Potent Bivalent Thrombin Inhibitors: Replacement of the Scissile Peptide Bond at P_1-P_1' with Arginyl Ketomethylene Isosteres▽

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We have designed highly potent synthetic bivalent thrombin inhibitors, which consist of an active site blocking segment, a fibrinogen recognition exosite blocking segment, and a linker connecting these segments. The bivalent inhibitors bind to the active site and the fibrinogen recognition exosite simultaneously. As a result, the inhibitors showed much higher affinity for thrombin than the individual blocking segments. Various arginyl ketomethylene isosteres ArgΨ-[CO-CH_2-X]P_1' were incorporated into the bivalent inhibitors as P_1-P_1' segment to eliminate the scissile bond. The P_1' residue is a natural or unnatural amino acid; specifically, the incorporation of mercaptoacetic acid exhibited superiority in synthesis and affinity for thrombin. Inhibitor **16**, (D-cyclohexylalanine)-Pro-ArgΨ[CO-CH_2-S]Gly-$(Gly)_4$-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Tyr-cyclohexylalanine-(D-Glu)-OH, showed the lowest K_i value of $3.5 \pm 0.5 \times 10^{-13}$ M, which is comparable to that ($K_i = 2.3 \times 10^{-13}$ M) of recombinant hirudin. Consequently we successfully reduced the size of the inhibitor from ∼7 kDa of recombinant hirudin to ∼2 kDa without losing the affinity.

Introduction

The trypsin-like serine protease thrombin (EC 3.4.21.5) is a primary target in developing successful anticoagulants due to its central functions in the process of hemostasis and thrombosis. Thrombin converts soluble circulating fibrinogen into clottable fibrin and amplifies its own generation through the activation of other coagulation enzymes such as factors V and VIII.[1,2] In addition, thrombin activates factor XIII, which stabilizes the clot by cross-linking fibrin, and stimulates platelet secretion and aggregation through a proteolytic activation of the thrombin receptor on the platelets.[3] Thrombin also mediates a negative-feedback regulation of the coagulation cascade by activating protein C upon binding to the endothelial cell surface protein thrombomodulin.[1]

The importance of blocking thrombin activity in prophylaxis of thromboembolic disorders in high-risk patients was described elsewhere.[4,5] Anticoagulants such as heparin and antiplatelet agents such as aspirin are routinely used during coronary angioplasty.[6] They are also used in combination with thrombolytic therapy induced by urokinase, streptokinase, or tissue plasminogen activator[7] to prevent rethrombosis.[8] Thrombin released from thrombus seems to be one of the major components responsible for the reocclusion observed in 10−20% of patients despite heparinization.[9,10] The modest benefit of heparin in combination with thrombolytic therapy seems to be due to the inaccessibility of heparin to the thrombin molecules in the thrombus.[8,11,12]

Various small active-site thrombin inhibitors for chronic dosing have been extensively designed over nearly three decades. However, there has been no compound in clinical use that clearly satisfies all of the necessary criteria of high and reproducible oral bioavailability, long duration, selectivity, and satisfactory pharmacokinetic properties.[13] In acute treatment, hirudin, a 65-amino acid protein from the leech *Hirudo medicinalis*, may have some advantages over the low-molecular-weight inhibitors or heparin.[14] Hirudin, due to its bivalent binding mode, has a high selectivity and a high affinity for human α-thrombin ($K_i = 2.2 \times 10^{-14}$ M):[15] the N-terminal globular hirudin(1−48) contacts the thrombin surface adjacent to the active site, and simultaneously, the C-terminal hirudin(55−65) interacts with the fibrinogen recognition exosite (FRE) of thrombin.[16] Another advantage of hirudin is that hirudin reduces fibrin polymerization rates and the content of fibrin−fibrinogen in the blood clots more efficiently than the covalent small active-site inhibitor PPACK.[17] This suggests that the FRE accelerates the fibrin clot assembly as a cofactor. Furthermore, hirudin, but not heparin, neutralized both thrombin in solution and fibrin-bound thrombin in the coagulation activated during thrombolysis, which was induced by a plasminogen activator.[18,19]

The high selectivity and affinity of hirudin motivated the design of synthetic bivalent inhibitors in which the bulky hirudin(1−48) was replaced by a small active-site-directed inhibitor segment, e.g., D-Phe-Pro-Arg-Pro, which was then connected to the FRE blocking segment r-hirudin(55−65) through an oligopeptide spacer.[20,21] Substitution of the N-terminal D-Phe by D-cyclohexylalanine (D-Cha) improved not only affinity but also

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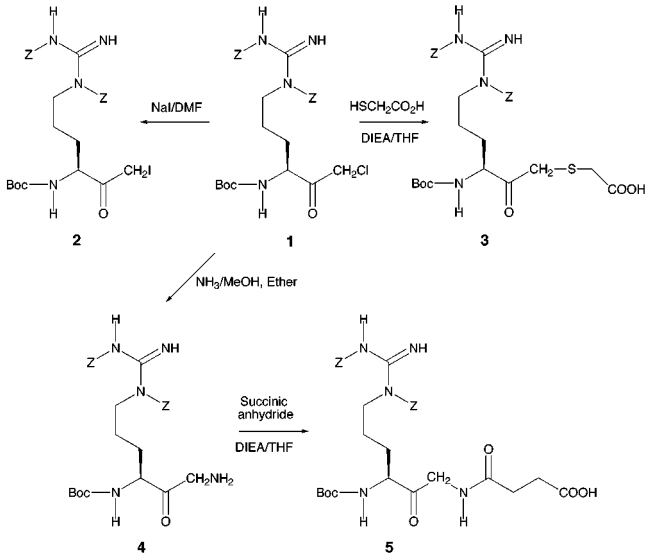
Scheme 1



