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N-SUBSTITUTED GLYCINES AS REPLACEMENTS FOR PROLINE IN TRIPEPTIDE ALDEHYDE THROMBIN INHIBITORS

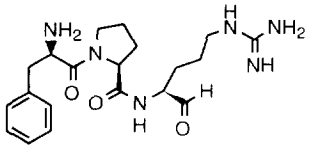
Aaron L. Schacht,* Michael R. Wiley, Nickolay Chirgadze, David Clawson, Trelia J. Craft, William J. Coffman, Noel D. Jones,[a] Donnetta Gifford-Moore, Jennifer Olkowski, Robert T. Shuman, Gerald F. Smith, Leonard C. Weir

Lilly Research Laboratories
A Division of Eli Lilly and Company
Lilly Corporate Center, Indianapolis, IN 46285

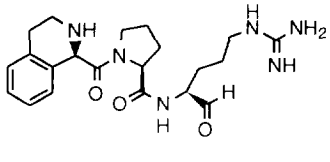
Abstract: We have prepared a series of tripeptide arginine aldehydes in which the P2 proline has been replaced with a variety of *N*-substituted glycines. The effects of these modifications on thrombin inhibitory potency and serine protease selectivity were evaluated.

Thrombin is a serine protease which is produced during activation of the blood coagulation pathways.[1] It is responsible for proteolytically activating plasma fibrinogen for fibrin formation,[2,3] and is the most potent agonist[4] for platelet aggregation. These two activities place thrombin in a central role in the coagulation process.[5,6] Imbalances in coagulation can result in thrombus formation leading to occlusive events which are responsible for significant morbidity and mortality. Therefore, thrombin inhibitors represent a new class of anticoagulant agents with potential utility in the treatment of thrombosis.[7] An important consideration in the development of thrombin inhibitors is the selectivity versus related fibrinolytic enzymes, as a non-selective agent could interfere with endogenous or therapeutic clot dissolution.

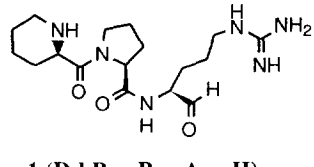
Thrombin cleaves its natural substrates on the C-terminal side of an arginine residue. Using the substrate analog approach,[8] Bajusz, et al., showed that D-phenylalanyl-prolyl-arginine aldehyde (**GYKI 14166**, D-Phe-Pro-Arg-H) is a potent inhibitor of thrombin.[9] Moreover, they showed that proline is the optimal natural residue for the P2 position in this class of inhibitors.[10] Another similar inhibitor of thrombin, **LY288570**, has been co-crystallized with bovine thrombin and examined by X-ray crystallography to provide important structural details.[11]



GYKI 14166



LY288570



1 (D-hPro-Pro-Arg-H)

[a] Present address: Molecular Structure Corporation, The Woodlands, TX 77381-4238

With respect to steric considerations, proline is complementary in size and shape to the S2 binding site of thrombin, defined by residues Tyr60A, Trp60D, Ile99, and His57. Also, proline induces a type II beta turn in the inhibitor, which places the P1 and P3 residues into position to bind with their respective sites in the enzyme. It has been shown that *N*-substituted glycines, e.g., sarcosine (*N*-methyl glycine), are replacements for proline in a variety of small peptide systems,[12,13] as the *N*-substituted glycine induces similar peptide backbone conformations to proline.[14] On the basis of these studies and inspection of the crystal structure of the **LY288570**:thrombin[11] complex, we proposed that *N*-substituted glycines would be suitable replacements for proline in the tripeptide aldehyde thrombin inhibitors. We hypothesized that not only would the *N*-substituted glycines provide similar conformational effects to proline, but that the Gly nitrogen could serve as a point of attachment from which we could tether substituents designed to interact with either the S3 or S2 hydrophobic binding sites (see Figure 1). We report here a study in which the P2 proline of a tripeptide aldehyde is replaced with a variety of *N*-substituted glycines.

