Active Site-Directed Synthetic Thrombin Inhibitors: Synthesis, in Vitro and in Vivo Activity Profile of BMY 44621 and Analogs. An Examination of the Role of the Amino Group in the D-Phe-Pro-Arg-H Series

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The serine protease thrombin has long been known to be a key mediator in venous thrombosis. Recent studies also suggest it's role in the pathogenesis of arterial thrombosis as well. The development of agents to inhibit thrombin in prethrombotic situations could aid in lowering the incidence of thrombus-induced myocardial and cerebral ischemia. As a result, thrombin inhibition has become an attractive therapeutic target.[1] A number of benzamidine and arginine derivatives such as N^{α}-[(2-naphthylsulfonyl)glycyl]-D,L-amidinophenylalanine piperidide[2] and argatroban (MD-805)[3] have been described as potent, reversible thrombin inhibitors, however, these compounds are not orally active.

Synthetic peptides, based on the fibrinogen scissile bond and the adjacent amino acid sequence, established the importance of the Phe-Pro-Arg sequence in thrombin active site recognition.[4] Introduction of electrophilic groups at the carboxy-terminus of the fibrinogen-like sequence D-Phe-Pro-Arg resulted in a number of potent irreversible and reversible "transition state analog" inhibitors.[5] In these compounds, the tetrahedral intermediates mimic the transition state of the substrate hydrolysis.[5] More recently, Bajusz et al. reported the aqueous instability of a promising thrombin inhibitor lead[6a] D-Phe-Pro-Arg-H (2).

In previous studies of D-Phe-Pro-Arg-H related inhibitors,[7] the corresponding L-isomer of 2 was reported as at least 2 orders of magnitude less potent than 2 implying rigid stereochemical requirements at the P3 site.[7] From the analysis of the X-ray crystal structure of the enzyme–inhibitor complex and modeling studies, this loss in potency was attributed to an unfavorable steric hindrance exerted by the L-isomer at the hydrophobic pocket.[8] In our ongoing effort to develop promising drug candidates as anticoagulants, as well as to further understand the structural and mechanistic details of inhibitor–enzyme interactions, we synthesized a series of thrombin inhibitors designed to address the role of the P3 amino group in D-Phe-Pro-Arg-H series. Herein, we wish to report the synthesis and biological activity of a new series of dipeptide arginine aldehydes in which non-amino acid P3 residues were introduced. The lead candidate in this series is the reversible inhibitor (vide infra) BMY 44621 (1a), which is stable in aqueous medium and also exhibits in vitro and ex vivo anticoagulant and in vivo antithrombotic activities.[9]

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Results and Discussion

The preparation of BMY 44621 and related analogs is illustrated in Scheme I. Synthesis began with the reaction of benzyl chloroformate and N^{α}-Boc-arginine to produce the key lactam intermediate 3 after an acid-mediated deprotection. Standard amino acid coupling with N-Boc-proline followed by the removal of the Boc group resulted in the formation of Pro-Arg lactam 5 in good yield. Coupling of the appropriate hydrophobic unit produced the lactam 6. Hydride reduction followed by hydrogenolysis of the CBz groups under acidic condition provided the target aldehydes 1a–h as hydrochloride salts. Alternatively, the proline derivative 7 can also be prepared prior to coupling with the arginine lactam 3 to obtain 6.

The compounds prepared as part of this study are listed in Table I. Aldehydes 1a–h were evaluated for their ability to (a) inhibit thrombin hydrolysis[10] of the chromogenic substrate S-2238 (EC_{50}, Table I) and (b) prolong clotting time in human platelet poor plasma (in vitro)[10] and in rats (ex vivo).[10]

The desamino-Phe-Pro-Arg-H, 1a (BMY 44621), was found to possess an EC_{50} of 0.39 μM in the in vitro chromogenic assay. Increase or decrease in chain length between the hydrophobic group and the proline residue (1b,c) resulted in diminished activity since homologation in either direction reduced potency 2-fold. Introduction of a hetero atom in the carbon chain or in the phenyl group (1d–f) resulted in some reduction in thrombin inhibitory potency. Interestingly, the cyclohexyl analog (1g) displayed a 2–3 fold increase in potency demonstrating that the phenyl group is not important for activity. However, when the amide carbonyl at the P3 site of 1g was replaced with a methylene group (1h), a dramatic 100-fold reduction in potency was observed suggesting the importance of the amide linkage to properly orient the hydrophobic side chain at the P3 site. Furthermore, compound 1h in it's protonated form at the proline nitrogen may also experience adverse interactions at the hydrophobic P2-P3 pocket.[11a]

