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Small Peptides Derived from Penetratin as Antibacterial Agents

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The synthesis, *in vitro* evaluation and conformational study of several small-size peptides acting as antibacterial agents are reported. Among the compounds evaluated, the peptides Arg-Gln-Ile-Lys-Ile-Trp-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH_2, Arg-Gln-Ile-Lys-Ile-Arg-Arg-Met-Lys-Trp-Arg-NH_2, and Arg-Gln-Ile-Trp-Trp-Trp-Trp-Gln-Arg-NH_2 exhibited significant antibacterial activity. These were found to be very active antibacterial compounds, considering their small molecular size. In order to better understand the antibacterial activity obtained for these peptides, an exhaustive conformational analysis was performed, using both theoretical calculations and experimental measurements. Molecular dynamics simulations using two different media (water and trifluoroethanol/water) were employed. The results of these theoretical calculations were corroborated by experimental circular dichroism measurements. A brief discussion on the possible mechanism of action of these peptides at molecular level is also presented. Some of the peptides reported here constitute very interesting structures to be used as starting compounds for the design of new small-size peptides possessing antibacterial activity.

Keywords: Antibacterial activity / Peptide syntheses / Rational drug design

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Introduction

Although there are numerous antibacterial agents available in the therapeutic armamentarium, undoubtedly, one main issue that must be addressed is the problem of resistance to the most traditional antibacterial agents. One of the WHO global strategies for the control of antimicrobial resistance is to encourage development of appropriate new drugs and vaccines [1]. This strategy has resulted in the identification of novel molecules which could be useful for future developments. Among them, some natural peptides have been reported as antimicrobial compounds; some of them have shown capability to inhibit a broad spectrum of pathogenic microorganisms [2–5]. In fact, the antibacterial properties of peptides have been studied for nearly five decades, but during the past 15 years, interest in their antibacterial nature

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has increased due to the following reasons: potential for broad-spectrum activity, rapid bactericidal activity, and low propensity for resistance development, whereas possible disadvantages include their high cost, limited stability (especially when composed of L-amino acids), and lack of knowledge about their toxicology and pharmacokinetics. Initial barriers to their success are being increasingly overcome with the development of stable, more cost-effective, and potent broad-spectrum synthetic peptides [6].

Antimicrobial peptides (AMPs) vary widely in their sources; although most are of natural origin, there is an increasing number of semisynthetic or totally synthetic compounds under study. These are linear or cyclic structures with hydrophobic or amphipathic properties, acting by lysis [7], by binding to, and by disruption of the outer membrane. Others penetrate the membrane and interact with specific internal targets or cause pore formation resulting in leakage of important intracellular material [8].

We are particularly interested in the small cationic peptides [9, 10] which have been described as "nature's antimicrobials" or "cationic antimicrobial peptides". These generally possess 12 to about 50 amino acids with 2–9 positively charged lysine or arginine residues and up to 50% hydrophobic amino acids. These peptides are folded in three dimensions so that they have both a hydrophobic face comprising non-polar amino acids and a hydrophilic face of polar and positively charged residues, and consequently these molecules have amphipathic nature.

The physicochemical properties of these peptides may play a key role in producing their antibacterial effects, so far it is not clear what are the most important properties for this purpose. For example, several bioinformatics methods have been proposed to identify and predict the activity of AMPs based on simple descriptors like hydrophobicity, amphipathicity, charge, or helicity. These have been partially successful but the lack of connection to a physical mechanism limits their applicability and ultimate usefulness [11]. On the other hand, the experiments have revealed that small changes in the primary structure of a peptide may lead to drastic changes in its specificity and activity [12]. In turn, other authors have observed that features of the sequence of longer peptides contribute to the predictability of peptide bioactivity, whereas in shorter peptides, the predictive power is more dominated by amino acid composition [13].

Penetratin is a synthetic peptide possessing 16 amino acids from the third helix of Antennapedia homeodomain [14]. It is a cationic amphipatic peptide which might penetrate cell membrane via the postulated "inverted micelle" pathway. However, the mechanism of membrane translocation is not well known to date. The question is whether the peptide is internalized via endocytosis which is energy-dependent or via direct transport. While the latter mechanism is scarcely known at present, it is believed that the process is non-receptor-mediated [15–17]. Penetratin has been proposed as a universal intracellular delivery vehicle [18].

Our research group was the first to report the antifungal activity of penetratin [19] and also that of some peptides structurally related to this well-known cell-penetrating peptide [20, 21]. Bahnsen et al. [22] reported that penentratin and some of its analogs also possess antibacterial activity. More recently, we reported the antibacterial activity of several peptides structurally related to penetratin [23]. Among them, peptides Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met(O)-Lys-Trp-Lys-Lys-NH_2 and Arg-Gln-Ile-Lys-Ile-Phe-Phe-Gln-Asn-Arg-Arg-Met(O)-Lys-Phe-Lys-Lys-NH_2 displayed the most interesting inhibitory effect against *Escherichia coli* ATCC 25922, LM_1-*E. coli*, LM_2-*E. coli*, MI-*Salmonella enteritidis*, and *Salmonella* sp. (LM). Another significant contribution of this paper was a detailed conformational and electronic study which helped us to identify a possible "biologically relevant conformation" for these peptides. Our theoretical and experimental results indicated that a particular combination of cationic and hydrophobic residues adopting a definite spatial ordering appears to be the key parameter for the transition from hydrophilic to hydrophobic phase, which could be a necessary step to produce the antibacterial activity in these small-sized peptides. However, one of the limitations of our previous work was that we were able to obtain just only one peptide with nine residues, having a significant antibacterial activity.

