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Structure-based design and synthesis of pyrazinones containing novel P_1 'side pocket' moieties as inhibitors of TF/VIIa

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Abstract—We describe the structure-based design, synthesis, and enzymatic activity of a series of substituted pyrazinones as inhibitors of the TF/VIIa complex. These inhibitors contain substituents meta to the P_1 amidine designed to explore additional interactions with the VIIa residues in the so-called 'S_1 side pocket'. A crystal structure of the designed inhibitors demonstrates the ability of the P_1 side pocket moiety to engage Lys192 and main chain of Gly216 via hydrogen bond interactions, thus, providing additional possibility for chemical modification to improve selectivity and/or physical properties of inhibitors.

Cardiovascular disease remains the most common cause of mortality in the western world. The use of standard therapies such as heparin or warfarin is severely limited due to slow onset of action and lack of selectivity leading to bleeding side effects that requires close patient monitoring.[1] The search for safer more efficacious antithrombotics has focused on direct inhibitors targeted to specific inhibition of enzymes within the extrinsic coagulation cascade—the ultimate goal being discovery of a drug with an improved safety profile and pharmacodynamics over existing treatments, specifically, better oral bioavailability and specificity.

Several enzymes within the coagulation cascade have been targets for antithrombotic therapies including thrombin,[2] Xa,[3] and IXa.[4] Recently, TF/VIIa has emerged as an attractive target.[5] Factor VIIa (TF/VIIa) is the initiator of the extrinsic coagulation cascade. Plaque rupture exposes tissue factor (TF) to zymogen VII in circulating plasma, which eventually forms the active TF/VIIa complex. The assembled TF/VIIa complex is responsible for activation of IX to IXa and X to Xa through proteolytic cleavage. This ultimately results in the amplified conversion of prothrombin to thrombin and fibrinogen to fibrin in the latter stages of clot formation.

Several approaches have been described in the literature for antagonizing the activity of TF/VIIa complex. These include active site inhibited factor VIIa (VIIai), tissue factor mutants, antibodies directed against TF or VIIa, naturally occurring protein-based inhibitors (for example, tissue factor pathway inhibitor or TFPI, hookworm anticoagulant protein, and NAPc2), peptide-based exosite inhibitors, and small molecule active site inhibitors.[5d]

Evidence from our laboratories and others indicate that selective inhibition of the TF/VIIa complex may provide prevention of thrombosis with the advantage of a lower risk of bleeding side effects versus other coagulation cascade targets.[6]

Recent papers from our laboratories, both published and in progress, describe the design and synthesis of compounds that inhibit the TF/VIIa complex.[7] These articles discuss optimization of the core structure and

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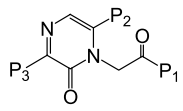


Figure 1. Pyrazinone core structure.

subsequent optimization at the P_1, P_2, and P_3 sites of the inhibitors with ultimate achievement of low nanomolar, pyrazinone-based inhibitors (Fig. 1). In this article, we report the design, synthesis, and biological activity of new inhibitors containing a 'side pocket' residue on P_1. These compounds arose from further optimization of the P_1 moiety based on some of our most potent analogs.

Our approach to the design of these inhibitors originated from examination of the crystal structure of existing active compounds described elsewhere.[7d] The common feature of all the structures is the anchoring interaction of benzamidine via bidentate ionic interaction with conserved Asp189 located deeply in the S_1 pocket of VIIa (as shown in Figs. 2 and 3). The benzamidine also forms good hydrogen bonds with main chain carbonyl of Gly219, a structural water and a weak hydrogen bond with the Ser190 side chain.

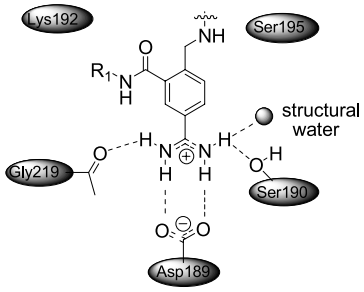


Figure 2. Schematic representation of the binding mode of the P_1 moiety of inhibitor with TF/VIIa complex.

