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Complete biosynthetic pathways of ascofuranone and ascochlorin in Acremonium egyptiacum

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Ascofuranone (AF) and ascochlorin (AC) are meroterpenoids produced by various filamentous fungi, including Acremonium egyptiacum (synonym: Acremonium sclerotigenum), and exhibit diverse physiological activities. In particular, AF is a promising drug candidate against African trypanosomiasis and a potential anticancer lead compound. These compounds are supposedly biosynthesized through farnesylation of orsellinic acid, but the details have not been established. In this study, we present all of the reactions and responsible genes for AF and AC biosyntheses in A. egyptiacum, identified by heterologous expression, in vitro reconstruction, and gene deletion experiments with the aid of a genome-wide differential expression analysis. Both pathways share the common precursor, illicicolin A epoxide, which is processed by the membrane-bound terpene cyclase (TPC) AscF in AC biosynthesis. AF biosynthesis branches from the precursor by hydroxylation at C-16 by the P450 terpene cyclase (TPC) AscF in AC biosynthesis. AF biosynthesis (ascABCDEG) and a transcriptional factor (ascR) form a functional gene cluster, whereas those involved in the late steps of AF biosynthesis (ascHIl) are present in another distantly located cluster. AF is therefore a rare example of fungal secondary metabolites requiring multi-locus biosynthetic clusters, which are likely to be controlled by the single regulator, AscR. Finally, we achieved the selective production of AF in A. egyptiacum by genetically blocking the AC biosynthetic pathway; further manipulation of the strain will lead to the cost-effective mass production required for the clinical use of AF.

Acremonium egyptiacum

Ascofuranone (AF) and ascochlorin (AC) are fungal natural products with similar chemical structures, originally isolated from Acremonium egyptiacum. Both have many useful biological properties; in particular, AF is a promising drug candidate against the tropical disease, African trypanosomiasis. However, the difficulty of the synthetic method and the inaccessibility of bioengineering methods have inhibited industrial production. This study identified all of the genes required for the branched biosynthetic pathways of AF/AC, which are clustered at two separate loci in the genome. In addition, we established the A. egyptiacum strain selectively producing AF, by genetically blocking the AC biosynthetic pathway. This study benefits the field of combinatorial biosynthesis through presenting biocatalysts and paves the way to cost-effective AF production with bioengineering.

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Significance

Ascofuranone (AF) and ascochlorin (AC) are fungal natural products with similar chemical structures, originally isolated from Acremonium egyptiacum. Both have many useful biological properties; in particular, AF is a promising drug candidate against the tropical disease, African trypanosomiasis. However, the difficulty of the synthetic method and the inaccessibility of bioengineering methods have inhibited industrial production. This study identified all of the genes required for the branched biosynthetic pathways of AF/AC, which are clustered at two separate loci in the genome. In addition, we established the A. egyptiacum strain selectively producing AF, by genetically blocking the AC biosynthetic pathway. This study benefits the field of combinatorial biosynthesis through presenting biocatalysts and paves the way to cost-effective AF production with bioengineering.
Interestingly, these compounds have been isolated from various fungi; for example, 2 from *Cylindrocladium ilicicola* (15, 16), *Cylindrocarpon* sp. (17), *Fusarium* sp. (18), *Microcera* sp. (19), and *Nectria coccinea* (20), and 1 from *Paecilomyces variotii* (21) and *Verticillium hemipterigenum* (22), indicating the broad distribution of the biosynthetic pathways. Compounds related to 1 and 2 are considered to be synthesized through the prenylation of orsellinic acid (3) and terminal cyclization via epoxidation (17, 18, 23) (Fig. 2A), but the details of their biosyntheses have not been established. Recently, we reported that *Stachybotrys bisbyi* PYH05-7 encodes the 3-producing polyketide synthase (PKS) StbA, the UbiA-family prenyltransferase StbC, which produces ilicicolinic acid B (4) (Fig. 2A), and the nonribosomal peptide synthetase (NRPS)-like reductase StbB for the synthesis of LL-Z1272 (ilicicolin B) (5), a putative precursor of 1 and 2 (24). By analogy to the biosynthetic pathways of other fungal meroterpenoids, such as pyripyropene, paxilline, and aflatrem (25), the terminal olefin of the prenyl chain of 5 is thought to be epoxidized by a flavin monooxygenase (FMO), and then cyclized by a membrane-bound terpene cyclase (TPC). Considering the different cyclization patterns of 5, two distinct TPCs were assumed to exist in *A. egyptiacum* (26). However, it is difficult to predict the relevance of genes with novel functions and to find biosynthetic clusters separated at multiple loci. For plant metabolites, in contrast, coexpression analyses have been effective in identifying nonclustered biosynthetic genes (28, 29); naturally, such a transcriptome-based approach is also promising in the case of fungal multilocus clusters (30). Since previous studies showed that 1 and 2, as well as other related compounds, suppress respiratory chain activities at multiple targets (31, 32), the fungal production of 1 and 2 is likely to be tightly regulated to avoid inhibiting fungal growth. Therefore, we can reasonably anticipate that determining the gene clusters that are differentially expressed in association with 1 production will lead to the identification of the responsible genes.

