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Biologically Active Secondary Metabolites from the Fungi

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ABSTRACT Many Fungi have a well-developed secondary metabolism. The diversity of fungal species and the diversification of biosynthetic gene clusters underscores a nearly limitless potential for metabolic variation and an untapped resource for drug discovery and synthetic biology. Much of the ecological success of the filamentous fungi in colonizing the planet is owed to their ability to deploy their secondary metabolites in concert with their penetrative and absorptive mode of life. Fungal secondary metabolites exhibit biological activities that have been developed into life-saving medicines and agrochemicals. Toxic metabolites, known as mycotoxins, contaminate human and livestock food and indoor environments. Secondary metabolites are determinants of fungal diseases of humans, animals, and plants. Secondary metabolites exhibit a staggering variation in chemical structures and biological activities, yet their biosynthetic pathways share a number of key characteristics. The genes encoding cooperative steps of a biosynthetic pathway tend to be located contiguously on the chromosome in coregulated gene clusters. Advances in genome sequencing, computational tools, and analytical chemistry are enabling the rapid connection of gene clusters with their metabolic products. At least three fungal drug precursors, penicillin K and V, mycophenolic acid, and pleuromutilin, have been produced by synthetic reconstruction and expression of respective gene clusters in heterologous hosts. This review summarizes general aspects of fungal secondary metabolism and recent developments in our understanding of how and why fungi make secondary metabolites, how these molecules are produced, and how their biosynthetic genes are distributed across the Fungi. The breadth of fungal secondary metabolite diversity is highlighted by recent information on the biosynthesis of important fungus-derived metabolites that have contributed to human health and agriculture and that have negatively impacted crops, food distribution, and human environments.

INTRODUCTION

Fungi, plants, and bacteria are the major kingdoms of life with well-developed secondary metabolism. About 500,000 secondary metabolites (also referred to as natural products) have been described to date. About 100,000 of these are derived from animals, 350,000 are from plants, and 70,000 are from microbes (1, 2). Roughly 33,500 bioactive microbial metabolites have been described (2). Of these 33,500 microbial metabolites, about 12.5% (4,200) are metabolites of unicellular bacteria and cyanobacteria, 41% (13,700) are products of Actinomycete fermentations, and about 47% (15,600) are of fungal origin (1). Furthermore, the rate of discovery of new fungal metabolites has accelerated significantly in the past two decades relative to the rate of discovery in the actinomycetes, filamentous bacteria that traditionally have been the richest source of microbial natural products (2). This complex and rich secondary metabolism is highly developed in the

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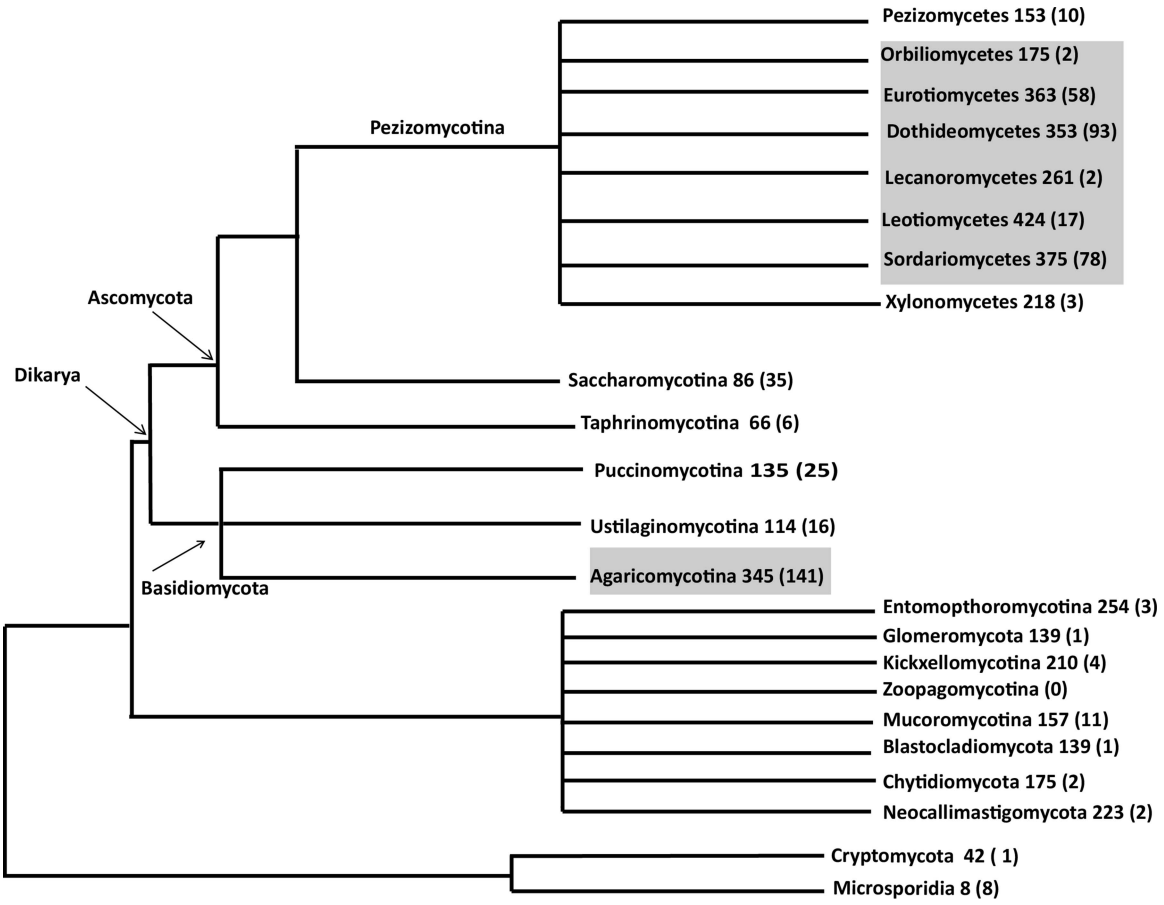
filamentous Ascomycota and Basidiomycota, while it is underdeveloped in the unicellular forms of the Ascomycota and Basidiomycota and in the Zygomycota, Blastocladiomycota, and Chytridiomycota (Fig. 1). The diversity of fungal species, particularly in the Ascomycota and Basidiomycota, and the accompanying diversification of biosynthetic genes and gene clusters points to an almost limitless potential for metabolic variation. In fact, one can argue that much of the ecological success of the filamentous fungi in colonizing virtually all habitats on the planet is owed to their ability to deploy arrays of secondary metabolites in concert with their penetrative and absorptive life forms. This dependence of the fungi on secondary metabolites to conquer diverse habitats and sustain their existence within them is evidenced by the facts that most species make multiple types of secondary metabolites, their expression is orchestrated with the life cycle and environment, and significant portions of their genomes are devoted to encoding and regulating the production of these products.

Secondary metabolites display an incredibly broad range of biological activities. Some have afforded society-changing benefits, while others are associated with serious, almost intractable problems. This dichotomy is indicative of the diversity of natural products that fungi can produce. Fungal metabolites with the greatest negative impact include mammalian toxins commonly known as mycotoxins. As many as 1,000 fungal compounds have been afforded this label, with the most widely known including aflatoxins, trichothecenes, fumonisins, ochratoxin, cytochalasins, and various indole-terpene tremor-

FIGURE 1 Simplified phylogeny of the phyla and classes of the Fungi. Numbers after phylum or class indicate the average number of secondary metabolite, transport, and catabolism genes recognized by the Clusters of Orthologous Groups of Proteins Classification (KOGs) from sequenced genomes (number in parentheses) of fungi at the Joint Genome Institute's Mycosm Project (January 15, 2016). Taxa shaded in gray are known to produce secondary metabolites with high frequency. Branch length reflects relatedness of taxa.



genic compounds (3, 4). Organisms in all domains of life produce toxins, including many that are more toxic than those from fungi, but mycotoxins tend to be more problematic because of their widespread occurrence as contaminants of food for humans and livestock, as well as mold-contaminated indoor environments (5). Understanding mycotoxin chemistry is essential to improving the ability to monitor and reduce the levels of exposure to such compounds. Fungi that produce mycotoxins and phytotoxins associated with crop diseases can cause considerable economic losses and require significant investment in their control. Metabolites that function as phytotoxins and small-molecule virulence factors produced by plant pathogenic species are responsible for many of the damaging effects of plant diseases (6).

On the other hand, many important pharmaceuticals have been discovered through studies of fungal chemistry (Table 1). Natural products continue to be among the most important therapeutic agents and lead compounds in medicine and have been particularly important in the development of effective therapies for cancer, malaria, bacterial and fungal infections, neurological and cardiovascular diseases, and autoimmune disorders (7). Many agricultural chemicals are also natural product-derived (8, 9). Fungi are particularly prolific sources of bioactive secondary metabolites (10) and have contributed in spectacular and indispensable ways to improvements in human and animal health (Table 1). The best known examples are the β-lactam antibiotics, which include penicillins and cephalosporins (Table 1). It is difficult to overstate the tremendous effect these compound classes have had on global health because of their effectiveness against bacterial infections. Moreover, the success of penicillins effectively spawned the development of major technological advances in microbiology, chemistry, biochemistry, and engineering and contributed in many ways to the establishment of the modern pharmaceutical industry. Many other fungal metabolites with valuable activities are known (Table 1), and further examples are incorporated into the discussion below.

One ongoing question regarding the dimensions of fungal biodiversity relates to judging the potential for discovery of even more novel and useful secondary metabolites, as well as the likelihood of further applications for those that are already known. Often one reads provocative statements such as "To date, fewer than 7% of the more than 1.5 million species of fungi thought to exist have been investigated for bioactive components" (11). Such statements are based on the number of metabolites described in the literature and the number of species from which those metabolites have been reported (12). Ultimately, no one really knows what percentage of the total number of species has been tested for biologically active compounds, but it is in all probability much higher. Our experience and that of our colleagues who have worked in the pharmaceutical and agrochemical industries tells us that far more species have been surveyed for secondary metabolites of interest over the decades in discovery screening programs than the literature would indicate, because inactive, strongly toxic, redundant, or unproductive fermentation extracts and products are rarely pursued, let alone published or otherwise documented (13, 14). Recognition of already known compounds in fungal fermentations—a process referred to as dereplication (15, 16)—is a vital step in natural product discovery processes. The high frequency of encounters with known compounds in screening programs represents a substantial resource cost that generally affords little value. Although improvements in the efficiency of dereplication processes continue to develop with advances in relevant analytical technologies (16), the costs in time and effort associated with "rediscovery" of known metabolites pose a significant obstacle to natural product discovery efforts. Such findings generally result in disinterest, and certainly not in publication. Other phenomena, such as compound decomposition or loss during separation efforts or the possibility that mixtures may exert effects not detected in purified/individual metabolites, may ultimately prevent identification of secondary metabolites that may be present.

Fermentations of most strains in major service collections have probably been examined for biologically active compounds at some level (17), yet most of this work likely is undocumented. For example, in the past, pharmaceutical companies would routinely buy strains from major service collections when the collection staff made routine transfers of their stock collections. It is impossible to know how many fungi have really been screened or how many compounds have been produced, detected, purified, and partially or fully characterized but then discarded for lack of potency and target specificity in the assays being employed for screening. To complicate such speculation even further, the window of desired biological activity in a given screening program often may be quite narrow, e.g., inhibition of bacterial type II topoisomerases, and negative data from these efforts are seldom considered worthy of publication. What is clear is that the numbers of possible applications for fungal metabolites in medicine, agriculture, cell biology, and consumer products are limitless and constantly evolving. Even well-known metabolites, previously discarded for one application, e.g., an antibiotic,

TABLE 1 Fungal metabolites that have been developed into pharmaceutical, agrochemical, and cosmetic products

