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STRUCTURE-BASED DESIGN AND SYNTHESIS OF NOVEL THROMBIN INHIBITORS BASED ON PHOSPHINIC PEPTIDE MIMETICS

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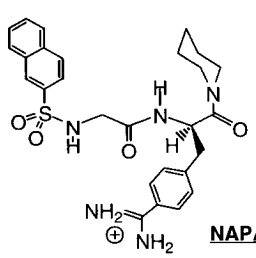
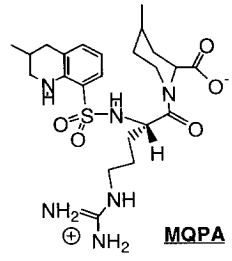
Abstract: Based on the structure of the thrombin--NAPAP complex, phosphinic dipeptide mimetics were designed as novel thrombin inhibitors. Synthesis and evaluation of these inhibitors revealed a promising lead with an IC_{50} of 0.6 μM. © 1999 Elsevier Science Ltd. All rights reserved.

Thrombin, the last protease in the blood coagulation cascade, plays an important role in thrombosis and hemostasis. Among the other physiological functions, this trypsin family serine protease converts fibrinogen into fibrin by selective cleavage of two Arg-Gly bonds among 181 Arg/Lys-Xaa bonds in fibrinogen. The resulting fibrin clot is further stabilized through crosslinking by factor XIIIa which itself is also activated by the limited proteolysis of thrombin. Because of its key role, thrombin has become an attractive target for the development of synthetic small molecule inhibitors.[1] Of the vast numbers of synthetic inhibitors, most can be categorized into three types of structures (i.e. derivatives of benzamidine, derivatives of arginine, and peptide inhibitors based on the optimized "fibrinogen-like" sequence of D-Phe-Pro-Arg). Two of the most potent ones emerging from the first and second types are NAPAP and MQPA (Agatroban), respectively.[2] MQPA was approved in Japan as a parenteral antithrombotic and is currently in clinical trial in the United States. However, these compounds are not orally active. A majority of the compounds in the third type were constructed by replacing the carboxyl of Arg with an aldehyde, boronate, or phosphonate functionality, which are expected to form a transition-state analog with the active site serine (Ser-195) through covalent interactions. The peptidyl phosphonates, unlike the aldehydes and boronates, form the tetrahedral intermediates by the attack of Ser-195 to the already crowded tetrahedral phosphonate, forming a covalent link.[3] It is not surprising that these inhibitors showed slow-binding behavior and that inhibition was at least partially irreversible.[4]



NAPAP



MQPA (Argatroban)

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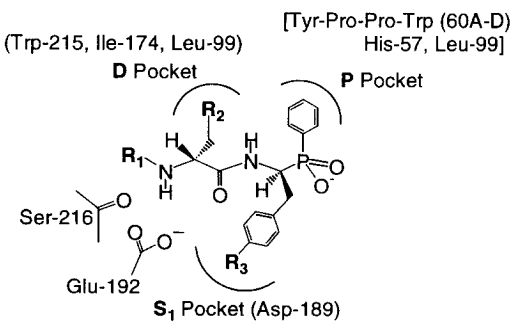
We report here a novel type of noncovalent, active site thrombin inhibitors using α-aminophosphinates, rather than phosphonates, as the key building blocks in a dipeptide mimetic design. In contrast to α-aminophosphonates, which have been widely employed in the peptidyl mimetic design, the phosphinates have received far less attention. By using computer modeling, we find that the phosphinate moiety provides a good template upon which several important structural elements can be placed in order to satisfy the crucial interactions revealed by the crystallographic studies.[5] Our designed phosphinates were found to display no slow-binding behavior in thrombin inhibition.



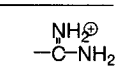
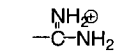
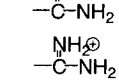
Inhibitor	R_1	R_2	R_3
1	Acetyl		
2	CBZ		
3	H		
4	CBZ		–OMe

Figure 1. Structures of the designed phosphinic dipeptide mimetics and their expected interactions inside the active site of thrombin.

