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POTENT AND SELECTIVE BICYCLIC LACTAM INHIBITORS OF THROMBIN: PART 2: P1 MODIFICATIONS

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Abstract: The synthesis and antithrombotic activity of a series of nonpeptide bicyclic thrombin inhibitors is described. We have explored the SAR with modifications to the P1 site. The introduction of arginine mimetics at the P1 site led to potent and selective thrombin inhibitors.

Introduction:

Thrombosis is a disease state typified by abnormal coagulation and remains a major cause of mortality in western cultures. One particularly debilitating indication manifests itself in the form of deep vein thrombosis. In this situation restricted blood flow and contact with foreign material (such as in hip replacements) may result in the formation of venous fibrin rich thrombi. In the event that emboli detach from this network of fibrin and travel to pulmonary arteries, perfusion of the lung is prevented, ultimately leading to death.[1] In an attempt to address this aberrant coagulatory response a number of anticoagulants and antithrombotics, such as coumarin and heparin,[1b] and direct thrombin inhibitors, such as hirudin[1c] and hirulog,[1c] have been assessed clinically. Side effects, such as excessive bleeding and difficulties of drug monitoring, associated with the current therapies have led to considerable activity in the search of novel orally active small molecule thrombin inhibitors.[2]

A key feature in the development of potent thrombin inhibitors is the ability to selectively inhibit thrombin over other serine proteases. For example, the homologous fibrinolytic enzymes (plasmin, t-PA, and urokinase) have important regulatory functions, inhibition of these enzymes along with thrombin could interfere with endogenous or therapeutic clot dissolution.[3] Furthermore, oral bioavailability coupled with a long duration of action would be particularly beneficial for the chronic treatment of venous thrombotic disorders.

A series of potent and novel thrombin inhibitors with a bicyclic core at the P2 site[4] was recently identified. The previous communication[5] described the SAR at the P3 site of these novel inhibitors. The current communication describes modifications at the P1 site with a view to developing potent and selective thrombin inhibitors. Structure-activity studies that explore features important for potency, selectivity, and antithrombotic activity will be described.

Chemistry:

The compounds listed in Table 1 were synthesized using a convergent strategy that involved coupling the bicyclic pyrrolidine **8**[4,5] with the appropriate keto-heterocycle (Scheme 1). In general, the keto-heterocycles

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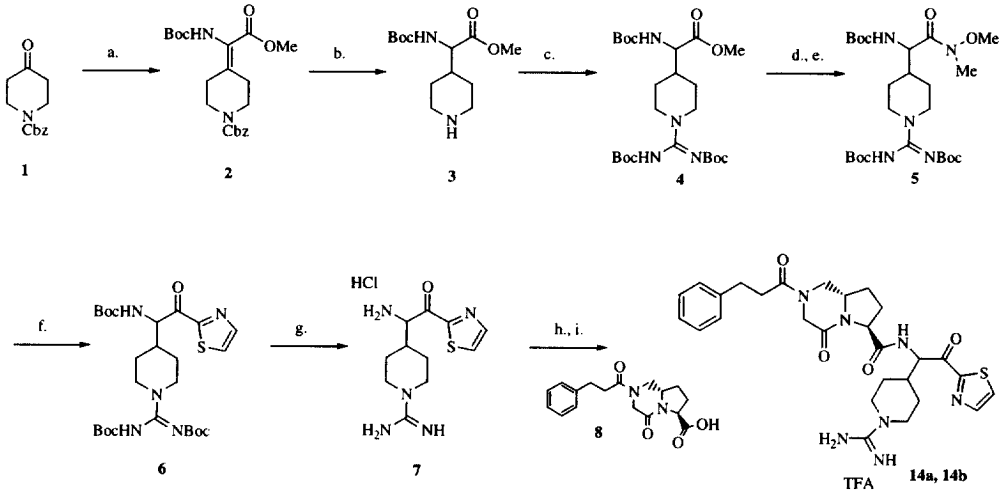
were derived from the Weinreb amides of the appropriate amino acid derivative.[6] The synthesis of the keto-heterocycle corresponding to compound **14** is shown in Scheme **1**, N-Cbz-4-piperidone was treated with N-Boc-phosphono glycine trimethyl ester and DBU to yield the olefin **2** in 70% yield. Hydrogenation afforded the piperidine derivative **3,** which was treated with N,N-(*tert*-butoxycarbonyl)-1*H*-pyrazole-1-carboxamidine and DIEA in THF yielding the protected guanidine derivative **4** (82%). Hydrolysis of the methyl ester and treatment with N,O-dimethylamine hydrochloride/BOP reagent and DIEA afforded the Weinreb amide **5** (60% over two steps). The addition of the lithiated heterocycle to Weinreb amide **5** at -78 °C, warming to -40 °C, and quenching at -78 °C afforded the keto-heterocycle **6** in 80% yield. Deprotection of **6** was accomplished with 4N HCl/dioxane in quantitative yield. Condensation of the amine, **7**, with the bicyclic pyrrolidine derivative, **8**, afforded the target compound. The isomers were separated by preparative reverse-phase HPLC. As expected the the L-isomer, **14a**, was more potent than the D-isomer, **14b**. Compounds **9- 13** (Table 1) were synthesized in a similar manner starting from the appropriate amino acid derivatives.[7-9]

