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Fungal Genetics, Genomics, and Secondary Metabolites in Pharmaceutical Sciences

Abstract

Filamentous fungi produce a plethora of bioactive natural products. These metabolites display a broad range of useful activities for pharmaceutical purposes, exemplified best by the antibiotic penicillin. Yet, many more have been isolated, characterised, and tested, and some have made their way in clinical trials and into pharmaceutical practice. Through genomics, we become increasingly aware that the biosynthetic abilities for natural products are much richer than expected. The first part of our review highlights selected metabolites that filamentous fungi offer to pharmacists for drug development. This is followed by a summary on the potential of fungal genetics and genomics for pharmaceutical sciences and natural product research.

Key words
Fungi · natural products · genomics · gene cluster

Introduction – Fungi in the History of Pharmacy

Natural products represent the traditional source of new drug candidates [1]. A global overview on the metabolic wealth of fungal secondary metabolites – prominent and less prominent ones – is provided in diverse compilations [2], [3]. From the systematic viewpoint, the most prolific producers of pharmaceutically relevant molecules are the ascomycetes. Their evolutionary counterpart, the basidiomycetes, are equally rich and often unique in their secondary metabolism, however, they cannot compare for significance in terms of clinically used compounds. We open our review with spotlights in the history of pharmacy to underline the Janus-faced nature of fungal metabolites.

The Eleusinian Mysteries, initiation ceremonies in the ancient Greek culture that have been established about 1500 BC, climaxed when the initiates reached a visionary state. Their revelatory state of mind was mediated by a drink called "kykeon". Today's research attributes this preparation to rye, parasitised by the ergot fungus *Claviceps* – a classic in the history of pharmacy. Ergot fungi are known to produce hallucinogenic lysergic acid amides [4]. In the medieval Europe, the sclerotia of the same fungus posed a severe threat to human health when continued ingestion of contaminated bread caused horrible symptoms, like gangrene of the extremities. The order of St. Anthony, in particular, cared for those who were ailing from these symptoms colloquially referred to as "holy fire" or "St. Anthony's fire". Created in 1515 for an Antonitarian monastery, one painting of the Isenheim Altar symbolises the suffering and the pain of those affected by the toxic metabolites of the ergot fungus. The Isenheim Altar is one of the most outstanding pieces of art ever and marked the onset of the Renaissance. Starting in the 18th century *Claviceps* sclerotia were pharmaceutically used to induce contractions of the uterus. In the 1930 s, the ergot alkaloids served as a molecular template for the synthesis of the lysergic acid diethylamide (LSD) by Albert Hofmann and co-workers at the San-

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doz Laboratories. The ramifications of this work have been told many times.

In the 20th century, the discovery of the penicillins and their introduction into clinical use saved countless lives. During and shortly after World War II, breeding and fermentation techniques were developed to increase the mediocre titres of the producer strains. However, as the bulk of the production was reserved for military use, the civil demand exceeded the supply by far: penicillin became an item on European black markets in the early post-war era. This situation is adopted in the movie "The Third Man" with Orson Welles in the role of Harry Lime, a Vienna black marketeer.

Highlighted Bioactive Compounds from Fungi

The most famous fungal metabolites in successful clinical use include the β-lactams, e.g., penicillins G (**1**) and V (**2**), the antihypercholestemic drug lovastatin (**3**) and its derivatives, the immunosuppressant cyclosporin (**4**), and ergotamine (**5,** Fig. **1**). Pharmaceutical and medical aspects of these valuable drugs have been reviewed comprehensively. Therefore, we refer the reader to a number of articles and textbooks [5], [6], [7], [8], [9]. In the following section, we sift through the metabolic realm of fungi for less prominent fungal metabolites and mainly focus on those relevant for two selected areas: anti-infectives, including antivirals, and antitumour compounds.

Antifungal drugs

As a result of a steadily rising number of immunocompromised individuals, e.g., AIDS patients or patients with organ xenografts, infections with pathogenic fungi have turned into a major problem in medical science. Pathogens increasingly resistant to current antifungal drugs further exacerbate the situation [10]. In sharp contrast, hardly any new antifungal agent with a different mode of action has become available during the last decades. However, two new classes of antifungals derived from fungal lead structures, the echinocandins and sordarins, offer entirely new modes of action and, therefore, these compounds hold potential for antifungal therapy [11], [12].

Identification of echinocandin B (**6**) as a fungicide from *A. nidulans* var. *echinulatus* dates back to the 1970 s [13]. This compound laid the foundation for the semi-synthetic derivatives caspofungin (**7**, Cancidas®) [14], the recently FDA-approved anidulafungin (**8**, Eraxis™) [15], [16], and micafungin (**9**, Mycamine™) (Table **1**, Fig. **2**) [17].

