

Biosynthesis of Terpenoid Natural Products in Fungi

Claudia Schmidt-Dannert

Abstract Tens of thousands of terpenoid natural products have been isolated from plants and microbial sources. Higher fungi (Ascomycota and Basidiomycota) are known to produce an array of well-known terpenoid natural products, including mycotoxins, antibiotics, antitumor compounds, and phytohormones. Except for a few well-studied fungal biosynthetic pathways, the majority of genes and biosynthetic pathways responsible for the biosynthesis of a small number of these secondary metabolites have only been discovered and characterized in the past 5–10 years. This chapter provides a comprehensive overview of the current knowledge on fungal terpenoid biosynthesis from biochemical, genetic, and genomic viewpoints. Enzymes involved in synthesizing, transferring, and cyclizing the prenyl chains that form the hydrocarbon scaffolds of fungal terpenoid natural products are systematically discussed. Genomic information and functional evidence suggest differences between the terpenome of the two major fungal phyla—the Ascomycota and Basidiomycota—which will be illustrated for each group of terpenoid natural products.

Keywords Ascomycota • Basidiomycota • Isoprenoid • Terpene synthase • Natural products • Terpenoids • Prenyl transferase • Terpene cyclase • Sesquiterpenoids • Diterpenoids • Biosynthesis • Pathways • Gene cluster

Abbreviations

TPS	Terpene synthase
TPC	Terpene cyclase
PT	Prenyltransferase
FPP	Farnesyl diphosphate
IPP	Isopentenyl diphosphate
IPPS	Isopentenyl diphosphate synthase

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DMAPP	Dimethylallyl diphosphate
GPP	Geranyl diphosphate
GGPP	Geranylgeranyl diphosphate
CPP	Copalyl diphosphate
ABBA	beta/alpha-fold with antiparallel beta strands
Me	Methyl
Ac	Acetyl
NPs	Natural products

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1 Introduction

The fungal kingdom includes an enormous diversity of organisms, of which only a fraction ($\sim 100,000$ species) has been described [1]. Conservative calculations estimate that fungal diversity exceeds the number of described species by at least one order of magnitude (~ 1.5 million species), making the fungal kingdom the second largest kingdom after the bacteria [2–4]. The phylum Ascomycota (filamentous fungi) accounts for ~ 60 % of the described species. This group includes many serious human, animal, and plant pathogens [5, 6], as well as fungi (e.g. *Aspergillus*, *Fusarium*, *Trichoderma*) with a long tradition in industrial biotechnology [7–10] and strains that are investigated for their secondary metabolism [11–13]. Basidiomycota (including mushroom-forming fungi) make up ~ 30 % of the known fungal species. Very few species of this phylum have been characterized, despite playing important roles in the decomposition of plant material (e.g. wood-rotting or saprophytic fungi) or as plant symbionts (e.g. mycorrhizal fungi) [14, 15]. Considering the enormous diversity of basidiomyceteous fungal species, very few of these fungi have yet been characterized, and biosynthesis of natural products has been investigated for an even smaller subset of taxa [16–27].

Studies aimed at deciphering secondary metabolic pathways on molecular and biochemical levels have been almost exclusively focused on Ascomycota (e.g. *Aspergillus*, *Penicillium*, *Fusarium*), which can be grown readily in the laboratory and are genetically tractable [9, 13, 28, 29]. The fungi remain a largely uncharted territory for the discovery of new natural products and their biosynthetic pathways, including isoprenoid-derived secondary metabolites. This is particularly true for the Basidiomycota; these fungi are typically difficult to grow or cannot be grown at all under laboratory conditions; except for a few species, they are not amenable to genetic manipulation. Yet, mushrooms have been used for millenia in traditional medicine and are known to make a range of bioactive compounds, including a plethora of antimicrobial, cytotoxic, and anticancer compounds [30–37].

The slow progress made over the past decades in fungal natural products pathway identification and characterization will certainly accelerate drastically during the coming decade. Rapid progress in fungal genome sequencing and the development of synthetic biology approaches for heterologous refactoring of complex biosynthetic pathways with synthetic genes will drive the discovery process. Together with advances in bioinformatics and metabolomics, these approaches will eventually facilitate high-throughput discovery of natural products pathways from genomic sequence information alone independent of fungal cultivation. Identification of many fungal natural products pathways is also greatly aided by the fact that their genes are physically co-localized as clusters that facilitate efficient co-regulation of their expression by the fungus [29, 38–43].

This chapter reviews the current progress in the biosynthesis of isoprenoid natural products in fungi. After providing an overview of the different pathways and enzymes involved in generating isoprenoid precursor molecules that are then converted into complex biomolecules, the current knowledge on fungal biosynthesis of sesquiterpenoids, diterpenoids, and triterpenoids is discussed. Natural products of mixed biosynthetic origin, such as meroterpenoids and indole-diterpenoids, that contain a terpenoid derived moiety are discussed elsewhere in this series; their biosynthesis is only discussed here as it relates to the enzymes that install the terpenoid moieties in these compounds. Fungal carotenoid biosynthesis by basidiomycetous yeasts, such as the well-known carotenoid producers *Rhodotorula* and *Xanthophyllomyces* (*Phaffia*), has been described extensively in the literature [44, 45] and will therefore not be reviewed in this chapter.

Special emphasis is given to the differences observed between the isoprenoid natural products repertoire of the two major fungal phyla Asco- and Basidiomycota. This review concludes by discussing current bottlenecks in the identification and characterization isoprenoid natural products' biosynthetic pathways, stressing the significance of genomic-and bioinformatics-driven pathway discovery as well as opportunities offered through synthetic biology approaches for heterologous refactoring of natural products pathways.

2 Fungal Terpenoid Natural Product Biosynthesis

2.1 Types of Isoprenoid Biosynthetic Enzymes

2.1.1 Overview

All fungal terpenoid natural products are derived from the common five-carbon isoprenyl diphosphate intermediates isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are synthesized from acetyl-CoA through the mevalonate pathway shown in Fig. 1. Successive head-to-tail 1'-4 condensation of one to three IPP extender units to DMAPP catalyzed by *all-trans* isoprenyl

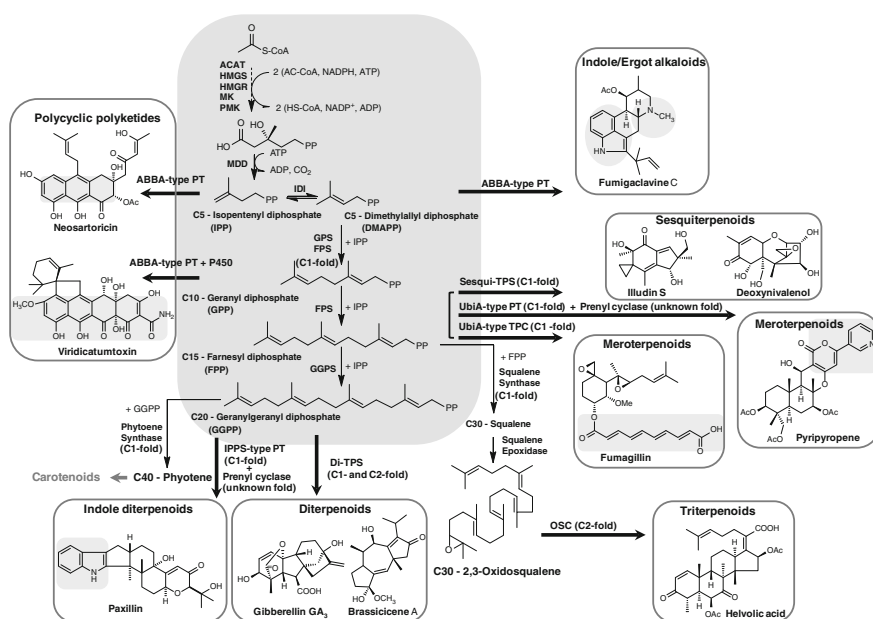


Fig. 1 Overview of isoprenoid derived natural products biosynthesis in fungi. The mevalonate pathway generates the isomeric C-5 isoprenoid chain precursors isopentenyl and dimethylallyl diphosphate (IPP and DMAPP). Head-to-tail condensation of these C-5 units yields prenyl-diphosphate chains of different lengths. Longer prenyl chains are formed by head-to-head condensation of two prenyl diphosphate chains. Linear prenyl chains are the substrates of prenyl transferases and cyclases as well as terpene synthases, which form the terpenoid scaffolds of different natural products classes, for which example compounds are shown. Prenyl chain modifying enzymes can be divided by structural folds (C1- and C2-fold, ABBA-type; see text) and by catalytic function. Abbreviations: ACAT: acetoacetyl-CoA thiolase, HMGS: 3-hydroxy 3-methylglutaryl CoA synthase, HMGR: 3-hydroxy 3-methylglutaryl CoA reductase, MK: mevalonate kinase, PMK: phosphomevalonate kinase, MDD: mevalonate diphosphate decarboxylase, IDI: isopentenyl diphosphate isomerase, GPS, FPS or GGPS: geranyl-, farnesyl- or geranylgeranyl diphosphate synthase, PT: prenyl transferase, TPS: terpene synthase, TPC: terpene cyclase, OSC: oxidosqualene synthase

diphosphate synthases (IPPS) gives rise to isoprenyl diphosphates with ten (C-10, geranyl, GPP), fifteen (C-15, farnesyl, FPP), or twenty (C-20, geranylgeranyl, GGPP) carbons. Longer chains (C-30 and C-40) are formed by a 1'-1 head-to-head condensation of two FPP (squalene synthase) or GGPP (phytoene synthase) molecules catalyzed by another group of prenyl chain synthases.

These linear chains are the substrates of different enzymes that either transfer a prenyl chain to another molecule, typically an aromatic compound, or trigger prenyl chain cyclization, thereby generating tens of thousands different isoprenoid-derived natural products [46]. Different types of prenyl transferases and cyclases have evolved in fungi and seem to be associated with specific natural products classes. Some biosynthetic pathways, such as the paxilline indole-diterpene pathway [47], involve two types of prenyl transferases that catalyze different prenylation reactions.

The different classes of characterized terpenoid natural products can be distinguished based on whether their scaffolds are derived solely from isoprenyl units or are of mixed biosynthetic origin. The first group is divided into mono-, sesqui-, di-, or triterpenoids, which contain two to six C-5 isoprene units. This group also includes the carotenoids and rare sesterterpenoids (C-25). The second group includes the meroterpenoids, the indole diterpenoids, and the structurally and biosynthetically diverse group of prenylated aromatic natural products.

As discussed in more detail elsewhere in this book series, the majority of characterized prenyl chain synthases, transferases (PTs), and cyclases share one of two structural folds (C1- and C2-fold) and associated reaction mechanisms (shown in Fig. 1) [48–50]. It should be noted, however, that despite clear structural homology and inferred evolutionary relatedness, sequence homology between these different enzymes is often not obvious.

A large group of aromatic prenyltransferases has a different fold (ABBA-fold) and uses a different reaction mechanism [51–53]. No structural or detailed mechanistic information is available for prenyl cyclases involved in the biosynthesis of indole diterpenoids [47, 54–56] and of some meroterpenoids [57–59].

The first major class of isoprenoid biosynthesis enzymes (class I) is characterized by an α -helical bundle fold (C1-fold, referred to as α -domain) that forms a hydrophobic active site cavity. Two aspartate-rich motifs (D(D/E)XX(D/E) and NSE/DTE) are situated at the active site entrance and coordinate binding of the substrate diphosphate group via Mg^{2+} . Catalysis is initiated by heterolytic cleavage of the diphosphate group, generating a reactive allylic carbocation for electrophilic addition to an electron-rich nucleophile. In the case of terpene synthases, the carbocation reacts with a C–C double bond, triggering in the active site a cascade of cyclization and rearrangement reactions of the prenyl chain until final quenching of the carbocation by proton abstraction or, in some cases, an electrophilic reaction with water. In the case of the *trans*-IPPS enzymes, a new C–C bond is formed between the carbocation of DMAP (or of GPP and FPP in subsequent chain extension) (head) and the terminal double-bond of IPP (tail). The head-to-head condensation of two FPP or GGPP molecules catalyzed by squalene or phytoene synthase, respectively, proceeds via a cyclopropyl diphosphate intermediate that is

subsequently cleaved. Prenyl transferases (C1-fold and ABBA-type, see below) catalyze electrophilic alkylation between a prenyl diphosphate chain and an electron-rich indole or polyketide moiety.

