Identification of Diterpene Biosynthetic Gene Clusters and Functional Analysis of Labdane-Related Diterpene Cyclases in Phomopsis amygdali


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Two diterpene biosynthesis gene clusters in the fusicoccin-producing fungus, Phomopsis amygdali, were identified by genome walking from PaGGS1 and PaGGS4 which encode the geranylgeranyl diphosphate (GGDP) synthases. The diterpene cyclase-like genes, PaDC1 and PaDC2, were respectively located proximal to PaGGS1 and PaGGS4. The amino acid sequences of these two enzymes were similar to those of fungal labdane-related diterpene cyclases. Recombinant PaDC1 converted GGDP mainly into phyllocladan-16α-ol via (+)-copalyl diphosphate (CDP) and trace amounts of several labdane-related hydrocarbons which had been identified from the P. amygdali F6 mycelia. Since phyllocladan-16α-ol had not been identified in P. amygdali F6 mycelia, we isolated phyllocladan-16α-ol from the mycelia. Recombinant PaDC2 converted GGDP into (+)-CDP. Furthermore, we isolated the novel diterpenoid, phyllocladan-11α,16α,18-triol, which is a possible metabolite of phyllocladan-16α-ol in the mycelia. We propose that genome walking offers a useful strategy for the discovery of novel natural products in fungi.

Key words: biosynthesis; cyclase; diterpene; gene cluster; Phomopsis amygdali

The cyclic diterpenoids are derived from geranylgeranyl diphosphate (GGDP) through cyclization and chemical modification. Fungi produce a variety of diterpenoids with unique biological activities, e.g., gibberellin is a phytohormone, aphidicolin is a specific inhibitor of DNA polymerase α, and fusicoccin A has not only potent plant plasma membrane H⁺-ATPase-activating activity but also exerts novel effects on amphibian embryogenesis. The biosynthetic genes of gibberellins and aphidicolin, including a gene that encodes GGDP synthase (GGS), have recently been found to be clustered in the fungal genome. This suggested that the diterpene biosynthetic gene cluster could be obtained by isolating the GGS genes and subsequent genome walking. We have previously isolated six cDNA fragments that were derived from GGS-like genes (PaGGS1 to 6) from Phomopsis amygdali N2, and carried out genome walking from GGS to identify a cDNA that encodes fusicoccin-2,10(14)-dien synthase and the fusicoccin biosynthetic gene cluster. As a result, we isolated a chimera diterpene synthase gene that encodes fusicoccin-2,10(14)-dien synthase (PaFS), which possesses a terpene cyclase domain at the N-terminus and a prenyltransferase domain at the C-terminus. These results indicate that genome walking is a useful strategy for isolating cDNAs that encode unknown diterpene biosynthetic enzymes.

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genome walking of two additional diterpene biosynthetic gene clusters, which include the labdane-related diterpene cyclase genes PaDC1 and PaDC2, respectively located around PaGGS1 and PaGGS4, in P. amygdali. In addition, we determined the structures of the two products converted from GGDP by recombinant PaDC1 and PaDC2, indicating that PaDC1 and PaDC2 encode phyllocladan-16α-ol synthase and (+)-copalyl diphosphate (CDP) synthase, respectively. Based on these evidences, we also identified from the P. amygdali mycelia the novel diterpene, phyllocladan-11α,16α,18-triol, which is a possible metabolite of phyllocladan-16α-ol. Thus, genome walking facilitated the discovery of two novel diterpene synthases and a novel diterpenoid.

Materials and Methods

Chemicals. Isopentenyl diphosphate (IPP), dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GDP), farnesyl diphosphate (FDP), and GGDP were purchased from Sigma-Aldrich. [1-14]CIPP (CFA476, 2.15 GBq/mmol) was obtained from Amersham Bioscience. (+)-Copalol was synthesized by Toshima et al., and converted to the diphosphate ester, (+)-CP, according to the method described previously.1) Fungal strains. Phomopsis amygdali strains N2 and F6 were respectively presented by Dr. Kanematsu (Apple Research Center, National Institute of Fruit Tree Science) and Professor A. Graniti (Bari University).

