
Making Use of Genomic Information to Explore the Biotechnological Potential of Medicinal Mushrooms

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Abstract

Fruiting bodies of fungi are rich in multiple types of bioactive compounds with (potential) pharmaceutical effects. Many kinds of mushrooms are thus highly valued in traditional medicine in different cultures over the world for treatment of diseases and maintenance of good health. Modern science has uncovered functional principles in many medicinal species and assigned beneficial activities (antimicrobial, antiviral, anti-oxidative, immunomodulatory, anti-inflammatory, anti-tumorous, hypotensive, hepatoprotective, antidiabetic/hypoglycemic and hypocholesterolemic, mitogenic/regenerative, etc.) to a wealth of secondary metabolites, peptides, proteins, and sugar-based polymers. Compared to the extensive lists of bioactive compounds and the description of their distinctive effects, the pathways of their biosynthesis and the genes behind are largely understudied. This can now become changed by the many genomes which are provided by large-scale fungal sequencing programs. Among are many assembled genomes for important edible and medicinal mushroom species which can be used in genome mining for genes of interest, both for the synthesis of known products and for the synthesis of novel, so far undetected compounds. Also, genomes of other species offer possibilities to predict genes for the biosynthesis of formerly unnoticed bioactive fungal products of either biochemically already known or novel structure. We present here examples of recent

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identification of genes and gene clusters for bioactive compounds (different terpenoids, phenolics, polyketides, cyclic peptides, aegerolysins, lectins, protease inhibitors, and ribosome-inactivating proteins) in medicinal and edible fungi. Genome comparisons and gene mining identify related genes for similar products in other species. Usually, genes for medicinally interesting products are found in only a restricted range of species, inconsistently distributed over the fungal taxa. Some of the recognized medicinal species probably have genes for a higher variety of bioactive products than species which are estimated purely for their good edible value or species being commonly neglected for exploitation as food and medicine.

Keywords

Agaricomycetes • *Ascomycota* • Secondary metabolites • Cyclic peptides • Lectins • Aegerolysins • Fungal genomes

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Abbreviations

atf Gene for acetyltransferase
cyc Gene for terpene cyclase

<i>ggs</i>	Gene for geranylgeranyl pyrophosphate synthase
<i>p450</i>	Gene for cytochrome P450
<i>sdr</i>	Gene for short-chain dehydrogenase/reductase

13.1 Introduction

The fungal subkingdom *Dikarya* splits into the two phyla *Ascomycota* and *Basidiomycota* that represent the higher fungi, many of which grow in mycelial form and produce compact fruiting bodies with meiotic spores for sexual reproduction (Hibbett et al. 2007). Specific genera of the *Ascomycota*, in particular within the order *Pezizales* (e.g., *Helvella*, *Morchella*, *Terfezia*, *Tirmania*, *Tuber*), and in full extend the class *Agaricomycetes* within the phylum *Basidiomycota* give rise to larger mushrooms (Hibbett 2007; Schoch et al. 2009). Mushrooms are fruiting bodies of macroscopic size, can be seen by the naked eye and be picked by hand, and may grow above (epigeous) or below (hypogeous) ground in soil or a respective growth substrate (Chang and Miles 1992). There is a tremendous variety in mushroom morphologies, such as in their overall shapes, sizes, colors, consistencies, tissue structures, etc. (Schoch et al. 2009; Kües and Navarro-Gonzalés 2015; Sherratt et al. 2005; Halbwachs et al. 2016). Mushrooms attracted humans since ancient times not only by their often beautiful eye-catching looks but for reasons of exploitations such as for food, health care, and use as hallucinogens, among in spiritual practices (Kües and Liu 2000; Boa 2004; Jo Feeney et al. 2014; Sayin 2014; de Mattos-Shiplely et al. 2016).

Mushrooms are commonly distinguished into edible, nonedible (e.g., due to no or too bad taste or by too hard structure), medicinal, psychedelic, and poisonous species, with sometimes fuzzy overlaps between the categories (Boa 2004; Guzman 2008; Garibay-Orijel et al. 2009; Gonmori et al. 2011; Grienke et al. 2014; Jo et al. 2014; Santiago et al. 2016). Although controversially discussed, the best known fly agaric *Amanita muscaria*, for example, is reported to be eaten upon decoction in certain locations of the world, possibly in the lack of alternative better and safer food (Rubel and Arora 2008; Viess 2012). The consumption of this unmistakable fungus for the experience of its hallucinogenic effects is also disseminated in certain populations and cultures (Feeney 2010; Viess 2012; Sayin 2014), while (accidental) poisonings with the fly agaric (pantherina-muscaria syndrome based on ibotenic acid as the most potent toxin in the fruiting bodies) are also recorded. However, fatal outcomes are rare (2–5% of cases) by advanced diagnosis and good medical treatment (Michelot and Melendez-Howell 2003; Marciniak et al. 2010; Vendramin and Brvar 2014; Mikaszewska-Sokolewicz et al. 2016).

Global numbers of total fungal species and of different higher fungal taxa are still challenged with contemporary estimates for species between lowest 0.7 and highest 5.1 million, with 1.5–3.0 million being the current working figure (Schmitt and Mueller 2007; Blackwell 2011; Hawksworth 2012; Tedersoo et al. 2014; Dai et al. 2015). In the so far most comprehensive molecular genetic survey of soil

mycobiomes from different continents, latitudes, and ecosystems, about 50% of all detected fungal species (in total 80,486) were *Agaricomycetes* and 1.8% *Pezizomycetes*, with over 40% of the taxa being still unknown (OTUs, operational taxonomic units; Tedersoo et al. 2014). Earlier conservative estimates stated that within a total of 1.5 million fungi, about 140,000 macrofungi exist worldwide of which 10 % were known at the time (Hawksworth 1991; Chang 2001). Of the then known mushroom species, about 7000 and >2000 are considered to be edible and to be entirely safe, respectively (Hawksworth 1991; Chang 2001), while a small percentage (2–3%) of the known species (at least 170) is poisonous to deadly toxic (Gonmori and Yoshioka 2003; Boa 2004). However, the numbers of named fungal taxa (especially genera and species) are rapidly increasing, including that important edible and medicinal fungal species complexes (e.g., within the genera *Morchella*, *Ganoderma*, and *Inonotus*) become better resolved and subdivided into new lineages (Dai et al. 2015).

Regardless of an actual edible status, mushrooms can have many useful medicinal properties, including psychedelic and potentially lethal species which may provide medicinal power also by their Janus-faced toxins (Wasser 2002; Jo et al. 2014; Carhart-Harris et al. 2016; Rahi and Malik 2016). Substantial pharmacological properties were recorded for over 700 species. However, the extensive medicinal potential offered by mushrooms is likely by far not fully perceived and certainly not yet utterly exploited (Wasser and Weiss 1999; Wasser 2002, 2011; Lindequist et al. 2005; Badalyan 2012; De Silva et al. 2013; El Enshasy et al. 2013). Best established in cancer cell line and animal test systems are positive effects of preparations of fungal β -glucans and proteoglycans such as of *Trametes versicolor* (the 100 kDa polysaccharide-K, PSK, krestin), *Schizophyllum commune* (the 45–50 kDa schizophyllan), *Lentinula edodes* (the 45–50 kDa lentinan), *Grifola frondosa* (the 100 kDa maitake polysaccharide D fraction, respectively, purified MD fraction, grifoldan), *Ganoderma lucidum* (the 40 kDa polysaccharide PS-G preparation, ganopoly), and *Phellinus* species (fractions variably sized 8.9–2000 kDa) which are marketed as alternative medicine and used as anticancer therapeutics in Asian countries (Kidd 2000; Lindequist et al. 2005; Lemieszek and Rzeski 2012; El Enshasy and Hattikaul 2013; De Silva et al. 2013; Huang and Nie 2015; Yan et al. 2017). *L. edodes* and *G. frondosa* are widely eaten gourmet mushrooms; *S. commune* fruiting bodies are included in human diets in some tropical countries (e.g., Thailand, Malaysia, and Mexico), whereas the tough sporocarps of *T. versicolor* and the conks of *G. lucidum* are edible but unpalatable and the latter in addition is too bitter (Boa 2004; Chang and Lee 2004; Ruán-Soto et al. 2006). Many versatile medicinal mushrooms, such as from the *Ganoderma* species complex, are therefore applied as food additives (nutriceuticals; Chang and Buswell 2001) in the form of tonics, teas, soups, and alcoholic beverages or as mushroom powders, frequently filled into capsules or pressed into pills, in order to make use of their good ingredients for benefits of human health (De Silva et al. 2012a; Bishop et al. 2015).

Medicinal mushrooms and their multiple bioactive compounds are listed to function in human health care for instance antiviral, antibacterial, antifungal, anti-oxidative, immunomodulatory and immunosuppressive, anti-inflammatory,

anti-tumorous, hypotensive, hepatoprotective, antidiabetic/hypoglycemic and hypocholesterolemic, anti-obese, mitogenic/regenerative, anti-dementia, and more (Wasser and Weiss 1999; Wasser 2002, 2011; Poucheret et al. 2006; Badalyan 2012; Chang and Wasser 2012; Hassan et al. 2015; Phan et al. 2017). Such claims, however, need a good clinical survey to substantiate these potentials in practice (Chung 2006; Hapuarachchi et al. 2016; Money 2016; Wasser 2017).

Functional compounds of mushrooms, their biochemical nature, their principles of actions, and their production and safe applications are a matter of concern. A wealth of organic bioactive molecules has been identified in worldwide research from mushrooms to have medicinal effects, including different bioactive proteins (e.g., aegerolysins, lectins, ribosome-inactivating proteins, and protein inhibitors), ribosomal and non-ribosomal peptides, polysaccharides, peptidoglycans, alcohols, phenolics, different types of terpenoids (sesquiterpenes, diterpenes, triterpenes, meroterpenes of mixed biosynthetic origin, less often monoterpenes), lipids, steroids, alkaloids, polyketides, and other compounds (Wasser and Weiss 1999; Wasser 2002; Ferreira et al. 2010; Anke and Antelo 2011; Schöffler and Anke 2011; De Silva et al. 2013; Grienke et al. 2014; Duru and Çayan 2015; Sabotič et al. 2016; Dickschat 2017). Functional compounds for applications might be isolated from collected or cultivated mushrooms (Tang et al. 2007; Stadler and Hoffmeister 2015; Bedlovicova et al. 2016; Tang et al. 2016a; Lau and Abdullah 2017) or, when possible, obtained from mycelial fermentation (Zhong and Xiao 2009; De Silva et al. 2012b; Elisashvili 2012; El Enshasy and Hatti-Kaul 2013; Chen et al. 2016a). Not all mushroom species can grow in culture and such therefore fall short for fermentations (Badalyan 2012; Stadler and Hoffmeister 2015). However, polysaccharides (mainly β -glucans) and peptidoglycans as ordinary constituents of fungal cell walls and their external mucilage layers might abundantly be produced in large mycelial fermentation from well-growing species (Fazenda et al. 2008; Wasser 2011; Castillo et al. 2015; Chen et al. 2016a; Money 2016), but also some other bioactive compounds, e.g., phenolic antioxidants and certain terpenoids, can be obtained from cultures (Lorenzen and Anke 1998; Asatiani et al. 2007; Carvajal et al. 2012; Thongbai et al. 2015; Tešanović et al. 2017). Not uncommonly, mycelia produce bioactive compounds which are not seen in the fruiting bodies (Hartley et al. 2009; Thongbai et al. 2015). In other instances, specific proteins and peptides, secondary metabolites, and other potent bioactive molecules might confine purely to the fruiting bodies and even to specific tissues and stages in the development (Enjalbert et al. 1999; Boulianne et al. 2000; Walser et al. 2005; Hu et al. 2012; Li et al. 2014a; Lu et al. 2014; Yilmaz et al. 2015; Zhang et al. 2015a; Sabotič et al. 2016), or specific conditions need to be established for induction of targeted metabolite production in liquid cultures (Xu et al. 2010). Favoring measures can be addition of methyl jasmonate, lipids, or surfactants (Ren et al. 2013a; Xu et al. 2016a,b), addition of suitable precursor molecules (Hu et al. 2014, 2016) or specific known inducers (Liang et al. 2010), addition of effective deregulators of the general metabolism (Ren et al. 2014) and of other severe stressors (You et al. 2013; Cao et al. 2017), application of specific pH control (Wang et al. 2016b) or temperature shifting strategies (Feng et al. 2016), or limitation of oxygen (Zhang and Zhong 2013). Such

environmental and physiological management strategies can become further expanded by genetic engineering of the fungal producers by smart modification of improving expression of genes for the synthesis of precursor molecules and of the compounds of concern and by interrupting gene expression of competitive pathways and unwanted pathway branches (Qin et al. 2015; Xu and Zhong 2015).

Fungal research with the twenty-first century experienced an unprecedented boost by whole genome sequencing programs over the fungal kingdom, many of which are provided to the public in assembled form with gene annotations on the MycoCosm page of the JGI (Joint Genome Institute, Walnut Creek, CA; <http://genome.jgi.doe.gov/programs/fungi/index.jsf>; Grigoriev et al. 2014) or can be found deposited in the NCBI databases (<https://www.ncbi.nlm.nih.gov/>). Fungal genome sequencing programs provide extraordinary resources for identifying new genes for the synthesis of bioactive compounds from mushrooms, both of biochemically already known substances and of unforeseen compounds. With respect to edible and medicinal mushrooms, sequencing so far provided from *Ascomycota* in particular the genomes of precious edible truffles such as *Tuber melanosporum* (Martin et al. 2010), *Tuber borchii*, *Terfezia boudieri*, and *Terfezia claveryi*, of the morels *Morchella conica* and *Morchella importuna*, and of the false morel *Gyromitra esculenta* (MycoCosm status from April 2017). Especially many mushroom genomes are published from the *Agaricomycotina* (see MycoCosm page and NCBI databases for full list), among from the edible *Agaricus bisporus* (Morin et al. 2012), *Amanita jacksonii* (Sánchez-Ramírez et al. 2014), *Armillaria mellea* (Collins et al. 2013), *Auricularia subglabra* (Floudas et al. 2012), *Boletus edulis* (MycoCosm page), *Flammulina velutipes* (Park et al. 2014), *Hypholoma sublateritium* (Kohler et al. 2015), *Laetiporus sulphureus* (Nagy et al. 2016), *Lentinula edodes* (Chen et al. 2016b), *Pleurotus eryngii* (Yang et al. 2016b), *Pleurotus ostreatus* (Riley et al. 2014), and *Volvariella volvacea* (Bao et al. 2013), also from the less often eaten *Coprinopsis cinerea* (Stajich et al. 2010) and *S. commune* (Ohm et al. 2010), and as further important medicinal mushrooms from *Amanita bisporigera* and *Amanita phalloides* (Pulman et al. 2016), *Amanita muscaria* (Kohler et al. 2015), *Taiwanofungus camphoratus* (synonym *Antrodia cinnamomea*; Lu et al. 2014), *Fistulina hepatica* (Floudas et al. 2015), *Fomitopsis pinicola* (Floudas et al. 2012), various *Ganoderma* species (Kües et al. 2015; Merciere et al. 2015; Zhu et al. 2015), *Lignosus rhinocerotis* (Yap et al. 2014)), *Omphalotus olearius* (Wawrzyn et al. 2012), *Punctularia strigosozonata* (Floudas et al. 2012), *Serpula lacrymans* (Eastwood et al. 2011), *Stereum hirsutum* (Floudas et al. 2012), *Suillus luteus* (Kohler et al. 2015), *Trametes versicolor*, and *Wolfiporia cocos* (Floudas et al. 2012). More fungal genomes of all categories of mushrooms are likely to come, all of which await exploitation.

