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Substrate specificities of wild and mutated farnesyl diphosphate synthases: Reactivity of allylic substrate homologs having hydrophilic groups at ω-position

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ABSTRACT

To investigate substrate specificities of wild and mutated types of farnesyl diphosphate synthases from *Bacillus stearothermophilus*, we have examined the reactivities of methoxymethoxydimethylallyl- and propoxygeranyl diphosphates as allylic substrate homologs.

The wild type farnesyl diphosphate synthase reaction of methoxymethoxydimethylallyl and propoxygeranyl diphosphates with isopentenyl diphosphate gave methoxymethoxygeranyl and propoxyfarnesyl diphosphates which stopped at the first stage of the condensation.

Using a mutated farnesyl diphosphate synthase (Y81D FPS), the reaction of methoxymethoxydimethylallyl diphosphate with isopentenyl diphosphate gave only methoxymethoxyfarnesyl diphosphate as single condensation product. Moreover, both of mutated farnesyl diphosphate synthase reaction with propoxygeranyl diphosphate of isopentenyl diphosphate gave propoxyfarnesyl- and propoxygeranylgeranyl diphosphate.

1. Introduction

A great many isoprenoids with varieties of structural diversity, such as steroids, carotenoids, prenyl quinones, and natural rubber, occur in nature. All of these biosynthetic precursors are constructed by the action of prenyl chain elongating enzymes [1–4]. These enzymes can be divided roughly into two major families, *E*- and *Z*-prenyl chain elongating enzyme, each of which are further classified into short-chain-, medium-chain-, and long-chain prenyl diphosphate synthases as shown in Scheme 1 [3,4].

Geranyl diphosphate-, farnesyl diphosphate- and geranylgeranyl diphosphate synthases are included in groups of *E*-short prenyl chain elongating enzymes (***E*-I**) [5–8]. Hexaprenyl diphosphate- and heptaprenyl diphosphate synthases are in *E*-medium prenyl chain elongating enzymes (***E*-II**) [9–12]. Moreover, *E*-long prenyl chain elongating enzymes (***E*-III**) include octaprenyl diphosphate- and solanesyl diphosphate synthases [13,14].

Among the *Z*-prenyl chain elongating enzymes (*Z*)-farnesyl diphosphate synthase is in the *Z*-short prenyl chain elongation enzyme (***Z*-I**) [15,16]. Decaprenyl diphosphate- and undecaprenyl diphosphate synthases are included in a group of *Z*-medium prenyl chain elongating enzymes (***Z*-II**) [17–19]. Furthermore, dehydrodolichyl diphosphate- and natural rubber synthases are included in the *Z*-long prenyl chain elongating enzymes (***Z*-III**) [20–22].

(*E*)-Farnesyl diphosphate synthase (FPP synthase) [EC 2.5.1.10], which has been widely studied for many years, catalyzes the stereospecific condensation of isopentenyl diphosphate (IPP, **1**) with dimethylallyl diphosphate (DMAPP, **2**) *via* geranyl diphosphate (GPP, **3**) to give (all-*E*)-farnesyl diphosphate (FPP, **4**) as the final product as shown in Scheme 2.

Tarshis et al. have reported the detailed research on recombinant avian FPS [23,24]. They have reported that the benzyl groups of F112 and F113 in the avian enzyme have played the especially important role for chain length determination. Furthermore, they reported that longer-chain prenyl diphosphates were afforded by mutated enzyme which replaced the phenylalanine by alanine and/or serine.

