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Biosynthesis of Steroidal Antibiotic Fusidanes: Functional Analysis of Oxidosqualene Cyclase and Subsequent Tailoring Enzymes from *Aspergillus fumigatus*

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Abstract: Three putative oxidosqualene cyclase (OSC) genes exist in the genome of the fungus *Aspergillus fumigatus* that produces a steroidal antibiotic, helvolic acid. One of these genes, *Afu4g14770*, designated *AfuOSC3*, is clustered with genes of cytochrome P450 monooxygenases (P450s), a short-chain dehydrogenase/reductase (SDR), and acyltransferases, which presumably function in triterpene tailoring steps, suggesting that this gene cluster codes for helvolic acid biosynthesis. *AfuOSC3* was PCR amplified from *A. fumigatus* IFO8866 genomic DNA and expressed in yeast. The yeast transformant accumulated protosta-17(20)*Z*,24-dien-3β-ol, an established precursor for helvolic acid. Its structural isomer, (20*R*)-protosta-13(17),24-dien-3β-ol, was also isolated from the transformed yeast. To further identify the function of triterpene tailoring enzymes, four P450 genes (*CYP5081A1*−*D1*) and a SDR gene (*AfuSDR1*) in the cluster were each coexpressed with *AfuOSC3* in yeast. As a result, coexpression of AfuSDR1 gave a 3-keto derivative of protostadienol. On the other hand, coexpression with CYP5081A1 gave protosta-17(20)*Z*,24-diene-3β,29-diol and protosta-17(20)*Z*,24-dien-3β-ol-29-oic acid. These metabolites are in well accord with the oxidative modification involved in helvolic acid biosynthesis. AfuSDR1 and CYP5081A1 presumably function together to catalyze demethylation of C-29 methyl group. These results provided a firm ground for identification of the present gene cluster to be involved in helvolic acid biosynthesis.

Introduction

Triterpenoids are a large class of natural products mainly present in higher plants. Extensive pharmacological studies revealed their important biological activities. For example, ginsenosides, tetracyclic dammarene-type triterpene saponins of *Panax ginseng*, show interesting activities including central nervous system-stimulating (or -suppressing) and anticancer activities,[1] while betulinic acid, a pentacyclic lupane-type triterpene of *Betula platyphylla*, shows anticancer and anti-HIV activities.[2] Triterpene skeletons derive from a common precursor, (3*S*)-2,3-oxidosqualene, via a cyclization reaction catalyzed by oxidosqualene cyclase (OSC). OSCs catalyze one of the most fascinating chemical reactions found in nature, producing multiple fused ring systems with numerous stereogenic centers in a single transformation involving carbocationic intermediates.[3] Including the precursors for sterols, over 100 different types of triterpene skeletons are known from natural sources.[4] Recent studies have led to the identification and characterization of a number of OSC genes. In addition to OSCs responsible for the biosynthesis of sterol precursors, lanosterol (fungi and animals) and cycloartenol (plants), they include OSCs for plant-specific triterpenes.[5]

Although plants are the major producers of bioactive triterpenes, some microorganisms are known to produce unique triterpenes. Bacteria and archaea produce hopanoids, which directly derive from squalene via squalene-hopene cyclase (SHC).[3] They utilize these hopanoids as membrane component, just like a surrogate of cholesterol in mammals. Yet most of these microorganisms, with a few exceptions,[6] lack OSC genes and do not produce any other triterpenes. On the other hand, eukaryotic microorganisms, such as yeast, filamentous fungi, and mushrooms, possess lanosterol synthase in common for ergosterol biosynthesis. Besides, a few of them produce triterpenes other than lanosterol.[7] Fusidanes, steroidal antibiotics produced by several fungal species including *Cephalosporium caerulens*, *Fusidium coccineum*, and *Aspergillus fumigatus*, are one such example.[8,9] A tetracyclic ring system of fusidanes is a representative of protostane-type triterpene and takes an ABC-

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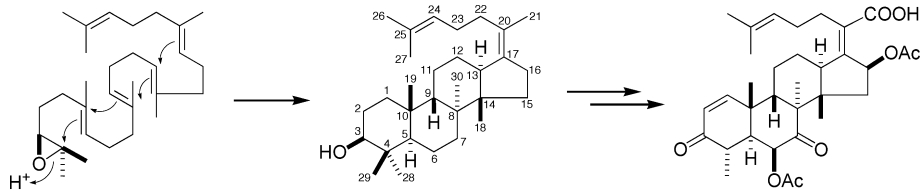
Scheme 1. Biosynthetic Pathway of Helvolic Acid[a]



[a] Carbon numbering is shown in the structure of **1**.

ring chair−boat−chair conformation with a number of oxidative modifications. The presence of a carboxylic acid-bearing side chain is another feature of fusidanes that include helvolic acid, cephalosporin P_1, and fusidic acid. The precursor of fusidanes is supposed to be protosta-17(20)*Z*,24-dien-3β-ol (**1**), which is formed through cyclization of 2,3-oxidosqualene folded in pre chair−boat−chair conformation (Scheme 1). After formation of a tetracyclic protosteryl cation, the reaction terminates by loss of H-17α proton without any methyl and/or hydride shifts.[10] Except for lanosterol synthase and cycloartenol synthase for sterol biosynthesis, cucurbitadienol synthase is the only identified OSC that involves protosteryl cation as an intermediate.[11] So far, fungal OSC producing triterpenes other than lanosterol has not been identified yet.

Search for such OSC in fungi may have additional benefit to identify genes involved in triterpene tailoring steps, such as oxidation, acylation, and glycosylation, mainly because genes involved in secondary metabolism are generally clustered in the genomes of microorganisms. For the formation of mature bioactive triterpenes, these modifications are essential, and even a single hydroxyl group introduction to the OSC products could lead to a dramatic enhancement of biological activity, as exemplified by protopanaxadiol.[2] To obtain more comprehensive understandings of the biosynthesis of bioactive triterpenes, cloning of genes involved in such tailoring steps is highly desired. Besides, these genes are necessary for the engineered production of bioactive triterpenes in heterologous hosts such as in yeast. In contrast to the rapid progress in the studies on OSC genes involved in skeletal formation, however, little is known about the subsequent tailoring reactions. Until now, CYP93E1, a cytochrome P450 monooxygenase (P450) of *Glycine max*,[12] CYP88D6 of *Glycyrrhiza uralensis*,[13] and UGT74M1, a family 1 glycosyltransferase of *Saponaria vaccaria*,[14] have been the only examples of hydroxylases and a glycosyltransferase acting on triterpene substrates, respectively. Difficulty in identifying triterpene tailoring enzymes from plants is mainly attributed to the fact that these genes comprise large gene families in plants. For example, *Arabidopsis thaliana* genome contains up to 246 P450 genes[15] and 112 family 1 glycosyltransferase genes,[16] many of them being expected to be involved in secondary metabolism.

Recently, the whole genome sequences of several filamentous fungi including *A. fumigatus*, a helvolic acid producer,[17] have become available. By searching and identifying OSC genes, the discovery of triterpene tailoring genes in the vicinity is highly expected. Here, we report the discovery of the helvolic acid biosynthetic gene cluster from *A. fumigatus* through functional identification of a protosta-17(20)*Z*,24-dien-3β-ol synthase, and two tailoring gene products, a short-chain dehydrogenase/reductase (SDR) family member and a cytochrome P450, responsible for the oxidative modification of triterpene skeleton, by rigorous structural analysis of the enzymatic products.

