

RESEARCH ARTICLE

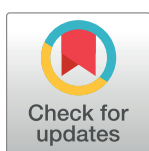
Bovistol B, bovistol D and strossmayerin: Sesquiterpene metabolites from the culture filtrate of the basidiomycete *Coprinopsis strossmayeri*

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OPEN ACCESS

Citation: Banks AM, Song L, Challis GL, Bailey AM, Foster GD (2020) Bovistol B, bovistol D and strossmayerin: Sesquiterpene metabolites from the culture filtrate of the basidiomycete *Coprinopsis strossmayeri*. PLoS ONE 15(4): e0229925. <https://doi.org/10.1371/journal.pone.0229925>

Editor: Minou Nowrouzian, Ruhr-Universitat Bochum, GERMANY

Received: September 11, 2019

Accepted: February 18, 2020

Published: April 6, 2020

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Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: This work was supported by the Engineering and Physical Sciences Research Council (<https://epsrc.ukri.org/>) under Grant EP/M027546/1 (AMB, AMB, GDF); and the Biotechnology and Biological Sciences Research Council (<https://bbsrc.ukri.org/>) under Grant BB/L01386X/1 (AMB). The funders had no role in

Abstract

Basidiomycete fungi are a rich source of natural products with a diverse array of potentially exploitable bioactivities. Two dimeric sesquiterpenes, bovistol B (**1**) and D (**2**), and one monomeric sesquiterpene, strossmayerin (**7**), were isolated from the culture filtrate of the basidiomycete fungus *Coprinopsis strossmayeri*. The structures were determined through a combination of MS and 1D/2D NMR spectroscopic techniques. Likely monomeric precursors, identified on the basis of HRMS analysis, allow a plausible biosynthetic pathway to be proposed for the biosynthesis of **1** and **2**, involving the dimerisation of the monomer through a hetero-Diels-Alder mechanism. A gene cluster, including a putative sesquiterpene 1–11 cyclase, was identified through phylogenetic and RNA-seq analysis, and is proposed to be responsible for the biosynthesis of **1** and **2**.

Introduction

The basidiomycete fungi are a phylum of organisms with a hugely diverse range of biological capabilities [1]. Although the secondary metabolism potential of basidiomycetes has long been known, inherent difficulties associated with the cultivation of such species, for example limited genetic tractability [2], have hindered extensive investigation. In recent years, some of these practical constraints have been overcome [3], making it timely to reconsider this understudied phylum.

We have recently re-evaluated the coprinoid basidiomycete *Coprinopsis strossmayeri* [4], previously *Coprinus quadrididus* [5]; a species in which antimicrobial activity had been briefly reported, though not thoroughly investigated [6]. The coprinoid fungi encompass the *Coprinopsis*, *Coprinus*, *Coprinellus*, and *Parasola* genera in the Psathyrellaceae family. Generally favouring a habitat in herbivore dung, these organisms have evolved to live in highly competitive environments. Notable bioactive metabolites previously reported from coprinoid species

study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

include: the antimicrobial sesquiterpene illudins, illudin I, I2, J and J2, from *Coprinopsis episcopalis* [7,8]; the diterpene heptemerones A-G from *Coprinus heptemerus* [9], as well as the sesquiterpene antibiotic coprinol from fermentations of *Coprinus* sp. [10]. Basidiomycetes are prolific producers of terpenoid compounds, in particular the sesquiterpenes [11]—a group of highly bioactive compounds with a wide range of activities and applications ranging from pharmaceutical to agricultural [12].

In our ongoing search for novel antimicrobials, investigations of the culture filtrate of *C. strossmayeri* were carried out. This led to the isolation of two novel compounds, **2** and **7**, as well as the identification of **1** previously isolated from *Cyclocybe aegerita* [13], the structures of which were determined based on spectroscopic data. Compounds were screened for antimicrobial activity against *Bacillus subtilis* ATCC 6633, *Escherichia coli* DH5 α and *Saccharomyces cerevisiae* Y10000. RNA-seq data led to the identification of a proposed gene cluster responsible for the biosynthesis of **1** and **2**.

