Anti-cryptococcal activity of preussolides A and B, phosphoethanolamine-substituted 24-membered macrolides, and leptosin C from coprophilous isolates of Preussia typharum

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Abstract: Cryptococcus neoformans is a serious human pathogen with limited options for treatment. We have interrogated extracts from fungal fermentations to find Cryptococcus-inhibiting natural products using assays for growth inhibition and differential thermostability. Extracts from fermentations of four fungal strains from wild and domestic animal dung from Arkansas and West Virginia, USA were identified as Preussia typharum. The extracts exhibited two antifungal regions. Purification of one region yielded new 24-carbon macrolides incorporating both a phosphoethanolamine unit and a bridging tetrahydrofuran ring. The structures of these metabolites were established mainly by analysis of high-resolution mass spectrometry and 2D NMR data. Relative configurations were assigned using NOESY data, and the structure assignments were supported by NMR comparison with similar compounds. These new metabolites are designated preussolides A and B. The second active region was caused by the cytotoxin, leptosin C. Genome sequencing of the four strains revealed biosynthetic gene clusters consistent with those known to encode phosphoethanolamine-bearing polyketal macrolides and the biosynthesis of dimeric epipolythiodioxopiperazines. All three compounds showed moderate to potent and selective antifungal activity toward the pathogenic yeast C. neoformans.

Keywords: Antifungal, Cryptococcus, Ethanolamine phosphate transferase, Epipolythiodioxopiperazine, Polyketides, Sporormiaceae

Introduction

Cryptococcus neoformans and C. gattii are basidiomycete yeasts found in many varied regions of the world. These species are frequent opportunistic pathogens in highly immunocompromised patients, especially those with late-stage HIV infection. Given their ability to remain clinically dormant after initial infection, these fungal pathogens can re-emerge from latently infected sites in the setting of impaired CD4-mediated immunity, most often resulting in lethal infections of the central nervous system (Rajasingham et al., 2017). Although many immunosuppressed populations are at risk for infections due to Cryptococcus species, there were an estimated 220 000 annual cases of C. neoformans infections in 2017 worldwide, and up to a 75% mortality rate, specifically in patients with AIDS (Bongomin et al., 2017; Rajasingham et al., 2017). Treatment routinely depends on decades-old antifungal agents (azoles, amphotericin B, flucytosine) that are associated with poor outcomes due to drug toxicity, elevated recurrence rates, the need for long-term suppressive therapy, and emergence of drug resistance (Brown et al., 2012; Perfect, 2017). In part, these drawbacks can be associated with the fact that antifungal compounds identified by whole-cell screening have generally targeted pathogenic fungi other than Cryptococcus, especially the ascomycetes Candida albicans and Aspergillus fumigatus. Therefore, targeting the basidiomycete C. neoformans for discovery of new antifungal agents could reveal overlooked molecules with potential as novel drug candidates (Butts et al., 2013; Krysan, 2015).

Among strategies and techniques for discovery of antifungal agents, fungal metabolites represent a rich source of intrinsically antifungal molecules with one of the highest probabilities for success due to the likelihood for their interaction with fungal-specific targets (Roemer et al., 2011). Dung-inhabiting fungi, also known as coprophilous fungi, are a specialized ecological assemblage of fungi adapted to colonize, decompose, and reproduce in animal dung (Dix & Webster, 1995; Doveri, 2004). They often exhibit traits that aid in gaining access to and completing their life cycle in the dung habitat, including rapid reproduction, forcibly discharged spores or sporangia that stick to plant surfaces, holothallic mating systems, and strong enzyme systems for degradation of plant polymers (Dix & Webster, 1995). There is some evidence that certain species need passage through the gut to activate spore germination, although, they are thought not to be active in the internal gut microbiome. However, once dung is deposited, members of the coprophilous assemblage quickly
we describe the morphology and phylogenetic placement of the strain with its respective biosynthetic gene clusters (BGCs). Herein we refer to selective antifungal activity toward C. albicans. Preussia species are rich sources of bioactive natural products (Ahmed & Cain, 1972; Doveri, 2004; Furuya & Udagawa, 1972; Gonzalez-Menendez et al., 2017; Poch & Gloer, 1991; Rangel-Grimaldo et al., 2017; Talontsi et al., 2014). Thus, they are readily accessible microbes for exploring for metabolites that mediate behavior of other organisms (Bills et al., 2013) and for enzymes that degrade plant polymers (Peterson et al., 2011; van Erven et al., 2020).

