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Enhanced antimicrobial activity of a peptide derived from human lysozyme by arylation of its tryptophan residues

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Antimicrobial peptides are valuable agents to fight antibiotic resistance. These amphipatic species display positively charged and hydrophobic amino acids. Here, we enhance the local hydrophobicity of a model peptide derived from human lysozyme (107RKWVWWRNR115) by arylation of its tryptophan (Trp) residues, which renders a positive effect on *Staphylococcus aureus* and *Staphylococcus epidermidis* growth inhibition. This site-selective modification was accessed by solid-phase peptide synthesis using the non-proteinogenic amino acid 2-aryltryptophan, generated by direct C-H activation from protected Trp. The modification brought about a relevant increase in growth inhibition: *S. aureus* was fully inhibited by arylation of Trp 112 and by only 10% by arylation of Trp 109 or 111, respect to the non-arylated peptide. On the other hand, *S. epidermidis* was fully inhibited by the three arylated peptides and the parent peptide. The minimum inhibitory concentration was significantly reduced for *S. aureus* depending on the arylation site. Copyright © 2016 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: peptide; human lysozyme; arylation; tryptophan; antimicrobial activity

Introduction

According to the World Health Organization priority programs, microbial resistance is a threat for public health because of the possible re-emergence of past diseases. The therapeutic management of infections caused by resistant microorganisms is a challenging issue in medicine, mainly because of the exhaustion of the classical antibiotic arsenal. In this way, the development of new entities targeting cell components difficult to modify to generate resistance (such as the cell membrane) represents a strategic approach for the pharmaceutical industry [1].

In this context, the fragment 107–115 of the C-terminus of the human lysozyme possesses a relevant antimicrobial activity [2,3]. The attributes of antimicrobial peptides are the presence of positively charged and hydrophobic amino acids and an amphiphilic helical conformation. There are many precedents in the literature, which illustrate that these features give rise to antimicrobial peptide–membrane interactions with subsequent bacterial membrane disruption [4]. They exert their destructive power by variety of mechanisms including the generation of pores; therefore arguably, the development of microbial resistance should be extremely difficult. The anchorage of an antimicrobial peptide to the bacterial membrane depends on its amphipatic character [1,5]. The substitution of amino acids in a peptide with known antimicrobial activity can dramatically affect its biological activity [6]. Consequently, when alanine in positions 108 and 111 of this peptide are replaced by lysine and tryptophan (Trp), respectively, the antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* is enhanced by fourfold and 20-fold, respectively [7,8]. Accordingly, its bioactivity could be enhanced by locally increasing its hydrophobicity.

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This paper is dedicated to the memory of Prof. Nancy B. Iannucci, a dear friend and an exceptional scholar.

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In this respect, the site-selective arylation of hydrophobic residues appears as the method of choice to perform such modification. Using state of the art C-H activation transformations [9] upon indole substrates via palladium-catalyzed processes [10], we have recently developed methods for the selective arylation of Trp residues in peptides at C-2indole [11]. This protocol has been extended to a new stapling technique for Trp-Phe peptides [12]. In these transformations, we can introduce a variety of diversely substituted aryl groups in N-protected Trp, and the ensuing adducts are suitable for solid-phase peptide synthesis [13]. This formal post-synthetic modification has important structural consequences, directly altering the physicochemical properties and, more importantly, the biological activity of the adducts [14]. In this context, it has recently been described that the antifungal activity of the natural product aureobasidin can be improved through the functionalization of phenylalanine residues by iridium-catalyzed borylation [15].

The aim of this paper was to enhance the hydrophobicity of the antimicrobial peptide RKWVWWRNR by programmed and selective arylation of its Trp residues directly from the leader sequence. We planned to assess this effect on the antimicrobial activity against two microorganisms of clinical relevance (*S. aureus* and *Staphylococcus epidermidis*). 2-Arylated Trp was prepared in a protected form, amenable to solid-phase peptide synthesis as previously described through Pd-catalyzed C-H activation from Fmoc-Trp and 2-iodobenzene [13] and directly used to synthesize three peptides, each with an aryl-Trp residue at positions 109, 111 or 112, respectively.

Materials and Methods

Reagents and microorganisms

Nα-Fmoc-Trp, iodobenzene, palladium (II) acetate and *N,N*-dimethylformamide anhydrous (DMF) were from Aldrich. Fmoc-Rink-Amide AM resin was from Iris-Biotech. *O*-benzotriazole-*N,N,N′,N′*-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3,-tetramethyl uroniumhexafluorophosphate (HATU) were from Fluorochem. *N,N*-dimethylformamide was from Panreac AppliChem. *N,N*-diisopropylethylamine (DIEA) and silicagel were from Merck Biosciences. Müller-Hinton culture medium was from Oxoid. Bacterial strains *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 12228 were from The American Type Culture Collection (Manassas, VA, USA).

