Substrate Specificities of Wild and Mutated Farnesyl Diphosphate Synthases from Bacillus Stearothermophilus with Artificial Substrates

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To determine the substrate specificities of wild and mutated types of farnesyl diphosphate (FPP) synthases from Bacillus stearothermophilus, we examined the reactivities of 8-hydroxygeranyl diphosphate (HOGPP) and 8-methoxygeranyl diphosphate (CH₃OGPP) as aliphatic substrate homologs.

The wild-type FPP synthase reaction of HOGPP (and CH₃OGPP) with isopentenyl diphosphate (IPP) gave hydroxyfarnesyl- (and methoxyfarnesyl-) diphosphates that stopped at the first stage of condensation.

On the other hand, with mutated type FPP synthase (Y81S), the former gave hydroxygeranylgeranyl diphosphate as the main double-condensation product together with hydroxyfarnesyl diphosphate as a single-condensation product and a small amount of hydroxygeranyl-farnesyl diphosphate as a triple-condensation product. Moreover, the latter gave a double-condensation product, methoxygeranylgeranyl diphosphate, as the main product and only a trace of methoxyfarnesyl diphosphate was obtained.

Key words: wild and mutated farnesyl diphosphate synthases; substrate specificity; homologs of isopentenyl diphosphate; 8-hydroxygeranyl diphosphate and 8-methoxygeranyl diphosphates; Bacillus stearothermophilus.

Farnesyl diphosphate synthase [EC 2.5.1.10] is one of the basic enzymes in the biosynthesis of isoprenoids, and it is widely distributed from higher organisms to bacteria. It catalyzes the “head-to-tail” condensations of two molecules of isopentenyl diphosphate (IPP, 1) with dimethylallyl diphosphate (DMAPP, 2) as a primer substrate via geranyl diphosphate (GPP, 3) as the intermediate to give the final product, (all-E)-farnesyl diphosphate (FPP, 4), as shown in Scheme 1.1,2 The carbon skeletons of most isoprenoids compounds, including biologically active substances, are biosynthesized from the two simple precursors IPP and DMAPP by the catalytic reaction of FPP synthase. Naturally occurring insect pheromones such as faranal, the trail mark pheromone of the Pharaoh’s ant,3,4 the aggregation pheromone of red flour beetles,5,6 and all of the insect juvenile hormones7–17 are biologically active molecules involved in the synthesis of isoprenoid compounds. Hydroxygeraniol is also a biologically active isoprenoid, acting as a homolog of pheromones and self-defence substances in insects such as Danaus chrysippus and Plagiodesma versicolora.18,19 Maki and co-workers have reported the synthesis of the butterfly hair pencil pheromone, methoxymethoxygeraniol, using FPP synthase,20,21 employing methoxy- or hydroxyl-homologs of GPP or DMAPP.

We have recently reported the applicability of the prenyl chain elongating reaction to the synthesis of antiproliferative terpenes, including (2Z)-4,8-dimethyl-non-2-en sodium sulfate, which was isolated from a sea squirt,22) by the use of the substrate specificities of several prenyl chain elongating enzymes with respect to an artificial IPP homolog, 4-methyl-4-pentenyl diphosphate.23

The genes encoding the FPP synthase of Bacillus...
**Materials and Methods**

**Analysis.** The prenyl alcohols produced by alkaline phosphatase treatment of the products from enzymatic reactions were measured by HPLC. The HPLC conditions, using a Hitachi type L-6000, equipped with Hitachi L-7420 (LaChrom) type UV–VIS detector with a ChromatoDAQ II (ULVAC) and with a LichroCART (Merck) column with the eluent of solvent mixture of hexane:2-propanol (20:1 (A) and 80:1 (v/v) (B)), were similar to those previously reported. Identification of the reaction products was carried out using GC–MS, a JMS-AM II 50 type GC Mass spectrometer connected with an HP 5890 series II Gas chromatograph equipped with Ultra-alloy-1 (S). The column temperature was programmed from 90 °C to 280 °C with a linear gradient temperature increase at a rate of 15 °C/min finally held at 280 °C for 3 min. The yields of FPP synthase reactions were determined relative to those of FPP derived from IPP and GPP.

The IR spectra were taken using a Hitachi 260-10 and a BIO-RAD FTS-30.