Results

Search for Protostadienol Synthase from *A. fumigatus* Genome Database. *A. fumigatus* is known as a producer of helvolic acid.[18] Since the total genome sequence of *A. fumigatus* has become available,[17] a whole gene set for helvolic acid biosynthesis could be found if the relevant genes are clustered. From the structure of helvolic acid, the cluster is predicted to include the genes for several oxygenases and acetyltransferases in addition to an OSC. Therefore, we first searched for OSC genes in the genome. Three OSC genes, *Afu4g12040*, *Afu4g14770*, and *Afu5g04080*, were found by database search using known OSC genes from plants as a query. *Afu4g12040* and *Afu5g04080* show high sequence identity (55−63%) to the only one known fungal lanosterol synthase OSLC from *C. caerulens*.[19] These genes are not accompanied by any tailoring genes in the vicinity of the genome. Therefore, these genes may code for lanosterol synthase required for ergosterol biosynthesis in *A. fumigatus*. On the other hand, *Afu4g14770*, which we denoted *AfuOSC3*, shows significant but lower sequence identity (ca. 40%) to fungal lanosterol synthase genes including the above two. Moreover, putative cytochrome P450 and acyltransferase genes were found clustered with this OSC gene on the genome (Figure 1 and Table 1). This putative gene cluster expands 16.3 kb on the chromosome 4 and contains 9 genes in total: four putative P450s, two putative acyltransferases, one putative SDR, and one putative 3-ketosteroid Δ^1-dehydrogenase (KSTD) (Table 1). Furthermore, upstream of the OSC gene is located an ABC transporter-like gene (*Afu4g14760*), which may be involved in sequestering the antibiotic out of the cells. On the other hand, no other protein encoding sequence was found downstream of the putative KSTD gene for up to 15 kb,

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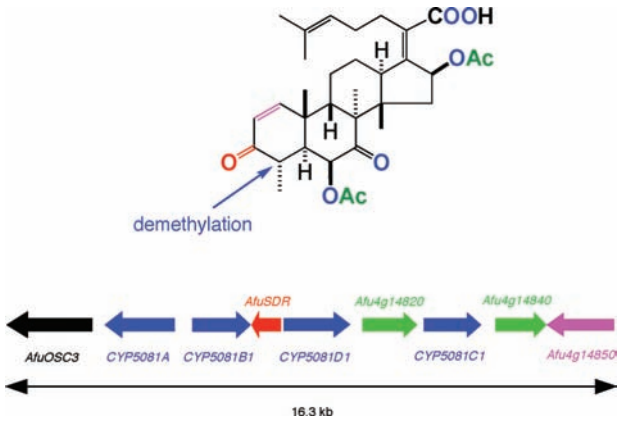

Figure 1. Gene organization of the putative helvolic acid biosynthetic gene cluster from *A. fumigatus*. Black arrow: OSC gene. Blue arrow: P450 genes. Red arrow: SDR gene. Green arrow: acyltransferase genes. Pink arrow: KSTD gene. Each of the coded enzymes are predicted to install the indicated functionalities (written with the same color) of helvolic acid shown above.

suggesting that the aforementioned 9 genes may define the complete set of the cluster. The composition of the cluster well matches the structure of helvolic acid (Figure 1). Especially noteworthy is the existence of a gene homologous to KSTD. This gene was first reported in bacteria to be responsible for degradation of steroids by introduction of an olefin at C1−C2 position.[20,21] The same Δ^1 structure can be found in helvolic acid with 3-keto functionality. All of this evidence points to that this gene cluster is responsible for helvolic acid biosynthesis. Therefore, we first set out a functional analysis of this OSC gene (*AfuOSC3*).

Functional Expression of AfuOSC3 in Yeast. The whole genome sequence is now available for *A. fumigatus* strain Af293, which is not known for helvolic acid productivity. Initial PCR was carried out with genomic DNA from *A. fumigatus* IFO8866. This strain is not known for helvolic acid productivity as well. This PCR amplified the 2.2 kb full length *AfuOSC3* without intron whose sequence was completely identical to the database sequence for Af293 strain. The full length *AfuOSC3* was cloned into yeast expression vector pESC(Ura) under the control of *GAL10* promoter to produce pESC-*AfuOSC3* as described before.[22] With this plasmid, a lanosterol synthase-deficient *Saccharomyces cerevisiae* strain GIL77 was transformed. After induction and resting cultures, the transformant cells were harvested, lysed, and extracted with hexane. The extract was analyzed by silica gel TLC to reveal a transgene specific spot at the R_f value of triterpene monoalcohols. This spot was further analyzed by GC−MS. In GC, two peaks in a ratio of about 3:1 were observed (Figure 2A). Both of them gave molecular ion peaks at *m*/*z* 426 in EI−MS, demonstrating them to be triterpenes. From a large-scale culture (3 L), 8 mg of triterpene mixture was obtained by silica gel column and further separated on $AgNO_3$ impregnated silica gel TLC to yield 6 mg of the major and 2 mg of the minor product. The ^{1}H NMR spectrum (in $CDCl_3$) of the major compound revealed 8 singlet methyls (3 vinylic and 5 aliphatic), a proton (δ 3.25, dd, J = 12.0, 5.0 Hz) characteristic for 3α-H, and an olefinic proton at δ 5.11. Extensive NMR analyses (^{1}H, ^{13}C, HMBC, and HMQC) established the structure of the major product to be **1** with complete ^{13}C assignment (Table 2). This product with characteristic *Z* configuration at $\Delta^{17(20)}$ has been isolated as a presumed biosynthetic precursor of helvolic acid from helvolic acid producers, *C. caerulens* and *F. coccineum*,[23] although detailed NMR characterization was lacking. The minor product, on the other hand, showed 6 aliphatic methyl signals with one as a doublet (δ 0.95, J = 7.5 Hz), and 2 vinylic methyls in the ^{1}H NMR spectrum. Careful comparison of the spectrum with that of **1** suggested it to have Δ^{13} structure, an olefinic isomer of **1**. Extensive NMR characterizations confirmed its structure to be (20*R*)-protosta-13(17),24-dien-3β-ol (**2**) with complete ^{13}C assignment except for two positions (Table 2). This minor product was also isolated from helvolic acid producing fungi *C. caerulens* and *F. coccineum* again with no detailed NMR data.[23] Although the configuration of C-20 could not be determined by NMR alone, we deduced it to be 20*R* on the basis of rationale of the cyclization mechanism described below. These results characterized AfuOSC3 as a protostadienol synthase producing **1** as a precursor of helvolic acid, together with its olefin isomer **2** (Scheme 2). Previous isolation of **2** from helvolic acid producing fungi supports that **2** was not produced by an aberrant expression of AfuOSC3 in yeast but rather represents the native function of this OSC.

During the course of this structural study, we observed the presence of **2** in a stored NMR sample (at 4 °C in $CDCl_3$) of **1**. We suspected that acid-catalyzed olefin isomerization might have taken place. To test this, purified **1** was dissolved in $CHCl_3$, a few drops of concentrated HCl were added, and the mixture was stirred at room temperature for 24 h. As a result, **1** was isomerized into **2** (Supporting Information). Facile isomerization of **1** into **2** under acidic conditions indicates **2** to be more thermodynamically stable than **1**. It is quite remarkable to note that this isomerization takes place stereospecifically producing only 20*R* isomer and none of 20*S*. A similar isomerization of $\Delta^{17(20)}$ into Δ^{13} was reported under catalytic hydrogenation condition (H_2/Pd−C) of **1**.[23] To test whether **2** is an artifact produced during extraction or not, once-purified **1** was exposed under identical extraction procedure to detect none of **2** on GC−MS analysis, indicating that **2** most likely is an enzymatic product of AfuOSC3 and not an artifact.