Metabolite	Source species	Structural classification	Commercial product(s)	Mode of action	Indication(s)
Penicillins G and V	*Penicillium rubens*, *Penicillium chrysogenum*	Nonribosomal peptide	Benzylpenicillin and phenoxymethylpenicillin; 6-aminopenicillanic acid derived from penicillin G is acylated to make semisynthetic penicillins with diverse side chains, e.g., piperacillin, amoxicillin, and ampicillin	Interferes with biosynthesis of cell wall peptidoglycans	Deep-seated infections caused by Gram-positive and some Gram-negative bacteria
Cephalosporin C	*Acremonium chrysogenum*	Nonribosomal peptide	The 7-amino cephalosporanic acid core is used to produce numerous derivatives, including cephalothin, cephalexin, cephradine, and cefadroxil	Interferes with biosynthesis of cell wall peptidoglycans	Treatment of Gram-positive and some Gram-negative bacterial infections
Pleuromutilin	*Clitopilus passeckerianus*, other *Clitopilus* spp.	Diterpene	Precursor for the partial chemical synthesis of topical antibiotic retapamulin (Altabax) and animal antibiotics tiamulin and valnemulin (Econor)	Targets peptidyl transferase center of bacterial ribosome	Topical treatment of infections from Gram-positive bacteria, treatment of Gram-positive bacterial infections in livestock
Fusidic acid	*Acremonium fusidioides*	Triterpene	Usidin, Fucidin, Fucicort, Fucibet, Taksta, and others	Prevents translocation of the elongation factor G from the ribosome	Topical antibiotic for Gram-positive bacterial infections including methicillin-resistant *Staphylococcus aureus*; under development for prosthetic joint infections
Strobilurins A–D and other strobilurins and oudemansins	*Strobilurus tenacellus*	Polyketide with benzoyl CoA starting unit	Templates for synthetic family of β-methoxyacrylate strobilurins including azoxystrobin, fenamidone, fluoxastrobin, kresoxim methyl, and trifloxystrobin	Targets the mitochondrial cytochrome b and interferes with respiratory electron transport	Fungicides for broad range of plant pathogenic fungi
Griseofulvin	*Penicillium griseofulvum*, *Penicillium aethiopicum*, *Penicillium Coprophilum*, and other *Penicillium* spp.	Halogenated polyketide	Fulcin, Fulsovin, Grisovin, and others	Inhibits mitosis by binding to tubulin and preventing tubulin polymerization	Treatment of fungal infections of the skin, hair, and nails
Pneumocandin B_0	*Glarea lozoyensis*	Nonribosomal peptide acylated to polyketide	Precursor of the partial chemical synthesis of caspofungin (Cancidas)	Inhibits β-1,3 glucan synthase and cell wall biosynthesis	Treatment of systemic fungal infections
FR901379	*Coleophoma cylindrospora* (= *C. empetri*)	Nonribosomal peptide acylated to fatty acid	Precursor of the partial chemical synthesis of micafungin (Mycamine, Fungiguard)	Inhibits β-1,3 glucan synthase and cell wall biosynthesis	Treatment of systemic fungal infections
Echinocandin B	*Aspergillus pachycristatus* and other *Aspergillus* spp. of section *Nidulantes*	Nonribosomal peptide acylated to fatty acid	Precursor of the partial chemical synthesis of anidulifungin (Eraxis)	Inhibits β-1,3 glucan synthase and cell wall biosynthesis	Treatment of systemic fungal infections
Enfumafungin	*Hormonema carpetanum*	Triterpene glycoside	Precursor of the partial chemical synthesis of experimental drug Scy-078 that is in phase II clinical trials	Inhibits β-1,3 glucan synthase and cell wall biosynthesis	Treatment of systemic fungal infections

Lovastatin (monacolin K)	*Aspergillus terreus*, *Monascus purpureus*, also occurs in basidiomata of *Pleurotus ostreatus*	Polyketide, product of two highly reducing polyketide synthases	Mevacor Simvastatin (Zocor, Simvacor, Simvastin, and other brand names) is made by semisynthesis from lovastatin	Inhibits 3-hydroxy-3-methylglutaryl-coenzyme A reductase, prevents conversion of mevalonate into cholesterol	Treatment of hypercholesterolemia to reduce risk of cardiovascular disease
Compactin (Mevastatin)	*Penicillum citrinum*, *Penicillum solitum*, and other *Penicilium* spp.	Polyketide, product of two highly reducing polyketide synthases	Although recognized as the first statin, never approved as a drug. Used for biotransformation or partial chemical synthesis from mevastatin (Pravachol, Selektine, Lipostat, and other brand names)	Inhibits 3-hydroxy-3-methylglutaryl-coenzyme A reductase, prevents conversion of mevalonate into cholesterol	Treatment of hypercholesterolemia to reduce risk of cardiovascular disease
Cyclosporin A	*Tolypocladium inflatum*	Nonribosomal peptide	Cyclosporine A (Sandimmune, Neoral, Restasis, Gengraf, and other names)	Binds to cyclophilin A, resulting in calcineurin inhibition, which activates transcription of interleukin 2. Also inhibits lymphokine production and interleukin release	Prevention of organ transplant and tissue graft rejection
Mycophenolic acid	*Penicillium brevicompactum* and other *Penicillium* spp.	Meroterpenoid	Used to produce mycophenolate mofetil (Cellcept), mycophenolate sodium (Myfortic)	Inhibits inosine monophosphate dehydrogenase (IMPDH). T and B lymphocytes are heavily dependent on the activity of IMPDH to make DNA to proliferate	Prevention of organ rejection following kidney, liver, or heart transplants
Myriocin (ISP-I)	*Isaria sinclairii*	Amino acid lipid	Structural template for synthesis of fingolimod (Gilenya)	Activated form binds to extracellular G protein-coupled receptors and prevents release of lymphocytes that enter the CNS	Treatment of multiple sclerosis
Ergotamine	*Claviceps purpurea*, *Claviceps fusiformis*, and *Claviceps paspali*	Prenylated nonribosomal peptide	Ergotamine tartrate (Ergomar, Migril, and others), dihydroergotamine mesylate (Migranal), or combined with caffeine (Cafergot, Cafetrate, and other names), dihydroergotamine (DHE 45)	Neurotransmitter mimic, agonists of 5-OH tryptamine (5-HT2) receptor 1B, dopamine, norepinephrine, and serotonin receptors	Vasoconstrictor used as antimigraine agent, also combined with belladonna and phenobarbital for relief from menopausal hot flashes
Ergometrine (ergonovine)	*C. purpurea*, *C. fusiformis*, and *C. paspali*	Prenylated nonribosomal peptide	Ergometrine maleate (Ergotrate, Ergovin, and others); Methylergometrine is a synthetic version, sometimes combined with oxytocin (Syntometrine) for veterinary usage	Serotonin antagonist, activating (or blocking) 5-HT2 receptors	Treatment of postpartum haemorrhage
			Used for partial chemical synthesis of lysergic acid diethylamide, LSD-25, LSD (Delysid)		Hallucinogen, treatment of psychosis and depression
Ergocryptine	*C. purpurea*, *C. fusiformis*, and *C. paspali*	Prenylated nonribosomal peptide	Partial chemical synthesis of bromocriptine (Parlodel and other names)	Agonist of dopamine D_2 receptors and various serotonin and adrenergic receptors	Treatment of reproductive disorders, e.g., galactorrhoea, prolactindependent mammary carcinoma, amenorrhoea, acromegaly or anovulation, complications, e.g., osteoporosis. Bromocriptine is also used in the treatment of Parkinson's disease

(continued)

TABLE 1 Fungal metabolites that have been developed into pharmaceutical, agrochemical, and cosmetic products *(continued)*

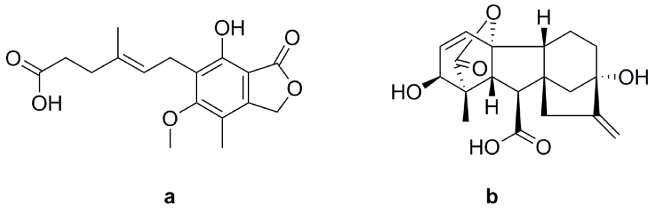
Metabolite	Source species	Structural classification	Commercial product(s)	Mode of action	Indication(s)
Mizoribine	*Penicillium brefeldianum*	Imidazole nucleoside	Bredinin and other names	Competitive inhibitor of IMPDH	Immunosuppressant used for renal transplants in Japan, Korea and China
Kojic acid	*Aspergillus oryzae. Aspergillus tamarii, Aspergillus flavus*	Pyrone derived from glucose	Often formulated as kojic dipalmitate to increase stability and shelf life	Inhibits tyrosinase and suppresses melanogenesis	Antioxidant in cosmetic products, used to lighten skin color and treat abnormal hyperpigmentation
PF1022A	*Rosellinia* sp.	Nonribosomal peptide	Emodepside is the bis-paramorphonyl-derivative of PF1022A	Disrupts target nematode neuromuscular function due to interactions with the latrophilin-like HC110-R G-protein coupled and SLO-1 potassium channels	Combined with praziquantel, another anthelmintic drug, to treat roundworm, hookworms, and tapeworms in cats as Profender
Fumagillin	*Aspergillus fumigatus*	Meroterpenoid	Bicyclohexyl ammonium fumagillin	Inhibits methionine aminopeptidase2 (MetAP2)	Control of nosema disease in honey bees caused by *Nosema apis*
Gibberellic acid (GA)	*Fusarium fujikuroi*	Diterpene	Produced by fermentation of *F. fujikuroi*	GA signaling promotes plant growth by overcoming DELLA-mediated growth restraint	Plant growth hormone for plant tissue culture and applications for certain high-value crops
α-Zearalanol (Zeranol)	*Fusarium* spp., including *Fusarium culmorum*, *Fusarium sporotrichioides*, *Fusarium equiseti*, and *Fusarium semitectum*	Polyketide, product of a highly reducing and a nonreducing polyketide synthase	Produced by fermentation of zearalenone, then hydrogenation to form α-zearalanol, formulated as Ralgro implants	An estrogen mimetic that binds to mammalian estrogen receptors α (ER α) and ER β	An anabolic agent used to increase the rate of weight gain and feed conversion in cattle in North America

can suddenly become recognized for new and exciting biology. In our opinion, exploration of fungal chemistry with the aim of finding new applications is likely to be far more productive than surveying what one believes are new fungi using dated screening methods, e.g., growth inhibition of typical model strains of human pathogens, and certainly more productive than simple chemical screening with no biological detection method at all.

No example illustrates the unforeseen value of "ordinary" fungal metabolites better than mycophenolic acid (MPA) (Fig. 2a, Table 1) (18). MPA was discovered by Bartolomeo Gosio from *Penicillium brevicompactum* in 1893, and it has the distinction of being the first antibiotic to be purified in crystalline form (19). It inhibited the growth of *Bacillus anthracis*, but quantities were insufficient for further investigation. Its medical application as an antibiotic was later investigated by C. L. Alsberg and O. M. Black in 1912 (20) and by Howard Florey's team at Oxford in 1946 (21), but it was discarded as a potential antibiotic due to its toxicity. During the 1960s and 1970s, MPA was recognized to have remarkable biological activities against viruses and certain tumor types, and its mode of action as an inhibitor of inosine 5′-monophosphate dehydrogenase, an enzyme required for the biosynthesis of guanine, was elucidated. Depletion of the cellular guanine derivative pool, therefore, accounted for many of MPA's effects. These mechanistic studies led to explorative studies of MPA as an oral treatment for psoriasis (22). Inhibition of this critical metabolic pathway in more susceptible human T and B lymphocytes was later exploited in the development of the immunosuppressive agent mycophenolate mofetil, marketed under the names CellCept (Hoffmann LaRoche) and Myfortic (Novartis).

Beyond its impact as the precursor for this important new class of immunosuppressant drugs, MPA illustrates the current transformation underway in modern natural product research. The complete biosynthetic pathway has been characterized in two producing fungi, *P. brevicompactum* (23, 24) and *Penicillium roqueforti* (25).

FIGURE 2 Structures of **(a)** mycophenolic acid and **(b)** gibberellic acid.



a b

MPA production is catalyzed by interaction of MpaA, a prenyl transferase, with a polyketide synthase (PKS), MpaC, producing 5-methylorsellinic acid, and MpaDE, which is a newly recognized hybrid enzyme, fusing a cytochrome P450 domain and a hydrolase domain, catalyzing the production of 5,7-dihydroxy-4-methylphthalide (23). By reconstituting the entire gene cluster in a heterologous host, *Aspergillus nidulans*, the functions of the genes encoding and regulating MPA biosynthesis have been characterized, and the feasibility of employing *A. nidulans* as a microbial cell factory for MPA has been established (23).

This review will briefly summarize general aspects of fungal secondary metabolism and recent developments in our understanding of how and why fungi make secondary metabolites, how these molecules are encoded, and how their encoding genes are distributed across the Fungi. Readers seeking in-depth and detailed works on fungal metabolites and their biosynthesis should consult information from more comprehensive works (3, 26–31) and a new database on functionally characterized secondary metabolite gene clusters (32). In addition, we will illustrate the breadth of fungal secondary metabolite diversity by highlighting recent information on the biosynthesis of some of the most important fungus-derived metabolites that have contributed to human health and agriculture, as well as those that have negatively impacted crop production, food distribution, and human-built environments.

WHAT ARE SECONDARY METABOLITES AND THEIR FUNCTIONS?

Classical definitions of secondary metabolites convey the idea that these compounds are not essential for growth or survival of the producing organism. While primary metabolism is generally shared by most organisms, a general characteristic of secondary metabolites is that they have a biased phylogenetic distribution due to multiple factors that have driven extinction and diversification of their encoding genes and gene clusters along narrower evolutionary lineages. In the laboratory, genes encoding the primary catalytic enzymes for secondary metabolite synthesis can often be silenced by gene disruption with relatively minor effects on vegetative growth or sporulation (33–35). However, the essentiality of secondary metabolism is frequently contextual, and some secondary metabolites that appear to fall into this category are indispensable to survival in nature, e.g., pigments, sunscreens, siderophores, and pheromones. What is clear is that their importance to the

organisms that produce them is underestimated because of their incomplete inventory and their unidentified or misunderstood functions. It has been argued that secondary metabolites and other small bioactive molecules of biogenic origin are so vital to the organisms that produce them that they should form the fourth pillar of "the central dogma of biology" along with DNA, RNA, and proteins (36). The line between secondary and primary metabolism can be blurry, and sometimes primary metabolites may behave like secondary metabolites, e.g., oxalic acid (37–39). Depending on their perceived essentiality and functions, secondary metabolites can include simple molecules derived directly from primary metabolism, such as alcohols, sugars, and organic acids. As an example, the discovery and characterization of quorum-sensing molecules and related systems in bacteria, and more recently in yeasts, have changed popular notions of the roles of low-molecular-weight compounds in microbial communities (40).

