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# Chemical diversity and cellular effects of antifungal cyclic lipopeptides from cyanobacteria

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## Abstract

Cyanobacteria produce a variety of chemically diverse cyclic lipopeptides with potent antifungal activities. These cyclic lipopeptides have an amphipathic structure comprised of a polar peptide cycle and hydrophobic fatty acid side chain. Many have antibiotic activity against a range of human and plant fungal pathogens. This review article aims to summarize the present knowledge on the chemical diversity and cellular effects of cyanobacterial cyclic lipopeptides that display antifungal activity. Cyclic antifungal lipopeptides from cyanobacteria commonly fall into four structural classes; hassallidins, puwainaphycins, laxaphycins, and anabaenolysins. Many of these antifungal cyclic lipopeptides act through cholesterol and ergosterol-dependent disruption of membranes. In many cases, the cyclic lipopeptides also exert cytotoxicity in human cells, and a more extensive examination of their biological activity and structure–activity relationship is warranted. The hassallidin, puwainaphycin, laxaphycin, and anabaenolysin structural classes are unified through shared complex biosynthetic pathways that encode a variety of unusual lipoinitiation mechanisms and branched biosynthesis that promote their chemical diversity. However, the biosynthetic origins of some cyanobacterial cyclic lipopeptides and the mechanisms, which drive their structural diversification in general, remain poorly understood. The strong functional convergence of differently organized chemical structures suggests that the production of lipopeptide confers benefits for their producer. Whether these benefits originate from their antifungal activity or some other physiological function remains to be answered in the future. However, it is clear that cyanobacteria encode a wealth of new cyclic lipopeptides with novel biotechnological and therapeutic applications.

## 1 | INTRODUCTION

Cyclic lipopeptides with long fatty acid side chains are a common group of natural products synthesized by a range of bacterial genera (Arima et al., 1968; Cochrane & Vederas, 2016). Cyclic lipopeptides typically possess fatty acids with β-hydroxy or β-amino residues and form macrolactone and macrolactam rings (Figure 1). These compounds have received considerable attention for their often potent antagonistic activity against a range of human and plant pathogenic organisms (Ongena & Jacques, 2008). Cyclic lipopeptides include membrane-active compounds, such as surfactin, fengycin, iturins, and daptomycins, some of which find use as surfactants, antifungal agents,

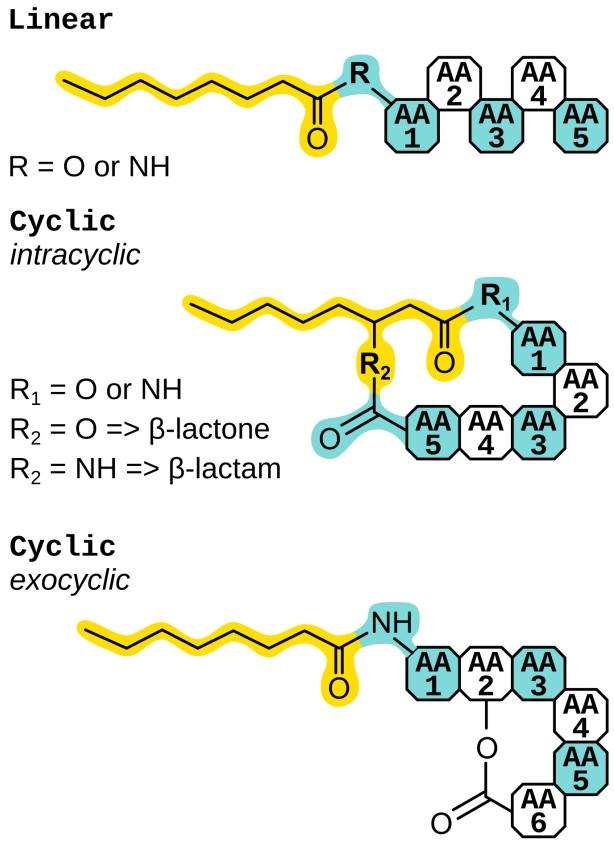


**FIGURE 1** A schematic figure showing generalized chemical structures for linear and cyclic lipopeptides. The substituent responsible for cyclization of the intracyclic lipopeptides may be present in other positions than the β-carbon of the modified fatty acid residue. AA, amino acid

or antibiotics (Arima et al., 1968; Taylor & Palmer, 2016). Their complex chemical structure makes their physico-chemical properties more diverse compared to other cyclic peptides, and they are therefore likely to have unique properties and bioactivities. Cyclic lipopeptides can harbor different bioactivities towards prokaryotic and eukaryotic cells (Minagawa et al., 2011; Shishido, Humisto, et al., 2015). The differing specificities can be mostly ascribed to the amphipathic molecular nature of many cyclic lipopeptides (Ongena & Jacques, 2008). This promotes integration into membranes in the target organisms but can also favor interaction with lipid-associated structures, enzymes, or other proteins that have amphiphilic substrates. Such cyclic lipopeptides are also used for biotechnological applications, for instance, as biocontrol agents in plant diseases due to their antagonistic activity against a wide range of potential phytopathogens (Malviya et al., 2020; Ongena & Jacques, 2008). Cyanobacteria are an emerging source of structurally diverse cyclic lipopeptides (Dittmann et al., 2015; Demay et al., 2019; Jones et al., 2021; Niedermeyer, 2015; Shishido, Humisto, et al., 2015), some of which have already been tested in disease models and show promise as drug candidates. However, there is a need for deeper knowledge on how cyclic lipopeptides affect biological processes and structure–activity relationship studies to be able to identify possible drug leads and new fields for applications. Furthermore, due to their complex chemical structure, understanding their biosynthesis might aid us in generating variants that have improved activities. In this review article, we present the current knowledge on the structural diversity, biosynthesis, and bioactivities of cyclic antifungal lipopeptides from cyanobacteria, to highlight their potential beneficial use in pharmaceutical and biotechnological applications.

## 2 | CHEMICAL STRUCTURE AND BIOSYNTHESIS OF CYANOBACTERIAL LIPOPEPTIDES

The chemical structure of cyanobacterial lipopeptides follows the chemical structures found in other prokaryotic lineages. Linear lipopeptides, cyclic lipopeptides, and cyclic lipopeptides with linear exocyclic amino-acid moieties have been reported from cyanobacteria, as exemplified by jahanyne (Iwasaki et al., 2015), laxaphycin (Frankmölle, Knubel, et al., 1992), and hassallidin (Vestola et al., 2014), respectively. The fatty acyl moiety is typically connected to a peptide backbone via a peptide bond in both linear and cyclic lipopeptides with exocyclic peptide moieties (see the schematic depiction in Figure 1). In many cyclic lipopeptides, the fatty acid residue is intercalated directly into the peptide macrocycle (Frankmölle, Knubel, et al., 1992; Hrouzek et al., 2012; Vestola et al., 2014). This can happen via two peptide bonds if the fatty acid residue is functionalized by an amino group, typically at the β carbon of the fatty acid, or by a peptide and an ester bond in cases where the fatty acid residue is functionalized by a hydroxyl group instead (Figure 1). Cyclic lipopeptides with fatty acid intercalated in the peptide cycle are commonly reported to possess antifungal properties (Demay et al., 2019; Jones et al., 2021; Niedermeyer, 2015; Shishido, Humisto, et al., 2015). Anabaenolysins, hassallidins, balticidins, puwainaphycins, minutissamides, muscotoxins, trichormamides, lobocyclamides, scytocyclamides, and laxaphycins have proved to possess significant antifungal effects, usually paired with the cytotoxic activities in human cells (Frankmölle, Knubel, et al., 1992; Gregson et al., 1992; Jokela et al., 2012; Neuhof et al., 2005). The structure and the fatty acid moiety of cyanobacterial antifungal cyclic lipopeptides differ substantially. A fully saturated fatty acid residue can be found in puwainaphycins, minutissamides, muscotoxins, and laxaphycins (Frankmölle, Knubel, et al., 1992; Gregson et al., 1992; Neuhof et al., 2005), while a set of polyunsaturated fatty acid is found in anabaenolysins (Jokela et al., 2012).

