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Dioxygenases, Key Enzymes to Determine the Aglycon Structures of Fusicoccin and Brassicicene, Diterpene Compounds Produced by Fungi

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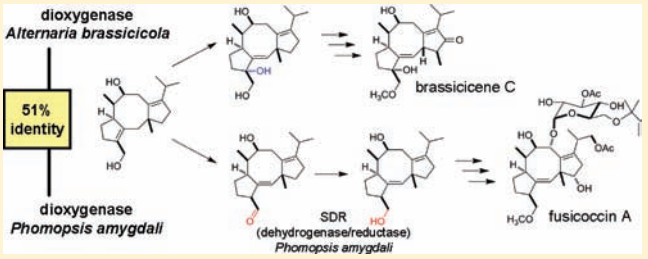
Supporting Information

ABSTRACT: Fusicoccin A and cotylenin A are structurally related diterpene glucosides and show a phytohormone-like activity. However, only cotylenin A induces the differentiation of human myeloid leukemia cells. Since the cotylenin A producer lost its ability to proliferate during preservation, a study on the relationship between structure and activity was carried out and a modified fusicoccin A with hydroxyl group at the 3-position showed a similar biological activity with that of cotylenin A. We then searched for an enzyme source that catalyzes the introduction of a hydroxyl group into the 3-position and found that brassicicene C, which is structurally related to fusicoccin A with hydroxyl group at the 3-position, was produced by *Alternaria brassicicola* ATCC96836. We recently cloned a brassicicene C biosynthetic gene cluster including the genes encoding fusicocca-2,10(14)-diene synthase and two cytochrome P450s, which were responsible for the formation of fusicocca-2,10(14)-diene-8β,16-diol. In this study, we report that a α-ketoglutarate dependent dioxygenase, the gene coding for which was located in the cluster, catalyzed a hydroxylation at the 3-position of fusicocca-2,10(14)-diene-8β,16-diol. On the other hand, a α-ketoglutarate-dependent dioxygenase, which had been identified in a fusicoccin A biosynthetic gene cluster, catalyzed the 16-oxidation of fusicocca-2,10(14)-diene-8β,16-diol to yield an aldehyde (8β-hydroxyfusicocca-1,10(14)-dien-16-al), although both dioxygenases had 51% amino acid sequence identity. These findings suggested that the dioxygenases played critical roles for the formation of the fusicoccin A-type and cotylenin A-/brassicicene C-type aglycons. Moreover, we showed that short-chain dehydrogenase/reductase located in the fusicoccin A biosynthetic gene cluster catalyzed the reduction of the aldehyde to yield fusicocca-1,10(14)-diene-8β,16-diol.



INTRODUCTION

Fusicoccin A (**1**)[1] and cotylenin A (**2**),[2] which are produced by *Phomopsis amygdali* and *Cladosporium* sp. 501-7W, respectively, are diterpene glucosides that have similar structures (Scheme 1). Both compounds activate plasma membrane H^+-ATPase leading to phytohormone-like activity.[3] Crystallographic analysis of a ternary complex comprised of a plant 14-3-3 protein, fusicoccin A (**1**), and a phosphopeptide derived from the C-terminus of H^+-ATPase revealed that fusicoccin A (**1**) stabilizes interaction between 14 and 3-3 protein and H^+-ATPase, resulting in continuous activation of H^+-ATPase.[4] However, it was recently shown that only cotylenin A (**2**) induces the differentiation of human myeloid leukemia cells and acts synergistically with IFN-α to induce apoptosis in a wide array of cancer cells.[5]

A preliminary study on the relationship between structure and activity showed that the presence of the hydroxyl group at the 3-position in cotylenin A (**2**) was perhaps important for inducing the differentiation of human myeloid leukemia cells. Since the cotylenin A (**2**) producer, *Cladosporium* sp. 501-7W, lost its ability to proliferate during preservation on a slant, we could not obtain enough cotylenin A (**2**) to be used for further analysis. Therefore, we searched for an enzyme source that catalyzes the introduction of a hydroxyl group into the 3-position. We found that *Alternaria brassicicola* ATCC96836, whose partial genome sequences have been released, had a gene cluster composed of 11 genes (Figure 1), including an orthologue of the fusicocca-2,10(14)-diene (**3**) (the first intermediate in fusicoccin A (**1**) biosynthesis) synthase and various oxygenases, namely five P450s and one dioxygenase.[6] Moreover, we confirmed that the strain ATCC96836 produced brassicicene C (**4**) which has a hydroxyl group at the 3-position (Scheme 1).

To date, we revealed that *orf8* and *orf6*, which were identified in the brassicicene C (**4**) biosynthetic gene cluster, encoded

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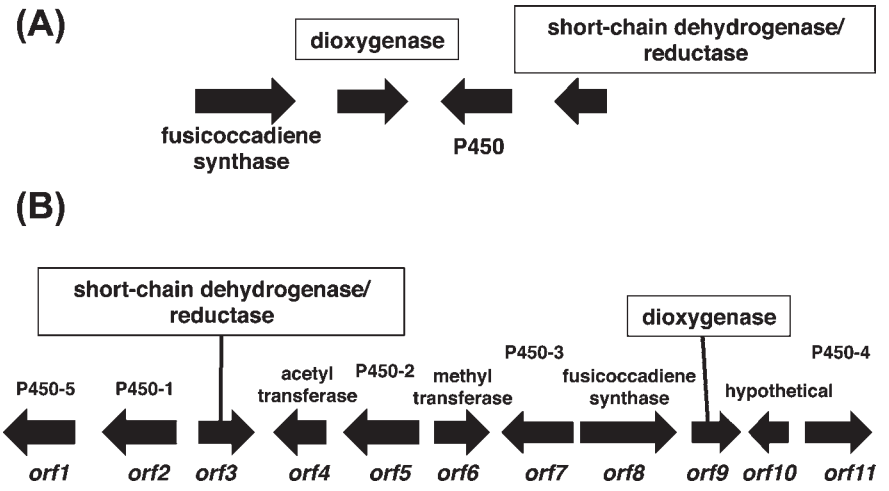
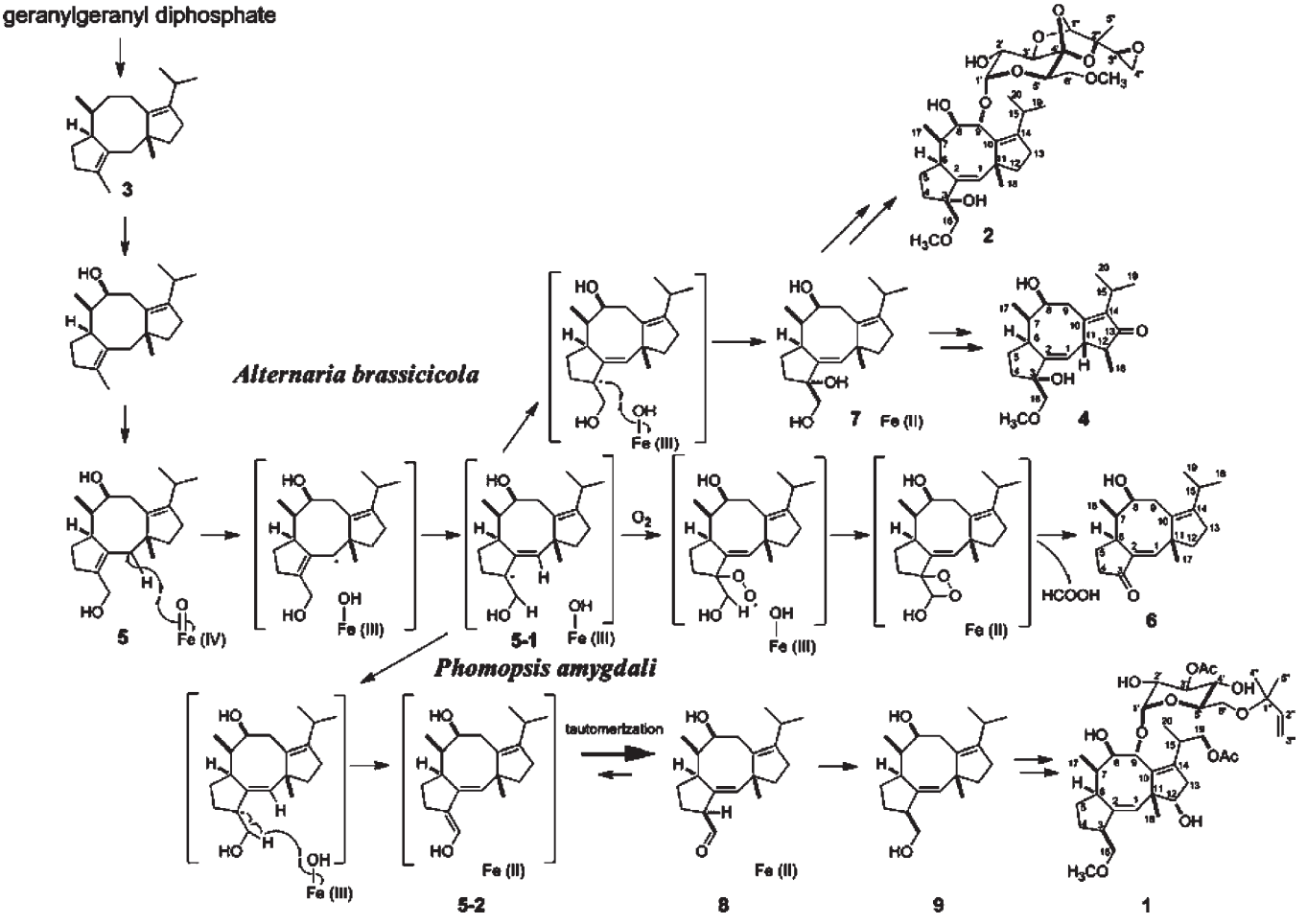