Fungi can release their secondary metabolites from cells as volatiles, excrete them into the environment, or incorporate them into structural elements of the cell, or they can remain cell-bound. The term "extrolites" has been suggested as an alternative descriptor for such compounds based primarily on the fact that their biological functions are assumed to be outwardly directed (41), but because the functions and sites of cellular biosynthesis for so many remain unknown, because some have self-directed functions, and also for the sake of continuity, we prefer to employ the classical term "secondary metabolite."

From a chemical standpoint, secondary metabolites are on one end of a continuum of metabolites and are categorized as compounds biosynthesized by pathways that employ primary metabolites as building blocks to assemble more complex molecules, such as polyketides, terpenoids, nonribosomal peptides, nuclear-encoded ribosomal peptides, and molecules of mixed biogenic origin arising from hybrid pathways. These categories, representative examples of each, and related recent developments are discussed in separate sections below. Thousands of secondary metabolites are known and catalogued in various compendia and databases (12). Many of them have been encountered and identified because of their association with a biological activity or phenomenon detected outside of laboratory settings (e.g., mycotoxins, phytotoxins, pigments). In a few groups of fungi, secondary metabolite profiles of fungal reproductive structures or cultures have been characterized and correlated with taxonomic and phylogenetic classifications (42–46). However, much of our knowledge of such chemistry has arisen through extensive screening programs conducted in the pharmaceutical and agricultural industries, as well as in academic laboratories, in search of small molecules with potentially useful effects. Bioassays of various kinds are typically used to guide the isolation of secondary metabolites from complex fermentation extracts using various chromatographic methods, and they are identified using spectroscopic techniques, most commonly nuclear magnetic resonance, mass spectrometry, and X-ray crystallography. These tools, along with databases that store spectral and other physical data, have grown rapidly in their capabilities, greatly expanding the ability to purify and identify such compounds and to detect and quantitate them in various samples.

Secondary metabolites that have been reported from both fungi and plants are not common. Of course, differences in secondary metabolism would be expected from these two domains of eukaryotic organisms due to differences in their basic metabolism and evolutionary origins of their respective biosynthetic pathways. Some of these differences stem from initial steps in biosynthetic processes that influence the prevalence of certain precursors. For example, decarboxylation of amino acids is much more common in plants than in fungi, affording amine precursors that are ultimately incorporated into many classes of plant alkaloids that have not been encountered among the fungi. The phenylpropanoid pathway so common among plants is not as prevalent among fungi. Similarly, secondary metabolic differences also occur between fungi and other microorganisms. For example, polyketide production in fungi generally involves poly-1,3-dicarbonyl precursors generated from acetyl and malonyl CoA. Bacteria also produce polyketides but can also utilize propionyl and butyryl CoA in such processes, leading to different kinds of end products. The dominant type of PKSs in plants are type III PKSs, such as chalcone synthase, a well-known plant type III PKS that catalyzes the first committed step in flavonoid biosynthesis.

Some secondary metabolites are made by both plants and fungi, such as gibberellins, diterpenoid plant hormones that can also be produced by certain fungi, albeit via different enzymatic pathways. The intensively studied gibberellic acids (GAs) (e.g., Fig. 2b) represent the best known unequivocal case of convergent secondary metabolites between plant-inhabiting fungi and plants. They are based on the tetracyclic *ent*-gibberellane terpenoid skeleton and are produced by plants, as well as the plant pathogen *Fusarium fujikuroi*, and are occasionally reported from other fungi (47, 48). In plants, the

GAs are a major family of hormones that promote and regulate plant growth. The commercial manufacture of GAs via fungal fermentation has enabled the development of these natural plant hormones into high-value plant growth regulatory hormones for plant tissue culture, horticultural, and biotechnological applications (Table 1). In *F. fujikuroi*, the GA gene cluster contains a pathway-specific geranylgeranyl diphosphate synthase gene (ggs2) that seems to form geranylgeranyl diphosphate exclusively for GA biosynthesis. In addition, a bifunctional CPS/KS (copalyl diphosphate synthase, CPS/*ent*-kaurene diphosphate synthase, KS)-encoding gene, *cps/k*s, catalyzes the cyclization steps to *ent*-kaurene, and four P450 monooxygenase-encoding genes and a desaturase gene carry out the oxidative steps and introduce a double bond, respectively.

The question of whether endophytic fungi may biosynthesize secondary metabolites in physiologically relevant quantities in living plants has received considerable attention. Ergot alkaloids are mycotoxins characteristically produced by fungi in the Clavicipitaceae that form biotrophic infections of grasses and some species of the *Convolvulaceae* (morning glories and relatives). The fungi and their metabolites can increase host plant resistance to herbivores and cause toxicity to consuming livestock (49). Some species of *Convolvulaceae* are infected by endophytic clavicipitaceous fungi (*Periglandula* spp.) that produce high concentrations of ergot alkaloids in seeds—up to 1,000-fold greater than in infected grasses. Swainsonine is an indolizidine alkaloid and is the toxic component that causes livestock poisoning upon consumption of locoweeds (*Astragalus*, *Oxytropis*, and *Swainsona* spp. of the *Fabaceae*) (50). It can also be found in certain species of *Ipomea* and *Turbina* (*Convolvulaceae*) and *Sida carpinifolia* (*Malavaceae*). In a manner analogous to ergot alkaloid production in grasses, the swainsonine in plant tissues results from infection by seed-transmitted endophytic fungi of the genus *Undifilum* (Dothideomycetes, Pleosporales) that produce swainsonine in living tissues of locoweeds. Swainsonine production in plants of the *Convolvulaceae* appears to be associated with an endosymbiotic fungus of the order Chaetothyriales (270).

Biosynthesis of secondary metabolites may be tightly regulated. Their expression is often triggered by chemical or environmental stimuli and coordinated with the development and morphogenesis of the producing organism (51–54). For example, early studies showing that certain bioactive secondary metabolites were strongly associated with *Aspergillus* sclerotia have been supported by more recent investigation of tissue-specific regulation of gene expression in sclerotia versus mycelia (52). Even so, details regarding their functions in nature remain elusive. In some cases, expression may be stimulated or repressed by association with other organisms (55, 56). Because some secondary metabolites are products from pathways that are expressed in a particular context or life stage, our perception of secondary metabolism in the laboratory depends on whether the context-dependent stimulus or life stage can be artificially replicated and whether pathways are expressed under typical laboratory environmental and nutritional conditions. Some products are easily produced in culture and thus are repeatedly encountered, while other pathways remain silent in artificially cultured strains. The long history of fermentation technology has documented that growing strains under varied nutrient and aeration regimes can profoundly change metabolite compositions and titers (57–59), e.g., plant-derived media versus defined media (60) or liquid versus solid media (61). Experimental efforts to capitalize on these phenomena have remained largely empirical. This knowledge gap has been a key rate-limiting factor in the identification of the natural functions of secondary metabolites, as well as in the discovery of new applications of secondary metabolites in medicine and agriculture.

Secondary metabolite gene clusters are the product of selection of complex traits over millions of years of evolution. Mechanisms contributing to the diversification of the end products include gene duplication, fusion, neofunctionalism, transposition, recruitment and loss of additional genes, and in some cases, horizontal gene transfer (HGT) (62, 63). During the past decade, apparent discontinuous distributions of many secondary metabolite pathways has been attributed to HGT, or in other words, the acquisition and stable inheritance of foreign genes by a recipient organism outside of the process of karyogamy. Although HGT is prevalent in prokaryotes and was responsible for the origin of eukaryotic mitochondria and plastids, through endosymbiosis, the degree to which HGT has impacted eukaryotic evolution remains controversial. In principle, the introduction of foreign DNA into fungal genomes might occur during unicellular or early developmental stages, when their nuclear DNA is relatively exposed to potential sources of HGT, but as of yet, the mechanisms of these proposed eukaryote HGT processes remain unknown. Nonetheless, if genes for a novel metabolic process move by HGT, they endow the recipient organism with the possibility of acquiring a new selective advantage that might promote the survival of the recipient and therefore also of the genes. Clustering of

biosynthetic, regulatory, and resistance genes would favor dispersal of entire pathways. Evidence for HGT can be particularly strong because the putatively transferred gene carries a nucleotide composition of the donor genome at the time of transfer from the donor organism and is often easily recognized in the case of bacterial-to-eukaryotic HGT. For example, the partially reducing, 6-methylsalicylic acid (6-MSA)-type polyketide synthases that are widespread across the Fungi may have originated from Actinomycete ancestor proteins (64). However, in the case of hypothetical interascomycete transfers, the direction of transfer is usually not apparent.

The principal method for identification of HGT is phylogenetic incongruence as measured by strong statistical conflict between the phylogenies of the putatively transferred gene and of the background genes of the organism. These types of analyses are highly dependent on the availability of genomes and sampling of species. What at first seems to be HGT of a biosynthetic cluster between phylogenetically distant species may later be recognized as congruent as new gene cluster orthologues from newly sequenced genomes become available. An especially high-profile example was the report of a putative HGT of the sterimatocystin gene cluster from *A. nidulans* (Eurotiales) to *Podospora anserina* (Sordariales) (65). The authors of this report verified that sterigmatocystin genes were transcribed, but they never tested whether *P. anserina* produced sterigmatocystin. Subsequently, we have independently observed sterigmatocystin production in at least one freshly isolated strain of *P. anserina* (J. Gloer, G. Bills, unpublished data). A broader analysis of fungal genomes inferred that the donor organism of the *P. anserina* sterigmatocystin gene cluster may have been a species in the Dothidiomycetes, e.g., *Rhytidhysteron rufulum* (66).

Neither of these reports took into account the widespread reports of sterigmatocystins or their biosynthetic analogues in other fungi belonging to the Sordariomycetes, including *Chaetomium* spp. (Sordariales) (67), *Aschersonia coffeae* (Hypocreales) (68), *Monicillium nordinii* (Hypocreales) (69, 70), *Calcarisporium arbuscula* (Hypocreales) (71), or other fungi in the Dothidiomycetes, including an unidentified species in the Microthyriaceae (72). Furthermore, orthologues of genes in aflatoxin-sterigmatocystin gene clusters have been recently detected in the genomes of the causal agent of chalkbrood disease of bees, *Ascosphaera apis* (Onygenales) (73), the soil fungus *Ochroconis mirabilis* (Venturiales) (74), and the tomato pathogen *Cladosporium fulvum* (Capnodiales) (75). Thus, sterigmatocystin-aflatoxin pathway genes appear to be more widely distributed across the Dothidiomycetes, Eurotiomyctes, and Sordariomycetes than previously thought (67), and the gaps will likely continue to be filled in as more genomes are sequenced. One wonders if new phylogenetic reconstructions based on the sterigmatocystin-aflatoxin pathway genes and associated housekeeping genes will eventually approach congruency once the analyses include data from the gene clusters from all of these fungi and others that have yet to be discovered. For the time being, our preference is to treat these putative discontinuous distributions as HGT hypotheses because unequivocal evidence is lacking and relatively few fungal genomes have been fully sequenced. As more fungal genomes become available, we predict that alternative evolutionary models will emerge that will explain these reticulate patterns of secondary metabolite distributions.

The events leading to secondary metabolite diversification are undoubtedly linked with speciation and niche radiation, but how selective pressures determine the genetic origins and distribution of these pathways, and their feedback on species fitness, remains obscure (76). Some secondary metabolites are critical to the ecological success of the Fungi because they mediate micronutrient acquisition and transport, virulence, intra- and interspecies communications, defensive mutualisms, protection against abiotic stress, and reproductive development and form pigments, among other functions (77). A puzzling aspect of the prevalence of secondary metabolism is that only a tiny fraction of secondary metabolites have a demonstrated effect that can be linked to a competitive advantage to the producer (78, 79). Design and interpretation of laboratory experiments to identify the natural function of a secondary metabolite discovered out of its natural context are notoriously difficult (79–81). Therefore, progress in dissecting the functions of microbial chemistry in nature has been limited to relatively few systems, such as plant and animal defensive mutualisms and host-pathogen relationships. Classic hypotheses regarding interference competition or defense have contributed explanations of the vast chemical repertoire of fungi and other microorganisms by incremental and reciprocal adaptations and counteradaptations between fungi and their symbionts, parasites, hosts, and predators, shaped by mutual selection (82–86). These hypotheses have underpinned fungal metabolite discovery projects since the discovery of the first antibiotics in the late 19th century and continue to guide modern fungal natural products research within the pharmaceutical and agrochemical industries.

Concepts of some secondary metabolites as agents of chemical defense or offense are further supported by observations that antibiotic resistance genes are

common in nature and that the organisms that produce them often have complex self-resistance mechanisms (87–89). As in bacterial secondary metabolite gene clusters, evidence that fungal gene clusters can encode auxiliary target proteins to evade self-toxicity from their own metabolites is accumulating. One of the best examples of self-resistance genes for a fungal secondary metabolite occurs in the pathways for the hydroxymethylglutarate (HMG)-CoA reductase inhibitors, lovastatin and compactin (Table 1). These metabolites are weakly antifungal because they block production of mevalonate, which in turn affects steroid and terpene synthesis. The lovastatin and compactin gene clusters in the producing fungi carry an ancillary HMG-CoA reductase gene (*Aspergillus terreus*, *lvrA*; *Monascus pilosus*, *mokG*; *Penicillium citrinum*, *mlcD*) which confers resistance to the pathway product by producing the mevalonate precursor during compactin and lovastatin biosynthesis (90, 91). A strain of *P. citrinum* with mutated *mlcD* was 200-fold more sensitive to compactin and produced lower ergosterol levels than the parent strain (90). *Aspergillus fumigatus* produces the epipolythiodioxopiperazine gliotoxin from a bimodular nonribosomal peptide synthetase (NRPS) as an immunosuppressive virulence factor during pathogenesis (92). Disruption of the *gliT* gene, encoding a gliotoxin reductase, in the biosynthetic gene cluster resulted in reduced production of gliotoxin and sensitization of the mutants toward gliotoxin (93). The immunosuppressive metabolite MPA, discussed above, potently inhibits inosine-5′-monophosphate dehydrogenase (IMPDH). Characterization of the MPA biosynthetic gene clusters from *P. brevicompactum* and *P. roqueforti* revealed an auxiliary copy of an IMPDH-encoding gene (*mpaF*) embedded within the cluster that is resistant to MPA (24, 25). Self-toxicity also may be mitigated by tight regulation of product biosynthesis during the critical stages of the life cycle (53), by transport proteins specific for biosynthetic pathways, and by product-specific trafficking in specific cells and intracellular compartments (89, 94).