13.2 The Value of Fungal Genomes

In the past, discoveries of metabolic gene functions were commonly activity driven by encoded enzymes and their products; via appointing mutants, smart selection methods, and function-based genetic screenings; and through elucidation of metabolic synthesis pathways by methods such as of isotope and precursor feedings combined with chemical structure clarifications (Casselton and Zolan 2002; Keller et al. 2005; Bills and Gloer 2016).

The established fungal genomes however offer now in addition invaluable platforms for advanced genome mining, for example, for searching of biosynthesis gene clusters (BGCs) for natural bioactive products generated from multienzyme pathways, applying first in silico sequence searches and suitable bioinformatics methods (Wawryzn et al. 2015; Bills and Gloer 2016; van der Lee and Medema 2016) which subsequently can be combined with experimental activity research on identified genes (Bills and Gloer 2016). The latter might be addressed through heterologous gene expression, e.g., in *Escherichia coli* (e.g., Agger et al. 2009; Quin et al. 2013a; Sun et al. 2016; Yang et al. 2016a; Zhou et al. 2016; Alberti et al. 2017; Braesel et al. 2017; Lin et al. 2017), *Saccharomyces cerevisiae* (Walser et al. 2004; Agger et al. 2009; Ishiuchi et al. 2012; Alberti et al. 2017; Nielsen and Nielsen 2017), *Pichia pastoris* (Xue et al. 2008; Bastiaan-Net et al. 2013; Lin et al. 2013, 2016), or *Aspergillus* species (Yaegashi et al. 2014; Alberti et al. 2017; Braesel et al. 2017). Recently, for the first time, a complete gene cluster from a mushroom has thus been functionally expressed in *Aspergillus oryzae* (Bailey et al. 2016; Alberti et al. 2017). Alternatively, if a transformation system exists for a fungus (in the basidiomycetes still rare), gene functions might be elucidated through homologous expression via in vitro-modified vector constructs providing, e.g., efficient promoters of superior regulatory schemes to cloned genes encoding biosynthesis enzymes (Bailey et al. 2016; Alberti et al. 2017) or gene-specific regulatory proteins (Fox and Howlett 2008; Brakhage and Schroeckh 2011), or respective genes might be knocked out (Bailey et al. 2016; Sun et al. 2016; Yu et al. 2016).

Importantly, genome mining can be used to find the biosynthetic genes for already known products but also for novel products of hitherto unknown existence. Examples of all situations can be found in this article. Different approaches for finding new genes by genome mining are possible. Whole genome comparisons, for example, can identify genes and gene clusters unique to a species as potential candidates for the production of unusual, “exotic,” bioactive compounds not found in other species (Wang et al. 2016c). Guesses on gene products, e.g., type of encoded enzyme families, and in the case of gene clusters on the combinations of enzymes provided can direct further research to eventually identify the synthesized metabolic products and their intermediates. Moreover, computer programs can be trained to specifically identify BGCs with relevant (highly conserved) genes but also to identify BGCs with more obscure genes and cluster organization. Motif-independent prediction programs make use of, e.g., transcriptomic data. On the other hand, a known bioactive secondary metabolite might be assigned to genes potentially responsible for their production. Where enzymes for biosynthesis of similar

metabolic products or for potential common precursors and intermediates are known, guesses can be made on the type of genes that should participate in the synthesis of the compound of interest. Genome searches with the respective enzymes of known functions will help by sequence identities and similarities to find candidate genes encoding the desired enzymes for a chemically defined metabolite (Umemura et al. 2013, 2015; Koczyk et al. 2015; Li et al. 2016c; van der Lee and Medema 2016).

Genome search approaches with amino acid sequences can of course also be performed with other, nonenzymatic bioactive proteins and peptides. Sometimes, however, revealing amino acid stretches of identity or similarity between proteins or peptides are very short or key amino acids in motifs are individually spread over larger sequence distances. Various bioinformatics tools are being developed to elegantly address such cases in genome mining. Known structural information from similar proteins and peptides in addition can be included into computer-directed searches and, of course, any possible further knowledge such as related to other genes expected to be positioned in respective BGCs (Nagano et al. 2016; van der Lee and Medema 2016; Hetrick and van der Donk 2017). Nevertheless, busy manual genome mining can be very helpful to (first) identify any informative motifs, and it is sometimes superior and more complete as compared to bioinformatics methods with discrete algorithms designed for finding small motifs (Niculita-Hirzel et al. 2008; Kües et al. 2015; Pulman et al. 2016; van der Velden et al. 2017).

In tendency in fungi, genes for a multienzyme biosynthesis pathway for given secondary metabolites localize together (Brakhage and Schroeckh 2011; Wiemann and Keller 2013; Yaegashi et al. 2014; Li 2016c), although in the so far understudied *Basidiomycota* this might be less true than in the *Ascomycota* (see opposing examples in the text below), or it might depend on the biochemical type of the metabolites of concerns (Schmidt-Dannert 2015). Many novel fungal metabolic gene clusters identified via genome mining appear at first sight to be silent (referred to as cryptic or orphan BGSs), failing to provide any interesting metabolic product(s). However, strategies are presented as for how to activate silent gene clusters through the application of types of stresses, such as co-culturing, e.g., with bacteria, other fungi, or nematodes (Zheng et al. 2011; Plaza et al. 2014; Yao et al. 2016; Tauber et al. 2016). Induced gene expression can then be followed up by transcriptomics, proteomics, and metabolomics (Kuan et al. 2013; Ren et al. 2013a; Plaza et al. 2014; Yap et al. 2015a,b; Yu et al. 2015; Martinez et al. 2016; Yao et al. 2016; Tauber et al. 2016) and coexpression correlations (Umemura et al. 2013, 2015).

In the following, we are reviewing the recent progress made in identification and characterization of genes involved in the production of specific bioactive secondary metabolites, peptides, and proteins with (potential) pharmaceutical value through the now available genomes of many of the interesting mushrooms.

13.3 Bioactive Secondary Metabolites and Their Enzymes for Biosynthesis

13.3.1 Sesquiterpene Synthases

Sesquiterpenes (C₁₅ terpenoids) are volatile terpenes (isoprenoids) with a skeleton of three isoprene (2-methyl-1,3-butadiene) units. They are produced from the mevalonate pathway-derived five-carbon precursors dimethylallyl pyrophosphate (DMAPP) and **isopentenyl pyrophosphate** (IPP) through the 10-carbon fusion product **geranyl pyrophosphate** (GPP) and another IPP via the 15-carbon compound **farnesyl pyrophosphate** (FPP). Sesquiterpene synthases dephosphorylate and cyclize the linear intermediate FPP to a multiplicity of different cyclic sesquiterpene scaffolds (>300) with the formula C₁₅H₂₄ (Miller and Allemann 2012; Li and Wang 2016). Sesquiterpenes might be monocyclic, bicyclic, or tricyclic, and they further differ by rearrangements and by side chain modifications through actions of a multitude of cytochrome P450 monooxygenases, oxidoreductases, further oxygenases, and group transferases (Quin et al. 2014). The wealth of different sesquiterpenes described from macrofungi and their medicinal potential is thus stupendous (Lorenzen and Anke 1998; Abraham 2001; Christianson 2006; Ajikumar et al. 2008; Fraga 2011; Li and Wang 2016).

The first sesquiterpene synthase genes from a sequenced mushroom to be cloned were from *C. cinerea* (Agger et al. 2009). This helped to find further sesquiterpene synthase genes in other genomes (>1000 genes in ~100 fungal genomes by year 2014) and, where present, associated gene clusters with genes for precursor biosynthetic enzymes, sesquiterpene-modifying enzymes, transcription factors for putative cluster regulation, and metabolite-related transporters (Wawrzyn et al. 2012; Quin et al. 2014). **Table 13.1** gives an overview on so far cloned and characterized sesquiterpene synthases from the *Agaricomycetes* with their specific products as the first entry into the very complex and variable enzymology of sesquiterpene synthesis from FPP in this fungal class. Enzymes differ in their modes of cyclization (by 1,6-, 1,10-, or 1,11-ring closures, respectively). Some of the cloned sesquiterpene synthases share the same products, while others generate sesquiterpene scaffolds of quite different structure. Some of the enzymes are quite specific with only one or mainly one product, and these can be assigned to a specific function, while others are variable promiscuous in their product range. Importantly, however, not all of the detected sesquiterpenes are direct products of the cloned enzymes but result from secondary chemical rearrangements (**Table 13.1**).

Sesquiterpene synthases are specifically identified by the two characteristic aspartate-rich motifs DDXXD/E and NDE/DTE located at the entrance of the catalytic enzyme center to coordinate a trinuclear Mg²⁺ cluster for binding the pyrophosphate moiety of the FPP substrate, positioning its isoprenyl chain into the pocket, and triggering the pyrophosphate cleavage (Schmidt-Dannert 2015). Enzymatic specificity is then influenced by amino acids in the conserved H- α 1 loop which is found C-terminal to the NDE/DTE motif and caps the active site of the enzymes upon FPP binding. Accordingly, amino acid changes in Cop3 and Cop4 of

Table 13.1 Cloned sesquiterpene synthases from the *Agaricomycetes* and detected products from FPP

Fungus	Compounds produced by fungus		Sesquiterpene synthase		Reference(s)
	Name	Bioactivities	Gene and enzyme	Enzymatic activities	
<i>Amillaria gallica</i>	Melleolides, composed of a protoilludane esterified to an orsellinic acid moiety	Antibacterial, antimicrobial, cytotoxic, phytotoxic	<i>pro1</i> ; protoilludene synthase Pro1 (AGR34199)	Forms exclusively $\Delta 6$ -protoilludene, involved in biosynthesis of melleolides	Amore et al. (1986), Obuchi et al. (1990), Bohnert et al. (2011, 2014), Engels et al. (2011), and Kobori et al. (2015)
<i>Boreostereum vibrans</i>	Cadinane sesquiterpenoids boreovibrans A-G	Boreovibrans E and F are weak inhibitory to 11 β -hydroxysteroid dehydrogenase activity	<i>BvCS</i> ; δ -cadinol synthase (KU668561)	Forms mainly δ -cadinol and some germacrene D-4-ol in the biosynthesis pathway of boreovibrans	Ding et al. (2012), Zhao et al. (2013), and Zhou et al. (2016)
<i>Coprinopsis cinerea</i>	Lagopodin, hydroxylagopodin B, coprinastatins, coprinol, oxazolinone, illudosins, armillols	Antibiotic, anti-tumorous	<i>cop1</i> ; germacrene A synthase <i>cop2</i> ; germacrene A synthase	Forms β -elemene ^a , α -muurolene, δ -cadinene, germacrene D Forms β -elemene ^a , α -muurolene, δ -cadinene, respectively, the alcohols α -cadinol, germacrene D-4-ol	Bottom and Siehr (1975), Bu'Lock and Darbyshire (1976), Johansson et al. (2001); Wihlborg et al. (2008), Agger et al. (2009), Lopez-Gallegro et al. (2010a, b), Pettit et al. (2010a, b), and Lauchli et al. (2014)
	Volatile sesquiterpenes from culture: pentalenene, α -muurolene, α -cuprenene, δ -cadinene, β -caryophyllene, coprinastatin 1, coprinol, 7,7a-diepicoprinastatin 1, 14-hydroxy-5-desoxy-2S,3S,9R-illudosin, 4,5-dehydro-5-deoxyarmillol		<i>cop3</i> ; α -muurolene synthase <i>cop4</i> ; promiscuous δ -cadinene synthase	Forms α -muurolene, (major product), β -elemene ^a , α -muurolene, germacrene D, δ -cadinene Forms δ -cadinene (major product), β -cubebene, sativene, β -copaene, cubebol; forms with <i>cis</i> -FPP as substrate α -acoradiene (major product), some β -bisabolene and others	
			<i>cop5</i> ; activity unknown <i>cop6</i> ; specific α -cuprenene synthase	No product detected Forms nearly exclusively α -cuprenene in the biosynthesis pathway of lagopodin; forms with <i>cis</i> -FPP as substrate a major unidentified product, α -acoradiene, amorpho-4,11-diene	

<i>Fomitopsis pinicola</i>	<p>Δ6-Protoilludene; α-barbatene, β-barbatene, α-muurolene, γ- muurolene, γ- cadinene, δ-cadinene, α-copaene; β-copaene; and several more</p>		ID84944; specific α-cuprenene synthase	Forms nearly exclusively α-cuprenene	Fäldt et al. (1999), Rösecke et al. (2000), and Wawrzyn et al. (2012)
<i>Omphalotus olearius</i>	<p>Illudin-S, illudin-M, illudosin, illudol, illudosone, illudiolone, isomphadiolone, omphadiol</p> <p>Major volatile sesquiterpenes from culture: Δ6-protoilludene, pentalene, african-2-ene, african-3-ene, α-barbatene, δ-cadinene, <i>trans</i>-dauca-4(11),8-diene</p>	Antimicrobial, cytotoxic, anti-tumorous	<p><i>omp6/Δ6</i>-protoilludene synthase</p> <p><i>omp7/Δ6</i>-protoilludene synthase</p> <p><i>omp1/selective</i> α-muurolene synthase</p> <p><i>omp3/β</i>-elemene α-muurolene synthase</p> <p><i>omp4</i>; promiscuous δ-cadinene synthase</p> <p><i>omp5a</i>; δ-cadinol synthase</p> <p><i>omp5b</i>; δ-cadinol synthase</p> <p><i>omp9</i>; α-barbatene synthase</p> <p><i>omp10</i>; carotene synthase</p>	<p>Forms in the absence of Ca²⁺ exclusively Δ6-protoilludene in the biosynthetic pathway of illudins; promiscuous in the presence of Ca²⁺; Δ6-protoilludene, β-elemene^a, (<i>E</i>)-β-caryophyllene, 4,11-selinadiene, β-selinene, α-selinene</p> <p>Forms in the absence of Ca²⁺ mainly Δ6-protoilludene in the biosynthetic pathway of illudins, some pentalene; promiscuous in the presence of Ca²⁺; Δ6-protoilludene, β-elemene^a, (<i>E</i>)-β-caryophyllene, β-selinene, α-selinene</p> <p>Forms α-muurolene</p> <p>Forms β-elemene^a, α-muurolene (major product), selina-4,7-diene, δ-cadinene</p> <p>Forms primarily δ-cadinene and small amounts of 16 other sesquiterpenes</p> <p>Forms β-elemene^a, γ- cadinene, <i>epi</i>-zонаrene (minor product)</p> <p>Forms β-elemene^a, γ- cadinene</p> <p>Forms α-barbatene (major product), β-barbatene</p> <p>Forms daucene, <i>trans</i>-dauca-4(11),8-diene</p>	<p>McMorris and Anchel (1965), McMorris et al. (1971, 2000, 2002), Morisaki et al. (1985), Arnone et al. (1991), McCloud et al. (1996), Mayer et al. (1997), Engler et al. (1998), Schobert et al. (2008), Wawrzyn et al. (2012), Quin et al. (2013b, 2015), and Yang et al. (2016a)</p>

(continued)

Table 13.1 (continued)