Figure 1. Fusicoccin A (**1**) (A) and brassicicene C (**4**) (B) biosynthetic gene clusters.

Scheme 1. Biosynthetic Pathways for Fusicoccin A, Cotylenin A, and Brassicicene C



fusicocca-2,10(14)-diene (**3**) synthase and 16-*O*-methyltransferase, respectively.[6] We also showed that *orf1* and *orf7*, both of which coded for cytochrome P450s, catalyzed 8β-hydroxylation and successive 16-hydroxylation, respectively, by heterologous expression of these genes in *Saccharomyces cerevisiae* and structural analysis of the compounds produced by the transformants.[7] In the cluster, there were four candidate genes that could catalyze 3-hydroxylation, three of which were cytochrome P450s (*orf2*, *orf5*, and *orf11*) and one which was a dioxygenase (*orf9*) (Figure 1).

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In this study, we examined which enzymes in the brassicicene C (**4**) biosynthetic gene cluster catalyzed a hydroxylation at the 3-position. We found that a α-ketoglutarate dependent dioxygenase catalyzed a hydroxylation at the 3-position of fusicocca-2,10(14)-diene-8β,16-diol (**5**). In contrast, a dioxygenase, which had been identified in a fusicoccin A biosynthetic gene cluster and 51% amino acid sequence identity with brassicicene-dioxygenase, catalyzed the 16-oxidation of fusicocca-2,10(14)-diene-8β,16-diol (**5**) to yield 8β-hydroxyfusicocca-1,10(14)-dien-16-al (**8**). Compound **8** was then converted into fusicocca-1,10(14)-diene-8β,16-diol (**9**) by a short-chain dehydrogenase/reductase located in the fusicoccin A biosynthetic gene cluster. Overall, these findings provide significant insights into the biosynthesis of the fusicoccin A-type and cotylenin A-/brassicicene C-type aglycons.

RESULTS AND DISCUSSION

Identification of Enzyme Catalyzing a Hydroxylation at the 3-Position of Fusicocca-2,10(14)-diene-8β,16-diol (5). First, we examined the function of cytochrome P450s (*orf2*, *orf5*, and *orf11*) by the same method as described above. Plasmids carrying the fusicocca-2,10(14)-diene (**3**) synthase gene (*orf8*), the two cytochrome P450 genes (*orf1* and *orf7*), one of the three cytochrome P450 genes (*orf2*, *orf5*, and *orf11*), and the cytochrome P450 reductase gene were constructed and introduced into *S. cerevisiae*. However, despite several attempts, we could not detect any specific products except for fusicocca-2,10(14)-diene-8β,16-diol (**5**). Moreover, we did not detect any products by an in vitro enzyme assay with fusicocca-2,10(14)-diene-8β,16-diol (**5**) and microsomes, which were prepared from *S. cerevisiae* carrying one of the cytochrome P450 gene (*orf2*, *orf5*, or *orf11*) and the cytochrome P450 reductase gene, although we recently succeeded in identifying a cytochrome P450 gene which catalyzed a hydroxylation of the 9-position of fusicocca-1,10(14)-diene-8β,16-diol (**9**) with this assay system (unpublished work).

Next, we examined the function of the brassicicene-dioxygenase (Figure 1, *orf9*). Recombinant enzyme was purified (Figure 2) and incubated with fusicocca-2,10(14)-diene-8β,16-diol (**5**) (Figures S1 and S2) in the presence of $FeSO_4$, α-ketogulutarate, and ascorbate. We detected the reaction product using HPLC analysis (Figure 3) and determined its structure (Scheme 1, Figures S3, S4, and S8). The product, 8β-hydroxy-16-norfusicocca-1,10(14)-dien-3-one (**6**), was plausibly a shunt product, formed by the elimination of the carbon at 16-position from the substrate (Scheme 1). However, this result showed that the enzyme accepted fusicocca-2,10(14)-diene-8β,16-diol (**5**) as a substrate. Therefore, we carefully searched for a product by varying analytical conditions of HPLC and found an additional product besides to the shunt product (Figure 3). The structure of the product was determined to be fusicocca-1,10(14)-diene-3,8β,16-triol (**7**) by comparing NMR and LC−MS data with those of authentic compound (Figures 3, S3, S5, and S8). The result clearly showed that brassicicene-dioxygenase catalyzed the 3-hydroxylation reaction (Scheme 1).

