PhytoMycology

PhytoMycology (2026) 2: 2003
https://doi.org/10.65390/phtomyc.2026.2003

Review Article

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Strategies for mining new secondary metabolites from fungi: from Classical Approaches to Genomics-Based Mining

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Received: 9 December 2025 / Accepted: 13 April 2026 / Published: 26 May 2026

Abstract

Fungi are prolific producers of structurally diverse secondary metabolites, which play significant ecological and pharmacological roles. However, the rate of discovering new fungal natural products has slowed in recent decades, highlighting the need for innovative approaches to unlock their biosynthetic potential. Recent advances in genome sequencing and bioinformatics have revealed the complexity of fungal biosynthetic gene clusters, enabling the development of strategies to activate silent pathways and improve the efficiency of compound identification. This review synthesizes current methodologies for mining novel fungal metabolites, including classical approaches that modify culture conditions, genomics-based genome mining, and dereplication techniques to efficiently distinguish known from unknown compounds. We further highlight integrated, multi-strategy frameworks that maximize discovery efficiency and discuss emerging directions such as multi-omics integration and machine learning-assisted pathway prediction. By providing a comprehensive and forward-looking overview, this work aims to guide the effective exploitation of fungal resources in the search for new natural products.

Key words: Fungal secondary metabolites, Genome mining, Biosynthetic gene clusters (BGCs), Dereplication, Culture condition optimization, Natural product discovery

Citation: Zhang, L.-J., Jayawardena, R.S., Ausana, M., Liu, N.-G., Ma, J., Xiao, X.-J., Arttapon, W., Xiao, Y.-P., Hyde, K.D., Lu, Y.-Z., Sun, J.-Z. (2026) Strategies for mining new secondary metabolites from fungi: from Classical Approaches to Genomics-Based Mining. PhytoMycology (2026) 2: 2003 https://doi.org/10.65390/phytomyc.2026.2003

Introduction

Fungi host an extensive repository of secondary metabolites, which are bioactive compounds that enable adaptation to their environment and host (Fig. 1). These metabolites often serve as defense mechanisms, allowing fungi to compete with other microorganisms. Notably, penicillin and cephalosporins, first identified as antibiotics, exemplify this function. Penicillin, produced by *Penicillium* species, was the first antibiotic demonstrated to effectively inhibit Gram-positive bacteria (Ligon 2004). Cephalosporins, initially obtained from the fermentation products of *Cephalosporium acremonium* (now known as *Acremonium chrysogenum*), marked the discovery of the first broad-spectrum antibiotic (Brotzu, 1945; Abraham, 1979; Bo, 2000). Additionally, many fungi produce potent toxins that aid in colonizing other organisms. For instance, aflatoxins, produced by *Aspergillus flavus*, are toxic to plants and carcinogenic to animals and humans (Amaike & Keller 2011; Bartholomew et al. 2021). These toxins not only suppress host defenses but also facilitate fungal colonization.

In response to environmental stress, fungi adapt by producing secondary metabolites. For example, some fungi secrete antifreeze secondary metabolites that enable survival in low-temperature environments (Xiao et al. 2010). Additionally, certain fungi produce glutathione and antioxidants to resist oxidative stress (Ogbe et al. 2020). Fungal secondary metabolites (SMs) not only serve as defense mechanisms for fungi but also have significant applications in human health and medicine. Caspofungin, derived from *Glarea lozoyensis*, is one of the safest and most effective antifungal drugs (Balkovec et al. 2014). Statins, obtained from *Penicillium compactum* and *Aspergillus terreus*, remain among the most effective lipid-lowering drugs (Yadav & Mohite 2020). Furthermore, the entomogenous fungi act as natural regulators of

insect populations, particularly in the absence of predators or under adverse environmental conditions, where they control insect populations by infecting and killing them. *Tolypocladium inflatum*, a soil fungus and insect pathogen, produces the potent immunosuppressant cyclosporin, which typically targets scarab beetle larvae (Yang et al. 2018; Dong et al. 2022). The mechanism behind such interaction helps to maintain insect population balance, benefiting agriculture and forestry (Sharma et al. 2020). Additionally, macrofungi serve as natural decomposers, playing a crucial role in breaking down complex organic materials like dead plants and wood (Niego et al. 2023). For example, *Ganoderma lucidum*, a medicinal and edible mushroom, is rich in triterpenes and polysaccharides, which exhibit hepatoprotective and anti-tumor effects (Galappaththi et al. 2022).

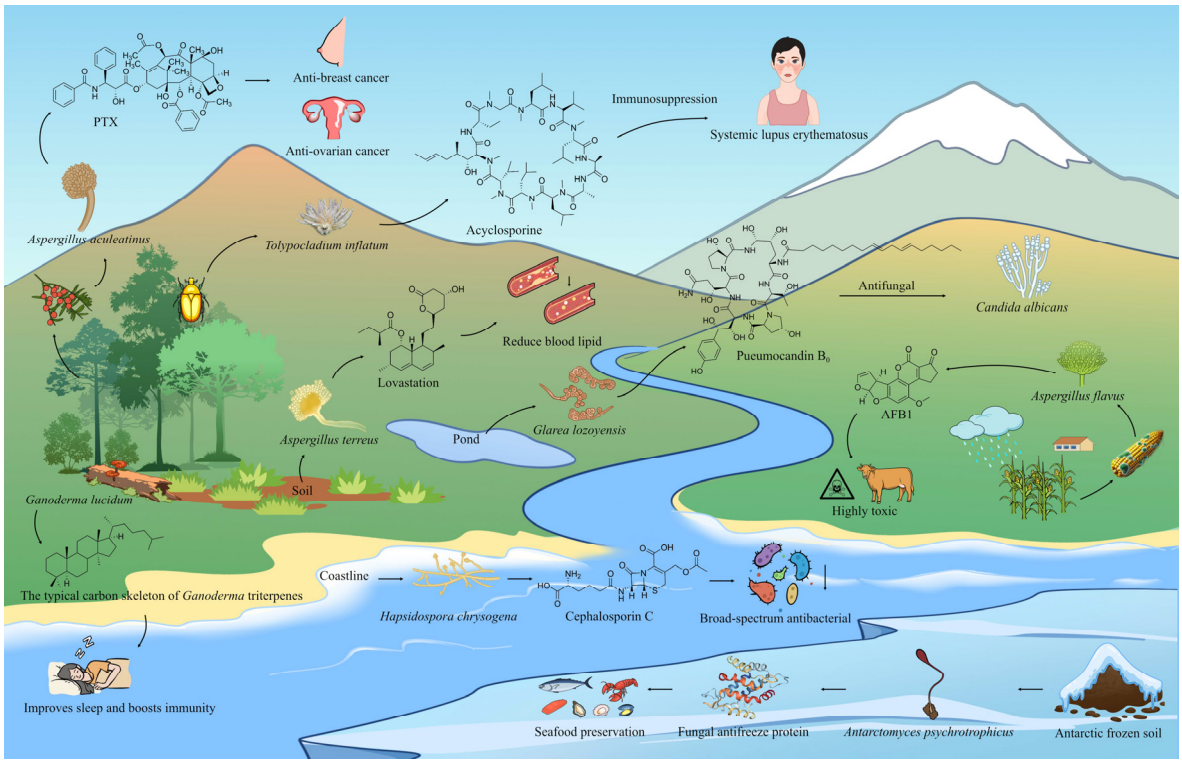

Fig. 1 Fungal secondary metabolites in various ecological environments.

Despite the diverse functions of fungal secondary metabolites, opportunities for discovering new natural products from fungi are diminishing (Lyu et al. 2020; 2022). This trend is primarily due to the vast number of undiscovered fungal species and the tendency of biosynthetic gene clusters responsible for secondary metabolite production in many wild strains to remain dormant under laboratory conditions (Lyu et al. 2020; 2022). This review is structured as follows. First, we classify methods of activating silent gene clusters into two categories: strategies to indirectly activate biosynthetic gene clusters by optimizing culture conditions, detailed in Section 2, and approaches involving gene mining, discussed in Section 3. Second, we systematically address dereplication strategies in Section 4. Section 5 provides examples of integrated strategies for novel secondary metabolite discovery. This review comprehensively explores methods for leveraging fungal resources to discover new natural products (Fig. 2).

Strategies for activating fungal silencing biosynthesis gene clusters based on changing culture conditions

It is well-established that environmental and cultural factors significantly influence the biosynthesis of fungal secondary metabolites. Conventional culture methods are insufficient to address the increasing demand for novel chemical entities. While replicating natural conditions in the laboratory presents challenges, researchers can activate latent biosynthetic gene clusters and stimulate secondary metabolite production by modifying culture parameters, such as light exposure, temperature, pH, carbon sources, and nitrogen sources. The discovery of novel secondary metabolites through cultural adjustments encompasses various techniques, including the One Strain/Many Compounds approach, co-culturing with other microorganisms, and chemical epigenetic modifications.

One strain/many compounds' strategies (OSMAC)

The "One Strain/Many Compounds" methodology was introduced by Bethe and Fuchser (Bethe 1994; Fuchser & Zeeck 1997) and later formalized by Hans-Jörg, who coined the term OSMAC (Schieve & Zeeck 1999). This strategy leverages the latent biosynthetic capacities of microorganisms to produce a diverse range of compounds by

modulating culture conditions, including medium composition, environmental parameters, and cultivation techniques (Pan et al. 2019). Key adjustable factors include nutrient composition, temperature, oxygen levels, pH, light exposure, metal ion concentrations, physical growth medium (solid vs. liquid), agitation, salinity, and culture maturity (Pan et al. 2019; Salim et al. 2021).

Changes in culture medium composition

Carbon and nitrogen sources are primary nutrients that significantly influence microbial metabolism, providing essential building blocks for the biosynthesis of secondary metabolites (Satyanarayana 2013; Xie et al. 2019). These studies studied the deep-sea-derived fungus *Penicillium allii-sativi* (MCCC 3A00580) and identified three androstanes from rice fermentation products. When the rice culture medium was replaced with an oat culture medium, nine new androstones (**1–9**) were discovered (Xie et al. 2021). This finding suggests that optimizing the carbon-to-nitrogen (C/N) ratio enhances the exploration of andrastones. Among these compounds, compound **1** features a novel hemiketal moiety, while compound **2** represents the first reported example of a tetrahydrofuran moiety linked via C-7 and C-15 (Fig. 3) (Xie et al. 2021).

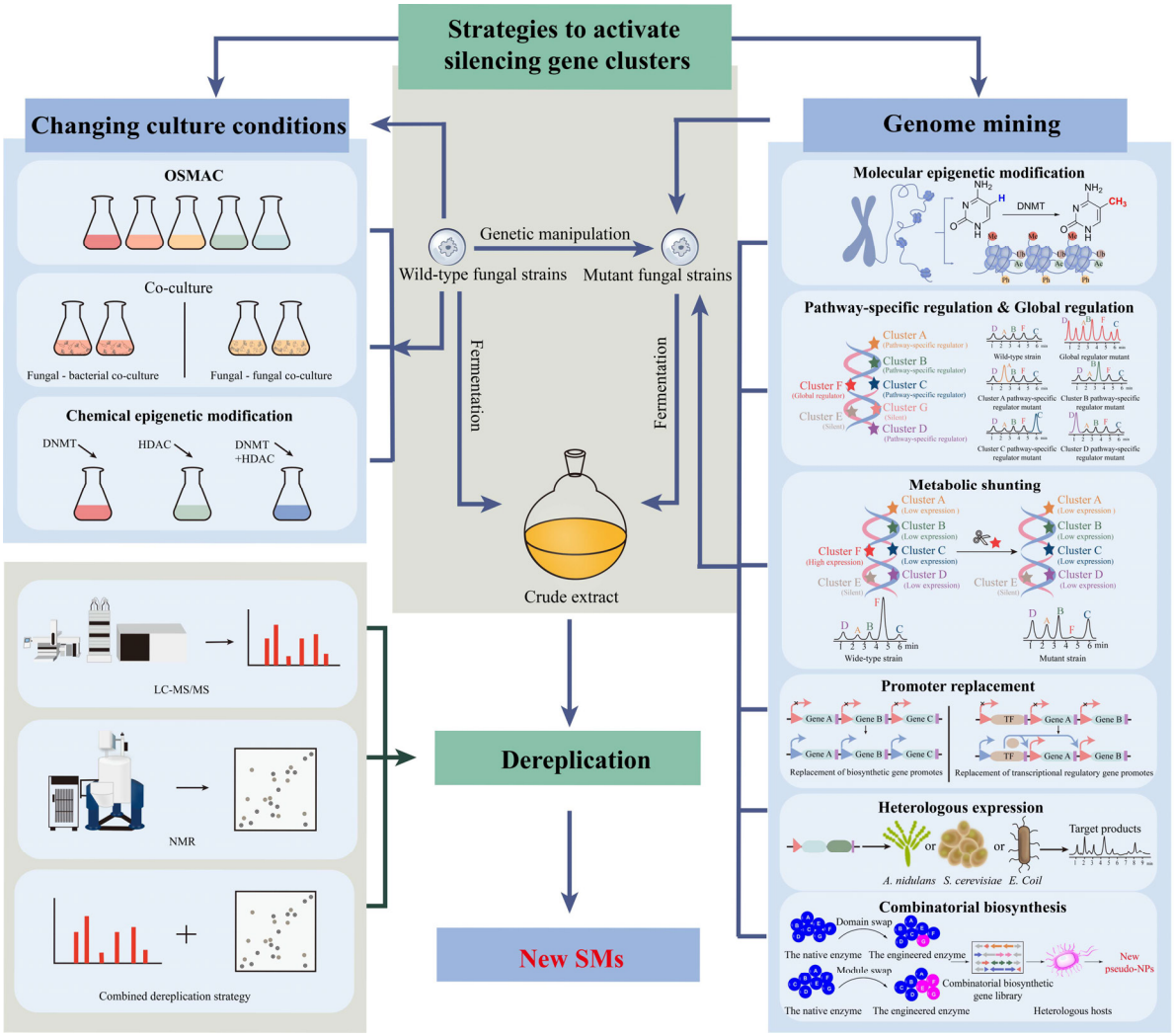

Fig. 2 Strategies for mining new secondary metabolites from fungi.

Altering pH

The pH is a key factor influencing fungal growth and the production of secondary metabolites. The production of trichothecene in *Fusarium graminearum* depends on the pH of the medium and is regulated by the pH regulatory factor *pac* gene (Merhej et al. 2011). Siridechakorn et al. (2017) also demonstrated that the endophytic fungus *Rhytidhysteron rufulum* AS21B produces significantly more diverse spirobisnaphthalene derivatives (**10–11**) (Fig. 4) under acidic conditions (pH ~ 5) compared to neutral conditions.

Adjusting fermentation time

Lin et al. (2009) employed One Strain/Many Compounds strategies to study the marine-derived fungus *Spicaria elegans*, identifying various secondary metabolites at different fermentation times. After 8 days of fermentation, they isolated spicochalasin A (**12**) (Fig. 5), five new aspochalasins (M–Q)

(**13–17**) (Fig. 5), and two known aspochalasins. Extending the fermentation period to 14 days resulted in the discovery of three new aspochalsins (R-T) (**18–20**) (Fig. 5). These findings were further supported in a subsequent study by Lin et al. (2010).

Altering culture media

Xie and colleagues (2022) investigated the rice fermentation products of the soil-derived fungus *Phialocephala* sp. YUD18001 and identified its capacity to produce lactones featuring 12-, 6-, and 5-membered rings. Upon transitioning the culture medium from rice to PDB, they discovered two novel pyranonaphthoquinones, phialoyxinones A (**21**) and B (**22**), along with the new 18-membered ring lactone, phialoyxtone (**23**), and five known pyranonaphthoquinone derivatives, as shown in Fig. 6 (Xie et al. 2023). Compound **22** demonstrated significant in vitro cytotoxicity against five human cancer cell lines (*HL-60, SMMC-7721, A549, MCF-7,* and *SW480*), with IC_{50} values ranging from 11.80 to 19.32 μM. Furthermore, compounds **22** and **23** exhibited moderate acetylcholinesterase (AChE) inhibitory activity, as previously reported (Xie et al. 2023).

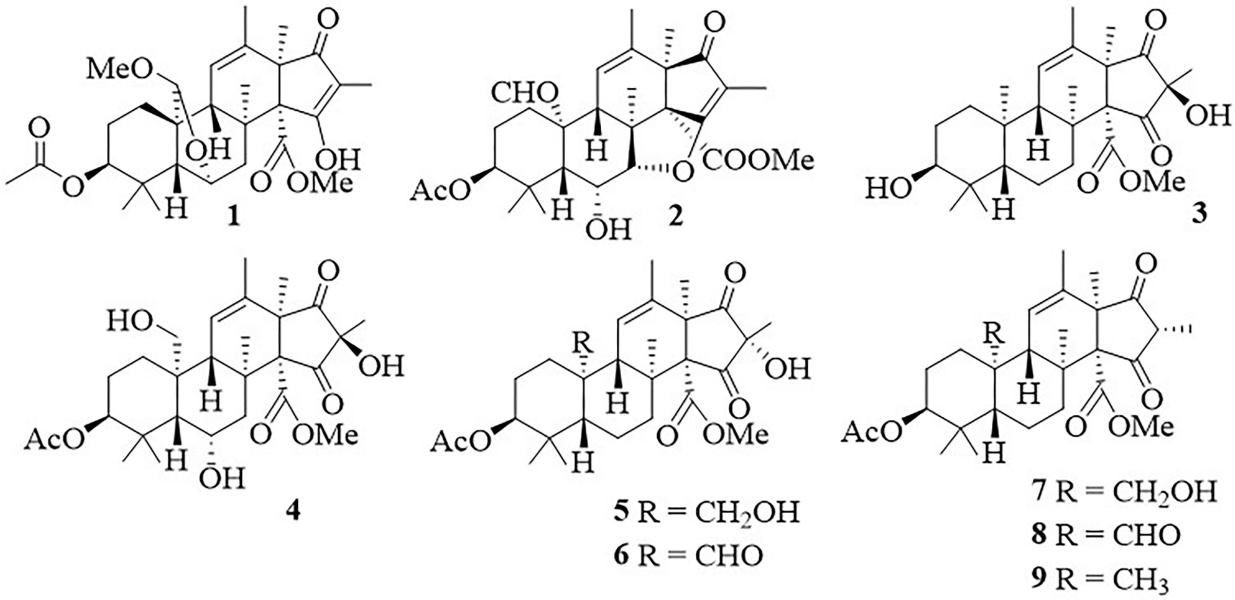

Fig. 3 New natural products discovered using OSMAC strategies by replacing rice with oats.

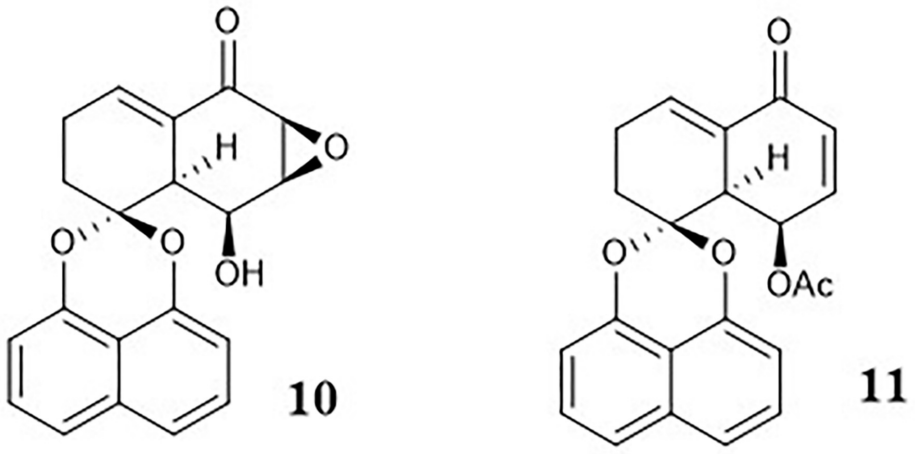

Fig. 4 New natural products discovered by modifying pH levels.

Adjusting oxygen concentration

Oxygen levels significantly influence microbial respiration and metabolism. Research has shown that early-branching fungi produce a variety of secondary metabolites under anaerobic conditions (Swift et al. 2021). Guo et al. (2013) changed the fermentation method of the deep-sea-derived fungus *Penicillium* sp. F23–2 from static to agitated culture in YPG medium, resulting in the discovery of five new nitrogen-containing sorbicillinoids, sorbicillamines A–E (**24**–**28**), via shaker fermentation (Fig. 7).

Altering fermentation temperature

Temperature significantly influences fungal metabolism. Investigations into the antimicrobial properties of fungi isolated from Arctic and Antarctic regions revealed that their antimicrobial activity is dependent upon secondary metabolite profiles, which vary across different temperatures (Yogabaanu et al. 2017). In a study by Liu et al. (2018), the fermentation temperature of *Penicillium raistrickii* was reduced from 28 °C to 15 °C, resulting in the isolation of five novel polyketides (PKs), namely raistrickiones A–E. (**29**–**33**) (Fig. 8).

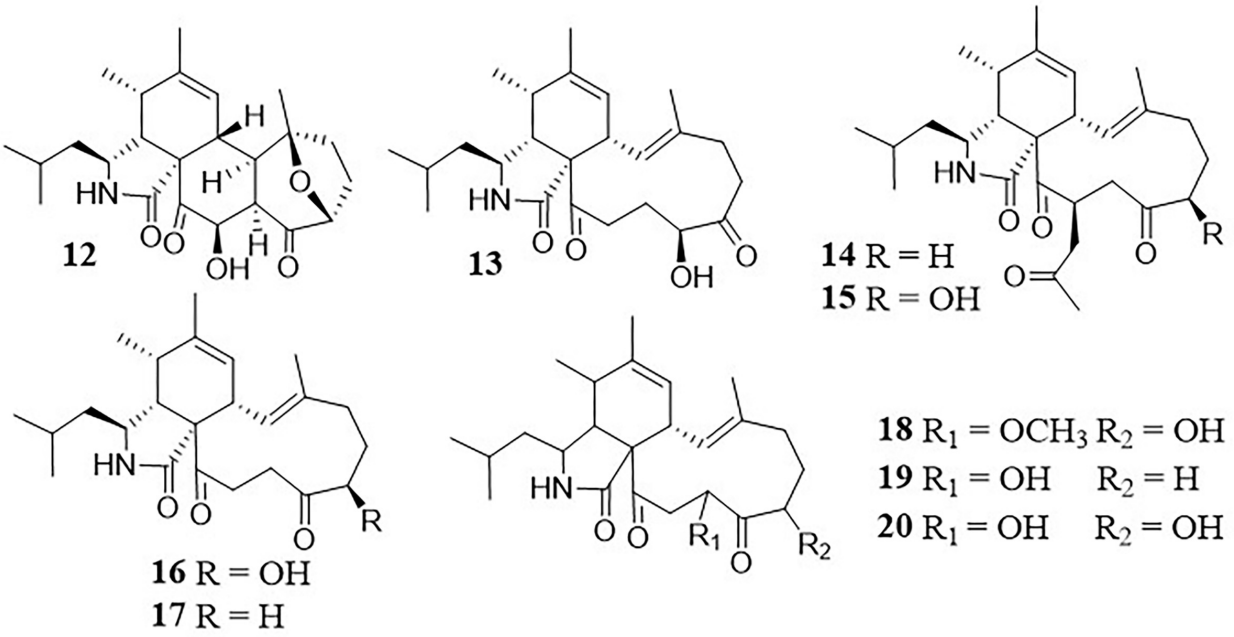

Fig. 5 New natural products discovered by modifying fermentation time.

