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Alexandra Brand

A.Brand@exeter.ac.uk

University of Exeter <https://orcid.org/0000-0002-7381-131X>

Ben Rutter

University of Aberdeen

Michael Herrera

University of Edinburgh

Gustavo Perez-Ortiz

king's college London

Claudio Greco

Swansea University <https://orcid.org/0000-0003-3067-0999>

Ben Ashley

Hai Deng

University of Aberdeen <https://orcid.org/0000-0002-0047-0622>

Abigail Hay

Pirbright Institute <https://orcid.org/0000-0002-8143-2109>

Cameron Bedford

University of Exeter

Marius Wenzel

University of Aberdeen

Stephen Mondo

United States Department of Energy Joint Genome Institute / Lawrence Berkeley National Laboratory

<https://orcid.org/0000-0001-5797-0647>

Zachary Konkel

MycoOmics LLC

Jason Slot

Ohio State University

Rhys Farrer

University of Exeter <https://orcid.org/0000-0001-8456-7458>

Dominic Campopiano

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Evolution, structure and function of the biosynthetic gene cluster of myriocin, a potent inhibitory sphingolipid

Ben Rutter¹, Michael Herrera², Gustavo Perez Ortiz², Claudio Greco³, Ben Ashley², Hai Deng⁴, Abigail Hay^{1,5}, Cameron Bedford⁶, Marius Wenzel^{7,8}, Stephen Mondo⁹, Zachary Konkel¹⁰, Jason C Slot¹⁰, Rhys A. Farrer⁶, Dominic Campopiano^{*2} & Alexandra C Brand^{*†1,6,11}

¹ School of Medicine, Medical Sciences & Nutrition, University of Aberdeen, Aberdeen, UK

² School of Chemistry, University of Edinburgh, Edinburgh, UK

³ Department of Biosciences, Swansea University, Swansea, UK

⁴ Department of Chemistry, School of Natural & Computing Sciences, University of Aberdeen, Aberdeen, UK

⁵ The Pirbright Institute, Woking, UK

⁶ Medical Research Council Centre for Medical Mycology at the University of Exeter, Exeter, UK

⁷ Centre for Genome-Enabled Biology & Medicine, University of Aberdeen, Aberdeen, UK

⁸ School of Biological Sciences, University of Aberdeen, Aberdeen, UK

⁹ Joint Genome Institute, Walnut Creek, CA 94598, USA.

¹⁰ Department of Plant Pathology, The Ohio State University, USA

¹¹ Living Systems Institute, University of Exeter, Exeter, UK

*Co-senior authors

†Corresponding author: Alexandra Brand a.brand@exeter.ac.uk

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ABSTRACT

Myriocin is a fungal secondary metabolite exploited worldwide as a powerful inhibitor of sphingolipid biosynthesis through its structural similarity to sphingosine. We identify the myriocin biosynthesis gene cluster (BGC) through *de novo* sequencing of two producing fungi, *Isaria sinclairii* and *Mycelia sterilia*, yielding genomes of 25.2 Mb and 34.2 Mb encoding 27 and 20 secondary metabolite BGCs, respectively. BGCs #5 in *I. sinclairii* and #18 in *M. sterilia* shared the Polyketide Synthase (PKS) and alpha oxo-amine synthase (AOS) predicted for myriocin biosynthesis, with 74 % and 79 % sequence similarity, respectively. Analysis of a 2,236 fungal genome database suggests the pathway originated in the *Sordariomycete* ancestor, presenting in two major clades distinguished by PKS gene orientation. The placement of thermophilic *M. sterilia* suggests myriocin BGC acquisition through horizontal gene transfer, but its origin in *I. sinclairii* is ambiguous. Heterologously-expressed IsMyrA bound amino-malonate and a protein-protein docking interface were identified between the acyl carrier protein and IsMyrA. A model of PKS domain function, the roles of the PKS and AOS genes and the synteny of the myriocin biosynthetic gene cluster across 34 carrier species of *Ascomycetes* is presented.

INTRODUCTION

Myriocin is a fungal secondary metabolite with a sphingosine-like structure – a long-chain fatty acid with an amino head group. It is also known as thermozymocidin and ISP-1: ((2*S*, 3*R*, 4*R*)-(*E*)-2-amino-3, 4-dihydroxy-2-hydroxymethyl-14-oxoeicos-6-enoic acid). Myriocin was first identified in independent screens for antifungal activity from natural products from the fungi *Myriococcum albomyces*¹ (now *Melanocarpus albomyces*), *Mycelia sterilia*² and *Melanconis flavovirens*³. A screen for fungal metabolites with immunosuppressant activity identified a compound from *Isaria sinclairii* that inhibited graft rejection in a rat dorsal skin transplant model and exhibited 10 – 100-fold more potency than cyclosporin A⁴. This compound, ISP-1, was found to be identical to myriocin. By binding an aldimine within the active site of Serine Palmitoyltransferase (SPT), myriocin inhibits the condensation reaction between serine and Palmitoyl-CoA that generates 3-ketosphinganine. Conserved in yeast and mammals⁵, this is the first committed step in the biosynthesis of complex sphingolipids that are important components of lipid membranes and cellular signalling⁶⁻⁸. Myriocin therefore shares a similar mode of action to the sphingofungins, which are potent SPT inhibitors first isolated from *Aspergillus fumigatus* and *Paecilomyces variotii* in the 1990s^{9,10}. Another well-known class of sphingolipid inhibitors are the fumonisins, which block the formation of complex sphingolipids by inhibiting Ceramide Synthase (CerS)¹¹.

Crucially, the potent bioactivity of myriocin, sphingofungins and fumonisins stems from their structural and chemical resemblance to sphingolipids.

Myriocin has been synthesised through a derivatization programme¹² into a water-soluble form, Fingolimod ('Gilenya', Novartis) and was licensed in 2010 as the first oral drug against the relapsing-remitting autoimmune disease, multiple sclerosis¹³. In the human body, Fingolimod is phosphorylated to form Fingolimod-P, which out-competes sphingosine in binding Sphingosine-1-phosphate 1 (S1P₁) receptors on central memory T-cells (TCM), inducing their internalisation and degradation. This renders TCMs insensitive to the S1P gradient that directs their migration from the lymph node to the bloodstream, from whence they attack myelinated axons in the central nervous system.

Myriocin as an inhibitor of sphingolipid metabolism is also relevant to the study of viral infections including Hepatitis B¹⁴, Hepatitis C¹⁵ and Herpes Simplex Virus (HSV) replication¹⁶, where there is a potential application for myriocin in the development of anti-tumour (HSV)-based vectors. As an antifungal, myriocin is of interest in the field of medical mycology and shows strong activity against invasive fungal pathogens such as *Candida albicans* (SC5314) (MIC₅₀ = 0.12 µg /mL)¹⁷, *Aspergillus fumigatus* (Af293) (MIC₅₀ = 8 µg/mL)¹⁸ and *C. auris* (MIC₅₀ = ≤ 0.5 µg/mL)¹⁹.

The primary myriocin-producing fungi have been identified as members of the *Sordariomycetes* class of Ascomycota. Although genome sequences are available for many of these species, the gene cluster responsible for myriocin biosynthesis has not previously been identified. *I. sinclairii* (teleomorph *Ophiocordyceps sinclairii*) is a haploid entomopathogen native to Asia⁴. *I. sinclairii* spores infect insect larvae of the orders *Hymenoptera* and *Lepidoptera*, where growth within the host leads to the death of the insect. *Mycelia sterilia*, which has not been phylogenetically classified, produces three times the amount of myriocin produced by *I. sinclairii*²⁰. However, '*Mycelia sterilia*' is a generic name for the artificial taxonomic group of fungi that do not produce spores, instead growing as fluffy colonies thought to propagate through fragmentation. As a result of the phylogenetic analysis undertaken in this study, we can place *M. sterilia* (ATCC20349) in the *Chaetomiaceae* family of the *Sordariomycetes* class of fungi.

Even though myriocin is recognised as a potent secondary metabolite with valuable research and clinical applications, its biosynthetic pathway has not been elucidated. Here we assembled *de novo* the genomes of two myriocin-producing fungi, *I. sinclairii* (ATCC24400) and *M. sterilia* (ATCC20349), using combined Illumina and Oxford Nanopore sequencing to identify the biosynthetic gene cluster (BGC) responsible for myriocin production. Comparative genomics revealed that the two fungi shared a single homologous BCG

comprising 6 – 7 core genes, which we designate as *myrA*, *B*, *C*, *D*, *F*, *G* and *H*, where *myrB* and *myrA* encode a Polyketide Synthase and PLP-dependent alpha oxo-amine synthase (AOS), respectively, that are predicted to be required for myriocin biosynthesis. Heterologous expression of *IsMyrA* and protein modelling confirmed preferential binding, *in vitro* and *in silico*, of a C18 fatty acid backbone to amino-malonate, and not to L-serine, placing myriocin within an emerging functional class of fungal secondary metabolites that inhibit sphingolipid and ceramide biosynthesis. We term these ‘Sphinganine-Like Mycotoxins (SLMs)’ that include fumonisin and phytosphingosine, which also act on the sphingolipid/ceramide biosynthetic pathway. Examination of published fungal genomes revealed that homologs of the myriocin BGC are ancestral in two major orders of *Ascomycota* – *Xylariales* and *Eurotiales* – where each order exhibits a different general arrangement of the BGC. However, placement of *I. sinclairii* within *Ophiocordycipitaceae* (*Hypocreales*) and *M. sterilia* within the *Chaetomiaceae* (*Sordariales*) provided exemplars of the insertion into these clades of other species carrying the myriocin BGC, consistent with a broader distribution of this cluster and multiple potential horizontal gene transfer events that have introduced the BGC outside the two main orders.

RESULTS

***I. sinclairii* undergoes temperature-dependent morphogenesis and *M. sterilia* is a constitutively-filamentous thermophile**

Beyond their shared ability to produce myriocin, little is known of the biology of *I. sinclairii* or *M. sterilia*. To understand more about their lifestyles, the effect of temperature and growth medium on cell morphology and colony growth were determined. *M. sterilia* did not grow on either solid potato dextrose agar (PDA) or tryptic soy agar (TSA) at 25 °C but colony diameter increased with temperature at 37 °C and 45 °C, reaching the plate periphery within 5 d (Fig. 1a). The faster growth rate at 45 °C compared to 37 °C classifies *M. sterilia* as a thermophilic fungus²¹. In contrast, the growth of *I. sinclairii* negatively correlated with temperature on solid media, with maximal growth at 25 °C and none at 45 °C (Fig. 1b). Although both fungi grew faster on potato dextrose than on tryptic soy agar, the growth of *I. sinclairii* on both media was slow compared to *M. sterilia* and colonies did not reach the plate periphery in 24 d. The two fungi also exhibited differential susceptibility to antifungal compounds: *I. sinclairii* was sensitive to the antifungal drugs, fluconazole and hygromycin B, but not to nourseothricin, while *M. sterilia* was sensitive to hygromycin B and nourseothricin, but not to fluconazole (Fig. S1). These results point to hygromycin B as a shared potential

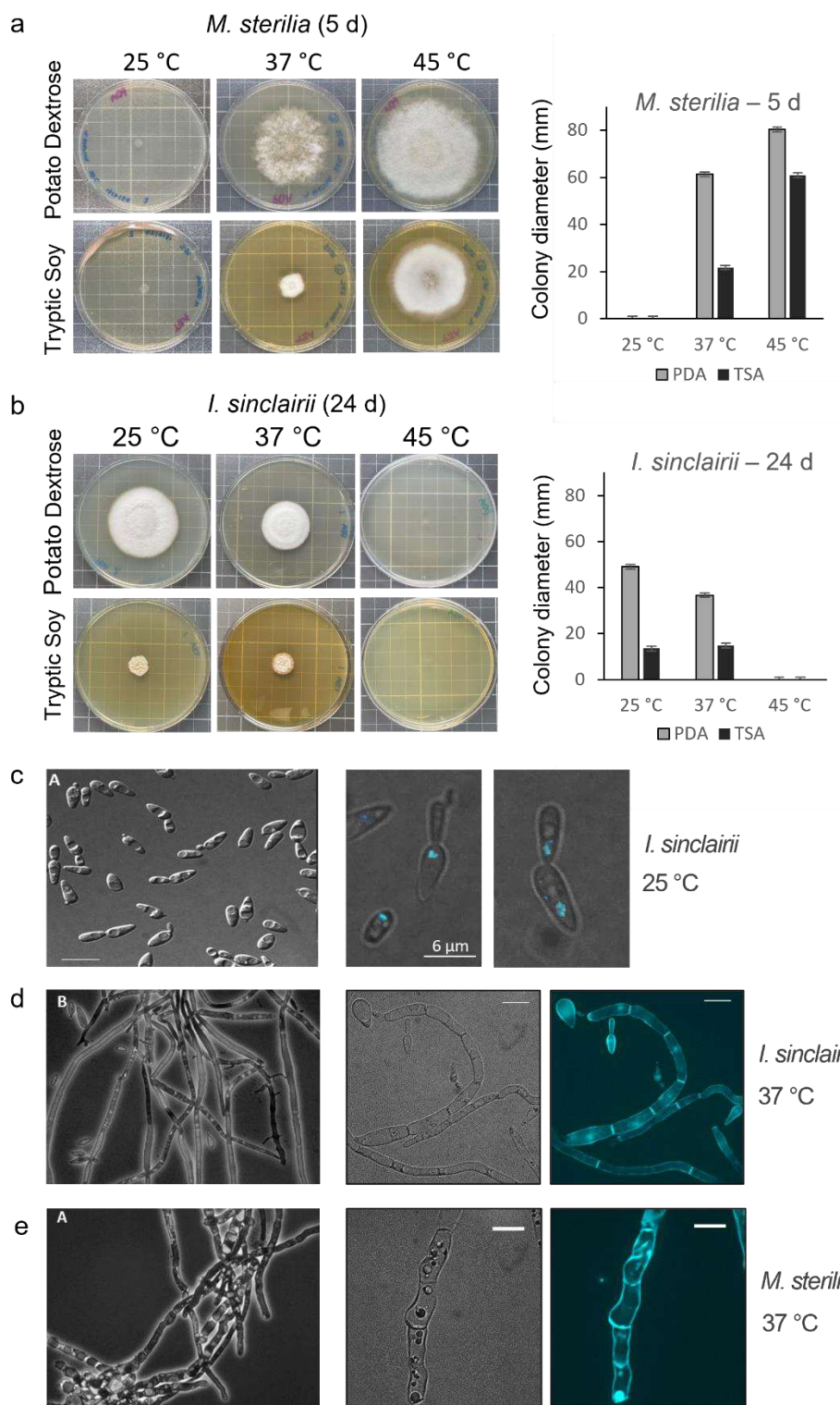


Fig. 1 Temperature-dependent dimorphism of *I. sinclairii* and the constitutively filamentous growth of thermophilic *M. sterilia*. a.b. The effect of growth medium and temperature on colony growth by a. *M. sterilia* at 5 d and b. *I. sinclairii* at 24 d, on potato dextrose agar (grey bars) or tryptic soy agar (black bars) at 25, 37, or 45 °C. c. In liquid culture at 25 °C, *I. sinclairii* formed elongated budding yeast cells. Staining with DAPI revealed cells were mononucleate. d. At 37 °C, *I. sinclairii* formed hyphal filaments. Staining with Calcofluor White showed strong fluorescence at hyphal septa relative to the parallel-sided cell wall. e. Growth of *M. sterilia* was constitutively filamentous, exhibiting septal-bound compartments of variable width and irregular morphology. Bars = 10 µm.

selectable marker for genetic modification approaches in these two fungi, although *M. sterilia* was up to 10-fold more sensitive to this drug than *I. sinclairii*.

In liquid fermentation medium at 25 °C, *I. sinclairii* grew as a mononucleate elongated yeast (Fig. 1c). No growth for *M. sterilia* was observed at this temperature, as seen on solid medium. At 37 °C both fungi grew as hyphal filaments (Fig. 1d,e), indicating that *I. sinclairii* undergoes a yeast-to-hyphal switch at the higher temperature. *I. sinclairii* hyphae were parallel-sided and septa stained more strongly than the hyphal cell wall with Calcofluor White, suggesting that septa have a relatively high chitin content in this fungus. Under all conditions tested, *M. sterilia* grew only in the hyphal form, which formed spherical hyphal aggregates in liquid media. Hyphae were non-parallel-sided with expanded regions in the walls adjacent to septa. Both fungi therefore grew as strings of non-separated cell compartments, as opposed to undergoing the coenocytic cytoplasmic streaming observed in many filamentous fungi. Together these findings indicate that, although *M. sterilia* and *I. sinclairii* both maintain a myriocin BGC, their lifestyles are otherwise divergent.

