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Multiple Oxidative Modifications in the Ophiobolin Biosynthesis: P450 Oxidations Found in Genome Mining

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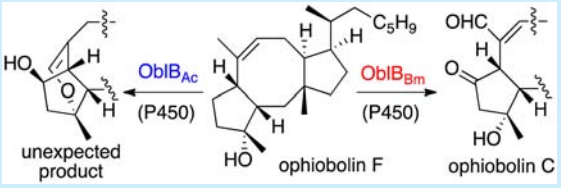
Supporting Information

ABSTRACT: Heterologous expression of four candidate genes found in ophiobolin gene clusters from three fungal strains was employed to elucidate the late-stage biosynthetic pathway of phytotoxin ophiobolin. Expression of $oblB_{Ac}$ (cytochrome P450) from the cryptic gene cluster gave unexpected products, and that of $oblB_{Bm}$/$oblB_{Ev}$ from the gene cluster of ophiobolin producers, with $oblD_{Bm}$ as the transporter, yielded intermediate ophiobolin C through an unusual four-step oxidation process. The observation made in this study may provide a useful guideline for the elucidation of genuine biosynthetic pathways of natural products.



Sesterterpene represents a relatively minor family of terpenoids even though more than 400 members have been reported.[1] The first member of this family is ophiobolin A (**1**), which was isolated as a phytotoxin from *Bipolaris oryzae* (*Ophiobolus miyabeanus*).[2] To date, a number of structural homologues such as ophiobolins C (**2a**), B (**2c**), K (**3a**), and F (**4**) have been isolated from various fungal species (Figure 1).[3] Until recently, no sesterterpene synthase had ever been characterized, unlike most of the other universal terpene synthases, largely described in the 1990s.[4] During our study on the genome mining of novel diterpene synthases related to bifunctional fusicoccadiene synthase,[5] we unexpectedly found the first sesterterpene synthase, ophiobolin F synthase (AcOS), from *Aspergillus clavatus*.[6] Notably, no previous report ever mentioned that this fungus produced ophiobolins. Since this finding, three sesterterpene synthases have been characterized by heterologous expression of genes homologous to *AcOS* using *A. oryzae*.[7] We recently proposed classification of di/sesterterpene synthases by phylogenetic analysis and the rapid cloning of novel terpene synthase by clade-specific gene expression.[7a]

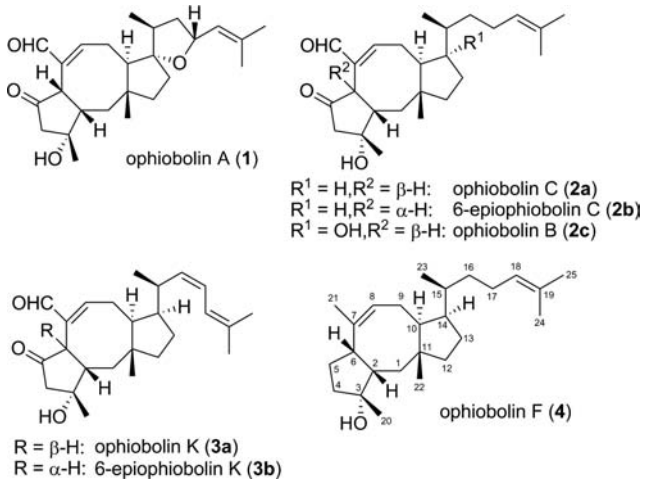


Figure 1. Representative ophiobolins.

Previous studies on the ophiobolin biosynthesis suggested that the first intermediate **4** was oxidized to **1** via **2a** and **2c**.[8] However, functional analysis of biosynthetic genes responsible for these transformations has not been carried out. In the *A. clavatus* genome, we found an *obl* gene cluster consisting of genes encoding terpene synthase ($oblA_{Ac}$, *AcOS*), cytochrome P450 monooxygenase ($oblB_{Ac}$), MFS transporter ($oblD_{Ac}$), and flavin-dependent oxidase ($oblC_{Ac}$) (Figure 2). In this paper, we report on the late-stage oxidative transformations from **4** to **2a** and discuss heterologous expression of "cryptic" and "genuine" ophiobolin gene clusters from several fungal strains.