Bacterial cyclic lipopeptides are synthesized through complex secondary metabolic pathways (Chooi & Tang, 2010; Roongsawang et al., 2010; Zhong et al., 2021). Fatty acyl chains are the first monomers incorporated into the peptidyl backbone via a process known as lipoinitiation (Chooi & Tang, 2010; Zhong et al., 2021). The lipid residue can be incorporated in a number of ways (Figure 2, Chooi & Tang, 2010). The lipopeptides are typically extended through the successive additions of both proteinogenic and nonproteinogenic amino acids by nonribosomal peptide synthetases (NRPSs) (Roongsawang

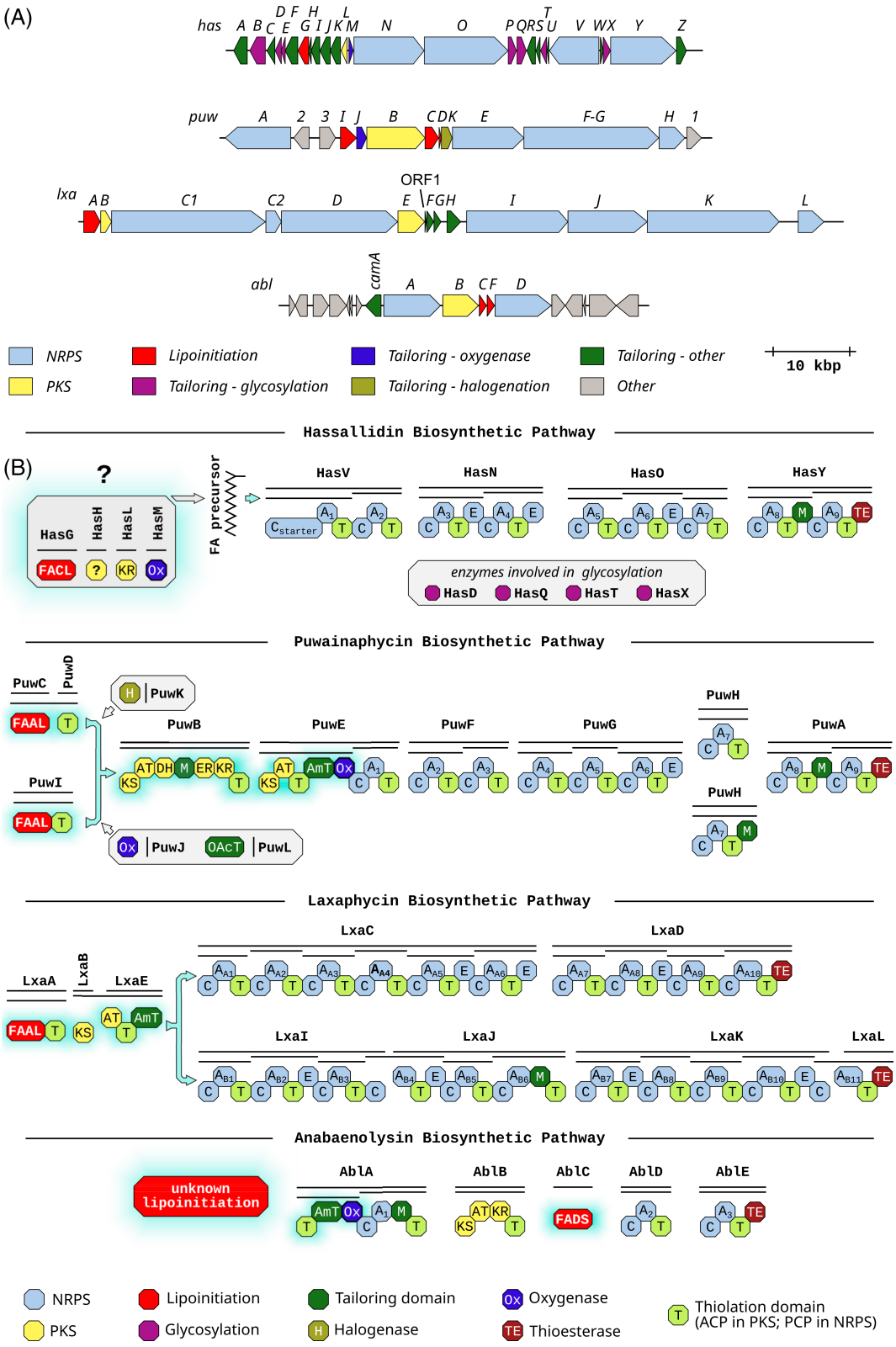


**FIGURE 2** Biosynthesis of cyanobacterial cyclic lipopeptides that display antifungal activity. (A) Organization of the hassallidin (*has*), puwainaphycin (*puw*), laxaphycin (*lxa*), and anabaenolysin (*abl*) biosynthetic gene clusters. (B) Hassallidin, puwainaphycin, laxaphycin, and anabaenolysin biosynthetic schemes showing variation in the lipoinitiation mechanisms, unusual branched biosynthesis, and tailoring enzymes to achieve the observed chemical variation. Enzymes involved in fatty acid biosynthesis and modification are highlighted in cyan. A adenylation domain; AmT, aminotransferase domain; AT, acyltransferase domain; C, condensation domain; DH, dehydratase domain, ER, enoylreductase domain; E, epimerase domain; FAAL, fatty acyl-AMP ligase; FACL, fatty acyl CoA ligases; FADS, fatty acid desaturase; KR, ketoreductase domain; KS, ketosynthtase domain; M, methylation domain; Ox, oxidase domain; T, thiolation domain; TE, thioesterase domain

et al., 2010). NRPSs are multimodular enzymes that catalyze the biosynthesis of diverse peptides with a wide variety of activities (Sieber & Marahiel, 2005). NRPS modules, which typically include a condensation (C) domain, an adenylation (A) domain, and a thiolation (T) domain, incorporate a single amino acid into a growing peptide chain, forming the peptidyl backbone by sequential condensations (Sieber & Marahiel, 2005). Bacterial lipopeptide biosynthesis may also involve modular polyketide synthase (PKS) enzymes, which can further increase the structural diversity of synthesized products, either by extending and tailoring the fatty acid chain or by modification of amino acids incorporated into the macrocycle. The chemical diversity of such natural products can be further increased through enzymes that supply dedicated precursor substrates or catalyze tailoring reactions (Fewer & Metsä-Ketelä, 2020). Bacterial lipopeptide biosynthetic pathways are typically organized in self-contained gene clusters that can be over 100 kb in length and encode over 40 biosynthetic enzymes (Fewer & Metsä-Ketelä, 2020; Heinilä et al., 2020). Biosynthetic pathways have been characterized for four structural classes of antifungal cyclic lipopeptides from cyanobacteria; hassallidins, laxaphycins, puwainaphycins, and anabaenolysins (Figure 2).