Characterization of a Dioxygenase-Like Gene Located in the Fusicoccin A (1) Biosynthetic Gene Cluster. We previously cloned the fusicocca-2,10(14)-diene (**3**) synthase gene from *P. amygdali* and found that some genes related to fusicoccin A (**1**) biosynthesis were located in a flanking region (Figure 1). Curiously, a dioxygenase (*orf2* product) that had a 51% amino acid sequence identity with that of brassicicene-dioxygenase (Figure 4) was included in this cluster, even though fusicoccin A (**1**) has no hydroxyl group at the 3-position. Therefore, we

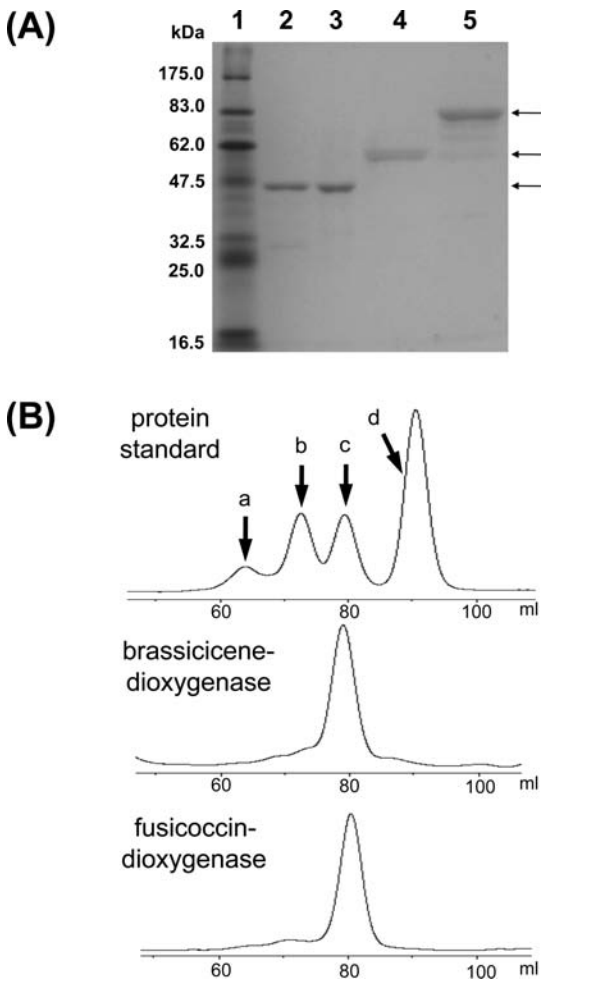

Figure 2. (A) SDS-PAGE of purified enzymes. Molecular mass marker (lane 1), brassicicene-dioxygenase (lane 2), fusicoccin-dioxygenase (lane 3), GST-tagged brassicicene-short-chain dehydrogenase/reductase (lane 4), and 6xHis-Trigerfactor (derived from pColdTF vector) -tagged fusicoccin-short-chain dehydrogenase/reductase (lane 5) were analyzed on an SDS-PAGE (10.0%). (B) Elution profile of the standard proteins (upper), the purified brassicicene-dioxygenase (middle), and the purified fusicoccin-dioxygenase (lower) are shown. Molecular weight standards [aldolase (a, 158 kDa), conalbumin (b, 75.0 kDa), ovalbumin (c, 43.0 kDa), chymotrypsinogen A (d, 25.0 kDa)] were used.

examined the function of this fusicoccin-dioxygenase with a recombinant enzyme. Recombinant enzymes were prepared by the same method as those of brassicicene-dioxygenase (Figure 2) and used for the in vitro assay. Under the same assay condition as that of brassicicene-dioxygenase, we again detected the formation of the shunt product, 8β-hydroxy-16-norfusicocca-1,10(14)-dien-3-one (**6**) (Figure 3). However, no 3-hydroxylated product such as fusicocca-1,10(14)-diene-3,8β,16-triol (**7**) was formed. Therefore, we tried to identify the intrinsic product by varying the reaction conditions, and we detected a new peak by HPLC analysis within 5 min of the reaction. A prolonged incubation resulted in the dominant formation of (**6**) (Figure 3). Since the amounts of the new product formed by the enzyme reaction were very small and the product was unstable, it was difficult to determine the structure. By NMR analysis, however, the compound was suggested to be 8β-hydroxyfusicocca-1,10(14)-dien-16-al (**8**). To confirm this, we chemically synthesized **8** (Figure S1, S6, and S8). By comparing NMR data, we concluded that the compound was indeed **8**.

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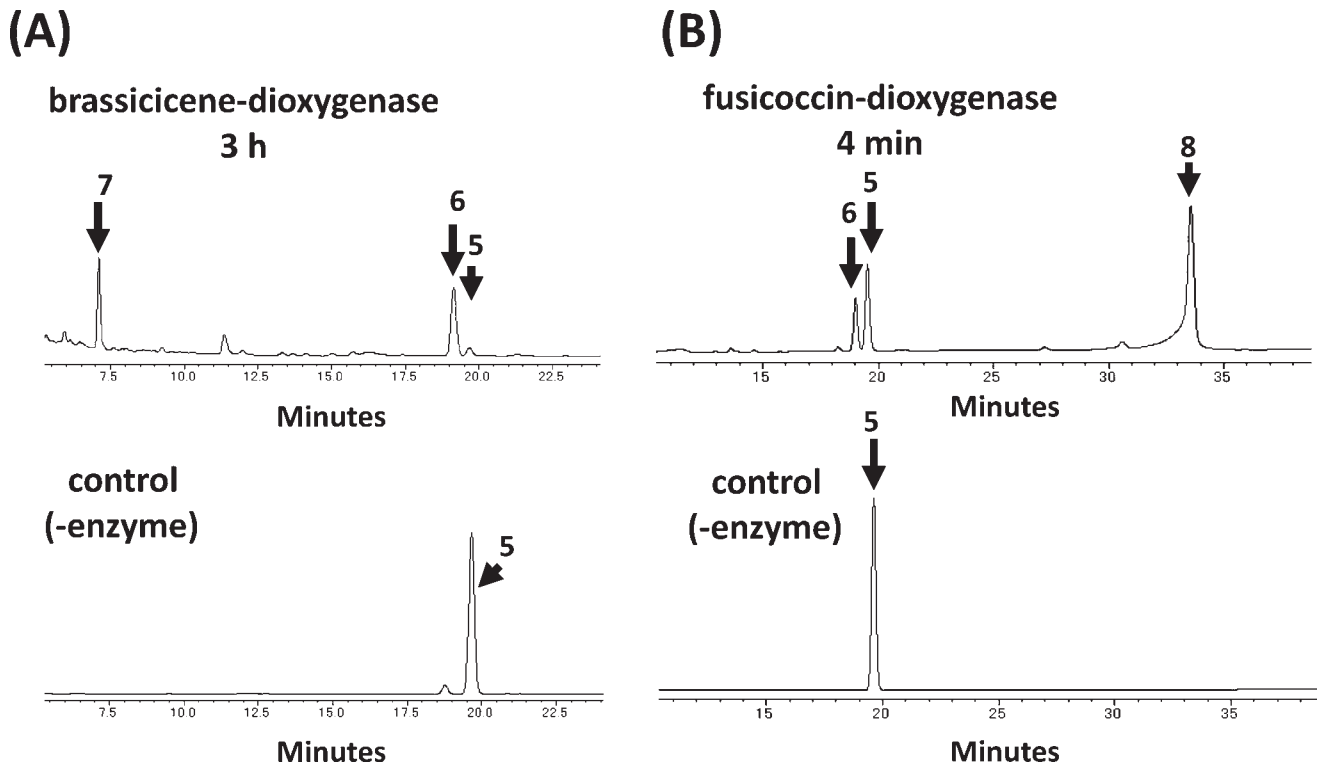

Figure 3. HPLC analyses of the products formed from fusicocca-2,10(14)-diene-8β,16-diol (**5**) by brassicicene-dioxygenase (A) and fusicoccin-dioxygenase (B). The products formed by 3 h (A) and 4 min (B) incubation were analyzed by HPLC. The product detected in (A) was confirmed to be fusicocca-1,10(14)-diene-3,8β,16-triol (**7**) with LC−MS analysis (Figure S3, S5, and S8). The product detected in (B) was confirmed to be 8β-hydroxyfusicocca-1,10(14)-dien-16-al (**8**) with NMR analysis (Figure S1, S6, and S8).

