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Selective 3-Amino-2-pyridinone Acetamide Thrombin Inhibitors Incorporating Weakly Basic Partially Saturated Heterobicyclic P_1-Arginine Mimetics

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Abstract—Novel, highly selective and potent thrombin inhibitors were identified as a result of combing the 3-benzylsulfonylamino-2-pyridinone acetamide P_2–P_3 surrogate with weakly basic partially saturated heterobicyclic P_1-arginine mimetics **1**–**8**. The design, synthesis, biological activity, and the binding modes of non-covalent thrombin inhibitors featuring P_1-4,5,6,7-tetrahydroindazole, 5,6,7,8-tetrahydroquinazoline, and 4,5,6,7-tetrahydrobenzothiazole moieties are described.

The serine protease thrombin plays a central role in the blood coagulation pathway, directly triggering the production of insoluble fibrin while at the same time providing the stimulus for platelet aggregation. The key position of thrombin in the blood coagulation cascade has made it a popular target for antithrombotic therapy.[1] Intravenously administered direct inhibitors of thrombin, such as argatroban,[2] have been shown to be clinically effective antithrombotics. However, the main effort has been focused on achieving the more difficult goal of an orally active, low molecular weight direct thrombin inhibitor.[3]

Our approach to the design of thrombin inhibitors based on the D-Phe-Pro-Arg sequence was focused mainly on replacing the arginine moiety with less basic, conformationally restricted, partially saturated heterobicyclic arginine side chain mimetics.[4] These contain a five- or six-membered *N*-heterocyclic ring optionally substituted by an amino group mimicking the guanidino moiety of arginine, annulated to a cyclohexane ring mimicking the arginine trimethylene side chain. We anticipated that the bulky and lipophilic cyclohexane ring could confer selectivity for thrombin against trypsin, since the S_1 pocket in thrombin is slightly larger and more hydrophobic than the one in trypsin.

Previous work in our laboratory on the D-Phe-Pro-Arg motif-based thrombin inhibitors which incorporated weakly basic, partially saturated heterobicyclic P_1-arginine side-chain mimetics, proline at the P_2 part, and various P_3-lipophilic moieties, resulted in the identification of a series of potent and selective thrombin inhibitors.[4,5] The development of thrombin inhibitors containing non-basic arginine side-chain mimetics **1**–**8** was coupled with the desire to overcome the limitations imposed by the amidine and guanidine moieties, whose high basicity reduces both bioavailability following peroral application, and selectivity for thrombin against trypsin.[6] According to our structure activity relationship studies, we were therefore interested in preparing a series of 2-(3-amino-6-methyl-2-oxo-2*H*-pyridin-1-yl)-acetamide template based thrombin inhibitors incorporating arginine side-chain mimetics **1**–**8** at the P_1 part of the inhibitor (Fig. 1). The 3-amino-2-pyridinone acetamide template has been applied several times as a non-peptide P_2 moiety[6b,7,13] giving in combination with suitable P_1 arginine mimics subnanomolar bioavailable thrombin

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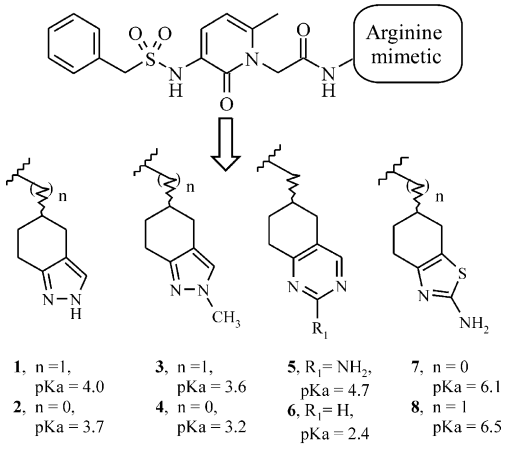


Figure 1. Evolution of 3-amino-2-pyridinone acetamide thrombin inhibitors incorporating weakly basic, partially saturated heterobicyclic P_1-arginine mimetics **1**–**8**, with their calculated pK_a values.[16a,16e]

inhibitors.[7] The design, synthesis, in vitro activity and the binding modes of thrombin inhibitors incorporating 3-benzylsulfonylamino-2-pyridinone acetamide as a P_2–P_3 surrogate, as well as weakly basic, partially saturated heterobicyclic P_1-arginine side-chain mimetics will be presented in this letter.

