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SERINE PROTEASE SELECTIVITY OF THE THROMBIN INHIBITOR D-PHE-PRO-AGMATINE AND ITS HOMOLOGS

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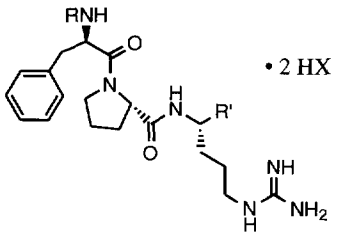
Abstract: Analogs of D-Phe-Pro-Agmatine were assayed for inhibititory activity versus thrombin, trypsin, plasmin, n-tPA and urokinase. The X-ray structure of the thrombin/D-Phe-Pro-Agmatine co-crystal revealed that the agmatine and analogous arginals have very similar bound conformations.

Agents that inhibit thrombin are important targets in the search for novel antithrombotics.[1] Like trypsin, thrombin is a serine protease which utilizes an Asp-His-Ser catalytic triad to hydrolyze peptide bonds on the C-terminal side of arginine residues. However, there are other endogenous "trypsin-like" serine proteases that serve important regulatory functions.[2] Thus, in the development of thrombin inhibitors for use as therapeutic agents, control over the selectivity of enzyme inhibition is an important issue. In particular, selectivity should be high for the inhibition of thrombin relative to the fibrinolytic enzymes (plasmin, t-PA and urokinase) since their activity is required for the *dissolution* of blood clots.[3]

A common theme in the design of serine protease inhibitors is the substrate analog strategy.[4] In this approach, peptide sequences found in the vicinity of the scissile bond in natural substrates of the target enzyme serve as the starting point for inhibitor development. To provide enzyme inhibitory potency, the amide at the cleavage site is replaced by an electrophilic group which forms a covalent bond with the serine of the catalytic triad. The resulting complex resembles the tetrahedral transition state of a proteolysis reaction. This strategy was used by Bajusz, et al. in the development of the potent thrombin inhibitors R-D-Phe-Pro-Arg-H (**1**, R=H; **2**, R=Me),[5] where the peptide sequence simulates the interactions of the A-chain of fibrinogen with the active site region of thrombin[6] and the arginine aldehyde is used to form a hemiacetal with the serine of the catalytic triad.[7a]

In 1982, Bajusz, et al. reported the synthesis and anticoagulant activity of D-Phe-Pro-Agmatine (Agm) (**3**),[8] in which the C-terminal aldehyde was replaced by a hydrogen. Although the agmatine derivative was less potent than analogous arginals, we were intrigued that significant anticoagulant activity was retained in (**3**), both *in vitro* and *in vivo*, despite the loss of the covalent bond between thrombin and the inhibitor. In order to deter-



• 2 HX

1 - R = H, R' = CHO

2 - R = Me, R' = CHO

3 - R = H, R' = H

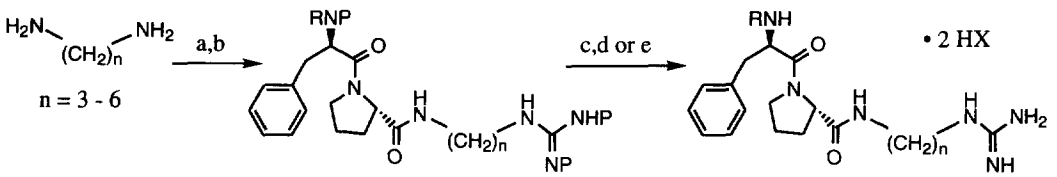
mine the thrombin-bound conformation of D-Phe-Pro-Agm (**3**), for comparison with the structures of covalently bound peptide thrombin inhibitors,[7] **3** was co-crystallized with thrombin and the resulting crystals were subjected to X-ray analysis.[9] Figure 1 represents the active site region of the D-Phe-Pro-Agm (**3**)/human α-thrombin complex. Agmatine (like the side chain of arginine) fits into the thrombin specificity pocket and its guanidinium ion engages the carboxylate of Asp 189.[10] The proline ring of the inhibitor fits into the hydrophobic pocket defined by His 57, Tyr 60A, Trp 60D, and Leu 99. The sidechain of the N-terminal residue (D-Phe) fits into another hydrophobic region presented by residues Trp 215, Ile 174 and Leu 99. The peptide backbone of the inhibitor interacts with thrombin segment Ser 214 - Gly 216 by formation of an anti-parallel β-pleated sheet. In summary, the thrombin-bound conformation of D-Phe-Pro-Agm is very similar to the structures of covalently bound peptide thrombin inhibitors.

