Articles

Design and Synthesis of Novel Biologically Active Thrombin Receptor Non-Peptide Mimetics Based on the Pharmacophoric Cluster Phe/Arg/NH_2 of the Ser_{42}-Phe-Leu-Leu-Arg_{46} Motif Sequence: Platelet Aggregation and Relaxant Activities

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The identification of the thrombin receptor has promoted the interest for the development of new therapeutic agents capable of selectively inhibiting unwanted biological effects of thrombin on various cell types. In this study we have designed and synthesized two series of new thrombin receptor antagonists based on the thrombin receptor motif sequence $S_{42}FLLR_{46}$, one possessing two (Phe/Arg) pharmacophoric groups and the other possessing three (Phe/Arg/NH_2). *N*-(6-Guanidohexanoyl)-*N*′-(phenylacetyl)piperazine (**1**), *N*-(phenylacetyl)-4-(6-guanidohexanoylamidomethyl)piperidine (**2**), and *N*-(phenylacetyl)-3-(6-guanidohexanoylamido)pyrrolidine (**3**) (group A) carry the two pharmacophoric side chains of Phe and Arg residues incorporated on three different templates (piperazine, 4-aminomethylpiperidine, and 3-aminopyrrolidine). Compounds with three pharmacophoric groups (group B) were built similarly to group A using the same templates with the addition of an extra methylamino group leading to (*S*)-*N*-(6-guanidohexanoyl)-*N*′-(2-amino-3-phenylpropionyl)piperazine (**4**), (*S*)-*N*-(2-amino-3-phenylpropionyl)-4-(6-guanidohexanoylamidomethyl)piperidine (**5**), and (*S*)-*N*-(2-amino-3-phenylpropionyl)-3-(6-guanidohexanoylamido)pyrrolidine (**6**). Compounds were able to inhibit thrombin-induced human platelet activation even at low concentrations. In particular, among compounds in group A, compound **3** was found to be the most powerful thrombin receptor activation inhibitor, showing an IC_{50} of approximately 0.11 mM on platelet aggregation assay. Among compounds in group B, compound **4** was the most powerful to inhibit thrombin-induced platelet aggregation, showing an IC_{50} of approximately 0.09 mM. All compounds were also found to act as agonists in the rat aorta relaxation assay. Interestingly, the order of potency of these compounds as agonists of the endothelial thrombin receptor was the inverse of the order of potency of the same compounds as antagonists of the platelet thrombin receptor. Such compounds that are causing vasodilation while simultaneously inhibiting platelet aggregation would be very useful in preventing the installation of atherosclerotic lesions and deserve further investigation as potential drugs for treating cardiovascular diseases. The above findings coupled with computational analysis molecular dynamics experiments support also our hypothesis that a cluster of phenyl, guanidino, and amino groups is responsible for thrombin receptor triggering and activation.

Introduction

Thrombin is a multifunctional serine protease that plays a central role in thrombosis and hemostasis, as well as in atherosclerotic and inflammatory diseases.[1−3] Thrombin acts on a specific receptor, a member of the seven transmembrane receptor family,[4] and causes a limited proteolytic cleavage, leading to the formation of a new N-terminus capable of activating the receptor via intramolecular interaction.[5−8] The identification of such proteolytically activated tethered-ligand thrombin receptor sequences on a variety of cell types (platelets, vascular smooth muscle cells, endothelial cells, fibroblasts) has increased our understanding on how thrombin signals are mediated within the cells[5,9−10] and

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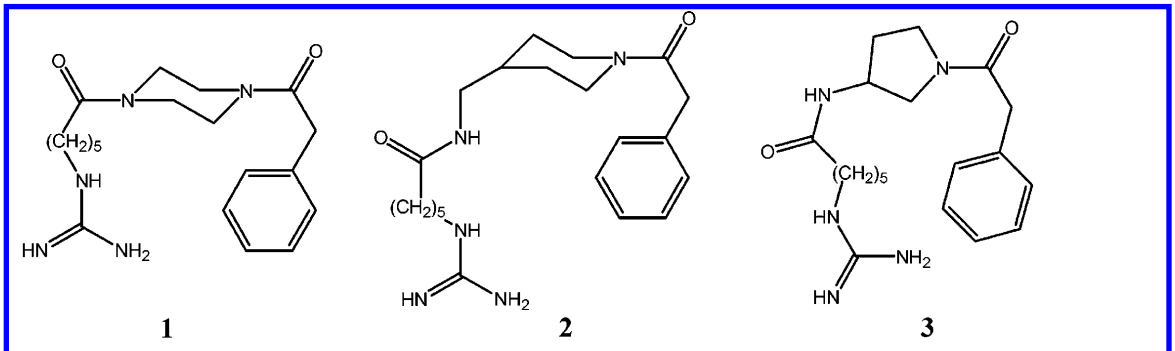
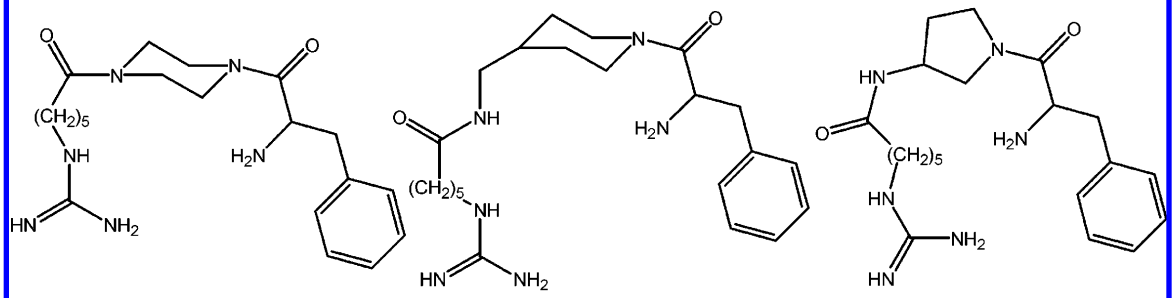
Scheme 1. Thrombin Receptor Non-Peptide Mimetics



1 **2** **3**

A) Chemical structures of Group A thrombin receptor mimetics.
N-(6-guanidohexanoyl)-*N*′-(phenylacetyl)piperazine (**1**)
N-(phenylacetyl)-4-(6-guanidohexanoylamidomethyl)piperidine (**2**)
N-(phenylacetyl)-3-(6-guanidohexanoylamido)pyrrolidine (**3**)



4 **5** **6**

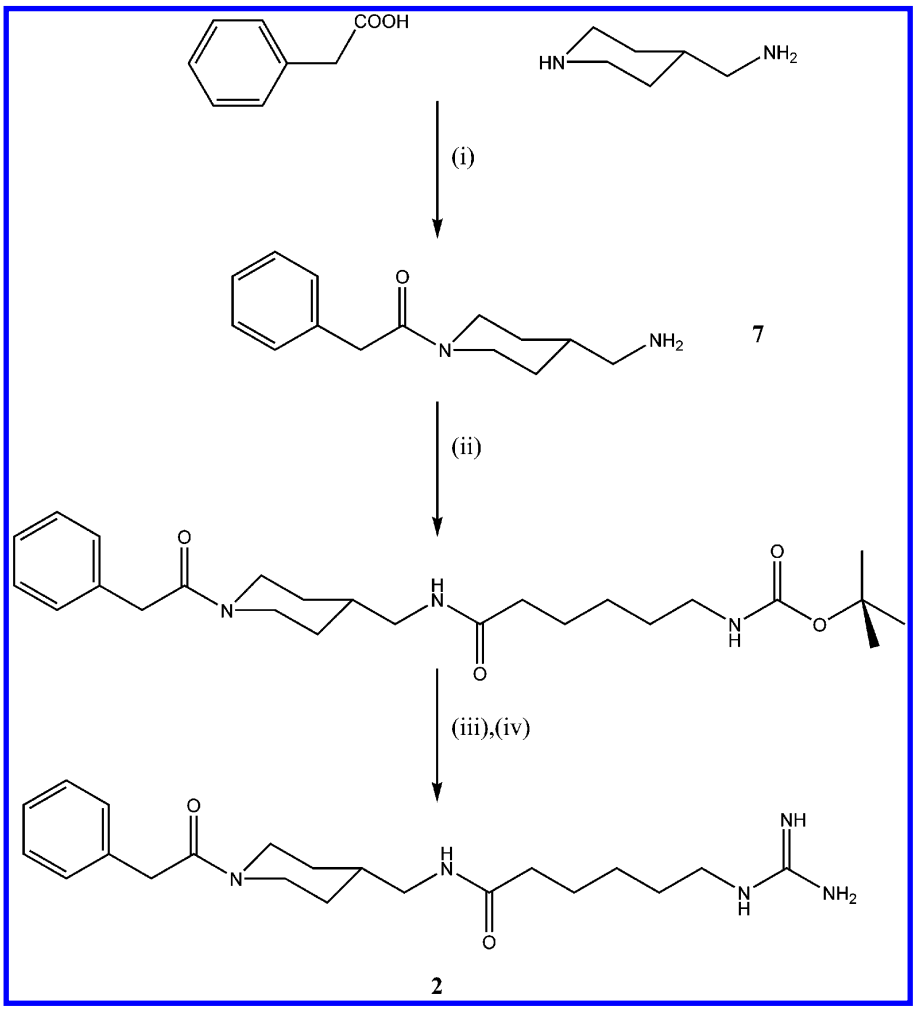
B) Chemical structures of Group B thrombin receptor mimetics.
(*S*) *N*-(6-guanidohexanoyl)-*N*′-(2-amino-3-phenylpropionyl)piperazine (**4**)
(*S*) *N*-(2-amino-3-phenylpropionyl)-4-(6-guanidohexanoylamidomethyl)piperidine (**5**)
(*S*) *N*-(2-amino-3-phenylpropionyl)-3-(6-guanidohexanoylamido)pyrrolidine (**6**)

stimulated our interest in synthesizing new thrombin receptor antagonists.[11]

About a decade ago, synthetic thrombin receptor activating peptides (TRAPs) comprising only the first five amino acids ($S_{42}FLLR_{46}$) of tethered ligand have been shown to be sufficient to mimic human receptor activation by thrombin.[12−15] Structure−activity relationships (SAR) and amino acid substitutions coupled with NMR studies and molecular modeling have determined the specific role of each amino acid in the native SFLLR sequence.[12,16,17] Early studies also indicated that a positively charged NH_2-terminus is important for full agonist activity.[18−20] By using cyclic TRAPs that we have designed and synthesized in our laboratories, we demonstrated that the Phe/Arg relative conformation is an important recognition motif in triggering the receptor, suggesting that a comparable cyclic conformation may be responsible for the interaction of linear TRAPs with the thrombin receptor.[20−22] Furthermore, we have demonstrated that this active motif is augmented by the presence of a primary amino group in the cyclic peptides.[20,21]

In this study we have designed and synthesized novel small-size non-peptide TRAP mimetics based on our SFLLR conformational model[17,20,21] using solution- and solid-phase organic synthesis. In particular we have synthesized and investigated the following compounds: *N*-(6-guanidohexanoyl)-*N*′-(phenylacetyl)piperazine (**1**), *N*-(phenylacetyl)-4-(6-guanidohexanoylamidomethyl)-piperidine (**2**), *N*-(phenylacetyl)-3-(6-guanidohexanoyl-amido)pyrrolidine (**3**), (*S*)-*N*-(6-guanidohexanoyl)-*N*′-(2-amino-3-phenylpropionyl)piperazine (**4**), (*S*)-*N*-(2-amino-3-phenylpropionyl)-4-(6-guanidohexanoylamidomethyl)piperidine (**5**), and (*S*)-*N*-(2-amino-3-phenylpropionyl)-3-(6-guanidohexanoylamido)pyrrolidine (**6**). These compounds carrying the essential pharmacophoric groups of Phe, Arg and of Phe, Arg, NH_2 incorporated onto three different small, bifunctional templates (piperazine, 4-aminomethylpiperidine, and 3-aminopyrrolidine) (Scheme 1) were subsequently tested for biological activity in platelet-rich plasma and perfused aortic rings. Data show strong potency in both assays, with compound **4** being the most active rendering these compounds potential substances for further investigation and possible development in the cardiovascular field. The conformation of the most active non-peptide antagonist in the platelet aggregation (compound **4**) was assessed using simulation and modeling methods in keeping with our previous studies.[17,20,21,23]

Scheme 2. Synthetic Procedure for *N*-(Phenylacetyl)-4-(6-guanidohexanoylamidomethyl)piperidine (**2**)[a]



[a] Reagents: (i) DCC, HOBt, $CHCl_3$; (ii) Boc-ϵ-Ahx-OH, DCC, HOBt, DIEA, CH_2Cl_2; (iii) 30% TFA/CH_2Cl_2; (iv) 1*H*-pyrazole-1-carboxamide, DIEA, DMF.

