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FACULTY OF CHEMICAL AND SYSTEM ENGINEERING
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DISSERTATION SYNOPSIS

SYNTHESIS AND BIOLOGICAL ACTIVITY OF TEMPORIN ANALOGUES

For the acquisition of an educational and scientific degree "Doctor" in the field of higher education 5. Technical Sciences, Scientific Specialty 5.11. Biotechnology
(Technology of biologically active substances)

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The dissertation is written on 134 pages, contains 33 figures, 2 graphs and 27 tables. 110 sources are cited.

The presented dissertation was discussed and accepted for defense at a meeting of the Scientific Council of the Scientific Unit of the Department of Biotechnology, held on 17.04.2026.

The public defense of the dissertation will be held on 25.06.2026 bym 11 hours in Hall 301, building "A" UCTM.
The materials are available to those interested on the website of UCTM and in the Department of Scientific Activities, room 406, floor 4, building "A" of UCTM.

INTRODUCTION

Since the introduction of antibiotics to present day, they have been one of the most prescribed drugs in medical practice and are produced in huge quantities, using them for various purposes. The development of chemical and biotechnological methods for their production makes these compounds more accessible, which facilitates their use. An undeniably negative result of the long-term selection pressure resulting from the use of antibiotics is the development of resistant strains to existing antibiotics and their wide distribution throughout the biosphere. In addition to overuse and misuse by humans, one of the main factors contributing to the development and advancement of antimicrobial resistance is the use of antibiotics in the livestock sector and agriculture.

The ability of microorganisms to withstand and remain viable when exposed to antimicrobial agents is known as antimicrobial resistance. It currently poses one of the greatest dangers to public health and threatens to become the pandemic of the 21st century. Infections with resistant microorganisms are not only difficult to treat, but also carry the risk of subsequent serious illnesses and even death. The rapid spread of multi-resistant bacteria that cause such diseases is a serious cause for concern. It is necessary to search for alternative and new means to combat them. Antimicrobial peptides (AMPs) are a promising alternative to existing antibiotics on the market and of great interest. These compounds have the potential to be effective against infections caused by bacteria, as well as other pathogens that are resistant to various available medicines, and thanks to their low molecular weight, they are easily obtained through chemical and biotechnological approaches. Based on natural antimicrobial peptides, scientists are developing modified analogues in order to improve their pharmacokinetics, pharmacodynamics and preserve or enhance their biological activity.

AIM AND OBJECTIVES

The aim of this dissertation is to synthesize new structural analogues based on the antimicrobial peptide temporin A with potential antibacterial activity and to investigate their biological properties.

To achieve this goal, the following tasks were set:

1. Design, synthesis and characterization of new analogues of temporin A.
2. Determination of the hydrolytic stability of newly synthesized analogues of temporin A.
3. Determination of antibacterial activity of newly synthesized analogues of the antimicrobial peptide temporin A.
4. Determination of cytotoxicity and phototoxicity of newly synthesized analogues of the antimicrobial peptide temporin A.
5. Determination of the antiproliferative activity of the newly synthesized analogues of the antimicrobial peptide temporin A.
6. Derivation of some SAR dependencies based on the results obtained.

EXPERIMENTAL PART

1. Synthesis and characterization

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1. Synthesis and characterization

The target peptides were obtained by solid-phase peptide synthesis by Fmoc(9-fluorenylmethoxycarbonyl)/O*t*-Bu strategy on Fmoc-Rink-Amide-MBHA resin (Figure 10). Removal of the Fmoc group was carried out with a 20% piperidine solution in DMF. To monitor condensation and unblocking reactions, both standard tests were applied - Kaiser test and Chloranil test.

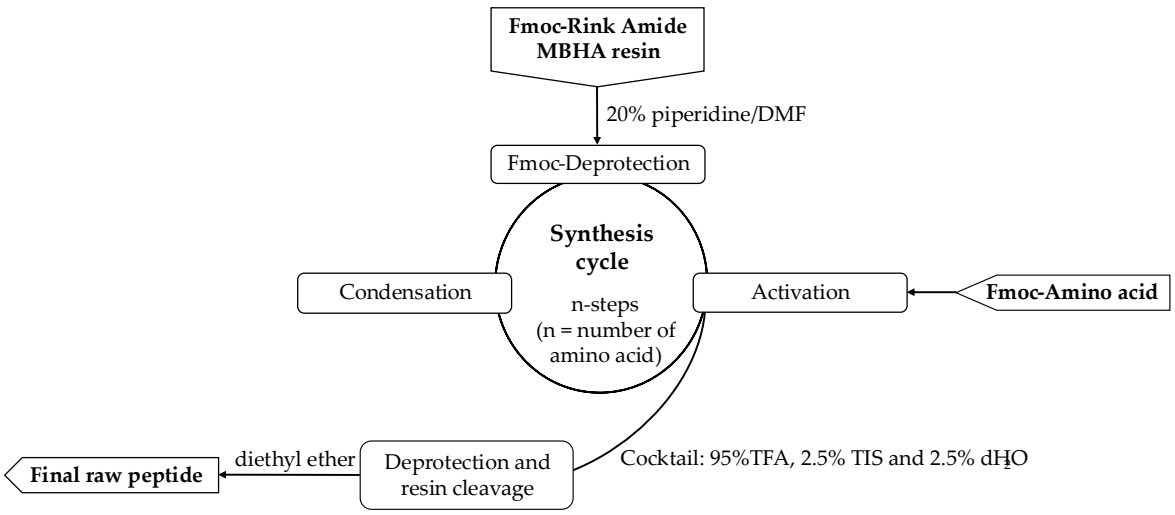


Figure 1 General Solid-Phase Peptide Synthesis Procedure (Dimitrova et al., 2024)

To remove the target peptide from the polymer carrier and unblock all protection groups of the amino acid side chains, the resulting final target peptide-resin is treated with a mixture of 95% TFA:2.5%TIS:2.5%H2O for a minimum of 4 hours. The peptide solution in TFA evaporates to dry under vacuum of a rotary vacuum evaporator. To completely remove TFA, the raw product is re-evaporated several times with a mixture of n-hexane and DCM in a 1:1 ratio. Diethyl ether cooled in the refrigerator chamber is added to the resulting oil and it crystallizes. Leave overnight in the refrigerator to completely settle the crystals. The diethyl ether is then filtered and the resulting peptide is dried to a constant weight in a vacuum dryer without heating.

To determine the purity of the obtained peptides, a liquid chromatograph Shimadzu LC MS/MS 8045 was used. Especially for the purpose of this dissertation, the following gradient system with mobile phase A: H_2O/10% AcCN/0.1% HCOOH and mobile phase B: AcCN/5% H_2O/0.1% HCOOH has been developed. Electrospray ionization mass spectrometry in SCAN/ESI+ mode was used to prove the structures.

The melting points of a semi-automatic apparatus Krüss Optronic GmbH (M3000) were determined. The optical activity of the newly synthesized peptides was investigated by determining the angle of optical rotation of the compounds, measured on an automatic polarimeter Polamat A, Carl Zeiss, Jena at c = 1 in methanol. Circular dichroism (CD) was used to study the secondary structure of the newly synthesized peptides. The spectra were recorded on a Jasco J-1500 spectropolarimeter (JASCO Corporation, Tokyo, Japan). The peptides were dissolved in a solution of 10% ethanol in water, then diluted in water to the required concentration of 1 μM. Measurements were carried out in a quartz cuvette with a path length of 1 mm, covering a wavelength range of 190–300 nm at 25°C.

To study the hydrolytic stability of the newly synthesized compounds, three model pH systems were simulated, meeting the conditions in the stomach (pH=2), blood plasma (pH=7.4) and small intestine (pH=9) of the human body. All solutions were prepared in accordance with the European Pharmacopoeia, tenth edition. They were also further modified with specific enzymes capable of hydrolyzing peptides. Hydrolytic stability was observed when using the Reverse Phase Perkin-Elmer Series 200 HPLC (Waltham, MA, USA) on a Lichrospher RP-8 nonendcaped column with a length of 150 mm, an inner diameter of 4.6 mm and a pore size of 5 μm (Alltech, Lexington, KY, USA) at 254 nm with a Perkin-Elmer Series 200 detector (Waltham, MA, USA).

2. Biological studies

Biological studies have been carried out with all synthesized compounds in order to determine their antibacterial and antimycotic activity, cytotoxicity, phototoxicity and antiproliferative activity.

2.1. Antibacterial/antimycotic tests

The antibacterial and antimycotic activity of the obtained analogues were studied using two methods - disc-diffusion (for determination of the inhibitory zone in mm on a solid agar medium) and microdilution in broth (for determination of the minimum inhibitory concentration (MIC) in μg/mL). Antimicrobial properties will be tested against model strains

Escherichia coli NBIMCC 8785, *Bacillus subtilis* NBIMCC 3562, *Arthrobacter oxydans* NBIMCC 9333, *Pseudomonas aeruginosa* NBIMCC 3700 and *Candida albicans* NBIMCC 74.

2.2. Determination of cytotoxicity, phototoxicity and antiproliferative activity

The BALB 3T3 (mouse embryonic fibroblasts) and MCF-12F (human breast epithelial cells) cell lines were used as healthy tissue models. The MCF-7 and MDA-MB-231 cell lines were used as *in vitro* models for luminal A- and basal B-type breast cancer, respectively.

Cytotoxicity/phototoxicity testing was conducted as described in the OECD Chemical Testing Guide, Section 4, Test No. 432: *in vitro* 3T3 NRU phototoxicity test (OECD, 2019) with the BALB 3T3 cells, Branch A31. In the phototoxicity tests, 96-well plaques were irradiated (+Irr) with a dose of 2.4 J/cm^2 by the Helios-iO artificial sunlight simulator (Seric Ltd., Tokyo, Japan). Cytotoxicity/phototoxicity was expressed as CC_{50} values (concentrations required for 50% cytotoxicity).

The antiproliferative activity study was conducted on cell cultures using the MTT-dye reduction assay. In the experiments, MCF-12F, MCF-7 and MDA-MB-231 cell lines were used. Antiproliferative activity was expressed as IC_{50} values (concentrations required for 50% inhibition of cell growth).

RESULTS AND DISCUSSION

For this dissertation, temporin A and 13 of its analogues were synthesized. All peptide analogues were grouped into three series. Figure 19 shows a summary of the modifications made in the structure of temporin A. The purpose of the introduction of various proteinogenic and non-proteinogenic amino acids is to improve pharmacokinetics, expressed in improved hydrolytic stability, and pharmacodynamics, expressed in improved biological activity of newly synthesized peptides, as well as to establish which side chain characteristic as basicity, charge, length and aroma is relevant in the selected positions for the activity of the peptide.

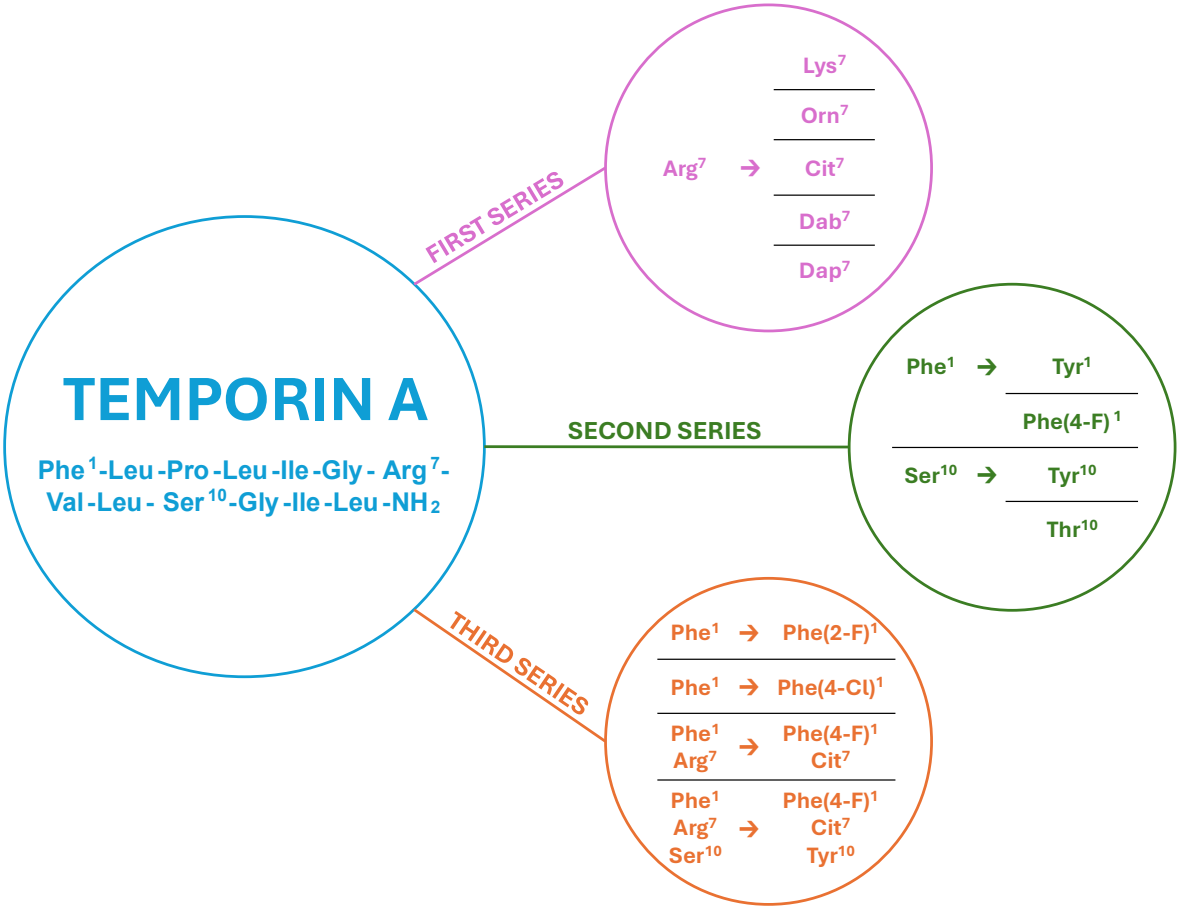


Figure 2 Summary of the modifications made to temporin A

The first series includes the parent peptide temporin A, the already published analogue with substitution at position 7 with Lys – temporin F, and 4 new analogues with substitution at position 7 with non-proteinogenic amino acids. In the second series, analogues with modifications in positions 1 and 10 are grouped. The created analogues in the third series are based on the results obtained from the first and second series, including peptides with two or more modifications, as well as halogenated analogues.

I. First series of analogues

1. Design

The first series of newly synthesized analogues of temporin A includes modifications to its structure in the seventh position, i.e. the arginine residue located in this position in the natural peptide was replaced. In addition to the parent peptide, we also chose to synthesize the already published by Wade et al. (2000) Temporin F, in which Arg was replaced by Lys (Wade et al., 2000), with the two peptides serving as model compounds for comparing the biological activity of the other analogues relative to them. For the remaining analogues of this series, the non-proteinogenic amino acids ornithine (Orn), citrulline (Cit), 2,4-diaminobutaryc acid (Dab) and 2,3-diaminopropionic acid (Dap) were used (Figure 20). Through these changes in the temporin A molecule, we investigated the influence of the basicity and volume of the side chain of the amino acid in the seventh position on hydrolytic stability, antibacterial and antiproliferative activity.

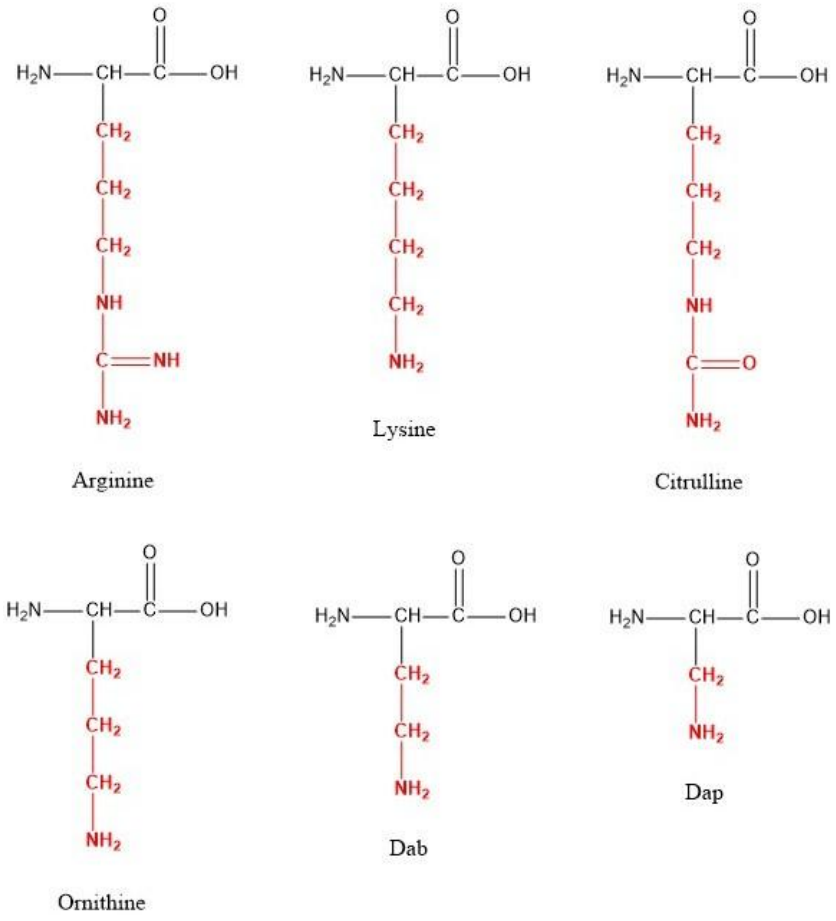


Figure 3 Structures of amino acids used at position 7 of temporin A (Dimitrova et al., 2024)

The positively charged amino acids Arg and Lys play an important role in peptide activity due to their ability to generate electrostatic attraction with the negatively charged membrane of microorganisms such as bacteria. At the same time, however, the propensity of AMPs to protease degradation contributes to the low stability of the majority of peptides in biological fluids, including serum and the gastrointestinal tract. Enzymes such as chymotrypsin recognize the aromatic residues Trp, Phe and Tyr, and serine proteinases such as trypsin are extremely specific to the basic amino acids Arg and Lys. Other proteinases specific to hydrophobic residues such as Val, Ala, Phe, and Leu include elastase and aspartate proteinase pepsin (Arias et al., 2018).

