides electrostatic steering and pre-orientation for substrates\textsuperscript{19}.

The **Exosite-II** is located towards the west of the active site and is the more electropositive region\textsuperscript{19}. Acidic molecules like glycosaminoglycans bind to this site and the primary residues present in this site are His\textsuperscript{91}, Arg\textsuperscript{93}, Arg\textsuperscript{101}, Arg\textsuperscript{126}, Arg\textsuperscript{165}, Lys\textsuperscript{236} and Lys\textsuperscript{240} (Figure 4A). These residues play a crucial role in interaction of thrombin with heparin. Also not every residue present in the exosite are significant for any interactions that occur on the site.

Thrombin utilizes both exosite-I and II for molecular recognition\textsuperscript{21,22}. Anionic molecules bind to either of the sites exclusively. The specificity of these molecules towards exosites depend on their ionic charge. For exosite-I, hydrophobic contacts provide most of the binding energy and electrostatics are involved in orienting the complementary hydrophobic surfaces. Example: Hirudin which is an exosite-I ligand for thrombin, 20\% of the binding energy originates from ionic interactions\textsuperscript{23-26}, whereas exosite-II interactions are primarily ionic in nature. Example: for heparin-thrombin interactions, 80\% of the binding energy is ionic\textsuperscript{27}. It has been reported that both the exosites modulate the binding of each other based on changes of fluorescence of fluorescein-FPR-thrombin on binding with peptides\textsuperscript{28} but, Bock \textit{et al.} have reported no change in $K_D$ values upon binding of these peptides\textsuperscript{29}.

The binding of ligands to both of the exosites induces conformational changes in the active site\textsuperscript{30, 31}. This changes the specificity and reactivity of thrombin towards macromolecular substrates. Exosite-I ligands influence the catalytic efficiency ($k_{\text{CAT}}/K_M$)
of thrombin towards hydrolysis of peptide substrates, whereas exosite-II ligands like GAG’s have no influence on the catalytic efficiency of peptide hydrolysis. However, allosteric modulation of the catalytic triad through exosite-II has been observed by chemo-enzymatically prepared lignin derivatives.

**Sodium binding site:** The Na⁺ binding site is located to the center of the 222 loop, which is situated behind the S1 specificity pocket near Asp¹⁸⁹. The Na⁺ ion is coordinated octahedrally by carbonyl oxygens or Arg²²¹ A and Lys²²⁴ and by four internal water molecules. This is further stabilized by the negative charges Asp²²¹ and Asp²²². Sodium is an important allosteric modulator of α-thrombin. Two allosteric states have been characterized kinetically in absence and presence of the Na⁺ ion – the slow and the fast form. The fast form displays procoagulant, prothrombotic and prosignalling properties whereas the slow form exhibits more anticoagulant properties.
**Figure 4A  Exosite-II of thrombin** (Figure was created in SYBYL 8.1 from protein structure 1XMN)

![Figure 4A](image1)

**Figure 4B  Exosite-II of fXa** (Figure was created in SYBYL 8.1 from protein structure 1HCG)

![Figure 4B](image2)
1.3.2 The structure of Factor Xa

Factor Xa is structurally similar to the other trypsin like serine proteases in the coagulation cascade. It is a Vitamin K–dependent glycoprotein that is synthesized from the liver and is secreted into the blood as a zymogen. The mature circulating form of factor X has a light chain (139 residues) and a heavy chain (303 residues) held together by a single disulfide bond. For activation a specific arginyl – isoleucyl (Arg–Ile) is cleaved in the N-terminal of the heavy chain resulting in fXa (390 residues). The new N-terminal Ile folds into the interior of the protein and forms ion pair with an Asp residue near the active site Ser195 (Figure 2B). The first structure of fXa determined by X-ray crystallography was in 1990 (PDB code 1HCG).

The various functional domains in fXa are:

**Active site S1;** where the heavy chain has the residues of the catalytic triad: Ser195, His57, Asp102 (chymotrypsinogen numbering). The overall folding resembles thrombin. The absence of the thrombin Tyr60A – Thr60I insertion loop makes the catalytic triad more accessible to substrates and inhibitors. Therefore the active cleft is described as more open and groove–like, when compared to the canyon–like cleft in thrombin. An Asp189 is present in the base of the S1 pocket which recognizes Arg like residues in the P1 site of substrates and inhibitors. A hydrophobic “disulfide pocket” is found adjacent to S1 formed by Gln192, Cys191, Cys220 and Gly218. This has been otherwise termed as the “ester pocket” due to accommodation of such groups in co – crystallization experiments.

**S2 subsite;** here a Tyr side chain obstructs the S2 pocket in fXa and thereby only small aliphatic residues like glycine can interact.
**S3 subsite:** Here an amide group of the conserved Gly$^{216}$ can provide hydrogen–bonding interactions with the P3 part of a substrate/inhibitor.

**S4 subsite,** where the residues Tyr$^{99}$, Trp$^{215}$ and Phe$^{174}$ form an aromatic box that serves as the S4 subsite. Moreover, the carbonyl groups of Lys$^{96}$, Glu$^{97}$ and Thr$^{98}$ form a cation hole at the back of the aromatic box. Aromatic interactions in the S4 pocket are crucial for the design of inhibitors that are selective to fXa over thrombin.

**Exosite-1** is the region in fXa that corresponds to the fibrinogen recognition exosite in thrombin encompasses Asn$^{35}$-Phe$^{41}$ and Asp$^{70}$-Ala$^{81}$. Whereas this region in thrombin is decorated with basic residues, in fXa has eight acidic groups with only one Ala. This region in fXa is crucial for prothrombin recognition and does not recognize fibrinogen or other exosite-I inhibitors of thrombin$^{101}$.

**Exosite-II:** Of the eleven basic residues seen in the exosite-II in thrombin, seven are found to be conserved in fXa. These residues are Arg$^{93}$, Lys$^{96}$, Arg$^{125}$, Arg$^{165}$, Lys$^{169}$, Lys$^{236}$ and Arg$^{240}$ (chymotrypsinogen numbering). This region is otherwise called as the “Heparin binding Exosite”. This region is found towards the west of the active site on exposed surface loops (Figure 4B). Full length high affinity heparin can interact with this site to produce ~200-300 fold greater acceleration of the reaction with antithrombin. Additionally, residues in this site have been implicated in binding of fXa with fVa and formation of the prothrombinase complex$^{101,102}$.

**Ca$^{2+}$ binding site:** fXa has a three Ca$^{2+}$ binding sites. There is one on the N-terminus Gla domain which is required for binding to fVa in the prothrombinase complex. The other
two sites are on the epidermal growth factor – like domain (EGF-1) and the C-terminal catalytic domain, which includes conserved loop spanning residues 70 – 80\textsuperscript{103-105}.

**Na\textsuperscript{+} binding site;** the 225 loop in fXa has been identified as the sodium binding site which is similar to thrombin. Studies in which the residue Tyr\textsuperscript{225}→Pro, displayed that specific binding of Na\textsuperscript{+} to this loop increases the catalytic activity of fXa towards small synthetic substrates and antithrombin\textsuperscript{106,107}.

Furthermore, it has been shown that there exists an allosteric linkage between the Na\textsuperscript{+} binding loop and the Ca\textsuperscript{2+} binding (Glu\textsuperscript{70}-Glu\textsuperscript{80}) that modulates the structure and function of fXa\textsuperscript{108}.

### 1.4 Rationale for inhibition of thrombin and fXa

Thrombin is the final serine protease involved in the coagulation cascade. It converts fibrinogen to fibrin and activates factor XIII which cross links the fibrin monomers. Thrombin amplifies its own generation by activation of factor VIII and factor V which are cofactors for the intrinsic tenase and prothrombinase complex and also by indirectly activating factor IX - the enzyme constituent of the intrinsic tenase. Additionally thrombin acts as a potent platelet agonist. By recruiting platelets to the site of vascular injury, these activated platelets further add to thrombin generation. With this significant role in thrombosis and hemostasis, thrombin is a major point for inhibition in the cascade (Figure 5). Moreover, data from clinical trials with direct thrombin inhibitors indicate that short term and long term inhibition of thrombin is both safe and effective\textsuperscript{41,42}.
Factor X can be activated by the contact pathway or by the tissue factor/VIIa pathway. Thereby initiation of coagulation by either pathway in response to any vascular injury activates factor X to fXa. So, pharmaceutically it is a desired intervention point in the development of anticoagulants. The activated factor X with its cofactor fVα combine on anionic surfaces (phospholipid membranes) to form the prothrombinase complex which converts prothrombin to thrombin.

Factor X deficiency manifests as severe bleeding diatheses. Patients display very low antigen and activity levels of fXa. A number of variants of fXa deficiency suggested that factor X activity levels must be less than 5% of normal to display bleeding tendencies. This proved that activity of factor X can be suppressed distinctly without affecting hemostasis. Thus an ideal anticoagulant could stop thrombosis without causing systemic hypocoagulation.

Studies carried out by Eisenberg et al. showed that fXa in thrombi is enzymatically active and was able to convert prothrombin to thrombin. Also, fXa is accountable more than thrombin for clot associated procoagulant activity. The procoagulant activity of thrombi is due to the de novo conversion of prothrombin to thrombin and not due to circulating thrombin. These data suggest that in thrombi fXa is active and direct inhibition of thrombus associated fXa would be an effective approach to prevention of thrombus growth.
Figure 5  A simplified scheme of the coagulation cascade. The intervention point for fXa and fIIa inhibitors is shown (Figure was adapted from Leadley, R. J., Jr Coagulation factor Xa inhibition: biological background and rationale. *Curr. Top. Med. Chem.* 2001, 1, 151-159).
1.5 Inhibitors of Thrombin

1.5.1 Indirect inhibitors

These molecules exploit an intermediate physiological protein to inhibit the procoagulant protease.

1.5.1.1 Heparin and its derivatives

Unfractionated heparin (UFH) is an acidic linear polysaccharide composed of alternating 1-4 linked uronic acid and glucosamine residues (Figure 6). It has a MW of ~15000 Da. Low molecular weight heparins (LMWH) are produced or fractionated from UFH by chemical or enzymatic depolymerization techniques\(^4^3\). They usually retain a structure similar to UFH although the different methods used in their preparation increase their structural heterogeneity. UFH is the strongest acid in human physiology due to the presence of numerous sulfates and carboxylates that span the saccharide chain (~3.7 negative charges per disaccharide). The pentasaccharide sequence, which is composed of 5 residues DEFGH, mediates the interaction with antithrombin (Figure 7). Antithrombin (AT), a serine protease inhibitor (SERPIN), is a plasma glycoprotein which inhibits its target proteases by a serpin mouse trap mechanism. It uses its P1-P1’ residues in its reactive center loop (RCL) to bind to the target serine protease. After binding of the protease to the RCL of AT to form a Michaelis - Menten complex (E:AT), the scissile bond P1-P1’ is cleaved to form an acyl-enzyme intermediate (E-AT), which rearranges to disrupt the catalytic triad of the enzyme (E\(^\ast\)-AT\(^\ast\)). A competing pathway called the substrate pathway may operate in parallel where in mutational changes in AT may cause hydrolysis of the acyl – enzyme intermediate (E-AT) to yield an active enzyme (E) and a
cleaved inhibitor (AT<sub>c</sub>) (Figure 8). The target proteases for AT are fIIa, fXa, fIXa. In presence of heparin, the inhibition rate constants of AT for these proteases increase 10<sup>2</sup>-10<sup>6</sup> fold<sup>11,44</sup>.

AT inhibits the procoagulant proteases by two separate mechanisms. The first one is the allosteric activation of AT by the sequence DEFGH. After recognition of the pentasaccharide sequence by AT, a change in conformation of the partially inserted RCL occurs<sup>11,45,46</sup>. This induces an accelerated cleavage of the RCL and explains the rate acceleration of fXa inhibition by ~300 fold in absence of Ca<sup>2+</sup>.

Inhibition of thrombin by AT in presence of UFH is by the bridging mechanism. Binding of AT to the UFH chain is followed by thrombin binding at non-specific positions along the polysaccharide chain. Thrombin then diffuses along the chain to form the Michaelis-Menten complex (E:AT), resulting in a 10<sup>2</sup>-10<sup>6</sup> fold increase in the inhibition rate constants<sup>27</sup> (Figure 9). LMWH and the pentasaccharide sequence by itself cannot inhibit thrombin as the minimum chain length of ~18 residues is required for the bridging mechanism<sup>44,47</sup>. 
**Figure 6** General structure of heparin (Figure adapted from Henry, B. L.; Desai, U. R. Recent research developments in the direct inhibition of coagulation proteinases--inhibitors of the initiation phase. *Cardiovasc. Hematol. Agents Med. Chem.* 2008, 6, 323-336).

