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Review on the Doctoral Thesis of Jan Hájek: "Structural variability of cyclic cyanobacterial lipopeptides: their biosynthesis and features affecting their bioactivity".

Cyanobacteria are rich sources of bioactive small molecules. Their secondary metabolites impact human life both positively (drugs) and negatively (toxins). Discovery, structure elucidation, bioactivity evaluation and biosynthesis investigations are current areas of dynamic research in the field of natural products, and cyanobacteria are no exception. However, despite the impact that some of these cyanobacterial metabolites have had in fundamental and applied knowldege, the body of work on cyanobacterial natural products is dwarved by those on other organisms such as actinobacteria or fungi. Several reasons may explain this, including the difficulty/time needed to grow these organisms to large scales and our inability to genetically manipulate most of them. There is, therefore, much more to be known and discovered from these organisms, namely new structures, new biochemical transformations in their biosynthesis and potent bioactivites.

In the thesis presented by the candidate Jan Hájek, the author summarizes their contributions to the field of cyanobacterial natural products, in particular the detection and structural analysis of lipopeptide from these organisms. The first part of the thesis corresponds to an introduction to lipopetides, in particular those from cyanobacteria, mostly focused on structural diversity (and quite exhaustive at it) but also covering biological activity/function and distribution. A second part of the thesis provides a general discussion on the findings and implications of the contributions in the thesis, while the third part comprises the four different contributions, which are original and have all been published in peer-reviewed publications and span a period of eight years (2014-2021).

Jan Hájek contributed to the four experimental studies mostly from an analytical chemistry perspective, detecting/discovering and performing MS/MS-based structure elucidation of several new variants of cyanobacterial lipopetides. In addition, Jan Hájek has also performed semi-synthesis of several lipopeptide analogs and tested their biological activity. In the first contribution (Paper I), a series of new puwainaphycins were detected and characterized as part of a genome mining effort that revealed the biosynthetic gene cluster associated with these metabolites. The analytical and structural characterization effort was critical to understand the function of several of the enzymes/enzymatic domains acting in the biosynthesis of the puwainaphycins, and therefore to extensively characterize how these molecules are put together inside cyanobacterial cells. This study likely motivated the second contribution (Paper II), which was idealized by the candidate, and reports on a new MS/MS based method to detect lipopeptide variants that contain a beta-amino-fatty acyl moiety. This ingenious strategy, based on higher-than-usual fragmentation energies for MS/MS analysis, proved very useful in revealing multiple new lipopetides and is an important addition to the scientific literature. By analyzing and characterizing the metabolomes and biosynthetic gene clusters of additional puwainaphycin/minutissamide-producing strains, Jan Hájek and co-authors have (in Paper III)

revealed that alternative starter units can be used (inclusively in the same strain) to expand the diversity of fatty acyl moieties in their lipopeptide reportoires. This contribution provides a great example of how lipopeptide biosynthetic pathways can evolve to generate chemical diversity. In the final experimental contribution (Paper IV), in which Jan Hájek is the co-first author, a structure activity relationship (SAR) study on pwas carried out using the puwainaphycin/minutissamide scaffold. This involved isolation of pure natural products, organic synthesis modification of their scaffolds and extensive biological activity testing. The study revealed important guidelines for modification of these and other lipopeptides.

In the introductory part of the thesis, Jan Hájek carries out an impressive, systematic, and extremely exhaustive review of cyanobacterial lipopeptide diversity (structural and biological activity). Moreover, the candidate devised a nomenclature system for the fatty acyl moieties of these compounds that allowed for the categorization of the lipopetides into discrete structural groups. This is extremely important since, traditionally, natural products are reported with trivial names and this sort of manual curation is required to provide an accurate representation of the chemical diversity of lipopetides within the phylum. This reviewer wishes to stress that this contribution is of high quality and, despite having noted that at least one particular group of lipopeptide was not included (hapalosins), considers that this work should be published in a peer-reviewed outlet of ample distribution. Such a publication will be very useful to both those in the field and those that are new to it.

The introductory part is important to more fully grasp the body of work presented in the four papers presented. The General Discussion is appropriate and places this body of work into context, while highlighting the major contributions of the candidate and their importance and implications for the field.

The work presented contains all the formal requirements to be considered a PhD thesis, is of high quality, is easy to read, contributes strongly to the knowledge in the field, cites appropriate references. It is also clear from this work that the candidate is knowledgeable in the topic and trained in different methodologies (from organic synthesis to bioinformatics), albeit with a strong focus on LC-HRESIMS/MS analytical chemistry.

I have the following questions for the discussion:

1. Taking into account the diversity of cyanobacterial lipopeptides and associated biosynthetic pathways, how confident can one be when reporting structures of lipopeptides based on HRESIMSn analysis, using one or even more than one reference compounds? In particular, positional assignments of additional (vs. reference) CH_2 (14 a.m.u.) units, Cl atoms, olefins, hydroxyl groups or amino groups, or even subtraction of such groups?

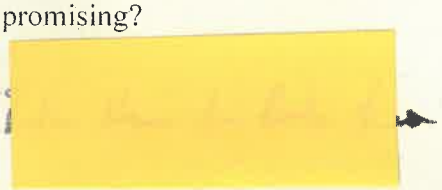
2. What are the biggest advantages of having a specific nomenclature system dedicated to lipopeptide fatty acyl moieties, when compared to the already existing IUPAC

nomenclature? Would a nomenclature system such as the one proposed in this thesis be easily incorporated in chemoinformatics workflows?

3. In light of the advances brought about by GNPS or other molecular networking/metabolomics approaches, as well as MS/MS spectra annotation software, which advantages would you say the LC-HRESIMS/MS method reported in this thesis has for detection of lipopetides in screening of cyanobacteria (or other organisms) extracts? Could the identification of immonium ions formed from fragmentation of novel lipopeptide scaffolds with a beta-amino-fatty acyl moiety be automated in MS analysis workflows?

4. There is considerable variation, for some groups of lipopeptides, like the puwainaphycins/minutissamides, on the decorations present in the fatty acyl moiety – what could be speculated regarding the role of fatty acid halogenation and oxygenation?

5. Regarding the SAR study, which implications would it have for future medicinal chemistry of other cyanobacterial lipopeptide scaffolds? Which scaffolds/moieties would be more ameanable for modification? Which scaffolds would be more promising?



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