The second major class of isoprenoid biosynthetic enzymes (class II) share a double α -helical barrel fold (C2-fold) with a hydrophobic active site cavity located between the two alpha-barrel domains (commonly referred to as $\gamma\beta$ -domains). Catalysis is initiated by the addition of a proton from a conserved aspartate residue to a C–C double bond (e.g. copalyl diphosphate synthase domain of bifunctional diterpene synthases) or to an epoxide-ring (e.g. oxidosqualene lanosterol synthase). Formation of the ensuing carbocation triggers a cyclization cascade along the prenyl chain. If the substrate is a prenyl diphosphate (e.g. GGPP), the carbocation is generated at the distal double bond located at the tail end of the prenyl-chain, which leaves the diphosphate group attached. The conserved aspartate residue (bold) is typically located in a **DXDD** motif in eukaryotic class II enzymes that protonate a C–C double bond, whereas epoxide ring protonating enzymes contain a catalytic aspartate in a different motif (e.g. **DCTAE** in oxidosqualene synthases) [60, 61].

2.1.2 Aromatic Prenyltransferases

Known prenyl transferases in fungi fall into three general groups: the ABBA-, IPPS- or UbiA-type prenyltransferases (PTs), as shown in Fig. 1. The latter two share a common C1-fold, but while the IPPS-type synthases appear to be soluble and contain a canonical D(D/E)XX(D/E) motif, the UbiA-type prenyl transferases are integral membrane proteins.

The C-5 isoprenyl diphosphate DMAPP is the substrate of a large group of aromatic ABBA-type PTs identified in Ascomycota, which are mainly involved in the biosynthesis of diverse bioactive indole alkaloids, including many important pharmaceuticals and toxins made by strains of *Aspergillus*, *Penicillium*, *Claviceps*, and *Neosartorya* (reviewed in [62]). Most of these enzymes catalyze the regio- and stereoselective transfer of DMAPP to any position of a tryptophan-derived indole ring via condensation to either the C-1 or C-3 position of the prenyl chain [63–65]. Some enzymes catalyze DMAPP and GPP (C-10) prenyl transfer to other aromatic substrates, such as polycyclic polyketides and phenols (e.g. xanthenes) [66, 67] or to O- and N-functional groups of aromatic moieties [68, 69]. More recently, an enzyme was identified that catalyzes O-prenylation of a glucose moiety of the diterpene fusicoccin A [70]. Mechanistic and structural studies revealed that these proteins adopt a new β -barrel fold containing repeating $\alpha\beta\beta\alpha$ -secondary structure elements, which gives this enzyme family their name: ABBA-type. This fold is conserved between bacterial and fungal aromatic PTs [51, 71]. These enzymes are soluble and do not contain aspartate-rich motifs that coordinate binding of the prenyl diphosphate group through Mg^{2+} .

IPPS-type PTs (e.g. LtmC, PaxC and AtmC) catalyze the transfer of GGPP to an indole group to create the core scaffold of the structurally diverse indole-diterpene natural products (NPs) isolated from Ascomycota, which include many potent

mammalian mycotoxins [47, 54–56, 72–74]. In vitro studies have been only very recently carried out with a purified prenyl transferase (PaxC) from the paxilline pathway and confirm transfer of GGPP to the C3 position of tryptophan [47]. Surprisingly, the tryptophan precursor indole-3-glycerophosphate was the preferred substrate and C3 prenylation resulted in simultaneous elimination of glyceraldehyde-3-phosphate. Concurrent prenylation and elimination has only been known for a different type of prenyl transferase, MenA, which catalyzes naphthol octaprenylation with simultaneous decarboxylation.

A third type of prenyl transferases (UbiA-type PT in Fig. 1) catalyzes the transfer of FPP to the polyketide-derived aromatic core in the biosynthesis of numerous bioactive meroterpenoids. Prenyl transferases (Trt2, Pyr6, AusN, and MpaA) have been identified in biosynthetic gene clusters for terretonin, pyripyropene, austinol, and mycophenolic acid synthesis [57–59, 75–77]. These enzymes are integral membrane proteins and belong to the UbiA/Coq2 quinone prenyl transferase group, which also includes enzymes involved in the secondary metabolite biosynthetic pathways of various plants [78]. Although these enzymes are integral membrane proteins, a homology model was built for UbiA based on a class I terpene synthase structure. Aspartate-rich motifs located at the entrance of the active site of UbiA and in a plant prenyl transferase homolog have subsequently been identified to bind Mg^{2+} and coordinate diphosphate binding of the prenyl substrate [79, 80]. Very recently, the structure of UbiA from *Aeropyrum pernix* was solved and shown to indeed possess an alpha-helical fold that is made up of nine transmembrane helices and three helices that cap a large active site cavity [81]. Aspartate residues located in two of the helices are involved in Mg^{2+} and diphosphate binding. The active site opens laterally into the membrane, thereby allowing binding of long-chain prenyl substrates and release of hydrophobic prenylated products directly into the membrane. The UbiA structure suggests that this enzyme may indeed be evolutionarily related to C-1 fold, IPPS-type PTs.

2.1.3 Prenyl Chain Cyclases

A large number of class I and class II terpene synthases have been characterized from plants and, to a lesser extent, from microorganisms, including fungi. These enzymes create the cyclic hydrocarbon scaffolds of many bioactive terpenoid antibiotics, toxins, and pheromones [82]. Enzymes that cyclize FPP or GGPP into structurally diverse sesqui- or diterpenoids have been characterized from several fungi, and structures are available for two fungal sesquiterpene synthases [83, 84]. As discussed later in more detail, the majority of biochemically characterized fungal terpene synthases are sesquiterpene synthases. Relatively few diterpene synthases have been characterized from fungi, and fungal triterpenoid natural products biosynthesis is even less studied. So far, no *bona fide* monoterpene synthases have been identified in fungi, although in vitro biochemical studies have shown that fungal sesquiterpene synthases are able to cyclize GPP in addition to their native

substrate FPP [22]. This is in stark contrast to the large number of mono-, sesqui-, di-, and triterpene synthases characterized from plants [85–87].

Until recently, it was thought that the enzymes that cyclize isoprenoid diphosphate molecules belong to one of the prototypical class I or II terpene synthase families [48]. Tang and co-workers, however, discovered a new, noncanonical type of sesquiterpene synthase in *Aspergillus fumigatus* that cyclizes FPP into the monocyclic β -trans-bergamotene, which is the biosynthetic terpenoid precursor of the meroterpenoid fumagillin [88] (Fig. 1). This enzyme is membrane-bound and shares a low, but recognizable, sequence similarity with UbiA-type prenyltransferases. Additional examples and biochemical studies are needed to gain insights into its role in the biosynthesis of fungal natural products, as well as its evolutionary relationship to the canonical class I terpene synthases. UbiA-type terpene cyclases could be the result of convergent evolution, or they could represent the common ancestor of all canonical class I terpene synthases.

Yet another group of isoprenyl-chain cyclizing enzymes acts on the farnesyl- and geranylgeranyl-chains attached to the polyketide and indole moieties during meroterpenoid [57–59, 75, 76] and indole-diterpene [47, 54, 56, 72, 74, 89–91] natural products biosynthesis (see also Sect. 2.5). Heterologous reconstitution of the pyripyropene (Fig. 1) meroterpenoid biosynthetic pathway from *A. fumigatus* in *A. oryzae* [59] identified a protein (Pyr4) predicted to be an integral membrane protein as farnesyl cyclase. This protein is unusually small (242 aa) and does not share any homology with known proteins in the National Center for Biotechnology Information (NCBI) database. It also lacks the aspartate-rich motifs commonly found in prenyl transferases and cyclases. Homologs are present in other meroterpenoid (e.g. Trt1, AusL, Pyr4) and indole-diterpenoid (e.g. PaxB, AtmB, LtmB) biosynthetic gene clusters. Each of these clusters also encodes an FAD-dependent monooxygenase. This enzyme was shown to catalyze epoxidation of the terminal (C10–C11) double-bond of the farnesyl moiety to allow for protonation initiated prenyl chain cyclization by the transmembrane cyclase, similar to the reaction catalyzed by oxidosqualene synthase [59]. Site-directed mutagenesis identified two residues (E62 and D218) conserved in all transmembrane cyclase homologs that when mutated abolished *in vitro* activity of Pyr4 [59]. These acidic residues are proposed to catalyze epoxide protonation in a similar fashion to oxidosqualene synthase [59]. Heterologous reconstitution of the indole-diterpene paxilline (Fig. 1) pathway from *Penicillium paxillii* in *A. oryzae* has confirmed epoxidation-dependent prenyl chain cyclization. Intriguingly, formation of the tricyclic terpenoid moiety of paxilline was shown to involve stepwise epoxidation by PaxM of the terminal and the C10–11 double bond of the prenyl moiety; each oxidation was followed by protonation-dependent cyclization of parts of the prenyl chain by PaxB [47].

The viridicatumtoxin biosynthetic gene cluster appeared to have no terpene cyclase that could cyclize the geranyl chain attached to the polyketide-derived naphthacene scaffold [67, 92, 93] (Fig. 1). Instead, a cytochrome P450–encoded by *vrtK* was identified through gene deletion in the native producer *P. aethiopicum* and heterologous expression in yeast as the enzyme responsible for prenyl cyclization [93]. Vrtk is

proposed to initiate carbocation formation by oxidation of the allylic C4 of the geranyl chain. This leads to the formation of an allylic cation that triggers subsequent prenyl chain cyclization via a proposed tertiary carbocation intermediate [93].

The identification of novel cyclases that have little in common with canonical class I and II terpene synthases, except that they all take advantage of the reactivity of the allylic prenyl chain to create a carbocation intermediate, shows the diversity of potential enzyme active sites and protein scaffolds available in nature for catalysis of reactions with similar outcomes. Fungal natural product gene clusters tend to have multiple P450 enzymes as well as predicted genes with no conserved domains. These unknown and uncharacterized genes provide a rich source for the discovery of novel types of enzymes, as discussed above for the recently discovered prenyl cyclases.

2.1.4 Isoprenoid Biosynthetic Genes in Ascomycota and Basidiomycota

The majority of studies on the enzymes discussed in the above sections have been done with enzymes and pathways from filamentous fungi (Ascomycota). Terpenoid-derived natural products of mixed biosynthetic origin (prenylated indole-alkaloids, indole-diterpenoids, meroterpenoids) have so far received the most attention. Relatively few studies describe the identification and characterization of enzymes and pathways that give rise to sesqui-, di- and triterpene natural products in filamentous fungi. Based on literature reports and a survey in SciFinder, polyketides (PK) and non-ribosomal peptides (NRP) synthesized by thiotemplate mechanism synthetases (PKSs and NRPSs) clearly appear to be the major classes of natural products made by Ascomycota [94–96]. A similar survey for natural products isolated from Basidiomycota, however, returned less than 100 potentially PKS-or NRPS-derived compounds, whereas close to 500 reports (~1,000 compounds) described the isolation of terpenoids. Furthermore, although filamentous fungi tend to have between 20 and 50 NRPS and PKS biosynthetic gene clusters in their genomes [38], Basidiomycota typically have fewer than 10 PKS or NRPS genes, of which only a few have been expressed and characterized [16, 24, 25, 97–99].