While there is considerable overlap between the thrombin-bound conformations of D-Phe-Pro-Agm and analogous arginals, the conformational similarities may not extend to the complexes of other enzymes. Thus, we were interested to learn how the difference in enzyme inhibitory potency between agmatine and arginal based peptides varies with serine proteases related to thrombin. Since the selectivity of enzyme inhibition was not explicitly addressed in previous studies with D-Phe-Pro-Agm,[8] we directly compared **3** , the N-Me analog **9** (Scheme 1), and the corresponding arginals (**1**) and (**2**), with respect to thrombin inhibitory potency and selectivity versus tryspin and the fibrinolytic enzymes plasmin, t-PA and urokinase.

Figure 1

The agmatine derivatives were prepared according to the sequence illustrated in Scheme 1. Symmetrical diamines were monoguanylated,[11] coupled with a dipeptide acid, and the desired products were deprotected. Where necessary, the final products were then purified by preparative reverse phase HPLC.[12]

Scheme 1[a]



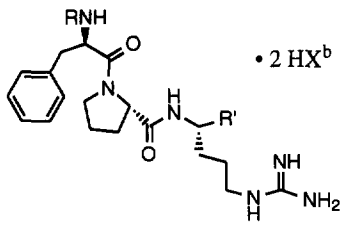
	n	R	P		n	R
(4)	4	H	Boc	**(3)**	4	H
(5)	4	Me	Cbz	**(9)**	4	Me
(6)	3	H	Boc	**(10)**	3	H
(7)	5	H	Boc	**(11)**	5	H
(8)	6	H	Boc	**(12)**	6	H

[a] (a) MeSC(NP)NHP, H_2O/DMF; (b) P-N-R-D-Phe-Pro-OH, EDC, HOBT, DIEA, DMF; (c) HCl, EtOAc; (d) 5 % anisole, TFA; (e) H_2, 10 % Pd/C, EtOH, H_2O, HCl;

Table 1 shows the apparent association constants[13] of the tripeptide arginals D-Phe-Pro-Arg-H (**1**) and N-Me-D-Phe-Pro-Arg-H (**2**), along with the corresponding tripeptide agmatines (**3**) and (**9**) for human α-thrombin, as well as selectivity ratios for the thrombin association constants versus those of trypsin, plasmin, t-PA and urokinase. The data illustrate that there is about a two order of magnitude difference in the thrombin association constants of both aldehydes relative to their corresponding agmatines. Thus, in the D-Phe-Pro-Arg series, the C-terminal aldehyde contributes approximately 3 kcal/mol[14] of association energy to the inhibitor-thrombin complex. Alternatively, the aldehyde contributes 4.2 kcal/mol to the binding energy in the case of plasmin and only 2.3 kcal/mol to the stability of the t-PA complexes, as estimated by the difference between the K_{ass} of compounds **1** and **3** or **2** and **4**.

As expected for compounds based on the D-Phe-Pro-Arg sequence,[5,6] all of the inhibitors have higher affinity for thrombin than trypsin or the fibrinolytic enzymes. However, as a result of the variable contribution of the C-terminal aldehyde to association energy, there are differences between the selectivity profiles of the arginals and agmatines. The most interesting and potentially useful difference is the order of magnitude improvement in plasmin selectivity which comes in each case with the loss of the C-terminal aldehyde. There is also some improvement in trypsin selectivity for each agmatine relative to the corresponding arginal. Alternatively with n-tPA, selectivity is slightly compromised with the agmatines, although the thrombin/t-PA selectivity ratio is still about three orders of magnitude for both **3** and **9**. With respect to urokinase, excellent selectivity is observed with all of the compounds. So although the agmatines **3** and **9** are less potent thrombin inhibitors than the corresponding arginals **1** and **2**, they appear to have better overall selectivity profiles.

Table 1.[a]



				Trypsin		Plasmin		n-tPA		Urokinase	
Cmpd.	R	R'	Thrombin[c]	Potency[c]	Ratio[d]	Potency[c]	Ratio[d]	Potency[c]	Ratio[d]	Potency[c]	Ratio[d]
(1)	H	CHO	$5.4x10^8$	$1.1x10^8$	5.1	$1.4x10^6$	390	$2.9x10^5$	1,800	$4.2x10^4$	13,000
(3)	H	H	$5.5x10^6$	$1.7x10^5$	33.	$1.2x10^3$	4,600	$6.0x10^3$	920	$1.8x10^2$	31,000
(2)	Me	CHO	$4.6x10^8$	$8.8x10^7$	5.2	$1.8x10^6$	260	$5.0x10^4$	8,500	$3.2x10^4$	14,000
(9)	Me	H	$2.4x10^6$	$2.4x10^5$	10.	$1.1x10^3$	2,100	$1.1x10^3$	2,100	$1.8x10^2$	13,000

[a]All values are the average of at least 3 separate experiments with a standard deviation of less than 20%. [b]The sulfate salt of compound **1** was tested; for all other compounds, the hydrochloride salt was used. [c]K_{ass} in L/mol. [d]Ratio of Thrombin K_{ass}/Enzyme K_{ass}.