Genome walking from GGS. Genome walking was carried out by using the Universal Genome Walker kit (Clontech). Genomic DNA was extracted from the mycelia by using the Nucleon PhytoPure kit (Amersham Bioscience). The genome walker library was constructed with genomic DNA and was used for a nested polymerase chain reaction (PCR) according to the manufacturer’s protocol. Primers for the first walking were designed from the cDNA fragment sequences of PaGGS1 and PaGGS4.

Rapid amplification of cDNA ends (RACE). To determine the sequences of PaGGS1 and PaGGS4, 5′-RACE and 3′-RACE were performed by using gene-specific primers based on the corresponding genomic DNA sequences according to the methods described previously.10) Functional analysis for prenyltransferase. To amplify the PaGGS1 and PaGGS4 ORF cDNAs, the following pairs of primers: for PaGGS1, sense 5′-GGATCCATGACCTCCGGGTCAAAGC-3′ (BamHI site underlined) and antisense 5′-CTGCAGATTGGTACTGCATTTTCTTAT-3′ (PstI site underlined). The PCR product was digested with BamHI and PstI and subcloned into the pQE30 vector (Qiagen), and the plasmid was introduced into E. coli BL21 (DE3). All the subsequent procedures have been described previously.11) The allylic substrates used were DMAPP, GDP, and FDP. The products derived from the allylic substrates with [14C]IPP were dephosphorylated and subjected to reversed-phase thin-layer chromatography (TLC), using LKC-18 and developing with acetone/H2O (9:1).

Functional analysis for cyclase. To amplify the cDNAs for the ORFs of PaDC1 and PaDC2, reverse transcription (RT)-PCR was carried out by using the following pairs of primers: for PaDC1, sense 5′-CCCGGGGATGACATATTAGGACATCGA-3′ (SmaI site underlined) and antisense 5′-CCGGCCGCT-TACGTGACGACATTGGAC-3′ (NolI site underlined); and for PaDC2, sense 5′-AGATCTATGGAGTT-CGATGAACAC-3′ (BglII site underlined) and antisense 5′-CCGGGCGACGGCCAGATCAGG-3′ (SmaI site underlined). The PCR products were digested with SmaI/NolI for PaDC1 and BglII/SmaI for PaDC2, and ligated into the pGEX-4T-3 vector (Amersham Bioscience) to generate a glutathione S-transferase (GST) fusion protein. The plasmid was transformed into E. coli XL1-Blue. The procedures used for the growth of the E. coli cells, induction of gene expression, extraction and purification of recombinant enzymes, and measurement of cyclase enzyme activity have been described previously.12) GDP, FDP and GGDP (20 μg) were used as the substrates. The hydrocarbon product and dephosphorylated derivative from the diphosphate product were analyzed as described previously.12)

General procedures. The melting point (mp) data are uncorrected. Optical rotation values were measured with a Horiba SEPA-300 polarimeter, and the IR, UV and CD spectra were recorded with Jasco J-20A and Jasco J-20A spectrophotometers. Gas chromatography-mass spectrometry (GC-MS) was performed with an Agilent 6890N GC-5973N mass selective detector system. Mass spectra were obtained with a JMS-700 instrument, and 1H- and 13C-NMR spectra were acquired with a Jeol EX-400 or Bruker DPX 400 spectrometer.

Extraction and isolation of phyllocladan-16α-ol from the mycelia of P. amygdali F6. A hexane extract (ca. 83.4 mg) from 95 g of P. amygdali F6 mycelia obtained from 10 culture flasks, each containing 100 ml of the culture medium (5.0% glucose, 1.0% Pharmamedia, 0.5% KH2PO4, and 0.1% MgSO4 in deionized water), was separated by silica gel flash chromatography (Wakogel FC-40, 2 cm i.d. × 22 cm). From the fraction (7 mg) eluted with a mixture of hexane:EtOAc (10:1), a colorless oil (0.2 mg) with an Rf value of 0.48 was
obtained by preparative TLC on silica gel (Kieselgel 60 F254, Merck) by using a mixture of hexane:EtOAc (2:1); this compound was identified as phillocladan-16α-ol (see text).