Myriocin content in *I. sinclairii* and *M. sterilia* cells and supernatant

The known structure of myriocin (Fig. 2a) and availability of a commercial standard allowed us to compare production and secretion of myriocin by *M. sterilia* and *I. sinclairii* at their two permissive growth temperatures. *M. sterilia* has previously been reported to secrete myriocin at detectable levels after two-stage culture for 14 d at 40 °C in fermentation medium⁴. For a direct comparison, the two fungi were incubated for 24 d in fermentation medium at 25 °C and 37 °C for *I. sinclairii* or 37 °C and 45 °C for *M. sterilia*, before extraction of 100 mg of desiccated cell pellets and 1 mL fermentation broth with ethyl acetate, followed by liquid chromatography-high resolution mass spectroscopic (UHPLC-HRMS) analysis. A metabolite eluting at 3.1 min with the mass-to-charge ratio (m/z) of 402.2853 $[M+H]^+$ was identified in the cell pellet and fermentation broth of both fungi (calcd: 402.277, Δ = 0.46 ppm), corresponding to myriocin ($C_{21}H_{39}O_6N$) (Fig 2a,b). The metabolite had the same retention time and mass spectrum as that of the myriocin chemical standard (Fig. 2b). Both fungi also produced a myriocin derivative with an additional hydroxyl group ($C_{21}H_{41}O_7N^+$, obsd, 428.22796, calcd, 418.2799, Δ = -0.54 ppm) eluting at 1.9 min as a minor metabolite (Fig. S2b), as previously reported²⁰. Myriocin production was quantified in both strains against a chemical standard and by integrating the area of the peak in the extracted ion chromatogram. In *I. sinclairii*, myriocin production was ~2-fold higher per 100 μ g biomass and per 1 mL culture medium at 25 °C compared to 37 °C (Fig. 2c). Surprisingly, in *M. sterilia*, myriocin production was nearly 50 % higher at 37 °C than at 45 °C, even though

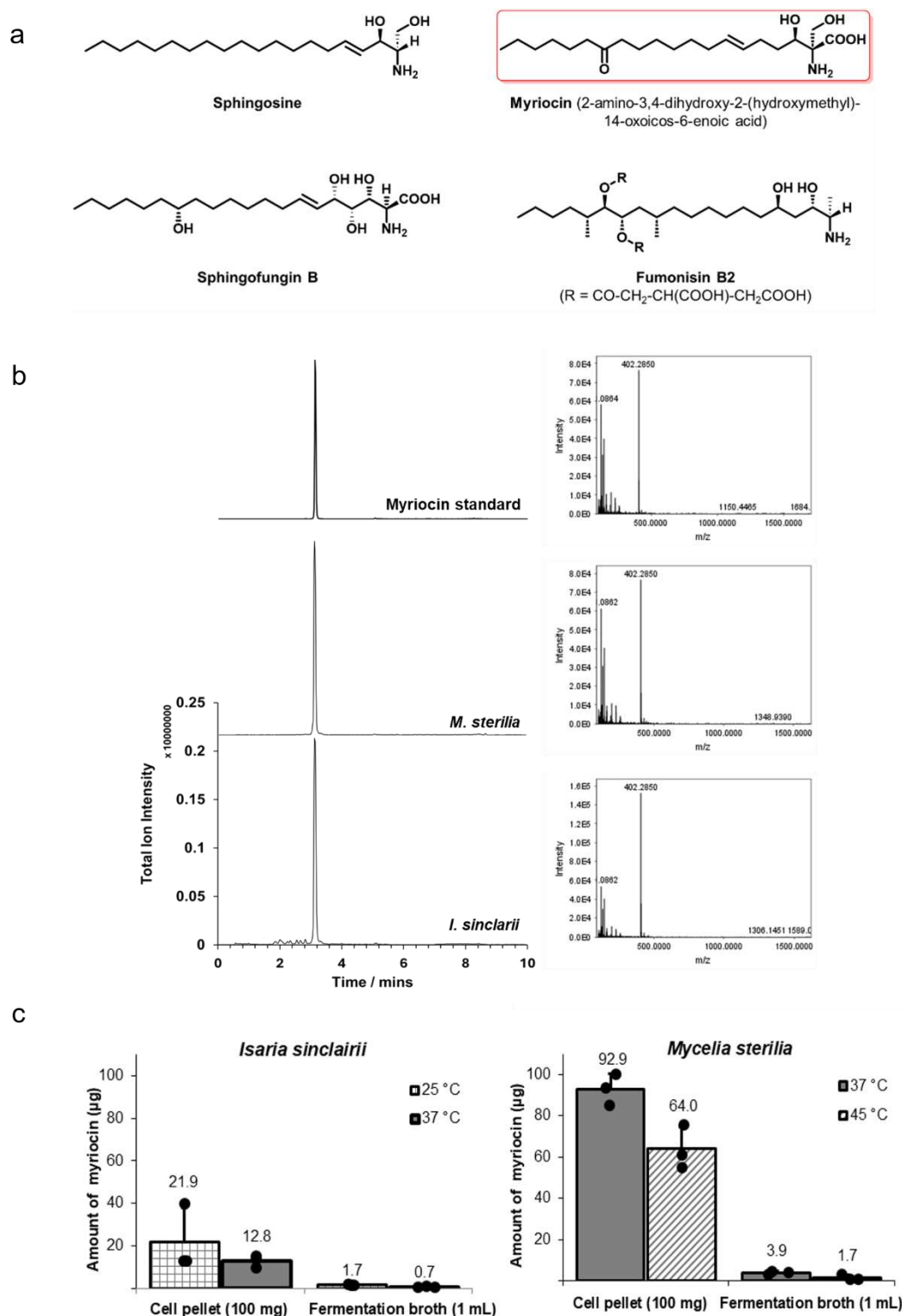


Fig. 2: Detection and quantification of myriocin in fungal cell pellets and supernatant. a. Chemical structures of sphingosine, myriocin (boxed) and two Sphinganine-Like Mycotoxins (SLMs), sphingofungin B and fumonisin B2. b. Detection of myriocin using UHPLC-HRMS analysis: Extracted ion chromatograms (402.285) in positive mode of myriocin chemical standard, *M. sterilia* and *I. sinclairii* organic extracts with their relative mass spectra. LCMS method: 30-98% (MeCN:H₂O), 10 mins. c. Quantification of myriocin extracted from the cell pellet or fermentation broth by *I. sinclairii* at 25 °C and 37 °C (left) or *M. sterilia* at 37 °C and 45 °C (right) at 24 d. The mean weight of myriocin (μg per 100 mg dried cells or per 1 mL culture medium) is reported above each bar (n ≥ 3).

growth on agar plates was faster at the higher temperature. A comparison of the mean cell biomass produced by the two fungi in 50 mL fermentation medium grown at 37 °C for 24 d showed that *I. sinclairii* generated significantly more biomass (0.33 mg) than *M. sterilia* (0.25 mg) ($p \leq 0.03$, $n = 5$) but yielded ~ 5-fold less myriocin per 50 mL culture. Overall, *M. sterilia* produced more than 7-fold the amount of myriocin per 100 µg cell pellet than *I. sinclairii*, and secreted 5.5-fold more per 1 mL of supernatant. Retention of such high levels of myriocin in the cell pellets relative to secreted amounts suggests these fungi have a highly-effective resistance mechanism to the myriocin they produce.

De novo sequencing and genome assembly for *I. sinclairii* and *M. sterilia*.

To identify the myriocin BGC, the genomes of *I. sinclairii* and *M. sterilia* were sequenced using the Illumina MiSeq and the Oxford NanoPore MinION sequencing platforms. The k-mer spectra of the Illumina data indicated a single well-defined coverage peak for both species and suggested a haploid genome size of c. 20 Mbp for *I. sinclairii* and c. 30 Mbp for *M. sterilia*, with little evidence for diploidy in either species (heterozygosity was estimated as 0.37 and 1.71 %, respectively). However, read coverage also suggested an increase in sequence redundancy in the *M. sterilia* assembly compared to *I. sinclairii* (7.32 % vs 4.04 % redundant read alignments; 1.37 % vs 0.65 % redundant assembly length), so a small degree of heterozygosity in a diploid *M. sterilia* genome cannot be ruled out. High-quality genome assemblies were achieved with total lengths of 25.2 Mb for *I. sinclairii* and 34.2 Mb for *M. sterilia* (Fig. S3a), which fall within the range for Ascomycota (up to 63 Mbp)²². The assemblies were highly contiguous ($N_{50} = 1.4$ Mb and 817 kb for *I. sinclairii* and *M. sterilia*, respectively) and had BUSCO completeness scores of 98.6 % and 99.3 %, respectively. In both genome assemblies, raw read coverage per contig clearly identified a single high-coverage contig derived from the mitochondrial genome (*I. sinclairii*: 158,893bp [contig Segkk24]; *M. sterilia*: 180,440 bp [contig Segkk118]). We identified 8,167 and 8,706 protein-coding genes, including 27 and 20 putative secondary metabolite BGCs for *I. sinclairii* and *M. sterilia*, respectively (Fig S3b,c). The *I. sinclairii* genome contained a broader range of secondary metabolite BGCs than *M. sterilia*, with putative representatives of 7 of 8 classes, including 3 RiPP (ribosomally-synthesized and post-translationally modified peptides (reviewed in²³) BGCs, compared to 5 classes and no RiPP BGCs in the latter.

Myriocin biosynthetic gene cluster prediction and identification

The structure of myriocin suggests that it is derived from an amino acid head-group and an C₁₈ fatty acid-like precursor (Fig. 2a). Long-chain fatty acids in secondary metabolites

are assembled by megasynthases that are usually either a fatty acid synthase (FAS) or a polyketide synthase (PKS). The fatty acid intermediate produced by the megasynthase is then combined with an amino acid by a pyridoxal 5'-phosphate-dependent (PLP-dependent) alpha-oxo-amine synthase (AOS)-like enzyme. As genes encoding the enzymes involved in secondary metabolite pathways tend to cluster within the genome, we searched for coding sequences in the genomes of both fungi for possible PKS and AOS genes that were paired within a BGC. The search revealed a PKS – AOS gene pair, which shared high sequence identity, in *I. sinclairii* Cluster 5 and *Mycelia sterilia* Cluster 18 (Fig. 3a). Within these pairs, one gene encodes a fully reducing iterative type I polyketide synthase (iPKS) of 2500-2513 aa (73.5 % identity, 99 % coverage), while the other encodes a predicted malonyl-CoA selective acyltransferase (AOS/AT) of 480 aa (78.8 % identity). Identification of this shared gene pair within similar clusters across two myriocin-producing species strongly suggested that they function in myriocin biosynthesis. Furthermore, Clusters 5 and 18 also contained 5 further genes with high similarity and synteny (Fig. 3b), assigned as *myrC*, *myrD*, *myrF*, *myrG*, *myrH* (see below), which strongly suggested that these clusters share a function and are involved in myriocin biosynthesis.

Comparative and structural analysis of myriocin biosynthesis genes, *myrB*, *myrA* and *myrF*

We undertook comparative and structural analyses of three key enzymes from the identified clusters that are predicted to be involved in the first steps of myriocin biosynthesis, the iPKS, the AOS and the ketodihydrosphingosine reductase (KDSR).

The myriocin iterative Polyketide Synthase (iPKS, *myrB*): The iPKSs from both fungi share high sequence identity with several fungal synthases known to produce long-chain fatty acids. These include the lovastatin diketide synthase (LovF) homologues from *Aspergillus* species (65-66 %, ≥99 % coverage), homologues identified in *Hypoxyylon* species (68-70 %, ≥99 % coverage) and in *Hypomontagnella monticulosa* (≥68 %, 99 % coverage) (Fig. 3c). We therefore predicted that the iPKSs identified in *I. sinclairii* and *M. sterilia* are responsible for the C_{18:1} carbon scaffold of the myriocin precursor, formed by the iterative Claisen-like condensation of one acetyl-CoA starter unit with eight malonyl-CoA extender units (Fig. 4a). A similar PKS enzyme, SphB, was recently identified in the biosynthetic pathway of sphingofungin, an inhibitor of sphingolipid biosynthesis expressed by *Aspergillus fumigatus*²⁴. In accordance with this nomenclature, the two iPKSs we identified are hereby

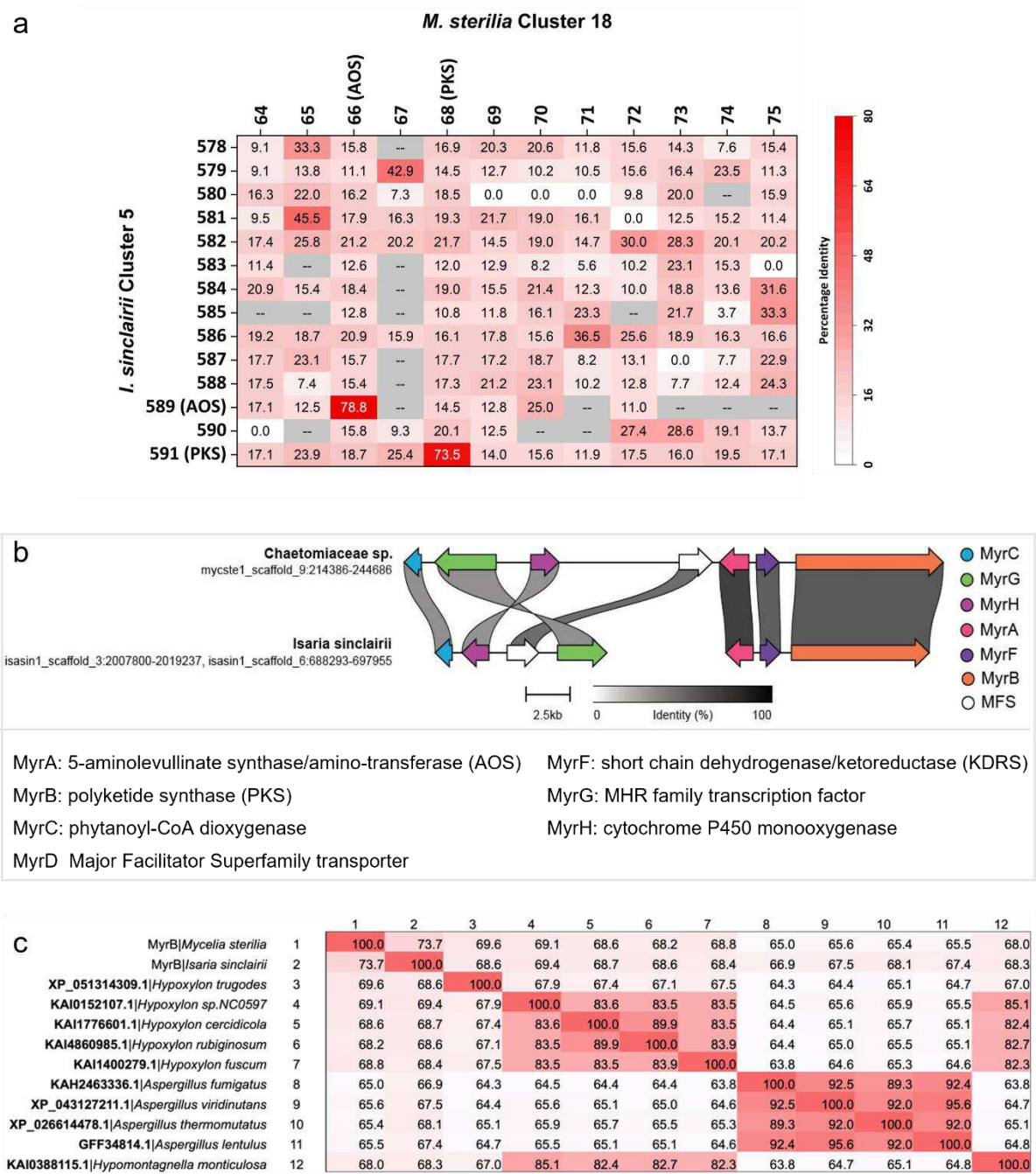


Fig. 3: Identification and synteny in *I. sinclairii* and *M. sterilia* genomes of a shared gene cluster containing the iPKS and AOS required for myriocin biosynthesis. a. Heatmap indicating the percentage identities for each pair of open reading frames (ORFs) within the two clusters predicted to produce myriocin in *M. sterilia* (Cluster 18) and *I. sinclairii* (Cluster 5). The pairwise percentage identities between the AOS (PLP-dependent enzymes) and iPKS sequences indicated the highest homology (78.8 % and 73.5 % identity), respectively). b. Synteny plot for the 7 core genes (listed) shared between *I. sinclairii* and *M. sterilia*. c. Percent identity matrix between *MyrB* (PKS) and fungal homologues. NCBI accession numbers are highlighted in bold.

annotated as putative pre-myrriocin synthase genes, *IsmyrB* and *MsmyrB*, from *I. sinclairii* and *M. sterilia*, respectively.

Protein modelling shows that the MyrBs exhibit canonical fatty acid synthase (FAS)-like architectural features^{25,26}, including a predicted homomeric interface comprising the β -ketoacyl synthase (KS), dehydratase (DH) and enoyl reductase (ER) domains (Fig. S4a). Of note, both MyrBs possess an S-adenosylmethionine-dependent methyltransferase (SAM-MT) domain bearing key active site mutations, including a T→R mutation situated in the highly conserved S-adenosylmethionine binding pocket (R₁₄₀₃ in *IsMyrB* and R₁₄₂₄ in *MsMyrB* (Fig. S5a,b)²⁷⁻³⁰. The occlusion of this binding site by a bulky, charged sidechain likely inactivates the MyrB SAM-MTs and may explain the lack of methyl branching in myriocin; these domains may instead serve a solely architectural role in the iPKSs.

