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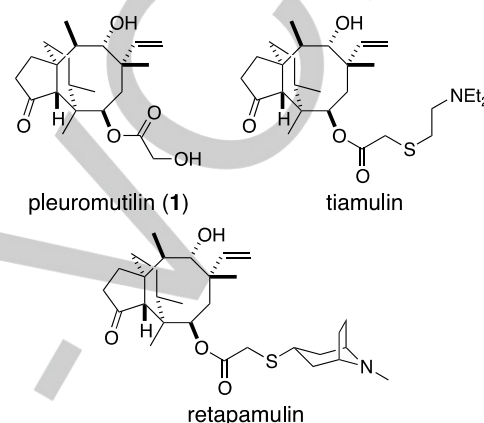
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Biosynthetic machinery of diterpene pleuromutilin isolated from basidiomycete fungi

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Dedication ((optional))

Abstract: The diterpene pleuromutilin is a ribosome-targeting antibiotic isolated from basidiomycete fungi, such as *Clitopilus pseudo-pinsitus*. Here, we report the functional characterization of all biosynthetic enzymes, involved in pleuromutilin biosynthesis, and propose a biosynthetic pathway. In vitro enzymatic reactions and mutational analysis revealed that a labdane-related diterpene synthase, Ple3, catalyzes two rounds of cyclization from geranylgeranyl diphosphate to premutilin possessing a characteristic 5-6-8-tricyclic carbon skeleton. Biotransformation experiments utilizing *Aspergillus oryzae* transformants possessing modification enzyme genes allowed us to propose the biosynthetic pathway from premutilin to pleuromutilin. The present study sets the stage for the enzymatic synthesis of natural products isolated from Basidiomycete fungi, which are a prolific source of structurally diverse and biologically active terpenoids.



Scheme 1. Structure of pleuromutilin and its derivatives.

Pleuromutilin (**1**) is a diterpene natural product (Scheme 1) originally isolated from *Pleurotus mutilus* and *P. passeckerianus* in 1951.¹ The structure features a “propellane-like” tricyclic skeleton composed of 5-, 6-, and 8-membered rings each sharing two of their carbon atoms. **1** and its derivatives are antibiotic agents that inhibit bacterial protein synthesis by binding to the peptidyltransferase center of the 50S ribosomal subunit.²⁻⁴ Therefore, much effort has been focused on the semi-synthesis of new ribosome-targeting analogues based on the unique tricyclic skeleton of **1**. These efforts have resulted in the development of two veterinary drugs, tiamulin and retapamulin, by replacing the C14-glycolic acid chain of **1** with a more hydrophobic thioacetate-containing side chain (Scheme 1).⁵ Structural analysis of the 50S ribosomal subunit from *Deinococcus radiodurans*, in complex with tiamulin, revealed that the side chain interfered with proper positioning of the initiator tRNA at the P-site.⁶ Notably, retapamulin was approved for human use in 2007 by the Food and Drug Administration (FDA).^{7,8} Subsequently, three additional derivatives, BC-3781, BC-3205, and BC-7013, were also developed for human use.^{9,10} In addition to these semi-synthetic studies focusing on the biological activities, the unique structure has attracted the

attention of researchers in the fields of synthetic chemistry. To date, numerous synthetic studies, including two racemic^{11a,11b} and one enantioselective^{11c} total synthesis, have been reported.^{11d}