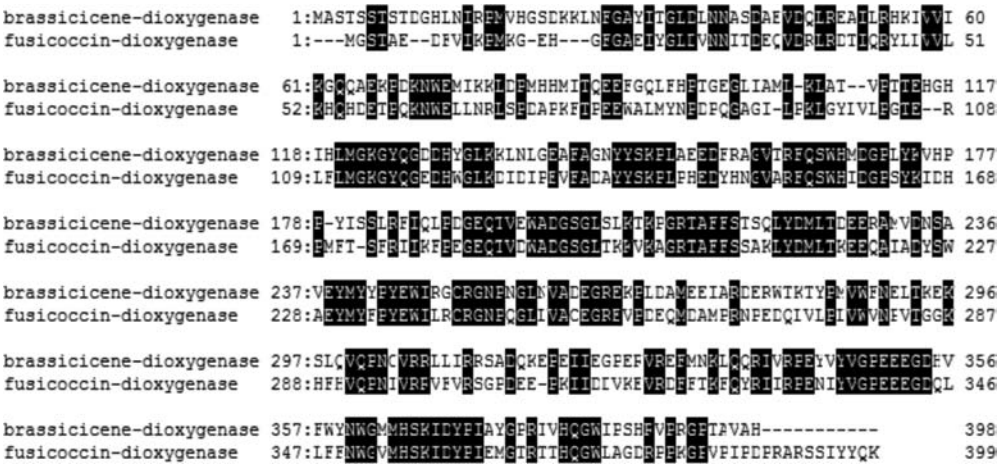

Figure 4. Alignment of brassicicene-dioxygenase and fusicoccin-dioxygenase.

Enzymatic Properties of the Brassicicene-Dioxygenase and Fusicoccin-Dioxygenase. As described, fusicoccin-dioxygenase and brassicicene-dioxygenase had a 51% amino acid sequence identity and accepted fusicocca-2,10(14)-diene-8β,16-diol (**5**) as a substrate. However, the reaction products were different from each other. We therefore investigated enzymatic properties of both enzymes. We first investigated the quaternary structure of recombinant dioxygenases because monomeric, dimeric and trimeric dioxygenase enzymes were reported.[8] The purified fusicoccin-dioxygenase (46.0 kDa) and brassicicene-dioxygenase (45.7 kDa) were subjected to gel filtration. A major peak, corresponding to molecular masses of approximately 40 kDa (the former enzyme) and 46 kDa (the latter enzyme), was detected, (Figure 2), suggesting both dioxygenases existed as a form of monomer.

The optimum pH was measured in several buffers at various pH values (final concentration of 0.05 M): citrate buffer, pH 5.0−5.8; bis-tris (Good's buffer) buffer, pH 5.8−6.9; HEPES (Good's buffer) buffer, pH 6.9−8.2; Tris buffer, pH 8.2−9.0. The optimum pH of brassicicene-dioxygenase is 6.0 and that of fusicoccin-dioxygenase is 6.3. The effect of temperature on enzyme activity was also investigated over a range of 20−45 °C in 0.05 M bis-tris buffer, pH 6.0 (brassicicene-dioxygenase) or pH 6.3 (fusicoccin-dioxygenase). Both of enzyme activities were maximal at 30 °C. Kinetic properties of the both enzymes were studied under the

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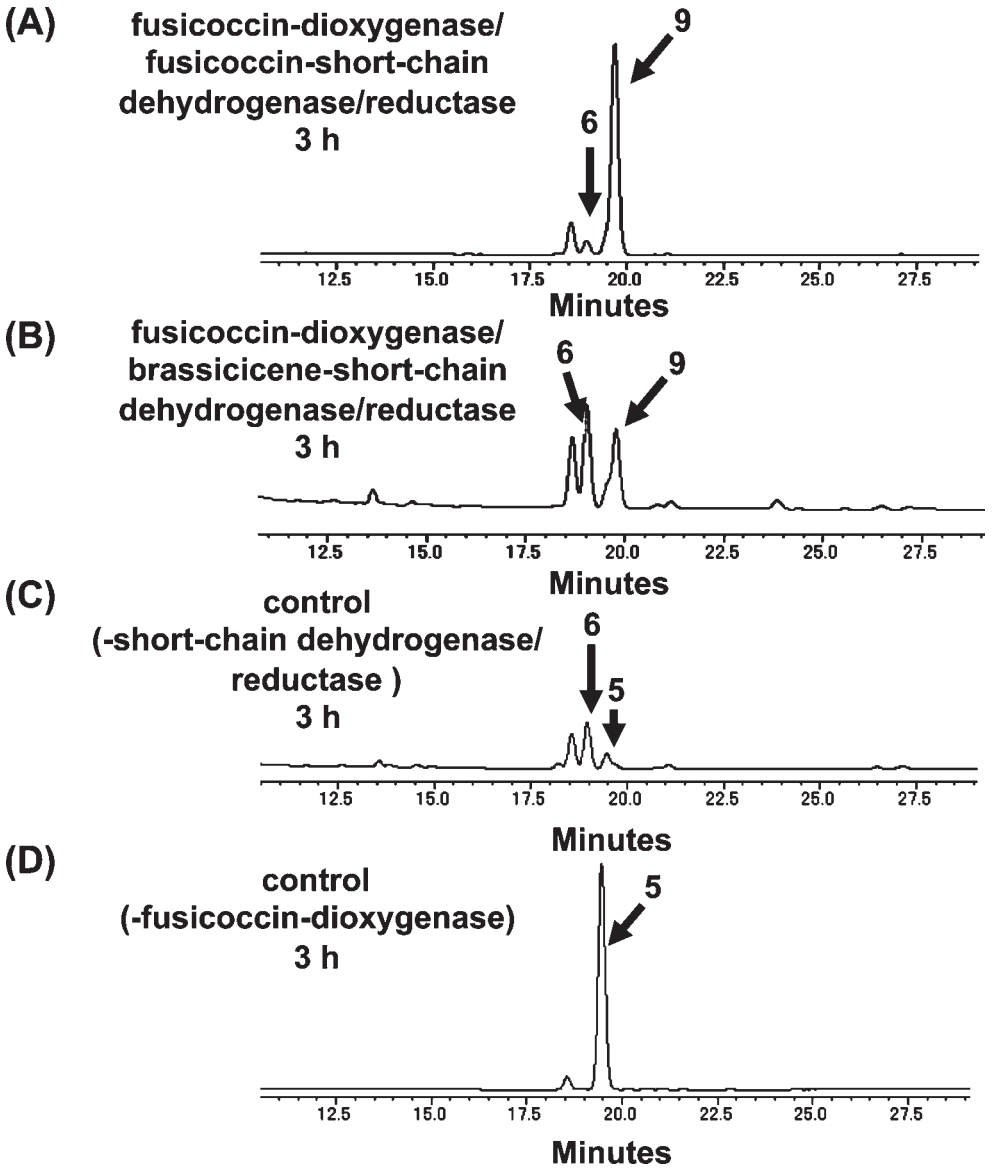

