Cloning and Characterization of an Armillaria gallica cDNA Encoding Protoilludene Synthase, Which Catalyzes the First Committed Step in the Synthesis of Antimicrobial Melleolides*

Received for publication, July 19, 2010, and in revised form, December 10, 2010 Published, JBC Papers in Press, December 10, 2010, DOI 10.1074/jbc.M110.165845

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Melleolides and related fungal sesquiterpenoid aryl esters are antimicrobial and cytotoxic natural products derived from cultures of the Homobasidiomycetes genus Armillaria. The initial step in the biosynthesis of all melleolides involves cyclization of the universal sesquiterpene precursor farnesyl diphosphate to produce protoilludene, a reaction catalyzed by protoilludene synthase. We achieved the partial purification of protoilludene synthase from a mycelial culture of Armillaria gallica and found that 6-protoilludene was its exclusive reaction product. Therefore, a further isomerization reaction is necessary to convert the 6–7 double bond into the 7–8 double bond found in melleolides. We expressed an A. gallica protoilludene synthase cDNA in Escherichia coli, and this also led to the exclusive production of 6-protoilludene. Sequence comparison of the isolated sesquiterpene synthase revealed a distant relationship to other fungal terpene synthases. The isolation of the genomic sequence identified the 6-protoilludene synthase to be present as a single copy gene in the genome of A. gallica, possessing an open reading frame interrupted with eight introns.

Terpenes are the largest and most diverse group of natural products, with more than 50,000 known structures (1). The biosynthesis of some terpenes has been studied in detail, particularly those from plants (e.g. menthol, artemisinin, and taxol), but less attention has been paid to terpenes from other sources. Fungi are a rich source of physiologically active natural products, and mushroom-forming fungi and other Basidiomycota are particularly well known for the synthesis of numerous bioactive sesquiterpenoids and, to a lesser extent, diterpenoids (2, 3). The biosynthesis of all terpenes begins with the cyclization and rearrangement of one of three universal precursors, geranyl diphosphate, farnesyl diphosphate, or geranylgeranyl diphosphate, to yield monoterpenoids, sesquiterpenoids, or diterpenoids, respectively. These cyclization reactions are among the most complex chemical reactions known in nature, and they are catalyzed by terpene synthases (also known as terpene cyclases). Following the terpene synthase reaction, which establishes the complete terpene carbon scaffold, additional reactions typically catalyzed by cytochrome P450-dependent mono-oxygenases generate further structural diversity (4–6).

Several terpene synthases have been isolated from plants (7, 8), but only a few microbial and fungal terpene synthases have been reported (9–21). A limited number of fungal sesquiterpene synthases have also been cloned and functionally characterized, including trichodiene synthase, aristolochene synthase, presilphiperfolan-8β-ol synthase and, recently, six sesquiterpene synthases from Coprinopsis cinerea (Coprinus cinereus) that yield germacrene A, α-murolene, δ-cadinene, and α-cuprenene as major products (11, 13, 19–26). Generally, plant and fungal terpene synthases show only low level sequence identity, and whereas genes related to plant terpene biosynthesis pathways are often scattered throughout the genome, fungal terpene synthase genes are frequently clustered together, along with genes encoding terpene-modifying enzymes such as cytochrome P450 mono-oxygenases.

The Basidiomycota, a subdivision of the Dicarya, comprises > 30,000 known species and represents approximately one-third of the phylum Eumycota (fungi) (27). This is a particularly rich source of complex, structurally diverse, and bioactive sesquiterpenoids (2, 3). Semisynthetic derivatives of pleuromutilin such as tiamulin, valnemulin, and retapamulin are already used as drugs to treat mycoplasma infections and bacterial pathogens that affect livestock (28).

