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# Design, synthesis, and evaluation of oxyanion-hole selective inhibitor substituents for the S1 subsite of factor Xa

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**Abstract**—We have designed, synthesized, and evaluated the factor Xa inhibitory activities of *p*-amidinophenyl-sulfones, amines, and alcohols intended to take advantage of the polarity and hydrogen-bonding potential of the oxyanion hole region of the S1 specificity pocket. We demonstrate that placement of an anionic group within the oxyanion hole region of the catalytic site substantially enhances activity, with small flexible groups favored over bulkier ones. Ab initio p$K_a$ calculations suggest that the hydroxyl substituent frequently used for benzamidine moieties may be ionized to form an anionic group, consistent with the general trend. One nonamidine based substituent also shows promising activity.


Factor Xa, the penultimate enzyme in the coagulation cascade, has received increasing attention in the development of new anticoagulant agents.[1–6] The enzymes of the blood coagulation cascade, including thrombin, factor Xa, and factor VIIa are known to have specificity pockets (S1 subsites) very similar to those of other serine proteases such as trypsin and plasmin. The Asp189 residue at the bottom of the pocket binds ionically with arginyl or benzamidinium ionic groups. To enhance affinity and specificity, it is desirable to generate additional hydrogen bonding or ionic interactions with other functional groups within the S1 specificity pocket. In prior theoretical analysis, we have proposed that hydrogen bonding with the catalytic Ser 195 hydroxyl in the oxyanion hole region can provide an additional stabilization mechanism for a properly positioned H-bond donor.[7] More recently, we have shown that addition of an anionic phosphinic acid moiety to benzamidine as a tetrahedral analog in the vicinity of the oxyanion hole enhances inhibitory affinity by factors of 5–10 against several serine proteases.[8] Other workers have also shown empirically that addition of hydrogen bonding groups (OH or NH),[9–14] amine,[14] carboxyl,[15,16] and sulfonyl[17] functionalities at positions within or adjacent to the S1 subsite also enhance activity. However, to date there has been no systematic evaluation of the optimum ionic/H-bonding functionality, or of the best positioning of such a group within the S1 subsite. Accordingly, we have undertaken such a survey, using the well-characterized benzamidine group as an anchor to the Asp 189 side chain. This moiety has been demonstrated to bind in the S1 specificity pocket with very little variation in positioning for a variety of benzamidine-based factor Xa inhibitors.[9,12,18–22] We have also explored the effects of a couple of benzamidine replacements that have been incorporated into factor Xa inhibitors, including isoquinoline[23,24] and phenyl-dimethylamine[25,26] groups.

Whenever satisfactory literature syntheses were located for proposed substrates they were followed (**1**,[27,28] **8**,[29,30] **17**,[30,31] **18**,[30,32] and **19**[28,33]), with the other members of the series obtained by the routes set out below.

In general, in the following syntheses, the Pinner reaction was the first method assayed for conversion of nitriles to amidines. When this failed, transformation of the nitrile group to the thioamide with diethyldithiophosphate–$H_2O$ followed by *S*-methylation then aminolysis was the method of choice.[8] In the event that neither of

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these methods was satisfactory, resort was made to the conversion of nitrile groups to *N*-acetoxy amidines with hydroxylamine and acetic anhydride followed by hydrogenation.[34]

4-Cyanobenzyl methyl sulfone (**2**) was prepared from 4-cyanobenzaldehyde,[27] which was treated with diethyl dithiophosphate–$H_2O$[8] to afford the thioamidate **3**. Alkylation with methyl iodide, then exposure to $NH_4OAc$ finally afforded the amidine **4** (Scheme 1).

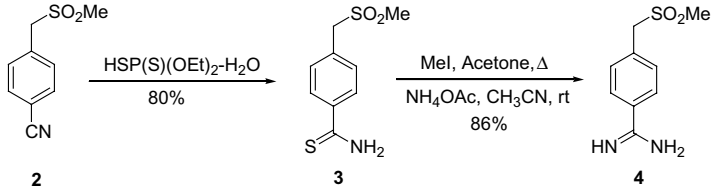
The homologous sulfone **7** was prepared from bromide **5** by a sequence beginning with sodium methyl sulfide followed by $H_2O_2$ oxidation.[27,28] The resulting nitrile **6** was treated with diethyl dithiophosphate–$H_2O$ to afford the thioamidate, which following methylation and subsequent aminolysis with $NH_4OAc$, gave amidine **7** (Scheme 2).