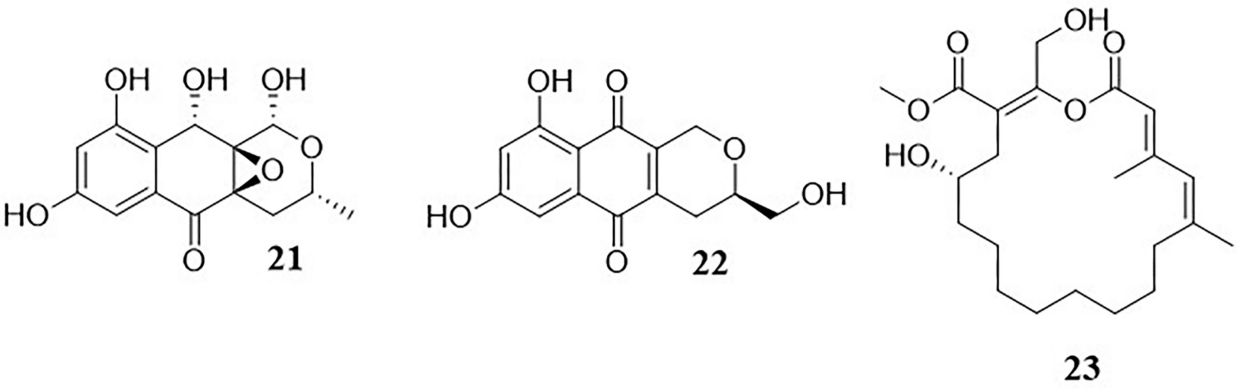

Fig. 6 New natural products discovered by altering culture media.

Adding halide ions

Salinity exerts a significant influence on microorganisms, particularly in aquatic environments, affecting their cellular osmotic pressure and capacity for environmental adaptation. Chen et al. (2022) investigated the effects of salinity on the mangrove-derived fungus *Phomopsis* sp. QYM-13 by incorporating 3% NaBr or 3% KI into its potato liquid medium. This approach facilitated the discovery of 12 novel cytochalasins, designated as phomopchalasins D–O (**34**–**45**) (Fig. 9). Notably, compounds 34 and 38 represent the first reported iodinated cytochalasins. Additionally, compound **35** demonstrated substantial cytotoxic activity against the human cancer cell line MDA-MB-435, with an IC_{50} value of 7.4 ± 1.5 μM.

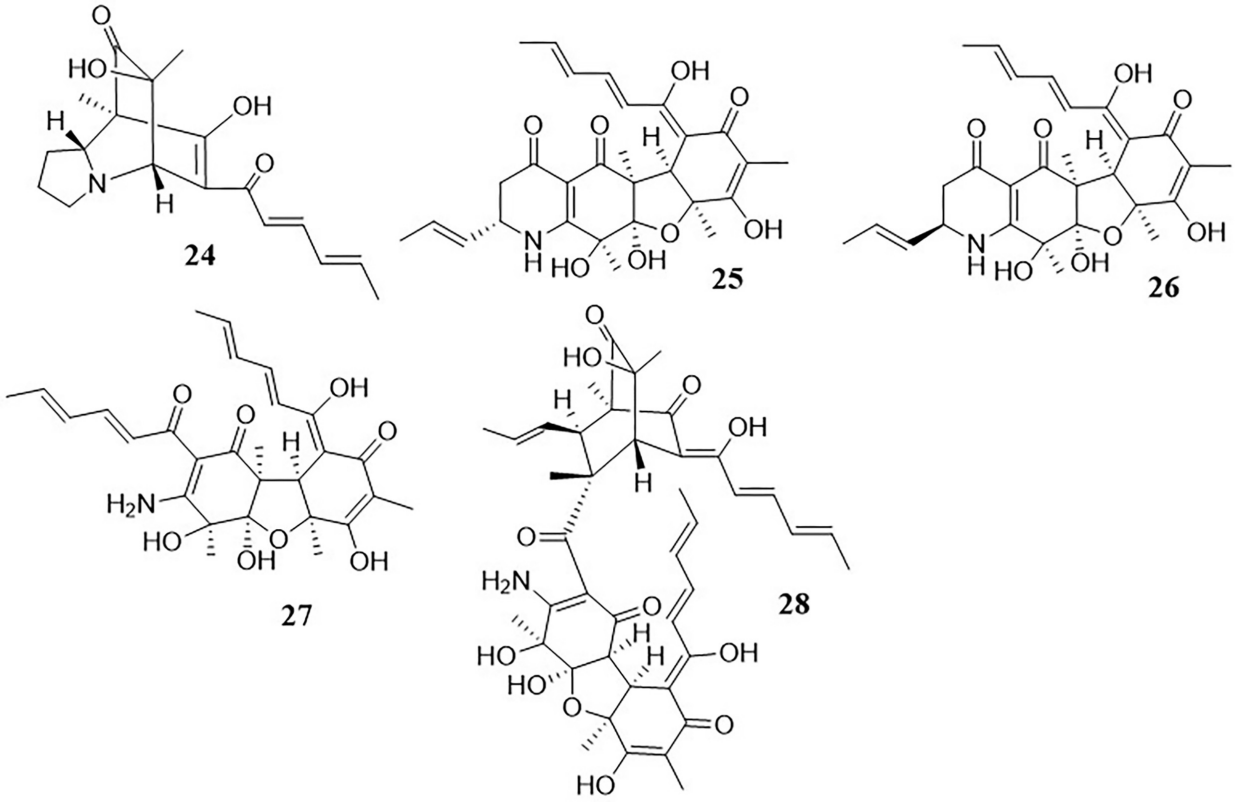

Fig. 7 New natural products discovered by increasing oxygen concentration.

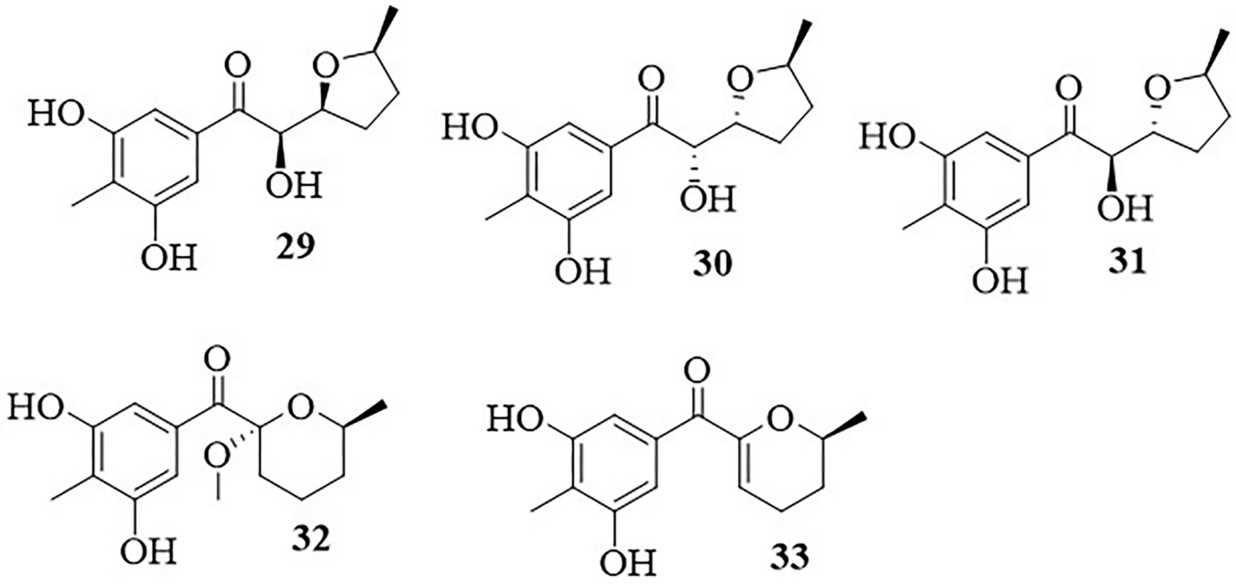

Fig. 8 New natural products discovered by altering fermentation temperature.

Adding metal ions

Metal ions are essential trace elements in microbial metabolism and serve as important cofactors for many enzymes involved in fungal secondary metabolite synthesis (Satyanarayana 2013). In a study by Lin et al. (2022), a rice medium supplemented with 0.1% $FeSO_4{\cdot}7H_2O$ and 3% sea salt was used to culture a coral-derived fungus, *Stachybotrys chartarum*. This led to the discovery of six novel dimeric phenylspirodrimanes, named distachydrimanes A–F (**46**–**51**) (Fig. 10). Notably, three compounds (**45**, **50**, and **51**) exhibited significant inhibitory effects on cell proliferation.

Adding organic small-molecule precursors

The incorporation of biosynthetic precursors, such as amino acids or solvents, can significantly influence metabolic pathways. In a study by Yamazaki et al. (2015), the natural seawater medium of the marine-derived fungus *Trichoderma* sp. strain TPU199 was supplemented with dimethyl sulfoxide

(DMSO), leading to the production of an unprecedented trithio-derivative of epidiketopiperazine, specifically chlorotrithiobrevamide (**52**) (Fig. 11). Similarly, Yuan et al. (2019) supplemented the glucose–peptone–yeast (GPY) medium of the marine fungus *Pseudallescheria boydii* with L-tryptophan, L-phenylalanine, L-methionine, and L-threonine. This modification resulted in the isolation of two new bisindole alkaloids, pseudobindole A (**53**) and pseudobindole B (**54**), as well as 11 known indole alkaloids (Fig. 12).

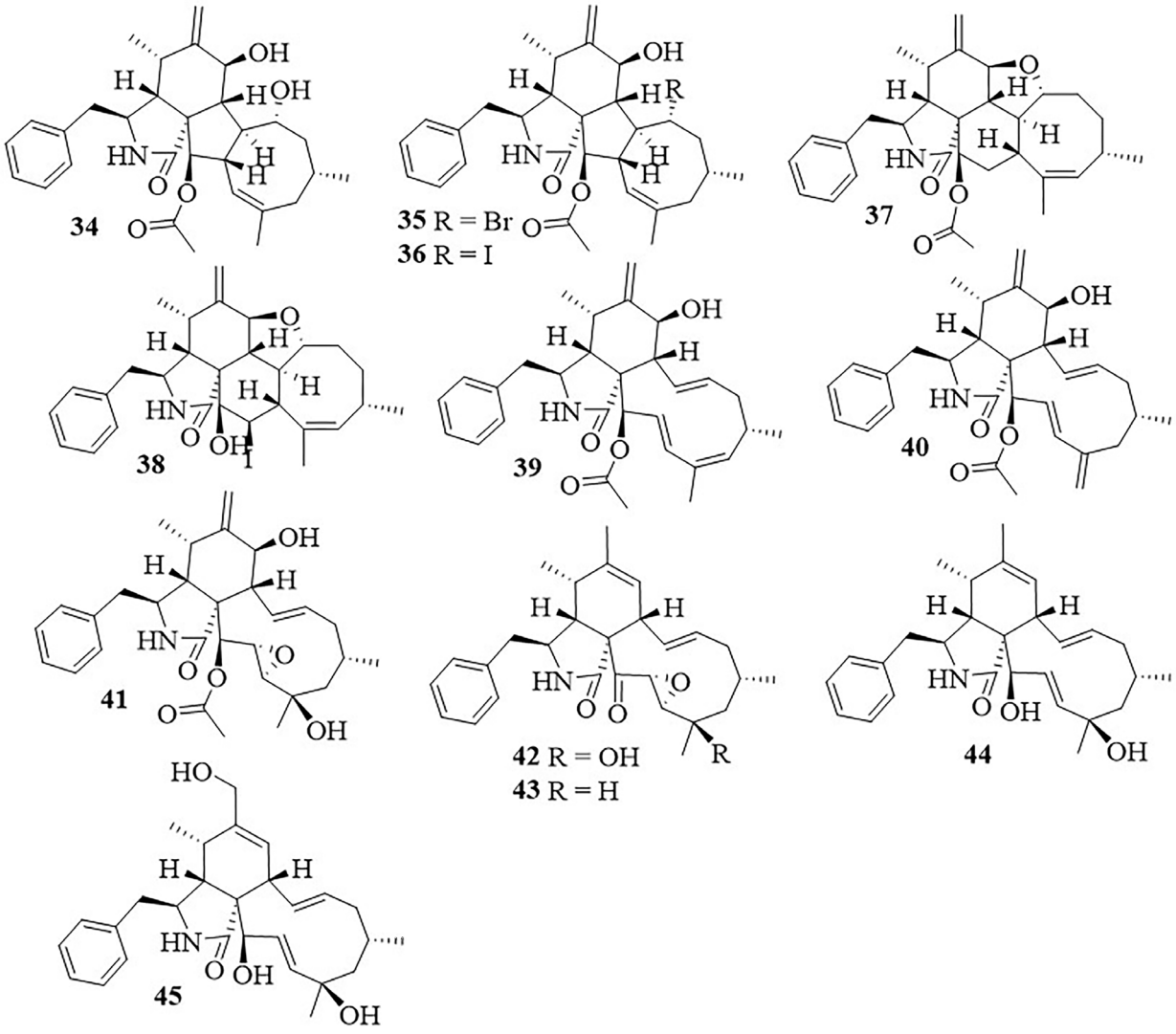

Fig. 9 New natural products discovered by adding halide ions.

Emerging techniques in OSMAC

Experimental manipulation of culture conditions can stimulate microbial strains to produce diverse secondary metabolites (Bode et al. 2002; Pan et al. 2019). However, this approach requires substantial laboratory resources, including space, consumables, and personnel, particularly when applied to large-scale solid or flask-based fermentation processes. These challenges are compounded by slow-growing microbial strains (Salim et al. 2021). To address these limitations, the micro-matrix microbioreactor system has been developed. This innovative system enables the parallel execution of complex and time-intensive experiments, facilitating rapid and reproducible microbial cultivation under various media conditions in shaken, static liquid, and solid phases (Salim et al. 2021; D'Ambrosio et al. 2021). Kankanamge et al. (2023) utilized a miniaturized 24-well plate system (MATRIX) for cultivation profiling, optimizing the growth conditions for the marine-derived fungus *Aspergillus noonimiae* strain CMB-M0339. This approach yielded the identification and characterization of a new series of indole diterpenes, noonindoles G–L (**55–60**) (Fig. 13) (Kankanamge et al. 2023).

Microbial interactions (co-culture)

While fungi are often cultivated individually in laboratory settings, they naturally inhabit complex communities crucial for ecosystem functioning and stability. The long-term co-evolution of these microbial communities has fostered diverse interaction types, including symbiosis, mutualism, collaboration, competition, parasitism, and predation (Webster 2014). Secondary metabolites, though not essential for microbial growth, are pivotal signaling molecules facilitating metabolic exchange and responses to external stimuli. These metabolites act as a "Rosetta Stone" for

interspecies communication (Phelan et al. 2012; Zhang et al. 2022b). Recognizing this, co-culture strategies have been developed to activate silent biosynthetic gene clusters, aiding in the discovery of novel natural products (Bertrand et al. 2014; Peng et al. 2021). Fungal co-culture systems are primarily categorized into fungal-bacterial and fungal-fungal co-cultures based on community composition and ecological relationships (Steffan et al. 2020; Knowles et al. 2022).

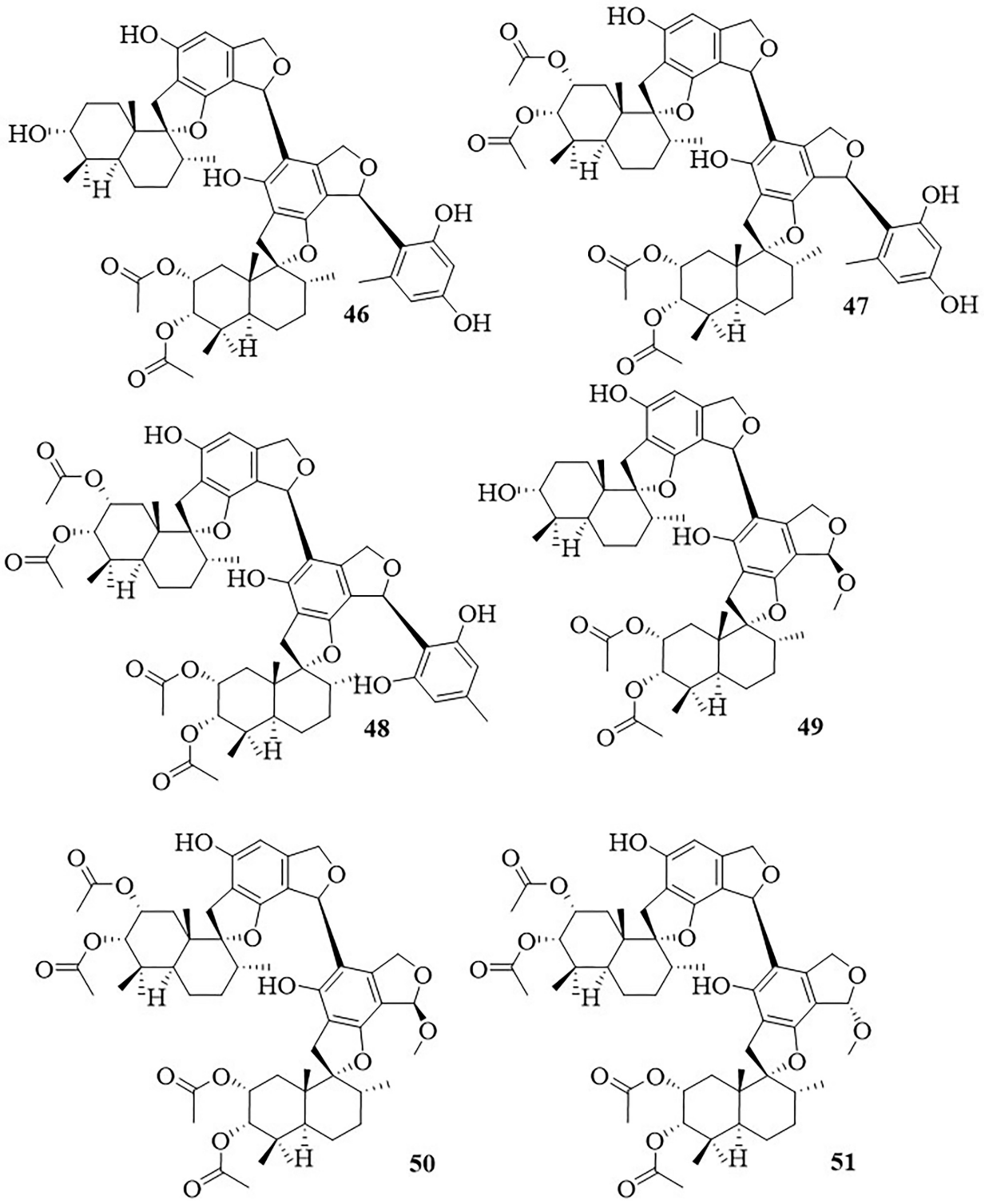

Fig. 10 New natural products discovered by adding metal ions.

Fungal–bacterial co-culture

Fungi and bacteria coexist in complex natural communities that play crucial roles in ecology, agriculture, and human health (Steffan et al. 2020; Espinosa-Ortiz et al. 2022). Numerous studies have shown that co-culturing fungi with different bacteria in laboratory settings can trigger the expression of silent gene clusters, leading to the production

of novel secondary metabolites (Ran & Yin 2023). The famous example is the accidental discovery of penicillin in a culture of *Staphylococcus aureus* contaminated by *Penicillium notatum* (Fleming 1929). Since then, hundreds of co-cultivation studies have been reported for the mining of natural products, especially new molecules not detected in monocultures. For instance, Wakefield et al. (2017) co-cultured the marine-derived fungus *Aspergillus fumigatus* strain MR2012 with the hyper-arid desert bacterium *Streptomyces leeuwenhoekii* strain C34, which induced the production of a new luteoride derivative, luteoride D (**61**), and a new pseurotin derivative, pseurotin G (**62**) (Fig. 14). Additionally, co-culturing the same fungus with *Streptomyces leeuwenhoekii* strain C58 doubled the titer of chaxapeptin compared to monocultures of the bacterium (Wakefield et al. 2017). Another example involves the co-cultivation of *Pantoea aggolomerans*, a bacterium associated with durum wheat roots, and *Penicillium citrinum*, a fungus derived from date palm leaves, which led to the isolation of two new pulicatin derivatives (**63**–**64**) (Fig. 14) (Thissera et al. 2020).

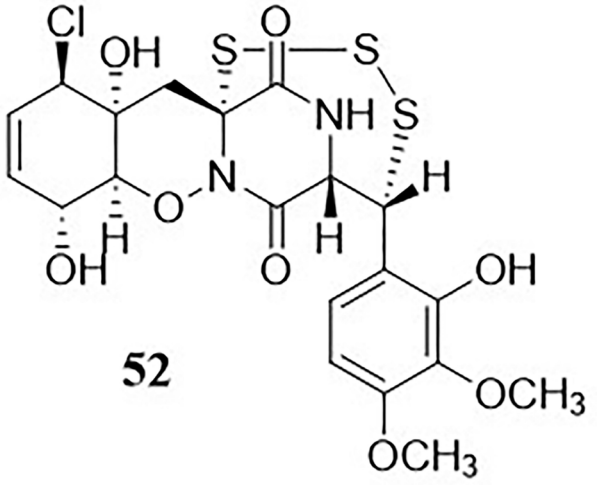

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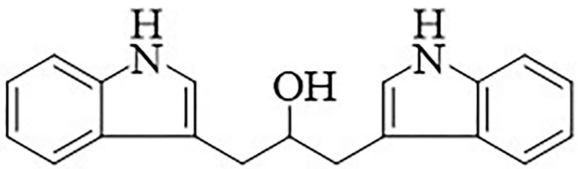
Fig. 11 New natural product discovered by adding DMSO.



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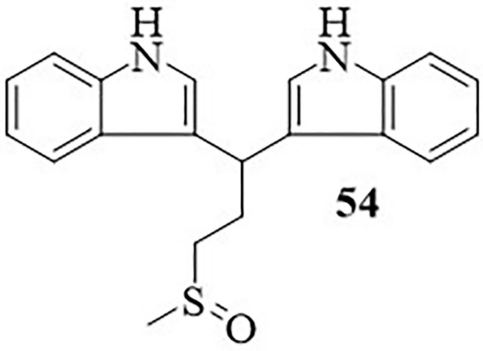

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Fig. 12 New natural products discovered by adding an amino acid.

Fungal–fungal co-culture

Fungi are ubiquitous in nature and naturally compete with one another in their environments. This competition frequently induces the biosynthesis of secondary metabolites for defense (Knowles et al. 2022). For example, citrifelins (**65**-**66**) (Fig. 15), which possess a unique tetracyclic framework, were characterized from the co-culture of *Penicillium citrinum* with *Beauveria felina* (Meng et al. 2015). A co-culture of two marine fungi demonstrated the activation of a rare class of 2-alkenyl-tetrahydropyran, chaunopyran A (**67**) (Fig. 15), and the deactivation of the antifungal metabolite pyridoxatin, leading to the formation of methyl-pyridoxatin. This observation highlights a complex offensive and defensive interaction between fungi (Shang et al. 2017). When co-cultured, the endophytic fungus *Phoma* sp. strain YUD17001 (isolated from *Gastrodia elata*) with *Armillaria* sp. in a liquid nutrient medium produced five novel secondary metabolites including two phenolic compounds, hexanediol A and B (**68** and **69**) (Fig. 15), and three aliphatic ester derivatives, phomesters A–C (**70**–**72**) (Fig. 15). The co-cultivation of the endophytic fungus *Epicoccum dendrobii* with the model fungus *Aspergillus nidulans* and other filamentous fungi resulted in significant changes in secondary metabolites (Wang et al. 2022), offering new insights into the exploration of fungal secondary metabolites during fungal-fungal interactions.

