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STRUCTURE/FUNCTION ASPECTS OF NEUTRAL P1 RESIDUE PEPTIDE INHIBITORS OF THROMBIN

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Control of thrombin by its inhibition in indications such as myocardial infarction, unstable angina or stroke has been demonstrated to be therapeutically valuable. However restoration of hemostasis by targeting thrombin while avoiding its fellow serine proteinases, (e.g. plasmin, trypsin), remains a challenge of medicinal chemistry. Tripeptide-boronates and -phosphonates with neutral P1 side chains meet these criteria. Development of novel, high yielding chemical routes furnishes a wide range of un-natural P1 functionalities, demonstrating that this indeed is a class effect with selectivity conferred by the uncharged P1 residue. For example *N*-benzyloxycarbonyl-D-phenylalanylprolyl- 1-(3-methoxypropyl) boroglycine ester (**1**) has a K_i value for thrombin of 7 nM and greater than two order of magnitude higher with all other serine proteinases tested. The ester group determines the kinetics of inhibition by tripeptide phosphonates, with diphenylphosphonates being slow tight binding inhibitors, showing 50% reversibility of inhibition. Therefore this design of inhibitors offers a facile strategic approach to development as thrombin specific pharmaceutical agents.

KEY WORDS: Thrombin inhibitors, tripeptide, boronates, phosphonates, un-natural amino acid synthesis, specificity, slow binding, competitive.

INTRODUCTION

Thrombin is a trypsin-like protease which preferably cleaves polypeptide substrates at Arg/Lys-Xaa bonds.[1–3] Unlike trypsin, thrombin displays highly restricted specificity, exhibiting particular preference for hydrophobic P2–P4 residues. The requirements have been used in the design of a number of potent inhibitors of thrombin built around a P1 arginine or arginine analogue, considered to be obligatory for binding to the primary specificity pocket of the enzymes.[4,5] Previously, we have reported[6–8] that peptide phosphonates containing a neutral side chain at P1 were selective potent inhibitors of thrombin. Peptide boronic acids with a similar group at P1 were also found[8,9] to have a high binding affinity for thrombin (K_i 7 nM, compounds **1**), with high specificity[10] as regards inhibition of a number of other trypsin-like proteinases (trypsin, plasmin, kallikrein). Here we report our ongoing work which suggests that thrombin inhibitors lacking a positive charged residue at the P1 site are innately specific. Modification can be carried out without drastic loss of potency. Such an approach may be generally applicable to the development of specific inhibitors of trypsin-like

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proteases. Thrombin exists in blood as a precursor present at a concentration of 1.5 μM.[11] During the clotting of plasma, all the prothrombin is consumed with the release of a large pulse of thrombin formed over 3–4 minutes and reaching a peak of concentration of 0.2 to 0.5 μM. Thrombin inhibitors developed as therapeutic agents must neutralize such a pulse of thrombin. Concentrations of inhibitors will need to attain a level of at least 0.2 μM to act as an antithrombotic agents.[12] At these levels a less specific inhibitor (for example Z-D-Phe-Pro-boro-Irg, compound **2**) although perhaps potent as a thrombin inhibitor will inhibit many other vital processes due to trypsin-like proteases (e.g. fibrinolytic system, complement system) and act as a toxic rather than curative compound.

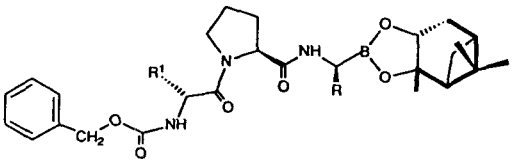
PEPTIDE BORONIC ACIDS

Mechanism of Action

Peptide boronic acids which function as reaction intermediates are highly effective reversible inhibitors of serine proteases. Functionalities of reduced electron density as substitutes of the P1 (e.g. Arg) carbonyl have been introduced to improve binding as reviewed.[13] Disadvantageously this reduces *in vivo* stability, for example aldehydes[14,15] and α-diketones[16] exist in equilibrium as predominantly the inactive hydrate form under physiological conditions, while halomethyl ketones are deactivated by non-specific interactions. Acidity of the α-carbon favours rapid base induced racemisation of trihalomethylketones.[17] The boronic acid warhead is ideal to favour acylation of the active site serine, while the acidity of the P1 α-carbon is not increased greatly, as evidenced by chemical shifts of δ2–3 for the α-protons. Accordingly we have observed no racemisation in physiological conditions.

