Amino Acids (1998) 15:211–220

Amino
Acids

A comparative SAR study of thrombin receptor derived non peptide mimetics: Importance of phenyl/guanidino proximity for activity

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Accepted October 29, 1997

Summary. Thrombin, the most potent physiological platelet agonist interacts with cells through a specific G protein-coupled receptor which has been cloned and sequenced. Synthetic thrombin receptor peptides (TRAPs) comprising the first 5 amino acids (SFLLR and SFLLR-NH_2) of the new N-terminus tethered ligand of the thrombin receptor that is generated by thrombin's proteolytic activity were found to cause full platelet aggregation. During the screening of novel thrombin receptor derived non-peptide mimetics in the platelet aggregation assay we found that 1-phenylacetyl-4-(6-guanidohexanoyl)-piperazine (**1**) and 1-(6-guanidohexanoyl)-4-(phenylacetylamidomethyl)-piperidine (**2**) exerted *in vitro* antagonist activities (56% and 40% correspondingly) as it is depicted by the platelet aggregation assay. Using Molecular Modeling, the synthetic compounds were overlayed with SFFLR. All three superimposed low energy structures had Phe and Arg aminoacids in spatial close proximity. The superimposition results revealed that **1** resembled more the stereoelectronic environment of SFLLR than **2**. This difference may be related to their different antagonist efficacy.

Keywords: Amino acids – Thrombin – Tethered ligand – TRAPs – Peptide mimetics – Platelet aggregation

Introduction

Thrombin is able to elicit many cellular responses (e.g. thrombotic, inflammatory, proliferative and atherosclerotic) that are mediated by proteolytic activation of a specific cell surface receptor known as tethered ligand receptor (Vu et al., 1991; Rasmussen et al., 1991; Zhong et al., 1992; Bahou et al., 1993;

McNamara et al., 1993; Glembotski et al., 1993; Park et al., 1994). The thrombin receptor has seven transmembrane-spanning domains and belongs to a family of G-protein-coupled receptors (Vu et al., 1991; Schwartz, 1994). Activation of the receptor occurs by thrombin cleavage of an extracellular N-terminal domain. The new N-terminus through intramolecular interaction activates the receptor (Vu et al., 1991; Coughlin, 1993; Van Obberghen-Schilling and Pouyssegur, 1993; Brass et al., 1994). Synthetic thrombin receptor activating peptides (TRAPs) comprising the 6–14 amino acids of the tethered ligand were found to activate platelets equally with thrombin itself and are considered to be full agonists (Vu et al., 1991; Vassallo et al., 1992; Coller et al., 1992; Chao et al., 1992; Rasmussen et al., 1993). In contrast, only the first five amino acids ($S_{42}FLLR_{46}$) are required for activation of the platelet thrombin receptor (Scarborough et al., 1992; Hui et al., 1992). Structure activity studies (SAR), NMR experiments and molecular modeling have determined the specific requirements for each amino acid in SFLLR (Matsoukas et al., 1997; Natarajan et al., 1995). Furthermore, using cyclic TRAPs it was found that the Phe/Arg relative conformation is important for activity (Matsoukas et al., 1996; Panagiotopoulos et al., 1996). Based on the previous data, small-molecule non-peptide based antagonists are now in the step of development. At this step the essential amino acid side chains (Phe and Arg) are positioned onto a scaffold which is preferably a small, difunctional ring of defined stereochemistry. Such compounds are shown in Scheme 1. These compounds were tested in the platelet aggregation assay as described by Born (1962) and their comformational properties were investigated using Molecular modeling methods (Mavromoustakos et al., 1995).

