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MINI REVIEW

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Complexity of fungal polyketide biosynthesis and function

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Abstract

Where does one draw the line between primary and secondary metabolism? The answer depends on the perspective. Microbial secondary metabolites (SMs) were at first believed not to be very important for the producers because they are dispensable for growth under laboratory conditions. However, such compounds become important in natural niches of the organisms, and some are of prime importance for humanity. Polyketides are an important group of SMs with aflatoxin as a well-known and well-characterized example. In *Aspergillus* spp., all 34 *afl* genes encoding the enzymes for aflatoxin biosynthesis are located in close vicinity on chromosome III in a *so-called* gene cluster. This led to the assumption that most genes required for polyketide biosynthesis are organized in gene clusters. Recent research, however, revealed an enormous complexity of the biosynthesis of different polyketides, ranging from individual polyketide synthases to a gene cluster producing several compounds, or to several clusters with additional genes scattered in the genome for the production of one compound. Research of the last decade furthermore revealed a huge potential for SM biosynthesis hidden in fungal genomes, and methods were developed to *wake up* such *sleeping* genes. The analysis of organismic interactions starts to reveal some of the ecological functions of polyketides for the producing fungi.

KEYWORDS
Alternaria alternata, antibiotic, Aspergillus nidulans, mycotoxin, polyketide, secondary metabolites

1 | INTRODUCTION

Many secondary metabolites (SMs) which are used as pharmaceuticals, or are important as food contaminants, are of fungal origin (Greco et al., 2019). SMs are not only used to treat human and animal infections (e.g., penicillins, griseofulvin) or diseases (e.g., lovastatin) but are also used as fungicides for plant protection (e.g., strobilurins) (Nofiani et al., 2018). Most filamentous fungi produce a blend of different metabolites, for many of which the natural function is still unknown and not easy to decipher. Meanwhile, hundreds of fungal genomes have been sequenced, and most contain numerous genes encoding enzymes for SM biosynthesis (Caesar et al., 2020; Robey et al., 2021). However, under laboratory conditions, most SM genes are silent. Therefore, many tools and strategies have been developed over the years to assign functions to the *sleeping genes* and/or to discover novel SMs.

Fungi produce different types of SMs, among which polyketides represent a large and diverse class. In this review, we will focus on fungal polyketides and the recent advancements in understanding their biosynthesis and biological functions as well as further perspectives for their exploration.

2 | BIOSYNTHESIS—FROM SIMPLE TO COMPLEX

"The biosynthesis of fungal polyketides is encoded in gene clusters in the genome, and one gene cluster is responsible for the production of one SM." This statement was mainly based on the aflatoxin biosynthesis in *Aspergillus flavus* and *Aspergillus nidulans*, where more than 30 genes are coregulated and are all required for the formation of aflatoxin or sterigmatocystin, respectively (Brown et al., 1996). The central enzyme is a polyketide synthase (PKS) which produces a molecular backbone to which so-called tailoring enzymes may add residues and functional groups, or further modify the structure (Figure 1a). Meanwhile simpler, but also much more complex pathways have been discovered.

PKSs are multidomain enzymes similar to fatty acid synthases (FAS); the two pathways are likely to have diverged early on in evolution (Hertweck, 2009). The reactions are catalyzed by the covalently fused catalytic domains, which constitute discrete functionalities of the polyketide synthase (Hertweck, 2009). The structural diversity of polyketides is very high and is due to multiple factors, such as length of the polyketide chain, oxidation state and stereochemistry of the β-keto groups, mechanism of offloading and chain release, as well as post-PKS tailoring steps (extensively reviewed in Hertweck, 2009; Cox, 2023).

PKSs can be divided into several classes based on their general architecture and mode of product assembly. The majority of fungal PKSs belong to the type I iterative class, meaning that the same domains are reused in a cyclic fashion to catalyze successive rounds of elongation. These PKSs can be further divided into nonreducing (nr-PKS), partially reducing (pr-PKS), and highly reducing (hr-PKS) systems (Hertweck, 2009). The iterative type I PKSs are incredibly intriguing, and several biosynthetic aspects remain unclear, such as the logic behind the timing and order of domain-catalyzed reactions, as well as the number of iterative cycles or degree of reduction during a given extension cycle. Significant progress has been made in understanding the programming of nr-PKSs, in particular, starter unit selection, chain-length control, and stereochemistry following the polyketide cyclization. The least understood are the programming mechanisms of hr-PKSs, despite their high sequence, domain, and structure similarity to FASs (extensively reviewed in Cox, 2023). In contrast to type I iterative PKSs, FASs are not programmed, in