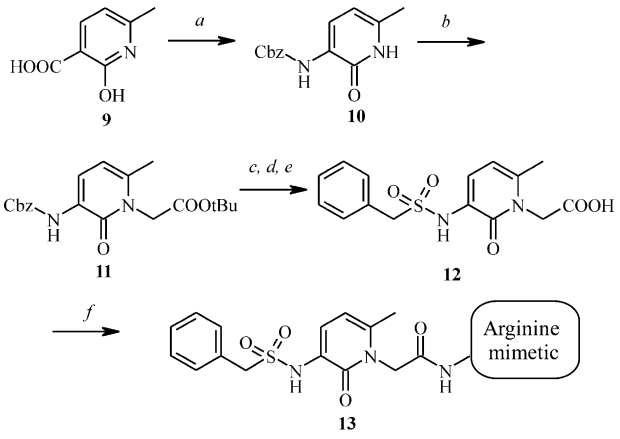
The thrombin S_1 site is a deep pocket with Asp189 at the bottom, capable of forming ionic and hydrogen-bond interactions with positively charged residues such as the guanidine moiety of arginine, and is slightly larger and more lipophilic than that of trypsin, due to the presence of an alanine residue at position 190, in contrast to serine in trypsin and chymotrypsin.[8] The prospect of making optimal use of the lipophilicity and size of the thrombin S_1 pocket, that is apparently not fully exploited by arginine, presented an opportunity to obtain good binding and selectivity for thrombin using novel P_1 arginine mimetics with appropriate basicity.[6]

In order to overcome the high basicity of the P_1 guanidine, amidine and aliphatic amine moieties, various heterocycles were investigated as P_1 arginine mimetics in designing of thrombin inhibitors. Among them, some bicyclic aromatic heterocycles, for example 1-isoquinolinamine,[9] 3-benzisoxazolamine,[10] indole,[11] benzimidazole,[11] imidazo[1,2-*a*]pyridine,[12] indazole,[11,13] pyrrolo[3,2-*b*]pyridine,[14] benzotriazole[15] and 2-aminobenzothiazole[15] were recently employed as P_1 moieties in thrombin inhibitors.

As summarized in Figure 1, our strategy was to explore the S_1 selectivity pocket of thrombin with the diverse, partially saturated heterobicyclic arginine side-chain mimetics **1**–**8** of low basicity (calculated pK_a = 2.4–6.5).[16a] Ideally, such P_1-arginine mimetics would preserve important hydrogen bonding interactions with Asp189 and glean additional hydrophobic interactions at the S_1 pocket of thrombin. The 3-amino-2-pyridinone acetamide peptidomimetic template was used as the P_2 moiety, maintaining intrinsic potency via β-sheet hydrogen bonding interactions with Gly216 and hydrophobic interactions at the YPPW loop of the S_2 site. The benzylsulfonyl group at P_3 is flexible enough to allow the phenyl group to fill the S_3 distal pocket.[7b] The arginine mimetics investigated include 4,5,6,7-tetrahydroindazoles, 5,6,7,8-tetrahydroquinazolines and 4,5,6,7-tetrahydrobenzothiazoles (Fig. 1). The calculated pK_a values,[16a] listed in Figure 1, range from weakly basic 2-amino-4,5,6,7-tetrahydrobenzothiazoles **7** (pK_a = 6.1) and **8** (pK_a = 6.5) to the effectively non-basic 5,6,7,8-tetrahydroquinazoline **6** (pK_a = 2.4).