proteolytic stability against thrombin.[22] Pro at the P_1' residue also improved proteolytic stability against thrombin; however, the Arg−Pro peptide bond was still slowly cleaved by thrombin.[21] Complete proteolytic stability against thrombin was achieved by introducing homoarginine ArgΨ[CH_2-CO-NH]Gly or a reduced bond ArgΨ[CH_2-NH]Gly, although they also reduced the affinity of the inhibitors 3−10-fold compared to the corresponding inhibitor with Arg-Pro.[23] Ketomethylene pseudopeptide bonds ArgΨ[CO-$(CH_2)_n$]Gly (n = 1−4), on the other hand, were successfully introduced with full proteolytic stability against thrombin and no loss of affinity. This class of inhibitors achieved a K_i value of as little as 1.4×10^{-10} M.[24,25] Ketoamide transition-state mimetics ArgΨ[CO-CO-NH]Gly were further introduced to stimulate the affinity of the inhibitor, i.e., the K_i value of the inhibitor CVS995 in this class, which reached 1×10^{-12} M; however, it should be recalled that synthesis of the building unit of the transition-state analogue, 6-nitroguanidino-3(*S*)-*t*-Boc-amido-2(*R*,*S*)-hydroxyhexanoic acid, is labor-intensive.[26]

In this article, we introduced a series of ketomethylene pseudopeptide bonds containing substituted or nonsubstituted heteroatoms (N or S)[27−29] into the bivalent thrombin inhibitors. These are ArgΨ[CO-CH_2-NH]-Gly, ArgΨ[CO-CH_2-N(CH_3)]Gly, ArgΨ[CO-CH_2-N(acetyl)]-Gly, ArgΨ[CO-CH_2-NH-CO-CH_2]Gly, and ArgΨ[CO-CH_2-S]Gly. The incorporation of ArgΨ[CO-CH_2-S]Gly has advantages in the yield of the synthesis as well as in the affinity for thrombin.

Chemistry

Three types of pseudopeptide bonds were introduced in the bivalent thrombin inhibitors. The Arg side chain was protected by two benzyloxycarbonyl (Z) groups in all syntheses to eliminate side reaction during the synthesis. The P_2 and P_3 amino acids were coupled as a dipeptide, e.g., Boc-(D-Phe)-Pro-OH, for all inhibitors in this article. Compound **1**, Boc-Arg(Z_2)-CH_2Cl, was synthesized by procedures described elsewhere[30−32] with slight modifications. Compound **1** was further converted to **2**, **3**, and **5** (Scheme 1), which were then used in solid-phase synthesis.

The type I pseudopeptide bonds ArgΨ[CO-CH_2-NR]-Xaa, where R is H, CH_3, or acetyl, were used in inhibitors **6**−**13**. They were generated by coupling Boc-Arg(Z_2)-CH_2I (**2**) to the N-terminus of P_1'-linker-(the FRE blocking segment)-Pam resin, while the side chains of the peptides on the resin were still protected. The reaction was never completed, even with a large excess of the iodomethyl ketone (10 times), at high temperature (37 °C), and with a prolonged coupling time of 1 week. The unreacted amino groups were capped with acetic anhydride. No capping was performed for peptides **6** and **11** in order to avoid acetylation of the secondary amine at the P_1' residue. The coupling yield of **2** was 15−55% based on the HPLC profiles of the crude products. A low yield (∼25%) of alkylation was also reported in the solution synthesis of a furine inhibitor with a similar ArgΨ[CO-CH_2-NH]Ala building unit.[33] The coupling of the P_3-P_2 dipeptide to H-Arg(Z_2)Ψ[CO-CH_2-NR]Xaa-linker-(the FRE blocking segment)-Pam resin avoided the formation of the N-terminal unprotected dipeptidyl ketone intermediate, which has a strong tendency to undergo intramolecular cyclization.[31,34,35]

The type II pseudopeptide bond ArgΨ[CO-CH_2-NH-CO-CH_2]Gly has an interesting feature in that -NH-CH_2- of the original P_1' glycine residue is reversed and the NH group of the normal P_2' glycine residue is replaced by a methylene group. Unlike the type I pseudopeptide bond, there is no insertion of a methylene group at the P_1' residue. The chloromethyl ketone **1** was slowly converted to the aminomethyl ketone **4** with an excess of NH_3 in absolute diethyl ether. Compound **4** was reacted with succinic anhydride to give compound **5** (Scheme 1). Building unit **5** was then incorporated into the inhibitors by using conventional solid-phase peptide synthesis. The HPLC profile of the crude peptide **17** showed two major peaks (∼1:1 ratio) at 24.6 and 25.0 min (peptides **17a** and **17b**). The peptides showed the same molecular mass (2096, MH^+) and the same profile in the amino acid analysis. Peptide **17a** inhibited thrombin in the nanomolar range of the K_i value, whereas **17b** showed no significant inhibition even at a concentration of 1 μM. Based on the results of the HPLC profile, the molecular mass, the amino acid composition, and the inhibition constant, inhibitor **17** seems to be racemized, most likely during the synthesis of the aminomethyl ketone **4** because of the large excess of NH_3 used.

The type III pseudopeptide bond has a formula of ArgΨ[CO-CH_2-S]Gly. Unlike the low alkylating yield of the type I pseudopeptide bond, chloromethyl ketones easily alkylate mercapto groups such as dithioerythritol.[36] Thus, the intermediate Boc-Arg(Z_2)Ψ[CO-CH_2-S]-Gly (**3**) was quantitatively produced in the presence of diisopropylethylamine (Scheme 1). Compound **3** was incorporated in the bivalent inhibitors by standard solid-phase peptide synthesis procedure. Similar type III pseudopeptide bonds have been reported in active-site-directed thrombin inhibitors[37] and angiotensin-converting enzyme (ACE) inhibitors.[27,29]

Table 1 lists the sequences of the bivalent thrombin inhibitors used in this article.

