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 Nonpeptide Thrombin Receptor Mimetics†

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The novel cyclic analogues cyclo(Phe-Leu-Leu-Arg-ϵLys-Dap) (**1**) and cyclo(D-Phe-Leu-Leu-Arg-ϵLys-Dap) (**2**), which differ only in the absolute conformation of Phe, have been designed and synthesized based upon the minimal peptide sequence Phe-Leu-Leu-Arg which has been found to exhibit biological activity for the thrombin receptor. Compound **1**, in which all amino acids have the L-configuration, exhibited higher activity in the rat aorta relaxation and rat longitudinal muscle bioassays compared to compound **2**, in which the Phe residue is in the D-configuration. This is attributed to the spatial proximity of the Phe and Arg in compound **1** which does not exist in its diastereomeric compound **2**, as is depicted from a combination of NMR studies and computational analysis. Structure−activity studies (SAR) showed that the Phe and Arg side chains along with a primary amino group form an active recognition motif that is augmented by the presence of a second primary amino group in the cyclic peptide. We suggest that a comparable cyclic conformation may be responsible for the interaction of linear TRAPs with the thrombin receptor. The validity of this proposition was tested by the synthesis of four active nonpeptide thrombin receptor mimetics. Substance (*S*)-*N*-(6-guanidohexanoyl)-*N′*-(2-amino-3-phenylpropionyl)piperazine (**3**), in which the pharmacophoric phenyl, guanidino, and amino groups were incorporated onto a piperazine template, was found to be the most active compared to the other synthesized compounds which lack the amino pharmacophoric group.

Introduction

Thrombin, a multifunctional serine protease generated at sites of vascular injury, plays a central role in blood coagulation. Thrombin is also a powerful agonist for a variety of cellular responses.[1,2] Most of these biological activities of thrombin are mediated through its specific G-protein-linked functional receptors, which have been cloned and shown to be present on a variety of cells such as human platelets and endothelial cells[3] and rat vascular smooth muscle cells.[4] According to a novel mechanism of receptor activation, thrombin binds to its receptor's N-terminal extension cleaving the peptide bond between Arg[41] and Ser[42] within the sequence LDPR[41]/S[42]FLLRNPNDKYEPF.[3,5,6] This proteolytic event unmasks a new N-terminal domain that acts as an anchored ligand to stimulate receptor function. The synthetic decatetrapeptide SFLLRNPNDKYEPF which mimics this new N-terminus possesses thrombin mimetic activity in both vascular and nonvascular smooth muscle preparations.[7−9] Based on a gastric smooth muscle contractile bioassay for SFLLRNPNDKYEPF, it has been shown that only the first five amino acids of this decatetrapeptide (i.e. SFLLR) are required to exhibit contractile activity and that the intrinsic activity of the peptide resides in the sequence FLLR.[8−10] Furthermore, structure−activity (SAR) analysis has singled out the key importance of the Phe[43] and Arg[46] residues for the biological activity of the receptor-activating peptides (TRAPs), not only in smooth muscle[9] but also in a human platelet assay.[11,12] The alanine scan experiment indicated that residues other than Phe and Arg were not critical for the biological activity of SFLLR.[9,11]

From previous SAR studies,[9] it was observed that the peptide *N*-propionyl-FLLR was active in the gastric contractile bioassay, indicating that the primary pharmacophores of the TRAPs resided in the sequence FLLR. Further SAR and NMR studies made by us have indicated the importance of the SFLLR cyclic conformation wherein the Phe and Arg residues cluster together.[13] Recent studies have also indicated that a positively charged NH_2-terminus of SFLLR is important for full agonist activity.[14,15] On the basis of the above

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information, we proceeded in the synthesis of diastereomeric cyclic hexapeptides containing the active core tetrapeptide FLLR with Phe in its L- or D-configuration, as well as Lys and diaminopropionic acid. In particular we synthesized, using Fmoc methodology and the acid-sensitive 2-chlorotrityl resin as solid support, the following two cyclic analogues: cyclo(Phe-Leu-Leu-Arg-ϵLys-Dap) (**1**) and cyclo(D-Phe-Leu-Leu-Arg-ϵLys-Dap) (**2**), where Dap = diaminopropionic acid and ϵLys denotes that the ϵ-amino group of Lys was coupled to the α-carboxyl group of Arg. In these analogues two free α-amino groups from Lys and Dap are available, while the key Phe (L or D) and Arg side chains are constrained in the same or opposite sides.

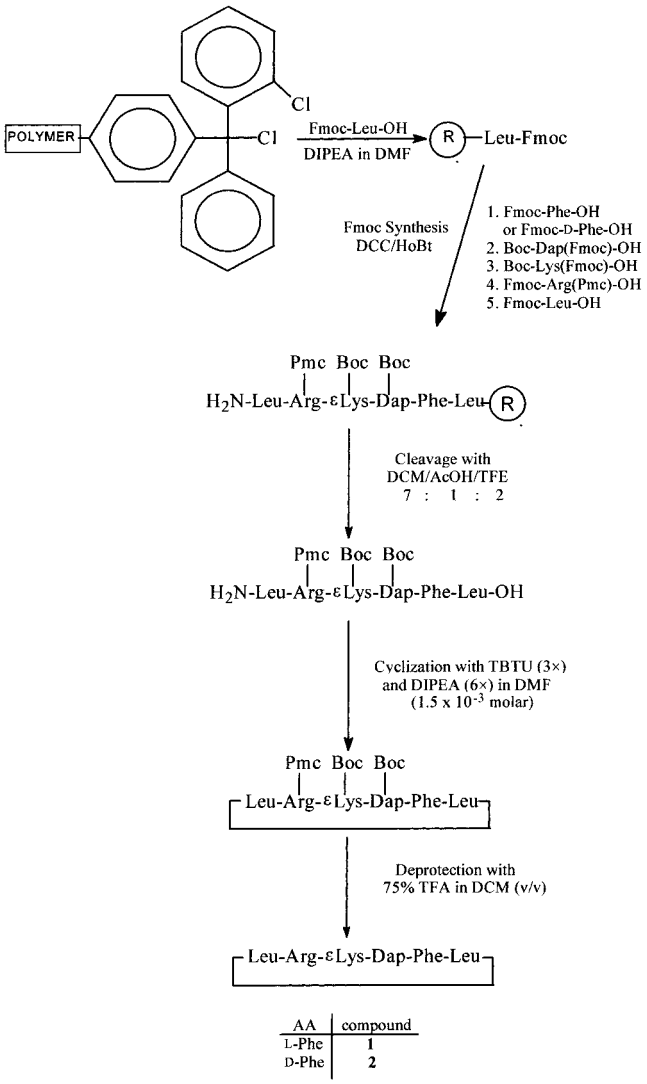
The two cyclic compounds were assayed for their biological activity in the rat aorta relaxation and rat gastric longitudinal smooth muscle (LM) contraction assays,[16] and their activity was compared with the activity of the linear TRAP SFLLR-NH_2. The cyclic compound **1** mimicked the relaxant activity of SFLLR-NH_2 in the rat aorta relaxation assay, and its activity was 100-fold higher compared to the activity of cyclic compound **2** in which the Phe/Arg side chains are constrained in opposite sites. In the rat gastric contractile assay, compound **1** exhibited 8% contraction, while no contractile effects were observed for compound **2**. The comparable activity of cyclic analogue **1** with that of SFLLR-NH_2 and the substantially higher activity compared to cyclic analogue **2** show the importance of a Phe/Arg cluster for receptor activation and of a primary amino group for augmenting activity. The Phe/Arg relative conformation of the two cyclic analogues was also assessed using NMR spectroscopy and molecular dynamics.

To validate the Phe/Arg/NH_2 cluster conformational model, we designed four novel nonpeptide mimetic substances. These substances were also tested in the rat aorta relaxation assay and they were found to stimulate the endothelial thrombin receptor in a concentration-dependent manner, showing a similar pharmacological profile with cyclic compound **1**. (*S*)-*N*-(6-Guanidohexanoyl)-*N′*-(2-amino-3-phenylpropionyl)piperazine (**3**), containing the three pharmacophoric groups, was the most active compared to the other compounds which lack the amino pharmacophoric group. The comparable activity of cyclic TRAP analogue **1** and nonpeptide TRAP analogue **3** confirms our hypothesis that a cluster of phenyl, guanidino, and amino groups is responsible for thrombin receptor triggering and activation.

Results

Chemistry. SFLLR-NH_2 was synthesized and purified using methods which have been detailed previously.[17] The cyclic analogues, based on the FLLR motif (compounds **1**, **2**), were synthesized using the 2-chlorotrityl chloride resin (CLTR)[18] that was used previously for the synthesis of novel cyclic amide-linked analogues of angiotensin II and III,[19] TRAPs,[14,20] and myelin basic protein epitope MBP_{72-85}.[21] For the successful cyclization of the precyclic linear peptides, it was necessary to initiate the synthesis using the Leu[2] residue of the linear Phe[1]-Leu[2]-Leu[3]-Arg[4] motif, as outlined in Scheme 1. If Arg was used for the first step of synthesis, the

Scheme 1. Synthetic Procedure for Cyclic Peptides **1** and **2**



resulting linear-protected peptide failed to cyclize, presumably because of steric hindrance. Using the 2-chlorotrityl resin and mild conditions, it was possible to cleave the peptide−resin bond and subsequently to cyclize the desired protected peptide, which was then deprotected with 75% trifluoroacetic acid in dichloromethane (Scheme 1). For the synthesis of compounds **1** and **2** the δ-amino group of N^{α}-Boc-protected Dap was coupled to the α-COOH group of N^{α}-Boc (N^{ϵ}-Fmoc) of Lys and subsequently the ϵ-amino group of N^{ϵ}-Boc-protected Lys was coupled to the α-COOH group of Arg in the course of the synthesis of H-Leu-Arg(Pmc)-ϵLys-(Boc)-δDap(Boc)-Phe-Leu-OH as outlined in Scheme 1. For cyclization of the protected peptides of the general composition H-Leu-Arg-Lys-Dap-Phe-Leu-OH, it was used either benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate (BOP) or 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and *N*,*N*-diisopropylethylamine (DIPEA) in a solvent of dimethylformamide (DMF). As monitored using a ninhydrin test of the synthesized cyclic peptide, resolved by thin-layer chromatography with a chloroform−methanol (6:1, v/v) solvent system, cyclization was more efficient using TBTU as a coupling reagent compared with BOP. The success of the cycliza-