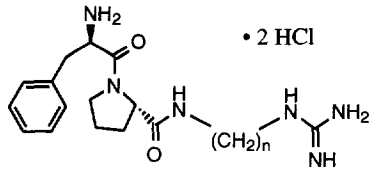
In order to evaluate the dependence of potency and selectivity on the length of agmatine's carbon chain, the homologs **10 - 12** were prepared (Scheme 1).[15] Since the carbon chain length in agmatine is the same as arginine, it is not suprising that D-Phe-Pro-Agm (**3**) is the most potent thrombin inhibitor in the series illustrated in Table 2. However, relative to agmatine **3**, only about 0.8 kcal/mol in binding energy is sacrificed by the removal (**10**) or the addition (**11**) of one carbon to the agmatine tether. This is particularly interesting in light of the selectivity ratios for **10** and **11**, which demonstrate that manipulation of the tether length can provide some additional control over serine protease specificity. Increasing the tether length to 5 carbons eliminates inhibitory activity against all of the fibrinolytic enzymes, and also improves the thrombin to trypsin selectivity ratio to 480. As a result of these attributes, compound **11** has the most attractive profile in the series.

Conclusion

In theory, optimal selectivity is achieved when inhibitors derive their binding energy from interactions with unique structural elements of the target enzyme. The crystal structure represented above confirms that D-Phe-Pro-Agm, like the corresponding arginals, derives binding energy from non-covalent interactions with the thrombin specificity pocket and the neighboring hydrophobic binding sites, mimicking interactions of the natural substrate fibrinogen. However, unlike the arginals, D-Phe-Pro-Agm does not derive binding energy from a covalent interaction with the serine of the catalytic triad. Since the Asp-His-Ser catalytic triad and the adjacent oxyanion hole are the defining structural features of all serine proteases, it may be advantageous to avoid covalent interactions with this highly conserved structural region in the design of *selective* thrombin inhibitors.

In any case, the data demonstrate that agmatine and its homologs are useful tools for controlling the serine protease selectivity of peptide thrombin inhibitors. Future work will focus on strategies to improve the thrombin inhibitory potency of the agmatine series while retaining the selectivity profile.[16]

Table 2.[a]



			Trypsin		Plasmin		n-tPA		Urokinase	
Cmpd.	n	Thrombin[b]	Potency[b]	Ratio[c]	Potency[b]	Ratio[c]	Potency[b]	Ratio[c]	Potency[b]	Ratio[c]
(10)	3	$1.4x10^6$	$1.4x10^4$	100.	$<2.5x10^2$ [d]	>5,600	$4.1x10^3$	341	$<2.5x10^2$ [d]	>5,600
(3)	4	$5.5x10^6$	$1.7x10^5$	33.	$1.2x10^3$	4,600	$6.0x10^3$	920	$1.8x10^2$	31,000
(11)	5	$1.3x10^6$	$2.7x10^3$	480.	$<2.5x10^2$ [d]	>5,200	$<2.5x10^2$ [d]	>5,200	$<2.5x10^2$ [d]	>5,200
(12)	6	$2.4x10^4$	$6.0x10^2$		$<2.5x10^2$ [d]		$<2.5x10^2$ [d]		$<2.5x10^2$ [d]	

[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. [c]Ratio of Thrombin K_{ass}/Enzyme K_{ass}. [d]Since no enzyme inhibition was observed at an inhibitor concentration of 200 μM, the Kass for this complex must be less than $2.5x10^2$.