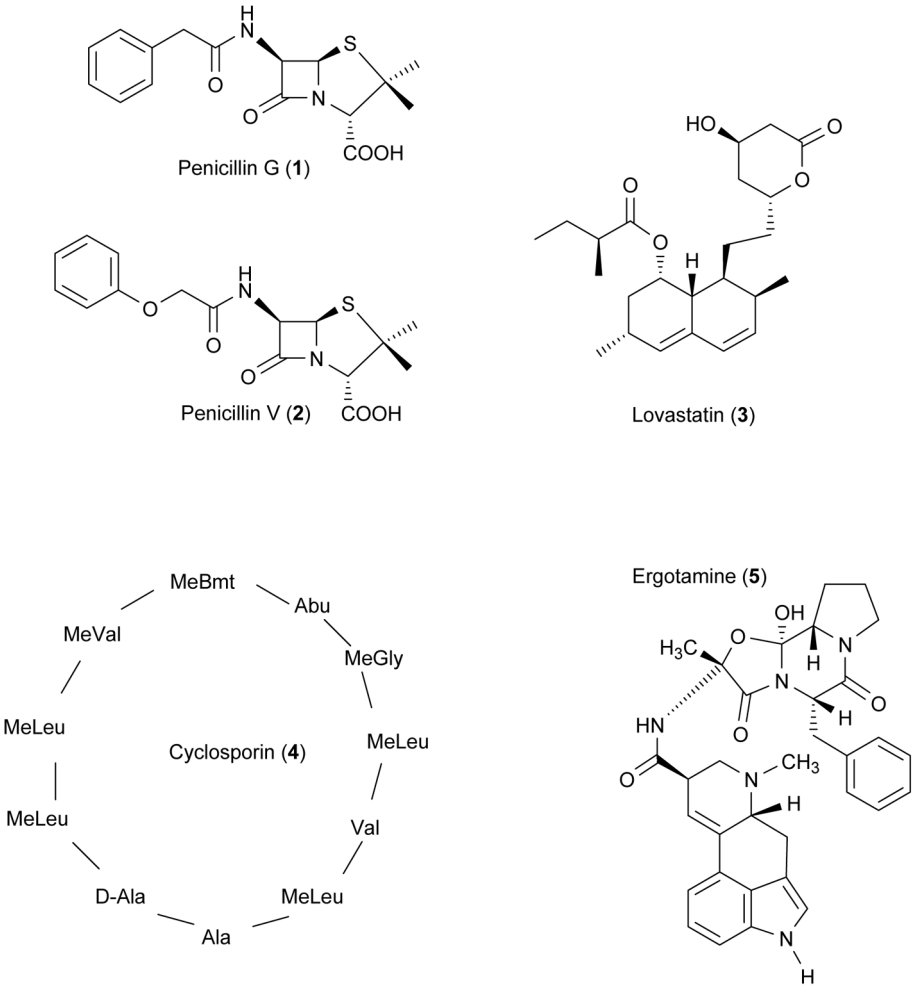


Fig. **1** Chemical structures of clinically used fungal secondary metabolites. Abbreviations for amino acids: MeBmt = (4*R*)-4-[(*E*)-2-butenyl]-4-methyl-l-threonine, Abu = 2-aminobutyric acid, MeGly = *N*-methylglycine, MeLeu = *N*-methylleucine, MeVal = *N*-methylvaline.

Table **1** Echinocandins approved for clinical use

Drug	*Company*	*Approval*	*Indication*
Caspofungin (**7**, Cancidas®)	Merck	USA, EU	Candidaemia, oesophageal candidasis, invasive aspergillosis (if refractory to other therapies), presumed fungal infections with febrile neutropenia
Anidulafungin (**8**, Eraxis™)	Vicuron	USA	Candidaemia, oesophagial candidesia, intraabdominal abcess and peritonitis due to infection with *Candida*
Micafungin (**9**, Mycamine™)	Fujisawa	USA, Japan; EU (expected for 2008)	Oesophageal candidasis, prophylaxis of fungal infections in haematopoietic stem cell transplantations

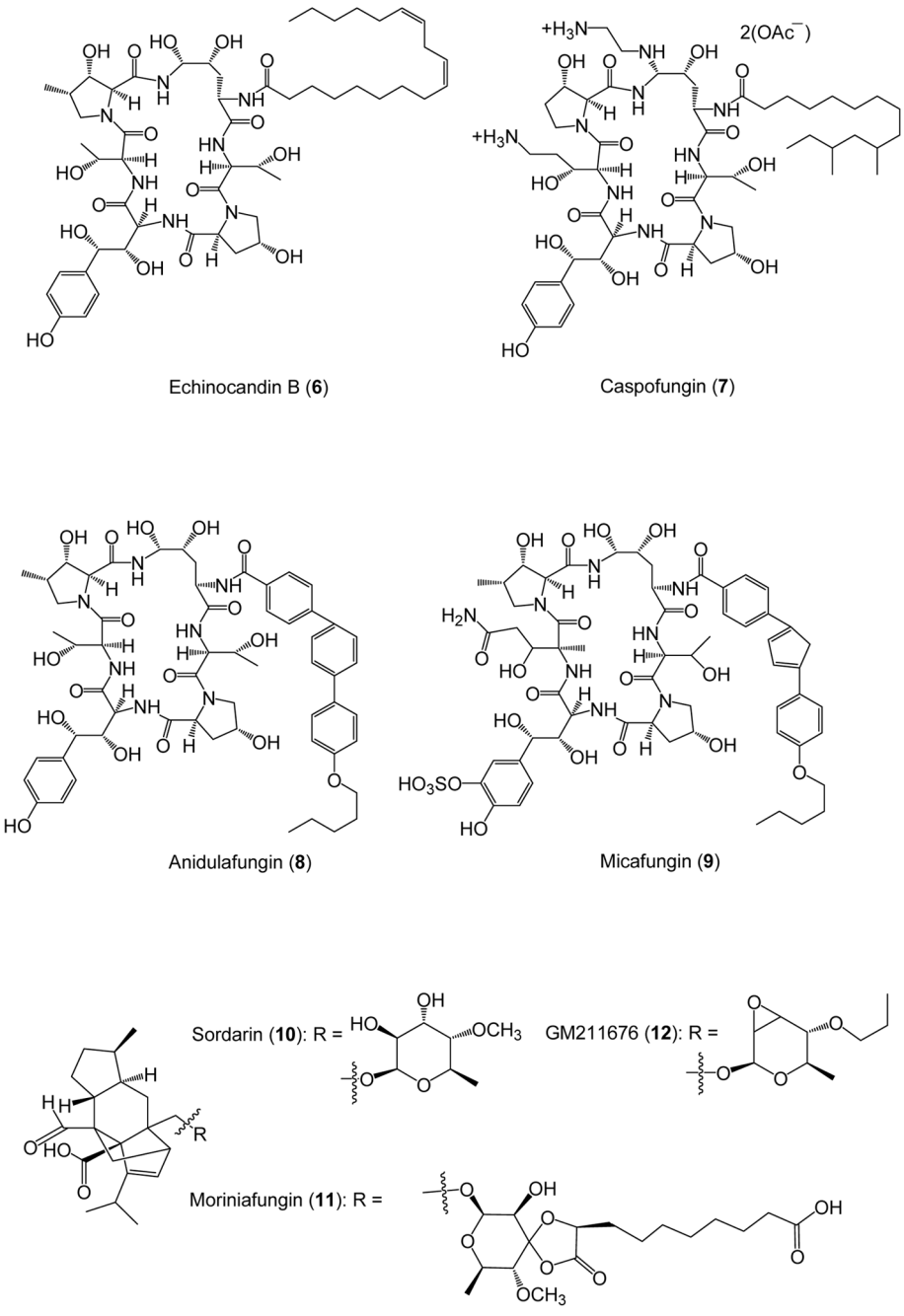


Fig. **2** Selected natural compounds from fungi with antifungal activity.

In several clinical trials, echinocandins have been studied for the efficacy in treatment of fungal infections compared with azoles and amphothericin B [18], [19], [20]. Chemically speaking, echinocandins are cyclic *N*-acylated hexapeptides, which groups them with the lipopeptide natural products. Derivatives of echinocandin differ in either substitutents within the hexapeptide core or in the lipid side chain. Echinocandins target the membrane-associated protein complex β-1,3-D-glucan synthase. Poly-

saccharides like β-1,3-glucan are essential constituents of fungal cell walls and are responsible for structural integrity and stability. Mammalian cells do not contain β-1,3-glucans. The non-competitive inhibitory effect of the echinocandins is exerted by binding to the glucan synthase subunit FKS1. However, many details of the molecular interactions still remain elusive.