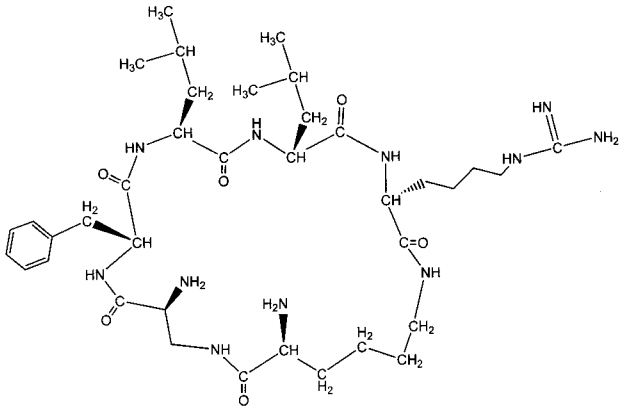
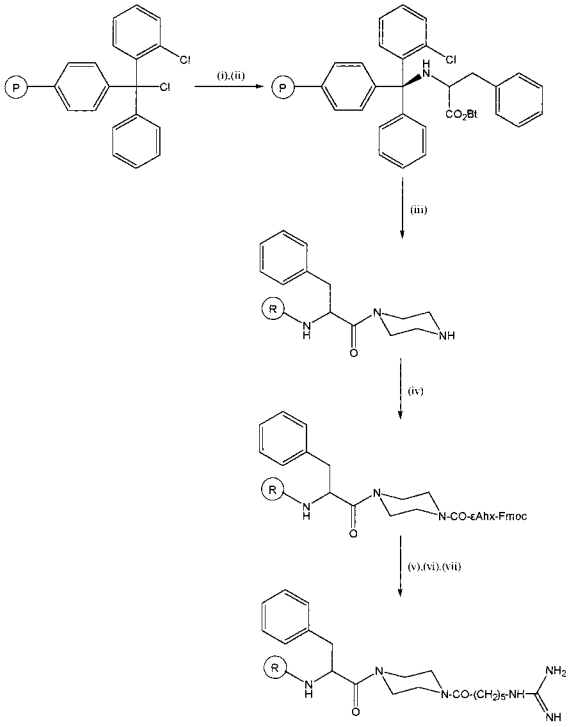


Figure 1. Chemical structure of compound **1** [cyclo(Phe-Leu-Leu-Arg-ϵLys-Dap)].

Scheme 2. Solid-Phase Organic Synthesis of (*S*)-*N*-(6-Guanidohexanoyl)-*N′*-(2-amino-3-phenylpropionyl)piperazine (**3**)[a]



[a] (i) Phe-OH, $(CH_3)_3SiCl$, TEA, CH_2Cl_2; (ii) DIC, HOBt, THF; (iii) piperazine, DIEA, DMF; (iv) Fmoc-ϵAhx-OH, DIC, HOBt, DMF; (v) 20% piperidine in DMF; (vi) 1*H*-pyrazole-1-carboxamide, DIEA, DMF; (vii) 10% TFA/CH_2Cl_2.

tion procedure, as indicated by the lack of a reaction with ninhydrin, was confirmed by fast atom bombardment (FAB) mass spectrometry and NMR spectroscopy. The structures of the two peptides were confirmed by amino acid and FAB analysis (Table 1). The chemical structure of compound **1** is shown in Figure 1.

The synthesis of the nonpeptide thrombin receptor analogue **3** (Scheme 2) was accomplished using also solid-phase methods and the 2-chlotrityl chloride resin as solid support. Attachment of the amino group of Phe (L-Phe) to the resin (2.27 mequiv 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 $(CH_3)_3$-SiCl. The loading of Phe to the resin was certified by the Kaiser ninhydrin test. The Phe attached to the resin

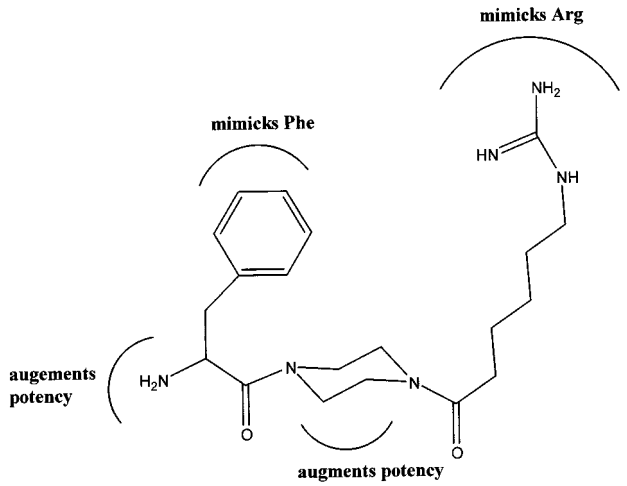


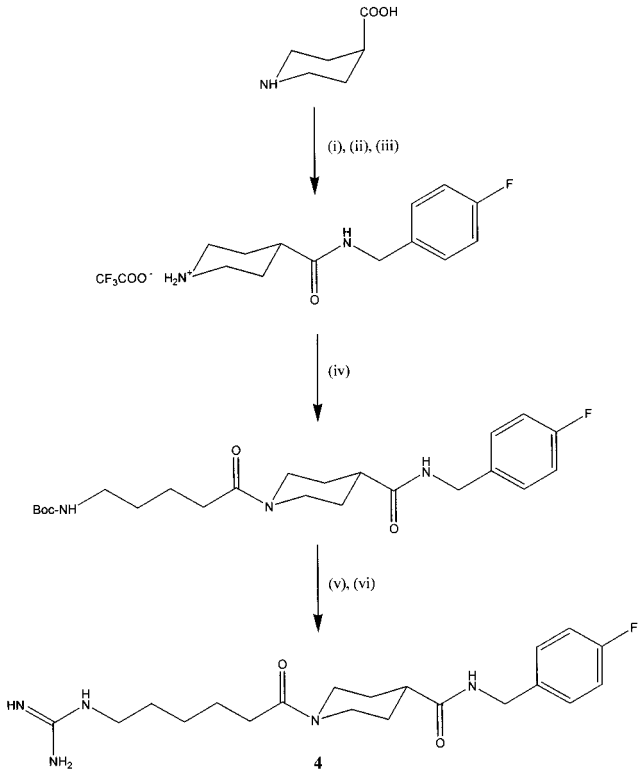
Figure 2. Chemical structure and functionality of pharmacophoric groups of compound **3**.

was first activated with DIC and HOBt; then it was coupled with piperazine in DMF and in the presence of DIEA. Couplings with Fmoc-ϵAhx-OH (2.5 equiv) were aided by the use of DIC and HOBt under basic conditions (DIEA) using minimum of DMF. Fmoc group removals were carried out by treatment with 20% piperidine/DMF for 30 min. The guanidino group was incorporated using 1*H*-pyrazole-1-carboxamide hydrochloride in DMF/DIEA[22] in the last solid-phase chemistry step before cleavage. The resin was cleaved with 10% TFA/CH_2Cl_2 for 15 min at room temperature. The crude product was purified by HPLC. The chemical structure of compound **3** is shown in Figure 2.

The general methodology for the synthesis of *N*-(6-guanidohexanoyl)-4-(4-fluorophenylmethylamidoacetyl)-piperidine (**4**) is depicted in Scheme 3. First the *N*-amino group of isonipecotic acid was Boc-protected, and then the Boc analogue was coupled with 4-fluorobenzylamine using DCC and HOBt as coupling reagents. After Boc deprotection with trifluoroacetic acid, ϵ-aminohexanoic acid (ϵAhx) was incorporated at the free amine salt aided by the use of DCC and HOBt under basic conditions (DIEA). Boc deprotection was then followed, and guanylation of the primary amine using 1*H*-pyrazole-1-carboxamide hydrochloride[22] afforded compound **4**. Most of the synthetic intermediates were purified by flash chromatography (Merck silica gel 60, 230−400 mesh) and the final product by recrystallization (MeOH/acetone/Et_2O). The synthesis of *N*-phenylacetyl-*N′*-(6-guanidohexanoyl)-1,4-diaminobutane (**5**) and *N*-(4-fluorophenyl)acetyl-*N′*-(6-guanidohexanoyl)-1,4-diaminobutane (**6**) was carried out by an analogous procedure as shown in Scheme 4. The chemical structures of compounds **4**−**6** are shown in Figure 3.

Bioassay Data. The relaxant activities of cyclic compounds **1** and **2** and nonpeptide compounds **3**−**6** were compared with that of the amidated linear pentapeptide SFLLR-NH_2 (Figure 4). SFLLR-NH_2 represents the shortest TRAP that exhibits a potency greater than that of the originally described receptor-activating tetradecapeptide.[3,8−10] The relative potency (R_{EC}) for each compound was determined from the dose−response curves.[10] R_{EC} represents the ratio between each of the concentrations of the six TRAP analogues and the concentration of SFLLR-NH_2 necessary for producing

Scheme 3. Synthetic Procedure for *N*-(6-Guanidohexanoyl)-4-(4-fluorophenylmethylamidoacetyl)piperidine (**4**)[a]

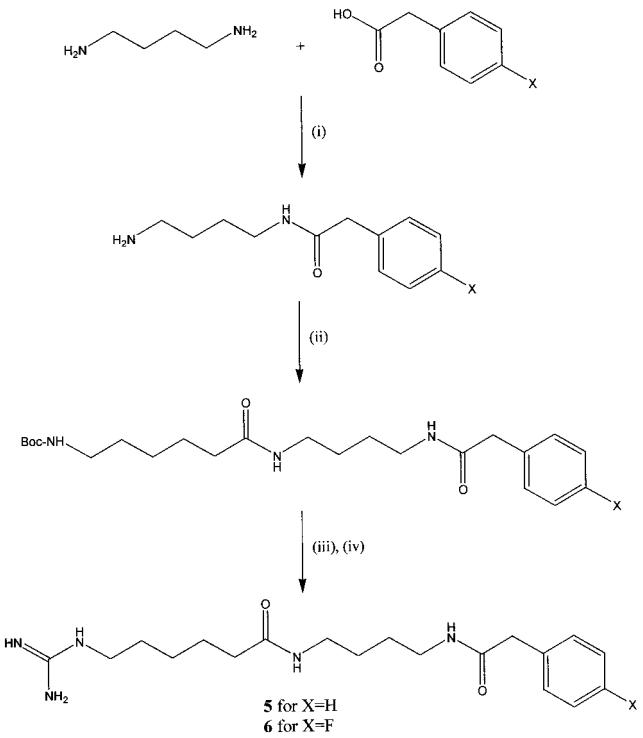


[a] (i) Boc_2O, dioxane, H_2O; (ii) 4-fluorobenzylamine, DCC, HOBt, CH_2Cl_2; (iii) 30% TFA/CH_2Cl_2; (iv) Boc-ϵAhx-OH, DCC, HOBt, DIEA, CH_2Cl_2; (v) 30% TFA/CH_2Cl_2; (vi) 1*H*-pyrazole-1-carboxamide, DIEA, DMF.

an equal relaxing effect on the isolated aortic rings (R_{EC} = $EC_{TRAP} \div EC_{SFLLR-NH2}$). The R_{EC} value of compound **1** was 3.30 ± 0.68 (the compound was 3.3 times less potent than SFLLR-NH_2). In contrast, the R_{EC} value of compound **2** was higher than 500 (the compound was at least 500 times less active than SFLLR-NH_2 and at least 100 times less active than compound **1**). The R_{EC} values of nonpeptide compounds **3**−**6** were 32.8 ± 7.3, 37.3 ± 4.0, 68 ± 6.8, and 50.6 ± 3.1, respectively (Table 2). These R_{EC} values show that compound **3** is the most active nonpeptide analogue. The relative potency of compound **3** is 3 times smaller than that of compound **1** and 32.8 times smaller that that of SFLLR-NH_2.

