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CYPArm2 is a CYP450 Monooxygenase with Protoilludene 13-Hydroxylase Activity Involved in the Biosynthesis of Armillyl Orsellinate-Type Sesquiterpenoids

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The melleolides are a family of structurally and functionally diverse sesquiterpenoids with potential applications as fungicides, antimicrobials, and cancer therapeutics. The initial and terminal steps of the biosynthesis pathway in *Armillaria* spp. have been characterized, but the intermediate steps are unclear. Biosynthetic gene clusters in *A. mellea* and *A. gallica* were shown to encode a terpene cyclase, a polyketide synthase, and four CYP450 monooxygenases. We have characterized CYPArm3, which is responsible for the hydroxylation of Δ-6-protoilludene, but the functions of the other CYP450s remain to be determined. Here we describe CYPArm2, which accepts Δ-6-protoilludene and 8α-hydroxy-6-protoilludene as substrates. To investigate the products in more detail, we generated recombinant *Saccharomyces cerevisiae* strains overexpressing CYPArm2 in combination with the previously characterized protoilludene synthase and 8α-hydroxylase. Using this total biosynthesis approach, sufficient quantities of product were obtained for NMR spectroscopy. This allowed the identification of 8α,13-dihydroxy-protoilludene, confirming that CYPArm2 is a protoilludene 13-hydroxylase.

Introduction

The melleolide family of sesquiterpene aryl esters is an intriguing class of fungal secondary metabolites with diverse biological activities. In the earliest observations, *Armillaria mellea* culture fluids were shown to inhibit the growth of *Pseudomonas pyocanea*, indicating an antibiotic activity.[1] Other melleolide derivatives were found to inhibit Gram-positive bacteria, fungi and cancer cell lines, and yet others showed no apparent activity. Interestingly, *A. mellea* tablets are used in Chinese medicine for a range of neurological indications such as neurasthenia, insomnia, vertigo, and headaches.[2] More than 60 chemotypes have been described thus far.[3] The relationship between melleolide structural variants and specific activities has been investigated in detail under controlled conditions (see Supplement Table S1).

The mode of action of melleolides has been thoroughly investigated in mammalian cell cultures, cancer cell lines, and the model fungus *Aspergillus nidulans*.[4] For example, the exposure of Jurkat T cells to 10 μM arnamial for 6 h caused cell blebbing and shrinkage, reflecting the 18.1-fold induction of caspase-3 activity compared to untreated cells. In contrast, exposure to 0.3 μM actinomycin D (a known activator of apoptosis) for the same duration induced caspase-3 activity by only 5.7-fold. Similar levels of DNA fragmentation were observed in both treatments, indicating that arnamial has pro-apoptotic activity.[4a] Better characterization of the enzymatic steps involved in the synthesis of these compounds could help to identify structural properties associated with specific activities and thus facilitate the production of individual melleolide chemotypes.

The first committed step in the melleolide biosynthesis pathway is the cyclization of farnesyl pyrophosphate to Δ-6-protoilludene by a terpene synthase named protoilludene synthase (encoded by the *Pro1* gene).[5] Genomic sequencing of *Armillaria gallica* strain FU02472 revealed a melleolide, armillyl orsellinate, and armillane biosynthetic gene cluster containing the genes for protoilludene synthase and four cytochrome P450 monooxygenases (CYP450s). The homologous proteins were named CYPArm1–CYPArm4, although CYPArm2 and CYPArm3 are much more similar to each other than either is to the two other enzymes (Figure 1).[6] Each protein was expressed in an engineered *S. cerevisiae* strain already expressing *Pro1*, and ^{3}H-6-protoilludene feeding assays showed that only CYPArm2 and CYPArm3 were able to utilize the substrate. Nuclear magnetic resonance (NMR) spectroscopy was used to characterize the products of CYPArm3, and it was designated as a protoilludene-8α-hydroxylase. Here we describe the further characterization of CYPArm2 by feeding assays in *S. cerevisiae* and the structural analysis of the metabolic products by NMR spectroscopy. This