Figure 5. HPLC analysis of the products formed from 8β-hydroxyfusicocca-1,10(14)-dien-16-al (**8**) by sequential reaction assays using fusicoccin-dioxygenase/short-chain dehydrogenase/reductase (A) and fusicoccin-dioxygenase/brassicicene-short-chain dehydrogenase/reductase (B). Both of the products were confirmed to be fusicocca-1,10(14)-diene-8β,16-diol (**9**) with NMR analysis (Figure S1, S7, and S8).

optimum reaction conditions. The K_m value and k_{cat}value of brassicicene-dioxygenase were calculated to be $113 \pm 14\ \mu$M for the substrate (**5**) and $1.5 \times 10^{-1} \pm 1.6 \times 10^{-2}$/s, respectively. On the other hand, The K_m value and k_{cat} value of fusicoccin-dioxygenase were $124 \pm 4.1\ \mu$M and $5.7 \times 10^{-1} \pm 4.1 \times 10^{-2}$/s, respectively. The two enzymes showed almost the same K_m values. In contrast, the k_{cat} value of the fusicoccin-dioxygenase was 4 times as high as that of brassicicene-dioxygenase. These results were in good agreement with the rapid substrate-consumption/product-formation by fusicoccin-dioxygenase (we could detect **6** and **8** within a few minutes, Figure 3B).

Characterization of a Short-Chain Dehydrogenase/Reductase Gene Located in the Fusicoccin A (1) Biosynthetic Gene Cluster. Considering the structure of fusicoccin A (**1**), aldehyde at 16-position of 8β-hydroxyfusicocca-1,10(14)-dien-16-al (**8**) should be reduced to give alcohol, fusicocca-1,10(14)-diene-8β,16-diol (**9**). We previously identified a short-chain dehydrogenase/reductase gene (Figure 1, *orf4*) close to the dioxygenase gene (*orf2*). Therefore, we examined whether this gene product was responsible for the reduction of 8β-hydroxyfusicocca-1,10(14)-dien-16-al (**8**). Recombinant fusicoccin-short-chain dehydrogenase/reductase was expressed and used for an in vitro assay after purification (Figure 2). Since 8β-hydroxyfusicocca-1,10(14)-dien-16-al (**8**) was unstable as described above, we performed a sequential enzymatic assay with the fusicoccin-dioxygenase and fusicoccin-short-chain dehydrogenase/reductase as the catalysts and fusicocca-2,10(14)-diene-8β,16-diol (**5**) as the substrate. Considering the structural similarity between the substrate, fusicocca-2,10(14)-diene-8β,16-diol (**5**) and the plausible product, fusicocca-1,10(14)-diene-8β,16-diol (**9**), we expected that both compounds would have similar retention times when eluted using HPLC analysis. To overcome this, we used a high concentration of the fusicoccin-dioxygenase for the assay. Under this condition, all of the substrate (**5**) was consumed by the fusicoccin-dioxygenase

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alone (Figure 5C). Using the sequential enzymatic assay, a reaction product was detected by HPLC at almost the same retention time as that of the substrate (Figure 5A) and its structure was confirmed to be fusicocca-1,10(14)-diene-8β,16-diol (**9**) (Figure S1, S7, and S8). In summary, the overall reaction catalyzed by the fusicoccin-dioxygenase and fusicoccin-short-chain dehydrogenase/reductase was the transfer of the double bond from the C-2,3 position to the C-1,2 position.

Characterization of a Short-Chain Dehydrogenase/Reductase Gene Located in the Brassicicene C (4) Biosynthetic Gene Cluster. In the brassicicene C (**4**) biosynthetic gene cluster, we found a putative short-chain dehydrogenase/reductase gene (Figure 1, *orf3*) that did not have similarity to the above-mentioned short-chain dehydrogenase/reductase gene (*orf4*). However, as described, the brassicicene-dioxygenase catalyzed the 3-hydroxylation of fusicocca-2,10(14)-diene-8β,16-diol (**5**) to yield fusicocca-1,10(14)-diene-3,8β,16-triol (**7**), and the hydroxyl group at the 16-position is destined to be methylated to form brassicicene C (**4**). Therefore, no short-chain dehydrogenase/reductases are required for the biosynthesis of brassicicene C (**4**). To know the function of the brassicicene-short-chain dehydrogenase/reductase, we examined if the gene (*orf3*) was transcribed in the brassicicene C (**4**) producer. We were able to obtain cDNA with a poly-T, suggesting that the gene was indeed transcribed. Then, we examined whether recombinant brassicicene-short-chain dehydrogenase/reductase has the same catalytic activity as that of the fusicoccin-short-chain dehydrogenase/reductase. The above-mentioned sequential enzymatic assay was conducted with recombinant brassicicene-short-chain dehydrogenase/reductase instead of the fusicoccin-enzyme. We detected the formation of fusicocca-1,10(14)-diene-8β,16-diol (**9**) (Figure 5B) though the amount of product was smaller than that with the fusicoccin-enzyme. The result suggested that a trace amount of 8β-hydroxyfusicocca-1,10(14)-dien-16-al (**8**) might be formed by the brassicicene-dioxygenase in addition to fusicocca-1,10(14)-diene-3,8β,16-triol (**7**) and thus formed **8** might be converted into fusicocca-1,10(14)-diene-8β,16-diol (**9**) by the brassicicene-short-chain dehydrogenase/reductase. Therefore, we carefully examined if the brassicicene-dioxygenase could convert fusicocca-2,10(14)-diene-8β,16-diol (**5**) into 8β-hydroxyfusicocca-1,10(14)-dien-16-al (**8**). Consequently, using HPLC analysis, we detected a very small peak that eluted at the same retention time as that of **8** (Figure 6). The amount of product was too small to determine its structure even by LC−MS analysis. Considering that the brassicicene-short-chain dehydrogenase/reductase gene (*orf3*) was transcribed in vivo and the recombinant enzyme showed the same catalytic function as that of the fusicoccin enzyme, the biosynthetic route from fusicocca-2,10(14)-diene-8β,16-diol (**5**) to fusicocca-1,10(14)-diene-8β,16-diol (**9**) might also be operating as a minor pathway even in the brassicicene C (**4**) producer.

