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Chemical Inhibitors Suggest Endophytic Fungal Paclitaxel Is Derived from Both Mevalonate and Non-mevalonate-like Pathways

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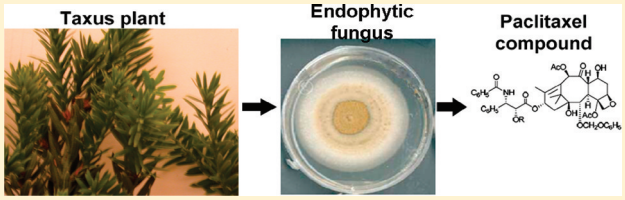
Supporting Information

ABSTRACT: *Taxus* trees possess fungal endophytes reported to produce paclitaxel. Inhibitors that block early steps in plant paclitaxel biosynthesis were applied to a paclitaxel-producing fungus to determine whether these steps are shared. The plant paclitaxel backbone is reportedly derived from the non-mevalonate terpenoid pathway, while the side chain is phenylalanine-derived. Evidence that the shikimate pathway contributes to fungal paclitaxel was shown by decreased paclitaxel accumulation following inhibition of phenylalanine ammonia lyase. Expression of another shikimate pathway enzyme, 3-dehydroquinate synthase, coincided with paclitaxel production. The importance of the mevalonate pathway in fungal paclitaxel biosynthesis was shown by inhibition of fungal paclitaxel accumulation using compactin, a specific inhibitor of 3-hydroxy-3-methyl-glutaryl-CoA reductase. Expression of another mevalonate pathway enzyme, 3-hydroxy-3-methyl-glutaryl-CoA synthase, coincided with fungal paclitaxel accumulation. Unexpectedly, results from using fosmidomycin suggested that fungal paclitaxel requires 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), an enzyme in the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway normally found in bacteria/plants. Additional lines of evidence support this finding; first, a plant DXR antibody recognized a fungal peptide of the correct size; second, expression of an apparent fungal DXR ortholog correlated to changes in paclitaxel production; finally, BLAST searching identified a gene putatively encoding 1-deoxy-D-xylulose-5-phosphate synthase, the first enzyme in the MEP pathway in *Aspergillus*.



Paclitaxel, a major diterpenoid anticancer drug,[1−3] is found in very low quantities (0.4 g/kg)[4] in its original plant source, the bark of yew trees (*Taxus* spp.),[1] prompting the search for alternative sources. In 1993, a fungal endophyte, *Taxomyces andreanae*, was discovered living within the inner tissues of *Taxus brevifolia*[5] that was subsequently reported to produce paclitaxel *in vitro*. The identity of fungal paclitaxel was confirmed using mass spectrometry, NMR, and recognition by a plant paclitaxel monoclonal antibody.[6,7] Some have questioned the authenticity of fungal-derived paclitaxel, and controversy remains whether plant compounds are needed for its biosynthesis.[8] Several reports have demonstrated, however, that fungal paclitaxel is produced independently of the plant following *in vitro* culturing.[6,9,10] At least 18 different fungal genera are currently claimed to produce paclitaxel.[11]

Fundamental questions remain about the fungal paclitaxel pathway, including whether the biosynthetic pathway is the same as that used by plants.[5,14] Plant paclitaxel consists of a taxane ring system with a phenylalanine-derived side chain.[15,16] The plant biosynthetic pathway involves multiple enzymatic steps, and most of these steps are well characterized,[15,16] although important gaps remain. The precursor of the taxane ring system of paclitaxel is the diterpene geranylgeranyl diphosphate (GGPP), which is derived from isopentenyl pyrophosphate (IPP)[17,18] (Figure 1). The first committed step in the biosynthetic pathway of the taxane ring is the conversion of GGPP to taxa-4(5),11(12)-diene by taxadiene synthase (TS) (Figure 1). Recent reports have claimed the isolation of a fungal gene encoding taxadiene synthase,[8,19,20] the sequence similarity (96%) being surprisingly high when compared to plants.[8] We have independently confirmed the presence of a protein of the expected molecular weight (110 kDa) of TS in a paclitaxel-producing fungal strain using a plant anti-TS antibody (S. Soliman and M. Raizada, unpublished data). Following multiple enzymatic steps, taxadiene is then converted to baccatin III, an intermediate to paclitaxel that lacks the phenylisoserine side chain.[15,16]

In general, two distinct pathways can generate IPP and GGPP, the precursors of the taxane ring of paclitaxel: the classical mevalonate (MVA) pathway, which is cytosolic and active in all organisms including fungi, and the 2-C-methyl-D-erythritol-4-phosphate (MEP or DXP) pathway, which is chloroplastic (plastidic) and bacterial. The MVA pathway generates the precursors for sesquiterpenes, including steroids and triterpenes, and it also generates the precursor GGPP for diterpenoids in fungi and yeast.[1] The MEP pathway directs the