The NMR spectra were recorded on JEOL JNMGX 270 FT NMR and JEOL JNM-ECA 500 FT NMR spectrometers using TMS as an internal standard in CDCl$_3$.

**Chemicals.** Syntheses of 8-hydroxygeranyl and 8-methoxygeranyl diphosphates. 8-Hydroxygeranyl chloride (HOGCl, yield: 83 mg, 0.44 mmol) was prepared by oxidation with selenium dioxide in the presence of t-BuOOH from geranyl chloride (2.1 mmol), which was derived from the chlorination of geraniol (2.6 mmol).

1$^H$ NMR (CDCl$_3$, TMS) of HOGCl was as follows: $\delta$ 1.67 (3H, s.), 1.73 (3H, s.), 2.10 (2H, t. $J = 6.0$ Hz), 2.16 (2H, dd. $J = 6.5$ Hz), and 5.40 (2H, dd. $J = 7.6$ Hz), and 5.40 (2H, dd. $J = 7.9$ Hz). $^{13}$C NMR (CDCl$_3$, dept) was as follows: $\delta$ 14.0 (CH$_3$), 28.7 (CH$_2$), 29.0 (CH$_2$), 31.8 (CH$_2$), 65.9 (CH$_2$), 128.9 (CH), 130.9 (CH), and 132.4 (C, C). GC–MS (rel. int.) of HOGCl: $m/z$ 188 (M$^+$) (0.1%), 152 (3.6), 134 (31.4), and 91 (base peak).

Then HOGCl was converted to the corresponding diphosphate by Davison's method.

The hydroxyl function of geraniol (GOH) was protected by treatment with dihydroxypropane in the presence of a small amount of pyridinium-toluene sulfonate as tetrahydroxypyranyl (THP) ether. The ether was oxidized...
with selenium dioxide to give 8-hydroxygeranyl-OTHP ether. Then 8-methoxygeranyl-OTHP ether was obtained by the methylation of 8-hydroxygeranyl-OTHP ether with methyl iodide and sodium hydride. Subsequently, deprotection of 8-methoxygeranyl-OTHP ether was carried out with PPTS (pyridinium p-toluenesulfonate) to give 8-methoxygeraniol. 1H NMR (CDCl3, TMS) of 8-methoxygeraniol was as follows: δ 1.57 (3H, s.), 1.63 (3H, s.), 1.64 (3H, s.), 1.66 (3H, s.), 2.05 (3H, s.), 2.0–2.3 (12H, m.), 3.98 (1H, t. J = 6.5 Hz), 4.59 (2H, d. J = 7.2 Hz), 5.10 (2H, t. J = 7.2 Hz), 5.34 (1H, t. J = 7.0 Hz), and 5.38 (1H, d.d. J = 5.5, 7.0 Hz). GC–MS (rel. int.) of 8-methoxygeraniol (HOGGOAc) was obtained by hydrolysis of geranylgeranyl acetate (1.70 mmol), similarly to the procedure described above; it was derived from the acetylation of geranylgeraniol (1.72 mmol). 1H NMR (CDCl3, TMS) of HOGGOAc was as follows: δ 1.60 (3H, s.), 1.63 (3H, s.), 1.64 (3H, s.), 1.66 (3H, s.), 2.05 (3H, s.), 2.0–2.3 (12H, m.), 3.98 (1H, t. J = 6.5 Hz), and 3.66 (2H, s.), 3.98 (2H, d. J = 8 Hz), and 5.25 (2H, t. J = 8 Hz). IR ʋmax (KBr) cm−1 was as follows: 3,400, 2,980, 1,660, 1,440, 1,380, 1,190, 1,090, 1,030, and 960. Then chlorination and diphosphorylation of 8-methoxygeraniol were carried out to give 8-methoxygeranyl diphosphate.