![Diagram of heparin structure]

lichalc: X = −SO₂⁻ or −H; Y = −SO₃⁻ or −COCH₃
Z = −SO₂⁻ or −H; W = −SO₃⁻ or −H

**UFH**: $M_R \approx 15,000$ Da; **LMWH**: $M_R \approx 5,000$ Da

**Figure 7** The pentasaccharide binding sequence.

![Diagram of pentasaccharide binding sequence]
Figure 8  A model of the antithrombin inhibition mechanism (Figure is adapted from Desai, U. R. New antithrombin-based anticoagulants. *Med. Res. Rev.* 2004, 24, 151-181).
1.5.1.2 Warfarin

Warfarin is an indirect oral anticoagulant that inhibits vitamin K dependent post translational modification of glutamic acid to γ-carboxyl glutamic acid, which are present on the N terminus of Vitamin K dependant proteins eg: factor II, VII, IX and protein C, S and Z\textsuperscript{38} (Figure 10).
1.5.1.3 Limitations of indirect anticoagulants

All anticoagulants pose an excessive risk for bleeding. UFH can cause heparin-induced thrombocytopenia (HIT), which is a significant drop in platelet count that happens between 4-14 days of therapy\(^ {49} \). HIT has been classified into two; Type 1 and Type II. The type II HIT is the most severe and is characterized by the development of antibodies against heparin-platelet factor 4 (PF4) complex\(^ {50} \). Platelet factor 4 is a protein found on platelets and interacts with heparin to from a tetrameric complex. Once the heparin-PF4 complex is formed it is recognized by antibodies on the platelet surface. In due course, these platelets form a thrombus effectively being removed from circulation\(^ {51} \). Usually, the majority of HIT episodes occur with UFH, although cases with LMWH are also reported\(^ {52} \). Additionally, UFH displays significant intra- and inter-patient dose variability. Moreover, heparins do not neutralize clot bound thrombin, which increases the possibility of reactivating clotting at a later time\(^ {53,54} \).

In 2007-2008, reports of serious allergy and hypersensitivity-type reactions surfaced following the use of UFH resulting in at least 81 cases of death. Investigations revealed
that the UFH used was contaminated with oversulfated chondroitin sulfate (OSCS) and the source originated in China. It is not sure whether the contamination was intentional or due to faulty processing of pig intestines\textsuperscript{55, 56}.

Warfarin suffers from a narrow therapeutic index and many drug–drug or drug–food interactions. Clinically used warfarin is a racemic mixture of R- and S-enantiomers, with the R-enantiomer being more active. However it is metabolized more quickly than the S-enantiomer. Single nucleotide polymorphisms (SNP’s) in the gene encoding the enzyme that metabolizes S-warfarin have been found which translate to significantly enhance anticoagulation effect\textsuperscript{57-60}. From 1990 to 2000, warfarin was ranked in the top ten for the most number of serious and adverse events received by the FDA. Also in 2006, a “black box” warning was placed on warfarin by FDA related to its high risk of bleeding\textsuperscript{61, 62}.

### 1.5.2 Direct Thrombin Inhibitors (DTI)

These molecules inhibit thrombin directly either through the active site or by an allosteric mechanism of action. A major advantage of DTI’s are that both circulating and clot bound thrombin can be theoretically inhibited.

These are classified into naturally occurring compounds and synthetic compounds.
1.5.2.1 Naturally occurring compounds

**Hirudin** was extracted from the salivary glands of the medicinal leech *Hirudo medicinalis* in 1957. It is a polypeptide having 60 residues and is the most potent DTI known till date having an Ki of ~20 fM. The primary sequence of hirudin has a sulfated Tyr\(^{63}\) (**Figure 11**). The binding of hirudin to thrombin is a slow process involving many steps\(^{63}\).

Recombinant hirudins were made due to the high affinity of natural hirudin and differ at only the three residues in the carboxyl terminus from hirudin. These recombinant peptides (desiuridin, lepiuridin and bivaluridin) have Ki values of 60 fM, 200 fM and 1-2 nM\(^{64,65}\). These molecules are bivalent thrombin inhibitors binding to the exosite-I and the hydrophobic region near the active site.

**Triabin** is also another natural peptide that inhibits thrombin\(^{66}\). **Nazumamide A**, **A90720A** are also natural products that have an arginine which recognize Asp\(^{189}\) in the S1 specificity pocket of thrombin\(^{67,68}\).

**Theromin**, a 67 amino acid peptide produced by the Rhynchobdellid leech, is the most potent inhibitor of thrombin described till date (Ki = 12 ± 5 fM). The molecule had no sequence homology with any other animal thrombin inhibitor characterized so far. It is suggested that dimerization is required to give the protein an active folded configuration that can bind to thrombin; however, the monomers are found to have inhibitory activity\(^{69}\).
**Figure 11**  The sequence of naturally occurring hirudin. Recombinant hirudins do not have a sulfate at Tyr$^{63}$. The residues in red consist of the C-terminal exosite-1 recognition sequence and the residues in blue recognize the active site. (Figure adapted from Rydel, T. J.; Ravichandran, K. G.; Tulinsky, A.; Bode, W.; Huber, R.; Roitsch, C.; Fenton, J. W., 2nd The structure of a complex of recombinant hirudin and human alpha-thrombin. *Science* 1990, 249, 277-280.

1.5.2.2 Synthetic compounds

1.5.2.2.1 Irreversible Covalent Inhibitors

The peptide chloromethyl ketone D-Phe-Pro-Arg-CH$_2$Cl (PPACK, 1) is used as an affinity label for the active site of thrombin. It irreversibly acylates His$^{57}$ of the catalytic triad and inhibits thrombin$^{70}$. However these molecules show no selectivity over other serine proteases.
1.5.2.2.2 Reversible Covalent Inhibitors

These class of molecules have an electrophillic moiety (serine trap), which is susceptible to attack by the active site serine forming a reversible hemiacetal. Several electrophillic groups which have been used are ketones, trifluoromethyl ketones, $\alpha$-diketones, $\alpha$-keto heterocycles, $\alpha$-keto amides, $\alpha$-keto esters, organoboronic acids and organophosphonic acids\textsuperscript{71-73}. Some of the molecules under clinical evaluation are compound 2 (GYKI-14766, $K_i = 1.2\ nM$), compound 3 (CVS-1123), compound 4 (DUP-714, $K_i = 40\ pM$)\textsuperscript{74-77}.

However these compounds have a reactive functional group, which may make it less selective against other coagulation proteases, more prone to metabolism and more toxic.
1.5.2.2.3 Reversible Non–Covalent Inhibitors

The lack of any serine trap is a characteristic of non covalent DTI’s. This reduces a potential for toxicity and the molecule can bind to the enzyme subsites by electrostatic and hydrophobic interactions. Due to these reasons, several research groups have concentrated their work on this class of compounds.147
1.5.2.2.3.1 Guanidine or Amidine based molecules

The first compounds developed, compound 6 (NAPAP, Ki = 6 nM), and compound 7 (argatroban, Ki = 39 nM) were derived from the arginine derivative N-α-tosylarginine methyl ester (TAME, 5)\textsuperscript{78-80}. All of the molecules in this class have a guanidine or arginine in its P1 position, which was based on the rationale to have binding interactions with Asp\textsuperscript{189} in the S1 pocket of the protease. Argatroban was the first rationally designed DTI to reach the clinic, however it is not orally bioavailable due to the highly basic guanidine moiety.
The researchers at Astra Zeneca developed compound 8 (inogatran, $K_i = 15$ nM)$^{81}$. Systematic modifications resulted in compound 9 (melagatran, $K_i = 2$ nM)$^{82, 83}$. The benzamidino group of melagatran goes in the S1 pocket, while the azetidine and the cyclohexyl ring are buried in the S2 and S3 pockets. Due to the low oral bioavailability of melagatran, compound 10 (ximelagatran) was developed as a prodrug$^{84, 85}$. Ximelagatran is transformed to melagatran by two intermediates, which involves hydrolysis of the ester and reduction of the amidoxime group. Due to the increased hydrophobicity of ximelagatran, it was found to be more orally available. Despite the high efficacy and bleeding profile of ximelagatran in clinical trials, FDA rejected its approval due to its off target hepatotoxicity.
Boehringer Ingelheim developed compound 11 (dabigatran etexilat), which is a prodrug of 12 (dabigatran, Ki = 4.5 nM)\textsuperscript{86}. It is acted upon by esterases in vivo to generate dabigatran. It is based on a 1,2,5 trisubstituted benzimidazole core scaffold. The molecule is under advanced phases of clinical trials. In the Bistro I and II study dabigatran etexilat demonstrated an acceptable safety profile and satisfactory antithrombotic potential\textsuperscript{87,88}.
Compound 13 (UK 156,406, Ki = 0.46 nM) developed by Allen et al. was modified from the lead argatroban\textsuperscript{89}. The molecule had a tetrahydroisoquinoline group at P3 and a carboxylic acid and a olefin residue in the piperidine moiety at P2. The changes resulted in good oral bioavailability and the drug is in clinical trials\textsuperscript{89}.

The most potent synthetic inhibitor till date is compound 14 (LB-30812, Ki = 3 pM). However due to the basic amidine group at P1 position, it is only moderately bioavailable\textsuperscript{90}.
The BMS group used a piperidine unit at P1 to obtain compound 15 (BMS-189090, Ki = 3.6 nM), which is in preclinical development\textsuperscript{91}.
1.5.2.3.2 Non–amidine based molecules

The realization that the lipophilicity of the S1 pocket can be utilized for designing inhibitors spurred an interest for compounds which have a lesser basic P1 group and thereby be more orally bioavailable.

Compound 16 (SSR-182289A, $K_i = 31$ nM) is an extremely potent DTI whose lead was argatroban. The P1 unit was replaced from guanidine in argatroban to a 3-aminopyridine moiety and thereby the molecule is orally bioavailable. The compound is under phase-II clinical trials\(^92\).

![SSR-182289](image)

Oh et al. designed the compound 17 (LB-30057, $K_i = 0.38$ nM) which has a less basic benzamidrazone in its P1 position. Also, it was shown to be orally active in dog models\(^93\).
Kane et al. optimized the P1 position of argatroban utilizing the lipophilicity of the S1 pocket which has an Ala¹⁹⁰ in addition to an Asp¹⁸⁹. A lipophilic group benzothiazole was used in the P1 position to obtain compound 18 (CGH 752, Ki = 0.026 nM)⁹⁴.
Sanderson et al. used 3-amino-2-pyridinone and 5-amino-6-pyridinone acetamide scaffolds with an amino pyridine in P1 to get compound 19 (Ki = 0.85 nM) with an improved pharmacokinetic profile\textsuperscript{95}.

![Image of compound 19]

Replacement of the pyridinone core with a more stable 3-alkylaminopyrazinone biostere lead to compound 20 (L-375,378, Ki = 0.8 nM), which was further selected for clinical trials\textsuperscript{96}.

![Image of compound 20]
1.6 Factor Xa Inhibitors

1.6.1 Synthetic Direct Non Covalent fXa inhibitors

Initially the first molecules identified were based on bisbenzamidines. The early molecules originated were from thrombin inhibitor programs. The presence of basic amidine groups on the P1 and the P4 positions of the scaffold made the compounds poorly bioavailable. The first bisbenzamidines, which were identified as potent molecules were compound 21 (DABE, Ki = 573 nM) and compound 22 (BABCH, Ki = 610 nM)\textsuperscript{116,117}.

![DABE](image1.png)

DABE

21

![BABCH](image2.png)

BABCH

22

Upon replacing one of the basic benzamidine/amidine moieties on the scaffold (either P1 or P4) with a lesser basic functional group resulted in higher oral bioavailability. But this lowered the selectivity of the molecule towards fXa against other serine proteases.
However, when the remaining benzamidine/amidine was substituted with another lesser basic group highly potent, selective and orally bioavailable inhibitors were discovered. Compound 23 (rivaroxaban, $Ki = 2.1 \text{ nM}$) is currently the most advanced inhibitor in clinical trials. In 2008, it was approved by the European commission for prevention of venous thromboembolism (VTE) after total knee and hip replacement. The cocrystal structure of with fXa (PDB code 2W26) shows that the oxazolidinone ring serves as the core to direct the morpholine residue into the S4 pocket and the chlorothiophene into the S1 pocket. Hydrogen bonds are seen between amide N-H of Gly$^{219}$ and the carbonyl of the oxazolidinone and between the carbonyl of Gly$^{219}$ and the N-H of the thiophene carboxamide$^{118-121}$. In the S1 pocket the important interaction is between the chlorine and the aromatic ring of Try$^{228}$.

Phenylglycinamide series of compounds with a rigid P4 unit and an indole in the P1 position displayed Ki values in the lower nM range. Among the series, compound 24 (LY-517717, $Ki = 4.6 \text{ nM}$) showed 25% to 82% oral bioavailability and a 1000 fold selectivity against other serine proteases. The compound was advanced to phase-II clinical trials$^{122,123}$. 
Among a set of compounds with a pyrazole scaffold and benzamidine as the P1 substituent, replacement of the P1 substituent with less basic or neutral scaffolds was explored. It was found that lowering the pKa of the P1 position systematically improved oral bioavailability. Substituting the P1 with a 3-aminobenzisoxazole moiety and optimizing the P4 with dimethylaminomethylimidazole gave the compound 25 (razaxaban, Ki = 0.19 nM). The cocrystal structure of 25 with fXa shows that the aminobenzisoxazole binds to the S1 pocket and the amino group interacts with Asp\textsuperscript{189} and the carbonyl of Gly\textsuperscript{218}. The imidazole binds in the S4 pocket with a hydrogen bond to Glu\textsuperscript{97}. The compound passed phase-II trials; however, it was withdrawn for undisclosed reasons\textsuperscript{124,125}. 