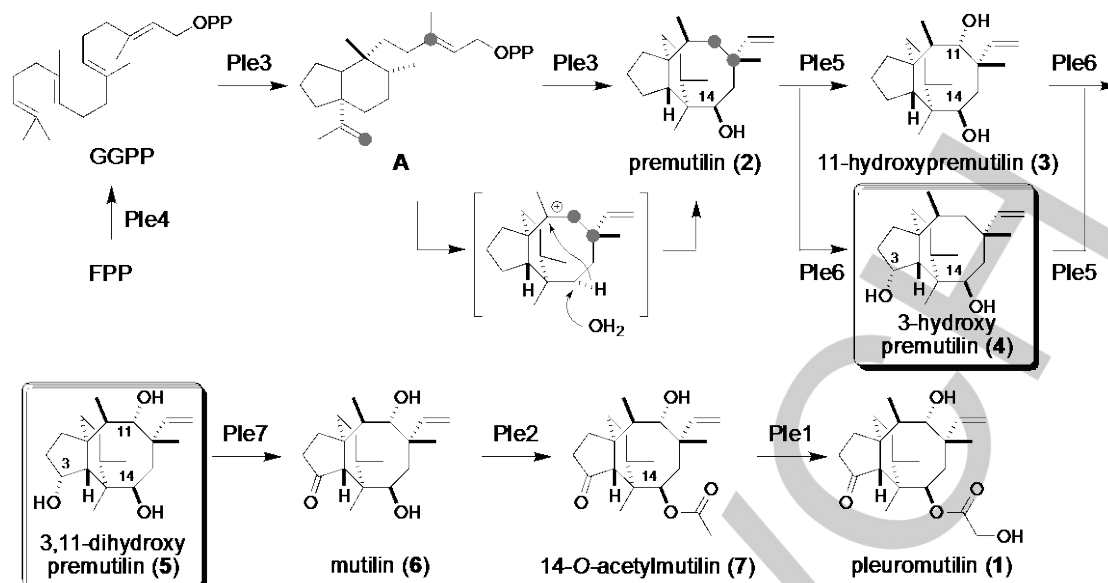
Since the late 1970s, biosynthetic studies of **1** have also been conducted. The biosynthetic pathway was originally proposed by the ETH group on the basis of feeding experiments with ¹⁴C-labeled compounds such as 11-desoxy-3-desoxo mutilin **2** (named as premutilin), 11-hydroxypremutilin **3**, and mutilin **6** (Scheme 2).¹² In 2007, we synthesized ²H-labeled intermediates from **1** and conducted feeding experiments with the **1**-producing strain, *C. pseudo-pinsitus*. Effective incorporation of the deuterium atoms into **1** further confirmed the intermediacy of these compounds.¹³ Recently, the biosynthetic gene cluster of **1** was identified in *Clitopilus passeckerianus*, and reconstitution of the biosynthetic machinery in *Aspergillus oryzae* revealed the involvement of 7 genes in the biosynthesis.¹⁴ However, the function of each enzyme gene remains unclear. Understanding of the detailed biosynthesis would facilitate genetic manipulation to synthesize derivatives of **1**, which represents promising precursors for the semi-synthesis.

In the last few years, we have successfully elucidated the biosynthetic pathways for natural products such as di- and sesterterpenes (aphidicolin, ophiobolin, sesterfisheric acid),¹⁵ indoleterpenes (paxilline, aflatrem, penitrem, and shearinine),¹⁶ polyketides (betaenone and didymellamide B),¹⁷ and ribosomal peptide (ustiloxin)¹⁸ by harnessing the biosynthetic machinery in *A. oryzae*. Artificial reconstitution of the biosynthesis allowed us to isolate biosynthetic intermediates of these natural products. In the present study, we report the isolation and characterization of all biosynthetic intermediates in the biosynthesis of **1**, in combination with in vitro enzymatic reaction and biotransformation experiments, enabling us to propose the detailed biosynthetic pathway.

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Scheme 2. Proposed biosynthetic pathway of **1**: New intermediates, **4** and **5**, isolated in this study are enclosed by solid squares.

We independently conducted draft-genome sequencing of *C. pseudo-pinsitus*. As exemplified in the biosynthetic studies of diterpenes, such as aphidicolin¹⁹ and gibberellin²⁰, a geranylgeranyl diphosphate (GGPP) synthase gene is highly conserved in the biosynthetic gene cluster of diterpenes.²¹ Local BLAST search of the draft-genome sequencing data utilizing GGPP synthase revealed a single cluster (*ple*) possessing a GGPP synthase (*ple4*), a terpene synthase (*ple3*), a cytochrome P450 (*ple1*, *ple5*, and *ple6*), a short-chain dehydrogenase/reductase (*ple7*), and an acyltransferase (*ple2*). The gene organization closely resembled that previously reported (Figure S1).¹⁴

Heterologous expression of biosynthetic genes derived from basidiomycete fungi has generally been examined using corresponding cDNA sequences prepared from the mRNA of the natural product-producing strain.²² Accordingly, we isolated and sequenced cDNA clones of each enzyme genes. Full-length cDNA sequences of *ple2* (1,134 bp), *ple3* (2,880 bp), *ple4* (1,053 bp), and *ple7* (762 bp) indicated that 3 or 4 introns were present. In contrast, the number of introns in three cytochrome P450s, *ple1* (1,572 bp; 10 introns), *ple5* (1,572 bp; 13 introns), and *ple6* (1,578 bp; 11 introns), was much higher than that in the other genes described above (Figure S2). Of particular note, each P450 included 1-4 microexons (Figure S2), of which presence features cytochrome P450 genes from basidiomycete fungi.²³

