Rational Design of True Hirudin Mimetics: Synthesis and Characterization of Multisite-Directed α-Thrombin Inhibitors[1]

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We describe here the design, synthesis, and activity of a novel class of α-thrombin inhibitors named hirunorms. They were rationally designed to interact through their N-terminal end with the α-thrombin active site in a nonsubstrate mode and to specifically bind the fibrinogen recognition exosite. An appropriate spacer that is able to properly orient the N-terminal end in the active site was also selected. This spacer allowed the size of the inhibitors to be reduced to about one-third of the amino acid residues in the hirudin sequence. Hirunorms specifically inhibit the amidolytic action of human α-thrombin toward a small chromogenic substrate. The most active compounds of the series, hirunorms IV and V, inhibit α-thrombin catalyzed hydrolysis of Tos-Gly-Pro-Arg-*p*-nitroanilide with $K_i = 0.09$ and $K_i = 0.21$ nM, respectively. Comparison of the anticoagulant properties of hirunorms, natural hirudin from the European leech *Hirudo medicinalis*, and the synthetic analog hirulog-1 revealed that hirunorm IV is about 10-fold and 3-fold more active, on a molar base, than hirudin and hirulog-1 in increasing the aPTT, PT, and TT of normal human plasma. The peculiar structure of hirunorms makes them stable to the amidolytic action of thrombin without the introduction of any peptide bond modification. These molecules display long-lasting activity in human plasma, due to the presence of several unnatural amino acids in susceptible positions. Hirunorms are potential candidates for injectable anticoagulants, due to their potency, specificity of action, long-lasting activity, and safety profiles.

Introduction

Thrombin (EC 3.4.21.5) is a trypsin-like serine protease with the primary function of activating fibrinogen to fibrin and thus being responsible for fibrin clot formation.[2] It has also several regulatory functions in the coagulation process and induces platelet aggregation.[3] Its central role in the coagulation cascade has brought several scientists to develop highly potent and selective α-thrombin inhibitors that may be used with broader efficacy over existing antithrombotic agents. Among the many inhibitors developed until now, hirudin[4] and hirudin-like molecules[5] have been considered for clinical trials.

Hirudin,[4] a small protein of 65 amino acids from the leech *Hirudo medicinalis*, is the most potent natural α-thrombin inhibitor known to date. It binds specifically to α-thrombin with a K_i value of 2.2×10^{-14} M and thereby inhibits the cleavage of fibrinogen and fibrin clotting.[6]

The design of small synthetic α-thrombin inhibitors based on the hirudin sequence has been recently achieved in order to mimic the distinctive mechanism of hirudin.[5] The first compounds of this type were developed when it was recognized that the C-terminal end of hirudin binds in a site distinct from the catalytic site.[7] Maraganore *et al.* developed the hirulogs[5c] by combining in a single molecule the small fragment D-Phe-Pro-Arg-Pro and the C-terminal hirudin $Asp^{55'}$-$Gln^{65'}$ (the residue numbering of the hirudin sequence is primed), spaced by a multiple glycine linker of variable size. The N-terminal tetrapeptide fragment was expected to bind the α-thrombin active site and the C-terminal undecapeptide to penetrate the fibrinogen recognition exosite when a flexible spacer of appropriate length was chosen. Hirulogs, as well as hirutonins,[5d] which were successively developed, actually bind to α-thrombin both to the active site and to the fibrinogen recognition exosite, but they only partly mimic the hirudin−α-thrombin interaction mechanism. The more recent elucidation of the three-dimensional structure of recombinant hirudin (variant 2, Lys^{47}),[8] hirulogs,[9] and hirutonins[10] complexed with human α-thrombin revealed their different mode of interaction.

The main structural features of recombinant hirudin−human α-thrombin interactions are (i) the hirudin N-terminal segment is located near the α-thrombin active site and it is oriented in a non-natural mode, because the peptide chain aligns parallel to the α-thrombin residues Ser^{214}-Gly^{216}, whereas the natural mode of substrate interaction corresponds to an antiparallel alignment; (ii) the hirudin C-terminal 3_{10} helical segment (Pro^{60}-Gln^{65}) interacts with the α-thrombin fibrinogen recognition exosite; (iii) the hirudin segment Pro^{48}-Ile^{59} is in an extended conformation; (iv) the hirudin core Cys^{6}-Lys^{47} interacts with the α-thrombin surface at only a few points.

The main structural features of hirulog− and hirutonin−human α-thrombin complexes can be summarized as follows. The inhibitor N-terminal segment is located in the α-thrombin active site. It is oriented in a natural mode (substrate-like) because the peptide

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chain aligns antiparallel to the α-thrombin residues Ser^{214}-Gly^{216}, similarly to other active site directed inhibitors and in a mode that is common to other serine proteases.[11] The inhibitor C-terminal end interacts with the α-thrombin fibrinogen recognition exosite similarly to the hirudin segment $Asp^{55'}$-$Gln^{65'}$.

Very recently it was shown that tripeptide thrombin active site inhibitors also bind in a nonsubstrate manner.[12]

We report here the design of a series of low molecular weight hirudin analogues, named hirunorms. They are proposed to be true hirudin mimetics because they presumably act as multisite-directed α-thrombin inhibitors which penetrate the α-thrombin active site in a nonsubstrate-like mode (parallel mode) and bind to the fibrinogen recognition exosite. Therefore, hirunorms may bind to α-thrombin similarly to hirudin, but differently than other hirudin-like molecules, such as hirulogs and hirutonins.

We also report here the inhibition of human α-thrombin amidolytic activity on a tripeptide *p*-nitroanilide substrate by hirunorms and their ability to increase the coagulation parameters aPTT, PT ,and TT of normal human plasma.

Results and Discussion

The three-dimensional structure of human α-thrombin is characterized by the presence on its surface of several clefts or canyons and bulges. They might be at least partly responsible of the large variety of α-thrombin activities.[13] These cleft and bulges may function as series of keys or locks in the interaction with different target proteins or substrates. Hirudin binds to α-thrombin covering some of these peculiar surface motifs. The hirudin N-terminal pentapeptide segment penetrates the active site cleft, strongly interacting with the bulge of the insertion loop 60A-60D[8] (the numbering of the human α-thrombin residues is based on the chymotrypsin sequence[14]). Hirudin also penetrates into the canyon of the fibrinogen recognition exosite with its C-terminal undecapeptide.[8] The central compact core of the hirudin molecule is in contact with the α-thrombin surface at only few points with respect to the N- and C-terminal ends.[8]

Since hirudin is the most potent natural anticoagulant agent known so far, and its potency is certainly dependent on the mode of interaction with α-thrombin and presumably on the number and type of occupied surface recognition sites, hirunorms were designed to accurately reproduce the hirudin mechanism of action and its biological properties. Thus, the development of analogs of sufficiently reduced size which may allow an easy low-cost chemical synthesis has been achieved.