Many known sesquiterpenoids such as illudane, marasmane, cerapicane, and, to a lesser extent, lactarane and sterpane (29) are structurally related to the protoilludene skeleton. Although some of these compounds are thought to be synthesized uniquely in the basidiocarps of symbiotic fungi, others are produced in large quantities by the mycelial cultures of saprotrophic or facultative parasitic species, which are easier to culture under laboratory conditions. To study fungal terpenoids and investigate the genetic background that has led to the phenotypic diversity in the synthesis of such compounds, we studied the important cosmopolitan genus Armillaria.
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![FIGURE 1. Plausible scheme of melleolide I biosynthesis involving the cyclization of farnesyl diphosphate to 6-protoilludene, oxygenation reactions, and the side chain attachment.](image)

*Armillaria* (commonly known as honey mushrooms and currently classified in the family Physalaciaceae). *Armillaria* species are not only regarded as edible mushrooms, but many species are also notorious forest parasites, with a host range of the most virulent species, primarily *Armillaria mellea* and *Armillaria ostoyae*, comprising >600 species worldwide (30). This is also reflected by their ability to form rhizomorphs that allow them to grow across nutrient-poor areas located between large food sources such as tree stumps and, finally, to infect entire forests. Smith *et al.* (31) have even postulated that *Armillaria bulbosa* (current name: *Armillaria gallica*) may be among the oldest and largest organisms on Earth.

The characteristic secondary metabolites produced by *Armillaria* species are protoilludene-type sesquiterpenoids, potent antimicrobial molecules also known as melleolides. Some of the examined melleolides, like melleolide B, C, and D, possess remarkable antibacterial activity against *Bacillus cereus*, *Bacillus subtilis*, and *Escherichia coli* (32). For armillaric acid, antimicrobial activity against *Microcococcus luteus*, *B. subtilis*, *Candida albicans*, and *Staphylococcus aureus* has been reported (33). There are ~50 known melleolides, produced almost exclusively by this fungal genus. Each molecule comprises a tricyclic sesquiterpenoid skeleton linked to an orsellinic acid-like polyketide side chain via an ester bond (34).

The biosynthesis of protoilludene is thought to involve cyclization of the universal sesquiterpenoid precursor farnesyl diphosphate to protoilludene followed by further modification by cytochrome P450 mono-oxygenases and subsequent attachment of the polyketide side chain (Fig. 1). In this study, we describe the partial purification and characterization of *A. gallica* protoilludene synthase and the cloning and expression of the corresponding cDNA. We found that *A. gallica* protoilludene synthase exclusively produces 6-protoilludene as a reaction product, requiring a double bond rearrangement from the 6–7 to the 7–8 position encountered in the melleolides.

**EXPERIMENTAL PROCEDURES**

*Strains and Growth Conditions—Armillaria gallica* strain FU02472 was established from basidiocarps collected near Traunsee, Austria and was propagated in submerged culture in 500-ml Erlenmeyer shake flasks containing 200 ml of YM medium at 23 ºC with agitation at 140 rpm, as described previously (35). Mycelia were harvested from the culture broth by filtration, shock-frozen with liquid nitrogen, and stored at −80 ºC. *E. coli* strain TOP10 (Invitrogen) was used for cloning, and strain BL21(DE3) CodonPlus (Agilent) was used for heterologous protein expression, along with the Gateway® compatible vector pDEST14 (Invitrogen).

