Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 2767–2772

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

THE DESIGN OF POTENT, SELECTIVE, NON-COVALENT, PEPTIDE THROMBIN INHIBITORS UTILIZING IMIDAZOLE AS A S1 BINDING ELEMENT

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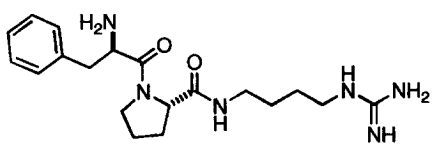
Received 1 June 1999; accepted 19 August 1999

Abstract: Modeling of neutral or mildly basic functional groups in the S1 site of thrombin led to the targeting of imidazole as a S1 binding element and correctly predicted the optimal chain length for connecting this group with the S2 and S3 binding elements. Derivatives of 4-(3-aminopropyl)-imidazole can be selective inhibitors of thrombin demonstrating potent anticoagulant activity.

Among the numerous strategies for the control of thrombosis that are currently under investigation, direct inhibition of the trypsin-like serine protease thrombin has been the most popular approach due to thrombin's central role in the control of blood coagulation.[1] As part of a program to identify potent and selective inhibitors of thrombin, we previously reported on the selectivity of the peptide thrombin inhibitor D-Phe-Pro-Agmatine **1**,[2] as well as the enhancement in potency which is achieved upon replacement of agmatine with the S1 binding element amidinobenzylamine, as in compound **2**.[3] Although such peptides display both potent anticoagulant activity and a high level of serine protease selectivity, their activity is predicated upon the interaction of Asp 189 in the S1 site with a highly basic functional group. Given the barrier that such functional groups can provide to transport across membranes, we were interested in looking for alternative S1 binding elements, which are significantly less basic than amidines or guanidines.

In order to identify neutral or mildly basic functional groups with affinity for the S1 site, a series of model probes were computationally constructed and evaluated for their complementarity with the binding pocket.[4] Each probe was equipped with at least one hydrogen bond donating group and a methyl substituent, which was included to insure that a point of attachment was available for connecting the S1 binding element to the S2 and S3 binding elements of the inhibitor. In this report we present results for the 4-methyl imidazolium ion.[5]



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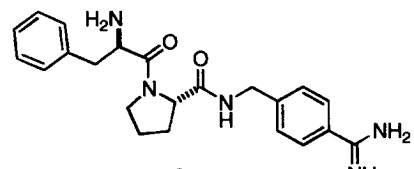

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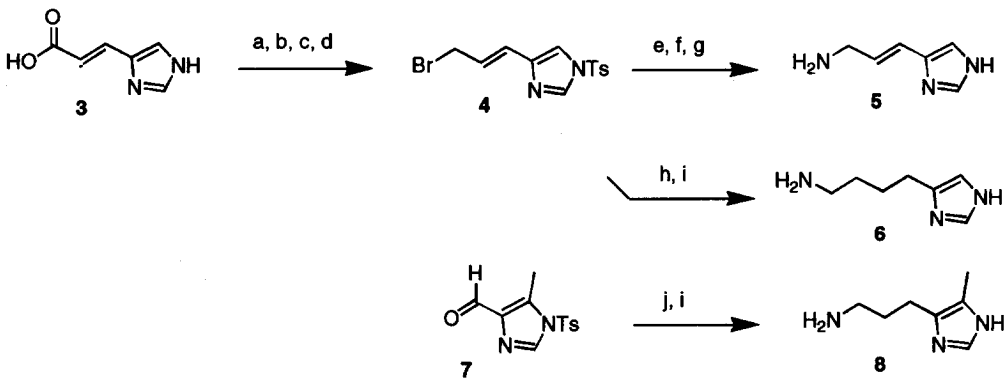
PII: S0960-894X(99)00459-X

Figure 1. Molecular modeling results for the human α-thrombin active site. **1A** and **1B**: The two energy minimized positions for 4-methyl imidazolium ion (orange) in the S1 pocket. The D-Phe-Pro-NH scaffold (green) from the starting X-ray structure[2] was used. **1C**: The energy minimized complex for the three carbon tethered analog (green). In each figure, possible hydrogen bonds are shown as thin dashed lines.