In this study, we aimed to find the biosynthetic genes for 1 by using a comparative transcription analysis of *A. egyptiacum* cultures exhibiting all-or-none production. Taking advantage of heterologous expression systems in *Aspergillus* spp., in vitro reconstruction assays, and a newly established gene disruption method for *A. egyptiacum*, we identified the complete biosynthetic enzymes of 2 encoded by the most prominently induced gene cluster. We then discovered another gene cluster at a discrete locus involved in the remaining steps of AF production, by virtue of a combination of motif-based and differential expression approaches. In addition, we analyzed the transcriptional factor regulating the biosynthetic genes of both compounds located in separate chromosomal regions.

### Results and Discussion

**Discovery of the asc-1 Cluster.** First, we performed a genome-wide transcription analysis using the *A. egyptiacum* strain F-1392, to identify the differentially expressed gene clusters possibly responsible for the biosynthesis of 1 or 2. The production of 1 and 2 in *A. egyptiacum* varied dependent on the culture medium, and two media, designated as F1 and AF here, showed the virtual all-or-none difference. We thus prepared poly(A) selected RNAs from mycelia grown in F1 and AF media, which yielded 0.96 and 399 mg of 1 from mycelia grown in F1 and AF media, respectively, RNA-seq reads, respectively. Although the experiment was preliminary, in terms of read numbers and lacking replicates, mapping the differential expression profiles to the 31-Mb draft genome (*SI Appendix*, Table S1) shed light on a 32-kb region on

![Fig. 1. Chemical structures of ascofuranone (1) and ascochlorin (2).](image)

![Fig. 2. Summary of ascofuranone (1) and ascochlorin (2) biosyntheses.](image)
scaffold 2 (named the asc-1 cluster; DNA Data Bank of Japan (DDBJ)/European Nucleotide Archive (ENA)/GenBank accession LC406756) (Fig. 3A and SI Appendix, Fig. S1). The transcription of eight genes (ascABCDEFG) in the asc-1 cluster was more strongly induced (log10 values ≥ 2.5) in AF medium than in F1 medium, and some of them were predicted to encode characteristic enzymes required for the biosyntheses of 1 and 2; that is, PKS, prenyl transferase, and halogenase (SI Appendix, Table S2). Therefore, we named the eight genes ascR and ascA-G as candidates for the 1 or 2 biosynthetic genes (Fig. 3A).