## 3 | HASSALLIDINS AND BALTICIDINS

Hassallidins A–E are cyclic glycosylated lipopeptides produced by a range of cyanobacteria with a fatty acid chain, a peptide ring of eight amino acids, an exocyclic amino acid, and 1–3 sugar moieties (Neuhof et al., 2005; Neuhof, Schmieder, et al., 2006; Pancrace et al., 2017; Vestola et al., 2014). Hassallidin chemical variants differ considerably in fatty acid length (C14–C18), as well as in glycosylation of the molecule, which takes place at the Thr residues of the peptide cycle as well as at the β-hydroxyl residue of the modified fatty acid (Figure 3, Table S1). Closure of the eight-membered peptide macrocycle via an ester bond links the exocyclic Thr residue to the fatty acid moiety (Neuhof et al., 2005). Hassallidins were first reported from a strain of the genus *Hassallia* (Neuhof et al., 2005; Neuhof, Schmieder, et al., 2006). Hassallidins have been subsequently reported from the genera *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Nostoc*, *Planktothrix*, and *Tolypothrix* (Neuhof et al., 2005; Pancrace et al., 2017; Vestola et al., 2014). Balticidins A–D share near chemical identity with hassallidins identified from *Anabaena cylindrica* Bio33 from the Baltic Sea (Bui et al., 2014) and clearly belong to the same family as hassallidins. Members of the hassallidin family share potent antifungal activity (Bui et al., 2014; Neuhof et al., 2005; Neuhof, Schmieder, et al., 2006; Vestola et al., 2014), including antifungal activity against several opportunistic pathogenic yeasts on humans (Neuhof et al., 2005; Vestola et al., 2014).

Hassallidins are produced using a complex nonribosomal biosynthetic pathway encoded in 48–59 kb biosynthetic gene clusters (Pancrace et al., 2017; Vestola et al., 2014). The hassallidin biosynthetic pathway encodes four NRPS proteins (HasO, HasN, HasV, and HasY), comprising nine modules catalyzing the incorporation of amino

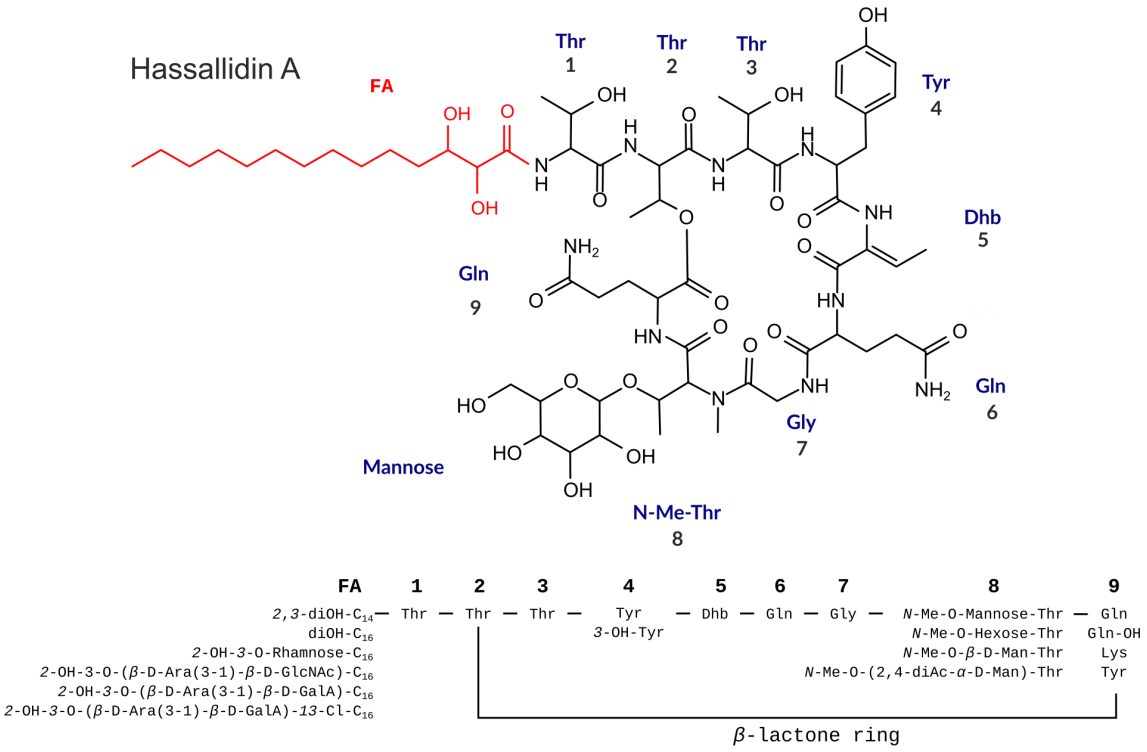


**FIGURE 3** Structure of hassallidin A and schematic general structure of the hassallidin type of cyclic lipopeptides. Hassallidins are cyclic glycosylated lipopeptides with a fatty acid chain, a peptide ring of eight amino acids, an exocyclic amino acid, and 1–3 sugar moieties. See Table S1 for the exact stereochemistry of particular residues. Ara, arabinose; Dhb, dehydrobutyrine; FA, fatty acid; GalA, galacturonic acid; GlcNAc, N-acetylglucosamine, man, mannose

acids into the hassallidin peptide backbone via a thiotemplate mechanism (Vestola et al., 2014). Interestingly, hassallidin biosynthetic pathways are rich in glycosyltransferases (HasB, HasD, HasE, HasP, HasQ, HasT, and HasX) that could potentially catalyze the incorporation of sugars (Vestola et al., 2014). HasR has been reported as an acyltransferase that may catalyze the acetylation of hassallidin sugars from *Anabaena* strains (Vestola et al., 2014). The first step in the biosynthesis of hassallidins is postulated to be the *N*-acylation of the exocyclic Thr by HasV (Vestola et al., 2014). The *N*-acylation of lipopeptides using starter condensation domains has been reported from other lipopeptide biosynthetic pathways, including surfactin, lichenysin, fengycin, and arthrofactin (Zhong et al., 2021). A putative biosynthetic scheme for the lipidation of hassallidins implicates three proteins (HasG, HasH, and HasL) in the addition of lipids (Figure 2, Vestola et al., 2014). The combination of variation in the chain length of the fatty acid moiety and lipoinitiation in the glycosylation patterns leads to extensive variation in hassallidin chemical structure even within the same strain (Vestola et al., 2014).

