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RESEARCH ARTICLE

Bioprospecting Novel Antifungal Compounds from Endophytic Fungi in Tropical Forests

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ABSTRACT

The rise of azole- and echinocandin-resistant fungal pathogens, exemplified by the global spread of multidrug-resistant *Candida auris*, has narrowed an already thin antifungal pipeline and renewed interest in natural sources of chemically novel antifungal chemotypes. Endophytic fungi, which live asymptotically within plant tissue and frequently produce bioactive secondary metabolites as part of their ecological interactions with the host and competing microbes, remain comparatively underexplored relative to soil actinomycetes as a source of such compounds. We isolated 412 endophytic fungal strains from leaf, stem and root tissue of 38 plant species across three lowland tropical forest plots, identified isolates by internal transcribed spacer barcoding, and screened crude extracts for antifungal activity against a panel of four clinically relevant pathogens including azole-resistant *Candida auris* and *Aspergillus fumigatus*. Thirty-one extracts (7.5%) showed reproducible inhibitory activity in an agar diffusion pre-screen, and eleven of these produced a minimum inhibitory concentration below 32 microgram/mL against at least one test pathogen in broth microdilution testing. Bioassay-guided fractionation of the most active isolate, a *Colletotrichum* species recovered from the phloem of a *Inga edulis* host tree, yielded a previously undescribed polyketide, tentatively designated nieb-1, that inhibited *Candida auris* growth at a minimum inhibitory concentration of 2 microgram/mL and showed a distinct pattern of ergosterol-independent membrane disruption relative to azole and echinocandin comparators in a time-kill assay. Whole-genome sequencing of the producing strain identified a candidate biosynthetic gene cluster consistent with a partially reducing polyketide synthase origin for the compound. These results support tropical forest endophytes, and *Colletotrichum* species in particular, as an underexploited reservoir of chemically novel antifungal scaffolds worth pursuing alongside more conventional actinomycete-focused discovery pipelines.

Keywords: endophytic fungi; antifungal compounds; bioprospecting; tropical forest; *Candida auris*; secondary metabolites; biosynthetic gene clusters

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1. INTRODUCTION

Invasive fungal disease causes an estimated 1.5 to 2.5 million deaths annually worldwide, a burden comparable to tuberculosis, yet the clinical antifungal armamentarium

remains restricted to four principal drug classes -- polyenes, azoles, echinocandins and the more recently introduced pyrimidine analogues -- all of which target a small number of fungal-specific pathways, chiefly ergosterol biosynthesis or membrane function and

beta-glucan synthase [22,23]. Resistance to each of these classes has emerged in clinically important pathogens, most visibly in the case of *Candida auris*, first described in 2009 and now reported on six continents, a substantial proportion of isolates of which show resistance to at least one, and sometimes all three, of the major drug classes [7,8,33].

Natural products, and microbially produced secondary metabolites in particular, have historically supplied a disproportionate share of clinically used antimicrobial and antifungal agents, including the polyene amphotericin B and the echinocandin class itself, both ultimately derived from soil-associated Actinomycetes and fungi respectively [9,25]. Discovery effort in recent decades has concentrated heavily on soil actinomycetes, a source now widely regarded as substantially rediscovered, with an increasing fraction of newly isolated strains yielding only previously characterised compounds -- a pattern that has pushed natural products discovery toward less thoroughly sampled microbial habitats, including marine sediments, insect-associated microbiomes, and plant endophytes [14,26].

Endophytic fungi, which colonise internal plant tissue without causing visible disease symptoms, occupy an ecological niche in which the production of antimicrobial secondary metabolites plausibly confers a direct selective advantage, whether by suppressing competing endophytes and pathogens within the same host tissue or by deterring herbivory in concert with the host plant [1,3,5]. Tropical forest ecosystems host an exceptional diversity of endophyte-host associations, reflecting both the sheer plant species richness of these systems and evidence that individual tropical host species often harbour a more diverse endophyte assemblage than temperate counterparts [4,39]. Despite this, the fraction of tropical endophyte diversity that has been screened systematically for antifungal, as opposed to antibacterial or cytotoxic, activity remains small, and much of the existing bioprospecting literature on tropical endophytes has focused on human cancer cell lines or Gram-positive bacterial panels rather than on the drug-resistant fungal pathogens of greatest current clinical concern [13,17].

