Functional Analysis of Eubacterial Diterpene Cyclases Responsible for Biosynthesis of a Diterpene Antibiotic, Terpentecin* 

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Eubacterial diterpene cyclase genes had previously been cloned from a diterpenoid antibiotic terpentecin producer (Dairi, T., Hamano, Y., Kuzuyama, T., Itoh, N., Furihata, K., and Seto, H. (2001) <i>J. Bacteriol.</i> 183, 6085–6094). Their products, open reading frame 11 (ORF11) and ORF12, were essential for the conversion of geranylgeranyl diphosphate (GGDP) into terpentinetriene (TTE) that had the same basic skeleton as terpentecin. In this study, functional analyses of these two enzymes were performed by using purified recombinant enzymes. The ORF11 product converted GGDP into a cyclized intermediate, terpentediene diphosphate (TDP), which was then transformed into TTE by the ORF12 product. Interestingly, the ORF12 product directly catalyzed the conversion of GGDP into three olefinic compounds. Moreover, the ORF12 product utilized farnesyl diphosphate as a substrate to give three olefinic compounds, which had the same structures as those formed from GGDP with the exception of the chain lengths. These results suggested that the ORF11 product with a DXDD motif converted GGDP into TDP by a protonation-initiated cyclization and that the ORF12 product with a DDXXD motif completed the transformation of TDP to the olefin, terpentinetriene by an ionization-initiated reaction followed by deprotonation. The kinetics of the ORF12 product indicated that the affinity for TDP and GGDP were higher than that of farnesyl diphosphate and that the relative activity of the reaction converting TDP into TTE was highest among the reactions using TDP, GGDP, or farnesyl diphosphate as the substrate. These results suggested that an actual reaction catalyzed by the ORF12 was the conversion of TDP into TTE in vivo.

Isoprenoids are the largest single family of compounds found in nature with over 22,000 known examples and can be classified into several groups based on the number of C5 unit derived from isopentenyl diphosphate (1). The successive condensation of isopentenyl diphosphate with dimethylallyl diphosphate gives rise to geranyl diphosphate (C<sub>10</sub>), farnesyl diphosphate (FDP) (C<sub>15</sub>), and geranylgeranyl diphosphate (GGDP, C<sub>20</sub>), which are usually cyclized by organism-specific cyclases to produce the parent skeletons of monoterpenes (C<sub>10</sub>), sesquiterpenes (C<sub>15</sub>), and diterpenes (C<sub>20</sub>) (1, 2).

Until now, >50 isoprenoid cyclase genes and a few isoprenoid cyclase genes have been cloned from eukaryotes and prokaryotes, respectively (2). The cyclization mechanisms of their products were extensively studied by using recombinant enzymes. Triterpene cyclases from either eukaryotes (3–10) or prokaryotes (11–17) are representatives. As for diterpene cyclases, reaction mechanisms of enzymes from eukaryotes such as abietadiene synthase (18–23), (−)-copalyl diphosphate synthase (24), and ent-kaurene synthase (25) have been recently and extensively studied.

We have previously cloned and identified diterpene cyclase genes from <i>Streptomyces griseolosporeus</i> MF730-N6, a diterpenoid antibiotic terpentecin (Fig. 1, compound 4) producer for the first time from prokaryotes (26). GGDP (compound 1) was converted into terpentinetriene (compound 3), which had the same basic skeleton as terpentecin, in the presence of their products ORF11 and ORF12 (26), which were redesignated as Cyc1 and Cyc2, respectively, in this study. The Cyc1 has a weak homology with the N-terminal half of diterpene cyclases from plants and fungi; a sole protein homologous to the Cyc2 is the pentalenane synthase from <i>Streptomyces</i> sp. UCS319 (27). The reaction mechanism of these cyclases, which was investigated by switching the isopentenyl diphosphate-forming pathway, one-shot labeling with perdeuterated mevalonate, and 1H NMR spectroscopy suggested that GGDP was cyclized into a clerodane skeleton through an A/B chair-boat conformation followed by a series of concerted methyl and hydride shifts to afford an intermediary carbenium ion (2).