For the design of hirunorms, we used the X-ray structure of recombinant hirudin (variant 2, Lys^{47}) complexed with human α-thrombin as the template structure.[8a] Particularly interesting for the design is the α-thrombin active site cleft which is partly covered by a bulge[14] involving the insertion loop Tyr^{60A}-Pro^{60B}-Pro^{60C}-Trp^{60D}. This loop is mainly hydrophobic in nature and determines a best fit of substrates or inhibitors which are hydrophobic on one side of their surface. Other hydrophobic residues are also present at the border of the active site cleft such as Leu^{99}, Trp^{215}, and Ile^{174}. In addition, optimum substrate or inhibitor binding is achieved when these hydrophobic interactions are coupled with dipoles and/or charge interactions. Hydrogen bonds between the inhibitor backbone and the α-thrombin *β*-extended segment Ser^{214}-Gly^{216} stabilize the inhibitor in the active site. Salt bridge partnership between an Arg residue in the P1 site and Asp^{189} further stabilizes the inhibitor−α-thrombin complex. Many of the known α-thrombin inhibitors show this dualistic nature.[15] The N-terminal segment of hirudin from the European leech *Hirudo medicinalis* shows part of these inhibitor features, except for the lack of a positively charged residue that may interact with Asp.[189] However, a favorable charge interaction $Glu^{5'}$---Arg^{221A}, shortly outside the active site cleft, partly confirms the dualistic nature of the hirudin N-terminal segment that interacts with the α-thrombin active site. Furthermore, hirudin P6 from *Hirudinaria manillensis*[16] actually contains an Arg residue at position 2′ (which presumably may interact with Asp^{189}), and its inhibitory activity is comparable to that of hirudin from the leech *Hirudo medicinalis*. In addition, α-thrombin is capable of binding molecules in the active site with two different mechanisms. These molecules can either penetrate in a nonsubstrate mode because the peptide chain aligns parallel to the α-thrombin residues Ser^{214}-Gly^{216} or in a natural mode because of the antiparallel alignment. In an attempt to optimize the strength of the interaction with the active site, hirunorms were designed to align parallel to the α-thrombin *β*-extended segment Ser^{214}-Gly,[216] and containing Ile or Chg at position 1″ (hirunorm residue numbering is double primed), Val or Arg at position 2″, and Tyr, Phe or 2-Nal at position 3″. The replacement of $Ile^{1''}$ with $Chg^{1''}$ and $Tyr^{3''}$ with $Phe^{3''}$, or $2\text{-}Nal^{3''}$ would progressively increase the hydrophobicity of the N-terminal end. Site-specific substitution of the hirudin amino-terminal residues,[17] as well as novel hirudin mimetics,[5e] indicate the effect of hydrophobicity in affecting the α-thrombin amidolytic activity. $Arg^{2''}$ was initially selected because it can penetrate the S1 site and form an ion pair with Asp,[189] similarly to hirudin P6. Site-specific substitution of the first five amino acids of hirudin[18] indicated the beneficial effect of the $Arg^{2'}$ residue in strengthening the hirudin−α-thrombin interaction. However, $Val^{2''}$ was subsequently selected to reduce the putative toxicity and blood pressure reducing side effect.[19] When superimposing the backbone atoms of $Chg^{1''}$-$Arg^{2''}$-$2\text{-}Nal^{3''}$ tripeptide segment with the N-terminal tripeptide of hirudin, the small hydrophobic cavity between the active site and the insertion loop 60 is completely filled with the $Chg^{1''}$ and $2\text{-}Nal^{3''}$ side chains, and the $Arg^{2''}$ residue can easily enter the S1 site and interact with Asp.[189] Simple modeling of the $Arg^{2''}$ side chain with a *gauche*(−) χ_2 angle allowed us to bring the $Arg^{2''}$ guanidinium group and the Asp^{189} carboxy function in a typical orientation for ion pairing. Figure 1 describes the result of the modeling of hirunorm IV 1″−5″ N-terminal segment in the α-thrombin active site. The parallel backbone alignment with a typical hydrogen bond pattern of the $Chg^{1''}$-$Arg^{2''}$-$2\text{-}Nal^{3''}$ tripeptide with the α-thrombin Ser^{214}-Trp^{215}-Gly^{216} does not cause any severe steric hindrance. The $Thr^{4'}$-$Asp^{5'}$ segment protrudes outward from the active site cleft of α-thrombin[8] and was kept as such in the sequence of hirunorms for the known high propensity of Thr residue to be accommodated in *β*-extended

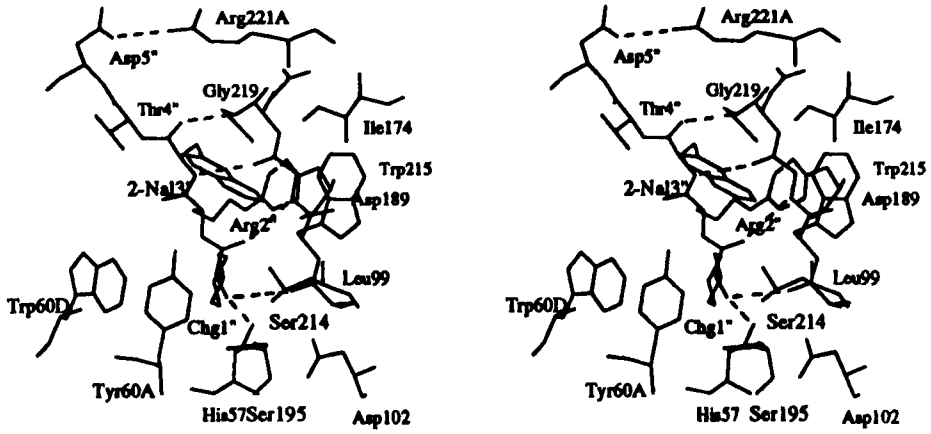

Figure 1. Stereoview of the expected mode of interaction with the α-thrombin active site of hirunorm IV Chg$^{1''}$-Asp$^{5''}$ segment, as obtained from molecular modeling. Hydrogen bonds are indicated by dashed lines.

