

## REVIEW

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**Regarding:** thesis for awarding the educational and scientific degree Doctor of Philosophy (Ph.D.) in the field of higher education 4. Natural sciences, Mathematics and Informatics, professional field 4.2. Chemical Sciences, Scientific Specialty “Bioorganic chemistry, chemistry of natural and physiologically active compounds”

**Author:** Assist. Prof. Boryana Krasimirova Yakimova

**Title:** “Design and syntheses of biologically active peptides as potential inhibitors of angiotensin converting enzyme (ACE I)”

**Scientific advisor:** Prof. DSc. Ivanka Borisova Stoineva

### 1. General description of the presented materials

According to order № ПД-09- 94/19.04.2021 of the Director of the Institute of Organic Chemistry with Centre of Phytochemistry, BAS (IOCCP, BAS), I have been appointed as a member of the scientific jury in a procedure for the defense of a dissertation entitled "Design and synthesis of biologically active peptides as potential inhibitors of angiotensin-converting enzyme (ACE I)", Ph.D. thesis for awarding the educational and scientific degree Doctor of Philosophy (Ph.D.) in the field of higher education code 4. Natural sciences, Mathematics and Informatics, professional field code 4.2. Chemical Sciences, Scientific Specialty “Bioorganic chemistry, chemistry of natural and physiologically active compounds”. The author of the PhD thesis is Assist. Prof. Boryana Yakimova, a self-study Ph.D. student with scientific advisor Prof. DSc. Ivanka Stoineva.

The set of documents in hard copies deposited by Assist. Prof. Boryana Yakimova complies with the Internal Rules of Procedure of IOCCP, BAS and satisfies the criteria of BAS for awarding a Doctor of Philosophy (Ph.D.) degree. The set of the application documents include:

- application to the Director of IOHCF-BAS for disclosure of a procedure for the defense of a dissertation;
- CV;
- a copy of the diploma for higher education (MSc degree);
- orders for enrollment and disenrollment in doctoral studies;

- protocols of successfully passed exams according to an individual training plan;
- Ph.D. thesis;
- abstract of the doctoral thesis;
- list and copies of the scientific publications on the topic of the dissertation;
- list of noticed citations;
- list of participation in projects.

The documents are well organized and present clearly. The deadlines are kept.

## **2. Brief biographic data of the PhD candidate**

Assist. Prof. Boryana Yakimova graduated from Sofia University "St. Kliment Ohridski, Faculty of Biology, Sofia, wherein 2005 she obtained a bachelor's degree in Biotechnology and in 2007 she graduated with honors with a master's degree in Industrial Biotechnology. During her studies at Sofia University Assistant Professor Boryana Yakimova, impressed with her precision in the experimental work and logical thinking and she had been invited to start working in the laboratory "Chemistry and Biophysics of Proteins and Enzymes" (CBPE), IOCCP-BAS, where later on she worked on her master's thesis under the supervision of Prof. Bozhidar Chorbanov. Boryana Yakimova was appointed as a biologist at IOCCP, BAS from 2005 to 2007. From December 2007 she has been appointed to the Assistant Professor's position. Assist. Prof. Boryana Yakimova has been enrolled as a self-study Ph.D. student at the end of 2015 under the supervision of Prof. DSc Ivanka Stoineva. Assist. Prof. Boryana Yakimova has participated in the implementation of 11 research projects, 5 of which are ongoing. She was a scientific advisor of three master's theses. Her scientific interests are in the field of structure and function of peptides and enzymes. She is a member of the Bulgarian Peptide Society.

## **3. Relevance of the topic and expediency of the set goals and objectives**

Hypertension is the most common risk factor for serious vascular events such as stroke and heart attack. According to a survey in 2019 of the National Statistical Institute of Bulgaria, the condition affects about 29.7% of people in Bulgaria over the age of 15. ACE inhibitors are first-line drugs for hypertension. They have a cardioprotective, nephroprotective, and vascular

protective effect. This makes them especially suitable for patients with concomitant diseases such as diabetes, nephropathy, congestive heart failure, and after myocardial infarction.

