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Unearthing fungal chemodiversity and prospects for drug discovery

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Abstract

Natural products have drastically improved our lives by providing an excellent source of molecules to fight cancer, pathogens, and cardiovascular diseases that have revolutionized medicine. Fungi are prolific producers of diverse natural products and several recent advances in synthetic biology, genetics, bioinformatics and natural product chemistry have greatly enhanced our ability to efficiently mine their genomes for the discovery of novel drugs. In this article, we provide an overview of improved heterologous expression platforms for targeted production of fungal secondary metabolites, of advances in chemical and bioinformatics dereplication, and of novel bioinformatic platforms to discover biosynthetic genes involved in the production of metabolites with specific bioactivities. These advances, coupled with the presence of vast numbers of biosynthetic gene clusters in fungal genomes whose natural products remain unknown, have revitalized efforts to mine the fungal treasure chest and renewed the promise of discovering new drugs.

Graphical abstract

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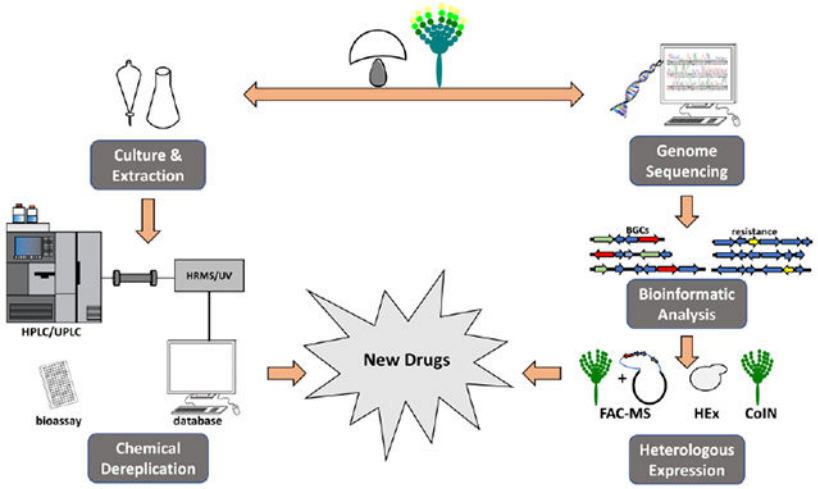

Introduction

The rampant rise of antimicrobial resistance to known drugs is a major challenge for medicine and agriculture [1,2]. Antibiotics, immunosuppressants, anticancers, cholesterol-lowering and other drugs are largely derived from natural products, which are compounds that are naturally synthesized by an organism [3]. Many natural products are also called secondary or specialized metabolites (SMs), because, unlike primary metabolites, they are not essential for survival under laboratory conditions, they are generally uniquely produced by one or a small group of closely related organisms, and often have low molecular weight and potent bioactivities [4,5].

Fungi are an rich source of SMs and several important drugs have been isolated from diverse fungal organisms [6]. But with only a small slice of SM pathways functionally characterized [7], we are just starting to tap into fungal chemodiversity and appreciate its full potential. Usually the genes involved in the synthesis of a specific SM are clustered together in the genome, forming a biosynthetic gene cluster (BGC) [8]. There is an estimated 5.1 million extant species of fungi, and genome sequencing and bioinformatics analysis have shown that the genomes of species in ascomycetes, arguably the largest of the fungal phyla with an estimated 500,000–3 million species, can encode for up to 80 BGCs each [9]. Furthermore, population genomic studies examining within-species variation in BGCs have shown that the genomes of individual isolates do not always capture the entire spectrum of BGCs present in a species [7,10], further increasing the genomic potential of fungal chemodiversity.

This review offers an overview of different approaches to fungal drug discovery, focusing on three areas with exciting recent advances: building efficient platforms for heterologous expression of BGCs whose products remain unknown, developing novel techniques to target chemical and bioinformatic identification of known SMs to address dereplication issues of ‘re-discovery’, and developing bioinformatic tools for efficiently identifying BGCs involved in the production of SMs with specific bioactivities.

