School of Doctoral Studies in Biological Sciences
University of South Bohemia in České Budějovice
Faculty of Science

Structural variability of cyclic cyanobacterial lipopeptides: their biosynthesis and features affecting their bioactivity

Ph.D. Thesis

Jan Hájek

Supervisor: RNDr. Pavel Hrouzek, PhD.

Institute of Microbiology, Czech Academy of Sciences

České Budějovice 2021

This thesis should be cited as:

HÁJEK, J., 2021: Structural variability of cyclic cyanobacterial lipopeptides: their biosynthesis and features affecting their bioactivity. Ph.D. Thesis Series, No. 18. University of South Bohemia, Faculty of Science, School of Doctoral Studies in Biological Sciences, České Budějovice, Czech Republic, 170 pp.

Annotation

This thesis is devoted to the diversity of cyanobacterial cyclic lipopeptides and their bioactivity. Special class of cyclic cyanobacterial lipopeptides puwainaphycins and minutissamides was studied in terms of their structural diversity, biosynthesis and structure-activity relationship. Chemical diversity of observed variants in cyanobacterial strains was correlated with the genetic background for their biosynthesis from which the probable cores of biosynthesis were inferred. Biological activity (mainly the cytotoxicity and anti-fungal activity) of major natural puwainaphycin and minutissamide variants was studied in detail. Finally, semi-synthetic puwainaphycin and minutissamide variants were prepared in order to study the relationship between distinct functional moieties and their effect on the bioactivity of the lipopeptide.

Declaration

I hereby declare that I have written the presented thesis by myself using the literature provided in the list of references.

_____

Jan Hájek

České Budějovice

19.9.2021

This thesis originated from a partnership of **Faculty of Science, University of South Bohemia**, and **Institute of Microbiology AS CR**, supporting doctoral studies in the **Molecular and Cell Biology and Genetics** study programme.







Financial support

This work was supported by Czech Science Foundation grant no. 16-09381S (**Bioactive cyanobacterial lipopeptides: genome mining, detection, and structure-activity relationships**), and by the Ministry of Education, Youth and Sports of the Czech Republic, **National Program of Sustainability I**, ID: LO1416 project ALGAMIC (ID: CZ.1.05/2.1.00/19.0392). International cooperation was supported by Ministry of Regional Development of the Czech Republic—Cross-Border cooperation **Czech-Bavaria project no. 41.**

Acknowledgements

This work would be never finished without a huge help of many people. First I would like to thank my supervisor Pavel Hrouzek for his support and encouragement during all stages of my PhD studies, for all his knowledge and experiences he shared with me in all different fields of science and lab work. Similarly, I would like to thank also all my lab members from Algatech, especially Petra Urajová for our long discussions about MS data interpretation and Káťa Voráčová for the introduction into cell based assays. I would like to express my thanks to Jan Mareš for giving me an insight into the bioiformatics and genome mining. Least but not last, a major part of my PhD thesis was done in cooperation with TUM Straubing and I would never had such a nice results without Sebastian Bieringer and Herbert Riepl, thank you so much for the introduction into the chemical modification!
Further, the study was supported by the collaboration with Technical University of Munich, Campus Straubing.



List of scientific publication and the author's contribution (ISO 690)

The thesis is based mainly on the following papers (listed chronologically):

I. MAREŠ, J., **HÁJEK, J.**, URAJOVÁ, P., KOPECKÝ, J. and HROUZEK, P. A Hybrid Non-Ribosomal Peptide/Polyketide Synthetase Containing Fatty-Acyl Ligase (FAAL) Synthesizes the β-Amino Fatty Acid Lipopeptides Puwainaphycins in the Cyanobacterium *Cylindrospermum alatosporum*. *PLoS One*, 2014, 9(11), e111904. **IF 3.234** (2014)

JH performed cultivation of cyanobacterial strain, extraction of lipopeptides from the biomass and their structural analysis using HPLC-HRMS. He also purified Min A for NMR analysis and assisted in the data interpretation. (30%)

II. URAJOVÁ, P., **HÁJEK, J.**, WAHLSTEN, M., JOKELA, J., GALICA, T., FEWER, D.P., KUST, A, ZAPOMĚLOVÁ-KOZLÍKOVA, E., DELAWSKÁ, K., SIVONEN, K. and HROUZEK, P.: A liquid chromatography-mass spectrometric method for the detection of cyclic beta-amino fatty acid lipopeptides. *Journal of Chromatography A*, 2016, 1438, 76-83. **IF 3.981** (2016)

JH proposed a new HPLC-HRMS method for universal detection of ß-amino containing lipopeptides using 2 different fragmentation energies, this method was further tested and validated by Petra Urajová. (50%)

III. MAREŠ, J., **HÁJEK, J.**, URAJOVÁ, P., KUST, A., JOKELA, J., SAURAV, K., GALICA, T., ČAPKOVÁ, K., MATTILA, A., HAAPANIEMI, E., PERMI, P., MYSTERUD, I., SKULBERG, O.M., KARLSEN, J., FEWER, D.P., SIVONEN, K., TONNESEN, H.H. and HROUZEK, P. Alternative Biosynthetic Starter Units Enhance the Structural Diversity of Cyanobacterial Lipopeptides. *Applied and Environmental Microbiology*, 2019, 85(4), e02675-18. **IF 4.016** (2019)

Using HPLC-HRMS, JH identified puwainaphycins and minutissamides in 6 cyanobacterial strain under study and performed structural analysis of all 95 detected variants. (30%)

IV. **HÁJEK, J.**, BIERINGER, S., VORÁČOVÁ, K., MACHO, M., SAURAV, K., DELAWSKÁ, K. FIŠER, R., MIKUŠOVÁ, G., CHEEL, J. FEWER, D.P., VU, D.L., PAICHLOVÁ, J., RIEPL, H. and HROUZEK, P. Semi-synthetic puwainaphycin/minutissamide cyclic lipopeptides with improved antifungal activity and limited cytotoxicity. *RCS Advances*, 2021,**11**, 30873-30886 **IF 3.361** (2021)

JH cultivated, isolated and purified 6 variants of puwainaphycins and minutissamides and tested their cytotoxic activity on HeLa cells using MTT assays and LDH assays. Additionally, he assisted Sebastian Bieringer in chemical modification of Min A and Min C and synthesis of new cyclic lipopeptides with prolonged and branched fatty acid chain and 4 different diesters of Min A. He also contributed to conceptualization of the study. (60%)

Co-author agreement

The corresponding authors of the publications listed in the scientific publication section acknowledge the contribution of Jan Hajek as stated above:

_____

RNDr. Pavel Hrouzek, Ph.D.

_____

Prof. Dr. Herbert Riepl

Table of Contents

PART I: INTRODUCTION **1**

Cyanobacteria as a producer of secondary metabolites 1
Nonribosomal peptide synthetases 4
NRPS-domains *5*
Core biosynthetic domains 5
Tailoring domains and tailoring enzymes 7
Polyketide synthases 9
Cyclic cyanobacterial lipopeptides 10
Amino acid composition *11*
Fatty acid chain variation *12*
Cyanobacterial cyclic lipopeptide classes 15
S-class lipopeptides: fatty acid attached as a side group 15
Class S-1 (cyanopeptolin type) 16
Class S-2 (hassallidin type) 18
O Classes and N Classes lipopeptides: fatty acid incorporated in the core cycle 20
O Classes: Cyanobacterial cyclic lipopeptide with β-hydroxy-fatty acids 23
Class O-1 (yanucamide type) 23
Class O-2 (veraguamides, palmyramides and similar) 24
Class O-3 (cocosamides etc.) 36
Class O-4 (dithiazole type) 39
N Classes: Cyanobacterial cyclic lipopeptide with β-amino-fatty acids 43
Class N-1 (anabaenolysin type) 44
Class N-2 (porpoisamides, malevamides etc.) 46
Class N-3 (muscotoxins) 53
Class N-4 (minutissamide type) 58
Class N-5 (laxaphycins) 62
General remarks on the cyanobacterial cyclic lipopeptide toxicity 70

PART II: GENERAL DISCUSSION **78**

Structural variability and biosynthesis of minutissamides and puwainaphycins. 79
Cytotoxicity and other bioactivities of minutissamides and puwainaphycins 86
Summary 92

ABBREVIATIONS **93**

BIBLIOGRAPHY (ISO 690) **95**

PART III: PUBLICATIONS **109**

Paper I: A Hybrid Non-Ribosomal Peptide/Polyketide Synthetase Containing Fatty-Acyl Ligase (FAAL) Synthesizes the b- Amino Fatty Acid Lipopeptides Puwainaphycins in the Cyanobacterium *Cylindrospermum alantosporum*. 111
Paper II: A liquid chromatography-mass spectrometric method for the detection of cyclic b-amino fatty acid lipopeptides 123
Paper III: Alternative Biosynthetic Starter Units Enhance the Structural Diversity of Cyanobacterial Lipopeptide 133
Paper IV: Semi-synthetic puwainaphycin/minutissamide cyclic lipopeptides with improved antifungal activity and limited cytotoxicity 151

CURRICULUM VITAE **167**

Part I: Introduction

Cyanobacteria as secondary metabolites producers

Cyanobacteria are one of the most widespread organisms in the world. They represent a diverse and highly specialized group of organisms well adapted to almost all environmental conditions. They can be found in various ecosystems including freshwater and marine phytoplankton and benthos, terrestrial environments such as microbial soil crusts, and also take part in symbioses with various other organisms[1].

Cyanobacteria have been studied for their unique phylogenetic position in the tree of life, mechanism of photosynthesis, ability to fix nitrogen, and further for their production of unique secondary metabolites. Secondary metabolites are considered as not being essential for the normal growth and function, but to improve properties of the producing organism and provide benefits in surviving competition with other organisms. Some of them are applicable in medicine and biotechnology as antibacterial[2], antifungal[3], anticancer[4], cholesterol- and triglyceride-lowering[5], immunosuppressive[6], antiparasitic[7], and diagnostic agents. Nonetheless, the exact biochemical and physiological role and mechanism of action of the majority of these secondary metabolites still remains unknown[8].

Cyanobacterial secondary metabolites are commonly divided into structural classes related to their biosynthetic pathways, although this classification has a strong limitation due to the fact that some secondary metabolites contain building blocks from different biosynthetic pathways. The common classes are alkaloids[9,10], terpenoids[11,12], various heterocyclic structures[13], and finally the largest groups consisting of polyketides and non-ribosomal peptides[14,15]. As mentioned above, the building blocks of synthetic pathways occur in all classes (especially peptide-coding blocks). The majority of the cyanobacterial secondary metabolites are peptides or

compounds containing peptide substructures. Up to date more than 600 different peptides or peptidic metabolites[16] have been described from various taxa of cyanobacteria.

While some of these peptides are biosynthesized via standard ribosomal translation (e.g. microviridins[17]), their major portion is produced via two unique pathways. First, the non-ribosomal peptide synthetase pathway (NRPS) is responsible for the creation of the majority of peptides and operates at the nucleic acid-free level solely by the action of peptide synthetases[18]. The second pathway, polyketide synthase (PKS), creates linear and cyclic polyketones (including macrolides and derived compounds[19]). Both of those pathways are often combined into a hybrid NRPS/PKS pathways[14] resulting in structures that are not achievable by usual metabolic pathways. An example of such a compound produced by this hybrid NRPS/PKS pathway are, among many others, cyanobacterial cyclic lipopeptides. NRPS, PKS, as well as NRPS/PKS clusters are found in the genomes of many organism including bacteria, archaea, and eukarya[20].

Since the discovery of cyclic lipopeptides they have attracted the attention of researchers because of their broad bioactivities and possible environmental impact[21]. While the majority of the lipopeptides isolated from cyanobacteria are cytotoxic (e.g. lyngbyabellins[22], muscotoxins[23], pitipeptolides[24], puwainaphycins[25]), many other possess antifungal activities (e.g. Calophycin[26], hassalidins[27], laxaphycins[28]). Rarely, cyanobacteria also produce lipopeptides with antibacterial activities (e.g. tiahuramides[29], lyngbyazothrins[30]).

Generally, the structure of cyclic lipopeptides is cyclized via peptide or ester bonds and consists of a peptidic part and a longer or shorter acyl chain which results in the amphiphilic nature of the molecule. The non-ribosomal origin of lipopeptide is responsible for a broad variability of structures and allows the incorporation and modification of rare amino acids as well as the incorporation of various fatty acid moieties.

The variability of the lipopeptide structures, the composition of amino acids, their modification as well as unsaturation, substitution, and the length and branching of the fatty acid chain are responsible for a broad range of biological actions. Lipopeptides mostly exert a biological effect connected for example to membrane permeabilization[51,70], protein interaction or inhibition[32] and actin reorganization[22] .

The interaction of lipopeptides with biological membranes is the most deeply studied lipopeptide mode of action. Based on research performed on bacterial cyclic lipopeptides (namely in Daptomycin[33], Viscosin[34] and Iturin[35]), the mode of action is mainly based on interaction with different cell membranes via the aliphatic part of the molecule followed by changes in biological and physico-chemical properties of the membrane. Alteration in the fluidity, stiffness, and curvature of the membrane causes permeabilization and solubilization of the membrane, and very fast cell death[33,35].

Additionally, the aggregation of cyclic lipopeptide in the biological and artificial membrane is described for several compounds and can lead to self-assembly and the formation of multimeric structures. Iturin A, a potent antifungal lipopeptide, forms after its aggregation transmembrane pores with an average diameter of 32 Å[36]. These conductive pores facilitate the leakage of ions, mostly K^+ and Ca^{2+}[35] through the membrane.

While the biological effects of many of bacterial cyclic lipopeptides are well described and some of them are used in clinics (e.g. bacterial lipopeptide Daptomycin is used under the commercial name Cubicin© as a new class of antibiotics against Gram-positive organisms[33]); the cyclic lipopeptides from cyanobacteria remain understudied. Further, the exact structure-function relationship remains unknown for many bacterial as well as cyanobacterial cyclic lipopeptides. In this thesis I will focus on the variability of the cyclic cyanobacterial lipopeptide, their structural types and biological activities.

Nonribosomal peptide synthetases

Nonribosomal peptide synthetases (NRPS) are large multifunctional protein complexes that incorporate free amino acid (both proteinogenic and rare) without the need for a nucleic-acid template[18,37]. The specific process of condensation of two amino acids (or structurally related organic acids) while forming a new (mostly peptide) bond is directed and regulated by the enzyme itself. Individual steps of the amino acid addition to the molecule are catalyzed by an adequate biosynthetic module. Each module is composed of several catalytic domains separated by short linkers (up to 15 amino acids). The minimal length of genetic sequence for a single module is around 3–3.5 kbp. The modules are assembled to form large multifunctional proteins. As a quite substantial number of modules can be embedded in one ORF, NPRS genes are one of the largest known genes not only in the cyanobacterial world[31,37].

So-called core biosynthetic domains are responsible for the peptide chain elongation. Namely the adenylation domain (A-domain) recognizes and activates specific residues, the thiolation domain or peptidyl carrier protein (T-domain or PCP) binds the nascent peptide and transfers it between the individual domains, and the condensation domain (C-domain) catalyzes the formation of peptide/ester bond[20]. At least one module with core biosynthetic domains is obligatory for each NPRS. Additionally, the thioesterase domain as the last core biosynthetic domain is present mostly in the very last module and releases the biosynthesized peptide in the form of a cyclic or linear structure.

Tailoring domains and tailoring enzymes further modify the biosynthesized structure by oxidation, epimerization, internal cyclisation, or

other substitution. These enzymes can be part of the NRPS (tailoring domains) or co-translated as individual ORFs (tailoring enzymes).

Individual NRPS genes are assembled together to form a biosynthetic gene clusters, which allows the bioinformatic prediction of the hypothetical NRPS product.

NRPS-domains

Core biosynthetic domains

Core biosynthetic domains handle single amino acids and elongate the nascent peptide with additional building blocks, while ATP is consumed. Typical possible building blocks are common or rare amino acids (amino-fatty acid can be also comprehended as rare amino acid), imino acids (OH-Pro), hydroxy acid (HIVa, hydroxy-fatty acids etc.), as well as carboxylic acids.

The adenylation domains comprising of about 550 amino acids[37] are keystones of non-ribosomal peptide diversity and recognize and activate substrates required for their further incorporation into peptides. The domain is specific for each single amino/imino/hydroxy/carboxylic acid (or a very limited set of similar building blocks) and therefore the order of modules/adenylation domains in NRPS determines the primary structure of the whole peptide[16]. The selectivity of an adenylation domain is caused by the geometry of a binding pocket that allows only a specific substrate to enter into the catalytic site. Consequently some domains can incorporate slightly different substrates into the nascent peptide (as known e.g. for NRPS/PKS of Puwainaphycin F/G, where the adenylation domain activates Gln as well as Asn[15]). Up to now about 200 adenylation domains have been identified in cyanobacterial genes[16].

The biosynthetic function of thiolation domains (sometimes called PCP, peptidyl carrier protein) resides in the transfer of activated substrate from the adenylation domain to the condensation domain. In contrast to adenylation domains, thiolation domains are highly conserved across known NPRS. This very small domain of about 80 amino acids[37] includes a cofactor, 4′-phosphopantetheine, acting as a "swinging arm"[16]. This cofactor, covalently bound to serine residue[38], possesses a free thiol group and thus allows the binding of activated carboxyl-containing substrates. Further, thiolation domains are required for the interaction of the reaction intermediate with tailoring domains[39].

The module is terminated by a condensation domain of about 450 amino acids[37]. The mechanism of the action is analogous to general ribosomal synthesis and the ribosomal condensation process. Briefly, domains catalyze the nucleophilic attack of an amino/imino/hydroxy side group of activated substrate with nascent product, forming a peptide or ester bond. Currently more than 160 condensation domains are described[16].

Thioesterase domains (TE) are attached to the core biosynthetic domains[37] of the very last module and are considered as a termination domain for the whole non-ribosomal synthetic pathway. Thioesterase domains are also responsible for the final release and/or cyclisation of non-ribosomal peptides. The release of the peptide from NRPS enzyme is achieved via two separate reactions. At first, peptidyl-O-TE is formed, followed by a nucleophilic attack by water or the peptide itself[40]. The nucleophilic attack by water results in a linear peptide, the attack by a side group of biosynthesized peptide leads to cyclisation and the formation of cyclic peptides.

Tailoring domains and tailoring enzymes

As mentioned above, tailoring domains and tailoring enzymes are not obligatory parts of NRPS, but they are present in the majority of NRPS and rapidly increase the structural variability of cyanobacterial secondary metabolites through forming structures not accessible via conservative pathways. Tailoring domains are placed mostly between the thiolation and condensation domain, thus the selective modification of one substrate is feasible. Similarly, tailoring enzymes as single enzymes/enzymatic complexes are attached externally between the thiolation and condensation domain of the module.

Heterocyclization domains (often called simply cyclisation domains) catalyze the internal cyclisation of cysteine, serine or threonine residue, while forming thiazoline or oxazoline rings. Peptides containing heterocycles are common among almost all cyanobacterial strains, although the formation of a thiazole heterocycle is not restricted only to the non-ribosomal synthetic pathways (as revealed for ribosomally synthesized and post-translationally modified peptides, e.g. cyclic thiazole-containing patellamide[41]).

The *N*-methylation domain catalyzes the transfer of the methyl group to the nitrogen atom of the peptide bond[42]. Especially in the structure of cyclic lipopeptides, multiple *N*-methylation is a common modification.

Epimerization domains that largely re-assemble terminal condensation domains are frequent in NRPS. Amino acid is epimerized while still attached to PCP, the equilibrium of the L/D stereoisomers remains until final condensation. The formation of the specific complex of epimerization and condensation domain is responsible for D- stereoisomer production[42]. Though some D-forms can be directly incorporated directly at the level of the

adenylation domain as discovered for microcystin biosynthesis where D-Glu is a direct precursor[43] and the specific adenylation domain is present.

Common domains modifying the non-ribosomal peptides are also the oxidation and reduction domains and the formyl-transferase domain[18].

The variability of NRPS cyanobacterial secondary metabolites is further increased by the presence of other cooperating (tailoring) enzymes. The most important are oxygenases, amidases, halogenases, and *O*-methyl transferases[18]. The screening of secondary metabolites for halogen atoms reveals the presence of chlorine in more than 22% structures[44], however, there is almost no information about the origin and mechanism of their substitution. Many secondary metabolites are chlorinated at the tyrosine moiety. Lipopeptides are often substituted with one (Puwainaphycin C[45]) or more (Lyngbyabellin[3, 46]) chlorine atom on the aliphatic chain. The mechanism of halogenation as studied in bacteria (e.g. Balhimycin[47] produced by *Amycolatopsis sp.*) assumes that chlorination is performed on a peptidyl carrier protein-bound intermediate[48]. Alternatively, free-standing $FADH_2$ dependent halogenases and non-heme iron halogenases are described[49]. Only a few NRPS genes with incorporated putative halogeneses are reported (e.g. BarB modules in cyanobacterial triple chlorinated barbamide[50]).

Bromination on the aliphatic fatty acid moiety is also reported (Veraguamide A[51]). Similarly, the hydroxylation of amino acids is described in some non-ribosomal peptides. Interestingly, specific domains responsible for oxygenase recruitment and binding are described in several NPRS[52].

Polyketide synthases

Polyketide synthases (PKS) are also a modular types of enzyme in which modules catalyze two-carbon linear extensions and keto-group processing reactions[53]. PKS enzymes typically involve β-carbonyl-processing catalytic domains (an interesting exception is the nostophycin gene cluster containing PKS with an α-carbonyl-processing domain[54]). Similar to NPRS, each PKS module contains several enzymatically active domains, namely an acyl carrier protein (ACP, functionally similar to PCP in NRPS), acyl transferase (AT, transferring activated acyl building blocks to ACP), and finally ketosynthase (KS, catalyzing the formation of a new carbon-carbon bond). The thioesterase domain releases again the biosynthesized polyketone from the PKS enzyme. Additionally, facultative domains present in the modules can modify the polyketide. These domains mainly interact with the ketone group. Ketoreductase (KR) facilitates the reduction of ketone to hydroxyl. The dehydratase domain (DH) forms a double bond via water elimination and acts immediately after the KR. The additional employment of the enoylreductase domain (ER) after the action of KR and DH leads to the biosynthesis of unsubstituted saturated carbon-carbon systems[53]. Last but not least, the aminotransferase domain (AmT) catalyzes the exchange of the ketone group with an amino group.

PKS play a crucial role in the biosynthesis and modification of fatty acids incorporated in cyanobacterial cyclic lipopeptides. The substitution of mainly *β*-carbon (alternatively other positions) of the fatty acid moiety by the amino or hydroxyl group is crucial to the formation of the second peptide or ester bond, respectively (as mentioned later).

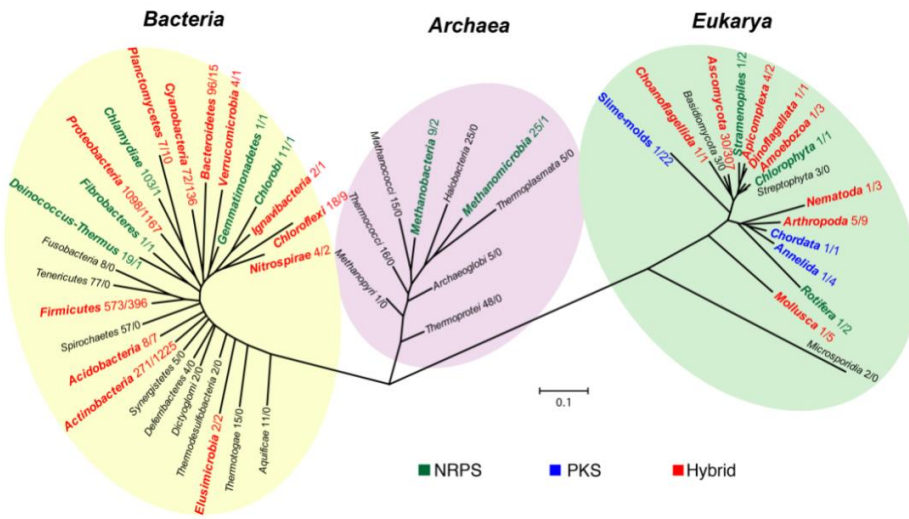


Figure 1: Distribution of NRPS, PKS and combined NRPS/PKS clusters in genomes of various organisms[20]

Cyclic cyanobacterial lipopeptides

As mentioned above, cyclic cyanobacterial lipopeptides are synthesized mostly by a unique biosynthetic pathway, combining NRPS/PKS elements. This mechanism allows formation of a huge variability and a wide range of different cyclic structures. Cyanobacterial lipopeptides share some similarities with structures produced by other bacterial groups[55] (e.g. the well-known powerful biosurfactant and antibiotic Surfactin[56]) and fungi (e.g. Isiradin with antimalarial and antibacterial activity[57]) or were found in other organisms such as molluscs (dolastatin 17 with anticancer properties[58]).[1]*

[1]* There is some evidence that compounds originally isolated from mollusc *Dolabella auricularia* are products of cyanobacteria (*Lyngbya majuscula*). However, it is proven that this sea hare can modify molecules originating in (symbiotic) red and brown algae and biosynthesize its own secondary metabolites, while using building blocks from the algal metabolism[139].

The majority of the cyclic cyanobacterial lipopeptides consist of a core structure containing both a common and rare amino acid and aliphatic lipidic chain. The peptide cycle is formed by at least 4 amino acid residues as in the case of anabaenolysins[59] up to 14 amino acid residues as found in malevamides[60,61] (larger cores are expected but not described yet).

According to the fatty acid position, two subgroups of cyclic cyanobacterial lipopeptides can be defined. The lipidic chain can be attached either as a side group to an exocyclic amino-acid via a simple peptide bond (as in case of cyanopeptolin[62] or hassallidin[63,64]) or can be inserted directly as part of the cyclic core structure (minutissamides[65] and many other) via two distinct peptides bonds or a peptide and an ester bond while forming a macrolactam and macrolactone ring, respectively.

For this review, the term "lipopeptide" describes a compound with at least 3 amino/imino/hydroxy acid residues bound into a cyclic structure via a peptide/ester bond or via forming a thiazole/oxazole ring. The term "fatty acid" is reserved for a carboxylic acid the in which the linear part consists of at least 5 carbons (thus is longer than Ile/Leu) and exhibits hydrophobic nonpolar properties typical for lipids[66][2*].

Amino acid composition

While cyanobacterial ribosomal peptides and proteins contains mostly unmodified common proteogenic amino acids and other amino acids are an exception (e.g. RIPPs), many rare and modified amino acids are involved in the NRPS/PKS biosynthesis of cyclic lipopeptides. Usual modifications are *N*-methylation (malevamide C[60] as tetradecapeptide consisting of a total 5

[2]* Based on this definition, microcystins and nostophycins should also be considered as lipopeptides. However, their assignment among peptides only is set by previous studies. Hence, microcystins and nostophycins are not included in this review.

N-methyl-amino acids, another is *O*-methylated), the dehydration of threonine to dehydrothreonine (Dhb), or chlorination (chloro-phenylalanine in cyanopeptolins[62]). Additionally, uncommon, unique, and rare amino acids can be found in the lipopeptide structures. Some of these amino acids are derived from the proteogenic (hSer, hPhe, *β*-methyl-Asp, OH-Pro, OH-Leu, OH-Asn, and β-amino-2-methylbutanoic acid based on Val) other variants present new structures unique to cyanobacteria, such as β-amino-6-hydroxy-2-piperidone (Ahopip) present in cyanopeptolins or 2-(β-amino-5-oxotetrahydrofuran-2-yl)-2-hydroxyacetic acid (AOFHa) found in anabaenolysins. Special case are hydroxy acids such as HIVa (2-hydroxyisovaleric acid) or HMPa (2-hydroxymethylprentanoic acid) present in cyclic peptides and cyclic lipopeptides (cocosamides[67] and many others). Moreover, the high abundance of neutral amino acids (Val, Leu/Ile, dhb, Gln/Asn) is significant, while the incorporation of basic amino acids is rare (one of the examples is Arg found in cyanopeptolins).

Fatty acid chain variation

Similar variability as observed in the amino acid composition can be found in the fatty acid moiety.

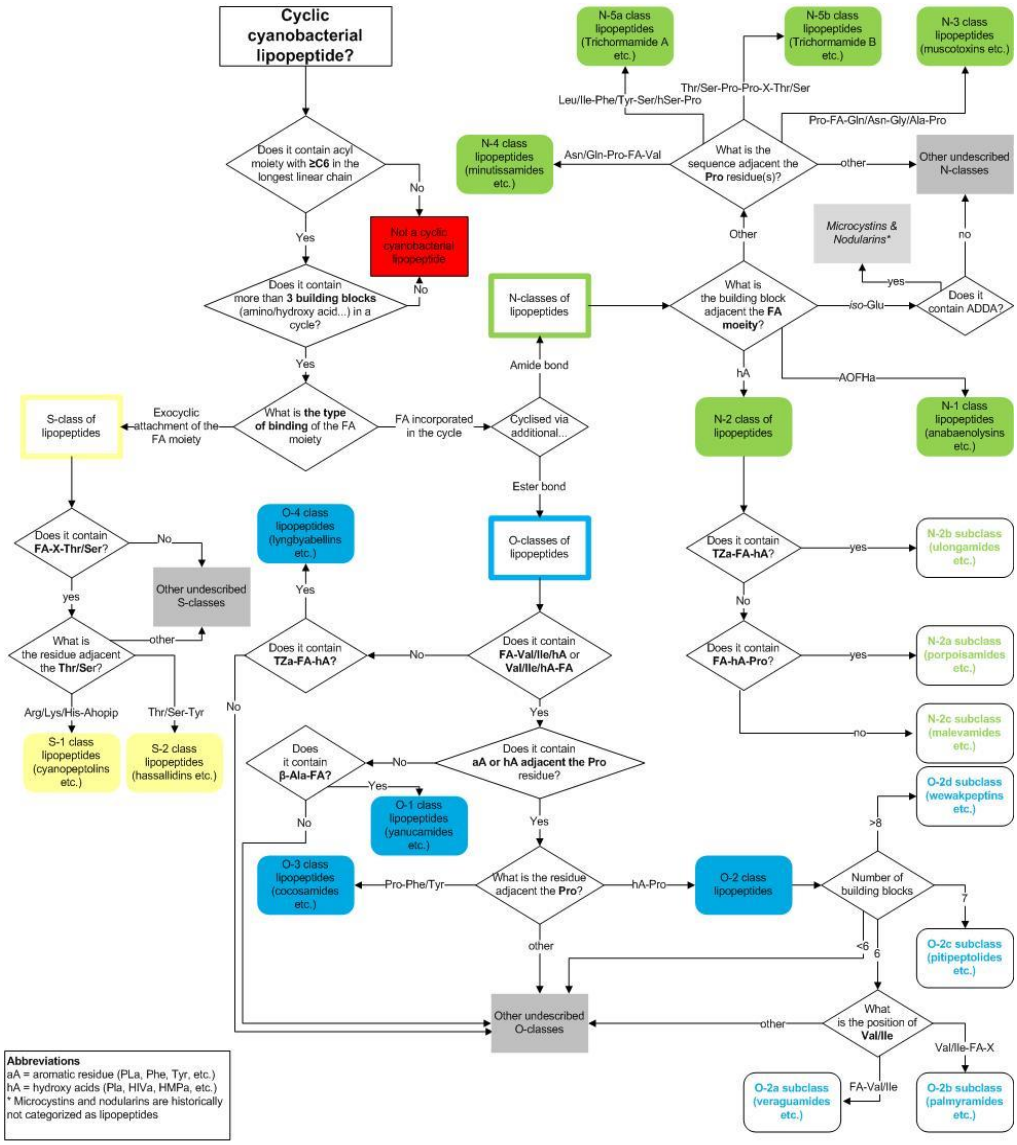
Generally, fatty acids present in cyclic cyanobacterial lipopeptides possess 5-18 carbons in their longest linear part, and facultative unsaturation and/or multiple substitution (including halogenation, methylation, hydroxylation, etc.) can occur. The hydroxylation or amination of β-carbon (or other carbon) is required for fatty acid moiety incorporation and second chemical bond formation. Also, FA α-carbon is frequently substituted, usually by the hydroxylation or methylation. Additionally, hydroxylated fatty acids can be further substituted with saccharides[27] or amino acids[68].

Lipopeptide nomenclature in this study

The lipopeptide nomenclature has yet to be established and thus lipopeptides with almost identical structures are described by various researchers under different names. Therefore, the establishment of universal nomenclature assuring the definition of lipopeptides classes and designation of their structural variants is highly demanding. Our approach to the creation of the novel nomenclature is motivated by the statement: *"Since a one-letter code is restricted to 26 amino acids, it is not applicable when residues like formic, acetic(…) acids, and others have to fit into the same scheme. In summary, we think that a simple but efficient naming system such as that used for microcystins is not applicable to most other peptide classes and that other systems have to be developed."*[16] Lipopeptides mentioned in this review are therefore described with the following rules and classified in specific classes based on their structure, number of residues, and the type of fatty acid. The trivial names of described lipopeptides were maintained in their original form, however, the description of their chemical structure follows the rules mentioned below. The number of residues is not defined by the number of peptide/ester bonds, but generally by the presence of characteristic building blocks (thiazole-containing peptides without peptide bonds or fatty acid moieties are also considered as residues), non-proteinogenic acids are described using unified nomenclature (e.g. Dhb as dehydrothreonine/dehydrobutyric acid) and finally, an incorporated fatty acid moiety is annotated using newly-established general coding (see below). Names for entire classes (puwainaphycins, etc.) are written with the initial letter in the lower case, names for specific compounds (Puwainaphycin A) with the initial letter in the upper case. The primary structure is expressed in abbreviated form starting with FA (fatty acid moiety), three letter code is used for the description of adjacent proteogenic and rare amino and hydroxy acids in COOH→NH_2/OH order. Letter code for non-proteogenic amino acids is listed in the abbreviation section and, X stands for variable residue(s). Cyclic structure is indicated by asterisk (e.g. *FA-X-X-Ala*-X stands for lipopeptide cyclized via FA-Ala bond).

Coding of lipid moiety

Because of the high variability in length, substitution, (un)saturation, and branching of the fatty acid moiety, the following nomenclature has been established in this work. For *β*-hydroxy-fatty acids, letter codes start with **H**, for *β*-amino-fatty acids letter codes start with **A**. The following small letter indicates substitution on the α-carbon. (**Hh** stands for *β*-hydroxy-α-hydroxy-fatty acids and **Hm** for *β*-hydroxy-α-methyl-fatty acids. If multiple substitution occurs, a lower index is added, e.g. Hm_2). The length of the fatty acid chain is defined using $\mathbf{C_x}$ standing for carbon and the number in lower index indicates the length of the longest linear carbon-carbon part (Hm_2C_{12} indicates *β*-hydroxy-α-dimethyldodecanoic acid). The suffix **aa, ea, or ya** shows the fatty acid (un)saturation. If not terminal, the position of the unsaturated bond is indicated in the lower index. Full coding $AhC_8e_{4,6}a$ stands for *β*-amino-α-hydroxy-oct-4,6-dienoic acid. Additional substitution is further expressed using prefixes such as 5-OH-AhC_8ya (*3*-amino-2,5-dihydroxy-octynoic acid).



For purpose of this flowchart, building block modification (methylation, hydroxylation, formylation...) do not play a role if not mention specifically

Figure 2: Classification of cyclic cyanobacterial lipopeptides used in this work. Each class and subclass is established using decision points based on the common motifs and/or structural similarities

Cyanobacterial cyclic lipopeptide classes

S-class lipopeptides: fatty acid attached as a side group

Attachment of the fatty acid as a peptide or protein side chain is a common mechanism of cyanobacterial posttranslational lipidation and acylation processes (e.g. palmitoylation of enzymes[69]). NRPS/PKS derived cyanobacterial cyclic lipopeptides are encoded by genetic clusters containing genes for fatty acid handling and/or biosynthesis. Moreover, in the majority of cases, the lipopeptide biosynthesis is started by lipoinitiation (the activation of the FA is chronologically the first biosynthetic step). NRPS/PKS lipopeptides with FA attached as a side chain are classified as S-class cyanobacterial cyclic lipopeptides (*S* stands for side-chained). Widely studied cyanopeptolins and hassallidins are typical representatives.

Fatty acids found in the S-class lipopeptides are present mainly as saturated aliphatic chains. A new chemical bond between the FA terminal carboxyl group and free amino group (typically exocyclic amino acid) is established. In the case of cyanopeptolins, the fatty acid moiety is probably not essential for bioactivity, while in the case of hassallidins, the substituted fatty acid directly interacts with the membrane and is responsible for their membrane permeabilization effect.

Class S-1 (cyanopeptolin type)

Cyanopeptolins

Cyanopeptolins are peptides widely distributed in many cyanobacterial strains (e.g. *Microcystis*, *Planktothrix* or *Anabaena*). Earlier described Cyanopeptolins A, B, C, and D[70] isolated from *Microcystis* strain possess a relatively conserved structure based on the core cycle with one exocyclic amino acid and attached fatty acid chain (see Figure 3). The cyclic core structure is formed by 6 amino acids. The ring is enclosed with an ester bond between the hydroxy-group of Thr and carboxyl of Val making an amine group of threonine accessible for binding of side amino acid (mostly Glu or Asp). Uncommon amino acid Ahopip present in the molecule is formed from L-Glutamic acid-γ-aldehyde and the terminal amino group of the next amino acid (Leu). Some members of the cyanopeptolin family still possess a conserved cyclic amino acid core structure, but some of them lack the fatty acid moiety (Cyanopeptolin 954[62]). Streptopeptolins, structural analogs of cyanopeptolins, generally share an amino acid composition with cyanopeptolins, but possess only very short acyl chains on side chain amino acid.

S-1

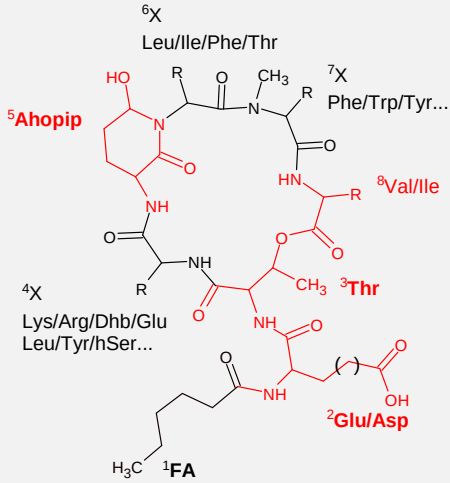


Figure 3: General structure (FA)-Glu/Asp-*Thr-X-Ahopip-X-X-Val/Ile* of lipopeptides belonging to Class S-1. Acylation of Glu residue is only facultative, cyanopeptolins without FA moiety are also described.

The 30-kb cyanopeptolin NRPS/PKS gene cluster contains two novel domains (a putative glyceric acid-activating A-domain and a facultative sulfotransferase domain[71]) and seven classical non-ribosomal peptide synthetase modules. Additionally, in the fatty-acid-moiety containing cyanopeptolins, the first module contains a condensation starter domain (C-starter domain). While in the vast majority of cyanobacterial NRPS gene clusters, the first module starts with an adenylation domain, a C-starter domain is highly unusual and is usually connected to the N-acylation (attachment of fatty acid moiety)[72]. An analogous mechanism of acylation is found in hassallidins as well as other bacterial lipopeptides[73].

While some cyanopeptolin members seems to lack bioactive properties such as cyanopeptolin VW-1 or VW2[74](the structure of these cyanopeptolins is not fully revealed), many other possess significant biological activity and e.g. inhibit serine proteases such as chymotrypsin or trypsin[75]. For cyanopeptolin 1020 a very potent picomolar trypsin inhibition activity (IC_{50} = 670 pM) has been determined as well as an acute toxicity against the crustacean *Thamnocephalus platyurus* (LC_{50} 8.8μM)[76]. Furthermore, global transcriptomic analysis of Zebrafish revealed the downregulation or interruption of many crucial biological pathway including DNA damage recognition and repair, response to light, and heme metabolism etc.[77]

Some cyanopeptolins do not possess the fatty acid part and still retains the protein inhibition activity as the e.g. cyanopeptolin 954 shows chymotrypsin inhibition with an IC_{50} 45 nM[62]. Accordingly, **we can assume that in the case of cyanopeptolins, the fatty acid part does not play a crucial role in the bioactivity and the overall mode of action depends on the amino acid composition and 3D structure.** This theory is supported by

the role of the residue adjacent to Ahopip. If positively charged arginine is present at position 4 of the lipopeptide, the molecule strongly inhibits trypsin with IC_{50} 0.24-0.26μM (e.g. cyanopeptolin 962), while the inhibition of chymotrypsin remains mild (IC_{50} 3.1-3.8μM). Analogously, in case of aromatic tyrosine residue presence on the position 4 (eg. cyanopeptolin 985), cyanopeptolin preferentially inhibits chymotrypsin (IC_{50} 0.26μM)[78]. Generally, cyanopeptolin are thought to lack cytotoxic activity such as for e.g. cyanopeptolin 963 and 985 no cytotoxicity against MCF-7 breast cancer cells are reported.

Class S-2 (hassallidin type)

Hassallidins and balticidins

Hassallidins[63,64,79] and balticidins[27] are glycosylated lipopeptides isolated from many cyanobacteria including *Hassallia*, *Nostoc* and

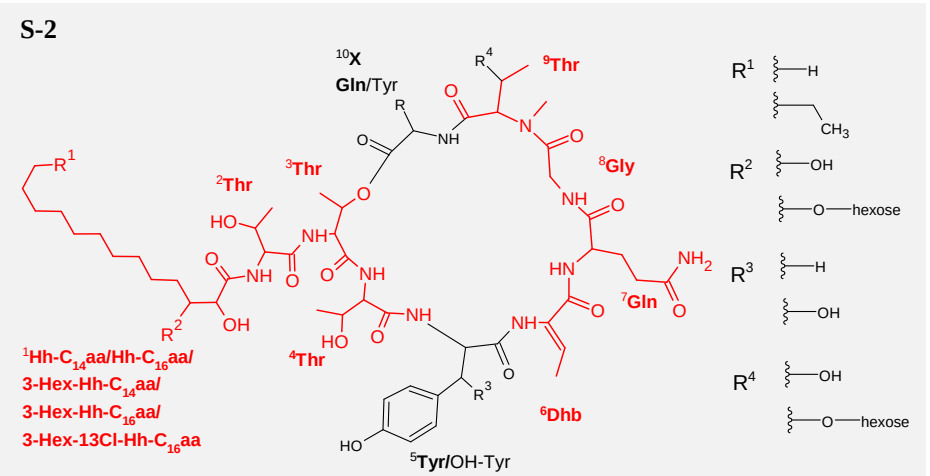


Figure 4: General structure FA-Thr-*Thr-Thr-X-Dhb-Gln-Gly-Thr-X* of hassallidins and balticidins.

Dolichospermum strains and *Anabaena cylindrica* Bio33. This class of lipopeptides comprises decapeptides in which 8 amino acids form a cyclic

structure with one exocyclic amino acid, and Hh-C_{14}aa (α,β-dihydroxy-tetradecanoic acid) attached to exocyclic amino acid via a peptide bond (see Figure 4) and possess general structure **FA-Thr-*Thr-Thr**-X-**Dhb-Gln-Gly-Thr**-X*. Similarly to smaller cyanopeptolins, the core structure is mostly cyclized via an ester bond between the hydroxyl group of Thr and the carboxyl group of Gln. Two uncommon amino acids can be incorporated into the cycle, OH-Tyr (hydroxytyrosine) and Dhb (dehydrothreonine).

Hassallidins possess a varying pattern of glycosylation on the Thr^2, Thr^9, and FA hydroxyl. Because of this feature, additional Thr residue and free hydroxyl groups on FA, hassallidins, and balticidins show a higher polarity than other cyanobacterial cyclic lipopeptides. The presence of free hydroxyl on the exocyclic part of the FA is rare in cyanobacterial cyclic lipopeptides. In the case of hassallidin C, both hydroxyl groups on FA are unsubstituted, while in hassallidin B, the β-hydroxy group is glycosylated by monosaccharide and in hassallidin C, D, and balticidins C, D by disaccharide. The fatty acid moiety seems to be conserved among hassallidins and balticidins, but the amino acid composition is highly variable including the unusual amino acids hTyr and Dhb. Multiple variants are found to be produced by a single strain, as e.g. a total of 14 hassallidins have been identified in Nostoc *sp*. CENA 219[(80)].

The reported 59-kb long gene cluster responsible for the biosynthesis of hassallidins was characterized in *Anabaena sp.* SYKE748A[(79)]. The biosynthesis starts with an activation of dihydroxylated acyl chain and its condensation to Thr residue via the action of the C-starter domain, which is followed by regular NPRS modules facilitating the amino acid ligation and cyclisation by the thioesterase domain. The glycosylation takes place via the action of 4 glycosyl-transferases (HasD,Q,T and X).

All described hassallidins and balticidins are reported as antifungal lipopeptides, with IC_{50} 0.3-4μM against *Candida* strains, however no antibacterial activity was detected (e.g. against *Bacillus subtillis, E.coli)*[63,64,79]. Interestingly, Hassallidin B shows cytotoxicity against acute T-Cell leukemia with IC_{50} 0.2 μg/ml[81]. Recent studies have proved that hassallidins are highly active on cell membranes, causing lysis of the cells with a selectivity to cholesterol rich regions[82]. **These findings prove that the fatty acid moiety of hassallidins and balticidins interacting with membranes is one of the crucial parts of the molecule responsible for their bioactivity.** The different compositions of fungal, bacterial, and human cell membranes, especially with regards their sterol components and lipid rafts, can further explain their selective effect (strong antifungal and no antibacterial bioactivity).

O Classes and N Classes lipopeptides: fatty acid incorporated in the core cycle

In the majority of cyanobacterial cyclic lipopeptides, the fatty acid is inserted into the peptide core. The formation of two bonds is required for successful incorporation. The modification of FA residue during NRPS/PKS biosynthesis allows several possibilities of ring closure.

Cyanobacterial cyclic lipopeptides with incorporated fatty acid represent a broad group of different structures, however, similar building blocks can be found across the classes (Table 1)[3*]. In general, two lipopeptide families defined based on the incorporated fatty acids can be established: lipopeptides with ***β*-hydroxy**-fatty acids incorporated via peptide and ester

[3*] This classification was created for this thesis only and cannot be considered as official classification of cyanobacterial cyclic lipopeptides.

bond (O-classes); and lipopeptides with ***β*-amino**-fatty acids lipopeptides cyclized via two peptide bonds (N-classes) (Figure 5).

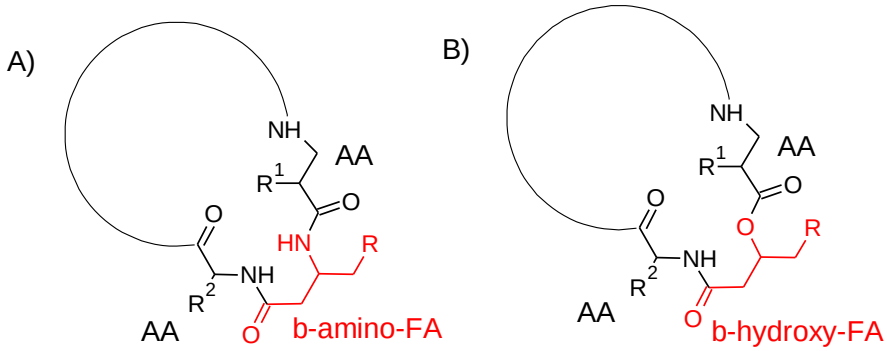


Figure 5: Two schematic illustrations of different incorporation of FA into the peptidic core A) Incorporation of β-amino FA via two peptide bonds, typical for N-class of lipopeptides. B) Incorporation of β-hydroxy FA via peptide and ester bond, typical for O-class of lipopeptides.

β-amino modified fatty acid is the most abundant type of fatty acid present in cyclic cyanobacterial lipopeptides. Its structure with one carboxyl and one free amine group on *β*-carbon allows formation of two peptide bonds, analogously to well-known iturin-class of bacterial cyclic lipopeptides[(35)]. Similarly, *β*-hydroxy-fatty acid forms an ester bond between free hydroxyl group on *β*-carbon and terminal carboxyl group of amino acid. This group is structurally alike well-known bacterial peptide surfactin with antibacterial and antiviral properties[(34)]. Also dolastatin 17, depsipeptide isolated from sea hare *Dolabella auricularia*[(35)], exhibiting human cancer cell growth inhibitory activity (GI_{50} 0.45-0.74 μg/mL) falls within this group (however, dolastatin 17 is not reviewed in this work because of its uncertain origin).

The fatty acids present in N and O classes are introduced to the structure via acyl ligase/transferase (e.g. FAAL, FACL, C-starter domain) and subsequently might be modified by PKS machinery. Consequently, aliphatic chains with even carbon number in the longest linear chain dominate. High

variability in substitution is observed on the FA α carbon, that is in many cases hydroxylated or (di)methylated. β-hydroxy FA are slightly more conserved, unbranched, and more often contains triple acetylene bond on the aliphatic chain terminus (AmC_6ya etc.) compare to β-amino FA.

Table 1: Classification of cyanobacterial cyclic lipopeptides with incorporated FA. X stands for highly variable residues, asterisk indicates places of the cyclisation.

β-hydroxy-fatty acid (O classes)

Class	Sub-class	General structure	Peptides
O-1		***FA-Val/Ile-HIVa/HMPa/PLa-*N*MePhe-βAla***	*Yanucamide*
O-2	O-2a	***FA-Val/Ile**-X-**HIVa/HMPa/PLa-Pro**-X**	*Veraguamides, Antanapeptins, Hantupeptins, Trungapeptins, Radamide B, Naopeptin*
	O-2b	***FA**-X-**PLa-Pro**-X-**Val***	*Palmyramide*
	O-2c	***FA-Val/Ile**-X-**HIVa/HMPa/PLa-Pro**-X-X**	*Pitipeptolides, Viequeamides, Kohamamides, Tiahuramides,*
	O-2d	***FA-Ile/Val**–(***N*MeVal/*N*MeAla**)$_n$-**HIVa/HMPa/PLa-Pro$_n$**-Xx;**	*Wewakpeptins*
O-3		***Fa-X-X-(X)-Pro-*N*MePhe/*N*MeTyr-Ala/Gly***	*Cocosamide, Benderamide, Dudawalamides, Guineamide*
O-4		***FA-dHMBa/dHMPa/HIVa-TZa**-(X)-X-**TZa***	*Lyngbyabellin, Hectochlorin*

β-amino-fatty acids (N classes)

Class	Subclass	General structure	Peptides
N-1		***FA-AOFHa-X-Gly***	*Anabaenolysins*
N-2	**N-2a**	***FA-HMPa/La/HIVa**-X-***N*MePhe/*N*Me**X-**Ala/X***	*Porpoisamide,*
	N-2b	***FA-La/HMPa**-X-*N*MeX-**Ala-TZa***	*Ulongamides*
	N-2c	***FA-HMPa/La/HIVa**-X-X-***N*MeX**-(X-X)-***N*Me-X-Ala/Leu/Ile/Val**-(X-X-X)-(X)-***N*MeX***	*Ulongapeptins, Companeramides, Malevamide B,C*
N-3		***FA-Gln-Gly-Pro/mPro**-X-X-**Ser/Thr-Dhb-Ser/Thr**-X-**Pro/mPro***	*Muscotoxin, Portoamides, Schizotrin A, Pahayokolides*
N-4		***FA-Val**-X-X-X-**Asn/Gln-Ala/Gly**-X-***N*MeAsn-Pro***	*Minutissamides, Puwainaphycins, Calophycin*
N-5	N-5a	***FA**-X-X-**Pro/OH-Pro-Ser/hSer-Phe/Tyr-Leu**-X-X-X-**Gly***	*Trichormamide A,D , Laxaphycin A, Lobocyclamide A and similar*
	N-5b	***FA**-X-**OH-Leu**-X-X-**Gln-*N*MeIle**-X-**Thr-Pro/OH-Pro**-X-**Thr***	*Trichormamide B-C, Laxaphycin B, Lobocyclamide B and similar*

O Classes: Cyanobacterial cyclic lipopeptide with β-hydroxy-fatty acids

Generally, cyanobacterial cyclic lipopeptides containing β-hydroxy-fatty acids are rather small (4-9 residues). The mode of action highly depends on the overall lipopeptide structure as no specific chemical moieties seems to be responsible for their bioactivity. Some O-class lipopeptides are cytotoxic, others exhibit antiparasitic or antifungal properties. Four main subclasses based on the structure, biosynthesis, and activity can be assigned. Classes O-1, O-2, and O-3 comprise cyanobacterial kulolide super family members[83], class O-4 is unique for cyanobacteria and contains chlorinated lipopeptide with thiazole moieties (Thz).

Class O-1 (yanucamide type)

Class O-1 represented by yanucamides is defined by a general structure of ***FA-Val/Ile-HIVa**/HMPa/PLa-X-**βAla***(see Figure 6), and

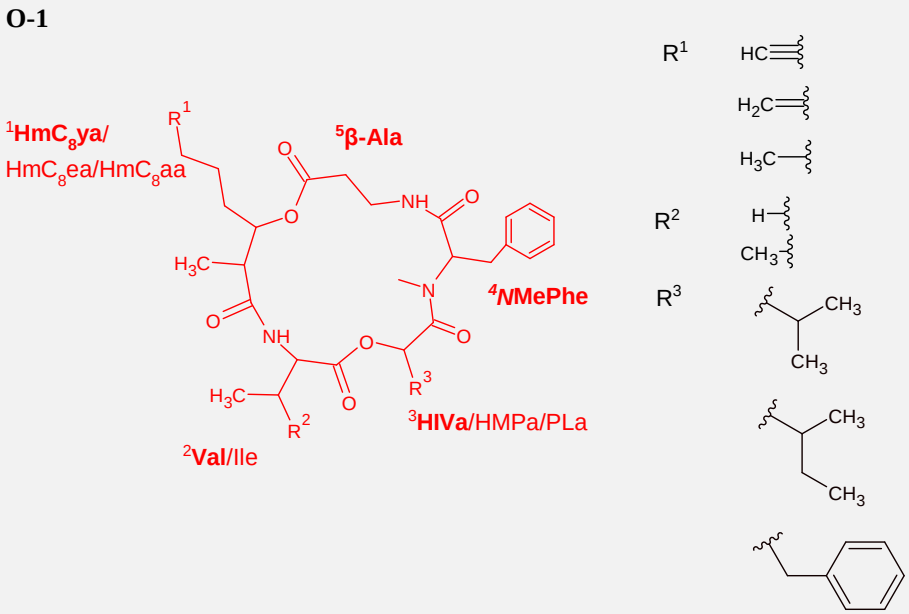


Figure 6: General structure *FA-Val/Ile-HIVa/HMPa/PLa-X-βAla* of lipopeptides belonging to Class O-1

includes the smallest known β-hydroxy-fatty acid containing cyclic lipopeptides. With only 5 residues and short Hm_2C_8ya fatty acid the molar mass does not exceed 650 Da. As the only one structural type (yanucamides) is known, the definition of this group is relatively weak. However, similar to members of Class O-2 and O-3 and (cyano)bacterial kulolides, the presence of Val/Ile adjacent to the fatty acid moiety seems to be important. Additionally, hydroxy acid HIVa and rare βAla are incorporated. While lipopeptides with only Hm_2C_8ya are known, tentative variants with Hm_2C_8ea or -aa can be expected but so far remain undiscovered. Similarly, variants with other hydroxy acids such as HMPa or PLa instead of HIVa are expected, but not yet described.

Yanucamide A and B isolated from the Yanuca Island collection of *Lyngbya majuscula* and Schizothrix *sp.* as the only representatives possess strong brine shrimp toxicity (LD_{50} 5 ppm)[84]. No additional bioactivity has yet been described.

Class O-2 (veraguamides, palmyramides and similar)

Class O-2 contains cyanobacterial cyclic hexapeptides, heptapeptides, and nonapeptides with a general structure of ***FA**-X-(X)-**Hx**-**Pro**-X-(X)-(X-X)* (see Figure 7, 8, 9 and 10) where Hx represents HIVa, HMPa, PLa, or Phe. Additionally, Val/Ile is adjacent the FA. Incorporated FA in described variants is either β-hydroxy-α-methyl-octanonic acid (HmC_8aa), or unsaturated HmC_8ea or HmC_8ya. Additional halogenation can take place usually at the FA terminus. Remaining amino acids (marked as X) are variable and their positions are in the majority of known O-2 class members occupied by non-polar amino acids such Val, Gly, Ile, or Phe. Similarly to the structures in the bacterial kulolide family, unpolar residue Val/Ile is adjacent to FA with

the exception of palmyramide A (class O-2b member) which possess LA at this position. The overall structure is highly nonpolar with an unusually high content of branched amino acids (at least 40% of amino acids are Ile, Leu, or Val). Incorporated fatty acid moiety is of medium length with no additional hydroxylation except for β-hydroxylation. At least one residue is hydroxy acid (HIVa, HMPa and PLa derived from Val, Ile and Phe, respectively). Typical representatives are the hexapeptide Veraguamides A-J[51,85] or heptapeptides pitipeptolides[24,86].

All O-2 class lipopeptides share similar biological and biophysical properties as well as biosynthetic origin in tentative NRPS chemical machinery. Subclasses are defined by the order of putative modules (hexapeptide O-2a vs O-2b), the presence of an additional module (heptapeptide O-2c) or multiple copies of a distinct module (nonapeptides O-2d)

The cytotoxicity ranges from non-bioactive antanapeptins (no brine shrimp toxicity, sodium channel modulation, or bioactivity in antimicrobial bioassays) and mild brine shrimp toxicity (Trungapeptin A), to a highly cytotoxic compound with an effect against cancer cell lines in nanomolar concentration (Viequeamide A). The level of (un)saturation of the FA-terminal bond seems to largely affect the lipopeptide cytotoxicity. The most potent lipopeptides of this class possess fatty acid moiety reduced to octynoic acid (HmC_8ya). A comparison of similar structures proves the possible partial importance of this acetylene modification. Pitipeptolide A with HhC_8ya is reported to have six times lower cytotoxicity than Pitipeptolide C with HhC_8aa. A similar relationship was found in other O-2 class lipopeptides (e.g. Viequeamide A vs. Viequamide C). However, this relation cannot be fully generalized, as Hantupeptin C with HhC_8aa possesses a slightly higher

cytotoxicity than Hantupeptin A with unsaturated HmC_8ya and, similarly, Kohamamide B with HmC_8ea is more active than Kohamamide A with HmC_8ya. Additionally, the (un)saturated fatty acid is not the only part of the molecule responsible for the bioactivity.

Interestingly, both Veraguamide D and F contain HhC_8ya, their cytotoxicities against HeLa cells differs by two orders. The difference between these two structures is the presence of PLa residue instead of HMPa and *N*MeVal instead of *N*MeIle in less potent Veraguamide F[51]. A similar difference in cytotoxicity can be observed for viequeamides, where aromatic PLa containing Viequeamide B-D shows no activity in relation to cancer cells, and Viequeamide A containing HMPa instead of PLa was observed as a potent cytotoxic compound[87]. The loss of bioactivity cannot be easily explained by the presence of an aromatic residue, as Hantupeptin A containing HhC_8ya as well as PLa maintains its bioactivity[88]. Further, the brominated fatty acid moiety can significantly increase cytotoxicity as observed in the most active Veraguamide A with bromo-HhC_8ya. The mollusk-derived cyclic lipopeptides kulomo'opunalide-1 and -2 share identical or very similar amino acid composition to Veraguamide A, lack bromination, and exhibit only a moderate bioactivity[89]. **Taken together, the bioactivity and cytotoxicity of Class O-2 seems to be highly affected both by the incorporated fatty acid residue as well as the peptide core composition; it is likely that the 3D structure also plays a crucial role in interaction with various cellular targets.** Nonetheless, the mechanism of O-2 class lipopeptides action and their molecular targets remain unknown.

Subclass O-2a (Veraguamide type)

Cyanobacterial lipopeptides belonging to subclass O-2a are hexapeptides and possess a general structure of *FA-Val/Ile-X-HIVa/HMPa/PLa-Pro-X* (see Figure 7). This subclass contains for example antanapeptins, veraguamides, and Radamamide B.

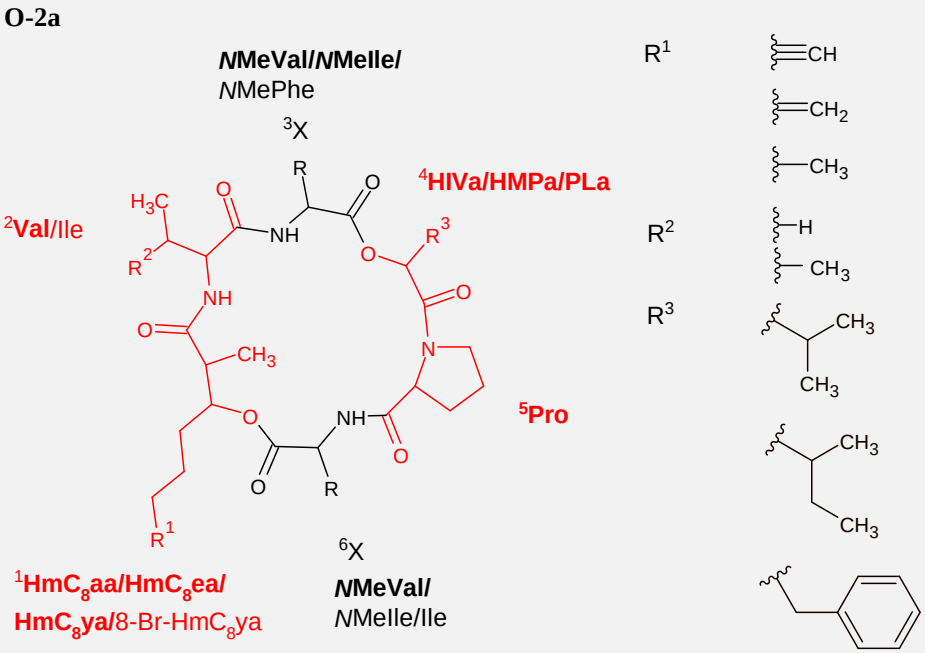


Figure 7: General structure *FA-Val/Ile-X-HIVa/HMPa/PLa-Pro-X* of lipopeptides belonging to Subclass O-2a

Antanapeptins

Antanapeptins A-D are hexadepsipeptides isolated from *Lyngbya majuscula* (from Antany Mora) with a general structure of ***FA**-Val-*N*MePhe-**HIVa**-**Pro**-*N*MeIle/*N*MeVal*. The antanapeptins were evaluated in several biological assays (brine shrimp toxicity, sodium channel modulation, antimicrobial essay against *Candida albicans*, *Pseudomonas aeruginosa*, and *Escherichia coli*), however, no bioactivity was associated with these structures[(90)].

Veraguamides and Radamamide B

Veraguamides A-J are hexadepsipeptides isolated from a dolastatin-16-producing strain of marine cyanobacterium *Symploca cf. hydnoides*[51,85]. The general structure of these lipopeptides ***FA**-Val/Ile-*N*MeVal/Ile-**HIVa/HMPa/Pla-Pro**-*N*MeVal/Ile* is very similar to antanapeptins. The fatty acid moiety could be further halogenated. The typical terminal modification also includes the presence of the alkynyl, alkynyl bromide, and vinyl group (for the exact structures, see Table 2, section O-2b). Veraguamides show a strong to very weak cytotoxic activity against HT29 colorectal adenocarcinoma and HeLa cervical carcinoma cell lines with IC_{50} for HeLa cells ranging from 0.54μM (Veraguamide D) to 49 μM (Veraguamide F).

Radamamide B, isolated from a collection of *Lyngbya majuscula*, from Madagascar shares a general structure of veraguamides with sequence ***FA**-Ile-*N*MeVal-**HMPa-Pro**-*N*MeIle*. This compound was tested in several inhibitory activity assays (thrombin, trypsin, and Leu aminopeptidase) without showing any bioactivity[91], similarly, no toxicity against brine shrimp or sodium channel modulation was observed.

Hantupeptins

Hantupeptins A-C were isolated from *Lyngbya majuscula* (from Pulau Hantu Besar)[88,92] and actually might be assigned to the veraguamides group as the general structure ***FA**-Val-*NMe*Ile-**PLa-Pro**-*N*MeVal* is identical to Veraguamide F, however hantupeptins contain fatty acids with different saturation not described in Veraguamide F (HmC_8ea and -aa).

Hantupeptin A showed cytotoxicity against MOLT-4 leukemia cells and MCF-7 breast cancer cells with IC_{50} values of 32 and 4.0 μM, respectively[88]. The most active Hantupeptin B exhibits the cytotoxicity against identical cell lines with IC_{50} 0.2 and 0.5 μM, respectively, Hantupeptin C with IC_{50} 3.0 and 1.0 μM, respectively[92].

Trungapeptins and Naopopeptin

Trungapeptins A-C isolated from *Lyngbya majuscula* (from Trung Province)[93] are, similarly to hantupeptins, the only analogues of Veraguamide F with the replacement of *N*MeVal by Ile (general structure ***FA**-Val-*N*MeVal-**PLa-Pro**-Ile*). Trungapeptin A showed mild brine shrimp toxicity and ichthyotoxicity but no toxicity against KB and LoVo cells.

Naopopeptin isolated from Papua New Guinea collections of the cyanobacterium *Lyngbya majuscula* is similar to Trungapeptin C with *N*-Methylation on Ile and possesses interesting antiparasitic properties (IC_{50} 2.7 μM and 5.8 μM against *Plasmodium falciparum* and *Leishmania donovani*, respectively) and it has been found to be an inhibitor of calcium oscillations in murine cortical neurons (IC_{50} 11.7 μM)[94].

Subclass O-2b (Palmyramide type)

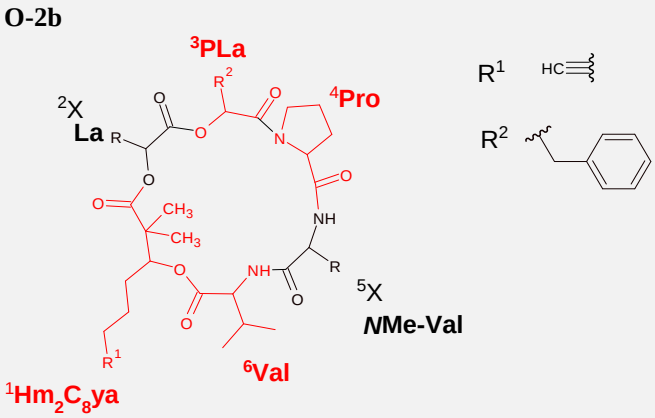


Figure 8: General structure *FA-X-PLa-Pro-X-Val* of lipopeptides belonging to Subclass O-2b

The lipopeptides of subclass O-2b contain similar building blocks to subclass O-2a, however, they are assembled in a different order (see Figure 8). Val/Ile residue adjacent to fatty acid typical for all members of Class O-2 is attached on the *β*-hydroxy-side instead of the carboxy-side of the fatty acid.

Palmyramide

Only one member of the palmyramide group with a structure of ***FA**-La-**PLa**-**Pro**-*N*MeVal-**Val*** is currently described (Palmyramide A[95]). It contains three amino acids and two hydroxy acids (one of them is the rare La, a hydroxy acid derived from Ala) and an unsaturated fatty acid moiety. Palmyramide was isolated from *Lyngbya majuscula* (from Palmyra Atoll).

Palmyramide A showed sodium channel blocking activity in neuro-2a cells and cytotoxic activity against H-460 human lung carcinoma cells with IC_{50} 17 µM and 39.7 µM, respectively[95].

Subclass O-2c (Pitipeptolide type)

Subclass O-2c of cyanobacterial cyclic lipopeptides is probably evolutionary and biosynthetically derived from Class O-2a and O-2b. It

contains the majority of described cyanobacterial kulolide superfamily members[83]4* with a general structure of ***FA-Val/Ile**-X-**HIVa/HMPa/PLa-Pro**-X-X** (see Figure 9), where FA stands again for short HmC_8aa, ea or ya.

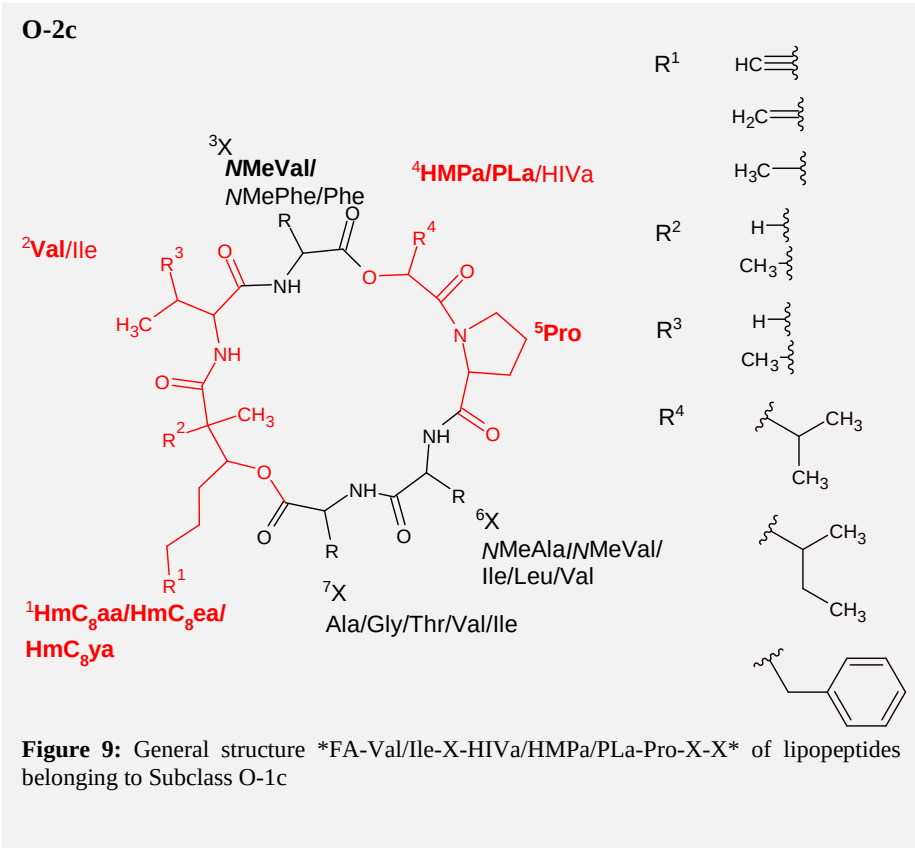


Figure 9: General structure *FA-Val/Ile-X-HIVa/HMPa/PLa-Pro-X-X* of lipopeptides belonging to Subclass O-1c

The structures often possess hydroxy acids at position 4. Adjacent to this residue, various *N*-methylated amino acids are present (*N*MeVal, *N*MeIle, *N*MePhe) which seem to be conserved and play a role in the compound bioactivity. Concerning pitipeptolides, the bioactivity strongly depends on the length of the amino acid side chain; variants with Ile instead of Val or HMPa instead of HIVa exhibit higher bioactivity.