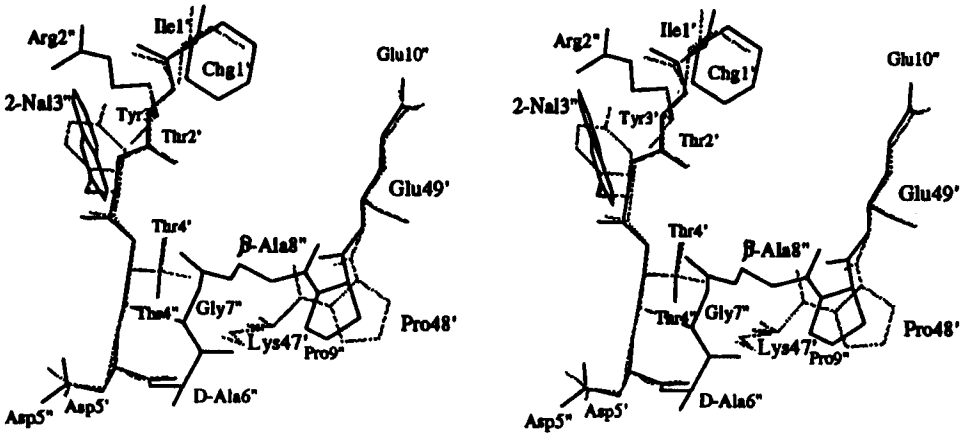

Figure 2. Stereoview of the backbone atoms superimposition between recombinant hirudin variant 2 Lys^{47} from Ile$^{1'}$ to Asp$^{5'}$ and from Lys$^{47''}$ to Glu$^{49'}$ (broken lines) and hirunorm IV model structure from Chg$^{1''}$ to Glu$^{10''}$. The hydrogen bond NH-Gly$^{7''}$CO-Thr$^{4''}$ involved in the type II β-turn (-Thr$^{4''}$-Gly$^{7''}$-) is reported as dashed line.

conformation[20] and for the Asp$^{5'}$ interaction with Arg^{221A} of α-thrombin.

From Cys$^{6'}$ to Lys$^{47'}$ the hirudin structure is characterized by a compact core that participates with a few of contacts with the α-thrombin surface,[8] when compared with the N- and C-terminal end. This core folds in such a way to bring the Lys$^{47'}$ back into proximity of the active site cleft. The Lys$^{47'}$ Cα atom is 8.5 Å apart from Asp$^{5'}$ Cα atom. This Cys$^{6'}$-Lys$^{47'}$ 42 residue hirudin segment was replaced with a tripeptide segment in the hirunorms. Simple modeling of a D-Ala$^{6''}$-β-Ala$^{7''}$-β-Ala$^{8''}$ or D-Ala$^{6''}$-Gly$^{7''}$-β-Ala$^{8''}$ segment allowed us to connect Asp$^{5'}$ and Pro$^{48'}$ in a reasonable low-energy conformation. The Thr$^{4''}$-Asp$^{5''}$-D-Ala$^{6''}$-β-Ala$^{7''}$/Gly$^{7''}$ segment was modeled in a type II β-turn conformation stabilized by an $i \leftarrow i + 4$ hydrogen bond. The successive β-Ala$^{8''}$ residue was inserted for its known flexibility to be adapted in a folded conformation.[21] Figure 2 describes the results of the modeling of the Thr$^{4''}$-Asp$^{5''}$-D-Ala$^{6''}$-Gly$^{7''}$-β-Ala$^{8''}$. It should, however, be mentioned that hirunorms, when compared to hirulog and hirutonin, contain an effective linker (from Arg$^{2''}$ to β-Ala$^{8''}$) of 18 (hirunorm II, III and V) or 19 (hirunorm I and V) bonds long, and as with hirulog and hirutonin, the linker in the hirunorms could then be disordered, especially as β-Ala increases flexibility.

At this point the hirudin molecule is completely out of the α-thrombin active site and begins to cover with the extended peptide Pro$^{48'}$-Gly$^{54'}$ segment the natural postulated entrance of fibrinogen in the active site.[22] The Pro$^{48'}$-Glu$^{49'}$-Ser$^{50'}$-His$^{51'}$ segment was kept identical in the hirunorm sequences because these residues show a large number of contacts in the hirudin-α-thrombin complex.[8] A single amino acid substitution Ser$^{50'}$ → Asn$^{11''}$ was performed in this segment to evaluate the effect of hydrogen bonding with α-thrombin Glu^{192}. This amino acid replacement seemed compatible because hirudin P6 contains an Asn residue in this position.[16] The flexible sequence Asn$^{52'}$-Asn$^{53'}$-Gly$^{54'}$ was instead replaced with the h-Phe$^{13''}$-Gly$^{14''}$-Gly$^{15''}$ sequence. It was reported that methionine residue in position 52$'$ improves the affinity of hirudin for α-thrombin[23] and thus h-Phe, which is a non-natural hydrophobic amino acid of comparable size to Met, was preferred. The Gly-Gly sequence was also preferred because the Asn ↔ *iso*-Asn interconversion frequently occurs in the Asn-Gly segment and gives the undesired *iso*-Asn derivative during the synthesis.[24]

Starting from Asp$^{55'}$, hirudin penetrates the long canyon corresponding to the fibrinogen recognition exosite which is positively charged on its surface.[8] Asp$^{55'}$-Phe$^{56'}$-Glu$^{57'}$-Glu$^{58'}$-Ile$^{59'}$-Pro$^{60'}$ sequence was also kept as such in the hirunorm sequences, because of the large number of contacts for this segment with α-thrombin, except for a Tyr residue replacing Phe$^{56'}$ which improves the affinity of other known inhibitors for α-thrombin.[25] Furthermore, Glu$^{61'}$ and Glu$^{62'}$ residues do not participate directly to the interaction with

Table 1. Sequences of Synthetic Hirunorms[a]

hirudin	hirunorm I	hirunorm II	hirunorm III	hirunorm IV	hirunorm V
$Ile^{1'}$	$Ile^{1''}$	Ile	Ile	Chg	Chg
$Val^{2'}$	$Arg^{2''}$	Arg	Arg	Arg	Val
$Tyr^{3'}$	$Tyr^{3''}$	Phe	Phe	2-Nal	2-Nal
$Thr^{4'}$	$Thr^{4''}$	Thr	Thr	Thr	Thr
$Asp^{5'}$	$Asp^{5''}$	Asp	Asp	Asp	Asp
|	D-$Ala^{6''}$	D-Ala	D-Ala	D-Ala	D-Ala
|	β-$Ala^{7''}$	Gly	β-Ala	Gly	Gly
|	β-$Ala^{8''}$	β-Ala	β-Ala	β-Ala	β-Ala
$Pro^{48'}$	$Pro^{9''}$	Pro	Pro	Pro	Pro
$Glu^{49'}$	$Glu^{10''}$	Glu	Glu	Glu	Glu
$Ser^{50'}$	$Asn^{11''}$	Ser	Ser	Ser	Ser
$His^{51'}$	$His^{12''}$	His	His	His	His
$Asn^{52'}$	$Asn^{13''}$	h-Phe	h-Phe	h-Phe	h-Phe
$Asx^{53'}$	$Asn^{14''}$	Gly	Gly	Gly	Gly
$Gly^{54'}$	$Gly^{15''}$	Gly	Gly	Gly	Gly
$Asp^{55'}$	$Asp^{16''}$	Asp	Asp	Asp	Asp
$Phe^{56'}$	$Phe^{17''}$	Tyr	Tyr	Tyr	Tyr
$Glu^{57'}$	$Glu^{18''}$	Glu	Glu	Glu	Glu
$Glu^{58'}$	$Glu^{19''}$	Glu	Glu	Glu	Glu
$Ile^{59'}$	$Ile^{20''}$	Ile	Ile	Ile	Ile
$Pro^{60'}$	$Pro^{21''}$	Pro	Pro	Pro	Pro
$Glu^{61'}$	$Aib^{22''}$	Aib	Aib	Aib	Aib
$Glu^{62'}$	$Aib^{23''}$	Aib	Aib	Aib	Aib
$Tyr^{63'}$	$Tyr^{24''}$	Tyr	Tyr	Tyr	Tyr
$Leu^{64'}$	$Leu^{25''}$	Cha	Cha	Cha	Cha
$Gln^{65'}$	$Glu^{26''}$	D-Glu	D-Glu	D-Glu	D-Glu

[a] The first column reports the corresponding residues in the recombinant hirudin variant 2, Lys^{47}, which was used as template structure for the design of hirunorm.