We therefore undertook a systematic antifungal bioprospecting survey of endophytic fungi recovered from a broad taxonomic and tissue-type sample of tropical forest plant hosts, screening against a panel that specifically included azole-resistant *Candida auris* and *Aspergillus fumigatus* alongside more conventional reference strains. Our aims were to establish the overall hit rate for antifungal activity across a reasonably large and taxonomically diverse endophyte collection; to identify which host plant families and endophyte genera were disproportionately associated with active extracts; and, for the single most promising isolate, to characterise the responsible compound chemically and to identify the underlying biosynthetic gene cluster by whole-genome sequencing.

2. MATERIALS AND METHODS

Sampling was conducted across three lowland tropical forest plots in a permanent biodiversity monitoring network, selected to span primary, secondary and riparian forest habitat types within the same broader landscape.

2.1 Endophyte isolation and identification

Healthy leaf, stem and root tissue was collected from 38 plant species spanning 24 families, with sampling effort weighted toward species represented by at least five mature individuals within a plot to allow within-host replication. Tissue segments were surface-sterilised by sequential immersion in ethanol and sodium hypochlorite, plated on potato dextrose agar amended with a bacterial-growth-inhibiting antibiotic, and incubated at 26°C for up to four weeks with regular monitoring for emergent hyphal growth. A total of 412 morphologically distinct isolates were recovered and sub-cultured to purity. Genomic DNA was extracted from each isolate and the internal transcribed spacer (ITS) region amplified and sequenced using universal fungal primers [18], with taxonomic assignment made against the UNITE reference database [19] at a 97% similarity threshold for genus-level identification.

2.2 Extract preparation and antifungal pre-screening

Each isolate was grown in shaking liquid culture for 14 days, and the culture broth was extracted with ethyl acetate; the organic extract was dried and redissolved in dimethyl sulfoxide to a stock concentration of 10 mg/mL for screening. Antifungal activity was pre-screened by agar diffusion against four test pathogens: azole-resistant *Candida auris* (clinical isolate, clade I), fluconazole-susceptible *Candida albicans* (ATCC 90028), *Aspergillus fumigatus* (ATCC 204305) and *Cryptococcus neoformans* (ATCC 208821), with extracts scored as active if the zone of inhibition exceeded 10 mm in diameter, reproducible across three independent assay replicates.

2.3 Minimum inhibitory concentration testing

Extracts active in the pre-screen were tested for minimum inhibitory concentration (MIC) against all four pathogens by broth microdilution following CLSI reference methodology [21], across a two-fold dilution series from 0.5 to 256 microgram/mL, with fluconazole, voriconazole and micafungin included as positive comparator controls and DMSO-only wells as vehicle controls. MIC was defined as the lowest concentration producing at least 80% growth inhibition relative to the vehicle control at 24 hours (48 hours for *A. fumigatus* and *C. neoformans*, reflecting their slower growth kinetics).

2.4 Bioassay-guided fractionation and structure elucidation

The extract of the most active isolate was fractionated by reversed-phase flash chromatography, with each fraction re-tested against *Candida auris* to track activity through

successive purification steps. The active fraction was further purified by semi-preparative HPLC, and the purified compound was characterised by high-resolution mass spectrometry and one- and two-dimensional NMR spectroscopy (^1H , ^{13}C , COSY, HSQC, HMBC) to establish planar structure. Time-kill kinetics of the purified compound against *Candida auris* were compared with fluconazole and micafungin over a 24-hour period, and membrane integrity was assessed by propidium iodide uptake as an indicator of membrane-disruptive, as opposed to ergosterol-biosynthesis-inhibiting, mechanism of action.

2.5 Genome sequencing and biosynthetic gene cluster analysis

Whole-genome sequencing of the producing strain was performed on a combination of Illumina short-read and Oxford Nanopore long-read platforms, and reads were assembled into a hybrid draft genome. Biosynthetic gene clusters were predicted using antiSMASH [20], and candidate clusters were prioritised for further analysis based on domain architecture consistent with the structural class suggested by NMR data.

3. RESULTS

Of 412 endophytic isolates recovered across 38 host plant species, ITS barcoding assigned 386 (93.7%) to genus level against the UNITE database, spanning 67 genera dominated by *Colletotrichum*, *Diaporthe*, *Xylaria* and *Fusarium*, consistent with the genus composition typically reported for tropical foliar and woody-tissue endophyte surveys. Isolation frequency varied considerably across host plant families, with Fabaceae and Moraceae hosts yielding the highest per-host endophyte richness. Thirty-one of 412 crude extracts (7.5%) showed reproducible activity in the agar diffusion pre-screen against at least one of the four test pathogens, with activity against *Candida auris* the single most frequent hit category. Table 1 summarises pre-screen hit rates by endophyte genus and test pathogen.