Diterpene cyclases are classified into two major types with respect to their modes of cyclization (29). One type of the reaction is initiated by ionization of GGDP to an allylic carbonation followed by cyclization and deprotonation to the olefin (2, 30). Casbene synthase (31–33) and taxadiene synthase (34–36) are representatives of this class. The other type of the reaction is initiated by protonation at the 14,15-double bond of GGDP (37–39) in a similar manner to that of squalene (hopene) cycla-

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§ The abbreviations used are: FDP, (E,E)-farnesyl diphosphate; GGDP, (E,E,E)-geranylgeranyl diphosphate; HPLC, high pressure liquid chromatography; TDP, terpentediene diphosphate; MES, 4-morpholinethanesulfonic acid; MOPS, 4-morpholinopropanesulfonic acid; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]aminoethanesulfonic acid; TBQ, tera Bq; GC-MS, gas chromatography-mass spectrometry; TTE, terpentinetriene; AS, abietadiene synthase.

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ses. The former class and the latter class of enzymes are known to possess DxDD and DxDD motifs, which mediate substrate binding by chelation of a divalent metal ion. Considering that the Cyc1 and the Cyc2 have a DxDD motif and a DxDD motif, respectively, the Cyc1 was suggested to convert GGDP into the intermediary carbonyl ion by the protonation-initiated cyclization, and the intermediate would be transformed into terpentene by the ionization-initiated reaction by the Cyc2. In this study, we examined this hypothetical enzymatic reaction by using the purified recombinant enzymes. The unique property of the Cyc2, which was able to accept GGDP and FDP as substrates in addition to the intrinsic substrate, was also reported as revealed by kinetic data.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—(E,E,E)-[1-1H]GGDP (1.48 TBq mmol⁻¹), (E,E)-[1-3H]FDP (1.48 TBq mmol⁻¹), and (E,E)-[1-13C]GGDP (0.74 TBq mmol⁻¹) were obtained from American Radiolabeled Chemicals, Inc. GGDP was purchased from Sigma and further purified by column chromatography to remove impurities such as Mg²⁺. The purification procedure was essentially the same as that for terpentedienol diphosphate (see below). No metal contamination was confirmed by a mode ICPS-1000III sequential mode-sensitive heteronuclear single quantum coherence, field gradient NMR data obtained by phase-sensitive DQF-COSY, and 31P NMR (202 MHz). C-P couplings were observed. Values in parentheses represent ¹H-¹H coupling constants in hertz. ²Groups in parentheses indicate those of terpentene, and the numbering corresponds to that in Fig. 1. ³J-C couplings were observed.

**Structure Determination of Terpentedienol Diphosphate (TDP)—**To purify the product formed by the Cyc1, the enzyme reaction was scaled up directly. The reaction mixture (1000 ml) including EDTA (100 mM) was subjected to column chromatography on a Diaion HP-20 column (Mitsubishi Chemical, Japan), which had been equilibrated with 100 mM EDTA (pH 8.0). After washing the column with 100 mM EDTA (10 liters, pH 8.0) to remove Mg⁴⁺, the material was eluted with methanol followed by filtration and concentrated to dryness. The dried material was dissolved in a small volume of 25 mM NH₄HCO₃/CH₃OH (3:7) and then fractionated by preparative HPLC (Merck Mightsil RP-18 column (250 × 10 mm), mobile phase of 20% acetonitrile, flow rate of 4.726 ml/min, and detection at 210 nm). After the removal of the organic solvent, the aqueous layer was dried to give the compound as a white powder.