On the other hand, we considered that tyrosin-81 of *Bacillus stearothermophilus* FPS is equivalent to this portion (F112, F113) of

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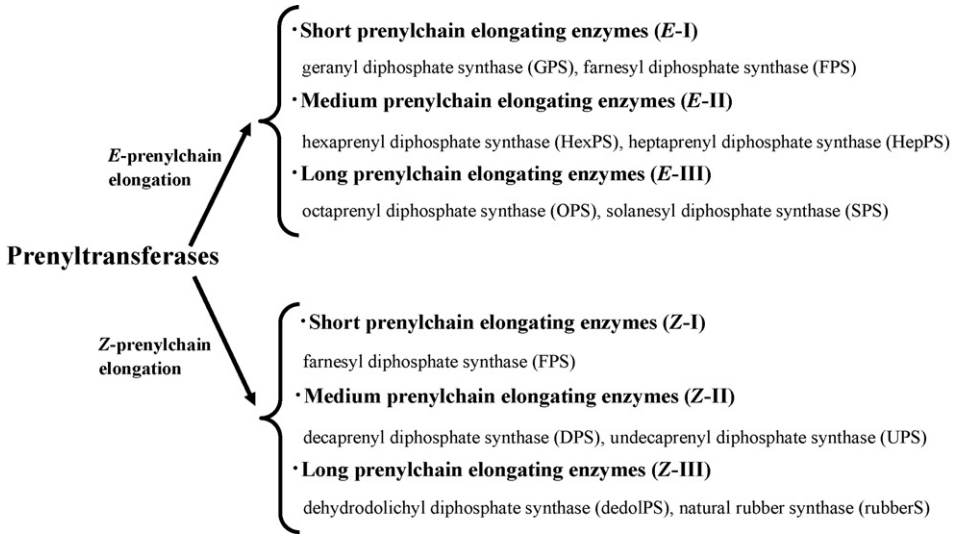
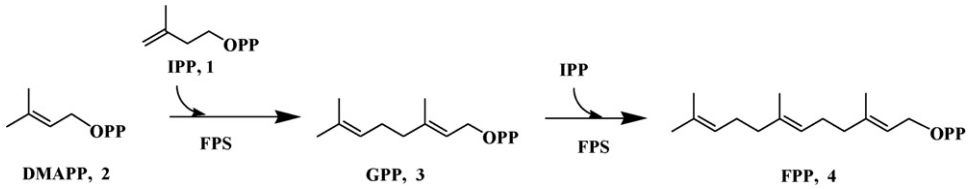


Scheme 1. A classification of prenyl chain elongating enzymes.



Scheme 2. Farnesyl diphosphate synthase reactions of dimethylallyl diphosphate with isopentenyl diphosphate.

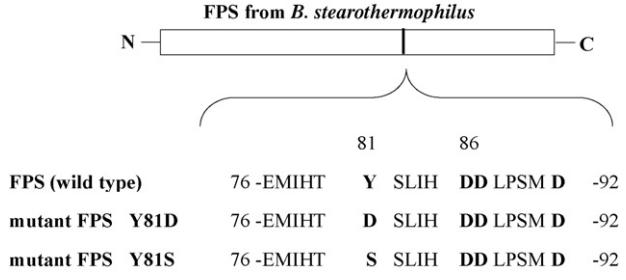
avian enzyme. As shown in Scheme 3, we constructed two kinds of mutated type FPP synthases of *B. stearothermophilus* in which tyrosine-81 was substituted with aspartate (Y81D), and with serine (Y81S), and recently reported the substrate specificities of wild and the mutated farnesyl diphosphate synthases with GPP homologs [25].

In this paper, we describe the substrate specificities of the wild and mutated FPP synthases with respect to two kinds of allylic substrate homologs having a hydrophilic group at ω-position, methoxymethoxydimethylallyl diphosphate (MOMODMAPP) and propoxygeranyl diphosphate (propoxyGPP) as substrates.

2. Experimental

2.1. 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 (80:1) were similar to those previously reported [25,27,28]. Identification of the reaction products was carried out using GC–MS, a JMS-AM II 50 type mass spectrometer connected with HP 5890 series II Gas chromatograph equipped with Ultra-alloy-1 (S). The column temperature was programmed from 40 to 280 °C with a linear gradient temperature increase at a rate of 15 °C/min and then held at 280 °C for 5 min. The yields of FPP synthase reactions were determined as relative to those of FPP derived from IPP and GPP.