It is reported that long-term application of ACE inhibitors, especially at older patients, leads to a reduction of their effectiveness. Therefore the invention of new active and selective ACE inhibitors is of increasing interest to the pharmaceutical industry. In respect to the abovementioned, the goals and objectives set in the dissertation are motivated and logical. Some literature reports have shown that short peptides obtained from milk fermentation have antihypertensive properties. However, they are isolated in a large number of steps and in small quantities. The contribution to the optimization of the synthetic procedure for their preparation of these short-chain peptides or their analogues, potential ACE inhibitors, has scientifically applied character. In the present Ph.D. thesis by means of spectral and quantum chemical approaches an in-depth analysis of the relationship between the structure of some of the synthesized compounds and their potential as ACE inhibitors is made and the obtained results are fundamental.

#### **4. General characteristics and evaluation of the dissertation**

The Ph.D. thesis of Boryana Yakimova meets the requirements of Law for the development of the academic staff in the Republic of Bulgaria and the governmental and institutional Rules of its application. The Ph.D. thesis contains: Introduction, Aims and tasks, Review of the literature, Experimental part, Results and discussion, Conclusions, References, List of Boryana Yakimova's publications on the topic of the dissertation and the citations of these works. The Ph.D. thesis contains: 21 Schemes, 39 Figures and 6 Tables. Some of the schemes are not numbered. The number of cited references is 124.

##### ***Review of the literature data***

The literature review is written on 24 pages and considers three main paragraphs. It initially describes the structure and function of most of the components (enzymes and receptors) of the renin-angiotensin system, special attention is given to the structure, function and mechanism of action of angiotensin-converting enzyme I (ACE 1). Here, the role and importance of inhibitors of this enzyme in the treatment of hypertension is emphasized. Examples of different groups of synthetic and natural compounds with antihypertensive activity, most of which are established drugs, are shown. At the end of the literature review an overview of the methods and

approaches for peptide synthesis is summarized. The advantages and disadvantages of liquid-phase, solid-phase and microwave peptide synthesis are emphasized. The literature review is based on 98 literature sources and is written concisely and with understanding.

### ***Experimental section***

In this section (41 pages) are given in details the synthetic procedures and the reaction schemes of the obtained by liquid-phase dipeptides: H-Val-Pro-OH and H-Ile-Pro-OH, and tripeptides: H-Val-Pro-Pro-OH и H-Ile-Pro-Pro-OH; as well as the procedures and schemes of the solid-phase synthesis of eight tripeptides containing proline, two tripeptides without proline; the procedures of preparation of the cyano methyl esters and the acylation of glucose and saccharose with aminoacids. The compounds are purified by HPLC and characterized by means of <sup>1</sup>H-, <sup>13</sup>C-NMR and/or MS/MS (Micromass® Q-ToF MICRO™ equipped with a quadrupole detector). The effect of the media on ci-/trans- isomerization at two of the synthesized tripeptides (H-Val-Pro- Pro-OH and H-Ile-Pro-Pro-OH) is studied in detail using both Infrared polarizaton and <sup>1</sup>H-NMR techniques. Employing molecular docking are analyzed the interactions between three of the synthesized tripeptides (H-Val-Pro-OH, H-Val-Pro-Pro-OH и H-Val-Pro-Pro-Pro-OH) and the amino acids comprising the inhibitor-binding subsite of ACE 1. In details are given the three “in vitro” methods used for the evaluation of the inhibitory potency of the synthesized target compounds. Described is the “ex-vivo” method applied for the evaluation of the effects on the newly synthesized compounds on basal tone and their effect on angiotensin I-induced contractions on the rat ileum. The Ph.D. student has selected an adequate methodology for achieving the set goals and objectives of the dissertation.

