Poznań, September 2, 2024

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Review of the doctoral dissertation of Mr. MASROOR AHMED KHAN entitled "*Synthesis of bioluminogenic substrates of firefly and NanoLuc® luciferases and validation of their response to analytes involved in redox homeostasis*"

performed at the Institute of Bioorganic Chemistry of the Polish Academy of Sciences under the supervision of Dr. Hab. Jacek Kolanowski, co-supervisor Dr. Dorota Jakubczyk

Synthetic work in the area of organic chemistry is still extremely interesting and relevant. Research conducted in fields of science as chemistry, pharmacy, or medicine is focused, and therefore justified – on the search for new biologically active chemical compounds. The development of a convenient, fast, and inexpensive synthesis of new chemical compounds is the basis of this search. Unfortunately, in many cases, it is a time-consuming process. However, determining and developing another (often new) synthetic goal always leads to improvement of previous work. All this means that a significant part of the threats associated with pathogenic factors in the current era of civilization is solved or minimized. However, it is still necessary to take into account the evolutionary development of microorganisms, which leads to their significant resistance to developed and used biocidal agents. In this respect, the “fight” of mankind against pathogenic factors is still ongoing.

One of the important and extremely interesting aspects of such a fight may be bioluminescence, which consists of the emission of light as a result of the oxidation of the luciferin molecule by the luciferase enzyme. It is a sensitive, non-invasive, and real-time monitoring method. Bioluminescence can be used successfully to detect analytes involved in physiological processes, including pathological ones, using probes based on a chemical reaction with the analyte. Therefore, in my opinion, the goal of the work chosen by Mr. Masroor Ahmed Khan, MSc., consisting of developing the synthesis and characterization of bioluminescent probes with the intention of expanding the palette of bioluminescent tools for simultaneous detection of many analytes seems fully justified.

The doctoral dissertation submitted for review is a 190-page manuscript and has a typical layout for this type of work, consisting of the following: Abstract (3 pages); Summary in Polish (3 pages); Abbreviations (3 pages); Introduction (15 pages); Objectives (1 page); Results and Discussion (54 pages); Conclusions and Perspectives (2 pages); Materials and Methods (31 pages); Bibliography (10 pages, 107 items); Supplementary Information (57 pages) and List of Publications (1 page). It is a pity

that the Author did not compile a list of tables, figures and diagrams, which would undoubtedly make it easier to find them in the text.

The scientific achievements of the PhD student consist of 2 published works: (1) a review in Postępy Biochemii (2024) and (2) an original work in Antibiotics (2019). It is worth highlighting that the work from 2019 was not carried out in the team of Dr. Hab. Jacek Kolanowski. In addition, one paper is currently under review (Frontiers in Chemistry journal). It is also worth noting that the research work was financed by the National Science Center SONATA grant no. 2017/26/D/NZ1/01234. Unfortunately, I did not find any information related to the PhD student's participation in domestic or foreign conferences, on presented posters or oral communications. I would like to ask at this point whether the PhD student actively participated in conferences. If so, in which ones and in what role? I am convinced that the PhD student may continue to pursue research topics that are interesting from an experimental point of view in the future. This will certainly increase his bibliographic results to date.

In the literature section, the Author accurately, factually and correctly discusses issues related to the topic, such as the phenomenon of bioluminescence, its mechanism and applications in biological research, luciferin-luciferase systems, issues related to in vivo bioluminescence imaging or strategies for designing bioluminescent probes. In my opinion, this part of the work is presented comprehensively and, more importantly, interestingly. The discussed examples and issues were selected precisely. Perhaps a short historical thread regarding luciferin and luciferase is missing, which, if only related to the issue of the name of the compounds, is quite interesting.

The aim of the work, which is the second and at the same time the most important part of the dissertation, was to design and synthesize bioluminescent probes for simultaneous detection of many analytes involved in redox homeostasis. The basic building blocks in the series of syntheses were structures based on firefly luciferin and furimazine, which are substrates for small NanoLuc® luciferase. The PhD student focused on analytes related to redox homeostasis, in particular iron, gamma-glutamyltransferase (GGT) and nitroreductase (NTR), because, as he himself states, "the importance of their mutual interaction in diseases such as cancer is crucial and still not fully understood". The PhD student's work consisted of developing:

1) a probe compatible with the split luciferin strategy for detecting iron(II) ions;

2) three probes responsive to nitroreductase (NTR), compatible with the split luciferin system and NanoLuc®. It is worth emphasizing here the development of two variants based on 2-cyano-6-hydroxybenzothiazole and D-cysteine, and that the first bioluminescent probe based on furimazine was obtained;

3) a dual-analyte probe based on the aminoluciferin skeleton, which simultaneously detected the presence of nitroreductase (NTR) and gamma-glutamyltransferase (GGT).