The myriocin AOS (*myrA*): As MyrB encodes no identifiable chain-terminating domain such as a thioesterase or a thioester reductase, the release of the polyketide product must therefore be mediated by a *trans*-acting enzyme. It is known that release of the dimethyl-branched C₁₈ fumonisin precursor from the iPKS in *F. verticillioides*, Fum1, is facilitated by the AOS, Fum8^{31,32}, which catalyses the PLP-dependent condensation of the precursor with L-alanine. Recently, Bissell *et al.* reported a similar AOS-dependent release mechanism in the producers of another SLM, sphingofungin, in which *A. fumigatus* and *Paecilomyces variotii* utilise 2-aminomalonnate (rather than L-alanine or L-serine) to yield the sphingofungin precursor, 3-keto-presphingofungin²⁴. The unusual AOS was named SphA. We therefore hypothesised that the head of the myriocin molecule may originate via a similar AOS-mediated condensation between the C_{18:1} polyketide and an amino substrate. The genes encoding AOS homologues in *I. sinclairii* and *M. sterilia* BGCs are hereafter named *IsmyrA* and *MsmyrA*, respectively.

While the two MyrA sequences are highly similar to each other, they bear little relation to the AOS involved in fumonisin biosynthesis, Fum8 (< 20 % sequence identity). In contrast, *IsMyrA* and *MsmyrA* share > 60 % sequence identity with SphA from *A. fumigatus* (AfSphA) and *P. variotii* (PvSphA). Additional close homologues were identified in *Penicillium polonicum*, *Monosporascus sp. CRB9-2* and *Neonectria ditissima*. Structurally, MyrA is predicted to resemble several 8-amino-7-oxononanoate synthases (BioF) and serine palmitoyltransferases (SPT) that are deposited in the Protein Data Bank (pLDDT ≥94.0, pTM = 0.93, Fig. S6a). Using these structural models, L-serine, 2-aminomalonnate and 2-amino-2-(hydroxymethyl)malonnate were docked into the active site as PLP-bound intermediates (Fig. 4b), thereby enabling the identification of the key catalytic lysine (K₂₇₁ in *IsMyrA* and

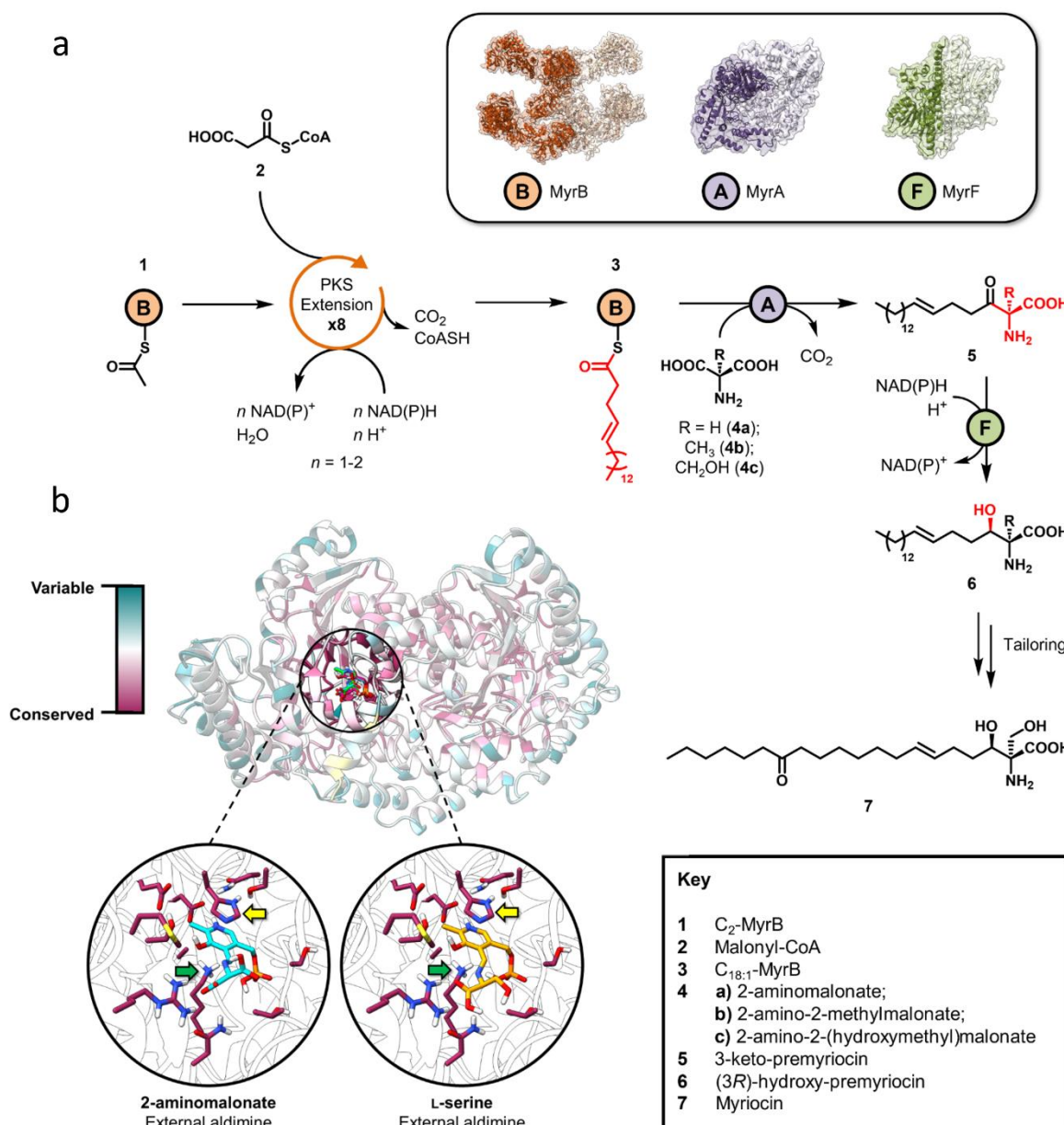


Fig. 4: Myriocin structure and proposed biosynthetic pathway a. Hypothetical biosynthetic cascade towards myriocin via MyrB, MyrA and MyrF. The C_{18:1} scaffold is produced by MyrB and released by MyrA via condensation with 2-aminomalonate (or derivative). Following ketoreduction by MyrF, the final molecule is furnished by oxidative tailoring, likely by MyrH and MyrC. b. Substrate binding to MyrA, the α -oxo-amine synthase (AOS) involved in the condensation of an amino headgroup with a fatty acid chain. Molecular docking experiments using Autodock Vina. 2-aminomalonate and L-serine were docked as PLP external aldimines. Binding affinities (kcal mol⁻¹) using the Vina forcefield are shown. Residues are coloured by conservation; maroon residues are highly conserved, whereas teal residues are mutable. Green and yellow arrows show the position of the catalytic L₂₇₁ and H₁₅₂, respectively.

MsMyrA) and conserved pocket residues (including N₆₃, H₁₅₂, M₂₀₉, A₂₃₁, D₂₃₇ and R₄₁₃). Furthermore, a plausible protein-protein docking interface was identified between the MyrB acyl carrier protein (ACP) and MyrA from both species (pTM = 0.88), with the catalytic serine of the ACP positioned directly above the substrate tunnel leading to the catalytic K₂₇₁ of MyrA (approximately 3.4-3.8 nm away, Fig. S7a). The overall orientation of the protomers compares favourably (albeit not identically) to the recently solved *E. coli* BioF/crypto-ACP crosslink by Chen *et al* (PDB: 8DLE)³³. Furthermore, the AOS-ACP docking interface shows electrostatic complementarity between the electropositive surface of MyrA and the acidic MyrB ACPs, which may underpin the mutual recognition between these two proteins as observed in other ACP-dependent biosynthetic cascades (Fig. S7b,c)^{34,35}.

The myriocin KDSR-like enzyme (*myrF*): Like sphingosine (and sphingosine analogues), myriocin features an (*R*)-hydroxy substituent at the C₃-position. Therefore, the 3-keto-premyriocin must be reduced by a 3-ketodihydrosphingosine reductase (KDSR)-like biocatalyst to furnish (3*R*)-hydroxy-premyriocin. Such KDSRs were identified in both *I. sinclairii* and *M. sterilia* BGCs and are hereafter named *IsMyrF* and *MsMyrF*, respectively. As with *myrB* and *myrA*, *IsmyrF* and *MsmyrF* share high sequence identity with each other (72.3 %, 99 % coverage) and with homologues from other ascomycetous fungi (including *Aspergillus*, *Hypoxylon* and *Penicillium* species). The MyrFs were modelled as homodimers (pLDDT ≥93.9, pTM ≥0.95, Fig. S8a,b) and feature Rossmann folds ubiquitous across the NAD(P)H-binding short-chain dehydrogenase/reductase (SDR) oxidoreductase family. A 600-650 Å³ binding pocket was identified by topological analysis of the MyrFs, each encompassing the glycine-rich nucleotide binding motif TGxxGxxG (T₁₅GGSGGLG₂₂ in *IsMyrF* and T₁₃GGSGGLG₂₀ in *MsMyrF*); NADP⁺ was docked in these binding pockets in a productive conformation, comparable to homologous SDR crystal structures, including the recently-solved KDSR from *Cryptococcus neoformans* (PDB: 8JAT, 6D9Y, 3P19, Fig. 9a,b)^{36,37}.

Taken together, our analysis suggests that myriocin production follows a similar biosynthetic logic to sphingofungin and fumonisins (Fig. 4). In a hypothetical biosynthetic cascade, the monounsaturated C_{18:1} polyketide scaffold is first synthesised by MyrB and released by MyrA via a Claisen-like condensation with 2-aminomalonate (or a derivative thereof), yielding 3-keto-premyriocin. The subsequent ketoreduction is performed by MyrF to furnish (3*R*)-hydroxy-premyriocin. Additional tailoring reactions are required to introduce the (4*R*)-hydroxy and 14-keto functional groups. As oxidation at these positions cannot be achieved by a polyketide synthase, these modifications are mostly likely catalysed by a pair

of dedicated tailoring enzymes. By homology, we suggest that the cytochrome P450 monooxygenase, MyrH, and the phytanoyl-CoA dioxygenase homolog, MyrC, may provide this oxidative tailoring. Overall, we hypothesise that the combination of iPKS, AOS and KDSR-like biocatalysts act as a fingerprint for the biosynthesis of sphingosine analogues across many fungal species.

MyrA expression and substrate binding

To interrogate the functionality of the myriocin AOS enzyme, the N- and C- terminals of the *IsMyrA* protein coding sequence were identified through analysis of published transcriptomics studies previously undertaken in fungi with an identified myriocin BGC homolog (Fig. S10a and see below). The *IsMyrA* protein carrying a C-terminal His-tag was synthesised and expressed in *Escherichia coli*. The expressed recombinant *IsMyrA* protein was soluble and was purified by affinity and size-exclusion chromatography (Fig. 5a). *IsMyrA* displayed the characteristic UV-VIS spectrum associated with a PLP-dependent enzyme with a maximum absorbance at 420 nm (Fig. 5b). The ability of the enzyme to generate the external aldimine form by binding L-serine was measured by the addition of increasing concentrations of the amino acid (0 to 80 mM). Addition resulted in increased absorbance at 420 nm suggesting *IsMyrA* bound this amino acid. By analogy with SphA, titration of aminomalonate with *IsMyrA* led to a small shift of a few nm to a lower wavelength (410 nm) and a decrease in the intensity of the signal (Fig. 5b). This is consistent with the prediction that *IsMyrA* binds this unusual amino acid²⁴. The predicted product of the condensation of the *IsMyrB* polyketide intermediate with an amino acid substrate by the AOS, *IsMyrA*, is 3-keto-premyriocin²⁴ (Fig. 4a). In the absence of recombinant acylated *IsMyrB*, acyl-CoA thiol esters were used as substrates in an enzymatic assay to determine if *IsMyrA* was catalytically active. C₁₆ and C₁₈ acyl chains were used as they are closer in length to the predicted natural substrate. Prompted by the work of Bissell *et al.*, both aminomalonate and L-serine were tested as amino acid substrates for the decarboxylative Claisen condensation (Fig. 5c)²⁴.

Using an indirect assay that monitors the release of CoASH from the acyl-CoA substrate (Fig. S10e)³⁸, the optimal amino acid and acyl-CoA substrate combination was aminomalonate, where C₁₈-CoA gave the highest activity (Fig. S10f). To further support the activity observed in the indirect assay, Liquid-Chromatography-Mass Spectroscopy (LCMS) was used to identify the products formed. The two expected decarboxylated products (Fig. 5d) were observed, with the greatest product formation detected for a key C₂₀ myriocin

amino-ketone intermediate that contains a carboxylated head-group (Fig. 5e). Unlike SphA from the biosynthesis of sphingofungin, which only accepts aminomalonate, *IsMyrA* also catalysed the reaction using L-serine, since we also detected the C₂₀ product when this amino acid was used (Fig 5d). However, based on the increased product generated when

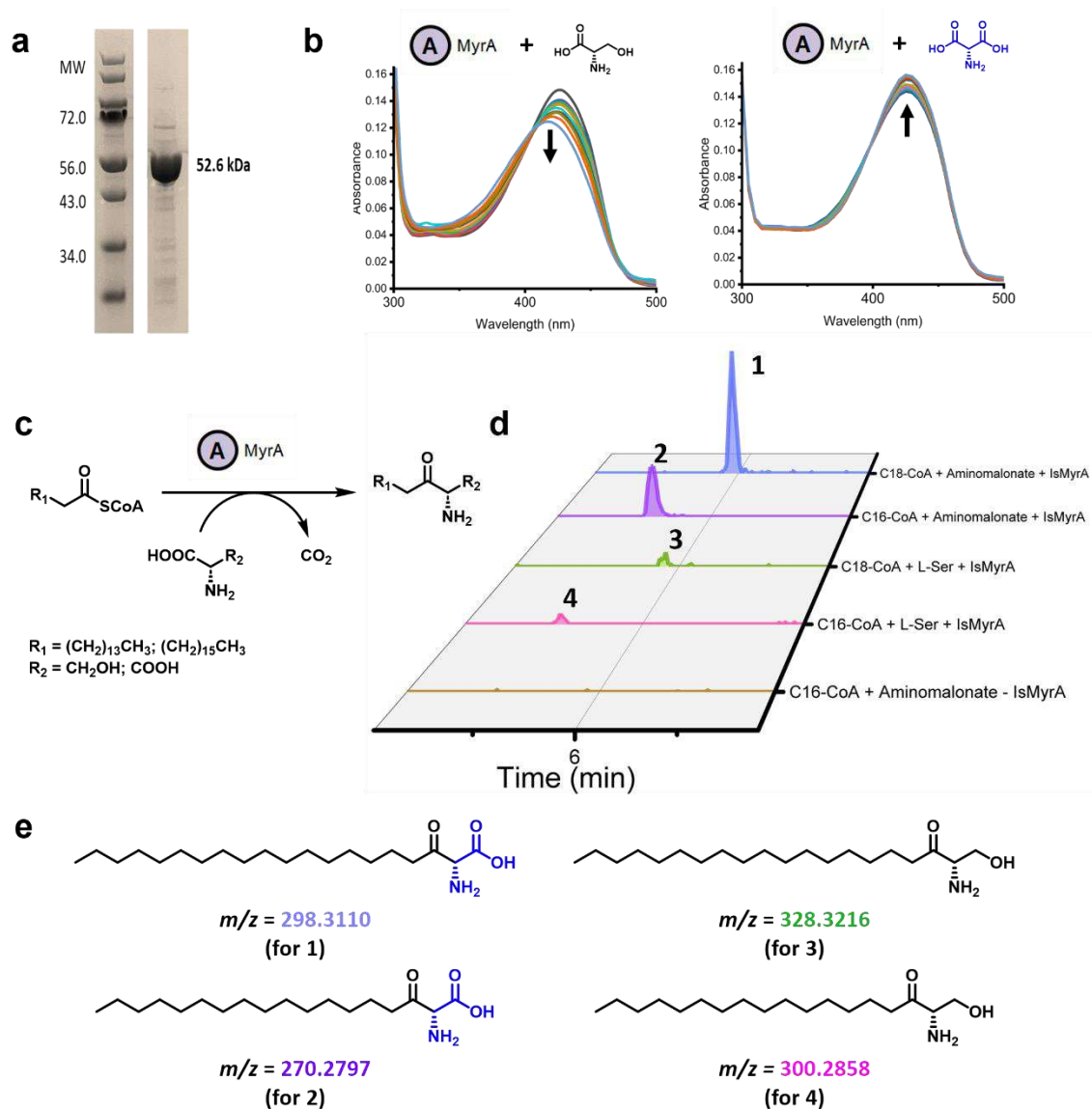


Fig. 5: Aminomalonate is the preferred substrate of *IsMyrA* *in vitro*. a. SDS-PAGE gel of purified recombinant *IsMyrA*. b. UV-vis spectrum of 40 μ M *IsMyrA* in increasing concentrations of L-serine (left) and aminomalonate (right). c. Reaction of *IsMyrA* with C16 and C18 acyl-CoA thioester substrates to sphingosine products with L-Ser and aminomalonate. d. Extracted ion chromatograms (EIC) overlays of the reaction of *IsMyrA* with acyl-CoAs and amino acids. e. The corresponding $+[m/z]$ ion mass observed for each product and its molecular formula.

aminomalonate was used as the substrate, we predict this to be the most suitable substrate for *IsMyrA* for production of a 3-keto-premyriocin intermediate by a decarboxylative Claisen condensation reaction. These data are supported by docking studies where the key PLP:amino acid external aldimine intermediates are shown to bind in a highly conserved active site (Fig 4b). Given the high sequence identity (78.8 %, Fig. 3b) between *IsMyrA* and *MsMyrA*, it is likely that they use the same substrate combination.