Having interesting structural information and based on these starting sequences, our challenge now is to find new peptides with stronger antibacterial activities, but, at the same time, maintaining the small size of these compounds. In fact, one of our goals is to reduce the size of the peptide, but preserving or enhancing the antibacterial effect.

Here, we report a new series of small peptides possessing 13, 11, and also 9 amino acids that have a strong antibacterial activity. To our knowledge, these peptides are the most active small peptides reported until now. In addition to their synthesis, we also report a conformational study using molecular dynamics simulations and experimental studies by using circular dichroism (CD). A brief discussion of their possible mechanism of action is also included at the end of this work.

Results and discussion

Previous studies have shown that the C-terminal segment of penetratin (10–7-mer analogs) was necessary and sufficient for efficient cell membrane translocation [25]. Indeed, Fischer et al. [24] have shown that successive truncation from the N-terminus of penetratin until the 7-mer analog is sufficient for this purpose. Therefore, in principle, it seems reasonable to consider that this property could be associated in some way with the antibacterial activity in this type of peptides. Thus, as a first approximation to obtain new small size antibacterial peptides, we synthesized peptide **1** (Table 1). This peptide has the structural basis of penetratin, in which three residues were removed (F, Q, and N). It is noteworthy that

Table 1. Antibacterial activity of peptides derived from penetratin.

Sequences	MIC[a] (μg/mL)							
	S.a. (ms)	S.a. (mr)	E.c.	LM1-E.c.	LM2-E.c.	PI-Y.e.	MI-S.e.	S. sp. (LM)
Arg-Gln-Ile-Lys-Ile-Trp-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH_2 (**1**)	50	25	6.25	6.25	5	6.25	12.5	5
Arg-Gln-Ile-Lys-Ile-Trp-Arg-Arg-Met(O)-Lys-Trp-Lys-Lys-NH_2 (**2**)	>50	50	12.5	6.25	5	12.5	12.5	12.5
Arg-Gln-Ile-Lys-Ile-Arg-Arg-Met-Lys-Trp-Arg-NH_2 (**3**)	>50	50	12.5	6.25	5	6.25	50	5
Arg-Gln-Ile-Lys-Ile-Arg-Arg-Met(O)-Lys-Trp-Arg-NH_2 (**4**)	>50	50	25	12.5	6.25	50	25	25
Arg-Gln-Ile-Ile-Arg-Arg-Met-Trp-Arg-NH_2 (**5**)	>50	>50	>50	25	25	50	>50	>50
Arg-Gln-Ile-Ile-Arg-Arg-Met(O)-Trp-Arg-NH_2 (**6**)	>50	>50	>50	50	50	50	>50	>50
Arg-Gln-Ile-Ile-Arg-Arg-Met-Gln-Arg-NH_2 (**7**)	>50	>50	>50	>50	>50	>50	>50	>50
Arg-Gln-Ile-Ile-Arg-Arg-Ile-Gln-Arg-NH_2 (**8**)	>50	>50	>50	>50	>50	50	>50	>50
Arg-Gln-Ile-Arg-Arg-Trp-Trp-Gln-Arg-NH_2 (**9**)	50	25	25	12.5	12.5	25	25	25
Arg-Gln-Ile-Arg-Arg-Trp-Trp-Met(O)-Arg-NH_2 (**10**)	>50	>50	25	6.25	12.5	12.5	12.5	12.5
Arg-Gln-Ile-Arg-Arg-Trp-Trp-Met-Arg-NH_2 (**11**)	>50	>50	>50	>50	>50	>50	>50	>50
Arg-Gln-Ile-Arg-Arg-Ile-Ile-Gln-Arg-NH_2 (**12**)	>50	>50	>50	>50	>50	50	>50	>50
Arg-Gln-Ile-Trp-Trp-Trp-Trp-Gln-Arg-NH_2 (**13**)	50	>50	6.25	6.25	6.25	25	12.5	6.25
Cefotaxime	0.5	0.5	0.5	5	0.5	0.5	12.5	0.5

[a] The minimal inhibitory concentrations (MIC) of the peptides were determined in lM ($n = 3$) for S.a. (ms), *Staphylococcus aureus* methicillin-sensitive ATCC 29213; S.a. (mr), *Staphylococcus aureus* methicillin-resistant ATCC 43300; E.c., *Escherichia coli* ATCC 25922; LM1-E.c., LM1-*Escherichia coli*; LM2-E.c., LM2-*Escherichia coli*; PI-Y.e., PI-*Yersinia enterocolitica*; MI-S.e., MI-*Salmonella enteritidis*; S. sp. (LM), *Salmonella* sp. (LM).

