Fungal natural products – organismal diversity, seen from the (bio)chemical angle

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Abstract: Fungi are ingenious producers of complex natural products which show a broad range of biological activities. We encounter these products e.g. as bright colors or pungent tastes of carpophores, or as toxins that exert strong pharmacological effects on human or mammalian cells. On the beneficial end, we find pharmaceutically used drugs, such as antibiotics and lipid-lowering agents, while other fungal metabolites possess potent toxic or carcinogenic properties and threaten human, animal, or plant health. Along with bioactivity, perhaps the most intriguing aspect is the diversity of chemical structures that are produced in often species- or genus-specific metabolic pathways. This article presents an overview on the biosynthetic principles underlying assembly of the three major groups of natural product compounds, that is, the terpenoids, the polyketides and the amino acid-derived natural products.

1. Introduction

Besides the organismic, morphological and ecological diversity of fungi, a remarkable feature of fungal biology is the capacity to collectively produce a virtually limitless diversity of small yet structurally complex and often bioactive natural products. Just looking at the basidiomycetes, we encounter such products e.g. as bright colors or pungent tastes of carpophores. Every mycologist is familiar with and enjoys the golden to orange color of species of the Dacrymycetales or *Cantharellus cibarius*, or the amazing color reactions, when a bolete turns blue upon cutting or bruising the fruit body. Other fungal natural products are toxic and exert strong pharmacological effects on human or mammalian cells, e.g, muscarin, which is the toxic principle of *Inocybe* and other genera. The amanitins are infamous mushroom toxins, too, and named after the genus *Amanita*, but occur erratically in basidiomycetes, e.g. also in *Galerina* species (Luo et al. 2012). However, the potential of fungal metabolism was also harnessed by pharmacists, with invaluable drugs and pharmacophores found among fungal natural products, such as the β-lactam antibiotics, e.g. penicillins, the lipid-lowering agent lovastatin, i.e. *Penicillium* and *Aspergillus* metabolites. More recently, the antibiotic retapamulin, which derives from pleuromutilin, a product isolated from *Clitocybe passeckerianus*, was approved for clinical use (Kilaru et al. 2009). Another notable basidiomycete compound is the strobilurine family of compounds, whose antifungal activity has been used for decades in plant protection (Sauter et al. 1999).

Knowledge on fungal natural products also helps support and recognize evolutionary relationships via chemotaxonomic approaches. Textbook examples are the detection of 4-nitrophenol and 2-chloro-4-nitrophenol in both *Lindtneria trachyspora* and *Stephanospora caroticolor* (Lang et al. 2001)

or grevillin pigments, which define the genus *Suillus* chemically (Gill & Steglich 1987).

Primary metabolism is rather uniform and secures fundamental cellular functions, such as energy production, synthesis of membrane lipids, amino acids and cell wall material, etc. In contrast, it is the hallmark of the so-called secondary metabolism to be diverse, often species-specific, and to create an incredible diversity of compounds which may appear at times confusing at first glance. A number of excellent reviews have appeared, to which the readers are referred. These articles, among them are reviews by Gill and Steglich 1987, Gill 2003, Ziawiony 2004, Zhou and Liu 2010, Jiang et al. 2011, and Schueffler and Anke 2014, cover various aspects of fungal small molecules. This contribution highlights that the diversity of secondary metabolism is basically rooted in mainly three biosynthetic strategies which are presented here.

2. Biosynthetic mechanisms of fungal natural product formation

2.1. Terpenoids

The terpenoids represent a repertoire of incredible 30,000 natural products and include volatile compounds of essential oils, membrane sterols, hormones, such as estrogen or testosterone, phytol alcohol as building block of the chlorophylls, or ubi-/plastoquinone as components of the respiratory chain or photosynthetic light reaction. The yellow to red pigments of the carotenoid family of natural products are also made via the terpene metabolism which leads us back to the basidiomycetes, as it is the carotenes that confer the golden or orange color of *Dacrymyces* or *Cantharellus cibarius* fruiting bodies, mentioned above, or in urediniospores and aeciospores of rust fungi. A recent review that highlights the capacity of fungi to produce terpenes is highly recommended (Quin et al. 2014).