There were concerns about the pyrazole carboxamide core present in compound. It might hydrolyze in physiological conditions to release a potentially mutagenic biarylaniline. One of the approaches to this issue was to replace the nitrogen of the carboxamide scaffold with a methylene group. Further optimization led to compound 26 (apixaban, \( Ki = 0.08 \text{ nM} \)). The compound was found to be highly selective for fXa against thrombin and trypsin. The cocrystal structure of compound bound to fXa (PDB code 2PI6) showed that the p-methoxy group goes in the S1 pocket, the pyrazole interacts with Gln\(^{192} \) and the carboxamide carbonyl interacts with Gly\(^{216} \). The lactam is found in the S4 pocket interacting with Tyr\(^{99} \) and Phe\(^{174} \). This compound showed an oral bioavailability of 58% and has advanced to phase-III trials\(^{126,127} \).
In a set of amino acid derivatives of fXa inhibitors synthesized, a compound was reported to have a Ki of 9.4 nM. Removal of the phenyl group and SAR exploration around P4 lead to the compound 27 (otamixaban, Ki = 0.4 nM). Moreover, the compound was found to be highly selective against other serine proteases. The cocrystal structure of compound 27 bound to fXa (PDB code 1KSN) showed that the benzamidine is in the S1 pocket and the heterobiaryl group is in the P4 pocket. A hydrogen bond is found between the carbonyl of the amide and N-H of Gly\textsuperscript{219}. The compound was advanced to phase-II clinical trials\textsuperscript{128,129}. 

![APIXABAN](attachment:image.png)
In a series of pyrazoles, compound 28 (DPC-423, Ki = 0.15 nM) showed an improved pharmacokinetic profile compared to others in the series. It has a benzylamine in the P1 position and a substituted phenyl group in the P4 position and a pyrazole carboxamide core. Compound 28 showed an oral bioavailability of 57%. The molecule was selective against other coagulation proteases however, not against trypsin and kallikrein. DPC-423 is in phase-I clinical trials. \(^{130}\)
Compound 29 (KFA-1411, Ki = 1.7 nM) has been found to be a highly potent and selective inhibitor of fXa. It has an amidine moiety both at the P1 and P4 positions. Compound 29 is under phase-I clinical trials\textsuperscript{131}.

Compound 30 (DX-9065a, Ki = 41 nM) is a naphthyl-amidine based fXa inhibitor, which inhibits free fXa, clot bound fXa, fXa in the prothrombinase complex and clot bound prothrombinase complex. The naphthyl-amidine moiety binds to the Asp\textsuperscript{189} in the S1 pocket and the pyrrolidine goes to the S4 pocket. However it is not orally bioavailable and its prodrug DU-176b is in phase-II clinical trials\textsuperscript{132}.
1.6.2 Limitations of Direct Inhibitors

The hirudins are associated with major bleeding episodes, which limits their use in patients refractory to UFH\textsuperscript{134, 135}. As hirudins are foreign peptides, they are potentially immunogenic and may cause life threatening disorders. Due to the high potency of hirudins for thrombin, there is no antidote for their use\textsuperscript{49}. As a result bivalirudin was developed however, it has a shorter half life (25 min) because thrombin cleaves its Arg\textsuperscript{3}-Pro\textsuperscript{4} bond. Argatroban, approved for patients having a high risk for HIT, has a shorter half life and is associated with bleeding risks. Moreover, a significant limitation for the direct inhibitors is the presence of the highly basic guanidine or the arginine moiety which doesn’t make them orally bioavailable. To counteract this problem ximelagatran was developed as a pro-drug of melagatran and was approved for clinical use in Europe. But US FDA rejected its application in 2004 due to concerns of hepatotoxicity\textsuperscript{136}. Recent studies also show that inhibitors which use a basic group as the P1 moiety may cause degranulation of mast cells resulting in histamine release\textsuperscript{136,137}. Therefore, a lot more challenges persist to develop a direct inhibitor which addresses all these issues.

1.7 Rationale for the work

With a goal of developing dual fXa and thrombin inhibitors, which can mimic heparins but are less polyanionic and more hydrophobic, our lab had designed sulfated dehydropolymers (DHP) from enzymatic coupling of 4-hydroxy cinnamic acids followed by the chemical sulfation of the free phenolic groups giving sulfated dehydropolymers. An example of such a molecule is CDSO3 (Figure 12). The average molecular weight of
CDSO3 was found to be 3320 and the range of oligomeric chain length varied from 5-13 monomeric units. CDSO3 was found to contain an average of 0.4 sulfate groups per monomer\textsuperscript{37}.

Inhibition studies on thrombin and fXa in presence and absence of AT indicated that CDSO3 inhibited both the proteases preferentially by a direct mechanism, i.e. in the absence of AT with an IC\textsubscript{50} value of 18 nM and 34 nM for thrombin and fXa, respectively. Michaelis – Menten kinetics indicated that K\textsubscript{m} was unaffected in the presence of these inhibitors, whereas V\textsubscript{max} decreased steadily. Thus, CDSO3 did not affect the formation of the Michaelis – Menten complex, while the forward rate constant of hydrolysis of the substrate (k\textsubscript{cat} or k\textsubscript{2}) is significantly reduced. These results suggest a non – competitive mechanism of enzyme inhibition, wherein the catalytic apparatus of the enzyme is disrupted by an allosteric mechanism\textsuperscript{36}. Moreover, in whole blood and plasma coagulation assays (PT and APPT), CDSO3 displayed anticoagulation properties similar to the clinically used anticoagulant enoxaparin\textsuperscript{138}.

Being a polyanionic molecule, CDSO3 can theoretically bind either to the exosite-I or exosite-II of thrombin, both of which are known to bind anionic molecules. Competitive binding studies with exosite-I and exosite-II ligands indicate that CDSO3 binds in or near the anion binding exosite-II\textsuperscript{36}. In a similar manner CDSO3 is expected to bind at the heparin binding exosite-II in fXa, as the exosite-I is cationic in character.
**Figure 12**  **Preparation of CDSO3.** The oxidative polymerization of caffeic acid by horseradish peroxidase and sulfation by triethyamine sulfur trioxide complex gives the heterogeneous CDSO3. The $\beta$-5 and $\beta$-$O$-4 linkages are highlighted.

CDSO3 has been characterized as of having $\beta$-5, $\beta$-$O$-4, $\beta$-$\beta$ and 5-5 inter-monomeric linkages where the $\beta$-5 and $\beta$-$O$-4 are predominant in its structure. In addition, CDSO3 is heterogeneous with variable numbers of sulfates, carboxylates, chiral centers and variable chain length. To study the role of these structural units require a large library of aromatic molecules with multiple sulfates, carboxylates and stereo-centers. Chemically, the structure of CDSO3 can be thought of as dihydrobenzofuran and phenoxypropanoic acid units, which could be successively, alternatively or randomly linked with each other. We hypothesized that delineation of key structural features of CDSO3 can be achieved by
accessing the importance of each contributing units, i.e., dihydrobenzofuran and phenoxypropanoic acid individually. Thus, we sought initially to synthesize a library of dihydrobenzofuran-like molecules and evaluate their inhibitory potential towards the two proteases – factor Xa and thrombin.

The β-5 monomer of CDSO3 is a dihydrobenzofuran moiety with a carboxylate at position-3, sulfate at position-7 and two stereo-centers at C-2 and C-3. With a goal of simplifying the structure, we removed the chiral-centers and introduced aromaticity to arrive at a benzofuran unit. Keeping an eye on the synthetic accessibility resulted in 5,6-dihydroxy-benzofuran-3-carboxylic acid ethyl ester (1E, Figure 13).
Figure 13  The rationale behind synthesis of monomer 1E

```
  dihydro-benzofuran          phenoxy-propanoic acid

  CDSO3
  R= H, OH, OSO₃

  Simplify structure
  -Remove chirality

  Synthetically accessible
```

1E
Monomer 1E served as the starting point to differentially introduce multiple sulfate and carboxylate groups on the benzofuran scaffold so as to study the structure-activity relationship (SAR) (Figure 14). The hydroxyl groups can be sulfated to their O-sulfates and the ethyl ester can be hydrolyzed to the corresponding carboxylic acid. In compounds where only one phenolic sulfate is present, the other hydroxyl group was sought to be protected as a methoxy group. Also we sought to study the effect of carbon homologation at the C-3 position, wherein the distance between the C-3 carboxylic acid and the ring was to be varied by one carbon. In this manner, a small focused library of compounds was designed.
Figure 14  The SAR sought to be explored around compound 1E
2 Synthesis and Biological Evaluation of β-5 like Monomers

2.1 Synthesis of β-5 like monomers

2.1.1 Synthesis of ethyl ester 1E

The initial ethyl ester monomer 1E was synthesized by a laccase mediated oxidative coupling of catechol and ethyl-4-chloro acetoacetate in presence of the Lewis acid scandium triflate and SDS to solubilize the reactants in the aqueous buffer\textsuperscript{139}. 43% yield of compound 1E was obtained, which is comparable to the literature yield of 46%. The compound was characterized by both \textsuperscript{1}H and \textsuperscript{13}C NMR, where in the characteristic two aromatic protons of 1E were distinguished.

\[
\begin{align*}
\text{HO} & \quad \text{+} \quad \begin{array}{c} \text{O} \\ \text{Cl} \end{array} \\ \text{HO} & \quad \text{CH}_3 \quad \text{O} \\ \text{Et} & \quad \text{O} \\
\end{align*}
\]

2.1.2 Synthesis of 1A

The ethyl ester of 1E was sought to be hydrolyzed under traditional basic hydrolytic conditions in which either NaOH or LiOH in ethanol or methanol is used to expose the carboxylate group. However, these conditions gave a mixture of undesirable degradative
products as had been observed by $^1$H NMR (Table 1). It is possible that metal-catalyzed $o$-quinol oxidation to the corresponding $o$-quinone, which might have further facilitated the benzofuran ring opening, resulting in degradation.

Table 1  Reagents and conditions tried for synthesis of 1A

<table>
<thead>
<tr>
<th>Reagents and conditions</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH/EtOH, $60^\circ$C</td>
<td>Degradation</td>
</tr>
<tr>
<td>NaOH/EtOH, $0^\circ$C</td>
<td>Degradation</td>
</tr>
<tr>
<td>LiOH/MeOH, $0^\circ$C</td>
<td>Degradation</td>
</tr>
<tr>
<td>Na t-BuO, H$_2$O, DMSO</td>
<td>Degradation</td>
</tr>
</tbody>
</table>

Then an alternative synthetic plan was devised. The catechol was protected with dichlorodiphenylmethane followed by hydrolysis of the ethyl ester under basic hydrolytic conditions in one pot and isolation of the protected acid 8 in 63% overall yield. $^1$H NMR indicated the presence of the carboxylic acid proton at 12.97 δ and twelve aromatic protons in 7.36-7.56 δ suggesting the formation of 8. Then, under aqueous acidic conditions, which is described in literature for the removal of the diphenylmethane protecting group, the compound 1A was isolated in 69% yield.
2.1.3 Synthesis of 6ES

6ES was synthesized by a microwave based sulfation protocol developed in our lab that uses trimethylamine sulfur trioxide complex and triethylamine base in acetonitrile under microwave, followed by purification using size-exclusion chromatography\textsuperscript{140}. The trialkylamine salt of the sulfate was exchanged to the sodium salt using cation-exchange columns to give the corresponding sulfate 6ES in 90% yield. Characterization was performed by using $^1$H NMR wherein a characteristic downfield shift of the aromatic protons is observed when two sulfates are introduced in the product. These protons are seen at 7.42-7.73 $\delta$, whereas they resonate at 6.95-7.37 $\delta$ in the un-sulfated precursor 1E.
2.1.4 Synthesis of 6AS

Conversion of the ester into the corresponding acid worked with the anhydrous ester hydrolysis protocol previously used in our lab for compounds with sulfates. The method uses potassium t-butoxide in anhydrous DMSO having one equivalent of H₂O to hydrolyze the ester, followed by size-exclusion chromatography to purify the compound. Traditional hydrolytic conditions like NaOH in ethanol and HCl in ethanol resulted in the breakdown of the sulfate groups. Compound 6AS was obtained in 90% yield and was characterized by ¹H and ¹³C NMR, which showed the absence of an ester group, and mass spectrometry, which indicates the parent ion peak.
2.1.5 Synthesis of sulfates 4ES and 5ES

To protect either one of the phenolic groups as a methoxy group, the ethyl ester monomer 1E was treated with methyl iodide and cesium carbonate in DMF to give the regioisomers 2E and 3E in 53% and 30% yield respectively.