Results and Discussion

Slow Inhibition. Slow binding inhibition was observed in some inhibitors such as **9**−**11** (Figure 1).[38]

Table 1. Sequences of the Bivalent Thrombin Inhibitors

inhibitor	active site blocking segment	linker	FRE blocking segment
6	(D-Phe)-Pro-ArgΨ[$COCH_2NH$]Gly	$(Gly)_4$	Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln-OH
7	(D-Phe)-Pro-ArgΨ[$COCH_2NCH_3$]Gly	$(Gly)_4$	Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln-OH
8	(D-Phe)-Pro-ArgΨ[$COCH_2NCH_3$]Gly	$(Gly)_4$	Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Tyr-Cha-Asp-OH
9	(D-Cha)-Pro-ArgΨ[$COCH_2NCH_3$]Gly	$(Gly)_4$	Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Tyr-Cha-Asp-OH
10	(D-Cha)-Pro-ArgΨ[$COCH_2NAc$]Gly	$(Gly)_4$	Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Tyr-Cha-Asp-OH
11	(D-Cha)-Pro-ArgΨ[$COCH_2NH$]Ala	$(Gly)_4$	Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Tyr-Cha-Asp-OH
12	(D-Cha)-Pro-ArgΨ[$COCH_2NCH_3$]Gly	$(Gly)_4$-Asn-Gly	Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH
13	(D-Cha)-Pro-ArgΨ[$COCH_2N$]Pro	$(Gly)_4$-Asn-Gly	Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH
14	(D-Phe)-Pro-ArgΨ[$COCH_2S$]Gly	$(Gly)_4$	Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln-OH
15	Ac-(D-Phe)-Pro-ArgΨ[$COCH_2S$]Gly	$(Gly)_4$	Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln-OH
16	(D-Cha)-Pro-ArgΨ[$COCH_2S$]Gly	$(Gly)_4$	Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH
17	(D-Phe)-Pro-ArgΨ[$COCH_2NHCOCH_2$]Gly	ϵAhx-Gly	Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln-OH

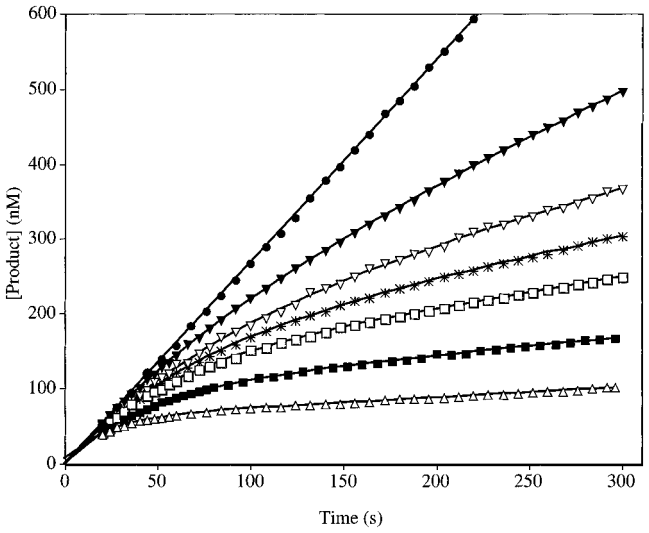


Figure 1. Slow inhibition of human α-thrombin by inhibitor **9** as monitored by the hydrolysis of the fluorogenic substrate tosyl-Gly-Pro-Arg-AMC (40 μM). The solid lines are the nonlinear least-squares fitting of the data (● at [I] = 0 M, ▼ at [I] = 5.1×10^{-11} M, ▽ at [I] = 8.5×10^{-11} M, * at [I] = 1.19×10^{-10} M, □ at [I] = 1.70×10^{-10} M, ■ at [I] = 2.6×10^{-10} M, and △ at [I] = 5.1×10^{-10} M) using eq 2.

Table 2. Inhibition Constants and Inhibitor Class of the Bivalent Thrombin Inhibitors[a]

inhibitor	K_i (M)	inhibitor class[b]
6	$8.3 \times 1.5 \times 10^{-11}$	classical
7	$8.3 \times 0.8 \times 10^{-11}$	classical
8	$3.5 \times 0.7 \times 10^{-11}$	classical
9	$2.0 \times 0.3 \times 10^{-12}$	slow binding
10	$1.7 \times 0.3 \times 10^{-12}$	slow binding
11	$1.5 \times 0.2 \times 10^{-12}$	slow binding
12	$8.7 \times 0.3 \times 10^{-13}$	slow, tight binding
13	$5.7 \times 0.7 \times 10^{-12}$	slow binding
14	$9.4 \times 1.2 \times 10^{-12}$	slow binding
15	$1.06 \times 0.10 \times 10^{-9}$	hyperbolic[c]
16	$3.5 \pm 0.5 \times 10^{-13}$	slow, tight binding
17	$7.3 \pm 0.4 \times 10^{-9}$	hyperbolic[c]

[a] Values are shown as mean ± SE at pH 7.8 and 23 °C. [b] The inhibitors are classified according to Morrison and Walsh[38] except hyperbolic inhibition. [c] The hyperbolic inhibition was analyzed as described elsewhere.[47] K_r values are 0.0078 and 0.013 for inhibitors **15** and **17**, respectively.