Functional Analysis of AfuSDR1. With the AfuOSC3 function being identified, the cluster does now seem to code for genes involved in helvolic acid biosynthesis. Therefore, we then turned our attention to identify genes involved in successive tailoring steps. Apparently, the next step after formation of **1** should be the oxidative step introducing either hydroxyl, carboxylic acid, or ketone functionality. Plausible candidate enzymes catalyzing these reactions could be the products of the four P450 genes and the one SDR gene of the cluster. To test this, these five genes were each coexpressed with AfuOSC3 in yeast GIL77 to identify any metabolite derived from **1**. The full length SDR gene (*Afu4g14800*), which we designated *AfuSDR1*, was again amplified from *A. fumigatus* IFO8866 genomic DNA and introduced into pESC to produce pESC-*AfuOSC3,AfuSDR1*. The sequence from this amplificaton contained no intron and matched perfectly with the database sequence for Af293 strain. Coexpression of AfuSDR1 with AfuOSC3 in GIL77 gave a

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Table 1. Putative Function of Each Gene in the Cluster

gene name	putative function	homologous gene with known function (organism) [NCBI no.]	% identity
Afu4g14770 (*AfuOSC3*)	oxidosqualene cyclase	lanosterol synthase (*Cephalosporium caerulens*) [AF327881]	40
Afu4g14780 (*CYP5081A1*)	cytochrome P450	*TRI11* (*Fusarium sporotrichioides*) [AF011355]	23.9
Afu4g14790 (*CYP5081B1*)	cytochrome P450	*bcaba2* (*Botrytis cinerea*) [AJ851088]	22.9
Afu4g14800 (*AfuSDR1*)	short-chain dehydrogenase/reductase	*Bt-adh* (*Bacillus thermoleovorans*) [AB040809]	28.1
Afu4g14810 (*CYP5081D1*)	cytochrome P450	*TRI4* (*Fusarium sporotrichioides*) [FSU22462]	22.8
Afu4g14820	acyltransferase	AsHHT (*Avena sativa*) [AB076980]	20.6
Afu4g14830 (*CYP5081C1*)	cytochrome P450	*TRI11* (*Fusarium sporotrichioides*) [AF011355]	25.4
Afu4g14840	acyltransferase	*TRI101* (*Fusarium sporotrichioides*) [AY032747]	20.2
Afu4g14850	3-ketosteroid-Δ^1-dehydrogenase	*TesH* (*Comamonas testosteroni*) [AB076368]	36.2

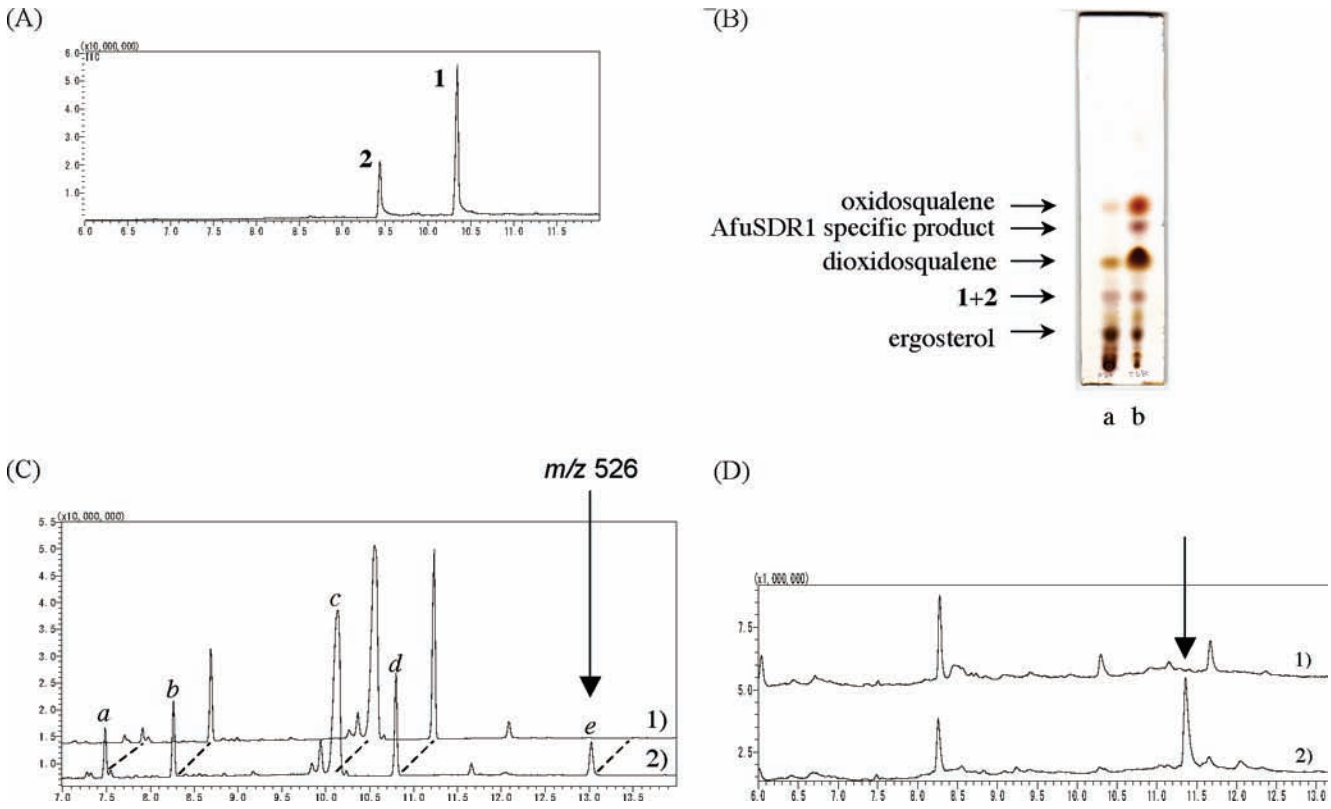


Figure 2. Functional expression of AfuOSC3, AfuSDR1, and CYP5081A1 in yeast. (A) GC−MS total ion chromatogram of AfuOSC3 triterpene products. Both peaks gave molecular ion peaks at *m*/*z* 426. Retention time is shown in min on the horizontal axis. (B) TLC analysis of hexane extract from mycelia expressing AfuOSC3 and AfuSDR1. Lane a, pESC-*AfuOSC3*; lane b, pESC-*AfuOSC3,AfuSDR1*. Products are labeled on the left. TLC was developed with hexane:ethylacetate = 9:1 and heated after spraying with 10% H_2SO_4. (C) GC−MS total ion chromatogram of extract from mycelia expressing AfuOSC3 and CYP5081A1 after acetylation. (1) pESC-*AfuOSC3*, (2) pESC-*AfuOSC3,CYP5081A1*. Product *e* gave *m*/*z* = 526, which was only seen in (2). *a*, oxidosqualene; *b*, dioxidosqualene; *c*, ergosterol acetate; *d*, 3β-acetoxy-protosta-17(20)*Z*,24-diene. Retention time is shown in min on the horizontal axis. (D) GC−MS total ion chromatogram of extract from resting culture medium expressing AfuOSC3 and CYP5081A1 after methylation. (1) pESC-*AfuOSC3*, (2) pESC-*AfuOSC3,CYP5081A1*. The peak with an arrow gave *m*/*z* = 470, which was only seen in (2). Retention time is shown in min on the horizontal axis.

product specific to *AfuSDR1* (Figure 2B) in addition to **1** and **2**. This new product was less polar than **1** and upon GC−MS analysis showed a molecular ion at *m*/*z* 424 that corresponds to dehydrogenated product. 3 L of culture of this cotransformant yielded 5 mg of the product. NMR analysis indicated the presence of a ketone and disappearance of an oxymethine proton at C-3. By comparison of its NMR data with those of **1**, the structure was established to be protosta-17(20)*Z*,24-dien-3-one (**3**) with complete chemial shift assignment except for two positions (Table 2, Figure 3). This metabolite has never been reported and is a novel natural compound. It should be noted here that AfuSDR1 did not dehydrogenate **2**, a minor product of AfuOSC3. AfuSDR1 is thus a specific dehydrogenase of **1**to produce a 3-ketone, which is well accommodated in the helvolic acid biosynthetic pathway. This is the first characterization of any dehydrogenase that catalyzes the oxidation of triterpenes.