Distribution and evolution of homologs of myriocin/sphingolipid BGCs across *Pezizomycotina*

Close homologs of myriocin and sphingofungin BGCs are broadly, but sparsely distributed across *Sordariomycetes* (*Xylariales*, *Hypocreales*, and *Chaetomiaceae*), *Dothideomycetes* (*Pleosporales* and *Mycosphaerellales*), and *Eurotiomycetes* (*Eurotiales*, *Onygenales*, and *Chaetothyriales*). A phylosynteny analysis using a database that included 1,112 *Pezizomycotina* genomes demonstrates homology between myriocin BGCs and sphingofungin BGCs (Fig. 6). The 170 members of the BGC family that include the central PKS, *myrB*, were detected through a query of six genes with shared synteny between *I. sinclairii* and *M. sterilia* (*myrA,B,C,F,G,H*) plus sphingofungin BGC genes *sphD* (encoding a Major Facilitator Superfamily transporter) and *sphE* (encoding a ketoreductase, KDRS). These BGCs were inferred to contain between 4 and 8 homologous genes. Similarity in gene order among BGCs largely follows two syntenic patterns that correlate with the two clades in the *MyrB* gene tree (Fig. 6). Clade I (supported by 100 rapid bootstrap replicates [BS]) is composed largely of *Xylariales*. The majority of myriocin BGCs in Clade I contained homologs of 6 genes (*sphD*, *myrH*, *myrA*, *myrF*, *myrB*, and *myrG*), while *myrC* (a phytanoyl-CoA dioxygenase) was not detected. The bulk of the genes encoding the 'accessory' enzymes in Clade I myriocin BGCs (*myrA,F,H*, *sphD*) lie upstream of the PKS, *myrB*, in the genome. In contrast, Clade II (also supported by 100 BS) is composed largely of *Eurotiomycetes* and mostly contained BGCs with homologs of all 8 query genes. Accessory enzyme genes in Clade II tend to be downstream of *myrB*, except for *myrA*.

Gene order in *M. sterilia* is unique, but similar to two BGCs with which it forms the clade (94 % BS) adjacent (without support) to Clade I. The *M. sterilia* BGC contains a large gap between clusters of three (*myrC,G,H*) and four (*sphD*, *myrAFB*) genes. Interestingly, the BGCs correspond to a similar grouping in the six gene homologs in *I. sinclairii*, which also form two clusters of four (*myrG,sphD*, *myrH,C*) and three (*myrA,F,B*) genes. However, while

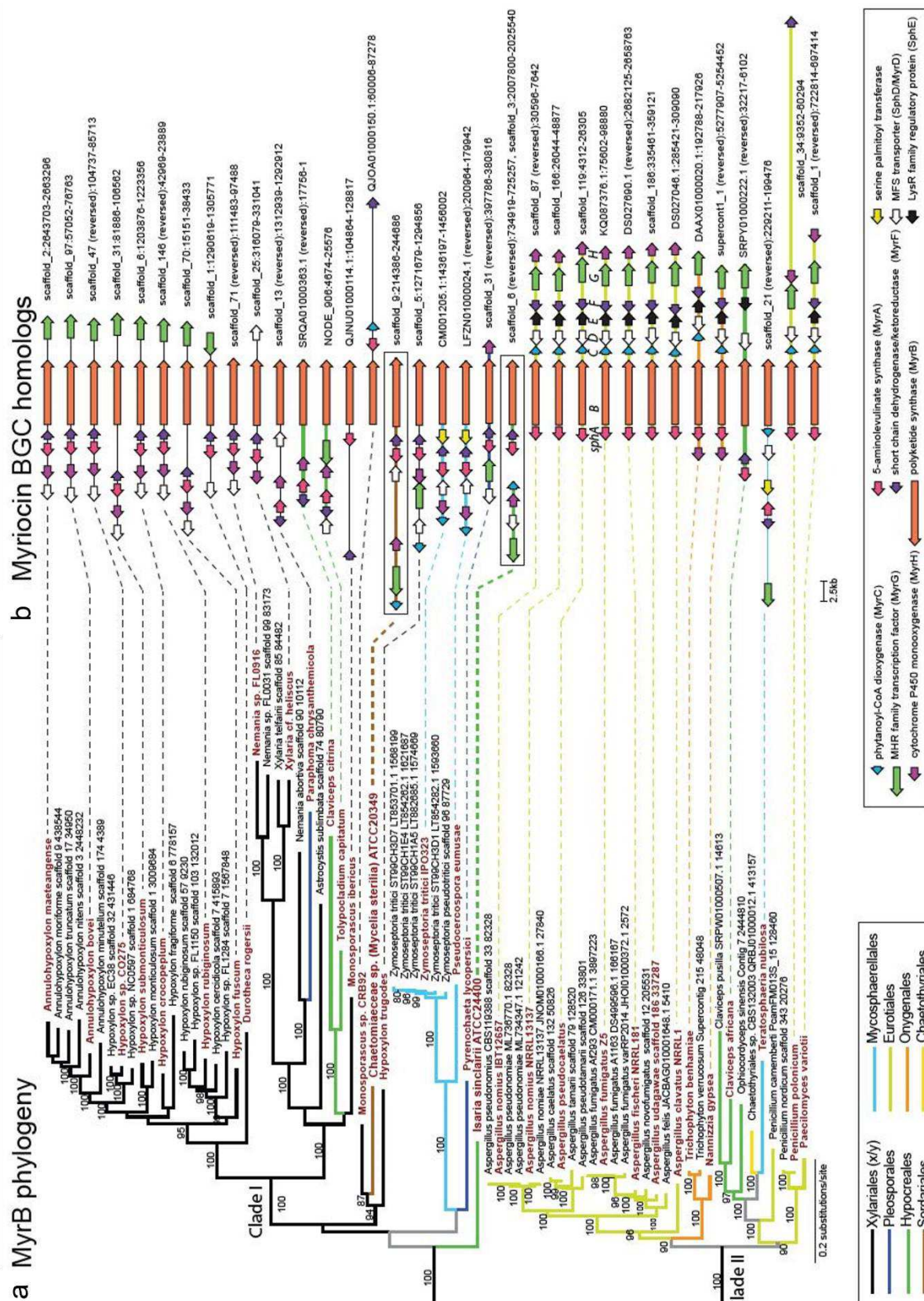


Fig. 6: Phylosynteny of the myriocin biosynthetic gene cluster. a. Maximum likelihood phylogeny of putatively functional MyrB sequences in Pezizomycotina. Red taxon names indicate MyrB sequences from representative BGCs in (b), sampled across the phylogeny. BGC coordinates are provided for those not shown in (b). Support values are the percentage of 1000 ultrafast bootstraps in

IQTREE and only values ≥ 80 are shown. The scale bar and branch lengths signify the number of substitutions per site. b. Representative BGCs are aligned at the MyrB gene to demonstrate shared gene order among BGCs associated with MyrB clades. Gene homologs encoding the sphingofungin pathway (sphA,B,C,D,E,F,G,H) are indicated above the *A. fumigatus* Z5 cluster. Taxonomy at the level of order of genomes containing MyrB is reconstructed on tree branches and shown in connections to BGCs and scaffolds as shown in the key.

in *M. sterilia* the clusters are split by a gap, in *I. sinclairii* the clusters are each on separate scaffolds that correspond to the split in the *M. sterilia* cluster. While one of these scaffolds shares synteny with *M. sterilia* and most of clade I, the other has a unique gene order. The only core enzyme-encoding gene that does not occur in all major clades is *myrC* (phytanoyl-CoA-dioxygenase), which was not found in any clade I BGCs. We also failed to detect homologs of the LysR family regulatory protein (*sphE*) outside of Clade II. A serine palmitoyl transferase was detected only in *Dothideomycetes* BGCs, but in both Clade I and in Clade II.

The dominance of *Xylariales* in *myrB* Clade I and *Eurotiomycetes* in *myrB* Clade II, and general correspondence between the *myrB* tree (Fig. 6) and the species tree within these clades (Fig. 7) (exceptions noted below), suggests myriocin and sphingofungin BGCs have long histories within those two lineages, respectively. We note that a few fungi from other lineages are nested within each clade, suggesting their recent myriocin biosynthesis origins were the result of horizontal gene transfer (HGT). For example, two *Hypocreales* BGCs, in *Claviceps citrina* and *Tolypocladium capitatum*, are placed with strong support in Clade I, while those of three others (*Claviceps pusila*, *Claviceps africana*, and *Ophiocordyceps sinensis*) are placed with strong support in Clade II. The first of these putative HGTs is reproduced in the *myrA* and *myrF* gene trees, although those trees are not well supported overall (Supplementary Data S11: konkel_slot_tables). Similarly, there are two *Pleosporales* (Dothideomycetes) BGCs, one placed deep in Clade I among *Xylariales* species, and the other in an unsupported position sister to an unplaced clade of *Mycosphaerellales* (Dothideomycetes) BGC. The *Teratosphaeria nubilosa* (Mycosphaerellales) BGC is sister to Chaetothyriales sp. CBS132003 (Eurotiomycetes) within Clade I.

The placement of the *M. sterilia* (Sordariales) myriocin BGC in a clade adjacent to Clade I, close to and with similar gene order to the BGC in *Hypoxylon trugodes* (*Xylariales*), suggests an HGT from *Hypoxylaceae* to *Chaetomiaceae*. By contrast, the fragmented BGC in *I. sinclairii* (*Hypocreales*) is not particularly close to any other taxa in the MyrB phylogeny, leaving its origin cryptic. Overall, the myriocin BGC family appears to have diversified through multiple horizontal transfer events punctuating vertical descent in two main orders

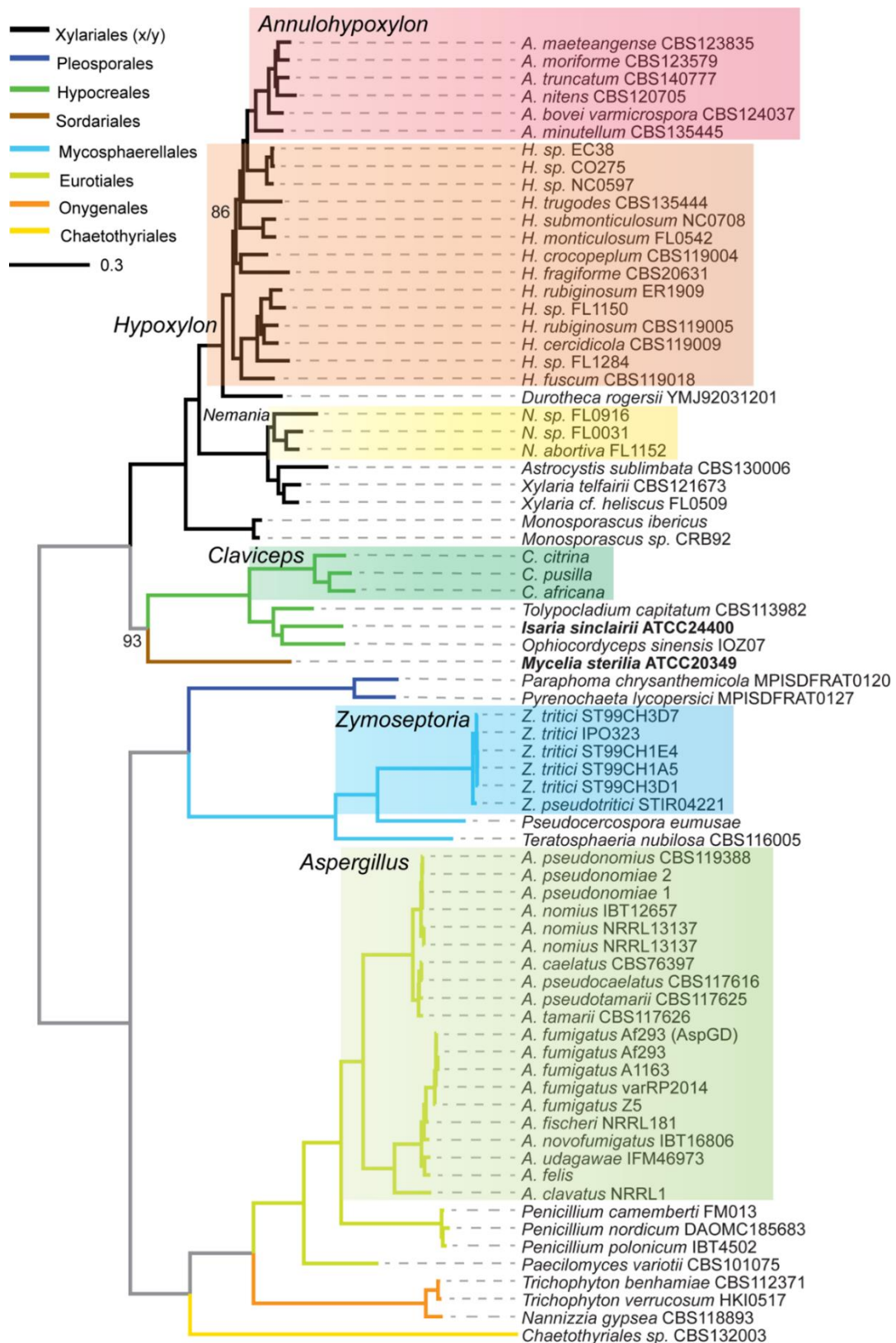


Fig. 7: Species phylogeny for all taxa in the MyrB tree. Maximum likelihood tree inferred by IQ-TREE (v.2.2.6 COVID-edition) using 752 single copy orthologs. All branches were supported by 100% of 1000 ultrafast bootstrap replicates, except for the two nodes indicated by their bootstrap percent values. The scale bar and branch lengths signify the number of substitutions per site.

(*Eurotiales* and *Xylariales*). It is likely that many BGCs have experienced gene loss and potentially reconfiguration into other gene networks because *myrA* and *myrF* trees contain orthologs of clustered genes from relatives of taxa in the *myrB* tree, and because multiple *myrB* genes were not included due to multiple stop codons in their coding sequences, indicating loss of function.

Discussion

By sequencing two myriocin-producing fungi, *Isaria sinclairii* and *Mycelia sterilia*, we have identified their shared orthologous BGC involved in myriocin biosynthesis and defined the core genes involved in the biosynthesis of the major Sphinganine-Like Mycotoxins (SLMs): myriocin, sphingofungin and fumonisin B. This group of myriocin-like secondary metabolites inhibit specific stages of the sphingosine biosynthetic pathway in eukaryotic cells, suggesting that they have evolved because this pathway has proved a successful target during fungal confrontation within the environment. However, each mycotoxin exerts differential effects against other fungi. For example, myriocin is not equally effective against the 5 clades of *Candida auris*, and viridifungins inhibit serine palmitoyltransferase in *C. albicans* but not in *Saccharomyces cerevisiae*^{19,39}. Overall, the specificity of each mycotoxin may reflect its required level of efficacy against environmentally-relevant adversaries.

Beyond the shared presence of the myriocin BGC, there appears to be little similarity in the lifestyles of *M. sterilia* and *I. sinclairii*. *M. sterilia* (ATCC24400) was isolated from a soil sample and is a generic name as no inducing conditions for spore formation or a morphological transition from the hyphal form have been identified. It can be classed as a thermophile due to its higher growth rate at 45 °C than at 37 °C in permissive media. Genome analysis places *M. sterilia* in the *Chaetomiaceae* family, which includes thermophilic species important for the production of industrially-relevant bioactive metabolites⁴⁰. Indeed, the biotechnology industry exploited thermophilic *Chaetomium globosum* and *C. thermophilum* species for myriocin production until the 2010-20 decade, when its production became fully synthetic due to its increased use as a pharmaceutical and the consequent requirement for stricter regulatory control. The ability of *M. sterilia* to produce significantly greater amounts of myriocin by biomass than *I. sinclairii* is therefore consistent with its placement within this industrially-relevant *Chaetomiaceae* sp. (*Sordariomycetes*) family.