According to literature, the regioisomer 2E would be the product in the highest yield due to the increased acidity of the 6-hydroxy group when compared with the 5-hydroxy group. Interestingly, the Rf values of the regioisomers were different on silica, hence they could be purified using flash chromatography on silica. ¹H and ¹³C spectra for the compounds indicated an obvious difference in the ppm values of the isomers. A NOESY experiment, which correlates proton-proton through space coupling, was used to confirm the structure of 2E. The ¹H NMR spectrum of 2E shows peaks at 7.44, 6.95, 5.58, 4.48, 3.96, 2.75 and 1.46 δ. The NOESY spectrum shows cross-peaks between 7.44 and 1.46 δ protons, suggesting that the aromatic proton ‘a’ is in close proximity to ester –CH₃- ‘g’ (Figure 15). This correlation was used to assign the aromatic resonances and confirm the compound 2E.
Sulfates were introduced onto the free phenolic groups of 2E and 3E using the base-catalyzed, microwave-assisted protocol described earlier to give 4ES and 5ES in yields of 94% and 92%, respectively. 1H NMR was used to confirm the compounds in which a shift of one of the aromatic protons that is adjacent to the introduced sulfate group is observed.
(7.97 δ in 4ES when compared to 7.44 δ in 2E and 7.13 δ in 5ES when compared to 7.40 δ in 3E).

2.1.6 Synthesis of 4AS and 5AS

The ethyl esters of 4ES and 5ES were hydrolyzed using the anhydrous ester hydrolysis protocol using potassium t-butoxide in DMSO. Compounds 4AS and 5AS were isolated in yields of 90% each and were confirmed by \( ^1H, ^{13}C \) NMR and mass spectroscopy. The absence of the ester protons and carbons in the \( ^1H \) and \( ^{13}C \) NMR was used to confirm the compounds. Moreover, mass spectrometry indicated the parent ion peak of these compounds.
2.1.7 Synthesis of 2A

The hydrolysis of the ethyl ester in compound 2E occurred uneventfully under basic hydrolytic conditions to give compound 2A in 90% yield. The compound was confirmed by $^1$H and $^{13}$C NMR wherein the ester protons and carbons were absent. Also, mass spectrometry indicated the parent ion peak of the product 2A.
2.1.8 Synthesis of 1XS

To make compound 1XS, a synthetic plan was devised in which the ethyl ester in 2E was reduced to an alcohol by a hydride delivering agent and then sulfates are introduced on both of the hydroxyl groups. The compound 1X was isolated on reduction of 1E with LAH in a yield of 84%. The structure of 1X was confirmed by $^1$H and $^{13}$C NMR wherein the characteristic benzylic protons at 4.65 δ are observed.

However, sulfation of 1X to obtain 1XS did not succeed as 1XS was found to be unstable in aqueous solution. The reason for this unstability is unclear at this time.
2.1.9 Synthesis of 8ES and 8AS

To study the effect of homologation by an additional carbon at the C-3 position, a synthetic strategy was envisioned such that disconnection of the CH$_2$-COOH bond of the target 8AS by the Wittig reaction leads to the benzofuran-3-aldehyde precursor 12 and methoxymethylene triphenylphosphorane (MMTPP). The synthesis of 12 would commence from the compound 2E by reduction of the ester to the aldehyde 12 by a sterically hindered hydride delivery agent, followed by protection of the phenolic group and Wittig with MMTPP to give the compound 11. The acidic hydrolysis of the Wittig derivative to give the aldehyde, followed by sulfation of the free phenolic group and oxidation of the aldehyde to the corresponding carboxylic acid would lead to 8ES and 8AS (Figure 16).
The reduction of the compound 2E to the aldehyde derivative 10 proved to be problematic as no reaction occurred with the common hydride reducing agent DIBAL. Hence for synthesizing compound 10, one more step was added in the sequence: reduction of the ester to the alcohol with LAH and oxidation of the alcohol to the aldehyde. The protection of the phenol to a silyl derivative was carried out before the reduction because of ease of handling the now protected derivative 9 (Figure 17).
The compound 2E was converted into the silyl derivative and the ester was reduced into the alcohol 9 using LAH. Compound 9 was obtained in 80% yield and confirmed by $^1$H NMR, which shows the presence of the -CH$_2$- protons at 4.69 δ. However, the oxidation of compound 9 to the aldehyde 10 proved to be difficult with common mild oxidants like chromate salts and Dess Martin periodinane, giving unsatisfactory yields. Experiments with TEMPO (2,2,6,6-Tetramethylpiperidine-1-oxyl) gave satisfactory yields to give the compound 10 (Table 2), which was characterized by $^1$H NMR. The characteristic aldehyde proton at 10.15 δ and the absence of the -CH$_2$- protons observed in 9 were used to confirm the product 10.
Table 2  
**Optimization of Oxidation Conditions**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>MnO₂</td>
<td>No conversion</td>
</tr>
<tr>
<td>PCC, DCM, rt</td>
<td>10%</td>
</tr>
<tr>
<td>PCC, DCM, µw</td>
<td>17%</td>
</tr>
<tr>
<td>DMP, DCM</td>
<td>40%</td>
</tr>
<tr>
<td>TEMPO, NCS, DCM</td>
<td>80%</td>
</tr>
</tbody>
</table>

The Wittig reaction proceeded with the aldehyde to obtain a compound 11 that was a mixture of both isomers. However the the acidic hydrolysis of 11 resulted in degradation.

2.1.10 Synthesis of 7ES

Due to the degradation of the Wittig derivative 11, an alternative approach was devised in which, instead of a one carbon homologation, a two carbon increase in the linkage between the ester and the aromatic scaffold was devised to give the new target 7ES. Again disconnection of the target 7ES at the CH₂-CH₂ bond by Wittig and subsequent hydrogenation of the olefin gives compound 10 and (carbethoxy) methylenetriphenyl phosphorane (CEMTPP) (Figure 18).
Figure 18  Retrosynthetic analysis of 7ES

Then, from compound 10, the scheme described above could be followed. The Wittig reaction occurred and the trans isomer was isolated from the reaction mixture in 96% yield. The double bond was reduced by hydrogen over palladium to give the crude 12. $^1$H NMR of the crude 12 indicated a mixture, which suggested that partial de-silylation had occurred. The silyl group was subsequently removed using TBAF to give the compound 7E. The structure of 7E was determined by $^1$H NMR, wherein the four protons of -CH$_2$-CH$_2$- were distinctly observed at 2.59 and 2.87 δ. The ester protons were also distinguished at 1.22 and 4.12 δ. Following, microwave-based sulfation gave the compound 7ES in 39% yield and was confirmed by $^1$H NMR wherein the downshifted aromatic proton which resonated at 7.50 δ when compared to 6.94 δ for the unsulfated precursor 7E.
2.1.11 Synthesis of 7AS

The ethyl ester present in 7ES was subsequently hydrolyzed by the anhydrous ester hydrolysis protocol to give the compound 7AS in 91% yield. The compound was confirmed by $^1$H NMR, wherein the absence of the ester protons confirmed the presence of 7AS. Moreover, mass spectrometry indicated the parent ion peak of 7AS.
2.1.12 Synthesis of 7A

Under basic hydrolytic conditions the ethyl ester present in compound 7E was hydrolyzed to compound 7A in 50% yield. The structure of 7A was confirmed by $^1$H, $^{13}$C NMR and mass spectrometry.

![Reaction Scheme]

2.2 Direct inhibition of thrombin and factor Xa (Work performed by Dr. Liang)

To determine whether the β-5-like monomers synthesized inhibit the coagulation proteases – fXa and thrombin, the residual enzymatic activity of the proteases was measured by following spectrophotometrically the initial rate of hydrolysis of the appropriate chromogenic substrate. Spectrozyme TH and S-2772 were used as substrates for thrombin and fXa. Relative residual protease activity (in %) at each concentration of the β-5 like monomer was calculated as a ratio of the slopes measured in the presence of the inhibitor to that in its absence. From the residual protease activity, the percentage of the enzyme inhibited was determined.

Out of the seventeen molecules screened, only seven of them (1A, 2A, 4AS, 5AS, 6AS, 7A and 7AS) exhibited any inhibition potential (Figure 19). The minimal concentration of these compounds necessary to display inhibition was approximately 400 μM. All the other derivatives in the library were found to be inactive below 4.3 mM.
Figure 19  Inhibition of Thrombin (upper plot) and Factor Xa (lower plot) by the β-5 like monomers 1A, 2A, 4AS, 5AS, 6AS, 7A and 7AS. The compounds were present at 2.9 mM (thrombin) or 2.6 mM (fXa), except for the asterisk (*) labeled cases where concentration was 4.3 mM. The standard error in the experiments was less than 10%.
2.3 **Mechanism of Factor Xa Inhibition** (Work performed by Dr. Liang)

To determine the mechanism of inhibition of factor Xa by the synthesized β-5-like monomers, the kinetics of S-2772 hydrolysis by factor Xa in the presence and the absence of the compound 5AS were determined. The initial rate of hydrolysis varied in a hyperbolic manner with the concentration of the substrate from which the Michaelis constant ($K_M$) and maximal velocity of the reaction ($V_{MAX}$) were derived (**Figure 20**). The $K_M$ and $V_{MAX}$ for S-2772 in the absence of compound 5AS was found to be $441±86 \, \mu M$ and $197±22 \, mAUmin^{-1} \mu M^{-1}$ respectively. In the presence of 5AS the values changed to $61±13 \, \mu M$ and $54±4 \, mAUmin^{-1} \mu M^{-1}$ respectively.

**Figure 20**  **Hydrolysis of substrate S-2772 by factor Xa in the presence (□) and absence (●) of 2.6 mM 5AS.** The solid lines represent the fits to Michaelis – Menten equation to derive $K_M$ and $V_{MAX}$ values
2.4 Discussion

Of the seventeen β-5 like monomers made, only 1A, 2A, 4AS, 5AS, 6AS, 7A and 7AS displayed inhibitory properties. This indicates that only selected monomeric units with appropriate substitutions carry the property of direct inhibition of thrombin and factor Xa. The minimal concentration required to display inhibition was 400 μM, which is fairly high. However interesting structure-activity relationships could be deduced from the study.

All ester containing monomers exhibited no inhibition, suggesting an important role for the –COO⁻ group. Whereas no molecule displayed more than 40% inhibition of thrombin at 2.9 mM, at least four out of seven (4AS, 5AS, 6AS and 7A) inhibit factor Xa better (>40%) at 2.6 mM. This suggests a slight preference of the benzofuran scaffold for inhibition of factor Xa.

Importantly, inhibitors 5AS and 6AS display 86% and 75% inhibition of factor Xa respectively, which is better than all the other inhibitors. This suggests a significant selectivity of recognition of these molecules by the enzyme. The common pharmacophore present in these two molecules is the 3-COO⁻ and the 6-OSO₃⁻ unit, which is absent in all the other molecules. Interestingly, the molecule 4AS, which is the regioisomer of 5AS, only inhibits factor Xa 42%, when compared to 5AS (86%), supporting a preferential recognition hypothesis. 5AS and 6AS more closely resemble the native CDSO3 dihydro-benzofuran monomer than molecules 7A and 7AS, which have a non-native extended COO⁻ bearing linker.

The decrease of the $K_M$ value from 441±86 μM to 61±13 μM and the $V_{MAX}$ from 197±22 mAUmin⁻¹μM⁻¹ to 54±4 mAUmin⁻¹μM⁻¹ for the hydrolysis of S-2772 by factor Xa in
presence of 5AS indicate that in presence of the inhibitor the binding of the substrate to the active site of the protease is significantly affected. Specifically the interaction of 5AS with factor Xa results in a 7-fold increase in affinity of the substrate towards the enzyme and a 3.6 fold decrease in the catalytic rate of hydrolysis. Thereby, the presence of 5AS introduces structural changes in the active site of factor Xa that alters the formation of the factor Xa-S-2772 Michaelis complex as well as induces dysfunction of the catalytic apparatus of the enzyme. Considering that the lead, i.e., CDSO3, binds to the exosite-II of thrombin, these results support the hypothesis that these β-5-like monomers also inhibit thrombin and factor Xa through exosite-II mediated allosteric dysfunction of the catalytic triad.

2.5 Experimental section

2.5.1 General methods

Reagents/chemicals were purchased from Sigma-Aldrich (unless specified otherwise) and used as supplied. Analytical thin layer chromatography (TLC) was performed using UNIPLATE™ silica gel GHLF 250 μm pre-coated plates (ANALTECH, Newark, DE) with a fluorescence indicator (254 nm). Column chromatography was performed using silica gel (60 Å, 200-400 mesh, Sigma-Aldrich). Microwave-assisted sulfation reactions were performed using CEM-Discover (Matthews, NC) synthesizer in sealed reaction vessels (7 ml). The reaction condition was set to ramp to 100°C at 50 W and was maintained by cooling with nitrogen at 45 psi.
Sephadex G10 chromatography and SP Sephadex-Na chromatography (cation exchange) were performed using flex columns (KIMBLE/KONTES, Vineland, NJ) of dimensions 170 × 1.5 cm and 75 × 1.5 cm. Cation exchange was performed using 30 fold excess of sodium ion equivalents. Samples were chromatographed at a controlled flow rate of 0.5 ml/min using water or 20% EtOH/H₂O as the eluent. Fractions of 5 mL each were collected and analyzed by RP-HPLC or capillary electrophoresis.

