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Total Biosynthesis of Antiangiogenic Agent (−)-Terpestacin by Artificial Reconstitution of the Biosynthetic Machinery in *Aspergillus oryzae*

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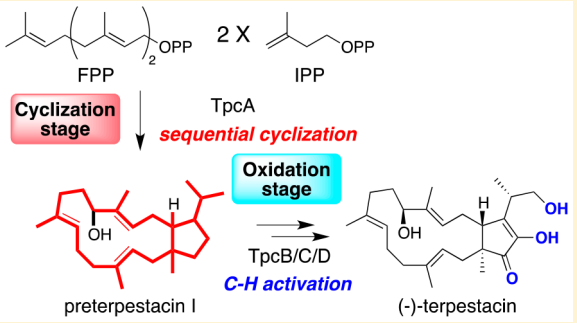
Supporting Information

ABSTRACT: The total biosynthesis of (−)-terpestacin was achieved by heterologous expression of four biosynthetic enzyme genes (*tpcA*−*D*) in *Aspergillus oryzae*. After construction of preterpestacin I by the action of bifunctional terpene synthase (TpcA), two cytochrome P450s (TpcBC) activate inert C−H bond to install three hydroxyl groups on the A-ring in stereo- and regioselective manners. Subsequently, a flavin-dependent oxidase (TpcD) catalyzes oxidation of the vicinal diol moiety to give a α-diketone, which undergoes an enolization to furnish terpestacin. The successful synthesis of structurally elaborated terpestacin showed that a reconstitution approach that harnesses several biosynthetic enzyme genes in *A. oryzae* could be a promising alternative to the current chemical synthesis of natural terpenoids.



INTRODUCTION

The sesterterpene terpestacin (**1**) (Figure 1), originally isolated from *Arthrinium* sp. SF1744 in 1993, is a potent inhibitor of the formation of cellular syncytia, which is one of the major cytopathic effects induced by human immunodeficiency virus (HIV) infection.[1,2] Compound **1** also exhibited antiangiogenic activity by binding to the 13.4 kDa subunit (UQCRB) of mitochondrial complex III.[3] Intriguingly, this antiangiogenic activity was enhanced by combined treatment with **1** and the Ca^{2+}/calmodulin antagonist, HBC.[4] On the other hand, the C24-acetoxy derivative of **1**, fusaproliferin (**2a**) (Figure 1),[5] is known to be a mycotoxin that shows toxic activity against brine shrimp.[6] Besides the toxicity, **2a** is cytotoxic to the lepidopteran cell line SF-9 and the human nonneoplastic B-lymphocyte cell line IARC/LCL 171.[6] Recently, **1** and **2a** demonstrated significant growth inhibition activity against *Alternaria brassicicola*, *Botrytis cinerea*, and *Fusarium graminearum*, suggesting that these sesterterpenes could be allelopathic agents to those phytopathogenic fungi.[7] The diverse biological activities of these structurally related sesterterpenes **1** and **2a** are highly interesting.

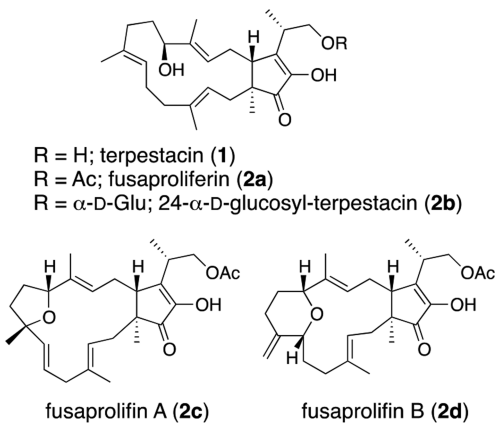


Figure 1. Structures of terpestacin (**1**) and structurally related natural sesterterpenes **2a**−**d**.[5]

Apart from the biological activities of **1** and its derivatives (Figure 1), the unique structure featuring the 5−15 trans-fused ring system and the vicinal carbonyl groups on the A ring have imposed significant challenges for the chemical synthesis. To date, six studies on enantioselective total synthesis and several synthetic studies have been reported.[8−10] The enantioselective total syntheses of (−)-terpestacin/(−)-fusaproliferin by Myers's and Jamison's groups resolved the controversy regarding the absolute configuration of these sesterterpenes.[8,9] On the other hand, the biosynthetic pathways for **1** and **2** remain to be elucidated.