Major fungal pathogens that can be targeted by echinocandins include *Candida* spp., *Aspergillus* spp. and *Pneumocystis carinii* [21], [22], [23]. Against most *Candida* species, including azole-resistant strains, echinocandins show potent fungicidal effects with minimal inhibitory concentrations (MICs), typically in the range of 0.001 – 0.03 mg/L [24]. However, variation in susceptibility of different *Candida* strains has been reported, and about 10% of all *Candida* isolates are resistant. Studies for two less susceptible species, *C. parapsilosis* and *C. guillermondii*, reported MICs of 1 – 2 mg/L (comparable to amphothericin B).

Despite a significant amount of 1,3-β-glucan in cell walls of *Aspergillus,* echinocandins display only fungistatic activity against *Aspergillus* species. Apparently, the compound lyses only actively growing *Aspergillus* hyphae, microscopically observed as morphologically altered hyphal growth, such as branched hyphal tips, balloon-like cells and swollen germ tubes as a consequence of a weakened cell wall [25]. No activity was detected against *Cryptococcus neoformans*, *Fusarium* spp., *Trichosporon* spp., or any zygomycete.

Due to their unique mode of action and because azole-resistance conferring efflux pumps do not recognise echinocandins, cross-resistance with polyenes and azoles has not been observed. However, *Candida albicans* and *C. parapsilosis* strains have already emerged in which a mutated FKS1 or rho1 gene, the latter coding for the regulatory subunit of β-1,3-glucan synthase, confers resistance [26]. With an absorption rate of less than 3%, echinocandins fail as orally available medication. Therefore, intravenous administration is required once daily. So far, clinical studies have indicated a good drug tolerance for echinocandins.

Sordarin (**10,** Fig. **2**), the prototypical compound of this class of potential future antifungals [27], [28], was isolated from the ascomycete *Sordaria araneosa* [29]. The tetracyclic diterpene glycoside is composed of two structural components: the aglycone sordaricin, *O*-glycosidically linked to sordarose (6-deoxy-4-*O*-methyl-D-altrose) as sugar moiety. Like with many anti-infectives, the sugar plays an important role for ligand-target interactions and the spectrum of activity.

As sordarin shows only weak activity, as assessed in yeast cell assays, major pharmaceutical companies made continued screening efforts to discover and develop new derivatives. Novel sordarins were either found from fungal sources or were synthesised semi-syntheticially [30], [31]. New derivatives primarily differ in the sugar moiety and show a superior antifungal activity. One of these, moriniafungin (**11**) [32], was recently found in *Morinia pestalozzioides* and showed a broader antifungal spectrum yet slightly less activity compared to GM211676 (**12**) as a semi-synthetic member of the sordarin family (Table **2**).

Table **2** Antifungal activities for sordarin antifungals against *Candida* species. The strains used to determine IC_{50} values for GM211676 were not identical with those used for sordarin and moriniafungin

Strain	*IC_{50} (μg/mL)*		
	Sordarin (10)	*Moriniafungin (11)*	*GM211676 (12)*
C. albicans (MY 1055)	0.4	0.9	
C. glabrata (MY 1381)	8	1.8	
C. krusei (ATCC 6258)	> 100	21	
C. albicans (2005E)			< 0.005
C. glabrata (2375E)			0.01
C. krusei (2374E)			100

Sordarins exert their antifungal activity by interaction with elongation factor 2 (EF-2) in complex with the ribosome. This stabilisation leads to a specific inhibition of translocation and – eventually – of ribosomal protein synthesis. Although EF-2 is an extremely conserved protein across the entire eukaryotic kingdom, sordarins exclusively block the fungal protein synthesis and do not affect mammalian or plant cells. A unique conformational change of fungal EF-2 upon interaction with the ribosome has been proposed to create the specific sordarine binding site [33].

Sordarins are active against most common fungal pathogens in immunocompromised patients. They show remarkable activity against *Candida albicans*, including azole-resistant isolates, and activity against *C. tropicalis*, *C. kefir* and *Cryptococcus neoformans* [27]. However, some *Candida* species, such as *C. krusei* and *C. parapsilosis*, are completely resistant to most sordarins with the notable exception of moriniafungin. Sordarins are also effective against filamentous fungi, such as *Aspergillus* spp., and *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Blastomyces dermatitidis*, and *Coccidioides immitis* [27]. Due to the unique mode of action, sordarins are extremely potent against *Pneumocystis carinii*, which is resistant to all current ergosterol biosynthesis inhibitors. So far, sordarins have not entered clinical trials, but studies on pharmacokinetics and toxicological effects were carried out in animal models [34], [35].

Antibiotics

The great success of penicillins spurred efforts to screen a huge number of new fungal metabolites for antibacterial properties. However, only a disappointingly small number of compounds were considered for further development and entered the pipeline. Among those are tiamulin (tiamutin®, **13**, Fig. **3**), which is successfully used in veterinary medicine. This peptidyltransferase inhibitor is a semi-synthetic derivative of the basidiomycete tricyclic diterpene antibiotic pleuromutilin (**14**), which was initially found in *Clitopilus scyphoides* (syn. *Pleurotus mutilus)* [36]. Interactions and binding sites between tiamulin and the 50S ribosomal subunit were investigated by crystallographic studies [37], [38]. Retapamulin (**15**), a novel pleuromutilin derivative, has been developed into a dermal antibiotic. MIC_{90} values for *Staphylococcus aureus* and *Streptococcus pyogenes* are 0.12 μg/