At the end of its side chain, Arg has a guanidine group, while Lys has an amino group that gives peptides strong basic properties that affect their antimicrobial activity. In most studies done, the introduction of Lys has been associated with reduced antimicrobial activity. Wade et al. (2000) also report a reduced antimicrobial capability of Temporin F. This trend can be explained by the greater number of hydrogen bonds that the guanidine group of Arg can form compared to the amino group of Lys, as well as by the significantly weaker bonds of this type, due to the lower basicity of the ε-amino group of Lys compared to the guanidine group of Arg. Side chains of Orn, Dab and Dap are altered not only in terms of their nature, i.e. their underlying strength, but also in terms of the length of the side chain, relative to that of Arg and Lys (Arias et al., 2018; Wade et al., 2000). In contrast to all of them, in the Cit molecule, the basic grouping (guanidine or simple amino group) in the side chain is replaced by a urea residue, which has neutral properties, i.e. in it we have a complete lack of charge in the side chain.

At neutral pH, Arg is a basic amino acid with a guanidine group in its side chain and a positive charge. Under normal circumstances, the human body produces enough Arg to maintain muscle mass and connective tissue, making it a semi-essential amino acid. However, the endogenous synthesis of Arg is often insufficient to meet the high needs during times of stress. Under these circumstances, Arg becomes a key amino acid for maintaining positive nitrogen balance and promoting optimal growth. Protein metabolism and metabolism provide the majority of plasma Arg. About 60% of these reserves are produced in the kidneys, which are the main site for net Arg synthesis. The main substrate is Cit, which is produced in the small intestine as a by-product of the metabolism of dietary amino acids such as Pro, Gln, and Gly (Tong and Barbul, 2004). From a chemical point of view, the difference between Cit and Arg is that the side chain of Cit has a ketone group (C = O) instead of an imine group (= NH), thus removing the basicity (Johnson et al., 2022).

Cit is a non-essential amino acid that the human body produces and is also found in some foods. Cit is involved in the synthesis of Arg in the body, which is essential for the immunological response, nitric oxide production, and protein synthesis. The urea cycle, which is responsible for detoxifying ammonia in the body, is highly dependent on Cit. Like other amino acids, Cit has both a positively charged amino group and a negatively charged carboxyl group at physiological pH, allowing it to exist as a zwitterion in aqueous solutions (Baião et al., 2025).

2. Characterization analyses

Temporins A and F are synthesized in the first series, as well as new amided C-terminal analogues with a common structure FLPLIG-**X**^{7}-VLSGIL-NH_2, where X = Arg, Lys, Cit, Orn, Dab and Dap. Each peptide was prepared following the Fmoc/O*t*-Bu strategy for solid-phase peptide synthesis. The physicochemical properties of the newly synthesized compounds are summarized in Table 7. Appendix 1 presents the HPLC and MS profiles of the compounds of series 1 in Figures 1.1 – 1.12. The chromatographic purity of all newly synthesized peptides is over 95%.

Table 1 Series 1 - structure with a one-letter amino acid code, molecular formula and analytical data from HPLC-MS analysis, determination of optical rotation and determination of the melting point of newly synthesized peptides

Peptide	Structure	Molecular Formula	MM $_{exact}$ g/mol	$[M+H]^+$ observed g/mol	$[M+Na]^+$ observed g/mol	RT min	α_D^{20} [°]*	M.p. [°C]
DTA	FLPLIG-**R**-VLSGIL-NH_2	$C_{68}H_{117}N_{17}O_{14}$	1395.90	1397.00	1418.95	4.513	-38	158 ± 2
DTF	FLPLIG-**K**-VLSGIL-NH_2	$C_{68}H_{117}N_{15}O_{14}$	1367.89	1368.80	1390.75	4.501	-40	155 ± 3
DTCit	FLPLIG-**Cit**-VLSGIL-NH_2	$C_{68}H_{116}N_{16}O_{15}$	1396.88	1397.75	1419.75	5.793	-34	182 ± 2
DTOrn	FLPLIG-**Orn**-VLSGIL-NH_2	$C_{67}H_{115}N_{15}O_{14}$	1353.87	1354.70	1376.70	4.364	-42	123 ± 2
DTDab	FLPLIG-**Dab**-VLSGIL-NH_2	$C_{66}H_{112}N_{14}O_{15}$	1340.84	1340.75	1362.70	4.191	-52	138 ± 4
DTDap	FLPLIG-**Dap**-VLSGIL-NH_2	$C_{65}H_{110}N_{14}O_{15}$	1326.83	1326.65	1348.70	4.077	-48	140 ± 1

*methanol (c=1)

Hydrolytic stability is one of the most important properties of all newly synthesized molecules for their successful application in medical practice. Therefore, the stability of the newly synthesized peptides was studied for 24 hours in three model systems that mimic different parts of the human body: pH 2 (stomach), pH 7.4 (blood plasma) and pH 9 (small intestine). The enzymes pepsin and trypsin were added to the developed model systems at concentrations of 0.5 mg/mL and 0.1 mg/mL, respectively, in peptide/pepsin ratios of 1:20 and 1:100 (Mistry et al., 2007). The compounds tested had a concentration of 1.0 mg/mL. The parent peptide DTA showed stability in all three pH systems for the 24-hour period tested. All peptides

tested were stable at acidic and neutral pH. Analogues DTF and DTCit were fully hydrolyzed in 24 hours at alkaline pH (Figure 21).

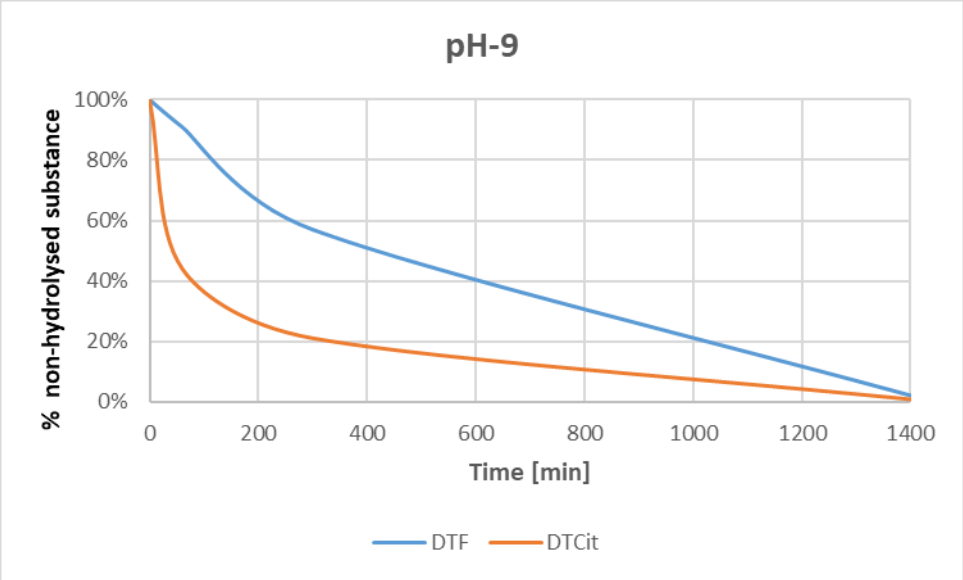


Figure 4 Series 1 - hydrolysis of DTF and DTCit analysis in 24 hours under alkaline pH.

The circular dichroism spectra of all peptides show relatively low ellipticity and lack the characteristic elements of well-defined secondary structures (Figure 22). In particular, none of the spectra show the double minimum at approximately 208 and 222 nm, which are indicative of α-helical conformations, nor is a pronounced negative peak observed near 216–218 nm, which would suggest β-sheet formation (Woody, 1995). Instead, spectra are dominated by broad, weak signals, corresponding to mostly disordered or random folds in solution (Rodger and Marshall, 2021).

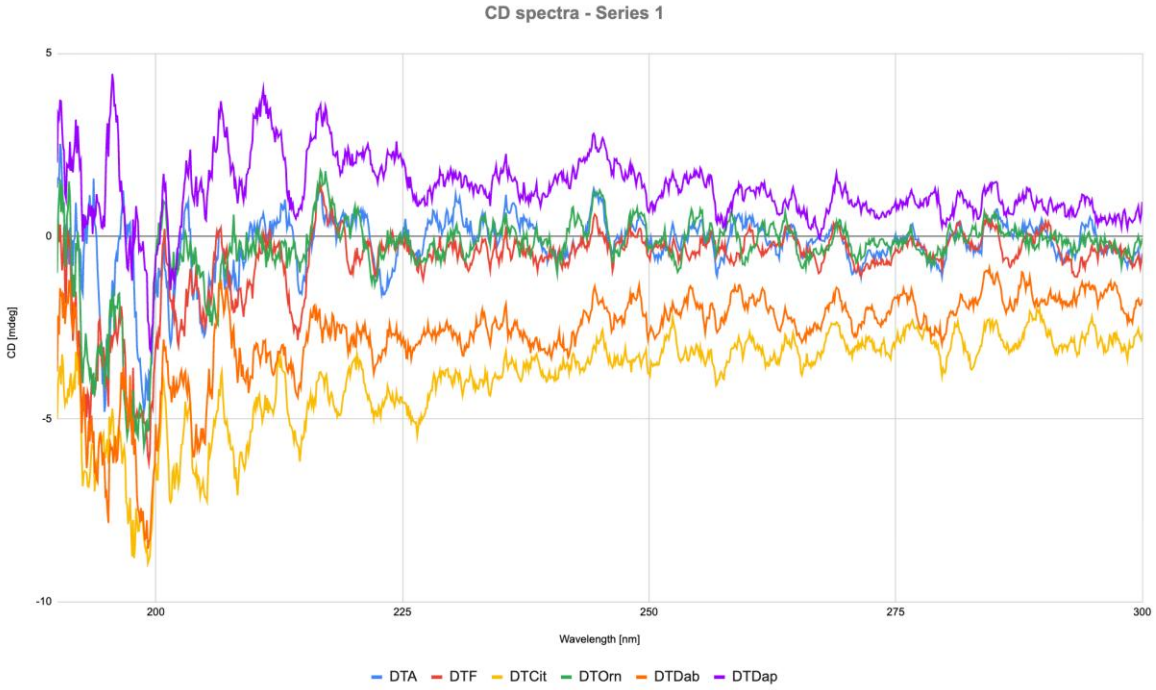


Figure 5 CD spectra - series 1

The resulting spectrum of DTA is comparable to those obtained by Wade et al. (2000) and D'Abramo et al. (2006) as the parent peptide shows a minimum of about 200 nm and a general shape suggesting a random fold conformation (D'Abramo et al., 2006; Wade et al., 2000). The peptides DTA, DTF and DTOrn exhibit very similar spectral profiles, with ellipticity values oscillating around zero above 205 nm and a shallow negative minimum near 195–200 nm. These characteristics are typical of highly flexible peptides with a weakly stable secondary structure. Minor differences between these spectra are likely due to the specific effects of amino acid side chains, and not significant changes in the conformation of the peptide chain. In contrast, the DTDab and DTCit peptides show a more pronounced negative ellipticity with deeper minimum around 195–200 nm, possibly due to enhanced electrostatic interactions or binding between the side chain and the main chain originating from the amino acids Dab and Cit. A significantly different spectrum is possessed by the peptide DTDap, which is characterized by predominantly positive ellipticity over a large part of the 205–250 nm range. This different behavior indicates a significantly altered electronic and conformational environment relative to other peptides. Although this spectrum does not correspond to a classical folded secondary structure, it suggests that DTDap adopts a sequence-dependent conformational structure in solution.

3. Biological studies

3.1. Antibacterial/antifungal studies

In order to establish the sensitivity of the tested strains to both the newly synthesized analogues and to the maternal DTA and the already published DTF, two methods were used – disc diffusion method and broth microdilution, against two model Gram-positive bacterial strains, two model Gram-negative bacterial strains and one fungal strain. The selected strains are among the most common microorganisms that cause a number of diseases. *P. aeruginosa* causes respiratory tract infections (Sharma et al., 2014), *C. albicans* causes mucosal tract infections (Kim and Sudbery, 2011), and *E. coli* causes bloodstream and urinary tract infections (Muhldorfer et al., 2002). Although *B. subtilis* is generally considered non-pathogenic, there are some reports of infections of the central nervous system caused by these bacteria (Tsonis et al., 2018). In addition, when combined with other strains of *Bacillus*, it becomes pathogenic (La Jeon et al., 2012). The species *Arthrobacter* as well as *C. albicans* can act as opportunistic pathogens, especially in immunocompromised hosts, according to clinical reports of them in humans and animals (Gobbetti and Rizzello, 2014).

For the purposes of the disc-diffusion study, microbial cultures were distributed in a thin layer (100 μL with a concentration of 0.5 McFarland) on sterile petri dishes with a solid nutrient medium. 20 μL of peptide solutions with concentrations of 1.4 mg/mL and 10 mg/mL were pipetted on sterile paper discs. A strain-specific antibiotic was used for positive control, and a 10% ethanol solution in water was used as a negative control. The studies were conducted in three repetitions, and the diameter of the inhibition zones (IZ) measured in millimeters was indicative as an average of the three results. In addition, the standard deviation (SD) is calculated and displayed. The obtained values are shown in Table 8 for the Gram-positive strains *B. subtilis* 3562 and *A. oxydans* 9333 and Table 9 for the Gram-negative strains *E. coli* 8785 and *P. aeruginosa* 3700. The inhibition rate for *B. subtilis* 3562, *A. oxydans* 9333 and *P. aeruginosa* 3700 at both concentration levels was calculated for each synthesized analogue. The antibiotic inhibition zone was taken as 100% inhibition and the percentage for each peptide analogue was then extrapolated. Table 10 shows the summary values from the experiment. All the resulting zones can be seen in the figures presented in Appendix No. 2, Table 1.1.

Table 2 Series 1 - Zones of inhibition (mean in mm with standard deviation) of synthesized peptides and associated antibiotics against Gram (+) strains of *B. subtilis* 3562 and *A. oxydans* 9333.

Peptide	Structure	*B. subtilis* 3562			*A. oxydans* 9333		
		1.4 mg/mL	10 mg/mL	Chloramphenicol [30 µg/disc]	1.4 mg/mL	10 mg/mL	Gentamicin [10 µg/disc]
DTA	FLPLIG-**R**-VLSGIL-NH_2	8.8 ± 0.3	8.8 ± 0.8	26.0	6.8 ± 0.3	9.7 ± 0.6	18.0
DTF	FLPLIG-**K**-VLSGIL-NH_2	8.5 ± 0.5	12.7 ± 0.6	25.0	6.7 ± 0.3	11.5 ± 0.5	20.0
DTCit	FLPLIG-**Cit**-VLSGIL-NH_2	0.0	0.0	25.0	0.0	0.0	19.0
DTOrn	FLPLIG-**Orn**-VLSGIL-NH_2	0.0	8.2 ± 0.3	25.0	0.0	8.3 ± 0.6	19.0
DTDab	FLPLIG-**Dab**-VLSGIL-NH_2	0.0	11.3 ± 0.6	25.0	8.2 ± 0.3	14.5 ± 0.5	19.0
DTDap	FLPLIG-**Dap**-VLSGIL-NH_2	0.0	0.0	26.0	0.0	9.8 ± 0.3	18.5

Table 3 Series 1 - Zones of inhibition (mean in mm with standard deviation) of synthesized peptides and associated antibiotics against Gram (-) strains *E. coli* 8785 и *P. aeruginosa* 3700.