Analysis of structures also revealed a solvent exposed region outside of the S_1 pocket formed by Gln143, Asp146, Arg147, Lys192, Gly216, Gly219, and Cys220 ('S_1 side pocket') that could be reached off of the ortho position of the P_1-needle (Fig. 3). Analysis of crystal structures of related serine proteases revealed that a similar binding pocket exists in thrombin, factor Xa, and trypsin. However, only VIIa has a lysine at position 192. This is replaced by a glutamine in Xa and trypsin, while thrombin has a glutamic acid at this position. The S_1 side pocket is very shallow in trypsin while thrombin has a bulky tryptophan residue that forms one of the walls of the pocket. Interestingly, the side pocket in Xa appears to be very similar to that in VIIa. We believed that exploration of this region could provide additional specific interactions with VIIa leading to enhanced potency, selectivity, and improved physical properties.

We began with synthesis of compound **4b**, a pyridyl substituted P_1. The pyridyl residue can participate in hydrogen bonds with Lys192 through the nitrogen of the pyridine ring as well as through its amide carbonyl.

Preparation of **4b** began with compound **1**[8] (Scheme 1). Protection of the basic nitrogens using di-*tert*-butyldicarbonate gave us a mixture of mono and bis-protected products, **2a** and **b**.[9] We carried both of these forward to the mono and bis protected versions of compound **3.** This was achieved through activation of the carboxylate **2** using HBTU followed by reaction with 4-pyridylamine. Final deprotection using TFA followed by reverse phase HPLC purification afforded us compound **4b** as the trifluoroacetate salt. Despite the overall low

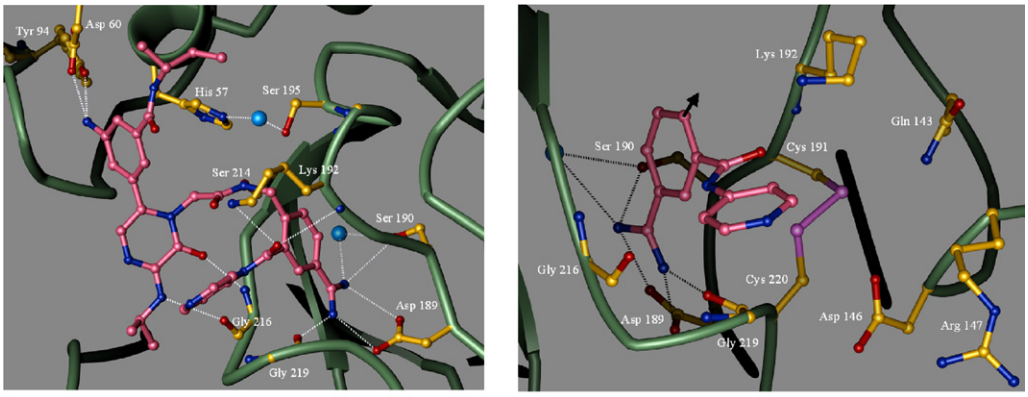
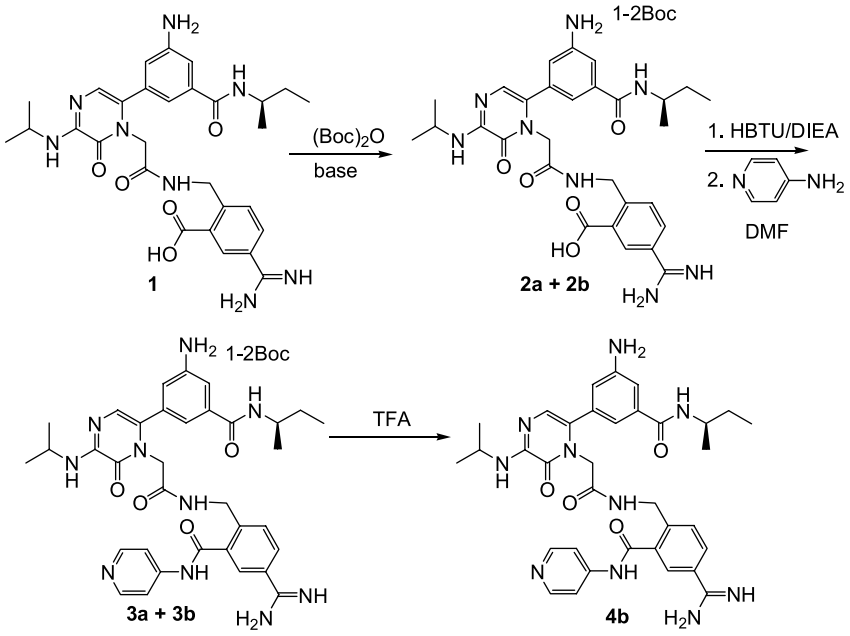


