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D-Phe-Pro-Arg Type Thrombin Inhibitors: Unexpected Selectivity by Modification of the P1 Moiety

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Abstract—Synthesis of thrombin inhibitors and their binding mode to thrombin is described. Modification of the P1 moiety leads to an increased selectivity versus trypsin. The observed selectivity is discussed in view of their thrombin–inhibitor complex X-ray structures.

Thrombin is a multifunctional serine protease that is involved in the final step of the cascade-like activation of blood coagulation. As a trypsin-like endopeptidase it mediates the conversion of soluble fibrinogen into fibrin and the activation of platelets.[1] Thromboembolic diseases like deep vein thrombosis, myocard infarction, unstable angina, pulmonary embolism, and ischaemic stroke are a major cause of morbidity and mortality in the industrialized world. Several pharmaceutical companies have therefore very intensely pursued the search for low molecular weight active site thrombin inhibitors over the past years.[2]

Many thrombin inhibitors share the D-Phe-Pro-Arg motif mimicking the binding region of the natural substrate fibrinogen (cf. Fig. 1). The guanidine moiety of the Arg forms a salt bridge with the Asp189 at the bottom of the S1 pocket.[3] The Pro and D-Phe increase the affinity to thrombin by hydrophobic interactions in the S2 and S3 pockets, respectively.

The 3D structures of thrombin and trypsin, although very related, differ in various regions. Especially, the S2 and S3 pockets in trypsin are not as constrained as in thrombin. The S1 pockets of both enzymes are very similar due to their specificity towards arginine in P1. The walls of these pockets are mainly hydrophobic and Asp189 at the bottom creates a hydrophilic environment. An important difference, however, is the replacement of Ser190 in trypsin by alanine in thrombin. This renders the S1 pocket of thrombin somewhat bigger and more hydrophobic.[4]

An ideal thrombin inhibitor should be potent, orally bioavailable, and selective versus related serine proteases, like trypsin. As part of our effort towards effective

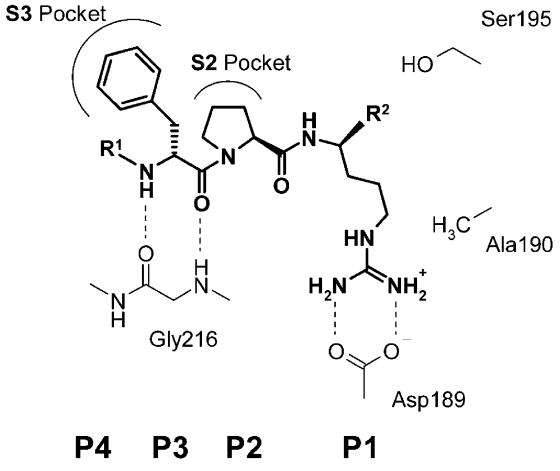

Figure 1. Schematic representation of the binding mode of a D-Phe-Pro-Arg type inhibitor to the active site of thrombin.

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thrombin inhibitors with increased selectivity versus trypsin we initiated the following synthetic program for active site thrombin inhibitors of the D-Phe-Pro-Arg type.[5]

To probe the influence of the P1 moiety on the selectivity of our inhibitors, we decided to conserve the hydrophobic moieties D-cyclohexylalanine and dehydroproline in P2 and P3 and modify only the P1 building block. It was expected that by exploiting the differences of the two enzymes with regard to their S1 pockets we could obtain thrombin inhibitors with increased selectivity.[6]

We decided to probe this hypothesis by using substituted five-membered hetaryl amidines as P1 building blocks. These hetaryl moieties give a different spatial display of the substituents than the often used para-aminomethylbenzamidines.[7] An additional small substituent on the heterocyclic ring was expected to result in a disfavorable interaction with the Oγ atom of Ser190 in trypsin thereby increasing the selectivity towards thrombin.

Chemistry

Several P1 building blocks of this type were prepared and incorporated in thrombin inhibitors. Two examples with *N*-methyl pyrroles **8** and **10** as P1 building blocks are depicted in Scheme 1.[5,8] The P1 building blocks **8** and **10** were readily attached to the P4-P3-P2 fragment **11**[5] by a TOTU mediated peptide coupling[9] and after deprotection the desired thrombin inhibitors **12** and **13** were obtained (Scheme 2).

