How a single non-conventional amino acid can modify the effectiveness of amphibian antimicrobial peptides

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Background

Esculentin(1-21) [Esc(1-21)] is a short-sized cationic antimicrobial peptide (AMP) corresponding to the N-terminal region of the frog-skin AMP esculentin-1a. It is endowed with a fast rate of killing activity, especially against Gram-negative bacteria, by a membrane perturbing mechanism of action.

Esculentin-1a

GIFSKLAGKKIKNLLISGLKNVGKEVGMDVVRTGIDIAGCKIKGEC

Esc(1-21) GIFSKLAGKKIKNLLISGLKG-NH_2 +6 Basic residues

- **Short size**
- **A large spectrum of activity (Gram$^-$, Gram$^+$, fungi)**
- **A potent activity against planktonic and biofilm forms of *Pseudomonas aeruginosa* (Gram$^-$).**

Rana esculenta

Antimicrobial activity of Esc(1-21) against reference strains and clinical isolates of *P. aeruginosa*

[PEPTIDE] CAUSING 99,9% KILLING OF PLAKTONIC CELLS	[PEPTIDE] CAUSING 95% KILLING OF BIOFILM CELLS
1 µM	12.5-25 µM

RESULTS

Design and Synthesis of Glycine-Replaced Derivative of Esc(1-21)

Fig. 1 *Primary structure of Esc(1-21) and its derivative*

GIFSKLAGKKIKNLLISGLKG-NH_2 **Esc(1-21)**

↓

GIFSKLAAibKKIKNLLISGLKG-NH_2 ***[Aib^8]***

Residue variation is highlighted in red.
The last glycine is amidated on the C-terminus.

With the aim to increase the biological activity of Esc(1-21) against Gram-positive bacteria we explored the effect of the **replacement** of Glycine amino acid residue in **position 8** with a non-coded amino acid, (**Fig. 1**) **α-aminoisobutyric acid, *[Aib^8].***

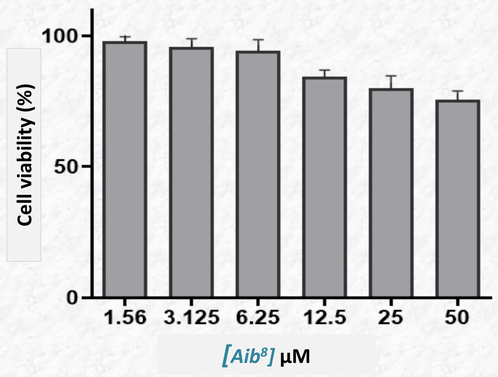
Antimicrobial activity of [Aib^8]

Strain	MIC (µM) Esc (1-21)	MIC (µM) [Aib^8]
Gram-Negative		
E. coli ATCC 25922	1.56	0.78
A. baumannii ATCC 19606	1.56	0.78
P. aeruginosa ATCC 25853	3.12	3.12
Gram-Positive		
S. epidermidis ATCC 12228	3.12	0.78
S. aureus ATCC 25923	>100	12.5
S. aureus MDR #1	>100	12.5
S. aureus MDR #2	100	12.5
S. aureus MDR #3	100	12.5
S. aureus MDR #4	>100	6.25

Tab. 1 *Antimicrobial activity of Esc-peptides*

The parent peptide Esc (1-21) did not show any antibacterial effect against *S. aureus*. On the contrary, ***[Aib^8]*** showed strong activity against the Gram-positive *S. epidermidis* strain, and it was also effective against the Gram-positive *S. aureus* ATCC 25923 and multi-drug resistant strains **(Tab. 1)**.

Effect of [Aib^8] on Cell Viability



[Aib^8] did not induce any significant cytotoxic effect on HaCaT cells up to a concentration of 25 µM after 24h of treatment on metabolically active cells **(Fig. 2)**.

Fig. 2 *Effect of [Aib^8] on the viability of HaCaT cells was evaluated using the MTT assay after 24 h treatment. All data are expressed as percentage with respect to the untreated control cells.*

Antibiofilm activity of [Aib^8]