Scheme 3. Wild and mutated type of farnesyl diphosphate synthases.

The IR spectra were taken using a Hitachi 260-10 and a BIO-RAD FTS-30. The NMR spectra were recorded on a JEOL JNMGX 270 FT NMR and JEOL JNM-ECA 500 FT NMR spectrometers using TMS as an internal standard in $CDCl_3$.

2.2. Chemicals

2.2.1. Syntheses of methoxymethoxydimethylallyl- and methoxymethoxygeranyl diphosphates

Ethyl 4-methoxymethoxy-3-methyl-2*E*-butenoate was prepared by alkylation of ethyl 4-hyroxy-3-methyl-2*E*-butenoate (600 mg, 4.2 mmol) with chloromethyl methyl ether (8.3 mmol) in the presence of diisopropyl ethyl amine (4.2 mmol) according to a previously reported protocol [25]. The yield was 535 mg (68.4%). ^{1}H NMR ($CDCl_3$, TMS) of ethyl 4-methoxymethoxy-3-methyl-2*E*-butenoate was as follows: δ 1.27 (3H, t, J = 7.1 Hz), 2.05 (3H, s), 3.28 (3H, s), 4.07 (2H, d, J = 7.1 Hz), 4.52 (2H, s), 5.82 (1H, s). IR ν_{max} (neat) cm^{-1}: 1710, 1660, and 1100.

4-Methoxymethoxy-3-methyl-2*E*-butenol (MOMODMAOH) was given by the reduction of ethyl 4-methoxymethoxy-3-methyl-2*E*-butenoate (828 mg, 4.4 mmol) with lithium aluminium hydride (250 mg, 6.6 mmol) in dry ether. The yield was 214 mg (33.3%). ^{1}H

NMR ($CDCl_3$, TMS) of 4-methoxymethoxy-3-methyl-2*E*-butenol was as follows: δ 1.65 (3H, s), 3.25 (3H, s), 3.82 (2H, d, J = 7.2 Hz), 4.02 (2H, s), 4.47 (2H, s), 5.52 (1H, t, J = 7.2 Hz). IR ν_{max} (neat) cm^{-1}: 3400 and 1100.

4-Methoxymethoxy-3-methyl-2*E*-butenyl chloride (MOMODMACl) was prepared by the chlorination of 4-methoxymethoxy-3-methyl-2*E*-butenol (220 mg, 1.5 mmol) according to the similar method [25]. The yield was 170 mg (67%). ^1H NMR ($CDCl_3$, TMS) of 4-methoxymethoxy-3-methyl-2*E*-butenyl chloride: 1.70 (2H, s), 3.23 (3H, s), 3.87 (2H, d, J = 7.6 Hz), 4.03 (2H, s), 4.45 (2H, s), 5.60 (1H, t, J = 7.6 Hz). IR ν_{max} (neat) cm^{-1}: 1250 and 1700.

Then, methoxymethoxydimethylallyl diphosphate (MOMODMAPP, **2a**), was synthesized by the diphosphorylation of MOMODMACl according to the method reported previously [29]. The yield was 69 mg (20.3%).

The 8-methoxymethoxygeranyl acetate (MOMOGOAc, 235 mg, 0.92 mmol) was prepared by alkylation of 8-hydroxygeranyl acetate (1.2 mmol) with chloromethyl methyl ether (2.4 mmol) in the presence of diisopropylethyl amine (1.8 mmol) from hydroxygeranyl acetate (1.2 mmol) according to a previously reported protocol [25]. ^1H NMR ($CDCl_3$, TMS) of MOMOGOAc was as follows: δ 1.63 (3H, s), 1.76 (3H, s), 1.99 (3H, s), 2.11 (2H, br. m.), 3.24 (3H, s), 3.80 (2H, s), 4.37 (2H, s) 4.46 (2H, d, J = 6.7Hz) and 5.23 (2H, t, J = 5.6 Hz). IR ν_{max} (KBr) cm^{-1}: 2900, 1720, 1430, 1360, 1220, 1140, 1090, 1010 and 920.