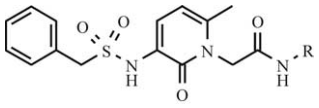
A convenient synthetic approach to arginine mimetics **1**–**8**, containing a five- or six-membered *N*-heterocyclic ring optionally substituted by amino, methyl and guanidino group, has already been reported by us.[17–20] The synthetic route to the (2-oxo-2*H*-pyridin-1-yl) acetic acid intermediate **12**[21] and elaboration to targets **13** is summarized in Scheme 1. Following the published procedure, intermediate **12** was prepared from the commercially available 2-hydroxy-6-methylpyridine-3-carboxylic acid (**9**) by Curtius rearrangement and subsequent treatment with benzyl alcohol to give Cbz protected amine **10**. Alkylation of the ring nitrogen of **10** with *tert*-butyl bromoacetate gave **11**, whereupon hydrogenolysis of the Cbz group followed by sulfonylation of the free amine and deprotection, afforded benzyl sulfonamide **12**. Coupling reactions between **12** and the various arginine mimetics **1**–**8** using *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC), 1-hydroxybenzotriazole (HOBt) as amide bond forming reagents, *N*-methyl morpholine as a base and DMF as solvent at room temperature afforded compounds **13**.

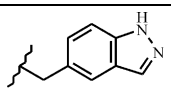
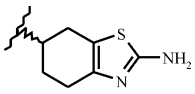
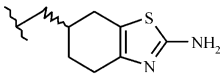
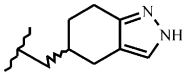
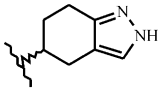
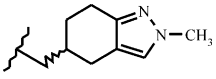
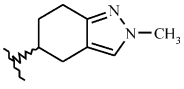
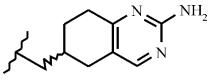
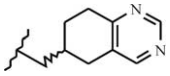
The in vitro inhibitory activities of targets **14**–**21** are summarized in Table 1. The ability of new thrombin inhibitors to inhibit the enzymatic action of thrombin, trypsin and factor Xa was measured with the amidolytic enzyme assays using chromogenic substrates.[22a] Values of K_i were calculated according to Cheng and Prusoff,[22b] based on IC_{50} values, or from a relation between reaction velocity equations in the absence and presence of inhibitor using the relevant K_m.[22c] The selectivity for thrombin over trypsin was compared on



Scheme 1. Reagents and conditions: (a) DPPA, Et_3N, dioxane, reflux 16 h, then BnOH, Et_3N, reflux 24 h; (b) NaH, $BrCH_2COOtBu$, THF, 2 h; (c) H_2 (100 psi), $Pd(OH)_2$, EtOH/H_2O, 2 h; (d) $BnSO_2Cl$, pyridine, rt, 1 h; (e) HCl_g, EtOAc, 0 °C, 1 h; (f) arginine mimetics **1**–**8**, DMF, HOBt, *N*-methylmorpholine, EDC, rt, 12 h.

Table 1. Inhibitory potencies of compounds **14–21**



Compd	R	K_i (μM)			Selectivity thrombin/trypsin	APTT	PT	TT
		Thrombin	Trypsin	FXa		(μM)		
Corvas **NC1**[15]		0.0087	—	—	—		—	
New compounds								
14		0.12	260	92	2166	36.5	41.4	6.6
15		2.5	380	106	152	121	334	44
16		0.17	Inactive	103	Selective	20.5	30.6	5.2
17		4.7	438	99	93	272	>400	105
18		11.8	557	95	47	318	>400	105
19		25	807	90.2	32	>200	>400	375
20		6.9	640	87.3	93	219	>400	84
21		34	179	ND	5.2	ND	ND	ND

APTT, concentration of inhibitor required to double the activated partial thromboplastin time in human plasma; PT, concentration of inhibitor required to double the prothrombin time in human plasma; TT, concentration of inhibitor required to double the thrombin time in human plasma; ND, not determined.

the basis of the ratios $K_{i(trypsin)}/K_{i(thrombin)}$. The inhibitors were also tested in standard clotting assays including the thrombin time (TT), activated partial thromboplastin time (APTT) and prothrombin time (PT) determinations, which were used as qualitative in vitro indicators of potential antithrombotic activity.