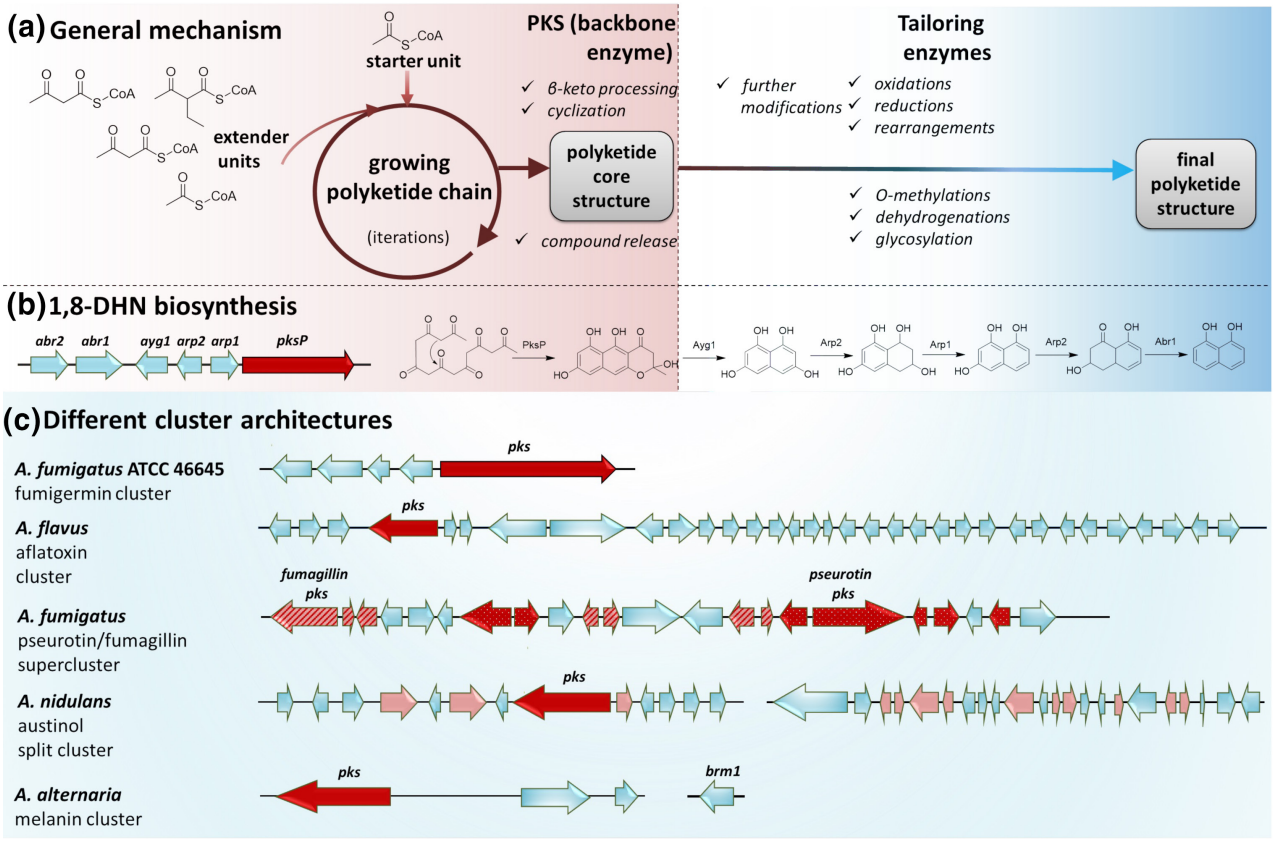

FIGURE 1 Fungal polyketide biosynthesis by main enzyme and tailoring enzymes. (a) General mechanism of iterative polyketide assembly and enzyme activities. The core PKS enzyme catalyzes the condensation of a core polyketide compound from simple precursors such as acetyl-CoA and malony-CoA, and releases the compound for further modifications by tailoring enzymes, until the final product of cluster is formed. (b) Biosynthesis of the polyketide 1,8-dihydroxynapththalene (DHN) as an illustrative example of a typical SM gene cluster consisting of a core PKS and tailoring enzyme-encoding genes (Langfelder et al., 2003). (c) Different cluster architectures (top to bottom): PKS alone sufficient for compound production and release, all genes in one cluster, intertwined cluster leading to formation of two unrelated different compounds, one cluster split into two parts located on different chromosomes, one cluster with additional enzymes scattered across the genome.