Advances in co-culture techniques

While co-culture strategies effectively activate silent gene clusters, they are labor-intensive and require extensive preliminary screening. Recently, the development of modular

co-culture engineering and microfluidic platforms has successfully overcome the technical barriers of traditional co-culture methods, leading to significant breakthroughs, particularly in high-throughput screening and quantitative biology (Park et al. 2011; Bai et al. 2018; Tan et al. 2020; Wang et al. 2020b).

Modular co-culture engineering. Moular co-culture engineering represents a novel and promising approach for the advanced engineering of biosynthetic pathways (Zhang & Wang 2016). This strategy has facilitated the development of sophisticated artificial microbial co-cultures. Compared to monoculture systems, modular co-culture engineering reduces the metabolic burden on individual strains, thereby enhancing overall bioproduction and bioconversion performance. The diverse cellular environments provided by multiple strains enable the functional expression of different pathway genes. For instance, the biosynthesis of taxol precursors was significantly improved using co-cultures of *Escherichia coli* and *Saccharomyces cerevisiae* (Zhou et al. 2015). This approach also minimizes unwanted interactions between pathway modules and offers flexibility in balancing biosynthetic activities among them. Researchers have successfully implemented modular co-culture engineering by designing, regulating, and optimizing metabolic pathways in model strains of *E. coli* and *S. cerevisiae*, leading to the production of various natural products (Wang et al. 2020b). Although modular co-culture engineering presents significant advantages, challenges such as maintaining the co-existence of constituent strains and stabilizing their population ratios remain obstacles to large-scale industrial applications.

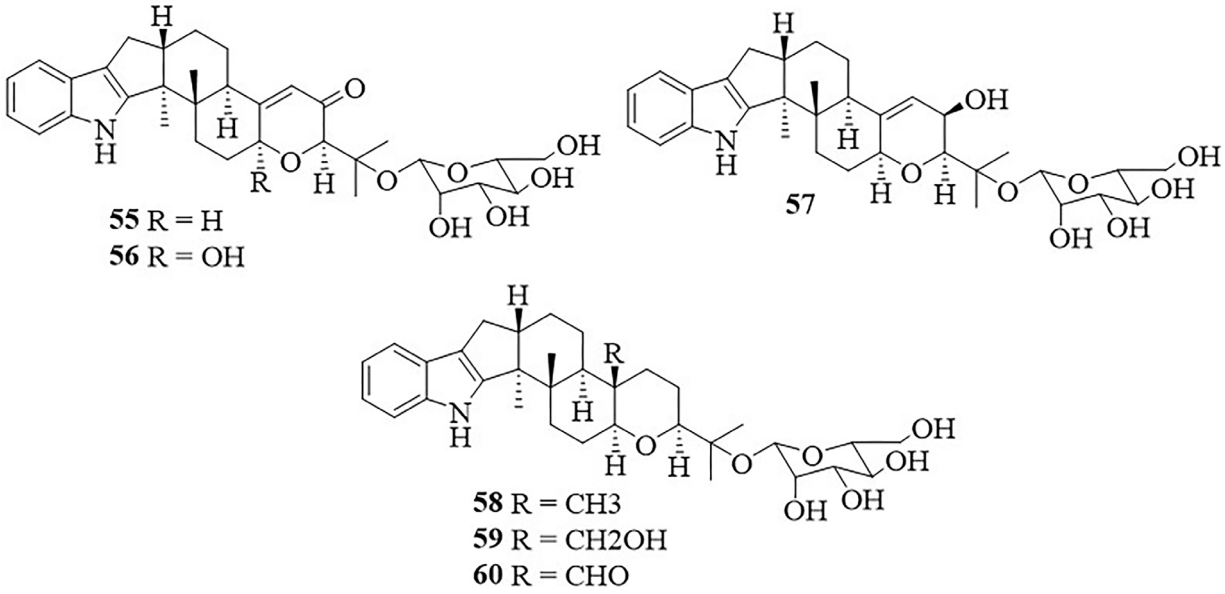

Fig. 13 New natural products discovered by MATRIX.

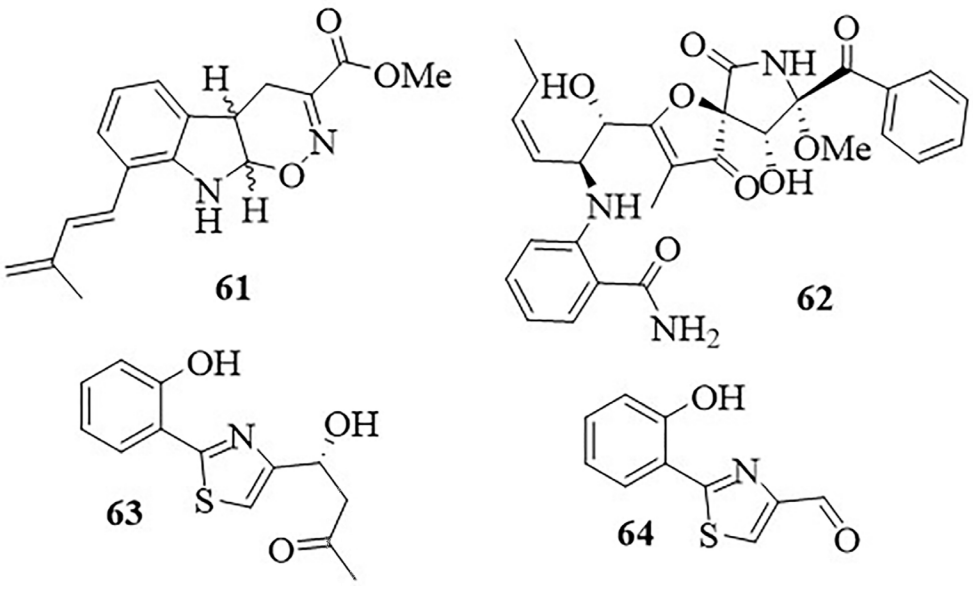

Fig. 14 New natural products discovered by fungalbacterial co-culture strategies.

Microfluidics co-culture platform. Current microfluidic platforms are primarily categorized into droplet-based microfluidics and channel-based microfluidics (Bai et al. 2018). The droplet-based microfluidic co-culture platform uses microfluidic droplets as nanoscale bioreactors to culture microorganisms, enabling the creation of artificial microbial communities, including those involving previously "uncultivable" microorganisms (Park et al. 2011; Jackman et al. 2019; Tan et al. 2020). This platform supports high-throughput studies of interspecies interactions. In contrast, the channel-based microfluidic co-culture platform enables precise manipulation of microscale continuous flow within microchannels and chambers, allowing for accurate control of biological samples (Bai et al. 2018; Burmeister & Grünberger 2020). It facilitates both qualitative and quantitative studies of microbial interactions, thereby expanding the capabilities of quantitative biology. Xu et al. (2024) developed a customized microfluidic chip and integrated it with a mathematical model to precisely regulate the biosynthesis of secondary metabolites in the model fungus *Aspergillus nidulans*, enabling the quantitative synthesis of dendrobiums. Furthermore, the specialized microfluidic chip designed for filamentous fungi can accommodate up to eight strains simultaneously.

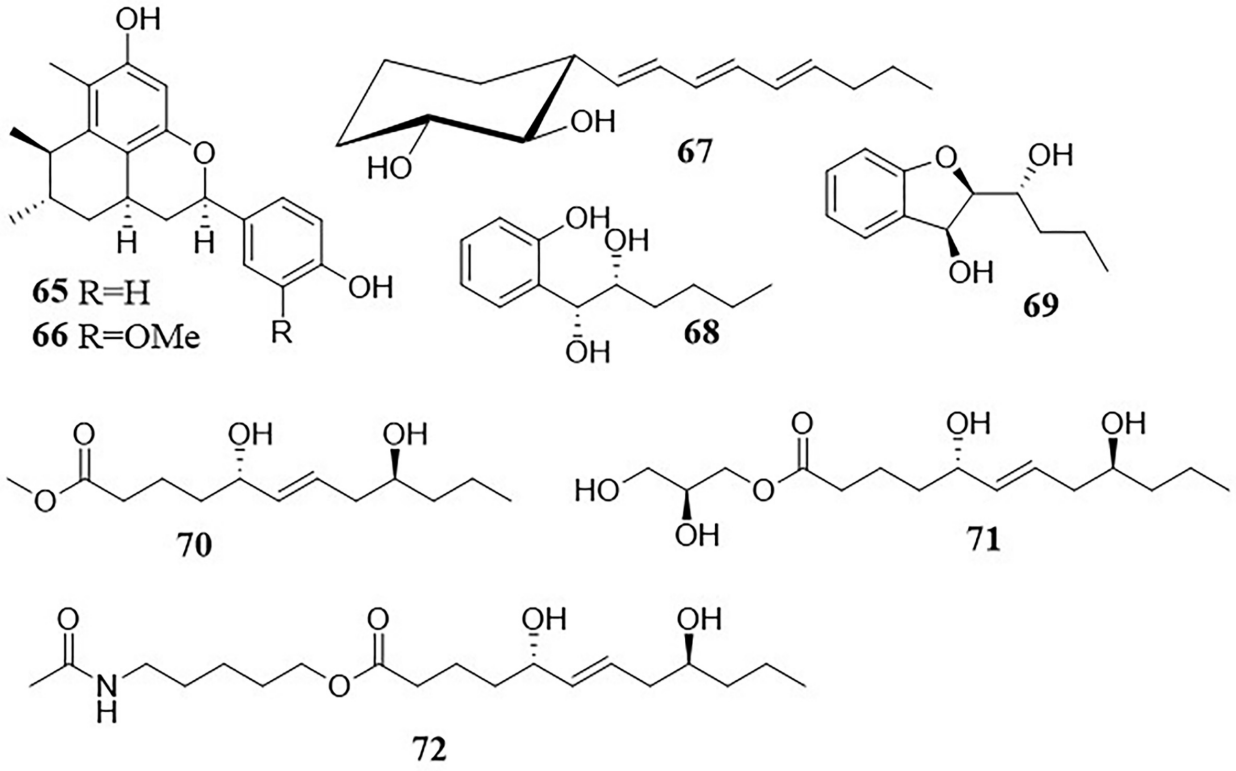

Fig. 15 New natural products discovered by fungalfungal co-culture strategies.

Challenges in co-culture strategies. Despite their promise, co-culture strategies face several challenges. First, selecting and combining interacting strains in microbial co-culture systems is largely random, requiring extensive preliminary screening, which introduces uncertainty in the activation of silent gene clusters. Second, microbial interactions are complex and dynamic, often influenced by culture conditions, leading to instability in metabolite production. Finally, the bioactive molecules secreted during these interactions are often present in trace amounts, making detection and identification difficult.

Epigenetic regulation strategy

The concept of epigenetics was first proposed by Waddington (2012). It involves the regulation of gene expression at the chromatin level, affecting cellular and organismal characteristics without altering the DNA sequence (Marø 2020). Key epigenetic mechanisms include DNA methylation, histone modifications, and non-coding RNAs (Li et al. 2020). These processes enable cells to adapt to environmental changes and life experiences by adjusting gene expression without modifying the genome. Since epigenetics influences all stages of growth and development, interventions targeting fungal epigenetics can significantly impact the biosynthesis of their secondary metabolites. Studies on the epigenetic regulation of fungal secondary metabolites have shown that adding small-molecule epigenetic enzyme inhibitors to culture media or employing molecular epigenetic modification techniques can stimulate fungi to produce novel secondary metabolites (Bind et al. 2022; Xue et al. 2023). Molecular epigenetic modification strategies involve genetically transforming strains by knocking out or overexpressing genes encoding epigenetic-related enzymes. These transformed strains are then cultured to produce new secondary metabolites or enhance the yields of specific target products (Xue et al. 2023). A detailed discussion of this topic is provided in Chapter 3.

Chemical epigenetic modification

Chemical epigenetic modification involves using enzyme inhibitors to influence fungal gene expression. These inhibitors primarily fall into two categories: DNA methyltransferase inhibitors (DNMTs) and histone deacetylase inhibitors (HDACs) (Prakash et al. 2024).

DNA methyltransferase (DNMT) inhibitors. DNA methylation is a chemical modification where a methyl group (-CH_3) attaches to cytosine in DNA, forming 5-methylcytosine. This process, catalyzed by DNA methyltransferases (DNMTs), plays a critical role in regulating gene expression and influencing cellular differentiation, development, and function (Prakash et al. 2024). DNMT inhibitors suppress DNA methylation, thereby disrupting fungal epigenetic inheritance and reactivating silenced genes. Notable inhibitors include 5-azacytidine, 5-aza-2'-deoxycytidine, and N-phthalyl-L-tryptophan (RG108). For additional applications, consult Xue et al. (2023) and Prakash et al. (2024). Wang et al. (2010) investigated the effects of 5-azacytidine (5-Aza) on *Penicillium citreonigrum* by comparing fermentation products from cultures treated with and without the compound. Their study identified two novel meroterpenes (**73** and **74**) (Fig. 16) and seven known compounds in the 5-Aza-treated medium, except pencolide (see Fig. 16). Similarly, Guo et al. (2020) demonstrated that the addition of 5-Aza-2'-deoxycytidine (10 mg/L; RG108) to the culture medium of *Penicillium herquei* induced the production of three new α-pyrone derivatives (**75–77**) (Fig. 16).

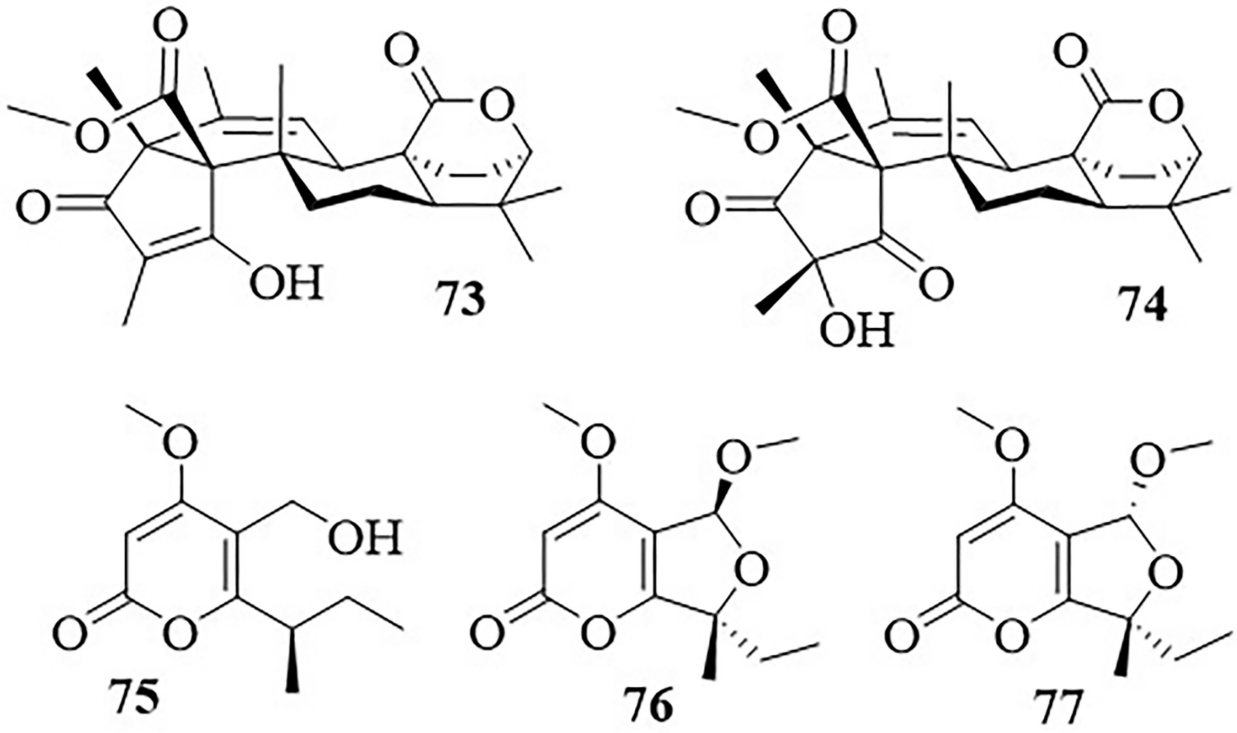

Fig. 16 New natural products induced by DNA methyltransferase (DNMT) inhibitors.

Histone deacetylase (HDAC) inhibitors. Histone acetylation and deacetylation regulate histone-DNA interactions, significantly impacting gene expression in fungi (Zhu & Zhang 2024). HDAC inhibitors, such as suberoylanilide hydroxamic acid (SAHA) and suberohydroxamic acid (SBHA), influence histone acetylation levels, thereby modulating gene expression and cellular functions. For additional examples of their applications, refer to studies by Xue et al. (2023) and Prakash et al. (2024). Chung et al. (2013) demonstrated that the addition of SAHA (0.5 mM) to the growth medium of *Beauveria felina* induced the production of three novel cyclodepsipeptides (**78–80**) (Fig. 17) and five known compounds (Fig. 17). Among these, compound **80** featured a cyclodepsipeptide ring containing N-methylbutyric acid, while compounds **78** and **79** exhibited anti-inflammatory activity. Similarly, Asai et al. (2013b) utilized SBHA (500 μM) to activate silent polyketone biosynthesis gene clusters (*pksCH-1* and *pksCH-2*) in *Chaetomium indicum*, resulting in the production of six novel prenylated aromatic polyketides, chaetophenols A–F (**81–86**) (Fig. 17), with compounds **83**, **84**, and **85** exhibiting unprecedented polycyclic skeletons (Asai et al. 2013b). In another study, Asai et al. (2013a) exploited nicotinamide-treated fermentation media to induce the production of six novel benzophenones, cephalanones A–F (**87–92**), and one known compound from *Graphiopsis chlorocephala* (Fig. 17). Additionally, Zhen et al. (2018) treated *Penicillium chrysogenum* HLS111 with sodium valproate (SVP, 10 μM), leading to the isolation of three novel heterodimeric tetrahydroxanthone-chromanone lactones, chrysoxanthones A–C (**93–95**) (Fig. 17), along with 17 known compounds. Chrysoxanthones A–C demonstrated moderate antibacterial activity against *Bacillus subtilis*. Similarly, Feng et al. (2022) tested several epigenetic modifiers on *Phomopsis asparagi* DHS-48 and obtained two novel compounds (**96–97**) (Fig. 17) and nine known compounds from cultures treated with

sodium butyrate. Notably, compound **94** exhibited potent cytotoxicity against human cancer cell lines *HeLa* and *HepG2*.

Other chemical epigenetic modifiers. Additional chemical epigenetic modifiers used in fungal secondary metabolism studies include proteasome inhibitors (e.g., bortezomib) (VanderMolen et al. 2014), histone acetyltransferase inhibitors (e.g., anacardic acid) (Mafezoli et al. 2018), and histone methyltransferase inhibitors (e.g., BRD4770) (Nishad et al. 2021).

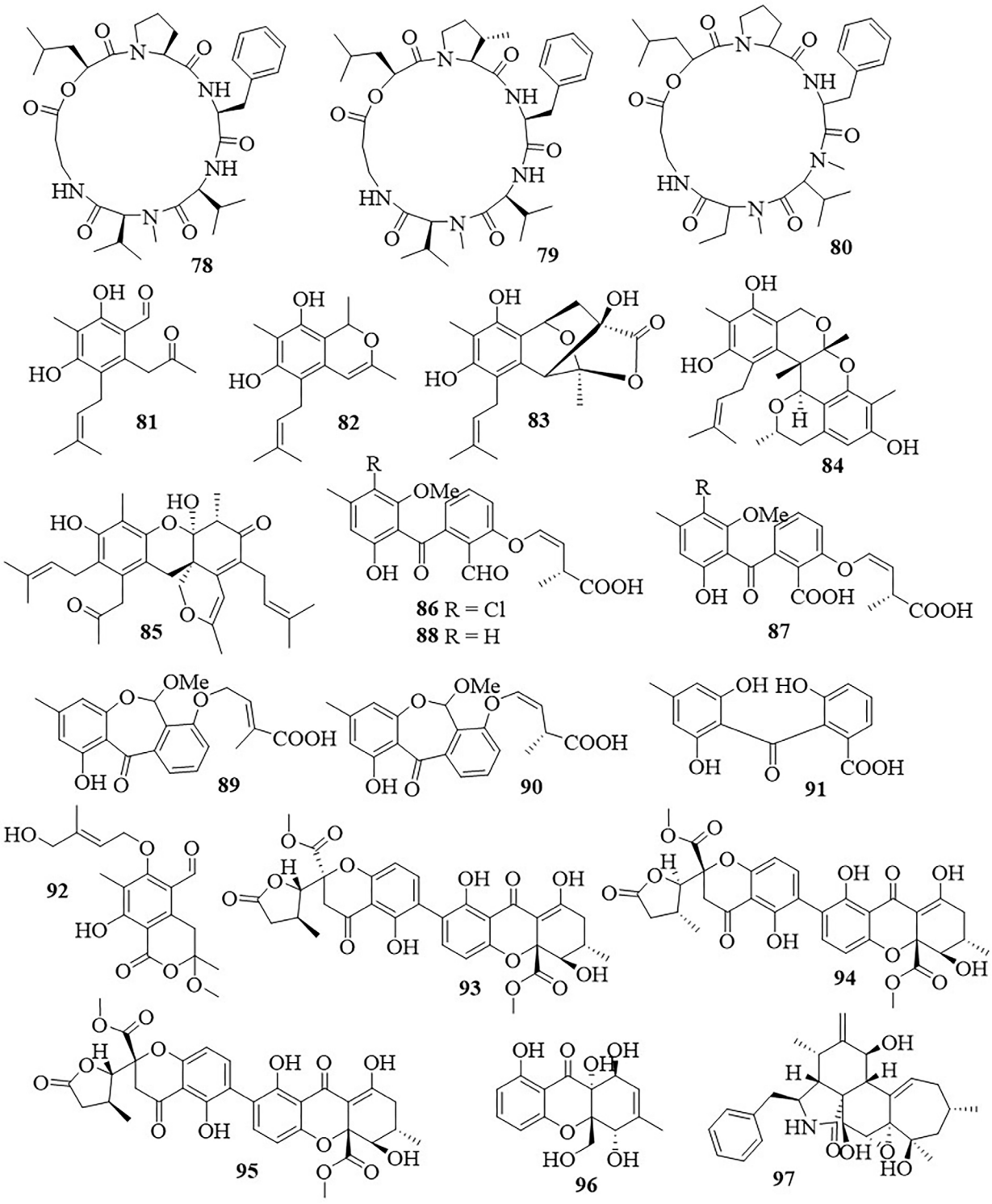

Fig. 17 New natural products induced by Histone deacetylase (HDAC) inhibitors.