4* This superfamily describes depsipeptides originating from all possible biological sources with NPRS/PKS origin, cyclic peptide Kulolide was isolated from the mollusc *Philinopsis speciosa* [83]

Similar to previous subclasses, the bioactivity depends on the overall 3D structure, showing weak to moderate bioactivities in various bioassays (pitipeptolides possess antimycobacterial bioactivity, while Viequeamide A is highly cytotoxic to lung cancer cells).

Pitipeptolides and Kohamamides

Pitipeptolides A-F were isolated from *Lyngbya majuscula* (from Piti Bomb Holes)[24,86]. The general structure of ***FA-Val**-Phe-**HIVa/HMPa**-**Pro**-Val/Ile-Gly** shows again some similarity to smaller trungapeptins from Class O-2a with similar occupation of the variable positions of the peptide sequence (Ile/Leu, Thr/Val/Ile etc.). Additionally, *N*-methylation of Phe is observed.

Pitipeptolides have been previously shown to exhibit weak to moderate cytotoxicity against cancer cells as well as moderate antimycobacterial activities. While pitipeptolides A and B show moderate cytotoxicity against HT-29 and MCF-7 cancer cell lines with IC_{50} around 13 μM, pitipeptolides C-F exhibit no significant bioactivity[24,86]. Interestingly, there is only a small structural difference between individual pitipetolides. The most active pitipeptolides, A and B, contain in both positions 4 and 7 longer side-chained residue, HMPa instead of HIVa in position 4 and Ile instead of Val in position 7; and *N*-methylation of Phe. Loss of any of these additional CH_3 groups probably leads to the decrease of bioactivity.

Kohamamides A-C[96] isolated from the marine cyanobacterial strain *Okeania sp*. are structurally related to pitipeptolides, *N*MePhe is replaced by *N*MeVal, Ile is replaced by the structurally similar Leu and Gly by Ala. Kohamamides were tested against HeLa and HL60 cells. Interestingly, Kohamamide B with Hm_2C_8ea was shown to have a weak to moderate

cytotoxic activity (as the most potent kohamamide variant) IC_{50} 23 μM and 6 μM, respectively; Kohamamide A with the usually more active Hm_2C_8ya shows reduced activity with IC_{50} of 29 μM and 19 μM, and Kohamamide C with saturated Hm_2C_8aa 18 μM and 11 μM, respectively[96].

Viequeamides and Tiahuramides

Vierqueamides A-F were isolated from marine *Rivularia* sp. (from the island of Vieques)[87]. Amino acid composition of different vierqueamides is not as conserved as in previous pitipeptolides. The general structure of ***FA-Val**-*N*MeVal-**HMPa/Pla**-**Pro**-*N*MeVal/NMeAla-Val/Thr** shows the presence of free-hydroxyl-group containing amino acid Thr that is not as common for small cyclic cyanobacterial lipopeptides.

The toxicity of viequeamides varies according to particular structure. While Viequeamide A was found to be highly toxic to H460 human lung cancer cells with IC_{50} 60 nM, all other variants of viequeamides were inactive[87].

Tiahuramides isolated from *Lyngbya majuscula* (from a collection from French Polynesia) are highly similar to viequeamides[29]. While all viequeamides possess Hm_2C_8ya, ea or aa, Tiahuramides possess an identical peptide sequence to Viequeamide E, but Hm_2C_8ya is replaced by HmC_8ya, ea or aa. The bioactivity assay against SH-SY5Y shows IC_{50} 14 and 6 μM for Tiahuramide A and B, respectively. Tiahuramide C with HmC_8ya was inactive even at 100 μM[29]. Interestingly, antibacterial essays against various bacteria (including *Aeromonas salmonicida, Vibrio anguillarum, Shewanella baltica*, *Escherichia coli*, and *Micrococcus luteus*) show the highest effect of Tiahuramide C with MIC 6.7-17 μM, while the least active Tiahuramide A shows MIC starting at 27 μM for *A.salmonicida* and >50 μM for *S.baltica*[29].

Radamamide A

Radamamide A is the second novel compound (after Radamamide B) isolated from a collection of *Lyngbya majuscula* from Madagascar[91]. While Radamamide B shares its structure with the veraguamide type of O-2a class lipopeptides, Radamamide A is a hexapeptide belonging to the O-2c class. Structural similarities are observed between Pitipeptolide A and Radamamide A, however, the position of aromatic residue and branched amino acid residue is exchanged. Pitipeptolide A (mentioned aboved) contains *N*MePhe in position 3 and Ile in position 6, while Radamamide A has *N*MeVal and *N*MePhe in identical positions. (for the structures, see Table 2, section O-2c).

Similarly to Radamamide B, no bioactivity against brine shrimp or in sodium channel modulation assays was observed for Radamamide A. However, weak antimalarial activity is described[91].

Subclass O-2d (Wewakpeptin type)

Subclass O-2d is defined by multiple copies of building blocks, namely *N*MeVal/*N*MeAla and Pro, which together with other residues forms a nonapeptidic structure. The general structure of this subclass can be defined as ***FA-Val/Ile-(*N*MeVal/*N*MeAla)$_n$-HIVa/HMPa/PLa-(Pro)$_n$**-X* (see Figure 10). The cytotoxicity varies based on the overall structure from nM to μM similarly to members of the previously mentioned classes O-1 and O-2. In subclass O-2d lipopeptides, variants with Hm_2C_8ya and Hm_2C_8aa are described, Hm_2C_8ea is expected but not discovered yet. Likewise, variants with Val instead of Ile are expected in the position adjacent to the FA moiety.

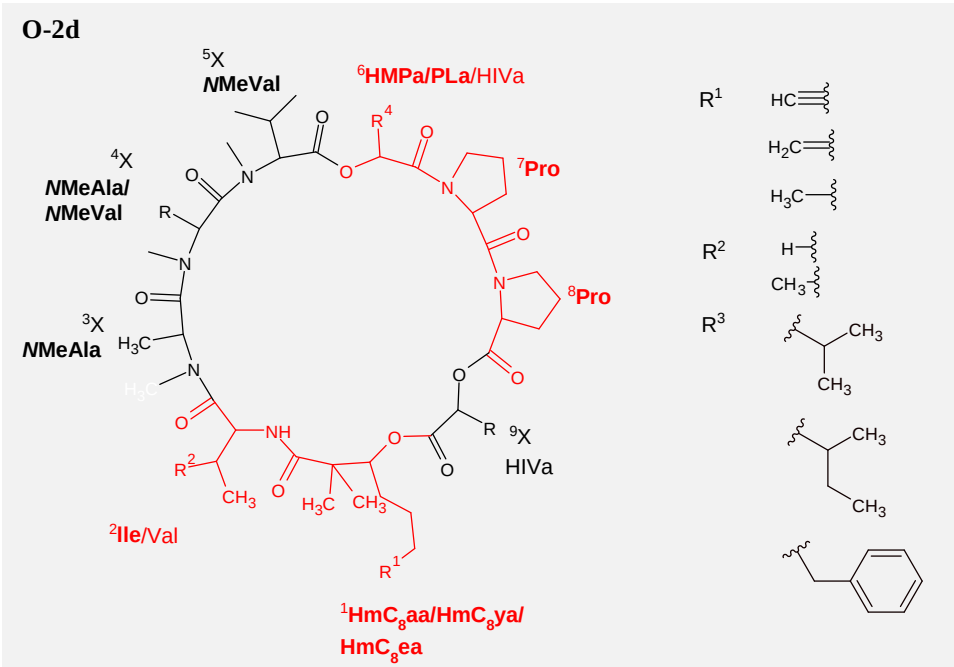


Figure 10: General structure *FA-Val/Ile-(NMeVal/NMeAla)$_n$-HIVa/HMPa/PLa-(Pro)$_n$-X* of lipopeptides belonging to Subclass O-2d

Wewakpeptins A-D with a fully determined structure as the only known members of this subclass possess a general structure of ***FA**-Ile-***N*MeAla-*N*MeAla/*N*MeVal-*N*MeVal-HMPa/PLa/HIVa-Pro**$_n$-HIVa* and up to now represent the largest β-hydroxy-fatty-acid containing cyclic lipopeptide described in cyanobacteria. The variants with a lower number of *N*MeVal/*N*MeAla are expected but not yet purified. These lipopeptides are found and isolated from marine *Lyngbya semiplena* (Papua New Guinea collection).

Wewakpeptins A-D are cytotoxic to the NCI-H460 human lung tumor and neuro-2a mouse neuroblastoma cells with LC_{50} around 0.5µM[97].

Class O-3 (cocosamides etc.)

Class O-3 of the cyclic cyanobacterial lipopeptides, possessing a β-hydroxy fatty acid moiety and 6-7 residues, is only weakly defined. Some of the compounds are partially similar to classes O-1 and O-2 (a medium length fatty acid with possible acetylene termination and a different number of residues in the cycle). While Class O-2 is defined by the presence of hydroxy-acid adjacent to the Pro residue (typically HMPa/HIVa/PLa-Pro), Class O-3 contains a characteristic Pro-Phe/Tyr motif. Additionally, while the position adjacent to the fatty acid moiety occupied in Class O-1 and O-2 lipopeptides by Ile or Val only, in Class O-3 this position is more structurally variable (including HIVa or HMPa). Taken all together, the general structure of Class O-3 lipopeptides can be defined as ***FA**-X-X-(X)-**Pro-*N*MePhe/*N*MeTyr-Ala/Gly**** (see Figure 11). The probable NRPS biosynthetic pathway of these

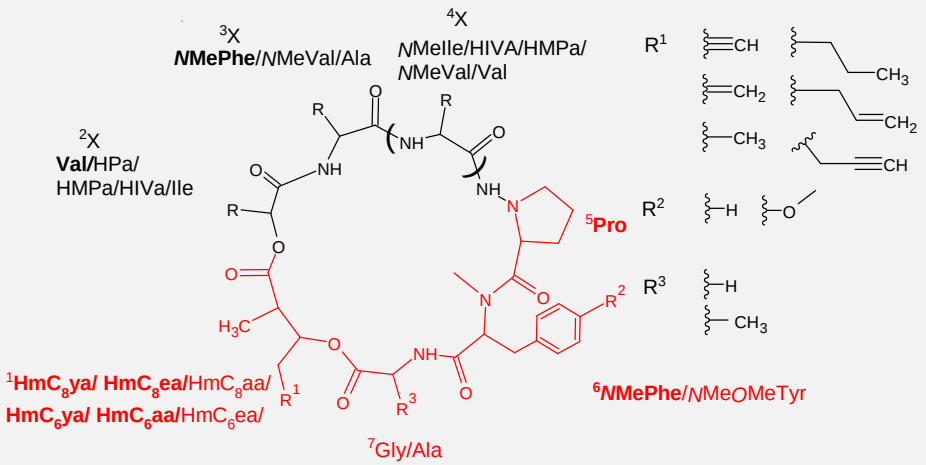


Figure 11: General structure *FA-X-X-(X)-Pro-Phe/Tyr-Gly/Ala* of lipopeptides belonging to Subclass O-3

lipopeptides is likely branched or allows alternating mechanisms because hexapeptide and heptapeptides are biosynthesized in the single cyanobacterial strain simultaneously. The NRPS assemblies likely differ at position 4, before

Pro residue. Both position 3 and 4 are highly variable, however, while methylated residues *N*MePhe or *N*MeVal and unmodified Ala is found at position 3, in some O-3 class lipopeptides position 4 is occupied by either Val, Ile, HIVa, or HMPa or the corresponding NRPS module is missing and the assembly continues directly with the addition of Pro at the next position. Generally, Hm_2C_8ya, Hm_2C_8ea, or Hm_2C_8aa are incorporated and form a hydrophobic part together with the Pro-*N*MePhe-Ala/Gly motif.

Similarly to all O-1 and O-2 cyclic lipopeptides, the bioactivity of members of O-3 strongly depends on the overall structure, since cocosamide A containing Ala shows weak cytotoxicity compare to Benderamide A with Gly instead of Ala which is non active against the same cell line[98]. However, Dudawalamide A, a heptapeptide similar to cocosamides, containing Gly in the identical position, but HPa in position 2 and non-methylated Ala in position 3, exhibits weak cytotoxicity. In addition, moderate antiparasitic activity is reported for Dudawalamide A[7].

Analogously to Class O-2, in Class O-3 we can conclude that **the lipopeptide bioactivity depends highly on the overall compound structure, especially the effect of the methylation pattern is observed.** The mechanism of action and molecular targets of Class O-3 remain unknown due to the existence of many different compounds with different observed bioactivities.

Cocosamide and Benderamide

Hexapeptides Cocosamides A-B isolated from *Lyngbya majuscula* (from Cocos Lagoon) have a general structure of ***FA**-Val-***N*MePhe-Pro**-*N*MePhe-Gly/Ala* and shows weak to moderate cytotoxicity against MCF-7 breast cancer and HT-29 colon cancer cells with IC_{50} 11 μM, and 39 μM,

respectively[67]. Benderamide A isolated from *Lyngbya* sp. (Singapore Collection of Marine Cyanobacteria) is almost identical to cocosamide B with Ala instead of Gly[98] and does not present cytotoxic activity against the MCF-7 cell line.

Dudawalamides

Dudawalamides A-D were isolated from the marine cyanobacterium *Moorea producens* collected from Dudawaly Bay, Papua New Guinea[7]. The general structure of ***FA**-hPa/Val-X-(X)-**Pro-*N*MePhe/*N*MeTyr**-Gly/Ala* resemble cocosamides and benderamides. Dudawalamides A and D with Gly in position 6 were potent against *Plasmodium falciparum* (IC_{50} 3.5-3.6 μM). Dudawalamide B with Ala in the same position preserves antiparasitic activity against *Plasmodium falciparum* with IC_{50} 8 μM. Dudawalamide D was also selectively active against *Leishmania donovani* (IC_{50} 2.6 μM), while other duwalamides did not show any effect[7].

Note:

A special case is presented by guineamides. Guineamide is a common name for a group of peptides from Lyngbya majuscula isolated from a collection from Papua New Guinea, but only Guineamide C-F contain incorporated fatty acid moiety, β-hydroxy as well as β-amino fatty acid (Hm_2C_6aa, AmC_6aa, and AmC_6ya). Heptapeptide Guineamide E and hexapeptide Guineamide F are similar to Dudawalamide A and Dudawalamide B, respectively, possessing Hm_2C_6aa, methylated residues and important Gly adjacent to FA moiety. However, Guineamide C and D do not contain the typical Pro-Phe/Tyr residue, additionally, FA present in their structure are β-amino fatty acids, AmC_6aa and AmC_6ya, and thus should belong to N-class lipopeptides[99] *(for the structures, see Table 2, section O-3).*

Class O-4 (dithiazole type)

Class O-4 is an example of broad structural variability of cyclic cyanobacterial lipopeptides. This class comprising small chlorinated "pentapeptides" or "hexapeptides" is structurally related to dolabellin isolated from the mollusc *Dolabella auricularia*[100]. The formation of thiazole ring(s) by the cyclisation of cysteine residue leads to the incorporation of nitrogen participating in the peptide bond into the heterocycle, and, consequently, the "loss" of the peptide bond. Lipopeptides of Class O-4 contain unusual *β*-hydroxy-carboxylic acid, namely dHMBa (2,3-dihydroxy-3-methylbutanoic acid) or dHMPa (2,3-dihydroxy-3-methylpentanoic acid) and two thiazole

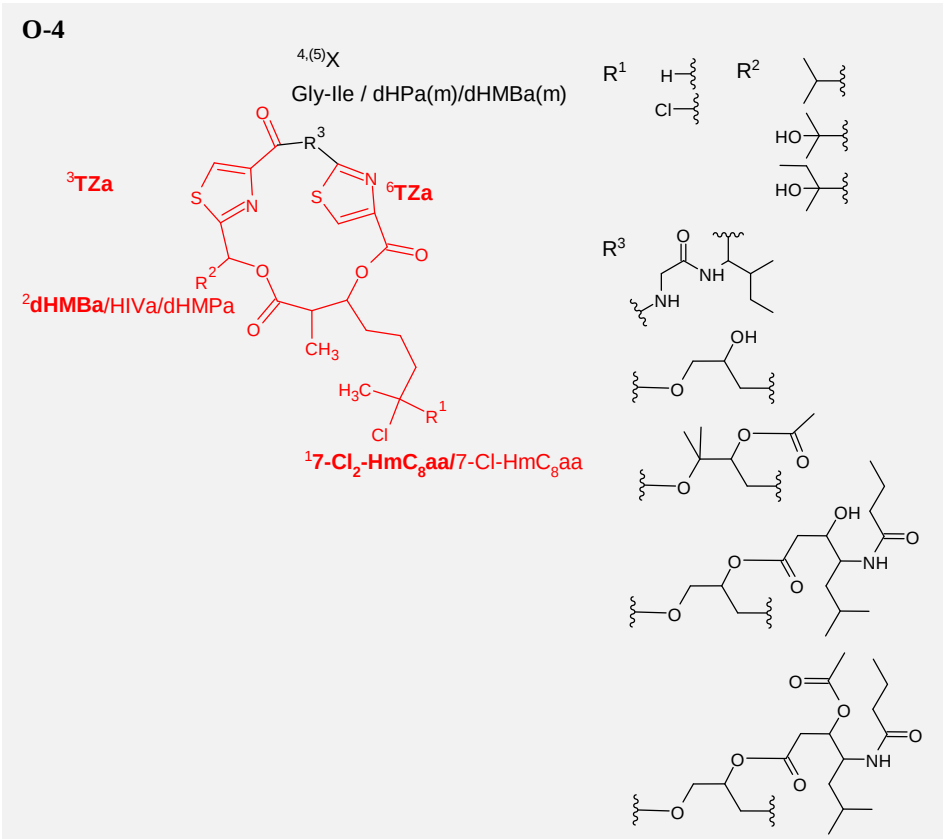


Figure 12: General structure *FA-dHMBa/dHMPa/HIVa-Tza-X-(X)-TZa* of lipopeptides belonging to Subclass O-4

units (TZa) originating from Cys via the action of a specific heterocyclization domain present in the NRPS. The general structure can be defined as ***FA-dHMBa/dHMPa-TZa**-(X)-X-**TZa*** (see Figure 12).

Two main structural types within this class are described so far. The first, represented by Lyngbyabellin A, contains a dipeptide Gly-Ile between thiazole units. The second, represented by hectochlorin, contains dHPa (2,3-dihydroxypropanoic acid) or dHMBa in this position with possible substitution either by acetate or a complex side chain (Figure 12).

The gene cluster responsible for hectochlorin biosynthesis contains 8 ORFs, among others, one PKS and two bimodular NPRS modules. Extremely atypical is the presence of a KR domain between A and PCP in both NRPS modules. The biosynthesis is initiated by the activation of hexanoic acid[(101)]. Interestingly, the hectochlorin gene cluster contains two ORFs for P-450 monooxygenase biosynthesis, which is believed to be responsible for the hydroxylation of dHMBa residue[(101)].

Due to their highly modified and unique structures, these lipopeptides target specific molecular target(s). Hectochlorins as well as Lyngbyabellin A are proven to affect actin polymerization and surprisingly compromise the cell in the G2/M phase of the cell cycle[(102)]. As a result of microfilament perturbation and the consequent apoptosis, these compounds are active against different organisms including fungi and parasites.

Despite the quite large structural differences between the penta- and hexapeptides of this group, the bioactivity based on actin disruption remains similar among all described structures.

The anticancer activity of lyngbyabellins and hectochlorins is also described. Interestingly, the cytotoxicity seems to be highly dependent on the overall structure. Cyclic hexapeptide Dehydroxylyngbyabellin A exhibits

prominent cytotoxicity in the micromolar range, while similar cyclic pentapeptide Lyngbybelin G shows almost no cytotoxicity against the identical cell line, and cyclic pentapeptide Lyngbyabellin H with a highly modified substituted dHPa maintains a similar bioactivity to dehydroxylyngbyabellin A[103] (for the structures, see Table 2, section O-4). Cytotoxicity is further influenced by cyclisation of the peptide as linear lyngbyabellins exhibit a slightly lower bioactivity compared to cyclic variants[22]. However, some linear lyngbyabellins preserve their bioactivity as lyngbyabellin D and I are both cytotoxic within the 0.1-1μM range.

The bioactivity of O-4 lipopeptides is mainly connected with the inhibition of actin polymerization, however, the mechanism of action has not yet been discovered. Additionally, cytotoxicity depends on the overall structure and functionalization of the particular lipopeptide. While the number of residues does not play a crucial role as heptapeptides can be almost as equally active as hexapeptide. The substitution of hydroxy acid between TZa seems to be important for the bioactivity.

Lyngbyabellin A and B

Hexapeptides Lyngbyabellin A and B isolated from a non-specified Guamanian strain of the *Lyngbya majuscula* contains Gly and Ile/Val in place of variable amino acids (***FA-dHMBa-TZa**-Gly-Ile/Val-**TZa***)[3,22].

Lyngbyabellin A and B show prominent cytotoxicity against multiple human cancer lines including KB human nasopharyngeal carcinoma cells and LoVo human colon adenocarcinoma cells (IC_{50} 0.05μM and 0.07μM, respectively)[3,102]. Similarly, Lyngbyabellin B was tested against HT29, HeLa, Ca46, and PtK2 with IC_{50} 0.1-1.1μM[103]. Interestingly, some of the linear lyngbyabellins show similar bioactivity, namely Lyngbyabellin D with

IC_{50} 0.1μM against KB cells and Lyngbyabellin I with IC_{50} 0.7-1.0 μM against H460 and Neuro-2a cells[103].

Lethal *in vivo* doses of Lyngbyabellin A have been determined ranging between 2.4-8.0 mg/kg. Additionally, a strong antimalarial effect is described for Lynbyabellin A with IC_{50} 0.0015 and 0.3μM[103] against the liver stage and intraerythrocytic stage of *Plasmodium falciparum*, respectively.

Hectochlorines and other Lyngbyabellins

"Pentapeptides" hectochlorins isolated from *Lyngbya majuscula* (Hector Bay, Jamaica, and Boca del Drago Beach, Bocas del Toro, Panama) represent unique cyclic "lipopeptides" [104] with a general structure of ***FA-dHMBa/dHMPa/HIVa-TZa**-dHMBa/dHPa-**TZa***. They contain a second hydroxyl acid in the only variable amino acid position. This second dhMBa causes "loss" of the very last peptide bond (other peptide bonds are lost during the cyclisation of cysteine into thiazole heterocycle). Therefore, it is questionable whether hectochlorins and pentapeptide lyngbyabellins should be considered lipopeptides as they often lack peptide bonds, however, the individual building blocks originate from amino and hydroxy acids.

Similarly to hexapeptidic lyngbyabellins, hectochlorins show bioactivity against fungi, with a moderate zone of inhibition against *Candida albicans*[3]. Additionally, the affect on actin polymerization in the human cells has been reported[104]. Hectochlorins A-D show further broad antiproliferative activity with an average GI_{50} 5.1 μM against 60 different cell lines including colon, melanoma, ovarian, and renal cell lines[104].

Structurally related pentapeptide lyngbyabellins vary in both dHMBa residues and instead can possess HIVa, DHMPa, or dHPa residue, also bearing complex modification. Their cytotoxicity varies with the substitution

of the hydroxyl group in the above mentioned residues. If bioactive, their anticancer potencies vary, with IC_{50} values ranging from 0.005 to 6μM.

Interestingly, Lynbyabellin K and L, structurally similar to other pentapeptide lyngbyabellins and hectochlorins with hydroxylated hydroxy acid instead of the second dHMBA residue, do not exhibit any significant cytotoxicity against cancer cell lines at tested concentrations[105]. Lyngbyabellin G shows sell line selective anticancer activity and it reaches IC_{50} values around 3μM against H460 and Neuro-2a cells while it possess extremely weak activity again against MCF7 (IC_{50}=120μM).

Lyngbyabellin E with a highly modified free OH group at residue 5 (Figure 12) shows cytotoxicity against NCT-H460 human lung tumor cells and mouse neuroblastoma cell lines with LC_{50} 0.4-1.2 μM. Structurally similar Lyngbyabellin N is further proved to be cytotoxic in the nanomolar range against human colon HCT 116 cells with IC_{50} 40 nM[106]. Antimalarial activity is reported for Lyngbyabellin G only.

As mentioned above, it is obvious that the substitution of the hydroxy acid located between both TZa residues plays a crucial role in the bioactivity and further research is necessary to explain this phenomenon.

N Classes: Cyanobacterial cyclic lipopeptide with β-amino-fatty acids

Cyclic cyanobacterial lipopeptides with incorporated β-amino-FA are very variable in terms of size and thus Class N includes very small lipotetrapeptides as well as large molecules with 13 residues. Their chemical structures are in general slightly more polar compared to lipopeptides containing β-hydroxy-fatty acid, as many lipopeptides of this class contain a free hydroxyl group(s). Generally, the cyclic core is formed mostly by amino acids and hydroxy acids are present exceptionally only in Class N-2 and N-5

(malevamides, companeramides etc.). The classification of these lipopeptides into 5 classes is mostly based on the specific amino acid composition and/or number of residues in the cycle.

Class N-1 (anabaenolysin type)

Class N-1 contains the smallest known cyclic cyanobacterial lipopeptides, consisting of 4 residues only. The characterization of this class is based on described variants of tetrapeptides anabaenolysins[59]. Their structure can be characterized by the general sequence of ***FA-AOFHa**-X-Gly** (see Figure 13) where FA is polyunsaturated β-amino-α-hydroxy-octadecanoic acid (mostly $AhC_{18}e_{5,7,9,13}a$ or $AhC_{18}e_{5,7,9,13,15}a$) and AOFHa is a lactone containing 2-(β-amino-5-oxytetrahydrofuran-2-yl)-2-hydroxy acetic acid. Among 13 known anabaenolysins, the variation is found mainly on the fatty acid moiety (different level of unsaturation, different fatty acid chain length) and a single amino acid Gly (in Anabaenolysin A-B) can be exchanged by glutamine (Anabaenolysin C-D). The biosynthetic cluster responsible for anabaenolysin production was described in 2015 in *Anabaena* sp. XPORK 15F and contains NRPS modules biosynthesizing the main amino acid core. However, no enzymes responsible for FA activation can be found in the cluster. The predicted genes of anabaenolysis synthesis contains PKS

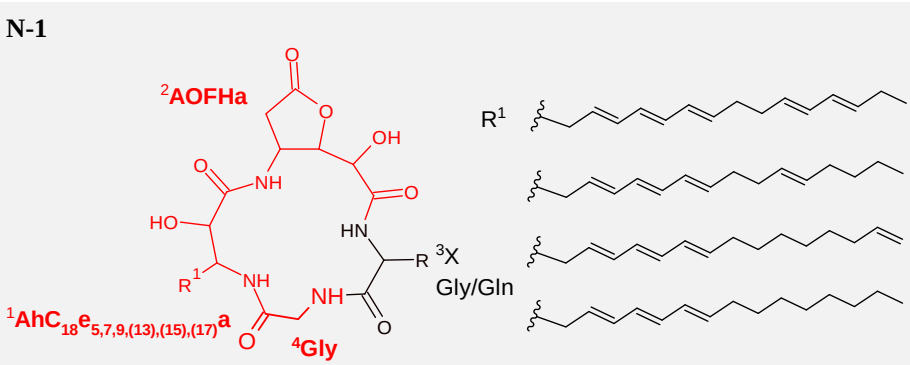


Figure 13: General structure *FA-AOFHa-X-Gly* of lipopeptides belonging to Subclass O-3

for FA extension, aminotransferase and oxygenase domains responsible for the formation of the alpha-hydroxy beta-amino FA motif. The FA is further modified by fatty acid desaturase[80].

Anabaenolysins A-D were isolated from cyanobacterial strains of the genus *Anabaena* and show high cytolytic activity in mammal cell lines, the LC_{50} values ranging from 3.7 μM to 17 μM[59]. Due to the long unsaturated fatty acid and presence of two free hydroxyl groups in the amino acid core, the overall properties of anabaenolysis are highly amphiphilic. Oftedal et al.[107] showed that anabaenolysins act as amphipathic detergents and permeabilize liposomes in a cholesterol dependent manner followed by a necrotic type of cell death. The mitochondrial membranes remain intact which further strengthens the hypothesis on cholesterol involvement in the anabaenolysis effect. Therefore, the mechanism of action is very similar to a common bacterial cyclic β-hydroxy-fatty acid containing lipopeptide surfactin[31]. However, while surfactin is primarily active in the phospholipid region of the membranes[108] anabaenolysins are preferentially active in cholesterol rich areas due to the presence of a highly unsaturated fatty acid chain. The unsaturated acyl chain of anabaenolysins can probably be structurally aligned with cholesterol in the membrane[107].

In general, the cytotoxicity of anabaenolysin is caused by the structural similarity of the whole structure (the unsaturated acyl chain and free OH groups) to the building block of the membrane. Moreover, it is strengthened by interaction with sterols. Incorporation of these molecules causes ion leakage via membrane permeabilisation and subsequent cell death.

Class N-2 (porpoisamides, malevamides etc.)

Class N-2 represents a β-amino-fatty acid analogues to Class O-2 and contains structures with a general structure of ***FA-HIVa/Pla/HMPa**-X-X-X_n* (see Figure 14, 15 and 16). The number of variable amino acid positions can reach up to 13 for class N-2c (***FA-HIVa/HMa/Pla**-X-X-X-X-X-X-X-X-X-X-X-(X)*). A characteristic is the presence of hydroxy acid adjacent to the FA moiety and a high number of N-methylated residues. Additionally, a dipeptide sequence NMeX-Ala/Val/Ile is also a distinctive feature of this class. Among all N classes of lipopeptides, structures belonging to N-2 are the only ones containing not only amino acids but hydroxy acids inside the peptidic core as well.

Unfortunately, no biosynthetic cluster has yet been described for these lipopeptides. Lipopeptides can be classified as N-2 if they match the general sequence ***FA-HIVa/HMPa/La**-$(X)_n$-*N*MeX-Ala/Val/Ile-$(X)_n$*, where FA stands for short unsubstituted fatty acid, including AmC_6aa or AmC_8ya.

Having a similar cytotoxicity to the O-2 and O-3 type, the mode of action of N-2 lipopeptides seems to be independent of the type of incorporated fatty acid (β-hydroxy/β-amino) while the FA length, substitution, and saturation is likely more important. Put simply, the exchange of β-amino-FA for β-hydroxy-FA (in Class N-2 class versus Class O-2) seems to have a marginal effect on the lipopeptide bioactivity.

Subclass N-2a (porpoisamide type)

The smallest lipopeptides belonging to Class N-2 contains pentapeptides with general sequence of ***FA-HMPa/La/HIVa**-X-***N*Me-Phe/*N*Me-X-Ala*** (see Figure 14), with AmC_8aa as FA. The only described

part of the molecule adjacent to the fatty acid is HMPa. However, La and HIVa can be expected in the same position as they are found in N-2b and N-2c, i.e. adjacent to FA. Similarly, lipopeptide with AmC_8ea or AmC_8ya are contemplated.

N-2a

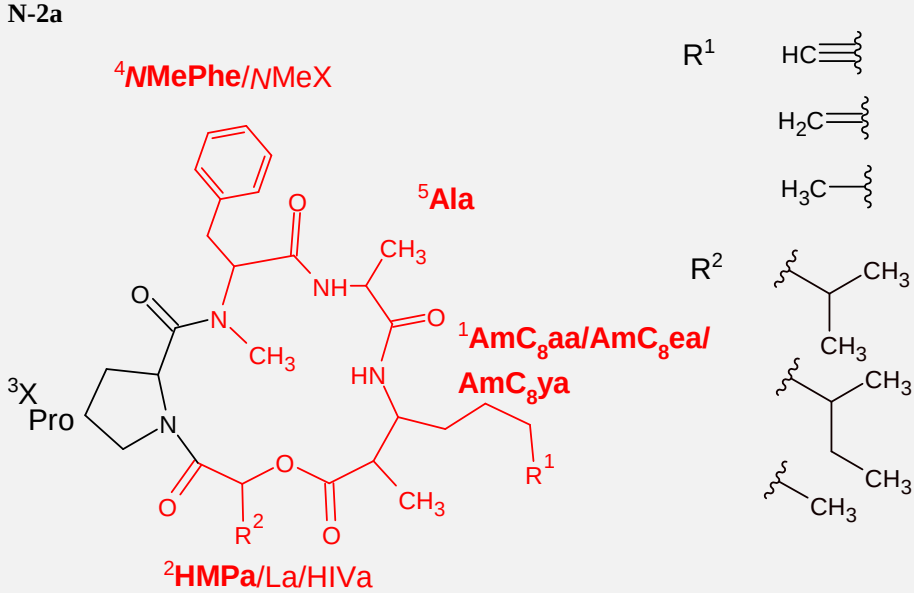


Figure 14: General structure *FA-HMPa/La/HIVa-X-NMePhe/NMeX-Ala* of lipopeptides belonging to Subclass N-2a

This subclass is based on the structure of Porpoisamide A-B[109] isolated from *Lyngbya* sp. (from Florida Keys) containing AmC_8aa. While O-2b lipopeptide Palmyramide A[95] with Hm_2C_8ya shows selective sodium channel blocking activity, no concrete mechanism of action was described for porpoisamides, however, no deep research has been been performed in this respect. In spite of that, porpoisamides and palmyramides were found to possess similar cytotoxicity, while palmyramide possess weak to moderate cytotoxicity against neuro-2a and H-460 cells (IC_{50} 17 μM and 40 μM, respectively), Porpoisamides A and B exhibit similar potency against HCT-

116 colorectal carcinoma with IC_{50} 25 µM and 21 µM, respectively and U2OS osteosarcoma cells with IC_{50} 28 µM and 22 µM, respectively[109].

Interestingly, porpoisamides possess Ala next to the fatty acid moiety, which has been previously discussed to lower the bioactivity in Class O-2 lipopeptides. **Based on the current knowledge and bioactivities observed in porpoisamides and palmyramides we can estimate that the mode of action of these lipopeptides is largely independent of the structure of the fatty acid moiety and that the overall 3D structure plays a more significant role.** Some evidence suggests that the presence of aromatic side chains is important (see N-2b), but this is not described for porpoisamides.

Subclass N-2b

Subclass N-2b is characterized by the presence of the motif TZa-FA-HMPa/HIVa/La. This subclass contains only Ulongamides A-F isolated from apratoxin-producing cyanobacterium *Lyngbya* sp. NIH 309 and possess the

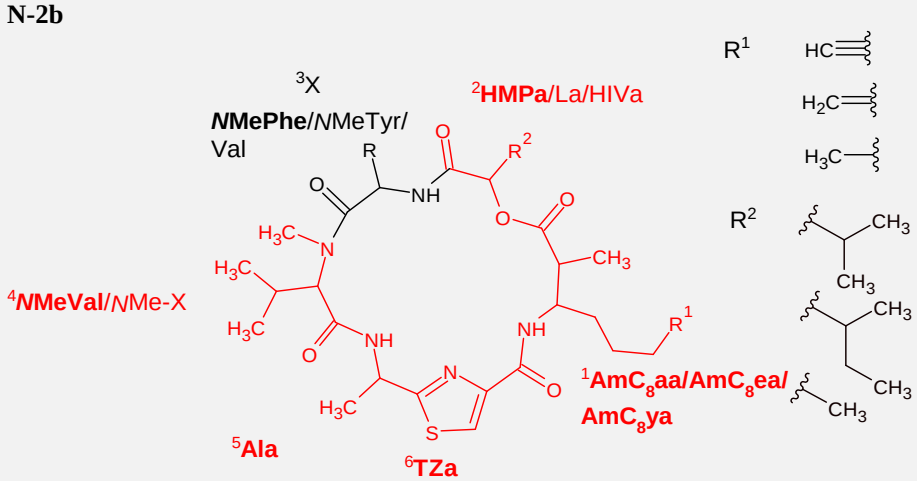


Figure 15: General structure FA-HMPa/La/HIVa-X-*N*MeX-Ala-TZa of lipopeptides belonging to Subclass N-2b

general structure of ***FA-HMPa/La**-X-*N*MeX-**Ala-TZa***[110] (see Figure 15). While *N*MePhe-Ala is found in Subclass N-2a, N-2b contains mostly a *N*MeVal-Ala dipeptide sequence.

Interestingly, a similar chemical structure was described as obyanamide[111], a cyanobacterial pentapeptide with a structure of Apa-La-*N*MeVal-*N*MePhe-Ala-TZa where Apa represents β-amino pentanoic acid. As lipopeptides in this work are defined as structures containing fatty acids with more than five carbons in the longest linear chain, this compound cannot be considered a cyclic lipopeptide. Despite the shorter aliphatic chain, obyanamide exhibits strong to moderate cytotoxicity against KB and Lovo cells (IC_{50} 1 μM and 5.2 μM, respectively), which is almost identical to Ulongamides A-E (IC_{50} 1.0 μM and 5.0 μM for KB and Lovo, respectively[110]). While the observed variation in the fatty acid moiety does not significantly change bioactivity, variation in amino acids shows a more significant effect. Especially the presence of aromatic residue seems to be important as Ulongamide A-E containing Phe or Tyr retains a similar cytotoxicity, while Ulongamide F (possessing Val instead of aromatic residue) does not exhibit any activity even in the highest concentration tested (10 μM). Interestingly, some ulongamides (Ulongamide B-E) contain residue with a free OH group (Tyr instead of Phe), but this increase of polarity has no effect on cytotoxicity. **Therefore, we can assume that regarding Ulongamides the presence of aromatic functionalization is essential for bioactivity while fatty acid moiety can vary.**

Subclass N-2c

The last subclass, N-2, contains lipopeptides of various sizes, ranging from the relatively small heptapeptides ulongapeptins up to the biggest known cyclic cyanobacterial lipopeptide Malevamides C. These lipopeptides contain hydroxy acid adjacent to the incorporated beta-amino fatty acid and do not fulfill the requirement for being classified as N-2a or N-2b. These lipopeptides share a general structure of ***FA-HMPa/La/HIVa**-X-X-***NMe-X**-(X-X)-***NMeX-Ala/Leu/Ile/Val**-(X-X-X)-(X)-***NMeX*** (see Figure 16)

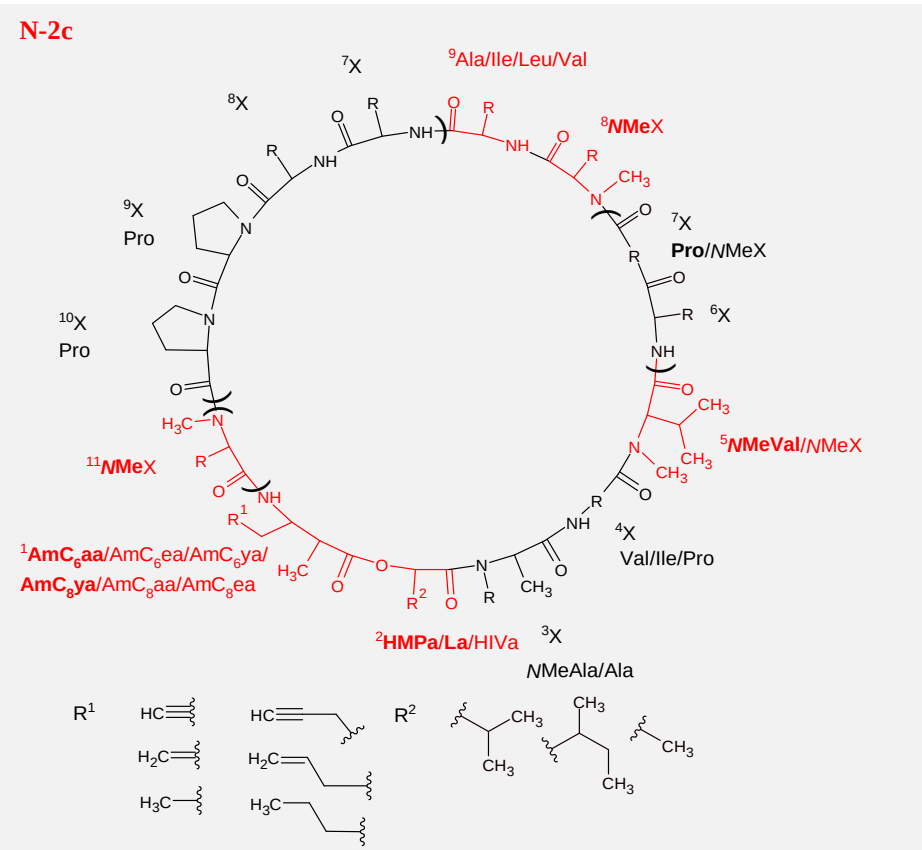


Figure 16: General structure *FA-HMPa/La/HIVa-X-X-*N*MeX-(X-X)-*N*MeX-Ala/Leu/Ile/Val-(X-X-X)-(X)-NMeX* of lipopeptides belonging to Subclass N-2b

where X represents either Ala/Val/Ile/Leu, Pro, or *N*-methylated residues. The number of the residues in the cycle varies with ulongapeptins containing

7 residues[112], companeramides 11 residues[113], and Malevamide B 14 residues[60]. It is remarkable that in all of these compounds, regardless of size, residue 5 is occupied by conserved *N*-methylated Val.

Incorporated fatty acids are short (hexanoic to octanoic acid, optionally with a terminal triple bond, $AmC_{6/8}$aa or -ya) and unsubstituted. The presence of such a FA residue together with multiple *N*-methylation further supports the overall nonpolar and hydrophobic character of these compounds.

The cytotoxicity of these compounds is anticipated to be similar to lipopeptides belonging to O-2 and O-3 classes, however, the majority of these lipopeptides has been tested against human cancer cell lines only at a very low concentration of 1 μM. Because many other lipopeptides from the above-mentioned classes exhibit bioactivities in concentrations above 1 μM it is not possible to make relevant comparisons and conclusions. Similarly, ulongapeptin has not been tested against *Plasmodium falciparum*, while the similar structure belonging to O-classes possesses this bioactivity. There is a huge insufficiency in published data even for the biggest lipopeptides Malevamide B and C.

Subclass N-2c represents an interesting group of compounds, **but the absence of cytotoxic assays at higher concentration** (1-20 μM and above) **leads to incertitude in the parts of the molecules responsible for the action and further research is required.**

Ulongapeptin

Ulongapeptin A, the smallest member of N2c, isolated from the Palauan marine cyanobacterium *Lyngbya* sp. is a cyclic lipopeptide with a high number of Val residue, and fatty acid with an acetylene termination. Compared to other N-2c members ulongapeptin does not contain incorporated

Pro. Additionally, no *N*-methylated residue adjacent to the fatty acid is present. Ulongapeptin A has proven to be cytotoxic against KB cells with a IC_{50} value of 0.63 μM[112]. Antiparasitic properties have yet to be evaluated.

Companeramides

Recently discovered companeramides are decapeptides with a general structure of ***FA-HIVa**-*N*MeAla-Ile/Val-***N*MeVal**-Ile-Pro-*N*MeIle/*N*MeVal-Ala/Val-***N*MeVal***, where FA stands for AmC_8ya[113]. Companeramide A and B isolated from a marine cyanobacterium *Leptolyngbya* collected from the Coiba National Park, Panama. Companeramides show antiparasitic activity against *Plasmodium falciparum* (IC_{50} 230 nM for companeramide A and 1 μM for companeramide B) but were not cytotoxic to four human cancer cell lines (H460, SF-295, MDA-MB-231 and SK-OV3) at concentrations below 1 μM[113].

Malevamide B-C

While malevamides A, D, and E are linear hepta- or octapeptides with an attached fatty acid chain, malevamides B and C are cyclic tri- or tetradecapeptides with a general structure of ***FA-HMPa/La/HIVa**-X-X-***N*Me-X**-X-X-***N*Me-X-Ala/Leu/Ile/Val**-X-X-Pro-(X)-***N*MeX***[60,61,124].

Malevamides represent a group of cyclic lipopeptides with a particular disproportion between the length of the fatty acid moiety (6-8 carbons) and number of residues (13-14). The compounds consequently do not possess amphiphilic properties typical for other lipopeptide structures. The hydrophobicity of the structure is further supported by the absence of charged amino acid and the methylation of free hydroxyl groups as well as the multiple methylation of peptide bonds.

Malevamide B-C were isolated from the marine cyanobacterium *Symploca laete-viridis*. Only a simple bioactive screening has been performed where malevamides B-C were found to be inactive against P-388, A-549, and HT-29 cancer cells at the highest concentration tested (1.4 μM)[60].

Class N-3 (muscotoxins)

Class N-3 comprises a wide range of undecalipopeptides with a general structure of ***FA-Gln-Gly-Pro/mPro**-X-X-**Ser-Dhb-Ser/Thr**-X-**Pro/mPro*** (see Figure 17) and introduce a unique cyanobacterial class of cyclic lipopeptides that have probably no analogous structures in the bacterial and fungal strains. The fatty acid moiety bears multiple hydroxylation and additionally a complex substitution of C5 hydroxyl of FA is described. Similarly, two hydroxyl-containing amino acids present in positions 7 and 9 are usually separated by Dhb. The conservation of this region among different N-3 lipopeptides is in contrast with the high variability of neighbouring amino acid positions (Phe/Pro at position 4, Ile/Phe/*O*Me-hTyr at position 10). Incorporated FA is either 5-OH-Ahc$_{10}$aa (muscotoxins[23]) or 5,7,8-(OH)$_3$-10-Me-Ahc$_{11}$aa (other N-3 lipopeptides). In pahayokolides[115,116], Schizotrin A[117] and portamides[118] hydroxyl at 5C carbon is facultatively modified by *N*-methylation and simultaneously *N*-acylated amino acid residue is bound via an ester bond. Last but not least, in tychonamides[68], fatty acid is terminated by an aromatic benzene ring making the residue similar to Adda found in microcystins and nodularins. While microcystins and nodularins are historically not considered as lipopeptide but rather hybrid peptides, Tychonamides with a structure of amino acid sequence identical to portoamides should be seen as muscotoxin type N-3 of cyclic cyanobacterial lipopeptides.

Cyanobacterial lipopeptides of Class N-3 clearly show the important relationship between the structure of fatty acid moiety and overall bioactivity. Muscotoxins with only slightly modified and unbranched 5-OH-AhC$_{10}$aa permeabilize biological membranes likely based on the interaction with the plane of the biological membrane, as artificial liposomes composed solely of phospholipids components are also affected[23].

On the other hand, lipopeptides with 5,7,8-(OH)$_3$-10-Me-C$_{14}$a lack the general

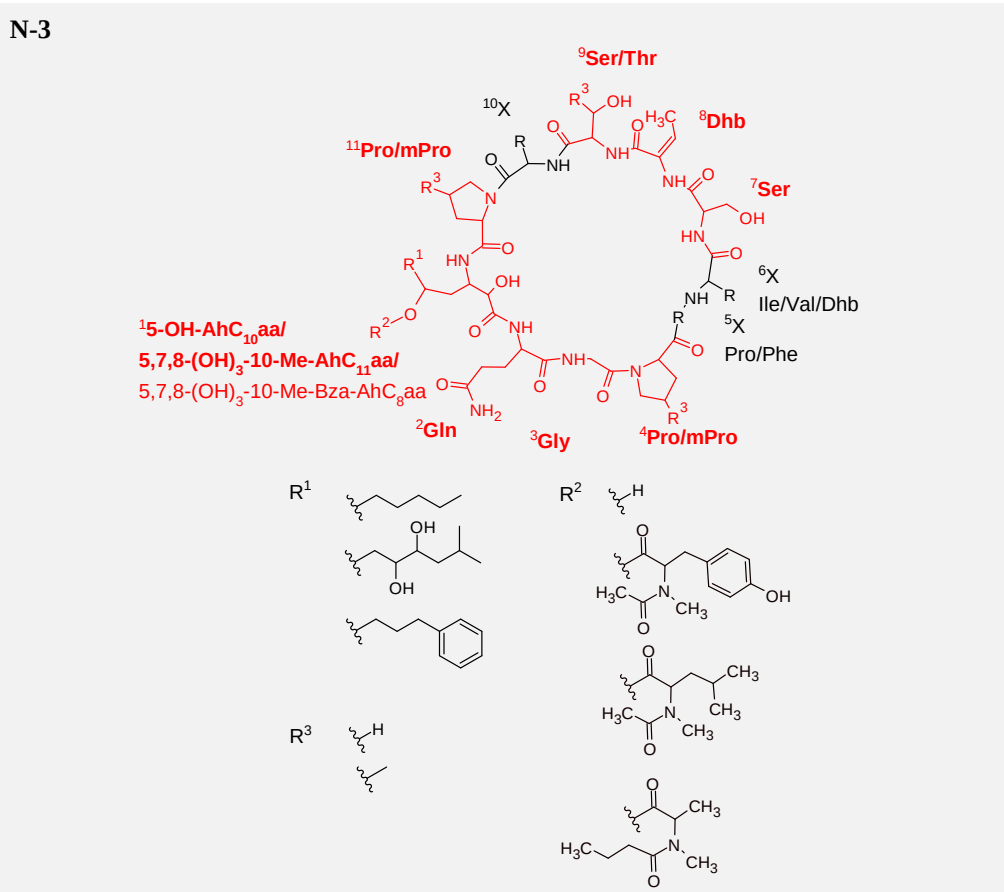


Figure 17: General structure *FA-Gln-Gly-Pro/mPro-X-X-Ser-Dhb-Ser/Thr-X-Pro/mPro* of lipopeptides belonging to Subclass N-3

membrane permeabilization effect, affect mitochondria in human cells, and show high selectivity and unique antibacterial activity among cyanobacterial

cyclic lipopeptides. Antibacterial properties are significantly influenced by the amino acyl modification of the 5-OH group which results in a different mechanism of the action compared to muscotoxin. Ribeiro et al.[118] suggest that portoamides with substituted FA mostly affect the energetic metabolism in human cells via a bias of the mitochondrial electron transport chain, showing an increase in mitochondrial polarization and a decrease in ATP production which, however, does not cause oxidative stress or an increase of ROS or mito-toxicity. **Based on recent research we can conclude that the mode of action of muscotoxin type lipopeptides depends largely on FA moiety properties and its substitution.**

Muscotoxins

The recently discovered Muscotoxin A-C[23,117] group was isolated from the soil cyanobacterium *Desmonostoc muscorum* (wet meadow soil, Chelčice, Czech Republic) with a general structure of ***FA-Gln-Gly-Pro/mPro-Phe**-Ile-**Ser**-**Dhb**-**Ser**-Ile-**Pro/mPro***. The only alteration in the structure beside the Ile/Val variation is the facultative γ-methylation of the first, second, or both proline residues. Muscotoxin A is cytotoxic to YAC-2, SP/2, and HeLa cells with IC_{50} 13.2 μM, 11.3 μM, and 9.9 μM, respectively[23], causing membrane disruption in a cholesterol-independent way. The incorporation of muscotoxin into the membrane causes a decrease in both membrane elasticity and fluidity. Further, at higher concentrations (5-25 μM), an increase in intracellular Ca^{2+} concentration is observed[23]. Consequently, the fatty acid part of the molecule is essential for bioactivity. Muscotoxins also show antifungal activity against the plant pathogens *Alternaria alternata*, *Monographella cucumerina,* and *Aspergillus fumigatus*, with minimum inhibitory concentration values of 0.48, 1.93, and 1.93 μM[119] respectively.

Portoamides and Lyngbyazothrins

Portoamides A-D, recently isolated from cyanobacterium *Oscillatoria sp.*[118,120] with a general structure of ***FA-Gln-Gly-Pro**-Pro-Ile-**Ser-dhb-Thr**-hPhe/hTyr-**Pro*,** share most of their amino acid building units with muscotoxins, and possess, however, more substituted fatty acids (5,7,8-OH_3-10Me-C_{14}aa). Portoamides have a prominent effect on the proliferation of human cancer cell lines as well as on immortalized, non-carcinogenic cells: MG-63, HCT116, RKO, HT-29, hCMEC-D3 and HaCaT (IC_{50} 6.03 μM, 3.38 μM, 3.02 μM, 0.98 μM, 3.61 μM, and 2.82 μM, respectively). Exposure to portoamides influences the expression of cytoplasmic and mitochondrial proteins, mainly proteins involved in the mitochondrial respiratory chain and pentose phosphate cycle, and thus affecting the mitochondrial morphology and metabolism (protein NDFUFS3 and 14-3-3 proteins decrease the production of ATP and influence the mitochondrial redox potential)[118]. However, their effect is not connected to mito-toxicity and they do not cause mitochondrial dysfunction via oxidative stress.

Additionally, portoamides show a significant synergic effect of both known portoamide variants in algae. While the pure compounds exhibited almost no algicidal activity, a mixture of Portoamide A and Portoamide B exhibit weak bioactivity and completely inhibits the growth of *Chlorella vulgaris* at 20 μM. Additionally, growth inhibition of the microalgae *Ankistrodesmus falcatus*, *Chlamydomonas reinhardtii*, and cyanobacterium *Cylindrospermopsis raciborskii* with IC_{50} of around 8.4 μM[120] was recorded.

Lynghbyazotrhins A-D are isolated from *Lyngbya* sp. 36.91 as a mixture of A+B and C+D variants are structurally fully identical to portoamides. Consequently, these mixtures show similar activity. Mixture A+B possess only low antimicrobial activity against *Micrococcus flavus,*

mixture C+D shows a moderate effect in agar diffusion against *Bacillus subtilis,* and *Escherichia coli* and low activity against *Pseudomonas aeruginosa and Serratia marcescens.* Neither mixtures A+B nor C+D were active against yeast *Candida maltosa.*[30]

Pahayokolides

Pahayokolides A-B[115] purified from a new species of cyanobacteria of the genus *Lyngbya* possess a general structure of *__FA__-__Gln__-__Gly__-__Pro__-Phe-Dhb-__Ser__-__Dhb__-__Thr__-hPhe-__Pro__*. Further Pahayokolide A includes *N*-acetyl-*N*MeLeu attached via an ester bond to 5-OH of the fatty acid moiety. Pahayokolide A was shown to inhibit a number of cancer cell lines over a large range of concentrations (IC_{50} varies from 2.13 μM to 44.57 μM) depending on the cell-type. The highest cytotoxicities were observed against H460, A498, SK-OV-03, SK-MEL-28, and CEM human cancer cell lines (IC_{50} 2.13, 2.61, 2.76, 3.27, and 5.74 μM, respectively)[116], lower cytotoxicity was reported for U251 and SKBR3 cells (IC_{50} 13.33 μM, and 16.70 μM, respectively) and a low effect was observed in HT29 (IC_{50} 44.57 μM). At concentrations higher than 3.5 μM, pahayokolide A was toxic to zebrafish embryos, while brine shrimp toxicity was observed only at the highest measured concentration (2.5 mM). The mechanism of action of pahayokolides is not described, however because of the structural similarity with Portoamides, we can assume that even the mechanism of pahayokolides will be similarly related to mitochondrial dysfunction.

Schizotrin A

Schizotrin A is the major active metabolite of the cultured cyanobacterium *Schizotrix* sp. with a structure of *__FA__-__Gln__-__Gly__-__Pro__-Phe-

Val-**Ser-dhb-Ser**-hTyr-**Pro***[117]. Its structure is very similar to pahaykolides and the main difference seems to be the presence of branched Val instead of unsaturated Dhb. Schizotrin A possesses moderate antibacterial activity against *Bacillus subtillis* and weak activity against *Saccharomyces cerevisiae, Sclerotium rolfsii, Fusarium oxysporum, Colletotrichum gloeosporioides* but the additional cytotoxicity tests have yet to be performed on human cell lines[117].

Tychonamides

Tychonamides A-B purified from cyanobacterium *Tychonema* sp.[68] (isolated from a wastewater pond, Braunschweig, Germany) possess the same amino acid sequence as portoamides. The only distinction is a different acyl substitution of the fatty acid moiety (Figure 17). Tychonamides showed strong cytotoxic potencies against multiple human cancer cell lines. In total, activity against 37 lines has been tested in propidium iodide essays with IC_{50} 0.2-0.9 μM for Tychonamide A and 0.7-9.5 μM for Tychonamide B. Additionally, the antiproliferative activity of Tychonamide B was evaluated on 22 human tumor xenografts with IC_{50} 0.1-2.7 μM[68]. Both Tychonamide A and B possess antiprotozoal activity against *Plasmodium falciparum, Trypanosoma cruzi, Leishmania donovani,* and *Trypanosoma brucei* with IC_{50} 0.07-2.8 μM and 0.16-3.7 μM, respectively[68].

Class N-4 (minutissamide type)

Class N-4 includes exclusively decapeptides similar to the bacterial lipopeptides of Iturin group[35]. Their general sequence is ***FA-Val**-X-X-X-**Gln/Asn-Gly/Ala**-X-***N*MeAsn-Pro*** (see Figure 18). It contains two groups of lipopeptides, minutissamides[65,122] and puwainaphycins[25,122]

biosynthetized via NRPK/PKS machinery[15]. Despite the different designation in name, both types of lipopeptides possess a similar variation in amino acid and fatty acid composition. Incorporated modified β-amino-fatty acid is further hydroxylated on C2 carbon and methylated on C4 carbon in all known puwainaphycins and minutissamides (4-Me-AhC$_{12\text{-}18}$aa).

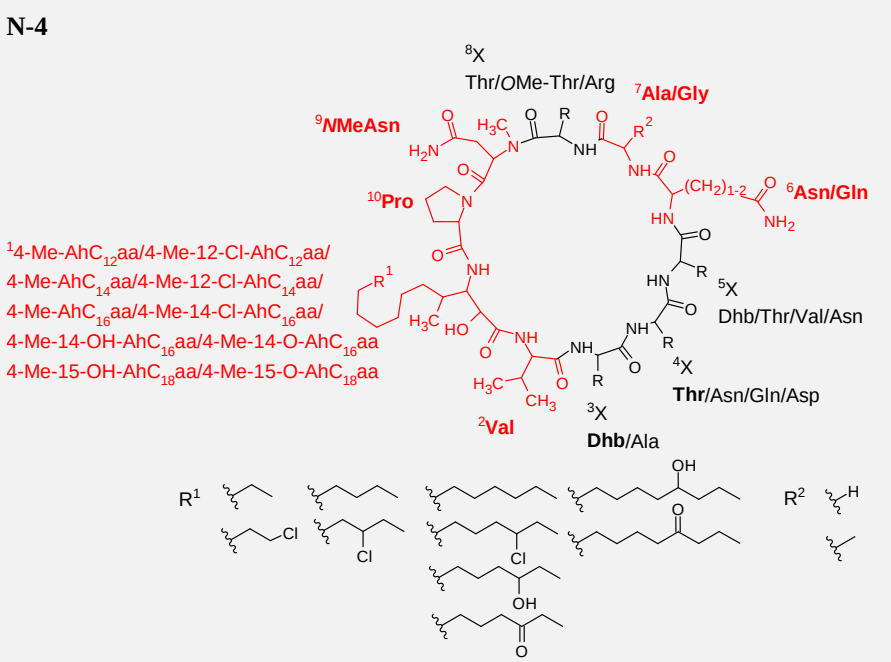


Figure 18: General structure *FA-Val-X-X-X-Gln/Asn-Gly/Ala-X-NMeAsn-Pro* of lipopeptides belonging to Subclass N-4

While the amino acids adjacent to the fatty acid are conserved among the N-4 group members, creating an *N*MeAsn-Pro-FA-Val invariable region, the other residues highly vary. Hydroxy-containing Thr and nonpolar Asn might alternate in position 4, while in position 5 Dhb, Thr, and Val can be found. These variations alter the overall hydrophobic/hydrophilic properties (introduction or removal of free OH groups), yet the cytotoxicity remains similar. The only exception is Calophycin with Asp and Arg residues forming

a significantly more hydrophilic amino acid core which likely results in a broad antifungal activity.

Generally, modifications to the fatty acid chain play a more distinct role in biological activity compared to amino acid composition. The introduction of hydrophilic substituent near the FA terminus significantly lowers cytotoxic activity, while prolongation, chlorination or the presence of ketone does not have a measurable effect. This behavior was partially explained by Hrouzek et al.[25], who proved that the bioactivity is based on the membrane permeabilization effect as shown in Puwainaphycin F/G. These lipopeptides interact with cell membranes. Once incorporated, the increased fluidity and subsequent permeabilization of the membrane is followed by an elevation of intracellular Ca^{2+} similarly to bacterial lipopeptide Daptomycin[33]. Consequently, the presence of a polar group on the fatty acid does not allow incorporation of the lipopeptide into the membrane due to its charge and the formation of hydrogen bridges and thus lowers overall cytotoxicity. Additionally, some puwainaphycin congeners possess cardioactive properties.

To conclude, the effect of minutissamides and puwainaphycins depends primarily on the β-amino-α-hydroxy-fatty acid moiety and its interaction with cell membranes. The mechanism of action of Calophycin remains unclear.

Puwainaphycins A-G

Puwainaphycins are isolated from soil *Anabaena*, *Symplocastrum* and *Cylindrospermum*[25,122], and share a general structure of ***FA-Val**-Dhb-X-X-**Gln/Asn-Gly/Ala**-X-***N*MeAsn-Pro***. Apart from chlorinated Puwainaphycin C being characterized by an ionotropic effect with ED_{50} 0.1ppm in mouse atria[45] and puwainaphycin F/G causing necrotic cell death via membrane disruption[25] with IC_{50} 2-3 μM against HeLa cells, little is known about the cytotoxic effect of other puwainaphycins on mammalian cells.

Calophycin

A single structural variant of Calophycin was isolated from *Calotrix fusca* EU-10-1 (freshwater stream, Oahu island, Hawai)[26]. While possessing an unsubstituted *β*-amino fatty acid, namely, 4-Me-AhC_{16}aa and the *N*MeAsn-Pro-FA-Val motiff, the structure *FA-Val-Ala-Asp-Asn-Gln-Gly-Arg-*N*MeAsn-Pro* of Calophycin differs from puwainaphycins and minutissamides. Calophycin is together with Cyanopeptolin the only reported cyclic cyanobacterial lipopeptide with basic amino acid residue (Arg). Additionally, acidic residue is also present (Asp). The overall properties of the cyclic peptidic core possess a significant net charge and much higher polarity compared to minutissamides and puwainaphycins. Calophycin is reported as a broad-spectrum fungicide, with MIC 1.25-2.5 μg/ml against *Candida albicans, Trichophyton mentagrophytes, and Aspergillus fumigatus.* Disc-diffusion assays further show a moderate inhibition of *Aspergillus oryzae, Penicillium notatum, and Saccharomyces.cerevisiae* at very low concentration 1.2 μg/disc. The cytotoxicity of Calophycin is reported against KB cells with IC_{50} of 0.24 μM[26].

Minutissamides A-L

Minutissamides, decalipopeptides isolated from *Anabaena minutissima*, *Cylindrospermum* sp. and other strains[65,122] share a general structure of ***FA-Val-Dhb**-Asn/Thr-Thr/Val-**Gln/Asn-Ala/Gly**-Thr-***N*MeAsn-Pro***. Minutissamides have been evaluated for their antiproliferative activities in several human cancer cell lines. Minutissamides A-D are characterized as cytotoxins with biological activity against HT-29 human colon cancer cell lines (IC_{50} 2.0, 20.0, 11.8 and 22.7 μM, respectively)[65], Minutissamide E-J manifest their activity in the MDA-MB-435 human melanoma cancer cell line (IC_{50} 2.9, 1.2, 9.9, 1.1, 2.9, and 2.6 μM, respectively)[121], as well as a mixture of Minutissamide K and L showing cytotoxicity against MDA-MB-435 (IC_{50} 2.9μM)[121]. From the above summarized data we can conclude that the lowest cytotoxicity is observed for hydroxylated variants Minutissamide D and Minutissamide G and the variants possessing short-chained substituted FA (Minnutisamide B).

Class N-5 (laxaphycins)

The last group of lipopeptides, class N-5, is based on two completely different compounds, Laxaphycin A and Laxaphycin B[123] and can be characterized by two general structures:***FA**-X-X-**Pro/OH-Pro-hSer-Phe-Leu**-X-X-X-**Gly*** for Subclass N-5a (see Figure 19) and ***FA**-X-X-X-X-**Gln-Ile**-X-**Thr-Pro**-X-**Thr*** for Subclass N-5b (see Figure 20). As is obvious from the general structure, laxaphycin types of lipopeptides contain many rare hydroxylated amino acids (OH-Leu, OH-Asn, OH-Pro) as well as unusual hSer, therefore their overall structure is highly polar and represents one of the most hydrophilic non-glycosylated cyanobacterial cyclic lipopeptides.

Members of both subclasses are co-biosynthesized via one biosynthetic gene cluster as described by Heinilä et al.[124]. The NRPS/PKS cluster contains a common starter FAAL-containing module and PKS enzyme elongation and modification of the FA by the β-amino group. Subsequently the biosynthetic pathway is branched into Laxaphycin A (Subclass N-5a) and Laxaphycin B type (Subclass N-5b) of NRPS assembly, resulting in the co-biosynthesis of compounds from both compound types in one strain[124]. Comparing both subclasses, Laxaphycin A type possesses in general a lower amount of free hydroxyl groups (2-4 free hydroxyls) than Laxaphycin B type (4-7 hydroxyls). Interestingly, the mixture of compounds generated by the individual pathways shows a synergic antifungal effect as demonstrated for many members of Class N-5 lipopetides. A 1:1 mixture of Lobocyclamide A and B shows MIC 10-30 μg/ml (in microbroth dilution assay against *Candina Albicans* 96-489) compared to the non-antifungal activity of pure Lobocyclamide A and the weak antifungal effect of pure Lobocyclamide B (MIC 30-100 μg/ml)[125]. The same relationship is found for scytocyclamides, where pure scytocyclamides exhibit no to very low antifungal activity, while their mixtures possess moderate antifungal activity (15mm inhibition in *Aspergillus flavus* compared to a hardly recognizable inhibition in pure scytocyclamides)[125]. However, an exception is found in trichormamides, where no synergic cytotoxic or antifungal effect of Trichormamide C and D was observed despite Trichormamide C belonging to N-5b and Trichormamide D to Subclass N-5a[126].

Subclass N-5a (Laxaphycin A type)

Subclass N-5a is defined by a general structure of ***FA**-X-X-**Pro/OH-Pro-Ser/hSer-Phe/Tyr-Leu**-X-X-X-**Gly*** (see Figure 19)**,** where FA stands for fully saturated AC_8aa or AC_{10}aa bearing only β-amino and no other

substitution. The residues adjacent to fatty acid are mostly Gly and hSer/Ser bound to the amino- and carboxyl- groups of FA, respectively. Only in the case of Trichormamide D[126] and Scytocyclamide A[127] is Gln present, bound to the FA carboxyl group. A third residue is conserved (Dhb) in all known Laxaphycin A type lipopeptides except Trichormamide A. Generally, part of the molecule opposite the fatty acid is highly conserved (Pro/OH-Pro-Ser/hSer-Phe/Tyr-Leu). This part of the molecule possesses a high ratio of polar substituents, which is unusual for cyclic lipopeptides. Hydroxyl groups positioned in this area seem to be important as compounds possessing hydroxylation of Pro (forming OH-Pro) lack hydroxylation on Phe (forming Tyr) and vice versa. The exception is Lobocyclamide A containing both

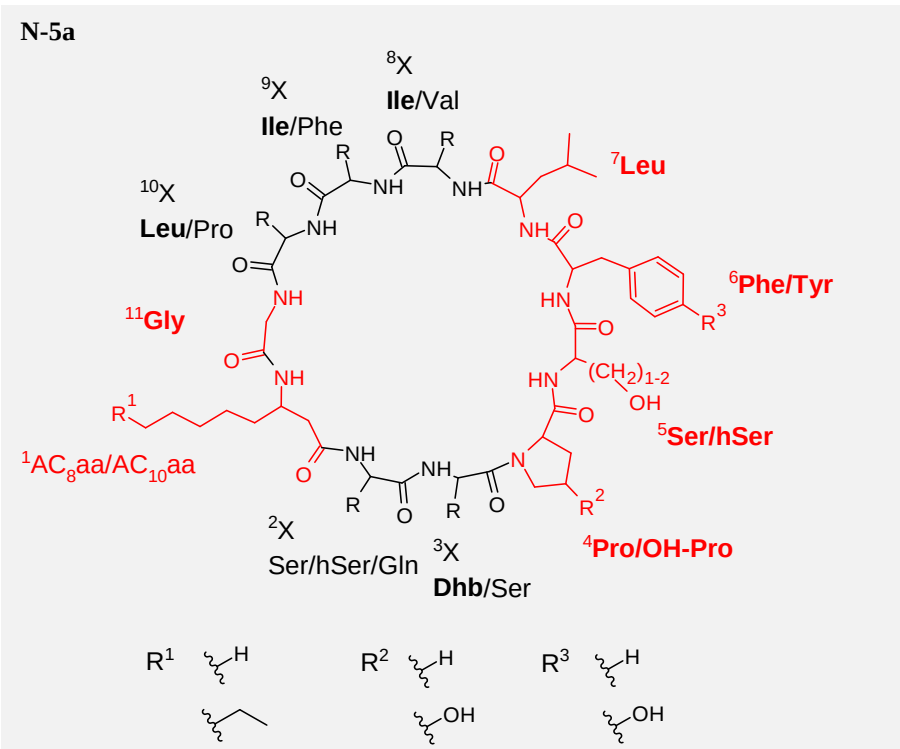


Figure 19: General structure *FA-X-X-Pro/OH-Pro-Ser/hSer-Phe/Tyr-Leu/X-X-X-Gly* of lipopeptides belonging to Subclass N-5a

hydroxyproline and Tyr[125]. However, a distinct positioning of hydroxylation

seems not to be crucial for the effect in human cells as Laxaphycin A and Trichormamide A possess similar anticancer activity while differing in OH substitution. Scytocyclamide A has been proven to cause cell membrane permeabilization (suggesting membrane damage and necrotic cell death). However, further experiments proved asymmetric distribution of phosphatidylserine in the plasma membrane and the reduction of mitochondrial membrane potential visible together with observed "blebbing" after the treatment apoptotic type of cell death.

Laxaphycins (A type)

Laxaphycins A is one of the major components in an antifungal mixture of cyclic peptides from the terrestrial cyanobacterium *Anabaena laxa*[28,125] and marine *Lyngbya majuscula*[128]. Besides the major variant, Laxaphycin A, congener A2 (Ile => Val exchange) and E (AC_8aa is replaced by AC_{10}aa) are described[129]. Laxaphycin A and A2 show only weak cytotoxicity against HCT-116 cancer cells (IC_{50} 23.0 and 29.0μM, respectively)[129].

