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Identification of Chimeric αβγ Diterpene Synthases Possessing both Type II Terpene Cyclase and Prenyltransferase Activities

Takaaki Mitsuhashi[a], Masahiro Okada[a], and Ikuro Abe*[a]

Abstract: We identified two unusual diterpene synthases composed of three domains (α, β, and γ) from fungal *Penicillium* species. They are the first enzymes found to possess both Type II terpene cyclase (TC) and prenyltransferase (PT) activities. These enzymes were characterized by heterologous expression in *Aspergillus oryzae* and *in vitro* experiments with wild-type, mutated, and truncated enzymes. The results revealed that the α domain in the C-terminal region of these enzymes was responsible for the PT activity, while the βγ domains in the N-terminal region composed the Type II TC, and formed copalyl diphosphate **2**. Additionally, between the α and βγ domains, there is a characteristic linker region in which minimal secondary structure is predicted. This linker does not exist in the characterized three domain (αβγ) terpene synthases known as the mono functional Type I or Type II TCs, or the bifunctional Type I TC + Type II TC enzymes. Therefore, both the catalytic activities and protein architecture substantially differentiate these new enzymes from the previously characterized terpene synthases.

Prenyltransferase (PT) and terpene cyclase (TC) are key enzymes in the formation of the basic carbon skeleton of terpenoids, which constitute one of the most structurally and stereochemically diverse families of natural products with more than 75,000 members. Despite the vast chemodiversity of the terpenoids, they all originate from simple C_5 substrates, dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP).[1] In the biosynthesis of terpenoids, the PTs catalyze a chain elongation reaction utilizing DMAPP and IPP to form linear isoprenoid precursors, such as (C_{10}) geranyl diphosphate (GPP), (C_{15}) farnesyl diphosphate (FPP), (C_{20}) geranylgeranyl diphosphate (GGPP), and (C_{25}) geranylfarnesyl diphosphate (GFPP).[2] TCs are the enzymes responsible for the cyclization of the linear isoprenoid precursors to form the complex carbon skeletons of various cyclic terpenoids.[1, 3] The TCs are categorized into two distinct classes (Type I and Type II), depending on their strategies to initiate catalysis. The Type I TCs initiate cyclization by heterolytic cleavage of the diphosphate moiety, which generates a reactive carbocation intermediate. The Type II TCs produce the carbocation intermediate by the protonation of a C=C double bond or an epoxide ring. Based on prior structural and mechanistic studies of PTs and TCs, various combinations of the three basic domains (α, β, and γ) form the structures of these enzymes.[4] Typically, both the PTs and microbial Type I TCs contain only an α domain. This structural homology between PTs and microbial Type I TCs suggests that they may share common evolutionary origins.[5] In contrast, the plant Type I TCs are normally composed of αβ or αβγ domains. The Type II TCs are enzymes with either βγ or αβγ domains.

Indeed, some TCs and PTs are bifunctional enzymes fused with another domain that constitutes a distinct active site for successive reactions.[6] For example, the bifunctional αβγ TCs, found in plants and fungi, can catalyze tandem Type II and Type I TC reactions.[7] In this case, the Type I TC active site is in the α domain, and the Type II TC active site is at the interface of the βγ domains. Another example of bifunctional catalysis is a group of di- and sesterterpene synthases from fungi, composed of αα domains. The N-terminal α domain works as a Type I TC, while the C-terminal α domain is a PT that forms a substrate used by the N-terminal Type I TC domain.[8] Other αα terpene synthases were found in several Gram-positive soil bacteria.[9] The N-terminal α domain catalyzes the Type I TC reaction to form a cyclized sesquiterpenoid, and uniquely, the C-terminal α domain is responsible for the protonation, cyclization, and fragmentation of the cyclized product. Actually, the fusion of the TC or PT domain with another domain, which catalyzes sequential reactions, may provide a catalytic advantage, because the physical proximity of the two active sites can enhance product flux.[10] For the αα di- and sesterterpene synthases composed of a Type I TC and a PT, this catalytic advantage seems to be enhanced through an agglomeration effect,[11] considering that one of them, fusicoccadiene synthase from *Phomopsis amygdali*, proved to be a hexamer.[12]