α-thrombin,[8] but they are involved in a 3_{10} helical structure allowing a correct folding of the C-terminal residues in contact with the fibrinogen recognition exosite. Side chain to side chain cyclization at the C-terminal end of hirudin fragments was also reported in the literature with the aim of rigidifying the helical conformation.[26] $Glu^{61'}$ and $Glu^{62'}$ residues were replaced in the hirunorm sequences with two Aib residue, because Cα,α-dialkylated residues favor a 3_{10}-helical structure.[27] In addition to this, the presence of the unnatural Aib residues would lower the hydrolysis by plasma proteases that has been observed between $Glu^{61'}$ and $Glu^{62'}$.[28] Finally, $Tyr^{24''}$-$Cha^{25''}$-$Glu^{26''}$/D-$Glu^{26''}$-OH end was selected as C-terminal due to the beneficial effects of these residues in strengthening the interaction of other known inhibitors with α-thrombin.[25]

Table 1 reports the hirunorm sequences here described. Figure 3 reports the result of the modeling of hirunorm IV on the X-ray structure of recombinant hirudin[8] when complexed with human α-thrombin. When superimposing the backbone atoms of hirunorm IV with the corresponding atoms in the 4HTC structure (see Table 1), a root mean square displacement < 0.6 Å was calculated. Figure 4 depicts the expected mode of interaction of hirunorm IV with human α-thrombin.

The synthesis of hirunorms was performed using the standard Boc strategy solid phase method. The peptide chains were assembled on the PAM resin. Deprotection and cleavage from the resin was obtained by treatment with anhydrous HF. The extracted crude materials were purified to homogeneity (>98.5%) by preparative RP-HPLC with a water/acetonitrile (0.1% TFA) linear gradient. Fab mass spectra gave the expected molecular ion peak values. The yields were satisfactory for all peptides. Table 2 reports the molecular weights, HPLC retention times, and overall final yields of the purified synthetic hirunorms based on the initial resin substitution. Large-scale synthesis of hirunorm V was recently developed.[29]

Hirunorms are potent human α-thrombin inhibitors. We investigated the effect of hirunorms on the amidolytic action of human α-thrombin toward the small substrate Tos-Gly-Pro-Arg-*p*-nitroanilide. Human α-thrombin-catalyzed hydrolysis of the chromogenic substrate showed a K_m value of $(14.24 \pm 0.98) \times 10^{-6}$ M in our experimental conditions. Substrate concentrations ranging between 2 and 10 times K_m were used in the inhibition studies. The resulting Dixon plots, which are reported in Figure 5, showed that hirunorms act as competitive inhibitors of human α-thrombin. These findings are in agreement with the results obtained by Mura[30] in the kinetic studies of human α-thrombin inhibition by hirunorms, and with the previously reported competitive inhibition on α-thrombin by hirulog-1.[31] Inhibition constants K_i for human α-thrombin-catalyzed hydrolysis of the chromogenic substrate by hirunorms and hirulog-1 are reported in Table 3. The K_i value for hirulog-1 is in good agreement with previously reported values.[5c,32] Hirunorms I, II, and III show K_i in the nanomolar range whereas K_i for hirunorm IV and V are in the picomolar range. Hirunorm IV was the most active in inhibiting the amidolytic activity of human α-thrombin; its potency is about 100-fold higher than hirulog-1. Hirunorms IV and V differ for a single amino acid ($Arg^{2''}$ or $Val^{2''}$), and hirunorm V is only 2-fold less active than hirunorm IV. Furthermore, K_i values were determined at different enzyme−inhibitor preincubation time (0−4 h). Interestingly, hirunorms are stable to the amidolytic action of α-thrombin for at least 4 h, because the K_i are independent from the preincubation time (within the experimental errors), whereas hirulog-1 inhibitory activity drops down after 1 h of preincubation time.[31] These findings are in agreement with the expected mechanism of action of hirunorms. They all bind α-thrombin in the active site because they inhibit the α-thrombin amidolytic activity toward small substrates and presumably they enter the active site in a nonsubstrate mode because they are not hydrolyzed by α-thrombin.

Hirunorm V is also highly selective, being unable to inhibit plasmin, t-PA, and trypsin up to 10 mM concentration.[33]

The potency of hirunorms to affect the coagulation parameters aPTT, PT, and TT of normal human plasma was also evaluated in *in vitro* experiments. Figure 6a−c reports the percentage of the increase in TT (a), PT (b), and aPTT (c), with respect to control values, induced by hirunorms. The activity of native hirudin and hirulog-1 is also reported for comparison: their anticoagulant potency was found to be comparable to previously reported data.[5c,34] The effect on the aPTT and PT of hirunorm I is not reported because it was about 4-fold and 10-fold less active (on a molar basis) than hirudin and hirulog-1, respectively, in these anticoagulant *in vitro* assays. A dose-dependent effect of hirunorms on coagulation parameters was observed. Hirunorms II, III, IV, and V are more effective than hirudin at increasing the TT of normal human plasma, whereas hirunorm I produces a weaker effect than hirudin. Hirunorms II, III, and V are as effective as hirulog-1. Conversely, hirunorm IV was more potent than all the other compounds investigated: an increase