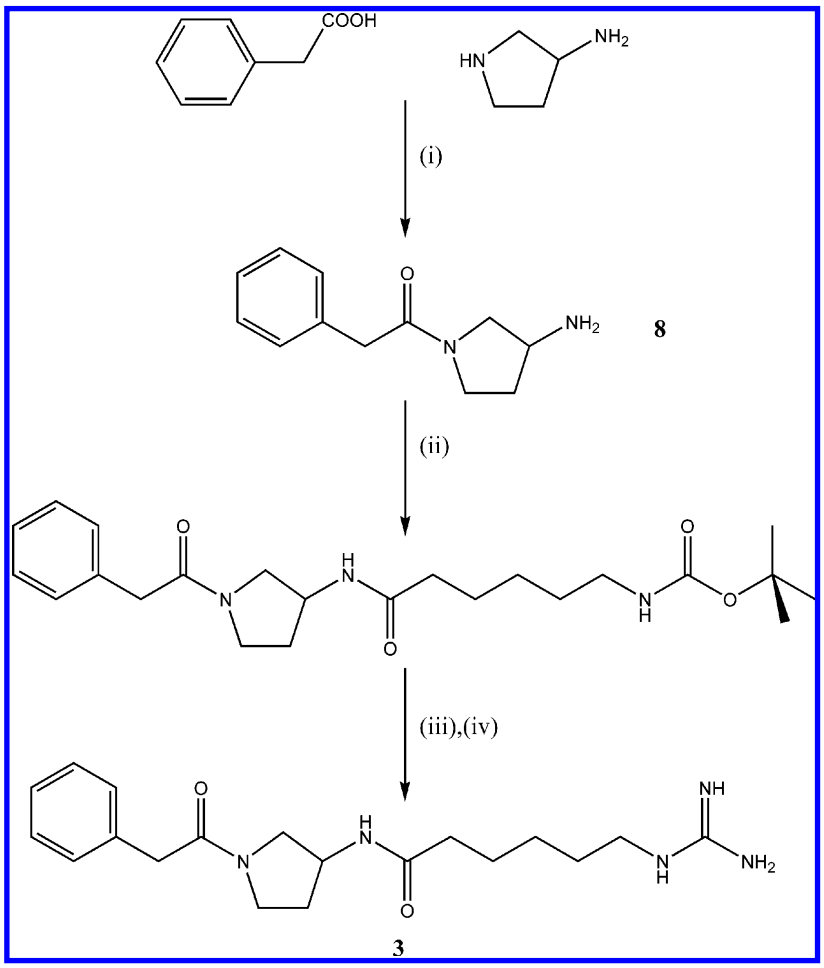
Results

Chemistry. Compound **1** of group A has been synthesized by methods previously described,[24] while synthesis of compound **2** was accomplished as is shown in Scheme 2. First, 4-aminomethylpiperidine was reacted with phenylacetic acid (2:1 molar excess of diamine to carboxylic acid) using DCC and HOBt as coupling reagents. Only one of the two possible regioisomers, **7a** and not **7b** (see Scheme 6), was obtained as identified by NMR and HPLC analysis. Boc-ϵ-aminohexanoic acid was then incorporated in the free primary amino group of piperidine, aided by the use of DCC and HOBt under basic conditions (DIEA). Boc deprotection was accomplished with trifluoroacetic acid, giving the free amine salt. Guanylation of the primary amine using 1*H*-pyrazole-1-carboxamide hydrochloride afforded compound **2**, which was purified by recrystallization (MeOH/acetone/Et_2O). Compound **3** was synthesized in a similar way starting from 3-aminopyrrolidine (Scheme 3). Again, only one of the two possible regioisomers **8a** and **8b** (see Scheme 6) was obtained after the first alkylation step and identified by NMR and HPLC analysis to be isomer **8a**.

Compound **4** has been synthesized by methods recently described,[20] while compounds **5** and **6** were synthesized by an analogous procedure accomplished using solid-phase methods and the 2-chlotrityl chloride resin as solid support (Scheme 4). Briefly, attachment of the amino group of phenylalanine (L-Phe) to the resin (2.27 mequiv of Cl^-/g of resin) was achieved by refluxing using TEA in CH_2Cl_2 solution and after protection of the carboxyl group of Phe with $(CH_3)_3SiCl$. The loading of Phe to the resin was certified by the Kaiser ninhydrin test. The carboxyl group of Phe attached to the resin through the amino group was first activated with DIC and HOBt and then divided among two reaction vessels where it was coupled with two different diamines (4-aminomethylpiperidine and 3-aminopyrrolidine) in DMF and in the presence of DIEA. Couplings with Fmoc-ϵ-aminohexanoic acid (2.5 equiv) were aided by the use of DIC and HOBt under basic conditions (DIEA) using a minimum of DMF. Fmoc group removals were carried out by treatment with 20% piperidine/DMF for 30 min. The amino group was converted to the guanidino group by using 1*H*-pyrazole-1-carboxamide hydrochloride in DMF/DIEA in the last solid-phase chemistry step before cleavage. The resin was treated with 10% TFA/CH_2Cl_2 for 15 min at room temperature to provide crude products **5** and **6**, which were purified by HPLC.

To determine definitely the stereochemistry of compounds **2**, **3**, **5**, and **6**, we resorted to selective chemical reactions differentiating primary and secondary amine

Scheme 3. Synthetic Procedure for *N*-(Phenylacetyl)-3-(6-guanidohexanoylamido)pyrrolidine (**3**)[a]



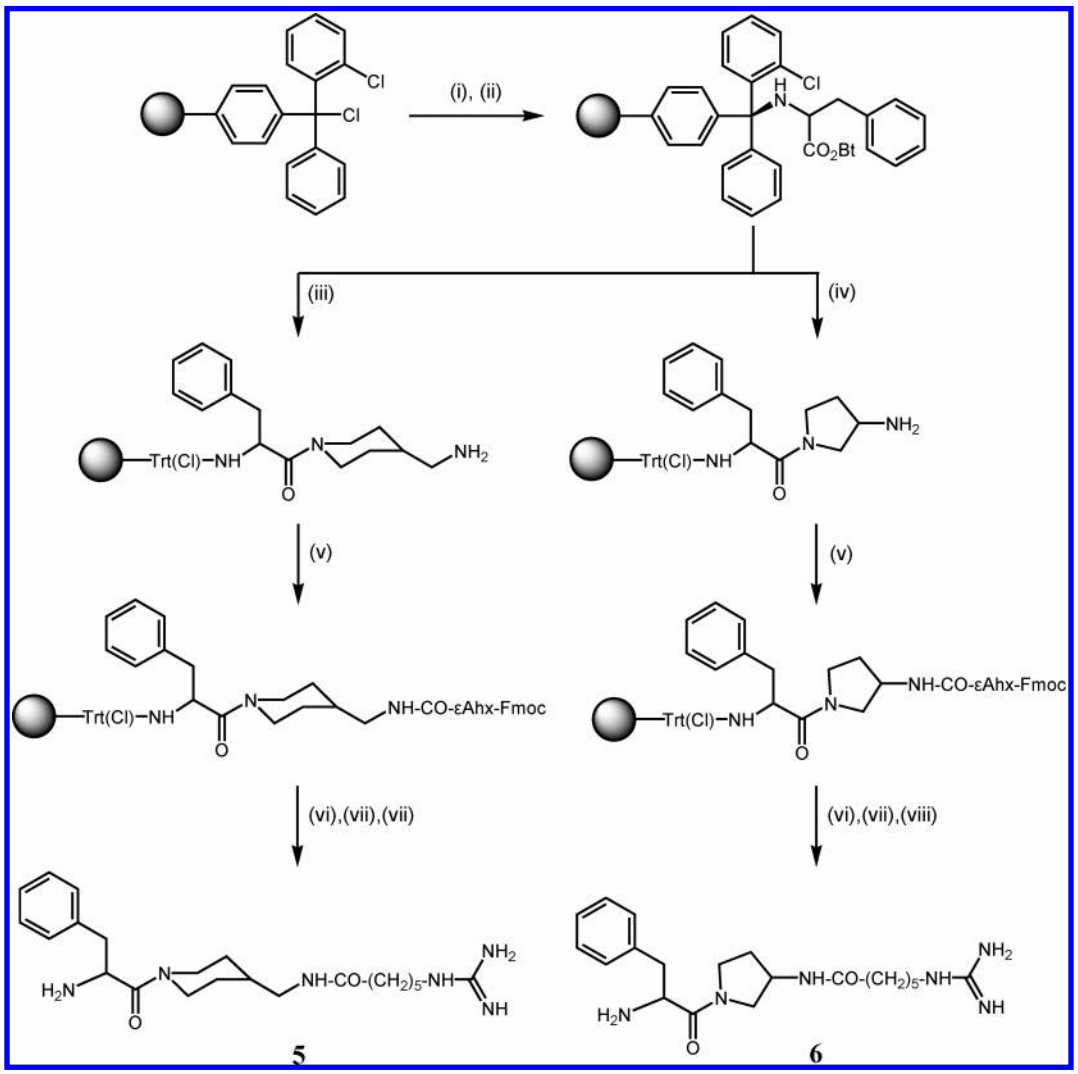
[a] Reagents: (i) DCC, HOBt, $CHCl_3$; (ii) Boc-ϵ-Ahx-OH, DCC, HOBt, DIEA, CH_2Cl_2; (iii) 30% TFA/CH_2Cl_2; (iv) 1*H*-pyrazole-1-carboxamide, DIEA, DMF.

groups. Thus, we repeated the first acylation step with phenylacetic acid using as starting material the primary amine protected *N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl] (Dde) derivatives of 4-aminomethylpiperidine and 3-aminopyrrolidine. The selectivity of the Dde protecting group towards primary amines has been demonstrated by other groups[25] and is presumably a function of the stabilization provided by a strong intramolecular hydrogen bond (N*H* $\delta = 13-14$ ppm) which is not possible with secondary amines (Scheme 5). Thus, the two diamines 4-aminomethylpiperidine and 3-aminopyrrolidine reacted readily with 2-acetyldimedone to afford the corresponding primary amine protected Dde derivatives. Phenylacetic acid was then coupled to the above Dde-protected diamines, using DCC and HOBt as activating reagents. Hydrazinolysis of the *N*-Dde afforded the free primary amine analogues **7a** and **8a** (Scheme 5), identified also by ^{1}H NMR and HPLC analysis.

Furthermore, using as standards the compounds **7a** and **8a**, we compared their ^{1}H NMR spectra with the relative spectra of the synthesized compounds **7** and **8** and found them to be identical. The TLC and HPLC profiles of each pair **7**−**7a** and **8**−**8a** were also identical. The above described method provides an alkylation procedure of 4-aminomethylpiperidine and 3-aminopyrrolidine (2-fold or more molar excess of diamine to carboxylic acid), which in short reaction time leads to high yields of free primary amine derivatives. N-acylation selectivity is due to the higher nucleophilicity of the secondary nitrogen in these molecules.

Bioassay Data. Non-peptide TRAP mimetics were tested on human platelet-rich plasma in order to determine whether such compounds could affect thrombin receptor activation on the platelet surface. By the use of this assay, it was found that all tested compounds were able to inhibit thrombin-induced platelet aggregation in a dose-dependent manner. As shown in Figure 1, non-peptide mimetics, compounds **1** and **3** of group A, were able to inhibit thrombin-induced platelet aggregation at a concentration of thrombin as high as 0.3 IU/mL. In contrast, the inhibitory effect of compound **2** was demonstrated when thrombin was used at a concentration less than 0.25 IU/mL while the effect was lost at higher (0.3 IU/mL) concentrations. Compound **3** was clearly the most effective among the three compounds of group A, with an IC_{50} of 0.11 mM at a thrombin concentration of 0.20 IU/mL (Table 1). As shown in Figure 2, compound **5** of group B showed a biphasic response. At lower concentrations, it not only failed to inhibit thrombin-induced platelet aggregation but also increased it, whereas at higher concentrations it showed clearly an inhibitory effect. Compound **4** was the most effective inhibitory compound ($IC_{50} = 0.09$ mM for a thrombin concentration of 0.25 IU/mL) followed by compound **6**.