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**Enzyme Assay of the Cyc1—**An enzyme reaction to search for a product produced by the Cyc1 was conducted by the same methods as described previously (26). The reaction mixture (500 ml) contained 50 mM Tris-HCl (pH 6.8), 1 mM MgCl₂, 5 mM 2-mercaptoethanol, 0.1% (w/v) Tween 80, 20% (v/v) glycerol, 50 mM G6PD, and 150 µM EDTA. The purified Cyc1 (Table I) was used as the enzyme source (Bio-Rad) using bovine serum albumin as a standard. The apparent molecular mass was estimated by gel filtration using a G3000SWXL (7.8 × 300 mm) column (TOSOH, Tokyo, Japan) in D₂O. The 1H and 13C NMR data were collected with an A500 NMR spectrometer (JEOL, Tokyo, Japan) in D₂O.

**Enzyme Assay of the Cyc2—**Enzyme reactions to search for a product formed by the Cyc2 were carried out under the same conditions as those for the Cyc1 with the exception of substrate and pH 8.0. [¹³C]GGDP (1.48 TBq mm⁻¹), [¹H]GGDP (1.48 TBq mm⁻¹), and [³¹P]GGDP (1.48 TBq mm⁻¹) were used as substrates. [¹³C]GGDP (1.48 TBq mm⁻¹) was used as the substrate instead of cold GGDP. Appropriate amounts of the substrates ([¹H]labeled substrate and unlabeled substrate (1.28867 µl)) were included.

![Fig. 1. Structures of terpentedienol diphosphate (2), terpentetriene (3), and terpentin (4).](image-url)
FIG. 2. Gel filtration chromatography of Cyc1 and Cyc2. Elution profile of the standard proteins (A) and a mixture of the Cyc1 and the Cyc2 (B) are shown. A, molecular mass standards of glutamate dehydrogenase (a, 290 kDa), lactate dehydrogenase (b, 142.0 kDa), enolase (c, 67.0 kDa), myoglobin (d, 110 kDa), and cyt. The structures of the three olefinic compounds generated from FDP (compound 8) by the Cyc2 were also determined in a manner similar to those formed from GGDP and were confirmed to be 7,11-dimethyl-3-methyleneoctadeca-1,6,10-triene-(E) (compound 9), 3,7,11-trimethyldodeca-1,3,6,10-tetraene-(E) (compound 10), and 3,7,11-trimethyldodeca-1,3,6,10-tetraene-(E) (compound 11).

RESULTS

GGDP Was Converted into Terpentetriene via an Intermediate—We have previously reported that GGDP was converted into terpentetriene (TTE) in the presence of both the Cyc1 and the Cyc2 (26). Recently, we have also clarified that GGDP was cyclized into TTE through an A/B chair-boat conformation followed by a series of concerted methyl and hydride shifts to afford an intermediary carbenium ion. Therefore, this intermediate might be detectable if the Cyc1 and the Cyc2 had no interaction with each other. On the other hand, if both of the enzymes formed a heterodimer, which was tightly associated, no intermediates would be detected. To examine whether Cyc1 and Cyc2 formed a heterodimer under enzymatically active conditions, the assay mixture, which contained the His-tagged Cyc1 with a calculated molecular mass of 55 kDa and the Cyc2 with a mass of 37 kDa, was subjected to gel filtration. As shown in Fig. 2, two major peaks corresponding to molecular masses of 70 kDa (Fig. 2B, peak f) and 50 kDa (Fig. 2B, peak g) were detected, and no proteins with a molecular mass of >92 kDa, which corresponds to the molecular mass of the heterodimer, were eluted. Both of the peaks were fractionated and used for an SDS-PAGE analysis and the enzyme assay. The peaks f and g were found to contain the Cyc2 and the Cyc1, respectively, by the SDS-PAGE (data not shown), suggesting that the former and the latter would be a homodimer and a monomer, respectively. By the enzyme assay, GGDP was confirmed to be converted into TTE only when both of the fractions were used as enzyme sources. To obtain direct evidence that the Cyc1 and the Cyc2 did not associate with each other, an enzymes assay was performed. The Cyc1 was incubated with GGDP followed by inactivation by boiling, and then the enzyme reaction was continued by adding the Cyc2 into the reaction mixture. Under this reaction condition, the formation of TTE was confirmed by HPLC analysis. On the other hand, TTE was not formed by the