Synthesis of authentic 16-hydroxygeranylgeraniol. 16-Hydroxygeranylgeranyl acetate (HOGGOAc, yield: 27.0 mg, 0.08 mmol) was prepared by oxidation with selenium dioxide in the presence of t-BuOOH from geranylgeranyl acetate (1.70 mmol), similarly to the procedure described above; it was derived from the acetylation of geranylgeraniol (1.70 mmol), similarly to the procedure described above; it was derived from the acetylation of geranylgeraniol (1.72 mmol). 1H NMR (CDCl3, TMS) of HOGGOAc was as follows: δ 1.57 (3H, s.), 1.63 (3H, s.), 1.64 (3H, s.), 1.66 (3H, s.), 2.05 (3H, s.), 2.0–2.3 (12H, m.), 3.98 (1H, t. J = 6.5 Hz), 4.59 (2H, d. J = 7.2 Hz), 5.10 (2H, t. J = 7.2 Hz), 5.34 (1H, t. J = 7.0 Hz), and 5.38 (1H, d.d. J = 5.5, 7.0 Hz). GC–MS (rel. int.) of HOGGOAc: m/z 348 (M+) (0.01%), 330 (0.6), 288 (0.1), 270 (14.5), 203 (2.4), 135 (63.5), and 93 (base peak). 16-Hydroxygeranylgeraniol (HOGGOH) was obtained by hydrolysis of HOGGOAc with potassium carbonate in ethanol. The yield of HOGGOH was 9.0 mg (37.9%). 1H NMR (CDCl3, TMS) of HOGGOH was as follows: δ 1.60 (3H, s.), 1.61 (3H, s.), 1.67 (3H, s.), 1.68 (3H, s.), 2.0–2.1 (12H, m.), 2.12 (2H, br. s.), 4.00 (2H, s.), 4.16 (2H, d. J = 7.0 Hz), 5.11 (2H, t. J = 7.0 Hz), 5.39 (1H, t. J = 7.7 Hz), and 5.42 (1H, dd. J = 6.3 Hz). GC–MS (rel. int.) of HOGGOH: m/z 306 (M+) (0.2), 288 (0.5), 270 (28.0), 202 (8.1), 134 (19.1), and 93 (base peak).

Purification of FPP synthases.

Purification of wild-type FPP synthase. Purification of wild-type FPP synthase from B. stearothermophilus was carried out according to the method reported previously.23,28

Purification of mutated FPP synthases. We purified mutated FPP synthases (Y81D and Y81S) from Escherichia coli DH5α cells harboring the plasmids that carry corresponding mutated FPP synthases,27 which were kindly provided by Dr. Hemmi and Dr. Nakayama. The cells were harvested, and disrupted by sonication in 50 mM Tris–HCl buffer (pH 7.0) containing 10 mM 2-mercaptoethanol and 1 mM EDTA. The homogenate was heated at 55°C for 60 min and then centrifuged at 100,000 × g for 10 min. Further purification of the mutated enzyme was carried out according to the method described previously.24

Conditions of the enzymatic reaction. The incubation mixture for the B. stearothermophilus FPP synthase reaction contained, in a total volume of 1 ml, 100 mmol of Tris–HCl buffer (pH 8.5), 10 μmol of MgCl2, 50 μM β-mercaptoethanol, 5 μmol of KCl, 0.5 μmol of an allylic substrate (HOFP and methoxyGPP) to be examined, 0.5 μmol of IPP, and wild and mutated FPP synthases (about 25 μg). After incubation at 55°C for 3 h, the reaction mixture was treated with alkaline phosphatase for 5 h, and extracted with pentane and analyzed by HPLC and GC–MS.23

Results and Discussion

To clarify the reactivities of allylic substrates having a hydroxyl or a methoxy group, we examined the substrate specificities of wild and mutated FPP synthases of B. stearothermophilus.

Reaction of hydroxygeranyl diphosphate (3a) with IPP (1) using wild-type FPP synthase

As shown in Scheme 3, the hydrolysate derived from alkaline phosphatase treatment of the product obtained from a wild-type FPP synthase reaction of hydroxygeranyl diphosphate (3a) with IPP (1), which eluted at a peak of 35.5 min on HPLC (elucent A), was subjected to GC–MS analysis. The MS spectrum of the alcohol showed a molecular ion at m/z 238 (rel. int. 0.03%), corresponding to C15H26O2, together with main fragment ions at m/z 220 (M+ – 18) (2.3), 202 (M+ – 18 – 18) (25.2), 135 (M+ – 18 – 18 – 67) (5.0), and 93 (base peak), indicating that the product had a 12-hydroxy-3,7,11-trimethyltrideca-2,6,10-trien-1-ol (12-hydroxyfarnesol, HOFOH) structure. It is reasonable to assign the product to HOFOH 4a-OH, considering the nature of the enzymatic reaction. As shown in Table 1, the relative yield of the product 4a-OH was 11.2% of that of IPP derived from the reaction with GPP and IPP. Our re-examination of the action of the porcine liver FPP synthase-catalyzed reaction of 3a with 1 gave the same product in a yield of 29.2% under the same conditions for the bacterial enzyme, except that the incubation temperature was 37°C.