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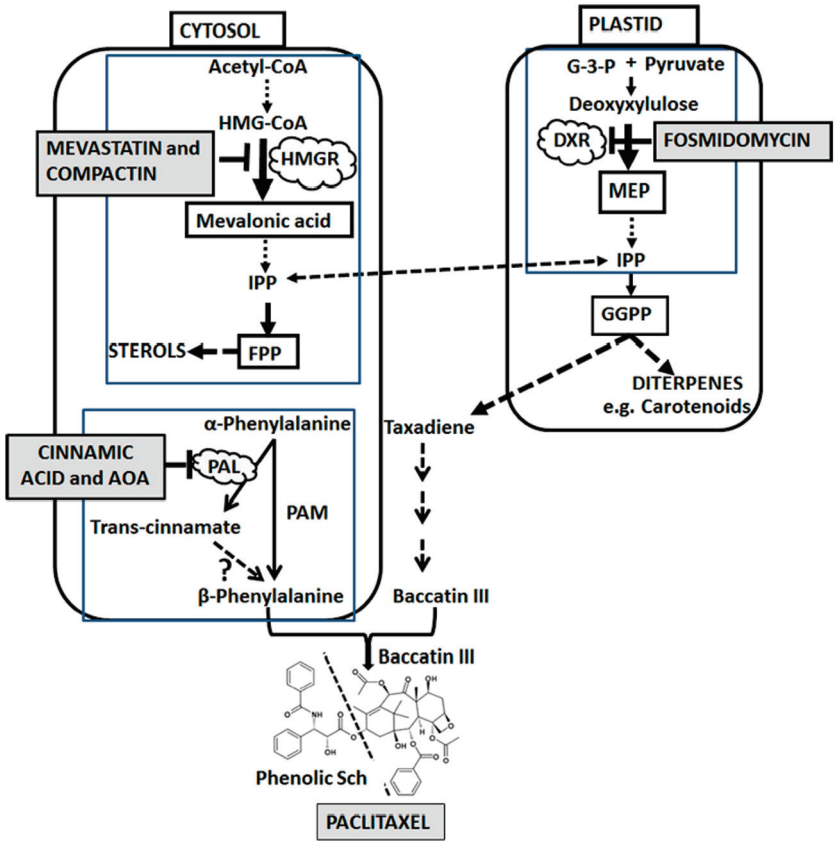

Figure 1. Hypothesized biosynthetic pathway of plant paclitaxel from the terpenoid precursor geranylgeranyl pyrophosphate (GGPP) and aromatic precursor phenylalanine. The cytosolic MVA pathway and plastid MEP pathway contribute terpenoid precursors for paclitaxel. The enzyme targets of specific inhibitors for each pathway are indicated by arrows. G3P, glyceraldehyde-3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; HMG-CoA, hydroxymethyl glutyryl CoA; IPP, isopentenyl diphosphate; GGPP, geranylgeranyl diphosphate; FPP, farnesyl diphosphate; DXR, DXP reductoisomerase; HMGR, HMG-CoA reductase; PAL, phenylalanine ammonia lyase; PAM, phenylalanine ammonia mutase; Sch, side chain.

supply of precursors for monoterpenes, diterpenes, and tetraterpenes.[17,18]

To distinguish whether the taxane ring system of plant paclitaxel is derived primarily from the cytosolic MVA or plastidic MEP pathways or both, previous studies employed chemical inhibitors targeting enzymes specific to each of these pathways (Figure 1). For example, in suspension cultures of *Taxus chinensis*, lovastatin and fosmidomycin were used to block 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) and 1-deoxy-D-xylulose-5-phosphate reducto-isomerase (DXR), key enzymes in the MVA (cytosolic) and MEP (plastid) branches, respectively, of the terpenoid biosynthetic pathway (Figure 1).[21] Paclitaxel production was lowered by ~16% by the lovastatin (1 mmol/L) treatment and ~40% by fosmidomycin (200 mmol/L), suggesting that both MVA and MEP pathways contribute to plant paclitaxel biosynthesis, with the latter plastid pathway being the main source of IPP.[21] This was confirmed using mevastatin, an analog of lovastatin.[22]

Recent evidence suggests that phenylalanine ammonia lyase (PAL; EC 4.3.1.5) catalyzes the nonoxidative deamination of alpha-phenylalanine to form *trans*-cinnamic acid; cinnamic acid derivatives could be intermediates for phenylalanine ammonia mutase (PAM) in an amination reaction to release beta-phenylalanine for the synthesis of the phenylisoserine side chain.[22,23] PAL is of particular interest, because cinnamic acid, a specific feedback inhibitor that reduced PAL activity by 40–50% without affecting total protein levels, reduced plant paclitaxel accumulation by 90%.[24] Another PAL inhibitor, alpha-aminooxyacetic acid (AOA), almost entirely shut down plant paclitaxel production at both 0.5 and 1.5 mM.[24] The effects of these two inhibitors provide strong evidence for the contribution of the PAL pathway in the biosynthesis of the paclitaxel side chain. PAL is found in plants[25] and fungi.[26,27]

In the absence of convincing molecular data from fungi, the availability of well-characterized inhibitors of the plant paclitaxel biosynthetic pathway created an opportunity to characterize the fungal paclitaxel pathway and to determine whether key enzyme targets are shared between plants and fungi.

RESULTS AND DISCUSSION

Culturing and Identification of an Endophytic Fungus from *Taxus media* Plants. The internal structures of old *Taxus media* branches were cultured on potato dextrose agar (PDA) media following bleach/ethanol surface sterilization and removal of outer bark. Only one fungal endophyte was recovered. The fungus was identified as belonging to the genus *Paraconiothyrium*, with 99% 18S rDNA sequence identity to *P. variabile*, and was designated as *Paraconiothyrium* strain SSM001 (Figure S1A). The 18S rDNA sequence has been deposited into Genbank (HQ324134). Strain SSM001 has been deposited into ATCC (strain #ATCC MYA-4697). Strain SSM001 was found to be stably paclitaxel-producing *in vitro* in liquid culture using thin layer chromatography (TLC) (Figure S1B). SSM001 was used for all subsequent experiments.