Scheme 4. Solid-Phase Organic Synthesis of Compounds **5** and **6** of Group B[a]



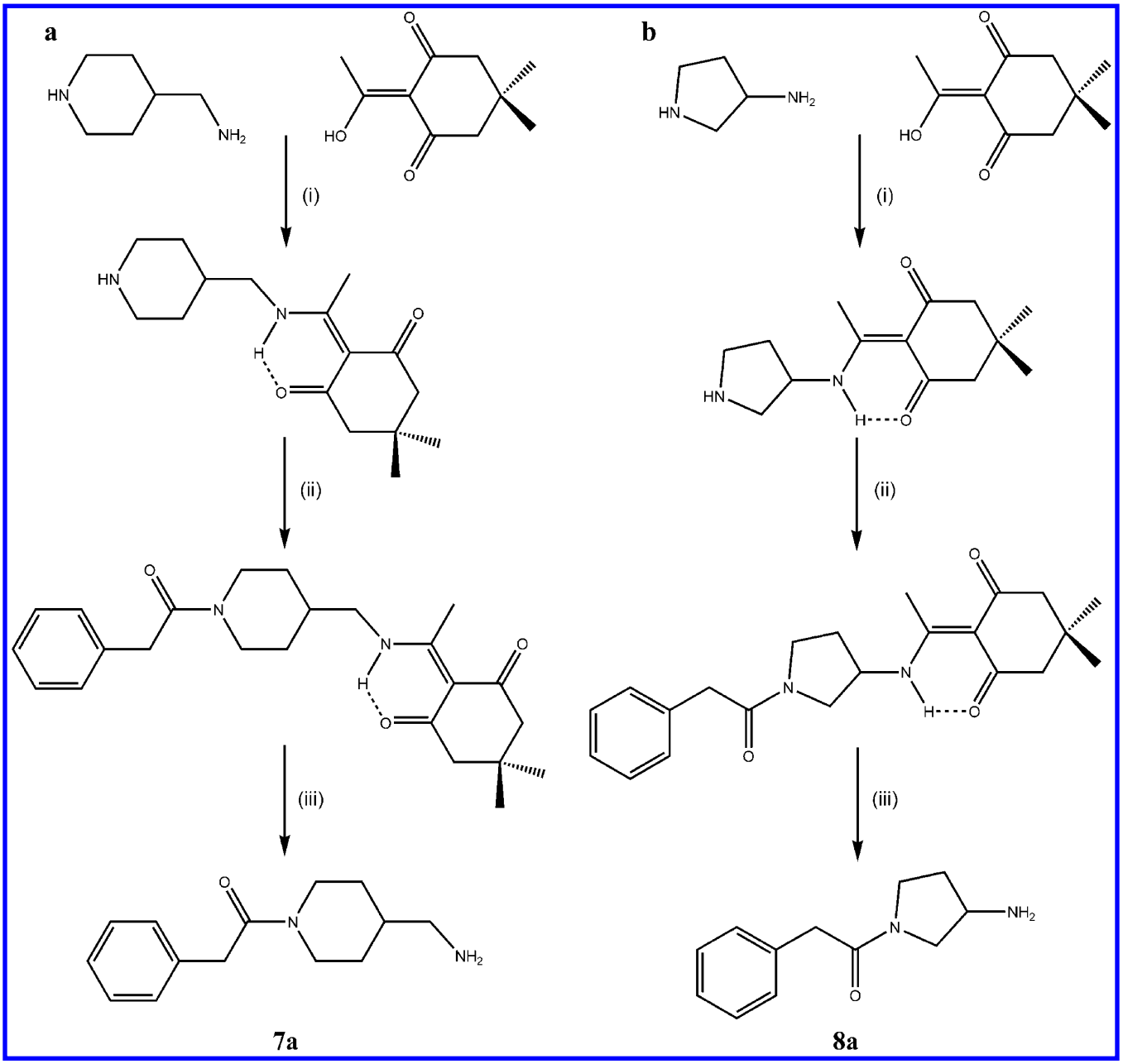
[a] Reagents: (i) Phe-OH, $(CH_3)_3SiCl$, TEA, CH_2Cl_2; (ii) DIC, HOBt, THF; (iii) 4-aminomethylpiperidine, DIEA, DMF; (iv) 3-aminopyrrolidine, DIEA, DMF; (v) Fmoc-ϵ-Ahx-OH, DIC, HOBt, DMF; (vi) 20% piperidine in DMF; (vii) 1*H*-pyrazole-1-carboxamide, DIEA, DMF; (viii) 10% TFA/CH_2Cl_2.

As shown in Table 1, the addition of an amino group improved the inhibitory effect of non-peptide mimetic **4** with a piperazine template but failed to improve and in fact worsened the inhibitory effect of the other two non-peptide mimetics **5** and **6**. None of the tested compounds were able by itself to produce any appreciable platelet aggregation when incubated at various concentrations with stirred platelet-rich plasma. In addition, none of the tested compounds were able to cleave the chromogenic substrate S-2238, indicating that these compounds lack thrombin enzymatic activity.[26,27]

The relaxant activities of compounds **1**−**6** were compared with that of the amidated linear pentapeptide SFLLR−NH_2. SFLLR−NH_2 represents the shortest receptor-activating peptide that exhibits a potency greater than that of the originally described receptor-activating tetradecapeptide.[5] The relative potency (R_{EC}) for each compound was determined from the dose−response curves. R_{EC} represents the ratio of each of the concentrations of the six TRAP analogues to the concentration of SFLLR−NH_2 necessary for producing an equal relaxing effect on the isolated aortic rings (R_{EC} = $EC_{TRAP}/EC_{SFLLR-NH2}$). The R_{EC} values for compounds **1**−**6** were 0.19, 0.06, 17.3, 38.2, 13.6, and 44, respectively (Table 2).

As shown in Figure 3, all compounds in group A induced a concentration-dependent relaxation of the aortic rings. In particular, compound **1** produced a concentration-dependent relaxation of the aortic rings precontracted with 1 μM phenylephrine. The maximal relaxing effect (81%) was obtained at a concentration of 100 μM. The relaxing effect of compound **1** was completely abolished by pretreatment of the aortic rings with L-NAME (300 μM). Moreover, in aortic rings without endothelium, compound **1** was without relaxing effect. This proves that the relaxing effects of compound **1** in aortic rings are mediated by nitric oxide release. Compound **2** relaxed the aortic rings precontracted with 1 μM phenylephrine with a maximal efficacy (91.4%) at 100 μM. The relaxing effects of compound **2** were absent in aortic rings pretreated with 300 μM L-NAME or with lacking of endothelium. Compound **3** relaxed the aortic rings precontracted with phenylephrine in a concentration-dependent manner, with a maximal efficacy (37.5%) at 100 μM. The relaxing effects of compound **3** were abolished by removing the endothe-

Scheme 5. (a) Synthesis of *N*-Phenylacetyl-4-aminomethylpiperidine (**7a**) and (b) Synthesis of *N*-Phenylacetyl-3-aminopyrrolidine (**8a**)[a]



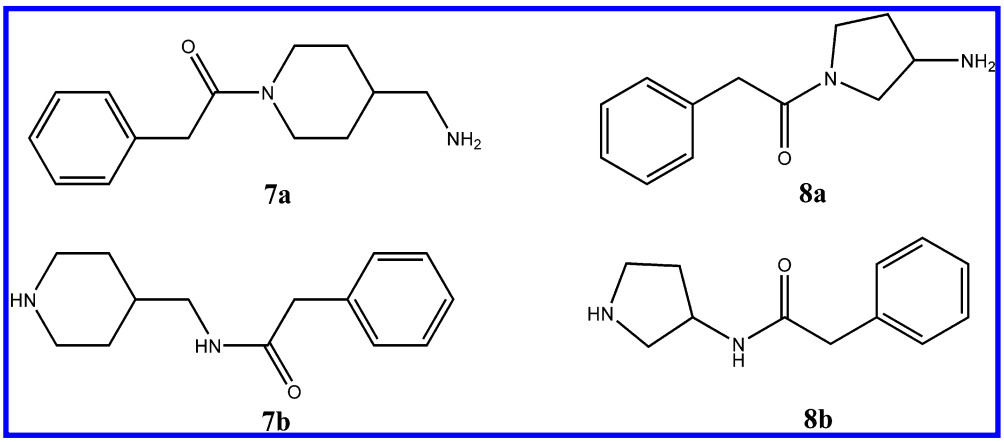
[a] Reagents and conditions: (i) EtOH, room temp, 2 h; (ii) phenylacetic acid, DCC, HOBt, DIEA, CH_2Cl_2; (iii) 2% v/v methanolic hydrazine solution, 30 min.

lium or by pretreatment with 300 μM L-NAME. Compound's **3** efficacy and potency were significantly lower than the ones of compound **1** and **2**, which did not differ structurally significantly from each other.

All compounds in group B were also found to stimulate the endothelial thrombin receptor in a concentration-dependent matter as seen in Figure 3. The biological activity of compound **4** has been previously described.[20] Compound **5** (0.1−100 μM) induced a concentration-dependent relaxation of the aortic rings with endothelium precontracted with 1 μM phenylephrine, by 1.3−42.7%. Pretreatment of these aortic rings with *N*ω-nitro-L-arginine (L-NAME) reduced the maximal relaxing response to 17−18%. In the aortic rings without endothelium, compound **5** (0.1−100 μM) also induced a concentration-dependent relaxation by 2.3−14%. The nitric oxide dependent relaxing effect of compound **5** (0.1−100 μM) was thus found to be from 0% to 25%. Compound **6** (0.1−100 μM) induced a concentration-dependent relaxation of the aortic rings with endothelium precontracted with 1 μM phenylephrine, by 1.8−40.7%. Pretreatment of these aortic rings with L-NAME induced the disappearance of the relaxing response. At concentrations of 10 and 100 μM, compound **6** induced a transient contraction of 2−3% in the aortic rings without endothelium. A comparison of the dose−response curves of the non-peptide TRAPs, performed by two-way repeated measures of ANOVA analysis, revealed that compounds from group A had statistically significant higher effects than compounds from group B.

Molecular Modeling. In a previous publication[17] the molecular modeling of SFLLR was built on the basis of NMR data. These data revealed that SFLLR probably

Scheme 6. Regioisomers of Compounds **7a**,**b** and **8a**,**b**



favors cyclic conformation characterized by reduced mobility of the Arg and Phe side chain residues, which have been shown to be essential for the biological activity of the pentapeptide. On the basis of the synthesis of novel potent cyclic analogues and the fact that the amide SFLLR−NH_2 with the higher biological potency assumes a predominately cyclic conformation, we have suggested that probably cyclic conformation of SFLLR represents its bioactive conformation. To further analyze the proposed model, we studied the superimposition properties of the synthesized compounds and SFLLR. From the superimposition studies, compound **4** gave the best results. Therefore, we will focus on this structure because this was also of the most biological significance. The starting structure of compound **4** was built to be extended with the phenyl and guanidino groups positioned far away. This structure was minimized using a combination of steepest descent and Newton−Ramphson algorithms. The alkyl chain was turned in a way such that the phenyl ring established a spatial vicinity with the guanidino group. To this turn the presence of the carbonyl group was mainly contributed, which favors electrostatic interaction with the positively charged guanidino one. To further explore the conformational space that compound **4** can adopt, a short molecular dynamics experiment was performed at the high temperature of 1000 K to allow high flexibility of the alkyl chain and thus to adopt a great variety of possible conformers.

The simulated high-energy structures were further minimized using $\epsilon = 45$ to simulate a receptor environment and 2000 iterations using the steepest descent algorithm. This intermediate dielectric constant simulates an amphipathic environment which is the most putative one to represent a receptor environment. Three family structures were generated from the 100 minimized structures. The lowest energy structures derived from these family structures differ in energy only by 1−3 kcal/mol. The major characteristic of these structures is the high flexibility of the alkyl chain. Thus, the alkyl chain either can be in an extended form or can turn toward the phenyl ring. By this turning, the ϵ-NH of the guanidino group approaches the carbonyl group and stabilizes the molecule by an electrostatic interaction. The guanidino group also comes in the vicinity of the phenyl ring. The observed conformers in the dynamic experiment with a proximity of the phenyl rings to the guanidino group predominate (constitute 85% of the simulated ones) and seem to represent the bioactive conformer of compound **4**. The final structure was derived by imposing distance constraints between the phenyl and guanidino groups and further subjected to minimization procedures using the steepest descent and Newton−Raphson algorithms. The approach of the guanidino group toward the phenyl ring would be even more stable if the amino group was not positively charged. However, in a receptor environment the amino group may interact with a carbonyl group as is suggested in a recent paper, which deals with potent inhibitors incorporating a phenyl group as a peptide mimetic and aminopyridines as guanidine substitutes.[28,29] Such an interaction will allow the guanidino group to approach even closer the phenyl ring and stabilize interactions of ϵ-NH of the guanidino group to the carbonyl group (Figure 4).

Subsequently, the proposed bioactive conformer among the low-energy structures derived from molecular dynamics experiment was superimposed with SFLLR using rigid-body superimposition. During the superimposition the phenyl and guanidino groups of SFLLR were matched with the corresponding ones of compound **4** (Figure 5), showing rms distances of only 0.15 Å between the matched groups. This indicates that the proposed bioactive conformer of compound **4** can mimic the two requirements demanded by SFLLR to exert its biological activity. Moreover, other molecular characteristics were also matched. The $-NH_2$ group of **4** was in spatial proximity with serine $-NH_2$ and the serine−phenylananine amide bond. The nitrogen of piperazine and the carbonyl groups in the vicinity of $-NH_2$ for compound **4** are matched with the amide bond between phenylananine and leucine. These superimpositions resulted in an increase of rms to 1.5 Å.