this compound maintains the 7-mer peptide in its sequence. This peptide showed strong antibacterial activity against all species tested. Specifically, this peptide displayed a strong antibacterial activity against *E. coli* ATCC 25922, LM1-*E. coli*, LM2-*E. coli*, *Salmonella* sp. (LM), PI-*Yersinia enterocolitica*, and MI-*S. enteritidis*, with MICs values from 5 to 12.5 μg/mL. It should be noted that this peptide also exhibited antibacterial activity against methicillin-resistant *Staphylococcus aureus* ATCC 43300, although in this case its activity was only moderate (Table 1). In order to gain insight about their activity spectrum, peptide **1** was tested against a second panel of clinical isolates of *E. coli* (Table 2), which were moderately inhibited with MIC values between 12.5 and 50 μg/mL. The *E. coli*-784A was the most susceptible strain (MIC = 12.5 μg/mL).

In our previous work [23], we reported the octapeptide Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH_2, which is the 8-mer analog of penetratin. This shortened penetratin analog was completely inactive, and therefore, the comparison between both results, the ones previously reported and the ones obtained here (peptide **1**), clearly indicates that the presence of the 7-mer peptide in the sequence might be a structural

Table 2. Antibacterial activity of peptides 1–4, 10, and 13 against a panel of clinical isolates of *E. coli*.

Sequences	*E. coli* clinical isolates, MIC[a] (μg/mL)							
	E.c.-3	E.c.-15	E.c.-121	E.c.-122	E.c.-712	E.c.-782A	E.c.-784A	E.c.-786H
Arg-Gln-Ile-Lys-Ile-Trp-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH_2 (**1**)	25	25	25	50	25	25	12.5	25
Arg-Gln-Ile-Lys-Ile-Trp-Arg-Arg-Met(O)-Lys-Trp-Lys-Lys-NH_2 (**2**)	50	50	50	>50	25	50	12.5	25
Arg-Gln-Ile-Lys-Ile-Arg-Arg-Met-Lys-Trp-Arg-NH_2 (**3**)	50	50	>50	50	>50	25	25	50
Arg-Gln-Ile-Lys-Ile-Arg-Arg-Met(O)-Lys-Trp-Arg-NH_2 (**4**)	50	50	>50	>50	50	50	50	>50
Arg-Gln-Ile-Arg-Arg-Trp-Trp-Met(O)-Arg-NH_2 (**10**)	50	50	>50	>50	50	50	12.5	>50
Arg-Gln-Ile-Trp-Trp-Trp-Trp-Gln-Arg-NH_2 (**13**)	>50	>50	>50	>50	50	>50	12.5	50
Cefotaxime	0.1	0.1	0.1	2.5	0.05	0.5	0.1	0.5

[a] The minimal inhibitory concentrations (MIC) of the peptides were determined in lM ($n = 3$) for E.c.-3, *Escherichia coli*-3; E.c.-15, *Escherichia coli*-15; E.c.-121, *Escherichia coli*-121; E.c.-122, *Escherichia coli*-122; E.c.-712, *Escherichia coli*-712; E.c.-782A, *Escherichia coli*-782A; E.c.-784A, *Escherichia coli*-784A; E.c.-786H, *Escherichia coli*-786H.

requirement necessary but not sufficient by itself to produce the antibacterial effect.

Since **1** is a peptide derived from penetratin having the highest antibacterial activity obtained so far, we decided to take its sequence as a starting structure. We previously reported that an increase of polarity enhances the antibacterial activity. In consequence, we decided to replace Met residue in **1** by Met(O) (methioninesulfoxide or methionine sulfone) which yielded compound **2** (Table 1). While this peptide showed antibacterial activity, an increase in activity over its predecessor was not observed. In order to get smaller peptides but maintaining their antibacterial effect, we synthesized peptide **3**. This compound has 11 residues and was obtained by removing a Trp residue and the terminal Lys residue. In addition, as it has been reported that Arg is better than Lys for antibacterial effect [25], we decided to replace Lys by Arg (see the terminal portion of **3**). Peptide **3** showed a powerful antibacterial activity, being one of the small-sized peptides with the highest antibacterial effect reported so far. It should be noted that this peptide possesses six cationic residues. To increase its polarity, we substituted the Met residue by Met(O) obtaining peptide **4**. The antibacterial activity of peptide **4** was similar to **3**, but in some bacteria its effect was somewhat weaker. In an attempt to further reduce the molecular size of peptide **3** we removed two Lys residues, producing peptide **5** which possesses nine residues, four of them of cationic nature (charge +4). Unfortunately, peptide **5** has lower antibacterial activity compared to **3**. Even weaker activities were obtained for compound **6**, which was produced by the replacement of Met by Met(O) in peptide **5**. Trying to perform other structural modifications on **5**, we obtained compounds **7** and **8**, in which we substituted Trp by Gln and Met by Ile, respectively. None of these compounds showed significant antibacterial activity. Thus, we arrived at a situation of stagnation, since we could not improve the antibacterial activity of these peptides, maintaining or decreasing the number of amino acids in their sequences. At this stage of our study, we decided to change the starting structure and we chose the nonapeptide Arg-Gln-Ile-Arg-Arg-Trp-Trp-Gln-Arg-NH_2 (**9**) reported in our previous paper [23], which was the compound with the strongest antibacterial activity in that series. In the same study, we reported that a single amino acid substitution of Met by Met(O) in some compounds increased the antibacterial activity. So, first we replaced Gln by Met(O) in order to obtain peptide **10**. This compound exhibited strong antibacterial activity, which was significantly enhanced with respect to its predecessor. To confirm that the presence of Met(O) residue is an important structural requirement for **10**, we substituted Met(O) by Met, obtaining compound **11**. This compound was practically inactive, clearly indicating the importance of Met(O) residue in the sequence of peptide **10** to produce its antibacterial effect. On the other hand, several previous studies have reported the importance of the presence of Trp in this type of antibacterial peptides. To verify this hypothesis in this type of peptides, two Trp residues of **9** were replaced by Ile, obtaining compound **12**, which was totally inactive. These results are in complete agreement with those reported in Refs. [26] and [27]. Based on these results, we decided to replace two Arg by Trp residues. By doing so, nonapeptide **13** was obtained, showing a strong antibacterial activity; it was, in fact, one of the most active peptides in this series with similar effect to those obtained for peptides **1** and **3**.

