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# Discovery of novel fungal RiPP biosynthetic pathways and their application for the development of peptide therapeutics

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**Abstract**

Bioactive peptide natural products are an important source of therapeutics. Prominent examples are the antibiotic penicillin and the immunosuppressant cyclosporine which are both produced by fungi and have revolutionized modern medicine. Peptide biosynthesis can occur either non-ribosomally via large enzymes referred to as non-ribosomal peptide synthetases (NRPS) or ribosomally. Ribosomal peptides are synthesized as part of a larger precursor peptide where they are posttranslationally modified and subsequently proteolytically released. Such peptide natural products are referred to as ribosomally synthesized and posttranslationallly modified peptides (RiPPs). Their biosynthetic pathways have recently received a lot of attention, both from a basic and applied research point of view, due to the discoveries of several novel posttranslational modifications of the peptide backbone. Some of these modifications were so far only known from NRPSs and significantly increase the chemical space covered by this class of peptide natural products. Latter feature, in combination with the promiscuity of the modifying enzymes and the genetic encoding of the peptide sequence, makes RiPP biosynthetic pathways attractive for synthetic biology approaches to identify novel peptide therapeutics via screening of *de novo* generated peptide libraries and, thus, exploit bioactive peptide natural products beyond their direct use as therapeutics. This review focuses on the recent discovery and characterization of novel RiPP biosynthetic pathways in fungi and their possible application for the development of novel peptide therapeutics.

## Introduction

Bioactive natural products have been of great interest for therapeutic health care throughout human history. While many natural products have been successfully used for thousands of years, only fairly recent advancements have allowed elucidating their structure, biosynthesis and mode of action (Dias et al. 2012). Meanwhile, the search for novel natural products with pharmacological activities continues. High-throughput screens using molecular and cellular bioassays allow the evaluation of large numbers of natural products and synthetically generated compound libraries for bioactivities of interest. Statistical analysis of the complementarity of natural products and synthetic compounds showed that 40% of chemical scaffolds of natural products are absent in synthetic compound libraries (Henkel et al. 1999), demonstrating that the biomedical relevance of natural products lies in their wide range of structural diversity and complexity. Thus, natural products continue to play a significant role in drug development. From the 1940s to 2014, 40% of small molecules approved for cancer treatment were natural products or derivatives thereof (Newman and Cragg 2016). Plants and microorganisms are the main sources of bioactive natural products (Dias et al. 2012). The vast dimension of 'microbial dark matter' suggested by genomic microbiome studies promises an enormous variety of bioactive natural products yet to be discovered (Solden et al. 2016). This is also true for fungi where it is assumed that only about one-twentieth of all existing fungi have been described and an even smaller fraction has been cultured successfully in the lab (Jiang and An 2000; Hawksworth and Lücking 2017). In addition to the direct use of natural products for pharmacological purposes, their chemical structures might serve as an inspiration for structural motifs in synthetic chemistry, and the knowledge about the biosynthetic pathways leading to distinct natural products might allow to biotechnologically produce libraries of new-to-nature compounds for screening(Jiang and An 2000; Boecker et al. 2016; Bozhüyük et al. 2018).

Natural products can be divided into different structural classes including alkaloids, terpenoids, polyketides and peptides (Cragg and Newman 2013). Among these classes, peptides cover the largest chemical space due to their polymeric nature and the large number and variety of building blocks (20 in case of proteinogenic amino acids). Thus, a large fraction of bioactive natural products used as therapeutics are peptides (Lau and Dunn 2018). As prominent examples, the discoveries of two fungal peptide natural products, the antibiotic penicillin in the late 1920s and the immunosuppressant cyclosporin in the early 1970s, have revolutionized modern medicine (Bills and Gloer 2016). Therapeutic peptides are recognized to be efficient and selective while being, at the same time, well tolerated and safe (Fosgerau and Hoffmann 2015). On the other hand, peptides are comparatively large molecules with usually poor chemical and physical properties: They have a short life span due to degradation by proteases and a limited membrane permeability. Their binding specificity is favorable compared to typical small-molecule drugs, but off-target effects e.g. due to adoption of different conformations can still pose a risk (Bruno et al. 2013). The pharmacological applicability of peptide natural products depends on their properties, e.g. their serum stability and oral availability, which are influenced by modifications of the peptide side chains and backbone (Beutler 2009).

The biosynthesis of peptides can occur either non-ribosomally or ribosomally. Non-ribosomal biosynthesis is mediated by so-called non-ribosomal peptide synthetases (NRPSs), large modular enzymes that accept also non-proteinogenic amino acids and often contain amino acid-modifying modules (Süssmuth and Mainz 2017). Non-ribosomally produced peptide natural products include above mentioned examples (penicillin and cyclosporin) from fungi and many examples from bacteria e.g. vancomycin and actinomycin. Conversely, ribosomal biosynthesis involves the translation of a precursor peptide which is then posttranslationally processed to the mature peptide (Fig. 1a). Since the discovery of the first family of such ribosomally synthesized and post-translationally modified peptides (RiPP) in 1988, over 20 additional families from all domains of life have been discovered to date (Arnison et al. 2013). The interest in this class of natural products is increasing both from a basic and applied research point of view due to two main reasons: First, the amino acid sequence of RiPPs is genetically encoded and thus, the peptide-encoding gene (including the usually proximal genes for the modifying enzymes) can be easily identified in genome sequences and also modified in order to change the sequence of the peptide (McIntosh et al. 2009). Second, some of the recently identified posttranslational modifications of RiPPs were previously regarded as hallmarks of non-ribosomally produced peptides, affect the peptide backbone and significantly improve the pharmacological properties of these peptides e.g. their proteolytic stability and membrane permeability (Freeman et al. 2012; Van Der Velden et al. 2017; Müller 2018). These features, together with the promiscuity of the RiPP modifying enzymes, may allow to exploit these pathways for the

biotechnological production of new-to-nature libraries of peptides with advantageous pharmacological properties.

This history of fungal RiPPs is very recent with the first family discovered only in 2007 (Hallen et al. 2007). In this review, we describe the commonalities and the differences between these RiPP biosynthetic pathways and discuss their potential for the development of novel peptide therapeutics, having the successful history of non-ribosomal fungal peptide natural products in mind.

## General biosynthetic pathway of RiPPs

RiPPs share several features with regard to their biosynthetic pathways. Usually, these pathways are encoded in the genome by gene clusters, where one gene encodes the peptide precursor while neighboring genes encode enzymes that will modify the precursor (Yang and Van Der Donk 2013). In RiPPs, common posttranslational modifications are single residue modifications such as methylation, hydroxylation, acetylation, epimerization and glycosylation, as well as crosslinks between residues via disulfide, lanthionine, thiazole and oxazole bridges (Arnison et al. 2013). The peptide precursor is usually 20-110 residues long and composed of a N-terminal leader sequence and the C-terminal core peptide (Yang and Van Der Donk 2013). The core peptide will undergo various posttranslational modifications followed by proteolytic cleavage of the RiPP from the leader sequence. In some cases the core peptide is followed by a recognition sequence at the C- terminus, which is relevant in excision and cyclization of the core peptide (Koehnke et al. 2012; Luo et al. 2014). In eukaryotes, a signal sequence preceding the leader peptide can regulate the transport of the precursor to cellular compartments.