Figure 3. (a) Crystal structure of compound **4b** bound in the active site of TF/VIIa complex. The structure has been refined to an R_{free} of 26.8% at 2.0 A resolution ($R_{crystal}$: 22.3%). Some of the key side chains of factor VIIa are displayed (C: gold, N: dark blue, O: red, and S: pink). The carbon atoms of the inhibitor are shown in dark pink color. The hydrogen bonds formed by the inhibitor are shown in dotted (white) lines. Two bound solvent molecules are shown as cyan spheres. One of them is bound near the active site serine and histidine side chains while the other solvent molecule (structure water) is bound in the S_1 pocket (b) close-up view of the S_1 side pocket (multi-colored as shown in Fig. 3a). This figure is rotated relative to Figure 3a to clarify the position of the S_1 side pocket. Also, for clarity, only the benzamidine and the pyridyl amide of the inhibitor are shown. The black arrow indicates the approximate directionality of the connection between the benzamidine moiety and the rest of the inhibitor. The hydrogen bonds are shown in black dotted lines.



Scheme 1. Synthesis of compound **4b**.

yield of **4b**, probably due to low reactivity of the aminopyridyl nucleophile, we obtained sufficient quantities for biological testing and X-ray structure determination.

The new inhibitor **4b** containing the P_1 pyridyl side pocket moiety maintained low nanomolar activity against TF/VIIa (9.4 nM) as well as excellent selectivity against Xa (>30 μM) and thrombin (>30 μM).

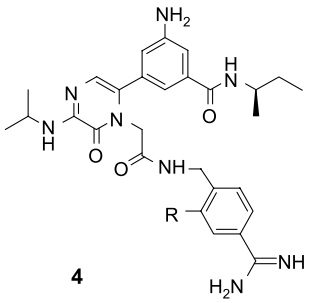
The crystal structure of compound **4b** bound to TF/VIIa is shown in Figure 3a.[10] The benzamidine moiety of the inhibitor forms an ion-pair with the carboxylate of Asp189 as observed in the structures of other pyrazinone complexes with TF/VIIa.[7b,d] In addition, the amidine moiety also forms hydrogen bonds to the side chain of Ser190 and the main chain carbonyl of Gly219. The inhibitor forms three hydrogen bonds to main chain atoms of Ser214 and Gly216 of VIIa as observed in other serine protease structures.[11] The meta amine of the phenyl group of P_2 forms hydrogen bonds with the side chains of Asp60 and Tyr94 and the main chain carbonyl of Thr99, and provides enhanced potency and selectivity against factor Xa and thrombin as described previously.[7d] Factor VIIa has a well-defined S_2 pocket that is electronegative due to the presence of Asp60. The S_1 amide linker also forms a hydrogen bond to main chain carbonyl of His57.

The amide moiety from the P_1 ortho phenyl position and the pyridyl group extend toward the solvent exposed S_1 side pocket. While the pyridyl ring is oriented almost orthogonal to the P_1 phenyl ring of the inhibitor (112°), the amide of the P_1 side chain is involved in three hydrogen-bonding interactions. One hydrogen bond is formed between the amide nitrogen and main chain of Gly216 (3.3 Å). The two others are between the amide carbonyl and the main chain nitrogen (3.4 Å) and the terminal protonated nitrogen of Lys192 (3.6 Å). The interactions formed by the inhibitor with the lysine side chain result in a well-defined conformation of the side chain. The side chain of Lys192 is disordered in most of the previous crystal structures of TF/VIIa with pyrazinone inhibitors (unpublished observation). While stabilization of one specific conformation of the disordered side chain of Lys192 is entropically unfavorable, it could be compensated by enthalpic gains afforded by specific interactions between the amino acid side chain and the inhibitor.

Figure 3b shows a close view of the S_1 side pocket of factor VIIa formed by residues Gln143, Asp146, Arg147, Cys191, Lys192, Gly216, Gly219, and Cys220. As predicted, the pyridyl moiety from the meta position of the P_1 phenyl fills up most of the S_1 side pocket.