HPLC analysis was carried out on a Shimadzu chromatography system using Waters Atlantis dC18 column (5 µm, 4.6 × 250mm). The mobile phase consisted of acetonitrile – water mixture (2:8 v/v) run at a flow rate of 0.5 ml/min. Analysis was carried out using a UV-VIS detector at 254 nm.

Capillary electrophoresis was performed using a P/ACE MDQ™ Beckman capillary electrophoresis system (Fullerton, CA). The experiments were performed at a constant voltage of 8 kV and constant pressure of 0.1 psi. An uncoated fused silica capillary (I.D. 75 µm) of 31.2 cm total length with a 5 mm detection window at 21.0 cm length from the injection point was used. During electrophoresis, the capillary was held at a constant temperature of 25 °C. Sequential washes of 1 M HCl for 10 min, high purity water for 3 min, 1 M NaOH for 10 min and high purity water for 3 min at 20 psi were used to activate a new capillary. Before each electrophoretic run, the capillary was rinsed with the run buffer for 3 min at 20 psi. Run electrolytes containing 50 mM sodium phosphate buffer, pH 3.0. Sample solution in deionized water was injected at the cathodic end using 0.5 psi pressure for 5 s. Electrophoresis was monitored at the anodic end by following absorbance at 254 nm.
$^1$H NMR and $^{13}$C NMR were recorded on Varian Mercury-300 MHz spectrometer in CDCl$_3$, DMSO-d$_6$, CD$_3$OD, acetone-d$_6$, or D$_2$O. All signals are reported in ppm with the residual chloroform, DMSO, acetone, and H$_2$O signals at 7.26, 2.50, 3.31, 2.05, and 4.79, respectively, as standards. The data is reported as: chemical shifts (ppm), multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), coupling constant(s) (Hz), and integration. ESI mass spectra were recorded using a Micromass ZMD 4000 single quadrupole spectrometer. Samples were dissolved in 5% formic acid in methanol and infused at a rate of 10 μL/min. Mass scans were obtained in the range of 100-900 amu at a scan rate of 400 amu/s. Ionization conditions were optimized for each compound to maximize ionization of the parent ion. The capillary voltage was varied between 3.0 and 5.0 V, while the cone voltage ranged from 20 to 85 V. For all experiments the extractor voltage was set to 3.0, the Rf lens voltage to 0.1 V, the source block temperature to 100$^0$C and the desolvation temperature to 150$^0$C.

Proteins — Human coagulation factors Xa and IIa ($\alpha$-thrombin) were purchased from Haematologic Technologies (Essex Junction, VT). Stock solutions of proteins were prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl and 2.5 mM CaCl$_2$ (AT and thrombin) or 5 mM MES buffer, pH 6.0 (factor Xa). Chromogenic substrates Spectrozyme TH was purchased from American Diagnostica (Greenwich, CT), while substrate Chromogenix S-2772 was from DiaPharma group, Inc (West Chester, Ohio).
2.5.2 Synthesis of 1E

To a solution of catechol 1 (550 mg, 5 mmol, 1.0 equiv) in sodium phosphate buffer (150 ml, 0.1 M, pH 7) were added laccase (25 mg, 500 U), chloro ethyl acetoacetate 2 (Alfa Aesar) (1.385 ml, 10 mmol, 2.0 equiv), scandium triflate (492 mg, 1 mmol, 0.2 equiv), and sodium dodecyl sulfate (Fischer Biotech) (288.38 mg, 1 mmol, 0.2 equiv). The reaction mixture was vigorously stirred. After 4 hours the reaction mixture was acidified to pH 2 using HCl and filtered on celite. The residue was dissolved in ethyl acetate, dried using an. Na₂SO₄ and concentrated to a powder, which was purified by flash chromatography on silica gel (30% EtOAc in hexanes) to give 1E (510 mg, 2.16 mmol, 43%). ¹H NMR (acetone-d₆, 300 MHz) δ 1.36 (t, J = 3 Hz, 3H, CH₃), 2.63 (s, 3H, CH₃), 4.31 (q, J = 6 Hz, 2H, CH₂), 6.95 (s, 1H, Ar-H), 7.37 (s, 1H, Ar-H), 7.99 (s, 2H, OH) (Figure 21). ¹³C NMR (CDCl₃) δ 14.79, 14.96, 60.54, 98.53, 106.62, 108.83, 117.56, 144.09, 144.92, 147.79, 161.79, 164.48 (Figure 22).

2.5.3 Synthesis of 1A

To 1E (100 mg, 0.423 mmol, 1.0 equiv) in a two necked flask fitted with a reflux condenser was added dichlorodiphenyl methane (0.08 ml, 0.423 mmol, 1.0 equiv) under nitrogen. The reaction mixture was heated to 180°C. After 30 minutes the reaction mixture was allowed to cool to room temperature and ethanol (4.0 ml) and sodium hydroxide (0.8 ml from 2N soln) were added, and the mixture was heated to 80°C. After 16 hours the reaction mixture was allowed to cool to room temperature and quenched with 2N HCl and extracted with ether and ethyl acetate. The organics were dried (Na₂SO₄), concentrated and purified using flash chromatography on silica gel (20-90% EtOAc in hexanes) to give 8.
(100 mg, 0.27 mmol, 63%). $^1$H NMR (DMSO-d$_6$, 300 MHz) δ 2.63 (s, 3H, CH$_3$), 7.36-7.56 (m, 12H), 12.97 (s, 1H, OH) (Figure 23).

To 8 (78.0 mg, 0.209 mmol, 1.0 equiv) in a two-necked flask fitted with a reflux condenser was added 80% AcOH/H$_2$O (3 ml) and the mixture was refluxed. After 16 hours the mixture was cooled to room temperature and extracted with water and ethyl acetate. The organic extract was dried (Na$_2$SO$_4$), concentrated in vacuo and purified using flash chromatography on silica gel (10-60% EtOAc in hexanes) to give 1A (30 mg, 0.144 mmol, 69%). $^1$H NMR (acetone-d$_6$, 300 MHz) δ 2.67 (s, 3H, CH$_3$), 6.97 (s, 1H, Ar-H), 7.41 (s, 1H, Ar-H) (Figure 24). $^{13}$C NMR 13.63, 97.64, 106.38, 108.91, 118.62, 143.11, 144.11, 148.17, 161.89, 164.93 (Figure 25). ESI (-ve) m/z calcd for C$_{10}$H$_8$O$_5$ [(M-H)$^-$] 207.04, found 206.88 (Figure 26). Capillary electropherogram (Figure 57B).
Figure 21 \( ^1\text{H NMR of compound 1E} \)
Figure 22  $^{13}$C NMR of compound 1E
Figure 23  $^1$H NMR of compound 8
Figure 24  $^1$H NMR of compound 1A
Figure 25  $^{13}$C NMR of compound 1A
Figure 26  ESI mass spectra for 1A
2.5.4 Synthesis of 6ES

To a solution of 1E (30 mg, 0.127 mmol, 1.0 equiv) in acetonitrile (0.8 ml) in a microwave tube were added triethylamine (0.31 ml, 2.286 mmol, 18.0 equiv) and trimethylamine-sulfur trioxide complex (282.5 mg, 2.03 mmol, 16.0 equiv) under nitrogen. The mixture was exposed to microwaves (50 W, 30 min, 100°C). The process was repeated, the resulting mixture was concentrated in vacuo and 2 ml of water was added with stirring. The mixture was loaded onto a Sephadex G-10 column and was chromatographed with 20% EtOH-water as eluent. Fractions were monitored using reverse phase HPLC, pooled according to similarity of migration profiles, concentrated and reloaded onto a SP Sephadex C-25 column. Appropriate fractions were pooled and lyophilized to give 6ES (101 mg, 0.23 mmol, 90%). 1H NMR (D2O, 300 MHz) δ 1.23 (t, J =6 Hz, 3H, CH3), 2.34 (s, 3H, CH3), 4.13 (q, J =9 Hz, 2H, CH2), 7.42 (s, 1H, Ar-H), 7.73 (s, 1H, Ar-H) (Figure 27).

2.5.5 Synthesis of 6AS

6ES (50 mg, 0.114 mmol, 1.0 equiv) was dissolved in DMSO (1.1 ml). To this solution were added potassium tert-butoxide (TCI AMERICA) (38.4 mg, 0.342 mmol, 3.0 equiv) and H2O (3.1 µl, 0.171 mmol, 1.5 equiv) under N2. The reaction was monitored on reverse phase HPLC and after 3 hours dibasic sodium phosphate (161.8 mg, 1.14 mmol, 10.0 equiv) was added and stirred for an additional 30 minutes. The reaction mixture was loaded onto a Sephadex G-10 column and eluted with deionized water. The fractions containing the product were identified using a reverse phase HPLC, lyophilized to obtain
6AS (44.51 mg, 0.10 mmol, 90%). \(^1\)H NMR (D\(_2\)O, 300 MHz) \(\delta\) 7.46 (s, 1H, Ar-H), 7.77 (s, 1H, Ar-H), 2.54 (s, 3H, CH\(_3\)) (Figure 28). \(^{13}\)C NMR 13.56, 105.84, 114.05, 114.87, 125.63, 139.94, 140.16, 150.13, 162.27, 172.10 (Figure 29). ESI (+ve) m/z calcd for C\(_{10}\)H\(_5\)Na\(_3\)O\(_{11}\)S\(_2\) [(M+H)\(^+\)] 435.24, found 435.20 (Figure 30). Capillary electropherogram (Figure 57A).

### 2.5.6 Synthesis of 4ES and 5ES

To a solution of 1E (340 mg, 1.44 mmol, 1.0 equiv) in DMF (12 ml) were added cesium carbonate (234 mg, 0.72 mmol, 0.5 equiv) and methyl iodide (0.45 ml, 7.20 mmol, 5.0 equiv) under N\(_2\). The reaction mixture was stirred for 15 hours, was quenched with 2N HCl and extracted into EtOAc. The organic extract was dried using an Na\(_2\)SO\(_4\), evaporated and the residue purified on silica gel (10-25% EtOAc in hexanes) to give 2E (192 mg, 0.77 mmol, 53%) and 3E (108 mg, 0.43 mmol, 30%). 2E; \(^1\)H NMR (CDCl\(_3\), 300 MHz) \(\delta\) 1.46 (t, \(J=\)6 Hz, 3H, CH\(_3\)), 2.75 (s, 3H, CH\(_3\)), 3.96 (s, 3H, CH\(_3\)), 4.39 (q, \(J=\)6 Hz, 2H, CH\(_2\)), 5.58 (s, 1H, OH) 6.95 (s, 1H, Ar-H), 7.44 (s, 1H, Ar-H) (Figure 31). ESI (+ve) m/z calcd for C\(_{13}\)H\(_{14}\)O\(_5\) [(M+H)\(^+\)] 251.25, found 251.17; 3E; \(^1\)H NMR (CDCl\(_3\), 300 MHz) \(\delta\) 1.42 (t, \(J=\)6 Hz, 3H, CH\(_3\)), 2.69 (s, 3H, CH\(_3\)), 3.94 (s, 3H, CH\(_3\)), 4.40 (q, \(J=\)9 Hz, 2H, CH\(_2\)), 5.83 (s, 1H, OH), 7.00 (s, 1H, Ar-H), 7.40 (s, 1H, Ar-H) (Figure 32), NOESY for 2E (Figure 33).

To a solution of 2E (34 mg, 0.136 mmol, 1.0 equiv) in acetonitrile (0.45 ml) in a microwave tube were added triethylamine (0.18 ml, 1.3 mmol, 10 equiv) and
trimethylamine-sulfur trioxide complex (151.4 mg, 1.08 mmol, 8.0 equiv) under nitrogen. The reaction mixture was exposed to microwaves (50 W, 30 min, 100°C). The mixture was concentrated in vacuo and purified by flash chromatography on silica gel (0-20% MeOH in DCM) and dried to give the triethylamine-sulfate salt. This was dissolved in 20% EtOH-water and loaded onto a Sephadex C-25 column. Appropriate fractions were pooled and lyophilized to give **4ES** (45 mg, 0.13 mmol, 94%). $^1$H NMR (DMSO d$_6$, 300 MHz) δ 1.35 (t, $J$ = 6 Hz, 3H, CH$_3$), 2.52 (s, 3H, CH$_3$), 3.78 (s, 3H, CH$_3$), 4.32 (q, $J$ = 6 Hz, 2H, CH$_2$), 7.25 (s, 1H, Ar-H), 7.97 (s, 1H, Ar-H) (**Figure 34**).