During our efforts to identify fungal-produced antifungal metabolites active against the pathogenic yeast C. neoformans, we have identified a pair of new 24-carbon macrolides bearing a distinctive phosphoethanolamine substituent (1 and 2) and a previously described dimeric epipolythiodioxopiperazine (ETP), leptosin C (3), from coprophilous strains of Preussia typharum (Dothideomycetes, Sporormiaceae). These compounds (Fig. 1) exhibit selective antifungal activity toward C. neoformans compared to C. albicans. Preussia is an ascomycete genus with a cosmopolitan distribution, and the species are primarily associated with herbivore dung, although they can also be recovered from soil, living plants, and plant litter (Ahmed & Cain, 1972; Doveri, 2004; Furuya & Udagawa, 1972; Gonzalez-Menendez et al., 2017; Mapperson et al., 2014). Frequent previous reports indicate that Preussia species are rich sources of bioactive natural products (Bergstorn et al., 1995; Chen et al., 2009, 2020; Du et al., 2012, 2014; Gonzalez-Menendez et al., 2017; Noumeur et al., 2017; Poch & Gloer, 1991; Rangel-Grimaldo et al., 2017; Talontsi et al., 2014; Weber et al., 1990; Weber & Gloer, 1988, 1991; Xu et al., 2019; Zhang et al., 2012). However, to the best of our knowledge, no studies have yet associated secondary metabolites produced by any Preussia strain with its respective biosynthetic gene clusters (BGCs). Herein we describe the morphology and phylogenetic placement of the producing fungal strains identified as P. typharum, along with their fermentation, isolation, and the structure elucidation, and biological activity of the antifungal molecules. Furthermore, we identify the putative BGCs responsible for the macrolide and EPT assembly from draft genome sequences.

Materials and Methods
General Experimental Procedures

Extracts were fractionated on a Grace Reveleris X2 flash chromatography system equipped with UV and ELSD detectors. HPLC–DAD–MS analysis employed an Agilent 1260 LC coupled to an Agilent 6120 single quadrupole MS with 0.1% aqueous acetonitrile (A) and 0.1% aqueous formic acid (B) as mobile phases. Chromatographic profiles were monitored by scanning from 190 to 400 nm, and by positive and negative ESI (electrospray ionization)-MS from m/z 160–1500. NMR data were collected at 298 K on a Bruker 500-MHz NMR instrument equipped with a 5-mm triple resonance cryoprobe, with CD3OD as solvent and tetramethylsilane used as internal standard (TMS δH 0).

Fig. 1 Compounds 1–3 isolated from strains of Preussia typharum from Arkansas and West Virginia, USA.
CuSO₄ · 5H₂O in 1 000-ml deionized H₂O and MFP was replaced for GS [glucose 25 g, corn starch 10.0 g, blackstrap molasses (Brer Rabbit) 5.0 g, casein hydrolysate 4.0 g, meat peptone 1.0 g, 1 000-ml deionized H₂O]. TTI-0926 and TTI-1099 were grown in the same media as TTI-0947, except that GS medium was replaced by GLX medium (10.0-g peptone, 21.0-g malt extract, 40.0-g glycerol, 1.0-g carboxymethyl cellulose in 1 000-ml deionized H₂O). Cultures on the Wheat1, CY580, and CLA media were incubated statically with vials slanted at a 45° angle; cultures on other media were agitated at 220 rpm.

**Genome Sequencing and Annotation of Putative Preussolid and Leptosin Gene Clusters**

Strains TTI-0926, TTI-0947, TTI-1045 and TTI-1099 were grown in a static culture of 100-ml SMY for 14 days at 23°C. Mycelium was filtered, pressed dry, frozen at −80°C, and lyophilized. Genomic DNA was purified from ground mycelial powder with a Zymo Research Corporation Quick-DNA™ Fungal/Bacterial Miniprep Kit. For preparation of sequencing libraries, 500 ng of total genomic DNA were used as the template and processed using the KAPA HyperPlus Kit for PCR-free workflows (Roche, Switzerland) according to the manufacturer’s instructions. Sequencing libraries were size selected for 600–800-bp fragments using a LightBench (Coastal Genomics, Canada). Whole genomes were sequenced on a HiSeq 4000 Sequencing System (Illumina, USA). The genome was assembled by SPAdes using standard parameters (Bankevich et al., 2012).

The putative preussolid (GenBank MW147207) and leptosin C (GenBank MW161056) gene clusters in TTI-0926 and TTI-1099 were predicted by submitting the unannotated scaffold sequences for antiSMASH analysis (https://fungismash.secondarymetabolites.org/) (Blin et al., 2019). The ORFs of the BGCs were further refined by a combination of gene predictions from Augustus (Stanke et al., 2006) and FGENESH (Solovyev et al., 2006) using Preussia sp. as the reference genome followed by manual correction of ORFs. Possible functions of predicted hypothetical proteins were explored by analysis of catalytic domains at the Protein Data Bank (https://www.rcsb.org/). Reciprocal BLAST searches of predicted proteins and annotated scaffolds with sequences from previously determined core genes of the CLA genome as TTI-0947, except that GS medium was replaced by GLX medium (10.0-g peptone, 21.0-g malt extract, 40.0-g glycerol, 1.0-g carboxymethyl cellulose in 1 000-ml deionized H₂O). Cultures on the Wheat1, CY580, and CLA media were incubated statically with vials slanted at a 45° angle; cultures on other media were agitated at 220 rpm.