Fungus	Compounds produced by fungus		Sesquiterpene synthase		Reference(s)
	Name	Bioactivities	Gene and enzyme	Enzymatic activities	
<i>Stereum hirsutum</i>	<p>δ-Cadinol, hirsutenes, hirsutenols, hirsutic acid C, sterins, sterhirtustin C and D, chlorosterone, complicatic acid</p> <p>From <i>Stereum</i> sp. strains: stereumins, sterpuranes, sterostreins, cadmanes, sterelactones</p> <p>Major volatile sesquiterpenes from culture: 1,11-cyclization products Δ6-protoilludene, α-humulene, hirsutene, pentalene; 1,10-cyclization products β-elemene, δ-cadinene; 1,6-cyclization product sesquisabinene A, (E)-β-caryophyllene</p>	<p>Anti-oxidative, nitric oxide inhibitory, antimicrobial, antiviral, immunosuppressive, cytotoxic, apoptosis inducing, autophagy inducing</p> <p>Nematicidal, antimicrobial</p>	<p>ID25180; Δ6-protoilludene synthase (1,11-cyclization)</p> <p>ID64702 and ID73029; Δ6-protoilludene synthases (1,11- and 1,10-cyclization)</p> <p>ID159379; β-barbatene synthase (1,6-cyclization)</p> <p>ID128017; δ-cadinene synthase (1,10- and 1,6-cyclization?)</p>	<p>Forms in the absence of Ca²⁺ exclusively Δ6-protoilludene; possibly involved in biosynthesis of sterostreins; promiscuous in the presence of Ca²⁺. Δ6-protoilludene, β-elemene^a, (E)-β-caryophyllene, β-selinene, α-selinene</p> <p>Forms Δ6-protoilludene (major product), β-elemene^a</p> <p>Forms β-barbatene (major product), α-barbatene, α-cuprene</p> <p>Forms δ-cadinene (major product; possibly involved in biosynthesis of stereumins), β-coparene, sativene, γ-muuroolene, α-muuroolene, β-cubebene, germacrene D, sesquisabinene A</p>	<p>Heatley et al. (1947), Ainsworth et al. (1990), Yun et al. (2002a, b), Yoo et al. (2005, 2006), Li et al. (2006, 2008, 2011a), Opatz et al. (2008), Liermann et al. (2010), Liu et al. (2010), Isaka et al. (2011), Quin et al. (2013a, 2015), Yang et al. (2013), Zheng et al. (2013), Ma et al. (2014), Qi et al. (2014, 2015), and Tian et al. (2016)</p>

<i>Taiwanofungus camphoratus</i>	Antrocin, antroquinonols as sesquiterpene derivatives, among various other sesquiterpenes in culture	Cytotoxic, apoptosis inducing, anti-tumorous	M_Fcontig14619; AcTPS1	Forms δ -cadinol	Geethangili and Tzeng (2011), Rao et al. (2011), Tsai et al. (2011, 2012), Xia et al. (2011), Yeh et al. (2013), Sulake and Chen (2015), Chui et al. (2016), and Lin et al. (2017)
	Labdane derivatives	Neuroprotective	M_Fcontig13346; AcTPS2	Forms acyclic α -farnesol	
			M_Fcontig26676; AcTPS3	Forms acyclic α -farnesol	
			M_Fcontig40411; AcTPS4	Forms α -cubebene, sibirene, γ -amorphene, γ -cadinene, zonarene, acyclic α -farnesol	
			M_Fcontig40579; AcTPS5	Forms α -cubebene, γ -muurolene, γ -amorphene, γ -cadinene, δ -cadinene, T-cadinol, α -cadinol, acyclic α -farnesol	
			M_Fcontig28068; AcTPS6	Forms nerolidol	
			M_Fcontig36944; AcTPS7	Forms α -cubebene, nerolidol, acyclic α -farnesol	
			M_Fcontig47706; AcTPS9	Forms α -cubebene, sibirene, γ -amorphene, cubebol, 1-epi-cubanol, acyclic α -farnesol	
			M_Fcontig62520; AcTPS10	Forms nerolidol, α -cadinol, acyclic α -farnesol	
			M_Fcontig44443; AcTPS11	Forms acyclic α -farnesol	

^aHeat-induced Cope-rearrangement product from germacrene A (Selzer 2008)

C. cinerea and replacing the loop of Cop4 by that of Cop6 changed product profiles of the enzymes, whereas mutations in the H- α 1 loop of Cop6 and introduction of the Cop4 H- α 1 loop into Cop6 had no effects on the product outcomes (Lopez-Gallego et al. 2010a, b). Single amino acid replacements in Cop2 outside the functional center (L59H, S310Y, T65A) improved the selectivity of the enzyme for product germacrene D-4-ol (Lauchli et al. 2014).

Phylogenetic analysis can help to predict specific reactions from sesquiterpene synthases obtained from genome mining. *Basidiomycota* sesquiterpene synthases split by overall sequence conservation into five main clusters which reflect different types of cyclization mechanisms (e.g., 1,6-cyclization; 1,10-cyclization; 1,11-cyclization) and make predictions for their expected types of products possible (Wawrzyn et al. 2012; Quin et al. 2013a; de Sena Filho et al. 2016; Tao et al. 2016).

The analysis on the diverged sesquiterpene biosyntheses in the fungi is however still in its infancy, with only a few cases reported where enzyme activities subsequent to the syntheses of sesquiterpene backbones were identified. The sesquiterpene synthase gene *Pro1* from *Armillaria gallica*, for example, forms Δ 6-protoilludene (Engels et al. 2011) to which after oxygenation reactions an orsellinic acid moiety is esterified to give antimicrobial and cytotoxic hybrid terpenoids termed melleolides (Bohnert et al. 2011, 2014; see also Sect. 13.3.4). The monocyclic polyketide orsellinic acid is produced by a seven-domain nonreducing polyketide synthase (NR-PKS) ArmB (Lackner et al. 2013; Sect. 13.3.4). The melleolide-biosynthetic gene cluster with *Pro1* has been deduced from the established genome of *A. mellea* which includes also the characterized gene *ArmB* for the orsellinic acid synthase and 14 other genes for putative cytochrome P450 monooxygenases, NAD⁺-dependent oxidoreductases, a flavin-dependent oxidoreductase, and an *O*-methyltransferase (Lackner et al. 2013; Wick et al. 2016). However, outside the gene cluster spread over four different genomic locations, there are additional genes for melleolide modification, *ArmH1* to *ArmH5* for FAD-dependent halogenases with a canonical signature sequence (FW[A/V]W[F/L]I). These five halogenases were all able to chlorinate melleolide F as the compound experimentally tested for such a post-PKS-biosynthetic step. In addition, bromination activity was demonstrated for ArmH4 as one selected enzyme (Misiek et al. 2011; Wick et al. 2016). In the satellite gene cluster for melleolide modification together with *armH1* and *armH2*, a gene *armA* is found as another characterized gene which encodes a tridomain enzyme for adenylation of primarily L-leucine and L-threonine. Although the gene is expressed under melleolide production conditions, any biochemical link to melleolide production is not obvious (Misiek et al. 2011).

Of the six identified sesquiterpene synthase genes in *C. cinerea* (Table 13.1), only the *cop6* gene appears to be part of a biosynthetic gene cluster. *cop6* is flanked by two genes for cytochrome P450 monooxygenases (*cox1*, *cox2* for α -cuprenene oxidases). The P450 enzyme Cox2 oxidizes the α -cuprenene produced by the highly specific enzyme Cop6 to α -cuparene and α -cuparophenol, while Cox1 oxidizes α -cuprenene to unknown hydroxy or ketone derivatives, respectively, and likely also α -cuparophenol into a keto-derivative (Agger et al. 2009). *C. cinerea* produces blue-stained antibiotic lagopodins (Bottom and Siehr 1975; Bu'Lock and Darbyshire

1976) which can principally be generated from α -cuprenene through multiple oxidations to which Cox1 and Cox2 likely contribute (Agger et al. 2009).

O. olearius has in total 11 genes for sesquiterpene synthases (Table 13.1). *omp1*, *omp6*, and *omp7* are located in distinct gene clusters. *omp6* is found in the largest cluster of about 25 kb with four P450 genes and 14 other genes for a selection of putative oxidoreductases, transferases, drug transporters, a GTPase1 anthranilate synthase-related enzyme, and a poly-galactonurase. *omp7* clusters with one P450 gene and one gene for a FAD-binding protein, and they originate possibly from a partial duplication of *omp6* cluster genes. *Omp6* and *Omp7* both produce $\Delta 6$ -protoilludene. *omp1* for a selective α -muurolene synthase groups with a single P450 gene and three genes for enzymes suggested to act in the modification of α -muurolene. α -Barbatane synthase *Omp9* falls into the same clade of enzymes as seven of the total 17 different sesquiterpene synthases of *F. pinicola* (Table 13.1). In this species, only enzyme *Fomp1* is encoded in a small gene cluster with two P450 genes (Wawrzyn et al. 2012). α - and β -Barbatane are products of *F. pinicola* (Rösecke et al. 2000). However, *Fomp1* is a highly specific α -cuprenene synthase similar to *Cop6* of *C. cinerea* (Wawrzyn et al. 2012).

Cluster predictions were also provided for *S. hirsutum*. Genes 159379 for β -barbatene synthase and 128017 for δ -cadinene synthase (Table 13.1) are located in distinct clusters together with genes for a range of oxidoreductases, but there is no P450 gene. δ -Cadinene is the likely precursor for the antibacterial stereumin. P450 monooxygenases required for stereumin synthesis may thus be provided from satellite gene clusters or individual genes still to be defined (Quin et al. 2013a). Notably, the *Agaricomycetes* commonly possess large armies of potential P450 genes (>100, e.g., Doddapaneni et al. 2005; Wawrzyn et al. 2012; Syed et al. 2014; Kües et al. 2015; Qhynya et al. 2015; Mgbeahuruike et al. 2017). Genes 25180, 64702, and 73029 for the three functional $\Delta 6$ -protoilludene synthases in *S. hirsutum* (Table 13.1) in contrast are all located in larger gene clusters together with genes for a variety of scaffold-modifying enzymes including for several P450 monooxygenases. Some of these are closely related to the P450 enzymes encoded in the *omp6* and *omp7* gene clusters of *O. olearius*, which suggests shared origins of these clusters of the different species. The cluster in *S. hirsutum* with gene 64702 contains also a gene for a transporter. Cytotoxic sterostreins could be products of the three *S. hirsutum* clusters with the functional $\Delta 6$ -protoilludene synthase genes (Quin et al. 2013a, b). Altogether, there appears to be a trend in the *Agaricomycetes* that genes for $\Delta 6$ -protoilludene synthases associate with biosynthetic gene clusters (Schmidt-Dannert 2016).

The newest whole genome analysis for sesquiterpene synthase genes is from *T. camphoratus* (Lin et al. 2017; Table 13.1). In this fungus, one or two P450 genes localize in the vicinity of the genes for the enzymes AcTPS1, AcTPS2, AcTPS3, and AcTPS6, the genes for enzymes AcTPS4 and AcTPS9 are linked, and one to a few genes for potential modification have been identified in the closer and wider genomic environments of all sesquiterpene genes. However, there are usually several additional genes for other unrelated or unknown functions, and genes from a shared chromosomal region appear to be not much in parallel regulated in mycelial

growth and during fruiting (Lin et al. 2017), shedding doubt on that many of these genes sit in true sesquiterpene biosynthetic gene clusters. In conclusion, a gene cluster situation for sesquiterpene synthesis is not necessarily the canonical situation in the *Agaricomycetes*.

13.3.2 The Pleuromutilin Gene Cluster

Pleuromutilin and its natural and semisynthetic derivatives are antibacterial tricyclic diterpenes (C₂₀ terpenoids) from *Clitopilus* species (Hartley et al. 2009) which block bacterial ribosome function by binding to the peptidyl transferase component of the larger ribosomal subunit (Lolk et al. 2008; Eyal et al. 2016). Some of these compounds are long appointed in veterinary medicine, and some are considered for human use (Paukner and Riedl 2017).

A gene cluster for the synthesis of pleuromutilin has recently been identified from the saprotrophic agaricomycete *Clitopilus passeckerianus* by homologous and heterologous gene expression studies (Bailey et al. 2016). Here, the authors made successful use of the repeated observation that fungal genes for enzymes of secondary metabolite biosynthesis pathways often come in clusters (Brakhage and Schroeckh 2011; Wiemann and Keller 2013; Yaegashi et al. 2014). They had further the biochemical expectation that a gene for a geranylgeranyl pyrophosphatase (GGPP) synthase (GGS; a diterpene synthase for GGPP production from FPP and IPP as a key step in the synthesis of terpenes) should be implicated in the pleuromutilin biosynthesis pathway. The authors designed degenerate primers to successfully screen a genomic λ phage library for GGS genes and identified by subsequent sequencing and expression studies the complete cluster with seven genes in total (Fig. 13.1). The authors did not provide the actual sequences of the genes and the encoded proteins (PI-P450-3, cytochrome P450; PI-ATF, acetyltransferase; PI-CYC, terpene cyclase; PI-GGS; PI-P450-1 and PI-P450-2, cytochrome P450s, PI-SDR, short-chain dehydrogenase/reductase), but a table supplied with the closest known homologues from other organisms (Bailey et al. 2016) allowed us to use these in genome mining of other mushrooms in order to find related gene clusters.

In pBlast searches of the *Agaricomycetes* entries in GenBank at NCBI, all proteins but the *Gibberella fujikuroi* terpene cyclase (*ent*-kaurene synthase; Q9UVY5) gave longer lists of significant hits in various fungal species. However, sequence evidence for potential CYC functions for a two-step cyclization of GGPP was only found in *P. strigosozonata*, *S. lacrymans*, *Gymnopus luxurians*, *Moniliophthora roreri* (partial sequences), and *Moniliophthora perniciosa* (partial sequences; see also Mondego et al. 2008; Fischer et al. 2015). The *cyc* genes in *P. strigosozonata* and *S. lacrymans* cluster with *ggs*, *p450*, and *sdr* genes which resembles the gene composition of the pleuromutilin biosynthesis cluster (Fig. 13.1), unlike the three potential *cyc* genes in *G. luxurians* which locate to fully different gene contexts. A similar analysis of the genomic environment is not yet possible for the two *Moniliophthora* species (synonyms *Crinipellis roreri* and *Crinipellis perniciosa*) by too short available scaffold lengths. However, these fungi synthesize gibberellin-like diterpenoid

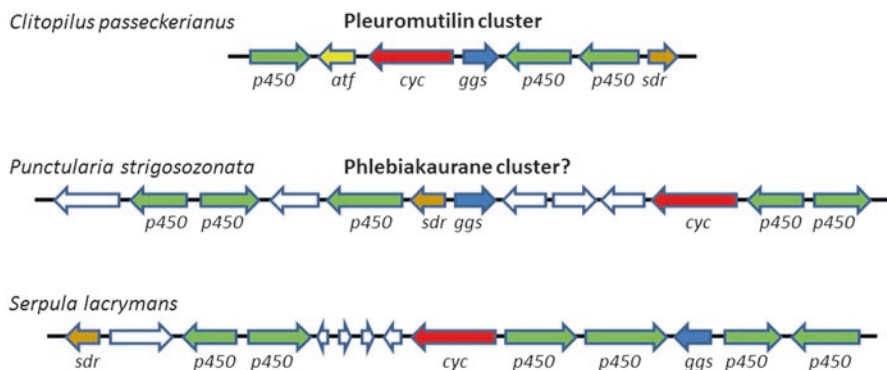


Fig. 13.1 Gene arrangements in the pleuromutilin cluster of *C. passeckerianus* (Bailey et al. 2016) and putative antibiotic biosynthesis gene clusters of *P. strigosozonata* and *S. lacrymans* S7.9 (as deduced from the MycoCosm page, April 2017). *Note:* A shared color indicates same types of gene functions, the arrows direction of transcriptions. *ggs* encodes geranylgeranyl pyrophosphate synthase, *cyc* terpene cyclase, *p450* cytochrome P450, *atf* acetyltransferase, *sdr* short-chain dehydrogenase/reductase; *white arrows*: other functions

acids which in analogy of gibberellin biosynthesis is expected to happen via *ent*-kaurene (Mondego et al. 2008). In the past, red and violet terphenyl quinone pigments (phlebiarubrones) and phlebiakauranes with antibacterial and selective antifungal activities have been reported from cultures of *P. strigosozonata* and *Punctularia atropurpurascens* (Lisy et al. 1975; Anke et al. 1984, 1987), and there is also a report on phlebiakaurane production in addition to crinipellins (tetraquinane diterpenoids) by a strain *Crinipellis* sp. 113 (Li and Shen 2010). Synthesis of kaurane diterpenes employs a terpene cyclase and P450 oxygenases and monooxygenases for post-kaurene modifications (Takahashi et al. 2014), and the identified *P. strigosozonata* gene cluster might thus be responsible for phlebiakaurane production. The *S. lacrymans* CYC is reported to give rise to the tricyclic diterpene *ent*-kauranol, differentially from the *C. passeckerianus* enzyme which forms the unique tricyclic pleuromutilin core with a C8 ring from GGPP (Proteau et al. 2012). We are however not aware of any kaurane diterpenes described from *S. lacrymans*.