Drug discovery using new heterologous expression platforms

In recent years, fungal genome sequencing and the development of specialized software for the prediction of fungal BGCs such as antiSMASH, SMURF and FunGeneClusterS [11–13], have revealed that most filamentous fungi contain dozens of BGCs in their genomes and have the potential to produce many SMs [7]. However, the major challenge hindering efforts to discover novel SMs is that the products of most of these BGCs remain unknown and are difficult to activate under laboratory conditions [3]. One common approach for tackling this challenge is BGC activation using gene overexpression, deletion/overexpression of chromatin modifying enzymes [14]. But the techniques involved are not always efficient and can only be performed on genetically amenable fungi, making their adoption across the Fungal Kingdom difficult. To overcome this issue, several different strategies have been developed, such as growth under diverse nutritional media, co-culture with other organisms, and use of epigenetic modifiers [15–18]. A common characteristic of all these strategies is that they require study of BGCs in their native organisms. An alternative approach is to study BGCs outside their native genomes. This can be achieved through genetic engineering and heterologous expression of BGCs in well-developed platforms in a variety of organism hosts, such as filamentous fungi in the genus *Aspergillus* (subphylum Pezizomycotina, phylum Ascomycota) [19–22], or budding yeasts in the subphylum Saccharomycotina (phylum Ascomycota), such as the baker's yeast *Saccharomyces cerevisiae* [23] or the methylotrophic yeast *Komagataella (Pichia) pastoris* [24].

One recently developed and highly promising platform is the co-inducible nitrate (CoIN) expression system for SMs in *Aspergillus nidulans* [14]. This platform takes advantage of the sterigmatocystin (ST) BGC, in which the transcription factor (*aflR*) and its cofactor (*aflS*) can effectively regulate the other ST biosynthetic genes. The *aflR* and *aflS* promoters were substituted with a bidirectional nitrate-inducible promoter (*niaD/niiA*) which in turn can regulate the expression of the promoter regions within the ST BGC. In addition, the genes of interest are flanked with small DNA regions to direct homologous recombination at the genomic loci *wA* and *yA* associated with spore pigmentation, which enables for facile selection of the transformants. An *A. nidulans* strain devoid of ST was designed and as proof-of-concept, the three genes involved in β-carotene biosynthesis were constructed with specific ST promoters. The strain produced β-carotene with an excellent yield at the desired induction. This is a promising platform for the study of BGCs whose SM products remain unknown, especially when these products may be toxic for the host because it utilizes an inducible promoter (Figure 1).

Another robust platform called Heterologous Expression (HEx) was recently developed in *S. cerevisiae* (Figure 1) [25]. For this platform 30 auto-inducible, coordinated and sufficiently unique HEx promoters have been designed. A new *S. cerevisiae* strain (DHY) was engineered with increased mitochondrial stability and high sporulation. In addition, this strain features the *A. nidulans npgA* gene encoding for a 4′-phosphopantetheinyl transferase, and a P450 reductase from *Aspergillus terreus* to facilitate the biosynthesis of SMs derived from non-ribosomal synthetases (NRPSs) and polyketide synthases (PKSs). To prove the versatility of this system, the authors computationally predicted BGCs containing PKSs or UbiA-type sesquiterepene cyclases from every ascomycete and basidiomycete

genome available in GenBank and used phylogenetic analyses to select 41 unique BGCs. They used yeast homologous recombination to build vectors containing the 41 BGCs and transformed them in the DHY strain. Excitingly, more than half (22/41) of these BGCs produced detectable levels of different compounds.