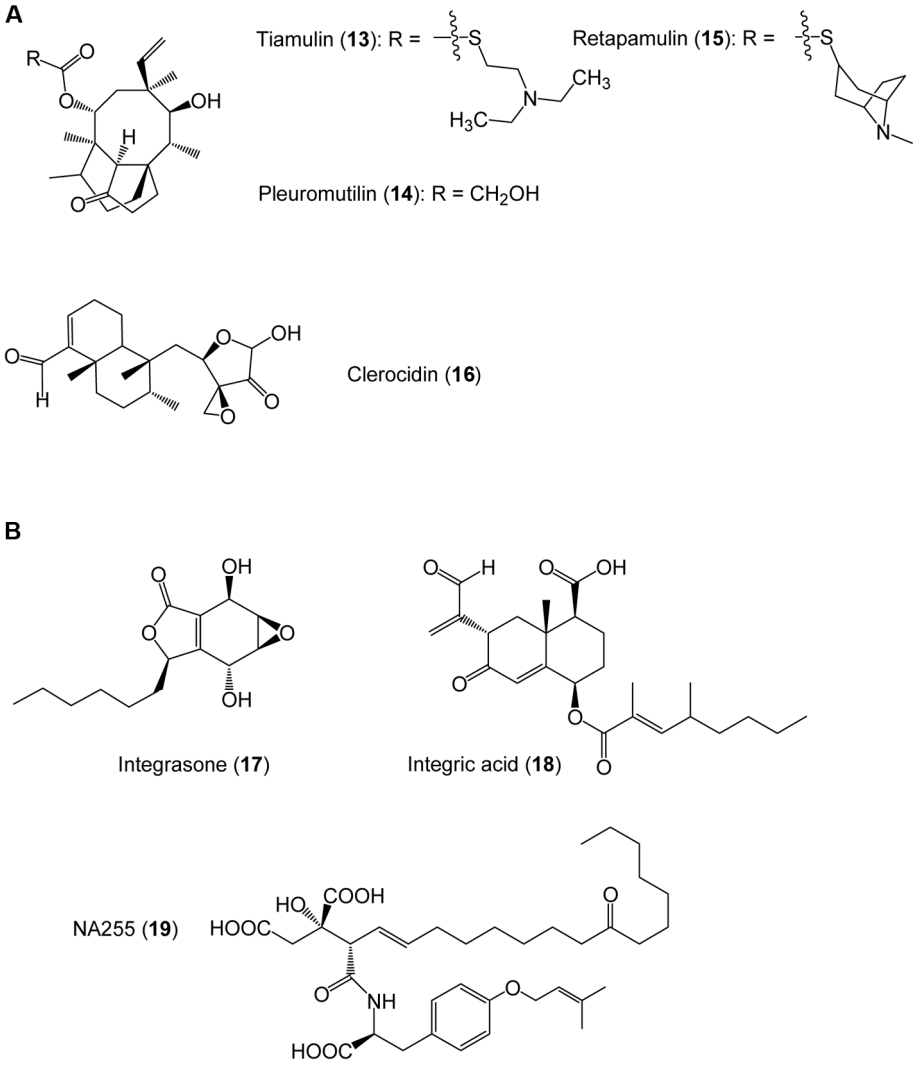


Fig. **3** Highlighted fungal secondary metabolites with **A**) antibacterial and **B**) antiviral activity.

mL and 0.03 μg/mL, respectively [39], [40]. Randomised, double-blind multicentre studies of topical retapamulin versus oral cephalexin treatment of infected skin lesions showed equal clinical success rates [41], [42].

Clerocidin (**16**), a diterpene isolated from *Fusidium viride*, represents a new class of gyrase inhibitors [43], [44]. Compared to quinolone antibiotics, its mode of action differs in the interaction with a topoisomerase IV cleavage complex leading to irreversible DNA breaks. Unfortunately, clerocidin is not sufficiently specific for the bacterial gyrase, but also targets the eukaryotic topoisomerase II, which limits its potential for drug development and therapeutic benefit.

Plectasin differs from the bulk of antibiotics in that it does not belong to the "small molecules". Instead, plectasin is a member of a class of small antimicrobial peptides (AMPs), dubbed defensins [45]. They feature a conserved six disulphide-linked cysteine motif. The small peptide plectasin, 95 amino acids (aa) in length, is composed of three distinct regions: a signal sequence (23 aa), a pro-region (32 aa) and the structural domain (40 aa). The past years saw identification of AMPs in plant and animal tissues and organs, now extended by the discovery of plectasin as the first fungal AMP, isolated from the ascomycete *Pseudoplectania nigrella*. Defensins are particularly attractive due to a rapid bactericidal effect against a wide range of microorganisms including Gram-positive and Gram-negative bacteria, protozoa, yeast, fungi, and viruses, and the low potential resistance pathogens could acquire against AMPs.

One model for explaining the AMP mode of action includes the amphiphilic and cationic nature of AMPs, which promote binding to and pore formation in the cell membrane [46]. However, this effect does not account for all antimicrobial effects observed. For plectasin, first *in vivo* studies in mice confirmed excellent activity against *Pneumococcus* species. Major concerns against the use of defensins as therapeutic agents are, among others, yield and purity and, above all, little experience with systemic administration of native proteins as well as a narrow therapeutic index. The specific advantage of plectasin as defensin of fungal origin lies is its systemic activity and available protocols for production in high yield and purity in *S. cerevisiae* [47].

Antivirals

Fungal secondary metabolism has also been tapped as a source of new antiviral agents. Here, we present recent developments

of how natural products of fungal origin could contribute to the treatment of hepatitis C and HIV infections. Significant progress to combat HIV has been made during the past decade. However, this virus is known to notoriously acquire resistance to current medication. Furthermore, the absence of any therapeutic option toward a complete remission of viraemia underlines the continued challenge pharmaceutical scientists are facing.

Three enzymes critical for the HIV life cycle, among which is HIV-integrase, constitute the targets for antiviral drugs [48]. Intensive screening efforts for inhibitors of the integrase have been carried out and identified a multitude of small molecules, including fungal natural products, some of which have already entered preclinical trials [49]. Scientists at Merck Research Laboratories identified fungal polyketides, integrasone (**17**, Fig. **3**) and integric acid (**18**), as potential lead structures for integrase inhibitors (IC_{50} = 41 μM for integrasone) which interfere with the strand transfer step, i.e., integration of viral DNA into host chromosomes [50], [51]. The neighbouring hydroxy and keto groups are predicted to chelate a divalent metal ion in the integrase catalytic site of the integrase-DNA complex [52].

NA255 (**19**, Fig. **3**) designates a highly reduced fungal polyketide-amino acid hybrid which was recently discovered from a *Fusarium* species during a screen for antiviral compounds [53]. NA255 prevents hepatitis C virus (HCV) from replication by inhibiting the host's serine palmitoyltransferase, an enzyme crucial for the first step in sphingolipid synthesis. Sphingolipids are essential elements of lipid rafts, i.e., microdomains in intracellular membranes. During HCV replication, viral non-structural proteins are closely associated with the sphingolipids. Inhibition of *de novo* sphingolipid synthesis effectively interferes with HCV replication. Preliminary data indicate the potential of NA255 as a promising alternative to current IFN-α-based therapies [53].