Materials and methods

Producing organism

Isolate CBS 177.39, listed as *Coprinus quadrifidus*, was obtained from The CBS Fungal Biodiversity Centre and its internal transcribed spacer region (S1 Text in [S1 File](#)) confirmed the isolate as *Coprinopsis strossmayeri*, showing 99.69% identity to *C. strossmayeri* isolate PK7630 (accession number MH752456.1). The fungus was maintained on potato dextrose agar (potato dextrose broth 24 g/L, agar 15 g/L) at 20°C.

Fermentation and isolation

A 1 L culture of *C. strossmayeri* was grown in potato dextrose broth (PDB) (potato dextrose broth 24 g/L) for 14 days at 20°C at 170 rpm. Fungal material was removed *via* filtration through Miracloth, culture filtrate was acidified to pH 3.0 with HCl, EtOAc was added to the culture filtrate at a 1:1 (v:v) ratio and mixed for 30 minutes then vacuum filtered to remove remaining mycelial fragments. The filtrate was separated using a separation funnel and the organic phase collected. The aqueous phase was extracted two further times and the extracts combined. Water was removed from the pooled solvent fraction using anhydrous MgSO₄ before drying using a rotary evaporator. This yielded 400 mg total crude extract.

A 90 mg sample of crude extract was initially assessed and fractionated using preparative HPLC. A Waters 2767 Sample Manager with Waters 2545 pump system, Phenomenex LUNA column (5 μ m, C₁₈, 100 Å, 10 $\times$ 250 mm) was used for reverse-phase chromatography, with Phenomenex Security Guard precolumn (Luna C₅ 300 Å). UV absorbance was detected between 200–400 nm with Waters 2998 diode array detector; mass spectrometry with Waters Quattro Micro; and approximate target compound abundance evaluated with Waters 2424 for ELSD. Mobile phases consisted of A: water with 0.05% formic acid; and B: acetonitrile with 0.05% formic acid. A gradient of 20% B to 90% B in 30 minutes was employed with flow rate of 5 mL/min. Compounds from the five major peaks were collected across five fractions (A–E) giving the following yields: A = 1.6 mg, B = 0.5 mg, C = 1.3 mg, D = 2.8 mg, E = 3 mg.

LC-MS analysis was performed on the fractions with Dionex 3000RS UHPLC coupled with Bruker MaXis Impact Q-TOF mass spectrometer. An Agilent Zorbax Eclipse plus column (C₁₈, 100 $\times$ 2.1 mm, 1.8 μ m) was used. Mobile phases consisted of A: water with 0.1% formic acid; and B: acetonitrile with 0.1% formic acid. After 5 minutes of isocratic run at 5% B, a gradient of 5% B to 100% B in 15 minutes was employed with flow rate at 0.2 mL/min, UV was set at 210 nm. Mass spectrometer was operated in electrospray positive ion mode with a scan range 50–3,000 *m/z*. Source conditions are: end plate offset at -500 V; capillary at -4,500 V;

nebulizer gas (N₂) at 1.4 bar; dry gas (N₂) at 8 L/min; dry temperature at 180°C. Ion transfer conditions as, ion funnel 1 RF at 200 Vpp; ion funnel 2 RF at 200 Vpp, hexapole RF at 200 Vpp; quadrupole ion energy at 5 eV, quadrupole low mass set at 55 *m/z*; collision energy at 5.0 eV; collision RF ramping from 800 to 1500 Vpp; transfer time set at from 100 to 155 μ s; pre-Pulse storage time set at 5 μ s. Calibration was done with sodium formate (10 mM) through a loop injection of 20 μ L of standard solution at the beginning of each run.

Each compound was further purified using an Agilent Zorbax C18 column (100 x 21.1 mm, 5 μ m) connected to an Agilent 1100 HPLC at a flow rate of 5 mL/min, monitoring absorbance at 210 nm. Mobile phases consisted of A: water containing 0.1% formic acid; and B: acetonitrile containing 0.1% formic acid. The following program was used to elute the column: 0 min, 80% B; 5 min, 80% B; 25 min, 100% B; 30 min, 100% B; 33 min 80% B; 38 min 80% B. Fractions containing target compounds were identified using ESI-HR-Q-TOF-MS and pooled. Organic solvent was removed using a rotary evaporator and the resulting aqueous solutions freeze dried yielding 2 mg each of **1** and **2** and 1 mg of **7**. Samples were analysed immediately by NMR spectroscopy.

