# *Notes*

## 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[▽]

John M. Matsoukas,*,[†] Dimitris Panagiotopoulos,[†] Maria Keramida,[†] Thomas Mavromoustakos,[‡] Raghav Yamdagni,[§] Qiao Wu,[§] Graham J. Moore,[⊥] Mahmoud Saifeddine,[⊥] and Morley D. Hollenberg[⊥]

*Department of Chemistry, University of Patras, 26110 Patras, Greece, National Hellenic Research Foundation, Institute of Organic and Pharmaceutical Chemistry, 11635 Athens, Greece, and Departments of Chemistry and Pharmacology and Therapeutics, The University of Calgary, Calgary, Alberta, Canada T2N 4N1*

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Based on the minimal peptide sequence (Phe-Leu-Leu-Arg) that has been found to exhibit biological activity in a gastric smooth muscle contractile assay for thrombin receptor-activating peptides, the cyclic peptide analogues cyclo(Phe-Leu-Leu-Arg-Acp) (**1**), cyclo(Phe-Leu-Leu-Arg-ϵLys) (**2**), and cyclo(Phe-Leu-Leu-Arg-Gly) (**3**) have been synthesized by the solid-phase method using benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluoroborate or 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate as cyclization reagents. The contractile activities of compounds **1**−**3** have been compared with that of the linear thrombin receptor-activating peptide (TRAP) Ser-Phe-Leu-Leu-Arg-$NH_2$ (compound **4**) using a gastric smooth muscle strip assay. Compound **2**, wherein the ϵ-amino group of lysine was coupled to the α-carboxyl of arginine, exhibited a contractile activity comparable to that of the linear TRAP, compound **4**. However compound **1**, wherein the aminocaproic linker group yielded a ring size the same as for compound **2** but without a primary amino group, exhibited a contractile activity 600−1000-fold lower than compounds **2** and **4**. Compound **3**, which exhibited partial agonist activity, was about 100-fold less potent than either compound **2** or **4**. NMR spectroscopy of compound **2** revealed a proximity of the Phe and Arg side chains, leading to a molecular model generated by distance geometry and molecular dynamics, wherein the Phe and Arg residues are shown in proximity on the same side of the peptide ring. We conclude that the Phe and Arg side chains along with the primary amino group form an active recognition motif that is augmented by the presence of a 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.

### Introduction

In addition to its role as a coagulation factor, thrombin is now known to regulate cell function via the proteolytic activation of a specific G-protein-linked cell surface receptor.[1−3] The novel mechanism whereby thrombin activates its receptor involves the proteolytic exposure of a tethered *N*-terminal self-activating ligand,[1,2] beginning with $S_{42}$FLLRNPNDKYEPF$_{55}$ in the human receptor. Remarkably, synthetic peptides based on this revealed *N*-terminal sequence (so-called thrombin receptor-activating peptides or TRAPs) are able on their own to activate the thrombin receptor, so as to mimic the actions of thrombin in a variety of target tissues ranging from platelets[1,2] to vascular and gastric smooth muscle preparations.[4−7] On the basis of data obtained using a gastric smooth muscle bioassay, we concluded that only the first five amino acids of the tethered receptor-activating sequence (i.e., Ser-Phe-Leu-Leu-Arg) were required for thrombin-mimetic contractile activity.[5] Further work singled out the importance of the phenylalanine and arginine residues of this pentapeptide for its contractile activity in smooth muscle[6] and for its ability to aggregate platelets.[8,9] Alanine scans of the pentapeptide motif indicated that residues other than Phe and Arg were not critical for the biological activity of the peptide.[6,8]

In our initial structure−activity (SAR) survey,[6] we observed that the peptide *N*-propionyl-Phe-Leu-Leu-Arg-OH was active in the gastric contractile bioassay, indicating that the primary pharmacophores of the receptor-activating peptides resided in the sequence Phe-Leu-Leu-Arg. That observation prompted us to consider synthesizing cyclic analogues of the active core tetrapeptide Phe-Leu-Leu-Arg, in which the key phenylalanine and arginine side chains would be constrained. We therefore synthesized the following cyclic analogues of this sequence (Table 1): cyclo(Phe-Leu-Leu-Arg-Acp) (compound **1**, wherein Acp = aminocaproic acid), cyclo(Phe-Leu-Leu-Arg-ϵLys) (compound **2**, wherein ϵLys denotes that the ϵ-amino group of lysine was coupled to the α-carboxyl group of arginine), and cyclo(Phe-Leu-Leu-Arg-Gly) (compound **3**). The three

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* Corresponding author.
[†] University of Patras.
[‡] Institute of Organic and Pharmaceutical Chemistry.
[§] Department of Chemistry, The University of Calgary.
[⊥] Department of Pharmacology and Therapeutics, The University of Calgary.

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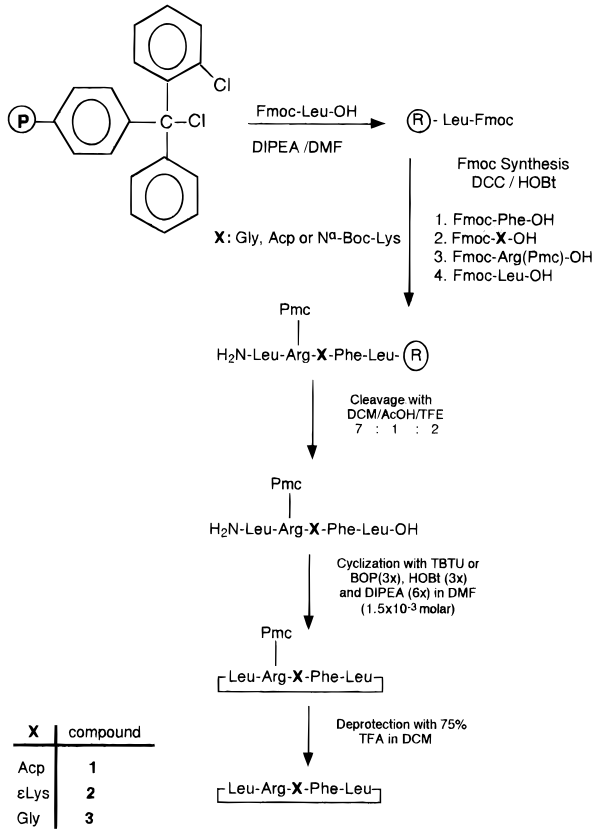
**Table 1.** Chemical Data on Cyclic TRPs

