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D-PHE-PRO-*p*-AMIDINOBENZYLAMINE: A POTENT AND HIGHLY SELECTIVE THROMBIN INHIBITOR

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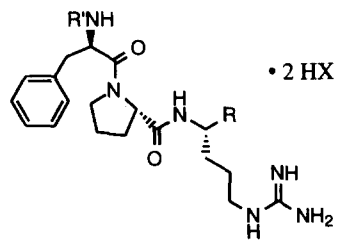
Abstract: The design, synthesis, and enzyme inhibitory profile of *D*-Phe-Pro-*p*-Amidinobenzylamine are presented. This compound has inhibitory activity equivalent to *D*-Phe-Pro-Arg-H, two orders of magnitude more potent than *D*-Phe-Pro-Agmatine. The results indicate that binding energy provided by the covalent bond of a transition-state analog can be replaced with noncovalent interactions.

Because of its central role in the control of blood clot formation, the "trypsin-like" serine protease, thrombin, is an important target for therapeutic intervention in thrombotic disease, and a number of strategies have been successfully applied towards the development of potent thrombin inhibitors.[1] However, for a protease inhibitor to be therapeutically useful, potency is only one criterion; the level of selectivity versus homologous enzymes becomes an important consideration. This is certainly true for thrombin, since there are other "trypsin-like" serine proteases which serve important regulatory roles.[2] In this regard, the fibrinolytic enzymes (plasmin, tissue plasminogen activator (t-PA), and urokinase) are of particular importance, since their activity (clot dissolution) will undoubtedly be required in patients who are candidates for antithrombotic therapy.[3]

In a recent report,[4] we described selectivity studies on the peptide-derived thrombin inhibitor *D*-Phe-Pro-Agmatine (**1**)[5] and several of its homologs. Agmatine derivatives are attractive targets as peptide thrombin inhibitors relative to the arginine-based transition-state analogs such as the arginine aldehydes (i.e., **2** and **3**).[6] In



1 - R = H, R' = H

2 - R = CHO, R' = H

3 - R = CHO, R' = CH_3

Figure 1. D-Phe-Pro-ArgH and D-Phe-Pro-Agm

addition to the attractive selectivity profile they possess, agmatine derivatives are easily prepared and characterized and they lack the (potentially labile) stereogenic center present in the arginine-based transition-state analogs. However, the affinity of agmatine derivatives for thrombin is ~ 3 to 4 Kcal/mol weaker than the corresponding tripeptide arginine aldehydes (**2** and **3**). This abated activity results from the loss of a covalent interaction between the C-terminal aldehyde and the serine of thrombin's Asp-His-Ser catalytic triad.[7] In order to improve on the properties of the agmatine lead, while maintaining the advantages of this series, we sought to replace the thrombin inhibitory potency that was lost upon removal of the C-terminal electrophile through noncovalent interactions.