Hormothamnin is a lipopeptide isolated from the tropical marine cyanobacterium *Hormothamnion enteromorphoides*. Hormothamnin A is the compound responsible for cytotoxic activity of the strain extract[130]. Its structure is identical to Laxaphycin A and E differing only in the stereochemistry of Dhb (*Z*-Dhb in case of Hormothamnin, *E*-Dhb in case of Laxaphycin A)

Lobocyclamides (A type)

Lobocyclamide A is produced by *Lyngbya confervoides*[125] isolated from a benthic mat in the Bahamas. While the lobocyclamide cytotoxic activity was not studied in detail the antifungal properties were observed against *Candida albicans* 96-489. Lobocyclamide A shows the smallest inhibition zone 7 mm (150μg/disk) compared to lobocyclamides belonging to B-type (see below). Lobocyclamide A is the only known member of N-5a class possessing two free hydroxyl groups in the region opposite the fatty acid (OH-Pro as well as Tyr).

Trichormamide (A type)

Trichoramides A and D were isolated from the cultured freshwater cyanobacterium *Trichormus* sp. IUC 10339[126,131] isolated from Raven Lake, North Wisconsin. Trichormamide A is the only known member of N-5a class with Ser instead of Dhb in position 3, showing weak cytotoxicity and antiproliferative activities against HT-29 and MDA-MB-435 (IC_{50} 16.9 μM and 9.9 μM, respectively)[131]. Similar cytotoxicity was reported for Trichormamide D (IC_{50} 11.5 μM and 11.7 μM, for the above-mentioned cell lines, respectively)[126]. Trichormamide D contains Gln instead of Ser/OH-Ser on position 2, thus having slightly more nonpolar characteristics, however, the effect of such substitution on bioactivity is neglectable.

Scytocyclamide A (A type)

Scytocyclamide A is one of the bioactive metabolites in *Scytonema hofmanni* PCC7110[127] isolated from the limestone Crystal Caves in Bermuda. The cytotoxic potency of the compound was not determined, however, exposition of Scytocyclamide A at a concentration of 10 μM causes

rapid blebbing and membrane disruption in 3T3 cells. Only weak antifungal properties are observed.

Subclass N-5b (Laxaphycin B type)

Despite sharing the identical gene cluster with A type laxaphycins (as mentioned above), subclass N-5b is defined by a totally different peptide sequence, with a general structure of ***FA**-X-**OH-Leu**-X-X-**Gln**-***N*MeIle**-X-**Thr-OH-Pro/Pro**-X-**Thr*** (see Figure 20)**.** Longer fatty acid ($AC_{10}aa$) present in Laxaphycin B and other B analogues[123], Lobocyclamides A-B[125] and Trichormamide B-C[131,132] is more frequently incorporated compared to

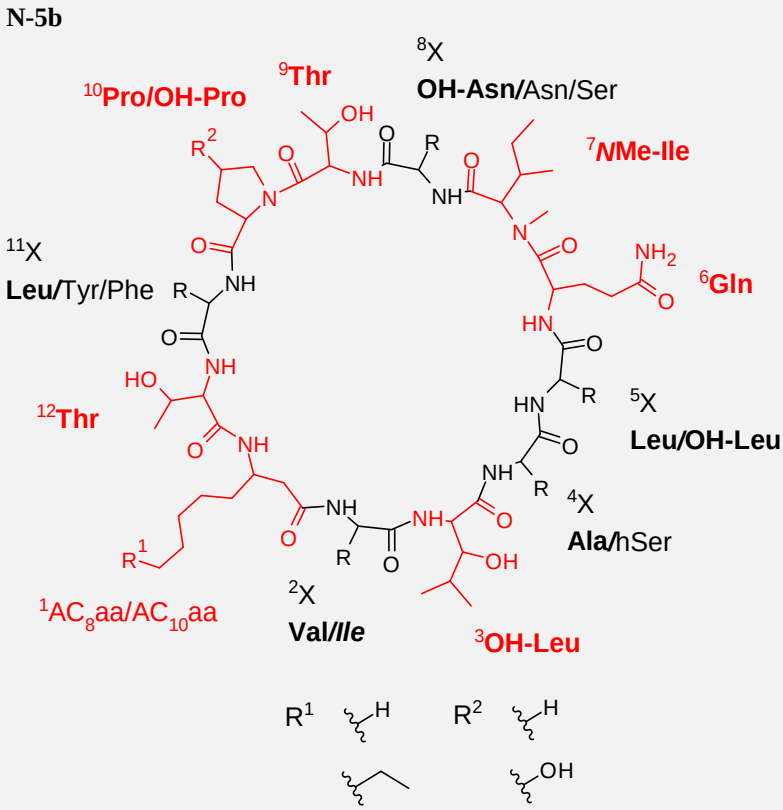


Figure 20: General structure *FA-X-OH-Leu-X-X-Gln-*N*MeIle-X-Thr-OH-Pro/Pro-X-Thr* of lipopeptides belonging to Subclass N-5b

AC_8aa acid present in Laxaphycin D, Lobocyclamide C, and Scytocyclamides B-C. Lipopeptide within Class N-5b frequently contains rare amino acids, especially unusual hydroxylated residues (OH-Leu, OH,OH-Leu, OH-Asn, hSer, or OH-Pro). Generally, these lipopeptides contain 4-7 free hydroxyl groups and thus have a high dipole moment between the nonpolar FA chain and the polar amino acid core which results in an anomalously amphiphilic lipopeptide molecule. However, a difference in OH-substitution seems not to play a crucial role for the bioactivity, as e.g. Trichormamide B containing seven free hydroxyl groups possesses a similar cytotoxicity to Trichormamide C with five free hydroxyl groups.

Laxaphycins (B type)

Laxaphycins B and D are additional components of a cyclic lipopeptide antifungal mixture in the terrestrial cyanobacterium *Anabaena laxa*[28] or marine representative *Lyngbya majuscula*[128]. Laxaphycin B and an additional 5 congeners B2-B6 has been described to be mostly differing in variable residue 3 (Ile/Val), 5 (Leu/OH-Leu), 8 (Asn/OH-Asn), and 10 (Pro/OH-Pro). Cytotoxicity seems again not to be dependent on the amino acid composition and a number of free hydroxyl groups such as Laxaphycin B (5 free hydroxyl groups and Ala at position 4) and Laxaphycin B4 (7 free hydroxyl groups containing OH-Ser on the same position) exhibit similar bioactivity against HCT116 with IC_{50} 1.11 μM and 1.7 μM for the B and B4 variant, respectively[123].

Lobocyclamides (B type)

Lobocyclamide B-C are produced by *Lyngbya confervoides* together with Lobocyclamide A[125]. Lobocyclamide B-C share identical structure except for the fatty acid moiety, AC_{10}aa vs. AC_8aa acid is present in Lobocyclamide B and C, respectively. No cytotoxic data are available for neither Lobocyclamide B nor C, however, slightly higher antifungal activity against *Candida albicans* 96-489 compared to Lobocyclamide A is reported (150 μg/Disk, inhibition zone 8mm and 10mm)[125].

Lyngbyacyclamides

Lyngbyacyclamides A-B were isolated from the Okinawan marine cyanobacterium *Lyngbya* sp.[132]. They exhibit cytotoxicity against B16 mouse melanoma cells with IC_{50} 0.7 μM, but no toxicity in brine shrimp assay up to the highest concentration tested (70 μM) [132].

Trichormamide (B type)

Trichoramide B and C[126], congeners isolated together with other trichormamides from the cultured freshwater cyanobacterium *Trichormus* sp. IUC 10339, do not share a common amino acid composition. Variation is observed at 4 different residues and includes substitution of Ala by OH-Ser, Ser by Asn, or Tyr by Leu, amino acids differing in size, charge, and polarity. However, the cytotoxicity of the variants remains similar independently of these variations. Trichormamide B (IC_{50} 0.8 μM and 1.5 μM against MD-MB-435 and HT-29, respectively) has a comparable cytotoxicity to Trichormamide C (IC_{50} 1.7 μM and 1.0 μM)[131,132].

Scytocyclamide B (B type)

Scytocyclamide B and C[127] are isolated from *Scytonema hofmanni* PCC7110 and are structurally similar to Laxaphycin D. They possess an almost identical structure to AC_8aa, except for residue 5, where Leu is not hydroxylated. The cytotoxicity for B-C variants was not determined, but at a concentration of 10 μM causes rapid blebbing and membrane disruption in 3T3 cells identically to Scytocyclamide A[127].

General remarks on the cyanobacterial cyclic lipopeptide toxicity

Cyclic lipopeptides are widely distributed among different cyanobacterial species, particularly *Lyngbya majuscula* is a valuable source of abundant miscellaneous lipopeptide structures. Based on this review, we are able to find many structural patterns affecting the bioactivity of this group of compounds.

At first, many of the cyclic lipopeptides are reported as membrane disrupting molecules[25,35,59,79]. The cytotoxicity of these molecules is tightly bound with their lipidic chain, interacting with the cell membrane. Interestingly, all of these bioactive lipopeptides contain a free hydroxyl-group in the proximity of the FA carboxy-terminus. In the case of hassallidins it is represented by α,β-dihydroxyl of the FA residue or free hydroxyls of the saccharide bound to the β-hydroxy group. In the case of anabaenolysins, puwainaphycins and muscotoxins the –OH is present on the FA α-carbon. This indicates the relevance of such a polar group adjacent to the FA moiety likely in interaction with the membrane components. Generally, the IC_{50} values of these lipopeptides range from 0.1 to 25 μM and are not highly cell specific.

Secondly, brine shrimp toxicity, antiparasitic toxicity, and cell-specific cytotoxicity is described for many cyanobacterial cyclic lipopeptides[51,67,90,112,113] with relatively small amino acid cyclic cores. These lipopeptides usually further contain the terminally reduced fatty acids as e.g. C_8ya, C_8ea, and C_6ya (β-amino- as well as β-hydroxy fatty acid). The vast majority of these lipopeptides contain hydroxy acids (HIVa, HMPa, PLa etc.) and a range of branched acids (Val, Leu, Ile, HIVa, HMPa) and aromatic acids (Phe, PLa). Interestingly, among all members of O Classes and N Classes, alternation of the branched Ile/Val by aromatic Phe/PLa is described with no effect on the bioactivity. A specific case is PLa residue. While in some lipopeptide classes the aromatic character is important for the resulting effect (and variation between PLa and Phe is often observed), in other lipopeptides the presence of hydroxyl group containing residue forming the ester bond is more important (and variation between PLa, HIVa, and HMPa is observed). No combined variability (PLa/Phe/HIVa/HMPa) on particular position is described within one lipopeptide class. The structural variability between the lipopeptide classes differs in the size of the ring and in the residue composition. However, some structural motifs remain conserved. Except for N-3 and N-5a, all cyclic cyanobacterial lipopeptides (both O and N Class lipopeptides) contain branched residue adjacent to the fatty acid moiety, mostly amino acid Val/Ile or hydroxy acid HIVa/HMPa.

Table 2: List of known cyclic cyanobacterial lipopeptides

Cyclic lipopeptides with FA as side chain

	(ref)	FA	R2	R3	R4	R5	R6	R7	R8	R9	R10
Class S-1		**FA**	**Asp/Glu**	**Thr**	**X**	**Ahopip**	**X**	**X**	**Val/Ile**		
Cyanopeptolin A	[70]	C_6aas	Asp	Thr	Arg	Ahopip	Val	*N*MePhe	Val		
Cyanopeptolin B	[70]	C_6aa	Asp	Thr	Lys	Ahopip	Val	*N*MePhe	Val		
Cyanopeptolin C	[70]	C_6aa	Asp	Thr	*N*MeLys	Ahopip	Val	*N*MePhe	Val		
Cyanopeptolin D	[70]	C_6aa	Asp	Thr	*N*,*N*Me_2Lys	Ahopip	Val	*N*MePhe	Val		
Class S-2		**FA**	**Thr**	**Thr**	**Thr**	**X**	**Dhb**	***Gln***	**Gly**	**Thr**	**X**
Balticidin C	[27]	Hh-C_{16}aa	Thr	Thr	Thr	OH-Tyr	Dhb	Gln	Gly	Man-*N*Me Thr	Gln
Balticidin D	[27]	3-Ara-Gal-13-Cl-Hh-C_{16}aa	Thr	Thr	Thr	OH-Tyr	Dhb	Gln	Gly	Man-*N*Me Thr	Gln
Hassallidin A	[79]	Hh-C_{14}aa	Thr	Thr	Thr	Tyr	Dhb	Gln	Gly	Man-*N*Me Thr	Gln
Hassallidin B	[79]	3-Rha-Hh-C_{14}aa	Thr	Thr	Thr	Tyr	Dhb	Gln	Gly	Man-*N*Me Thr	Gln
Hassallidin C	[79]	3-Hex-Pen-Hh-C_{16}aa	Thr	Thr	Thr	Tyr	Dhb	Gln	Gly	Hex-*N*Me Thr	Gln
Hassallidin D	[79]	3-Hex-Pen-Hh-C_{16}aa	Thr	Thr	Thr	Tyr	Dhb	Gln	Gly	Hex-*N*Me Thr	Tyr

Cyclic lipopeptides with FA incorporated into the cycle:
β-hydroxy-fatty acid containing lipopeptides

Class O-1		**FA**	**Val/Ile**	**HIVa/HPMa / Pla**	***N*MePhe**	***β*Ala**					
Yanucamide A	[84]	Hm_2C_8ya	Ile	HIVa	*N*MePhe	*β*Ala					
Yanucamide B	[84]	Hm_2C_8ya	Val	HIVa	*N*MePhe	*β*Ala					
Class O-2a		**FA**	**Val/Ile**	**X**	**HIVa/HMPa/PLa**	**Pro**	**X**				
Antanapeptin A	[90]	HmC_8ya	Val	*N*MePhe	HIVa	Pro	*N*MeIle				
Antanapeptin B	[90]	HmC_8ea	Val	*N*MePhe	HIVa	Pro	*N*MeIle				
Antanapeptin C	[90]	HmC_8aa	Val	*N*MePhe	HIVa	Pro	*N*MeIle				
Antanapeptin D	[90]	HmC_8ya	Val	*N*MePhe	HIVa	Pro	*N*MeVal				
Veraguamide A	[51]	8-Br-HmC_8ya	Val	*N*MeVal	HMPa	Pro	*N*MeVal				
Veraguamide B	[51]	8-Br-HmC_8ya	Val	*N*MeVal	HIVa	Pro	*N*MeVal				
Veraguamide C	[51]	HmC_8ya	Val	*N*MeVal	HMPa	Pro	*N*MeVal				
Veraguamide D	[51]	HmC_8ya	Val	*N*MeVal	HMPa	Pro	*N*MeIle				
Veraguamide E	[51]	HmC_8ya	Ile	*N*MeIle	HMPa	Pro	*N*MeVal				
Veraguamide F	[51]	HmC_8ya	Val	*N*MeVal	PLa	Pro	*N*MeVal				
Veraguamide G	[51]	HmC_8ya	Val	*N*MeVal	HMPa	Pro	*N*MeVal				
Veraguamide H	[133]	HmC_8ya	Val	*N*MeVal	HIVa	Pro	*N*MeVal				
Veraguamide I	[133]	HmC_8aa	Val	*N*MeVal	HMPa	Pro	*N*MeVal				

Veraguamide J	(133)	HmC_8ya	Val	*N*MeVal	HIVa	Pro	*N*MeVal				
Radamide B	(91)	HmC_8ya	Ile	*N*MeVal	HMPa	Pro	*N*MeIle				
Hantupeptin A	(88)	HmC_8ya	Val	*N*MeIle	PLa	Pro	*N*MeVal				
Hantupeptin B	(92)	HmC_8ea	Val	*N*MeIle	PLa	Pro	*N*MeVal				
Hantupeptin C	(92)	HmC_8aa	Val	*N*MeIle	PLa	Pro	*N*MeVal				
Trungapeptin A	(93)	HmC_8ya	Val	*N*MeVal	PLa	Pro	Ile				
Trungapeptin B	(93)	HmC_8ea	Val	*N*MeVal	PLa	Pro	Ile				
Trungapeptin C	(93)	HmC_8aa	Val	*N*MeVal	PLa	Pro	Ile				
Naopopeptin	(94)	HmC_8ya	Val	*N*MeVal	PLa	Pro	*N*MeIle				
Class O-2b		**FA**	**X**	**PLa**	**Pro**	**X**	**Val**				
Palmyramide A	(95)	Hm_2C_8ya	La	PLa	Pro	*N*MeVal	Val				
Class O-2c		**FA**	**Val/Ile**	**X**	**HIVa/ HMPa/ PLa**	**Pro**	**X**	**X**			
Kohamamide A	(96)	Hm_2C_8ya	Val	*N*MeVal	HMPa	Pro	Leu	Ala			
Kohamamide B	(96)	Hm_2C_8ea	Val	*N*MeVal	HMPa	Pro	Leu	Ala			
Kohamamide C	(96)	Hm_2C_8aa	Val	*N*MeVal	HMPa	Pro	Leu	Ala			
Pitipeptolide A	(24)	Hm_2C_8ya	Val	*N*MePhe	HMPa	Pro	Ile	Gly			
Pitipeptolide B	(86)	Hm_2C_8ea	Val	*N*MePhe	HMPa	Pro	Ile	Gly			
Pitipeptolide C	(86)	Hm_2C_8aa	Val	*N*MePhe	HMPa	Pro	Ile	Gly			
Pitipeptolide D	(86)	Hm_2C_8ya	Val	Phe	HMPa	Pro	Ile	Gly			
Pitipeptolide E	(86)	Hm_2C_8ya	Val	*N*MePhe	HIVa	Pro	Ile	Gly			
Pitipeptolide F	(86)	Hm_2C_8ya	Val	*N*MePhe	HMPa	Pro	Val	Gly			
Radamamide A	(91)	Hm_2C_8ya	Val	*N*MeVal	HMPa	Pro	*N*MePhe	Gly			
Tiahuramide A	(29)	HmC_8ya	Val	*N*MeVal	PLa	Pro	*N*MeAla	Ile			
Tiahuramide B	(29)	HmC_8ea	Val	*N*MeVal	PLa	Pro	*N*MeAla	Ile			
Tiahuramide C	(29)	HmC_8aa	Val	*N*MeVal	PLa	Pro	*N*MeAla	Ile			
Viequeamide A	(87)	Hm_2C_8ya	Val	*N*MeVal	PLa	Pro	*N*MeVal	Thr			
Viequeamide B	(87)	Hm_2C_8ya	Val	*N*MeVal	PLa	Pro	*N*MeAla	Val			
Viequeamide C	(87)	Hm_2C_8ea	Val	*N*MeVal	PLa	Pro	*N*MeAla	Val			
Viequeamide D	(87)	Hm_2C_8aa	Val	*N*MeVal	PLa	Pro	*N*MeAla	Val			
Viequeamide E	(87)	Hm_2C_8ya	Ile	*N*MeVal	PLa	Pro	*N*MeAla	Val			
Viequeamide F	(87)	Hm_2C_8ya	Val	*N*MeVal	PLa	Pro	*N*MeAla	Ile			
Class O-2d		**FA**	**Ile/Val**	**(*N*MeVal/*N*MeAla)$_n$**			**HIVa/HMPa/ PLa**	**(Pro)$_n$**		**X**	
Wewakpeptin A	(97)	Hm_2C_8ya	Ile	*N*MeAla	*N*MeVal	*N*MeVal	HIVa	Pro	Pro	HIVa	
Wewakpeptin B	(97)	Hm_2C_8aa	Ile	*N*MeAla	*N*MeVal	*N*MeVal	HIVa	Pro	Pro	HIVa	
Wewakpeptin C	(97)	Hm_2C_8aa	Ile	*N*MeAla	*N*MeAla	*N*MeVal	PLa	Pro	Pro	HIVa	
Wewakpeptin D	(97)	Hm_2C_8aa	Ile	*N*MeAla	*N*MeAla	*N*MeVal	PLa	Pro	Pro	HIVa	
Class O-3		**FA**	**X**	**X**	**(X)**	**Pro**	***N*MePhe/Tyr**	**Ala/Gly**			
Cocosamide A	(67)	Hm_2C_8ya	Val	*N*MePhe		Pro	*N*MePhe	Gly			
Cocosamide B	(67)	Hm_2C_8ea	Val	*N*MePhe		Pro	*N*MePhe	Gly			
Benderamide A	(98)	Hm_2C_8ya	Val	*N*MePhe		Pro	*N*MePhe	Ala			
Dudawalamide A	(7)	Hm_2C_8ya	hPa	Ala	*N*MeIle	Pro	*N*MePhe	Gly			
Dudawalamide B	(7)	Hm_2C_8ya	Val	*N*MePhe		Pro	*N*Me*O*MeTyr	Ala			
Dudawalamide C	(7)	Hm_2C_8ya	Val	*N*MeVal	HIVa	Pro	*N*MePhe	Gly			
Dudawalamide D	(7)	Hm_2C_6ya	Val	*N*MeVal	HMPa	Pro	*N*MePhe	Gly			
Guineamide D	(99)	*AmC_6aa*	*HIVa*	*NMeVal*	*HIVa*		*NMeOMeTyr*	*Gly*			
Guineamide E	(99)	Hm_2C_6aa	HIVa	Ala	*N*Me-Val	Pro	*N*MePhe	Gly			
Guineamide F	(99)	Hm_2C_6aa	Val	*N*MePhe		Pro	*N*MePhe	Gly			

Class O-4		FA	dHMBa/dHMPa/HIVa	TZA	(X)	X	TZa				
Lyngbyabellin A	(22)	7-Cl_2-HmC_8aa	dHMBa	TZa	Gly	Ile	TZa				
Lyngbyabellin B	(22)	7-Cl_2-HmC_8aa	dHMBa	TZa	Gly	Val	TZa				
Deoxylyngbyabellin A	(22)	7-Cl_2-HmC_8aa	HMBa	TZa	Gly	Ile	TZa				
Lyngbyabellin C	(134)	7-Cl_2-HmC_8aa	dHMPa	TZa		dHPa	TZa				
Lyngbyabellin E	(134)	7-Cl_2-HmC_8aa	dHMBa	TZa		dHPa(m)	TZa				
Lyngbyabellin H	(134)	7-Cl_2-Hm C_8aa	HIVa	TZa		dHPa(m)	TZa				
Lyngbyabellin G	(134)	7-Cl_2-HmC_8aa	dHMBa	TZA		dHPa	TZa				
Lyngbyabellin J	(103)	7-Cl_2-HmC_8aa	dHMPa	TZa		dHPa(m)	TZa				
Lyngbyabellin K	(105)	7-Cl_2-HmC_8aa	HIVa	TZa		dHPa	TZa				
Lyngbyabellin L	(105)	7-Cl-HmC_8aa	HIVa	TZa		dHPa	TZa				
Lyngbyabellin N	(105)	7-Cl_2-HmC_8aa	HIVa	TZa		dHPa(m)	TZa				
Homohydroxydolabellin	(134)	7-Cl_2-HmC_8aa	dHMBa	TZA		dHPa	TZa				
Hectochlorin A	(104)	7-Cl_2-HmC_8aa	dHMBa	TZa		Ac-DHMBa	TZa				
Hectochlorin B	(135)	7-Cl_2-HmC_8aa	dHMBa	TZa		DHMBa	TZa				
Hectochlorine C	(135)	7-Cl-HmC_8aa	dHMBa	TZa		Ac-DHMBa	TZa				
Hectochlorine D	(135)	7-Cl_2-Hm$_2$$C_8$aa	dHMBa	TZa		Ac-DHMBa	TZa				

Cyclic lipopeptides with FA incorporated into the cycle:

β-amino-fatty acid containing lipopeptides

Class N-1		FA	AOFHa	X	Gly									
Anabaenolysin A	(59)	AhC_{18}e$_{5,7,9,13,15}$a	AOFHa	Gly	Gly									
Anabaenolysin B	(59)	AhC_{18}e$_{5,7,9,13}$a	AOFHa	Gly	Gly									
Anabaenolysin C	(136)	AhC_{18}e$_{5,7,9,17}$a	AOFHa	Gln	Gly									
Anabaenolysin D	(136)	AhC_{18}e$_{5,7,9}$a	AOFHa	Gln	Gly									
Class N-2a		**FA**	**HMPa/HIVa/La**	**X**	***N*Me-Phe**	**Ala**								
Porpoisamide A	(109)	AmC_8aa	HMPa	Pro	*N*MePhe	Ala								
Porpoisamide B	(109)	AmC_8aa	HMPa	Pro	*N*MePhe	Ala								
Class N-2b		**FA**	**HMPa/HIVa/La**	**X**	***N*Me-Phe/*N*Me-Tyr**	**Ala**	**TZa**							
Ulongamide A	(110)	AmC_6aa	La	*N*MePhe	*N*MeVal	Ala	TZa							
Ulongamide B	(110)	AmC_6aa	La	*N*MePhe	*N*MeVal	Ala	TZa							
Ulongamide C	(110)	AmC_6aa	La	*N*MeTyr	*N*MePhe	Ala	TZa							
Ulongamide D	(110)	AmC_6aa	HIVa	*N*MeTyr	*N*MeVal	Ala	TZa							
Ulongamide E	(110)	AmC_6aa	HMPa	*N*MeTyr	*N*MeVal	Ala	TZa							
Ulongamide F	(110)	AmC_6aa	HMPa	Val	*N*MeVal	Ala	TZa							

Class N-2c		**FA**	**HIVa/HMPa/PLa**	**X**	**X**	***N*Me-Val**	**(X)**	**(X)**	***N*Me-X**	**Ala/Ile/Val**	**(X)**	**(X)**	**(X)**	**(X)**	***N*Me-X**
Ulongapeptin A	[112]	AmC_8ya	La	*N*MeVal	Val	*N*MeVal			*N*Me Phe	Val					
Companeramide A	[113]	AmC_8ya	HIVa	*N*MeAla	Ile	*N*MeVal	Ile	Pro	*N*Me Ile	Ala					*N*Me Val
Companeramide B	[113]	AmC_8ya	HIVa	*N*MeAla	Val	*N*MeVal	Ile	Pro	*N*Me Ala	Val					*N*Me Val
Malevamide B	[60]	AmC_6aa	HIVa	*N*MeAla	Val	*N*MeVal	*N*MeVal	Pro	*N*Me Val	Ile	Pro	*N*Me Val	Pro		*N*Me Phe
Malevamide C	[60]	AmC_8ya	HIVa	Ala	Pro	*N*MeVal	*N*Me *O*MeSer	*N*Me Phe	*N*Me Ala	Ala	*N*Me Val	Leu	Pro	Pro	*N*Me Ile
Class N-3		**FA**	**Gln**	**Gly**	**Pro/mPro**	**X**	**X**	**Ser/Thr**	**Dhb**	**Ser/Thr**	**X**	**Pro/mPro**			
Muscotoxin A	[119]	5-OH-$AhC_{10}aa$	Gln	Gly	Pro	Phe	Val	Ser	Dhb	Ser	Ile	Pro			
Musxotoxin B	[119]	5-OH-$AhC_{10}aa$	Gln	Gly	Pro	Phe	Ile	Ser	Dhb	Ser	Ile	mPro			
Muscotoxin C	[119]	5-OH-$AhC_{10}aa$	Gln	Gly	mPro	Phe	Val	Ser	Dhb	Ser	Ile	mPro			
Lyngbyazothrin A	[30]	5,7,8-$(OH)_3$-10-Me-$AhC_{11}aa$	Gln	Gly	Pro	Pro	Ile	Ser	Dhb	Thr	*O*Me-hTyr	Pro			
Lyngbyazothrin B	[30]	5,7,8-$(OH)_3$-10-Me-$AhC_{11}aa$	Gln	Gly	Pro	Pro	Ile	Ser	Dhb	Thr	hPhe	Pro			
Lyngbyazothrin C	[30]	5,7,8-$(OH)_3$-10-Me-$AhC_{11}aa$(m)	Gln	Gly	Pro	Pro	Ile	Ser	Dhb	Thr	*O*Me-hTyr	Pro			
Lyngbyazothrin D	[30]	5,7,8-$(OH)_3$-10-Me-$AhC_{11}aa$(m)	Gln	Gly	Pro	Pro	Ile	Ser	Dhb	Thr	hPhe	Pro			
Portoamide A	[106]	5,7,8-$(OH)_3$-10-Me-$AhC_{11}aa$(m)	Gln	Gly	Pro	Pro	Ile	Ser	Dhb	Ser	*O*Me-hTyr	Pro			
Portoamide B	[106]	5,7,8-$(OH)_3$-10-Me-$AhC_{11}aa$(m)	Gln	Gly	Pro	Pro	Ile	Ser	Dhb	Ser	hPhe	Pro			
Portoamide C	[106]	5,7,8-$(OH)_3$-10-Me-$AhC_{11}aa$	Gln	Gly	Pro	Pro	Ile	Ser	Dhb	Ser	*O*Me-hTyr	Pro			
Portoamide D	[120]	5,7,8-$(OH)_3$-10-Me-$AhC_{11}aa$	Gln	Gly	Pro	Pro	Ile	Ser	Dhb	Ser	hPhe	Pro			
Pahayokolide A	[115]	5,7,8-$(OH)_3$-10-Me-$AhC_{11}aa$(m)	Gln	Gly	Pro	Phe	Dhb	Ser	Dhb	Thr	hPhe	Pro			
Pahayokolide B	[115]	5,7,8-$(OH)_3$-10-Me-$AhC_{11}aa$	Gln	Gly	Pro	Phe	Dhb	Ser	Dhb	Thr	hPhe	Pro			
Schizotrin A	[117]	5,7,8-$(OH)_3$-10-Me-$AhC_{11}aa$(m)	Gln	Gly	Pro	Phe	Val	Ser	Dhb	Ser	*O*Me-hTyr	Pro			

Tychonamide A	(68)	5,7,8-OH_3-C_8-Bza-AhC_8aa(m)	Gln	Gly	Pro	Pro	Ile	Ser	Dhb	Thr	*O*Me-hTyr	Pro			
Tychonamide B	(68)	5,7,8-$(OH)_3$-C_8-Bza-AhC_8aa (m)	Gln	Gly	Pro	Pro	Ile	Ser	Dhb	Thr	hPhe	Pro			
Class-N4		**FA**	**Val**	**X**	**X**	**X**	**Asn/Gln**	**Ala/Gly**	**X**	***N*MeAsn**	**Pro**				
Minutissamide A	(65)	4-Me-AhC_{12}aa	Val	Dhb	Asn	Dhb	Asn	Ala	Thr	*N*MeAsn	Pro				
Minutissamide B	(65)	4-Me-12-Cl-AhC_{12}aa	Val	Dhb	Asn	Dhb	Asn	Ala	Thr	*N*MeAsn	Pro				
Minutissamide C	(65)	4-Me-14-O-AhC_{16}aa	Val	Dhb	Asn	Dhb	Asn	Ala	Thr	*N*MeAsn	Pro				
Minutissamide D	(65)	4-Me-14-OH-AhC_{16}aa	Val	Dhb	Asn	Dhb	Asn	Ala	Thr	*N*MeAsn	Pro				
Minutissamide E	(121)	4-Me-15-O-AhC_{18}aa	Val	Dhb	Thr	Thr	Gln	Ala	*O*MeThr	*N*MeAsn	Pro				
Minutissamide F	(121)	4-Me-12-Cl-AhC_{14}aa	Val	Dhb	Thr	Thr	Gln	Ala	*O*MeThr	*N*MeAsn	Pro				
Minutissamide G	(121)	4-Me-15-OH-AhC_{18}aa	Val	Dhb	Thr	Thr	Gln	Ala	*O*MeThr	*N*MeAsn	Pro				
Minutissamide H	(121)	4-Me-15-O-AhC_{18}aa	Val	Dhb	Thr	Val	Gln	Ala	*O*MeThr	*N*MeAsn	Pro				
Minutissamide I	(121)	4-Me-15-O-AhC_{18}aa	Val	Dhb	Thr	Thr	Gln	Gly	*O*MeThr	*N*MeAsn	Pro				
Minutissamide J	(121)	4-Me-12-Cl-AhC_{14}aa	Val	Dhb	Thr	Thr	Gln	Gly	*O*MeThr	*N*MeAsn	Pro				
Minutissamide K	(121)	4-Me-15-OH-AhC_{18}aa	Val	Dhb	Thr	Thr	Gln	Gly	*O*MeThr	*N*MeAsn	Pro				
Minutissamide L	(121)	4-Me-15-O-AhC_{18}aa	Val	Dhb	Thr	Val	Gln	Gly	*O*MeThr	*N*MeAsn	Pro				
Puwainaphycin A	(122)	4-Me-14-O-AhC_{18}aa	Val	Dhb	Thr	Thr	Gln	Gly	*O*MeThr	*N*MeAsn	Pro				
Puwainaphycin B	(122)	4-Me-14-O-AhC_{18}aa	Val	Dhb	Thr	Val	Gln	Gly	*O*MeThr	*N*MeAsn	Pro				
Puwainaphycin C	(122)	4-Me-14-Cl-AhC_{16}aa	Val	Dhb	Thr	Thr	Gln	Gly	*O*MeThr	*N*MeAsn	Pro				
Puwainaphycin D	(122)	4-Me-14-Cl-AhC_{16}aa	Val	Dhb	Thr	Val	Gln	Gly	*O*MeThr	*N*MeAsn	Pro				
Puwainaphycin E	(122)	4-Me-AhC_{16}aa	Val	Dhb	Thr	Thr	Gln	Gly	*O*MeThr	*N*MeAsn	Pro				
Puwainaphycin F	(25)	4-Me-AhC_{14}aa	Val	Dhb	Asn	Dhb	Asn	Ala	Thr	*N*MeAsn	Pro				
Puwainaphycin G	(25)	4-Me-Ah-C_{14}aa	Val	Dhb	Gln	Dhb	Asn	Ala	Thr	*N*MeAsn	Pro				
Calophycin	(26)	4-Me-AhC_{16}aa	Val	Ala	Asp	Asn	Gln	Gly	Arg	*N*MeAsn	Pro				
Class N-5a		**FA**	**X**	**X**	**Pro/ OH-Pro**	**Ser/ hSer**	**Phe/Tyr**	**Leu**	**X**	**X**	**X**	**Gly**			
Laxaphycin A	(123)	AC_8aa	hSer	Dhb	OH-Pro	hSer	Phe	Leu	Ile	Ile	Leu	Gly			

Laxaphycin A2	[124]	AC_8aa	hSer	Dhb	OH-Pro	hSer	Phe	Leu	Val	Ile	Leu	Gly			
Laxaphycin E	[124]	$AC_{10}aa$	hSer	Dhb	OH-Pro	hSer	Phe	Leu	Ile	Ile	Leu	Gly			
Hormothamnin A	[130]	AC_8aa	hSer	Dhb	OH-Pro	hSer	Phe	Leu	Ile	Ile	Leu	Gly			
Lobocyclamide A	[137]	$AC_{10}aa$	Ser	Dhb	OH-Pro	hSer	Tyr	Leu	Ile	Ile	Pro	Gly			
Trichormamide A	[131]	$AC_{10}aa$	Ser	Ser	Pro	Ser	Tyr	Leu	Ile	Ile	Pro	Gly			
Trichormamide D	[126]	$AC_{10}aa$	Gln	Dhb	Pro	Ser	Tyr	Leu	Val	Phe	Leu	Gly			
Scytocyclamide A	[127]	AC_8aa	Gln	Dhb	OH-Pro	hSer	Phe	Leu	Ile	Ile	Leu	Gly			
Class N-5b		**FA**	**X**	**OH-Leu**	**X**	**X**	**Gln**	***N*MeIle**	**X**	**Thr**	**OH-Pro/ Pro**	**X**	**Thr**		
Laxaphycin B	[124]	$AC_{10}aa$	Val	OH-Leu	Ala	OH-Leu	Gln	*N*MeIle	OH-Asn	Thr	Pro	Leu	Thr		
Laxaphycin B2	[124]	$AC_{10}aa$	Val	OH-Leu	Ala	Leu	Gln	*N*MeIle	OH-Asn	Thr	Pro	Leu	Thr		
Laxaphycin B3	[124]	$AC_{10}aa$	Val	OH-Leu	Ala	OH-Leu	Gln	*N*MeIle	OH-Asn	Thr	OH-Pro	Leu	Thr		
Laxaphycin B4	[124]	$AC_{10}aa$	Val	OH-Leu	hSer	OH-Leu	Gln	*N*MeIle	OH-Asn	Thr	OH-Pro	Leu	Thr		
Laxaphycin B5	[124]	$AC_{10}aa$	Ile	OH-Leu	Ala	OH-Leu	Gln	*N*MeIle	Asn	Thr	Pro	Tyr	Thr		
Laxaphycin B6	[124]	$AC_{10}aa$	Ile	OH-Leu	Ala	Leu	Gln	*N*MeIle	Asn	Thr	Pro	Tyr	Thr		
Laxaphycin D	[124]	AC_8aa	Val	OH-Leu	Ala	OH-Leu	Gln	*N*MeIle	OH-Asn	Thr	Pro	Leu	Thr		
Lobocyclamide B	[137]	$AC_{10}aa$	Val	OH-Leu	Ala	OH-Leu	Gln	*N*MeIle	OH-Asn	Thr	OH-Pro	Leu	Thr		
Lobocyclamide C	[137]	AC_8aa	Val	OH-Leu	Ala	OH-Leu	Gln	*N*MeIle	OH-Thr	Thr	OH-Pro	Leu	Thr		
Lyngbyacyclamide A	[132]	$AC_{10}aa$	Val	OH-Leu	hSer	Leu	Gln	*N*MeIle	OH-Asn	Thr	Pro	Phe	Thr		
Lyngbyacyclamide B	[132]	$AC_{10}aa$	Val	OH-Leu	hSer	Leu	Gln	*N*MeIle	OH-Asn	Thr	OH-Pro	Phe	Thr		
Trichormamide B	[131]	$AC_{10}aa$	Ile	OH-Leu	hSer	OH-Leu	Gln	*N*MeIle	Ser	Thr	Pro	Tyr	Thr		
Trichormamide C	[131]	$AC_{10}aa$	Val	OH-Leu	Ala	OH-Leu	Gln	*N*MeIle	Asn	Thr	Pro	Leu	Thr		
Scytocyclamide B	[127]	AC_8aa	Val	OH-Leu	Ala	OH-Leu	Gln	*N*MeIle	OH-Asn	Thr	Pro	Leu	Thr		
Scytocyclamide C	[127]	AC_8aa	Val	OH-Leu	Ala	Leu	Gln	*N*MeIle	OH-Asn	Thr	Pro	Leu	Thr		

Part II: General discussion

As demonstrated in the introductory part, cyanobacterial cyclic lipopeptides represent a broad group of diverse structures with a variety of biological activities. However, no focused research aiming to uncover the structure-activity relationships within the group has been performed up to now. Moreover, different sets of bioactivities are available for particular lipopeptide classes which limits their mutual comparison and prevent to draw the general conclusions. An additional limitation is the lack of systematic classification regarding cyanobacterial lipopeptides. This leads to disorganization in the nomenclature as newly discovered lipopeptides are assigned with new, unique names despite sharing many similarities with already known and described structures. Minutissamides and puwainaphycins are clear examples. In 1992 Gregson et al. described new cyanobacterial cyclic lipopeptides Puwainaphycin A-E[122] and analogous Puwainaphycin F and G has been discovered by Hrouzek et al. in 2012[25]. However, structurally similar compounds found by Kang et al. in 2011[65] and 2012[121] were entitled Minutissamide A-L. All of these compounds share an identical general structure and belong to N-3 class lipopeptides as defined in this study. Only a minor amino acid or FA variation is observed between distinct puwainaphycins and minutissamides. Consequently, I have initially made an overview of all known cyanobacterial cyclic lipopeptides and tried to create classes based on their structural similarities. This literature review helped me to identify the parts of the molecule that are shared between different classes with similar biological effects and to estimate which moieties might be responsible for their bioactivities. Additionally, this overview was used as a

background to propose the chemical modifications of the puwainaphycin and minutissamides aiming to study their mechanism of action and demonstrate which part of the molecules are crucial for their bioactivity.

Structural variability and biosynthesis of minutissamides and puwainaphycins.

Many cyclic lipopeptide structures are produced or estimated to be produced by the NRPS/PKS machinery. We have successfully identified the biosynthetic NRPS/PKS cluster responsible for puwainaphycin biosynthesis in *Cylindrospermum alatosporum* **(Paper I)**, which contains 10 ORFs and expands over 57kbp. The biosynthesis starts with a FAAL catalytic domain which activates the fatty acid residue by adenylation and forms a substrate for canonical PKS. Two distinct PKS modules (encoded in *puwB* and *puwE*) extend the fatty acid chain by 4 carbon. The first PKS module (*PuwB*) contains a methyltransferase domain responsible for side chain methylation. Tailoring domains present in catalytic modules of *PuwE*, namely the putative aminotransferase and (mono)oxygenase, form a modified FA with α-hydroxylation and *β*-amino substitution accepted by NPRSs. The first NRPS module is encoded together with the second PKS in the same hybrid gene (*PuwE*). An additional 8 NRPS modules are encoded in 4 genes (*puw A,F,G,H*). Finally, the thioesterase domain present in the very last NPRS module synthetizes and cyclizes the final product by forming an additional peptide bond between the β-amino group of the fatty acid chain and the terminal carboxyl of the last amino acid (Pro).

The initiation of the biosynthetic processes via activation of fatty acid is known as lipoinitiation, is relatively common among NRPS peptides, and well described in bacterial NPRS products[138,139]. The mechanism of

initiation via FAAL allows the activation of fatty acids with different chain lengths. For example, the biosynthetic pathway of amphi-enterobactins contains the FAAL domain that can activate saturated C10-C14 FA as well as their unsaturated and/or hydroxylated counterparts[138]. Experiments with purified FAAL enzyme from *Escherichia coli have* proven the presence of various acyl adenylates, mostly C12 and C14 fatty acids[139]. Similar behavior is observed in the biosynthesis of puwainaphycins and minutissamides, congeners with different fatty acid moieties were widely detected (**Paper I** and **Paper II**). The FAAL domain is not the only enzyme connected with lipoinitiation. FACL enzymes represent an alternative starter unit. While FAAL activates the fatty acid via adenylation, FACL uses a conjugation with CoA. Similarly to FAAL, FACL are well described in NRPS peptides (e.g. bacterial surfactin[140] or cyanobacterial hassallidin[79]).

NRPS/PKS machineries are known to generate several natural product analogs via one biosynthetic assembly due to the enhanced substrate specificity of particular adenylation domains. For puwainaphycins, such variability has already been shown by Hrouzek et al.[25] and Kang et al.[65,121], showing that several amino-acid positions can be occupied by variable amino acids (Gln/Asn, Ser/Thr etc.). During our surveys, the cyanobacterial strain *Cylindrospermum alatosporum* was found to produce congeners also differing in the length of the FA moiety (**paper I**); HPLC-HRMS analysis showed, besides Puwainaphycin F and Puwainaphycin G, ten additional compounds varying in the length and substitution of the fatty acid, some of them being identical to previously described as minutissamides. The exact structure of these variants was later confirmed by NMR studies[146]. The compound appeared to have the following structures: puwainaphycin containing $AhC_{12}aa$ (Puwainaphycin F), $AhC_{14}aa$ (Minutissamide A), OH-4-

Me-AhC$_{12}$aa PUW F, OH-4-Me-AhC$_{14}$aa PUW F, Cl-4-Me-AhC$_{12}$aa PUW F, and Cl-4-Me-AhC$_{14}$aa PUW F[146] . This evidence clearly shows that the FAAL domain in puwainaphycin biosynthetic gene cluster, similarly to the A domain, possesses enhanced substrate specificity similarly to previously described enterobactins.

The observed variation in PUW/MIN structure suggested that their actual structural variability might be even higher. Thus we proceeded to characterize all variants in the PUW/MIN producing strains. The cyanobacterial culture extracts are often rich in many different compounds and thus finding exclusively lipopeptide compounds is highly challenging. Therefore, a new method for the targeted screening and identification of lipopeptides in cyanobacterial extract based on HPLC-HRMS/MS has been developed (**Paper II**). Puwainaphycins and minutissamides possess a peptide cycle, thus they are easily accessible to fragmentation at lower fragmentation energies facilitating the determination of their primary amino-acid structure. The assignment of the fragmentation spectra of an unknown compound, such as a lipopeptide, is yet more complicated. However, in this case a fragmentation using higher collision energy might provide additional data. While at 5-50eV, peptide bonds are preferentially cleaved, at a fragmentation energy of 90eV and higher, most of the C-O and C-N bonds are broken down resulting in fragments mostly below the *m/z* threshold detected by many mass spectrometers. The majority of substituents bound to the fatty acid (including α-hydroxyl and terminal carbonyl group) are cleaved off as well except for the β-amino group. The stability of the acyl chain and the presence of the β-amino group results in the formation of specific diagnostic immonium ions detectable by the MS. Accordingly, fragmentation at high energies allows us to perform selective screening to find possible β-amino FA containing

lipopeptides. The method was not tested on β-hydroxy FA containing lipopeptides as it is expected that β-hydroxyl will be cleaved off at higher energies and consequently form unsubstituted aliphatic chain, which make it difficult to detect using ESI-HRMS methods used in this study. This new analytical method was verified on β-amino FA containing lipopeptide Iturin A standard solution and revealed 11 putative Iturin A variants with diagnostic immonium fragments from 128 Da (corresponding to AhC_{10}aa-Iturin A) up to 226 Da (corresponding to AhC_{17}aa-Iturin A). The FA corresponding to detected immonium ions contained both an even and odd number of carbons. However, concerning the lipopeptides containing FA chain with odd number of carbons, we are not able to distinguish between linear and branched FA chains based on the HRMS/MS data (e.g. Me-AhC_{16}aa and AhC_{17}aa). Since the FAAL domain is capable of activating different fatty acids (Paper I), there is an expectation that this domain could activate linear fatty acid as well as *iso*- and *anteiso*-FA. As mentioned in the introductory section, despite the majority of lipopeptides containing an even carbon chain, lipopeptides with linear fatty acids containing an odd number of carbons are known as well (e.g. portoamides with 5,7,8-OH_3-10Me-C_{11}aa). However, the HPLC-HRMS analysis further proved a strong linear correlation between the acyl chain length of lipopeptides and their retention on the C18 reverse column. Based on the data available, the retention time of linear FA and *iso*-FA differs[137]. Accordingly, the retention time of lipopeptides with linear and branched fatty acid should differ as well. This suggests that the Iturin variant with *m/z* 1086Da is indeed the variant containing FA with an odd number of carbons (C_{17}aa-Iturin A). Regrettably, the absolute amount of these odd-fatty-acid containing variants was too low for the purification and identification of these compounds using NMR. To prove that this method is also applicable for β-

amino FA lipopeptides with substantially different peptide cycles this screening method was used for the analysis of extract of *Anabaena* sp. SPORK 15F, anabaenolysin producing strain. Because the structure of anabaenolysin contains substantially different FA moiety (e.g. AhC$_{18}$e$_{5,7,9,13,15}$a) and also differs in the size of the amino-acid cycle the method has to be slightly adjusted (fragmentation energy 40eV for MS and 100eV for MS-MS). After this optimalization interpretable diagnostic fragments at m/z 228-244Da were obtained and clearly identified 6 anabaenolysins congeners (including hydroxylated and dihydroxylated fatty acids).

Finally, the developed analytical method was used to complement the data on puwainaphycin and minutissamide production from known producers of these lipopeptides (*Anabaena minutissima* UTEX B1613 and *Cylindrospermum alatosporum* CCALA 988) as well as for strains in which the lipopeptide production was proved using this method (*Cylindrospermum moravicum* CCALA 993 and CCALA 994, *Anabaena sp.* UHCC-0399, and *Symplocastrum muelleri* NIVA CYA 644). It resulted in the detection of 61 unique puwainaphycin or minutissamide congeners (**Paper III**). The observed variation includes differing FA lengths (AhC$_{10}$-C$_{18}$aa) as well as various functionalizations. Fatty acid substituted with hydroxyl (e.g. OH-AhC$_{12}$aa or OH-AhC$_{18}$aa), chlorine (e.g. Cl-AhC$_{12}$aa or Cl-AhC$_{14}$aa), acetyl (*O*Ac-OH-AhC$_{16}$aa and *O*Ac-OH-AhC$_{16}$aa), and oxo-functional groups (O-AhC$_{16}$aa and O-AhC$_{18}$aa) has been found to be incorporated in the puwainaphycin/minutissamide molecules. Besides the variation in the fatty acid part, alternation in the peptidic core was detected as well with the following variable amino acid positions: Asn/Gln/Thr at variable position 4, Dhb/Thr/Val at position 5, Asn/Gln at position 6, Ala/Gly/Ser at position 7,

and Thr/Ser/*O*Me-Thr at position 8. It is especially notable that the amino acid adjacent to the FA moiety remains conserved and forms the *N*MeAsn-Pro-FA-Val-Dhb motif in all described variants suggesting that these residues are important for their compound bioactivity.

Interestingly, variation in the fatty acid part moiety was not equally distributed among strains (**Paper III**) suggesting that the process of lipoinitiation differs. The genomic analysis showed that indeed the puwainphycin/minutissamide biosynthetic clusters differed in the FAAL starter units and that three principal arrangements exist in the studied strains. While strains CCALA 988, CCALA 993, and CCALA 994 possess type-I starter unit consisting of a standalone of FAAL (gene *puwC*) and separate ACP (gene *puwD*), NIVA CYA 644 contains a type-II starter unit formed by a fusion of FAAL and ACP (gene *puwI*) (**Paper II**). Finally, both *Anabaena* strains contain both starter units (of type I and II). The substrate specificities of the individual starter modules were subsequently inferred from the structural diversity detected in particular strains. Accordingly, starter unit I is responsible for the activation and incorporation of shorter unsubstituted fatty acid ($AhC_{10\text{-}16a}$aa) which can bear hydroxyl- or chloro substitution while starter unit II incorporates longer fatty acid exclusively substituted with the oxo- or hydroxyl groups (O-$AhC_{16\text{-}18}$aa). The functionalization of FA by the oxo and hydroxyl groups in the case of the type-II starter unit can be explained by the presence of a putative cytochrome P450-like oxidase (*puwJ*). All of the biosynthetic clusters with type-I starter units lack only this gene. In the case of the *Symplocastrum* strain the hydroxyl substituent might be further modified by O-acetylation, likely by the action of O-acyltransferase present in the cluster (*PuwL*). An alternative explanation might be the activation of the FA already bearing the substitution as observed in the biosynthesis of

surfactin. The surfactin biosynthetic pathway starts with lipoinitiation by a FACL (Fatty acid CoA ligase) and activation of 3-hydroxy myristic acid[140]. Consequently, the FAAL domain of the type-II starter unit could hypothetically activate hydroxyl-containing FA and an undescribed tailoring enzyme could be responsible for the reduction to ketone. However, the conserved position of the substitution shows a more plausible role for specific oxygenase modifying loaded unsubstituted fatty acid. Moreover, naturally occurring fatty acids hydroxylated in the proximity to the CH_3 terminus are rare. The halogenation of FA in the case of *Anabaena minutissima* UTEX B1613 likely results from the action of the dedicated halogenase (*PuwK*) present in the respective biosynthetic gene cluster. In all of the above-mentioned cases of FA modification, the plausible explanation will be that the FAAL directly cooperates with the tailoring enzymes to activate and modify the FA residue during the lipoinitiation. The manner by which these enzymes cooperate in the synthesis of the FA intermediate should be subjected to further research, however, it is reasonable to expect direct protein-protein interaction as observed for example in the recruitment of P450-like oxidases [141]. The X-domain described in some NRPS pathways, especially those involving glycopeptide biosynthesis, is located mostly between the terminal PCP and TE domain and is responsible for the binding of P450s and consequently cyclisation of glycopeptides. Additionally, aminoacyl-PCP domains with hydroxylated substrates and the possible involvement of P450s contain highly conserved surface residues and hypothetical interaction with P450s is expected. It is worth mentioning that even lipopeptides biosynthesized via the type-I starter unit are found to be substituted (hydroxylated or chlorinated, e.g. OH-AhC_{12}aa). As no dedicated enzymes are found within the biosynthetic cluster of *Cylindrospermum alatosporum*

CCALA 988 we suppose that this modification occurs after the synthesis on the finalized compound. Several experimental studies have proven that halogenation can occur either on free substrate (e.g. rebeccamycin[142]) or an intermediate bound to NRPS enzymes (e.g. clorobiocin[144]); specific halogenases are consequently tightly associated with corresponding NRPS gene clusters (such as is the case of *PuwK*). However, in some NRPS products, genomic analysis does not reveal any halogenase within the genome of the producing organism as can be exemplified in the production of chlorinated Oscillaginin[143].

Cytotoxicity and other bioactivities of minutissamides and puwainaphycins.

As previously demonstrated, puwainaphycins/minutissamides interact directly with biological membranes causing their enhanced fluidity and disruption[23,25]. Although there is no direct mechanistic evidence for this, it can be inferred that the FA moiety is responsible for the biological effect. Such evidence has been for example obtained in other β-amino FA lipopeptide iturins where the analogs missing FA moiety lack biological activity[36]. In the previous chapters I have demonstrated that FA length can vary substantially in puwainaphycins which should affect the bioactive properties of the particular congeners. In the case of puwainaphycins/minutissamides it is notable that all studied variants as well as many other cytotoxic cyanobacterial lipopeptides possess such an FA functionalized with the α-hydroxy group, which suggests its involvement in the bioactivity of the compound. Due to these reasons I aimed to study the effect of FA length and the presence of the α-hydroxyl group on the bioactive properties of puwainaphycins/minutissamides.

At the first instance we have compared the bioactive properties of the most abundant congeners, Minutissamide A and Puwainaphycin F, differing only in the length of the FA and bearing AhC_{12} and AhC_{14}. Despite the long-term cytotoxicity of these compounds is similar (IC_{50} of 2.8±0.5μM and 3.15±0.5μM, respectively), the dynamics of the cytotoxic effect of these compounds differed (**Paper IV**). HeLa cells treated with Minutissamide A recovered fully even after a 3h treatment and the resulting viability was very similar to negative controls. In case of Puwainaphycin F the cells were not able to recover even after the shortest treatment (5 min). This is supported by the evidence on the large membrane damage as assessed by lactate dehydrogenase (LDH) leakage. In the case of Minutissamide A, extracellular LDH is highly increased no earlier than after 3 hours, while LDH leakage in cells treated with Puwainaphycin F shows 40% of leakage within 30 minutes after treatment. These experiments suggested that lipopeptides interact with the membranes in a different way. As the less potent Minutissamide A contains shorter 4-Me-AhC_{12}aa, we can assume that its interaction with the membrane is much weaker and that there is a dynamic equilibrium between lipopeptides bound to the membrane and free lipopeptides allowing the lipopeptide to leave and re-enter the membrane. Consequently, Puwainaphycin F with longer 4-Me-AhC_{14}aa creates a much stronger interaction with phospholipids bilayer due to its longer FA chain and that this results in a firm anchoring of the lipopeptide, which is why the wash-out experiments are unsuccessful.

To fully prove this theory, the concentration of lipopeptides in cells was measured directly using HPLC-MS. The data confirmed the fast entry of Puwainaphycin F into the cell compared to Minutissamide A as the concentration of Puwainaphycin F and Minutissamide A in cells reaches

approximately 500 ag/cell and 150 ag/cell after 5 minutes of treatment, respectively. It is however notable that the concentration of Minutissamide A reached 700 ag/cell at 2 h with no apparent cellular damage. In contrast, a comparable concentration (500 ag/cell) was sufficient for Puwainaphycin F to manifest the effect at the earlier time points. In the latest time point (8 hours), concentration of Minutissamide A reached 6000 ag/cell compared to only 2000 ag/cell of Puwainaphycin F (**Paper IV**). The three times lower concentration of Puwainaphycin F can be explained by the more rapid disruption effect of this compound connected to cell membrane damage causing the removal of compound together with cellular debris during the wash-out. The different uptake rates of Minutissamide A and Puwainaphycin F into the cell membrane was also demonstrated by Vašíček et al. on in Caco-2 human intestinal barrier model. After treatment with 2.5μM concentration, the concentration of Minutissamide A and Puwainaphycin F dropped to 80% and 15%, respectively, further proving the more rapid incorporation of Puwainaphycin F[(147)].

Class N-3 and N-4 cyclic cyanobacterial lipopeptides are believed to permeabilize the membrane via incorporated fatty acid. As discussed above, the length of the fatty acid plays a distinct role in the compound mechanism of the action. We used Minutissamide C with 4-Me-14-O-AhC_{16}aa as a substrate for the chemical modification of the fatty acid moiety via Grignard reactions and synthesized minutissamides with branched and prolonged fatty acid moiety containing an additional double bond, namely 14-Et-4-Me-Ah$C_{18}e_{13}$a and 14-Et-4-Me-Ah$C_{22}e_{13}$a. The treatment of HeLa cells with these variants shows a decrease in cytotoxicity compared to Puwainaphycin F and Minutissamides A and C which manifest moderate effect with IC_{50} values ranging ~2-3 μM. The dose response curve of synthetic 14-Et-4-Me-AhC_{18}ea

variant shows that this compound cannot reach higher inhibition than 50% at the highest concentration tested (20 μM). The second compound, 14-Et-4-Me-Ahc$_{22}$ea variant, proves to be more potent with a drop in viability in sub-micromolar concentrations, reaching 50% viability even at the lower concentration range (1.2-10 μM) and almost 100% inhibition at 20 μM (**Paper IV**). In consequence of this, we cannot clearly prove that the longer incorporated fatty acid increased the cytotoxic potential of the lipopeptide. The principle of chemical modification did not allow us to create unbranched and fully saturated FA. Accordingly, we cannot specify whether the decrease in cytotoxicity is caused by the longer chain, the presence of the double bond, or branching. Concerning FA length, some natural variants possessing C18 substituted FA maintain the IC_{50} values ~2 μM[145] and thus the length itself should not be a reason for lower cytotoxicity. Also, the presence of a double bond should not hamper the cytotoxicity as anabaenolysins possessing multiple conjugated double bonds (e.g. Ahc$_{18}$e$_{5,7,9,13,15}$a) and maintain strong cytotoxic effects. The branching of the FA moiety by the methyl group was proved to affect lipopeptide bioactivity[135] to a high degree and so the introduction of the ethyl group in the semi-synthetic variants can play a crucial role. Surprisingly, a decrease in cytotoxicity was accompanied by the increase of antifungal activities. Pure Minutissamide A and Puwainaphycin F exhibit moderate to weak antifungal properties against *Aspergillus fumigatus* (MIC 2.34 μM and 37.5 μM, respectively) while the variants containing 14-Et-4-Me-Ahc$_{18}$ea and 14-Et-4-Me-Ahc$_{22}$ea show inhibition with MIC 0.5 μM and 3.8 μM, respectively. This antifungal activity seems however to be strain specific, as significant improvements in antifungal activities were found against the *Aspergillus fumigatus* and *Alternaria alternata* and a decrease in

antifungal properties in *Monographella cucumerina* and *Chaetomium globosum*. (**Paper IV**).

The second moiety of the N-3 and N-4 class molecules which we believe to be important for the incorporation of cyclic lipopeptide into the cell membrane is the hydroxyl group bound to α-carbon of the FA moiety. As all molecules from N-3 to N-4 class of lipopeptides possess this substitution[23,65,115,117,121] it can be assumed that this polar and hydrophilic group facilitate the incorporation of the membrane. While the hydrophobic fatty acid acyl chain interacts with the lipid bilayer, the hydrophilic OH group can interact with phosphate groups. Accordingly, we prepared semi-synthetic variants acylated on both OH groups (α-hydroxy of FA as well as hydroxy-group of Thr) **(Paper IV)**. For acylation, anhydrides of acetic acid, butanoic acid, hexanoic acid, and decanoic acid were used. As expected, none of these variants of Minutissamide A exhibit any relevant cytotoxicity against HeLa cells even at the highest concentration. Similar to the prolonged and branched FA, even this modification surprisingly increases the antifungal properties that seem again to be strain specific. MIC values against *Aspergillus fumigatus* was 4.7 μM, 9.4 μM, 75 μM, and 37 μM for acetyl-, butanoyl-, hexanoyl-, and decanoyl- modified Minutissamide A, respectively. The unmodified Minutissamide A exhibits antifungal activity with MIC 37.5 μM. While variants with shorter acylation were more potent, acylation with the hexanoyl- and decanoyl- chain do not significantly affect or increase the MIC against *Aspergillus fumigatus*. The most potent antifungal effect of these semi-synthetic variants was recorded against the plant pathogen *Alternaria alternata* with MIC values ranging from 0.2 to 0.6 μM with no clear relation to the length of acyl substituent. For other fungal strains such as *Trichoderma harzianum, Bipolaris sorokiniana, Monographella cucumerina, Chaetomium*

globosum, and *Candida friedrichii*, all semi-synthetic variants exhibit a mild increase of antifungal effect.

Summary

Based on the experimental evidence obtained within this study we can conclude the following facts. Puwainaphycins and minutissamides as cyclic cyanobacterial lipopeptides are biosynthesized via specific NRPS/PKS pathways involving the FAAL domain(s) responsible for the lipoinitiation of the biosynthesis. Two different FAAL starter units have been described in several cyanobacterial strains. The first starter unit activates the mainly shorter aliphatic chains, the second starter unit activates longer fatty acid moieties and further allows their modification by a hydroxyl group or oxo group performed by accessory enzymes. The increased substrate specificity of the FAAL domains together with the presence of alternating modules are the reason for the wide variability of incorporated fatty acid moiety in different puwainaphycin and minutissamide variants. Minutissamide A and Puwainaphycin F are confirmed as membrane disrupting compounds, however, the dynamics of their incorporation into the membrane depends strongly on fatty acid length. Further, FA α-hydroxylation is crucial for this bioactivity (probably via interaction with charged phosphate groups) as the acylation of free hydroxyl groups leads to a total loss of cytotoxic bioactivity. However, selective antifungal properties are observed in puwainaphycins lacking free α-hydroxyl functionality. Consequently, increasing lipopeptide antifungal activity by the alternation of hydrophobicity is a feasible way of generating novel fungicides, since the cytotoxicity of these modified variants is strongly diminished. While the prolongation of the FA moiety depends on the presence of a ketone or other functional group(s), acylation of the hydroxyl group present in the majority of known membrane permeabilizing lipopeptides is an easy way of lipopeptide modification.

Abbreviations

4-Me-10-Cl-AhC$_{12}$aa - β-amino-α-hydroxy-4-methyl-10-chloro-dodecanoic acid
4-Me-12-Cl-AhC$_{14}$aa - β-amino-α-hydroxy-4-methyl-12-chloro-tetradecanoic acid
4-Me-14-O-AhC$_{16}$aa - β-amino-α-hydroxy-4-methyl-14-keto-hexadecanoic acid
4-Me-14-OH-AhC$_{16}$aa - β-amino-α-hydroxy-4-methyl-14-hydroxy-hexadecanoic acid
4-Me-15-O-AhC$_{18}$aa - β-amino-α-hydroxy-4-methyl-15-keto-octadecanoic acid
4-Me-15-OH-AhC$_{18}$aa - β-amino-α-hydroxy-4-methyl-15-hydroxy-octadecanoic acid
4-Me-AhC$_{12}$aa - β-amino-α-hydroxy-4-methyl-dodecanoic acid
4-Me-AhC$_{14}$aa - β-amino-α-hydroxy-4-methyl-tetradecanoic acid
4-Me-AhC$_{16}$aa - β-amino-α-hydroxy-4-methyl-hexadecanoic acid
AC$_{10}$aa - β-amino-decanoic acid
AC$_{8}$aa - β-amino-octanoic acid
AhC$_{10}$aa - β-amino-α-hydroxy-decanoic acid
AHDHa - 4-amino-β-hydroxy-2,5-dimethylheptanoic acid
AhC$_{18}$e$_{5,7,9,13,15}$a - Ahopa, β-amino-2-hydroxyoctadeca-5,7,9,13,15-pentaenoic acid
Ahopip - β-amino-6-hydroxy-2-piperidone
AhC$_{18}$e$_{5,7,9,13}$a - Ahota, β-amino-2-hydroxyoctadeca-5,7,9,15-tetraenoic acid
AmC$_{6}$aa - β-amino-α-methyl-hexanoic acid
AmC$_{6}$ea - β-amino-α-methyl-hexenoic acid
AmC$_{6}$ya - β-amino-α-methyl-hexynoic acid
AmC$_{8}$aa - β-amino-α-methyl-octanoic acid
AmC$_{8}$ea - β-amino-α-methyl-octenoic acid
AmC$_{8}$ya - β-amino-α-methyl-octynoic acid
AOFHa - 2-(β-amino-5-oxotetrahydrofuran-2-yl)-α-hydroxyacetic acid
Ara – Arabinose
Bza – benzene ring substitution (phenyl)
C$_{6}$aa - hexanoic acid
Dhb – dehydrothreonin, dehydrobutyric acid
dHMBa - 2,3-dihydroxy-3-methylbutanoic acid
dHMPa - 2,3-dihydroxy-3-methylpentanoic acid
dHPa - 2,3-dihydroxypropanoic acid (glyceric acid)
FA – fatty acid
FAAL – fatty acyl-AMP ligase
Gal - Galactose
Hex - Hexose
Hh-C$_{14}$aa - α,β-dihydroxy-hexadecanoic acid
Hh-C$_{14}$aa - α,β-dihydroxy-tetradecanoic acid
HIVa - hydroxyisovaleric acid
Hm$_2$C$_{10}$aa - β-hydroxy-α-dimethyl-decanoic acid
Hm$_2$C$_{10}$ea - β-hydroxy-α-dimethyl-decenoic acid
Hm$_2$C$_{10}$ya - β-hydroxy-α-dimethyl-decynoic acid
Hm$_2$C$_{8}$aa - β-hydroxy-α-dimethyl-hexanoic acid
Hm$_2$C$_{8}$ea - β-hydroxy-α-dimethyl-hexenoic acid
Hm$_2$C$_{8}$ya - β-hydroxy-α-dimethyl-hexynoic acid
HmC$_{10}$aa - β-hydroxy-α-methyl-decanoic acid
HmC$_{10}$ea - β-hydroxy-α-methyl-decenoic acid
HmC$_{10}$ya - β-hydroxy-α-methyl-decynoic acid
HmC$_{8}$aa - β-hydroxy-α-methyl-hexanoic acid
HmC$_{8}$ea - β-hydroxy-α-methyl-hexenoic acid
HmC$_{8}$ya - β-hydroxy-α-methyl-hexynoic acid
HMPa - hydroxymethylpentanoic acid
hPa - α-hydroxy-propanoic acid
HPCCC – Hight performace countercurrent chromatography
HPLC – High performance liquid chromatography
HRMS – High resolution mass spectrometry

hSer - homoserine
hTyr - homotyrosine
La - Lactic acid
(m) – modified residue
Man – Mannose
NMR – Nuclear magnetic resonance
NRPS – Non-ribosomal peptide synthetase
OH_3-10-Me-C_{11}aa - trihydroxy-10-methyl-undecanoic acid
OH_3-C_8-Bza - trihydroxy-8-phenyl-octanoic acid
OH-Asn - hydroxyasparagine
OH-Leu – hydroxyleucine
OH-Pro – hydroxyproline
Pen - Pentose
PKS – polyketide synthase
PLa - Phenyllactic acid
Rha – Rhamnose
RIPPs - Ribosomally synthesized and post-translationally modified peptides
TZa – Thiazole

Bibliography (ISO 690)

1. **WHITTON, B.A**., and POTTS, M. *The Ecology of Cyanobacteria : Their Diversity in Time and Space* [online]. Dordrecht: Kluwer Academic Publishers, 2002. ISBN 0-7923-4735-8. Available at: doi:10.1007/0-306-46855-7
2. **VOLK, R.-B.** and FURKERT, F. H. Antialgal, antibacterial and antifungal activity of two metabolites produced and excreted by cyanobacteria during growth. *Microbiological Research* [online]. 2006, **161**(2). ISSN 09445013. Available at: doi:10.1016/j.micres.2005.08.005
3. **MILLIGAN, K. E.,** MARQUEZ, B. L., WILLIAMSON, R. T. and GERWICK, W. H. Lyngbyabellin B, a Toxic and Antifungal Secondary Metabolite from the Marine Cyanobacterium *Lyngbya majuscula*. *Journal of Natural Products* [online]. 2000, **63**(10). ISSN 0163-3864. Available at: doi:10.1021/np000133y
4. **LEÃO, P. N.,** COSTA, M., RAMOS, V., PEREIRA, A. R., FERNANDES, V. C., DOMINGUES, V. F., GERWICK, W. H., VASCONCELOS, V. M. and MARTINS, R. Antitumor Activity of Hierridin B, a Cyanobacterial Secondary Metabolite Found in both Filamentous and Unicellular Marine Strains. *PLoS ONE* [online]. 2013, **8**(7). ISSN 1932-6203. Available at: doi:10.1371/journal.pone.0069562
5. **IWATA, K.,** INAYAMA, T. and KATO, T. Effects of Spirulina platensis on plasma lipoprotein lipase activity in fructose-induced hyperlipidemic rats. *Journal of Nutritional Science and Vitaminology* [online]. 1990, **36**(2). ISSN 0301-4800. Available at: doi:10.3177/jnsv.36.165
6. **WRIGHT, P. C.,** BURJA, A. M., BANAIGS, B., ABOU-MANSOUR, E. and BURGESS, J. G. Secondary metabolites produced by the tropical marin filamentous cyanobacterium, Lyngbya majuscula - a prolific source of marine natural products. *Tetrahedron*. 2001, **57**(46), 9347–9377. ISSN 0040-4020. Available at:
7. **ALMALITI, J.,** MALLOY, K. L., GLUKHOV, E., SPADAFORA, C., GUTIÉRREZ, M. and GERWICK, W. H. Dudawalamides A–D, Antiparasitic Cyclic Depsipeptides from the Marine Cyanobacterium *Moorea producens*. *Journal of Natural Products* [online]. 2017, **80**(6). ISSN 0163-3864. Available at: doi:10.1021/acs.jnatprod.7b00034
8. **HOLLAND, A.** and KINNEAR, S. Interpreting the Possible Ecological Role(s) of Cyanotoxins: Compounds for Competitive Advantage and/or Physiological Aide? *Marine Drugs* [online]. 2013, **11**(7). ISSN 1660-3397. Available at: doi:10.3390/md11072239
9. **ROGERS, E. H.,** HUNTER, E. S., MOSER, V. C., PHILLIPS, P. M., HERKOVITS, J., MUÑOZ, L., HALL, L. L. and CHERNOFF, N. Potential developmental toxicity of anatoxin-a, a cyanobacterial toxin. *Journal of Applied Toxicology* [online]. 2005, **25**(6). ISSN 0260-437X. Available at: doi:10.1002/jat.1091
10. **VASAS, G.,** BORBELY, G., NANASI, P. and NANASI, P. P. Alkaloids from Cyanobacteria with Diverse Powerful Bioactivities. *Mini-Reviews in Medicinal Chemistry* [online]. 2010, **10**(10). ISSN 1389-5575. Available at: doi:10.2174/138955710792007231
11. **PRINSEP, M. R.**, THOMSON, R. A., WEST, M. L. and WYLIE, B. L. Tolypodiol, an Antiinflammatory Diterpenoid from the Cyanobacterium *Tolypothrix nodosa*.