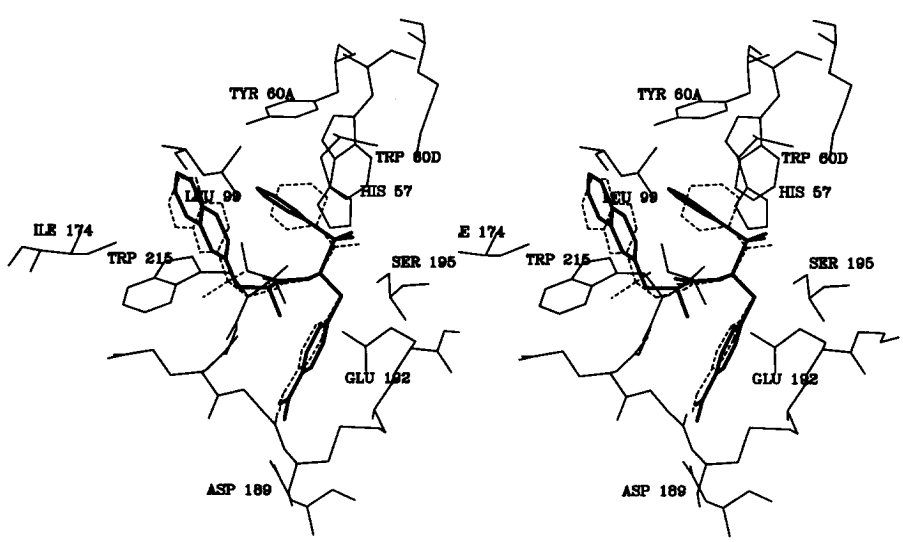


Figure 2. Minimum energy conformer of inhibitor **3** (thick line) overlaid with the crystal structure of NAPAP (dotted line) and thrombin (thin line) complex.

Design of the Inhibitors

The designed phosphinic dipeptide mimetics are shown in Figure 1 in which expected key interactions are illustrated. The binding of these compounds in the active site of thrombin was modeled using NAPAP as a template[5]. As an example, Figure 2 displays the superposition of the minimum energy conformer of inhibitor **3** upon NAPAP in the thrombin active site. The overall conformation of the inhibitor resembles that of NAPAP, especially the orientation of the backbone. An important antiparallel hydrogen bond is also formed between the inhibitor backbone and the O and N of thrombin Gly-216. Other critical interactions are also satisfied, which include the primary recognition pocket (S1 pocket), the "P-pocket" comprised by His-57, Tyr-60A, Trp-60D, and part of Leu-99, and the "D-pocket" consisting of Leu-99, Ile-174, and Trp-215[6]. Hence, the side chain of the phosphinic moiety extends into the S1 pocket with the amidino group forming a salt bridge with Asp-189, the phenyl group occupies the unique "P-pocket", and the naphthyl or indole ring locates in the "D-pocket" (Figures 1 and 2). In principle, a variety of rings can be incorporated onto the phosphorus including aromatic, aliphatic, or heterocyclic rings. For the initial design, we used the phenyl group to occupy the P pocket, because of the ready availability of the corresponding starting material.

Apart from these satisfied interactions, a unique feature of the design is the negative charge retained by the phosphinate moiety which may impart favorable characteristics into the designed molecules. First of all, the negative charge could interact favorably with the partial positive charge of His-57 and it is in the right range to hydrogen bond with the OH of the active site serine (Ser-195). The zwitterion nature of the inhibitors due to the presence of the negative charge of the phosphinates should make these compounds transport more easily through cell membrane than uniformly charged species.