In order to further understand the above experimental results, a conformational study of the peptides reported here was performed using different approaches. These results are presented in the following section.

Conformational analysis and possible mechanism of action

Based on the results obtained from molecular dynamics simulations, we have recently proposed a four-step molecular mechanism for these small cationic peptides acting as antibacterial agents [28, 29]. Such mechanism includes the following steps:

(a) Peptides folding and induction of the phospholipid segregation.
(b) Peptides protruding into domains with poor charged phospholipid content.
(c) Membrane collapse and pore formation.
(d) Peptides transposition and, then, the recovery of the bilayer structure.

Our simulations indicated that the existence of some patches in the membrane in which the insertion of peptides is more favorable than in others (from a thermodynamic point of view), together with the mechanical inhomogeneities associated with its lipid composition, seem to be a key factor in explaining the molecular activity of these small cationic peptides. On the other hand, our findings suggest that the lytic effect of small cationic peptides should be considered as a dynamic picture of structural transformations adopted by the lipid–peptide mixture depending on the relative ratio of the two species, rather than a static picture of pores induced in the membrane by the presence of peptides.

An important consideration, from the structural viewpoint, for these peptides is that in the vicinity of the membrane they must be able to adopt a helical structure enabling them to accommodate the two differentiated facades, so that both portions (charged and hydrophobic face) are located operatively. In order to determine the conformational properties of the peptides reported here, we conducted a comprehensive conformational study using theoretical methods (MD simulations) and experimental measurements (CD).

The structure determination from MD simulations may be used to monitor changes induced by the variation in the polarity of the molecular environment. The conformational behavior of peptides Arg-Gln-Ile-Lys-Ile-Trp-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH_2 (**1**), Arg-Gln-Ile-Lys-Ile-Arg-Arg-Met-Lys-Trp-

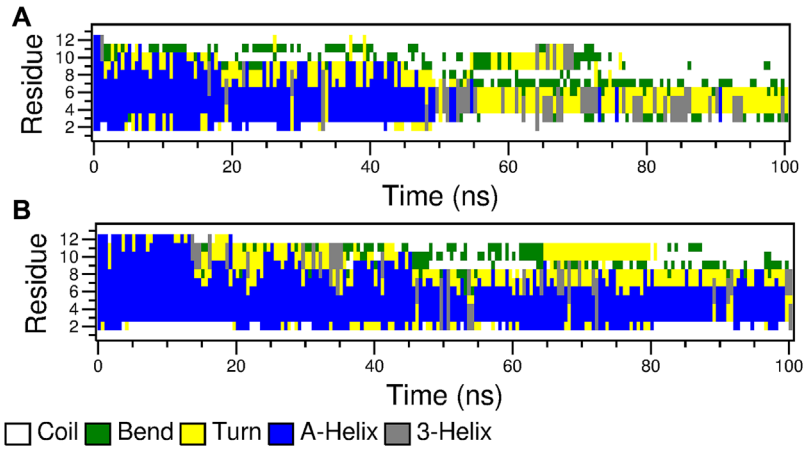

Figure 1. Change in the secondary structure during molecular dynamics simulations (100 ns) for peptide **1**: (A) in water and (B) in TFE/water system.

Arg-NH_2 (**3**), and Arg-Gln-Ile-Trp-Trp-Trp-Trp-Gln-Arg-NH_2 (**13**) were simulated using an extracellular matrix-mimetic environment as well as a membrane-mimetic environment. While the aqueous solution simulated the extracellular matrix, the solutions containing TFE mimicked a membrane-like molecular environment. Thus, the molecular dynamics simulations were carried out in water and in a mixture of TFE and water (3:7).