Functional Analysis of CYP5081A1. We then examined whether any one of the four P450s in the cluster would catalyze the oxygenation of **1**. All four genes are classified in a unique CYP5081 family (A1−D1) (http://drnelson.utmem.edu/CytochromeP450.html) with no other members among fungal P450 families. These P450s exhibit 43−49% sequence identity to each other, indicating a close evolutional relationship among them. Careful examination of the annotated sequence in the genome revealed that *CYP5081A1* (*Afu4g14780*) has a longer N-terminal sequence of ca. 50 residues as compared to the other three P450s (Supporting Information). As microsomal P450s generally contain a stretch of hydrophobic residues as membrane anchor followed by a basic residue such as Arg or Lys and then by a conserved Pro-rich region in the N-terminal portion,[24] we suspected that Met-52 from the annotated N-terminus might be the true start of a polypeptide. Therefore, the construct of

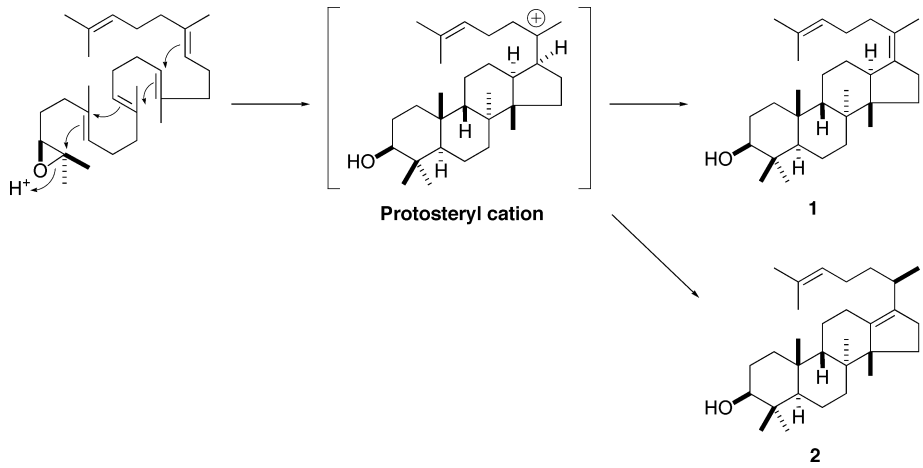
Table 2. ^{1}H and ^{13}C NMR Chemical Shift Assignments for **1**−**5**[a]

	1		**2**		**3**	
position	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1		32.91		33.84		31.34
2		29.12		28.93	2.28 (1H, ddd, J = 16.0, 10.0, 3.0 Hz), 2.67 (1H, ddd, J = 16.0, 12.5, 6.0 Hz)	*(33.71 or 33.74)
3	3.25 (1H, dd, J = 12.0, 5.0 Hz)	79.32	3.22 (1H, dd, J = 12.0, 5.0 Hz)	79.25		220.48
4		39.16		39.31		47.01
5	1.46 (1H, m)	47.55	1.39 (1H, m)	47.61	2.15 (1H, m)	46.99
6		18.31		18.37	1.42 (1H, m), 1.20 (1H, m)	19.82
7		34.69		33.33		33.89
8		38.85		40.11		38.98
9	1.52 (1H, m)	45.29	1.62 (1H, m)	47.20	1.56 (1H, m)	42.87
10		36.70		36.83		35.98
11		22.87		23.06		22.47
12		27.23		*(23.27 or 28.67)		27.04
13	2.30 (1H, d, J = 12.0 Hz)	46.80		139.01	2.32 (1H, d, J = 12.0 Hz)	46.81
14		50.58		57.42		50.39
15		30.29		30.95		30.31
16		29.25		*(23.27 or 28.67)		29.19
17		136.74		135.25		136.56
18	0.75 (3H, s)	16.92	1.05 (3H, s)	22.61	0.78 (3H, s)	16.81
19	0.93 (3H, s)	22.59	0.96 (3H, s)	23.96	0.80 (3H, s)	23.49
20		126.36	2.45 (1H, m)	31.58		126.66
21	1.58 (3H, brs)	20.81	0.95 (3H, d, J = 7.5 Hz)	20.07	1.59 (3H, m)	20.84
22		33.69	1.29 (2H, m)	35.67	2.06 (1H, m), 1.99 (1H, m)	*(33.71 or 33.74)
23	1.95 (1H, m), 2.04 (1H, m)	28.05		26.34	2.07 (1H, m), 1.90 (1H, m)	28.04
24	5.11 (1H, m)	124.68	5.07 (1H, m)	124.98	5.11 (1H, m)	124.63
25		131.13		130.95		131.18
26	1.68 (3H, s)	25.76	1.67 (3H, s)	25.73	1.68 (3H, s)	25.76
27	1.60 (3H, s)	17.66	1.56 (3H, s)	17.60	1.60 (3H, s)	17.67
28	0.99 (3H, s)	29.06	0.98 (3H, s)	28.87	1.07 (3H, s)	29.40
29	0.79 (3H, s)	16.08	0.80 (3H, s)	15.99	1.04 (3H, s)	19.54
30	1.12 (3H, s)	22.02	0.94 (3H, s)	22.91	1.18 (3H, s)	21.53

	4		**5**	
position	^{1}H	^{13}C	^{1}H	^{13}C
1	1.43 (1H, m)	32.49	1.47 (1H, m)	32.59
2		29.44		29.81
3	3.47 (1H, m)	80.84	3.19 (1H, m)	78.37
4		43.59		48.30
5	1.56 (1H, m)	48.33	1.53 (1H, m)	50.06
6		18.48		20.53
7	1.16 (1H, m), 1.97 (1H, m)	35.04	1.18 (1H, m), 2.00 (1H, m)	35.27
8		38.83		38.82
9	1.48 (1H, m)	45.20	1.59 (1H, m)	44.15
10		36.42		37.03
11		22.83		23.02
12		27.15		27.09
13	2.29 (1H, d, J = 12.0 Hz)	46.74	2.30 (1H, d, J = 10.5 Hz)	46.72
14		50.58		50.51
15	1.12 (1H, m), 1.45 (1H, m)	30.27	1.14 (1H, m), 1.45 (1H, m)	30.27
16		29.22		29.20
17		136.60		136.59
18	0.74 (3H, s)	16.91	0.77 (3H, s)	16.82
19	0.89 (3H, s)	23.15	0.91 (3H, s)	20.13
20		126.46		126.54
21	1.58 (3H, m)	20.81	1.58 (3H, m)	20.83
22	2.06 (1H, m)	33.69	2.06 (1H, m)	33.71
23	1.97 (1H, m), 2.05 (1H, m)	28.04	1.97 (1H, m), 2.05 (1H, m)	28.05
24	5.10 (1H, m)	124.65	5.10 (1H, m)	124.64
25		131.17		131.19
26	1.68 (3H, s)	25.76	1.68 (3H, s)	25.76
27	1.60 (3H, s)	17.67	1.60 (3H, s)	17.68
28	1.22 (3H, s)	23.15	1.46 (3H, s)	24.26
29	3.29 (1H, dd, J = 11.5, 9.0 Hz, *pro-S*), 4.22 (1H, dd, J = 11.5, 2.5 Hz, *pro-R*)	64.27		181.89
30	1.12 (3H, s)	22.17	1.12 (3H, s)	22.25
29-OH	2.45 (1H, dd, J = 9.0, 2.5 Hz)			

[a] Asterisks on compounds **2** and **3** indicate that the assignments of these carbons cannot be determined.