Discussion

Thrombin interacts with the cells through a specific G-protein-coupled receptor that has been cloned and sequenced.[5] Receptor activation occurs when thrombin cleaves the N-terminus between Arg_{41} and Ser_{42}, exposing a new amino terminus that functions as a tethered peptide ligand.[1,5] In support of this model, peptides of only five amino acids (SFLLR) have been shown to mimic the effects of thrombin on platelets[13] and smooth muscle,[30] and antibodies directed against this domain have been shown to inhibit the effects of thrombin.[31] Furthermore, it has been recently shown that replace-

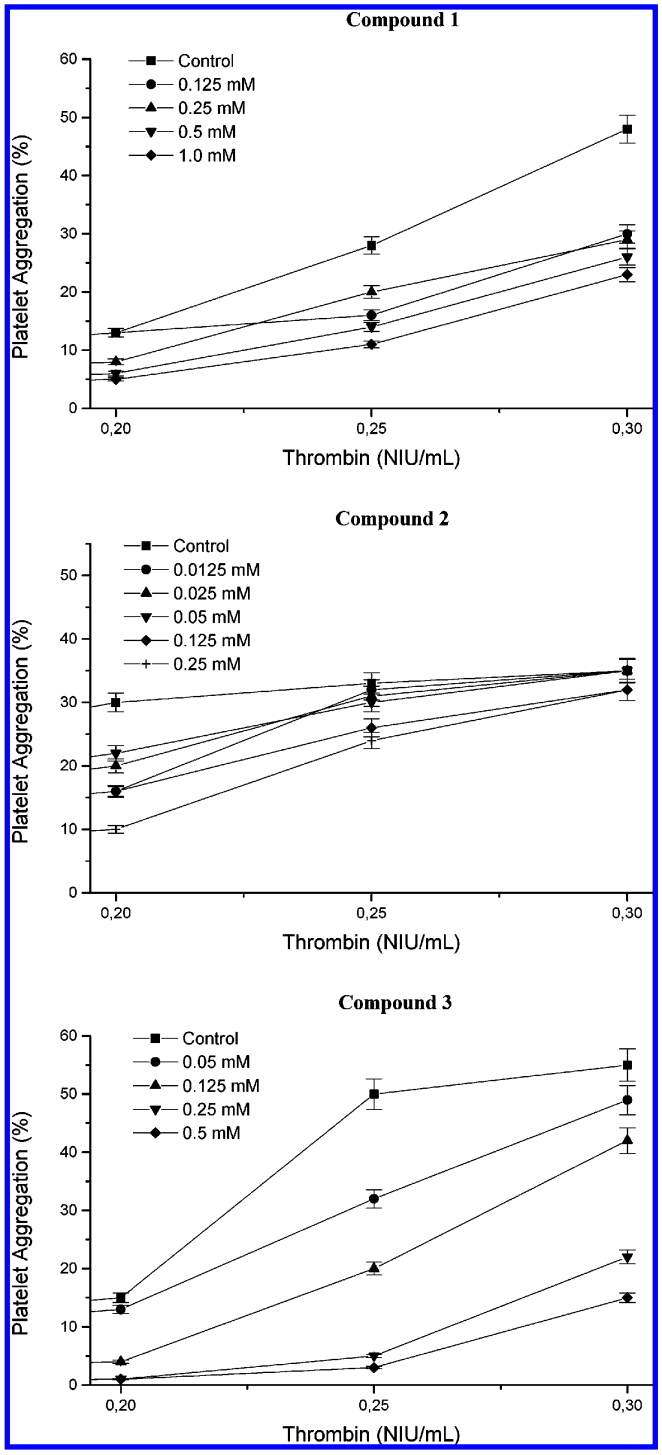


Figure 1. Effect of group A non-peptide thrombin mimetics on inhibition of thrombin-induced platelet aggregation in human PRP after activation with different concentrations of thrombin.

ment of the aliphatic residues of the active peptide SFLLR with small bifunctional rings retains the activity only when the pharmacophoric groups of Phe and Arg remain in place.[20]

In this work, the structural requirements of the native pentapeptide SFLLR have been incorporated onto different molecular scaffolds providing structure rigidity (piperazine), flexibility (4-aminomethylpiperidine and 3-aminopyrolidine), and in a particular spatial order that is in agreement with the previously defined bio-

Table 1. Effect of Thrombin Receptor Non-Peptide Mimetics on Human Platelet Aggregation in Vitro

SFLLR control	IC_{50} (mM) 0.20 IU/mL TRAP	0.25 IU/mL TRAP	0.30 IU/mL TRAP
1	0.46	0.51	0.44
2	0.14	>0.25	>0.25
3	0.11	0.12	0.21
4	0.12	0.09	0.24
5	>1.0	>1.0	0.87
6	0.42	0.34	0.39

active topology. The described compounds have been designed rationally on purpose to mimic the active cyclic conformation adopted by SFLLR,[32,33] because they contain key pharmacophoric groups of Phe, Arg, and the primary NH_2 group. The guanidino group is at the end of the linear lipophilic chain of five methylene groups, since it has been found through SAR that such a long alkyl chain gives the advantage to these molecules to maximize hydrophobic interaction as it happens with the leucine side chain of SFLLR.[23]

SFLLR mimetics were found to be able to prevent thrombin-induced platelet aggregation. All three compounds in group A inhibited thrombin-induced platelet activation when thrombin was used at low concentration (up to 0.2 IU/mL or 2 nM) which requires thrombin's anion-binding exosite and occurs via activation of the thrombin tethered-ligand receptor.[34,35] However, at higher concentrations of thrombin (up to 0.3 IU/mL or 3 nM), where platelet activation was achieved independent of thrombin's exosite and mediated by a moderate affinity (KD = 10 nM) binding site,[34] thrombin could overcome such inhibition for both compounds **1** and **2** but not for compound **3**. Thus, at higher concentrations of thrombin, induced platelet activation was suppressed effectively with compound **3** but not with compounds **1** and **2**.

In group B, platelet activation with low concentrations of thrombin was almost totally prevented with the three SFLLR mimetics of group B at a relatively high concentration (0.5−1 mM) for both compounds **5** and **6** but not for compound **4**. Compound **4** inhibited permanently thrombin-induced platelet activation even at a 4-fold smaller concentration (0.125 mM) compared to the other two SFLLR mimetics **5** and **6**. At even higher concentrations (up to 0.3 IU/mL), thrombin could partly overcome all three SFLLR inhibitory activities when SFLLR mimetics were used at low concentrations. By contrast, higher concentrations of SFLLR mimetics (up to 1 mM for compounds **5** and **6** and 0.5 mM for compound **4**) completely inhibited thrombin-induced platelet activation even when thrombin was used up to 3 nM. Therefore, compounds **5** and **6** have limited efficacy in suppressing a thrombin-stimulated platelet activation when used up to 0.25 mM even though compound **5** up to 0.25 mM enhanced weak platelet activation. Compound **4** at 0.25−0.5 mM caused a complete and permanent blockage of thrombin-induced platelet activation.

SFLLR mimetics were also tested for biological activity in isolated aortic rings with intact endothelium precontracted with phenylephrine and were found capable of producing a dose-dependent relaxation at concentrations as low as 1−10 μM. Of note, nitric oxide

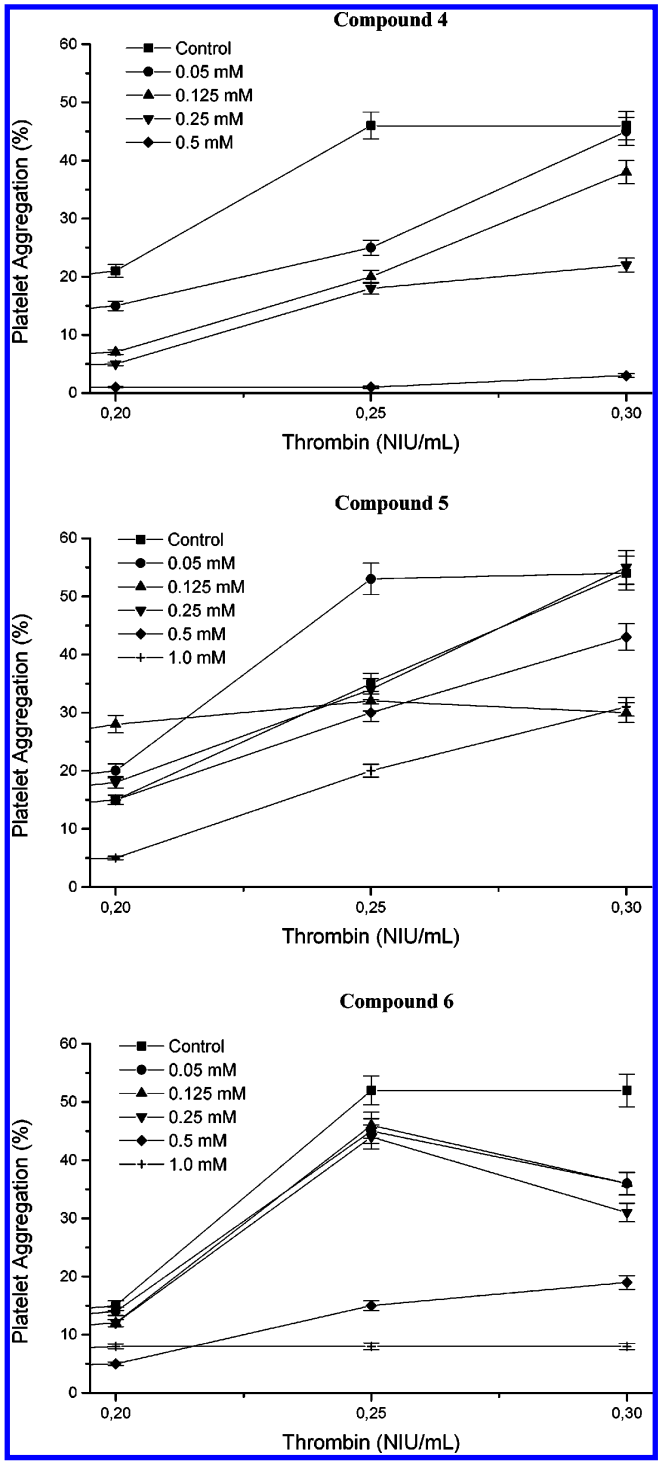


Figure 2. Effect of group B non-peptide thrombin mimetics on inhibition of thrombin-induced platelet aggregation in human PRP after activation with different concentrations of thrombin.

synthase blocker L-NAME inhibited SFLLR-induced aortic relaxation to a different extent. Generally, the magnitude of the relaxation induced by a given SFLLR mimetic was higher in preparations with endothelium than in de-endothelized preparations. The two types of relaxation were very close or equal in the preparations pretreated with L-NAME, and this provides an additional way of separating the endothelium-dependent effects from the endothelium-independent effects for the same SFLLR mimetic.

Table 2. Bioassay Relaxation Results for Non-Peptide TRAPs (Mean ± SE) in Isolated Aortic Rings (Smooth Muscle ED_{50})

compound	relative potency (R_{EC})
SFLLR−NH_2	1
1	0.19
2	0.06
3	17.3
4	38.2
5	13.6
6	24.0

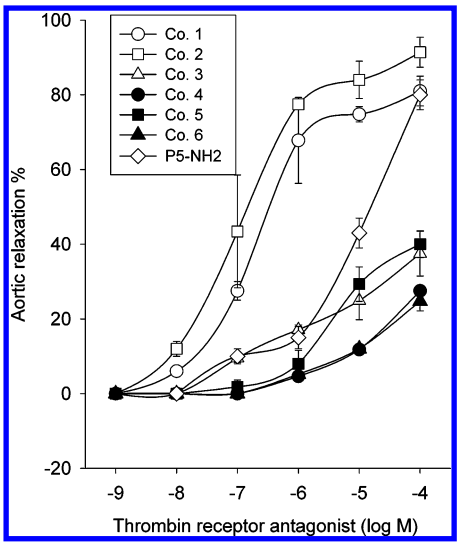


Figure 3. Concentration-dependent relaxing effects of group A (compounds **1**−**3**) and group B (compounds **4**−**6**) SFLLR mimetics and SFLLR−NH_2 on the phenylephrine-induced contraction of aortic rings with intact endothelium. Results are expressed as the mean ± SE ($n = 6$).

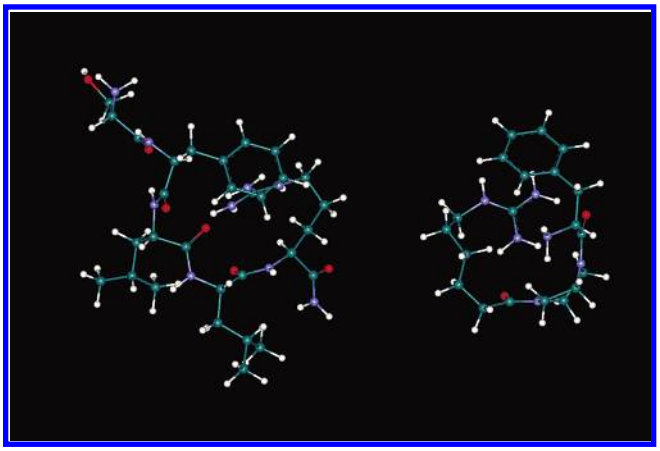


Figure 4. Low-energy conformers of compound **4** derived after short molecular dynamics coupled with minimization algorithms.