*Protein Purification and Biochemical Characterization—Shock-frozen* *A. gallica* mycelia were powdered using a mortar and pestle and transferred into 5 volumes (w/v) of extraction buffer (50 mM MES, 20 mM MgCl$_2$, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol, and 0.1 g/g mycelia poly(vinylpyrrolidone), pH 6.5). After additional treatment with an Ultraturrax, the protein extract was cleared by centrifugation. Protein concentration was determined using the Quick Start Bradford Protein Assay (Bio-Rad). *E. coli* protein extracts were prepared by pelleting overnight cultures and resuspendling the cell pellet in extraction buffer (see above), followed by lysis under constant cooling by two rounds of microfluidizer treatment. Protoilludene synthase activity was determined using [1-3H]-farnesyl diphosphate (20 Ci/mmol) (Biotrend, Cologne) in assay buffer (50 mM MOPS, 20 mM MgCl$_2$, 5 mM 2-mercaptoethanol, pH 7.2). Standard protoilludene synthase activity measurements were performed with 500 nM of [1-3H]-farnesyl diphosphate for 2 min followed by quenching with ethyl acetate. Part of the organic extract was then spotted onto silica-gel TLC plates and separated using 9:1 cyclohexane:ethyl acetate as the solvent, prior to analysis in a radio-TLC reader (Raytest). To determine $K_m$ values, the reactions were stopped by quenching with 100 mM EDTA (final concentration) followed by extraction with n-pentane, purification by silica gel column chromatography and quantitation by liquid scintillation counting. All kinetic activity assays were performed in triplicate. Mass spectrometry analysis of solvent extracts was performed on a QP2010S quadrupole mass spectrometer (Shimadzu, Duisburg) equipped with an Rxi$^\text{TM}$, 5 ms (0.25-mm inner diameter and 30-m length) column (Restek, Bad Homburg) using the following temperature program: 80 °C for 20 min, followed by heating the column at a rate of 15 °C/min to 300 °C with a final constant temperature of 300 °C for 4 min. Fragmentation was achieved by electric ionization at 1 keV.

*cdDNA Library Construction and Sequencing—An A. gallica CloneMiner$^\text{TM}$ cdDNA library (Invitrogen) was constructed according to the manufacturer’s protocol using a cesium chloride density gradient and *A. gallica* strain FU02472 mRNA purified by Oligotex (Qiagen). Recombinant *E. coli* were selected on 2YT-agar plates containing 50 µg/ml kanamycin, and 2800 randomly picked colonies were transferred to 96-well microtiter plates containing 200 µl 2YT medium per well with 50 µg/ml kanamycin for selection. The plates were incubated at 37 °C with continuous shaking at 160 rpm for ~12 h. *A. gallica* cDNAs were amplified directly from the culture.
using forward primer (5’-CTC GGG TAA CAG ATA GCA TGG ATG-3’) and reverse primer (5’-GAG CAC CCA TGG TCA TAG ATC-3’). PCR products were cleaned and sequenced by Raphael Soeur (Fraunhofer IME Aachen, Functional and Applied Genomics Group) using primer (5’-CA GGG CGG CCA GCT TTA AGC TCG GGC-3’) on an Applied Biosystems 3730 DNA Analyzer. Sequence data were analyzed using CLC Combined Workbench software (version 3; CLC bio), the Lasergene Package (DNASTAR), NCBI BLASTx, and Local BLAST.

Heterologous Expression of A. gallica Protoilludene Synthase in E. coli—Where CDNA sequencing identified potential terpene synthase clones, the corresponding pENTRY vectors were used in LR recombination reactions involving the pDEST14 destination vector. The resulting expression constructs were then introduced into E. coli BL21(DE3) CodonPlus cells (Stratagene) for heterologous expression. Recombinant bacteria were cultivated in Fernbach-baffled flasks and were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside when the A600 reached 0.5. The induced bacteria were maintained at 28 °C for 8 h with constant shaking at 160 rpm. Cells were then harvested by centrifugation, resuspended in prototulludene assay buffer, and lysed by a microfluidizer.

Genomic DNA Isolation and Southern Blot Hybridization—A. gallica genomic DNA was isolated using the cetyltrimethylammonium bromide method, and 120 μg was digested with 50 units of BamHI, EcoRI, or HindIII (New England Biolabs), as appropriate, for 8 h. The digested DNA was fractionated by agarose gel electrophoresis at a constant 50 V overnight, transferred to a positively charged nylon membrane (Roche Applied Science) and prehybridized with Roti® Hybri-Quick (Roth) containing single-stranded salmon sperm DNA. Two nucleic acid probes (~400 bp) were synthesized by PCR using forward primer (5’-CCT TCC TGA TAC TCT TGC CAA CTG-3’) and reverse primer (5’-CCT CCT CCG TCG AGA CGT CCG AGT AC-3’) for probe 1 and forward primer (5’-GTC ATC AAT CAT CCG GTT ATC AAA G-3’) and reverse primer (5’-CTT GGG CAT CAG CGT TAT CCA CCT C-3’) for probe 2. These products were labeled with [α-32P]dATP (Hartmann Analytic, Braunschweig) using the DecaLabel™ DNA Labeling kit (Fermentas) according to the manufacturer’s recommendations.