While *M. sterilia* is a constitutively filamentous fungus, *I. sinclairii* undergoes a yeast-to-hypha transition between 25 °C and 37 °C. This temperature-directed switch is also seen

in *Candida albicans*, which lives as a commensal yeast in humans but undergoes morphogenesis to generate invasive hyphae in its pathogenic form. This switch is the reverse of the major disease-associated pathogens, *Cryptococcus neoformans*, *Paracoccidioides brasiliensis*, *Histoplasma capsulatum* and *Coccidioides immitis*, which are filamentous in the environment but grow as yeast at human body temperature. As *I. sinclairii* is an entomopathogen, it is possible that the filamentous form is required for host invasion and the formation of external fruiting bodies for wind-mediated spore dispersal outside the host.

Myriocin and other SLMs are produced by fungi with very differing lifestyles so it seems unlikely that these sphingolipid inhibitors are targeted directly at a single specific species. For example, the production of sphingofungin by *Aspergillus fumigatus* was not used as a specific defence against predation by amoeba²⁴. Instead, the diverse host range of the myriocin BGC suggests that it confers a competitive advantage to fungi as a defensive toxin against other fungi within their environment. Both *M. sterilia* and *I. sinclairii* retained and tolerated greater amounts of intracellular myriocin than they secreted, suggesting they have an internal resistance mechanism. *M. sterilia* may have co-evolved resistance to myriocin as it lies within a related clade of BGC carriers. However, HGT of a BGC that encodes a toxic metabolite is expected to favour recipient hosts with pre-existing tolerance, such as over-expression of, or mutations in, the SPT subunits, or the ability to modify myriocin to reduce binding to SPT⁴¹. Some BGCs carry an intrinsic resistance mechanism in the form of enzymes that act in both the toxin and native sphingolipid/ceramide synthase pathways. This ensures that self-production of sphingosines is maintained through an increase in output^{42,43}, thus avoiding self-poisoning during horizontal gene transfer. It is plausible that additional alternative SPTs, such as those in *Mycosphaerellales* BGCs, may replace the function of impaired native SPTs, enhancing the fitness of the pathway during HGT⁴⁴. However, the resistance mechanisms in *I. sinclairii* and *M. sterilia* remain to be elucidated.

Our finding that aminomalonate is the substrate preference for MyrA, instead of L-serine, is consistent with recent findings for sphingofungin biosynthesis in *A. fumigatus*²⁴, where successful heterologous expression of the sphingofungin BGC in a non-producing fungus, *A. niger*, suggested that aminomalonate may be a readily-available precursor in fungi. Further, replacement of the *A. fumigatus* SphA with PvSphA, its ortholog from *Paecilomyces variotii*, produced several sphingofungin variants. Therefore, even though AfSphA could accept only aminomalonate as a substrate, PvSphA was less specific and was able to exploit other aminomalonate derivatives available in *A. fumigatus*. Our work showed that, while aminomalonate is the preferred substrate for IsSphA, it is also able to accept L-

serine to produce a C20 product. Therefore, while the horizontal gene transfer of an SPT-inhibitor cluster can only succeed if a functional self-protection mechanism is in place. For example, contained within the BGC or inherent in the recipient species, the specificity of the AOS and the availability of substrates and any derivatives will determine the number of variants produced and, hence, the effective external range within the environment.

Analyses of the *myrB* distribution and phylogeny combined with comparisons of gene composition and order in homologous BGCs containing *myrB* suggest the myriocin BGC is part of a broadly, but sparsely distributed BGC family of Sphingangine-Like Mycotoxins that also includes sphingofungin BGCs, as described in *Aspergillus fumigatus* and *Paecilomyces variotii*²⁴. The *myrB* phylogeny suggests two main clades in the SLM BGC family are predominantly found in Xylariales (Clade I) and Eurotiales (Clade II). Within these clades, placement of specific clusters from alternative taxa is consistent with occasional HGT events. Specifically, the myriocin BGC identified in *Chaetomiaceae sp. (Sordariomycetes)* is strongly supported in a clade of *Xylariales* adjacent to Clade I.

The origin of the BGC family remains ambiguous because all genes except the *cis* regulatory gene are distributed through most of the tree. Because there is no support for the placement of Clade I, it is ambiguous as to whether Clade I represents an ancestral state that lacked *myrC*, or the ancestral cluster had all enzymes, but *myrC* was lost. It is also curious how enzymes are similarly sorted in subclusters in *Chaetomiaceae sp.* and *I. sinclairii* genomes in a way not seen in any others. Given the placement of *I. sinclairii* at the base of the *myrB* tree, it could represent two smaller ancestral BGCs that later merged to become the progenitor of the BGC family. Alternatively, sub-clusters in these two species might represent branch points in metabolism where alternative genetic networks are driving genomic architecture. Metabolic rewiring could also be suggested by the failure to detect the regulatory protein SphE, but again, it is currently unclear whether such a difference reflects its gain in Clade II vs loss in Clade I.

In summary, this study has defined the core biosynthetic gene cluster and associated pathway towards myriocin, highlighting the key enzymes responsible for the formation of the fatty acid backbone and the characteristic head group. Moreover, determination of the genome sequences of two myriocin-producing fungi allowed a deep phylogenetic analysis that revealed the widespread presence of this BGC across many other fungi. Furthermore, a similar biosynthetic logic was recently discovered in *Pseudomonas fluorescens* that produces an SLM that protects *C. elegans* from attack by a pathogenic *Bacillus*⁴⁵. Our discovery suggests that many other cross-kingdom organisms have the potential to

synthesize myriocin and other SLMs, whose structures and functions remain to be elucidated.

MATERIALS AND METHODS

Cell culture and imaging *I. sinclairii* (ATCC24400) and *M. sterilia* (ATCC20349) were obtained from the American Type Culture Collection. *I. sinclairii* was stored at -80 °C as 500 µL yeast cells in Tryptic Soy Broth (TSB) (3 % (w/v) tryptic soy (Fisher)) in 500 µL 50 % glycerol. *M. sterilia* was stored as agar cubes (from excised from a potato dextrose agar plate (PDA)) in 500 µL 50 % glycerol. *I. sinclairii* yeast was grown at 25 °C in TSB or on tryptic soy 2 % agar (TSA) and hyphae induced in TSB at 37 °C. Cubes of *M. sterilia* were thawed at room temperature (RT) and placed on PDA or in liquid PD (0.039 % (w/v) (Sigma)) at 37 °C with shaking at 200 rpm. *I. sinclairii* nuclei were stained with VECTASHIELD® with DAPI (Vector Laboratories, CA). Fungal cell walls and septa were imaged at 63x magnification on a fluorescence microscope (Zeiss Axiovision), after addition of 10 µg/mL Calcofluor White, centrifugation and washing in ddH₂O. To measure colony growth, *I. sinclairii* was cultured as yeast and *M. sterilia* hyphae were picked from an plate and fragmented by vortexing in ddH₂O. Cells (10 µL) were inoculated onto TSA or PDA plates and incubated at 25, 37 or 45 °C. Colony diameter was measured at 24 d for *I. sinclairii* or 5 d for *M. sterilia*.

Genome sequencing and assembly Genomic DNA was extracted using a QIAGEN QIAamp kit and sequenced on the Illumina MiSeq (2x300bp) and Oxford NanoPore MinION (R9.4) platforms (see Supp. Methods for Genome Sequencing). Genomes were assembled, scaffolded and polished using a hybrid strategy and assembly completeness was estimated against the OrthoDB v8 fungal reference set of 290 single-copy genes (see Supp. Methods for Genome Assembly).

Identification of myriocin gene cluster using FungiSMASH. The genomes of *I. sinclairii* and *M. sterilia* were analysed using antiSMASH fungal version 7.1.0; the detection strictness was set to “relaxed” and the extract features “KnownClusterBlast,” “ClusterBlast,” “SubClusterBlast,” “MIBiG cluster comparison,” “ActiveSiteFinder,” “REFinder,” and “Cluster Pfam analysis” were used⁴⁶. The genes predicted in biosynthetic gene clusters (BGCs) were further searched using NCBI BLAST. The genomic locations were extracted from the contigs using SAMTOOLS FAIDX⁴⁷. The sequences of the two assemblies were then aligned and inspected in GENEIOUS. In *I. sinclairii*, the myriocin cluster is designated as Cluster 5 and is located on contig Segkk0; in *M. sterilia*, myriocin is Cluster 18 and is located on contig Segkk40.

BGC distribution and diversity. Homologs of the myriocin BCG locus were detected in a database of 2,236 fungal genomes (Supplemental data S11: `konkel_slot_tables`) using *CLOCI* v0.1.11 with default parameters as described in Konkel *et al.*⁴⁸. Briefly, *CLOCI* circumscribes gene cluster families by identifying homologous loci with unexpectedly shared microsynteny compared to null distributions of randomly sampled loci. Gene cluster family 1920, corresponding to myriocin, was extracted by identifying the similarity network of homologous loci that contain close homologs of MyrB (Supplemental data S11 – `konkel_slot_tables`). Best gene models were then determined for each locus using exonerate v.2.2.0 with the protein2genome:bestfit model, minimum identity of 23 %, and maximum intron size of 200 bp, using manually curated sequences of MyrA, MyrB, MyrC, MyrF, MyrG, MyrH, SphE, and SphD as queries. Putative pseudogenes were identified by manually checking for at least 2 stop codons in predicted conserved runs of amino acid sequences, and additional sequences with one stop codon were removed after verifying truncation > 30 % in preliminary alignments and/or long branches in preliminary phylogenetic analyses. Gene homology and alignment for 34 representative BGCs across the MyrB phylogeny (see below) were determined with clinker v.0.0.28 using exonerate gene coordinates and a minimum amino acid identity of 22.5 %.

Phylogenetic analyses. Two species trees were generated for this study. To define taxonomic placement of *Mycelia sterilia*, we inferred a phylogenomic tree of 483 *Sordariomycetes* genomes derived from an alignment of 51 near single-copy genes (Supplemental data S11 – `konkel_slot_tables`). *Mycelia sterilia* was provisionally identified as *Chaetomiaceae* sp. based on placement in a clade of *Chaetomiaceae* species with 100 % ultrafast bootstrap support. To identify the genes for phylogenomic reconstruction we clustered protein sequences from all proteomes using MMseqs v.14.7e284 implemented in *db2hgs* (Mycotools v.0.30.36) and defined near single-copy genes as those present in *Mycelia sterilia* and missing from at most one genome, with a median copy number per genome of one, and copy number standard deviation less than 0.5. To identify the best hit protein for these sequences in genomes with multiple copies, we aligned each near single-copy sequence group with mafft v.7.487, constructed profile hidden Markov models of alignments using hmmbuild v.3.3.2, and identified the best hit for each model in all genomes the models were present in using hmmsearch implemented in *db2search* (Mycotools). We built the phylogenomic tree by aligning the final sequence set in mafft v.7.487, trimming with ClipKIT v.1.3.0, and inferring the multi-gene tree with best evolutionary models applied to each gene partition via IQ-TREE v.2.2.6 COVID-edition.

To compare the MyrB tree to the 74 taxa where it was deemed functional, a phylogenomic tree was generated with these genomes from single-copy orthologs (SCO)

determined by OrthoFinder v.2.3.12. Individual amino acid trees were computed for 752 SCO by aligning with mafft (v.7.487), trimming via ClipKIT (v.1.3.0), and inferring the maximum likelihood topology in IQ-TREE (v.2.2.6 COVID-edition) with 1000 ultrafast bootstrap replicates. 95 SCO had an average bootstrap support of 90% and were concatenated for phylogenomic analysis (Supplemental data S11: konkel_slot_tables). The phylogenomic tree was determined using IQ-TREE with best evolutionary models for each partition determined by ModelFinder and support was evaluated by 1000 ultrafast bootstrap replicates. To generate gene trees for MyrA, myrB, and MyrF, amino acid sequences were aligned with mafft v.7.407 with default parameters, trimmed with trimal v.1.4 using the -automated1 method, and maximum likelihood phylogeny inferred using IQ-TREE v. 1.6.11 with models determined by ModelFinder (MyrA=LG+I+G4, MyrB=JTT+F+R5, MyrF=JTT+I+G4) and branch support evaluated by 1000 ultrafast bootstrap replicates (Supplemental data S11: konkel_slot_tables).

Generation of cluster heatmaps. Pairwise percentage identities for the PKS genes of both fungi were generated using Clustal OMEGA⁴⁹. The PKS open reading frame (ORF) was translated using standard codon usage. Percentage identity for each pairing was used to produce heat maps with 10 % bins (0-10 %, 11-20 % etc.). Where the sequence of key biosynthetic genes was split across more than one ORF, all ORFs were run and the ORF producing the highest percentage identities across all its comparisons was used in the heat map. To investigate whether there was extended homology in the putative myriocin clusters, Clustal OMEGA was used to determine percentage identities for each pairwise comparison of the ORFs within the two clusters. BLASTp searches were performed for each of the ORFs annotated as containing “central biosynthetic genes” for each cluster with an assigned type. For clusters where there was more than one central biosynthetic gene ORF, each was run independently. Putative clusters were not examined. The BLASTp search database was restricted to fungi. The top 5 hits returned for each search were examined to determine whether there were any validated proteins. Any proteins denoted as putative or hypothetical were excluded. The top hit, out of hits fitting these criteria, was selected.

DNA sequences Segkk0.cluster005 and Segkk0.cluster018 were processed using ExPASy Translate. Open reading frames encoding PKS domains were identified by BLASTp analysis and searched against ClusterCAD⁵⁰ and UniProt to define domain boundaries (with the exception of the inactive SAM-dependent methyltransferase). Nine of the closest myriocin PKS homologues were selected for sequence alignment following identification by BLAST analysis, accounting for both percent identity and query coverage. Sequence

alignment, phylogeny and percent identity were performed using Clustal Omega and visualised using ESPript3.0 and Microsoft Excel.

Sequence Analysis BLASTp was configured to search non-redundant sequences (NRS), UniProt KB/Swiss-Prot or Protein Data Bank (PDB) databases for homologous sequences. MyrB domain boundaries were determined using a combination of ClusterCAD⁵¹ and BLASTp. Multiple sequence alignments (MSA) and percent identity matrices were computed using Clustal Omega⁴⁹ using default parameters. MSAs were visualised using ESPript 3⁵². Theoretical molecular weights (MWs) and isoelectric points (pI) were computed using ProtScale⁵³. *IsMyrA* evolutionary conservation analysis was performed using the ConSurf server configured to build MSAs using MAFFT; 133 homologous sequences with identities ranging from 30-95% were compiled from UNIREF90 using the HMMER search algorithm, and conservation scores were calculated via the Bayesian method and visualised using UCSF ChimeraX (v. 1.6)⁵⁴.

Structure Prediction All structural predictions were performed using ColabFold^{55,56}. In brief, a deep MSA was generated using MMSeqs2 prior to structure prediction using AlphaFold 2 (structural templates were not utilised for prediction). When appropriate, ColabFold was configured to perform multimeric prediction. The output of the AlphaFold 2 structure module was recycled up to 3 times for refinement. For each sequence, a total of 5 models were generated and ranked by Predicted Template Model score (pTM); Predicted Local Distance Difference Test (pLDDT) scores were also computed for each model to evaluate fold-level confidence. The best model was subsequently relaxed to eliminate steric clashes. Visual inspection was performed in UCSF ChimeraX (v1.6) and PyMOL (v2.5.4), and electrostatic potentials were computed using APBS Electrostatics⁵⁷. Topological analysis was performed using the CASTp 3.0 server⁵⁸. For molecular docking studies, both the ligand and receptor were prepared using AutoDockTools, and ligand docking was performed using AutoDock Vina (v1.1.2)⁵⁹. Ligand-receptor hydrophathy surfaces were computed in BIOVIA Discovery Studio 2020.

Myriocin detection by LC-MS. Myriocin biosynthesis was induced by growing *I. sinclairii* or *M. sterilia* in 50 mL fermentation medium (FM) (3 % (w/v) D-glucose (anhydrous), 0.5 % (w/v) Bacto peptone (Difco No. 0118-01), 0.3% (w/v) Yeast extract (Oxoid), 0.03 % (w/v) KH₂PO₄ (Monobasic), 0.03 % (w/v) K₂HPO₄ (dibasic) and 0.03 % (w/v) MgSO₄·7H₂O. pH was adjusted to 5.5 with HCl prior to autoclaving)) for 24 d. *I. sinclairii* was incubated with shaking at 25 or 37 °C and *M. sterilia* at 37 or 45 °C⁶⁰. Cells were pelleted at 12000 rpm and supernatants removed. Cells were desiccated at – 105 °C for 2 d (Labogene ScanVac CoolSafe) in pre-weighed tubes for determination of dry-weight biomass.

