SYNTHESIS AND STUDY OF MODIFIED TEMPORIN ANALOGS WITH UNNATURAL AMINO ACIDS CITRULLINE AND ORNITHINE AS POTENTIAL ANTIBACTERIAL AGENTS

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Introduction

Antimicrobial resistance (AMR) has reached almost critical levels and is listed by the WHO as one of top ten global health threats. In 2019 AMR was clearly accountable for 1.27 million deaths globally and contributed to 4.95 million deaths. The search for new alternatives in the treatment of bacterial infections has become a primary objective [1].

A promising new substitute are antimicrobial peptides (AMPs), which are biologically active compounds with low toxicity, high selectivity and can be administered alone or in combination with other antimicrobial agents for a synergistic effect [2]. They are highly hydrophobic and have a positive net charge, a wide range of activity against Gram-negative and Gram-positive bacteria, fungi, and yeast, as well as some additional anticancerogenic properties. In addition, they appear to be non-toxic against the human red blood cells [2, 3].

Temporin A (FLPLIGRVLSGIL-NH_2) is an AMP secreted from the European red frog *Rana temporaria*, with a length of 13 amino acids, hydrophobic, basic and amidated at the C-terminus. It is proven to be effective against gram-positive bacteria, including methicillin- and vancomycin-resistant strains [4, 5].

Results

The new analogs are fully characterized, and the results are summarized in **Table 1.** The HPLC profiles and MS data for the determination of the structure and purity are shown in **Fig. 2a** and **2b.**

Table 1 Structure and characteristics of the synthesized peptides

Entry	Chemical Formula	MM $_{exact}$ [g/mol]	$[M+H]^+_{observed}$ [g/mol]	$[M+Na]^+_{observed}$ [g/mol]	RT [min]	α_D^{20} [°]*	M.p. [°C]
DTA	$C_{68}H_{117}N_{17}O_{14}$	1395,90	1397,00	1418,95	4,513	-38	158 ± 2
DTCit	$C_{68}H_{116}N_{16}O_{15}$	1396.88	1397.75	1419.75	5.793	-34	182 ± 2
DTOrn	$C_{67}H_{115}N_{15}O_{14}$	1353.87	1354.70	1376.70	4.364	-42	123 ± 2

*methanol (c=1)

Fig. 2a HPLC profiles: Temporin TA, Temporin TThr and Temporin TTyr4 (from left to right)

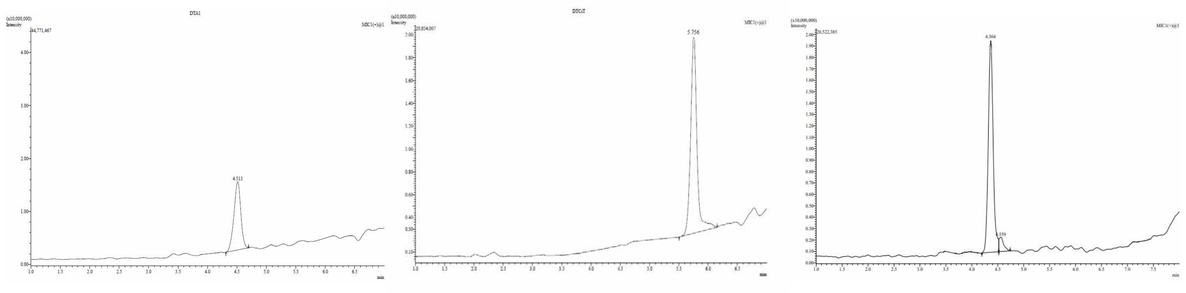
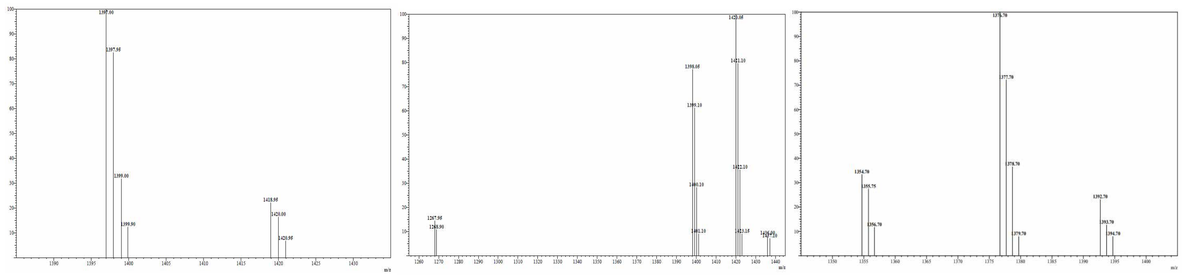