The functions of AscR and AscA-G were deduced by bioinformatics analyses, as follows. AscR possessed a ZnCys2 binuclear cluster for DNA binding and was presumably a transcription regulator. AscA, AscB, and AscC exhibited more than 50% amino acid identities with the ilicicolin B (5) biosynthetic enzymes (StbABC) in S. bisbryi (24). A flavin-binding enzyme, AscD, was thought to be a halogenase, because it shares 68% amino acid identity with the halogenase that catalyzes the 5-chlorination of ilicicinic acid B (4) (33). The Pfam motifs indicated that AscG is a membrane-bound P450 monoxygenase, and AscE is a P450 monoxygenase/P450 reductase fusion protein, such as the bacterial soluble P450 monoxygenase BM3 (34). AscF was predicted to be a membrane-bound TPC, sharing 29% amino acid identity to AndB, a TPC in anditomin biosynthesis in Emeriella variecolor (35). The multiple alignment of AscF, AndB, and other meroterpenoid TPCs, Pyr4 in pyrpyrione biosynthesis (36), and PabB in paclitaxel biosynthesis (37), revealed that the active-site E63 and D218 residues of Pyr4 (36) were also conserved in AscF (SI Appendix, Fig. S2). Although three other genes (gene-1, -2, and -3) of unknown function are located within the asc-1 cluster, we excluded them from the analysis since they exhibited little or no expression in the AF medium.

Characterization of the asc-1 Cluster Genes. To investigate the biosynthesis, we constructed Aspergillus oryzae transformants expressing ascA-D genes under the starch-inducible amyB promoter (38). Comparison of MS and NMR data with literature (24) indicated that 5 (m/z 355, [M−H]−) was produced in the strain expressing ascCAB (yield, 0.71 mg/L) (SI Appendix, Figs. S3 and S4 and Table S3). We further confirmed that AscA, AscB, and AscC in this order catalyzed each reaction as expected from the homology, based on the detection of the specific products 3 and 4 in the transformants expressing ascC and ascCA (SI Appendix, Figs. S4 and S5 and Table S3). Subsequently, the A. oryzae transformant harboring ascCABD (SI Appendix, Figs. S4 and S5 and Table S3) was confirmed to be responsible for the biosynthesis of 6.

Next, we performed an in vitro reconstruction of the succeeding steps, since the meager production of 6 complicated the analysis of the downstream metabolites in A. oryzae transformants. To prepare the Asc-E-G proteins, we employed an Aspergillus sojae high-copy expression system in which the pyrG selective marker with a truncated promoter enables the transformation of high-copy plasmids (SI Appendix, Fig. S7) (39). Since AscF and AscG are membrane-bound proteins and difficult to purify, a cell-free homogenate was used for the in vitro enzyme reaction. We hypothesized that 6 was epoxidized into ilicicolin A epoxide (7) by AscE or AscG before cyclization by AscF, as in the case of other meroterpenoid biosynthesis in which terpene cyclization follows epoxidation of the prenyl group (25). This is also supported by the previous report of accumulation of 7 in A. egyptiacum (41) mutant strain obtained through random chemical mutagenesis (23). However, in the in vitro assay showed that 7 was not detected with either the Asc-E- or Asc-G-containing homogenate; alternatively, a compound (11) with an m/z of 423 ([M−H]−) was detected only with the AscE-containing homogenate (Fig. 3B and SI Appendix, Fig. S8) (39). The m/z suggested that 11 was a diol, hydroxylized from the epoxide 7 (m/z 405, [M−H]−), so we considered that AscE catalyzes the epoxidation to produce 7. Since 11 is unlikely to participate in the production of 1 or 2 in view of the reaction mechanisms, the diol 11 was presumed to be a shunt product formed by endogenous hydrolysis in A. sojae, as previously observed in the A. oryzae system (36, 40). These assumptions were later confirmed by our deletion experiment (see below). Another reaction product (8) was detected when a mixed homogenate containing AscE and AscF was incubated with 6 (Fig. 3B). Compound 8 was deduced to be ilicicolin C from the high-resolution (HR)-MS data (SI Appendix, Fig. S8 and Table S7), which was supported by the fact that ilicicolin C was isolated from A. egyptiacum (41). Finally, when the protein extracts containing AscE, AscF, and AscG were incubated with 6, the reaction product was identified as ascochlorin (2) (Fig. 3B). Confirmation of the structure was obtained by direct comparison of the MS/MS fragmentation pattern, and by coinjection with the authentic standard.