The results obtained in the isolated aortic smooth muscle bioassay indicate that the presence of piperidine or piperazine groups in the structure of the thrombin receptor analogues of group A (compounds **2** or **1**, respectively) increases their potency as agonists of the endothelial thrombin receptor up to 0.1−0.5 μM, whereas their substitution with the pyrrolidine group (compound **3**) decreases the agonistic potency by approximately 10 times. The R_{EC} of compounds **1** and **2** were therefore significantly lower compared to that of compound **3**, and the order of potency of compounds from group A was Co**2** > Co**1** > Co**3**. The maximal relaxing effects of

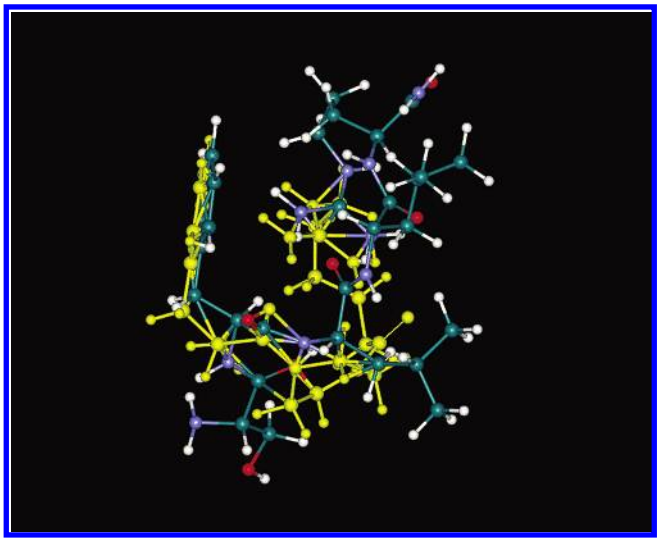


Figure 5. Superimposition of compound **4** with SFLLR. The major characteristic of this superimposition is the excellent matching between the phenyl and their guanidino groups.

compounds **1** and **2** were significantly higher (by 2−3 times) compared to compound **3** but different from each other.

The introduction of an amino group in the structure of the SFLLR mimetics **1**−**3** reduced significantly their potency and efficacy in perfused aortic rings. Thus, the R_{EC} values of compounds **1**−**3** were significantly lower than the ones of compounds **4**−**6**, respectively. The maximal relaxing effects of compounds **4**−**6** were significantly smaller than the ones of compounds **1** and **2** but not from that of compound **3**.

The association between an amino group and a piperazine group in the structure of compound **4** from group B of thrombin analogues resulted in the lowest agonistic potency in the aortic smooth muscle bioassay. The highest potency from compounds in group B was observed for compound **5** containing the piperidine group. The order of potency of thrombin analogues from group B was Co**5** > Co**6** > Co**4**.

By comparison of activities, the order of potency of thrombin receptor analogues as agonists of the endothelial thrombin receptor was the inverse of the order of potency of the same compounds as blockers of the platelet thrombin receptor. Thus, in group A the order of potency of thrombin receptor analogues as agonists of the endothelial receptor was Co**2** > Co**1** > Co**3**, whereas as antagonists in platelet aggregation, the order of potency was inverted: Co**3** > Co**1** > Co**2**. In group B the order of agonistic potency of thrombin analogues as agonists of the endothelial receptor was Co**5** > Co**6** > Co**4**, whereas as antagonists in platelet aggregation, the order of potency was Co**4** > Co**6** > Co**5**. This indicates the existence of a certain degree of functional analogy between the endothelial and platelet thrombin receptor although the present and previous studies indicate that thrombin analogues exhibit a very low activity (if any) as agonists of platelet thrombin receptors.

Compounds **4** and **3** with three (Phe/Arg/NH_2) and two (Phe/Arg) pharmacophoric groups, respectively, which show the highest inhibitory effects in platelet aggregation while preserving agonist activity in the rat aorta assay, are the most promising for further development. Compound **6** with a reasonably strong inhibitory effect in platelet aggregation and simultaneous moderate relaxant activity in rat aorta assay is also a suitable candidate for further development.

All compounds were readily soluble in both platelet aggregation and rat aorta relaxation assays. In the platelet aggregation assay the concentrations of the compounds used in the experiments were identified between a range of lower and higher concentrations to evaluate their capacity to inhibit thrombin-induced platelet activation in vitro. These concentrations, however, did not affect platelet activation by other agonists such as collagen, ADP, ristocetin, and arachidonic acid, indicating that the concentrations used act directly and therefore a non-target-related effect is excluded. Additionally, compounds **1** and **4** inhibited platelet activation caused by the amide SFLLR−NH_2, 2.5 μM (IC_{50} = 0.78 and 0.37 mM, respectively), indicative that these compounds act directly toward thrombin receptor PAR-1. Regarding the rat aorta relaxation assay, there are few chances that the response obtained in aortic bioassays at the highest concentration (100 μM) was nonspecific because of the fact that the largest part of the relaxing effects (70−80%) was already obtained at the next lowest concentration (10 μM). On the other hand, it is known that TRAPs are active at high micromolar concentrations.[11] Moreover, the specificity of the effect is proved by the dramatic changes in activity of the compounds induced by slight changes in their structures (presence or absence of the amino group).

Superimposition studies based on NMR/NOE constraints showed that compound **4** mimicked most the cyclic conformation of SFLLR and this may explain its biological activity. A pharmacological profile for drug therapy requires a strong inhibitory effect in platelet aggregation with simultaneous muscle vasodilatation.

Conclusion

This study describes the synthesis and activities for rationally designed thrombin receptor mimetics bearing Phe/Arg and Phe/Arg/NH_2 pharmacophoric groups on three different templates (piperazine, 4-aminomethylpiperidine, and 3-aminopyrrolidine). All of the synthesized compounds were found to be active in the rat aorta relaxation assay, and the platelet aggregation study indicated that those bearing an extra amino group were more active as inhibitors in platelet aggregation. Compound **2** was found to have significant NO relaxing properties, whereas compound **4** was found to be the most powerful inhibitor of thrombin-induced platelet thrombin receptor activation. The use of piperazine as a template in compound **4** appears to offer the molecule more rigidity, with the guanidino, phenyl, and amino groups in the vicinity, required for the interaction with the thrombin receptor. In contrast, the use of 4-aminomethylpiperidine and the 3-aminopyrrolidine as templates in compounds **5** and **6** provides for a flexible conformation that allows inhibition of thrombin receptor activation only at higher concentrations. These findings further support the suggestion that a cluster of two groups (phenyl, guanidine) together with an adjacent primary amino group is important for the expression of maximum biological activity. Such compounds are po-

tential thrombin receptor antagonists and might be efficacious in drug therapy.

Experimental Section

I. Methods. Chemistry. The ^{1}H NMR spectra were recorded on a Bruker ACF-300 spectrometer operating at 7.05 T. All NMR spectra were obtained in dilute DMSO-d_6, $CDCl_3$, or CD_3OD solutions. All chemical shifts were referenced to tetramethylsilane (TMS). Mass spectra were obtained on FAB instrumentation operating on a AEI M29 mass spectrometer and on a TSQ 7000 spectrometer (ESIMS). The FAB gun was run at 1 mA discharge current at 8 kV. The FAB matrix used was a mixture of dithiothreitol/dithioerythritol (6:1) (Cleland Matrix). The accurate M^+ of the synthesized compounds was obtained on a Kratos Profile HV-3 mass spectrometer operating with a mass range of 0−1200 amu at 4 kV and resolution variable from nominal mass (600) to accurate mass (10 000). The ESIMS spectra were run on a TSQ 7000 spectrometer (electrospray mode) by direct infusion. A solution of the sample (1 μg/1μL) in methanol was introduced in the ESI probe at a flow rate of 5 μL/min with a Harvard syringe. The capillary temperature was at 80 °C, and the sheath gas was at 45 units while the spray needle voltage was +4.5 KV. Most of the compounds involved in the solution synthesis were purified by flash chromatography using Merck silica gel 60 (230−400 mesh). Solid-phase organic synthesis was achieved on the 2-chlorotrityl chloride resin using a manually handled reaction vessel (2 cm^2 × 12 cm) equipped with a porous G filter (size 2) and a tap connected with a water aspirator. Preparative HPLC was performed with a Waters system equipped with a 600E system controller using a Nucleosil C-18 reversed-phase semi-preparative column (250 mm × 10 mm) with 7 μm packing material. Solvents used for HPLC elution were (A) water containing 0.1% TFA and (B) acetonitrile containing 0.1% TFA. Separations were achieved with a stepped linear gradient of solvent A going to 50% solvent A in solvent B over 60 min at a flow rate of 3 mL/min. The crude product (20 mg) was dissolved in methanol (450 μL), and this solution was injected using a Waters U6K injector with a 2.0 mL sample loop. Fractions were manually collected at 0.5 min intervals; the elution time of the major product was 8−10 min. Eluted product was determined simultaneously from the absorbances at 254 and 230 nm (Waters 996 photodiole array detector). Fractions containing the major product peak were pooled, and the acetonitrile was removed using a rotary evaporator. After lyophilization, the product was stored at −20 °C.

The purity of each intermediate and final product was assessed by analytical HPLC returns (Nucleosil C-18, 250 mm × 4 mm) and thin layer chromatography (TLC). Analytical HPLC was done by the procedure described above. TLC was performed using Merck precoated silica gel 60 F-254 glass plates in an *n*-butanol/acetic acid/water (4:1:1) (BAW) solvent system. Visualization was accomplished by using a combination of UV lamp and ninhydrin spray. All synthetic reagents were purchased from the Aldrich-Sigma Chemical Company and were used without purification. 2-Chlorotrityl chloride resin was supplied by CBL Patras. Solvents were analytical reagent grade or higher purity and were used as supplied. A Buchi RE 111 rotavapor was utilized for the removal of the solvents in vacuo. Melting point determinations were performed on a Buchi 530 melting point apparatus.

a. Synthesis of *N*-(Phenylacetyl)-4-(6-guanidohexanoylamidomethyl)piperidine (2). a.1. *N*-Phenylacetyl-4-aminomethylpiperidine (7). Phenylacetic acid (950 mg, 6.7 mmol) and HOBt (920 mg, 6.8 mmol) were suspended in $CHCl_3$ (20 mL). DCC (1.4 g, 6.8 mmol) was then added followed by DMF (2 mL). The mixture was stirred for ∼30 min at room temperature to give a white suspension. The mixture was transferred slowly to a cold solution of 4-aminomethylpiperidine (1.47 g, 13 mmol) in 30 mL of $CHCl_3$. The reaction mixture was stirred for 3 h at room temperature, the white suspension (DCU) was filtered, and the filtrate was acidified with 2 M HCl (3 × 30 mL). The HCl extracts were basified with 2 M NaOH, extracted with $CHCl_3$ (4 × 50 mL), dried over anhydrous Na_2SO_4, and reduced in vacuo to give 1.4 g (6.0 mmol, 90%) of a white waxy solid product: R_f = 0.35 (4:1:1, ButOH/H_2O/AcOH); ^{1}H NMR (CD_3OD) δ 0.95 (dm, 2H), 1.61 (m, 1H), 1.72 (dd, 2H), 2.48 (d, 2H), 2.82 (dm, 2H), 3.78 (s, 2H), 4.29 (dd, 2H), 7.21−7.33 (m, 5H); MS (nominal) *m/z* 204 (M^+), 91 ($C_6H_5CH_2^+$); MS (accurate) *m/z* M^+ calculated 204.1263, M^+ found 204.1260.

a.2. *N*-(Phenylacetyl)-4-[(6-(*tert*-butoxycarbonylamino)hexanoylamidomethyl)]piperidine. *N*-Phenylacetyl-4-aminomethylpiperidine (460 mg, 2.0 mmol), Boc-ϵ-Ahx-OH (486 mg, 2.1 mmol), HOBt (310 mg, 2.3 mmol), and DIEA (1 mL) were dissolved in 15 mL of CH_2Cl_2. The mixture was cooled to 0 °C, and DCC (475 mg, 2.3 mmol) was added. The reaction mixture was monitored by TLC (5% MeOH/$CHCl_3$) and showed completion after 12 h. The supernatant liquid was filtered in vacuo, dried over anhydrous Na_2SO_4, and concentrated in vacuo to give an orange-yellow solid as a crude product. Flash chromatography (2% MeOH/$CHCl_3$) yielded a waxy white solid (535 mg, 1.2 mmol, 60%): R_f = 0.74 (15% MeOH/$CHCl_3$); ^{1}H NMR (DMSO-d_6) δ 0.9 (m, 2H), 1.2 (m, 2H), 1.3 (s, 9H), 1.3−1.5 (m, 4H), 1.5 (m, 2H), 1.6 (m, 3H), 2.0 (t, 2H), 2.7 (dt, 2H), 2.9 (m, 2H), 3.7 (s, 2H), 4.2 (dd, 2H), 6.7 (brs, 1H), 7.2−7.3 (m, 5H), 7.7 (brs, 1H); MS (nominal) *m/z* 446 (M^+).

a.3. *N*-(Phenylacetyl)-4-(6-aminohexanoylamidomethyl)piperidine Trifluoroacetic Salt. The above Boc derivative (1.0 mmol) was dissolved in 3 mL of a 30% TFA/CH_2Cl_2 solution, and the mixture was stirred for 1 h. The solution was evaporated to dryness in vacuo and the residue was triturated with dry ether, giving a colorless oil (0.97 mmol, 97%): R_f = 0.09 (15% MeOH/$CHCl_3$); ^{1}H NMR (DMSO-d_6) δ 0.9 (m, 2H), 1.2 (m, 2H), 1.4−1.6 (m, 4H), 1.6 (m, 3H), 2.0 (t, 2H), 2.7 (dt, 2H), 2.8 (m, 2H), 2.9 (m, 2H), 3.7 (s, 2H), 4.2 (dd, 2H), 7.2−7.3 (m, 5H), 7.7 (brs, 3H), 7.8 (t, 1H); MS (nominal) *m/z* 346 (M^+ − tfa).