Production of kaurane diterpenes is generally rare in basidiomycetes (Anke et al. 1987; Shen et al. 2009; Yang et al. 2012). An interesting further observation is thus that the GGSs from the putative antibiotic clusters of *P. strigosozonata* and *S. lacrymans* (and also *C. passeckerianus*?) are of the same origin than a larger group of ascomycetous enzymes, unlike the other identified basidiomycetous GGSs coming from *G. luxurians*. An origin from horizontal gene transfer is therefore suggested for the clusters (Fischer et al. 2015). Some *Chytridiomycota*, *Mucoromycota*, and insect GGSs appear also to be closely related (our unpublished observations). A further analysis of the evolutionary origin of the GGSs and the whole gene clusters could thus be very interesting.

13.3.3 Ganoderic Acid

Ganoderma species are especially rich in secondary metabolites (Peterson 2006; Sanodoya et al. 2009; Batra et al. 2013; Xia et al. 2014; Baby et al. 2015; Duru and Çayan 2015; Richter et al. 2015). From the multitude of bioactive triterpenoids in *Ganoderma* species (>150 different triterpenoids have been isolated at different fungal developmental stages; Chen et al. 2012; Duru and Çayan 2015), ganoderic acids and derivatives are best known as highly oxygenated lanostane-type triterpenoids (C30 terpenoids) with potent anticancer activities (Cheng et al. 2010; Shi et al. 2010; Xu et al. 2010; De Silva et al. 2013; Wu et al. 2013), inhibition effects on cholesterol synthesis (Komoda et al. 1989; Hajjaj et al. 2005), and antioxidant, antidiabetic, hepatoprotective, and other activities (Zhu et al. 1999; Shi et al. 2010; Peng et al. 2013; Liu et al. 2015b; Tang et al. 2016c).

As for other terpenoids, ganoderic acid synthesis takes its origin in the mevalonate pathway, via FPP, squalene, and lanosterol as intermediates (Shiao 2003; Shi et al. 2010; Chen et al. 2012). Thirteen genes for the 11 enzymes (for two enzymes there are two genes in *G. lucidum*) of the mevalonate pathway up to lanosterol cyclization by the 2,3-oxidosqualene-lanosterol cyclase (lanosterol synthase) are known (Chen et al. 2012; Liu et al. 2012a; Kües et al. 2015; Zhu et al. 2015). Genetic engineering to overexpress various cloned genes from the upstream mevalonate pathway successfully leads to improved ganoderic acid synthesis (Shi et al. 2010; Xu et al. 2012, 2015; Ren et al. 2013b; Zhou et al. 2014; Xu and Zhong 2015; Zhang et al. 2017), as did introduction of heterologous chaperones as protectants against accompanied oxidative stress (Li et al. 2016a).

Lanosterol is at a branch point of sterol and terpenoid biosynthesis. The fungal-unique essential membrane organizer ergosterol is produced from lanosterol by actions of the P450 enzyme lanosterol 14 α -demethylase (CYP51A; lanosterol synthase) (Lepesheva and Waterman 2008; Shi et al. 2010; Chen et al. 2012; Liu et al. 2012a). A series of oxidations, reductions, and acylation reactions are expected to lead alternatively from lanosterol to the different forms of ganoderic acids (Chen et al. 2012). However, genes for biochemical reactions after lanosterol cyclization are not yet been clearly identified. P450 enzymes and also UDP-glucosyltransferases (with six identified genes for potential transfer of sugar moieties to triterpenoid backbones; Liu et al. 2012a) are discussed to act as candidate enzymes in later steps of ganoderic acid synthesis. Compared to any other fungi (ascomycetes and basidiomycetes), *Ganoderma* species have an exceptional high number of genes for oxidative and hydroxylating P450 enzymes containing heme cofactors (cytochromes P450, CYPs), i.e., nearly 200 and more different functional ones with an additional number of pseudogenes (Chen et al. 2012; Liu et al. 2012a; Syed et al. 2014; Zhu et al. 2015). The P450 proteins cluster into 42 different families. Some have only a single member in *Ganoderma* sp. and others multiple members, some are presented with new subfamilies, and some of the families are novel and unique to *Ganoderma* sp. (Chen et al. 2012; Liu et al. 2012a; Kües et al. 2015). The plenty of *p450* genes makes it difficult to predict their individual enzymatic substrate and product specificities. Expression of *p450* genes is variably differentially regulated, with

subgroups (e.g., 15 *CYP512* and one *CYP5144* gene) which correlate in regulation with the lanosterol synthase gene (Chen et al. 2012). A total of 24 gene clusters with three or more different *CYP* genes (three larger clusters longer than 200 kb might split into two or more different biosynthetic gene clusters) were identified in the genome of *G. lucidum* (Chen et al. 2012; Liu et al. 2012a; Kües et al. 2015). Two of these gene clusters show some parallels to the expression of lanosterol synthase, whereas ten other *p450* genes linked to the lanosterol synthase gene do not (Chen et al. 2012). *G. sinense* distinguishes in the number of gene clusters from *G. lucidum* with 29 in total identified gene clusters. Further, the number of *p450* genes in shared gene clusters can vary between the species. Many of the *p450* genes appear to originate from gene duplications, with options of neofunctionalization, subfunctionalization, and increased gene-dosage advantage (Zhu et al. 2015). Because reliable transformation methods and gene silencing techniques for *G. lucidum* are at hand (Mu et al. 2012; Xu and Zhong 2015), it should be possible with time to address the important *p450* genes in ganoderic acid production. Comparison of the P450omes of different species can possibly give some insight into which candidate genes to select for further study (Lu et al. 2014).

13.3.4 Polyketide Synthases

Prenylphenols are hybrid molecules of the monocyclic polyketide orsellinic acid and a prenyl side chain of terpene origin. A steraceous basidiomycete BY1 produces the novel prenylphenol cloquetin. The fungus has a gene cluster with two functional genes for nonreducing polyketide synthases, *PKS1* and *PKS2*, for the synthesis of orsellinic acid and an unlinked gene *BYBP* for a regiospecific prenyltransferase that attaches a prenyl group to orsellinic acid (Braesel et al. 2017). *S. hirsutum* possesses a highly similar gene (69% protein identity to *BYBP*; XP_007308836; Floudas et al. 2012) and is as other *Stereum* species also a prenylphenol producer (Omolo et al. 2002; Yun et al. 2002b; Braesel et al. 2017). Other mushrooms (e.g., *A. bisporus*, XP_007334149; *A. muscaria*, KIL64777; *A. subglabra* XP_007349181; *C. cinerea*, XP_001839609, XP_001839607; *G. frondosa*, OBZ66374; *G. luxurians*, KIK57132, KIK57133; *P. strigosozonata*, XP_007385948; *S. commune*, XP_003029237; *S. lacrymans*, XP_007322388, XP_007323929) have genes for less similar, yet uncharacterized prenyltransferases (around 25–40 % identity to *BYBP*). In general, prenyltransferases catalyze regiospecific and stereoselective prenylations of aromatic compounds such as tryptophan, tryptophan-containing peptides, indole derivatives, tyrosine, and also nitrogen-free aromates. Among others, FPP synthases and GGPP synthases producing the precursors for sesquiterpene and triterpene synthesis, respectively (Sects. 13.3.1 and 13.3.2), are prenyltransferases (Winkelblech et al. 2015).

Other than *PKS1* and *PKS2* of strain BY1, only a few other genes for polyketide synthases (PKSs) have been characterized in the *Basidiomycota*, i.e., for the production of orsellinic acid in *T. camphoratus* (Yu et al. 2016; Chou et al. 2017), *A. mellea* (Lackner et al. 2012, 2013; ArmB; see Sect. 13.3.1 above) and *C. cinerea* (Ishiuchi

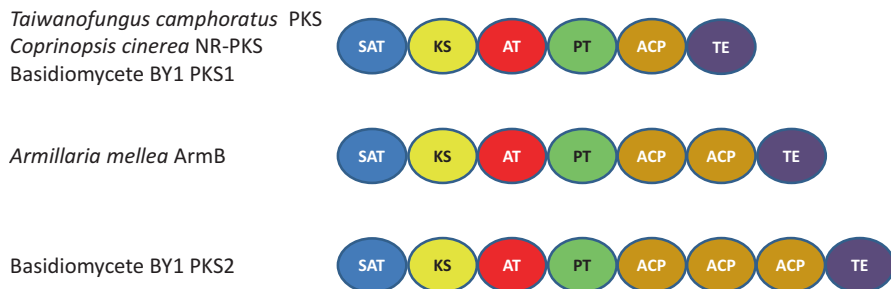


Fig. 13.2 Domain structures of nonreducing polyketide synthases for the monocyclic polyketide orsellinic acid production from the *Agaricomycetes* (Lackner et al. 2012, 2013; Yu et al. 2016; Braesel et al. 2017) and of the highly reducing polyketide synthases PPS1 and PPS2 for polyene production of the basidiomycete BY1 (Brandt et al. 2017). *Note:* SAT starter unit acyl carrier protein transacylase, KS β -ketosynthase, AT acyl (malonyl-CoA) transferase, PT product template domain, ACP acyl carrier protein, transacylase, TE thioesterase, DH dehydratase, KR β -ketoreductase, MT methyltransferase. Note that orsellinic acid synthases from different fungi differ in numbers of ACP domains

et al. 2012). These enzymes have multi-domain structures as typical for fungal type I nonreducing iterative polyketide synthases (NR-PKSs; Fig. 13.2), distinct from modular highly reducing polyketide synthases (HR-PKSs) of clade I (Fig. 13.2) which have additional other domains, from highly reducing polyketide synthases of class II and from hybrid clades I, II, and III polyketide synthases, respectively (Lackner et al. 2012; Du and Lou 2010; Liu et al. 2015a). Basidiomycetous NR-PKSs of class I are monophyletic and divide into two major subclasses (Lackner et al. 2013). Genes for similar modular enzymes as PKS1 and PKS2 from strain BY1 are, for example, present in *A. muscaria* (KIL57002), *F. hepatica* (KIY47273), *G. frondosa* (BAO20284), *G. luxurians* (KIK51473, KIK52832), *Hebeloma cylindrosporium* (KIM41076), *P. strigosozonata* (P_007386370), *S. commune* (XP_003038401), *S. lacrymans* (EGN93845), *S. hirsutum* (XP_007307184, XP_007300189; Floudas et al. 2012), *S. luteus* (KIK37237), and *T. versicolor* (XP_008041566). Of these, orsellinic acid metabolites are known from *Stereum* species (Li et al. 2006; Braesel et al. 2017).

Orsellinic acid was further detected in co-culture of *Ganoderma applanatum* and *T. versicolor*. The authors put the production onto *G. applanatum* (Yao et al. 2016), while a respective conserved polyketide synthase gene can also be detected in *T. versicolor* (Koczyk et al. 2015; XP_008041566). The enzyme from gene *pks63787* in *T. camphoratus* is responsible for the synthesis of orsellinic acid from acetyl-CoA and malonyl-CoA. Orsellinic acid is then in parallel routes farnesylated by different CoQ (coenzyme Q)-type synthesizing enzymes (i.e., Coq2, Coq3, and Coq6 which were identified from a pool of total 10 different *coq* genes) to give with additional ring modifications the benzoquinone ring precursors for a variety of antroquinonols with anticancer activities and for the cytotoxic meroterpene 4-acetylanthroquinonol B (Yu et al. 2016; Chou et al. 2017).

4-Acetylanthroquinol B production is intimately linked to CoQ synthesis. Production of 4-acetylanthroquinol B can be enhanced by feeding either CoQ or the CoQ precursor 4-hydroxybenzoic acid from the shikimate pathway and by the addition of oleic acids to enhance the mevalonate pathway (Yang et al. 2017).

Most recently, the first modular HR-PKSs have been characterized with the 2736 amino acid long polyene pigment synthases PPS1 and PPS2 of basidiomycete BY1 (Fig. 13.2) from an own clade of fungal PKSs. These enzymes are 99% identical in sequence, likely allelic, and the only HR-PKSs found to be encoded in the host genome. Their genes do not reside in a recognizable gene cluster (Brandt et al. 2017). Genes for enzymes of similar modular structure occur in *M. roreri* and *S. hirsutum* (Brandt et al. 2017), *Fibroporia radiculosa* (XP_012183873, XP_012185398, XP_012183840), *Gloeophyllum trabeum* (XP_007869847), *Jaapia argillacea* (KDQ50123), and *L. sulphureus* (KZT06902, KZT06920). PPS1 was expressed in *Aspergillus niger* and shown to confer production of yellow nematoxic polyene pigments (18-methyl-19-oxoicosaoctanoic acid, 20-methyl-21-oxodocosanoic acid) with an unusual shifted conjugated C-C double-bond pattern (Brandt et al. 2017).

Another striking observation from genome mining is that false reports in the literature on functional compounds from mushrooms can be wiped out through genome mining. Together with highlighting misleading flaws in analytical methods used in reports of flavonoid productions of mushrooms, Gil-Ramírez et al. (2016) showed in such manner that mushrooms might have genes for phenylalanine ammonia lyases (e.g., *A. bisporus*, XP_006461241; *P. ostreatus*, KDQ28180) but that they do not have like plants any genes for chalcone synthases and for chalcone isomerases required for flavone synthesis. Distantly related to plant chalcone synthase genes (around 30% amino acid identities between gene products) are exceptional genes (1 or 2 pro genome) for fungal type III polyketide synthases in the mushrooms *Calocera cornea* (KZT59672.1), *Calocera viscosa* (KZO96206), *Dacryopinax primogenitus* (EJT98013), *Exidia glandulosa* (KZW02173), *Phanerochaete carnosia* (XP_007391992, XP_007391993), *Phlebia centrifuga* (OKY58712, OKY58713), and *Sporotrichum laxum* (AMW87979, AMW87980).

Gene knockouts and heterologous expression in *E. coli* proved that the *S. laxum* Pks2 (AMW87980) is an alkylresorcinol synthase which elongates palmitoyl-CoA or palmitoyl-ACP by three malonyl-CoAs and cyclizes the product via a 2,11 intramolecular aldol condensation, with fatty acid acyl-primed triketide and tetraketide pyrenes as by-products (Sun et al. 2016). The alkylresorcinols are precursors of spiro-laxine (Sun et al. 2016) which acts as plant growth inhibitor (Arnone et al. 1990), antibacterial (Blaser 1992), cholesterol-lowering (Robinson and Brimble 2007), and inhibitory toward cancer cell lines (Tsukamoto et al. 1998; Gianni et al. 2004). Antibacterial analogs are known from *Phanerochaete velutina* (Dekker et al. 1997). The two *pks* genes in *S. laxum*, *P. carnosia*, and *P. centrifuga* are tail-to-tail arranged and locate in a cluster with a gene for a membrane-bound *O*-acyltransferase (MBOAT) family protein (Sun et al. 2016; XP_007391994 and OKY58711). However, genes for other enzymes expected to participate in the biosynthesis of spiro-laxine, and analogs are not present in the cluster (Wang et al. 2013; Sun et al. 2016).