Compound identification

¹H, COSY, HSQC and HMBC NMR spectra were acquired in d₄-MeOH (180 μ L in 3 mm tube) on a Bruker Avance II 700 MHz spectrometer equipped with a TCI cryoprobe at 298 K. The solvent peak was used to calibrate the spectra.

Antimicrobial screening

Chemical fractions obtained by preparative HPLC were dissolved to a concentration of 1 mg/mL in dimethyl sulfoxide. Microbial plates were prepared in 90 mm Petri dishes by resuspending microbial cells in the appropriate growth medium supplemented with 2, 3, 5-triphenyl-2H-tetrazolium chloride (200 μ g/mL)–*B. subtilis* ATCC 6633 (tryptic soy broth 30 g/L, agar 5 g/L); *E. coli* DH5 α (LB Broth, Miller 25 g/L, agar 5 g/L); *S. cerevisiae* Y10000 (yeast extract 10 g/L, peptone 20 g/L, D-glucose 20 g/L, adenine hemisulphate 40 mg/L, agar 5 g/L). 50 μ L of each fraction was aliquoted into centrally bored wells in each assay plate and the plates incubated appropriately (*B. subtilis* and *S. cerevisiae* 48 hours at 28°C, *E. coli* 24 hours at 37°C). Antimicrobial activity was determined by the presence of a zone of inhibition surrounding the central well.

Transcriptome analysis

RNA was extracted from *C. strossmayeri* mycelium, harvested from a two-week culture grown in PDB, using the E.Z.N.A.[®] Fungal RNA Kit (OMEGA bio-tek). Isolated RNA was quality checked using RNA Analysis ScreenTape[®] (Agilent). Approximately 500 ng of total RNA was prepared for sequencing using the Illumina TruSeq Total RNA LT Kit (Illumina). The data were processed using RTA version 1.18.64, with default filter and quality settings. The reads were demultiplexed with CASAVA 1.8.4, allowing no mismatches. This was carried out at the Bristol Genomics Facility. RNA-seq data were processed using Galaxy QC and manipulation tools to trim the sequences followed by the TopHat RNA analysis tool to map the RNA-seq reads to the assembled genomes. These data were then viewed in Artemis to evaluate relevant expression levels of genes of interest. Partek[®] Genomics Suite was also used to map RNA-seq reads to assembled genomes and to genes of interest. RNA-seq data are available on the NCBI SRA database under the accessions: STUDY: PRJNA604530; SAMPLE: CBS 177.39 (SAMN13973684); EXPERIMENT: C.s (SRX7684385); RUN: AB_C_ACAGTG_L001_R1_001.fastq (SRR11032120).

Results and discussion

Crude ethyl acetate extract from the culture filtrate of *C. strossmayeri* was fractionated using preparative HPLC. Five fractions were obtained, A-E, each containing one of the major peaks. These were examined for inhibitory activity against *B. subtilis* ATCC 6633, *E. coli* DH5 α and *S. cerevisiae* Y10000 using plate-based bioassays. Weak antimicrobial activity was detected in fractions A and E against *B. subtilis* (S20 Fig in [S1 File](#)) but not *E. coli* or *S. cerevisiae*. No antimicrobial activity was observed in fractions B-D.

Initial preparative HPLC fractions were analysed using LC-MS and major components were further purified with semi-preparative HPLC. Structural elucidation was carried out with a combination of HRMS and 1D/2D NMR spectroscopic techniques; this led to the identification of **1** from fraction E and two novel compounds, **2** and **7**, from fractions D and C respectively. Fraction E was identified as the dimeric sesquiterpene bovistol B (**1**) and fraction D the related bovistol D (**2**), both yellowish-white solids. Fraction C was identified as a sesquiterpene monomer strossmayerin (**7**). Compounds present in fractions A and B were produced at too low titre to yield sufficient product for structural elucidation.