Boronic acids are Lewis acids, while the pK for the ionisation process $RB(OH)_2 + H_2O = RB(OH)_{-3} + H^+$ is dependent on the nature of the compounds[18]. That the pK of compound (BoroMpg, **1**), has been determined as ~8.6, indicates that at physiological pH the species interacting with the active site is the neutral, trigonal boronic acid,[18] and contrasts with phosphonates and sulphonates which are charged Brönsted acids. Upon interaction with the Ser-195 the incipient negative charge on the boronic acid in the transition state stabilises the residual charge on His-57 for non-peptidic[19] and peptide based[20] boronates, analogously to the intermediate for peptide bond hydrolysis. For phosphonate and aldehyde based inhibitors, for which the transition state (TS) is uncharged, the conformation of His-57 must change[21] with concomitant disruption of the configuration of the active site triad and loss of binding energy. This explains the higher initial binding affinity observed with tripeptide boronic acids in relation to homologous sulphonic and phosphonic esters and aldehyde functionalised compounds. The trigonal boronic acid forms a tetrahedral complex with the active site serine.[22,23] Evidence for the formation of this complex comes from crystallographic data.[24,25] However, certain other experimental data and characteristics of these inhibitors suggests that histidine adducts may be formed.[25–27] The most potent of these inhibitors as have been developed for elastase[28–31] and thrombin (H-D-Phe-Pro-Arg-boro-Arg)[32] exhibit slow tight binding inhibition in which an

TABLE 1
Structure activity relation of inhibition of thrombin by peptide boronates with neutral P1 residues.



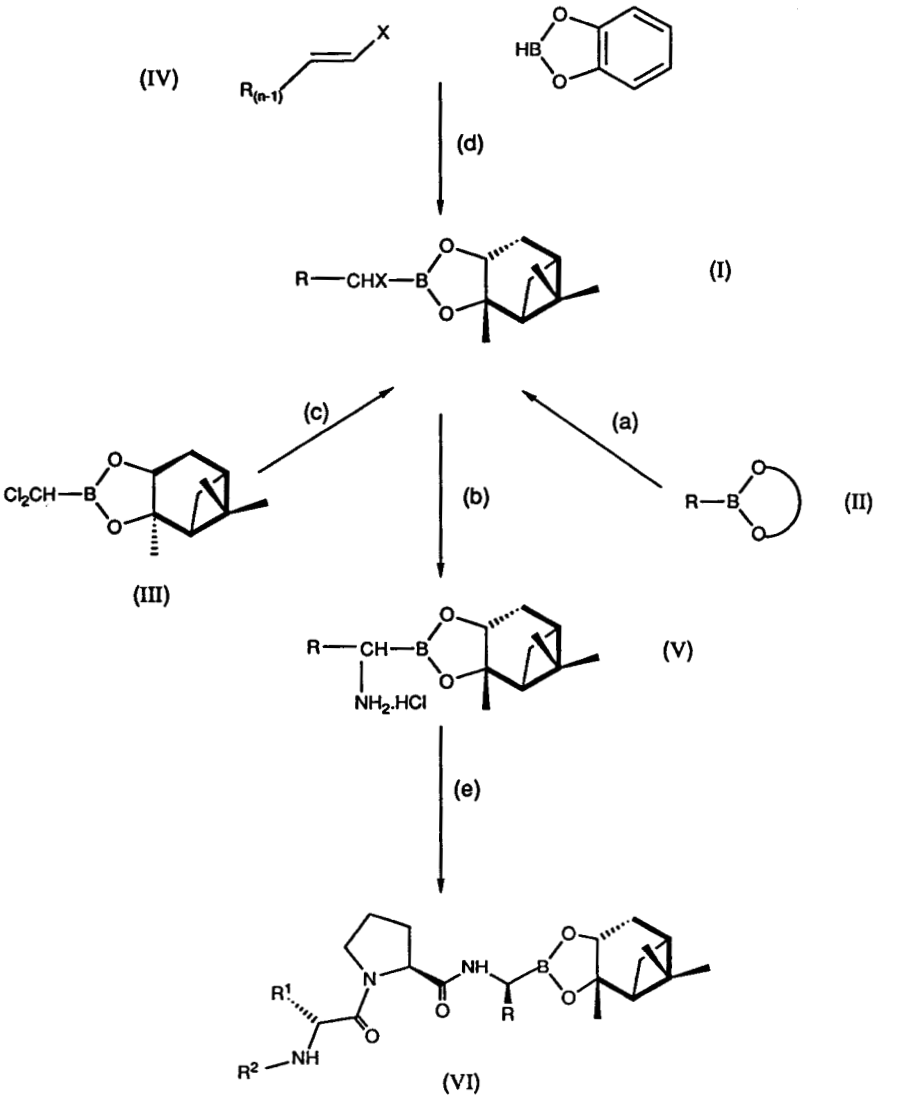
No.	R[a]	R[1]	formula[b]	K_i-Thr (nM)
3	CH_2CH_3	CH_2Ph	$C_{35}H_{46}N_3BO_6$	1000
4	$(CH_2)_4CH_3$	CH_2Ph	$C_{38}H_{52}N_3BO_6$	19
6	$(CH_2)_4CH_3$	$CHPh_2$	$C_{40}H_{53}N_3BO_6$	42
1	$(CH_2)_3OMe$[b]	CH_2Ph	$C_{37}H_{50}N_3BO_7$	7
5	$(CH_2)_3OMe$[b]	$CHPh_2$	$C_{54}H_{54}N_3BO_7$	3
2	$(CH_2)_3SC(NH)NH_2$	CH_2Ph	$C_{37}H_{50}N_5BO_6S.HBr$	1[c]