Another new heterologous platform also uses *A. nidulans* but this time with a fungal artificial chromosome (FAC) self-replicating vector [26,27]. This system works by shearing the DNA in an unbiased fashion in large DNA fragments (30–100 kb), which can contain entire BGCs. The DNA fragments are then assembled together with the FAC vector that contains the *E. coli* F replicon and the *Aspergillus* autonomously replicating sequence (AMA1). The group was able to produce a library of FACs containing 156 BGCs from three *Aspergillus* species, which were confirmed by insert end-sequencing and alignment to the reference genome. Of these FACs, 56 contained uncharacterized BGCs; these were transformed in *A. nidulans* and the strains were cultured, extracted and analyzed by liquid chromatography (LC) coupled with high-resolution mass spectrometry (HRMS). Metabolomic scoring was used to identify novel SMs produced by the transformant strains. To confirm that the BGC was involved in the biosynthesis of the SM, for each strain that produced novel SMs, *A. nidulans* was also transformed with the FAC vector containing the BGC with the synthase gene disrupted. In this work, 17 metabolites were produced by 15 different FACs (Figure 1).

Dereplication strategies for drug discovery

Recent advances in purification methods, extremely sensitive analytical techniques and cheaper sequencing are flooding databases with ever increasing amounts of data, rendering systematic analysis and prioritization challenging. In the context of drug discovery, two key questions are how one can take advantage of these sophisticated analytical techniques to prioritize promising novel SM candidates with interesting bioactivity from a pool of (known and unknown) metabolites (chemical dereplication) and how one can navigate large genomic databases to identify BGCs involved in producing new SMs (bioinformatic dereplication) (Figure 2).

Chemical dereplication

One of the main challenges in discovering novel SMs is in distinguishing unknown from known compounds. Chemical dereplication is essential for avoiding re-characterization of already known SMs. In recent years, great advances in chemical separation by liquid chromatography and detection by HRMS and diode array in combination with the use of databases have facilitated a faster chemical dereplication [28]. El-Elimat *et al.* have optimized a protocol that utilizes a short chromatographic method (10 minutes) with a deconvoluting program to analyze complex mixtures (ACD/IntelliXtracts) and an in-house database containing HRMS, MS/MS and UV data for each SM. This method allowed the group to identify that, among all the analyzed fungal crude extracts with activity against cancer cells, only 50% contained novel SMs [29]. The importance of a good database was highlighted by the work of Kildergaard *et al.,* who were able to identify bioactive compounds from marine-derived fungi and show how fractionation of crude extracts and a

comprehensive library that contains HRMS, MS/HRMS, and UV data facilitated the purification of novel SMs and derivatives of known SMs with activities against cancer cells, fungi and bacteria [30,31] (Figure 2). They also implemented their dereplication strategy by using an unbiased peak picking algorithm which further improved the identification of unknown SMs. Zani and Carroll have developed an open access nuclear magnetic resonance (NMR) and MS database for SMs called DEREP-NP [32]. To be able to use this database, the crude extracts need to be fractionated or purified, but it allows for quick identification of known SMs.

Open-access databases of compounds exist (MetLin, MassBank) [33,34], but they feature a limited number of microbial SMs and should be further implemented with HRMS, MS fragmentation and UV data to allow characterization of known SMs. Recently, a new database called Global Natural Products Social molecular networking (GNPS) was developed where researchers can upload raw or processed MS/MS data [35]. This database offers various features, such as molecular explorer, which provides all the datasets available for a specific molecule and its analogues [35]. Similarly, the molecular networking feature allows users to correlate and visualize a set of spectra for similar molecules. Naman *et al.* have applied this to cyanobacteria and shown that this tool, in combination with bioactivity screening, was extremely useful for the identification of SMs with novel chemical features and led to the characterization of a novel cyclic peptide active against a range of cancer cells [36]. Another novel strategy to induce production of cryptic SMs was developed by Oakley and co-workers. Their idea was to minimize the production of known SMs by gene deletion of the main BGCs, and it was called ‘genetic dereplication’ [37]. In this case, eight BGCs were deleted in *A. nidulans,* and HPLC-UV/MS analysis showed a very simple metabolite profile. The strain was now able to produce a novel metabolite derived from NRPS called aspercryptin. In addition, heterologous expression of BGCs in this *A. nidulans* strain could lead to an easier SM identification as the background from the host metabolites is considerably reduced. Together with advances in genetic modifications in fungi, this strategy offers novel way to discover new secondary metabolites.