Bovistol B: High resolution MS data established **1** with the molecular formula C₃₀H₃₈O₅, (measured m/z 479.2793, calculated (C₃₀H₃₈O₅+H)⁺ 479.2792) with 12 degrees of unsaturation. The UV λ_{max} at 290 nm also indicates the presence of an aromatic ring or conjugated multiple double bonds. ¹H, ¹³C, COSY, HSQC and HMBC NMR spectra were acquired in d₄-MeOH (S2-S5 Figs in [S1 File](#)). There are 2 methyl groups appearing as a singlet (1.14 and 1.16 ppm) connected to saturated quaternary carbons, while the third methyl group (2.13 ppm), also appears as a singlet, is probably connected to an aromatic ring or substituted carbon-carbon double bond. There are two isolated oxygenated methylene signals H10 (3.40 ppm) and H10' (3.41 ppm) and five further isolated methylene signals (H6, H8, H6', H8' and H12') with no COSY correlations observed to any other signals. COSY correlations established the connectivity between H14 (3.50 ppm) and H13 (2.80 ppm), H14' (0.80/1.13 ppm) and H13' (0.26/1.38 ppm) and H15 (2.77 ppm) with H15' (1.87/2.19 ppm). HMBC correlations observed from H10 and H11 to C6, C7 and C8 confirmed the presence of a five membered ring. Further HMBC correlation from H12 to C3, C5, from H8 to C1, C9 and from H13 to C2, C4 and C14 established coupling of the aromatic ring with the 5-membered ring and complete the right-hand part of the structure. The left-hand part of the structure was also deduced mainly from HMBC correlations, similar correlations confirmed the presence of a 5-membered ring. Different to the right part of the structure, there is an isolated olefinic CH₂ signal (5.25 ppm with carbon chemical shift at 113.3 ppm) instead of a methyl group connected to C4', HMBC correlation from H12' to C3' and C5' confirmed the presence of the isolated double bond. The relatively high field methylene signal at 0.26/1.38 ppm (H13') and 0.80/1.13 ppm (H14') suggest the presence of a cyclopropane group. HMBC correlation from H13' and H14' to C4', C3' and C2' established the left-hand side of the structure. Key HMBC correlation observed from H15' to C1', C2' and C3' from the left side and C15, C2 from right-hand side established the connectivity between the two monomers.

Therefore, the planar structure of **1** is proposed as a dimeric sesquiterpene; the structure of **2** was elucidated as the oxidised form of **1**, where the C10 hydroxymethyl group is oxidised to a carboxyl group. **1**, previously isolated from *C. aegerita*, has been characterised by MS and NMR spectroscopy and the relative configurations assigned by ROSEY correlations [13]. The closely related compound bovistol (**8**) has also undergone extensive structural characterisation [14]. **7** showed high similarity with the right-hand side of compound **1** and appears to be derived from delta 6-protoilludene **3** (Fig 1; S1, S7 and S13 Figs in [S1 File](#)).

1 and **2** appear to be dimeric sesquiterpenes, a proposed pathway for their biosynthesis is presented in Fig 2. Pathway intermediates to support this, identified from HRMS data, are