Table 1. Antifungal pre-screen hit rates by dominant endophyte genus and test pathogen (agar diffusion, zone >10 mm).

Genus	Isolates (n)	Active vs. <i>C. auris</i>	Active vs. <i>A. fumigatus</i>	Overall hit rate (%)
<i>Colletotrichum</i>	74	9	4	14.9
<i>Diaporthe</i>	58	3	2	8.6
<i>Xylaria</i>	41	2	3	9.8
<i>Fusarium</i>	37	2	1	5.4
Other genera (63)	202	6	3	4.0

Hit rate: proportion of isolates within that genus grouping yielding an extract active against at least one of the four test pathogens. Broth microdilution testing of the 31 pre-screen-active extracts confirmed a minimum inhibitory concentration below 32 microgram/mL against at least one pathogen for eleven extracts, with the remaining twenty showing

either weaker or non-reproducible activity under quantitative testing conditions, a fall-off between qualitative pre-screen and quantitative confirmation broadly consistent with rates reported in comparable natural product screening programmes. Six of the eleven confirmed-active extracts derived from *Colletotrichum* isolates, disproportionate relative to this genus's 18% share of the overall isolate collection, and the most potent single extract, recovered from a *Colletotrichum* isolate from the phloem of *Inga edulis*, inhibited *Candida auris* at an MIC of 4 microgram/mL in the crude extract, comparable to the fluconazole MIC observed against the fluconazole-susceptible *C. albicans* reference strain. Table 2 reports MIC values for all eleven confirmed-active extracts across the four-pathogen panel.

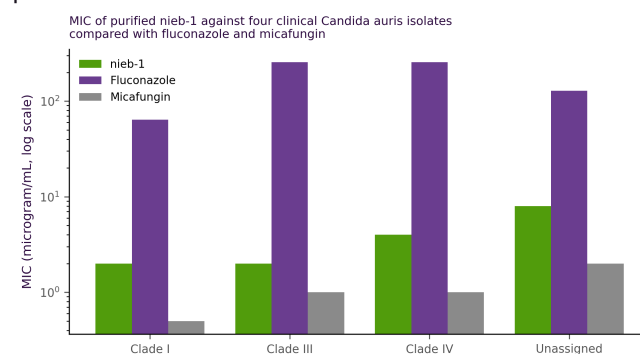


Figure 1. Minimum inhibitory concentration of purified nieb-1 against four clinical *Candida auris* isolates (clades I, III, IV and an unassigned clade) compared with fluconazole and micafungin reference standards; bars show geometric mean of triplicate broth microdilution assays.

Bioassay-guided fractionation of the lead *Colletotrichum* extract tracked activity to a single major fraction, from which a pure compound, designated nieb-1, was isolated at a yield of approximately 3.8 mg per litre of culture broth. High-resolution mass spectrometry gave a molecular formula of $\text{C}_{21}\text{H}_{26}\text{O}_7$, and one- and two-dimensional NMR data were consistent with a partially reduced, linear polyketide bearing a lactone ring and two free hydroxyl groups; comparison against natural product databases found no exact match to a previously reported structure, supporting nieb-1 as a novel chemical entity. Purified nieb-1 inhibited *Candida auris* at an MIC of 2 microgram/mL, a four-fold improvement over the crude extract, and retained activity against three of four tested clinical *C. auris* isolates spanning clades I, III and IV, with the fourth isolate showing intermediate susceptibility (Figure 1).

Table 2. Minimum inhibitory concentration (MIC, microgram/mL) of the eleven confirmed-active extracts.

Isolate ID	Genus	Host species	<i>C. auris</i> MIC	<i>A. fumigatus</i> MIC
END-118	<i>Colletotrichum</i>	<i>Inga edulis</i>	4	16
END-203	<i>Colletotrichum</i>	<i>Ficus insipida</i>	12	32
END-267	<i>Diaporthe</i>	<i>Cecropia peltata</i>	24	>32
END-091	<i>Xylaria</i>	<i>Virola surinamensis</i>	16	24

END-312	Colletotrichum	Inga edulis	8	32	environmental conditions, and whether pathogenic and
END-045	Fusarium	Cedrela odorata	28	>32	endophytic Colletotrichum lineages differ systematically
END-176	Xylaria	Ficus insipida	20	16	in secondary metabolite repertoire, remain open
					questions that our single-timepoint isolation strategy