| compd | structure | mol wt FABMS | TLC $R_f$ BAW | TLC $R_f$ CMA | amino acid analysis Arg | Leu | Phe | Gly | Lys | Ser |
|---|---|---|---|---|---|---|---|---|---|---|
| **1** | cyclo(Phe-Leu-Leu-Arg-Acp) | 643 | 0.62 | 0.57 | 1.00 | 2.12 | 0.92 | | | |
| **2** | cyclo(Phe-Leu-Leu-Arg-$\epsilon$Lys) | 658 | 0.45 | 0.39 | 1.00 | 2.09 | 0.93 | | 0.99 | |
| **3** | cyclo(Phe-Leu-Leu-Arg-Gly) | 587 | 0.57 | 0.49 | 1.00 | 2.10 | 0.92 | 0.98 | | |
| **4** | Ser-Phe-Leu-Leu-Arg-$NH_2$ | 635 | 0.35 | 0.42 | 1.00 | 1.98 | 0.93 | | | 1.05 |

**Scheme 1.** Synthetic Procedure for Cyclic TRPs




cyclic compounds were assayed for their biological activity in the gastric longitudinal muscle contractile assay,[4,5] and their activity was compared with the activity of the linear receptor-activating peptide Ser-Phe-Leu-Leu-Arg-$NH_2$ (compound **4**). The conformation of the most active cyclic peptide (compound **2**) was assessed using nuclear magnetic resonance spectroscopy, employing dimethyl sulfoxide as a solvent, in keeping with our previous studies.[10−13]

## Results

**Chemistry.** The linear receptor-activating peptide Ser-Phe-Leu-Leu-Arg-$NH_2$ (compound **4**) was synthesized in solid phase using conventional Fmoc procedures followed by purification to homogeneity using gel filtration and HPLC chromatography. The cyclic analogues, based on the Phe-Leu-Leu-Arg motif (compounds **1**−**3**), were synthesized using the 2-chlorotrityl chloride resin[14] that was used previously for the synthesis of novel cyclic amide-linked analogues of angiotensin-II and -III.[15] We found that 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-Leu[2]-Leu[3]-Arg motif, as outlined in Scheme 1.[16] Using the 2-chlorotrityl resin, the mild conditions possible for cleaving the peptide−resin bond allowed for the release from the resin and subsequent cyclization of the desired protected peptide, which was then deprotected with 75% trifluoroacetic acid in dichloromethane (Scheme 1). Unexpectedly, we found that using arginine for the first step of the synthesis yielded a linear protected peptide that failed to cyclize, presumably because of steric hindrance. For the synthesis of compound **2**, the $\epsilon$-amino group of $N^{\alpha}$-Boc-protected Lys was coupled to the α-COOH group of Arg, in the course of the synthesis of H-Leu-Arg(Pmc)-$\epsilon$Lys(Boc)-Phe-Leu-OH, as outlined in Scheme 1. Compounds **1** and **3** were also synthesized as outlined in Scheme 1.

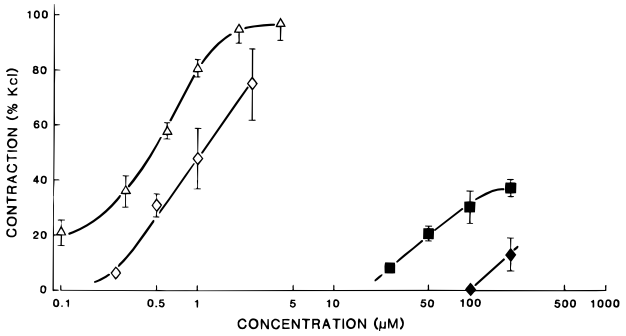



**Figure 1.** Contractile actions of cyclic thrombin receptor-derived peptides. Compounds **1** (♦), **2** (◇), and **3** (■) were measured in a rat gastric longitudinal muscle preparation as previously described[4−6] in comparison with the contractile action of the linear receptor-derived peptide, compound **4** (△). Values represent the means ± standard error (bars) for 3−12 estimates at each petide concentration. Error bars smaller than the symbols are not shown.

Two conditions for cyclization of the protected peptides of the general composition H-Leu-Arg-X-Phe-Leu-OH were evaluated, the first using benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) and the second using 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 cyclization procedure, as indicated by the lack of a reaction with ninhydrin, was confirmed by fast atom bombardment mass spectrometry and nuclear magnetic resonance spectroscopy. The structures of the four peptides evaluated by bioassay are shown in Table 1.

**Bioassay Data.** The contractile activities of cyclic compounds **1**−**3** were compared with that of the amidated linear pentapeptide compound **4** (Figure 1). Compound **4** represents the shortest receptor-activating peptide that exhibits a potency greater than that of the originally described receptor-activating tetradecapeptide.[1,5−7] The contractile potency of cyclic compound **2** was comparable to that of the linear peptide compound **4**. However, the potency of compound **3**, which behaved

**Chart 1.** Chemical Structure of Compounds 1 and 2

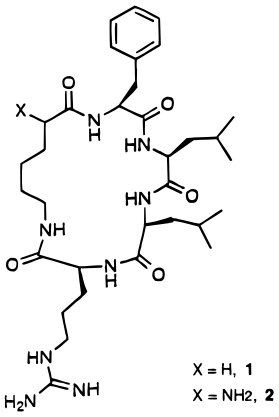



**Table 2.** Relative Biological Activities in the Gastric Contractile Assay[a]

| compd | activity: $R_{EC}$ value relative to compound **4** |
|---|---|
| **1** | >1000 |
| **2** | 2.4 ± 0.1 |
| **3** | 390 ± 10 |
| **4** | 1.0 |