Bioinformatic dereplication

As more fungal genomes become available and software for the mining of BGCs improve their accuracy of detection, the possibility of distinguishing unknown from known compounds can also take place at the level of BGCs. For example, sequence similarity searches and evolutionary analyses harbor tremendous potential for mining genomes to identify BGCs that uniquely appear in one or a few species or to identify all the organisms (including ones that are potentially experimentally more tractable) that contain a BGC of interest.

One key requirement for this bioinformatic dereplication approach are databases that contain functionally characterized BGCs and their products. Toward this end, Medema et al. recently established a community standard for the information necessary to create a high-quality repository of BGCs and the SMs they encode [38]. Furthermore, they implemented this community standard as part of the MIBiG repository, on an online database that allows individual users to upload new data as well as to obtain currently known BGCs [39]. As of

the end of 2018, the MIBiG repository contains more than 1,800 complete and partial entries for BGCs and their products from diverse fungi, bacteria and plants (Figure 2).

But how does one analyze genomic data to identify BGCs that are unlike those already in MIBiG? One solution to the challenge is offered by a newly developed computational tool named Biosynthetic Gene Similarity Clustering and Prospecting Engine (BiG-SCAPE) [40]. BiG-SCAPE is a similarity-based algorithm aimed at constructing the network of BGC sequence similarities; examination of such a network can reveal “families” of BGCs that are far apart from or do not contain BGCs already present in the MIBiG repository.

Drug discovery with a target in mind

One highly interesting feature of BGCs is that they sometimes contain a self-resistance gene for self-protection against the SM produced; in recent years, several such self-resistance genes have been identified in fungal BGCs, including transporter genes that accelerate SM secretion, genes that detoxify the SM by changing its chemical structure, or additional copies of the target protein that carry mutations that render them insensitive to the SM’s effects [41]. Great examples of BGCs with extra copy of the target protein are the lovastatin BGC, which contains an extra copy of the (3S)-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase [42], the mycophenolic acid BGC, which contains an additional IMP dehydrogenase [43], the fumagillin BGC which contains two copies of the methionine aminopeptidase [44,45], and the sporiofungin A BGC, which contains an additional copy of the β-1,3-glucan synthase FKS1 [46] (Table 1).

Efforts to identify BGCs containing such self-resistance genes have increased because their functional characterization allows for the identification of not only a novel SM but also its specific gene target (as well as clues about the molecular processes or pathways that the SM likely affects). Recently, an exciting pipeline was developed for Actinobacteria that identified resistance genes in the context of a BGC, called Antibiotic Resistance Target Seeker (ARTS) [47]. This platform identifies resistance genes by looking for a copy of a housekeeping gene within a BGC and evidence of horizontal gene transfer. The platform was shown to be able to identify several BGCs containing resistance genes, including already discovered examples.

More recently, the Fungal Resistance Gene-directed Genome mining (FRIGG) pipeline was created to identify resistance gene within BGC in fungi [48]. This pipeline does not require any knowledge of the resistance gene, but it is designed to identify BGCs that contain a homolog to a housekeeping gene present elsewhere in the genome through a strict search aimed at limiting false-positive hits for resistance genes. Testing of FRIGG on the genomes of 51 *Aspergillus* and *Penicillium* species resulted in the identification of 72 unique resistance genes within BGCs, one of which was the already characterized fellutamide B BGC, demonstrating the applicability of this platform [49,50].

Self-resistance genes can also be employed to guide discovery of drugs that affect them. Yan *et al.* were interested in identifying novel herbicide leads that could inhibit dihydroxyacid dehydratase (DHAD), an enzyme that is part of the branched chain amino acid (BCAA)

pathway [51]. Since the BCAA pathway is also found in fungi, the authors searched fungal genomes for BGCs containing a DHAD homolog; strikingly, they identified DHAD homologs within four different BGCs across different fungi, one of which was *A. terreus.* Expression of the *A. terreus* BGC in *S. cerevisiae* showed that it produced aspterric acid, which was effective at inhibiting the growth of *Arabidopsis thaliana.* The work was able to establish the mode of action of this compound and showed by *in vivo* and *in vitro* experiments that it is a competitive inhibitor of DHAD.