Biology

Both compounds **12** and **13** inhibit thrombin at nanomolar concentrations and compare favorably with melagatran in terms of potency and selectivity (Table 1).[10] Thrombin inhibitor **13** is slightly less potent than **12**, but more selective versus trypsin. Inhibitors structurally analogous to *N*-methylpyrrole derivative **13** with other substituted five-membered hetaryl rings were equally potent and selective.[11] D-Phe-Pro-Arg-type inhibitors with a benzamidine moiety in P1, like melagatran,[12] often lack selectivity versus trypsin.[13] The observed selectivity of **12** and **13** could be rationalized by comparison of their thrombin–inhibitor complex X-ray structures (Fig. 2).[14]

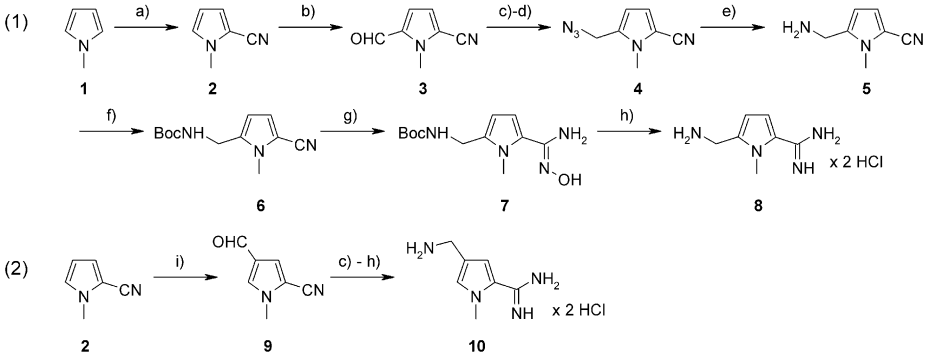
Results and Discussion

The only recognizable difference between trypsin and thrombin in the S1 pocket is the Ala/Ser exchange in position 190. Docking of molecule **12** into the binding pocket of thrombin using Quanta (Accelrys, San Diego, USA) suggested that the methyl group of the pyrrole ring would come close to Ala190, which was confirmed

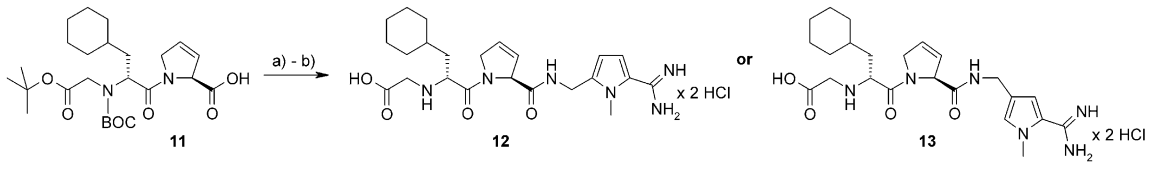
Table 1. In vitro data

Compd	Thrombin inhibition IC_{50} (nM)[a]	Trypsin inhibition IC_{50} (nM)[a]
12	6.2	16
13	11	306
Melagatran	69.2	11.9

[a]For detailed assay protocols see ref 10.



Scheme 1. Synthesis of the P1 building blocks: (a) CSI, CH_3CN/DMF, −78 °C then rt, 61%; (b) (1) LDA, (2) DMF, 54%; (c) $NaBH_4$, EtOH, 91%; (d) (1) CBr_4, PPh_3, (2) NaN_3, 63%; (e) H_2, Pd/C, MeOH, 68%; (f) Boc_2O, NEt_3, DCM, 94%; (g) $NH_2OHxHCl$, DIEA, MeOH/DCM, 69%; (h) (1) H_2, Raney-Ni, CH_3COOH, MeOH, (2) HCl, DCM, 94%; (i) Cl_2CHOCH_3, $AlCl_3$, CH_3NO_2/DCM, 91%; (yields given for (c) to (h) refer to eq 1 and were comparable for building block **10** shown in eq 2, 11.3% overall).



Scheme 2. Synthesis of the thrombin inhibitors **12** and **13**: (a) **8** or **10**, NMM, TOTU, DMF, 0 °C; (b) HCl, DCM (45% from **8**, 42% from **10**).

by the X-ray structure of the thrombin–inhibitor complex (distance CH_3 Cα 4.0 Å). The distance to the Oγ of Ser190 in trypsin was expected to be smaller than 3.5 Å. This could lead to an unfavorable steric interaction forcing the heterocyclic ring to rotate slightly away from the Oγ, which would lead to a dislocation of the buried water molecule. The affinity of **12** to trypsin and to thrombin is, however, comparable (Table 1).

From docking studies with the structural isomer **13** it was deduced that there should be no significant difference in binding affinity between thrombin and trypsin. The methyl group is located in a region with high structural conservation between thrombin and trypsin. For both enzymes a close contact between carbonyl oxygen of Gly219 and the methyl group of the pyrrole ring was postulated, which should lead to a reduction of the affinity. As expected the affinity for trypsin dropped by a factor of 20. To our surprise the affinity towards thrombin was only affected by a factor of 2 (Table 1). Therefore, the 2,4-substituted pyrrole **13** is a highly potent thrombin inhibitor with increased selectivity versus trypsin while the 2,5-substituted pyrrole **12** is an equally potent, but less selective thrombin inhibitor.