Tryptophan arylation

The arylation of the indolic C2 of Trp was carried out by activation of this carbon catalyzed by palladium as described by Ruiz-Rodríguez *et al.* [11] and Preciado *et al.* [13]. Briefly, the Nα-Fmoc-Trp, activated with $Pd(OAc)_2$ was treated with iodobenzene in a microwave at 90° C and purified by flash chromatography on silicagel as described by Preciado *et al.* [14]. The product was identified by nuclear magnetic resonance and electrospray mass spectrometry, and the yield was 56% (see Supporting Information for details).

Synthesis of arylated peptides

The preparation of 2-aryl-Nα-Fmoc-Trp was performed as described from 4-iodobenzene and Nα-Fmoc-Trp [1]. The resulting arylated amino acid was used in solid-phase peptide synthesis without any special requirement. The solid-phase method was performed according to Kates and Albericio [16]. Fmoc chemistry and Fmoc-Rink-Amide AM resin were used. HBTU and DIEA were used as coupling reagents for arginine, alanine, Trp, valine and asparagine. According to previous observations, HATU and DIEA were used for aryl Trp coupling to ensure its total incorporation. After side-chains removal, peptides were cleaved from the resin with trifluoroacetic acid/water/triisopropylsilane. Purification of peptides was performed by RP-HPLC. Identification was carried out by MALDI-MS and MALDI-MS/MS (see Supporting Information for details).

Scanning electron microscopy

Samples were fixed with 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0, for 10 min, then were dehydrated with ethanol washes (50–100% ethanol) and immersed in ethanol/hexamethyldisilazane (1 : 1) and 100% hexamethyldisilazane for 60 min. Afterwards, samples were dried, mounted on scanning electron microscope (SEM) stubs and coated immediately in a sputter coater with gold/palladium (Cressington Scientific Instruments). The micrographies were obtained with a Zeiss Supra 40 scanning electron microscope.

Growth inhibition assay

Bacteria from −80 °C frozen glycerol stock were grown in Müller-Hinton (MH) agar plates for 16 h at 37 °C. Individual colonies were inoculated in Erlenmeyer flasks containing 50 ml MA broth and incubated at 37 °C and 200 RPM until a density of 1×10^8 CFU/ml was reached. Bacteria suspensions were diluted to 2.0×10^5 CFU/ml with 2X MA broth for the assay.

The 0.5 ml peptide solutions were twofold diluted with water from 2.50 to 0.16 mg/ml and filter sterilized through 0.22 μm filters. Sterile peptide dilutions were then mixed with the same volume of 2X MH medium containing 2.0×10^5 CFU/ml and incubated at 37 °C for 21 h in sterile capped glass tubes. Bacterial growth was determined by measuring the absorbance at 600 nm. Positive control (100% growth) was performed in absence of peptide (0.5 ml of sterile water), and negative control was carried out in absence of bacteria (0.5 ml 2X MH broth).

Minimum inhibitory concentration (MIC) determination

Minimum inhibitory concentration determination was performed based on a micro-dilution assay [17]. Briefly, 100 μl of 2.0 mg/ml peptide sterile solution in water was diluted twofold with 2X MH broth in well 1 and twofold serially diluted with MH broth up to well 12.

One-hundred microliter of bacterial suspension containing 2.0×10^5 CFU/ml in MH broth was added to each well, and plates were incubated for 16–20 h at 37 °C. Growth was measured at 600 nm in a FlexStation 3 Multi-Mode Microplate Reader. A positive control without peptide (100% growth) and a negative control without bacteria (0% growth) were included in the assay.

Minimum inhibitory concentration is defined as the minimum concentration of peptide producing total inhibition of the growth under these conditions. Each peptide was assayed in triplicate.

Hemolysis assay

Hemolysis was performed using human red blood cells (RBC), basically as described by Helmerhorst *et al.* [18]. Briefly, RBC from heparinized blood were washed three times with phosphate-buffered saline (PBS) and resuspended at 0.5% in the same buffer. 500 μl of peptide

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solution in concentrations of 15, 50 and 125 μg/ml was added to equal volume of RBC suspension. After 1 h at 37 °C, the mixture was centrifuged at 2800 rpm for 5 min, and the absorbance of the supernatant at 414 nm was measured. PBS and 1% Triton X-100 in water were the negative and positive controls, respectively. The hemolysis percentage was calculated by the formula:

$$\left[\left(A_{peptide}\text{-}A_{PBS}\right)/A_{Triton}\text{-}A_{PBS}\right)\right]\times 100$$

Results and Discussion

We efficiently synthesized all three peptides selectively monophenylated at indole position 2 of each Trp (see Figure 1). Once the peptides were prepared, the purification and spectroscopic analyses confirmed the expected structures (see Supporting Information).