[a] All D configuration at P1. [b]All compounds show MS and ^1H and ^{13}C NMR consistent with the assigned structures. [c]Initial K_i.

initial EI complex is formed (K_i 1 nM) which slowly tightens (K_i final 1 pM). It is considered that the formation of the initial complex is due to a freely reversible boro-His adduct which decays to the tight binding boro-Ser adduct observed in crystallographic studies.

Chemical Synthesis

The general structure of the inhibitors of thrombin was based upon the peptide H-D-Phe-Pro-Arg first developed in the form H-D-Phe-Pro-Arg-pNA as a highly efficient and selective substrate for thrombin. As was demonstrated empirically and then confirmed by the crystallographic studies of Bode *et al.*[33–35] this structure fits to a hydrophobic binding area adjacent to the active site of the thrombin molecule; the D-configuration of the phenylalanine and the conformational rigidity of the proline conferring a high binding affinity for the enzyme (K_d 1 μM) (Table 1). In designing the novel peptide boronic acids we have used several P3 groups including D-Phe and D-β, β-Diphenylalanine (Dpa), with different amino blocking groups. A range of different neutral side chains at P1 have been synthesized to investigate their influence on the properties of the inhibitor. In order to introduce these different P1 side chains to the boronic acid we used three different routes, all having an α-chloro boronic ester as intermediate (see Figure 1) Matteson's facile route[36] to α-amino boronic acids, (route a, Figure 1) uses (dichloromethyl) lithium and a boronate to form the



(a) $LiCHCl_2$, -100°C, ~90% (b) $LiN(SiMe_3)_2$, -78°C; 3N HCl, 3eq., -78°C , 50-60% (c) RLi or RMgX, -78°C
(d)~90°Cor $RhCl(PPh_3)_3$ 0.05-0.5mol%, 5-72h;pinanediol,dist. 54-98% (e) ^{i}BUOCOCl, $R^2NHCHR^1COPROOH$,60-80%.

FIGURE 1 Preparation of peptide boronic acids.

borate complex which rearranges to give α-chloro boronic ester (II, Figure 1) in good yield. This route, however, requires hydroboration of an alkene to form the substrate boronate (I, Figure 1) and necessarily gives only residues with at least a two carbon alkyl chain. To provide inhibitors with sterically crowded β-positions, not available by the above method, we therefore developed[37] a route (c, Figure 1) from (dichloromethyl) borate (III, Figure 1) allowing the introduction of a range of substituents at the α-carbon via Grignard or alkyllithium reagents. This proceeds via an 'ate' complex of opposite stereochemistry to that of the homologation. In order to give, after the inversion during the S_N2 amination (b, Figure 1), the prequisite D-boronates, a chiral director is required of opposite diastereoselectivity (e.g. (−)- rather than (+)-pinanediol). In the presence of $ZnCl_2$ this gives equal enantiomeric excess to the homologation and the α-chloro boronic (II, Figure 1) esters were isolated in excellent yields. The above routes require cryogenic conditions and are not convenient for scaling up. Therefore, we developed[38] a novel route (d, Figure 1) via hydroboration of 1-haloalkenes (IV, Figure 1, X=halogen), which we found occurs with high regiospecificity. In the cases of some unreactive halo-alkenes, where electron withdrawal disafavours addition of the boron as with β-bromostyrene we found[39] that catalysis by Rh(1) complexes lowered the activation energy sufficiently to give high yields of products. The α-haloboronates (II, Figure 1) obtained above were then aminated using lithium hexamethyldisilylamide and the α-aminoboronic ester (V, Figure 1), isolated as the hydrochloride, was then coupled to the required peptide via the mixed anhydride method to give the tripeptide boronate (VI, Figure 1).