Figure 3. Functional characterizations of AscA-G. (A) Schematic representation of the asc-1 cluster, found by the differential expression analysis. The expression change was indicated with log10 value for each gene. (B) HPLC profiles of the in vitro reaction products of ilicicolin A (6) as a substrate when incubated with the buffer (i), the homogenate of the A. sojae wild-type strain (ii), the homogenates containing either AscE (iii) or AscG (iv), and the mixed homogenates containing AscE+AscF (v), AscE+AscG (vi), or AscE+AscF+AscG (vii), and authentic ascochlorin (viii). (C) HPLC profiles of authentic ascochlorin (i), authentic ascochlorin (ii), mycelium extracts of A. egyptiacum F-1392 (iii), ΔascE strain (iv), authentic ilicicolin A (v), and mycelium extract of ΔascE strain (vi). The yields of the compounds are summarized in SI Appendix, Table S8.
Characterization of the ascF-2 Cluster Genes. To link the candidate genes ascHIJ to AF biosynthesis, each candidate gene was disrupted in the A. egyptiacum ΔascF strain accumulating the precursor 7. HPLC analyses of these double disruptants showed that ΔascF/ΔascH and ΔascF/ΔascI could not produce 1, indicating that AscH and AscI are essential for AF biosynthesis, although ΔascF/ΔascI could produce it (0.41 g/L; SI Appendix, Table S8) (Fig. 4B). In contrast, the cell-free homogenate from the A. sojae strain expressing ascE, H, and I did not convert 6 into 1, while the addition of the ascH homogenate to the mixture sufficed to produce 1 (SI Appendix, Fig. S11). Taken together, AscI is also required for the biosynthesis, although its function is compensated by the endogenous dehydrogenase in A. egyptiacum. These results clearly demonstrated that AscHIJ are responsible for AF biosynthesis.

The double disruptant ΔascF/ΔascI specifically accumulated a new compound 9 (0.05 g/L, SI Appendix, Table S8) (Fig. 4B), which was isolated from the large-scale culture, and analyzed by HR-MS and HR-MS(T). Its molecular formula was established as...
C22H33ClO3s from the HR-MS data (m/z 421.1799, calc. 421.1787; SI Appendix, Fig. S8 and Table S7), indicating that it contains an additional oxygen compared with 7. The NMR data revealed that 7 and 9 are very similar to each other, but one methylene signal (δH: 2.01/2.05 ppm; δC: 36.4 ppm) disappeared and one oxygen methine signal (δH: 4.20 ppm; δC: 75.5 ppm) appeared in 9 (SI Appendix, Fig. S12 and Table S4). Based on the association with the spin system of H-17/H-18, we assigned this signal as H-16. Notably, under acidic condition, 9 was non-enzymatically converted into ascofuranol (10), whose planar structure was identified by HR-MS and NMR analyses, and its absolute configuration was determined as (16S,18S)-10 by the Mosher and NOESY analyses (SI Appendix, Figs. S13 and S14 and Table S6). The absolute configurations of 7 and 9 were thus the same to be (16S)-7 and (16S,18S)-9, respectively.

We further conducted in vitro assays to attribute the succeeding reactions to the candidate genes. The microsomal fraction of the yeast expressing ascI efficiently converted 9 into 10, while that from the yeast harboring a blank vector did not afford any product (Fig. 4C). The Km and Vmax values of AscI were 50.4 ± 11.1 μM and 129 ± 13 nmol/min, respectively. In addition, we found that the cell-free homogenate of A. sojae expressing ascI dehydrogenizes 10 into 1 (Fig. 4D). Thus, we demonstrated that 7 is hydroxylated by the P450 monoxygenase AscH, and the resultant 9 is cyclized by AscI to 10, which is oxidized into 1 by AscJ (Fig. 2).