[a] An activity ratio ($R_{EC} = EC_{peptide} \div EC_{compound4}$) was calculated from the concentration−effect curves as outlined previously[6,7] and in the Experimental Section, based on the concentrations of each agonist ($EC_{peptide}$) causing a contraction equal to that of a known concentration of compound **4** ($EC_{compound4}$). Values represent the mean ± standard error estimates of the $R_{EC}$ at four positions on the concentration−effect curves. The value for compound **1** was prorated from its activity relative to compound **2**.

as a partial agonist, was about 2 orders of magnitude lower than that of compound **2**. Compound **1**, having the same ring size as compound **2** but lacking a primary amino group (Chart 1), had an even lower potency, with contractile activity measurable only at concentrations greater than 100 $\mu$M (Figure 1). Further, compound **1**, at a concentration of 100 $\mu$M, failed to alter the contractile activity of 1 $\mu$M compound **4** (data not shown), indicating that it did not exhibit appreciable antagonist activity. Due to solubility problems, it was not possible to study concentrations of compound **1** higher than about 200−300 $\mu$M. Because of the markedly different intrinsic activities of the cyclic compounds **1**−**3**, it was not possible to obtain p$A_2$ values with any confidence. Their activities were therefore determined relative to that of compound **4**, using the concentration−effect curves, as we have done previously for other receptor-activating peptides[6,7] (Table 2).

**Conformational Studies of Cyclo(Phe-Leu-Leu-Arg-$\epsilon$Lys).** Since the cyclic compound **2** exhibited activity comparable to that of the linear peptide, compound **4**, we decided to assess the conformation of compound **2**. In compound **2**, the Phe and Arg residues, shown by us and by others to play key roles in the biological activity of the receptor-derived peptides,[6−9] would be more constrained than in the linear receptor-activating peptides. In the present study, we conducted 2D-COSY, ROESY, and 1D-NOE experiments (Figures 2−4) using DMSO-$d_6$ as a solvent, as we have done previously for the conformational analysis of angiotensin-II-related peptides.[10−13] $^1$H assignment was achieved by combining information from the COSY spectra and the 1D-NOE experiments. The 1D-NOE and ROESY experiments provided distance information for the backbone $C_\alpha$, amide, and side chain protons.

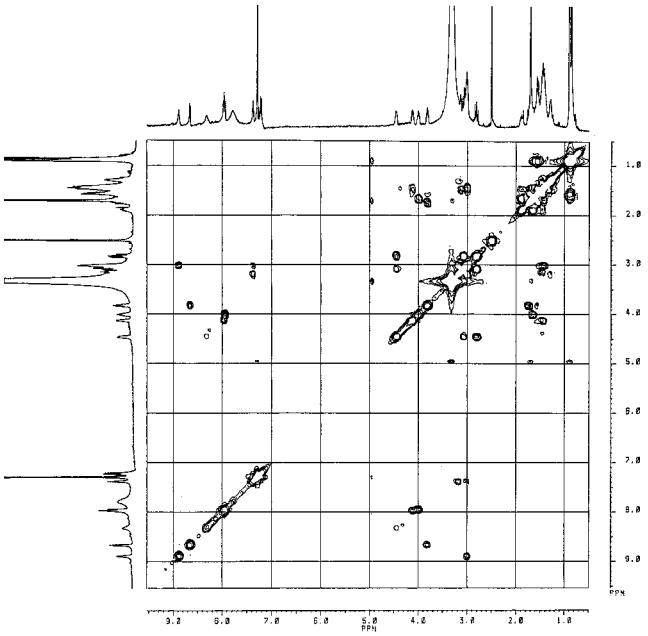


**Figure 2.** Two-dimensional contour plot of a 400 MHz COSY using 5 mM compound **2** in DMSO-$d_6$.

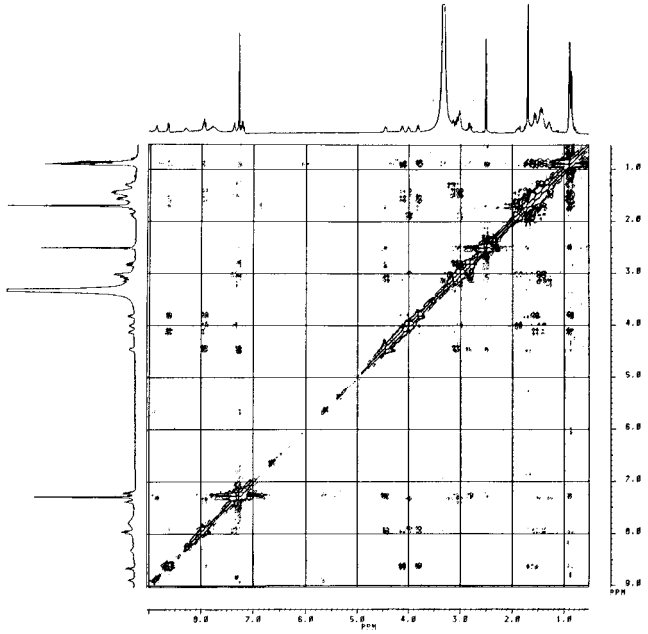


**Figure 3.** Two-dimensional contour plot of a 400 MHz ROESY using 5 mM compound **2** in DMSO-$d_6$.

**Assignment of Proton Resonances.** Figure 4 shows the assignment of proton resonances for compound **2**. For the Phe residue, the NH and $C_\alpha$ proton resonances were readily assigned at $\delta = 8.32$ and 4.46 ppm, respectively, through intense COSY peaks of the $C_\alpha$ proton with the characteristic $C_{\beta\beta'}$ protons at $\delta = 3.02$ and 2.82 ppm (Figure 2). For the Arg residue, the $N_\alpha$, $C_\alpha$, $C_{\beta\beta'}$, $C_{\gamma\gamma'}$, $C_{\delta\delta'}$, and $N_\epsilon$ proton resonances were readily assigned through intraresidue COSY cross-peaks, based on the well-established interaction between Arg $C_\delta$ and $N_\epsilon$ proton resonances at $\delta = 3.05$ and 8.90 ppm, respectively. The broad peak at $\delta = 7.78$ was assigned to $Arg_{\omega\omega'}$ protons. For Leu$^2$ and Leu$^3$, the respective NH, $C_\alpha$, and $C_{\beta\beta'}$ proton resonances were assigned through cross-peaks of vicinal protons starting from the characteristic Leu Me resonances at $\delta = 0.88$ ppm. Differentiation between Leu$^2$ and Leu$^3$ proton reso-