**Phylogenetic Tree Construction**

To reconstruct the approximate phylogenetic position of strains TTI-0926, TTI-0947, TTI-1045, and TTI-1099, genomic DNA was purified from lyophilized mycelial powder of strains TTI-0926 and TTI-0947 with a Zymo Research Corporation Quick-DNA™ Fungal/Bacterial Miniprep Kit. The rDNA region containing the internal transcribed spacer (ITS) region with primers ITS1 and ITS5. PCR products were amplified from 30-μl reaction mixtures containing 1.0-μl DNA template, 1.0-μl each forward and reverse primers, 15 μl of 2 × MasterMix (Promega), and 12 μl of H₂O. The PCR parameters were 94°C for 40 s, followed by 40 cycles at 54°C for 60 s, 72°C for 90 s, and a final extension at 72°C for 10 min. Partial sequences obtained from sequencing reactions were assembled with Geneious (version R11. https://www.geneious.com/). ITS sequences amplified from TTI-0926 and TTI-0947 (MW256658, MW256659) were used to search genomic assemblies from strains TTI-1095 and TTI-1099 to locate their respective ITS sequences (GenBank MW256656, MW256657).

A phylogenetic tree was constructed based on alignments of the ITS region. The DNA sequences from other Preussia species retrieved from GenBank were aligned by using ClustalW implemented in MEGA X (Kumar et al., 2018), and the resulting alignment was manually trimmed. Phylogenies were inferred by the maximum likelihood (ML) method implemented in MEGA X under a Kimura 2-parameter model with gamma distribution of evolutionary rates. Bootstrap supports for tree nodes were calculated using the default options in MEGA X with 1 000 replicates per run.

**Screening for Antimicrobial Activity and Identification of Bioactive Compounds**

For initial detection of antifungal activity from previously untested fungal strains, extracts from 12-ml fermentations were tested in agar zone of inhibition assays. Each fermentation was extracted by the addition of an equal volume of methyl ethyl ketone (MEK) followed by shaking for 2 hr. The MEK layer was evaporated under vacuum. Residues were dissolved in DMSO at 10 × the original culture volume and 10 μl of each DMSO extract was applied to a 4-mm well aspired from a plate of YM agar (malt extract 10 g, yeast extract 2 g, agar 20 g, in 1 000 ml of deionized H₂O) seeded with an overnight culture of C. neoformans ATCC 10231 or C. neoformans H99. Plates were incubated at 25°C and examined after 24–48 hr, and zones of inhibition (ZI) were photographed.

The antifungal activity produced by strains was tracked by HPLC microfractionation followed by liquid growth inhibition assay directly in the plates used for fractionation as described previously (Perlatti, Nichols, Lan, et al., 2020). Briefly, the HPLC eluents from an Ace Equivalence C18 150 × 4.6 mm, 5 μm, 35°C, 10–100% A over 20 min, holding 100% for 4 min, 1.0 ml min−1 were collected with a fraction collector. Aliquots of 250 μl were collected in 96-well plates and vacuum-dried. An overnight culture of C. neoformans was diluted to O.D. 0.8 in sterile H₂O and then diluted another 500-fold in YM media. An aliquot of 90 μl was transferred to each well, and the plate incubated for 36 hr, afterwards 10 μl of PrestoBlue (Invitrogen) dye was added and incubated for another 12 hr to differentiate growing versus non-growing wells. Antifungal compounds were traced to wells where growth of C. neoformans was completely inhibited.

**Isolation and Purification of Antifungal Compounds**

Strain TTI-1095 was selected for scale-up and was grown in 20 × 250-ml baffled flasks containing 50 ml of MMK2 medium. A 2.5-ml aliquot of a 4-day seed culture in SMY was transferred to each flask and incubated at 23°C and 220 rpm. After 14 days of growth, the whole cultures were extracted by adding 50 ml of MEK to each flask, followed by agitation for 3 hr at 220 rpm. The organic phase was separated, filtered, and evaporated to dryness. The resulting crude extract was dissolved in MeOH:H₂O 9:1, and extracted with hexane. The methanic fraction was dried, adsorbed in C18 and submitted to flash chromatography (Reveleris C18 RP 40-g cartridge, 20% MeOH for 2 min, 20–40% MeOH in 2 min, held for 1 min, 40–60% MeOH in 2 min, held for 2 min, 60–90% MeOH in 3 min, held for 1.5 min, 90–100% MeOH in 1 min and held for 20 min at 220 rpm. Vials were slanted at 45° angle; cultures on other media were agitated at 220 rpm.
Table 1 1H and 13C NMR Data for 1 and 2 (CD3OD; 500 and 125 MHz for 1H and 13C, Respectively)

