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Beyond ergosterol: Strategies for combatting antifungal resistance in *Aspergillus fumigatus* and *Candida auris*

Ricardo Cruz, William M. Wuest*

Department of Chemistry, Emory University, 1515 Dickey Dr, Atlanta, GA, 30322, USA

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B S T R C T

Aspergillus fumigatus and *Candida auris* are historically problematic fungal pathogens responsible for systemic infections and high mortality rates, especially in immunocompromised populations. The three antifungal classes that comprise our present day armamentarium have facilitated ef cacious treatment of these fungal infections in past decades, but their potency has steadily declined over the years as resistance to these compounds has accumulated. Importantly, pan-resistant strains of *Candida auris* have been observed in clinical settings, leaving affected patients with no treatment options and a death sentence. Many compounds in the ongoing antifungal drug discovery pipeline, similar to those within our aforementioned trinity, are predicated on the binding or inhibition of ergosterol. Recurring accounts of resistance to antifungals targeting this pathway suggest optimization of ergosterol-dependent antifungals is likely not the best solution for the long-term. This review aims to present several natural products with novel or underexplored biological targets, as well as similarly underutilized drug discovery strategies to inspire future biological investigations and medicinal chemistry campaigns.

1 Introduction

The golden age of antimicrobials has reaped a bleak present and future as antimicrobial resistance (AMR) continues to challenge treatment options in all settings. In 2014, AMR was linked to at least 700,000 deaths worldwide per year with a strong likelihood of reaching well over 10 million by 2050 if current trends persist [1]. As of 2019, the Centers for Disease Control and Prevention (CDC) reports nearly 3 million infection events and 36,000 resultant deaths in the United States attributable to AMR in bacteria and fungi, suggesting our current efforts in antimicrobial development are insuf cient [2]. Although bacterial infections contribute to the bulk of these gures, it is paramount to consider the situation beyond the United States as, worldwide, over 150 million fungal infections and 1.7 million annual deaths were reported in 2017 alone [3].

The development of AMR is one of the greatest challenges in the drug discovery effort. Pathogens employ a variety of mechanisms that ultimately increase the minimum inhibitory concentration (MIC) of a given antimicrobial. Following entry into the cell, the drug can be modi ed or degraded by enzymatic action, as seen in the action of β-lactamases against penicillin [4]. Alternatively, rather than targeting the drug, modi cation of the drug target through mutation can lead to decreased drug af nity and consequent loss of activity. Overexpression of the drug target demands higher drug quantities to achieve the same inhibitory effect, and overexpression of efflux pumps shuttle drugs out of the cell. Another key defensive measure used by microbes is the formation of bio lm – an amalgamation of extracellular polymeric substances such as polysaccharides, proteins, and nucleic acids [5]. Bio lms aid in cellular adhesion and denying intracellular access, signi cantly increasing pathogenicity.

Both the CDC and the World Health Organization (WHO) have recently identi ed the human fungal pathogens *Aspergillus fumigatus* and *Candida auris* as high priority health threats based on several key variables, such as global mortality rates, diagnostic availability, and reports of antifungal resistance [6]. To demonstrate the ubiquity of *A. fumigatus*, it is estimated that the average person inhales several hundred of this species conidial spores daily [7]. Fortunately, the human immune system can ef ciently clear these antigens, with symptoms manifesting as mild allergies [8]. Previously, the immunocompromised were most at-risk for developing

Dedicated to Prof. John Wood for his steadfast dedication to the art of natural product total synthesis and the mentorship of the next generation organic chemists.

* Corresponding author.
E-mail address: wwuest@emory.edu (W.M. Wuest).

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invasive aspergillosis, where infection was accompanied with an alarmingly high mortality rate of 80–90 in leukemia patients [9]. However, the recent increase in immunosuppressive therapies has left a larger portion of the population vulnerable to infection, making *A. fumigatus* the most common airborne fungal pathogen [10]. Agricultural overuse and environmental spillage of antifungal agents has caused widespread resistance to common antifungals to develop across the majority of strains, making treatment of *A. fumigatus* infections more dif cult relative to other fungal pathogens [11].

The fungal yeasts *Candida albicans* and *Candida glabrata* are the most common cause of vaginal candidiasis, otherwise known as yeast infection. Candidiasis rarely progresses in severity as commercially available treatments remain ef cacious against the aforementioned major causes of candidiasis, with few reports of signi cant resistance development. Although relatively much rarer, candidiasis brought on by *C. auris* has the potential to be insurmountably problematic. Despite being a rarity in the time following its initial identi cation in Japan in 2009, *C. auris* has become far more prevalent following a 318 increase in cases reported in 2018 alone [12]. With some isolated strains of *C. auris* having been found to be resistant to all classes of available commercial antifungals, the necessity for new antifungals has never been clearer [13].

2 Current antifungals and resistance mechanisms

Compared to antibacterial drug development, antifungal development is accompanied by some additional challenges. As fungi are eukaryotic, proposed compounds must be capable of selectively inhibiting fungal machinery over those of humans, a consideration that does not have to be made in drug design targeting prokaryotes. For the past several decades, the fungal sterol ergosterol has been leveraged to access this desired selectivity. Ergosterol is important for cellular membrane structure and causes defects in membrane integrity in its absence, leading to eventual cell death [14].

There are currently only three major antifungal compound classes for invasive fungal infections, and two of them are dependent on ergosterol in some manner for their desired activity (Fig. 1). Polyenes were the rst of these major classes to be developed and are large, glycosylated macrocycles with highly unsaturated alkyl regions. The rst polyene, nystatin (**1**), was isolated from *Streptomyces noursei* in 1950 and briefly used in clinics prior to the adoption of amphotericin B (AmB, **2**) by 1958 [15]. AmB and other polyenes cause fungal cell death by binding to ergosterol and subsequently disrupting the cellular membrane through intercalation of the polyene region [16]. Expectedly, mutations in ERG3 that slightly alter sterol structure confers resistance to AmB by reducing membrane binding af nity [17]. Unfortunately, approximately 66 of *C. auris* isolates collected in the United States were found to be resistant to AmB treatment [18]. On the other hand, AmB resistance in *Aspergillus* species outside of *A. terreus* is exceedingly rare, thus AmB is the rst-line treatment option for patients suffering from aspergillosis [19]. In spite of this, widespread use of AmB is precluded by its low oral bioavailability restricting administration to intravenous delivery, as well as severe dose-dependent toxicities [20]. Recent efforts to modify AmB formulations to minimize these caveats have been met with preliminary success, suggesting that there is still room for optimization of this decades-old drug [21].

The next antifungal class to be developed are the azoles, beginning with the synthetic lead compound ketoconazole (**3**) in 1981 [22]. Celebrated as a broad-spectrum antifungal, the imidazole and piperazine moieties of ketoconazole were soon simpli ed to the achiral triazole fluconazole (**4**), the current azole antifungal standard, in 1985 [23]. Several fluconazole derivatives and optimizations would be developed over the following years, but their mechanism of action remained consistent – inhibition of cytochrome P450 enzyme 51 (CYP51), the enzyme responsible for demethylation of lanosterol in the synthesis towards ergosterol, leads to cytotoxic depletion of ergosterol [24]. Resistance to azoles was observed as early as 1989 in *Candida krusei*, where overexpression and mutation of ERG11 decreases azole binding ef cacy [25]. Overexpression of the efflux-related genes *MDR1*, *FLU1*, *CDR1*, and CDR2 has also been found to be a major contributor to azole resistance [26]. Due to environmental and clinical overuse, fluconazole is almost completely ineffective against a number of fungal pathogens. Approximately 93 of *C. auris* isolates studied are resistant to fluconazole, while the gure for *A. fumigatus* approaches 100 [11]. Although there have been recent attempts at developing novel azole antifungals to rescue their bioactivity against these resistant pathogens, the consequences of our misuse will likely forever limit azole ef cacy and force us to explore other pharmacophores.