In particular, cyclo(Phe-Leu-Leu-Arg-ϵLys-Dap) induced a concentration-dependent relaxation of the aortic rings with intact endothelium, precontracted with phenylephrine (1 μM) (Figure 5). This effect became visible at a concentration of 0.1 μM and reached a maximum of 54 ± 2% (n = 6) at a concentration of 0.5 mM. The relaxing effect was abolished by pretreatment of the aortic rings with N^{ω}-nitro-L-arginine methyl ester (L-NAME) (300 μM). Compound **1** also induced contractile effects in the aortic rings without endothelium, at concentrations equal to or higher than 0.1 mM (maximum 8% at 0.05 mM). Cyclo(D-Phe-Leu-Leu-Arg-ϵLys-Dap) exerted reduced relaxation, in a concentration-dependent manner, in which the aortic rings with intact endothelium were precontracted with phenylephrine (1 μM). The lowest concentration of the drug which induced relaxation was 10 μM, while the maximal relaxing effect of 15 ± 3% (n = 6) was observed at 0.1 mM. The

Scheme 4. Synthetic Procedure for *N*-Phenylacetyl-*N*′-(6-guanidohexanoyl)-1,4-diaminobutane (**5**, for X = H) and *N*-(4-Fluorophenyl)acetyl-*N*′-(6-guanidohexanoyl)-1,4-diaminobutane (**6**, for X = F)[a]



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

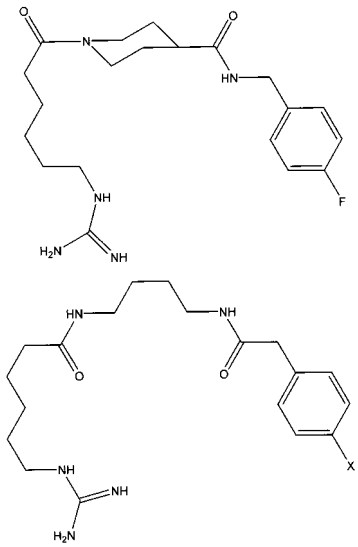


Figure 3. Chemical structures of compounds **4** (top), **5** (bottom; for X = H), and **6** (bottom; for X = F).

appearance of this relaxing effect was prevented by pretreatment with L-NAME (300 μM). No contractile effects of compound **2** were observed in the aortic rings without endothelium, even at higher concentrations of the compound (0.5 mM). The analysis of the dose−response by two-way repeated measure of analysis of variance (ANOVA) followed by the Student Newman-Keuls test revealed that there was a significant difference in the responses induced by the two thrombin mimetics at concentrations of 0.1 mM or higher.

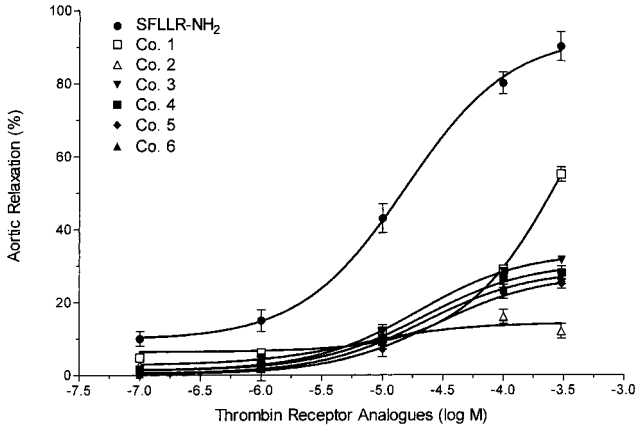


Figure 4. Concentration-dependent relaxing effects of SFLLR-NH_2, cyclo(Phe-Leu-Leu-Arg-ϵLys-Dap) (**1**), cyclo(D-Phe-Leu-Leu-Arg-ϵLys-Dap) (**2**), (*S*)-*N*-(6-guanidohexanoyl)-*N*′-(2-amino-3-phenylpropionyl)piperazine (**3**), *N*-(6-guanidohexanoyl)-4-(4-fluorophenylmethylamidoacetyl)piperidine (**4**), *N*-phenylacetyl-*N*′-(6-guanidohexanoyl)-1,4-diaminobutane (**5**), and *N*-(4-fluorophenyl)acetyl-*N*′-(6-guanidohexanoyl)-1,4-diaminobutane (**6**) upon the phenylephrine-induced contraction of aortic rings with intact endothelium. Results are expressed as means ± SE ($n = 6$).

The above data show that the activity of cyclo(D-Phe-Leu-Leu-Arg-ϵLys-Dap) appeared to be lower compared to that of cyclo(Phe-Leu-Leu-Arg-ϵLys-Dap), indicating that an Arg/Phe side chain cluster is essential for maximum activity. Substances **1** and **2** have respectively the two pharmacophoric groups Phe and Arg on the same or different sides of the cyclic backbone. Furthermore, two adjacent primary amino groups provided by Lys and Dap were introduced in the chemical structure to confirm previous findings suggesting a combined action of Phe/Arg/NH_2 groups for maximum activity. Our work illustrates also that cyclic peptides based on the pentapeptide motif of the active thrombin receptor-derived sequences containing the two pharmacophores (Phe and Arg) and an adjacent free amino group exhibit high contractile and relaxant activity in tissues.

Compound **3** (0.1−300 μM) produced a concentration-dependent relaxation of the aortic rings with endothelium, precontracted with phenylephrine (1 μM), by 1.5−59% ($n = 7$; Figure 4). Pretreatment of the aortic rings with L-NAME (300 μM) reduced the maximum relaxing effect of compound **3** to 26−28%, which represents the value of the nitric oxide-dependent component of the relaxation. Compound **3** (0.1−300 μM) also induced the relaxation of the aortic rings without endothelium precontracted with phenylephrine (1 μM) by 1.2−31%. The relaxing effects of compound **3**, in aortic rings with and without endothelium, were reversible through administration of phenylephrine (1 μM). In aortic rings with endothelium, but not in the ones without endothelium, repeated administrations of compound **3** reduced the amplitude of the phenylephrine-induced contraction by 23−25%. This effect was reverted by administration of L-NAME (300 μM). At concentrations of 10 and 100 μM, compound **3** induced a transient contraction of 2−3% in the aortic rings without endothelium.

The nonpeptide TRAPs **4**−**6** (0.1−300 μM) produced concentration-dependent relaxing effects on aortic rings with endothelium precontracted with phenylephrine (1 μM) (Figure 4). Pretreatment of the aortic rings with L-NAME (300 μM) and experiments with aortic rings without endothelium showed that the relaxing effects produced by these compounds, as the ones of substance **3**, were both endothelium-dependent and endothelium-independent. For all these compounds the endothelium-dependent relaxing effects appeared at a concentration of 0.1 μM and were blocked by L-NAME (300 μM). The dose−response curves for the endothelium-dependent relaxing effects of compounds **3**−**6**, presented in Figure 4, were significantly different when analyzed by two-way ANOVA ($p < 0.01$). The R_{EC} values (Table 2) indicate an order of potency **3** > **4** > **6** > **5**. The endothelium-independent effects appeared at concentrations of 10 μM, had a dose-dependent character, and had values ranging between 1.43−12% for **4**, 0.4−5% for **5**, and 3.83−15% for **6**.

NMR Studies. In the present study 2D-TOCSY and 1D-NOE experiments in DMSO-d_6 for cyclo(Phe-Leu-Leu-Arg-ϵLys-Dap) were conducted in an attempt to relate structure with biological activity. ^{1}H assignment was achieved by combining information from TOCSY spectra and 1D-NOE experiments. The 1D-NOE experiments provided also distance information for the side chain protons of Arg and Phe. For the Phe residue, the C^α proton resonance was readily assigned at $\delta = 4.46$ ppm through intense TOCSY peaks of the C^α proton with the characteristic $C^{\beta\beta}$, protons at $\delta = 3.02$ and 2.82 ppm. For the Arg residue, the NH^α, C^α, $C^{\beta\beta'}$, $C^{\gamma\gamma'}$, $C^{\delta\delta'}$, N^ϵ, and N^ω proton resonances were readily assigned through intraresidue TOCSY cross-peaks, based on the well-established interaction between Arg C^δ and N^ϵ proton resonances at $\delta = 3.05$ and 7.98 ppm, respectively. The Phe/Arg side chain proximity in cyclic peptide **1** was indicated in the 1D-NOE experiments. Saturation of the Phe ring protons at $\delta = 7.28$ ppm resulted in enhancements of the Arg N^ϵ proton at $\delta = 7.98$ ppm (13.4%) and of the Arg N^ϵ protons at $\delta = 6.65$ ppm (17.2%), indicating this proximity. This NOE phenomenon was a reversible one. Thus, saturation of the Arg N^ϵ proton resulted in enhancement of the Phe ring protons at $\delta = 7.28$ ppm (9.2%) confirming the Phe/Arg side chains interaction. Such enhancements were not observed in cyclic peptide **2** upon saturation of Phe ring protons and Arg N^ϵ proton.