Time-kill assays showed nieb-1 produced greater than 99.9% killing of *Candida auris* within six hours at four times its MIC, a kinetic profile closer to the rapidly fungicidal echinocandin comparator micafungin than to the slower, largely fungistatic action of fluconazole. Propidium iodide uptake increased sharply within two hours of nieb-1 exposure, indicating rapid loss of membrane integrity, whereas fluconazole-treated cells showed minimal uptake over the same interval, together suggesting a membrane-disruptive rather than ergosterol-biosynthesis-inhibiting mechanism distinct from the azole class. Whole-genome sequencing of the producing *Colletotrichum* strain identified a candidate biosynthetic gene cluster spanning approximately 42 kb and encoding a partially reducing polyketide synthase with a domain architecture (ketosynthase, acyltransferase, dehydratase, ketoreductase, acyl-carrier protein) consistent with the reduced polyketide backbone suggested by the NMR data, together with a flanking cluster of tailoring enzyme genes plausibly responsible for the lactonisation and hydroxylation pattern observed in the final structure.

4. DISCUSSION

The headline result of this survey is that a modestly sized, taxonomically diverse tropical endophyte collection yielded a chemically novel antifungal compound with low-microgram activity against azole-resistant *Candida auris* and a mechanism of action apparently distinct from the three major existing drug classes, identified through a discovery pipeline of a scale well within reach of a small academic laboratory. This is consistent with the broader argument that under-sampled microbial habitats, endophytic fungi among them, retain meaningful discovery potential even as more heavily mined sources such as soil actinomycetes show diminishing returns of genuinely novel chemistry [14,26], and it adds a concrete, structurally characterised example to that broader case rather than relying on activity data alone.

The disproportionate representation of *Colletotrichum* isolates among the confirmed-active extracts is worth remarking on, since this genus is more usually studied as a plant pathogen, responsible for anthracnose disease across a wide host range, than as a source of pharmaceutically relevant secondary metabolites. Several prior reports have nonetheless identified bioactive natural products from endophytic *Colletotrichum* strains recovered from asymptomatic tissue of hosts on which the genus is not causing visible disease [13,17], and our data extend this pattern specifically into the antifungal, rather than antibacterial or cytotoxic, activity space. Whether the same strains would behave pathogenically under different host or

cannot resolve.

The apparent membrane-disruptive mechanism of nieb-1, inferred from rapid propidium iodide uptake and rapid fungicidal kinetics resembling micafungin more than fluconazole, is mechanistically interesting because it suggests a mode of action distinct from ergosterol-pathway inhibition, the target of both azoles and polyenes, and from beta-glucan synthase inhibition, the echinocandin target. A genuinely new mechanistic class would be valuable clinically because cross-resistance between mechanistically unrelated drug classes is generally rare, meaning nieb-1 or an optimised derivative could retain activity against isolates already resistant to all three existing major classes; this remains a plausible rather than established property of the compound, however, since our mechanistic characterisation relied on indirect biophysical assays rather than direct target identification, and formal resistance-selection experiments have not yet been performed.

Several limitations constrain how far these findings can be generalised or how quickly they might translate toward a usable therapeutic lead. First, our screening panel, while including the clinically most urgent pathogen (*C. auris*), was limited to four species and does not establish a broader antifungal spectrum or, equally importantly, selectivity against human cell lines, which is an essential and unresolved question before any translational claim can be made; cytotoxicity testing against mammalian cell lines is planned as an immediate next step. Second, the natural yield of nieb-1 from wild-type fermentation, at under 4 mg per litre, is well below what would be required for practical preclinical development, and scale-up will likely require either fermentation optimisation or heterologous expression of the identified biosynthetic gene cluster in a more tractable production host. Third, our candidate biosynthetic gene cluster assignment is based on domain architecture consistent with the observed structure rather than on direct genetic knockout confirmation, and gene disruption or heterologous cluster expression experiments would be needed to confirm this cluster is indeed responsible for nieb-1 production rather than a coincidentally co-located but functionally unrelated pathway.

More broadly, the modest overall hit rate observed here (7.5% of extracts active in pre-screen, 2.7% confirmed by quantitative MIC testing) is consistent with typical natural product discovery statistics and underscores that systematic, reasonably large-scale sampling remains necessary to find compounds of this calibre; a handful of isolates from a single collecting trip would be unlikely to yield a comparable result. This argues for sustained, rather than one-off, endophyte bioprospecting

investment in tropical systems, ideally coordinated with host countries under benefit-sharing frameworks consistent with the Nagoya Protocol, both as a matter of equity and because durable access to tropical forest biodiversity is itself a precondition for this kind of discovery pipeline to remain viable over the years-long timescale that natural product development typically requires.