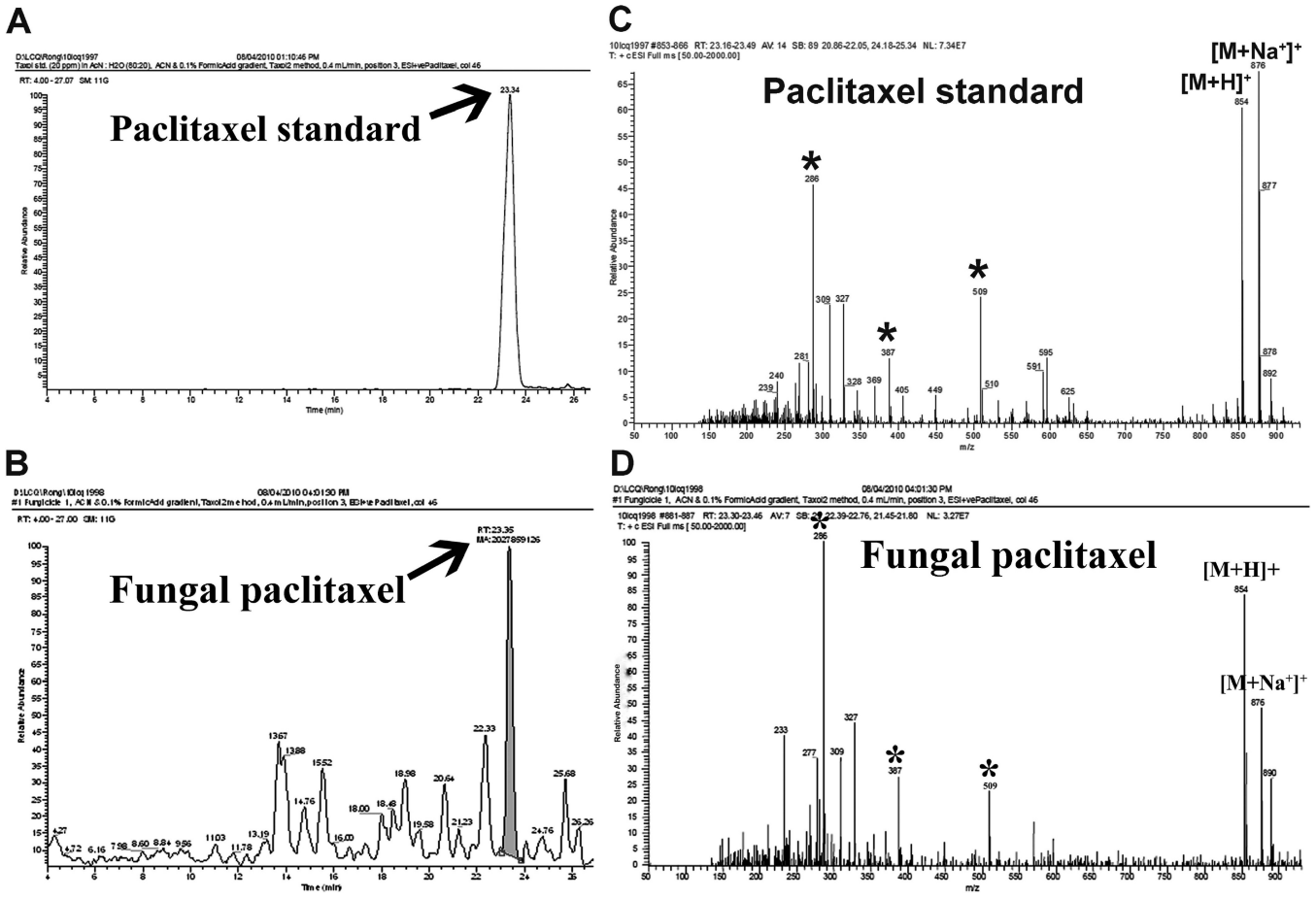

Figure 2. Identification of fungal paclitaxel. HPLC analysis of (A) pure paclitaxel standard, in comparison to (B) a three-week-old SSM001 fungal liquid culture extract. The HPLC for the fungal extract shows the same retention time (23.3 min) as the pure paclitaxel standard using selective ion mode (SIM). Mass fragmentation of (C) the paclitaxel standard and (D) proposed paclitaxel from fungal strain SSM001. The asterisks indicate the diagnostic mass fragments.

Fungal Strain SSM001 Produces Paclitaxel Autonomously Based on Independent Diagnostic Assays. The filtered fungal liquid culture extract showed the same chromatographic properties as that of authentic paclitaxel by TLC (Figure S1B). The fungal compound was identified as paclitaxel using high-performance liquid chromatography-electrospray ionization-mass spectroscopy (HPLC-ESIMS) (Figure 2A–D): the fungal liquid fraction showed a strong peak with the same retention time as that of authentic paclitaxel at 23.3 min (Figure 2A,B). The mass fragmentation pattern of the paclitaxel standard was similar to that of the fungal liquid extract compound (Figure 2C,D; characteristic paclitaxel peaks have an asterisk). The fungal paclitaxel fragmentation showed the ESIMS (m/z), $[M + H]^+$ calculated for $C_{47}H_{52}NO_{14}$, as 854.509 for $[M - ScH$, side chain of paclitaxel$]^+$ and 286 for $[ScH + H]^+$. In addition, the fungal liquid fraction was identified as possessing paclitaxel using the surface antigen properties of paclitaxel: this fraction reacted positively in a quantitative paclitaxel monoclonal antibody assay compared to controls (Figure S2). To buffer against interfering fungal metabolites in the fungal extract, each immunoassay was compared to a standard curve of paclitaxel standards added into a fungal extract from non-paclitaxel-producing *Fusarium* (Figure S2C). The specificity of the paclitaxel immunoassay was confirmed by testing different concentrations of diverse taxanes including baccatin III and cephalomannine either alone or added to wells containing paclitaxel; only cephalomannine in combination with paclitaxel and at high concentration showed minor effects (Figure S3A–C). Because the HPLC of total fungal *Paraconiothyrium* extract showed no peaks corresponding to taxane standards except paclitaxel (Figure S3D,E), the minor effects of cephalomannine should not be considered.

It should be noted that the fungus could produce paclitaxel independently of plant tissues following two cycles of *in vitro* hyphal tip transfer and inoculation into liquid media, where the fungus grew >1000-fold in biomass (as indicated in the Experimental Section) prior to peak paclitaxel production, all in the absence of any plant tissues or extracts. We conclude that endophytic fungal strain SSM001 produces paclitaxel, and it does so autonomously.