Heterologous expression in *Aspergillus oryzae* has become a standard tool to elucidate the biosynthetic pathways of fungal natural products.[9] To examine the function of P450 $oblB_{Ac}$, we

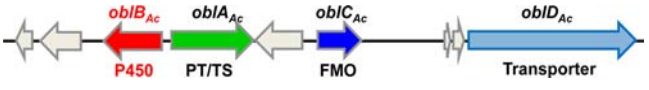


Figure 2. Ophiobolin gene cluster from *A. clavatus*.

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introduced the gene into the transformant AO-*oblA*$_{Ac}$[6] with the plasmid pUSA2-*oblB*$_{Ac}$. We identified four products in the transformant's extract, which showed characteristic spots on TLC (Figure S1). LC−MS analysis of the extracts from the resultant AO-*oblA*$_{Ac}$*B*$_{Ac}$ showed four peaks representing products whose molecular formulas were determined by HR-MS analysis (**5**: $C_{25}H_{40}O_2$, **6**: $C_{25}H_{42}O_3$, **7**: $C_{25}H_{40}O_4$, **8**: $C_{25}H_{42}O_2$), suggesting that all of them were oxygenated ophiobolin derivatives (Figure 3).

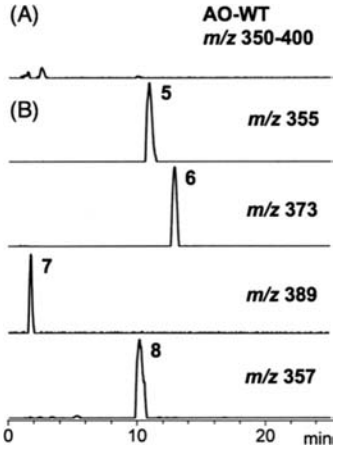


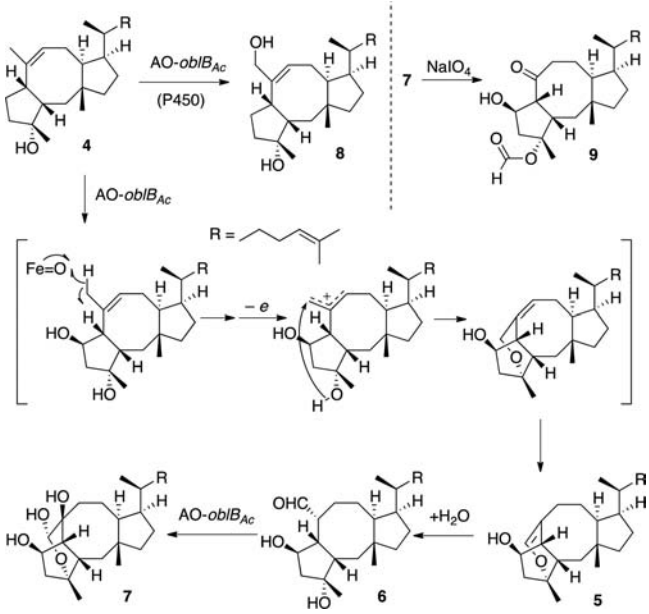
Figure 3. LC−MS analytical data of *A. oryzae* culture extracts. (A) *A. oryzae* NSAR1 (wild type), SIM: *m*/*z* 350−400. (B) Metabolites produced by transformant AO-*oblA*$_{Ac}$*B*$_{Ac}$: *m*/*z* 355, 373, 389, 357.

In the ^{1}H and ^{13}C NMR spectra of major product **5**, several signals were newly observed [oxymethine (δ_H 4.21 (dd); δ_C 75.4) and [olefin (δ_H 5.93 (s); δ_C 138.0 and 111.4)]. On the basis of COSY and HMBC analysis (especially characteristic correlation between C3 and H21), the presence of a C5-hydroxyl group and cyclic enol ether was proposed. Extensive NMR analysis including COSY, HMQC, HMBC, and NOESY enabled us to determine the structure of enol ether **5** as shown in Scheme 1 (Figure S4 and Table S3).