To a solution of **3E** (50 mg, 0.2 mmol, 1.0 equiv) in acetonitrile (0.7 ml) in a microwave tube were added triethylamine (0.27 ml, 2.0 mmol, 10 equiv) and trimethylamine-sulfur trioxide complex (222.6 mg, 1.6 mmol, 8.0 equiv) under nitrogen. The reaction mixture was exposed to microwave energy (50 W, 30 min, 100°C). The mixture was concentrated in vacuo and purified by flash chromatography on silica gel (0-20% MeOH in CH$_2$Cl$_2$) and dried to give the triethylamine-sulfate salt. This was dissolved in 20% EtOH-water and loaded onto a Sephadex C-25 column. Appropriate fractions were pooled and lyophilized to give **5ES** (65 mg, 0.18 mmol, 92%). $^1$H NMR (D$_2$O, 300 MHz) δ 1.13 (t, $J$ = 6 Hz, 3H, CH$_3$), 2.18 (s, 3H, CH$_3$), 3.56 (s, 3H, CH$_3$), 3.94 (q, $J$ = 9 Hz, 2H, CH$_2$), 6.63 (s, 1H, Ar-H), 7.13 (s, 1H, Ar-H) (**Figure 35**).
Figure 27  $^1$H NMR of 6ES
Figure 28  $^1$H NMR of 6AS
Figure 29  $^{13}$C NMR of 6AS
Figure 30  ESI mass spectra of 6AS

TH1SA in 50% 5% FA in MEOH 3.36 57 3 100/150 +ve
JU 22.1 (1.017)
Figure 31  $^1$H NMR of 2E
Figure 32  $^1$H NMR of 3E
Figure 33  NOESY for 2E with distinctive correlation’s in yellow
Figure 34 \( ^1H \text{NMR of 4ES} \)
Figure 35  $^1$H NMR of 5ES
2.5.7 Synthesis of 4AS and 5AS

**4ES** (45 mg, 0.123 mmol, 1.0 equiv) was dissolved in DMSO (1.3 ml). To this solution were added potassium tert-butoxide (TCI AMERICA) (45.1 mg, 0.369 mmol, 3.0 equiv) and H$_2$O (3.3 μl, 0.184 mmol, 1.5 equiv) under nitrogen. The reaction was monitored on reverse phase HPLC and after 3 hours dibasic sodium phosphate (174.6 mg, 1.23 mmol, 10.0 equiv) was added and stirred for an additional 30 minutes. The reaction mixture was loaded onto a Sephadex G-10 column and eluted with deionized water. The fractions with the product were identified using reverse phase HPLC and lyophilized to obtain **4AS** (38.3 mg, 0.11 mmol, 90%). $^1$H NMR (D$_2$O, 300 MHz) $\delta$ 2.45 (s, 3H, CH$_3$), 3.73 (s, 3H, CH$_3$), 7.04 (s, 1H, Ar-H), 7.61 (s, 1H, Ar-H) (Figure 36). $^{13}$C NMR 13.37, 56.44, 96.32, 113.81, 114.75, 120.20, 137.10, 149.21, 151.28, 160.18, 172.46 (Figure 37). ESI (+ve) m/z calcd for C$_{11}$H$_8$Na$_2$O$_8$S [(M+H)$^+$] 346.97, found 347.15 (Figure 38). Capillary electropherogram (Figure 57A)

**5ES** (73 mg, 0.199 mmol, 1.0 equiv) was dissolved in DMSO (2.0 ml). To this solution were added potassium tert-butoxide (TCI AMERICA) (73 mg, 0.598 mmol, 3.0 equiv) and H$_2$O (5.3 μl, 0.298 mmol, 1.5 equiv) under nitrogen. The reaction was monitored on reverse phase HPLC and after 3 hours dibasic sodium phosphate (282.5 mg, 1.99 mmol, 10.0 equiv) was added and stirred for an additional 30 minutes. The reaction mixture was loaded onto a Sephadex G-10 column and eluted with deionized water. The fractions with the product were identified using reverse phase HPLC and lyophilized to obtain **5AS** (62 mg, 0.18 mmol, 90%). $^1$H NMR (D$_2$O, 300 MHz) $\delta$ 2.50 (s, 3H, CH$_3$), 3.78 (s, 3H, CH$_3$), 7.32 (s, 1H, Ar-H), 7.39 (s, 1H, Ar-H) (Figure 39). $^{13}$C NMR 13.60, 56.67, 104.48, 114.75, 120.20, 137.10, 149.21, 151.28, 160.18, 172.46 (Figure 37). ESI (+ve) m/z calcd for C$_{11}$H$_8$Na$_2$O$_8$S [(M+H)$^+$] 346.97, found 347.15 (Figure 38). Capillary electropherogram (Figure 57A)
105.74, 113.99, 125.63, 137.24, 146.75, 148.54, 161.61, 172.44 (Figure 40). ESI (+ve) m/z calcd for C₁₁H₈Na₂O₈S [(M+H)⁺] 346.97, found 347.15 (Figure 41). Capillary electropherogram (Figure 57A)

**2.5.8 Synthesis of 2A**

To a solution of 2E (50 mg, 0.2 mmol, 1.0 equiv) in EtOH (2 ml) in a 2-necked flask fitted with a condenser was added 2N NaOH (0.3 ml) and the mixture was heated to 80°C. After 16 hours, the completion of the reaction indicated by TLC, the reaction mixture was cooled to room temperature and quenched, acidified with 2N HCl and extracted with ethyl acetate. The organic extract was dried (an. Na₂SO₄), concentrated *in vacuo* and purified using flash chromatography on silica gel (50% EtOAc in hexanes) to give 2A (40 mg, 0.18 mmol, 90%). ¹H NMR (CH₃OD, 300 MHz) δ 2.66 (s, 3H, CH₃), 3.88 (s, 3H, CH₃), 7.05 (s, 1H, Ar-H), 7.32 (s, 1H, Ar-H) (Figure 42). ¹³C NMR 13.16, 55.51, 94.55, 106.08, 108.83, 119.12, 144.00, 146.66, 148.03, 162.37, 166.49 (Figure 43). ESI (-ve) m/z calcd for C₁₁H₁₀O₃ [(M-H)⁻] 221.05, found 220.98 (Figure 44). Capillary electropherogram (Figure 57B)
Figure 36  $^1H$ NMR for 4AS

![NMR spectrum image]
**Figure 37**  $^{13}$C NMR for 4AS

![NMR spectrum for 4AS](image-url)

- **Residual Spin Count:** 1.000 sec
- **Pulse Delay:** 1.000 sec
- **Pulse Width:** 90 degrees
- **Number of Scans:** 64
- **Decoupling Off:**
- **Sample:** 3.032 g
- **Diluent:** DMSO-d$_6$
- **Solvent:** CDCl$_3$
- **Sweep:** 120.00 MHz
- **Field Strength:** 9.4 T
- **Temperature:** 298 K
- **Proton Decoupling:** 
- **Data Processing:**
- **FT Time:** 11.1 sec
- **Total Time:** 8 hr, 2 min, 49 sec
Figure 38   ESI mss spectra for 4AS

092908_JENSON_DESAI_BSMSC 232 (4.399) Cm (212:273-2:95)
Figure 39  $^1$H NMR for 5AS

![NMR spectrum of 5AS]

**Chemical Structure:**
- **a**: Hydroxyl group (OH)
- **b**: Sulfate group (SO$_4^{2-}$)
- **c**: Sodium ion (Na$^+$)
- **d**: Carbonyl group (C=O)

**NMR Data:**
- **Resonance at 5.00 ppm**: 
  - **a**: Water signal
- **Resonance at 3.53 ppm**: 
  - **b**: Sulfate group
- **Resonance at 3.51 ppm**: 
  - **c**: Sodium ion
- **Other resonances**
  - **d**: Carbonyl group

**Conditions:**
- **Solute concentration**: 1.000 g/L
- **Sample volume**: 4.00 mL
- **Temperature**: 298 K
- **Spectrometer frequency**: 300.2 MHz
- **Solvent**: DMSO-d$_6$
Figure 40  $^{13}$C NMR for 5AS

[Image of a chemical structure with a proton NMR spectrum]
Figure 41  ESI mass spectra of 5AS
Figure 42  $^1$H NMR for 2A
Figure 43  $^{13}$C NMR for 2A

[Chemical structure and NMR spectrum image]

Prime Sequence: 62pm
Sweep: 40000 Hz
Power: 25 Hz
Shift: 0.0 ppm
Frequency: 123.26 ppm
Proton: 2H, 1H, 3H
Power: 25 Hz
Shift: 0.0 ppm
Frequency: 123.26 ppm
Proton: 2H, 1H, 3H
Total Line Width: 3.6 Hz
Figure 44  ESI mass spectra of 2A
2.5.9 Synthesis of 1X

To a solution of LiAlH₄ (15.18 mg, 0.4 mmol, 5.0 equiv) in THF (0.8 ml) was added compound 2E (20 mg, 0.08 mmol, 1.0 equiv) in THF (0.8 ml) drop wise, slowly under nitrogen. The reaction mixture was stirred for ten hours after which 6 μl of H₂O, 6 μl of 15 % w/v NaOH and 18 μl of H₂O were added successively. Afterwards ethylacetate was added to the mixture and was stirred for 30 minutes. The crude was filtered over celite and dried (an. Na₂SO₄), concentrated in vacuo and purified on silica gel (0-40% EtOAc in hexanes) to obtain 1X (14 mg, 0.067 mmol, 84%, ) ¹H NMR (acetone-d₆, 300 MHz) δ 2.35 (s, 3H, CH₃), 3.88 (s, 3H, CH₃), 4.65 (s, 2H, CH₂), 7.02 (s, 1H, Ar-H), 7.05 (s, 1H, Ar-H) (Figure 45). ¹³C NMR 10.87, 54.01, 55.52, 94.41, 103.66, 114.74, 121.31, 142.87, 145.14, 147.50, 150.36 (Figure 46).

2.5.10 Synthesis of 7E and 7ES

To a solution of 2E (910 mg, 3.63 mmol, 1.0 equiv) in DMF (30 ml) was added imidazole (1.20 g, 18.15 mmol, 5.0 equiv) and TBDMSCl (3.2 g, 21.83 mmol, 6.0 equiv) and the mixture was stirred. After 16 hours the reaction mixture was diluted with water, extracted with diethyl ether and the organic extract was dried (an. Na₂SO₄), concentrated in vacuo and purified by flash chromatography on silica gel (0-15% EtOAc in hexanes) to obtain the silyl protected derivative (1.19 g, 3.26 mmol, 89%).

A two-necked flask was charged with a solution of lithium aluminum hydride (520 mg, 13.71 mmol, 5.0 equiv) in THF (27 ml). To this solution under an atmosphere of nitrogen was added in a dropwise manner (over a period of 20 minutes) a solution of the silyl
derivative (1.0 g, 2.74 mmol, 1.0 equiv) in THF (27 ml). The mixture was stirred for 16 hours. After the completion of the reaction H₂O (0.52 ml), 15% w/v aqueous NaOH (0.52 ml) and H₂O (1.56 ml) were sequentially added. Then, the reaction mixture was poured into diethyl ether and stirred for 30 minutes. The organic extract was filtered through celite, dried (an. Na₂SO₄), concentrated in vacuo and purified using flash chromatography on silica gel (10-30% EtOAc in hexanes) to give 9 (710 mg, 2.2 mmol, 80%). ¹H NMR (CDCl₃, 300 MHz) δ 0.14 (s, 6H, CH₃), 1.01 (s, 9H, CH₃), 2.41 (s, 3H, CH₃), 3.82 (s, 3H, CH₃), 4.69 (s, 2H, CH₂), 6.93 (s, 1H, Ar-H), 7.01 (s, 1H, Ar-H) (Figure 47).

To a solution of 9 (100 mg, 0.310 mmol, 1.0 equiv) in CH₂Cl₂ (3.1 ml) were added TEMPO (9.68 mg, 0.062 mmol, 0.2 equiv), tetrabutyl ammonium chloride (17.23 mg, 0.062 mmol, 0.2 equiv), NaHCO₃ solution (3.1 ml, 0.5 M) and N-chloro succinimide (124.18 mg, 0.93 mmol, 3.0 equiv). The reaction mixture was allowed to stir for 4 hours and after the completion of the reaction as indicated on TLC, the mixture was extracted with CH₂Cl₂ and water. The organic layer was dried (an. Na₂SO₄), concentrated in vacuo and purified using flash chromatography on silica gel (20% EtOAc in hexanes) to give 10 (80 mg, 0.25 mmol, 81%). ¹H NMR (CDCl₃, 300 MHz) δ 0.16 (s, 6H, CH₃), 1.01 (s, 9H, CH₃), 2.71 (s, 3H, CH₃), 3.84 (s, 3H, CH₃), 6.95 (s, 1H, Ar-H), 7.54 (s, 1H, Ar-H), 10.15 (s, 1H, CH) (Figure 48).