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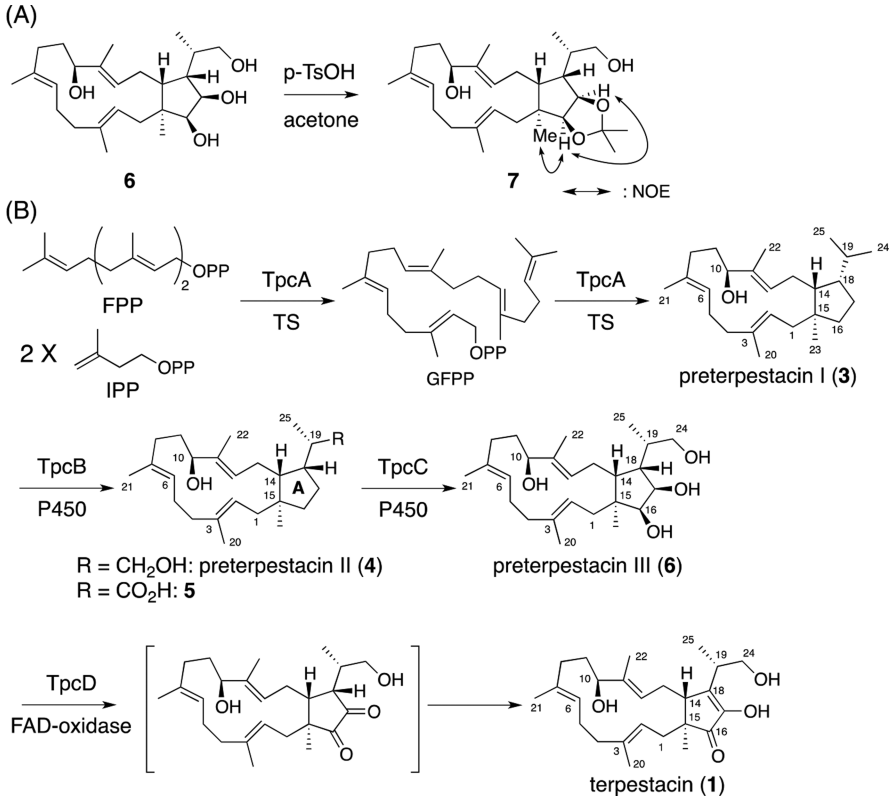
Scheme 1. (A) Key NOE Correlations of Acetonide Derivative 7. (B) Proposed Biosynthetic Pathway of Terpestacin



During the course of genome mining of fungal sesterterpenes, we identified a bifunctional terpene synthase, preterpestacin I synthase (BmTS3), from *Bipolaris maydis*, that catalyzes a chain elongation and a cyclization to afford **3** (Scheme 1).[11] The structure corresponded to the hydrocarbon skeleton of **1**, suggesting that **3** is an intermediate in the biosynthetic pathway to **1**. We postulated that oxidative modifications from **3** to **1** could be catalyzed by enzymes encoded by genes adjacent to *BmTS3* (renamed as *tpcA* in this report). Indeed, two cytochrome P450 genes (*tpcB*: COCHEDRAFT_1223366 and *tpcC*: COCHEDRAFT_1212392) and a single flavin-dependent oxidase gene (*tpcD*: COCHEDRAFT_1171747) are present in this gene cluster (Figure 2, Table S1). Herein, we characterized the function of these modification enzymes and achieved total biosynthesis of **1** by artificial reconstitution of the biosynthetic machinery in *Aspergillus oryzae*.

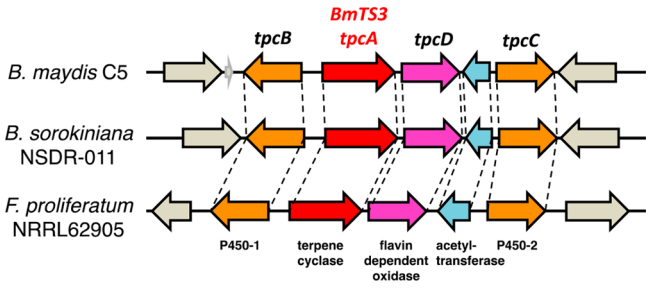


Figure 2. Biosynthetic gene clusters of **1** in *Bipolaris maydis*, *Bipolaris sorokiniana*, and *Fusarium proliferatum*.