Figure 1 shows the change in the secondary structure during 100 ns of MD simulation in water for peptide **1** while Fig. 2 shows a snapshot of the location of peptide **1** taken at 80 ns of simulation. Figure 1A displays that the starting helical structure was destroyed in order to form a mixture of β-turn, bend, and coil after 50 ns of simulation. For compounds **3** and **13**, a similar result was obtained; however, in these cases, also a mixture of α- and 3_{10}-helices was obtained from 20 ns of simulation (Supporting Information Figs. S1A and S2A). These conformational behaviors were observed until the end of the simulation. The initial and final amino acids appear to have a random coil structure because of the flexibility of these residues. These results are very similar to those previously reported for Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met(O)-Lys-Trp-Lys-Lys-NH_2 [23], Arg-Gln-Ile-Lys-Ile-Phe-Phe-Gln-Asn-Arg-Arg-Met(O)-Lys-Phe-Lys-Lys-NH_2 [23], and also for penetratin [19]. In contrast, the results obtained from MD simulations performed in a TFE/water system are very different. In general, all the simulations performed using the TFE/water environment yielded very similar results. Residues 2–15 adopt a helix-like conformation, with the α-helix being the predominant form. Once again the initial and final residues appear to have a random coil structure. Figure 1B shows these changes in the secondary structure during 100 ns for peptide **1**. Simulations obtained for peptides **3** and **13** are shown in Supporting Information Figs. S1B and S2B.

To corroborate the above theoretical results, in the next step, we evaluated the peptide structure based on CD spectroscopic measurements in both water and a mixture of TFE and water (3:7). Such measurements were carried out for peptide **1**, which is representative of the entire series of antibacterial peptides reported here.

This peptide was measured at room temperature using the following conditions (pH adjusted by HCl/NaOH solutions): concentration: 0.023 mM; pH = 6.7. Our results indicate that in water, peptide **1** is predominantly a random coil structure (Fig. 3, black lines). The "U"-type CD spectra reflected the presence of a very large number of different local conformations in an average time. Our results also indicate that in water and from the shape of these U-type CD curves, we extracted just a little of characteristic secondary structure content for this peptide (black lines). When the same peptide

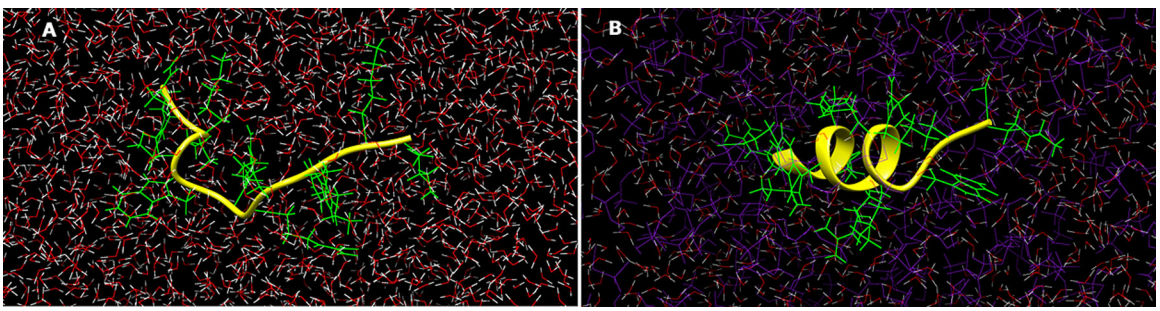

Figure 2. Snapshot of the location of peptide **1** taken at 80 ns of simulation: (A) in water and (B) in TFE/water system.

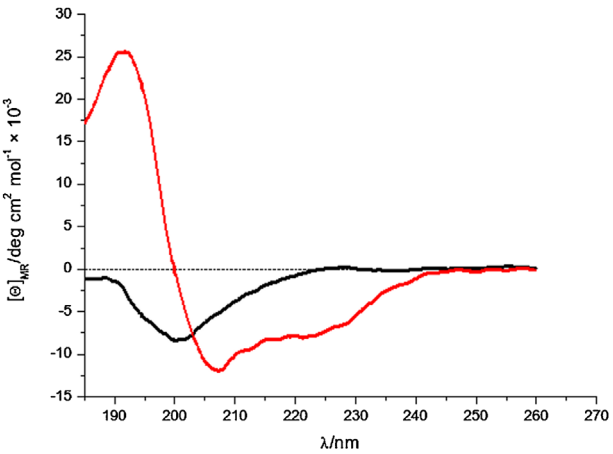

Figure 3. The CD spectrum of peptide **1** (RQIKIWRRMKWKK-NH_2) in water (black line) and in trifluoroethanol (TFE)/H_2O (3:7) (red curve).

was recorded in the solvent mixture of 30% TFE and 70% H_2O, significant changes in the shape of both curves were observed. The CD curve obtained for peptide **1** (see red line in Fig. 3) had spectral features similar to those of a C-type CD curve. Thus, the "red curve" most probably reflected a conformational ensemble composed of α- or 3_{10}-helix combined with type I/III β-turns plus some percentage of still unstructured (or highly mobile) backbone foldamers. These results indicate that in the presence of a considerable amount of TFE, this peptide has a clear tendency to adopt an increased amount of helical and/or type I/III β-turn secondary structure. These results are in agreement with those previously obtained for penetratin [30, 31], *retro-inverso* of penetratin [32] and other small-size peptides derived from penetratin [20]. Thus, our experimental measurements are in complete agreement with our MD simulations.