The echinocandins ended a 15-year drought in antifungal development following the introduction of caspofungin (**5**) in the early 2000s, previously isolated from the fungus *Glarea lozoyensis* in the late 1980s [27]. Making a notable departure from the dependence on previously discussed for polyenes and azoles, echinocandins act as non-competitive inhibitors of β-1,3-glucan synthase. This enzyme is responsible for the production of β-1,3-glucan, an integral component of the fungal cellular wall. Resistance to the echinocandins in *C. auris* has been found to originate from mutations in FKS1, altering the drug binding site at a small tness cost to the pathogen [28]. Interestingly, benzylic dehydroxylation has been found to restore echinocandin activity against mutant glucan synthases [29]. Nevertheless, only about 7 of *C. auris* isolates studied were found to carry this resistance, and thus the echinocandins are the rst-line treatment option against *C. auris* infection [30]. However, approximately 1 of *C. auris* isolates are resistant to echinocandins, AmB, and fluconazole, leaving us with no treatment options for a small percentage of pan-resistant *C. auris* that will likely to increase over time as more echinocandin regimens are made necessary. The echinocandins have some notable advantages over other antifungal classes – low toxicity and few drug-drug interactions make the very act of treatment less problematic, as seen in the recently developed echinocandin rezafungin (**6**) [31]. One major limitation of the echinocandins is poor oral bioavailability, making intravenous administration necessary – similar to AmB, recent efforts at improving echinocandin bioavailability stand to make signi cant improvements to these decades-old compounds.

Although rarely administered as a monotherapy for invasive fungal infections, 5-fluorocytosine (5-FC, **7**) is sometimes employed in combination with the above antifungal classes. Introduced in 1968, 5-FC is an orally available prodrug that is metabolized *in vivo* to both fluorouridine triphosphate (**8**), a protein synthesis inhibitor mimicking uridylic acid, and 5-fluorodeoxyuridine monophosphate (**9**), a strong inhibitor of thymidylate synthase [32]. 5-FC is one of the few antifungals whose ef cacy does not entirely depend on ergosterol, but in order to compensate for the poor selectivity it offers, it is usually co-administered with AmB to ease entry into fungal cells over human cells. Mutations in metabolic and transporter enzymes, as well as upregulation of native pyrimidines, confers resistance to 5-FC in *Candida* species [33]. Furthermore, the prohibitive cost of 5-FC treatments has also contributed to the lack of widespread adoption of this particular therapeutic [34].

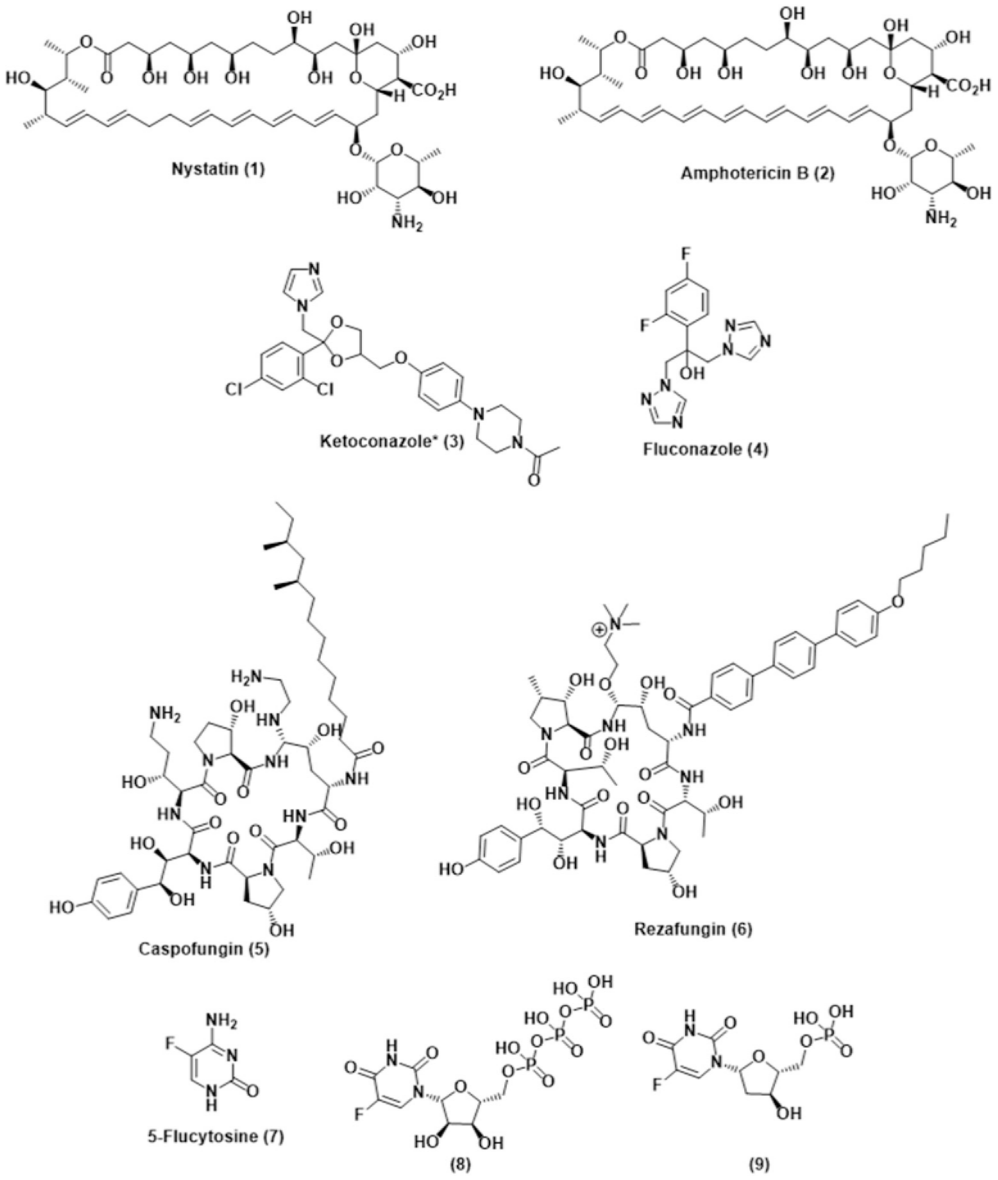

Fig 1 Representative compounds from our current armamentarium. *Ketoconazole is typically prepared and administered as a racemic mixture.

3 Recent efforts

Although our current antifungal armament is largely based on the foundations laid several decades ago, the optimization of these foundations has continued well into the present (Fig. 2). Treatment of recurrent candidiasis with fluconazole has been met with widespread resistance development and off-target toxicities exacerbated by repeated use. Oteseconazole (**10**), sold under the brand name Vivjoa, is a tetrazole-containing optimization of the azole scaffold that has been developed to address these concerns [35]. Although the mechanism of action remains consistent with other azoles, up to a 40-fold increase in potency has been observed against *Candida* species, including *C. auris*, relative to fluconazole, suggesting tighter binding or the presence of a distinct binding site. This is further supported by the 2200-fold selectivity observed for fungal CYP51 inhibition over the human homolog [36]. The related triazole opelconazole (**11**) was similarly developed as a solution for recurrent pulmonary aspergillosis [37]. Delivery through inhalation of aerosolized opelconazole yields high concentrations of the therapeutic in the lungs while maintaining low systemic concentrations, facilitating inhibition of azole-resistant *Aspergillus* residing in pulmonary cavities. Although these new additions to the azole antifungal family ll important clinical niches, continuing to target CYP51, a known locus for resistance development, stands to limit the long-term ef cacy of these new drugs.