Methods of Computer Modeling

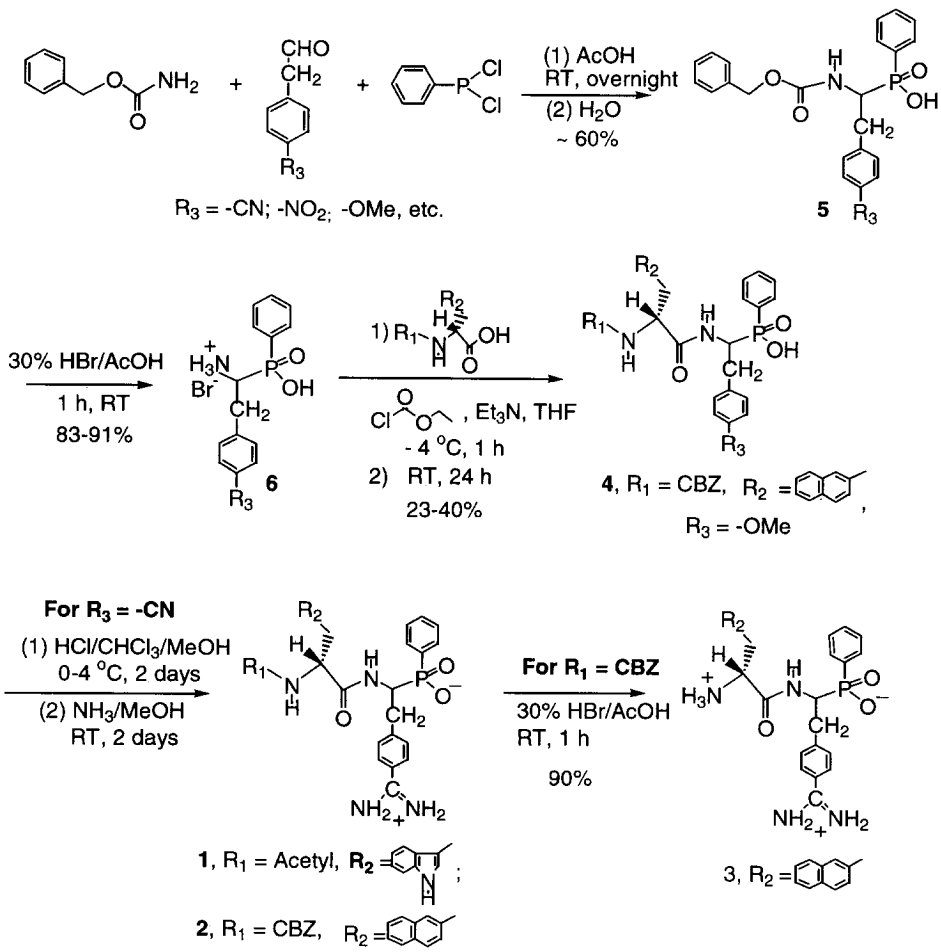
The conformational analyses for inhibitors **1** and **3** were carried out using the MacroModel/BatchMin program.[7] The truncated models, with the amidino groups removed, were used for searches due to their limited influence on the overall conformation and to their low quality parameters in the force field. Five thousand systematic pseudo Monte Carlo searches were conducted on the six torsional angles (t1--t6) of inhibitors **1** and **3**, followed by 500 iterations of conjugate gradient energy minimization with the MM2 force field. The torsional angles of the benzamidine side chain were constrained to those of NAPAP, since it was rationalized that the side chain would adopt the same bound conformation as that of NAPAP. The conformers generated within a 6.0 kcal/mol energetic window from that of the lowest energy conformer were collected and subjected to a further 500 iterations of energy minimization to remove some duplicates, which resulted in less than 50 conformers within a 2.5 kcal/mol energy window for both inhibitors. These conformers, with the amidino group added back, were docked into the thrombin active site using NAPAP as the template to position the amidinobenzyl of the inhibitors into S1 pocket. The coordinates of the thrombin-NAPAP complex[5] were obtained from the Brookhaven Protein Databank. The results were visualized using INSIGHTII (Biosym Technologies) running on a Silicon Graphics Indigo 2 workstation.