Supernatants were filtered using Nalgene Rapid-Flow filter unit (Thermo Scientific) with a 0.2 µm pore size PES membrane and stored at – 80 °C. Dried cell pellets (100 mg) were powdered, resuspended in ethyl acetate (5 mL) and sonicated for 10 min. Water (5 mL) was added to wash the ethyl acetate layer, which was then removed and the aqueous solution extracted with 5 mL ethyl acetate. Ethyl acetate extracts were combined, dried under reduced pressure and resuspended in 1 mL of acetonitrile/water (90:10), which was further diluted (1:100) for UHPLC-HRMS analysis and myriocin quantification. For culture supernatants, water was added to a total of 40 mL to counter variable evaporation during culture, and 1 mL extracted with ethyl acetate (2 x 5 mL). The combined organic extracts were dried under reduced pressure and resuspended in 1 mL acetonitrile/water (90:10), which was further diluted (1:10) for UHPLC-HRMS analysis and myriocin quantification. Myriocin standard (Sigma, M1177-5MG) was diluted in acetonitrile/water (90:10) to final concentrations of 1, 0.5, 0.25, 0.1, 0.05 and 0.025 µg/mL and analysed by UHPLC-HRMS. The extracted ion chromatogram (EIC) for mass 402.285 was used to detect myriocin. The area of the peak was integrated and plotted against the known concentration to obtain the calibration curves. The areas of the peak for the EIC (402.258) were used to quantify the amount of myriocin in the extracted samples. See Supplementary Methods for analytical instrument and method used.

Functional analysis of *IsMyrA*: The 480 aa sequence for *IsMyrA* (Fig. S10a) was synthesised by GeneArt inserted in a pET18 plasmid. Primers *IsMyrA*-F (5'-ctttaagaaggagatataccatggccgctgcatcctttc-3') and *IsMyrA*-R (5'-ccctgaaaataaagattctctgcgttgccgcccacag – 3') were used to amplify *IsMyrA* and 21 nt overlaps with a linearized pET28 plasmid to generate the pET28*IsAOSV3* vector using Gibson assembly: 1 µL plasmid (35 ng), 1 µL gene insert (70 ng) (1 to 2 ratio), 5 µL HiFi DNA Assembly Master Mix (New England Biolabs), 2.5 µL ddH₂O, incubated for 45 min at 50 °C. The assembly reaction (2 µL) was added to NEB *E. coli* 5-alpha cells, incubated according to general protocols and plated on LB agar plates (30 µg/mL⁻¹ Kanamycin). Positive colonies were confirmed by restriction digest using 300 ng DNA, 2.5 µL (1X) CutSmart buffer 10X (NEB), 0.5 µL *Xba*I (NEB), and/or 0.5 µL *Pst*I enzyme (NEB), ddH₂O up to 25 µL incubated for 1 h at 37 °C, and analysed by agarose gel. Positive plasmids were sequenced (Eurofins). The resulting *IsMyrA* product of 52.6 kDa contained a C-terminal TEV-His6 tag and was used for both interaction and modelling studies (Fig S10b).

Large Scale Overexpression of *IsMyrA*: A single *E. coli* BL21(DE3) colony harboring pET28*IsAOSv3* (kanamycin 30 µg/mL) was grown in 1 L LB at 37 °C to OD₆₀₀ = 0.6, then cooled on ice. Protein expression was induced with 1 mM IPTG and incubated overnight at 20 °C with shaking. Cells were pelleted (4000 rpm, 20 min),

combined, and re-pelleted (8000 rpm, 20 min), yielding a yellow-green pellet. Cells were resuspended in cold Buffer A (20 mM phosphate pH 7.5, 150 mM NaCl, 10 mM imidazole, 25 mM PLP) and lysed by sonication (30 s pulse, 30 s off, 10 cycles). The lysate was pelleted by centrifugation (14,000 rpm, 45 min, 4 °C) and the cell-free extract collected. His-tagged *IsMyrA* was purified using a HisTrap (1 mL) Co-IMAC column, washed with Buffer A and eluted with a gradient of 0 to 100% in 25 mL with Buffer B (20 mM phosphate pH 7.5, 150 mM NaCl, 300 mM imidazole, 25 mM PLP) in 2 mL fractions (Fig. S10b). Fractions were diluted in Buffer C (20 mM phosphate pH 7.5, 150 mM NaCl, 25 mM PLP) (1 to 10) and concentrated using a spin column 30 kDa concentrator to a final volume of 1 – 2 mL. The enzyme was injected into a Superdex 200 size-exclusion chromatography (SEC) column previously equilibrated with buffer C and eluted with buffer C (0.5 mL/min) (Fig. S10c). Fractions containing *IsMyrA* were combined and concentrated. Buffer D (20 mM phosphate (pH 7.5), 150 mM NaCl, 25 mM PLP, 50% v/v glycerol) was added to purified *IsMyrA* to a final glycerol concentration of 25 % v/v. *IsMyrA* was flash frozen and stored at -80 °C. The concentration of purified enzyme as the yield after SEC purification was estimated by Bradford assay as ~ 11 mg/L bacterial culture.

UV-Vis Spectrophotometry: *IsMyrA* was converted to the holo-form by 1 h dialysis at 4 °C in 20 mM potassium phosphate (pH 7.5) with 150 mM NaCl and 25 mM PLP. Excess PLP was removed, and protein concentrated to 10–20 mg/ mL using a 30 kDa VivaSpin filter. UV-Vis spectra (200–800 nm) of 150 µL samples (20 mM phosphate buffer, pH 7.5; 10 µM *IsMyrA*-TEv-His₆) were recorded in quartz cuvettes (Varian Cary 50) (Fig. 5a).

Substrate interactions: Potential substrates, L-serine and aminomalonate, were prepared as stock solutions of 0.1 - 50 mM. In quartz cuvettes 1 µL of each substrate concentration was added to 150 µL *IsMyrA* enzyme (10 µM) in 20 mM phosphate buffer (pH 7.5), mixed by pipetting. Solutions were incubated for 10 min at room temperature and the UV-Vis spectrum plotted using Cary WinUV software (Varian) (Fig 5a). The black line in each spectrum is the holo-form of the enzyme (10 mM MyrA, 20mM potassium phosphate buffer (pH 7.5), 150mM NaCl, 25 °C). Increasing concentrations of L-serine were added (0, 0.1, 0.5, 1, 2, 5, 10, 20, 40, 60, and 80 mM; colour lines), and the spectrum was recorded after 10 min. For aminomalonate, concentrations were 0, 0.05, 0.1, 0.5, 1, 2, 5, 8, 12, 16 mM.

DTNB assay: SPT activity was determined by monitoring the release of CoASH from the reaction of C16-CoA/C18-CoA with L-serine or aminomalonate (Merck and Santa Cruz Biotechnology), as previously reported³⁸, where the thiol group on CoA reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent) to break the disulphide bond and generate the coloured TNB-anion measured at 412 nm ($\lambda_{\text{max}} = 412\text{nm}$; $\epsilon = 14,150/\text{M}$ -

1cm 1) (Fig. S10e). Kinetic assay: in a 96-well plate containing DTNB (0.4 mM), *IsMyrA* enzyme (0.8 mg/mL), L-serine or aminomalonate (5 mM), C16-CoA or C18-CoA (200 mM), phosphate buffer (20 mM), pH 7.5 was added to a final volume of 100 μ L. The reaction mix was preincubated at 30 °C for 30 min before addition of the enzyme to initiate the reaction. The concentration of C₁₆-CoA/C₁₈-CoA was maintained at 200 mM. Absorbance was read at 412 nm every 5 s for 30 min in a plate reader (Fig. S10f).

Activity assay by LCMS: *IsMyrA*-TEV-His₆ (8 mg/mL) was incubated with L-serine or aminomalonate (5 mM) and C16-CoA or C18-CoA (200 μ M) in 20 mM phosphate buffer (pH 7.5, 100 μ L total volume). Reactions were run for 180 min at 30 °C with shaking (800 rpm, ThermoMixer C). Heat-inactivated enzyme (15 min, >100 °C) served as a negative control. Reactions were quenched with 100 μ L acetonitrile, centrifuged (14,000 rpm, 15 min), and diluted 1:1 with water containing 3 % formic acid. Samples were analysed by LC-HRMS (Waters Acquity UPLC Class I Plus with Synapt G2-Si Q-TOF HDMS) using a Premier CSH C18 column (1.7 μ m, 2.1 $\times$ 100 mm) and VanGuard FIT cartridge. Column temp: 55 °C; flow rate: 0.2 mL/min. Gradient: 95 %A:5 %B (0–1.5 min), 5% A:95 %B (1.5–11 min), 95 %A:5 %B (11.0–12 min); A: water + 0.1 % FA; B: acetonitrile + 0.1 % FA.

Statistical analysis was carried out using a 2-tailed Student's *t*-test in Excel.

DATA AVAILABILITY

Genome sequences are deposited in NCBI GenBank (Accession numbers: RXP000000000 (*M. sterilia*) and RXP000000000 (*I. sinclairii*), BioProject PRJNA509326, and on MycoCosm (Joint Genomes Institute) (*M. sterilia* - <https://genome.jgi.doe.gov/Mycester1> and *I. sinclairii* - <https://genome.jgi.doe.gov/lsarsinc1>).

SUPPLEMENTAL MATERIAL

Supplementary Methods

Supplementary Data: S11: `konkel_slot_tables`

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AUTHOR CONTRIBUTIONS

AB and DC designed the project and obtained funding. AB, DC, MH and JS wrote the manuscript. BR and CB undertook fungal growth experiments and imaging. BR and MW assembled the genomes. CG and HD carried out gene cluster identification and myriocin LC-MS analysis. AH analysed gene clusters and generated heatmaps. MH modelled enzyme structure and function. SM curated the genome data on MycoCosm. GPO expressed MyrA and determined its substrate. JS and ZK analysed the boundaries, distribution, and evolution of gene clusters. RF assisted with bioinformatic analysis and interpretation.

DISCLOSURE STATEMENT: The authors declare no competing interests.

ORCID identifiers:

Michael Herrera: 0009-0001-4122-1374
 Gustavo Perez-Ortiz: 0000-0002-6521-3096
 Claudio Greco: 0000-0003-3067-0999
 Hai Deng: 0000-0002-0047-0622
 Abigail Hay: 0000-0002-8143-2109
 Marius Wenzel: 0000-0003-0093-368X
 Jason Slot: 0000-0001-6731-3405
 Zachary Konkel: 0000-0003-3628-8336
 Stephen Mondo: 0000-0001-5797-0647
 Rhys Farrer: 0000-0001-8456-7458
 Dominic Campopiano: 0000-0001-8573-6735
 Alexandra Brand: 0000-0002-7381-131X

Supplementary Figures and Tables

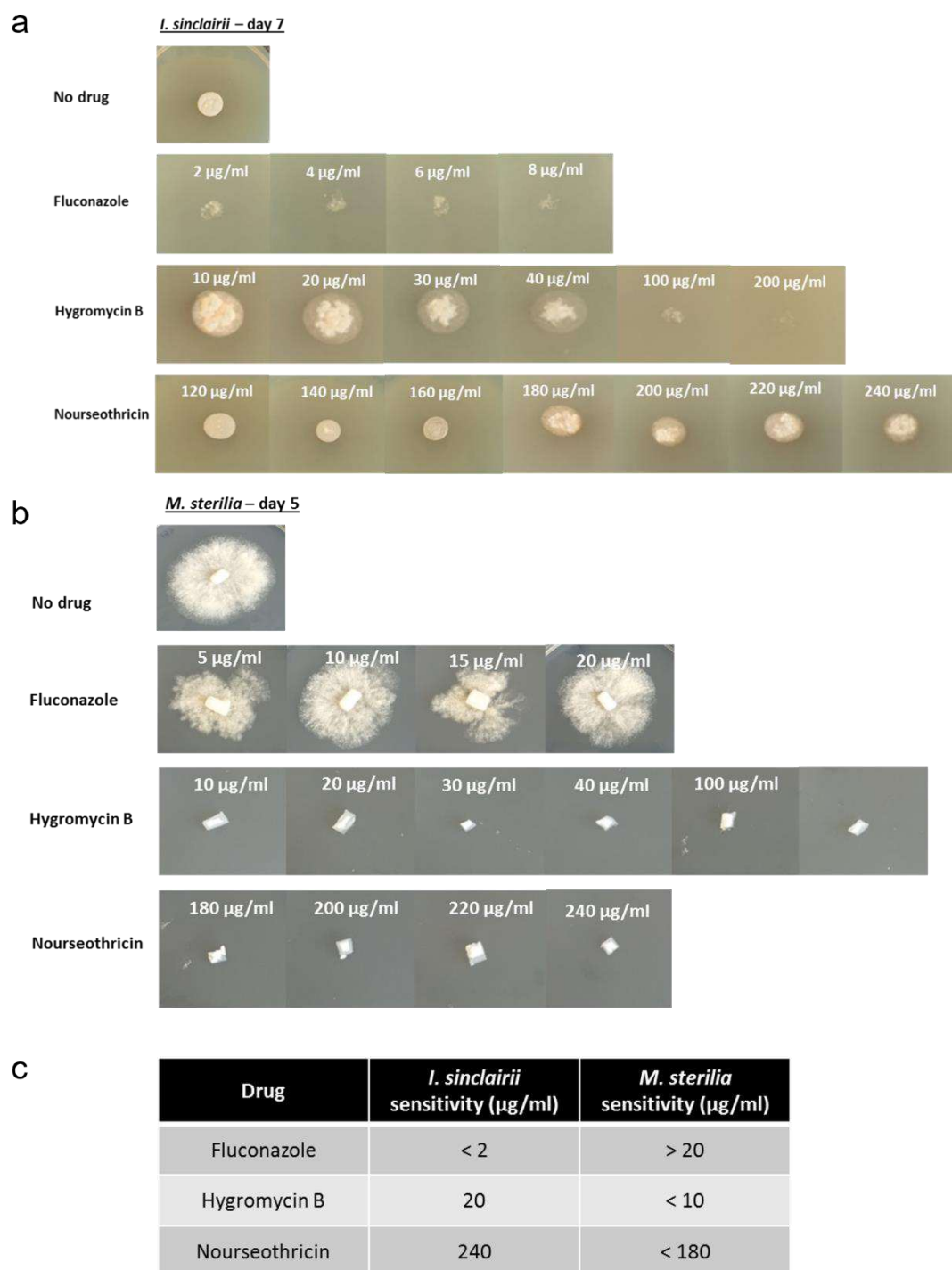
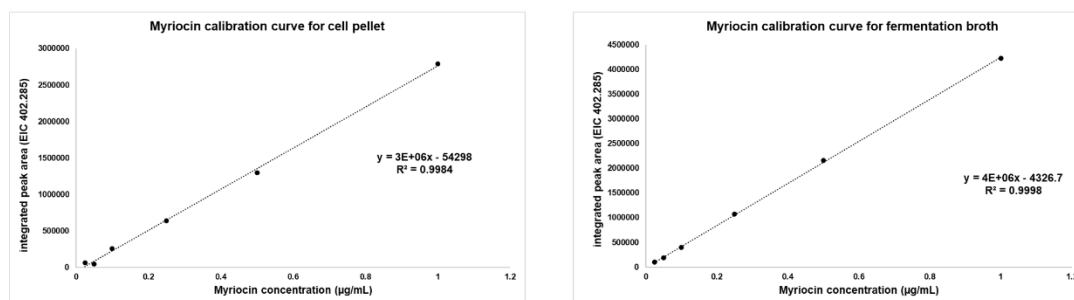


Fig. S1: Susceptibility of *I. sinclairii* and *M. sterilia* to fluconazole, hygromycin B and nourseothricin. *I. sinclairii* or *M. sterilia* were inoculated onto tryptic soy agar or potato dextrose agar, respectively, containing increasing concentrations of the antifungal agents fluconazole, hygromycin B or nourseothricin. A) *I. sinclairii* plates were incubated at 25 °C for 7 d. B) *M. sterilia* plates were incubated at 37 °C for 5 d. C) Sensitivity was quantified by measuring the ratio of colony diameter compared to the no-drug control.

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b

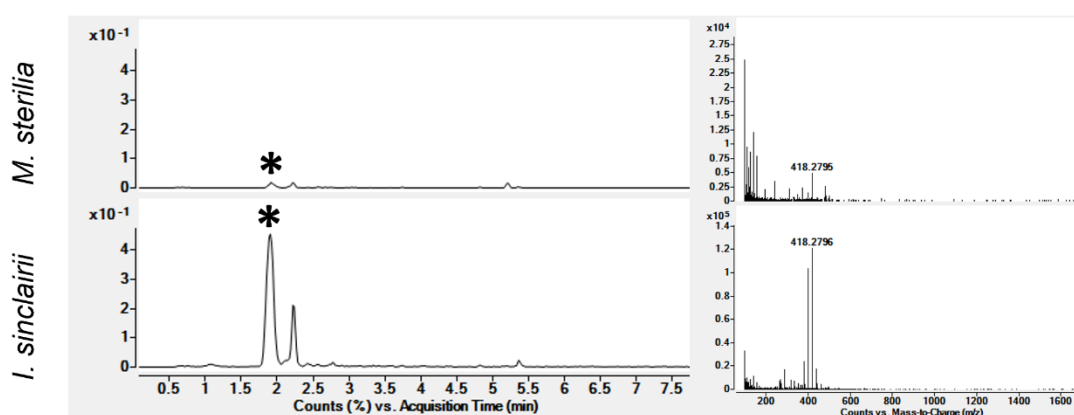


Fig. S2: Myriocin quantification and detection of myriocin derivatives a. Calibration curves for the quantification of myriocin for the cell pellet (left) and for the fermentation broths (right). b. UHPLC-HRMS analysis in positive mode of the organic extracts for the myriocin derivative. EIC for 418.28, noted with an * (left) and mass spectra (right) for *M. sterilia* (top) and *I. sinclairii* (bottom).