Computational Analysis. To extend the observations made using NMR, theoretical calculations were performed. The most striking difference in the biological activity was between cyclic peptides **1** and **2**. As explained above, this can be attributed to the spatial orientation of the phenyl ring of the amino acid Phe. In compound **1** the phenyl ring orients in such a way to spatially approximate the Arg amino acid. This is not observed with compound **2** and the phenyl ring orients away from the Arg amino acid. Therefore, **2** lacks the π interactions between the Arg and Phe rings. This may explain adequately the dramatic loss of biological activity of **2** relative to **1**. The low-energy conformations of the two compounds are shown in Figure 6a. A superimposition of compound **1** with the parent compound SFLLR-NH_2, already discussed in previous papers,[13,14] shows that the two peptides have their phenyl and guanidino groups in spatial proximity (rms = 1 Å) (see

Table 1. Chemical Data for the Synthesized Cyclic TRAPs

peptide	R_f[a] (%)	MW FAB-MS	amino acid analysis Phe	Leu	Arg	Lys
1, cyclo(Phe-Leu-Leu-Arg-ϵLys-Dap)	0.57	587	0.93	2.10	1.00	0.98
2, cyclo(D-Phe-Leu-Leu-Arg-ϵLys-Dap)	0.56	587	0.90	2.12	1.00	0.94

[a] Retention fraction from TLC with BAW.

Table 2. Bioassay Results for Cyclic and Nonpeptide TRAPs (means ± SE)

compd	rel potency (R_{EC})	compd	rel potency (R_{EC})
SFLLR-NH_2	1	**4**	37.3 ± 4.0
1	3.3 ± 0.68	**5**	68 ± 6.8
2	>500	**6**	50.6 ± 3.1
3	32.8 ± 7.3		

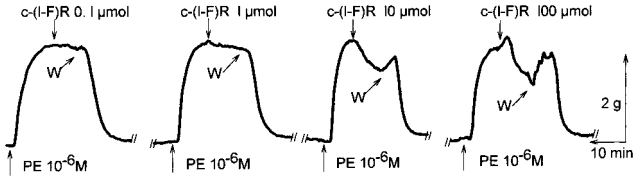


Figure 5. Concentration-dependent effects of cyclo(Phe-Leu-Leu-Arg-ϵLys-Dap) upon isolated aortic rings precontracted with phenylephrine (PE), 1 μM. After TRAP administration, the preparations were washed (W) and left to recover for 30 min.

Figure 6b). However, the cyclic peptide mimics only part of SFLLR-NH_2. This may explain their different activity.

A superimposition between active cyclic peptide **1** and the most active peptide mimetic **3** is shown in Figure 7a. The comparative groups used for superimposition were: (a) phenyl ring of **1** with phenyl ring of **3**; (b) amide bond between Phe-Dap of **1** with amino group of **3**; (c) Arg of **1** with Arg of **3**; (d) two amino groups of Dap in **1** with piperazine of **3**. The results show that the superimposition between peptide **1** and peptidomimetic **3** using the above equivalent groups has an rms of 0.96 Å. This represents a good match and may explain the comparative activity of **3** relative to **1**. The much less activity of **3** prompts us to design and synthesize more novel compounds in the future that would better fit the parent SFLLR-NH_2. This information may be valuable for synthetic chemists who are willing to attempt this design for synthesis of drugs with a better biological profile. A superimposition between peptidomimetics **3** and **4** (Figure 7b) shows a deviation of 0.24 Å between the matched groups. This deviation is reflected in the rms value of 1.34 Å when compound **4** is superimposed with cyclic peptide **1.** Superimposition of peptidomimetics **5** and **6** with cyclic peptide **1** showed rms values of 1.56 Å. The higher activity of **6** relative to **5** may relate to the CH/π interactions of the phenyl ring with thrombin receptor. Compound **6** has the phenyl ring *para* substituted with fluorine, and this is well-known to cause an increase of the CH/π interactions with the receptor.[23]

These superimposition studies suggest that peptide mimetics that show best mimicking of active peptide **1** closely approach its biological activity.

Discussion

Thrombin receptor is activated by a novel mechanism in which thrombin cleaves its receptor's N-terminal extension to reveal a new N-terminus that functions as a tethered peptide ligand.[3] Synthetic TRAPs, comprising only the first five amino acids ($S^{42}FLLR^{46}$) of tethered ligand, have been shown to be sufficient to possess thrombin mimetic activity.[11,12,24] Recent conformational studies have shown that a cyclic conformation for SFLLR in which the Phe and Arg residues cluster together to form a primary pharmacophore motif may be required for the biological activity of the peptide.[13] The above proposed cyclic model was testified since cyclic derivatives of SFLLR were found to be biologically active.[14,20] To further investigate the bioactive conformation of SFLLR, we proceeded in the synthesis of novel cyclic TRAP analogues in which two free ϵ-amino groups from Lys and Dap would be available and the key Phe and Arg residues would be constrained in the same or opposite sides.

In our SAR survey we tested the conformationally restricted analogues cyclo(Phe-Leu-Leu-Arg-ϵLys) (**1**) and cyclo(D-Phe-Leu-Leu-Arg-ϵLys-Dap) (**2**) in which the Phe/Arg side chains are respectively on the same or different sides of the peptide ring in the rat aorta relaxation and rat gastric longitudinal smooth muscle contraction assays. In analogue **1**, in which the two pharmacophoric groups cluster together, the activity is high and comparable to that exerted by linear SFLLR-NH_2. In contrast, in analogue **2**, wherein the two pharmacophoric groups are located at different sites of the peptide ring, the activity is much lower (100 times less) indicating the importance of the Phe/Arg cluster for maximum activity. This activity is augmented by the presence of the two α-amino groups provided by Lys and Dap residues. Indeed, the existence of a primary NH_2 group has been proved critical for receptor activation by TRAPs,[14,15] while an extra α-amino group probably enhances the positive charge of the Phe/Arg/NH_2 cluster, thus increasing the activity.

The bioassays performed in the isolated aortic rings with intact endothelium, precontracted with phenylephrine (1 μM), show that both cyclic TRAPs induce relaxation by releasing nitric oxide (the relaxing effect was blocked by pretreatment of the isolated vessels with nitric oxide synthase inhibitor L-NAME). However, the relaxing effect was significantly lower (100 times less) for cyclic analogue **2**, compared to cyclic analogue **1** at concentrations of 0.1 mM or higher, which indicates a lower capacity of the first compound to stimulate the receptor (lower efficacy or intrinsic activity). Cyclic compound **1** was also found to exhibit contractile activity in aortic rings without endothelium, while compound **2** did not exert such activity. Therefore, these results suggest that the main function of Phe^{43} is the activation of the thrombin receptor, whereas its role in binding to the receptor seems to be less important. A similar conclusion was reached by Scarborough et al.,[24] using a different technical approach (substitution of Phe^{43} with other amino acids).

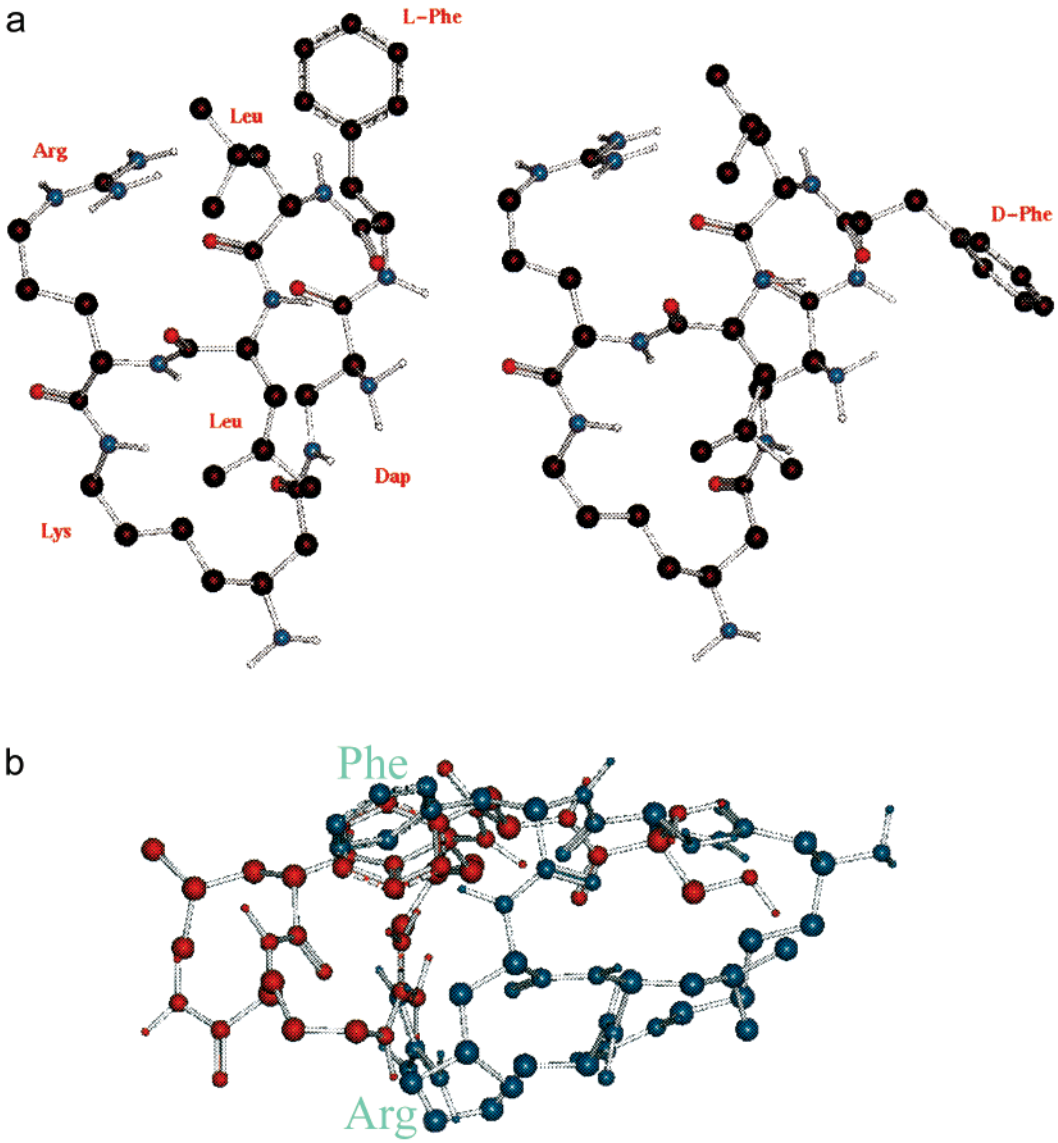


Figure 6. (a) Low-energy conformations of cyclo(Phe-Leu-Leu-Arg-ϵLys-Dap) (left) and cyclo(D-Phe-Leu-Leu-Arg-ϵLys-Dap) (right). (b) Superimposition of cyclic peptide **1** with SFLLR-NH_2.