| Position | δC, mult | δH, mult (J in Hz) | δC, mult | δH, mult (J in Hz) |
|----------|-----------|---------------------|-----------|---------------------|
| 1        | 170.2, C  | –                   | 169.8, C  | –                   |
| 2        | 91.4, CH  | 5.30, br s          | 91.5, CH  | 5.27, br s          |
| 3        | 176.1, C  | –                   | 176.3, C  | –                   |
| 4        | 38.5, CH2 | Ha: 3.58, d (19)    | 38.5, CH2 | Ha: 3.55, d (19)    |
|          |           | Hb: 3.22, ddd (19, 6.4, 1.4) | Hb: 3.06, ddd (19, 5.8) |
| 5        | 75.7, CH  | 5.02, t (6.4)       | 75.7, CH  | 5.01, d (6.5, 5.8)  |
| 6        | 93.3, CH  | 4.3, d (9.3)        | 93.3, CH  | 4.29, d (9.4)       |
| 7        | 69.5, CH2 | 3.26, td (9.3, 1.2) | 68.7, CH2 | 3.11, ddd (116, 9.4, 1.2) |
| 8        | 41.5, CH2 | Ha: 1.89, ddd (139, 7.5, 1.2) | 42.7, CH2 | Ha: 1.81, ddd (13, 11, 1.2) |
|          |           | Hb: 1.55, m         | Hb: 1.68, ddd (13, 11, 3.8) |
| 9        | 69.9, CH  | 3.83, dt (13, 5.3)  | 71.1, CH  | 4.21, ddd (11, 8.0, 3.8) |
| 10       | 36.8, CH2 | 1.44, m             | 133.3, CH | 5.32, m             |
| 11       | 25.2, CH2 | 1.34, m             | 134.1, CH | 5.65, dt (15, 6.7)  |
| 12       | 30.7, CH2 | 1.26, m             | 32.7, CH2 | 2.03, m             |
| 13       | 30.5, CH2 | 1.27, m             | 30.4, CH2 | 1.30, m             |
| 14       | 29.4, CH2 | 1.25, m             | 29.4, CH2 | 1.25, m             |
| 15       | 30.6, CH2 | 1.29, m             | 30.5, CH2 | 1.29, m             |
| 16       | 30.1, CH2 | 1.33, m             | 30.2, CH2 | 1.34, m             |
| 17       | 33.2, CH2 | 1.98, m             | 33.4, CH2 | 1.98, m             |
| 18       | 131.6, CH | 5.35, m             | 131.4, CH | 5.35, m             |
| 19       | 131.8, CH | 5.36, m             | 131.9, CH | 5.36, m             |
| 20       | 33.0, CH2 | 1.93, m             | 33.2, CH2 | 1.92, m             |
| 21       | 27.0, CH2 | 1.35, m             | 26.6, CH2 | 1.37, m             |
| 22       | 36.8, CH2 | Ha: 1.58, m         | 36.6, CH2 | Ha: 1.59, m         |
|          |           | Hb: 1.48, m         | Hb: 1.46, m |
| 23       | 70.8, CH  | 4.88, m             | 70.4, CH  | 4.93, m             |
| 24       | 20.7, CH2 | 1.19, d (6.2)       | 20.6, CH2 | 1.18, d (6.2)       |
| 25       | 62.9, CH2 | 4.04, m             | 63.0, CH2 | 4.03, m             |
| 26       | 41.6, CH2 | 3.17, m             | 41.7, CH2 | 3.15, m             |

Minimum Inhibitory Concentration

To quantify the inhibitory concentrations of compounds 1–3 for strains of fungal and bacterial pathogens, minimum inhibitory concentrations (MICs) were measured using species-specific modifications to standard CLSI testing methods (Alexander, 2017). Strains tested included Staphylococcus aureus ATCC 43300, C. albicans ATCC 10231, A. fumigatus AF239, and C. neoformans H99, with the latter tested at both 23 and 37°C. Briefly, overnight cultures were sequentially diluted to O.D. 600 of 0.8 in phosphate buffered saline and again by 1 000-fold in RPMI-1640 buffered with MOPS (Sigma-Aldrich). The final cell suspension was incubated with serial dilutions of selected compounds dosed at range of 0.5–256 μg/ml for preussolides and 0.03125–32 μg/ml for leptosin C. Growth was assessed by adding 10% alamarBlue (Bio-Rad) followed by incubation for 24 hr (S. aureus) or 48 hr (C. albicans, A. fumigatus, and C. neoformans).

Macrophone Cytotoxicity Assay

The cytotoxicity of compounds against the macrophage-like murine cell line J774A.1 was evaluated as described previously (Perlatti, Nichols, Alspaugh, et al., 2020). Macrophage J774.1 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) to 70% confluency, harvested, transferred to 96-well tissue culture plates at 105 cells well−1, and incubated overnight at 37°C and 5% CO2. Compounds 1 and 3 were added to the DMEM medium in serial twofold dilutions to achieve a dose range of 0.03125–32 μg/ml−1. The macrophage medium was replaced with the DMEM medium containing compound and incubated for 24 hr at 37°C and 5% CO2. Macrophage viability was assessed by the addition of 10% alamarBlue and incubating for 3 hr at 37°C and 5% CO2 prior to fluorescence measurement (BMG FLUORstar Optima plate reader).
Results and Discussion

Identification of Strains

The four strains producing compounds 1–3 were judged to be conspecific based on multiple criteria. All four strains were recovered from animal dung, TTI-0926 and TTI-0947 from pony dung from Arkansas, and TTI-1095 and TTI-1099 from white-tailed deer dung from West Virginia. Fermentation extracts of these strains yielded similar patterns of antifungal and antibacterial activity (Fig. 2). HPLC–UV–MS analysis of these extracts indicated that they shared some major metabolites. Mycelial growth and pigments observed among cultures on a set of four different media indicated the strains were highly similar (Supplementary Fig. S1). Sporulation was more or less consistent among the strains, with strains, TTI-0926, TTI-947, and TTI-01099 (Supplementary Figs. S2–S4) producing both membranaceous pycnidia (often referred to as spermagonia (Cain, 1961)) bearing minute, hyaline, globose conidia (Supplementary Fig. S3) and ascomata with four-celled melanized ascospores typical of the genus Peussia (Supplementary Figs. S2–S4). Strain TTI-1095 only produced the pycnidial state and never produced fertile ascomata (not shown).