Optimal positions for the imidazolium ion probe within the specificity pocket were investigated by placing it in several distinct starting orientations at the entrance of the S1 pocket. For each orientation, the probe was energy minimized in the environment of the rigid protein. Two well-defined orientations resulted, each hydrogen bonded to the carbonyl of Gly 218 and the side-chain carboxyl of Asp 189 (Figure 1A and 1B). The difference between these is found in the orientation of the methyl substitutent. In the model depicted in Figure 1B, the methyl group points directly toward the entrance of the S1 pocket. Alternatively, in the model shown in Figure 1A, this methyl fits into the small hydrophobic hole defined by residues Ala 190, Val 213 and Tyr 228, suggesting that substitution on the 5-position of the imidazole ring will be tolerated (**13**, Table 1). Next, a graphical assessment was made to determine how many methylene groups would optimally be needed to attach the probe to the dipeptide scaffold (Figure 1A and 1B). In each case, the scaffold and probe were edited to produce analogs having tethers of one to four methylenes, and the active site complexes were solvated and energy minimized. Analysis of the resulting structures revealed that as the tether length was increased from one to three methylenes, both the imidazolium ion/protein hydrogen bonds and the overall original binding orientation of the scaffold were better maintained. Therefore the three carbon tether was predicted to have the most favorable binding (Figure 1C). The model also suggested that introduction of a double bond in conjugation with the imidazole ring would be productive through rigidification of the molecule with minimal perturbation of the bound conformation.

To test the modeling hypothesis, imidazole derivatives were prepared with an amine tethered two to four carbons from the 4-position of the imidazole. Compounds containing a two carbon tether were prepared from

Scheme 1.[a]



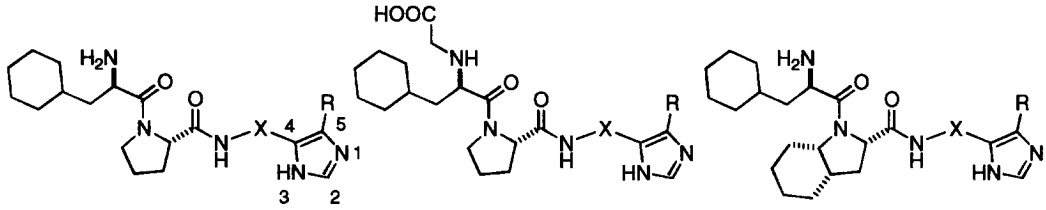
[a] (a) MeOH, HCl; (b) TsCl; (c) Dibal; (d) CBr_4, PPh_3; (e) $HN(Boc)_2$, NaH; (f) HOBT, THF; (g) TFA; (h) NaCN; (i) H_2, PtO_2; (j) $(EtO)_2POCH_2CN$, NaH.

commercially available histamine. As shown in Scheme 1, intermediates containing a three or four carbon tether were prepared from urocanic acid (**3**) in a similar procedure to that reported by Schunack.[6] The key allylic bromide intermediate **4** was prepared via esterification of the carboxylic acid, followed by tosylation of the imidazole, reduction of the ester and then bromination of the resulting allylic alcohol. The three carbon tethered intermediate **5** was prepared by displacement of the bromide (**4**) with the sodium salt of $HN(Boc)_2$ followed by deprotection. The four carbon tethered intermediate **6** was prepared by cyanation of bromide **4**, followed by exhaustive hydrogenation. The 5-methyl imidazole derivative **8** was prepared by a Horner - Emmons olefination of aldehyde **7**, followed by hydrogention. The various imidazole containing intermediates were coupled to dipeptide acids by standard methods, and the desired products were deprotected and purified by RPHPLC.[7]