Despite the existence of chimeric Type I TC + PT enzymes[8] and their catalytic advantage, Type II TC + PT terpene synthases have not been identified so far. Here, we report two unusual αβγ diterpene synthases, as the first bifunctional proteins consisting of a Type II TC and a PT. Characteristically, the amino acid sequences of these enzymes suggest that a linker region exists between the α domain and the βγ domains. This type of linker region is not present in the other αβγ terpene synthases.[1, 3] These enzymes were characterized by heterologous expression in *Aspergillus oryzae* and *in vitro* experiments with wild-type, mutated, and truncated enzymes.

We searched for Type II TC + PT enzymes by a genome-based approach. We tried to find a gene encoding a chimeric protein composed of a Type II TC homolog and a PT homolog. This attempt afforded two candidates in fungi. One was a 963 amino acid residue protein from *Penicillium verruculosum* TPU1311[13] (accession: LC316181). The other was from *Penicillium fellutanum* ATCC 48694, and contained 935 amino acid residues (JGI Protein ID: 403578). Based on their amino acid sequences, the βγ domain architecture for the Type II TC is predicted in the N-terminal regions of these enzymes (aa 1-539 and 1-538, respectively), because of the ~25% sequence

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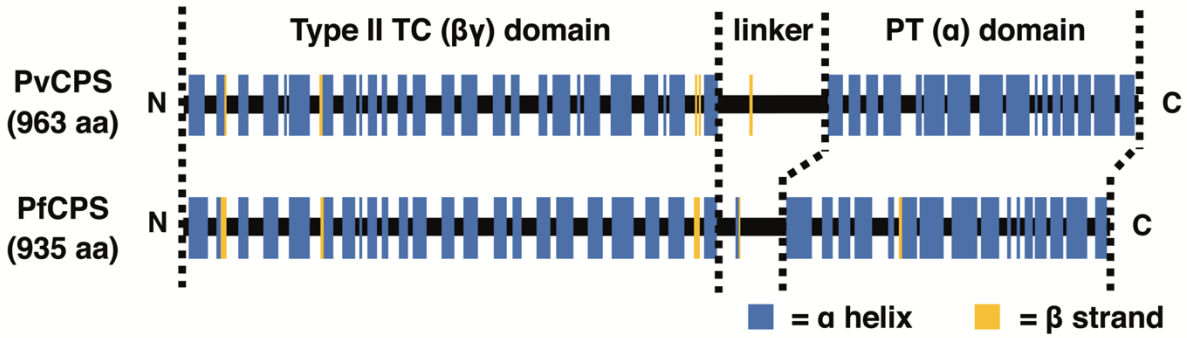
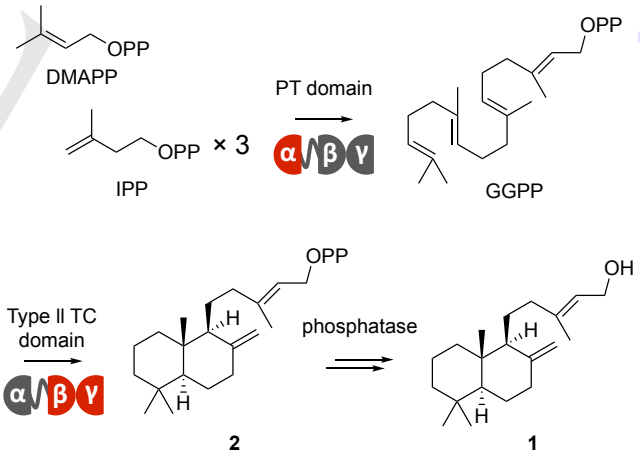


Figure 1. Secondary structure predicted in the amino acid sequences of PvCPS and PfCPS. The α helix and the β strand are shown as blue and orange boxes, respectively. The linker region is inserted between the N-terminal βγ domain and the C-terminal α domain.