We then prepared five plasmids (pUSA2-*ple34*, pUARA2-*ple5*, pUARA2-*ple6*, pTASU03-*ple56*, pTASU03-*ple567*, and pUARA2-*ple12*) for heterologous expression in *A. oryzae*. We constructed an *Escherichia coli-Saccharomyces cerevisiae-A.*

oryzae shuttle vector pTASU03 harboring three cloning sites by referring to the plasmid constructed by the Lazarus group (Figure S3).²⁴ Three target genes, *ple5*, *ple6*, and *ple7*, were then introduced into the shuttle vector to give pTASU03-*ple567*. Other plasmids possessing less than two genes were constructed by inserting target genes into either pUARA2 (*argB* marker) or pUSA2 (*sC* marker) vector. Resultant plasmids were then used for transformation of *A. oryzae* NSAR1 to afford AO-*ple34*, AO-*ple5*, AO-*ple6*, AO-*ple56*, AO-*ple567*, and AO-*ple12*.

Using the expected transformants, we conducted in vivo synthesis and biotransformation experiments to characterize the function of each enzyme gene. Semi-synthetic intermediates, such as **2**, **3**, and **6**,¹² and natural products, such as **7** and **1**, isolated from *C. pseudo-pinsitus* were used as standards. We initially focused on a cyclization from a linear precursor GGPP to **2**. GC-MS analysis of the metabolites of AO-*ple34* showed production of a new metabolite with *m/z* 290, whose retention time and mass spectrum were identical to those of authentic premutilin (isolated yield; 37 mg/L in MPY medium) (Figures S4, S5). After purification, the structure was confirmed by ¹H NMR analysis in comparison with premutilin.^{12,13} These experiments proved that *ple3* encodes a premutilin synthase. We then employed in vitro analysis of Ple3. The *ple3* was cloned into the pColdI vector and expressed as an N-terminal His₆-tagged fusion protein. Recombinant Ple3 was purified by Ni-NTA column chromatography (Figure S6). When incubating Ple3 with GGPP in the presence of Mg²⁺, **2** was detected as the sole product (Figure 1 (A)). Similar to other terpene synthases, no cyclization product was detected in the absence of Mg²⁺ (Figure S4).

The amino acid sequence of Ple3 indicated that Ple3 has two catalytic domains, a *N*-terminal class II terpene cyclase domain and a *C*-terminal class I terpene cyclase domain. Similar domain organization was found in labdane-related fungal diterpene synthases, such as *ent*-kaurene synthase (CPS/KS)²⁵ and aphidicolan-16 β -ol synthase (PbACS)²⁶, which catalyze two rounds of cyclization via an *ent*-copalyl diphosphate and a *syn*-copalyl diphosphate, respectively. Each catalytic domain has a conserved aspartate/glutamate-rich motif, a DxDD and DDxxD motif, that is responsible for the cyclization (Figure S7). Mutations in aspartates, indicated by underlining, abolished the enzymatic activity, demonstrating that these are essential for catalysis.²⁷ Corresponding motifs, Dx³¹¹DM and ⁶⁴⁹DDxxD, were conserved in Ple3 (Figure S7), although natural mutation of the third aspartic acid into a methionine was found in the conserved DxDD motif. In order to examine the involvement of each catalytic domain in catalysis, mutational analyses were conducted. Two aspartic acids (D311 and D649) were independently mutated to alanine and the mutants, D311A and D649A, were purified using similar procedures as those employed for wild-type Ple3 (Figure S6). When each mutant was separately incubated with GGPP, no production of **2** was observed by GC-MS analysis (Figure 1 (B) and (C)). On the other hand, the cyclization activity was restored by incubation of both mutants with GGPP (Figure 1 (D)). These results indicated the importance of the aspartate/glutamate-rich motifs for the catalysis of two rounds of cyclization by Ple3. Taking the previous isotope labeling experiments in consideration, compound **A**, which possesses a 5-6 bicyclic system, may represent an intermediate of this cyclization (Scheme 2). To our knowledge, Ple3 is a unique labdane-related diterpene synthase, which catalyzes a ring contraction leading to the 5-6 bicyclic ring system during the catalysis.