the sense that all domains are active in all cycles. Therefore, in general, it is very difficult to predict the final compound arising from an iterative PKS enzyme (Cox, 2023; Hertweck, 2009). The growing polyketide chain often circularizes and is released from the enzyme by the thioesterase domain of the PKS. An example is the formation of fumigermin in *Aspergillus fumigatus* (Stroe et al., 2020). Although the PKS-encoding gene *fgnA* is embedded into a gene cluster and co-expressed with tailoring enzyme-encoding genes, apparently only FgnA is required for fumigermin biosynthesis (Figure 1c). A fascinating example is that of CalA, the PKS responsible for the biosynthesis of calbistrin. CalA is a dual-function PKS that produces two separate portions which are finally joined together by the acyltransferase CalD (Tao et al., 2021). Therefore, PKSs themselves can generate complexity, depending on their type, module, and domain composition. To further complicate matters, it has been recently discovered that some PKSs are multi-chain length synthases that preferentially produce differing compounds. A pair of "twin" basidiomycete PKSs, CoPKS1, and CoPKS4, have been shown to produce different compounds, with CoPKS4 producing both hepta- and octa-ketides, despite the PKSs sharing more than 88% sequence similarity (Löhr et al., 2022). The authors identified the β-ketoacyl synthase domain of each PKS as that determining whether a hepta- or an octa-ketide is the predominant product. Additional complexity therefore stems from various polyketide chain termination modes, catalyzed either by additional discrete enzymes (trans-acting), or intrinsic domains of the PKS itself. Further examples include a C-terminal thioesterase-like PKS domain which intercepts the intermediate to produce shorter chain-length products (Watanabe & Ebizuka, 2004), a ketoreductase domain-catalyzed polyketide chain release (Yang et al., 2023), a discrete acyltransferase-mediated release (Xie et al., 2009) and a discrete thiohydrolase-controlled chain length (Zabala et al., 2014).

PKSs are therefore not always acting alone. A gene cluster is typically characterized by genes that are all co-expressed under the same conditions. If a cluster-specific transcription factor is encoded inside the cluster, this is the first gene to be expressed and in turn activates all other cluster genes. In addition, global regulators like LaeA are required for the coordinated expression of the genes (Keller et al., 2006; Tannous et al., 2023). Applying these parameters to whole-genome transcriptomic approaches helps to identify novel gene clusters. However, another level of complexity was added with the discovery that a cluster with co-regulated genes was able to produce two different SMs, as is the case for the fumagillin and pseurotin *supercluster* in *A. fumigatus* (Wiemann et al., 2013).

There are also examples where two separate clusters are required for the biosynthesis of one compound, as is the case of austinol in *A. nidulans* (Lo et al., 2012). On the other hand, an intermediate from one pathway may be used in a branched biosynthesis in order to produce different metabolites, as for altertoxin (ATX) biosynthesis in *Alternaria alternata*, which shares most of its enzymes with melanin biosynthesis. 1,8-DHN is the last common intermediate and is either further converted to melanin or it dimerizes to produce a perylene quinone which is then transformed into ATX (Gao et al., 2022) (Figure 1b,c). Likewise, in the nematode-trapping fungus *Arthrobotrys flagrans*, the same pathway is shared for pigment and arthrosporol production (Yu et al., 2021). Another example is the biosynthesis of the bis-anthraquinones skyrin and rugulosin A (Han et al., 2021). Both compounds share a common intermediate, emodin, from which the intertwined *rug* cluster simultaneously leads to the biosynthesis of skyrin and rugulosin A in the endophytic fungus *Talaromyces* sp. YE3016. All these variations are probably the result of complex genomic re-arrangements and evolutionary transformations that the clusters are subjected to over time.