Alignment of the rDNA ITS region of the four strains indicated sequences were 99–100% similar. BLAST searches of public databases with the ITS sequences of all four strains retrieved many sequences with >98% similarity. The majority of the assigned names were associated with the P. funiculata clade of the genus Peussia (Kruys & Wedin, 2009), including P. funiculata, P. aemulans, P. vulgaris, P. typharum, and P. fleischhakii, but no coherent pattern with respect to the nomenclature of these strains could be inferred.

To understand the relationships of these four coprophilous strains more precisely, we aligned ITS sequences from strains in and near the P. funiculata group and built a phylogenetic tree using the ML method (Supplementary Fig. S5). Our strains fell into a clade with a large number of sequences that again corresponded to the P. funiculata group (Kruys & Wedin, 2009), and this clade has been observed in other surveys of environmental isolates of Peussia species (Gonzalez-Menendez et al., 2017; Massimo et al., 2015). Notably, this clade also included the ITS sequence (GenBank JX143871) from a soilborne strain from Oklahoma, USA designated as P. typharum that produced leptosin C (Supplementary Fig. S5) (Du et al., 2014). Therefore, this Oklahoma strain is likely conspecific with our strains. None of the strains included within the terminal clade with our isolates were type strains, and furthermore, living type strains for the core species of the P. funiculata clade, P. funiculata, P. aemulans, P. vulgaris, P. typharum, are non-existent because they were described during the 19th century (Cain, 1961).

Thus, we were unable to confidently associate rDNA sequences from these strains with those of unambiguously named reference strains. Rather than arbitrarily select a species name, we related morphological features of the strains to authoritative illustrated descriptions from the literature. Diagnostic features included the elongated bases of the ascii, four-celled ascospores with transverse septa perpendicular to the longitudinal axis of the spore, each ascospore cell with a longitudinal germ slit, and tapered ascospore terminal cells (Supplementary Figs. S2–S4). The combination of features conformed to the morphological concept of P. typharum (Cain, 1961; Doveri, 2004), therefore, we have applied this name to the strains.

Detection of Bioactivity, Isolation, and Characterization of Active Compounds

Organic extracts obtained from strains TTI-0926, TTI-0947, TTI-1095, and TTI-1099 grown in different culture media produced clear inhibitory zones against Staphylococcus aureus, Candida albicans, and C. neoformans. Bioactivity, as assessed by zone sizes, varied with strain, medium, and test pathogen. The largest inhibition zones were observed for C. neoformans. HPLC microfractionation guided by liquid C. neoformans bioassay at 37°C enabled tracking of the bioactivity to two regions of the chromatogram, where two distinct peaks were observed in wells E5 (r.t. 13.0 min) and F5 (r.t. 16.75 min), respectively (Fig. 2), with similar assay results for the four strains of P. typharum.

Strain TTI-1095 was chosen for investigation of the antifungal components because its extracts produced the largest and clearest inhibition zones against C. neoformans H99. Scaled-up growth of strain TTI-1095 in MMK2 medium provided adequate material for the purification of compounds 1 and 3, which corresponded to wells E5 and F5 (Fig. 2), respectively. They were produced in sufficient amounts for detailed characterization, along with a third, minor compound 2 with a UV profile similar to that of the peak for 1 observed in well E5.

