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

The PhD thesis presented by Masroor Ahmed Khan was carried out at the Institute of Bioorganic Chemistry, Polish Academy of Sciences in Poznań in the Department of Molecular Probes and Prodrugs and the Centre for Chemical Biology (Laboratory of Medicinal Chemistry) under the supervision of Dr. Hab. Jacek L. Kolanowski and the auxiliary supervision of Dr. Dorota Jakubczyk.

The scientific aim of his work was the design, synthesis and early evaluation of proluciferins as chemical probes capable of undergoing selected enzyme-based activation. From such compounds and following this activation, the corresponding luciferins are then generated and become detectable by the action of their respective luciferases. The resulting light signal is thus providing an analytical proof for the actual presence of the selected enzymes (or chemical process) in the medium studied. Quite a few probes were designed and prepared in the course of these research.

- The first probe focused on the detection of iron II. This was achieved by preparing, in five chemical steps, a precursor of the firefly D-luciferin featuring an iron II binding component. Upon binding of iron on this moiety and a subsequent reaction with oxygen, the release of the precursor of the firefly luciferin can then undergo a reaction with D-cysteine to give D-luciferin. Then, in the presence of the firefly luciferase this substrate would lead to the production of light and thus to a tool capable of detecting iron II in biological media. In collaboration with an array of research teams, this probe was then subject of extensive validation studies. To illustrate the results obtained, part of the conclusion from Masroor Ahmed Khan dissertation could be quoted here:*" In conclusion, probe 4 exhibited a clear iron(II) response in vitro and was able to qualitatively detect even endogenous levels of iron(II) in cell lysates and live cells. However, high variability of that response, caused by the multitude of factors and interferents, exacerbated by the split-luciferin design that multiplies the complexity of transformations leading to signal generation, all compromise its cellular applicability*." Indeed, these results are an illustration of the ups and down one can encounter in the course of the design of biological tools useful for studies *in cellulo* and even more difficult, *in vivo*.

- The second set of probes was based on the same principle with the design of a precursors of the firefly D-luciferin featuring nitroreductase-sensitive components. Two approaches were undertaken, the classic one in which the nitroreductase-sensitive component was attached to 6-hydroxy-2-cyanobenzothiazole and an original one in which the nitroreductase-sensitive component was attached to D-cysteine. In both cases, upon the action of the nitroreductase, the release of these precursors allowed them to react with their respective complement to give the D-luciferin and thus provide a light signal. The second probe was assessed *in vitro* and as stated in the dissertation "Compound 10 demonstrated a clear and specific response to NTR activity in the split-luciferin assay."

- A third project, also focusing on the detection of nitroreductase, involved the use of furimazine, a luciferin completely different from the firefly D-luciferin with a far more complex synthesis. However, the very high intensity reported for the bioluminescence obtained with furimazine and the luciferase, derived from the shrimp *Oplophorus gracilirostris*, NanoLuc provided a very good intensive to work with this system. As stated in the dissertation "considerable amounts of optimizations were required" and in at least ten synthetic steps, two nitroreductase-sensitive probes were obtained following these optimizations. Unfortunately, by the end of this difficult work, one of the probes was reported by a different research group (see: *Shi, X. et al. An Efficient Probe for Bacterial Nitroreductase Imaging and Detection Based on NanoLuc-Furimazine Bioluminescent Pair. Chinese J. Chem. 42, 1373–1380 (2024)*).

- Then, the research focused on the design of "dual" probes which would lead to a luciferin and thus to a light signal, only upon the successive action of two distinct enzymes. This approach provided a D-aminoluciferin-derived probe as well as a D-luciferin-derived one. Each of them were prepared in six synthetic steps with some requiring a degree of optimizations. Concerning their bioluminescence properties, if the second one does not appear to have been evaluated yet, the first one is indeed sensitive to the action of nitroreductase followed by gamma-glutamyltransferase and only then lead to the occurrence of D-aminoluciferin and thus, in the presence of a firefly luciferase, to a light signal.

This concluded the PhD project which succeeded in the design and synthesis of seven different probes, six of them were then demonstrated to be effective in detecting their biologically relevant targets, at the least in *in vitro* experiments. In doing so, Masroor Ahmed Khan certainly acquired many research skills as well as extensive insights in the difficulties (and sometime vagaries) of organic chemistry which indeed remains an experimental science.

Concerning the manuscript itself, the text succeeds in, describing the aims of the projects in, providing comments and hypothesis explaining the difficulties encountered as well as in giving insights in the approaches adopted to solve them. One statement, on page 56, on the respective nucleophilic power of an amine and the potassium salt of a carboxylic acid will certainly be at the source of an uncomfortable question from this jury but this is, in my opinion, the only (mild) problem with this dissertation. Moreover, and as for every PhD report, few typos as well as some rather awkward sentences were noticed but contrary the French system, in which the official version of a PhD dissertation will have extensively benefited from the jury attention, the Polish rules insist on retaining the original version. In the present case, this is not really a

problem and indeed, history as well as the next generation of young chemists will be able to see how much stressed their PhD advisors were when they wrote their dissertation. In the present case the sentence "*The solvent was evaporated and the crude was mixture was purified via RP-PLC*" will only illustrate some of the possible results of long hours spent on typing such text!

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 allows it to further steps in PhD defense process.

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