A careful analysis of the data presented in Table 1 shows that most of the compounds have comparable binding affinities for VIIa and trypsin. This is consistent with the crystal structures of these two serine proteases. The active site in trypsin is relatively open with a shallow S_1 side pocket and S_2 sub site. While trypsin does not have an aspartic acid in the S_2 pocket, comparable to Asp60 of VIIa, the side chain of Tyr94 and the carbonyl oxygen of Ser96 are optimally positioned for forming good hydrogen bonding interactions with the aniline moiety of the inhibitor. Similarly, the shallow S_1 side pocket in trypsin could accommodate bulky substituents from the P_1 position of the inhibitor without any steric clashes. Also, both trypsin and factor VIIa contain a serine at position 190 in the S_1 pocket that is replaced by an alanine in thrombin and Xa. These structural similarities in the active sites of VIIa and trypsin could explain the difficulties encountered in engineering selectivity over trypsin in our current series of inhibitors.

Table 1. TF/VIIa Inhibition by **4**



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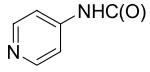
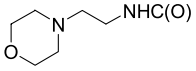
Compd	R	IC_{50} (μM)			
		VIIa	Xa	IIa	Trypsin
4a	$C(O)NH_2$	0.0062	>30	13.7	0.020
4b		0.0094	>30	>30	0.028
4c	$CF_3CH_2NHC(O)$	0.012	>30	22.6	0.015
4d		0.020	>30	16.7	0.021
4e	$HO_2CCH_2NHC(O)$	0.021	>30	>30	0.016
4f	$MeO_2CCH_2NHC(O)$	0.023	>30	26.7	0.031
4g	C(O)OH	0.152	>30	>30	0.050
4h	H	0.0017	>30	6.1	0.015

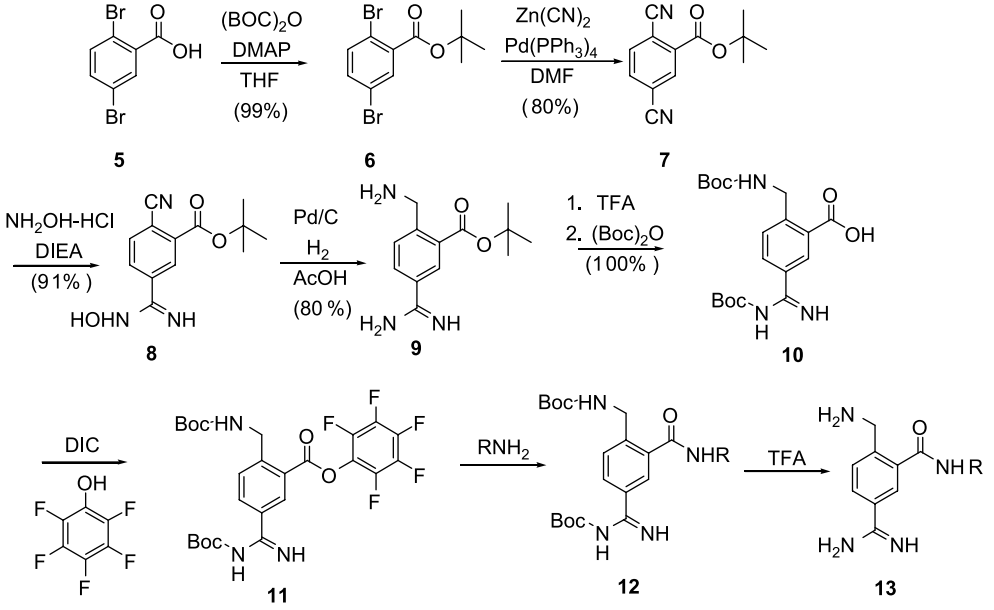
Based on the biological and structural evidence from compound **4b**, the next logical step was to prepare a series of analogs to further probe this region. To accomplish this goal a more parallel friendly synthesis for the P_1 side pocket compounds was developed.

Thus, the carboxylic acid functionalized P_1 benzamidine **10** was prepared in a protected form followed by coupling of the P_1 to the desired amine, deprotection, and coupling to the pyrazinone core (Schemes 2 and 3). This allowed rapid assembly of side pocket amides from a single intermediate. In addition, this would allow us to attach our P_1 analogs to other interesting cores as well. The commercially available dibromocarboxylic acid **5** was transformed in five high yielding steps to the key intermediate **10** (Scheme 2). One particularly interesting step in this pathway involves differentiation of the two nitriles of compound **7**. Interestingly, we obtained reaction of hydroxyl amine exclusively at the less hindered nitrile to give **8**, even though the nitrile ortho to the acid is fully conjugated and hence more electron poor. A more in-depth discussion of the selectivity of this reaction will be presented at a later date.