Properties as Thrombin Inhibitors

Over 30 compounds have been synthesized and results are presented here for six examples which clearly demonstrate that the neutral side chain at P1 does confer affinity for thrombin. The compound Z-D-Phe-Pro-boro-Etg-OPin **3** is a weak competitive inhibitor of thrombin (K_i 1 μM) (Table 1). Increasing the length of the aliphatic chain (**4**, Table 1) increased the potency as a decrease in K_i of 50 fold. Introduction of a methoxy group also enhances the affinity of the inhibitor (compound **1**, Table 1, K_i 7 nM). The hydrophobic group can also have an influence on the potency according to the side chain at P1 (e.g. Dpa increase potency of Mpg containing inhibitor, (**5**, Table 1) but Dpa, (**6**, Table 1) decreases the potency of Pg1 containing inhibitors). All the inhibitors of this type tested were observed to act as competitive inhibitors of thrombin. This is in contrast to the properties of similar compounds containing a positive charge at P1. Z-D-Phe-Pro-boro-isothiouronium (**2**, Table 1 was synthesized as a slow tight binding inhibitor (K_i initials 1 nM, K_i final 2 pM, k_2 4 sec^{-1}).

Properties as Specific Inhibitors of Thrombin

The peptides were tested against a range of trypsin-like proteinases viz trypsin, plasmin, factor Xa, kallilrein, urokinase, protein Ca and a number of other serine proteases with preference for neutral aliphatic or aromatic side chains at P1, namely elastase, cathepsin G and chymotrypsin. The results showed that these compounds

have a remarkable specificity for thrombin. This is clearly demonstrated in Figure 2, in which the K_i values with each enzyme for the boroMpg compound **1** and boroPgl compound **4** are compared to that of the boroIrg compound **2**. For all other enzymes the Mpg compound **1** shows at least two orders of magnitude higher binding affinity than that of thrombin unlike the positively charged peptide **2** which is equally potent in inhibiting trypsin and other trypsin-like proteases as thrombin. The specificity of these compounds can be described as innate since variation of the side chain exhibits very little change in the potency towards thrombin in relation to other proteases. The advantages of such a specificity are demonstrated clearly in Figure 3 in which the effect of Z-D-Phe-Pro-boro-Mpg and Z-D-Phe-Pro-boro-Irg upon the lysis of clots by urokinase is tested. It is to be expected from the data in Figure 2 that Z-P-Phe-Pro-boro-Irg will act as an inhibitor of plasmin and urokinase. In Figure 3 the lysis time of clots is prolonged to infinity at concentrations greater than 100 nM unlike the neutral charged peptide which shows little prolongation at concentrations greater than micromolar.