Relative activities were anticipated considering the molecular models of compounds **1** and **2** shown in Figure 6a. In compound **1** (Figure 6a, left) the conformational model shows that Phe and Arg side chains are in a spatial close proximity. These two key amino acids are protruding in a different phase in relation to the alkyl chains of the two Leu. In the case of compound **2** (Figure 6a, right) the spatial vicinity between Phe and Arg no longer exists. In this conformation, both alkyl chains of Leu and Arg are in the same phase while the phenyl ring of Phe is in an opposite phase. It appears that distinct conformational preferences can exist between the two cyclic peptides.

The molecular dynamics approach, in which the structure was manipulated to fulfill the imposed constraints and NOE data, yielded a model with the Phe and Arg residues on the same side of the cyclic ring of analogue **1** (Figure 6a). In terms of the *cis* orientation of the Phe and Arg side chains, this model is consistent with previous suggestions in which the two pharmacophoric groups are on the same side in TRAPs.[13,14,20] Our findings would suggest further that the active conformation of TRAPs may bring the Arg and Phe residues into closer proximity in a quasicyclic structure. Our data appear to argue against other models that have suggested unstructured or (*S*)-like conformations for the TRAPs.[25−27]

To test the proposed cyclic model, four novel nonpeptide thrombin receptor analogues have been designed rationally[28−32] to mimic the active cyclic conformation adopted by SFLLR. (*S*)-*N*-(6-Guanidohexanoyl)-*N*′-(2-amino-3-phenylpropionyl)piperazine (**3**) is carrying the essential pharmacophoric groups of Phe, Arg, and NH_2 incorporated onto the piperazine template (Figure 2). *N*-(6-Guanidohexanoyl)-4-(4-fluorophenylmethylamidoacetyl)piperidine (**4**), *N*-phenylacetyl-*N*′-(6-guanidohexanoyl)-1,4-diaminobutane (**5**), and *N*-(4-fluorophenyl)-acetyl-*N*′-(6-guanidohexanoyl)-1,4-diaminobutane (**6**) were built onto two different small bifunctional templates carrying the essential pharmacophoric groups of Phe

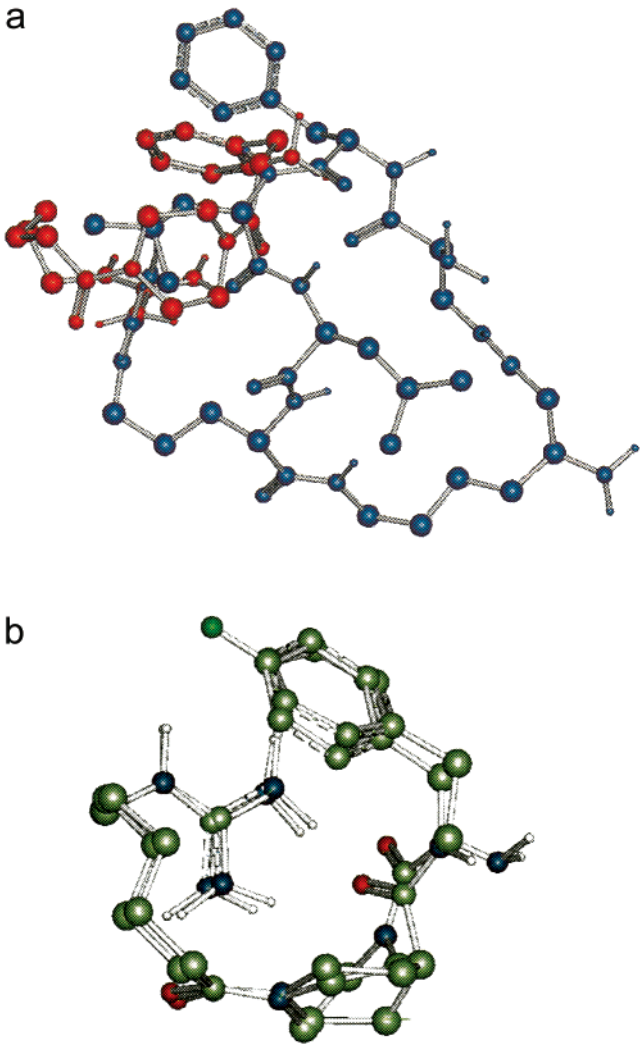


Figure 7. (a) Superimposition of nonpeptide mimetic **3** with cyclic peptide **1**. (b) Superimposition of nonpeptide mimetic **3** with nonpeptide mimetic **4**.

and Arg but missing the NH_2 pharmacophoric group. Compound **4** was built onto a piperidine template (isonipecotic acid) having attached a guanidino linear chain of five methyl groups and a phenyl group having a fluorine atom in the *para* position (Figure 3). Introduction of a fluorine atom in the *para* position of a phenyl group is known to result in an increment of activation of TRAPs.[33] Compounds **5** and **6** were built onto a linear template (1,4-diaminobutane) having attached the pharmacophoric groups of Phe and Arg with the difference in the presence of a fluorine atom in the *para* position of the phenyl group in compound **6** (Figure 3). These nonpeptide TRAP mimetics were also tested for biological activity in perfused aortic rings (Figure 4).

Compounds **3**−**6** relaxed in a concentration-dependent manner the isolated aortic rings with intact endothelium, precontracted with phenylephrine. Generally, the magnitude of the relaxation induced by a given TRAP mimetic was higher in preparations with endothelium than in deendothelized preparations. The two types of relaxation were very close or equal in the preparations pretreated with L-NAME, which comprises an additional way of separating the endothelium-dependent from endothelium-independent effects for the same TRAP mimetic. The relaxing effects of the nonpeptide thrombin receptor mimetics became visible at concentrations as low as 1−10 μM. The ED_{50} values for compounds **3**−**6** were 17.7, 21.7, 27.3, and 22.1 μM, respectively. The ED_{50} values were in accordance with their R_{EC} values (Table 2) showing an order of potency **3** > **4** > **6** > **5**.

Superimposition studies showed that compound **3** mimicked the best cyclic compound **1**, and this explains their comparative activities. The activities of the other synthetic peptide mimetic analogues paralleled the degree of mimicry of cyclic analogue **1**. It appears that the combination of NMR spectroscopy with computational analysis can give a plausible explanation of the observed biological activities for the nonpeptide mimetic analogues.

Compound **1** is still less active than SFLLR-NH_2. While the two models of the two compounds have in close proximity both phenyl and Arg moieties, other structural features are not in spatial vicinity (for example their hydrophobic parts). This observation suggests that a novel cyclic peptide compound that better mimics SFLLR-NH_2 is in need. Such a peptide cyclic compound may generate other classes of peptide mimetics which in turn have better biological activity. The present promising biological activities of the synthetic nonpeptide mimetic analogues make this future effort a reasonable goal.

Conclusion

Two cyclic analogues of the thrombin receptor peptide SFLLR containing Lys, Dap, and L- or D-Phe have been synthesized using TBTU as cyclization reagent, and their potency has been compared with that of linear SFLLR-NH_2 in the rat aortic smooth muscle relaxation and contraction assays. The cyclic analogue Phe-Leu-Leu-Arg-ϵLys-Dap in which the two pharmacophoric groups Phe and Arg are on the same side of the peptide ring showed comparable activity with linear SFLLR-NH_2 and higher activity compared to cyclic analogue D-Phe-Leu-Leu-Arg-ϵLys-Dap in which the two groups are fixed on opposite sides. The key function of Phe[43] is the activation of the thrombin receptor, whereas its role in binding to the receptor seems to be less important. These findings confirm the suggestion that a cluster of the two groups (phenyl, guanidine) together with an adjacent primary amino group is important for expression of maximum biological activity by thrombin receptor-derived peptides. A comparable cyclic conformation may be responsible for the interaction of linear TRAPs with the thrombin receptor.

This model was used as a basis for the design of nonpeptide thrombin receptor mimetics. (*S*)-*N*-(6-Guanidohexanoyl)-*N*′-(2-amino-3-phenylpropionyl)piperazine which contains the structural requirements of SFLLR (Phe/Arg/NH_2) on the molecular template of piperazine was found to be the most active nonpeptide TRAP analogue in the rat aorta relaxation assay, confirming our proposal for the interaction of TRAPs with the thrombin receptor. These findings may open new avenues in the design of potent thrombin receptor mimetics that might be efficacious in drug therapy.

Experimental Section

Abbreviations. Abbreviations used are in accordance with the rules of the IUPAC−IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem.* 1984, *138*, 9−37; *J. Biol.*

Chem. 1989, *264*, 663−673). Abbreviations: AcOH, acetic acid; DCM, dichloromethane; Et_2O, diethyl ether; TFE, 2,2,2-trifluoroethanol; TFA, trifluoroacetic acid; TEA, triethylamine; DMF, *N*′,*N*′-dimethylformamide; *i*-PrOH, 2-propanol; MeOH, methanol; Boc, (*tert*-butyloxy)carbonyl; Fmoc, (9-fluorenylmethoxy)carbonyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; DMSO, dimethyl sulfoxide; Ahx, aminohexanoic acid; DIC, *N*,*N*′-diisopropylcarbodiimide; DCC, *N*,*N*′-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; THF, tetrahydrofuran; LM, longitudinal muscle; TRAPs, thrombin receptor-activating peptides; L-NAME, *N*′-nitro-L-arginine methyl ester; ANOVA, analysis of variance; ®, poly(styrene)-2-chlorotrityl resin.

Solid-Phase Peptide Synthesis. The synthesis of precyclic thrombin receptor-derived peptides H-Leu-Arg-ϵLys-Dap-Phe-Leu-OH and H-Leu-Arg-ϵLys-Dap-D-Phe-Leu-OH was accomplished by the Fmoc methodology utilizing the 2-chlorotrityl chloride resin as solid support as reported by Barlos et al.[18] Attachment of the first N-Fmoc-amino acid (Leu) to the resin (1.4−1.6 mequiv Cl^-/g resin) was achieved by a simple, fast and racemization-free reaction using DIPEA in DMF solution at room temperature. The loading of the first amino acid per gram of substituted resin was calculated by weight and by a quantitative photometric Kaiser test. Solid-phase peptide synthesis was achieved on the 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. 2.5 equiv of Fmoc-protected amino acid was preactivated with DCC in the presence of HOBt at 0 °C in each coupling step. The solution was filtered to remove the remaining DCU and poured into the vessel. The coupling was maintained for 150 min and completion of the coupling was verified by the ninhydrin (Kaizer) test and TLC. A second coupling under the same conditions was employed in cases of incomplete coupling. The Fmoc protective groups were removed by incubation in 20% piperidine in DMF (v/v), for 30 min. The amino acids used in Fmoc synthesis were: Fmoc-Leu-OH, Fmoc-Phe-OH (or Fmoc-D-Phe-OH), N^{α}-Boc-Dap(N^{β}-Fmoc)-OH, N^{α}-Boc-Lys(N^{ϵ}-Fmoc)-OH and N^{α}-Fmoc-Arg($N^{\omega/\omega'}$-Pmc)-OH. After the final coupling−deprotection step, the obtained peptide resin was dried in vacuo and then treated with the splitting mixture DCM/AcOH/TFE (7:1:2, v/v/v; 15 mL/g resin) for 45 min at room temperature to remove the peptide from the resin. The mixture was filtered off and the resin washed with the splitting mixture and DCM several times. The solvent was removed on a rotary evaporator and the obtained oily product precipitated from cold diethyl ether. The protected precyclic peptide was neutralized with TEA in DMF and cyclized using TBTU as cyclization reagent in the presence of DIPEA in DMF solution (10^{-3} M). The cyclization process was easily monitored by the ninhydrin test of the synthesized peptide resolved by TLC. The successfully cyclized products were ninhydrin test negative and final products were identified by FAB-MS and NMR spectroscopy.