bated with the freshly prepared Cyc2 (5 μg/ml). The assay was linear with respect to protein concentration up to 5 μg/ml for 15-min incubation, and all assays were carried out under these linear conditions. Control reactions without enzyme and with boiled enzyme were also performed at the various substrate concentrations used in the kinetic assay to evaluate actual enzyme activities. After incubation at 30 °C for 10 min, the reaction mixtures (200 μl) were terminated by the addition of EDTA (83 mM) and were extracted twice more with 500 μl of n-hexane. The combined organic layers were washed with water and then analyzed by a liquid scintillation counter (Beckman). Triplicate sets of enzyme assays were performed at each substrate concentration, and a Lineweaver-Burk plot was used for the estimation of kinetic constants. One unit of the Cyc2 activity was defined as the amount of the enzyme that catalyzes the formation of 1 nmol of terpentetriene, diterpene olefin, or sesquiterpene olefin compounds/min at 30 °C.

Structure Determination of Compounds Produced by the Cyc2—For the structure determination of olefinic compounds formed from GGDP or FDP by the Cyc2, a large scale reaction (1000 ml) was carried out under the condition that the substrate was completely consumed. After extraction of the compound with n-hexane, the material was concentrated to dryness. Thus, the obtained olefinic material was directly analyzed by NMR and GC-MS, because almost no impurities existed in that preparation as judged by HPLC analysis.

HPLC analysis showed that at least two compounds were formed from GGDP by the Cyc2. The compounds were next analyzed by GC-MS, the conditions of which were as follows. Shimadzu GC-17A (MS, QP-5000) equipped with a DB-VRX capillary column (0.25 mm inner diameter × 60 m, J&W Scientific) was used. The column temperature was maintained at 80 °C for 1 min, increased at the following rates: 30–200 °C/min and 5–250 °C/min, and then held at 250 °C for 40 min. Separations were made under a constant flow of 0.7 ml of He/min. By comparing obtained data with those of the Shimadzu Class 5000 software libraries, the compounds were suggested to be 1,3-(20),6,10,14-phytapentaene (Figs. 3 and 4, compound 5) and α-springene (compound 6). These compounds were also verified by comparison of the mass spectrum and retention time with those of authentic standards. To obtain conclusive evidence, NMR analysis was also performed. Because we could not separate 1,3-(20),6,10,14-phytapentaene (compound 5) and α-springene (compound 6) by any preparative chromatographic method, the NMR spectra were recorded by using a mixture of these compounds. Surprisingly, the spectral data exhibited a complicated situation, because three compounds were suggested to be involved in the sample. One compound was relatively abundant and could be determined to be 1,3-(20),6,10,14-phytapentaene (compound 5) by the 1H and 13C NMR analyses. Other two compounds were suggested to have similar structures to 1,3-(20),6,10,14-phytapentaene (compound 5) and were confirmed to be α-springene (compound 6) and 3,7,11,15-tetramethyldodeca-1,3,6,10,14-pentaene-(E,E,E) (compound 7) by phase-sensitive DQF-COSY, phase-sensitive HSQC, FG-HMBC, and CT-HMBC experiments.

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enzyme reaction in which the Cyc2 was firstly used followed by inactivation, and then the Cyc1 was added. These results suggested that the Cyc1 and the Cyc2 were not associated and that an intermediate could be formed from GGDP by the Cyc1. Therefore, we tried to find the intermediate compound.