Reaction Mechanism Catalyzed by the Two Dioxygenases. We summarized the putative reaction mechanisms of the two dioxygenases in Scheme 1. In cotylenin A (**2**) and brassicicene C (**4**) biosynthesis, Fe(IV)═O species abstracts an H-atom at the C-1 position to generate a substrate radical and Fe(III)−OH species. After isomerization of the radical species to **5**−**1**, brassicicene-dioxygenase/Fe(III)−OH catalyzes the hydroxylation of **5**−**1** to afford key intermediate **7**. In contrast, in fusicoccin A (**1**) biosynthesis, fusicoccin-dioxygenase/Fe(III)−OH abstracts the second H-atom at the 16-position of **5**−**1** to form dienol **5**−**2**. Then, it is converted into keto-form

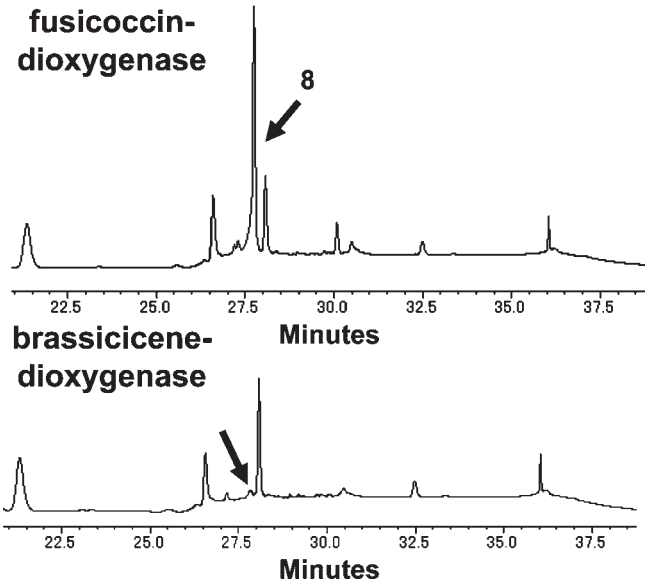

Figure 6. HPLC analysis of the products formed from fusicocca-2,10(14)-diene-8β,16-diol (**5**) by brassicicene-dioxygenase. The reaction mixture was incubated for 3 min, and the products were immediately analyzed by HPLC.

8β-hydroxyfusicocca-1,10(14)-dien-16-al (**8**) by keto−enol tautomerism. Once **8** is formed, fusicoccin-short-chain dehydrogenase/reductase might immediately reduce the aldehyde moiety of **8** into the alcohol to form fusicocca-1,10(14)-diene-8β,16-diol (**9**) as exemplified by the sequential enzymatic assay, because β, γ-unsaturated aldehyde **8** can easily be isomerized to the thermodynamically stable α, β-unsaturated aldehyde. The presence of intermediate **5**−**1** in both dioxygenase reactions was suggested by the formation of 8β-hydroxy-16-norfusicocca-1,10(14)-dien-3-one (**6**). The reaction mechanism of the formation of **6** was thought to be that shown in Scheme 1. Both dioxygenases catalyze the incorporation of molecular oxygen to afford a peroxide intermediate in a manner similar to that of FtmOx1, which was recently shown to catalyze the insertion of an endoperoxide bond between two prenyl moieties in verruculogen biosynthesis,[10] and then the elimination of formic acid from the peroxide intermediate yields the side product 8β-hydroxy-16-norfusicocca-1,10(14)-dien-3-one (**6**).

■ CONCLUSION

It was unclear what enzyme introduced the hydroxyl group at the 3-position and how the C-1,2 double bond was formed from fusicocca-2,10(14)-diene-8β,16-diol (**5**) which has a C-2,3 double bond. In this study we clearly showed that the dioxygenases played critical roles in the formation of the fusicoccin A-type and cotylenin A-/brassicicene C-type aglycons. Moreover, in the case of fusicoccin A (**1**) biosynthesis, fusicoccin-short-chain dehydrogenase/reductase was shown to also be the key enzyme to make the biosynthetic progress against the thermodynamic stability.

■ EXPERIMENTAL PROCEDURES

Materials. α-Ketoglutarate, ascorbate, and $FeSO4\cdot7H_2O$ were purchased from Wako Pure Chemical (Japan). NADPH and NADH were from Oriental Yeast Co., Ltd. (Japan).

Strains, Cultures, Media, and Preparation of cDNA. *P. amygdali* Niigata-2 (N2) and *A. brassicicola* ATCC96836 were used

for the preparation of cDNAs. The former strain was grown in 100 mL of medium containing 5% sucrose, 0.7% soy flour, 0.5% KH_2PO_4 and 0.1% $MgSO_4 \cdot 7H_2O$ at 25 °C for 4 days in a reciprocal shaker (120 strokes/min). The latter strain was cultivated in 100 mL of medium containing 0.5% Pharmamedia (Traders Protein, U.S.A), 0.2% Yeast Extra (Oriental Yeast), 3% saccharose, 0.5% KH_2PO_4, and 0.1% $MgSO_4 \cdot 7H_2O$ at 25 °C for 4 days in a reciprocal shaker (120 strokes/min). Then 6 mL of the preculture was inoculated into a medium containing 0.5% Yeast Extra (Oriental Yeast), 5% saccharose, 0.5% KH_2PO_4, and 0.1% $MgSO_4 \cdot 7H_2O$ and grown at 25 °C for 6 days in a reciprocal shaker (120 strokes/min). The mycelia were filtered using a plastic filter and dried on paper towels. They were then frozen and homogenized with an SK-mill (Tokken Inc., Japan). The total RNA of the strain was isolated using the TRIzol reagent (Invitrogen, U.S.A.) according to the manufacture's protocol. The fragments containing 5′- or 3′-termini of cDNA were obtained using the SMART RACE cDNA Amplification Kit (Clontech, U.S.A.) and GeneRacer Kit (Invitrogen).

Cloning and Construction of Expression Plasmids of Brassicicene-Dioxygenase, Fusicoccin-Dioxygenase, Fusicoccin-Short-Chain Dehydrogenase/Reductase and Brassicicene-Short-Chain Dehydrogenase/Reductase. The coding region of the brassicicene-dioxygenase (accession no. AB570430) gene was determined using the RACE (rapid amplification of cDNA end) method. Then, approximately 1.2-kb of full length DNA fragment was amplified with *A. brassicicola* 5′-Ready cDNA as the template and a primer set of 5′-GCTCTAGAGAAGATCTCGCTTCCACCAGTTC-CACTAGCACGGAT-3′ and 5′-GGGGGTACCCCTTAATGAGCC-ACCGCTGTTGGCCCTCG-3′. The *Xba*I-*Kpn*I-digested fragment was ligated to the same enzymes-digested pUC118 to yield pUCBCDOX. After the sequence was confirmed, the *Bgl*II-*Kpn*I fragment from pUCBC-DOX was ligated to the same site of pRSFDuetI to make pRSFBCDOX.

The 1.2-kb fragment including the fusicoccin-dioxygenase gene (accession no. AB570428), the coding region of which was determined using the RACE method, was amplified with *P. amygdali* 5′-Ready cDNA as the template and a primer set of 5′-GGGGGATCCCATATGGGATCGAC-TGCTGAAGACT-3′ and 5′-AAAACTGCAGCTCGAGTCACTTCT-GATAATAGATG-3′. The *Bam*HI-*Pst*I-digested PCR product was ligated to the same site of pUC118 to yield pUCFCDOX. After the sequence was confirmed, the *Nde*I-*Xho*I fragment from pUCFCDOX was ligated to the same site of pRSFDuetI to make pRSFFCDOX.

The coding region of the fusicoccin-short-chain dehydrogenase/reductase gene (accession no. AB570429) was determined using the RACE method. The 1.0-kb fragment was amplified with *P. amygdali* 5′-Ready cDNA as the template and a primer set of 5′-CGGGAGCTCCC-GATGTTCGGCAGCTTCAACTCC-3′ and 5′-CCCAAGCTTGGC-TAGACATACGGCTTGACTTGTTC-3′. The *Sac*I-*Hin*dIII-digested PCR product was ligated to the same site of pUC118 to yield pUCF-CSDR. After the sequence was confirmed, the *Sac*I-*Hin*dIII fragment from pUCFCSDR was ligated to the same site of pColdTF to make pCTFFCSDR.