13.3.5 The Atromentin Gene Cluster

Atromentin is an NRSP (non-ribosomal peptide synthetase)-like derived terphenyl-quinone with antibacterial activity and apoptosis-inducing activity in leukemia cell lines (Zeng et al. 2006; Hee and Lee 2009). Atromentin is a central precursor of variegatic acid and various related pigments, for example, for diarylcyclopentenone pigments in *Paxillus involutus* (Braesel et al. 2015) and pulvinic acid-derived pigments in *Suillus* species (Wackler et al. 2012). Variegatic acid and the diarylcyclopentenone pigment involutin have roles in redox cycling during Fenton chemistry in the breakdown of lignocellulosic organic matter (Eastwood et al. 2011; Braesel et al. 2015; Shah et al. 2015).

The synthesis pathway of atromentin has first been elucidated in *Tapinella panuoides*. Atromentin derives from L-tyrosine which is deaminated by the pyridoxal 5'-phosphate-dependent L-tyrosine:2-oxoglutarate aminotransferase AtrD into 4-hydroxyphenylpyruvic acid. Two 4-hydroxyphenylpyruvic acid molecules are then adenylated by the NRSP-like quinone synthetase AtrA (a tridomain enzyme reminiscent to non-ribosomal peptide synthetases, NRPS; see Sect. 13.4.5), ester-bonded to the enzyme, and finally condensed through symmetric C-C bond formation into atromentin. Genes *atrA* and *atrD* are clustered with an *adh* gene (for alcohol dehydrogenase) in between (Schneider et al. 2008). The same genetic arrangement is found in several other basidiomycetes including in *O. olearius*, *Paxillus* species, *S. lacrymans*, and *Suillus* species, and the gene promoters have specific conserved sequence motifs with a potential regulatory function (Wackler et al. 2012; Braesel et al. 2015; Tauber et al. 2016). Confrontation with soil bacteria can elucidate expression of these genes (Tauber et al. 2016).

13.3.6 Enzymes for Bioactive Metabolites Evolved from Conventional Fungal Functions

Clavric acid is a triterpenoid (C₃₀H₄₈) from *H. sublateritium* with an antitumor activity which reversibly inhibits farnesyltransferase activities competitive to Ras (Jayasuriya et al. 1998; Lingham et al. 1998). It is synthesized from squalene alternatively to lanosterol, an essential intermediate in the typical fungal ergosterol pathway. Synthesis occurs in four steps via 2,3-oxidosqualene, squalene, 2,2:22,23-dioxidosqualene, and clavarinone. Steps 1 and probably also 2 are performed by the ERG1 squalene epoxidase, and step 1 is shared with lanosterol production (compare Sect. 13.3.3). Step 3 is performed by the specific oxidosqualene cyclase OCC (Godio and Martin 2009) and step 4 presumably by a clavric acid synthase. Genes *erg1* and *occ* gene with their encoded products are known. These are unlinked. OCC distinguishes from the lanosterol cyclases by a VSDCVGE motif in its active center (Godio et al. 2007; Godio and Martin 2009). As the only other related enzyme in the NCBI database in April 2017, KIM38979 of *H. cylindrosporum* has the same motif.

13.3.7 Others

The FAD-binding monooxygenase and aromatic ring hydrolase VibMO1 of *Boreostereum vibrans* were identified to convert prenyl 4-hydrobenzoate into prenylhydroquinone, likely as one step in the biosynthesis of the unusual fused β -lactone-type metabolites vibrallactones and related meroterpenoids (Yang et al. 2016c). Vibrallactones can act as lipase inhibitors (Liu et al. 2006) and can have antifungal activities (Schwenk et al. 2016). The gene for the protein closest and related to VibMO1 is from *S. hirsutum* (XP_007305833: 84% identical, 98% similar). Genes for FAD-binding monooxygenases are not uncommon in *Agaricomycetes*. Identity and similarity values for FAD-binding monooxygenases from other mushrooms range around 40% and 60%, respectively. Prenyl hydroquinone is a secondary metabolite also known from *Ganoderma* species (Baby et al. 2015).

A gene *dodA* for a 4,5-DOPA (3,4-dihydroxyphenylalanine) dioxygenase in *A. muscaria* is long known from biosynthesis of the yellow betalain-related pigment muscaflavin with a 7-membered nitrogen heterocycle. DodA opens DOPA either by 4,5-ring cleavage or by an extra 2,3-extradiol cleavage activity to give unstable *sec*-DOPA intermediates which spontaneously recyclize to betalamic acid and muscaflavin, respectively (Hinz et al. 1997; Mueller et al. 1997). DOPA is derived from tyrosine through oxidation by a tyrosinase (a monophenol monooxygenase) restricted to colored mushroom tissues (Mueller et al. 1996). *A. muscaria* has two tyrosinase genes (KIL63081; KIL63082) found in tandem at another chromosomal position than gene *dodA* (our unpublished observation). Betalain pigments are better known from the suborder *Chenopodinae* of the plant order *Caryophyllales* and are of interest for their antioxidant activities and as food colorants. Muscaflavin is not formed in plants because their enzymes lack significant 2,3-ring cleavage activity (Gandía-Herrero and García-Carmona 2013; Slimen et al. 2017). In the fungi, betalains occur distinctively in species of the three genera *Amanita*, *Hygrocybe*, and *Hygrophorus* (Steglich and Strack 1991; Stintzing and Schliemann 2007; Babos et al. 2011). Many other of the *Agaricomycetes* have also genes for tyrosinases, e.g., DOPA production (Halaouli et al. 2006), and importantly, also genes for potential 4,5-DOPA dioxygenases highly identical in sequence to *A. muscaria* DodA (between 60 and 80% identity and >70 to >90% similarity; e.g., *A. bisporus* XP_007326059: 68% identical, 85% similar). Whether these potential 4,5-DOPA dioxygenases promote 2,3-extradiol cleavage of DOPA or whether they are more stringent in their activities like the plant enzymes remains to be tested.

13.4 Peptides: Linear and Cyclic, Ribosomal and Non-ribosomal

13.4.1 Ribosomal Cyclic Peptides of the α -Amanitin and Phalloidin Family

Amatoxins and phallotoxins bind to RNA polymerase II and F-actin, respectively (Wieland 1986; Vetter 1998; Göransson et al. 2012). This group of toxins occur in a range of toxic *Amanita*, *Conocybe*, *Galerina*, and *Lepiota* species, with α -amanitin



Fig. 13.3 Precursor proteins for the bicyclic toxins α -amanitin and phalloidin from the MSDIN family of RiPPs (Pulman et al. 2016). *Note:* The conserved leader and follower regions are shown in different shading to the interior core sequences of the toxins. The bows mark amino acids involved in macrocyclization and Trp-Cys cross-bridging, respectively

as best known example (Pomilio et al. 2006; Li et al. 2014a, b; Sgambelluri et al. 2014; Pulman et al. 2016; Tang et al. 2016b). They are bicyclic peptides generated from ribosomally produced short precursor peptides (between 33 and 39 amino acids long; Pulman et al. 2016) by the action of a specialized prolyl oligopeptidase B (POPB) of the serine protease family S9a (Luo et al. 2009, 2010, 2014). The precursors are characterized by a conserved 10-aa leader region and a conserved 17-aa follower region with an internal core of different length (between 6 and 10 aa) and sequence which represents the mature cyclopeptide (Fig. 13.3). The conserved sequence of the leader region gave this family of RiPPs (ribosomally synthesized and posttranslationally modified peptides) the name MSDIN family of cyclic peptides (Hallen et al. 2007; Pulman et al. 2016), while the primary sequences outside the core region from *Amanita* species and *Galerina marginata* diverged, and the latter species has only two α -amanitin genes (Luo et al. 2012, 2014; Pulman et al. 2016). Two conserved prolines, one terminal to the conserved leader and one terminal to the core sequence, are required for proteolytic processing (Pulman et al. 2016; Fig. 13.3).

Toxic *Amanita* species have several MSDIN genes (*A. phalloides* and *A. bisporigera* each ~30 with little overlap between the species) mostly for toxins or in some cases also nontoxic cyclopeptides (cycloamanides), but mature products are only found from genes whose predicted precursors have the two conserved prolines (Pulman et al. 2016; Fig. 13.3). The peptide chains are processed and macrocyclized to yield a backbone macrolactam by head-to-tail peptide bonds through peptide bond hydrolysis and transpeptidation by POPB (Luo et al. 2009, 2010; Truman 2016) and further by an unusual internal Trp-Cys cross-bridge via a sulfoxide or a sulfide link for amanitins and phalloidins, respectively (Battista et al. 2000; Göransson et al. 2012). The bicyclic toxins can further diversify by hydroxylations of side chains of their amino acids (Sgambelluri et al. 2014). The *ppob* gene clusters with two *ama* genes for α -amanitin in the *G. marginata* genome and on a genomic cosmid from an *Amanita* species (likely not *A. bisporigera*) with a gene for an MSDIN family cyclic peptide with the sequence GAYPPCPMP (Luo et al. 2010; Pulman et al. 2016).

Another member of the MSDIN family of cyclic peptides is antamanide from *A. phalloides* which is a monocyclic decapeptide (cyclic sequence = cVPPAFFPPFF) with a strong antidote activity against amatoxins and phallotoxins. Moreover, it

shows antitumor and immunosuppressive activities and inhibits the mitochondrial permeability transition pore as an effector of cell death induction through binding of the pore regulator cyclophilin D (Siemion et al. 1992; Azzolin et al. 2011). Immunosuppressive activity has also been reported for *A. phalloides* cycloamanide A (VFFAGP) and B (SFFFPIP) (Wieczorek et al. 1993). Less studied are the monocyclic virotoxins (cVTSPAW) from *A. phalloides* and *Amanita virosa* which bind to actin similar as the phallotoxins (Faulstich et al. 1980; Vetter 1998; Hossain and Park 2016).

13.4.2 The Omphalotin Gene Cluster

Omphalotins are cyclic *N*-methylated depsipeptides (WVIVVGVIGVIG) with nematocidal activity from *O. olearius* (Mayer et al. 1997; Sterner et al. 1997). These are members of a new (fourth) class of fungal RiPPs and are produced by a suicidal enzyme OphA. This 399 amino acid-long enzyme has an N-terminal SAM-dependent methyltransferase domain with which it methylates iteratively the amino acids in the depsipeptide motif located at its C-terminus (Fig. 13.3). Methylation occurs intermolecularly. The biochemical process of macrocyclization of the *N*-methylated omphalotins out of their larger protein precursor and the responsible endopeptidase remain to be uncovered. A candidate prolyl oligopeptidase is encoded by gene *ophp* in a gene cluster together with *ophA* and genes *ophB1* and *ophB2* (monooxygenases), *ophC* (NTF2-like), *ophD* (*O*-acyltransferase), and *ophE* (F-box/RNI-like) for putative posttranslational modification functions of the omphalotin core peptide (van der Velden et al. 2017). Posttranslational modifications of the tryptophan residue (to a hexahydropyrrolo[2,3-*b*]indole 2-carboxamide) in the omphalotin core peptide are reported as well as oxidations of other residues (Buchel et al. 1998; Liermann et al. 2009; Ruiz-Sanchez et al. 2011). Importantly, it is possible to exchange amino acids in the depsipeptide motif and its border region without loss of methylation function indicating flexibility in the process (van der Velden et al. 2017).

A related enzyme to OphA with an autocatalytic *N*-methylation function of a similar C-terminal core peptide (Fig. 13.4) has been found in the basidiomycete *Dendrothele bispora* (van der Velden et al. 2017). NCBI pBlast searches with the *D. bispora* protein detect one or more candidate enzymes from a few other agaricomycetes for similar reactions (see selected examples in Fig. 13.3) which could offer potential for further natural cyclic peptides of the new class of RiPPs with the proposed name borosins (van der Velden et al. 2017). Notably, for further attention, a dioxygenase domain with aromatic-ring-cleavage activity is predicted for some of the well-conserved enzymes in between the N-terminal SAM-dependent methyltransferase domain and the C-terminus (around amino acids 315–375). Borosin candidates might also be certain unprecedented highly *N*-methylated cyclopeptides from other agaricomycetes such as the gymnopeptides from *Gymnopus fusipes* and the pteratides from *Pterula* sp. which have antiproliferative activities on human cancer cell lines (Chen et al. 2006; Ványolós et al. 2016).

<i>Omphalotus olearius</i> (van der Velden et al. 2017)	GFPWVIVVGVIGVIGSVMSTE
<i>Dendrothele bispora</i> (ID 765759)	GFPWVIVTGIVGVIGSVVSSA
<i>Lentinula edodes</i> (GAW09067)	GFPWIIIVGVVGVVGSVVSSA
<i>Rhizopogon vinicolor</i> (OAX31299)	GFPTVLVILPTVIVVLLIGRE
<i>Moniliophthora roreri</i> (XP_007857024.1)	GKPTAFLSAVVIATIIIAL
<i>M. roreri</i> (KTB34288)	GKPTAFVGLVVI IAVVV
<i>Moniliophthora perniciosa</i> (EEB90231)	GKPVAFLSAVVIATIIIAL
<i>Fomitiporia mediterranea</i> (EJD06538)	GTPHPALALLVVIICLI
<i>Hydnomerulius pinastri</i> (KIJ64101)	NEPAALTTMINIHVTHV
<i>R. vinicolor</i> (OAX33483)	NGPQGLGTIIILVWHTVHGIA
<i>R. vinicolor</i> (OAX39229)	DGPEGLAVVVIVLVATVALLALLV

Fig. 13.4 The C-terminal ends with short sequences for (putative) cyclopeptides (borosins) from a family of some (potentially) autocatalytic SAM-dependent methyltransferases from different agaricomycetes. *Note:* The known sequence of the omphalotins is shaded in gray. JGI and NCBI accession numbers are added where known

13.4.3 More Potential Cyclic RiPPS

Gene clusters for the synthesis of other fungal cyclic RiPPs (e.g., ustiloxins and phomopsins, antimetabolic cyclic mycotoxins which inhibit microtubule assembly and may be of interest for antitumor drug development; Koiso et al. 1994; Cormier et al. 2008) and functions of first genes in these clusters have recently been described in filamentous ascomycetes (Umemura et al. 2014; Tsukui et al. 2015; Ding et al. 2016; Nagano et al. 2016; Ye et al. 2016). Among genes for regulation, transport, and types of posttranslational modifications, these clusters include the genes for protein precursors with repeated peptide motifs and genes for DUF3328 proteins which represent a novel class of oxidases with function in peptide cyclization (Ding et al. 2016; Nagano et al. 2016; Ye et al. 2016). Genes for DUF3328 oxidases (family oxidase ustYa) were found in many ascomycetes and also in a subset of *Agaricomycotina*, often near candidate genes for protein precursors with repeated peptide motifs (Ding et al. 2016; Nagano et al. 2016). Two examples of putative precursors of novel cyclic RiPPs from the *Agaricomycetes* identified via genome mining by DUF3328 sequences are shown in Fig. 13.5. The cyclized products of such precursors were termed dikaritins after the fungal subkingdom of *Dikarya* (Ding et al. 2016).