A two necked flask fitted with a reflux condenser was charged with a solution of 10 (120 mg, 0.374 mmol, 1.0 equiv) in benzene (2.5 ml). To this was added (carbethoxymethylene) triphenylphosphorane (196 mg, 0.562 mmol, 1.5 equiv) under an atmosphere of nitrogen.
The mixture was refluxed for 3.5 hours. Afterwards the reaction was brought to room temperature and was diluted with CH$_2$Cl$_2$. The mixture was concentrated in vacuo and purified by flash chromatography on silica gel (10% EtOAc in hexanes) to obtain the trans isomer (140 mg, 0.36 mmol, 96%). $^1$H NMR (CDCl$_3$, 300 MHz) $\delta$ 0.16 (s, 6H, CH$_3$), 1.03 (s, 9H, CH$_3$), 1.36 (t, $J$ = 6Hz, 3H, CH$_3$), 2.53 (s, 3H, CH$_3$), 3.84 (s, 3H, CH$_3$), 4.30 (q, $J$ =9Hz, 2H, CH$_2$), 6.36 (d, $J$=15Hz, 1H, CH), 6.95 (s, 1H, Ar-H), 7.18 (s, 1H, Ar-H), 7.77 (d, $J$ =18Hz, 1H, CH) (Figure 49).

A solution of the trans isomer (230 mg, 0.588 mmol, 1.0 equiv) in ethanol (6 ml) was hydrogenated in Parr shaker apparatus over 46 mg of 10% Pd/C at room temperature and at an initial pressure of 35 psig over a period of 4 hours. The catalyst was removed by filtration over celite and washed with ether and acetone. The solvents were concentrated under vacuo to obtain 260 mg of the crude compound, which NMR indicated partial de-silylation had occurred. Crude 12 was used for the next step without purification.

To a solution of the crude 12 (176 mg, 1.0 equiv) in THF (2.61 ml) was added tetrabutyl ammonium fluoride in THF (1 M in THF, 1.79 ml, 4.0 equiv) and stirred for a period of 4 hours. Afterwards the reaction was quenched with saturated ammonium chloride solution and extracted into EtOAc. The organic extract was concentrated in vacuo and purified using flash chromatography on silica gel to obtain 7E (130 mg, 0.47 mmol). $^1$H NMR (CDCl$_3$, 300 MHz) $\delta$ 1.22 (t, $J$ = 6Hz, 3H, CH$_3$), 2.36 (s, 3H, CH$_3$), 2.59 (t, $J$ = 6Hz, 2H, CH$_2$), 2.87 (t, $J$ = 9Hz, 2H, CH$_2$), 3.91 (s, 3H, CH$_3$), 4.12 (q, J = 6Hz, 2H, CH$_2$), 5.49 (s, 1H, OH), 6.93 (s, 1H, Ar-H), 6.94 (s, 1H, Ar-H) (Figure 50).
To a solution of 7E (34 mg, 0.122 mmol, 1.0 equiv) in acetonitrile (0.42 ml) in a microwave tube were added triethylamine (0.16 ml, 1.22 mmol, 10 equiv) and trimethylamine-sulfur trioxide complex (136.0 mg, 0.977 mmol, 8.0 equiv) under nitrogen. The reaction mixture was exposed to microwaves (50 W, 30 min, 100°C). The mixture was concentrated in vacuo and purified by flash chromatography on silica gel (0-10% MeOH in DCM) and dried to give the triethylamine-sulfate salt. This was dissolved in 20% EtOH-water and loaded onto a Sephadex C-25 column. Appropriate fractions were pooled and lyophilized to give 7ES (18 mg, 0.047 mmol, 39%). $^1$H NMR (D$_2$O, 300 MHz) δ 1.18 (t, $J = 6$Hz, 3H, CH$_3$), 2.34 (s, 3H, CH$_3$), 2.62 (t, $J = 9$Hz, 2H, CH$_2$), 2.87 (t, $J = 9$Hz, 2H, CH$_2$), 3.85 (s, 3H, CH$_3$), 4.08 (q, $J = 9$Hz, 2H, CH$_2$), 7.06 (s, 1H, Ar-H), 7.50 (s, 1H, Ar-H) (Figure 51).

2.5.11 Synthesis of 7AS

7ES (18 mg, 0.047 mmol, 1.0 equiv) was dissolved in DMSO (0.5 ml). To this solution were added potassium tert-butoxide (TCI AMERICA) (15.93 mg, 0.142 mmol, 3.0 equiv) and H$_2$O (1.5 μl, 0.0705 mmol, 1.5 equiv) under nitrogen. The reaction was monitored on reverse phase HPLC and after 3 hours dibasic sodium phosphate (66.72 mg, 0.47 mmol, 10.0 equiv) was added and stirred for an additional 30 minutes. The reaction mixture was loaded onto a Sephadex G-10 column and eluted with deionized water. The fractions with the product were identified using reverse phase HPLC, lyophilized to obtain 7AS (16 mg, 0.043 mmol, 91%). $^1$H NMR (D$_2$O, 300 MHz) δ 2.12 (s, 3H, CH$_3$), 2.22 (t, $J = 6$Hz, 2H, CH$_2$), 2.61 (t, $J = 6$Hz, 2H, CH$_2$), 3.66 (s, 3H, CH$_3$), 6.94 (s, 1H, Ar-H), 7.23 (s, 1H, Ar-H)
(Figure 52). ESI (+ve) m/z calcd for C_{13}H_{12}Na_{2}O_{8}S [(M+H)^+] 375.00, found 375.17 (Figure 53). Capillary electropherogram (Figure 57A).

2.5.12 Synthesis of 7A

To a solution of 7E (27 mg, 0.097 mmol, 1.0 equiv) in EtOH (0.9 ml) in a 2-necked flask fitted with a condenser was added sodium hydroxide (0.15 ml from 2N soln) and the mixture was heated to 80°C. After 6 hours, the completion of the reaction indicated by TLC, the reaction mixture was cooled to room temperature and quenched, acidified with 2N HCl and extracted with ethyl acetate. The organic extract was dried (an. Na_{2}SO_{4}) and concentrated in vacuo and purified using flash chromatography on silica gel (5% MeOH in EtOAc) to give 7A (12 mg, 0.048 mmol, 50%). \(^1\)H NMR (CH_{3}OD, 300 MHz) δ 2.32 (s, 3H, CH_{3}), 2.55 (t, J = 6Hz, 2H, CH_{2}), 2.85 (t, J = 6Hz, 2H, CH_{2}), 3.86 (s, 3H, CH_{3}), 6.85 (s, 1H, Ar-H), 6.98 (s, 1H, Ar-H) (Figure 54). \(^13\)C NMR 10.66, 19.10, 33.85, 55.59, 94.86, 103.29, 112.66, 121.69, 142.80, 145.83, 148.28, 150.00 (note: one carbon missing probably because of an overlap) (Figure 55). ESI (-ve) m/z calcd for C_{13}H_{14}O_{5} [(M-H)^-] 249.08, found 249.00 (Figure 56). Capillary electropherogram (Figure 57B).
Figure 45  $^1$H NMR of 1X

NMR Spectroscopy: 
Solvent: Acetone
Spectrometer: Varian

Proton NMR spectral data:
Chemical shifts (ppm): 3.08, 2.74, 3.70, 2.80
Resonance assignments:
A: 3.08; B: 2.74; C: 3.70; D: 2.80
Figure 46  

$^{13}$C NMR of 1X
Figure 47  $^1$H NMR of compound 9
Figure 48  
$^1$H NMR of compound 10
Figure 49  $^1$H NMR of compound 11
Figure 50  $^1$H NMR of compound 7E
Figure 51  $^1$H NMR of compound 7ES
Figure 52  $^1$H NMR of compound 7AS

![NMR Spectrogram]

- Proton frequency: 1.063 Hz
- Inversion angle: 45°
- Flip angle: 90°
- Number of scans: 15
- Time per sweep: 0.115047 MHz
- Data points: 2048
- Total time: 3 min. 6 sec.
Figure 53  ESI mass spectra of 7AS
Figure 54  $^1$H NMR of compound 7A
Figure 55  $^{13}$C NMR of compound 7A
Figure 56  ESI mass spectra of 7A
Figure 57A  Capillary electropherogram’s for 4AS, 5AS, 6AS and 7AS

Figure 57B  Capillary electropherogram’s for 1A, 2A and 7A
2.5.13 Direct Inhibition of factor Xa and thrombin (Work performed by Dr. Liang)

Direct inhibition of thrombin and factor Xa by the benzofuran derivatives was determined using chromogenic substrate hydrolysis assays. The buffer used in these experiments was 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM CaCl₂, and 0.1% polyethylene glycol (PEG) 8000. In these assays, 3 to 134 μL of a stock solution of benzofuran derivatives in DMSO was diluted with appropriate volume of the buffer in PEG 20,000-coated polystyrene cuvettes to give 0.4 to 4.3 mM inhibitor concentration in a final incubation volume of 1000 μL. Then 5 μL of the stock enzyme solution was added to give 4.8 nM thrombin or 1.1 nM factor Xa, followed by 20 μL of 1 mM Spectrozyme TH or 100 μL of 4.1 mM S-2772. The residual enzyme activity was determined from the initial rate of increase in absorbance at 405 nm. Control experiments with DMSO without the benzofuran derivative were performed to assess the loss of enzyme activity. Relative residual proteinase activity (in %) at each concentration of the benzofuran derivative was calculated as a ratio of the slopes measured in the presence of the inhibitor to that in its absence.

2.5.14 Michaelis-Menten Kinetics of S-2772 Hydrolysis by Factor Xa in the Presence of 5AS (Work performed by Dr. Liang)

The initial rate of S-2772 hydrolysis by 1.1 nM factor Xa was monitored from the linear increase in absorbance at 405 nm. The initial rate was measured as a function of various concentrations of substrate S-2772 (20.5–492.0 μM) in the presence of 2.6 mM 5AS in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM CaCl₂, and 0.1% PEG8000 at 25 °C. The data were fitted by the Michaelis-Menten equation to determine $K_M$ and $V_{MAX}$. 
CHAPTER 3 Virtual screening of the synthesized β-5 like Compounds

3.1 Rationale

Designing non-sugar heparin mimics that recognize targets like antithrombin, thrombin and fXa is a major challenge. Non-sugar scaffolds bearing multiple sulfate and carboxylate groups of high chain length, e.g., of the size of heparin, are difficult to model. Modeling such scaffolds is fraught with problems of specificity because of the surface exposed, shallow heparin-binding sites on proteins with high density of positively charged residues, which induces recognition of practically any collection of negative charges in these molecules. Our lab had earlier designed the dual filter strategy for ligand docking using GOLD, which consists of an “affinity” and “specificity” filter. This strategy successfully reproduced the binding geometry of a crystallized sequence–specific heparin pentasaccharide to within 2.5 Å for antithrombin-heparin interaction\textsuperscript{143}.

Results described in chapter 2 show that selected β-5 like molecules inhibit fXa. Moreover, a slight preference for the benzofuran scaffold was observed for inhibition of fXa when compared to thrombin.

We sought to explore if the GOLD-based protocol of ligand docking, which was developed for antithrombin-heparin interaction would help in our case, i.e., for explaining the observed inhibition profile of the synthesized β-5 like molecules against factor Xa.
Furthermore, kinetic studies showed that these molecules inhibit fXa by an allosteric mechanism of action. Based on the structural domains present in fXa, the synthesized β-5 like molecules would bind to the exosite-II in fXa. Therefore, it was an important point to elucidate how these molecules interact with the exosite-II in fXa, and which residues of the exosite-II are involved in binding.

3.2 Coordinates for the synthesized β-5 like molecules

The seven synthesized molecules which were observed to inhibit fXa; 1A, 2A, 4AS, 5AS, 6AS and 7AS, were drawn in SYBYL. The atom type of sulfur and oxygen atoms in SO₃ was modified to S.o2 and O.co2, and the atom type for oxygen and carbon in carboxylates was modified to O.co2 and O.Co2 respectively. The bond type between these atoms was modified to aromatic. Each of the compounds in the library was minimized using the Tripos force field with Gasteiger-Hückel charges, a fixed dielectric constant of 80, and a nonbonded cutoff radius of 8 Å. Minimization was carried out for a maximum of 10000 iterations subject to a termination gradient of 0.05 kcal/(mol.A).

3.3 Protein coordinates

The exosite in fXa is a surface-exposed, shallow, solvent exposed surface lined by basic residues. Therefore, to define the binding site on fXa in GOLD, wherein the genetic algorithm (GA) can sample all possible conformations of the ligand, the cavity detection algorithm in GOLD cannot be used. This is because the exosite-II is not a hydrophobic cavity where a ligand can be docked. Therefore, the binding site must be defined using a
ligand in the bound site which implies it is crystallized with the protein. However, no crystal structure has been deposited in the PDB with a ligand crystallized on the exosite-II of fXa.