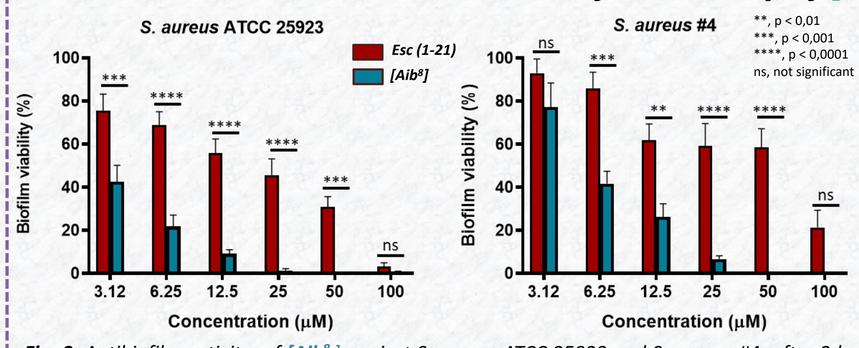


Fig. 3*. Antibiofilm activity of [Aib^8] against S. aureus ATCC 25923 and S. aureus #4, after 2 h of treatment. Biofilm viability was evaluated by MTT assay and expressed as percentage compared to that of untreated samples (bacterial biofilm not treated with the peptide, 100% viability).*

Esc (1-21) was able to reduce ~90% of biofilm viability at 100 μM against the reference strain of *S. aureus* ATCC 25923. On the contrary, ***[Aib^8]*** was able to reduce more than 90% biofilm viability of both reference and clinical isolates at concentrations of 25 μM, which are only 2- or 4-fold higher than the corresponding MIC against the two strains **(Fig. 3)**.

Stability of [Aib^8] in Serum

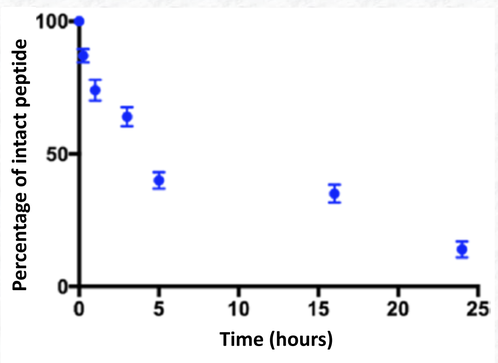


Fig. 4 *Stability of [Aib^8] in 50% bovine at different incubation times at 37°C. The percentage of non degraded peptide (%) after 1, 3, 4, 5, 16, and 24h incubation.*

To investigate the stability of the promising ***[Aib^8]*** derivate in biological fluids, the amount of intact peptide was monitored within 24h of incubation at 37°C in the presence of 50% fresh bovine serum (**Fig 4**).

The percentage of intact peptide was ~90%, ~75%, and ~65% after 1, 3, and 4h of incubation, respectively. Interestingly, after 5h treatment the nondegraded amount of peptide was ~40% and the same percentage of intact peptide was detected even after 16h.

Structural Characterization of [Aib^8]

Circular Dichroism Analysis in POPG/POCL LUVs

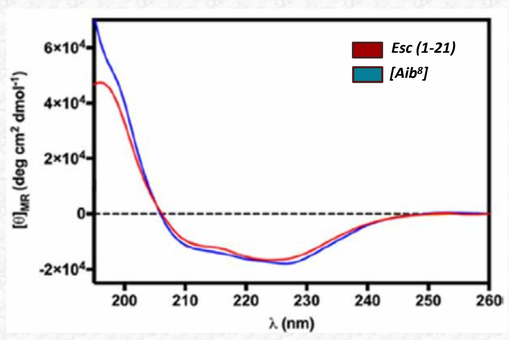


Fig 5. *Circular dichroism spectra of Esc-(1-21) and [Aib^8] at 20 µM measured in the presence of POPG/POCL (6:4 mol/mol) LUVs (500 µM).* **POPG**: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol **POCL**: 1',3'-bis[1-palmitoyl-2-oleoyl-sn-glycero-3-phospho]-glycerol

In the presence of liposomes mimicking the Gram-positive bacterial membrane, both CD spectra were characteristic of a helical structure. The intensity ratio between the two minima was greater than 1.0, indicating a helical conformation in its oligomeric state **(Fig. 5)**.

NMR analysis in the presence of POPG/POCL bicelles

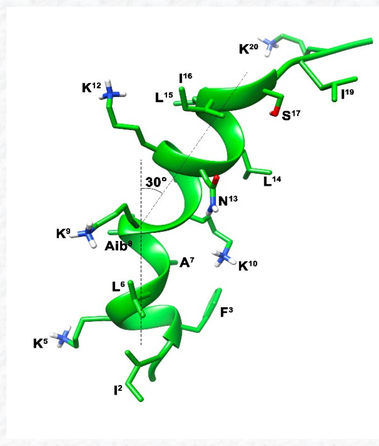


Fig.6. *Representative structure of [Aib^8] peptide. Backbone is shown as ribbon and helical axes are shown as dotted lines.*

An α-helix from Phe^3 to Leu^6 can be observed followed by a 3_{10} helix from Ala^7 to Lys^9 and again by an α-helix from Lys^{10} to Ile^{16};
C-terminal tail has also tendency to the helix but turns out to be more flexible.
The structure can be described as a distorted helix, bent on the Aib^8 residue (**Fig. 6**).