RUDIMENTARY SECONDARY METABOLITES: EVOLUTION OF THE FIRST BIOACTIVE SMALL MOLECULES

The origins of secondary metabolites are likely to coincide with the origins of self-replicating biological molecules (95–97). One can easily imagine how simple amino acids reacted with each other and other organic molecules to form new molecules that interacted with the earliest macromolecular processes; when these rudimentary secondary metabolites exerted positive effects on macromolecular processes and ultimately on reproduction of the cell, they were retained as a new cellular process. Many consider that the early chemical and structural interaction sites of primordial amino acids, which gradually evolved into polypeptides, still retain some level of that specificity today (98). Indeed, the antagonistic activity of antibiotics, among their other varied small-molecule interactions, could simply be one facet of their primordial origins. And while not constituting proof of the role of secondary metabolites in nascent biochemical evolution, their ubiquity is consistent with the belief that bioactive small molecules have been critical to forging diversification of all organisms throughout molecular history (36, 96).

Many of these rudimentary metabolites (e.g., Fig. 3) are amino acid derivatives or by-products of amino acid biosynthesis. Given the importance of amino acids in cell composition and function and as building blocks of many secondary metabolites, the nonribosomal peptides may be the oldest secondary metabolites (97). One of the simplest fungal secondary metabolites is hadacidin (*N*-formyl hydroxyaminoacetic acid) (Fig. 3a), a compound found in *Penicillium frequentans* and other *Penicillium* spp. (99). Its name is based on its inhibition of human adenocarcinoma (100, 101). Furthermore, it potently inhibits plant growth (102). Hadacidin inhibits purine biosynthesis by inhibition of adenylosuccinate synthetase. Isotope labeling studies indicated that it is formed by *N*-oxygenation of glycine to yield *N*-hydroxyglycine, followed by *N*-formylation to give the hydroxamic acid. Another intriguing example of primitive secondary metabolites is provided by cyclic dipeptides (CDPs; diketopiperazines or dioxopiperazines) formed by the linkage of two amino acids. CDPs have been suggested to be among the most ancient classes of signaling molecules (98). These compounds are ubiquitous in biological systems and have even been found in extraterrestrial meteorites (103). In abiotic systems, CDPs are produced from unprotected linear dipeptides, and once formed, they remain relatively stable. The lack of free C- and N-terminal groups enables CDPs to resist human digestion, so they are attractive templates for the development of protein-based drugs.

Aside from spontaneous formation in primordial stews, they are produced by two major biosynthetic routes: as products of dedicated NRPSs (see below) or, more interestingly, by a recently discovered enzymatic process employing cyclodipeptide synthases (CDPSs) (104–106). CDPSs commandeer aminoacyl-tRNAs and employ them in the formation of a cyclodipeptide link

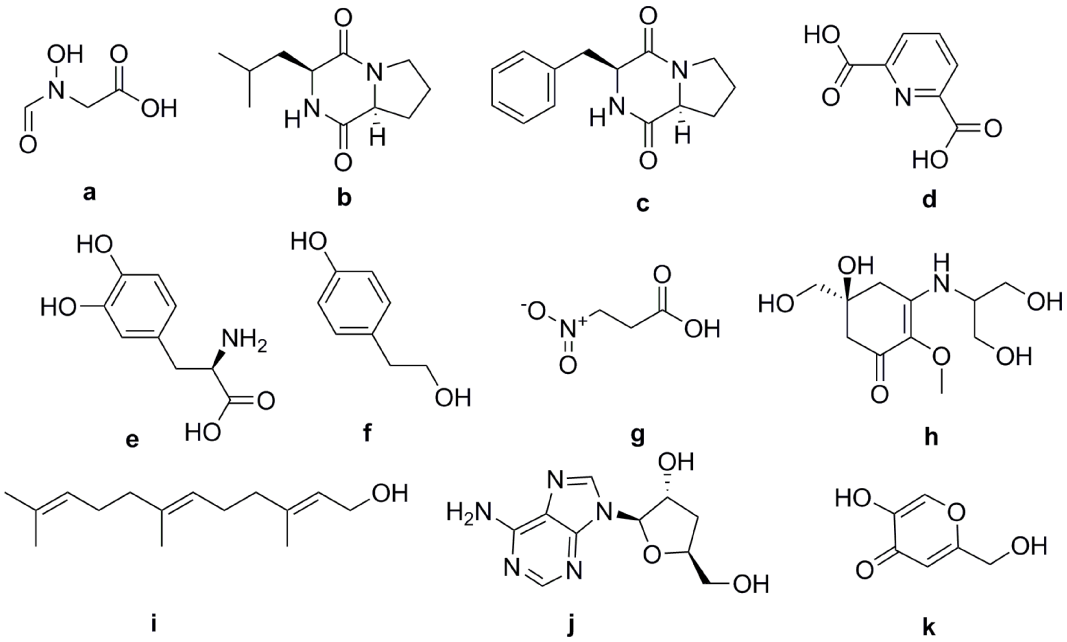


FIGURE 3 Some rudimentary fungal metabolites. **(a)** Hadicidin. **(b)** Cyclo (L-leucine-L-proline). **(c)** Cyclo (L-proline-L-phenylalanine). **(d)** Dipicolinic acid. **(e)** L-DOPA. **(f)** Tyrosol. **(g)** 3-Nitropropionic acid. **(h)** Mycosporine serinol. **(i)** Farnesol. **(j)** Cordycepin. **(k)** Kojic acid.

from two aminoacyl transfer RNAs using a ping-pong mechanism that transfers an aminoacyl moiety of the first aminoacyl tRNA onto a conserved serine residue, yielding an aminoacyl enzyme. At the same time, CDPSs divert the flow of loaded tRNAs away from the ribosomal machinery (Fig. 4). The covalently bound intermediate recognizes the aminoacyl-binding site of the cognate aminoacyl tRNA and reacts with the aminoacyl moiety of the second aminoacyl tRNA, forming a dipeptidyl enzyme. This novel enzyme family represents an interesting intermediate between amino acid tRNA-synthetase-based protein synthesis and other nonribosomal processes for peptide bond formation (Fig. 4). Genome mining indicated the presence of CDPS genes in more than 50 bacteria and in at least four *Fusarium* species (104), indicating the presence of a novel mode of fungal peptide synthesis in these fungi.

Many CDPs display biological activities such as inhibition of growth of fungi, bacteria, and human tumor cells. For example, a single fermentation of *A. fumigatus* can produce at least seven CDPs—cyclo(L-Leu-L-Pro) (Fig. 3b), cyclo(L-Phe-L-Pro) (Fig. 3c), cyclo(Gly-L-Pro), cyclo(L-Pro-L-Pro), cyclo(L-Pro-L-Val), cyclo(L-Leu-L-trans-4-OH-Pro), and cyclo(L-Phe-L-trans-4-OH-Pro)—most of which had antibacterial activity. Cyclo(L-Phe-L-Pro), a phytotoxin from *Rosellinia necatrix* (107), attenuated the development of physical dependence on morphine (108). CDPs also provide structural cores for a number of complex fungal secondary metabolites that incorporate diketopiperazine units, including chaetocin, gliotoxin, verticillin B, and many more.

Another simple amino-acid-derived metabolite is dipicolinic acid (pyridine-2,6-dicarboxylic acid) (Fig. 3d), a by-product of the lysine biosynthetic pathway. The synthesis of dipicolinic acid in fungi is thought to be similar to that in bacteria and may occur by condensation of aspartyl-β-semialdehyde with pyruvate to form 2-keto-6-aminopimelic acid, followed by cyclization to form 3,5-dihydro-4-hydroxyl-dipicolinc acid, a hypothetical precursor to dipicolinic acid (109, 110). It is a major component of bacterial endospores, and it is also found in *Penicillium* spp. and various entomopathogenic fungi of the Hypocreales (111). Its role as an extracellular metabolite in *Penicillium* spp. is thought to involve scavenging of external metal ligands, e.g., Mg^{2+}, while in hypocrealean fungi, it may act as an insect toxin (111).

The nonproteinogenic amino acid L-3,4-dihydroxyphenylalanine (L-DOPA) (Fig. 3e) can function as a secondary metabolite and a virulence factor in fungi. *Cryptococcus neoformans* causes invasive infections in patients with immune systems impaired by AIDS, cancer chemotherapy, or immunosuppressive drug treatments. The fungus produces melanin by transforming shikimic acid to aromatic amino acids, resulting in the formation of L-DOPA. Melanization occurs during growth in its natural habitat in bird excrement and during host

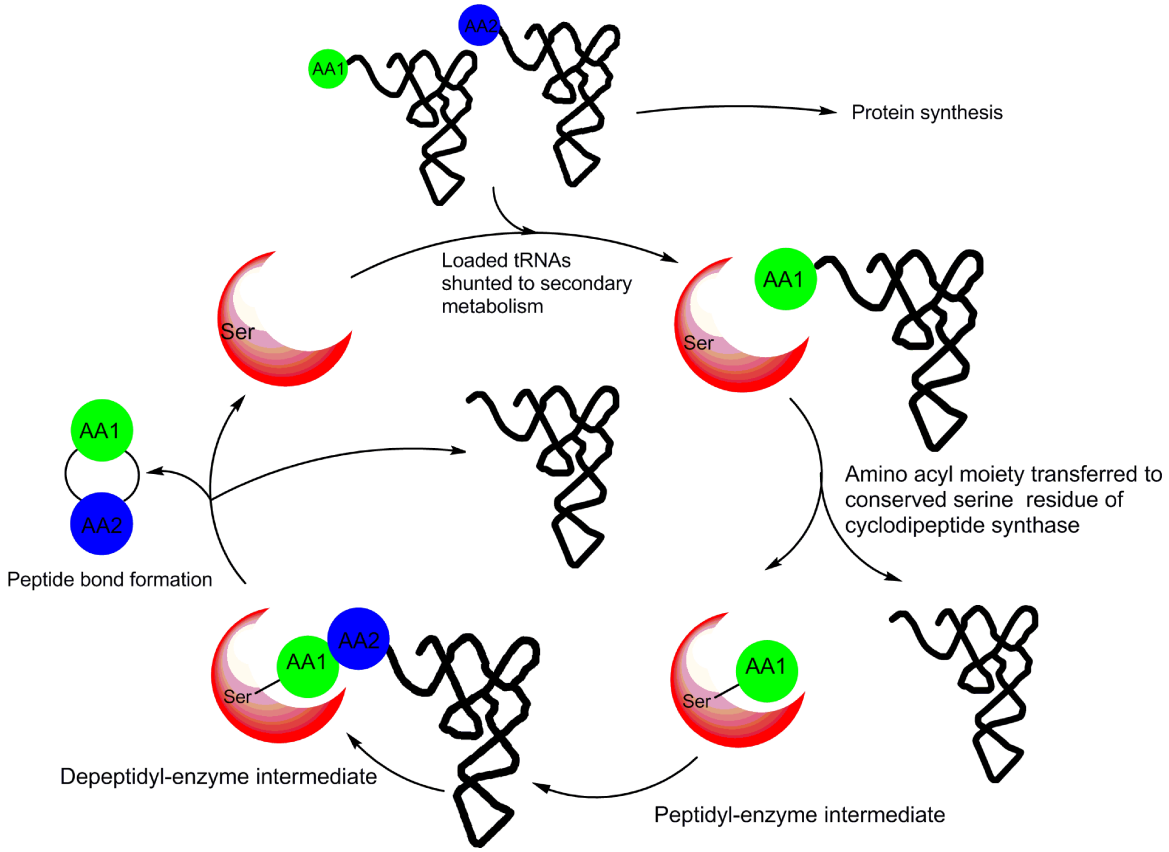


FIGURE 4 Schematic representation of a cyclodipeptide synthase biosynthetic pathway. A cyclodipeptide synthase (red) binds aa-tRNAs (black) via a serine residue (Ser) to produce cyclodipeptides. aa-tRNAs are generated from an amino acid, ATP, and tRNAs.

infection by the action of a laccase on a variety of precursors, including L-DOPA, D-DOPA, dopamine, and norepinephrine (112). Disruption of genes essential for melanin production in *C. neoformans* caused a reduction in fungal dissemination and lethality in murine infection models (113, 114). Tyrosol (Fig. 3f) is another amino acid-derived metabolite produced by *Candida albicans*, presumably through the decarboxylation of tyrosine; it acts as a quorum-sensing molecule by stimulating the yeast-to-hypha conversion (115).

