

OPINION

by Dr. Romyana Ivanova Bakalska,

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in the area of higher education 4. Natural sciences, mathematics and informatics

professional field 4.2. Chemical sciences

scientific specialty Organic Chemistry

For awarding the educational and scientific degree "Doctor"

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Author: *Miglena Zlatkova Milusheva*

Thesis theme: *"Synthesis of new antispasmodics affecting memory functions in experimental animals"*

PhD supervisors: *Assoc. Prof. Dr. Stoyanka Nikolova Atanasova from the "Paisii Hilendarski" University of Plovdiv and Assoc. Prof. Dr. Iliyana Stefanova Kancheva from the Medical University of Plovdiv.*

1. General description of the submitted materials

By order No. PD 22-1134 of 15.05.2025 of the Rector of the Plovdiv University "Paisii Hilendarski," I am appointed as a member of the scientific jury for ensuring a procedure for the defense of a dissertation on the topic "Synthesis of new antispasmodics affecting memory functions in experimental animals" for the acquisition of the educational and scientific degree "doctor" in the field of higher education 4. Natural sciences, mathematics and informatics; professional field 4.2. Chemical sciences, doctoral program in organic chemistry. The author of the dissertation is Miglena Zlatkova Milusheva, a doctoral student in full-time study at the Department of Organic Chemistry, with scientific supervisors Assoc. Prof. Dr. Stoyanka Nikolova Atanasova from the Plovdiv University "Paisii Hilendarski" and Assoc. Prof. Dr. Iliyana Stefanova Kancheva from the Medical University of Plovdiv.

The submitted thesis and all accompanying documents are in complete accordance with the requirements of the Law for the Development of the Academic Staff in the Republic of Bulgaria and the internal regulations of the Plovdiv University.

The *Scopus* report shows that the PhD student Miglena Milusheva is a co-author of 11 articles published in prestigious scientific journals and 2 book chapters from international publishers, as well as 5 more articles in non-refereed publications. For the award of the educational and scientific degree "Doctor," the candidate participated with 3 articles in journals with a quartile Q1 and a high impact factor, each of which carries 25 points, or a total of 75 points. In addition, the completed dissertation work carries 50 points. Thus, with 125 points, the PhD student significantly exceeds the minimum requirements under the law on the development of the scientific staff and the regulations of PU "P. Hilendarski" on the procedure for acquiring the educational and scientific degree "Doctor."

2. Brief biographical data for the PhD candidate

Miglena Milusheva received her secondary education at the "Romain Rolland" high school with foreign language teaching in the town of Stara Zagora, with profiled study of English, German, and biology. She spent the last year of her studies in an English-speaking environment - at Darlington School, Rome, Georgia (USA)—with an intensive study of chemistry, biology, mathematics, and English language, which ended with a certificate of excellent success and a certificate of the highest success in the anatomy and physiology graduation. The PhD student is a graduate of the Faculty of Chemistry of the "Paisiy Hilendarski" university, majoring in medicinal chemistry (bachelor's), with a successfully defended thesis in the Department of Organic Chemistry. During her studies as a medicinal chemist, she also completed a pedagogical specialization as a chemistry teacher. Her master's degree is in the field of food chemistry. In the next 5 years, she worked in her specialty at Mondi EAAD, Stamboliyski, and then briefly in a medical diagnostic laboratory as a clinical laboratory assistant, as well as in the field of drug trade. From May 2021 until today, Miglena Milusheva is an assistant at the Medical University in Plovdiv, where she conducts chemistry laboratory exercises with students from the medicine and dentistry majors in Bulgarian and English. From March 2023, she is a full-time doctoral student at the Department of Organic Chemistry of the University of Plovdiv and was awarded the right of defense in May 2025. During the period of her studies, the doctoral student completed the educational part of her individual plan in full. She attended over 20 specialized courses and seminars related to her direct scientific interests, such as "Retrosynthesis" by the Oxford professor M. Moloney, "Organic Stereochemistry" by Prof. F. Alonso of the University of Alicante, and "Biofunctionalized Heterocyclic Compounds" by Assoc. Prof. Statkova; an intensive course in "Nuclear Pharmacy" with an emphasis on imaging diagnostics and treatment with the help of radionuclide-labeled chemical compounds; and the very useful courses for PhD students organized by *Yuventutis* Academy. Ms. Milusheva receives the "Doctoral Student of the Year 2024" award.