Scheme 2. Formation of **1** and **2** by AfuOSC3 through Protosteryl Cation Intermediate



CYP5081A1 was made from the corresponding ATG codon. Full length sequence of these four P450 genes was amplified from genomic DNA of strain IFO8866 (none of them contain an intron) and introduced into pESC to produce pESC-*AfuOSC3,CYP5081A1*−*D1*. As for *CYP5081A1*, the sequence obtained was completely identical to the database sequence for Af293 strain. These plasmids were used to transform GIL77 to see any oxygenated product of **1**. Prior to GC−MS analysis, extracts were acetylated (Ac_2O/pyridine) or methylated (TMS-diazomethane) to detect any hydroxylated or carboxylic acid derivatives, respectively. In the acetylated extract of a cotransformant with CYP5081A1, a product with molecular mass of triterpenediol diacetate (*m*/*z* 526) was detected in GC−MS analysis (Figure 2C, peak *e*). Furthermore, from the methylated extract of the same cotransformant, a product with *m*/*z* 470 that corresponds to triterpene monoalcohol mono-oic acid methyl ester was found (Figure 2D). The majority of this product was found in the culture medium. These products were not detected in the cotransformants with the other P450s nor in controls. To elucidate the structure of these oxygenated products, ca. 1 mg of each of the underivatized samples was obtained from a large-scale culture (2 L) by silica gel column chromatography followed by preparative HPLC.

The ^{1}H NMR spectrum of a putative diol compound exhibited carbinol protons at δ 3.29 (dd, J = 11.5, 9.0 Hz) and 4.22 (dd, J = 11.5, 2.5 Hz) in addition to δ 3.47 (m) for 3α-H. Comparison with the spectrum of **1** indicated that methyl signals for C-18, 19, 21, 26, 27, and 30 were retained intact. However, a signal for either C-28 or 29 disappeared, and the other was found shifted downfield to δ 1.22. These results revealed that one of the C-4 methyl groups of **1** was hydroxylated. To aid structure elucidation of a scarce product, [2-^{13}C]acetate was fed to enrich some of the carbons for ^{13}C NMR measurements. According to the conventional mevalonate pathway operating in yeast, all of the methyl groups in triterpenes derive from C-2 of acetate. The ^{13}C NMR spectrum showed the presence of 18 enriched signals as predicted and 12 low intensity signals of natural abundance. In accord with ^{1}H NMR data, a new oxygen-bearing carbon at δ 64.27 was observed in addition to δ 80.84 for C-3. Extensive 2D-NMR analysis provided the complete assignment of carbons as shown in Table 2 and determined its structure as protosta-17(20)*Z*,24-diene-3β,29-diol (**4**) (Figure 3). A critical NOE was observed from C-19 methyl (δ 0.89) to one of the carbinol protons at δ 4.22 (and also to C-9 proton at δ 1.48) (Supporting Information), confirming that a hydroxyl group resides on C-29 (4β-side). Because NOE was observed between C-19 methyl group and only one carbinol proton at δ 4.22, we assigned this proton as *pro-R* hydrogen, while the one at δ 3.29 we assigned as *pro-S* (Figure 4). This product **4** was previously identified from helvolic acid producing fungi, again with no detailed NMR information.[25]

Another product, a putative carboxylic acid derivative, showed further downfield shift of one methyl (δ 1.46) signal and disappearance of carbinol protons in ^{1}H NMR. The ^{13}C NMR spectrum of [2-^{13}C]acetate-enriched sample indicated the presence of a carboxylic acid (δ 181.89). Extensive NMR analysis established the structure as protosta-17(20)*Z*,24-dien-3β-ol-29-oic acid (**5**) (Table 2, Figure 3). Co-occurrence of this product with **4** suggested C-29 (4β carbon) to be a carboxylic acid. This compound has not been described and represents a novel natural metabolite.

Structure assignment of two of the CYP5081A1 products shows that CYP5081A1 catalyzes oxidation of C-29 methyl group of **1** into carbinol and then to carboxylic acid, presumably through an aldehyde. These products are in accord with the helvolic acid biosynthetic pathway as well. Here again, we were unable to detect any oxygenated product derived from **2**. These

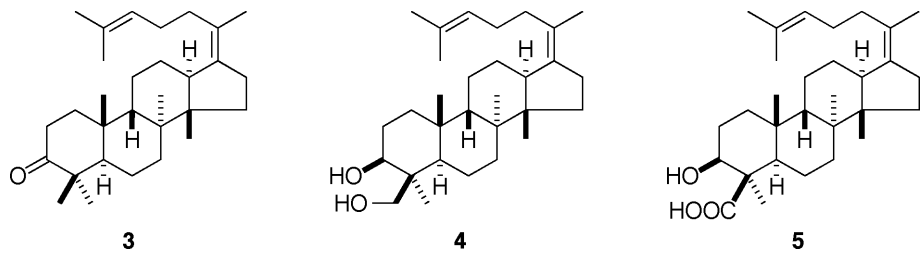


Figure 3. Structures of oxidized derivatives **3**, **4**, and **5**.

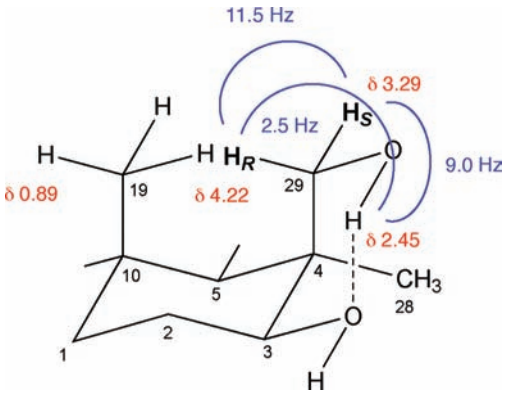


Figure 4. [1]H correlation around A ring of **4**.

results together establish that the present gene cluster does code for helvolic acid biosynthesis in *A. fumigatus*.

Discussion

Recent genome sequencing projects of several fungal species have greatly facilitated discovery of many important genes responsible for biosynthesis of secondary metabolites including polyketides and terpenoids with important biological activities. Our search for gene clusters for triterpene biosynthesis from fungi successfully identified the one for helvolic acid biosynthesis. This gene cluster is the first one from any organism for triterpenoid biosynthesis. While the search for genes involved in triterpene tailoring steps in plants is greatly hampered by the presence of large gene families, especially for P450s, the present study represents a novel and an efficient way of identifying triterpene tailoring genes. These genes would be valuable not only for the production of important antibiotic fusidanes, but also for the production of useful plant-derived triterpenoids by manipulating the coded enzymes to accept broader substrates or by altering the reaction specificity.

The three characterized genes participate in the early steps of helvolic acid biosynthesis. After the formation of the basic carbon skeleton **1** by AfuOSC3, both AfuSDR1 and CYP5081A1 presumably function together to catalyze the demethylation of C-29 (Scheme 3). Because both enzymes apparently accepted **1** as a substrate in the in vivo coexpressions with AfuOSC3, the exact order of the two reactions remains yet established, and a metabolic grid formation is left as a possibility.

The C-4 demethylation process is well-known in sterol biosynthesis. In yeast, this step is catalyzed by two enzymes, ERG25 and ERG26 (Scheme 4).[26] ERG25, a nonheme di-iron enzyme, catalyzes oxidation of 4α-methyl group of 4,4-dimethylzymosterol (**6**) into a carboxylic acid (**7**),[27] while ERG26, a SDR, converts 3β-hydroxyl into a ketone (**8**), thus triggering the decarboxylation.[28] Conversely, in helvolic acid biosynthesis, 4β-methyl is oxidized into a carboxylic acid by a P450, although the final product (**10**) harbors the same 4α-methyl group. In both processes, the configuration of methyl group at C-4 is determined by a protonation at C-4 during enol to keto tautomerization following the decarboxylation of β-keto acid. Consequently, in yeast sterol biosynthesis, ERG26 is responsible for establishing the 4α-methyl stereochemistry of the product (**9**). It would be interesting to see which of the two enzymes determines the configuration at C-4 in helvolic acid biosynthesis.