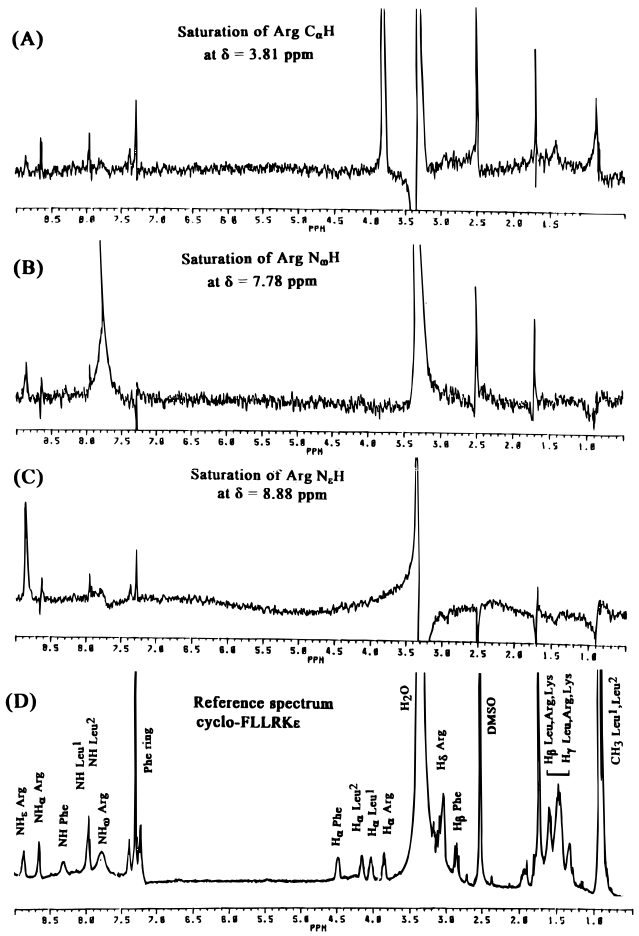



**Figure 4.** Reference spectrum (D) and NOE difference spectra for compound **2** in DMSO-$d_6$ obtained upon saturation of proton lines for Arg $N_\epsilon H$ (C), Arg $N_\omega H$ (B), and Arg $C_\alpha H$ (A).

nances was possible from the ROESY experiment and the observed sequential $i-(i+1)$ crosspeak interaction between Arg $C_\alpha$ and Leu$^3$ $C_\alpha$ protons.

**Conformational Properties.** The ROESY experiment was recorded at a spin−lock time of 200 ms (Figure 3). A weak interaction observed between a Phe ring proton and a Leu methyl proton indicates proximity of the Phe side chain with the Leu$^2$ side chain above the ring plane. A weak interaction of the Phe ring proton (ortho) with the Phe $C_{\beta\beta'}$ protons and a strong interaction of the same Phe ring proton with the Phe $C_\alpha$ proton indicates reduced mobility. Interaction of this Phe ring proton with the Arg $N_\epsilon$ proton, clearly seen in the ROESY spectrum, also indicates proximity of the Phe/Arg side chains. The Phe/Arg side chain proximity was indicated further in the 1D-NOE experiments. The Arg $N_\epsilon$ proton resonance was unequivocally assigned at $\delta = 8.88$ ppm, through COSY cross-peaks with the Arg $C_\delta$ proton at $\delta = 3.00$ ppm. Saturation of the Arg $N_\epsilon$ proton resulted in enhancement of the Phe ring protons at $\delta = 7.30$ ppm (15.8%) indicating proximity of the Phe/Arg side chains. Saturation of a broad resonance at $\delta = 7.78$ ppm, which had been assigned to Arg $N_\omega$ protons, produced an enhancement of the Phe ring protons further confirming the Phe/Arg interaction. Although no cross-peaks could be observed between the $N_\epsilon/N_\omega$ protons, the 1D-NOE experiments indicated that the broad resonance at $\delta = 7.78$ ppm was related to the Arg $N_\omega$ protons. Thus, saturation of the Arg $N_\epsilon$ resonance at $\delta = 8.88$ produced enhancement of the broad resonance at $\delta = 7.78$ ppm, assigned to the neighboring Arg $N_\omega$ proton. Conversely, saturation of the broad proton resonance at $\delta = 7.71$ ppm, assigned to Arg $N_\omega$ protons, resulted in enhancement of the 8.88 ppm resonance known to be the Arg $N_\epsilon$ proton resonance. In summary, the one-dimensional nuclear Overhauser effect (1D-NOE) spectroscopy, a more sensitive technique for conformational studies, allowed for a confirmation of the interactions detected by ROESY and revealed additional weak interactions which might be difficult to identify by the ROESY technique. Under the experimental conditions which we used for the NOE experiments (low power, different preirradiation times, saturation of control areas), spin diffusion and partial saturation were visibly minimized for the interactions under consideration.

**Table 3.** NOE Enhancements for Cyclo(Phe-Leu$^2$-Leu$^3$-Arg-ϵLys)

| proton(s) saturated | chemical shift (ppm) | enhancements | chemical shift (ppm) | % proton |
|---|---|---|---|---|
| Arg $N_\epsilon H$ | 8.88 | Arg $N_\alpha H$ | 8.65 | 8.5 |
| Arg $N_\epsilon H$ | 8.88 | Arg $N_\omega H$ | 7.78 | 9.4 |
| Arg $N_\epsilon H$ | 8.88 | Phe ring | 7.30 | 15.8 |
| Arg $N_\omega H$ | 7.78 | Arg $N_\epsilon H$ | 8.88 | 13.7 |
| Arg $N_\omega H$ | 7.78 | Arg $N_\alpha H$ | 8.65 | 2.2 |
| Arg $N_\omega H$ | 7.78 | Phe ring | 7.30 | 4.6 |
| Arg $C_\alpha H$ | 3.81 | Arg $N_\epsilon H$ | 8.88 | 3.1 |
| Arg $C_\alpha H$ | 3.81 | Arg $N_\epsilon H$ | 8.65 | 3.3 |
| Arg $C_\alpha H$ | 3.81 | Leu$^3$ NH | 7.95 | 5.1 |
| Arg $C_\alpha H$ | 3.81 | Phe ring | 7.30 | 9.1 |
| Arg $C_\alpha H$ | 3.81 | Leu$^3$ $CH_3$ | 0.88 | 26.3 |