The presence of the leader sequence is usually required for the core peptide modifications. Deletion of the leader sequence resulted in a reduction or absence of core peptide modifications (Ortega and Van Der Donk 2016). However, the exact function of the leader sequence is still unclear and might differ between different peptide precursors. It has been suggested that the leader sequence supports the posttranslational modification steps as a recognition motif for the modifying enzymes and assists the export of the core peptide from the producing cell as a non-classical secretory signal. In addition, the leader sequence probably plays a role in the folding, stabilization and activation of the bound modifying enzyme (Ortega and Van Der Donk 2016). For such a role, the leader sequence could either induce a conformational shift in the enzyme and thereby activate enzyme activity, or it could trap enzymes in a specific, active conformation (Ortega and Van Der Donk 2016). The importance of the leader sequence was demonstrated in an experiment where the core peptide sequences of precursors were exchanged with foreign sequences. The foreign core peptides were still posttranslationally modified suggesting that the modifying enzymes only recognized the leader and not the core peptide sequence (Ortega and Van Der Donk 2016). This promiscuity of the modifying enzymes towards the modified core sequence is regarded as a hallmark of RiPP biosynthetic pathways. In addition, posttranslational modifications of the core peptide usually proceed in a processive manner from the N- to the C-terminus (Kelleher et al. 1999; Lee et al. 2009; Lubelski et al. 2009) with some instances proceeding in the opposite direction (Krawczyk et al. 2012; Melby et al. 2012). The processivity of these modifications indicates that the intermediates are kept bound to the modifying enzyme after every reaction (Ortega and Van Der Donk 2016).

## Fungal RiPPs

Fungi have been shown to be a valuable source of peptide natural products exemplified by (non-ribosomally produced) penicillin and cyclosporin (Bills and Gloer 2016). However, to date, only four families of RiPPs have been found in fungi: amatoxins/phallotoxins (Hallen et al. 2007), borosins (Van Der Velden et al. 2017), dikaritins* (Umemura et al. 2014; Nagano et al. 2016; Ding et al. 2016) and epichloëcyclins (Fig. 2) (Johnson et al. 2015). See Table 1 for a summary of all known fungal RiPP families, their representatives and their activities.

### *Amatoxins/Phallotoxins*

Amatoxins and phallotoxins are structurally similar peptides synthesized by several species of the basidiomycete genera *Amanita*, *Galerina*, *Lepiota* and *Conocybe* (Hallen et al. 2007; Luo et al. 2009; Walton et al. 2010; Luo et al. 2012; Sgambelluri et al. 2014). Amatoxins, exemplified by α-amanitin, are highly toxic to insects, nematodes, and mammals and many of the amatoxin-synthesizing fungi, such as the death cap (*Amanita phalloides*), are infamous for causing fatal mushroom poisonings. Amatoxins selectively inhibit RNA polymerase II which leads to a stop of mRNA transcription, a halt of cell metabolism and ultimately to cell death (Chafin et al. 1995; Rudd and

Luse 1996; Bushnell et al. 2002). In spite of their structural similarity, phallotoxins, exemplified by phallacidin, rarely exert toxicity due to their poor absorption in the gut (Hallen et al. 2007). If injected in the bloodstream, they inhibit actin polymerization primarily in liver cells which results in cell membrane dysfunction and severe liver damage (Lengsfeld et al. 1974). The ecological function of these peptides is not clear but based on their toxicity and their induction upon initiation of fruiting body formation it is assumed that they protect the early stage fruiting bodies from fungivores (Spiteller 2015; Zhang et al. 2018).

Amatoxins and phallotoxins consist of eight respectively seven amino acid residues, forming a bicyclic peptide (Fig. 2a) (Walton et al. 2010). The bicyclic structure is established through a crosslink between a cysteine and a tryptophan residue, referred to as tryptathionine (May and Perrin 2007). Nine amatoxins and seven phallotoxins have been identified so far: α-/β-/γ-/ε-amanitin, amanullin, amanullinic acid, amaninamide, amanin and proamanullin, respectively phallacidin, phalloidin, prophalloin, phalloin, phallisin, phallacin and phallisacin (Table 1) (Wieland 1987; Baumann et al. 1993). The variants share basically the same peptide backbone (with variations at one or two positions) and differ in hydroxylations at different positions. Phallotoxins contain one residue in D-configuration, which can be either D-hydroxy-Asp or D-Thr (Hallen et al. 2007).

A number of closely related, monocyclic peptides have been found in *Amanita* that seem to belong to the RiPP family of amatoxins/phallotoxins. The so-called virotoxins, consisting of the peptides alaviroidin, viroisin, deoxoviroisin, viroidin and deoxoviroidin, were isolated from *A. phalloides* (Table 1) (Baumann et al. 1993; Vetter 1998). Additionally, a monocyclic nonapeptide termed amanexitide of unknown function was recently identified in *A. exitialis* from China (Xue et al. 2011). Its structure is related to antamanide, a monocyclic decapeptide with antidote activity against amatoxins and phallotoxins previously identified in *A. phalloides* (Wieland et al. 1978). Antamanide was recently shown to be immunosuppressive and bind, similar to cyclosporin A, the peptidyl prolyl cis-trans isomerase cyclophilin D (Siemion et al. 1992; Azzolin et al. 2011). Immunosuppressive activity has also been reported for several monocyclic hepta- and octapeptides, referred to as cycloamanides A-F, identified from different *Amanita phalloides* specimens (Gauhe and Wieland 1977; Wieczorek et al. 1993; Pulman et al. 2016).