Experimental

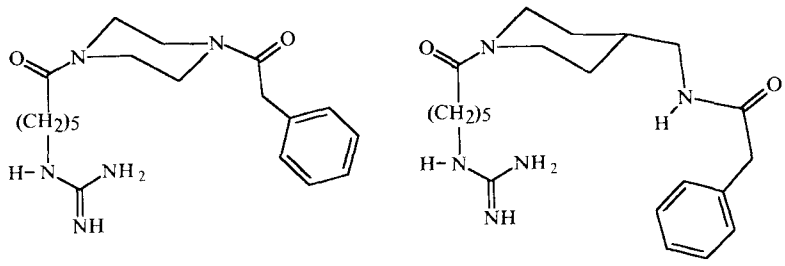
Chemistry

Materials

The synthesis of novel 1-(6-guanidohexanoyl)-4-(phenylacetylamidomethyl)-piperidine (**2**) was carried out as shown in Scheme 2. Briefly, 4-(phenylacetylamidomethyl)-piperidine was synthesized by coupling 4-(aminomethyl)-piperidine and phenylacetic acid with DCC and HOBt as coupling reagents. Boc-ε-Aminohexanoic acid was then incorporated in the NH of piperidine aided by the use of DCC and HOBt under basic conditions (DIEA). Boc-deprotection was accomplished with trifluoroacetic acid giving the free-amine salt. Guanylation of the primary amine using 1*H*-pyrazole-1-carboxamide hydrochloride afforded compound **2**. All synthetic intermediates were purified by flash chromatography (Merck Silica Gel 60, 230–400 mesh) and the final product (**2**) by recrystallization (MeOH/Acetone/Et_2O). The synthesis of 1-phenylacetyl-4-(6-guanidohexanoyl)-piperazine (**1**) was carried out by an analogous procedure.

Methods

All synthetic intermediates and final products **1** and **2** were monitored by thin layer chromatography (TLC) using the following solvent systems: methanol-chloroform (MC) and *n*-butanol-acetic acid-water (4:1:1) (BAW). The purity of compounds **1** and **2** were



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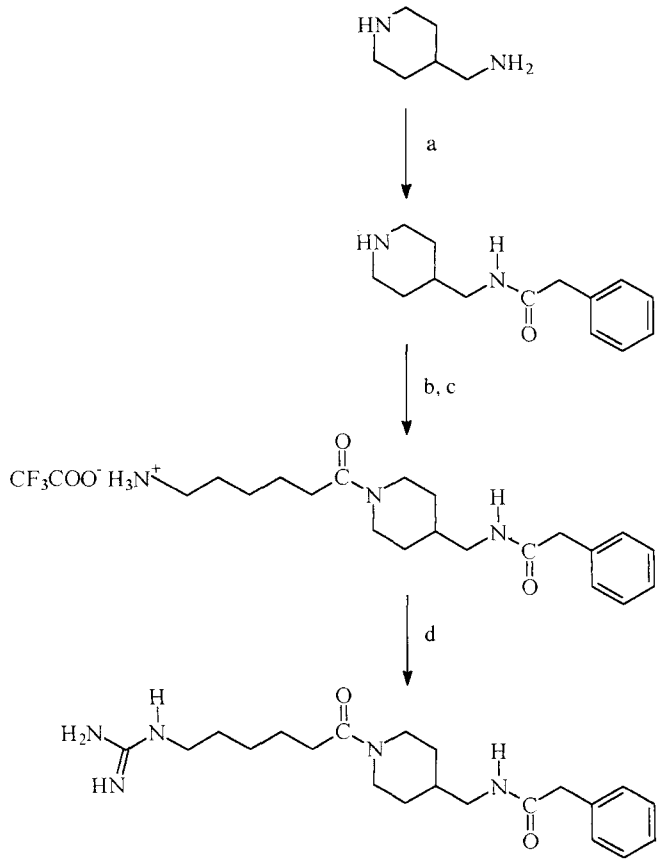
Scheme 1. Thrombin receptor non-peptide mimetics



Scheme 2. Synthesis of 1-(6-guanidohexanoly)-4-(phenylacetylamidomethyl)-piperidine. **a** $C_6H_5CH_2COOH$, HOBt, DCC, $CHCl_3$, DMF, 3h, (70%), **b** Boc-εAhx-OH, HOBt, DCC, DIEA, CH_2Cl_2, 24h, (62%), **c** 30% CF_3COOH/CH_2Cl_2, 1h, (95%), **d** 1H-pyrazole-1-carboxamide hydrohloride, DMF, DIEA, 24h, (53%)

assessed by HPLC. Analytical reversed-phase HPLC was performed with a Waters system equipped with a 600E system controller using a Techsil C-18 reversed-phase analytical column (250 × 4.6 mm) with 5 μm packing material. HPLC runs were carried out with a stepped linear gradient of 0%–50% acetonitrile (0.1% TFA) in water (0.1% TFA) over 30 min at rate of 1 ml/min. Compound **2** (0.1 mg) was dissolved in methanol (5 μl) and this solution was injected using a Waters U6K injector with a 20 μl sample loop. Compound **2** was eluted in 22.5 min as determined simultaneously from the absorbances at 214 and 254 nm (Waters 996 photodiode array detector).