Isolation of an A. gallica Protoilludene Synthase Genomic Clone—The genomic clone encompassing the A. gallica protoilludene synthase gene was isolated by amplifying 100 ng of A. gallica genomic DNA using forward primer (5’-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CTA AGG AGA TAG AAC CAT GTC TCA ACG CAT CCT TCC TG-3’), reverse primer (5’-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT TAG AGA TGA AAT CCG TCA ACA ATT TGA GG-3’) and Herculase® II Fusion DNA Polymerase (Stratagene) in a 50-μl reaction. The PCR product was purified and sequenced as described above.

RESULTS

Characterization of Melleolide Biosynthesis by A. gallica Cell Culture—To isolate and characterize a putative honey mushroom (A. gallica) protoilludene synthase, we established a cell culture (FU02472) from a mushroom specimen collected near Traunsee, Austria. To examine secondary metabolite formation, the culture was cultivated by fermentation in liquid YM6.3 medium for 500 h to promote melleolide production. The culture was then harvested, and the melleolide product profile was characterized by LC-UV-MS using appropriate reference substances. The major melleolides produced by FU02472 were identified as melleolide I and armillaridin (Fig. 2). We also investigated the melleolide accumulation profile and changes in protoilludene synthase activity over time by sampling the culture at different time points (Fig. 3).

Protoilludene Synthase Activity—Enzyme activity was tested by incubating soluble enzyme extracts from the culture with radioactively labeled farnesyl diphosphate. Organic extracts from these reactions were then analyzed by radio-TLC (Fig. 3) Variable levels of protoilludene synthase activity were observed in all the crude protein extracts we tested, generating a strongly nonpolar product with an RF value of 0.7 (tentatively identified as 6-protoilludene) and a product with an RF value of 0.1 (tentatively identified as farnesol). The identity of both products was later confirmed by GC-MS (see below). Thermally inactivated control fractions were unable to convert farnesyl diphosphate into nonpolar products. The highest protoilludene synthase activity was observed after 185 h in culture, and this time point was therefore chosen for enzyme purification. To confirm that the putative protoilludene synthase activity in the soluble protein fraction was indeed due to a protoilludene synthase enzyme present in the fungal extract, cold farnesyl diphosphate was spiked with tritium-labeled material and incubated with the FU02472 soluble protein extract for 12 h. The radioactive fraction was then analyzed by radio-TLC and GC-MS to confirm the RF values and identities of the products. The mass spectrum of the extracted product yielded ions at an m/z of 175 (100%), 119 (91%), 105 (59%) 133 (35%), 91 (40%), 189 (17%), 161 (15%), and 147 (14%), with the molecular parent ion at an m/z of 204 (24%), which matched the fragmentation pattern previously reported for 6-protoilludene (Fig. 4) (36, 38). Surprisingly, there was no...
evidence indicating the presence of 7-protoilludene among the reaction products.

**Enzymatic Characterization of A. gallica Protoilludene Synthase**—Further characterization of the enzyme was carried out using partially purified A. gallica protoilludene synthase, tritium-labeled farnesyl diphosphate and radio-TLC analysis. These experiments revealed a $K_m$ for farnesyl diphosphate of 0.53 $\mu$M. As expected, we found that the enzyme activity was absolutely dependent on divalent metal ions. The highest protoilludene synthase activity was achieved in the presence of 5 mM MgCl$_2$, and it fell by 75% when MgCl$_2$ was replaced with MnCl$_2$. The temperature optimum was 22 °C, with nearly complete loss of activity at temperatures >35 °C. Several buffering systems were tested at 50 mM, with optimal activity at pH 7.2 (MOPS buffer) and pH 8.5 (Tris buffer). The enzyme was also sensitive to the presence of ethanol, with concentrations as low as 5% causing a dramatic reduction in activity. Attempts to purify the A. gallica protoilludene synthase to homogeneity and determine the N-terminal amino acid sequence by Edman sequencing proved unsuccessful, but they showed that the enzyme was likely a 45-kDa monomer.