Amidine **11** was obtained from the known azide **9**[34,35] by a straightforward procedure (Scheme 3).

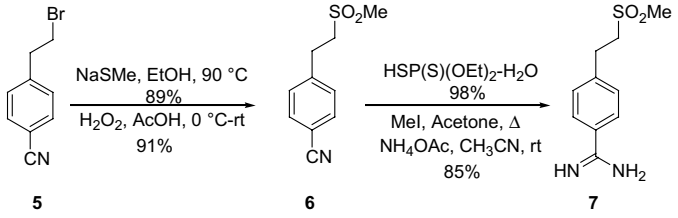
The homologous aminoamidine **16** was obtained from **12**[36] by the sequence shown in Scheme 4. Thus, Curtius rearrangement of **12** afforded *N*-Boc nitrile **13**. This was converted to the *N*-acetoxyamidine **14**, hydrogenation of which afforded **15**. Finally, removal of the Boc group with TFA yielded **16** (Scheme 4).

Palladium mediated phosphorylation[37] of 6-bromo-1-amino-isoquinoline[24] **20** afforded phosphonate **21**, which on removal of the benzoyl and isopropyl groups under acidic conditions gave aminoisoquinoline **22** (Scheme 5).

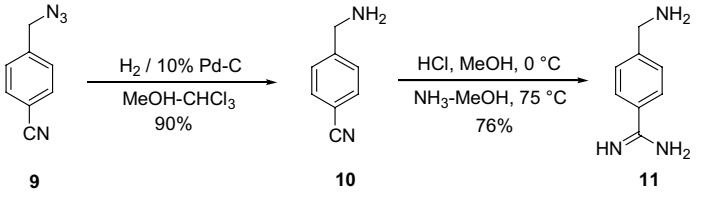
The *N*,*N*-dimethylamino phosphinic acid **28** was obtained by metallation of commercially available **24**, fol-



