Lodz University of Technology
Institute of Organic Chemistry

Evaluation of PhD Thesis
"DESIGN, SYNTHESIS, AND BIOLOGICAL INVESTIGATION OF NEW PEPTIDES AND PEPTIDOMIMETICS OF COSMECEUTICAL INTEREST"

Presented by: **Patrycja Ledwoń**

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The aim of the Doctoral Thesis was to design and prepare new inhibitors of human neutrophil elastase and tyrosinase, potentially useful as anti-wrinkle and anti-hyperchromic cosmeceuticals. This topic is important for all members of human population, without any exception, determining their personal wellbeing, and when excessive, leading to pathological processes. To achieve the goal already known small organic molecules possessing activity against elastase (1,2,4-triazoles) and tyrosinase (thiosemicarbazones) respectively were coupled with carefully selected peptides expecting that this transformation improve activity and the penetration of the conjugates into the *stratum corneum.* The strategy based on application of peptides as modifiers of the leading active structure is inventive because diversified properties of biocompatible amino acid residues decrease the toxicity and enable composition of desired physiological properties of conjugates but the danger of excessive enzymatic degradation should not to be ignored. A simultaneous search for inhibitors of two completely different enzymatic processes is rather unusual approach, but in this case it is justified by similarity of synthetic pathways leading to conjugates and analogous procedures used in structure-activity studies.

The structure of the Dissertation in most of the cases is in agreement with the classic recommendations. The chapters are comprehensive and proportional. The thesis is divided into 7 chapters. Following lists of abbreviation, figures, schemes, tables, discussed compound and abstract the literature review, the Chapter 2 dedicated to literature review is presented. Its structure is not following scheme typical for review publications, with exactly defined single theme, but consist the survey of multidisciplinary literature data focused on successful designing the pathway leading to the main goal of the thesis.

The Literature section presents a comprehensive and detailed background for future studies. It is composed from 3 parts. The first one is presenting potential of peptides used as cosmeceuticals. The second one is dedicated to the collagen degradation and is presenting representative groups of elastase inhibitors. As mentioned above, this fragment is not simply presenting the huge number of highly diversified structures already described inhibitors but consists the compilation of literature information suitable for successful reaching the main goal of the Thesis. The third fragment of literature chapter is devoted respectively to presentation of the structure and activity of tyrosinase and its inhibitors. The introductions to all three parts are adequately long and complex and the research question are clearly formulated.

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The confusing is however, diversified pattern of presentation of structural formulas of the sets of homologues structures. The uniform arrangement of the core fragment of formula surely facilitate perceiving by the readers what is crucial for understanding the nature of this structure-relations. Not complete is the list of only two categories of inhibitors presented on page 36. The omitted remained "suicide inhibitors" and inhibitors interacting outside of active cavity of enzymes but forcing so strong modification of catalytic fragment that function of enzyme is not possible. The existence of alternative binding modes and receptor flexibility was documented by X-ray crystallography and NMR spectroscopy (Appelt et al., *J. Med. Chem.***1991**, *34*(7) 1925-1934). The following reference was omitted: B. Donarska, K.Z. Łączkowski, Recent advances in the development of elastase inhibitors, *Future Med. Chem.* **2020**, *12*(20) 1809-1813, doi: 10.4155/fmc-2020-0163. Problematic is also information presented on page 37: "In a control group, 900 % loss of cellular matrix is accompanied….by further increase of 800% within next week" and on page 58, where Table 8 presents R substituents of acetophenone fragment as formulas without defining attachment points.

The Experimental part of the thesis is presented in Chapter 4. In my opinion its structure should be remodeled. Sub-fragment 4.1 should be either transferred to the Chapter 3: **Aims and objectives**" or as an Introduction to the "Chapter 5: "**Results and discussion**". All other fragments of **Experimental part** are wholly informative and confirm the proficiency and experience in laboratory experiments. The methods are clearly defined and their use is justified. Compounds described in the Thesis were obtained with up-to-date techniques. Appropriate analytical methods: HPLC, LC/MS, ^{1}H- and ^{13}C-NMR, confirmed their purity and structure. The biological activity of synthesized compounds was carefully evaluated by *in vitro* assays. The conformation, spatial structure and interaction with the target enzymes was studied by CD, molecular modeling and docking and biological activity of synthesized compounds determined by *in vitro* assays. The results are reliable and of high scientific relevance, which was already confirmed by the publication of them in international peer-reviewed journals characterized by high for this discipline Impact Factors.