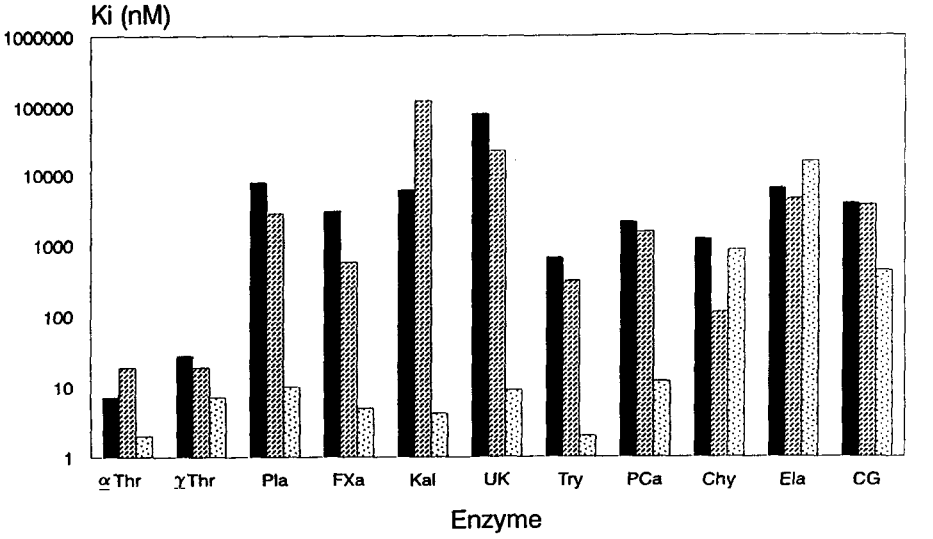


FIGURE 2 Specificity of neutral P1 peptide boronates **1** and **4** for thrombin compared to other serine proteases in comparison to P1 Arg-like compound **2**.
Chromogenic subtrates S-2238 (H-D-Phe-Pip-Arg-pNA) for Thrombin (Thr), S-2222 (Bz-Ile-Glu-Gly-Arg-pNA) for Factor Xa (FX), S-2586 (MeO-Suc-Arg-Pro-Tyr-pNA) for α-Chymotrypsin (α-Chym), S-2288 (H-D-Ile-Pro-Arg-pNA) for Kallikrein (Kal), S-2444 (Glu-Gly-Arg-pNA) for Urokinase (UK), S-2251 (H-D-Val-Leu-Lys-pNA) for Plasmin (Pla) and S-2366 (Glu-Pro-Arg-pNA) for Protein C (PCa), (Suc-Ala-Ala-Pro-Phe-pNA) for Cathepsin G. (CG) and (Suc-Ala-Ala-Ala-pNA) for Elastase (Ela). A solution of each enzyme was added to a series of 10-fold dilutions of each inhibitor in the presence of a fixed concentration of the appropriate chromogenic substrate. The velocity of the reaction was measured over 2 min on a Molecular Devices Corporation Thermomax plate reader at 25°C. Percentage inhibition of the enzyme was calculated for each inhibitor coneentration and this data was used to determine the inhibitor concentration neeessary to give 50% inhibition of the enzyme. Each experiment was repeated after the enzyme and inhibitor had been pre-incubated at room temperature for 2 h. BoroMpg (compound **1**) ■; BoroPgl (compound **4**) □; BoroIrg (compound **2**) □. The first bar refers to Human α-thrombin and the second to γ-thrombin.

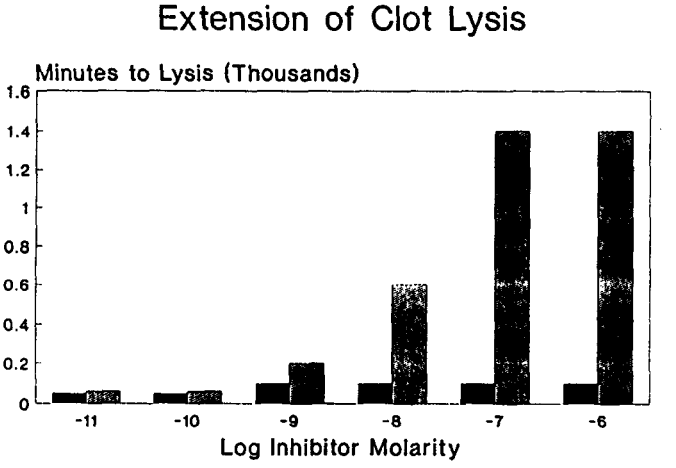


FIGURE 3 Extension of clot lysis.
Effect of compound **1** wih a neutral P1 side chain and a compound with P1 Arg-like residue **2** on the time required for urokinase mediated clot dissolution. An aliquot (0.5 ml) of human fibrinogen (2 mg/ml in 0.05M sodium phosphate buffer pH 7.0) was clotted by the addition of 50 μL of human α-thrombin (final concentration 1.5 NIH units/ml) in round bottomed polstyrene tubes (5×0.3 cm). After incubation for 15 min. the clot was overlaid with a solution containing urokinase (50 μg/ml, Abbot Labs.) and varying concentrations of inhibitor. The time to complete dissolution of the clot (lysis time) was estimated visually. ■ compound **1**; □ Arg-like residue compound **2**.

PEPTIDE PHOSPHONIC ACIDS

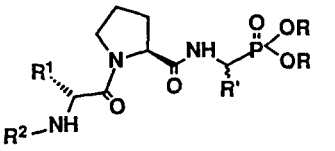
Mechanism of Action

Phosphorylating agents are the prototypical inactivators of serine proteases[40] as exemplified by diisopropyl phosphofluoridate (DFP). Phosphorylating agents act by forming a covalent adduct with the active site serine hydroxyl group. As essentially irreversible inhibitors of serine proteases, phosphorylating agents may have distinct advantages as inhibitors of thrombin; however, simple agents of this type suffer from an inability to discriminate between various enzymes and are therefore highly toxic. Phosphonamidate peptide analogues were first shown to be useful as inhibitors of chymotrypsin.[41] In our study[6,7,42,43] we extended the use of a neutral side chain at P1 to the development of highly specific inhibitors of thrombin.