BMY 44621 represents the lead prototype in this series. Furthermore, in contrast to D-Phe-Pro-Arg-H, this compound displayed enhanced aqueous stability when stored over a period at 40 °C. This was shown by thrombin clotting time measurements taken at various time intervals (Figure 1). This is not surprising since removal of the amine moiety in the D-Phe group (as in BMY 44621) prevents the first cyclization step involved in the cascade of reactions leading to an inactive heterocycle.[6] Thus, the biochemical and pharmacological characterization of this candidate in various in vitro and in vivo anticoagulants and antithrombotic models were examined in some detail.[9b]

Table II shows the selectivity profile of the inhibitor 1a. While 1a and most of the inhibitors in Table I showed good selectivity for thrombin over plasmin, only moderate to poor selectivity to trypsin was observed. This may be attributed to an adverse effect in the removal of the amino group in D-Phe which was thought to be involved in an antiparallel H-bond with the enzyme. Perhaps, appropriately housed basic functionality at the P3 residue may be a factor in enhancing selectivity for thrombin over trypsin since the potential role of the Glu-202 residue of thrombin in substrate specificity is known.[11b]

Compound 1a exhibited clean competitive inhibitory kinetics with no time-dependence to thrombin inhibition

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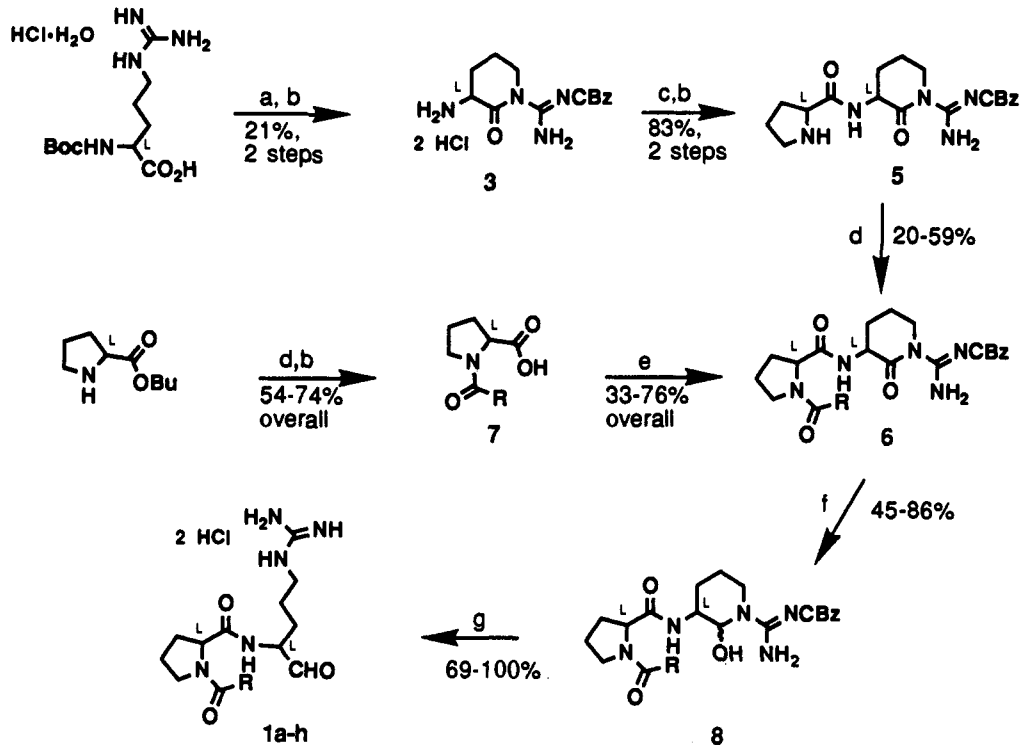
Scheme I[a]



[a] 2 equiv CBzCl, TEA, THF; [b] HCl/EtOAc; [c] Boc-L-Pro-OH, iBCF, TEA, NMM, THF; [d] RCO_2H, iBCF, TEA, NMM, DMF or RC(O)Cl, TEA, DMF; [e] iBCF, TEA, NMM, THF; [f] *LAH*, THF; [g] 10% Pd/C, H_2, 1 N HCl, THF.