3 | COMPARTMENTALIZATION OF POLYKETIDE BIOSYNTHESIS INCREASES CHEMICAL DIVERSITY

The concept of *superclusters* already disproved the dogma of *one cluster–one compound*; additional complexity may arise from biosynthetic steps taking place at different rates or at different times in different fungal hyphae. Individualism of bacterial colonies is a well-documented phenomenon (Schreiber et al., 2016); there is some evidence that the situation is similar for fungi, with examples showing the same protein being differentially produced in discrete areas of fungal hyphae (Tegelaar et al., 2020). Returning to the case of polyketides, the *A. alternata* ATX and 1,8-DHN are not produced simultaneously in the same hyphae: 1,8-DHN is produced in aerial hyphae, while ATX biosynthesis is restricted to substrate hyphae (Gao et al., 2022). In *Botrytis cinerea*, polyketide synthases BcPks12 and BcPks13 catalyze the initial step of 1,8-DHN biosynthesis in sclerotia and conidia, respectively (Schumacher, 2016). Another example of such diversification of hyphae is the formation of arthrosporols and 6-methylsalicylic acid (6-MSA) in the nematode-trapping fungus *A. flagrans*. The production of arthrosporols requires 10 enzymes, but not all enzymes are expressed in the same place in one hypha. The PKS is expressed mainly at hyphal tips and produces 6-MSA, whereas all enzymes are expressed in the rear of the hyphae where 6-MSA is then further converted to arthrosporols (Yu et al., 2021).

Relatively few reports have focused on the subcellular compartmentalization of biosynthesis steps. Enzymes required for the biosynthesis of fungal SMs may reside in the cytosol, peroxisome, and other delimited membrane-bound organelles, like vacuoles, vesicles, endosomes, and toxisomes (Skellam, 2022). The complexity of fungal secondary metabolism may therefore also stem from the individuality of the producing hyphae, but so far it is unclear whether such differential spatial expression of biosynthetic enzyme-encoding genes is the norm or the exception.

4 | SM MINING AND "BIOSYNTHETIC DARK MATTER"

The success story of microbial SMs was largely due to the discovery of natural products in microbes under different cultivation

conditions. Although tedious, this strategy led to the discovery of many lead structures for modern medicine. After the "low-hanging fruits" were collected, the success rate decreased over time, but the post-genomic era and development of new strategies for combinatorial biosynthesis or designer-made drugs led to a renaissance of organism-based drug discovery. For instance, an early publication reported the biosynthesis of several statin derivatives by mutating *lovD*, the acyl transferase of lovastatin biosynthesis; changes in the sequence of LovD led to increased promiscuity in the substrate acceptance of the enzyme, which allowed for the formation of 22 unnatural statin compounds with differing side chains (Xie et al., 2006).

On the other hand, many research groups focused on underinvestigated niches for new isolates with new biosynthetic capacities. Examples are anaerobic fungi, whose genomes are poorly characterized and have not been extensively mined. A recent study investigated the biosynthetic potential of anaerobic gut fungi, which belong to the class Neocallimastigomycetes, and revealed 146 genes encoding biosynthetic enzymes for diverse types of natural products, including polyketides such as the styrylpyrone baumin, a compound unique to anaerobic fungi according to molecular networking analysis (Swift et al., 2021). Endophytic fungi are another interesting group to be studied. A recent publication combined the OSMAC approach with metabolomic, statistical, and networking analyses to reveal antibacterial properties of endophytic *Penicillium* sp. and found the polyketides austalides and viridicatumtoxin A, a rare tetracycline (da Silva et al., 2023).

The rapidly increasing number of genomic sequences from fungi revealed that the potential for SM production has been vastly underestimated. The model organism *A. nidulans* is one of the best-characterized fungal species, but despite this special status, over half of its biosynthetic gene clusters (BGCs) remain uncharacterized. Thus, the "biosynthetic dark matter" accounts for more than half of BGCs of thoroughly studied fungi, and anywhere between 50% and 100% of more "exotic" or uncultured fungi from unexplored habitats (Caesar et al., 2020). To this extent, a new bioinformatic meta-analysis set out to use the full capability of all available fungal genomes at the time (1037 published genomes in 2021) for the annotation of fungal BGCs and comparison with bacterial ones. The authors describe 15,213 fungal compounds and conclude that the chemical space occupied by fungal-derived compounds does not overlap with that occupied by bacterial-derived compounds, which again emphasizes the unique biosynthetic potential of fungi (Robey et al., 2021). With 5, 453 available bacterial genomes, another conclusion that can be drawn from the work of Robey et al. is that there is a clear discrepancy between the amount of data available for fungal species compared to bacterial species. A direct consequence is that *in-silico* tools for mining fungal BGCs do not receive enough training material, compared to bacterial applications. The popular tool antiSMASH ("antibiotics and secondary metabolite analysis shell") is the most widely used tool for mining of BGCs. Since going online in 2011, the tool has seen seven iterations (Blin et al., 2023). While the tool manages bacterial BGCs perfectly well, fungal BGCs are often missed or incorrectly assigned. Nowadays, machine- and deep-learning approaches mix with such classical algorithms for an improved identification and annotation of BGCs, as is the case for DeepBGC, which claims to reduce false-positive rates and to better identify novel BGCs which the algorithm has not yet encountered (Hannigan et al., 2019). However, this tool is now only well established for bacterial BGCs—a tool suitable for fungal BGCs should follow soon.