Hassallidins are so far the only known cyanobacterial antifungal cyclic lipopeptides with an exocyclic peptide moiety (Neuhof et al., 2005; Neuhof, Schmieder, et al., 2006). In this sense, they are analogous to the bacterial lipopeptide fengycin, which is shown to act on both bacteria and fungi but does not affect mammalian cells (de Souza Freitas et al., 2020; Desmyttere et al., 2019; Lin et al., 2020). Hassallidins were reported to possess promising antifungal activities with low minimum inhibitory concentration values ranging from 0.29 to 8.0 μM against *Candida* strains (Neuhof et al., 2005; Neuhof, Schmieder, et al., 2006; Neuhof, Seibold, et al., 2006; Shishido, Humisto, et al., 2015; Shishido, Jokela, et al., 2015). However, it was later shown that hassallidin D variants were equally potent inducers of cell death in mammalian cells (Humisto et al., 2019). The cell death in the mammalian cell was lytic, meaning that cell death was caused by disruption of the cell membrane as evidenced by rapid internalization of the propidium iodide counterstain into cells (Humisto et al., 2019). However, mitochondria appeared to be functional at concentrations that caused disruption of the outer cell membrane (Humisto et al., 2019). This was shown to be due to the presence of low amounts of cholesterol in the mitochondria, since liposomes containing only phospholipids showed higher tolerance to hassallidin D compared to liposomes consisting of both phospholipids and cholesterol (Humisto et al., 2019). The antifungal effect of hassallidin may thus be attributed to the presence of ergosterol in the fungal membrane because liposomes containing ergosterol in the lipid membrane were equally sensitive to hassallidin D as those containing cholesterol (Humisto et al., 2019). It was shown that the $EC_{50}$ for internalization of propidium idodide after hassallidin-exposure in *Candida* was slightly higher than that of mammalian cells, which was believed to be due to the presence of the fungal cell wall (Humisto et al., 2019). Computer simulation of the interaction of hassallidin with membranes with or without cholesterol suggested that the strict organization of membranes lacking cholesterol hampered the insertion of the acyl-chain of hassallidin D into the membrane bilayer (Humisto et al., 2019). However, any temperature dependency with respect to the transition temperature of the membranes from the rigid gel phase to the more disorganized liquid crystalline phase, has not been studied. The difference in membrane organization can also affect the ability of biodetergents to insert into the membrane, in addition to the presence of cholesterol.

## 4 | PUWAINAPHYCINS AND MINUTISSAMIDES

Puwainaphycins A–G and minutissamides A–L are structurally homologous amphipathic cyclic lipopeptides featuring a β-amino fatty acid and a nine-membered peptide ring (Gregson et al., 1992; Hrouzek et al., 2012; Kang et al., 2011; Kang et al., 2012). These two cyclic lipopeptides are distinguished by differences in the lengths and substitutions of the fatty acid chain (Figure 4, Table S2). The peptide sequence includes mostly proteinogenic amino-acids together with modified *N*-methyl asparagine (*N*MeAsn) and dehydrobutyric acid (Dhb) (Figure 4). All reported chemical variants contain a stable *N*MeAsn-Pro-(FA)-Val-Dhb motif adjacent to the fatty acid (Figure 4). Furthermore, both lipopeptide types exhibit considerable variability in terms of length and functionalization of the fatty acid side chain (Mareš et al., 2019). Puwainaphycins A–G (Gregson et al., 1992; Hrouzek et al., 2012) and minutissamides A–L (Kang et al., 2011; Kang et al., 2012) have been reported from the genera *Cylindrospermum*, *Symplocastrum*, and *Anabaena* (Mareš et al., 2019). A wide range of bioactivities has been reported for puwainaphycins and minutissamides, including cardiovascular activity, antiproliferative activity, and antifungal activity (Gregson et al., 1992; Hrouzek et al., 2012; Kang et al., 2011; Kang et al., 2012).

Puwainaphycins and minutissamides share a common biosynthetic origin involving a hybrid NRPS/PKS enzyme complex accompanied by a number of tailoring enzymes (Mareš et al., 2019). Lipointiation in the puwainaphycin and minutissamide biosynthetic pathway involves unusual fatty acyl-AMP ligase (FAAL) starter units (Figure 2, Mareš et al., 2014, 2019). Puwainaphycin and minutissamide chemical diversity is generated largely by the presence of two alternate fatty acyl-AMP ligase starter units, PuwC, and PuwI (Figure 2), which allow the incorporation of fatty acids of differing length (Mareš et al., 2019). Such alternate branched biosynthesis is rare in the biosynthesis of natural products and further enhanced by the unusually broad specificity for fatty acids substrates of various lengths by PuwI (Mareš et al., 2019). Following activation, the fatty acid residue is extended via two PKS modules encoded by PuwB and PuwE, which also introduce additional methyl, hydroxyl, and amino substitutes, of which the latter allows cyclization (Mareš et al., 2014). The incorporation of amino acids into the puwainaphycin/minutissamide peptide backbone is catalyzed by nine NRPS modules found in proteins PuwA, PuwE, PuwF, PuwG, and PuwH (Mareš et al., 2014, 2019). In addition, the PuwK putative halogenase, the PuwL *O*-acetyltransferase, and the PuwJ monooxygenase, are present in the biosynthetic gene clusters of some strains and contribute to the chemical diversity of this family of cyclic lipopeptides (Mareš