Journal of Natural Products [online]. 1996, **59**(8). ISSN 0163-3864. Available at: doi:10.1021/np9602574

12. **PATTANAIK, B.** and LINDBERG, P. Terpenoids and Their Biosynthesis in Cyanobacteria. *Life* [online]. 2015, **5**(1). ISSN 2075-1729. Available at: doi:10.3390/life5010269
13. **NUNNERY, J. K.**, MEVERS, E. and GERWICK, W. H. Biologically active secondary metabolites from marine cyanobacteria. *Current Opinion in Biotechnology* [online]. 2010, **21**(6). ISSN 0958-1669. Available at: doi:10.1016/j.copbio.2010.09.019
14. **KEHR, J.-C.,** GATTE PICCHI, D. and DITTMANN, E. Natural product biosyntheses in cyanobacteria: A treasure trove of unique enzymes. *Beilstein Journal of Organic Chemistry* [online]. 2011, **7**. ISSN 1860-5397. Available at: doi:10.3762/bjoc.7.191
15. **MAREŠ, J.**, HÁJEK, J., URAJOVÁ, P., KOPECKÝ, J. and HROUZEK, P. A Hybrid Non-Ribosomal Peptide/Polyketide Synthetase Containing Fatty-Acyl Ligase (FAAL) Synthesizes the β-Amino Fatty Acid Lipopeptides Puwainaphycins in the Cyanobacterium Cylindrospermum alatosporum. *PLoS ONE* [online]. 2014, **9**(11). ISSN 1932-6203. Available at: doi:10.1371/journal.pone.0111904
16. **WELKER, M.** and VON DÖHREN, H. Cyanobacterial peptides — Nature's own combinatorial biosynthesis. *FEMS Microbiology Reviews* [online]. 2006, **30**(4). ISSN 1574-6976. Available at: doi:10.1111/j.1574-6976.2006.00022.x
17. **ISHITSUKA, M. O.**, KUSUMI, T., KAKISAWA, H., KAYA, K. and WATANABE, M. M. Microviridin. A novel tricyclic depsipeptide from the toxic cyanobacterium Microcystis viridis. *Journal of the American Chemical Society* [online]. 1990, **112**(22). ISSN 0002-7863. Available at: doi:10.1021/ja00178a060
18. **SIEBER, S. A.** and MARAHIEL, M. A. Molecular Mechanisms Underlying Nonribosomal Peptide Synthesis: Approaches to New Antibiotics. *Chemical Reviews* [online]. 2005, **105**(2). ISSN 0009-2665. Available at: doi:10.1021/cr0301191
19. **REIN, K. S.** and SNYDER, R. V. The Biosynthesis of Polyketide Metabolites by Dinoflagellates. *Advanced Applied Microbiology*. 2006, 59, [online]. 2006. Available at: doi:10.1016/S0065-2164(06)59004-5
20. **WANG, H.**, FEWER, D. P., HOLM, L., ROUHIAINEN, L. and SIVONEN, K. Atlas of nonribosomal peptide and polyketide biosynthetic pathways reveals common occurrence of nonmodular enzymes. *Proceedings of the National Academy of Sciences* [online]. 2014, **111**(25). ISSN 0027-8424. Available at: doi:10.1073/pnas.1401734111
21. **DELCAMBE, L**. Iturine, new antibiotic produced by Bacillus subtilis. *Comptes rendus des seances de la Societe de biologie et de ses filiales*. 1950, **144**(19–20), 1431–1434. ISSN 0037-9026.
22. **LUESCH, H**., YOSHIDA, W. Y., MOORE, R. E., PAUL, V. J. and MOOBERRY, S. L. Isolation, Structure Determination, and Biological Activity of Lyngbyabellin A from the Marine Cyanobacterium *Lyngbya majuscula*. *Journal of Natural Products* [online]. 2000, **63**(5). ISSN 0163-3864. Available at: doi:10.1021/np990543q
23. **TOMEK, P.**, HROUZEK, P., KUZMA, M., SÝKORA, J., FIŠER, R., ČERNÝ, J., NOVÁK, P., BÁRTOVÁ, S., ŠIMEK, P., HOF, M., KAVAN, D. and KOPECKÝ, J. Cytotoxic Lipopeptide Muscotoxin A, Isolated from Soil Cyanobacterium *Desmonostoc muscorum*, Permeabilizes Phospholipid Membranes by Reducing

Their Fluidity. *Chemical Research in Toxicology* [online]. 2015, **28**(2). ISSN 0893-228X. Available at: doi:10.1021/tx500382b

24. **LUESCH, H.**, PANGILINAN, R., YOSHIDA, W. Y., MOORE, R. E. and PAUL, V. J. Pitipeptolides A and B, New Cyclodepsipeptides from the Marine Cyanobacterium *Lyngbya majuscula*. *Journal of Natural Products* [online]. 2001, **64**(3). ISSN 0163-3864. Available at: doi:10.1021/np000456u
25. **HROUZEK, P.**, KUZMA, M., ČERNÝ, J., NOVÁK, P., FIŠER, R., ŠIMEK, P., LUKEŠOVÁ, A. and KOPECKÝ, J. The Cyanobacterial Cyclic Lipopeptides Puwainaphycins F/G Are Inducing Necrosis via Cell Membrane Permeabilization and Subsequent Unusual Actin Relocalization. *Chemical Research in Toxicology* [online]. 2012, **25**(6). ISSN 0893-228X. Available at: doi:10.1021/tx300044t
26. **MOON, S. S.**, CHEN, J. L., MOORE, R. E. and PATTERSON, G. M. L. Calophycin, a fungicidal cyclic decapeptide from the terrestrial blue-green alga Calothrix fusca. *The Journal of Organic Chemistry* [online]. 1992, **57**(4). ISSN 0022-3263. Available at: doi:10.1021/jo00030a013
27. **BUI, T.-H.**, WRAY, V., NIMTZ, M., FOSSEN, T., PREISITSCH, M., SCHRÖDER, G., WENDE, K., HEIDEN, S. E. and MUNDT, S. Balticidins A–D, Antifungal Hassallidin-Like Lipopeptides from the Baltic Sea Cyanobacterium *Anabaena cylindrica* Bio33. *Journal of Natural Products* [online]. 2014, **77**(6). ISSN 0163-3864. Available at: doi:10.1021/np401020a
28. **FRANKMÖLLE, W. P.**, KNÜBEL, G., MOORE, R. E. and PATTERSON, G. M. L. Antifungal cyclic peptides from the terrestrial blue-green alga Anabaena laxa. II. Structures of laxaphycins A, B, D and E. *The Journal of Antibiotics* [online]. 1992, **45**(9). ISSN 0021-8820. Available at: doi:10.7164/antibiotics.45.1458
29. **LEVERT, A.**, ALVARIÑO, R., BORNANCIN, L., ABOU MANSOUR, E., BURJA, A. M., GENEVIÈRE, A.-M., BONNARD, I., ALONSO, E., BOTANA, L. and BANAIGS, B. Structures and Activities of Tiahuramides A–C, Cyclic Depsipeptides from a Tahitian Collection of the Marine Cyanobacterium *Lyngbya majuscula*. *Journal of Natural Products* [online]. 2018, **81**(6). ISSN 0163-3864. Available at: doi:10.1021/acs.jnatprod.7b00751
30. **ZAINUDDIN, E. N.**, JANSEN, R., NIMTZ, M., WRAY, V., PREISITSCH, M., LALK, M. and MUNDT, S. Lyngbyazothrins A−D, Antimicrobial Cyclic Undecapeptides from the Cultured Cyanobacterium *Lyngbya* sp. *Journal of Natural Products* [online]. 2009, **72**(8). ISSN 0163-3864. Available at: doi:10.1021/np8007792
31. **CARRILLO, C.**, TERUEL, J. A., ARANDA, F. J. and ORTIZ, A. Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. *Biochimica et Biophysica Acta (BBA) - Biomembranes* [online]. 2003, **1611**(1–2). ISSN 0005-2736. Available at: doi:10.1016/S0005-2736(03)00029-4
32. **KURTZ, M. B.** and DOUGLAS, C. M. Lipopeptide inhibitors of fungal glucan synthase. *Medical Mycology* [online]. 1997, **35**(2). ISSN 1369-3786. Available at: doi:10.1080/02681219780000961
33. **MILLER, W. R.**, BAYER, A. S. and ARIAS, C. A. Mechanism of Action and Resistance to Daptomycin in *Staphylococcus aureus* and Enterococci. *Cold Spring Harbor Perspectives in Medicine* [online]. 2016, **6**(11). ISSN 2157-1422. Available at: doi:10.1101/cshperspect.a026997
34. **GEUDENS, N.**, NASIR, M. N., CROWET, J.-M., RAAIJMAKERS, J. M., FEHÉR, K., CENYE, T., MARTINS, J. C., LINS, L., SINNAEVE, D. and DELEU, M.

Membrane Interactions of Natural Cyclic Lipodepsipeptides of the Viscosin Group. *Biochimica et Biophysica Acta (BBA) - Biomembranes* [online]. 2017, **1859**(3). ISSN 00052736. Available at: doi:10.1016/j.bbamem.2016.12.013

35. **MAGET-DANA, R.** and PEYPOUX, F. Iturins, a special class of pore-forming lipopeptides: biological and physicochemical properties. *Toxicology* [online]. 1994, **87**(1–3). ISSN 0300483X. Available at: doi:10.1016/0300-483X(94)90159-7
36. **ARANDA, F. J.**, TERUEL, J. A. and ORTIZ, A. Further aspects on the hemolytic activity of the antibiotic lipopeptide iturin A. *Biochimica et Biophysica Acta (BBA) - Biomembranes* [online]. 2005, **1713**(1). ISSN 00052736. Available at: doi:10.1016/j.bbamem.2005.05.003
37. **FINKING, R.** and MARAHIEL, M. A. Biosynthesis of Nonribosomal Peptides. *Annual Review of Microbiology* [online]. 2004, **58**(1). ISSN 0066-4227. Available at: doi:10.1146/annurev.micro.58.030603.123615
38. **LAMBALOT, R. H.**, GEHRING, A. M., FLUGEL, R. S., ZUBER, P., LACELLE, M., MARAHIEL, M. A., REID, R., KHOSLA, C. and WALSH, C. T. A new enzyme superfamily - the phosphopantetheinyl transferases. *Chemistry & Biology* [online]. 1996, **3**(11). ISSN 10745521. Available at: doi:10.1016/S1074-5521(96)90181-7
39. **WEBER, T.**, BAUMGARTNER, R., RENNER, C., MARAHIEL, M. A. and HOLAK, T. A. Solution structure of PCP, a prototype for the peptidyl carrier domains of modular peptide synthetases. *Structure* [online]. 2000, **8**(4). ISSN 0969-2126. Available at: doi:10.1016/S0969-2126(00)00120-9
40. **KOHLI, R. M.**, TRAUGER, J. W., SCHWARZER, D., MARAHIEL, M. A. and WALSH, C. T. Generality of Peptide Cyclization Catalyzed by Isolated Thioesterase Domains of Nonribosomal Peptide Synthetases. *Biochemistry* [online]. 2001, **40**(24). ISSN 0006-2960. Available at: doi:10.1021/bi010036j
41. **SCHMIDT, E. W.**, NELSON, J. T., RASKO, D. A., SUDEK, S., EISEN, J. A., HAYGOOD, M. G. and RAVEL, J. Patellamide A and C biosynthesis by a microcin-like pathway in *Prochloron didemni*, the cyanobacterial symbiont of Lissoclinum patella. *Proceedings of the National Academy of Sciences* [online]. 2005, **102**(20). ISSN 0027-8424. Available at: doi:10.1073/pnas.0501424102
42. **STEIN, D. B.**, LINNE, U. and MARAHIEL, M. A. Utility of epimerization domains for the redesign of nonribosomal peptide synthetases. *FEBS Journal* [online]. 2005, **272**(17). ISSN 1742-464X. Available at: doi:10.1111/j.1742-4658.2005.04871.x
43. **SIELAFF, H.**, DITTMANN, E., TANDEAU DE MARSAC, N., BOUCHIER, C., VON DÖHREN, H., BÖRNER, T. and SCHWECKE, T. The mcyF gene of the microcystin biosynthetic gene cluster from Microcystis aeruginosa encodes an aspartate racemase. *Biochemical Journal* [online]. 2003, **373**(3). ISSN 0264-6021. Available at: doi:10.1042/bj20030396
44. **GUYOT, M.**, DORÉ, J. C. and DEVILLERS, J. Typology of secondary cyanobacterial metabolites from minimum spanning tree analysis. *SAR and QSAR in Environmental Research* [online]. 2004, **15**(2). ISSN 1062-936X. Available at: doi:10.1080/10629360410001665866
45. **MOORE, R. E.**, BORNEMANN, V., NIEMCZURA, W.- P., GREGSON, J. M., CHEN, J. L., NORTON, T. R., PATTERSON, G. M. L. and HELMS, G. L. Puwainaphycin C, a cardioactive cyclic peptide from the blue-green alga Anabaena BQ-16-1. Use of two-dimensional carbon-13-carbon-13 and carbon-13-nitrogen-15 correlation spectroscopy in sequencing the amino acid units. *Journal of the American Chemical Society* [online]. 1989, **111**(16). ISSN 0002-7863. Available

at: doi:10.1021/ja00198a021

46. **LUESCH, H.**, YOSHIDA, W. Y., MOORE, R. E. and PAUL, V. J. Isolation and Structure of the Cytotoxin Lyngbyabellin B and Absolute Configuration of Lyngbyapeptin A from the Marine Cyanobacterium *Lyngbya majuscula*. *Journal of Natural Products* [online]. 2000, **63**(10). ISSN 0163-3864. Available at: doi:10.1021/np000104n
47. **NADKARNI, S. R.**, PATEL, M. V., CHATTERJEE, SUGATA, VIJAYAKUMAR, E. K. S., DESIKAN, K. R., BLUMBACH, J. and GANGULI, B. N. Balhimycin, a new glycopeptide antibiotic produced by Amycolatopsis sp. Y-86,21022. Taxonomy, production, isolation and biological activity. *The Journal of Antibiotics* [online]. 1994, **47**(3). ISSN 0021-8820. Available at: doi:10.7164/antibiotics.47.334
48. **KITTILÄ, T.**, KITTEL, C., TAILHADES, J., BUTZ, D., SCHOPPET, M., BÜTTNER, A., GOODE, R. J. A., SCHITTENHELM, R. B., VAN PEE, K.-H., SÜSSMUTH, R. D., WOHLLEBEN, W., CRYLE, M. J. and STEGMANN, E. Halogenation of glycopeptide antibiotics occurs at the amino acid level during non-ribosomal peptide synthesis. *Chemical Science* [online]. 2017, **8**(9). ISSN 2041-6520. Available at: doi:10.1039/C7SC00460E
49. **VAN PÉE, K.-H.** Enzymatic Chlorination and Bromination. *Methods in Enzymology* [online]. 2012, 516. ISSN 0076-6879. Available at: doi:10.1016/B978-0-12-394291-3.00004-6
50. **CHANG, Z.**, FLATT, P., GERWICK, W. H., NGUYEN, V.-A., WILLIS, C. L. and SHERMAN, D. H. The barbamide biosynthetic gene cluster: a novel marine cyanobacterial system of mixed polyketide synthase (PKS)-non-ribosomal peptide synthetase (NRPS) origin involving an unusual trichloroleucyl starter unit. *Gene* [online]. 2002, **296**(1–2). ISSN 0378-1119. Available at: doi:10.1016/S0378-1119(02)00860-0
51. **SALVADOR, L. A.**, BIGGS, J. S., PAUL, V. J. and LUESCH, H. Veraguamides A−G, Cyclic Hexadepsipeptides from a Dolastatin 16-Producing Cyanobacterium *Symploca* cf. *hydnoides* from Guam. *Journal of Natural Products* [online]. 2011, **74**(5). ISSN 0163-3864. Available at: doi:10.1021/np200076t
52. **HASLINGER, K.**, PESCHKE, M., BRIEKE, C., MAXIMOWITSCH, E. and CRYLE, M. J. X-domain of peptide synthetases recruits oxygenases crucial for glycopeptide biosynthesis. *Nature* [online]. 2015, **521**(7550). ISSN 0028-0836. Available at: doi:10.1038/nature14141
53. **DUTTA, S.**, WHICHER, J. R., HANSEN, D. A., HALE, W. A., CHEMLER, J. A., CONGDON, G. R., NARAYAN, A. R. H., HÅKANSSON, K., SHERMAN, D. H., SMITH, J. L. and SKINIOTIS, G. Structure of a modular polyketide synthase. *Nature* [online]. 2014, **510**(7506). ISSN 0028-0836. Available at: doi:10.1038/nature13423
54. **FEWER, D. P.**, ÖSTERHOLM, J., ROUHIAINEN, L., JOKELA, J., WAHLSTEN, M. and SIVONEN, K. Nostophycin Biosynthesis Is Directed by a Hybrid Polyketide Synthase-Nonribosomal Peptide Synthetase in the Toxic Cyanobacterium Nostoc sp. Strain 152. *Applied and Environmental Microbiology* [online]. 2011, **77**(22). ISSN 0099-2240. Available at: doi:10.1128/AEM.05993-11
55. **RAAIJMAKERS, J. M.**, DE BRUIJN, I., NYBROE, O. and ONGENA, M. Natural functions of lipopeptides from *Bacillus* and *Pseudomonas* : more than surfactants and antibiotics. *FEMS Microbiology Reviews* [online]. 2010, **34**(6). ISSN 1574-6976. Available at: doi:10.1111/j.1574-6976.2010.00221.x
56. **SEYDLOVÁ, G.** and SVOBODOVÁ, J. Review of surfactin chemical properties

and the potential biomedical applications. *Open Medicine* [online]. 2008, **3**(2). ISSN 2391-5463. Available at: doi:10.2478/s11536-008-0002-5

57. **ZHAO, P.**, XUE, Y., LI, X., LI, J., ZHAO, Z., QUAN, C., GAO, W., ZU, X., BAI, X. and FENG, S. Fungi-derived lipopeptide antibiotics developed since 2000. *Peptides* [online]. 2019, **113**. ISSN 0196-9781. Available at: doi:10.1016/j.peptides.2019.02.002
58. **PETTIT, G.**, XU, J.-P., HOGAN, F. and CERNY, R. L. Isolation and structure of dolastatin 17. *Heterocycles*. 1998, **47**(1), 491–496. ISSN 0385-5414
59. **JOKELA, J.**, OFTEDAL, L., HERFINDAL, L., PERMI, P., WAHLSTEN, M., DØSKELAND, S. O. and SIVONEN, K. Anabaenolysins, Novel Cytolytic Lipopeptides from Benthic Anabaena Cyanobacteria. *PLoS ONE* [online]. 2012, **7**(7). ISSN 1932-6203. Available at: doi:10.1371/journal.pone.0041222
60. **HORGEN, D. F.**, YOSHIDA, Y. W. and SCHEUER, P. J. Malevamides A−C, New Depsipeptides from the Marine Cyanobacterium Symploca laete-viridis. *Journal of Natural Products*. 2000, **63**(4), 461–467. ISSN 0163-3864. Available at: doi.org/10.1021/np990449+
61. **HORGEN, F. D.**, KAZMIERSKI, E. B., WESTENBURG, H. E., YOSHIDA, W. Y. and SCHEUER, P. J. Malevamide D: Isolation and Structure Determination of an Isodolastatin H Analogue from the Marine Cyanobacterium *Symploca hydnoides*. *Journal of Natural Products* [online]. 2002, **65**(4). ISSN 0163-3864. Available at: doi:10.1021/np010560r
62. **VON ELERT, E.**, OBERER, L., MERKEL, P., HUHN, T. and BLOM, J. F. Cyanopeptolin 954, a Chlorine-Containing Chymotrypsin Inhibitor of *Microcystis a eruginosa* NIVA Cya 43. *Journal of Natural Products* [online]. 2005, **68**(9). ISSN 0163-3864. Available at: doi:10.1021/np050079r
63. **NEUHOF, T.**, SCHMIEDER, P., PREUSSEL, K., DIECKMANN, R., PHAM, H., BARTL, F. and VON DÖHREN, H. Hassallidin A, a Glycosylated Lipopeptide with Antifungal Activity from the Cyanobacterium *Hassallia* sp. *Journal of Natural Products* [online]. 2005, **68**(5). ISSN 0163-3864. Available at: doi:10.1021/np049671r
64. **NEUHOF, T.**, SCHMIEDER, P., SEIBOLD, M., PREUSSEL, K. and VON DÖHREN, H. Hassallidin B - Second antifungal member of the Hassallidin family. *Bioorganic & Medicinal Chemistry Letters* [online]. 2006, **16**(16). ISSN 0960-894X. Available at: doi:10.1016/j.bmcl.2006.05.094
65. **KANG, H.-S.**, KRUNIC, A., SHEN, Q., SWANSON, S. M. and ORJALA, J. Minutissamides A–D, Antiproliferative Cyclic Decapeptides from the Cultured Cyanobacterium *Anabaena minutissima*. *Journal of Natural Products* [online]. 2011, **74**(7). ISSN 0163-3864. Available at: doi:10.1021/np2002226
66. **WYNN, J.** Lipids, Fatty Acids. In: *Comprehensive Biotechnology* [online]. Pergamon: Elsevier, 2011. Available at: doi:10.1016/B978-0-444-64046-8.00005-7
67. **GUNASEKERA, S. P.**, OWLE, C. S., MONTASER, R., LUESCH, H. and PAUL, V. J. Malyngamide 3 and Cocosamides A and B from the Marine Cyanobacterium *Lyngbya majuscula* from Cocos Lagoon, Guam. *Journal of Natural Products* [online]. 2011, **74**(4). ISSN 0163-3864. Available at: doi:10.1021/np1008015
68. **MEHNER, C.**, MÜLLER, D., KRICK, A., KEHRAUS, S., LÖSER, R., GÜTSCHOW, M., MAIER, A., FIEBIG, H.-H., BRUN, R. and KÖNIG, G. M. A Novel β-Amino Acid in Cytotoxic Peptides from the CyanobacteriumTychonema sp. *European Journal of Organic Chemistry* [online]. 2008, **2008**(10). ISSN 1434193X.

Available at: doi:10.1002/ejoc.200701033
69. **GALLON, J. R.**, CHENG, J., DOUGHERTY, L. J., GALLON, V. A., HILZ, H., PEDERSON, D. M., RICHARDS, H. M., RÜGGEBERG, S. and SMITH, C. J. A novel covalent modification of nitrogenase in a cyanobacterium. *FEBS Letters* [online]. 2000, **468**(2–3). ISSN 0014-5793. Available at: doi:10.1016/S0014-5793(00)01229-1
70. **MARTIN, C.**, OBERER, L., INO, T., KÖNIG, W. A., BUSCH, M. and WECKESSER, J. Cyanopeptolins, new depsipeptides from the cyanobacterium Microcystis sp. pcc 7806. *The Journal of Antibiotics* [online]. 1993, **46**(10). ISSN 0021-8820. Available at: doi:10.7164/antibiotics.46.1550
71. **ROUNGE, T. B.**, ROHRLACK, T., TOOMING-KLUNDERUD, A., KRISTENSEN, T. and JAKOBSEN, K. S. Comparison of Cyanopeptolin Genes in Planktothrix, Microcystis, and Anabaena Strains: Evidence for Independent Evolution within Each Genus. *Applied and Environmental Microbiology* [online]. 2007, **73**(22). ISSN 0099-2240. Available at: doi:10.1128/AEM.01475-07
72. **TOOMING-KLUNDERUD, A.**, ROHRLACK, T., SHALCHIAN-TABRIZI, K., KRISTENSEN, T. and JAKOBSEN, K. S. Structural analysis of a non-ribosomal halogenated cyclic peptide and its putative operon from Microcystis: implications for evolution of cyanopeptolins. *Microbiology* [online]. 2007, **153**(5). ISSN 1350-0872. Available at: doi:10.1099/mic.0.2006/001123-0
73. **ZHONG, L.**, DIAO, X., ZHANG, N., LI, F., ZHOU, H., CHEN, H., BAI, X., REN, X., ZHANG, Y., WU, D. and BIAN, X. Engineering and elucidation of the lipoinitiation process in nonribosomal peptide biosynthesis. *Nature Communications* [online]. 2021, **12**(1). ISSN 2041-1723. Available at: doi:10.1038/s41467-020-20548-8
74. **NEUMANN, U.**, CAMPOS, V., CANTARERO, S., URRUTIA, H., HEINZE, R., WECKESSER, J. and ERHARD, M. Co-Occurrence of Non-toxic (Cyanopeptolin) and Toxic (Microcystin) Peptides in a Bloom of Microcystis sp. from a Chilean Lake. *Systematic and Applied Microbiology* [online]. 2000, **23**(2). ISSN 07232020. Available at: doi:10.1016/S0723-2020(00)80004-1
75. **BISTER, B.**, KELLER, S., BAUMANN, H. I., NICHOLSON, G., WEIST, S., JUNG, G., SÜSSMUTH, R. D. and JÜTTNER, F. Cyanopeptolin 963A, a Chymotrypsin Inhibitor of *Microcystis* PCC 7806. *Journal of Natural Products* [online]. 2004, **67**(10). ISSN 0163-3864. Available at: doi:10.1021/np049828f
76. **GADEMANN, K.**, PORTMANN, C., BLOM, J. F., ZEDER, M. and JÜTTNER, F. Multiple Toxin Production in the Cyanobacterium *Microcystis* : Isolation of the Toxic Protease Inhibitor Cyanopeptolin 1020. *Journal of Natural Products* [online]. 2010, **73**(5). ISSN 0163-3864. Available at: doi:10.1021/np900818c
77. **FALTERMANN, S.**, ZUCCHI, S., KOHLER, E., BLOM, J. F., PERNTHALER, J. and FENT, K. Molecular effects of the cyanobacterial toxin cyanopeptolin (CP1020) occurring in algal blooms: Global transcriptome analysis in zebrafish embryos. *Aquatic Toxicology* [online]. 2014, **149**. ISSN 0166-445X. Available at: doi:10.1016/j.aquatox.2014.01.018
78. **MAZUR-MARZEC, H.**, FIDOR, A., CEGŁOWSKA, M., WIECZERZAK, E., KROPIDŁOWSKA, M., GOUA, M., MACASKILL, J. and EDWARDS, C. Cyanopeptolins with Trypsin and Chymotrypsin Inhibitory Activity from the Cyanobacterium Nostoc edaphicum CCNP1411. *Marine Drugs* [online]. 2018, **16**(7). ISSN 1660-3397. Available at: doi:10.3390/md16070220

79. **VESTOLA, J.**, SHISHIDO, T. K., JOKELA, J., FEWER, D. P., AITIO, O., PERMI, P., WAHLSTEN, M., WANG, H., ROUHIAINEN, L. and SIVONEN, K. Hassallidins, antifungal glycolipopeptides, are widespread among cyanobacteria and are the end-product of a nonribosomal pathway. *Proceedings of the National Academy of Sciences* [online]. 2014, **111**(18). ISSN 0027-8424. Available at: doi:10.1073/pnas.1320913111
80. **SHISHIDO, T.**, HUMISTO, A., JOKELA, J., LIU, L., WAHLSTEN, M., TAMRAKAR, A., FEWER, D., PERMI, P., ANDREOTE, A., FIORE, M. and SIVONEN, K. Antifungal Compounds from Cyanobacteria. *Marine Drugs* [online]. 2015, **13**(4). ISSN 1660-3397. Available at: doi:10.3390/md13042124
81. **NEUHOF, T**. Lipopeptides having pharmaceutical activity. EP 1 698 638 A1. 2006.
82. **HUMISTO, A.**, JOKELA, J., TEIGEN, K., WAHLSTEN, M., PERMI, P., SIVONEN, Kaarina and HERFINDAL, Lars. Characterization of the interaction of the antifungal and cytotoxic cyclic glycolipopeptide hassallidin with sterol-containing lipid membranes. *Biochimica et Biophysica Acta (BBA) - Biomembranes* [online]. 2019, **1861**(8). ISSN 0005-2736. Available at: doi:10.1016/j.bbamem.2019.03.010
83. **REESE, M. T.**, GULAVITA, N. K., NAKAO, Y., HAMANN, M. T., YOSHIDA, W. Y., COVAL, S. J. and SCHEUER, P. J. Kulolide: A Cytotoxic Depsipeptide from a Cephalaspidean Mollusk, *Philinopsis speciosa* [1]. *Journal of the American Chemical Society* [online]. 1996, **118**(45). ISSN 0002-7863. Available at: doi:10.1021/ja9620301
84. **SITACHITTA, N.**, WILLIAMSON, R. T. and GERWICK, W. H. Yanucamides A and B, Two New Depsipeptides from an Assemblage of the Marine Cyanobacteria *Lyngbya majuscula* and *Schizothrix* Species. *Journal of Natural Products* [online]. 2000, **63**(2). ISSN 0163-3864. Available at: doi:10.1021/np990466z
85. **MEVERS, E.**, LIU, W.-T., ENGENE, N., MOHIMANI, H., BYRUM, T., PEVZNER, P. A., DORRESTEIN, P. C., SPADAFORA, C. and GERWICK, W. H. Cytotoxic Veraguamides, Alkynyl Bromide-Containing Cyclic Depsipeptides from the Marine Cyanobacterium cf. *Oscillatoria margaritifera*. *Journal of Natural Products* [online]. 2011, **74**(5). ISSN 0163-3864. Available at: doi:10.1021/np200077f
86. **MONTASER, R.**, PAUL, V. J. and LUESCH, H. Pitipeptolides C–F, antimycobacterial cyclodepsipeptides from the marine cyanobacterium Lyngbya majuscula from Guam. *Phytochemistry* [online]. 2011, **72**(16). ISSN 0031-9422. Available at: doi:10.1016/j.phytochem.2011.07.014
87. **BOUDREAU, P. D.**, BYRUM, T., LIU, W.-T., DORRESTEIN, P. C. and GERWICK, W. H. Viequeamide A, a Cytotoxic Member of the Kulolide Superfamily of Cyclic Depsipeptides from a Marine Button Cyanobacterium. *Journal of Natural Products* [online]. 2012, **75**(9). ISSN 0163-3864. Available at: doi:10.1021/np300321b
88. **TRIPATHI, A.**, PUDDICK, J., PRINSEP, M. R., LEE, P. P. F. and TAN, L. T. Hantupeptin A, a Cytotoxic Cyclic Depsipeptide from a Singapore Collection of *Lyngbya majuscula*. *Journal of Natural Products* [online]. 2009, **72**(1). ISSN 0163-3864. Available at: doi:10.1021/np800448t
89. **NAKAO, Y.**, YOSHIDA, W. Y., SZABO, C. M., BAKER, B. J. and SCHEUER, P. J. More Peptides and Other Diverse Constituents of the Marine Mollusk *Philinopsis speciosa*. *The Journal of Organic Chemistry* [online]. 1998, **63**(10). ISSN 0022-

3263. Available at: doi:10.1021/jo9719867
90. **NOGLE, L. M.** and GERWICK, W. H. Isolation of Four New Cyclic Depsipeptides, Antanapeptins A−D, and Dolastatin 16 from a Madagascan Collection of *Lyngbya majuscula*. *Journal of Natural Products* [online]. 2002, **65**(1). ISSN 0163-3864. Available at: doi:10.1021/np010348n
91. **MEDINA, R. A.** *Biologically Active Cyclic Depsipeptides from Marine Cyanobacteria*. [online]. Corvallis, OR, 2009. Oregon State University. [cit. 2021/09/01] Available at: https://ir.library.oregonstate.edu/concern/graduate_thesis_or_dissertations/w3763873b
92. **TRIPATHI, A.**, PUDDICK, J., PRINSEP, M. R., LEE, P. P. F. and TAN, L. T. Hantupeptins B and C, cytotoxic cyclodepsipeptides from the marine cyanobacterium Lyngbya majuscula. *Phytochemistry* [online]. 2010, **71**(2–3). ISSN 0031-9422. Available at: doi:10.1016/j.phytochem.2009.10.006
93. **BUNYAJETPONG, S.**, YOSHIDA, W. Y., SITACHITTA, N. and KAYA, K. Trungapeptins A−C, Cyclodepsipeptides from the Marine Cyanobacterium *Lyngbya majuscula*. *Journal of Natural Products* [online]. 2006, **69**(11). ISSN 0163-3864. Available at: doi:10.1021/np050485a
94. **MALLOY, K. L.** *Structure elucidation of biomedically relevant marine cyanobacterial natural products*. San Diego, CA, 2011. University of California San Diego.
95. **TANIGUCHI, M.**, NUNNERY, J. K., ENGENE, N., ESQUENAZI, E., BYRUM, T, DORRESTEIN, P. C. and GERWICK, W. H. Palmyramide A, a Cyclic Depsipeptide from a Palmyra Atoll Collection of the Marine Cyanobacterium *Lyngbya majuscula*. *Journal of Natural Products* [online]. 2010, **73**(3). ISSN 0163-3864. Available at: doi:10.1021/np900428h
96. **IWASAKI, A.**, SHIOTA, I., SUMIMOTO, S., MATSUBARA, T., SATO, T. and SUENAGA, K. Kohamamides A, B, and C, Cyclic Depsipeptides from an *Okeania* sp. Marine Cyanobacterium. *Journal of Natural Products* [online]. 2017, **80**(6). ISSN 0163-3864. Available at: doi:10.1021/acs.jnatprod.7b00256
97. **HAN, B.**, GOEGER, D., MAIER, C. S. and GERWICK, W. H. The Wewakpeptins, Cyclic Depsipeptides from a Papua New Guinea Collection of the Marine Cyanobacterium *Lyngbya s emiplena*. *The Journal of Organic Chemistry* [online]. 2005, **70**(8). ISSN 0022-3263. Available at: doi:10.1021/jo0478858
98. **DING, C.**, ONG, J., GOH, H., COFFILL, C. and TAN, L. T. Benderamide A, a Cyclic Depsipeptide from a Singapore Collection of Marine Cyanobacterium cf. Lyngbya sp. *Marine Drugs* [online]. 2018, **16**(11). ISSN 1660-3397. Available at: doi:10.3390/md16110409
99. **TAN, L. T.**, SITACHITTA, N. and GERWICK, W. H. The Guineamides, Novel Cyclic Depsipeptides from a Papua New Guinea Collection of the Marine Cyanobacterium *Lyngbya majuscula*. *Journal of Natural Products* [online]. 2003, **66**(6). ISSN 0163-3864. Available at: doi:10.1021/np020492o
100. **SONE, H.**, KONDO, T., KIRYU, M., ISHIWATA, H., OJIKA, M. and YAMADA, K. Dolabellin, a Cytotoxic Bisthiazole Metabolite from the Sea Hare Dolabella auricularia: Structural Determination and Synthesis. *The Journal of Organic Chemistry* [online]. 1995, **60**(15). ISSN 0022-3263. Available at: doi:10.1021/jo00120a021
101. **RAMASWAMY, A. V.**, SORRELS, C. M. and GERWICK, W. H. Cloning and

Biochemical Characterization of the Hectochlorin Biosynthetic Gene Cluster from the Marine Cyanobacterium *Lyngbya majuscula*. *Journal of Natural Products* [online]. 2007, **70**(12). ISSN 0163-3864. Available at: doi:10.1021/np0704250

102. **COSTA, M.**, COSTA-RODRIGUES, J., FERNANDES, M. H., BARROS, P., VASCONCELOS, V. and MARTINS, R. Marine Cyanobacteria Compounds with Anticancer Properties: A Review on the Implication of Apoptosis. *Marine Drugs* [online]. 2012, **10**(12). ISSN 1660-3397. Available at: doi:10.3390/md10102181
103. **FATHONI, I.**, PETITBOIS, J. G., ALARIF, W. M., ABDEL-LATEFF, A., AL-LIHAIBI, S. S., YOSHIMURA, E., NOGATA, Y., VAIRAPPAN, C. S., SHOLIKHAH, E. N and OKINO, T. Bioactivities of Lyngbyabellins from Cyanobacteria of Moorea and Okeania Genera. *Molecules* [online]. 2020, **25**(17). ISSN 1420-3049. Available at: doi:10.3390/molecules25173986
104. **MARQUEZ, B. L.**, WATTS, K. S., YOKOCHI, A., ROBERTS, M. A., VERDIER-PINARD, P., JIMENEZ, J. I., HAMEL, E., SCHEUER, P. J. and GERWICK, W. H. Structure and Absolute Stereochemistry of Hectochlorin, a Potent Stimulator of Actin Assembly. *Journal of Natural Products* [online]. 2002, **65**(6). ISSN 0163-3864. Available at: doi:10.1021/np0106283
105. **CHOI, H.**, MEVERS, E., BYRUM, T., VALERIOTE, F. A. and GERWICK, W. H. Lyngbyabellins K-N from Two Palmyra Atoll Collections of the Marine Cyanobacterium Moorea bouillonii. *European Journal of Organic Chemistry* [online]. 2012, **2012**(27). ISSN 1434193X. Available at: doi:10.1002/ejoc.201200691
106. **QAMAR, H.**, HUSSAIN, K., SONI, A., KHAN, A., HUSSAIN, T. and CHÉNAIS, B. Cyanobacteria as Natural Therapeutics and Pharmaceutical Potential: Role in Antitumor Activity and as Nanovectors. *Molecules* [online]. 2021, **26**(1). ISSN 1420-3049. Available at: doi:10.3390/molecules26010247
107. **OFTEDAL, L.**, MYHREN, L., JOKELA, J., GAUSDAL, G., SIVONEN, K., DØSKELAND, S. O. and HERFINDAL, L. The lipopeptide toxins anabaenolysin A and B target biological membranes in a cholesterol-dependent manner. *Biochimica et Biophysica Acta (BBA) - Biomembranes* [online]. 2012, **1818**(12). ISSN 0005-2736. Available at: doi:10.1016/j.bbamem.2012.07.015
108. **BOUFFIOUX, O.**, BERQUAND, A., EEMAN, M., PAQUOT, M., DUFRÊNE, Y.F., BRASSEUR, R. and DELEU, M. Molecular organization of surfactin–phospholipid monolayers: Effect of phospholipid chain length and polar head. *Biochimica et Biophysica Acta (BBA) - Biomembranes* [online]. 2007, **1768**(7). ISSN 0005-2736. Available at: doi:10.1016/j.bbamem.2007.04.015
109. **MEICKLE, T.**, GUNASEKERA, S. P., LIU, Y., LUESCH, H. and PAUL, V. J. Porpoisamides A and B, two novel epimeric cyclic depsipeptides from a Florida Keys collection of Lyngbya sp. *Bioorganic & Medicinal Chemistry* [online]. 2011, **19**(22). ISSN 0968-0896. Available at: doi:10.1016/j.bmc.2011.05.051
110. **LUESCH, H.**, WILLIAMS, P. G., YOSHIDA, W. Y., MOORE, R. E. and PAUL, V. J. Ulongamides A−F, New β-Amino Acid-Containing Cyclodepsipeptides from Palauan Collections of the Marine Cyanobacterium *Lyngbya* sp. *Journal of Natural Products* [online]. 2002, **65**(7). ISSN 0163-3864. Available at: doi:10.1021/np0200461
111. **WILLIAMS, P. G.**, YOSHIDA, W. Y., MOORE, R. E. and PAUL, V. J. Isolation and Structure Determination of Obyanamide, a Novel Cytotoxic Cyclic Depsipeptide from the Marine Cyanobacterium *Lyngbya confervoides*. *Journal of Natural*

Products [online]. 2002, **65**(1). ISSN 0163-3864. Available at: doi:10.1021/np0102253

112. **WILLIAMS, P. G.**, YOSHIDA, W. Y., QUON, M. K., MOORE, R. E. and PAUL, V. J. Ulongapeptin, a Cytotoxic Cyclic Depsipeptide from a Palauan Marine Cyanobacterium *Lyngbya* sp. *Journal of Natural Products* [online]. 2003, **66**(5). ISSN 0163-3864. Available at: doi:10.1021/np030050s
113. **VINING, O. B.**, MEDINA, R. A., MITCHELL, E. A., VIDEAU, P., LI, D., SERRILL, J. D., KELLY, J. X., GERWICK, W. H., PROTEAU, P. J., ISHMAEL, J. E. and McPHAIL, K. L. Depsipeptide Companeramides from a Panamanian Marine Cyanobacterium Associated with the Coibamide Producer. *Journal of Natural Products* [online]. 2015, **78**(3). ISSN 0163-3864. Available at: doi:10.1021/np5007907
114. **ADAMS, B.**, PÖRZGEN, P., PITTMAN, E., YOSHIDA, W. Y., WESTENBURG, H. E. and HORGEN, F. D. Isolation and Structure Determination of Malevamide E, a Dolastatin 14 Analogue, from the Marine Cyanobacterium *Symploca laete-viridis*. *Journal of Natural Products* [online]. 2008, **71**(5). ISSN 0163-3864. Available at: doi:10.1021/np070346o
115. **AN, T.**, KUMAR, T. K. S., WANG, M., LIU, L., LAY, J. O., LIYANAGE, R., BERRY, J., GANTAR, M., MARKS, V., GAWLEY, R. E. and REIN, K. S. Structures of Pahayokolides A and B, Cyclic Peptides from a *Lyngbya* sp. *Journal of Natural Products* [online]. 2007, **70**(5). ISSN 0163-3864. Available at: doi:10.1021/np060389p
116. **BERRY, J. P.**, GANTAR, M., GAWLEY, R. E., WANG, M. and REIN, K. S. Pharmacology and toxicology of pahayokolide A, a bioactive metabolite from a freshwater species of Lyngbya isolated from the Florida Everglades. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* [online]. 2004, **139**(4). ISSN 1532-0456. Available at: doi:10.1016/j.cca.2004.11.005
117. **PERGAMENT, I.** and CARMELI, S. Schizotrin A; a novel antimicrobial cyclic peptide from a cyanobacterium. *Tetrahedron Letters* [online]. 1994, **35**(45). ISSN 0040-4039. Available at: doi:10.1016/S0040-4039(00)74436-4
118. **RIBEIRO, T.**, LEMOS, F., PRETO, M., AZEVEDO, J., SOUSA, M. L., LEÃO, P. N., CAMPOS, A., LINDER, S., VITORINO, R., VASCONCELOS, V. and URBATZKA, R. Cytotoxicity of portoamides in human cancer cells and analysis of the molecular mechanisms of action. *PLoS ONE* [online]. 2017, **12**(12). ISSN 1932-6203. Available at: doi:10.1371/journal.pone.0188817
119. **CHEEL, J.**, HÁJEK, J., KUZMA, M., SAURAV, K., SMÝKALOVÁ, I., ONDRÁČKOVÁ, E., URAJOVÁ, P., VU, D. L., FAURE, K., KOPECKÝ, J. and HROUZEK, P. Application of HPCCC Combined with Polymeric Resins and HPLC for the Separation of Cyclic Lipopeptides Muscotoxins A–C and Their Antimicrobial Activity. *Molecules* [online]. 2018, **23**(10). ISSN 1420-3049. Available at: doi:10.3390/molecules23102653
120. **LEAO, P. N.**, PEREIRA, A. R., LIU, W.-T., NG, J., PEVZNER, P. A., DORRESTEIN, P. C., KONIG, G. M., VASCONCELOS, V. M. and GERWICK, W. H. Synergistic allelochemicals from a freshwater cyanobacterium. *Proceedings of the National Academy of Sciences* [online]. 2010, **107**(25). ISSN 0027-8424. Available at: doi:10.1073/pnas.0914343107
121. **KANG, H.-S.**, STURDY, M., KRUNIC, A., KIM, H., SHEN, Q., SWANSON, S. M. and ORJALA, J. Minutissamides E–L, antiproliferative cyclic lipodecapeptides

from the cultured freshwater cyanobacterium cf. Anabaena sp. *Bioorganic & Medicinal Chemistry* [online]. 2012, **20**(20). ISSN 0968-0896. Available at: doi:10.1016/j.bmc.2012.08.017

122. **GREGSON, J. M.**, CHEN, J.-L., PATTERSON, G. M. L. and MOORE, R. E. Structures of puwainaphycins A–E. *Tetrahedron* [online]. 1992, **48**(18). ISSN 0040-4020. Available at: doi:10.1016/S0040-4020(01)92264-1
123. **BORNANCIN, L.**, ALONSO, E., ALVARIÑO, R., INGUIMBERT, N., BONNARD, I., BOTANA, L. M. and BANAIGS, B. Structure and biological evaluation of new cyclic and acyclic laxaphycin-A type peptides. *Bioorganic & Medicinal Chemistry* [online]. 2019, **27**(10). ISSN 0968-0896. Available at: doi:10.1016/j.bmc.2019.03.046
124. **HEINILÄ, L. M. P.**, FEWER, D. P., JOKELA, J. K., WAHLSTEN, M., JORTIKKA, A. and SIVONEN, K. Shared PKS Module in Biosynthesis of Synergistic Laxaphycins. *Frontiers in Microbiology* [online]. 2020, **11**. ISSN 1664-302X. Available at: doi:10.3389/fmicb.2020.578878
125. **MACMILLAN, J. B.**, ERNST-RUSSELL, M. A., DE ROPP, J. S. and MOLINSKI, T. F. Lobocyclamides A−C, Lipopeptides from a Cryptic Cyanobacterial Mat Containing *Lyngbya confervoides*. *The Journal of Organic Chemistry* [online]. 2002, **67**(23). ISSN 0022-3263. Available at: doi:10.1021/jo0261909
126. **LUO, S.**, KANG, H.-S., KRUNIC, A., CHEN, W.-L., YANG, J., WOODARD, J. L., FUCHS, J. R., HYUN CHO, S., FRANZBLAU, S. G., SWANSON, S. M. and ORJALA, J. Trichormamides C and D, antiproliferative cyclic lipopeptides from the cultured freshwater cyanobacterium cf. Oscillatoria sp. UIC 10045. *Bioorganic & Medicinal Chemistry* [online]. 2015, **23**(13). ISSN 0968-0896. Available at: doi:10.1016/j.bmc.2015.04.073
127. **GREW, J. Ch.** *Inaugural-Dissertation: Cyanopeptoline und Scytocyclamide: Zyklische Peptide aus Scytonema hofmanni PCC 7110 Struktur und biologische Aktivität*. Freiburg im Breisgau, 2005. Albert-Ludwigs-Universität, available at: https://freidok.uni-freiburg.de/fedora/objects/freidok:2320/datastreams/FILE1/content.
128. **GBANKOTO, A.**, VIGO, J., DRAMANE, K., BANAIGS, B., AINA, E. and SALMON, J.-M. Cytotoxic Effect of Laxaphycins A and B on Human Lymphoblastic Cells (CCRF-CEM) Using Digitised Videomicrofluorometry. *In Vivo*. 2005, **19**(3), 577–582. ISSN 1791-7549
129. **CAI, W.**, MATTHEW, S., CHEN, Q.-Y., PAUL, V. J. and LUESCH, H. Discovery of new A- and B-type laxaphycins with synergistic anticancer activity. *Bioorganic & Medicinal Chemistry* [online]. 2018, **26**(9). ISSN 0968-0896. Available at: doi:10.1016/j.bmc.2018.03.022
130. **GERWICK, W. H.**, JIANG, Z. D., AGARWAL, S. K. and FARMER, B. T. Total structure of hormothamnin A, A toxic cyclic undecapeptide from the tropical marine cyanobacterium hormothamnion enteromorphoides. *Tetrahedron* [online]. 1992, **48**(12). ISSN 0040-4020. Available at: doi:10.1016/S0040-4020(01)88753-6
131. **LUO, S.**, KRUNIC, A., KANG, H.-S., CHEN, W.-L., WOODARD, J. L., FUCHS, J. R., SWANSON, S. M. and ORJALA, J. Trichormamides A and B with Antiproliferative Activity from the Cultured Freshwater Cyanobacterium *Trichormus* sp. UIC 10339. *Journal of Natural Products* [online]. 2014, **77**(8). ISSN 0163-3864. Available at: doi:10.1021/np5003548
132. **MARU, N.**, OHNO, O. and UEMURA, D. Lyngbyacyclamides A and B, novel

cytotoxic peptides from marine cyanobacteria Lyngbya sp. *Tetrahedron Letters* [online]. 2010, **51**(49). ISSN 0040-4039. Available at: doi:10.1016/j.tetlet.2010.06.105

133. **ATTA-UR-RAHMAN**, ed. *Studies in Natural Products Chemistry: Bioactive Natural Products (Part J)*. Elsevier, 2003. 29. ISSN 1572-5995
134. **HAN, B.**, McPHAIL, K. L., GROSS, H., GOEGER, D. E., MOOBERRY, S. L. and GERWICK, W. H. Isolation and structure of five lyngbyabellin derivatives from a Papua New Guinea collection of the marine cyanobacterium Lyngbya majuscula. *Tetrahedron* [online]. 2005, **61**(49). ISSN 00404020. Available at: doi:10.1016/j.tet.2005.09.036
135. **BOUDREAU, P. D.**, MONROE, E. A., MEHROTRA, S., DESFOR, S., KOROBEYNIKOV, A., SHERMAN, D. H., MURRAY, T. F., GERWICK, L., DORRESTEIN, P. C. and GERWICK, W. H. Expanding the Described Metabolome of the Marine Cyanobacterium Moorea producens JHB through Orthogonal Natural Products Workflows. *PLoS ONE* [online]. 2015, **10**(7). ISSN 1932-6203. Available at: doi:10.1371/journal.pone.0133297
136. **SHISHIDO, T. K.**, JOKELA, J., KOLEHMAINEN, C.-T., FEWER, D. P., WAHLSTEN, M., WANG, H., ROUHIAINEN, L., RIZZI, E., DE BELLIS, G., PERMI, P. and SIVONEN, K. Antifungal activity improved by coproduction of cyclodextrins and anabaenolysins in Cyanobacteria. *Proceedings of the National Academy of Sciences* [online]. 2015, **112**(44). ISSN 0027-8424. Available at: doi:10.1073/pnas.1510432112
137. **MACMILLAN, J. B.**, ERNST-RUSSELL, M. A., DE ROPP, J. S. and MOLINSKI, T. F. Lobocyclamides A-C, lipopeptides from a cryptic cyanobacterial mat containing Lyngbya confervoides. *Journal of Organic Chemistry* [online]. 2002, **67**(23). ISSN 0022-3263. Available at: doi:10.1021/jo0261909
138. **KEM, M. P.** and BUTLER, A. Acyl peptidic siderophores: structures, biosyntheses and post-assembly modifications. *BioMetals* [online]. 2015, **28**(3). ISSN 0966-0844. Available at: doi:10.1007/s10534-015-9827-y
139. **ZHANG, Z.**, ZHOU, R., SAUDER, J. M., TONGE, P. J., BURLEY, S. K. and SWAMINATHAN, S. Structural and Functional Studies of Fatty Acyl Adenylate Ligases from E. coli and L. pneumophila. *Journal of Molecular Biology* [online]. 2011, **406**(2). ISSN 0022-2836. Available at: doi:10.1016/j.jmb.2010.12.011
140. **KRAAS, F. I.**, HELMETAG, V., WITTMANN, M., STRIEKER, M. and MARAHIEL, M. A. Functional Dissection of Surfactin Synthetase Initiation Module Reveals Insights into the Mechanism of Lipoinitiation. *Chemistry & Biology* [online]. 2010, **17**(8). ISSN 1074-5521. Available at: doi:10.1016/j.chembiol.2010.06.015
141. **CRYLE, M. J.**, MEINHART, A. and SCHLICHTING, I. Structural Characterization of OxyD, a Cytochrome P450 Involved in β-Hydroxytyrosine Formation in Vancomycin Biosynthesis. *Journal of Biological Chemistry* [online]. 2010, **285**(32). ISSN 0021-9258. Available at: doi:10.1074/jbc.M110.131904
142. **POMMEREHNE, K.**, WALISKO, J., EBERSBACH, A. and KRULL, R. *The antitumor antibiotic rebeccamycin - challenges and advanced approaches in production processes* [online]. 2019. ISSN 1432-0614. Available at: doi:10.1007/s00253-019-09741-y
143. **SANO, T.** and KAYA, K. A 3-amino-10-chloro-2-hydroxydecanoic acid-containing tetrapeptide from Oscillatoria agardhii. *Phytochemistry* [online]. 1997, **44**(8). ISSN 0031-9422. Available at: doi:10.1016/S0031-9422(96)00767-4

144. **EUSTÁQUIO, A. S.**, GUST, B., LUFT, T., LI, S. M., CHATER, K. F. and HEIDE, L. Clorobiocin biosynthesis in Streptomyces: Identification of the halogenase and generation of structural analogs. *Chemistry and Biology* [online]. 2003, **10**(3). ISSN 1074-5521. Available at: doi:10.1016/S1074-5521(03)00051-6
145. **SAURAV, K.** *Fatty acid substitution affecting the cytotoxicity of puwainaphycin/minutissamide isolated from marine benthic cyanobacterium Nodularia harveyana UHCC-0300*. in preparation, 2021.
146. CHEEL, J., URAJOVÁ, P., **HÁJEK, J.**, HROUZEK, P., KUZMA, M., BOUJU, E., FAURE, K. and KOPECKÝ, J. Separation of cyclic lipopeptide puwainaphycins from cyanobacteria by countercurrent chromatography combined with polymeric resins and HPLC. *Analytical and Bioanalytical Chemistry*, 2017, 409(4), 917-930.
147. VAŠÍČEK, O., **HÁJEK, J.**, BLÁHOVÁ, L., HROUZEK, P., BABICA, P., KUBALA, L. and ŠINDELÁŘOVÁ, L. Cyanobacterial lipopeptides puwainaphycins and minutissamides induce disruptive and pro-inflammatory processes in Caco-2 human intestinal barrier model. *Harmful Algae*, 2020, 96,101849.

Part III: Publications

Paper I: A Hybrid Non-Ribosomal Peptide/Polyketide Synthetase Containing Fatty-Acyl Ligase (FAAL) Synthesizes the β-Amino Fatty Acid Lipopeptides Puwainaphycins in the Cyanobacterium *Cylindrospermum alantosporum*

A Hybrid Non-Ribosomal Peptide/Polyketide Synthetase Containing Fatty-Acyl Ligase (FAAL) Synthesizes the β-Amino Fatty Acid Lipopeptides Puwainaphycins in the Cyanobacterium *Cylindrospermum alatosporum*

Jan Mareš[1,2,3], Jan Hájek[1,2,4], Petra Urajová[1], Jiří Kopecký[1], Pavel Hrouzek[1]*

1 Institute of Microbiology AS CR, v.v.i., Department of Phototrophic Microorganisms – ALGATECH, Třeboň, Czech Republic, **2** Biology Centre of AS CR, v.v.i., Institute of Hydrobiology, České Budějovice, Czech Republic, **3** University of South Bohemia, Faculty of Science, Department of Botany, České Budějovice, Czech Republic, **4** University of South Bohemia, Faculty of Science, Department of Molecular Biology and Genetics, České Budějovice, Czech Republic

OPEN ACCESS Freely available online

PLOS ONE

A Hybrid Non-Ribosomal Peptide/Polyketide Synthetase Containing Fatty-Acyl Ligase (FAAL) Synthesizes the β-Amino Fatty Acid Lipopeptides Puwainaphycins in the Cyanobacterium *Cylindrospermum alatosporum*

Jan Mareš[1,2,3], Jan Hájek[1,2,4], Petra Urajová[1], Jiří Kopecký[1], Pavel Hrouzek[1]*

1 Institute of Microbiology AS CR, v.v.i., Department of Phototrophic Microorganisms – ALGATECH, Třeboň, Czech Republic, **2** Biology Centre of AS CR, v.v.i., Institute of Hydrobiology, České Budějovice, Czech Republic, **3** University of South Bohemia, Faculty of Science, Department of Botany, České Budějovice, Czech Republic, **4** University of South Bohemia, Faculty of Science, Department of Molecular Biology and Genetics, České Budějovice, Czech Republic

Abstract

A putative operon encoding the biosynthetic pathway for the cytotoxic cyanobacterial lipopeptides puwainphycins was identified in *Cylindrospermum alatosporum*. Bioinformatics analysis enabled sequential prediction of puwainaphycin biosynthesis; this process is initiated by the activation of a fatty acid residue via fatty acyl-AMP ligase and continued by a multidomain non-ribosomal peptide synthetase/polyketide synthetase. High-resolution mass spectrometry and nuclear magnetic resonance spectroscopy measurements proved the production of puwainaphycin F/G congeners differing in FA chain length formed by either 3-amino-2-hydroxy-4-methyl dodecanoic acid (4-methyl-Ahdoa) or 3-amino-2-hydroxy-4-methyl tetradecanoic acid (4-methyl-Ahtea). Because only one puwainaphycin operon was recovered in the genome, we suggest that the fatty acyl-AMP ligase and one of the amino acid adenylation domains (Asn/Gln) show extended substrate specificity. Our results provide the first insight into the biosynthesis of frequently occurring β-amino fatty acid lipopeptides in cyanobacteria, which may facilitate analytical assessment and development of monitoring tools for cytotoxic cyanobacterial lipopeptides.

Citation: Mareš J, Hájek J, Urajová P, Kopecký J, Hrouzek P (2014) A Hybrid Non-Ribosomal Peptide/Polyketide Synthetase Containing Fatty-Acyl Ligase (FAAL) Synthesizes the β-Amino Fatty Acid Lipopeptides Puwainaphycins in the Cyanobacterium *Cylindrospermum alatosporum*. PLoS ONE 9(11): e111904. doi:10.1371/journal.pone.0111904

Editor: Avadhesha Surolia, Indian Institute of science, India

Received July 11, 2014; **Accepted** October 9, 2014; **Published** November 4, 2014

Copyright: © 2014 Mareš et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. DNA sequence data are available from the NCBI database (accession number KM078884 and BioProject PRJNA261005). All remaining relevant data are within the paper and its Supporting Information files.

Funding: This study was supported by the Grant Agency of the Czech Republic, project "Toxic potential, Evolution of Toxin Synthesis and Factors Driving Anatoxin-a Production in Benthic and Soil Nostocacean Cyanobacteria" (No. 14-18067S) and by the Center for Algal Biotechnology Třeboň - ALGATECH (CZ. 1.05/21.00/03.0110). The NMR experiments were performed at the Upper Austrian - South Bohemian Research Infrastructure Center in Linz, co-financed by the European Union in the context of the project "RERI-uasb", EFRE RU2-EU-124/100-2010 (ETC Austria-Czech Republic 2007–2013, project M00146). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: hrouzekp@gmail.com

Introduction

Analogous to many other bacterial groups, cyanobacteria possess a unique biosynthetic apparatus capable of generating enormous structural diversity in various secondary metabolites [1–3]. Large multidomain enzymes of non-ribosomal peptide synthetase (NRPS) machinery and polyketide synthetases (PKS) can be reassembled to generate an almost infinite number of chemical structures. Some of the end-products of this machinery have been found to be important pharmaceuticals; by contrast, others are recognized as important environmental toxins. In cyanobacteria, hepatotoxic microcystins [4], nodularins [5], cylindrospermopsin [6] and neurotoxic alkaloids anatoxin [7], and saxitoxins [8] are produced by the NRPS/PKS pathways. This finding has initiated intensive research on secondary metabolite biosynthesis in cyanobacteria. Moreover, these results also brought about the possibility of detecting cyanotoxins using molecular markers [6–9].

As one of the less explored cyanobacterial secondary metabolites, cyclic lipopeptides may be important due to their broad biological effects in various organisms, which also raises questions on their possible toxicity to humans [10,11]. Thus far, approximately 80 structural variants derived from several core structures have been isolated from cyanobacteria. The peptide cycle may comprise four amino acid residues, as in the case of anabaenolysins [12], or up to 14 amino acid residues, as in malevamides [13]. The lipidic part of the molecule may be formed by a modified fatty acid (FA) and connected to the ring by a carboxyl group via peptide bond formation, as in hassallidins [14]. Alternatively a β-amino FA may be incorporated into the cycle by two peptide bonds formed from a carboxyl group and a β-amino group, as found in the majority of described cyanobacterial lipopeptides. Some 16 core structures of cyanobacterial β-amino FA lipopeptides have been identified thus far. They display a broad range of bioactivities including cytotoxicity, as in the case of laxaphycins [15], hormothamnin A [16], minutissamides [17], pahayokolides [18]

and lyngbyacyclamides [19], or antifungal and/or antibacterial activity [20–25].

The chemical structures of cyanobacterial lipopeptides share some similarities with those produced by other bacterial groups. The well-known bacterial lipopeptide surfactin and members of the iturin lipopeptide family (bacillomycin, mycosubtilin, lichenysin) comprise a seven-member ring of D- and L-amino acids and a FA side chain of varying length [26–30]. In the surfactin molecule, the FA chain is connected to the ring by a peptide bond on the carboxyl side and an ester bond between the β-hydroxy group of the unusual FA and the isoleucine of the peptide cycle. In the case of iturin family members, β-amino FA is present; thus, it is incorporated via a standard peptide bond, similar to the majority of cyanobacterial lipopeptides. Finally, the FA chain may be connected via an ester bond to the linear part of the peptide molecule, as in fengycin [31], which is the structural analog of the cyanobacterial metabolite hassallidin. The majority of reported bacterial lipopeptides have been isolated from *Bacillus subtillis* and display a broad range of biological activities including antibiotic and antifungal effects [32].

The biosynthesis pathway of small bacterial lipopeptides was initially elucidated for mycosubtilin, an iturin lipopeptide family member produced by *Bacillus subtillis* ATCC6633 [33]. Insertional mutagenesis of an operon encoding four ORFs (*fenF*, *mycA-C*) proved the operon to be responsible for mycosubtilin biosynthesis. The operon encodes MycA, a hybrid enzyme combining domains involved in polyketide, FA and peptide synthesis. This multifunctional enzyme activates a FA residue, elongates it, modifies it by placing an amino moiety on the β position, catalyzes asparagine addition and passes it on for further synthesis of the peptide cycle by two peptide multisynthetases (MycB and MycC). The mycosubtilin biosynthesis is finalized by forming a peptide bond between the β-amino group of the FA and the last asparagine of the cycle. Similarly, in the case of surfactin [34], biosynthesis is initiated by transfer of a β-hydroxy FA mediated by acyl transferase SrfD. Eventually, the peptide cycle is closed by forming an ester bond of the hydroxyl with a leucine of the peptide backbone [35].

In cyanobacteria, an operon encoding the enzymes involved in the synthesis of the hassallidin type of lipopeptides has been recently characterized [36]. The authors clarified the formation of the peptide backbone and predicted the modules responsible for glycosylation and acetylation of the molecule. As mentioned above, apart from the hassallidin (sfingomycin) type of lipopeptides, cyanobacteria produce structurally diverse cyclic peptides containing β-amino fatty acids that possess cytotoxic and antifungal bioactivities and that may potentially be toxic to humans. Thus, knowledge of their biosynthetic pathways is essential for the understanding of chemical variability in their structures and for providing screening markers for toxic lipopeptides in cyanobacteria.

In this report, we describe the characterization of the biosynthetic gene cluster encoding the cytotoxic cyanobacterial lipopeptides puwainaphycins and describe novel structural variants of puwainaphycins synthesized by a single enzyme complex. For the first time, we identify an operon of a β-amino lipopeptide in cyanobacteria, comparing its features to the lipopetide variants produced, and discussing the presence of genes that may be essential for the biosynthesis of cyclic bacterial lipopetides in general.

Materials and Methods

Cultivation of cyanobacterial biomass

The uni-cyanobacterial strain *Cylidrospermum alatosporum* CCALA 988 (C24/1989), previously shown to be a typical member of the *Cylidrospermum* cluster [37], was cultivated in 350-mL glass tubes in liquid Allen Arnon medium [38] and bubbled with 2% CO_2-enriched air at a constant temperature of 28°C, under 50 $W.m^{-2}$ continuous illumination. Following 5–7 days of cultivation, the culture was harvested by centrifugation, stored at –70°C and freeze-dried.

Extractions

Eight grams of lyophylized *Cylindrospermum alatosporum* biomass was extracted using methanol/water (70/30, v/v) for 1 hour and then centrifuged at 1920 *g*. Supernatant was collected, and the volume was reduced using a rotary evaporator where the temperature did not exceed 38°C. The final solution was extracted in a glass funnel using H_2O:n-hexan (4:1). The water phase was collected and concentrated under the same conditions using a rotary evaporator and finally dissolved in 2 mL pure MeOH for high-performance liquid chromatography (HPLC) purification.

To quantify 4-methyl-Ahdoa-PUW-F and 4-methyl-Ahtea-PUW-F (Puwainaphycin F), 200 mg of freeze-dried *Cylindrospermum alatosporum* biomass was extracted using methanol/water (70/30, v/v) solution for one hour. Following centrifugation at 1920 *g* for 10 minutes, the supernatant was concentrated under a vacuum at 38°C to dryness, and the residue was then dissolved in 1 mL of methanol/water (70/30, v/v) solution. After centrifugation, the pellet was extracted once again using the same procedure. The solutions were 10x diluted prior to LCMS analysis. All samples were prepared in triplicate. Purified compounds used as standards were prepared in our laboratory.

HPLC purification

The concentrated extract was purified using a HPLC-MS Agilent 1260 Infinity series equipped with preparative pumps, a multiwavelength detector, automatic fraction collector and a mass spectrometer ESI-Quadrupole (Agilent 6120). The first step was performed using a semi-preparative Eclipse XDB-C18 column (9.4×250 mm) with methanol/water gradient, see Table S1, and at a flow rate of 3.5 $mL.min^{-1}$. Fractions were collected using an automatic fraction collector at 1-min intervals. The fractions containing puwainaphycin analogs with *m/z* 1118.6, 1134.6, 1146.6, 1152.6, 1162.6 and 1180.6 were collected in separate vials. A second purification step was performed using a semi-preparative Reprosil 100 Phenyl column (250×8 mm) with methanol/water gradient, see Table S2, at a flow rate of 2.5 $mL.min^{-1}$. Desired fractions were collected again using the automatic fraction collector. The fractions obtained were dried under nitrogen.

HPLC–high-resolution mass spectrometry (HRMS) analysis and MS/MS experiments

Cylindrospermum alatosporum 70% methanolic extracts were analyzed using a Thermo Scientific Dionex UltiMate 3000 UHPLC+ equipped with a diode-array detector. Separation of compounds was performed on a reversed-phase Phenomenex Kinetex C18 column (150×4.6 mm, 2.6 μm) using H_2O (A)/acetonitrile (B), both of which contained 0.1% HCOOH as a mobile phase, at a flow rate of 0.5 $mL.min^{-1}$. The gradient was as follows: A/B 85/15 (0 min), 85/15 (in 1 min), 0/100 (in 20 min), 0/100 (in 25 min) and 85/15 (in 30 min). The HPLC was connected to a Bruker Impact HD high-resolution mass

spectrometer with electrospray ionization. The following settings were used: dry temperature, 200°C; drying gas flow, 12 L.min^{-1}; nebulizer, 3 bar; capillary voltage, 4500 V; endplate offset, 500 V. The spectra were collected in the range 20–2000 *m/z* with a spectra rate of 3 Hz. Spectra were calibrated using both LockMass 622 internal calibration solution and sodium formate clusters at the beginning of each analysis. The summary formulas of molecular peaks obtained were calculated using Smart Formula in Bruker Compass DataAnalysis software (version 4.2). The fractions obtained from preparative chromatography were used for manual direct infusion to a Bruker Impact HD spectrometer at the following settings: dry temperature, 180°C; drying gas flow, 4 L.min^{-1}; nebulizer, 0.4 bar; capillary voltage, 4500 V; endplate offset, 500 V. The spectra were collected in the range 20–2000 *m/z* at a spectra rate of 3 Hz. The fractions were dissolved in methanol containing 0.05% formic acid for more effective ionization, and molecular peaks were isolated in quadrupole with window 3 *m/z*, and the collision energy was set manually from 0 to 150 eV.

Nuclear magnetic resonance

Pure samples were diluted in 500 µL of DMSO. For NMR measurements, a 700 MHz AvanceIII spectrometer with an Ascend magnet and a TCI cryoprobe for high sensitivity and high resolution was used. For structure determination, ^{1}H, $^{1}H^{13}C$ heteronuclear single quantum coherence NMR spectroscopy (HSQC), $^{1}H^{15}N$ HSQC and TOSCY spectra were recorded. Data were analyzed using Bruker TopSpin 3.0 and MestReNova 6.0.

Molecular and bioinformatic analysis

Single filaments of *Cylindrospermum alatosporum* CCALA 988 were isolated using the glass capillary technique [39] using an Olympus CX31 microscope (×200–400 magnification) to exclude minor bacterial contaminants from the subsequent molecular analyses. Suitable filaments were washed using 10 droplets of TE buffer, and finally, each was placed in a single 0.2-mL PCR tube. All instruments and the TE buffer were sterilized by autoclaving and UV, and the microscope work area was cleaned using 100% ethanol and treated with UV light for one hour prior to the start of the isolation. After freezing (–20°C) for 3 days and thawing for the initiation of cell lysis, the *C. alatosporum* filaments were used as a template for whole-genome amplification (WGA). Multiple displacement amplification (MDA) with Phi29 polymerase was conducted using Repli-g Mini Kit (Qiagen) according to the manufacturer's protocol. MDA products were tested by PCR for the cyanobacterial 16S rRNA gene [40]. Positive samples (16 MDA products) were then pooled to create a template for WGA sequencing, to reduce possible unequal MDA amplification throughout the genome. The DNA was sent for commercial *de novo* genome sequencing (Macrogen, Inc.) using Illumina HiSeq2000 (Illumina) with a ~500-bp insert Pair-End library and 100-bp reads (approximately 9 Gbp data yield). The sequence data were deposited in the NIH Sequence Read Archive (http://trace.ncbi.nlm.nih.gov/Traces/sra/) and they are accessible under the NCBI Bio Project PRJNA261005, BioSample SRS700947.

Raw data from *de novo* WGS were assembled using CLC Bio Genomics Workbench (CLC Bio). The genomic contigs were loaded into Geneious Pro R6 (Biomatters; available from http://www.geneious.com) and investigated for NRPS genes using blastp searches with several known cyanobacterial amino acid adenylation domains (A-domains) as queries. Contigs yielding high similarity hits for A-domains were then analyzed using the Glimmer 3 [41] algorithm to discover putative ORFs. Functional annotation of the ORFs (Table S3) was conducted by applying a combination of blastp searches against the NCBI database, and by HMMER 3 [42] and InterPro Scan 5 [43] searches in available protein databases. The organization of the individual NRPS/PKS modules and A-domain amino-acid specificity was assessed using PKS/NRPS Analysis [44], NRPSpredictor2 [45] and NaPDoS [46] online applications.