identity and ~40% sequence similarity with PtmT2 from *Streptomyces platensis* CB00739,[14] which is a Type II TC with the βγ domain architecture (Protein Data Bank accession code: 5BP8, Figure S1).[15] In contrast, the C-terminal regions of these enzymes (aa 651-963 and 609-935, respectively) exhibited ~40% sequence identity and ~60% sequence similarity with the PT domain of fusicoccadiene synthase from *Phomopsis amygdali*,[8h] which forms an α domain architecture (Protein Data Bank accession codes: 5ERN and 5ERO, Figure S1).[12] Thus, the C-terminal regions seem to be the α domain for the PT. Remarkably, the N-terminal Type II TC (βγ) domain and the C-terminal PT (α) domain are separated by linker regions (aa 540-650 and 539-608, respectively), which are not found in the typical αβγ terpene synthases.[3] Minimal secondary structure is predicted to exist in the linker regions, based on an analysis of the amino acid sequences (Figure 1). Contrarily, in the typical αβγ terpene synthases, the α and βγ domains are connected by an alpha helix straddling these domains, according to prior X-ray crystal structural analyses (Figure S2).[16] Notably, between these two unusual αβγ enzymes, the amino acid sequences of the linkers exhibit relatively low similarity (Figure S3), as compared with the high homology in the N- and C-terminal domains (61% sequence identity and 73% sequence similarity for N-terminal βγ domains, and 74% sequence identity and 88% sequence similarity for C-terminal α domains). The insertion of a linker between two distinctive active sites is also found in the αα terpene synthases.[8, 9] The similarity of the linkers is also low among the αα enzymes consisting of the Type I TC and PT domains (Figure S4 and Table S4).

To characterize these two unusual αβγ enzymes, at first, we determined the structures of their products. For this purpose, they were heterologously expressed in *A. oryzae* NSAR1,[17] a quadruple auxotrophic mutant strain that has been successfully utilized for biosynthetic studies of fungal natural products.[18] The mycelial extract of each *A. oryzae* transformant was analyzed by GC-MS, and consequently, both transformants produced new metabolites with *m/z* 290 $[M]^+$. These metabolites were expected to be a diterpene alcohol and exhibited the same retention times and mass spectra (Figure S5). After their isolation, the NMR analysis (Tables S2 to S3 and Figures S10 to S21), the optical rotation determination ($[\alpha]^{23}_D$ +25.4 (c 0.33, $CHCl_3$) and $[\alpha]^{31}_D$ +22.3 (c 1.00, $CHCl_3$), respectively), and a comparison with a previous report[19] revealed that both metabolites are the same compound, (+)-copalol **1**. This investigation indicated that these enzymes are responsible for the formation of copalyl diphosphate **2**, and an endogenous phosphatase in *A. oryzae* seemed to convert **2** to **1** (Scheme 1). Such dephosphorylation has previously been reported when another kind of copalyl diphosphate synthase was introduced into an *Escherichia coli* heterologous expression system.[20] Therefore, these enzymes were designated as *Penicillium verruculosum* copalyl diphosphate synthase (PvCPS) and *Penicillium fellutanum* copalyl diphosphate synthase (PfCPS).



Scheme 1. The reactions catalyzed by PvCPS and PfCPS. They catalyze the same reaction. The domains (α or βγ) responsible for each reaction are highlighted in red.

For more detailed characterization, *in vitro* experiments with the purified enzymes were performed. First, the wild-type enzymes were incubated with the C_5 substrates, DMAPP and IPP. After the incubation, the reaction products were hydrolyzed by a phosphatase, and then analyzed by GC-MS. The detection of **1** indicated that PvCPS and PfCPS exhibit both Type II TC and PT activities (Scheme 1, Figure 2A). Additionally, these

enzymes could also utilize GPP+IPP, FPP+IPP, and GGPP as substrates to produce **2** (Figures S6 to S7). However, GFPP, a C_{25} substrate, was not converted to a cyclized sesterterpenene (data not shown). Thus, the TC domains of these enzymes proved to be C_{20}-specific cyclases.