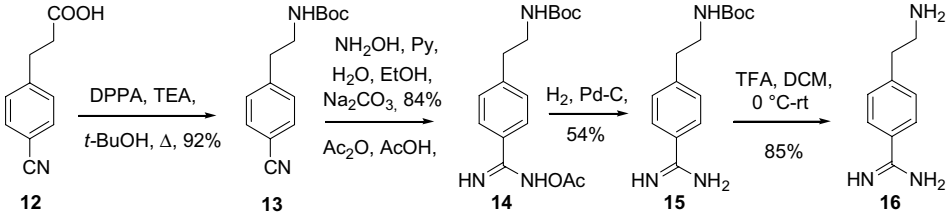

**Scheme 1.**



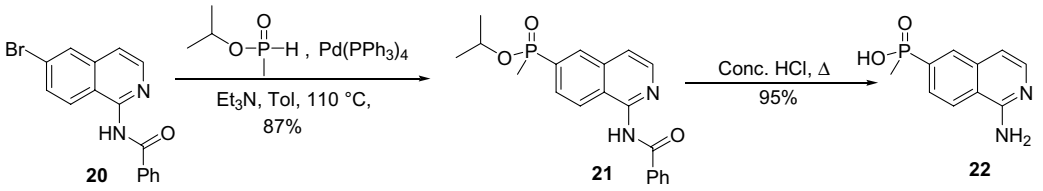

**Scheme 2.**



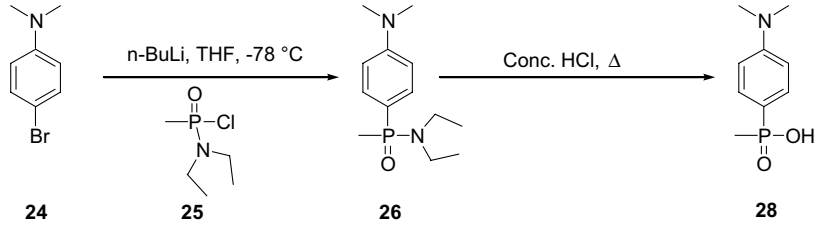

**Scheme 3.**




**Scheme 4.**




**Scheme 5.**

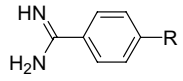


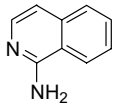
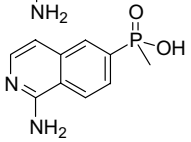
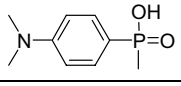

**Scheme 6.**

lowed by quenching with the phosphoryl amidate[38] **25**, and final amide hydrolysis in concd HCl at reflux. This protocol was found to be more convenient than an alternative literature synthesis for this compound (Scheme 6).

Compounds were evaluated for their potency as FXa inhibitors using the chromogenic substrate, MeO-CO-D-CHG-Gly-Arg-*p*NA. Activities were determined as the initial rate of cleavage of the peptide *p*-nitroanilide by the enzyme. All substrate concentrations used were equal to the $K_m$ values under the present assay conditions; $K_i$ values are listed in Table 1.

**Table 1.** In vitro inhibitory activities of benzamidine-based analogs and related compounds against factor Xa




| Compound no. | R-group or inhibitor structure | FXa $K_i$ (μM) |
|---|---|---|
| **27** | R = H | 349 |
| **1** | R = $SO_2CH_3$ | 1800 |
| **4** | R = $CH_2SO_2CH_3$ | >5000 |
| **7** | R = $(CH_2)_2SO_2CH_3$ | 193 |
| **8** | R = $NH_2$ | 197 |
| **11** | R = $CH_2NH_2$ | 429 |
| **16** | R = $(CH_2)_2NH_2$ | 750 |
| **17** | R = OH | 30 |
| **18** | R = $CH_2OH$ | 55 |
| **19** | R = $(CH_2)_2OH$ | 607 |
| **23** |  | 226 |
| **22** |  | >5000 |
| **28** |  | 4100 |

The sulfone **1** should be essentially isosteric with the previously described **AMPA** system,[8] but lacks the negative charge. Modeling calculations suggest that the sulfone should be positioned in the oxyanion hole region similar to the phosphinic acid of **AMPA**. It exhibits about a 5-fold higher $K_i$ than benzamidine **27**, in contrast to the 5-fold decrease in $K_i$ (enhancement of activity) for **AMPA** over benzamidine,[8] confirming that charge is an important determinant of binding affinity in this region. Adding an ethylene spacer between the sulfone and the phenyl ring (**7**) increases activity by about a factor of 10, but a single methylene spacer (**4**) results in the loss of activity, indicating that moving the sulfone out of the oxyanion hole with a flexible spacer of sufficient length allows the formation of a more favorable contact interaction between the sulfone and the protein, but that a short spacer probably induces bumping collisions that cannot be compensated by conformational rearrangement.

Placing an amine in the oxyanion hole (**8**) also produces about a twofold decrease in $K_i$ over benzamidine (**27**). Aniline exhibits a p$K_a$ of about 4.6, and the strong electron-withdrawing character of the amidine group *para* to the amine should substantially further reduce the p$K_a$ of **8**, thus producing a hydrogen-bonding group that will have no cationic character. However, adding one (**11**) or two (**16**) methylene spacers between the phenyl and the amine produces subsequent ~2-fold and ~4-fold reductions in activity, respectively. The methylene spacers will separate the amine from the electron-withdrawing effects of the benzamidine, with the p$K_a$ of the resulting alkyl amine probably in the 8–10 range, producing a protonated amine cationic group in the vicinity of the oxyanion hole that is consequently disadvantageous to binding.