Similar to other echinocandins, ibrexafungerp (**12**), sold under the name Brexafemme, affects fungal cell death through the inhibition of β-1,3-glucan synthase [38]. In contrast to previous echinocandins, however, optimization of the echinocandin scaffold signi cantly increases oral bioavailability and affords good activity against azole-resistant *Candida* and *Aspergillus* species [39]. Interestingly, this activity was conserved against echinocandin-resistant strains as ibrexafungerp interacts differently than other echinocandins.

Rather than investing resources into continually soon-to-be-obsolete antifungals from the 1980s, the development of new drugs with novel targets, good fungal selectivity, and potent activity against clinically relevant *Candida* and *Aspergillus* species is

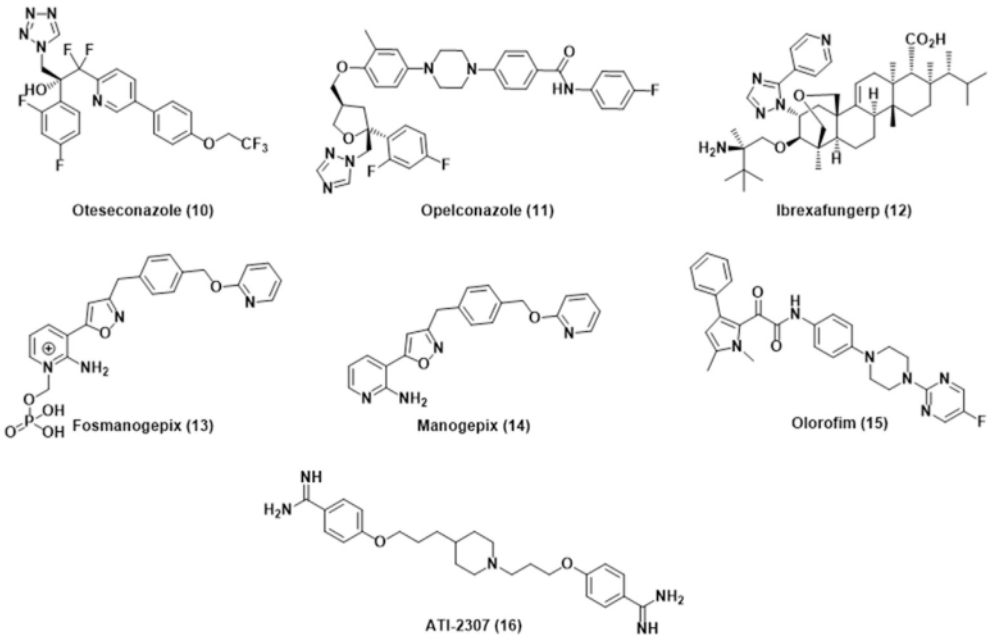

Fig 2 Antifungals recently approved for use or currently in clinical trials.

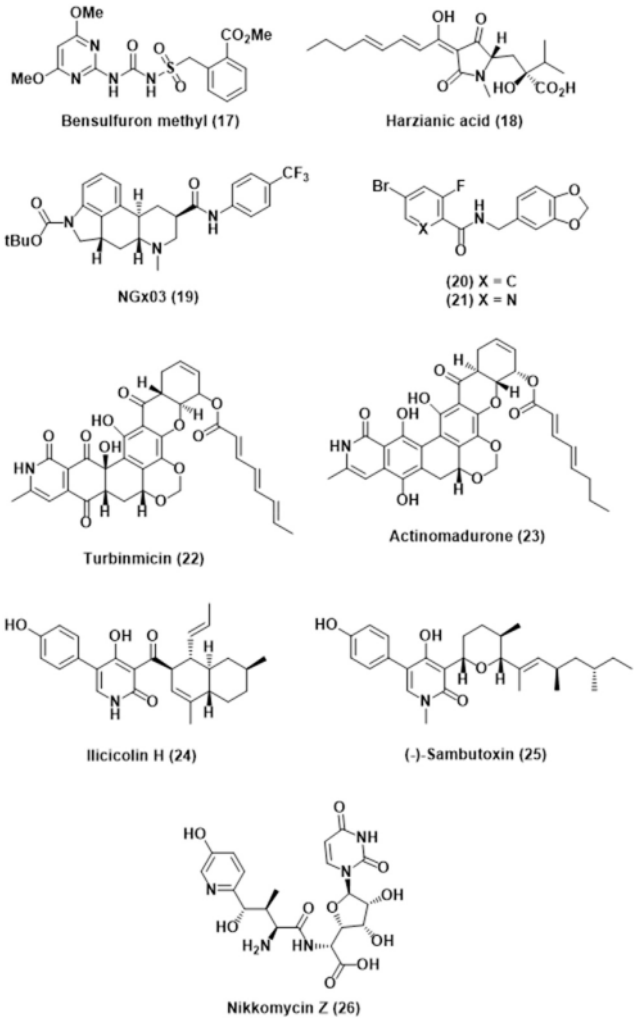

Fig 3 Compounds targeting novel or understudied fungal machinery.

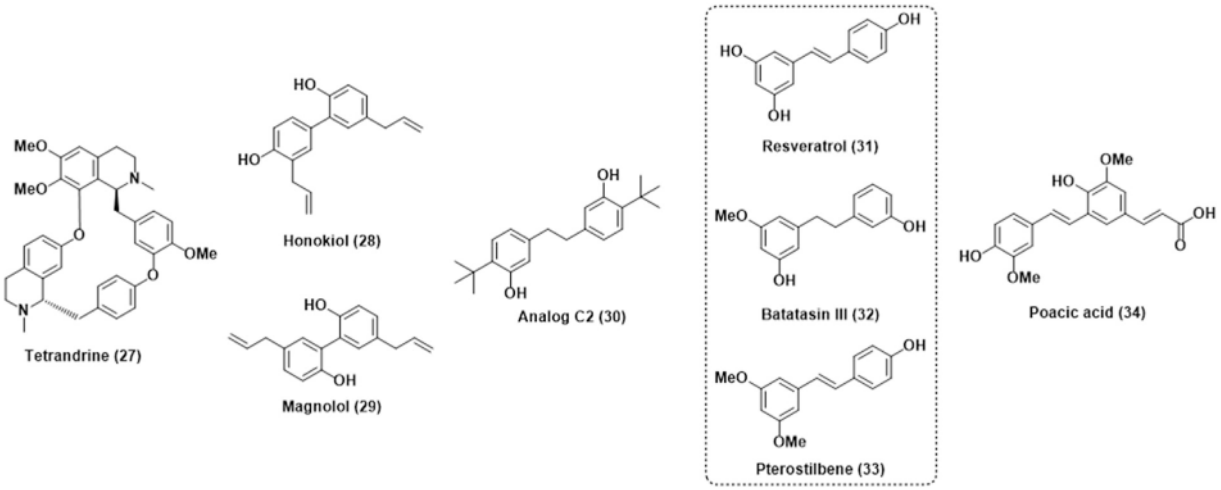

Fig 4 Anti-virulence antifungals and related stilbenoid natural products.

more likely to overcome antifungal resistance. Fortunately, several such antifungals have recently entered clinical studies. Similar to 5-FC, the prodrug fosmanogepix (**13**) is metabolized to the active antifungal manogepix (**14**) that then inhibits Gwt1, a vital protein involved in the transportation and anchoring of mannoproteins [40]. The resulting inhibition of several virulence factors, such as bio lm formation and adhesion, was observed in *C. auris* [41]. As expected, point mutation of the enzymatic target and over-expression of the MDR1 superfamily transporter gene conferred resistance in *Candida* by decreasing binding af nity and increasing efflux, respectively [42,43].