Each progress curve of the slow inhibition was fitted to eq 2 (see the Experimental Section). The steady-state velocity v_S was then incorporated into eq 1 to estimate the K_i value (Table 2). The parameter k_{obs} increased linearly with the inhibitor concentration (Figure 2). The slope and the intercept are $k_{on}/(1 + [S]/K_m)$ and k_{off}, respectively (eq 3). The association rate constant k_{on} of

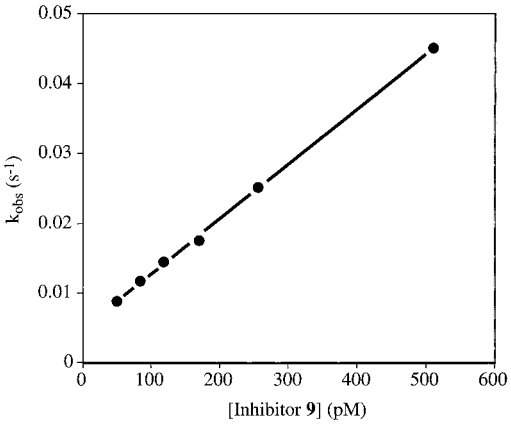


Figure 2. Observed first-order rate constant k_{obs} at various concentrations of inhibitor **9**.

inhibitor **9** was estimated as $9.5 \pm 1.4 \times 10^8$ M^{-1} s^{-1}, which is 7 times faster than that ($k_{on} = 1.37 \pm 0.03 \times 10^8$ M^{-1} s^{-1}) of r-hirudin.[39] Site-directed mutagenesis of r-hirudin at the FRE blocking segment, e.g., substitutions of GluH57, GluH58, GluH61, and GluH62 by Gln, predominantly reduced the k_{on} value with little effect on the k_{off} value.[39] Similarly, the improved k_{on} value of **9** may be due to its 10-fold higher affinity for the FRE blocking segment compared to that of r-hirudin(55−65).[40] The k_{on} values, which are close to a diffusion-controlled process, were also observed in inhibitors **10** and **11**. Thus, the slow inhibition of these inhibitors is simply due to the low inhibitor concentrations ($5-50 \times 10^{-11}$ M) in the assays.

FRE Inhibitor Segment. Krstenansky et al.[40] extensively made single or multiple substitutions of the FRE inhibitor fragment of r-hirudin. For example, single substitutions of PheH56 by Tyr, of GluH58 by Pro, and of GlnH65 by Asp or D-Glu improved the IC_{50} of the r-hirudin-based FRE inhibitors by 10%, 70%, and 60%, respectively. Although the effect of each substitution was relatively small, the combined multiple substitutions improved the IC_{50} value up to 10-fold, e.g., a multiple substitution of PheH56, GluH58, LeuH64, and GlnH65 by Tyr, Pro, Cha, and Asp, respectively, and a multiple substitution of PheH56, GluH58, TyrH63, LeuH64, and GlnH65 by Tyr, Pro, Ala, Cha, and D-Glu, respectively. The IC_{50} values of the N-terminal acetylated FRE inhibitors **18**−**21**, which were incorporated into the bivalent inhibitors in this article, are listed in Table 3.

N-Terminal P_3 Residue. X-ray structures of thrombin in complex with D-Phe-Pro-Arg-based inhibitors showed that the side chain of the P_3 residue D-Phe binds

Table 3. Inhibition (IC_{50}) and Sequences of the FRE Inhibitors[a]

inhibitor	sequence	IC_{50} (M)
18	Ac-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln-OH	$1.46 \pm 0.10 \times 10^{-6}$
19	Ac-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Tyr-Cha-Asp-OH	$2.8 \pm 0.2 \times 10^{-7}$
20	Ac-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH	$1.35 \pm 0.10 \times 10^{-7}$
21	Ac-Asn-Gly-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH	$1.59 \pm 0.22 \times 10^{-7}$

[a] Values are shown as mean ± SE at pH 7.52 and 37 °C.

to a hydrophobic cavity, the so-called aryl binding site,[41] which is formed by Leu99, Ile174, Trp215, and Tyr60A. Inhibitor **9** improved the K_i value ~20-fold by substituting N-terminal D-Phe of **8** with D-Cha. This is consistent with the ~30-fold improvement of hirulog-B2 by replacing N-terminal D-Phe in hirulog-1 with D-Cha.[22] The bivalent thrombin inhibitors with an N-terminal acetyl-D-Phe- (hirutonin-2)[42] or D-Cha residue (P596)[43] insert the side chain of D-Cha or D-Phe into the same S_3 subsite according to their crystal structures; however, the cyclohexyl ring was slightly rotated with respect to the plane of the phenyl ring. Rehse et al.[43] suggested that the improved affinity of D-Cha is due to the stronger van der Waals interactions of the saturated and nonplanar ring, which points directly toward the aliphatic side chains of Ile174 and Leu99. Another drastic effect of the P_3 residue is the acetylation of the N-terminal amino group. Inhibitor **15** with an N-terminal acetyl group has low affinity ($K_i = 1.06 \pm 0.10 \times 10^{-9}$ M) for thrombin, and the inhibition was hyperbolic. Removal of the N-terminal acetyl group resulted in inhibitor **14**, with ~100-fold improved K_i value ($K_i = 9.4 \pm 1.2 \times 10^{-12}$ M) and slow inhibition mode. Similar large effects of the N-terminal acetylation were reported in active-site-directed inhibitors based on the D-Phe-Pro-Arg sequence[44] and in the k_{cat}/K_m values of the chromogenic substrates.[45] In the crystal structures of hirutonin-2[42] and P596,[43] both the N-terminal amide and the amino protons of hirutonin-2 and P596, respectively, form a hydrogen bond to the carbonyl oxygen of Gly216 of thrombin; however, the amide hydrogen bond is generally weaker than that of the free amino group.[45,46] In addition, the N-terminal amino group in the P596−thrombin complex was well-hydrated by several bound water molecules, whereas no bound water molecule was observed around the N-terminal acetyl group in the hirutonin-2−thrombin complex. The weaker hydrogen bond and the larger dehydration energy may contribute to the weaker affinity of inhibitor **15** compared to that of inhibitor **14**.