AfuOSC3 represents the first protostadienol synthase to be identified. Historically, the finding of both fusidanes and **1** as natural products has established the idea that protosteryl cation exists as an intermediate enroute to lanosterol. Since then, an explanation of the mechanism for C-17(20) double bond formation has been the subject of intensive research.[29] At one time, a stable intermediate, such as the one produced by capture of C-20 cation by X group of the enzyme or one with C-20(22) double bond, was postulated for lanosterol biosynthesis, but was later proven to be absent. The AfuOSC3 reaction involves tetracyclic protosteryl cation as an intermediate from which loss of H-17α yields the product **1**. This cationic intermediate is common to lanosterol and cycloartenol synthase reactions that require further extensive Wagner−Meerwein shifts of methyl groups and hydrides. The sequence of AfuOSC3 was compared to those of lanosterol synthases. Based on the crystal structure of human lanosterol synthase complexed with product lanosterol,[30] none of the residues of AfuOSC3 seems to be an obvious catalytic base to abstract a proton from C-17 at protosteryl cation stage. The closest residue of lanosterol synthase located toward H-17α is Phe696, which occupies the α face of lanosterol around D-ring and C-20 position. As this Phe residue is also conserved in AfuOSC3, we speculate that AfuOSC3 may have an additional space between C-17 and the Phe residue, which allows a solvent water molecule to occupy this space and serve as a catalytic base. Some sequence differences around Phe696 may be responsible for such a structural difference. For example, highly conserved Asn697 and Cys700 residues of lanosterol synthase are replaced by Ala and Gly in AfuOSC3, respectively. Mutational studies on these residues should clarify our hypothesis.

The other characteristic feature of **1** is the *Z* geometry of $\Delta^{17(20)}$, which implies that the side chain is folded in the active site in such a way that does not require substantial C-17 and C-20 bond rotation prior to deprotonation as once proposed. Perhaps, such side-chain folding is conserved in lanosterol and cycloartenol synthases as well. This geometry with α-face hydride migration from C-17 well accounts for 20*R* configuration found in lanosterol. In fact, all natural tetracyclic triterpenes generated from protosteryl cation possess 20*R* configuration as exemplified by cycloartenol and cucurbitadienol. These arguments are in good agreement with the 20*R* configuration of a minor product **2**, which is formed in the same active site that generates **1**. The unique 20*R* configuration among these triterpenes indicates that OSCs that produce protosteryl cation as an intermediate strictly constrain the side-chain geometry within their active sites. Restricted rotation of the side chain around C-17(20) bond had also been documented in a nonenzymatic system as well.[31] In contrast, plant tetracyclic

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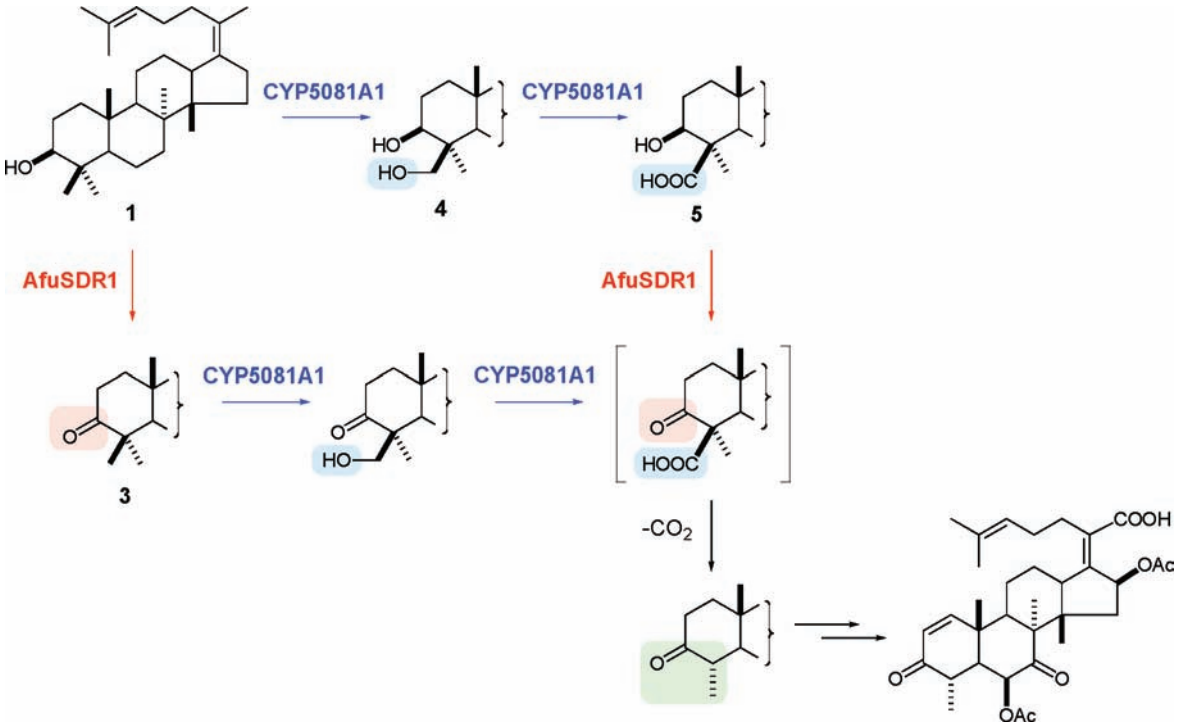
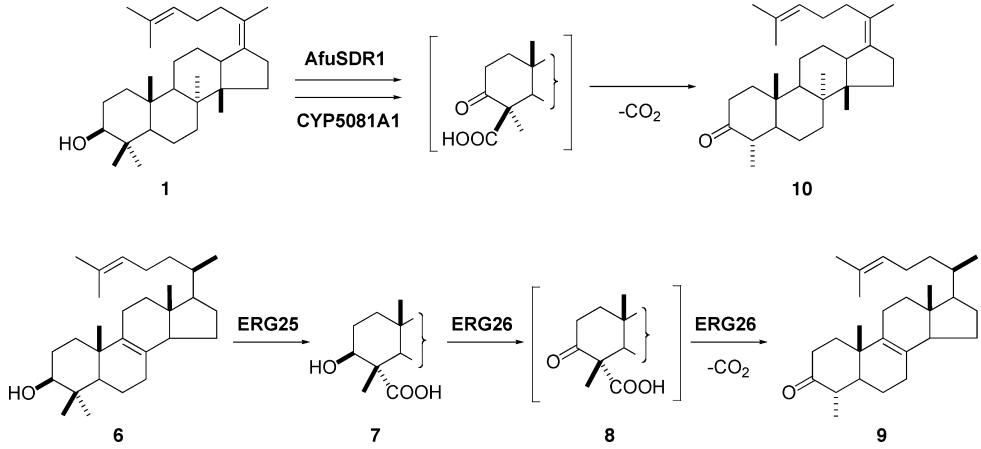
Scheme 3. C-4 Demethylation Step Catalyzed by AfuSDR1 and CYP5081A1 during Helvolic Acid Biosynthesis



Scheme 4. Comparison of C-4 Demethylation Processes in *A. fumigatus* Helvolic Acid and in Yeast Sterol Biosyntheses[a]



[a] Top figure represents the proposed helvolic acid case catalyzed by AfuSDR1 and CYP5081A1. Bottom figure shows the yeast sterol case catalyzed by ERG25 and ERG26.

triterpenes derived from dammarenyl cation have both 20*R* and 20*S* configurations in their side chains. Euphanes and tirucallanes are representative examples, respectively (Supporting Information). Perhaps, a hydride migration from C-17 with or without rotation of the side chain may install either 20*R* or 20*S* configuration. Production of a minor compound **2** by AfuOSC3 may point to an incomplete enzymatic control in deprotonation from H-17α to yield the $\Delta^{17(20)}$ product. As evident from facile isomerization of **1** into **2** under acidic conditions, **1** is indeed less stable than **2**. Perhaps, nature has failed to make AfuOSC3 specific enough to produce the desired product **1** for helvolic acid biosynthesis, allowing H-17α to migrate to C-20 followed by deprotonation from C-13 yielding the thermodynamically favored product **2**. Again, the previous isolation of **2** from helvolic acid producing fungi indicates that **2** was not formed by an aberrant expression of AfuOSC3 in a heterologous host.

