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# Recombinant expression, antimicrobial activity and mechanism of action of tritrpticin analogs containing fluoro-tryptophan residues☆

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ABSTRACT

The increase in antibiotic-resistant bacterial infections has prompted significant academic research into new therapeutic agents targeted against these pathogens. Antimicrobial peptides (AMPs) appear as promising candidates, due their potent antimicrobial activity and their ubiquitous presence in almost all organisms. Tritrpticin is a member of this family of peptides and has been shown to exert a strong antimicrobial activity against several bacterial strains. Tritrpticin's main structural characteristic is the presence of three consecutive Trp residues at the center of the peptide. These residues play an important role in the activity of tritrpticin against *Escherichia coli*. In this work, a recombinant version of tritrpticin was produced in *E. coli* using calmodulin as a fusion protein expression tag to overcome the toxicity of the peptide. When used in combination with glyphosate, an inhibitor of the endogenous synthesis of aromatic amino acids, this expression system allowed for the incorporation of fluorinated Trp analogs at very high levels (>90%). The antimicrobial activity of the 4-, 5- and 6-fluoro-Trp-containing tritrpticins against *E. coli* was as strong as the activity of the native peptide. Similarly, the tritrpticin analogs exhibited comparable abilities to perturb and permeabilize synthetic lipid bilayers as well as the outer and inner membrane of *E. coli*. Furthermore, the use of $^{19}$F NMR spectroscopy established that each individual fluoro-Trp residue interacts differently with SDS micelles, supporting the idea that each Trp in the original tritrpticin plays a different role in the perturbing/permeabilizing activity of the peptide. Moreover, our work demonstrates that the use of fluoro-Trp in solvent perturbation $^{19}$F NMR experiments provides detailed site-specific information on the insertion of the Trp residues in biological membrane mimetics. This article is part of a Special Issue entitled: Antimicrobial peptides edited by Karl Lohner and Kai Hilpert.



## 1. Introduction

Antimicrobial peptides (AMPs) constitute a large family of peptides with microbicidal properties [1–3]. These molecules have been extensively studied over the past three decades due to the need for new antibiotics that can counteract the rising number of antibiotic resistant bacterial strains [4–7]. As part of the innate immune system of multicellular organisms (insects, plants and animals), AMPs have been identified as important elements of the first line of defense against infections [1,8–10]. However, AMPs have also been recognized as important mediators/modulators of the adaptive immune system in higher organisms, helping to regulate the immune response [11–13]. The direct antimicrobial activity exhibited by these peptides is often linked to their ability to perturb and permeabilize the membrane of susceptible organisms [14]. Nevertheless, other mechanisms of action involving intracellular targets such as DNA, RNA and proteins have been proposed for those AMPs with very low membrane perturbing activity [15,16]. A considerable amount of the research in this field has focused on understanding the structure–function relationships of AMPs [17–19]. At the amino acid sequence level, the importance of several amino acid residues has been identified. Positively charged residues, such as Arg and Lys, are essential for targeting the AMPs towards the negatively charged phospholipid headgroups of bacterial membranes. Hydrophobic residues are important to create a structure with an amphipathic character allowing the peptides to interact with the non-polar components of the lipid bilayer [17].

Another amino acid of particular interest is Trp, which is present in almost 34% of all AMP sequences known to date [20–22]. In fact,

*Abbreviations:* AMPs, antimicrobial peptides; ePC, egg-derived L-α-phosphatidylcholine; ePG, egg-derived L-α-phosphatidylglycerol; IPTG, isopropyl β-D-thiogalactopyranoside; LUVs, large unilamellar vesicles; MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; NMR, nuclear magnetic resonance; NCF, nitrocefin; ONPG, 2-nitrophenyl-β-D-galactopyranoside; SDS, sodium dodecyl sulfate; TEV, tobacco etch virus.

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peptides with more than one Trp and Arg residues have been grouped in the Trp/Arg-rich family of AMPs [23]. For most members of this family, Trp residues have been identified as crucial elements for the antimicrobial activity of the peptides [19,23–30]. From a biophysical perspective, the presence of Trp residues in the primary structure of AMPs also allows the use of intrinsic fluorescence spectroscopy, which constitutes a powerful biophysical and sensitive approach for the study of peptide–membrane interactions [31,32]. However, the presence of multiple Trp residues limits the interpretation of fluorescence experiments, as the signals constitute the average of all Trps present in the peptide.

Tritrpticin is a member of the Trp/Arg-rich family of AMPs with a strong antimicrobial activity against bacteria and fungi [33]. Its sequence is almost palindromic and it encompasses a cluster of three consecutive Trp residues at the center of the peptide, flanked by four Arg residues [34]. The C-terminal amidation of tritrpticin produces a slightly more potent bactericidal peptide called Tritrp1 [24]. Tritrp1's mechanism of action has been linked to the permeabilization of bacterial membranes [24,29,30,35], although the possibility of intracellular targets has also been proposed [29,36]. Several mutational studies have addressed the importance of the Trp residues for the membrane perturbing and antimicrobial activities of the peptide. Trp to Phe mutations resulted in a peptide variant with a similar antimicrobial activity but reduced membrane perturbation properties [24], while Trp to Tyr or Ala mutations produced less active peptide analogs [24,29,30]. A more conservative modification of all three Trp residues, involving the addition of a hydroxyl group to the indole side chain (5-hydroxy-Trp), reduced the membrano-lytic ability of the peptide without considerably affecting the antimicrobial activity [37]. These results indicated that well-defined chemical alterations of the Trp indole ring not only can affect the antimicrobial activity of the peptide but can also modulate its mechanism of action.