In 2007, α-amanitin and phallacidin were found to be derived from ribosomally produced peptide precursors encoded in the genome of *A. bisporigera* (destroying angel) and to represent the first fungal RiPP family (Hallen et al. 2007). The peptide precursors consist of 35 respectively 34 amino acid residues and comprise a 10-residue leader sequence, the 8/7-residue core peptide and a 17-residue C-terminal recognition sequence (Fig. 2b) (Hallen et al. 2007). Amatoxin and phallotoxin precursors do not contain a signal sequence for classical secretion and are thus synthesized and processed in the cytoplasm, eventually close to the vacuole (Luo et al. 2010). Analysis of the draft genome sequences of *A. bisporigera* and *A. phalloides* suggests that the two species code together for over 50 unique small, cyclic peptides of this family (Pulman et al. 2016). The encoded peptide precursors are very conserved in the leader and C-terminal recognition sequences but vary significantly in the length and the sequence of the core peptide. Due to a conserved MSDIN motif in the leader sequence, these peptides are also referred to as MSDIN family of peptides. Remarkably, the two different *Amanita* species share only three peptides, α-amanitin, phallacidin and an uncharacterized MSDIN peptide, demonstrating the sequence and species diversity within this RiPP family. Based on the transcriptomes of the two *Amanita* species, it can be assumed that many of the detected precursor genes are expressed. Accordingly, two novel monocyclic, but otherwise not posttranslationally modified hepta and nonapeptides, referred to as cycloamanide E and F, could be identified from *A. phalloides* based on the core peptide sequences of the respective peptide precursors. It should be noted, however, that this RiPP family is only present in species of *Amanita* sect. *Phalloideae* and not in other, more distantly related *Amanita* species. Similarly, the genome sequences of the distantly related mushrooms *Galerina marginata* and *Lepiota brunneoincarnata* contain only two precursor genes each, both coding for α-amanitin and no extended MSDIN-like peptide family (Luo et al. 2012; Luo et al. 2018). The leader and C-terminal recognition sequences of these precursors are of the same length as the ones of the different *Amanita* precursors but the individual residues are less conserved.

Regarding the modifying enzymes of the amatoxin/phallotoxin RiPP family, thus far, a prolyloligopeptidase (POP) of serine protease family S9a responsible for the dual function of core peptide release and macrocyclization has been identified and characterized both biochemically and structurally (Luo et al. 2014; Czekster et al. 2017;

Czekster and Naismith 2017). The POP-encoding gene clusters with one of the two α-amanitin precursor genes of *G. marginata* was shown to be required for α-amanitin production in this mushroom (Luo et al. 2014). Accordingly, homologs of this gene are found in the genomes of all amatoxin- and phallotoxin-producing basidiomycetes and the gene product colocalizes with α-amanitin in the same cells of *Amanita* fruiting bodies(Luo et al. 2010). The POP involved in the amatoxin/phallotoxin biosynthetic pathway (POPB) may have evolved from a related, housekeeping POP (POPA) that is found in all basidiomycetes (Zhang et al. 2005; Luo et al. 2010). In vitro studies with recombinant *G. marginata* POPB demonstrated that the enzyme processes the peptide precursor in two consecutive steps: In a first step, it removes the leader sequence of the 35-residue α-amanitin precursor by proteolytic cleavage after the terminal and conserved Pro-residue of the leader sequence; in a second step, the enzyme performs a transpeptidation reaction between the generated N-terminus of the shortened peptide precursor and the second conserved Pro-residue at the end of the core peptide, thereby releasing the C-terminal recognition sequence (Luo et al. 2014). Based on kinetic studies and substrate-bound structures of the enzyme, it was proposed that the enzyme recognizes the original peptide precursor via its leader sequence, releases both the leader sequence and the truncated precursor upon the first proteolytic cleavage and rebinds the truncated precursor to catalyze the transpeptidation (Czekster et al. 2017; Czekster and Naismith 2017). In accordance with such a mechanism, the leader sequence of the peptide precursor was shown to be required for the first proteolytic cleavage and the C-terminal recognition sequence for the second proteolytic cleavage and the transpeptidation (Luo et al. 2014; Czekster et al. 2017). None of the enzymes catalyzing any of the other known modifications of members of this RiPP family, including the formation of the tryptathionine crossbridge and the oxidation, L-to-D-epimerization or hydroxylation of specific residues, has been identified yet. Hence, it is not clear whether these enzymes act at the level of the peptide precursor or on the released and cyclized core peptide. At least in vitro, the POP does not seem to depend on any additional modification of the core peptide for its activity which rather argues for latter option.

*Borosins*

The fungal RiPP family of borosins was recognized only in 2017 (Ramm et al. 2017; Van Der Velden et al. 2017). Omphalotin A, the founding member of this RiPP family, was isolated already in 1997 and 1998 from the basidiomycete *Omphalotus olearius* in a screen for natural products from submerged cultures of asco- and basidiomycetes exerting toxicity against the plant parasitic nematode *Meloidogyne incognita* (Mayer et al. 1997; Sterner et al. 1997; Büchel et al. 1998; Liermann et al. 2009). Further analysis revealed that this toxicity was higher than the one of the bacterial nematotoxin ivermectin and very specific for this nematode species with very low or no toxicity towards mammalian cells, plants, insects, other nematode species, fungi and bacteria (Mayer et al. 1997). Omphalotin A is a cyclic dodecapeptide with backbone N-methylations on 9 of the 12 amino acid residues (Fig. 2a) (Mayer et al. 1997; Sterner et al. 1997). The peptide consists mainly of hydrophobic amino acids and all residues are in L-configuration (Büchel et al. 1998). Eight other derivatives of omphalotin called omphalotin B-I, varying in additional modifications i.e. hydroxylations, acetylations and an unusual tryptophan modification, have been discovered (Table 1) (Büchel et al. 1998; Liermann et al. 2009). Similar to the amatoxins/phallotoxins, omphalotins are not secreted and have to be extracted from *O. olearius* cells (Mayer et al. 1997). In contrast to the amatoxins/phallotoxins, however, omphalotins are exclusively produced in the vegetative mycelium of *O. olearius* and not in its fruiting bodies (Anke and Schüffler 2018). Accordingly, submerged cultures of monokaryotic strains of *O. olearius* are efficient producers of omphalotins (Liermann et al. 2009).

Backbone N-methylation was long thought to be an exclusive modification of non-ribosomally produced peptides exemplified by the immunosuppressant cyclosporin A (Dreyfuss et al. 1976), the antibiotic actinomycin S (Hollstein 1974) and several depsipeptides (Maharani et al. 2015). In all these cases, the methylation of the nitrogen occurs on the level of the primary amine before formation of the peptide bond which makes sense given the low reactivity of a secondary amine in particular in the context of the delocalization of its free electron pair in a peptide bond (Scherer et al. 1998). Hence, it came as a surprise when omphalotin A was shown to be genetically encoded and derived from the ribosomally produced precursor OphMA** (Ramm et al. 2017; Van Der Velden et al. 2017). This peptide precursor is, with 417 residues, unusually long and its N-terminus (residues 12 to 219) shows sequence homology to SAM-dependent methyltransferases (Fig. 2b) (Ramm et al. 2017; Van Der Velden et al. 2017). This domain was shown to be responsible for the backbone N-methylation of the omphalotin core peptide located at the C-terminus of the OphMA precursor (Ramm et al. 2017; Van Der Velden

et al. 2017). The modified residues of the OphMA core peptide were identical to the ones of the natural product omphalotin A demonstrating that omphalotin A was a RiPP and that enzymatic backbone N-methylation of the nitrogen in preformed peptide bonds is possible (Van Der Velden et al. 2017). The omphalotin precursor is so far the only example where a RiPP modifying enzyme is covalently coupled to the modified core peptide. An enzymatic domain was reported for the precursors of a bacterial RiPP family but this domain appears to function rather in export than in modification of the core peptide and is not catalytically active (Haft et al. 2010). In analogy to the automethylating activity of the OphMA precursor, the name "borosins" was proposed for this novel fungal RiPP family, after the ancient mythological symbol Ouroboros depicting a serpent biting its own tail (Van Der Velden et al. 2017).