Functional identification of AfuSDR1 from this gene cluster marks the first characterization of a dehydrogenase in triterpene biosynthesis from any organism. It would be interesting to see whether AfuSDR1 could catalyze similar dehydrogenation of triterpene substrates found in plants. Substrate specificity of this enzyme, however, might be strict as none of the 3-keto derivative of **2** was detected in the present study. Sequence comparison of AfuSDR1 with other SDR members, for instance a bacterial 3β/17β-hydroxysteroid dehydrogenase, shows rather low overall sequence identity, although all of the catalytic residues and cofactor binding motifs are well conserved (Supporting Information).[32] According to the classification of SDR family, AfuSDR1 belongs to a classical SDR of cD1d subfamily, which utilizes NAD(H) as a cofactor.[33] It must await further in vitro experiment to see if AfuSDR1 does utilize this cofactor or not.

On the other hand, CYP5081A1 is the third example of P450s that catalyze an oxygenation of triterpene substrates next to CYP93E1, a β-amyrin 24-hydroxylase, from *G. max*[12] and CYP88D6, a β-amyrin 11-oxidase.[13] CYP5081A1 catalyzes oxidation of C-29 methyl group into an alcohol and a carboxylic acid. This three-step oxidation from methyl to carboxylic acid presumably involves an aldehyde, although we failed to detect it in the present study. A similar three-step oxidation has been found in diterpene biosynthesis. For example, CYP701A3 from *A. thaliana* converts *ent*-kaurene into *ent*-kaurenoic acid.[34] The identification of **4** in helvolic acid producing fungi supports that the same three-step oxidation by P450 is operational in vivo as well. As the four P450 genes in the present cluster form a distinct CYP5081 family and cannot be grouped to any other families of fungal P450s, remaining *CYP5081B1*−*D1* are also expected to participate in helvolic acid biosynthesis. If so, the entire family is devoted to the same biosynthetic pathway. These P450s are presumed to install C-6 OH, C-7 ketone, C-16 OH, and C-21 carboxylic acid (Figure 1). The presence of only three P450s for four potential oxygenation sites suggests that one of them may have a dual function oxidizing different positions. Such dual function P450s are known in fungi that participate in gibberellin biosynthesis.[35]

In the present studies, we cloned the genes from *A. fumigatus* IFO8866 strain whose helvolic acid productivity is not known. As mentioned before, deduced amino acid sequences of *AfuOSC3*, *AfuSDR1*, and *CYP5081A1* were completely identical to those of the Af293 strain. Later, we obtained a helvolic acid producing strain ATCC10894. All three genes together with *CYP5081B1*−*D1* were PCR amplified from genomic DNA of this strain, and their sequences were compared to those from the IFO8866 strain. When translated, there are two different residues in AfuOSC3 and one in CYP5081A1 (Supporting Information). The sequence for AfuSDR1 was completely identical. Our studies indicate that these amino acid discrepancies may not have a decisive effect on the enzyme activity. We also found sequence differences in CYP5081B1, whereas C1 and D1 sequences were exactly the same in two strains. Whether the difference in the sequence of CYP5081B1 has a critical influence on the enzyme activity is the subject of our next studies.

The successful production of oxygenated triterpenes in yeast coexpression system described here demonstrates the feasibility of using yeast as a platform for a production of useful triterpenoids. It may be possible to produce helvolic acid in yeast when a simultaneous expression methodology of 9 genes of the cluster becomes available. The information provided here would contribute to a cloning of biosynthetic genes for other fusidanes from different fungi whose genome sequences are not available and hopefully lead to generation, by a combinatorial biosynthesis fashion, of novel structural variants of fusidanes having different functionality in different positions.

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Conclusion

Studies on triterpene tailoring steps have been severely retarded due to the presence of large gene families of oxygenases and glycosyltransferases in the genomes of higher plants, the major triterpenoid producers. By taking advantage of the fungal genome sequences, a triterpene biosynthetic gene cluster was discovered for the first time from *A. fumigatus*. The cluster is expected to contain all of the genes for triterpene tailoring enzymes. Functional expression of *AfuOSC3* in the cluster in yeast confirmed it to encode a novel OSC producing **1**, an established precursor of helvolic acid and its congener fusidane antibiotics. The reaction catalyzed by this OSC closely resembles that of lanosterol synthase up to protosteryl cation but is terminated by a deprotonation without any methyl and/or hydride shifts required for the latter. Therefore, AfuOSC3 could be an ideal case to identify amino acid residues that control such rearrangement steps. By coexpressing an accompanying SDR family gene in the cluster with this OSC in yeast, AfuSDR1 was shown to catalyze the dehydrogenation of protostadienol to yield the 3-keto derivative. This is the first functional identification of a dehydrogenase acting on triterpene substrate. Furthermore, a similar coexpression with one of the P450 genes (*CYP5081A1*) in the cluster gave 29-hydroxylated and 29-oic acid derivatives. CYP5081A1 is the third example of P450s acting on triterpene substrates. AfuSDR1 and CYP5081A1 may function together for the oxidative removal of the 4β-methyl group of **1** and comprise the early part of helvolic acid biosynthesis. Efficient identification of triterpene tailoring genes from fungi and production of modified triterpenes in yeast demonstrated in this study would contribute for further understandings of triterpene tailoring steps and surely lead to future engineering of the pathways to produce not only fusidanes but also useful bioactive triterpenoids in yeast.

Experimental Section

Fungal Strains. The fungus *A. fumigatus* strain IFO8866 was obtained from IAM culture collection (Institute of Molecular and Cellular Biosciences, The University of Tokyo). *A. fumigatus* ATCC10894 was purchased from ATCC. These *A. fumigatus* strain were cultivated at 30 °C in YPD medium (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$, pH 5.5) for 2 days.

PCR and Sequence Analysis. Synthesis of oligo DNAs was done by Nihon Bioservice (Saitama, Japan). PCR was performed by PTC-200 Peltier Thermal Cycler (MJ Research). Sequencing was carried out via an ABI PRISM 3100 genetic Analyzer (Applied Biosystem).