Cyclization Procedure. Preparation of Cyclo(Leu-Arg-(Pmc)-ϵLys(Boc)-Dap(Boc)-Phe-Leu) (1a). To a solution of the linear precyclic pentapeptide H-Leu-Arg(Pmc)-ϵLys(Boc)-Dap(Boc)-Phe-Leu-OH (0.3 mmol) in DMF (3 mL) was added TEA (0.3 mmol) and the solution was stirred for 5 min at room temperature. The neutralized linear protected hexapeptide was triturated with H_2O, filtered off, washed with H_2O (2 × 10 mL) and diethyl ether (2 × 10 mL) and dried in vacuo for 16 h. The neutralized linear protected peptide (0.3 mmol) was dissolved in DMF (15 mL) and added dropwise to a solution of TBTU (0.9 mmol) and DIPEA (0.3 mL, 1.8 mmol) in DMF (170 mL) at room temperature, over 45 min. The cyclization was completed in an additional 30 min, as determined by TLC and analytical reversed-phase HPLC. The solvent was removed under reduced pressure affording a light yellow oily residue. The cyclic protected hexapeptide **1a** was precipitated from H_2O and dried in vacuo for 16 h.

Preparation of Cyclo(Leu-Arg-ϵLys-Dap-Phe-Leu) ≡ Cyclo(Phe-Leu-Leu-Arg-ϵLys-Dap) (1). The cyclic protected hexapeptide **1a** was treated with 75% TFA in DCM (1 mL/100 mg peptide) for 4 h at room temperature. The resulting solution was concentrated to a small volume and the final free cyclic peptide was precipitated as a light yellow amorphous solid, upon addition of cold diethyl ether (purity ≥ 79%, as determined by HPLC).

Solid-Phase Organic Synthesis. Preparation of HO-Phe-2-chlorotrityl resin. Phe (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 cooling 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.

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 and they were all added to the resin and the mixture was stand 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.

Synthesis of Piperazine-Phe-2-chlorotrityl resin. BtO-Phe-resin (500 mg, 0.8 mmol/g) was swelled and saturated with DMF. Piperazine (86 mg, 1 mmol), dissolved in the minimum volume of DMF, and DIEA (0.4 mL) were added to the above resin and the mixture was shaken overnight. 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.

Synthesis of Fmoc-ϵAhx-piperazine-Phe-2-chlorotrityl resin. Piperazine-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-ϵAhx-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.

(*S*)-*N*-(6-Guanidohexanoyl)-*N*′-(2-amino-3-phenylpropionyl)piperazine (3). Fmoc-ϵAhx-piperazine-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-ϵAhx-piperazine-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 which was then triturated with dry ether. The crude product was purified by preparative HPLC as described below to yield 33 mg (0.05 mmol, 13% overall yield) of a waxy white solid product: R_f 0.47 (4:1:1, ButOH:H_2O:AcOH); ^{1}H NMR (DMSO-d_6) δ 1.3 (t, 2H), 1.5 (m, 4H), 2.3 (m, 2H), 2.6 (m, 2H), 3.1−3.6 (m, 8H), 3.1 (m, 2H), 3.5 (t, 1H), 6.9−7.4 (brs, 4H), 7.2−7.3 (m, 5H), 7.5 (brs, 1H), 8.3 (brs, 3H); MS/FAB 389 [$(M + 1)^+ - 2$TFA].

Synthesis of *N*-(6-Guanidohexanoyl)-4-(4-fluorophenylmethylamidoacetyl)piperidine (4). *N*-(*tert*-Butoxycarbonyl)-4-piperidinecarboxylic Acid. A solution of isonipecotic acid (2.6 g, 20 mmol) in a mixture of dioxane (40 mL),

water (20 mL) and 1 N NaOH (20 mL) was stirred and cooled in an ice−water bath. Di-*tert*-butyl pyrocarbonate (4.7 g, 21 mmol) was added and stirring was continued at room temperature for 3 h. The solution was concentrated in vacuo to about 10−15 mL, cooled in an ice−water bath, covered with a layer of ethyl acetate (30 mL) and acidified with a dilute solution of HCl to pH 3−4. The aqueous phase was extracted with ethyl acetate (2 × 15 mL) and the extracts were pooled, washed with water (2 × 30 mL), dried over anhydrous Na_2SO_4 and evaporated in vacuo. The crude product was purified by recrystallization from EtOAc/hexane to give 3.15 g (13.8 mmol, 69%) of a white solid: mp 115−116 °C; R_f 0.58 (10% MeOH/$CHCl_3$); ^{1}H NMR (DMSO-d_6) δ 1.4 (s, 9H), 1.7−1.9 (m, 4H), 2.4 (m, 1H), 2.8 (m, 2H), 3.4 (m, 2H); MS (nominal) 229 (M^+), 156 ($M^+ - (CH_3)_3CO^\bullet$); MS (nominal) 229 (M^+).

***N*-(*tert*-Butoxycarbonyl)-4-(4-fluorophenylmethylamidoacetyl)piperidine**. *N*-(*tert*-Butoxycarbonyl)-4-piperidinecarboxylic acid (1.5 g, 6.5 mmol), 4-fluorobenzylamine (810 mg, 6.5 mmol), HOBt (900 mg, 6.7 mmol) and DIEA (2 mL) were dissolved in CH_2Cl_2 (20 mL). The mixture was cooled to 0 °C and DCC (1.4 g, 6.8 mmol) was added. The reaction solution was stirred at this temperature for ∼30 min and then at room temperature for 3 h. The suspension (DCU) was filtered and washed with 50 mL CH_2Cl_2. The filtrate was washed with 8% citric acid (2 × 40 mL), 50% saturated $NaHCO_3$ solution (2 × 40 mL) and H_2O (2 × 40 mL), dried over anhydrous Na_2SO_4 and concentrated in vacuo to give a gray solid as a crude product. Flash chromatography (50% EtOAc/hexane) yielded a white solid (1.55 g, 4.6 mmol, 71%): mp 92−93 °C; R_f 0.80 (10% MeOH/$CHCl_3$); ^{1}H NMR (DMSO-d_6) δ 1.4 (s, 9H), 1.4 (m, 2H), 1.7 (m, 2H), 2.3 (m, 1H), 2.7 (brt, 2H), 3.9 (d, 2H), 4.2 (d, 2H), 7.1−7.3 (m, 4H), 8.3 (t, 1H); MS (nominal) 336 (M^+).

4-(4-Fluorophenylmethylamidoacetyl)piperidine Trifluoroacetic Salt. The above Boc derivative (2.4 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 white solid (2.1 mmol, 89%): mp 120−121 °C; R_f 0.07 (10% MeOH/$CHCl_3$); ^{1}H NMR (DMSO-d_6) δ 1.7−1.9 (m, 4H), 2.4 (m, 1H), 2.9 (dt, 2H), 3.3 (dd, 2H), 4.2 (d, 2H), 7.1−7.3 (m, 4H), 8.4 (t, 1H), 8.6 (brs, 2H); MS (nominal) 236 (M^+ − TFA).

***N*-[6-(*tert*-Butoxycarbonylamino)hexanoyl]-4-(4-fluorophenylmethylamidoacetyl)piperidine.** The above salt (600 mg, 1.9 mmol), Boc-ϵAhx-OH (460 mg, 2.1 mmol), HOBt (290 mg, 2.1 mmol) and DIEA (1.5 mL) were dissolved in 18 mL of a 5:1 CH_2Cl_2/DMF solution. The mixture was cooled to 0 °C and DCC (460 mg, 2.2 mmol) was added. The reaction was monitored by TLC (5% MeOH:$CHCl_3$) at room temperature and showed completion after 24 h. The supernatant liquid was filtered in vacuo, dried over anhydrous Na_2SO_4 and concentrated in vacuo to give a gray solid as a crude product. Flash chromatography (2% MeOH/$CHCI_3$) yielded a white solid (500 mg, 1.1 mmol, 60%): mp 106−109 °C; R_f 0.57 (10% MeOH/$CHCI_3$); ^{1}H NMR (DMSO-d_6) δ 1.2 (m, 2H), 1.3 (s, 9H), 1.3−1.5 (m, 4H), 1.4 (m, 2H), 1.6 (m, 2H), 2.2 (t, 2H), 2.4 (m, 1H), 2.7 (dt, 2H), 2.9 (m, 2H), 4.2 (d, 2H), 4.2 (dd, 2H), 6.7 (brt, 1H), 7.1−7.3 (m, 4H), 8.3 (t, 1H); MS (nominal) 449 (M^+), 376 ($M^+ - (CH_3)_3CO^\bullet$).

***N*-(6-Aminohexanoyl)-4-(4-fluorophenylmethylamidoacetyl)piperidine Trifluoroacetic Salt.** The Boc group removal took place according to general procedure for Boc deprotection with trifluoroacetic acid. A waxy white solid resulted (1.00 mmol, 95%): R_f 0.28 (4:1:1, ButOH:H_2O:AcOH); ^{1}H NMR (DMSO-d_6) δ 1.3−1.6 (m, 6H), 1.4 (m, 2H), 1.7 (m, 2H), 2.2 (t, 2H), 2.4 (m, 1H), 2.7 (dt, 2H), 2.8 (m, 2H), 4.2 (d, 2H), 4.2 (dd, 2H), 7.1−7.3 (m, 4H), 7.7 (brs, 3H), 8.3 (t, 1H); MS (nominal) 340 (M^+ − TFA).