Beyond the cluster paradigm

Although most fungal SM pathways are encoded by BGCs, examination of fungal genomes suggests that there is substantial variation in the genomic arrangement of fungal SM pathways [7]. On one hand, SM pathways can be split into two or more BGCs, such as in the case of dothistromin [52], cephalosporin [53], and the meroterpenoids austinol and dehydroaustinol [54]. On the other hand, SM pathways can be intertwined, as in the case of the genes involved in the biosynthesis for the SMs pseurotin and fumagillin, which are found as part of a single supercluster [45]. Irrespective of their genomic arrangement, a hallmark characteristic of genes in SM pathways is that they are all part of the same regulatory network [55]. Thus, an alternative approach is to use global co-expression data sets to identify sets of genes, including encoding for enzymes such as PKSs and NRPSs, that are co-expressed across a range of different conditions or experiments [55], irrespective of whether these genes are part of a BGC or not.

This gene co-expression network approach has been recently successfully employed in both plants [55], which are not always organized into BGCs [7], and fungi [56,57]. For example, a recent study used 155 transcriptomic experiments to construct the gene co-expression network of *Aspergillus niger* [56]. Examination of co-expression subnetworks that contain BGCs revealed two transcription factors that are not parts of BGCs but whose over-expression led to the up-regulation of several BGCs in the *A. niger* genome [56], suggesting that they are involved in the global regulation of SM in this species.

Conclusion and outlook

Fungi have for a long time been considered as a great source of drugs, but only with the sequencing of many fungal genomes and the discovery that they contain many uncharacterized BGCs, we realized that their true potential in producing bioactive molecules is still largely untapped. In order to exploit this great resource, new platforms for heterologous expression have been designed in different organisms with success in expressing natural products from traditionally hard to mine fungi, such as Basidiomycetes [21]. These different platforms have shown to be very robust in expressing BGCs from a range of different fungi, which allowed the identification of novel metabolites. These platforms have great potential for drug discovery and they can provide examples to further improve existing platforms. Progress has been made in the development of pipelines that allow efficient identification of unknown molecules with bioactivities. Chemical dereplication can offer a systematic way to process crude extracts. Efforts in developing comprehensive and publicly available chemical databases with detailed data about

metabolites (e.g. HRMS, HRMS/MS, and UV spectra) need to be made to facilitate drug discovery. New bioinformatic tools have been developed to identify novel strategies to mine the growing number of fungal genomes available. These novel bioinformatic tools allow to better identify BGCs and provide some information about their products. This is an exciting time to discover new compounds from fungi and this review has highlighted some of the recent advances that will facilitate this process.

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special interest (●) or outstanding interest (●●)

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Highlights

- Fungi are an excellent source of drugs and their full potential in producing different metabolites is still largely untapped.
- Several heterologous platforms have been developed to produced novel metabolites.
- Chemical and bioinformatic pipelines can successfully identified unknown metabolites.
- Bioinformatic tools can identify fungal biosynthetic gene clusters involved in the biosynthesis of novel bioactive compounds.