3-Nitropropanoic acid (3-NPA) (Fig. 3g) has been widely found in some leguminous plants (116) and in some fungi, including *Penicillium* (117) and *Aspergillus* (118) spp. and as a necrotrophic toxin from *Septoria cirsii* (119). Consumption of moldy sugar cane contaminated with 3-NPA produced by *Arthrinium* spp. has led to human deaths in China (120, 121). Its cell toxicity is caused by irreversible inhibition of mitochondrial succinate dehydrogenase (122). Current proposals regarding its biosynthesis are based on the incorporation of ^{14}C-, ^{15}N-, and ^{18}O-labeled substrates into 3-NPA and its derivatives in cultures of *Penicillium atrovenetum*. These experiments showed that ^{14}C is incorporated when the fungus is fed L-[4-^{14}C]aspartate and that an ammonium ion rather than nitrate is involved in forming the nitro group of 3-NPA. The process possibly occurs through a series of oxidations resulting in conversion of the amino group of aspartate to a nitro group with concomitant loss of the aspartate C1 as CO_2. *Phomopsis* species and other endophytic strains excrete high levels of 3-NPA in their culture media, thus raising questions about whether 3-NPA accumulation detected in living plant tissues could be of fungal origin (123).

To resist high-intensity ultraviolet (UV) radiation, cyanobacteria and fungi have evolved biosynthetic pathways to produce hybrid amino acid-containing metabolites called mycosporines to protect vital macromolecules from degradation (124, 125). Mycosporine-like amino acids, e.g., mycosporine serinol (Fig. 3h), were first identified in fungi because of their role in mediating UV-induced sporulation (126). Mycosporine-like amino acids are small (<400 molecular weight), colorless, water-soluble compounds composed of a cyclohexenone or cyclohexenimine chromophore that is

derived from a pentose phosphate pathway intermediate, e.g., sedoheptulose 7-phosphate, conjugated with the amino group of an amino acid or its imino alcohol. They are widespread in both the Ascomycota and Basidiomycota (127). Along with melanins, mycosporines absorb UV light, providing photo-protection for the organism. They may also play roles in osmotic regulation (11) and defense against oxidative stress (12). Recently, the biosynthetic pathways for mycosporine and mycosporine-like amino acid formation have been elucidated in cyanobacteria (128) and Actinomycetes (11). Genomic analysis revealed that orthologous biosynthetic potential exists in fungi, suggesting that fungal mycosporines are likely to be encoded by similar gene clusters (128).

Quorum sensing is a means of cooperative intercellular communication via accumulation of small messenger molecules in the environment. As the number of cells increases, the production of messenger molecules increases, and when messenger concentration reaches a threshold, the behavior of the population changes. Quorum sensing was first discovered in various bacteria, but more recently it has been recognized in fungi and has been shown to be relevant for the control of virulence factor expression in *C. albicans*. Supernatants from 2-day cultures of *C. albicans* inhibit the switch from yeast to hyphal growth in new cultures (129). Farnesol (Fig. 3i), a linear sesquiterpene alcohol derived from mevalonate, is the causative agent. *C. albicans* continuously releases farnesol into the environment during growth so that its concentration in the medium is roughly correlated with cell density (129). Farnesol is thought to be secreted by the cells to inhibit germ tube formation in the late stage of biofilm development where there is a high density of interwoven filamentous cells, and therefore, it promotes the dispersal of yeast cells to colonize new environments. Not only is morphological switching essential to *C. albicans* pathogenesis, but farnesol can act as a virulence factor of *C. albicans* by negatively affecting innate immune cells to promote inflammation and a nonprotective and dysfunctional response that fails to activate T cells and produce Th1-promoting cytokines (130). Farnesol can modulate the activity of a transcriptional regulator in *Pseudomonas aeruginosa* when applied as a pure compound or when produced during *C. albicans* coculture, resulting in decreased production of the secondary metabolites pyocyanin and *Pseudomonas* quinolone signal (2-heptyl-3-hydroxy-4-quinolone) while diminishing swarming (131, 132). Farnesol has also recently been implicated in mediating the yeast-hyphal transition in *Ophiostoma piceae* (133). Additionally, farnesol can induce apoptosis in *Aspergillus* and mammalian cells and inhibits conidial germination and growth in a broad array of fungi and bacteria. Although farnesol is derived from farnesyl pyrophosphate, a central metabolite in steroid and terpene metabolism, the enzyme responsible for hydrolysis to farnesol remains uncharacterized. However, enzyme extracts from *C. albicans* can catalyze formation of farnesol (134).

Nucleoside analogues further exemplify modified primary metabolites that function like secondary metabolites. Cordycepin (3′-deoxyadenosine) (Fig. 3j) is a naturally occurring toxic adenosine analogue and one of the bioactive constituents isolated from *Cordyceps militaris* (135) and *Ophiocordyceps sinensis* (136). Cordycepin enters the cell and is converted to 3′-dATP, which functions as both an RNA chain terminator and a competitive inhibitor of polyadenylate polymerase via interference with subunits of the cleavage and polyadenylation complex (137, 138). A putative biosynthetic pathway for cordycepin was recently proposed based on transcriptome analysis of *O. sinensis*. The analysis identified a putative adenosine kinase, an adenylate kinase, and a 5′-nucleotidase that mediate phosphorylation and dephosphorylation in the process, followed by reduction of adenosine diphosphate to 3′-dADP by a putative ribonucleotide reductase and dephosphorylation to cordycepin (139).

Kojic acid (Fig. 3k) was one of the earliest fungal metabolites to be characterized. It was first isolated from solid-state koji cultures of *Aspergillus oryzae* on rice (140). Kojic acid is a weak, broad-spectrum antibacterial (141), antioxidant, and tyrosinase inhibitor, but its role in the fungal life cycle remains obscure. Early isotope labeling studies suggested that glucose was converted directly to kojic acid by only two or three enzymes and that polyketide or amino acid pathways were not involved (142–145). Recently, its biosynthetic genes were identified by statistical analysis of comparative gene expression profiles (146). Biosynthesis was associated with a small coregulated gene cluster consisting of an oxidoreductase (*kojA*), a transporter (*kojT*), and a transcription factor (*kojR*).

STRUCTURAL CLASSES OF SECONDARY METABOLITES

Shikimate-Derived Metabolites

Shikimate is the deprotonated form of shikimic acid—a trihydroxy cyclohexenecarboxylic acid that provides the carbon skeletons for the aromatic amino acids

L-tryptophan, L-phenylalanine, and L-tyrosine via a multistep process leading through chorismate (the shikimate pathway) (147). Thus, any metabolites that incorporate aromatic amino acids in their construction are effectively shikimate-derived. The shikimate pathway is well studied because these essential aromatic amino acids are not produced by mammals and thus constitute important dietary requirements (148). This ancient eukaryotic pathway is highly conserved, because it is found in fungi, bacteria, and plants, and is to a substantial degree an important process involved in primary amino acid metabolism. However, the pathway has undergone diverse evolutionary processes leading to branches that produce a considerable array of secondary metabolites as well, many of which are aromatics and quinones. As noted above, shikimate is also involved in the formation of melanins, as well as mycosporine and mycosporine-like amino acids. Chorismate and subsequent products formed via the shikimate pathway are precursors for the biosynthesis of important isoprenoid quinones such as ubiquinones and menaquinone, which serve important roles in electron transport and as antioxidants. Secondary metabolic products of the pathway are particularly common in plants (flavonoids, coumarins, phenylpropanoids). Fungi tend to display somewhat different kinds of preferences among their shikimate-derived secondary metabolic products (149), because the enzymatic process in fungi is somewhat different from that in plants and bacteria. For example, in fungi, a single complex (AroM) performs five consecutive reactions of the seven basic steps in the pathway, converting 3-deoxy-D-arabino-heptulosonate-7-phosphate (initially formed from phosphoenol pyruvate and D-erythrose-4-phosphate) to 5-enol pyruvyl-shikimate 3-phosphate, which is converted to chorismate in the final step (150).

Simple aromatics often encountered among the fungi, such as benzoic acid derivatives, are commonly derived from this pathway and could be considered as further examples of rudimentary secondary metabolites. *Para*- and *ortho*-hydroxylation patterns tend to occur among shikimate-derived aromatics, in contrast with the *meta*-hydroxylation patterns typical of phenolics arising via the polyketide pathway, and can help in deducing the likely biosynthetic origin of an aromatic compound or unit. More complex shikimate-derived metabolites have been implicated in a variety of roles. For example, involutin (Fig. 5a), a diarylcyclopentenone secreted by the ectomycorrhizal fungus *Paxillus involutus*, reportedly plays a key role as an Fe^{3+} reductant in Fenton-based decomposition of organic matter by mobilizing embedded nutrients, thereby increasing their accessibility to the host plant (151, 152). Structurally related metabolites, including aryl benzyl lactones such as pulvinic and vulpinic acids, and diaryl quinones, such as polyporic acid, are often encountered as lichen or basidiomycete metabolites (42, 153). *A. terreus* builds related products from *p*-hydroxyphenyl pyruvic acid, e.g. butyrolactones and aspulvinones, from indole pyruvic acid, e.g., asterriquinones (154), and from phenyl pyruvic acid, e.g., phenguignardic acid (155). These types of compounds serve as pigments and antioxidants and can show other bioactivities. Compounds of mixed biogenetic origin that incorporate simple aryl substituents such as phenyl groups (e.g., the antifungal strobilurins, Table 1) are also commonly constructed in part from shikimate-derived precursors.

RiPPs: Ribosomally Synthesized and Posttranslationally Modified Peptides

The influx of sequenced fungal genomes during the past decade has revealed that certain fungal peptide toxins are encoded directly in precursor proteins that are ribosomally synthesized and posttranslationally modified to create toxic peptides (RiPPs). RiPPs are produced in the *Eukaryota*, *Bacteria*, and *Archaea*. Their biosynthetic genes are ubiquitous in genomes, and their products exhibit a vast structural diversity (106, 156, 157). Many RiPPs have cyclic structures that confer the advantage of evading degradation by exopeptidases while increasing structural rigidity that enhances accuracy for binding to their molecular receptors, leading to high binding affinity and biological activity. Furthermore, cyclic RiPPs can often resist denaturation upon heating; thus, their activity is often unaffected by cooking.

The amatoxins and phallotoxins are infamous mushroom toxins produced by various species in the genera *Amanita*, *Galerina*, *Lepiota*, and *Conocybe* (Agaricomycotina) (158–160). They consist of N-to-C cyclized peptides of eight and seven residues, respectively, that also contain several other posttranslational modifications including an unusual 2′-indole thioether bond between the Cys and Trp residues, resulting in a unit referred to as tryptathionine that forms an inner ring structure. The genes (*AMA1* and *PHA1* in *Amanita bisporigera*) encode proproteins of 35 and 34 amino acids, respectively, in which the amino acid sequences found in the mature toxins are flanked by conserved amino acid sequences. The proproteins are processed by proteolytic cleavage to produce linear peptides comprising the amino acids found in the mature toxins (161). The precursor peptides contain a 10-residue leader peptide, an 8- or 7-residue core peptide for amatoxins

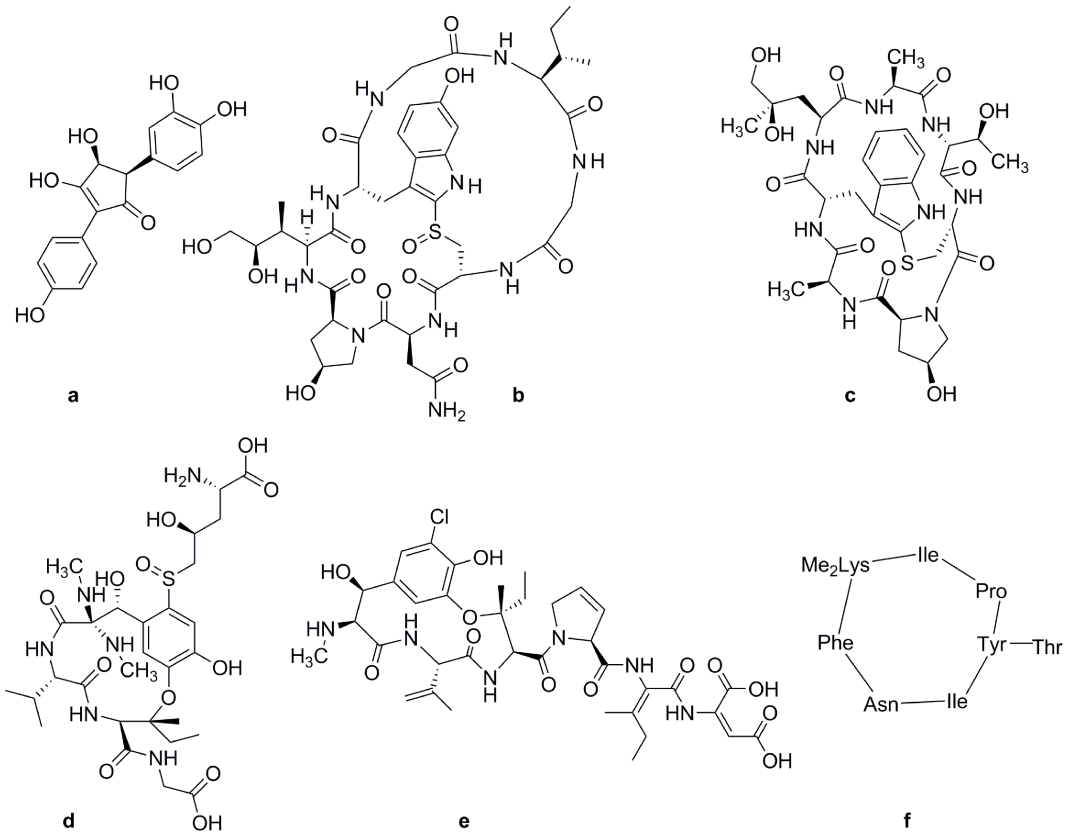


FIGURE 5 Some fungal metabolites derived from the shikimic acid pathway and ribosomally synthesized and posttranslationally modified peptides (RiPPs). **(a)** Involutin. **(b)** α-Amanitin. **(c)** Phalloidin. **(d)** Ustiloxin A. **(e)** Phomopsin A. **(f)** Epichloëcyclin A.

and phallotoxins, respectively, and a C-terminal recognition sequence. The tryptathionine in the amatoxin α-amanitin (Fig. 5b) is oxidized to the sulfoxide and hydroxylated on the indole. Other subunits displaying posttranslational modifications include a bishydroxylated isoleucine and *cis*-4-hydroxy proline. Despite their similar structures, the biological activities of amatoxins and phallotoxins are surprisingly different. Amatoxins are potent inhibitors of RNA polymerase II. Phalloidin and phallacidin bind to F-actin (162); thus, they have been developed into commercial kits in which they are conjugated with fluorescent dyes to visualize the cytoskeleton.