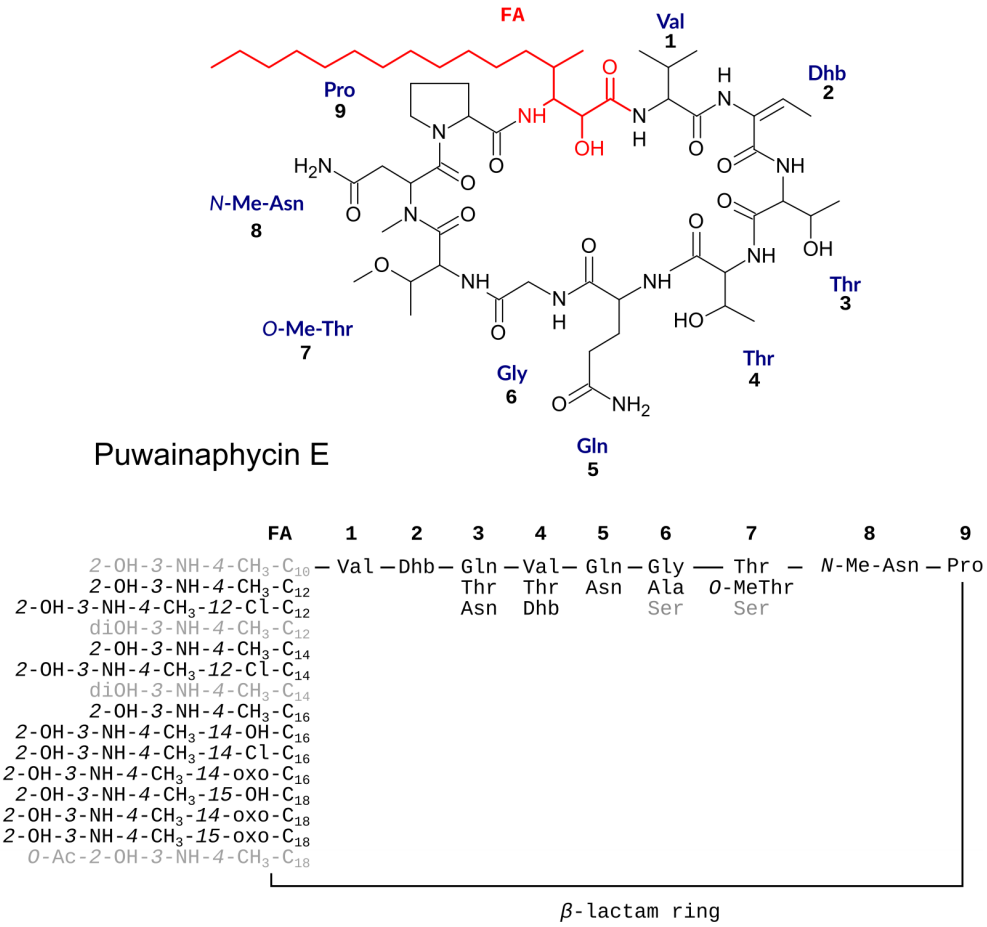


**FIGURE 4** Structure of puwainaphycin E and schematic general structure of the puwainaphycin and minutissamide type of cyclic lipopeptides. Puwainaphycins and minutissamides are structurally homologous amphipathic cyclic lipopeptides featuring a β-amino fatty acid and a nine-membered peptide ring. Residues depicted in gray were described using high-resolution mass spectrometry only. In addition to depicted structures also minor variants bearing fatty acids with odd carbon number ($C_{11}$,$C_{13}$, and $C_{17}$) were detected (Mareš et al., 2019). See Table S2 for the exact stereochemistry of particular residues. Dhb, dehydrobutyric acid; FA, fatty acid

et al., 2019). These fatty acid-tailoring enzymes, in combination with the alternate broad-specificity FAAL starter units, results in the observed high chemical diversity of the hydrophobic moiety of puwainaphycin/minutissamide, on which chloro-, acetyl-, and oxo−/hydroxyl substituents can be present in various combinations (Figure 4, Table S2).

Puwainaphycins have antagonistic effects on a variety of fungal strains. Puwainaphycin A displays weak antifungal activity against *Candida* strains (Mareš et al., 2019), but quite prominent inhibition effects were observed against the plant and human pathogens *Alternaria alternata*, and *Aspergillus fumigatus*, with minimum inhibitory concentration values of 0.58, and 2.37 μg $mL^{-1}$ (Hrouzek, 2021). Puwainaphycins were shown to affect the membranes of other eukaryotic cells, including human cells, in vitro (Hrouzek et al., 2012; Vašíček et al., 2020). The cytotoxic effect was studied in detail in case of puwainaphycin F/G which induce $Ca^{2+}$ influx and necrotic cell death eventually leading to vast membrane damage and LDH-leakage with $EC_{50}$ values between 1 and 10 μM (Hrouzek et al., 2012; Vašíček et al., 2020). The fact that a similar phenotype was observed in HeLa (human cervical cells), Caco2 (human colon adenocarcinoma) as well as primary human fibroblasts suggests that puwainaphycin exerts a rather general and nonspecific cytotoxic effect (Hrouzek et al., 2012; Vašíček et al., 2020). The cytotoxic effect is clearly connected to their membrane effect as these molecules were found to act on large unilamellar vesicles composed of lipids only (Tomek et al., 2015). Cells treated with puwainaphycin F/G undergo a rapid tyrosine phosphorylation (Hrouzek et al., 2012) and stimulate the production of the pro-inflammatory interleukin 8 (Vašíček et al., 2020). However, whether these cellular responses are a secondary consequence of the membrane effects of the lipopeptides, or due to other cellular targets is not yet known. Puwainaphycin F and minutissamide C also affected an immune response and were pro-inflammatory at noncytotoxic concentrations in an intestinal barrier model (Vašíček et al., 2020). It was speculated that these lipopeptides might increase the toxicity of cyanobacterial hepatotoxin microcystin (Vašíček et al., 2020), which could indicate that lipopeptides potentiate the toxic effect of the hepatotoxins. This potentiation could be due to loss of integrity in the intestinal wall, caused by the inflammatory response, but further studies need to be conducted to reveal the connection between these cyclic lipopeptides and microcystin poisoning.

## 5 | LAXAPHYCINS

The laxaphycins constitute a large family of cyclic lipopeptides, which are characterized by a rare β-amino fatty acid with a short linear chain of eight or 10 carbons (Figure 5, Tables S3, S4). Thirty-three reported lipopeptides from the genera *Scytonema*, *Anabaena*, *Hormothamnion*,