Antitumour

Irofulvene (**20**, hydroxymethylacylfulvene, Fig. **4**) is a semi-synthetic derivative of illudin S (**21**), a highly toxic sesquiterpene isolated from the homobasidiomycete *Omphalotus illudens* (the Jack O'Lantern mushroom). The toxicity and severe side effects of illudin S spurred semi-synthetic derivatisation efforts which led to irofulvene and other acylfulvenes, whose greater efficacy is based on their slower and more selective action [54], [55]. IC_{50} values found in the MV522 cell assay are 3 nM for illudin S, 350 nM for acylfulvene, and 73 nM for irofulvene.

As an alkylating agent, irofulvene's toxicity stems from its electrophilic character: once the α,β-unsaturated ketone has been activated by reduction, the cyclopropane ring is attacked by cellular nucleophiles, such as DNA or proteins [56], [57]. Adducts formed in this reaction lead to inhibition of DNA synthesis, cell cycle arrest, DNA strand breaks, and induce apoptosis. Surprisingly, irofulvene is active against tumour cell lines with multidrug-resistant phenotypes. Unlike many other alkylating agents, the presence of the DNA repair machinery enzymes or absence of p53 does not impact irofulvene activity. The precise mode of action of irofulvene is still poorly understood and under investigation.

When exposed for less than two hours to irofulvene, selective attack of a range of human carcinoma cells, such as myelocytic leukaemia, epidermoid, lung, ovarian, and breast cancer cells has been observed. This selectivity depends on a fast energy-dependent uptake mechanism which exists in sensitive cells, contrasting passive diffusion in relatively resistant cells (e.g., bone marrow progenitor cells or some solid tumour cells) [58]. Irofulvene has entered diverse phase I and II clinical trials to investigate the treatment of hormone-refractory prostate cancer, ovarian cancer, metastatic thyroid cancer and inoperable liver cancer [59], [60], [61]. Furthermore, irofulvene has been studied for synergistic effects when used in combination with gemcitabine, cisplatin, and others to treat patients suffering from a variety of solid tumours [62], [63]. Adverse effects from irofulvene therapy are comparable to current chemotherapy.

Fumagillin (**22**, Fig. **4**), a natural product from *Aspergillus fumigatus*, has attracted interest in recent years due to its antiangiogenetic properties and has served as the template for a range of semi-synthetic derivatives. One of those, TNP-470 (**23**), entered phase I/II trials as a candidate agent to treat brain, prostate, and breast cancer, and Kaposi's sarcoma. Fumagillin, TNP-470 and fumarranol (**24**) irreversibly bind to the methionine aminopeptidase 2 as target and therefore interfere with intracellular signalling [64], [65]. Due to the same antiproliferative mechanism, the fumagillin analogue PPI-2458 (**25**) has been investigated for the treatment of rheumatoid arthritis [66]. Fumagillin and ovalicin (**26**) from *Pseudorotium ovalis* have also been considered for use as antimicrosporidial agent as they proved to be broadly efficient in mice, yet moderate in toxicity [67].

The tryprostatins, which are metabolic products from *Aspergillus fumigatus* as well, belong to the widespread diketopiperazine class of natural products. Specifically, tryprostatin A (**27,** Fig. **4**) inhibits cell cycle progression by interfering with microtubule assembly at concentrations of about 20 μM [68]. Cytotoxicity has also been shown for diastereomers [69]. The structurally related diketopiperazine fumitremorgin C (**28**) was found to reverse multidrug resistance, mediated by the breast cancer resistance protein BCRP, at 5 μM [70].

The asterriquinones (Fig. **4**) comprise fungal tryptophan-derived, often prenylated bisindolylquinone alkaloids, with di-demethylasterriquinone D (**29**) as structural prototype. Initially, asterriquinones were discovered in *Aspergillus terreus* (hence the name) [71], yet, numerous derivatives were also isolated from other genera of filamentous fungi. Asterriquinones were first investigated as antitumour agents [72], [73]. More recently, the focus shifted toward other activities: along with epidermal growth factor receptor protein tyrosine kinase inhibition, which accounts for the antitumour activity of the semicochliodinols A (**30**) and B (**31**) from *Chrysosporium merdarium*, HIV-1 protease inhibition (IC_{50} = 0.17μM for semicochliodinol A) was found [74]. Molecular modelling suggests hydrogen-bonding of the quinone core to both active site aspartic acids of this homodimeric protease. Asterriquinones received particular interest after the discovery of L-783,281 (**32**), hinnuliquinone (**33**), and demethylasterriquinone B1 (**34**) as orally available insulin receptor agonists [75], [76]. In the insulin receptor tyrosine kinase assay and at concentrations ranging from 3 – 6 μM, L-783,281 induced