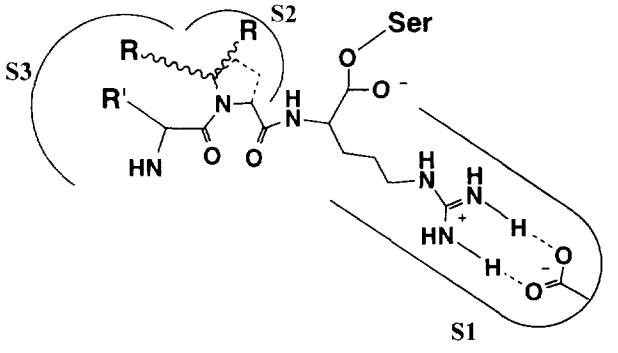
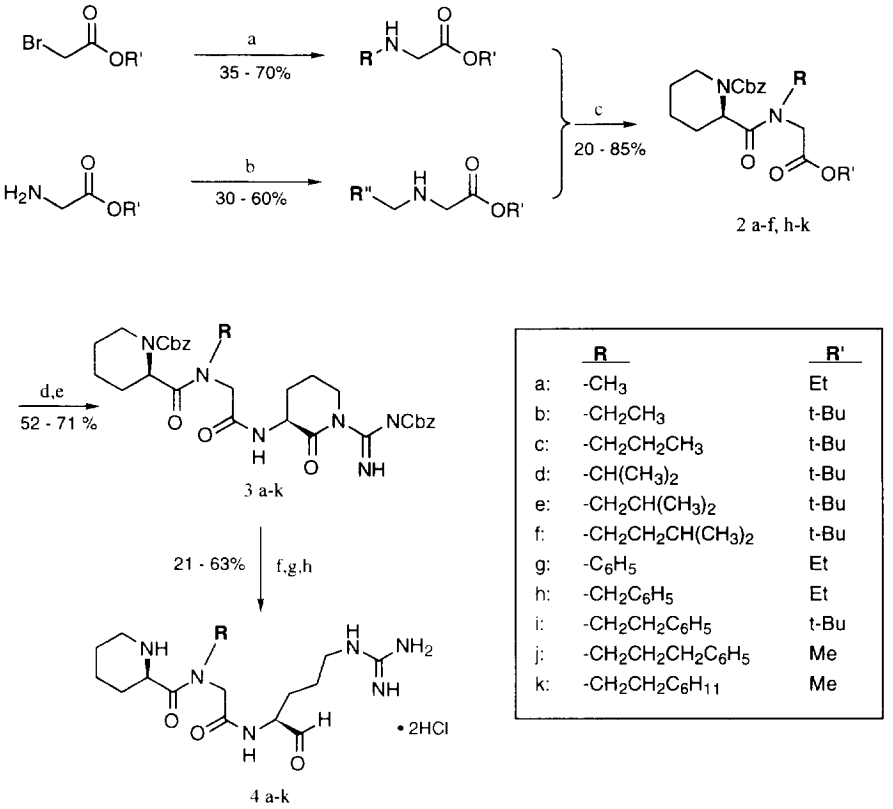


Figure 1: Cartoon depicting binding of inhibitor in thrombin active site.

Chemistry

The synthesis and characterization of inhibitors generally followed procedures as described in reference 15. We chose D-homoproline (D-hPro) as the N-terminal residue since this is readily available as an enantiomerically pure material, and the small size of the homoproline ring should not preclude the opportunity for interaction of the *N*-substituent with the S3 pocket. D-hPro-Pro-Arg-H (**1**) had been previously prepared in our laboratories and was shown to be a potent thrombin inhibitor *in vitro*.[16] Several *N*-substituted glycines were prepared by alkylation of the appropriate alkyl amine with a bromoacetate, while others were obtained by reductive amination utilizing a glycine ester and the appropriate aldehyde. Scheme 1 illustrates the general synthetic route for the preparation of the tripeptide arginals incorporating the *N*-substituted glycines at P2 (**2 a-f, h-k**). In the case of the N-phenylglycine derivative **2g**, coupling of Cbz-D-homoproline to N-phenylglycine ethyl ester hydrochloride failed under EDC coupling conditions. As an alternative approach, Cbz-D-homoproline was first coupled to aniline, and the dipeptide ester was then obtained by deprotonation of the anilide with NaH in the presence of excess ethyl bromoacetate in THF at 0 °C (96%).

Scheme 1.



	R	R'
a:	$-CH_3$	Et
b:	$-CH_2CH_3$	t-Bu
c:	$-CH_2CH_2CH_3$	t-Bu
d:	$-CH(CH_3)_2$	t-Bu
e:	$-CH_2CH(CH_3)_2$	t-Bu
f:	$-CH_2CH_2CH(CH_3)_2$	t-Bu
g:	$-C_6H_5$	Et
h:	$-CH_2C_6H_5$	Et
i:	$-CH_2CH_2C_6H_5$	t-Bu
j:	$-CH_2CH_2CH_2C_6H_5$	Me
k:	$-CH_2CH_2C_6H_{11}$	Me

(a) RNH_2, EtOH, DIEA; (b) R"CHO, $NaBH_3CN$, MeOH; (c) Cbz-D-homoproline, EDC, HOBT, DIEA, dichloromethane; (d) LiOH, dioxane/H_2O 0 °C or TFA 0 °C; (e) isobutylchloroformate, N-methyl morpholine, arg(Cbz)lactam•2HCl, DMF, -15 °C; (f) LAH, THF, -78 °C; (g) H_2, Pd/C, EtOH, H_2O, HCl; (h) RP-HPLC (elution with gradient of 0.05 N HCl/CH_3CN)

