Design and synthesis of cationic Aib-containing antimicrobial peptides: conformational and biological studies

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Abstract: Development of antimicrobial peptides has attracted considerable attention in recent years due to the excessive use of antibiotics, which has led to multiresistant bacteria. Cationic amphiphilic Aib-containing peptide models Ac-(Aib-Arg-Aib-Leu)$_n$-NH$_2$, $n = 1$–4, and sequential cationic polypeptides (Arg-X-Gly)$_n$, X = Ala, Val, Leu, were prepared and studied for their antimicrobial and hemolytic activity, as well as for their proteolytic stability. Ac-(Aib-Arg-Aib-Leu)$_n$-NH$_2$, $n = 2$, 3 and the polypeptide (Arg-Leu-Gly)$_n$ exhibited significant antimicrobial activity, and they were nontoxic at their MIC values and resistant, in particular the Aib-peptide models, to enzymatic degradation. The conformational characteristics of the peptide models were studied by circular dichroism (CD). Structure-activity relationship studies revealed the importance of the amphipathic α-helical conformation of the reported peptides in inducing antimicrobial effects. It is concluded that peptide models comprising cationic amino acids (Arg), helicogenic and noncoding residues (Aib) and/or hydrophobic and helix-promoting components (Leu) may lead to the development of antimicrobial therapeutics. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cationic Aib-containing peptides; antimicrobial activity; proteolytic stability; hemolytic assay; circular dichroism

INTRODUCTION

The widespread use of antibiotics, in recent years, has led to the development of antibiotic-resistant microbial strains, resulting in an urgent need for new antibiotics. Antimicrobial peptides are used as the first defensive barrier of the organism against microbial infection. They possess a broad spectrum of antimicrobial activity against gram-positive and gram-negative bacteria, fungi and protozoa [1–3]. It has been shown that some of these peptides exhibit antiviral and anticancer activity, promote wound healing and modulate the innate immune response [4–9].

Currently, more than 700 antimicrobial peptides have been isolated from a wide range of organisms, which are classified, on the basis of their structure, into four major classes: α-helical, β-sheet, loop and extended peptides [1,10]. Most of the natural antimicrobial peptides carry a positive charge and a substantial portion of hydrophobic residues, and adopt an amphipathic conformation with opposing hydrophobic and positively charged faces when they are in contact with the bacterial membranes. Their mode of action is usually the disruption of the bacterial membranes. Two general mechanisms have been proposed to explain the membrane disruption: the 'barrel stave' mechanism and the 'carpet' mechanism [1,11–14]. According to the barrel stave model, the amphipathic peptides orient perpendicular to the membrane and align in a manner in which the hydrophobic side chains face outwards into the lipid environment, while the polar side chains align inwards to form transmembrane pores. These pores disrupt the membrane potential and allow the leakage of cytoplasmic components. In the carpet model, the peptides align parallel to the bilayer and interact with the negatively charged phospholipids of the membrane. Local disturbance in membrane stability causes the formation of large cracks and disintegration of the membrane. On the basis of the above characteristics, peptide models have been designed and studied for the development of new antibiotics. Elements desirable for therapeutics include antimicrobial activity, low toxicity and proteolytic stability [15,16].

It is well documented that incorporation of C^α-tetrasubstituted amino acids like α-aminoisobutyric acid (Aib) into a peptide sequence results in the formation of α- or 3_{10}-helices depending on its length and the relative Aib content. In peptides of eight or more residues, a pure α-helix is observed if Aib does not exceed 50%. Shorter peptides and peptides containing more than 50% Aib prefer the 3_{10} conformation [17–21]. The preference of Aib for specific dihedral angles (φ, ψ) favors α-helical structures of the peptides in the phospholipid membranes [22]. Furthermore, since Aib does not belong to the coded amino acid repertoire, Aib-containing peptides may be more resistant to proteases, overcoming thus one of the common problems of peptides, the poor stability *in vivo*. The relationship between biological functions of Aib-containing peptides (ion transfer, lipid–peptide interaction, antimicrobial activity) and their secondary and/or their super secondary

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structures has been the stimulus for intensive efforts [23–28].