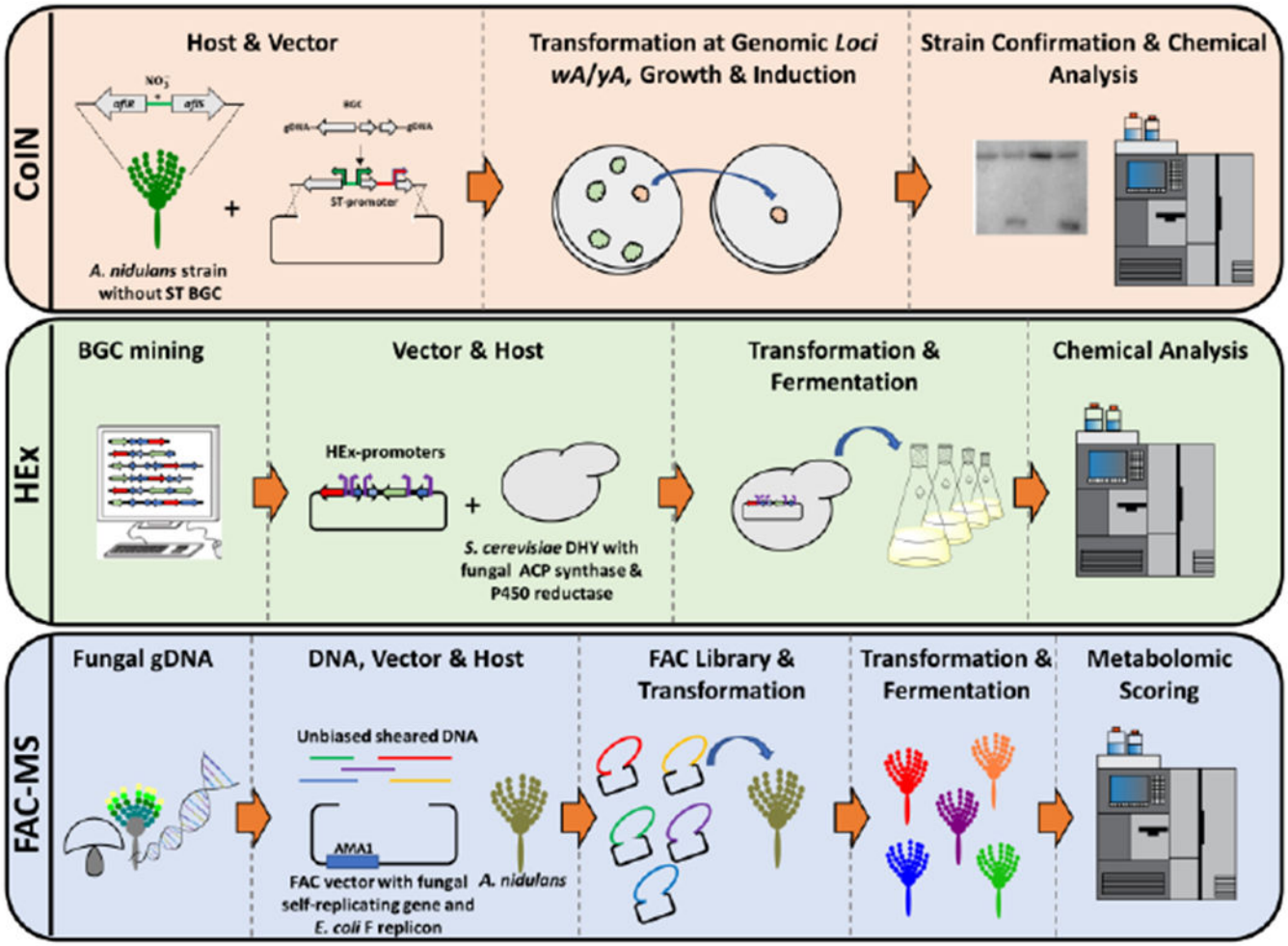

Figure 1.

Schematic of three heterologous platforms recently developed to characterize novel fungal secondary metabolites [14,25,26].