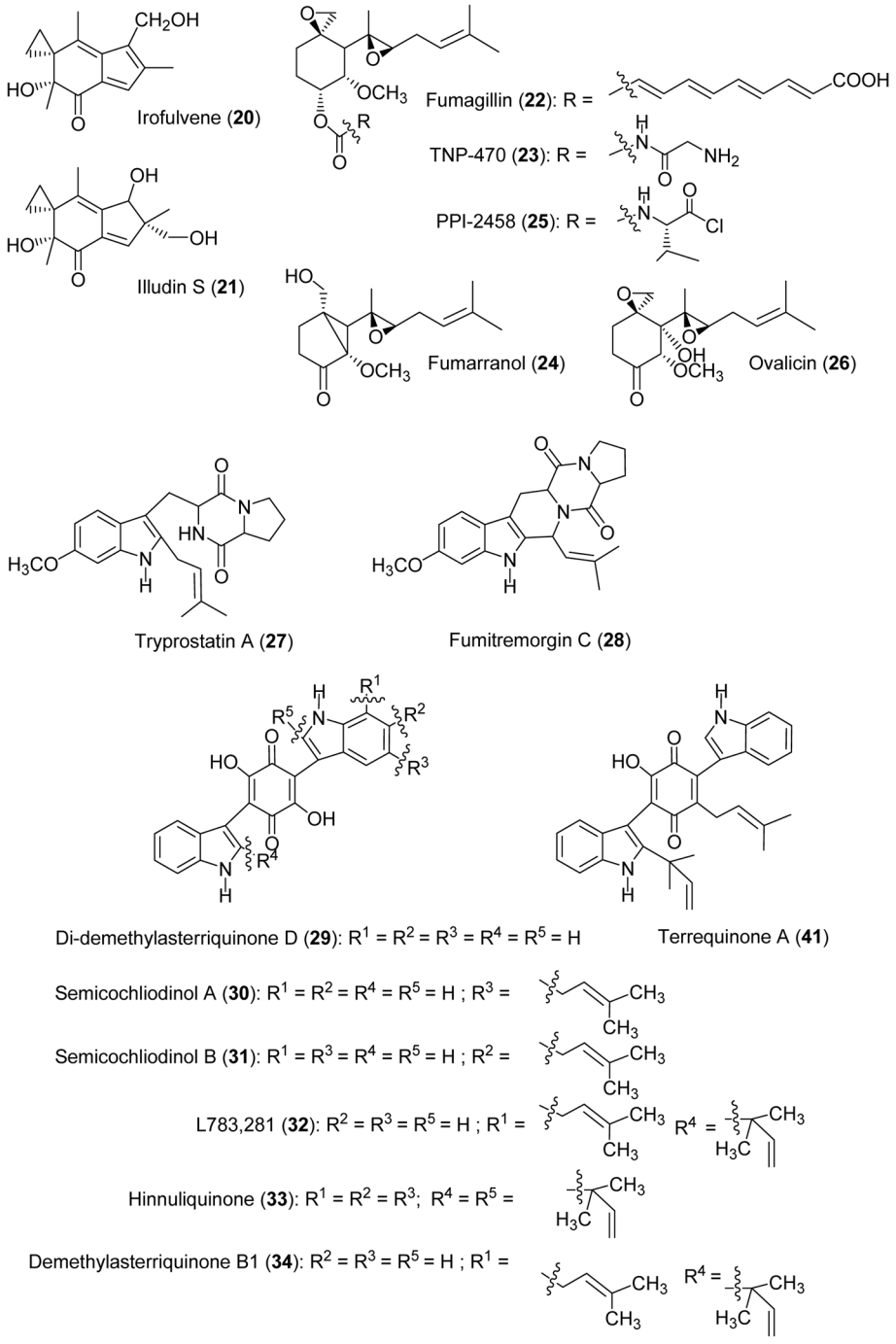


Fig. **4** Fungal compounds with anticancer properties. The bisindolylquinones (lower part) were first investigated as antitumour agents, yet have become more prominent for antidiabetes and antiretroviral activities (see text).

50% of the maximum insulin effect. In mice, L-783,281 corrected substantially hyperglycaemia at therapeutic doses [75].

The Genetics behind Fungal Compounds

For about 15 years, recombinant DNA techniques have been applied to understand natural product formation in fungi. Accumulating data from these studies on known secondary metabolite biosynthetic genes dispelled an original premise that fungal metabolic genes would be scattered throughout the genome. Although eukaryotic, the hallmark of fungal secondary metabolite genes is that they are (in most cases) clustered on one chromosomal locus [77], [78]. This contrasts eukaryotic organisation of genes involved in primary metabolism. Models to explain this phenomenon include hypotheses that horizontal transfer and uptake of prokaryotic genetic material accounts for the clustering. More recent evidence, however, points to regulatory mechanisms as an evolutionary driving force [79], also, clustering of natural product genes increases the probability of co-mobilisation and may confer selective advantage to the gene cluster itself [80], [81] which encompasses biosynthetic genes, and the genes for autoresistance, regulation and transport.

Many backbone structures of fungal natural products are polyketides, small peptides, or a combination of both. They derive from

acetyl-CoA/malonyl-CoA and amino acids and are assembled by dedicated multifunctional multidomain enzymes, referred to as polyketide synthases (PKSs) non-ribosomal peptide synthetases (NRPSs), or hybrid NRPS-PKS systems. A number of excellent reviews on PKSs and NRPSs provide in-depth insight into these molecular assembly lines [82], [83], [84]. Fungal PKSs are monomodular multidomain enzymes, which act multiple times ("iteratively") on a growing ketide. Each iteration extends the growing ketide by a two-carbon, i.e., acetate unit. NRPSs are organised in many successive modules within the enzyme. In either case, one domain harbours one catalytic activity, each module harbours several monocatalytic domains. One module provides all necessary activities to extend the nascent peptide or ketide by one building block, i.e., an amino acid or one acetate unit. Consequently, most gene clusters include one (or multiple) PKS or NRPS genes which can serve as markers during genome-wide analyses on the metabolic potential of a given species. From the researcher's perspective, these gene clusters greatly facilitate cloning of biosynthetic genes as it was realised that many of the structural genes involved in secondary metabolism are highly conserved and could be cloned by hybridisation probing or amplified from the fungal genome by use of degenerate PCR-primers [85]. This conservation of DNA and protein sequences, coupled with the cluster motif of metabolic pathways, greatly facilitated the assignment of putative fungal secondary metabolite genes.

As a textbook example of a microbial compound that made an illustrious career in pharmacy, β-lactam biosynthesis has been the object of scientific research for decades [86], [87]. In brief, penicillin and cephalosporin assembly starts with biosynthesis of the linear tripeptide ACV (**35,** Fig. **5A**) from L-α-aminoadipic acid, L-cysteine and L-valine (the latter epimerised to D-valine), catalysed by the *pcbAB*-encoded ACV-synthetase. This enzyme is a three-module NRPS of 3792 aa [88]. The labile β-lactam moiety is created when IpnA, the *pcbC*-encoded isopenicillin *N*-synthase, cyclises ACV to isopenicillin N (**36**). Pathways to penicillins and cephalosporins, e.g., cephalosporin C (**37**), diverge after isopenicillin N formation. Specific to the penicillin pathway is a replacement event, where L-α-aminoadipic acid is substituted for phenylacetic or phenoxyacetic acid by isopenicillin *N*-acyltransferase (IAT), encoded by the *penDE* gene. All three genes (*pcbAB*, *pcbC* and *penDE*) are clustered on one locus (Fig. **5A**). Genetic analyses have shown that high-performance strains used in industrial production carry up to 14 copies of this cluster in their genome. Recently, the gene for a CoA-ligase has been cloned, whose product – phenylacetyl-CoA – serves as substrate for IAT [89]. The genetic region adjacent to the three penicillin genes has been studied and harbours genes that seem to be involved in – but not strictly indispensable for – penicillin production [90]. In the cephalosporin producer *Acremonium cephalosporium* the genes specific for cephalosporin production (*cefEF* and *cefG*) are located in a separate microcluster on chromosome II, while *pcbA* and *pcbC* are arranged on chromosome VI.

Two crystal structures exist for β-lactam pathway enzymes: firstly for IpnA, which closes the β-lactam ring and subsequently forms the thiazolidine ring [91]. A second crystal structure was obtained for the "expandase", the *cefEF* encoded deacetoxycephalosporin C-synthase, which catalyses the expansion to the six-membered dihydrothiazine ring [92].