Purified compound 1 was a light-brown solid. HRMS analysis was consistent with the molecular formula C_{26}H_{46}NO_{9}P. A set of 1D (1H and 13C) and 2D (COSY, HSQC, HMBc, and NOESY) NMR data was acquired for 1 in CD_{3}OD (Supplementary Figs. S6–S11, Table 1). 1H, 13C, and multiplicity-edited HSQC NMR data showed the presence of 26 carbons, of which five are sp²-hybridized, attributed to a carboxyl at δ 169.8, an oxygen-bearing double bond at δ 91.4 and δ 176.3, and another pair of olefinic carbons at δ 131.5 and δ 131.8. The remaining (sp³) carbons were identified as 5 oxymethines, 1 oxymethylene, 1 aminomethylene, 13 other methylenes, and 1 methyl group. COSY cross-peaks allowed the observation of spin-systems corresponding to the H-2-H-7-H-11 and
H₂-16–H₄-24 units of 1. Correlations between H₄-12 and H₄-15 were not specifically assigned due to extensive overlap. An HMBC correlation between oxymethine H-23 and C-1 allowed the establishment of the 24-membered macrolide ring. Surprisingly, measurable coupling between H-4a/H-5 and H-5/H-6 was not detected in ¹H NMR, although very weak corresponding correlations were observed in the COSY spectrum. This suggests a dihedral angle close to 90° in each case, and strong NOESY interactions were observed between each of these pairs. HMBC correlations of H-4a (δ 3.58) to C-3, C-5, and C-6, of H-4b (δ 3.22) to C-2 and C-3, of H-5 (δ 5.02) to C-3 and C-6, and of H-6 (δ 4.30) to C-3, C-4, C-5, C-7, and C-8 allowed the establishment of a trisubstituted 3,4,5-trihydrofuran ring bridging C-3 and C-6. This structural unit is rare, and it appears that the only prior report of a metabolite possessing this feature was described in a European patent application that disclosed a compound referred to as SM 140 I discovered from an unidentified Penicillium strain (Zeeck et al., 1992). The structure of SM 140 I corresponds well with the gross substructure of 1, and the relevant ¹H and ¹³C NMR data for 1 match well with those described (Zeeck et al., 1992). A COSY correlation between oxymethylenes H-25 (δ 4.11) and aminomethylene H-26 (δ 3.17) protons indicated the existence of an ethanolamine moiety, insulated from the rest of the molecule as evidenced by the lack of other observable HMBC correlations for their ¹H and ¹³C NMR signals. The doublet signals in the proton-decoupled ¹³C NMR spectrum of 1 for carbons at C-5 (δC 75.7, J_C_ = 4.6 Hz) and C-25 (δC 62.9, J_C_ = 4.6 Hz), and the adjacent carbons C-4 (δC 38.5, J_C_ = 2.9 Hz), C-6 (δC 93.6, J_C_ = 5.0 Hz), and C-26 (δC 41.6, J_C_ = 5.4 Hz) indicated that the ethanolamine unit is part of a phosphoethanolamine group connected to the macrolide at C-5. The remaining methylenes at C-12–C15 were set based on the molecular formula, although the corresponding ¹H NMR signals were not precisely located due to extensive overlap. The Z-geometry of the double bond at C-2 was determined based on NOESY correlations between H-2 and H-4a/b. Because the Δ18,19 olefin ¹H NMR signals were overlapped, the corresponding J_HH value was not measured, but it was assigned the E geometry on the basis of the δ values of the corresponding allylic carbons (C-17 at δC 33.2 and C-20 at δC 33.0) and by comparison of these shift values with those of similar compounds (Kozone et al., 2009; Morishita et al., 2020).

The relative configuration of 1 was difficult to assign with confidence by NMR analysis alone due to the flexibility of the macrolide ring. Unfortunately, efforts to crystallize a sample of 1 were unsuccessful. Strong NOESY correlations of H-7 with both H-5 and H-9 suggested an all-cis relative configuration of H-5, H-7 and H-9. The relative configuration of C-2 was proposed as shown based on the absence of NOESY correlations to H-6. These assignments, and proposal of the relative configuration shown at remote stereocenter C-23, would be consistent with those recently elucidated for products of heterologous expression of the akml and cinl BGCs of A. luchuensis and C. incanum, respectively (Morishita et al., 2020), and proposed analogy with the corresponding biosynthetic pathway as discussed below. We have named compound 1 as preussolide A. Notably, the presence of a dialkyl phosphoric acid OH group together with a free amine in the molecule indicates that the structure likely exists in the zwitterionic form. The structure shown does not incorporate this feature in order to be consistent with prior reports of this structure type.

Compound 2 exhibited a similar UV profile to that of 1, and HRMS analysis indicated a formula 2 Da lower than the formula of 1. The NMR data for 2 (Supplementary Figs. S12–S17, Table 1) indicated a structure similar to that of 1 (Fig. 3). However, the methylene signals for H₄-10 and H₄-11 observed in the data for 1 were absent, and the signals for H-9 (δ 4.21) and H-12 (δ 2.03) in 2 were deshielded compared to the corresponding signals in the spectrum of 1 (δ 3.83 and δ 1.26 for H-9 and H-12, respectively).

Furthermore, new COSY correlations, with H-9 correlating to a hydrogen at δ 5.32 (H-10) and H-12 showing a correlation to δ 5.65 (H-11) indicated the presence of a C-10/C-11 double bond in 2. In this case, the E-geometry of the double bond at C-10 could be assigned based on the H-10/H-11 J-value (15.2 Hz). All other data acquired aligned well with those of preussolide A. The compound (2) was named preussolide B.

Compound 3 was identified based on its UV, HRMS, [α]D, and ¹H NMR data (Supplementary Fig. S18) as leptosin C, a cytotoxic dimeric EPT affecting mammalian cells that has been previously reported from strains identified as Leptosphaeria sp. (Takahashi et al., 1994; Yanagihara et al., 2005) and P. typharum (Du et al., 2014). However, antifungal activity, although predictable based on the structural class, has not previously been reported for leptosin C (Wang, Li et al., 2017; Yanagihara et al., 2005).

Identification of Gene Clusters Capable of Encoding Biosynthesis of Preussolides and Leptosin