Spectroscopy

FAB MS, ^{1}H NMR

The identity of compounds **1** and **2** was established by mass spectrometry (FAB MS) and ^{1}H NMR. FAB spectra were carried out on a AE1 M29 mass spectrometer with the FAB gun run at 1 mA discharge current and at 8 kV. The obtained mass spectrum of compound **2** showed the following molecular ions: 386 ($M^+ - 1$), 343 [($M^+ - 1$) − $H_2NC(NH)NH^{\bullet}$)]. One- and two-dimensional (ROESY) NMR experiments were performed using a 300 MHz NMR spectrometer. Three milligrams of compound **2** were dissolved in 0.33 ml of d_6-DMSO. Chemical shifts were reported relative to tetramethylsilane (TMS). 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: Matsoukas et al. (1997). The protons assignment was helped by the ROESY spectrum (Fig. 1) and is as follows: δ 0.9 (m, 2H), 1.3 (m, 2H), 1.5 (m, 4H), 1.6 (m, 3H) 2.1 (t, 2H), 2.7 (t of t, 2H), 2.9 (m, 2H), 3.1 (m, 2H), 3.7 (s, 2H), 4.2 (d of d, 2H) 6.9–7.5 (br.s, 3H), 7.2–7.3 (m, 5H), 7.7 (br.s, 1H), 7.8 (t, 1H).

Pharmacology

Platelet aggregation assay

TRAP analogues SFLLR (P5), SFLLR-NH_2 (P5-NH_2), compound **1** and compound **2** were dissolved in phosphate buffered saline (PBS) at concentration 10 mM, aliquoted and stored frozen (−80°C). Platelet aggregation assay was used to study the effect of TRAP analogues on human blood platelets. Briefly, blood was collected into trisodium citrate (0.38% final concentration) and platelet rich plasma was prepared by centrifugation at 200 g for 15 minutes. After adjustment of platelet count of 200,000/μl using autologous platelet poor plasma an 225 μl aliquot was stirred (1000 RPM, 37°C) in cuvette of Chrono-log lumi-aggregometer (Chrono-log, USA) and aggregation induced by 25 μl of TRAP analogue (0, 1, 10, 100, 1000 μM final concentration). If there was not any response, after 2 minutes of recording, the aggregation was induced in the same cuvette using 10 μM of the human TRAP SFLLRNP-NH_2 (P7-NH_2) and inhibition of platelet aggregation in comparison to sample with PBS instead TRAP analogue evaluated. All samples were run in dublicates and the functionality of platelets at the beginning and at the end of every measurement were controlled by evaluation of aggregation after 10 μM of P7-NH_2.

Molecular modeling

Theoretical calculations were performed on a Silicon Graphics 4D/35 model using the Quanta 3.3 version of Molecular Simulations. The constructed structures of compounds **1** and **2** were first minimized. Constraints on the minimized structures were imposed in order to achieve a close proximity between Phe and Arg. The superimposition of pairs of