Our theoretical and experimental results suggested a helical feature for these small-sized antibacterial peptides in TFE/water environment. This spatial ordering might be important in order to produce the biological response which is in concordance with our previously reported model for a putative action mechanism for these small-size cationic peptides derived from penetratin.

Given that these peptides might adopt a helical secondary structure, at least when they are approaching to the membrane and that such conformation would be important for the antibacterial activity, we used the Edmundson wheel representations in order to better characterize the spatial orientation of these peptides. Thus, Edmundson wheel representations for peptides **1**, **3**, and **13** were plotted (Fig. 4). These results are representative for the whole series reported here. Previously, we have reported that for the CPP peptides, a balanced electronic distribution not "too cationic" and not "too hydrophobic" is necessary to produce the antifungal effect [19, 20] and the antibacterial activity as well [23].

The results obtained for the peptides reported here are very similar to those previously reported for other peptides derived from penetratin [19, 20, 23]. Thus, our results indicated that such electronic distribution pattern appears to be necessary for the antibacterial activity of these compounds. Figure 4 shows that the wheel representations obtained for these peptides displayed two clearly differentiated facades: The "charged face" (denoted in dashed blue line) and a more extended "non-charged face" (denoted in full green line). The first face identifies the cationic residues which account for the coulombic portion of the molecule, and the second face, which is more extensive, is formed by hydrophobic and polar residues. We cannot ensure that all peptides with a homogeneous charge distribution and hydrophobicity might have a significant antibacterial effect; however, it is also important to remark that all compounds with antibacterial effect have their distribution of charge and hydrophobicity perfectly balanced. Thus, considering that all peptides having significant antibacterial and antifungal activities show a characteristic Edmundson wheel representation, this type of study might be used as a

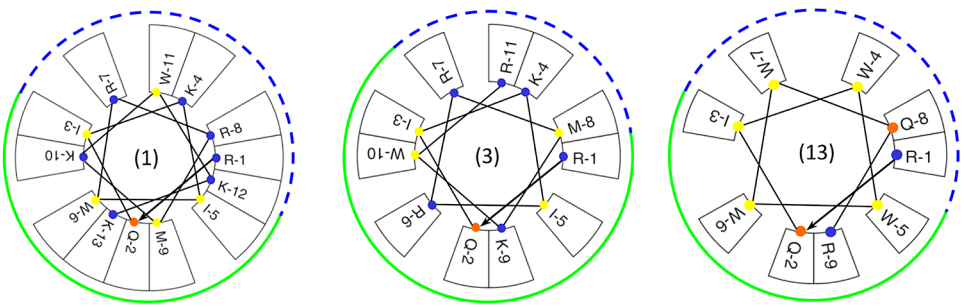

Figure 4. Edmundson wheel representations of peptides **1**, **3**, and **13**. The number in the center of the wheel corresponds to the peptide number. The "charged" and "non-charged" faces are shown in blue dashed lines and full green lines, respectively. Positively charged amino acids are denoted with blue dots, the polar ones with orange, and the hydrophobic ones with yellow.

molecular descriptor to evaluate the potential antimicrobial effect in this sort of peptides. It is evident that this kind of study should be considered only as preliminary and exploratory, but at the same time, it might help to guide *a priori* whether a peptide of this type may have antibacterial effects or not.

Conclusion

On the basis of the premise: While shorter is the peptide, better is for the development of a new antibacterial agent, we are satisfied with the results obtained with this series of peptides. In part because we have had success in that we could not achieve in our previous work [23]. In this paper, we report the synthesis and the antibacterial activity of some small-sized peptides. We obtained six new peptides possessing significant antibacterial effect. Among the tested peptides, compound **1** (Arg-Gln-Ile-Lys-Ile-Trp-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH_2), **2** (Arg-Gln-Ile-Lys-Ile-Trp-Arg-Arg-Met(O)-Lys-Trp-Lys-Lys-NH_2), **3** (Arg-Gln-Ile-Lys-Ile-Arg-Arg-Met-Lys-Trp-Arg-NH_2), **4** (Arg-Gln-Ile-Lys-Ile-Arg-Arg-Met(O)-Lys-Trp-Arg-NH_2), **10** (Arg-Gln-Ile-Arg-Arg-Trp-Trp Met(O)-Arg-NH_2), and **13** (Arg-Gln-Ile-Trp-Trp-Trp-Trp-Gln-Arg-NH_2) displayed the stronger inhibitory effect against *E. coli* ATCC 25922, LM1-*E. coli*, LM2-*E. coli*, PI-*Y. enterocolítica*, MI-*S. enteritidis*, and *Salmonella* sp. (LM). Some of the compounds reported here might provide a new source of lead structures.

On the other hand, our theoretical simulations and experimental measurements by using CD provide useful information about the preferred conformations and molecular flexibility of these compounds, which might be useful to better understand the biological response of these small-sized peptides. From our results we can conclude that, in general, these methods predict a helical structure for these peptides at the TFE/water environment.