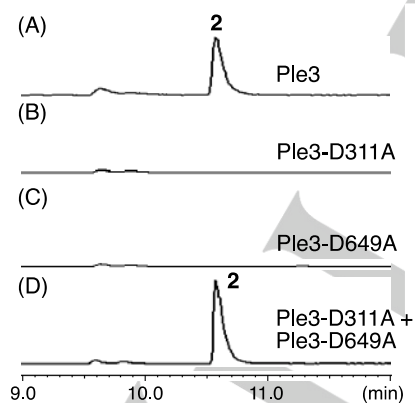


Figure 1. GC-MS profiles (m/z 149) of the enzymatic cyclization: (A) Reaction of Ple3 with GGPP and Mg^{2+} ; (B) Reaction of Ple3-D311A with GGPP; (C) Reaction of Ple3-D649A with GGPP; (D) Coupling reaction of Ple3-D311A and Ple3-D649A mutants with GGPP.

We then examined the oxidative modifications of **2** by biotransformation experiments. When incubating **2** with AO-*ple5*, AO-*ple6*, AO-*ple56*, and AO-*ple567*, we found new metabolites, **3**, **4**, **5**, and **6** by GC-MS analysis (Figures 2 (A)-(C) and S5). Among these, mass spectra and retention times of **3** and **6** were identical to those of standards, 11-hydroxy-premutilin and mutilin respectively. HR-MS analysis revealed the molecular formula of **4** and **5** as $C_{20}H_{34}O_2$ and $C_{20}H_{34}O_3$, respectively. The molecular formula and the 1H -NMR spectrum of **5** showed that **5**

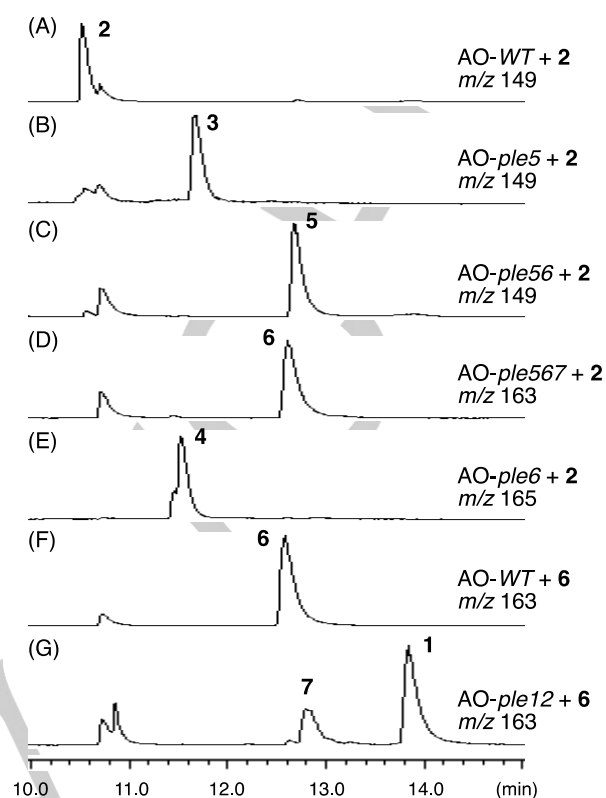


Figure 2. GC-MS profiles: (A) metabolites produced by transformant AO-WT with **2**; (B) AO-*ple5* with **2**; (C) AO-*ple56* with **2**; (D) AO-*ple567* with **2**; (E) AO-*ple6* with **2**; (F) AO-WT with **6**; (G) AO-*ple12* with **6**.

has three oxymethine protons ($\delta_H = 4.53, 4.32,$ and 3.23). The 1H -NMR spectrum was in good agreement with that of a reduction product of **6** with DIBAL-H ($R/S = 10/1$), indicating that **5** is C3,C11-dihydroxy-premutilin. NOE correlation between H3 and H15 showed the stereochemistry at C3 position to be of R configuration. Further extensive NMR analyses, including COSY, HSQC, HMBC, and NOESY, supported the structure (Figure S7). The 1H -NMR spectrum of **4** closely resembled that of **5**, except that only two oxymethine protons ($\delta_H = 4.54$ and 4.20) were observed. Further detailed NMR analysis allowed us to determine its structure as C3-hydroxy-premutilin (Figure S8). The intermediacy of **4** was confirmed by successful biotransformation from **4** to **5** in AO-*ple5* (Figure S9). These results revealed two oxidation pathways from **2** to **5** involved in the biosynthesis. Metabolic grids in the oxidative modification processes have been reported in the biosynthetic pathways of several natural products, including gibberellin,²⁸ trichothecene,²⁹ penitrem,^{16c} and chaetoglobosin.³⁰ It should be noted that Ple6 abstracts the sterically hindered α -hydrogen at C3 of **2/3** (Figure S10).