Scheme 1



Reagents and Conditions: a. N-Boc-α-phosphono glycine trimethyl ester, DBU, CH_2Cl_2, 24 h b. H_2/ Pd/C, MeOH, 2.5 h c. DIEA, N,N-(*t*-butoxycarbonyl)-1H-pyrazole-1-carboxamidine, CH_2Cl_2, 24 h d. LiOH, H_2O/MeOH (1/5), 2 h e. MeNH(OMe) HCl, BOP reagent, DIEA, CH_2Cl_2, 4 h f. *n*-BuLi, thiazole, TMEDA. THF -78 °C, 3 h g. 4 N HCl/dioxane, 3 h h. BOP reagent, DIEA, CH_3CN, 2 h i. TFA note: BOP reagent (Benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate)

Biological Assays: The compounds prepared were evaluated for their ability to inhibit the serine proteases thrombin and trypsin (Table 1).[10] Compounds **9- 14a** were evaluated for antithrombitic efficacy in a rat model of acute arterial thrombosis.[11]

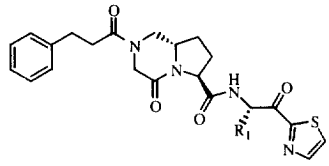
Results and Discussion:

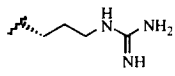
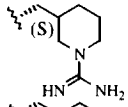
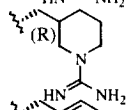
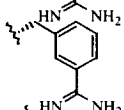
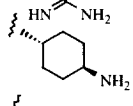
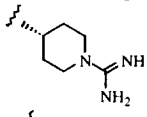
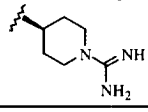
The initial compound in this series, **9**, showed excellent inhibition of thrombin with an IC_{50} of 5 nM while inhibiting trypsin with an $IC_{50} < 1nM$.[12] Given our goal to prepare potent and selective thrombin inhibitors, we chose to explore P1 modifications of **9** to improve the selectivity over trypsin. Four arginine mimetics were placed in P1 yielding the diastereomers **10** and **11,** and the compounds **12, 13** and **14a, 14b**. The two isomers **10** (IC_{50} = 30nM) and **11** (IC_{50} = 137nM) showed marked differences in activity against thrombin. X-ray crystallographic studies of **10** complexed with thrombin demonstrated that compound **10** contained the S stereochemistry and hence compound **11** was assigned the R stereochemistry. More importantly, **10** and **11** were 145- and 260-fold selective over trypsin, respectively. The X-ray crystal structures of **9** and **10** complexed with both thrombin and trypsin led to some interesting findings regarding the origin of the selectivity. The crystal structures of **10** showed that, in both the thrombin and trypsin structures, the P1 3-amidino piperidine fits tightly into the S1 specificity pocket of each enzyme forming a double salt bridge with the Asp189 in the bottom of the pocket (Figure 1a). Ser195 also forms a covalent bond to the carbonyl of the keto-thiazole in both cases. In order to determine the origin of selectivity we compared the crystal structure of **9** complexed to trypsin with that of **10** with trypsin (Figure 1b). In the crystal structure of **9** with trypsin there exists a hydrogen bond between Gln192 and the amide carbonyl of the inhibitor. The crystal structure of **10** with trypsin shows that the piperidine ring methylene units disrupts this Gln192 - carbonyl hydrogen bond. In thrombin, residue 192 is a Glu residue which does not normally form a hydrogen bond with the inhibitor. Hence the piperidine ring does not disturb an important hydrogen bond in thrombin, as seen in trypsin. Thus taken together, the structure and potency data suggest that the noted selectivity arises from a loss of a strong hydrogen bond between **10** and trypsin.