Combining multiple epigenetic modifiers. When a single chemical epigenetic modifier fails to sufficiently activate silent gene clusters, combining multiple modifiers with different mechanisms can yield better results. Asai et al. (2012) combined SBHA and RG-108 to induce the entomopathogenic fungus *Isaria tenuipes* to produce tenuipyrone (**98**) (Fig. 18), featuring a unique tetracyclic spiroketal structure. Niu et al. (2021) co-treated a deep-sea-derived fungus, *Eutypella* sp.,

with SBHA and 5-Aza, activating a sesquiterpene biosynthetic gene cluster and producing 17 novel compounds, eutypeterpenes A–Q (**99–115**) (Fig. 18), along with four known compounds.

Discussion

Strategies for activating fungal silent gene clusters by modifying culture conditions are relatively simple and often yield unexpected results. However, these approaches are inherently uncertain and have limitations. (i) Limited genomic knowledge: Less than 1% of fungal genomes have been characterized, leaving significant untapped potential and opportunities for discovery (Lei & Zhao 2019). (ii) Suitability for complex fungi: This strategy is particularly effective for studying secondary metabolites in fungi that are difficult to genetically manipulate, such as multinucleate, heterokaryotic, or basidiomycetes that do not readily produce spores (Lei & Zhao 2019).

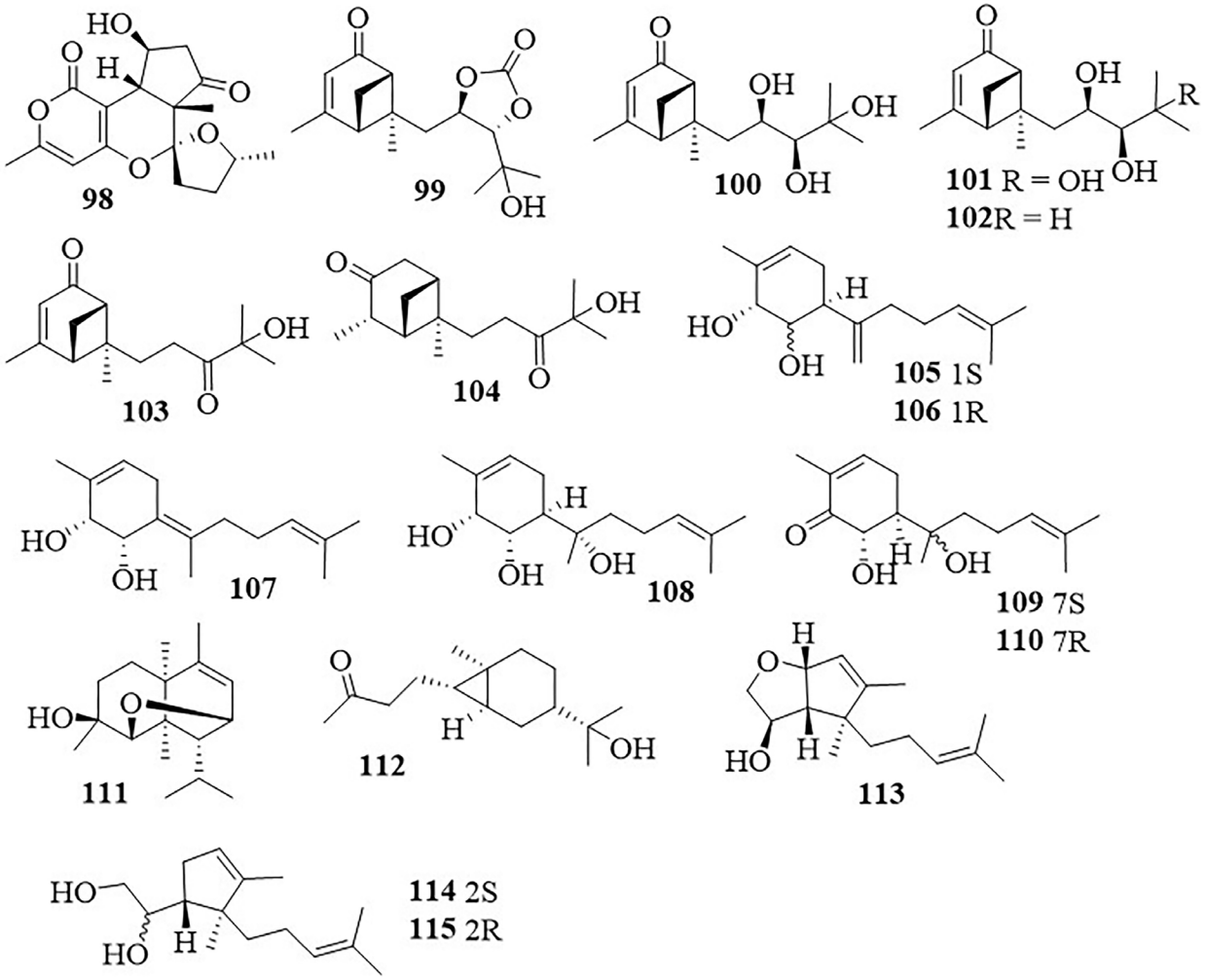

Fig. 18 New natural products induced by combined DNA methyltransferase (DNMT) inhibitors and Histone deacetylase (HDAC) inhibitors.

Strategies for activating fungal silencing biosynthesis gene clusters based on genome mining

Obtaining new natural products traditionally involves extensive, labor-intensive isolation and analysis efforts. However, relying solely on these conventional methods fails to meet the needs of modern drug development. Recent advancements in sequencing technologies and bioinformatics have significantly enhanced our ability to identify fungal secondary metabolite (SM)-encoding genes, which are frequently arranged in clusters on chromosomes, a configuration known as biosynthetic gene clusters (BGCs) (Medema et al. 2015; Rokas et al. 2020). Under standard culture conditions, most fungal SM gene clusters primarily remain silent, masking a wealth of potentially bioactive compounds with novel structures that have yet to be discovered (Lyu et al. 2020). Therefore, activating these silent BGCs to enhance their expression has emerged as a promising strategy for exploring new secondary metabolites. This chapter provides a comprehensive review of recent approaches for activating silent gene clusters in fungi through genome mining efforts.

Bioinformatics Analysis of Silent BGCs. The surge in full microbial genome sequences and related resources has driven the development of BGC prediction algorithms, albeit with varying precision and capacity to identify novel BGCs (Hannigan et al. 2019; Wang et al. 2025). Fungal genome analyses typically depend on homology comparisons with

annotated fungal genomes. Researchers can access several widely used BGC databases, such as NCBI (Sharma et al. 2018), SMURF (Khaldi et al. 2010), and antiSMASH (Blin et al. 2023). In addition, numerous algorithms have been developed to investigate more elusive BGCs (Hannigan et al. 2019; Li et al. 2024; Li et al. 2024). Deep learning has emerged as a powerful tool in the discovery of SMs, leveraging its capabilities in data processing and pattern recognition. It enables the identification of BGCs associated with SM biosynthesis, prediction of compound structures, screening of compound activities, and exploration of SM biosynthetic pathways. For instance, DeepRiPP integrates the NLPP recursor, BARLEY, and CLAMS modules to analyze microbial genome and metabolomics data, facilitating the efficient screening and discovery of novel ribosomally synthesized and post-translationally modified peptides (RiPPs) (Merwin et al. 2020). Similarly, TOUCAN employs classifiers, such as support vector machines (SVM), multilayer perceptron, logistic regression (LR), and random forests (RF), to identify BGCs in fungi (Almeida et al. 2020). BioNavi-NP, the first deep learning-based retrosynthetic inference tool, predicts biosynthetic pathways for SMs (Zheng et al. 2022). In the post-genomic era, AI-driven bioinformatics tools offer transformative potential for SM discovery, significantly enhancing genome quality and mining efficiency of BGCs.

Activation strategies based on molecular epigenetic modification

As mentioned above, in fungi, many metabolic elicitors promote epigenetic modification. Histone deacetylase inhibitors and DNA methylation inhibitors are particularly potent activators of otherwise repressed BGCs (Gacek & Strauss 2012). Small-molecule inhibitors represent a straightforward and convenient approach for targeting epigenetic regulation; however, their limited specificity often leads to off-target effects, potentially disrupting fungal growth and metabolism (Pfannenstiel et al. 2019; Lyu et al. 2020). Therefore, gene-editing techniques that selectively knock out or overexpress genes encoding epigenetic modification enzymes in fungi offer a more precise regulatory strategy (Lyu et al. 2020; 2022). The efficacy of this approach lies in the ability to disrupt or overexpress genes involved in DNA methylation or histone modifications, thereby inducing chromatin remodeling. This remodeling activates biosynthetic gene clusters (BGCs) located in heterochromatin regions (Lyu et al. 2020; 2022). For instance, Wu et al. (2016) utilized gene knockout techniques to target two epigenetically relevant genes, *PfCclA* and *PfHdaA*, in the endophytic fungus *Pestalotiopsis fici*. This intervention yielded 15 novel polyketides, including pestaloficiols T–W (**116–119**), and 11 macrodiolide ficiolides A–K (**120–130**) (Fig. 19). Similarly, Fan et al. (2017) disrupted the histone acetyltransferase (HAT) gene *Hat1* in the entomopathogenic fungus *Metarhizium robertsii*, leading to the discovery of nine new polyketides (meromusides A–I, **131–139**) and two novel peptides (meromutides A and B, **140** and **141**) (Fig. 19).

Activation strategies based on pathway-specific regulation factors

Transcriptional regulatory factors are abundant in the genome and play a crucial role in controlling the transcription and expression of functional genes. By regulating specific transcription factors, silent BGCs can be activated and novel secondary metabolites can be discovered. The expression of many specialized metabolite BGCs is controlled by pathway-specific transcriptional activators or repressors (Rutledge & Challis 2015). The genes encoding such regulators are often located within the BGC that they regulate. Activating pathway-specific activating genes or knocking out pathway-specific inhibiting genes are effective ways to activate silent BGCs. For instance, Zhang et al. (2018) revealed an unconventional paraherquonin-like meroterpenoid BGC in the chromosome of *Neosartorya glabra* through genome mining. By constitutively expressing the pathway-specific regulator gene *berA*, two new berkeleyacetal derivatives, Berkeleyacetal D (**142**) and 11-epi-Berkeleyacetal C (**143**), were isolated (Fig. 20) (Zhang et al. 2018). Similarly, overexpression of the transcriptional regulator *aspE* in *Aspergillus* sp. strain CPCC400735 led to the discovery of 11 new asperphenalenone derivatives (**144–154**) with significant anti-influenza A virus activity (Fig. 20) (Zhang et al. 2022a).

Activation strategies based on global regulatory factors

Unlike pathway-specific regulators, global regulators influence the expression of multiple gene clusters simultaneously, impacting the biosynthesis of numerous fungal secondary metabolites. One of the most studied global regulators, *LaeA*, achieves transcriptional regulation by modulating chromatin modification (Lyu et al. 2020). Overexpression of *LaeA*-like genes have facilitated the discovery of novel isocoumarin analogs (compounds **155–156**, Fig. 21) from *Trichoderma afroharzianum* (Ding et al. 2020) and cytotoxic acetaminobutyric acid derivatives (versicolor A, compound **157**, Fig. 21) from *Aspergillus versicolor* (Zhang et al. 2020a). Knockout studies of *LaeA* homologs from *Aspergillus flavipes* have also led to the identification of unique metabolites, including piperazine derivatives flavipamides A and B (**158** and **159**) (Fig. 21) (Liu et al. 2024b).

In addition to *LaeA* and its homologous genes, researchers have also discovered other global regulators affecting fungal secondary metabolite biosynthesis, such as *MetR* (Yu et al. 2021), *FnVeA* (Qin et al. 2022b), histone deacetylase *UvHST2* (Liu et al. 2023), *VeA* (Moon et al. 2023), etc. Still, they have not been further developed to obtain new natural products.

Activation strategies based on metabolic shunting

Metabolic shunting refers to a strategy that indirectly activates the remaining BGCs by knocking out the key genes of biosynthesis of major metabolites in strains, allowing more biosynthetic precursors to flow to other biosynthetic pathways (Chiang et al. 2016; Chen et al. 2019; Lyu et al. 2022). This approach, also called “genetic dereplication”, helps avoid rediscovery of known compounds. For example, Chiang et al. (2016) deleted eight of the most highly expressed SMs BGCs from *Aspergillus nidulans* to create a “genetic dereplication” strain, obtained a new compound, aspercryptin (**160**) (Fig. 22). Similarly, knockout of *thtri5*, a key gene for high triterpene production in *Trichoderma hypoxylon,* led in the discovery of rare polycyclic lactones and sesquiterpenes, such as tricholactones A and B (**161–162**)

(Fig. 22) and tricinoloniol acids (TRAs) A–C (**163–165**) (Fig. 22) (Chen et al. 2019; Liu et al. 2020)

Activation strategies based on promoter replacement

Natural promoters are typically classified into constitutive and inducible types. Constitutive promoters enable continuous and stable gene expression, making them ideal for the production of enzymes, antibodies, and other proteins (Yu et al. 2021). In contrast, inducible promoters are regulated by various factors and are activated only under specific conditions, which makes them particularly suitable for the production of natural products (Xu et al. 2019). Promoter replacement refers to the process of substituting the native promoter of a gene with a different one, thereby modulating the promoter's activity and enabling precise control over the expression level, timing, and method of target gene expression (Jin et al. 2019). In synthetic biology, promoter replacement can be leveraged to optimize metabolic pathways, activate silent gene clusters, and enhance the ability of microorganisms to produce specific SMs (Lin et al. 2019; Viggiano 2020; Yu et al. 2021).

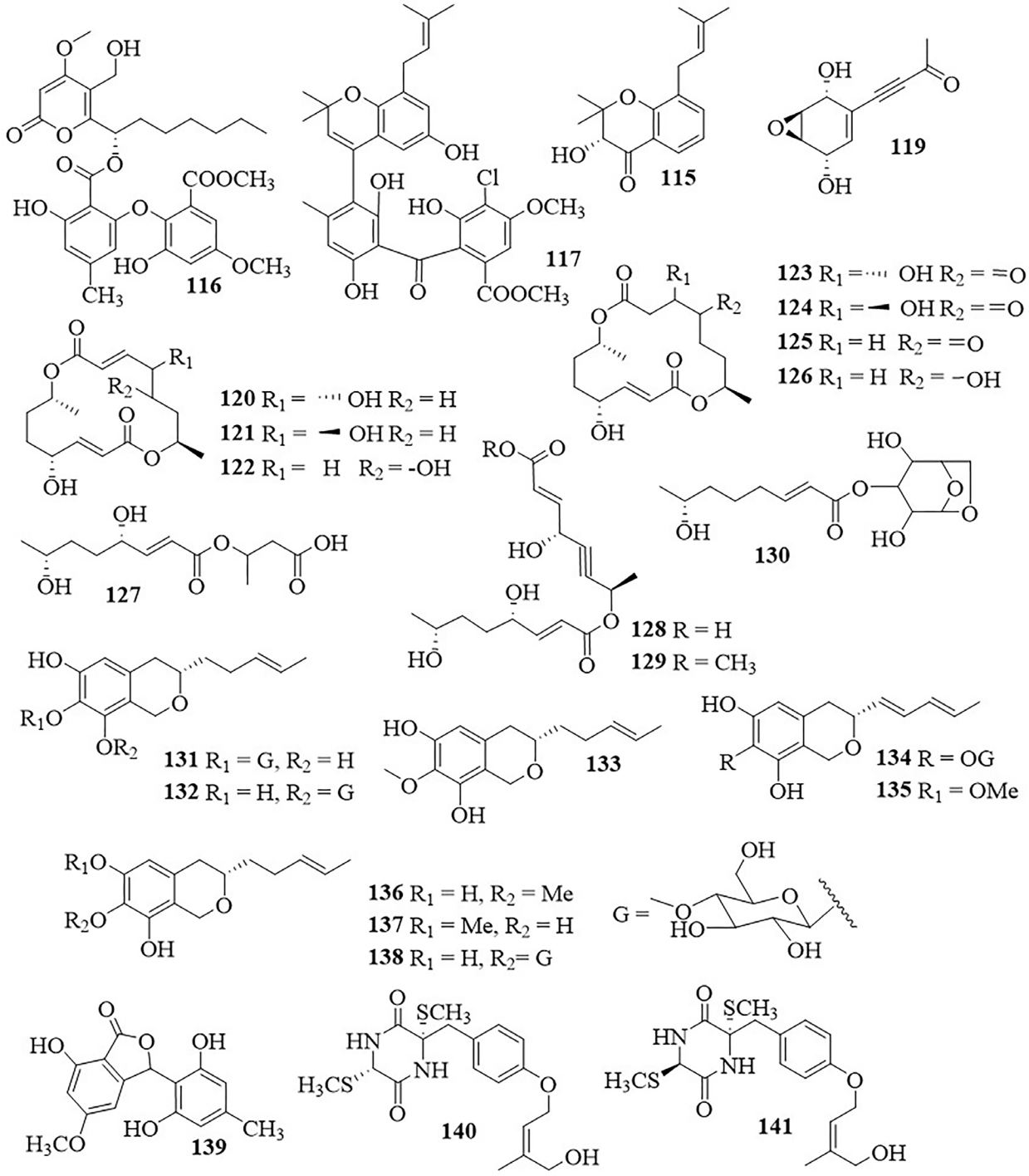

Fig. 19 New natural products discovered based on epigenetic-related gene knockout strategies.

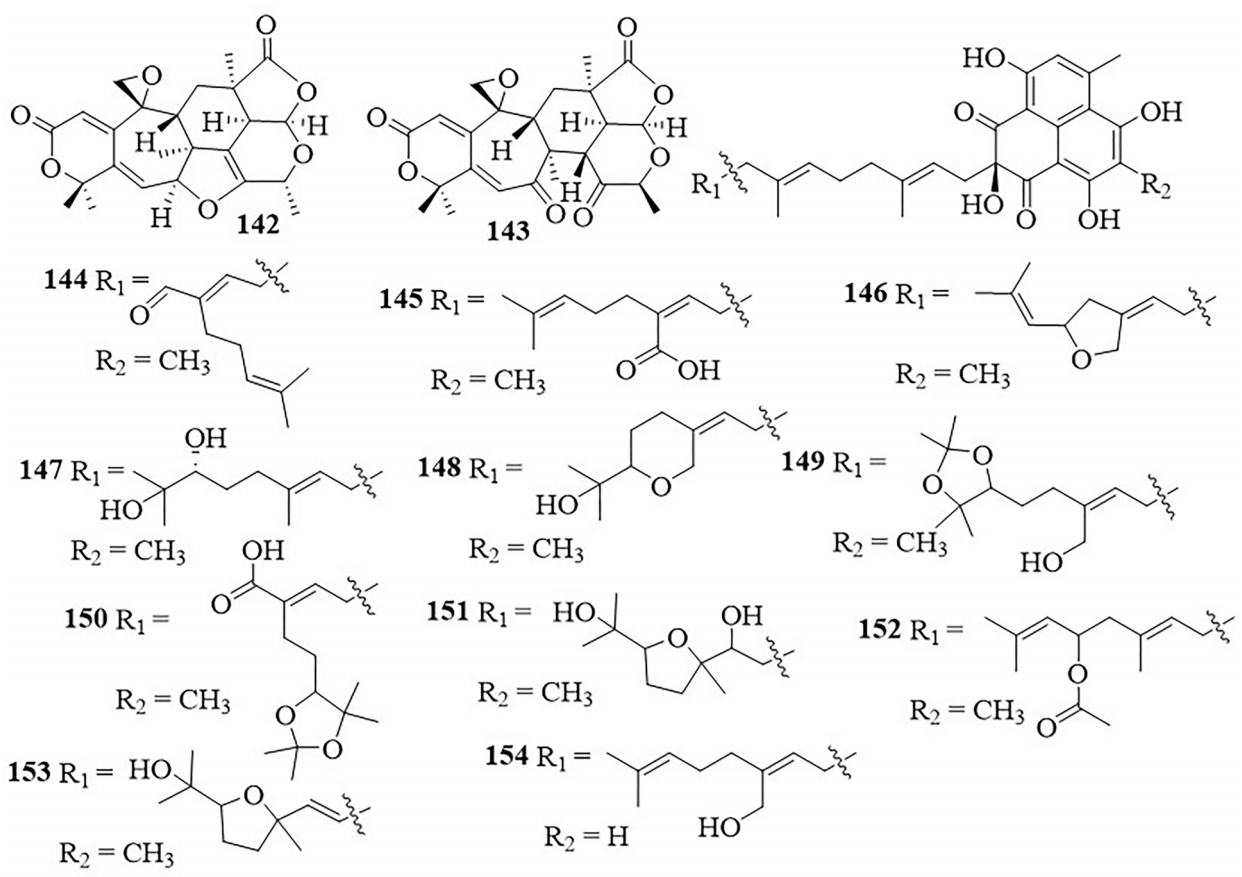

Fig. 20 New natural products discovered by pathway-specific regulator modification strategy.

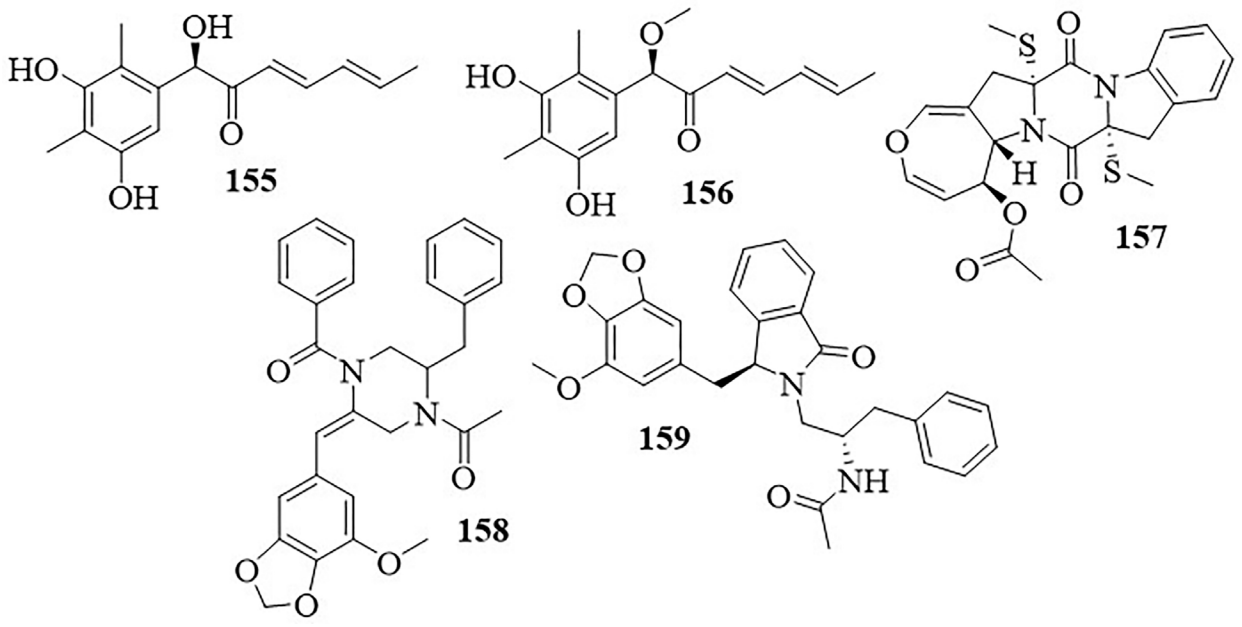

Fig. 21 New natural products discovered based on the global regulator modification strategy.