Location and Timing of Fungal Paclitaxel Accumulation *in Vitro*. As noted above, TLC analysis showed that fungal paclitaxel accumulated in the liquid media fraction not in fungal mycelia (Figure S4A). This was confirmed using the paclitaxel antibody immunoassay (Figure S4B). In terms of the timing of production, a 10 mg fungal inoculum in 500 mL of yeast-peptone-dextrose (YPD) liquid media grew up to 10 g in three weeks and was able to produce 40 μg of paclitaxel as measured by TLC and the paclitaxel immunoassay, with almost no detectable paclitaxel accumulation in the liquid media after two weeks (Figure S4A,C). The three-week liquid media fraction was used in all subsequent experiments.

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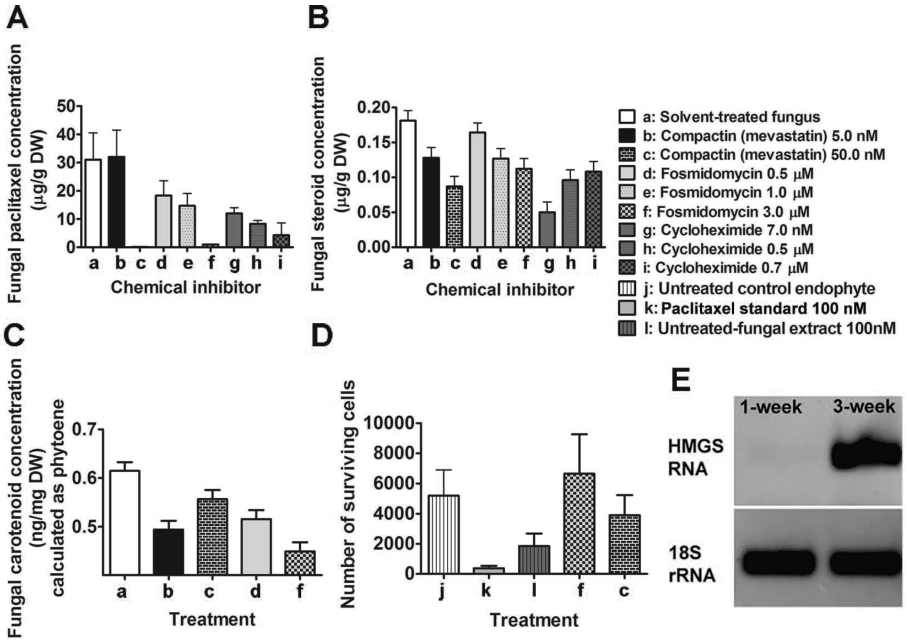

Figure 3. Effects on fungal paclitaxel accumulation of chemical inhibitors previously shown to target enzymes in the MVA or MEP terpenoid precursor pathways. Effects of inhibitors on fungal (A) paclitaxel and (B) steroid accumulation three weeks after inoculation. Shown are the effects of compactin, a terpenoid cytosolic MVA pathway inhibitor; fosmidomycin, an inhibitor of the plastid/bacterial MEP pathway; and cycloheximide, a eukaryotic cytosol translation inhibitor. (C) Corresponding effects on fungal carotenoid concentration (ng/mg dry wt). (D) Corresponding effects on PEER cancer cells exposed to fungal extracts pretreated with chemical inhibitors. (E) RT-PCR expression analysis of fungal HMGS from one- and three-week-old fungal cultures. High HMGS expression at three weeks corresponded to the timing of peak paclitaxel accumulation.

Steroids were also quantified, as they were later used to confirm the uptake of chemical inhibitors by the fungus: unlike paclitaxel, the fungal mycelia steroid content showed a steady decline after culturing (Figure S4D) as previously observed.[28]

Elucidation of Early Steps in the Biosynthetic Pathway of the Taxane Ring Component of Fungal Paclitaxel Using Chemical Inhibitors Specific for Plant Terpenoid Biosynthesis. Prior to the presumptive onset of fungal paclitaxel biosynthesis (Figure S4A,C), chemical inhibitors were applied to 10-day-old fungal liquid cultures. For all chemical inhibitors, DMSO (dimethylsulfoxide)/ethanol was found to be an effective, universal solvent[29,30] (Figure S5). Surprisingly, fosmidomycin, a specific inhibitor of DXR in the plastid/bacterial nonmevalonate pathway, caused ≤99% inhibition of fungal paclitaxel accumulation by both TLC (Figure S6A, lane 6) and the paclitaxel immunoassay (Figure 3A; statistics shown in Table S1). The quantitative effects of all inhibitors are listed (Supplementary Tables 1 and 2). Compactin (mevastatin), an inhibitor of hydroxymethyl glutyryl CoA reductase in the cytosolic mevalonate pathway, resulted in the appearance of two new blue spots on the TLC not in the control fungal culture (Figure S6A, lane 4). Using the paclitaxel immunoassay, compactin caused a >99.9% decline in antigen-reactive paclitaxel (Figure 3A).

An impact by compactin was expected but not by fosmidomycin. Confirming the specificity of the two inhibitors for the terpenoid pathway, both caused declines in fungal steroids that are derived from a downstream branch of the terpenoid pathway[31] (Figure 3B). Compactin and fosmidomycin slightly inhibited the biosynthesis of phytoene, a carotenoid derived from the terpenoid pathway (Figure 3C). Finally, both compactin- and fosmidomycin-treated fungal extracts caused dramatically less inhibition of lymphoblastic T cells (PEER cells)[32] than controls (Figure 3D) presumably due to less paclitaxel being present.

We conclude that the terpenoid-derived taxane ring of fungal paclitaxel can be blocked or altered by chemicals known to inhibit HMGR (mevalonate pathway) and DXR, the latter being the rate-limiting step in the bacterial and plant plastidic MEP pathways.

A Mevalonate Pathway Enzyme Shows Peak Expression Coincident with Fungal Paclitaxel Production. Compactin inhibition suggests that the mevalonate pathway is critical for fungal paclitaxel production. We performed expressed sequence tag (EST) library sequencing and gene annotation of SSM001 hyphae (data not shown) and were able to identify an ortholog of HMGCoA synthase (HMGS), a fungal-specific mevalonate pathway enzyme and the immediate precursor step for HMGR, the target of compactin. The *Paraconiothyrium* SSM001 EST fragment showed high similarity to several fungal HMGCoA synthases (Figure S7A). Using the SSM001 sequence for polymerase chain reaction (PCR) primer design, RT-PCR demonstrated that HMGS expression dramatically increased in three-week-old liquid cultures (Figure 3E), consistent with peak paclitaxel production (Figure S4B).