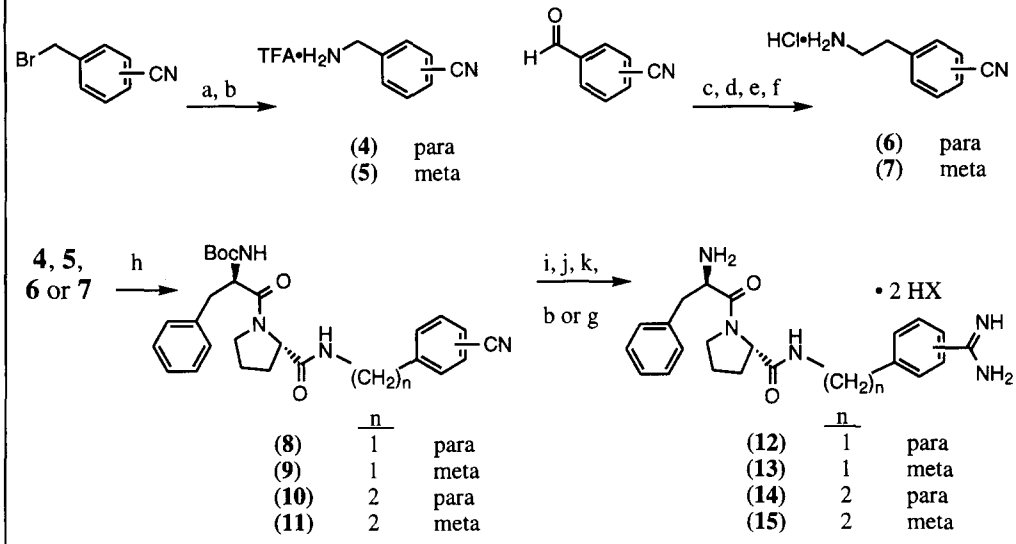
Upon consideration of the crystal structure of the *D*-Phe-Pro-Agm/thrombin complex,[4] the interaction between agmatine and the thrombin specificity pocket was identified as a potential opportunity for improvement. Over the years, several laboratories have studied the structure-activity relationship of the inhibitory activity of small organic bases for the "trypsin-like" serine proteases.[8] This database serves as a useful experimental model for improving the interaction of the sidechain of arginine-based thrombin inhibitors with the thrombin specificity pocket. For example, the thrombin inhibitory activity of benzamidine[8b] is ~ 2 orders of magnitude more potent than tosylagmatine.[8c] As a result, benzamidine has been incorporated into the sidechain of potent, amino acid derived thrombin inhibitors such as NAPAP.[1] Since the difference in binding energy between tosylagmatine and benzamidine is similar to the difference between compounds **1** and **2**, we hypothesized that replacement of the alkyl guanidine portion of *D*-Phe-Pro-Agm with an appropriately tethered benzamidine could improve the thrombin inhibitory potency of the agmatine series to rival the analogous tripeptide arginals.

As an initial test of this hypothesis, the para substituted benzamidine **12** (Scheme 1) was targeted. *p*-Amidinobenzylamine was chosen as the replacement for agmatine based on its similarity in size and spatial orientation relative to the thrombin-bound conformation of agmatine in the **1**-thrombin crystal structure.[4] In order to obtain experimental confirmation that *p*-amidinobenzylamine presents the optimal benzamidine orientation, the meta isomer **13** was targeted for comparison, along with the corresponding phenethylamine derivatives **14** and **15**. Scheme 1 illustrates the synthetic sequence used to prepare compounds **12**-**15**. The cyanobenzylamines **4** and **5** and the cyanophenethylamines **6** and **7** were prepared and coupled to Boc-*D*-Phe-Pro-OH. The resulting benzonitriles (**8**-**11**) were then elaborated to the desired products by sequential treatment with H_2S, MeI, and NH_4OAc,[9] followed by deprotection and purification by preparative reverse phase HPLC.

As the data in Table 1 illustrate,[10] the improvement in thrombin inhibitory activity predicted for the incorporation of benzamidine was realized with the para substituted derivative **12**, with an association constant of 6.8 x 10^8 (L/mol) for human α-thrombin, virtually identical to *D*-Phe-Pro-Arg-H (**2**). When the amidine is moved from the para (**12**) to the meta (**13**) position, potency decreases by 3 orders of magnitude. Also, the one carbon tether of the benzylamine derivative **12** provided a significant potency advantage relative to either of the phenethylamine derivatives (**14** or **15**).

Fortunately, the increase in potency achieved with **12** does not come at the expense of serine protease selectivity. In addition to retaining 130-fold selectivity for thrombin over trypsin, the incorporation of *p*-amidinobenzylamine actually improves control over selectivity for thrombin versus the important fibrinolytic enzymes (e.g., 26,000-fold over plasmin, 170,000-fold over t-PA, and 400,000-fold over urokinase).