Structural analysis of OphMA has revealed that the precursor forms a dimer with an unusual catenane-like arrangement in which the methyltransferase domain of one protomer methylates the core peptide of the other protomer (Ongpipattanakul and Nair 2018; Song et al. 2018). Analysis of the methylation states of reaction intermediates and the structure of the enzyme suggest an overall mechanism in which the core peptide is coordinated into and processively pulled, from the N- to the C-terminus, through a substrate tunnel which is formed at the interface between the two protomers and contains the active site (Van Der Velden et al. 2017; Song et al. 2018). The structure of the active site and computational reaction modelling suggest a base-catalyzed reaction mechanism that involves a protein-mediated clamping of the amide nitrogen towards the methyl group of SAM and stabilization of the reaction intermediate (Ongpipattanakul and Nair 2018; Song et al. 2018). Methylation analysis of hybrid precursors where the core peptide of OphMA was replaced by unrelated sequences revealed a considerable promiscuity of the enzyme with regard to its substrate (Van Der Velden et al. 2017). These results also suggested a minor role of the six-residue C-terminal recognition sequence in methylation as methylation also occurred when this sequence was deleted - although the last two positions of the core peptide remain unmethylated in this case(Van Der Velden et al. 2017). In accordance with this result, at least in case of the *D. bispora* OphMA homolog, no binding of synthetic peptides corresponding to the C-terminal recognition sequence to the OphMA methyltransferase domain could be detected (Ongpipattanakul and Nair 2018). In addition, recombinant full length OphMA methylates 1-2 residues in the C-terminal recognition sequence beyond the core peptide (Ramm et al. 2017; Van Der Velden et al. 2017). All these results suggest that the C-terminal recognition sequence has a role in the completion of the methylation of the core peptide but it is not clear whether this effect is sequence-specific.

The *ophMA* gene is part of a gene cluster of *O. olearius* where neighbouring genes are likely responsible for the release, macrocyclization and additional modifications of the core peptide (Ramm et al. 2017; Van Der Velden et al. 2017). Thus far, only the role of one of these genes has been elucidated: *ophP* codes for a prolyloligopeptidase of the same serine protease family as POPB, that is involved in the amatoxin/phallotoxin biosynthetic pathway, and its coexpression with *ophMA* in the yeast *Pichia pastoris* lead to formation of both linear and cyclic forms of omphalotin A (Ramm et al. 2017). These results suggest that OphP is sufficient for the release and the macrocyclization of the methylated core peptide similar to the role of POPB in amatoxin/phallotoxin biosynthesis. Accordingly, the residue preceding the core peptide is a proline but the terminal residue is a glycine. So far, the specificity of OphP with regard to the sequence, methylation state or flanking residues of the core peptide has not been addressed experimentally.

BLAST searches revealed homologous gene clusters in the basidiomycetes *Dendrothele bispora* and *Lentinula edodes* (Ramm et al. 2017; Van Der Velden et al. 2017; Ongpipattanakul and Nair 2018). The coding region of the *D. bispora* OphMA precursor was expressed in *Escherichia coli* and the core peptide of the recombinant protein was shown to be methylated in the same pattern as OphMA from *O. olearius* despite three sequence variations each therein (Van Der Velden et al. 2017; Ongpipattanakul and Nair 2018). Additional homologs of the *ophMA* precursor gene were found in genomes of many other basidiomycetes suggesting that borosins are widespread in this fungal phylum (Van Der Velden et al. 2017). The genome coding for the highest number of predicted borosin precursors is thus far the one of the ectomycorrhizal fungus *Rhizopogon vinicolor* (unpublished results) The C-termini of all these predicted borosin precursors differ considerably from the one of *O. olearius*, *D. bispora* and *L. edodes* but are generally also rich in hydrophobic residues. In none of these fungi, backbone N-methylated peptides have been described so far. On the other hand, such peptides have been described for several fungi for which no genome sequences are available at the moment. Examples are the dictyonamides, linear dodecapeptides from an unknown marine-derived fungus (Komatsu et al. 2001), the RHM family of linear

octapeptides from a sponge-derived ascomycota (Boot et al. 2006), the verrucamides from the plant-pathogenic ascomycete *Myrothecium verrucaria* (Zou et al. 2011) and the gymnopeptides, cyclic octadecapeptides from the wood-degrading basidiomycete *Gymnopus fusipes* (Ványolós et al. 2016). It can be assumed that at least some of these peptides will turn out to be new members of the borosin RiPP family.

*Dikaritins*

In contrast to the cytoplasmic localization of the amatoxin/phallotoxin and borosin precursors and their processing proteases, the precursors of the other known fungal RiPP families are secreted and proteolytically processed by the Golgi-localized endopeptidase kexin that is present in most fungi (Yoshimi et al. 2016; Li et al. 2017). The name of the dikaritin RiPP family was proposed based on the production of these cyclopeptides by the fungal subkingdom Dikarya comprising the phyla Ascomycota and Basidiomycota (Ding et al. 2016). Hitherto characterized examples include the ustiloxins, phomopsins and asperipins originally identified in the ascomycetes *Ustilaginoidea virens, Phomopsis leptostromiformis* and *Aspergillus flavus*, respectively (Tsukui et al. 2014; Umemura et al. 2014; Nagano et al. 2016; Ding et al. 2016). A common feature of all dikaritins is their cyclization via ether bridges between the hydroxyl group of tyrosine and the Cβ of isoleucine (ustiloxins and phomopsins) or phenylalanine/tyrosine residues (asperipins) (Fig. 2a). This special type of peptide cyclization is catalyzed by DUF3328-containing oxidases encoded by all dikaritin-encoding gene clusters (Ye et al. 2016; Ye et al. 2019). In case of ustiloxins and phomopsins, the cyclizing hydroxyl group of the tyrosine residue is provided by a tyrosinase. Other modifications besides cyclization include methylation of the N-terminus (ustiloxin and phomopsin), addition of the non-proteinogenic amino acid norvaline through a sulfoxide bond (ustiloxin), hydroxylation (ustiloxin) and dehydrogenation of side chains (phomopsin).