Compounds 1 and 2 belong to a class of natural products known as macrolides that can comprise of macrocyclic lactone rings of varying sizes and a wide range of substituents. Most notably, 14- and 16-membered macrolides produced mainly by actinobacteria have been successfully used as antibacterial, antifungal, immunosuppressive, antiparasitic, and anticancer compounds (Dinos, 2017; Ōmura, 2003, Park et al., 2010; Zuckerman et al., 2011). Fungal macrolides likewise are common and often exhibit potent biological properties (Ackland et al., 1985; Greve et al., 2008; Morishita et al., 2019; Singleton et al., 1958; Stierle et al., 2017; Surup et al., 2018; Xu et al., 2014). Compounds 1 and 2 have a 24-membered macrolactone ring, a structure observed in other classes of molecules such as macroactins (Gustafson et al., 1989), archazolids (Sasse et al., 2003), patellazoles (Kwan et al., 2012; Zabriskie et al., 1988), lejimalides (Kobayashi et al., 1988), and lecythomycin (Sugijanto et al., 2011). The presence of a phosphoethanolamine moiety or its derivatives is uncommon and only observed thus far in fungi, including eushearilide from Penicillium shearii (= Eupenicillium shearii) (Hosoe et al., 2006), JBIR-19 and JBIR-20 from Metarhizium sp. fE61 (Kozone et al., 2009), and two compounds from A. luchuensis IFO 4308 and C. incanum MAFF 238704 obtained by heterologous expression of the akml and cinl BGCs, respectively (Morishita et al., 2020). To the best of our knowledge, this is the first report of a 24-membered macrolide incorporating both a phosphoethanolamine unit and a bridging tetrahydrofuran ring. Bioinformatic triangulation using the akml and cinl...
Table 2 Predicted Proteins of Putative Preussolide Cluster (ptml) in Preussia typharum and Amino Acid Similarity to akml Cluster from Aspergillus luchuensis

| Gene   | Function                        | Protein ortholog in Aspergillus luchuensis | Similarity % |
|--------|---------------------------------|-------------------------------------------|--------------|
| ptmlA  | Polyketide synthase             | akmlA                                     | 64           |
| ptmlB  | Thioesterase                    | akmlB                                     | 55           |
| ptmlC  | GPI-ethanolamine transferase    | akmlC                                     | 41           |
| ptmlD  | P450                            | akmlD                                     | 60           |
| ptmlE  | NTF2 domain-bearing protein     | Absent                                    | –            |
| ptmlF  | MFS transporter                 | akmlF                                     | –            |

Fig. 4 Graphic representation of putative and proven macrolide-type gene clusters and their microsynteny. The preussolide (ptml) gene cluster from two of the four strains of Preussia typharum are illustrated and aligned with the akml and ciml gene clusters.

gene clusters (Morishita et al., 2020) as probes identified a putative BGC (ptml) in all the genomes of the four P. typharum strains with high sequence similarities and similar gene content as akml and ciml. The ptml BGC consists of ptmlA, a highly reducing polyketide synthase (HR-PKS), a separately encoded thioesterase, ptmlB, a P450 ptmlD, and a glycosylphosphatidylinositol-ethanolamine phosphate transferase homologue (GPI-EPT) ptmlC, that forms the phosphate ester linkage between the phosphoethanolamine group and the macrolide C-5 hydroxyl (Table 2, Fig. 4). Furthermore, in the preussolide, akml and ciml gene clusters an extra enzyme was evident not mentioned in previous studies (Morishita et al., 2020), a MFS transporter protein encoded by ptmlF (Table 2, Fig. 4). Another predicted enzyme, ptmlE, absent in the akml and ciml gene clusters, did not retrieve clear results from protein BLAST. Nonetheless, a search for possible catalytic domains in the Protein Data Bank and alignment of amino acid sequences indicated its distant homology to NTF2 (nuclear transport factor 2)-like enzymes, including AusH (Mori et al., 2017), Trt14 (Matsuda et al., 2015), and PrhC (Matsuda et al., 2016) involved in austinol, terretomin, breviamine, and paraherquonin biosynthesis, respectively, and BvnE, a semipinacolase participating in the biosynthetic pathway of the indole alkaloid breviamine (Ye et al., 2020). Despite their seemingly low sequence or functional similarity, all these enzymes catalyze the activation of hydroxyl or water molecules and subsequent intramolecular nucleophilic addition. Structural comparison of the putative enzyme product of this gene with those from AusH, Trt14, PrhC, and BvnE revealed a high similarity in their predicted three-dimensional structures (Supplementary Fig. S19).

Based on the structural evidence and the identification of the putative BGC in P. typharum, we propose one possible biosynthetic route for the production of 1 and 2, noting that the timing and sequence of the individual reactions could vary (Fig. 5).

Using the same triangulation approach as above, we were able to identify a putative leptosin C BGC (ptver) in all four strains of P. typharum that exhibited remarkably similar gene content and order as the previously characterized EPT BGCs encoding the biosynthesis of verticillin and chaetocin (Fig. 6, Table 3).

Antifungal and Cytotoxic Activity of Preussolides and Leptosin C

The results of MIC dilution assays for compounds 1–3 are displayed in Table 4. All three compounds selectively inhibited the growth of C. neoformans H99 relative to parallel growth inhibition
assays with C. albicans ATCC 10231. Leptosin C (3) was most potent toward C. neoformans at 37°C.