The X-ray structure of the inhibitor–thrombin complex of **13** revealed that the expected unfavorable interaction with Gly219 was accommodated by a movement of the main chain oxygen, adapting the steric requirements. In Figure 3 inhibitor **13** is shown with the amino acids Asp189 and Gly219. Also shown is Gly219 of thrombin-inhibitor complex of **12** after superposition of the active site residues with Quanta. The shift between the two oxygen atoms amounts to 0.85 Å.

Malikayil et al.[15] described a thrombin inhibitor with a bulky indole P1 moiety exhibiting a 400-fold selectivity over trypsin. They observed a 90° rotation of the whole the peptide bond between Gly219 and Cys220 in the X-ray structure of the thrombin–inhibitor complex. They attributed the obtained selectivity to size differences of the S1 pockets in thrombin and trypsin.

A more bulky and more hydrophobic P1 substituent, however, does not by itself explain the higher selectivity as shown for the inhibitors **12** and **13**. We concluded that the amino acid Gly219 is more flexible in thrombin than in trypsin. In Figure 4 the Cα traces of amino acids 213 to 226 and the supporting loop of thrombin (red) and trypsin (blue) are shown, respectively. For thrombin this second loop comprising amino acids 184 to 188 has an insertion of three amino acids (Asp186A, Glu186B and Gly186C) and protrudes into the solvent (Fig. 4). Since in trypsin this loop is much shorter it has not the same flexibility and might be responsible for a more rigid Gly219 loop in trypsin.

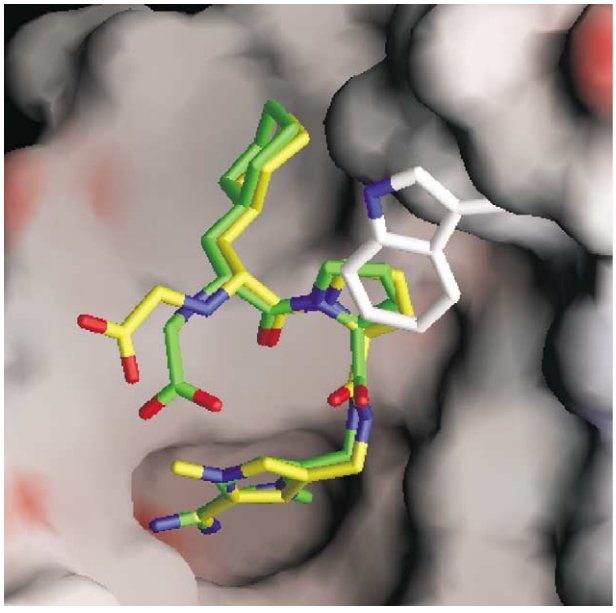


Figure 2. Superposition of **13** (green) and **13** (yellow) in the thrombin pocket as determined by X-ray crystallography. Water accessible surface for Trp 60D was omitted for the sake of clarity.

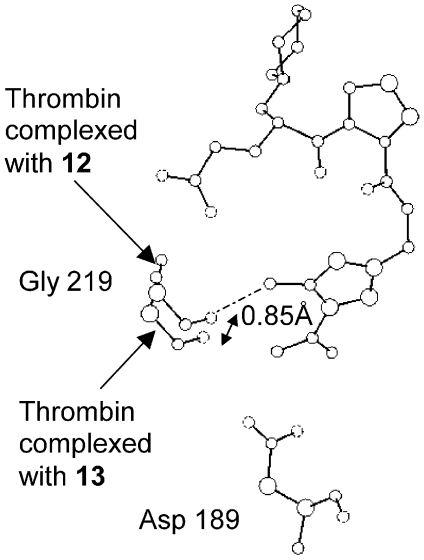


Figure 3. X-ray structure of thrombin–inhibitor complex of **13**. The inhibitor and Asp189 and Gly219 of thrombin are depicted along with the superpositioned Gly219 of the X-ray structure of the thrombin–inhibitor complex of **12**.

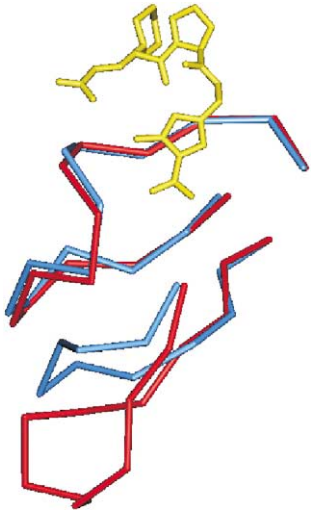


Figure 4. Inhibitor **13** (yellow) and Cα-traces of thrombin (red) and trypsin (blue) showing the loops involved in Gly219 fixation.