Oloro m (**15**) similarly evades ergosterol dependence and represents the rst advancement towards the development of a new antifungal class, the orotomides. The orotomides inhibit dihydroorotate dehydrogenase, a vital enzyme implicated in pyrimidine synthesis, preventing DNA and glucan synthesis [44]. Oloro m is ineffective against the Class 2 DHODH isoforms observed in *Candida*, *Cryptococcus*, and humans, limiting the scope of activity to *Aspergillus* species for now. Otherwise, mutations within DHODH confer resistance to oloro m in *A. fumigatus* at a signi cant tness cost to the pathogen [45]. The diamidine ATI-2307 (**16**) selectively inhibits fungal mitochondrial complexes III and IV, leading to membrane potential collapse [46]. Low ng/mL MICs were reported against several *Candida* and *Cryptococcus* yeasts, while activity was signi cantly lower yet comparable to azole and echinocandin controls in lamentous *A. fumigatus* [47].

4 Antifungal natural products

Nature has long been an abundant source of bioactive compounds and has provided us with the inspiration and innovation needed to engineer new antifungals. As stated previously, these new antifungals should ideally be both potent and selective inhibitors of fungal machinery without depending on ergosterol. There are several strategies antifungal candidates can employ to achieve this – for example, inhibition of novel enzymatic targets, as seen with the orotomides, stands to introduce wholly new drug classes and signi cantly bolster current treatment options (Fig. 3). Another potential antifungal development strategy involves abandoning the traditional desire for fungicidality and instead opting for an anti-virulence approach (Fig. 4). Several *Candida* species, including *C. auris*, have been observed to be dimorphic and can alternate between a commensal yeast form and a pathogenic hyphal form in response to environmental stimuli. Although discovered compounds are rarely tested against *C. auris*, results attained by testing against *C. albicans* will be cautiously employed as a proxy on account of their homology [48]. The absence of biological data against *C. auris* is another major contributor to the current fungal crisis, but inhibitors of other *Candida* species stand to be effective against this deadly pathogen.

Acetohydroxyacid synthase (AHAS) is the rst enzyme within branched chain amino acid synthesis [49]. The biological importance of this enzyme, as well as its absence in animals, has previously been leveraged in the design of AHAS-inhibiting herbicides developed in the late 1980s [50]. In addition to their original indication, several of these compounds were recently found to be similarly active against fungal AHAS, inhibiting the growth of several *Candida* species [51]. A follow-up study then investigated these herbicides for activity against *C. auris* in particular, and the sulfonylurea herbicide bensulfuron methyl (**17**) was found to potently inhibit wild-type *C. auris* with an MIC of 0.391 μM [52]. The herbicide also retained good activity when tested against an azole- and AmB-resistant strain while maintaining a therapeutic index greater than 100. Point mutations of a key proline residue within AHAS was found to confer resistance to these commercial herbicides. Produced by *Trichoderma afroharzianum*, the known siderophore harzianic acid (HA, **18**) was later found to similarly inhibit wild-type AHAS while also retaining activity against AHAS mutants [53]. Structural analysis of an AHAS-HA complex revealed that HA binds differently to AHAS than any other commercial herbicide, explaining the retention of activity. Therapeutic parameters of HA beyond fungal activity remain to be investigated, but these preliminary results suggest HA has the potential to develop into a promising candidate.

The phosphatidylinositol and phosphatidylcholine transfer protein Sec14p is exclusive to yeasts and implicated in several cellular processes critical to growth and virulence, such as fatty acid synthesis and hyphal transition, respectively [54]. A high-throughput screen searching for activity against Sec14p using chemogenomic pro ling found that the ergoline NGx03 (**19**) inhibited Sec14p in fluconazole-resistant *Cryptococcus neoformans* with an IC_{50} of 16 μg/mL through binding competition with native substrates [55]. A subsequent study similarly investigated a library of picolinamide and benzamide compounds using the same pro ling methodology and ultimately identi ed the benzamide **20**, as well as its picolinamide analog **21**, as inhibitors of Sec14p [56]. Unfortunately, potent inhibition of Sec14p did not translate to potent activity against several fluconazole-resistant fungal strains

as the alternative azole posaconazole vastly outclassed compounds **20** and **21**. Although resistance to Sec14p inhibitors can be conferred by Sec14p mutation and one of seven possible genetic loss-of-function events, these changes have nevertheless been found to negatively impact lipid metabolism and generally decrease cellular tness.

Recently, the marine natural product turbinmicin (**22**) was isolated from *Micromonospora* bacteria contributing to a sea squirt microbiome [57]. Structurally similar polyketides such as actinomadurone (**23**) have previously been shown to have fungal activity but no mechanism of action had been elucidated [58]. In accordance with these ndings, turbinmicin notably possessed an MIC of 0.25 μg/mL against pan-resistant *C. auris*, suggesting a mechanism of action unique from current antifungals. Indeed, treatment of a knockout library with turbinmicin revealed the selective inhibition of Sec14p as the cause of fungicidal activity. A subsequent investigation studied the effects of turbinmicin on *Candida* bio lm and found that in vitro bio lm inhibition was observed at concentrations as low as 0.125 μg/mL in *C. auris* [59]. Synergy between fluconazole and turbinmicin was also observed in murine models, further supporting the claim of bio lm inhibition. These ndings are consistent with established outcomes for Sec14p inhibition as hyphal transition is a prerequisite for bio lm formation in *Candida*. Although Sec14p has been validated time and time again as a rational target for antifungal drug design, no such inhibitors have been approved for clinical use nor extensively studied in biological systems. Fortunately, ongoing studies with turbinmicin may eventually culminate in its introduction to clinics if fungal activity and human cell toxicity continues to impress.

Although the pyridone scaffold has been extensively explored across several different medical indications, the 4-hydroxy-2-pyridone subfamily has remained largely biologically unexplored [60]. Ilicicolin H (**24**) was originally isolated from the plant pathogen *Cylindrocladium ilicicola* and found to be the least cytotoxic towards mammalian cells among the ilicicolin co-isolates [61]. The antifungal ef cacy of ilicicolin H was soon investigated and found to have potent activity against both *C. albicans* (MIC 0.04–0.31 μg/mL) and *A. fumigatus* (MIC 0.08 μg/mL). Another similarly cytotoxic and structurally related *Fusarium* isolate (−)-sambutoxin (**25**) was also found to have potent antifungal activity [62]. Studies investigating the mechanism of action of these pyridones determined the target to be the Q_n site of mitochondrial cytochrome bc_1 reductase, compromising electron transport and rationalizing the observed potent cytotoxicity [63]. A subsequent structure-activity relationship (SAR) campaign of ilicicolin H corroborated these ndings and concluded that the 5-aryl-4-hydroxy-2-pyridone and β-diketone moieties are responsible for the observed selective inhibition of fungal cytochrome at sub-μg/mL quantities [64]. Unfortunately, these essential moieties also conferred high plasma protein binding that signi cantly decreased the potency and bioavailability of ilicicolin H *in vivo*, blockading further progression into clinical trials. Additional research towards the minimization of these compounds plasma protein af nity could promote them to drug candidacy, especially as several other 4-hydroxy-2-pyridones remain to be extensively studied in biological systems.