Ustiloxins are mycotoxins produced by the plant pathogen *U. virens*. The fungus infects the spikelets of rice, leading to the so-called "rice false smut disease" which is known as one of the most destructive fungal diseases in rice (Koiso et al. 1992; Koiso et al. 1994; Koiso et al. 1998; Wang et al. 2017). It was shown that ustiloxins cause phytotoxic effects in rice, wheat and maize, such as growth reduction of radicles and plumules (Koiso et al. 1998; Abbas et al. 2014). Ustiloxins inhibit the polymerization of tubulin and suppress mitosis, causing a range of symptoms in domestic animals that were fed with contaminated rice. Observed symptoms were diarrhea, hemorrhage, poor growth, abortion and organ damage (Wang et al. 2017). Until today, contamination of food crops with ustiloxins remains a serious safety risk and financial burden (Wang et al. 2017). Detection and analysis of ustiloxin production in the mold *A. flavus* revealed that these peptide natural products are RiPPs (Umemura et al. 2014; Tsukui et al. 2014). The cyclic tetrapeptides of the sequence "Y-V/A-I-G" are modified with methyl groups, hydroxyl groups and norvaline (Fig. 2a) (Umemura et al. 2014). Seven different ustiloxins have been discovered, called ustiloxin A-G (Table 1) (Koiso et al. 1992; Koiso et al. 1998; Ding et al. 2016). All *A. flavus* ustiloxin variants are derived from precursor *ustA*, consisting of a signal peptide for classical secretion and a 16-fold repeat of the core peptide "YAIG" (Fig. 2b). Each core peptide is flanked by a leader peptide at the N-terminus and a recognition sequence at the C-terminus. These peptide units are interspersed with dibasic residues "KR" which are recognized by the Golgi endoproteinase kexin (Umemura et al. 2014). The *ustA*-encoding gene is part of a gene cluster that encodes most of the enzymes that are needed for biosynthesis of ustiloxin B (Umemura et al. 2014). As a result of a detailed genetic and biochemical analysis, the biosynthetic pathway could be established as follows: Upon kexin-mediated cleavage of the precursor protein UstA, the tetrapeptides YVIG and YAIG are cyclized by the tyrosinase-homolog UstQ and the DUF3328-containing UstYa/Yb. Upon N-methylation of the terminal amino group by the methyltransferase UstM, UstC (a cytochrome P450) probably installs a cysteine residue by oxidative substitution. After the substitution, UstF1 (a Flavin-containing monooxygenase [FMO]) catalyzes oxygenation of the sulfur atom to a sulfoxide, and the subsequent N-hydroxylation by another FMO, UstF2, results in an aldoxime. Finally, UstD, a PLP-dependent enzyme homologous to cysteine desulfurase, catalyzes condensation of a C3 unit derived from aspartate (Ye et al. 2016),. Although Kexin plays a vital role in the ustiloxin biosynthesis by cleaving the repeating units after the second basic residue, it is not encoded by the cluster but elsewhere in the genome (Umemura et al. 2014; Yoshimi et al. 2016). Analysis of biosynthetic intermediates accumulating in gene deletion mutants allowed the establishment of the biosynthetic pathway (Ye et al. 2016). However, the subcellular localization of the various reactions remains, with the exception of the kexin-mediated endoproteolytic cleavage of the precursor in the Golgi, unclear.

Phomopsins are mycotoxins produced by a number of legume-infesting ascomycetes including *Phomopsis leptostromiformis* which causes stem blight in lupins (Battilani et al. 2011). Similar to ustiloxins, phomopsins bind tubulin and prevent it from polymerization, leading to inhibition of spindle formation during mitosis and mitotic arrest (Battilani et al. 2011). Affected are mainly hepatocytes, as seen in the occurrence of liver damage in livestock fed with phomopsin-contaminated crops (Battilani et al. 2011). Inspired by the related structure (Fig. 2a) and the ribosomal biosynthesis of ustiloxins, Ding *et al*. recently established that phomopsins are derived from the ribosomally produced precursor *phomA* in *P. leptostromiformis* (Ding et al. 2016). This precursor contains, similar to the ustiloxin precursor *ustA*, a signal peptide followed by eight repeats of the phomopsin core peptide "Y-V-I-P-I/F-D" (Fig. 2b). The core peptide in each repeating sequence is flanked by an N-terminal leader sequence and the C-terminal dibasic residue "K-K/R". The gene coding for the precursor PhomA is part of the gene cluster *phom* encoding 18 modifying enzymes (Ding et al. 2016). These enzymes include peptidases that might cleave the leader and the dibasic residues from the core peptide, a tyrosinase that catalyzes the cyclization of the peptide scaffold possibly together with several DUF3328-containing oxidases, a SAM-dependent methyltransferase that methylates the amino-terminus and several enzymes with unknown functions (Ding et al. 2016). Gene clusters that generate phomopsin-like cyclic peptides are widespread in fungal genomes, including plant-pathogenic, animal parasitic and saprobic ascomycetes but also basidiomycetes (Ding et al. 2016). Based on these biocomputational findings, the name dikaritins was suggested for this RiPP family.

At the time of its discovery, the structure of the precursor peptide *UstA* with its 16-fold repeat of the core tetrapeptide sequence seemed rather unique, since RiPP precursors usually contain only one single core peptide sequence (Umemura et al. 2014). However, the previous discovery of cyanobactins in bacteria (Gu et al. 2018) and the subsequent discoveries of the phomopsins and the epichloëcyclin RiPP family demonstrate that RiPP precursors with repetitive peptide sequences are more common than previously thought (Fig. 2b). The acquisition of several repeats of the core sequence was suggested to increase the production of the peptide natural product and allow diversification of the peptide sequence in terms of its bioactivity. The presence of highly repetitive core peptides also simplifies the biocomputational detection of new RiPP precursor genes with different core peptide sequences. An algorithmic approach for the identification of secreted, kexin-cleavable and highly repetitive precursor genes clustering with DUF3328-encoding genes in the *Aspergilli* genomic database (AspGD) (Cerqueira et al. 2014) suggested an average of 70 candidate precursors per *Aspergillus* genome and 94 candidate gene clusters coding for novel dikaritins (Nagano et al. 2016). Gene deletions in one of these clusters in *A. flavus* lead to the identification of asperipin-2a, a bicyclic hexapeptide of the sequence "FYYTGY" (Fig. 2). In contrast to ustiloxins and phomopsins, the cyclization of asperipin occurs via the para-hydroxyl group of two tyrosine residues and is mediated by a single DUF3328-containing oxidase encoded in the respective gene cluster without the involvement of a tyrosinase (Ye et al. 2019). Although the biological activity of asperipin-2a has not been characterized yet, the results of these studies demonstrate that in particular ascomycete genomes encode a large variety of dikaritins some of which are likely to have other targets than ustiloxins and phomopsins.