Biosynthetic gene promoter replacement

Since each gene in fungi usually requires a promoter to drive expression (Blumenthal 2004), when using the promoter replacement strategy to activate silent SMs BGCs, it is necessary to replace the promoters of all biosynthetic genes in the gene cluster. Manmeet Ahuja et al. (2012) systematically replaced all promoters of non-reducing polyketide synthase (NR-PKS) genes in *Aspergillus nidulans* with a regulatable *alcA* promoter. This approach led to the activation of silent gene clusters and the production of seven novel polyketides (**166–172**) (Fig. 23).

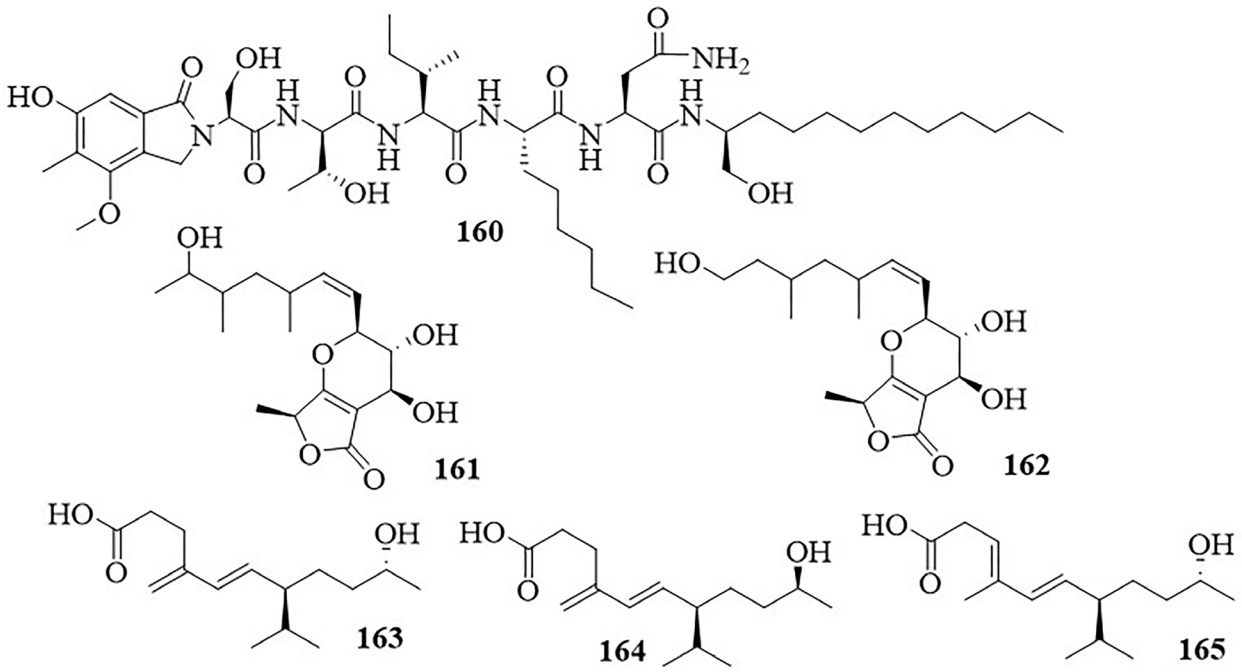

Fig. 22 New natural products discovered based on metabolic shunting strategy.

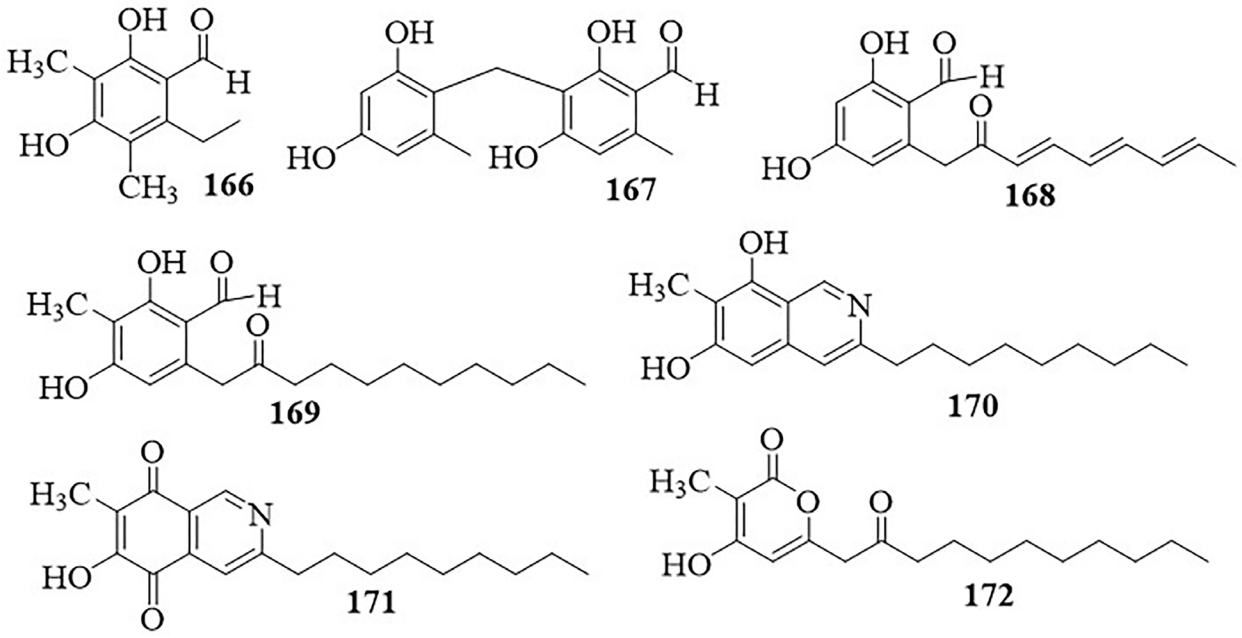

Fig. 23 New natural products discovered by replacement of biosynthetic gene promoters.

Transcriptional regulatory gene promoter replacement

When a silent gene cluster contains a transcriptional regulatory gene, the cluster can be activated by replacing only the promoter of the regulatory gene, without the need to alter the promoters of all the biosynthetic genes within the cluster (Lyu et al. 2020). Since bacterial genetic manipulation is relatively simple, replacing the promoter of the transcriptional regulatory gene is comparatively easy to achieve.

Heterologous expression strategies

Heterologous expression involves the introduction of target genes, gene cassettes, or even entire biosynthetic gene clusters into genetically tractable heterologous hosts (such as *Saccharomyces cerevisiae*, *Aspergillus* sp., etc.) for expression, effectively accelerating the production of natural products (Zhang et al. 2019; Oikawa 2020). Through heterologous expression, it is possible to break through the limitations of natural production conditions and achieve efficient, controllable, and large-scale production of target metabolites and the discovery of new metabolites.

Prokaryotic systems

Prokaryotic expression systems are widely regarded as ideal hosts for heterologous expression due to their simple culture requirements, short growth cycles, and well-characterized genetic backgrounds. Common systems include *E. coli* (Wang et al. 2020b), *Streptomyces coelicolor* (Qian et al. 2020) and *Bacillus subtilis* (Zhao et al. 2024). These systems are predominantly used for the production of recombinant proteins (Souza et al. 2021) and the expression of SMs BGCs derived from bacteria (Liu et al. 2024a). However, certain transmembrane proteins from fungi and plants tend to misfold in *E. coli*, limiting their effectiveness for such applications (Ma et al. 2021).

Eukaryotic (yeast) systems

Saccharomyces cerevisiae is a widely used eukaryotic model organism in molecular and cellular biology. Its well-characterized nature and clear genetic background make it an excellent tool for studying fungal natural product biosynthetic pathways and developing novel natural products (Wang et al. 2021). Beyond *S. cerevisiae*, non-traditional yeast species, such as *Komagataella phaffii*, *Yarrowia lipolytica*, and *Schizosaccharomyces pombe*, have also demonstrated potential as hosts for producing proteins and natural products (Khlebodarova et al. 2024). The sesquiterpene synthase FgJ03939 from *Fusarium graminearum* was utilized in *S. cerevisiae* engineered to overexpress farnesyl diphosphate. This strategy resulted in the biosynthesis of novel sesquiterpenes, including fusariumdiene (**173**), epi-fusagramineol (**174**), and fusagramineol (**175**) with 5/7 bicyclic and 5/6/3 tricyclic ring systems (Fig. 24) (Bian et al. 2018).

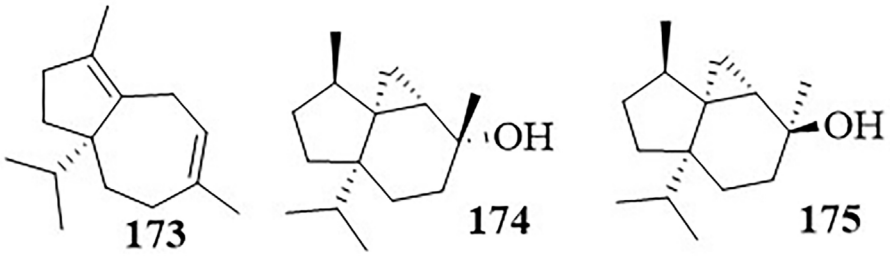

Fig. 24 New natural products discovered using heterologous expression (*Saccharomyces cerevisiae* as host.

Filamentous fungal systems

Advancements in molecular biology have enabled the development of filamentous fungi as hosts for heterologous expression of proteins and small-molecule compounds. Compared to *E. coli* and yeast hosts, filamentous fungi offer a distinct advantage: they can express entire biosynthetic gene clusters for fungal natural products without requiring intron removal during cloning (He et al. 2018). Through genome mining, Zhang et al. (2020b) identified a potential 4-hydroxy pyridone biosynthetic gene cluster in the endophytic fungus *Tolypocladium* sp. strain 49Y. The cluster was introduced into *Aspergillus oryzae* NSAR1, leading to the production of two rare 4-hydroxy pyridones, tolypyridone C (**176**) and tolypyridone D (**177**) (Fig. 25). Similarly, Hu et al. (2024) discovered a BGC *alt* for alternapyrone in the sponge-derived fungus *Arthrinium arundinis* strain ZSDS-F3. Heterologous expression of *alt* in *Aspergillus nidulans* A1145 ΔSTΔEM resulted in the isolation of a series of anti-inflammatory linear polyketides, including two new compounds (**178–179**) and four known ones (Fig. 25).

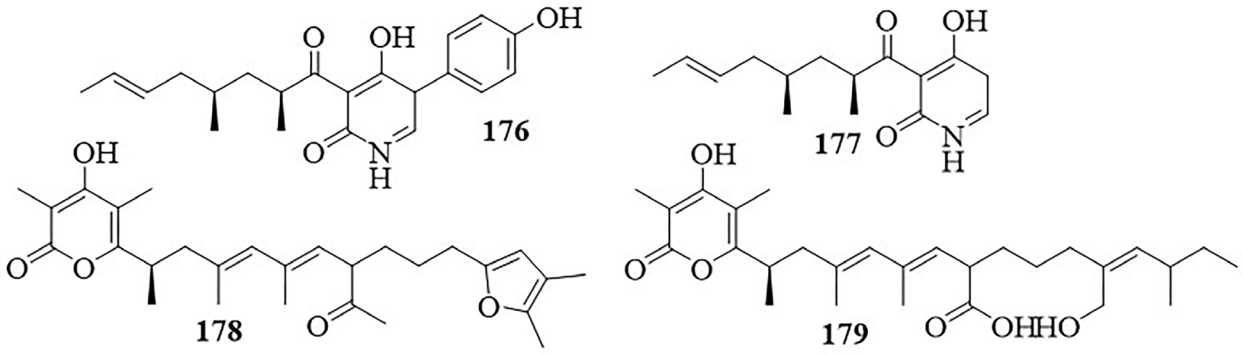

Fig. 25 New natural products discovered using heterologous expression (*Aspergillus* spp. as host).

Activation strategies based on combinatorial biosynthesis (CBT)

The concept of combinatorial biosynthesis (CBT) was first proposed by Floss (2006). This strategy involves using genetic engineering to modify biosynthetic pathways or assemble new ones to produce novel compounds. By reengineering and combining specific enzymes involved in secondary metabolite biosynthesis, new natural products can be generated through customized pathways (Skellam et al. 2024). For instance, *Omphalina mutila* demonstrates significant potential for producing new pleuromutilin analogs. To enhance chemical diversity, Guo et al. (2022) reconstituted diterpene cyclases and modification enzymes involved in

pleuromutilin biosynthesis and expressed them heterologously in yeast. This effort resulted in the production of three novel pleuromutilin analogs (**180–182**) (Fig. 26) (Guo et al. 2022).

Discussion

The activation of silent gene clusters in fungi through genome mining has revolutionized the discovery of fungal natural products, leading to the identification of numerous compounds with novel structures and remarkable bioactivities. However, a fundamental prerequisite for implementing this strategy is the establishment of a genetic manipulation system for the target strain.

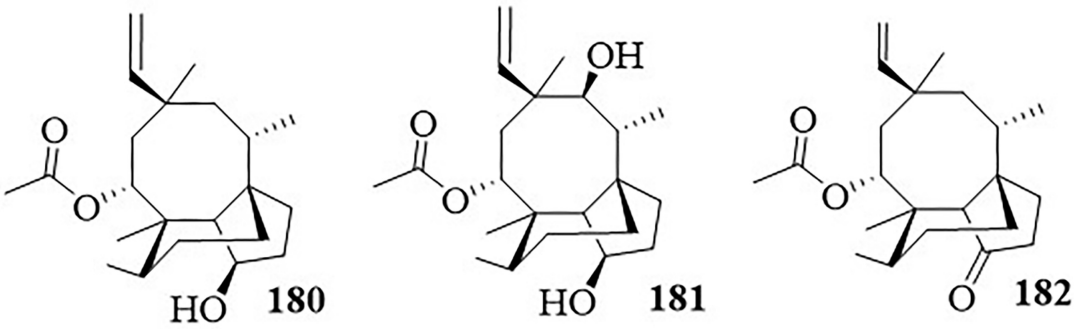

Fig. 26 New natural products discovered using a combinatorial biosynthesis strategy.

This paper presents seven strategies for uncovering new fungal secondary metabolites via genome mining, each with distinct advantages and limitations. No single strategy is sufficient to fully activate all silent gene clusters. For instance, molecular epigenetic modification, global regulator activation, and metabolic shunting are relatively straightforward in terms of genetic manipulation but often yield unpredictable results. Metabolic shunting, in particular, is effective only in strains with a high abundance of main metabolites.

Conversely, pathway-specific transcription factor activation, heterologous expression, promoter replacement, and combinatorial biosynthesis strategies provide more predictable outcomes but require precise and complex genetic engineering. However, these methods face technical challenges. For example, the pathway-specific transcription factor activation strategy is limited to specific gene clusters; the effectiveness of heterologous expression and promoter replacement strategies depends heavily on the genetic tractability of the target strain; and combinatorial biosynthesis requires detailed knowledge of biosynthetic pathways (Ran & Yin 2023). Despite these challenges, the complementary nature of these approaches offers great potential for advancing fungal secondary metabolite research and expanding the chemical diversity of bioactive compounds.

Natural products dereplication strategies

The study of natural products (NPs) has captivated researchers for decades. A persistent challenge in the discovery of new bioactive compounds from complex natural mixtures is avoiding the re-isolation of known compounds (El-Elimat et al. 2013). To address this, the researchers tried various methods, first by comparing ultraviolet-visible spectra (UV-Vis spectra) and mass spectra (MS), and later by activity-directed separation (Lopez-Perez et al. 2007; El-Elimat et al. 2013; Gaudêncio & Pereira 2015). While these methods demonstrated some efficacy, they fell short of meeting the rapidly evolving demands of the pharmaceutical industry and the urgent need for novel therapeutics. Among these strategies, MS remains a cornerstone of dereplication (El-Elimat et al. 2013). However, its application is often restricted by data-sharing limitations, as results are typically confined to publications or individual research teams, leaving other investigators to confront redundant efforts.

Recent advancements in modern analytical technologies, metabolomics, bioinformatics, artificial intelligence, and the interdisciplinary convergence of various fields have led to the emergence of several innovative strategies and methods for discovering and studying new natural products (Reher et al. 2020; Zhang et al. 2023). Techniques such as molecular networking (MN) using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and Small Molecule Accurate Recognition Technology (SMART) based on nuclear magnetic resonance (NMR) are redefining the field. These methods enable the targeted identification of structurally unique natural products and have been successfully applied in diverse areas, including plants (Bai et al. 2020), microorganisms (Hou et al. 2019a), and marine organisms (Freire et al. 2022). Consequently, these innovations have reinvigorated natural product research. This chapter reviews prominent strategies for discovering new natural products derived from fungi (Table 1).

Efficient discovery strategies for new natural products based on LC-MS/MS

The LC-MS/MS technology provides unparalleled sensitivity, selectivity, and versatility in chemical analysis, making it an indispensable tool across diverse scientific fields. The development of various software tools, algorithms, and databases for dereplication based on mass spectrometry has significantly advanced the field of natural product discovery.

Table 1 List of the effective strategies for the discovery of new natural products from fungi

Types of data	Tool	Features	Ref.
LC-MS/MS	GNPS	Sharing, analysis, processing, annotation, and visualization of global natural product mass spectrometry data	Wang et al. 2016; Qin et al. 2022
	GNP	A genome-guided natural products discovery tool	Johnston et al. 2015
	DEREPLICATOR	An algorithm for identifying peptidic natural products using GNPS	Mohimani et al. 2017
	DEREPLICATOR+	An algorithm for identifying polyketides, terpenes, benzenoids, alkaloids, flavonoids, and other classes of natural products using GNPS	Mohimani et al. 2018
	METLIN	A technology platform for the identification of known and unknown metabolites and other chemical entities	Guijas et al. 2018
	SIRIUS 4	A powerful chemical computational tool for predicting the structure of the compound	Dührkop et al. 2019
	ZODIAC	A new network-based algorithm for the de novo annotation of molecular formulas	Ludwig et al. 2020
	MolDiscovery	A mass spectral database search method based on a probabilistic model	Cao et al. 2021
	CANOPUS	A chemical classification prediction tool based on a deep neural network	Dührkop et al. 2021
	SNAP-MS	A structural similarity Network Annotation Platform for Mass Spectrometry	Morehouse et al. 2023
	LUMIOS	A software by preprocessing molecular information from mass spectra and dereplicates molecules through comparison with a collection of chemical databases	Vieira et al. 2024
^{1}H-NMR & ^{13}C-NMR	Spektraris-NMR	A database that can match multiple spectra data queries with database entries	Fischedick et al. 2015
^{1}H-NMR	1D-TOCSY	A one-dimensional TOCSY-based dereplication method	Diaz-Allen et al. 2021; Diaz-Allen 2022
	Using PCA to analyze ^{1}H-NMR data	An algorithm that uses the principal component analysis (PCA) loadings values to analyze ^{1}H-NMR data of mixtures	Selegato et al. 2016
^{13}C-NMR	NAPROC-13	A database containing ^{13}C spectral information allows for fast identification of known compounds present in the crude extracts and provides insight into the structural elucidation of unknown compounds.	Lopez-Perez et al. 2007
	Pattern Recognition Strategy	A dereplication strategy for pattern recognition of ^{13}C NMR data using hierarchical cluster analysis (HCA)	Hubert et al. 2014
	Computer-Aided ^{13}C NMR Profiling of Crude Natural Extracts	An algorithm evaluates the quality of the matching between experimental ^{13}C NMR data and predicted ^{13}C NMR data by calculating a score function.	Bakiri et al. 2017
	MixONat	A software based on a freely distributed algorithm that can be used to decipher complex mixtures using ^{13}C-NMR	Bruguière et al. 2020; Bruguière et al. 2021
	Prediction of compound structure with ^{13}C-NMR data	A database retrieves and analyzes compound structures recorded in acd_lotusv7 using ^{13}C-NMR data	Bruguière et al. 2018; Nuzillard 2021, 2022; Kuhn and Nuzillard 2023
2D-NMR	SMART	A tool for the efficient discovery of natural products based on Non-Uniform Sampling (NUS) HSQC techniques and deep Convolutional Neural Networks (CNNs)	Zhang et al. 2017; Reher et al. 2020
	MADByTE	A new platform to identify common structural features between samples in complex extract libraries using two-dimensional NMR spectra	Egan et al. 2021
	Tomic Novelty Scoring Technology	A method based on sorting atomic novelty to identify new features of secondary metabolites	Duggan et al. 2020
	DOSY	A tool to predict MW by NMR	Kleks et al. 2021

Types of data	Tool	Features	Ref.
MS and NMR	DEREP-NP	A database to match structure by counting the number of times one or more of these structural features occur in an unknown compound	Zani and Carroll 2017
	ELIAN	a ^{1}H NMR-MS workflow based on the HetCA of ^{1}H NMR spectra	Grienke et al. 2019

Molecular networking (MN) technology

In 2012, Prof. Pieter Dorrestein's group at the University of California, San Diego, introduced molecular networking (MN), a groundbreaking technique leveraging tandem mass spectrometry (MS/MS) for the discovery and analysis of microbial natural bioactive molecules (Watrous et al. 2012). MN-based dereplication enables the identification of both identical and structurally related molecules. It is compatible with various types of MS/MS data, and can be seamlessly integrated into the standard NPs discovery workflow (Yang et al. 2013).

By 2016, with the joint support of the University of California, San Diego and multiple other teams, Global Natural Products Social Molecular Networking (GNPS) had been created, enabling the sharing, analysis, processing, annotation, and visualization of global natural product mass spectrometry data (Wang et al. 2016). GNPS serves as an open-source and open-access knowledge base for community-wide organization and sharing of raw, processed, or identified tandem mass (MS/MS) spectrometry data (Wang et al. 2016). Since the emergence of Global Natural Product Social Molecular Networking (GNPS), the efficiency of natural product deduplication has been greatly improved, greatly saving research time, energy, and cost.

With the further development of metabolomics, computer science and bioinformatics, molecular networking has since evolved from its classical molecular networks (CLMN) to incorporate advanced approaches such as substructure-based MN (MS2LDA) (van Der Hooft et al. 2016), bioactive MN (BMN) (Nothias et al. 2018; Baskiyar et al. 2022), the feature-based MN (FBMN) (Nothias et al. 2020), ion-identifying MN (IIMN) (Schmid et al. 2021), building blocks-based molecular network (BBMN) (He et al. 2021), Integrated Molecular Networking Workflow for NP Dereplication (IMN4NPD) (Sheng et al. 2024), see Table 2 for details. These advancements have led to the discovery of numerous novel compounds, demonstrating the utility and adaptability of GNPS-based strategies (Table 2).