**Extraction and isolation of phillocladan-11α, 16α, 18-triol from the mycelia of P. amygdali F6.** For seed fermentation, *Phomopsis amygdali* F6 was grown in two 500-ml Sakaguchi flasks that contained 100 ml of a medium consisting of 3.0% glucose, 0.5% Pharamedia, and 0.1% peptone. The inoculated flasks were incubated at 25 °C for 6 d on a rotary shaker. The culture broth from the two flasks was inoculated into 4500 ml of a medium containing 4% sugar, 0.5% soybean meal, 0.2% Pharamedia, 0.5% KH2PO4, and 0.1% MgSO4·7H2O in a jar fermenter (TS-A-7L; Takasago Co., Tokyo, Japan) and cultured for 14 d. The culture broth was fermented with agitation at 25 °C, 180 rpm, and aeration at 2500 ml/min. After the incubation period, the culture supernatant was purified by filtration. The filtrate was basified with Na2CO3 and extracted with EtOAc. The organic layer was concentrated in vacuo to give an oily residue (8.8 g) which was subjected to silica gel column chromatography using stepwise elution with mixtures of CHCl3–EtOH. The CHCl3–EtOH (9:1) eluate (63.4 mg) was crystallized from MeOH to afford colorless needles (14.4 mg) which were identified as phillocladan-11α,16α,18-triol (see the text).

**Phillocladan-11α,16α,18-triol**: mp 295–300 °C; [α]D20 20 = +3.5° (c 0.1, MeOH); IR (KBr) νmax cm–1: 3292, 1382, 1357, 1057, 1018; HRFABMS m/z [M + Na]+: calcld. for C20H32O6Na, 345.2406; found, 345.2411; 1H-NMR δH (CDCl3): 0.89 (3H, s, Me-19), 1.29 (3H, s, Me-20), 1.25 (1H, dd, J = 12.7, 7.8 Hz, 6-H), 1.31 (1H, t, J = 10.8 Hz, H-14), 1.42 (overlapped signals, H-1 and H-3), 1.48 (1H, d, J = 9.7 Hz, H-9, 1.54 (1H, m, H-2), 1.57 (3H, s, Me-17), 1.64 (1H, m, 6-H), 1.70–1.74 (overlapped signals, H-5 and H-7), 1.72 (1H, d, J = 14.7 Hz, H-15), 1.78 (1H, m, H-2), 1.81 (1H, td, J = 9.7, 2.9 Hz, H-12), 1.93 (1H, td, J = 13.7, 3.9 Hz, H-3), 2.10 (1H, m, H-13), 2.32 (1H, d, J = 14.7 Hz, H-15), 2.36 (1H, m, H-12), 2.46 (1H, ddd, J = 10.8, 6.8, 3.9 Hz, H-14), 3.08 (1H, br. d, J = 13.7 Hz, H-1), 3.22 (1H, dd, J = 10.8, 5.4 Hz, H-18), 3.68 (1H, dd, J = 10.8, 5.4 Hz, H-18), 4.27 (1H, m, H-11), 5.30 (1H, d, J = 7.3 Hz, OH-11), 5.43 (1H, s, OH-16), 5.96 (1H, t, J = 5.4 Hz, OH-18); 13C-NMR δC (CDCl3, assigned by HSQC and HMBC data): 16.4 (q, C-20), 18.6 (q, C-19), 18.7 (t, C-2), 20.4 (t, C-6), 24.9 (q, C-17), 35.9 (t, C-3), 38.3 (s, C-4), 39.8 (s, C-10), 40.9 (t, C-12), 42.1 (t, C-1), 43.2 (t, C-7), 45.6 (s, C-8), 48.1 (d, C-13), 49.4 (t, C-14), 49.5 (d, C-5), 51.5 (t, C-15), 63.5 (d, C-9), 67.4 (d, C-11), 71.8 (t, C-18), 80.5 (s, C-16).