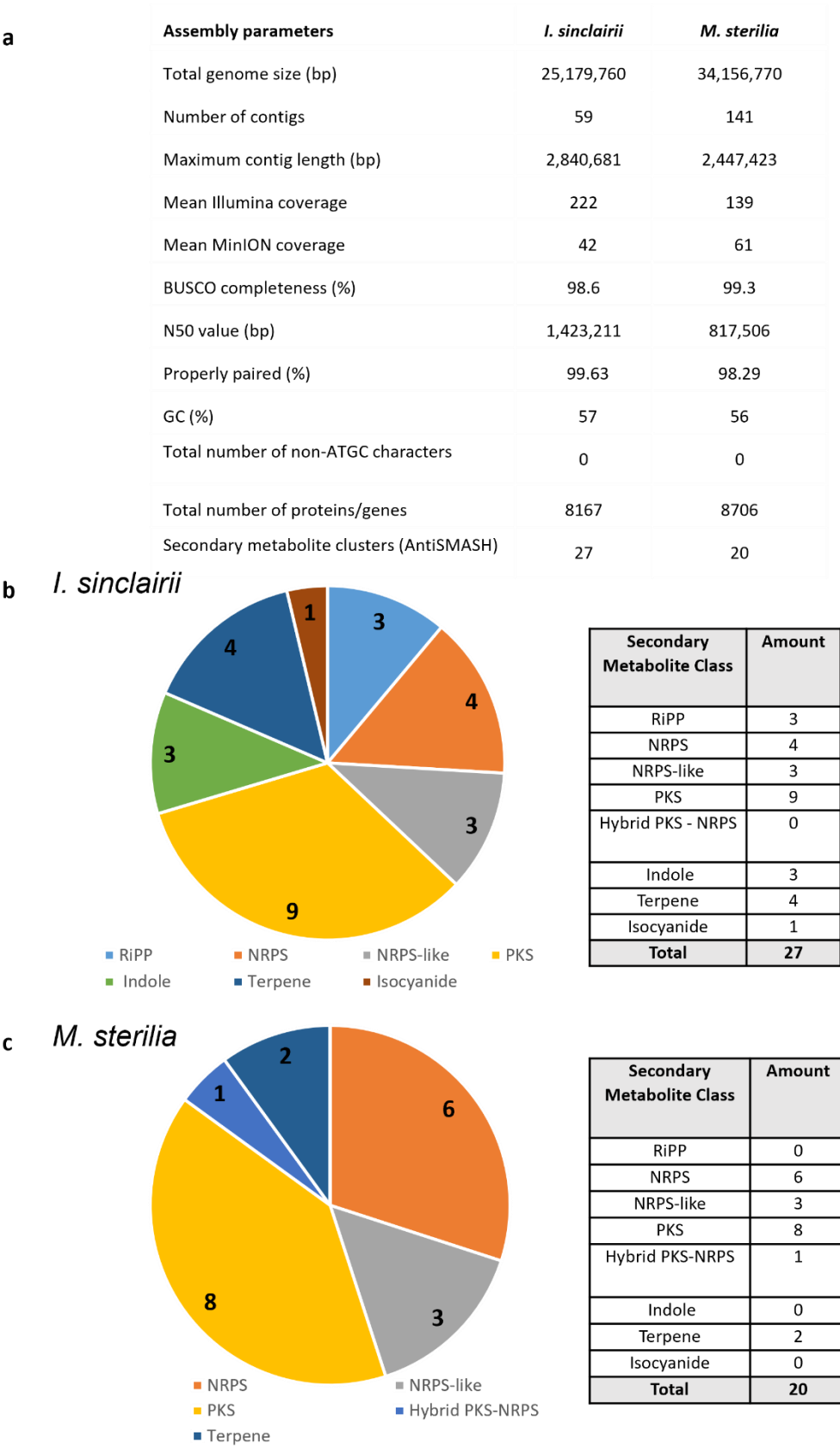


Fig. S3: Genome assembly parameters and analysis of secondary metabolite gene clusters by class
a. Comparison of genome assembly parameters for *I. sinclairii* and *M. sterilia*. b,c. Breakdown of secondary metabolite biosynthesis gene clusters by class using AntiSMASH V7.1.0.

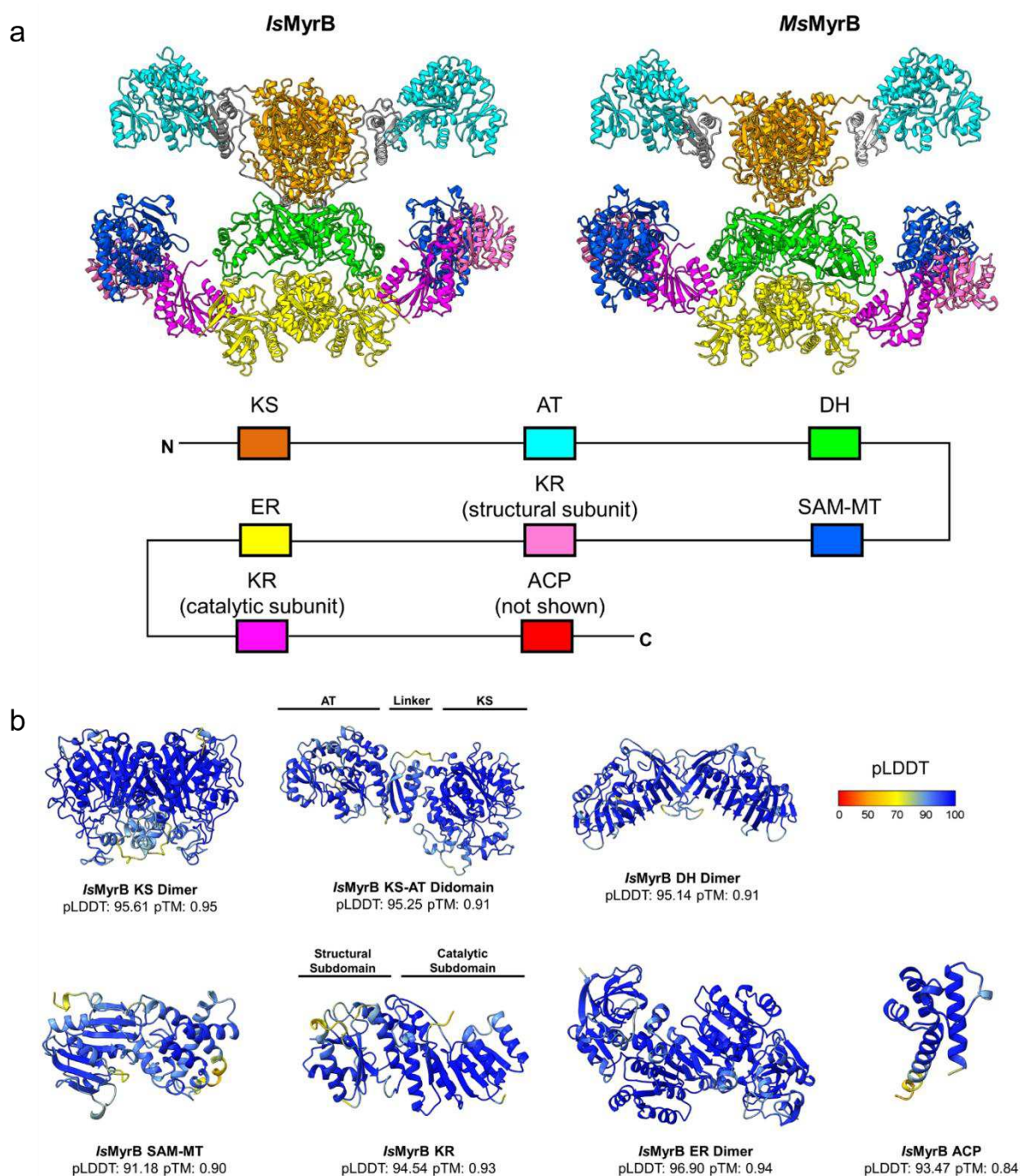


Fig. S4: a. Composite predicted structural models showing the relative domain organisation of the *IsMyrB* and *MsMyrB* homodimers. Mammalian fatty acid synthase (PDB: 2VZ8) was used as a template guide, using structural alignment tools in PyMOL. Acyl Carrier Proteins (ACPs) have been omitted. Colours indicate active domains: KS = Ketoacyl Synthase, AT = acyltransferase, DH = dehydratase, SAM-MT = S-adenosylmethionine-dependent methyltransferase. KR = ketoreductase, ER = enoylreductase. b. The constitutive domains of *IsMyrB*, coloured by pLDDT confidence score (0-100).

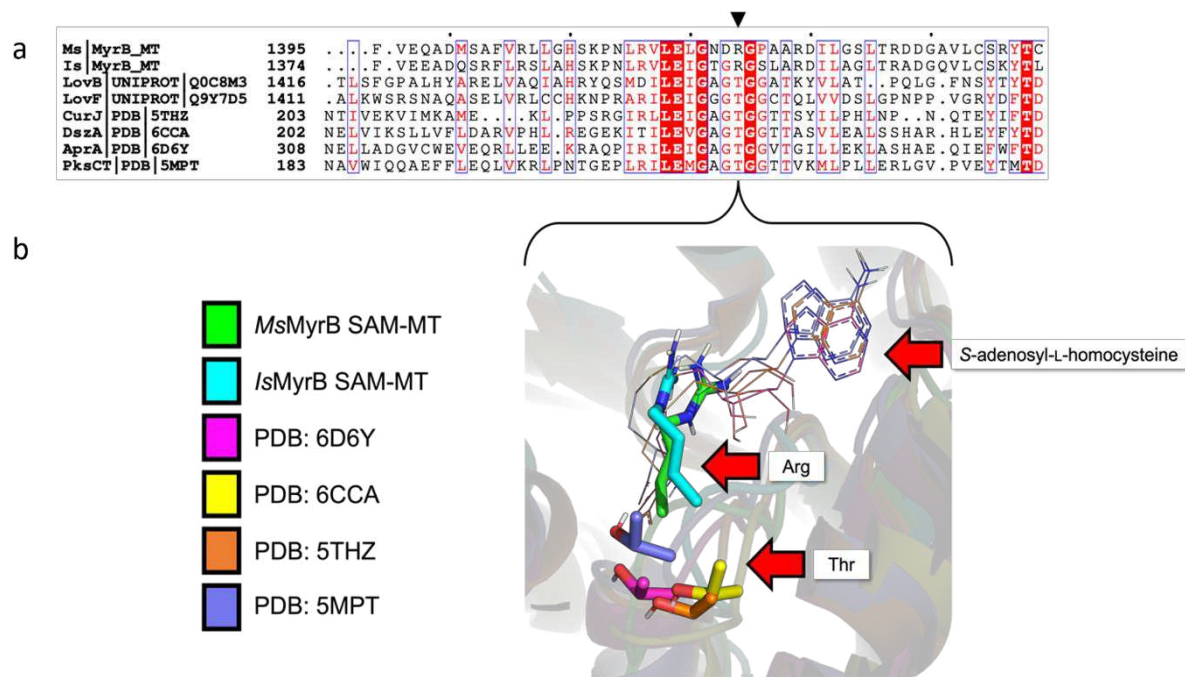


Fig. S5: Occlusion of the *IsMyrB* and *MsMyrB* S-adenosylmethionine binding pocket by a key threonine → arginine mutation. a. Comparison with active, homologous S-adenosylmethionine methyltransferases (SAM-MTs) from the Protein Data Bank revealed a shared mutation in *I. sinclairii* and *M. sterilia* where the 3-carbon threonine is substituted by a 6-carbon arginine (black arrow). b. Modelling suggests that this substitution occludes the S-adenosylmethionine binding pocket, which likely inactivates the domain and correlates with the lack of methyl branching in myriocin. PDB = Protein Data Base.

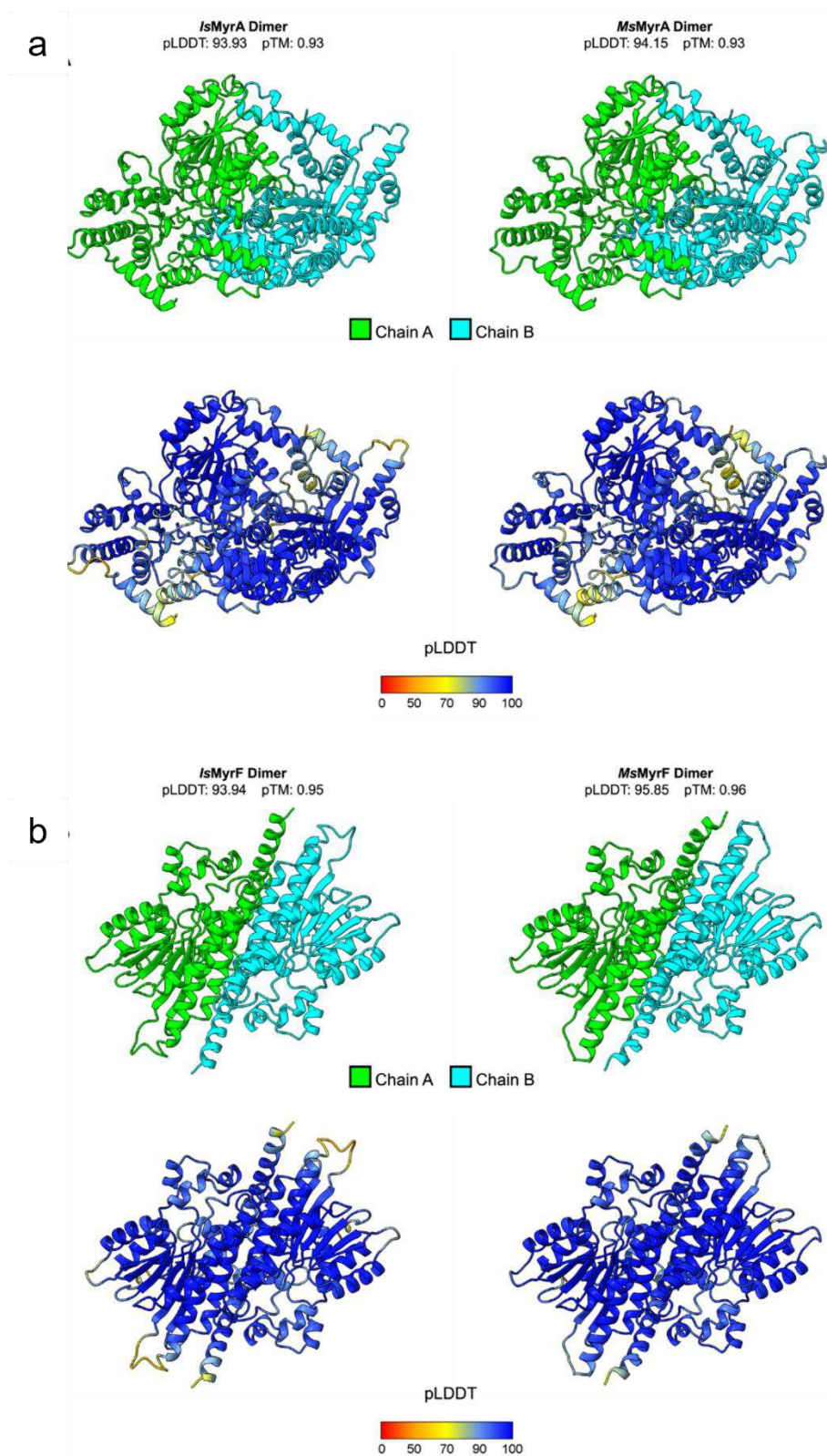


Fig. S6: Structural models of MyrA (AOS) and MyrF (ketodihydrosphingosine reductase – KDRS). a. Structural models of IsMyrA and MsMyrA, coloured by chain (top) and pLDDT confidence score (bottom, 0-100). b. Structural models of IsMyrF and MsMyrF, coloured by chain (top) and pLDDT confidence score (bottom, 0-100).