These surveys indicate that Ascomycota and Basidiomycota may have evolved different arsenals of natural products. Ascomycota seem to rely on PK, NRP, and indole-derived compounds as their predominant natural product classes, while terpenoids are the major class made by Basidiomycota. To support this conclusion further, we performed a BLAST survey of all Ascomycota and Basidiomycota genomes available at the Joint Genome Institutes (JGI) Fungal Genome database at the time of writing, with known sequences of the different prenyl transferases, prenyl cyclases, and terpene synthases discussed in this chapter. As shown in Fig. 2, sesquiterpene synthases constitute the most abundant group of enzymes in Basidiomycota, with four times as many sequences identified per genome than in Ascomycota genomes. In contrast, Basidiomycota have comparatively few ABBA-type prenyltransferases involved in the biosynthesis of prenylated polyketides and indole alkaloids. It also appears that homologs of UbiA-type prenyl transferase and

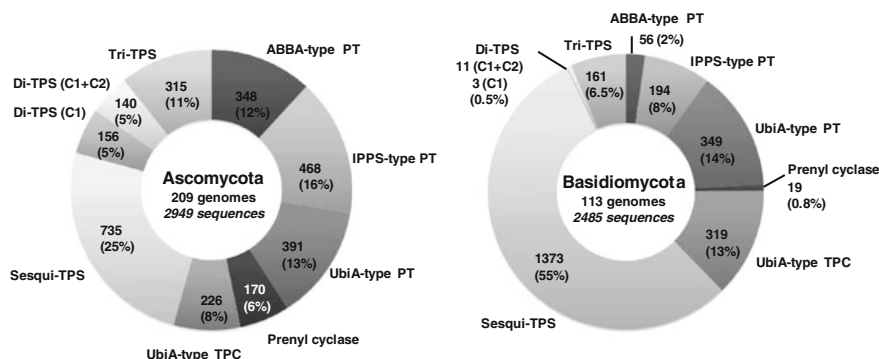


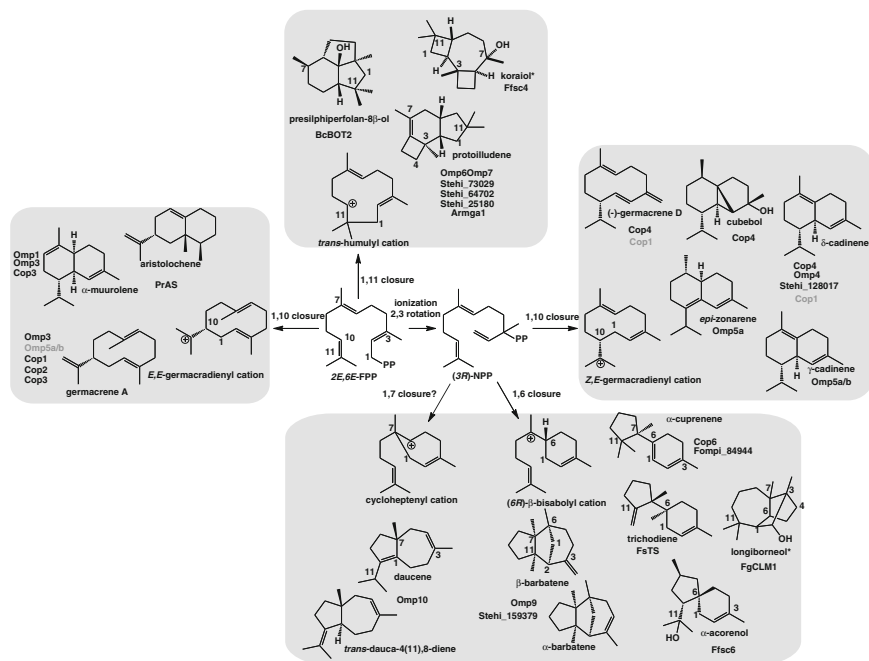
Fig. 2 Abundance of prenyl-chain modifying enzyme homologs identified in Ascomycota and Basidiomycota genomes. Shown are numbers (and rounded percentages) of homologs of enzymes identified in fungal genomes available at JGI using BLAST analysis with sequences of functionally characterized prenyl transferases, prenyl cyclases, and terpene synthases and cyclases discussed in this review. BLAST hits from different searches were cross-analyzed for duplicates and manually inspected for alignment coverage and scores. Enzyme abbreviations correspond to those in Fig. 1

cyclases are relatively more abundant in Basidiomycota compared to Ascomycota. Because none of these putative enzymes have been characterized, we do not know whether they catalyze similar reactions to the enzyme characterized from Ascomycota or are involved in the biosynthesis of yet-to-be-identified natural products. Notable is the scarcity of diterpene synthase homologs (both mono- and bifunctional enzymes) in genomes from both fungal phyla. For example, only 11 sequences were identified in six Basidiomycota genomes out of over 100 searched. Homologs of prenyl cyclases involved in meroterpenoid and indole-diterpene biosynthesis in filamentous fungi are likewise rare in Basidiomycota, suggesting that these compounds either represent a minor group of natural products in Basidiomycota or their synthesis involves a different type of cyclizing enzyme.

2.2 Sesquiterpenoids

2.2.1 Overview

The C-15-hydrocarbon scaffolds of the thousands of structurally diverse sesquiterpenoid natural products isolated from plants, bacteria, and fungi are synthesized from FPP by sesquiterpene synthases [46]. These enzymes bind the pyrophosphate group (PP) of FPP at the entrance of the active site via a Mg^{2+} cluster, which is coordinated by two conserved aspartate-rich motifs, DDXXD/E and NSE/DTE. Upon binding of the PP group, the prenyl chain becomes oriented in the hydrophobic active site cavity of the enzyme [100] and a conformational change is triggered, which results in the closure of the active site and concurrent PP cleavage to generate



an initial *transoid* allylic carbocation [101–103], shown in Fig. 3. This carbocation is then transferred along the isoprenyl chain and eventually quenched either by a water molecule or through proton abstraction. The binding pocket determines folding of the isoprenyl chain and chaperones the reactive carbocation intermediates until the final quenching step [104], thereby defining the product profile of a particular sesquiterpene synthase.

Sesquiterpene synthases catalyze different initial cyclization reactions generating secondary or tertiary cyclic carbocation intermediates, as shown in Fig. 3. For example, the C–C bond formation between C1 and C11 of the primary farnesyl carbocation yields a *trans*-humulyl-carbocation, which is a 1,11-cyclization product. This secondary carbocation can then undergo additional cyclizations and rearrangements until carbocation quenching in the active site and subsequent release of

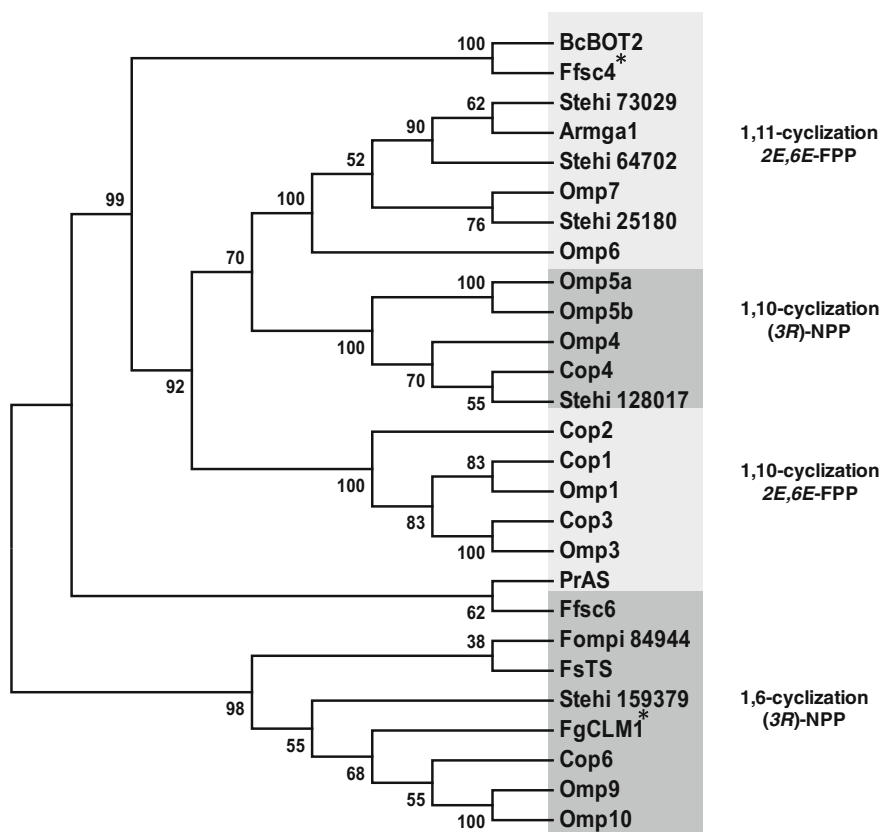


Fig. 4 Phylogenetic analysis of characterized fungal sesquiterpene synthases. Sesquiterpene synthases form different clades based on their cyclization mechanisms. The unrooted neighbor-joining phylogram was built in MEGA6 [114] with the sequences listed in Fig. 3. Branches are labeled with their bootstrap values

the final sesquiterpenoid scaffold by the enzyme. Some sesquiterpene synthases catalyze first *trans-cis* isomerization of the 2,3-double-bond of (2*E*,6*E*)-FPP, which yields a *cisoid*, allylic nerolidyl-carbocation after PP_i cleavage. The *cis*-configuration of the 2,3-double bond now allows initial cyclizations between the C1 and C6 or C7 as well as C10. Figure 3 shows the major cyclization products of all currently cloned and characterized fungal sesquiterpene synthases. Phylogenetic analysis indicates that the majority of these enzymes form distinct clades related to their cyclization mechanisms (Fig. 4).

Crystal structures have been solved for several microbial and plant sesquiterpene synthases [83, 84, 115–121]. Aristolochene synthase from *Penicillium roqueforti* and *Aspergillus terreus* [84, 117, 122, 123] and trichodiene synthase from *Fusarium sporotrichioides* [83, 103, 124, 125] are the only two fungal enzymes for which

crystal structures are known; structures are available in both the open (ligand-free) and closed (substrate/substrate analog-complexed) conformations.

The active site of all sesquiterpene synthases is located in a α -helical bundle (α -domain), which is characteristic for ionization-dependent class I terpene synthases as discussed above. Plant sesquiterpene synthases have catalytically inactive domains that are believed to be remnants from an ancient fusion event between the α -bundle domain of class I terpene synthase and the double α -barrel domains ($\gamma\beta$ -domains) of a protonation-dependent class II terpene synthase [50]. As described below, all three domains are present in diterpene synthases and both are functional in bifunctional enzymes. Plant sesquiterpene synthases have retained either one (β -domain) [118] or both α -barrel domains ($\gamma\beta$ -domains) [120], in addition to the catalytically active α -domain.

2.2.2 Sesquiterpenoid Biosynthetic Pathways in Ascomycota and Basidiomycota

Filamentous fungi, such as *Fusarium*, *Botrytis*, *Aspergillus*, and *Penicillium*, produce potent mycotoxins that can pose major health risks for humans and animals if these fungi infect plants or grow on feeds or foods (reviewed in [12]). Many well-known mycotoxins are sesquiterpenoids that are produced by plant pathogenic fungi such as *Fusarium* and *Botrytis* strains, and by fungi such as *P. roqueforti* or *Aspergillus oryzae* used in food processing. These toxins often play an important role in fungal virulence.

Fusarium strains infect cereals, which causes head blight or crown and root rot; it also leads to contamination of cereal crops with trichothecene mycotoxins, as shown in Fig. 5. Not surprisingly, trichothecene biosynthesis has been intensely studied. Trichodiene synthase, which creates the tricyclic sesquiterpenoid scaffold for these compounds, was among the first fungal sesquiterpenoid synthases to be purified, characterized, and subsequently crystallized (trichothecene biosynthesis is reviewed in [126, 127]). This enzyme catalyzes the 1,6-cyclization of a *cisoid*, allylic nerolidyl-carbocation, followed by additional cyclization and rearrangement reactions that yield the 5,6-membered bicyclic trichodiene structure shown in Fig. 5. *Fusarium* strains modify this scaffold into toxic trichothecenes, which include deoxynivalenol and nivalenol made by *F. graminearum* and the T-2 toxin produced by *F. sporotrichoides* (Fig. 5) [126]. Most of the biosynthetic genes are found in one gene cluster locus, but in several strains three additional genes are located in two loci outside this core cluster.