Inhibitors with an ArgΨ[CO-CH_2-NR]Gly Segment. Peptides **6** and **7** with ArgΨ[CO-CH_2-NH]Gly or ArgΨ[CO-CH_2-N(CH_3)]Gly at the P_1-P_1' residues, respectively, were classical competitive inhibitors with K_i values of $8.3 \pm 1.5 \times 10^{-11}$ and $8.3 \pm 0.8 \times 10^{-11}$ M, respectively. Both **6** and **7** bound to thrombin 25 times tighter than hirulog-1, D-Phe-Pro-Arg-Pro-$(Gly)_4$-(r-hirudin(53−64)) ($K_i = 2.3 \times 10^{-9}$ M).[20] It should be noticed that the P_1' residues of inhibitors **6** and **7** are basic due to their secondary and tertiary amines, respectively. If these amines were protonated in the thrombin−inhibitor complex, their electron-withdrawing effect on the carbonyl carbon of the P_1 residue would stabilize the transition-state analogue binding mode with enhanced affinity for thrombin. On the other hand, the corresponding secondary or tertiary amines of the ACE inhibitors containing PheΨ[CO-CH_2-NH]Gly or PheΨ-[CO-CH_2-N(CH_3)]Gly were suggested to be a hydrogen bond acceptor; i.e., the amines were not protonated in the enzyme−inhibitor complexes.[29] We investigated the amines further by preparing a reference inhibitor **10** in which the amine of the P_1' residue is acetylated with no possibility of protonation. The acetylation had no effect on affinity, with a K_i value of $1.7 \pm 0.3 \times 10^{-12}$ M compared to that ($2.0 \pm 0.3 \times 10^{-12}$ M) of the reference inhibitor **9**. Thus, it is unlikely that inhibitors **6** and **7** are protonated at the amine of the P_1' residue and act as transition-state mimetics.

Inhibitor with an ArgΨ[CO-CH_2-NH-CO-CH_2]Gly Segment. Inhibitors **6**−**16** have a methylene group inserted between the P_1 and P_1' residues, shifting the peptide chain of the P′ residues by one atom. This shift was eliminated in inhibitor **17**, where the ArgΨ[CO-CH_2-NH-CO-CH_2]Gly segment corresponding to the $P_1-P_1'-P_2'$ residues have the same number of main chain atoms as the natural amino acid residues. The K_i value ($7.3 \pm 0.4 \times 10^{-9}$ M) of inhibitor **17** was surprisingly high, and the inhibition was hyperbolic. This is ~100-fold less active than the similar inhibitor **6**. Despite the significant affinity loss of **17**, there is no obvious atom in inhibitor **17** which may disturb the binding to thrombin; i.e., the NH of the P_1' residue of inhibitor **17** is located at the same position as that in inhibitors **6** and **11**; the carbonyl of the P_1' residue is located at the same position as that of the substrates; and 6-aminohexanoic acid (ϵAhx) was well-accepted in the thrombin S′ subsites with almost no effect on the affinity for thrombin.[47]

Inhibitors with an ArgΨ[CO-CH_2-S]Gly Segment. Inhibitor **14** replaces the ArgΨ[CO-CH_2-NH]Gly pseudopeptide bond of inhibitor **6** by ArgΨ[CO-CH_2-S]-Gly and improves the K_i value ~9-fold, in contrast to the negative effects reported previously where incorporation of the corresponding PheΨ[CO-CH_2-S]Gly at the P_1-P_1' residues of ACE inhibitors reduced in vitro activity significantly or inactivated the inhibitor in vivo.[27,29] Further substitutions of the P_3 residue and of the FRE blocking segment led to a potent bivalent thrombin inhibitor **16** with a K_i value of $3.5 \pm 0.5 \times 10^{-13}$ M, comparable to that (2.3×10^{-13} M) of r-hirudin.[39] The advantages of α-thiomethyl ketone are not only the improved affinity of the inhibitor but also, and more importantly, the straightforward synthesis of Boc-Arg$(Z)_2$Ψ[CO-CH_2-S]Gly-OH (**3**) with a high yield of 89%, compared to the synthesis of other pseudopeptide bonds which are often tedious with low yield. Furthermore, it provides an option to incorporate amino acid mimetics other than Gly mimetic at the P_1' residue; e.g., 2-mercaptopropionic acid is a mimetic of Ala, 3-mercaptopropionic acid substitutes for β-Ala, and mercaptosuccinic acid mimics Asp.

Conclusion

Arginyl methyl ketone derivatives were incorporated as P_1-P_1' residues in the bivalent thrombin inhibitors by a combination of solution and solid-phase peptide synthesis and eliminated the scissile peptide bond. The use of a pseudopeptide bond based on an α-aminomethyl ketone moiety (ArgΨ[CO-CH_2-NR]Xaa) resulted in potent inhibitors with picomolar K_i values. Although inhibitors **9** and **11** contain basic amines of ArgΨ[CO-CH_2-N(CH_3)]Gly and ArgΨ[CO-CH_2-NH]Gly, respectively, it is unlikely that these amines are protonated in the enzyme−inhibitor complex or that the inhibitors act as transition-state analogues. Pseudopeptide bond ArgΨ[CO-CH_2-S]Gly exhibited dual advantages in inhibitor design; i.e., Boc derivative of ArgΨ[CO-CH_2-S]-Gly was easily synthesized with a high yield of 89%, and inhibitor **16** containing this pseudopeptide bond bound to human α-thrombin with the highest affinity ($K_i = 3.5 \pm 0.5 \times 10^{-13}$ M), comparable to that ($K_i = 2.3 \times 10^{-13}$ M) of r-hirudin.