**Structural Analysis of the Intermediate**—To find the compound formed from GGDP by the Cyc1, GGDP was incubated with the purified Cyc1 or a heat-denatured Cyc1 under the standard reaction condition, and the reaction mixture was analyzed by reversed-phase HPLC using an ion-pair reagent. A new product, which was eluted with a retention time shorter than that of GGDP, was specifically detected in the reaction mixture using the active Cyc1 (Fig. 3A). The product was purified, and its structure was determined as column 2 in Fig. 1. We propose to name this new product as TDP based on structural similarities to terpentetriene and the presence of two double bonds and a diphosphate.

**Characterization of Cyc1**—We investigated the effects of metal ions on the enzyme activity, because many terpenoid cyclases analyzed so far required Mg$^{2+}$. The Cyc1 (Fig. 4, Reaction 1, and Table II) was dialyzed with 50 mM Tris-HCl (pH 6.8) containing 10 mM EDTA for 24 h and then diluted with the same buffer without EDTA 20 times. Thereafter, 1 or 10 mM divalent cations were added, and the enzyme activity was assayed. No formation of TDP was detected in the absence of Mg$^{2+}$. The enzyme activity of the Cyc1 was highest at a concentration of 1 mM but slightly inhibited at a concentration of 10 mM (decreased 40%). The addition of 10 mM EDTA resulted in the almost complete loss of the enzyme activity. No activity was detected with other divalent metal ions such as Ca$^{2+}$, Co$^{2+}$, Cu$^{2+}$, Fe$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$ at both 1 and 10 mM.

The optimum pH was measured in several Good's buffers at various pH values (final concentration of 0.05 M): MES (pH 5–6.5); MOPS (pH 6.5–7.9); TES (pH 6.8–8.2); and Tris-HCl (pH 6.5–9.0). The Cyc1 showed higher activity around neutral pH with each of the buffers. Maximum activity was observed at pH 6.8 (Tris-HCl) and was rapidly lost with either decreasing or increasing pH. The effect of temperature on the enzyme activity was also investigated over the range of 25–70 °C in 0.05 M Tris-HCl buffer (pH 6.8). The enzyme activity was maximal at 25–30 °C and was not detected at above 50 °C. The Cyc1 retained full activity after incubation at 30 °C in 0.05 M Tris-
HCl buffer (pH 6.8) for 1 h. Under the optimum pH and temperature conditions, other additives that enhanced the enzyme activity were also investigated. Finally, 20% glycerol, 5 mM 2-mercaptoethanol, and 0.1% Tween 80 were found to be required for the full activity of the Cyc1.

The kinetic properties of the Cyc1 were studied under the optimum reaction conditions. The $K_m$ and $V_{max}$ values were calculated to be 64.2 $\pm$ 5.7 $\mu$M for GGDP and 94.7 $\pm$ 6.9 units/mg, respectively. The enzyme activity was inhibited by GGDP at a concentration of $>50$ $\mu$M as had been reported for other terpenoid cyclases (18, 24, 40).

Characterization of Cyc2—As described above, GGDP was converted into TDP by the Cyc1. Therefore, the Cyc2 (Fig. 4, Reaction 2, and Table II) was expected to catalyze a reaction from TDP to TTE. To verify this assumption, TDP was incubated with the Cyc2 or the heat-denatured Cyc2 under the standard reaction conditions, and the reaction mixture was analyzed by reversed-phase HPLC with an authentic compound as a control. When TDP was incubated with the Cyc2, TTE was specifically formed (Fig. 3B). The formed compound was also confirmed to be TTE by GC-MS and NMR analyses showing that TDP is the real intermediate converted from GGDP by the Cyc1.