Reaction of hydroxygeranyl diphosphate (3a) with IPP (1) using mutated FPP synthases

The alcohols were derived from the mutated FPP Y81D (Y81S) and Y81S) from Escherichia coli DH5α cells harboring the plasmids that carry corresponding mutated FPP synthases,27 which were kindly provided by Dr. Hemmi and Dr. Nakayama. The cells were harvested, and disrupted by sonication in 50 mM Tris–HCl buffer (pH 7.0) containing 10 mM 2-mercaptoethanol and 1 mM EDTA. The homogenate was heated at 55°C for 60 min and then centrifuged at 100,000 × g for 10 min. Further purification of the mutated enzyme was carried out according to the method described previously.24
15,19-pentamethylicosa-2,6,10,14,18-pentaen-1-ol (20-hydroxygeranylfarnesol, HOGFOH) structure. It is reasonable to assign the product to HOGFOH\(6a\text{-OH}\) according to Poulter’s mechanism of the FPP synthase reaction.\(^{30}\) This result suggests that the mutated FPP Y81D (and Y81S) synthase reaction with \(3a\) and \(1\) stops at the stage of triple condensation of IPP.

Furthermore, the MS spectrum of the second product (34.7 min) showed a similar cleavage pattern, at \(m/z = 238\) (rel. int. 3.3%), corresponding to \(C_{15}H_{26}O_2\), and other main fragment ions at \(m/z = 220, 202, 135,\) and 93 (base peak), with some differences in relative intensity, which also indicates a \(4a\text{-OH}\) structure.

The MS spectrum of the third product (37.6 min) showed a distinct dehydoration (\(M^+ – 18\)) at \(m/z = 288\) (rel. int. 0.3%), corresponding to \(C_{20}H_{32}O_2\), although the molecular ion was ambiguous. The other fragment ions at \(m/z = 270\) (\(M^+ – 18 – 18\) (15.9), 203 (\(M^+ – 18 – 18 – 18\) (4.7), 135 (\(M^+ – 18 – 18 – 67\) (16.1), and 93 (base peak), indicate that the alcohol had a 16-hydroxy-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraen-1-ol (16-hydroxygeranylgeraniol, HOGGOH) structure. Therefore, this product can also be assigned to \(5a\text{-OH}\), suggesting that chain elongation stopped after the condensation of two molecules of \(1\). When we injected \(5a\text{-OH}\) with an authentic sample of HOGGOH, only a single peak, at 40.3 min, was obtained, which indicates that \(5a\text{-OH}\) was HOGGOH.

\(4a\text{-OH}\) gave 0.8% of the yield of the product derived from the reaction with \(3a\) and \(1\) using mutated FPP synthase. However, the main product, \(5a\text{-OH}\) by double condensation with them, was obtained in a yield of 38.2%, and then \(6a\text{-OH}\) by triple condensation was obtained in 1.4% yield.

The enzymatic reaction between the GPP-homolog and \(1\) using FPP synthase should afford the product FPP-homolog. However, when mutated FPP Y81D (and Y81S) synthase was used, the condensation products with two and three molecules of \(1\) (\(5a\) and \(6a\)), which were not obtained easily using the wild-type enzyme, were obtained.

By creating these mutated FPP synthases, Nishino and co-workers confirmed that Y81 of the wild-type enzyme played a key role in determining the length of the chain. Furthermore, they confirmed that the function of these recombinant enzymes was changed to that of a GPP synthase.\(^{24,25}\) As shown in Scheme 4, the mechanism of chain-length elongation is as follows: when the bulky Y81 five upstream of FARM (first aspartate rich motif), is replaced with a smaller amino acid such as serine or aspartate, the benzyl group of the tyrosine is not present to cause an obstruction and it is as if the stopper has come off, thus allowing longer prenyl diphosphates to be synthesized.