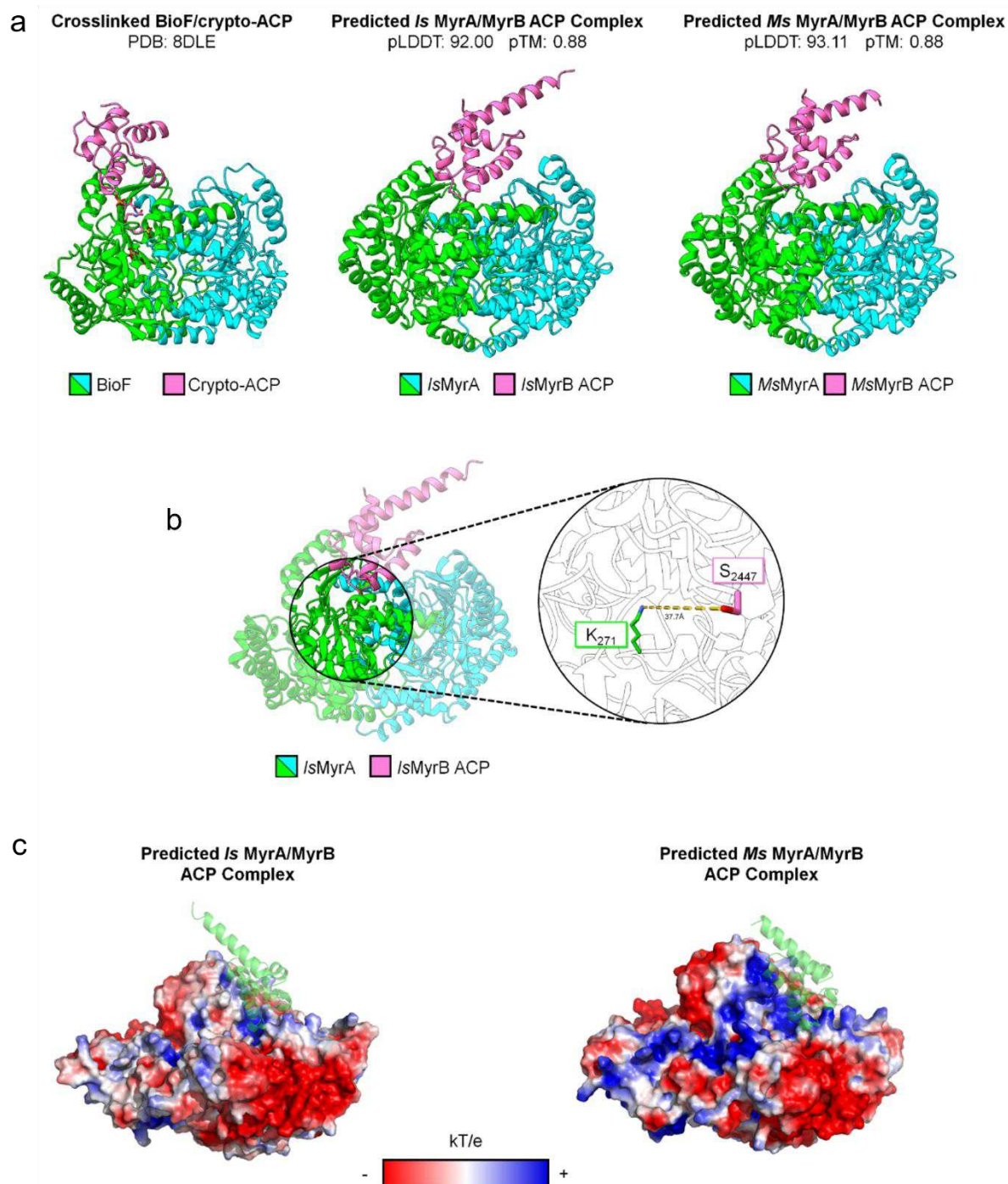
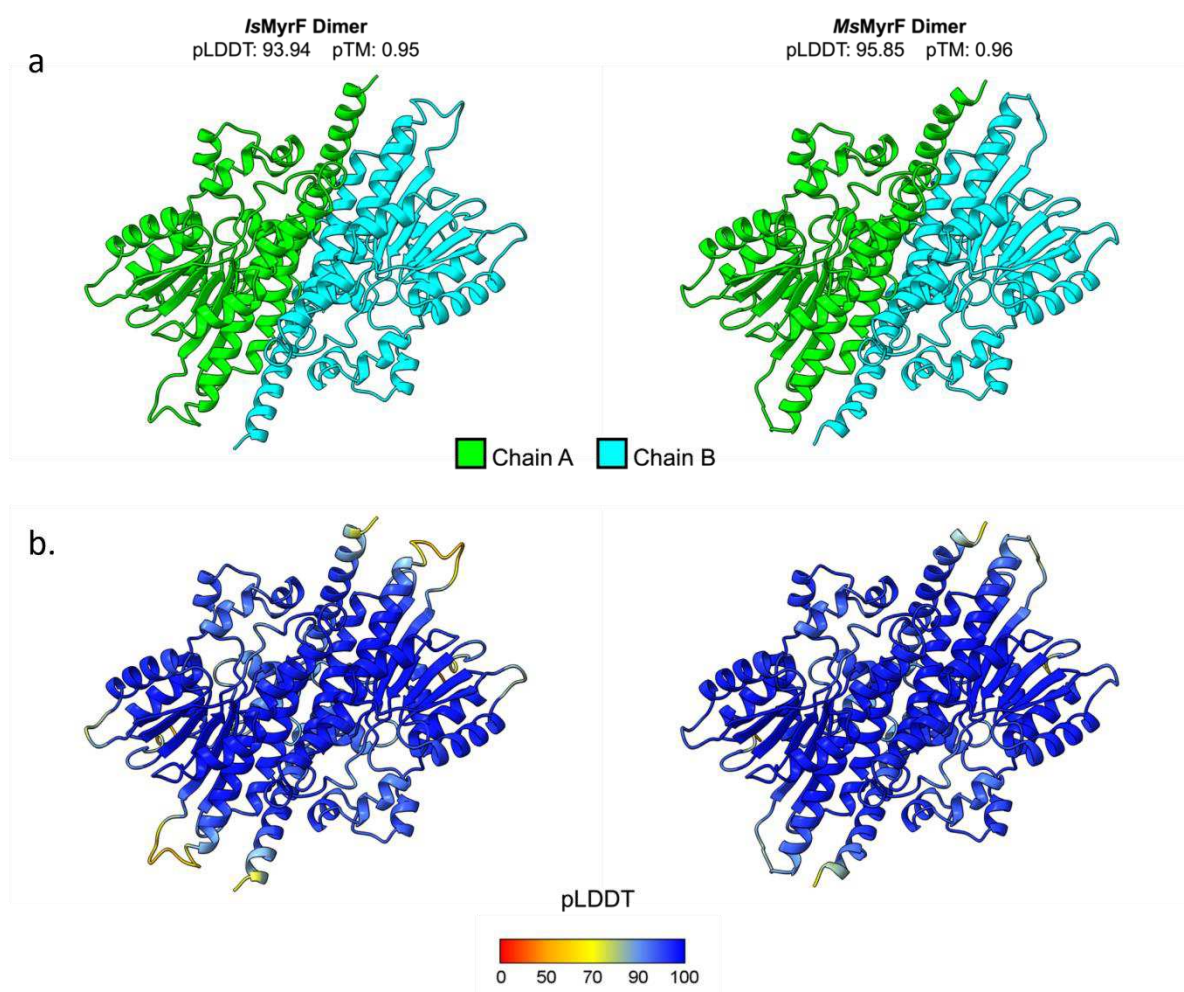


Fig. S7: Interaction between the predicted MyrA (AOS) and MyrB (iPKS) acyl carrier protein complexes a. Comparison between *E. coli* BioWF/crypto-acyl carrier protein (ACP) experimental structure and the predicted MyrA and *Is*MyrB ACP complexes. b. Predicted docking interface between *Is*MyrA and *Is*MyrB ACP, showing the catalytic *Is*MyrB S₂₄₄₇ positioned directly above the substrate binding tunnel of *Is*MyrA. c. Computed MyrA APBS electrostatic surface potentials, identifying patches of electropositivity around the AOS-ACP docking interface. MyrB ACPs are shown in green.



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940 **Fig. S8: Structural models of *IsMyrF* and *MsMyrF* homodimers.** a. Models coloured by chain.

941 b. Models colours by pLDDT confidence score, as shown.

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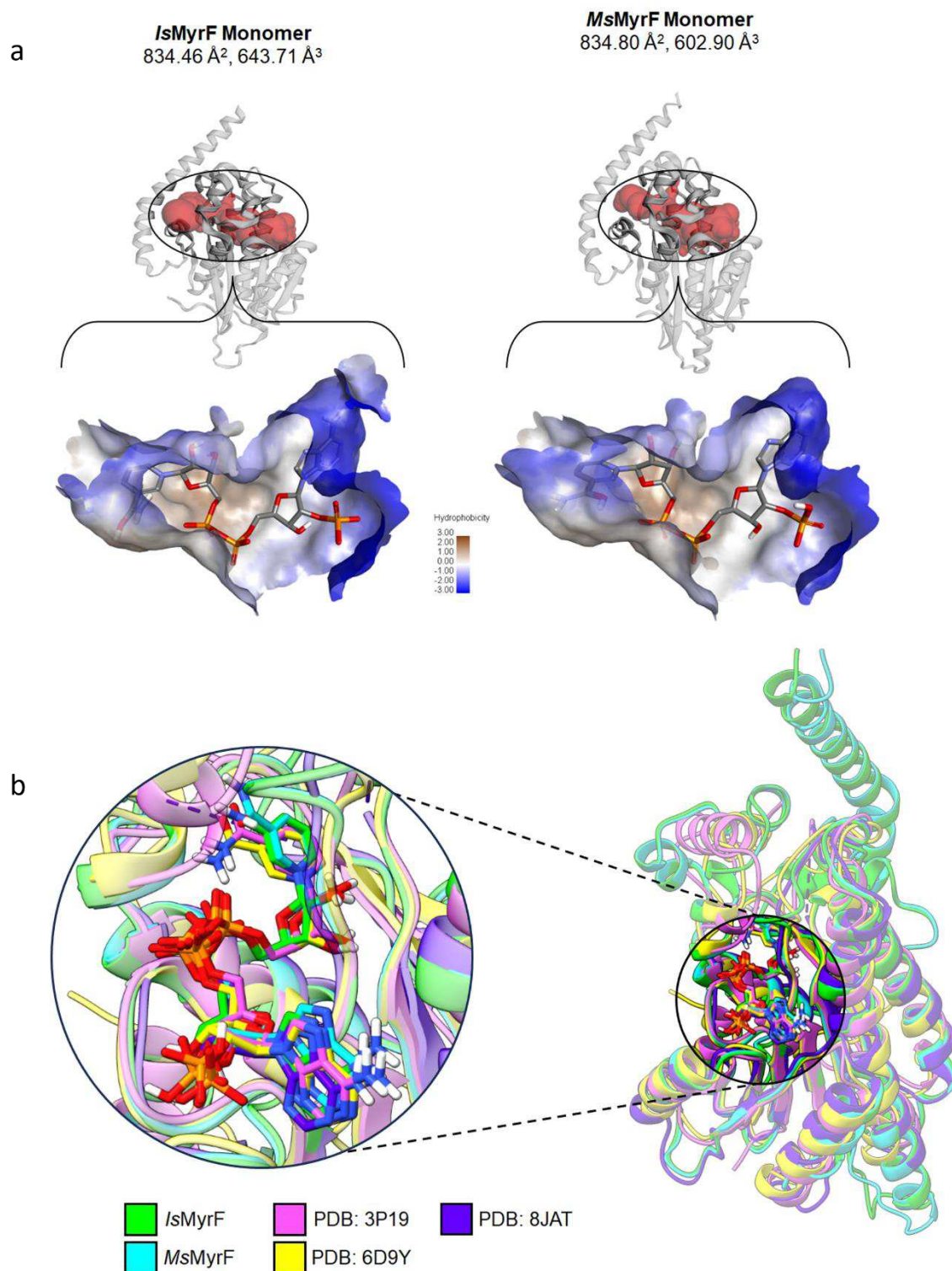


Fig. S9: Structural analysis of *IsMyrF* and *MsMyrF* (myriocin ketodihydrosphingosine reductase (KDSR)) a. Identification of a binding pocket in MyrF by topological analysis (CASTp3.0). NADP⁺ was docked in the identified binding pockets (~ 12.5 kcal mol⁻¹) and Kyte-Doolittle hydropathy surfaces were visualised in BIOVIA Discovery Studio 2020. b. Superimposition of homologous, NAD(P)-bound crystal structures over MyrF (with NADP⁺ docked).

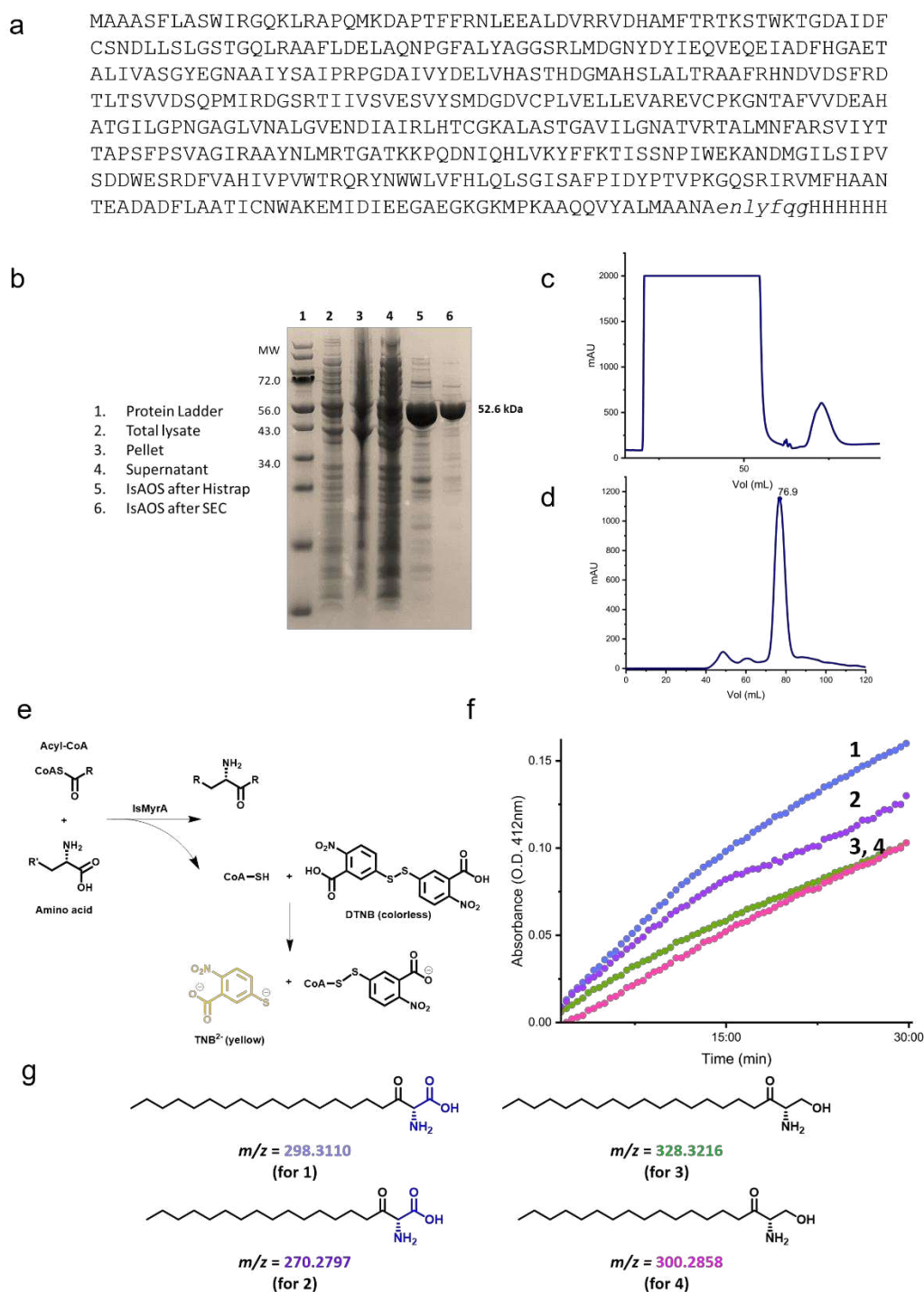


Fig. S10: IsMyrA protein purification and DTNB activity assay a. Sequence of recombinant IsMyrA construct. b. IsMyrA IMAC chromatogram. Lysate was loaded using an AKTA start onto a 5 mL Nickel His-Trap FF column and eluted with a 10 – 300 mM imidazole gradient. Protein was detected at 280 nm. c. SEC purification of IsMyrA using a HiLoad 16/600 Superdex 200 pg (120 mL) SEC column, monitored by UV-vis (280 nm). d. SDS-PAGE gel of purification of IsMyrA. HiMark Pre-stained Protein Standard was used as ladder. Lanes 2 to 6 show each step of purification from transformed *E. coli* BL21 (DE3) 18+ hours post-induction using IPTG. e. DTNB assay general reaction. The activity of IsMyrA was detected by capturing the CoASH released with 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB) and the spectrophotometric detection of the TNB²⁻ anion (412 nm). f. IsMyrA activity against a acyl-CoAs (C₁₆ and C₁₈) and amino acids aminomalonate and L-serine, detected using the DTNB assay. g. Molecular formula of the respective product of each reaction.

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