The effects of metal ions on the activity of the Cyc2 were studied. The metal ion-free Cyc2, which was prepared by the same method as that for the Cyc1, was used for the enzyme
The Cyc2 Reacted Even with GGDP and FDP—The optimum pH of both the Cyc1 and Cyc2 were 6.8 as described above. When GGDP was incubated with both the Cyc1 and the Cyc2 at pH 7.5 at which the Cyc1 and the Cyc2 showed a weak activity and a high activity, respectively, at least two unknown products, both of which were eluted with a retention time slightly shorter than that of TTE by HPLC analysis, were found to be formed in the reaction mixture in addition to TTE. When the Cyc1 was incubated with GGDP at pH 7.5, the remaining GGDP and TDP formed were the only detectable compounds. On the other hand, the incubation of GGDP with the Cyc2 resulted in the formation of unknown compounds. Finally, the formation of these products was found to be completely dependent on the presence of both the Cyc2 and GGDP, suggesting that the Cyc2 catalyzed not only the conversion of TDP into TTE but also the reaction from GGDP to the unknown compounds (Fig. 3C). By GC-MS and NMR analyses, the compounds were found to be a mixture of the following three compounds: 1,3-(20),6,10,14-phytapentaene (compound 5) and 3,7,11,15-tetramethylhexadeca-1,3,6,10,14-pentaene-(E,E,E) (compound 7) (42), all of which were previously reported. Considering the structures of these three compounds, the Cyc2 with the DBXXD motif would react with GGDP by ionization-initiated reaction to an allylic carbocation followed by deprotonation to the olefin. Therefore, we expected that the Cyc2 might react with other prenyl diphosphates such as FDP (compound 8) and GDP (compound 12), which are one and two C₂ units shorter than that of GGDP. When the Cyc2 was incubated with FDP, at least two new compounds were also detected by HPLC analysis in a similar manner as those with the GGDP. These compounds were purified, and their structures were determined. As shown in Fig. 4, three olefinic compounds (compounds 9, 10, and 11), which had the same structures as those formed from GGDP with the exception of the chain lengths and which had previously been reported (43–45), were found to be formed. On the other hand, GDP was inactive as a substrate.

Optimization of the Reactions Catalyzed by Cyc2 with GGDP and FDP as Substrates—Optimum conditions of the reactions catalyzed by the Cyc2 with GGDP (Fig. 4, Reaction 3, and Table II) and FDP (Reaction 4) as the substrates were investigated. Mg²⁺ ion was essential, and an optimum concentration was determined to be 1 mM for both of the reactions. The enzyme activities of both of the reactions were also detected with 1 mM Fe²⁺ and Mn²⁺, the relative activities of which were 59 and 30% with GGDP and 57 and 34% with FDP in the reaction with 1 mM Mg²⁺. Interestingly, the preferable metal ions were in the reverse order in the reaction with TDP as the substrate, and Co²⁺, which was effective on the reaction with TDP as the substrate, was inactive. No activity was detected with Ca²⁺ and Cu²⁺. The optimum pH and the temperature of both of the reactions were investigated with the same buffers as those used for characterization of the Cyc1. Both of the reactions had a pH optimum of 8.0 with Tris-HCl buffer. The optimum temperatures were the same as those of the reaction with TDP as the substrate. The additives described above were also required for maximum activity.

Kinetic Properties of the Reactions Catalyzed by Cyc2—The kinetic studies of the reactions catalyzed by Cyc2 with TDP (Fig. 4, Reaction 2), GGDP (Reaction 3), and FDP (Reaction 4) as the substrates were performed. As described above, the optimum pH of Reactions 2, 3, and 4 were 6.8, 8.0, and 8.0, respectively. However, the assay mixtures adjusted at pH 8.0 for Tris-HCl were used in all kinetic studies, because these reactions could kinetically be comparable at the same pH and the Cyc2 showed 80% of activities in Reaction 2 at pH 8.0. Kinetic parameters are summarized and shown in Table II. The Kₘ values of the Cyc2 were calculated to be 7.6 ± 0.6 μM for TDP, 7.9 ± 0.6 μM for GGDP, and 61.7 ± 3.0 μM for FDP, indicating that the enzyme has a higher affinity for C₁₀₀ substrates than for C₁₅ substrates. The Vₘₐₓ value of Cyc2 for TDP was 13-fold higher than that for GGDP and 7-fold higher than that for FDP. The calculated Vₘₐₓ/Kₘ value of Reaction 2 was 15-fold higher than that for Reaction 3 and 58-fold higher than that for Reaction 4. These results suggested that the conversion of TDP into TTE was probably a reasonable reaction among those catalyzed by the Cyc2 and that diterpene olefins and sesquiterpene olefins, which were formed by Reactions 3 and 4, respectively, were artifacts generated in vitro. In fact, we could not detect such olefinic compounds in the culture broth of TP producer and of Streptomyces lividans in which the Cyc1 and Cyc2 genes were heterologously expressed (26).