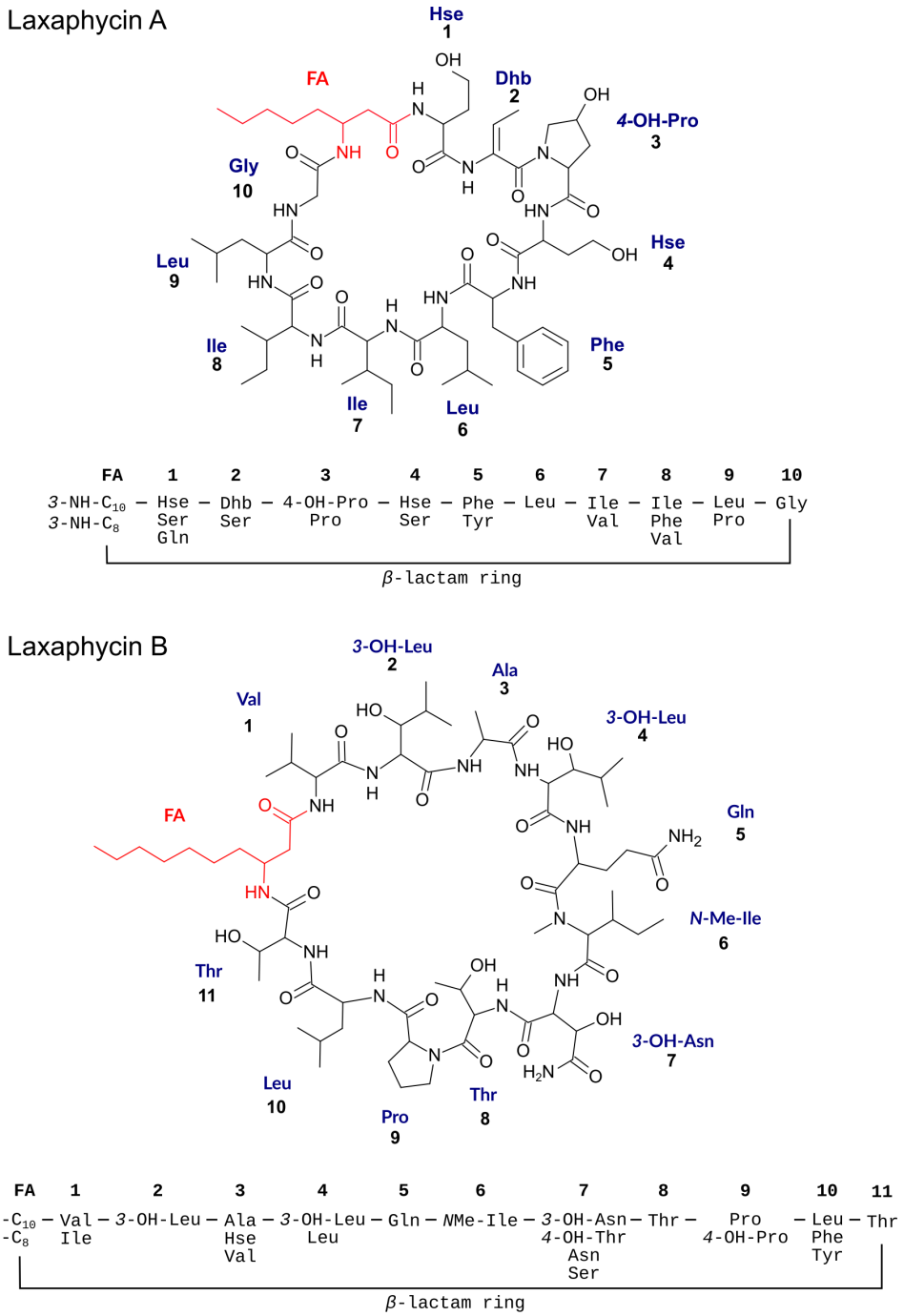


**FIGURE 5** Structure of laxaphycin A and B and a schematic general structure of the laxaphycin type of cyclic lipopeptides. The laxaphycins are a large family of cyclic lipopeptides, which are characterized by a rare β-amino fatty acid with a short linear chain of 8 or 10 carbons and complex nomenclature. See Tables S3, S4 for the exact stereochemistry of particular residues. Dhb, dehydrobutyric acid; FA, fatty acid; Hse, homoserine

*Trichormus*, *Lyngbya*, and *Oscillatoria* can be assigned to laxaphycin family of cyclic lipopeptides (Heinilä et al., 2020). Laxaphycins divide into two distinct groups, cyclic undecapeptides and dodecapeptides, the major representative of each class being laxaphycin A and B, respectively (Frankmölle, Larsen, et al., 1992; Luo et al., 2015). Laxaphycin A and B compounds are frequently isolated from the same cyanobacterium. Laxaphycin nomenclature is complicated because new variants are usually named after the producing organisms even though the two distinct macrocycle types are considered chemical variants of a single family (Figure 5, Tables S3, S4). Laxaphycin A and B chemical variants contain a range of nonproteinogenic amino acids, including 4-hydroxyproline, Dhb, 3-hydroxyleucine, hydroxythreonine, and hydroxyasparagine (Figure 5, Tables S3, S4). Laxaphycin A variants are characterized by segregation of hydrophobic and hydrophilic residues, while laxaphycin B variants have alternating hydrophobic and hydrophilic residues (Cai et al., 2018). However, the only common structure between the two classes is the β-amino fatty acid and amino acids compared by position to position are not only different but belong in many cases to opposite polar groups (Tables S3, S4). The chemical structures of the laxaphycin A and B types are highly conserved and act in synergy to produce antifungal and antiproliferative activity (Cai et al., 2018; Frankmölle, Knubel, et al., 1992).

Laxaphycins were predicted to be produced by distinct hybrid NRPS/PKS biosynthetic pathways based on their chemical structures (Bornancin et al., 2015, 2019). However, laxaphycin A and B chemical variants are now known to be produced simultaneously through an unusual branched biosynthetic pathway encoding a hybrid NRPS/PKS enzyme complex encoded in a single 96-kb biosynthetic gene cluster, as reported for scytocyclamides A and B (Heinilä et al., 2020). Lipointiation in the scytocyclamide biosynthetic pathway is catalyzed by the LxaA fatty acyl-AMP ligase that is predicted to activate a fatty acid and load it to an acyl carrier protein (Figure 2, Heinilä et al., 2020). Following activation, the fatty acid unit is extended by a PKS module, which is split over the LxaB and LxaE proteins (Heinilä et al., 2020). The biosynthesis of scytocyclamide A and B variants branches at this point (Figure 2, Heinilä et al., 2020). LxaC-D encodes 10 NRPS modules that extended the fatty acid by 10 proteinogenic and nonproteinogenic amino acids to produce scytocyclamide A variants (Heinilä et al., 2020). Alternatively, LxaI-L encodes 11 NRPS modules that extend the fatty acid by 11 proteinogenic and nonproteinogenic amino acids to produce scytocyclamide B variants (Heinilä et al., 2020). Scytocyclamide A and B variants contain 3-OHLeu, 3-OHAsn, 4-OHPro, which are believed to be supplied to the synthetase as precursors, while Dhb is produced through the dehydration of Thr by the $LxaC_3$ condensation domain (Heinilä et al., 2020). Laxaphycin A and B chemical variants frequently contain homoserine (Frankmölle, Knubel, et al., 1992; Luo et al., 2015). However, the scytocyclamide biosynthetic pathway does not encode an obvious enzyme for the supply of homo amino acids to the pathway, and homoserine is likely to be supplied to the laxaphycin peptide synthetase as an intermediate of amino acid biosynthesis. Cyclization of the scytocyclamide A and B peptide intermediates is achieved by the thioesterase domain of LxaD and LxaL, respectively (Heinilä et al., 2020).

Individual laxaphycin chemical variants are reported to have weak antifungal activity (Frankmölle, Knubel, et al., 1992). However, a crude mixture of all chemical variants (A–E) of both laxaphycin types leads to a substantial potentiation of their antifungal effect (Frankmölle, Knubel, et al., 1992). This study also showed that laxaphycin B and C had the most potent antifungal activity, with activity towards most or all fungi tested (Frankmölle, Knubel, et al., 1992). These also showed moderate activity towards two human cell lines, whereas the other variants (laxaphycin A, D, and E) showed low or no activity (Frankmölle, Knubel, et al., 1992). The biological effects of the laxaphycins in mammalian cells have been investigated and differ somewhat from the detergent-like activity seen in other lipopeptides. It appears that there is a large variation in the potency of the different laxaphycins towards mammalian cells, with reports of $EC_{50}$ values as low as 0.8 μM in for instance, Laxaphycin B3 (Alvariño et al., 2020) and 0.6 μM for [L-$Val^8$] laxaphycin A (Bornancin et al., 2019). Interestingly, linear laxaphycins appeared to be nontoxic (Alvariño et al., 2020). Laxaphycin B and B3 induced apoptotic cell death as evidenced by annexinV and propidium idodide staining as well as activation of caspase 3, but was also found to activate the autophagic machinery (AMP-activated kinase and LC3 affection) in SH-SY5Y neuroblastoma cells (Alvariño et al., 2020). This is in line with the findings of Bornancin et al., who found that the metabolic rate of cells was inhibited at lower concentrations than those inducing leakage of lactate dehydrogenase (Bornancin et al., 2019). Laxaphycins apparently have a variety of molecular targets, since laxaphycins A and B have also been shown to cause alteration of topoisomerase II activity (Gbankoto et al., 2005). In this study, only laxaphycin B was active, but laxaphycin A potentiated the effect of laxaphycin B (Gbankoto et al., 2005).