Table 1 shows the sequences of the leader peptide, the K108-W111 intermediary sequence **1** and the three aryl-peptides **2**, **3** and **4**, each with one phenyl-Trp respectively.

Table 1. Peptides used in this work

Sequence[a]	Formula[b]	Name
RAWVAWRNR-NH$_2$	107–115 hLz	Leader
RKWVWWRNR-NH$_2$	[K^{108}W^{111}] 107–115 hLz	1
RKW(Ar)VWWRNR-NH$_2$	[K^{108}W(Ar)^{109}W^{111}] 107–115 hLz	2
RKWVW(Ar)WRNR-NH$_2$	[K^{108}W(Ar)^{111}] 107–115 hLz	3
RKWVWW(Ar)RNR-NH$_2$	[K^{108}W^{111}W(Ar)^{112}] 107–115 hLz	4

[a]R, arginine; A, alanine; W, tryptophan; W(Ar), 2-phenyltryptophan;V, valine; N, asparagine; -NH2, amidated C-terminal group.
[b]hLz, human lysozyme.

Table 2. Characterization of the peptides used in this work

Peptide[a]	RT[b] (min)	Experimental MW[c] (theoretical)
1	4.7	1384.79 (1384.76)
2	5.2	1461.05 (1460.86)
3	5.2	1461.02 (1460.86)
4	5.2	1461.07 (1460.86)

[a]Purities of the pure peptides are around 94-98% according to HPLC profiles (see SI).
[b]RT, retention time.
[c]MW, molecular mass weight.

Table 2 shows the results of the characterization by RP-HPLC and MALDI-MS for the intermediary peptide and the three aryl-peptides.

The retention time of the aryl-peptides **2**, **3** and **4** in RP-HPLC was longer than that of the non-arylated intermediary peptide **1**, thus evidencing the increase in hydrophobicity because of the addition of a phenyl group. Besides, the experimental molecular weight matched with that of the theoretical one, and the sequence analysis by MALDI-MS/MS allowed identifying unambiguously the Trp in the preselected position. Next, we tackled the biological assays of the antimicrobial activity of these compounds. To this end, we performed growth inhibition assays and MIC determination of each arylated peptide and compared the results with those obtained with the parent non-arylated peptide **1** (Figure 1).

Figure 2 shows the results of the inhibition assays performed on the microorganisms studied, where different degrees of inhibition are evident. Full inhibition of *S. epidermidis* growth was evidenced regardless of which Trp was arylated. Moreover, the non-arylated intermediary peptide **1** was almost equally active. In contrast, for the more pathogenic *S. aureus*, the arylation of Trp 109 or 111 (peptides **2** and **3**, respectively) caused little effect on the antimicrobial activity of the intermediary peptide, whereas arylation at position 112 (peptide **4**) brought about a relevant increase in the antimicrobial activity of the intermediary peptide **1**.

Preliminary calculations showed that the molecular mechanics optimized geometries (Merck Molecular Force Field implemented in a Spartan'14 suite) of the arylated peptides display a common conserved structure, similar to that of the native peptide, sharing relatively close hydrogen bond networks and helical motifs (see Supporting Information). The newly incorporated phenyl group does not seem to modify the preferred conformation of the biomolecules drastically, but rather is preferentially located outward in the periphery in all three arylated peptides. Simultaneously, the incorporated hydrophobic aryl moiety should moderately increase the LogP, thus partially justifying the observed effects. Although more refined computational approaches, for instance Molecular Dynamics, may bring a better understanding of the structural features of the aryl-peptides, at this point, it may be risky to rely mainly on conformational factors taking into account the variety of mechanisms of action for antimicrobial peptides, as stated earlier [1,4,5]. We took an empirical approach as the starting point and future experimentation (polyarylated peptides, exploration of other sequences/residues, and Molecular Dynamic simulations) will shed more light in these issues.

Figure 3 shows the SEM pictures of *S. aureus* and *S. epidermidis* treated with the phenyl-peptides synthesized, where the damage in the microbial membrane due to the treatment can be seen.

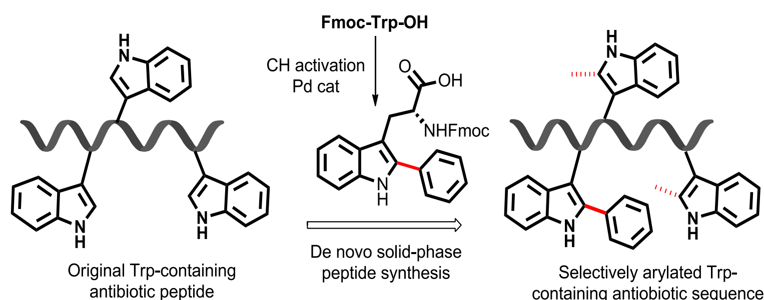

Figure 1. Synthetic approach for the selective arylation of the antimicrobial peptide RKWVWWRNR.