The monomeric building block of the terpenes is isopentenyl diphosphate (IPP, Fig. 1) i.e. a branched and unsaturated five-carbon unit, which is enzymatically isomerized to dimethylallyl diphosphate (DMAPP). In fungi, IPP is assembled from acetyl-CoA. The diversity of terpenes is established by three processes: first, the number of building blocks that are assembled into a non-cyclic precursor. Two building blocks, i.e. DMAPP as a starter unit, extended once, twice, or three times by IPP yield geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP), respectively (Fig. 1), which represent ten-, fifteen-, or twenty-carbon precursors en route to the mono-, sesqui-, or diterpene natural products. Homodimerization of FPP or GGPP yields C_{30} or C_{40} units to elaborate tri- and tetraterpenes. The second criterium of terpenoid diversity is the mode and stereochemical course how the precursors are cyclized by terpene cyclases (reviewed e.g. by Christian-

son 2006, Fig. 1). In addition, the third mechanism to diversify the terpenoid backbone structure are ring cleavage and rearrangements, and oxidations or reductions that occur after the terpene backbone is assembled.

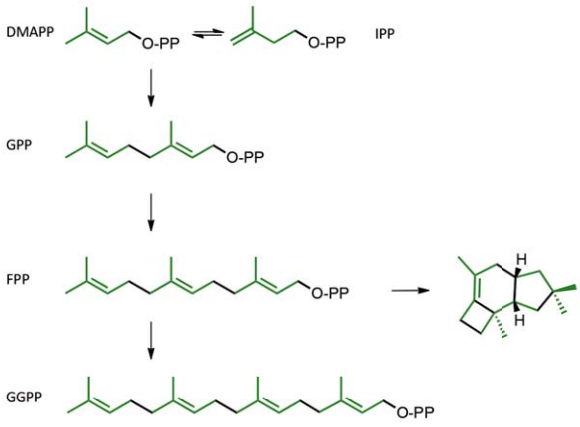

Fig. 1: Schematic of terpene biosynthesis. Abbreviations are: DMAPP: dimethylallyl diphosphate; IPP: isopentenyl diphosphate; GPP: geranyl diphosphate; FPP: farnesyl diphosphate; GGPP: geranylgeranyl diphosphate. The individual five-carbon building blocks are shown in green. An example for a terpene cyclase reaction is shown at the sesquiterpene level for the tricyclic protoilludene backbone.

Yet another level of structural diversity with small molecules is achieved by combining DMAPP or longer terpenoid units with polyketides (see below). This is, e.g. the case with the melleolides, i.e. *Armillaria* natural products (Fig. 2). Also, amino-acid derived molecules may also be decorated (and hence, diversified) with terpenoid units. A prominent example is the tetracyclic ergoline backbone as structural moiety of d-lysergic acid (Fig. 3) and countless ergot alkaloids (reviewed by Jakubczyk et al. 2014, Fig. 3) which is created from prenylated l-tryptophan.

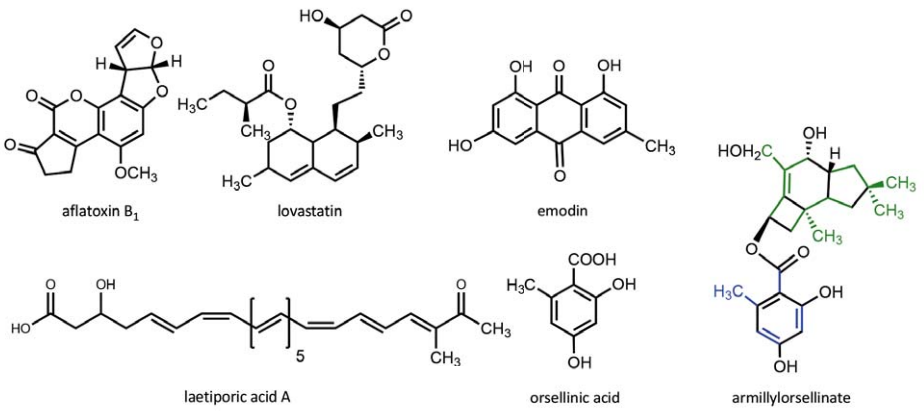

Fig. 2: Chemical structures of fungal polyketides. For the terpene-polyketide hybrid molecule armillylorsellinate, which is a member of the melleolide family of compounds, acetate units incorporated by PKS domains are in blue, carbon atoms of the terpenoid moiety are shown in green.