As Table 1 indicates, the compounds containing a three carbon tether were indeed found to be optimal for this series. Compounds **10** and **11** are about an order of magnitude more potent than both the two carbon tethered compound (**9**) and the four carbon tethered compound (**12**). Although no significant increase in activity was achieved with the more rigid tether (**11**), titration studies revealed that the introduction of an olefin substituent onto the imidazole decreased the basicity of the heterocycle by almost an order of magnitude. The pK for the imidazole ring in compound **11** was found to be 5.94 vs 6.74 for the saturated derivative **10**.[8] Thus, it is possible that the favorable decrease in entropy from conformational constraint in compound **11** was offset by the unfavorable shift in the acid-base equilibrium. The table also illustrates that substitution of a methyl group at the 5-position of the imidazole (**13**) is tolerated as predicted by modeling studies.

The data for compounds **14** - **17** illustrate that structural modifications at both the P2 and P3 residues of the inhibitors can be used to achieve further increases in thrombin inhibitory activity. At the P3 position, affinity is increased by an additional order of magnitude by incorporation of a carboxymethyl group[9] at the *N*-terminus

Table 1.[a]



9 - 13 **14 - 15** **16 - 17**

Compound	R	X	Human α–Thrombin K_{ass} (x 10^6 L/mol)
9	-H	$-CH_2CH_2-$	4.4
10	-H	$-CH_2CH_2CH_2-$	20.
11	-H	-CH_2-*trans*-CHCH-	16.
12	-H	$-CH_2CH_2CH_2CH_2-$	1.3
13	$-CH_3$	$-CH_2CH_2CH_2-$	23.
14	-H	$-CH_2CH_2CH_2-$	100.
15	-H	-CH_2-*trans*-CHCH-	84.
16	-H	$-CH_2CH_2CH_2-$	34.
17	-H	-CH_2-*trans*-CHCH-	7.0

[a]All values are the average of at least three separate experiments with a standard deviation of less than 20%.

whether the tether is saturated (**14**) or unsaturated (**15**). At the P2 position, an increase in activity is observed with the more hydrophobic octahydroindole residue,[10] but only in combination with the saturated tether (**16**). The more rigid tether in **17** appears to cause greater sensitivity to structural manipulation at the P2 residue.

The X-ray crystal structure of the human α-thrombin/**14** complex[11] (Figure 2) confirms the proposed binding mode. The inhibitor binds in a similar orientation to that reported for other peptidomimetics, such as **1**[2] and **2**[3]. As expected for **14**, the imidazole forms hydrogen bonds to both the carboxylic acid of Asp 189 and the carbonyl of Gly 218. The proline ring occupies the S2 pocket and the P3 cyclohexyl ring is positioned in the hydrophobic S4 region, perpendicular to the Trp 215 indole side chain. The N-terminal carboxyl group points away from the active site and is predominately exposed to solvent.

The serine protease selectivity profiles for compounds **10**, **14**, and **16** are reported in Table 2, along with the tripeptide arginal efegatran for comparison. The data illustrate that the very high selectivity seen with other non-covalent peptide inhibitors[2,3] is retained with the imidazole series. Selectivity for thrombin over trypsin was at least several hundred-fold for each of the compounds in this series, and over 10,000-fold selectivity was achieved for thrombin versus the fibrinolytic enzymes and activated protein C.