Next, we investigated the reaction mechanism of the meroterpenoid TPC AscI, which lacks significant sequence similarity to any known TPCS. Sequence alignment revealed that AscI also harbors several conserved acidic residues that are thought to be essential for the catalysis (36) (SI Appendix, Fig. S15). Indeed, the alanine substitutions of AscI D61, E103, D296, E353, and D355 resulted in significant loss of activities (SI Appendix, Fig. S16). Notably, D61, D296, and E353 are also conserved in AurD (SI Appendix, Fig. 2C). Collectively, the three genes, ascH-J, in the asc-2 cluster encode the late-step biosynthetic enzymes, indicating that AF biosynthesis represents another rare example of a fungal multilocus biosynthetic cluster. Although there have been a few reports (51-54), there is still no established strategy for exploring such clusters. In the cases of prenyl xanthone and austinol, seeking the gene candidates based on the homology of the characteristic enzymes, a nonreducing PKS and a prenyltransferase, successfully led to the identification of the separate clusters, because there were only a few paralogs in the genome (52, 54). This was not the case for 1, since more than 100 genes encode P450 monoxygenases in the genome of A. egyptiacum, and the TPC, which is usually considered as a characteristic core enzyme, has not been known. The expression analysis was shown to be useful for the motif-independent identification of the fungal biosynthetic gene clusters, although previous studies exploited many sample conditions for the expression analysis and dedicated algorithms (55, 56). This study indicated that, even with a simple comparison of producing and nonproducing conditions, the expression analysis is a powerful method to identify the split gene clusters when combined with a motif-based approach.

Coregulation of the Multilocus Gene Clusters by AscR. Last, we analyzed the coregulation of the multilocus AF biosynthetic genes. Since the putative DNA-binding protein AscR was highly expressed in AF medium (SI Appendix, Table S2), it is predicted to be a transcription factor positively regulating the expression of the asc-1 cluster genes. An A. egyptiacum strain constitutively expressing AscR was constructed to verify this, and it produced both 1 and 2 in F1 medium (SI Appendix, Fig. S17A), suggesting the coregulation of the asc-1 and asc-2 clusters by AscR. This was further corroborated by the fact that all of the promoters of ascA-J possessed the shared sequence motif of TGGGYGNNNTW detected by MEME (SI Appendix, Fig. S17B), containing the CGG nucleotide triplet essential for the DNA binding of transcription factors with the same ZnCys2 binuclear cluster (57). In fact, this motif was not present in the promoters of the three preceeding genes in the asc-1 cluster, which showed little or no expression in AF medium. We thus consider that AscR positively regulates the expression of both the asc-1 and -2 cluster genes by binding to this motif.

The evolutionary origin of fungal multilocus biosynthetic clusters has also been a matter of debate. In the cases of the biosyntheses of aflatoxin and austinol, ancestrally single-locus clusters were presumably divided into two separated loci by chromosomal rearrangement, supported by the presence of the relict copy in one cluster and the functional gene in the other cluster (51, 52). However, phylogeny and synteny analyses of trichothecene biosynthesis genes revealed that ancestrally separated genes could work with the major biosynthetic cluster as a coregulated pathway, and, depending on the species, were later merged into the major cluster (53). For AF biosynthesis, there was no trace of past duplication suggesting a split into the asc-1 and -2 clusters. BLAST searches revealed partially syntenic clusters of the asc-1 cluster in several related fungi of the class Sordariomycetes, but there is no asc-2 syntenic cluster. Interestingly, the asc-1 syntenic cluster in Coniella lustricola also contains the ascH ortholog (PSR83571; 50% amino acid identity), whereas the fungus does not encode a plausible ascI ortholog. Since the orthologs of the ascH and asc-1 cluster genes are widely distributed among Sordariomycetes, ascH is likely to play another role in the biosynthesis of AC-related compounds. Given that the report of AF-producing fungi (13) and the distribution of ascH orthologs were biased to the class Eurotiomycetes, we assume that ascH was horizontally transferred and grafted upon the preexisting ascH to make a coregulated asc-2 cluster, although the elucidation will require further detailed investigation including synteny and phylogeny analyses using a wide range of fungal species.