*Epichloëcyclins*

Epichloëcyclins are cyclic nonapeptides produced by ascomycetes of the genus *Epichloë* (Johnson et al. 2015). These fungi form a symbiotic relationship with certain grasses by growing in the intercellular space of stems, leaves, inflorescences and seeds. The infected grasses benefit in several ways from the fungal endophyte: Fungal alkaloids contribute to the plant defense against herbivores, while other products secreted by the fungus increase the plants stress resistance and growth (Johnson et al. 2003; Khan et al. 2010; Ambrose and Belanger 2012). One of the most abundant fungal transcripts in infected plants is *gigA* (Johnson et al. 2015). *gigA* encodes a precursor that consists of a signal sequence, followed by four repeats of 27 amino acids. Each repeat contains a dibasic "KR" motif which is highly conserved between species and probably cleaved by the two kexin proteases of *Epichloë*, kexA and kexB. The repeating units in *gigA* are not identical but slightly variable. Accordingly, metabolic analysis of *gigA*-expressing *Epichloë* showed that six different Epichloëcyclins (A-F) are produced, corresponding to a slightly variable core nonapeptide within the repeating units of the *gigA* gene (Table 1) (Johnson et al. 2015). These core peptides are cyclized by a bridge between the conserved tyrosine residue at position 7 and the aminoterminal proline or isoleucine residue and are in addition modified by a dimethylation of the conserved lysine residue at position 4. Since the chemical nature of the cyclizing bridge has not been characterized yet and no information about the composition of the respective gene cluster is available, epichloëcyclins are at the moment categorized as separate RiPP family and not as additional representatives of

dikaritins. The high expression of *gigA* during infection suggests a role of the epichloëcyclins in the symbiosis with grass. However, the biological function of epichloëcyclins remains unknown (Johnson et al. 2015).

*Beyond RiPPs: Peptides derived from Kex2-processed repeat proteins (KEPs)*

A recent biocomputational analysis of 250 fungal genomes for the occurrence of secreted, kexin-processed and highly repetitive proteins suggests that such proteins, referred to as KEX2-processed repeat proteins (KEPs), are very common in all fungal phyla and give rise to a large diversity of yet uncharacterized, secreted fungal peptides (Le Marquer et al. 2019). Some of the peptides derived from such precursors do not need additional modifications for bioactivity, and do therefore not meet the definition of a RiPP. Characterized examples are α-factor, one of the two types of the *Saccharomyces cerevisiae* mating pheromones (Brake et al. 2015) and candidalysin, a recently identified virulence factor of the mucosal pathogen *Candida albicans* (Moyes et al. 2016). These ribosomally synthesized peptides arenot further discussed here.

**Application of RiPP biosynthetic pathways for the development of peptide therapeutics**

In the light of the blockbuster therapeutics penicillin and cyclosporin A, there is a strong interest in exploiting fungi for the development of novel peptide therapeutics. In this regard, RiPP biosynthetic pathways have several advantages over the non-ribosomal biosynthesis of peptides which can be exploited using two main approaches:

*Discovery approach*: The examples of penicillin and cyclosporin A show that fungal peptide natural products can sometimes be used directly as therapeutics without significant alterations. Thus, it is worthwhile to search for novel peptide natural products in fungi as they may be used, with minor alterations, as peptide therapeutics. In this regard, precursors of new members of already characterized RiPP families can be easily identified by biocomputational analyses of the rapidly increasing number of available fungal genome sequences. Based on the available knowledge about the processing of the precursor in the respective RiPP family, a putative sequence of the core peptide can be derived. This information, together with data on the expression of the precursor gene, helps in the subsequent identification of the respective RiPP natural product and the elucidation of its structure. Latter analysis is further aided by the fact that the various modifications of a core peptide are usually mediated by individual enzymes which are encoded by genes clustering around the precursor-encoding gene. The biosynthetic pathway of a novel fungal RiPP can thus be dissected by deletion or heterologous expression of the individual biosynthetic genes and biochemical characterization of the encoded enzymes. The new compounds can then be tested for their biological and/or pharmaceutical activities. A nice example for this approach is the recent identification and characterization of asperipin-2a whose biological activity is not known yet (Nagano et al. 2016; Ye et al. 2019). Once a novel RiPP natural product with an interesting biological activity is identified, variants of the compound could be easily generated by genetic alteration of the core peptide sequence in the precursor and/or modification of the biosynthetic pathway by removing one or more modifying enzymes.

*Synthetic biology approach:* The promiscuity of the modifying enzymes for the core peptide sequence may be exploited for the development of peptide therapeutics beyond the direct use of the natural products. The idea of this synthetic biology approach is to use these enzymes, along with engineered precursors, to generate, *in vitro* or *in vivo*, libraries of new-to-nature peptides with the respective posttranslational modifications. These libraries can then be screened, in vitro or in vivo, for desired biological activities and the hits can serve as leads for novel peptide therapeutics. A scheme of this strategy is shown in Fig. 1b. As an example, Sgambelluri *et al.* managed to create *de novo* libraries of macrocyclic peptides using chemically synthesized or *E. coli*-produced linear precursor peptides. The peptides were cyclized in vitro using *S. cerevisiae*-produced POPB from *G. marginata* (Sgambelluri et al. 2018). Such libraries are of interest as macrocyclization is, together with backbone N-methylation, one of the favourite strategies to improve the pharmacological properties of peptides. Both modifications increase the metabolic stability, structural rigidity, target specificity, cell permeability and oral availability of peptides (Pattabiraman and Bode 2011; Chatterjee et al. 2013). Since retroactive modification of peptides often destroys their biological activity, it would be preferable if the peptide library to be screened for a biological activity, would already contain these modifications. Both of these modifications are difficult to achieve by chemical synthesis (Pattabiraman and Bode 2011) and engineering of respective NRPSs, such as the one for cyclosporin A, is a difficult task (Winn et al. 2016), in particular for the production of a peptide library. Thus,

alternative approaches are wanted. One such approach is based on *in vitro* translation and reprogramming of codons from proteinogenic to non-proteinogenic amino acids (Passioura and Suga 2017). A key element of this system are promiscuous, ribozyme-based aminoacyl-tRNA-synthetases that accept a large variety of chemically synthesized, non-proteinogenic amino acids, including α-N-methylated ones, and thereby massively increase the chemical space covered by ribosomally synthesized peptides. Coupling of this reprogrammed ribosomal synthesis with ribosomal display and screening technologies allowed the recent identification of a natural product-like macrocyclic N-methyl peptide inhibitor of a ubiquitin ligase (Yamagishi et al. 2011). A possibility for another biotechnological approach is based on the promiscuity of the methyltransferase and prolyloligopeptidase involved in omphalotin A biosynthesis. In this regard, Van der Velden et al. demonstrated that the methyltransferase domain of the omphalotin A precursor OphMA functions in the cytoplasm of *E. coli* and accepts omphalotin A-unrelated sequences in its core peptide region (Van Der Velden et al. 2017). Ramm et al. demonstrated that OphMA also functions in the cytoplasm of *P. pastoris* and that coexpression of the prolyloligopeptidase OphP results in formation of the peptide natural product in this organism (Ramm et al. 2017). If OphP also shows promiscuity for the core peptide region of OphMA, libraries of macrocyclic, backbone N-methylated peptides could be generated in vivo by coexpression of OphP and hybrid OphMAs containing a randomized core peptide region. This in vivo approach would, in comparison to above mentioned in vitro approach, have the advantages that it would allow a higher number of backbone N-methylations per peptide and a coupling of the production and the screening of the peptide in the same microbial cell. Such systems were constructed for bacterial RiPP pathways and recently lead to the identification of natural-product-like peptides (Yang and Van der Donk 2016). Further characterization of the promiscuity of OphMA and OphP is needed to judge the feasibility of such an approach.