The second major product **7** had the highest polarity among oxidation products. In the ^{1}H and ^{13}C NMR spectra of **7**, acetal and three oxygenated signals were observed [oxymethine (δ_H 4.65 (m); δ_C 70.3), acetal (δ_H 4.59 (s); δ_C 97.4)] and [oxygenated moieties (δ_C 72.6, 83.8)]. On the basis of further NMR analysis, a structure was proposed (Scheme 1), which was confirmed by periodate oxidation of **7** to give formyl ester **9**. Similarly, minor components **6** and **8** were determined by detailed NMR analysis as shown in Scheme 1. Compound **6** contains aldehyde unconjugated with olefin and secondary and tertiary alcohol moieties at C5 and C3, while **8** is a C21-oxidation product of **4**.

The reaction mechanism of OblB$_{Ac}$ is proposed as shown in Scheme 1. Initial hydroxylation at C5 is followed by abstraction of the C21-hydrogen, and the resultant allylic radical reacts with closely located C3-OH to give an unstable cyclic ether (Figures S5 and S6). Due to severe ring strain, 1,3-rearrangement may occur to give more stable enol ether **5**. Hydrolysis of **5** yields saturated aldehyde **6**, which is subsequently converted into **7** by further C7-hydroxylation. Alternative C21-hydroxylation of **4** affords **8**. After determination of the structures of novel metabolites, we recovered cDNA of OblB$_{Ac}$ from the AO-*oblA*$_{Ac}$*B*$_{Ac}$ and resequenced it. No mutation was found in the sequence. Although OblB$_{Ac}$ catalyzes multistep oxygenations at

Scheme 1. Oxidation Mechanism of Cytochrome P450 OblB$_{Ac}$



C5 and C21/C7 in a relatively efficient manner (>140 mg/kg medium), it was unable to convert **4** to **2a**. Considering all experimental results, we speculated that this inability might be originated from *OblB*$_{Ac}$, which is found in the cryptic gene cluster.

Using the ophiobolin gene clusters from *A. clavatus* as a query of a BLAST (Basic Local Alignment Search Tool) search, we found highly homologous clusters in the genomes of fungi *Bipolaris maydes* (ophiobolin A (**1**) producer)[10] and *Emericella variecolor* (ophiobolin K (**3**) producer)[11] (Figures S7 and S8). Comparing structures **1** and **3a**, we speculated that **2a** is a common intermediate in the biosynthesis of **1** and **3a**. In these gene clusters, we found a single common oxidation enzyme gene *oblB*$_{Bm}$/*oblB*$_{Ev}$ homologous to *oblB*$_{Ac}$. At first, we introduced oblA$_{Bm}$ and oblA$_{Ev}$ genes into the AO-wild type. The resultant AO-*oblA*$_{Bm}$ and AO-*oblA*$_{Ev}$ produced **4**, confirming that these clusters are responsible for the biosynthesis of **1** and **3a**. We then introduced *oblB*$_{Bm}$ to the AO-*oblA*$_{Ac}$. Although LC−MS analysis of the transformant did not reveal any new peaks, obvious consumption of **4** was observed. Together with the fact that the *A. oryzae* wild-type growth was inhibited in the presence of **2a**, we speculated that the transporter OblD$_{Ac}$/OblD$_{Bm}$/OblD$_{Ev}$ is necessary for their production, unlike for the heterologous production of other metabolites that are toxic to eukaryotic cell.[9] To examine this hypothesis, we introduced the transporter gene *oblD*$_{Bm}$ into the AO-*oblA*$_{Ac}$*B*$_{Bm}$. In the LC−MS analysis, the resultant AO-*oblA*$_{Ac}$*B*$_{Bm}$*D*$_{Bm}$ gave new products which were identical to **2a**, 6-epi-isomer **2b**, and anhydro derivative **10** (Figure 4).[11] Similarly, AO-*oblA*$_{Ac}$*B*$_{Ev}$*D*$_{Bm}$ gave essentially the same results (Figure S2). These were confirmed by the fact that **2a** was converted into **2b** and **10** by incubation in the buffer (pH 5) (Figure S3).[12] Together with the observation that **2a**, **2b**, and **10** were produced by *E. variecolor* in solid medium,[11] we concluded that cytochrome P450 monooxygenase OblB$_{Bm}$ catalyzes four-step oxidation of **4** to give **2a** and that **2b** and **10** are artifacts derived from **2a**. In addition, the results also

