Appendix 12a

REVIEW

of dissertation for the acquisition of:

educational and scientific degree " **doctor** "	X
scientific degree **"Doctor of Science"**	
	the true is indicated by the sign "X"

Author of the dissertation:

Assist.		Dilyana	Vasileva	Dimitrova	University of Chemical Technology and Metallurgy
academic position	scientific degree	name	middle name	last name	workplace

Topic of the dissertation:

SYNTHESIS AND BIOLOGICAL ACTIVITY OF TEMPORIN ANALOGUES

Scientific area:

5.	Technical Sciences
code	name

Professional area:

5.11.	Biotechnology
code	name

Scientific specialty:

Technology of biologically active substances

The review was written by:

Assoc. prof.	PhD	Zlatka	Miltcheva	Alexieva	Institute of microbiology, Bulg. Acad. Sciences, retiree
academic position	scientific degree	name	middle name	last name	workplace

1. Completion of the provided documents:

A) The dissertation and the competition documents are in full compliance with the Regulations.	4 points	**X**
B) The documents are complete but do not fully comply with the requirements of the Regulations.	2 points	

C) The documents are not completed in accordance with the requirements of the Regulations.	0 points	
		one of the answers given is marked with the sign "X"

Missing documents and violated standards must be described if response C is marked.

2. Meeting the minimum requirements under the Regulations:

A) The candidate meets the minimum requirements	20 points	**X**
B) The candidate doesn't meet the minimum requirements	0 points	
		one of the answers given is marked with the sign "X"

It must be filled in if answer B is marked. The publication activity of the candidate is analyzed. The response of the results achieved (quoted) is analyzed.
The author has presented 3 scientific publications directly related to the topic of the dissertation. Two of the publications are in the journal Pharmaceutics, which has an IF of 5.5 and is Q1 in the field of pharmaceutical science, and the third publication is a review and published in the Journal of Chemical technology and metallurgy, with Q3 in the field of chemical engineering and industrial production, focused on chemical technologies, metallurgy, biotechnology and environmental protection and SJR – 0.205. In all three publications, Assoc. Prof. Dimitrova is the first author. With the required number of 30 points to satisfy the minimum requirements of the Regulations, the doctoral student has accumulated 33 points with the presented articles.

3. The relevance of the topic of the dissertation:

A) The topic is relevant and new (there are no known results on the topic by other authors)	8 points	X
B) The topic is relevant and results from other authors are known	6 points	
C) The topic is not relevant, but results from other authors are known	2 points	
D) The topic is not relevant and no results from other authors are known	1 point	
E) The topic does not correspond to the level of dissertation	0 points	
		one of the answers given is marked with the sign "X"

The evaluation of the relevance of the dissertation must be substantiated
Solving the problem of antibiotic resistance of microorganisms is a global contemporary problem that concerns scientists from various professional fields. An important aspect of the developments is the

search for alternatives to antibiotic drugs that can solve the problems of treating infections caused by antibiotic-resistant microorganisms. The creation of effective antimicrobial peptides is one of the important directions in this field. In this context, temporins are one of the widely studied and modified short-chain peptides that have shown significant activity against gram-positive bacteria, even multidrug-resistant strains and fungi. The synthesis of new structural analogues of antimicrobial peptides, such as temporins, is one of the latest trends in attempts to create therapeutic drugs with increased antibacterial activity. Due to its high potential, one of the most studied and promising temporins is Temporin A, which is also the main subject of the dissertation. The data obtained are original and make a significant contribution to the topic under development. An important and topical aspect of the dissertation work is the study of antitumor activity of newly synthesized antimicrobial peptides.
In recent years, synthesis of target peptides on a solid polymer support has been preferred and applied - an approach that is used in the present work.
The topicality of the presented work is also emphasized by the percentage of cited literary sources from the last 5 years, which is almost 18% of the entire list.

4. Knowledge of the problems, subject of research in the dissertation:

A) The doctoral student knows in detail the achievements of other authors on the topic of the dissertation	8 points	X
B) The doctoral student is partially familiar with the achieved results on the topic of the dissertation	4 points	
C) The doctoral student has no prior knowledge of the status of the problems in the dissertation	0 points	
		one of the answers given is marked with the sign "X"

The evaluation must be substantiated if answer C is marked.

Assoc. Prof. Dimitrova has presented a literature review that contains detailed information on all aspects of the developed topic. Significant attention is paid to the causes, mechanisms and consequences of the emergence of microorganisms resistant to antimicrobial substances, especially sulfonamides and antibiotics. The history of the discovery and use of different groups of antibiotics is described in detail. As an alternative, detailed information is given about another group of compounds, such as natural antimicrobial peptides, with special attention being paid to temporins and especially Temporin A.
These sections are supported by a large number of scientific works by authoritative authors and scientific publications. They convincingly prove the need for new developments in the field of synthesis and modification of antimicrobial peptides such as Temporin A, which is the main object of interest in the dissertation. The presented literature review covers 110 literary sources, a significant part of which have been published in the last 5 years. 3 of them cite publications with the participation of the author of the dissertation, which is not necessary, since the results are part of the presented material.
In the following parts of the literature review, the author has shown in-depth knowledge of peptide synthesis approaches and existing strategies for performing solid-phase peptide synthesis, as well as for modifying natural peptides.
Both the information presented in the literature review and the description of the experimental methods and approaches to achieve the goals, as well as the discussion of the obtained results, convincingly show that the doctoral student knows in detail what has been achieved by other authors on the topic of the dissertation.