From Table 1, it can been seen that 4,5,6,7-tetrahydrobenzothiazole and 4,5,6,7-tetrahydroindazole inhibitors **14** and **16** are highly selective and the most potent in the series, with the K_i for thrombin of 120 and 170 nM, respectively. In the tetrahydroindazole type inhibitors, a methylene linker between the heterocycle and the amino group, which allows substantial rotational freedom of the P_1 part, was beneficial for inhibitory activity against thrombin. Thus, inhibitor **16** exhibited a 28-fold greater inhibitory potency than compound **17**. Contrary to our expectation, a methylene linker was not beneficial for the inhibitory potency of 2-amino-4,5,6,7-tetrahydrobenzothiazole type inhibitors. Thus, compound **15** exhibited a 21-fold lower inhibitory potency for thrombin than inhibitor **14**. This is probably due to the increased length of the molecule, which is responsible for a shift of the P_2 and P_3

groups from the sites of optimal interaction with the active site residues.

Regarding the structure–activity relationship of inhibitors with different aminomethylene linked P_1 heterobicyclic residues, the in vitro potency decreased as a function of the P_1 group in the following order: tetrahydroindazole (**16**, $K_i = 170$ nM, $pK_a = 4.0$) > 2-aminotetrahydrobenzothiazole (**15**, $K_i = 2.5$ μM, $pK_a = 6.5$) > 2-aminotetrahydroquinazoline (**20**, $K_i = 6.9$ μM, $pK_a = 4.7$) > *N*-methyltetrahydroindazole (**18**, $K_i = 11.8$ μM, $pK_a = 3.6$) > tetrahydroquinazoline (**21**, $K_i = 34$ μM, $pK_a = 2.4$). Replacement of the tetrahydroindazole or tetrahydrobenzothiazole P_1 moieties by the larger 2-aminotetrahydroquinazoline or by the tetrahydroquinazoline resulted in drastic decrease of inhibitory potency against thrombin. It is evident that, among the P_1 arginine side-chain mimetics tested, the 4,5,6,7-tetrahydroindazole and 4,5,6,7-tetrahydrobenzothiazole moieties are preferred for binding in the S_1 selectivity pocket of thrombin.

Comparison of antithrombotic activity of a structurally related Corvas inhibitor **NC1**[15] (Table 1) with that of our 4,5,6,7-tetrahydroindazole counterpart **16** shows that the potency is reduced considerably, due to replacing the indazole by 4,5,6,7-tetrahydroindazole. The explanation for this is not clear and may suggest a preference for planar groups in the S_1 pocket. Based on analogy with thrombin inhibitors studied by Dullweber et al.[16d] using a combination of isothermal titration calorimetry and X-ray crystallography it would appear that binding of an unsaturated P_1 substituent is entropically disfavored whereas the binding of saturated cyclohexyl fragment is favorable. However, in order to explain the lower potency of **16** (Table 1) the presence of two enantiomers in **16** and different interactions of the P_1 heterocyclic moieties of **16** and **NC1** in the S_1 pocket of thrombin should be taken into consideration.

Inhibitors **14** and **16** exhibited excellent selectivity against trypsin. This selectivity might be due to the exchange of Ala190 in thrombin for Ser190 in trypsin. This makes the trypsin S_1 pocket more polar and slightly smaller. Inhibitors with the more hydrophobic and bulkier 4,5,6,7-tetrahydroindazole and 4,5,6,7-tetrahydrobenzothiazole moieties will therefore be less favorable for interaction with trypsin. Although greater inhibitory potency usually results as a consequence of increasing P_1-basicity, in this work we showed that retention of the intrinsically favorable P_2–P_3 substituted pyridinone acetamide template allows for efficient accommodation of neutral or weakly basic bicyclic arginine mimetics. These heterobicycles possess favorable topography and ability to participate in key hydrogen bonding and hydrophobic interactions at the S_1 pocket of thrombin.