Experimental Section

Materials. 7-Aminomethylcoumarin (AMC) was obtained from Aldrich (Milwaukee, WI). Human α-thrombin (3000 NIH units/mg), bovine fibrinogen (70% protein, 85% of protein clottable), tosyl-Gly-Pro-Arg-AMC·HCl salt, poly(ethylene glycol) 8000, and Tris were purchased from Sigma (St. Louis, MO). Boc-Cha-OH, Boc-D-Cha-OH, Boc-sarcosine-OH, and Boc-Arg(Z_2)-OH were purchased from BaChem BioScience Inc. (Philadelphia, PA). All other amino acid derivatives for peptide synthesis were obtained from Advanced ChemTech (Louisville, KY). The side chain protecting groups for Boc-amino acids are benzyl for Glu, cyclohexyl for Asp, and 2-bromobenzyloxycarbonyl for Tyr. Boc-Gln-Pam resin and Boc-Asp(OBzl)-Pam resin were purchased from Applied Biosystems Inc. (Foster City, CA). Boc-D-Glu(OBzl)-Pam resin was purchased from Peninsula Laboratories Inc. (Belmont, CA). The solvents for peptide synthesis were purchased from Anachemia Chemical Inc. (Rouses Point, NY) and Applied Biosystems Inc. HF and TFA were purchased from Matheson Gas Products Inc. (Secaucus, NJ) and Halocarbon Products Co. (North August, SC), respectively. All other chemicals were from Aldrich (Milwaukee, WI).

General. Melting points were determined on a micro hot plate according to BOETIUS and were uncorrected. Optical rotations were measured with a Polamat (Carl Zeiss Jena, Germany). The molecular mass of the compounds was determined using a SCIEX API III mass spectrometer (Sciex, Concord, ON, Canada). A Beckman model 6300 amino acid analyzer (Beckman, Fullerton, CA) was used to determine the amino acid composition and the peptide content of the inhibitors. The bivalent inhibitors were purified by a preparative HPLC (Vydac C_4 column, 46 × 250 mm, 33 mL/min flow rate, linear gradient of 15−45% acetonitrile in 0.1% TFA in 80 min, monitored at 220 nm) and, if necessary, further by a semi-preparative HPLC with the same gradient (Vydac C_{18} column, 20 × 250 mm, 13 mL/min flow rate). Analytical HPLC experiments were performed on a Waters HPLC (Vydac C_{18} column, 4.6 × 250 mm) with a linear gradient of acetonitrile (10−70% in 60 min) containing 0.1% TFA at a flow rate of 1 mL/min. The ^{13}C NMR spectra were recorded on an AC-250 (Bruker) at 62 MHz and referenced to internal tetramethylsilane.

Boc-(D-Phe)-Pro-OH. The dipeptide was prepared from Boc-D-Phe-OH and H-Pro-OBzl in solution using a mixed anhydride procedure, followed by hydrogenation catalyzed by palladium on activated carbon (Pd 5%).[48] The product was crystallized from ethyl acetate/hexane: mp 171−174 °C; $[\alpha]^{23}_D = -91.0°$ (*c* 0.56, acetic acid); t_R 31.6 min; ^{13}C NMR ($CDCl_3$) 172.86, 172.66, 155.14, 135.96, 129.34, 128.53, 127.18, 80.17, 59.70, 53.84, 47.33, 39.76, 28.27, 27.66, 24.25.

Boc-D-Cha-Pro-OH. The dipeptide was synthesized using the same method as that for Boc-D-Phe-Pro-OH described above: mp 158−162 °C; $[\alpha]^{23}_D = -48.3°$ (*c* 0.63, acetic acid); t_R 38.9 min; ^{13}C NMR ($CDCl_3$) 174.27, 172.76, 155.65, 79.98, 59.98, 49.94, 47.37, 40.30, 34.04, 33.89, 32.55, 28.25, 27.93, 26.35, 26.21, 26.00, 24.63.

Boc-Arg(Z_2)-CH_2Cl (1). Isobutyl chloroformate (2.21 mL, 17 mmol) was added to a solution of Boc-Arg(Z_2)-OH (8.68 g, 16 mmol) and *N*-methylmorpholine (1.87 mL, 17 mmol) in 75 mL of dry tetrahydrofuran (THF) at −15 °C. After 15 min, the precipitated salt was removed by filtration and the mixed anhydride solution was cooled to −70 °C. The diazomethane generated from Diazald was distilled at 30−35 °C into the cooled (−70 °C) mixed anhydride solution. The reaction mixture was then kept overnight at 4 °C. The solvent and the excess of diazomethane were removed in vacuo. The remaining crystalline diazomethyl ketone was redissolved in 250 mL of dry THF and cooled to 0 °C. The diazomethyl ketone was converted to **1** by adding 8 mL of 4 N HCl/dioxane dropwise over a period of 10 min at 0 °C. The reaction solution was further incubated for 10 min at 0 °C before the solvent was evaporated. **1** was redissolved in ethyl acetate and washed three times with half-saturated $NaHCO_3$ solution and three times with saturated NaCl solution. The solution was dried over Na_2SO_4, and the solvent was evaporated. The product was crystallized from ethyl acetate/hexane and recrystallized from ethanol: yield 59%; mp 67−69 °C; $[\alpha]^{23}_D = -17.1°$ (*c* 0.50, acetic acid); t_R 53.5 min; MS 575 (MH^+); ^{13}C NMR ($CDCl_3$) 201.61, 163.55, 160.52, 155.67, 155.56, 136.54, 134.51, 128.92, 128.83, 128.46, 128.36, 128.11, 128.03, 80.14, 69.03, 67.14, 57.2446.85, 44.02, 28.26, 26.92, 24.55.