Fusarium strains also produce tricyclic diols, such as the antifungal culmorin, that are proposed to follow a similar 1,6-cyclization pathway than trichodiene. A sesquiterpene synthase that makes the mono-hydroxylated culmorin precursor longiborneol has been identified in *F. graminearum* [111] (Fig. 3). However, the surrounding genomic region of the gene that encodes this protein (ACY69978, FG_10397, Broad Institute *F. graminearum* genome sequence) does not appear to include predicted genes that encode for a P450 or other oxidoreductase that could

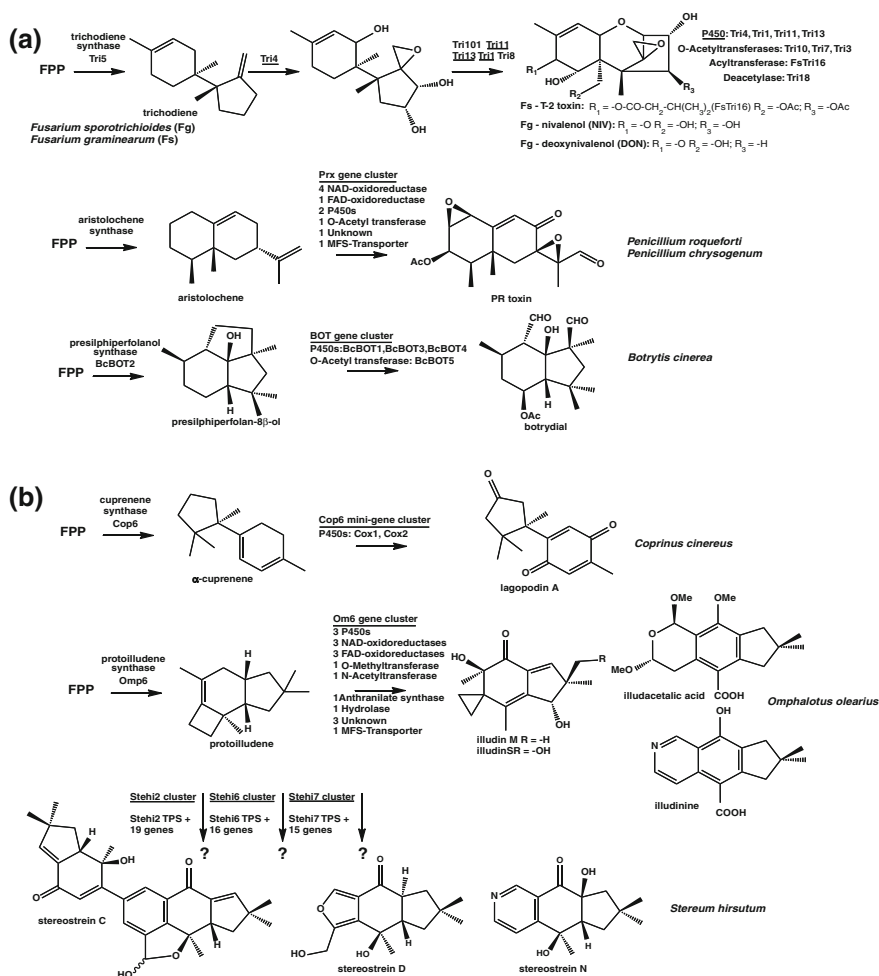


Fig. 5 Identified sesquiterpenoid biosynthetic gene clusters in Ascomycota **(a)** and Basidiomycota **(b)**. Terpene synthases cyclize FPP into sesquiterpene hydrocarbon scaffolds that are modified by tailoring enzymes into the final products shown. Function of the majority of biosynthetic genes shown (except for the trichodiene and cuprenene gene clusters) has been inferred based on sequence homology only. The gene clusters identified in *Stereum hirsutum* and *Omphalotus olearius* are expected to make a range of structurally similar compounds that have been isolated from these fungi. See text for details

yield the dihydroxylated culmorin. The closest homolog among the nine sesquiterpene synthases found in the genome of this fungus, FG_06444, appears to be the only other putative sesquiterpene synthase in addition to the characterized trichodiene synthases that may be part of a gene cluster that contains P450s.

The rice pathogen *Fusarium fujikuroi* is well known for its production of diterpenoid phytohormones, as discussed in the next section [112]. It also produces a

number of volatile sesquiterpenoids, and its genome contains putative sesquiterpene synthases [113]. Gene deletion studies have identified two terpene synthases responsible for the production of α -acorenol and koraiol [113]. Although α -acorenol is derived from an initial 1,6-cyclization intermediate, koraiol synthesis likely proceeds through an initial 1,11-cyclization reaction to generate a humulyl cation (Fig. 3).

Synthesis of the sesquiterpenoid alcohols longiborneol, koraiol, and α -acorenol by their respective fungal terpene synthases involves quenching of the final carbocation intermediate by water. Formation of such a sesquiterpenoid alcohol by a fungal sesquiterpenoid synthase was first characterized for an enzyme identified in the biosynthetic gene cluster responsible for the production of the phytotoxin botrydial by the gray mold *Botrytis cinerea* [106, 107] (Fig. 5). This enzyme catalyzes a 1,11-cyclization reaction, which is followed by additional cyclizations to yield the tricyclic alcohol presilphiperfol-8 β -ol [107] (Fig. 3).

Other filamentous fungi, such as *Aspergillus* and *Penicillium* strains, produce sesquiterpenoid toxins, including the PR-toxin, sporogen-AO1, and phomenone, which are derived from the aristolochene scaffold [128] (Fig. 3). The corresponding 1,10-cyclizing sesquiterpene synthases were cloned more than a decade ago [101, 129], but only recently have studies begun to elucidate the biosynthesis of these toxins [130, 131]. A gene cluster encoding the PR-toxin biosynthetic pathway has been identified in the blue cheese mold *P. roqueforti* by screening a genomic phage library and subsequent comparison with orthologous genes identified in the sequenced genome of a related strain, *P. chrysogenum* [131] (Fig. 5).

2.2.3 Sesquiterpenoid Biosynthetic Pathways in Basidiomycota

Until recently, no sesquiterpene synthase had been cloned and characterized from Basidiomycota. This lack of biosynthetic information is surprising considering that this fungal phylum is known to produce a plethora of structurally unique and bio-active sesquiterpenoids that likely are responsible of the medicinal properties of many traditionally used mushrooms [33–35, 107, 132–135]. Many of the isolated sesquiterpenoids have unique skeletons derived from the humulyl pathway that are not made by other organisms [35, 132, 136–139]. For example, protoilludenes have only been identified in one Ascomycete [140], a fern [141], and an octacoralia [142].

More than 60 genera of Basidiomycota (e.g. *Boletales*, *Cantharellales*, *Lactarius*, *Laccaria*, *Amanita*) are ectomycorrhizal fungi that associate with tree roots and form a symbiotic partnership with their hosts, which is crucial to plant nutrition in terrestrial ecosystems [15]. *Lactarius* species are known to produce modified lactarane- and protoilludane-derived sesquiterpenoids that have plant growth promoting activities [143–145]. Although the mechanism is not known, they may play similar roles as the diterpenoid gibberellic acids produced by filamentous fungi discussed later.

As shown in Fig. 3, different sesquiterpene synthases catalyze the 1,11-cyclization of FPP into various tricyclic sesquiterpene scaffolds. Strained rings may then undergo secondary rearrangements triggered by other enzymes, such as P450s.

Recognizing Basidiomycota as virtually unexplored territory for the discovery of novel terpenoid biosynthetic pathways, we chose to investigate sesquiterpenoid biosynthesis in the model basidiomycete *Coprinus cinerea*, which was the first mushroom-forming basidiomycete for which a genome sequence had been released [2]. We cloned and characterized six sesquiterpene synthases (Cop1-6) [23]. Three of the enzymes—Cop3, Cop4, and Cop6—had new activities shown in Fig. 3. Biochemical studies as well as structural modeling and mutagenesis provided new insights into their cyclization mechanism as well as their product specificities [21, 22]. Cuprenene-synthase Cop6 proved to be a highly product-specific enzyme, while all the other Cop enzymes were less selective; in the case of Cop4, product selectivity could be influenced by reaction conditions as well as site-directed mutagenesis [21, 22]. Cop6 was also the only enzyme located in an apparent mini-cluster that includes to P450s. Co-expression of Cop6 with its two P450s in *S. cerevisiae* [23] led to the discovery of the pathway for the biosynthesis of the antimicrobial NP lagopodin [146] (Fig. 5).

Although we detected the production of the 1,11-cyclization product pentalenene by *C. cinerea*, none of the functionally cloned enzymes produced sesquiterpenoids that could be derived from the humulyl-cation intermediate (Fig. 3). One enzyme, Cop5, was nonfunctional and is hypothesized to be responsible for this cyclization activity. Because humulyl-cation derived sesquiterpenoids are the major bioactive sesquiterpenoid natural products isolated from Basidiomycota (Fig. 6), we continued

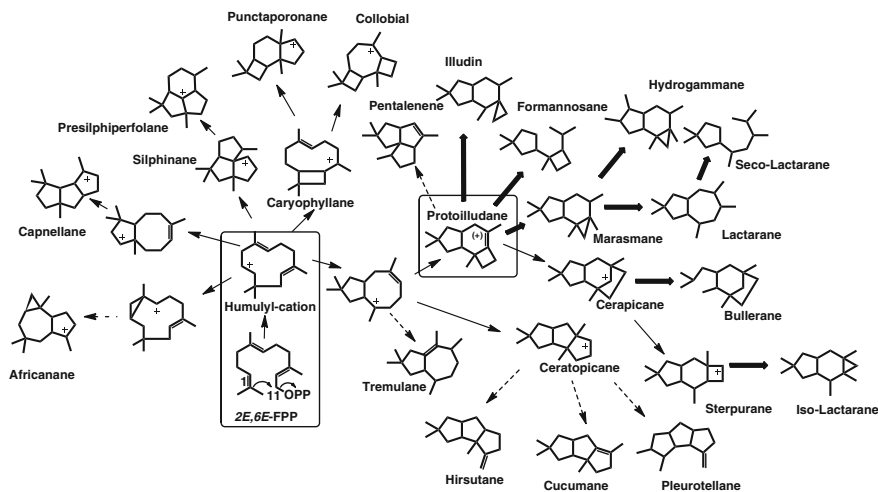


Fig. 6 Structurally diverse tricyclic sesquiterpenoid scaffolds known to be made by Basidiomycota. Possible cyclization routes and cyclic intermediates are shown. Fungal enzymes catalyzing cyclization of FPP to presilphiperfolane, protoilludane, and pentalenene have been biochemically characterized (see Fig. 3). **Bold arrows** indicate secondary ring-opening and ring-contraction reactions that are proposed to be catalyzed by terpenoid scaffold-modifying enzymes, such as cytochrome P450s. In the absence of any characterized enzymes that catalyze these reactions, only the undecorated, rearranged terpenoid hydrocarbon scaffolds are shown

our quest to identify 1,11-cyclizing sesquiterpene synthases in other Basidiomycota, which would facilitate the discovery of the corresponding biosynthetic gene clusters for these compounds.

Illudane-type sesquiterpenoids are among the best-known bioactive compounds made by Basidiomycota. They exhibit potent antitumor, antiviral, and antibacterial activity [147–152] and have been isolated from several mushrooms [153–166]. *Omphalotus olearius* was identified decades ago as a prolific producer of anticancer illudin M and S [152, 167–169]. Illudins are derived from the protoilludane scaffold (Fig. 6). We aimed to identify the corresponding sesquiterpene synthase and expected surrounding genes encoding the illudin biosynthetic pathway.

We sequenced the genome of *O. olearius* (deposited at JGI) and discovered a surprisingly large sesquiterpene synthase family (Omp1-10) [20]. The recombinant enzymes catalyze all but one possible initial cyclization reaction of FPP, shown in Fig. 3. Several sesquiterpene synthases catalyze new cyclization reactions, including synthesis of Δ^6 -protoilludene by Omp6 and 7, and of barbatene and daucene by Omp9 and 10, respectively. Unlike many characterized sesquiterpene synthases, protoilludene synthases Omp6 and 7 are highly product specific. Omp6 is part of a large biosynthetic cluster comprised of 18 genes that are proposed to synthesize the different illudin compounds isolated from *O. olearius* (Fig. 5) [20].