We report now on the design and synthesis of amphipathic Aib-containing peptide models of various chain length, Ac-(Aib-Arg-Aib-Leu)$_n$-NH$_2$, $n = 1$–4, (Ac = acetyl), as antimicrobials. The presence of Aib, which induces and stabilizes α-helical structures, in combination with the positively charged Arg side chains, which may interact with the negatively charged membrane phospholipids, is expected to lead to the development of new antimicrobial therapeutics. Apart from the peptide models that encompass amino-isobutyric acid, cationic α-helical sequential polypeptides (Arg-X-Gly)$_n$, where X = Ala, Val, Leu [29,] were also tested for their antimicrobial activity. High proteolytic stability and low hemolytic activity, a key issue that potentiates the antimicrobial function, were investigated. The conformational characteristics of the peptide models, studied by CD, were evaluated to estimate the structure–activity relationship.

MATERIALS AND METHODS

Peptide Synthesis

The synthesis of the peptides Ac-(Aib-Arg-Aib-Leu)$_n$-NH$_2$ ($n = 1$–4) was carried out by the stepwise solid-phase synthesis procedure SPPS [30–32] on a Rink Amide AM resin (0.72 mmole/g resin) using the Fmoc methodology. Arginine was introduced as Fmoc-Arg(Pbf)-OH (Pbf: 2,2,4,6,7-pentamethyl-dihydrobenzofurane-5-sulfonyl). Fmoc groups were removed using 20% piperidine in DMF. The coupling reactions were performed using an Fmoc-amino acid/HBTU/HOBt/DIEA/resin molar ratio of 3/3/3/9/1. DMF used for couplings was distilled in the presence of ninhydrin to remove traces of amines. Completion of the coupling reactions was ensured by the use of ninhydrin Kaiser test. Acetylation was performed using acetic anhydride in pyridine. The peptides were cleaved from the resin by treatment with TFA/TIS/H$_2$O (95/2.5/2.5). The resin was removed by filtration, the filtrate was evaporated under reduced pressure and the product was precipitated with cold diethyl ether. Yields ranged from 70 to 80%. The crude peptides were purified by semipreparative reverse-phase HPLC on a C$_{18}$ column. Appropriate programmed gradients were applied using eluants A (H$_2$O/0.1%TFA) and B (CH$_3$CN/0.1%TFA). The purity of the peptides was checked by analytical HPLC and the correct molecular masses were confirmed by ESI-MS.

Ac-Aib-Arg-Aib-Leu-NH$_2$, expected M$^+$:

499.6, found M$^+$: 499.75.

Ac-(Aib-Arg-Aib-Leu)$_2$-NH$_2$, expected M$^+$:

939.13, found M$^+$: 939.50.

Ac-(Aib-Arg-Aib-Leu)$_3$-NH$_2$, expected M$^+$:

1380.68, found M$^+$: 1378.90.

Ac-(Aib-Arg-Aib-Leu)$_4$-NH$_2$, expected M$^+$:

1821.22, found M$^+$: 1818.39.

The sequential polypeptides (Arg-X-Gly)$_n$, where X = Ala, Val, Leu, were synthesized by polymerization of the penta-chlorophenyl esters of the appropriate tripeptides as reported in a previous study. Molecular weight of the polypeptides ranges from 12 000 to 20 000 and the estimated n from 40 to 67 [29].

Circular Dichroism

The CD spectra were recorded at 25 °C on a Jasco J-710 spectropolarimeter using a 0.1-cm path length quartz cell and at a peptide concentration 10^{-4} M. Spectra were obtained from 260 to 180 nm by signal-averaging three scans at a scan speed of 50 nm/min. Experiments were performed in a phosphate buffer (pH 7.4), as well as in TFE/H$_2$O (trifluoroethanol/water) mixtures and in the presence of SDS. The CD spectra were smoothed after subtraction of the solvent contribution. All CD spectra are reported in terms of ellipticity units per mole of peptide residue, $[\theta]$ in deg cm^2/dmol. The CD deconvolution (CDNN program) described by Bohm *et al.* [33] was used for estimating the percentage helical contact according to the Neural Network Analysis method. The CD spectra data analysis was performed on the basis of the program's protein reference set (option: Net using 13 basespectra).

BIOLOGICAL ASSAYS

Antimicrobial Activity

The peptides were tested for their antimicrobial activity against gram-negative bacteria *Escherichia coli* DH5a, *Pseudomonas aeruginosa* PAO and *Zymomonas mobilis* ATCC 10988, and gram-positive bacteria *Mycobacterium smegmatis* mc^{2}155 and *Bacilus subtilis* DELTA. *E. coli* is a representative of potent pathogens, and *P. aeruginosa* and *Z. mobilis* possess natural multidrug resistance. The selection of the gram-positives was based on differences of their membrane lipid components. A suspension of 2×10^7 log phase cell/ml obtained from microorganisms grown in the appropriate culture medium was aliquoted into microtubes and varying amounts of peptide stock solution (5–500 μg/ml) were added. Control microtubes contained all the components except the peptide. The experiments were done in triplicate. Inhibition of growth was determined by measuring the absorbance at 600 nm after incubation for 24 h at the appropriate temperature for each microorganism. The antimicrobial activities were expressed as the MIC at which 100% inhibition of growth was observed [34,35].