Scheme 1[a]



[a] (a) Boc_2NH, NaH, THF; (b) 5 % anisole, TFA; (c) $(MeO)_2POCH_2COOMe$, NaH, THF; (d) H_2, 5 % $Pd/BaSO_4$, toluene; (e) LiOH, p-dioxane, water; (f) DPPA, TEA, t-BuOH, Δ; (g) HCl, EtOAc; (h) Boc-D-Phe-Pro-OH, EDC, HOBT, DIEA, CH_2Cl_2; (i) H_2S, pyridine; (j) MeI, acetone; (k) NH_4OAc, MeOH.

Table 1.[a]

		------ Trypsin ------		------ Plasmin ------		-------- n-tPA --------		----- Urokinase -----	
no.	Thrombin[b]	Activity[b]	Selectivity[c]	Activity[b]	Selectivity[c]	Activity[b]	Selectivity[c]	Activity[b]	Selectivity[c]
(2)	5.4×10^8	1.1×10^8	5.1	1.4×10^6	390	2.9×10^5	1,800	4.2×10^4	13,000
(1)	5.5×10^6	1.7×10^5	33.	1.2×10^3	4,600	6.0×10^3	920	1.8×10^2	31,000
(12)	6.8×10^8	5.3×10^6	130.	2.6×10^4	26,000	4.0×10^3	170,000	1.7×10^3	400,000
(13)	9.7×10^5	2.8×10^4		$< 2.5 \times 10^2$ [d]		6.6×10^3		$< 2.5 \times 10^2$ [d]	
(14)	8.1×10^6	8.8×10^4		4.3×10^3		1.7×10^2		7.2×10^2	
(15)	1.3×10^6	1.7×10^4		1.3×10^3		7.6×10^2		2.6×10^2	

[a] All values are the average of at least 3 separate experiments with a standard deviation of less than 20%. [b] K_{ass} in L/mol.[10] [c] Ratio of Thrombin K_{ass} / Enzyme K_{ass}. [d] Since no enzyme inhibition was observed at an inhibitor concentration of 200 μM, the K_{ass} for this complex must be less than 2.5×10^2.

In order to prove that the increase in potency and selectivity in **12** came as a result of the predicted improvement in noncovalent interactions with the thrombin specificity pocket, we obtained crystals of the **12**-thrombin complex for X-ray analysis.[11] A partial representation of the resulting structure appears in Figure 2. Like agmatine in the thrombin complex of **1**,[4] the benzamidine of **12** binds in the thrombin specificity pocket, and the amidine hydrogen bonds to Asp 189.[12] The benzene ring is sandwiched between two main chain loops, Ser 214 - Glu 217 and Asp 189 - Glu 192, and is parallel to the latter. The proline residue of **12** is located in the hydrophobic pocket formed by His 57, Tyr 60A, Leu 99, and Trp 60D. The rest of the inhibitor fits into another hydrophobic region presented by residues Trp 215, Ile 174, and Leu 99, with the phenyl ring of the inhibitor's N-terminal residue (D-Phe) oriented perpendicular to the indole ring of Trp 215. Finally, the peptidyl chain of **12** interacts with thrombin segment Ser 214 - Gly 216 by formation of an anti-parallel β-pleated sheet. This binding orientation is analogous to the bound conformation of related peptide thrombin inhibitors based on the *D*-Phe-Pro-Arg sequence,[4,7,13] which simulate the interaction of thrombin's active site with the natural substrate fibrinogen.[14] Thus, the structure confirms that the only significant difference between the thrombin complexes of *D*-Phe-Pro-Agm (**1**) and *D*-Phe-Pro-*p*-amidinobenzylamine (**12**) is the improved complementarity of the benzamidine for the thrombin specificity pocket.