The previous substitution studies motivated the use of other non-natural Trp-analogs in order to further investigate the ability of the Trp residues to modulate the mechanism of action of tritrpticin and therefore regulate its antimicrobial activity. Fluoro-Trp substitutions have been used in proteins and peptides for the study of protein–protein interactions, as well as for structural stability and dynamics measurements [38–43]. The recognition of these Trp analogs by tryptophanyl-tRNA-synthase permits the production of fluoro-Trp containing proteins by biosynthetic methods [44,45]. In addition, fluorine constitutes an excellent NMR probe due to several properties of the fluorine-19 nucleus, which include large chemical shift dispersion, high sensitivity to its local environment, and low background noise due to the absence of fluorine in biological molecules [43,46]. Several fluorinated amino acid analogs have been used for the chemical synthesis of modified AMPs resulting in peptides with increased stability [47–49] and/or modulated biological activities [47]. In addition, the synthetically fluoro-labeled peptides have allowed the study of peptide-membrane interactions by solution-state [50–52], and solid-state $^{19}$F NMR [53–55], thereby providing insight into the mechanism of action of these AMPs.

In this work, we set out to produce the AMP tritrpticin, where its Trp residues are substituted with 4-, 5- and 6-fluoro-Trp, respectively. Unfortunately, the cost of chemical synthesis of peptides containing only L-fluoro-Trp is considerable, mostly due to difficulties in the separation of the D- and L-enantiomers from a racemic mixture. Therefore, an *Escherichia coli* recombinant approach was adopted, using the calmodulin (CaM) fusion-protein system, recently developed by Dr. Hiroaki Ishida in our laboratory, in order to overcome the innate toxicity of the AMP during biosynthesis. The use of this system in combination with a high-density media [56,57], and the aromatic amino acid synthesis inhibitor glyphosate [58], allowed us to produce tritrpticin analogs with a high level of incorporation of fluoro-Trp. These peptide analogs preserved the antimicrobial activity and more importantly the membrano-lytic mechanism of action of native tritrpticin. In addition, the use of $^{19}$F NMR spectroscopy allowed us to establish the behavior of the individual Trp residues within a membrane mimetic, namely that all three Trp residues interact in a different manner with the membrane.

## 2. Materials and methods

### 2.1. Materials, bacterial strains and synthetic peptides

Fluoro-DL-tryptophans and N-(Phosphonomethyl)glycine (glyphosate) were obtained from Sigma-Aldrich (St. Louis, MO). Nitrocefin (NCF) and 2-nitrophenyl-β-D-galactopyranoside (ONPG) were purchased from EMD Millipore Corporation (Billerica, MA) and Sigma-Aldrich (St. Louis, MO) respectively. Sodium dodecyl sulfate (SDS-$d_{25}$) was obtained from Cambridge Isotopes Laboratories, Inc. (Andover, MA). Cholesterol, egg-derived L-α-phosphatidylcholine (ePC) and L-α-phosphatidylglycerol (ePG) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO). Tobacco etch virus (TEV) protease was expressed from the pRK793 plasmid from Addgene (Cambridge, MA) and purified as previously described [59].

*E. coli* ATCC 25922 was purchased from the American Type Culture Collection (Manassas, VA). *E. coli* ML35p was kindly provided by Dr. Robert Lehrer at David Geffen School of Medicine at UCLA (Los Angeles, CA).

The peptide Tritrp1 was obtained from GenScript, Inc. (Piscataway, NJ) and was synthesized by solid phase methods, its purity (>95%) and molecular weight were confirmed by HPLC and mass spectrometry, respectively.

### 2.2. Recombinant production of tritrpticin and its analogs containing fluoro-tryptophans

The recombinant version of tritrpticin (recTritrp) was expressed in *E. coli* BL21(DE3) as a fusion protein with 6xHis-CaM containing a tobacco each virus (TEV) proteolysis cleavage site. This expression method was recently developed in our laboratory by Dr. Hiroaki Ishida and colleagues.[2] Briefly, *E. coli* BL21(DE3) was transformed with the pET15b plasmid encoding the fusion protein (6xHis-CaM-Tritrp). The bacterial culture was grown overnight at 37 °C in non-inducing media MDAG [56], which contained 0.36% (w/v) of all amino acids (without Cys and 0.005% Tyr) and was supplemented with ampicillin (100 mg/L). The cells were then collected by centrifugation and used to inoculate 100 ml of fresh auto-inducing media MDA-5052 [56], supplemented with ampicillin. The culture was grown overnight at 25 °C allowing for protein expression. Fluoro-Trp exhibited low levels of incorporation in recTritrp when the regular auto-induction method was used [56] (data not shown). Therefore, an isopropyl β-D-thiogalactopyranoside (IPTG) induction method in the presence of the aromatic amino acid synthesis inhibitor, glyphosate, was used [57,58,60]. Briefly, an overnight culture was prepared as described above and used to inoculate 100 ml of fresh MDAG (without Trp) and supplemented with L-Trp (20 mg) and ampicillin. When the $OD_{600}$ reached 4–5 the cells were collected by centrifugation and resuspended in a fresh 100 ml of MDAG (without Trp) supplemented with glyphosate (1 g/L). After 1 h incubation at 37 °C, intended to consume any remaining L-Trp, fluoro-DL-Trp (40 mg) and IPTG (0.5 mM) were added and the culture was induced overnight at 25 °C. The cells were then collected by centrifugation and stored at −20 °C until use.

The cells pellets were resuspended in binding buffer (Tris 20 mM pH 7.4, NaCl 500 mM, Imidazole 10 mM) supplemented with PMSF (3 mM) and DNase I. Cell lysis was performed by French pressing (1000 PSI) three times. Soluble proteins (supernatant) and inclusion

[2] Manuscript submitted for publication.