Molecular Cloning. Genomic DNA of *A. fumigatus* IFO8866 was extracted as usual and used as a template in the following PCR. Each gene was amplified by PCR using the following sets of primers: AfuOSC3-N-*Spe*I (5′-CTTGCCT**ACTAGT**ATGGCGACAGACAGCAGCATG-3′) and AfuOSC3-C-*Bgl*II (5′-GTACCAA**GATCT**TTATATAGCTAGACATTCATTTCCATGG-3′) for *AfuOSC3*, AfuSDR1-N-*Bam*HI (5′-CCGC**GAATTC**ACCATGAACACGGCC-3′) and AfuSDR1-C-*Xho*I (5′-CTAA**CTCGAG**CTACGTATATCCATCATGTATTTTGATCCCC-3′) for *AfuSDR1*, CYP5081A1-N-*Bam*HI (5′-CTATTACT**GGATCC**ATGATTAGAGTCGC-3′) and CYP5081A1-C-*Kpn*I (5′-CATTAA**GGTACC**CTAGCAAGGTGAATACTCTCTTGC-3′) for *CYP5081A1*, CYP5081B1-N-*Bgl*II (5′-GCTGA**AGATCT**ATGGCCCTCCCCATTATTCTC-3′) and CYP5081B1-C-*Kpn*I (5′-GTTATATTCAG**GGTACC**CTAGGTCTTTGG-3′) for *CYP5081B1*, CYP5081C1-N-*Bam*HI (5′-CACAC**GGATCC**ATGGTAGAAATCCTCCGTTTCATCCCC-3′) and CYP5081C1-C-*Kpn*I (5′-GCACG**GGTACC**TTACTGCCTGCGAGACACGACCAAC-3′) for *CYP5081C1*, and

CYP5081D1-N-*Bgl*II (5′-CTTCAT**AGATCT**ATGGCCGTCGCCACC-3′) and CYP5081D1-C-*Kpn*I (5′-CATG**GGTACC**TCACTCTGTAATTCTCACCGACAATACCATTCG-3′) for *CYP5081D1* (each restriction enzyme site is in bold; underline indicates start or stop codons). PCR was carried out for 40 cycles with a program (98 °C, 10 s, 65 °C, 15 s, 72 °C, 30 s/kb, and final extension at 72 °C, 10 min) using Phusion DNA polymerase (Finnzymes) with dNTP (0.2 mM) in a final volume of 50 μL following the manufacturer's protocol. Amplified *AfuOSC3* was digested with *Spe*I and *Bgl*II, then ligated into the corresponding site (MCS1) of pESC-Ura (Stratagene) to construct the plasmid pESC-*AfuOSC3*. On the other hand, each amplified P450 gene was digested with appropriate restriction enzymes (*Bgl*II or *BamH*I, and *Kpn*I) and ligated into the site between *BamH*I and *Kpn*I (MCS2) of pESC-Ura. The constructed pESC-*P450* was then digested with *Spe*I and *Bgl*II and ligated with correspondingly digested *AfuOSC3* to produce coexpression plasmids pESC-*AfuOSC3*,*P450*. Similarly, amplified *AfuSDR1* was digested with *BamH*I and *Xho*I and ligated into the corresponding site (MCS2) of pESC-Ura, and the constructed pESC-*AfuSDR1* was then digested with *Spe*I and *Bgl*II, which was ligated with correspondingly digested *AfuOSC3* to produce a coexpression plasmid pESC-*AfuOSC3*,*AfuSDR1*.

Functional Expression of AfuOSC3 in Yeast. Lanosterol synthase-deficient yeast mutant strain GIL77 (*erg7*, *ura3*−*167*, *hem3*−*6*, *gal2*) was transformed with pESC-*AfuOSC3* using Frozen-EZ Transformation II kit (ZYMO RESEARCH). The transformant was inoculated in 20 mL of synthetic complete medium without uracil (SC-U), containing ergosterol (20 μg/mL), hemin chloride (13 μg/mL), and Tween 80 (5 mg/mL), and incubated at 30 °C for 2 days. Media were changed to SC-U with the same supplements and 2% galactose in place of glucose, and further incubated at 30 °C for 2 days. Cells were collected and resuspended in 10 mL of 0.1 M KPB (pH 7.0) supplemented with 2% glucose and hemin chloride (13 μg/mL) and further cultured for 24 h at 30 °C. Cells were collected and refluxed with 2 mL of 5% KOH/50% EtOH aqueous for 10 min. After extraction with the same volume of hexane, the extract was concentrated and analyzed by TLC plate (Merck #5715) with benzene:acetone = 19:1 as a solvent system. For preparative scale, 3 L of culture was prepared. Induction and resting cultures were performed as described above. After being refluxed with 500 mL of 20% KOH/50% EtOH aqueous for 1 h, the mixture was extracted with 500 mL of hexane three times, combined, and concentrated. The extract was purified by silica gel column chromatography with benzene as an eluent. The fractions with a spot migrating between dioxidosqualene and ergosterol on TLC were collected and concentrated. For the major and minor products, the fraction containing both was further separated by $AgNO_3$-impregnated TLC plate (Merck #11798) with benzene as a developing solvent. The lower major band and higher minor band were scraped off separately and extracted with acetone. An aliquot of each product was applied to GC−MS as described below to confirm the separation was appropriately done.

Functional Coexpression of AfuOSC3 and SDR in Yeast. GIL77 harboring pESC-*AfuOSC3*,*AfuSDR1* was cultured, and cells were extracted as described above. The extract was concentrated and analyzed by TLC plate (Merck #5715) with hexane:ethylacetate = 9:1 as a solvent system. For preparative scale, 3 L of culture was prepared. The extract was purified by silica gel column chromatography with hexane:ethylacetate = 19:1 as an eluent. The fractions with a spot migrating between oxidosqualene and dioxidosqualene on TLC were collected and concentrated.

Functional Coexpression of AfuOSC3 and P450s in Yeast. GIL77 harboring pESC-*AfuOSC3*,*CYP5081A1*−*D1* were cultured, and cells were extracted as described above except hexane:ethylacetate = 3:1 was used as a solvent. In addition to extraction of triterpenes from the cells, resting culture medium was also extracted with the same volume of hexane:ethylacetate = 3:1. For preparative scale, 3 L of culture of GIL77/pESC-*AfuOSC3*,*CYP5081A1* was prepared. The extract was purified by silica gel column chromatography with benzene:acetone from 19:1 to 9:1 as an eluent. The fractions showing lower R_f value than ergosterol on TLC were collected and concentrated. Further purification was carried out by preparative HPLC using ODS-80T_M column (7.8 × 300 mm) (TOSOH) with 96% CH_3CN(aq) as a solvent for a diol compound and 93% CH_3CN(aq) with 0.01% TFA for a carboxylic acid derivative (flow rate 2.5 mL/min, detection UV 202 nm) at room temperature. In case of [2-^{13}C]acetate feeding, [2-^{13}C]sodium acetate (99% atom ^{13}C, Cambridge Isotope Laboratories, Inc.) 250 mg/L was added during galactose induction and resting culture.

GC−MS Analysis. The collected fractions were applied to GC−MS (Shimadzu, GCMS-QP2010) equipped with a Restec Rtx-5MS glass capillary column (30 m in length, 0.25 mm in diameter, 0.25 μm film thickness) and He as a carrier gas (45 cm/min) on the program (held at 240 °C for 2 min, then temperature increased at the rate of 10 °C/min until 330 °C). The temperature of the ionization chamber was 250 °C, with electron impact ionization at 70 eV. In the case of coexpression of AfuOSC3 and P450s, the extract was treated with TMS-diazomethane (50 μL of 0.6 M hexane solution added to samples dissolved in 100 μL of benzene and 100 μL of methanol and stirred at room temperature for 1 h) or acetylated (samples dissolved in 200 μL of acetic anhydride and 40 μL of pyridine and stirred at room temperature for 12 h) prior to GC−MS analysis.

NMR Analysis. All of the NMR spectra were recorded on a JEOL ECA-500 spectrometer with chloroform-*d* (99.8% atom ^{2}H, ISOTEC) as a solvent. A solvent signal δ 7.26 for ^{1}H and δ 77.0 (t) for ^{13}C was used as references for chemical shifts.

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Supporting Information Available: Complete ref 17, phylogenetic tree, GC−MS data, ^{1}H and ^{13}C NMR spectra, and NMR assignments for **1**−**5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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