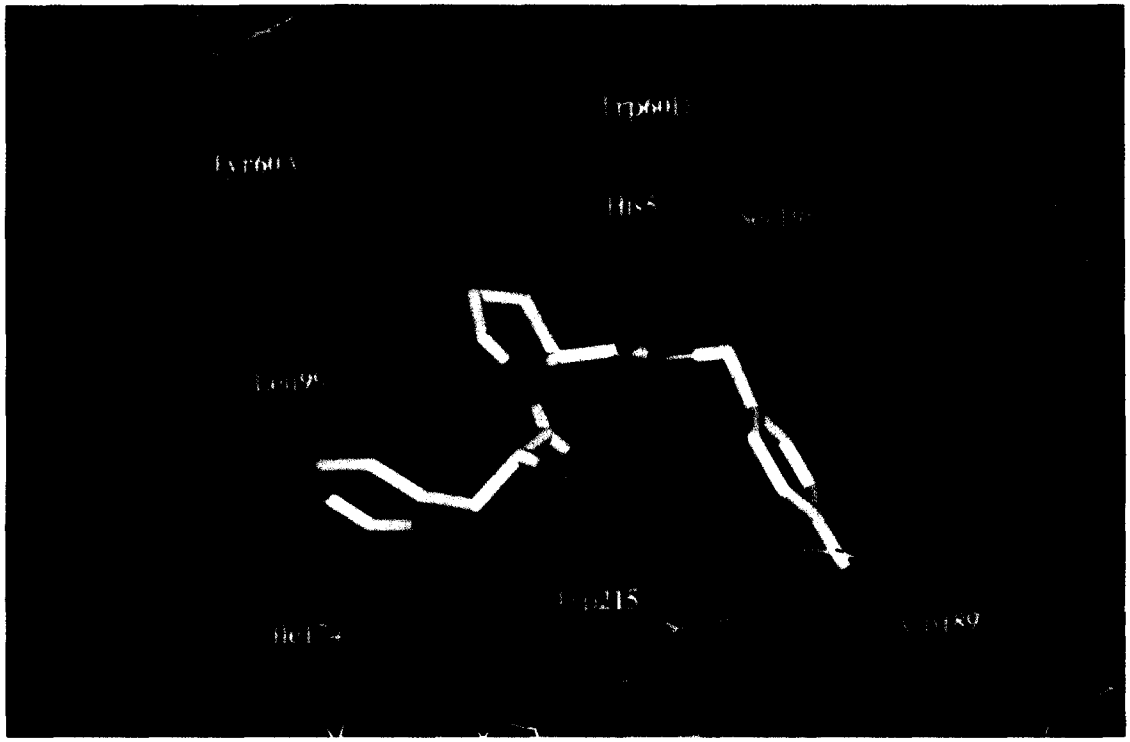


Figure 2. Representation of the structure of the **12**-thrombin complex

Conclusion

At the outset, our goal was to improve the thrombin inhibitory potency of *D*-Phe-Pro-Agm to the level of analogous tripeptide arginals, while retaining the high selectivity of the agmatine series. This goal was achieved by the replacement of agmatine with *p*-amidinobenzylamine, which serves as a very useful alternative to arginine-based transition-state analogs. The increase in binding energy achieved by enhancing the noncovalent interaction of agmatine with the thrombin specificity pocket completely compensates for the loss of the C-terminal aldehyde from *D*-Phe-Pro-Arg-H, and high selectivity for thrombin versus trypsin and the fibrinolytic enzymes is retained. In addition to these biological attributes, derivatives of *p*-amidinobenzylamine are simple, robust molecules which are readily accessibile from inexpensive, commercially available starting materials.

In summary, transition-state analogs are an excellent source of first-generation enzyme inhibitors. With a covalent bond serving as a powerful source of binding energy, they can be used as a platform to study the interactions of residues on the inhibitor with corresponding regions of the target protein. However, once significant potency is achieved, we find that the covalent bond of the transition-state analog can be sacrificed and replaced with noncovalent interactions in order to improve the pharmacological/chemical properties of the inhibitor.[15]

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