Next, site-directed mutagenesis was performed to determine whether the N- and C-terminal domains of these enzymes are actually responsible for the Type II TC and PT activities, respectively. In general, Type II TCs and PTs contain characteristic aspartate-rich motifs necessary for the catalytic activity and constituting part of the active site.[21] The Type II TCs possess a DXDD motif, in which the central aspartic acid serves as a general acid to initiate cyclization.[21a] The PTs contain two DDXX(D,N) motifs involved in substrate binding and catalysis, via chelation with Mg^{2+}.[21b] In fact, the DXDD motif is present in the N-terminal domains of PvCPS and PfCPS ($D^{311}ADD^{314}$ and $D^{313}ADD^{316}$, respectively). Meanwhile, each enzyme possesses two DDXX(D,N) motifs in the C-terminal domain ($D^{727}DIQD^{731}$ and $D^{851}DYLN^{855}$ in PvCPS, and $D^{699}DIED^{703}$ and $D^{823}DYLN^{827}$ in PfCPS). To examine the role of the DXDD motif in the N-terminal domain, the central aspartate residue was substituted with the nonpolar residue, alanine: D313A for PvCPS and D315A for PfCPS. These mutants lacked the Type II TC activity but retained the PT activity, producing GGPP. Conversely, mutations in the C-terminal aspartate-rich motif (D727A for PvCPS and D699A for PfCPS) resulted in the loss of the PT activity; however, these mutants still exhibited the Type II TC activity, converting GGPP into **2**. Therefore, these analyses indicated that the N-terminal domain is a Type II TC domain, and the C-terminal domain is a PT domain (Figures 2B and 2C and Figures S8 to S9).

Truncation variants of these enzymes were prepared, to determine whether the Type II TC and PT domains could still function when they were separated from each other (Figure 2). To characterize the N-terminal TC domains (βγ), two truncated mutants, PvCPS-N559 (1-559 aa) and PfCPS-N548 (1-548 aa), were constructed. These mutants exhibited TC activity, but not PT activity. The PT domains (α) also retained their activity, when we analyzed the TC domain deletion mutants, PvCPS-C363 (601-963 aa) and PfCPS-C348 (588-935 aa). Therefore, the TC and PT domains are both able to catalyze their own reactions without the other domain (Figures S8 to S9). Notably, this feature is dissimilar from those of other previously reported αβγ terpene synthases. For example, AgAS, the abietadiene synthase from *Abies grandis* (αβγ bifunctional Type I + Type II TC), shows high interdependence between the two catalytic domains, because the separate α and βγ domain constructs are both catalytically inactive.[22] Additionally, in the case of TbTS, the taxadiene synthase from *Taxus brevifolia* (αβγ mono functional Type I TC), both the α and β domains are necessary for its activity, despite the fact that its active site exists solely in the α domain.[23] In the case of FCPS/KS, the ent-kaurene synthase from *Phaeosphaeria sp.* L487 (αβγ bifunctional Type I + Type II TC), although it was possible to construct truncated mutants that exhibit only one of its two activities,[24] some interdependence between two catalytic domains still exists, since the amino acid sequences necessary for the activities of the N- and C-termini overlap each other. Actually, the region composed of 172 amino acid residues (359-530 aa) of FCPS/KS, which contains 946 amino acid residues in total, is required for both the N- and C-terminus truncated mutants to exhibit their activities. In contrast, the amino acid sequences of the N- and C-terminus truncated mutants of PvCPS and PfCPS do not overlap. Indeed, aa 560-600 of PvCPS and aa 549-587 of PfCPS are not necessary for both the TC and PT activities (Figures 2B and 2C). Thus, for PvCPS and PfCPS, the degrees of the interdependence between the α domain and the βγ domains are different from those of the previously characterized αβγ terpene synthases.