Chemistry

An ideal serine protease phosphorus inhibitor represents a delicate balance between several competing factors which determine its specificity and activity. The balance between reactivity and chemical stability is determined by the electron density at the phosphorus atom. Very electronegative leaving groups on the phosphorus atom will increase activity toward serine proteases, but also decrease stability

TABLE 2
Kinetic analysis of thrombin inhibition by peptide phosphates. A range of dilutions of each inhibitor were mixed with human α-thrombin (final concentration 15 pM) and chromogenic substrate S-2238 (final concentration 200 μM) in a 96-well microtitre plate. All components were diluted with buffer containing 0.1 M NaPhosphate, 0.2 M NaCl, 0.5% PEG, 0.02% Tween 20, 0.02% NaAzide, PH 7.4). The mixture was incubated at 25°C and the OD at 405 nm was followed for up to 20 h using a Molecular Devices corporation Thermomax plate reader.



No.	R[a]	R^1	R^2	R	formula[b]	K_i (nM)	k_2 (s^{-1})	k_{-2} (s^{-1})	K_i^* (nM)
7	$(CH_2)_3OMe$	$CHPh_2$	H	Ph	$C_{37}H_{41}N_{306}P.HOAc$	10.5	3.00×10^{-4}	1.55×10^{-5}	0.52
8	$(CH_2)_3OMe$	CH_2Ph	H	Ph	$C_{31}H_{36}N_{306}P.HOAc$	2820	1.88×10^{-3}	9.27×10^{-6}	13.84
9	$(CH_2)_4CH_3$	$CHPh_2$	Z	$CH(CH_3)_2$	$C_{40}H_{54}N_3O_7P$	11.7[c]	–	–	–

[a]D,L configuration at P1. [b]All compounds show MS and ^1H, ^{13}C, ^{31}P NMR consistent with the assigned structures. [c]Competitive inhibition kinetics.

toward hydrolysis, which is an obstacle toward development of practically useful inhibitors. For example, difluorophophonates are hydrolysed spontaneously in water and diphenylphosphonates (**7** and **8**, Table 2) can be considered as active esters. Decreasing the electronegativity of the leaving group, as we have demonstrated by incorporation of dialkoxy groups (**9**, Table 2), substantially increases the chemical stability of the inhibitor, but at the cost of lower inactivation rates. Greater selectivity could be achieved when there are structural and stereochemical similarities between a natural serine protease substrate and the potential organophosphorus inhibitor, and could provide the ability to discriminate between different serine proteases. If the choice of the leaving group is appropriate, these inhibitors will retain enough activity to inactivate their target enzyme effectively. Diphenyl esters of peptide phosphonates have been investigated as inhibitors of a number of serine proteases and they demonstrate high selectivity and potency, but previously no thrombin inhibitors of this type had been published. Diphenyl α-aminophosphonates were prepared[6] by a three component, Arbuzov-like, reaction (a, Figure 4) from triphenyl phosphite, the appropriate aldehyde and benzyl carbamate, then deprotected by hydrogenation on Pd/C (b, Figure 4). To obtain α-aminophosphonates (I, Figure 4) for which the esters are not compatible with catalytic hydrogenation we have developed[42] reduction (e, Figure 4) of 1-hydroxyimino-alkanephosphonates (II, Figure 4), which occurs in 40–95% yield, using nascent borane-THF, generated *in situ* by $LiBH_4/Me_3SiCl$.