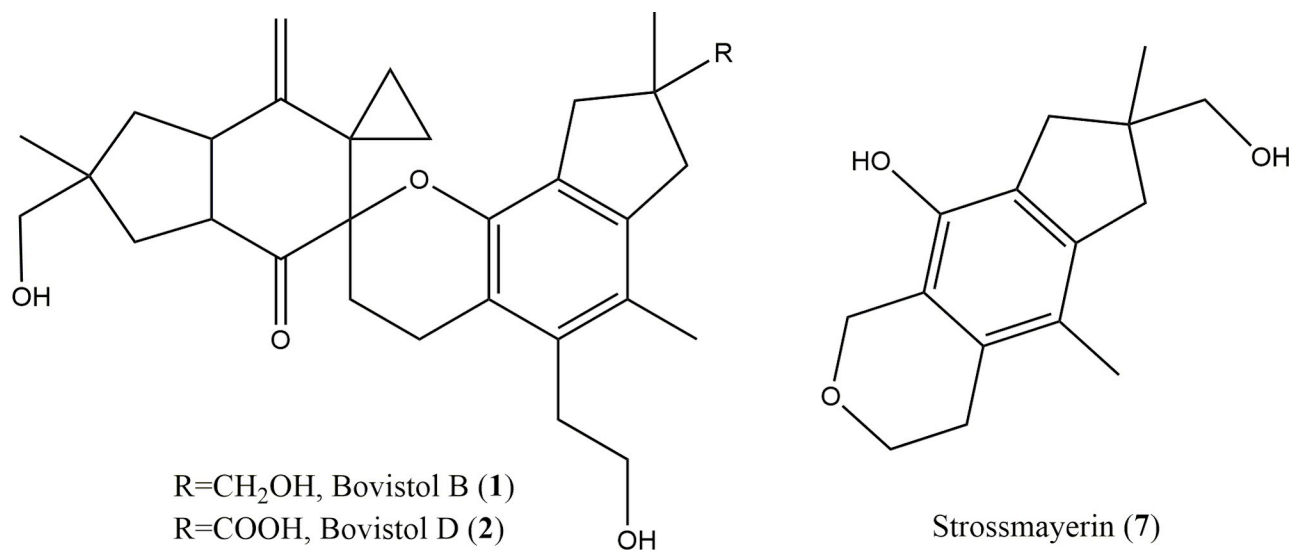


Fig 1. Novel sesquiterpenes produced by *C. strossmayeri*. Structures of bovistol B (1), D (2) and strossmayerin (7).