***Paraconiothyrium* SSM001 Fungus Does Not Host a DXR-Producing Bacterial Endophyte.** MEP pathway enzymes have not been reported in the fungal literature. One hypothesis to explain the presumptive presence in strain SSM001 of DXR, an enzyme common to the bacterial/plastid MEP pathway, was that the fungus possessed endophytic bacteria.[33,34] This hypothesis was rejected on the basis of four independent experiments: cycloheximide, an inhibitor of eukaryotic cytosolic ribosomal protein synthesis,[35] showed a dramatic decrease (~70%) in fungal paclitaxel production by TLC (Figure S8A, lanes 3–5) and the paclitaxel immunoassay

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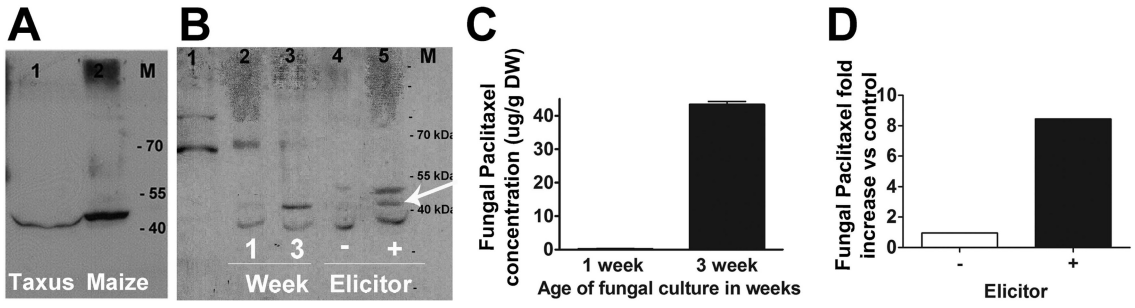

Figure 4. Molecular evidence for MEP pathway enzyme DXR in *Paraconiothyrium* SSM001 fungus. (A, B) Western blot identification and expression analysis of DXR using a maize polyclonal anti-DXR antibody; (C, D) corresponding fungal paclitaxel production. Anti-DXR Western blot analysis of total protein extracts from (A) plants (positive controls) and (B) fungi. The white arrow indicates the expected 43 kDa DXR protein. (A) Plants: lane 1, *Taxus* needles; lane 2, maize leaves. (B) Fungi: lane 1, *Fusarium graminearum* (negative control); lane 2, one-week-old fungal SSM001 culture; lane 3, three-week-old fungal SSM001 culture; lane 4, nonelicited SSM001 fungus; lane 5, chloromethane-elicited SSM001 fungus. (C) Paclitaxel production peaked in three-week-old liquid cultures compared to one-week-old cultures. (D) Fungal paclitaxel production was induced by chloromethane elicitor versus control.

(Figure 3A). Treatment with the antibiotic streptomycin caused no significant decrease in paclitaxel production (data not shown). Plating of fungal liquid media and fungal mycelia fractions on multiple types of bacterial growth media did not result in any bacterial growth (data not shown). Finally, specific bacterial 16S rDNA primers[36] did not amplify PCR products of the correct size or expected sequence (Figure S8B).

***Paraconiothyrium* SSM001 Fungus Expresses a Protein That Cross-Reacts to an Anti-DXR Antibody.** We tested the alternative hypothesis that fungal strain SSM001 itself encodes for a DXR-like enzyme. A polyclonal antibody for plant DXR, which detected 43 and 52 kDa bands in *Taxus* plant needles and maize leaves (Figure 4A),[37] cross-reacted with extracts from the paclitaxel-producing SSM001 fungus (Figure 4B, lanes 2−5) but not nonpaclitaxel-producing *Fusarium graminearum* (Figure 4B, lane 1). Furthermore, there was higher expression of the correct 43 kDa band (shown by white arrow) in a three-week-old fungal SSM001 culture than at one week (Figure 4B, lanes 2, 3) and upon treatment with an elicitor of fungal paclitaxel production, chloromethane (Figure 4B, lanes 4, 5), coincident with peak paclitaxel release at three weeks (Figure 4C) and following chloromethane elicitor treatment (Figure 4D), respectively.

***Aspergillus* Fungus Has a Putative MEP Pathway Ortholog.** Partial EST sequencing did not identify a MEP-pathway gene in *Paraconiothyrium* fungus. However, BLAST analysis identified a putative ortholog of 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (DXS), the enzyme immediately prior to DXR in the plant MEP pathway,[37] in the fungus *Aspergillus terreus* (Figure S7B). A ClustalW alignment showed conservation of amino acid residues along the entire 372 amino acid length between the candidate fungal DXS and bacterial DXS enzymes (Figure S7B). Together with the DXR antibody result, this evidence suggests that some fungi may possess MEP pathway genes(s).