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References and Notes

1. Markwardt, F.; Hauptmann, J. In *Advances in Experimental Medicine and Biology, Volume 340: The Design of Synthetic Inhibitors of Thrombin*; Claeson, G.; Scully, M. F.; Kakkar, V. V.; Deadman, J., Eds.; Plenum: New York, 1993; pp 143-171.
2. Nevrath, H. *J. Cellular Biochemistry* **1986**, *32*, 35.
3. Francis, C. W.; Marder, V. J. In *Hemostasis and Thrombosis: Basic Priciples and Clinical Practice, 2nd Ed.*; Colman, R. W.; Hirsh, J.; Marder, V. J.; Salzman, E. W. Eds.; J. B. Lippincott Co.: Philadelphia, 1987; Chapter 22.
4. Coggins, J. R.; Kray, W.; Shaw, E. *Biochem. J.* **1974**, *137*, 579.
5. Bajusz, S.; Szell, E.; Bagdy, D.; Barabas, E.; Horvath, G.; Dioszeg, M.; Fittler, Z.; Szabo, G.; Juhasz, A.; Tomori, E.; Szilagyi, G. *J. Med. Chem.* **1990**, *33*, 1729.

6. (a) Claeson, G.; Aurell, L. *Ann. N. Y. Acad. Sci.* **1981**, *370*, 789; (b) Ni, F.; Konishi, Y.; Frazier, R. B.; Scheraga, H. A. *Biochemistry* **1989**, *28*, 3094.
7. (a) Chirgadze, N. Y.; Clawson, D. K.; Gesellchen, P. D.; Hermann, R. B.; Kaiser, R. E., Jr.; Olkowski, J. L.; Sall, D. J.; Schevitz, R. W.; Smith, G. F.; Shuman, R. T.; Wery, J. P.; Jones, N. D. *American Crystallographic Association Meeting, August 9-14*; University of Pittsburgh, **1992**, *20*, 116. (b) Bode, W.; Turk, D.; Karshikov, A. *Protein Science* **1992**, *1*, 426.
8. Bajusz, S.; Szell, H.; Barabas, E.; Bagdy, D.; Nagy, Z. M. US Patent 4,346, 078, August 24, 1982.
9. The crystal structure of the complex of human α-thrombin with an exosite binder [hirudin peptide (54-65)][7b] and the active site inhibitor **3** was crystallographically refined at 2.0 Å resolution using the simulated annealing method presented by the X-PLOR[17] program and the restrained least-squares technique using PROLSQ.[18] A visual inspection and manual model correction was done with the interactive graphic program FRODO.[19] An experimental data set of 16,438 unique reflections, representing 70% of all theoretically possible reflections in the resolution range of 6.0-2.0 Å with Rmerge=7.3% was used. The position of the inhibitor in the active site cleft was determined by different Fourier electron density distribution. The final model includes 2,840 non-hydrogen atoms, of which 263 are water oxygen atoms, and has a crystallographic R-factor of 19.9% with bond length standard deviation from ideal by 0.019 Å.
10. The numbering of thrombin residues is obtained on the basis of topological equivalences with chymotrypsinogen. Hartley, B. S.; Shotton, D. M. In *The Enzymes*; (Boyer, P. D., Ed.), Academic: New York, 1971; Vol III, pp 323-373.
11. Bergeron, R. J.; McManis, J. S. *J. Org. Chem.* **1987**, *52*, 1700.
12. All new compounds were characterized by combustion analysis, NMR, IR, MS, OR and amino acid analysis.
13. Smith, G. F.; Shuman, R. T.; Craft, T. J.; Gifford, D. S.; Kurz, K. D.; Jones, N. D.; Chirgadze, N.; Hermann, R. B.; Coffman, W. J.; Sandusky, G. E.; Roberts, E.; Jackson, C. V. *Seminars In Thrombosis and Hemostasis*, **1995**, *in press.*
14. The estimation of the free energy of binding is made using the apparent K_{ass} in the equation $\Delta G = -RT \ln K$. For discussions on the contribution of individual functional groups to the total binding energy of enzyme inhibitors: (a) Kati, W. M.; Acheson, S. A.; Wolfenden, R. *Biochemistry* **1992**, *31*, 7356; (b) Liu, S.; Hanzlik, R. P. *Biochim. Biophys. Acta* **1993**, *1158*, 264.
15. With the 3 carbon tether, the monoguanylated intermediate was not concentrated by rotary evaporation to avoid cyclization.

16. One approach to improving potency in the agmatine series has recently been reported. Teger-Nilsson, A. C.; Gyzander, E.; Andersson, S.; Englund, M.; Mattson, C.; Ulvinge, J. C.; Gustafsson, D. *Thromb. and Haemostas.* **1995**, *73*, 1325.
17. Brunger, A. T. *Acta Crystallogr. A* **1990**, *46*, 46.
18. Hendrickson, W. A.; Konnert, J. H. In *Biomolecular Structure, Function, Conformation and Evolution*: Srinivasan, R., Ed.; Pergamon: Oxford, 1981; pp 43-57.
19. Jones, T. A. *J. Appl. Crystallogr.* **1978**, *11*, 268.

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