***N*-(6-Guanidohexanoyl)-4-(4-fluorophenylmethylamidoacetyl)piperidine (4).** TFA salt (450 mg, 0.97 mmol), 1*H*-pyrazole-1-carboxamide hydrochloride (220 mg, 1.5 mmol) and DIEA (0.7 mL) were dissolved in 2 mL DMF. The mixture was stirred under nitrogen for ∼24 h at room temperature. Then, dry ether (10 mL) was added and the product appeared as gel at the bottom of the flask, while the supernatant liquid was decanted. The crude product was purified by recrystallization from MeOH/Acetone/Et_2O to give 180 mg (0.46 mmol, 47%) of a waxy white solid: R_f 0.48 (4:1:1, ButOH:H_2O:AcOH); ^{1}H NMR (DMSO-d_6) δ 1.3−1.6 (m, 6H), 1.4 (m, 2H), 1.7 (m, 2H), 2.3 (t, 2H), 2.4 (m, 1H), 2.7 (dt, 2H), 3.1 (m, 2H), 4.2 (d, 2H), 4.2 (dd, 2H), 6.8−7.5 (brs, 3H), 7.1−7.3 (m, 4H), 7.7 (brs, 1H), 8.4 (t, 1H); MS/FAB 392 ($M^+ + 1$).

Synthesis of *N*-Phenylacetyl-*N*′-(6-guanidohexanoyl)-1,4-diaminobutane (5). *N*-Phenylacetyl-1,4-diaminobutane. Phenylacetic acid (820 mg, 6.0 mmol) and HOBt (840 mg, 6.2 mmol) were suspended in $CHCl_3$ (20 mL). DCC (1.28 g, 6.2 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 precold solution of 1,4-diaminobutane (2.64 g, 30 mmol) in 40 mL $CHCl_3$. The reaction was stirred for 3 h at room temperature and then the white suspension (DCU) was filtered and the filtrate acidified with 2 M HCl (2 × 40 mL). The HCl extracts were basified to pH = 9 with 2 M NaOH, extracted with $CHCl_3$ (4 × 30 mL), dried over anhydrous Na_2SO_4 and reduced in vacuo to give a pale orange solid as a crude product. The crude product was purified by recrystallization from EtOAc/hexane to give 660 mg (3.2 mmol, 53%) of a white solid: mp 110−113 °C; R_f 0.45 (4:1:1, ButOH:H_2O:AcOH); ^{1}H NMR (DMSO-d_6) δ 1.4 (m, 4H), 2.5 (t, 2H), 3.0 (q, 2H), 3.4 (s, 2H), 7.1−7.3 (m, 5H), 8.0 (brs, 1H); MS (nominal) 206 (M^+), 177 ($M^+ - H_2NCH^\bullet$), 115 ($M^+ - C_6H_5CH_2^\bullet$).

***N*-Phenylacetyl-*N*′-[6-(*tert*-butoxycarbonylamino)hexanoyl]-1,4-diaminobutane.** *N*-Phenylacetyl-1,4-diaminobutane (600 mg, 2.9 mmol), Boc-ϵAhx-OH (700 mg, 3.0 mmol), HOBt (420 mg, 3.1 mmol) and DIEA (1 mL) were dissolved in 20 mL of a (3:1) CH_2Cl_2/DMF solution. The mixture was cooled to 0 °C and DCC (640 mg, 3.1 mmol) added. The reaction was stirred at this temperature for ∼30 min and then at room temperature for 6 h. The suspension (DCU) was filtered and washed with 25 mL CH_2Cl_2. The filtrate was dried over Na_2SO_4 and concentrated in vacuo to give an orange-yellow solid as a crude product. Flash chromatography (3% MeOH/$CHCI_3$) yielded a white solid (900 mg, 2.1 mmol, 75%): mp 125−126 °C; R_f 0.83 (4:1:1, ButOH:H_2O:AcOH); ^{1}H NMR (DMSO-d_6) δ 1.2 (m, 2H), 1.4 (s, 9H), 1.4 (m, 4H), 1.5 (m, 4H), 2.0 (t, 2H), 2.9 (q, 2H), 3.0 (m, 2H), 3.0 (m, 2H), 3.4 (s, 2H), 6.7 (t, 1H), 7.2−7.3 (m, 5H), 7.7 (t, 1H), 8.0 (t, 1H); MS (nominal) 419 (M^+), 345 ($M^+ - (CH_3)_3CO^\bullet + 1$).

***N*-Phenylacetyl-*N*′-(6-aminohexanoyl)-1,4-diaminobutane Trifluoroacetic Salt**. The above Boc analogue (2.0 mmol) was dissolved in 4 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 white solid (1.8 mmol, 91%): mp 107−109 °C; R_f 0.36 (4:1:1, ButOH:H_2O:AcOH); ^{1}H NMR (DMSO-d_6) δ 1.3 (m, 2H), 1.4 (m, 4H), 1.5 (m, 4H), 2.0 (t, 2H), 2.8 (m, 2H), 3.0 (m, 2H), 3.0 (m, 2H), 3.4 (s, 2H), 7.2−7.3 (m, 5H), 7.7 (t, 1H), 8.0 (t, 1H); MS (nominal) 319 (M^+ − TFA), 261 (M^+ − TFA − $H_2NCH_2CH_2CH_2^\bullet$).

***N*-Phenylacetyl-*N*′-(6-guanidohexanoyl)-1,4-diaminobutane (5).** Following the guanylation procedure of synthesis of compound **4** a white solid of compound **5** was obtained at 58% yield: mp 101−103 °C; R_f 0.44 (4:1:1, ButOH:H_2O:AcOH); ^{1}H NMR (DMSO-d_6) δ 1.2 (m, 2H), 1.4 (m, 4H), 1.5 (m, 4H), 2.0 (t, 2H), 3.0 (m, 2H), 3.0 (m, 2H), 3.1 (m, 2H), 3.4 (s, 2H), 6.9−7.3 (brs, 3H), 7.0−7.3 (m, 5H), 7.6 (brt, 1H), 7.8 (t, 1H), 8.0 (t, 1H); MS/FAB 362 ($M^+ + 1$).

Synthesis of *N*-(4-Fluorophenyl)acetyl-*N*′-(6-guanidohexanoyl)-1,4-diaminobutane (6). *N*-(4-Fluorophenyl)acetyl-1,4-diaminobutane. The synthesis was similar to the one applied for *N*-phenylacetyl-1,4-diaminobutane, except that 4-fluorophenylacetic acid was used instead of phenylacetic acid. The crude product was purified by recrystallization from EtOAc/hexane giving a white solid at 58% yield: mp 109−114 °C; R_f 0.46 (4:1:1, ButOH:H_2O:AcOH); ^{1}H NMR (DMSO-

d_6) δ 1.4 (m, 4H), 2.5 (t, 2H), 3.0 (q, 2H), 3.4 (s, 2H), 7.0−7.3 (m, 4H), 8.0 (brs, 1H); MS (nominal) 224 (M^+), 195 ($M^+ - H_2NCH^\bullet$).

***N*-(4-Fluorophenyl)acetyl-*N′*-[6-(*tert*-butoxycarbonylamino)hexanoyl]-1,4-diaminobutane.** The synthetic procedure was similar to the one applied for the nonfluorinated analogue and gave a white solid at 40% yield: mp 128−129 °C; R_f 0.85 (4:1:1, ButOH:H_2O:AcOH); ^{1}H NMR (DMSO-d_6) δ 1.3 (m, 2H), 1.4 (s, 9H), 1.4 (m, 4H), 1.5 (m, 4H), 2.0 (t, 2H), 2.9 (q, 2H), 3.0 (m, 2H), 3.0 (m, 2H), 3.4 (s, 2H), 6.7 (t, 1H), 7.0−7.3 (m, 4H), 7.7 (t, 1H), 8.0 (t, 1H); MS (nominal) 437 (M^+).

***N*-(4-Fluorophenyl)acetyl-*N′*-(6-aminohexanoyl)-1,4-diaminobutane Trifluoroacetic Salt.** Following the general procedure for Boc deprotection with trifluoroacetic acid, a white solid was afforded at 93% yield: mp 122−124 °C; R_f 0.38 (4:1:1, ButOH:H_2O:AcOH); ^{1}H NMR (DMSO-d_6) δ 1.3 (m, 2H), 1.4 (m, 4H), 1.5 (m, 4H), 2.0 (t, 2H), 2.8 (t, 2H), 3.0 (m, 2H), 3.0 (m, 2H), 3.4 (brs, 3H), 3.5 (s, 2H), 7.0−7.3 (m, 4H), 7.8 (t, 1H), 8.0 (t, 1H); MS (nominal) 337 (M^+ − TFA), 279 (M^+ − TFA − $H_2NCH_2CH_2CH_2^\bullet$).

***N*-(4-Fluorophenyl)acetyl-*N′*-(6-guanidohexanoyl)-1,4-diaminobutane (6).** Guanylation of the above TFA salt took place by a procedure analogous to the one applied for the synthesis of compound **5** and gave a white solid at 61% yield: mp 119−122 °C; R_f 0.46 (4:1:1, ButOH:H_2O:AcOH); ^{1}H NMR (DMSO-d_6) δ 1.3 (m, 2H), 1.4 (m, 4H), 1.5 (m, 4H), 2.0 (t, 2H), 3.0 (m, 2H), 3.0 (m, 2H), 3.1 (m, 2H), 3.4 (s, 2H), 6.9−7.3 (brs, 3H), 7.0−7.3 (m, 4H), 7.6 (brt, 1H), 7.8 (t, 1H), 8.0 (t, 1H); MS (FAB) 380 (M^+ + 1).

Preparative Reversed-Phase HPLC. Compounds were purified on a Waters HPLC system equipped with a 600E system controller and using a Lichrosorb RP-18 reversed-phase preparative column (250 × 10 mm) with 7-μm packing material. Separations were achieved with a stepped linear gradient of acetonitrile in 0,1% aqueous TFA at a flow rate of 3 mL/min. The crude peptide material (18 mg) was dissolved in water (450 mL), clarified by centrifugation and the solution was injected through a Rheodyne 7125 injector with 500-μL sample loop. The mobile phases used were A: 0.1% aqueous TFA and B: 0.1% TFA in acetonitrile, with a linear gradient from 20%B to 65%B over 60 min (3 mL/min). Fractions were manually collected at 0.5-min intervals and the elution time of the major product was 25−30 min. Elution of the peptide was determined simultaneously from the absorbances at 220, 230, 257 and 280 nm (Waters 996 photodiode array detector). Fractions determined to be pure (214 nm) by analytical reversed-phase HPLC (Techsil C18, 250 × 4.6 mm) were combined and acetonitrile was removed on a rotary evaporator. After lyophilization the product was stored at −20 °C.