The sesquiterpene synthase families characterized from *C. cinereus* (Cop1-6) and *O. olearius* (Omp1-10) provided a diverse set of sequences for bioinformatics analysis of Basidiomycota genomes, with the goal of developing a framework for the prediction of sesquiterpenoid biosynthesis in these fungi. BLAST analysis of the 40 Basidiomycota genomes available at JGI's Fungal Genomes database [170] in 2011–2012 led to the identification of 500 putative STS (this number has since more than doubled, with currently more than 100 genomes sequenced; see Fig. 2), which could be grouped into five distinct clades based on sequence homology and cyclization mechanism [20]. Protoilludene synthases Omp6 and 7 are located in one clade (clade III), suggesting that all of its members carry out an initial 1,11-cyclization of FPP (Fig. 3).

Considering that the vast majority of bioactive sesquiterpenoids in Basidiomycota are derived from the 1,11-cyclization pathway, sesquiterpene synthases in clade III should therefore be considered as prime candidates for the identification of gene clusters associated with the biosynthesis of bioactive sesquiterpenoid natural products. To test our prediction, we recently set out to identify terpenoid pathways in the published genome sequence of *Stereum hirsutum* [171]. This wood-rotting mushroom was primarily sequenced to elucidate its lignin decomposition biochemistry. However, it is also among the few Basidiomycota with a sequenced genome for which a number of bioactive natural products have been isolated, including many humulyl-pathway derived sesquiterpenoids [138, 172–178].

We identified 16 putative sesquiterpene synthase sequences in *S. hirsutum* and predicted their cyclization mechanisms [19]. Seven enzymes were predicted to catalyze 1,11-cyclization of FPP, while the remaining enzymes were predicted to catalyze other cyclizations of FPP. Representative sequences, including three sequences predicted to encode 1,11-cyclizing sesquiterpene synthases (Stehi2, 6, and

7), were cloned and the recombinant enzymes characterized [19]. Stehi2, 6, and 7 proved to be highly specific protoilludane synthases that are each located in large gene clusters, which are presumed to be responsible for the synthesis of protoilludane-derived terpenoids previously isolated from *Stereum* (Fig. 6) [139, 174]. Other representative enzymes cloned from *Stereum* catalyzed as predicted 1,6- and 1,10-cyclizations of FPP [19].

BLAST searches of Ascomycota and Basidiomycota genome sequences available at JGI for putative sesquiterpene synthase sequences uncovered major differences between these two fungal phyla. Not only do Basidiomycota genomes have an average of 2–3 times larger families of sesquiterpene synthases (10–20 sequences compared to 4–10 in Ascomycota), but their genomes typically encode several predicted protoilludene synthase homologs, of which one or more are located in biosynthetic gene clusters. Only about a dozen sequence homologs of 1,11-cyclizing enzymes (BLAST searches with Omp6, 7, and Stehi2, 6, 7 sequences; E-value cutoff: $<10^{-50}$) are found scattered in different Ascomycota genomes, compared to about one-fifth of all sesquiterpene sequences identified in Basidiomycota (Fig. 2). The sequenced Ascomycota genomes seem to have a relatively large complement of 1,6-cyclizing enzymes; representatives include the characterized trichodiene, longiborneol and α -acorenol synthases from *Fusarium* strains. However, only trichodiene synthase appears to be located in a biosynthetic gene cluster.

Mining of fungal genomes for sesquiterpene synthase homologs revealed that only a few of the homologs in each fungal genome are part of identifiable biosynthetic gene clusters that could synthesize modified and probably nonvolatile products. In Basidiomycota, 1,11-cyclizing enzymes are frequently located in predicted biosynthetic gene clusters. A random survey of different Ascomycota genomes did not reveal a similar preference for one particular clade of sesquiterpene synthase homologs. Enzymes that are not part of gene clusters in a fungus are probably responsible for the production of an array of volatile terpene products; their biological significance is currently unknown, but they could potentially play a role in signaling, such as for interactions with insects [179].

2.3 Diterpenoids

2.3.1 Overview

The C-20 scaffolds of tens of thousands of known diterpenoid compounds [82] are generated from geranylgeranyl diphosphate via two different cyclization routes involving either one or two separate cyclase activities (Fig. 7).

Diterpenoids cyclized by the first, one-step route involve a monofunctional class I diterpene synthase that catalyzes ionization-dependent diphosphate cleavage and subsequent carbocation migration and quenching using a mechanism similar to sesquiterpene synthases, except the prenyl chain is now longer by one isoprene unit.

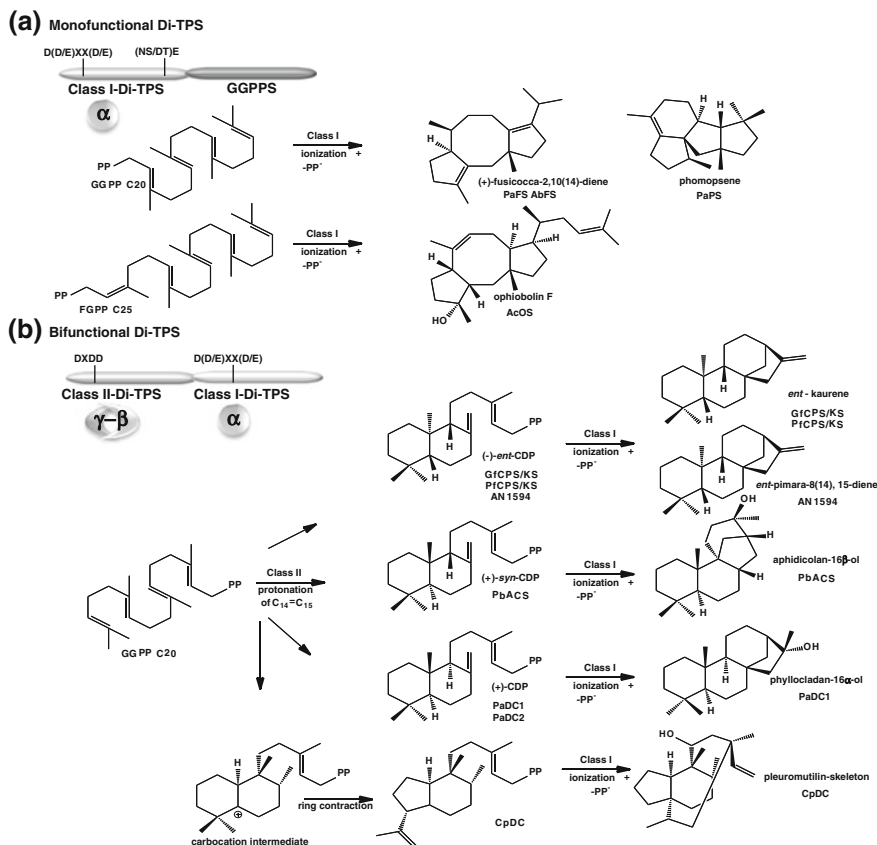


Fig. 7 Cyclization of GGPPS (FGPP) by characterized fungal diterpene synthases (Di-TPS). **a** Monofunctional class I Di-TPS fused to GGPP synthase catalyzes a one-step ionization dependent cyclization of GGPP or FGPP to generate tricyclic (5-8-5 ring system) or tetracyclic (5-6-5-5) hydrocarbon scaffolds. **b** Bifunctional Di-TPS catalyzes a two-step cyclization that is performed by two separate enzyme functions involving a bicyclic diphosphate intermediate that leads to the labdane-related diterpenoids. The N-terminal class II domain catalyzes a protonation dependent cyclization that yields a bicyclic 6-6 copalyl- or 5-6 diphosphate. The C-terminal class I domain subsequently catalyzes ionization-dependent cyclization to yield the final cyclic products. Note that different copalyl diphosphate stereoisomers are generated. Cartoons illustrate domain organizations (class I: C1-fold or α -bundle fold, referred to as α -domain; class II: C2-fold or α -barrel fold, referred to as $\beta\gamma$ -domains) and conserved catalytic motifs. Enzyme names refer to fungal source organisms and cyclization activities described in the text. CDP: copalyl diphosphate

Conserved aspartate-rich motifs in these enzymes likewise facilitate Mg-ion mediated binding of the diphosphate group.

Diterpenoids generated by the second, two-step route involve two separate enzyme activities. First, a class II-type, protonation-dependent mechanism generates a carbocation at the terminal C14-C15 double bond of the prenyl diphosphate chain that is cyclized into a bicyclic diphosphate characteristic of labdane-related

diterpenoids [180]. Second, a class I ionization-dependent cleavage of the diphosphate group is followed by carbocation-triggered cyclization to yield the final cyclic scaffold. In fungi, these two class I and class II activities are present in one large bifunctional enzyme. The N-terminal region contains the α -domain of class I terpenoid synthases, whereas the C-terminal domain contains the α -barrel (or $\gamma\beta$ -) domains of class II terpenoid synthases (Fig. 7).

It is believed that these three domain ($\gamma\beta\alpha$) proteins found in fungi and plants are the result of an ancient fusion event of bacterial class I (α) and class II ($\gamma\beta$) terpenoid synthases. In fact, typical bacterial diterpene synthases are not fused, and cyclization involves class II ($\gamma\beta$) and class I (α) terpenoid synthases for this two-step cyclization mechanism [181]. In plants, however, many three-domain ($\gamma\beta\alpha$) diterpene synthases are monofunctional and catalyze either a protonation-or an ionization-dependent cyclization reaction (on a bicyclic diphosphate or GGPP) because the other domain is not functional [180]. Structures have been solved for the monofunctional taxadiene synthase [182], which converts GGPP directly into taxadiene; bifunctional abietadiene synthase [183], which has two functional domains like the fungal enzymes; and a monofunctional copalyl diphosphate synthase [184]. Although the characterized fungal bifunctional diterpene synthases share little sequence homology with plant enzymes except for some conserved motifs (Fig. 7), structural modeling of the fungal proteins in Phyre2 [185] reveals that they have the same three-domain structure as abietadiene and taxadiene synthase.

2.3.2 Fusicoccanes and Other Diterpenoids Made by Monofunctional Enzymes

Fusicoccanes are potent phytotoxins known to be synthesized by a few fungal species [186]. *Phomopsis amygdali* causes wilting disease in trees and produces the diterpenoid fusicoccin A that binds and permanently activates plasma membrane H^+ -ATPase [186], which causes severe physiological effects in plants. A related compound, cotylenin A, made by the fungus *Cladosporium* sp. was shown to have the same mode of action (structures shown in Fig. 8). Related fusicoccane phytotoxins, brassicene A-F, were subsequently isolated from the phytopathogenic fungus *Alternaria brassicicola* that causes leaf spot in Brassica plants [186]. Fusicoccanes bind to a highly conserved family of 14-3-3 proteins in eukaryotes, which regulate a wide range of cellular functions. Interaction of cotylenin A with 14-3-3 proteins has been shown to induce differentiation of leukemia cells and apoptosis of cancer cells [186].