Peptide	Structure	*E. coli* 8785			*P. aeruginosa* 3700		
		1.4 mg/mL	10 mg/mL	Gentamicin [10 µg/disc]	1.4 mg/mL	10 mg/mL	Gentamicin [10 µg/disc]
DTA	FLPLIG-**R**-VLSGIL-NH_2	0.0	0.0	18.0	7.8 ± 0.3	9.3 ± 0.6	17.0
DTF	FLPLIG-**K**-VLSGIL-NH_2	0.0	0.0	18.0	7.0 ± 0.5	10.3 ± 0.8	16.0
DTCit	FLPLIG-**Cit**-VLSGIL-NH_2	0.0	0.0	18.0	0.0	0.0	16.0
DTOrn	FLPLIG-**Orn**-VLSGIL-NH_2	0.0	0.0	18.0	0.0	11.3 ± 0.6	16.0
DTDab	FLPLIG-**Dab**-VLSGIL-NH_2	0.0	0.0	18.0	7.7 ± 0.6	14.3 ± 0.6	16.0
DTDap	FLPLIG-**Dap**-VLSGIL-NH_2	0.0	0.0	18.0	0.0	8.8 ± 0.8	17.0

Table 4 Series 1 - percentage inhibition of the tested microorganisms by peptide analogues compared to associated antibiotics. Antibiotics are taken as a 100% inhibition.

Strains	***B. subtilis* 3562**		***A. oxydans* 9333**		***P. aeruginosa* 3700**	
Concentration / **Peptide**	**1.4 mg/mL**	**10 mg/mL**	**1.4 mg/mL**	**10 mg/mL**	**1.4 mg/mL**	**10 mg/mL**
			Inhibition (%)			
DTA	33.8	33.8	37.8	53.9	45.9	54.7
DTF	34.0	50.8	33.5	57.5	43.8	64.4
DTCit	0.0	0.0	0.0	0.0	0.0	0.0
DTOrn	0.0	32.8	0.0	43.7	0.0	70.6
DTDab	0.0	45.2	43.2	76.3	48.1	89.4
DTDap	0.0	0.0	0.0	53.0	0.0	51.8

The newly synthesized analogues showed the same trend in antibacterial activity as those reported by Romero et al. (2020), namely - they are more active against Gram-positive bacteria (Romero et al., 2020). Both the parent peptide DTA and the analogue with Lys (DTF) showed the same characteristics, namely greater activity against the Gram (+) bacteria A. *oxydans* 9333 and *B. subtilis* 3562, and the inhibitory capacity increases with an increase in the concentration of the peptide. However, larger inhibition zones suggest that DTF has slightly stronger antibacterial activity at the higher concentration of 10 mg/mL. The most noticeable difference is with *B. subtilis* 3562, where DTA at 10 mg/mL inhibits at 33.8% and DTF at

50.8%. Also in *P. aeruginosa* 3700, there is a nearly 10% difference in inhibition in favor of DTF. Therefore, antibacterial activity is enhanced when Arg is replaced by a less basic residue - Lys.

Removing the positive charge in the side chain of Cit results in a complete loss of activity against all tested strains. Analogues containing Orn, Dab, and Dap showed similar or greater activity against Gram-positive bacteria. DTOrn is not active at low concentrations, but at high concentrations it shows an inhibition rate against *B. subtilis* 3562 of 32.8% similar to the parent peptide of 33.8%. Against *A. oxydans* 9333, DTOrn has a weaker rate of inhibition compared to DTA, while against *P. aeruginosa* 3700 it shows a 15.9% higher inhibition. The analogue DTDab showed the best antibacterial effect of the new analogues, and against *A. oxydans* 9333 and *P. aeruginosa* 3700 it showed enviably higher activity than the parent peptide DTA. DTDab forms zones of 14.3 ± 0.6 mm in size or 89.4% inhibition, which is quite close to the values obtained from the antibiotic gentamicin. Subsequent shortening of the side chain in DTDap resulted in a lack of antibacterial activity against *B. subtilis* 3562, as well as in the lower concentration with *A. oxydans* 9333 and *P. aeruginosa* 3700. At a concentration of 10 mg/mL, the rate of DTDap inhibition was nearly 23.3% lower against *A. oxydans* 9333 and 37.6% lower against *P. aeruginosa* 3700 compared to DTDab.

All peptides did not form zones of inhibition against the Gram-negative strain of *E. coli* 8785. According to Mangoni and Shai (2009) and Rosenfeld et al. (2006), upon contact with the outer membrane of the cell, the peptide oligomerizes, which may be the cause for the inactivity of temporin A against Gram-negative bacteria (Mangoni and Shai, 2009; Rosenfeld et al., 2006).

The second method by which analogues were evaluated based on their antimicrobial activity was the determination of MIC by broth microdilution. For this purpose, microbial cultures diluted to 0.5 McFarland were dripped into the 96-well microplates and peptide solutions with concentrations in the range from 0 to 320 μg/mL were added to them. After a 24-hour incubation period, absorption was measured at 630 nm. The study was conducted in three repetitions. Table 11 summarizes the MIC values obtained from the experiment.

Table 5 Series 1 - MIC values of temporin A and new analogues [μg/mL]

Peptide	Structure	*B. subtilis* 3562	*E. coli* 8785	*A. oxydans* 9333	*P. aeruginosa* 3700	*C. albicans* 74
DTA	FLPLIG-**R**-VLSGIL-NH_2	80 μg/mL	320 μg/mL	80 μg/mL	320 μg/mL	320 μg/mL
DTF	FLPLIG-**K**-VLSGIL-NH_2	80 μg/mL	320 μg/mL	80 μg/mL	160 μg/mL	320 μg/mL
DTCit	FLPLIG-**Cit**-VLSGIL-NH_2	NI	NI	160 μg/mL	NI	NI
DTOrn	FLPLIG-**Orn**-VLSGIL-NH_2	160 μg/mL	NI	160 μg/mL	NI	NI
DTDab	FLPLIG-**Dab**-VLSGIL-NH_2	160 μg/mL	320 μg/mL	320 μg/mL	320 μg/mL	NI
DTDap	FLPLIG-**Dap**-VLSGIL-NH_2	320 μg/mL	NI	NI	NI	NI

*NI – no inhibition.

From the MIC data, it is evident that the lowest value for all strains is 80 μg/mL against the Gram-positive bacteria *B. subtilis* 3562 and *A. oxydans 9333*. These results are fully consistent with those obtained by Capparelli et al. (2009), where lower MIC values of the peptides were also against Gram-positive bacteria (Capparelli et al., 2009). However, according to the literature data published by Paduszynska et al. (2020), temporin A demonstrated activity against the Gram-negative bacteria *of P. aeruginosa*. While in the study of Paduszynska et al. MIC for *P. aeruginosa* ATCC 9029 was found at 512 mg/L, DTA demonstrated strong activity against the test strain selected in our study against *P. aeruginosa* 3700 at 320 μg/mL (Paduszynska et al., 2020).

The minimum bactericidal concentration for the strains *B. subtilis* 3562, *A. oxydans* 9333 and *P. aeruginosa* 3700 was established. The parent peptide DTA MBC values for *P. aeruginosa* 3700 and *B. subtilis* 3562 were 320 μg/mL. The DTOrn analogue demonstrated a bactericidal effect at 320 μg/mL against *B. subtilis* 3562 and 160 μg/mL against *A. oxydans* 9333. Also in DTCit, MBC against *A. oxydans* 9333 was reported to be 160 μg/mL.

All newly synthesized analogues showed no activity against the Gram-negative bacteria *E. coli* 8785 by the disc-diffusion method. However, MIC values for DTA, DTF and DTDab were established. This can be explained by the differences between the two techniques for studying antibacterial activity. According to Mercer et al. (2020), Kunin and Edmondson (1968), and Lehrer et al. (1991), the disc-diffusion method greatly underestimates or completely masks the actual activity when the positively charged AMPs interact with the negatively charged agar components. The same trend is observed in the interaction of Gram (+) bacteria *A. oxydans* 9333 and the analogue DTCit (Kunin and Edmondson, 1968; Lehrer et al., 1991; Mercer et al., 2020). The way the peptide interacts with the environment and bacterial cells in the methods themselves is different. In microdilution in broth, the peptides are in direct contact with the strain deep in the culture medium, where the interaction is direct, thus obtaining easier penetration through the cell membrane. In the disc-diffusion method, the peptide interacts with

both the strain that grows on the surface of the agar and the agar itself and its components, and weaker antibacterial activity is observed (Mercer et al., 2020).

According to the research of Rollins-Smith et al. (2003), the parent peptide DTA shows activity against *C. albicans* (Rollins-Smith et al., 2003). Our studies have shown that in addition to DTA, the Lys-substituted analogue DTF has antifungal activity. Therefore, the results presented in this dissertation correlate with those obtained from them, and it has been established that the antifungal potential of temporin analogues depends on the higher basicity of the Arg and Lys.

DTDab showed the strongest antimicrobial activity of the newly synthesized analogues. From the data collected, it can be seen that the antibacterial activity was positively affected by the shortening of the side chain to two methylene groups in the side chain. The additional shortening of the side chain of the DTDap analogue resulted in a reduced antimicrobial effect at low concentrations and showed no activity against *A. oxydans* 9333 and *P. aeruginosa* 3700, but at higher concentrations it showed inhibition. Interestingly, however, the same analogue did not show activity when using the disc diffusion method to the other Gram (+) bacterium *B. subtilis* 3562, although it had a MIC value of 320 μg/mL.

It is possible to conclude that a bulkier, longer and therefore more basic side chain at position 7 is needed to obtain a lower MIC value after examining the resulting MIC values and taking into account the variations between the six amino acids. Larger areas of inhibition are provided by an amino acid that is shorter and less basic, such as Dab. Furthermore, the antibacterial qualities observed in the DTCit analogue are lost when the positive charge in the side circuit is removed.

3.2. Determination of cytotoxicity, phototoxicity and antiproliferative activity

Several zwitterionic structures, including phosphatidylethanolamine and phosphatidylcholine, are present in the phospholipid bilayer of healthy mammalian cells. They all possess a neutral total charge and reduce the attraction of cationic antimicrobial peptides to these cells (Mai et al., 2001; Schweizer, 2009). From the perspective of this fact, numerous research teams have investigated peptides with proven antimicrobial properties for potential antiproliferative effects (Deslouches and Di, 2017; Javadpour et al., 1996; Mader and Hoskin, 2006; Marqus et al., 2017; Mistry et al., 2007).

According to published studies, temporins are toxic to healthy mammalian cells at IC_{50} concentrations between 50 and 100 μM (Mäntylä et al., 2005; Rinaldi et al., 2002). However, in order to improve their safety, methods and possibilities for changing temporins are being

investigated. Compared to the original peptide (DTA), four of the studied peptide analogues of temporin A (DTCit, DTOrn, DTDab and DTDap) demonstrated markedly lower cytotoxicity. The observed effect (cytotoxicity/phototoxicity) is of a dose-dependent type (Figure 23). Based on the sigmoidal curves obtained, the average CC_{50} values for each study were calculated for every peptide (Table 12).

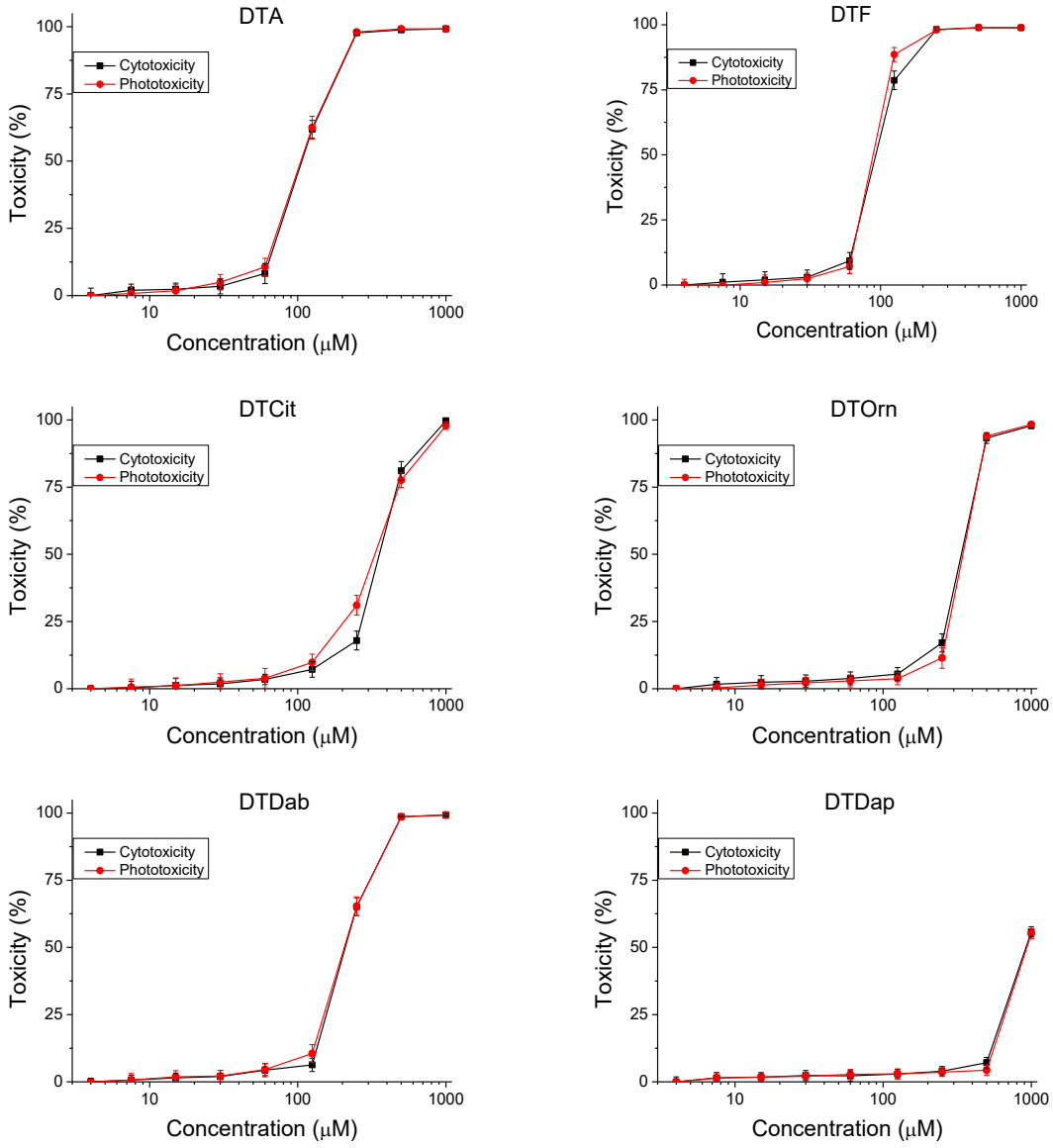


Figure 6 Series 1 - dose-response curves for cyto- and phototoxicity of peptide analogues determined in cell line BALB 3T3 clone A31. Values are averages ± SD, $n = 6$.

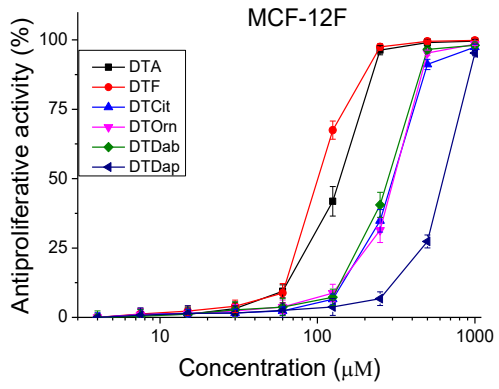
Furthermore, the phototoxicity test revealed that the peptide analogues examined were not phototoxic. These results demonstrate the potential for safe topical and systemic administration of these peptides. With the lowest cytotoxicity, the peptide DTDap stood out with a value of $CC_{50} = 923.84 \pm 21.56$.

Table 6 Series 1 - cytotoxicity/phototoxicity in a cell line BALB 3T3 clone A31, mean values of CC_{50} and PIF factor.

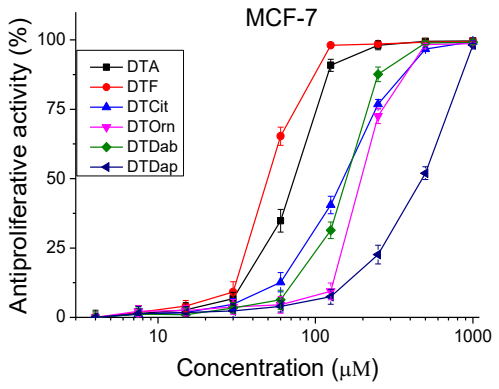
Peptide	Mean CC_{50} ± SD (µM)		PIF**
	- Irr	+ Irr*	
DTA	106.32 ± 4.18	105.40 ± 4.88	1.01
DTF	92.40 ± 2.82	88.35 ± 1.48	1.05
DTCit	356.09 ± 7.64	331.78 ± 12.30	1.07
DTOrn	337.27 ± 7.09	345.1 ± 6.41	0.98
DTDab	209.57 ± 6.69	206.53 ± 7.41	1.01
DTDap	923.84 ± 21.56	933.3 ± 20.7	0.99

* Irr—Irradiation; ** PIF—Photo-Irritation Factor. PIF < 2—not phototoxic; 2 < PIF < 5—possible phototoxicity; PIF > 5—phototoxic.