13.4.4 Linear Peptides

Linear oligopeptides with cytotoxic activities against cell lines [pterulamides I–VI, highly *N*-methylated pentapeptides consisting of modified valine, isoleucine, and alanine residues (sequences: AIVVL; VVVVI) from *Pterula* sp. mushrooms; Lang et al. 2006] were reported, and several inhibitors with variable short sequences (between 3 and 17 amino acids) of specific enzymatic activities (angiotensin I-converting enzyme) with antihypertensive effects and anti-oxidative actions from

<i>Aspergillus flavus</i> Ustiloxin (XP_002381318)	<i>Phomopsis leptostromiformis</i> Phomopsin (AMR44282)	<i>Leatiporus sulphureus</i> KZT04758	<i>Galerina marginata</i> KDR68284
MKLLITLLVSLGICALAAPAAKR	MRFTPAIVIAAFCSLAVAPAA	MKFFVALSMKLIALAPLVALAVHAL	MQISRVFLALLPLTISVAI
DGVEDYAIGIDKGR	KAIARSP	PTSGTRGPTLKR	PLESAKAGSAPSRVIRE
NSVEDYAIGIDKGR	SEAVEDYVIFIDKGR	GDLVDDTPEIYKGR	ELEGPEVGGIKFEYKGR
NSVEDYAIGIDKGR	GEAVEDYVIFIDKGR	EGLVDDTPEIYKGR	EIGSEEDGIKFEYKGR
NSVEDYAIGIDKGR	GEAVEDYVIFIDKGR	EGLVDDTPEVYKGR	EIGSSEEDGIKFEYKGR
NSVEDYAIGIDKGR	GEAVEDYVIFIDKGR	GDLVDDTPEFVYKGR	EIGNEKEDGIKFEYKGR
NTVEDYAIGIDKGR	GEAVEDYVIFIDKGR	EDLTDDTPEIYKGR	EIGSEEDGIKFEYKGR
NSVEDYAIGIDKGR	GEAVEDYVIFIDKGR	EGLVDDTPEIYKGR	EEDGIKFEYKGR
NSVEDYAIGIDKGR	GEAVEDYVIFIDKGR	ATKR	EIGTEEDGIKFEYKGR
NSVEDYAIGIDKGR	GEAVEDYVIFIDKGR	EDLIDDTPEIYRR	EIGSEEDGNFVAFG
NSVEDYAIGIDKGR		SDLVDDTPEIYRR	GNALKLTLTTS
GGSVEDYAIGIDKGR		DDLVDTPSPVYKGR	
NSVEDYAIGIDKGR		GDLVDDTPEVYKGR	
NSVEDYAIGIDKGR			
GGSVEDYAIGIDKGR			
GTVEDYAIGIDKGR			
GGSVEDYAIGIDKGR			
HGGH			

Fig. 13.5 Precursor sequences for cyclic mycotoxins (dikaritins) from the *Ascomycota* and newly reported precursor molecules for putative ribosomally synthesized cyclic peptides from two mushrooms. *Note:* The (predicted) core sequence for peptide cyclization are shaded in *gray* (Tsukui et al. 2015; Ding et al. 2016) and dipeptides for potential proteolytic processing by KEX proteases are given in *bold*

different mushrooms were described (Kang et al. 2013; Geng et al. 2016). Their origins (ribosomal or non-ribosomal) are currently unclear.

13.4.5 Non-ribosomal Peptides

Non-ribosomal peptide synthetases (NRPSs) are multifunctional enzymes with multiple core domains (Fig. 13.6) which resemble polyketide synthases by a modular multi-domain structure (Du and Lou 2010; Wang et al. 2015; compare Fig. 13.2). NRPSs assemble their products (non-ribosomal peptides, NRPs) progressively while moving the growing peptide chain directionally along the protein template. A typical single NRPS elongation module therefore includes all reactive domains required for incorporation of an element to the chain (at minimum adenylation domain A with a non-ribosomal specific code for selection and activation of a monomeric substrate, thiolation domain T to hold the activated substrate, and condensation domain C to catalyze amide bond formation between adjacent T-domain bonded substrates). An NRPS can have a single A-T-C module (monomodular) or several (multimodular). An initiation module used to initiate the peptide synthesis misses the condensation domain. However, NRPS can be very diverse in a number of domains and by the inclusion of various other types of domains for product modifications (Finking and Marahiel 2004; Du and Lou 2010; Kalb et al. 2013; Singh et al. 2017; (Fig. 13.6).

A first NRPS gene cloned and also the first gene cluster detected in a basidiomycete were *fsoI* and the ferrichrome A siderophore gene cluster of *O. leariius* with the additional genes *atoI* and *omoI*, respectively (Welzel et al. 2005). Ferrichrome A is made up of a glycine, two serines, and three *trans*-(α -methyl)-glutaconic acid-acylated N^5 -hydroxyornithine amino acid residues (Zalkin et al. 1966). The synthesis occurs in at least three steps: hydroxylation of L-ornithine by an



Fig. 13.6 Domain structures of the multimodular non-ribosomal peptide synthetase (NRPS) Fso1 for hydroxamate siderophore production in *Omphalotus olearius* (Welzel et al. 2005). *Note:* Fso1 is compared to the monomodular L-tyrosine:oxoglutarate aminotransferase AtrD from *Tapinella panuoides* (Schneider et al. 2008; Sect. 13.3.3). A adenylation, T thiolation, TE thioesterase/cyclase, C condensation

L-ornithine- N^5 -monooxygenase, acylation of N^5 -hydroxy-L-ornithine by an acyl-CoA: N^5 -hydroxy-L-ornithine N -acyl-transferase, and incorporation of other amino acids by a modular NRPS. *omol* of *O. olearius* encodes an L-ornithine- N^5 -monooxygenase and *atol* an acetylase. The NRPS Fso1 is 4548 amino acids long with a weight of about 500 kDa. It consists of repeated A-T-C and T-C modules (Welzel et al. 2005; Fig. 13.6). Adenylation (A) domains are the gatekeepers for NRPSs and select, activate, and load the substrate onto thiolation domains (the molecular carrier) for subsequent condensation reactions. A domains have signature sequences for the selection of cognate substrates. Attempts are made to define non-ribosomal codes for amino acid selection of different A domains (Schwecke et al. 2006; Kalb et al. 2013; Lee et al. 2015). A selective sequence of DIITITATLR has been deduced for the third A domain (A3) in Fso1 for N^5 -hydroxy- N^5 -methylglutaconyl-L-ornithine (MGHO) binding (Kalb et al. 2013). Basidiomycete mushroom genomes, in general, do not contain as many genes for modular NRPSs, NRPS-like enzymes, and also polyketide synthases as genomes of ascomycetes. In average, there are about four genes for such modular enzymes in a respective genome, with 0 up to 9 different genes for NRPSs spread into different gene clusters (Lackner et al. 2012; Liu et al. 2012a; Wawrzyn et al. 2012; Riley et al. 2014; Sasso et al. 2014).

13.5 Proteins

13.5.1 Aegerolysins and Other Pore-Forming Proteins

Aegerolysins represent an aegerolysin superfamily (Pfam 06355) of short microbial proteins (ca 140 amino acids, molecular weights of 15–20 kDa) with two conserved cysteines and potential hemolytic activities. They are named after the primordia-specific protein Aa-Pri1 from *Agrocybe aegerita* (Fernandez Espinar and Labarère 1997). Well-known other members of this family are erylysin A from *P. eryngii* (Shibata et al. 2010) and pleurotolysin A (ostreolysin) of *P. ostreatus* (Berne et al. 2002) which come together with the larger 59 kDa MACPF (membrane attack

complex/perforin; Reboul et al. 2016) proteins erylysin B and pleurotolysin B, respectively (Shibata et al. 2010; Ota et al. 2013). Typically in mushrooms, these types of proteins are expressed in stages of fruiting body development and in specific cells of the fruiting bodies (Fernandez Espinar and Labarère 1997; Berne et al. 2002; Schlumberger et al. 2014) or in multicellular sclerotia such as in *Lignosus rhinocerotis* (Yap et al. 2015b).

A. aegerita aegerolysin and the *Pleurotus* proteins are reported to be hemolytic acting against mammalian erythrocytes (Berne et al. 2002). However, erylysin A and pleurotolysin A exert such activities only in bicomponent complexes with the MACPF perforins erylysin B and pleurotolysin B, respectively (Ota et al. 2013; Schlumberger et al. 2014). Erylysin A and pleurotolysin A are pore-forming proteins which interact with lipid rafts in cholesterol- and sphingomyelin-rich cellular membranes and insert into cellular membranes (Berne et al. 2009; Hullin-Matsuda et al. 2016; Yamaji-Hasegawa et al. 2016). Erylysin A and pleurotolysin A need to bind to the membranes to then recruit the perforins erylysin B and pleurotolysin B, respectively, and to further assemble as heteromeric complexes with β -barrel protein shapes the transmembrane pores in the cellular membranes (Tomita et al. 2004; Ota et al. 2013; Schlumberger et al. 2014; Lukoyanova et al. 2015; Skočaj et al. 2016). The assembled pore complexes influence transport processes of the cells such as increasing Ca^{2+} influx, thereby disturbing Ca^{2+} homeostasis of the cells (Vrecl et al. 2015a, b).

Aegerolysins occur in about 20% of the *Agaricomycetes*, inconsistently distributed over the species (Lakkireddy et al. 2011; Nayak et al. 2013; Novak et al. 2015), among in *G. marginata* (KDR71454, KDR68296, KDR71457), *H. sublateralitium* (KJA14257, KJA14258), and *T. versicolor* (XP_008043601). Of these species, *T. versicolor* (XP_008043602) also has an MACPF perforin-type protein. Apart of a link to fruiting body development, the biological functions of the proteins for the mushrooms are unclear (Berne et al. 2009; Lakkireddy et al. 2011; Schlumberger et al. 2014).

Other types of membrane-integrating pore-forming mushroom toxins are hemolytic chimerolectins (Lakkireddy et al. 2011; Sabotič et al. 2016). *L. sulphureus* has a family of chimerolectins of which the hemolytic LSL has been studied in detail (Mancheno et al. 2010). The 315 amino acid-long LSL consists of an N-terminal β -trefoil-type lectin domain and a C-terminal aerolysin-like pore-forming domain. LSL binds to β -galactosides such as lactose and LacNAc. The crystal structure of LSL resembles that of bacterial aerolysins. LSL is therefore believed upon insertion into membranes to adopt an oligomeric β -barrel protein structure like the aegerolysins (Mancheno et al. 2004, 2005, 2010; Angulo et al. 2011). Genes for LSL-like proteins occur singly or as families inconsistently distributed over the *Agaricomycetes* (Lakkireddy et al. 2011). Highly similar, full-length proteins, for example, exist in *G. marginata* (KDR66599), *G. frondosa* (OBZ73067), *F. pinicola* (EPT04861), and *Sphaerobolus stellatus* (KIJ34088). Protein CC1G_11805 of a family of LSL homologues of *C. cinerea* (Lakkireddy et al. 2011) has been shown to be nematotoxic (Plaza et al. 2014).

A further novel family of mushroom lectins (referred to as FB_lectin superfamily) with structural similarities to hemolytic actinoporins is presented by the

insecticidal XCL of *Xerochomus chrysesteron* which binds to *N*-acetyl-galactosamine (Bleuler-Martinez et al. 2011; Yan et al. 2012). XCL forms tetramers in solution. Upon internalization into insect and human cells by a clathrin-dependent pathway, lectin XCL is delivered to endosome compartments, and it induces changes to the actin cytoskeleton (Francis et al. 2003; Birck et al. 2004). Other characterized lectins of this family are ABL from *A. bisporus*, SRL from *Athelia rolfsii*, and BEL from *B. edulis* (Lakkireddy et al. 2011; Sabotič et al. 2016). FB_lectin genes are more common in species of the *Agaricomycetes*, while the presence of such genes (singly or in families) is also irregularly distributed over the mushrooms (Lakkireddy et al. 2011).

13.5.2 Fungal Immunomodulatory Proteins (FIPs)

A first fungal immunomodulatory protein (FIP, Fve superfamily) identified from mushrooms is the well-studied 111-aa-long asparagine-rich protein LZ-8 (FIP-glu) from *G. lucidum* (Kino et al. 1989) with anti-allergy, wound-healing, and manifold antitumor activities with partially understood mechanisms (Li et al. 2011b; Wang et al. 2012; Lin et al. 2014; Wu et al. 2015). Similar immunomodulatory 13 kDa proteins (110–114 amino acids) are available from other *Ganoderma* species (Lin et al. 1997, 2010; Zhou et al. 2009; Wang et al. 2012; Yu et al. 2015), *Chroogomphus rutilus* (Lin et al. 2016), *Dichomitus squalens* (Li et al. 2017), *F. velutipes* (Ko et al. 1995), *L. rhinocerotis* (Pushparajeh et al. 2016), *Postia placenta* (Li et al. 2015), *T. versicolor* (Li et al. 2012), and *V. volvaceae* (Hsu et al. 1997; Wang et al. 2016c). There are also genes for FIPs in *A. muscaria* (KIL61952, KIL58061), *A. subglabra* (XP_007340731, an immunomodulatory 13.4 kDa protein has been isolated from *Auricularia polytricha*, Sheu et al. 2004), *Botryobasidium botryosum* (KDQ10166), *G. subvermispora* (EMD41188, EMD41189, EMD41190), *H. sublateralitium* (C-terminal linked to a protein kinase domain, KJA26560), *Obba rivulosa* (C-terminal linked to a protein kinase domain, OCH90019), *P. carnosa* (XP_007392586), *Schizopora paradoxa* (C-terminal linked to a protein kinase domain, KLO20748), *Trametes pubescens* (OJT03424, OJT03425), and more (Lakkireddy et al. 2011), while they are extremely rare in the *Ascomycota* with only two host species detected so far (Bastiaan-Net et al. 2013; Li et al. 2016b). FIPs of closer-related species might be up to 100% identical and sequences from more distantly related fungi between 56 and 75%. Depending on the species, FIPs are variably asparagine- and valine-rich (Li et al. 2011b, 2017; Lin et al. 2016; Pushparajeh et al. 2016).

Importantly, the sequences of the FIPs show similarities to sequences of immunoglobulins (Igs; Tanaka et al. 1989; Ko et al. 1997) although they usually lack cysteine, methionine, and histidine residues which are regularly present in Igs (Paaventhath et al. 2003; Huang et al. 2008). FIPs adopt a Ig-like β -sandwich fold from the seven β -sheets in their C-terminal fibronectin III-type domain like Igs, and they form homodimers or homotetramers (FIP-gmi from *Ganoderma microsporium*) via dimerization through an N-terminal α -helix stabilized by hydrophobic amino acid interactions and assisted by a β -sheet formed by the two dimerizing proteins

from the β -strand following directly after the N-terminal α -helix (Lin et al. 1997; Paaventhan et al. 2003; Wu et al. 2007; Huang et al. 2008; Pushparajah et al. 2016). Variably strong in their cytotoxic, mitogenic, and anticancer activities (Li et al. 2011b; Wang et al. 2012; Ou et al. 2015), FIPs are capable to perform Ig-like reactions and stimulate, e.g., proliferation of lymphocytes and macrophages and modify the production of cytokines by T cells (e.g., Ko et al. 1997; Hsieh et al. 2003, 2007; Hsu et al. 2008; Ou et al. 2009; Lin et al. 2010; Lee et al. 2013; Li et al. 2015; Wu et al. 2015), and they can be inhibitory to virus infections (Chang et al. 2014).

FIPs' activities partially depend on their 3D structure and homodimerization (Lin et al. 1997; Huang et al. 2008, 2014b). The difference in activities between FIPs probably base on the variable amino acid sequences found in loopDE and loopFG of the Ig-like β -sandwich structure (Huang et al. 2008; Wang et al. 2016c) and possibly on strength and structural differences of the α -helices mediating the N-terminal dimerization (Lin et al. 1997; Wang et al. 2016c). The proteins provide a carbohydrate-binding pocket (CBM, family CBM34-like; key residues in *F. velutipes* FIP-fve: W24, T28, D34, R90, I91, W111) by the β -sandwich structure, and the folded proteins can bind to complex cell-surface sugars such as dextrin, cyclodextrin, and *N*-acetyl neuraminic acid (Kino et al. 1989; Ko et al. 1995; Liu et al. 2012b; Pushparajeh et al. 2016). FIPs therefore have a lectin character and can cause blood cell aggregation, while individual FIPs differ in specificity to agglutinate different animal (rat, mouse, sheep) or human red blood cells (Li et al. 2012; Liu et al. 2012b; Lin et al. 2016; Pushparajah et al. 2016).