The coding region of the brassicicene-short-chain dehydrogenase/reductase gene (accession no. AB570249) was determined using the RACE method. The 0.8-kb fragment was amplified with *A. brassicicola* 5′-Ready cDNA as the template and a primer set of 5′-GGAATTCGAG-CTCTCGCAATCCACTCTAGTGATAA-3′ and 5′-AAAACTGCAG-CTAGGAGCTAGTATCCCTGCTAAGG-3′. The *Eco*RI-*Pst*I-digested PCR product was ligated to the same site of pUC118 to yield pUCB-CSDR. After the sequence was confirmed, the *Eco*RI-*Pst*I fragment from pUCBCSDR was ligated to the same site of pET41a to make pETBCSDR.

Expression and Purification of Brassicicene-Dioxygenase and Fusicoccin-Dioxygenase. *E. coli* BL21 (DE3) cells harboring pRSFBCDOX or pRSFFCDOX were grown overnight at 37 °C (5 mL of L-broth containing 50 μg/mL of kanamycin) and then used for inoculation. The scaled-up cultures (1 L of L-broth containing 50 μg/mL of kanamycin) were grown at 37 °C until the absorbance at 600 nm reached a value of 0.5−1.0. IPTG was then added to a final concentration of 1 mM, and the culture was grown overnight at 37 °C. Cells were harvested by centrifugation, washed, and resuspended in lysis buffer (10 mM MOPS (Good's buffer) (pH 6.9), 10% (w/v) glycerol). After disruption by sonication, the lysate was centrifuged at 12,000 rpm at 4 °C for 30 min (R19A rotor, Hitachi, Japan). The supernatant was subjected to DEAE sepharose column chromatography (GE Healthcare, US) at 4 °C. The recombinant enzymes were eluted with a linear gradient from 0 to 0.4 M NaCl in lysis buffer. The fractions containing brassicicene-dioxygenase or fusicoccin-dioxygenase were collected and desalted using an Ultracel YM-10 membrane (Millipore, U.S.A.). The protein concentration was determined using a NanoVue Spectrophotometer (GE Healthcare). Purity of the enzymes was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The apparent molecular mass was estimated by gel filtration using a HiLoad 16/60 Superdex 200 prep grade (16 mm ×600 mm) column (GE Healthcare) that had been equilibrated with 0.02 M MOPS buffer (pH 6.9) containing 0.15 M NaCl.

Expression and Purification of Fusicoccin-Short-Chain Dehydrogenase/Reductase and Brassicicene-Short-Chain Dehydrogenase/Reductase. Fusicoccin-short-chain dehydrogenase/reductase and brassicicene-short-chain dehydrogenase/reductase were also prepared by essentially the same methods as those of the dioxygenases except for the following: the supernatant of fusicoccin-short-chain dehydrogenase/reductase was subjected to the ProfiniaProtein Purification System (Bio-Rad, US) at 4 °C. His-tagged enzyme was eluted and desalted using the standard protocol. The supernatant of brassicicene-short-chain dehydrogenase/reductase was subjected to glutathione sepharose 4B (GE Healthcare) and GST-tagged enzyme was eluted and desalted using the standard protocol. After glycerol (final concentration of 10% (w/v)) was added into the enzyme solutions, it was frozen and stored at −80 °C.

Dioxygenase Assay and Dioxygenase/Short-Chain Dehydrogenase/Reductase Sequential Enzymatic Reaction Assay. The activities of dioxygenases were measured using a modified α-ketoglutarate dependent dioxygenase assay.[11] The reaction mixtures contained 20 mM MOPS buffer (pH 6.9), 10% (w/v) glycerol, 111 μM fusicocca-2,10(14)-diene-8β,16-diol (**5**), 250 μM FeSO4·7H_2O, 2 mM α-ketoglutarate, 1 mM ascorbate, and appropriate enzymes in a final volume of 100 μL. After incubation at 30 °C, the reaction was terminated by adding 100 μL of methanol and mixed by vortex for 30 s. After centrifugation (15,000 rpm, 4 °C for 10 min, RA50-JS rotor, Kubota, Japan), part of the supernatant was used for HPLC analysis.

The activitiy of short-chain dehydrogenase/reductase was measured using a sequential enzymatic assay with dioxygenase as the catalysts and fusicocca-2,10(14)-diene-8β,16-diol (**5**) as the substrate. fusicoccin-dioxygenase and fusicoccin-short-chain dehydrogenase/reductase (or brassicicene-short-chain dehydrogenase/reductase) were added to the reaction mixture (100 μL) containing 50 mM Tris-HCl (pH 8.0), 300 mM KCl, 10% (w/v) glycerol, 111 μM fusicocca-2,10(14)-diene-8β,16-diol (**5**), 250 μM $FeSO_4 \cdot 7H_2O$, 2 mM α-ketoglutarate, 1 mM ascorbate, 1 mM NADH and 1 mM NADPH. The incubation conditions and assay method were the same as those of the dioxygenase assay. The HPLC was used for analysis of reaction product and its condition was as follows: Meark Mightisil RP-18GP Aqua column (250 mm × 4.6 mm) (Kanto Chemicals, Japan); mobile phase where the concentration of acetonitrile in water was stepwise increased (0 min, 45%; 35 min, 65%, 40 to 45 min, 100%); flow rate, 1.0 mL/min; detection, 205 nm.

Semisynthesis of Fusicocca-2,10(14)-diene-8β,16-diol (5), 8β-Hydroxyfusicocca-1,10(14)-dien-16-al (8), and Fusicocca-1,10(14)-diene-8β,16-diol (9). Synthesis procedures for the substrate, **5**, and authentic samples of the enzymatic products, **8** and **9**, have been reported in the Supporting Information of our previous report.[12] All of these compounds were semisynthetically prepared from

fusicoccin H,[1] a minor metabolite of *P. amygdali*. The synthesis procedures are summarized in Figure S1 and will be reported elsewhere in detail.

Fusicocca-2,10(14)-diene-8β,16-dio (**5**): colorless prisms (recrystallized from *n*-hexane/Et_2O); mp 167.7−169.2 °C; ^{1}H NMR ($CDCl_3$, 400 MHz. Because of the conformational mobility of **5**, NMR signals are heavily broadened. Therefore, only diagnostic signals are recorded here.): δ 0.91 (3H, s), 0.92 (6H, d, *J* = 6.8 Hz), 1.00 (3H, br d, *J* = 6.8 Hz), 2.13 (1H, dd, *J* = 13.1, 12.1 Hz), 2.37 (1H, dm, *J* = 13.1 Hz), 3.82 (1H, dt, *J* = 11.8, 4.4 Hz), and 4.18 (2H, br s); ^{1}H NMR (C_6D_6, 70 °C, 400 MHz. Because of the conformational mobility of **5**, NMR signals are heavily broadened. Therefore, only diagnostic signals are recorded here.) δ 0.90 (3H, d, *J* = 7.0 Hz), 0.92 (3H, s), 0.97 (3H, d, *J* = 6.8 Hz), 0.99 (3H, d, *J* = 7.3 Hz), 2.64 (1H, sept, *J* = 6.8 Hz), 3.75 (1H, dt, *J* = 11.6, 4.4 Hz), 4.04 (1H, dd, *J* = 12.6, 8.6 Hz), and 4.05 (1H, br d, *J* = 12.6 Hz); HR-ESI-MS: *m*/*z* calcd for $C_{20}H_{32}O_2Na$ $[M + Na]^+$ 327.2300, found 327.2278. X-ray crystallographic analysis of a single crystal of **5** was shown in Figure S2.