The ergot alkaloids from *Claviceps purpurea* are irreversibly linked to pharmacy and its history, and still keep attracting the attention of pharmaceutical biologists and chemists. Deciphering the ergot-alkaloid biosynthetic machinery started in the 1950s with radioisotope feeding experiments, later followed by enzyme purification [93], [94]. It was not until recently that genetic studies revealed a textbook example of how mother nature evolved natural product diversity. Ergot alkaloid biosynthesis was thought to rely on only two non-ribosomal peptide synthetases: LPS1 and LPS2, encoded by *cpps1* and *cpps2*, respectively (Fig. **5B**). LPS2, a 140 kDa protein serves to adenylate D-lysergic acid (**38**, LSA) and catalyses the formation of the first amide bond with L-alanine. LPS1, a 370 kDa multidomain enzyme encompasses three modules to complete ergotamine (**5**) assembly through extension of LSA with L-alanine, L-phenylalanine, and L-proline. In an interesting enzymatic interplay, LPS2 provides the catalytic activity *in trans* to close the amide bond between the LPS2-activated starter molecule LSA and L-alanine, activated by LPS1. Two further putative genes (*cpps3* and *cpps4*) coding for NRPSs (LPS3 and LPS4) adjacent to the core ergotamine biosynthesis cluster have been identified by bioinformatic means [95]. Excitingly, LPS4 is an extremely similar duplicate of LPS1 (85% identical amino acids). Minimal, yet important differences in their primary sequences may, however, impact upon substrate selection. The current model explains the coexistence of LPS1 and LPS4 as basis for the concurrent biosyntheses of ergotamine and ergocryptines, e.g., α-ergocryptine (**39**). Both the LPS1 and the LPS4 assembly line cooperate with LPS2, and, thus, share the LSA starter but diverge thereafter in the amino acid extension as directed by modules 1 and 2 of LPS1 and LPS4, respectively, toward the products LSA-alanine-phenylalanine-proline (ergotamine) and LSA-valine-leucine/isoleucine-proline (ergocryptines) [96]. The biosynthetic function of LPS3 remains to be elucidated.

Cyclosporin A (**4**, INN: ciclosporin) marketed as Sandimmun® is a calcineurin inhibitor and is produced by the filamentous fungus *Beauveria nivea*. The enormous pharmaceutical relevance of this peptide stems from its use as an immunosuppressive drug to prevent allograft rejection after organ transplants, to treat psoriasis and rheumatoid arthritis. The key biosynthetic event is the oligomerisation of the amino acid building blocks into the cyclic peptide structure catalysed by a massive NRPS enzyme, the cyclosporin synthetase SimA [97], [98]. It is encoded in an open reading frame spanning 45.8 kb of chromosomal DNA [99], which makes this gene one of the longest ever found. The calculated mass of this NRPS is 1,689,243 Da and includes eleven modules with an equal number of adenylating and a total of seven methylation domains, consistent with the peptide structure, plus a twelfth small cyclisation module.

As a potent 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitor, lovastatin (**3**) is of immense value as a lipid-lowering agent, both therapeutically and commercially: global sales of the best-selling medicine (Lipitor®) exceeded $10 billion in 2005. Two gene clusters for lovastatin (from *Aspergillus terreus*, Fig. **5C**) and the closely related compound compactin (**40**, from *Pencillium citrinum*) reported [100], [101], which revealed an unusual mechanism for polyketide assembly, i.e., the curious combination of an iterative multifunctional type I PKS of conventional architecture (the *lovB* encoded LNKS) and an auxiliary pro-

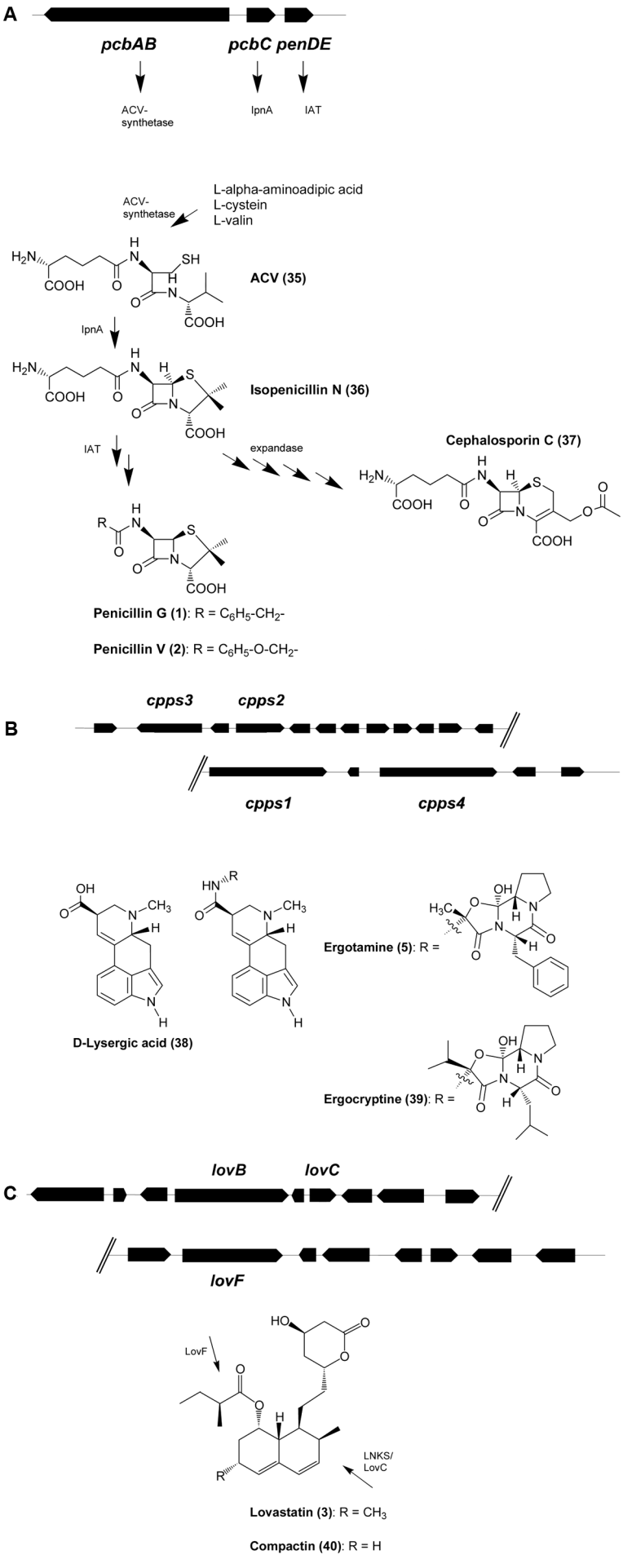


Fig. **5** Organisation of genetic loci dedicated to natural product biosyntheses in filamentous fungi. Arrows represent individual genes and indicate the transcriptional direction. **A**) The penicillin core gene cluster in *Aspergillus nidulans*. Intermediate compounds are shown. **B**) The ergotamine/ergocryptine gene cluster in *Claviceps purpurea*. **C**) The lovastatin cluster in *Aspergillus terreus*.

tein, LovC, acting as monofunctional enoylreductase. Together, these two proteins assemble the nonaketide core. Deletion of the *lovC* gene corrupted lovastatin biosynthesis and led to the production of shorter and not properly reduced shunt products. Furthermore, a non-iterative type I PKS (LovF) provides the lovastatin diketide moiety 2-methylbutyrate. In *P. citrinum* nine genes on 30 kb were identified, all of those homologous to their *lov* counterparts. For lovastatin, 17 genes on a 64 kb stretch of the *A. terreus* genome were identified.