Table I. In Vitro EC_{50} for Thrombin Inhibition[a] and Doubling of Clotting Time[b] for BMY 44621 (**1a**) and Analogous *N*-Acyl-L-prolyl-L-arginine Aldehydes[c]

no.	R[d]	EC_{50} (μM)[a]	EC_{50} (μM) (DTT)[b]
1a	Ph*CH_2CH_2CO	0.39	0.46
1b	$PhCH_2CH_2CH_2CO$	0.88	0.5
1c	$PhCH_2CO$	0.59	0.75
1d	$PhOCH_2CO$	0.84	1.0
1e	Pyr*CH_2CH_2CO	1.17	1.0
1f	Thl*CH_2CH_2CO	0.59	0.5
1g	Chl*CH_2CH_2CO	0.15	0.3
1h	$ClCH_2CH_2CH_2$	56	75
2	D-Phe**	0.049	0.4

[a] Concentration required to inhibit thrombin cleavage of the chromogenic substrate S-2238 by 50% (ref 10). [b] DTT is the concentration required to double the clotting time (TT) as described.[10] [c] All compounds discussed herein are characterized by ^1H and ^{13}C NMR IR, mass spectrometry, and elemental analysis, or high resolution mass spectrometry. [d] (*)Ph = phenyl; Pyr = 3-pyridyl; Chl = cyclohexyl; Thl = 2-thienyl; (**) D-Phe = D-phenylalanyl.

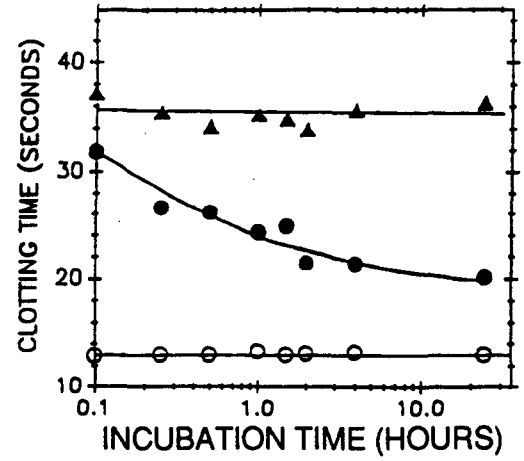


Figure 1. Stability of the D-Phe-Pro-Arg aldehyde (filled circles) and BMY 44621 (filled triangles) compared to control (open control). Compounds were prepared as stock solutions (50 μM) in deionized water. The stock solutions were incubated at 40 °C for the indicated times after which aliquots were withdrawn for thrombin time determinations (average of duplicate determinations). The compound was added in concentrations that initially caused prolongation of clotting time approximately three times control value (the initial concentrations used in this study were 0.2 μM for D-Phe-Pro-Arg aldehyde and 0.5 μM for BMY 44621).

(as opposed to D-Phe-Pro-Arg-H which was time-dependent in its inhibition and could be characterized as a slow-tight binder). This suggests compound **1a** is a reversible inhibitor. The K_i of thrombin inhibition averaged 0.14 μM (obtained from Dixon plots see Figure 2). A modified thrombin time (TT) was used to monitor the direct inhibition of human thrombin activity in plasma-containing samples. In this assay, BMY 44621 doubled thrombin clotting time in vitro at 0.46 ± .12 μM (n = 3). This compound also prolonged both activated partial thromboplastin time (APTT) and prothrombin time (PT).[9b]

Ex vivo clotting time measurements were used as a measure to determine the oral activity of BMY 44621. Prolongation of thrombin clotting time in rats ex vivo was found to be dose dependent (Figure 3). Doubling clotting time was observed at a dose of approximately 60 mg/kg. BMY 44621 terminated thrombin clotting time (TT) at 150 mg/kg. In iv studies, this compound doubled TT at lower doses (0.5 mg + 30 μg/kg per min, n = 9; $p < 0.01$).[9b]

The competitive thrombin inhibitor BMY 44621 produced dose-dependent inhibition of arterial and venous thrombosis in pentobarbital-anesthetized rats. Thrombosis was induced in the carotid artery by electrical stimulation and in the vena cava by a combination of blood stasis and endothelial disruption caused by hypotonic saline infusion.[9] Threshold and near complete inhibition of vena cava thrombi was achieved at a dose of 3 mg/kg

Table II. Selectivity of BMY 44621 for Thrombin Inhibition[a]

protease	EC_{50} (μM)	
	BMY 44621	D-Phe-Pro-ArgH
Thrombin	0.39	0.05
Plasmin	7.65	2.35
Trypsin	0.026	0.22

[a] Inhibition of thrombin using 10 μM S-2238 was measured as described in ref 10. Inhibition of bovine pancreatic trypsin was measured in an assay containing 2 mM $CaCl_2$, 50 mM Tris pH 8.0 using 100 μM carbobenzoxy-Val-Gly-Arg-pNA. The compound was combined with trypsin in assay buffer and incubated for 3 min, after which the substrate was then added and absorption was measured using a microplate reader (Molecular Devices V_{max}) at 405 nm. Inhibition of human plasmin was measured in an assay containing 145 mM NaCl, 5 mM KCl, 1 mg/mL polyethylene glycol (PEG-8000) 30 mM *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid, pH 7.4 and 100 μM S-2251 (D-Val-Leu-Lys-pNA). The compound was incubated with the enzyme for 3 min, after which, 100 μM S-2251 was added and the rate of hydrolysis was measured as for trypsin and thrombin. This data for BMY 44621 represents the average of duplicate determinations with less than 25% variability between determinations.