Results and discussion: Chapter 5 presents the answer to the research question formulated in the **Aim of the work** and in **Introduction**. The most interesting are results of evaluation of the biological activity of synthesized compounds by *in vitro* assays presented in two independent parts. In the first one, focused on the search for elastase inhibitors, 1,2,4-triazole building blocks based on *Safirinium* fluorescent dyes and their conjugates with pentapeptides MGKVV and/or PGKVV were examined. The prepared compounds presented several interesting structural and physiochemical properties such as improved solubility promoted by the hydrophilicity of the peptide chain and the presence of charged quaternary nitrogen atom, the presence of functional groups offering further modification of structure, and fluorescence, so beneficial in biological studies and bioimaging experiments. However, the contrary to expectations, most of obtained compounds not exhibited significant inhibitory activity or were totally inactive in experiments with Porcine Pancreas Elastase. The only exception was Fmoc deprotected compound **5a** designated as H-Lys[Nε**(5)**]-OH exhibiting 25% of inhibition at 50 μM concentration. It is worthy to underline, that this observation confirmed the relevance of lysine residue in conjugates since the core compound **5** alone was found inactive.

The second part of Chapter 5 is focused on the search of tyrosinase inhibitors. The starting point in this search was already known thiosemicarbazone motif. Three analogues selected after careful analysis of structure-activity relations reported in the literature, denoted as in thesis as **TSC 43-45**, were decorated with tripeptides Phe-Xxx-Tyr, with Xxx limited to 3 coded aromatic amino acid residue (excluding His). All 9 conjugates and 3 thiosemicarbazones designed for the studies inhibited tyrosinase activity. Four of them were substantially more potent then kojic acid, used as the standard in the assay. The less active was **TSC 44** and its conjugates, what was justified by the bulk of fragment derived from benzophenone.

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To understand the nature of influence of thiosemicarbazone conjugates on melanogenesis the studies on proliferation of B16F0 murine melanoma cell line were undertaken. The results shown, for most of the compounds studied, that effect on cell proliferation dominate on the inhibition of melanogenesis. The most interesting is the attentive discussion of this outcome by Defendant presenting complexity of the problem.
Page 120, replace **TSC 43** by **TSC 44**.
Questions to be discussed with Defendant are as follow:
1). It has been noted the absence of the explanation what is the reason of rather low yield obtained in the syntheses of conjugates of peptides with 1,2,4-triazoles (as well as with thiosemicarbazones) on the solid support. In the syntheses of elastase inhibitors no attempts to explain the cause of this is presented. In the second case, problems with purification of thiosemicarbazones conjugates is offered as the only explanation. The question is as follow: could it happen, that intensively hydrophobic products with electron deficient aromatic rings are binding to aromatic core of the resin by π stacking, so strongly, that final products of syntheses were just not eluted wholly from the resin under standard synthetic procedure?
2). Why the electron withdrawing effect of carboxylic group attached directly *via* phenyl ring to the thiosemicarbazone moiety in **43** and **45** and their derivatives respectively was neglected as the factor influencing inhibitory activity?
Conclusion: The thesis as a whole is an original in its concept, multifaceted, presents a comprehensive approach to the research problems and provided very interesting results which give new insight into innovative cosmeceuticals. The way of preparing the thesis, the presentation and interpretation of the results, as well as rising new research questions and opportunities proves that the PhD candidate has not only extensive knowledge concerning the subject of research but also analytical skill and ability to precisely evaluate results of own work. Altogether 42 compounds were synthesized, purified, characterized, and evaluated towards their bioactivity as enzymes inhibitors. The work contain comprehensive discussion of the methods and results. The obtained results are compared with the literature data and are clearly conclusive.
In my opinion, the reviewed doctoral dissertation meets all the requirements of the Art. 187 on Scientific Degrees and Academic Titles and Degrees, from 20 July 2018 (Journal of Laws of 2021, item 478). In view of the above, I hereby apply to the High Council of the Discipline Chemical Sciences, Wrocław University of Sciences and Technology (Politechnika Wrocławska) to admit M.Sc. Patrycja Ledwoń to further stages of the doctoral procedure.
In recognition of the importance, wide range of research and valuable, original results, I recommend honoring of the thesis.

W mojej opinii przedstawiona do recenzji rozprawa doktorska spełnia wszelkie wymagania określone w artykule 187 ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz.U. z dnia 2021 r. pozycja 478 z późniejszymi zmianami). W związku z powyższym, Radzie Dyscypliny Naukowej Nauki Chemiczne Politechniki wniosek o dopuszczenie mgr Patrycji Ledwoń do dalszych etapów przewodu doktorskiego.
Jednocześnie, w uznaniu szerokiego zakresu badań i wartościowych, oryginalnych wyników, rekomenduję wyróżnienie rozprawy.

Date: 03/04/2023

Signature:
Zbigniew Kamiński

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