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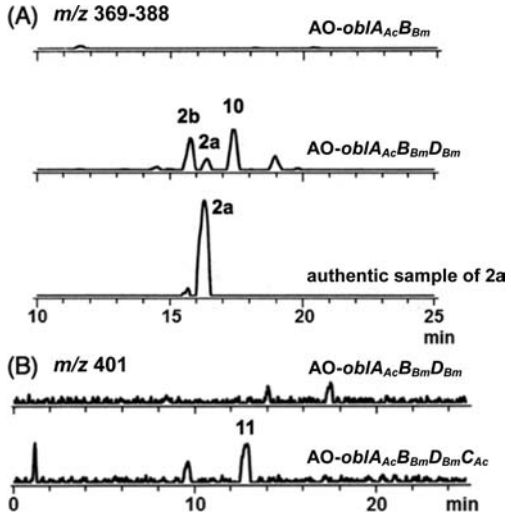


Figure 4. LC−MS analytical data. (A) Metabolites produced by transformants AO-*oblA*$_{Ac}$*B*$_{Bm}$*D*$_{Bm}$: *m*/*z* 369−388. (B) Metabolites produced by transformants AO-*oblA*$_{Ac}$*B*$_{Bm}$*D*$_{Bm}$*C*$_{Ac}$: *m*/*z* 401.

suggested that the common *oblD* genes found in three gene clusters encoded a putative ophiobolin specific transporter.

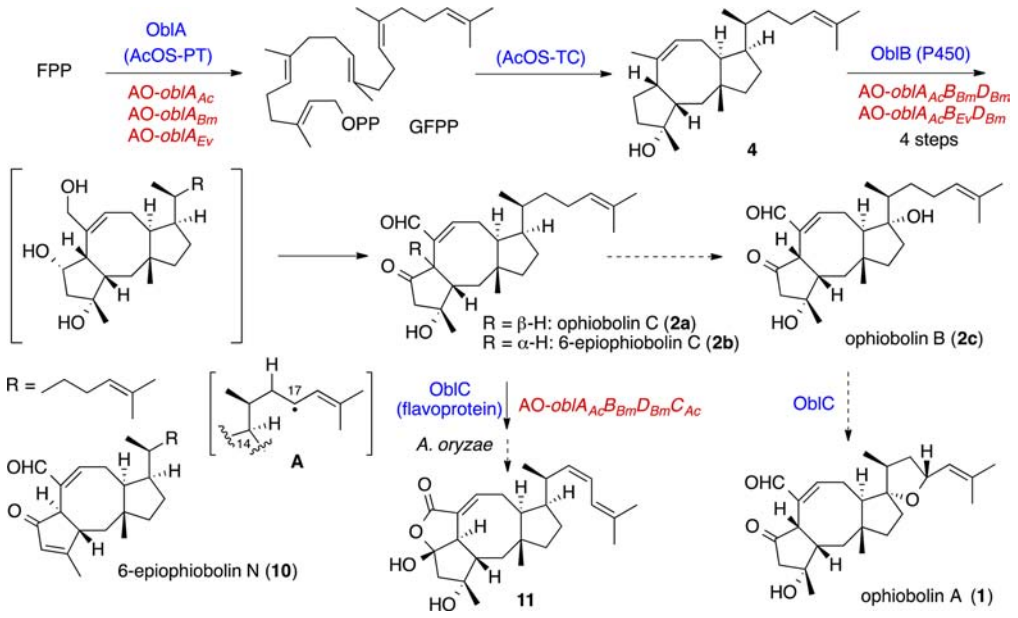
For the final conversion from **2a** to ophiobolin A (**1**), only *oblC*$_{Ac}$ encoding flavin-dependent oxidase remained in the ophiobolin gene clusters from both *A. clavatus* and *B. maydes*, presumably catalyzing the conversion of **2a** to **1**. To examine this hypothesis, we introduced *oblC*$_{Ac}$ into the AO-*oblA*$_{Ac}$*B*$_{Bm}$*D*$_{Bm}$ strain. In the LC−MS analysis, the resultant quadruple transformant gave a new peak that was different from either **2c** or **1** (Figure 4). The HR-ESI-MS of oxidation product **11** revealed the molecular formula composition of $C_{25}H_{36}O_4$. ^{1}H and ^{13}C NMR analysis suggested that **11** has a conjugated diene (δ_H 5.11 (t), 6.00 (d), and 6.06 (t); δ_C 122.9 (CH), 126.7 (CH), 138.3 (CH) and 139.0 (C)) at the side chain and ester and acetal moieties (δ_C 174.0, 114.7). Further detailed NMR analysis allowed us to determine its structure as shown in Scheme 2. Interestingly, the structure of **11** corresponds to the oxidation product of 6-epiophiobolin K (**3b**).[11]