The predicted biosynthetic pathway for puwainaphycins contained two minor gaps in the DNA sequence, which were covered by Sanger sequencing (SeqMe, Dobříš, Czech Republic) using all combinations of custom specific primers located near to contig ends. The complete sequence of the predicted biosynthetic pathway was uploaded to the NCBI GenBank database under accession number KM078884.

Results

Predicted biosynthetic pathway

After the final assembly of Illumina and Sanger sequencing data, the predicted puwainaphycin biosynthesis pathway was recovered in the middle of a ~100-kbp long contig, with >20 kbp flanks at each side to ensure that the full pathway was sequenced. The putative biosynthetic gene cluster was 56,728 bp long and comprised 10 protein-coding ORFs. The ORFs were transcribed starting from a bi-directional promoter region, with *orf2*, *puwA* and *orf1* transcribed in one direction and *puwB-H* in the opposite direction (Figure 1). The functional annotation of the individual enzymes and catalytic domains is summarized in Figure 1 and Table S3.

The reconstructed course of puwainaphycin biosynthesis, as visualized in Figure 2, starts with the *puwC* gene product. This protein contains conserved catalytic domains typical for fatty acyl-AMP ligases (FAALs), enzymes that activate a fatty acyl by adenylation to subsequently serve as a substrate for polyketide synthetases. PuwC also showed a relatively high amino acid sequence identity (approximately 60%) to several annotated cyanobacterial fatty-acyl acyl carrier protein (ACP) ligases. After activation of the fatty acyl and its ligation to the first ACP encoded by *puwD*, it is forwarded directly to the PKS machinery. The first unimodular PKS (encoded by *puwB*) contains a canonical sequence of acyltransferase, dehydratase, methyltransferase, enoylreductase and ketoreductase domains, resulting in an α-methylated product. The next enzyme (PuwE) is an unusual hybrid comprising one PKS module, a putative aminotransferase and a monooxygenase domain, and a terminal single NRPS module. The PKS module solely elongates the acyl chain with no additional modifications; however, the aminotransferase and monooxygenase located between the PKS and NRPS modules of the enzyme apparently catalyze the tailoring of the chain into a 3-amino-2-hydroxy-4-methyl-acyl typical for this group of lipopeptides. The predicted aminotransferase and monooxygenase domains show sequence similarity (50–70%) to several class-III aminotransferases and flavin-utilizing monooxygenases from cyanobacteria. The terminal NRPS module includes an A-domain activating valine as the first amino acid member of the oligopeptide ring. Valine was chosen as the most probable amino acid based on a nearest-neighbor search in NRPSPredictor2 (90%); the closest blast hits were uncharacterized cyanobacterial A-domains. Subsequently, the intermediate is transferred to another NRPS enzyme (PuwF) that comprises two modules, each adding one amino acid - dehydrothreonine and asparagine/glutamine. The A-domain putatively responsible for the incorporation of dehydrothreonine shows an amino acid-specific structural motif with considerable similarity to known threonine-activating

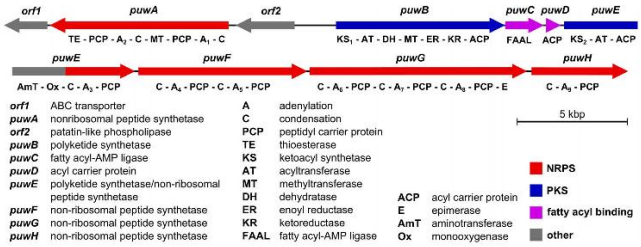


Figure 1. Gene arrangement, functional annotation and domain structure of the *puw* gene cluster (56.7 kbp) from *Cylindrospermum alatosporum* CCALA 988.
doi:10.1371/journal.pone.0111904.g001

A-domains (100% threonine in nearest-neighbor search, 64–66% pairwise identity to McnB and OciE, which are involved in cyanopeptolin synthesis in *Microcystis* and *Planktothrix*). Because no additional tailoring enzymes with amino-acid dehydration function were found in the pathway, this domain is possibly specific directly for dehydrothreonine. The second A-domain of PuwF was recovered as specific (100%) to asparagine in the nearest-neighbor analysis; however, the closest, weakly similar blast hit (54% amino acid sequence identity) was the glutamine-activating McnA protein included in cyanopeptolin biosynthetic pathways. Considering the puwainaphycin F/G variants reported in this study, the A-domain appears to accept both asparagine and glutamine as a substrate at a defined rate, as further demonstrated by MS measurements. The following biosynthesis steps are catalyzed by the *puwG* product that comprises three NRPS modules predicted to incorporate dehydrothreonine, asparagine and alanine. The dehydrothreonine-incorporating module is highly identical (97%) to that in PuwF, as described earlier. The other two modules activate asparagine and alanine, based on a nearest neighbor search (100%). The first shows similarity (55%) to the asparagine-activating A-domain in NosC nostopeptolide synthetase from *Nostoc*, and the second is similar (63% identity) to the alanine-activating domain in the jamaicamide synthesis gene JamO (*Lyngbya majuscula*). The last module of PuwG includes an epimerization domain consistent with the occurrence of both optical isomers (L/D-alanine) in the puwainaphycin molecules. The addition of threonine, the next member of the oligopeptide ring, is catalyzed by another protein (PuwH) that possesses a single NRPS module (100% threonine in a nearest-neighbor search, 65% identity with threonine-incorporating OciE). The last enzyme involved in the peptidyl elongation is PuwA. It clearly comprises two NRPS modules, the first containing a specific motif in the A-domain predicted to activate asparagine (90% asparagine in a nearest-neighbor search, 57% identity with NosC asparagine-activating A-domain) and a methyltransferase domain obviously linked to N-methylation of that asparagine. The second module is predicted to incorporate proline (80% proline in a nearest-neighbor search, 63% identity with NpnC proline-activating domain involved in nostophycin synthesis) and a thioesterase domain in its terminal part that cleaves the finished puwainaphycin chain from the peptidyl carrier protein, thus promoting its cyclization.

The function of the *orf2* product could not be confidently linked to any step in puwainaphycin biosynthesis. Its amino acid sequence shows considerable similarity (40–55%) to cyanobacterial patatin-like family phospholipases, enzymes capable of fatty-acyl cleavage from membrane phospholipids. The last ORF that appears to be part of the biosynthetic cluster is *orf1*. The encoded protein shows similarity (53–58%) to the ABC-transporter family proteins in cyanobacteria; thus, it may be involved in the transport of puwainaphycin to its destination in the cell. No specific enzymes linkable to chlorination and hydroxylation of the fatty acyl chain were noted in the puwainaphycin biosynthetic cluster or its vicinity, and no protein coding sequence with predicted halogenase activity was recovered from the WGS data. Thus, the mechanism of these modifications for a minor part of the puwainaphycin produced remains unexplained by the genomic data.

High-resolution HPLC-MS analysis of puwainaphycins analogs

The HPLC-HRMS analysis of crude *Cylindrospermum alatosporum* extract revealed about 30 pseudomolecular ions in the region 1050–1200 Da, which were attributed to putative puwainaphycin analogs. Puwainaphycin F (*4-methyl-Ahtea*-PUW-F), together with the congener of *m/z* 1118.6, were found to be dominant based on UV and MS detection, other variants were observed in trace amounts based on their MS signals (Figure 3B). Of these we have selected eight more prominent peaks for purpose of this study. Their exact masses were measured with high precision (0.0–4.9 ppm), enabling calculation of the elemental composition (Table 1). The MS/MS experiments provided product ions corresponding to identical losses of *N*-methyl asparagine (Δ 128), dehydrated threonine (Δ 83), alanine (Δ 71), asparagine (Δ 114) and dehydrothreonine (Δ 83) in all compounds detected (Figure S1). Furthermore, the fragmentation pattern of the pseudomolecular ions with *m/z* 1118.6, 1152.6, 1180.6, 1134.6 and 1162.6 showed clear loss of an additional asparagine and thus have the same amino acid composition as *4-methyl-Ahtea*-PUW-F (Figure S1A, B). The loss of glutamine was recognized in compounds *m/z* 1126.6, 1132.6, 1176.6, 1194.6, and 1166.6; thus, their amino acid sequence is identical to puwainaphycin G (*4-methyl-Ahtea*-PUW-G; Figure S1C, D). Moreover, the fragmentation of all of these compounds led to the formation of the diagnostic fragments *m/z* 101.0, 186.1, 198.1, 269.2, 281.2, which were identical for all precursor ions and further confirm the identical primary structure of the cyclic part of the molecule. The ion 281.2 corresponded to fragment $C_{12}H_{16}N_4O_4+H^+$ (Pro-X^1-Val-dThr, where X^1 corresponds to the 3-amino-2-oxopropanoyl fragment of the 3-amino-2-hydroxy-4-methyl-fatty acid but where the major part of the aliphatic chain was missing. The remaining diagnostic fragments were derived from ion 281.2. These results

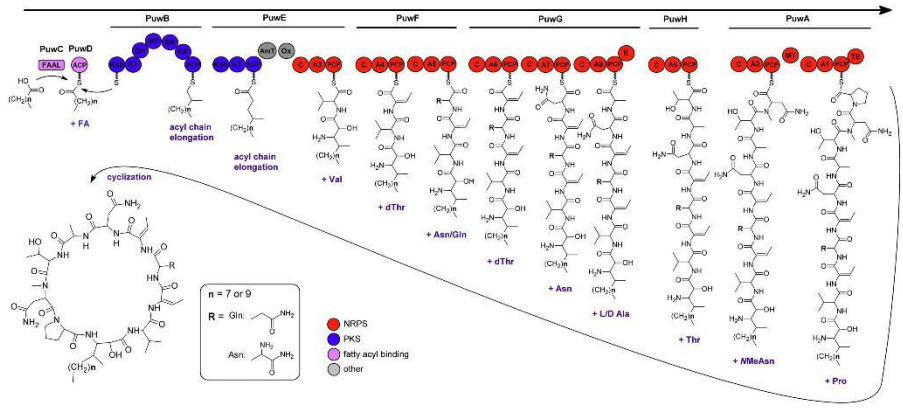


Figure 2. Proposed biosynthesis of puwainaphycins. A, adenylation domain; ACP, acyl carrier protein; AmT, aminotransferase; AT, acyltransferase; C, condensation domain; DH, dehydratase; E, epimerase; ER, enoylreductase; FAAL, fatty acyl-AMP ligase; MT, methyltransferase; NRPS, non-ribosomal peptide synthetase; KR, ketoreductase; KS, ketosynthetase; Ox, monooxygenase; PCP, peptidyl carrier protein; PKS, polyketide synthetase; TE, thioesterase.
doi:10.1371/journal.pone.0111904.g002

confirm that non-identical parts of the puwainaphycin variants are situated in the unusual FA aliphatic chain whereas the remaining part of the molecule is identical for all the puwainaphycin analogs, based on the PUW-F/PUW-G structures. Indeed, within the fragmentation spectra of *4-methyl-Ahtea*-PUW-F and its analog 1118, the intense fragment ions *m/z* 535 and 507, corresponding to sequence Pro-X-Val-DhB (with X as modified FA), were recognized (Figure 4). The mass difference of 28.0291 Da corresponded well with $-(CH_2)_2$ on the FA chain (2.2 ppm), and this mass difference was noted in all fragments containing the FA residue (Table S4 A–F). The presence of 3-amino-2-hydroxy-4-methyldodecanoic (*4-methyl-Ahdoa*) instead of 3-amino-2-hydroxy-4-methyltetradecanoic acid (*4-methyl-Ahtea*) in the congener 1118.6 was further confirmed by NMR measurements (see Table S5). The 2D NMR spectra (Figure S2) of congener 1118.6 were almost identical to the previously measured NMR spectra of *4-methyl-Ahtea*-PUW-F [10]. The only non-identical part of the spectrum was noted in the HSQC region set by a ^{13}C shift of 15–34 ppm and a ^{1}H shift of 0.5–1.5 ppm. Although in *4-methyl-Ahtea*-PUW-F $^{1}H^{13}C$, the HSQC crosspeaks X-CH_2^1 to X-CH_2^9 were identified, in the case of *4-methyl-Ahdoa*-PUW-F, the crosspeaks X-CH_2^8 and X-CH_2^9 were clearly missing (see Figure 5). An identical MS/MS fragmentation pattern was also provided by the ion 1132.6 (Figure S1 C, D), a structural analog of *4-methyl-Ahtea*-PUW-G; thus, the *4-methyl-Ahdoa*-PUW-G was confirmed as the variant of puwainaphycin G with tetradecanoic acid replaced by dodecanoic acid (see Table S4). The $^{1}H^{13}C$ HSQC crosspeaks unique for Gln^4 in *4-methyl-Ahdoa*-PUW-G were also identified in the sample; however, the full assignment of this variant was not successful due to overlapping of very similar signals with *4-methyl-Ahdoa*-PUW-F and its low concentration (Table S5). Additionally, in some of less intensive molecular ions observed, the fragmentation analysis suggests presence of analogs with shorter (<C12) and longer (>C14) FA chains (data not shown).

MS/MS spectra were also obtained from other puwainaphycin congeners that showed a constant shift in mass of the fragments possessing FA moiety compared with other congeners. In the pseudomolecular ions 1180.6 and 1152.6, the fragments containing FA chains differed by 33.96 Da from *4-methyl-Ahtea*-PUW-F and *4-methyl-Ahdoa*-PUW-F, respectively. This mass difference fits with replacing one hydrogen bonded on the FA chain by chlorine (2.2 ppm). The presence of chlorine on the aliphatic FA chain has already been described in puwainaphycin variants C–D [45]. The fragmentation of ion 1180.6 showed not only a constant shift in mass but also a new fragment ion of *m/z* 533.4. This fragment ion may be assigned to Pro-X^1-Val-DhB, where X^1 represents monounsaturated 4-methyl-Ahtea formed by the loss of –Cl and subsequent double bond formation. Analogously, the ion of *m/z* 505.3 observed in the fragmentation spectrum of ion 1152.6 corresponds well with Pro-X^2-Val-DhB, where X^2 is monounsaturated 4-methyl-Ahdoa (Figure 4, Table S3 C, D). Identical fragmentation leading to similar product ions was observed in PUW-G analogs (1194.6 and 1166.6) and thus also chlorinated *4-methyl-Ahdoa*-PUW-G and *4-methyl-Ahtea*-PUW-G were confirmed to be produced by the *Cylindrospermum* strain. Finally, the presence of hydroxylated variants of all puwainaphycin congeners may be explained by the constant shift of 16 Da on the lipidic chain and the formation of a fragment with monounsaturated 4-methyl-Ahdoa/Ahtea of *m/z* 533.4 and 505.3 (Figure 4, Table S3 E, F).

Quantitative analysis and ratio of puwainaphycin variants

Quantitative analysis was performed using a calibration curve with a range of concentrations of *4-methyl-Ahtea*-PUW-F and *4-methyl-Ahdoa*-PUW-F of 10–500 $\mu g.mL^{-1}$. The MS peaks, belonging to *4-methyl-Ahdoa*-PUW-F and *4-methyl-Ahtea*-PUW-F, were eluted at 12.9 and 14.6 min, respectively. The content of *4-methyl-Ahdoa*-PUW-F and *4-methyl-Ahtea*-PUW-F in freshly harvested biomass was 13.9 ± 7.3 $mg.g^{-1}$ of dry biomass and 7.7 ± 3.9 $mg.g^{-1}$ of dry biomass, respectively. The content of the

Table 1. Exact masses of puwainaphycin analogs and their elemental composition.

m/z	Molecular Formula	Puwainaphycin Variants
1118.6248	$C_{61}H_{83}N_{13}O_{15}+H^+$	*4-methyl-Ahdoa*-Puw-F
1132.6355	$C_{62}H_{85}N_{13}O_{15}+H^+$	*4-methyl-Ahdoa*-Puw-G
1134.6146	$C_{61}H_{83}N_{13}O_{16}+H^+$	*hydroxy-4-methyl-Ahdoa*-Puw-F
1148.6342	$C_{62}H_{85}N_{13}O_{16}+H^+$	*hydroxy-4-methyl-Ahdoa*-Puw-G
1146.6517	$C_{63}H_{87}N_{13}O_{15}+H^+$	*4-methyl-Ahtea*-Puw-F **(Puwainaphycin F)**
1160.6727	$C_{64}H_{89}N_{13}O_{15}+H^+$	*4-methyl-Ahtea*-Puw-G **(Puwainaphycin G)**
1152.5822	$C_{61}H_{82}ClN_{13}O_{15}+H^+$	*chloro-4-methyl-Ahdoa*-Puw-F
1166.5953	$C_{62}H_{84}ClN_{13}O_{15}+H^+$	*chloro-4-methyl-Ahdoa*-Puw-G
1162.6451	$C_{63}H_{87}N_{13}O_{16}+H^+$	*hydroxy-4-methyl-Ahtea*-Puw-F
1176.6648	$C_{64}H_{89}N_{13}O_{16}+H^+$	*hydroxy-4-methyl-Ahtea*-Puw-G
1180.6136	$C_{63}H_{86}ClN_{13}O_{15}+H^+$	*chloro-4-methyl-Ahtea*-Puw-F
1194.6296	$C_{64}H_{88}ClN_{13}O_{15}+H^+$	*chloro-4-methyl-Ahtea*-Puw-G

doi:10.1371/journal.pone.0111904.t001

two congeners was greater compared with biomass stored seven years at laboratory temperature, the concentrations of which were 9.8 ± 4.0 mg.g^{-1} of dry biomass and 5.7 ± 2.1 mg.g^{-1} of dry biomass. The ratio in content of both congeners remained almost constant during storage. Although the ratio of *4-methyl-Ahdoa*-PUW-F/*4-methyl-Ahtea*-PUW-F was 1.83 in freshly harvested biomass, the ratio changed to 1.74 in the aged biomass. Furthermore, the ratio of asparagine vs. glutamine variants of the different congeners was verified by HPLC-HRMS. The ratio was calculated by comparing the integrated area of peaks on an extracted ion chromatogram ($[M+H]^+$, $[M+Na]^+$, $[M+2H]^{2+}$) of every single congener. The proportion of asparagine vs. glutamine variants was similar for all puwainaphycin analogs and varied from 1:0.15 to 1:0.23 (Table 2). However, the lower ratio was observed in the variants that were low in concentration; thus, the peak integration may have contained greater error.

Discussion

Utilizing a combination of DNA sequencing, bioinformatics analyses and analytical chemistry (HRMS, NMR) approaches, we elucidated the genetic background and probable course of biosynthesis of the cytotoxic cyanobacterial lipopeptides puwainaphycins [10]. Puwainaphycins represent a large group of cyanobacterial β-amino FA containing lipopeptides with unknown biosynthetic pathways [12,13,15,16,18,22,23,25,47]. Thus, our results are the first step in clarifying the origin of an entire family of natural secondary metabolites. Some of these compounds have strong membrane disruption effects on human cells [10–12]. Interestingly, puwainaphycins possess some structural traits analogous to antibiotics of the iturin family produced by *Bacillus subtilis*, the biosynthesis of which has already been characterized [33,48,49]. However, the β-amino FA linked by two peptide bonds, which is present in the iturin family of antibiotics, lacks some of the modifications typical for puwainaphycins and the majority of cyanobacterial lipopeptides. This especially relates to the 2-hydroxy and 4-methyl functional groups. The puwainaphycin biosynthetic operon also shows features similar to that of the iturin family members iturin A, mycosubtilin and bacillomycin D. Most importantly, the AMP-dependent fatty acyl ligase (FAAL) and aminotransferase domains present in MycA, ItuA and BamA have been proposed to be responsible for the formation of the β-amino FA part of the molecule [33,48,49]. The enzyme was reported to also include a PKS and NRPS module in a single ORF in addition to FAAL and aminotransferase. In the puwainaphycin cluster, the organization is highly divergent from that of the iturin

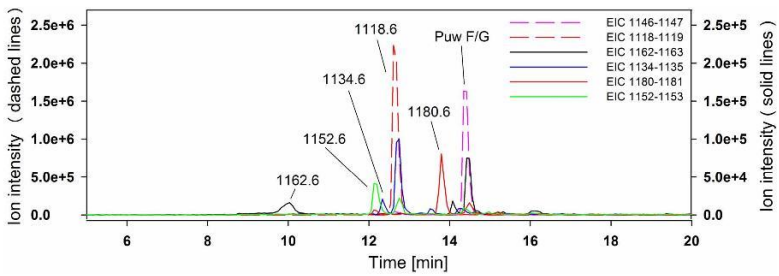


Figure 3. HPLC-HRMS analysis of *Cylindrospermum alatosporum* CCALA 988 extract. Extracted ion chromatograms of puwainaphycin analogs are shown, indicating their *m/z* values using different scales for major (left axis) and minor variants (right axis).
doi:10.1371/journal.pone.0111904.g003

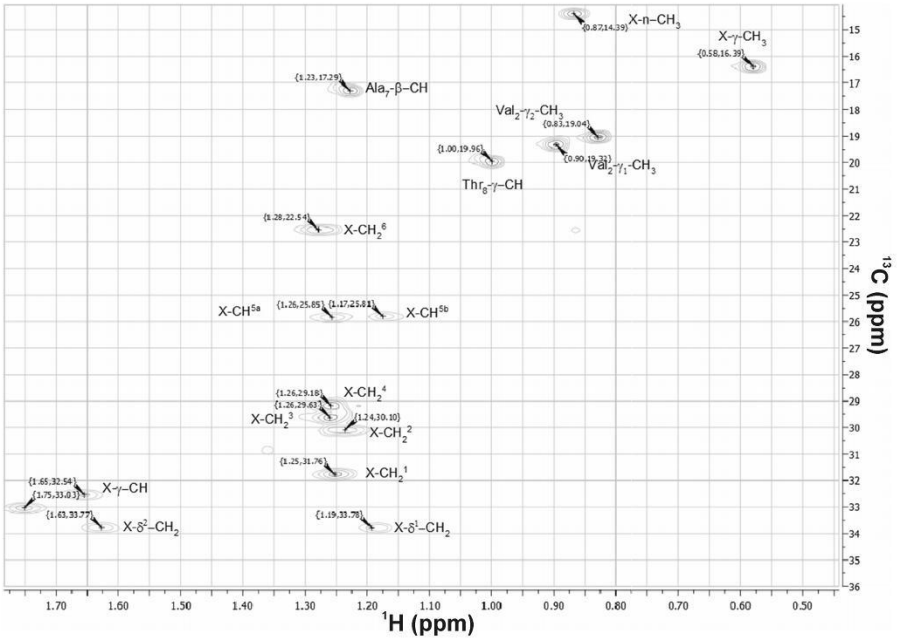


Figure 4. $^{1}H^{13}C$ HSQC spectrum of 4-methyl-Ahdoa puwainaphycin F at a range of shifts 15–34 ppm for C^{13} and 0.5–1.5 ppm for ^{1}H. Assignment of the peaks shows an aliphatic chain containing 7 carbons ($X\text{-}CH_2^{1-6}$, $X\text{-}CH_3$) and additional peaks $X\text{-}CH_2^{7-8}$ found in 4-methyl-Ahtea puwainaphycin F are missing.
doi:10.1371/journal.pone.0111904.g004

family. The FAAL enzyme and the ACP, to which the FA is ligated at the very beginning of puwainaphycin synthesis, are encoded in separate ORFs (*puwC-D*), whereas the PKS subunit (PuwB) that elongates and methylates the fatty acyl is another gene. Moreover, the next ORF in the biosynthetic cascade (*puwE*) encodes a hybrid PKS/NRPS enzyme that comprises the aminotransferase and oxygenase domains that modify the methylated fatty acyl into a 3-amino-2-hydroxy-4-methyl-acyl typical for this family of lipopeptides. Based on HRMS measurements, a small portion of the puwainaphycin variants produced was found to be chlorinated or hydroxylated on the fatty acyl chain. This type of halogenation has been repeatedly reported from peptides possessing FA chain in cyanobacteria [1,17,50] including puwainaphycins [47]. In unusual chlorinated lipopetides barbamides, the chlorination was associated with a halogenase encoded within the biosynthetic pathway in *Lyngbya majuscula* [51]. However, as demonstrated for a linear lipopeptide oscillaginin A in *Planktothrix rubescens*, chlorination of the FA chain may also occur when no known type of halogenase is noted in either the biosynthetic cluster or the entire genome [52]. Similarly, in the case of the puwainaphycins, the mechanism of halogenation in these compounds remains unexplained. Perhaps a non-specific post-synthesis mechanism is involved, as further supported by the relatively low ratio of chlorinated puwainaphycin forms. However, the hydroxylation may be performed by a number of oxygenases putatively expressed by *Cylindrospermum*. Nevertheless, none were predicted as a standalone dedicated enzyme or catalytic domain within the *puw* cluster.

Based on the evidence presented above, we can conclude that all 12 detected puwainaphycin congeners produced by *Cylindrospermum alatosporum* CCALA 988 are synthesized by a single NRPS/PKS synthetase. The chemical structure of the previously characterized puwainaphycins A-E is similar to the F and G variants studied hereby, maintaining a 10-membered ring and sharing valine, dehydrothreonine, N-methylasparagine, and proline in the positions 2, 3, 9 and 10, respectively. However in the positions 4–8 the aminoacid residues can vary. In the position 4 asparagine/glutamine is replaced by threonine in puwainaphycin A-E. The position 5, which is occupied by dehydrothreonine in puwainaphycin F and G, is held by threonine or valine in puwainaphycin A-E. Alanine residue (position 6) in puwainaphycin F and G is replaced by glycine, and finally the threonine residue in the position 7 is replaced by O-methyl threonine in puwainaphycin A-E. Although this is the first report of a β-amino FA lipopeptide operon in cyanobacteria, we suggest that this diversity is most likely obtained by the replacement of the individual NRPS modules within the synthetase with modules activating alternative amino acids. The variation between asparagine and glutamine in the third amino acid position, the only difference between puwainaphycin F and G core cyclic structures,

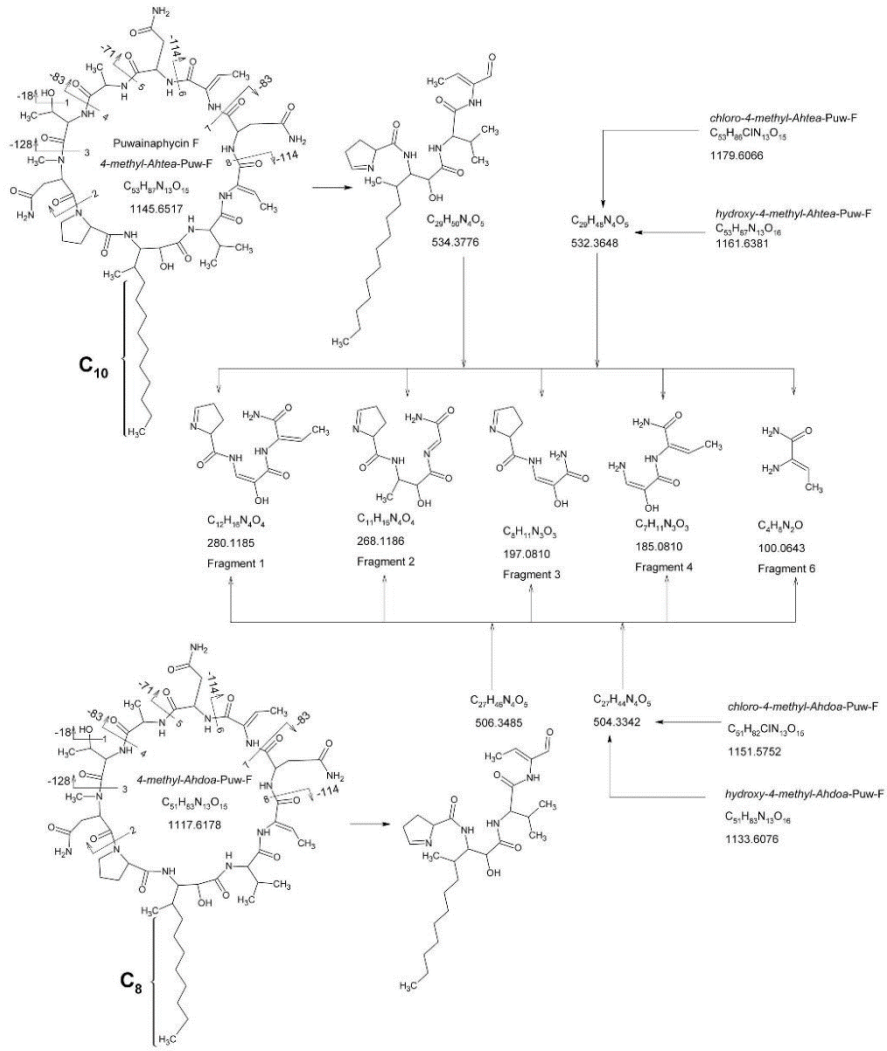


Figure 5. Fragmentation of 4-methyl-Ahdoa and 4-methyl-Ahtea puwainaphycin F and their chlorinated and hydroxylated analogs. The observed fragmentation losses are denoted by the corresponding amino acid residues. The diagnostic fragments common to all puwainaphycin analogs are marked as Fragments 1–6.
doi:10.1371/journal.pone.0111904.g005

is most likely caused by extended substrate specificity of the amino acid activation domain of the PuwF subunit of the NRPS. Due to high 3D structural similarity, glutamine and asparagine may be complementary to the active site of the enzyme and therefore the

Table 2. Relative ratio of asparagine[4]- vs. glutamine[4]-containing puwainaphycin variants.

Fatty acid	EIC PUW-F [milions of ions]	EIC PUW-G [milions of ions]	Ratio
4-methyl-Ahtea	38.97	8.3	1:0.21
4-methyl-Ahdoa	48.94	11.28	1:0.23
chloro-4-methyl-Ahtea	8.2	1.73	1:0.21
chloro-4-methyl-Ahdoa	6.77	1.35	1:0.20
hydroxy-4-methyl-Ahtea	0.84	0.17	1:0.20
hydroxy-4-methyl-Ahdoa	1.81	0.28	1:0.15

The only deviation from the ratio 1:0.2 was recorded in the case of hydroxy-4-methyl-Ahdoa PUW F/G, which may be explained by its low ion intensity and the presence of coeluting compounds.
doi:10.1371/journal.pone.0111904.t002

extended substrate specificity is not surprising. The multispecificity of several adenylation domains has been shown already [50,53]. The HPLC-MS data further showed that all variants based on the core cyclic structure of puwainaphycin F and G are produced at a ratio of 1:0.150.23. This ratio is consistent with previously published data obtained by NMR on mixed fractions of the F and G variant where the molar ratio was noted to be 1:0.24 [10]. Thus, we propose that the adenylation domain of PuwF incorporates one glutamine per four asparagine molecules. A similar mechanism most likely plays a role in the addition of the FA. Apparently, enanthic (C7) or pelargonic (C9) acid may be activated by the FAAL domain and subsequently elongated and modified to the final 3-amino-2-hydroxy-4-methyl-fatty acid. Based on quantitative HPLC-HRMS analyses, the *4-methyl-Ahdoa*-PUW-F and *4-methyl-Ahtea*-PUW-F are produced in a ratio of approximately 1:0.5, which reveals the different rate of enanthic and pelargonic acid incorporation. Multispecificity of the FAAL domain may also be clearly observed in the biosynthesis of iturin family members, where congeners differing in FA length were reported. The reported length of the FA ranges are C14C16 for iturin and bacillomycin and C16–C17 for mycosubtilin [32].

Because this is the first report of a complete biosynthetic pathway for lipopeptides containing β-amino FA in cyanobacteria, a direct comparison of biosynthetic steps employed in the synthesis of other lipopeptide variants in cyanobacterial cells is impossible. In the recently suggested biosynthesis of hassallidins, little explanation was provided on the mechanism of incorporation of the FA into the peptide cycle [36]. However, a FAAL enzyme was also annotated within the putative gene cluster (HasG); thus, the process may be analogous to the one we have reconstructed in our study. A structural motif (3-amino-2-hydroxy-4-methyl fatty acid) identical to puwainaphycin was reported from minutissamides, in which even the length of the FA varied from C14–C18 and furthermore, chlorination and hydroxylation of the FA chain was present [17]. In many cyanobacterial cyclic lipopeptides, the 2-hydroxy group and 4-methyl groups are missing compared with puwainaphycins, and the β-amino FA chain bears one or more hydroxyls, for example, as in pahyokolides [18] and largamide H [21]. Alternatively, some variants of cyanobacterial lipopeptides occur where a sole β-amino group is present in the FA, as was described for laxaphycins [15]. It may be expected that this diversity is generated by different combinations of the FAAL/PKS/oxygenase/aminotransferase enzymatic domains at the beginning of the synthetic pathway. Taking into consideration the multispecificity of the FAAL domain and possible post-synthetic modifications, it is obvious that a large number of lipopeptides with identical amino acid cycles that differ only in FA chains may be generated. Moreover, the substituents on the α and γ carbons of the FA may vary. Thus, we propose designating the lipopeptide by a prefix that denotes the length of the FA and the substituents on the α and β carbon in alphabetical order. All substituents starting from the γ-carbon should be mentioned separately.

Given the similar (although highly modified) mechanism of biosynthesis of puwainaphycins and iturin family antibiotics, it appears that a combination of FAAL and PKS enzymes containing an aminotransferase may be characteristic of bacterial β-amino-lipopeptide synthetases in general. Interestingly, a very similar machinery has recently been proposed to take part in cyanobacterial olefin synthesis [54]. A hybrid enzyme comprising FAAL, ACP, and a PKS was suggested to be employed in one of the two common mechanisms of producing hydrocarbons in cyanobacteria. It can be easily imagined that β-amino-lipopeptide synthesis may have shared a common evolutionary origin with olefin synthesis, differing by the addition of an aminotransferase (and other) domains to the extant enzyme complex. Supporting this hypothesis, the FAAL domain of an olefin synthetase from *Moorea bouillonii* [54] was among the best-scoring blastp hits for the PuwC FAAL enzyme (59% protein sequence identity).

Our results provide the first attempt to describe the biosynthesis of β-amino lipopeptides in cyanobacteria. The only other known type of cyanobacterial lipopeptide operons encodes a different class of lipopeptides, hassallidins. However, the obvious similarity to known lipopeptide biosynthesis machineries in bacteria enables us to discuss the enzymatic steps putatively universal in this kind of compounds and their numerous variants. Once additional data are collected on other similar biosynthetic clusters, molecular markers specific for FAAL and other characteristic enzymatic domains may serve as environmental-monitoring tools for toxic lipopeptide producers or for identification of bioactive lipopeptide producers in antimicrobial research.

Supporting Information

Figure S1 Mass spectrum displaying the fragmentation pattern of different puwainaphycin analogs. (A) 4-methyl-Ahdoa-Puw-F; (B) 4-methyl-Ahtea-Puw-F; (C) 4-methyl-Ahdoa-Puw-G; (D) 4-methyl-Ahtea-Puw-G.
(PDF)

Figure S2 NMR spectra of 4-methyl-Ahdoa-Puw-F in DMSO. (A) ^{1}H NMR spectrum; (B) 2D $^{1}H^{13}C$ HSQC spectrum; (C) 2D $^{1}H^{15}N$ HSQC spectrum.
(PDF)

Table S1 Gradient used for pre-purification of the puwainaphycin analogs on preparative C18-column.
(PDF)

Table S2 Gradient used for second purification step of the puwainaphycin analogs on semi-preparative Phenyl-column.
(PDF)

Table S3 Deduced functions of the open reading frames in the *puw* gene cluster.
(PDF)

Table S4 Product ions obtained by high resolution mass spectrometry (MS/MS) experiments performed with different puwainaphycin analogs. Diagnostic fragments are highlighted. (A) 4-methyl-Ahtea-Puw-F; (B) 4-mehtyl-Ahdoa-Puw-F; (C) chloro-4-methyl-Ahtea-Puw-F; (D) chloro-4-methyl-Ahdoa-Puw-F; (E) hydroxy-4-methyl-Ahtea-Puw-F; (F) hydroxy-4-methyl-Ahdoa-Puw-F.
(PDF)

Table S5 NMR assignment of different puwainaphycin variants. The values marked as grey were not proved because of the signal overlap in the mixture of particular F/G variants of puwainaphycin analogs.
(PDF)

Acknowledgments

The skillful technical assistance of Lada Samcová is gratefully acknowledged. We greatly appreciate the help with NMR measurements provided by Prof. Norbert Müller.

Author Contributions

Conceived and designed the experiments: JM PH JH PU. Performed the experiments: JM PH JH PU. Analyzed the data: JM JH PH PU. Contributed reagents/materials/analysis tools: JK. Contributed to the writing of the manuscript: JM PH JH PU.

References

1. Welker M, von Dohren H (2006) Cyanobacterial peptides – Nature's own combinatorial biosynthesis. FEMS Microbiol Rev 30: 530–563.
2. Dittmann E, Fewer DP, Neilan BA (2013) Cyanobacterial toxins: biosynthetic routes and evolutionary roots. FEMS Microbiol Rev 37: 23–43.
3. Tillett D, Dittmann E, Erhard M, von Dohren H, Borner T, et al. (2000) Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide-polyketide synthetase system. Chem Biol 7: 753–764.
4. Dittmann E, Neilan BA, Erhard M, vonDohren H, Borner T (1997) Insertional mutagenesis of a peptide synthetase gene that is responsible for hepatotoxin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806. Mol Microbiol 26: 779–787.
5. Moffitt MC, Neilan BA (2004) Characterization of the nodularin synthetase gene cluster and proposed theory of the evolution of cyanobacterial hepatotoxins. Appl Environ Microb 70: 6353–6362.
6. Mihali TK, Kellmann R, Muenchhoff J, Barrow KD, Neilan BA (2008) Characterization of the gene cluster responsible for cylindrospermopsin biosynthesis. Appl Environ Microb 74: 716–722.
7. Rantala-Ylinen A, Kana S, Wang H, Rouhiainen L, Wahlsten M, et al. (2011) Anatoxin-a Synthetase Gene Cluster of the Cyanobacterium *Anabaena* sp. Strain 37 and Molecular Methods To Detect Potential Producers. Appl Environ Microb 77: 7271–7278.
8. Kellmann R, Michali TK, Neilan BA (2008) Identification of a Saxitoxin Biosynthesis Gene with a History of Frequent Horizontal Gene Transfers. J Mol Evol 67: 526–538.
9. Jungblut AD, Neilan BA (2006) Molecular identification and evolution of the cyclic peptide hepatotoxins, microcystin and nodularin, synthetase genes in three orders of cyanobacteria. Arch Microbiol 185: 107–114.
10. Hrouzek P, Kuzma M, Černý J, Novák P, Šíša R, et al. (2012) The Cyanobacterial Cyclic Lipopeptides Puwainaphycins F/G Are Inducing Necrosis via Cell Membrane Permeabilization and Subsequent Unusual Actin Relocalization. Chem Res Toxicol 25: 1203–1211.
11. Oftedal L, Myhren L, Jokela J, Gausdal G, Sivonen K, et al. (2012) The lipopeptide toxins anabaenolysin A and B target biological membranes in a cholesterol-dependent manner. BBA-Biomembranes 1818: 3000–3009.
12. Jokela J, Oftedal L, Herfindal L, Permi P, Wahlsten M, et al. (2012) Anabaenolysins, Novel Cytolytic Lipopeptides from Benthic *Anabaena* Cyanobacteria. Plos One 7.
13. Horgen FD, Yoshida WY, Scheuer PJ (2000) Malevamides A-C, new depsipeptides from the marine cyanobacterium *Symploca laete-viridis*. J Nat Prod 63: 461–467.
14. Neuhof T, Schmieder P, Preussel K, Dieckmann R, Pham H, et al. (2005) Hassallidin A, a glycosylated lipopeptide with antifungal activity from the cyanobacterium *Hassallia* sp. J Nat Prod 68: 695–700.
15. Frankmolle WP, Knubel G, Moore RE, Patterson GML (1992) antifungal cyclic-peptides from the terrestrial blue-green-alga *Anabaena laxa*. 2. structures of laxaphycin-A laxaphycin-B, laxaphycin-D and laxaphycin-E. J Antibiot 45: 1458–1466.
16. Gerwick WH, Jiang ZD, Agarwal SK, Farmer BT (1992) total structure of hormothamnin-A, a toxic cyclic undecapeptide from the tropical marine cyanobacterium *Hormothamnion enteromorphoides*. Tetrahedron 48: 2313–2324.
17. Kang HS, Sturdy M, Krunic A, Kim H, Shen Q, et al. (2012) Minutissamides E-I, antiproliferative cyclic lipodecapeptides from the cultured freshwater cyanobacterium cf. *Anabaena* sp. Bioorgan Med Chem 20: 6134–6143.
18. An TY, Kumar TKS, Wang ML, Liu L, Lay JO, et al. (2007) Structures of pahayokolides A and B, cyclic peptides from a *Lyngbya* sp. J Nat Prod 70: 730–735.
19. Maru N, Ohno O, Uemura D (2010) Lyngbyacyclamides A and B, novel cytotoxic peptides from marine cyanobacteria *Lyngbya* sp. Tetrahedron Lett 51: 6384–6387.
20. Moon SS, Chen JL, Moore RE, Patterson GML (1992) Calophycin, a fungicidal cyclic decapeptide from the terrestrial blue-green-alga *Calothrix fusca*. J Org Chem 57: 1097–1103.
21. Plaza A, Bewley CA (2009) Largamides A-H, Unusual Cyclic Peptides from the Marine Cyanobacterium *Oscillatoria* sp. (vol 71, pg 6898, 2006). J Org Chem 74: 486–486.
22. MacMillan JB, Ernst-Russell MA, de Ropp JS, Molinski TF (2002) Lobocyclamides A-C, lipopeptides from a cryptic cyanobacterial mat containing *Lyngbya confervoides*. J Org Chem 67: 8210–8215.
23. Pergament I, Carmeli S (1994) Schizotrin-a - a novel antimicrobial cyclic peptide from a cyanobacterium. Tetrahedron Lett 35: 8473–8476.
24. Helms GL, Moore RE, Niemczura WP, Patterson GML, Tomer KB, et al. (1988) Scytonemin-a, a novel calcium-antagonist from a blue-green-alga. J Org Chem 53: 1298–1307.
25. Zainuddin EN, Jansen R, Nimtz M, Wray V, Preisitsch M, et al. (2009) Lyngbyazothrins A-D, Antimicrobial Cyclic Un-decapeptides from the Cultured Cyanobacterium *Lyngbya* sp. (vol 72, pg 1373, 2009). J Nat Prod 72: 2080–2080.
26. Kakinuma A, Hori M, Isono M, Tamura G, Arima K (1969) Determination of amino acid sequence in surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*. Agr Biol Chem Tokyo 33: 971–&.
27. Kakinuma A, Sugino H, Isono M, Tamura G, Arima K (1969) Determination of fatty acid in surfactin and elucidation of total structure of surfactin. Agr Biol Chem Tokyo 33: 973–&.
28. Kakinuma A, Hori M, Sugino H, Yoshida I, Isono M, et al. (1969) Determination of location of lactone ring in surfactin. Agr Biol Chem Tokyo 33: 1523–&.
29. Kakinuma A, Ouchida A, Shima T, Sugino H, Isono M, et al. (1969) Confirmation of structure of surfactin by mass spectrometry. Agr Biol Chem Tokyo 33: 1669–&.
30. Peypoux F, Pommier MT, Marion D, Ptak M, Das BC, et al. (1986) Revised structure of mycosubtilin, a peptidolipid antibiotic from *Bacillus subtilis*. J Antibiot 39: 636–641.
31. Vanittanakom N, Loeffler W, Koch U, Jung G (1986) Fengycin - a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. J Antibiot 39: 888–901.
32. Stein T (2005) *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol Microbiol 56: 845–857.
33. Duitman EH, Hamoen LW, Rembold M, Venema G, Seitz H, et al. (1999) The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: A multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase. Proc Natl Acad Sci USA 96: 13294–13299.
34. Nakano MM, Magnuson R, Myers A, Curry J, Grossman AD, et al. (1991) SrfA is an operon required for surfactin production, competence development, and efficient sporulation in *Bacillus subtilis*. J Bacteriol 173: 1770–1778.
35. Yao SY, Gao XW, Fuchsbauer N, Hillen W, Vater J, et al. (2003) Cloning, sequencing, and characterization of the genetic region relevant to biosynthesis of the lipopeptides iturin A and surfactin in *Bacillus subtilis*. Curr Microbiol 47: 272–277.
36. Vestola J, Shishido TK, Jokela J, Fewer DP, Aitio O, et al. (2014) Hassallidins, antifungal glycolipopeptides, are widespread among cyanobacteria and are the

end-product of a nonribosomal pathway. Proc Natl Acad Sci USA 111: E1909–E1917.
37. Johansen JR, Bohunická M, Lukešová A, Hrčková K, Vaccarino MA, et al. (2014) morphological and molecular characterization within 26 strains of the genus *Cylindrospermum* (Nostocaceae, Cyanobacteria), with descriptions of three new species. J Phycol 50: 187–202.
38. Allen MB, Arnon DI (1955) Studies on nitrogen-fixing blue-green algae.1. Growth and nitrogen fixation by *Anabaena cylindrica* LEMM. Plant Physiol 30: 366–372.
39. Zapomělová E, Hisem D, Řeháková K, Hrouzek P, Jezberová J, et al. (2008) Experimental comparison of phenotypical plasticity and growth demands of two strains from the *Anabaena circinalis/A. crassa* complex (cyanobacteria). J Plankton Res 30: 1257–1269.
40. Boyer SL, Flechtner VR, Johansen JR (2001) Is the 16S–23S rRNA internal transcribed spacer region a good tool for use in molecular systematics and population genetics? A case study in cyanobacteria. Mol Biol Evol 18: 1057–1069.
41. Delcher AL, Bratke KA, Powers EC, Salzberg SL (2007) Identifying bacterial genes and endosymbiont DNA with Glimmer. Bioinformatics 23: 673–679.
42. Finn RD, Clements J, Eddy SR (2011) HMMER web server: interactive sequence similarity searching. Nucleic Acids Res 39: W29–W37.
43. Jones P, Binns D, Chang H-Y, Fraser M, Li W, et al. (2014) InterProScan 5: genome-scale protein function classification. Bioinformatics 30: 1236–1240.
44. Bachmann BO, Ravel J (2009) Methods for in silico prediction of microbial polyketide and nonribosomal peptide biosynthetic pathways from DNA sequence data. In: Hopwood DA, editor. Complex Enzymes in Microbial Natural Product Biosynthesis, Part A: Overview Articles and Peptides. 181–217.
45. Roettig M, Medema MH, Blin K, Weber T, Rausch C, et al. (2011) NRPSpredictor2-a web server for predicting NRPS adenylation domain specificity. Nucleic Acids Res 39: W362–W367.
46. Ziemert N, Podell S, Penn K, Badger JH, Allen E, et al. (2012) The Natural Product Domain Seeker NaPDoS: A Phylogeny Based Bioinformatic Tool to Classify Secondary Metabolite Gene Diversity. Plos One 7: e34064.
47. Gregson JM, Chen JL, Patterson GML, Moore RE (1992) Structures of puwainaphycins A-E. Tetrahedron 48: 3727–3734.
48. Tsuge K, Akiyama T, Shoda M (2001) Cloning, sequencing, and characterization of the iturin A operon. J Bacteriol 183: 6265–6273.
49. Moyne AL, Cleveland TE, Tuzun S (2004) Molecular characterization and analysis of the operon encoding the antifungal lipopeptide bacillomycin D. FEMS Microbiol Lett 234: 43–49.
50. Fewer DP, Jokela J, Paukku E, Osterholm J, Wahlsten M, et al. (2013) New Structural Variants of Aeruginosin Produced by the Toxic Bloom Forming Cyanobacterium *Nodularia spumigena*. Plos One 8: e73618.
51. Chang ZX, Flatt P, Gerwick WH, Nguyen VA, Willis CL, et al. (2002) The barbamide biosynthetic gene cluster: a novel marine cyanobacterial system of mixed polyketide synthase (PKS)-non-ribosomal peptide synthetase (NRPS) origin involving an unusual trichloroleucyl starter unit. Gene 296: 235–247.
52. Rounge TB, Rohrlack T, Nederbragt AJ, Kristensen T, Jakobsen KS (2009) A genome-wide analysis of nonribosomal peptide synthetase gene clusters and their peptides in a *Planktothrix rubescens* strain. BMC Genomics 10: 396.
53. Kurmayer R, Dittmann E, Fastner J, Chorus I (2002) Diversity of microcystin genes within a population of the toxic cyanobacterium *Microcystis* spp. in Lake Wannsee (Berlin, Germany). Microb Ecol 43: 107–118.
54. Coates RC, Podell S, Korobeynikov A, Lapidus A, Pevzner P, et al. (2014) Characterization of Cyanobacterial Hydrocarbon Composition and Distribution of Biosynthetic Pathways. Plos One 9: e85140.

Paper II: A liquid chromatography-mass spectrometric method for the detection of cyclic β-amino fatty acid lipopeptides

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

A liquid chromatography–mass spectrometric method for the detection of cyclic β-amino fatty acid lipopeptides

Petra Urajová[a], Jan Hájek[a,b,c], Matti Wahlsten[d], Jouni Jokela[d], Tomáš Galica[a,b], David P. Fewer[d], Andreja Kust[a,b,c], Eliška Zapomělová-Kozlíková[c], Kateřina Delawská[a,b], Kaarina Sivonen[d], Jiří Kopecký[a,b], Pavel Hrouzek[a,b,*]

[a] Centre Algatech, Institute of Microbiology, The Czech Academy of Sciences (CAS), Opatovický mlýn, 379 81, Třeboň, Czech Republic
[b] University of South Bohemia, Faculty of Science, Branišovská 1760, České Budějovice, Czech Republic
[c] Biology Centre of CAS, v.v.i., Institute of Hydrobiology, Na Sádkách 7, 370 05 České Budějovice, Czech Republic
[d] Department of Food and Environmental Sciences, Viikki Biocenter 1, University of Helsinki, FI-00014 Helsinki, Finland

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

A liquid chromatography–mass spectrometric method for the detection of cyclic β-amino fatty acid lipopeptides

Petra Urajová[a], Jan Hájek[a,b,c], Matti Wahlsten[d], Jouni Jokela[d], Tomáš Galica[a,b], David P. Fewer[d], Andreja Kust[a,b,c], Eliška Zapomělová-Kozlíková[c], Kateřina Delawská[a,b], Kaarina Sivonen[d], Jiří Kopecký[a,b], Pavel Hrouzek[a,b,*]

[a] Centre Algatech, Institute of Microbiology, The Czech Academy of Sciences (CAS), Opatovický mlýn, 379 81, Třeboň, Czech Republic
[b] University of South Bohemia, Faculty of Science, Branišovská 1760, České Budějovice, Czech Republic
[c] Biology Centre of CAS, v.v.i., Institute of Hydrobiology, Na Sádkách 7, 370 05 České Budějovice, Czech Republic
[d] Department of Food and Environmental Sciences, Viikki Biocenter 1, University of Helsinki, FI-00014 Helsinki, Finland

ARTICLE INFO

Article history:
Received 29 October 2015
Received in revised form 12 January 2016
Accepted 2 February 2016
Available online xxx

Keywords:
Lipopeptides
Bacteria
Cyanobacteria
LC-HRMS
Fatty acid
Peptide

ABSTRACT

Bacterial lipopeptides, which contain β-amino fatty acids, are an abundant group of bacterial secondary metabolites exhibiting antifungal and/or cytotoxic properties. Here we have developed an LC-HRMS/MS method for the selective detection of β-amino fatty acid containing cyclic lipopeptides. The method was optimized using the lipopeptides iturin A and puwainaphycin F, which contain fatty acids of similar length but differ in the amino acid composition of the peptide cycle. Fragmentation energies of 10–55 eV were used to obtain the amino acid composition of the peptide macrocycle. However, fragmentation energies of 90–130 eV were used to obtain an intense fragment specific for the β-amino fatty acid ($C_nH_{2n+2}N^+$). The method allowed the number of carbons and consequently the length of the β-amino fatty acid to be estimated. We identified 21 puwainaphycin variants differing in fatty acid chain in the crude extract of cyanobacterium *Cylindrospermum alatosporum* using this method. Analogously 11 iturin A variants were detected. The retention time of the lipopeptide variants showed a near perfect linear dependence ($R^2 = 0.9995$) on the length of the fatty acid chain in linear separation gradient which simplified the detection of minor variants. We used the method to screen 240 cyanobacterial strains and identified lipopeptides from 8 strains. The HPLC-HRMS/MS method developed here provides a rapid and easy way to detecting novel variants of cyclic lipopeptides.

© 2016 Published by Elsevier B.V.

1. Introduction

Bacteria are a well-known source of low-molecular weight secondary metabolites [1]. These compounds represent an important source of novel chemical structures for pharmacy and biotechnology [1,2]. Intensive pharmacological screenings for novel chemical structures have identified immense number of novel microbial secondary metabolites [1,2]. Many microbial secondary metabolites are peptides or have a peptidic portion and are produced through the action of non-ribosomal peptide synthetases (NRPS) [3]. Based on the genome mining studies it is assumed that the NRPS biosynthetic clusters of known chemical structures represent only a small portion of the total cluster number found [4]. Taken together the known metabolites represent only a small portion of the real microbial metabolite chemical diversity.

Cyclic lipopeptides are an important class of bacterial secondary metabolites and include many antibiotics and antifungal agents [1,5–9]. Their structures contain peptide macrocycle with a fatty acyl residue side chain. There are several basic structural types of bacterial cyclic lipopeptides. The carboxyl group of the fatty acid can form a peptide bond with an amino-acid residue situated on the side chain of the lipopeptide molecule as found in bacterial antibiotics fengycin [6] and daptomycin [7] and the anti-fungal lipopeptides hassallidins [8] and balticidins [9]. Alternatively, the fatty acid can be incorporated into the peptide backbone *via* cyclization (most frequently on C_2 or C_3 carbon) by amine- or hydroxyl-groups [10–12]. The 3-OH substituted fatty acid of surfactin is bound by a peptide bond between the carboxyl and *via* ester bond by the hydroxyl group [13]. If an amino group is introduced during biosynthesis the fatty acid residue allows the formation of two

* Corresponding author at: Centre Algatech, Institute of Microbiology, The Czech Academy of Sciences (CAS), Opatovický mlýn, 379 81, Třeboň, Czech Republic.
E-mail address: hrouzekp@gmail.com (P. Hrouzek).

http://dx.doi.org/10.1016/j.chroma.2016.02.013
0021-9673/© 2016 Published by Elsevier B.V.

Please cite this article in press as: P. Urajová, et al., A liquid chromatography–mass spectrometric method for the detection of cyclic β-amino fatty acid lipopeptides, J. Chromatogr. A (2016), http://dx.doi.org/10.1016/j.chroma.2016.02.013

peptide bonds (or an isopeptide bond in case of β-NH_2) with C- and N-terminus of the peptide forming a cycle [10,12].

The structure of iturin family of lipopeptide antibiotics was described already 40 years ago [5]. However, novel structural types of lipopeptides are still reported highlighting their rich chemical diversity in the nature and especially in cyanobacteria [13–15]. Cyanobacteria produce a large diversity of β-amino fatty acid lipopeptides varying in the type and number of amino acids in the cycle as well as the length and functionalization of the fatty acid moiety [16–25]. Usually C_8–C_{18} long fatty acid can be found in cyanobacterial lipopeptides sometimes methylated [18,20], hydroxylated [18,19], halogenated [12,18] or unsaturated [23]. Cyanobacterial cyclic β-amino fatty acid lipopeptides display a broad range of bioactivities including cytotoxicity [16–22], cytolytic activity [26], as well as antifungal and/or antibacterial activity [24,25,27]. Some cyanobacterial lipopeptides permeabilize human plasma membranes and are potentially toxic to humans [20,21].

To date no selective screening method allows the targeted screening of novel lipopeptides from bacteria. Here we report the development of a rapid and selective liquid chromatographic mass spectrometric method for β-amino fatty acid lipopeptides analysis based on the detection of a characteristic fatty acid immonium ion. This method allows rapid screening for novel lipopeptides from various biological samples.

2. Experimental

2.1. Materials

Methanol for extraction (p.a.) was obtained from Analytika (Czech Republic), acetonitrile and water for HPLC-HRMS/MS analyzes were obtained either from Sigma–Aldrich (Germany) or Merck (New Jersey, USA) and were of LC–MS grade purity. The iturin A standard was obtained from *Bacillus subtilis* (≥95%) and contained mainly C_{14}–C_{15} fatty acids (with traces of C_{13}–C_{17}) according to manufacturer instructions (Sigma, Germany). 4-Methyl-Ahdoapuwainaphycin F and anabaenolysin A were isolated from crude cyanobacterial biomass by HPLC from *Cylindrospermum alatosporum* CCALA 988 and *Anabaena* sp. XPORK 15F, respectively. Hexakis(*1H,1H,2H*-perfluoroethoxy) phosphazene (LockMass Tuning Mix ES-TOF 622 Da, 97%) was purchased from ABCR GmbH & Co., KG, Germany).

2.2. Methods and procedure

2.2.1. Cultivation of cyanobacterial biomass

C. alatosporum CCALA 988 and strains for lipopeptide screening were cultivated in 350 mL glass tubes or Erlenmeyer flasks on liquid Allen Arnon medium [28] and bubbled with 1.5% CO_2-enriched air at constant temperature of 26 °C, under continuous illumination of 50 μmol m^{-2} s^{-1}. Strains used in lipopeptide screening originate from culture collections of the Institute of Hydrobiology, Biological Centre CAS and Culture collection of phototrophic microorganisms (CCALA) and the University of Helsinki (HAMBI Culture collection). *Anabaena* sp. XPORK 15F was grown in Z8 medium without a nitrogen source [29] under continuous light (8–14 μmol m^{-2} s^{-1}) at 20–25 °C for 20–40 days. The cultures were harvested by centrifugation (1250 × *g*, 15 min) after 5–7 days of cultivation, stored at −70° C and freeze-dried (Leybold–Heraeos freeze-drier Lyovac GT 3).

2.2.2. Extraction

200 mg of dried biomass of *C. alatosporum* CCALA 988 and *Anabaena* sp. XPORK 15F was extracted with 10 mL of methanol/water (70/30, v/v) for 1 h and centrifuged for 10 min. at 1920 × *g*. The supernatant was evaporated until dryness on rotary evaporator, while the temperature did not exceed 40 °C. The residue was redissolved in 1 mL of extraction solvent. 5–30 mg of lyophilized biomass from the 240 cyanobacterial strains was homogenized with sand in Eppendorf tube and 100 μl of 70% MeOH. The samples were centrifuged (12,000 × *g*, 7 min.) in a Minispin Eppendorf centrifuge and transferred into vials with inserts for LC-HRMS analysis.

2.2.3. HRMS/MS analysis by direct infusion

Methanolic solutions (acidified by 0.1% HCOOH) of iturin A, puwainaphycin F and anabaenolysin A were analyzed by direct infusion HRMS/MS using collision energy varying from 0 to 200 eV. The experiments were performed in triplicate.

2.2.4. HPLC-HRMS/MS analysis and MS/MS experiments

A standard solution of iturin A (0.1 mg/mL), *C. alatosporum* CCALA 988 and *Anabaena* sp. XPORK 15F 70% methanol extracts were analyzed on a Dionex UltiMate 3000 UHPLC+ (Thermo Scientific, Sunnyvale, CA, USA) equipped with a diode-array detector. Separation of compounds was performed on reversed phase C_{18} column (Phenomenex Kinetex, 150 × 4.6 mm, 2.6 μm, Torrance, CA, USA) using H_2O (A)/acetonitrile (B) both containing 0.1% HCOOH as a mobile phase with the flow rate of 0.5 mL. min^{-1}. The gradient was as follows: A/B 85/15 (0 min), 85/15 (in 1 min), 0/100 (in 39 min), 0/100 (in 44 min) and 85/15 (in 49 min). The HPLC was connected to an Impact HD high resolution mass spectrometer (Bruker, Billerica, Massachusetts, USA) with electrospray ionization. The following settings were used: drying temperature 200 °C; drying gas flow 12 l. min^{-1}; nebulizer gas pressure 3 bar; capillary voltage 3.8 kV; endplate offset 500 V. The spectra were collected in the range *m/z* 20–2000 with spectra rate of 3 Hz. Collision energy alternated from 35 eV or 50 eV to 100 eV. Mass spectrometer was calibrated with sodium formate clusters and LockMass Tuning Mix ES-TOF (622 Da) was used as the lockmass calibrant. The formulas of obtained molecular peaks and fragments were calculated using Smart Formula in Bruker Compass DataAnalysis software (version 4.2) and MassLynx software (version V4.1). Only fragments with maximal *m/z* difference 1 mDa between theoretical and measured value were taken into consideration. This corresponds to ppm values bellow 10 ppm in MS2 for all detected ions. However, the majority of the detected ions had values were below 5 ppm (Tables 1–3).

240 strains of cyanobacteria were analyzed by the HPLC-HRMS/MS method as described above. The resulting data were analyzed for characteristic β-amino fatty acid immonium ion from C_8 to C_{16} both saturated ($C_xH_{(2x+2)}N^+$) and with one double bond ($C_xH_{2x}N^+$).

3. Results

3.1. HRMS/MS analysis of selected lipopeptides

The HRMS/MS method was optimized by obtaining diagnostic fragment ion from iturin A preparate (*m/z* 1043.5520 $[M+H]^+$), puwainaphycin F (*m/z* 1146.6517 $[M+H]^+$) and anabaenolysin A (*m/z* 559.2762 $[M+H]^+$) isolated from cyanobacterial crude extracts in direct infusion mode using collision energies from 0 to 200 eV. All of the selected lipopeptides possess at least C_{14} unit long β-amino acid chain (Fig. 1), but differ in the composition of their peptide core structure and the level of the fatty acid chain saturation.

Iturin A and puwainaphycin F exhibit similar fragmentation patterns. Lower collision energies (~50 eV) led to fragments indicating loss of amino acids, while higher collision energies resulted in formation of diagnostic β-amino fatty acid immonium ions for both tested compounds (Fig. 2). The diagnostic β-amino fatty acid ion for

Please cite this article in press as: P. Urajová, et al., A liquid chromatography–mass spectrometric method for the detection of cyclic β-amino fatty acid lipopeptides, J. Chromatogr. A (2016), http://dx.doi.org/10.1016/j.chroma.2016.02.013

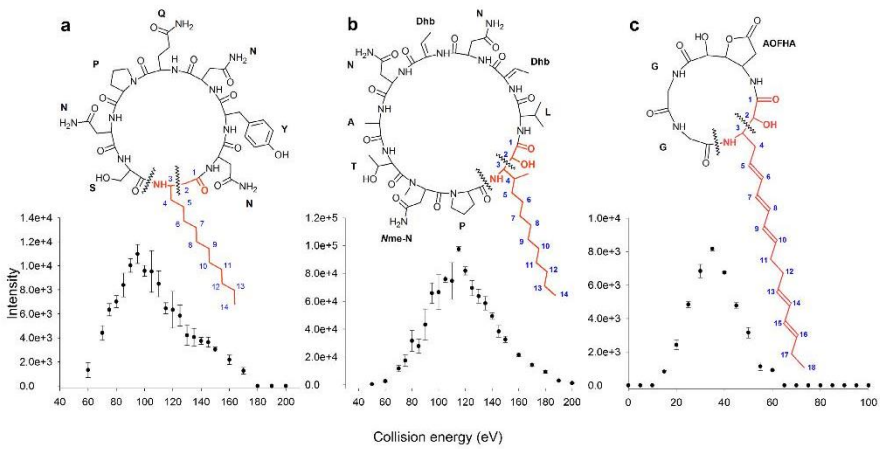


Fig. 1. The structure of the lipopeptides included in this study and the intensity of their diagnostic FA immonium ion $C_xH_{2x}N^+$ at varying collision energies. a) Ahtea-Itu A provided a diagnostic fragment at *m*/*z* 184.2064 with a maximal intensity at 95 eV. b) 4-Methyl-Ahtea-Puw F provided a diagnostic fragment at *m*/*z* 198.2220 with a maximal intensity at 115 eV and c) in Ahopa-Abl A the diagnostic fragment at *m*/*z* 230.1900 was found at a collision energy of 35 eV. The modified β-amino fatty acids are highlighted in red and the diagnostic immonium FA fragments lacking the C_1 and C_2 carbons are separated by wavy line. Itu stands for iturin, Puw stands for puwainaphycin, Abl stands for anabaenolysin.

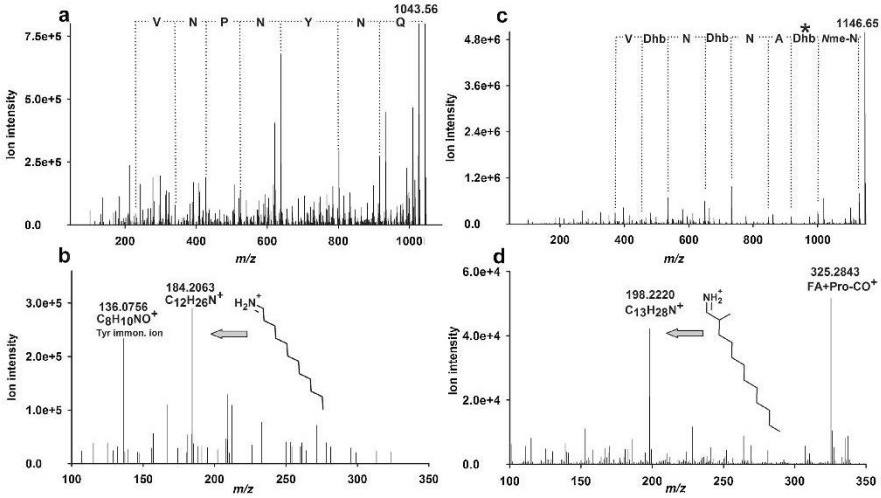


Fig. 2. Fragmentation of Ahtea-Itu A (a, b) and 4-methyl-Ahtea-Puw F (c, d) at different collision energies. a, c) Fragmentation at 50 eV showing the peptide sequence of the macrocycle. b, d) Fragmentation at 100 eV showing the presence of modified β-amino fatty acid diagnostic immonium ion. The interpretation of the amino acid sequence of 4-methyl-Ahtea-Puw F was derived from $[M - H_2O + H]^+$ and thus the loss of additional Dhb instead of Thr takes place (marked by asterisk). Itu stands for iturin and Puw stands for puwainaphycin.

iturin A has a *m*/*z* 184.2063 with the formula $C_{12}H_{26}N^+$. It reached maximal absolute intensity at 95 eV and in the range 85–135 eV it was the base peak of MS/MS spectra (Fig. 1). Although the diagnostic ion consisted of 12 carbon atoms, the itutin A fatty acid chain itself is 14 carbon atoms. We obtained similar results for puwainaphycin F. The diagnostic β-amino fatty acid ion was at *m*/*z* 198.2216 with formula $C_{13}H_{28}N^+$. The maximal intensity was reached at 120 eV (Fig. 1) and it was the most intense ion in the range 110–160 eV. Despite having the same length of the fatty acid chain (C_{14}) the diagnostic fragments of iturin A and puwainaphycin F differ by a

Please cite this article in press as: P. Urajová, et al., A liquid chromatography–mass spectrometric method for the detection of cyclic β-amino fatty acid lipopeptides, J. Chromatogr. A (2016), http://dx.doi.org/10.1016/j.chroma.2016.02.013

$-CH_2$, which is due to the methylation of the puwainaphycin F on the carbon C4. Both diagnostic fragments lacked the first two carbons of the fatty acid chain (Fig. 1). This made it possible to recognize the presence of β-amino fatty acid chain in the molecule, but it was impossible to determine the exact fatty acid chain length due to branching occurring in some lipopeptides. Although amino acids adjoining the β-amino fatty acid chain differ in iturin A and puwainaphycin F, it is possible to observe fatty acid diagnostic ions by using collision energy of 100 eV for both lipopeptides.

Anabaenolysin A exhibited a different fragmentation behavior. The immonium ion of the fatty acid chain (m/z 230.1903) with formula $C_{16}H_{24}N^+$ appeared at a collision energy of 20 eV, reached its maximum abundance at 35 eV (relative intensity 67%) and was no longer detectable at 60 eV, but was not the base peak in any MS/MS spectra (Fig. 1).

3.2. HPLC-HRMS/MS analysis of iturin A variants

The HPLC-HRMS/MS analysis of the commercial iturin A standard, was composed mainly of variants with a fatty acid chain length C_{14}–C_{15} (traces of C_{13}–C_{17}) according to information provided by manufacturer, revealed the presence of eleven protonated molecules in the range m/z 987–1086 (Table 1). MS/MS spectra of these ions obtained using low collision energy (50 eV) confirmed the amino-acid sequence of iturin family members (Fig. 2a). Eight variants had common structure FA-Asn-Tyr-Asn-Gln-Pro-Asn-Ser as described for iturin A and in three variants the sequence FA-Gln-Tyr-Asn-Gln-Pro-Asn-Ser were found, showing an exchange of Asn to Gln at the position adjacent to fatty acid chain.

Diagnostic β-amino fatty acid fragment ions of the variants obtained using collision energy 100 eV identified the fatty acid moiety of the lipopeptide (Fig. 2b). The general formula was $C_xH_{(2x+2)}N^+$, where x represents the length of fatty acid chain reduced of the first and the second carbon. In all cases, the diagnostic β-amino fatty acid fragment ions represent the dominant fragment ions in the MS/MS spectra (Fig. S1a) and are well recognizable by the extracted ion chromatogram. We were able to detect minor iturin A variants with C_{10}–C_{17} fatty acid chains in the sample analyzed using this approach (Table 1).

All iturin A variants eluted in region between 12 and 23 min, corresponding to linear increase of organic solvent in the mobile phase. When analyzed by linear regression the retention times of lipopeptides showed perfectly linear relationship with the length of amino acid chain. The retention time y of eight iturin A variants increased with equation $y = 1.475x - 2.45$ (x = number of fatty acid carbons) and $R^2 = 0.9941$ and only slightly worse fit was found in Asn/Gln substituted variants ($y = 1.300x + 0.1$, $R^2 = 0.9826$). While the change in presence of one $-CH_2$ group in fatty acid chain caused marked shift in retention time, the Asn to Gln substitution in the cycle had almost no effect.