To further complicate matters, the potential of SM production varies among fungi. The analysis of fungal genomes allowed studies on the evolution of the clusters and the chemo-diversity across species and surprisingly revealed heterogeneity even among isolates of the same species (Rokas et al., 2020). Generally, there is a literature bias towards ascomycete-derived polyketides, but significant progress has been made regarding basidiomycete PKSs as well, for instance, the discovery of melledonol produced by a basidiomycete orsellinic acid synthase-type PKS (Lackner et al., 2013), or the discovery of a PKS producing pigments and benzenoid metabolites responsible for the antioxidant activity of an important medicinal mushroom (Yu et al., 2016). Furthermore, more recent studies identified the first basidiomycete hr-PKS, producing polyenes (Brandt et al., 2017) and two PKSs producing atrochrysone, a compound also produced by *Aspergillus* spp., indicating that the basidiomycete and the ascomycete synthases evolved convergently (Löhr et al., 2022). In other basidiomycete fungi, like the plant pathogenic *Ustilago maydis*, only five polyketide BGCs have been found. They are all involved in the biosynthesis of the black fungal pigment but are divided into one main cluster (*pks1* and *pks2*) and one additional, alternative cluster encoding PKSs 3 to 5 (Reyes-Fernandez et al., 2021).

In order to access the dormant biosynthetic potential, approaches aiming to *wake up* such *sleeping* gene clusters have been developed. One popular strategy that bypasses the slow growth of some organisms is to move the PKS genes or cluster in a heterologous host, such as *Escherichia coli* or *Saccharomyces cerevisiae*, or more commonly, filamentous ascomycete expression platforms. For instance, *Aspergillus oryzae* is a convenient host, because it is a GRAS (generally regarded as safe) organism and produces none or very few own polyketides. It has been used to decipher the alternariol biosynthesis (Wenderoth et al., 2019) and more recently for the production of basidiomycete polyketides (Han et al., 2023). Another host organism is *A. nidulans*, which has been engineered by deletion of eight entire native BGCs from its genome for a cleaner background and less crosstalk between native and heterologous genes (Chiang et al., 2022). Expression systems have also been developed for *Aspergillus niger*, for instance, the terrein-based system (Gressler et al., 2015), the ATNT-system (Geib & Brock, 2017), or a system suitable for the expression of very long PKSs derived from mushroom-forming fungi and early diverging fungi (Kirchgaessner et al., 2023). *Waking up* gene clusters may help to understand their natural relevance, which in turn will let us hope to find new compounds with new pharmacological activities.

5 | BIOLOGICAL FUNCTIONS OF POLYKETIDES—FROM SELF-PROTECTION TO SIGNALING AND MORPHOGENESIS CONTROL

The concept of *SMs* assumes that those molecules are not very important for the survival of the organism under laboratory conditions. However, in nature, SMs may have important functions that are slowly being revealed (Figure 2). Polyketide-derived pigments are found in many fungi and are important for UV protection. Black melanin is a typical pigment of this class, but green pigments such as that of *A. nidulans* can also serve a protective function and also require a polyketide synthase for their biosynthesis. In another example of polyketides with protective roles, antilarval compounds were shown to be produced by a basidiomycete in response to physical damage (Brandt et al., 2017). This example shows that polyketides may