Interesting for future studies could be functional analyses on the potentially bifunctional proteins of *H. sublateritium*, *O. rivulosa*, and *S. paradoxa* with a protein kinase domain linked to one or two FIP units, because FIP-fve has been shown to activate protein kinase C- α in human peripheral blood mononuclear cells (Ou et al. 2009).

13.5.3 Other Lectins

The catalog of different lectins, i.e., carbohydrate-binding proteins which are also known as hemagglutinins, is large in mushrooms as are the lists of types of sugars they interact with and the potential medicinal functions attributed to them including antitumor, mitogenic/antimitogenic, immunomodulatory, antiviral, and other activities (Lakkireddy et al. 2011; Erjavec et al. 2012; Hassan et al. 2015; Singh et al. 2015; Sabotič et al. 2016). Moreover, 3D structures have been established for several distinct fungal lectins, and different structural families of fungal lectins have been defined (Varrot et al. 2013; Hassan et al. 2015; Sabotič et al. 2016). Related genes for the diverse types of lectins are often found in only selected species scattered over the *Agaricomycetes*, commonly not much linked to any taxa relationships. Sometimes, the distribution of related genes within different mushrooms is very restricted to only a low number of species (Lakkireddy et al. 2011; Sabotič et al. 2016).

By the wealth of different lectins which occur in mushrooms and which are moreover possibly even characterized, we can present here only a few selected examples of different groups of lectins and document the quite irregular dissemination of respective lectin genes over the species ranges. The examples discussed should emphasize the value of looking into more (types of) lectins of more mushroom species. The chimeric lectins and the FIPs discussed in Sects. 13.5.1 and 13.5.2 belong to other families of lectins, and further lectin-like proteins with protease inhibitory functions and protease activities are presented in Sect. 13.5.4.

Genes for galectins binding β -galactosides are, for example, present in *Agrocybe cylindracea* (ACG; Yagi et al. 1997, 2001; Ban et al. 2005), *A. aegerita* (the fruiting body-specific AAG; Yang et al. 2009; Luan et al. 2010), *C. cinerea* (the fruiting body-specific Cgl1 and Cgl2; the related Cgl3 binds LacdiNac and chitobiose; Cooper et al. 1997; Boulianne et al. 2000; Walser et al. 2004; Wälti et al. 2008; Plaza et al. 2014), *Heterobasidion irregulare*, *Laccaria amethystina*, *L. bicolor* (Lakkireddy et al. 2011; Lyimo et al. 2011), *G. marginata* (KDR68111), *Pisolithus microcarpus* (KIK30469), and a few others (Sabotič et al. 2016). The fungal galectins adopt a typical β -sandwich structure composed of two antiparallel, six-stranded β -sheets. CGL2 and CGL3 of *C. cinerea* form homotetramers; ACG and AAG from *Agrocybe* species form homodimers (Walser et al. 2004; Ban et al. 2005; Wälti et al. 2008; Yang et al. 2009). CGL1 and CGL2 show toxic activity against nematodes, mosquitos, and amoebae, by binding to *N*-glycans of glycoproteins of attacked organisms (Butschi et al. 2010; Bleuler-Martinez et al. 2011). ACG recognizes specifically *N*-sialoglycans specific to human leukemic cells in a comparably well-understood binding mode (Hizukuri et al. 2005; Parasuraman et al. 2015). Antitumor activities and binding to tumor-related glycan antigens have been demonstrated for AAL (Zhang et al. 2015b; Jin et al. 2016; Liu et al. 2017).

The β -trefoil family of fungal lectins (from the ricin-B superfamily) is much larger with the majority of *Agaricomycetes* having one or more members. However, overall sequence identities and similarities between individual members of this family can be very low with values of 7–26% and 25–40%, respectively. The lectins of the β -trefoil family also vary in their carbohydrate-binding specificity and are often fruiting body- or sclerotia-specific (Lakkireddy et al. 2011; Žurga et al. 2014; Sabotič et al. 2016). Well-studied examples for the β -trefoil family of lectins are the fruiting body-specific CCL1 and CCL2 of *C. cinerea* which have nematocidal activities like many other members of this family, while others are acting entomotoxic (Schubert et al. 2012; Plaza et al. 2014; Sabotič et al. 2016). CCL1 and CCL2 are 55% identical and 73% similar in sequence. Most similar proteins, e.g., to CCL2 from other mushrooms, are from *S. lacrymans* (XP_007323761: 43% identical, 60% similar), *Coniophora puteana* (XP_007765699: 43% identical, 59% similar), *Rhizopogon vinicolor* (OAX40927: 45% identical, 56% similar), *S. stellatus* (KIJ40569: 43% identical, 55% similar), *P. ostreatus* (KDQ26460: 45% identical, 59% similar), and *L. amethystina* (KIJ95877: 41% identical, 54% similar). CCL2 binds to a GlcNAc- β 1,4-(Fuc- α 1,3)GlcNAc (anti-HRP, anti-horseradish peroxidase) epitope *N*-glycans of nematodes (Schubert et al. 2012; Stutz et al. 2015). Another well-studied lectin from the β -trefoil family in the *Agaricomycetes*, the

entomotoxic agglutinin RSA from *Rhizoctonia solani* (Candy et al. 2001; Hamshou et al. 2013), interacts in contrast with Gal-/GalNAc-containing insect glycans (Hamshou et al. 2012; Walski et al. 2014). RSA is shown to also bind to proteins α -2-macroglobulin and IgA from human serum (Van Leuven et al. 1993). The nematotoxic ricin B-like lectin CNL from *Clitocybe nebularis* (26% identical, 40% similar to RSA; Sabotič et al. 2016) immunostimulates dendritic cells via the toll-like receptor 4 pathway (Svajger et al. 2011; Pohleven et al. 2012). A lectin in other species closest to CNL is from *Leucoagaricus* sp. (KXN92490: 32% identical, 50% similar), while other related lectins from *P. placenta* and *C. cinerea* are only 26% identical and 46–44% similar to CNL (Lakkireddy et al. 2011).

Another group of interesting lectins is represented by the 42 kDa PVL from *Lacrymaria (Psathyrella) lacrymabunda*. This is an integrin-like protein which adopts a six-bladed β -propeller fold with six carbohydrate-binding sites for terminal GlcNAc and sialic acid residues (Ueda et al. 2002; Cioci et al. 2006; Audfray et al. 2015). This lectin binds truncated *N*-glycans on human cancer cells (Audfray et al. 2015). A highly similar lectin PAL from *Lacrymaria (Psathyrella) asperospora* of high sequence identity (87%) and similarity (92%) and with a similar 3D structure also binds terminal GlcNAc and sialic acid. PAL has a strong cytotoxic effect on human colon cancer cells and on monkey kidney cells (Rouf et al. 2014; Ribeiro et al. 2017). PVL- and PAL-related lectins are however also not as widely distributed in other mushroom species (Lakkireddy et al. 2011). *A. aegerita*, *C. cinerea*, and *L. bicolor* each have one gene (Lakkireddy et al. 2011; Rouf et al. 2014; Ren et al. 2015), and there are related genes in *G. marginata* (KDR83047, KDR74277), *H. cylindrosporium* (KIM35067, KIM47177, KIM35070), *H. sublateralitium* (KJA19519), and *L. amethystina* (KIK03462, KIJ92371, KIJ96935, KIJ93857).

Tectonins are lectins of an interesting structure by having tandem β -propeller repeats which form blades by a four-stranded antiparallel β -sheet (Low et al. 2009). Tectonin Le-Tec2 of *L. bicolor* agglutinates *E. coli* and recognizes *O*-methylated mannose and fucose residues, typically present in the outer bacterial lipopolysaccharide layers (Wohlschlagler et al. 2014). Genes for tectonins appear not to be much distributed over all the various mushroom species, but they are repeatedly found in species with an ectomycorrhizal lifestyle (Sabotič et al. 2016) such as in *H. cylindrosporium* (KKIM38814, KIM38815, KIM38816, KIM38829, KIM38830, KIM38831, KIM35453, KIM35454, KIM35464, KIM35465), *L. amethystina* (KIJ90107, KIJ94531, KIJ94903), *L. bicolor* (XP_001876432, XP_001877906), *Paxillus rubicundulus* (KIK93906), while the saprotrophic *G. marginata* (KDR71733, KDR71773) has also gene copies.

13.5.4 Proteinase Inhibitors and Lectins with Protease Activities

Another type of small proteins found often specifically expressed in stages of fruiting body development and in specific mushroom tissues concerns protein inhibitors. While many mushroom crude extracts have been shown to confer proteinase-inhibiting activities, few inhibitors have been isolated and characterized. Among

these are mycospains (e.g., cospin of *C. cinerea* and cnispin from *C. nebularis*) as serine proteinase inhibitors and mycopins (clitopypins from *C. nebularis* and macrocypins from *Macrolepiota procera*) as cysteine protease inhibitors (Renko et al. 2012; Dunaevsky et al. 2013; Sabotič et al. 2016). Cospin as an example of the mycospain family is a 150 amino acid-long antibiotic protein (Sabotič et al. 2012), cnispin is 146 amino acid-long (Avanzo Caglič et al. 2014), and LeSP1 from *L. edodes* 152 amino acid-long (Odani et al. 1999). These proteins belong by sequence similarities all to the ricin B-like superfamily and adopt a β -trefoil structure with 12 β -strands and 11 loops, and they show high activity against trypsin (Odani et al. 1999; Avanzo Caglič et al. 2014). Cnispin inhibits proteases by its β 11- β 12 loop with Lys-127 as the P1 amino acid which binds to the active site in trypsin (Avanzo Caglič et al. 2014). In contrast, loop β 2- β 3 with Arg-27 is responsible for the inhibitory activity of cospin (Sabotič et al. 2012). The mycopins macrocypin 1 and clitopycin are also members of the large fungal ricin B-like family. However, they block proteases through interaction with their β 5- β 6 loops. Mycopins are strong inhibitors of papain-like proteases (Sabotič et al. 2007, 2009; Renko et al. 2010). Genes for the superfamily of ricin-like proteins are widely distributed in the *Agaricomycetes* and often occur in the species in larger diverse families (Lakkireddy et al. 2011; Sabotič et al. 2016). Fungal β -trefoil proteases and fungal ricin B-like lectins of β -trefoil structure interact with each other which is considered as a regulatory mechanism of respective protein activities (Žurga et al. 2015).

The second group of β -trefoil chimerolections encloses MOA from *M. oreades*. This lectin consists of an N-terminal lectin domain and a C-terminal cysteine protease domain. MAO binds to terminal Gal- α 1,3-Gal/GalNAc- β epitopes (Grahn et al. 2007, 2009; Wohlschlager et al. 2011). The homologous lectins PSL1a from *P. squamosus* and SCA from *S. commune* in contrast recognize terminal Neu5Acc- α 2,6-Gal- β epitopes (Kadirvelraj et al. 2011; Wohlschlager et al. 2011). MAO forms dimers of a dumbbell shape with the dimerized cysteine protease domain connecting the two β -trefoil lectin domains. Protease activity requires binding of divalent ions such as Ca^{2+} for induction of conformational changes (Cordara et al. 2011, 2016; Wohlschlager et al. 2011). When taken up into mammalian cells, MOA inhibits protein and DNA biosynthesis and induces degradation of β 1-integrin, disruption of integrin-dependent cell adhesion signaling, rearrangements of the cytoskeleton, and cell death (Cordara et al. 2014; Juillot et al. 2016). Toxic activities toward mammalian cells depend on the protease domain (Manna et al. 2017). Also the presence of genes for MAO-like genes is inconsistently spread over the *Agaricomycetes*. Related genes (protein similarity ca 35 to >40%) were further found in *Peniophora* sp. (KZV64829, KZV65628), *Plicaturopsis crispa* (KII87094), *Polyporus umbellatus* (ANC28063), *Pycnoporus coccineus* (OSC96843, OSD05564), *S. lacrymans* (EGN95167), *S. stellatus* (KIJ35645), and *Trametes cinnabarina* (CDO76070).

13.5.5 Ribosome-Inactivating Proteins (RIPs)

Ribosome-inactivating proteins (RIPs) are *N*-glycosidases which depurinate an adenine residue in the conserved α -sarcin/ricin loop of eukaryotic 28S rRNA, thereby blocking the binding of elongation factor 2 (EF-2) and in consequence protein biosynthesis. Type 1 RIPs (chimero-RIPs) are enzymes with a single RNA *N*-glycosidase activity domain and molecular weights from ca 20 up to 40 kDa. Type 2 RIPs consist of two different chains of distinct function, an *N*-glycosidase A-chain (similar to type 1 RIPs) and a lectin-like B-chain (for cell binding and transport into cells), which are interlinked by a disulfide bridge. Type 3 RIPs are atypical RNA *N*-glycosidases with two domains, an N-terminal type-1 RIP-like domain and a C-terminal domain of unknown function which must be removed to activate the enzymatic function of the N-terminus (Girbes et al. 2004; Akkouch et al. 2015; Wang et al. 2016a). RIPs are best known from plants (Akkouch et al. 2015; Wang et al. 2016a) while ribosome-inactivating effects have also been attributed to several proteins isolated from various mushrooms (Table 13.2). These are however all not fully characterized. Enzymatic depurination activities on 28S rRNA are deduced for marmorin of *H. marmoreus* and volvatin of *V. volvaceae* from gels showing a 28S rRNA fragmentation band of about 0.4 kb which was similar in size to the specific “Endo’s” bands generated from 28S rRNA through depurination by plant RIPs (Endo et al. 1988; Yao et al. 1998; Wong et al. 2008). Indication for a member of the classical RIP superfamily in fungi could come indirectly from a comparison of full-length protein sequences (Lapadula et al. 2013). Significantly, the sequence of a RIP from maize (2PQI_A) detected genes for RIP-like proteins only in exceptional cases in very few fungal plant and insect pathogens from the *Ascomycota* and in *S. stellatus* (KIJ38555; KIJ48721) as the only basidiomycete (Lapadula et al. 2013; this report).

Usually, the N-terminal sequences of reported fungal ribosome-inactivating proteins and the protein sizes as determined by migration in gel electrophoresis were published (Table 13.2), whereas the actual nature of the proteins always remained open by the lack of complete sequences. Now the published N-terminal sequences can be used in tBlastn or pBlast searches in the databases against the genomic sequence or against the proteome deduced from a fungal genome, respectively. We have done this here for a range of fungal ribosome-inactivating proteins (Table 13.2). Even with complete genomes available for *H. marmoreus* (GCA_001605315) and two distinct strains of *F. velutipes* (Park et al. 2014; BDAN01000000; both only little annotated), we could not detect 100% perfect sequence matches with any of the experimentally established N-terminal peptide sequences. However, the *H. marmoreus* marmorin and *F. velutipes* velin peptides hit the N-termini of matured secreted proteins of expected sizes which have suggested lectin functions. Marmorin belongs thus to a novel family of fungal lectins together with a crystallized α -galactosyl-binding lectin of *Lyophyllum decastes* and some proteins from a few ascomycetes and basidiomycetes (Goldstein et al. 2007; van Eerde et al. 2015; this study). It is striking that the *L. decastes* lectin shares galabiose-binding activity with the Shiga toxin from the RIP superfamily (Goldstein et al. 2007). Another interesting finding concerns flammin. It appears to be related to or be in fact the formerly characterized *F. velutipes* endo- β -1,3-galactanase FcEn3GAL (Kotake et al. 2011).