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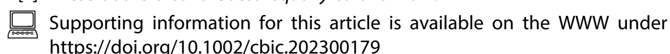
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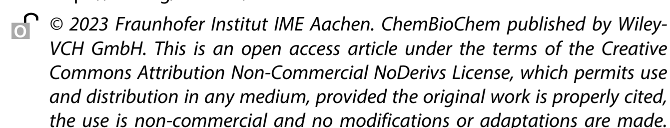
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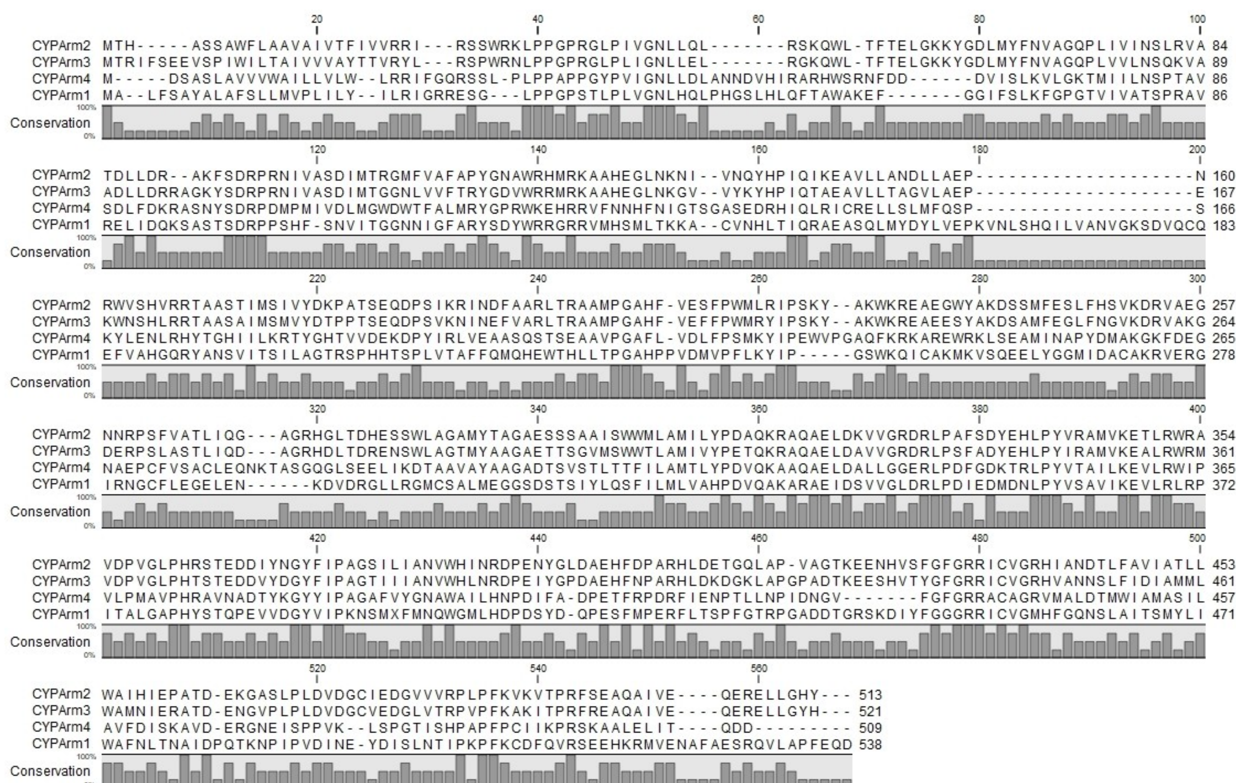

Figure 1. Amino acid sequence alignment of four cytochrome P450 monooxygenases encoded by the melleolide gene cluster in *Armillaria gallica* (GenBank accession number MT277003). CYPArm2 and CYPArm3 are much more closely related to each other, than either is to CYPArm1 or CYPArm4.

allows us, for the first time, to define the position of CYPArm2 in the melleolide biosynthesis pathway.

Results and Discussion

Characterization of CYPArm2 in *S. cerevisiae*: 6-protoilludene feeding assays

Our earlier investigations of the melleolide biosynthesis pathway led to the creation of three yeast strains expressing the *Taxus chinensis* NADPH:cytochrome P450 reductase together with CYPArm2, CYPArm3, or the empty vector. We established cultures of each strain in 250 mL baffled shake flasks containing 50 mL YPD medium. Later, we switched them to galactose containing YPG medium to induce gene expression and fed them with 1 mg of purified Δ-6-protoilludene, the product of the pathway committing protoilludene synthase. After overnight incubation, the cultures were processed by solid-phase extraction (SPE) followed by GC-MS analysis. This confirmed the absence of 6-protoilludene, reflecting either its conversion or integration into lipid membranes due to its nonpolar structure (Figure 2). As expected, no conversion was observed for the empty vector control strain (Figure 2a), whereas the previously characterized CYPArm3 converted the substrate into the hydroxylated product 8α-hydroxy-6-protoilludene with a retention time (RT) of 10.1 min (*m/z*=220, 205, **191**, 173, 163, 149, 135, 121, 109, 91, 79, 67, 55; base peak in bold) (Figure 2c). In contrast, CYPArm2 converted the substrate into a novel hydroxylated 6-protoilludene product with a RT of 9.2 min (*m/z*=220, **205**, 191, 173, 159, 135, 121, 107, 93, 79, 67, 55) (Figure 2b). We therefore set out to generate a fermentation strain to produce this unknown compound in amounts sufficient for structural elucidation.

Generation and analysis of a novel protoilludene alcohol fermentation strain

For the functional characterization of CYPArm2 we created the expression strain CEN-Arm2, which co-expresses the *T. chinensis* P450 reductase, a truncated *S. cerevisiae* HMG-CoA reductase, the protoilludene synthase (*Pro1*), and CYPArm2. We established duplicate 100 mL cultures of CEN-Arm2 and the empty vector strain. One of each duplicate was cultivated in YPD for 2 days, whereas the other was cultivated in YPD for 1 day and then switched to YPG to induce gene expression. Analysis of the cytosolic and microsomal fractions by western blot confirmed the presence of the Pro1-His_6 (40 kDa) and CYPArm2-His_6 (56 kDa) enzymes (see Supplement Figure S1, lane 4).