RESULTS AND DISCUSSION

Heterologous expression in *A. oryzae* has become a promising method to characterize the function of biosynthetic genes of fungal metabolites. A transformant produces metabolites that reflect the function of the introduced biosynthetic genes, enabling the isolation of all biosynthetic intermediates and the final product. Applying this expression system, the biosynthetic pathways of fungal terpenoids,[12] such as indolediterpenes,[13] meroterpenoids,[14] polyketides,[15] and ribosomal peptide,[16] have been elucidated. We applied this expression system to characterize the function of the putative modification enzyme genes *tpcBCD*. We initially cloned these genes into the expression vectors such as pUARA2 and pUSA2 to prepare three expression plasmids pUSA2-*tpcAB*, pUARA2-*tpcC*, and pUARA2-*tpcCD*. The resulting plasmids were then used for transformation of *A. oryzae* NSAR1 to afford three transformants, AO-*tpcAB*, AO-*tpcABC*, and AO-*tpcABCD*. Those transformants were cultured either in MPY medium supplemented with maltose or in rice medium to induce expression of target genes.

We initially analyzed the metabolites from the quadruple *A. oryzae* transformant AO-*tpcABCD*. LC−MS analysis showed that the transformant produced a new metabolite with m/z 403 ($[M + H]^+$) (11 mg/kg of rice medium) (Figure 3A). HR-MS analysis revealed the molecular formula to be $C_{25}H_{38}O_4$. The NMR data and the optical rotation value of the metabolite were in good agreement with those of **1** (Table 1).[8,9] These results showed that TpcBCD are involved in the oxidative modifications of **3** (renamed as preterpestacin I) to give **1**.

To characterize the function of each enzyme gene, we then analyzed the metabolites from AO-*tpcAB* and AO-*tpcABC*. LC−MS analysis revealed that three new metabolites were produced: preterpestacin II (**4**) and the corresponding

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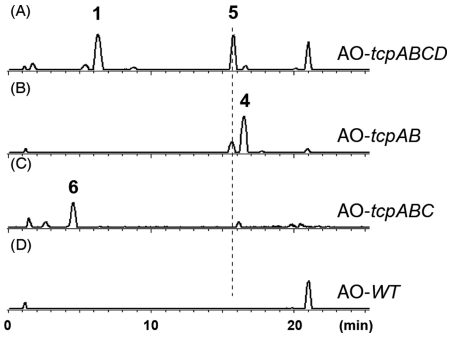

Figure 3. HPLC profiles (m/z 350−410) of metabolites from (A) AO-*tpcABCD*, (B) AO-*tpcAB*, (C) AO-*tpcABC*, and (D) AO-*WT*.

carboxylic acid **5** by AO-*tpcAB* and preterpestacin III (**6**) by AO-*tpcABC* (Figures 3B and 3C). HR-MS analysis revealed the molecular formulas of **4**, **5**, and **6** as $C_{25}H_{42}O_2$, $C_{25}H_{40}O_3$, and $C_{25}H_{42}O_4$, respectively. The ^{1}H NMR spectra of **4** closely resembled that of **3**. The spectrum showed three oxymethine protons (δ_H 3.22 (dd, 1H, H25), 3.62 (dd, 1H, H25), and 3.92 (dd, 1H, H10)), three allylic methyl protons (δ_H 1.54 (s, 3H, C11-Me), 1.57 (s, 3H, C3-Me), and 1.59 (s, 3H, C7-Me)), and two methyl protons (δ_H 0.94 (s, 3H, C15-Me) and 0.98 (d, 3H, C19-Me)) (Table 1), suggesting that a hydroxylation occurs on the isopropyl moiety of **3**. Further 2D-NMR analysis, including COSY, HSQC, HMBC, and NOESY, allowed us to determine its structure as 24-hydroxypreterpstacin I (= preterpestacin II) (Figure S1). Given the fact that AO-*tpcABCD* produced **1**, the hydroxyl group was installed on the pro-*S* methyl group. Comparison of the ^{1}H and ^{13}C NMR spectra for **4** and **5** suggested that **5** has a carboxyl group (δ_C 180.5) instead of the hydroxymethyl group of **4** (Table 1). Extensive 2D-NMR analysis supported this structure (Figure S1). The ^{1}H NMR spectrum of **6** resembled that of **4**, except that two additional oxymethine protons (δ_H 3.64 (m, 1H, H16) and 3.99 (m, 1H, H17)) were observed (Table 1). The COSY spectrum showed correlations between those oxymethine protons and between H18 and the oxymethine proton (H17), suggesting the presence of a vicinal diol moiety on the A ring. This structure was supported by 2D-NMR analysis (Figure S1). NOE correlations between H23 and H16 and between H24 and H17 suggested a cis-configuration of the vicinal diol moiety (Figure S1). The stereochemistries were further confirmed by detecting an NOE correlation between H23 and H16 and between H16 and H17 of the acetonide derivative **7** (Scheme 1A). Overall, the structure of **6** was established as 16,17-dihydroxypreterpestacin II (= preterpestacin III).