a.4. *N*-(Phenylacetyl)-4-(6-guanidohexanoylamidomethyl)piperidine (2). TFA-salt of *N*-(phenylocetyl)-4-(6-aminohexanoylamidomethyl)piperidine (260 mg, 0.64 mmol), 1*H*-pyrazole-1-carboxamide hydrochloride (130 mg, 0.9 mmol), and DIEA (0.5 mL) were dissolved in 1.5 mL of DMF. The mixture was stirred under nitrogen for ∼24 h at room temperature. Then, dry ether (15 mL) was added and the product appeared as a gel at the bottom of the flask. The supernatant liquid was decanted. The crude product was purified by recrystallization from MeOH/acetone/Et_2O to give 174 mg (0.45 mmol, 70%) of a white solid: R_f = 0.13 (15% MeOH/$CHCl_3$); ^{1}H NMR (DMSO-d_6) δ 0.9 (m, 2H), 1.3 (m, 2H), 1.5 (m, 4H), 1.6 (m, 3H), 2.1 (t, 2H), 2.7 (dt, 2H), 2.9 (m, 2H), 3.1 (m, 2H), 3.7 (s, 2H), 4.2 (dd, 2H), 6.9−7.5 (brs, 3H), 7.2−7.3 (m, 5H), 7.8 (brs, 1H), 7.9 (t, 1H); MS (nominal) *m/z* 387 (M^+)

b. Synthesis of *N*-(Phenylacetyl)-3-(6-guanidohexanoylamido)pyrrolidine (3). b.1. *N*-Phenylacetyl-3-aminopyrrolidine (8). Compound **8** was synthesized using the same procedure that was followed for the synthesis of compound **7**, and it was obtained in 87% yield as a white waxy solid: R_f = 0.37 (4:1:1, ButOH/H_2O/AcOH); ^{1}H NMR (CD_3OD) δ 1.96−2.17 (m, 1H), 2.27−2.46 (m, 1H), 3.32 (brs, 2H), 3.54−3.94 (m, 5H), 3.74 (s, 2H), 7.24−7.35 (m, 5H); MS (nominal) *m/z* 204 (M^+), 91 ($C_6H_5CH_2^+$); MS (accurate) *m/z* M^+ calcd 204.1263, M^+ found 204.1260.

b.2. *N*-(Phenylacetyl)-3-[(6-(*tert*-butoxycarbonylamino)hexanoylamido)]pyrrolidine. The same procedure described above for the synthesis of the relative piperidine analogue was followed. The product was obtained pure in 65% yield as a waxy white solid after flash chromatography (2% MeOH/$CHCl_3$): R_f = 0.66 (10% MeOH/$CHCl_3$); ^{1}H NMR (DMSO-d_6) δ 1.2 (m, 2H), 1.3 (s, 9H), 1.3 (m, 2H), 1.5 (m, 2H), 1.6−2.1 (m, 2H), 2.0 (t, 2H), 2.9 (m, 2H), 3.1−3.7 (m, 4H), 3.7 (s, 2H), 4.1−4.3 (m, 1H), 6.7 (brs, 1H), 7.2−7.3 (m, 5H), 8.0 (t, 1H); MS (nominal) *m/z* 417 (M^+), 344 [($M^+ + 1$) − $(CH_3)_3CO^•$)].

b.3. *N*-(Phenylacetyl)-3-(6-aminohexanoylamido)pyrrolidine Trifluoroacetic Salt. After deprotection of the above Boc derivative with 30% TFA/CH_2Cl_2 solution, a color-

less oil was obtained in 95% yield: $R_f = 0.38$ (4:1:1, ButOH/H_2O/AcOH); ^1H NMR ($CDCl_3$) δ 1.2 (m, 2H), 1.3−1.6 (m, 4H), 1.7−2.1 (m, 2H), 2.0 (t, 2H), 2.8 (m, 2H), 3.2−3.6 (m, 4H), 3.6 (s, 2H), 4.1−4.3 (m, 1H), 7.2−7.3 (m, 5H), 7.7 (brs, 3H), 8.0 (t, 1H); MS (nominal) *m/z* 317 (M^+ − tfa).

b.4. *N*-(Phenylacetyl)-3-(6-guanidohexanoylamido)pyrrolidine (3). The compound was prepared with the same guanylation procedure that was followed for the synthesis of compound **2**. The crude product was purified by recrystallization from MeOH/acetone/Et_2O to give a white solid in 79% yield: $R_f = 0.42$ (4:1:1, ButOH/H_2O/AcOH); ^1H NMR (DMSO-d_6) δ 1.2 (m, 2H), 1.4−1.5 (m, 4H), 1.6−2.1 (m, 2H), 2.1 (t, 2H), 3.1 (m, 2H), 3.1−3.7 (m, 4H), 3.6 (s, 2H), 4.1−4.3 (m, 1H), 6.9−7.4 (brs, 3H), 7.2−7.3 (m, 5H), 7.7 (brt, 1H), 8.1 (t, 1H); MS (nominal) *m/z* 360 ($M^+ + 1$).

c. Solid-Phase Organic Synthesis. c.1. Preparation of HO-Phe-2-chlorotrityl Resin. Phenylalanine (375 mg, 2.27 mmol) and $(CH_3)_3SiCl$ (0.86 mL, 2.50 mmol) were dissolved in CH_2Cl_2 (8 mL), and the mixture was stirred at room temperature for 30 min. TEA (0.72 mL, 5.20 mmol) was then added dropwise to the solution, and the mixture was refluxed for 20 min in a water bath. After the mixture was cooled, 2-chlorotrityl chloride resin (1.0 g, 2.27 mequiv of Cl^-/g resin) and CH_2Cl_2 (7 mL) were added and refluxing of the mixture was followed for 1 h. The reaction was continued under stirring for another 24 h at room temperature. The HO-Phe resin was filtered, subsequently was washed with CH_2Cl_2 (5 × 10 mL), CH_2Cl_2/CH_3OH/DIEA (85:10:5) (3 × 10 mL), CH_2Cl_2 (2 × 10 mL), DMF (2 × 10 mL), DMF/H_2O (2 × 10 mL), H_2O (2 × 10 mL), DMF (2 × 10 mL), *i*-PrOH (3 × 10 mL), and *n*-hexane (5 × 10 mL) and then dried in vacuo for 24 h at room temperature.

c.2. Preparation of BtO-Phe-2-chlorotrityl Resin. HO-Phe resin (1.37 g, 2.27 mmol theoretical) was washed with THF (2 × 10 mL). HOBt (600 mg, 4.50 mmol) and DIC (0.7 mL, 4.50 mmol) were dissolved in the minimum volume of THF, they were all added to the resin, and the mixture stood for 24 h. The BtO-Phe resin was filtered, subsequently washed with DMF (3 × 10 mL), *i*-PrOH (2 × 10 mL), DMF (2 × 10 mL), *i*-PrOH (2 × 10 mL), and *n*-hexane (3 × 10 mL) and then dried in vacuo for 24 h at room temperature.

c.3. Synthesis of 4-Aminomethylpiperidine-Phe-2-chlorotrityl Resin. BtO-Phe resin (500 mg, 0.8 mmol/g) was swelled and saturated with DMF. 4-Aminomethylpiperidine (114 mg, 1 mmol) was dissolved in the minimum volume of DMF, DIEA (0.4 mL) was added to the above resin, and the mixture was shaken for 8 h. The resin was then filtered and subsequently was washed with DMF (3 × 10 mL), *i*-PrOH (2 × 10 mL), DMF (2 × 10 mL), *i*-PrOH (2 × 10 mL), and *n*-hexane (3 × 10 mL) and then was dried in vacuo for 24 h at room temperature.

c.4. Synthesis of 3-Aminopyrrolidine-Phe-2-chlorotrityl Resin. A procedure similar to the one described above was followed except 3-aminopyrrolidine was used instead of 4-aminomethylpiperidine.

c.5. Synthesis of Fmoc-ϵ-Ahx-4-(aminomethyl)piperidine-Phe-2-chlorotrityl Resin. 4-Aminomethylpiperidine-Phe resin (0.4 mmol of compound theoretical) was swelled and saturated with DMF. DIC (0.23 mL, 1.5 mmol) was added to a solution of Fmoc-ϵ-Ahx-OH (350 mg, 1.0 mmol) and HOBt (200 mg, 1.5 mmol) in DMF, and the mixture was poured into the above resin. The reactants were shaken for 24 h, and then the resin was filtered and subsequently was washed with DMF (3 × 10 mL), *i*-PrOH (2 × 10 mL), DMF (2 × 10 mL), *i*-PrOH (2 × 10 mL), and *n*-hexane (3 × 10 mL) and then was dried in vacuo for 24 h at room temperature.

c.6. Synthesis of Fmoc-ϵ-Ahx-3-aminopyrrolidine-Phe-2-chlorotrityl Resin. The procedure described above was followed for the coupling of Fmoc-ϵ-Ahx-OH to 3-aminopyrrolidine-Phe resin.

c.7. (*S*)-*N*-(2-Amino-3-phenylpropionyl)-4-(6-guanidohexanoylamidomethyl)piperidine (5). Fmoc-ϵ-Ahx-4-aminomethylpiperidine-Phe resin (0.4 mmol of compound theoretical) was swelled and saturated with DMF and then was treated twice with 20% piperidine in DMF (20 min, 30 min) at room temperature. After removal of excess reagent, the H-ϵ-Ahx-4-aminomethylpiperidine-Phe resin was washed liberally with DMF and treated with 1*H*-pyrazole-1-carboxamide hydrochloride (730 mg, 5 mmol) and DIEA (1 mL) diluted to 1.5 mL with DMF and the reaction was allowed to proceed at 47 °C for 3 h. The resulting guanylated resin was washed with DMF (3 × 10 mL), *i*-PrOH (2 × 10 mL), DMF (2 × 10 mL), *i*-PrOH (2 × 10 mL), and *n*-hexane (3 × 10 mL), was dried in vacuo, and then was treated twice with 10% TFA in CH_2Cl_2 for 15 min. Solvent and excess of TFA were removed under reduced pressure, affording a light-yellow solid that was then triturated with dry ether. The crude product was purified by preparative HPLC as described in the Experimental Section to yield 146 mg (0.35 mmol, 35% overall yield) of a white waxy solid product: $R_f = 0.46$ (4:1:1, ButOH/H_2O/AcOH); ^1H NMR (DMSO-d_6) δ 0.9 (m, 2H), 1.3 (m, 2H), 1.5 (m, 4H), 1.6 (m, 3H), 2.3 (t, 2H), 2.6 (m, 2H), 2.6 (d of t, 2H), 3.0 (m, 2H), 3.1 (m, 2H), 3.8 (m, 1H), 4.1 (d of d, 2H), 6.9−7.4 (br s, 4H), 7.2−7.3 (m, 5H), 7.6 (t, 1H), 8.3 (br s, 3H), 8.4 (t, 1H); MS/FAB 417 [$(M + 1)^+$ − 2TFA], 357 [$(M + 1)^+$ − 2TFA − $H_2NC(NH)NH_2$].

c.8. (*S*)-*N*-(2-Amino-3-phenylpropionyl)-3-(6-guanidohexanoylamido)pyrrolidine (6). The procedure described for the synthesis of **5** was followed for the synthesis of **6**, giving after preparative HPLC purification a white waxy solid (17% overall yield): $R_f = 0.42$ (4:1:1, ButOH/H_2O/AcOH); ^1H NMR (DMSO-d_6) δ 1.3 (m, 2H), 1.4−1.5 (m, 4H), 1.6−2.1 (m, 2H), 2.2 (m, 2H), 2.6 (m, 2H), 3.1 (m, 2H), 3.1−3.7 (m, 4H), 3.8 (m, 1H), 4.1−4.3 (m, 1H), 6.9−7.4 (br s, 4H), 7.3−7.4 (m, 5H), 7.6 (br s, 1H), 8.3 (br s, 3H), 8.6 (m, 1H); MS/FAB *m/z* 389 [$(M + 1)^+$ − 2TFA], 329 [$(M + 1)^+$ − 2TFA − $H_2NC(NH)NH_2$].

d. Synthesis of *N*-Phenylacetyl-4-aminomethylpiperidine (7a). 4-[1-(4,4-Dimethyl-2,6-dioxocyclohexylidene)-ethylamino]methylpiperidine. 4-Aminomethylpiperidine (460 mg, 4.0 mmol) and 2-acetyldimedone (810 mg, 4.5 mmol) were dissolved in absolute ethanol (20 mL), and the solution was stirred for 12 h at room temperature. The solvent was rotary-evaporated, and the yellowish residue was triturated with dry ether. The crude product was purified by recrystallization from MeOH/ether to give a pale-yellow solid (840 mg, 3.0 mmol, 75%): mp 104−107 °C; $R_f = 0.20$ (4:1:1, ButOH/H_2O/AcOH); ^1H NMR ($CDCl_3$) δ 1.04 (s, 6H), 1.24 (m, 2H), 1.76 (m, 1H), 1.79 (d, 2H), 2.36 (s, 2H), 2.39 (s, 2H), 2.56 (s, 3H), 2.62 (t, 2H), 3.13 (d, 2H), 3.28 (t, 2H), 13.6 (s, 1H); MS (nominal) *m/z* 279 ($M^+ + 1$).