Results and Discussion

Compounds **4a-k** were evaluated *in vitro* for their inhibitory potency versus thrombin, trypsin, plasmin and n-tPA. These values are reported in Table 1, which gives the determined association constant[17] (K_{ass}) of the inhibitor with thrombin as well as the selectivity ratios toward the other enzymes. Replacement of proline with sarcosine, as in **4a**, results in only a 10-fold loss of thrombin inhibitory potency (K_{ass} = 22 x 10^6 L/mol), despite the loss of an element of conformational restriction and the elimination of the interaction between the S2 subsite and two methylenes of the inhibitor. This modification also results in a 7-fold increase in selectivity for thrombin versus trypsin and a 3-fold improvement versus plasmin, while selectivity for thrombin versus n-tPA remains high (5,500).

Systematically increasing the size of the *N*-substituent from methyl (**4a**) to ethyl (**4b**) to propyl (**4c**) shows little effect on activity. Incorporation of branching either α, β, or γ to the nitrogen (**4d-f**) again provides

little or no change in potency, with some effects on selectivity. Notable is the fact that all of the *N*-alkyl substituted glycine derivatives maintain or enhance selectivity relative to the proline containing counterpart (**1**).

Substituting the P2 gly nitrogen with a phenyl or benzyl group offers no increase in thrombin inhibitory potency, and with the benzyl derivative (**4h**) selectivity for thrombin versus trypsin is eliminated, while selectivity for thrombin versus plasmin decreases 9-fold and selectivity for thrombin versus tPA decreases 5-fold. Tethering the phenyl ring out one methylene further, as in **4i**, results in an improvement in potency. In fact, the anticoagulant activity of **4i** is nearly indistinguishable from that of **1**, as measured by the concentration required to double the thrombin clotting time,[17] 120 nM for **4i** versus 110 nM for **1**. Extending the chain again to the phenpropyl analog (**4j**) reduces activity back to the level of the *N*-alkyl derivatives. Saturating the ring of the phenethyl derivative yields an inhibitor (**4k**) with diminished potency and selectivity.

Table 1. Summary of in vitro evaluation.

No.	R	K_{ass}* Thrombin	K_{ass} Thr/ K_{ass} Try	K_{ass} Thr/ K_{ass} Pla	K_{ass} Thr/ K_{ass} ntPA
1		200	3.3	220	6700
4a	$-CH_3$	22	24	730	5500
4b	$-CH_2CH_3$	33	25	3300	6600
4c	$-CH_2CH_2CH_3$	34	14	680	8500
4d	$-CH(CH_3)_2$	10	20	10000	10000
4e	$-CH_2CH(CH_3)_2$	24	15	480	24000
4f	$-CH_2CH_2CH(CH_3)_2$	16	2.7	270	4000
4g	$-C_6H_5$	16	15	400	4000
4h	$-CH_2C_6H_5$	10	1	77	1000
4i	$-CH_2CH_2C_6H_5$	87	32	200	14500
4j	$-CH_2CH_2CH_2C_6H_5$	24	4	200	4000
4k	$-CH_2CH_2C_6H_{11}$	5.4	0.9	16	770

* All K_{ass} values are in units of L/mole x 10^{-6}, and were determined according to the procedures detailed in Reference 17.

In order to understand the interactions responsible for activity in this series, the most potent analog (**4i**) was co-crystallized with thrombin and examined by X-ray crystallography. A crystal structure was solved at 2.2 Å resolution.[18] Inspection of the active site region of this complex revealed several interesting features (Figure 2). First, the inhibitor molecule fits in the active site in a similar fashion to other related arginals.[11] The guanidinium group forms a salt bridge with the carboxyl of Asp189 which is buried in the specificity (P1) pocket. Covalent bond formation between the carbonyl carbon of the inhibitor and the oxygen of Ser195 is indicated by a strong, well-defined, continuous electron density (C-O distance is approximately 1.4 Å). The P3 D-homoproline residue sits in the hydrophobic region formed by side chains of residues Leu99, Ile174 and Trp215, engaging in an anti-parallel hydrogen bond pair with Gly 216. However, the S2 subsite which is defined by residues Tyr60A, Trp60D, Leu99 and His57, is not occupied, in contrast to the case where proline is the P2 residue. Instead, a water molecule rests in the vicinity of carbon 3 of the proline ring. Finally, the

most interesting aspect of this structure is the binding of the phenethyl sidechain of the *N*-substituted glycine. This sidechain appears to have two equally populated binding modes, each with well-defined electron density. In one mode, the phenyl group rests in a portion of the S3 pocket that is formed by Leu99, Ile174, Trp215, and the P3 homoproline residue. In the alternate mode, the phenyl group is tightly sandwiched between the P3 piperidine of the inhibitor and the Trp60D of the thrombin insertion loop, engaging in an edge-face aromatic interaction.