Hemolytic Assay and Proteolytic Stability

The hemolytic activity of the peptides was determined using fresh human red blood cells (RBC) [34]. Peptide concentrations causing 50% hemolysis (EC$_{50}$) were derived from the dose–response curve.

For studying their proteolytic stability, peptides were dissolved in PBS buffer, pH 7.4, and aliquoted

into Eppendorf tubes. Trypsin was mixed with the peptides in the ratio 1 : 250 (enzyme/peptide, w/w). The enzymatic degradation of peptides by trypsin was carried out at 37 °C for 4 h and stopped by adding a trypsin inhibitor type II-S, soybean (Sigma) at different time points. The samples were tested for their residual antimicrobial activity and were also analyzed using RP-HPLC [35,36].

RESULTS AND DISCUSSION

Conformational Study by CD

CD experiments on the peptides Ac-(Aib-Arg-Aib-Leu)$_n$-NH$_2$ ($n = 1–4$) were performed in phosphate buffer (PBS) (pH 7.4), as well as in TFE/H$_2$O mixtures (from 0 to 100%) and SDS (concentrations ranged
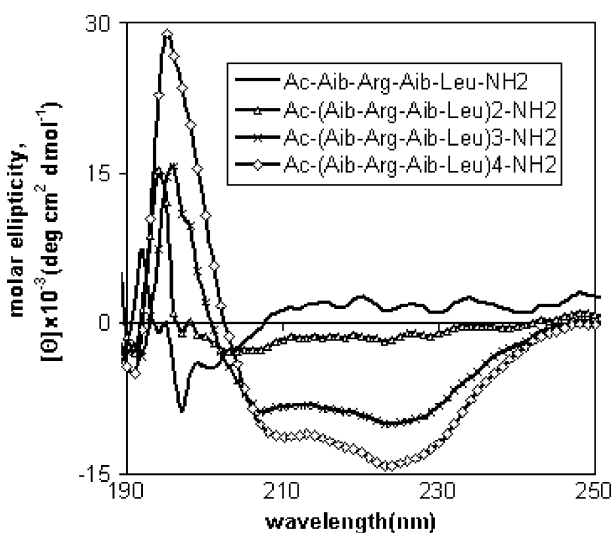
Figure 3 CD spectra of Ac-(Aib-Arg-Aib-Leu)$_n$-NH$_2$, $n = 1–4$, (100 μM) in 5 mM SDS.