Regarding a probable pharmacophoric pattern, we can assume that a particular combination of cationic and hydrophobic residues, adopting a definite spatial ordering, appears to be the key parameter for the transition from hydrophilic to hydrophobic phase, which could be a necessary step for these small-size peptides to produce the antibacterial activity.

It is interesting to remark that these experimental results are in line with our model for the mechanisms of action of these compounds proposed on the basis of MD simulations and molecular modeling [28, 31]. On the other hand, our results also clearly show that antibacterial activity within the series is mainly dominated by amino acid residues composition. These results are in complete agreement with those reported previously by Mooney et al. [13].

In short we have reported three peptides, **1** (Arg-Gln-Ile-Lys-Ile-Trp-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH_2), **3** (Arg-Gln-Ile-Lys-Ile-Arg-Arg-Met-Lys-Trp-Arg-NH_2), and **13** (Arg-Gln-Ile-Trp-Trp-Trp-Trp-Gln-Arg-NH_2), which are small-sized peptides with the strongest antibacterial activity reported until now. We believe that these peptides deserve further attention for future studies.

Experimental

Synthetic methods

The synthesis of the peptide RQIRRWWQR-NH_2 (**9**) was already reported in a previous work, details about the synthesis procedure can be obtained from Ref. [18].

Solid phase synthesis of the peptides was carried out manually on a *p*-methylbenzhydrylamine resin (MBHA, 1.06 mmol/g) with standard methodology using Boc-strategy. Side chain protecting groups were as follows: Arg (Tos), Lys (2Cl-Z) Glu(OcHex), and Asp(OcHex), while the side chain of the other amino acids was unprotected. All amino acids were coupled in CH_2Cl_2 (5 mL) using DCC (2.5 equiv.) and HOBt (2.5 equiv.) until completion (3 h) judged by Kaiser ninhydrin test. After coupling of the appropriate amino acid, Boc-deprotection was effected by use of TFA/CH_2Cl_2 1:1, 5 mL) for 5 min first then repeated for 25 min. Following neutralization with 10% TEA/CH_2Cl_2 1:1, 5 mL) three times (5.5 mL of each), the synthetic cycle was repeated to assemble the resin-bond protected peptide. The peptides were cleaved from the resin with simultaneous side chain deprotection by acidolysis with anhydrous hydrogen fluoride (5 mL) containing 2% anisole, 8% dimethyl sulfide, and indole at 5°C for 60 min. The crude peptides were dissolved in aqueous acetic acid and lyophilized.

The purification of the crude peptides was performed on an LKB apparatus (preparative HPLC column: Lichrosorb RP C18, 7 μm, 250 × 16 mm, gradient elution 0–40%, 80 min, mobile phase 80% acetonitrile, 0.1% TFA, flow rate 4 mL/min, 220 nm).

Analytical HPLC of the purified peptides was done on a Phenomenex Luna 5C 18(2), 250 × 4.6 mm column, using different gradient elution, mobile phase 80% acetonitrile, 0.1 % TFA, flow rate 1.2 mL/min except for peptide **11** (flow rate 1 mL/min), 220 nm.

ESI-MS: Finnigan TSQ 7000. Peptides were dissolved in 50% aqueous methanol-0.5% acetic acid and analyzed by TSQ 7000 triple-quad mass spectrometer (Finnigan). The instrument was equipped with an electrospray ion source (ESI), and the measurement was performed in positive mode. Spray voltage was 4.0 kV, capillary temperature set at 240°C. The acquisition range was from 100 to 200 *m/z*. Data are shown in Table 3.

Microorganisms

For the antibacterial evaluation of peptides, strains ATCC *S. aureus* methicillin-sensitive ATCC 29213, *S. aureus*, methicillin-resistant ATCC 43300, *E. coli* ATCC 25922, and clinical isolated from human patients of enterobacteria *E. coli*: LM1-*E. coli*, LM2-*E. coli* (LM: Laboratorio de Microbiología, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Mendoza, Argentina); *E. coli*-3, *E. coli*-15, *E. coli*-121, *E. coli*-122, *E. coli*-712, *E. coli*-782A, *E. coli*-784A, *E. coli*-786H provided

Table 3. Experimental data obtained for the synthesized peptides.