**DISCUSSION**

In this study, we performed functional analysis of two enzymes, Cyc1 and Cyc2, both of which are essential for the conversion of GGDP into TTE. By using the purified recombinant enzymes, we suggest that the Cyc1 and the Cyc2 would be a monomer and a homodimer, respectively, and that the Cyc1 product with a DXDD motif converts GGDP into TDP by protonation-initiated cyclization and the Cyc2 product with a DDXXD motif and then completes the transformation by ioni-
zation of 2 to an allylic carbonation followed by deprotonation to the olefin. These reactions are analogous to those catalyzed by (−)-copalyl diphosphate synthase (kaurene synthase A) (37, 39) and ent-kaurene synthase (kaurene synthase B) (28, 46) of the gibberellin biosynthetic pathway in plants, although the Cyc2 does not catalyze a cyclization reaction.

Peters and Croteau (18) recently reported a detailed assessment of the protonation-initiated cyclization reaction catalyzed by abietadiene synthase (AS), which converts GGDP into a mixture of abietadiene double bond isomers by two sequential cyclizations, protonation-initiated and ionization-initiated reactions, at separate active sites. They constructed 16 mutated enzymes in which 10 charged or aromatic residues around a double bond at C-12 and C-13 when the reaction pH is varied. However, Cyc2 produced only the stereochemically pure TTE from TDP at various pH levels. These results suggested that the formation of a mixture of the three compounds from GGDP or FDP by the Cyc2 was mainly dependent on an alternative deprotonation of the carbocation in a similar manner as that of AS.