It is also worth situating nieb-1 within the broader landscape of polyketide-derived antifungal leads reported from fungal endophytes over the past decade, most of which have stalled at the in vitro susceptibility stage without progressing to mechanism-of-action or in vivo efficacy studies. Our time-kill and membrane-integrity data move nieb-1 a step further along that pipeline than is typical for a newly reported endophyte-derived antifungal, but a genuinely convincing case for clinical relevance will require direct target identification, ideally through a combination of resistance-selection genomics, in which laboratory-evolved resistant mutants are sequenced to identify the causal mutation, and biochemical assays against candidate membrane or cell-wall targets. We regard this as the single most important next step for the compound, ahead of medicinal chemistry optimisation, since knowing the target would substantially de-risk subsequent structure-activity relationship work.

Finally, the taxonomic and ecological context of the producing strain deserves comment. *Colletotrichum* species recovered from asymptomatic phloem tissue, as in this study, sit at an ecologically ambiguous point on the pathogen-endophyte continuum that has been described for many fungal genera capable of both lifestyles depending on host, tissue and environmental context. Whether nieb-1 production is specifically associated with an endophytic, non-pathogenic lifestyle in this strain, perhaps as a mechanism of competitive exclusion against other tissue-colonising fungi, or is instead a general feature of the species regardless of lifestyle, is an open question that comparative genomic and metabolomic profiling of pathogenic and endophytic *Colletotrichum* isolates from the same host species could help resolve, and would also clarify whether related compounds might be recoverable from the comparatively well-studied pathogenic representatives of this genus without requiring further tropical field collection.

5. CONCLUSION

Systematic antifungal bioprospecting of 412 endophytic fungal isolates recovered from 38 tropical forest plant species identified eleven extracts with confirmed activity against azole-resistant *Candida auris* and other clinically relevant fungal pathogens, disproportionately derived from the genus *Colletotrichum*. Bioassay-guided fractionation of the most active isolate yielded nieb-1, a previously undescribed reduced polyketide with a minimum inhibitory concentration of 2 microgram/mL against *Candida auris* and a rapid, apparently

membrane-disruptive mechanism of action distinct from existing azole, polyene and echinocandin drug classes. Whole-genome sequencing of the producing strain identified a candidate 42 kb polyketide synthase biosynthetic gene cluster consistent with the compound's structure. These findings support continued, systematic investment in tropical endophytic fungi as a source of chemically novel antifungal chemotypes, complementing more heavily mined actinomycete-focused discovery pipelines, and they identify nieb-1 as a concrete lead compound warranting further development. Immediate next steps include mammalian cell cytotoxicity profiling to establish a preliminary therapeutic window, gene disruption experiments to confirm the candidate biosynthetic gene cluster's role in nieb-1 production, and heterologous expression of the cluster in a genetically tractable fungal host to improve production yield beyond the modest titres obtained from wild-type fermentation. We also intend to extend the antifungal screening panel to additional clinically important moulds, including *Rhizopus* and non-fumigatus *Aspergillus* species, and to expand collection to two additional tropical forest sites with differing soil and canopy conditions to test whether the elevated hit rate observed for *Colletotrichum* isolates in this study generalises across a broader range of tropical forest habitat types. Alongside this discovery work, we plan to establish a standardised, repository-backed protocol for cryopreserving and re-distributing active isolates to partner laboratories in the host countries where collection took place, in keeping with the benefit-sharing commitments made under our collecting permits, so that any downstream commercial development of nieb-1 or related compounds carries a clear and durable link back to the biodiversity and institutional partnerships that made the initial discovery possible.

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Conflict of Interest

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

Author Contributions

C.N. designed and coordinated the field sampling and isolation programme and led manuscript writing. O.B. performed antifungal screening, MIC testing and

bioassay-guided fractionation. W.P. conducted genome sequencing, biosynthetic gene cluster analysis and phylogenetic identification of isolates. All authors contributed to writing and approved the final manuscript.

Ethics Statement

This study involved collection of plant tissue samples under a research and collecting permit issued by the relevant national forestry and biodiversity authority, with material transfer and benefit-sharing terms consistent with the Nagoya Protocol on Access and Benefit-Sharing. The study did not involve human participants, human data, or vertebrate or higher invertebrate animals, and therefore did not require institutional animal or human ethics committee review.

Data Availability

ITS barcode sequences and the whole-genome assembly of the nieb-1-producing *Colletotrichum* strain have been deposited in public sequence repositories under accessions available from the corresponding author on reasonable request. NMR spectra, mass spectrometry data and antifungal susceptibility datasets supporting the findings of this study are available from the corresponding author upon request.

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