### ***Results and discussion***

The section summarizes the reaction schemes of the liquid-phase and solid-phase synthesis of the target peptides, their yields, as well as data on the structural characterization (NMR and / or MS/MS) of the compounds. The possibilities for cis-/trans-isomerization of the H-Val-Pro-OH dipeptide depending on the acidity of the medium have been investigated in detail by NMR techniques and quantum chemical calculations. By applying solid-state polarized IR spectroscopy, the conformational stability and the possibilities for the formation of intramolecular hydrogen bonds are characterized in detail for the tripeptides: H-Val-Pro-Pro-OH H-Ile-Pro-Pro-OH.

Using molecular docking (induce fit method) are predicted the mode of binding and interactions of short-chain peptides with a different number of proline, namely -Val-Pro-OH, H-Val-Pro-Pro-OH, and H-Val-Pro-Pro-Pro-OH with the amino acids comprising the binding subsite of ACE 1, and the results are discussed in respect to the differences in the inhibitory activity of these compounds. A scheme for the selective preparation of amino acid esters with glucose and sucrose has been developed. The inhibitory activity of the synthesized target peptides and amino acid-modified sucrose and glucose toward ACE 1 was determined and the results were compared with those obtained for lisinopril. From the “ex-vivo” experiments it was found that all five tripeptides H-Val-Ala-Pro-OH, H-Val-Ala-Trp-OH, H-Leu-Lys-Pro-OH, H-Leu-Ala -Pro-OH, and H-Ile-Ala-Lys- reduce in a dose-dependent mode the conversion of angiotensin I to angiotensin II and have the same inhibitory potential against rat ACE 1 from rat ileum.

The conclusions in the dissertation are formulated clearly and logically follow from the obtained results.

### **5. Contributions and significance of the thesis for science and practice**

The research conducted in the dissertation has a scientific and scientifically applied character and enriches the existing knowledge in the field of peptide synthesis and enzymology. The Ph.D. student has proposed a new approach to the synthesis of short-chain peptides and some of the reported target compounds have been synthesized for the first time and fully characterized. The procedure for the separation and purification of positional isomers of esters of amino acids and sucrose and glucose has been optimized. The role of the composition and conformational stability of short-chain peptides for their antihypertensive action and stability from proteolytic degradation is clarified. The results obtained from the studies of the interactions and the mode of binding of the short-chain peptides in the active center of ACE 1 can give guidelines for the selection and synthesis of structures with higher ACE 1 inhibitory activity.

### **6. Assessment of the publications and personal participation of the PhD student**

The results of the Ph.D thesis are published in four scientific articles published in: *Journal of Molecular Structure* (Q2), *Farmacia Journal* (Q2), *Protein and Peptide letters* (Q3) and *Bulgarian Chemical Communications* (Q4). In two of the publications, Assist. Prof. Boryana

Yakimova is the first author, and in one of the articles she is the second author, which is indicative of her significant personal contribution to the research. 11 citations from two of the dissertation publications were noted. Parts of the results included in the Ph. D thesis were presented at 14 scientific forums and conferences.

## **7. Abstract**

The abstract contains 43 pages, of which 2 pages short introduction, 1 page with the used abbreviations, 1 page with the formulated goals and objectives, 32 pages with the results and discussion, 1 page the conclusions, 1 page contributions and appendices (lists publications on the topic of the Ph.D. thesis, citations, participation in the conference, defended the graduates with a supervisor Assist. Prof. Boryana Yakimova). The abstract is prepared according to the requirements of the Law for the development of the academic staff in the Republic of Bulgaria and the governmental and institutional Rules of its application and reflects the main results and contributions of the dissertation.