8β-Hydroxyfusicocca-1,10(14)-dien-16-al (**8**): colorless prisms (recrystallized from *n*-hexane/Et_2O); mp 112.5−114.2 °C; ^{1}H NMR (Figure S6, $CDCl_3$, 400 MHz): δ 0.76 (3H, d, *J* = 7.0 Hz), 0.98 (3H, d, *J* = 6.8 Hz), 1.02 (3H, d, *J* = 6.8 Hz), 1.12 (3H, s), 1.44−1.88 (6H, m), 1.94−2.14 (2H, m), 2.11 (1H, dd, *J* = 12.9, 3.7 Hz), 2.11 (1H, dd, *J* = 12.9, 11.6 Hz), 2.30 (1H, ddd, *J* = 13.1, 3.1, 1.5 Hz), 2.80 (1H, sept, *J* = 6.8 Hz), 2.81 (1H, m), 3.16 (1H, dddd, *J* = 7.8, 6.0, 2.0, 1.9 Hz), 3.89 (1H, dt, *J* = 11.6, 3.7 Hz), 5.56 (1H, t, *J* = 1.9 Hz), and 9.56 (1H, d, *J* = 2.0 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 8.44, 20.83, 20.99, 24.29, 26.73, 27.41, 27.52, 28.55, 36.78, 40.29, 41.23, 45.03, 53.57, 61.42, 76.24, 133.43, 134.12, 137.66, 145.42, and 202.44; HR-ESI-MS: *m*/*z* calcd for $C_{20}H_{30}O_2Na$ $[M + Na]^+$ 325.2143, found 325.2171.

Fusicocca-1,10(14)-diene-8β,16-diol (**9**): colorless prisms (recrystallized from *n*-hexane/Et_2O); mp 117.5−121.9 °C; ^{1}H NMR (Figure S7, $CDCl_3$, 400 MHz): δ 0.82 (3H, d, *J* = 7.0 Hz), 0.98 (3H, d, *J* = 6.8 Hz), 1.01 (3H, d, *J* = 6.8 Hz), 1.11 (3H, s), 1.40−1.52 (2H, m), 1.52−1.85 (6H, m), 1.95 (1H, m), 2.08 (1H, m), 2.06 (1H, dd, *J* = 13.3, 3.9 Hz), 2.11 (1H, dd, *J* = 13.3, 11.4 Hz), 2.32 (1H, ddd, *J* = 13.5, 3.2, 1.7 Hz), 2.56 (1H, tdd, *J* = 8.1, 3.5, 1.5 Hz), 2.75 (1H, ddd, *J* = 8.8, 6.7, 2.1 Hz), 2.80 (1H, sept, *J* = 6.8 Hz), 3.51 (1H, dd, *J* = 10.6, 8.1 Hz), 3.54 (1H, dd, *J* = 10.6, 8.1 Hz), 3.90 (1H, dt, *J* = 11.4, 3.9 Hz), and 5.40 (1H, dd, *J* = 2.2, 1.5 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 8.65, 20.83, 21.01, 26.60, 27.23, 27.49, 27.71, 28.57, 35.87, 40.76, 42.01, 44.30, 51.29, 53.26, 64.87, 76.41, 133.02, 134.85, 137.68, and 145.57; HR-ESI-MS: *m*/*z* calcd for $C_{20}H_{32}O_2Na$ $[M + Na]^+$ 327.2300, found 327.2318.

Semisynthesis of 8β-Hydroxy-16-norfusicocca-1,10(14)-dien-3-one (6) and Fusicocca-1,10(14)-diene-3α,8β,16-triol (7). Authentic samples of 8β-hydroxy-16-norfusicocca-1,10(14)-dien-3-one (**6**) and fusicocca-1,10(14)-diene-3α,8β,16-triol (**7**) were semisynthetically prepared from fusicoccin A (**1**),[13,14] a major metabolite of *P. amygdali*. The synthesis procedures are summarized in Figure S3 and will be reported elsewhere in detail.

8β-Hydroxy-16-norfusicocca-1,10(14)-dien-3-one (**6**): colorless prisms (recrystallized from *n*-hexane/Et_2O); mp 136.5−138.7 °C; ^{1}H NMR (Figure S4, $CDCl_3$, 400 MHz): δ 0.81 (3H, d, *J* = 7.2 Hz), 0.98 (3H, d, *J* = 6.8 Hz), 1.06 (3H, d, *J* = 6.8 Hz), 1.16 (3H, s), 1.65−1.76 (3H, m), 2.05−2.46 (7H, m), 2.07 (1H, d, *J* = 13.6, 11.6 Hz), 2.80 (1H, sept, *J* = 6.8 Hz), 3.17 (1H, dm, *J* = 8.9 Hz), 3.97 (1H, ddd, *J* = 11.4, 4.6, 2.9 Hz), and 6.73 (1H, d, *J* = 2.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 7.70, 20.91 (2C), 26.04, 27.04, 27.47, 28.63, 29.65, 36.27, 37.87, 39.60, 46.24, 53.17, 76.12, 134.16, 134.27, 145.19, 146.56, and 209.36; HR-ESI-MS: *m*/*z* calcd for $C_{19}H_{28}O_2Na$ $[M + H]^+$ 289.2168, found 289.2196.

Fusicocca-1,10(14)-diene-3α,8β,16-triol (**7**): colorless prisms (recrystallized from *n*-hexane/Et_2O); mp 117.5−121.9 °C; ^{1}H NMR (Figure S5, $CDCl_3$, 400 MHz): δ 0.79 (3H, d, *J* = 7.2 Hz), 0.98 (3H, d, *J* = 6.8 Hz), 1.00 (3H, d, *J* = 6.8 Hz), 1.15 (3H, s), 1.21−1.46 (2H, m), 1.66 (1H, dt, *J* = 12.1, 8.6 Hz), 1.71−1.82 (2H, m), 1.89−2.07 (4H, m), 2.10 (1H, dd, *J* = 13.5, 11.9 Hz), 2.32 (1H, dm, *J* = 13.2 Hz), 2.79 (1H, sept, *J* = 6.8 Hz), 2.89 (1H, td, *J* = 8.6, 2.0 Hz), 3.41 (1H, dd, *J* = 11.4, 2.6 Hz), 3.52 (1H, dd, *J* = 11.4, 8.8 Hz), 3.89 (1H, dm, *J* = 10.8 Hz), and 5.54 (1H, d, *J* = 2.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 7.65, 20.84, 20.93, 26.52, 27.18, 27.44, 28.47, 31.44, 35.14, 40.40, 42.08, 44.46, 52.84, 67.31, 76.19, 82.92, 132.45, 135.37, 140.21, and 146.00; HR-ESI-MS: *m*/*z* calcd for $C_{20}H_{32}O_3Na$ $[M + Na]^+$ 343.2249, found 343.2249.

■ ASSOCIATED CONTENT

Supporting Information. The semisynthesis procedures of the intermediate compounds and their NMR. This material is available free of charge via the Internet at http://pubs.acs.org.

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