**Molecular Dynamics.** To extend the observations made using NMR, a theoretical modeling approach was used. Theoretical calculations were performed as described previously[13,17] using a Silicon Graphics 4D/35 workstation and Quanta 3.3 version of MSI. The structure of compound **2** was first minimized using a combination of steepest descent and Newton Raphson algorithms. The resulting structure was manipulated in order to mimic the observed NOEs shown in Table 3. Thus, the Phe ring was located in such a way as to communicate in space with Arg guanidino group. The Phe residue was situated in a position which also permitted an interaction with the Leu$^2$ side chain. After the construction of a rough model, distance constraints were applied using the NOE data in order to refine the model. Thus, the structure of compound **2** was first minimized with the constraints on and then subjected to dynamics. The resulting conformation for compound **2** (Figure 5), showing a proximity of the Phe and Arg side chains, represents a local energy minimum in the potential energy surface obtained by searching from extended conformations and is a valid candidate for the biological conformation of this cyclic analogue.

## Discussion

The main finding of our study was that the NMR data for the conformationally restricted analogue (compound **2**), which exhibited a contractile activity comparable to that of the linear receptor-activating peptide Ser-Phe-Leu-Leu-Arg-$NH_2$ (compound **4**), indicated a proximity of the Phe and Arg side chains. The molecular dynamics approach, in which the structure was manipulated to fulfill the observed NOE data, yielded a model with the Phe and Arg residues on the same side of the cyclic ring (Figure 5). In terms of the cis orientation of the Phe

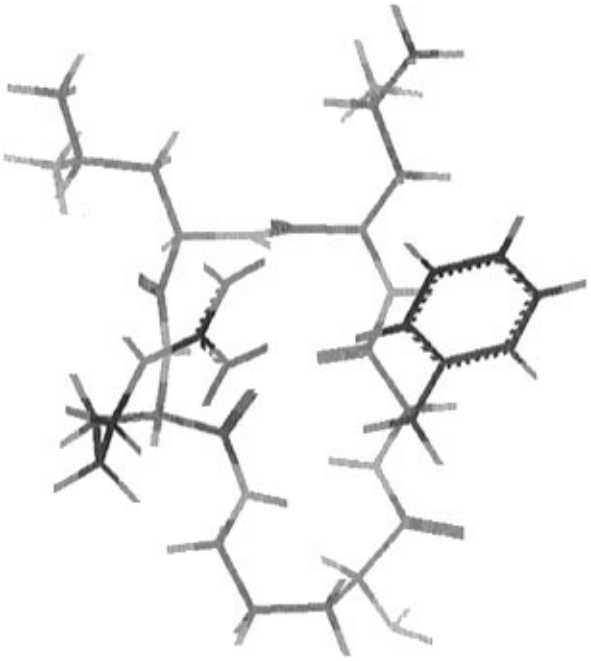


**Figure 5.** Model of cyclo(Phe-Leu-Leu-Arg-$\epsilon$Lys) generated by distance geometry and molecular dynamics.

**Table 4.** Proton−Proton ROESY Interactions Identified for Cyclo(Phe-Leu$^2$-Leu$^3$-Arg-$\epsilon$Lys) in DMSO-$d_6$[a]

| Phe (F) | Leu ($L^2$) | Leu ($L^3$) | Arg (R) |
|---|---|---|---|
| | Intraresidue Interactions | | |
| | α:N | | |
| $F_P$:α | α:$\beta$ | α:N | α:N |
| $F_P$:$\beta$ | $\beta$:$\gamma$ | α:$\beta$ | α:$\beta$ |
| α:$\beta$ | α:$\gamma$ | $\beta$:$\gamma$ | $\beta$:$\gamma$ |
| $\beta$:N | $\beta$:N | $\gamma$:$\delta$ | |
| | Interresidue Interactions | | |
| $F_P$:$L^2{}_M$ | $L^2{}_M$:$F_P$ | $L^3{}_\alpha$:$R_\alpha$ | $R_\alpha$:$L^3{}_\alpha$ |
| $F_P$:$R_\delta$ | | | $R_\delta$:$F_P$ |
| $F_P$:$R_\epsilon$ | | | $R_\epsilon$:$F_P$ |
| $F_P$:$L^2{}_\alpha$ | $L^2{}_\alpha$:$F_P$ | | |
| $F_P$:$R_\delta$ | | | $R_\delta$:$F_P$ |

[a] M stands for methyl group protons; P stands for Phe ring protons which overlap and are not individually assigned; N stands for amide NH protons.

and Arg side chains, this model would be consistent with a previous *ab initio* evaluation of the putative conformational state of thrombin receptor-activating peptides.[9] In that study, based on a search of the crystallographic database for the conformation of the Ser-Phe-Leu/Phe-Leu-Arg-Asn motif and on energy minimization/molecular dynamics calculations, it was suggested that the sequence assumes an extended helical structure in which the side chains of the Phe and Arg are on the same side, looking down the axis of an extended helix. Our data would suggest further that the active conformation of the linear receptor-activating peptides may bring the Arg and Phe residues into closer proximity in a quasi-cyclic structure. Our data would appear to argue against other models that have suggested unstructured or S-like conformations for the receptor-activating peptides.[18−20]

It was of interest to observe that although compounds **1** and **2** have the same ring size, compound **2**, having a free primary amino group, was very active, whereas compound **1**, lacking a free amino group in the ring, exhibited low activity as an agonist and did not exhibit appreciable antagonist activity. Since compound **1** exhibited neither appreciable agonist nor antagonist activity, we concluded that it did so most likely because of a lack of affinity for the receptor conferring contractile activity. Our data thus substantiate the importance for biological activity of the primary amino group on the amino side residue preceding Phe, as has been found previously for linear thrombin receptor-activating peptides.[1,8,9] Alanine scans of the linear pentapeptide motif ($Ser_1$-$Phe_2$-$Leu_3$-$Leu_4$-$Arg_5$), including observations by us[6] and others,[8] have indicated that residues other than $Phe_2$ and $Arg_5$ play relatively minor roles in conferring biological activity. It would appear that the recognition motif, comprising a conformation with the Phe and Arg side chains oriented toward the receptor, can be enhanced by the presence of a primary amino group as an added element. A comparable situation has been pointed out for the opiate peptides, wherein the *N*-terminal amino group, added to the primary cluster groups (Phe and Tyr side chains) in a cyclic conformation, may enhance biological activity.[21] It remains to be determined if the contractile activity of the linear thrombin receptor-activating peptides is due to a cyclic conformation, akin to the one we propose for compound **2**.