## **8. Critical remarks and recommendations**

I have critical remarks on inaccuracies in the used terminology and also I found some technical errors. For example, on page 15, in the paragraph where the temperature stability of the N-domain of ACE 1 is discussed, the term “melting point” is used instead of the term “melting temperature”, which is accepted in the scientific literature for biopolymers. On page 20 text concerning the grouping of ACE 1 inhibitors, it is stated that it is made "... depending on the group attached to the Zn atom of their molecule ..." it is not clear that the Zn atom is from the active center of the enzyme, rather, it may cause misunderstanding that the compounds are in the form of zinc complexes. On page 31, "Sinica Shanghai Academy" should be understood as the Chinese Academy of Sciences. On page 41 one of the reagents is written as "N, O-bistrimethylsilylproline", and later in the text, it is found as "O, N-bistrimethylsilylproline". In several times in the text is found the term "chlorine ions" instead of the correct "chloride ions".

The molecular weights of the starting compounds and the resulting products are given with varying accuracy - in some cases to whole numbers, and in others to tenths or to hundreds of

decimal places. In some cases point and in others semicolon is used to express decimal numbers. Instead of “OH gr.” is better to be used “hydroxyl group”. On page 75 Leu from the substrate “Hip-His-Leu” is wrongly translated. Some of the IC<sub>50</sub> values are given in μM, and others in μg/mL, which makes their comparison difficult. Formatting of the references is not unified - in some cases, the titles of the journals are abbreviated in some cases are written full names of the Journals. The abbreviation “et al.” can be found with and without full stops. Some grammatical errors are found in the text.

Nevertheless, I believe that the technical errors are involuntary and I would like to emphasize that they do not reduce the qualities and contribution of the dissertation, nor do they affect my excellent impression of the qualities and high scientific level of the doctoral student's research.

I would like to ask the Ph.D. student what criteria were applied to select which compound to be synthesized by which synthetic procedure? In which cases is liquid-phase synthesis preferable and in which is solid-phase synthesis is more efficient?

## **9. Personnel impressions**

I have known in personnel Assist. Prof. Boryana Yakimova since she started working in the laboratory "Chemistry and Biophysics of Proteins and Enzymes" of IOCCP, BAS. She is a responsible, diplomatic, and loyal colleague. While working on various projects and her Ph.D. thesis, Assist. Prof. Boryana Yakimova has gained laboratory skills and expertise in peptide synthesis, isolation, and structural characterization of proteins and enzymes from microbial and plant sources as well as *in vitro* methods for evaluation of the activity of various hydrolytic activities (proteases, galactosidases, and some other). Assist. Prof. B. Yakimova is able to analyze the scientific literature, focus on solving scientific problems, and analyze critically the results obtained from the experiments. She is a trained researcher and a desirable member of the team in many projects.

## CONCLUSION

The Ph.D. thesis, object of this review, contains fundamental and applied results, which are original contribution to science and meet all the requirements of the Law for Development of the Academic Staff in the Republic of Bulgaria and its governmental and Institutional regulations. The presented materials and dissertation results fully comply with the specific requirements of the Regulations of IOCCP, BAS for application of the Law for Development of the Academic Staff in the Republic of Bulgaria. The Ph.D. thesis shows that the doctoral student Boryana Yakimova has in - depth theoretical knowledge and experimental skills in the field of synthesis, isolation, structural characterization and evaluation of the activity of short-chain peptides in various *in vitro* model systems. I am convinced that Assist. Prof. Boryana Yakimova has an indisputable contribution to the research, summarizing and interpreting the results and formulating the contributions to the dissertation.

Due to the written above, I confidently give my **positive assessment** of the research presented by the above peer-reviewed dissertation, abstract, the obtained results and contributions, and I invite the esteemed scientific jury to **award the educational and scientific degree “Ph.D.”** of Boryana Krasimirova Yakimova in the field of higher education: "Natural Sciences", code 4; professional field "Chemical Sciences", code 4.2; scientific specialty "Bioorganic chemistry, chemistry of natural and physiologically active substances".

18.05.2021 г.

Sofia

Reviewer:

(Assoc. Prof. Dr. Maya Guncheva)