2.2. Polyketides

The polyketides represent a class of compounds that are named after a theorhetical biosynthetic intermediate that carries multiple keto groups. Textbook representatives of fungal polyketides are the aflatoxins (Fig. 2), i.e. *Aspergillus flavus* and *A. parasiticus* natural products that bind to DNA and, thus, are highly mutagenic. In contrast, another polyketide, lovastatin (Fig. 2) of *Aspergillus terreus*, is clinically used as a lipid-lowering drug.

Polyketides are assembled out of activated precursor building blocks, i.e. acetyl-CoA for initiation and malonyl-CoA, which, after decarboxylation, yields a two-carbon unit for chain elongation. Therefore, polyketide biosynthesis is mechanistically related to fatty acid biosynthesis and, consequently, the enzymes of these metabolic pathways resemble each other. In fungi, polyketide assembly is catalyzed by large multi-domain, multi-functional enzymes, the polyketide synthases (PKSs) which resemble fatty acid synthases (FASs). The fundamental difference between fatty acids and polyketides is the degree of how the nascent carbon chain is reduced. Whereas fatty acids are fully reduced during biosynthesis, polyketides are not. Therefore, double bonds, alcohol or carbonyl groups remain in the product when it is offloaded from the PKS enzyme after assembly. Like fatty acid synthases, PKSs are organized in domains that catalyze loading of monomeric building blocks, formation of the carbon-carbon bond between monomers, and release of the product. The domains of these so-called iterative type I PKSs are used several times per molecule to be assembled, and each round extends the growing polyketide by one monomer, i.e. two carbon atoms.

Typically, fungi feature two subcategories of PKSs, reducing and non-reducing ones. The former possess extra domains for reduction and produce non-aromatic compounds, such as laetiporic acid (Fig. 2) and related compounds, which are the yellow pigment of *Laetiporus sulphureus* and various other basidiomycetes. Also, the lovastatin molecule is composed of two polyketides, both of which are the product of a reducing PKS. In contrast, the non-reducing PKSs lack these reductive domains and generally produce aromatic compounds, such as emodin (Fig. 2) and numerous other anthranoids which are the pigments of various *Cortinarius* species, or orsellinic acid (Fig. 2), which appears as aromatic moiety of the above mentioned melleolides. To learn about mechanistic details of PKSs, the reader is referred to excellent reviews, among them those authored by Cox (2007), Crawford & Townsend (2010), Townsend (2014) and Vederas (2014).

While the polyketide biosynthetic principle – oligomerization of two-carbon units – sounds simple, the structural diversity that can be generated is breathtaking and maximized using various strategies:

i) the choice of the building block that serves as starter unit. By incorporating molecules other that acetate, diversity is achieved during chain initiation,
ii) the number of two-carbon units (formal acetate units) that are oligomerized, i.e. the number of extension steps which translates in a given chain length,
iii) cyclization of the linear PKS product may occur,
iv) reduction steps may occur, as mentioned above,
v) the mode of product release from the synthase.

Yet another layer of molecular diversification, the so-called post-PKS tailoring, happens during or after polyketide assembly, as the PKS product may undergo further modification, catalyzed by separate stand-alone enzymes or as domains integral to PKSs. Examples for such modifications are halogenation, esterification, or methylation, all of them seen again with the melleolides.

2.3. Amino acid-derived compounds

The term "amino acid-derived natural products" summarizes the third immensely diverse group of fungal natural products. They fall into the categories non-peptidic products versus those that have a peptidic structure i.e. that include one (or multiple) amide bonds in the compound. Figure 3 illustrates the chemical structures of the compounds highlighted in this chapter.