Enzyme activity was confirmed by cultivating the CEN-Arm2 test strain, the empty vector control strain, and the previously described positive control CEN-22 strain (pCM183::*P450-Red-tc*, pRS315 *tHMGR* S.c., pRS423::*Pro1*His6) and pYES-DEST52::*CYP-*

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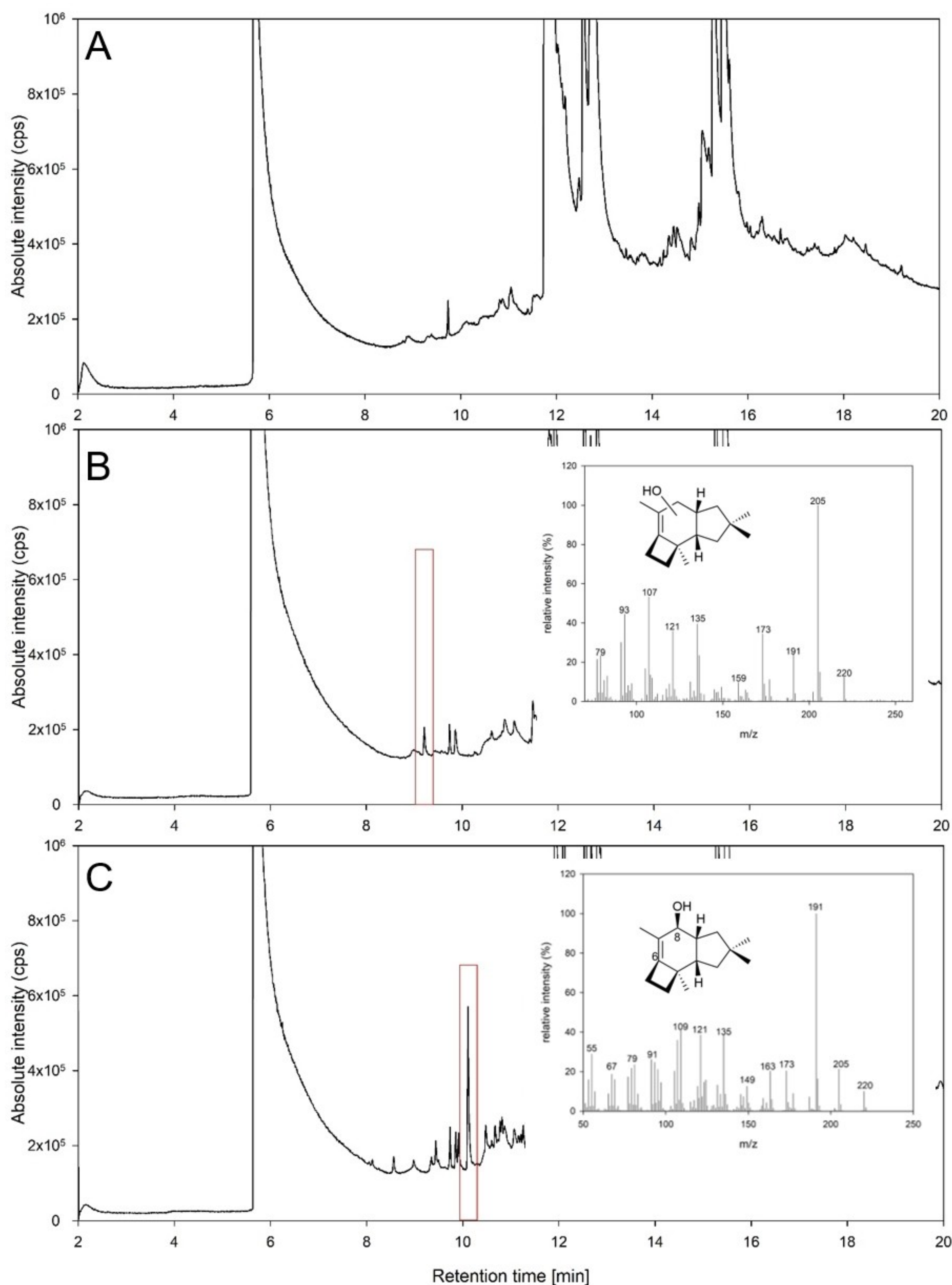

Figure 2. GC-MS total ion count chromatograms of a Δ-6-protoilludene feeding assay. The 50 ml cultures were fed with 1 mg 6-protoilludene. (A) CEN.PK2-1C (pCM183::P450-Red-tc and pYESDEST52::Empty). (B) CEN.PK2-1C (pCM183::P450-Red-tc and pYES-DEST52::CYPArm2His6). (C) CEN.PK2-1C (pCM::P450-Red-tc and pYES-DEST52-CYPArm3His6). Boxes surround confirmed monooxygenated 6-protoilludenes.

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*Arm3*His.[6] We established duplicate 100 mL cultures of each strain in YPD and, as above, maintained one in YPD but switched the other to YPG. The cells were pelleted and the broth was processed by SPE for the analysis of hydroxylated protoilludenes. The melleolide precursor 8α-hydroxy-6-protoilludene (RT = 10.1 min) was readily identified in the positive control strain (see Supplement Figure S2a) whereas the CEN-Arm2 strain produced the same novel fermentation product (RT = 9.2 min) identified in the feeding assays (see Supplement Figure S2b). The latter confirmed the results of the feeding assays, which suggested that the activity of CYPArm2 was inferior to that of CYPArm3. As anticipated, Δ-6-protoilludene and the novel product no longer accumulated in the fermentation broth when the test and positive control strains were cultivated under non-inducing conditions. Furthermore, neither of these products was found in the extracts of the induced or non-induced empty vector negative control strain.