3.3. HPLC-HRMS/MS analysis of Cylindrospermum alatosporum CCALA 988 extract

We have previously shown that cyanobacterium *C. alatosporum* CALA 988 produces the cytotoxic lipopeptides puwainaphycins [12,20]. Now we further analyzed the crude extract of *C. alatosporum* CALA 988 in order to test the method for lipopeptide screening in a complex matrix. The HPLC-HRMS/MS analyzes of the extract allowed us to detect 21 molecular ions in the region m/z 1090–1200 (Table 2). Each of these ions could be attributed to puwainaphycin F/G variants based on the amino-acid sequence obtained by fragmentation in lower collision energy at 50 eV (Fig. 2c). Fragmentation spectra obtained at a collision energy of 100 eV revealed diagnostic fragment ions $C_xH_{(2x+2)}N^+$ as in the case of iturin A. Based on the presence of the diagnostic immonium ion $C_xH_{(2x+2)}N^+$ we were able to prove the presence of puwainaphycin F/G variants with fatty acid chain lengths ranging from C_{10} to C_{17} (Fig. S1b). Some puwainaphycin variants are hydroxylated or chlorinated on the fatty acyl chain [12]. All four detected fatty acid substituted puwainaphycin variants (chloro/hydroxy-Ahdoa puwainaphycin F and chloro/hydroxy-Ahtea puwainaphycin F) have provided common diagnostic fragment $C_xH_{2x}N^+$ when fragmentation energies 100–150 eV were used (Fig. S2). These sum formula of these fragments perfectly matches with presence of a double bond created after dehydration/dechlorination of the fatty acid chain during fragmentation (Table 2).

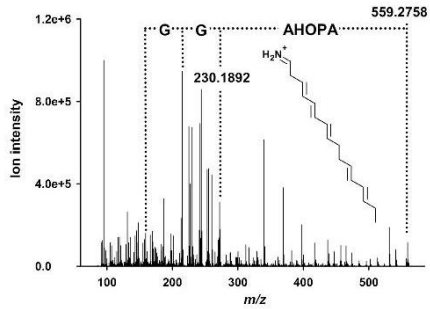


Fig. 3. MS/MS spectrum of anabaenolysin A at collision energy of 35 eV. The presence of modified β-amino fatty acid diagnostic immonium ion $C_{16}H_{24}N^+$ and fragments corresponding to amino acids loses are highlighted.

The retention time of puwainaphycin F/G variants on C_{18} reversed phase column is only partially dependent on the amino acid composition but strongly dependent on the length of the fatty acid chain as found for iturin A variants. While the shift in retention time of 4-methyl-Ahdoa-puwainaphycin F and 4-methyl-Ahdoa-puwainaphycin G (differing in Asn–Gln substitution) is almost negligible (max 0.1 min), the retention time distinctly shifts with the length of fatty acid chain. A similar shift in retention time with increasing fatty acid chain length was also observed for chlorinated and hydroxylated variants. The relationship between retention time and the length of the fatty acid chain within the gradient used is almost perfectly linear with only a slight deviation from the linear trend in the 4-methyl-Ahhea-puwainaphycin F variant. The increasing chain length allows rotation of the fatty acid chain resulting in intramolecular interactions affecting the retention time. The regression curve for this relationship was determined excluding 4-methyl-Ahhea-puwainaphycin-F $y = 1.516x + 0.434$ with R^2 value 0.9999 ($y = 1.440x + 1.323$ with R^2 value 0.9959 including 4-methyl-Ahhea-puwainaphycin F).

3.4. HPLC-HRMS/MS analysis of Anabaena sp. XPORK 15F extract

In order to test the method also on lipopeptides containing polyunsaturated β-amino fatty acids we analyzed 70% methanolic extract of cyanobacterium *Anabaena* sp. XPORK 15F previously shown to produce anabaenolysin variants [23]. The separation was performed by the same HPLC-HRMS/MS method as for the puwainaphycin and iturin variants. Since anabaenolysin A exhibited different fragmentation behavior in direct infusion experiments in comparison to puwainaphycin and iturin (Fig. 3) we applied a fragmentation energy of 35 eV.

Six anabaenolysin lipopeptides in the range of m/z 550–600 were found in the XPORK 15F extract using HPLC-HRMS

Please cite this article in press as: P. Urajová, et al., A liquid chromatography–mass spectrometric method for the detection of cyclic β-amino fatty acid lipopeptides, J. Chromatogr. A (2016), http://dx.doi.org/10.1016/j.chroma.2016.02.013

Table 1
HPLC-HRMS/MS data of iturin A standard solution. Itu stands for iturin.

Retention time (min)	$[M+H]^+$	Formula	Error (ppm)	Diagnostic immonium ion (m/z)	Diagnostic immonium ion formula	Error (ppm)	Compound	β-amino fatty acid length
12.1	987.4905	$C_{44}H_{67}N_{12}O_{14}^+$	1.0	128.1426	$C_8H_{18}N^+$	6.0	Adoa-Itu A	C_{10}
13.5	1001.5052	$C_{45}H_{69}N_{12}O_{14}^+$	0.1	142.1590	$C_9H_{20}N^+$	0.0	Auda-Itu A	C_{11}
15.7	1015.5202	$C_{46}H_{71}N_{12}O_{14}^+$	0.5	156.1749	$C_{10}H_{22}N^+$	1.4	Adea-Itu A	C_{12}
16.7	1029.5355	$C_{47}H_{73}N_{12}O_{14}^+$	0.9	170.1901	$C_{11}H_{24}N^+$	1.4	Atda-Itu A	C_{13}
18.4	1057.5685	$C_{49}H_{77}N_{12}O_{14}^+$	0.8	184.2064	$C_{12}H_{26}N^+$	2.2	Atea-1-Gln Itu A	C_{14}
18.5	1043.5538	$C_{48}H_{75}N_{12}O_{14}^+$	1.7	184.2064	$C_{12}H_{26}N^+$	2.2	Atea-Itu A	C_{14}
19.4	1071.5860	$C_{50}H_{79}N_{12}O_{14}^+$	2.5	198.2222	$C_{13}H_{28}N^+$	3.1	Apda-1-Gln Itu A	C_{15}
19.6	1057.5691	$C_{49}H_{77}N_{12}O_{14}^+$	1.3	198.2222	$C_{13}H_{28}N^+$	3.1	Apda-Itu A	C_{15}
21.3	1071.5852	$C_{50}H_{79}N_{12}O_{14}^+$	1.7	212.2371	$C_{14}H_{30}N^+$	0.6	Ahda-1-Gln Itu A	C_{16}
21.0	1085.6018	$C_{51}H_{81}N_{12}O_{14}^+$	2.5	212.2371	$C_{14}H_{30}N^+$	0.6	Ahda-Itu A	C_{16}
22.3	1085.6001	$C_{51}H_{81}N_{12}O_{14}^+$	1.0	226.2532	$C_{15}H_{32}N^+$	1.2	Ahea-Itu A	C_{17}

Table 2
HPLC-HRMS data of *Cylindrospermum alatosporum* CCALA 988 extract. Puw stands for puwainaphycin.

Retention time (min)	$[M+H]^+$	Formula	Error (ppm)	Diagnostic immonium ion (m/z)	Diagnostic immonium ion formula	Error (ppm)	Compound	β-amino fatty acid length
12.4	1134.6170	$C_{51}H_{84}N_{13}O_{16}^+$	1.4	168.1853	$C_{11}H_{22}N^+$	0.4	Hydroxy-4-methyl-Ahdoa-Puw F	C_{12}
12.4	1148.6327	$C_{52}H_{86}N_{13}O_{16}^+$	1.5	168.1851	$C_{11}H_{22}N^+$	1.0	Hydroxy-4-methyl-Ahdoa-Puw G	C_{12}
15.0	1162.6478	$C_{53}H_{88}N_{13}O_{16}^+$	1.0	196.2064	$C_{13}H_{26}N^+$	2.1	Hydroxy-4-methyl-Ahtea-Puw F	C_{14}
15.0	1176.6648	$C_{54}H_{90}N_{13}O_{16}^+$	2.1	196.2065	$C_{13}H_{26}N^+$	2.5	Hydroxy-4-methyl-Ahtea-Puw G	C_{14}
15.5	1090.5917	$C_{49}H_{80}N_{13}O_{15}^+$	2.4	142.1591	$C_9H_{20}N^+$	0.8	4-Methyl-Ahdea-Puw F	C_{10}
15.5	1104.6074	$C_{50}H_{82}N_{13}O_{15}^+$	2.3	142.1585	$C_9H_{20}N^+$	3.4	4-Methyl-Ahdea- Puw G	C_{10}
17.1	1104.6067	$C_{50}H_{82}N_{13}O_{15}^+$	1.8	156.1747	$C_{10}H_{22}N^+$	0.1	4-Methyl-Ahuda-Puw F	C_{11}
17.1	1118.6193	$C_{51}H_{84}N_{13}O_{15}^+$	1.0	156.1751	$C_{10}H_{22}N^+$	2.6	4-Methyl-Ahuda-Puw G	C_{11}
17.9	1152.5815	$C_{51}H_{83}ClN_{13}O_{15}^+$	0.0	168.1745	$C_{11}H_{22}N^+$	1.0	Chloro-4-methyl-Ahdoa-Puw F	C_{12}
17.9	1166.5994	$C_{52}H_{85}ClN_{13}O_{15}^+$	1.9	168.1749	$C_{11}H_{22}N^+$	1.2	Chloro-4-methyl-Ahdoa-Puw G	C_{12}
18.7	1118.6194	$C_{51}H_{84}N_{13}O_{15}^+$	0.9	170.1907	$C_{11}H_{24}N^+$	2.4	4-Methyl-Ahdoa-Puw F	C_{12}
18.7	1132.6352	$C_{52}H_{86}N_{13}O_{15}^+$	0.8	170.1900	$C_{11}H_{24}N^+$	1.9	4-Methyl-Ahdoa-Puw G	C_{12}
20.1	1132.6363	$C_{52}H_{86}N_{13}O_{15}^+$	0.2	170.1907	$C_{11}H_{24}N^+$	2.4	4-Methyl-Ahdoa-Puw F	C_{12}
20.1	1146.6527	$C_{53}H_{88}N_{13}O_{15}^+$	0.9	170.1900	$C_{11}H_{24}N^+$	1.9	4-Methyl-Ahdoa-Puw G	C_{12}
20.6	1180.6146	$C_{54}H_{89}ClN_{13}O_{15}^+$	1.6	196.2058	$C_{13}H_{26}N^+$	0.9	Chloro-4-methyl-Ahtea-Puw F	C_{14}
20.6	1194.6284	$C_{55}H_{91}ClN_{13}O_{15}^+$	0.0	196.2058	$C_{13}H_{26}N^+$	0.6	Chloro-4-methyl-Ahtea-Puw G	C_{14}
21.6	1146.6545	$C_{53}H_{88}N_{13}O_{15}^+$	2.4	198.2220	$C_{13}H_{28}N^+$	2.0	4-Methyl-Ahtea-Puw F	C_{14}
21.6	1160.6701	$C_{54}H_{90}N_{13}O_{15}^+$	2.4	198.2222	$C_{13}H_{28}N^+$	3.0	4-Methyl-Ahtea-Puw G	C_{14}
24.7	1174.6838	$C_{55}H_{92}N_{13}O_{15}^+$	0.6	226.2529	$C_{15}H_{32}N^+$	0.1	4-Methyl-Ahhda-Puw F	C_{16}
24.7	1188.6992	$C_{56}H_{94}N_{13}O_{15}^+$	0.4	226.2525	$C_{15}H_{32}N^+$	1.8	4-Methyl-Ahhda-Puw G	C_{16}
25.4	1188.6977	$C_{56}H_{94}N_{13}O_{15}^+$	0.8	240.2678	$C_{16}H_{34}N^+$	3.4	4-Methyl-Ahhea-Puw F	C_{17}

Table 3
HPLC-HRMS/MS data of *Anabaena* sp. XPORK 15F extract.

Retention time (min)	$[M+H]+$	Formula	Error (ppm)	Diagnostic immonium ion (m/z)	Diagnostic immonium ion formula	Error (ppm)	Compound	β-amino fatty acid length
16.4	557.2617	$C_{28}H_{37}N_4O_8^+$	2.0	228.1744	$C_{16}H_{22}N^+$	1.0	Ahoha-Anabaenolysin A	C_{18}
16.8	573.2559	$C_{28}H_{37}N_4O_9^+$	0.8	244.1693	$C_{16}H_{22}NO^+$	1.3	Hydroxy-Ahoha-Anabaenolysin A	C_{18}
19.9	591.2672	$C_{28}H_{39}N_4O_{10}^+$	1.8	244.1696	$C_{16}H_{22}NO^+$	1.4	Dihydroxy-Ahoha-Anabaenolysin A	C_{18}
22.3	577.2863	$C_{28}H_{41}N_4O_9^+$	0.9	230.1903	$C_{16}H_{24}N^+$	0.1	Ahopa-Anabaenolysin A with Aofha lactone ring hydrolyzed	C_{18}
23.9	559.2758	$C_{28}H_{39}N_4O_8^+$	0.8	230.1900	$C_{16}H_{24}N^+$	1.3	Anabaenolysin A (Ahopa-Anabaenolysin A)	C_{18}
24.7	561.2927	$C_{28}H_{41}N_4O_8^+$	1.5	232.2064	$C_{16}H_{26}N^+$	1.7	Anabaenolysin B (AHOTA-Anabaenolysin A)	C_{18}

analysis. Their amino acid composition Gly-Gly-Aofha (Aofha—2-(3-amino-5-oxytetrahydrofuran-2-yl)-2-hydroxyacetic acid) was confirmed (Table S1). All variants had the same β-amino fatty acid chain length, but differed in the number of double bonds and substitution on the fatty chain. It was possible to detect characteristic immonium fragment ions. However, these ions exhibited lower intensity in comparison to puwainaphycin and iturin variants. Anabaenolysin variants with four (Ahota-anabaenolysin A (Ahota—3-amino-2-hydroxyoctadeca-tetraenoic acid)), five (Ahopa-anabaenolysin A (Ahopa—3-amino-2-hydroxyoctadeca-pentaenoic acid)) and six (Ahoha-anabaenolysin A (Ahoha—3-amino-2-hydroxyoctadeca-hexaenoic acid) double bonds on the fatty chain were found in the extract, so the diagnostic fragments showed general formula $C_{16}H_{22\text{-}26}N^+$ (Table 3). In addition, two variants with hydroxyl substitution of the fatty acid chain were detected, hydroxy-Ahoha-anabaenolysin A and dihydroxy-Ahoha-anabaenolysin A, and provided diagnostic immonium ion fragments $C_{16}H_{22}NO^+$ and $C_{16}H_{22}NO_2^+$, respectively. Anabaenolysin variants eluting at 22.3 min with m/z 577.2863 corresponds to Ahoha-anabaenolysin A with hydrolyzed lactone ring of the unusual amino acid Aofha (Table S1).

Please cite this article in press as: P. Urajová, et al., A liquid chromatography–mass spectrometric method for the detection of cyclic β-amino fatty acid lipopeptides, J. Chromatogr. A (2016), http://dx.doi.org/10.1016/j.chroma.2016.02.013

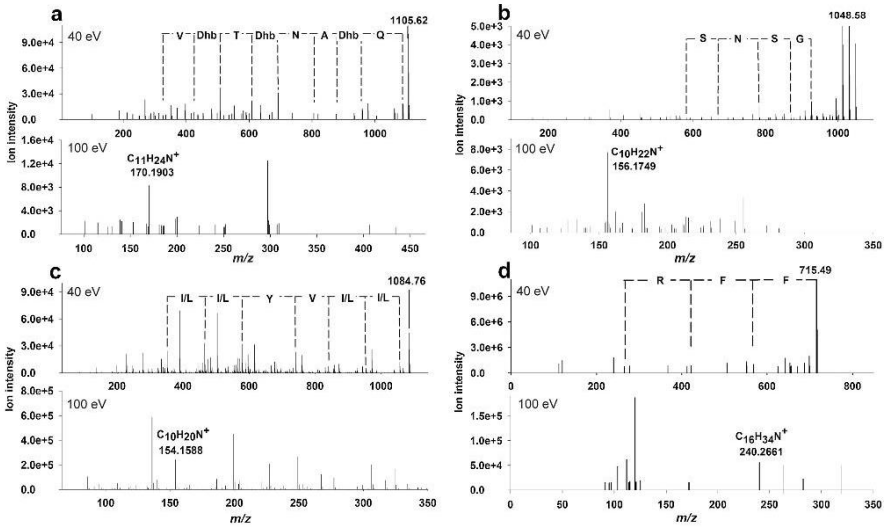


Fig. 4. Examples of novel putative lipopeptides detected in cyanobacterial strains with unknown metabolite profiles. The partial amino acid sequence was obtained by fragmentation at 40 eV. (a) Novel Puw variant (*m*/*z* 1105.62) detected in strain *Anabaena* sp. SMIX. The interpretation of the amino acid sequence was derived from $[M-H_2O+H]^+$ and thus the loss of additional Dhb instead of Thr takes place (marked by asterisk). (b) Novel lipopeptide (*m*/*z* 1048.58) detected in strain *Cylindrospermum* sp. CCALA 993. (c) Novel lipopeptide (*m*/*z* 1084.76) detected in strain *Cylindrospermum* sp. CCALA 1000. (d) Novel lipopeptide (*m*/*z* 715.49) detected in strain *Anabaena* sp. A-Pastvisko11. Puw stands for puwainaphycin.

3.5. Screening for lipopeptides in cyanobacteria with unknown metabolic profiles

We identified new lipopeptides from 240 cyanobacterial strains with an unknown metabolite content using the new method leading to the detection of 2 known and 3 unknown puwainaphycin variants and/or 9 putative lipopeptide structures in 8 cyanobacterial strains.

In *Cylindrospermum* spp. strains CCALA 993 and 994 we detected 4-methyl-Ahdoa-puwainaphycin F by presence of characteristic C_{12} β-amino fatty acid immonium ion (*m*/*z* 170.9005). In the third puwainaphycin producing strain (*Anabaena* sp. SMIX) we detected 4-methyl-Ahdoa-puwainaphycin F, 4-methyl-Ahtea-puwainaphycin F and three novel puwainaphycin variants (*m*/*z* 1105.3, 1177.4 and 1190.4). In variant 1105.3 characteristic fatty acid immonium ion fragment of elemental composition $C_{16}H_{34}N^+$ was detected at 100 eV (Fig. 4a) and peptide sequence of Val-Dhb-Thr-Dhb-Asn-Ala-Dhb-Glu was obtained at 40 eV (Fig. 4a). In variants 1177.4 and 1190.4 the fatty acid immonium ion fragment $C_{15}H_{30}N^+$ was detected and their peptide sequences were identical to 1105 and puwainaphycin F, respectively.

We detected 10 putative novel lipopeptide structures in the 240 strains analyzed (Table S2). These compounds contained the characteristic β-amino fatty acid immonium ion in fragmentation spectra obtained at 100 eV and at least 3 amino-acid residues were detected in fragmentation at 40 eV (see Table S2). Examples of fragmentation spectra of three structures are given in Fig. 4b–d. In *Cylindrospermum* spp. strains CCALA 993 and 994 we detected a protonated molecule at *m*/*z* 1048.6 which formed $C_{10}H_{22}N^+$ fatty acid immonium ion at 100 eV and partial peptide sequence Gly-Ser-Asn-Ser at 40 eV (Fig. 4b). A protonated molecule *m*/*z* 1084.8 detected from *Cylindrospermum* sp. CCALA 1000 formed a unsaturated fatty acid immonium ion $C_{10}H_{20}N^+$ (Fig. 4c) and the 40 eV fragmentation provided the peptide sequence Ile/Leu,Ile/Leu,Val,Tyr,Ile/Leu, Ile/Leu (Fig. 4c). Finally in the strain *Anabaena* sp. A-Pastvisko11 we detected the putative lipopeptide with characteristic fragment $C_{16}H_{34}N^+$ and short peptide sequence Phe-Phe-Arg (Fig. 4d).

These results demonstrates that the method developed here is capable of detecting not only already described lipopeptides but also variants of known lipopeptides and lipopepeptides with novel chemical structures.

4. Discussion

Bacteria are a prolific source of β-amino fatty acid lipopeptides typically in molecular range from 700–1400 Da [5–7,16–25]. The majority of known lipopeptide chemical variants possess fully saturated non-substituted β-amino fatty acid chain of fatty acid length C_8–C_{12} [16–25]. Some of the minor variants can be functionalized by hydroxyl-, keto-, chlorine or bromine group, but substituted variants can be also produced [21]. Complex branching and functionalization of the fatty acid chain have also been reported [19].

Characteristic fragments are routinely used in selective detection of many microbial metabolites as for example hepatotoxic microcystins [30,31]. However, no selective method for the detection of cyclic β-amino fatty acid lipopeptides is currently available. The peptidic nature of the compound is seen from neutral losses of amino acid residues and their immonium ions (the mass range of proteinogenic amino acid even number immonium ions is *m*/*z* 30–159) in the product ion spectra produced with collision energies typical for peptides. With higher collision energies intensities of higher mass immonium ions start to dominate which are diagnostic for β-amino fatty acids ($C_xH_{2x+2}N^+$ for non-functionalized and

Please cite this article in press as: P. Urajová, et al., A liquid chromatography–mass spectrometric method for the detection of cyclic β-amino fatty acid lipopeptides, J. Chromatogr. A (2016), http://dx.doi.org/10.1016/j.chroma.2016.02.013

$C_xH_{2x}N^+$ for those bearing one functionalization). Fully saturated non-derivatized β-amino C_8–C_{20} fatty acid form even numbered immonium ions which mass range is m/z 100–270. An even numbered ion mass directly shows the presence of one nitrogen in the fragment. Taking together, by coupling of the information from two separate HPLC-HRMS/MS analyzes and plotting the extracted ion chromatograms for the immonium ions of β-amino fatty acid chains (C_8–C_{12}, both saturated and once unsaturated) we can obtain information about the presence of β-amino fatty acid lipopeptides in the sample analyzed (for example Fig. S3).

Different derivatives of the fatty acid chain, how it is bound to the peptide part and the structure of the peptide part change the immonium ion formation dramatically in collision induced dissociation. This was demonstrated in case of anabaenolysin A. The immonium ion formed from the fatty acid of anabaenolysin A did not dominate similarly as all the immonium ions formed from iturin and puwainaphycin variants and was obtained at lower collision energies. Identification of the peptidic nature of the compound could be problematic in peptides with 3–5 amino acid residues especially if non-proteogenic amino acids are present since there are no easy assignment tools or databases available for identification of the rare amino acid in the mass spectra analysis software. In anabaenolysin the cyclic peptide part is containing only two proteogenic amino acids and one residue specific for anabaenolysin [23,27]. In anabaenolysin A the two amino acids are both glycines so only the loss of Gly residue from the molecular ion or the presence of Gly immonium ion in the product ion spectrum would straightforward reveal the compounds peptidic structure. In practice the probability of both these events to happening is negligible low. So in screening studies without existing knowledge it could be challenging to recognize lipopetides with more modified β-amino fatty acids and/or peptide part containing mostly nonproteogenic amino acids possibly together with polyketidic structures. It is also important to mention that the occurrence of multiple modifications on the fatty acid side chain including more complex branching, desaturation or multiple substitutions by ─OH and ─Cl groups will make the data interpretation more difficult and thus such type of compound will be hardly detected by the method presented.

The majority of characterized bacterial β-amino fatty acid lipopeptides contain mostly proteinogenic amino acids and have saturated fatty acid chain [5,16–25]. Therefore the method developed here has broad applications in bioprospection, pharmaceutical and environmental screening. We previously reported that *C. alatosporum* CCALA 988 is capable of synthesizing puwainaphycin variants with C_{10} and C_{14} fatty acid both chlorinated and hydroxylated [12]. However, the method described here and knowledge about the linear dependence of retention time and fatty acid length made it possible to to detect puwainaphycin variants from C_{10} to C_{17} including majority of their chlorinated and hydroxylated variants.

5. Conclusions

Here we report a method for the analysis of crude bacterial extracts by a HPLC-HRMS/MS technique using varying collision energies as a good tool for targeted cyclic β-amino-lipopeptides screening. We detected novel structural variants of puwainaphycin F and G with different β-amino fatty acid in *C. alatosporum* CCALA 988 extract using this method. Selected high collision energy (100 eV) is suitable for both puwainphycin and iturin A types of lipopeptides to generate diagnostic fragments. On the contrary, in anabaenolysin lower collision energy (35 eV) is needed to obtain the diagnostic β-amino fatty acid immonium ion. The proposed HPLC-HRMS/MS approach here provides rapid and easy method for detecting novel variants of bioactive cyclic lipopeptides as was demonstrated in studied cyanobacterial strains.

Acknowledgements

The work was supported by Ministry of Education of the Czech Republic—National Programme of Sustainability I, ID: LO1416 and the Czech Science Foundation (Project Nos. CSF 14-18067S and 16-09381S).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2016.02.013.

References

[1] J. Berdy, Bioactive microbial metabolites—a personal view, J. Antibiot. 58 (2005) 1–26.
[2] G.E. Chlipala, S.Y. Mo, J. Orjala, Chemodiversity in freshwater and terrestrial cyanobacteria—a source for drug discovery, Curr. Drug Targets 12 (2011) 1654–1673.
[3] M. Welker, H. von Dohren, Cyanobacterial peptides—nature's own combinatorial biosynthesis, Fems Microbiol. Rev. 30 (2006) 530–563.
[4] H. Wang, D.P. Fewer, L. Holm, L. Rouhiainen, K. Sivonen, Atlas of nonribosomal peptide and polyketide biosynthetic pathways reveals common occurrence of nonmodular enzymes, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 9259–9264.
[5] L. Delcambe, F. Peypoux, F. Besson, M. Guinand, G. Michel, Structure of iturin and iturin/like substances, Biochem. Soc. Trans. 5 (1977) 1122–1124.
[6] N. Vanittanakom, W. Loeffler, U. Koch, G. Jung, Fengycin—a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis*, J. Antibiot. 39 (1986) 888–901.
[7] J. Micklefield, Daptomycin structure and mechanism of action revealed, Chem. Biol. 11 (2004) 887–888.
[8] T. Neuhof, P. Schmieder, K. Preussel, R. Dieckmann, H. Pham, F. Bartl, H. von Dohren, Hassallidin A, a glycosylated lipopeptide with antifungal activity from the cyanobacterium *Hassallia* sp, J. Nat. Prod. 68 (2005) 695–700.
[9] T.H. Bui, V. Wray, M. Nimtz, T. Fossen, M. Preisitsch, G. Schroeder, K. Wende, S.E. Heiden, S. Mundt, Balticidins A-D antifungal hassallidin-like lipopeptides from the Baltic Sea Cyanobacterium *Anabaena cylindrica* Bio33 (vol 77, pg 1287, 2014), J. Nat. Prod. 78 (2015), 345–345.
[10] E.H. Duitman, L.W. Hamoen, M. Rembold, G. Venema, H. Seitz, W. Saenger, F. Bernhard, R. Reinhardt, M. Schmidt, C. Ullrich, T. Stein, F. Leenders, J. Vater, The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: a multifunctional hybrid between a peptide synthetase an amino transferase, and a fatty acid synthase, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 13294–13299.
[11] S.Y. Yao, X.W. Gao, N. Fuchsbauer, W. Hillen, J. Vater, J.S. Wang, Cloning sequencing, and characterization of the genetic region relevant to biosynthesis of the lipopeptides iturin A and surfactin in *Bacillus subtilis*, Curr. Microbiol. 47 (2003) 272–277.
[12] J. Mares, J. Hajek, P. Urajova, J. Kopecky, P. Hrouzek, A hybrid non-ribosomal peptide/polyketide synthetase containing Fatty-Acyl Ligase (FAAL) synthesizes the β-Amino fatty acid lipopeptides Puwainaphycins in the cyanobacterium *Cylindrospermum alatosporum*, PLoS One 9 (2014) E111904, 10.1371.
[13] A. Kakinuma, M. Hori, H. Sugino, I. Yoshida, M. Isono, G. Tamura, K. Arima, Determination of location of the lactone ring in surfactin, Agric. Biol. Chem. 33 (1969) 1523–1524.
[14] T.K. Shishido, A. Humisto, J. Jokela, L.W. Liu, M. Wahlsten, A. Tamrakar, D.P. Fewer, P. Permi, A.P.D. Andreote, M.F. Fiore, K. Sivonen, Antifungal compounds from cyanobacteria, Mar. Drugs 13 (2015) 2124–2140.
[15] J. Vestola, T.K. Shishido, J. Jokela, D.P. Fewer, O. Aitio, P. Permi, M. Wahlsten, H. Wang, L. Rouhiainen, K. Sivonen, Hassallidins, antifungal glycolipopeptides, are widespread among cyanobacteria and are the end-product of a nonribosomal pathway, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) E1909, 10.1073.
[16] W.P. Frankmolle, G. Knubel, R.E. Moore, G.M.L. Patterson, Antifungal cyclic peptides from terrestrial blue green alga *Anabaena laxa*. 2. Structures of laxaphycin A, B, D and E, J. Antibiot. 45 (1992) 1458–1466.
[17] W.H. Gerwick, Z.D. Jiang, S.K. Agarwal, B.T. Farmer, Total structure of hormothamnin A, a toxic cyclic undecapeptide from the tropical marine cyanobacterium *Hormothamnion enteromorphoides*, Tetrahedron 48 (1992) 2313–2324.
[18] H.S. Kang, M. Sturdy, A. Krunic, H. Kim, Q. Shen, S.M. Swanson, J. Orjala, Minutissamides E-L, antiproliferative cyclic lipodecapeptides from the cultured freshwater cyanobacterium cf. *Anabaena* sp, Bioorgan. Med. Chem. 20 (2012) 6134–6143.

Please cite this article in press as: P. Urajová, et al., A liquid chromatography–mass spectrometric method for the detection of cyclic β-amino fatty acid lipopeptides, J. Chromatogr. A (2016), http://dx.doi.org/10.1016/j.chroma.2016.02.013

[19] T.Y. An, T.K.S. Kumar, M.L. Wang, L. Liu, J.O. Lay, R. Liyanage, J. Berry, M. Gantar, V. Marks, R.E. Gawley, K.S. Rein, Structures of pahayokolides A and B, cyclic peptides from a Lyngbya sp, J. Nat. Prod. 70 (2007) 730–735.
[20] P. Hrouzek, M. Kuzma, J. Cerny, P. Novak, R. Fiser, P. Simek, A. Lukesova, J. Kopecky, The cyanobacterial cyclic lipopeptides puwainaphycins F/G are inducing necrosis *via* cell membrane permeabilization and subsequent unusual actin relocalization, Chem. Res. Toxicol. 25 (2012) 1203–1211.
[21] P. Tomek, P. Hrouzek, M. Kuzma, J. Sykora, R. Fiser, J. Cerny, P. Novak, S. Bartova, P. Simek, M. Hof, D. Kavan, J. Kopecky, Cytotoxic lipopeptide muscotoxin A, isolated from soil cyanobacterium desmonostoc muscorum permeabilizes phospholipid membranes by reducing their fluidity, Chem. Res. Toxicol. 28 (2015) 216–224.
[22] N. Maru, O. Ohno, D. Uemura, Lyngbyacyclamides A and B, novel cytotoxic peptides from marine cyanobacteria *Lyngbya* sp, Tetrahedron Lett. 51 (2010) 6384–6387.
[23] J. Jokela, L. Oftedal, L. Herfindal, P. Permi, M. Wahlsten, S.O. Doskeland, K. Sivonen, Anabaenolysins, novel cytolytic lipopeptides from benthic *Aanabaena* cyanobacteria, PLoS One 7 (2012) E41222, 10.1371.
[24] J.B. MacMillan, M.A. Ernst-Russell, J.S. de Ropp, T.F. Molinski, Lobocyclamides A-C, lipopeptides from a cryptic cyanobacterial mat containing *Lyngbya confervoides*, J. Org. Chem. 67 (2002) 8210–8215.
[25] A. Plaza, C.A. Bewley, Largamides A-H, unusual cyclic peptides from the marine *cyanobacterium oscillatoria* sp. (vol 71, pg 6898, 2006), J. Org. Chem. 74 (2009), 486–486.
[26] L. Oftedal, L. Myhren, J. Jokela, G. Gausdal, K. Sivonen, S.O. Doskeland, L. Herfindal, The lipopeptide toxins anabaenolysin A and B target biological membranes in a cholesterol-dependent manner, BBA-Biomembranes 1818 (2012) 3000–3009.
[27] T.K. Shishido, J. Jokela, C.T. Kolehmainen, D.P. Fewer, M. Wahlsten, H. Wang, L. Rouhiainen, E. Rizzi, G. De Bellis, P. Permi, K. Sivonen, Antifungal activity improved by coproduction of cyclodextrins and anabaenolysins in cyanobacteria, Proc. Natl. Acad. Sci. U.S.A. 112 (2015), 10.1073.
[28] M.B. Allen, D.I. Arnon, Studies on nitrogen-fixing blue-green algae. 1. Growth and nitrogen fixation by anabaena cylindrica LEMM, Plant Physiol. 30 (1955) 366–372.
[29] J. Kótai, Instructions for preparation of modified nutrient solution Z8 for algae, Blindern B–11/69 (Norwegian Institute for Water Research, Oslo).
[30] L. Spoof, P. Vesterkvist, T. Lindholm, J. Meriluoto, Screening for cyanobacterial hepatotoxins, microcystins and nodularin in environmental water samples by reversed-phase liquid chromatography–electrospray ionisation mass spectrometry, J. Chromatogr. A 1020 (2003) 105–119.
[31] J. Kohoutek, O. Adamovský, M. Oravec, Z. Simek, M. Paliková, R. Kopp, L. Bláha, LC–MS analyses of microcystins in fish tissues overestimate toxin levels-critical comparison with LC–MS/MS, Anal. Bioanal. Chem. 398 (2010) 1231–1237.

Please cite this article in press as: P. Urajová, et al., A liquid chromatography–mass spectrometric method for the detection of cyclic β-amino fatty acid lipopeptides, J. Chromatogr. A (2016), http://dx.doi.org/10.1016/j.chroma.2016.02.013

Paper III: Alternative Biosynthetic Starter Units Enhance the Structural Diversity of Cyanobacterial Lipopeptide

AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

GENETICS AND MOLECULAR BIOLOGY

Alternative Biosynthetic Starter Units Enhance the Structural Diversity of Cyanobacterial Lipopeptides

Jan Mareš,[a,b,c] Jan Hájek,[b,c] Petra Urajová,[b] Andreja Kust,[a,b,c] Jouni Jokela,[d] Kumar Saurav,[b] Tomáš Galica,[b,c] Kateřina Čapková,[a] Antti Mattila,[d] Esa Haapaniemi,[e,f] Perttu Permi,[e] Ivar Mysterud,[g] Olav M. Skulberg,[h] Jan Karlsen,[i] David P. Fewer,[d] Kaarina Sivonen,[d] Hanne Hjorth Tønnesen,[i] Pavel Hrouzek[b,c]

[a]The Czech Academy of Sciences, Biology Centre, Institute of Hydrobiology, České Budějovice, Czech Republic
[b]The Czech Academy of Sciences, Institute of Microbiology, Center Algatech, Třeboň, Czech Republic
[c]University of South Bohemia, Faculty of Science, České Budějovice, Czech Republic
[d]Department of Microbiology, Viikki Biocenter, University of Helsinki, Helsinki, Finland
[e]Department of Chemistry, University of Jyväskylä, Jyväskylä, Finland
[f]Department of Biological and Environmental Science, Nanoscience Center, University of Jyväskylä, Jyväskylä, Finland
[g]Department of Biosciences, University of Oslo, Oslo, Norway
[h]Norwegian Institute for Water Research (NIVA), Oslo, Norway
[i]School of Pharmacy, University of Oslo, Oslo, Norway

AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

GENETICS AND MOLECULAR BIOLOGY

Alternative Biosynthetic Starter Units Enhance the Structural Diversity of Cyanobacterial Lipopeptides

Jan Mareš,[a,b,c] Jan Hájek,[b,c] Petra Urajová,[b] Andreja Kust,[a,b,c] Jouni Jokela,[d] Kumar Saurav,[b] Tomáš Galica,[b,c] Kateřina Čapková,[a] Antti Mattila,[d] Esa Haapaniemi,[e,f] Perttu Permi,[e] Ivar Mysterud,[g] Olav M. Skulberg,[h] Jan Karlsen,[i] David P. Fewer,[d] Kaarina Sivonen,[d] Hanne Hjorth Tønnesen,[i] Pavel Hrouzek[b,c]

[a]The Czech Academy of Sciences, Biology Centre, Institute of Hydrobiology, České Budějovice, Czech Republic
[b]The Czech Academy of Sciences, Institute of Microbiology, Center Algatech, Třeboň, Czech Republic
[c]University of South Bohemia, Faculty of Science, České Budějovice, Czech Republic
[d]Department of Microbiology, Viikki Biocenter, University of Helsinki, Helsinki, Finland
[e]Department of Chemistry, University of Jyväskylä, Jyväskylä, Finland
[f]Department of Biological and Environmental Science, Nanoscience Center, University of Jyväskylä, Jyväskylä, Finland
[g]Department of Biosciences, University of Oslo, Oslo, Norway
[h]Norwegian Institute for Water Research (NIVA), Oslo, Norway
[i]School of Pharmacy, University of Oslo, Oslo, Norway

ABSTRACT Puwainaphycins (PUWs) and minutissamides (MINs) are structurally analogous cyclic lipopeptides possessing cytotoxic activity. Both types of compound exhibit high structural variability, particularly in the fatty acid (FA) moiety. Although a biosynthetic gene cluster responsible for synthesis of several PUW variants has been proposed in a cyanobacterial strain, the genetic background for MINs remains unexplored. Herein, we report PUW/MIN biosynthetic gene clusters and structural variants from six cyanobacterial strains. Comparison of biosynthetic gene clusters indicates a common origin of the PUW/MIN hybrid nonribosomal peptide synthetase and polyketide synthase. Surprisingly, the biosynthetic gene clusters encode two alternative biosynthetic starter modules, and analysis of structural variants suggests that initiation by each of the starter modules results in lipopeptides of differing lengths and FA substitutions. Among additional modifications of the FA chain, chlorination of minutissamide D was explained by the presence of a putative halogenase gene in the PUW/MIN gene cluster of *Anabaena minutissima* strain UTEX B 1613. We detected PUW variants bearing an acetyl substitution in *Symplocastrum muelleri* strain NIVA-CYA 644, consistent with an *O*-acetyltransferase gene in its biosynthetic gene cluster. The major lipopeptide variants did not exhibit any significant antibacterial activity, and only the PUW F variant was moderately active against yeast, consistent with previously published data suggesting that PUWs/MINs interact preferentially with eukaryotic plasma membranes.

IMPORTANCE Herein, we deciphered the most important biosynthetic traits of a prominent group of bioactive lipopeptides. We reveal evidence for initiation of biosynthesis by two alternative starter units hardwired directly in the same gene cluster, eventually resulting in the production of a remarkable range of lipopeptide variants. We identified several unusual tailoring genes potentially involved in modifying the fatty acid chain. Careful characterization of these biosynthetic gene clusters and their diverse products could provide important insight into lipopeptide biosynthesis in prokaryotes. Some of the variants identified exhibit cytotoxic and antifungal properties, and some are associated with a toxigenic biofilm-forming strain. The findings may prove valuable to researchers in the fields of natural product discovery and toxicology.

KEYWORDS biosynthesis, cyanobacteria, fatty acyl-AMP ligase, lipopeptides, nonribosomal peptide synthetase

Citation Mareš J, Hájek J, Urajová P, Kust A, Jokela J, Saurav K, Galica T, Čapková K, Mattila A, Haapaniemi E, Permi P, Mysterud I, Skulberg OM, Karlsen J, Fewer DP, Sivonen K, Tønnesen HH, Hrouzek P. 2019. Alternative biosynthetic starter units enhance the structural diversity of cyanobacterial lipopeptides. Appl Environ Microbiol 85:e02675-18. https://doi.org/10.1128/AEM.02675-18.

Editor Marie A. Elliot, McMaster University

Copyright © 2019 American Society for Microbiology. All Rights Reserved.

Address correspondence to Pavel Hrouzek, hrouzekp@gmail.com.

Received 5 November 2018
Accepted 28 November 2018
Accepted manuscript posted online 30 November 2018
Published 6 February 2019

Bacterial lipopeptides are a prominent group of secondary metabolites with pharmaceutical potential as antibacterial, antifungal, anticancer, and antiviral agents (1). Compounds like fengycin, the iturin family antibiotics, octapeptins, and daptomycin are important pharmaceutical leads, the latter of which is already in clinical use (1–3). Their biological activity is the result of an amphipathic molecular structure that allows micellar interaction within the cell membranes of target organisms (4).

Lipopeptides are widespread in cyanobacteria and possess cytotoxic and antifungal activities (5–8). Puwainaphycins (PUWs) and minutissamides (MINs) are lipopeptides featuring a β-amino fatty acid and a 10-membered peptide ring (5, 9–11). Both classes exhibit considerable structural variability in terms of length and functionalization of the fatty acyl (FA) side chain attached to the stable peptide core (10–14). Only minor discrepancies in the lengths and substitutions of the FA chain separate these two types of lipopeptides. A wide array of bioactivities has been reported for these compounds. PUW C is a cardioactive compound (15), as demonstrated by positive inotropic activity in mouse atria, while PUW F/G exhibit cytotoxicity against human cells *in vitro* through cell membrane permeabilization (5). MIN A to L exhibited antiproliferative effects over a range of concentrations when tested against human cancer cell lines, similarly to PUWs (10, 11). The overall structural similarity suggests that PUWs and MINs share a similar biosynthetic origin. However, the biosynthetic mechanisms generating the conspicuous chemical variability remain unknown.

PUWs are synthesized by a hybrid polyketide/nonribosomal peptide synthetase (PKS/NRPS) accompanied by tailoring enzymes (12). A characteristic feature of the PUW synthetase is the fatty acyl-AMP ligase (FAAL) starter unit (12). This enzyme specifically binds and adenylates FAs and passes the activated acyl-adenylate to a downstream phosphopantetheine arm of the PKS acyl carrier protein (ACP) for further processing (12). The whole process bears a resemblance to the biosynthesis of iturin family lipopeptides (16–19), as well as small lipopeptide-like cyanobacterial metabolites like hectochlorin (20), hapalosin (21), and jamaicamide (22), as discussed previously (23). Bacterial FAAL enzymes originate from basal cell metabolism and likely evolved from fatty acyl-coenzyme A (CoA) ligases (FACLs) following a specific insertion that hampered subsequent ligation to reduced CoA (CoASH) (24) or altered the catalytic conformation (25). FAAL enzymes play an important role in the assembly of other metabolites, including olefins (26) and unusual lipids (27), in addition to lipopeptide synthesis. The exact substrate-binding mechanism employed by FAALs was demonstrated experimentally in *Mycobacterium tuberculosis* using several homologous FAAL enzymes and FA substrates as models (28). The substrate specificity of these enzymes corresponds to the structure of the substrate-binding pocket (25, 28), although it overlaps among homologs.

Herein, we combined recently developed bioinformatics and high-performance liquid chromatography combined with high-resolution tandem mass spectrometry (HPLC–HRMS-MS) approaches (13, 23) to identify biosynthesis gene clusters for PUWs/MINs in five new cyanobacterial strains and characterized the chemical variability of their products. We discuss the specific structural properties of the lipopeptide variants identified and compare the predicted functions of synthetase enzymes.

RESULTS AND DISCUSSION

Structural variability versus common biosynthetic origin of PUWs and MINs. In the present study, we collected all known PUW/MIN producers (except for *Anabaena* sp. strain UIC10035). The strains, referred to herein as strains 1 to 6, were originally isolated from various soil habitats (Table 1). HPLC–HRMS-MS analysis detected multiple PUW and MIN variants in each of the strains studied (Fig. 1), ranging from 13 to 26 in strains 3 and 1, respectively (Table S1 in the supplemental material).

The MS-MS data acquired for crude extracts were used to create a molecular network (Fig. 2), analysis of which demonstrated that *Cylindrospermum* strains 1 to 3 and *Anabaena* strains 4 and 5 formed a single group with MIN A as the only variant common to all the strains (Fig. 2a). All major structural variants of these strains shared

TABLE 1 Strains analyzed for PUW/MIN production

Strain no. herein	Strain	Person who isolated strain	Date	Locality, habitat	Reference
1	*Cylindrospermum alatosporum* CCALA 988	A. Lukešová	1989	Canada, Manitoba, Riding Mountain National Park, soil	55
2	*Cylindrospermum moravicum* CCALA 993	A. Lukešová	2008	Czech Republic, South Moravia, Moravian Karst, Amaterska Cave, cave sediment	55
3	*Cylindrospermum alatosporum* CCALA 994	A. Lukešová	2011	Czech Republic, Moravian Karst, earthworms collected from soil above Amaterska Cave, earthworm casings	55
4	*Anabaena* sp. UHCC-0399	M. Wahlsten	N/A	Finland, Jurmo, Southwestern Archipelago National Park, copepods	56
5	*Anabaena minutissima* UTEX B1613	T. Kantz	1967	South Texas, USA, soil	57
6	*Symplocastrum muelleri* NIVA-CYA 644	O. M. Skulberg	2009	Norway, Møre og Romsdal county, Halsa municipality, western slope of Slettfjellet mountain in semiterrestrial alpine habitat, biofilm on turf in ombrotrophic blanket bog	40

the common peptide sequence FA^{1}-Val^{2}-dehydroaminobutyric acid (Dhb)^{3}-Asn^{4}-Dhb^{5}-Asn^{6}-Ala^{7}-Thr^{8}-*N*-methyl-L-asparagine (*N*MeAsn)^{9}-Pro^{10} (Fig. 3), described previously for PUW F and MIN A (5, 10). The pattern of variant production was almost identical in *Cylindrospermum* strains 2 and 3, which in addition to MIN A contained PUW F (Fig. 1, Table S1). In contrast, *Anabaena* strains 4 and 5 produced MIN C and D in addition to the major variant, MIN A (Fig. 1). The peptide core of the molecule was different in *Symplocastrum muelleri* strain 6 (Fig. 3), forming a separate group in the molecular network (Fig. 2b), with the general peptide sequence FA^{1}-Val^{2}-Dhb^{3}-Thr^{4}-Thr/Val^{5}-Gln^{6}-Ala^{7}-*O*-methyl-L-threonine (*O*MeThr)^{8}-*N*MeAsn^{9}-Pro^{10} (Fig. 3), identical to those of PUW A to D and MIN I, K, and L isolated previously from *Anabaena* sp. (9, 11).

The peptide cores of the variants included in the network differed to some degree, but most variation was detected in the FA moiety (Fig. 4) when crude extracts were analyzed for the presence of characteristic FA immonium fragments (13).

Accordingly, bioinformatic analysis identified putative PUW and MIN gene clusters in each of the five newly sequenced strains (Fig. 5, Table 2). Based on BLASTp, CDD, and AntiSMASH searches, these gene clusters exhibited synteny and functional homology with the previously characterized *puw* biosynthesis gene cluster in strain 1 (12) (Fig. 5). Therefore, our results strongly indicate a common biosynthetic origin of PUWs and MINs in cyanobacteria.

Variability in the peptide core. A common set of NRPS genes (*puwA* and *puwE* to *-H*) (Fig. 5) encoding a sequence of nine amino acid-incorporating modules (Fig. 6) was detected in all strains analyzed. Individual NRPS modules displayed variability in amino acid adenylation and tailoring domains that was generally congruent with the PUW/MIN peptide cores inferred using HPLC–HRMS-MS (Fig. 3). The two major types of peptide cores observed (represented by PUW A and PUW F, respectively) differed in the amino acids at positions 4 (Thr→Asn), 5 (Thr→Dhb), 6 (Gln→Asn), 7 (Ala→Gly), and 8 (Thr→*O*Me-Thr), as shown in Fig. 3 and Table S1. This was reflected in the predicted substrates of the corresponding adenylation domains (A domains) and by the presence of an *O*-methyltransferase domain in PuwH of *S. muelleri* strain 6, which is responsible for the methoxylation of Thr^{8} (Fig. 6, Table S2). In contrast to the variability observed at the previously noted amino acid positions, the two positions adjacent to both sides of the modified fatty acid [*N*MeAsn^{9}-Pro^{10}-(FA^{1})-Val^{2}-Dhb^{3}] are conserved in all known PUW/MIN variants described here and previously (5, 9, 13–15) (Fig. 3, Table S1). Accordingly, no functional variation in A domains corresponding to these positions was observed within the deduced PuwA, PuwE, and PuwF proteins (Table S2). This is interesting because these four hydrophobic amino acids surround the FA moiety, which is likely responsible for the membrane disruption effect suggested previously (5). Thus, we hypothesize that such an arrangement could further support hydrophobic interactions with the lipid layer of the plasma membrane.

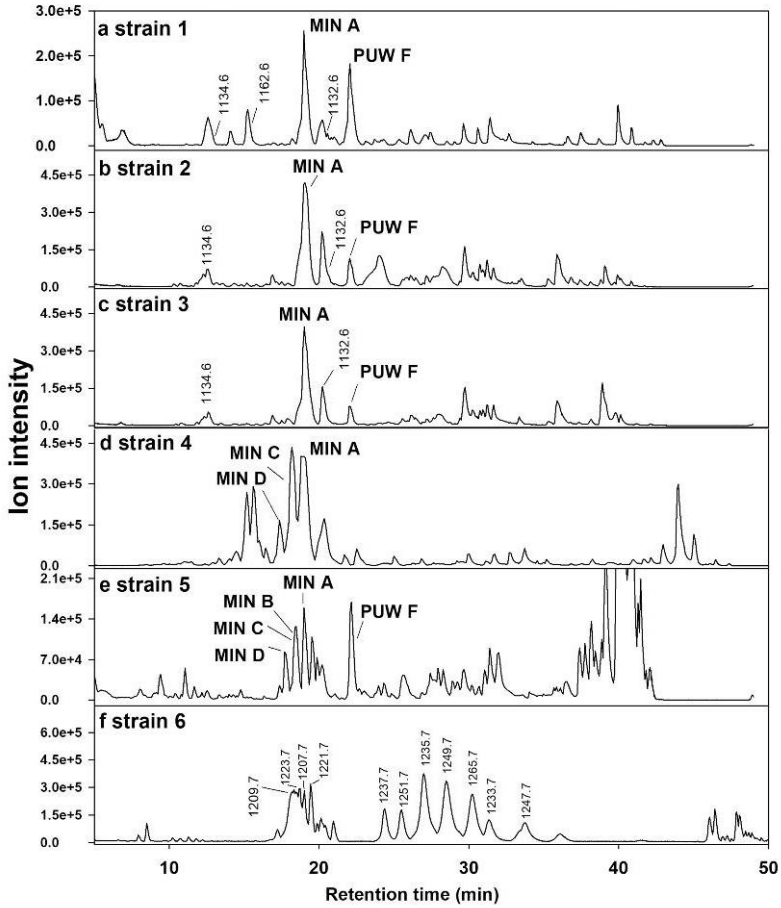


FIG 1 HPLC–HRMS-MS analysis of crude extracts from the strains investigated. Major puwainaphycin (PUW) and minutissamide (MIN) variants are highlighted. For variants without complete structural information, only *m/z* values are shown.

For some of the other positions, minor variants were observed involving substitution of amino acids similar in structure and hydrophobicity, including Asn-Gln at position 4, Thr-Val at position 5, Ala-Gly at position 7, and Thr-Ser at position 8 (Fig. 3, Table S1), indicative of probable substrate promiscuity in their respective adenylation domains (29). The A6 domains in strains 4 and 5 activated Ala as a major substrate and Gly to a lesser extent, even though *in silico* analysis predicted Gly as their main substrate (Table S2). In strain 6, Gly was incorporated, in agreement with the predicted substrate specificity. An epimerase domain was present in each of the sixth NRPS modules (i.e., C-A6-PCP) of the pathways (Fig. 6), indicating the probable formation of a D-amino acid enantiomer at position 7 of the peptide core. Indeed, the presence of D-Ala was previously confirmed in PUW F (5) and MIN A to H (10, 11), and D-Gly was identified in MIN I to L (10, 11). In two cases, the adenylation domains A3 (PuwF) and A6 (PuwG) are capable of incorporating significantly different amino acids, such as Asn^4-Thr^4 and Ala^7-Ser^7, respectively (Fig. 6). This degree of substrate promiscuity is relatively uncom-

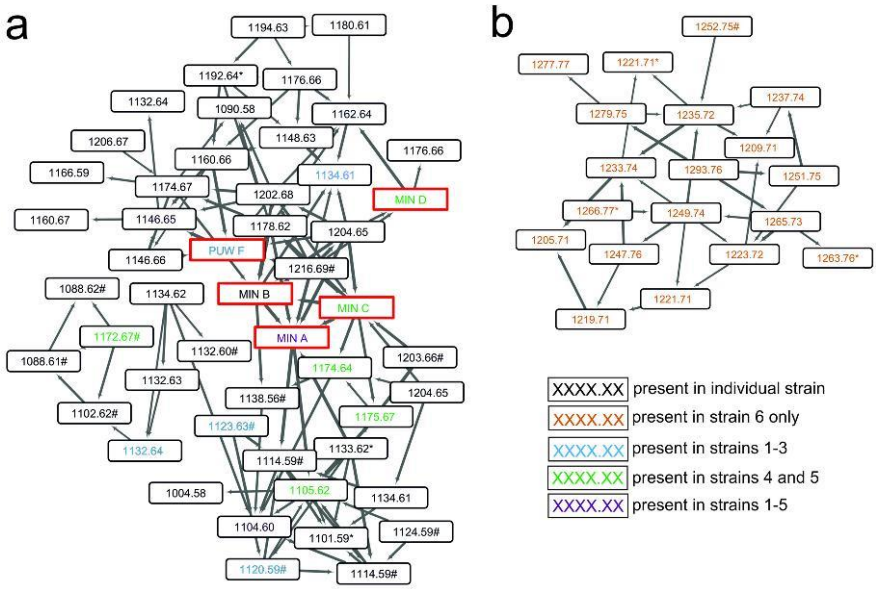
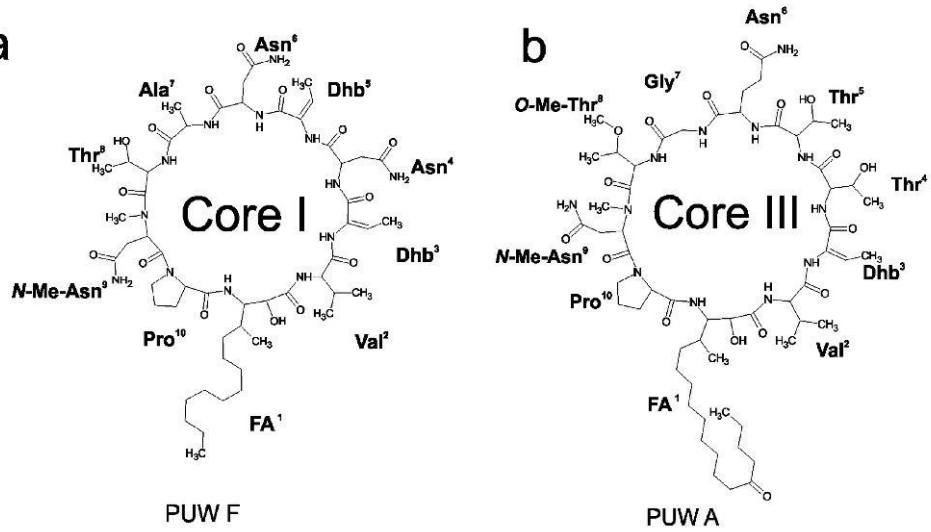


FIG 2 Molecular network created using the Global Natural Products Social Molecular Networking (GNPS) web platform. Two separate networks were obtained during GNPS analysis: a group containing *Cylindrospermum* strains 1 to 3 and *Anabaena* strains 4 and 5 (a), and a group containing only variants detected in *Symplocastrum muelleri* strain 6 (b). The separate groups differ mainly in the peptide core of the molecule. For variants without complete structural information, only *m/z* values are shown. *, compound present in trace amounts; #, compound for which MS-MS data failed to resolve the structural information.

mon. The activation of two divergent amino acids (Arg/Tyr) by a single adenylation domain, based on point mutations in just three codons, was previously demonstrated in the anabaenopeptin synthetase from the cyanobacterium *Planktothrix agardhii* (30). The substrate exchange of Ala versus Ser was previously reported from fungal class IV adenylate-forming reductases that contain A domains homologous to those of NRPS enzymes (31).

The last synthetase enzyme in the pathway (PuwA) is equipped with a terminal thioesterase domain (Fig. 6), which presumably catalyzes cleavage of the final product and formation of the cycle via a peptide bond between the terminal prolyl and the β-amino group of the FA chain, as previously suggested (12).

Two hypothetical starter units and their substrate range. The biosynthesis of bacterial lipopeptides is typically commenced by FA-activating enzymes (16, 18). Initiation of the biosythesis of PUW/MIN is performed by a FAAL enzyme (12) and allows a much broader array of activated substrates than the relatively conserved oligopeptide core (Fig. 4) (13). We identified three alternative arrangements of the putative FAAL starter units (Fig. 5 and 6), each corresponding to a different array of FA side chains detected by HPLC–HRMS-MS, which presumably reflects the range of FA substrates activated during their biosynthesis (Fig. 4). *Cylindrospermum* species strains 1 to 3 possess the type I putative starter unit consisting of a standalone FAAL enzyme, PuwC, and a separate ACP, PuwD (Fig. 5, Table 2). In contrast, the biosynthetic gene cluster of *S. muelleri* strain 6 contains the type II putative starter unit (PuwI) consisting of a FAAL fused to an ACP (Fig. 5, Table 2). *Anabaena* species strains 4 and 5 combine both type I and type II putative starter units in their biosynthetic gene clusters (Fig. 5, Table 2). Although the functions and substrate ranges of these hypothetical starter units require further confirmation by gene manipulation experiments, they are supported by the



c

	AA_1	AA_2	AA_3	AA_4	AA_5	AA_6	AA_7	AA_8	AA_9	AA_{10}	known compounds	variants found in strains 1-6
Core I	FA	Val	Dhb	Asn	Dhb	Asn	Ala	Thr	*N*-Me-Asn	Pro	MIN A, MIN B, MIN C, MIN D, PUW F	**MIN A, MIN B, MIN C, MIN D, PUW F,** Comp. 1,3,10,11,12,14,16,19,20, 25,26,28,30,31,37,45,51
Core II	FA	Val	Dhb	Gln	Dhb	Asn	Ala	Thr	*N*-Me-Asn	Pro	PUW G	**PUW G,** Comp. 2,6,13,15,17,27, 29,32
Core III	FA	Val	Dhb	Thr	Thr	Gln	Gly	*O*-Me-Thr	*N*-Me-Asn	Pro	PUW A, PUW C, PUW E, MIN I, MIN K	Comp. 38,46,50,52,53,56,58
Core IV	FA	Val	Dhb	Thr	Thr	Gln	Ala	*O*-Me-Thr	*N*-Me-Asn	Pro	MIN E, MIN G	Comp. 39,47,53,57,59
Core V	FA	Val	Dhb	Thr	Val	Gln	Gly	*O*-Me-Thr	*N*-Me-Asn	Pro	PUW B, PUW D, MIN L	Comp. 40,48,54,60
Core VI	FA	Val	Dhb	Thr	Val	Gln	Ala	*O*-Me-Thr	*N*-Me-Asn	Pro	MIN H	Comp. 41,49,55,61
Core VII	FA	Val	Dhb	Asn	Dhb	Asn	Gly	Thr	*N*-Me-Asn	Pro		Comp. 7,36,44
Core VIII	FA	Val	Dhb	Asn	Dhb	Asn	Ser	Thr	*N*-Me-Asn	Pro		Comp. 8,23,35
Core IX	FA	Val	Dhb	Asn	Dhb	Asn	Ala	Ser	*N*-Me-Asn	Pro		Comp. 9,18,24
Core X	FA	Val	Dhb	Thr	Dhb	Asn	Ala	Thr	*N*-Me-Asn	Pro		Comp. 5,34,43

FIG 3 Structural variability of the peptide cores of PUW/MIN variants. Examples of structural variants PUW F (a) and PUW A (b) with designated amino acid positions representing the two major peptide cores. (c) Table summarizing all types of the PUW/MIN peptide core found in known compounds reported in literature and compounds (Comp.) detected in studied strains. Columns shaded in gray highlight the conserved amino acid positions.

patterns of lipopeptide variants detected by HPLC–HRMS-MS (Fig. 4, Table S1). In *Cylindrospermum* strains 1 to 3, which exclusively contain the type I starter unit, the PUW/MIN products exhibited an almost-continuous FA distribution of between C_{10} and C_{15} (up to C_{17} in negligible trace amounts) (Fig. 4). In *S. muelleri* strain 6, the presence of the type II loading module resulted in the production of PUW/MIN variants with discrete FA lengths of C_{16} and C_{18}. Strains containing both type I and type II starter units (*Anabaena* strains 4 and 5) produced two sets of PUW/MIN products with no overlap (C_{12} to C_{14-15} for the type I pathway and C_{16} for the type II pathway) but exhibited a slightly shifted length distribution (Fig. 4). Based on these results, it seems plausible that PuwC/-D and PuwI represent two alternative FAAL starter modules capable of initiating PUW/MIN biosynthesis (Fig. 5 and 6). An analogous situation was previously described for the alternative NRPS starter modules in the anabaenopeptin synthetase (32).

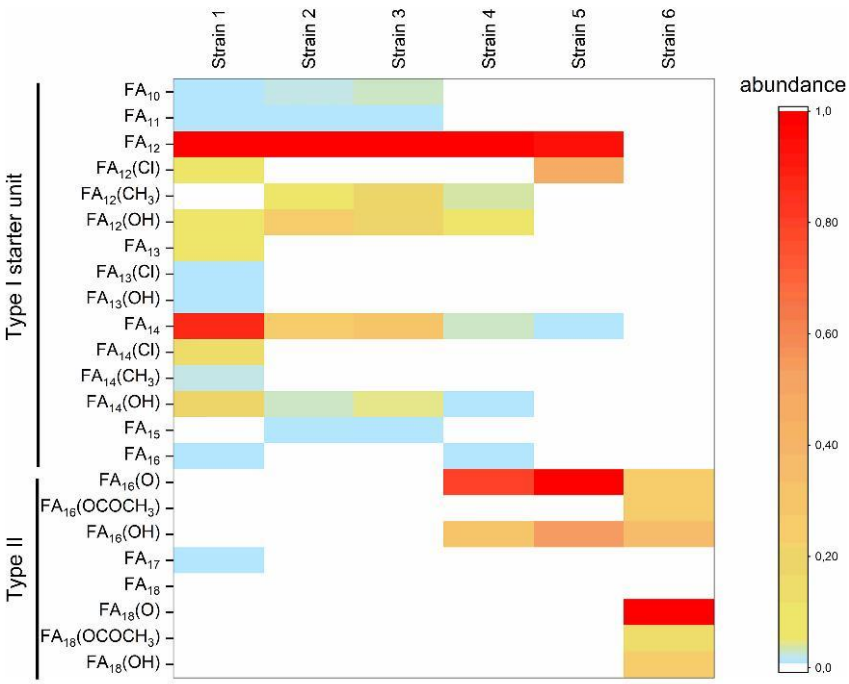


FIG 4 Structural variability of the FA moiety of PUW/MIN variants. The relative proportions of variants with differences in FA lengths and substitutions are depicted using a color scale. For comparison, the peak area of a given variant was normalized against the peak area of the major variant present in the strain (MIN A for strains 1 to 5 and *m/z* 1,235.7 for strain 6).

In the FA residue of the lipopeptide, proximal carbons in the linear aliphatic chain are incorporated into the nascent product by PKS enzymes (12). The PKS domains of PuwB and PuwE (Fig. 6, Table 2) catalyze two elongation steps. Therefore, the fatty acid is expected to be extended by four carbons. The substrate length specificities of the

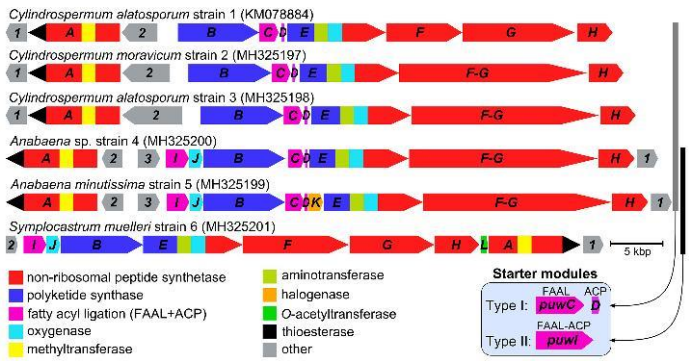


FIG 5 Structures of the *puw* gene cluster in the six cyanobacterial strains investigated. Gene arrangement and functional annotation of *puwA* to *-L* genes and selected PKS/NRPS tailoring domains are indicated by colored arrows. The distribution of the two types of putative starter modules (shaded boxes) observed is indicated by bars.

TABLE 2 Deduced proteins encoded by the *puw* gene cluster in six cyanobacterial strains, including length and functional annotation[c]

Protein	Length (aa) in strain no.:						Predicted function(s)[a]
	1	2	3	4	5	6	
ORF1	659	664	664	643	643	647	ABC transporter
PuwA	2,870	2,870	2,870	2,854	2,854	2,866	NRPS
ORF2	1,116	1,499	1,875	643	670	376	Patatin-like phospholipase
ORF3				696	696		Dynamin family protein
PuwI				709	702	711	FAAL, ACP
PuwJ				427	427	529	Cytochrome-like protein
PuwB	2,534	2,592	2,592	2,549	2,537	2,555	Hybrid PKS/NRPS, aminotransferase, oxygenase
PuwC	597	590	590	597	589		FAAL
PuwD	101	104	96	93	92		ACP
PuwK					465		Halogenase
PuwE	3,077	3,121	3,121	3,099	3,112	3,113	NRPS
PuwF	2,370	5,851[b]	5,851[b]	5,877[b]	5,871[b]	3,310	NRPS
PuwG	3,492					2,620	NRPS
PuwH	1,102	1,081	1,102	1,121	1,121	1,408	NRPS
PuwL						217	*O*-Acetyltransferase

[a]ACP, acyl carrier protein; FAAL, fatty acyl-AMP ligase; PKS, polyketide synthase; NRPS, nonribosomal peptide synthetase.
[b]The proteins PuwF and PuwG are encoded in a single ORF in this strain.
[c]The empty fields in the table indicate the absence of a particular protein.

FAAL enzymes in *Mycobacterium tuberculosis* were recently shown to be determined by the size and position of specific amino acid residues protruding into the FA-binding pocket (28). Experimental replacement of Gly or Ile by a larger Trp residue in the upper and middle parts of the pocket blocked the binding of the original C_{12} substrate, but shorter chains (C_2 or C_{10}, respectively) were still activated (28). Experimental data on FAAL substrate specificity in cyanobacteria are currently lacking. Alignment of amino acid residues from all putative PuwC and PuwI proteins demonstrates overall homology (Fig. S2a), including the positions corresponding to the FA-binding pocket, as previously shown in *Mycobacterium* (Fig. S2b) (28). Experimental evidence, such as *in vitro*

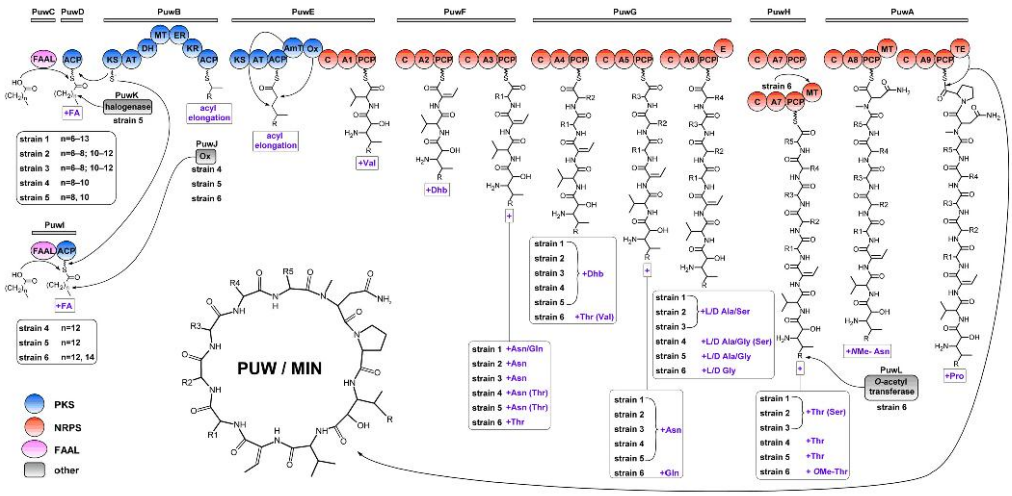


FIG 6 Schematic view of the proposed biosynthesis assembly line of puwainaphycins and minutissamides. Variable amino acid positions and the ranges of fatty acyl lengths incorporated by the two putative alternative starter units are listed for individual strains. A, adenylation domain; ACP, acyl carrier protein; AmT, aminotransferase; AT, acyltransferase; C, condensation domain; DH, dehydratase; E, enoylreductase; FAAL, fatty acyl-AMP ligase; MT, methyltransferase; NRPS, nonribosomal peptide synthetase; KR, ketoreductase; KS, ketosynthetase; Ox, monooxygenase; PCP, peptidyl carrier protein; PKS, polyketide synthetase; TE, thioesterase.

activity assays and crystallization of protein-ligand complexes, is required to explain the varying substrate specificities of PuwC versus those of PuwI. Also, we cannot exclude the possibility that the FA substrate length range is partially determined by the pool of free FAs available to the FAAL enzyme. Indeed, this possibility is supported by observations of *Cylindrospermum* strains 1 to 3, which share highly conserved PuwC proteins (Fig. S2a) with identical residues in the predicted FA-binding pockets (Fig. S2b) but display slightly different ranges and ratios of incorporated FAs in the PUW/MIN variants produced (Fig. 1 and 4).

FA tailoring reactions: oxidation, halogenation, and acetylation. Intriguingly, all products originating from biosynthesis initiated by the type II starter unit (variants with C_{16} and C_{18} FA tails in *Anabaena* strains 4 and 5 and *S. muelleri* strain 6) include the substitution of a hydroxy or oxo moiety (Fig. 6). For minutissamides C and D, this substitution takes place on the third carbon from the FA terminus (C_{14}), as described previously (10), and this position was confirmed by nuclear magnetic resonance (NMR) in variants produced by *Anabaena* sp. strain 4 in our study (Table S3, Fig. S3 to S6). In agreement with these hydroxy and oxo substitutions, the respective gene clusters each encode PuwJ, a putative cytochrome P450-like oxidase (Table 2), immediately downstream from the gene encoding the type II starter module. We therefore hypothesize that the PuwJ enzyme is responsible for hydroxylation of FA residues activated by PuwI (Fig. 6). However, the formation of the keto variant remains unexplained by our data.