Late-stage modifications from **6** to **1** were then examined by biotransformation with AO-*ple12*. GC-MS analysis revealed the production of two new metabolites: 14-*O*-acetyl mutilin **7** and **1** (Figures 2 and S5); the chromatographic behavior of both was identical to that of the standards. As only one oxidation enzyme gene, a cytochrome P450 *ple1*, was incorporated into the transformant, Ple1 catalyzed hydroxylation on the alpha position of the acetyl side chain of **7**.

Basidiomycete fungi are prolific producers of biologically active terpenoids such as illudin S (C15; antitumor activity), merulidial (C15; antifungal activity), aleurodiscal (C20; antifungal activity), ganoderic acid β (C30; anti-HIV-1 protease activity), and ganoderadiol (C30; antiviral activity). Database surveys revealed that these fungi have a large number of terpene synthase genes accompanying cytochrome P450 (CYP) genes in their genomes.³¹ These terpene synthases give structurally diverse hydrocarbon skeleton. Following skeletal construction, further oxidative modifications catalyzed by CYPs provide functionalized terpenoids. During the catalysis of oxidation reactions, CYPs receive two electrons from NADPH through a redox partner NADPH-cytochrome P450 oxidoreductase (CPR). Therefore, co-expression of CPR with CYP is necessary for functional analysis of CYP when utilizing *E. coli* and yeast as hosts.^{32,33} In contrast, in the present study, successful biotransformation was observed in the *A. oryzae* transformant without co-expression of CYP and CPR, indicating that the intrinsic CPR (Ao-CPR) in *A. oryzae* is functional to CYPs from basidiomycete fungi, although only two CPRs are found in the genome.³² Notably, Ao-CPR also mediates electron transfer to CYPs from several ascomycete fungi such as *Aspergillus*, *Alternaria*, *Biopolaris*, *Chaetomium*, and *Phoma* species,¹⁷⁻²⁰ suggesting that Ao-CPR is a versatile redox-partner for fungal CYPs.

Recently, reconstitution of biosynthetic machinery in *A. oryzae* has proven a reliable method for the synthesis of natural products from ascomycete fungi. Successful functional characterization of biosynthetic enzyme genes of pleuromutilin, a diterpene natural product from basidiomycete fungi, should facilitate in vivo synthesis of other terpenoids from these fungi in the near future. However, basidiomycete fungi are typically difficult to grow, or cannot be grown at all, under laboratory conditions, thus rendering impossible the preparation of cDNA clones of target enzyme genes. In such situations, a synthetic gene based on a cDNA sequence predicted by bioinformatics tools is required for heterologous expression. Therefore, accurate prediction of the cDNA sequence is crucial. In the case of *C. pseudo-pinsitus*, a public gene prediction program, Augustus,³⁶ provided correct cDNA sequences in *ple2/3/4/7*. However, this program failed to predict the cDNA sequence of CYPs, such as *ple1/5/6*, possibly due to the presence of microexons (Figure S2). These results suggest that careful re-analysis of the predicted sequence is required for CYPs. Currently, the conserved regions/motifs, such as a N-terminal transmembrane region,³⁷ a proline-rich motif (PPGPxxxP motif),³⁸ a ExxR motif in the K-helix region,³⁹ and a heme-binding motif⁴⁰ (Figure S11), may be used for validation. The availability of correct cDNA sequences of functionally active CYPs would provide opportunities for the further improvement of prediction programs.

In summary, we examined functional analyses of all enzyme genes involved in the biosynthesis of pleuromutilin through an approach combining in vitro enzymatic reactions of a labdane-related diterpene synthase Ple3 as well as in vivo biotransformation experiments utilizing *A. oryzae* transformants possessing modification enzyme genes. The present work sets the stage for the enzymatic synthesis of terpenoids isolated from basidiomycete fungi utilizing the *A. oryzae* expression system. Currently, the reconstitution of biosynthetic machinery for these

terpenoids, for which the gene clusters consist of less than 10 genes, is in progress.