Table 1. NMR Spectral Data of 1 and Isolated Compounds

	terpestacin (**1**)			preterpestacin II (**4**)			**5**			preterpestacin III (**6**)	
$CDCl_3$	δ_C	δ_H (multiplicity, J in Hz)	$CDCl_3$	δ_C	δ_H (multiplicity, J in Hz)	C_6D_6	δ_C	δ_H (multiplicity, J in Hz)	$CDCl_3$	δ_C	δ_H (multiplicity, J in Hz)
1	39.5	1.68−1.80 (m)	1	42.6	1.85 (dd, 8.7, 13.5)	1	43.1	1.89 (m)	1	34.7	2.05 (m)
		2.40 (dd, 10.4, 13.7)			2.08 (m)			2.21 (dd, 7.7, 13.4)			2.39 (dd, 8.3, 13.6)
2	121.7	5.25 (dd, 5.2, 10.1)	2	125.2	5.21 (m)	2	125.1	5.36 (t, 7.7)	2	122.7	5.28 (t, 7.7)
3	138.3		3	134.8		3	135.2		3	136.0	
4	40.5	1.90−2.04 (m)	4	39.8	2.05 (m)	4	40.2	2.10 (m)	4	39.9	2.11 (m)
		2.22−2.30 (m)									
5	24.0	2.22−2.30 (m)	5	23.9	2.10 (m)	5	24.1	2.13 (m)	5	24.0	2.11 (m)
											2.19 (m)
6	124.5	5.14 (m)	6	124.8	5.14 (t, 6.2)	6	125.0	5.30 (brt, 5.6)	6	125.4	5.02 (br)
7	133.1		7	132.6		7	133.0		7	132.8	
8	35.1	1.68−1.80 (m)	8	35.1	1.76 (m)	8	35.5	1.90 (m)	8	35.2	1.78 (m)
		2.09−2.12 (m)			1.99 (m)			2.06 (m)			2.04 (m)
9	30.0	1.68−1.80 (m)	9	29.8	1.62 (m)	9	30.5	1.71 (m)	9	29.7	1.61 (m)
					1.76 (m)			1.89 (m)			1.79 (m)
10	76.7	4.07 (dd, 4.0, 10.1)	10	77.0	3.92 (dd, 3.6, 10.2)	10	77.1	4.01 (dd, 3.2, 9.0)	10	77.2	3.94 (dd, 4.0, 9.9)
11	136.7		11	133.7		11	134.8		11	133.7	
12	129.1	5.41 (m)	12	132.3	5.18 (m)	12	131.5	5.30 (brt, 5.6)	12	131.9	5.17 (t, 5.8)
13	29.0	1.90−2.04 (m)	13	23.1	1.92 (t, 5.0)	13	24.4	1.93 (m)	13	25.0	1.94 (m)
		2.45 (d, 17.4)						2.13 (m)			2.05 (m)
14	49.8	2.72 (dd, 2.1, 11.3)	14	45.8	1.76 (m)	14	47.0	2.12 (m)	14	45.5	2.06 (m)
15	49.1		15	46.2		15	46.2		15	46.9	
16	208.0		16	41.2	1.38 (m)	16	41.7	1.36 (m)	16	80.7	3.64 (m)
					1.51 (m)			1.45 (m)			
17	146.8		17	28.0	1.23 (m)	17	27.6	1.02 (m)	17	72.1	3.99 (m)
					1.71 (m)			1.53 (m)			
18	149.0		18	46.5	1.73 (m)	18	47.2	2.33 (m)	18	49.2	2.31 (m)
19	37.3	2.68 (m)	19	36.7	1.64 (m)	19	41.4	2.39 (m)	19	33.8	1.89 (m)
20	15.5	1.64 (s, Me)	20	15.7	1.57 (s, Me)	20	15.8	1.64 (s, Me)	20	15.6	1.64 (s, Me)
21	15.8	1.65 (s, Me)	21	15.3	1.59 (s, Me)	21	15.6	1.58 (s, Me)	21	15.2	1.61 (s, Me)
22	10.7	1.58 (s, Me)	22	10.6	1.54 (s, Me)	22	10.9	1.64 (s, Me)	22	10.4	1.55 (s, Me)
23	16.4	1.01 (s, Me)	23	23.9	0.94 (s, Me)	23	24.2	1.00 (s, Me)	23	21.8	0.97 (s, Me)
24	66.3	3.83 (dd, 5.5, 10.4)	24	67.0	3.22 (dd, 8.2, 10.5)	24	180.5		24	68.8	3.55 (dd, 3.5, 11.1)
		3.90 (dd, 7.0, 10.4)			3.62 (dd, 3.6, 10.5)						3.63 (br)
25	14.6	1.30 (d, Me, 7.3)	25	16.5	0.98 (d, Me, 6.4)	25	17.7	1.12 (d, Me, 6.7)	25	14.4	1.14 (d, Me, 6.9)