Identification of the gene clusters encoding fusicoccane biosynthetic pathways followed a strategy that was previously successfully applied to the cloning of bifunctional diterpene synthases described below. All GGPP using biosynthetic pathways (including carotenoid, indole-diterpene, and diterpene pathways) require a dedicated GGPP synthase that adds one isoprene unit to FPP, which is a

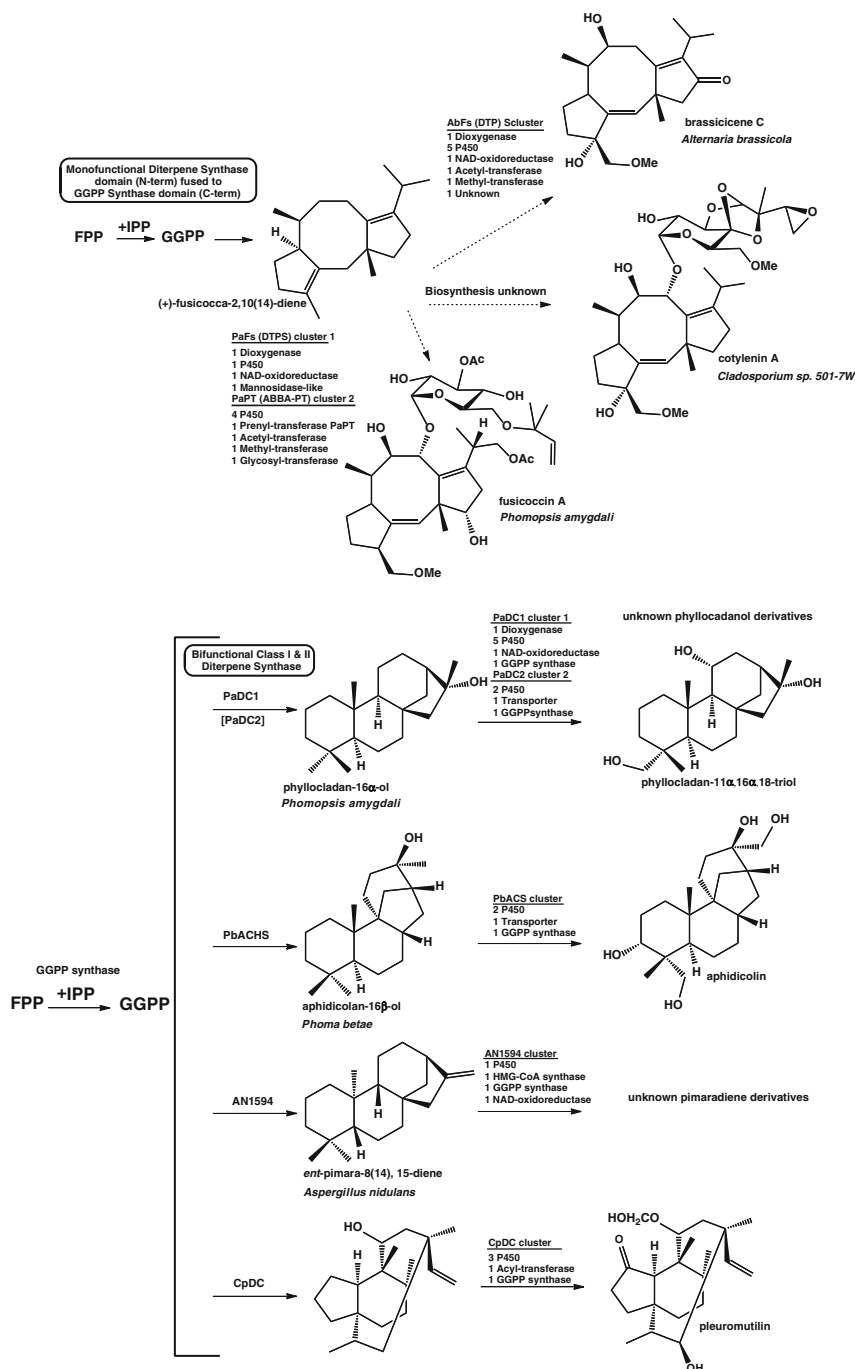


Fig. 8 Biosynthesis of fusicoccane- and labdane-related diterpenoids. **a** Fusicocca-2,10(14)-diene is modified into different fusicoccane compounds. **b** Bifunctional diterpene synthases make different labdane-related scaffolds that are modified into bioactive compounds. Biosynthetic gene clusters, if known, are shown and named based on their corresponding diterpene synthases (or prenyl-transferase in one case). See text and Fig. 7 for details

prenyldiphosphate made by all eukaryotic cells. In fungi (and bacteria), GGPP synthase genes are typically located in gene clusters (operons in bacteria) of GGPP requiring pathways. Reverse-transcriptase polymerase chain reaction with degenerate primers to amplify GGPP synthase cDNA sequences and subsequent genome walking to identify flanking regions therefore has been a successful strategy to clone several monofunctional diterpene synthases.

Fusicocca-2,10(14)diene synthase (PaFS) from *P. amygdali* was the first monofunctional diterpene synthase cloned and characterized in *E. coli* [187] (Fig. 7). This enzyme, like all other fungal monofunctional diterpene synthases characterized so far, is a chimeric protein where the GGPP synthase is fused to the C-terminus of the terpene synthase domain. Genome walking led to the identification of four additional biosynthetic genes clustered with the chimeric terpene synthase gene [187]. Enzymes responsible for oxidative modification of the hydrocarbon scaffold were subsequently biochemically characterized [188]. Later, a second gene cluster was identified that encodes nine additional genes needed for the biosynthesis of fusicoccin A [189] (Fig. 8). This cluster was identified in the draft genome sequence of *P. amygdali* based on a previously identified ABBA-type prenyltransferases that catalyzed in vitro the reverse O-prenylation of glucose with DMAPP [70], which is a prenylation that is also present in fusicoccin A. A combination of in vitro assays with recombinant enzymes and gene disruption allowed Daiiri's group to then establish the complete fusicoccin A pathway [189]. His group also identified and functionally characterized the brassicene C biosynthetic pathway from *A. brassicola*, which includes, in addition to a fusicoccadiene synthase (AbFs), a dioxygenase and oxidoreductase as well as five P450s [188, 190, 191].

Two additional fusicoccadiene synthase homologs (PaPS, AcOS) with new cyclization activities were identified and biochemically characterized (Fig. 7). A second chimeric diterpene synthase (PaPS) was cloned from *P. amygdali* that cyclizes GGPP into the tetracyclic (5/6/5/5) phomopsene. This tetracyclic hydrocarbon is the precursor for methylphomopsenonate made by *P. amygdali* and for related spirocyclic diterpenoids isolated from other fungi [192]. Mining of the genome of *Aspergillus clavatus* recently led to the identification of a chimeric sesterterpene (C-25) synthase (AcOS) [193]. The prenyldiphosphate synthase domain of AcOS converts GPP and FPP (not GGPP) into geranylarnesyl diphosphate (GFPP) to provide the substrate for subsequent cyclization by terpene synthase domain to ophiobolin F, which is a fusicoccadienol tricycle appended with a 5-carbon extension.

The few monofunctional diterpene synthases and associated biosynthetic genes characterized so far are all found in Ascomycota. Except for pleuromutulin biosynthesis (see below), no genes encoding diterpenoid biosynthetic enzymes from Basidiomycota have been identified. This may not be surprising considering that only a handful of homologs to known diterpene synthases (both monofunctional and bifunctional) can be found in currently available Basidiomycota genomes (Fig. 2).

The scarcity of diterpene synthase homologs in our genome survey seems to be supported by the relatively small number of diterpenoid compounds characterized

from Basidiomycota [194] compared to the large number of sesquiterpenoid natural products isolated from these fungi. However, the assumption that diterpenoids do not play a major role in the natural products portfolio of Basidiomycota may not be correct. So far, only relatively few genome sequences are available for Basidiomycota; the vast majority have been sequenced for reasons other than natural product discovery. Most of the well-known medicinal mushrooms [37] have not been genome sequenced, but a large number of the known diterpenoids from Basidiomycota have been isolated from these mushrooms (e.g. *Cyathus helence* [bird's nest fungus], *Hericium erinaceum* [lion's mane fungus], *Sarcodon scabrosus* [bitter tooth fungus]) [194]. Furthermore, our genome searches were carried out with known fungal diterpene synthase sequences, including the full chimeric sequences of fungal monofunctional enzymes, such as PaFS. Basidiomycota may not use chimeric diterpene synthases and may instead use enzymes that are currently grouped with other terpene/prenyl synthases/cyclase sequences. The most likely class of such enzymes would be the sesquiterpene synthases. In fact, except for a few examples for labdane-related diterpenoids, most of the identified diterpenoids from Basidiomycota have tricyclic structures, which suggest an ionization-dependent cyclization mechanism (Fig. 9).

2.3.3 Labdane-Related Diterpenoids Made by Bifunctional Enzymes

As mentioned above, biosynthesis of labdane-type diterpenoids requires a two-step cyclization pathway involving first a protonation dependent cyclization of GGPP to form the characteristic labdane bicycle and, in a second step, ionization-dependent cyclization at a separate active site to generate the final cyclic product (Fig. 7). Cyclization of GGPP to *ent*-CDP and then to the tetracyclic *ent*-kaurene generates the precursor for gibberellin (gibberellic acids, GA) phytohormones that are major regulators of plant growth and development. It is believed that because of its essential role in plants, *ent*-kaurene represents the ancestral diterpenoid cyclization pathway from which alternative cyclization routes evolved to generate the large diversity of labdane-type compounds known today [180]. In fact, it has been shown that single amino acid changes are sufficient to alter the product profile of the class-I *ent*-kaurene synthase to form new cyclic scaffolds [195, 196].

Despite their critical function in plants, GAs were first isolated in the early 20th century from a fungal phytopathogen, the rice pathogen *F. fujikuroi* (teleomorph: *Gibberella*). Following the realization of the importance of GAs for plant growth, biotechnological production methods were established. In the early to mid-1990s, GA biosynthetic genes, including dedicated *ent*-CDP and *ent*-kaurene synthases, were identified in plants (reviewed in [197–199]). Structural studies showed that both synthases are tri-domain proteins that share the same ancestor, but each enzyme has only one functional active site (see above) [182–184].

Identification of fungal GA biosynthetic genes followed later and uncovered fundamental differences between the fungal and plant pathways, pointing to a largely divergent evolution except for presumably a shared ancestral *ent*-CDP,

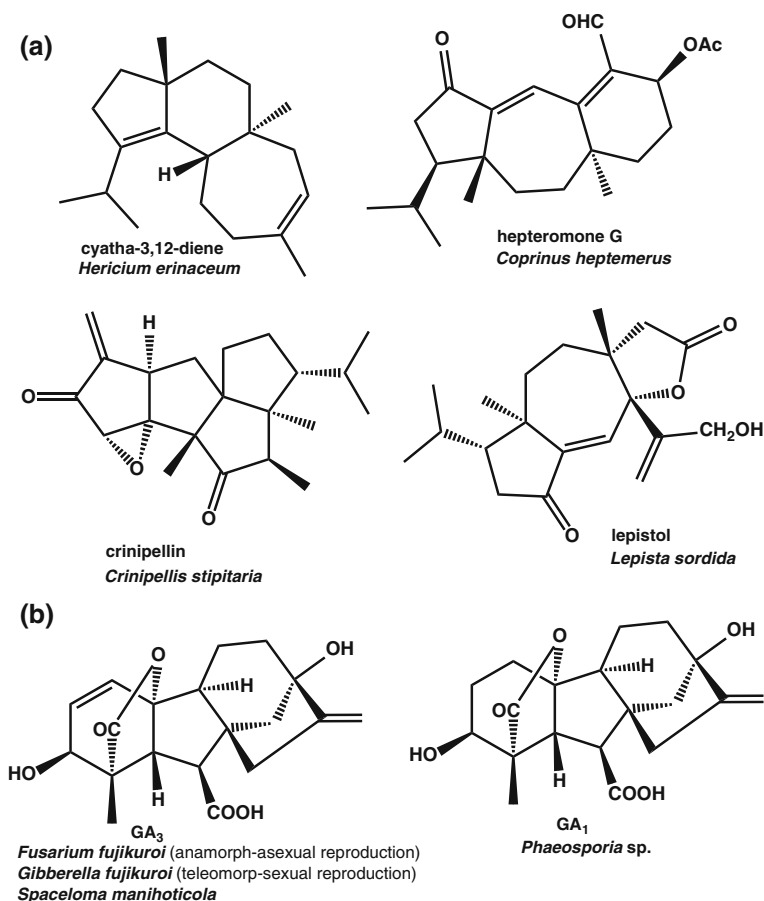


Fig. 9 Fungal diterpenoid natural products. **a** Bioactive tri- and tetracyclic diterpenes isolated from Basidiomycota. **b** Labdane-type gibberellic acid phytohormones

ent-kaurene diterpene synthase. The fungal diterpene synthase is bifunctional, retaining both class-I and class-II activity (GfCPS/KS, *G. fujikuroi* in Fig. 7) [200–203]. The GA pathways from plants and *F. fujikuroi* have been cloned and characterized; they are reviewed in detail in [197]. Notable are the many oxidation steps involved in the production of GAs. (Note that GAs are numbered according to their order of discovery; only few GA structures are bioactive). In GA₃ biosynthesis by *F. fujikuroi*, oxidations are carried out by four P450s and one desaturase; each P450 catalyzes multiple scaffold modifications. Functional GA pathways were also identified in *Phaeosphaeria* sp. (PfCPS/KS in Fig. 7), *Sphaceloma manihoticola*. These fungi produce less oxidized GAs (Fig. 9) because their biosynthetic gene clusters lack a desaturase or a desaturase and P450, respectively, which are present in the *F. fujikuroi* GA cluster [197].