We attempted to purify the novel product despite the low efficiency of 6-protoilludene substrate conversion. The CEN-Arm2 strain was therefore used to seed a 3 L fermentation process,[6] followed by SPE, silica column purification, and GC-MS analysis. The fractions were also concentrated, resuspended in methanol and purified by preparative HPLC. LC-MS/MS was carried out to identify the elution fraction containing the protoilluden-ol. After 20 rounds of purification, fractions containing the mono-ol were pooled, dried over anhydrous $MgSO_4$ and the methanol was evaporated in the presence of silica. The powder was then packed into a mini-column and eluted with methylene chloride, before a sample was taken again for GC-MS analysis. The solvent was then exchanged for deuterium chloroform to facilitate NMR spectroscopy, but accurate structural characterization was hindered by the low quantity of sample and residual impurities. Although we confirmed that CYPArm2 accepts 6-protoilludene as a substrate, we observed a very limited turnover to a more oxygenated product. GC-MS analysis showed that the CYPArm2 product differed from that of the previously characterized protoilludene-8α-hydroxylase produced by CYPArm3, suggesting that 6-protoilludene is not the native substrate of CYPArm2 but is closely related to it. We therefore determined whether CYPArm2 would accept the previously identified 8α-hydroxy-6-protoilludene alcohol as a substrate to yield a protoilluden-diol.[6]

For the diol production strain, we constructed a new plasmid (pRS423::*Pro1*His6::*CYP-Arm3*His6) by ligating the GAL1p-*CYP-Arm3*His6-CYC1t cassette from expression vector pYES-DEST52::*CYP-Arm3*His6 to the pRS423::*Pro1*His6 backbone. The heterologous yeast strain CEN-23 was then created by transforming strain CENPK2-1c with the plasmids pCM183::*P450-Red-tc*, pRS315::*tHMGR*-S.c., pRS423::*Pro1*-His6::*CYP-Arm3*His6 and pYES-DEST52::*CYP-Arm2*His6.

Biosynthesis, purification, and structural analysis of the hydroxylated protoilludenes

Yeast strain CEN-23 was cultivated in a 3-L batch process.[6] The cells were pelleted, and the fermentation broth was extracted with chloroform and compared to a 500 mL chloroform extract of the negative control (which produces 8α-hydroxy-6-protoilludene) and a standard of 8α-hydroxy-6-protoilludene standard (APCI spectrum *m/z* = 203, 175, 161, 147, 133, **119**, 105; base peak in bold) available from our previous investigation (see Supplement Figure S2). The presence of a di-oxygenated protoilludene was confirmed by the similarity of the spectrum at RT = 22.5 min (APCI spectrum *m/z* = 219, 201, 173, 159, **145**, 131, 119, 105; base peak in bold) and the characteristic loss of 18 amu (presumably water) (Figure 3a and 3b).

The extract was also analyzed using a mid-polar GC-MS column, which confirmed the presence of a putative diol product with the following mass spectrum at RT = 11.8 min (*m/z* = 236, 218, 205, 189, 175, 161, 147, 133, 119, **105**, 91, 79, 55; base peak in bold). Following final HPLC purification, the sample was analyzed by GC-MS as above (Figure 3c and 3d) before NMR spectroscopy, which confirmed the diol structure was 8α,13-dihydroxy-6-protoilludene. For structural determination both ^{1}H and ^{13}C NMR analysis were performed. The complete ^{1}H- and ^{13}C-NMR shifts and their assignments as a result of the two dimensional NMR spectra analysis are shown in Table 1 (see Supplement Figure S4). Both, ^{1}H and ^{13}C NMR resonances were similar to the 8α-hydroxy-6-protoilludene assigned previously.[6] The structure of the 8α,13-dihydroxy-6-protoilludene is confirmed in particular by HMBC and NOESY spectra. Unvaried, the hydrogen atom H-8 (4.07 ppm) and the hydroxyl group are bounded on carbon C-8 (74.81 ppm, T), whereas hydrogen atoms H-13 (4.15 ppm, s) and C-13 (58.99 ppm) were clearly assigned as an oxy-methine. Both, the 1H integration at H-13 and the ^{13}C DEPT-135 NMR spectrum shows at position 13 the presence of a methylene group instead of the methyl-group of 8α-hydroxy-6-protoilludene. In HMBC spectrum couplings from methylene group 13, were observed to carbons C-6, C-7, and C-8, respectively. The stereochemistry of the compound is confirmed by the observed Nuclear Overhauser Enhancement (NOE) effects in NOESY spectrum.

NOE's were observed clearly between hydrogen H-8 and H-10B and the methyl group 12, which confirms that these atoms are located on the same molecule side. A weak NOE was observed between H-13 and H-5B. The conversion of 8α-hydroxy-6-protoilludene to the novel di-oxygenated product 8α,13-dihydroxy-6-protoilludene confirms that CYPArm2 is a protoilludene 13-hydroxylase, and that its promiscuity enables it to accept both 6-protoilludene and 8α-hydroxy-6-protoilludene as substrates.