RiPPs are well known in other organisms, but several recent studies point to their widespread occurrence in the Ascomycota. Ustiloxins (e.g., ustiloxin A; Fig. 5d) are toxic cyclic peptides from rice false smut, a rice disease caused by *Villosiclava* (=*Ustilaginoidea*) *virens* (163), and also have been identified in *Aspergillus flavus* strains cultured on maize (164). They are generally constructed of a Tyr-Val/Ala-Ile-Gly tetrapeptide sequence and cyclized via an ether linkage of the sidechains of modified tyrosine and isoleucine units. It was long thought that ustiloxins A and B were biosynthesized in *V. virens* by a nonribosomal peptide synthetase (165). Analysis of coregulated transcripts in *A. flavus* identified a 15-gene cluster. The cluster's core gene, *ustA*, encoded UstA, a peptide with 16 repeats of a Tyr-Ala-Ile-Gly tetrapeptide unit that is converted into the cyclic moiety of ustiloxin B (164, 166). This highly repetitive sequence for the core peptide indicates that the *ustA* gene might have undergone selection for increased production of ustiloxin B. Adjacent to *ustA* in the gene cluster are gene(s) that encode a peptidase (*ustP1* and *ustP2* as a putative single gene), indicating that a peptide sequence served as the enzyme's substrate. Norvaline is then transferred to the activated aromatic ring of the ustiloxin tyrosine residue by a glutathione S-transferase (UstS). Sequence similarity searches with the ustiloxin biosynthetic gene cluster from *A. flavus* were used to probe the genome of *V. virens* (167) to identify a 15-gene cluster containing a gene encoding a precursor protein with five Tyr-Val-Ile-Gly and three Tyr-Ala-Ile-Gly repeats for ustiloxins A and B, respectively.

Phomopsins (e.g., phomopsin A; Fig. 5e) comprise a family of cyclic hexapeptide mycotoxins produced by *Diaporthe leptostromiforme* that infects lupins (*Lupus* spp.) (168). Phomopsins are comprised of a 13-membered macrocyclic ring formed by an ether bridge linking an isoleucine residue to the aromatic ring of a highly modified tyrosine unit. Lupin forage contaminated with phomopsins causes lupinosis, a liver disease of sheep and cattle. Phomopsins are potent microtubule inhibitors, and their toxicity is caused by their binding of the phomopsin tripeptide side chain to the vinca domain of tubulin, resulting in potent antimitotic activity (169). The ustiloxins contain a similar modified-tyrosine-containing macrocycle. The putative precursor peptide sequence (YVIPID) was used to search the genome of *D. leptostromiforme*, resulting in the identification of a precursor peptide gene, *phomA*, whose product contained five repeats of short peptides with the sequence YVIPID. The Phom gene cluster also included a copper-containing tyrosinase (PhomQ), an S41 family peptidase (PhomP1), an *S*-adenosylmethionine-dependent methyltransferase (PhomM), and other functionally unknown proteins (170). Disruption of PhomQ, the tyrosinase responsible for formation of the cyclic scaffold of phomopsins, abolished phomopsin biosynthesis.

A combination of two functionally unknown genes (*ustYa* and *ustYb*) and the *ust B* precursor gene were used to search genomes of other *Aspergillus* spp. At least 94 sets of precursor candidates were computationally identified and classified, indicating that RiPPs are likely to be widespread in the Ascomycota. Furthermore, one of these newly identified candidate RiPPs with two types of repeated sequences was identified by gene inactivation in *A. flavus* and found to be a novel bicyclic peptide that was named asperipin-2a (171). Similarity searches with the PhomA, PhomQ, and PhomM homologous proteins identified an additional 27 gene clusters similar to that involved in phomopsin biosynthesis in a variety of ascomycetes (170). Furthermore, Phom-like gene clusters were recognized in strains of *Beauveria bassiana* and *Metarrhizium aniosipliae*, leading to the fermentation, purification, and characterization of new phomopsin A analogs from *B. bassiana* (phomopsin Z) and *Metarhizium anisopliae* (metarisin A and B).

One of the most highly expressed *in planta* fungal transcripts in *Epichloë* endophyte-infected grasses, designated *gigA* (for grass-induced gene), was identified from tall fescue and perennial ryegrass associations (172). The protein sequence encoded by *gigA* was predicted to be a variable 27-amino acid repeat with an N-terminal signal sequence, indicating that it should be translocated into the Golgi apparatus. The region adjacent to *gigA* contained a kexin protease gene for processing GigA for the liberation of multiple cyclic nonapeptides, the epichloëcyclins, into the host plant. The structures of epichloëcyclins A to E (e.g., epichloëcyclin A; Fig. 5f) were determined in part by the sequences of individual repeat units within the GigA protein, which differed between fungal strains and within the proprotein itself. Such processing of a single protein into multiple different small peptides is a remarkable and novel mode for generating chemical diversity. Gene deletion of *gigA* led to the elimination of all epichloëcyclins, with no conspicuous phenotypic impact on the host grass (172). The *gigA* gene cluster has also been identified in other plant-symbiotic Clavicipitaceae including *Periglandula ipomoeae* and *Atkinsonella hypoxylon*, suggesting a possible widespread bioactive role in plant-fungus symbioses.

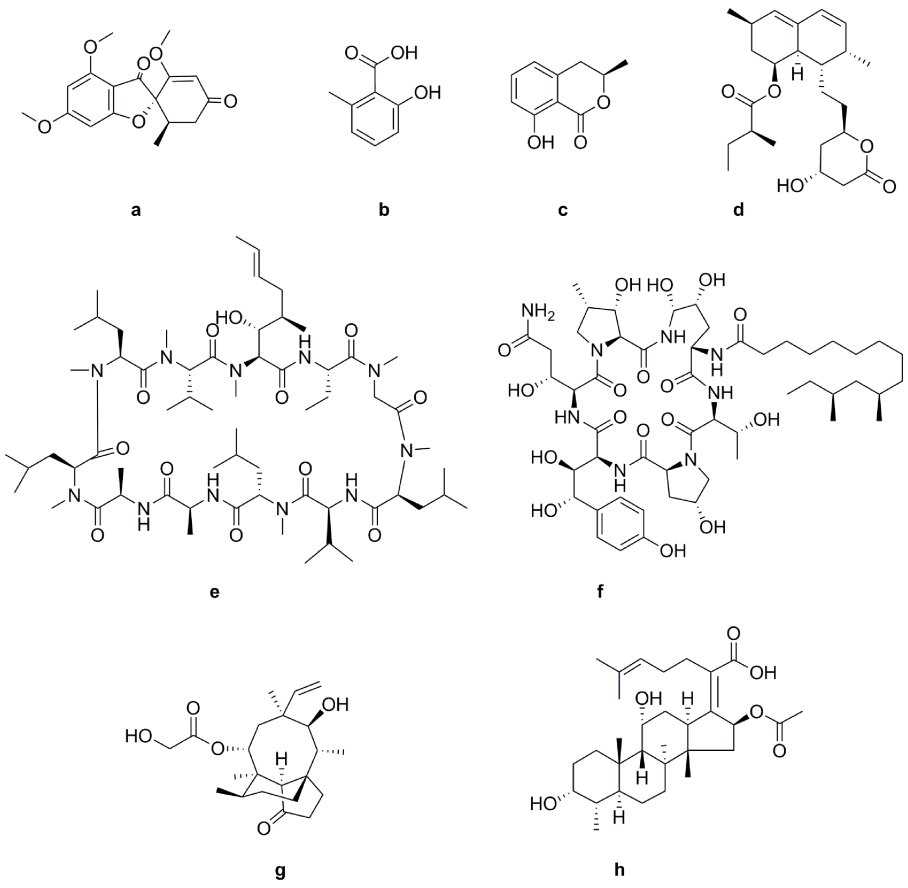
Polyketides

Polyketides are the largest and most structurally diverse group of fungal secondary metabolites. Their carbon scaffolds share a common biosynthetic origin and are derived from the polymerization of short-chain carboxylic acid units (e.g., acetate, malonate). Polyketides are biosynthesized by large multifunctional enzymes called polyketide synthases (PKSs) and can be classified into four main types according to the catalytic domains and enzymatic mechanisms involved in their formation (173). Fungal PKS enzymes are closely related to fatty acid synthases. In the Ascomycota and Basidiomycota, the prevalent types of PKSs belong to the iterative type I class in which the domains act repetitively on the growing polymer during each chain extension cycle. The carbon backbones are assembled by repetitive decarboxylative Claisen condensation catalyzed by β-ketoacylsynthase domains and employ malonyl thioesters as extender units. An acyl carrier protein domain acts as an anchor where the growing polyketide chain is covalently tethered. After each chain-extension step, the polyketide backbone is increased by one "ketide" unit. Additional domains including β-ketoreductases, dehydratases, and enoylreductases can selectively modify the newly formed polyketide. Unlike fatty acid synthases, in which most or all of the β-keto positions are completely reduced to methylenes, resulting in a fatty acyl product, the tailoring domains in PKSs act in preprogrammed patterns at each β-position, resulting in products of varied oxidation states. Furthermore, one or more methyl groups may be introduced at the α-position of the growing chain by C-methyltransferase domains. Finally, the fully extended chain is usually offloaded or cyclized by a thioesterase

domain, or in other cases, a terminal acyl carrier protein domain transfers the tethered product to an acyl transferase or other protein that shuttles it and covalently links it with another biosynthetic product. Thus, the collection of domains functions in an iterative fashion and follows integral programming rules that determine chain length, cyclization, reductive tailoring, and other structural features (174, 175).

Fungal iterative PKSs can be further grouped into three major subtypes: nonreducing (or aromatic) (NR-PKS), partial reducing (PR-PKS), and highly reducing (HR-PKS) PKSs, depending on the degree of programming and extent of α- and β-carbon processing. NR-PKSs are among the best-understood types of fungal PKSs. Griseofulvin (Fig. 6a, Table 1) was first characterized from *Penicillium griseofulvum* in 1939 and later was recognized for its antifungal effects, resulting in it being named the "curling factor" because it induced curling of fungal hyphae (176). Griseofulvin was developed at Glaxo Laboratories for the treatment of ringworm and other infections by dermatophytes. Genome scanning and gene deletion studies established that an NR-PKS, *gsfA*, encodes biosynthesis of the griseofulvin heptaketide backbone and that gsfI is the halogenase involved in chlorination of the final product (177, 178). Far fewer PR-PKSs have been characterized, with the 6-MSA (Fig. 6b) PR-PKS being a biosynthetically pivotal and widespread gene. The 6-MSA PR-PKS from *Penicillium patulum* was the first PKS to be purified and sequenced, and 6-MSA is an intermediate in the biosynthesis of the mycotoxin patulin (179). The *atX* gene from *A. terreus* and the *PKS2* gene from *Glarea lozoyensis* also encode for the 6-MSA PKS (180, 181). A fungal PR-PKS is responsible for the synthesis of the widespread metabolite (*R*)-mellein (Fig. 6c) in *Parastagonospora nodorum* (182) and probably many other fungi. It was proposed that biosynthesis of (*R*)-mellein was highly parallel to that of 6-MSA, requiring an additional chain elongation and keto reduction. These PR-PKS genes are likely important in encoding the biosynthesis of other dihydroisocoumarins

FIGURE 6 Some fungal polyketides, nonribosomal peptides, and terpenoids. **(a)** Griseofulvin. **(b)** 6-Methyl salicylic acid. **(c)** (R)-Mellein. **(d)** Lovastatin. **(e)** Cyclosporine A. **(f)** Pneumocandin A_0. **(g)** Pleuromutilin. **(h)** Fusidic acid.



and possibly the mycotoxin ochratoxin (183). Phylogenetic analyses have shown that the 6-MSAs and other uncharacterized PR-PKSs are more distantly related to the NR- and HR-PKSs and more closely resemble bacterial modular type I PKSs, suggesting a common origin from bacteria (184). However, divergence between the 6-MSA- and mellein-type PR-PKSs likely occurred during the course of fungal radiation (182).