Although only distantly related, these GA-producing fungi share conserved biosynthetic genes that are rearranged in their respective clusters. Horizontal gene transfer is presumed to be responsible for the spread of GA clusters among fungi. GA clusters (complete or remnants thereof) were also found in other fungi closely related to *F. fujikuroi*. However, GA production could only be established in a few strains; in some clusters, mutations were shown to render pathways nonfunctional (see detailed overview in [197]). This serves as a reminder that some biosynthetic gene clusters identified by in silico genome mining may be evolutionary junk. Careful sequence analysis and re-annotation should precede efforts toward functional characterization via heterologous means.

As hypothesized for labdane-diterpenoid biosynthesis in plants, ancestral GA-type pathways in fungi may have diversified to produce other bioactive compounds. New modifying enzymes may have been recruited into the cluster and activities of GA-biosynthetic cluster genes evolved to act on new labdane scaffolds. Although our genome survey suggests that more than 100 such putative clusters may be found in a quarter of the sequenced Ascomycota genomes (Fig. 2), only three such clusters have so far been characterized from these fungi; this is in contrast to the many labdane-type diterpenoid biosynthetic genes characterized from plants [204]. The first bifunctional diterpene synthase leading to alternative labdane skeletons cloned and characterized was the aphidicolan-16 β -ol synthase (PbACS in Fig. 7) [205] from *Phoma betae*. This enzyme generates the scaffold for the DNA polymerase inhibitor aphidicolin. Genome walking was used to identify and clone the clustered aphidicolin biosynthetic genes [206] (Fig. 8), and then heterologously express this pathway in *A. oryzae* [207].

A second fungal labdane-diterpenoid pathway was identified in the fusiccocin producing fungus *P. amygdali*. Several GGPP synthase sequences were amplified; genome walking led to the identification of three diterpene synthase sequences, including the monofunctional fusicoccadiene synthase (PaFS) described above and two bifunctional CDP/KS synthase homologs, PaDC1 and PaDC2 [208]. PaDC1 and PaDC2 (in Fig. 7) are each located in gene clusters; however, although only the CDP-synthase domain is functional in PaDC2, PaDC1 converts GGDP into a novel diterpenoid phyllocladan-16 α -ol (Fig. 8). The two gene clusters together are proposed to be responsible for the biosynthesis of phyllocladan-11 α ,16 α ,18-triol [208]. Most recently, a diterpene cluster has been identified through genome mining in *A. niger*. In this case, overexpression of a transcription factor located within the cluster resulted in the production of *ent*-pimara-8(14),15 diene, suggesting that the bifunctional diterpene synthase located in this cluster is a pimaradiene synthase [209].

Finally, only one labdane-type diterpene synthase gene has been cloned from Basidiomycota. A patent application described the identification of the biosynthetic pathway for pleuromutilin (Fig. 8) from *Clitopilus passeckeranus* [210]. This diterpenoid has been isolated from *Clitopilus* and related fungi [211]. Derivatives of pleuromutilin are commercially important antibiotics and are used in veterinary medicine (tiamulin, valnemurin) and for human treatments (retapamulin) [212, 213]. Transfer of the biosynthetic gene cluster into a heterologous production host is expected to yield increased production titers and allow modification of the

pleuromutilin scaffold to afford production of derivatives. A cyclization pathway forming the tricyclic pleuromutilin scaffold has been proposed in [180] and is thought to involve the generation of a rearranged bicycle (5-6) diphosphate, which is subsequently cyclized upon diphosphate cleavage into a tricyclic skeleton.

Homology searches in Basidiomycota genomes for bifunctional diterpene synthases, however, result in only 11 putative homologs in the genome sequences of five strains (Fig. 2). Few labdane diterpenoids have been isolated from Basidiomycota [194], which could mean that this class of diterpenoid metabolites does not play a significant role in these fungi.

2.4 Triterpenoids

2.4.1 Overview

Triterpenoids constitute a large group of natural products that are particularly ubiquitous in plants, and more than 100 different skeletons have been described (reviewed in [214, 215]). Class II triterpene cyclases catalyze proton-initiated cyclization of either squalene (bacterial squalene hopene cyclases) or oxidosqualene (eukaryotic cyclases) into mostly penta- or tetracyclic ring systems, although other cyclizations are also known [214, 216]. Triterpene cyclases are considered to catalyze among the most complex enzyme reactions by guiding in a single-step carbocation-driven multicycle formation with frequently exquisite product specificity.

The most basic function of triterpenoids is their essential role as membrane sterols in eukaryotes (and in some bacteria). The three eukaryotic kingdoms (plants, fungi, animals) make different types of sterols: phytosterols (sitosterol, stigmasterol, campesterol) in plants, cholesterol in animals, and ergosterol in fungi [217]. All of these structures are derived from cyclization of oxidosqualene into either cycloartenol in plants or lanosterol in animals and fungi (Fig. 10). Multiple oxidation steps subsequently generate the different sterols.

In addition to the housekeeping cycloartenol synthase, plants have additional cyclase homologs that make other triterpenoid skeletons. A large number of P450s have been identified that oxidize these skeletons into diverse secondary metabolites, which are frequently further glycosylated (reviewed in [218]). Lanosterol is the precursor to both ergosterol and triterpenoid metabolites in fungi. Some fungi cyclize oxidosqualene into the related protostadienol, which is modified into bioactive compounds (Fig. 10).

2.4.2 Fungal Triterpenoid Biosynthesis

A large body of publications describes triterpenoid biosynthesis and metabolic engineering in plants, while very little is known about the structural diversity,

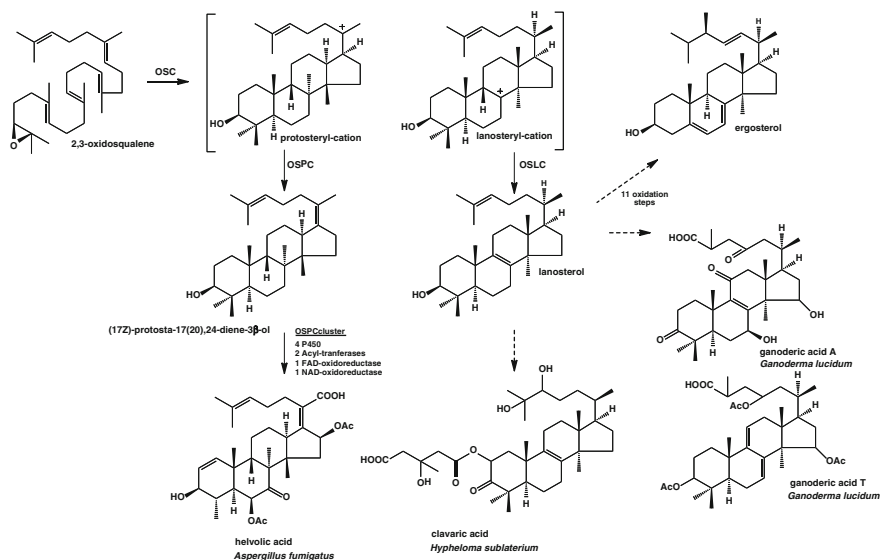


Fig. 10 Fungal triterpenoid biosynthesis. Oxidosqualene is cyclized by a cyclase (OSC) into tetracycles hydrocarbon scaffolds as biosynthetic precursors for the membrane sterol ergosterol and bioactive triterpenoids

biological significance, and biosynthesis of fungal triterpenoids. Bioactive triterpenoids, such as the fusidane antibiotics [219], have been identified from Ascomycota, but the vast majority of fungal triterpenoid natural products have been isolated from Basidiomycota [220]. Genera such as *Ganoderma*, *Inonotus*, (*Wolfi*) *Poria*, *Laetiporus*, *Anrodia*, and *Daedalea* are well-known producers of diverse lanosterane-type triterpenoids, with pharmacological properties that include anti-tumor, apoptotic, and antimalarial activities (reviewed in [220]). Ganoderic acids produced by the medicinal mushroom *Ganoderma lucidum* are among the best known fungal triterpenoids [221], and considerable effort has been devoted to the development of fermentative production processes [32].

Two fungal triterpenoid cyclases involved in secondary metabolite biosynthesis have so far been characterized on a molecular level. *A. fumigatus* produces fusidane-type triterpenoids, including the anticancer helvolic acid derived from the proto-stadienol scaffold (Fig. 10). The corresponding triterpene synthase and associated fusidane biosynthetic gene cluster has been identified by two groups [222, 223] (Fig. 10). It was shown that the cyclization outcome of this enzyme (as for diterpene synthases, see above) can be readily altered; substitution of one amino acid residue was sufficient to direct cyclization either towards lanosterol or protostadienol [224]. Two genes, oxidosqualene synthase and squalene epoxidase, were identified in the antitumor clavaric acid producer *Hypheoloma sublaterium* (a basidiomycete), and are expected to be required for the production of this secondary metabolite [225, 226].

In an effort to elucidate the biosynthesis of lanosterane-type triterpenoids in *Wolfiporia* and *Ganoderma*, genomic and transcriptomic studies were published by several groups [227–230]. These studies suggested that *Ganoderma* has two oxidosqualene-lanosterol cyclases, with one homolog dedicated to either ergosterol or triterpenoid natural product biosynthesis. Our genome survey (Fig. 2) also showed that Basidiomycota genomes commonly have one or two (sometimes three) triterpene cyclase homologs. Several Ascomycota genera, such as *Aspergillus* and *Neosartorya*, however, have between 2 and 6 homologs, of which some are part of predicted biosynthetic clusters. In Basidiomycota, triterpene cyclases seem not to be located in closely arranged gene clusters, but tailoring enzymes are likely to be coregulated with a dedicated secondary metabolic triterpene cyclase. In fact, genome analysis of *Ganoderma* revealed a large complement of cytochrome P450s (CYPome), including 24 P450 clusters and 78 P450s that appear to be co-regulated with lanosterol cyclase [228].

In plants, P450s are considered to be drivers of chemical diversity, and they play a major role in the diversification of triterpenoid secondary metabolism [231]. The vast majority of triterpenoid scaffold modifications are catalyzed by P450s. Although genes are typically not clustered in plants, notable exceptions have been identified in *Arabidopsis*, where triterpene cyclases and P450s form co-regulated gene clusters [232, 233]. Considering the massive expansion of the cytochrome P450 family in Basidiomycota, P450s likely play a similar role in triterpenoid secondary metabolism in these fungi. After plants, Basidiomycota have the largest and functionally most diverse CYPome, with 100–300 P450s per genome in the *Agaricomycotina* subdivision [234, 235], which includes ~70 % of the known Basidiomycota [4]. Only few Basidiomycota subdivisions, including pathogenic Basidiomycota such as *Puccinia*, *Ustilago*, and *Malessizia*, have a drastically reduced CYPome (less than 20), which is also reflected in a largely absent secondary metabolome in these fungi.