The polyoxin nucleoside antibiotic nikkomycin Z (**26**) was originally isolated from the soil bacterium *Streptomyces tendae* in the late 1970s, and later found to inhibit growth of clinically relevant *Coccidioides* fungal species [65]. The mechanism of action of nikkomycin Z against fungi was determined to be competitive inhibition of chitin synthase through mimicking of the native substrate, uridine diphosphate *N*-acetylglucosamine. Chitin is a polymeric accumulation of *N*-acetylglucosamine present in the vast majority of fungi and is integral to the stability of the fungal cell wall [66]. As a result, inhibition of chitin synthase by nikkomycin Z directly affects cellular integrity. The mechanistic novelty and fungal selectivity of nikkomycin Z have inspired further studies into its activity beyond *Coccidioides*. Synergy with the azole antifungals fluconazole and itraconazole was observed across a panel of fungal strains, particularly against *A. fumigatus* [67]. Recently, nikkomycin Z activity has also been studied against *C. auris*, yielding promising results as inhibitory concentrations are similarly low to those necessary to affect inhibition in *C. albicans* [68]. Notably, some of the *C. auris* strains were particularly resistant to treatment with nikkomycin Z, but the underlying resistance mechanism responsible has yet to be determined.

Although the discovery and development of fungicidal compounds dominate antifungal drug discovery efforts, resistance selection is greatest when the survival of the pathogen is at stake. Recently, a greater abundance of therapeutic strategies seeking to inhibit virulence factors like hyphal transition and bio lm formation have emerged (Fig. 4) [69]. Since inhibition of virulence factors tends to not directly endanger the fungus itself, there is less selective pressure driving resistance development, signi cantly boosting the longevity of these proposed compounds. Antivirulence treatments also avoid the adverse effects of disturbing the commensal microbiome, yet another advantage against traditional growth inhibition or cytotoxic approaches.

The traditional Chinese medicine tetrandrine (TET, **27**) was originally isolated from the roots of *Stephania tetrandra* and used to treat inflammation [70]. TET was found to synergize with azole antifungals like fluconazole and ketoconazole against azole-resistant *C. albicans*, suggesting that it is interfering with efflux-dependent resistance mechanisms and restoring the ef cacy of the azoles [71,72]. Indeed, RT-PCR analysis con rmed that TET affects genes coding for vital efflux machinery, including ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters [73]. A subsequent study found that TET also inhibits *C. albicans* hyphal transition through the downregulation of several hyphal-speci c genes vital to the Ras-cAMP-PKA pathway, a biochemical signaling pathway necessary for several key cellular processes, including morphological transition from yeast to hyphae [74]. Inhibition of hyphal transition also rationalizes the *C. albicans* bio lm inhibition that has also been observed following treatment with TET.

The phenolic natural products honokiol (**28**) and magnolol (**29**) have been isolated from the barks of several *Magnolia* species and found to have broad antibacterial and antifungal properties, most notably against bio lm formation in *Streptococcus mutans* and *C. albicans* [75–77]. The promising native activities of these compounds against the bacterium *S. mutans* originally inspired our group to develop honokiol analogs in an attempt to optimize the scaffold for the treatment of dental caries [78,79]. Our SAR campaign ultimately led us to Analog C2 (**30**) as the most potent *S. mutans* bio lm inhibitor within the series. The modi ed dihydrostilbene core of Analog C2 makes it more similar in structure to other stilbenoid natural products such as batatasin III (**31**), resveratrol (**32**), and pterostilbene (**33**). Pterostilbene is a known inhibitor of bio lm formation in *C. albicans*, and a recent SAR study found that reduction to the dihydrostilbene analog of pterostilbene mostly retained its potency against nascent bio lm, only suffering a 2-fold decrease in activity [80,81]. These ndings and the structural relationships between Analog C2 and the hydrogenated analog of pterostilbene suggest further structural optimization of a dihydrostilbene scaffold could pivot from known antibacterial activity and towards potent bio lm inhibition in fungal pathogens.

The stilbenoid poacic acid (**34**), a dehydrodiferulate-derived natural product isolated from the hydrolysate of *Poaceae* grasses, has also recently been found to have antifungal activity [82]. Rather

than exhibiting anti-virulence activity, however, chemical genomics and morphological analysis showed that poacic acid damaged fungal cell walls and caused detrimental cellular leakage, similar to echinocandins. By leveraging the intrinsic fluorescent capabilities of the natural product it was then determined that poacic acid causes cell wall damage by directly binding to surface β-1,3-glucan rather than inhibiting β-1,3-glucan synthase. Promising activity was reported against several agriculturally relevant fungal plant pathogens, and ef cacy against several *Candida* species was later determined in a follow-up study [83]. This study interestingly found that wild-type *C. albicans* was signi cantly more resistant to poacic acid treatment compared to *S. cerevisiae*, yet caspofungin-resistant mutant strains of *C. albicans* were more sensitive. This further supported the mechanistic differentiation between poacic acid and echinocandins and demonstrated the lack of overlap between the two compound classes despite both leveraging the biological importance of β-1,3-glucan. Unfortunately, wide variability in fungicidality against a panel of clinically relevant fungal pathogens, including *C. auris*, have precluded further development of poacic acid into a next-generation commercial antifungal.

Although traditional natural product isolation, characterization, and biological investigation efforts have forged the foundation of modern antimicrobial therapies, debilitating limitations to the methodology have signi cantly slowed drug discovery efforts in recent decades. As more and more natural reservoirs and their bioactive metabolites have been investigated and archived in the literature, discovery of novel natural products with relevant antimicrobial activity has dramatically increased in dif culty since unproductive re-isolation of known compounds is far more likely. The development and implementation of new methodologies to expedite drug discovery and circumvent re-isolation is paramount if we wish to abolish the current stagnation.

Candida albicans tness test

The concept of leveraging the eukaryotic phenomenon of haploinsuf ciency in drug discovery was rst introduced over two decades ago yet remains thoroughly underutilized in the search for novel antifungals [84]. This genomic pro ling methodology is based upon the treatment of a library of heterozygous deletion strains with a choice compound. Retention of one functional gene is most often suf cient for the organism to develop without phenotypic abnormalities. However, these deletion mutants are consequently hypersensitive to stressors targeting the deleted locus as there is no compensatory duplicate to aid in resistance efforts. Screening bioactive extracts or pure compound against these deletion libraries quickly elucidates the mechanism of action based on these observed hypersensitivities and enables identi cation of bioactive extract components with novel chemical structures based on similarly novel bioactivity, doing away with re-isolation complications. This system was originally developed in the model yeast *S. cerevisiae* but has recently been adapted to the more clinically relevant fungal pathogen *Candida albicans* [85,86]. The aforementioned *Candida albicans* tness test (*Ca*FT) has been used to simultaneously identify bioactive compounds in crude extracts and their biological targets in a handful of examples within recent literature (Fig. 5).