## Concluding remarks

In the light of the rapidly increasing number of available fungal genome sequences, the recent discovery of fungal RiPPs has revived the interest in fungi as sources of peptide natural products. There are two main reasons for this interest: First, the biocomputational identification of structural or sequence variants of characterized RiPP precursors is comparably easy and can serve as basis for the identification of novel peptide natural products with interesting biological activities. Second, the discovery of modifying enzymes that introduce unprecedented peptide modifications that may allow to produce peptide libraries. These libraries would contain new-to-nature peptides with favorable pharmacological properties which can be screened for interesting biological activities. Both of these approaches will speed up the identification and development of novel peptide therapeutics, some of which may be of similar clinical significance as the non-ribosomal peptides penicillin and cyclosporin A.

## Notes

*The authors prefer 'dikaritins' over 'ust-RiPS' suggested by Nagano et al 2016 as designation for the RiPP family comprising ustiloxins, phomopsins and asperipin-2a although the original definition of 'dikaritins' by Ding et al 2016 was restricted to peptides with sequence similarity to ustiloxins and would, thus, not include asperipin-2a.

**The authors suggest to use the designation 'OphMA' (as used in Ramm et al 2017 and Ongpipattanakul and Nair 2018) instead of 'OphA' (as used in van der Velden et al 2017 and Song et al 2018) for the omphalotin precursor to indicate the presence of the methyltransferase domain and to avoid dual designations in future publications.

## Complicance with Ethical Standards

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## Figure Captions

**Figure 1.** Schematic illustration of the general RiPP biosynthesis pathway (a) and its biotechnological application for the identification of leads for novel peptide drugs via generation and screening of new-to-nature peptide libraries (b). In RiPP biosynthesis, the precursor is ribosomally synthesized and consists of a leader sequence and the core peptide. Depending on the molecule, the precursor can also contain an N-terminal signal sequence or a C-terminal recognition sequence. The core peptide within the precursor is modified by enzymes that are encoded by large gene clusters comprising also the precursor gene. Afterwards, the peptide is proteolytically cleaved, optionally followed by additional modifications, resulting in the bioactive, mature form. Figure is based on image by Arnison *et al*. (Arnison et al. 2013). RiPP biosynthesis pathways may be applied for the de novo generation and screening of modified peptide libraries. A DNA library would serve as source for the translation of a large variety of precursor peptides with unique core peptides. Each core peptide would be modified individually and cleaved from the precursor, resulting in a library of new-to-nature mature peptides that display a unique combination of different residues and modifications. The mature peptides would be screened for specific bioactivities and hits would serve as lead peptides for the development of novel peptide drugs.

**Figure 2.** The hitherto identified families of fungal RiPPs and their precursors. a) Chemical structures of representative family members. α-Amanitin and phallacidin are representatives of the family of amatoxins/phallotoxins. Omphalotin A is the founding member of the recently discovered family of borosins with the characteristic backbone N-methylations indicated in red. Ustiloxin B, phomopsin A and asperipin-2a are members of the family of dikaritins. The N-methylations of the primary amines of the ustiloxin and phomopsin backbones are indicated in orange to distinguish them from the N-methylations of the secondary amines of the borosin backbones. Due to the lack of details about its chemical structure, epichloëcyclin constitutes its own family at the moment. b) Respective precursor sequences. Leader and C-terminal recognition sequences are indicated in gray, core sequences in black with recognition sequences for the kexin Golgi protease in bold. The schematic representation of the Omphalotin A-I precursor is based on image by Van der Velden *et al.* (Van Der Velden et al. 2017). The protein accession numbers for the peptide precursors are A8W7M4 for α-amanitin, A8W7M7 for phallacidin, XP_002381318 for ustiloxins, AMR44282 for phomopsins, XP_002377602 for asperipin-2a and KP797979 for epichloëcyclins. The JGI protein ID of the omphalotin precursor is 2087.

**Table 1**: Compilation of all currently known fungal RiPP classes, their representatives and their activities.

| RiPP family | Representatives | | Activity | References |
|---|---|---|---|---|
| Amatoxins/phallotoxins | MSDIN peptides | α-/β-/γ-/ε-amanitin, amanullin, amanullic acid, amaninamide, amanin, proamanullin | Inhibition of RNA polymerase II. Highly toxic to insects, nematodes and mammals. | (Baumann et al. 1993; Garcia et al. 2015) |
| | MSDIN peptides | Phallacidin, phalloidin, prophalloin, phalloin, phallisin, phallacin, phallisacin | Inhibition of actin polymerization. Low toxicity due to poor absorption by the gut of fungivores. | (Wieland et al. 1978; Garcia et al. 2015) |
| | MSDIN peptides | Antamanide | Antidote activity against amatoxins and phallotoxins. Immunosuppressive activity by binding to cyclophilin D. | (Wieland et al. 1978; Siemion et al. 1992; Azzolin et al. 2011) |
| | MSDIN peptides | Cycloamanides A-F | Immunosuppressive activity (cycloamanides A-D) | (Gauhe and Wieland 1977; Wieczorek et al. 1993; Pulman et al. 2016) |
| | MSDIN peptides | Amanexitide | Unknown activity | (Xue et al. 2011) |
| | Virotoxins[a] | Alaviroidin, viroisin, deoxoviroisin, viroidin, deoxoviroidin | Similarly to phallotoxins: Inhibition of actin polymerization. Low toxicity due to poor absorption by the gut of fungivores. | (Faulstich et al. 1980; Garcia et al. 2015) |
| Borosins | Omphalotin A-I | | Toxicity towards nematodes (omphalotin A-I). | (Büchel et al. 1998; Liermann et al. 2009) |
| Dikaritins | Ustiloxin A-G | | Suppression of mitosis by inhibition of tubulin polymerization (ustiloxin A-F). | (Koiso et al. 1994; Koiso et al. 1998; Ding et al. 2016) |
| | Phomopsins A-E, P | | Suppression of mitosis by inhibition of tubulin polymerization (phomopsin A, B, C, D). | (Allen and Hancock 1989; Battilani et al. 2011; Ding et al. 2016) |
| | Asperipin-2a | | Unknown activity | (Nagano et al. 2016; Ye et al. 2019) |
| Epichloëcyclins | Epichloëcyclin A-F | | Unknown activity. Only produced in endosymbiosis with grass. | (Johnson et al. 2015) |

[a]Likely also MSDIN peptides but precursor genes are not known yet.