Sequence	Calculated MS	Found MS	Assignment	Purity (%)
Arg-Gln-Ile-Lys-Ile-Trp-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH_2 (**1**)	1856.5	1855.3	1857.3[a] 929.05[b]	97.6
Arg-Gln-Ile-Lys-Ile-Trp-Arg-Arg-Met(O)-Lys-Trp-Lys-Lys-NH_2 (**2**)	1872.5	1870.8	1873.6[a] 937.25[b]	98.3
Arg-Gln-Ile-Lys-Ile-Arg-Arg-Met-Lys-Trp-Arg-NH_2 (**3**)	1569.9	1568.6	1571.0[a] 786.0[b]	98.5
Arg-Gln-Ile-Lys-Ile-Arg-Arg-Met(O)-Lys-Trp-Arg-NH_2 (**4**)	1586.1	1585.2	1586.9[a] 793.95[b]	991
Arg-Gln-Ile-Ile-Arg-Arg-Met-Trp-Arg-NH_2 (**5**)	1313.7	1312.2	1314.8[a] 657.9[b]	97.9
Arg-Gln-Ile-Ile-Arg-Arg-Met(O)-Trp-Arg-NH_2 (**6**)	1329.7	1328.4	1330.6[a] 665.8[b]	98.7
Arg-Gln-Ile-Ile-Arg-Arg-Met-Gln-Arg-NH_2 (**7**)	1255.4	1254.9	1256.7[a] 628.85[b]	99.3
Arg-Gln-Ile-Ile-Arg-Arg-Ile-Gln-Arg-NH_2 (**8**)	1237.6	1237.2	1238.4[a] 619.7[b]	97.8
Arg-Gln-Ile-Arg-Arg-Trp-Trp-Met(O)-Arg-NH_2 (**10**)	1402.7	1402.0	1403.4[a] 701.7[b]	98.6
Arg-Gln-Ile-Arg-Arg-Trp-Trp-Met-Arg-NH_2 (**11**)	1386.7	1386.2	1387.6[a] 694.3[b]	99.3
Arg-Gln-Ile-Arg-Arg-Ile-Ile-Gln-Arg-NH_2 (**12**)	1237.5	1237.3	1238.6[a] 619.3[b]	99.8
Arg-Gln-Ile-Trp-Trp-Trp-Trp-Gln-Arg-NH_2 (**13**)	1443.6	1443.1	1444.8[a] 722.9[b]	99.0

[a] {M+1H}+.
[b] {M+2H}2+/2.

by Laboratorio de Microbiología, Hospital Marcial Quiroga, San Juan, Argentina and PI-*Y. enterocolitica* (PI: Pasteur Institute), MI-*S. enteritidis* (MI: Malbran Institute), and LM-*Salmonella* sp. were used.

Antibacterial activity

Minimal inhibitory concentration (MIC) values were determined using the broth microdilution method according to the protocols of the Clinical and Laboratory Standards Institute (CLSI, 2012). All tests were performed in Müeller–Hinton broth, and cultures of each strain were prepared overnight. Microorganism suspensions were adjusted in a spectrophotometer with sterile physiological solution to give a final organism density of 0.5 McFarland scale (5.10^5 CFU/mL). Stock solutions of peptides in DMSO were diluted to give serial twofold dilutions that were added to each medium to obtain final concentrations ranging from 1.56 to 50 μg/mL. The final concentration of DMSO in the assay did not exceed 1%. The Cefotaxime® Argentia Pharmaceutica antimicrobial agent was included in the assays as a positive control. The plates were incubated for 24 h at 37°. Activity was evaluated at 620 nm using a Multiskan FC instrument. The MIC values were defined as the lowest compound concentrations showing no bacterial growth after the incubation time. Tests were done in triplicate.

CD spectroscopy

Far-UV ECD data were acquired on a Jasco J810 dichrograph in 0.1-cm quartz cells; temperature controlled by a Peltier-type heating system. Each spectrum was obtained by averaging a total of four scans. Before each measurement, the sample in the cell was allowed to equilibrate for 5–10 min at the adjusted temperature. The solvent reference spectra were used as baselines, automatically subtracted from the peptide CD spectra. CD band intensities were expressed in mean residue ellipticity, ($[Q]_{MR}$, deg × cm^2/dmol).

Computational methods

The conformational behavior of peptides **1**, **3**, and **13** was studied using MD simulations. Two different media were used, water and a mixed-solvent system (TFE/water). MD simulations were performed using the GROMACS 4.5.5 programs package [33–35] with a constant integration time step of 2 fs. OPLS [36] was the force field chosen. The electrostatic contribution was calculated using a long-range electrostatic interaction by the particle-mesh Ewald method [37]. On the one hand, a simulation box containing the peptide was filled with water molecules which were described by the simple point charge water model [38]. The total number of water molecules ranged from 2680 to 3464. On the other hand, the required number of TFE

molecules [39], to reach a concentration of 30% v/v TFE/water, was added to a simulation box containing the peptide, the next step was to fill the remainder of the box with water. Both systems were neutralized replacing water molecules in random positions by Cl^- ions. The MD protocol consisted of several preparatory steps: A steep descent energy minimization process was applied to the whole system before running the MD simulations, density stabilization (NVT conditions), and finally production of MD simulation trajectory. All production simulations were performed under NPT conditions at 300.0 K and 1.0 bar, using v-rescale coupling algorithm [40] ($\tau_T = 0.1$ ps) and Parrinello–Rahman approach [41] ($\tau_P = 2.0$), respectively. The compressibility was 4.5×10^{-5} bar^{-1}. All coordinates were saved every 500 ps. The SETTLE [42] algorithm was used to keep water molecules rigid during MD simulations. The LINCS [43] algorithm was used to constrain all length bonds in the preparatory steps. However, no restrains were used in production-MD simulations. A length of 100 ns was taken into account for all production-MD simulations.

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