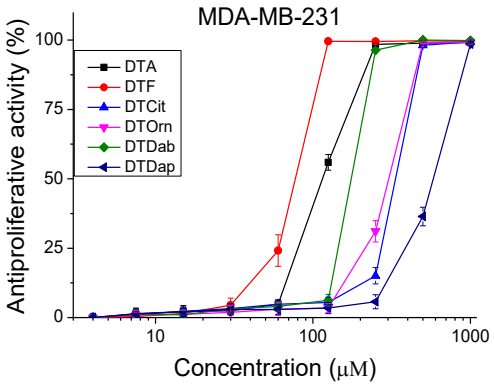
Human cell lines, an *in vitro* healthy tissue model (MCF-12F), and two forms of breast cancer (MCF-7 and MDA-MB-321) were used for the antiproliferative activity test (Figure 24). In the MCF-7 cell line (luminal breast cancer type A), the parent peptide (DTA), and the peptide with Lys at position seven (DTF) showed the strongest antiproliferative effect. We found that the Cit-substituted peptide (DTCit) exhibits selectivity (SI > 2). On the other hand, the peptide analogues studied did not exhibit selectivity (SI < 2) against the MDA-MB-231 cell line, which is the basal breast cancer type B. These findings confirm data from the literature on the antitumor activity of positively charged temporins and their analogues (Swithenbank et al., 2020; Wang et al., 2013), where the authors examined various tumor cell lines (A549, MCF-7, MDA-MB-231).



(a)



(b)



(c)

Фигура 7 Series 1 - antiproliferative activity of peptide analogues determined in (a) non-tumorogenic MCF-12F cells, (b) tumor cell lines MCF-7 and (c) MDA-MB-231, $n = 6$.

The antiproliferative effect of the peptide analogues studied was lower than that of DTA, except for DTF, which had an IC_{50} = 100.48 ± 3.30 μM for the MCF-12F cell line (Table 13). Treatment with the peptide analogue DTDap, which has the shortest side chain with the lowest basicity, resulted in the lowest antiproliferative effect against all cell lines tested, along with the lowest cytotoxicity and phototoxicity. Compared to MDA-MB-231, the MCF-7 cell line showed significantly higher sensitivity to the studied peptide analogue series. The maternal peptides DTF and DTA had the strongest antiproliferative effect in MCF-7 cells, with IC_{50} values of 49.75 ± 1.90 μM and 73.15 ± 3.36 μM, respectively. The peptides DTF and the analogue containing the non-essential amino acid Cit (DTCit) showed significant selectivity (SI > 2) in the MCF-7 cell line. The same analogue that has a non-basic side chain is not phototoxic and has relatively low cytotoxicity. Conversely, the peptide analogues studied did not show adequate selectivity against triple-negative basal breast cancer type B (MDA-MB-231). According to the MCF-7 cell line, the results obtained indicate that more significant selectivity is achieved by reducing the baseline of the side chain from DTA through DTF and DTCit. However, the antiproliferative effect of temporin A analogues decreases in the same direction as the basicity of their side chain decreases. These findings show a strong correlation with the AMPs defensins data collected by Zou et al. (2007) (Zou et al., 2007).

Table 7 Series 1 - average values of IC_{50} and selectivity index

Peptide	Mean IC_{50} ± SD (μM)			Selectivity index (SI)*	
	MCF-12F	MCF-7	MDA-MB-231	MCF-7	MDA-MB-231
DTA	138.65 ± 8.36	**73.15 ± 3.36**	115.13 ± 4.04	1.90	1.20
DTF	100.48 ± 3.30	**49.75 ± 1.90**	77.01 ± 2.92	**2.02**	1.30
DTCit	300.73 ± 10.49	149.69 ± 6.94	334.9 ± 4.37	**2.01**	0.90
DTOrn	305.59 ± 10.75	195.62 ± 3.63	302.87 ± 8.67	1.56	1.01
DTDab	280.25 ± 13.59	157.45 ± 4.97	174.91 ± 1.35	1.78	1.60

DTDap	**630.44 ± 9.97**	472.61 ± 16.21	580.97 ± 17.50	1.33	1.09

*selectivity index, SI = IC_{50} (MCF-12F)/ IC_{50} (tumor cells)

II. Second series of analogues

1. Design

In the second series, the following changes were made to the temporin A molecule:

- Ser^{10} was replaced by Tyr and Thr, two other hydroxyl-containing naturally occurring amino acids;
- Phe^{1} was replaced by Tyr, another proteinogenic aromatic amino acid, or non-proteinogenic fluorinated Phe (Phe(4-F)).

To investigate their importance for biological activity and stability, all modifications were made to mimic the naturally occurring amino acids in the primary structure of temporin A using comparable natural or non-proteinogenic amino acids (Figure 25). These particular amino acids were selected to examine the difference in the type of hydroxyl group – primary, secondary or aromatic, as well as the addition of fluorine on its antimicrobial and antiproliferative properties, as well as on hydrolytic stability.

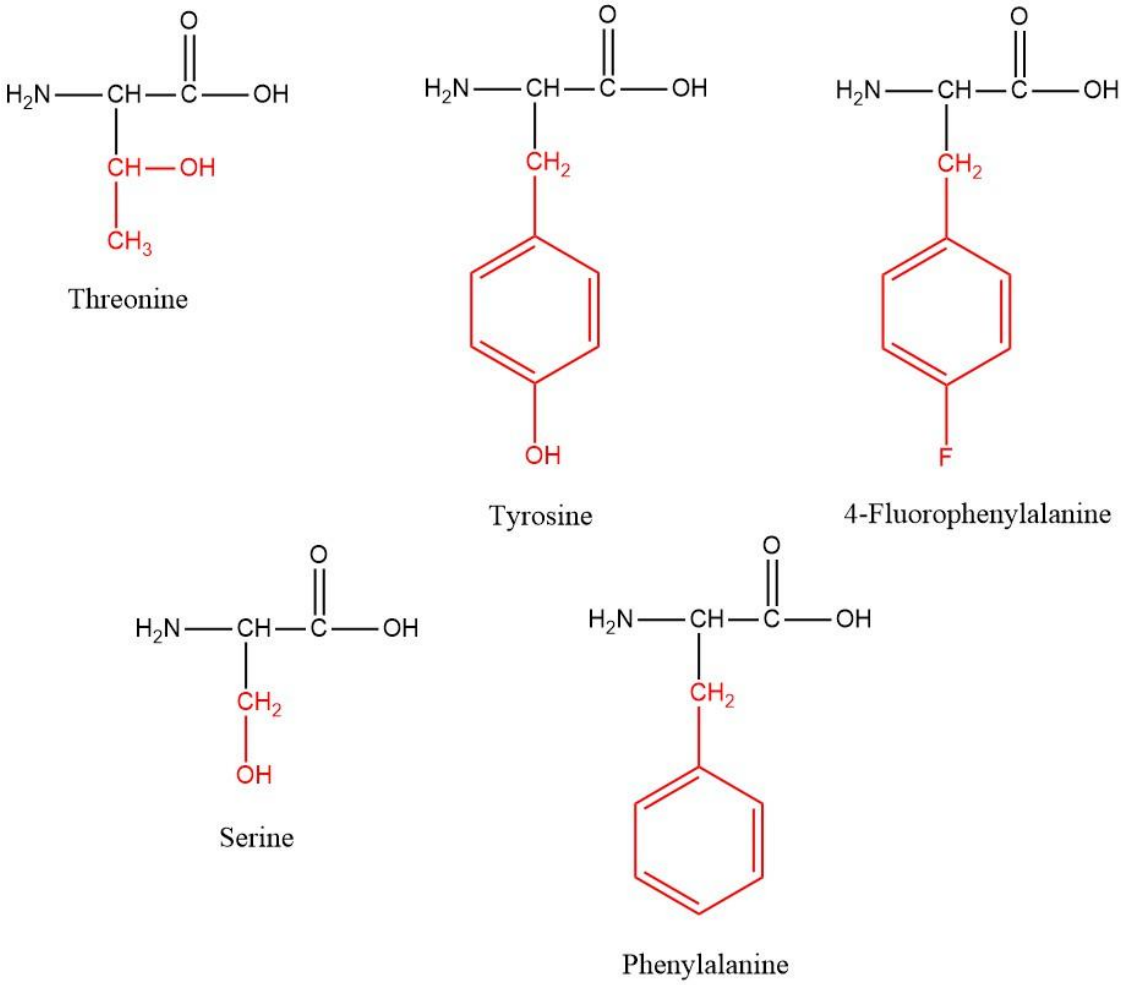


Figure 8 Structures of amino acids used at position 1 and 10 of temporin A (Dimitrova et al., 2025)

The side chains of four of the twenty naturally occurring α-amino acids (α-AK) have aromatic rings. L-Tyr is categorized as a conditionally essential amino acid, while L-Phe is an essential α-AK and cannot be produced by the human body. These amino acids have an aromatic ring attached to the C atom in their side chains, as a common structural feature (Hernández et al., 2010). The Phe side chain has a phenyl ring, which, due to the hydrophobic nature of the aromatic side chain, is classified as a neutral and non-polar amino acid (Das et al., 2018). The human body uses the liver enzyme phenylalanine hydroxylase (PAH), which converts the phenyl-phenol ring of the side chain to synthesize Tyr from Phe. The accumulation of Phe from PAH deficiency is transformed into phenylketone, resulting in phenylketonuria (PKU), a metabolic disorder. Tyr becomes a necessary amino acid in this situation and must be supplied to the body. Tyr is considered a biochemical precursor to a host of other molecules important to biology, including melanin, adrenaline, dopamine, and dihydroxyphenylalanine (DOPA). However, the hydroxyl group of Tyr allows the kinase intermediate to phosphorylate the

tyrosine side chain, a chemical change necessary for enzymatic regulation. One of the double helices of DNA is cut by topoisomerase I, an enzyme that can relax tense superhelical DNA by creating a reversible phosphothyrosine bond between a tyrosine hydroxyl group and a DNA phosphate group (Hernández et al., 2010). The phenolic ^{n}OH group of Tyr is capable of hydrogen bonds unlike Phe. In this regard, a local element, containing tyrosine, known as the "tyrosine angle," is present in a number of proteins, including immunoglobulin, fibronectin type III, and the β-barrel Greek key protein. The tyrosine angle is a conformation in which Tyr near the beginning or end of an antiparallel β-chain creates a hydrogen bond with the backbone amide of adjacent tyrosine residues, causing the nucleus to fold and stabilize (Lee et al., 2019).

Amino acids with aromatic side chains in the peptide chain are thought to be sites that are recognized by specific enzymes. For example, the enzyme chymotrypsin cleaves the peptide chain at a bond involving the residues Phe, Tyr, and Trp at the C-terminus (Hernández et al., 2010; Terra and Ferreira, 2012). In addition, these amino acids are essential for the physical characteristics of proteins and peptides. These amino acids help in the structural stabilization and self-assembly of short peptides, soluble proteins, and transmembrane proteins. Their side chain rings can stack on top of each other, and the Tyr and Trp rings can participate in hydrogen bonds through their OH and NH groups. The fluorescent effect of these amino acids has been used in recent years to develop sensitive methods that allow the collection of data on cell and tissue metabolism, as well as on the structural and dynamic characteristics of proteins and peptides (Hernández et al., 2010).

Tyr is a universal amino acid that is crucial for controlling the structural conformational changes of proteins. Its redox-active characteristic also facilitates the transport of protons and electrons along with metal ions to the active sites of enzymes. All living things depend on precisely regulated electron transfer processes that are controlled by complex protein/peptide complexes. The participation of tyrosine in multiple molecular interactions and biosynthetic transformations is possible thanks to its phenolic side chain and distinct chemical reactivity. With its ability to chemically join phenolic groups to form dithyrosine and transfer electrons, this reactivity offers an effective means of influencing protein activity. Special enzymatic reactions, interactions with redox-active metal complexes, radiolysis or photolysis can easily carry out such cross-linking reactions. The ability of tyrosine residues to bind to an oxidizing substance is vital for living organisms, but it can also lead to harmful protein conjugation. Such a pathology usually occurs as a result of oxidative stress, various pathological conditions and the natural aging process (Lee et al., 2019).

Halogenation can give compounds many beneficial characteristics, such as higher cytotoxicity to cancer cells, better affinity and selectivity towards the target, fewer side effects, and easier passage through cell membranes and blood-brain barriers. Additionally, it has the potential to significantly alter protein function (Sana et al., 2022). The stability, specificity, and activity of AMPs are significantly affected by halogenation (Huan et al., 2020). It is well known that the addition of halogens to peptide chemical structures usually makes molecules more hydrophobic (Molchanova et al., 2020). The basis for its successful application is the distinctive stereoelectronic properties of fluorine substitutions, which result from a combination of very low polarizability, minimal size, and the strongest inductive effect among known chemical elements (Salwiczek et al., 2012).. Fluorine substitution can improve efficacy and selectivity towards the target by changing pKa, conformation, hydrophobic interactions, lipophilicity, or a combination of these characteristics. In addition, fluoride has been used to deal with problems with drug metabolism. Also, fluorine can alter lipophilicity and limit conformation, which can improve metabolic stability (Gillis et al., 2015). When fluorine replaces hydrogen in the aromatic ring of Phe, the electrostatic potential is rearranged and the hydrophobicity of the aryl side chain increases (Salwiczek et al., 2012).

Polar amino acids easily interact with water because they contain functional groups that can form hydrogen bonds. Because they have a polar hydroxyl group, Ser, Thr, and Tyr can participate in hydrogen bonds, which is crucial for protein structure. In proteins, hydroxyl groups have additional uses. The OH-groups of Ser and Thr serve as places for binding carbohydrates (McKee and McKee, 2017).

2. Characterization analyses

The analogues of temporin A synthesized in the second series have a common structure FLPLIGRVL-$\mathbf{X^1}$-GIL-NH_2, where X_1 = Tyr or Thr, and $\mathbf{X^2}$-LPLIGRVLSGIL-NH_2, where X_2 stands for Tyr or Phe(4-F). Each peptide was prepared following the Fmoc/O*t*-Bu strategy for solid-phase peptide synthesis. The physicochemical properties of the newly synthesized compounds are summarized in Table 14. Appendix 1 presents the HPLC and MS profiles of the compounds of series 2 in Figures 2.1 – 2.9. All newly synthesized peptides, subjected to biological activity tests, have over 95% chromatographic purity.

Table 8 Series 2 - structure with a one-letter amino acid code, molecular formula and analytical data from HPLC-MS analysis, determination of optical rotation and determination of the melting point of newly synthesized peptides

Peptide	Structure	Molecular formula	MM $_{exact}$ [g/mol]	$[M+H]^+$ observed [g/mol]	$[M+Na]^+$ observed [g/mol]	RT [min]	α_d^{20} [°]**	M.p. [°C]
DTA*	FLPLIGRVL-**S**-GIL-NH_2	$C_{68}H_{117}N_{17}O_{14}$	1395.90	1397.00	1418.95	4.513	−38	158 ± 2
DTThr	FLPLIGRVL-**T**-GIL-NH_2	$C_{69}H_{119}N_{17}O_{14}$	1409.91	1410.75	1432.70	4.486	-40	135 ± 1
DTTyr10	FLPLIGRVL-**Y**-GIL-NH_2	$C_{74}H_{121}N_{17}O_{14}$	1471.93	1472.70	1494.70	4.712	-38	145 ± 1
DTTyr1	**Y**-LPLIGRVLSGIL-NH_2	$C_{68}H_{117}N_{17}O_{15}$	1411.89	1412.60	-	4.177	-58	123 ± 2
DT4F	**Phe(4F)**-LPLIGRVLSGIL-NH_2	$C_{68}H_{116}FN_{17}O_{14}$	1413.89	1415.05	1437.10	4.177	-64	141 ± 1

*DTA is fully characterized in Series 1; **methanol (c=1)

The hydrolytic stability of the resulting peptides was evaluated in model systems that reproduce the conditions of the stomach (pH 2.0), blood plasma (pH 7.4), and small intestine (pH 9.0) in the human body, in the same manner as the Series 1 analogues.

All peptides were found to be completely stable at pH 2 and 7 during the 24-hour test period. Peptides, which include fluorinated Phe at position 1 (DT4F) and Thr at position 10 (DTThr), were fully hydrolyzed in 24 hours at pH 9. The data obtained are summarised in Figure 26. The results are largely consistent with earlier findings using a different class of anti-cancer peptides that are analogues of natural somatostatin (Danalev et al., 2020). According to the findings, the addition of halogen atoms – i.e., fluorine or chlorine – reduces hydrolytic stability at a base pH. This effect is stronger for chlorine atoms and weaker for fluorine atoms. However, in the three pH values tested, analogues with a more hydrophobic aromatic side chain Tyr and Phe show good stability in 24 hours.