Conclusion

The melleolide gene cluster in *A. gallica* is almost identical to that in *A. mellea*, encoding a terpene synthase, four CYP450s, several dehydrogenases, and a polyketide synthase.[7] This indicates that similar biosynthesis pathways are likely present in both of these melleolide-forming basidiomycetes, given that they also produce the same spectrum of aryl esters.[8] Thus far, the terpene synthase, two of the CYP450s, and the polyketide

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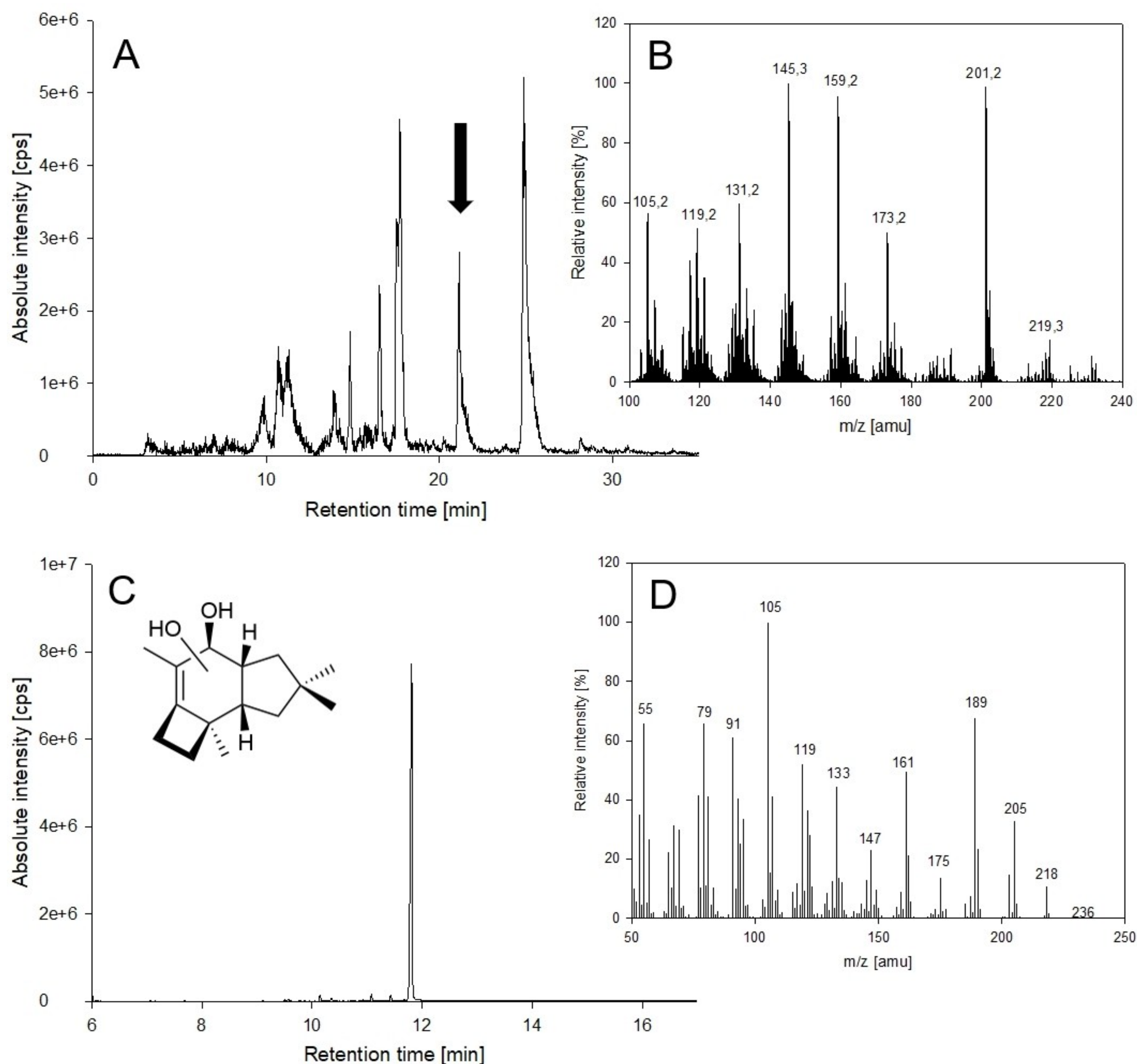

Figure 3. Compilation of LC-MS and GC-MS spectra of the dihydroxylated protoilludene fermentation product of strain CEN-23 (see Supplement Table S2), expressing both *A. gallica* cytochrome P450s CYPArm2 and CYPArm3. (A) Showing an extracted ion chromatogram (119 amu) of the chloroform extract. (B) APCI Mass Spectrum of the putative dihydroxy-protoilludene, identified as 8α,13-hdroxy-6-protoilludene. (C) GC-MS total ion count spectrum of purified 8α,13-hdroxy-6-protoilludene. (D) GC-MS mass spectrum of 8α,13-hdroxy-6-protoilludene.

synthase have been well characterized. However, the enzyme that synthesizes the 6-hydroxy-7-protoilludene early intermediate has not yet been identified, and until this is achieved all melleolides formed downstream of this pathway bifurcation will be difficult to acquire using a solely biological approach. Ichinose and Kitaoka demonstrated that 6-hydroxy-7-protoilludene can be autocatalytically derived from 8α-hydroxy-6-protoilludene in the presence of a strong acid.[9] We have confirmed that CYPArm2 does not produce this compound, but our work sheds light on the earliest stages of the melleolide biosynthesis pathway (Figure 4). Feeding assays with ^{3}H-6-protoilludene showed CYPArm3 and CYPArm2 are able to convert this substrate but CYPArm1 and CYPArm4 are not. We previously confirmed that CYPArm3 converts Δ-6-protoilludene into 8α-hydroxy-6-protoilludene,[6] and here we have shown that CYPArm2 can also accept 6-protoilludene, leading to the accumulation of a mono-oxygenated protoilludene in the fermentation broth. This agrees with the outcome of earlier feeding experiments with ^{3}H-labeled substrates.[5] Following the structural analysis of 8α,13-dihydroxy-6-protoilludene, and the comparison of GC-MS and LC-MS/MS data collected from the extracts of the CEN-Arm2 strain, we suggest that the product of