Structure of oxidation product **11** suggested that OblC$_{Ac}$ forms C17-allylic radical **A** through a oxidation process and then generates conjugated diene product (Scheme 2). The same radical intermediate may contribute to the formation of the cyclic ether found in **1** if the substrate has a C14-OH group. Oxidation of aldehyde to carboxylic acid found in **11** is likely catalyzed by an unidentified oxidase in the *A. oryzae* host. Overoxidation of product/intermediate is sometimes observed in heterologous production of fungal metabolites in *A. oryzae*.[13] Although we obtained the evidence that OblC$_{Ac}$ catalyzes allylic oxidation at the side chain, the enzyme responsible for C14-hydroxylation remains unknown.

In this study, we characterized a key enzyme, OblB$_{Bm}$, that installs oxygen functionalities at C5 and C21, which are found in most of the ophiobolin congeners.[1,3] In the late biosynthetic stage of fungal metabolites, similar multistep oxidations catalyzed by P450 were found in the biosynthesis of paxilline (PaxP, five steps),[14] gibberellin (P450-1, four steps),[15] trichothecene (Tri4, four steps),[16] and fumagillin (Fma-P450, four steps).[17] These examples indicated that a limited number of oxidation enzymes contributed to creating highly decorated fungal metabolites. Although the biosynthetic pathway of **1** is not completely determined, our study shows that complex structures of ophiobolins are likely produced by relatively few enzymes including OblA, OblB, and OblC.

As described here, genome mining approach is useful to discover novel molecules or previously unidentified enzymes that synthesize known natural products. During elucidation of the ophiobolin biosynthetic pathway, we encountered intriguing experimental results on "genuine" and "cryptic" ophiobolin clusters, which were found in the ophiobolin producing strain and in the nonproducing strain, respectively. While the genuine

Scheme 2. Proposed Biosynthetic Pathway of Ophiobolins[a]



[a]Solid arrows represent reactions experimentally confimed and dashed arrows represent reactions proposed. Key: PT, prenyltransferase; TC, terpene cyclase.

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cluster gave **2a**, the cryptic cluster provides quite different end products **5**–**8** in an efficient manner. This finding pointed out that the results obtained from the genome-mining approach need to be interpreted with caution depending on whether an elucidated pathway is a genuine one via a common intermediate or a cryptic, in other words, a "newly branched" one. This observation on P450 genes, $oblB_{Ac}$ and $oblB_{Bm}/oblB_{Ev}$, might be regarded as a part of evidence on creating molecular diversity in the evolution process.

In summary, we describe the heterologous expression of OblA (sesterterpene synthase), OblB (cytochrome P450), and OblD (MFS transporter) in *A. oryzae* and the resultant transformant catalyzed by a four-step oxidative transformation from ophiobolin F to ophiobolin C. This unambiguously confirmed that OblB introduces C5 and C21 oxygen functionalities that are characteristic among ophiobolin family members. In addition, highly homologous $OblB_{Ac}$ from the cryptic gene cluster in *A. clavatus* catalyzed unusual oxidations to yield structurally distinct enol ether and its derivatives. This observation points out the difference between cryptic and genuine gene clusters. The reconstitution strategy in *A. oryzae* host has again proven to be a powerful tool for genome mining of fungal natural products.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b00552.

Experimental procedures; analytical and spectral data (PDF)

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Notes

The authors declare no competing financial interest.

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