3. Relevance of the topic and appropriateness of the goals and objectives

The dissertation on the topic "Synthesis of new antispasmodics affecting memory functions in experimental animals" is in a very modern and rapidly developing area of the drug design, namely the

development of hybrid molecules combining pharmacophoric elements with different mechanisms of action within one chemical structure. The target of these efforts are functional and inflammatory disorders of the gastrointestinal tract, such as irritable bowel syndrome and inflammatory bowel disease, which continue to be a serious clinical challenge. The relevance of the problem is determined, on the one hand, by the still insufficiently clarified pathophysiology of these socially significant diseases, and on the other, by the limited effectiveness and unwanted side effects of currently administered antispasmodics. Despite their established use, existing drugs often do not achieve the desired clinical result, especially in patients with severe or refractory forms of the disease, and can cause side effects such as dry mouth, dizziness, or heart rhythm disorders. This highlights the need to discover new drug molecules with an improved pharmacological profile—higher efficacy, lower toxicity, and a wider spectrum of action.

As building blocks for the creation of the new hybrid molecules, the authors chose anthranilic acid and 2-phenylethylamine because they are well studied, present in a number of bioactive compounds and offer opportunities for chemical modification. The aim of the dissertation is adequately formulated, namely: synthesis of new derivatives of anthranilic acid as candidates for antispasmodics and substances influencing memory functions in experimental animals, as well as the tasks for its achievement. The value of this work is that the authors go through most of the steps in the investigation of potential drug candidates, starting from *in silico* modeling to predict the biological profile, then chemical synthesis of selected molecules and experimental biological testing. They apply *ex vivo* models to evaluate contractile activity and elucidate the mechanism of action, while *in vitro* tests reveal additional aspects such as anti-inflammatory and antimicrobial potential. The conducted *in vivo* studies in experimental animals make it possible to track systemic effects, including influence on cognitive parameters - a factor of increasing importance in chronic diseases associated with stress and altered quality of life. They compared the obtained results with established reference agents such as mebeverin, diclofenac, acetylsalicylic acid and prednisolone.

This dissertation presents original data on the synthesis, characterization and biological evaluation of new hybrid derivatives of anthranilic acid as potential antispasmodics with additional modulation of memory functions in experimental animals.

4. Knowledge of the problem

The literature review on the topic covers 285 literary sources from various fields of human knowledge - chemistry, medicine, pharmacology, biology, statistics, etc. Thanks to her alert mind and excellent knowledge, purposefully acquired in previous educational degrees, as well as her teaching work at the Medical University, the PhD student has managed to creatively handle the processing of information and demonstrates excellent knowledge of the problem, both in its chemical and biological

parts. I dare to confidently assert that Milusheva is a well-rounded specialist in the field, capable of searching, systematizing and critically analyzing scientific literature.

5. Research methodology

In the dissertation, modern and adequate methods were used to study and prove the properties of the obtained organic compounds and to study their biological activity. The chemical structure and purity of the compounds were proven by melting point, ^1H - and ^{13}C -NMR, HRMS analyses, and FT-IR spectroscopy. The study of the biological activity of the newly synthesized hybrid compounds was carried out by combining modern approaches - *in silico* modeling for predicting the biological profile, *ex vivo* models, and *in vitro* and *in vivo* tests. The obtained results were compared with established reference tools, and statistical processing of the results was performed. The chosen research methodology allows achieving the set goal and obtaining an adequate answer to the tasks solved in the dissertation work.

6. Characteristics and evaluation of the dissertation work.

The dissertation work is presented on 172 printed pages and includes an introduction (2 pages), a literature review (50 pages), a goal and objectives (1 page), own research (60 pages), an experimental part (28 pages), conclusions, and contributions (3 pages). It is illustrated with 13 schemes, 10 tables, and 32 figures. The bibliography includes 394 literary sources, mainly from recent years.