The isoxazolidinones parnafungin A (**35**) and B (**36**) were

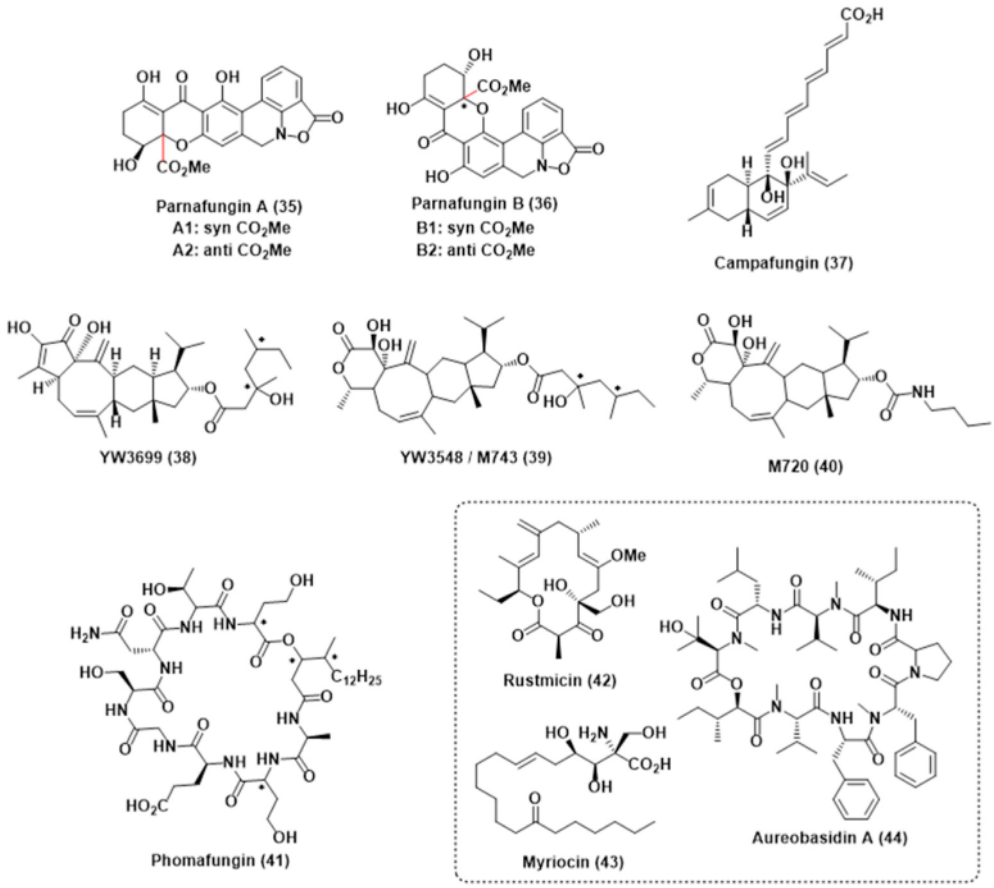

Fig 5 Antifungal natural products identi ed by the *Ca*FT. * represents an unde ned stereocenter.

isolated from a fermentation extract of the insect fungal pathogen *Fusarium larvarum* as a mixture of *syn* (A1, B1) and *anti* (A2, B2) diastereomers. Interestingly, these diastereomers were observed to freely interconvert between one another in DMSO through a proposed retro-Michael ring opening followed by rotation and ring closing [87]. Potent activity against *C. albicans* and *A. fumigatus* was observed with MICs of 0.014 μg/mL and 8−16 μg/mL, respectively. Notably, this activity against *C. albicans* was comparable to that of the caspofungin control and retained potency after addition of 50 mouse serum. Evaluation of these compounds using the *Ca*FT identi ed several heterozygous deletion mutants de cient in genes implicated in mRNA processing as hypersensitive to parnafungin treatment [88]. Indeed, the parnafungins were ultimately determined to inhibit the action of polyadenosine polymerase, preventing the polyadenylation of mRNA substrates. This polyadenylation event is crucial to the proper translation, traf- cking, and degradation of mRNA. As a result, its absence is signi cantly deleterious to the fungal cell. *In vivo* ef cacy showcased in a murine model suggests the parnafungins may be viable drug candidates in the future should mammalian cytotoxicity remain minimal.

The decalin polyketide campafungin (**37**) was isolated from the ascomycete *Plenodomus enteroleucus* and similarly passaged through the *Ca*FT [89]. MIC values against *C. albicans* and *A. fumigatus* were greater than desired, but the *Ca*FT pro le suggested the compound was targeting some aspect of the cAMP-PKA pathway based on the observed hypersensitivity of heterozygous mutants de cient in genes coding for vital components of the pathway. Although campafungin was limited to fungistatic activity at high concentrations, it was also found to compromise hyphal transition at concentrations as low as 10 μg/mL.

Fermentation of the fungus *Codinaea simplex* yielded the structurally unique sesterterpene natural product YW3699 (**38**), which was found to inhibit the biosynthesis of yeast glycosylphosphatidylinositol (GPI) at a MIC of 3.5 μM [90]. Modi cation of proteins with the phosphoglyceride GPI is necessary for proper transportation to the endoplasmic reticulum, and complications in fungal cell wall synthesis arise in its absence [91]. Although YW3699 was moderately active against yeast, the related terpene YW3548 (**39**) was found to have signi cantly more potent activity with a MIC of 3.4 nM. In order to better understand the mechanisms by which YW3548 (also known as M743) inhibits GPI synthesis, genomic pro ling was done using the *Ca*FT approach [90]. The resulting *Ca*FT pro le revealed that M743 prevents GPI synthesis by inhibiting ethanolaminephosphate transferase activity, stalling the biosynthetic pathway on a mannose precursor. Additionally, the ester moiety of M743 was found to be particularly unstable in a murine candidiasis model and thus, semisynthetically exchanged for a carbamate linkage, giving M720 (**40**), which was signi cantly more stable *in vivo* and retained its target and ef cacy based on con rmatory *Ca*FT pro ling.

The cyclic lipodepsipeptide phomafungin (**41**) was isolated from a plant pathogenic *Phoma* species and passaged through the *Ca*FT [92]. Observed hypersensitivities suggested phomafungin was targeting sphingolipid biosynthesis. However, comparison of the phomafungin *Ca*FT pro le against the known natural product sphingolipid inhibitors rustmicin (**42**), myriocin (**43**), and aureobasidin A (**44**) implied a novel mechanism of inhibition. Further investigation and development of phomafungin as an antifungal was halted by the observation of mammalian toxicity in a murine candidiasis model. Nevertheless, the *Ca*FT has enabled identi cation of several antifungal natural products with novel or underrepresented biological targets. Widespread incorporation of the *Ca*FT into antifungal drug discovery efforts is likely to signi cantly streamline the ongoing search for new options against resistant pathogens.

6 Biosynthetic gene cluster mining

Although microbial genomes encode an enormous number of proteins and metabolites, only a fraction of these compounds are expressed in any given environment. This limitation on isolable natural products has also signi cantly contributed to unproductive re-isolation and the current stagnation in natural product drug discovery [93]. Recently, genome mining and other genetic technologies have been increasingly leveraged to identify biosynthetic gene clusters within microbial genomes to elucidate previously undiscovered natural products and, potentially, natural analogs thereof (Fig. 6) [94,95].

To decipher the biosynthetic origins of the aforementioned natural product phomafungin, genome mining of an antifungal-producing *Phoma* species revealed the production of BII-rafflesfungin (**45**), a structurally similar cyclic lipodepsipeptide [96]. BII-Rafflesfungin was found to inhibit the growth of both *A. fumigatus* and azole-resistant *C. albicans* strains at MICs of 1.2 and 2.4 μM, respectively. Although a putative mechanism of action has yet to be determined for phomafungin and BII-rafflesfungin, they are likely to act similar to other antifungal cyclic depsipeptides. Syringomycin (**46**) binds to fungal membranes, inhibiting growth, while aureobasidin inhibits inositol phosphorylceramide, compromising sphingolipid biosynthesis [97,98]. Although a distinct mechanism of action has yet to be validated for both phomafungin and BII-rafflesfungin, the ability of genome mining to reveal previously undiscovered natural products with such antifungal potency makes the technique formidable in drug discovery.