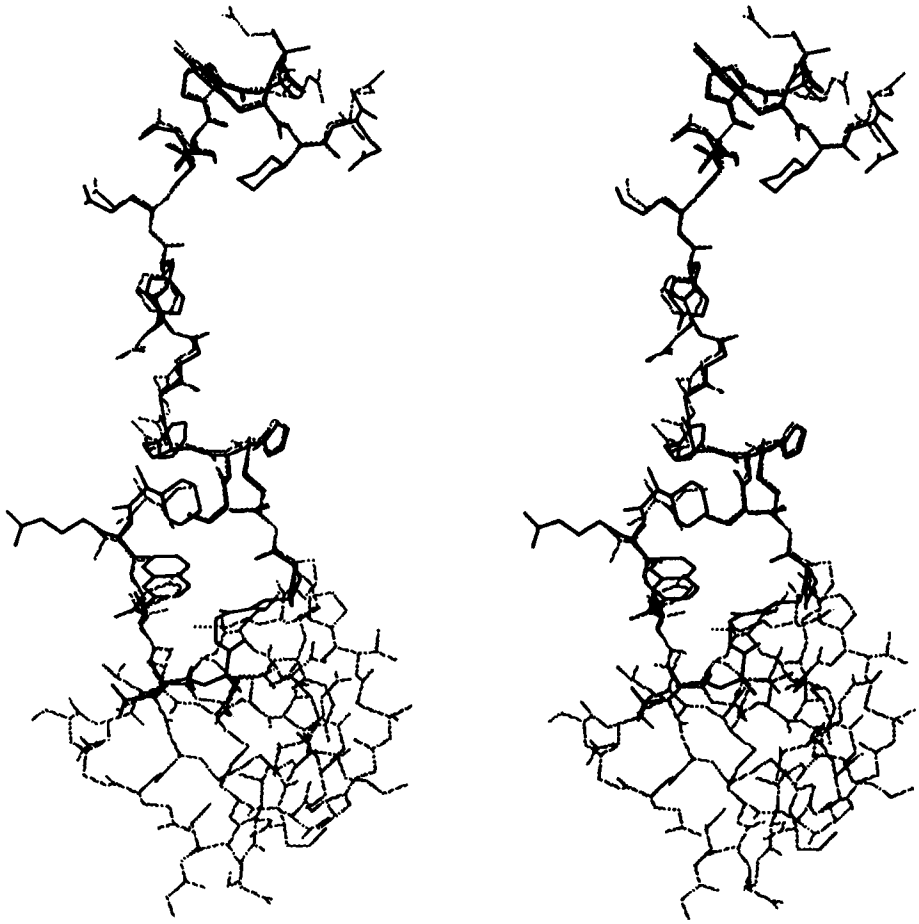

Figure 3. Stereoview of the backbone atoms superimposition between recombinant hirudin variant 2 Lys^{47} from $Ile^{1'}$ to $Asp^{5'}$ and from $Glu^{49'}$ to $Gln^{65'}$ (broken lines) and hirunorm IV model structure from $Chg^{1''}$ to $Asp^{5''}$ and from $Glu^{10''}$ to D-$Glu^{26''}$.

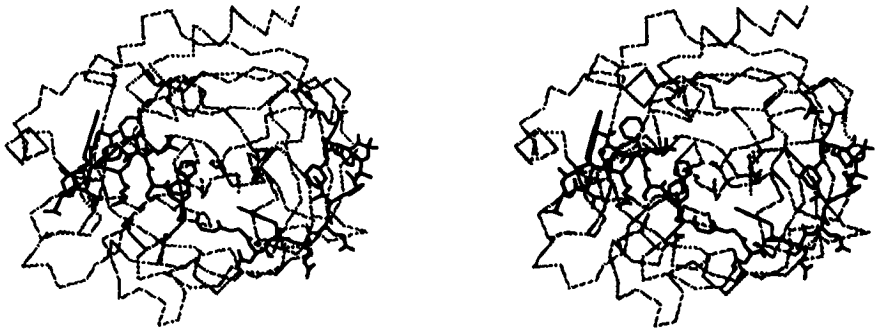

Figure 4. Stereoview of the hypothetical mode of interaction of hirunorm IV with α-thrombin (broken lines). Only Cα atoms of α-thrombin are reported for clarity.

Table 2. Molecular Weights, HPLC Retention Times (See the Text for the Experimental Conditions), and Overall Final Yields of the Purified Synthetic Hirunorms Based on the Initial Resin Substitution

compound	MW (amu)	t_R (min)	yield (%)
hirunorm I	2946	22.3	20
hirunorm II	2935	23.3	28
hirunorm III	2949	25.8	18
hirunorm IV	3011	21.5	14
hirunorm V	2954	22.1	16

in the TT to 300% of the control values was achieved at hirunorm IV concentrations 10-fold and 4-fold smaller than those required for hirudin and hirulog-1, respectively. Hirunorms II, III, IV, and V are also more effective than hirudin at increasing the aPTT of normal human plasma. Hirunorms II, III, and V exhibit an effectiveness comparable to hirulog-1, but hirunorm IV was 3-fold more active than hirulog-1 to increase the aPTT to 300% of the control values. Hirunorms II, III, IV, and V are more effective than hirudin to prolong the PT of normal human plasma, but hirunorms II and III are slightly less effective than hirulog-1. Conversely, hirunorm V, which has an effect on aPTT and TT of human plasma comparable to hirulog-1, increases the PT of human plasma to a larger extent than hirulog-1. Hirunorms IV and V, which are of comparable potency, are more potent than all the other compounds here investigated. An increase in the PT to 200% of control values was achieved at concentrations of hirunorms IV and V 20-fold and 3-fold smaller than those required for hirudin and hirulog-1, respectively.

The great ability to inhibit the amidolytic action of human α-thrombin, exhibited by hirunorms IV and V,