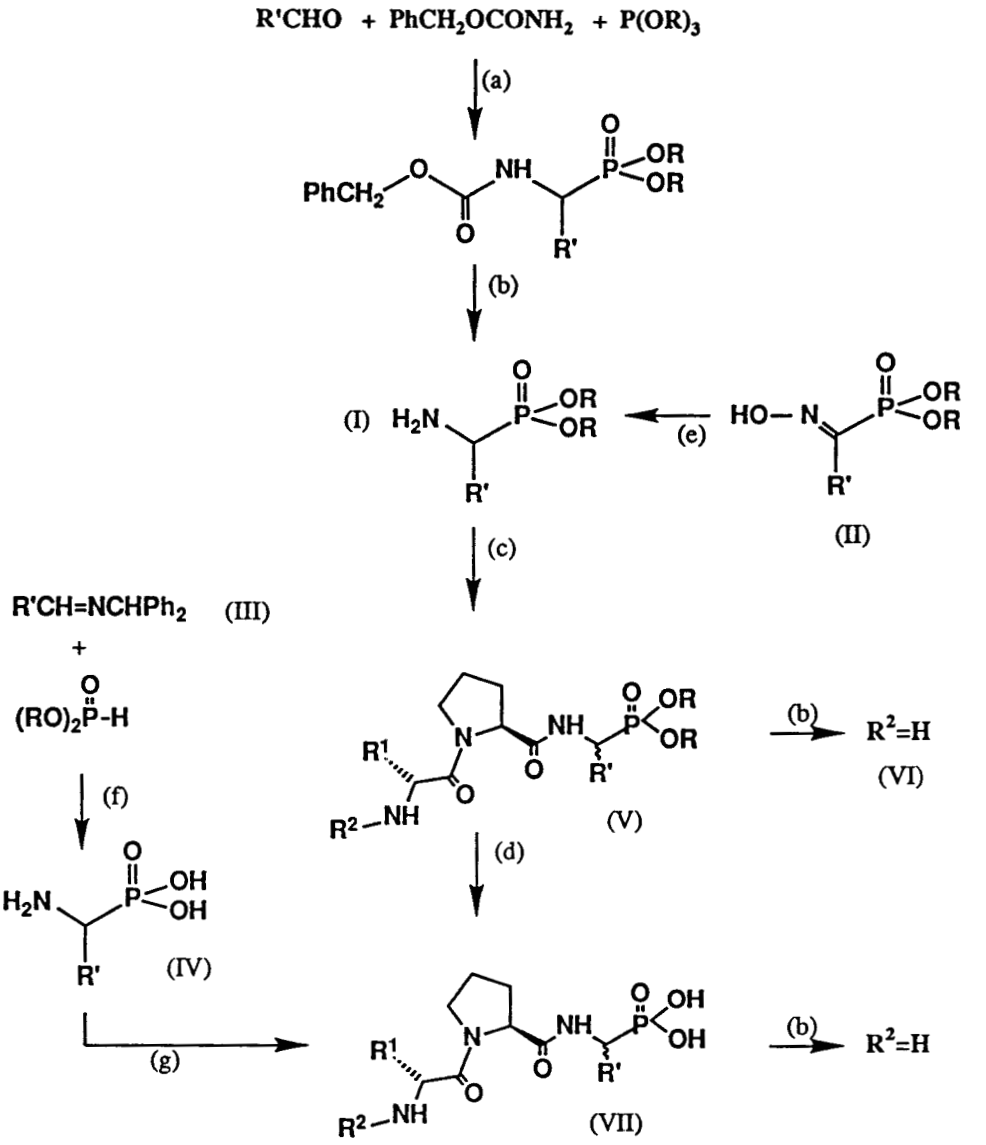


FIGURE 4 Preparation of peptide phosphonic acids.

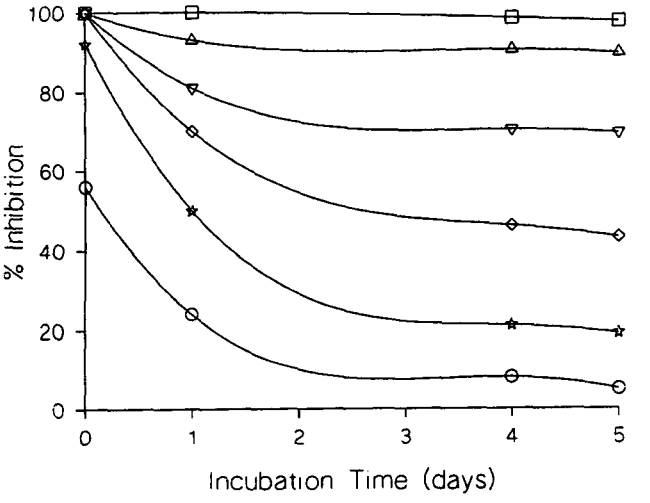


FIGURE 5 Reversibility of thrombin inhibition by phosphonic acid inhibitor **7** upon dilution. The inhibitor **7**-thrombin complex was prepared and incubated at 25°C over four days, then diluted to a final concentration of (□) 9.4 μM, (△) 3.1 μM, (▽) 940 nM, (◇) 310 nM, (⋆) 94 nM and (○) 31 nM.