The Genomic Perspective for Fungal Natural Product Research

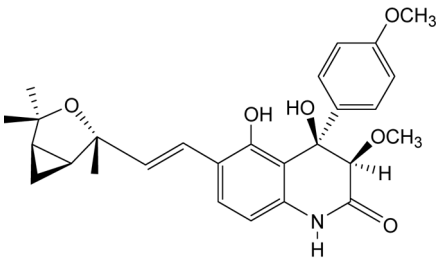
As yet, 55 fungal genomes have been completed, a similar number of projects is currently in progress. Given the vast significance for both biotechnology and pathogenicity, genomes from ascomycetous and basidiomycetous yeasts (e.g., ten *Saccharomyces* species, four *Candida* species, four *Cryptococcus neoformans* strains, as well as *Schizosaccharomyces pombe*, *Pichia pastoris* and others) have so far been dominating the sequencing initiatives. An up-to-date overview of publicly accessible fungal genomic sequences (annotated, finished, or at least at the draft status) is provided at internet servers hosted by non-profit organisations (The National Institutes of Health, The Broad Institute, The Institute for Genomic Research). The number of fungal genomes published in scientific journals, however, lags behind as a consequence of the challenging annotation process for fungal genes.

Other genomes of fungi have been (or are being) sequenced for medical purposes as these species may cause serious infections. These species include, among others, *Neosartorya fischeri* (*Aspergillus fischeri*), which can cause aspergillosis, *Coccidioides immitis* (coccidioidomycosis), *Chaetomium globosum*, a mycotoxin producer and human pathogen, and *Ajellomyces capsulatus* (= *Histoplasma capsulatus*). Viewed from the drug lead discovery viewpoint, most intriguing are the finished genomes of filamentous fungi, in particular from the *Aspergilli*. The finished genomes of *Aspergillus nidulans*, *A. fumigatus*, and *A. oryzae,* have recently been published [102], [103], [104] with *A. fumigatus* being also eminently important as a notorious human pathogen. Genomics also helps understand mycotoxin production and thus indirectly impacts on food safety and, eventually, on human health.

Using the numbers of genes for PKSs, NRPSs, and cytochrome P_{450} oxygenases as indicators, filamentous fungi are unexpectedly rich in genetic material presumably dedicated to secondary metabolism. For example, the *A. nidulans* genome harbours 27 genes for polyketide synthases, 14 for non-ribosomal peptide synthetases, and 102 for P_{450} monooxygenases [102]. Judged by the three *Aspergillus* genomes mentioned above, the number of genetic loci presumably dedicated to secondary metabolism and now revealed by genome sequencing often exceeds the number of known compounds from the particular species by far. Thus, traditional natural product chemistry and genomics find themselves in a stimulating and creative disparity. In a study on *A. nidulans* genomics, a strategy to a genome-wide mine for unknown biosynthetic capabilities to help resolve this discrepancy was devised [105]. It is based on a global transcriptional regulator, called LaeA, that selectively regulates genes that are implicated in secondary metabolism. As proof of principle, the first genetic locus coding for an asterriquinone biosynthetic pathway, the terrequinone A (**41,** Fig. **4**) gene cluster, was identified using this genomic approach, combined with microarray analyses. Thus, identification of the natural product gene locus preceded the discovery of the corresponding metabolite in a given species – the conventional approach turned upside down and lately been duplicated: As the *A. nidulans* genome codes for multiple anthranilate synthases, biosynthetic abilities for quinoline or quinazoline alkaloids were anticipated and verified by isolation of aspoquinolone A (**42,** Fig. **6**) and three derivatives, however, without relating their biosynthesis to a particular gene [106].

Notably, the *Aspergillus* genomes revealed a low degree of synteny between species regarding natural product gene clusters, so the majority of pathways identified through genomics is neither redundant in different species, nor duplicated within a given genome. Similar estimates of the metabolic wealth can also be made for other fungi, e.g., *Cochliobolus heterostrophus*, an important maize pathogen, which harbours 11 NRPS and NRPS-like genes. All NRPS genes, one at a time, have been inactivated which led to phenotypes susceptible to oxidative stress or attenuated virulence [107]. Through genomics, we may move towards a more profound understanding as to why secondary metabolites are produced, and what ecological relevance they have.

The impact of fungal genomics for pharmaceutical sciences reaches far beyond small molecule drug lead discovery, e.g., available genomes enable researchers to undertake transcriptome-wide studies of fungal responses after drug treatment. Also, whole genome analyses help identify essential genes as potential drug targets by screening a pathogen's genome for homologues in other fungi which are, however, absent in mammalian and the human genome. Such work has been carried out for *Candida albicans* and led to 228 genes/proteins which might represent targets for small molecule inhibition [108], [109]. Similarly, by expressing an inducible antisense cDNA library in *C. albicans*, 86 genes critical for growth were identified; more than half of them coded for enzymes with no obvious function [110]. Pharmaceutical aspects of fungal genomics have been reviewed [111]. Based on a comparative analysis with known fungal allergens or on modelled interactions with IgE, the *A. fumigatus* genome was screened for putative allergens and is predicted to code for as many as 58 allergens, such as secreted cellulases and proteases.



Aspoquinolone A (**42**)

Fig. **6** Chemical structure of aspoquinolone A.

In conclusion, our relationship with fungi is arguably described best as light *and* shade, as friend *and* foe. Given their biochemical ingenuity and overwhelming metabolic wealth, however, fungi will not let the drug lead pipeline run dry. Moreover, the major portion of the fungal organismic diversity still remains to be discovered. Consequently, a huge untapped reservoir of new natural products and intriguing enzymes as tools for chemoenzymatic syntheses appeals to pharmaceutical biologists and chemists as an open road ahead. With respect to the pharmaceutical sciences, the bright and friendly side of fungal biology prevails.

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