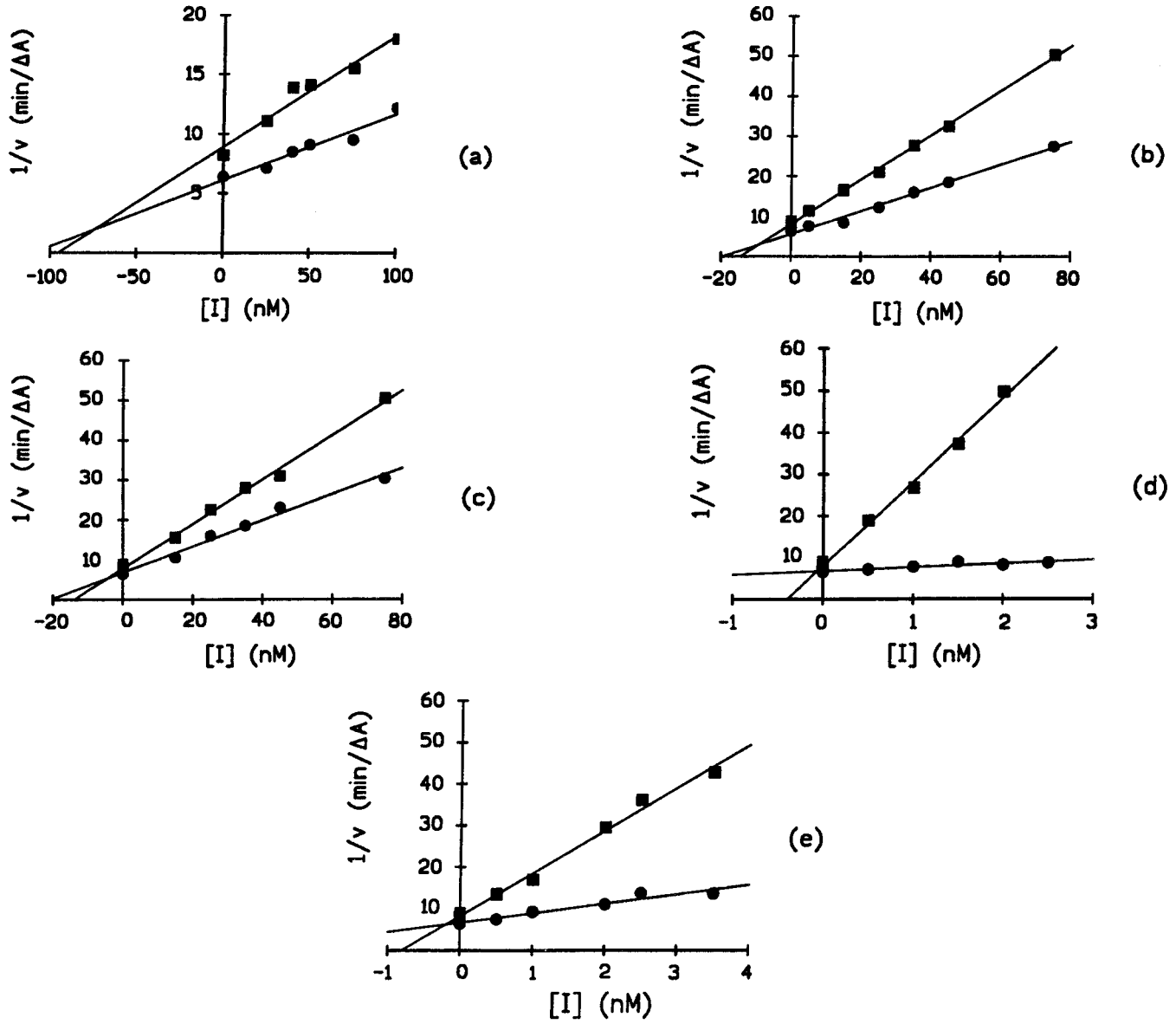

Figure 5. Dixon plots of human α-thrombin inhibition by hirunorms I−V (a−e); chromogenic substrate concentration: (■) 20 μM, (●) 125 μM. Experimental conditions are reported in the text. Each point represents the average of triplicate determinations. Linear regression correlation coefficients are >98% in all the experiments.

Table 3. K_i Values with esd for Human α-Thrombin-Mediated Hydrolysis of Tos-Gly-Pro-Arg-*p*-nitroanilide by Hirunorms and Hirulog-1

substance	K_i (nM)	substance	K_i (nM)
hirulog-1	6.02 ± 0.35	hirunorm-III	3.52 ± 0.21
hirunorm-I	72.1 ± 0.2	hirunorm-IV	0.09 ± 0.03
hirunorm-II	8.45 ± 0.58	hirunorm-V	0.21 ± 0.05

as compared to other hirudin analogs, well agrees with their strong potency in the anticoagulant assays.

The antithrombin activity displayed by hirunorms is in nice agreement with the design hypotheses and partly demonstrates the hypothetical mode of interaction of hirunorms with human α-thrombin. Hirunorm I contains only a few differences with the recombinant hirudin (variant 2, Lys^{47}) sequence (see Table 1). The most relevant are (i) $Val^{2'}$ has been replaced with $Arg^{2''}$ in the N-terminal region; (ii) $Glu^{61'}$ and $Glu^{62'}$ are replaced with Aib residues in the C-terminal region; (iii) the central core of hirudin ($Cys^{6'}$-$Lys^{47'}$) has been replaced with the tripeptide segment D-$Ala^{6''}$-β-$Ala^{7''}$-β-$Ala^{8''}$. Hirunorm I was a strong competitive inhibitor of human α-thrombin amidolytic activity toward small substrates with K_i in the nanomolar range (see Figure 5). This indicates that the prototype hirunorm I binds near the α-thrombin catalytic site. Furthermore, hirunorm I is stable to the amidolytic action of α-thrombin, and thus, in agreement with the design hypothesis, hirunorm I presumably binds near the catalytic site in a nonsubstrate-like mode. Hirunorms II and III are about 10-fold and 20-fold, respectively, more potent than hirunorm I in inhibiting the human α-thrombin amidolytic activity toward a small substrate. The increased potency can be ascribed to the beneficial effects of the amino acid substitutions $Tyr^{3''}$ → $Phe^{3''}$, $Asn^{13''}$-$Asn^{14''}$ → h-$Phe^{13''}$-$Gly^{14''}$, $Phe^{17''}$ → $Tyr^{17''}$, $Leu^{25''}$-$Glu^{26''}$ → $Cha^{25''}$-D-$Glu^{26''}$ in strengthening the hirunorm−α-thrombin interaction. Furthermore, it is reasonable to believe that a finer tuning of the hirunorm conformation in the linker segment D-$Ala^{6''}$-β-$Ala^{8''}$ can be achieved incorporating a β-Ala residue instead of a Gly residue. Finally, the increase in hydrophobicity of the N-terminal part of hirunorms, by replacing $Ile^{1''}$ with $Chg^{1''}$ and

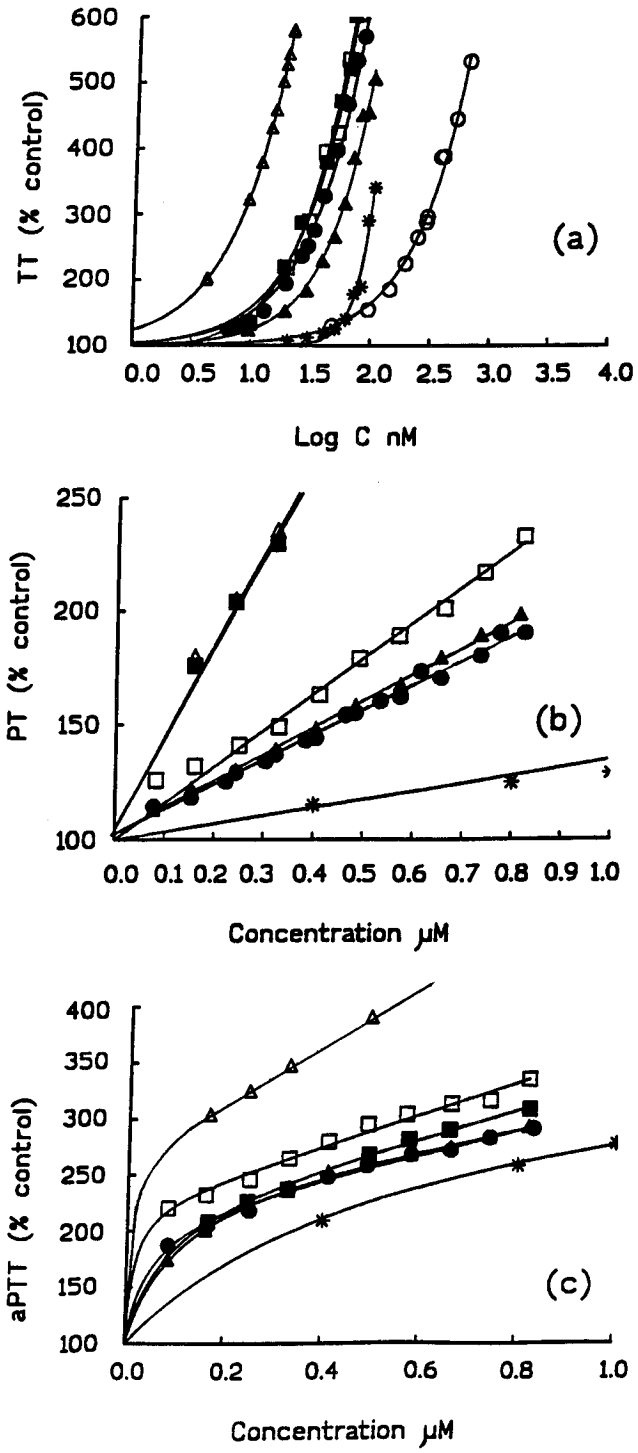

Figure 6. Percentage increase over control values of thrombin time (a), prothrombin time (b), and activated partial thromboplastin time (c) *versus* inhibitor concentration: (□) hirulog-1, (*) hirudin, (○) hirunorm I, (●) hirunorm II, (▲) hirunorm III, (△) hirunorm IV, (■) hirunorm V.