This view has been supported by two crystallographic findings. The two loops described above are tightly linked by three main chain hydrogen bonds between the atoms Ala221A O-Gly188 N, Gly226 O-Ala183 N, and Asn223 N-Leu185 O in trypsin,[16] whereas in thrombin no comparable main chain hydrogen bonds are present in this region. The temperature factors of these loops are much higher in thrombin than in trypsin. Both results are consistent with a higher flexibility of these loops in thrombin compared to trypsin. Therefore, the adaptation of Gly219 can be considered as an induced fit which to this extent is only possible in thrombin, but not in trypsin, leading to the observed selectivity.

In summary, an approach towards selective thrombin inhibitors of the D-Phe-Pro-Arg type exploiting the differences of the S1 pockets of thrombin and trypsin has been described. Although the observed selectivity is moderate the effect it is based on could potentially be used in the molecular modeling assisted design of highly selective thrombin inhibitors. Furthermore, the structural information disclosed herein provides the basis for future investigations in fields like molecular dynamics. It is expected that such studies will give a deeper insight into the underlying mechanism of the observed backbone flexibility in enzymes like thrombin and may also help to refine today's modeling tools,[17] which altogether would have failed to predict the observed selectivity and subtle changes in the protein structure induced by the inhibitor.

Acknowledgements

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10. (a) Thrombin assay: 1. Reagents: Thrombin from human plasma (No. T-8885, Sigma, Deisenhofen, Germany). 2. Substrate: H-D-Phe-Pip-Arg-pNA·2HCl (S-2238, Chromogenix, Mölndahl, Sweden).

(b) Trypsin assay: 1. Reagents: Trypsin from bovine pancreas (No T-8003, Sigma, Deisenhofen, Germany). 2. Substrate R^1-Ile-Glu-(O-OR^2)-Gly-Arg-pNA·HCl (S-2222, Chromogenix, Mölndahl, Sweden).

Buffer (for both assays): Tris 50 mmol/L, NaCl 154 mmol/L, pH 8.0.

Experimental procedure (for both assays): The chromogenic assay for the determination of thrombin activity (trypsin activity, respectively) was performed in 96-well microplates (No. 650101, Greiner, Nürtingen, Germany). 10 μL of substance solution in DMSO were added to 250 μL of buffer containing thrombin at a final concentration of 0.1 NIH-units/mL (or 0.1 mg/L trypsin) and incubated for 5 min at 20–28 °C. The assay was started by addition of 50 μL of substrate solution in buffer (final concentration 100 μmol/L) and stopped after a 5 min incubation period at 20–28 °C by addition of 50 μL of citric acid (35%). The absorbance in each well was measured against blank at 405/630 nm in a double beam microplate photometer (MR5000, Dynatech, Denkendorf, Germany).

The concentration effect curves and the IC_{50} values were calculated from data points by multiple iterations using a nonlinear sigmoidal least squares regression fit algorithm.
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Compound **12** was soaked into the preformed thrombin crystals. Diffraction data were collected on a conventional

rotating anode with CuK_α radiation with a Siemens CCD detector. Crystals of the ternary complex with **12** belong to the space group C2 with unit cell parameters a = 71.8 Å, b = 72.0 Å, c = 73.5 Å, β = 101.1°. A total of 29,541 observations were measured and yielded 12,245 unique reflections with an R_{merge} of 0.078 (0.32 for outer shell at 2.56–2.44 Å). Model inspection and correction between cycles of refinement and the refinement itself was performed with QUANTA and CNX (Accelrys, San Diego, USA). The final R-factor amounted to 0.199 (R_{free} 0.252) with excellent stereochemistry (rms deviation on ideal bond lengths 0.007 Å, on ideal angles 1.41°). The authors have deposited the coordinates with the Protein Data Bank: Deposition code 1O0D.

Thrombin crystals with **13** have been prepared and measured in an analogous manner. The unit cell parameters for this soaked crystal were a = 71.1 Å, b = 72.0 Å, c = 73.2 Å, β = 100.8°. A total of 41,789 observations were measured and yielded 17,026 unique reflections with an R_{merge} of 0.077 (0.277 for outer shell at 2.23–2.18 Å). The final R-factor amounted to 0.197 (R_{free} 0.242) with excellent stereochemistry (rmsd on bond 0.006 Å, on angles 1.28°). The authors have deposited the coordinates with the Protein Data Bank: Deposition code 1NZQ.

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