Large-scale preparation of the dephosphorylated derivative of the product converted from GGDP by recombinant PaDC2. The plasmid (pGEX-4T-3) that harbors PaDC2 was transformed into *E. coli* BL21 (DE3). Transformants were grown at 37 °C in a 2× YT medium that contained ampicillin (50 μg/ml). When the optical density at 600 nm had reached 0.6, isopropyl-1-thio-β-D-galactoside was added to a final concentration of 0.2 mM. The bacteria, which were grown in 5000 ml of the 2× YT medium, were collected by centrifugation. To the pellets, 150 ml of a 50 mM Tris–HCl buffer (pH 7.5) that contained 0.1% Triton X-100 was added, and the suspension was subjected to ultrasonication and centrifugation to obtain a supernatant containing the enzyme. The reaction mixture contained 3 mg of GGDP (44 μM), a homogenous cell-free extract (140 ml), 1 mM MgCl2, 0.5 mM EDTA, and 2 mM dithiothreitol in a total volume of 150 ml of a 50 mM Tris–HCl buffer solution (pH 7.5) plus 0.1% Triton X-100. After incubating at 25 °C for 15 h, and subjecting to a phosphatase treatment, the enzyme reaction was terminated by adding 100 ml of 15% KOH/MeOH and heating at 75 °C for 30 min, after which the lipopholic materials were extracted with hexane (150 ml × 3). The enzymatic product was purified by SiO2 column chromatography, using gradient elution with hexane/EtOAc (100:0–100:5), which produced 1.2 mg of a pure compound. The structure of this was determined by mass and 2D-NMR spectral analyses to be that of copalol (oil)19 with a specific rotation [α]D24 = +30.6° (c 0.12, CHCl3). HRMS m/z (M+): calcld. for C20H32O6Si, 290.2610; found 290.2601; 1H-NMR δH (400.13 MHz, CD2Cl2): 0.855 (3H, s, H-3), 1.38 (m, H-2), 1.48 (m, H-6), 1.53 (m, H-3), 1.53 (m, H-2), 1.60 (m, H-11), 1.65 (3H, s, H-16), 1.66 (m, H-2), 1.69 (m, H-9), 1.72 (m, H-11) 1.73 (m, H-6), 1.77 (m, H-1), 1.97 (m, H-12), 2.07 (m, H-7), 2.35 (m, H-12), 2.50 (m, H-7), 4.13 (2H, d, J = 6.4 Hz, H-15), 4.78 (bs, H-7), 5.07 (bs, H-17), 5.57 (bt, J = 6.8 Hz, H-14); 13C-NMR δC (100.6 MHz, CD2Cl2): 14.74 (C-20), 16.30 (C-16), 19.74 (C-2), 21.89 (C-19), 22.19 (C-11), 24.74 (C-6), 33.64 (C-4), 33.71 (C-18), 37.68 (C-7), 38.79 (C-12), 39.20 (C-1), 39.87 (C-10), 42.39 (C-3), 55.60 (C-5), 56.53 (C-9), 59.37 (C-15), 106.7 (C-17), 124.5 (C-14), 139.0 (C-13), 148.7 (C-8).

**Results and Discussion**

Identification of two diterpene biosynthesis gene clusters in *P. amygdali*

We identified by genome walking from *PaGGS1* and *PaGGS4* in *P. amygdali* N2 strain the genomic DNA sequences of about 26 kb and 14 kb, respectively (Fig. 1). These nucleotide sequences have been deposited in the databases with accession numbers AB254158 and AB254160, respectively. BLASTx searching identified a diterpene cyclase, five P450 monooxygenases, a dehydrogenase, and a dioxygenase-like gene proximal to *PaGGS1* (Fig. 1A), and a diterpene cyclase, two
P450 monooxygenases, and a transporter-like gene proximal to PaGGS4 (Fig. 1B). We have named the two diterpene cyclase-like genes PaDC1 and PaDC2, respectively. We determined the full-length cDNA sequences of PaGGS1 and PaGGS4 by RACE (nos. AB254158 and AB254160), and carried out a functional analysis of each recombinant protein. Recombinant PaGGS1 catalyzed the conversion by IPP of GDP or FDP, but not DMAPP, into GGDP, and PaGGS4 catalyzed the conversion by IPP of DMAPP, GDP or FDP into GGDP (Fig. 2). It thus appears that both PaGGS1 and PaGGS4 encode GGS, which suggests that both of these genomic DNA regions contain diterpene biosynthesis gene clusters.