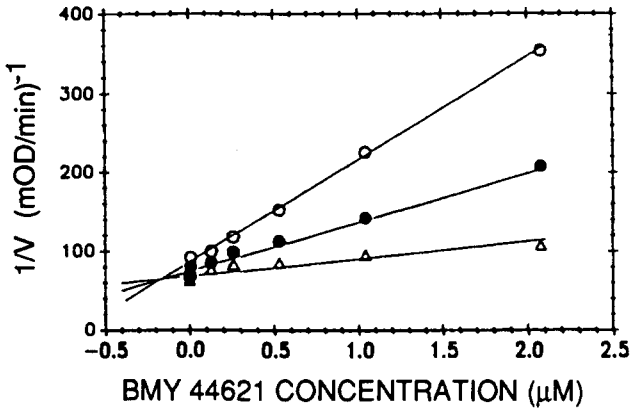


Figure 2. Dixon Plot for the determination of inhibitory K_i for BMY 44621. The enzyme velocity was measured using the synthetic substrate D-Phe-Pip-Arg-pNA (S-2238) at three different concentrations 100 μM (open triangles), 25 μM (filled circles), and 10 μM (open circles). Each of the above points represents the average of a triplicate determination. (This study is representative of two separate experiments, the results of which differed by less than 10%).

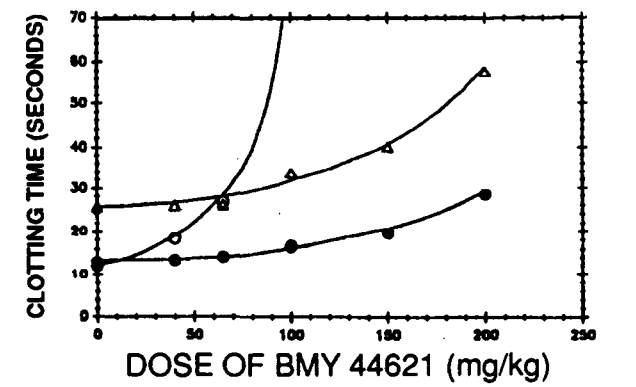


Figure 3. Oral activity of BMY 44621 (tested 2 hours after oral administration to rats). TT (open circles) measures the time required to clot plasma after the direct addition of human α-thrombin to rat platelet-poor plasma. APTT (open triangles) measures the intrinsic blood coagulation pathway. PT (filled circles) measures the extrinsic blood coagulation pathway. Each point represents the average of four determinations.

iv (1 mg/kg + 0.05 mg/kg per min).[9b] Threshold inhibition of carotid artery thrombosis was obtained at a dose of 8 mg/kg iv (2 mg/kg + 0.1 mg/kg per min).[9b] Maximal antithrombotic activity was correlated with increases in thrombin time of 2-fold for venous thrombosis and 5-fold for arterial thrombosis.

In conclusion, we have shown that removal of the primary amino group in **2** provides a compound that although weaker than **2** in measuring synthetic substrate hydrolysis (probably because the slow-binding inhibitor **2** can preincubate with thrombin) was equieffective in the clotting assay. Our results suggest that the carbonyl group at the P3 residue is of paramount importance for maximal binding. In addition, the stable desamino compound **1a** prolongs clotting time following iv and oral administration to rats. It is also effective in models of both arterial and venous thrombosis. Further pharmacological evaluation of BMY 44621 as well as an expanded structure–activity relationships in this series of arginine aldehydes will be the subject of future reports.

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(10) (a) Chromogenic substrate assay: The inhibitors (10 μL) were incubated with the assay buffer (270 μL of 145 mM NaCl, 5 mM KCl, 1 mg/mL polyethylene glycol (PEG-8000), 30 mM *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid, pH 7.4.) and human α-thrombin (10 μL of 3 units/mL) at room temperature for 3 min (for initial IC_{50} determinations). The enzymatic reaction was started with 10 μL of 3 mM s-2238 substrate and continued at room temperature. The change in optical density was measured at 405 nm. A kinetic microplate reader (Molecular Devices Corporation V_{max}) was used to measure the change in optical density over time. (b) Clotting time measurements were determined optically at 37 °C using an MLA 700 coagulation timer. Thrombin time was determined with 0.1 mL of pooled citrated human plasma (1/10th final volume of 0.129 M buffered citrate) added to 0.1 mL of Owren's buffer (125 mM NaCl, 28.4 mM sodium barbital, pH 7.35.) and stimulated with 0.1 mL of 10 units/mL human α-thrombin. The concentration of inhibitor that caused a doubling of thrombin clotting time was determined.

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