The most significant enhancement in activity results from placing a hydroxyl group in the oxyanion hole (**17**). It is unclear whether this is due simply to advantageous hydrogen bonding, or if it is due to deprotonation of the hydroxyl to form the *p*-benzamidinophenate

anion. The p$K_a$ of *p*-nitrophenol is about 7.2, and the amidine group should exhibit similar or stronger electron-withdrawing characteristics, suggesting that the hydroxyl of **17** may exist as an anion within the oxyanion hole. Accordingly, we have used ab initio methods[39,40] and Gaussian 03[41] to calculate the p$K_a$ for the hydroxyl of **17**, and find a value of 7.8, supporting this suggestion. Adding a single methylene to form **18** reduces the activity by about a factor of two, while an ethylene spacer reduces it by a further factor of 10, to approximately half that of benzamidine itself. A single methylene spacer will still permit hydroxyl hydrogen bonding within the S1 catalytic region, while the ethyl spacer pushes the hydroxyl up out of the S1 pocket, probably explaining their relative activities. Compared to benzamidine, the small *p*-hydroxyl group imparts about two times more activity than the bulkier *p*-phosphinic acid group that we have previously described.[8] Molecular modeling comparisons (not shown), indicate that *p*-hydroxybenzamidine is less likely to exhibit minor bumping collisions than either the phosphinic acid or its isosteric sulfone analog **1**.

Less polar replacements for the benzamidine have also been explored by a number of workers. To obtain a more quantitative analysis of the effectiveness of some such replacements, we have also evaluated two substitutions—the replacement of benzamidine with 1-aminoisoquinoline, and *N*,*N*-dimethylaniline. Although 1-aminoisoquinoline is much less basic than benzamidine, surprisingly **23** is nearly a factor of two more active than benzamidine (**27**). We speculate that the added aromatic bulk fills the S1 pocket somewhat more than benzamidine, with the enhanced van der Waals interactions compensating for any loss in ionic bonding. Disappointingly, addition of the methyl phosphinic acid at a position structurally equivalent to that in **AMPA** to form **22** eliminates activity in FXa, probably due to bumping collisions. (Interestingly, **22** has about 50% higher inhibitory activity for trypsin than **27**, suggesting that slight structural differences between the enzymes can drastically affect activity (data not shown). Replacement of the amidine with dimethylamine and addition of the methyl phosphinic acid group to form the **AMPA** analog **28** produces barely detectable activity, indicating that replacement of the cationic amidine with the formal charge of the dimethyl amine does not retain critical interaction with the anionic Asp189 side chain carboxylate.

One of the more puzzling aspects of the results in Table 1 is the very low activity of sulfone **1**. Referenced to benzamidine, it is nearly 25-fold less active than its structurally similar phosphinic acid analog.[8] To clarify this difference, we have determined the crystal structure of **1** (**AMSO**) in complex with trypsin, following protocols similar to that previously used for structure determination of trypsin complexed with *p*-amidinophenylmethylphosphinic acid (**AMPA**). The X-ray crystallographic data collection, processing and refinement statistics for the structure of bovine trypsin complexed with **AMSO** followed our published methods.[8] The electron density is well ordered and unambiguous for