The introduction skillfully guides the reader to the nature of the research, and in the literature review, the PhD student has paid special attention to familiarizing herself with inflammatory bowel diseases and irritable bowel syndrome, critically discussing the efficacy of currently applied therapeutic agents and their undesirable effects and limitations. This is followed by an introduction to the hybrid molecules and anthranilic acid, whose hybrid derivatives are the subject of the present dissertation, as well as to the experimental models in the preclinical evaluation of bioactive substances. I feel that the subsection on anthranilic acid could be shortened at the expense of extensive historical data. The goal of the research and the research tasks are clearly formulated. To achieve this goal, an initial screening and selection of active structures was performed by *in silico* computer models, on the basis of which the authors established that each of the compounds **3–8** could be considered as a potential therapeutic candidate. This is followed by the synthesis of the target molecules with very good yields and then of their amides using methods known from the literature. The synthetic approach chosen by the authors involved the reaction of isatoic anhydride with unsubstituted and differently substituted 2-phenylethylamines. The resulting amides, which are a "hybrid" of anthranilic acid and 2-phenylethylamine, are converted to diamide derivatives by an acylation reaction. When choosing acyl chlorides, the authors prefer those with phenyl and benzyl substituents over alkyl ones, based on the previously conducted computer modeling for toxicity and biological effects. The structure of all synthesized compounds was confirmed correctly by ^1H - and ^{13}C -NMR, HRMS analyses, and FT-IR

spectroscopy. The main part of the research on the dissertation work, including the synthesis and purification of the compounds, computer modeling, and determination of *in vitro* anti-inflammatory activity, was carried out at the Faculty of Chemistry of PU "Paisiy Hilendarski." Biological trials begin by examining the effect of the hybrid molecules on smooth muscle contractile function *ex vivo*. In order to determine the anti-inflammatory potential of the synthesized hybrid compounds, the authors evaluate their activity both *in vitro* conditions (as inhibition of albumin denaturation) and *ex vivo* conditions (through immunohistochemical evaluation). The obtained results are extremely interesting and encouraging. Anthranilic acid amides are known to be very good antimicrobial agents, which is why the authors also conducted an *in vitro* evaluation of the antimicrobial activity. Given that cognitive impairment often accompanies the use of some antispasmodics, the authors investigated the potential of three of the substances that showed the highest antispasmodic and anti-inflammatory activity in terms of their effect on memory functions in experimental animals. They found that the three studied compounds did not impair cognitive functions and did not suppress locomotor activity in experimental animals; even one of them, compound **6d**, showed the highest therapeutic potential, improving both learning and short-term and long-term memory. The experimental determination of the antispasmodic activity, the *ex vivo* anti-inflammatory activity, and the influence on the memory functions was carried out at the Medical University–Plovdiv under the valuable guidance of the other scientific supervisor, Dr. Iliyana Kancheva, Assoc. Prof. at Medical University.

7. Contributions and significance of the development for science and practice

The integration of results from *in silico* modeling, chemical synthesis, biological testing, and *in vivo* studies provides a comprehensive view of the pharmacological potential of the developed compounds. In this sense, the contributions of Miglena Milusheva's dissertation work are indisputable, and the most important can be summarized as follows:

Scientific contributions: 1) The design of small molecules as antispasmodics was carried out and synthetic approaches for their preparation were found; 2) The influence of the antispasmodic activity of the introduction of various substituents was theoretically studied; 3) Clear relationships between the molecular structure and biological activity were found for the synthesized compounds, which can serve as a basis for future rational design of biologically active compounds.

Scientific and applied contributions: 1) For the first time, using the proposed synthetic method, 18 compounds were obtained, of which 16 were new, previously unpublished in the scientific literature; 2) For the first time, the antispasmodic activity of all 18 synthesized compounds was measured, and for five of them this activity was better compared to the known antispasmodic mebeverine; 3) A degree of biological activity has been proven, characterizing one of the hybrids as a substance with functional

characteristics of an antispasmodic with an anti-inflammatory effect, positively affecting memory functions in experimental animals.

8. Evaluation of publications on the dissertation work

The results included in the dissertation have been published in 3 scientific articles in specialized international open access journals *International Journal of Molecular Sciences* (Q1, IF 4.9), *Pharmaceuticals* (Q1, IF 4.3) and in *Molecules* (Q1, IF 4.2). Currently, the number of independent citations in the *Scopus* and/or *Web of Science* databases significantly exceeds those indicated by the doctoral student 21, which is a sure criterion for recognition in the scientific community. All scientific publications are co-authored and are in English, which is common for the chemical field. As can be seen in the "Author's Contribution" for each article, Miglena Milusheva is an active participant not only in the experimental work on the synthesis, isolation, and purification of the compounds but also in writing the original draft, in reviewing and editing, and in visualizing the results. I found no evidence of plagiarism, and I believe that the data obtained is original, which is supported by the fact that the publications are in international peer-reviewed journals. For Miglena Milusheva, the calculated Hirsch index is 4, based on *Scopus* data.