5. Type of research:

A) Theoretical	4 points	X
B) Applied	4 points	

C) Theoretical with application elements	4 points	
D) It does not correspond to the level of dissertation	0 points	
		one of the answers given is marked with the sign "X"

The level of research must be substantiated if answer D is marked.
All research conducted is fundamental in nature, but it also creates a solid foundation for future application in medicine and pharmacy

6. Objectives of the research:

A) Realistic and of scientific and / or applied interest	8 points	X
B) Realistic, but not of scientific and / or applied interest	3 points	
C) Unattainable (unrealistic)	0 points	
		one of the answers given is marked with the sign "X"

Objectives must be specified. The type of the set objectives must be justified.
The aim of this dissertation is to synthesize new structural analogues of the antimicrobial peptide temporin A and to investigate their basic biological properties related to antibacterial and antiproliferative activity, cyto- and phototoxicity. The aim is realistic and defines research of a scientific nature, which also has applied significance. To achieve the aim, 6 main tasks are clearly formulated, which determine the logic and interrelationship of the planned research.

7.Methods of research:

A) Adequate to research and set objectives	8 points	X
B) Partially appropriate, enabling part of the scientific objectives and / or applications to be achieved	4 points	
C) Inappropriate methods	0 points	
		one of the answers given is marked with the sign "X"

Methods must be specified. The type of methods used is justified.
In the experimental part of the dissertation, classical and modern approaches were used. For the synthesis of antimicrobial peptides, analogues of temporin, the approach for solid-phase peptide synthesis using the Fmoc(9-fluorenylmethoxycarbonyl)/Ot-Bu strategy on a polymeric support Fmoc-Rink-Amide-MBHA resin was used. To prove the structures and purity of the newly synthesized target peptides, HPLC/MS analyses were applied. Important characteristics of the obtained products were determined, such as boiling point, optical activity, circular dichroism and hydrolytic activity. The second part of the experiments is devoted to the study of the biological activity of the newly synthesized compounds: antibacterial and antifungal activity, cytotoxicity, phototoxicity and

antiproliferative activity. The work carried out in the process includes cultivation of model strains Escherichia coli NBIMCC 8785, Bacillus subtilis NBIMCC 3562, Arthrobacter oxydans NBIMCC 9333, Pseudomonas aeruginosa NBIMCC 3700 and Candida albicans NBIMCC 74 on suitable nutrient media. To determine the inhibitory effect of the synthesized substances, compared to that of specific antibiotics used to suppress the growth and development of microorganisms, classical methods were used - disk diffusion and microdilution in broth. The concentrations of peptides used are in a wide range from 0 to 320 μg/mL. Their minimum inhibitory bactericidal and/or fungicidal concentrations were determined.
To determine the cytotoxicity of the obtained peptides, in vitro tests were conducted with mouse embryonic fibroblast (BALB 3T3) cell lines, human breast epithelial cells (MCF-12F), as well as in vitro models for luminal A (MCF-7) and basal B (MDA-MB-231) type of breast cancer. Phototoxicity tests were also conducted using an artificial sunlight simulator Helios-iO with a dose of 2.4 J/cm2. Absorption was measured at a wavelength of 540 nm. The CC50 concentrations for cyto- and phototoxicity of the tested peptides were determined.
To determine the antiproliferative activity, a colorimetric assay was applied, which includes the reduction of a yellow, water-soluble tetrazolium salt (MTT) by mitochondrial succinate dehydrogenase in living cells to an insoluble, purple formazan product, the concentration of which was determined spectrophotometrically. Cell lines (MCF-12F, MCF-7 and MDA-MB-231) were used in the experiments. The formazan absorption was determined at λ=540 nm. IC50 values for the antiproliferative activity of the studied peptides were determined. The results were subjected to statistical analysis by one-way ANOVA, followed by a post hoc Bonferroni test.
The applied methods are very well selected and fully correspond to the set scientific goals.

8. Contributions of the dissertation:

A) With lasting scientific and / or applied response, they form the basis for new research and applications	20 points	X
B) They are of significant scientific and / or applied interest, complete and / or summarize previous research	16 points	
C) They are of scientific and / or applied interest	12 points	
D) Lack of significant contributions	8 points	
E) Lack of contributions	0 points	
		one of the answers given is marked with the sign "X"

Contributions must be specified. The type of results achieved must be justified.