Phe$^{3''}$ with 2-Nal$^{3''}$, yields hirunorm IV which is the most active analog in inhibiting human α-thrombin amidolytic activity toward small substrates. Hirunorm V contains a single amino acid substitution (Arg$^{2''}$ → Val$^{2''}$), and it is only 2-fold less active than hirunorm IV in inhibiting human α-thrombin amidolytic activity toward small substrates. In contrast, Thr$^{2'}$Arg$^{2'}$ hirudin mutant is about 9-fold more potent than wild type.[18] Hirunorm V is of greater interest for application as antithrombotic drug, because it displays a better resistance to plasma proteases.[33]

In summary, the structure−activity relationship here described seems to confirm the design hypotheses of hirunorms. While the manuscript for this paper was under consideration for publication, we were able to solve the X-ray structure of human α-thrombin complexed with hirunorm V. The structure definitively proves the hirunorms−human α-thrombin interaction mechanism.

Conclusions

Hirunorms represent a new class of multisite-directed human α-thrombin inhibitors. They were rationally designed to interact through their N-terminal end with the thrombin active site in a nonsubstrate mode and to specifically bind the fibrinogen recognition exosite. An appropriate spacer that is able to properly orient the N-terminal end in the active site was also selected. Hirunorms are true hirudin mimetics because they reproduce more strictly the distinctive mechanism of action of hirudin. The synthesis was easily achieved by using the standard Boc strategy with solid phase method. Accordingly to the hypothetical mode of interaction with human α-thrombin, hirunorms were strong inhibitors of the amidolytic activity of α-thrombin toward a small chromogenic substrate. The most active analogs, hirunorms IV and V, showed the highest affinity toward α-thrombin with K_i values in the picomolar range. To the best of our knowledge, hirunorm IV is the most potent synthetic human α-thrombin inhibitor reported to date. A detailed study on the kinetics of human α-thrombin inhibition by hirunorm IV and V is presently under consideration for publication.[30] Similarly to hirudin, the peculiar structure of hirunorms makes them stable to the amidolytic action of α-thrombin without the introduction of any peptide bond modification. As expected, hirunorms showed high potency in the anticoagulant assays. Hirunorm IV was the most effective analog in prolonging aPTT, PT, and TT of normal human plasma: its potency was much greater than that shown by native hirudin or hirulog-1. Experimental pharmacology[33] on hirunorm V demonstrates its effectiveness as anticoagulant agent with antithrombotic properties in different rat models of thrombosis. Hirunorm V is resistant to proteolysis and shows low plasma clearance. Its elimination is less dependent on renal function than that of hirudin. Surprisingly hirunorm V compares favorably with recombinant hirudin against arterial thrombosis. Hirunorm V does not affect bleeding at antithrombotic doses.

Hirunorms are potential candidates for injectable anticoagulants, due to their potency, specificity of action, long-lasting activity, and safety profiles.

Experimental Section

Materials. *t*-Boc-D-Glu(OBzl)-OCH_2-PAM resin, *t*-Boc-Glu-(OBzl)-OCH_2-PAM resin, *t*-Boc amino acids (except *t*-Boc-Chg-OH, *t*-Boc-2-Nal)-OH, *t*-Boc-D-Ala-OH, and *t*-Boc-h-Phe-OH), and pyBop were purchased from Novabiochem (Läufelfingen, CH). *t*-Boc-Chg-OH was from Sigma (Sigma Chemical Co., St. Louis, MO); *t*-Boc-*β*-(2-naphthyl)-Ala-OH, *t*-Boc-D-Ala-OH, and *t*-Boc-h-Phe-OH were from Bachem (Bubendorf, CH). All the solvents and the reagents for the solid phase peptide synthesis were purchased from Applied BioSystem (Foster City, CA). H_2O and CH_3CN used in the peptides purification were HPLC grade and were supplied from LabScan Analytica (Dublin, Ireland). HF was from UCAR Union Carbide (Oevel, Belgium).

Highly purified human α-thrombin (3000 NIH units/mg) and bovine serum albumin (fraction V) (BSA) were purchased from Sigma, Tos-Gly-Pro-Arg-*p*-nitroanilide (Chromozym TH) and natural hirudin (1000 ATU/mg) from Boehringer (Mannheim, Germany), activated partial thromboplastin suspension and bovine thromboplastin from Organon Teknika (Duram, NC). Hirulog-1 was synthesized by conventional solid phase methods as described in the literature.[5c]

Peptide Design. Molecular design was performed on a Silicon Graphics workstation Personal Iris 4D/35 GT TURBO by using X-ray structural data for recombinant hirudin variant 2, Lys[47] human α-thrombin complex (PDB code 4HTC)[8a] from Protein Data Bank Brookhaven National Laboratory (Upton, NY),[35] and the Insight/Discover program package (Biosym).[36] Energy minimizations were performed using the DISCOVER program ver. 2.9, with the Consistent Valence Force Field,[37] and performing a template forcing of hirunorms backbone atoms over the hirudin structure as reference object. A force constant of 5 kcal/Å was used. Prior to energy minimization, side chain conformations of hirunorms, which were in common with recombinant hirudin variant 2, Lys,[47] were modeled in a conformation quite similar to those observed in the 3HTC structure, otherwise hand modeling was performed, while keeping all bonds staggered. Bump check of the modeled hirunorms with the α-thrombin structure was also performed using a 0.1 Å cutoff for the overlapping van der Waals radii.