d.1. *N*-Phenylacetyl-4-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethylamino]methylpiperidine. The above Dde-protected 4-aminomethylpiperidine (430 mg, 1.55 mmol), phenylacetic acid (211 mg, 1.55 mmol), HOBt (230 mg, 1.70 mmol), and DIEA (0.5 mL) were dissolved in 15 mL of CH_2Cl_2. The mixture was cooled to 0 °C, and DCC (350 mg, 1.70 mmol) was added. The reaction mixture was then stirred at room temperature for 18 h. The suspension (DCU) was filtered and washed with 25 mL of CH_2Cl_2. The filtrate was subsequently concentrated in vacuo to give a waxy yellowish solid as a crude product. Flash chromatography (1.5% MeOH/$CHCl_3$) yielded a waxy white solid (376 mg, 0.95 mmol, 61%): $R_f = 0.76$ (4:1:1, ButOH/H_2O/AcOH); ^1H NMR ($CDCl_3$) δ 1.04 (s, 6H), 1.05 (dm 2H), 1.79 (dd, 2H), 1.83 (m, 1H), 2.35 (s, 2H), 2.40 (s, 2H), 2.54 (s, 2H), 2.77 (dt, 2H), 3.24 (dm, 2H), 3.75 (s, 2H), 4.36 (dd, 2H), 7.24−7.35 (m, 5H), 13.6 (s, 1H); MS (nominal) *m/z* 397 ($M^+ + 1$).

d.2. *N*-Phenylacetyl-4-aminomethylpiperidine (7a). The previous amide (150 mg, 0.38 mmol) was dissolved in 10 mL of 2% v/v methanolic hydrazine solution. The reaction was monitored by TLC (10% MeOH/$CHCl_3$) and showed completion after ∼30 min. The excess of hydrazine was removed by rotary evaporation. The remaining white solid was dissolved in $CHCl_3$ (20 mL) and then washed with 2 M HCl (2 × 15 mL). The HCl extracts were basified with 2 M NaOH to pH 9, extracted with $CHCl_3$ (3 × 20 mL), dried over anhydrous Na_2SO_4, and reduced in vacuo to give 75 mg (3.2 mmol, 85%) of a colorless oil as a crude product. The crude product was purified by preparative HPLC to yield 60 mg (0.26 mmol, 68%) of a waxy white solid:

R_f = 0.35 (4:1:1, ButOH/H_2O/AcOH); ^{1}H NMR (CD_3OD) δ 1.02 (dm, 2H), 1.74 (dd, 2H), 1.86 (m, 1H), 2.78 (d, 2H), 2.85 (dm, 2H), 3.31 (brt, 2H), 3.78 (s, 2H), 4.32 (dd, 2H), 7.21−7.33 (m, 5H); MS (nominal) *m/z* 233 ($M^+ + 1$).

e. Synthesis of *N*-Phenylacetyl-3-aminopyrrolidine (8a). e.1. 3-[1-(4,4-Dimethyl-2,6-dioxocyclohexylidene)-ethylamino]pyrrolidine. 3-Aminopyrrolidine dihydrochloride (636 mg, 4.0 mmol), 2-acetyldimedone (810 mg, 4.5 mmol), and DIEA (1.4 mL) were dissolved in absolute ethanol (20 mL), and the solution was stirred for 12 h at room temperature. The solvent was rotary-evaporated, and the yellowish residue was triturated with dry ether. The crude product was purified by recrystallization from MeOH/ether to give a white solid (870 mg, 3.5 mmol, 87%): mp 206−208 °C; R_f = 0.26 (4:1:1, ButOH/H_2O/AcOH); ^{1}H NMR ($CDCl_3$) δ 1.04 (s, 6H), 2.35 (dm, 2H), 2.37 (s, 4H), 2.61 (s, 3H), 3.43 (dq, 2H), 3.57 (m, 1H), 4.23 (dq, 2H), 13.8 (s, 1H); MS (nominal) *m/z* 251 ($M^+ + 1$).

e.2. *N*-Phenylacetyl-3-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethylamino]pyrrolidine. The above Dde-protected 4-aminopyrrolidine (500 mg, 2.0 mmol), phenylacetic acid (280 mg, 2.05 mmol), HOBt (285 mg, 2.1 mmol), and DIEA (1 mL) were dissolved in 15 mL of CH_2Cl_2. The mixture was cooled to 0 °C, and DCC (435 mg, 2.1 mmol) was added. The reaction mixture was then stirred at room temperature for 18 h. The suspension (DCU) was filtered and washed with 25 mL of CH_2Cl_2. The filtrate was subsequently concentrated in vacuo to give a waxy yellowish solid as a crude product. Flash chromatography (1.5% MeOH/$CHCI_3$) yielded a waxy white solid (650 mg, 1.76 mmol, 88%): R_f = 0.80 (4:1:1, ButOH/H_2O/AcOH); ^{1}H NMR ($CDCl_3$) δ 1.04 (s, 6H), 2.23 (dm, 2H), 2.36 (s, 2H), 2.40 (s, 2H), 2.61 (s, 3H), 3.44−3.91 (m, 5H), 3.69 (s, 2H), 7.25−7.36 (m, 5H), 13.8 (s, 1H); MS (nominal) *m/z* 369 ($M^+ + 1$).

e.3. *N*-Phenylacetyl-3-aminopyrrolidine (8a). The above amide (70 mg, 0.19 mmol) was dissolved in 5 mL of 2% v/v methanolic hydrazine solution. The reaction was monitored by TLC (10% MeOH/$CHCl_3$) and showed completion after ~30 min. The excess of hydrazine was removed by rotary evaporation. The remaining white solid was dissolved in $CHCl_3$ (20 mL) and then washed with 2 M HCl (2 × 15 mL). The HCl extracts were basified with 2 M NaOH to pH 9, extracted with $CHCl_3$ (3 × 20 mL), dried over anhydrous Na_2SO_4, and reduced in vacuo to give 35 mg (3.2 mmol, 90%) of a colorless oil as a crude product. The crude product was purified by preparative HPLC to yield 28 mg (0.14 mmol, 72%) of a waxy white solid: R_f = 0.37 (4:1:1, ButOH/H_2O/AcOH); ^{1}H NMR (CD_3OD) δ 2.07 (m, 1H), 2.37 (m, 1H), 3.22 (m, 2H), 3.54−3.93 (m, 5H), 3.73 (s, 2H), 7.24−7.35 (m, 5H); MS (nominal) *m/z* 205 ($M^+ + 1$).

II. Biological Activity. a. Platelet Aggregation. Venous blood (30 mL) for the preparation of platelet-rich plasma (PRP) was withdrawn between 8 and 11 a.m. from healthy volunteers who had not taken any medication for the previous 2 weeks. Blood samples were subsequently centrifuged at 800 rpm at room temperature for 8 min. The supernatant platelet-rich plasma (PRP) was counted by a Coulter counter (Coulter Corp.) and was diluted with platelet-poor plasma (PPP) to the desired final concentration (2×10^8 platelets/mL).

Platelet aggregation was determined by the optical method in a four-channel platelet aggregometer (PAP-4, Bio/Data). Samples of platelet suspensions (450 μL) under stirring at 1100 rpm were activated at 37 °C with 50 μL of increasing concentrations (0.125−0.3 IU/mL) of bovine thrombin (Chromogenix, Sweden). Higher concentrations of thrombin could not be used because fibrin clot formation interfered with the measurement of aggregation on platelet-rich plasma.[36] On the other hand, less than 0.2 IU/mL thrombin concentration did not constantly cause proper platelet aggregation; therefore, platelet activation was carried out with a thrombin concentration greater than 0.2 IU/mL.

Light transmittance was measured and recorded for 4 min, at which time platelets had reached a maximal response to thrombin. Results were expressed as the percent change in light transmittance, with the light transmittance of plasma alone (PPP) taken as 100% and that of plasma with platelets in suspension (PRP) taken as 0%. To evaluate the compounds under investigation on platelet activation through the thrombin receptor, the PRP samples were incubated under stirring with increasing concentrations (0.05−1 mM) of each compound for at least 4 min prior to the addition of thrombin. Each study was completed within 1 h after blood collection. The response of platelet activation by thrombin varies tremendously between blood samples withdrawn from different blood donors. Each series of experiments was carried out using a different healthy blood donor.

b. Amidolytic Assay. The possibility that these compounds were direct thrombin inhibitors was investigated by an amidolytic assay using the chromogenic substrate S-2238 (D-Phe-Arg-pNA) (Chromogenix, Sweden) for thrombin. In a microelisa system (CERES 900C, Bio-Tec), the light emission was measured at 405 nm, blanking on air. The standard kinetics curve concerning the dissociation of the chromogenic substrate caused by thrombin was obtained. To determine whether the compounds under investigation influence the amidolytic capacity of thrombin, low and high concentrations of these compounds were preincubated with thrombin or the chromogenic substrate and the corresponding curve was compared with the standard one.

c. Rat Aorta Relaxation Assay. The experimental technique was described extensively elsewhere.[37] Briefly, rings of 2 mm width were obtained from the central part of the thoracic aorta of adult male Wistar. Two rings were used in each experiment, one with intact endothelium and the other with the endothelium mechanically removed. Both rings were placed in a horizontal organ chamber (0.5 mL volume) and continuously superfused at a rate of 1 mL/min, with a modified Tyrobe buffer (composition in mM/L: NaCl, 137; KCl, 2.7; $MgCl_2$, 0.69; $NaHCO_3$, 11.9; NaH_2PO_4, 0.4; $CaCl_2$, 1.8; glucose, 10). The preheated (37 °C) perfusate was oxygenated and maintained at pH 7.44 by bubbling with carbogene. Each aortic ring was connected to a Grass FT 03 isometric force transducer and stretched with a basal tension of 2 g. The contractile activity of the aortic rings was recorded with a Grass polygraph, model 79, and with a CODAS DATAQ DI-120 computer-based system (4/s sampling rate). The presence of the endothelium in both aortic rings was checked 1 h after isolation by measuring the relaxing action of 1 μM carbachol upon the contraction induced by 1 μM phenylephrine. The endothelium was considered intact if carbachol induced a relaxation of 90% or higher and was considered absent if no relaxation was observed.

The action of the TRAP mimetic compounds **4**−**6** (0.1−0.5 nM), upon the aortic smooth muscle was studied in preparations precontracted with 1 μM phenylephrine. Thrombin mimetics were applied 30 min after normal perfusate administration. The relaxing action of the two thrombin mimetics was also measured after pretreatment with 300 μM of the competitive inhibitor of nitric oxide synthase, *N*ω-nitro-L-arginine methyl ester (L-NAME).

All results are expressed as the mean ± SEM. The relaxing action of the thrombin mimetics is expressed as a percentage decrease of the contraction induced by 1 μM phenylephrine. The dose−response curves for all compounds were compared using two-way repeated measures of ANOVA, followed by the Student Newman−Keuls test in the case of significant differences ($p < 0.05$). The ED_{50} of the various compounds was calculated graphically from the dose−response curve.

III. Molecular Modeling. Theoretical calculations were performed on a Silicon Graphics computer using QUANTA from Molecular Simulations. The three-dimensional structure of compound **4** was built and was subjected to steepest descent (SD) and Newton−Raphson algorithms using an energy tolerance 0.01 kcal mol^{-1} $Å^{-1}$ in order to reach a local minimum conformer. To the obtained local minimum conformer, the molecular dynamics experiment was performed at 1000 K using 1 ps for heating, equilibration, and stimulation steps using $\epsilon = 45$. One-hundred structures from the simulated ones were minimized using 2000 iteration steps and the SD algorithm. The obtained low-energy structures were divided

into three family structures using the dihedral angle criterion (60°) ($R_{max} = 101.6$, $R_{min} = 0$). The lowest energy conformers from each family were considered as representative conformers. These structures were used for superimposition with the template, the cyclic conformer of SFLLR. The best superimposed of those were further optimized through SD and NR minimization algorithms. The details of the building of the SFLLR structure was given in a previous publication.[17] The superimposition of the four pairs of structures was performed using a rigid-body fit to the target method of molecular similarity software. Rigid-body fitting translates and rotates working structures to minimize the rms of the fit to the target structure.

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Appendix

Abbreviations. Abbreviations used are in accordance with the rules of IUPAC-IUB Commission on Biochemical Nomenclature: *Eur. J. Biochem.* **1984**, 9−37; *J. Biol. Chem.* **1989**, *264*, 663−673. AcOH, acetic acid; TFA, trifluoroacetic acid; TEA, triethylamine; DIEA, diisopropylethylamine; DMF, *N*,*N*′-dimethylformamide; *i*-PrOH, 2-propanol; MeOH, methanol; EtOH, ethanol; Fmoc (9-fluorenylmethoxy)carbonyl; DMSO, dimethyl sulfoxide; DIC, *N*,*N*′,diisopropylcarbodiimide; HOBt, 1-hydroxybenzotriazole; THF, tetrahydrofuran; Et_2O, diethyl ether; Boc, (*tert*-butyloxy)carbonyl; Fmoc, (9-fluorenylmethoxy) carbonyl; Ahx, aminohexanoic acid; DCC, *N*,*N*′-dicyclohexylcarbodiimide; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl; TRAPs, thrombin receptor activating peptides; FAB, fast atom bombardment; L-NAME, *N*ω-nitro-L-arginine methyl ester; ANOVA, analysis of variance; rms, root mean square; PRP, platelet-rich plasma; PPP, platelet-poor plasma.