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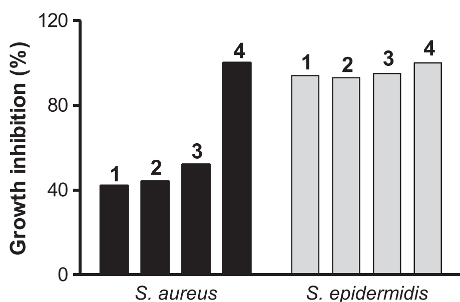

Figure 2. Inhibition assay at 0.1 mg/ml of the non-arylated intermediary peptide 1 and aryl-peptides at tryptophan (Trp) 109, Trp 111 and Trp 112 (peptides 2, 3 and 4, respectively) on *Staphylococcus aureus* and *Staphylococcus epidermidis*. Results are expressed as percentage.

A similar membrane disruption effect was demonstrated by Tan *et al.* [19] working with Gram negative bacteria and peptides with positive charge and different hydrophobicity degree.

Table 3 shows the MICs of the phenyl-peptides compared with those of the leader peptide and peptide **1**, against the two microorganisms studied. In *S. aureus* ATCC 29213, the MIC decreased twofold with peptide **2** and fourfold with peptides **3** and **4** respect to peptide**1**. In *S. epidermidis*, the arylation of Trp 109 (peptide **2**) brought about no changes in the MIC, while arylation of Trp 111 or Trp 112 (peptides **3** and **4**, respectively) decreased it by twofold respect to peptide **1**. In all cases, arylation of Trp of peptide **1** brought about a significant decrease in the MIC, but the inhibition degree was different if the arylated Trp was in positions 109 or 111–112. Moreover, the arylation of Trp in these peptides increased their antimicrobial activity, reaching MICs compatible with an effective therapeutic window and evidencing their excellent possibilities as active pharmaceutical ingredients for renovation of antimicrobial agents.

The results of the hemolysis assay shown in Table 4 are similar to those obtained with the intermediary peptide and become significant only at concentrations higher than tenfold their MICs, thus

Figure 3. Pictures of microorganisms treated with the antimicrobial peptides assayed in this work. (A) *Staphylococcus aureus* ATCC 29213. a) Control without peptide; b) Peptide **1**; c) Peptide **2**; d) Peptide **3**; e) Peptide **4**. (B) *Staphylococcus epidermidis* ATCC 12228. f) Control without peptide; g) Peptide **1**; h) Peptide **2**; i) Peptide **3**; j) Peptide **4**. All experimentation was performed at a peptide concentration of 60 μM (see Supporting Information).

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Table 3. Minimum inhibitory concentration (MIC) of the peptides used in this work

Peptide	*Staphylococcus aureus* ATCC 29213		*Staphylococcus epidermidis* ATCC 12228	
	μg/ml	μM	μg/ml	μM
Leader	250	206	ND[a]	
1	16	11	8	5
2	8	5	8	5
3	4	3	4	3
4	4	3	4	3

[a]ND, not determined.

Table 4. Hemolysis percentage of aryl-peptides at 15, 50 and 125 μg/ml

Peptide	15 μg/ml	50 μg/ml	125 μg/ml
1	2.6	5.4	30.7
2	2.7	6.1	30.9
3	2.7	5.8	31.6
4	2.9	5.6	29.8

evidencing the safety of the arylated peptides towards human red blood cells.

There are some relevant precedents in the literature regarding the effect of arylated histidines in antimicrobial peptides [20,21]; herein, we disclose a new methodology based on the preparation of an arylated Trp-peptide through C-H activation, leading to enhancement of its antimicrobial activity. This may open the door to further transformations of this type, aimed to improve the pharmacological action without disrupting the existing amphipatic nature of the peptide, but locally increasing the lipophilicity of specific residues. Although the herein described proof of concept has only explored the introduction of a naked phenyl group, access to peptides displaying diversely substituted aryl groups using the same methodology is open. To date, most biomolecule modifications of this type involve conjugation reactions that severely decrease the polarity of the original peptide by generating amide bonds from amino and acid groups present in the precursor peptide.

Conclusions

We have shown that the arylation of Trp-containing microbial peptides results in meaningful improvements of their biological activity, while keeping the hemolysis low. It is remarkable that a slight increment in the molecular weight (~5%) results in a fourfold decrease in the MIC, suggesting additional interactions between the extra aromatic group and the target. Importantly, because the microbial membranes are the biological targets of aryl-peptides, the possibility of generating resistance is seriously compromised. Thus, the eventual therapeutic use of these agents may display relevant advantages over conventional antibiotics to combat resistance. This proof of concept can open new avenues in the development of novel amphipatic peptides based on differently arylated Trp residues.

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Supporting Information

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