Yet, seven of the basic residues present on the exosite-II in fXa are conserved in thrombin also. These residues are Arg$^{93}$, Lys$^{96}$, Arg$^{125}$, Arg$^{165}$, Lys$^{169}$, Lys$^{236}$ and Arg$^{240}$. Additionally, the crystal structure of thrombin in complex with heparin octasaccharide was available (PDB code 1XMN). Thus, fXa (PDB code 1HCG) and thrombin were aligned by homology. The seven basic residues in the exosite-II of thrombin were spatially found to be similar in orientation in fXa (Figure 58).
The seven basic exosite-II residues in fXa are conserved in thrombin. The blue ribbon is fXa and the magenta is thrombin.

The next step in defining the binding site in GOLD was to place the heparin hexasaccharide crystallized with thrombin onto the same site of fXa. Thus, the coordinates of the heparin hexasaccharide were extracted and placed on the aligned structure of fXa.
and the binding site in GOLD was defined as 16 Å from every atom in the hexasaccharide (Figure 59). This definition of the binding site covers all important known heparin binding residues in fXa.

**Figure 59**  The binding site defined on fXa in GOLD. The binding site is shown as a solid surface colored by lipophilic potential. Green represents hydrophilic surface and red shows hydrophobic surface.
3.4 Docking of β-5 like synthesized molecules onto fXa

The docking of seven synthesized compound, onto fXa was done by GOLD 4.0. GOLD is a soft docking method that allows a small degree of interpenetration, or van der waals overlap of ligand and protein atoms. GOLD also optimizes the positions of hydrogen-bond donating atoms on Ser, Thr, Tyr and Lys residues as part of the docking process\textsuperscript{146}. For docking, the specificity screen developed in our lab was used. This involves screening of all possible sequences using 300000 genetic algorithm (GA) iterations, which is collectively termed as one GA run. This ensures sampling of the entire conformational space available in the predefined binding site of fXa. Here, GOLD starts with a population of 100 arbitrarily docked ligand orientations and evaluates them using the GA fitness function, which assigns them a GOLD score and improves their fitness by an iterative optimization procedure that is biased towards higher scores. The initial population is selected at random; hence several such runs are required to consistently predict bound conformations. 10 GA runs were used in this study with the GOLD score to evaluate the fitness and 10 such runs are collectively termed as one docking experiment. Furthermore, the GA was set to preterminate if the top two ranked solutions came within 2.5 A RMSD and the top two ranked solutions are saved. The docking was driven using the GOLD scoring function. The final analysis of the computed solutions was done using the modified GOLD score, which was found to correlate well with antithrombin binding affinities for pentasaccharide variants\textsuperscript{143}.

Modified GOLD score = HB\textsubscript{EXT} + 1.375 × VDW\textsubscript{EXT}

125
Where \( \text{HB}_{\text{EXT}} \) and \( \text{VDW}_{\text{EXT}} \) are the “external” (nonbonded interactions between the ligand and the receptor) hydrogen bonding and van der waals respectively.

The docking experiments were run in triplicate to ensure greater confidence in docked geometries. The top two solutions resulting from these runs (from each experiment) were considered for further analysis. Hence, a triplicate docking experiment would yield a maximum of six solutions. The RMSD between the six computed solutions were compared and convergence of these 3 independent experiments is observed if the RMSD matrix displays a set of 6 solutions within 2.5 Å of each other, indicating a high affinity-specificity sequence. Specificity here suggests a preference for a defined binding geometry; wherein a particular sequence among the entire set of compounds can recognize the protein binding site in a single, well-defined binding mode. Hence, all the seven compounds which displayed inhibitory potential were docked using the adapted docking algorithm developed in our lab (Figure 60).
Figure 60 Docking algorithm used for present study (adapted from Raghuraman et.al; *J. Med. Chem.* 2006, 49, 3553-3562).
3.5 **GOLD explains the observed inhibition profile of the monomers**

Out of the seven compounds, which were docked in triplicate, five of them (2A, 4AS, 5AS, 6AS and 7AS) were found to exhibit high specificity, i.e., the RMSD matrix indicated that RMSD difference between the computed docked poses of the experiments ran in triplicate came within 2.5 Å. Inspection of the computed binding mode of these 5 compounds revealed that two of these compounds (5AS and 6AS) bind in exactly the same manner interacting with the same residues (Lys$^{236}$, Arg$^{125}$, Lys$^{230}$, Gln$^{178}$) that came within hydrogen bonding distance (**Figure 61A and 61B**).

**Figure 61A**  **The docked pose of 5AS on exosite-II of fXa.** The red lines indicate hydrogen bonds
Interestingly, these two compounds, 5AS and 6AS, were found to inhibit fXa 86% and 75% respectively at 2.6 mM. Inspection of the docked pose of compound 4AS, which is a regioisomer of 5AS, indicates that it adopts a totally different docked pose on the exosite-II of fXa with interacting only with Arg$^{125}$, Lys$^{230}$ and Gln$^{178}$. (Figure 62A)

Figure 62A  The docked pose of 4AS. The red lines indicate hydrogen bonds
This likely explains why 4AS inhibits fXa much weaker (42% at 2.6 mM) when compared to 5AS (86% at 2.6 mM) and 6AS (75% at 2.6 mM). 4AS cannot adopt the docked pose assumed by 5AS and 6AS, because if 4AS is overlaid on 5AS and 6AS, the sulfate can interact with Arg^{125}, Lys^{230} and Gln^{178}. However, the carboxylate on position-3 of 4AS does not have any residue to interact with, so 4AS assumes a different docked pose that 5AS and 6AS (Figure 62B).

**Figure 62B** Overlay of docked poses of 4AS, 5AS and 6AS

Going over of the docked pose of 2A indicates that it adopts a totally different conformation on the protein. Only the carboxylate on the position-3 of the ring interacts with the backbone nitrogen of Lys^{236} (Figure 63).
Figure 63  Docked pose of 2A on fXa. Red lines indicate hydrogen bonds

The only interaction observed between 2A and fXa is just one hydrogen bond between the carboxylate and Lys\textsuperscript{236}. This explains the much weaker inhibition of fXa observed by 2A (34\% at 2.6 mM).

Compound 7AS adopts a quite interesting pose on fXa. The 5-O-SO\textsubscript{3} is seen to interact with Arg\textsuperscript{125}, Gln\textsuperscript{178} and Lys\textsuperscript{230}. Moreover, the extended carboxylate linker interacts with the backbone nitrogen of Lys\textsuperscript{236} (Figure 64A).
When this pose of 7AS is overlaid with the docked poses of 5AS and 6AS, interesting observations can be made (Figure 64B).

7AS has a docked pose almost similar to 5AS and 6AS. The sulfate interacts with the same residues as which 5AS and 6AS interact but, the carboxylate is poised on the other side and because of the extended linker; it is able to interact with the backbone nitrogen of Lys\textsuperscript{236}. This difference in the docked pose explains the weaker inhibition exhibited by 7AS (39\% at 2.6 mM).
3.6 Discussion

The design of allosteric inhibitors of fXa is a challenging task because of the inadequate information of the exact binding site to be targeted and due to lack of robust computational methods to solve this problem. Overall, we have the idea that the exosite-II in fXa is lined by arginines and lysines, but which factors generate specificity is an ongoing debate.

Screening of our synthesized β-5 like monomers against fXa, generates some information about which residues in the exosite-II play a critical role in driving specificity in binding. Looking for any specific compounds in the bioactive synthesized series, only 5 molecules were found to bind specifically, which are 2A, 4AS, 5AS, 6AS and 7AS. Interestingly, the most active compounds in the series, 4AS, 5AS and 6AS were found to exhibit higher specificity and affinity. Observing the calculated docked poses of the most potent molecules in this series, 5AS and 6AS drives some interesting conclusions. Both of these compounds are seen to interact with residues Lys\textsuperscript{236}, Arg\textsuperscript{125}, Lys\textsuperscript{230} and Gln\textsuperscript{178}. The 5-OSO\textsubscript{3} group in compound 5AS is seen to interact with no residue in the protease. Having a
common pharmacophore i.e. the 6-OSO₃ and the 3-COO⁻ among these compounds and both being docked consistently on the exosite-II supports and explains the observed inhibition profile of these compounds. Furthermore, the regioisomer of 5AS, which is 4AS having a much weaker inhibitory potential was found to be interesting during the biological evaluation of these compounds, as it was found to inhibit fXa much weaker than what was observed for 5AS. The docked pose of 4AS, 5AS and 6AS supports the biochemical study. In addition, it supports the preferential recognition hypothesis, as can be seen that 4AS interacts with Arg¹²⁵, Lys²³⁰ and Gln¹⁷⁸. The absence of the common pharmacophore in 4AS seen in 5AS and 6AS renders 4AS incapable of making any interaction with Lys²³⁶. Moreover, the 3-COO⁻ is not seen to interact with any residue in the protease explaining its weaker inhibitory profile.

Compound 2A is seen to interact only with Lys²³⁶. This explains why 2A inhibits the protease much weakly. Inspection of the docked pose of 7AS overlaid with 5AS and 6AS is exciting. The 5-OSO₃⁻ interacts with Arg¹²⁵, Lys²³⁰ and Gln¹⁷⁸. However, the carboxylate with the extended linker cannot assume a conformation such that it interacts with the side chain of Lys²³⁶. The carboxylate is observed to make contacts with the backbone nitrogen of Lys²³⁶. This explains the weaker inhibitory profile observed for this compound.

A careful examination of what residues in the exosite-II of fXa drive specificity show that the most potent compounds in the series (5AS and 6AS) interact with Lys²³⁶, Arg¹²⁵, Lys²³⁰ and Gln¹⁷⁸. Also, all the other compounds that were found to be specific in GOLD screening are seen to interact with the same above mentioned residues, but not necessarily
with all of them. Hence, a pharmacophore, which can interact with all the above mentioned residues, should be designed for displaying allostERIC inhibition towards fXa.
CHAPTER 4 Summary and Significance

A large number of inhibitors have been designed to target either fXa or thrombin. Nearly all of these compounds act as competitive inhibitors that target the active site of the proteases. However, nature favors a strategy that involves targeting the exosites on these coagulation proteases and inhibiting them. Examples being; heparin that binds to the exosite-II of thrombin and fXa and other peptides extracted from blood sucking leeches, which also target the exosite of these proteases. So, evolutionarily this seems like preferred tactic wherein exosites were evolved on these proteases for the modulation of their function. But, small molecules that act as allosteric inhibitors of factor Xa and thrombin are unknown and the molecules studied here are the first example of such allosteric inhibitors.

The molecules synthesized here are based on the β-5 like scaffold of CDSO3 and sulfates and carboxylates were introduced differentially on them to target the exosite-II of thrombin and fXa. The inhibition profile of these compounds indicated a preference for the β-5 like scaffold for fXa over thrombin. Docking of these compounds on the exosite-II of fXa explained the relevance of the pharmacophore that was extracted from the inhibition profile.

These compounds were found to display inhibition of thrombin and fXa in the millimolar range, which is fairly high. However, this is not too unexpected as these compounds are
fairly small and have suboptimal binding features. Michealis-Menten kinetics indicated an allosteric mechanism of enzyme inhibition, which is similar to the lead CDSO3. Considering that the exosite-II in fXa and thrombin is 15-20Å long, the inhibition profile exhibited by these compounds is quite promising. Moreover, the lead CDSO3 consists of 5-13 monomeric units and possible involves multiple dihydrobenzofuran and phenoxypropanoic acid units to generate the observed nanomolar affinity. The coupling of the most potent compounds in this study **5AS** and **6AS** with phenoxypropanoic acid units, which are found in CDSO3 would result in radically different molecules from the known ligands with high affinity.

Furthermore, the design of allosteric inhibitors for fXa and thrombin is difficult due to the inadequate information of the important residues critical for binding and the mode of binding. This inadequacy is amplified with heparin mimics because of the highly surface exposed interaction involving electrostatic interactions. Yet, the novel allosteric mechanism of these small synthetic molecules affords the opportunity to discover new molecules with possibly different pharmacological and toxicological profile. Thus, the synthetic molecules designed on the basis of CDSO3 scaffold may result in potent functional mimics of heparin.
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APPENDIX A

Abbreviations used: APC, activated protein C; AT, antithrombin; APTT, activated partial thromboplastin time; CA, caffeic acid; CEMTPP, (carbethoxy) methylene triphenyl phosphorane; CD, dehydropolymer of caffeic acid; CDSO3, sulfated dehydropolymer of caffeic acid; DEFGH, antithrombin-binding heparin pentasaccharide sequence; DHP, dehydrogenation polymer; DIBAL, diisobutylaluminum hydride; DMSO, dimethyl sulfoxide; GOLD, genetic optimization for ligand docking; HIT, heparin induced thrombocytopenia; HMWK, high molecular weight kininogen; HRP, horseradish peroxidase; IC50, concentration of inhibitor that results in 50% inhibition; LAH, lithium aluminum hydride; LMWH, low molecular weight heparin; MMTPP, methoxymethylene triphenyl phosphorane; Mw, weight average molecular weights; PT, prothrombin time; RCL, reactive center loop; RMSD, root mean squared deviation; TEMPO, 2,2,6,6-Tetramethylpiperidine-1-oxyl; TFPI, tissue factor pathway inhibitor; UFH, unfractionated heparin.
VITA

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