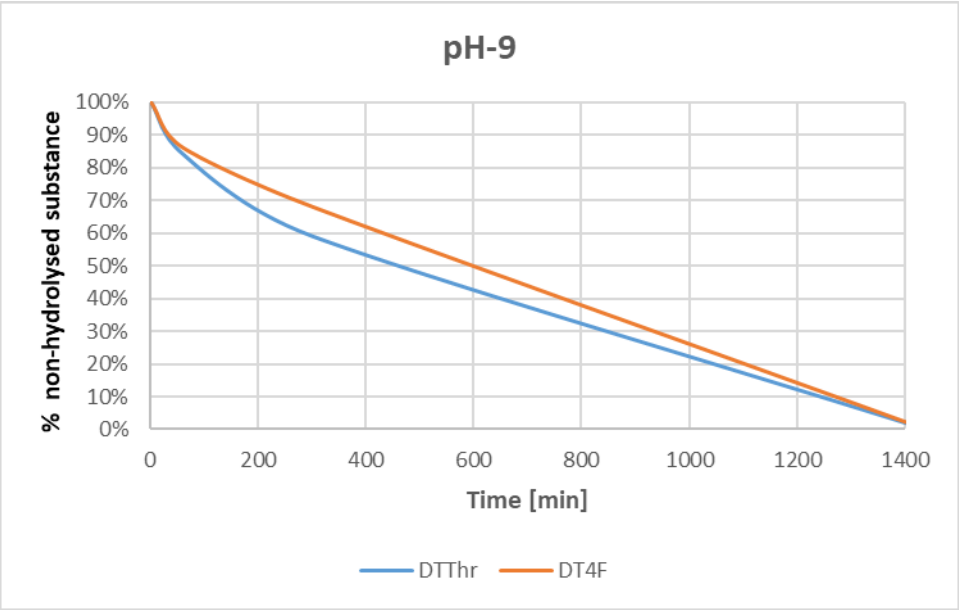


Figure 9 Series 2 - hydrolysis of DTThr and DT4F for a period of 24 hours at alkaline pH.

All CD spectra in this series exhibit relatively low ellipticity and are dominated by weak signals below approximately 200 nm (Figure 27). Above 200 nm spectra stabilize and fluctuate near zero ellipticity, indicating the absence of a well-defined secondary structure.

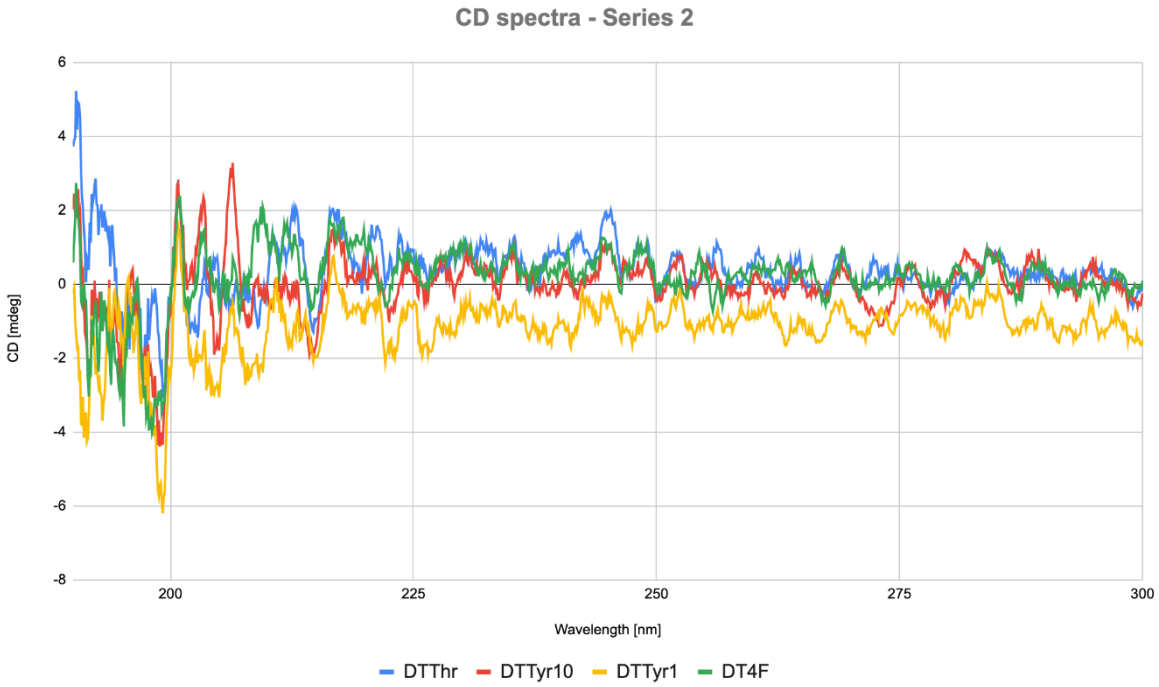


Figure 10 CD spectra - series 2

None of the peptides exhibit the characteristic CD characteristics of α-helical conformations. Instead, the observed profiles are consistent with mostly disordered or random folds in aqueous solution. Among the peptides, DTThr and DTTyr10 exhibit very similar spectral behavior, with ellipticity values ranging around zero in the range of 210–300 nm. These profiles suggest highly flexible structures with minimal peptide chain arrangement. The peptide DTTyr1 showed slightly more negative ellipticity over much of the measured range, especially between 200 and 260 nm, indicating a moderate increase in conformational deviation relative to DTThr and DTTyr10, although not yet sufficient to indicate the formation of a stable secondary structure. The spectrum of DT4F overlaps to a large extent with those of DTThr and DTTyr10, which supports the conclusion that substitutions in this peptide series do not induce substantial folding under the conditions studied. Minor variations between spectra are likely due to side-chain specific electronic effects rather than changes in the overall secondary structure. Overall, the CD spectra of the second series show that all peptides remain largely disordered in solution, with no detectable α-helix or β-sheet content. The close similarity between the spectra indicates that the introduced sequence modifications have only a limited impact on the global conformational preferences of these peptides under the experimental conditions used.

3. Biological studies

3.1. Antibacterial/antifungal studies

In order to establish the sensitivity of the tested strains to the newly synthesized analogues of the second series, two methods were used – disc diffusion method and microdilution in broth. In general, the results of both methods show that the new peptides do not exhibit activity against *E. coli* 8785.

For the purposes of the disc-diffusion study, the peptides were examined in a similar manner to those in Series 1. The obtained values are shown in Table 15 for the Gram-positive strains *B. subtilis* 3562 and *A. oxydans* 93333 and Table 16 for the Gram-negative strains *E. coli* 8785 and *P. aeruginosa* 3700. The inhibition rate for *B. subtilis* 3562, *A. oxydans* 9333 and *P. aeruginosa* 3700 at both concentration levels were calculated for each synthesized analogue. The antibiotic inhibition zone was taken as 100% inhibition and the percentage for each peptide analogue was then extrapolated. Table 17 shows the obtained values from the experiment. 2.1.

Table 9 Series 2 - zones of inhibition (mean in mm with standard deviation) of synthesized peptides and associated antibiotics against Gram (+) strains *B. subtilis* 3562 and *A. oxydans* 9333.

Peptide	Structure	*B. subtilis* 3562			*A. oxydans* 9333		
		1.4 mg/mL	10 mg/mL	Chloramphenicol [30 µg/disc]	1.4 mg/mL	10 mg/mL	Gentamicin [10 µg/disc]
DTA*	FLPLIGRVL-**S**-GIL-NH_2	8.8 ± 0.3	8.8 ± 0.8	26	6.8 ± 0.3	9.7 ± 0.6	18
DTThr	FLPLIGRVL-**T**-GIL-NH_2	7.2 ± 0.3	0	24	8	10.5 ± 0.5	21
DTTyr10	FLPLIGRVL-**Y**-GIL-NH_2	0	0	27	0	0	23
DTTyr1	**Y**-LPLIGRVLSGIL-NH_2	0	0	28	10	10	22
DT4F	**Phe(4F)**-LPLIGRVLSGIL-NH_2	8.2 ± 0.3	9.7 ± 0.3	24	10	12.8 ± 0.3	20

* DTA is fully characterized in Series 1;

Table 10 Series 2 - inhibition zones (mean in mm with standard deviation) of synthesized peptides and associated antibiotics against Gram (-) strains *E. coli* 8785 and *P. aeruginosa* 3700.

Peptide	Structure	*E. coli* 8785			*P. aeruginosa* 3700		
		1.4 mg/mL	10 mg/mL	Gentamicin [10 µg/disc]	1.4 mg/mL	10 mg/mL	Gentamicin [10 µg/disc]
DTA*	FLPLIGRVL-**S**-GIL-NH_2	0	0	18	7.8 ± 0.3	9.3 ± 0.6	17
DTThr	FLPLIGRVL-**T**-GIL-NH_2	0	0	17.5	0	0	17
DTTyr10	FLPLIGRVL-**Y**-GIL-NH_2	0	0	16.5	0	0	18
DTTyr1	**Y**-LPLIGRVLSGIL-NH_2	0	0	17	0	0	16
DT4F	**Phe(4F)**-LPLIGRVLSGIL-NH_2	0	0	17	9.2 ± 0.3	12.3 ± 0.6	18

* DTA is fully characterized in Series 1;

Table 11 Series 2 - percentage inhibition of the tested microorganisms by peptide analogues compared to associated antibiotics. Antibiotics are taken as a 100% inhibition.

Strain	*B. subtilis* 3562		*A. oxydans* 9333		*P. aeruginosa* 3700	
Concentration / Peptide	1.4 mg/mL	10 mg/mL	1.4 mg/mL	10 mg/mL	1.4 mg/mL	10 mg/mL
	Inhibition (%)					
DTA*	33.8	33.8	37.8	53.9	45.9	54.7
DTThr	30.0	0.0	36.4	47.7	0.0	0.0
DTTyr10	0.0	0.0	0.0	0.0	0.0	0.0
DTTyr1	0.0	0.0	47.6	42.9	0.0	0.0
DT4F	32.8	48.5	50.0	64.0	51.1	68.3

* DTA is fully characterized in Series 1;

The results of the disc diffusion test showed that fluorinated DT4F was the most active molecule against the two Gram-positive strains *B. subtilis* 3562 and *A. oxydans* 9333. With the highest inhibition rate of 51% at a lower concentration (1.4 mg/mL) and 68% at a higher concentration (10 mg/mL), DT4F was also effective against Gram-negative *P. aeruginosa* 3700. Therefore, fluorinated temporin A (DT4F) showed a higher inhibitory potential. especially against *P. aeruginosa* 3700, compared to the inhibitory zones of the parent peptide DTA (Series 1). The new compounds are again more active against Gram-positive strains than Gram-negative strains, which is consistent with research by Rosenfeld et al. (2006), Mangoni and Shai (2009), as well as current Series 1 results (Mangoni and Shai, 2009; Romero et al., 2020; Rosenfeld et al., 2006). In line with the previous series also, none of the new peptides studied showed activity against *E. coli* 8785 (Dimitrova et al., 2024).

The inhibition zones after treatment with DTThr have shown that the alkyl side chain of Thr has a better effect than the more hydrophobic and aromatic side chain of Tyr. Regarding DTThr and its activity against *B. subtilis* 3562, an intriguing conclusion can be drawn. At the lower tested concentration of 1.4 mg/mL, it forms an inhibition zone, and at the higher tested concentration of 10 mg/mL, however, this does not happen. This result is largely consistent with the finding of Zapadka et al. (2017) that a higher concentration of the peptide can negatively affect stability, making compounds less active or completely inactive (Zapadka et al., 2017).

The addition of hydroxyl containing an aromatic side chain in the tenth position with the replacement of Ser with Tyr (DTTyr10) results in a complete loss of antibacterial activity in the disc diffusion method. While modification of N-terminal Phe with Tyr (DTTyr1) results in a partial loss of the antibacterial properties of the peptide – DTTyr1 showed inhibition of 47.6% at a lower concentration (1.4 mg/mL) and 42.9% at a higher concentration (10 mg/mL).

The second method by which analogues were evaluated based on their antimicrobial activity was the determination of MIC by broth microdilution. The study was conducted

similarly to the same one from Series 1. Table 18 presents a summary of the obtained values of MIC.

Table 12 Series 2 - MIC values of temporin A and new analogues [μg/mL].

Peptide	Structure	*B. subtilis* 3562	*E. coli* 8785	*A. oxydans* 9333	*P. aeruginosa* 3700	*C. albicans* 74
DTA*	FLPLIGRVL-**S**-GIL-NH_2	80 μg/mL	320 μg/mL	80 μg/mL	320 μg/mL	320 μg/mL
DTThr	FLPLIGRVL-**T**-GIL-NH_2	160 μg/mL	NI	320 μg/mL	320 μg/mL	NI
DTTyr10	FLPLIGRVL-**Y**-GIL-NH_2	320 μg/mL	NI	320 μg/mL	320 μg/mL	NI
DTTyr1	**Y**-LPLIGRVLSGIL-NH_2	160 μg/mL	NI	320 μg/mL	320 μg/mL	NI
DT4F	**Phe(4F)**-LPLIGRVLSGIL-NH_2	80 μg/mL	NI	160 μg/mL	80 μg/mL	NI

* DTA is fully characterized in Series 1;**NI – no inhibition.

All target compounds demonstrated bacteriostatic properties against *B. subtilis* 3562, *A. oxydans* 9333 and *P. aeruginosa* 3700. According to the results obtained from the Series 1, only the parent peptide DTA exhibited bacteriostatic and fungistatic effects against the strains *E. coli* 8785 and *C. albicans* 74, respectively. Among the new analogues against *B. subtilis* 3562, *A. oxydans* 9333 and *P. aeruginosa* 3700, the analogue DT4F and by this method has the lowest MIC values. Fluorinated DT4F showed a potential comparable to or even higher than that of most compounds, compared to the MIC values of the parent peptide DTA. Its MIC value against *P. aeruginosa* 3700 was four times lower than that of DTA (320 μg/mL).

The DTTyr10 analogue showed inhibitory ability and MIC values of 320 μg/mL were found in *B. subtilis* 3562, *A. oxydans* 9333 and *P. aeruginosa* 3700. However, the same did not have inhibition zones against any of the tested strains. As mentioned above, the differences between the two methods in terms of the interaction of the peptide with the medium and bacterial cells could be one of the reasons for the observed results (Mercer et al., 2020).

The new spatially more constrained Thr residue (DTThr) has better antibacterial properties against *B. subtilis* 3562 and the same activity against the other Gram-positive strain, *A. oxydans* 9333, and the Gram-negative bacteria, *P. aeruginosa* 3700, compared to the effect of replacing the Ser residue at position 10 with Tyr.

3.2. Determination of cytotoxicity, phototoxicity and antiproliferative activity

The results showed a significant reduction in toxicity – six and more than ten times, respectively – when the parent peptide DTA was modified at positions 1 or 10 with Tyr (DTTyr1, DTTyr10). Therefore, it is possible to assume that the maternal DTA molecule with an aromatic ring and a hydroxyl function of the phenolic type is less toxic than one that is aliphatic (Ser^{10}) or lacks an OH group (Phe^{1}). Therefore, the peptides with the lowest cytotoxicity are DTTyr10 with a value of CC_{50} > 1000 μM and DTTyr1 with a value of CC_{50} = 668.98 ± 13.39 μM. On the other hand, cytotoxicity is doubled compared to the parent peptide

when fluorine is added to the primary structure of the peptide by replacing the proteinogenic Phe at position 1 with fluorinated Phe(4-F) (DT4F) (Figure 28 and Table 19). However, none of the new analogues of Temporin A showed phototoxicity.

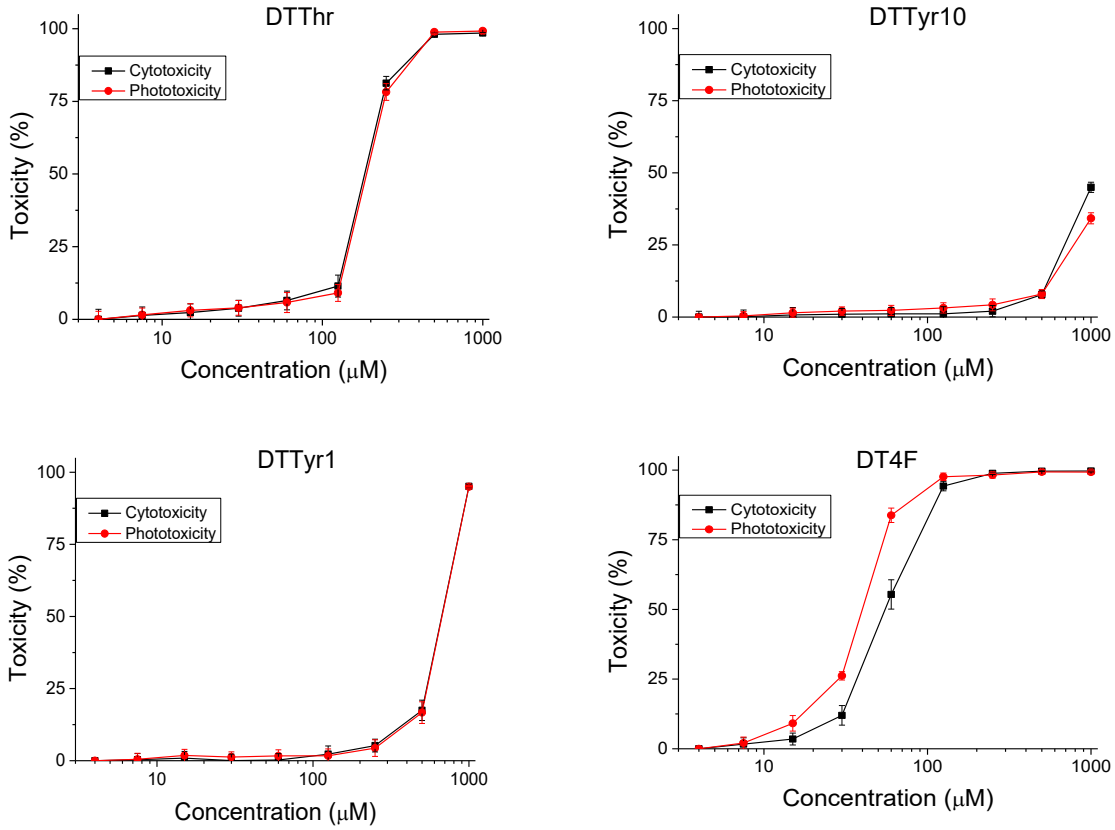


Figure 11 Series 2 - dose-response curves for cyto- and phototoxicity of peptide analogues determined in cell line BALB 3T3 clone A31. Values are averages ± SD, $n = 6$.