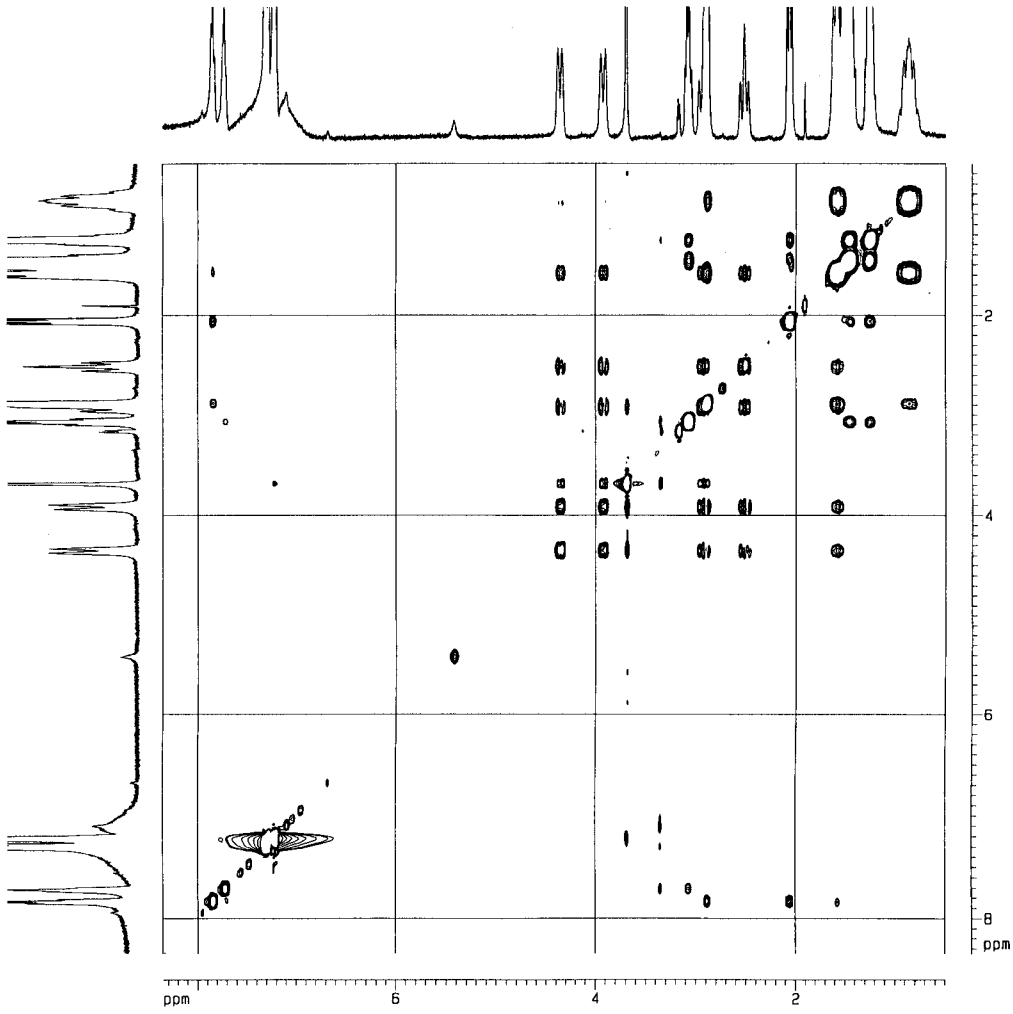


Fig. 1. ROESY spectrum of compound **2**

structures was performed using rigid body fit to target method of molecular similarity software. Rigid body fitting translates and rotates working structures (**1** and **2**) to minimize the RMS (root mean square) of the fit to the target structure (SFLLR). Details of this approach were given in our previous publications (Matsoukas et al., 1996; Mavromoustakos et al., 1995).

Results and discussion

Following the method of rational design cycle described by Adang et al. (1994) and Moore et al. (1995) we have synthesized two novel thrombin receptor derived-peptide analogues. At this stage the previously obtained insights from the study of SFLLR and SFLLR-NH_2 (Matsoukas et al., 1997),

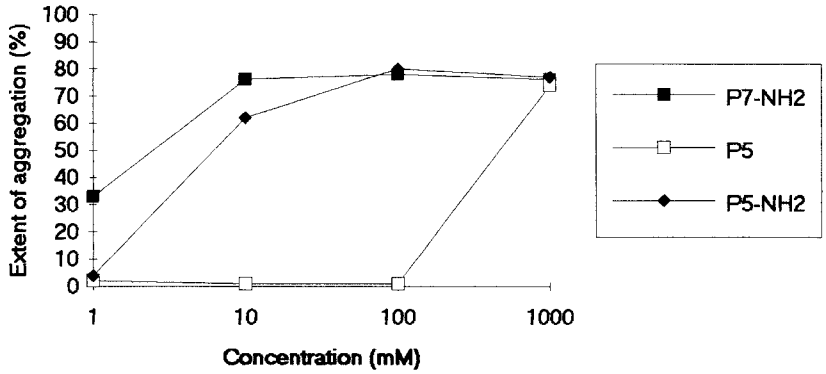


Fig. 2. Comparison of the extent of platelet aggregation after activation with different doses of TRAP analogues: SFLLR, SFLLR-NH_2 and SFLLRNP-NH_2