\textbf{Reactions of methoxygeranyl diphosphate (3b) with IPP using wild-type FPP synthase}

The alcohols derived from the products of wild-type
FPP synthase catalyzed the reaction of methoxygeranyl diphosphate (3b) with 1 and gave a peak at 63.7 min on HPLC (eluent B), and were subjected to GC–MS. The MS spectrum of the product showed a molecular ion at \( m/z = 252 \) (rel. int. 0.1%), corresponding to C_{18}H_{28}O_{2}, and other main fragment ions at \( m/z = 234 \) (M^{+} – 18) (3.7), 202 (M^{+} – 18 – 32) (44.4), 134 (M^{+} – 18 – 32 – 68) (30.6), and 91 (base peak), indicating that the product had a 12-methoxy-3,7,11-trimethyltetradeca-2,6,10-trien-1-ol (12-methoxyfarnesol, CH_{3}FOFOH) structure. It is reasonable to assign the alcohol to CH_{3}FOFOH 4b-OH considering the nature of the enzymatic reaction. The relative yield was 188.3% based on the product derived from the reaction between natural substrates 3 and 1. The relative yield exceeded 100%, which shows that the reactivity of FPP synthase with respect to 3b is excellent.

Reactions of methoxygeranyl diphosphate (3b) with IPP using mutated FPP synthases

The mutated FPP Y81D (and Y81S) synthase reaction of 3b with 1 afforded products that were hydrolyzed with phosphatase to two alcohols showing retention times on HPLC (eluent, B) of 54.4 and 63.1 min respectively. Each alcohol was purified and subjected to GC–MS.

The MS spectrum of the former showed an obvious dehydration ion (M^{+} – 18) at \( m/z = 302 \) (rel. int. 0.3%), corresponding to C_{21}H_{26}O, although the molecular ion was obscure. The other main fragment ions were at \( m/z = 270 \) (M^{+} – 18 – 32) (12.1), 203 (M^{+} – 18 – 32 – 67) (3.8), 135 (M^{+} – 18 – 18 – 67 – 68) (11.4), and 93 (base peak), indicating that the alcohol had a 16-methoxy-3,7,11,15-tetramethyldodeca-2,6,10,14-tetraen-1-ol (16-methoxygeranylgeraniol, CH_{3}OGGOH) structure. It can be assigned to alcohol CH_{3}OGGOH, 5b-OH. This result indicates that the mutated enzymatic reaction can extend the chain length up to the stage where the double condensation of GPP-homolog with 1 is completed. The relative yield was 204.7%, based on the product derived from the reaction between natural substrates 3 and 1. In addition, this homolog appears to have excellent reactivity, based on relative yield as compared with natural substrates.

The latter product gave a spectrum similar to that of a wild-type FPP synthase reaction product, showing a molecular ion at \( m/z = 252 \), corresponding to C_{16}H_{28}O_{2}, with fragment ions at \( m/z = 234 \) (M^{+} – 18), 202 (M^{+} – 18 – 32), 134 (M^{+} – 18 – 32 – 68), and 91 (base peak). This product can be reasonably assigned to 12-methoxyfarnesol, 4b-OH (relative yield, 16.1%). No product resulting from condensation of the three molecules of IPP was detected in this experiment.

**Conclusion**

Scheme 3 illustrates the farnesyl diphosphate synthase reaction of GPP-homolog, having either an 8-hydroxy or 8-methoxy group, examined in this study. As for the results, the wild-type FPP synthase reaction of 3a (or 3b) with 1 gave 4a-OH (or 4b-OH) as a single-condensation product. On the other hand, with mutated type FPP synthase (Y81S), the former gave 5a-OH as the main double-condensation product together with 4a-OH and a small amount of 6a-OH as a triple-condensation product. Moreover, the latter gave a double-condensation product, 5b-OH, as the main product, with only a trace of 4b-OH. Scheme 2 illustrates part of an amino acid sequence of the FARM neighborhood, which is regarded as the active site of wild and mutated FPP synthases. The results of this study suggest that prenyl diphosphate, in which the chain length is longer, was formed when Y81 five upstream of FARM was replaced with an amino acid with a smaller bulk such as aspartate or serine (Scheme 4).

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