Figure 1 CD spectra of Ac-(Aib-Arg-Aib-Leu)$_n$-NH$_2$, $n = 1–4$, (100 μM) in phosphate buffer (pH 7.4).
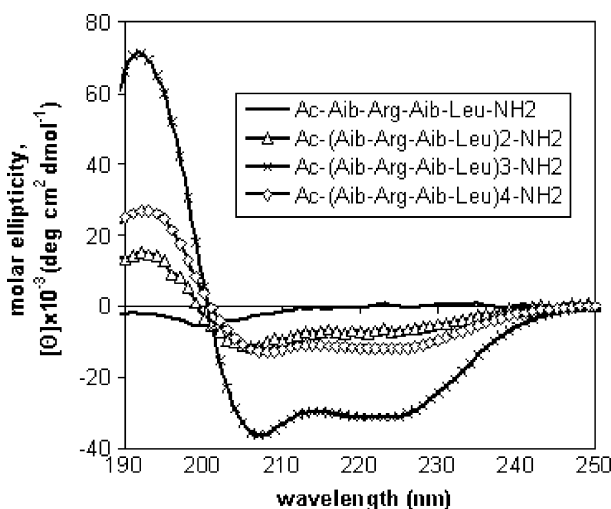
Figure 2 CD spectra of Ac-(Aib-Arg-Aib-Leu)$_n$-NH$_2$, $n = 1–4$, (100 μM) in TFE/H$_2$O (50/50).
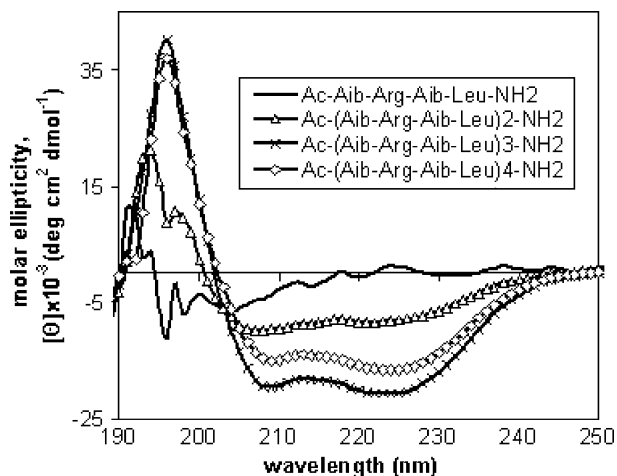
from 5 to 11 mM), which mimics the amphiphilic environment of the membranes. In Figure 1 the CD spectra of the peptides Ac-(Aib-Arg-Aib-Leu)$_n$-NH$_2$, $n = 1–4$, at 100 μM in phosphate buffer (pH 7.4) are shown. The peptides with $n = 3$ and 4 exhibited a positive band at 195 nm and two negative bands at 208 and 222 nm, typical of helical structures, showing that their helical content increased following the peptide elongation. The helical conformations of the peptides in TFE/H$_2$O (50/50, v/v) (Figure 2) increased substantially, compared to those in the phosphate buffer solutions. TFE favors the formation of more ordered conformations by lowering the dielectric constant of the dissolving medium and has been widely used as an α-helix-inducing cosolvent [12,19]. The percentage helical content of the 8-, 12- and 16-peptide was estimated by the CDNN program to be 30, 93 and 42% respectively. The ratio (R) of the negative bands of the CD spectrum, where $R = [\theta]_{n \to \pi^*}/[\theta]_{\pi \to \pi^*}$ is a generally accepted parameter used to distinguish a 3_{10}-helix from an α-helix. For an α-helix $R \approx 1$, while for a 3_{10}-helix $R \approx 0.4$ [17,21]. The ratio R for the 8-, 12- and 16-peptides are 0.68, 0.86 and 0.92 respectively, corresponding to 14% α-helix, 16% 3_{10}-helix (8-peptide); 71% α-helix, 22% 3_{10}-helix (12-peptide); and 36.5% α-helix, 5.5% 3_{10}-helix (16-peptide).

The effect of SDS, a membrane mimetic, on the conformation of the peptides was also studied (Figure 3). Upon addition of SDS at a concentration of 5 mM, below the CMC (8 mM), the helical characteristic bands appeared, indicating interaction with SDS, even below CMC, which brings out the α-helical character of the peptides. As estimated by the CDNN program, the percentage helical content of the 8-, 12- and 16-peptide is 27, 55 and 46% respectively. In fact, the ellipticity remained practically unchanged at 8 and 11 mM

Table 1 Minimum inhibitory concentration, MIC (µg/ml) of the peptides against various microorganisms

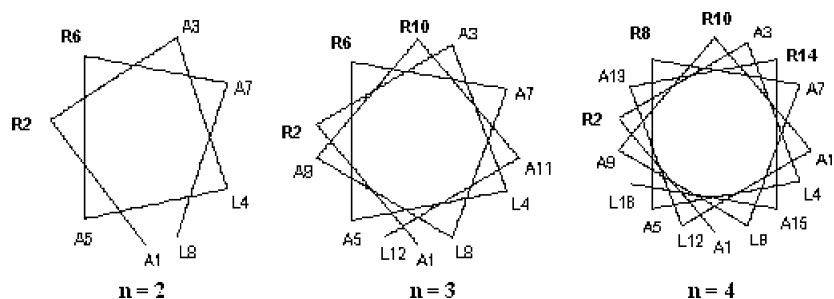| Microorganisms Peptides	*E. coli* DH5a	*P. aeruginosa* PAO	*Z. mobilis* ATCC 10988	*M. smegmatis* mc^{2}155	*B. subtilis* DELTA
Ac-Aib-Arg-Aib-Leu-NH$_2$	—	—	—	—	—
Ac-(Aib-Arg-Aib-Leu)$_2$-NH$_2$	250	—	—	50	50
Ac-(Aib-Arg-Aib-Leu)$_3$-NH$_2$	150	500	500	300	5
Ac-(Aib-Arg-Aib-Leu)$_4$-NH$_2$	—	—	—	—	—
(Arg-Ala-Gly)n	—	—	—	—	—
(Arg-Val-Gly)n	—	—	—	150	—
(Arg-Leu-Gly)n	150	—	—	5	—

(–): Not active.