Eushearilide, a phosphethanolamine-bearing polyketide, was shown to exhibit qualitative antifungal activity against a broad range of fungi, including pathogenic ascomycetous yeasts, dermatophytes, filamentous ascomycetes, Zygomycetes, and C. neoformans (Hosoe et al., 2006). The last three compounds of the cimlA pathway produced by heterologous expression (6–8) inhibited fungal hyphal growth of Trichophyton mentagrophytes IFM 62679 \([\text{minimum effective concentration } = \text{MEC}_{80} \text{ of } 6.25 \mu\text{M}(7), 12.5 \mu\text{M}(3), \text{and } 50.0 \mu\text{M}(4, 6, 8)]\), and compound 3 also inhibited of A. fumigatus IFM 62541 \([\text{MEC}_{80} \text{ of } 25.0 \mu\text{M}]\) (Morishita et al., 2020). Compounds 3 and 7 inhibited germination of conidia T. mentagrophytes IFM 62679 \([\text{MIC}_{80} \text{ of } 12.5 \mu\text{M}(7) \text{ and } 25.0 \mu\text{M}(3)]\) and A. fumigatus IFM 62541 \([\text{MIC}_{80} \text{ of } 50.0 \mu\text{M}(3, 7)]\) (Morishita et al., 2020).

Leptosin C (3) was found to selectively inhibit C. neoformans in the submicromolar range (Table 4). Other ETPs have also been reported as a selective antifungals against C. neoformans when compared to C. albicans (Li et al., 2016). Furthermore, leptosin C (3), like

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**Fig. 5** A possible biosynthetic pathway for preussolides in Preussia typharum. Domain structure for the ptlmA polyketide is represented graphically.

**Fig. 6** Graphic representation of putative and proven dimeric epidithiodiketopiperazine gene clusters and their microsynteny. The leptosin C gene cluster (ptver) from two of the four strains of Preussia typharum are illustrated and aligned with the verticillin (ver) and chaetocin (cha) gene clusters.
Table 3 Predicted Proteins of Putative Leptosin C Cluster (ptver) in Preussia typharum and Amino Acid Similarity to ver Cluster from Clonostachys rogersoniana

| Gene   | Function                        | Protein homolog in Clonostachys rogersoniana | Similarity % |
|--------|---------------------------------|-----------------------------------------------|--------------|
| ptverA | ABC multidrug transporter       | verA                                          | 64           |
| ptverB | Cytochrome P450                  | verB                                          | 49           |
| ptverC | Cytochrome P450 oxidoreductase   | verC                                          | 55           |
| ptverD | Glutathione S-transferase        | verG                                          | 74           |
| ptverE | Aminotransferase                 | verI                                          | 59           |
| ptverF | Membrane dipeptidase             | verJ                                          | 71           |
| ptverG | γ-Glutamyl cyclotransferase      | verK                                          | 68           |
| ptverH | Cytochrome P450                  | verL                                          | 71           |
| ptverI | O-Methyltransferase              | verM                                          | 67           |
| ptverJ | Methyltransferase                | verN                                          | 58           |
| ptverK | Nonribosomal peptide synthetase  | verP                                          | 46           |
| ptverL | Oxidoreductase                   | verT                                          | 61           |
| ptverM | Unknown                          | Hypothetical protein                          | –            |
| ptverN | Unknown                          | Hypothetical protein                          | –            |
| ptverO | C6 zinc finger domain protein    | verZ                                          | –            |

Table 4 Minimum Inhibitory Concentrations (MICs) of 1–3

| Organism                              | Compound (MIC; μg ml⁻¹) | Control* |
|---------------------------------------|-------------------------|----------|
| Staphylococcus aureus ATCC 43300       | >256 64 >256 0.05       |          |
| Candida albicans ATCC 10231            | 256 256 8 1.56          |          |
| Cryptococcus neoformans H99 (23°C)     | 8 32 0.25 0.78         |          |
| Cryptococcus neoformans H99 (37°C)     | 4 32 0.06 0.78         |          |
| Aspergillus fumigatus AF239            | 8 NT 0.125             |          |
| Macrophae J774A.1                      | 32 NT 0.125            |          |

*aChlorotetracycline Chlorotetracycline + streptomycin were the control for S. aureus. Amphotericin B was the control for fungal strains. NT: Not tested.

other dimeric ETPs, exhibited potent mammalian cell cytotoxicity (Table 4).

Summary

In conclusion, by assaying secondary-metabolite-enriched extracts of under-investigated coprophilous ascomycetes (Bills et al., 2013; Bills & Polishook, 1993; Wicklow & Hirschfield, 1979) for growth inhibition of the basidiomycete pathogen, C. neoformans, we have identified two new 24-membered phosphoethanolamine-esterified macrolides, preussolides A and B (1, 2), and the ETP dimer, leptosin C (3). In contrast to the recent report on the products of the akml and ciml BGCs as unexpressed in fungal cultures (Morishita et al., 2020), the preussolide macrolides were readily produced in liquid agitated and solid culture media. They were produced by four coprophilous strains of the P. funiculata complex with morphology conforming to P. typharum. Additionally, we have identified gene clusters from their draft genome assemblies that likely encode the biosynthesis of 1, 2, and 3. Consequently, this is the first report of a putative secondary metabolic BGCs from strains in the genus Preussia. All three compounds showed moderate to potent and selective antifungal activity toward the pathogenic yeast C. neoformans.

Supplementary Material

Supplementary material is available online at JIMB (www.academic.oup.com/jimb).

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Conflict of Interest

The sponsors were not involved in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to publish the article. All authors declare no conflict of interest. J.E.S. and C.J.B.H. are employees of Hexagon Bio which supported the genome sequencing. This work did not involve studies with human participants or animals.

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