Table 13 Series 2 - cytotoxicity/phototoxicity in a cell line BALB 3T3 clone A31, mean values of CC_{50} and PIF factor

Peptide	Mean CC_{50} ± SD (μM)		PIF**
	- Irr	+ Irr*	
DTA* **	106.32 ± 4.18	105.40 ± 4.88	1.01
DTThr	183.36 ± 2.91	188.42 ± 3.55	1.0
DTTyr10	> 1000	> 1000	-
DTTyr1	668.98 ± 13.39	670.76 ± 12.2	1.0
DT4F	55.41 ± 4.64	39.99 ± 0.78	1.4

* Irr – Irraditation; **Photo irritation factor: PIF < 2 = not phototoxic, 2 < PIF < 5 = possible phototoxicity, PIF > 5 = phototoxic.; *** Data are from Series 1 for DTA;

In vitro models of basal B-type (MDA-MB-231 cells) and luminal type A (MCF-7 cells) breast cancer were used to investigate the antiproliferative activity of the analogues in series 2 (Figure 29 and Table 20). The non-tumorogenic cell line MCF-12F was chosen as the healthy tissue model. For each peptide, the mean IC_{50} values were comparable in the basal type of breast cancer and the non-tumorogenic cell line. This indicates that in terms of the basal type of breast

cancer, the peptides tested did not have selectivity. On the other hand, much lower IC_{50} values were found in luminal type cancer. The peptide DTTyr10 showed the highest selectivity against MCF-7 cells with a selective index of 3.9. Furthermore, the proliferation of MCF-7 cells was 50% inhibited by the compound DTTyr10 at a concentration of 64.51 μM, while non-tumorogenic cells (MCF-12F) showed no antiproliferative activity at the same concentration. Therefore, the results suggest that DTTyr10 therapy may be helpful in the treatment of luminal breast cancer.

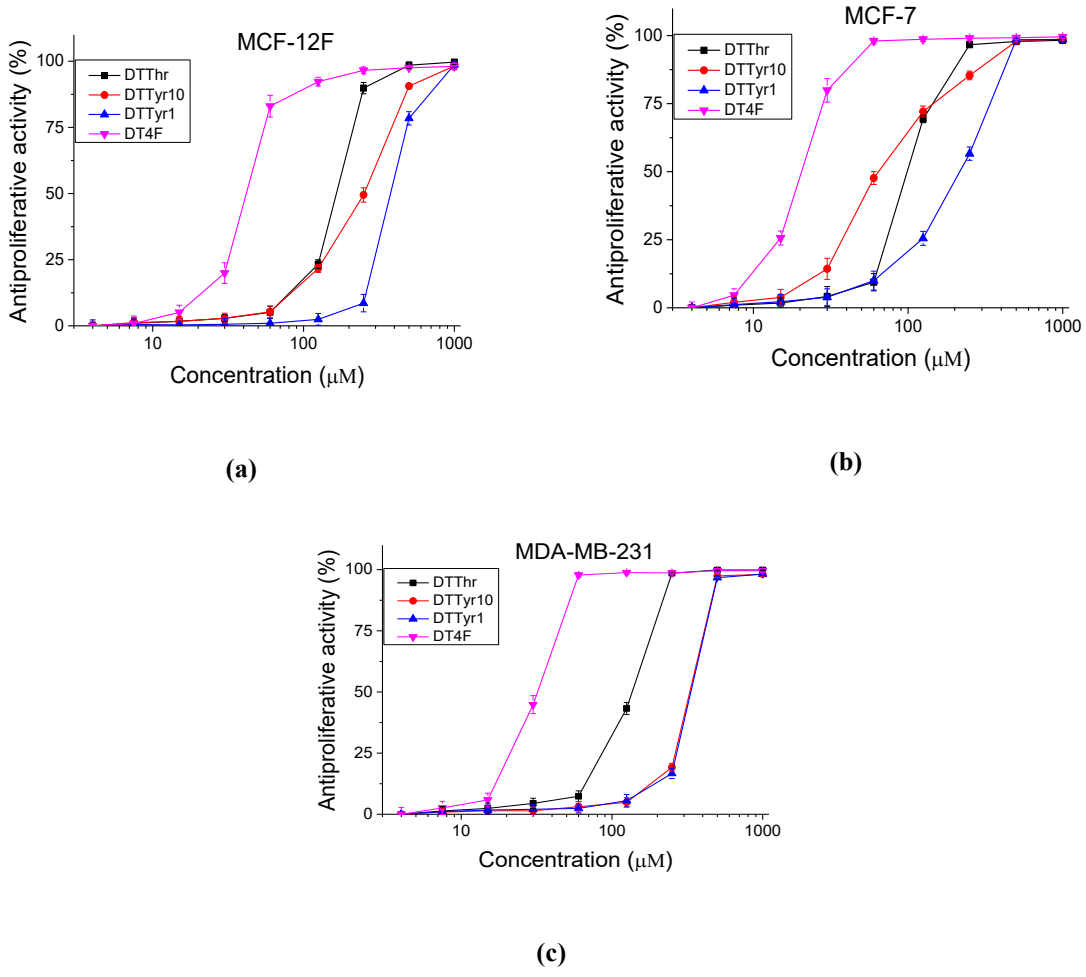


Figure 12 Series 2 - antiproliferative activity of peptide analogues determined in (a) non-tumorogenic MCF-12F cells, (b) MCF-7 tumor cell lines, and (c) MDA-MB-231, $n = 6$.

Table 14 Series 2 - average values of IC_{50} and selectivity index

Peptide	Mean IC_{50} ± SD (μM)			Selectivity index (SI)*	
	MCF-12F	MCF-7	MDA-MB-231	MCF-7	MDA-MB-231
DTA**	138.65 ± 8.36	**73.15 ± 3.36**	115.13 ± 4.04	1.90	1.20
DTThr	165.09 ± 2.21	98.57 ± 1.19	136.12 ± 3.61	1.67	1.21
DTTyr10	251.44 ± 11.81	**64.51 ± 3.93**	328.47 ± 2.62	**3.9**	0.77
DTTyr1	317.74 ± 9.79	216.37 ± 10.3	333.55 ± 3.72	1.75	1.13
DT4F	41.78 ± 1.64	**20.33 ± 0.60**	32.05 ± 1.43	**2.06**	1.3

* selectivity index, SI = IC_{50} (MCF-12F) / IC_{50} (tumor cells);** Data are from Series 1 for DTA

Due to their antitumor activity, halogenated amino acids – especially fluorinated amino acids – have attracted great interest for use in cancer therapy (Giese et al., 2008). The results obtained from the fluorinated peptide DT4F confirm the beneficial effect of the fluorine atom on antiproliferative activity. With a slightly higher selective index than the parent molecule, this analogue demonstrates significant selectivity with respect to luminal type A (MCF-7 cells) breast cancer.

III. Third series of analogues

1. Design

The modifications in the Series 3 were made after the full characterization and study of the previous two series in order to select the most promising modifications and continue work in this direction. For this purpose, analogues from the previous two series were selected, which show good antimicrobial activity, low cytotoxicity to healthy cells and/or significant antiproliferative activity. Modifications from the selected analogues were then combined to produce peptides with two or three modifications in their structure.

As described above, halogenation represents an interesting approach to enrich and/or improve the characteristics of peptides, which is why this line was continued with two new halogenated peptides. Phe(2F) was chosen to investigate the effect of the position of the fluorine atom in the side chain of Phe. Also, chlorinated Phe (Phe(4Cl)) was also used to evaluate the different properties of the halogen used in the vapor position of phenylalanine.

2. Characterization analyses

Each peptide was prepared following the Fmoc/O*t*-Bu strategy of solid-phase peptide synthesis. The physicochemical properties of the newly synthesized compounds are summarized in Table 21. In Appendix 1, HPLC and MS profiles of 3 series compounds are presented in Figures 3.1 – 3.8. All newly synthesized peptides, subjected to biological activity tests, are over 95% chromatographic purity.

Table 15 Series 3 - structure with a one-letter amino acid code, molecular formula and analytical data from HPLC-MS analysis, determination of optical rotation and determination of the melting point of newly synthesized peptides

Peptide	Structure	Molecular formula	MM exact [g/mol]	$[M+H]^+$ observed [g/mol]	$[M+Na]^+$ observed [g/mol]	RT [min]	α_d^{20} [°]***	M.p. [°C]
DTA*	FLPLIGRVL-**S**-GIL-NH_2	$C_{68}H_{117}N_{17}O_{14}$	1395.90	1397.00	1418.95	4.513	−38	158 ± 2
DT4F**	**Phe(4F)**-LPLIGRVLSGIL-NH_2	$C_{68}H_{116}FN_{17}O_{14}$	1413.89	1415.05	1437.10	4.177	-64	141 ± 1
DT2F	**Phe(2F)**-LPLIGRVLSGIL-NH_2	$C_{68}H_{115}FN_{16}O_{15}$	1413.89	1415.10	1437.05	4.487	-96	142 ± 3
DT4Cl	**Phe(4Cl)**-LPLIGRVLSGIL-NH_2	$C_{68}H_{116}CLN_{17}O_{14}$	1429.86	1430.85	1452.85	6.535	-36	144 ± 2
DT4FCi	**Phe(4F)**-LPLIG-**Cit**-VLSGIL-NH_2	$C_{68}H_{115}FN_{16}O_{15}$	1414.87	1416.00	1437.95	7.396	-40	182 ± 2
DT4FCiY	**Phe(4F)**-LPLIG-**Cit**-VL**Y**SGIL-NH_2	$C_{74}H_{119}FN_{16}O_{15}$	1490.90	1492.05	1514.00	7.761	-42	190 ± 2

*DTA is fully characterized in Series 1; **DT4F is fully characterized in Series 2; ***methanol (c=1)

The hydrolytic stability of the resulting peptides has been evaluated in model systems that reproduce the conditions of the stomach (pH 2.0), blood plasma (pH 7.4) and small intestine (pH 9.0) in the human body. The data obtained are summarised in Figure 30. All Series 3 peptides are stable at neutral and alkaline pH. An interesting behavior is exhibited by the chlorinated peptide DT4Cl, which is completely hydrolyzed at acidic pH, and remains stable at alkaline. While the fluorinated analogue DT2F has the exact opposite behavior and remains stable at pH 2.0.

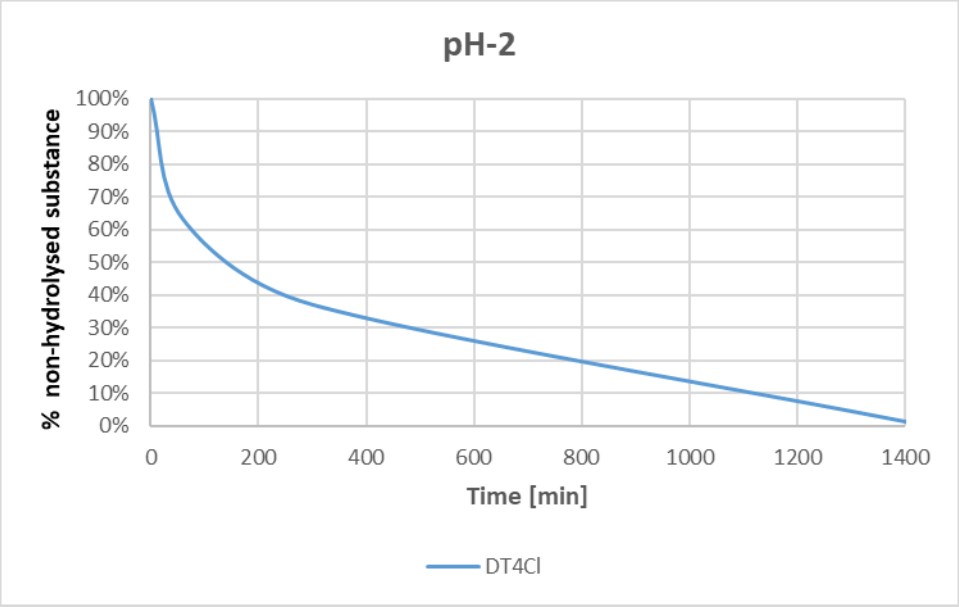


Figure 13 Series 3 - hydrolysis of DT4Cl for a period of 24 hours in acidic pH.

CD spectra in series 3 have improved signal stability above 200 nm, while in the deep far UV region below this wavelength, increased noise is observed (Figure 31). None of the peptides showed the characteristic CD markers of well-defined secondary structures.

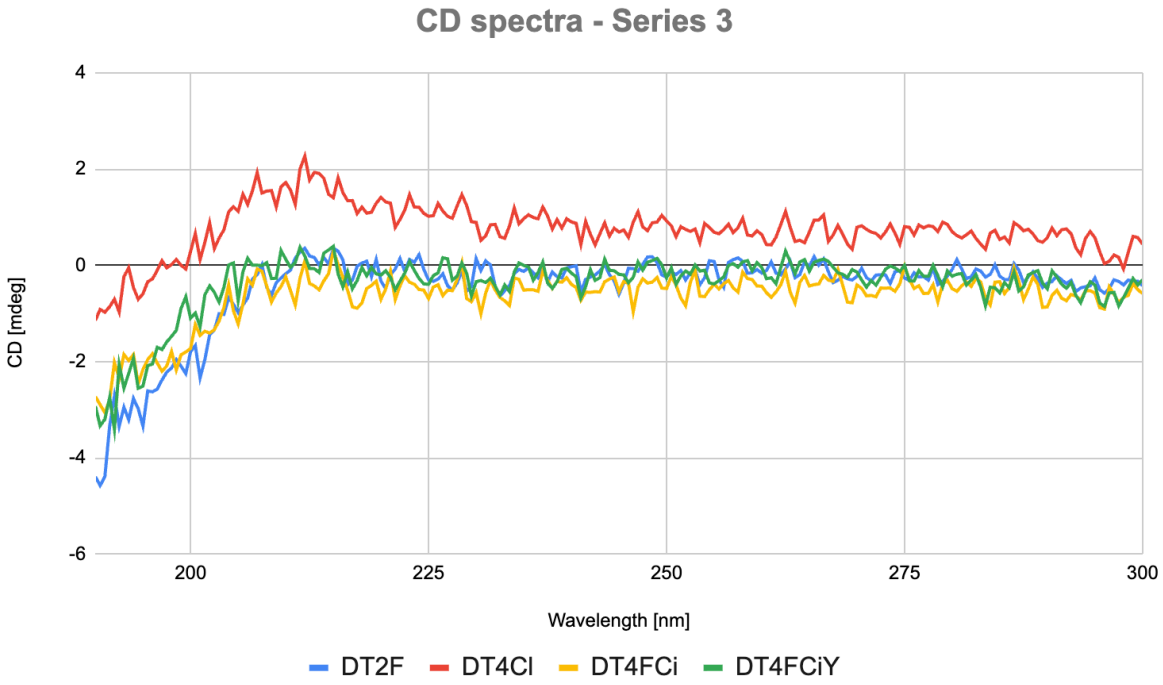


Figure 14 CD spectra - series 3

The DT2F, DT4FCi, and DT4FCiY peptides exhibit similar CD profiles, characterized by moderately negative ellipticity in the 190–200 nm region, followed by near-zero ellipticity in the range of 210 to 300 nm, corresponding to mostly disordered or random conformations. In contrast, the DT4Cl peptide exhibits a distinctly different profile, with positive ellipticity extending over a large part of the 205–300 nm region, and a wide maximum near 210–220 nm. Although this spectrum does not correspond to the markers typical of α-helical or β-sheet structures, the increased positive ellipticity, probably due to increased electrostatic interactions or coupling between the side chain and the backbone. Overall, the CD data indicate that the Series 3 peptides remain largely disordered under the experimental conditions used.

3. Biological studies

3.1. Antibacterial/antifungal studies

In order to establish the sensitivity of the tested strains to the newly synthesized analogues of the 3 series, two methods were used – disc-diffusion method and microdilution in broth. The Series 3 analogues were examined in a similar way to those from Series 1 and 2. It was found that, with the exception of the two halogenated analogues DT2F and DT4Cl, the modifications made to the other two analogues resulted in a complete loss of antimicrobial activity in both methods used.