Peptide Synthesis and Purification. The synthesis of hirunorms was achieved by the solid phase method (0.5 mmol scale) on an Applied BioSystems Model 430A automatic peptide synthesizer (Perkin-Elmer Corporation, Foster City, CA), employing the classical protocols for N-α-*t*-Boc strategy. The peptide chains were assembled on a *t*-Boc-D-Glu(OBzl)-OCH_2-PAM resin or *t*-Boc-Glu(OBzl)-OCH_2-PAM resin; the following amino acid side chain protections were used: Arg(Tos), Thr(Bzl), Asp(O*c*Hx), Glu(O*c*Hx), Ser(Bzl), His(Bom), Tyr(2-Br-Z). All amino acids (2 mmol), except *t*-Boc-Arg(Tos)-OH, *t*-Boc-His(Bom)-OH, and *t*-Boc-Aib-OH, were incorporated *via* symmetric anhydrides by using 2 mL (1 mmol) of a 0.5 M DCC in DCM solution, over 10 min. *t*-Boc-Arg(Tos)-OH and *t*-Boc-His(Bom)-OH were preactivated to the 1-hydroxybenzotriazole ester with 2 mmol of HOBt and 2 mmol of DCC as a 0.5 M solution in DCM, over 45 min. Finally, *t*-Boc-Aib-OH was activated *in situ*, according to the pyBop procedure:[38] 3 equiv of *t*-Boc-amino acid, 3 equiv of pyBop, and 6 equiv of DIEA in DMF. Each cycle consisted of a 45 min coupling step. Completeness of the reaction, after each coupling, was checked by the Kaiser test;[39] a recoupling step was necessary for all amino acids. *t*-Boc protecting groups were removed by a 18 min deprotection step with 50% TFA in DCM (v/v), followed by a 2 min neutralization step with 10% DIEA in DMF (v/v).

Peptides were fully deprotected and cleaved from the resin by treatment with anhydrous HF, anisole (10/1 v/v).[40] The crude products were extracted in aqueous acetic acid (50% v/v), and the resultant solutions were lyophilized. The crude materials were purified to homogeneity by preparative RP-HPLC on a Waters Delta Prep 3000 (Milford, MA) chromatographic system, equipped with a UV Lambda Max Mod. 481 detector. The samples were injected on a Vydac (The Separation Group, Hesperia, CA) C_{18} column (50 × 250 mm; 10 μm), eluted with a H_2O/0.1% TFA (A) and CH_3CN/0.1% TFA (B) solvent mixture. A linear gradient from 5 to 70% of B over 50 min at flow rate of 114 mL/min was employed. The effluent was monitored at 210 nm, and fractions were collected manually. The pooled fractions containing the desired products were lyophilized. The purity of the products (>98.5%) was ascertained by analytical RP-HPLC on a Varian 3000 LC Star System (Palo Alto, CA), equipped with a 9065 Polychrom and a 9095 auto sampler. Peptide samples (20−50 μg) were applied to a Vydac C_{18} column (4.6 × 150 mm, 5 μm); a linear gradient from 5 to 70% of B over 30 min at flow rate of 1 mL/min was achieved.

Fab mass spectra gave the expected values for the molecular ion peak $[M - H]^+$. They were obtained with a VG ZAB 2 SE double-focusing mass spectrometer equipped with a cesium gun operating at 25 kV (2 μA), at the "Servizio di Spettrometria di Massa, CNR, University of Napoli".

The sequences of the synthesized peptides are reported in Table 1. The yields of the purified peptides, their molecular weight, and the analytical RP-HPLC retention times are reported in Table 2.

Amidolytic Assay of Thrombin Activity. The human α-thrombin-catalyzed hydrolysis of Tos-Gly-Pro-Arg-*p*-nitroanilide was monitored at 405 nm on a Cary-1 double-beam spectrophotometer and recorded continuously as a function of time. The hydrolytic reactions were performed at 37 °C in 0.05 M Tris-HCl buffer, pH 7.8, containing 0.1 M NaCl and 0.1% polyethylene glycol 6000. The concentration of α-thrombin was determined spectrophotometrically by using an extinction coefficient at 280 nm of 1.83 $mL \cdot mg^{-1} \cdot cm^{-1}$.[41] The incubation mixtures contained 50 μL inhibitor ($0 \div 1 \times 10^{-7}$ M), 50 μL of α-thrombin (2.5×10^{-8} M), and buffer to 1.0 mL final volume. The reactions were initiated by addition of 50 μL of substrate ($2 \div 12.5 \times 10^{-5}$ M). Initial rate velocities were recorded and used to construct the Dixon plots: the relationship of (initial velocities)$^{-1}$ *versus* inhibitor concentrations were analyzed by using linear regression calculations included in the Varian software. K_i values were determined by standard procedures using equations for competitive and noncompetitive inhibition.[42] K_i values were also determined using different enzyme and inhibitor preincubation times in the 0−4 h interval.

aPTT, PT, and TT Measurements. aPTT, PT, and TT determinations were performed on pooled normal human plasma by using a Coag-A-Mate X2 instrument (Organon Technicon, Oklahoma City, OK). The aPTT, PT, and TT of the normal human plasma, derived from 20−30 healthy blood donors, were 35.0 ± 1.5, 11.4 ± 0.5, and 15.7 ± 0.4 s, respectively.

Stock solutions of inhibitors were prepared at 1.5−3.0 mg/mL concentrations in 5% BSA solution. BSA alone at the dose used in the assays had no effect on the aPTT, PT, and TT of normal human plasma.

Plasma (100 μL) and inhibitor solution (50 μL) were premixed prior to aPTT and PT determinations. After the addition of activated partial thromboplastin suspension, or bovine thromboplastin and calcium chloride, the final volume of the assay mixture was 350 μL. The data are reported as the percentage of increase in aPTT and PT with respect to control values in the absence of inhibitor. The final data are the mean of at least three different determinations, each performed in duplicate.

For TT measurements, human α-thrombin was freshly dissolved in 10 mM Tris, pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$ at a concentration of 3.5 NIH units/mL. A 50 μL sample of inhibitor solution and 200 μL of plasma sample were premixed prior TT determinations and were added to 100 μL of thrombin solution (final concentration 1 NIH unit/mL). The data are reported as the percentage of the increase in TT with respect to control values in the absence of inhibitor. They are the mean of six different determinations.

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(1) Abbreviations used for natural amino acids and peptides are according to IUPAC−IUB Commission on Biochemical Nomenclature (IUPAC−IUB, 1984; 1989): *t*-Boc, *tert*-butoxycarbonyl; PAM, (phenylacetamido)methyl; Chg, cyclohexyl-glycine; 2-Nal, β-(2-naphthyl)alanine; h-Phe, (+)-α-amino-4-phenylbutyric acid; Aib, α-aminoisobutyric acid; Cha, β-cyclohexylalanine; Tos, *p*-tolylsulfonyl; Bzl, benzyl; *c*Hx, cyclohexyl; Bom, (benzyloxy)-methyl; 2-Br-Z, 2-bromobenzyloxycarbonyl; PyBop, (benzotriazol-1-yloxy)trispyrrolidinophosphonium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DMF, dimethylformamide; DCM, dichloromethane; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid; RP-HPLC, reverse phase high-performance liquid chromatography; Fab-MS, fast atom bombardment mass spectroscopy; t_R, retention time; aPTT, activated partial thromboplastin time; PT, prothrombin time; TT, thromboplastin time; BSA, bovine serum albumine; ATU, antithrombin units.

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