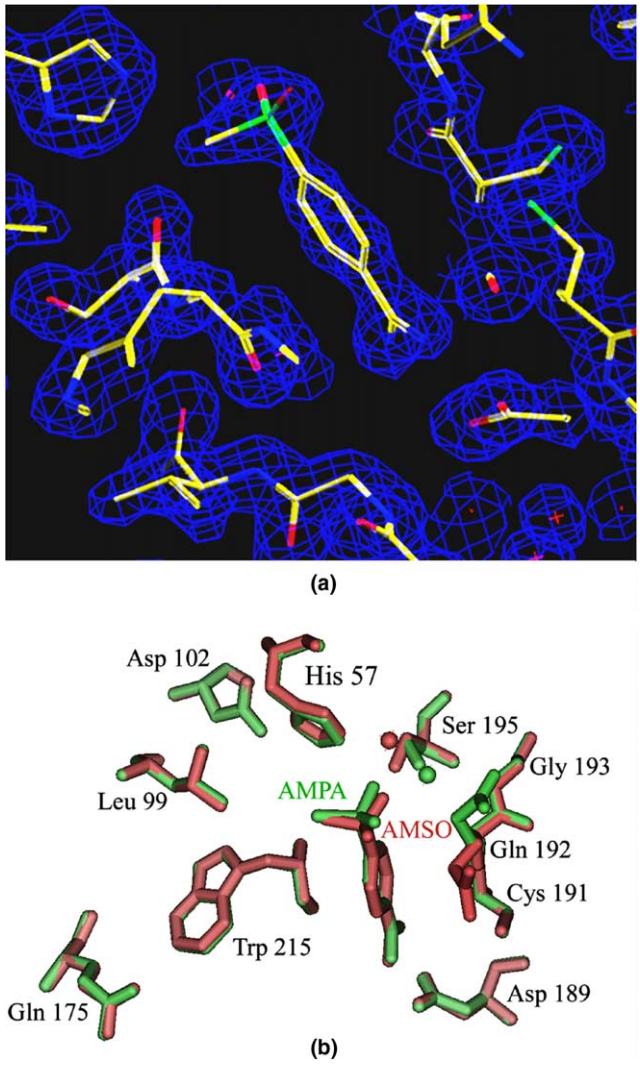



**Figure 1.** (a) $2F_o$–$F_c$ electron density map of trypsin–AMSO surrounding the S1 site. The electron density is contoured at the 1.2 $\sigma$ level. (b) Superposition of the structure of the trypsin–AMSO complex with the structure of trypsin–AMPA complex.[8] Residues of trypsin–AMSO are colored red while those of trypsin–AMPA are colored green. The AMPA–trypsin and AMSO–trypsin structures have been deposited with the protein data bank as 1TX7·pdb and 1TX8·pdb, respectively.

**AMSO** (Fig. 1a). The final structure has 169 water molecules and 1 $Ca^{2+}$. The amidino group of the inhibitor forms a salt bridge with Asp 189 located at the bottom of S1 pocket as usual. One of the oxygens of the sulfonyl group forms direct hydrogen bonds with the side chain oxygen of Ser 195 (2.45 Å) and the oxygen of a single water (2.79 Å). To compare the structure differences of the trypsin–**AMSO** complex with that of trypsin–**AMPA** complex, the two structures were superimposed in Figure 1b. The side chain of Gln 192 is flipped down in the trypsin–**AMSO** structure compared to the trypsin–**AMPA** structure, and a water molecular is in a different position, eliminating hydrogen bonds for one of the two sulfonyl oxygens. Since both oxygens of phosphinic acid in the trypsin–**AMPA** structure form hydrogen bonds with neighboring atoms,[8] the reduced hydrogen bonding for **AMSO** is probably a contributing factor for the lower inhibitory activity of **AMSO**, com-

pared to **AMPA**. The reduced hydrogen bonding is probably a consequence of its lower polarity, consistent with the hypothesis that polar interactions within or adjacent to the oxyanion hole region will enhance binding affinity and inhibitory activity. The near identity of the binding geometries for **AMSO** and **AMPA** thus suggest that the substantially reduced activity of **AMSO** is almost certainly the result of its lower polarity and lack of anionic character.

In summary, we have developed a systematic characterization of several 'building blocks' that have been used in the development of various FXa inhibitors. Comparison of the isosteric **AMSO** and **AMPA** indicate that placement of an anionic group within the oxyanion hole substantially enhances inhibitory activity. Surprisingly, we find that the small hydroxyl group is a more effective substituent for placement in the oxyanion hole region than the somewhat bulkier and highly acidic phosphinic acid group. Our Gaussian calculations suggest that the electron-withdrawing character of the benzamidine may shift the hydroxyl p$K_a$ to a value low enough for at least partial ionization, effectively providing a hydroxylic anion at the oxyanion hole. This is consistent with literature observations on potency enhancements resulting from 4-OH addition to benzamidine.[6,9,14] The bulkier 1-amino-isoquinoline also provides an effective replacement for benzamidine, although further derivatization can be problematic due to steric restrictions.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2004.07.054.

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