Elucidating the Importance of the Phenylalanine Pathway for Fungal Paclitaxel Biosynthesis. In addition to the taxane ring, plant paclitaxel has a phenylisoserine side chain derived from phenylalanine via phenylalanine ammonia mutase.[16] To determine whether fungal paclitaxel also utilizes a phenylpropanoid biosynthetic pathway, effects of two PAL inhibitors were investigated. First, aminooxy-acetic acid treatment showed no significant change in paclitaxel accumulation, but also no effect on the production of phenolics, demonstrating that AOA did not target fungal PAL (Figure S8A, lanes 6−8; Figure S9). Cinnamic acid caused a >99% decline in paclitaxel production as measured by TLC and the paclitaxel immunoassay (Figure S6B; Figure 5A; Table S2). On TLC plates, cinnamic acid treatment was associated with the appearance of a new blue spot with an R_f value of 0.47, not paclitaxel nor baccatin III (Figure S6B). Cinnamic acid also caused a 96% decline in fungal phenolics (Figure 5B), confirming that it targets PAL. Functionally, cinnamic acid-treated fungal extracts showed loss of paclitaxel inhibitory activity against lymphoblastic T cells (PEER cells) (Figure 5C). Though no PAL ortholog was found in fungal strain SSM001 by EST sequencing or by the use of degenerate PCR primers, transcriptome sequencing identified another gene in the fungal shikimate pathway, 3-dehydroquinate synthase (DHQS). The coding region showed high similarity to several fungal DHQS proteins using ClustalW (Figure S7C). Primers designed against this gene showed increased expression at three weeks in liquid culture compared to one week (Figure 5D), consistent with the timing of paclitaxel production. We conclude that the fungal shikimate pathway, likely via a PAL ortholog, contributes to fungal paclitaxel biosynthesis.

■ CONCLUSIONS

For the first time, we have identified candidate enzymes in the early terpenoid and phenylpropanoid pathways involved in fungal paclitaxel biosynthesis using chemical inhibitors, gene discovery, and gene expression studies. Inhibition by cinnamic acid identified phenylalanine ammonia lyase (PAL) activity as being important in paclitaxel biosynthesis, similar to plants, where it results in production of the phenylisoserine side chain. Identification and differential expression of a DHQS gene confirmed the importance of the shikimate pathway in fungal paclitaxel biosynthesis. Inhibition by compactin identified HMGR, a rate-limiting enzyme in the fungal mevalonate pathway, as being required for fungal paclitaxel biosynthesis, similar to plants, where it contributes to the terpenoid ring system. Similarly, identification and differential expression of a gene encoding HMGS confirmed the importance of the mevalonate pathway in fungal paclitaxel biosynthesis. Surprisingly, inhibition by fosmidomycin suggested that fungal paclitaxel production absolutely requires DXR, an enzyme in the MEP pathway normally found in plants and bacteria. Three additional types of evidence support the unexpected conclusion

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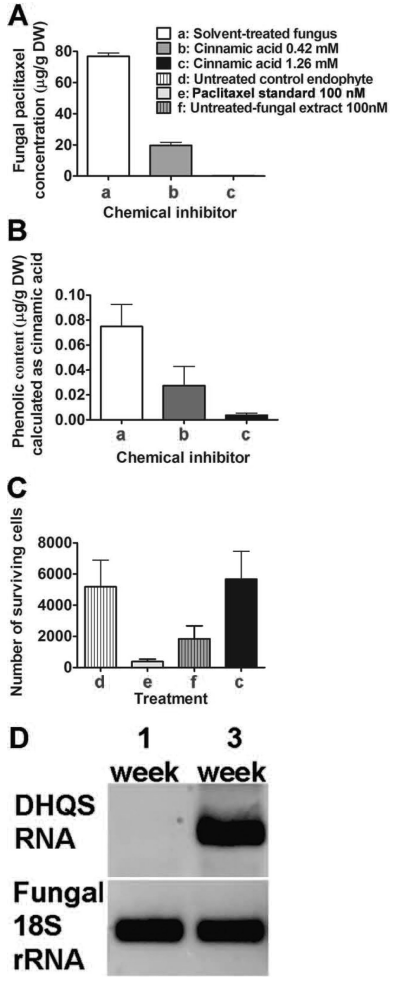

Figure 5. Effects of cinnamic acid, an inhibitor of phenylalanine ammonia lyase (PAL), critical to the phenylisoserine side chain of plant paclitaxel. Cinnamic acid was applied to three-week-old pure liquid cultures of fungal strain SSM001. (A) Quantification of fungal paclitaxel (μg/g dry wt) upon treatment with cinnamic acid. (B) Cinnamic acid effect on fungal phenolics, calculated as cinnamic acid (μg/g dry wt). (C) Corresponding effects on PEER cancer cells exposed to fungal extracts pretreated with cinnamic acid. (D) RT-PCR expression analysis of DHQS in one- and three-week-old SSM001 fungal cultures. Peak DHQS expression at three weeks corresponded to the timing of peak paclitaxel accumulation.

that a fungus might possess enzymes in the MEP pathway. First, a plant anti-DXR antibody cross-reacted with a fungal peptide of the correct molecular weight. Second, apparent fungal DXR expression correlated to changes in paclitaxel production based on elicitor treatment and fungal age. Finally, a gene encoding DXS, the enzyme that immediately precedes DXR in the MEP pathway, was identified in another fungus, *Aspergillus*. MEP enzymes in fungi could be the result of horizontal gene transfer from plants or microbes, and this will require more extensive investigation.

EXPERIMENTAL SECTION

General Experimental Procedures. The following reagents were from Sigma (USA), including taxane standards: paclitaxel (#T7402), baccatin III (#B8154), and cephalomannine (#C4991); fungal nutrient media: yeast-peptone-dextrose (YPD) (#Y1375) and potato-dextrose-agar (PDA) (#70139); and chemical inhibitors: compactin (mevastatin) (# 27696), *N*-Boc-aminooxy acetic acid (#15035), cinnamic acid (#C80857), and cycloheximide (#R750107). All solvents used for extraction, TLC, and HPLC were HPLC grade and obtained from Fisher Scientific. Fosmidomycin was provided by Dr. Hassan Jomaa and Dr. Jochen Wiesner (Universitatsklinikum Giessen and Marburg, Germany).