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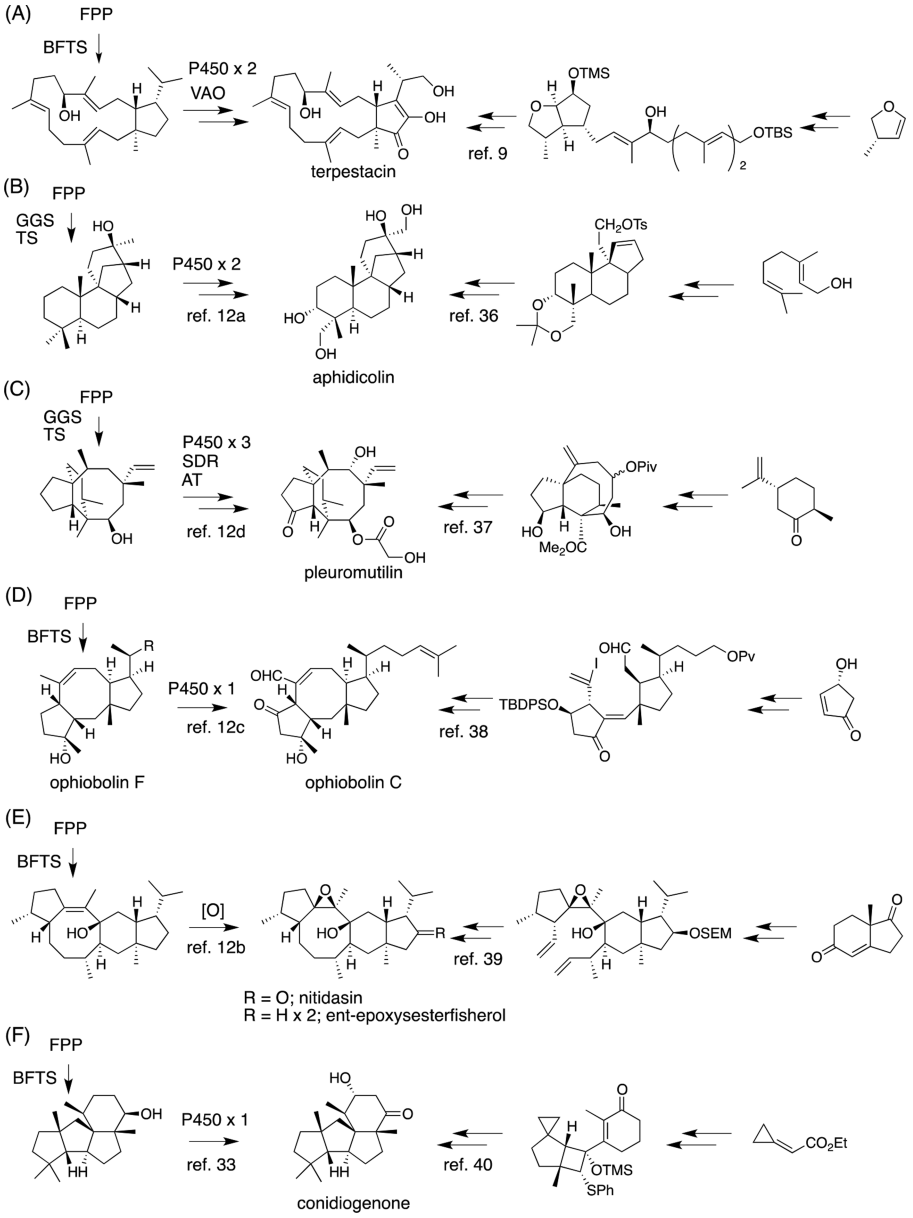

Figure 4. Comparison of synthetic biological and synthetic chemical preparation of (A) terpestacin,[9] (B) aphidicolin,[36] (C) pleuromutilin,[37] (D) ophiobolin C,[38] (E) nitidasin,[39] and (F) conidiogenone.[40] All abbreviations used in this figure are as follows: VAO, vanillyl alcohol oxidase flavoprotein; P450, cytochrome P450; SDR, short-chain dehydrogenase reductase; AT, acyltransferase.