This methodology has also recently been employed to study the biosynthesis of restricticin (**47**) and related molecules [99]. Produced by *Penicillium restrictum*, restricticin is thought to inhibit

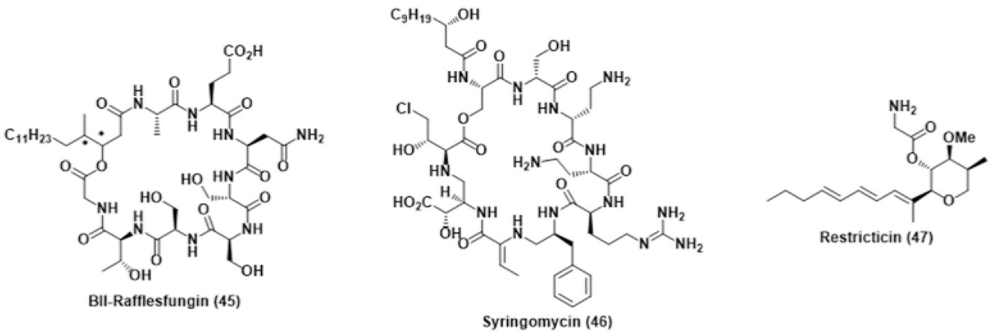

Fig 6 Antifungal natural products studied using genomics-based approaches. * represents an unde ned stereocenter.

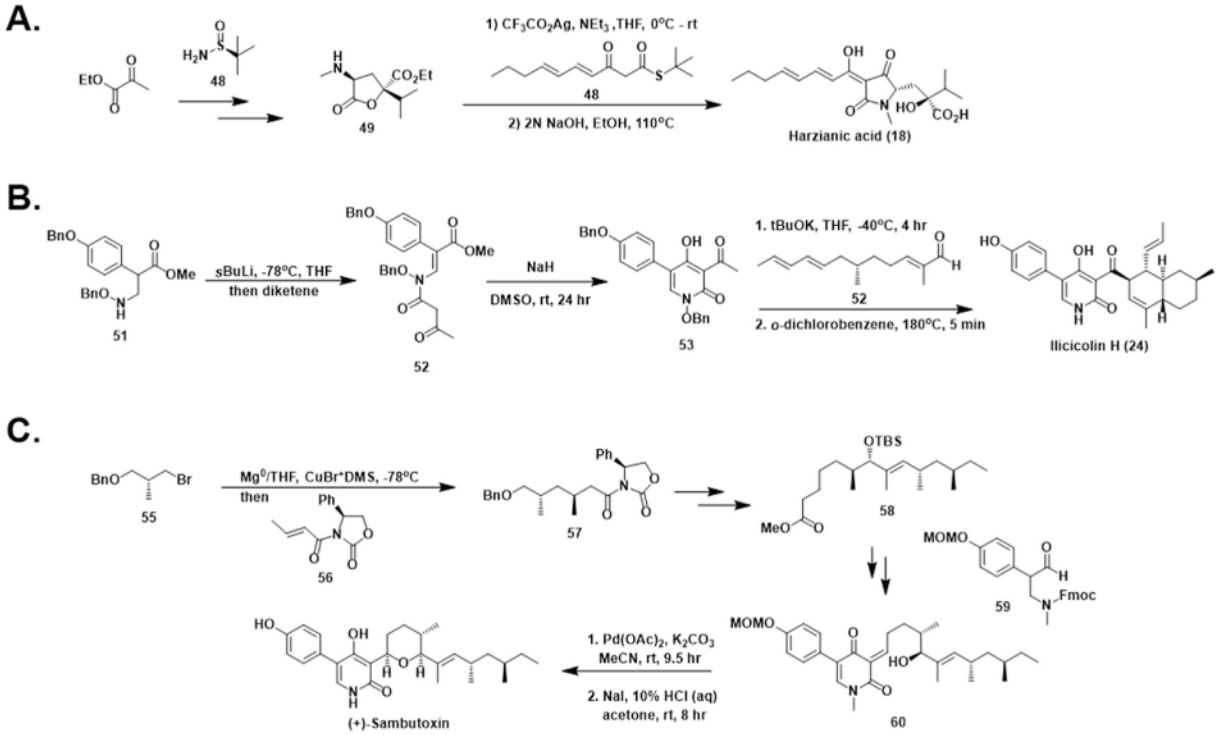

Fig 7 Total syntheses of harzianic acid (**A**), ( )-ilicicolin H (**B**), and (+)-sambutoxin (**C**).

CYP51 through coordination of the terminal amine to heme, rationalizing the observed similarities in bioactivity and antifungal spectrum with ketoconazole [100]. Production of an antifungal CYP51 inhibitor by a fungus dependent on CYP51 suggests the presence of an innate resistance mechanism. The restricticin biosynthetic gene cluster was found to be proximal to self-resistance genes encoding additional CYP51. Searching for similar gene clusters outside of *P. restrictum* found several fungal species also harbored the necessary genetic information to produce restricticin, previously unreported in these species. Application of this self-resistance-based approach to genome mining stands to surpass the limitations of traditional isolation and identify conserved biosynthetic clusters for novel antifungal natural products.

7 Synthetic efforts towards novel antifungals

The promising antifungal activity and structural complexity of many of the aforementioned natural products have inspired several total syntheses. Limited by the titer concentrations of these compounds in fermentation extracts, synthetic access to these natural products facilitates SAR studies and analog design that may otherwise be signi cantly more challenging or impossible through semisynthetic methodologies.

Work towards the construction of natural HA and its synthetic stereoisomers was completed by Westwood et al. in 2015 (Fig. 7A) [101]. Starting from ethyl pyruvate, condensation of the chiral sul namide **48** facilitated enantioselective aldol addition to ethyl dimethylpyruvate, yielding the key lactone intermediate **49** after lactonization and deprotection [102]. Treatment of this lactone with silver trifluoroacetate and thioester **50** gave HA following basic hydrolysis. Inverting the chirality of the sul namide reagent used afforded access to the other pair of diastereomeric lactones. Investigation of the activity of the four stereoisomers prepared against the fungal plant pathogens *Sclerotium rolfsii* and *Pythium ultimum* revealed that the synthetic analogs were more potent than natural harzianic acid yet remain to be evaluated against clinically relevant pathogens like *C. auris* or *A. fumigatus*.

Although the aforementioned SAR studies on ilicicolin H were recently conducted using material fermented from *Gliocladium roseum*, early synthetic efforts towards 4-hydroxy-2-pyridones resulted in a racemic synthesis as early as 1985 (Fig. 7B) [103]. The methodology for the construction of the pyridone core was reused from previous works targeting the structurally similar natural product tenellin [104]. Addition of aryl ester **51** to diketene generated acetoacetamide **52**, primed for intramolecular cyclization to the acylated pyridone **53** in basic conditions. Aldol condensation-dehydration with unsaturated aldehyde **54** followed by Diels-Alder cycloaddition established the eastern decalin scaffold and ultimately gave ( )-ilicicolin H after deprotection.

The landmark total synthesis of (+)-sambutoxin by Williams and Turske elucidated the previously unassigned stereochemical con guration of the structure (Fig. 7C) [105]. Addition of the organocuprate generated from chiral bromide **55** to Michael acceptor **56** gave **57**, establishing the desired *anti*-1,3-dimethyl con guration. Conversion of **57** to methyl ester **58** over several steps enabled Claisen condensation with aryl aldehyde **59**, yielding the 5-aryl-4-hydroxy-2-pyridone **60** after re-oxidation and deprotection at the nitrogen. Subsequent deprotection of the alcohol facilitated a *6-exo-trig* cyclization to construct the pyran. Deprotection of the phenol gave the nal compound, which was determined to be the antipode of the naturally occurring (−)-sambutoxin based on the observed optical rotation relative to the natural isolate.