## Conclusion

The intrinsic activity of the cyclic thrombin receptor-derived peptides suggest that the TRPs may approach the receptor site in a cyclic conformation rather than a linear one. The NMR data indicate spatial proximity of the Phe/Arg side chains which may be an important element for receptor recognition. The SAR data reveal that a free amino group present in the cyclo(Phe-Leu-Leu-Arg-$\epsilon$Lys) (compound **2**) but not in the cyclo(Phe-Leu-Leu-Arg-Acp) (compound **1**) of the same ring size is important for the biological activity in the rat gastric longitudinal muscle (contraction) assay. These findings suggest a contribution of the amino group of compound **2** in receptor recognition and consequently support a role for the *N*-terminal amino group of Ser-Phe-Leu-Leu-Arg in the pharmacophore cluster. Thus, a cyclic conformation of Ser-Phe-Leu-Leu-Arg would provide for the function of a triad of interacting groups, comprised of the Phe and Arg side chains and the primary amino group.

## Experimental Section

**Methods.** Preparative HPLC was performed with a Waters system equipped with a 600E system controller using a Lichrosorb RP-18 reversed-phase preparative column (250 × 10 mm) with 7 $\mu$m packing material. Separations were achieved with a stepped linear gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) over 60 min at a flow rate of 3 mL/min. The crude peptide material (20 mg) was dissolved in methanol (450 $\mu$L), 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 25−30 min. Elution of the peptide was determined simultaneously from the absorbances at 254 and 230 nm (Waters 996 photodiode array detector). Fractions containing the major peptide peak were pooled, and acetonitrile was removed using a rotary evaporator. After lyophilization the product was stored at −20 °C.

Peptide purity was assessed by amino acid analysis, analytical HPLC reruns (Techsil 5 C18, 250 × 4.6 mm), thin layer chromatography (TLC), and mass spectrometry (FABMS). Amino acid analysis was performed on a Beckman G300 high-performance analyzer. Appropriate molar ratios of amino

acids were observed for compounds **1**−**4**. Compositional analysis data were collected from 6 M HCl hydrosylates (150 °C, 1 h). Analytical HPLC was done by reruns of the procedure described above. Two TLC solvent systems were used as follows: *n*-butanol−acetic acid−water (4:1:1) (BAW) and chloroform−methanol−acetic acid (70:15:15) (CMA). FABMS 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).[22]

NMR experiments were performed using a Bruker 400 MHz NMR spectrometer. Three milligrams of compound **2** was dissolved in 0.33 mL of DMSO-$d_6$. The chemical shifts are reported relative to the undeuterated fraction of the methyl group of DMSO-$d_6$ at 2.50 ppm with respect to TMS. One-dimensional spectra were recorded with a sweep width of 4.600 Hz and 32 K (zero-filled to 64 K) data points and by methods previously described.[10−13] The COSY experiment provided contour plots which were symmetrized with respect to diagonal. One-dimensional NOE experiments were performed in the difference mode using multiple irradiation. The methods used for the COSY and NOE experiments were similar to those previously described.[23−27] The ROESY experiment required a basic 90° phase connection in $t_1$ before the phase tuning could be done. A carrier frequency of 6.2 ppm, a spin−lock time of 200 ms, and a 30° flip angle for the hard pulse spin−lock train were selected.

In the molecular modeling, theoretical calculations were performed on a Silicon Graphics 4D/35 work station using QUANTA 3.3 version of Molecular Simulations. The structure of compound **2** was built using the sequence builder (Figure 5). It was then minimized using steepest descent (SD) and adopted-basis Newton Raphson algorithms. Finally, a combination of dynamics with minimization was applied to obtain the lowest energy structures. In the structures obtained, molecular dynamics was performed at 500 K using 1, 2, and 2 ps time frame for heating, equilibration, and simulation steps. Two hundred structures from the simulated ones were minimized using iteration steps and an SD algorithm. Details of the above techniques have been recently described.[17]

The contractile activities of the peptides were assayed essentially as previously described,[5,6] using gastric longitudinal muscle (LM) strips obtained from male albino Sprague−Dawley rats (250−300 g). Tissue strips (3 × 10 mm) were pre-equilibrated in a Krebs−Henseleit buffer, pH 7.4, gassed with 95% $O_2$/5% $CO_2$. The integrity of each tissue preparation was assessed by a challenge of 1 $\mu$M carbachol and 50 mM KCl. Each tissue was also assessed for its sensitivity to 1 $\mu$M of the linear receptor-activating peptide compound **4**. In order to minimize degradation of the linear peptide by tissue aminopeptidase, amastatin (10 $\mu$M) was added to the organ bath 20 min prior to the addition of peptide; for consistency, 10 $\mu$M amastatin was also added to the organ bath for the assay of the cyclic peptides. Agonists were added to the organ bath at 30−35 min intervals; tissues were washed twice at approximately 10 and 15 min after the addition of agonists to the organ bath. Contractions were monitored isometrically, using Statham force-displacement transducers. Only tissues responding well to carbachol, KCl, and compound **4** were used for the assay of cyclic peptides. Upon standardizing a tissue to these agonists, the response to a single concentration of cyclic peptide was measured. The contractile response to each concentration of peptide was expressed as a percentage (% KCl) relative to the contraction caused by 50 mM KCl (1.2 ± 0.1 g tension, mean ± SEM for $n$ = 12). For each peptide concentration, data were pooled from experiments done with 3−12 individual tissue strips coming from two or more separate animals. The activities of the cyclic peptides, relative to that of compound **4**, were expressed as done previously,[6] based on the concentration−effect curves. Using several points on the curves, an average concentration ratio ($R_{EC}$) was calculated for each cyclic peptide relative to the concentration of compound **4** that caused an equal contractile response. This procedure allows for a comparison of agonist activities even when the concentration−effect curves do not permit an accurate comparison of $EC_{50}$s (or p$A_2$s).