Table 2 Dereplication strategies published from 2013 to 2024 based on GNPS technology

No	MN type	Advantage	Website	References
1	CLMN	It is crucial for the meta-analysis of large-scale datasets.	http://gnps.ucsd.edu	Watrous et al. 2012
2	MS2LDA	It can analyze and annotate molecules without reference spectra by analyzing their substructures.	https://ms2lda.org/	van Der Hooft et al. 2016
3	BMN	It can quickly identify bioactive compounds from complex natural product extracts.	https://github.com/DorresteinLaboratory/Bioactive_Molecular_Networks	Nothias et al. 2018
4	Molecular Networks Based on the t-SNE Algorithm	It can reduce the dimensions of complex, high-dimensional data and simplify it, making it easier for users to understand the relationships between compounds.	https://metgem.github.io	Olivon et al. 2018
5	FBMN	By distinguishing different retention times of similar MS2 spectra, FBMN enables the efficient visualization and annotation of isomers in LC–MS^2 datasets.	https://ccms-ucsd.github.io/GNPSDocumentation/featurebased-molecularnetworking/	Nothias et al. 2020
6	IIMN	It effectively reduces the redundancy of detecting multiple ion species per compound and can be used to reveal unknown ion-ligand complexes, enhancing annotation.	https://ccms-ucsd.github.io/GNPSDocumentation/fbmn-iin/	Schmid et al. 2021
7	BBMN	It is more selective and faster, helping to discover natural products with new skeletons.	/	He et al. 2021
8	IMN4NPD	It can integrate multi-source compound data, expedite the dereplication of large clusters, and effectively mitigate the occurrence of false negatives.	/	Sheng et al. 2024

Classical molecular networking (CLMN). Molecular network technology is a secondary mass spectrometry data visualization strategy that integrates modern mass spectrometry, bioinformatics, computational tools, and other technologies. The principle behind this approach is that molecules with similar structures tend to exhibit similar MS/MS (secondary mass spectrometry) fragmentation patterns. The GNPS molecular network clusters and visualizes similar molecules by calculating the similarity of their MS/MS fragments. In this process, the secondary mass spectra in the LC-MS/MS data are compared using a specific algorithm, with the cosine similarity value (ranging from 0 to 1) representing the degree of similarity between each spectrum. A higher cosine value indicates greater similarity between the spectra. Based on the calculated cosine values, spectra with higher similarity are grouped, and the results are presented as a visualized molecular network diagram. In the diagram, each node represents a compound, and its associated secondary mass spectrometry information can be depicted by various attributes such as the node's name, color, size, and shape. The edges connecting the nodes represent the structural correlations between the compounds, with the edge thickness indicating the strength of this correlation (Watrous et al. 2012). By constructing a molecular network diagram, complex secondary mass spectrometry data can be intuitively represented, revealing all compounds present in the sample as well as the relationships between their chemical structures. If the chemical structure of a node in the molecular cluster is identified, the structures of other nodes can be predicted by analyzing mass differences and characteristic ion fragments, enabling the efficient discovery and targeted isolation of new compounds. *Aspergillus pseudoviridinutans* strain TW58-5 was isolated from hydrothermal vent sediment collected in Kueishantao, Taiwan (Ding et al. 2023). CLMN-guided isolation led to the discovery of seven new cyclopentapeptides with anti-inflammatory potential, namely, pseudoviridinutans A–F (**183–189**) from the fungus (Fig. 27) (Ding et al. 2023).

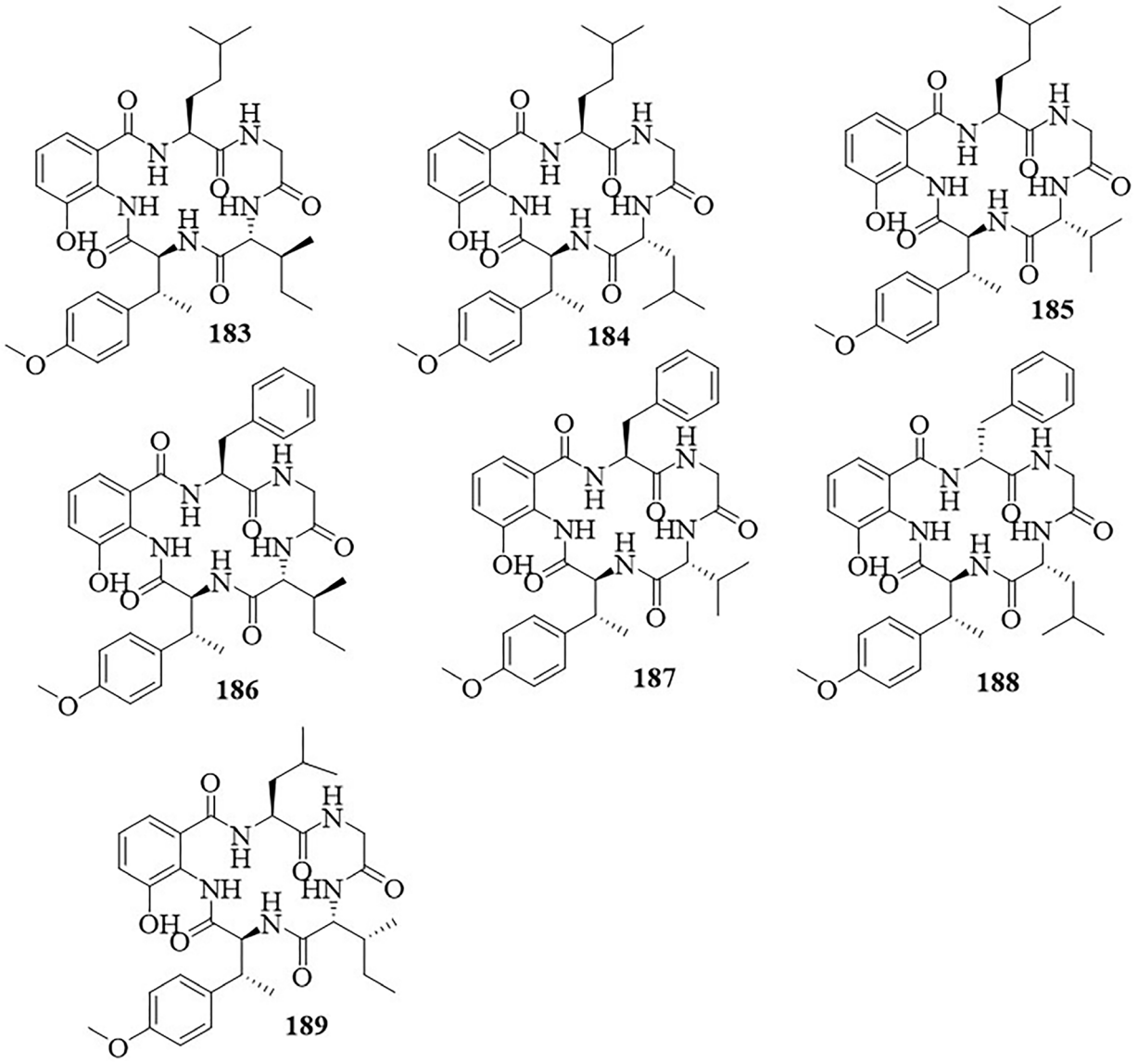

Fig. 27 New natural products discovered based on CLMN strategies.

Substructure-based molecular networking (MS2LDA). van der Hooft et al. (2016) introduced MS2LDA, a method that utilizes the Latent Dirichlet Allocation (LDA) model to extract recurring fragmentation and neutral loss patterns from MS/MS spectra, revealing biochemical relationships without requiring reference spectra. This method aids in identifying bioactive natural products and secondary metabolites, uncovering co-occurring metabolic patterns, and predicting unknown metabolite structures. Using MS2LDA, Liu et al. (2022) identified two rare tetracyclic alkaloids, perinadines B and C (**190** and **191**) (Fig. 28), from *Aspergillus* sp. (LS116), a marine sponge-derived fungus. Both compounds exhibited moderate antibacterial activity against *Bacillus subtilis*.

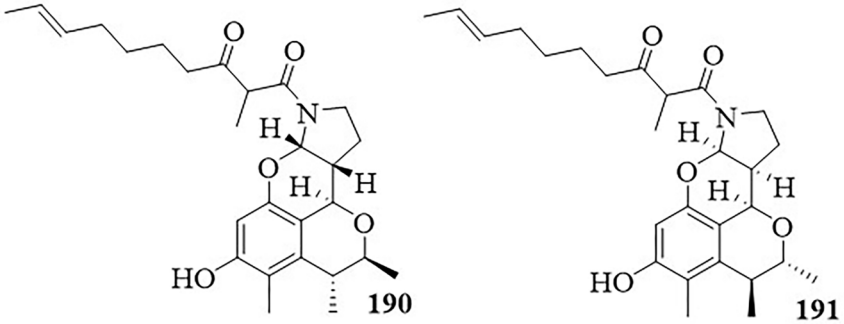

Fig. 28 Some new natural products discovered based on molecular network (MS2LDA) strategies.

Bioactivity-based molecular networking (BMN). During the separation of natural products using activity-oriented tracking and separation strategies, a phenomenon known as "activity disappearance" can occur. This means that while both the crude extract and its subfractions exhibit activity, the final isolated monomeric compound is inactive, or a known compound is repeatedly isolated (Nothias et al. 2018). To address this issue, in 2018, Pieter Dorrestein's group developed molecular network-based bioactivity (BMN) (Nothias et al. 2018). The BMN workflow involves three steps. First, tandem mass spectrometry data are collected from the sample after simple separation, followed by replication using CLMN. Next, the relative abundance and biological activity of molecules in each fraction are measured separately, and a bioactivity score is predicted. Finally, the bioactivity score prediction is integrated into the GNPS platform to generate the BMN. This strategy enables the rapid identification of bioactive compounds from complex natural product extracts, avoiding repeated separation and activity testing, while demonstrating strong targeting capabilities. Using BMN, three new decacarbonylspirotetraic acid derivatives, pyrenosetins A–C (**192**–**194**) (Fig. 29), were rapidly isolated from *Pyrenochaetopsis* sp. FVE-001 (Fan et al. 2020; Fan et al. 2022). Compounds **192** and **193** demonstrated strong anticancer activity against the malignant melanoma cell line A-375, which was consistent with the biological activity predicted by BMN.

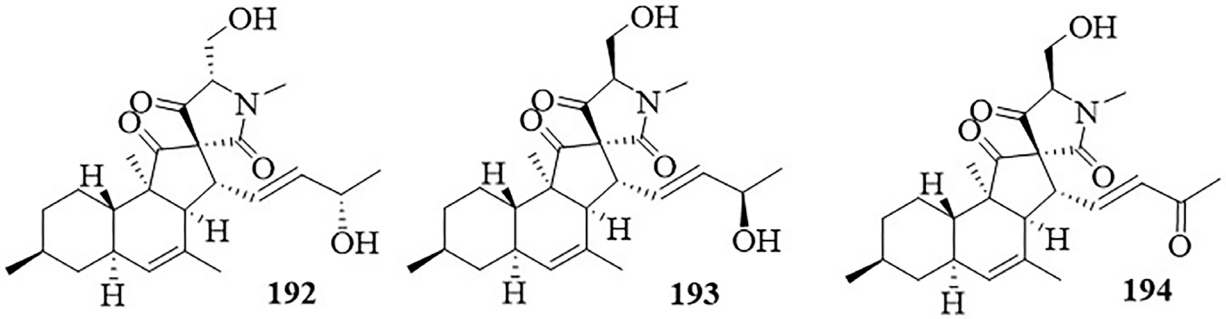

Fig. 29 New natural products discovered based on BMN.

Molecular networks based on the t-SNE algorithm. In a large amount of compound data, there are often some hidden patterns and structures, such as common substructures, similar mass spectra, etc. Using the t-SNE algorithm, such similar compounds can be clustered together to form clusters (Arora et al. 2018), helping researchers to better understand and analyze the relationship between compounds. Olivon et al. (2018) developed MetGem, which can perform parallel analysis of GNPs-style MNs and MNs of the t-SNE algorithm, simplifying complex high-dimensional chemical and biological data into a visual space, and accelerating the discovery process of new drugs or bioactive molecules. For instance, t-SNE MN analysis of *Penicillium Sclerotiorum* SNB-CN111, isolated from a termite nest in French Guiana, led to the identification of three novel azaphilones, chlorogeumasnol (**195**), peniazaphilone E (**196**), and 7-deacetylisochromophilone VI (**197**) (Fig. 30) (Hebra et al. 2021).

Feature-based molecular networking (FBMN). Building upon CLMN, Pieter Dorrestein's team introduced feature-based molecular networking (FBMN) to address key limitations of CLMN, such as its inability to differentiate isomers with similar MS^2 spectra and its lack of accurate relative quantification (Nothias et al. 2020). FBMN leverages retention time differences to distinguish similar MS^2 spectra, enabling efficient visualization and annotation of isomers in

LC–MS^2 datasets. Additionally, FBMN provides an accurate estimation of relative ion intensities, facilitating robust downstream statistical analyses in metabolomics. While FBMN excels in smaller-scale studies or single-sample analyses, CLMN remains indispensable for meta-analysis of large-scale datasets. The application of FBMN in comparative metabolomics and targeted isolation led to the discovery of two new stereoisomers of pyrenosetins E (**198**) and F (**199**) (Fig. 31), from *Pyrenochaetopsis* sp. FVE-001, which was isolated from *Fucus vesiculosus* (Fan et al. 2020; Fan et al. 2022). Notably, compound **198** exhibited significant anticancer activity against the malignant melanoma cell line A-375, with an IC_{50} value of 40.9 μM, consistent with bioactivity predictions (Fan et al. 2022). Recently, integrating Mass Spec Query Language (MassQL) with FBMN has resolved issues in clustering and annotation caused by multiple fragmentation events during MS analysis, further enhancing the precision of FBMN workflows (Selegato et al. 2023).

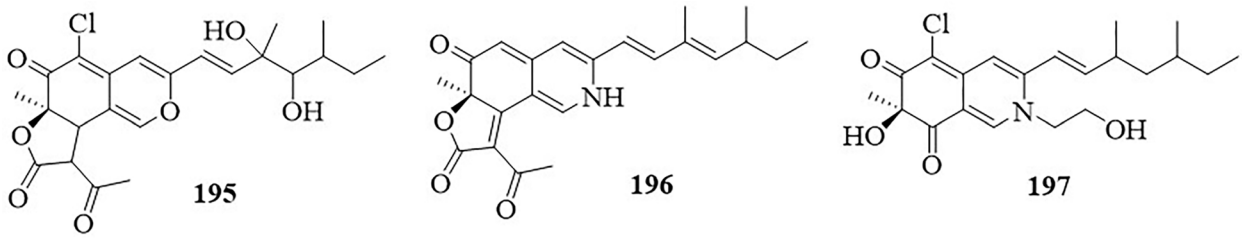

Fig. 30 New natural products from fungi discovered guided by t-SNE Molecular Networks.

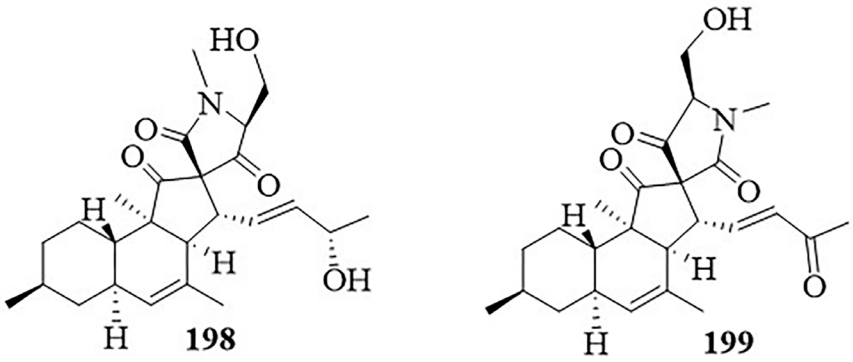

Fig. 31 New natural products from fungi discovered based on FBMN strategies.

Ion identity molecular networking (IIMN). Ionization often generates multiple ion forms of the same molecule, each displaying distinct fragmentation behaviors, which can fragment the tandem mass spectrometry molecular network of a single parent ion into separate clusters. To overcome this issue, Dorrestein's group developed Ion Identity Molecular Networking (IIMN), which integrates MS1 chromatographic peak shapes with MS2 spectral similarity (Schmid et al. 2021). This strategy reduces redundancy by consolidating data for multiple ion species of the same compound and enhances molecular network annotations. IIMN has proven effective in identifying unknown ion-ligand complexes and expanding spectral reference libraries. For example, the marine sponge-derived fungus *Stachylidium bicolor* strain 293 K04 produces cyclic tetrapeptides, known as endolides, which contain the rare amino acid N-methyl-3-(3-furyl)-alanine (Berger et al. 2024). Exploiting this unique structural feature, Berger et al. (2024) developed an endolide-specific molecular network annotated using MassQL, incorporating CLMN, FBMN, and IIMN workflows. This multimodal approach facilitated the discovery of two new proline-containing endolides, E (**200**) and F (**201**) (Fig. 32). Endolide F was identified as a moderate antagonist of the arginine vasopressin V1A receptor (Berger et al. 2024).

Building blocks-based molecular networking (BBMN). Natural products with similar structures typically follow analogous biosynthetic pathways. Biosynthetic building blocks serve as the foundational starting points for biogenic synthesis pathways and are essential for the synthesis of complex molecules. Organisms generate these building blocks through various biosynthetic routes and subsequently assemble them into target molecules (Walsh & Tang 2017). He et al. (2021) introduced a molecular network based on biosynthetic building blocks (BBMN) by integrating building block identification strategies with molecular network construction. Compared to CLMN and FBMN, the BBMN strategy offers higher selectivity and faster identification. This approach can recognize characteristic fragments derived from specific biogenetic building blocks by scanning for neutral loss/production events, enabling the selective identification of biogenetically relevant compounds. Additionally, the BBMN strategy has been employed to analyze metabolic pathways, identify known and novel biosynthetic gene clusters, and guide the isolation of new carbon skeleton compounds (He et al. 2021; Qin et al. 2022a). Li et al. (2024) applied BBMN to discover three unusual ajmaline-macroline type bisindole alkaloids from *Alstonia macrophylla*.

Integrated molecular networking workflow for NP dereplication (IMN4NPD). To integrate multi-source compound data, Jiang's group developed the integrated molecular networking workflow for natural product dereplication (IMN4NPD) (Sheng et al. 2024). Compared with previous molecular network strategies, IMN4NPD offers distinct advantages. By combining results from multiple computational tools, the workflow not only accelerates the dereplication of large clusters within the molecular network but also highlights features often overlooked by existing methods, such as self-looping or paired nodes. Furthermore, the integration of various spectral similarity measures enhances the comprehensiveness of the resulting t-SNE plot, effectively reducing false negatives.

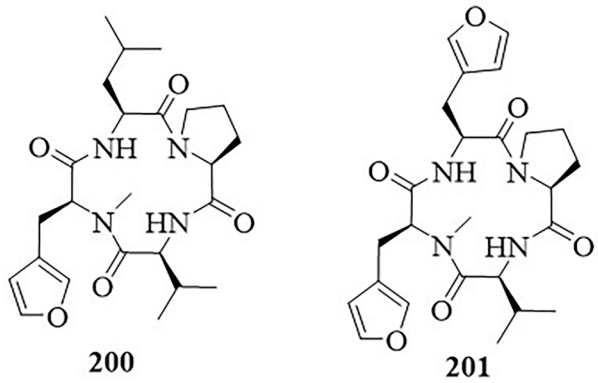

Fig. 32 New natural products from fungi discovered based on multimolecular network strategies.

An automated genomes-to-natural products platform (GNP)

The synthesis of secondary metabolites in fungi is typically governed by biosynthetic gene clusters. Johnston et al. (2015) introduced an accessible and automated tool named the Genomes-to-Natural Products platform (GNP, designed to predict natural products from genetic data and directly identify desired small molecules from liquid chromatography–MS/MS (LC-MS/MS) datasets (Johnston et al. 2015). Using the GNP platform, the researchers successfully identified and isolated six genetically predicted polyketides and nonribosomal peptides from bacterial sources.

DEREPLICATOR and DEREPLICATOR+

DEREPLICATOR+ (Mohimani et al. 2018) is an advanced algorithm that builds upon its predecessor, DEREPLICATOR (Mohimani et al. 2017), enhancing its ability to identify peptidic natural products. In addition, it expands the scope of natural product identification to include polyketides, terpenes, benzenoids, alkaloids, and flavonoids (Mohimani et al. 2018). DEREPLICATOR+ is notable for being the first database search tool for natural products capable of: (i) leveraging the entire GNPS molecular networking infrastructure to query large chemical structure databases and (ii) detecting variants of known metabolites through molecular networking (Mohimani et al. 2018).

METLIN

METLIN (METLIN) is a comprehensive technology platform designed for the identification of known and novel metabolites, originally developed as a database for identifying known metabolites (Guijas et al. 2018). By integrating reference standards, labeled stable isotope analogues, and fragment similarity search, METLIN facilitates both the characterization of unknown metabolites and the identification of known ones (Guijas et al. 2018).

SIRIUS 4

SIRIUS 4 (SIRIUS) is a sophisticated computational tool for analyzing complex mass spectrometry data to extract compound information and predict chemical structures (Dührkop et al. 2019). Its workflow involves steps such as mass spectrum analysis, rule-based fragment assembly, candidate structure generation, spectrum matching and ranking, and network analysis (Ludwig et al. 2020a). While highly effective for structural prediction and dereplication, SIRIUS 4 faces challenges in accurately identifying molecular formulas for compounds with molecular weights exceeding 500 Da or entirely novel formulas.

Organic compound determination by integral assignment of elemental compositions (ZODIAC)

Building on SIRIUS 4, Ludwig et al. (2020a) developed Organic compound Determination by Integral Assignment of elemental Compositions (ZODIAC), a network-based algorithm for molecular formula annotation. By combining split-tree calculations, Gibbs sampling, and Bayesian statistics, ZODIAC improves the accuracy of SIRIUS candidate formula ranking by a factor of 16 and is capable of annotating novel molecular formulas, such as $C_{24}H_{47}BrNO_8P$ (Ludwig et al. 2020a; 2020b). ZODIAC is particularly valuable for studying compounds with molecular weights above 500 Da.

MolDiscovery

Cao et al. (2021) introduced MolDiscovery, a mass spectral database search method that enhances the efficiency and accuracy of small molecule identification by employing a probabilistic model to match molecules with their mass spectra. Despite its advancements, MolDiscovery shares a common limitation with tools like DEREPLICATOR+ and

SIRIUS 4, as it exhibits reduced accuracy for molecules with masses below 600 Da.

Class assignment and ontology prediction using mass spectrometry (CANOPUS)

Class assignment and ontology prediction using mass spectrometry (CANOPUS) is a deep-learning-based tool for chemical classification that can predict 2,497 compound classes from fragmentation spectra, including all biologically relevant categories (Dührkop et al. 2021). It integrates seamlessly with tools such as SIRIUS 4 and GNPS, enabling comprehensive workflows from feature detection to compound classification (Dührkop et al. 2021).

Structural similarity network annotation platform for mass spectrometry (SNAP-MS)

SNAP-MS (Structural Similarity Network Annotation Platform for Mass Spectrometry, SNAP-MS) is an advanced prediction tool developed by Linington's team (Morehouse et al. 2023) that follows CANOPUS (Dührkop et al. 2021) in chemical similarity grouping. Unlike CANOPUS, SNAP-MS does not rely on experimental or calculated reference spectra, making it a complementary tool for fine-scale compound family annotation. This approach assigns compound families to molecular networking subnetworks. Subnetworks containing known families are then identified for dereplication, enabling efficient compound characterization. Compared to CANOPUS, SNAP-MS offers a more detailed level of annotation, enhancing the accuracy of natural product identification and enabling more precise dereplication.