## 6 | ANABAENOLYSINS

Anabaenolysins are cyclic lipopeptides featuring a rare unsaturated β-amino fatty acid with a conjugated triene structure and a four-membered peptide ring (Jokela et al., 2012; Shishido, Jokela, et al., 2015). The peptide ring also contains two proteinogenic amino acids and the unusual 2-(3-amino-5-oxotetrahydrofuran-2-yl)-2-hydroxyacetic acid moiety (Jokela et al., 2012). Anabaenolysins are reported from benthic strains of the genus *Anabaena* isolated from the Baltic Sea (Jokela et al., 2012; Shishido, Jokela, et al., 2015). These strains of *Anabaena* produce multiple anabaenolysin chemical variants that are distinguished by the length (C16–C19) and the nature of the conjugated trienic structure of the β-amino fatty acid (Figure 6, Table S5). Anabaenolysins have reported cytolytic activity against a number of mammalian cell lines (Oftedal et al., 2012) and antifungal activity against *Candida albicans* (Shishido, Jokela, et al., 2015).

Anabaenolysins are produced using a compact 23-kb hybrid PKS/NRPS enzyme complex (Shishido, Jokela, et al., 2015). The first module of the bimodular AblA protein is responsible for the elaboration of the unsaturated β-amino fatty acid (Shishido, Jokela, et al., 2015). However, there is no clear model for how lipoinitiation proceeds and the anabaenolysin biosynthetic gene cluster lacks an expected fatty acyl-AMP ligase (Figure 2). AblC is a fatty acid desaturase predicted to be responsible for the formation of double bonds in the 3-amino-2-hydroxyoctadecanoic acid (Shishido, Jokela, et al., 2015). The combined action of the second module of AblA and AblB are predicted to be responsible for the biosynthesis of the 2-(3-amino-5-oxytetrahydrofuran-2-yl)-2-hydroxyacetic acid moiety from asparagine or aspartic acid (Shishido, Jokela, et al., 2015). There is no plausible mechanism at present for the formation of the furanone moiety, although this is most likely to proceed through methylation and cyclization of aspartic acid. AblD catalyzes the incorporation of Gly or Gln into the peptide intermediate followed by AblE which incorporates Gly into the growing peptide chain, followed by cyclization by the thioesterase domain of AblE (Shishido, Jokela, et al., 2015).

Anabaenolysins have been shown to exhibit moderate antifungal activity (Shishido, Jokela, et al., 2015). Interestingly, the antifungal activity of anabaenolysins was improved by the presence of cyclodextrins, and it has been shown that all known producers of anabaenolysins also produce cyclodextrins (Besenicar et al., 2008; Shishido, Jokela, et al., 2015). The mechanism for this synergistic or

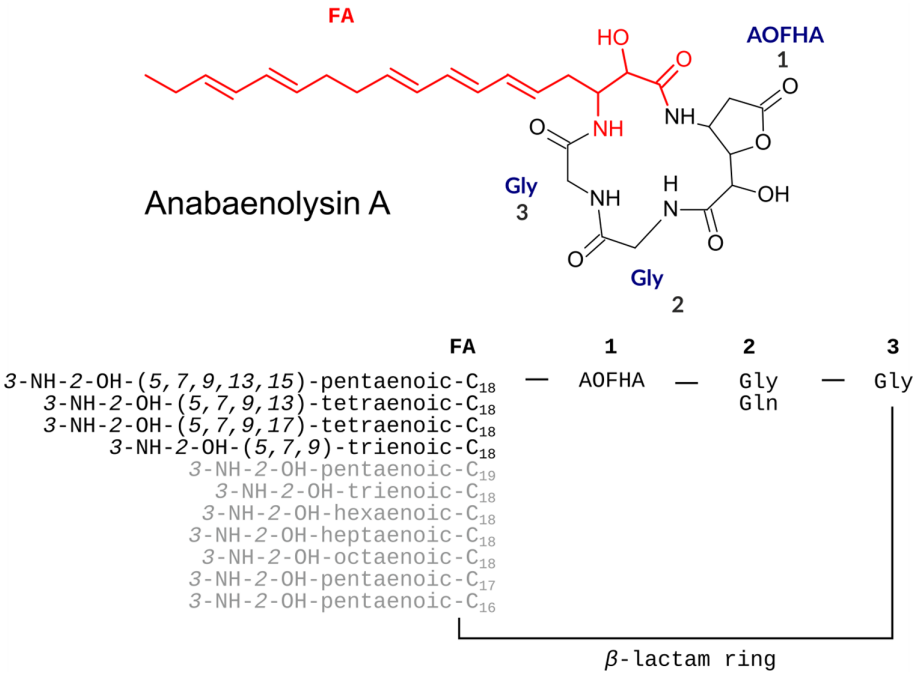


**FIGURE 6** Structure of anabaenolysin A and a schematic general structure of the anabaenolysin type of cyclic lipopeptides. Anabaenolysins are cyclic lipopeptides featuring a rare unsaturated β-amino fatty acid with a conjugated triene structure and a four-membered peptide ring. Residues depicted in gray were described using low-resolution mass spectrometry only. See Table S5 for the exact stereochemistry of particular residues. AOFHA, 2-(3-amino-5-oxotetrahydrofuran-2-yl)-2-hydroxyacetic acid moiety; FA, fatty acid

potentiating effect is not known, but it could be because the long acyl-chain of anabaenolysin is embedded into the cyclodextrins, which facilitates transport of the lipopeptide through the polar cell wall of the fungi. Another explanation may be that cyclodextrins themselves act on the membrane. Some cyclodextrins are known to extract sterols from membranes (Irie et al., 1982), and it could be that cyclodextrin deprives the fungal membrane of sterols. It is likely that extraction of ergosterol from the fungal membrane will affect viability, since one of the targets of antifungal drugs is ergosterol synthesis (Houšť et al., 2020). It has been shown that human erythrocytes that were pretreated with cyclodextrins had higher tolerance for anabaenolysins. This was explained by the fact that cyclodextrin pretreatment deprived the erythrocyte membrane of cholesterol, and since anabaenolysins is more active towards cholesterol-containing membranes, its ability to lyse the erythrocytes was reduced (Oftedal et al., 2012). This does not contradict the enhanced antifungal effect of cyclodextrins and anabaenolysins. The experiment of Oftedal et al. demonstrated the lysis of erythrocytes within minutes of treatment, and did not address the complex functions of a nucleated cell with a plethora of membrane-dependent mechanisms over days like an experiment where the aim is to determine the minimum inhibitory concentration (MIC). The activity of anabaenolysin has also been studied on mammalian cell lines, where it induces a lytic cell death similar to that described for hassallidins. Disruption of membranes was evident by positive trypan-blue staining (Jokela et al., 2012; Oftedal et al., 2012). Interestingly, the mitochondria appeared unharmed, judging both by electron microscopy, and lack of leakage of cytochrome C (Oftedal et al., 2012). There are other examples of cyclic lipopeptides that are membrane-active including surfactin. However, surfactin, originally isolated from *Bacillus subtilis* (Arima et al., 1968), has the opposite cholesterol-dependency compared to hassallidin and anabaenolysin, in that it is more active towards membranes lacking cholesterol (Carrillo et al., 2003; Oftedal et al., 2012).