The crystal structures of the ternary complexes of human α-thrombin with hirugen and with thrombin inhibitors featuring 4,5,6,7-tetrahydrobenzothiazole and 4,5,6,7-tetrahydroindazole at P_1 (**14** and **16**) were determined at resolutions of 2.0 and 1.9 Å, respectively (Fig. 2).[23] Electron density is well defined at 1 σ for both molecules over their entire course. As expected, the benzyl group

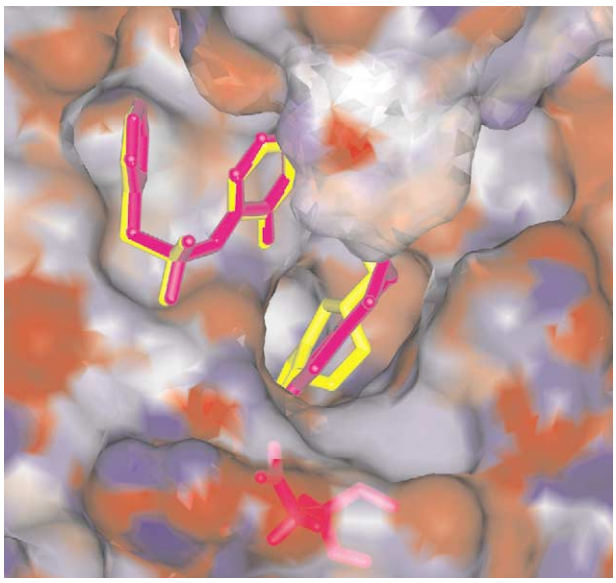


Figure 2. Connolly surface map of the X-ray structure of the human α-thrombin-inhibitors **14** and **16** complex at 2.0 and 1.9 Å resolution. The inhibitors are shown as sticks. The inhibitor **14** is shown in magenta and inhibitor **16** in yellow color.

fills the distal pocket, the pyridinone-6-methyl group occupies the proximal (S_2) site, and the tetrahydroindazole and tetrahydrobenzothiazole sit in the S_1 pocket.

Both inhibitors possess a stereogenic center: inhibitor **14** at position C6 of 4,5,6,7-tetrahydrobenzothiazole and inhibitor **16** at position C5 of 4,5,6,7-tetrahydroindazole ring, both located at the entrance of the S1 pocket (Figs. 3 and 4). The electron density around the stereogenic center is consistent with the *R* configuration in both cases. The 6-methyl substituted pyridinone ring in both cases lies below the YPPW loop of S2 and is coplanar with Trp60D and perpendicular to Tyr60A and His57. The pseudo-antiparallel β-sheet array of hydrogen bonds of **16** to the enzyme are formed between the sulfonamide nitrogen and the carbonyl oxygen of Gly216 (3.00 Å), the pyridinone oxygen and the amide nitrogen of Gly216 (3.05 Å), as well as between the acetamide

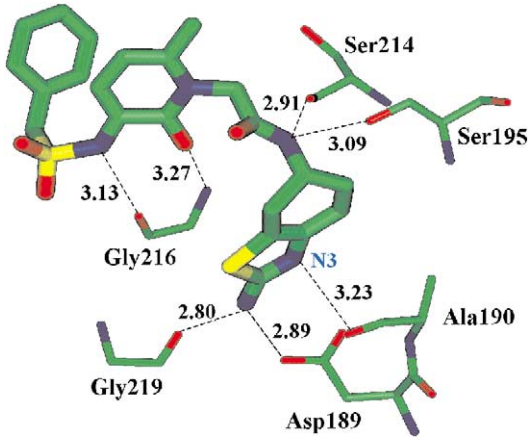


Figure 3. Schematic representation of inhibitor **14** bound in the active site of thrombin. Dashed lines indicate hydrogen bonds. Distances are given in Ångstroms.