Table 13.2 Fungal ribosome-inactivating proteins and their possible genes and functions

Fungus	Protein name/ best hit in database ^a	N-terminal sequence/ related sequence deduced from DNA sequences ^b	MW in kDa		Reference/ proposed function
			In gel	Calculated ^c	
<i>Calvatia caelata</i>	Calcaelin	ANPIYNI ^d DAFRV			Ng et al. (2003a)
	Hypsin KYQ37785	ITFQGLDLDARQVITNA ^d TRRKR ^d VDVRAA ITFQGCSPARQIVITNA ^d TRARADVRAA	20	36.19 (38.24) /18.25 (19.16) ^d	Lam and Ng (2001a) Peptidyl-Lys metalloendopeptidase
<i>Hypsizygus marmoris</i>	Marmorin	AEGTL ^d LGSRAT ^d CESGNSMY	10		Wong et al. (2008)
	KYQ33543	AA ^d TCWK ^d TSKCSP ^d CESANS ^d MY		10.7 (12.67)	Family of α -galactosyl binding lectins
	KYQ33548	A ^d TCWK ^d TSKCSP ^d CESANS ^d MY		10.61 (12.71)	
	KYQ33492	AQCFSQHGCC ^d NCESRD ^d TMY		10.95 (12.86)	
	KYQ33491	A ^d TCWER ^d WGCV ^d T ^d CESKDP ^d MY		11.1 (13.17)	
<i>Flammulina velutipes</i>	Flammin	SPV ^d IPAN ^d TFVAFRL ^d YEVGF ^d UPA	30		Ng and Wang (2004a)
	F7J1C8	ATV ^d IPANS ^d FS ^d FTY ^d WNN ^d F ^d Y ^d PW	30	24.5 (26.69)	Endo- β -1,3-galactanase Kotake et al. (2011)
	Flammulin	APS ^d HF ^d SH ^d PGVL ^d ADRA ^d Q ^d LD ^d FT ^d XGK ^d VNE ^d GAEP ^d W ^d X ^d SAYN	40		Wang and Ng (2000a)
<i>Gymnopus luxurians</i> KIK67684 ^e	BDAN01000649:	APS ^d V ^d TF ^d HP ^d GV ^d L ^d DR ^d AQ ^d LD ^d FL ^d K ^d DK ^d VNS ^d GAEP ^d W ^d A ^d SAYN			
	184794-184631	APS ^d TF ^d HP ^d GV ^d L ^d DR ^d AQ ^d LD ^d FL ^d K ^d GVNS ^d GAEP ^d W ^d T ^d SAYN			
	181700-181537	APS ^d TF ^d HP ^d GV ^d L ^d DR ^d AQ ^d LD ^d FL ^d K ^d GVNS ^d SAQP ^d W ^d T ^d SAYN			
	152142-152305	APS ^d TF ^d HP ^d GV ^d L ^d DR ^d AQ ^d LD ^d FL ^d K ^d GVNS ^d SAQP ^d W ^d T ^d SAYN			
	148068-147905	APS ^d TF ^d HP ^d GV ^d L ^d DR ^d AQ ^d LD ^d FL ^d K ^d GVNS ^d GAQP ^d W ^d T ^d SAYN			
		APA ^d TF ^d HP ^d GI ^d L ^d DR ^d Q ^d LD ^d FL ^d K ^d GVNS ^d GAEP ^d W ^d T ^d KAYN			44.45 (46.59)

	Velin BDAN01003789; 122030- 121947 <i>Moniliophthora roveri</i> XP_007856901 ^c	SGSPLTQAAQAEALLKPKQGL-AYSSGGNT SGSPLTQAAQAEALLKPKQGITAYSSGGCT SGTPLTQAAQAEALLIPQGITASTGGCT	19	16.97 (18.94)	Ng and Wang (2004a) Peptidoglycan-binding domain 1 protein Wang and Ng (2001a)
	Velutin BDAN01002178; 202395- 202327 <i>Fomitiporia mediterranea</i> XP_007269873	XHPDLFXXRPDNTASPKFEDPRLNP MSHPDLF . . RKGNTSPKFE SVRKGV MSHPDLY . . RSGNTTSPRFDNVREGT	13.8	27.78	Unknown
<i>Lentinus tuber-regium</i>	Pleuturing No meaningful hit	ARTQPGNIAPVGGDFTLYPNAPRQGHIVA	40		Wang and Ng (2001b)
<i>Lyophyllum shimeji</i>	Lyophyllin Serine metalloprotease KYQ37785 (<i>Hypsizygus marmoratus</i>)	ITFQGS PARQTVI TNAITFRARADVRAA IRFQ SASP ITFQGS PARQTVI TNAITFRARADVRAA	20 21	36.19 (38.24) /18.25 (19.16) ^d	Lam and Ng (2001b) Moon et al. (2014) Peptidyl-Lys metalloendopeptidase Ng and Wang (2004b)
<i>Polyporus adusta</i>	Adustin No revealing hit	ADVVED	16.5		

^aBest hits are either from the Basidiomycota protein databank at NCBI (pBLAST searches) or DNA sequence positions from whole genome sequences (tBLASTn searches)

^bAmino acids shared between an experimentally determined N-terminal sequence and a hit in the databases are marked in bold

^cFor proteins which have secretion signals, two distinct values are given for the precursor protein (number in brackets) and the mature protein

^dValues are given for a complete protein sequence as deduced from the whole open reading frame and for sequences of a truncated proteins from the 3' half of the putative gene

^eBest hit from another species

Notably, the gene for En3GAL was only found in one of the two released *F. velutipes* genomes (BDAN01000000; Table 13.2).

Further interesting cases are *H. marmoreus* hypsin and *Lyophyllum shimeji* lyophyllin. The experimentally determined N-termini of hypsin and lyophyllin (Lam and Ng 2001a, b) recognize in pBlast searches internal sequences of a larger *H. marmoreus* protein (KYQ37785) with an undefined N-terminal half and an M35-like Zn²⁺-metallopeptidase/deuterolysin domain in the C-terminal half (Table 13.2). Moreover, the N-terminal sequences match that of a 21 kDa serine metalloproteinase characterized from *L. shimeji* (Moon et al. 2014). The smaller size suggests either a posttranslational splitting of the two domains to give the experimentally determined N-terminus (the calculated protein size is 18.25 kDa), or the enzyme is produced from an ATG start codon in close vicinity to the coding sequence of the established N-terminus (the calculated size is 19.16 kDa). The N-terminal sequence analysis data as presented in Table 13.2 in effect suggest that none of the fungal ribosome-inactivating proteins are classical RIPs.

Most fungal ribosome-inactivating proteins have been classified as such by proof of inhibitory activities in cell-free translation assays, while RNA *N*-glycosidase activity is often claimed but not shown in the publications (Lam and Ng 2001a, b; Wang and Ng 2000a, 2001a, b; Ng et al. 2003; Ng and Wang 2004a, b). Looking at the shown or proposed biological functions of the diverse candidate proteins listed in Table 13.2, it is difficult to imagine that all of them should be true bioactive RIPs with specific 28S RNA cleavage function (see also the discussion by Lapadula et al. 2013). The proteins listed in Table 13.2 have variably been assigned further activities to, for example, being antiproliferative against cancer cell lines (hypsin, lyophyllin, marmorin), anti-mitogenic (calcaelin, hypsin, lyophyllin), antifungal (hypsin, lyophyllin), RNase active (calcaelin), HIV reverse transcriptase inhibiting (hypsin, lyophyllin, marmorin), and β -glucosidase and β -glucuronidase inhibiting (velutin), (Yao et al. 1998; Wang and Ng 2000a, 2001a, b; Lam and Ng 2001a, b; Ng et al. 2003; Ng and Wang 2004a; Wong et al. 2008). Such variability in multiple extra functions is shared with many other proteins isolated over the years from mushrooms and published under diverse headings such as antifungal, antibacterial, antiviral, and antitumor proteins (Akkouh et al. 2015; Ng et al. 2016). Strategies of their isolation and characterization are also commonly shared with the fungal ribosome-inactivating proteins, including that the protein identity is regularly defined via determination of N-terminal sequences. It is thus possible that also behind many of these yet to clearly identify proteins are such diverse kinds of proteins as suggested for the denoted fungal ribosome-inactivating proteins from the here presented peptide pBlast and tBlastn searches (Table 13.2). For further interest, tables with published N-terminal sequences of mushroom bioactive proteins of pharmaceutical interest are compiled, e.g., in Ng (2004) and Ng et al. (2016).

Other short mushroom proteins with reported antitumor-, deoxyribonuclease-, and ribosome-interfering activities are, e.g., the 8.5 kDa protein CULP from the puffball *Calvatia cealata* (Wang et al. 2003), the 9 kDa protein CCULP from *Cantharellus cibarius* (Wang et al. 2003), the 12.5 kDa glycosylated protein PULP

from *P. ostreatus* (Wang and Ng 2000b), and the 18.5 kDa RBUP from *Ramaria botrytis* (Zhou et al. 2017) from a longer catalog of ubiquitin-like proteins compiled from work of similar experimental systematics on many fungal species. No full-length protein sequence of any of the isolated and analyzed ubiquitin-like proteins seem to have ever been published, but obtained N-terminal sequences and MS/MS-covered internal peptides (69% in case of the *R. botrytis* protein) always match the ubiquitin of *Coprinellus congregatus* which as highly conserved protein is 99–100% identical in sequence to ubiquitins of other basidiomycetes and of ascomycetes.

13.5.6 Others

TFP of *Tremella fuciformis* fruiting bodies is a new 135 amino acid-long non-glycosylated homodimeric macrophage-activating protein. TFP cannot agglutinate blood cells and has likely no lectin activity. Its closest relatives are small expressed proteins from *S. commune* and *L. bicolor* classified as nonenzymatic proteins from the expansin superfamily which are plant-cell-wall binding and loosening proteins (Huang et al. 2014a).

Another novel immunomodulatory macrophage-activating protein is the glycosylated and cysteine-rich secreted ACA from mycelium of *T. camphoratus*. This protein belongs to the fungal phytotoxic cerato-platanin protein family and also shows no lectin agglutination properties. The ACA precursor is 136 amino acids long (Sheu et al. 2009). Members of the cerato-platanin family have been well studied in the *Ascomycota* (Gaderer et al. 2014; Pazzagli et al. 2014), while information in the *Basidiomycota* is scarce. Many of the *Agaricomycetes* do have variable genes for cerato-platanin-like proteins, among them are *Trametes* species (four genes in *T. cinnabarina*, five genes in *T. pubescens*, five genes in *T. versicolor*). *T. versicolor* protein YZP (alleles from different strains: AGH06133 and XP_008037058; 56% identical and 74% similar to ACA of *T. camphoratus*) stimulates B lymphocytes and induces production of interleukins IL-6 and IL-10 (Kuan et al. 2013). Notably, YZP is present in the Krestin polysaccharide-peptide extracts of *T. versicolor* and could be part of the effective principle of Krestin, similarly to lectin LZ-8 in *G. lucidum* polysaccharide PS-G (Yeh et al. 2010; Kuan et al. 2013).

A highly toxic protein for human cell lines via promotion of apoptosis is the 143 amino acid-long toxophallin from stems of *A. phalloides* mushrooms. It is structurally related to a functional amino oxidase of *L. bicolor* but has no mono- and diamine activity and acts as L-amino acid oxidase (L-AAO). Enzymatic activity generates aggressive H₂O₂ which is suggested to lead to activate apoptosis by a caspase-independent pathway (Stasyk et al. 2010). Similar enzymatic activities are known from snake venoms (Doley and Kini 2009). Other mushrooms including *L. bicolor* contain related genes for enzymes with 30–60% identity and 40–74% similarity to toxophallin.

13.6 Conclusions

In medicinal and other kinds of mushrooms, there is a fortune of unexploited secondary metabolites which have potential therapeutic values (Wasser and Weiss 1999; Wasser 2002; Anke and Antelo 2011; Schöffler and Anke 2011). Various unique types of proteins, both enzymes and non-enzymatic proteins, have also been reported to be linked to pharmaceutical effects of mushrooms (Lakkireddi et al. 2011; Xu et al. 2011; Erjavec et al. 2012; Sabotič et al. 2016). Modes of actions are being elucidated from many of fungal bioactive compounds, and the spectrum of activities is as broad as the biochemical nature of mushroom bioactive compounds.

Naturally by the high species number of the *Agaricomycetes* in nature as compared to the less studied ascomycetous mushrooms, most of the molecules discussed in this chapter originate from the *Agaricomycetes*. Although overlaps in the outfits of bioactive molecules are recorded between the *Agaricomycetes* from the *Basidiomycota* and the *Ascomycota*, there seems to be a tendency that different types of metabolites have preferentially been adopted in the two phyla of *Dikarya*. This has also been noted before. Comparably, much more polyketides and non-ribosomal peptides are found with corresponding genes in the ascomycetes and much more terpenoids and terpene synthase genes in contrast in the basidiomycetes (Bushley and Turgeon 2010; Chen et al. 2012; Liu et al. 2012a; Schmidt-Dannert 2015, 2016) and possibly (noncore) genes for lectins and lectin-like proteins (Sabotič et al. 2016). Production of secondary metabolites and specific proteins are often but not always connected to mushroom development. However, the vegetative mycelia may also produce the same or other bioactive compounds and proteins. Biological functions of mushroom compounds for the producer often remain elusive. Defense reactions against competing and adverse organisms are trendy postulates, while in some instances experimental evidence has been provided for such (Schwenk et al. 2014; Sabotič et al. 2016). Whatever the actual biological functions for the mushrooms are, their mode of actions might be exploited in tasks of pharmacology.

Research on bioactive metabolites and proteins classically starts by a screening of mushrooms for effective principles, possibly supported by ethnobotanical knowledge on traditional medicinal species. Now with the many fungal genomes at hand, there is a paradigm shift in approaches. Earlier in 2011, we performed a genome mining study with studied fungal lectins and pore-forming proteins with available genome sequences. Although complete at the time, the study was only on a small scale and finished quickly due to the restricted number of available sequences (Lakkireddy et al. 2011). Until recently, reports with sequences for metabolic genes were very rare and “anecdotal” (Schneider et al. 2008). When we agreed last year to write this chapter, we expected that this would not have changed as much. As documented in the chapter, genome-driven publications however now pop up like mushrooms in the forests. The recent flood of excellent papers in the field describing genes and their products indicates that a change has happened through the availability of the genomes. It is now much easier to target interesting genes and functions of pharmaceutical interest, both from medicinal species where such are

expected but also from genome mining in other mushrooms not considered before for potential medicinal applications. However, this makes classical experimental research on genes and compounds not obsolete.

We blasted with selected protein sequences (mostly by pBlast against defined protein sequences) the mushroom genomes deposited at NCBI (including all those mentioned in the introduction). A reoccurring observation is that usually only a few of all tested species contain specific genes of concern. Usually, genes of the same kind are distributed independently of clear species connections. Some of the species (e.g., *G. marginata* or *T. versicolor*) appeared more often in the searches than others mentioned in the text, and there are other species which were never hit. It is possible that recognized medicinal species are equipped with many more genes for pharmaceutically interesting secondary metabolites and unique type of proteins than other fungal species. This notion should be better elucidated in the future.

Detection of gene clusters in fungal genomes can be an entry point into the biosynthesis of known and of novel metabolites. However, on an average there seems to be fewer gene clusters to be present per species in basidiomycetes as compared to ascomycetes (Schmidt-Dannert 2015; Wawrzyn et al. 2015). Examples are accumulating from the *Agaricomycetes* that genes for a distinct biosynthetic pathway are not necessarily completely kept together in a single cluster (Wick et al. 2016; Braesel et al. 2017; this chapter). Moreover, there are also numerous examples of single genes in genomes (e.g., genes for sesquiterpene synthases; Sect. 13.3.1) suggesting that gene clustering for metabolic pathways in basidiomycetes might not be as strict as compared to ascomycetes. Possibly, this makes the systems more versatile and allows producing a much larger diversity of compounds. Also, this idea will need to become substantiated in the future.

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