Employing this structural information, additional modifications to P1 were explored to improve potency and selectivity. For example, the 3-amidinophenyl derivative **12** showed modest potency against thrombin with good selectivity. As previously observed, the aromatic ring disrupts the Gln192 -carbonyl hydrogen bond in trypsin, thereby making **12** a comparatively poorer trypsin inhibitor. Interestingly, compound **12** while imparting reasonable thrombin/trypsin selectivity, was ineffective in the rat arterial antithrombotic model. In fact, this lack of activity reasonably reflects the modest thrombin inhibitory activity.

Of considerable interest was derivative **13** which while incorporating a cyclohexylamine at P1, afforded remarkable potency and selectivity coupled with antithrombotic activity. However, it was not until we finally assessed the P1 subsitutuent in the form of 4-amidinopiperidine, **14**, that we saw excellent in vitro thrombin inhibitory acitivity and selectivity coupled with excellent antithrombotic activity

Table 1. In Vitro IC_{50}[a] Values (nM) and Selectivity Ratios against Thrombin (FIIa)[b] and Trypsin[c] and In Vivo Mean Occusion Times (MOT, min) of Compounds **9- 14**[d]



Compound	R_1	FIIa	Trypsin	Trypsin/FIIa	MOT(min)[e]
9		5	<1	0.2	42
10		30	4,360	145	>60
11		137	31,600	230	27
12		77	7,750	100	18
13		33	8,990	270	48
14a		1	11,940	11940	57
14b		150	31%@100μM	NA[f]	NT[g]

a. Concentration (nM) of **9- 14** necessary to inhibit enzymatic cleavage of the chromogenic substrates described in ref 10. by 50 %. b. Human Thrombin. c. Human Trypsin. d. All new target compounds were characterized by ^{1}H NMR, RP-HPLC and mass spectroscopy e. MOT in a group of control rats was 16.3+/- min. f. NA- not applicable. g. NT- not tested.

In Vivo Results:

Our most potent inhibitors were evaluated for their antithrombotic activity in the rat arterial thrombosis model. The nonselective inhibitor, **9**, possessed good anticoagulant and antithrombotic activity with a mean occlusion time of (MOT) >40 min. Several selective inhibitors (**10, 13,** and **14a**) also showed efficacy with MOTs >40 min. Compounds which demonstrated good antithrombotic activity were further evaluated for oral (30 mg/kg) bioavailability in the rat, unfortunately the compounds evaluated lacked significant activity when dosed in this manner.