Fig. 2b MS data: Temporin TA, Temporin TThr and Temporin TTyr4 (from left to right)



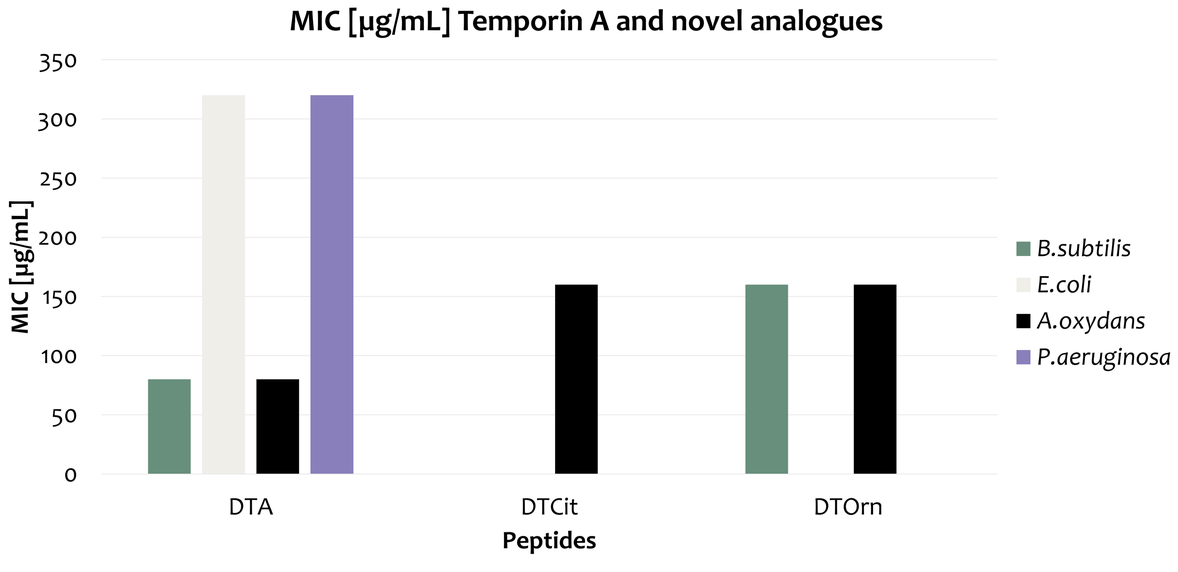
The novel analogs Temporin DTCit and DTOrn, as well as the parent peptide Temporin TA were studied for their antibacterial properties against *B. subtilis* strain 3562, *A. oxydans* 9333, *E. coli* 8785 *and P. aeruginosa* 3700. .The results are summarized in **Table 2.** The activity of the modified analogs were compared to that of the parent peptide in **Fig. 3**.

Table 2 Minimal inhibitory concentration (MIC) in µg/mL of DTA, DTCit and DTOrn

	Peptide	Sequence	*B. subtilis* 3562	*E. coli* 8785	*A. oxydans* 9333	*P. aeruginosa* 3700
1	**DTA**	Phe-Leu-Pro-Leu-Ile-Gly-**Arg**-Val-Leu-Ser-Gly-Ile-Leu-NH_2	80 µg/mL	320 µg/mL	80 µg/mL	320 µg/mL
2	**DTCit**	Phe-Leu-Pro-Leu-Ile-Gly-**Cit**-Val-Leu-Ser-Gly-Ile-Leu-NH_2	NI	NI	160 µg/mL	NI
3	**DTOrn**	Phe-Leu-Pro-Leu-Ile-Gly-**Orn**-Val-Leu-Ser-Gly-Ile-Leu-NH_2	160 µg/mL	NI	160 µg/mL	NI

*NI – no inhibition

Fig. 3 Comparison of the MIC values against tested strains



Materials & Methods

Here we report synthesis of new temporin A analogs with modification of Arg residue in position 7 with Citrulline (FLPLIG-**Cit**-VLSGIL-NH2) and Ornithine (FLPLIG-**Orn**-VLSGIL-NH2).