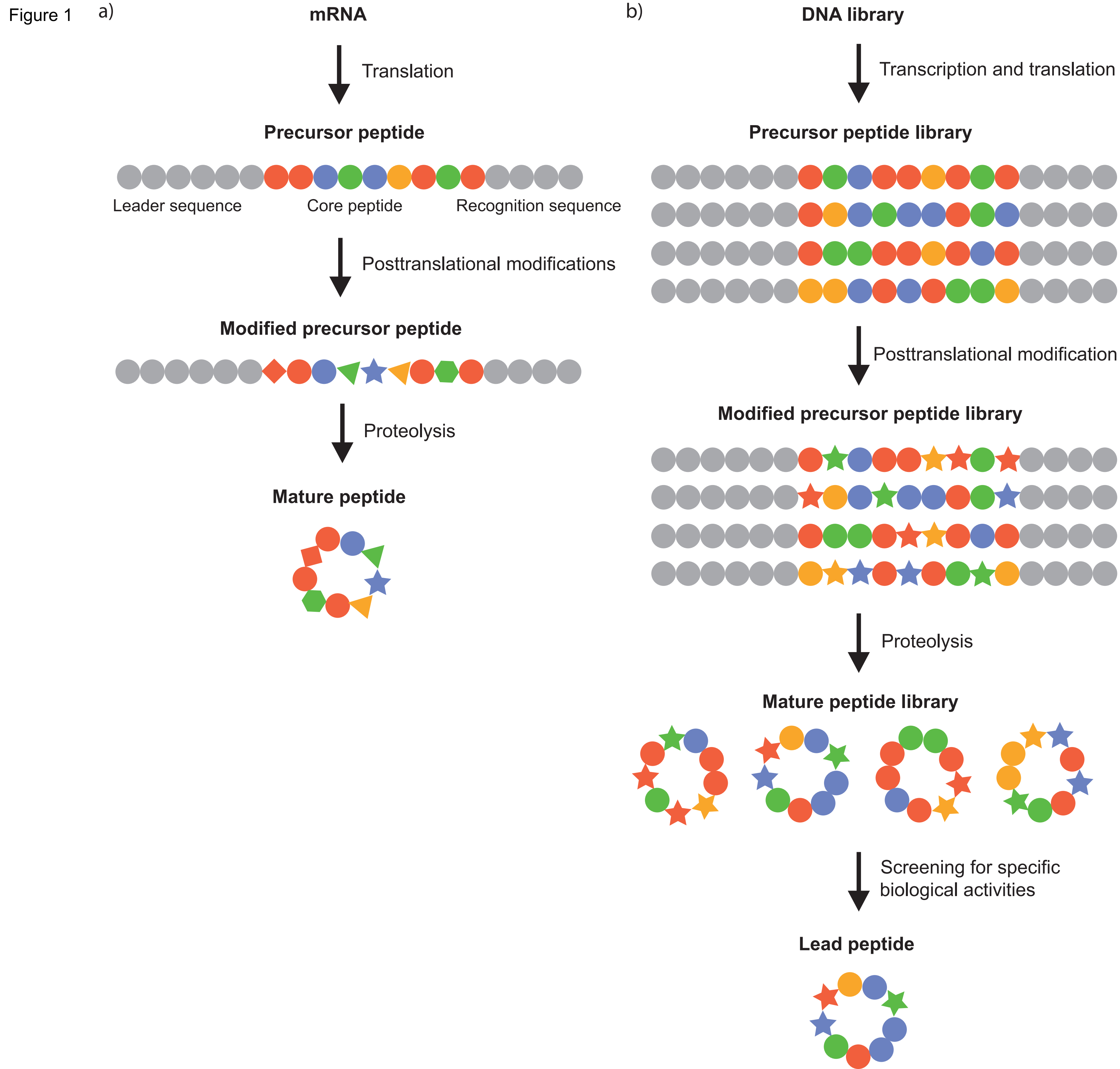

Figure 1
a)
mRNA
Translation
Precursor peptide
Leader sequence
Core peptide
Recognition sequence
Posttranslational modifications
Modified precursor peptide
Proteolysis
Mature peptide
b)
DNA library
Transcription and translation
Precursor peptide library
Posttranslational modification
Modified precursor peptide library
Proteolysis
Mature peptide library
Screening for specific
biological activities
Lead peptide

Figure 2 a)

α-amanitin

phallacidin

phomopsin A

omphalotin A

ustiloxin B

epichloëcyclin A

asperipin-2a

b) α-Amanitin precursor

MSDINATRLPIWGIGCNPCVGDDVTTLLTRGEALC

Phallacidin precursor

MSDINATRLPAWLVDCPCVGDDVNRLLTRGESLC

Omphalotin precursor

1 Methyltransferase domain 399 446

Leader sequence — Core peptide — Recognition sequence

...GFP-WVIVVGVIGVIG-SVMSTE

Ustiloxin precursor

MKLILTLLVSGLCALAAPAA**KR**DGVEDYAIGID**KR**NSVEDYAIGID**KR**NSVEDYAIGID**KR**NSVEDYAIGID**KR**NSVEDYAIGID**KR**
NTVEDYAIGID**KR**NSVEDYAIGID**KR**NTVEDYAIGID**KR**NSVEDYAIGID**KR**NSVEDYAIGID**KR**GGSVEDYAIGID**KR**NSVEDYAIGID**KR**
NSVEDYAIGID**KR**GSVEDYAIGIDK**KR**GTVEDYAIGID**KR**GGSVEDYAIGID**KR**HGGH

Phomopsin precursor

MRFTPAIVIAAFCSLAVAAPAAKAIARSPSEAVEDYVIPIDK**KR**GEAVEDYVIPIDK**KR**GEAVEDYVIPFD**KR**GEAVEDYVIPIDK**KR**
GEAVEDYVIPFD**KR**GEAVEDYVIPIDK**KR**GEAVEDYVIPFD**KR**GEAVEDYVIPIDK

Asperipin-2a precursor

MHLSRYIAVLLSASSFVSALPLQNDVISDDGSKPIDAIMATAMEHKVVNPENLDATPATPENPEDLD**KR**FYYTGY**KR**NAETPEDLD**KR**
FYYTGY**KR**NAETPEDLD**KR**FYYTGY**KR**NAETPEDLD**KR**FYYTGY**KR**NAETPEDLD**KR**FYYTGY**KR**
NAETPDDLD**KR**FYYTGY**KR**NAETPDDLD**KR**FYYTGY**KR**NAETPEDLD**KR**FYYTGY**KR**NAETPEDLD**KR**FYYTGY**KR**NAETPEDLD**KR**
FYYTGY**KR**NAETPEDLDK

Epichloëcyclin precursor

MQFTLIFFYATLAAFGLAAPSEQVGRDVVQEGDELD**KR**INFKIPYTGADLVDGDDVQEGDKLD**KR**
INFKIPYTGADMVDGDDVQEGDKLD**KR**IGFKLPYRGADMVDGDDVQEGDELA**KR**PNFKMPYKGADM