Label using a machine in organic samples (LUMIOS)

LUMIOS (Label Using Machine in Organic Samples) is a versatile Python-based software designed to assist both professionals and students in the computational exploration of NPs (Vieira et al. 2024). It preprocesses mass spectrometry data and performs dereplication by comparing molecules to a collection of chemical databases, enabling efficient identification of natural product candidates (Vieira et al. 2024).

Efficient discovery strategies for new natural products based on NMR

NMR technology is one of the most widely used and reliable methods for the structural characterization of natural products, offering atomic-level molecular information and playing an irreplaceable role in identifying unknown compounds (Edison et al. 2020). It is particularly valued for its ability to perform non-destructive analysis of complex samples, analyze samples that are difficult to ionize, and provide reproducible data and extensive structural insights. However, compared to mass spectrometry, NMR's lower sensitivity and longer analysis time are notable drawbacks (Pretsch et al. 2020; Yuan et al. 2021). Recent advancements, including higher magnetic field strengths in commercial NMR instruments and the use of ultra-low temperature probes, have significantly enhanced resolution and sensitivity. These improvements enable microgram-level sample testing while reducing analysis time. Additionally, two-dimensional NMR (2D NMR) addresses spectral overlap issues in 1D NMR, making it better suited for analyzing complex natural product structures (Ernst et al. 1990). Innovative spectrogram alignment algorithms have expanded the utility of NMR, particularly 2D NMR, in dereplication and mixture analysis. Notable methodologies include COLMAR Lipids Web Server, Small Molecule Accurate Recognition Technology (SMART), Diffusion-ordered NMR Spectroscopy (DOSY), Metabolomics and Dereplication by Two-dimensional Experiments (MADByTE), and Atomic Novelty Scoring Technology (Zhang et al. 2017; 2020; Duggan et al. 2020; Wang et al. 2020a; Kleks et al. 2021; Egan et al. 2021).

Dereplication strategy based on 1D-NMR

Spektraris-NMR. Spektraris-NMR is an online spectral resource that enables users to search multiple spectra simultaneously (Fischedick et al. 2015). The query results are ranked by the goodness of fit between the query data and database entries, and provide links to both ^{1}H NMR and ^{13}C NMR spectra, which can indirectly assist users in filtering out known compounds.

Dereplication strategy using PCA to analyze ^{1}H-NMR data. To analyze complex *Fusarium* extracts, Selegato et al. (2016) developed an algorithm that utilizes principal component analysis (PCA) loadings to analyze ^{1}H-NMR data from mixtures. This algorithm successfully differentiated the mycotoxins produced by *Fusarium solani* and *F. oxysporum*. By selecting key peaks that distinguish metabolic profiles from a complex NMR dataset, the algorithm extracts these peaks and compares them with in-house ^{1}H-NMR libraries and online databases, enabling the identification of important bioactive metabolites in the mixture.

One-dimensional total correlation spectroscopy (1D-TOCSY)

Total correlation spectroscopy (TOCSY) is an NMR experiment that reveals the relationships between all linked protons in a spin system (Diaz-Allen et al. 2021). First, the number of spin systems in the compound of interest must be determined, and then the connections between these spin systems form a unique "fingerprint". This fingerprint allows users to efficiently dereplicate known compounds from crude extracts and identify new compounds that are structurally related to known small molecules (Diaz-Allen et al. 2021; Diaz-Allen 2022). Using this approach, Diaz-Allen et al. (2021) discovered five new triterpenoids (**202–206**) (Fig. 33) from the lichen *Niebla homalea* and one new polyketone (**207**) (Fig. 33) from *Niebla* sp. However, the dereplication strategy based on 1D-TOCSY has its limitations, as it can only identify compounds with similar structures.

Dereplication strategy based on ^{13}C-NMR

^{13}C-NMR signals typically do not overlap easily and exhibit distinct peak shapes, making them an ideal data source for the development of various dereplication strategies (Sahayasheela et al. 2022).

NAPROC-13. Lopez-Perez et al. (2007) developed a ^{13}C-NMR spectral database comprising over 6,000 natural compounds, facilitating rapid identification of known components in crude

extracts and aiding structural elucidation of unknown compounds.

Pattern recognition strategy. Hubert et al. (2014) proposed a ^{13}C NMR-based pattern recognition strategy, where crude extracts are fractionated by centrifugal partition extraction (CPE), and the ^{13}C NMR spectra of each fraction are analyzed. Hierarchical cluster analysis (HCA) identifies chemical shift clusters, which are matched to a local database for dereplication and component identification. Oettl et al. (2014) identified six compounds in the lichen *Pseudevernia furfuracea* by utilizing an in-house ^{13}C chemical shift library and pattern recognition method.

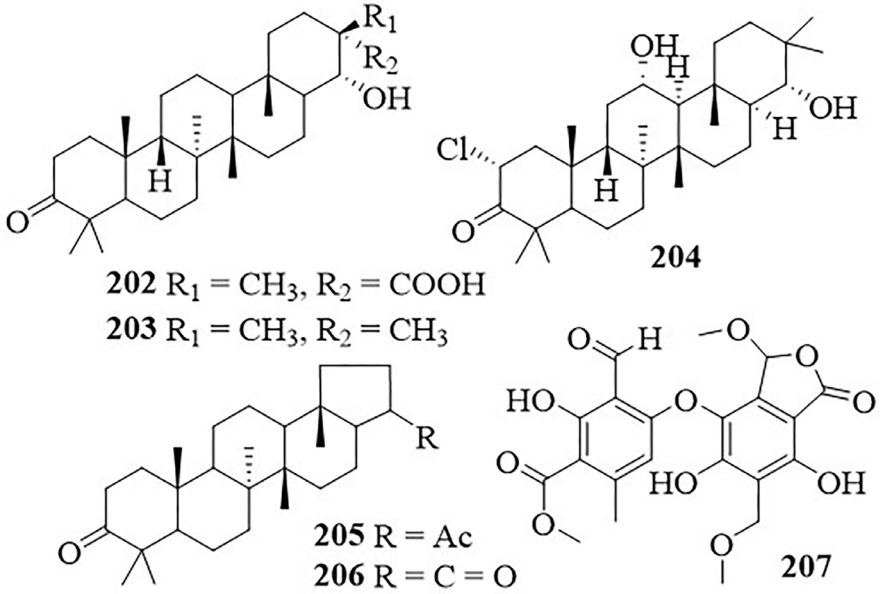

Fig. 33 New natural products from *Niebla* sp. guided by 1D-TOCSY strategies.

Computer-aided ^{13}C NMR profiling of crude natural extracts. Bakiri et al. (2017) developed a computer-aided ^{13}C NMR dereplication workflow that enables rapid identification of major constituents in natural mixtures without prefractionation. The algorithm compares experimental ^{13}C NMR data with predicted spectra from a natural metabolite database, calculating a score function to evaluate the match quality. This approach reduces isolation steps and directly analyzes crude extracts, streamlining the dereplication process.

MixONat software. MixONat is a software developed for the automated dereplication of natural products using ^{13}C-NMR data. It processes ^{13}C-NMR, DEPT-135, and DEPT-90 spectra to filter carbon types (i.e., CH_3, CH_2, CH, and C), helping to identify known major compounds in complex mixtures. The software compares experimental or predicted chemical shifts (δ_C) with relevant databases, producing interactive results that can be refined based on the user's phytochemical or metabolomic knowledge. MixONat is particularly useful for identifying stereoisomers and enhancing the efficiency of dereplication, and it is designed to work with both freely available and commercial δ_C datasets (Bruguière et al. 2020; 2021). Yin et al. (2023) employed the ^{13}C-NMR-based MixONat strategy in combination with 2D NMR techniques to efficiently dereplicate known secondary metabolites in *Aloe vera* and successfully identify two new chromones.

acd_lotusv7 database. Bruguière et al. (2018) used experimental ^{13}C-NMR data in ACD/Labs commercial software to predict δ_C with 73% accuracy, then matched the corresponding structures in a δ_C database to successfully identify Polycyclic Polyprenylated AcylPhloroglucinols (PPAPs) from *Garcinia bancana* extract. Nuzillard (2021) developed the free Python script CNMR_Predict, which supplements taxon-oriented search results from the LOTUS database (lotus.naturalproducts.net) with predicted ^{13}C-NMR data from the ACD/Labs CNMR predictor and database software (acdlabs.com), creating easily searchable databases. In 2023, Nuzillard's team further developed this concept into the acd_lotusv7 database (Kuhn & Nuzillard 2023). This open-source database integrates ^{13}C-NMR data and can be easily accessed through the nmrshiftdb2 online interface, enabling free retrieval and analysis of compound structures recorded in acd_lotusv7 (Kuhn & Nuzillard 2023).

Dereplication strategy based on 2D-NMR

Small molecule accurate recognition technology (SMART). Zhang et al. (2017) introduced Small Molecule Accurate Recognition Technology (SMART), a deep-learning approach that combines Heteronuclear Single Quantum Coherence (HSQC) spectra with convolutional neural networks (CNNs) for the efficient dereplication of natural products. SMART employs a Siamese neural network to train on a dataset of HSQC spectra from 2054 natural products, constructing a node space where compounds with similar structures are spatially close, while those with larger structural differences are distant. This allows for rapid identification and dereplication of compounds in complex natural product mixtures by comparing new analytes to this node space. Reher et al. (2020) expanded the dataset to include 25,434 HSQC spectra from the JEOL database (JEOL) and 27,642 predicted spectra from ACD/Labs software, which led to the release of SMART 2.0 (SMART) (Reher et al. 2020). The expansion significantly improved the ability of SMART to identify natural products with diverse structural types by increasing the number of training sets. Guided by SMART and

OSMAC strategies, eight new indole-benzodiazepine derivatives (asperdinones A–H, **208–215**) (Fig. 34) were isolated from the mangrove-derived fungus *Aspergillus spinosus* strain WHUF0344 (Lu et al. 2024). These new compounds featured a range of unique structural motifs, including dimeric alkaloids and a rare pentacyclic skeleton, with some showing moderate inhibitory effects against α-glucosidase (IC_{50} values: 24.65–312.25 μM).

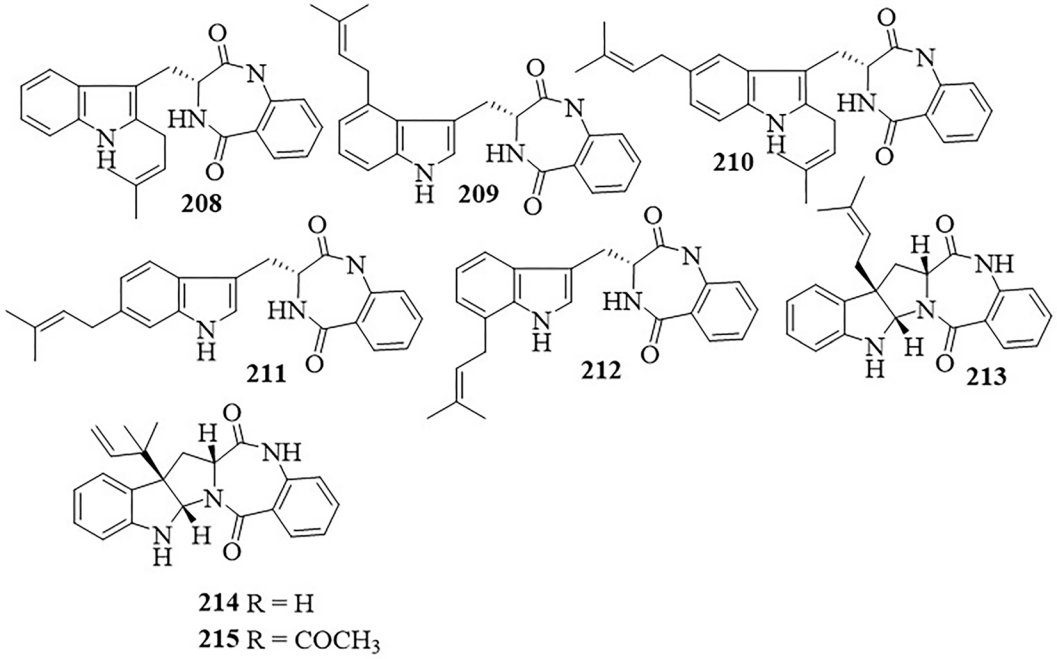

Fig. 34 New natural products from *Aspergillus spinosus* (WHUF0344) guided by OSMAC and SMART strategies.

Metabolomics and dereplication by two-dimensional experiments (MADByTE). Linington's group developed a new data analysis platform for complex mixture analysis, called MADByTE (Metabolomics and Dereplication by Two-Dimensional Experiments) (Egan et al. 2021). This platform combines TOCSY and HSQC spectra to identify spin system features in complex mixtures and constructs a chemical similarity network to achieve dereplication by identifying common proton spin coupling systems (Egan et al. 2021). To assess MADByTE's performance, Flores-Bocanegra et al. (2022) used the platform to detect two classes of fungal secondary metabolites: resorcylic acid lactones (RALs) and spirobisnaphthalenes. The platform successfully clustered these compounds from seven fungal extracts by generating a rotating system feature network. NMR-guided isolation led to the discovery of three new palmarumycins (**216–218**) (Fig. 35) (Flores-Bocanegra et al. 2022).

Tomic novelty scoring technology. Clair's team developed a method based on sorting atomic novelty to identify new features of secondary metabolites using HSQC data (Duggan et al. 2020). This approach involves listing 10,308 signal peaks from the Human Metabolome Database (HMDB) and BioMagResBank, then calculating the distance between each signal peak in the analyte and the nearest peak in the database to assign a score. The method efficiently identifies novel compounds with significant structural differences from known metabolites in the library. While effective in identifying novel compounds, false positives remain a challenge (Reher et al. 2020).

Diffusion ordered NMR spectroscopy (DOSY). In mixed samples, the diffusion rates of molecules in solution vary due to differences in their size and shape. Based on this principle, Carroll's group developed diffusion-ordered NMR spectroscopy (DOSY) to distinguish components of a mixture by their diffusion coefficients (D) (Morris & Johnson Jr 1992). By establishing a power law relationship between diffusion coefficient (D) and molecular weight (MW), molecular weights can be predicted from experimental diffusion values (D) (Kleks et al. 2021). Since many factors can influence D, Carroll's team developed multiple linear regression equations that incorporate various physicochemical properties to accurately correlate experimental and predicted diffusion coefficients (Kleks et al. 2021). These equations, when combined with structural features obtained from DOSY and NMR analysis, enable the dereplication of known natural products in a mixture, without the need for MS data, by matching the experimental D values with predicted diffusion coefficients and computational structural features in the DEREP-NP database (Zani & Carroll 2017).

Integration of MS and NMR in Natural Product Discovery

DEREP-NP

In 2017, Zani and Carroll developed a new platform, DEREP-NP (DEREP-NP), designed to analyze NMR and MS spectroscopic data for inferring structural features in natural products, enabling dereplication (Zani & Carroll 2017). By counting the occurrences of specific structural features in an unknown compound—deduced from the analysis of its NMR (^{1}H, HSQC, and/or HMBC) and/or MS data—the platform retrieves matching structures from the Universal Natural Products Database (UNPD) that share the same numeric combination of searched features (Zani & Carroll 2017). However, this methodology is limited to purified natural

products and fractions containing a small number of individual compounds.

Eliciting nature's activities (ELIAN)

Grienke et al. (2019) developed a ^{1}H NMR-MS workflow called ELIAN (Eliciting Nature's Activities), which utilizes statistical heterocovariance analysis (HetCA) of ^{1}H NMR spectra to identify chemical features linked to biological activity before undertaking complex separations. Using this approach, the authors successfully discovered steroid sulfatase-inhibiting lanostane triterpenes in an extract from the polypore fungus *Fomitopsis pinicola* (Grienke et al. 2019).

Discussion

Traditional methods for isolating and identifying natural products often involve extensive effort, with a high likelihood of rediscovering known compounds. To address this challenge, various dereplication strategies using LC-MS/MS and NMR data have been developed. Among these, the GNPS platform is particularly popular for its ability to annotate, classify, and visualize compounds in crude extracts (Qin et al. 2022a). While mass spectrometry offers high sensitivity and can detect trace amounts of compounds, low-concentration compounds may be difficult to isolate for structural characterization (Kleks et al. 2021).

^{1}H-NMR is less frequently used for dereplication due to the overlap of proton signals, which limits the development of proton-based dereplication strategies (Bruguière et al. 2020). In contrast, ^{13}C-NMR provides more distinct peak shapes, though it suffers from challenges such as low natural abundance, long acquisition times, and high costs (Bruguière et al. 2020). Despite these drawbacks, ^{13}C-NMR is particularly well-suited for identifying diastereomers (Clendinen et al. 2014; Wu et al. 2022).

Emerging artificial intelligence techniques, such as SMART 2.0, are overcoming bottlenecks in natural product research by enhancing the accuracy of 2D-NMR dereplication (Reher et al. 2020). Moreover, integrative strategies combining GNPS, MS2LDA, bioinformatics, and computational tools have further improved efficiency and facilitated the discovery of novel compounds (Reher et al. 2020; Egan et al. 2021; Hyde et al. 2024).

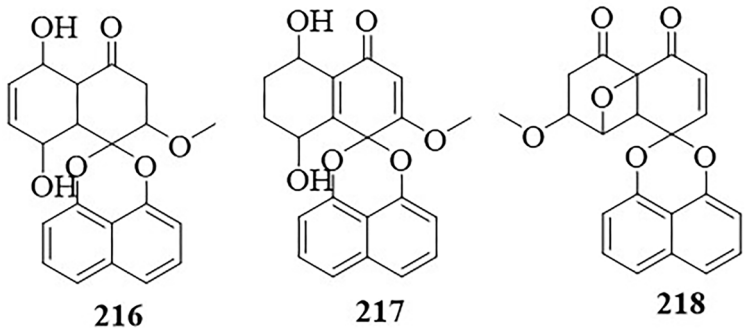

Fig. 35 New natural products from fungi guided by MADByTE strategies.

Combining multiple strategies for mining new natural products in fungi

In many cases, a single strategy may fail to produce satisfactory results. However, combining multiple approaches often leads to significant success. For instance, silent gene clusters are first successfully activated by altering culture conditions or through gene mining, followed by the accurate detection of new natural products using dereplication strategies based on LC-MS/MS and/or NMR, which ultimately enhances the effectiveness of targeted separation. Below are examples showcasing how different strategies have been combined to discover new fungal secondary metabolites.

GNPS + ^{1}H-NMR

Integrating the cheminformatics-based MS/MS molecular networking approach with highly sensitive ^{1}H-NMR allows for the elucidation of structural relationships while accelerating compound dereplication from crude extracts, thereby improving the efficiency of new compound discovery (Hou et al. 2019a). Using this combined technique, seven novel cyclohexadepsipeptides (**216**–**222**) (Fig. 36), chrysogeamides A–G, were identified from *Penicillium chrysogenum* CHNSCLM-0003, isolated from the gorgonian coral *Carijoa* sp. (GX-WZ-2010001) (Hou et al. 2019a). Among these, compounds **220** and **225** promoted angiogenesis in zebrafish at a concentration of 1.0 μg/mL, with no toxicity observed in zebrafish embryos at 100 μg/mL (Hou et al. 2019a). Notably, compound **222** contains a rare 3-hydroxy-4-methylhexanoic acid (HMHA) moiety (Hou et al. 2019a). Similarly, this approach led to the discovery of three new cycloheptapeptides, asperversiamides A–C (**226**–**228**) (Fig. 36), from the coral-derived fungus *Aspergillus versicolor* CHNSCLM-0063. All three compounds exhibited potent inhibitory activity against *Mycobacterium marinum* (Hou et al. 2019b).

LC-MS/MS + ^{1}H-NMR + Epigenetic modifiers

The combination of histone deacetylase inhibitor (SAHA) and DNA methyltransferase inhibitor (5-Aza) significantly altered the HPLC profile and ^{1}H-NMR spectra of the EtOAc extract from the marine-derived fungus *Aspergillus versicolor* strain XS-20090066, compared to the untreated control (Wu et al. 2020). MS/MS-based molecular networking led to the identification of two novel nucleoside derivatives, kipukasins K (**229**) and L (**230**), and a new bisabolane sesquiterpene, aspergillusene E (**231**) (Fig. 37). Compounds **229** and **231** exhibited antibacterial activity against *Staphylococcus epidermidis* and *Staphylococcus aureus*, with MIC values ranging from 8–16 μg/mL.

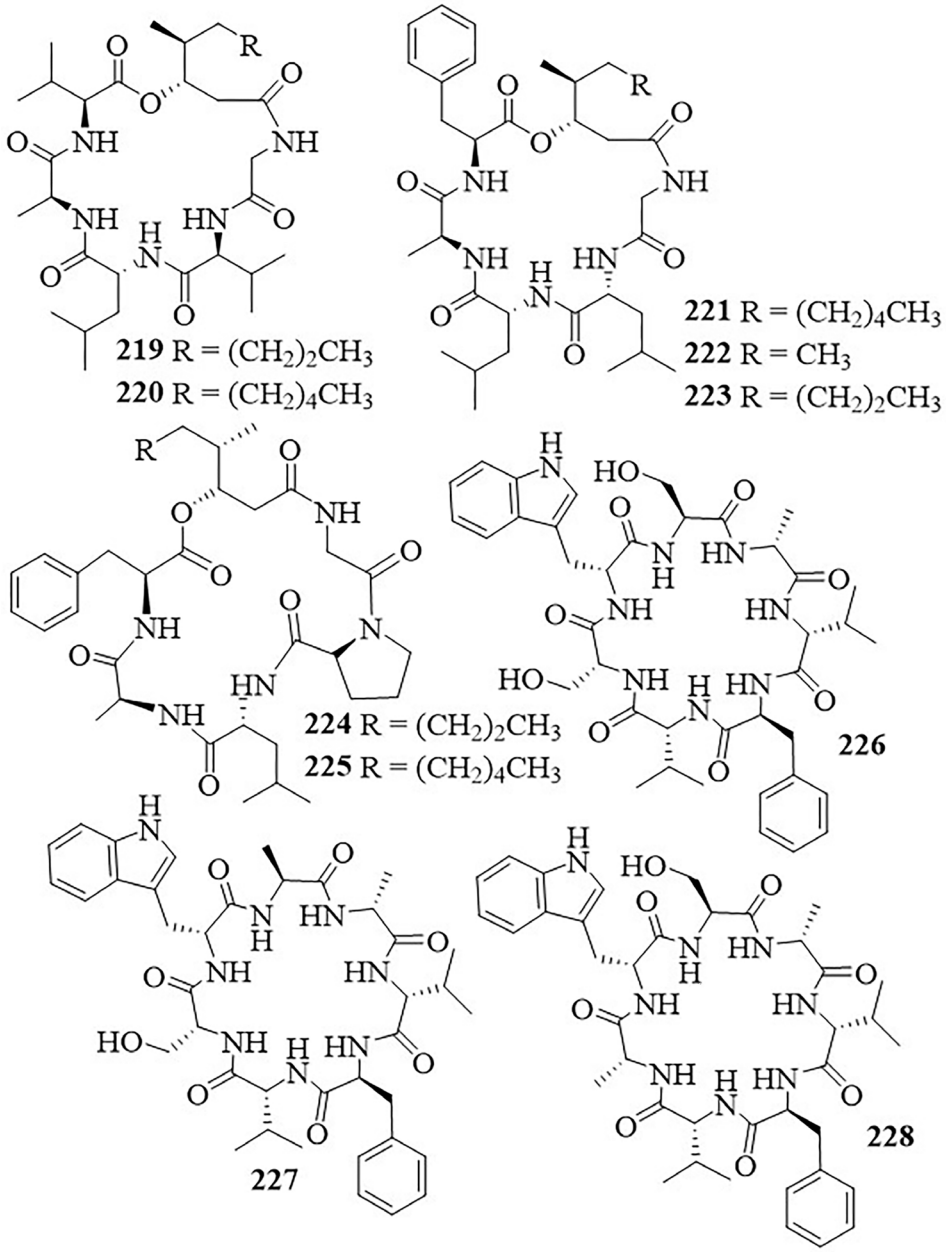

Fig. 36 New natural products discovered based on GNPS and ^{1}H-NMR strategies.