I would like to emphasize here that the syntheses performed are extremely interesting (including the Suzuki and Negishi coupling reactions, or the Horner–Wadsworth–Emmons reaction), while developing synthetic pathways was not always easy. It is worth noting that the PhD student not only provided the spectroscopic characteristics of the compounds (proton and carbon nuclear magnetic resonance and mass spectrometry), but also showed a catalog of nuclear magnetic resonance and mass spectrometry spectra. Here, as a reviewer, I must express some dissatisfaction. It is a pity that the Author did not provide the numbering of atoms in the structures placed in the spectra and did not correlate them with visible signals. This would give a full picture of the spectroscopic analysis. I would also like to ask what was the purpose of the mass spectra? Was it only to determine the composition and purity of the compounds obtained? Or were there attempts made to propose and determine the mass fragmentation patterns of selected compounds using other ionization techniques?

The probes obtained were tested in vivo and the probe obtained on the basis of D-cysteine showed significant activity in vitro. In turn, the dual analyte probe based on the aminoluciferin skeleton, which simultaneously detects the presence of nitroreductase (NTR) and gammaglutamyltransferase (GGT), made it possible to use a single molecular probe for the simultaneous monitoring of many analytes. As the Author of the dissertation states, " Additionally, a D-luciferin variant of this dual-analyte probe was developed, featuring a dual-analyte responsive motif for NTR and GGT as intermediate that can be conjugated to other fluorophores or bioluminophores. These results expand the available molecular toolset of multi-target bioluminescent probes". It can therefore be stated that the reviewed doctoral dissertation is one of the works in which the assumed goal was achieved. The PhD student designed and synthesized new bioluminescent probes used to detect various analytes related to redox homeostasis. The probes obtained certainly enrich the research workshop of biologists or chemists, in which studies of complex biological phenomena are often based on complex interactions of analytes.

At this point I would like to ask the PhD student some questions:

1) How can we interpret the presence of signals in the mass spectrum of compound 22 (Figure 4.21, p. 67) located at m/z 279.0909 and 557.1746?;
2) Can the reaction mechanism presented in Scheme 4.27 (p. 79) also have an alternative course?;
3) How can we explain the lack of a positive result for the first two reactions presented in Table 4.34 (p. 88);
4) What can explain the low yields of compounds 17 (17%), 18 (28%), 29 (7%), 34 (7%)?
5) Have you attempted molecular modeling of the compounds studied using semi-empirical methods?

6) Have you tried in silico studies? 7) What do you consider to be the greatest success in your work? As for the formal aspect of the work submitted for review, I would like to emphasize its clarity and good illustration of the theses derived with drawings, diagrams, and tables.

As a Reviewer, I must list the errors and imperfections noticed in the work. And so:

1) the work incorrectly uses the notation of substituents R1, R2, etc. The numeral is always written in superscript, not subscript, e.g. p. 22, Scheme 2.2; p. 26, Scheme 2.3;
2) in Table 2.4 on p. 28–30 in Ref. column, only the position number can be given;
3) in Schemes 4.13 (p. 54) and 4.14 (p. 55), the given reaction mechanism is missing protons that appear in the final product;
4) Figure 4.17 (p. 60) is rather unclear;
5) Scheme 4.19 (p. 65) contains two significant errors: (1) the base (B) should abstract the proton from the nitrogen atom, not oppositely (as indicated by the arrow in the drawing), and (2) the negatively charged oxygen atom should attack the electrophile (E), not oppositely. In reaction mechanisms, it is the negative charge (whether total or partial) that is the attacking factor;
6) I did not find a spectroscopic analysis of compound 4 (the synthesis of the compound is described on p. 97), while on pp. 142 and 143 there are 1H and 13C NMR spectra.

At the same time, I would like to emphasize that the above comments do not affect my positive assessment of the work in any way.

In summary, I declare that the dissertation being the subject of the review fulfills the conditions laid down in the Act of July 20, 2018, The Law on Higher Education and Science (Journal of Laws 2018, item 1668 as amended), the Act of July 3, 2018, Provisions Introducing the Act – The Law on Higher Education and Science (Journal of Laws 2018, item 1669 as amended), and The Rules of Proceeding in the Matter of Awarding the Doctoral Degree in the Institute of Bioorganic Chemistry PAS (Resolution of the Scientific Board of IBCH PAS No. 28/2024/Internet of March 20, 2024) and I recommend that the Scientific Board of the Institute of Bioorganic Chemistry PAS to admit Masroor Ahmed Khan, M.A., to further stages of the procedure for awarding the degree of doctor.

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