Isolation of ORF cDNAs of PaDC1 and PaDC2

To isolate the cDNA fragments that contain PaDC1 and PaDC2, we performed RT-PCR using a cDNA pool derived from the mycelia of P. amygdali as a template. The design of the 5′- and 3′-end primers for amplifying each ORF was based on the genomic DNA sequences. However, no band was amplified for PaDC1, whereas a band of the expected size was amplified for PaDC2 (Fig. 3, lane N). Furthermore, we could not amplify the PaDC1 band with certain combinations of other gene-specific primers (data not shown). In the mycelia of P. amygdali, fusicoccadiene-related hydrocarbons make up most of the hydrocarbon fraction.14) However, in the hydrocarbon fraction of the extract from the mycelia of P. amygdali F6, several labdane-related hydrocarbons derived from (+)-CDP were detected, in addition to the foregoing hydrocarbons.14) We therefore performed RT-PCR, using as

**Fig. 1.** Chromosome Map of Regions around PaGGS1 (A) and PaGGS4 (B).

The filled inverted triangle indicates the starting point for genome walking. These nucleotide sequences of A and B have been deposited in the databases with accession numbers AB254158 and AB254160, respectively. The restriction sites for Dros, EcoRV, PvuII and SstI used in the genome walking process are represented by the letters D, E, P and S, respectively. The direction and deduced region of transcription are represented by arrows. Deduced orf1, orf2, orf3, orf4, orf6, orf9, and orf10 represent P450 monooxygenase-like genes, and orf5, orf7, and orf8 represent dehydrogenase, dioxygenase, and transporter-like genes, respectively. Homology searches were performed by using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

**Fig. 2.** TLC Autoradiography of the Alcohols Obtained by Hydrolysis of the Products Derived by Recombinant PaGGS1 and PaGGS4.

The ‘+’ designation indicates the origin. DMADP (D), GDP (G), FDP (F), and GGDP (GG) were incubated with [14C]IPP. Spots for authentic standard alcohols are indicated by arrows. C10, geraniol; C15, farnesol; C20, geranylgeraniol; C30, farnesylfarnesol.

**Fig. 3.** Expression of PaDC1 and PaDC2 in Two Strains of Phomopsis amygdali.

RT-PCR was performed by using each set of gene-specific primers. F6 and N2 indicate template cDNAs derived from P. amygdali F6 and N2, respectively. Bands of approximately 3kb are amplified.
template the cDNA from *P. amygdali* F6 mycelia. A band of the expected size for *PaDC1* was amplified, as well as a band for *PaDC2* (Fig. 3, lane F). Both bands were subcloned and sequenced. *PaDC1* (accession number AB252834) and *PaDC2* (AB252835) were found to encode 1005 amino acids (113 kDa) and 984 amino acids (110 kDa), respectively. The nucleotide sequence of the coding region of *PaDC2* from N2 was identical to that of *PaDC2* from F6.

Homology searches indicated that both *PaDC1* and *PaDC2* were homologous to the labdane-related diterpene cyclases, *ent*-kaurene synthases from *Phaeosphaeria* (PICPS/KS)\(^{15}\) and *Gibberella fujikuroi* (GfCPS/KS),\(^{16}\) and aphidicolan-16β-ol synthase from *Phoma betae* (PbACS)\(^{17}\) (Fig. 4A). These bifunctional diterpene cyclases convert GGDP sequentially into (−)-*ent*-CDP or (+)-syn-CDP, and into the specific cyclic hydrocarbons. The former reaction is initiated by pro-
Functions of PaDC1 and PaDC2