The HR-PKSs have a higher degree of complexity in their programming relative to that of the NR-PKSs. T-toxin, the main virulence factor involved in southern corn leaf blight, a devastating disease caused by *Drechslera heterostrophus* race T that attacks maize, is a typical HR-PK. It consists of a linear polyketide whose biosynthesis is encoded by a nine-gene cluster that includes two cooperating HR-PKSs (185, 186). Fumonisins comprise a major group of mycotoxins produced by *Fusarium* spp. and certain black *Aspergillus* spp. (187). They act as ceramide synthase inhibitors. Coexpression of 15 contiguous genes defines a fumonisin biosynthetic gene cluster in *Fusarium moniliforme*, of which FUM1 is an HR-PKS that assembles the fumonisin backbone (19).

The compactin and lovastatin biosynthetic pathways are among the best-studied examples of HR-PKSs. These metabolites were among the first natural product enzyme inhibitors discovered for use in human health. As early as the mid-1960s, the biochemical pathway of cholesterol biosynthesis was fairly well understood, and the enzyme hydroxymethylglutaryl-CoA reductase (HMGR) was known to convert HMG-CoA to the intermediate mevalonate. Akira Endo at Sankyo in Japan developed a three-step assay (two phenotypic screens and a target-based assay) using liver tissues extracted from rats to screen fermentation broths for natural products that specifically inhibited HMGR (188). Using this approach, Endo discovered the compound ML-236B, later named compactin (Table 1), from a strain of *P. citrinum* (189). Although compactin was not developed as a drug, Endo and a group at Merck in the United States independently used strategies analogous to that used to find compactin to discover lovastatin, a methylated analog of compactin, from two fungi, *Monascus ruber* and *A. terreus*, respectively (188). Merck developed lovastatin (Fig. 6d), which became the first-in-class member of the statins (190), compounds that are widely and successfully used to treat hypercholesteremia. While this natural product was highly effective, a slight structural modification led to Zocor (simvastatin), a blockbuster drug product peaking at over $5 billion/year in sales during its patent lifetime. Using these compounds as models, chemists at Warner-Lambert designed the chemically distinctive compound atorvastatin (Lipitor), which became the best-selling drug of all time, with more than $125 billion in sales over its patent lifetime. These and other second-generation synthetic derivatives or analogues of compactin and lovastatin continue to be among the world's best-selling drugs.

Closely related to the products of HR-PKSs are a broad range of hybrid polyketide-amino acid metabolites formed by fusion of an HR-PK to an amino acid-binding domain and known as iterative PKS-NRPSs (174, 191). Functionally characterized fungal iterative PKS-NRPS hybrid gene clusters have been linked to the biosynthesis of tetramic acid-containing metabolites such as equisetin, the actin-binding cytochalasins, the immunosuppressive agent pseurotin A, the nematocidal thermolides, and the mycotoxins fusarin C and cyclopiazonic acid, among others.

NRPs: Nonribosomal Peptides

The Fungi offer one of the richest and most highly elaborated pools of NRPs among all organism types. This diversity reaches its zenith in the filamentous Ascomycota (192, 193). The corresponding NRPS megaenzymes synthesize a wide range of biologically active natural peptide compounds. NRPS-derived siderophores are involved in iron transport, storage and homeostasis, and virulence toward plants and animals (194–196). NRPSs are responsible for the biosynthesis of the iconic natural product-based drugs penicillins, cephalosporins, cyclosporine A, and echinocandins (Table 1). NRPSs are similar to PKSs in their modular structure and share an assembly line thiotemplate mechanism to achieve peptide synthesis (197). Like PKSs, minimal NRPS modules have an analogous set of three core functional domains: adenylation (A) domains that recognize and activate a specific amino acid or amino acid derivative by adenylation with ATP, peptidyl carrier protein domains (also known as thiolation [T] domains) that bind the activated substrate to a 4′-phosphopantetheine with a thioester bond and shuttle the substrate, and condensation (C) domains that catalyze peptide bond formation between adjacent monomer units on the NRPS complex.

The selection of a highly diverse set of monomers for peptide synthesis, including a plethora of nonproteinogenic amino acids, contributes to the overall complexity of NRPs. Although exceptions occur, generally, fungal multimodular NRPSs follow the collinearity rule, in which a complete A-C-T module corresponds to one amino acid in the NRP amino acid sequence. Further structural diversification accrues during chain initiation,

chain elongation, and chain termination of NRP biosynthesis. Some NRPSs, e.g., echinocandins, carry an N-terminal T and C domain (or starter C domain) before the first NRPS module to prime the synthesis of the peptide portion by addition of an aliphatic acyl chain. NRPSs may incorporate other catalytic domains which modify the substrate during biosynthesis, including (i) an epimerization (E) domain that inverts the chirality of the last amino acid in the growing peptide from the L- to the D-configuration, (ii) an *N*-methylation (M) domain (methyltransferase) that transfers a methyl group from an *S*-adenosylmethionine unit to the α-amino group of an amino acid substrate, and (iii) a specialized domain termed a cyclization (C) domain which catalyzes internal cyclization to oxazoline or thiazoline rings constructed from cysteine, serine, or threonine units. Often, additional tailoring enzymes may lie adjacent to the core NRPS and modify either the monomers or the final peptide product by acylation, glycosylation, hydroxylation, or halogenation. Offloading and release by macrocyclization is a common modality in fungal NRPSs and constrains the peptide backbone in a biologically active conformation. Fungal NRPSs characterized thus far universally terminate macrocyclic peptide synthesis with a condensation-like C_T domain that catalyzes the cyclization reaction.

The rapid development of therapeutic methods and industrial-scale processes for production of the β-lactam antibiotic penicillin during the 1940s were arguably the most significant milestones in the history of drug discovery research (198). Substantial increases in the human life span can be traced back to the introduction of the β-lactam antibiotics penicillin and cephalosporin and their modern derivatives (Table 1). The penicillin and cephalosporin biosynthetic gene clusters are among the best characterized of all NRPSs (199). Recently, the penicillin gene cluster from *Penicillium chrysogenum* was reconstituted as a single synthetic three-open reading frame polycistronic gene, and the construct was placed under the control of a single promoter (200). The open reading frames were separated by a sequence encoding the viral 2A peptide, which resulted in cotranslational cleavage, hence resulting in the formation of independent enzymes. After yeast-mediated homologous recombination, random genomic integration of this construct resulted in penicillin production in the heterologous host *A. nidulans* (200).

The introduction of the undecapeptide immunosuppressant drug cyclosporine A (Fig. 6e, Table 1) transformed and enabled the field of organ and tissue transplant methods. The genomic sequence of *Tolypocladium inflatum* and RNA-Seq transcriptome analysis has recently defined the complete gene cluster for cyclosporine biosynthesis (201). The formidable cyclosporine NRPS SimA, with its 11 amino acid-activating modules, seems to have evolved via duplication of simpler NRPSs that are apparently shared among other fungi of the Hypocreales. Several other NRPSs responsible for the biosynthesis of smaller cyclic depsipeptides in Hypocrealean fungi, such as enniatin, beauvericin, and bassianolide synthetases, and in part destruxin synthetase, appear to form a monophyletic lineage within the SimA clade (201). The biosynthetic pathways for the pneumocandins (e.g., Fig. 6f) in *G. lozoyensis* (202, 203) and echinocandin B in *Aspergillus pachycristatus* (204, 205), the starting metabolites for the semisynthesis of the lipopeptide antifungal drugs Cancidas and Eraxis, respectively (Table 1), have been mapped at the genetic level and functionally characterized to some extent. Phylogenetic studies from pathway gene clusters from multiple echinocandin-producing fungi have demonstrated that the echinocandin gene clusters have a common ancestor and formed a unique lineage among the families of fungal NRPSs (206).

Terpenoids

Fungi of the Ascomycota and Basidiomycota are prolific producers of terpenoids. In the Basidiomycota, terpenoids, especially diterpenoids and sesquiterpenoids, are the dominant biosynthetic class of secondary metabolites. In-depth information on the spectrum of fungal terpenoids can be found in recent reviews (26, 207, 208). Terpenoids are assembled from five-carbon precursor molecules: isopentenyl diphosphate and/or dimethylallyl diphosphate. In fungi, these two isomers are derived from acetyl-CoA via the mevalonate pathway (209). Condensation of isopentenyl diphosphate and dimethylallyl diphosphate monomers yields branched hydrocarbons in multiples of five carbons that are termed as being constructed of isoprene or prenyl units: geranyl pyrophosphate (C_{10}), farnesyl pyrophosphate (C_{15}), geranylgeranyl pyrophosphate (C_{20}), squalene (C_{30}), and phytoene/lycopene (C_{40}). These linear prenyl chains are further processed by prenyl transferases, terpene cyclases, and terpene synthases, resulting in a cascade of dephosphorylation, further condensations, and cyclizations which configure the carbon skeleton of the different classes of terpenoid natural products. Occasional loss of one or more methyl groups during biosynthesis results in norterpenoids. In addition, polyketide-, sugar-, or amino acid-derived elements are sometimes incorporated to yield molecules of mixed biosynthetic

origin. For example, a hybrid terpenoid-polyketide is often referred to as a meroterpenoid. We mention only a few representatives from this vast class of fungal metabolites below.

The topical antibacterial antibiotic retapamulin is synthesized from the diterpenoid pleuromutilin (Fig. 6g; Table 1) produced by the mushroom *Clitopilus passeckerianus* and some related species (210, 211). A patent application from Sandoz AG first described the pleuromutilin pathway as consisting of six transcriptionally coregulated genes, including a labdane-type diterpene synthase gene, a putative geranylgeranyl diphosphate synthase gene, three cytochrome P450 monooxygenase genes, and a putative acyltransferase-encoding gene (212). The cDNA sequences of seven genes (*Pl-ggs*, *Pl-cyc*, *Pl-atf*, *Pl-sdr*, *Pl-p450-1*, *Pl-p450-2*, and *Pl-p450-3*) constituting the gene cluster in *C. passeckerianus* were cloned and placed under control of constitutive promoters for expression in *A. oryzae* (213). One of the *A. oryzae* transformants gave a remarkable 20-fold increase in pleuromutilin titers compared to the native host. This outstanding achievement represents the first successful heterologous expression of an entire basidiomycete secondary metabolite gene cluster in an ascomycete host and paves the way for further improvement of the pleuromutilin-producing *A. oryzae* strain through classical mutagenesis and other genetic manipulations (213).

Fusidic acid (Fig. 6h, Table 1) is a fusidane-type triterpenoid produced by *Acremonium fusidioides* that has long been used as an oral antibiotic for treatment of Gram-positive bacterial infections (214). Although labeled isotope studies have established that its precursor is squalene (215), its gene cluster has yet to be characterized. Nevertheless, its biosynthesis is likely to be initiated by an oxidosqualene synthase similar to that found in *A. fumigatus*, *Metarhizium anisopliae* (216), and *Sarocladium caerulens* (217), all of which produce the closely related helvolic acid. In *A. fumigatus*, the helvolic acid gene cluster has been characterized and comprises a gene for an oxidosqualene cyclase (*AfuOSC3*) that is colocalized with genes of cytochrome P450 monooxygenases, a short-chain dehydrogenase/reductase, and an acyltransferase, which presumably function in triterpene-tailoring steps (218, 219).

The genetic basis of the biosynthesis of trichothecene-type mycotoxins, which share a common tricyclic 12,13-epoxytrichothec-9-ene core structure, has been identified in fusaria and other species of the Hypocreales (220, 221). The trichothecene family also illustrates the incompleteness of our knowledge of the distribution of fungal biosynthetic gene clusters; trichothecolone acetate was recently discovered in the apple sooty blotch fungus *Microcyclospora tardicrescens* (222). Indole-diterpenoid metabolites have been investigated intensively because of their neurotoxic and other biological effects. They are distributed sporadically among fungi of the Sordariomycetes and Eurotiomycetes. These metabolites consist of a diterpene backbone derived from geranylgeranyl diphosphate and an indole moiety derived from indole-3-glycerol phosphate (223). The biosynthetic gene clusters for paxilline (224, 225), aflatrem (226, 227), the lolitrems (228), penitrem (229), and terpendole E (230) have been functionally characterized. Finally, the heterologous expression of the products of several synthetic ascomycete monoterpene and sesquiterpene synthases has been achieved (231, 232), resulting in *in vitro* production of potentially valuable terpenoids, e.g., eucalyptol, pinenes, guaiene, caryophyllene, chamigrene, and gurjunene, for use as fuel additives, fragrances, and other commodities.

READING THE SECONDARY METABOLITE MENU AND BIOINFORMATICS-BASED DISCOVERY OF NATURAL PRODUCTS

Knowledge of microbial secondary metabolites is highly advanced due to the resources invested in controlling microbial toxins, crop virulence factors, and discovery of their chemical applications in medicine and agriculture. Studies of natural products produced by fungi have provided powerful probes for cell biology and drugs useful in medicine. Their importance is evidenced by their prominence as reagents and metabolic blockers for cellular metabolism and for labeling cell macromolecular structures. Understanding the biosynthetic logic of the principal pathways and how they operate can often enable straightforward recognition of the biogenetic origin of a given secondary metabolite. The metabolic precursors of metabolites can also be identified by feeding strains isotopically labeled precursors. Previous work in this area has served as the foundation for modern genetic biosynthetic studies (27, 233). Computational methods for decoding secondary metabolite pathways from sequenced genomes have transformed natural products research and have begun unfolding the roadmap to secondary metabolite biosynthetic genes and their regulation. Rapid advances in technology for genome sequencing and analysis are accompanied by parallel advances in relevant aspects of analytical chemistry. Applications of new techniques and informatics tools to mass spectrometric analysis are effec-

tively expanding the field of metabolomics to encompass secondary metabolites (234, 235). These techniques are also fostering efforts to explore the significance of secondary metabolites, e.g., by detecting and localizing them *in situ* during interspecies interactions (236). Similarly, nuclear magnetic resonance capabilities continue to improve in terms of sensitivity, speed, pulse sequence development, automated interpretation, and amenability to mixture analysis (237). For example, the gene clusters and essential biosynthetic steps have been characterized for most major mycotoxins and fungus-derived drugs. The tools are sufficiently accessible that discoveries of important new microbial metabolites are now accompanied by characterization of the corresponding encoding gene clusters (238, 239). Within the next few years, we expect to see a near complete catalogue of gene clusters of most of the important fungal secondary metabolites (32).