The formulated 9 conclusions and 4 contributions of the dissertation work are clearly formulated and actually reflect the results obtained from the experimental work. As a leading achievement, I would highlight the discovery of important correlations regarding the structure and biological activity of all newly synthesized analogues of temporin A regarding antimicrobial properties, antiproliferative effect, cytotoxicity and hydrolytic stability. An important contribution is the results of the studies of the structure-activity relationship, depending on the type and position of the included amino acid.
I would highlight the conclusion that the obtained double and triple substituted analogues (DT4FCi and DT4FCiY) are stable in all three pH systems (the conditions of the stomach (pH 2.0), blood plasma (pH 7.4) and small intestine (pH 9.0) in the human body). Their safety for use and the presence of significant selectivity towards the basal type of breast cancer MDA-MB-231, as well as promising antibacterial activity, have been established.
The peptide DTDab (with 2,4-diaminobutanoic acid) shows the strongest antimicrobial activity of the newly synthesized analogues against the two Gram-positive bacterial strains, B. subtilis 3562 and A. oxydans 9333, as well as against the Gram-negative *P. aeruginosa* 3700, which is due to the shortening of the side chain to two methylene groups. This peptide also showed full stability in the three pH values.

It is interesting to conclude that the aromatic side chain at position 10 enhances the antiproliferative activity, as well as reduces cytotoxicity to healthy cells. The scientific contributions of doctoral student Dilyana Dimitrova are original and of high scientific significance. The research is combined with the skillful application and logical combination of a number of state-of-the-art research methodological approaches.

9. Evaluation of the compliance of the dissertation summary with the dissertation:

A) Full compliance	4 points	X
B) Compliance of the main parts	2 points	
C) Lack of compliance of the main parts	0 points	
		one of the answers given is marked with the sign "X"

The evaluation must be substantiated if answer C is marked.
The Summaty meets the requirements of the law and accurately and completely reflects the content of the dissertation.

10. Participation of the doctoral student in the achievement of the results of the dissertation:

A) The doctoral student has at least an equal participation	8 points	X
B) The doctoral student has secondary participation	5 points	
C) The participation of the doctoral student is unnoticeable	0 points	
		one of the answers given is marked with the sign "X"

Critical notes must be provided if one of the items B or C is marked.
As a result of the results obtained during the development of this dissertation, 3 publications have been published in authoritative indexed scientific journals, in which the doctoral student is the first author. Two of the articles are in a journal with a rank of Q1 in the field of Pharmacology and Pharmacy and with an IF of 5.5. The third article has a rank of Q3 in the field of Chemical Engineering and SJR 2025 – 0.21. It should be emphasized that the development of the doctoral student as a scientist has been contributed by her solid educational background in the field of biotechnology: a bachelor's degree at the University of Natural Resources and Life Sciences in Vienna and a master's degree at the University of Technology and Engineering, Sofia. Her internship in several companies with biotechnological production and as a researcher (project BG-RRP-2.004 "BiOrgaMCT") at the University of Technology and Engineering, Sofia, has undoubtedly helped her methodological preparation. The PhD student has participated in 3 scientific projects, and has presented the results of her research at 12 scientific forums and international conferences with reports and poster presentations. These data are evidence of her development as an established young scientist in the field of biotechnology and give me reason to assess her participation as equal to that of her other colleagues.

11. Critical notes:

A) Lack of critical notes	8 points	X
B) Critical notes of a technical nature	7 points	
C) Critical notes that would partially improve the results achieved	4 points	
D) Significant critical notes	0 points	
		one of the answers given is marked with the sign "X"

Critical notes must be provided if one of the answers C or D is marked.
I have no critical remarks.

12. Conclusion

A) The evaluation of the dissertation is **POSITIVE**	This evaluation is assigned to a total number of at least 65 points	X 100 points
B) The evaluation of the dissertation is **NEGATIVE**	This evaluation is assigned to a total number below 65 points	
		one of the answers given is marked with the sign "X"

To be filled in at the request of the reviewer
The doctoral dissertation proposed for review has a current scientific topic, is filled with the application of modern methodological approaches. The results obtained are of an original nature and have a significant scientific contribution in the field of research on biologically active substances. The implementation of the set goals reveals a complex scheme for revealing key characteristics of the newly synthesized antimicrobial peptides, analogues of the antimicrobial peptide temporin A. The dissertation is richly illustrated with 33 figures, 2 graphs, as well as 2 appendices with graphic panels and rich photographic material. The data are summarized and demonstrated in 22 tables. The experiments combine complex structural and biological analyses, which convincingly show the advantages and disadvantages of each of the different series of analogues and each individual peptide studied. The combination and comparison of the obtained data brings out the most promising products for future development and application. The results obtained reveal great opportunities and prospects for new research on current problems aimed at discovering alternatives to antibiotic treatment, as well as the needs for creating new and selective antitumor agents. The presented scientific publications fully correspond to the scientific field and scientific direction of the dissertation work. Dilyana Dimitrova's scientometric data exceed the minimum national requirements of the Law on the Development of Academic Staff in the Republic of Bulgaria and the additional criteria from the relevant Regulations of the HTMU. The total point asset in the review is 100. Based on everything stated above in the review, I will confidently vote "FOR" awarding the Educational and scientific degree "Doctor" to Assistant professor Dilyana Dimitrova in Professional direction 5.11 Biotechnologies, scientific specialty Technology of Biologically Active Substances.

23.05.2026	The review was written by:	
date		signature