References

(1) Coughlin, S.; Vu, T. K.; Hung, D.; Wheaton, V. Characterization of a functional thrombin receptor. Issues and opportunities. *J. Clin. Invest*. **1992**, *89*, 351−355.
(2) Seiler, S. Thrombin receptor antagonists. *Semin. Thromb. Hemostasis* **1996**, *22*, 223−232.
(3) Grand, R.; Turnell, A.; Grabham, P. Cellular consequences of thrombin-receptor activation. *Biochem. J*. **1996**, *313*, 353−368.
(4) Schwartz, T. Locating ligand-binding sites in 7TM receptors by protein engineering. *Curr. Opin. Biotechnol*. **1994**, *5*, 434−444.
(5) Vu, T. K.; Hung, D.; Wheaton, V.; Coughlin, S. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **1991**, *64*, 1057−1068.
(6) Coughlin, S. Thrombin receptor structure and function. *Thromb. Haemostasis* **1993**, *70*, 184−187.
(7) Van Obberghen-Schilling, E.; Pouyssegur, J. Signalling pathways of the thrombin receptor. *Thromb. Haemostasis* **1993**, *70*, 163−167.
(8) Brass, L.; Ahuja, M.; Belmonte, E.; Pizarro, S.; Tarver, A.; Hoxie, J. The human platelet thrombin receptor. Turning it on and turning it off. *Ann. N. Y. Acad. Sci*. **1994**, *714*, 1−12.
(9) McNamara, C.; Sarembock, I.; Gimple, L.; Fenton, J.; Coughlin, S.; Owens, G. Thrombin stimulates proliferation of cultured rat aortic smooth muscle cells by a proteolytically activated receptor. *J. Clin. Invest*. **1993**, *91*, 94−98.
(10) (a) Bahou, W.; Coller, B.; Potter, C.; Norton, K.; Kutok, J.; Goligorsky, S. The thrombin receptor extracellular domain contains sites crucial for peptide ligand-induced activation. *J. Clin. Invest*. **1993**, *91*, 1405−1413. (b) Rasmussen, U.; Vouret-Graviari, V.; Jallat, S.; Schlesinger, Y.; Pages, G.; Pavirani, A.; Lecocq, J. P.; Pouyssegur, J.; Van Obberghen-Schilling, E. cDNA cloning and expression of a human α-thrombin receptor coupled to Ca^{+2} mobilization. *FEBS Lett*. **1991**, *288*, 123−128.
(11) Ahn, H.-S.; Chackalamannil, S. Nonpeptide thrombin receptor antagonists. *Drugs Future* **2001**, *26*, 1065−1085.
(12) Scarborough, R.; Naughton, M.; Teng, W.; Hung, D.; Rose, J.; Vu, T. K.; Wheaton, V.; Turck, C.; Coughlin, S. Tethered ligand agonist peptides: Structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. *J. Biol. Chem*. **1992**, *267*, 13146−13149.
(13) Vassallo, R.; Kieber-Emmons, T.; Cichowski, K.; Brass, L. Structure−function relationships in the activation of platelets thrombin receptors by receptor-derived peptides. *J. Biol. Chem*. **1992**, *267*, 6081−6085.
(14) Chao, B.; Kalkunte, S.; Maraganore, J.; Stone, S. Essential groups in synthetic agonist peptides for activation of the platelet thrombin receptor. *Biochemistry* **1992**, *31*, 6175−6178.
(15) Hui, K.; Jakubowski, J.; Wyss, V.; Angleton, E. Minimal sequence requirement of thrombin receptor agonist peptide. *Biochem. Biophys. Res. Commun*. **1992**, *184*, 790−796.
(16) Natarajan, S.; Riexinger, D.; Cambardella, M.; Seiler, S. "Tethered ligand" derived pentapeptide agonists of thrombin receptor: A study of side chain requirements for platelet aggregation. *Int. J. Pept. Protein Res*. **1995**, *45*, 145−151.
(17) Matsoukas, J.; Hollenberg, M.; Mavromoustakos, T.; Panagiotopoulos, D.; Alexopoulos, K.; Yamdagni, R.; Wu, Q.; Moore, G. Conformational analysis of the thrombin receptor agonist peptides SFLLR and SFLLR-NH_2 by NMR: Evidence for cyclic bioactive conformation. *J. Protein Chem*. **1997**, *16*, 113−131.
(18) Coller, B.; Ward, P.; Ceruso, M.; Scudder, L.; Springer, K.; Kutok, J.; Prestwich, G. Thrombin receptor activating peptides; importance of the N-terminal serine and its ionization state as judged by pH dependence, nuclear magnetic resonance spectroscopy and cleavage by aminopeptidase. M. *Biochemistry* **1992**, *31*, 11713−11722.
(19) Adrade-Gordon, P.; Maryanoff, B.; Derian, C.; Zhang, H.-C.; Addo, M.; Darrow, A.; Eckardt, A.; Hoekstra, W.; McComsey, D.; Oksenberg, D.; Reynolds, E.; Santulli, R.; Scarborough, R.; Smith, C.; White, K. Design, synthesis, and biological characterization of a peptide-mimetic antagonist for a tethered-ligand receptor. *Proc. Natl. Acad. Sci. U.S.A*. **1999**, *96*, 12257−12262.
(20) Alexopoulos, K.; Panagiotopoulos, D.; Mavromoustakos, T.; Fatseas, P.; Mihailescu, S.; Paredes-Carbajal, M.; Mascher, D.; Matsoukas, J. Design, synthesis and modeling of novel cyclic thrombin-receptor-derived peptide analogues of the Ser_{42}-Phe-Leu-Leu-Arg_{46} motif sequence with fixed conformations of pharmacophoric groups: Importance of a Phe/Arg/NH_2 cluster for receptor activation and implications in the design of non-peptide thrombin receptor mimetics *J. Med. Chem.* **2001**, *44*, 328−339.
(21) Matsoukas, J.; Panagiotopoulos, D.; Keramida, M.; Mavromoustakos, T.; Yamdagni, R.; Qiao, W.; Moore, G.; Saifeddine, M.; Hollenberg, M. Synthesis and contractile activities of cyclic thrombin receptor-derived peptide analogues with a Phe-Leu-Leu-Arg motif: Importance of the Phe/Arg relative conformation and the primary amino group for activity. *J. Med. Chem*. **1996**, *39*, 3585−3591.
(22) Panagiotopoulos, D.; Matsoukas, J.; Alexopoulos, K.; Zebeki, A.; Mavromoustakos, T.; Saifeddine, M.; Hollenberg, M. Synthesis and activities of cyclic thrombin-receptor-derived peptide analogues of the Ser_{42}-Phe-Leu-Leu-Arg_{46} motif sequence containing D-Phe and/or D-Arg. *LIPS* **1996**, *3*, 233−240.
(23) (a) Matsoukas, J.; Hondrelis, J.; Keramida, M.; Mavromoustakos, T.; Makriyannis, A.; Yamdagni, R.; Wu, Q.; Moore, G. J. Role of the NH_2-terminal domain of angiotensin II (ANG II)and [Sar1] angiotensin II on conformation and activity. *J. Biol. Chem.* **1994**, *269*, 5303−5311. (b) Tselios, T.; Probert, L.; Daliani, I.; Matsoukas, E.; Troganis, A.; Gerothanassis, I.; Mavromoustakos, T.; Moore, G.; Matsoukas, J. Design and synthesis of a potent cyclic analogue of the myelin basic protein epitope MBP72-85: importance of the Ala81 carboxyl group and of a cyclic conformation for induction of experimental allergic encephalomyelitis. *J. Med. Chem*. **1999**, *42*, 1170−1177. (c) Mavromoustakos, T.; Kolocouris, A.; Zervou, M.; Roumelioti, P.; Matsoukas, J.; Weisemann, R. An effort to understand the molecular basis of hypertension through the study of conformational analysis of Losartan and Sarmesin using a combination of nuclear magnetic resonance spectroscopy and theoretical calculations. *J. Med. Chem.* **1999**, *42*, 1714−1722. (d) Tselios, T.; Apostolopoulos, V.; Daliani, I.; Deraos, S.; Grdadolnik, S.; Mavromoustakos, T.; Melachrinou, M.; Thymianou, S.; Probert, L.; Mouzaki, A.; Matsoukas, J. Antagonistic effects of human cyclic MBP_{87-99} altered peptide ligands in experimental allergic encephalomyelitis and human T-cell proliferation. *J. Med. Chem.* **2002,** *45*, 275−283.

(24) Alexopoulos, K.; Fatseas, P.; Melissari, E.; Vlahakos, D.; Smith, J.; Mavromoustakos, T.; Saifeddine, M.; Moore, G.; Hollenberg, M.; Matsoukas, J. Design and synthesis of thrombin receptor-derived non peptide mimetics utilizing a piperazine scaffold. *Bioorg. Med. Chem*. **1999**, *7*, 1033−1041.
(25) (a) Nash, I.; Bycroft, B.; Chan, W. Dde−a selective primary amine protecting group: a facile solid-phase synthetic approach to polyamine conjugates. *Tetrahedron Lett*. **1996**, *37*, 2625−2628. (b) Bycroft, B.; Chan, W.; Chhabra, S.-R.; Hone, N. A novel lysine-protecting procedure for continuous flow solid phase synthesis of branched peptides. *J. Chem. Soc., Chem. Commun.* **1993**, 778−779.
(26) Gallimore, M. J.; Friberger, P. Chromogenic peptide substrate assays and their clinical applications. *Blood Rev*. **1991**, *5*, 117−127.
(27) Hageman, T. C.; Scheraga, H. A. Mechanism of action of thrombin on fibrinogen. *Arch. Biochem. Biophys*. **1974**, *164*, 707−715.
(28) Bone, R.; Tianbao, L.; Carl, R. I.; Soll, R. M.; Spurlino, J. C. Structural analysis of thrombin complexed with potent inhibitors incorporating a phenyl group as a peptide mimetic and aminopyridines as guanidine substitutes. *J. Med. Chem*. **1998**, *41*, 2068−2075.
(29) Mavromoustakos, T.; De-Ping, Y.; Theodoropoulou, E.; Makriyiannis, A. Studies of the conformational properties of the cannabimimetic-aminoalkylindole pravadoline using NMR and molecular modeling. *Eur. J. Med. Chem*. **1995**, *30*, 227−234.
(30) Hollenberg, M.; Laniyonu, A.; Saiffedine, M.; Moore, G. Role of amino- and carboxyl-terminal domains of thrombin receptor-derived polypeptides in biological activity in vascular endothelium and gastric smooth muscle: Evidence for receptor subtypes. *Mol. Pharmacol*. **1993**, *43*, 921−930.
(31) Brass, L.; Vassallo, R.; Belmonte, E.; Ahuija, M.; Cichowski, K.; Hoxie, J. Structure and function of the platelet thrombin receptor. Studies using monoclonal antibodies directed against a defined domain within the receptor N terminus. *J. Biol. Chem*. **1992**, *267*, 13795−13798.
(32) Adang, A.; Hermkens, P.; Linders, J.; Ottenheijm, H.; Staveren, C. Case histories of peptidomimetics: Progression from peptides to drugs. *J. R. Neth. Chem. Soc*. **1994**, *113*, 63−78.
(33) Moore, G.; Smith, J.; Baylis, B.; Matsoukas, J. Design and pharmacology of peptide mimetics. *Adv. Pharmacol.* **1995**, *33*, 91−141.
(34) Seiler, S. M.; Goldenberg, H. J.; Michel, I. M.; Hunt, J. T.; Zavoico, G. B. Multiple pathways of thrombin-induced platelet activation differentiated by desensitization and a thrombin exosite inhibitor. *Biochem. Biophys. Res. Commun*. **1991**, *181*, 636−643.
(35) Seiler, S. M.; Peluso, M.; Michel, I. M.; Goldenberg, H.; Fenton, J. W.; Riexinger, D.; Natarajan, S. Inhibition of thrombin and SFLLR-peptide stimulation of platelet aggregation. Phospholipase A2 and Na^+/H^+ exchange by a thrombin receptor antagonist. *Biochem. Pharmacol.* **1995**, *49*, 519−528.
(36) Jakubowski, J. A.; Maraganore, J. N. Inhibition of coagulation and thrombin-induced platelet activities by a synthetic dodecapeptide modeleted on the carboxy terminus of hirudin. *Blood* **1990**, *75*, 399−406.
(37) Paredes-Carbajal, M. C.; Juarez-Oporeza, M. A.; Ortiz-Mentoza, C. M.; Mascher, D. Effects of acute and chronic estrogenic treatment on vasomotor responses on aortic rings from ovariectomized rats. *Life Sci*. **1995**, *57*, 473−486.

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