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15 parvis René-Descartes
BP 7000, 69342 Lyon cedex 07
Tel. +33 (0)4 37 37 60 00
Fax +33 (0)4 37 37 60 60
www.ens-lyon.fr

JENS HASSERODT
PROFESSEUR DES UNIVERSITÉS

LABORATOIRE DE CHIMIE

Lyon, September 12, 2024

Review of manuscript for doctoral dissertation by Masroor Ahmed Khan entitled "Synthesis of bioluminogenic substrates of firefly and NanoLuc® luciferases and validation of their response to analytes involved in redox homeostasis".

The work contained in the above manuscript was carried out at the Institute of Bioorganic Chemistry, Polish Academy of Sciences in Poznan, under the supervision of Dr. Hab. Jacek L. Kolanowski and Dr. Dorota Jakubczyk (Auxiliary PhD advisor).

The thesis manuscript is composed of 125 pages. 55 pages of spectral characterization are attached as an annex. The main text comprises an introductory chapter of 14 pages, 1 page declaring the goals of the thesis, 54 pages presenting the results and discussion, 1 page of general conclusion, 30 pages of experimental protocols, and 9 pages of bibliography (107 references).

In his thesis, the candidate explores a general strategy to contribute to the field of bioluminescence detection of specific enzyme activity, without however advancing the work as far as tests with live cells or multicellular organisms. The principal results contained in the manuscript are in the realm of chemical synthesis of the objects of study, and certain insights into the required constitution of a luminescent detection experiment to deliver satisfactory results.

The work concentrates on the design of target molecules, their subsequent synthesis, and their evaluation as probes to detect select enzyme activities. More specifically, four probe designs are explored in four respective chapters. One only pretends to target a single analyte, the other three are meant to target two analytes that must act on the probe in succession. The latter approach is referred to as a “logic gate”, more specifically the category called “AND”, as it requires both analytes to be present to generate a signal. This concept has been reported by others prior to this thesis, namely in the context of fluorescence detection as the candidate points out himself. What is new is the candidate targeting a probe that causes a bioluminescent signal rather than a fluorescent one. This requires his future live cells or animals to produce the enzyme required to generate such a bioluminescent signal, luciferase. So, the thesis’ ultimate target is *transgenic* cells or animals harboring such luciferase activity.

Dual-analyte responsive probes are indeed of interest for the Life Sciences as they have the potential to deliver far higher detection specificity, for example in distinguishing cancer cells from healthy cells in a multi-cellular organism. To sum up the components of such an experiment envisaged above, the candidate must unite two target analytes, luciferase activity, his probe, and, for the split-luciferin

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approach of the two first probe designs, two probe molecules in a live cell where the bioanalytes and the luciferase activity reside. All in all, four or five chemical components must be present at sufficient quantities to generate a signal. To this adds the requirement for ATP (and oxygen) to see luciferin converted by Fluc (not NLuc), but these components are of course naturally present in live cells.

In chapter 4.1.5. (summary), the candidate recognizes the size of this challenge, particularly after encountering difficulties for this first of the four strategies. The probe no. 4 for this 1st strategy was obtained in a five-step synthesis with an overall yield of 15 percent. While the candidate did conduct the luminescence monitoring of in vitro incubations with pure components, the in vitro experiments with cell lysates (figures 4.6 and 4.7) were conducted by a collaborating team from biology (Master thesis).

In the 2nd approach, two probes (no. 8 and 10) for a split-luciferin, single-analyte assay were prepared, by a 1-step and 2-step synthesis, respectively. Probe 8 was not evaluated for its performance. An explanation for this decision should be added to the manuscript. Probe 10 was tested by the candidate in an *in vitro* setting with pure components. Inspection of the structure of 10 points out a potential vulnerability to spontaneous fragmentation. And indeed, the mass spectrum of 10 (page 150) shows a peak as prominent as that of the title compound corresponding to the thiazolidinone resulting from fragmentation (145.99, the carboxylate). However, figure 4.16 shows signal generation with probe 10 which would not have been possible if all of 10 would already have turned into the thiazolidinone. Future experimentation of the host team would need to establish whether fragmentation is a problem in physiological conditions, or not.