Isolation of Endophytic Fungi. Endophytic fungi were cultured from old branches of *Taxus media* plants cultivated on the University of Guelph Main Campus and Arboretum (Guelph, Canada). Fungi were isolated from fresh plant tissue immediately after harvesting. Tree branches were cut into 1 cm long × 0.5 cm diameter pieces and sterilized as follows: 2.5% sodium hypochlorite solution for 10 min; 70% ethanol for 5 min; washed in sterile double-distilled water three times. The outer bark was removed and the inner tissues were further sterilized using 70% ethanol for 5 min followed by flaming and washing three times with sterile double-distilled water. Each piece of tissue was then cut into smaller pieces (2 mm × 5 mm) and cultured on PDA media in Petri plates at 25 °C in the dark until the first fungal growth. Hyphal tips were then consecutively transferred twice onto fresh PDA media to ensure fungal culture purity.[6]

Fungal Genotyping. To ensure consistency between experiments, every fungal liquid culture was genotyped by PCR and DNA sequencing of the internal transcribed spacer regions (ITS) of 18S rDNA (ITS1, ITS4)[38] to confirm both strain identity and purity. The following conditions were used for PCR amplification: 94 °C for 2 min, followed by 35 cycles of 94 °C for 45 s, 45 °C for 1 min, and 72 °C for 2 min; with a final extension cycle of 4 min at 72 °C. The amplified fragment was cloned, sequenced, and aligned with strain SSM001 sequence using the Align-BLAST search tool.

Production and Extraction of Fungal Taxanes. Two-week-old pure fungal cultures grown on PDA plates were used for all subsequent experiments. Fungal tips (~10 mg) from two-week-old pure PDA plate cultures were transferred to 1 L Erlenmeyer flasks containing 500 mL of liquid YPD media and incubated for one week in the dark at room temperature without agitation and then for another two weeks at 25 °C in the dark, shaking at 100 rpm. The mycelia were filtered from the liquid media using cheesecloth, squeezed (final ~10 g), and washed using ddH_2O. The filtered liquid media was extracted three times with 50 mL of chloroform/methanol (9:1 v/v). The organic layers were separated, collected, and washed using distilled water, dried over anhydrous sodium sulfate (#AC21875-0025, Fisher Scientific, USA), and then evaporated at 45 °C using a Rotavapor until fully dried.[5] The same procedure was used separately to extract taxanes, at one week, two weeks, 18 days, and three weeks after inoculation.

Chromatographic and Spectroscopic Identification of Fungal Taxanes. For TLC, the residue from each extract was dissolved in 30 μL of methanol, and 8 μL applied onto silica gel plates (10 × 20 cm, Fisher Scientific #4861-320 and #11028) alongside the taxane standards, paclitaxel, cephalomannine, and baccatin III, each at a concentration of 25 μg/mL. TLC plates were then developed in solvent system A (chloroform/methanol, 5.0:0.5) or solvent system B (acetonitrile/methanol, 4:1). For compound visualization, TLC plates were either exposed to iodine vapor or dipped in 0.5% vanillin/sulfuric acid reagent for one minute.[5]

For HPLC identification of paclitaxel, fungal paclitaxel was purified from several TLC plates prior to LC-MS injection. A LCQ DECA ion trap LC-MS instrument (ThermoFinnigan, USA) was used, equipped with a Finnigan SpectraSystem UV6000LP UV detector and a RESTEK Ultra Phenyl column (250 × 2.1 mm, 5 μm). The binary mobile phase consisted of solvent A (0.1% formic acid) and solvent B (acetonitrile). A gradient program was used to run the analyzed samples as follows: isocratically at 80% A for 1 min, 80−30% A for 24 min, 30−80% A for 5 min, and isocratically at 80% A for 5 min. The flow rate was 0.4 mL/min. Paclitaxel was measured using the UV absorbance at 233 nm. Taxane standards were run in parallel. The electrospray (ESI) positive ion mode was used for ion detection. The system was operated as follows: shear gas and auxiliary flow rates were set at 96 and 12 (arbitrary units); voltage setting on the capillary; tube lens offset; multipole 1 offset; multipole 2 offset; lens and entrance lens were set at 32.50, 55.00, −4.40, −8.00, −14.00, and −58.00 V,

respectively; the capillary temperature was set at 350 °C; and the ion spray voltage was controlled at 5 kV.

Anticancer Assay (Alamar blue assay). On a 12-well microplate, into each well, 3 mL of complete media of 1×10^6 PEER cells (a human acute lymphoblastic T cell line)[32] and 100 nM paclitaxel standard or fungal paclitaxel (dissolved in DMSO, final concentrations) were added in triplicate.[41] The plate was incubated at 37 °C in the dark for 24 h, followed by centrifugation and washing the pellets with 5 mL of BPS. Each pellet was then resuspended in 3 mL of fresh complete media and seeded on a new 12-well microplate. Then 100 μL of the media was removed in triplicate and placed into a 96-well microplate along with 100 μL of 10% Alamar blue (Resazurin, Sigma, #R7017), mixed, and read at zero time in a fluorescence microplate reader (Ex516/20, Em590/35, Biosources, Canada). The plates were then incubated at 37 °C in the dark for 5 h, and the plate was read again. The background values were subtracted to obtain the actual reading. The Alamar blue assay was repeated every 24 h for up to 3 days to generate growth curves. The viability was expressed as a percentage of the negative control (solvent DMSO) value measured on day 2.

Fungal Paclitaxel Competitive Immunoassay and Specificity. Paclitaxel was identified and quantified using a competitive immunoassay procedure,[42] which employed a kit (#TA02, Hawaii Biotechnology Group, Inc., USA) in conjunction with a commercial paclitaxel monoclonal antibody (#SC-69899, Santa Cruz Biotechnology Inc., USA) (Figure S2). Manufacturer's instructions were followed.

In order to buffer against interfering fungal metabolites in the fungal extract, each immunoassay read was compared to a standard curve generated each time consisting of paclitaxel standards added into fungal extract from non-paclitaxel-producing *Fusarium* (Figure S2C). The specificity of the paclitaxel immunoassay was confirmed by testing different concentrations of diverse taxanes (baccatin III and cephalomannine) either alone or added to wells containing paclitaxel (Figure S3A-C). HPLC of total fungal *Paraconiothyrium* extract was compared to diverse taxane standards to determine if they were present (Figure S3D,E).