Conclusions

We clarified the entire biosynthetic pathways of ascofuranone (1) and aschochlorin (2) in A. egyptiacum (Fig. 2). The biosyntheses of 1 and 2 share the common pathway up to the generation of illicilocin A epoxide (7). Notably, the biosynthetic genes of 1 and 2 are localized at distinct chromosomal regions, but all of their promoters share a common conserved motif, and they are probably regulated by the transcriptional factor, AscR. This study thus contributes to increasing the knowledge on meroterpenoid biosynthesis and demonstrates the power of a differential expression analysis for exploring multilocus biosynthetic clusters. From a clinical viewpoint, the elucidated genes, as well as the established method for the genetic manipulation of the strain F-1392, will be the keys for the drug development of 1. We have already established the ascF-deleted strain producing exclusively 1, but not 2 (Fig. 3C), and further manipulation will lead to the cost-effective industrial production of ascofuranone.

Materials and Methods

A. egyptiacum (synonym: A. sclerotigenum) strain F-1392 (13, 14) is a derivative of the nitrosoguanidine-induced mutant no. 34, characterized in the original paper reporting 1 (2). The differential expression analysis was performed with an Ion PGM system, and the raw sequence reads and the expression profile per gene were deposited in the DDBJ under the accession nos. DR4008136 and E-GEAD-2822, respectively. A. egyptiacum gene disruptions were obtained from the newly established Δku70ΔpyrG strain by homologous recombination, after transformation by the protoplast–polyethylene glycol method (58). Aspergillus oryzae NSAR1 (59) (niaD+, sc–, ΔargB, adeA+) was used as the host for the heterologous coexpression of ascA-D, and Aspergillus sojae P6-1 (ΔpyrG) (39) was used as the host for the high-copy heterologous expression of ascJ. The products from mycelia of each transformant were extracted with acetone, and the in vitro reaction products were extracted with ethyl acetate. The extracts were analyzed by UV–HPLC and liquid chromatography–MS, using ODS columns with standard chromatographic methods. The purified products were monitored by NMR analyses, including 1H-NMR, 13CNMR, heteronuclear multiple bond coherence,
We thank Prof. Katsuya Gomi (Tohoku University) and Prof. Katsuhiko Kitamoto (The University of Tokyo) for kindly providing the expression vectors and the fungal strain. We also thank Dr. Keiko Gomi and Dr. Ryoichi Sakae (Kikkoman Corporation) for their helpful advice. This work was financially supported by Ministry of Education, Culture, Sports, Science, and Technology (METI)/Japan Society for the Promotion of Science (JSPS) KAKENHI Grants JP17H04763 (to T.A.), JP17KT0099S (to T.A.), JP17KT15679 (to Y.K.), 23117004 (to M.M.), JP16H06443 (to I.A.), JP18K19319 (to I.A.), and 26253025 (to K.K.). Japanese Initiative for Progress of Research on Infectious Disease for Global Epidemic [Grant JP18H02080020 (to Y.K.); Japan Science and Technology Agency/Natural Science Foundation of China Strategic International Collaborative Research Program (to I.A.); and Research Program on Emerging and Reemerging Infectious Diseases Grant 17k0108199J0001 (to K.K.)]. We also acknowledge support from the Program for Promotion of Basic and Applied Research Projects for Industrial innovation and from the Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries and Food Industry.

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heterogenous multiple quantum coherence, and COSY. Full experimental procedures are described in SI Appendix.