Until recently, discovery of fungal metabolites was largely an empirical process that was supported by ecological, phylogenetic, and physiological clues to what fungal strains were most likely to produce secondary metabolites and by knowledge of which laboratory conditions would most likely produce metabolite-rich fermentations. The universe of known fungal metabolites has been further expanded from studies of fungi of the Basidiomycota and Ascomycota that produced large or numerous fruiting structures that could be collected in the field and then extracted directly for chemical studies. Metabolites characterized directly from fruiting bodies of ectomycorrhizal fungi in many genera, e.g., *Cortinarius*, *Boletus*, *Lactarius*, *Hydnellum*, *Cantharellus*, and those directly extracted from lichenized fungi have provided a vast catalogue of secondary metabolites from species that have proven to be unculturable in the laboratory (42, 240). While the prokaryotic biology community sensationalized their realization of the "uncultured majority" in the 1980s and 1990s (241), fungus biologists have been aware of vast numbers of unculturable fungi since axenic cultures were introduced in the 1800s (242) and have generally assumed that unculturability was a normal phenomenon. Thus, issues of unculturability of fungi were acknowledged early on in the history of fungal chemical research and differ significantly from those associated with prokaryotic organisms. Unlike most prokaryotes, fungi can often be seen by eye or by low magnification microscopy, and their cells and tissues harvested for their DNA and chemistry. Consequently, the issue of unculturability has had less impact on efforts to evaluate biodiversity and chemical diversity in fungi, even though it remains a significant hurdle in the experimental study of biosynthesis and for practical scale-up of metabolite production.

Secondary metabolite gene clusters are among the most diverse and rapidly evolving features of fungal genomes and can be used to follow mechanisms of adaptive evolution, including evolution of pathogenic species (44, 243, 244). During the past decade, the search for fungal metabolites, like that for other microorganisms, and their corresponding biosynthetic genes has entered a transformative new era. The search for new and useful microbial metabolites is shifting from being an experience-guided empirical process to being a genomics-guided process in which identification of the biosynthetic genes precedes and informs isolation of the secondary metabolite. By examining historical data retrospectively, it is now possible to rationalize why a strain's genetic makeup dynamically interacts with fermentation methods to yield fluctuating patterns of bioactive molecules during screening and why the pool of new drug-like metabolites from certain kinds of common fungi appeared to have been depleted. The numbers of biosynthetic gene clusters in every filamentous genome examined to date is thought to exceed the number of detectable metabolites under a given set of laboratory conditions, indicating the existence of numerous unexpressed or poorly expressed pathways possibly encoding the biosynthesis of undescribed metabolites. These unexpressed gene clusters are commonly called "orphan" or "cryptic" gene clusters because their silence is likely an artifact caused by our inability to simulate the environmental conditions or chemical signals required for their expression, or even to grow the organism at all (245, 246). Regulation of secondary metabolism remains poorly understood across the diversity of secondary metabolite-producing organisms, and identification of a low-abundance discrete predicted metabolite from within a crude metabolome is a nontrivial challenge. Recognition that transcription and translation of these repressed genes can sometimes be induced has spawned an entirely new area of natural products research dedicated to finding chemical and genetic tools to break pathway silence and induce homologous expression in the native organism (71, 245, 247–249).

Genome sequencing has moved beyond model fungal species, major pathogens, and industrial organisms to encompass the full spectrum of fungal diversity. Consequently, as in other microorganisms, a global picture of the phylogenetic distribution of secondary metabolites encoding genes and gene cluster architecture in fungi is emerging. In principle, conceptual barriers no longer impede the future unlocking of cryptic secondary

metabolic gene clusters. The complex secondary metabolism of a given fungus can easily constitute 20 to as many as 80 different core secondary metabolite-encoding genes or gene clusters. On the other hand, fungi that have little history of secondary metabolite production, e.g., chytrids, yeasts, powdery mildews, vesicular-arbuscular mycorrhizae, and truffles, are now known to lack or have few secondary metabolite gene clusters because (i) they diverged prior to the evolution of secondary metabolism or (ii) during their descent from fungal groups with rich secondary metabolism, their genomes underwent a concurrent loss and reduction in unnecessary secondary metabolism due to stable environments provided by obligate parasitism (e.g., rusts, smuts, powdery mildews) (250). However, other biotrophic fungi, e.g., lichenized fungi, mycorrhizal mushrooms, and plant-associated Clavicipitaceae, have retained a rich secondary metabolism that is highly related to that of their metabolite-rich sister phylogenetic lineages. It is hypothesized that these fungi have retained their secondary metabolic capabilities in part because of the need for defense and the interorganism interactions of their conspicuous and often long-lived vegetative (lichens) or reproductive (mushrooms) structures, or to maintain their mutualistic relationships with host plants (Clavicipitaceae) (44, 240, 251–253).

Reading the biosynthetic menu for secondary metabolites from sequenced fungal genomes is a complex process, but it can be assisted by several computational tools that can identify signature motifs and genes associated with secondary metabolite biosynthetic gene clusters or, minimally, the primary catalytic backbone genes (254). A full range of software packages can be found at the Secondary Metabolite Bioinformatics Portal (http://www.secondarymetabolites.org). These software packages organize searches and categorize results so that identified genes and gene clusters are associated with their nearest known orthologues. In some instances, these searches can propose a hypothesis for a secondary metabolic product of a cluster through close homology with gene clusters that have been experimentally linked to a secondary metabolite.

The most widely used software tool is the Antibiotics & Secondary Metabolite Analysis Shell (antiSMASH, http://antismash.secondarymetabolites.org) (255). This Web-based tool enables users to easily identify and annotate pathways with typical repetitive domain structures directly from DNA or protein sequence data. Two complementary tools are designed to aid in organizing the results of AntiSMASH analyses. One is the ClusterMine360 database that stores the antiSMASH annotation of known gene clusters and links them to its own repository or to sequence references in the NCBI GenBank as well as to the structure of the natural product encoded by the gene cluster (256). The other is the recently released Minimum Information about a Biosynthetic Gene cluster (MIBiG), a Web-based community-annotated database which has cataloged about 200 unique natural product pathways including those corresponding to most classes of fungal metabolite. This effort is vital to guiding future genome mining and synthetic biology efforts aimed toward discovering novel fungal enzymes and metabolites. Other computational tools useful for fungi include Secondary Metabolite Unknown Regions Finder (SMURF, http://jcvi.org/smurf/index.php) (257); Cluster Assignment by Islands of Sites (CASSIS), a method for biosynthetic gene cluster prediction in eukaryotic genomes, and Secondary Metabolites by InterProScan (SMIPS), a tool for genome-wide detection of key secondary metabolite backbone enzymes (anchor genes), e.g., PKSs, NRPSs, and dimethylallyl tryptophan synthases (https://sbi.hki-jena.de/cassis/cassis.php) (258); ClustScan (http://bioserv.pbf.hr/cms/) (259, 260); and natural product searcher (NP.searcher, http://dna.sherman.lsi.umich.edu/) (261).

Currently, for fungal gene clusters, *de novo* structure prediction from gene cluster DNA sequence data may provide a structure hypothesis when the targeted genes exhibit high homology to a functionally characterized gene cluster of a known chemical product. Detailed manual prediction of structure from gene cluster data can often provide a reasonable estimate for the core structure encoded by a PKS or NRPS, but automated structure prediction is not yet possible.

More recently, alternative methods have been developed to identify clusters for which the genes are cooperatively regulated at transcriptional levels, thus allowing for the identification of novel gene clusters even when sets of canonical core genes are unrecognizable (254, 262). The cases of kojic acid and ustiloxin B, mentioned above, demonstrated that different computational techniques can be applied to identify biosynthetic gene clusters that do not contain any known types of core scaffold-synthesizing enzymes. Discovery of the corresponding gene clusters employing the MIPS-CG algorithm has been applied to two or more transcriptional data sets from producing and nonproducing conditions to identify chromosomally adjacent clusters of coexpressed genes over a 3- to 30-gene frame in a fungal genome. The advantage of this approach was that no assumptions were made regarding the patterns of the genes that would be identified which can help recognize

hypothetical new classes of biosynthetic gene clusters. The expanding use of these tools has led to the observation that most newly identified biosynthetic pathways are not readily associated with a known PK, NRP, terpenoid, or other product. The assignment of potential products to these orphan pathways is thus a major unaddressed challenge, as is the basic question of whether these newly recognized biosynthetic gene clusters are even functional (254).

A global overview of secondary metabolite gene cluster evolution and diversification in the Fungi is now emerging, and significant progress has been made in developing predictive frameworks for classifying fungal NRPSs, PKSs, and terpene synthases. Previously, the frequency of isolation of bioactive metabolites and the chemical complexity of organic extracts were used to measure the chemical potential of different fungal taxa. The genetic capacity of different groups of fungi can now be explored directly from genomic sequences to ask which taxa have the highest potential to produce large numbers of complex secondary metabolites with drug-like properties and which taxa have moderate or poor potential. Because most drug or drug-like metabolites are polyketides, nonribosomal peptides, or terpenoids or incorporate derivatives of dimethylallyltryptophan, the numbers of iterative PKS, NRPS, terpene synthase, prenyl transferase, and dimethylallyltryptophan synthase genes per genome can serve as a measurement of the potential to produce secondary metabolites. On this basis, the dikaryotic filamentous fungi are clearly the most productive sources of secondary metabolites compared to the basal fungal lineages. This conclusion correlates very well with historical data on how the numbers of known secondary metabolites are distributed across the Fungi.

The highest complexity of secondary metabolite pathways is concentrated within the filamentous Ascomycota, especially in the higher lineages of the Eurotiomycetes, Dothideomycetes, Sordariomycetes, Leotiomycetes, and Lecanoromycetes, while secondary metabolism is limited or absent altogether in the obligate biotrophic Erysiphales, the basal lineages of the Pezizomycetes, the Saccharomycotina, and the Taphrinomycotina (Fig. 1). All genomes of the higher ascomycete lineages examined to date have roughly a minimum of 5 to 10 core secondary metabolic pathways (pigments, siderophores) and may have as many as 80 (263), thus rivaling the complexity and richness of the Actinomycete genus *Streptomyces*. In particular, gene clusters for biosynthesis of polyketides and nonribosomal peptides have undergone significant expansion, and many of these pathways are ascomycete-specific. Furthermore, few of these core biosynthetic genes are fully conserved across ascomycete and basidiomycete fungi; therefore, when one takes into account the potential numbers of fungal species, the capacity for encoding chemical diversity in the Ascomycota seems limitless. The situation in the Basidiomycota appears to be nearly as complex, but with the chemistry and diversification of gene clusters skewed toward terpenoids. The Agaricomycotina have genomes that are often rich in terpene pathways, are moderately rich in PKSs, and incorporate only a few NRPSs (240, 264–266), while other Basidiomycota lineages have little or no secondary metabolism due to evolutionary contraction as a consequence of biotrophy and parasitism (6, 267, 268).

CONCLUDING REMARKS

There is an urgent a need for the development of new drugs to combat multidrug-resistant pathogens and for molecular templates for targeted cancer therapeutics. The capacity for sequencing microbial genomes is outpacing the ability to identify natural product gene clusters and, most importantly, to convert this sequence information into collections of testable molecules. Therefore, unlocking small-molecule chemodiversity encoded in microorganisms, including the Fungi, will be crucial for next-generation drug development. Genome sequencing projects covering the whole spectrum of environmental fungi are providing fertile ground for directed secondary metabolite discovery. A variety of new approaches have revitalized the field of fungal natural product discovery, particularly in the fields of elucidation of biosynthetic mechanisms, identification of biosynthetic gene clusters and their connections to new and known fungal secondary metabolites, induction of homologous expression of repressed pathways in the native organisms, and heterologous expression of pathways in genetic model hosts. A valuable approach would be to focus community resources on generating high-quality sequenced genomes of all fungi that produce biologically important and structurally distinctive secondary metabolites and to connect these secondary metabolite clusters to their corresponding annotated gene cluster. Such a collective community project would streamline the recognition of orthologous pathways in new genomes and enable mapping of widely and narrowly distributed families of fungal metabolites. Concurrently, the discovery of novel secondary metabolite pathways would be accelerated. It would be logical to assume that at some point these new platforms for fungal natural product discovery would be introduced into industrial

pharmaceutical and agrochemical discovery. Efforts to build industrial-scale genome-mining platforms for Actinomycetes are already under way (269). However, to the best of our knowledge, parallel efforts on the Fungi at an industrial scale have yet to be organized.

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