Fungal Steroid Extraction and Assay. Fungal steroid extraction was as previously described.[43] A modified Liebermann–Burchard steroid assay[44] was used for fungal steroid quantification.

Fungal Carotenoid Quantification. Fungal carotenoid extraction, assay, and quantification were as previously described.[45]

Fungal Phenolic Assays. Two methods for measuring fungal phenolics were employed: the *para*-hydroxybenzaldehyde method[46] and the Folin–Ciocalteu method.[47]

Chemical Inhibition of Fungal Paclitaxel. Ten-day-old fungal liquid cultures, incubated in the dark at 25 °C, with shaking at 100 rpm (inoculation conditions as described above), were subjected to the following chemical inhibitors (final concentrations noted): fosmidomycin (0.5, 1, and 3 μM), compactin (mevastatin) (5, 25, and 50 nM), (*N*-Boc-aminooxy)acetic acid (1, 2, and 5 mM), *dl*-cinnamic acid (0.42 and 1.26 mM), and cycloheximide (7 nM, 0.5 μM, and 0.7 μM). Each inhibitor was dissolved in 1 mL of DMSO with 4 mL of 70% aqueous ethanol as solvent. As a control, an equal volume of solvent only was added to a parallel liquid culture. All cultures continued their incubation at 25 °C in the dark, shaking at 100 rpm, for an additional 14 days, after which paclitaxel, steroids, carotenoids, and phenolics were extracted as described above. The results shown represent the mean of three independent experiments (three separate flasks).

Bacterial 16S rDNA PCR. To assay for the presence of bacterial endophytes within the fungal mycelia or fungal liquid culture, genomic DNA was isolated and subjected to PCR using degenerate 16S rDNA bacterial-specific primers:[36] 799f [5′-AACMGGATTAGATACCCKG-′3′] and 1525r [5′-AAGGAGGTGWTCCARCC-3′]. The PCR conditions were 95 °C for 3 min; then 30 cycles at 94 °C for 20 s, 53 °C for 40 s, and 72 °C for 40 s; followed by a final extension of 7 min at 72 °C.

Paclitaxel Detection and Quantification. Paclitaxel was verified and quantified either by a competitive immunoassay procedure[42] or by TLC spot densitometry.[48,49] Fungal paclitaxel was applied on standard TLC plates (10 × 20 cm, Fisher Scientific #4861-320) along with three different concentrations of standard paclitaxel (2, 4, 16 μg). The TLC plates were then developed in solvent system A (chloroform/methanol, 5.0:0.5). For compound visualization, the TLC plates were dipped in 0.5% vanillin/sulfuric acid reagent for 1 min, dried, and scanned using a CanoScan LiDE 600F scanner and saved in Tiff format. The colors of the plates were then enhanced, and the fungal paclitaxel spot densitometries were performed with a FluorChem 8800 with AlphaEaseFC FluorChem 8900 software (Alpha Innotech Corp.). The process was validated by comparing 10 different concentrations of paclitaxel standard on 10 different TLC plates. The process could be used to measure paclitaxel down to 1 μg. Assays were performed in triplicate.

Detection of a Fungal-like DXR Enzyme by Western Blot Analysis. Proteins were extracted from liquid nitrogen-ground fungal tissues using extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, and 1% [v/v] Nonidet P-40) supplemented with Complete, Mini, EDTA-free protease inhibitor cocktail (Roche). SDS-PAGE was performed using 12% acrylamide gels.[50] Protein was transferred to a nitrocellulose membrane (Costar Scientific).[51] Prior to immunoblotting, membranes were stained with Ponceau S (0.1% [w/v] Ponceau S and 5% [v/v] acetic acid) to ensure equal loading of protein. Polyclonal anti-DXR antibodies were used at approximately 1:1000 dilution for immunodetection using ECL Plus Western blotting detection reagents (GE Healthcare, Little Chatfont, UK) and according to the manufacturer's instructions for chemiluminescent detection of the antigen. The detection was followed by affinity-purified goat anti-rabbit IgG horseradish peroxidase-conjugated antibodies (Bio-Rad Laboratories) diluted to 1:10 000. ECL Plus Western blotting detection reagents (Amersham Biosciences) were used as substrate for the secondary antibodies, following the manufacturer's instructions.

RT-PCR. RNA was prepared using RNeasy Mini Kit (QIAGEN, Mississauga, ON, Canada), and genomic DNA was eliminated by loading RNase-free DNaseI onto the filter column (QIAGEN). First-strand cDNA was synthesized using oligo-dT primers with M-MuLV reverse transcriptase (Fermentas, USA). For 3-hydroxyl 3-methyl glutaryl CoA synthase, the following primers were used: HMGSF1, 5′ACACGAAGACTTAGCAGGTGGGTGCG-3′, and HMGSR1, 5′CGAGTACCCCGTCGTCGATGGTGGTC-3′. For 3-dehydroquinate synthase amplification, primers QDHSF1, 5′-TGTAGCCTTCGCGAGGATCTCCTCG-3′ and QDHSR1, 5′-ACTACACGAGCTTACTCCCGATGTGCC-3′ were used. The following conditions were used for PCR amplification: 94 °C for 2 min, followed by 33 cycles of 94 °C for 45 s, 56.6 °C for 1 min, and 72 °C for 2 min; with a final extension cycle of 1 min at 72 °C. The reaction was performed with the iCycler system (Biorad). 18S rRNA was used as an internal standard for normalization using primers[52] 18S rDNA-RtF; 5′GGCATCAGTATTCAGTTGTC-3′ and 18S rDNA-RtR; 5′-GTTAAGACTACGACGGTATC-3′. All PCR amplifications were conducted from replicate samples and in duplicate.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information concerning culturing the producing fungus, the paclitaxel immunoassay method and specificity, quantification of paclitaxel from the fungus and effect of age/location, effect of solvent and inhibitors on paclitaxel and other metabolite biosynthesis, and gene sequence for HMGS, DHQS, and DXS is provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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