The biosynthetic pathway of **1** was proposed as shown in Scheme 1B. Initially, preterpestacin I was constructed by the action of a bifunctional preterpestacin I synthase TpcA from farnesyl diphosphate (FPP) and two isopentenyl diphosphates (IPPs) via geranylfarnesyl diphosphate (GFPP).[11] The NMR spectrum of **3** showed that two methyl protons on the isopropyl moiety have different chemical shifts,[11] indicating that those methyl groups are not chemically equivalent. TpcB, a cytochrome P450, recognizes the difference and catalyzes a regioselective hydroxylation to afford **4**. Similar pro-*S* selective hydroxylation on the isopropyl side chain catalyzed by a cytochrome P450 is found in the biosynthesis of fusicoccin A.[17] Further oxidation of alcohol **4** gave carboxylic acid **5**. Significant accumulation of **5** only during long-term incubation (2 weeks) of AO-*tpcAB* suggests that **5** is a shunt product generated most likely by the action of an oxidase in *A. oryzae*. Unexpected oxidation of biosynthetic intermediates by *A. oryzae* was also reported in the heterologous production of desmethylbassianin[18] and prosolanapyrone I.[19] On the main biosynthetic pathway, two-step oxidation of **4** catalyzed by TpcC provided **6** possessing a vicinal diol moiety on the A ring. The dihydroxylation occurs on the sterically less-hindered beta-face, possibly due to the presence of neighboring isopropyl (C18 position) and methyl (C15 position) groups on the alpha-face. The iterative action of a single cytochrome P450 to install a vicinal *cis*-diol moiety in a stepwise manner has also been proposed in the biosynthetic pathways of echinocandin B and chaetoglobosin (Scheme S1).[20,21] Finally, TpcD, a flavin-dependent oxidase, catalyzed two rounds of alcohol oxidations to give an α-diketone intermediate that underwent an enolization to furnish **1**.

It should be noted that frequent occurrence of terpestacin in more than 20 fungal strains (*Arthrinium*,[1] *Cleistothelebolus*,[22] *Neogymnomyces*,[22] *Drechslera*,[23] *Ulocladium*,[24] and various

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Fusarium[25]) have been reported in the literature and that homologous gene clusters with *tpc* have been found in terpestacin/fusaproliferin producers such as *A. alternata*,[26] *B. sorokiniana*,[27] and *F. proliferatum*[28] (Figure 2). The wide distribution of the *tpc* gene cluster across species suggested the importance of terpestacin/fusaproliferin for those fungi. Since all of them are known as phytopathogenic fungi, it is likely that those sesterterpenes are involved in disease development in various plants.

Inspired by the logic of the terpene biosynthesis, Baran and co-workers proposed a "two-phase" approach for the synthesis of complex terpenoids.[29] This attractive strategy, involving a cascade cyclization and multiple C−H oxidations, has been successfully applied to the synthesis of highly elaborated terpenoids, such as ingenol[30] and phorbol.[31] In parallel to these achievements, we demonstrated a biochemical "two-phase" approach to synthesize the fungal terpenoids. With fungal genomic data and useful Web tools such as anti-SMASH[32] in hand, we can readily access to the biosynthetic gene clusters of target terpenoids. Usually, the gene clusters consist of a terpene synthase gene and a small number of modification enzyme genes such as those encoding cytochrome P450 monooxygenases. Heterologous expression of these biosynthetic genes with the genomic sequence in fungal hosts such as *A. oryzae* or *A. nidulans* enabled us to synthesize various terpenoids. Indeed, we demonstrated total biosynthesis of diterpenes such as aphidicolin,[12a] pleuromutilin,[12d] and conidiogenone[33] and sesterterpenes such as ophiobilin C,[12c] sesterfisheric acid,[12b] and terpestacin (Figure 4). Despite the complex structure of those terpenoids, functionalizations of the cyclized product are catalyzed by fewer than five modification enzymes, partially due to the multistep oxidations of a single P450, as exemplified in the total biosynthesis of aphidicolin (two steps) and ophiobolin (four steps). Without any optimization, introduced genes are sufficiently active to produce the final products at a level of 10−100 mg/L solely by screening for higher yielding strains. This methodology is quite simple and versatile compared with the corresponding synthetic approach depicted in the Figure 4. As Baran pointed out,[29] the difference between chemical and biochemical transformations is mainly due to oxidative C−H activation. In the case of enzymatic oxidations, the reaction proceeds in regioselective and stereoselective manners, thereby excluding the use of protecting groups.