Figure 4 Schiffer and Edmundson α-helical wheel projections of the 8-, 12- and 16-peptides. The nonproteinogenic amino acid Aib was replaced by the closest coded analog Ala (regarding size and physico-chemical properties).

SDS (not shown), suggesting that the micelle microenvironment at 5 mM is sufficient to induce maximum helical conformation to the peptides. It deserves to be mentioned that the helical content of the 16-peptide in TFE/H$_2$O (50/50) and SDS is lower than that of the 12-peptide.

CD conformational studies of the polypeptides (Arg-X-Gly)$_n$, where X = Ala, Val, Leu in various TFE/H$_2$O mixtures and in the presence of SDS (not shown) indicated that the degree of α-helical conformation of the polypeptides increased in the order Ala $\rightarrow$ Val $\rightarrow$ Leu and ranges from 20 to 30%.

BIOLOGICAL EXPERIMENTS

Antimicrobial Activity

The MIC of the peptides against gram-negative bacteria, *E. coli* DH5a, *P. aeruginosa* PAO and *Z. mobilis* ATCC 10988, and the gram-positive bacteria *M. smegmatis* mc^{2}155 and *B. subtilis* DELTA, are listed in Table 1.

Differences in antibacterial activities against the used strains could be due to the particular composition of the cell envelope of each selected strain. Gram-negative bacterial envelopes are complex structures composed of an inner and an outer membrane. On the contrary, gram-positive bacterial envelopes possess only an inner membrane, which could explain the best activity of the peptides against the gram-positive bacteria.

The Aib-containing 4-peptide, which has no α-helical conformation, was completely inactive against all the tested bacteria. Elongation of the chain length resulted in an increase of the activity, with MIC values of 50 and 5 µg/ml against gram-positive bacteria for the 8-peptide and 12-peptide, respectively, in accordance to their increasing α-helical content. Further extension of the chain length to 16 residues did not improve the antimicrobial activity. This peptide has lower α- and 3_{10}-helical content compared to the 12-peptide, and also, according to the Schiffer and Edmundson [37] helical wheel projection of the peptides (Figure 4), the 16-peptide, contrary to 8- and 12-peptide, cannot form an amphipathic helix. One may assume that the amphipathic α-helical conformation of the 8- and 12- peptides with opposing hydrophobic and positively charged faces favors the disruption of the bacterial membranes according to the 'barrel stave' model [1].

The (Arg-Ala-Gly)$_n$ polypeptide was completely inactive against the tested bacteria and the (Arg-Val-Gly)$_n$ polypeptide showed antimicrobial activity with MIC = 150 µg/ml against gram-positive bacteria, whereas the (Arg-Leu-Gly)$_n$ polypeptide exhibited high antimicrobial activity against gram-positive bacteria with a MIC = 5 µg/ml. It seems likely that the occurrence of Leu, a hydrophobic and α-helix-inducing component, enhances the α-helical conformation of (Arg-Leu-Gly)$_n$ compared to the rest of the polypeptides and accommodates its antimicrobial activity.
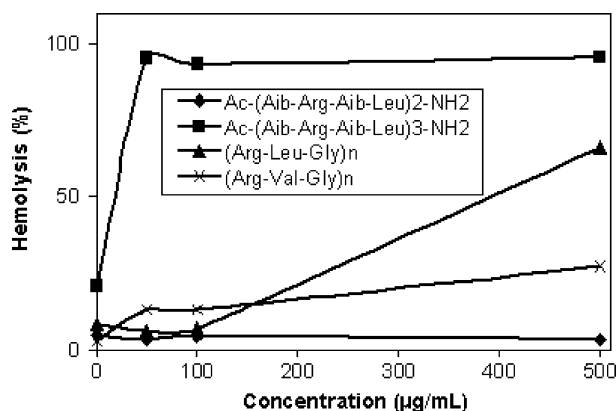
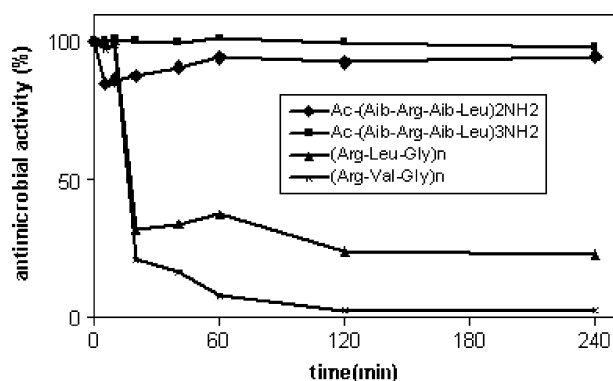**Figure 5** Hemolytic activity of the peptides.