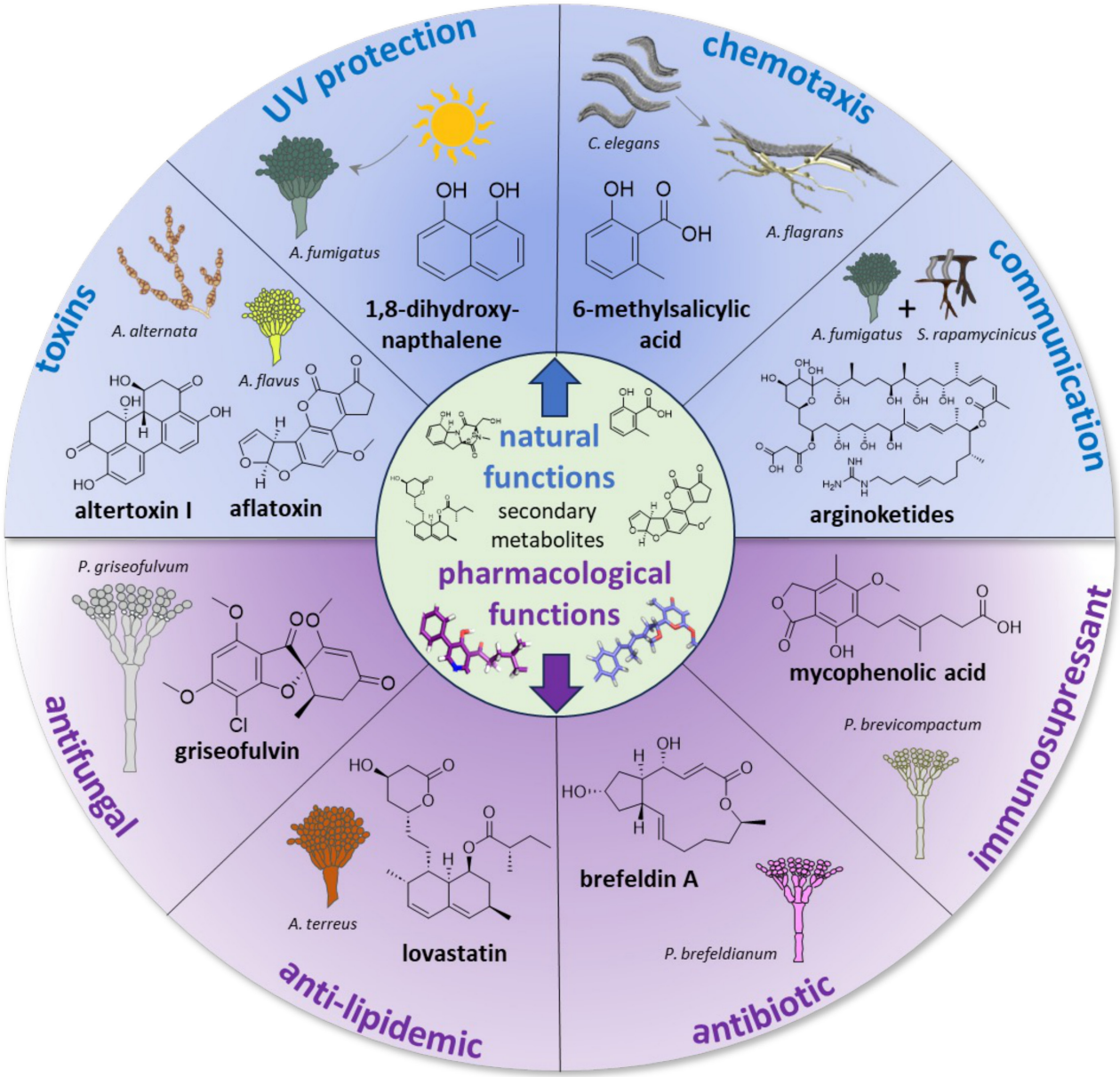

FIGURE 2 Diversity of fungal polyketide functions. Bottom, pharmacological functions assigned to fungal compounds, such as the mitosis-inhibiting antifungal compound griseofulvin, produced by *P. griseofulvum*; the HMG-CoA reductase inhibitor and hence cholesterol-lowering compound lovastatin, which led to the development of the statin blockbuster drugs; the protein-secretion inhibitor antibiotic compound brefeldin A, produced by *P. brefeldianum*; the purine-biosynthesis inhibitor mycophenolic acid produced by *P. brevicompactum*, which is used as an immunosuppressant drug. Top, recently discovered natural functions of fungal SMs, such as communication devices in the inter-kingdom species communication, as is the case for bacterial arginoketides recognized by *Aspergillus* fungi; volatile compounds such as the *A. flagrans*-produced 6-MSA which serves as a chemoattractant luring the nematodes into the fungal mycelial traps; 1,8-DHN, the monomer of the fungal black pigment melanin, which confers protection against UV damage; fungal toxins such as aflatoxin and altertoxin I, produced by *A. flavus* and *A. alternata*, respectively.