Synthesis of the novel Temporin A analogs

The peptides were synthesized by the Fmoc/OBu^t solid (9-fluorenylmethoxycarbonyl) phase peptide synthesis. Solid-phase peptide synthesis (SPPS) is a method for peptide synthesis, in which a peptide chain bonded to a polymer carrier is grown by presenting suitably protected amino acids (**Fig. 1**). The solid-phase carrier was Rink-amide MBHA resin, and the coupling agent was either N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) or Diisopropylcarbodiimide (DIC). The monitoring of the reactions of deprotection and coupling was carried out using Kaiser test. The peptides were then cleaved from the resin using a mixture of trifluoracetic acid (TFA), triisopropyl silane (TIS) and water. They were then crystalized. The structure and purity were determined using HPLC-MS technique.

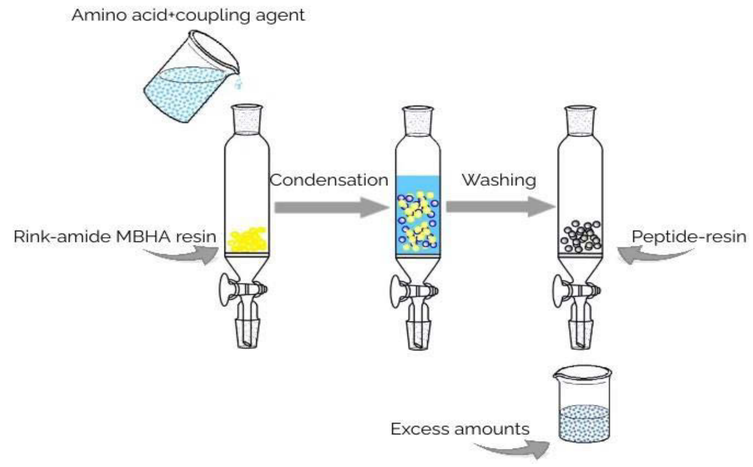


Fig. 1 General scheme for SPPS synthesis (Own drawing, modified based on [6])

Antibacterial activity of the novel Temporin A analogs

The minimal inhibitory concentration (MIC) of the peptides in concentration range from 0 to 320 µg/mL were determined using the broth dilution method. Stock solutions of the peptides with a concentration of 10 mg/mL in 10% EtOH/H_2O were prepared. Suspensions of the bacteria in the corresponding media were added to 96-well U-shaped bottom polystyrene plates and exposed to the peptide in concentration range from 0 to 320 µg/mL, as well as a 10% EtOH/H_2O control, diluted using the same scheme as the peptides. The plates were then incubated for 24h under aerobic conditions at the corresponding temperature. The MIC (µg/mL) was assumed as the lowest concentration of the antimicrobial, where there was visible inhibition of the strain. The absorbance was measured at 630 nm. To determine the minimal bactericidal concentration (MBC) 10 µl from concentrations greater than or equal to the MIC were placed on the corresponding solid nutrient medium. The Petri dishes were then incubated at 30 or 37° C for 24 h.

Discussion

The purity of the newly synthesized peptides is satisfactory as seen by the HPLC profiles. The desired sequences are also accomplished and proven by the MS data.

The peptides show lower MIC values against the Gram-positive bacteria *B. subtilis* 3562 and *A. oxydans* 9333. The parent peptide Temporin TA shows a MIC value of 80 µg/mL against the Gram(+) strains and 320 µg/mL against the Gram(-). The modification with Citrulline in position 7 proves to be redundant because the novel peptide shows no antibacterial activity in the chosen concentration range. An interesting observation can be made that DTCit shows inhibitory effect only against A. oxydans 9333. However, the substitution of the Arg residue with a Orn one, keeps the inhibitory effect, but at a higher concentration of the peptide. The analog DTOrn has a bactericidal effect at 320 µg/mL against *B. subtilis* 3562 and at 160 µg/mL against *A. oxydans* 9333. DTCit also has a MBC value of 160 µg/mL against *A. oxydans* 9333.

Conclusions

If looking over the obtained MIC values and taking into consideration the differences in the Arginine, Ornithine and Citrulline, a conclusion can de drawn that a bulkier, longer, and thus more basic side chain is needed at position 7 in order to have a lower MIC value. Additionally, the removal of positive charge in the lateral chain leads to a loss of the antibacterial properties evident with the analog DTCit.

Acknowledgement

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