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Table 1. NMR-analysis of 8α,13-dihydroxy-protoilludene: 1H and 13C chemical shifts (D in ppm) in relation to CHDCl2 (1H 5.32 ppm, 13C 54.00 ppm). Abbreviations: d and D: doublet, dd: doublet from doublets; dt: doublet derived from triplets, m: multiplet, Q: quartet, s and S: singlet, t and T: triplet).

Number	^{1}H-signal	^{13}C-signal	^{n}J coupling constant	NOE signal
1A	1.33 (ddd)	41.17 (T)	$^{2}J_{1A1B}=12.6$; $^{3}J_{1A,2}=7.6$; $^{3}J_{1B,2}=10.5$;	1_A–1_B; 1_A–2; 1_A–15
1B	1.23 (dd)		$^{4}J_{1A,10A}=1.7$	1_B–10_B; 1_B–12; 1_B–15
2	2.3 (ddd)	46.10 (D)	$^{3}J_{2,1A}=7.8$; $^{3}J_{2,1B}=10.5$; $^{3}J_{2,9}=11.7$	2–1_A; 2–15
3	–	45.61 (S)		–
4A	178 (2H, m)	36.13 (T)	$^{2}J_{4A,4B}=10.4$; $^{3}J_{4A,5B}=9.3$; $^{3}J_{4A,5A}=4.0$	4_{AB}–5, 4_{AB}–12
4B				
5A	2.71 (m)	24.83 (T)		5_A–5_B; 5_A–4; 5_A–12
5B	2.58 (m)			5_B–13
6	–	145.94 (S)	–	–
7	–	128.93 (S)	–	–
8	4.07 (m)	74.81 (D)		8–10_B; 8–12
9	2.25 (m)	51.05 (D)		9–10_A; 9–15
10A	1.71 (ddd)	46.32 (T)	$^{2}J_{10A,10B}=12.0$; $^{3}J_{10A,9}=7.2$; $^{4}J_{10A,1A}=1.7$	10_A–10_B; 10_A–15
10B	1.06 (dd)			10_B–1_B; 10_B–8
11	–	39.82 (S)		–
12	0.98 (3H, s)	20.22 (Q)		12–1_B; 12–4; 12–5_A; 12–8
13	4.15 (2H, s)	58.99 (T)		13–5_B
14	1.02 (3H, s)	29.55 (Q)		14–1_B; 14–15
15	0.89 (3H, s)	27.01 (Q)		15–1_A; 15–1_B; 15–2; 15–9; 15–10_A
–OH	~2.3 (broad)	–		