For the purposes of the disc-diffusion study, the peptides were examined in a similar manner to those in Series 1 and 2. The obtained values are shown in Table 22 for the Gram-positive strains *B. subtilis* 3562 and *A. oxydans* 9333 and Table 23 for the Gram-negative strains *E. coli* 8785 and *P. aeruginosa* 3700. The inhibition rate for *B. subtilis* 3562, *A. oxydans* 9333 and *P. aeruginosa* 3700 at both concentration levels were calculated for each synthesized analogue. The antibiotic inhibition zone was taken as 100% inhibition and the percentage for each peptide analogue was then extrapolated. Table 24 provides a summary of the values. All of the resulting zones are presented in Appendix No. 2, Table 3.1.

Table 16 Series 3 - zones of inhibition (mean in mm with standard deviation) of synthesized peptides and associated antibiotics against Gram (+) strains *B. subtilis* 3562 and *A. oxydans* 9333.

Peptide	Structure	*B. subtilis* 3562			*A. oxydans* 9333		
		1.4 mg/mL	10 mg/mL	Chloramphenicol [30 µg/disc]	1.4 mg/mL	10 mg/mL	Gentamicin [10 µg/disc]
DTA*	FLPLIGRVL-**S**-GIL-NH_2	8.8 ± 0.3	8.8 ± 0.8	26	6.8 ± 0.3	9.7 ± 0.6	18
DT4F**	**Phe(4F)**-LPLIGRVLSGIL-NH_2	8.2 ± 0.3	9.7 ± 0.3	24	10	12.8 ± 0.3	20
DT2F	**Phe(2F)**-LPLIGRVLSGIL-NH_2	10.2 ± 0.2	13.9 ± 0.3	29	10.8 ± 0.3	13.1 ± 0.5	20
DT4Cl	**Phe(4Cl)**-LPLIGRVLSGIL-NH_2	9.1 ± 0.2	9.7 ± 0.2	29	7.2 ± 0.3	8.3 ± 0.5	18

* DTA is fully characterized in Series 1; **DT4F is fully characterized in Series 2.

Table 17 Series 3 - inhibition zones (mean in mm with standard deviation) of synthesized peptides and associated antibiotics against Gram (-) strains *E. coli* 8785 and *P. aeruginosa* 3700.

Peptide	Structure	*E. coli* 8785			*P. aeruginosa* 3700		
		1.4 mg/mL	10 mg/mL	Gentamicin [10 µg/disc]	1.4 mg/mL	10 mg/mL	Gentamicin [10 µg/disc]
DTA*	FLPLIGRVL-**S**-GIL-NH_2	0	0	18	7.8 ± 0.3	9.3 ± 0.6	17
DT4F**	**Phe(4F)**-LPLIGRVLSGIL-NH_2	0	0	17	9.2 ± 0.3	12.3 ± 0.6	18
DT2F	**Phe(2F)**-LPLIGRVLSGIL-NH_2	0	0	22	8.2 ± 0.5	11.3 ± 0.3	17
DT4Cl	**Phe(4Cl)**-LPLIGRVLSGIL-NH_2	0	0	22	8.7 ± 0.2	11.7 ± 0.4	17

* DTA is fully characterized in Series 1; **DT4F is fully characterized in Series 2.

Table 18 Series 3 - percentage inhibition of the tested microorganisms by peptide analogues compared to associated antibiotics. Antibiotics are taken as a 100% inhibition.

Strain	*B. subtilis* 3562		*A. oxydans* 9333		*P. aeruginosa* 3700	
Concentration / Peptide	1.4 mg/mL	10 mg/mL	1.4 mg/mL	10 mg/mL	1.4 mg/mL	10 mg/mL
	Inhibition (%)					
DTA*	33.8	33.8	37.8	53.9	45.9	54.7
DT4F**	32.8	48.5	50.0	64.0	51.1	68.3
DT2F	33.8	33.8	37.8	53.9	48.2	66.5
DT4Cl	34.2	40.4	50.0	64.0	51.2	68.8

*DTA is fully characterized in Series 1; **DT4F is fully characterized in Series 2.

The results of the disc-diffusion method show that the position of the halogen in the side chain of Phe has an effect on the antibacterial activity of the peptide. In the fluorine analogue in the ortho-position of the benzene ring (DT2F) it inhibits to a lesser extent compared to the

analogue with fluorine in the para position (DT4F). Looking at the size of the zones between the chlorine analogue (DT4Cl) and the fluorine analogue (DT4F) in the para position, it becomes clear that fluorine is the more suitable halogen. Fluorine contributes with its smaller size, low mass, as well as higher reactivity to achieve better results (Gillis et al., 2015; Swallow, 2015).

The second method by which analogues were evaluated based on their antimicrobial activity was the determination of MIC by broth microdilution. The study was conducted similarly to the same one from Series 1 and 2. Table 25 shows the summarized MIC values obtained from the experiment.

Table 19 Series 3 - MIC values Temporin A and the new analogues [μg/mL].

Peptide	Structure	*B. subtilis* 3562	*E. coli* 8785	*A. oxydans* 9333	*P. aeruginosa* 3700	*C. albicans* 74
DTA*	FLPLIGRVL-**S**-GIL-NH_2	80 μg/mL	320 μg/mL	80 μg/mL	320 μg/mL	320 μg/mL
DT4F**	**Phe(4F)-**LPLIGRVLSGIL-NH_2	80 μg/mL	NI	160 μg/mL	80 μg/mL	NI
DT2F	**Phe(2F)**-LPLIGRVLSGIL-NH_2	160 μg/mL	NI	160 μg/mL	160 μg/mL	NI
DT4Cl	**Phe(4Cl)**-LPLIGRVLSGIL-NH_2	160 μg/mL	NI	160 μg/mL	160 μg/mL	NI

*DTA is fully characterized in Series 1; **DT4F is fully characterized in Series 2; ***NI – no inhibition.

The MIC values reinforce the conclusions from the determination of the inhibition rate. Fluorine is the more suitable halogen compared to chlorine, and its para position in the side chain of Phe gives the best MIC values.

3.2. Determination of cytotoxicity, phototoxicity and antiproliferative activity

The observed effect (cytotoxicity / phototoxicity) is of a dose-dependent type (Figure 32). Based on the obtained sigmoidal curves, the average CC_{50} values for each studied peptide are calculated (Table 26). The peptide with the lowest cytotoxicity is the peptide DT4FCiY with a value of CC_{50} = 3924.03 ± 147.66 μM. The studied peptides do not exhibit phototoxicity – there is no statistically significant difference in the CC_{50} values in the irradiated peptides versus the non-irradiated peptides.

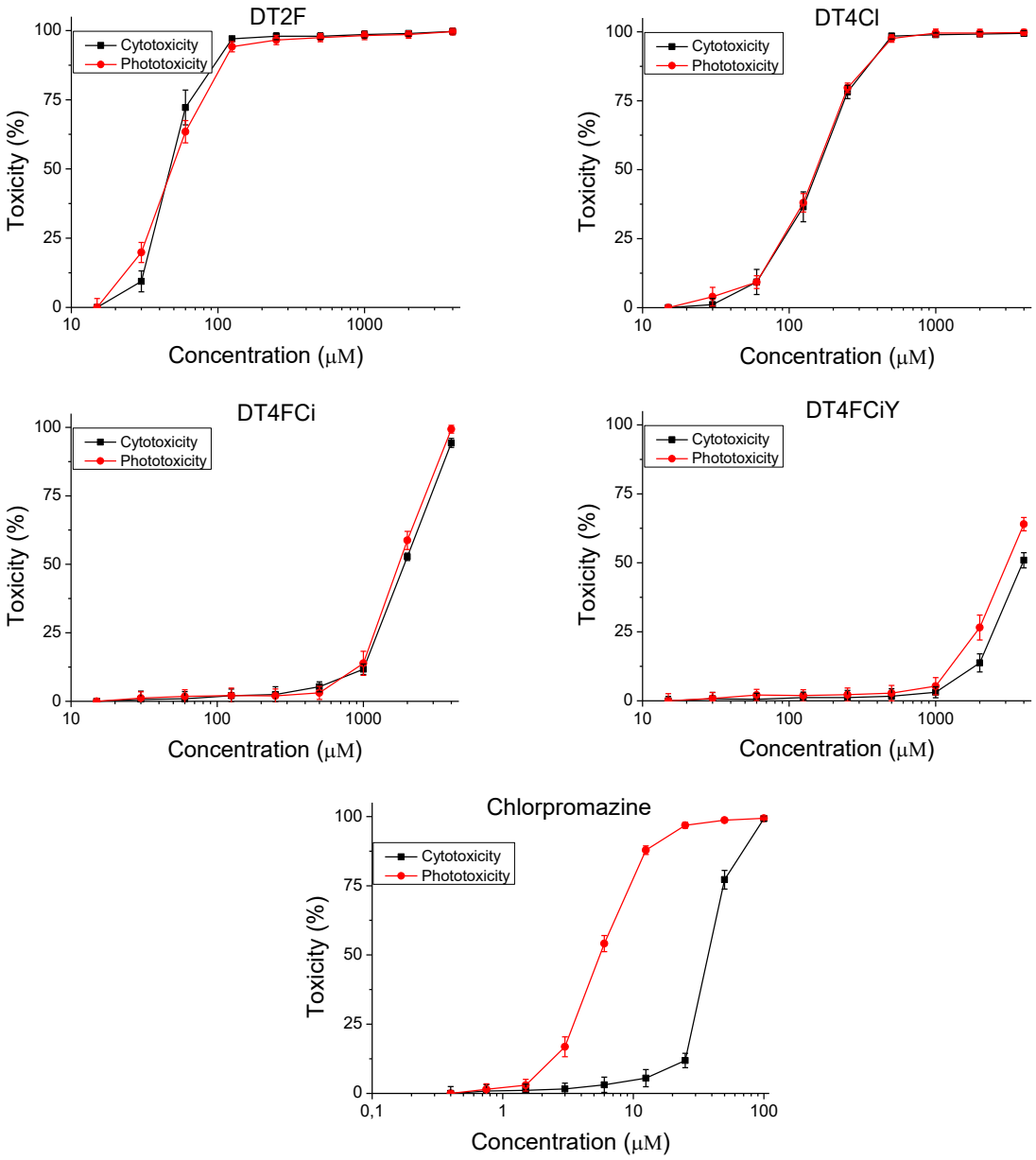


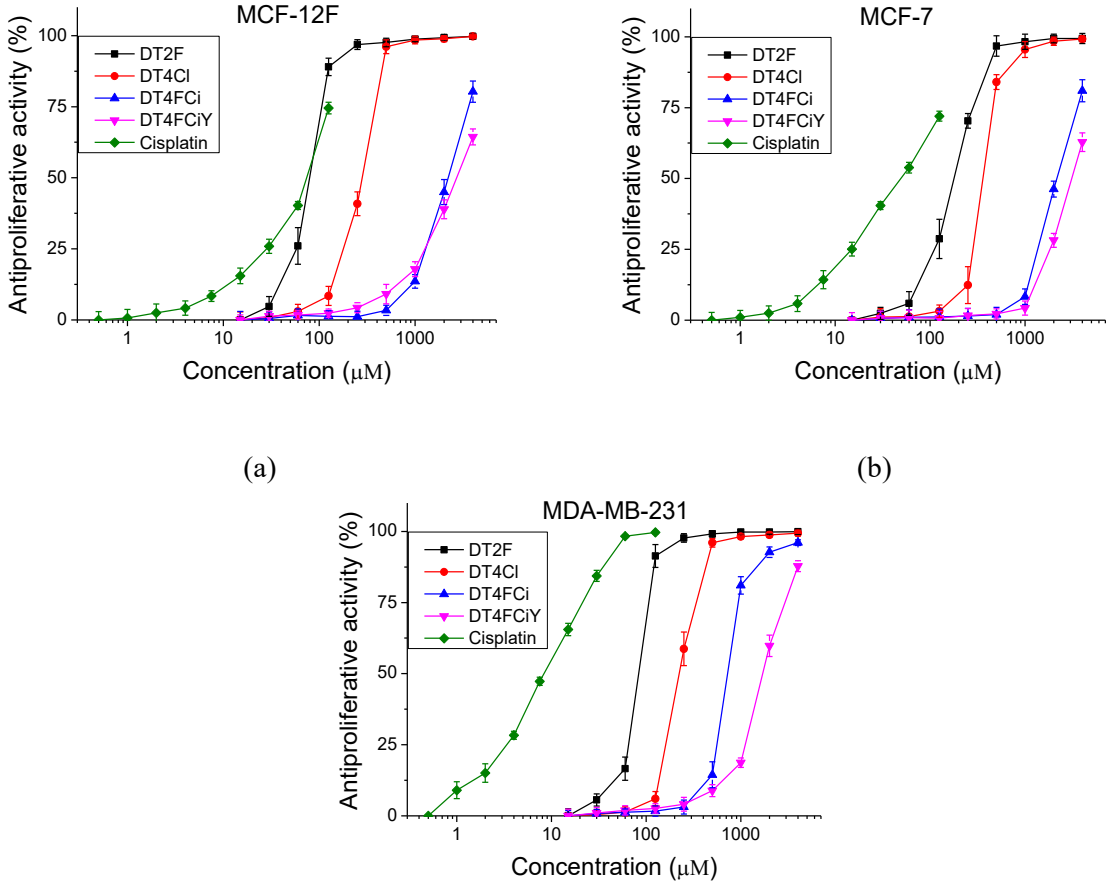
Figure 15 Series 3 - dose-response curves for cyto- and phototoxicity of peptide analogues determined in cell line BALB 3T3 clone A31. Values are averages ± SD, $n = 6$.

Table 20 Series 3 - cytotoxicity/phototoxicity in a cell line BALB 3T3 clone A31, mean values of CC_{50} and PIF factor

Peptide	Mean CC_{50} ± SD (μM)		PIF**
	- Irr	+ Irr*	
DTA* **	106.32 ± 4.18	105.40 ± 4.88	1.01
DT4F* **	55.41 ± 4.64	39.99 ± 0.78	1.4
DT2F	47.16 ± 2.49	48.66 ± 2.58	0.97
DT4Cl	155.87 ± 10.33	152.43 ± 5.65	1.02
DT4FCi	1909.78 ± 37.90	1747.76 ± 96.78	1.09
DT4FCiY	3924.03 ± 147.66	3084.02 ± 101.33	1.27
Chlorpromazine** **	37.47 ± 0.63	5.57 ± 0.27	6.73

* Irr – Irraditation; **Photo irritation factor: PIF < 2 = not phototoxic, 2 < PIF < 5 = possible phototoxicity, PIF > 5 = phototoxic.; *** Data are from Series 1 for DTA and from Series 2 for DT4F;
**** Chlorpromazine – positive control.

In vitro models of basal B-type (MDA-MB-231 cells) and luminal type A (MCF-7 cells) breast cancer were used to investigate the antiproliferative activity of the analogues in Series 3 (Figure 33 and Table 27). The non-tumorogenic cell line MCF-12F was used as a healthy tissue model. The average IC_{50} values for each peptide are comparable in luminal breast cancer and non-tumorogenic cell line. This shows that they do not have selectivity for this type of cancer. The peptides tested showed higher selectivity against the basal type of breast cancer.



(a) (b)

(c)

Figure 16 Series 3 - antiproliferative activity of peptide analogues determined in (a) non-tumorogenic MCF-12F cells, (b) MCF-7 tumor cell lines, and (c) MDA-MB-231, $n = 6$.

The peptide DT4FCi showed the highest selectivity against MDA-MB-231 cells, with a selective index of 3.03. Also, this analogue has a fairly high CC_{50} value and no phototoxicity, which reveals its potential for safe local and systemic application. Modification at position 10 of Ser with Tyr results in reduced selectivity of the peptide DT4FCiY to both tumor lines. This indicates that the introduction of Tyr with its bulky, non-polar aromatic benzene ring, being

less polar and more hydrophobic overall than the hydroxymethyl side chain of serine, results in a decrease in antiproliferant activity.