Experimental Section

General: All reagents commercially supplied were used as received. ¹H- and ¹³C-NMR spectra were recorded on a Bruker DRX-500 or a JEOL ECA-600 spectrometer. NMR spectra were recorded in CDCl₃ (99.8 atom % enriched, Kanto). ¹H chemical shift was reported in δ value based on internal CHCl₃ (7.26 ppm) as a reference. ¹³C chemical shift was reported in δ value based on chloroform (77.1 ppm) as a reference. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constant (Hz), and integration. Mass spectra were obtained with a JEOL JMS-T100LP (ESI mode). Oligonucleotides for polymerase chain reaction (PCR) were purchased from Hokkaido System Science Co., Ltd. Cell disruption was dealt with an ultrasonic disrupter UR-200P (TOMY SEIKO, Tokyo, Japan). Analysis of the samples during protein purification was performed using SDS-polyacrylamide gel electrophoresis, and the proteins were visualized by using coomassie brilliant blue staining.

Strain and Culture Conditions: *Escherichia coli* HST08 were used for cloning and following standard recombinant DNA techniques. *E. coli* BL21-Gold(DE3) was used for protein expression. *Clitopilus pseudo-pinsitus* ATCC20527, a pleuromutilin producing strain, was used for genomic DNA extraction. *Aspergillus oryzae* NSAR1, a quadruple auxotrophic mutant (*niaD*, *sC*, Δ *argB*, *adeA*), was used for heterologous expression of target genes.

Genomic DNA preparation: Genomic DNA of *Clitopilus pseudo-pinsitus* ATCC20527 was extracted according to the following method; the mycelia of fungus was collected and dried using paper towel. The dried mycelia was frozen in liquid nitrogen and crushed by SK-mill (Tokken). To the frozen powder was added extraction buffer (400 mM of Tris-HCl (pH 8.0), 500 mM of NaCl, 20 mM of ethylenediaminetetraacetic acid (EDTA) and 1% of sodium dodecyl sulfate) and the suspension was kept at room temperature for 5 min. To the suspension was added phenol:chloroform solution and the mixture was vortexed for 2 sec. After incubation at 65°C for 60 min, the reaction mixture was centrifuged at 12000 rpm for 5 min. The supernatant was then treated with RNase at 37°C for 90 min. To the reaction mixture was then added phenol:chloroform solution. After being vortexed for 2 sec, the mixture was centrifuged at 12000 rpm for 5 min. The supernatant was transferred to a new centrifuge tube and re-extracted twice with phenol:chloroform solution followed by chloroform. To the final supernatant was added cold-isopropanol and CH₃COONa solution and genomic DNA was recovered by centrifugation at 12000 rpm for 10 min. The pellet was then washed with 70% ethanol solution and dried for 15 min. Finally, the isolated DNA was resuspended in TE buffer (10 mM of Tris-HCl (pH 8.0) and 1mM of EDTA) and stored at -20°C for further use.

Genome Sequencing and Analysis: Genome sequencing of *C. pseudo-pinsitus* ATCC20527 was performed by Hokkaido System Science Co., Ltd. (Hokkaido, Japan) with an Illumina HiSeq 2000 system. Sequence assembly was performed with Velvet44 version 1.2.08 (<http://www.ebi.ac.uk/~zerbino/velvet/>) to yield 1617 contigs covering approximately 34.6 Mb. Gene prediction was then performed with BlastStation-Local (TM Software, Inc.) for the local BLAST search and 2ndFind (a Web-Based Support Tool to Find Secondary Metabolite Biosynthetic Gene Cluster, <http://biosyn.nih.gov.jp/2ndfind/>) for analysis of each contig.

Accession number: The sequences of genes for betaestacin biosynthesis have been deposited in the DNA Data Bank of Japan (DDBJ) with the accession number LC314149.

RT-PCR analysis: For RT-PCR analysis, *C. pseudo-pinsitus* ATCC20527 was grown on Potato-Glucose (PG) medium for 5 or 13 days at 30°C. Total RNA was extracted from each dried mycelia using

TRIZOL® Reagent (Invitrogen) according to the manufacturer's instructions and then treated with DNase I (Life Technologies) for reverse transcription. Complementary DNA (cDNA) was synthesized with PrimeScript™ II 1st strand cDNA synthesis kit (Takara) using the oligo dT primer according to the manufacturer's instructions.