Figure 6 Antimicrobial activity of peptides by trypsin treatment.

Hemolytic Assay

Peptides that exhibited sufficient antimicrobial activity were also tested for their toxicity against erythrocytes (Figure 5). Ac-(Aib-Arg-Aib-Leu)$_2$-NH$_2$ and (Arg-Val-Gly)$_n$ were nontoxic to erythrocytes, while (Arg-Leu-Gly)$_n$ exhibited low hemolytic activity at high concentrations. Ac-(Aib-Arg-Aib-Leu)$_3$-NH$_2$, which displays high antimicrobial activity (MIC = 5 µg/ml), was toxic at higher concentrations (EC$_{50}$ ≈ 25 µg/ml).

Lack of hemolytic activity of 8-peptide and (Arg-Val-Gly)$_n$ is possibly due to the differences between bacterial and erythrocyte membranes. The former possesses an anionic nature susceptible to lysis by cationic antimicrobial peptides, whereas the zwitterionic nature of the latter perhaps in this case renders their resistance to lysis. However, the 12-peptide, which exhibits higher antimicrobial activity, causes some hemolysis attributed to its enhanced hydrophobic content.

Proteolytic Stability

The proteolytic stability of the peptides to trypsin was determined at 37 °C for 4 h (Figure 6). The enzyme-treated samples collected at different time points after adding an appropriate enzyme inhibitor were tested for

their residual antimicrobial activity. The activity of the trypsin-treated 12- and 8-peptides was 100 and 90% of the control after 4 h, respectively. No degradation was observed by analyzing the trypsin-treated samples of the 8- and 12-peptides by RP-HPLC. These results suggest that the presence of Aib plays a crucial role in the proteolytic stability of the peptides.

In contrast, the antimicrobial activity of the (Arg-Leu-Gly)$_n$ decreased to 25–30% of the untreated control after 20 min and remained at about 20% of the control after 4 h, while the (Arg-Val-Gly)$_n$ lost all of its antimicrobial activity after 1 h.

CONCLUSIONS

Cationic amphiphilic Aib-containing peptide models Ac-(Aib-Arg-Aib-Leu)$_n$-NH$_2$, $n = 1$–4, and sequential cationic polypeptides (Arg-X-Gly)$_n$, X = Ala, Val or Leu, were prepared and studied for their antimicrobial activity, hemolytic activity and proteolytic stability.

The Ac-(Aib-Arg-Aib-Leu)$_3$-NH$_2$ peptide exhibited high antimicrobial activity against gram(+) bacteria (MIC = 5 µg/ml) and proteolytic stability, and it was toxic at concentrations higher than the MIC (EC$_{50}$ = 25 µg/ml). Ac-(Aib-Arg-Aib-Leu)$_2$-NH$_2$ peptide showed lower antimicrobial activity (MIC = 50 µg/ml) compared to the 12-peptide and it was stable to proteolysis and nontoxic. Among the cationic polypeptides, (Arg-Leu-Gly)$_n$ was the most effective antimicrobial (MIC = 5 µg/ml) and was less stable to enzymatic degradation compared to the Aib-containing peptides, with low toxicity.

Structure–activity correlations pointed out that the enhanced α-helical characteristics of the peptides studied by CD spectroscopy, attributed to the highly helicogenic Aib and/or to the presence of Leu, an α-helix inducer, strongly contribute to their antimicrobial properties. Furthermore, it is presumed that the amphipathic α-helical character of Ac-(Aib-Arg-Aib-Leu)$_n$-NH$_2$, $n = 2,3$, has a synergistic effect on the antimicrobial activity of the peptides. Consistent with this assumption is the fact that the absence of amphipathicity of Ac-(Aib-Arg-Aib-Leu)$_4$-NH$_2$ abrogates any possible antimicrobial function of the peptide.

It is concluded that Ac-(Aib-Arg-Aib-Leu)$_2$-NH$_2$, which exhibits high antimicrobial activity, is stable to proteolysis and non toxic, and constitutes a leader compound for the elaboration of antimicrobial therapeutics.

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