Another gene, *puwK*, encoding a putative halogenase, was associated with the type II starter module in *Anabaena* sp. strain 5 (Table 2). Although no conserved enzymatic domain was detected in the deduced protein, it shares similarity with proteins postulated to be involved in the halogenation of cyanobacterial chlorinated acyl amides known as columbamides (33) and *N*-oxygenases similar to the *p*-aminobenzoate *N*-oxygenase AurF (34–36). The possible functional designation of this enzyme as a halogenase is further supported by the fact that the ω-chlorinated product MIN B, originally described in *Anabaena* sp. strain 5 (10), was also detected in this study (Table S1) as one of the major variants, while no MIN B or any other chlorinated PUW/MIN products were detected in *Anabaena* sp. strain 4 (Fig. 1). *Anabaena* species strains 4 and 5 share identical organization across the entire gene cluster, and the lack of the putative halogenase gene *puwK* is the only difference between these two clusters in terms of the presence of genes (Fig. 5).

In *Cylindrospermum* species strains 1 to 3, whcih exclusively possess the type I starter unit, the presence of minor amounts of hydroxylated and chlorinated variants (Fig. 4) suggests the involvement of another biosynthetic mechanism unexplained by the current data. This ambiguity warrants experimental research, such as gene knockout experiments, to confirm the proposed functions of *puwJ* and *puwK*.

Finally, the gene cluster identified in *S. muelleri* strain 6 was the only one containing gene *puwL*. The deduced product of this gene shares 53.4% similarity with the *O*-acetyltransferase McyL (Table 2), which is involved in acetylation of the aliphatic chain of microcystin in cyanobacteria (37). Additionally, this gene is similar to those encoding chloramphenicol and streptogramin A *O*-acetyltransferases that serve as antibiotic resistance agents in various bacteria (38). The functional annotation of PuwL as a putative *O*-acetyltransferase is consistent with the detection of *O*-acetylated lipopeptide variants in *S. muelleri* strain 6 (Table 3, Fig. 7). Five PUW variants (*m/z* 1,265.7338, 1,279.7496, 1,277.7695, 1,291.7870, and 1,293.7654) that yield high-energy fragments proving the presence of an acetyl group bonded to the FA moiety have been detected. In the *m/z* 1,279.8 and 1,293.8 peaks, the high-energy-fragment ion at *m/z* 312 corresponds to the FA immonium ion bearing an acetyl group, and the fragment ion at *m/z* 439 corresponds to the prolyl-FA-acetyl fragment. The subsequent loss of an acetyl group resulted in the presence of ions at *m/z* 252 and 379, respectively (Table 3, Fig. 7). Similarly, analysis of the *m/z* 1,265.7 peak revealed analogous fragments at *m/z* 284/411 and 351/224 (Table 3).

TABLE 3 Fragmentation and amino acid composition of PUW variants from *Symplocastrum muelleri* strain 6 bearing acetyl substitutions on the FA moiety[a]

Level of fragmentation energy, peptide sequence or fragment obtained	Value when X, Y, and FA are as indicated														
	X, Ala; Y, Thr; FA, C_{16}			X, Gly; Y, Thr; FA, C_{18}			X, Ala; Y, Thr; FA, C_{18}			X, Gly; Y, Val; FA, C_{18}			X, Ala; Y, Val; FA, C_{18}		
	m/z	Δ(ppm)	Sum formula	*m/z*	Δ(ppm)	Sum formula	*m/z*	Δ(ppm)	Sum formula	*m/z*	Δ(ppm)	Sum formula	*m/z*	Δ(ppm)	Sum formula
Low fragmentation energy (60 eV)															
$[M+H]^+$	1,265.7338	+0.7	$C_{59}H_{101}N_{12}O_{18}$	1,279.7496	+0.9	$C_{60}H_{103}N_{12}O_{18}$	1,293.7654	+0.8	$C_{61}H_{105}N_{12}O_{18}$	1,277.7695	+1.6	$C_{61}H_{105}N_{12}O_{17}$	1,291.7870	+0.1	$C_{62}H_{107}N_{12}O_{17}$
$[M-CH_3OH]^+$	1,233.7170	−6.6	$C_{58}H_{97}N_{12}O_{17}$	1,247.7194	+4.1	$C_{59}H_{99}N_{12}O_{17}$	1,261.7494	−7.3	$C_{60}H_{101}N_{12}O_{17}$	Low int.		$C_{60}H_{101}N_{12}O_{16}$	Low int.		$C_{61}H_{103}N_{12}O_{16}$
$[M-CH_3OH$-*N*MeAsn$]^+$	1,105.6558	−4.9	$C_{53}H_{89}N_{10}O_{15}$	1,119.6619	+3.7	$C_{54}H_{91}N_{10}O_{15}$	1,133.681	+0.6	$C_{55}H_{93}N_{10}O_{15}$	1,117.6924	−5.0	$C_{55}H_{93}N_{10}O_{14}$	1,131.7307	−25.0	$C_{56}H_{95}N_{10}O_{14}$
$[M-CH_3OH$-*N*MeAsn-Dhb$]^+$	1,022.6180	−4.7	$C_{49}H_{84}N_9O_{14}$	1,036.6365	−7.3	$C_{50}H_{86}N_9O_{14}$	1050.6478	−3.1	$C_{51}H_{88}N_9O_{14}$	1,134.6603	−10.3	$C_{51}H_{88}N_9O_{13}$	1,048.6671	−1.7	$C_{52}H_{90}N_9O_{13}$
$[M-CH_3OH$-*N*MeAsn-Dhb-X$]^+$	951.5785	−2.5	$C_{48}H_{83}N_8O_{13}$	979.589	+18.8	$C_{48}H_{83}N_8O_{13}$	979.6041	+3.4	$C_{48}H_{83}N_8O_{13}$	977.6481	−20.5	$C_{49}H_{85}N_8O_{12}$	977.6518	−24.1	$C_{49}H_{85}N_8O_{12}$
$[M-CH_3OH$-*N*MeAsn-Dhb-X-Gln$]^+$	823.5253	−9.4	$C_{41}H_{71}N_6O_{11}$	851.5473	+1.8	$C_{43}H_{75}N_6O_{11}$	851.5478	+1.2	$C_{43}H_{75}N_6O_{11}$	849.5838	−16.7	$C_{44}H_{76}N_6O_{10}$	849.5589	+12.5	$C_{44}H_{77}N_6O_{10}$
$[M-CH_3OH$-*N*MeAsn-Dhb-X-Gln-Y$]^+$	722.4729	−4.2	$C_{37}H_{64}N_5O_9$	750.5005	+0.9	$C_{39}H_{68}N_5O_9$	750.5147	−18.1	$C_{39}H_{68}N_5O_9$	Low int.		$C_{40}H_{72}N_5O_8$	Low int.		$C_{40}H_{72}N_5O_8$
$[M-CH_3OH$-*N*MeAsn-Dhb-X-Gln-Y-Thr$]^+$	621.4223	−0.2	$C_{33}H_{57}N_4O_7$	649.4526	+1.4	$C_{35}H_{61}N_4O_7$	649.4539	−0.6	$C_{35}H_{61}N_4O_7$	649.465	−17.8	$C_{35}H_{61}N_4O_7$	649.4483	+8.0	$C_{35}H_{61}N_4O_7$
High fragmentation energy (100 eV)															
Fragment 1	411.3208	+2.2	$C_{23}H_{43}N_2O_4$	439.3559	−6.5	$C_{25}H_{47}N_2O_4$	439.3556	−5.8	$C_{25}H_{47}N_2O_4$	439.3556	−5.8	$C_{25}H_{47}N_2O_4$	439.3508	+5.1	$C_{25}H_{47}N_2O_4$
Fragment 1, $C_2H_4O_2$	351.3006	+0.0	$C_{21}H_{39}N_2O_2$	379.3334	−4.0	$C_{23}H_{43}N_2O_2$	379.3329	−2.6	$C_{23}H_{43}N_2O_2$	379.3328	−2.4	$C_{23}H_{43}N_2O_2$	379.3360	−10.8	$C_{23}H_{43}N_2O_2$
Fragment 2	284.2583	+0.4	$C_{17}H_{34}NO_2$	312.2919	−6.9	$C_{19}H_{38}NO_2$	312.2892	+1.6	$C_{19}H_{38}NO_2$	Low int.		$C_{19}H_{38}NO_2$	Low int.		$C_{19}H_{38}NO_2$
Fragment 2, $C_2H_4O_2$	224.2367	+2.6	$C_{15}H_{30}N$	252.2686	0.0	$C_{17}H_{34}N$	252.2687	−0.5	$C_{17}H_{34}N$	252.2684	+0.7	$C_{17}H_{34}N$	252.2677	+3.5	$C_{17}H_{34}N$

[a]Low int., low intensity (defined here as a signal-to-noise ratio lower than 2). For detailed methods, see Materials and Methods.

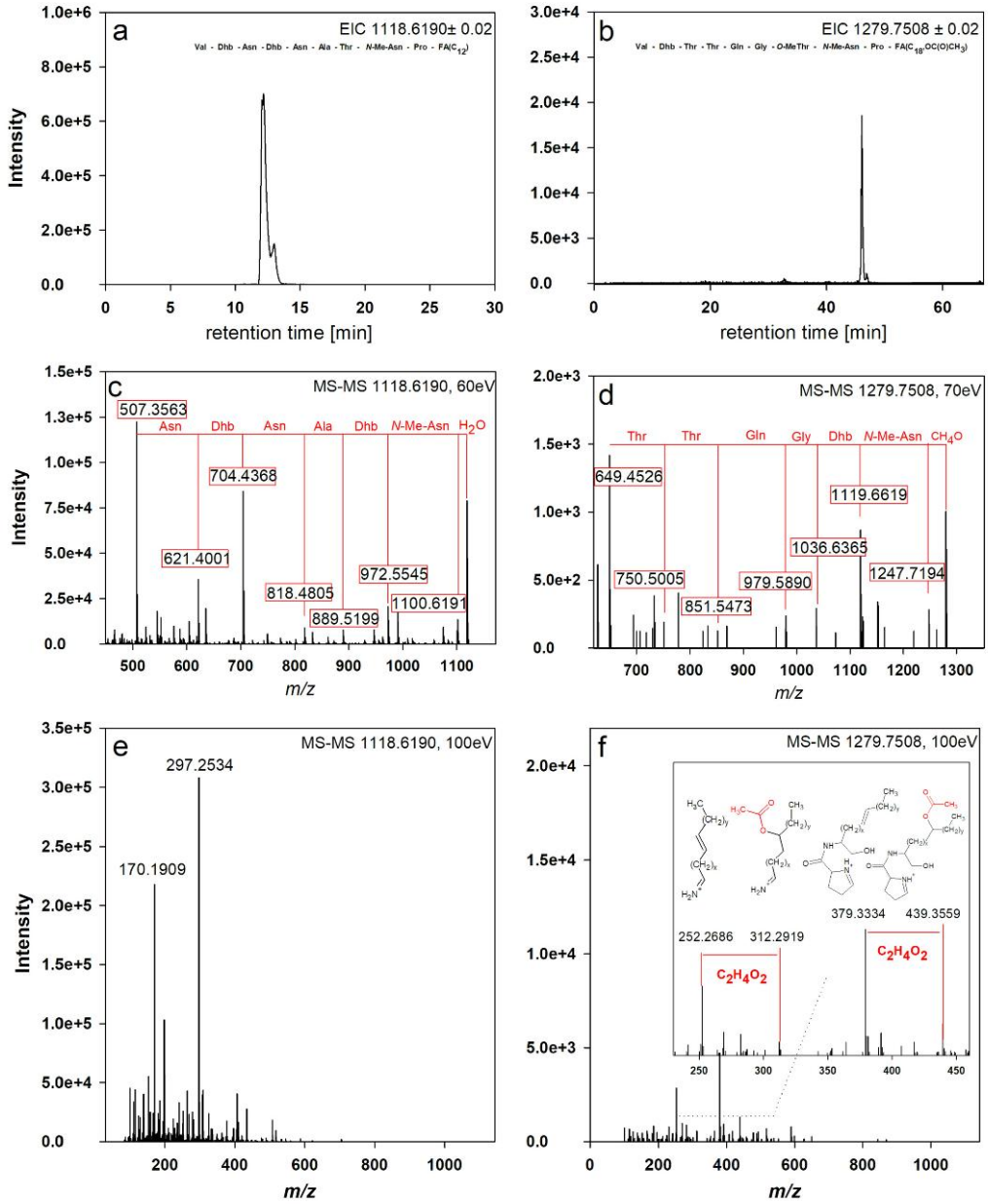


FIG 7 MS-MS fragmentation of MIN A (a, c, e) and the PUW variant at *m/z* 1,279 bearing an acetyl substitution of the fatty acid chain (b, d, f). (a, b) Base peak chromatograms. (c, d) Fragmentation of the protonated molecule at low fragmentation energy, yielding *b* series of ions, corresponding to the losses of particular amino acid residues. (e, f) Fragmentation of the protonated molecule at high energy (100 eV), yielding fragments characteristic for the β-amino fatty acid.

TABLE 4 Bacterial and yeast strains used for antimicrobial testing of PUW F and MIN A, C, and D

Test organism (HAMBI no.)[a]	Medium[b]	Incubation temp (°C)	Incubation time (h)	Gram stain reaction[c]
Pseudomonas sp. (2796)	TGY	28	24	−
Micrococcus luteus (2688)	TGY	28	24	+
Bacillus subtilis (251)	TGY	28	24	+
Pseudomonas aeruginosa (25)	TGY	37	24	−
Escherichia coli (396)	TGY	37	24	−
Bacillus cereus (1881)	TSA	28	24	+
Burkholderia cepacia (2487)	TSA	37	24	−
Staphylococcus aureus (11)	TSA	37	24	+
Xanthomonas campestris (104)	NA	28	24	−
Burkholderia pseudomallei (33)	NA	37	24	−
Salmonella enterica serovar Typhi (1306)	NA	37	24	−
Arthrobacter globiformis (1863)	NA	28	24	−
Kocuria varians (40)	NA	28	24	+
Candida albicans (261)	YM agar	37	24	Yeast
Cryptococcus albidus (264)	YM agar	28	24	Yeast
Saccharomyces cerevisiae (1164)	YM agar	28	24	Yeast

[a]HAMBI, culture collection of University of Helsinki, Faculty of Agriculture and Forestry, Department of Microbiology.
[b]The compositions of all media were obtained from the American Type Culture Collection (ATCC). TGY, tryptone glucose yeast; TSA, tryptic soy agar; NA, nutrient agar; YM agar, yeast malt agar.
[c]−, negative; +, positive.

Antimicrobial activity. Both PUWs and MINs possess cytotoxic activity against human cells *in vitro* (5, 10, 11). In the current study, we demonstrated that the major PUW/MIN variants (PUW F and MIN A, C, and D) did not exert antibacterial effects against either Gram-positive or Gram-negative bacteria in a panel of 13 selected strains (Table 4). PUW F was the only variant tested that manifested antagonistic activity against two yeast strains utilized in our experiment, namely, *Candida albicans* strain HAMBI 261 and *Saccharomyces cerevisiae* strain HAMBI 1164, with inhibition zones of 14 and 18 mm, respectively, and MICs of 6.3 μg ml^{-1} (5.5 μM) (Fig. 8). No antifungal activity was recorded for the MIN C and D variants, and only weak inhibition of the two yeast strains was recorded for MIN A (Fig. S7). PUW F differs only slightly from MIN A, by a -CH_2-CH_3 extension of the FA moiety, indicating that the FA length affects bioactivity. Furthermore, the lack of bioactivity for MIN C and MIN D suggests that hydroxy and oxo substitutions also compromise antifungal efficacy. As previously demonstrated, cytotoxicity is due to membrane permeabilization activity accompanied by calcium flux into the cytoplasm (5), consistent with the membrane effects documented for other bacterial lipopeptides (4). However, as is apparent from our data (Fig. 8), PUW/MIN products appear to be effective solely against eukaryotes (thus far tested only on human and yeast cells). This finding is in contrast to the typical antibacterial activity frequently described for many lipopeptides produced by Gram-positive bacteria (4). Analogously, the cyanobacterial lipopeptides anabaenolysin A and hassalidins

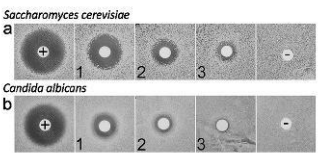


FIG 8 Antifungal activities of PUW F against yeast strains *Saccharomyces cerevisiae* HAMBI 1164 (a) and *Candida albicans* HAMBI 261 (b). Discs were treated with a range of concentrations from 25.2 μg ml^{-1} to 0.0394 μg/ml to determine the MIC. Numbers and symbols represent concentrations and controls, as follows: 1, 25.2 μg ml^{-1}; 2, 12.6 μg ml^{-1}; 3, 6.3 μg ml^{-1}; +, positive control (10 μg nystatin); −, negative control (10 μl methanol).

preferentially interact with cholesterol-containing membranes, hence their predisposition for activity against eukaryotic cells (6, 8).

Distribution of PUWs and MINs in cyanobacteria. PUWs and MINs form one of the most frequently reported groups of lipopeptides in cyanobacteria and have been isolated from heterocytous cyanobacteria, particularly members of the genera *Anabaena* and *Cylindrospermum* that inhabit soil (5, 9–11). Only a single study has mentioned the probable occurrence of puwainaphycins in a planktonic cyanobacterium (*Sphaerospermopsis*) (39). Our current comprehensive analysis of these lipopeptides and their biosynthetic genes further supports the hypothesis that lipopeptides occur predominantly in nonplanktonic biofilm-forming cyanobacteria (23). In this context, it is worth mentioning that *S. muelleri* strain 6 was isolated from a wetland bog in alpine mountains in coastal Norway (40). This strain is a toxigenic member of a biofilm microbiome and suspected to play a role in the development of severe hemolytic Alveld disease among outfield grazing sheep (41, 42). Biomass harvested from pure cultures of this strain exhibited strong cytotoxic activity toward primary rat hepatocytes (43, 44), which indicates the production of secondary metabolites with cytotoxic properties. Thus, the possible toxic potential of cyanobacterial lipopeptides, such as PUWs and MINs, in the environment warrants further attention.

Conclusions. Our study highlights and explores the extensive structural versatility of cyanobacterial lipopeptides from the PUW/MIN family by introducing previously unknown variants and newly sequenced biosynthetic gene clusters. Intriguingly, all variants are synthesized by a relatively conserved PKS/NRPS machinery with a common genetic origin. We hypothesize that chemical diversity is generated largely by the presence of two alternative fatty acyl-AMP ligase starter units, one of which exhibits an unusually broad specificity for FA substrates of various lengths. Additionally, putative halogenase and *O*-acetyltransferase genes were present in some gene clusters. This knowledge provides novel insight into the genetic background underpinning the biosynthesis of bacterial lipopeptides. The proposed biosynthetic mechanisms allow the studied microbes to generate a large pool of products that can be readily expanded by introducing relatively small genetic changes. This is consistent with the so-called "screening hypothesis" (45, 46), which predicts an evolutionary benefit for organisms producing a large chemical diversity of secondary metabolites at minimum cost. Accessory antimicrobial tests on bacteria and yeasts, together with previously published results, suggest a specific toxic effect of PUWs against eukaryotic cells. Thus, their toxic potential for humans and other animals clearly warrants further investigation, and their possible use as antifungal agents is ripe for exploration.

MATERIALS AND METHODS

Cultivation of cyanobacterial strains. Six cyanobacterial strains were included in the present study, *Cylindrospermum moravicum* CCALA 993 (strain 1), *Cylindrospermum alatosporum* CCALA 994 (strain 2), *Cylindrospermum alatosporum* CCALA 988 (strain 3), *Anabaena* sp. strain UHCC-0399 (previously *Anabaena* sp. strain SMIX 1; strain 4), *Anabaena minutissima* UTEX B 1613 (strain 5), and *Symplocastrum muelleri* NIVA-CYA 644 (strain 6). The origins of the strains are listed in Table 1. For chemical analysis, strains 1 to 5 were cultivated in BG-11 medium (47) in glass columns (300 ml) bubbled with air enriched in 1.5% CO_2 at a temperature of 28°C and constant illumination of 100 μmol photons m^{-2} s^{-1}. Strain 6 was maintained in culture using a custom liquid medium obtained by mixing 200 ml of Z8 medium (48), 800 ml distilled water, 30 ml soil extract, and common vitamin premix (according to SAG [Sammlung von Algenkulturen der Universität Göttingen], but without biotin). Cultivation was performed in 100- to 200-ml Erlenmeyer flasks at 20°C with a 16:8 light/dark photoperiod under static conditions. Cultures were kept at low irradiance (4 μmol m^{-2} s^{-1} of photosynthetically active radiation [PhAR], generated using red-green-blue light-emitting-diode [RGB LED] strips). Cells were harvested by centrifugation (3,125 × *g*), stored at −80°C, and subsequently lyophilized. Strain 4 was cultivated at a larger scale for purification of major lipopeptide variants in a 10-liter tubular photobioreactor under the above-mentioned conditions in BG-11 medium.

Molecular and bioinformatic analyses. Single filaments of strains 2, 3, 5, and 6 were isolated for whole-genome amplification (WGA) and subsequent preparation of a whole-genome sequencing (WGS) library as described previously (12). Briefly, the glass capillary technique was used to isolate filaments and exclude minor bacterial contaminants. A set of 20 filaments from each strain was then used as a template for WGA. Multiple displacement amplification (MDA) using a Repli-g minikit (Qiagen, Hilden, Germany) was followed by PCR and sequencing to monitor the cyanobacterial 16S rRNA gene using primers 16S387F and 16S1494R (49). Positive samples (7 to 10 MDA products yielding clear 16S rRNA gene

sequences of the respective genera) were then pooled to create a template for WGS. DNA samples were sent for commercial *de novo* genome sequencing (EMBL Genomics Core Facility, Heidelberg, Germany) using the Illumina MiSeq platform (Illumina, San Diego, CA, USA) with an ~350-bp average-insert-length paired-end library and 250-bp reads (~1.4-Gbp data yield per strain). Raw data from *de novo* WGS were assembled using CLC Bio Genomics Workbench version 7.5 (CLC Bio, Aarhus, Denmark). Genomic DNA was isolated from strain 4 as previously described (37), and the quality was assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and an Agilent TapeStation (Agilent Technologies, Santa Clara, CA, USA). High-molecular-weight DNA was used to construct an Illumina TruSeq PCR-free 350-bp library and sequenced using an Illumina HiSeq 2500 platform with a paired-end 100-cycle run. Genome data (1 Gb for each strain) were first checked using SPAdes version 3.7.1 (50) for read correction and removal of erroneous reads and then assembled using Newbler version 3.0 (454 Life Sciences, Branford, CT, USA). Genomic scaffolds were loaded into Geneious pro R10 (Biomatters) and investigated for FAAL and NRPS genes using BLASTp searches to identify putative lipopeptide synthetase gene clusters (23). FAAL and NRPS adenylation domains (A domains) from the single known PUW gene cluster (strain 1; KM078884) were used as queries since homologous gene clusters were expected. Contigs yielding high-similarity hits (E value of $<10^{-20}$) were then analyzed using the Glimmer 3 (51) algorithm to discover putative open-reading frames (ORFs). Functional annotation of ORFs was conducted by applying a combination of BLASTp/CDD searches against the NCBI database and using the antiSMASH 4.0 secondary metabolite gene cluster annotation pipeline (52, 53). Pairwise sequence identities and the presence of conserved residues in homologous putative proteins encoded in the gene clusters were assessed using Geneious pro software based on amino acid alignment (MAFFT plugin, default parameters). Minor assembly gaps were identified in the genomic scaffolds of all strains investigated, either directly after paired-end read assembly or based on mapping to the reference gene cluster from *C. alatosporum* CCALA 988. Gaps in PUW/MIN gene clusters were closed by PCR, and subsequent Sanger sequencing of PCR products was performed using custom primer annealing to regions adjacent to the assembly gaps.

Extraction and analysis of PUWs/MINs. To obtain comparable results, each strain was extracted using an identical ratio of lyophilized biomass (200 mg) to extraction solvent (10 ml of 70% methanol [MeOH], vol/vol). Extracts were evaporated using a rotary vacuum evaporator at 35°C and concentrated to 1 ml of 70% MeOH. The methanolic extracts were analyzed using a Thermo Scientific Dionex UltiMate 3000 ultra-high-performance liquid chromatography plus (UHPLC+) instrument equipped with a diode array detector connected to a Bruker Impact HD (Bruker, Billerica, MA, USA) high-resolution mass spectrometer with electrospray ionization. Separation of extracts was performed on a reversed-phase Phenomenex Kinetex C_{18} column (150 by 4.6 mm, 2.6 μm) using H_2O (A)-acetonitrile (B) containing 0.1% HCOOH as a mobile phase, at a flow rate of 0.6 ml min^{-1}. The gradient was as follows: A/B 85/15 (0 min), 85/15 (over 1 min), 0/100 (over 20 min), 0/100 (over 25 min), and 85/15 (over 30 min). For better resolution of minor PUW variants, another analytical method with a longer gradient (67 min) adopted from our previous study (13) was applied. The peptide sequence was reconstructed from the *b* ion series obtained after opening of the ring between the proline and *N*-methylasparagine residues, followed by the sequential loss of water and all the amino acids with exception of the last residue (Pro). The numbers of carbons in the FA moiety in PUW/MIN variants containing nonsubstituted and hydroxy-/chloro-substituted FA were determined using a method described previously by our team (13). Characteristic FA immonium fragments in oxo-substituted PUW/MIN variants were identified by applying this method to crude extracts of *Anabaena* strain 5 containing the oxo-substituted MIN D variant (10). Since a stable, prominent, and characteristic FA immonium fragment with the sum formula $C_{15}H_{30}NO^+$ was obtained for MIN D (Fig. S1), analogous fragments with the general formula $C_xH_{2x}NO^+$ were used to identify oxo-substituted components in unknown PUW/MIN variants from other strains investigated.

Molecular networking. A molecular network was created using the Global Natural Products Social Molecular Networking (GNPS) online workflow (54). Data were filtered by removing all MS-MS peaks within ±17 Da of the precursor *m/z*. MS-MS spectra were window filtered by choosing only the top six peaks in the ±50-Da window throughout the spectrum. Data were then clustered with MS-Cluster with a parent mass tolerance of 0.1 Da and an MS-MS fragment ion tolerance of 0.025 Da to create consensus spectra. Additionally, consensus spectra comprised of fewer than two spectra were discarded. A network was then created in which edges were filtered using a cosine score above 0.75 and more than three matched peaks. Additional edges between pairs of nodes were retained in the network only when both nodes were included in each other's respective top 10 most similar nodes. Spectra in the network were then searched against the GNPS spectral libraries, and library spectra were filtered in the same manner as the input data. All matches obtained between network spectra and library spectra were retained only when the score was above 0.7 and at least four peaks matched. Analog searching was performed against the library with a maximum mass shift of 200 Da.

Purification of MINs from *Anabaena* sp. strain 4 and its NMR analysis. Freeze-dried biomass of strain 4 (10 g) was extracted with 70% MeOH (500 ml). The extract was evaporated using a rotary vacuum evaporator to reduce the MeOH content, and the sample was subsequently diluted with distilled water to reach a final MeOH concentration of >5%. The diluted extract was prepurified using a Supelco C_{18} SPE cartridge (10 g, 60 ml) preequilibrated with 60 ml of MeOH and 120 ml of H_2O. After loading, retained components were eluted with 60 ml of pure MeOH, concentrated to dryness, and resuspended in 10 ml of pure MeOH. MIN A, C, and D were purified in two HPLC purification steps. The first step was performed on a preparative chromatographic system (Agilent 1260 Infinity series) equipped with a multiwavelength detector and automatic fraction collector. A preparative Reprosil 100 C_{18} column (252 by 25 mm) was employed for separation at a flow rate of 10 ml min^{-1} using the following gradient

of MeOH containing 0.1% HCOOH (A) and 10% MeOH containing 0.1% HCOOH (B): 0 min (100% B), 6 min (100% B), 15 min (43% B), 43 min (12% B), 45 min (0% B), 58 min (0% B), 60 min (100% B), and 64 min (100% B). Fractions were collected using an automatic fraction collector at 1-min intervals, and fractions were analyzed for MIN A, C, and D using the method described above. Fractions containing MIN A, C, and D were collected in separate vials and concentrated using a rotary evaporator.The second purification step was performed on a semipreparative HPLC (Agilent 1100 Infinity series) using a Reprosil 100 Phenyl column (250 by 8 mm) with acetonitrile containing 0.1% HCOOH (A) and water containing 0.1% HCOOH (B) using the following gradient: 0 min (60% B), 2 min (60% B), 6 min (50% B), 28 min (18% B), 30 min (0% B), 30 min (0% B), 32 min (0% B), 31 min (60% B), and 36 min (60% B). The flow rate was 1 ml min^{-1} throughout, fractions were collected manually, and the purity was analyzed using the HPLC-HRMS method described above. NMR spectra of minutissamides were measured in dimethyl sulfoxide (DMSO)-d_6 at 30°C. All NMR spectra were collected using a Bruker Avance III 500 MHz NMR spectrometer, equipped with a 5-mm-diameter broadband probe (BBI) head with actively shielded z gradient.

Antibacterial and antifungal assays. The antimicrobial activities of four major variants (PUW F and MIN A, C, and D) were tested against 13 bacterial and two yeast strains (Table 4) using disc diffusion assays (8) in three independent experiments with kanamycin/nystatin and MeOH as positive and negative controls, respectively. The antifungal activity of PUW F was further evaluated by determining the MICs against *Candida albicans* (HAMBI 261) and *Saccharomyces cerevisiae* (HAMBI 1164) as described previously (8). PUW F was isolated from *Cylindrospermum* strain 1 according to a protocol described previously (5), and isolation of MIN A, C, and D was performed as described above. The variants produced by *S. muelleri* strain 6 were impossible to isolate due to the slow growth of the cyanobacterium, which resulted in low biomass yields during the study period.

Accession number(s). The accession numbers for the newly sequenced complete putative biosynthetic gene clusters uploaded to the European Nucleotide Archive are MH325197 to MH325201.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02675-18.

SUPPLEMENTAL FILE 1, PDF file, 4.8 MB.

ACKNOWLEDGMENTS

This work was supported by Czech Science Foundation grant no. 16-09381S (Bioactive cyanobacterial lipopeptides: genome mining, detection, and structure-activity relationships), and by the Ministry of Education, Youth and Sports of the Czech Republic, National Program of Sustainability I, ID: LO1416 project ALGAMIC (ID: CZ.1.05/2.1.00/19.0392) and MSCA IF II project (CZ.02.2.69/0.0/0.0/18_070/0010493) as well as by Ministry of Regional Development of the Czech Republic—Cross-Border cooperation Czech-Bavaria project no. 41. Student participation (A. Kust and T. Galica) in the project was supported by The Grant Agency of the Faculty of Science, University of South Bohemia, grant GAJU 158/2016/P. Access to instruments and other facilities was supported by the Czech Research Infrastructure for Systems Biology (C4SYS; project no. LM2015055). This research was also supported by a grant from the NordForsk NCoE program NordAqua (project no. 82845) and by the Jane and Aatos Erkko Foundation (K. Sivonen and J. Jokela), which mostly supported the structural elucidation of PUW variants. The Norwegian participation was supported by grants from the Department of Agriculture and Forestry, the County Governor of Møre og Romsdal, the County Governor of Sogn og Fjordane, the University of Oslo, and the Norwegian Institute for Water Research.

The authors declare no conflict of interest.

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

REFERENCES

1. Cochrane SA, Vederas JC. 2016. Lipopeptides from *Bacillus* and *Paenibacillus* spp.: a gold mine of antibiotic candidates. Med Res Rev 36:4–31. https://doi.org/10.1002/med.21321.
2. Taylor SD, Palmer M. 2016. The action mechanism of daptomycin. Bioorg Med Chem 24:6253–6268. https://doi.org/10.1016/j.bmc.2016.05.052.
3. Velkov T, Roberts KD, Li J. 2017. Rediscovering the octapeptins. Nat Prod Rep 34:295–309. https://doi.org/10.1039/c6np00113k.
4. Ines M, Dhouha G. 2015. Lipopeptide surfactants: production, recovery and pore forming capacity. Peptides 71:100–112. https://doi.org/10.1016/j.peptides.2015.07.006.
5. Hrouzek P, Kuzma M, Černý J, Novák P, Fišer R, Šimek P, Lukešová A, Kopecký J. 2012. The cyanobacterial cyclic lipopeptides puwainaphycins F/G are inducing necrosis via cell membrane permeabilization and subsequent unusual actin relocalization. Chem Res Toxicol 25:1203–1211. https://doi.org/10.1021/tx300044t.
6. Oftedal L, Myhren L, Jokela J, Gausdal G, Sivonen K, Doskeland SO, Herfindal L. 2012. The lipopeptide toxins anabaenolysin A and B target biological membranes in a cholesterol–dependent manner. Biochim Biophys Acta Biomembr 1818:3000–3009. https://doi.org/10.1016/j.bbamem.2012.07.015.
7. Tomek P, Hrouzek P, Kuzma M, Sýkora J, Fišer R, Černý J, Novák P, Bártová S, Šimek P, Hof M, Kavan D, Kopecký J. 2015. Cytotoxic

lipopeptide muscotoxin A, isolated from soil cyanobacterium *Desmonostoc muscorum*, permeabilizes phospholipid membranes by reducing their fluidity. Chem Res Toxicol 28:216–224. https://doi.org/10.1021/tx500382b.
8. Vestola J, Shishido TK, Jokela J, Fewer DP, Aitio O, Permi P, Wahlsten M, Wang H, Rouhiainen L, Sivonen K. 2014. Hassallidins, antifungal glycolipopeptides, are widespread among cyanobacteria and are the end-product of a nonribosomal pathway. Proc Natl Acad Sci U S A 111:E1909–E1917. https://doi.org/10.1073/pnas.1320913111.
9. Gregson JM, Chen JL, Patterson GML, Moore RE. 1992. Structures of puwainaphycins A-E. Tetrahedron 48:3727–3734. https://doi.org/10.1016/S0040-4020(01)92264-1.
10. Kang HS, Krunic A, Shen Q, Swanson SM, Orjala J. 2011. Minutissamides A-D, antiproliferative cyclic decapeptides from the cultured cyanobacterium *Anabaena minutissima*. J Nat Prod 74:1597–1605. https://doi.org/10.1021/np2002226.
11. Kang HS, Sturdy M, Krunic A, Kim H, Shen Q, Swanson SM, Orjala J. 2012. Minutissamides E-L, antiproliferative cyclic lipodecapeptides from the cultured freshwater cyanobacterium cf. *Anabaena* sp. Bioorg Med Chem 20:6134–6143. https://doi.org/10.1016/j.bmc.2012.08.017.
12. Mareš J, Hájek J, Urajová P, Kopecký J, Hrouzek P. 2014. A hybrid non-ribosomal peptide/polyketide synthetase containing fatty-acyl ligase (FAAL) synthesizes the beta-amino fatty acid lipopeptides puwainaphycins in the cyanobacterium *Cylindrospermum alatosporum*. PLoS One 9:e111904. https://doi.org/10.1371/journal.pone.0111904.
13. Urajová P, Hájek J, Wahlsten M, Jokela J, Galica T, Fewer DP, Kust A, Zapomělová-Kozlíková E, Delawská K, Sivonen K, Kopecký J, Hrouzek P. 2016. A liquid chromatography-mass spectrometric method for the detection of cyclic beta-amino fatty acid lipopeptides. J Chromatogr A 1438:76–83. https://doi.org/10.1016/j.chroma.2016.02.013.
14. Cheel J, Urajová P, Hajek J, Hrouzek P, Kuzma M, Bouju E, Faure K, Kopecký J. 2017. Separation of cyclic lipopeptide puwainaphycins from cyanobacteria by countercurrent chromatography combined with polymeric resins and HPLC. Anal Bioanal Chem 409:917–930. https://doi.org/10.1007/s00216-016-0066-z.
15. Moore RE, Bornemann V, Niemczura WP, Gregson JM, Chen JL, Norton TR, Patterson GML, Helms GL. 1989. Puwainaphycin C, a cardioactive cyclic peptide from the blue-green alga *Anabaena* BQ-16-1. Use of two dimensional carbon-13-carbon-13 and carbon-13-nitrogen-15 correlation spectroscopy in sequencing the amino acid units. J Am Chem Soc 111:6128–6132. https://doi.org/10.1021/ja00198a021.
16. Duitman EH, Hamoen LW, Rembold M, Venema G, Seitz H, Saenger W, Bernhard F, Reinhardt R, Schmidt M, Ullrich C, Stein T, Leenders F, Vater J. 1999. The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: a multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase. Proc Natl Acad Sci U S A 96:13294–13299. https://doi.org/10.1073/pnas.96.23.13294.
17. Tsuge K, Akiyama T, Shoda M. 2001. Cloning, sequencing, and characterization of the iturin A operon. J Bacteriol 183:6265–6273. https://doi.org/10.1128/JB.183.21.6265-6273.2001.
18. Koumoutsi A, Chen XH, Henne A, Liesegang H, Hitzeroth G, Franke P, Vater J, Borriss R. 2004. Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. J Bacteriol 186:1084–1096. https://doi.org/10.1128/JB.186.4.1084-1096.2004.
19. Sood S, Steinmetz H, Beims H, Mohr KI, Stadler M, Djukic M, von der Ohe W, Steinert M, Daniel R, Müller R. 2014. Paenilarvins: iturin family lipopeptides from the honey bee pathogen *Paenibacillus larvae*. Chembiochem 15:1947–1955. https://doi.org/10.1002/cbic.201402139.
20. Ramaswamy AV, Sorrels CM, Gerwick WH. 2007. Cloning and biochemical characterization of the hectochlorin biosynthetic gene cluster from the marine cyanobacterium *Lyngbya majuscula*. J Nat Prod 70:1977–1986. https://doi.org/10.1021/np0704250.
21. Micallef ML, D'Agostino PM, Sharma D, Viswanathan R, Moffitt MC. 2015. Genome mining for natural product biosynthetic gene clusters in the subsection V cyanobacteria. BMC Genomics 16:669. https://doi.org/10.1186/s12864-015-1855-z.
22. Edwards DJ, Marquez BL, Nogle LM, McPhail K, Goeger DE, Roberts MA, Gerwick WH. 2004. Structure and biosynthesis of the jamaicamides, new mixed polyketide-peptide neurotoxins from the marine cyanobacterium *Lyngbya majuscula*. Chem Biol 11:817–833. https://doi.org/10.1016/j.chembiol.2004.03.030.
23. Galica T, Hrouzek P, Mareš J. 2017. Genome mining reveals high incidence of putative lipopeptide biosynthesis NRPS/PKS clusters containing fatty acyl-AMP ligase genes in biofilm-forming cyanobacteria. J Phycol 53:985–998. https://doi.org/10.1111/jpy.12555.
24. Arora P, Goyal A, Natarajan VT, Rajakumara E, Verma P, Gupta R, Yousuf M, Trivedi OA, Mohanty D, Tyagi A, Sankaranarayanan R, Gokhale RS. 2009. Mechanistic and functional insights into fatty acid activation in *Mycobacterium tuberculosis*. Nat Chem Biol 5:166–173. https://doi.org/10.1038/nchembio.143.
25. Liu Z, Ioerger TR, Wang F, Sacchettini JC. 2013. Structures of *Mycobacterium tuberculosis* FadD10 protein reveal a new type of adenylate-forming enzyme. J Biol Chem 288:18473–18483. https://doi.org/10.1074/jbc.M113.466912.
26. Coates RC, Podell S, Korobeynikov A, Lapidus A, Pevzner P, Sherman DH, Allen EE, Gerwick L, Gerwick WH. 2014. Characterization of cyanobacterial hydrocarbon composition and distribution of biosynthetic pathways. PLoS One 9:e85140. https://doi.org/10.1371/journal.pone.0085140.
27. Mohanty D, Sankaranarayanan R, Gokhale RS. 2011. Fatty acyl-AMP ligases and polyketide synthases are unique enzymes of lipid biosynthetic machinery in *Mycobacterium tuberculosis*. Tuberculosis (Edinb) 91:448–455. https://doi.org/10.1016/j.tube.2011.04.006.
28. Goyal A, Verma P, Anandhakrishnan M, Gokhale RS, Sankaranarayanan R. 2012. Molecular basis of the functional divergence of fatty acyl-AMP ligase biosynthetic enzymes of *Mycobacterium tuberculosis*. J Mol Biol 416:221–238. https://doi.org/10.1016/j.jmb.2011.12.031.
29. Villiers BRM, Hollfelder F. 2009. Mapping the limits of substrate specificity of the adenylation domain of TycA. Chembiochem 10:671–682. https://doi.org/10.1002/cbic.200800553.
30. Christiansen G, Philmus B, Hemscheidt T, Kurmayer R. 2011. Genetic variation of adenylation domains of the anabaenopeptin synthesis operon and evolution of substrate promiscuity. J Bacteriol 193:3822–3831. https://doi.org/10.1128/JB.00360-11.
31. Brandenburger E, Braga D, Kombrink A, Lackner G, Gressler J, Künzler M, Hoffmeister D. 2018. Multi-genome analysis identifies functional and phylogenetic diversity of basidiomycete adenylate-forming reductases. Fungal Genet Biol 112:55–63. https://doi.org/10.1016/j.fgb.2016.07.008.
32. Rouhiainen L, Jokela J, Fewer DP, Urmann M, Sivonen K. 2010. Two alternative starter modules for the non-ribosomal biosynthesis of specific anabaenopeptin variants in *Anabaena* (Cyanobacteria). Chem Biol 17:265–273. https://doi.org/10.1016/j.chembiol.2010.01.017.
33. Kleigrewe K, Almaliti J, Tian IY, Kinnel RB, Korobeynikov A, Monroe EA, Duggan BM, Di Marzo V, Sherman DH, Dorrestein PC, Gerwick L, Gerwick WH. 2015. Combining mass spectrometric metabolic profiling with genomic analysis: a powerful approach for discovering natural products from cyanobacteria. J Nat Prod 78:1671–1682. https://doi.org/10.1021/acs.jnatprod.5b00301.
34. Voráčová K, Hájek J, Mareš J, Urajová P, Kuzma M, Cheel J, Villunger A, Kapuscik A, Bally M, Novák P, Kabeláč M, Krumschnabel G, Lukeš M, Voloshko L, Kopecký J, Hrouzek P. 2017. The cyanobacterial metabolite nocuolin A is a natural oxadiazine that triggers apoptosis in human cancer cells. PLoS One 12:e0172850. https://doi.org/10.1371/journal.pone.0172850.
35. He J, Hertweck C. 2004. Biosynthetic origin of the rare nitroaryl moiety of the polyketide antibiotic aureothin: involvement of an unprecedented N-oxygenase. J Am Chem Soc 126:3694–3695. https://doi.org/10.1021/ja039328t.
36. Choi YS, Zhang HJ, Brunzelle JS, Nair SK, Zhao HM. 2008. In vitro reconstitution and crystal structure of p-aminobenzoate N-oxygenase (AurF) involved in aureothin biosynthesis. Proc Natl Acad Sci U S A 105:6858–6863. https://doi.org/10.1073/pnas.0712073105.
37. Fewer DP, Wahlsten M, Osterholm J, Jokela J, Rouhiainen L, Kaasalainen U, Rikkinen J, Sivonen K. 2013. The genetic basis for O-acetylation of the microcystin toxin in cyanobacteria. Chem Biol 20:861–869. https://doi.org/10.1016/j.chembiol.2013.04.020.
38. Murray IA, Shaw WV. 1997. O-acetyltransferases for chloramphenicol and other natural products. Antimicrob Agents Chemother 41:1–6.
39. Zapomělová E, Jezberová J, Hrouzek P, Hisem D, Řeháková K, Komárková J. 2009. Polyphasic characterization of three strains of *Anabaena reniformis* and *Aphanizomenon aphanizomenoides* (cyanobacteria) and their reclassification to *Sphaerospermum* gen. nov. (incl. *Anabaena kisseleviana*). J Phycol 45:1363–1373. https://doi.org/10.1111/j.1529-8817.2009.00758.x.
40. Skulberg OM, Mysterud I, Karlsen J, Tønnesen HH, Laane CMM, Schumacher T. 2012. Alveld research per annum 2012: searchlight on cyanobacteria we have minor knowledge of. Biolog 30:32–41. (In Norwegian.)
41. Tønnesen HH, Mysterud I, Karlsen J, Skulberg OM, Laane CMM, Schum-

acher T. 2013. Identification of singlet oxygen photosensitizers in lambs drinking water in an alveld risk area in West Norway. J Photochem Photobiol B 119:37–45. https://doi.org/10.1016/j.jphotobiol.2012.12.003.
42. Hegge AB, Mysterud I, Karlsen J, Skulberg OM, Laane CMM, Schumacher T, Tønnesen HH. 2013. Impaired secondary oxidant deactivation capacity and enhanced oxidative stress in serum from alveld affected lambs. J Photochem Photobiol B 126:126–134. https://doi.org/10.1016/j.jphotobiol.2013.07.005.
43. Heinze R. 1996. A biotest for hepatotoxins using primary rat hepatocytes. Phycologia 35:89–93. https://doi.org/10.2216/i0031-8884-35-6S-89.1.
44. Skulberg OM. 1996. Toxins produced by cyanophytes in Norwegian inland waters—health and environment, p 197–216. In Låg J (ed), Chemical data as a basis of geomedical investigations. The Norwegian Academy of Science and Letters, Oslo, Norway.
45. Jones CG, Firn RD. 1991. On the evolution of plant secondary chemical diversity. Philos Trans R Soc Lond B 333:273–280. https://doi.org/10.1098/rstb.1991.0077.
46. Firn RD, Jones CG. 2000. The evolution of secondary metabolism: a unifying model. Mol Microbiol 37:989–994.
47. Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J Gen Microbiol 111:1–61. https://doi.org/10.1099/00221287-111-1-1.
48. Skulberg R, Skulberg OM. 1990. Research with algal cultures—NIVA's Culture Collection of Algae. NIVA report. Norwegian Institute for Water Research, Oslo, Norway.
49. Taton A, Grubisic S, Brambilla E, De Wit R, Wilmotte A. 2003. Cyanobacterial diversity in natural and artificial microbial mats of Lake Fryxell (McMurdo dry valleys, Antarctica): a morphological and molecular approach. Appl Environ Microbiol 69:5157–5169. https://doi.org/10.1128/AEM.69.9.5157-5169.2003.
50. Bankevich A, Nurk S, Antipov D, Gurevich A, Dvorkin M, Kulikov AS, Lesin V, Nikolenko S, Pham S, Prjibelski A, Pyshkin A, Sirotkin A, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–497. https://doi.org/10.1089/cmb.2012.0021.
51. Delcher AL, Bratke KA, Powers EC, Salzberg SL. 2007. Identifying bacterial genes and endosymbiont DNA with Glimmer. Bioinformatics 23:673–679. https://doi.org/10.1093/bioinformatics/btm009.
52. Weber T, Blin K, Duddela S, Krug D, Kim HU, Bruccoleri R, Lee SY, Fischbach MA, Muller R, Wohlleben W, Breitling R, Takano E, Medema MH. 2015. antiSMASH 3.0—a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic Acids Res 43:W237–W243. https://doi.org/10.1093/nar/gkv437.
53. Blin K, Wolf T, Chevrette MG, Lu XW, Schwalen CJ, Kautsar SA, Duran HGS, Santos E, Kim HU, Nave M, Dickschat JS, Mitchell DA, Shelest E, Breitling R, Takano E, Lee SY, Weber T, Medema MH. 2017. antiSMASH 4.0—improvements in chemistry prediction and gene cluster boundary identification. Nucleic Acids Res 45:W36–W41. https://doi.org/10.1093/nar/gkx319.
54. Wang M, Carver JJ, Phelan VV, Sanchez LM, Garg N, Peng Y, Nguyen DD, Watrous J, Kapono CA, Luzzatto-Knaan T, Porto C, Bouslimani A, Melnik AV, Meehan MJ, Liu W-T, Crüsemann M, Boudreau PD, Esquenazi E, Sandoval-Calderón M, Kersten RD, Pace LA, Quinn RA, Duncan KR, Hsu C-C, Floros DJ, Gavilan RG, Kleigrewe K, Northen T, Dutton RJ, Parrot D, Carlson EE, Aigle B, Michelsen CF, Jelsbak L, Sohlenkamp C, Pevzner P, Edlund A, McLean J, Piel J, Murphy BT, Gerwick L, Liaw C-C, Yang Y-L, Humpf H-U, Maansson M, Keyzers RA, Sims AC, et al. 2016. Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. Nat Biotechnol 34:828–837. https://doi.org/10.1038/nbt.3597.
55. Johansen JR, Bohunická M, Lukešová A, Hrčková K, Vaccarino MA, Chesarino NM. 2014. Morphological and molecular characterization within 26 strains of the genus *Cylindrospermum* (Nostocaceae, Cyanobacteria) with descriptions of three new species. J Phycol 50:187–202. https://doi.org/10.1111/jpy.12150.
56. Tamrakar A. 2016. Isolation of benthic cyanobacteria and screening of bioactivities and natural products from culture collection strains. Master's Thesis. University of Helsinki, Helsinki, Finland.
57. Kantz T, Bold HC. 1969. Phycological studies IX. Morphological and taxonomic investigations of *Nostoc* and *Anabaena* in culture. Publication no. 6924. University of Texas, Austin, TX.

Paper IV: Semi-synthetic puwainaphycin/minutissamide cyclic lipopeptides with improved antifungal activity and limited cytotoxicity

Issue 49, 2021, Issue in Progress

Previous Article | Next Article



From the journal:
RSC Advances

Semi-synthetic puwainaphycin/minutissamide cyclic lipopeptides with improved antifungal activity and limited cytotoxicity†

Jan Hájek,‡[ab] Sebastian Bieringer,‡[cf] Kateřina Voráčová,[a] Markéta Macho,[ab] Kumar Saurav,[a] Kateřina Delawská,[ab] Petra Divoká,[a] Radovan Fišer,[d] Gabriela Mikušová,[d] José Cheel,[a] David P. Fewer,[e] Dai Long Vu,[a] Jindřiška Paichlová,[a] Herbert Riepl*[cf] and Pavel Hrouzek*[a]

RSC Advances

PAPER

View Article Online
View Journal | View Issue

Check for updates

Cite this: *RSC Adv.*, 2021, **11**, 30873

Semi-synthetic puwainaphycin/minutissamide cyclic lipopeptides with improved antifungal activity and limited cytotoxicity†

Jan Hájek,‡[ab] Sebastian Bieringer,‡[cf] Kateřina Voráčová,[a] Markéta Macho,[ab] Kumar Saurav,[a] Kateřina Delawská,[ab] Petra Divoká,[a] Radovan Fišer,[d] Gabriela Mikušová,[d] José Cheel,[a] David P. Fewer,[e] Dai Long Vu,[a] Jindřiška Paichlová,[a] Herbert Riepl*[cf] and Pavel Hrouzek*[a]

Microbial cyclic lipopeptides are an important class of antifungal compounds with applications in pharmacology and biotechnology. However, the cytotoxicity of many cyclic lipopeptides limits their potential as antifungal drugs. Here we present a structure–activity relationship study on the puwainaphycin/minutissamide (PUW/MIN) family of cyclic lipopeptides isolated from cyanobacteria. PUWs/MINs with variable fatty acid chain lengths differed in the dynamic of their cytotoxic effect despite their similar IC_{50} after 48 hours (2.8 μM for MIN A and 3.2 μM for PUW F). Furthermore, they exhibited different antifungal potency with the lowest MIC values obtained for MIN A and PUW F against the facultative human pathogen *Aspergillus fumigatus* (37 μM) and the plant pathogen *Alternaria alternata* (0.6 μM), respectively. We used a Grignard-reaction with alkylmagnesium halides to lengthen the lipopeptide FA moiety as well as the Steglich esterification on the free hydroxyl substituents to prepare semi-synthetic lipopeptide variants possessing multiple fatty acid tails. Cyclic lipopeptides with extended and branched FA tails showed improved strain-specific antifungal activity against *A. fumigatus* (MIC = 0.5–3.8 μM) and *A. alternata* (MIC = 0.1–0.5 μM), but with partial retention of the cytotoxic effect (~10–20 μM). However, lipopeptides with esterified free hydroxyl groups possessed substantially higher antifungal potencies, especially against *A. alternata* (MIC = 0.2–0.6 μM), and greatly reduced or abolished cytotoxic activity (>20 μM). Our findings pave the way for a generation of semi-synthetic variants of lipopeptides with improved and selective antifungal activities.

Received 23rd June 2021
Accepted 2nd September 2021

DOI: 10.1039/d1ra04882a

rsc.li/rsc-advances

Open Access Article. Published on 16 September 2021. Downloaded on 9/19/2021 12:02:23 PM.
This article is licensed under a Creative Commons Attribution-NonCommercial 3.0 Unported Licence.

1. Introduction

Cyclic lipopeptides produced by a variety of bacteria and fungi have attracted attention as drug leads due to their potent antifungal, antibacterial, and cytotoxic properties.[1–3] Their structure is composed of a peptide cycle linked with a fatty acid (FA) moiety that may be part of the peptide backbone or attached as an exocyclic moiety.[1] The composition of the peptide cycle and the structure of the FA moiety affect the propensity of cyclic lipopeptides to interact with biological membranes and different types of cell walls.[4] A number of cyclic lipopeptides contain positively charged amino acid residues, which promote interaction with negatively charged bacterial cell walls and membranes.[1,5] Antibacterial cyclic lipopeptides include polymyxins and daptomycin, which are approved for topical application or parenteral administration as treatment for various bacterial infections.[1] Cyclic lipopeptides containing neutral amino acid residues exhibit a range of bioactivities, including cytotoxicity against human cells as well as antifungal bioactivity.[1,6] The majority of known antifungal cyclic lipopeptides are produced by bacteria (*e.g.* iturins, surfactin, and fengycin) and form organized aggregates in biological membranes, which cause early membrane damage through ion leakage that subsequently leads to membrane disintegration.[6–8] However, in some cases the antifungal effect is achieved through enzyme inhibition rather than membrane damage, *e.g.* caspofungin exerts antifungal activity through selective inhibition of β-D-glucan synthase.[9]

[a]*Centre Algatech, Institute of Microbiology of the Czech Academy of Sciences, Opatovický mlýn, Novohradská 237, 379 81 Třeboň, Czech Republic. E-mail: hrouzek@alga.cz*

[b]*Faculty of Science, University of South Bohemia in České Budějovice, Branišovská 1760, 37005, České Budějovice, Czech Republic*

[c]*TUM Campus Straubing for Biotechnology and Sustainability, Technical University Munich, 94315 Straubing, Germany. E-mail: herbert.riepl@hswt.de*

[d]*Faculty of Science, Charles University, Viničná 5, 128 44 Prague 2, Czech Republic*

[e]*Department of Microbiology, University of Helsinki, Biocenter 1, Viikinkaari 9, FIN-00014 Helsinki, Finland*

[f]*Weihenstephan-Triesdorf University of Applied Sciences, Organic-analytical Chemistry, 94315 Straubing, Germany*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/d1ra04882a

‡ These authors contributed equally.

© 2021 The Author(s). Published by the Royal Society of Chemistry

Open Access Article. Published on 16 September 2021. Downloaded on 9/19/2021 12:02:23 PM.
This article is licensed under a Creative Commons Attribution-NonCommercial 3.0 Unported Licence.

Structure–activity relationship analysis (SAR) has been used extensively to study members of the iturin family,[6] which manifest significant antifungal activity (MIC values of 1.25–5 μM) against a broad range of fungi.[6,10–12] Their potency has been found to be substantially affected by both the amino-acid composition of the cyclic peptide as well as variation in the FA moiety length.[6] Members of the iturin family have a seven-membered ring containing an invariable Tyr residue, which is essential for compound folding[6] with *O*-methylation of Tyr reducing the potency of iturin.[13] The absence of a FA tail entirely abolishes the antifungal effect as demonstrated in bioassays using synthetic variants lacking FA moiety.[6] Natural variants bearing longer FA tails manifest stronger antifungal and haemolytic activity compared to those with shorter FA tails.[6] However, the effect of FA residue length on the bioactivity of cyclic lipopeptides has received only limited attention,[14] despite the large variation in FA tail length reported for the majority of antifungal cyclic lipopeptides.[15,16]

The structural diversity of cyclic lipopeptides found in cyanobacteria is similar to that found in other bacterial lineages.[17–23] Several classes of cyclic cyanobacterial lipopeptides are reported to possess potent antifungal potency,[24] namely hassallidins,[25] laxaphycins,[26] muscotoxins,[19,27] anabaenolysins,[28] puwainaphycins,[20,29] and minutissamides.[16,18] However, these lipopeptides also display prominent cytotoxic activity and/or haemolytic activity *in vitro*, which strongly contradicts use in human or veterinary medicine.

The puwainaphycin/minutissamide (PUWs/MINs) family of cyclic lipopeptides with incorporated β-amino-α-hydroxy-γ-methyl FA moiety exhibits structural similarities to members of the iturin family (Fig. 1).[17,18,20,29] A hybrid biosynthetic pathway combining enzymes of non-ribosomal peptide synthetases (NRPS) and polyketide synthase (PKS) is responsible for their biosynthesis. This pathway employs alternative starter modules containing fatty-acyl AMP-ligases capable of incorporating FAs with a variable chain length and accessory enzymes that can further modify the FA residue.[16,30] This biosynthetic machinery generates the wide structural variability of PUWs/MINs that includes variants bearing FA moiety consisting of C10-18 (ref. 15) that may be further functionalized with oxo-, hydroxy-, and chloro-substituents.[16] PUWs/MINs exhibit moderate cytotoxic effects in human cells connected to membrane damage resulting in necrotic cell death.[20] In addition, PUWs/MINs exhibit antifungal potency against *Saccharomyces cerevisiae* and *Candida albicans*.[16] Previous findings have suggested that the length of the FA residue may affect antifungal activity.[16] Despite structural variability, it is notable that the FA residue of PUW/MIN chemical variants contain both a conserved β-amino-α-hydroxy-γ-methyl motif and amino acids adjacent to the FA residue, suggesting that these residues are important for the resulting bioactivity.

Cyanobacterial cyclic lipopeptides have not previously been the subject of SAR studies or chemical improvement of their bioactivity properties. In the present study, we have performed a SAR study on the PUW/MIN group of cyclic lipopeptides in order to uncover the effect of FA moiety length on both antifungal and cytotoxic activity.

2. Experimental section

Cultivation of cyanobacterial biomass and extraction

The cyanobacterial strains *Cylindrospermum alatosporum* CCALA 988 and *Anabaena* sp. UHCC-0399 were cultivated in multiple 350 mL glass tubes in liquid BG-11 and bubbled with 1.5% CO_2-enriched air at a constant temperature of 28 °C under 50 W m^{-2} continuous illumination. After reaching the late

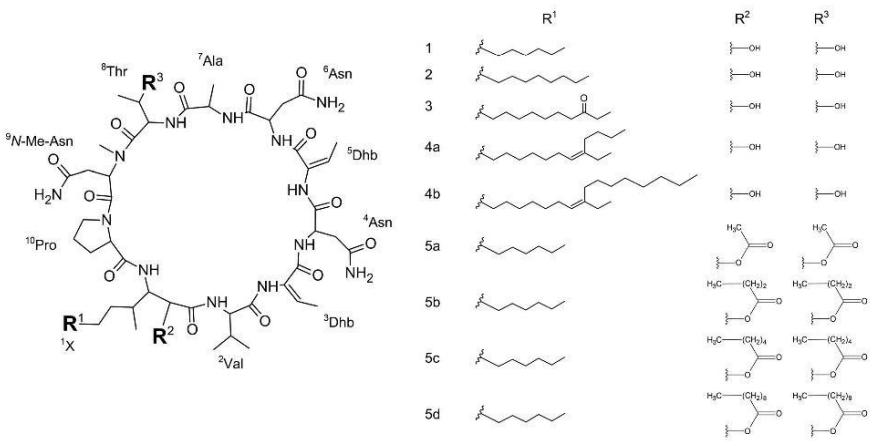


Fig. 1 Naturally occurring and semi-synthetic PUW/MIN variants. Compounds **1** (MIN A), **2** (PUW F), and **3** (MIN C) were isolated from cyanobacteria. Compounds **4a–5d** are semi-synthetic analogs prepared within this study.

© 2021 The Author(s). Published by the Royal Society of Chemistry

Open Access Article. Published on 16 September 2021. Downloaded on 9/19/2021 12:02:23 PM.
This article is licensed under a Creative Commons Attribution-NonCommercial 3.0 Unported Licence.

exponential phase the culture was transferred consecutively to 15 L and 100 L photobioreactors set to control identical temperature and illumination conditions. The culture was harvested by centrifugation after 10–14 days of cultivation and freeze-dried (Leybold-Heraeus freeze-drier Lyovac GT 3). 10 g of lyophilized biomass was extracted using MeOH/H_2O (70/30, v/v) for 1 hour and centrifuged at 4500 rpm. The supernatant was collected and evaporated using a rotary evaporator until dryness while temperature did not exceed 40 °C. The residue was dissolved in 5 mL of acidified MeOH.

HPLC purification of PUW F, MIN A and MIN C

PUW F and MIN A were isolated from *Cylindrospermum alatosporum* CCLA 988 using previously optimized protocols.[31] MIN C was isolated from biomass of *Anabaena* sp. UHCC-0399. Methanol extract solution (acidified by 0.1% v/v HCOOH) was purified on an HPLC Agilent 1260 infinity series chromatograph equipped with preparative pumps, a multi-wavelength detector, and an automatic fraction collector. A preparative Reprosil RP-C18 column (5 μm; 25 × 250 mm) with MeOH + 0.1% HCOOH (A)/10% MeOH aq. + 0.1% HCOOH (B) gradient at a flow rate of 10 mL min^{-1} was eluted using the following gradient: 0 min – 0% of A; 6 min – 0% of A; 15 min – 57% of A; 43 min – 76% of A; 45 min – 100% of A; 52 min – 100% of A; 60 min – 0% of (A). Fractions were collected using an automatic fraction collector at 1 min intervals and were checked for compound content using HPLC-HRMS/MS analysis. Fractions containing the desired PUW/MIN variants were evaporated on a rotary vacuum evaporator and resuspended in 1 mL of MeOH. The second purification step was performed using an Agilent 1100 Series modular HPLC system (Agilent Technologies 1100 Series) equipped with a diode array detector on semi-preparative Agilent Eclipse XDB-C18 column (5 μm; 9.4 × 250 mm) and ACN + 0.1% HCOOH (A)/H_2O + 0.1% HCOOH (B) gradient at a flow-rate of 2 mL min^{-1} (0 min – 30% of A; 2 min – 30% of A; 6 min – 42% of A; 15 min – 55% of A; 16 min – 100% of A; 20 min 100% of A; 21 min 30% of A). Fractions containing MIN C (collected at retention time range 8–12 min.) were collected manually using a UV detection set to 235 nm, evaporated on rotary evaporator and resuspended again in 1 mL of MeOH.

Chemical synthesis

General considerations. If not otherwise stated, reactions were performed in an argon atmosphere with dry solvents. All reagents and solvents were purchased from VWR, Sigma-Aldrich, Alfa Aesar, Acros Organics, and Abcr. The obtained products were purified by reversed phase HPLC on a Luna 250 × 15 mm Column (Phenomenex) with a Puriflash 4250–250 from Interchim. NMR-spectra were recorded on a JEOL ECS-400 (400 MHz) Nuclear Magnetic Resonance (NMR) spectrometer. Chemical shifts were reported in ppm and are referenced to residual protons in the deuterated solvent as the internal standard.

General procedure for the extension of the FA chain in MIN C

MIN C (50 mg, 0.042 mmol, 1 eq.) was dissolved in a flame-dried round bottom flask in dry THF (20 mL) under argon atmosphere. Two mL of a Grignard solution was added dropwise *via* syringe through a septum cap at 0 °C under stirring. The reaction was mixed by stirring at 0 °C for a further 5 min and then allowed to warm up to room temperature. The reaction was quenched after 2 h with 15 mL of aq. NH_4Cl. THF was removed *in vacuo* at 32 °C. The resulting mixture was loaded on a SPE-column packed with 40 g C18 silica and eluted with 30 mL of H_2O, 2 : 8 ACN/H_2O and 9 : 1 ACN/H_2O. Products were found in the 9 : 1 H_2O/ACN fraction. The products were used in the following steps with no further purification after removal of the volatiles under reduced pressure.

The material was dissolved in dry $CHCl_3$ (20 mL) in a flame-dried round-bottom flask under N_2 atmosphere. Subsequently, *p*-TsOH (45 mg, 0.29 mmol) was added under a constant flow of nitrogen. The reaction was mixed by stirring for 2 h at 35 °C. The reaction was quenched with H_2O (15 mL) and chloroform was removed under reduced pressure at 32 °C. The resulting mixture was loaded on a SPE-column packed with 40 g C18 silica and eluted with 30 mL of H_2O, 2 : 8 ACN/H_2O, and 9 : 1 ACN/H_2O. Products were found in the 9 : 1 ACN/H_2O fraction. The products were purified further by reversed phase HPLC (40 : 60 to 95 : 5 MeOH/H_2O, Luna 250 × 15 mm, C18 silica) following removal of the volatiles under reduced pressure.

Compound 4a. MIN C was converted with butylmagnesium chloride (1.5 M in THF/Toluene, 36 eq.) to give the title compound as white solid. Yield: 1.2 mg (0.001 mmol, 2%). Purity: 93.17% (UV/VIS base peak purity, 205–800 nm, $\int_{4a} = 341$, $\int_{contaminants} = 25$). HRMS: $[M + 2H^+]^{2+}$ 614.8684, calcd 614.8692 for $[C_{59}H_{97}N_{13}O_{15} + 2H^+]^{2+}$. ^1H NMR (400 MHz, CD_3OD) δ 7.30 (d, $J = 9.2$ Hz, 1H), 6.86 (d, $J = 8.7$ Hz, 1H), 5.91 (d, $J = 7.6$ Hz, 1H), 5.52 (d, $J = 7.6$ Hz, 1H), 5.36 (s, 2H), 4.59 (s, 1H), 4.46 (d, $J = 15.3$ Hz, 1H), 4.30 (d, $J = 6.0$ Hz, 1H), 4.14 (s, 2H), 3.84 (s, 2H), 3.67–3.61 (m, 9H), 3.50–3.48 (m, 1H), 3.14 (dt, $J = 3.2$, 1.8 Hz, 1H), 3.06 (s, 1H), 2.17 (t, $J = 7.6$ Hz, 4H), 1.99 (d, $J = 7.8$ Hz, 3H), 1.79 (d, $J = 7.8$ Hz, 2H), 1.60 (s, 5H), 1.44–1.28 (m, 50H), 1.13 (d, $J = 6.4$ Hz, 2H), 1.03 (d, $J = 6.9$ Hz, 2H), 0.95–0.88 (m, 9H), 0.76 (d, $J = 6.9$ Hz, 1H), 0.72 (s, 3H).

Compound 4b. MIN C was converted with octylmagnesium bromide (2 M in THF, 48 eq.) to give the title compound as white solid. Yield: 0.7 mg (0.5 μmol, 1%). Purity: 94.95% (UV/VIS purity, 205–800 nm, $\int_{4b} = 46\,944$, $\int_{cont.} = 2499$). HRMS: $[M + 2H^+]^{2+}$ 642.8986, calcd 642.9005 for $[C_{63}H_{105}N_{13}O_{15} + 2H^+]^{2+}$. ^1H NMR (400 MHz, CD_3OD) δ 8.64 (s, 1H), 7.72 (d, $J = 8.9$ Hz, 1H), 7.24 (d, $J = 10.5$ Hz, 1H), 5.89 (d, $J = 7.3$ Hz, 1H), 5.70 (d, $J = 8.9$ Hz, 1H), 5.51 (d, $J = 7.4$ Hz, 1H), 4.55 (d, $J = 16.8$ Hz, 2H), 4.45–4.41 (m, 2H), 4.28 (d, $J = 6.5$ Hz, 1H), 4.22 (s, 1H), 4.09 (s, 2H), 3.04 (s, 3H), 2.76–2.59 (m, 3H), 2.16 (s, 1H), 2.13 (s, 1H), 1.99 (dd, $J = 15.2$, 7.4 Hz, 9H), 1.77 (d, $J = 7.5$ Hz, 3H), 1.56 (d, $J = 6.8$ Hz, 3H), 1.37 (d, $J = 55.1$ Hz, 42H), 1.11 (d, $J = 6.4$ Hz, 3H), 1.01 (d, $J = 6.8$ Hz, 3H), 0.96–0.87 (m, 10H), 0.74 (d, $J = 6.6$ Hz, 3H).

General procedure for the esterification of free hydroxyl groups in MIN A

MIN A (20 mg, 0.018 mmol, 1 eq.) was dissolved in 15 mL dry ACN in a flame-dried round-bottomed flask under argon

© 2021 The Author(s). Published by the Royal Society of Chemistry

Open Access Article. Published on 16 September 2021. Downloaded on 9/19/2021 12:02:23 PM.
This article is licensed under a Creative Commons Attribution-NonCommercial 3.0 Unported Licence.

atmosphere. DMAP (240 mg, 2.13 mmol, 109 eq.) was added under a constant flow of argon. The respective carboxylic anhydride (0.54 mmol, 30 eq.) was added dropwise *via* syringe through a septum cap. The reaction was mixed by stirring for 20 h at room temperature and then quenched with H_2O. The mixture was loaded on a SPE-column packed with 40 g C18 silica after removal of ACN *in vacuo* at 32 °C and eluted with 30 mL of H_2O, 2 : 8 ACN/H_2O, and 9 : 1 ACN/H_2O. Products were found in the 1 : 9 H_2O/ACN fraction. The products were further purified by reversed phase HPLC (40 : 60 to 95 : 5 MeOH/H_2O, Luna 250 × 15 mm, C18 silica) after removal of the volatiles under reduced pressure.