Chemistry

The general synthesis is outlined in Scheme 1. A number of 4-substituted-phenylacetaldehydes were reacted, respectively, with dichlorophenylphosphine and benzyl carbamate in acetic acid to give the

corresponding CBZ-protected phosphinic amino acids (**5**) using a modified procedure of Chen et al.[8] Usually, these 4-substituted-phenylacetaldehydes were not readily available; they were either prepared from the corresponding 4-substituted-benzaldehydes (as in the case of 4-cyanophenylacetaldehyde) through a four step synthesis[9] or made from a direct oxidation of the corresponding 4-substituted-phenethyl alcohols (as in the case of 4-methoxyphenylacetaldehyde).[10] Compound **5** was deprotected in 30% HBr/AcOH[11] and then coupled with *N*-protected D-Trp or D-3-(2-naphthyl)alanine using the mixed anhydride method.[4a] The cyano group of the resulting dipeptide mimetics was then converted into an amidino group using a standard procedure.[9] Compound **2** was further deprotected to produce compound **3**.

Scheme 1.



Results and Discussion

The inhibitors synthesized according to this design were evaluated for their potency in thrombin inhibition by using the chromogenic substrate S-2238 and the releasing nitrophenol was monitored at 405 nm.[12] The assay results, listed in Table 1 for representative compounds, are in agreement with the modeling prediction.

Table 1. In Vitro Thrombin Inhibition by Phosphinic Dipeptide Mimetics (**1**--**4**)

Inhibitor	IC_{50} (μM)
1	7.5
2	3.6
3	0.6
4	70

As compared to the predicted binding mode for inhibitors **2** and **3** in the thrombin active site (only the binding mode of **3** is shown in Figure 2 as an example), inhibitor **1** behaves in an almost identical way except that the indole ring of **1** sticks somewhat out of the D pocket (result not shown), losing optimal shape complementary in regard to the pocket. Replacement of the indole ring by a 2-naphthyl ring as in **2** and **3** allows the aromatic group to fit into a more favorable position in terms of van der Waals interactions (Figure 2). This probably accounts for the observed activity enhancement with inhibitor **2**. The further increase in the potency of inhibitor **3** (IC_{50}=0.6 μM), which resulted from the deblocking at the N-terminus of **2**, could be explained by the fact that the positively charged amino group may have a favorable electrostatic interaction with the negatively charged Glu-192 side chain. Inhibitor **4**, which has a 4-methoxybenzyl group replacing the positively charged amidinobenzyl group, was designed on the notion that a neutral side chain in the S1 pocket might make the inhibitor more selective towards thrombin according to Cheng et al.[4a] Not surprisingly, it was found to be the least active inhibitor due to the lack of a salt bridge interaction in the S1 pocket.

The modeling suggests that the phenyl group of the phosphinate moiety might have unfavorably close contacts with Leu-99, which could account for the relatively lower inhibitory activities of the designed inhibitors. A more flexible and/or smaller ring, such as a cyclopentyl ring, may fit well in the P pocket without the worry of collision.

It was also determined from the modeling that both chiral centers in the designed inhibitors should be in the D configuration. Because of this and the fact that the only key amide linkage is surrounded by bulky side chains, it is reasonable to speculate that the amide linkage should be fairly resistant to in vivo hydrolysis. In other words, the phosphinate inhibitors may have reasonable in vivo half lives to be considered as potential orally active drug candidates.

In summary, novel thrombin inhibitors have been made within a relatively short period of time through computer aided rational design. Compound **3** was found to be the most potent among the phosphinic dipeptide mimetics synthesized, with an IC_{50} of 0.6 μM. This compound, in particular, provides a promising new lead for the design of more potent inhibitors in our on-going project aimed at discovering orally active therapeutic agents for the treatment of thrombosis.

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2. (a) Abbreviations: NAPAP, N^{α}-(2-naphthyl-sulphonyl-glycyl)-D-*p*-amidino-phenylalanyl-piperidine;[2b] MQPA, (2R,4R)-4-methyl-1-[N^{α}-(3-methyl-1,2,3,4-tetrahydro-8-quinolinesulphoyl)-L-arginine]-2-piperidine carboxylic acid.[2c] (b) Sturzebecher, J.; Markwardt, F; Voigt, B.; Wagner, G.; Walsmann, P. *Thromb. Res.* **1983**, *29*, 635. (c) Kikumoto, R.; Tamao, Y.; Tezuka, T.; Tonomura, S.; Hara, H.; Ninomiya, K.; Hijikata, A.; Okamoto, S. *Biochemistry* **1984**, *23*, 85.
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