Ple3 (mutant) cloning and expression in *Escherichia coli*: *ple3* was amplified using cDNA of *C. pseudo-pinositus* as template and primer sets as shown in Supplemental Table S1. The PCR product was directly inserted into the *Pst*I-digested pColdI to generate pColdI-*ple3*. Mutations were introduced into pColdI-*ple3* by PCR using respective primers as described in the Table S1 according to the manufacturer's protocol of PrimeSTAR Mutagenesis Basal Kit (Takara). These plasmids were separately introduced into *E. coli* BL21-Gold(DE3) for overexpression. The transformant was grown at 37°C at an OD₆₀₀ of ~0.6 in 500 mL flask. After cooling at 4°C, isopropyl β-D-thiogalactopyranoside (0.1 mM) was added to the culture. After incubation at 16°C for 17 h, the cells were harvested by centrifugation at 4000 rpm. Harvested cells were resuspended in disruption buffer (100 mM Tris-HCl (pH 8.0), 20% glycerol, 1 mM DTT) and disrupted by sonication. After centrifugation, the supernatant was applied to a Ni-NTA column to purify the expressed protein.

Ple3 Assays: Typical conditions are as follows; a reaction mixture (50 μL of Tris-HCl buffer (pH 8.0)) containing 100 μM of the substrate, 5.0 μM of DTT, 7.5 μM of MgCl₂, 5% glycerol, 1.64 μM of Ple3 was incubated at 30°C. The reaction was quenched by the addition of Hexane (100 μL) and the resultant mixture was vortexed and centrifuged at 12,000 x g. The supernatant was directly analyzed by a GC-MS QP2010 apparatus (Shimadzu, Kyoto, Japan) with a DB-1 MS capillary column (0.32 mm x 30 m, 0.25 μm film thickness; J&W Scientific, Folsom, CA). Each sample was injected onto the column at 80°C in the splitless mode. After isothermal hold at 100°C for 3 min, the column temperature was increased by 14°C min⁻¹ to 268°C. The flow rate of the helium carrier gas was 0.66 mLmin⁻¹.

Construction of a shuttle vector: A 6.1 kb of DNA fragment, containing the *argB* marker and promoter/terminator set, was amplified from pTAex3 vector with primer set as shown in Table S1. Another DNA fragment was prepared by digestion of pSU0 vector⁴¹ with restriction enzymes, BamHI and EcoRI. Co-transformation of these DNA fragments into MaV203 competent yeast cells were conducted according to the manufacturer's instructions of GeneArt High-Order Genetic Assembly System (ThermoFisher Scientific). The assembled DNA, a pTASU01, was recovered from the transformant.

The *PenoA*, *TagdA*, *PgpdA*, and *TamyB* were then amplified from genomic DNA of *A. oryzae* RIB40, *A. nidulans* ATCC58396, and pUARA2 vector with primer set as shown in Table S1. PCR reactions were performed with the KOD-Plus-Neo (TOYOBO). Co-transformation of these DNA fragments and Smal-digested pTASU01 into MaV203 competent yeast cells were conducted according to the manufacturer's instructions of GeneArt High-Order Genetic Assembly System (ThermoFisher Scientific). The assembled DNA, a pTASU03, was recovered from the transformant.

Preparation of expression plasmids: The *ple1-ple7* were amplified from cDNA of *C. pseudo-pinositus* ATCC20527 with primer set as shown in Table S1. PCR reactions were performed with the KOD-Plus-Neo (TOYOBO). Each PCR product was inserted into appropriate restriction site (site 1 and/or site 2) of pUARA2^{16b} or pUSA2^{16b} using In-Fusion Advantage PCR cloning kit (Clontech Laboratories) or NEBuilder HiFi DNA Assembly cloning kit (NEW ENGLAND Biolabs) to construct expression plasmids pUSA2-*ple34*, pUARA2-*ple5*, pUARA2-*ple6*, and pUARA2-*ple12*. Expression plasmids, pTASU03-*ple56* and pTASU03-*ple567*, were constructed by homologous recombination in yeast. The constructed plasmids are summarized in Table S2.

Transformation of *Aspergillus oryzae*: Transformation of *A. oryzae* NSAR1 (1.0 x 10⁸ cells) were performed by the protoplast-polyethylene glycol method reported previously¹⁶ to construct transformants AO-*ple34*, AO-*ple5*, AO-*ple6*, AO-*ple56*, AO-*ple567*, and AO-*ple12*.