**Heterologous Expression of Pro1 in E. coli**—Heterologous expression of the Pro1 gene in E. coli resulted in a crude soluble protein extract possessing sufficient sesquiterpene synthase activity to convert 80% of the 0.5 $\mu$M tritium-labeled farnesyl diphosphate substrate into a product matching the properties of 6-protoilludene ($R_f = 0.7$) within 5 min. Incubation of the same lysate with geranylgeranyl diphosphate did not produce significant amounts of a less polar product ($<1$%), and neither farnesyl diphosphate nor geranylgeranyl diphosphate was converted into less polar products when using E. coli control lysates derived from the empty vector control (Fig. 4). Characterization of the heterologous terpene synthase using cold farnesyl diphosphate, after pentane extraction and analysis of the organic extract by GC-MS, revealed the formation of a product with an identical retention time and mass spectrum as the native protoilludene synthase.

**Isolation of Genomic Protoilludene Synthase Gene Sequence**—Secondary metabolite biosynthetic pathways in microorganisms are often arranged in biosynthetic gene clusters. Thus, the isolation of the protoilludene synthase genomic clone would facilitate the isolation of a potential melleolide biosynthetic gene cluster in A. gallica. By using the 1270-bp full-length Pro1 cDNA clone, we designed primers to amplify the corresponding genomic DNA sequence (Fig. 5).
Analysis of the 1645-bp product revealed the presence of eight introns and nine exons, with some as short as 50 bp. The observed pattern of intron/exon structures differed significantly from the patterns observed in a previous analysis of plant terpene synthase genes (7). Southern blot hybridization, using oligonucleotide probes representing the 5' and 3' ends
of the Pro1 clone, respectively, revealed only a single copy of the Pro1 gene in the A. gallica genome (Fig. 6).

**DISCUSSION**

The emergence of multi-drug-resistant microbial pathogens, such as methicillin-resistant strains of *S. aureus*, has triggered a resurgence of interest in novel antibiotics with new mechanisms of action. Natural products traditionally represent a good source of antibiotic substances. Although terpenoids represent the largest as well as most diverse group of natural products, only two antibiotics based on a fungal sesquiterpenoid are currently in use.

Melleolides from the *Armillaria* species have long been known for their potent antibacterial activity (31, 32, 37). However, limited supplies of individual melleolides have prevented them from being more extensively examined and clinically developed. Metabolic pathway engineering in easily culturable microbial hosts such as *Saccharomyces cerevisiae* or *Aspergillus nidulans* may provide an efficient method of producing advanced melleolide precursors for semisynthesis or the desired product by total fermentation.

For successful metabolic engineering, the functional characterization and molecular cloning of the underlying enzymatic steps is a key prerequisite. The cyclization of the universal sesquiterpenoid building block farnesyl diphosphate to 6-protoilludene constitutes the initial pathway-committing step in the biosynthesis of all melleolides. Phytochemical analysis of *Formitopsis insularis* has led to the isolation of 6-protoilludene and 7-protoilludene-6-ol (38). Using a chemically synthesized reference, the analysis of the organic extract of *Formitopsis insularis* also yielded no indication of the formation of 7-protoilludene. Thus, the ob-
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cDNA in E. coli have provided important insight into the melleolide biosynthesis pathway in fungi and may contribute to the development of new strategies for the synthesis of novel antimicrobial compounds.

Acknowledgments—We are grateful for expert technical assistance from Raphael Soeur (Fraunhofer IME), Beate Schniescheck, Dirk Müller, and Stefan Heke (InterMed Discovery GmbH). We thank professor Ruth Seeger for providing the A. gallica culture.

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