In 2014, Paddon et al. successfully synthesized the antimalarial agent artemisinin by a chemoenzymatic process; 4 step-enzymatic synthesis generated artemisinic acid in 25 g/L yield, followed by 4 step chemical conversions of the intermediate gave artemisinin in 55% yield.[34] This indicated that improvement of the precursor supply by metabolic engineering and optimization of fermentation conditions dramatically increases the yield of target terpenoids from the laboratory scale to an industrial level, and integration of chemical and biochemical processes significantly improves production. The availability of late intermediates and natural terpenoids may open the door to diversify the natural product libraries by chemical transformation. The accumulated knowledge of P450 for terpenoid modifications may provide guidelines for engineering this useful enzyme as in the case of engineered P450 successfully applied in the total synthesis of nigelladine A.[35]

CONCLUSION

We identified genes for four biosynthetic enzymes, *tpcABCD*, for the antiangiogenic agent terpestacin from *B. maydis*. Stepwise introduction of the biosynthetic genes into *A. oryzae* facilitated isolation of two biosynthetic intermediates, preterpestacin II and preterpestacin III, and the natural product (−)-terpestacin. On the basis of these results, the biosynthetic pathway of terpestacin was firmly established. A database search revealed the wide distribution of homologous genes in several phytopathogenic fungi including terpestacin/fusaproliferin producing strains. The role of terpestacin and/or its derivatives in their phytopathogenesis is highly interesting. Currently, uncharacterized terpene synthase genes accompanying several modification enzyme genes are available in public databases. The global expression of those terpene synthase genes and coexpression of modification genes leads to the production of novel or known biologically active terpenoids.

EXPERIMENTAL SECTION

General Methods. All reagents commercially supplied were used as received. Column chromatography was carried out on 60N silica gel (Kanto Chemicals). Optical rotations were recorded on JASCO P-2200 digital polarimeter. ^{1}H and ^{13}C NMR spectra were recorded on Bruker DRX-500 or Bruker AMX-500 spectrometers (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR). NMR spectra were recorded in $CDCl_3$ (99.8 atom % enriched, Kanto) and C_6D_6 (99.5 atom % enriched, Kanto). ^{1}H chemical shifts were reported in δ based on residual chloroform (7.26 ppm) and benzene (7.15 ppm) as a reference. ^{13}C chemical shifts were reported in δ based on chloroform (77.1 ppm) and benzene (128.0 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. GC−MS analyses were conducted with MS-2010 (Shimadzu). Mass spectra were obtained with a JEOL JMS-T100LP (ESI mode) or a Waters ACQUITY QDa (ESI mode).

Oligonucleotides for polymerase chain reactions (PCRs) were purchased from Hokkaido System Science Co., Ltd. PCRs were performed with a BioRad S1000 thermal cycler.

Strain and Culture Conditions. *Escherichia coli* HST08 was used for cloning and following standard recombinant DNA techniques. A fungal host strain used in this study was *A. oryzae* NSAR1, a quadruple auxotrophic mutant (*niaD*$^{-}$, *sC*$^{-}$, Δ*argB*, *adeA*$^{-}$), for fungal expression.

Construction of *A. oryzae* Expression Plasmids. The genomic DNA of *B. maydis* C5 was prepared using a procedure similar to that in our previous report.[11] The *tpcA*, *tpcB*, *tpcC*, and *tpcD* were amplified from the genomic DNA with primer set as shown in Table S2. 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 or pUSA2 using In-Fusion Advantage PCR cloning kit (Clontech Laboratories) to construct expression plasmids.

Transformation of *A. oryzae*. Transformation of *A. oryzae* NSAR1 (1.0×10^{8} cells) was performed by the protoplast−polyethylene glycol method reported previously to construct the following transformants; AO-*tpcAB*, AO-*tpcABC*, and AO-*tpcABCD*.

Extraction of Oxidative Products from Transformants. Mycelia of transformant, AO-*tpcAB*, AO-*tpcABC*, or AO-*tpcABCD*, were inoculated into a solid medium containing polished rice (100 g) and adenine (10 mg) or MPY medium (3% maltose, 1% polypeptone, 0.5% yeast extract, 100 mL) in 500 mL Erlenmeyer flasks. Each culture was incubated at 30 °C for 10 days. After extraction with $CHCl_3$, the extract was concentrated in vacuo to afford crude extracts. The crude extracts were analyzed by LC−MS with a ZORBAX XDB-C18 column (50 mm × 2.1 mm) at the following condition; solvent: A/B = 50/50 → 0/100, 0−15 min; A/B = 0/100, 15−25 min; A/B = 0/100 → 50/50, 25−26 min; A/B = 50/50, 26−35 min (A: H_2O + 0.1% HCOOH, B: CH_3CN + 0.1% HCOOH) at a flow rate of 0.2 mL/min with 210 nm detection.