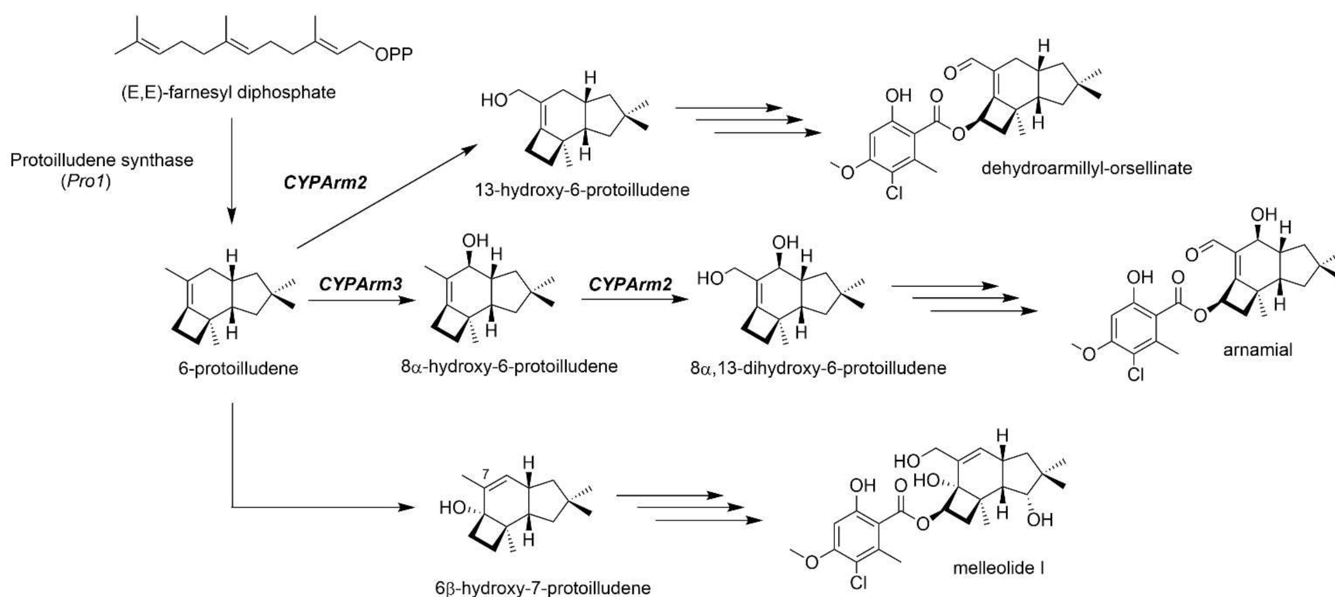

Figure 4. Complexity of the initial part of the melleolide biosynthetic pathway in *Armillaria gallica.* Single arrows represent single bioconversions and multiple arrows represent parts of the pathway including multiple reaction steps. Protoilludene synthase is encoded by the *Pro1* gene. CYPArm3 gene encodes a protoilludene 8α-hydroxylase and CYPArm2 encodes a protoilludene 13-hydroxylase. Melleolide I, arnamial and dehydroarmillyl-orsellinate represent possible corresponding metabolic end-products.

CYPArm2 is highly likely to be 13-hydroxy-6-protoilludene, a compound that has, to the best of our knowledge, been described only once before in the context of chemical synthesis.[10] This apparent lack of specificity is not surprising, given the plasticity of fungal CYP450s, with several known di-oxygenases also catalyzing the concomitant hydroxylation of closely related substrates.[11] Furthermore, the co-expression of CYPArm2 and CYPArm3 together with the protoilludene synthase produced a structurally confirmed novel protoillu-dane-diol (8α,13-dihydroxy-6-protoilludene), thereby enhancing our understanding of this fascinating pathway, which should facilitate the future heterologous production of melleolides and armillyl-orsellinates for the benefit of society. We are currently investigating the two remaining uncharacterized CYP450s in the gene cluster in order to define the pathway completely and develop a strategy for the production of these valuable sesquitepenoids so that their untapped potential can be realized.

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Experimental Section

Heterologous *S. cerevisiae* strains and plasmids

Engineered strains of *S. cerevisiae* were created by transformation using the lithium acetate method, starting with the background strain CEN.PK2-1C (MATa, ura3-52, trp1-289, Leu2-3_112, his3 △-1, MAL2-8c, SUC2) from EUROSCARF (University of Frankfurt, Frankfurt am Main, Germany). The CEN-Pro1 strain was created by transforming competent *S. cerevisiae* cells with plasmids pCM183::P450-Red-tc, pRS315 tHMGR S.c. and pRS423::*Pro1*His6 (see Supplement Table S2 and S3). The CEN-Arm2 strain overexpressing CYPArm2 was created by transforming competent CEN.PK2-1c cells with the plasmids pCM183::*P450-Red-tc*, pRS315 *tHMGR* S.c., pRS423::*Pro1*-His6, and pYES-DEST52::*CYP-Arm2*His6.[6] Plasmid pRS423::*Pro1*-His6::*CYP-Arm3*His6 was created by amplifying the CYP*Arm3*His6 cassette from pYESDEST52::*CYP-Arm3*His6 using forward primer 5′-CAC CGG CCG ACG GAT TAG AAG CCG CCG AGC-3′ and reverse primer 5′-CAG CGG CCG GAC CGA GCG CAG CGA GTC AGT G-3′ (95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1.5 min, and a final extension step at 72 °C 10 min). The product was ligated to the pRS423::*Pro1*His6 backbone digested with EagI-HF, and introduced into NEB 10β cells. Positive clones were screened using the same restriction enzyme, and those with the correct digestion profile for pRS423::*Pro1*-His6::*CYP-Arm3*His6 were sequenced before introduction into CENPK2-1c yeast cells along with pCM183::*P450-Red-tc*, pRS315 *tHMGR* S.c., and pYES-DEST52::*CYP-Arm2*His6 to create strain CEN-23.

Cultivation of *S. cerevisiae* strains and overexpression of CYP450s

Yeast cultures were seeded by inoculating a single colony or cryostab into 5 mL synthetic dropout (SD) minimal medium (20 mg mL^{-1} histidine and 20 mg mL^{-1} tryptophan to maintain auxotrophic selection, and 100 μg mL^{-1} ampicillin), followed by incubation at 28 °C, shaking at 160 rpm. We then used 4 mL of the pre-culture to inoculate 50 mL YPD medium containing 100 μg mL^{-1} ampicillin. The following day, the cells were pelleted by centrifugation at 3000×g for 5 min and the supernatant was discarded. The cells were resuspended in 50 mL fresh YPG medium containing 5 g L^{-1} raffinose, 20 g L^{-1} galactose, and 100 μg mL^{-1} ampicillin. These cultures were incubated with 1 mg Δ-6-protoilludene (dissolved in methanol) for 24 h before SPE and GC-MS analysis (see below). The same process was used for the 100 mL overexpression cultures except standard YPG medium (20 g L^{-1} galactose) was used for the final induction step and the cultures were incubated for 3 days at 28 °C, shaking at 160 rpm. Membrane-bound protein extracts were prepared from 20 mg of wet cell paste using the Mem-PER Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA) and the protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). We then used 60 μg of the hydrophobic protein extract for western blot analysis using an XCell SureLock Mini-Cell and XCell II Blot Module (Thermo Fisher Scientific). The samples were fractionated on NuPAGE Novex 10% Bis-Tris Protein Gels (Thermo Fisher Scientific) for 40 min at 200 V before transfer to a nitrocellulose membrane for 1 h at 30 V. After washing with PBS containing 0.05% Tween-20 (PBST), the membrane was incubated for 1 h with an anti-polyhistidine antibody (Sigma-Aldrich, St Louis, MO, USA), washed again with PBST, and the signal was visualized using the Pierce DAB Substrate Kit (Thermo Fisher Scientific).