2.5 Terpenoids of Mixed Biogenetic Origin

Many natural products are hybrid compounds built from precursors derived from different biosynthetic pathways. Ascomycota are well known for their ability to produce structurally diverse meroterpenoids and indole-diterpenoids that are hybrid scaffolds containing terpenoid and polyketide or indole domains. Their biosynthetic gene clusters encode enzymes for the generation and tailoring of both domains. In this section, we focus on the installation of the terpenoid domains shown in Fig. 11.

Two different types of prenyl transferases (see Sect. 2.1.2) catalyze transfer of a prenyl-chain to either a polyketide or indole precursor. UbiA-type prenyl transferases catalyze FPP transfer in the biosynthesis of meroterpenoids such as terretinin, pyripyropene, austinol, and mycophenolic acid [57–59, 75–77]. IPPS-type prenyl transferases catalyze the transfer of GGPP in the biosynthesis of a large group of structurally diverse indole-diterpenoids, such as the lolitrems made by

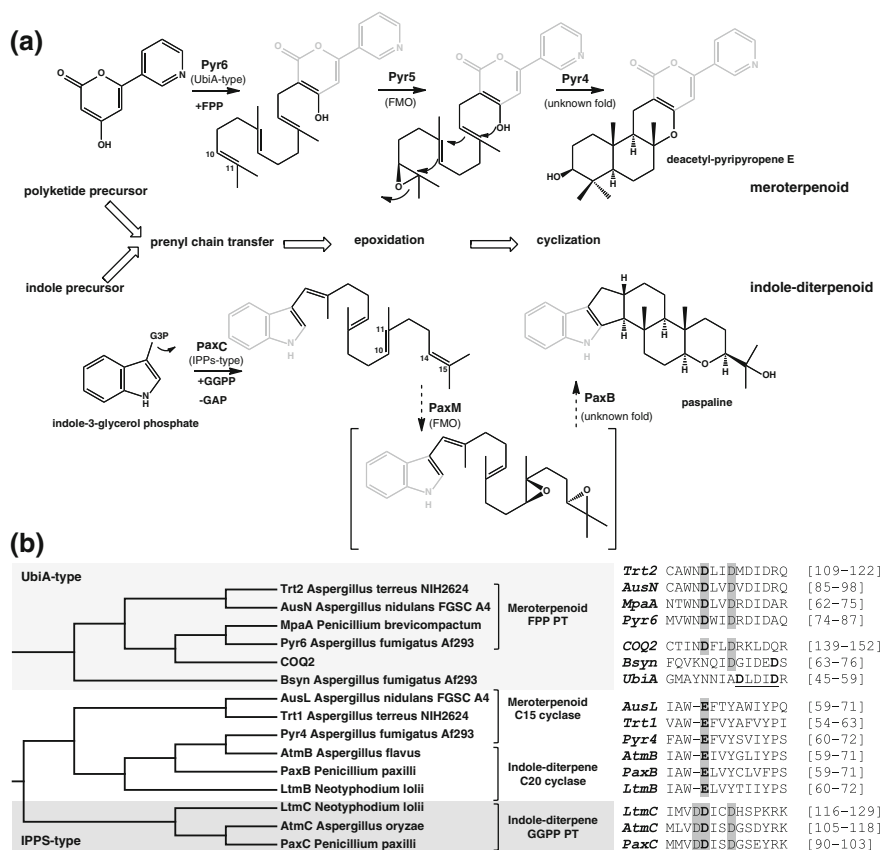


Fig. 11 Meroterpenoids and indole-diterpenoids are of mixed biogenetic origin. **a** Biosynthesis of most known hybrid compounds containing a terpenoid- and a polyketide or indole-derived moiety involves transfer, epoxidation, and cyclization of a prenyl chain (black) to a nonterpenoid precursor (gray). Shown are steps involved in pyripyropene and paxilline biosynthesis. **b** A dendrogram showing relatedness of different prenyl cyclases and transferases involved in indole-diterpene and meroterpenoid biosynthesis. Sequences were aligned by ClustalW (Gonnet) and subsequently subjected to phylogenetic analysis using the minimum evolution method in MEGA6 [114]. Conserved acidic residues (D/E) likely involved in catalysis (underlined: catalytic aspartate residues in UbiA structure [81], boxed: residue numbers) of each sequence are shown. Cyclases and prenyl transferases separate into three groups, quinone prenyltransferases, *E. coli* UbiA, and *S. cerevisiae* CoQ2, as well as β -trans-bergamotene cyclase Bsyn, are included as UbiA-type reference sequences [88]. NCBI sequence accession numbers: Trt2: XP_001209380, AusN: XP_682528, MpaA: ADY00128, Pyr6: XP_751272, COQ2: CAA96321, Bsyn: AGI05042, UbiA: AAC43134, AusL: XP_682526, Trt1: XP_001209379, Pyr4: XP_751270, AtmB: CAP53939, PaxB: ADO29934, LtmB: ABF20226, LtmC: ABF20225, AtmC: EIT82606, PaxC: AAK11529

grass endophytes and aflatrems, paxicilline mycotoxins produced by *Aspergillus* and *Penicillium*, respectively [47, 54–56, 72–74].

Cyclization of prenyl chains attached to either the polyketide or indole moieties presumably involves a protonation-dependent cyclization reaction catalyzed by membrane-bound cyclases (see Sect. 2.1.3) that have no homology to any known proteins. In each of the characterized biosynthetic gene clusters, a flavin-mono-oxygenase-type FMO is present that is required to catalyze prenyl-chain epoxidation as a prerequisite for subsequent cyclization (see Sect. 2.1.3).

Meroterpenoid and indole-terpenoid biosynthesis has so far only been studied in Ascomycota, and it is not known if these natural products are synthesized by Basidiomycota. A SciFinder search for indole-diterpenoids or meroterpenoids from Basidiomycota yielded no hits for the first class and less than 10 results that described the isolation of compounds that contain an aromatic ring attached to an acyclic farnesyl chain (e.g., [236]). These two groups of natural products may therefore not play an important role in Basidiomycota. A search of fungal genomes in the JGI Fungal Genome database with sequences of prenyl-chain cyclases (AtmB, PaxB, LtB, Pyr4, Trt1, AusL; Sect. 2.1.3) yielded only 19 hits in 5 Basidiomycota strains, whereas ~200 homologs are found in Ascomycota genomes. Half of these genomes have at least one homolog, whereas *Aspergillus* strains have 2–5 homologs.

3 Conclusions and Outlook

Fungi synthesize a vast number of diverse terpenoid natural products. Genome surveys show that we have just begun to scratch the surface of this biosynthetic diversity. The majority of explorations into the fungal terpenome have so far been in Ascomycota, whereas we have only recently begun to investigate the natural product potential of Basidiomycota using molecular and biochemical approaches. From our studies [19–23] and the observations described in this chapter, it is clear that the two fungal phyla have evolved different portfolios of terpenoid products and associated biosynthetic enzymes. Application of our current knowledge of secondary metabolite biosynthesis to searches of fungal genomes suggests that the secondary metabolome of Ascomycota is dominated by thiotemplated biosynthetic machineries. Sesqui- and triterpenoid natural products, on the other hand, seem to be playing major roles in the secondary metabolome of Basidiomycota.

It should be pointed out that these observed differences are based on the biosynthetic pathways and enzymes that have been characterized to date, as well as on the small number of available fungal genomes that represent just a tiny fraction of the fungal diversity. Many genes annotated in fungal genomes, and in particular in Basidiomycota genomes, have no known homologs. These “unknown” predicted or putative genes may well encode entirely new biosynthetic activities, as was seen for terpene cyclases discovered in indole-diterpene and meroterpenoid biosynthesis. Furthermore, many other genes may have been wrongly annotated and may catalyze

very different activities. Of particular note here is the discovery of a new UbiA-type sesquiterpene cyclase in the fumagillin biosynthetic pathway. Genome mining efforts with these new sequences will likely lead to new terpenoid natural products pathways.

To what extent we will be able to characterize the fungal terpenome is limited by our ability to biochemically characterize biosynthetic genes and their functions. Our current technologies for pathway identification and discovery are slow and inadequate to keep up with the massive influx of sequencing data. We need to develop strategies that will enable us to move rapidly from in silico biosynthetic pathway identification to high-throughput assembly and expression of predicted pathways, with concurrent analytical profiling of produced compounds. For this to happen, a number of bottlenecks and obstacles will need to be tackled. The development of such strategies will be crucial for reviving the natural products drug discovery pipeline.

The first step for the implementation of an in silico to metabolite integrated pipeline involves the accurate identification of natural products genes and pathways in genomes. A number of bioinformatics tools for genome mining (e.g. antiSMASH 2.0 [237]) have been developed for this purpose (for a recent review, see [238]), but these tools rely on algorithms trained with hidden Markov models derived from known biosynthetic genes—the majority of which come from Ascomycota and Actinomycetes. In our experience, when applied to terpenoid pathways from Basidiomycota, they tend to identify only a subset of the pathways and have difficulties identifying cluster boundaries.

Accurate structural annotation of biosynthetic genes will be crucial for any high-throughput synthetic biology workflow involving the assembly of pathways from synthetic genes based on in silico annotated gene structures. Our own experience has shown that gene predictions in the automated genome annotations of Basidiomycota are frequently incorrect, requiring manual reannotation and several attempts at amplifying spliced genes from cDNA. Basidiomycota have very intron-rich genomes [239], and we found many very small and unpredicted introns/exons (sometimes only 6–9 bps in size) in genes cloned from cDNA. Genes synthesized based on cDNA predictions are therefore often nonfunctional.

Deep RNA sequencing has been shown to significantly improve the accuracy of a large fraction of the gene models for *Laccaria bicolor* by finding all splice sites in genes through deep sequence coverage; one single transcriptome data set of 30 million reads provided 30 times more sequence coverage and hence much greater resolution for gene structure annotation than the EST libraries available for this fungus [240]. So far, only some of the more recently sequenced genomes have associated deep RNA sequence data [228, 230]. High-resolution transcriptomics analysis will be essential not only for direct translation of genomic information into synthetic gene assembly of pathway discovery, but also for improved functional annotation important for biosynthetic pathway identification.

Construction of gene co-expression networks built on the physical distance to natural biosynthesis product-associated seed genes represents a powerful tool for pathway discovery. The fact that natural product pathway genes are generally

co-regulated through levels of shared transcriptional control elements [241] represents yet another approach for network analysis within and also across species. Significant advances have been made in understanding the regulatory control elements of NP pathways in filamentous fungi, including the velvet family of regulatory proteins that are conserved among Ascomycota and Basidiomycota [38, 41, 242]. Genome analysis of *Ganoderma* and *Schizophyllum* [228, 243, 244] suggests high conservation of regulatory networks among mushroom-forming fungi, which could be exploited for network building. Yet, gene co-expression network analysis so far has been largely applied for the discovery of natural product genes in plants [245, 246]. Analysis based on co-expression arrays was only recently applied to natural product gene cluster analysis in *A. nidulans* [28]. Comprehensive deep transcriptome analysis of fungi grown under conditions that activate a broad range of natural products pathways [29, 39, 247, 248] should enable accurate prediction of complete pathways, including satellite or super-clusters of split pathways known from fungi [57, 249–251].

Deletion of biosynthetic genes represents the classic approach used for functional characterization of pathways, and it has been used for many of the pathways described in this chapter. However, this is a slow process, requiring that the pathway is expressed under given laboratory growth conditions; and most importantly, the fungal producer strain can be genetically manipulated. Alternatively, gene clusters may be transferred into a heterologous host if a compatible host is available. These strategies do not work for the vast majority of fungal strains for which no genetic approaches are available, are difficult to cultivate, and where the conditions to induce natural products biosynthesis are unknown. To access the natural product potential of these fungi, the development of synthetic biology approaches (discussed elsewhere in this book series) will be crucial for the rapid assembly of pathways from synthetic genes in suitable designed hosts. Such hosts could be a filamentous fungus or a yeast strain equipped with mechanisms to facilitate the expression of a potentially large complement of microsomal P450s and deal with the potential toxic metabolites produced by the pathways. Finally, refactoring of natural products pathways in heterologous platforms designed for high-throughput assembly and screening would provide the means for combinatorial approaches to explore an even larger diversity of natural products.

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