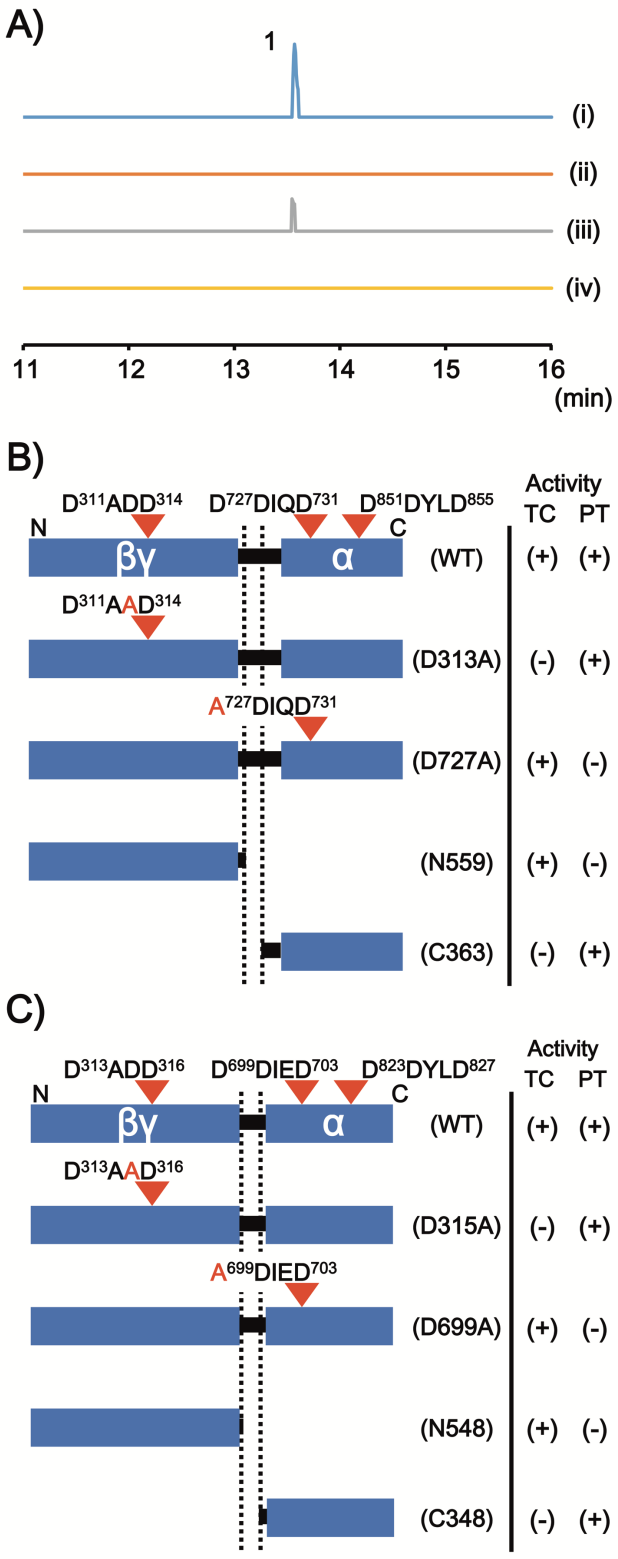


Figure 2. A) GC-MS profiles of products from *in vitro* enzymatic reactions of wild-type enzymes, (i) PvCPS, (ii) boiled PvCPS, (iii) PfCPS, (iv) boiled PfCPS, using DMAPP+IPP as substrates. The chromatograms were extracted at *m/z* 275 corresponding to $[M\text{-}Me]^+$. After the *in vitro* enzymatic reactions, the reaction products were dephosphorylated by an acid phosphatase from potato, and then analyzed by GC-MS. B) and C) The results of *in vitro* experiments with wildtype, mutated, and truncated enzymes. B) The results for PvCPS. C) The results for PfCPS. The experiments with wildtype enzymes indicated that PvCPS and PfCPS possess both Type II TC and PT activities. The results from the mutated enzymes suggested that the C-terminal (α) domain and the N-terminal (βγ) domain are responsible for the PT activity and the Type II TC activity, respectively. The investigation of truncated enzymes revealed that both the Type II TC and PT domains are able to exhibit their activities independently of the other domain.

The evolution of these unusual αβγ terpene synthases remains an open question. However, considering the hypothesis that the architecture of a typical αβγ terpene synthase arose from the fusion of ancestral genes encoding the discrete α and βγ proteins,[4] one possibility is that the domain architectures of PvCPS and PfCPS were also formed by the fusion of separate α and βγ proteins. The difference is whether the linker region exists between the two separate catalytic domains.