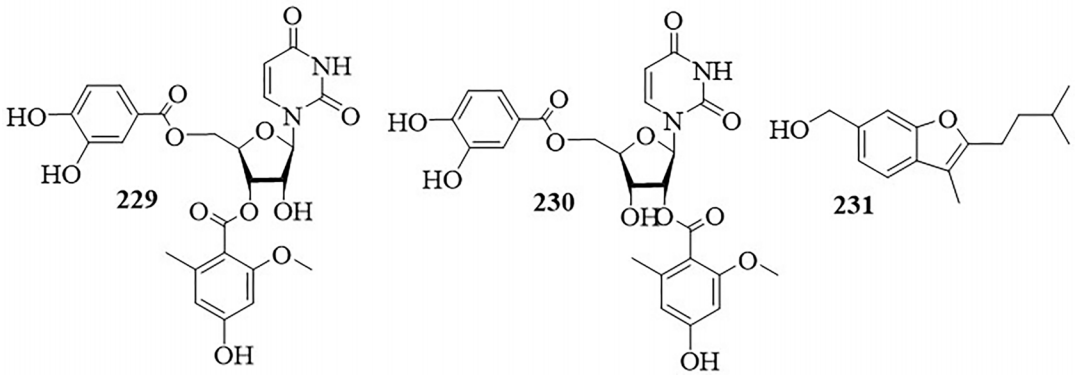

Fig. 37 New natural products discovered based on GNPS, ^{1}H-NMR and epigenetic modifiers strategies.

Co-culture + GNPS

Co-culturing of marine *Cosmospora* sp. with the phytopathogen *Magnaporthe oryzae*, coupled with molecular networking, led to the discovery of two new soudanones H and I (**232–233**) (Fig. 38) (Oppong-Danquah et al. 2022).

GNPS + OSMAC

Guided by molecular networking and the OSMAC approach, nine highly oxygenated meroterpenoids, peniciacetals A–I (**234–242**) (Fig. 39), were isolated from the mangrove-derived fungus *Penicillium* sp. HLLG-122 (Qin et al. 2023). Notably, peniciacetals A–B (**234–235**) feature a unique 6/6/6/6/5 pentacyclic structure, incorporating a 4,6-dimethyl-2,5-dioxohexahydro-6-carboxy-4*H*-furo[2,3-b]pyran moiety, while peniciacetals C–D (**236–237**) contain a rare 3,6-dimethyldihydro-4*H*-furo[2,3-b]pyran-2,5-dione unit with a fused 6/6/6/5/6 pentacyclic skeleton.

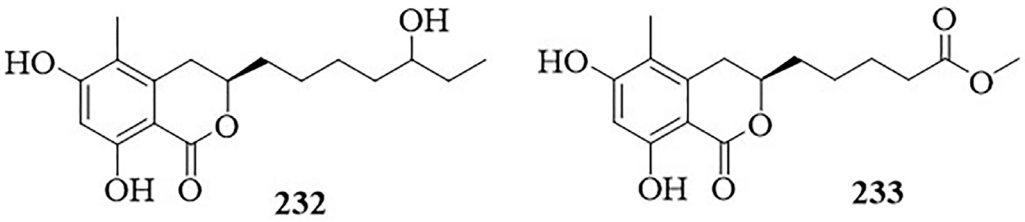

Fig.38 New natural products discovered based on co-culture and molecular network strategies.

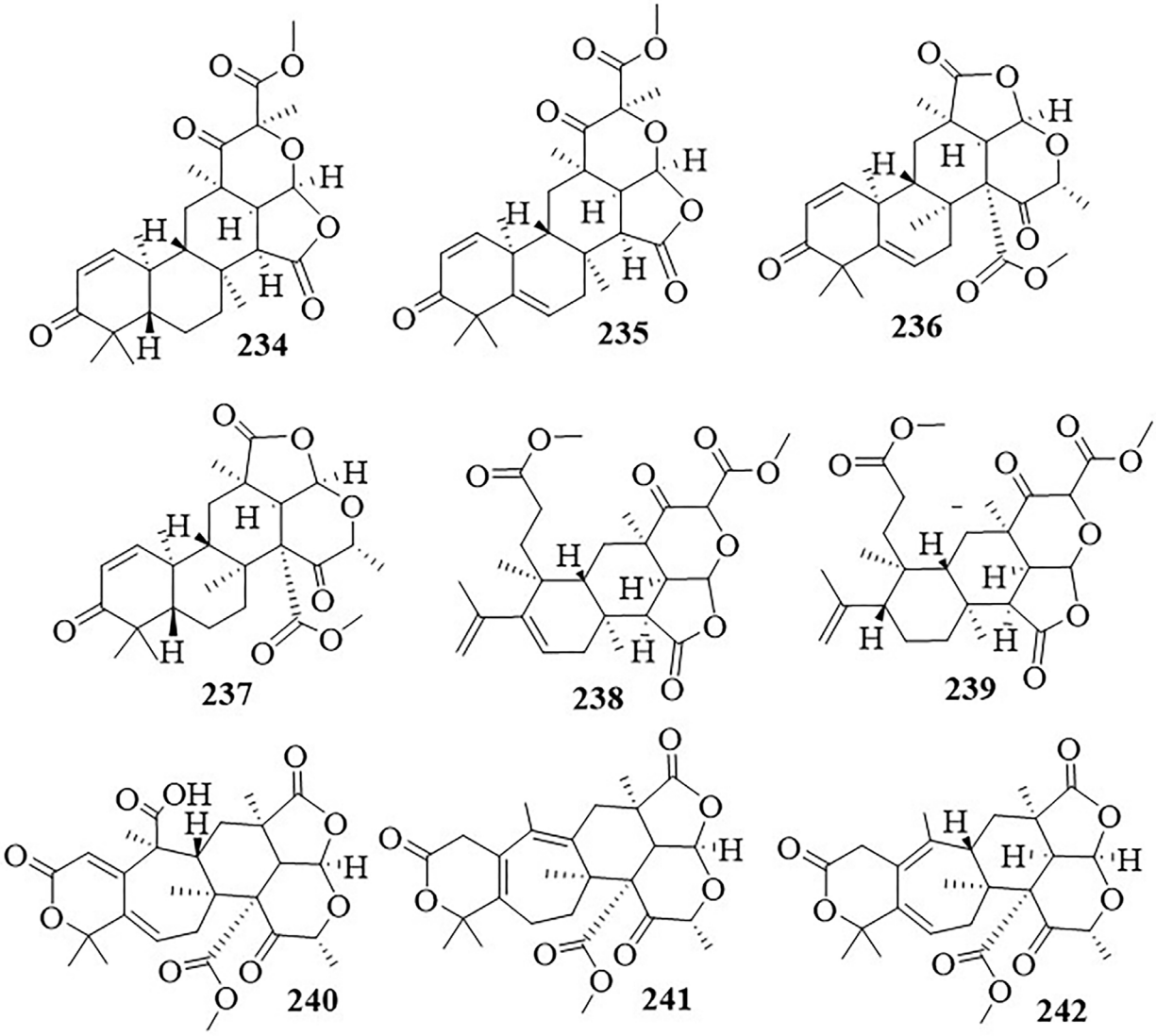

Fig. 39 New polyketides discovered based on OSMAC and molecular network strategies.

GNPS + Epigenetic modifiers

MS/MS networking revealed a large family of aspercryptins, with 13 members identified from a rpdA^{KD} mutant of *Aspergillus nidulans* that exhibits constitutively lower levels of the HDAC RpdA (AN4493). Among them, the novel aspercryptins A1 (**243**) and A2 (**244**) were isolated and characterized (Fig. 40) (Henke et al 2016). Interestingly, the lipid tails of these lipopeptides are located at the C-terminus.

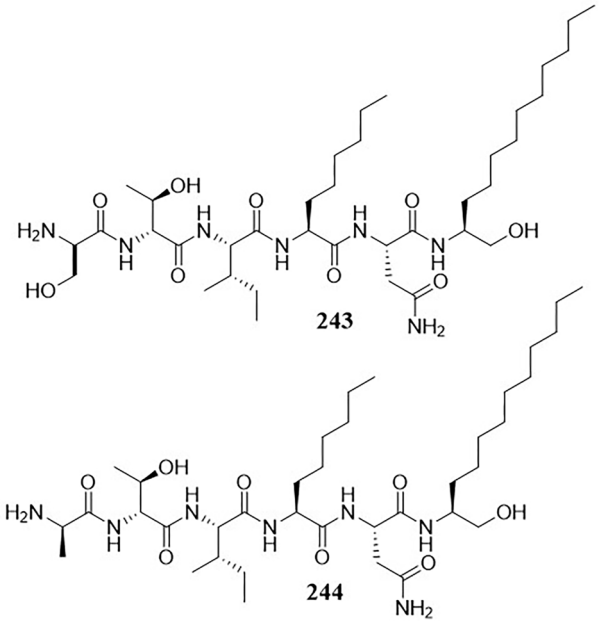

Fig. 40 New natural products discovered based on GNPS and epigenetic modifier strategies.

Heterologous expression + GNPS

Ten new asperterpenoids (**245–254**) (Fig. 41), possessing a rare 5/7/3/6/5 skeleton, were obtained from two *Aspergillus oryzae* transformants with heterologous expression of a terpene cyclase gene (*AstC*) and one or two P450 genes (AstB/A) under molecular networking guidance (Huang et al. 2019; Yang et al. 2022). Compounds **245–247** exhibited moderate to strong antimalarial activity against the chloroquine-sensitive *P. falciparum* strain 3D7, with EC_{50} values ranging from 2.1 to 19.3 μM. Five new indole diterpenoids (**255–259**) (Fig. 41) were activated by introducing the exogenous P450 gene (*AstB*) in *Aspergillus oryzae* transformants, also under molecular networking guidance (Yang et al. 2023).

Metabolic shunting + OSMAC

The endophytic fungus *Penicillium dangeardii*, isolated from the toxic plant *Lysidice rhodostegia*, predominantly produces rubratoxins under varied culture conditions, indicating that most other gene clusters are silent (Wei et al. 2021). Despite attempts to enhance rubratoxin production through epigenetic regulation and the OSMAC approach, high levels of rubratoxins were maintained. However, applying a metabolic shunting strategy—deleting the key gene *rbtJ* encoding the PKS responsible for rubratoxin biosynthesis—successfully activated multiple silent gene clusters, leading to the identification of 23 new compounds (**260–282**) (Fig. 42), including azaphilone monomers (**260–265**), glycosides (**266–267**), dimers (**268–275**), trimers (**276–280**), and two unclassified polyketides (**281–282**) (Wei et al. 2021).

Discussion

These cases demonstrate that combinatorial strategies not only improve the efficiency of discovering novel fungal natural products but also effectively minimize the redundant isolation of known compounds. However, several challenges remain. First, the activation of silent BGCs is inherently unpredictable—while genome mining can identify potential BGCs, their successful expression is not guaranteed. Second, the regulatory mechanisms governing fungal secondary metabolism are highly intricate. Third, dereplication strategies are still constrained by the limited coverage of current databases and the risk of misidentification due to signal overlap (Meunier et al. 2024). Fourth, Heterologous expression remains challenging, as fungal BGCs are typically large, making direct cloning and expression in heterologous hosts (e.g., *E. coli*, *S. cerevisiae*) difficult. Key obstacles include promoter incompatibility, differences in post-translational modifications, and potential toxicity of the expressed products (Keller 2019). Finally, integrating and interpreting multi-omics data remains a significant analytical challenge.

Conclusion and future perspectives

This review highlights the workflow and recent advancements in mining new natural products from fungi, reporting a total of 282 novel fungal secondary metabolites. Although traditional strategies are time-consuming, labor-intensive, and yield unpredictable results due to the complexity of factors influencing gene expression, they remain straightforward to implement, cost-effective, and broadly applicable. As such, these methods are promising for studying fungi whose genomes are not yet sequenced or fully characterized. Gene mining strategies, on the other hand, require genetic mani-

pulation of the target fungi and demand substantial research funding. However, these approaches are highly targeted, yield significant returns, and represent an inevitable trend in the future development of natural product discovery.

Fungi have a long history of use in medicine. With the rapid advances in bioinformatics and sequencing technologies, a vast number of uncharacterized BGCs in fungi have been identified, indicating that fungi's full potential in producing novel natural products remains untapped. The development of BGC prediction tools, such as antiSMASH (Blin et al. 2023), Cluster ASSignment by Islands of Sites (CASSIS) (Lakhani et al. 2020), and Prediction Informatics for Secondary Metabolomes (PRISM) (Skinnider et al. 2020), has made significant contributions to fungal gene mining. These innovative bioinformatics tools provide more accurate predictions and reliable datasets for identifying BGCs.

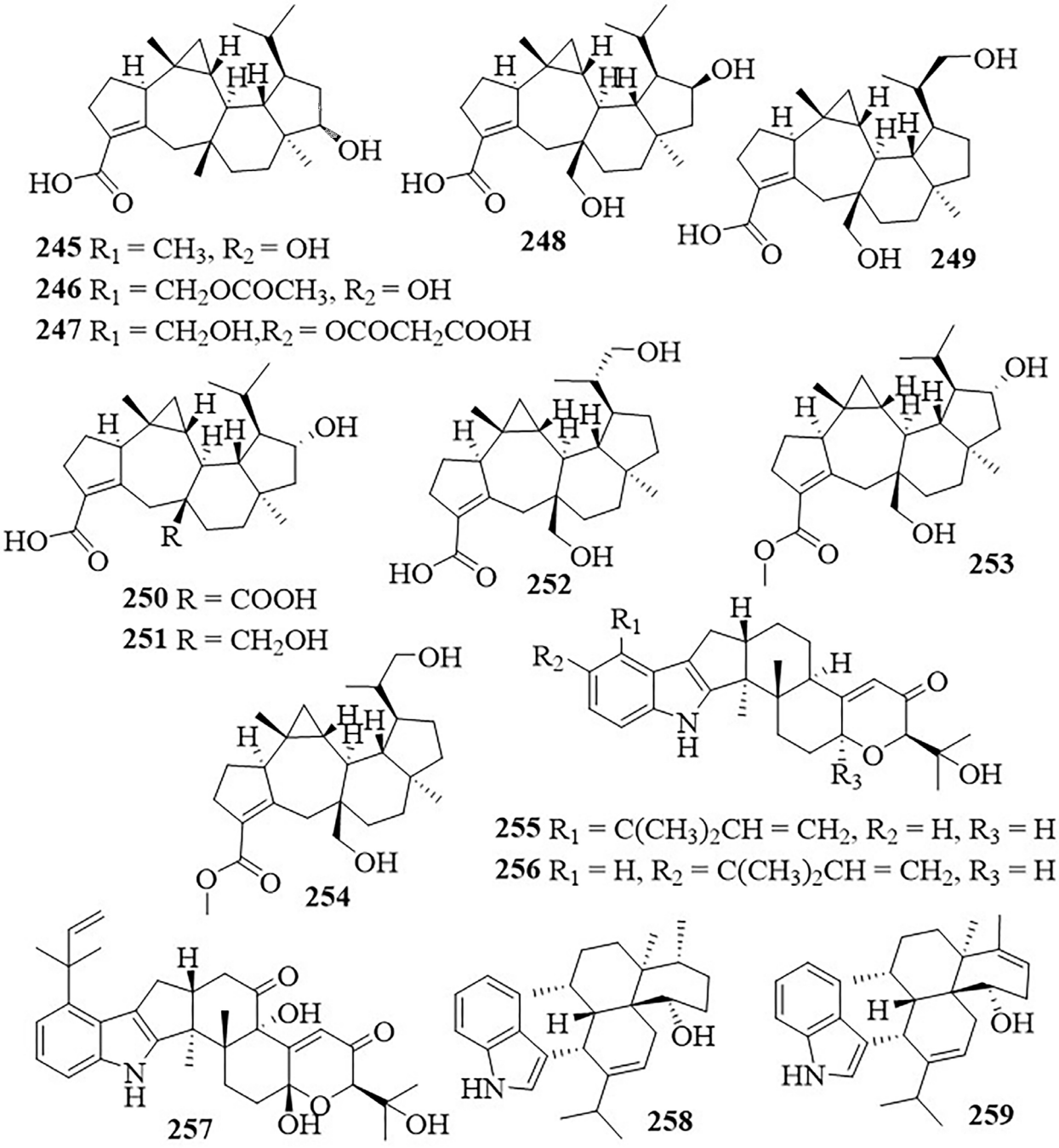

Fig. 41 New natural products discovered based on GNPS and heterologous expression strategies.

Whether using traditional approaches or gene mining strategies to obtain fungal natural products, the process invariably involves fermenting the strain, extracting metabolites, and performing separation and purification steps. This, in turn, inevitably leads to the challenge of redundant separation of known compounds. Meanwhile, dereplication strategies are advancing at an unprecedented pace. While foundational data acquisition still relies on NMR and LC-MS/MS, synthetic biology is rapidly transforming natural product production. In the future, enzyme-catalyzed synthesis of pseudo-natural products is expected to significantly broaden the chemical space (Vinogradov et al. 2022; Ye &

Zhang 2023). The integration of multiple disciplines, such as computer science, bioinformatics, and metabolomics, is propelling the field of natural product discovery to new heights. Notably, the development of artificial intelligence (AI) is accelerating and refining the application of these strategies (Sahayasheela et al. 2022). Recent research from the teams of Wenbing Yin and Chunxiong Luo has demonstrated the power of combining advanced microfluidic platforms with mathematical modeling (Xu et al. 2024). This integration has enabled the quantitative characterization of *Aspergillus nidulans* gene regulatory circuits and facilitated the precise production of novel bioactive natural products (Xu et al. 2024). As a vast reservoir of natural products, fungi represent an invaluable treasure trove with immense potential for future development.

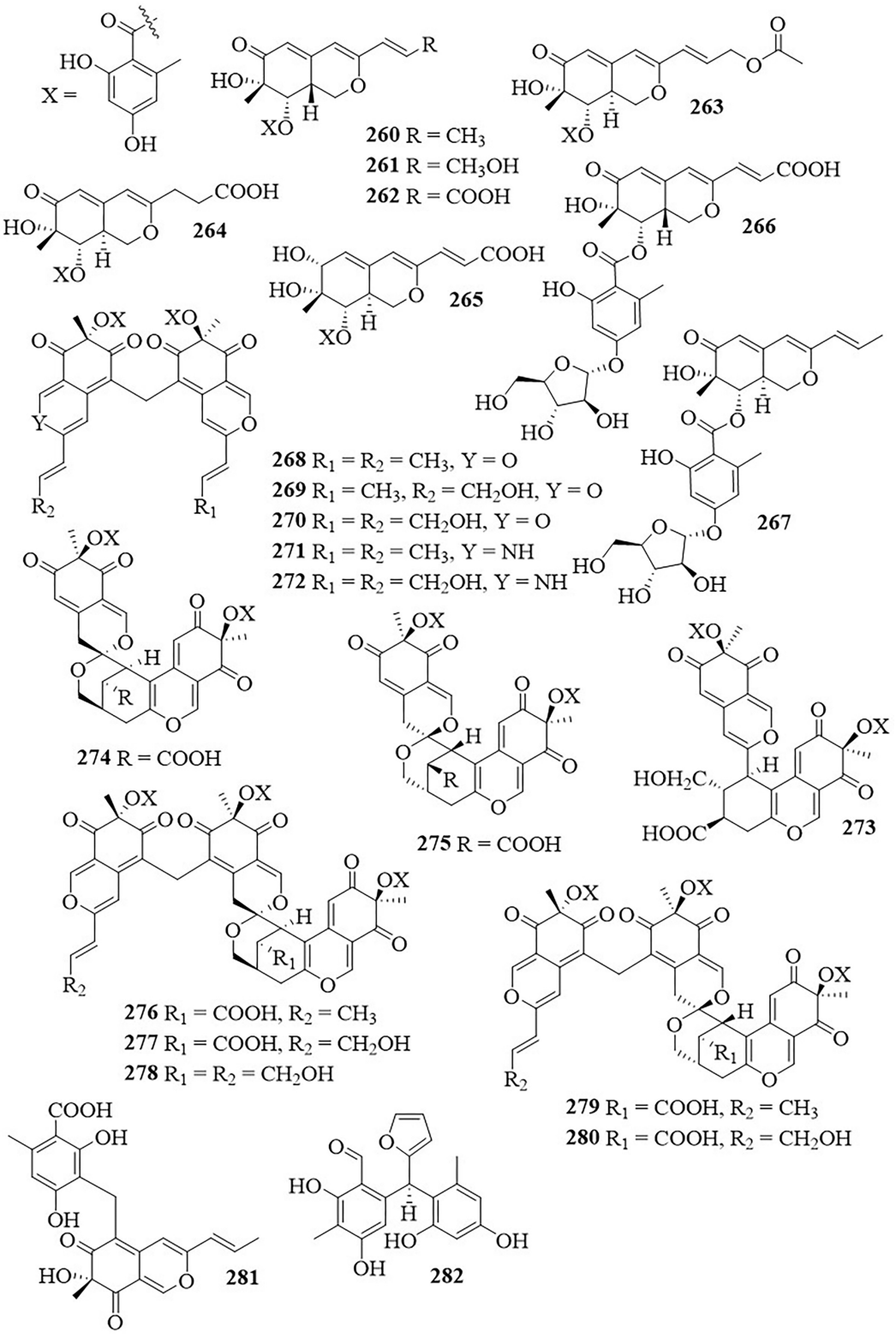

Fig. 42 New natural products discovered based on genome mining, combined with metabolic shunting and OSMAC strategy.

Acknowledgments

This work was financed by the National Natural Science Foundation of China (NSFC 32360011) and Guizhou Institute of Technology High-Level Talent Research Start-up Project (2023GCC069). K.D. Hyde was funded by the Distinguished Scientist Fellowship Program (DSFP), King Saud University, Kingdom of Saudi Arabia.

Author contributions

Conceptualization: Zhang LJ, Lu YZ, Sun JZ and Hyde KD; methodology: Zhang LJ, Jayawardena RS and Ausana M; formal analysis: Zhang LJ, Ma J and Xiao XJ; writing—original draft preparation: Zhang LJ, Jayawardena RS and Ausana M; writing—review & editing: Liu NG, Lu YZ, Sun JZ, Hyde KD, Xiao XJ, Xiao YP and Arttapon W; supervision: Lu YZ, Sun JZ and Hyde KD; project administration: Lu YZ and Sun JZ; funding acquisition: Lu YZ. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest statement

The author list includes members of the Editorial Board of PhytoMycology. They were not involved in the journal's review of, or decisions related to, this manuscript. The authors declare no competing interests.

Supplementary Information

The online version contains supplemental information available at https://doi.org/10.65390/phytomyc.2026.2003

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