Although semisynthetic efforts towards TET analogs for anticancer evaluation have previously been reported in the literature, not much attention has been placed on TET itself [106,107]. A recently published racemic synthesis of tetrandrine described a synthetic strategy allowing for rapid construction of the macrocyclic skeleton (Fig. 8A) [108]. Beginning from tetrasubstituted arene

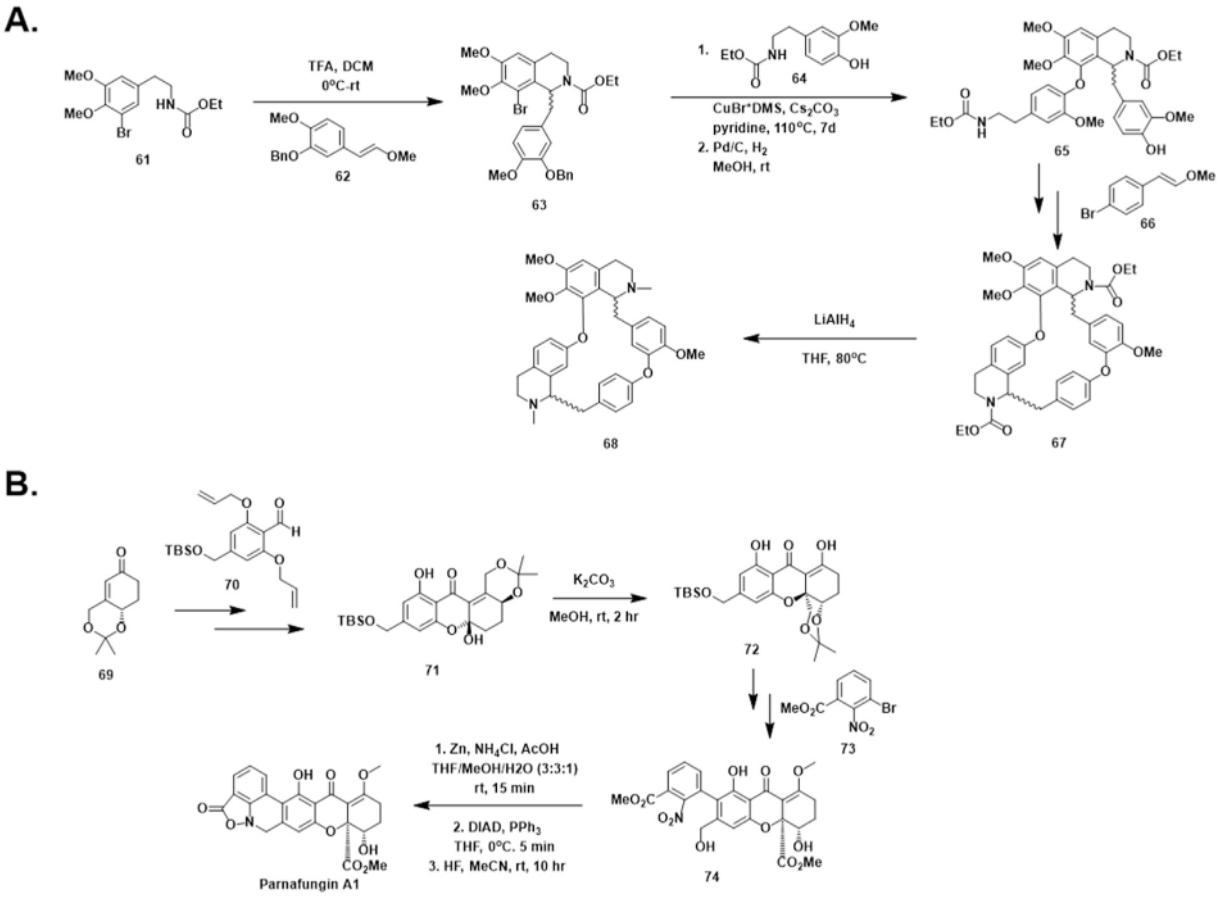

Fig 8 Total synthesis efforts towards the tetrandrines (**A**) and parnafungins (**B**).

61, a TFA-catalyzed Pictet-Spengler condensation with enol ether **62** gave isoquinoline **63**. A subsequent Ullman coupling with *N*-acylated arene **64** followed by reductive debenzylation afforded **65**. An additional iteration of the Pictet-Spengler/Ullman coupling sequence using enol ether **66** established the complete macrocycle **67**, and LAH-mediated reduction of the carbamates yielded a mixture of the four possible stereoisomers of tetrandrine (**68**).

Synthetic routes towards the syn-epimer of parnafungin A have recently been reported (Fig. 8B) [109]. Starting from known ,β-unsaturated cyclohexanone **69**, addition into aryl aldehyde **70** and re-oxidation which, after allyl deprotection, revealed the phenolic oxygen necessary for formation of the desired acetal **72**. However, only addition into the ketone was observed, generating the undesired hemiacetal **71**. Fortunately, treatment of **66** with base promoted ring-opening and Michael addition to afford acetal **72**. An iodination-borylation sequence facilitated aryl coupling with nitroarene **73** giving intermediate **74**. Reductive cyclization conditions constructed the unusual benzisoxazolinone moiety following conditions previously reported by Wierenga et al. [110] A Mitsunobu reaction promoted the nal cyclization and gave parnafungin A1 (**70**) after deprotection. Dissolution of synthetic parnafungin A1 in DMSO led to conversion of material to the three other possible stereoisomers in ratios closely matching those of the original isolation literature.

Unfortunately, the synthetic derivation of these antifungal natural products has not borne much fruit in regard to the advancement of our biological understanding. Ideally, initial triumphs in total synthesis ought to be succeeded by the development of methodology to facilitate analog design. The construction of analog libraries for these putative antifungals stands to greatly contribute to our knowledge of their biological targets and potency. Synthetic transformation or simpli cation of molecular scaffolds may serendipitously yield more potent analogs with less structural complexity. Guided by initial target identi cation results elucidated by technologies like the *Ca*FT, rational analog development for SAR studies is a powerful methodology for nature-inspired drug discovery efforts.

8 Conclusion and future outlook

The development of resistance against our limited antifungal options in several high-priority pathogens has signi cantly accelerated in recent years. Unfortunately, antifungal drug discovery efforts have not sped up to match this demand due to the inherent challenges associated with antifungal drug design. Traditional natural product isolation methodologies, such as bioactivity-guided fractionation, often lead to unproductive re-isolation of known compounds. The widespread adoption of newer approaches like the *Candida albicans* tness test and genome mining for underexplored biosynthetic gene clusters forego these limitations and instead celebrate ef cient identi cation of new natural products with novel and potent bioactivities. Several literature accounts of such compounds have been presented in hopes to inspire similar creativity in the next generation of antifungals. Pivoting modern antifungal development away from ergosterol dependence and towards novel, underexplored targets is our best hope in this crisis.

Antifungal drug discovery would also greatly bene t in more thorough biological evaluation of compounds of interest. Biological

studies of antifungals typically involve testing against the flagship fungal pathogens *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*. Although *Candida auris* has been considered a high-priority fungal pathogen for several years, it has still not yet been incorporated into initial inhibition reports, and activity reported against standard *C. albicans* strains cannot be expected to match that of *C. auris* on account of their vastly different antifungal resistance pro les. Beyond the void in biological investigation of *C. auris*, additional synthetic work towards the development of novel *C. auris* inhibitors is imperative. At the rate that *C. auris* infection rates have dramatically increased in recent years, it is very much so in our best interest to invest in solutions before a calamitous outbreak takes place.

Declaration of competing interest

The authors declare that they have no known competing nancial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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