Figure 1

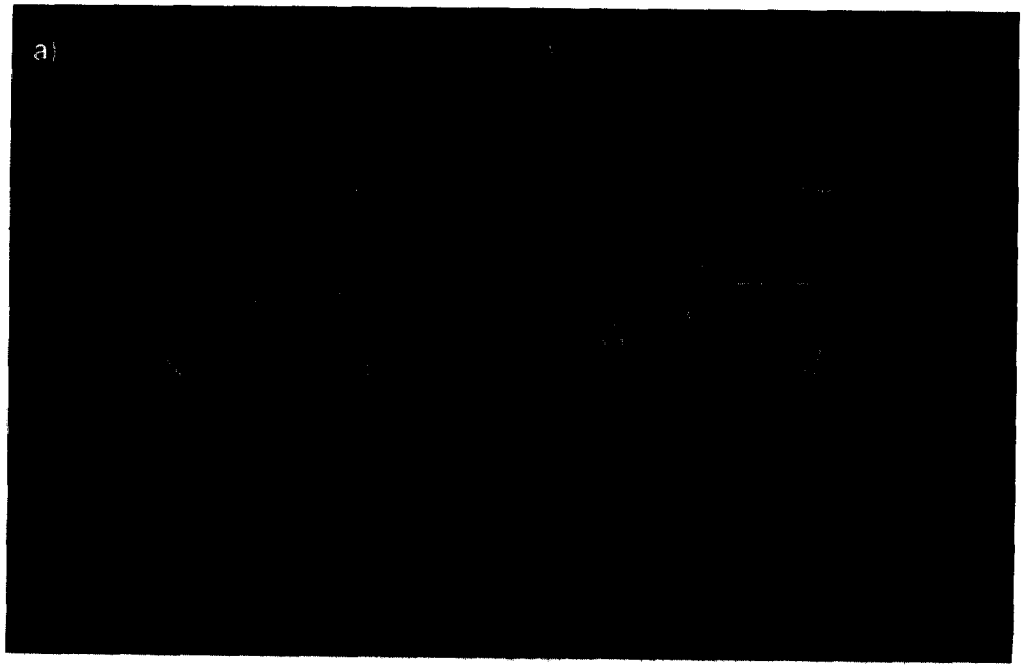


Figure 1. Stick rendition of (a) thrombin bound to compound **10** (colored by atom) and b) trypsin bound to compounds **9** (orange) and **10** (colored by atom). Inhibitors shown by thick lines; nearby protein residues are shown by thin lines. Color coding for atoms is: *red,* oxygen, *blue,* nitrogen, *yellow,* sulfur, *white,* carbon. *Green dashed* arcs denote van der Waals contacts, *purple dashed* lines denote electrostatic interactions between the protein and inhibitor. *Grey circled regions* denote P1, P1', P2 and dP3 binding pockets of enzyme.

Conclusions:

We have demonstrated that the modifications at the P1 position alter the activity and selectivity for thrombin over trypsin. Specifically, the arginine mimetics trans 4-amidinopiperidine glycine, 3-amidinopiperidine alanine, and 4-trans cyclohexylamine, at P1 yielded potent thrombin inhibitors with good to excellent selectivity over trypsin. X-ray crystallographic studies determined that the selectivity is achieved by the loss of a key hydrogen bond in trypsin. We demonstrated that guanidine or amidine at P1 is not necessary for potent thrombin inhibition, given that 4-*trans* cyclohexylamine at P1, **13** (IC_{50} = 33 nM), is a potent and selective inhibitor of thrombin. Antithrombotic efficacy in the rat was demonstrated with our most potent inhibitors via intravenous infusion administration.

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    In general the in vitro enzyme assays were performed according to the protocols describe below: all assays were run in duplicate with a confidence limit > or = to 99%.
    Thrombin Inhibition--Determination of IC_{50}. The ability of compounds to act as inhibitors of thrombin catalytic activity was assessed by determination of the concentration of test substance that inhibits by 50% (IC_{50}) the ability of thrombin to cleave the chromogenic substrate Chromozym TH (Tos-Gly-Pro-arg-p-nitroanilide acetate). Typically thrombin in 10 mM HEPES, 100 mM NaCl, 0.01%BSA, and 0.1% PEG-8000 and the test substance in DMSO (2% final) were incubated for 60 min. at room temperature. To this mixture was added Chromozym TH (2 x K_m) and the initial rate of Chromozym TH hydrolysis was measured by observing the changes in absorbance (OD_{405nm}) over 5 min.
    Trypsin Inhibition-- Determination of IC_{50}: The ability of compounds to act as inhibitors of trypsin catalytic activity was assessed by determination of the concentration of test substance that inhibits by 50% (IC_{50}) the ability of trypsin to cleave the chromogenic substrate S2222 (Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide HCl). Typically trypsin in 10 mM HEPES, 100 mM NaCl, 0.01%BSA, and the test substance in DMSO (2% final) were incubated for 60 minutes at 37 °C. To this mixture was added S2222 (2 x K_m) and the initial rate of S2222 hydrolysis was measured by observing the changes in absorbance (OD_{405nm}) over 5 min.
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