<https://doi.org/10.1371/journal.pone.0229925.g001>

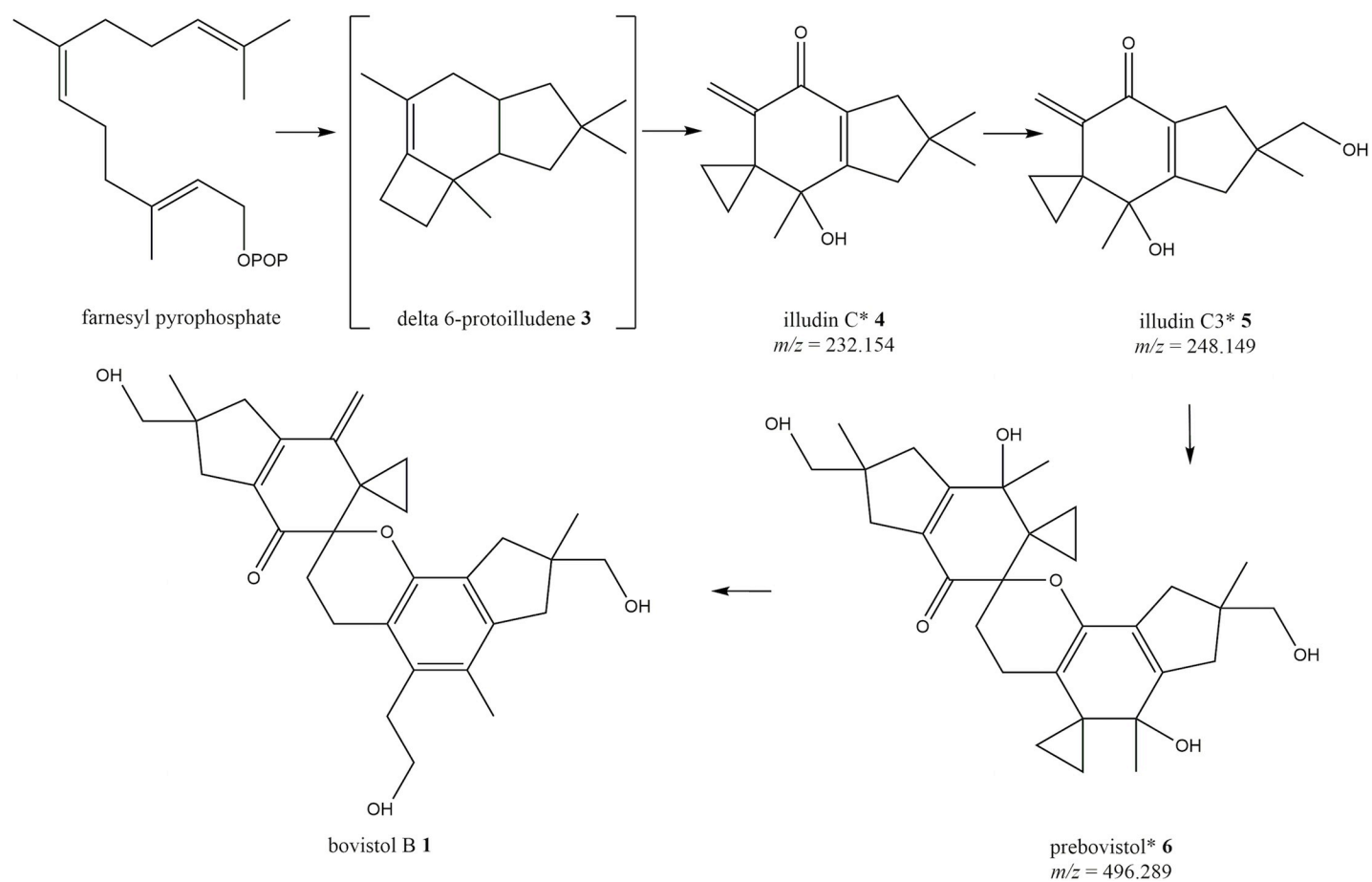


Fig 2. Proposed biosynthetic pathway for bovistol B (1). Accurate masses and formulae corresponding to the compounds indicated with an asterisk were identified in the HRMS data.

<https://doi.org/10.1371/journal.pone.0229925.g002>

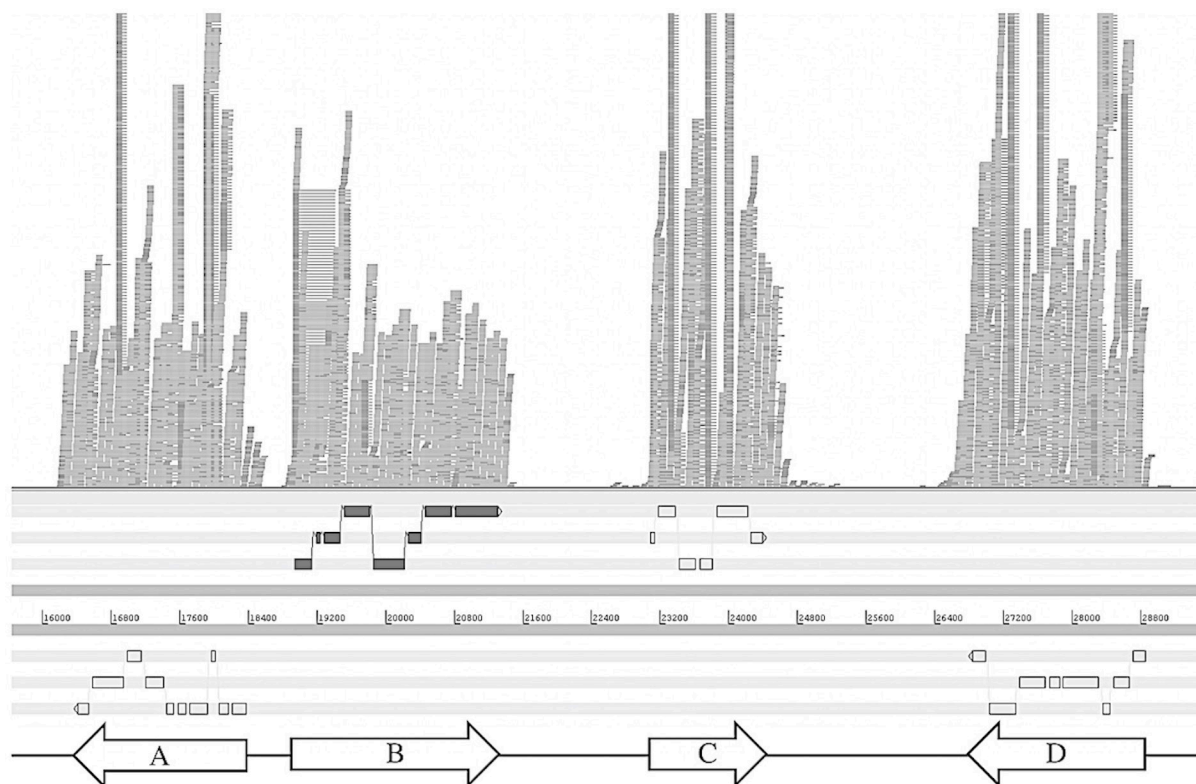


Fig 3. Putative gene cluster for the biosynthesis of 1. RNA-seq data aligned to the *C. strossmayeri* genome to identify cDNA sequences of the genes comprising the putative gene cluster for the biosynthesis of 1. Genes present include (A) FAD oxidoreductase, (B) sesquiterpene cyclase, (C) aldo-keto reductase, (D) P450-dependent oxidoreductase.