During this period of nearly 2 years, the PhD student has reported the results of her dissertation research at 10 thematic international and 8 national conferences, the most common form of presentation being an oral presentation or report. The PhD student is a member of the work team during this period of 5 projects, 2 of which are financed by the Scientific Research Fund at the Ministry of Education, Culture, Sports, and Science; 2 projects by the "Paisiy Hilendarski" PU; and one by the Medical University–Plovdiv.

9. Personal involvement of the PhD student

A chemist does not work alone in modern scientific pursuits. Usually, his expert skills are supplemented by fellow specialists in different scientific directions that are mutually necessary for the success of a work project. In this regard, as is evident from my assessment of the scientific production of the doctoral student above, Ms. Milusheva has worked in co-authorship with exactly the necessary specialists for the conduct and successful completion of each study. Her personal contribution is undoubted in each publication—she is the first author.

10. Abstract.

The abstract in a volume of 36 pages is made according to the requirements and presents the content of the dissertation in a concise and informative form. The goals and objectives, the main results and their discussion, the conclusions of the achievements, and the list of publications are reflected. It seems to me that the absence of a Materials and Methods section in it would only contribute to its tighter appearance. A pleasant impression is made by intelligently expressed thanks to people and institutions without which the present work would be impossible.

11. Critical remarks and recommendations.

I have no critical remarks about the materials presented to me; on the contrary, the documentation on the procedure is complete and neatly arranged, which not only facilitates the work of the scientific jury but also confirms the overall excellent impression of the candidate's creative presentation. I would like to ask 2 questions:

- “The dissertation describes 18 compounds, of which 16 are new compounds.” *Is the statement based on a check in Reaxis, another database, or in scientific periodicals?*
- “The activity of three of the compounds with very good antispasmodic activity was studied in influencing the memory functions of experimental animals, with one of them (**6d**) having a significant effect on memory and learning processes”. *What do you think are the next steps in assessing the biological profile of the compound with promising potential in order to apply it for therapeutic purposes?*

12. Personal Impressions.

I have known Miglena Milusheva since her student years as a lecturer in the basic course in organic chemistry at the Faculty of Chemistry of the PU. An alert and inquisitive mind of a critically thinking young person with a love for organic chemistry cannot go unnoticed! Very active, organized, and enthusiastic, and with that “broader” view of the world that people who have lived abroad acquire, she is not afraid to freely express her personal position or ask questions. Her good presentation and communication skills are based not only on her cheerful character and immediacy in communication but also on in-depth knowledge and excellent command of English (C1). I could only be proud that young people like her are carrying on the baton in science and in teaching chemistry.

CONCLUSION

Miglena Milusheva's dissertation is a thorough and systematic study in the current field of drug design and **contains original scientific studies** on the synthesis, characterization, and biological evaluation of new hybrid derivatives of anthranilic acid as potential antispasmodics with additional modulation of memory functions in experimental animals. **The scientific and applied results** in the dissertation work are related to the applied *ex vivo* models and *in vitro* and *in vivo* tests of the obtained compounds in experimental animals. The work performed is significant in volume and diverse in nature, which gives reason to assume that the doctoral student Miglena Zlatkova Milusheva **possesses** in-depth theoretical knowledge and professional skills in the scientific specialty of Organic Chemistry, **demonstrating** qualities and skills for independent conduct of scientific research.

In conclusion, the dissertation **meets all the requirements** of the Law on the Development of Academic Staff in the Republic of Bulgaria, the Regulations for the Implementation of the Law, and the relevant Regulations of the University "Paisiy Hilendarski."

Due to the above, I confidently give my **positive assessment** of the conducted research presented by the above-reviewed dissertation, abstract, achieved results, and contributions, and ***I propose to the esteemed scientific jury to award the educational and scientific degree "doctor" to Miglena Zlatkova Milusheva*** in the field of higher education. 4. Natural Sciences, Mathematics and Informatics; professional field 4.2. Chemical Sciences; doctoral program in Organic Chemistry.

06.06.2025

Reviewer:

/Assoc. Prof. Rumyana Bakalska, PhD/