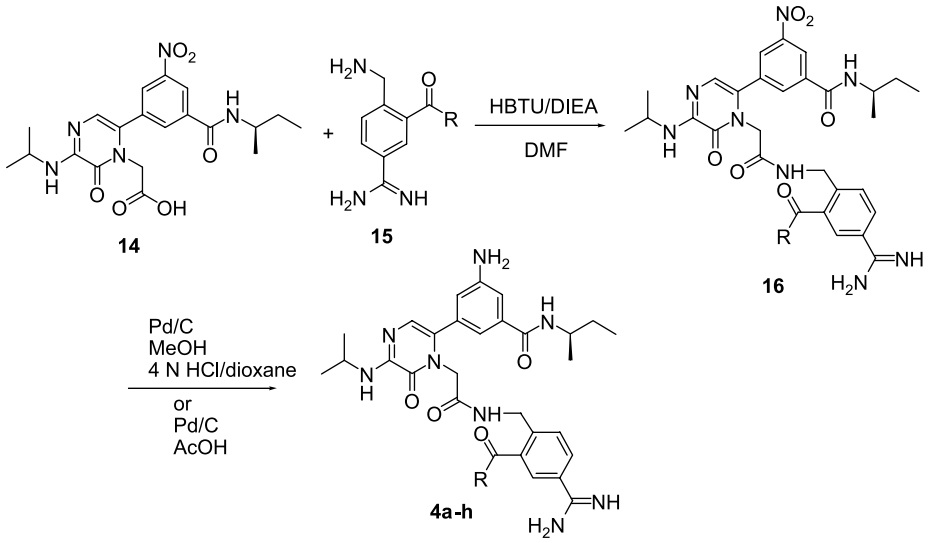
We explored several traditional amide bond forming reactions to generate **12**, including HBTU, DCC/HOBt, and EDC/HOBt. The cleanest, highest yielding reaction came from generation of the pentafluorophenol ester **11** followed by addition of the desired amine. In addition, the pentafluorophenol ester could be purified and stored, allowing for easy parallel functionalization at this position.

The pentafluorophenol ester was reacted with a small diverse set of amines with potential for H-bond interaction. The compounds were deprotected to give **13** and then coupled to the pyrazinone core (Scheme 3). The coupling reaction typically employed HBTU with DIEA in DMF at ambient temperature, with preactivation of the acid first followed by addition of the P_1 amine **15**. Catalytic hydrogenation of **16** in methanol with HCl/dioxane or in acetic acid yielded our final products that were purified by reverse phase chromatography. Products were isolated as the trifluoroacetate salts.

Table 1 summarizes the biological activity of the P_1 side pocket analogs. Excellent levels of TF/VIIa inhibition (6–21 nM) were observed for all amide side pocket ana-



Scheme 2. Synthesis of P1 side pocket analogs.



Scheme 3. Coupling of side pocket P_1 analogs to pyrazinone core.

logs. In addition, all compounds showed moderate to excellent selectivity versus Xa and thrombin.

For comparison we synthesized a non-amide analog, entry **4g**, and found that it was 24 times less potent than our most potent analog. The difference in potency probably arises from the fact that the amide carbonyl forms a better hydrogen bond to the enzyme than the carbonyl of the acid.

Previous studies indicate the amidine is responsible for poor oral bioavailability. In our search for more bioavailable compounds we have attempted to modulate the basicity of the amidine. Replacement of the amidine with less basic or non-basic groups has led to a drastic decrease of potency. It is possible that this newly discovered site of interaction within TF/VIIa could provide a starting point for making successful changes to the amidine.

In conclusion, we have identified yet another site of substitution of the P_1 benzamidine moiety able to engage additional interactions in the S_1 side pocket of factor VIIa. These additional molecular interactions can provide the basis for design of new potent and selective TF/VIIa inhibitors.

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9. Four possible products could be present in this mixture: mono-aniline protected, mono-amidine protected, bis-amidine protected, mono-aniline/mono-amidine. Ultimately, deprotection of the **3a** and **b** mixture would give us one compound, **4b**. Therefore, we did not pursue extensive characterization of these products.
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