Extraction of metabolites. Mycelia of AO-*ple34* was inoculated into a MPY medium (100 mL) in 500 mL Erlenmeyer flask. The culture was incubated at 30°C for 9 days. After extraction with acetone, the extract was concentrated in vacuo to afford crude extracts. The crude extracts was purified with silica gel column chromatography (hexane/EtOAc = 9) to give **2** (37.2 mg). [α]_D²⁷ +13.2 (c 1 CHCl₃). The optical rotation was in good agreement with the reported data.^{12,13}

Biotransformation experiments: Mycelia of *A. oryzae* transformants were inoculated into 4 mL of MPY medium containing appropriate nutrients in 10 or 20 mL Erlenmeyer flasks. Substrate (50-100 μg, mixture of hexane/ethyl acetate) was then administered to the culture medium. After an additional 3 days incubation at 30°C (200 rpm), the fermentation broth was soaked in acetone (4 mL) for 12 hr. After filtration, the filtrate was concentrated in vacuo. The residual water was extracted with EtOAc, and the organic layers were concentrated in vacuo. The crude extracts were passed through a short silica gel column chromatography before GC-MS analysis. The partially purified sample was analyzed by a GC-MS QP2010 apparatus (Shimadzu, Kyoto, Japan) with a DB-1 MS capillary column (0.32 mm x 30 m, 0.25 μm film thickness; J&W Scientific, Folsom, CA) at the same conditions with that for premutilin.

Large scale biotransformation: Mycelia of *A. oryzae* transformant were inoculated into 100 mL of MPY medium containing appropriate nutrients in 500 mL Erlenmeyer flasks. Substrate (1 mg each, total; 10 mg) was then administered to the culture medium. After an additional 3 days incubation at 30°C (200 rpm), the fermentation broth was soaked in acetone (4 mL) for 12 hr. After filtration, the filtrate was concentrated in vacuo. The residual water was extracted with hexane, and the organic layers were washed with sat. NaHCO₃, brine, dried over Na₂SO₄ and concentrated in vacuo.

3-hydroxypremutillin (4): Purification of the crude extracts by silica gel column chromatography (hexane/EtOAc = 2) gave **4** (1.6 mg). [α]_D²⁴ -11.8 (c 1 CHCl₃). APCI-HR-MS: calcd. for C₂₀H₃₄ClO₂ [M+Cl]⁻: 341.2253, found: 341.2255. ¹H and ¹³C NMR data are summarized in Table S3.

3,11-dihydroxypremutillin (5): Purification of the crude extracts by silica gel column chromatography (hexane/EtOAc = 1) gave **5** (4.2 mg). [α]_D²⁵ -23.4 (c 1 CHCl₃). APCI-HR-MS: calcd. for C₂₅H₃₄ClO₃ [M+Cl]⁻: 357.2202, found: 357.2202. ¹H and ¹³C NMR data are summarized in Table S3.

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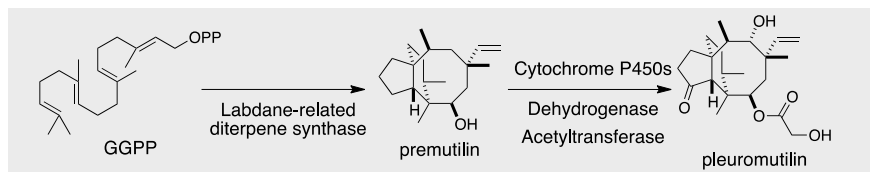
Keywords: Pleuromutilin • Diterpene • Biosynthesis • Heterologous expression • Basidiomycete fungi

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COMMUNICATION



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Chengwei Liu, Taro Ozaki, Ichiro
Takeuchi, Tae Tsukagoshi, Tetsuo
Tokiwano, Katsuya Gomi, and Hideaki
Oikawa*

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**Biosynthetic machinery of diterpene
pleuromutilin isolated from
basidiomycete fungi**

Biosynthetic machinery of pleuromutilin: Biosynthetic pathway of pleuromutilin was elucidated by in vitro enzymatic reactions and biotransformation experiments. Labdane-related diterpene synthase Ple3 and five modification enzymes, three cytochrome P450s (Ple156), a dehydrogenase (Ple7), and an acetyltransferase (Ple2), were functionally characterized.