Alternatively, to give direct access to the free α-amino phosphonic acids (IV, Figure 4), we have shown[43] that benzylidenediphenylmethane (III, Figure 4) undergo rapid nucleophilic addition of dialkylphosphite (f, Figure 4) and give, upon acid hydrolysis, 1-amino-1-arylphosphonic acids (IV, Figure 4). The phosphorus containing tripeptides (V, Figure 4) were obtained 1 through the coupling of dipeptides with the α-amino compound (I, Figure 4) by the mixed anhydride method (c, Figure 4) and isolated by flash chromatography on a silica gel column (2% methanol in chloroform as eluant) or on sephadex (MeOH eluant). Compounds **6** were obtained by removal of the Z protecting group through hydrogenation on Pd/C and purification by chromatography. Compounds **7** were obtained by the cleavage of the diphenyl groups on PtO_2/H_2 from **5** or directly (g, Figure 4) from (IV, Figure 4).

Properties as Inhibitors of Thrombin

All the diphenyl ester inhibitors synthesised (e.g. **7**,**8** Table 2) showed slow tight binding inhibition kinetics, while the dialky ester (**9**, Table 2) was a competitive inhihitor. The data curves for each concentration of inhibitor was solved by iterative fitting to the integrated equation of Cha[44] when E+I=EI=EI* [EI, primary binding complex, EI* final inhibitor complex]. The inhibitors have an initial K_i in the range 500 to 5 nM which tightens during slow binding by up to 60 fold to give final K_i's in the range 50 to 0.3 nM, (Table 2). Values for k_2 varied from 10_{-3} to 10_{-5} sec^{-1} and k_{-2} from 10^5 sec^{-1} to 10^{-7} sec^{-1}. Peptides containing D-Phe at the P3 position (**8**, Table 2) appear to have a higher initial K_i than those with D-Dpa (**7**, Table 2) at this position

but with a greater capacity for slow binding. The presence of a blocking group on the amino terminus greatly reduced the inhibitory capacity of these compounds. When the tightly bound thrombin complex in buffer is diluted and incubated for periods of up to 4 days at room temperature there is 50% reversibility (Figure 5). This suggests that the inhibition is partly irreversible perhaps related to the release or not of the phenyl group which is considered to increase reactivity of these phosphonic compounds.

Selectivity as Inhibitors of Thrombin

These compounds showed no reactivity towards other enzymes tested at concentrations up to 10 μM.

CONCLUSIONS

From earliest developments the design of synthetic inhibitors of thrombin has been based upon binding of arginine or arginine analogue at the primary specificity pocket P1. Because of the known preference for thrombin for Arg-Xaa bonds such a structure within these compounds was considered a *sine qua non*. By suitable modifications therefrom, highly specific and potent inhibitors of thrombin have been developed but usually after screening of very many different candidates and usually with only a single suitable compound chosen from this screen. For example, Okamoto *et al.*[45] developed the compound Argatroban using the thrombin substrate tosyl arginyl methyl ester (TAME)[46] as a starting compound. The need for starting with arginyl compounds has two inherent problems. Firstly it is well recognized that arginine containing compounds tend to be vasoactive and thus toxic.[47–49] Secondly, such compounds will tend to inhibit other trypsin-like proteinases. Trypsin itself, because of its open active site is particularly vulnerable but other vital proteases will also be liable to inhibition to a greater or lesser degree.[50]

The compounds described here with a neutral side chain at P1 show promise for fast track development of specific thrombin inhibitors. Using two warhead moieties, boronic acid and phosphonic acid, our results show such compounds as a group to be "innately" specific for thrombin. Two of the features of these compounds confer the high affinity of thrombin namely binding to the hydrophobic site and binding of the warhead moiety to components of the catalytic triad. Other trypsin-like proteinases which do not have the unique hydrophobic site of thrombin characterized by the ability to bind the peptide D-Phe-Pro show only weak interaction because the warhead moiety is not oriented for ready access to Ser and His. We consider that the primary binding of the inhibitors to this site on thrombin permits such interaction as a secondary event. It is interesting to consider that at this point, the neutral aliphatic group P1 can confer extra binding energy. For example, the compound **3** (Table 1) is more weakly bound than compound **4** (Table 1). Further binding energy is also conferred by an oxygen moiety in the P1 alkyl chain, suggesting possible formation of hydrogen bonding in the environs of the P1 site. Because of these properties the compounds are highly specific and it is possible to envisage that if they are developed as therapeutic agents their pharmacokinetic propertics could be "tuned"

by structural modifications without affecting their potency. At present this principle is being extended to the development of inhibitors with different warhead moieties using the same principles for constructing the P1 and P3 sites.

Acknowledgements

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