To study the functions of PaDC1 and PaDC2, terpene cyclase assays using recombinant proteins were performed. The results of the GC-MS analysis to identify a product of GST-PaDC1 are shown in Fig. 5. A functional analysis with the GST-PaDC1 recombinant enzyme, using GDP, FDP or GGDP as a substrate, showed that GGDP was converted into peak 1 at a retention time 23 min on GC (Fig. 5A), whereas there was no conversion of either GDP or FDP (data not shown). The retention time and mass spectrum of peak 1 (Fig. 5A and D) were identical to those of phyllocladan-16α-ol (11) (Fig. 6) isolated from the hexane extract of P. amygdali F6 mycelia (Fig. 5C and F, peak 3). The identification of phyllocladan-16α-ol (11) in the mycelia is described next. Recombinant GST-PaDC1 also converted (+)-CDP into phyllocladan-16α-ol (11) (Fig. 5B and E, peak 2). These results indicate that PaDC1 converted GGDP into phyllocladan-16α-ol (11) via (+)-CDP (Fig. 6). The aspartate-rich motifs, DADD (positions 321-324) and DEFME (positions 667-671), in PaDC1 (Fig. 4B) should be responsible for the conversion of GGDP into (+)-CDP (type B cyclization) and for the conversion of (+)-CDP into phyllocladan-16α-ol (11) (type A cyclization), respectively. In the reaction mixture, trace amounts of (+)-isopimara-8,15-diene (6) (15.2 min), pimara-8,15-diene (3) (15.5 min), (+)-pi- mara-8(14),15-diene (4) (15.9 min), (−)-sandaracopimaradiene (7) (16.3 min), (+)-phyllocladene (8) (17.7 min), and (−)-kaurene (5) (18.3 min) were detected as by-products (data not shown). These labdane-related compounds have also been identified in the hydrocarbon fraction from P. amygdali F6 mycelia.13) The general biogenetic pathways of these hydrocarbons are shown in Fig. 6A. (+)-CDP, which is converted from GGDP by type B cyclization, undergoes successive type A cyclization from re- or si-face attack of the C-13 olefin to generate tricyclic carbocations 1 and 2. Deprotonation of 10-H and 14-H in 1 gave 3 and 4, respectively, whereas sequential rearrangement of 1 provided 5. While deprotonation of 10-H and 14-H in 2 gave 6 and 7, respectively, sequential rearrangement of 2 afforded 8. During the rearrangement of 2, carbocation intermediate 10 was formed via 9, and deprotonation of 17-H in 10 provided 8, while stereoselective attack of the water molecules generated phyllocladan-16α-ol (11) (Fig. 6B). Therefore, PaDC1 is responsible for the synthesis of the labdane-related hydrocarbons in P. amygdali F6. Although labdane-related diterpene cyclase via (+)-CDP as an intermediate has previously been isolated from a plant, i.e., abietadiene synthase in grand fir,20) such a diterpene cyclase has not previously been identified in fungi.

The functional analysis conducted with GST-PaDC2 showed that GDP, FDP and GGDP were not converted into hydrocarbon (data not shown). Therefore, the reaction products were subjected to GC-MS after dephosphorylating with alkaline phosphatase. Peak 4 with a retention time of 23.6 min was detected on GC from the reaction mixture by using GGDP as the substrate (Fig. 7A). The retention time and mass spectrum of peak 4 (Fig. 7A and C), representing the reaction products derived from incubating GGDP with GST-PaDC2, were identical to those of authentic (+)-copalol previously synthesized6) (Fig. 7B and D, peak 5). It was not possible to distinguish between (+)-copalol and (−)-ent-copalol by our GC-MS system. We therefore determined the absolute stereostructure of the dephosphorylated derivative by measuring the specific rotation. The [α]D value for copalol derived from the reaction product was +30.6, which is similar to the previously published value of +29.8,21) demonstrating that PaDC2 converted GGDP into (+)-CDP, but not
Aspartate/glutamate-rich motif DVDD (positions 318-321; Fig. 4B) was expected to be responsible for the conversion of GGDP into (+)-CDP (type B cyclization). However, we did not detect any further cyclized product of (+)-CDP, even though PaDC2 contains another aspartate/glutamate-rich motif, DEFME (positions 656-660; Fig. 4B), which might be involved in type A cyclization. It is possible that a mutation occurred in the 3' region, outside the coding region of the DEFME motif, in PaDC2. These results indicate that PaDC2 encodes the (+)-CDP synthase. Labdane-related diterpenoids are biosynthesized from GGDP through specific diterpene hydrocarbon skeletal structures via a CDP intermediate, of which four common stereoisomers are known: ent-CDP, (+)-CDP, (+)-syn-CDP, and ent-syn-CDP. Although the cDNAs that encode ent-CDP synthase and syn-CDP synthase have been isolated from plants and bacteria, the cDNAs for (+)-CDP synthase and ent-syn-CDP synthase have not been identified in any organism. Thus,

Fig. 6. The Biogenetic Pathways to Diterpene Hydrocarbons and Phyllocladan-16α-ol. A, Formation of diterpene hydrocarbons from (+)-CDP by PaDC1. B, Formation of phyllocladan-16α-ol and phyllocladane by PaDC1.
mycelial extract, as described in a previous report, we

produce any labdane-related compounds, probably produces (+)-CDP as an end product. This is the first identification of a natural enzyme which produces (+)-CDP as an end product.