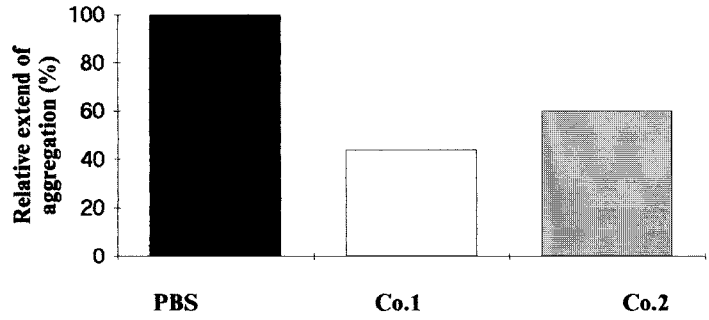


Fig. 3. The effect of incubation of platelets with 1 mM nonpeptide TRAP analogues **1** and **2** on platelet aggregation induced by 10μM SFLLRNP-NH_2 after of platelets. Platelet aggregation was reduced to 44% and to 60% respectively in comparison to platelets incubated with buffer only (*PBS*)

were used to position the pharmacophoric features of the essential amino acid side chains (Phe and Arg) onto a molecular template in a particular spatial order that is in agreement with the previously defined bioactive topology. Two small molecular scaffolds of defined stereochemistry were used: piperazine and 4-(aminomethyl)-piperidine.

The resulting compounds **1** and **2** were evaluated in the platelet aggregation assay. SFLLR and SFLLR-NH_2 were used as controls in this study. Only true TRAP peptides, SFLLR at 1 mM and SFLLR-NH_2 at 100μM concentration, caused full platelet aggregation comparable to 10μM SFLLRNP-NH_2 (Fig. 2). Neither compound **1** nor compound **2** caused notable platelet aggregation even in concentration 1 mM. In contrast, compound **1** (1 mM) caused 56% and compound **2** (1 mM) 40% inhibition of platelet aggregation induced by 10μM SFLLRNP-NH_2 (Fig. 3).

The above findings suggest that both compounds behave as thrombin receptor antagonists. Compound **1** has been also found recently to be active in

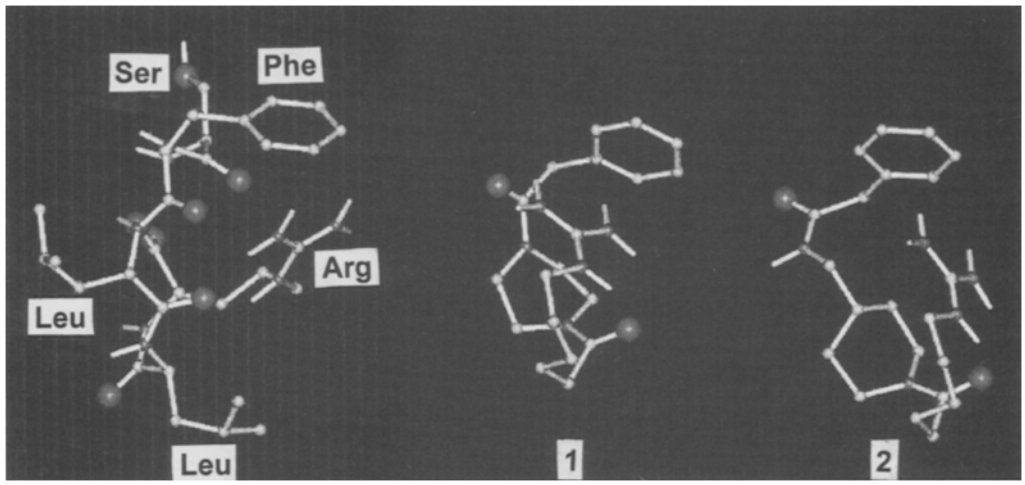


Fig. 4. Low energy conformers generated by imposing distance geometry constraints between Phe and Arg amino acids of SFLLR (left) and synthetic compounds **1** (middle) and **2** (right)

the rat aorta relaxation assay: Alexopoulos et al. (Manuscript in preparation). This finding further supports the suggestion that such compounds interact with the thrombin receptor providing additional information about the thrombin receptor activation mechanism. Thus, the importance of phenyl and guanidino groups for activation of the thrombin receptor is once more indicated. Small molecules bearing the above groups could be drug leads and could be further optimized under suitable modifications to give an orally active antithrombotic drug. The spatial arrangement of Arg/Phe seems to be of similar significance in the synthesized analogues, since both **1** and **2** can adopt equally energetically stable conformers that preserve the ring cluster of Arg and Phe. The low energy conformers of **1** and **2** as well as of SFLLR which is characterized by the spatial close proximity of the key amino acids Phe and Arg are shown in Fig. 4.