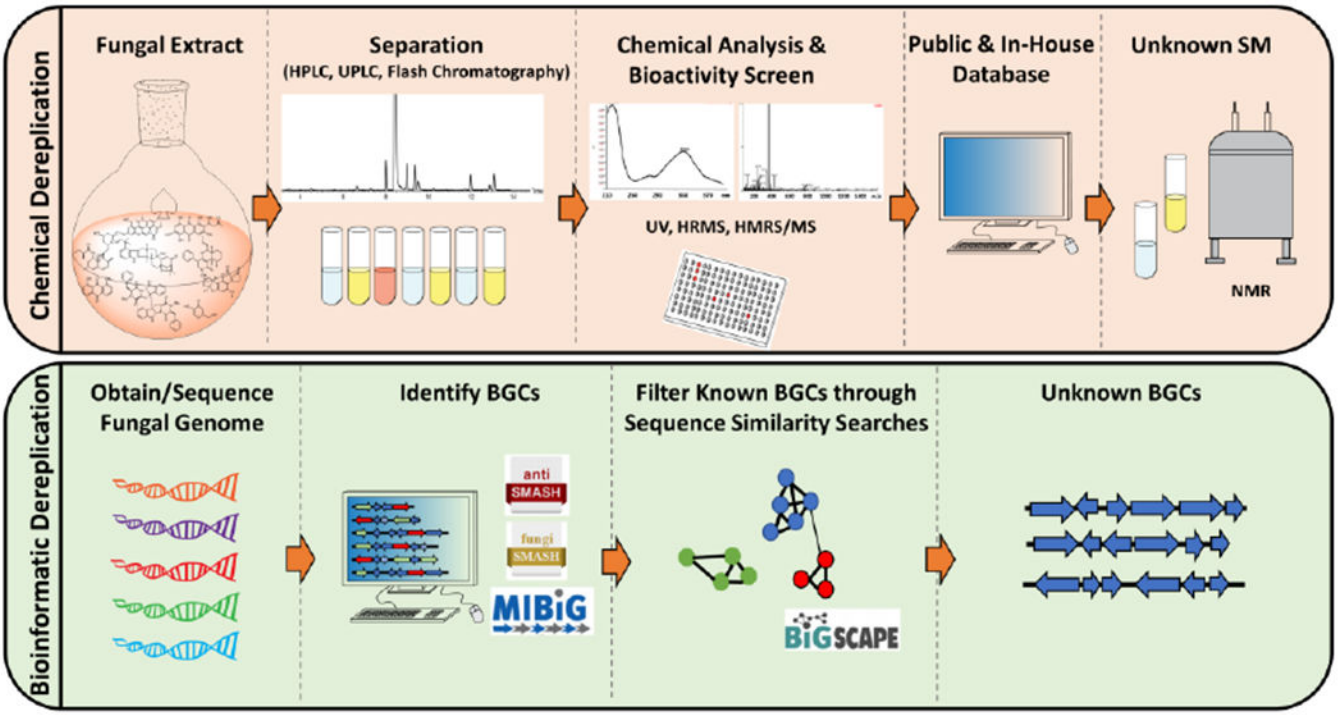

Figure 2.

Chemical and bioinformatic dereplication work-flow for the discovery of unknown secondary metabolites from fungi.

Table 1.

Natural products from fungi with a homolog of their target protein as the self-resistance gene within their BGC

Compound	Organism	Self-resistance gene	Activity	Ref.
Lovastatin	*A. terreus*	*IvrA* (HMG-CoA reductase)	Treats hypercholesterolemia by inhibiting HMG-CoA reductase	[42,48]
Mycophenolic acid	*Penicillium brevicompactum*	*mpaF*(IMP dehydrogenase)	Immunosuppressive	[43]
Fumagillin	*A. fumigatus*	AfuA_8g00460 (methionine amino peptidase I) AfuA_8g00410 (methionine amino peptidase II)	anti-angiogenesis	[44,45]
Sporiofungin	*Pezicula radicicola*	*prfks1a* (glucan synthase FKS1)	Antifungal	[46]
Fellutamide	*A. nidulans*	*inpE* (β6 proteasome subunit)	Proteasome inhibitor	[47]
Aspterric acid	*A. terreus*	*aspD* (dihydroxyacid dehydratase)	Herbicide – Inhibition of the DHAD enzyme involved in the branched-chain amino acid biosynthetic pathway in plants	[48]