Since we could not identify phyllocladan-16α-ol (11) in the hydrocarbon fraction of the P. amygdali F6 mycelial extract, as described in a previous report, we purified compound 11 from the hexane extract of P. amygdali F6 mycelia and performed a GC-MS analysis (Fig. 5C and F). Peak 3 gave the M⁺ ion at m/z 290, and characteristic fragment ions at m/z 272, 257, 217, 191, 147, 134 and 123. The mass spectrum of 11 was identical to that of phyllocladan-16α-ol listed in the mass spectral libraries (Vender Kit Wiley D.02.00; Agilent). Although 11 had very similar fragment ions to those of phyllocladan-16β-ol in the mass spectral libraries, the diagnostic ion at m/z 212 observed in phyllocladan-16β-ol was significantly different. Furthermore, the 1H-NMR chemical shifts of the methyl groups of 11 were in good agreement with those of phyllocladan-16α-ol [11: δH (400 MHz, CDCl₃) 1.33 (3H, s, Me-17), 0.87 (3H, s, Me-18), 0.85 (3H, s, Me-19), 0.80 (3H, s, Me-20)]. These data strongly support the designation of 11 as phyllocladan-16α-ol (Fig. 8).

In general, the cyclic diterpene synthase enzymes convert a common acyclic biosynthetic precursor (GGDP) to diterpenes which are modified, initially by hydroxylation and subsequently by oxidation, to their corresponding alcohols, aldehydes, and acids. In our studies of phyllocladane-related diterpenes from this fungal strain, hydroxylated phyllocladane derivative 12 (Fig. 8) was isolated in the form of colorless needles. The molecular formula for 12, C₂₀H₃₂O₃, which was determined by HRFABMS, has two additional oxygen atoms, as compared to 11. The 1H-NMR spectrum of 12 was similar to that of 11, except for the presence of oxymethine [δH 4.27 (1H, m, H-11)] and oxymethylene signals [δH 3.22 (1H, dd, J = 10.8, 5.4 Hz, H-18) and 3.68 (1H, dd, J = 10.8, 5.4 Hz, H-18)]. A careful comparison of the 13C-NMR spectra of 12 and phyllocladan-16β-ol revealed the presence of two additional hydroxyl groups at C-11 and C-18, based on the substitution effects at C-4, C-9, C-11, C-12, and C-18. This was confirmed by HMBC correlations between OH-11 and C-9, between OH-18 and C-18, and between Me-19 and C-18. The relative stereochemistry of 12 was inferred from the nuclear Overhauser effects (NOEs) from H-11 to H-15β, Me-17 and Me-20, and from Me-20 to Me-19 and H-15β.

Although the absolute configurations of 11 and 12 have not been confirmed, these compounds are presumed to be (+)-phyllocladane (13β-kaurane)-type diterpenoids on the basis of biosynthetic considerations, as well as by the fact that all natural cyclic diterpene hydrocarbons isolated from this fungal strain to date have been assigned to the (+)-kaurane series (Fig. 7A). We therefore conclude that 12 is phyllocladan-11α,16α,18-triol, which is reported here for the first time. Since the formation of 12 is thought to occur via 11, this being followed by hydroxylation, it is biogenetically plausible that cytochrome P450 oxygenase-like genes near the PaDC1 gene in the diterpene synthesis gene cluster (Fig. 1) are involved in the conversion of 11 into 12. The biological activities of

**Fig. 7.** GC-MS Results for the Dephosphorylated Derivatives of Products Converted from GGDP by GST-PaDC2. A. Total ion chromatograms of the products derived from the incubation of GGDP with recombinant GST-PaDC2. B. Total ion chromatogram of synthetic (+)-CDP. C. Full-scan mass spectrum of peak 4. D. Full-scan mass spectrum of peak 5.

**Fig. 8.** Structures of Phyllocladan-16α-ol (11) and Phyllocladan-11α,16α,18-triol (12).
11 and 12 have not been examined. However, 11 and/or 12 should possess unique biological activity, as it has been reported that a phyllocladanol-related compound from heartwood of Cryptomeria japonica exhibited termicidal activity.20

We identified in the present study two novel diterpene synthases, phyllocladan-16α-ol synthase and (+)-CDP synthase, and the novel diterpenoid metabolite, phyllocladan-11α,16α,18-triol. This is the first report of the identification of a novel terpenoid through the genome walking strategy. The identification of novel substances may indicate lead compounds for the creation of new medical and agricultural chemicals. We propose that genome walking offers a useful strategy for the discovery of novel natural products in fungi.

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