Compound 5a. MIN A was converted according to the general procedure with 57 μL acetic anhydride to give the title compound as white solid. Yield: 2.6 mg (0.002 mmol, 12%). Purity: 95.96% (UV/VIS purity, 205–800 nm, $\int_{\mathbf{5a}} = 2\,469\,933$, $\int_{\text{cont.}} = 103\,924$). HRMS: 1202.6405 $[M + H]^+$, calcd 1202.6421 for $[C_{55}H_{87}N_{13}O_{17} + H^+]^+$. ^{1}H NMR (400 MHz, CD_3OD) δ 7.72 (d, J = 8.2 Hz, 1H), 7.58 (d, J = 10.2 Hz, 1H), 6.59 (d, J = 7.3 Hz, 1H), 5.91 (q, J = 7.6 Hz, 1H), 5.67 (d, J = 12.6 Hz, 1H), 5.53 (d, J = 7.4 Hz, 1H), 5.48 (d, J = 4.8 Hz, 1H), 5.38–5.03 (m, 4H), 4.69–4.43 (m, 3H), 4.34–4.15 (m, 3H), 3.20–2.72 (m, 9H), 2.65 (s, 10H), 2.21 (d, J = 15.2 Hz, 3H), 2.09 (d, J = 2.1 Hz, 3H), 2.06–1.92 (m, 7H), 1.83 (d, J = 7.2 Hz, 2H), 1.75 (d, J = 7.5 Hz, 3H), 1.47–1.22 (m, 18H), 1.18 (d, J = 6.4 Hz, 3H), 1.00 (d, J = 6.8 Hz, 3H), 0.89 (d, J = 8.0 Hz, 6H), 0.72 (d, J = 6.7 Hz, 3H).

Compound 5b. MIN A was converted according to the general procedure with 110 μL butanoic anhydride to give the title compound as white solid. Yield: 10 mg (0.008 mmol, 44%). Purity: 93.48% (UV/VIS purity, 205–800 nm, $\int_{\mathbf{5b}} = 731\,946$, $\int_{\text{cont.}} = 51\,022$). HRMS: 1258.7102 $[M + H]^+$, calcd 1258.7047 for $[C_{59}H_{95}N_{13}O_{17} + H^+]^+$. ^{1}H NMR (400 MHz, CD_3OD) δ 5.91 (q, J = 7.3 Hz, 1H), 5.69 (dd, J = 12.0, 3.2 Hz, 1H), 5.54 (d, J = 7.5 Hz, 1H), 5.50 (d, J = 4.7 Hz, 1H), 5.25 (t, J = 6.2 Hz, 1H), 5.06 (dd, J = 9.7, 5.6 Hz, 2H), 4.63 (dd, J = 8.3, 3.4 Hz, 1H), 4.54 (d, J = 7.3 Hz, 1H), 4.50–4.46 (m, 1H), 4.29 (d, J = 7.2 Hz, 1H), 4.21 (dd, J = 11.3, 4.7 Hz, 1H), 3.35 (s, 3H), 3.19 (s, 3H), 3.08–2.76 (m, 6H), 2.36 (td, J = 7.3, 1.8 Hz, 2H), 2.27–2.18 (m, 4H), 1.99 (d, J = 7.4 Hz, 3H), 1.76 (d, J = 7.4 Hz, 3H), 1.71–1.65 (m, 3H), 1.60–1.52 (m, 2H), 1.41–1.28 (m, 20H), 1.19 (d, J = 6.4 Hz, 3H), 1.02–0.86 (m, 21H), 0.73 (d, J = 6.5 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 174.90, 174.78, 174.39, 173.79, 173.00, 172.51, 171.08, 170.64, 169.91, 168.24, 165.49, 165.38, 133.12, 129.67, 120.38, 73.12, 70.73, 61.48, 58.24, 55.82, 54.49, 51.94, 50.94, 50.18, 40.43, 36.88, 36.66, 35.10, 34.40, 33.89, 32.85, 31.06, 30.56, 30.21, 26.40, 24.91, 23.53, 19.45, 19.12, 19.09, 18.49, 17.31, 16.75, 16.10, 14.22, 13.82, 13.72, 13.59, 13.50.

Compound 5c. MIN A was converted according to the general procedure with 130 μL hexanoic anhydride to give the title compound as white solid. Yield: 13 mg (0.01 mmol, 55%). Purity: 94.67% (UV/VIS purity, 205–800 nm, $\int_{\mathbf{5c}} = 1\,088\,096$, $\int_{\text{cont.}} = 61\,248$). HRMS: 1314.7674 $[M + H]^+$, calcd 1314.7673 for $[C_{63}H_{103}N_{13}O_{17} + H^+]^+$. ^{1}H NMR (400 MHz, CD_3OD) δ 5.91 (q, J = 7.4 Hz, 1H), 5.69 (dd, J = 12.4, 3.2 Hz, 1H), 5.58–5.48 (m, 2H), 5.29–5.21 (m, 1H), 5.11–5.02 (m, 2H), 4.64 (dd, J = 8.4, 3.4 Hz, 1H), 4.54 (q, J = 7.3 Hz, 1H), 4.51–4.47 (m, 1H), 4.37–4.27 (m, 2H), 4.21 (dd, J = 11.3, 4.7 Hz, 1H), 3.43–3.33 (m, 1H), 3.19 (s, 3H), 3.10–2.74 (m, 6H), 2.37 (t, J = 7.5 Hz, 2H), 2.30–2.00 (m, 7H), 1.99 (d, J = 7.4 Hz, 3H), 1.96–1.86 (m, 2H), 1.83 (t, J = 6.9 Hz, 2H), 1.75 (d, J = 7.5 Hz, 3H), 1.71–1.48 (m, 5H), 1.48–1.24 (m, 30H), 1.19 (d, J = 6.4 Hz, 3H), 1.00 (d, J = 6.8 Hz, 3H), 0.97–0.85 (m, 15H), 0.73 (d, J = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 175.11, 175.00, 174.86, 174.62, 174.01, 173.18, 172.97, 172.72, 171.25, 170.85, 170.11, 168.43, 165.69, 165.57, 133.33, 130.14, 129.79, 120.58, 73.36, 70.89, 61.68, 58.45, 56.01, 54.67, 52.58, 51.17, 50.43, 40.70, 39.17, 37.13, 35.32, 35.14, 34.98, 34.60, 34.12, 33.06, 32.44, 32.34, 31.63, 31.26, 30.77, 30.41, 26.58, 25.61, 25.57, 25.17, 23.74, 23.43, 23.38, 19.66, 18.76, 17.52, 16.98, 16.32, 14.44, 14.28, 14.25, 13.82, 13.72.

Compound 5d. MIN A was converted according to the general procedure with 176 μL decanoic anhydride to give the title compound as white solid. Yield: 6 mg (0.004 mmol, 23%). Purity: 96.10% (UV/VIS purity, 205–800 nm, $\int_{\mathbf{5d}} = 556\,092$, $\int_{\text{cont.}} = 22\,574$). HRMS: 713.9501 $[M + 2H]^{2+}$, calcd 713.9502 for $[C_{71}H_{119}N_{13}O_{17} + H^{2+}]^{2+}$. ^{1}H NMR (400 MHz, CD_3OD) δ 7.57 (d, J = 10.1 Hz, 1H), 5.91 (q, J = 7.3 Hz, 1H), 5.69 (d, J = 10.1 Hz, 1H), 5.55 (t, J = 7.3 Hz, 1H), 5.50 (t, J = 5.0 Hz, 1H), 5.29–5.20 (m, 1H), 5.09–5.02 (m, 2H), 4.64 (dd, J = 8.5, 3.4 Hz, 1H), 4.61–4.45 (m, 3H), 4.36–4.17 (m, 4H), 3.19 (s, 3H), 3.04–2.80 (m, 4H), 2.37 (t, J = 7.4 Hz, 3H), 2.33–2.19 (m, 5H), 1.99 (d, J = 7.4 Hz, 4H), 1.75 (d, J = 7.4 Hz, 6H), 1.65 (t, J = 7.4 Hz, 3H), 1.41–1.26 (m, 58H), 1.19 (d, J = 6.4 Hz, 3H), 1.00 (d, J = 6.9 Hz, 3H), 0.90 (dt, J = 7.3, 3.6 Hz, 17H), 0.73 (d, J = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 174.80, 174.41, 173.81, 172.97, 172.74, 171.04, 169.90, 168.23, 165.47, 133.10, 129.94, 120.37, 70.65, 61.48, 58.25, 54.47, 50.98, 50.02, 40.56, 34.99, 34.82, 34.40, 33.93, 32.83, 31.07, 30.56, 30.37, 30.33, 30.28, 30.21, 30.05, 29.96, 26.36, 25.73, 25.66, 24.66, 23.53, 19.46, 18.59, 17.31, 16.80, 16.11, 14.24, 13.60, 13.50.

Purification of semi-synthetic variants

Fine purification of semi-synthetic PUW/MIN variants was performed on an Agilent 1100 Series modular HPLC system (Agilent Technologies) equipped with a MWD using a semi-preparative RP-C18 column with ACN + 0.1% HCOOH (A)/H_2O + 0.1% HCOOH (B) gradient at a flow-rate of 2.5 mL min^{-1} (0 min – 50% of A; 1 min – 50% of A; 25 min – 95% of A; 26 min – 100% of A; 36 min – 100% of A; 37 min – 50% of A). Fractions were collected manually using UV detection at 215, 235, 280, 440, and 680 nm, while 235 nm was mainly used due to the high absorption of both lipopeptides and possible contaminants at this wavelength. Because of the low ionizability of modified variants, the purity was determined based on the integration of the compound peak area in UV/VIS 205–800 nm chromatograms. In the case of **5a–d**, both lipopeptide isomers were taken together, in the case of **4a**, due to its low concentration, a UV/VIS base peak chromatogram was used.

HPLC-HRMS and HRMS/MS analysis

The purity of natural PUW/MIN as well as their semi-synthetic variants was monitored by HPLC-HRMS on Thermo Scientific Dionex UltiMate 3000 UHPLC+ (Sunnyvale, CA, USA) equipped with a diode-array detector. Separation of compounds was

© 2021 The Author(s). Published by the Royal Society of Chemistry

Open Access Article. Published on 16 September 2021. Downloaded on 9/19/2021 12:02:23 PM.
This article is licensed under a Creative Commons Attribution-NonCommercial 3.0 Unported Licence.

performed on reversed phase Phenomenex Kinetex C18 column (150 × 4.6 mm, 2.6 μm, Torrance, CA, USA) using H_2O (A)/ACN (B) both containing 0.1% HCOOH as a mobile phase with the flow rate of 0.5 mL min^{-1}. The gradient was as follows: A/B 85/15 (0 min), 85/15 (in 1 min), 0/100 (in 39 min), 0/100 (in 44 min), and 85/15 (in 49 min). The HPLC was connected to Bruker Impact HD high-resolution mass spectrometer (Bruker, Billerica Massachusetts, USA) with electrospray ionization. The following settings were used: dry temperature 200 °C; drying gas flow 12 L min^{-1}; nebulizer 3 bar; capillary voltage 4500 V; endplate offset 500 V. The MS/MS experiments were performed with identical instrument settings to confirm the chemical structure of the synthetized PUWs/MINs. Collision energy of 80 eV for single charged and 35 eV double charged ions was used. The spectra were collected in the range of 20–2000 *m/z* with a spectra rate of 3 Hz. Spectra were calibrated using both LockMass Tuning Mix ES-TOF (622 Da) internal calibration solution and sodium formate clusters at the beginning of each analysis.

Cytotoxicity testing using MTT and recovery experiments

Lipopeptide cytotoxicity was tested on a human cervical cancer cell line (HeLa cells) cultured in RPMI 1640 medium (supplemented with 10% FBS, 2 mM L-glutamine and 1% antibiotics). The cells were incubated at 37 °C and 5% CO_2 in humidified incubator. For dose–response curves, 10×10^3 cells per well (200 μL) were seeded into transparent 96-well plate (MTT assay) and allowed to adhere overnight. Lipopeptide solutions of desired concentrations were prepared in cultivation medium. For the cell treatment, the whole volume of the well was replaced with lipopeptide-containing media, incubated for 48 hours and the viability was assessed by MTT test. Briefly, 15 μL of MTT solution (4 mg mL^{-1} in PBS) was added per well and incubated for 4 h in the incubator. The plate was centrifuged (380 × *g*, 10 min), the supernatant was discarded and 200 μL of DMSO was added to the cells to dissolve formazan crystals. The absorbance was measured using a Tecan Sunrise reader at 590/640 nm. The viability was calculated as a ratio between absorbance of treated, control wells, and expressed in percent. To study the time needed to manifest the effect of compound **1** and **2**, the recovery experiments were performed. 20×10^3 cells per well (200 μL) were seeded in a 96-well transparent plate and allowed to adhere overnight. The cells were treated as stated above with 3 μM, 5 μM and 10 μM PUW F or MIN A and mixed multiple times by pipette. At defined time points (1–8 h), the lipopeptide-(exposure)-medium was exchanged for lipopeptide-free medium and the cells were further incubated for 48 hours. The MTT test was performed to check the viability and recovery of the cells.

Assessment of cellular morphology using light microscopy

Live cell imaging was carried out using an Axio Observer Inverted Microscope Z.1. (ZEISS) equipped with an incubation chamber supplied with 5% CO_2. HeLa cells grown in RPMI (Biowest) were seeded at 5×10^3 cells (100 μL) per well into a black 96-well A/2 poly-D-lysine-coated high content imaging plate with an 0.2 mm glass bottom (Corning® BioCoat™). The following day cells were treated, as described above, with different concentrations (20 μM, 5 μM, or 1.25 μM – final volume 100 μL per well) of **1** and **2**. Images were acquired after 48 h of treatment with **1** and **2** using a 100× Plan-Apochromat/1.40 Oil DIC M27 objective and camera adapter 0.63×.

Assessment of membrane damage caused by PUW F and MIN A

HeLa cells in densities of 1×10^4 were seeded on black 96-well plates. The cells were assessed at desired time points (30 min, 45 min, 1, 2, 3, and 6 h) after compound treatments and assessed for LDH-leakage using the CytoTOX One homogeneous membrane integrity assay (Promega) according to the protocol provided by the manufacturer. The provided lysis solution was used to generate maximum LDH leakage. The fluorescence ($560_{Ex}/590_{Em}$) obtained after the compound treatment were related to the maximum LDH leakage and expressed in percent.

HPLC-HRMS determination of PUW F and MIN A concentrations in HeLa cells

To determine the concentration of PUW/MIN in the cell during time 6×10^6 cells were seeded in 6-well plates in 6 mL media and let to adhere overnight. The cells were treated with the compounds at 0.5 and 5 μM concentrations. At the desired time points (5 min, 30 min, 2 h, and 6 h), the cells were harvested by trypsinization and pelleted in 15 mL falcon tubes, subsequently the cells were extracted using 2 mL of 60% ACN aq. containing 1.5% sodium cholate and centrifuged. The resulting samples were analyzed using HPLC-HRMS analysis described above and the concentration was calculated based on the external calibration curve of the pure **1** and **2** and the resulting concentration was expressed as Ag/cell.

Membrane permeabilization assay

Yeast cells (*Saccharomyces cerevisiae* P23) were grown aerobically in YEPG nutrient medium (yeast extract 5 g and peptone 10 g from Oxoid, glucose 20 g in 1 L of water) at 30 °C to mid log phase (OD_{450} nm ~0.5). Cells were harvested, washed, and resuspended (final OD_{450} nm ~0.2) in a citrate–phosphate buffer (0.15 M, pH = 6.0) containing 0.5% glucose and 10 μM propidium iodide (PI, Invitrogen). The tested compounds at final concentrations of 1, 5, and 10 μM were added to 0.2 mL of yeast suspension in a 96-well plate. Membrane permeabilization, as estimated by PI uptake into cells, was monitored as an increase in fluorescence intensity (excitation at 515 nm, emission at 620 nm with bandpass 5 and 10 nm, respectively) at 25 °C using FluoroMax-3 (Jobin Yvon, Horriba) spectrofluorometer with a MicroMax-384 accessory. We used optical filters (3RD500-530 and 3RD570LP, omega optical filters) for the suppression of light scattered by the cells. Ten μM melittin (Sigma) was added to the well as a positive control for cell permeabilization, whereas the buffer addition served as a baseline negative control. The presented data are the recorded intensities without background subtraction.

© 2021 The Author(s). Published by the Royal Society of Chemistry

Open Access Article. Published on 16 September 2021. Downloaded on 9/19/2021 12:02:23 PM.
This article is licensed under a Creative Commons Attribution-NonCommercial 3.0 Unported Licence.

Lipid bilayer experiments

Black lipid bilayer membranes were formed by painting a solution of 3% (w/v) DOPC/DOPE 2 : 1 (w/w, 1,2-dioleoyl-*sn-glycero*-3-phosphocholine, 1,2-dioleoyl-*sn-glycero*-3-phosphoethanolamine, Avanti lipids) in *n*-decane/butanol (9 : 1, v/v) across a circular aperture (0.5 mm in diameter) separating two compartments of a Teflon chamber. Both compartments contained 2 mL of 10 mM Tris, 1 M KCl, pH 7.4. The temperature was kept at 25 °C. Tested compound were added to the *cis* side of the membrane. The membrane current was measured with Ag/AgCl electrodes (applied voltage 50 mV, *cis* positive), amplified by LCA-200-100G or LCA-4k-1G amplifier (femto), and digitized by a KPCI-3108 card (Keithley). The signal was processed by QuB software.[32]

Antifungal activity

Antifungal activity was evaluated against *Candida friedrickii* (BCC020_2879), *Aspergillus fumigatus* (BCC020_2845), *Fusarium oxysporum* (BCC020_2866), *Trichoderma harzianum* (BCC020_0606), *Bipolaris sorokiniana* (BCC020_1571), *Monographella cucumerina* (BCC020_2872), *Chaetomium globosum* (BCC020_2527), and *Alternaria alternata* (BCC020_0609) using the broth two-fold microdilution method following the standard protocol of broth two-fold microdilution method (CLSI M100-S20).[33] In brief, for the broth microdilution method, the compounds (**1–5d**) were serially diluted at concentrations ranging from 75 to 0.036 μM in a Mueller Hinton broth with 2% glucose in 96-well plates. The inoculum (approximately 5×10^5 CFU mL^{-1} as final concentration) was prepared from an overnight culture and was added to each well containing the compounds. Culture broth (0.1 mL) was added to each well containing compounds. Plates were incubated aerobically at 30 °C for 48 h. Negative controls were prepared using culture media with 50% DMSO in methanol (the dissolving solvent). Fluconazole (32 to 0.0625 μg mL^{-1}) was used as the positive control for all the fungal isolates tested. After incubation, the well with the lowest concentration of the fraction/compound showing some inhibition in growth was taken as the MIC value for the respective organisms. All the experiments were done in triplicate.

3. Results

Cytotoxicity and antifungal properties evaluation of PUW F and MIN A

We initially compared minutissamide A (MIN A, **1**) and puwainaphycin F (PUW F, **2**) to assess the effect of FA length on cytotoxic and antifungal activity. Both lipopeptides possess identical FA functionalization in the proximity of the peptide cycle with a α-carbon substituted by a hydroxyl group, a β-carbon occupied by an amine group engaged in the peptide bond formation with the Pro of the peptide cycle, and a γ-carbon bearing a methyl group. No further FA substitution was observed in **1** and **2** with FA lengths of 12 or 14 carbons, respectively. Both compounds exhibited almost identical IC_{50} values against human HeLa cells as assessed by the MTT assay (Fig. 2A). The IC_{50} values were calculated as 2.8 ± 0.5 μM for **1** and 3.2 ± 0.5 μM for **2** (Fig. 2A). However, **2** exhibited a stronger effect at higher concentrations (5–20 μM), which reduced cell viability by 90–95%, while 70% inhibition was reached after exposure to **1** at this concentration range (Fig. 2A).

We studied the time progression of the cytotoxic effect of the tested compounds using recovery experiments at three concentrations (3, 5 and 10 μM) and evaluated cell viability by MTT, in order to better characterize the differences in the cytotoxic effect of these compounds. A rapid toxic effect on the cell population was observed at concentrations 5 and 10 μM of **2**, with no apparent recovery in cellular viability (after 48 h cultivation), even for the shortest exposure time tested (1 h). The same concentrations of **1** were easily tolerated by the cells for the first two hours, since their viability was comparable to that of the control, after the compound-washout and recovery period (Fig. 2B). Membrane damage, as measured by the lactate dehydrogenase (LDH) leakage, was more profound in **2** compared to **1** in accordance with the recovery experiment. The onset of extracellular LDH leakage was immediate and reached a maximum (60% of released LDH) 2 h after exposure (Fig. 2C) for compound **2** (Fig. 2C). No LDH leakage was recorded for compound **1** at early time points up to 2 h, and LDH started to accumulate in the medium (up to 30% of positive control) only after 3 h suggesting a greater degree of membrane damage (Fig. 2C). The cellular morphology was clearly affected by both **1** and **2** showing apparent cell shrinkage and membrane rupture after 48 h of exposure in concentrations ranging from 2.5 to 20 μM (Fig. 3, S1 and S2†).

We further estimated cellular PUW/MIN concentrations using HPLC-HRMS measurements (Fig. 2D). Compound **2** demonstrated a rapid entry into the cell resulting in a 3–10 times higher cellular concentration compared to **1** at the initial time points (5 to 30 min). The cellular concentration of **1** started to increase after 2 h when it reached a concentration comparable to that of **2** at the initial time points. Interestingly, even the two-fold higher concentration of **1** in the cell compared to **2** was detected at later time points (Fig. 2D). These data correlate well with the delayed cytotoxic effect of **1** as well as the LDH leakage.

The PUWs/MINs also differed substantially in their pore-forming activity as measured in planar DOPC/DOPE membranes. While **2** was able to induce membrane permeabilization at 5 μM concentration, **1** was almost completely inactive under the same conditions. At higher doses both lipopeptides induced detectable increases in membrane current but at considerably different scales (Fig. 4). The typical transmembrane electrical currents were about 10 pA for **1** and 500 pA for **2**, at 10 μM. Due to a great variability in individual pore events, it was not possible to characterize the PUW/MIN at the single-molecule level.

In general, the differences in cytotoxic activity were mirrored in antifungal activity as **2** exhibited substantially higher antifungal potency compared to **1**. We also observed a higher membrane permeabilization activity for **2** compared to **1** in *Candida glabrata* (data not shown) and *Saccharomyces cerevisiae* (Fig. 5) as followed by propidium iodide (PI) entry into cells. Compound **2** induced concentration dependent PI entry into the yeast cells within a few mins at 5 and 10 μM, comparable to

© 2021 The Author(s). Published by the Royal Society of Chemistry

Open Access Article. Published on 16 September 2021. Downloaded on 9/19/2021 12:02:23 PM.
This article is licensed under a Creative Commons Attribution-NonCommercial 3.0 Unported Licence.

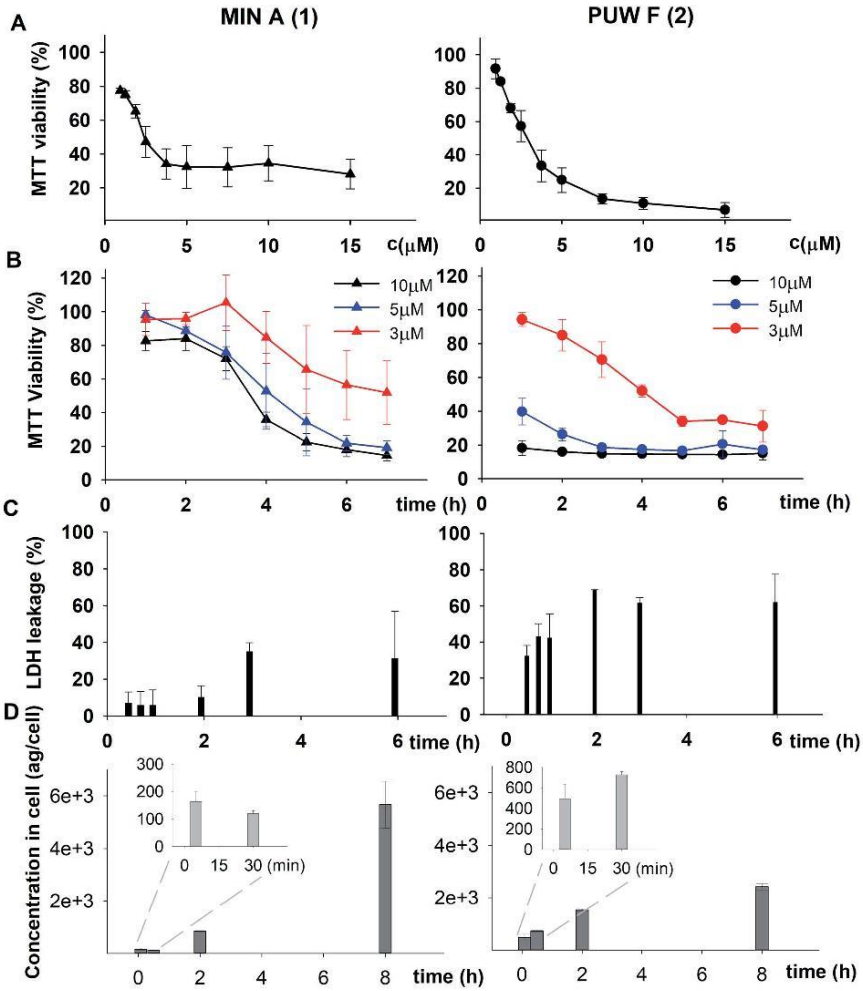


Fig. 2 Comparison of the cytotoxic activity of **1** and **2**: the dose–response curve of both compounds (after 48 h treatment) shows similar IC_{50} values but a stronger effect of **2** at higher concentrations (A). (B) Results of the recovery experiment (treatment followed by 48 h recovery) show more rapid effect on cell viability in **2**. (C) Membrane damage, as assessed by lactate dehydrogenase (LDH) leakage, shows the more potent effect of **2**. (D) Accumulation of **1** and **2** in the cell as assessed using HPLC-HRMS shows the faster entry of **2** in the cell.

the positive control melittin displaying a similar final fluorescence intensity. No membrane disruption was detectable under the same conditions for compound **1**. We tested both compounds for their activity against *Candida* strains and a small panel of filamentous fungi. Compound **2** exhibited a 3 to 129 times lower MIC values compared to **1** with the plant pathogen *A. alternata* and the facultative human pathogen *A. fumigatus* showing the highest sensitivity to both compounds (Table 1).

Preparation of PUW/MIN semi-synthetic variants

We prepared semi-synthetic variants with modified FA moieties based on the preliminary data obtained for compounds **1** and **2** showing that the length of the FA moiety affects both cytotoxicity

Open Access Article. Published on 16 September 2021. Downloaded on 9/19/2021 12:02:23 PM.
This article is licensed under a Creative Commons Attribution-NonCommercial 3.0 Unported Licence.

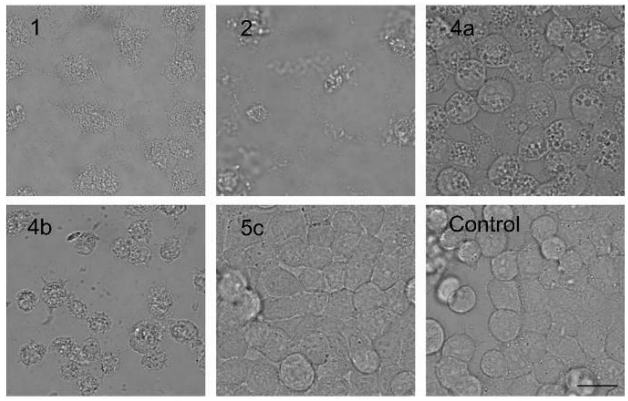


Fig. 3 The morphology of HeLa cells treated with 20 μM compounds under study. Semi-synthetic compound **4b** induced at this concentration clear membrane rupture comparable to natural compounds **1** and **2**. Under treatment of **4a**, the cells appeared stressed but with no membrane rupture at the end of the experiments. No morphology alteration was observed for any of the esterified variants **5a–d** (the figure shows an example morphology for **5c**). For morphological alteration observed at lower concentrations see Fig. S2.† The scale bar represents 20 μm.

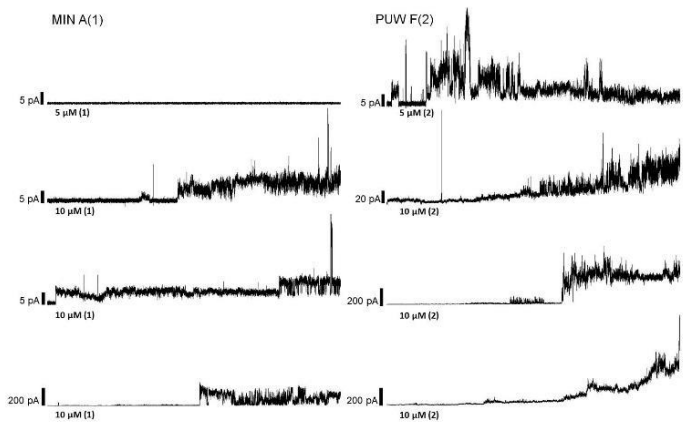


Fig. 4 Pore formation by compounds **1** and **2**. Typical ion current recordings at 5 and 10 μM treatment of **1** and **2** under 50 mV membrane voltage. The membrane was formed from DOPC/DOPE 2 : 1 (w/w) in *n*-decane/butanol (9 : 1, v/v), in a buffer containing 10 mM Tris, 1 M KCl, pH 7.4. Each current trace represents 120 s of recording. Note the different current scale for individual recordings.

and antifungal activity. We used minutissamide C (MIN C, **3**) bearing the oxo-substitution at the FA tail because the FA moieties of compounds **1** and **2** were not easily accessible to chemical modification (Scheme 1). The Wittig-type reaction, with phosphorus ylides preformed from triphenyl phosphonium salts and *n*-BuLi to allow the extension of the aliphatic chain of compound **3**, was initially tested. However, we observed only a small amount of product, which was formed by the side-reaction of *n*-BuLi with the ketone moiety of **3**. Instead, we sought to increase the chain length using other carbon nucleophiles such as alkylmagnesium halides. Moderate yields of the expected tertiary alcohol products were detectable by HPLC-HRMS analysis using this Grignard-reactant (Scheme 1). Reactions with butyl-, octyl-, and nonylmagnesium bromide allowed successful elongation of the FA moiety at C14 by 4, 8, and 9 carbons, respectively. The reaction with methylmagnesium bromide gave rise to a wide variety of side products and reaction with alkylmagnesium halides longer than C9 did not show conversion to the expected products. We performed

© 2021 The Author(s). Published by the Royal Society of Chemistry

Open Access Article. Published on 16 September 2021. Downloaded on 9/19/2021 12:02:23 PM.
This article is licensed under a Creative Commons Attribution-NonCommercial 3.0 Unported Licence.

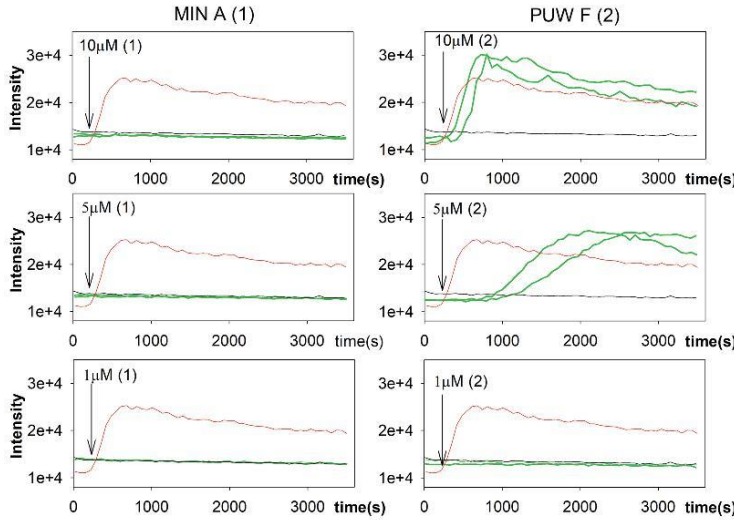


Fig. 5 Membrane permeabilization of *Saccharomyces cerevisiae*. The influx of propidium iodide (PI) across impaired membranes into cells was detected by an increase in PI fluorescence several seconds after the addition of compound **2** but not compound **1**. Green lines represent two sets of experiments; black line and red line represent the negative (buffer solution) and the positive control (melittin), respectively.

Table 1 Antifungal activity of natural and semi-synthetic PUW/MIN variants. The bioactivity of natural reference PUW/MIN variants is highlighted in grey. Abbreviations; NA, no activity at the highest concentration tested (75 μM)

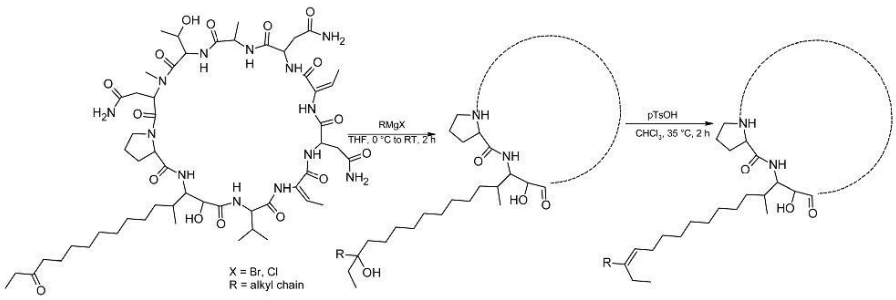
	Natural and semi-synthetic PUW/MIN variants							
	MIC (μM)							
Fungus/compound	**2**	**4a**	**4b**	**1**	**5a**	**5b**	**5c**	**5d**
A. fumigatus	2.34	0.5	3.8	37.5	4.7	9.4	75	37.5
F. oxysporum	75	NA	NA	NA	NA	NA	NA	NA
T. harzianum	37.5	15	NA	NA	75	NA	NA	NA
A. alternata	0.58	0.5	0.1	75	0.6	0.2	0.2	0.2
B. sorokiniana	NA	NA	NA	NA	NA	NA	NA	NA
M. cucumerina	6.25	7.5	30	NA	75	75	37.5	37.5
C. globosum	12.5	60	30	NA	75	75	75	75
C. friedrichii	75	7.5	NA	NA	NA	NA	NA	NA

a subsequent dehydration of the hydroxylated intermediate (Scheme 1) with *p*-TsOH because the formation of a hydroxyl group on the side chain of PUWs/MINs (Scheme 1) can diminish cytotoxicity. Unfortunately, elimination of water only took place at temperatures ≥35 °C resulting in significant decomposition of the compound. We suspected the limitation in time and temperature to be a major reason for the low overall yield of the procedure (1–2% over 2 steps). The elimination of water from the nonyl compound was not feasible before the total decomposition of the molecule. Theoretically, water elimination can occur in three different directions. The non-symmetrical chromatographic peak obtained using HPLC-HRMS indicated the formation of several dehydration products. However, it was not possible to separate these dehydration products and determine which final product was formed preferentially. In Scheme 1, the double bond is drawn tentatively in the direction of the peptide core following Zaitsev's rule. The dehydration of butyl- and octyl-compounds yielded compounds **4a** and **4b**, possessing branched mono-unsaturated FA moieties prolonged at C14 by 4 and 8 carbons, respectively, as confirmed by ^{1}H NMR (Fig. S3 and S4†). The identity of the compounds was validated using HRMS/MS measurements and the specific modified FA-Pro fragments with corresponding mass were obtained for all compounds (Fig. S12 and S13†).

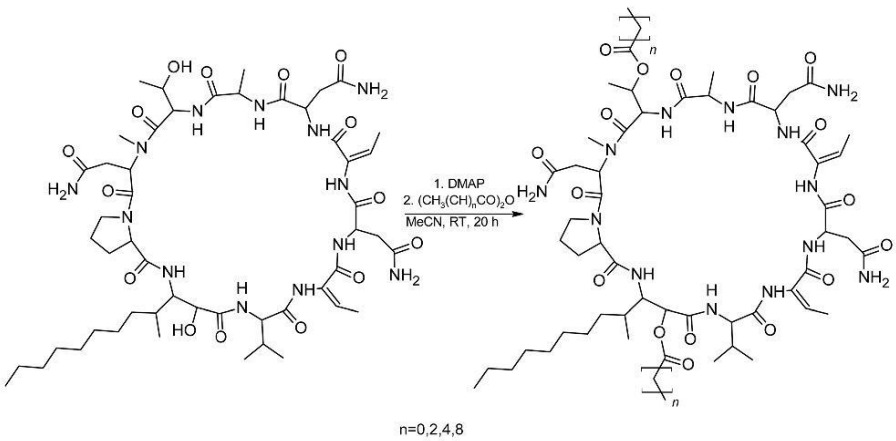
We suspected that hydroxylation might play an important role in bioactivity since the majority of known cyanobacterial cytotoxic lipopeptides contain a hydroxyl group on the FA moiety. Therefore, we attempted to modify the free hydroxyl groups in order to alter the hydrophobicity of the PUW/MIN molecule (namely the α-OH group of FA and –OH substituent at Thr^8 residues). A Steglich esterification with DMAP and carboxylic anhydrides (Scheme 2) is a mild procedure, which is especially suitable for sterically hindered alcohols.[34] This approach allowed the use of a variety of carboxylic anhydrides with **1** in a convenient one-step procedure with satisfying yields (40–60%). The reactions with acetic, butanoic, hexanoic, and decanoic anhydride led to the formation of double esterified adducts at the α-OH group of the FA moiety and at the OH– in the Thr^8 resulting in compounds **5a**, **5b**, **5c**, and **5d** (for compound characterization see Fig. S5–S11 and S14–S17†). The

© 2021 The Author(s). Published by the Royal Society of Chemistry

Open Access Article. Published on 16 September 2021. Downloaded on 9/19/2021 12:02:23 PM.
This article is licensed under a Creative Commons Attribution-NonCommercial 3.0 Unported Licence.



Scheme 1 Grignard-reaction of **3** with alkylmagnesium halides and subsequent dehydration with *p*-TsOH.



Scheme 2 Steglich esterification of **1** with DMAP and different acid anhydrides.

chromatographic behavior of all products is similar to those observed for **1**, **2**, and **3** (ref. 16) with minor conformational isomer eluting after the major chromatographic peak (Fig. S14–S17†). NMR analyses shows increased shifts of the OH–carbons of the new compounds **5b**, **5c**, and **5d** to 70.89 and 73.36 ppm, for Thr and FA, respectively (exemplary values from substance **3b**), indicating the formation of an ester. Further, the identity of the compounds was in all cases confirmed by the presence of corresponding esterified-FA fragments (Fig. S14–S17†). We did not obtain ^{13}C spectra data due to the high losses observed during the final repurification of **5a**. Instead, we confirmed the structure of the compound using a combination of ^{1}H spectra (Fig. S5†) and HRMS/MS fragmentation (Fig. S14†).

Biological activity of PUW/MIN semi-synthetic variants

The biological activity of semi-synthetic PUW/MIN variants was further evaluated and compared to the bioactivities of the natural PUWs/MINs **1** and **2**. The bioactive properties of **5a–d** were directly compared to compound **1**, used for their preparation. In the case of compounds **4a** and **4b** the original reactant, compound **3**, is oxo-substituted on the FA moiety, while the resulting compounds, **4a** and **4b**, do not contain any heteroatom substitution near the terminal acyl chain and thus these analogs are more similar to compounds **1** and **2**. Accordingly, we selected **2** as a reference compound based on the longer FA moiety of this compound.

The HeLa cells responded to the exposure of compounds **4a** and **4b** in a non-standard manner resulting in a biphasic dose response curve (Fig. 6). Subsequently, it was not possible to calculate the corresponding IC_{50} values. We observed lower viability values at concentration ranges from 0.3–2.5 μM following application of these semi-synthetic variants compared to reference compounds. This was in contrast to the cellular morphology, which appeared normal (Fig. S1 and S2†), with full confluence reached at the end of the experiment. Compound **4a** provided higher viabilities at a higher concentration range

© 2021 The Author(s). Published by the Royal Society of Chemistry

Open Access Article. Published on 16 September 2021. Downloaded on 9/19/2021 12:02:23 PM.
This article is licensed under a Creative Commons Attribution-NonCommercial 3.0 Unported Licence.

compared to compound **2**. Cells were fully inhibited only at 20 μM of compound **4a**, as also evidenced by cell shrinkage and apparent membrane rupture (Fig. 3). Compound **4b** reached a maximum of 50% inhibition even at a higher concentration range. After treatment with **4b**, the cell morphologies appeared comparable to those in control cells demonstrating no membrane disruption effect. The exception was the highest concentration where a clear cellular rounding and vacuolization was apparent (Fig. 3). Together, this demonstrated an attenuation of the cytotoxic activity of **4a** and **4b** compared to **2**. Consistent with this observation, we recorded membrane rupture, as visualized by microscopy, only at the highest concentrations of compound **4a** while in compound **2** membrane disruption progressed until concentrations of 2.5 μM (Fig. S1†). Surprisingly, cytotoxic activity was almost completely abolished or significantly diminished in treatments with compounds **5a–d** compared to **1** (Fig. 6). While we observed only a weak inhibition at the highest concentration of compounds **5a–c**, compound **5d** showed weak inhibition in the concentration range from 1.2 μM upwards. The cellular morphologies appeared normal across all concentrations (Fig. 3, and S2†) in all the compounds and cells reached full confluence as in the control wells.

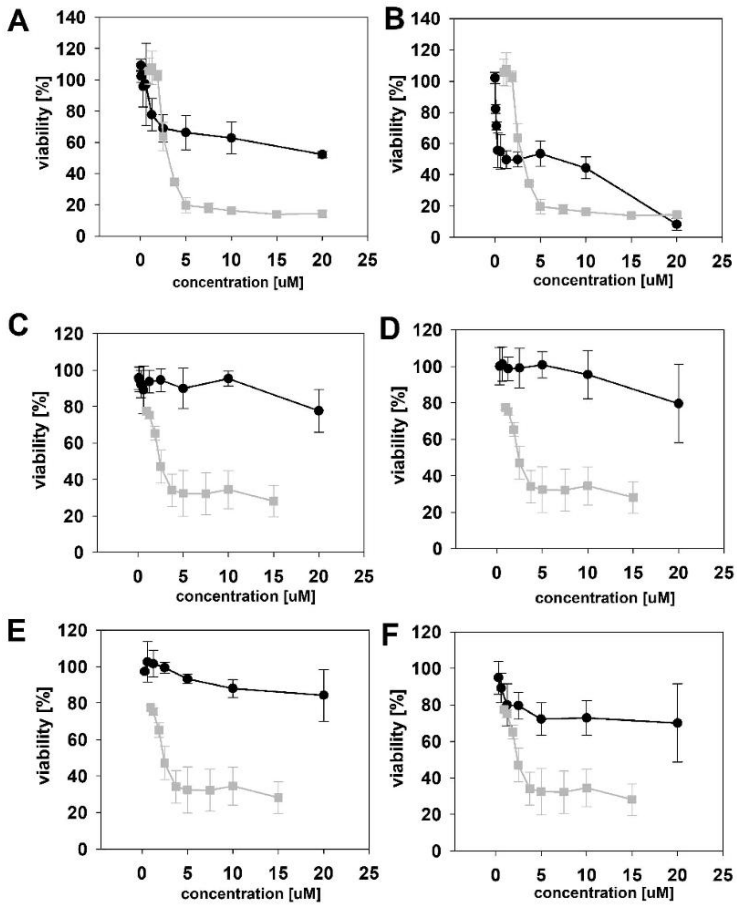


Fig. 6 The dose response curves of the semi-synthetic PUW/MIN variants (black line) in comparison to the reference natural PUW/MIN variant (grey line). Compounds with elongated FA moiety: compound **4a** (A), **4b** (B), and acylated PUW/MIN variants **5a** (C), **5b** (D), **5c** (E), and **5d** (F). Complex dose response curves or low cytotoxic activity prevented calculation of IC_{50} values for semi-synthetic variants in some cases.

© 2021 The Author(s). Published by the Royal Society of Chemistry

Open Access Article. Published on 16 September 2021. Downloaded on 9/19/2021 12:02:23 PM.
This article is licensed under a Creative Commons Attribution-NonCommercial 3.0 Unported Licence.

The alteration of the FA moiety substantially changed the antifungal properties of PUWs/MINs against most of the fungal strains tested albeit in a species-specific manner. The most profound differences between the natural PUW/MIN variant and the resulting semi-synthetic variants were found in the facultative human pathogen *A. fumigatus* and the plant pathogen *A. alternata*, which showed opposite sensitivities to **4a** and **4b** (Table 1). The FA chain elongation also led to a more profound activity in *T. harzianum* and *C. friedrichii*. The acylated semi-synthetic variants **5a–d** possessed substantially lower MIC values compared to the original compound **1**. We observed an 8 to 375 fold reduction of the MIC values of **4a** and **5a–d** against *A. alternata* and *A. fumigatus*, respectively. However, **5a–d** also caused higher effectivity in *M. cucumerina* and *C. globosum* **5a–d**, resulting in a drop in MIC. These results are notable given the almost complete abolishment of cytotoxic activity in **5a–d**.

4. Discussion

PUWs/MINs are representatives of a relatively large group of cyanobacterial β-amino-FA containing cyclic lipopeptides also comprising muscotoxins,[19,27] trichormamides,[35,36] lobocyclamides,[37] and laxaphycins.[22,23,38] These lipopeptides have biotechnological and pharmaceutical potential.[19,25,39] However, there is limited information available on how FA properties influence antifungal/cytotoxic potency. Natural sources of cyanobacterial cyclic lipopeptides have a restricted range of FA chain lengths, — PUWs/MINs (C10–C18), muscotoxins (C10), and laxaphycins (C8–C10) — which makes direct comparisons difficult. In addition, FA moieties are usually unbranched and FA chains of certain lengths are produced only in modified form (bearing functional groups), such as in PUWs/MINs where the variants with C16 and C18 FA are substituted by oxo or hydroxyl functional groups. Here, we have extended the FA moiety length of PUWs/MINs bearing FA with oxo substitution (compound **3**) by reaction with alkylmagnesium halides and subsequent dehydration using *p*-TsOH. This allowed us to study the effect of PUWs/MINs containing long chain FAs possessing an ethyl side branch and an adjacent double bond (C18 and C22). In addition, we prepared PUW/MIN variants bearing multiple acyl chains by modification of compound **1**. The stereochemistry of compound **1** and **3** was not determined. The stereochemistry of both MIN A and MIN C isolated from *Anabaena minutissima* (UTEX 1613) has previously been described.[17,18] However, in this study both compounds were isolated from different strains and consequently the actual absolute configuration may differ from the published structures. During the chemical modification, no stereogenic center was affected and the modified variants **4a–b** and **5a–d** share a stereochemistry identical to **1** and **3**, respectively. Therefore, the changes in bioactivity can be attributed to the FA modification or lipopeptide acylation.

Our initial results demonstrated that compounds **1** and **2**, differing in the length of their respective FA chains by two methylene groups, manifested similar long-term potency (Fig. 2). However, the application of **2** led to more rapid membrane damage followed by a decrease in the viability of HeLa cells. The actual PUW/MIN concentration within the cell is consistent with these results as the cyclic lipopeptide with a longer FA (**2**) shows more rapid association with the cell compared to **1**. It is notable that compound **1** with a shorter FA chain reached higher cellular concentration in the later time points compared to **2**. However, the cellular concentration of the compound itself does not fully explain its lytic potency. Compound **1** did not cause extensive membrane damage even at concentrations comparable to those of **2** manifesting cell membrane damage. Nevertheless, our results collectively suggest that the FA chain length plays a crucial role in the stability of PUW/MIN-membrane complexes and that even a small difference in the FA chain length affects the onset of the cytotoxic effect to a high degree.

Iturins, and related compounds, with longer FA chains display lower critical micellar concentrations that result in higher antifungal potency.[6] Similarly, compounds **1** and **2** differed in their membrane disruption activity against *Saccharomyces cerevisiae* with **2** promoting membrane disruption while **1** did not. Furthermore, these compounds had differing MIC values against tested fungi with compound **2** being again considerably more potent than compound **1**. Together, these results suggest that the FA chain length of PUWs/MINs plays a more profound role in antifungal activity compared to cytotoxicity. We also observed a similar trend for the semi-synthetic variants **4a** and **4b** with prolonged and branched FA tails. Extension of the FA main aliphatic chain by four (in compound **4a**) and eight carbons (in compound **4b**) led to a substantial decrease in the MIC values against *A. fumigatus* and *A. alternata*, respectively. Although these compounds retained some weak anti-proliferative effect as evidenced by MTT, the cell membrane perturbation was observed at the highest concentration only.

A comparison of natural variants bearing unsubstituted FA (**1** and **2**) with the semi-synthetic analogs **4a** and **4b** containing branched FA (with an ethyl side chain) is not straightforward. However, it is apparent that PUW/MIN variants containing modified FA display an improvement of antifungal activity in a species-specific manner while simultaneously exerting reduced cytotoxic activity. In this study, compounds **1** and **2** containing unsubstituted FA (C12 and C14), exert moderate cytotoxic effects with the resulting IC_{50} values of 2.8 and 3.15 μM. A similar range of IC_{50} was reported for C16 and C18 oxo-substituted FA. However, the semi-synthetic variants **4a** and **4b** possessing unsaturated C18 and C22 with an ethyl side chain do not exert relevant cytotoxicity. Thus, the cytotoxic activity of modified variants is likely diminished by the presence of an ethyl group and adjacent double bond. Neither **1** nor **2** contain double bonds in the exocyclic part of the FA chain. Consequently, the exact position of the unsaturation in compounds **4a** and **4b** is not significant for the presented SAR study as the double bond is newly introduced. For the bioactivity assays, a mixture containing highly enriched tentative isomers following Zaitsev's rule and a minor amount of two other isomers differing in the position of the double bond, was used. It is notable that side methyl FA branching is relatively common in cytotoxic lipopeptides and does not abolish the cytotoxicity against human cells as iturins exhibit cytotoxicity against HeLa

© 2021 The Author(s). Published by the Royal Society of Chemistry

Open Access Article. Published on 16 September 2021. Downloaded on 9/19/2021 12:02:23 PM.
This article is licensed under a Creative Commons Attribution-NonCommercial 3.0 Unported Licence.

cells with IC_{50} 1.73 μM and 7.8 μM for Iturin A and F_2, respectively.[40] Consequently, it is not the branching itself but rather the length of the side chain that affects the cytotoxic potential. The introduction of the double bond followed by small changes in the 3D structure of the acyl chain should not play a crucial role in the observed decrease of the cytotoxicity as highly potent cytotoxic anabaenolysins contain polyunsaturated FA moiety of a similar length (C18) and exhibit cytotoxicity against HeLa cells with IC_{50} 11 μM.[21] Nevertheless, the FA extension and ethyl branching was accompanied by a species-specific increase of antifungal properties with MIC values of 0.5 μM and 0.1 μM against *A. fumigatus* and *A. alternata* for **4a** and **4b**, respectively.

One of the main achievements of the study is a general improvement of the antifungal activity of PUW/MIN by increasing the hydrophobicity by modification of both the polar hydroxyl groups on Thr and an α –OH on the FA moiety. The double acylation of these functional groups **5a–d** fully diminishes the cytotoxic effect while substantially increasing antifungal activity. Even acylation with C10-mimicking FA tails similar to **1** and **2** abolished cytotoxicity in HeLa cells. Consequently, not only the hydrophobic FA properties but also polar and hydrophilic functional groups are important for the interaction of PUW/MIN with the human cell membrane and its permeabilization. We expect the α-OH on the FA residue to play a crucial role in such interaction as its position after PUW/MIN incorporation into the membrane will allow interaction with the polar phospholipid head. The vast majority of known membrane permeabilizing cyclic lipopeptides including polyunsaturated anabaenolysins[21] or polysubstituted trichormamides,[36] possessed the free α-OH on the FA chain. On the other hand, as apparent from our data, free α-OH is not essential for the antifungal properties of the cyclic lipopeptides. The observed species-specific antifungal properties suggest, that the effect of these modified cyclic lipopeptides (both with extended and branched FA as well as esterified) on the fungi cell is not as general as on the cytoplasmic membrane of human cells and is, moreover, connected to the overall increased hydrophobicity.

5. Conclusions

In the present study we have shown that length of the fatty acid moiety affects the dynamics of the cytotoxic effect in human cells as well the antifungal activity of the naturally occurring cyclic cyanobacterial lipopeptides puwainaphycins and minutissamides. Accordingly, we have modified the lipopeptide fatty acid moiety. In addition, we have acylated the free hydroxyl groups present in the puwainaphycin/minutissamides macrocycle to study their effect on compound bioactivity. Based on the data presented in our study we can conclude that increasing lipopeptide antifungal activity by alternation of hydrophobicity is a feasible way for new fungicides discovery, since the cytotoxicity of these modified variants is strongly diminished. While the prolongation of the FA moiety depends on the presence of a ketone or other functional group(s), the acylation of the hydroxyl group present in the majority of known membrane permeabilizing lipopeptides is an easy way of lipopeptide modification. In this study, we show esterification using DMAP as an effective approach providing satisfying yields.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This study was supported by the Ministry of Regional Development of the Czech Republic – EU Interreg ZIEL ETZ Cross-Border cooperation Czech-Bavaria project no. 41. Futher support was grated by the Ministry of Education, Youth and Sports of the Czech Republic, National Programme of Sustainability I, ID: LO1416 and MSCA IF II project (CZ.02.2.69/0.0/0.0/18_070/0010493).

References

1 S. A. Cochrane and J. C. Vederas, *Med. Res. Rev.*, 2016, **36**, 4–31.
2 G. Li, S. Kusari, C. Golz, C. Strohmann and M. Spiteller, *RSC Adv.*, 2016, **6**, 54092–54098.
3 A. Bahadoor, E. K. Brauer, W. Bosnich, D. Schneiderman, A. Johnston, Y. Aubin, B. Blackwell, J. E. Melanson and L. J. Harris, *J. Am. Chem. Soc.*, 2018, **140**, 16783–16791.
4 D. Balleza, A. Alessandrini and M. J. B. Garcia, *J. Membr. Biol.*, 2019, **252**, 131–157.
5 E. Sikorska, M. Dawgul, K. Greber, E. Ilowska, A. Pogorzelska and W. Kamysz, *Biochim. Biophys. Acta, Biomembr.*, 2014, **1838**, 2625–2634.
6 R. Magetdana and F. Peypoux, *Toxicology*, 1994, **87**, 151–174.
7 J. N. Horn, A. Cravens and A. Grossfield, *Biophys. J.*, 2013, **105**, 1612–1623.
8 C. Carrillo, J. A. Teruel, F. J. Aranda and A. Ortiz, *Biochim. Biophys. Acta, Biomembr.*, 2003, **1611**, 91–97.
9 D. Cappelletty and K. Eiselstein-McKitrick, *Pharmacotherapy*, 2007, **27**, 369–388.
10 E. Arrebola, R. Jacobs and L. Korsten, *J. Appl. Microbiol.*, 2010, **108**, 386–395.
11 H. Desmyttere, C. Deweer, J. Muchembled, K. Sahmer, J. Jacquin, F. Coutte and P. Jacques, *Front. Microbiol.*, 2019, **10**, 2327.
12 K. V. Pathak and H. Keharia, *3 Biotech*, 2014, **4**, 283–295.
13 F. Besson, F. Peypoux, G. Michel and L. Delcambe, *J. Antibiot.*, 1979, **32**, 136–140.
14 M. Deleu, J. Lorent, L. Lins, R. Brasseur, N. Braun, K. El Kirat, T. Nylander, Y. F. Dufrene and M. P Mingeot-Leclercq, *Biochim. Biophys. Acta, Biomembr.*, 2013, **1828**, 801–815.
15 P. Urajova, J. Hajek, M. Wahlsten, J. Jokela, T. Galica, D. P. Fewer, A. Kust, E. Zapomelova-Kozlikova, K. Delawska, K. Sivonen, J. Kopecky and P. Hrouzek, *J. Chromatogr. A*, 2016, **1438**, 76–83.
16 J. Mares, J. Hajek, P. Urajova, A. Kust, J. Jokela, K. Saurav, T. Galica, K. Capkova, A. Mattila, E. Haapaniemi, P. Permi, I. Mysterud, O. M. Skulberg, J. Karlsen, D. P. Fewer,

Open Access Article. Published on 16 September 2021. Downloaded on 9/19/2021 12:02:23 PM.
This article is licensed under a Creative Commons Attribution-NonCommercial 3.0 Unported Licence.

K. Sivonen, H. H. Tonnesen and P. Hrouzek, *Appl. Environ. Microbiol.*, 2019, **85**, e02675-18.
17 H. S. Kang, A. Krunic, Q. Shen, S. M. Swanson and J. Orjala, *J. Nat. Prod.*, 2011, **74**, 1597–1605.
18 H. S. Kang, M. Sturdy, A. Krunic, H. Kim, Q. Shen, S. M. Swanson and J. Orjala, *Bioorg. Med. Chem.*, 2012, **20**, 6134–6143.
19 P. Tomek, P. Hrouzek, M. Kuzma, J. Sykora, R. Fiser, J. Cerny, P. Novak, S. Bartova, P. Simek, M. Hof, D. Kavan and J. Kopecky, *Chem. Res. Toxicol.*, 2015, **28**, 216–224.
20 P. Hrouzek, M. Kuzma, J. Cerny, P. Novak, R. Fiser, P. Simek, A. Lukesova and J. Kopecky, *Chem. Res. Toxicol.*, 2012, **25**, 1203–1211.
21 J. Jokela, L. Oftedal, L. Herfindal, P. Permi, M. Wahlsten, S. O. Doskeland, S. O. and K. Sivonen, *PLoS One*, 2012, **7**, e41222.
22 L. Bornancin, F. Boyaud, Z. Mahiout, I. Bonnard, S. C. Mills, B. Banaigs and N. Inguimbert, *Mar. Drugs*, 2015, **13**, 7285–7300.
23 W. P. Frankmolle, G. Knubel, R. E. Moore and G. M. L. Patterson, *J. Antibiot.*, 1992, **45**, 1458–1466.
24 D. P. Fewer, J. Jokela, L. Heinilä, R. Aesoy, K. Sivonen, T. Galica, P. Hrouzek and L. Herfindal, *Physiol. Plant.*, 2021, 1–12.
25 J. Vestola, T. K. Shishido, J. Jokela, D. P. Fewer, O. Aitio, P. Permi, M. Wahlsten, H. Wang, L. Rouhiainen and K. Svonen, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**, E1909–E1917.
26 W. P. Frankmolle, L. K. Larsen, F. R. Caplan, G. M. L. Patterson, G. Knubel, I. A. Levine and R. E. Moore, *J. Antibiot.*, 1992, **45**, 1451–1457.
27 J. Cheel, J. Hajek, M. Kuzma, K. Saurav, I. Smykalova, E. Ondrackova, P. Urajova, D. L. Vu, K. Faure, J. Kopecky and P. Hrouzek, *Molecules*, 2018, **23**.
28 T. K. Shishido, J. Jokela, C. T. Kolehmainen, D. P. Fewer, M. Wahlsten, H. Wang, L. Rouhiainen, E. Rizzi, G. De Bellis, P. Permi and K. Sivonen, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, 13669–13674.
29 J. M. Gregson, J. L. Chen, G. M. L. Patterson and R. E. Moore, *Tetrahedron*, 1992, **48**, 3727–3734.
30 J. Mares, J. Hajek, P. Urajova, J. Kopecky and P. Hrouzek, *PLoS One*, 2014, **9**.
31 J. Cheel, P. Urajova, J. Hajek, P. Hrouzek, M. Kuzma, E. Bouju, K. Faure and J. Kopecky, *Anal. Bioanal. Chem.*, 2017, **409**, 917–930.
32 C. Nicolai and F. Sachs, *Biophys. Rev. Lett.*, 2013, **8**, 191–211.
33 K. Saurav, K. Macho, A. Kust, K. Delawska, J. Hajek and P. Hrouzek, *Folia Microbiol.*, 2019, **64**, 645–654.
34 B. Neises and W. Steglich, *Angew. Chem., Int. Ed.*, 1978, **17**, 522–524.
35 S. W. Luo, H. S. Kang, A. Krunic, W. L. Chen, J. L. Yang, J. L. Woodard, J. R. Fuchs, S. H. Cho, S. G. Franzblau, S. M. Swanson and J. Orjala, *Bioorg. Med. Chem.*, 2015, **23**, 3153–3162.
36 S. W. Luo, A. Krunic, H. S. Kang, W. L. Chen, J. L. Woodard, J. R. Fuchs, S. M. Swanson and J. Orjala, *J. Nat. Prod.*, 2014, **77**, 1871–1880.
37 J. B. MacMillan, M. A. Ernst-Russell, J. S. de Ropp and T. F. Molinski, *J. Org. Chem.*, 2002, **67**, 8210–8215.
38 I. Bonnard, M. Rolland, J. M. Salmon, E. Debiton, C. Barthomeuf and B. Banaigs, *J. Med. Chem.*, 2007, **50**, 1266–1279.
39 R. Alvarino, E. Alonso, L. Bornancin, I. Bonnard, N. Inguimbert, B. Banaigs and L. M. Botana, *Mar. Drugs*, 2020, **18**.
40 S. Son, S. K. Ko, M. Jang, J. W. Kim, G. S. Kim, J. K. Lee, E. S. Jeon, Y. Futamura, I. J. Ryoo, J. S. Lee, H. Oh, Y. S. Hong, B. Y. Kim, S. Takahashi, H. Osada, J. H. Jang and J. S. Ahn, *Mar. Drugs*, 2016, **14**.

© 2021 The Author(s). Published by the Royal Society of Chemistry

Curriculum Vitae

Personal information:
Name and surname: Jan Hájek
E-mail: zkoumi@gmail.com

Education:
Bc. – Program: Biochemistry (2007-2010), *Faculty of Sciences of Charles University in Prague*, Prague, Czech Republic

Mgr. – Program: Biological Chemistry (2010-2013), *Faculty of Science, University of South Bohemia in České Budějovice*, České Budějovice, Czech Republic

MSc. – Program: Biological Chemistry (2011-2013), *Faculty of Engineering and Natural Sciences of Johannes Kepler University*, Linz, Austria

PhD. – Program: Molecular and Cell Biology and Genetics (2014-present), *Faculty of Science, University of South Bohemia in České Budějovice*, České Budějovice, Czech Republic

Employment:
Laboratory technician (2012-2013, part-time), Laboratory of Photosynthesis (prof. RNDr. Josef Komenda, CSc., DSc.), Centre Algatech, *Institute of Microbiology of the Czech Academy of Sciences*, Třeboň, Czech Republic

PhD student (2014, part time), Laboraory of Phytoplankton Ecology (RNDr. Jan Mareš, PhD), *Institute of Hydrobiology Biology Centre, Czech Academy of Sciences*, České Budějovice, Czech Republic

Research assistant/PhD student (2013-present), Laboratory of algal Biotechnology (RNDr. Pavel Hrouzek, PhD.), Centre Algatech, *Institute of Microbiology of the Czech Academy of Sciences*, Třeboň, Czech Republic

Internships:
Research internship in research group David Fewer (7.4. – 5.5. 2015), University of Helsinki, Helsinky, Finland, supervisor Kaarina Sivonen.

Research internship in research group of Fatima Rivas (25.4.-24.6. 2016), St.Judes Children Research Hospital, Memphis, Tennessee, U.S., supervisor Taotao Ling.

Research internship in research group of Herbert Riepl (14.10.-16.12.2019), Campus Straubing for Biotechnology and Sustainability, Straubing, Austria, supervisor Sebastian Bieringer

Conferences:

HÁJEK J., URAJOVÁ, P. and HROUZEK, P.: Production of diverse ß-amino-fatty acid containing lipopeptides by soil cyanobacteria of genus *Cylindrospermum*. International Conference on Antimicrobial Research, 2014, Madrid, Spain

HÁJEK, J., URAJOVÁ P. and HROUZEK, P.: Biological activity of Puwainaphycin F and its congereners. Trend in Natural Products Research, 2017, Olomouc, Czech Republic

HÁJEK, J.: Cytotoxicity of cyclic lipopeptides Puwahiaphycins and minutissamides. International Summer School of Natural Products, 2017, Naples, Italy.

Publications:

MAREŠ, J., **HÁJEK, J.**, URAJOVÁ, P., KOPECKÝ, J. and HROUZEK, P. A Hybrid Non-Ribosomal Peptide/Polyketide Synthetase Containing Fatty-Acyl Ligase (FAAL) Synthesizes the β-Amino Fatty Acid Lipopeptides Puwainaphycins in the Cyanobacterium *Cylindrospermum alatosporum*. *PLoS One*, 2014, 9(11), e111904.

URAJOVÁ, P., **HÁJEK, J.**, WAHLSTEN, M., JOKELA, J., GALICA, T., FEWER, D.P., KUST, A, ZAPOMĚLOVÁ-KOZLÍKOVA, E., DELAWSKÁ, K., SIVONEN, K. and HROUZEK, P. A liquid chromatography-mass spectrometric method for the detection of cyclic beta-amino fatty acid lipopeptides. *Journal of Chromatography A*, 2016, 1438, 76-83.

VORÁČOVÁ, K., **HÁJEK, J.**, MAREŠ, J., URAJOVÁ, P., KUZMA, M., CHEEL, J., VILLUNGER, A., KAPUSCIK, A., BALLY, M., NOVÁK, P., KABELÁČ, M., KRUMSCHNABEL, G., LUKEŠ, M., VOLOSHKO, L., KOPECKÝ, J., and HROUZEK, P. The cyanobacterial metabolite nocuolin a is a natural oxadiazine that triggers apoptosis in human cancer cells. *PLoS One*, 2017, 12(3), e0172850.

CHEEL, J., URAJOVÁ, P., **HÁJEK, J.**, HROUZEK, P., KUZMA, M., BOUJU, E., FAURE, K. and KOPECKÝ, J. Separation of cyclic lipopeptide puwainaphycins from cyanobacteria by countercurrent chromatography combined with polymeric resins and HPLC. *Analytical and Bioanalytical Chemistry*, 2017, 409(4), 917-930.

VU, D.L., RANGLOVÁ, K., **HÁJEK, J.** and HROUZEK, P. Quantification of methionine and selenomethionine in biological samples using multiple reaction monitoring high performance liquid chromatography tandem mass spectrometry (MRM-HPLC-MS/MS). *Journal of Chromatography B*, 2018, 1084, 36-44.

KUST, A., MAREŠ, J., JOKELA, J., URAJOVÁ, P., **HÁJEK, J.**, SAURAV, K., VORÁČOVÁ, K., FEWER, D.P., HAAPANIEMI, E., PERMI, P., ŘEHÁKOVÁ, K., SIVONEN, K. and HROUZEK, P. Discovery of a Pederin Family Compound in a Nonsymbiotic Bloom-Forming Cyanobacterium. *ACS Chemical Biology*, 2018, 13(5), 1123-1129.

SKOTNICOVÁ, P., SOBOTKA, R., SHEPHERD, M., **HÁJEK, J.**, HROUZEK, P. and TICHÝ, M. The cyanobacterial protoporphyrinogen oxidase HemJ is a new b-type heme

protein functionally coupled with coproporphyrinogen III oxidase. *Journal of Biological Chemistry*, 2018, 293(32), 12394-12404.

CHEEL, J., **HÁJEK, J.**, KUZMA, M., SAURAV, K., SMÝKALOVÁ, I., ONDRÁČKOVÁ, E., URAJOVÁ, P., VU, D.L., FAURE, K., KOPECKÝ, J. and HROUZEK, P. Application of HPCCC Combined with Polymeric Resins and HPLC for the Separation of Cyclic Lipopeptides Muscotoxins A-C and Their Antimicrobial Activity. *Molecules*, 2018, 23(10), E2653.

MAREŠ, J., **HÁJEK, J.**, URAJOVÁ, P., KUST, A., JOKELA, J., SAURAV, K., GALICA, T., ČAPKOVÁ, K., MATTILA, A., HAAPANIEMI, E., PERMI, P., MYSTERUD, I., SKULBERG, O.M., KARLSEN, J., FEWER, D.P., SIVONEN, K., TONNESEN, H.H. and HROUZEK, P. Alternative Biosynthetic Starter Units Enhance the Structural Diversity of Cyanobacterial Lipopeptides. Applied and Environmental Microbiology, 2019, 85(4), e02675-18.

SAURAV, K., MACHO, M., KUST, A., DELAWSKÁ, K., **HÁJEK, J.** and HROUZEK, P. Antimicrobial activity and bioactive profiling of heterocytous cyanobacterial strains using MS/MS-based molecular networking. Folia Microbiologica, 2019, 64(5), 645-654.

VAŠÍČEK, O., **HÁJEK, J.**, BLÁHOVÁ, L., HROUZEK, P., BABICA, P., KUBALA, L. and ŠINDELÁŘOVÁ, L. Cyanobacterial lipopeptides puwainaphycins and minutissamides induce disruptive and pro-inflammatory processes in Caco-2 human intestinal barrier model. *Harmful Algae*, 2020, 96,101849.

NUPUR, KUZMA, M., **HÁJEK, J.**, HROUZEK, P., GARDINER, A.T., LUKEŠ, M., MOOS, M., ŠIMEK, P. and KOBLÍŽEK, M. Structure elucidation of the novel carotenoid gemmatoxanthin from the photosynthetic complex of Gemmatimonas phototrophica AP64. *Scientific Reports*, 2021, 11, 159764.

ČINČAROVÁ, D., **HÁJEK, J.**, DOBŘICHOVSKÝ, M., LUKEŠ, M. and HROUZEK, P. Recommendations on the quantitative analysis of pheophorbides, photosensitizers present in algal biomass intended as food supplement. Algal Research, 2021, 56, 102298

GALICA, T., BARBONE, N., MAREŠ, J., KUST, A., CASO, A., ESPOSITO, G., SAURAV, K., **HÁJEK, J.**, ŘEHÁKOVÁ, K., URAJOVÁ, P., CONSTANTINO, V. and HROUZEK, P. Cyanochelins, an Overlooked Class of Widely Distributed Cyanobacterial Siderophores, Discovered by Silent Gene Cluster Awakening. *Applied and Environmental Microbiology*, 2021, 87 (7), e03128-20

HÁJEK, J., BIERINGER, S., VORÁČOVÁ, K., MACHO, M., SAURAV, K., DELAWSKÁ, K. FIŠER, R., MIKUŠOVÁ, G., CHEEL, J. FEWER, D.P., VU, D.L., PAICHLOVÁ, J., RIEPL, H. and HROUZEK, P. Semi-synthetic puwainaphycin/minutissamide cyclic lipopeptides with improved antifungal activity and limited cytotoxicity. *RCS Advances*, 2021,**11**, 30873-30886

© for non-published parts Jan Hájek

zkoumi@gmail.com

Structural analysis of cyanobacterial cyclic lipopeptides regarding their biosynthesis and cell interaction
Ph.D. Thesis Series, 2021, No. 18

All rights reserved
For non-commercial use only

Printed in the Czech Republic by Typodesign
Edition of 10 copies

University of South Bohemia in České Budějovice
Faculty of Science
Branišovská 1760
CZ-37005 České Budějovice, Czech Republic

Phone: +420 387 776 201
www.prf.jcu.cz, e-mail: sekret-fpr@prf.jcu.cz