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REFERENCES
1. Connolly, J. D., and Hill, R. A. (1992) Dictionary of Terpenoids. Chapman & Hall, New York
2. Bohm, J., Moyer-Gauen, G., and Croteau, R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4126–4133
3. Abe, I., and Prestwich, G. D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9274–9278
4. Corey, K. J., Cheng, H., Baker, C. H., Matsuoka, S. P. T., Li, D., and Song, X. (1997) J. Am. Chem. Soc. 119, 1277–1288
5. Corey, K. J., Cheng, H., Baker, C. H., Matsuoka, S. P. T., Li, D., and Song, X. (1997) J. Am. Chem. Soc. 119, 1277–1288
6. Peters, R. J., and Croteau, R. B. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 580–584
7. Peters, R. J., Ravn, M. M., Coates, R. M., and Croteau, R. B. (2001) J. Am. Chem. Soc. 123, 8974–8978
8. Peters, R. J., Flory, J. E., Jetter, R., Ravn, M. M., Lee, H. J., Coates, R. M., and Croteau, R. B. (2000) Biochemistry 39, 15592–15602
9. Ravn, M. M., Coates, R. M., Flory, J. E., Peters, R. J., and Croteau, R. (2000) Org. Lett. 2, 573–576
10. Tippelt, A., Jahnke, L., and Croteau, R. (1998) Biochim. Biophys. Acta 1391, 223–232
11. Wendt, K., Roraille, K., and Schulz, G. E. (1997) Science 277, 1811–1815
12. Hoshino, T., and Sato, T. (2002) Chem. Commun. 4, 201–204
13. Peters, R. J., and Croteau, R. B. (2002) Biochemistry 41, 1836–1842
14. Peters, R. J., and Croteau, R. B. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 580–584
15. Peters, R. J., Ravn, M. M., Coates, R. M., and Croteau, R. B. (2001) J. Am. Chem. Soc. 123, 8974–8978
16. Ravn, M. M., Coates, R. M., Flory, J. E., Peters, R. J., and Croteau, R. (2000) Org. Lett. 2, 573–576
17. Vogel, B. S., Wildung, M. R., Vogel, G., and Croteau, R. (1996) J. Biol. Chem. 271, 23262–23268
18. Kawaide, H., Sassa, T., and Kamiya, Y. (2000) J. Biol. Chem. 275, 2277–2280
19. Kawaide, H., Imai, R., Sassa, T., and Kamiya, Y. (1997) J. Biol. Chem. 272, 21706–21712
20. Dairi, T., Hamano, Y., Kuzuyama, T., Inub, K., Furukita, H., and Seto, H. (2001) J. Bacteriol. 183, 6085–6094
21. Cano, E. D., Sobh, J. K., Lamberson, C. R., Rudnicki, S. M., Wu, Z., Lloyd, D. J., Oliver, J. S., and Hubbard, B. R. (1994) Biochemistry 33, 5846–5857
22. Raithel, I. J., Fellows, B., and West, C. A. (1984) Phytochemistry 23, 1261–1267
23. MacMillan, J., and Beale, M. H. (1999) Comprehensive Natural Products Chemistry, Vol. 2, pp. 217–243, Elsevier Science Publishing Co., Inc., New York
24. McCaskill, D., and Croteau, R. (1997) Adv. Biochem. Eng. Biotechnol. 55, 77–146
25. Mau, C. J. D., and West, C. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8497–8501
26. Hong, K., Huang, Q., and Scott, A. I. (1998) Arch. Biochem. Biophys. 352, 144–152
27. Hill, A. M., Cano, D. E., Mau, C. J. D., and West, C. A. (1996) Arch. Biochem. Biophys. 336, 283–289
28. Coepp, A. E., Hezari, M., Zajic, J., Vogel, B. S., LaFever, R. E., Lewis, N. G., and Croteau, R. (1995) J. Biol. Chem. 270, 8866–8890
29. Lin, X., Hezari, M., Coepp, A. E., Floss, H. G., and Croteau, R. (1996) Biochemistry 35, 2968–2971
30. Wildung, M. R., and Croteau, R. (1996) J. Biol. Chem. 271, 9201–9204
31. Ait-Ali, T., Swain, S. M., Reid, J. B., Sun, T. P., and Kamiya, Y. (1997) Plant 141, 443–454
32. Hedden, P., and Kamiya, Y. (1997) Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 413–460
33. Sun, T. P., and Kamiya, Y. (1994) Plant Cell 6, 1509–1518
34. Frat, R. G., and West, C. A. (1977) Plant Physiol. (Bethesda) 59, 22–29
35. Burger, B. V., Le Roux, M., Spies, H. S., Truter, V., and Bigalke, R. C. (1981) Z. Naturforsch C. Biosci. 36, 340–343
36. Waterhouse, J. S. Ke, J. Pickett, J. A., and Weldon, P. J. (1996) J. Chem. Ecol. 22, 1307–1314
37. Nabeta, K., Arata, A., Aoki, Y., and Miyake, M. (1990) J. Nat. Prod. 53, 1241–1248
38. Sasaoka, S., Yamamoto, T., Kinoshita, H., Inomata, K., and Kotake, H. (1985) Z. Naturforsch C. Biosci. 36, 340–343
39. Waterhouse, J. S. Ke, J. Pickett, J. A., and Weldon, P. J. (1996) J. Chem. Ecol. 22, 1307–1314
40. Abe, E., Ito, T., and Odaigter, S. (1980) Nippon Nogeikagaku Kaishi 54, 761–764
41. Duncan, J. D., and West, C. A. (1981) Plant Physiol. (Bethesda) 68, 1128–1134