## 7 | STRUCTURAL AND MECHANISTIC ASPECTS OF THE MEMBRANE ACTIVITY OF ANTIFUNGAL CYCLIC LIPOPEPTIDES

Membrane-disruption is the most frequently reported bioactivity associated with antifungal cyclic lipopeptides from cyanobacteria. The fatty acid residue is modified with the amino group at the β-carbon, which facilitates closure of the peptide ring in each of these compounds (Figures 3-6, Tables S1–S4). The presence of a hydrophobic acyl chain and a polar peptide-head creates an amphiphilic molecule and facilitates its insertion into biological membranes. A long acyl-chain comprised of 14–18 carbon atoms is a common feature of the two best-studied compounds, hassallidins and anabaenolysins (Jokela et al., 2012; Neuhof et al., 2005; Neuhof, Schmieder, et al., 2006; Neuhof, Seibold, et al., 2006). A slightly broader fatty acid range has been reported for puwainaphycins possessing 8–18 carbon atoms (Mareš et al., 2019; Urajová et al., 2016). However, these cyclic lipopeptides differ substantially in their peptide region. It is notable that compare to lipopeptides produced by other bacterial groups, cyanobacterial cyclic lipopeptides, in general, do not frequently contain charged amino acids (Ongena & Jacques, 2008). This structural feature may explain their low affinity for bacteria cells compared to fungal cells. Anabaenolysins have a relatively small peptide ring consisting of only three amino acids and one C18 β-amino acid (Jokela et al., 2012) while hassallidins have a much larger peptide part of eight amino acids forming the ring-structure, and the acyl-chain connected to a small linear peptide branch (Neuhof et al., 2005, 2006c).

Furthermore, the peptide ring may have a sugar attached, and the acyl-chain may have up to two sugars (Neuhof et al., 2005; Vestola et al., 2014). The role of the amino acids, or other constituents in the lipid head, in the membrane disrupting activity, has not been thoroughly investigated, but Humisto et al. demonstrated that the first contact between hassallidin and the membrane was by an interaction between amino acids and the polar head of the lipids (Humisto et al., 2019). The insertion of the acyl chain into the membrane happened at a later time-point, and appeared to be crucial for permanent attachment of the molecule to the membrane. The exact role of sterols has not been clarified, but the structural organization of the membrane has been suggested (Humisto et al., 2019). For anabaenolysin, it was shown that liposomes consisting of phosphatidylcholine with saturated fatty acids were more resistant to lysis compared to liposomes made from unsaturated soy phosphatidylcholine (Oftedal et al., 2012), confirming that the rigidity of the membrane plays an important role in the lytic activity of the lipopeptides. The polar heads of the cyclic lipopeptides also play a role in the lytic activity in that they generate a pulling force outwards, changing the membrane curvature, which eventually breaks the membrane (Frenkel et al., 2014; Nishikawa et al., 1984). Since the membrane-active lipopeptide has a much more conical shape compared to phospholipids, this force will be stronger compared to amphiphilic molecules with a small polar head, and this could explain their rapid effect on membranes at very low concentrations.

## 8 | OTHER ANTIFUNGAL CYCLIC LIPOPEPTIDES

Cyanobacteria produce additional cyclic lipopeptides with long hydrocarbon chains with antifungal and/or antiproliferative activity (Niedermeyer, 2015), that do not fall into any of these four structural classes. Muscotoxins A–C are cyclic lipopeptides consisting of a long fatty acid side chain and 14 amino acid residues produced by *Desmonostoc muscorum* (Cheel et al., 2018; Tomek et al., 2015). Muscotoxins A and B manifest quite prominent inhibition effects that were reached against the plant pathogens *Alternaria alternata*, *Monographella cucumerina*, and *Aspergillus fumigatus*, with minimum inhibitory concentration values of 0.58, 2.34, and 2.34 μg $mL^{-1}$ (Cheel et al., 2018). Muscotoxins are thought to permeabilize phospholipid membranes by reducing their fluidity (Cheel et al., 2018; Tomek et al., 2015). Nostofungicidine is an antifungal cyclic lipopeptide isolated from *Nostoc commune* containing a long fatty acid side chain and eight amino acids residues (Kajiyama et al., 1998). This cyclic lipopeptide demonstrates antifungal activity against *Aspergillus candidus* with a minimum inhibitory concentration of 1.6 μg $mL^{-1}$ (Kajiyama et al., 1998). Calophycin is a cyclic decapapetide with a long fatty acid side chain that was isolated from *Calothrix fusca* and displayed antifungal activity with minimum inhibitory concentration values of 1.25, 2.5, and 1.25 μg $mL^{-1}$ against *C. albicans*, *Trichophyton mentagrophytes*, and *Aspergillus fumigatus*, respectively (Moon et al., 1992). Calophycin also displayed cytotoxic against a human nasopharyngeal carcinoma cell line with an IC50 of 0.2 μM (Moon et al., 1992). The biosynthetic origins of muscotoxins, nostofungicidine, and calophycin are currently unknown. However, genome mining studies demonstrate that cyanobacteria encode a wide variety of cyclic lipopeptides that are unlikely to fall into the four structural classes reviewed here (Galica et al., 2017). Together, it follows that cyanobacteria produce a range of cyclic lipopeptides with antifungal and antiproliferative activity that defy current classification.

## 9 | CONCLUSIONS

Cyanobacteria are an underappreciated source of chemically diverse cyclic lipopeptides that display antifungal activity, and their full potential still remains to be unraveled. Many of the cyclic lipopeptides achieve this activity through disruption of membranes and act as biodetergents. Membrane disruption is in some cases cholesterol and ergosterol-dependent. However, it should be noted that most cyanobacterial cyclic lipopeptides are not extensively tested for biological activity. In many cases, these cyclic lipopeptides are also cytotoxic, and a more extensive examination of their biological activity and structure–activity relationship is warranted and justifies the chemical synthesis of improved cyclic lipopeptide analogs. The cyanobacterial cyclic lipopeptides can fill a gap in drug development, where there is an urgent need for novel molecular entities to face the increasing demand for new therapeutics, for instance in the treatment of cancer or fungal infections. The biosynthetic logic underpinning the biosynthesis and chemical diversity of cyanobacterial lipopeptides is slowly being unraveled. This approach now allows the classification of cyanobacterial lipopeptides into families of compounds that are likely to share similar biological activity. The genetic origins of cyclic lipopeptides and the mechanisms, which drive their structural diversification, are poorly understood. However, in the case of laxaphycins, pairs of lipopeptides act synergistically and their biosynthesis is shared in a unique organization of biosynthetic enzymes. It is clear from bioinformatics studies that cyanobacteria encode a wealth of lipopeptides that remain to be characterized opening to cyanobacteria as a rich source of new lipopeptides with novel biotechnological and therapeutic applications.

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### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

### DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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