Amino Acid Analysis, TLC, and FAB-MS. Amino acid analysis was performed on Beckman G300 high-performance analyzer. Compositional analysis data were obtained from 6 M HCl hydrosylates (150 °C, 1 h). The purity of products was established by analytical HPLC reruns and by thin-layer chromatography (TLC). Two solvent systems were used, 1-butanol−acetic acid−water (4:1:1) (BAW) and chloroform−methanol−acetic acid (70:15:15) (CMA). The identity of the desired products was established by FAB-MS using conditions previously described.[34] FAB spectra were run on a AEI M29 mass spectrometer. 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).

Bioassay Procedures. The experimental technique was described extensively by Paredes et al.[16] Briefly, rings of 2 mm width were obtained from the central part of the thoracic aorta of adult male Wistar rats. 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 Tyrode buffer (composition in mM: 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 a pH of 7.44 by bubbling with carbogene. Each aortic ring was connected to a Grass FT 03 isometric force transducer and stretched at 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 absent if no relaxation was observed.

The action of the cyclic and nonpeptide thrombin receptor mimetics, in aortic smooth muscle and at concentrations of 0.1 nM−0.5 mM, was studied in preparations precontracted with 1 μM phenylephrine. The administrations of the thrombin receptor mimetics were separated by 30-min periods of normal perfusate administration. The relaxing action of the three thrombin receptor mimetics was also measured after pretreatment with the competitive inhibitor of nitric oxide synthase L-NAME, 300 μM. All results are expressed as means ± SEM. The relaxing action of thrombin is expressed as percentage decrease of the contraction induced by 1 μM phenylephrine. The dose−response curves for both compounds were analyzed 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} values of compounds **3**−**6** were calculated graphically from their dose−response curves.

NMR Experiments. NMR experiments were carried out using a Bruker 400-MHz NMR spectrometer. Three milligrams of thrombin receptor analogue were dissolved in 0.33 mL of DMSO-d_6. The chemical shifts were reported relative to the undeuterated fraction of the methyl group of DMSO-d_6 at 2.50 ppm with respect to TMS. 1D-Spectra were recorded with a sweep width of 4600 Hz and 32 K (zero-filled to 64 K) data points and by methods previously described.[35−39] 1D-NOE experiments were carried out in the difference mode using multiple irradiation. The methods used for the TOCSY and NOE experiments were similar to those previously described.[37−39]

Molecular Modeling Methods. Theoretical calculations were performed as described previously[39,40] using a Silicon Graphics O2 workstation and Quanta version of MSI. Thus, structures of peptides SFLLR-NH_2 and cyclo(FLLR-ϵK-Dap) were built using the Sequence Builder while peptide mimetic structures used the Molecular Editor. The built structures were energy-minimized under distance constraints obtained by 1D-NOE spectroscopy and using the algorithms embedded in the software. Finally, dynamics was applied to choose the lowest-energy structures that are compatible with NMR data. Molecular dynamics was performed at 500 K using 1-, 2-, and 2-ps time frame for heating, equilibration, and simulation steps. The final structures were superimposed using Rigid Body that allows only translation and rotation of the molecules and not changes in their conformations.[39,40]

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References

(1) Bar-Shavit, R.; Kahn, A.; Wilner, G.; Fenton, J. Monocyte chemotaxis: Stimulation by specific exocite region in thrombin. *Science* **1983**, *220*, 728−731.
(2) Fenton, J. Regulation of thrombin generation and functions. *Semin. Thromb. Hemostasis* **1988**, *14*, 234−240.
(3) 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.

(4) 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.
(5) Coughlin, S. Thrombin receptor structure and function. *Thromb. Haemostasis* **1993**, *70*, 184−187.
(6) 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.
(7) Yang, S.-G.; Laniyonu, A.; Saifeddine, M.; Moore, G.; Hollenberg, M. Actions of thrombin and thrombin receptor peptide analogues in gastric and aortic smooth muscle: development of bioassays for structure activity studies. *Life Sci*. **1992**, *51*, 1325−1332.
(8) Hollenberg, M.; Yang, S.-G.; Laniyonu, A.; Moore, G.; Saifeddine, M. Actions of thrombin receptor polypeptide in gastric smooth muscle: identification of a core pentapeptide retaining full thrombin-mimetic intrinsic activity. *Mol. Pharmacol*. **1992**, *42*, 186−191.
(9) Hollenberg, M.; Laniyonu, A.; Saifeddine, M.; Moore, G. Role of the 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.
(10) Laniyonu, A.; Hollenberg, M. Vascular actions of thrombin receptor-derived polypeptides: structure−activity profiles for contractile and relaxant effects in rat aorta. *Br. J. Pharmacol*. **1995**, *114*, 1680−1686.
(11) 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.
(12) 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.
(13) 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.
(14) 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.
(15) 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.
(16) Paredes-Carbajal, M.; Juarez-Oropeza, M.; Ortiz-Mendoza, C.; Mascher, D. Effects of acute and chronic estrogenic treatment on vasomotor responses of aortic rings from ovariectomized rats. *Life Sci*. **1995**, *57*, 474.
(17) Matsoukas, J.; Agelis, G.; Hondrelis, J.; Yamdagni, R.; Wu, Q.; Ganter, R.; Smith, J.; Moore, D.; Moore, G. Synthesis and biological activities of angiotensin II, sarilesin and samersin analogues containing Aze or Pip at position 7. *J. Med. Chem*. **1993**, *33*, 904−911.
(18) Barlos, K.; Gatos, D.; Hondrelis, J.; Matsoukas, J.; Moore, G.; Schafer, W.; Sotiriou, P. Preparation of a new acid-labile resins of sec-alcohol type and their application in peptide synthesis. *Liebigs Ann. Chem*. **1989**, 951−955.
(19) Matsoukas, J.; Hondrelis, J.; Agelis, G.; Barlos, K.; Gatos, D.; Ganter, R.; Moore, D.; Moore, G. Novel synthesis of cyclic amide-linked analogues of Angiotensins II and III. *J. Med. Chem*. **1994**, *37*, 2958−2969.
(20) 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.
(21) 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 MBP_{72-85}: Importance of the Ala_{81} carboxyl group and of a cyclic conformation for induction of experimental allergic encephalomyelitis. *J. Med. Chem*. **1999**, *42*, 1170−1177.
(22) Bernatowicz, M.; Wu, Y.; Matsueda, G. 1H-Pyrazole-1-carboxamidine hydrochloride: an attractive reagent for guanylation of amines and its application to peptide synthesis. *J. Org. Chem*. **1992**, *57*, 2497−2502.
(23) Fujita, T.; Nose, T.; Matsushima, A.; Okada, K.; Asai, D.; Yamauchi, Y.; Shirasu, N.; Honda, T.; Shigehiro, D.; Shimohigashi, Y. Synthesis of a complete set of L-difluorophenylalanines, L-(F_2)Phe, as molecular explorers of the CH/π interaction between peptide ligand and receptor. *Tetrahedron Lett*. **2000**, *41*, 923−927.
(24) 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.
(25) Mathews, I.; Padmanabhan, K.; Ganesh, V.; Tulinsky, A.; Ishii, M.; Chen, J.; Turck, C.; Coughlin, S.; Fenton, J. 2nd. Crystallographic structures of thrombin complex with thrombin receptor peptides: existence of expected and novel binding modes. *Biochemistry* **1994**, *33*, 3266−3279.
(26) Smith, K. Trayer, I.; Grand, R. Structure around the cleavage site in the thrombin receptor determined by NMR spectroscopy. *Biochemistry* **1994**, *33*, 6063−6073.
(27) Bouton, M.; Jandrot-Perrus, M.; Moog, S.; Cazenave, J.; Guill, M.; Lanza, F. Thrombin interaction with a recombinant N-terminal extracellular domain of the thrombin receptor in an accellular system. *Biochem. J*. **1995**, *305*, 635−641.
(28) Alexopoulos, K.; Matsoukas, J.; Tselios, T.; Roumelioti, P.; Holada, K. A comparative SAR study of thrombin receptor derived nonpeptide mimetics: Importance of Phenyl/Guanidino proximity for activity. *Amino Acids* **1998**, *15*, 211−220.
(29) 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.
(30) Moore, G.; Smith, J.; Baylis, B.; Matsoukas, J. Design and pharmacology of peptide mimetics. *Adv. Pharmacol. (San Diego)* **1995**, *6*, 91−141.
(31) Giannis, A.; Rubsam, F. Peptidomimetics in drug design. *Adv. Drug Res*. **1997**, *29*, 1−78.
(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) Fugita, T.; Nose, T.; Nakajima, M.; Inoue, Y.; Casta, T.; Shimohigashi, Y. Design and synthesis of para-fluorophenylalanine amide derivatives as thrombin receptor antagonists. *J. Biochem*. **1999**, *1*, 174−179.
(34) Hogg, A. Conversion of mass spectrometers for fast-atom bombardment using easily constructed components. *Int. J. Mass Spectrom. Ion Phys*. **1983**, *49*, 24−34.
(35) Otter, A.; Scot, P.; Kotovych, G. Type I collagene alpha-1 chain c-teleopeptide: solution structure determined by 600 MHz proton NMR spectroscopy and implications for its role in collagen fibrillogenesis. *Biochemistry* **1988**, *27*, 3560−3567.
(36) Wuthrich, K. *NMR of proteins and nucleic acids*; John Willey and Sons: New York, 1983; pp 117−129.
(37) Matsoukas, J.; Bigham, G.; Zhou, N.; Moore, G. ^{1}NMR studies of [Des^1]angiotensin II conformation by nuclear Overhauser effect spectroscopy in the rotating frame (ROESY): clustering of the aromatic rings in dimethyl sulfoxide. *Peptides* **1990**, *11*, 359−366.
(38) Matsoukas, J.; Yamdagni, R.; Moore, G. ^{1}NMR studies of sarmersin and [Des^1]sarmesin conformation in dimethyl sulfoxide by the N- and C-terminal domains. *Peptides* **1990**, *11*, 367−374.
(39) Matsoukas, J.; Hondrelis, J.; Keramida, M.; Mavromoustakos, T.; Makriyiannis, A.; Yamdagni, R.; Wu, Q.; Moore, G. Role of the NH_2-terminal domain of angiotensin II and [Sar^1]angiotensin II on conformation and activity: NMR evidence for aromatic ring clustering and peptide backbone-folding compared to [$Des^{1,2,3}$]-angiotensin II. *J. Biol. Chem*. **1994**, *269*, 5303−5312.
(40) 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.

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