2.3.1. Non-peptidic amino acid-derived compounds

Non-peptidic amino acid-derived compounds derive from a single and, in the majority of cases, proteinogenic amino acid, which is then modified by decarboxylation, cyclization, oxidation, or other processes. The toxic principles of *Amanita muscaria* and *A. pantherina* are L-ibotenic acid and its follow-up product muscimol that derive from L-glutamine and act as unselective glutamate receptor agonists in the human central nervous system. Likewise, three toxins of *Trogia venenata* were identified as unusual, nonproteinogenic amino acids, i.e. 2*R*-amino-4*S*-hydroxy-5-hexynoic acid, 2*R*-amino-5-hexynoic acid and γ-guanidinobutyric acid). It is very likely that these compounds are the causative agents of the so-called Yunnan sudden unexplained death phenomenon (Zhou et al. 2012).

2.3.2. Peptidic amino acid-derived compounds

One would expect that ribosomes are involved to elaborate peptidic structures. However, this holds true only in a few cases, e.g. for the amanitins and the phallotoxins, i.e. cyclic oligopeptide toxins from *Amanita phalloides*, *Galerina marginata* and other species. The toxins derive from larger precursor proteins (35 and 34 amino acids, respectively), whose mRNA is translated by ribosomes. The precursor proteins are then cleaved by a prolyl oligopeptidase to release and cyclize the actual octa- and heptapeptide (Hallen et al. 2007, Luo et al. 2009, Luo et al. 2014).

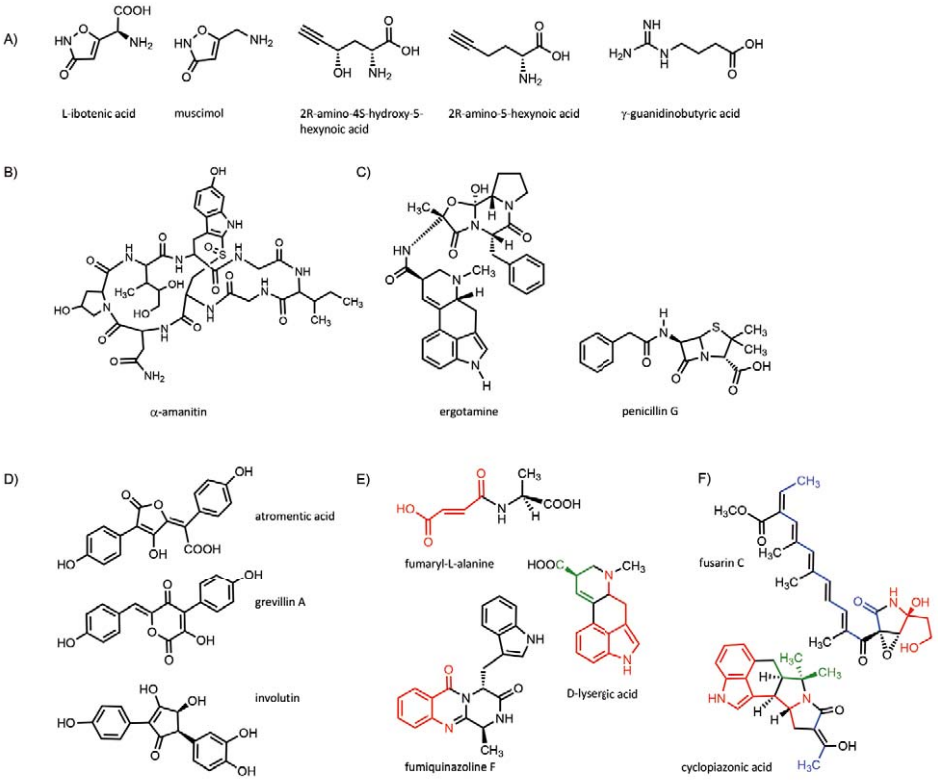

Fig. 3: Chemical structures of amino acid-derived compounds. A) non-peptidic compounds; B) α-amanitin; C) compounds made by non-ribosomal peptide synthetases (NRPSs); D) compounds made by NRPS-like enzymes that are derived from aromatic α-keto acids; E) anthranilic acid- and fumaric acid-containing fungal natural products (respective moieties highlighted in red) and D-lysergic acid (amino acid moiety in red, terpene moiety in green); F) examples for hybrid polyketide synthase/peptide synthetase-made natural products. Acetate units incorporated by PKS domains are shown in blue, amino acid-derived molecule portions incorporated by NPRS domains are shown in red, the terpene unit of cyclopiazonic acid is shown in green.