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Preterpestacin II (4) Isolated from AO-tpcAB. The crude extracts (299 mg from 1 L of MPY medium) were purified with silica gel column chromatography (hexane/EtOAc = 5:1). Purification of partially purified metabolites with HPLC equipped with Wakopak Navi C18−5 (ϕ 10 × 250 mm) at the conditions (100% acetonitrile at a flow rate of 5.0 mL/min) gave **4** (3.2 mg). $[\alpha]_D^{25}$ −24 (*c* 1.0, $CHCl_3$). ESI-HR-MS: calcd for $C_{25}H_{41}O$ $[M - H_2O + H]^+$ 357.3157, found 357.3188. ^1H NMR and ^{13}C NMR data are summarized in Table S3.

Compound 5 Isolated from AO-tpcABCD. The crude extracts (942 mg from 500 g of rice medium) were purified with silica gel column chromatography (hexane/EtOAc = 1). Purification of partially purified metabolites with HPLC equipped with Wakopak Navi C18−5 (ϕ 10 × 250 mm) at the conditions (100% acetonitrile at a flow rate of 5.0 mL/min) gave **5** (0.9 mg). $[\alpha]_D^{25}$ −13 (*c* 0.42, $CHCl_3$). ESI-HR-MS: calcd for $C_{25}H_{39}O_2$ $[M - H_2O + H]^+$ 371.2950, found 371.2989. ^1H NMR and ^{13}C NMR data are summarized in Table S3.

Preterpestacin III (6) Isolated from AO-tpcABC. The crude extracts (2.2 g from 1 kg of rice medium) were purified with silica gel column chromatography (hexane/EtOAc = 5:1). Purification of partially purified metabolites with HPLC equipped with Wakopak Navi C18−5 (ϕ 10 × 250 mm) at the conditions (100% acetonitrile at a flow rate of 5.0 mL/min) gave **6** (1.2 mg). $[\alpha]_D^{25}$ −15° (*c* 1.0, $CHCl_3$). ESI-HR-MS: calcd for $C_{25}H_{41}O_3$ $[M - H_2O + H]^+$ 389.3056, found 389.3013. ^1H NMR and ^{13}C NMR data are summarized in Table S3.

Terpestacin (1) Isolated from AO-tpcABCD. The crude extracts (383 mg from 200 g of rice medium) were purified with silica gel column chromatography (hexane/EtOAc = 5:1). Purification of partially purified metabolites with HPLC equipped with Wakopak Navi C18−5 (ϕ 10 × 250 mm) at the conditions (100% acetonitrile at a flow rate of 5.0 mL/min) gave **1** (2.2 mg). $[\alpha]_D^{25}$ −21 (*c* 0.1, MeOH). ESI-HR-MS: calcd for $C_{25}H_{39}O_4$ $[M + H]^+$ 403.2848, found 403.2829. ^1H NMR and ^{13}C NMR data (Supporting Information) are in good agreement with the reported data.[11]

Derivatization of Preterpestacin III (6). To a solution of **6** (1.2 mg, 3.0 μmol) in acetone (1.0 mL) was added *p*-TsOH (0.5 mg) in 0 °C, and the reaction mixture was stirred for 50 min. After addition of satd $NaHCO_3$, the reaction mixture was extracted with EtOAc. The organic layers were concentrated in vacuo. The crude extracts were purified with HPLC (Wakopak Navi C18−5 (ϕ 4.6 × 250 mm), 100% acetonitrile at a flow rate of 1.0 mL/min) to give **7** (0.70 mg, 1.6 μmol, 53%). ^1H NMR (500 MHz, $CDCl_3$): δ 5.23 (t, J = 8.1 Hz, 1H), 5.17 (t, J = 5.4 Hz, 1H), 5.12 (brt, J = 6.1 Hz, 1H), 4.34 (t, J = 6.5 Hz, 1H), 4.30 (d, J = 7.8 Hz, 1H), 3.93 (dd, J = 3.8 Hz, 10.3 Hz, 1H), 3.59 (dd, J = 3.8 Hz, 10.6 Hz, 1H), 3.33 (dd, J = 7.8 Hz, 10.6 Hz, 1H), 2.00−2.23 (m, 10H), 1.91 (m, 1H), 1.79−1.83 (m, 2H), 1.65 (m, 1H), 1.63 (s, 3H), 1.62 (s, 3H), 1.57 (s, 3H), 1.50 (s, 3H), 1.32 (s, 3H), 1.14 (d, J = 6.6 Hz, 3H), 1.11 (s, 3H). ESI-HR-MS: calcd for $C_{28}H_{45}O_3$ $[M - H_2O + H]^+$ 429.3369, found 429.3394.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.7b03220.

NMR data (PDF)

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Notes

The authors declare no competing financial interest.

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