Extraction and purification of melleolide precursors

The 50 mL feed cultures and small-scale 100 mL overexpression cultures were processed by SPE as previously described.[9] Briefly, acetonitrile was added to the cultures to a final concentration of 20% (v/v) before the cells were centrifuged for 5 min at 3000×g. The cells were discarded and the broth was applied under vacuum to an Oasis HLB 200-mg 5 cc Glass Vac Cartridge (Waters, Milford, MA, USA) preconditioned with 20% (v/v) acetonitrile in ddH$_2$O. The final eluate was dried under compressed air or using a SpeedVac and resuspended in methanol before GC-MS or LC-MS/MS analysis.

For 3 L scale extractions, cells and C18 silica gel were dried in a freeze drier at −60 °C for 72 h before the combined material was extracted with methylene chloride using a Soxhlet apparatus, and the extract was concentrated to ~3 mL in a rotary evaporator. This oily yellow concentrate was loaded onto a 30 cm silica 60 column before elution with pentane and methylene chloride. We collected 5 mL fractions for GC-MS analysis. Fractions containing the protoilludene alcohol were pooled and concentrated before resuspending them in methanol for further purification by HPLC.

To obtain the Δ-6-protoilludene substrate for feeding assays, the CEN-Pro1 strain was created by transformation with plasmids pCM183::P450-Red-tc, pRS315::tHMGR S.c., and pRS423::Pro1His6.[5] The CEN-Pro1 strain was cultured in self-induction medium (1.7 g L^{-1} yeast nitrogen base without amino acids, 5 g L^{-1} ammonium sulfate, 20 mg L^{-1} uracil, 40 mM succinate, 1 mM MgCl$_2$, 5 g L^{-1} raffinose, 20 g L^{-1} galactose, pH 6.5) containing 5 g L^{-1} C18 silica gel for extraction. After 3 days, the cells and silica were filtered and freeze dried before elution with *n*-pentane. Further concentration with nitrogen yielded almost pure Δ-6-protoilludene for feeding assays.

Semi-preparative HPLC purification of fermentation products

Extracts or pooled fractions were dried with compressed air and resuspended in methanol before separation by semi-preparative HPLC.[5] Briefly, the fermentation broth methanol extracts were fractionated using a Gemini 5 μm C18 110 Å HPLC column 250× 10 mm (Phenomenex, Aschaffenburg, Germany) in an LC-20A Prominence HPLC system (Shimadzu, Duisburg, Germany) equipped with a diode array detector (DAD; λ = 190–800 nm). Methanol and water were used as the mobile phases at a constant flow rate of 5 mL min^{-1}. The gradient started at 70% methanol and increased to 100% over 20 min.

GC-MS analysis

Crude SPE eluates were concentrated to 1 mL before up to 5 μL was injected into a Shimadzu GC-MS QP2010S quadrupole mass spectrometer equipped with a Rxi-5 ms (30 m) column (Restek, Bad Homburg, Germany) and an FS-OV-1701-CB-0.5 (25 m) column (CS-Chromatographie Service, Langerwehe, Germany). The sample was ionized at 1 keV, and gas-phase ions were separated on the Rxi-5 ms column using the following program: 2 min incubation at 80 °C, ramp to 300 °C at 15 °C min^{-1}, final incubation at 300 °C for 4 min. The resulting fractions were separated on the FS-OV-1701-CB-0.5 (25 m) column using the following program: 2 min incubation at 80 °C, ramp to 250 °C at 15 °C min^{-1}, final incubation at 250 °C for 4 min.

LC-MS/MS analysis

Samples containing hydrophilic, non-volatile analytes were characterized by LC-APCI-EMS. The samples were separated on a Curosil-

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PFP 3 μm LC column 100×4.6 mm (Phenomenex), ionized by atmospheric pressure chemical ionization (APCI) and 20 μL was injected into a 3200 Q TRAP MS (Applied Biosystems, Darmstadt, Germany). The APCI probe was used in tandem with an Agilent 1200 HPLC System (Agilent Biosciences, Santa Clara, CA, USA) fitted with a DAD (λ = 190–800 nm). The mobile phases were ddH_2O containing 5 mM ammonium acetate with 0.1% trifluoroacetate (TFA), and acetonitrile containing 0.1% TFA at a constant flow rate of 0.2 mL min^{-1}. The gradient started at 10% acetonitrile and increased to 100% over 24 min.

Sample preparation for NMR spectroscopy

HPLC fractions were pooled, dried with molecular sieves and anhydrous $MgSO_4$ and concentrated under N_2 to ~1 mL before resuspension in 0.6 mL d_2-methylene chloride and transfer to an NMR tube. The samples were analyzed using a DRX 500 MHz NMR instrument (Bruker, Billerica, MA, USA) using a 5 mm broadband observe (BBO) probe with a z-gradient. Data were processed using TopSpin v1.3 (Bruker) and MestReNova v8.0 (MestreLab Research, Santiago de Compostela, Spain).

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Conflict of Interests

The authors declare that they have no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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