To further examine the stereoelectronic factors that determine the biological activity of these compounds the experimental data were coupled with theoretical calculations. Thus, superimposition between active cyclic conformation of SFLLR and the two synthesized compounds was achieved using their minimized conformers. In these conformers we used distance constrains in order to keep in a close proximity the Phe and Arg amino acid moieties. Overlay of **1** and **2** with SFLLR (see Figs. 5 and 6 correspondingly) showed that both compounds could accommodate well their phenyl and guanidino group in a close proximity with the corresponding groups of Phe and Arg of SFLLR (overall RMS deviation 0.2 and 0.38 Å correspondingly). However, the stereoelectronic environment of **1** resembles more that of SFLLR. This is due to the fact that **1** contains piperidine *vis a vis* **2** which is substituted by the less polar ring piperazine. As a consequence of this, there is a mismatch between the electronic environment of **2** at piperazine ring and the equivalent

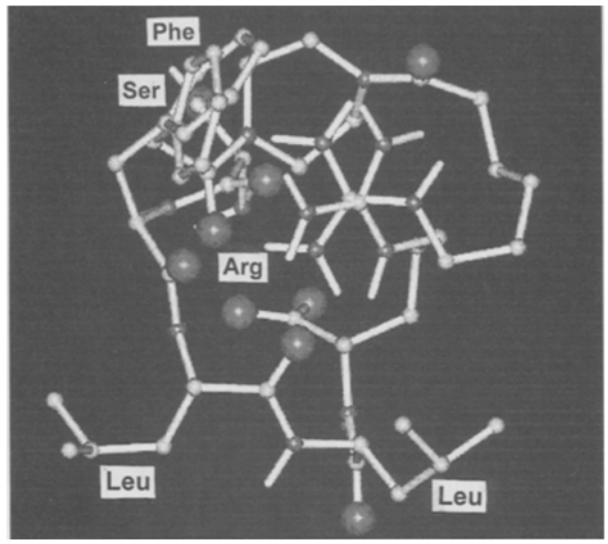


Fig. 5. Superimposition of the synthetic analogue **1** with SFLLR using rigid body fit to target method

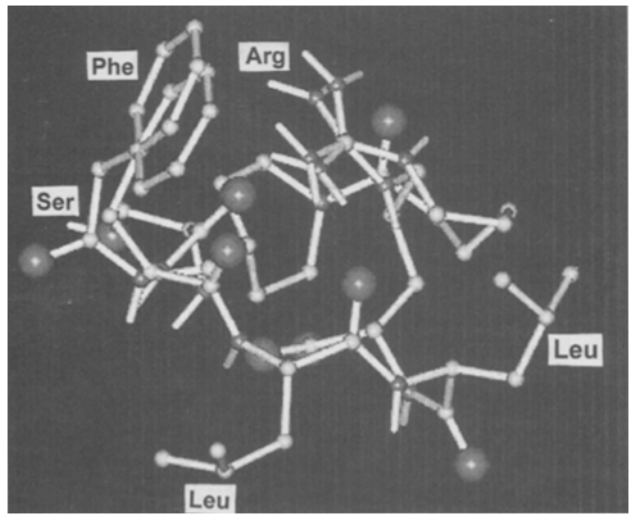


Fig. 6. Superimposition of the synthetic analogue **2** with SFLLR using rigid body fit to target method

in space environment of SFLLR (see Fig. 5). This only significant difference between **1** and **2** synthetic analogues may account for their different antithrombotic activity. A structure activity relationships on the piperidine template may provide further insight on the stereoelectronic requirements for optimum activity of these analogues.

In conclusion, this analysis showed that the design of antithrombotic agents with optimum activity needs both a close spatial relationship between

the important aminoacids Phe and Arg and mimic certain stereoelectronic features of the other aminoacids that constitute SFLLR.

Acknowledgement

This work was supported by European Community (EC) grants (BIOMED program No. 920038, BIOMED-PECO No. 930158, COPERNICUS No. 940238), the Ministry of Energy and Technology of Greece and the IGA MZ Prague, Czech Republic No. 2820-3.

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Received July 5, 1997