Boc-Arg(Z_2)-CH_2I (2). NaI (15 g, 100 mmol) was added to compound **1** (5 g, 8.7 mmol) solution in 200 mL of anhydrous dimethylformamide (DMF). The reaction mixture was incubated with stirring at 35−40 °C for 8 h in the dark before the solvent was evaporated. The product was then redissolved in ethyl acetate, washed three times with half-saturated NaCl solution, and dried over Na_2SO_4. After evaporating the solvent, the crude oily product, which still contained 5−10% of the starting material **1** (determined by analytical HPLC), was used in the solid-phase peptide synthesis without further purification: MS 667 (MH^+), t_R 55.4 min.

Boc-Arg(Z_2)Ψ[CO-CH_2-S]Gly-OH (3). Compound **1** (1 g, 1.74 mmol), diisopropylethylamine (DIEA) (0.45 mL, 2.6 mmol), and mercaptoacetic acid (0.185 mL, 2.6 mmol) were added to 15 mL of anhydrous THF and incubated under stirring at room temperature for 24 h. After evaporation of THF in vacuo, product **3** was dissolved in ethyl acetate and washed three times with saturated $NaHCO_3$ solution to remove the excess of mercaptoacetic acid. The product was further washed three times with saturated NaCl solution and dried over Na_2SO_4. After evaporation of the solvent in vacuo, product **3** was obtained as a white foam: yield 89%; t_R 46.5 min; MS 631 (MH^+); ^{13}C NMR ($CDCl_3$) 204.47, 173.47, 163.53, 160.48, 155.71, 136.60, 134.57, 128.88, 128.82, 128.42, 128.33, 128.06, 127.95, 80.11, 69.00, 67.11, 57.89, 44.16, 38.63, 33.91, 28.26, 27.51, 24.67.

Boc-Arg(Z_2)Ψ[CO-CH_2-NH-CO-CH_2]Gly-OH (5). Compound **1** (2 g, 3.5 mmol) was dissolved in 150 mL of anhydrous ether and mixed with 7 mL of 2 M ammonia in methanol at 0 °C. The reaction was continued with stirring at room temperature. The same volume of 2 M ammonia in methanol was further added twice at 24 and 48 h, respectively. The reaction was terminated after 3 days when more than 95% of the starting material **1** had reacted (based on the HPLC profile). The solvents were then evaporated. The aminomethyl ketone product was redissolved in ethyl acetate, washed three times with saturated NaCl solution, and dried over Na_2SO_4. After evaporation of the solvent, product **4** was characterized as MS 556 (MH^+) and t_R 39.7 min. Product **4** was redissolved in 25 mL of anhydrous THF and treated with succinic anhydride (3.5 g, 35 mmol) and DIEA (6.1 mL, 35 mmol) for 24 h. The unreacted anhydride was then converted to succinic acid by adding 15 mL of saturated $NaHCO_3$ solution and 25 mL of

THF with stirring for 12 h. After evaporation of the solvent in vacuo, the residue was redissolved in ethyl acetate, washed three times with saturated $NaHCO_3$ and NaCl solution, and dried over Na_2SO_4. The crude product **5** was used for solid-phase peptide synthesis without further purification; however, a small amount of product **5** was further purified by flash chromatography for identification (silica gel 60, benzene/acetone/acetic acid, 27/10/0.5): t_R 43.8 min; MS 656 (MH^+); ^{13}C NMR ($CDCl_3$) 201.93, 176.03, 171.43, 163.55, 160.18, 155.55, 154.43, 136.73, 134.51, 128.91, 128.81, 128.41, 128.35, 128.01, 127.83, 80.73, 73.24, 68.94, 66.95, 43.96, 30.59, 29.05, 28.06, 22.33, 21.91, 20.65.

Solid-Phase Peptide Synthesis. The bivalent thrombin inhibitors were prepared as described elsewhere,[49] except the N-terminal P_3, P_2, P_1, and P_1' residues. When the P_1' residue was -CH_2-S-CH_2-CO- or -CH_2-NH-CO-CH_2-CH_2-CO-, the P_1−P_1' building unit **3** or **5** was coupled to the side chain-protected linker-(the FRE blocking segment)-Pam-resin using conventional Boc chemistry. When the P_1' residue was -CH_2-sarcosine, -CH_2-Gly, -CH_2-N(acetyl)Gly, -CH_2-Pro, or -CH_2-Ala, the appropriate amino acid was first attached to the peptide resin using a standard Boc chemistry protocol. After deprotection of the Boc group, the resin was washed five times with dichloromethane (DCM). The peptide resin (∼300 mg) was then transferred into a polypropylene reaction column (5 mL volume, Abimed, Germany) and washed twice with 10% DIEA in DCM and three times with DCM. After being washed three times with DMF, the resin was treated with a 10-fold excess of compound **2** and shaken at room temperature in the dark for 3 days, followed by an extensive six-times-repeated wash to remove compound **2**. The resin was then capped by acetic anhydride, except for peptides **6** and **11**, where the resin was treated with a 10-fold excess of Z-Cl in the presence of DIEA. The P_3−P_2 residues were then coupled as a Boc-dipeptide. It should be mentioned that the neutralization and washing of the peptide resin after deprotecting the P_1 residue must be performed as quickly as possible in order to get a reasonable yield. All peptides were cleaved with HF containing 10% anisole (v/v) and 10% dimethyl sulfide (v/v) at −5 °C for 1 h. After evaporation of HF, the peptides were washed twice with ethyl ether, dissolved in 10% acetic acid, and lyophilized. The peptides were purified by the preparative HPLC. Only the fractions with >97% purity based on the analytical HPLC profiles were collected and lyophilized. All the peptides used have correct amino acid compositions and molecular masses.