Notably, such a linker region also exists in αα terpene synthase.[8, 9] Especially, the comparison with the Type I TC (α) + PT (α) terpene synthases[8] is significant, because the Type I TC + PT terpene synthases and the Type II TC + PT terpene synthases show high similarity in three ways. 1) Both of them are TC + PT enzymes, and only the type of the TC is different. 2) The TC domain exists in the N-terminal region of the enzyme, while the PT domain is in the C-terminal region, and they are connected by a linker. 3) In the linker regions, minimal secondary structure is predicted, according to the amino acid sequences. These facts suggest that nature adopted the same strategy with both the Type I TCs and Type II TCs, when combining the TC with the PT. Importantly, considering that the evolutionary origins of the Type I TCs and the Type II TCs seem to be different,[4] they might have independently become fused with a PT in the course of evolution. Additional studies are needed to determine the reason why such a highly similar manner is adopted for both the Type I TCs and Type II TCs. However, since the fusion of the TC and the PT seems to accelerate product flux,[10] we believe further investigations of these enzymes and their homologs will provide valuable insights into nature's strategy underlying the evolution of terpene synthases for the efficient production of terpenoids, which form one of the largest families of natural products.[1]

Regarding the genes located nearby the PvCPS and PfCPS genes, these Type II TC + PT terpene syntheses form clusters with genes encoding a variety of proteins, including cytochrome P450s, a short-chain dehydrogenase, an aldehyde dehydrogenase, and an α/β-hydrolase (Figures S22 to S23). We are also interested in the functional analyses of these genes.

In conclusion, we identified two unusual αβγ diterpene synthases and investigated them by *in vivo* experiments, heterologous expression in *A. oryzae*, and *in vitro* experiments with wild-type, mutated, and truncated enzymes. As a result, these enzymes exhibited both Type II TC and PT activities. The N-terminal (α) domain is responsible for the Type II TC activity to form **2**, and the C-terminal (βγ) domain has the PT activity to produce GGPP, the substrate for the N-terminal Type II TC domain. To our knowledge, these are the first terpene synthases that catalyze both Type II TC and PT reactions. In addition, they possess the characteristic linker regions between the α domain and the βγ domains. There is no such region in the known αβγ terpene synthases.[1, 3] Thus, not only the catalytic activities but also the protein architectures substantially differentiate these new enzymes, PvCPS and PfCPS, from the previously characterized terpene synthases.

Experimental Section

Strains and Media: *Penicillium verruculosum* TPU1311 was cultivated at 30 °C, 160 rpm in DPY medium (2% dextrin, 1% hipolypepton (Nihon Pharmaceutical Co., Ltd.), 0.5% yeast extract (Difco), 0.5% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$) for 2 days, and used as the source for the cloning of the PvCPS gene. *Penicillium fellutanum* ATCC 48694 was obtained from ATCC (Manassas, Virginia). *Penicillium fellutanum* ATCC 48694 was cultivated at 25 °C, 160 rpm in DPY medium for 3 days, and used as the source for the cloning of the PfCPS gene. *Aspergillus oryzae* NSAR1 (*niaD*$^-$, *sC*$^-$, *ΔargB*, *adeA*$^-$) was used as the host for fungal expression. Transformants of the *A. oryzae* strain were grown in shaking cultures in DPY medium at 30 °C and 160 rpm. The cells were then transferred into DPY medium with 0.01 % adenine, and the cultures were shaken further for three or four days. Standard DNA engineering experiments were performed using *Escherichia coli* DH5α, purchased from Clontech (Mountain View, CA). *E. coli* cells carrying each plasmid were grown in Luria-Bertani medium and were selected with appropriate antibiotics. *E. coli* Rosetta2 (DE3) pLysS (Novagen) was used for the expression of PvCPS and PfCPS.