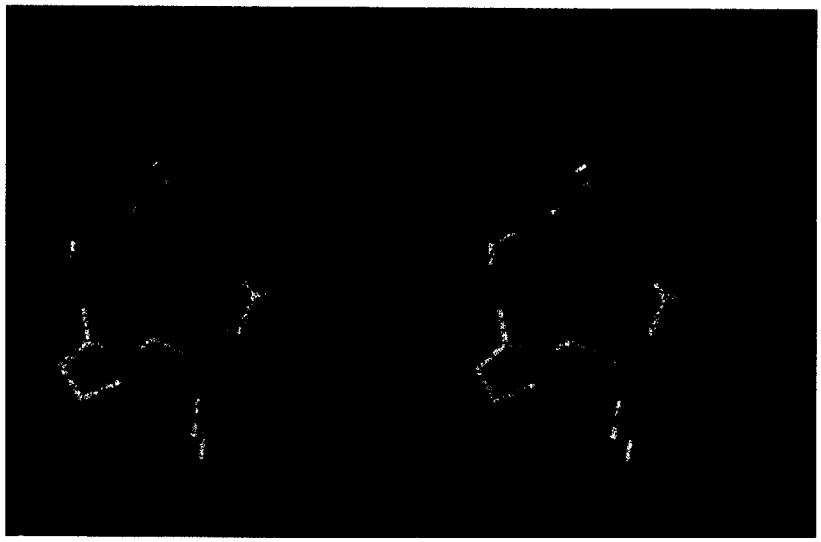


Figure 2. Stereoview of the X-ray crystal structure of **14** bound to the active site of human α-thrombin. The blue mesh represents electron density attributed to the inhibitor. The σ_A electron density map was calculated for a 20-2.3 Å resolution range and contoured at the 1.0 σ level.

Table 2.[a]

Compound	Enzyme K_{ass} (x 10^6 L/mol)					
	Thrombin	Trypsin	Plasmin	tPA	Urokinase	aPC
10	20.	0.01	< 0.01	< 0.01	< 0.01	< 0.01
14	100.	0.29	< 0.01	< 0.01	< 0.01	0.02
16	34.	0.04	< 0.01	< 0.01	< 0.01	0.01
D-Cha-Pro-Agm	30.	0.26	0.01	0.011	<0.01	0.01
efegatran	670.	91.	1.5	0.05	0.04	0.30

[a]K_{ass} values are the average of at least three separate experiments with a standard deviation of less than 20%.

Table 3.

Compound	Human α–Thrombin K_{ass} (x 10^6 L/mol)	2 x TT[a]	2 x APTT[b]	2 x PT[c]
10	20.	74.	660.	1,000.
14	100.	20.	170.	380.
16	34.	61.	470.	700.
D-Cha-Pro-Agm	30.	44.	490.	770.
efegatran	670.	41.	930.	1,000.

[a]Concentration of test compound (ng/mL) required to double the thrombin time. [b]Concentration of test compound (ng/mL) required to double the activated partial thromboplastin time. [c]Concentration of test compound (ng/mL) required to double the prothrombin time.

Finally, the anticoagulant profiles for these representative examples are illustrated in Table 3. The data show that for inhibitors in this series, thrombin inhibitory potency translates very well into potent anticoagulant activity in human plasma, also comparing favorably with efegatran.

In conclusion, molecular modeling was used to identify imidazole as a useful S1 binding element in non-covalent, peptide-derived thrombin inhibitors. A three-carbon tether was predicted and found to be optimal for connecting the peptide fragment to the 4-position of imidazole. These non-covalent imidazole based inhibitors have similar potency to corresponding agmatine derived inhibitors and are superior to corresponding transition-state analogs such as arginine aldehydes with respect to both selectivity and anticoagulant potency.