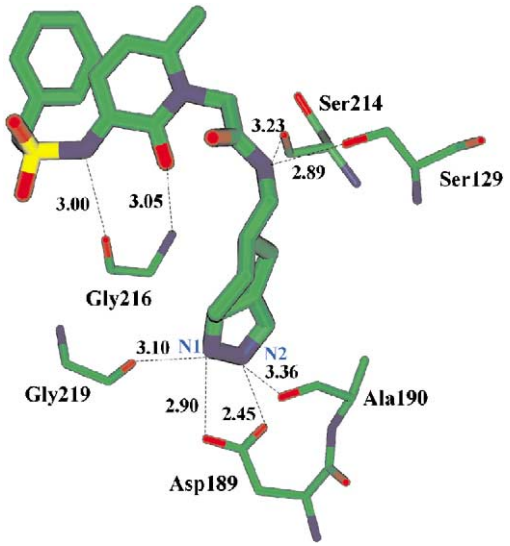


Figure 4. Schematic representation of inhibitor **16** bound in the active site of thrombin. Dashed lines indicate hydrogen bonds. Distances are given in Ångstroms.

nitrogen and the carbonyl oxygens of Ser214 (3.23 Å) and Ser195 (2.89 Å) (Fig. 4). The corresponding atomic separations in the crystal structure of **14** are 3.13, 3.27, 2.91 and 3.09 Å, respectively (Fig. 3).

The position and hydrogen bonding of the tetrahydroindazole of **16** in S_1 is similar to that of a related compound in the proline series.[4] It sits in such a way that N1 interacts with the Oδ2 of Asp189 (2.90 Å), with the carbonyl oxygen of Gly219 (3.10 Å) and with the carbonyl oxygen of Ala190 (3.36 Å). N2 interacts with the Oδ1 of Asp189 (2.45 Å) and the carbonyl oxygen of Ala190 (3.33 Å). The binding of the tetrahydrobenzothiazole moiety of **16** is similar to that of **14**, in which the 2-aminothiazole nitrogen interacts with the Oδ2 Asp189 (2.89 Å) and with the carbonyl oxygen of Gly216 (2.80 Å), whereas the nitrogen at position 3 interacts with the carbonyl oxygen of Ala190 (3.23 Å). The sulphur atom of the tetrahydrobenzothiazole of **14** is in an energetically unfavorable location making a close contact with the carbonyl oxygen of Gly219 (3.1 Å).

The location of the sulphur atom at the bottom of the S_1 pocket is clearly defined by its stronger density relative to that of the 3-nitrogen. This unfavorable contact is partially alleviated by the weak hydrogen bond to water molecule W252 (3.4 Å) as a hydrogen acceptor. The latter, however, forms no further contacts with the protein or with other water molecules.

As the thiazole 3-nitrogen of **14** has a higher proton affinity (calcd $pK_a = 6.1$)[16a] than the 2-amino nitrogen, the mean residence time of the proton is higher at the 3-nitrogen position,[16b,c] which results in the hydrogen bond to Oδ2 Asp189 having no ionic character. The same is true for the hydrogen bonds that form between the N1 and the N2 of the tetrahydroindazole[16b,c] and residue Asp189, since the pK_a value of the indazole nitrogen N1 is only 4.0.[16a] Although these bonds in **16** are short (N1–Oδ1 Asp189 measures 2.45 Å and N2–Oδ2 Asp189 measures 2.90 Å), they are not ionic. This could explain the lower potency of these inhibitors which, however, should possess a better pharmacokinetic profile than inhibitors having highly basic P_1 guanidine and amidine functionalities.

In conclusion, we have designed and evaluated a novel class of non-covalent thrombin inhibitors incorporating weakly basic, partially saturated heterobicyclic P_1-arginine side-chain mimetics and 3-amino-2-pyridinone acetamide P_2 template.

Acknowledgements

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