In constrast, most peptidic fungal products are biosynthesized in a ribosome-independent mode. Non-ribosomally assembled peptidic fungal natural

products are generated by means of multi-modular enzymes, the non-ribosomal peptide synthetases (NRPSs). Well-known examples for fungal metabolites that are made by NRPSs are the penicillin antibiotics and ergotamine, which is one of the toxins of the ergot fungus *Claviceps purpurea*. NRPSs are organized as assembly line-like multi-functional and modularly organized enzymes. Each module comprises several catalytic domains that include all necessary catalytic activities to initiate biosynthesis or elongate the growing natural product by one monomer (Hoffmeister & Keller 2007). The minimal set of domains are i) the adenylation domain (A domain) that serves substrate monomer recognition and activation through formation of an adenylate, ii) the thiolation domain (T domain), which accommodates the activated amino acid as a thioester, and iii) the condensation domain (C domain) that catalyzes formation of the amide. A domains are specific for one particular substrate, which they adenylate and load onto the T domain. Thus, the order of A domains within the synthetase is co-linear with the order of the building blocks in the final natural product.

The concept of NRPSs is a versatile strategy to create structural diversity, for various reasons: firstly, the number of modules is variable. It generally determines the number of monomers to be incorporated and, thus, product length. For example, the three-module NRPS Lps1 and the monomodular NRPS Lps2 which are the synthetases that cooperatively represent the assembly line to synthesize the ergotamine scaffold, possess a total of four modules that correspond to the four ergotamine building blocks D-lysergic acid, L-alanine, L-phenylalanine and L-proline (Riederer et al. 1996, Correia et al. 2003).

The second reason is that additional catalytic functionalities can be incorporated as extra domains into NRPSs, e.g. for *N*-methylation.

Lastly, NRPSs are not restricted to the canonical proteinogenic L-amino acids as substrates, as seen e.g. above for the ergotamine biosynthesis that begins with D-lysergic acid adenylation. NRPSs utilize a much larger substrate pool, totaling several hundreds of potential building blocks, which greatly helps generate structural and metabolic diversity (reviewed in Kalb et al. 2013). These substrates also include

i) non-proteinogenic amino acids, e.g. for siderophore assembly,
ii) α-keto acids. For example, the aromatic α-keto acid 4-hydroxyphenylpyruvic acid is the building block of Boletales pigments, such as a) pulvinic acid derivatives which turn blue upon oxidation after mycelial injury, b) the grevillins of *Suillus* species, and c) involutin and related diarylcyclopentenone pigments of *Paxillus involutus*. The molecular scaffolds of all of them are made by specialized NRPS-like synthetases lacking a condensation domain due to the absence of an amino group in the substrates.

iii) anthranilic acid (e.g. for fumiquinazoline assembly)

iv) non-amino acid substrates, such as D-lysergic acid (see above) or fumaric acid, that was recently identified as building block of *Aspergillus fumigatus* natural products (Steinchen et al. 2013).

Finally, as presented above, NRPS and PKS modules can be consolidated to hybrid multi-domain enzymes which account, e.g. for assembly of mycotoxins such as cyclopiazonic acid and fusarin C (reviewed by Boettger & Hertweck 2013).

Probably the most intriguing phenomenon in biology is organismic diversity. This contribution summarizes how fungi use a few biochemical strategies that collectively allow them to elaborate an essentially limitless chemical diversity of often highly functionalized and bioactive small molecule natural products that reflect organismic diversity on the chemical level.

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