Fibrin Clotting. The fibrin clotting assay was performed in 50 mM Tris·HCl buffer (pH 7.52 at 37 °C) containing 0.1 M NaCl and 0.1% poly(ethylene glycol) 8000 with 9.0×10^{-10} M (0.1 NIH unit/mL) and 0.03% (w/v) of the final concentrations of human α-thrombin and bovine fibrinogen, respectively, as reported elsewhere.[49] The clotting time was plotted against the inhibitor concentrations, and IC_{50} was estimated as the inhibitor concentration required to double the clotting time relative to the control.

Amidolytic Assays. Inhibition of the amidolytic activity of human α-thrombin was measured on a Hitachi F2000 fluorescence spectrophotometer (and Shimadzu RF-5001PC spectrofluorometer for inhibitor **16**) (λ_{ex} = 383 nm, λ_{em} = 455 nm) which was calibrated with AMC. The assay was performed with tosyl-Gly-Pro-Arg-AMC as a fluorogenic substrate in 50 mM Tris·HCl buffer (pH 7.8) containing 0.1 M NaCl and 0.1% poly(ethylene glycol) 8000 at 23 °C.[49] The final concentrations of the substrate and human α-thrombin were $2-40 \times 10^{-6}$ and 1.0×10^{-11} M, respectively. The final inhibitor concentrations varied depending on the K_i values (Table 2). Inhibitors **6**−**8** with K_i values of $2-10 \times 10^{-11}$ M were analyzed at concentrations of 1×10^{-10} M or higher and showed classical competitive inhibition. The linear steady-state velocities (v_S) were analyzed by using the method (eq 1) described by Segel[50] and a nonlinear squares fitting in UltraFit (Biosoft) or Microsoft Excel (Microsoft):

$$v_S = V_{max}[S]/(K_m(1 + [I]/K_i) + [S]) \quad (1)$$

where V_{max}, [S], [I], K_m, and K_i are the maximum velocity, the substrate concentration, the inhibitor concentration, the Michaelis constant, and the inhibition constant, respectively. Inhibitors **9**, **10**, **11**, **13**, and **14** with K_i values of $1-20 \times 10^{-12}$ M were analyzed at concentrations of 5×10^{-11} M or higher, and the progress curve characteristic of slow binding inhibition was observed. The progress curves were fitted to eq 2 by nonlinear least-squares fitting in Microsoft Excel:

$$[P] = v_S t + (v_0 - v_S)(1 - \exp(-k_{obs}t))/k_{obs} + c \quad (2)$$

$$k_{obs} = k_{on}[I]/(1 + [S]/K_m) + k_{off} \quad (3)$$

where [P], v_0, k_{obs}, c, k_{on}, and k_{off} represent the concentration of 7-(aminomethyl)coumarin product, the velocity observed in the absence of the inhibitor at the defined substrate concentration [S], the apparent first-order rate constant, the displacement of [P] from zero at $t = 0$, the association rate constant of the enzyme and the inhibitor, and the dissociation rate constant of the enzyme−inhibitor complex, respectively. The steady-state velocity v_S estimated from eq 2 was then incorporated into eq 1 to estimate the K_i values of the inhibitors by nonlinear least-squares fitting as described above. The association rate constant k_{on} of the inhibitors was estimated from eq 3. Inhibitors **12** and **16** with K_i values below 1×10^{-12} M were analyzed at concentrations of 1×10^{-11} M or higher, and the progress curve characteristic of slow tight binding inhibition was observed. The progress curves were fitted to the slow tight binding inhibition eq 4 by nonlinear least-squares fitting in Microsoft Excel:

$$[P] = v_S t + ((v_0 - v_S)(1 - \gamma)/\gamma k) \ln((1 - \gamma e^{-kt})/(1 - \gamma)) + c \quad (4)$$

$$v_S = v_0([E]_T - [I]_T - K_i' + Q)/(2[E]_T) \quad (5)$$

where $\gamma = (K_i' + [E]_T + [I]_T - Q)/(K_i' + [E]_T + [I]_T + Q)$; $Q = ((K_i' + [E]_T + [I]_T)^2 - 4[E]_T[I]_T)^{1/2}$, and $K_i' = K_i(1 + [S]/K_m)$, where k is the observed second-order rate constant for the interaction of the inhibitor with thrombin and v_0 is the velocity observed in the absence of the inhibitor at the defined substrate concentration [S].[15,51] The steady-state velocity v_S was then used to calculate the K_i values according to eq 5 using nonlinear least-squares fitting in Microsoft Excel, where $[E]_T$ and $[I]_T$ are the total enzyme and the total inhibitor concentrations, respectively.[15] For the regression, the observed steady-state velocities were weighted according to the reciprocal of their value. The hyperbolic inhibition of inhibitors **15** and **17** was analyzed using eq 6 and nonlinear least-squares fitting in Microsoft Excel as described elsewhere:[47]

$$v_S = V_{max}([S]/K_m + 1.6[I][S]K_r/K_mK_i)/(1 + [S]/K_m + (1 + 1/K_r)[I]K_r/K_i + 1.6[I][S]K_r/K_mK_i) \quad (6)$$

where K_r represents an equilibrium constant between the enzyme−inhibitor complex formed by simultaneous bindings of the FRE and the active site inhibitor segments to thrombin and the enzyme−inhibitor complex formed only through the interaction of the FRE inhibitor segment with thrombin (the active site inhibitor segment is exposed to the solvent).

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