**Preparation of $N^{\alpha}$-Fmoc-Leu-2-chlorotrityl Resin for Synthesis of Protected Linear Peptides.** 2-Chlorotrityl chloride resin (1 g, 1.5 mequiv of $Cl^-$/g of resin) in dry dimethylformamide (8 mL) was stirred in a round bottom flask. Diisopropylethylamine (DIPEA) (1.52 mL, 4.5 mmol) and $N^{\alpha}$-Fmoc-Leu-OH (355 mg, 1 mmol) were added, and the solution was stirred for 45 min at room temperature. A mixture of MeOH (0.8 mL) and DIPEA (0.2mL) was then added for endcaping and the mixture stirred for another 10 min at room temperature. The Fmoc-Leu-resin was filtered, subsequently washed with DMF (3 × 10 mL), 2-propanol (2 × 10 mL), and *n*-hexane (2 × 10 mL), and dried *in vacuo* for 24 h at room temperature. The loading of the amino acid per gram of substituted resin was 0.7 mmol of amino acid/g of resin, calculated by weight and amino acid analysis.

**Synthesis of Linear Peptide Intermediates H-Leu-Arg(Pmc)-X-Phe-Leu-OH: X = Acp (1A), X = $N^{\alpha}$-Boc-Lys (2A), X = Gly (3A).** $N^{\alpha}$-Fmoc-Leu-2-chlorotrityl resin (1 g, 0.7 mmol/g) was used for the synthesis of the linear peptide precursors following the protocol previously described.[15] The amino acids used in Fmoc synthesis were $N^{\alpha}$-Fmoc-Leu-OH, $N^{\alpha}$-Fmoc-Phe-OH, $N^{\alpha}$-Fmoc-Gly-OH, $N^{\alpha}$-Fmoc-Acp-OH, $N^{\alpha}$-Fmoc-$N^{\omega}$-Pmc-Arg-OH, and $N^{\alpha}$-Boc-$N^{\epsilon}$-Fmoc-Lys-OH. Protected peptide **2A** was formed by coupling of the $\epsilon$-amino Lys group with carboxyl group of Arg. The finished peptide resin (after Fmoc deprotection of the last amino acid) was dried *in vacuo* (1.25 g) and then treated with the splitting mixture dichloromethane−acetic acid−2,2,2-trifluoroethanol (12.5 mL, 7:1:2) for 1 h at room temperature to remove the peptide from the resin. The mixture was filtered off and the resin washed with the splitting mixture (×2) and DCM (×3). The solvent was removed on a rotary evaporator, and the obtained oily product precipitated from cold dry diethyl ether as a white solid (250 mg, 85%).

**Cyclization of Linear Protected Peptides.** To a solution of the linear protected peptides **1A**−**3A** in dry dimethylformamide (5 mL) was added triethylamine (1 equiv), and the solution stirred for 5 min at room temperature. The neutralized linear protected pentapeptide was precipitated from $H_2O$, washed with $H_2O$ (2 × 10 mL) and diethyl ether (1 × 10 mL), and dried *in vacuo* for 14 h. Two coupling methods were used for cyclization of linear peptides in solution.

**Method A:** To a solution of linear protected peptide **1A** (or **2A**, **3A**) (177 mg, 0.2 mmol) in DMF (46 mL), were added BOP (530 mg, 1.2 mmol) and DIPEA (0.7 mL), and the solution was stirred for 14−16 h at room temperature. The reaction was followed by the ninhydrin test employing thin layer chromatography (TLC) using $CHCl_3$−MeOH (6:1) as a solvent system. When the reaction had proceeded to completion, the solvent was removed under reduced pressure affording a light yellow oily residue. The cyclic protected pentapeptide was precipitated from $H_2O$ and dried *in vacuo* for 12 h (120 mg, 65%).

**Method B:** To a solution of linear protected peptide **1A** (or **2A**, **3A**) (100 mg, 0.11 mmol) in DMF (75.5 mL) were added TBTU (109.1 mg, 0.34 mmol), 1-hydroxybenzotriazole (52.2 mg, 0.34 mmol), and DIPEA (0.75 mL), and the solution was stirred for 2.5 h. The reaction was followed by the ninhydrin test on TLC using $CHCl_3$−MeOH (6:1) as an elutant. The solvent was removed from the reaction mixture under reduced pressure affording a light yellow oily residue. The cyclic protected pentapeptide was precipitated from $H_2O$ and dried *in vacuo* for 12 h (75 mg, 73%).

**Preparation of Cyclic Peptides: Cyclo(Phe-Leu-Leu-Arg-Acp) (1), Cyclo(Phe-Leu-Leu-Arg-$\epsilon$Lys) (2), and Cyclo(Phe-Leu-Leu-Arg-Gly) (3).** The dried residues from the cyclization reactions were treated with 75% (v/v) trifluoroacetic acid in dichloromethane (2 mL) for 4 h at room temperature. The resulting solution was concentrated under vacuum to a small volume (0.5 mL). Several drops of methanol were added, and the final free cyclic pentapeptide was precipitated as a light yellow amorphous solid by the addition of diethyl ether. The crude peptide product was purified further by preparative HPLC as outlined above.

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## References

(1) Vu, T.-K. H.; Hung, D. T.; Wheaton, V. I.; Coughlin, S. R. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **1991,** *64*, 1057−1068.

(2) Coughlin, S. R.; Vu, T.-K. H.; Hung, D. T.; Wheaton, V. I. Characterization of a functional thrombin receptor. *J. Clin. Invest.* **1992,** *89*, 351−355.

(3) Vouret-Craviari, V.; van Obberghen-Schilling, E.; Rasmussen, U. B.; Pavirani, A.; Lecocq, J. P.; Pouyssegur, J. Synthetic α-thrombin receptor peptides activate G-protein-coupled signalling pathways but are unable to induce mitogenesis. *Mol. Biol. Cell.* **1992,** *3*, 95−102.