Secondary structure prediction: For the prediction of secondary structure in the amino acid sequences of PvCPS and PfCPS, the SYMPRED server was utilized.[25]

Construction of fungal expression plasmids and transformation of *A. oryzae* NSAR1: The full-length PvCPS gene was amplified from *Penicillium verruculosum* TPU1311 genomic DNA with the primers pTAex3-PvCPS-F and pTAex3-PvCPS-R (Table S1), using the following program: 98 °C, 30 s; (98 °C, 10 s; 62 °C, 10 s; 72 °C, 100 s) × 30 cycles; 72 °C, 5 min. The full-length PfCPS gene was amplified from *Penicillium fellutanum* ATCC 48694 genomic DNA with the primers pTAex3-PfCPS-F and pTAex3-PfCPS-R (Table S1), using the following program: 98 °C, 30 s; (98 °C, 10 s; 61 °C, 10 s; 72 °C, 90 s) × 30 cycles; 72 °C, 5 min. The full-length PvCPS and PfCPS genes were purified, and ligated into the pTAex3 vector[26], to create pTAex3-PvCPS and pTAex3-PfCPS, using an In-Fusion® HD Cloning Kit according to the manufacturer's protocol. Transformation of *A. oryzae* NSAR1 was performed by the protoplast–polyethylene glycol method, as reported previously.[27]

Gas Chromatographic−Mass Spectrometric Analysis of metabolites from *A. oryzae* transformant: For the analysis of the mycelial extract from the *A. oryzae* transformant harboring PvCPS or PfCPS, the extract was subjected to a GC-MS analysis. The temperature of the ionization chamber was 260 °C, with electron impact ionization at 70 eV. Helium was used as the carrier gas, and its linear velocity was 44.9 cm/min. The program held the temperature at 100 °C for 3 min, increased the temperature at a rate of 14 °C/min up to 330 °C, and then held it at 330 °C for 15 min.

Isolation and purification of 1: For the purification of **1**, mycelia from four liters of the induction culture of the *A. oryzae* transformant with the PvCPS gene were extracted with acetone at room temperature overnight. The extract was concentrated, reextracted with hexane, and then subjected to silica-gel column chromatography, with elution using a hexane:ethyl acetate mixture (100:0 to 100:18), to yield 5.7 mg of **1** as a colorless oil: $[\alpha]^{23}_D$ +25.4 (c 0.33, $CHCl_3$); for MS spectrum see Figure S5; for NMR data see Table S2 and Figures S10 to S15; HR-ESI-MS found *m/z* 313.2498 $[M + Na]^+$ (calcd. 313.2502 for $C_{20}H_{34}NaO$). After the

cultivation of the *A. oryzae* transformant with the PfCPS gene in eight liters of the induction culture, **1** was purified in the same manner, to yield 55.6 mg of **1** as a colorless oil: $[\alpha]^{31}_{D}$ +22.3 (c 1.00, $CHCl_3$); for MS spectrum see Figure S5; for NMR data see Table S3 and Figures S16 to S21; HR-ESI-MS found *m/z* 313.2508 $[M + Na]^+$ (calcd. 313.2502 for $C_{20}H_{34}NaO$).