Characterization of Mechanism of Action of [Aib^8]

Cytoplasmic Membrane Perturbation

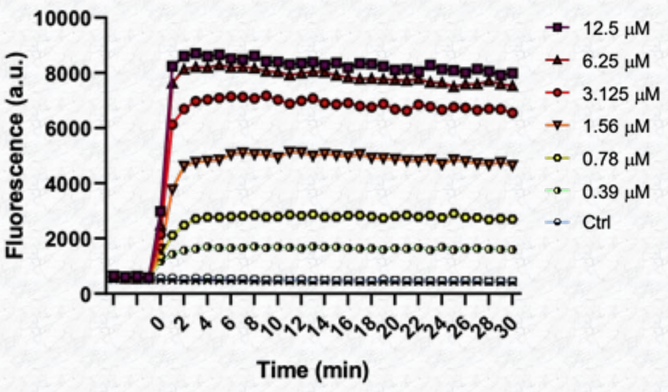


Fig 7*. Kinetics of cytoplasmic membrane permeabilization of S. aureus ATCC 25923. Sytox Green probe (1 µM) was able to bind nucleic acids upon the impairment of the cytoplasmic membrane, resulting in an increase of fluorescence intensity. Control is given by microbial cells without peptide.*

To verify the membrane perturbation mechanism of action of ***[Aib^8]*** against Gram-positive bacteria, the fluorescent probe Sytox Green was employed to carry out fluorescence studies on *S. aureus* ATCC 25923 during the first 30 minutes (**Fig.7**).
[Aib^8] induced a fast, and dose-dependent membrane perturbation process. Already within the first minutes from its addition, the highest values of fluorescence intensity were recorded at the concentration range of 6.25-12.5 µM.

Membranolytic Mechanism by CF Leakage from POPG/POCL LUVs

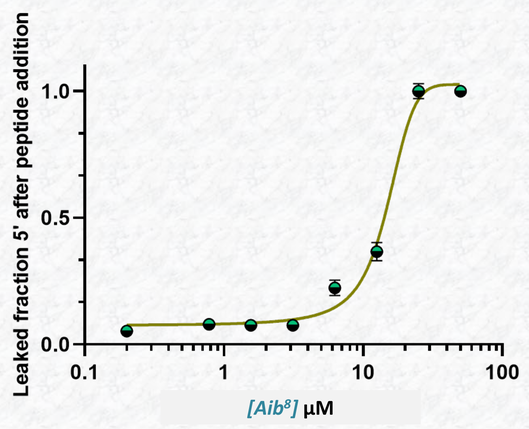


Fig 8. *Effect of different concentrations of [Aib^8] on the leakage of CF encapsulated into POPG/POCL (6:4, mol:mol) LUVs. LUVs were used at a final lipid concentration of 100 µM.*

The membranolytic mechanism of ***[Aib^8]*** and the extent of peptide-induced membrane injury were also explored by using artificial large unilamellar vesicles (LUVs), mimicking the composition of the membrane of Gram-positive bacteria and loaded with fluorescent probe carboxyfluorescein (CF).
[Aib^8] displayed a fast membrane-perturbing activity with a total CF leakage within 5 min from its addition to the LUVs at 25 µM (**Fig. 8**).

Conclusion

Our findings have contributed to explain how the incorporation of unconventional amino acids is a valid strategy to obtain promising candidates for the development of new anti-infective therapies.

References

Luca, V. et al. Esculentin(1−21), an amphibian skin membrane-active peptide with potent activity on both planktonic and biofilm cells of the bacterial pathogen Pseudomonas aeruginosa. *Cell. Mol. Life Sci.* 2013, *70*, 2773−2786.
Loffredo, MR. Et al. Strategic Single-Residue Substitution in the Antimicrobial Peptide Esc(1-21) Confers Activity against Staphylococcus aureus, Including Drug-Resistant and Biofilm Phenotype. ACS Infect Dis. 2024 Jun 7. doi: 10.1021/acsinfecdis.4c00130.
Di Grazia, A. et al. D-Amino acids incorporation in the frog skin-derived peptide esculentin-1a(1−21)NH2 is beneficial for its multiple functions. Amino Acids 2015, 47, 2505−2519.

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