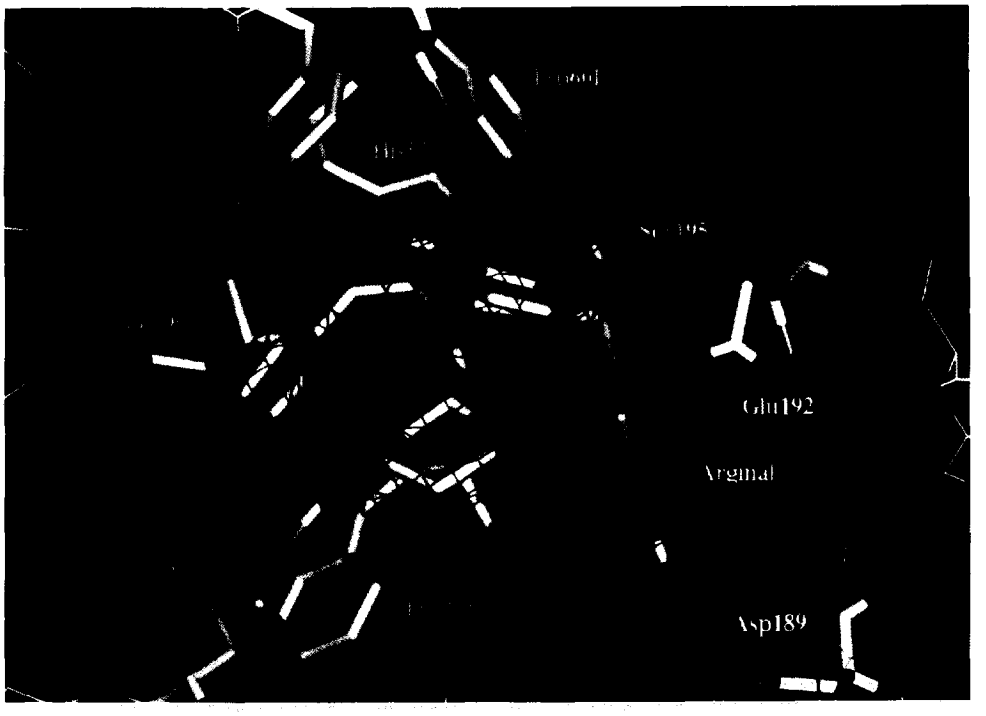


Figure 2. Active site region of the thrombin:4i complex. The principal side chains of thrombin which interact with the inhibitor are highlighted. A $2F_o$-F_c electron density map is contoured at the 1.5σ level. Both binding modes of the inhibitor are shown.

This crystal structure allows us to propose explanations concerning the activity of the inhibitors in this series. The alkyl substituents of **4b-f** are too small to derive benefit from binding to the S3 subsite. A phenyl ring can be positioned appropriately to bind in this site, with a two carbon tether providing the optimum distance. Reducing, or extending the tether length compromises the binding interaction. It may be that restriction of the conformation of the phenethyl side chain to the bound orientation could result in further enhancements of binding energy. Finally, since the phenyl ring of **4i** is in Van der Waals contact with Leu 99 and Trp215 as well as the N-terminal piperidine ring of the inhibitor, it is not surprising that increasing the width of the substituent by saturation of the phenyl ring (**4k**) destabilizes the complex.

In conclusion, we have demonstrated that proline can be replaced by readily available *N*-substituted glycines in a series of tripeptide aldehyde thrombin inhibitors. The nature of the *N*-substituent is important to

binding with thrombin, while the utility of *N*-substituted glycines to enhance selectivity versus related serine proteases appears to be general. D-hPro-(N$^{\alpha}$phenethyl)Gly-Arg-H, **4i**, is a potent thrombin inhibitor in both chromogenic and clotting assays, and has a superior selectivity profile. The X-ray crystal structure of the enzyme inhibitor complex has shown that the inhibitor has two distinct binding modes in the active site of thrombin. Finally, this structure could serve as a point of departure for further structure-based improvements in thrombin inhibitory potency and selectivity.

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18. Solved using the model described in [11]. Resolution ranged from 6.0 to 2.2Å. The position of the inhibitor molecule in the active site was determined by a difference electron density map. A final model of the complex has been crystallograpically refined up to R-factor value 15.2% with standard deviations in bonds length from ideal of 0.018 Å. R-factor = $\sum$ ||F_O| - |F_C|| / $\sum$ |F_O|, where |F_O| and |F_C| are the observed and calculated structure factor amplitudes respectively.

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