References and Notes

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3. Wiley M. R.; Chirgadze, N. Y.; Clawson, D. K.; Craft, T. J.; Gifford-Moore, D. S.; Jones, N. D.; Olkowski, J. L.; Weir, L. C.; Smith G. F. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2387.
4. Molecular modeling was performed using QUANTA/CHARMm, version 96 (Molecular Simulations Inc., San Diego, CA). Default settings were maintained and energy minimizations taken to convergence using the ABNR algorithm. The X-ray crystal structure of the D-Phe-Pro-Agm/human α-thrombin complex was used.[2] The protein system was prepared by removing the inhibitor, adding all hydrogens to the protein and resolved water molecules, and then optimizing the hydrogen positions with successive applications of the HBUILD procedure. Water molecules were then removed. Optimum positions for an imidazolium ion (+1 total charge) were investigated by energy minimization in the environment of the rigid protein structure. The D-Phe-Pro-NH scaffold was constructed by editing the original ligand X-ray structure. This scaffold was reinserted into the active site and geometric measurements made to determine the range of tethers to consider. Analogs were each processed, first by energy minimizing the introduced linkage and the imidazole group while the inhibitor scaffold portion and the protein were fixed in space. This was further processed by reinserting the crystallographic waters and then solvating the active site with a 20Å water sphere. For the final systems the total ligand, all protein residues having an atom within 14Å, and all waters within 20Å (of the active site center) were then energy minimized.
5. For other reports on the use of imidazole in S1 binding elements, see: (a) Dominguez, C.; Carini, D. J.; Weber, P. C.; Knabb, R. M.; Alexander R. S.; Kettner, C. A.; Wexler R. R *Bioorg. Med. Chem. Lett.* **1997**, *7*, 79. (b) Issacs, R. C. A.; Newton, C. L.; Solinsky, M. G.; Naylor-Olsen, A. M. *217th National ACS Meeting*, Anaheim, CA, March 1999, Abstract MEDI-005.
6. Sellier, C.; Buschauer, A.; Elz, S.; Schunack, W. *Liebigs Ann. Chem.* **1992**, 317.
7. Although the modeling studies described above were done using the S3-S2 binding elements (D-Phe-Pro) available from our D-Phe-Pro-Agm/Thrombin crystal structure,[5] the target molecules were prepared using D-Cha-Pro since this modification was known to produce some increase in thrombin affinity and yet maintain a similar overall binding orientation (Schacht, A.L.; Smith G. F.; Wiley, M. R. U. S. Patent 5,705,487; *Chem. Abstr.* **1998**, *128*, 128286). For experimental details of the synthesis of compounds **9** - **17**, see Klimkowski, V. J.; Wiley, M. R. US Patent 5,811,402; *Chem. Abstr.* **1998**, *129*, 265470.
8. Titrations were performed according to the method of Slater, B.; McCormack, A.; Avdeef, A.; Comer, J. E. A. *J. Pharm. Sci.* **1994**, *83*, 1280.
9. Teger-Nilsson, A.; Bylund, R.; Gustafsson, D.; Gyzander E.; Eriksson, U. *Thromb. Res.* **1997**, *85*, 133.
10. Chirgadze, N. Y.; Schacht, A. L.; Smith G. F.; Wiley, M. R. US Patent 5,599,793; *Chem. Abstr.* **1995**, *123*, 306600f.
11. The human α-thrombin/**14** cocrystal was prepared using conditions described in: Chirgadze, N. Y.; Sall, D. J.; Hermann, R.; Clawson, D. K.; Klimkowski, V. J., Smith, G. F.; Gifford-Moore, D. S.; Coffman, W. J. *Protein Science* **1997**, *6*, 1412. The crystals belong to the C2 space group with unit cell constants a = 71.0 Å, b = 71.9 Å, c = 73.0 Å, β = 100.7°, and have one molecular complex per asymmetric unit. An X-ray data set was collected using a conventional rotating anode with CuK_α radiation (λ = 1.54 Å). An imaging plate (R-AXISII) was used as a detector. A total of 50,385 observations were measured and yielded 15,281 unique reflections with an R_{merge} of 7.8% (24.6% for outer shell). This represents 95% of all reflections that are theoretically possible for resolution range of 30-2.3 Å. A final crystallographic R-value of 18.4% was obtained for 13,517 reflections, with $F > 2\sigma$ in the 20-2.3 Å resolution range. An R_{free} of 23.3% was calculated, using a random selection of approximately 5% of the data not involved in the crystallographic refinement. The root mean square deviation from ideal bond lengths was 0.010 Å, that from ideal angles 1.5°. Model inspection and correction between cycles of the refinement and crystallographic refinement itself was performed within the QUANTA97 and X-PLOR98 program suites (Molecular Simulations Inc., San Diego, CA).