(4) Yang, S.-G.; Laniyonu, A.; Saifeddine, M.; Moore, G. J.; Hollenberg, M. D. 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.

(5) Hollenberg, M. D.; Yang, S.-G.; Laniyonu, A. A.; Moore, G. J.; Saifeddine, M. Action 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.

(6) Hollenberg, M. D.; Laniyonu, A. A.; Saifeddine, M.; Moore, G. J. 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.

(7) Laniyonu, A. A.; Hollenberg, M. D. Vascular actions of thrombin receptor-derived polypeptides: structure-activity profiles for contractile and relaxant effects in rat aorta. *Brit. J. Pharmacol.* **1995,** *114*, 1680−1686.

(8) Chao, B. H.; Kalkunte, S.; Maraganore, J. M.; Stone, S. R. Essential groups in synthetic agonist peptides for activation of the platelet thrombin receptor. *Biochemistry* **1992,** *31*, 6175−6178.

(9) Vassalo, R. R.; Kieber-Emmons, T.; Cichowski, K.; Brass, L. F. Structure-function relationships in the activation of platelet thrombin receptors by receptor-derived peptides. *J. Biol. Chem.* **1992,** *267*, 6081−6085.

(10) Matsoukas, J. M.; Yamdagni, R.; Moore, G. J. $^1$H-NMR studies of sarmesin and [Des$^1$] sarmesin conformation in dimethylsulfoxide by the N- and C-terminal domains. *Peptides* **1990,** *11*, 367−374.

(11) Matsoukas, J. M.; Bigham, G.; Zhou, N.; Moore, G. J. $^1$H-NMR studies of [Sar$^1$]angiotensin II conformation by nuclear Overhauser effect spectroscopy in the rotating frame (ROESY): clustering of the aromatic rings in dimethylsulphoxide. *Peptides* **1990,** *11*, 359−366.

(12) Matsoukas, J. M.; Agelis, G.; Hondrelis, J.; Yamdagni, R.; Wu, Q.; Ganter, R. C.; Smith, J. R.; Moore, D.; Moore, G. J. Synthesis and biological activities of angiotensin II, sarilesin and sarmesin analogues containing Aze or Pip at position 7. *J. Med. Chem.* **1993,** *36*, 904−911.

(13) Matsoukas, J. M.; 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 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.

(14) Barlos, K.; Gatos, D.; Hondrelis, J.; Matsoukas, J.; Moore, G. J.; Schafer, W; Sotiriou, P. Preparation of new acid-labile resins of *sec*-alcohol type and their applications in peptide synthesis. *Liebigs Ann. Chem.* **1989,** 951−955.

(15) Matsoukas, J. M.; Hondrelis, J.; Agelis, G.; Barlos, K.; Gatos, D.; Ganter, R. C.; Moore, D.; Moore, G. J. Novel synthesis of cyclic amide-linked analogues of angiotensin II and III. *J. Med. Chem.* **1994,** *37*, 2958−2969.

(16) Panagiotopoulos, D.; Matsoukas, J. M.; Keramida, M.; Gatos, D.; Monteiro, L.; Maia, H. L. S.; Yamdagni, R.; Wu, Q.; Redstone, P.; Moore, G. J.; Laniyonu, A. A.; Saifeddine, M.; Hollenberg, M. D. Cyclic thrombin receptor-derived peptide analogues containing D-Phe and D-Arg. In *Peptides 1994*; Maia, H. L. S., Ed.; ESCOM: Leiden, 1995; pp 359−360.

(17) Mavromoustakos, T.; Yang, D. P.; Theodoropoulou, E. Studies of the conformational properties of the cannabimimetic-aminoalkylindole pravadoline using NMR and molecular modeling *Eur. J. Med. Chem.* **1995,** *30*, 227−234.

(18) Mathews, I. I.; Padmanabhan, K. P.; Ganesh, V.; Tulinsky, A.; Ishii, M.; Chen, J.; Turck, C. W.; Coughlin, S. R.; Fenton, J. W. 2nd. Crystallographic structures of thrombin complexed with thrombin receptor peptides: existence of expected and novel binding modes. *Biochemistry* **1994,** *33*, 3266−3279.

(19) Smith, K. J.; Trayer, I. P.; Grand, R. J. Structure around the cleavage site in the thrombin receptor determined by NMR spectroscopy. *Biochemistry* **1994,** *33*, 6063−6073.

(20) Bouton, M. C.; Jandrot-Perrus, M.; Moog, S.; Cazenave, J. P.; Guill, M. C.; Lanza, F. Thrombin interaction with a recombinant N-terminal extracellular domain of the thrombin receptor in an acellular system. *Biochem. J.* **1995,** *305*, 635−641.

(21) Moore, G. J.; Smith, J. R.; Baylis, B.; Matsoukas, J. M. Design and Pharmacology of peptide mimetics. *Adv. Pharmacol.* **1995,** *33*, 91−141.

(22) Hogg, A. M. Conversion of mass spectrometers for fast-atom bombardment using easily constructed components. *Int. J. Mass Spectrom. Ion Phys.* **1983,** *49*, 24−34.

(23) Marion, D. Rotating Frame Nuclear Overhauser Effect: A practical tool of the $^1$H-NMR study of peptides in solution. *FEBS Lett.* **1985,** *192*, 99−103.

(24) Marion, D.; Wüthrich, K. Application of phase sensitive two-dimensional correlated spectroscopy (COSY) for measurement of $^1$H- $^1$Hspin-spin coupling constants in proteins. *Biochem. Biophys. Res. Commun.* **1983,** *113*, 967−974.

(25) Otter, A.; Scot, P. G.; 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.

(26) Wüthrich, K. *NMR of proteins and nucleic acids*; John Wiley and Sons, Inc.: New York, 1983; pp 117−129.

(27) Wüthrich, K.; Wider, G.; Wagner, G.; Braun, W. Sequential resonance assignments as a basis for determination of spatial protein structures by high resolution proton nuclear magnetic resonances. *J. Med. Biol.* **1982,** *155*, 311−319.

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