The 3rd approach employs a substrate for another type of luciferase, called furimazin, a substrate that does not require ATP or oxygen to be present. The approach comprised the preparation of two probes 19 and 22, the former targeting a single analyte enzyme, NTR, the latter two enzymes. Only 19 was evaluated in an in vitro experiment by the candidate because 22 could not be obtained in sufficiently pure form. The response to NTR in the presence of luciferase was quite neat; no background luminescence was detected in the absence of the analyte; a feat in variance with the split-luciferin approach from above where sizeable background luminescence was observed. The candidate carried out a 9-step synthesis that was reported by another team for a close analogue of furimazin. Some adaptation of the experimental conditions for certain steps was needed. The target compound was obtained in an overall yield of 1%. Unfortunately, 19 was reported by another team before the candidate's team has gotten around to submitting this work for publication. The candidate also prepared two furimazin derivatives serving future in cellulo experiments as negative controls, compounds 20 and 21. As the synthesis of 19, 20, and 21 consumed roughly 20 mgs of furimazin each, the 1% overall yield for furimazin (the experimental protocol for furimazin 18 speaks of 18 mgs obtained in pure form) from

Phone : + 33 (0) 4 72 72 83 94 ; Fax : +33 (0) 4 72 72 88 66 ; jens.hasserodt@ens-lyon.fr; www.ens-lyon.fr/CHIMIE
membre de UNIVERSITÉ DE LYON

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above might have proven too low to carry forward enough material to be able to obtain a pure sample of 22.

The 4th and final strategy made again use of luciferin as the base system of a probe, but this time avoiding the split-substrate approach. The probe belongs to a dual-analyte approach again, this time targeting two enzymes, NTR and GGT, both associated with tumor development. Two probes were designed, cpds. 29 and 34, that incorporated amino-luciferin (an aniline) and D-luciferin (a phenol), respectively, to verify which of the two performs better. 29 was prepared by the candidate in a 6-step synthesis with a roughly 1.5% overall yield (the exp. protocol speaks of 9 mgs of product obtained). The candidate also conducted the in vitro evaluation of probe 29 during luminescence monitoring and was able to conclude that it performs as predicted, with negative control experiments (absence of one of the two enzyme activities) being truly negative. Probe 34 was also synthesized in 6 steps with roughly the same overall yield (1.2%). The experimental protocol speaks of 3 mgs of product obtained after prep. HPLC purification. No evaluation of the performance of probe 34 was provided. It is possible that the candidate was out of time prior to the manuscript's submission but this must be confirmed. The candidate emphasizes that probe 29 is the 2nd-ever instance of a dual-analyte responsive bioluminescent probe. It should be noted here that for the time being, this holds true only for in vitro conditions with all components of the assay added in pure form.

The candidate has already co-authored a review article with the team of his PhD advisor on the domain that his thesis' subject is part of. He also co-authored a manuscript for a scientific article currently under review that reports on his observations regarding the first strategy of this thesis, namely that the presence of iron salts can negatively impact the generation of bioluminescence by luciferases.

Regrettably, there are some shortcomings in the scientific work and presentation of the written thesis that need to be addressed during the formal defence of the thesis:

- P. 23: "Activity-based probes". This term is reserved for a separate molecular technology. As this review article says: "Activity based probes are composed of a functional group (warhead), linker and tag, which enable specific labeling and enrichment of target proteins." See Fonović M, Bogyo M. Activity-based probes as a tool for functional proteomic analysis of proteases. Expert Rev Proteomics. 2008.
- A few errors can be detected in the chemical formulae employed in the schemes (p. 27, p. 35)
- There are also a few factual errors detected in the argumentation, and the conclusions are oftentimes fairly speculative in the absence of hard experimental data.
- Quite a few results presented in this manuscript were elaborated by other researchers collaborating with the candidate: fig. 4.6, 4.7, 4.9, table 4.10, table 4.11, …, as the candidate correctly points out himself.
- Cpds in the supplementary information do NOT display the numbers given to them in the main text.

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- Each NMR spectrum needs to display the compound number, the identity of the deuterated solvent, and, if spectrometers of different field strength were used, the number in Mhz.

However, as comes across from the above, the candidate has achieved most goals he set himself on page 34 (except for the evaluation of *all* synthesized probes for their bioluminescent response to the target analytes through in vitro testing).

Thus, the dissertation being the subject of the review fulfils 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. 59/2023/Internet of March 29, 2023) and I recommend that the Scientific Board of the Institute of Bioorganic Chemistry PAS allows it to further steps in PhD defence process.

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membre de UNIVERSITÉ DE LYON