Expression and purification of Recombinant Proteins: The intron-free PvCPS gene was amplified with the primers pColdTF-PvCPS-F and pColdTF-PvCPS-R (Table S1), from the cDNA synthesized from RNA extracted from the *A. oryzae* transformant with the PvCPS gene, using the following program: 98 °C, 30 s; (98 °C, 10 s; 62 °C, 10 s; 72 °C, 90 s) × 30 cycles; 72 °C, 5 min. To obtain the intron-free PfCPS gene, the exons were amplified with the primers pColdTF-PfCPS-F1/ pColdTF-PfCPS-R1 and pColdTF-PfCPS-F2/ pColdTF-PfCPS-R2 (Table S1) from the pTAex3-PfCPS, using the following program: 98 °C, 30 s; (98 °C, 10 s; 61 °C, 10 s; 72 °C, 30 s) × 30 cycles; 72 °C, 5 min. After the amplification, the PvCPS cDNA and the PfCPS cDNA fragments were purified, and ligated into the pColdTF vector (TaKaRa), to create pColdTF-PvCPS and pColdTF-PfCPS, using an In-Fusion® HD Cloning Kit according to the manufacturer's protocol. The full-length plasmids for the production of mutated or truncated PvCPS and PfCPS were amplified with the appropriate primers (Table S1) from pColdTF-PvCPS and pColdTF-PfCPS, using the following program: 98 °C, 30 s; (98 °C, 10 s; 65 °C, 10 s; 72 °C, 128 s) × 30 cycles; 72 °C, 5 min, as the linear form. After the amplification, the fragments were purified, treated with DpnI (TAKARA), and converted into the circular form, using T4 Polynucleotide Kinase (TOYOBO) and a DNA Ligation Kit Ver. 2.1 (TAKARA). *E. coli* Rosetta2 (DE3) pLysS (Novagen) cells were transformed with each plasmid. *E. coli* Rosetta 2 (DE3) pLysS transformants were cultured to an OD_{600} of 0.5 in LB medium containing 100 mg/L ampicillin at 37 °C. Isopropyl β-D-1-thiogalactopyranoside was then added to a final concentration of 0.50 mM to induce gene expression, and the cultures were incubated further for 18 h at 15 °C. *E. coli* cells were harvested by centrifugation at 5,800 x g and resuspended in 50 mM Tris-HCl buffer (pH 8.4), containing 250 mM NaCl, 10% (v/v) glycerol, 5 mM $MgCl_2$, and 5 mM imidazole (buffer A). The cells were disrupted by sonication, and the lysate was centrifuged at 7,300 x g for 20 min. The supernatant was loaded onto a Cosmogel His-Accept (Nacalai Tesque) column equilibrated with buffer A. After the resin was washed with buffer A containing 10 mM imidazole, the recombinant proteins were subsequently eluted with buffer A containing 300 mM imidazole. The protein purification buffer was replaced with 50 mM Tris-HCl buffer (pH 8.4), containing 250 mM NaCl, 10% (v/v) glycerol, and 5 mM $MgCl_2$. All purification procedures were performed at 4 °C.

Reaction of Recombinant Proteins: The standard enzymatic reaction was performed in mixtures containing 100 mM Tris-HCl (pH 7.5), substrates (10 μg of DMAPP + 10 μg of IPP, 10 μg of GPP + 10 μg of IPP, 10 μg of FPP + 10 μg of IPP, 3 μg of GGPP or 10 μg of GFPP), 5 mM $MgCl_2$, 2 mM dithiothreitol, and 4.7 mM enzyme, in a final volume of 100 μL. When GGPP was utilized as the substrate, the final volume was 300 μL. In experiments with mutated or truncated enzymes, GGPP was used as the substrate to monitor the TC activity. When the PT activity was investigated, we used FPP+IPP as substrates. After an incubation at 30 °C overnight, the enzyme product was extracted with water-saturated butanol, and then the butanol was removed by evaporation. A dephosphorylation reaction was then performed in mixtures containing 20% (v/v) 1-propanol, 100 mM sodium acetate (pH 5.0), 0.1% (v/v) Triton X-100, and 0.13 units of acid phosphatase from potato (Sigma-Aldrich), in a final volume of 500 μL. After an incubation at 37 °C overnight and an extraction with hexane, the enzyme products were analyzed by GC-MS. The GC-MS conditions were the same as those for the analysis of the mycelial extracts from the *A. oryzae* transformants harboring PvCPS or PfCPS.

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Keywords: biosynthesis • terpenoids • bifunctional enzyme • terpene cyclase • prenyltransferase

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Layout 1:

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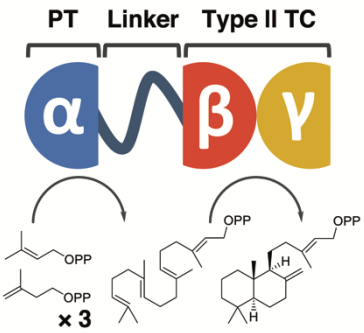
We identified two unusual diterpene synthases composed of three domains (α, β, and γ) and one characteristic linker inserted between α domain and βγ domain. They are the first enzymes possessing both Type II terpene cyclase (TC) and prenyltransferase (PT) activities.



*Takaaki Mitsuhashi, Masahiro Okada, and Ikuro Abe**

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Identification of Chimeric αβγ Diterpene Synthases Possessing both Type II Terpene Cyclase and Prenyltransferase Activities

Layout 2:

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