serve to inhibit competitors living in the same environment. Such an assumption was used to activate certain gene clusters in fungi upon co-cultivation with bacteria, which resulted in the discovery of fumigermin, a polyketide produced by *A. fumigatus* when it is challenged with a *Streptomyces* bacterial competitor co-inhabiting the same ecological niche (Stroe et al., 2020). A further research question was how *Aspergillus* spp. can sense the presence of the bacterial competitor. It was recently revealed that streptomycetes produce arginoketides (polyketide compounds derived from arginine) which function as communication signals that trigger the production of fungal antibacterial compounds, like fumigermin, in response to the presence of the bacterial competitors (Krespach et al., 2023). The latter example shows the complex interaction of microorganisms in natural habitats and that polyketides may be of prime importance in interkingdom communication.

Another example of polyketides with a role in interkingdom signaling is 6-MSA, which is produced by the nematode-trapping fungus *A. flagrans* as an attractive volatile compound luring nematodes into the fungal traps (Yu et al., 2021). 6-MSA can be further modified by *A. flagrans* into arthrosporols, which inhibit the development of trap formation by the nematode-trapping fungus. Once nematodes are present in large numbers and the fungus is starving, arthrosporol biosynthesis is inhibited and thereby trap formation is induced. Moreover, in *A. nidulans*, it was shown that xanthone biosynthesis is induced in the sexual structures of the fungus to protect them from predators feeding on the fungal biomass (Liu et al., 2021).

Polyketides may also facilitate or enable the colonization of hosts, as is the case of alternariol from *A. alternata*, which colonizes plants (Wenderoth et al., 2019). *Alternaria* spp. can produce more than 70 food-contaminating mycotoxins exerting mutagenic and estrogenic effects, such as alternariol, altenuene, tenuazonic acid, tentoxin, and the altertoxins (Crudo et al., 2019). A key area to follow here will be microbiome studies investigating how gut populations metabolize the ingested toxins.

6 | PERSPECTIVE

In summary, we have recounted several examples of different cluster architectures and functionalities of compounds. However, many questions remain unanswered, such as what the ecological functions of most metabolites are, how the compartmentalization of biosynthetic enzymes impacts compound diversity, or why SM genes are physically linked in fungi. The current holy grail of PKS research may be that of predicting the chemical structure of polyketides using bioinformatic analyses of genomic data. The programming logic of certain iterative PKSs, such as hr-PKSs, is only beginning to be understood, but there has been some progress in predicting the general class of compounds produced by such enzymes (Cox, 2023). Nevertheless, predicting any further compound details such as starter unit, chain length, presence of functional groups, and stereochemistry is currently far from reach. Despite the challenges, there is accumulating data (such as Minami et al., 2020) which allows us to be cautiously optimistic that AI-driven tools and machine-learning algorithms will contribute to this prediction issue. Another milestone of PKS research will be pinpointing the exact subcellular localization of compound biosynthesis, as well as the trafficking of natural products from fungal compartment to compartment. Such knowledge should improve compound production yields but also aid the optimization of heterologous expression techniques in commonly employed host organisms whose compartments may be inappropriate or even absent (such as *E. coli*). This crucial research question will hopefully be tackled by improved techniques in mass spectrometry imaging, which have already yielded some results in metabolomic research (Alexandrov, 2023; Yang et al., 2023). The fast development of modern informatics and analytical techniques, together with the ever-increasing number of annotated fungal genomes, will most certainly advance our understanding of fungal secondary metabolism and allow us to harness its full potential to our advantage.

AUTHOR CONTRIBUTIONS

Reinhard Fischer: Conceptualization; writing – original draft; writing – review and editing; project administration; supervision; validation; funding acquisition. **Maria Stroe:** Conceptualization; visualization; writing – original draft; writing – review and editing. **Jia Gao:** Investigation; validation. **Michael Pitz:** Investigation; validation.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have 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.

ETHICS APPROVAL STATEMENT

We followed the ethics guidelines of the journal.

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