<https://doi.org/10.1371/journal.pone.0229925.g003>

indicated with asterisks in Fig 2. The proposed biosynthesis starts with a 1,11 cyclisation of farnesyl diphosphate to give delta 6-protoilludene (3), this is then oxidised to illudin C (4), then illudin C3 (5). A dimerisation then takes place to yield prebovistol (6), this asymmetric dimerisation is predicted to proceed via an inverse-electron demand hetero-Diels-Alder (DA) mechanism.

Phylogenetic analysis has shown basidiomycete sesquiterpene cyclases to form distinct clades corresponding to the mode of cyclisation [15,16]. The *C. strossmayeri* genome [5] was mined for sesquiterpene biosynthesis genes using antiSMASH [17–19], revealing four sesquiterpene synthases. These genes were compared to other characterised basidiomycete sesquiterpene synthases [15,16], using neighbor-joining in MEGA 5.0 [20,21]. Protein sequences are available in S2 Text in S1 File. This revealed only one likely gene capable of carrying out the 1,11 cyclisation required (S19 Fig), this was located in contig 98 (FTPT010000098) of the genome assembly [5]. Analysis of genes adjacent to this sesquiterpene cyclase, along with RNA-seq analysis performed through Galaxy and Artemis [22–24], led to the determination of a candidate gene cluster responsible for the biosynthesis of 1 (Fig 3), along with the proposed cDNA sequences of each gene. This cluster comprises a core sesquiterpene cyclase (FTPT010000098 bp 18,942–22,551), FAD oxidoreductase (FTPT010000098 bp 14,417–18,361), aldo-keto reductase (FTPT010000098 bp 23,093–24,388), and cytochrome P450-dependent oxidoreductase (FTPT010000098 bp 26,838–28,847). Genomic DNA sequences are available in S3 Text in S1 File. If the DA reaction is enzyme mediated, the gene responsible was not immediately apparent. Few such enzymes have been characterised to date [25–27], so motifs responsible for such activity are yet to be defined.

We are aware that this is not the first report of dimeric sesquiterpenes from basidiomycete fungi (e.g. spirodienone) [28], however, to our knowledge this is the first where an inverse-electron demand hetero-DA mechanism has been proposed for the dimerisation. It is noteworthy that **5** has been reported by other authors without any observation of dimerisation, so it is feasible that the dimerisation is enzyme-mediated and if so, such mechanisms would certainly be of interest given the scarcity of DA enzymes reported to date. The pericyclase IccD from the ascomycete *Penicillium variable* catalyses the inverse-electron demand DA reaction in the biosynthesis of ilicicolin [29], however, no significant homology to IccD was found in the *C. strossmayeri* genome flanking the putative gene cluster for the biosynthesis of **1**. The S-adenosyl-L-methionine (SAM)-dependent enzyme, LepI from *Aspergillus nidulans*, can catalyse intramolecular DA and hetero-DA reactions involved in the biosynthesis of leporin B [30], but similarly, no significant homology was found with genes present in the *C. strossmayeri* genome when a tblastn search was performed. There are increasing reports of characterised intramolecular Diels-Alderses of natural origin [31], however many originate from bacterial or ascomycete hosts and are likely to differ significantly to those responsible for pericyclic activity in basidiomycete natural product biosynthesis. This work highlights the vastly understudied basidiomycetes as a promising source of novel antimicrobial compounds.

Supporting information

S1 File.
(PDF)

Author Contributions

Conceptualization: Andy M. Bailey, Gary D. Foster.

Formal analysis: Alice M. Banks, Lijiang Song.

Funding acquisition: Andy M. Bailey, Gary D. Foster.

Investigation: Alice M. Banks, Lijiang Song.

Methodology: Alice M. Banks.

Project administration: Andy M. Bailey, Gary D. Foster.

Resources: Lijiang Song, Gregory L. Challis.

Supervision: Gregory L. Challis, Andy M. Bailey, Gary D. Foster.

Visualization: Alice M. Banks, Lijiang Song.

Writing – original draft: Alice M. Banks.

Writing – review & editing: Andy M. Bailey, Gary D. Foster.

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