Table 21 Series 3 - average values of IC_{50} and selectivity index

Peptide	Mean IC_{50} ± SD (μM)			Selectivity index (SI)**	
	MCF-12F	MCF-7	MDA-MB-231	MCF-7	MDA-MB-231
DTA**	138.65 ± 8.36	73.15 ± 3.36	115.13 ± 4.04	1.90	1.20
DT4F**	41.78 ± 1.64	**20.33 ± 0.60**	32.05 ± 1.43	**2.06**	1.3
DT2F	79.19 ± 4.22	177.43 ± 13.95	83.25 ± 3.09	0.44	0.95
DT4Cl	279.24 ± 11.63	359.74 ± 11.95	224.38 ± 16.33	0.77	1.24
DT4FCi	2191.94 ± 164.95	2151.25 ± 105.47	723.73 ± 18.02	1.02	**3.03**
DT4FCiY	2703.96 ± 109.19	3102.74 ± 168.33	1707.14 ± 77.48	0.87	1.58
Cisplatin***	72.97 ± 2.11	49.28 ± 3.16	8.34 ± 0.47	1.48	8.75

*selectivity index, SI = IC_{50} (MCF-12F) / IC_{50} (tumor cells); ** Data are from Series 1 for DTA and from Series 2 for DT4F; *** cisplatin –positive control;

The halogenated analogues DT2F and DT4Cl have relatively low IC_{50} values, but do not exhibit sufficiently significant selectivity for both types of cancer. Comparing them with the analogue of the second series DT4F, it can be seen that it has almost 5 times higher selectivity for the luminal type of cancer compared to DT2F, showing that the position of fluorine in the phenylalanine molecule is essential for antiproliferative activity. Also, the type of halogen has an impact on selectivity. In conclusion, it can be said that the DT4F analogue replaced in the para-position by fluorinated phenylalanine shows the greatest potential of the halogenated analogues with a single substitution in the temporin A molecule.

SUMMARY

In this dissertation, three series of analogues of temporin A with the various modifications in positions 1, 7 and 10 with non-proteinogenic and other natural amino acids have been successfully obtained. Temporin A and its new analogues were synthesized by solid-phase peptide synthesis using the Fmoc/O*t*-Bu strategy. All created analogues, as well as temporin A, were characterized and subjected to biological studies to determine their antimicrobial activity, safety and antiproliferative activity. The characterization of the peptides was carried out on the basis of HPLC/MS analyses to prove the structure and purity, study of their hydrolytic stability, melting point and study of their optical activity by circular dichroism and determination of the angle of rotation. Biological studies include determining antimicrobial activity by two methods – disc diffusion and broth microdilution, as well as tests for cytotoxicity, phototoxicity and antitumor activity.

In Series 1, the basicity and volume of the side chain in position 7 are examined. The resulting compounds are of high purity and do not form specific secondary structures in an aqueous solution. The best antibacterial activity occurs with two methylene groups in the side chain (DTDab). This peptide also showed complete stability in the three pH values. DTF with Lys is the best analogue for combining antibacterial and antiproliferative activity, but with reduced stability at alkaline pH. Elimination of positive charge using Cit (DTCit) results in a lack of antibacterial properties and loss of stability at pH=9. To the MCF-7 cell line, this analogue has the highest selectivity, relatively low cytotoxicity, no phototoxicity, and adequate antiproliferative activity.

All changes to Series 2 are intended to mimic the naturally present amino acids in the primary structure of Temporin A with similar proteinogenic or non-proteinogenic amino acids in order to investigate their importance for biological activity and stability. The resulting compounds are of high purity and do not form specific secondary structures in an aqueous solution. According to the results of structure-activity studies, the fluorine atom-containing analogue DT4F shows high antiproliferative activity and selectivity against luminal type A (MCF-7 cells) breast cancer, as well as promising antibacterial activity when the results of both testing methods are taken into account. The DT4F analogue is a promising candidate for a medical drug that combines antimicrobial and antiproliferative activity, although it has higher cytotoxicity compared to the parent peptide DTA and lower hydrolytic stability at a basic pH of 9. The aromatic side chain at position 10 enhances antiproliferative activity as well as lowers

cytotoxicity to healthy cells. For antibacterial activity, Thr is a better option at position 10 than Ser.

For Series 3, the most promising peptide analogues of the previous series were selected and combined so as to obtain analogues with more than one substitution. Additional halogenated analogues were also made in order to study the influence from the site, as well as the type of halogen used. The results of different halogen substitutions and the position of fluorine in the benzene ring of Phe showed that fluorine in the para position in the side chain of Phe (DT4F) gave the strongest antibacterial activity. The combination of more than one replacement in the Series 3 resulted in a complete loss of antibacterial activity in the DT4FCi and DT4FCiY analogues. On the other hand, the obtained double and triple substituted analogues (DT4FCi and DT4FCiY) are completely safe for use, as well as with quite significant selectivity for the basal type of breast cancer MDA-MB-231. They are also stable in all three pH systems.

In all newly synthesized peptides of the three series, as well as the mother peptide DTA, the spectra from circular dichroism showed a lack of the characteristic elements of well-defined secondary structures.

CONCLUSIONS

Based on the experiments carried out in this dissertation, the following conclusions can be drawn:

1. Temporins A and F have been synthesized and characterized, as well as 4 new analogues with a common structure FLPLIG-**X^7**-VLSGIL-NH_2, where X^7 stands for Arg, Lys, Cit, Orn, Dab and Dap.
2. Two new analogues of temporin A with a common structure FLPLIGRVL-**X^{10}**-GIL-NH_2 have been synthesized and characterized, where X^{10} = Tyr or Thr.
3. Four new analogues of temporin A with a common **X^1**-LPLIGRVLSGIL-NH_2 have been synthesized and characterized, where X^1 is Tyr, Phe(4-F), Phe(2-F) or Phe(4-Cl).
4. Two new analogues of temporin A with two and three substitutions with the following structures have been synthesized and characterized**: Phe(4F)**-LPLIG-**Cit**-VLSGIL-NH_2 and **Phe(4F)**-LPLIG-**Cit**-VL-**Y**-SGIL-NH_2.
5. In analogues with a common structure FLPLIG-**X^7**-VLSGIL-NH_2, antimicrobial studies have shown that two methylene groups in the side chain are optimal for antibacterial activity (DTDab). When the positive charge is removed, antibacterial activity and stability at alkaline pH decreases over 24 hours, but selectivity is significantly improved with significant antiproliferative activity and low cytotoxicity (DTCit). The best combined antimicrobial and antiproliferative activity is obtained with a longer and amino-group side chain (DTF), but with reduced stability at alkaline pH.
6. In analogues with a common structure FLPLIGRVL-**X^{10}**-GIL-NH_2, antimicrobial studies showed that the presence of a secondary OH group in the amino acid side chain leads to improved antibacterial activity but weakened stability at basal pH (DTThr), while the aromatic side chain combined with an aromatic OH group enhances antiproliferative activity with preserved antibacterial activity against Gram-positive bacteria and Gram-negative *P. aeruginosa* 3700 (DTTyr10).
7. SAR studies of analogues with a common structure of **X^1**-LPLIGRVLSGIL-NH_2 showed that the introduction of fluorinated amino acids into the primary structure of the peptide enhances the antibacterial effect, but the presence of fluoride leads to reduced stability at alkaline pH and increased cytotoxicity compared to normal cell

line BALB 3T3 clone A31, combined with a high selective index to the luminal type of breast cancer MCF-7.

8. From the biological studies performed on the newly synthesized 2 new analogues with two and three substitutions with the following structures **Phe(4F)**-LPLIG-**Cit**-VLSGIL-NH_2 (DT4FCi) and **Phe(4F)**-LPLIG-**Cit**-VL-**Y**-SGIL-NH_2 (DT4FCiY), a loss of antibacterial activity was observed, with the DT4FCi analogue having a high selective index to the basal type of breast cancer MDA-MB-231 with preserved stability in the three model pH system.
9. The resulting spectra from the circular dichroism of temporin A and all new analogues do not show typical characteristics of secondary structures.

CONTRIBUTIOINS

1. For the first time, 9 analogues of the antimicrobial peptide temporin A with non-proteinogenic amino acids Dab, Dap, Cit, Orn, Phe(4-F), Phe(2-F) and Phe(4-Cl) have been synthesized and characterized.
2. Design, synthesis and characterization of 3 analogues of the antimicrobial peptide temporin A were made for the first time with the introduction of the natural amino acids Thr and Tyr in selected positions 1 and 10.
3. Important correlations have been revealed regarding the structure and biological activity of all newly synthesized analogues of temporin A regarding antimicrobial properties, antiproliferative effect, cytotoxicity and hydrolytic stability.
4. Studies have shown the following more significant SAR dependencies:
    - the introduction of Dab at position 7 results in an increase in antibacterial activity, while the introduction of Cit at the same position results in a loss of antibacterial activity, decreased stability in alkaline pH, but increased selectivity towards the luminal type of breast cancer;
    - the introduction of Tyr at position 10 results in low cytotoxicity against healthy tissue models, high antiproliferative activity and selectivity against the luminal type of breast cancer, which presents it as a potential therapeutic agent;
    - the introduction of Phe(4-F) at position 1 results in significantly higher antibacterial activity, as well as high antiproliferative activity and selectivity towards the luminal type of breast cancer, but with reduced stability at alkaline pH.

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LIST OF PUBLICATIONS FOR THE DISSERTATION

1. **Dimitrova, D**.; Nemska, V.; Foteva, T.; Iliev, I.; Georgieva, N.; Danalev, D. Synthesis and Biological Studies of New Temporin A Analogs Containing Unnatural Amino Acids in Position 7. *Pharmaceutics*, 2024, 16, 716, DOI: 10.3390/pharmaceutics16060716. (Q1, IF 5.8; 6.67)
2. **Dimitrova, D.;** Nemska, V.; Iliev, I.; Petrin, S.; Georgieva, N.; Danalev, D. New Temporin A Analogues Modified in Positions 1 and 10—Synthesis and Biological Studies. *Pharmaceutics,* 2025, 17, 396, DOI: 10.3390/pharmaceutics17040396. (Q1, IF 5.8; 6.67)
3. **Dimitrova, D.;** Georgieva, N. Antimicrobial Peptides And Temporin Family In The Context Of Rising Resistance - View On Current Development. *Journal of Chemical technology and metallurgy,* 2026, 61, 391-401. DOI**:** 10.59957/jctm.v61.i3.2026.1. (Q3, IF 1.4; 20)

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1. Ke Wan and Yucheng Su, Progress and biomedical applications of antimicrobial peptide-functionalized titanium surfaces, Front. Mater., 2026, 13:1827598. doi: 10.3389/fmats.2026.1827598

PARTICIPATION IN SCIENTIFIC CONFERENCES ON THE TOPIC OF THE DISSERTATION

Participation in scientific poster sessions and conferences in the country:

1. **D. Dimitrova**, Tsv. Foteva, N. Georgieva, D. Danalev, Synthesis and characterization of new antibacterial peptides, XX Scientific Poster Session for Young Scientists, PhD Students and Students, 23.06.2023, UCTM-Sofia
2. **D. Dimitrova**, Tsv. Foteva, N. Georgieva, D. Danalev, Synthesis and characterisation of new antimicrobial peptides, Workshop with international participation "Drug-molecule: stages in the discovery and development", 17-21.07.2023, Hisarya, Bulgaria
3. **D. Dimitrova**, Tsv. Foteva, N. Georgieva, D. Danalev, Synthesis and characterization of new antibacterial peptides, 10th International Peptide Conference - Bulgarian Peptide Society, 1-4.09.2023, Varna, Bulgaria
4. **D. Dimitrova**, Ts. Foteva, N. Georgieva, D. Danalev. synthesis and characterization of analogues of temporin A with substitution in the seventh position. X ePoster Scientific Session for Students, PhD Students and Young Scientists, 2.11.2023, UCTM-Sofia.
5. **D. Dimitrova**, V. Nemska, Tsv. Foteva, N. Georgieva, D. Danalev, Analogues of Temporin A with Potential Antimicrobial Activity, Seventeenth Spring Seminar "Interdisciplinary Chemistry", 23-25.04.2024, Sofia.

D. Dimitrova, Tsv. Foteva, V. Nemska, N. Georgieva, D. Danalev. Synthesis and study of new temporin analogs containing two unnatural amino acids with potential antibacterial properties. XXI Scientific Poster Session for Young Scientists, PhD Students and Students, 21.06.2024, UCTM-Sofia.

6. **D. Dimitrova**, Tsv. Foteva, V. Nemska, N. Georgieva, D. Danalev. Synthesis and study of new temporin analogs containing two unnatural amino acids with potential antibacterial properties. 70 years of the Department of Organic Synthesis, 28.06.2024, UCTM-Sofia.
7. **D. Dimitrova**, Tsv. Foteva, V. Nemska, N. Georgieva, D. Danalev, Effect of the unnatural amino acids Dab and Dap in temporin a molecule on antibacterial activity, XI ePoster scientific session for students, PhD students and young scientists, 8.11.2024, UCTM-Sofia
8. **D. Dimitrova**, Tsv. Foteva, V. Nemska, N. Georgieva, D. Danalev, Effect Of Modification of Temporin A With Fluorinated Phenylalanine on Antibacterial Activity,

International Conference on Bioactive, Organic and Inorganic Advanced Materials and Clean Technologies, 24-28.03.2025, Sofia, Bulgaria

9. **D. Dimitrova**, V. Nemska, Tsv. Foteva, N. Georgieva, D. Danalev, Synthesis and antibacterial study on modified Temporin A analogues with tyrosine, XXII Scientific poster session for young scientists, PhD students and students, 20.06.2025, UCTM-Sofia
10. G. Vasilev, B. Raychev, H. Mzik, **D. Dimitrova**, N. Georgieva, D. Danalev, Applications of antimicrobial peptides, XXII Scientific poster session for young scientists, PhD students and students, 20.06.2025, UCTM-Sofia

Participation in scientific poster sessions and conferences outside the country:

1. N. Georgieva, **D. Dimitrova**, D. Dimov, Tsv. Foteva, D. Danalev. New analogs of temporin modified with unnatural aminoacids. European biotechnology congress, 3-6.10.2023, Ljubljana, Slovenia.
2. V. Nemska,, N. Georgieva, **D. Dimitrova**, D. Danalev, Temporin A And F New Analogues Modified With Unnatural Amino Acids Dap And Dab, European biotechnology congress, 3-6.10.2023, Ljubljana, Slovenia.
3. **D. Dimitrova**, Tsv. Foteva, V. Nemska, N. Georgieva, D. Danalev, Newly synthesized Temporin A analogs with potential antimicrobial activity, Biologics 2024, 13-16.03.2024, London, UK.
4. N. Georgieva, **D. Dimitrova**, I. Iliev, R. Hristova, D. Danalev, Modification of temporin a with unnatural amino acids and study the effects on photo- and cytotoxicity, Mediterranean Congress on Mass Spectrometry and Its Applications – SPECTROMED,21-25.04.2024, Tunisia
5. Tsv. Foteva, I. Iliev, R. Hristova, **D. Dimitrova**, V. Nemska, N. Georgieva, D. Danalev. Cytotoxicity and phototoxicity effects of modified temporin analogs with unnatural aminoacids. 37 th EPS, European Peptide Symposium, 25-29.08.2024 Florence, Italy.
6. N. Georgieva, **D. Dimitrova**, Tsv. Foteva, V. Nemska, D. Danalev. Synthesis and study of modified temporin analogs with unnatural amino acids citrulline and ornithine as potential antibacterial agents. 37 th EPS, European Peptide Symposium, 25-29.08.2024 Florence, Italy.
7. V. Nemska, **D. Dimitrova**, N. Georgieva, D. Danalev, Synthesis and antibacterial activity of new Temporin A and F analogues modified with unnatural amino acids Dap and Dab, 20th International Symposium on Novel Aromatic Compounds, 11-16.08.2024, Toronto, Canada.

8. N. Georgieva, **D. Dimitrova**, I. Iliev, D. Danalev, Effect Of Modification Of Temporin A With Dab And Dap On Photo- And Cytotoxicity And Antiproliferative Activity, European Biotechnology Congress 2024, 3-5.10.2024, Istanbul, Turkey.
9. Ts. Foteva, **D. Dimitrova**, I. Iliev, N. Georgieva, D. Danalev. Study of antitumor activity of temporine derivatives, containing citrulline and ornithine. European Biotechnology Congress 2024, 3-5.10.2024, Istanbul, Turkey.
10. V. Nemska, **D. Dimitrova**, Tsv. Foteva, N. Georgieva, D. Danalev, New Temporin A and F analogues modified with unnatural amino acids Dap and Dab and antibacterial activity against Bacillus subtilis. European Biotechnology Congress 2024, 3-5.10.2024, Istanbul, Turkey.
11. Ts. Foteva, **D. Dimitrova**, N. Georgieva, D. Danalev. Investigation of antibacterial activity of temporin derivatives, containing 2,4-diaminobutyric acid and 2,3-diaminopropionic acid, International Conference on Environment, Biotechnology and Bioengineering Applications (ICEBBA-25), 17-18.07.2025, Zurich, Switzerland.
12. **D. Dimitrova**, N. Georgieva, D. Danalev. Synthesis and antibacterial investigation of tyrosine-modified Temporin A analogues. The European Peptide Synthesis Conference. 25-27.08.2025, Porto, Portugal.

PARTICIPATION IN SCIENTIFIC PROJECTS

1. BG-RRP-2.004-0002-C01, *"BiOrgaMCT" (Bioactive Organic and Inorganic Advanced Materials and Clean Technologies)* under the procedure: BG-RRP-2.004 – Establishment of a network of research universities in Bulgaria under the National Recovery and Sustainability Plan, 2023-2026
2. НИС No 239-01 headed by Prof. Dr. Nelly Georgieva on the topic "Biologically active products from waste plant mass of lavender", 2024
3. НИС No 403-29 headed by Prof. Dr. Nelly Georgieva on the topic "Synthesis and antibacterial activity of analogues of anoplin", 2025