The 8-methoxymethoxy-3,7-dimethylocta-2,6-dien-1-ol (MOMOGOH) was given by hydrolysis of MOMOGOAc (217 mg, 0.85 mmol) with KOH in methanol. The yield of MOMOGOH was 175 mg (95.8%). ^1H NMR ($CDCl_3$, TMS) of MOMOGOH was as follows: δ 1.60 (6H, s), 2.07 (4H, m.), 3.23 (3H, s), 3.80 (2H, s), 3.93 (2H, d, J = 6.0 Hz), 4.43 (2H, s) and 5.30 (2H, t, J = 6.5 Hz). IR ν_{max} (KBr) cm^{-1}: 3400, 2900, 1660, 1440, 1380, 1200, 1140,1100, 1050, and 920.

The methoxymethoxyfarnesyl diphosphate (MOMOFPP) was prepared from farnesol according to the similar method reported previously [25].

2.2.2. Synthesis of 8-propoxygeranyl diphosphate

8-Propoxygeranyl diphosphate was synthesized according to the method reported previously [25]. *O*-Propylation of 8-hydroxygeranyl-OTHP (3.1 mmol) derived from geraniol with propyl iodide (9.4 mmol) and sodium hydride (19 mmol) gave 8-propoxygeranyl-OTHP (yield: 47.1%). Then 8-propoxygeraniol was obtained by the deprotection of THP-group with *p*-toluenesulphonic acid in methanol. The yield of propoxygeraniol (propoxyGOH) was 150 mg (48.8%). ^1H NMR ($CDCl_3$, TMS) of propoxyGOH was as follows: δ 0.92 (3H, t, J = 7.4 Hz), 1.57 (2H, m), 1.64 (3H, s), 1.68 (2H, s), 2.08 (2H, t, J = 6.5 Hz), 2.17 (2H, m), 3.30 (2H, t, J = 7.0 Hz), 3.82 (2H, s), 4.13 (2H, d, J = 7.0 Hz), 5.37 (1H, t, J = 6.8 Hz) and 5.41 (1H, t, J = 6.4 Hz). IR ν_{max} (KBr) cm^{-1}: 3384, 2960, 1670, 1454, 1382, and 1010.

Then, chlorination and diphosphorylation of 8-propoxygeraniol was carried out to give of 8-propoxygeranyl diphosphate according to the similar method reported previously [25].

2.3. Purification of FPP synthases of *B. stearothermophilus* and porcine liver

The purification of wild type FPP synthase originally cloned from *B. stearothermophilus* ATCC 10149 [6] was carried out according to our method reported previously [5,25,27,28].

In the similar manner, we purified the site-directed mutants of the FPP synthase (Y81D and Y81S) overexpressed in *Escherichia coli* DH5α cells [26]. 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 [25].

Porcine liver FPP synthase was purified according to the method reported previously [27,30].

2.4. Conditions of the enzymatic reaction

The incubation mixture for *B. stearothermophilus* FPP synthase reaction contained, in a total volume of 1 mL, 200 μmol of Tris–HCl buffer (pH 8.5), 10 μmol of $MgCl_2$, 50 μmol of β-mercaptoethanol, 50 μmol of NH_4Cl, 5 μmol of KCl, 0.5 μmol of an allylic substrate (MOMODMAPP and propoxyGPP) to be examined, 0.5 μmol of IPP, and wild and mutated FPP synthases (ca. 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.

2.5. Estimation of the optimized structure

The estimation of optimized structures was performed by theoretical calculations as follows. On an Apple PowerBook G4 computer, the Chem3D Pro 5.0 energy minimization program was used to perform MM2 force field calculations of the energies associated with several potential conformations of model compounds as methoxymethoxyfarnesol and geranylgeraniol (GGOH).

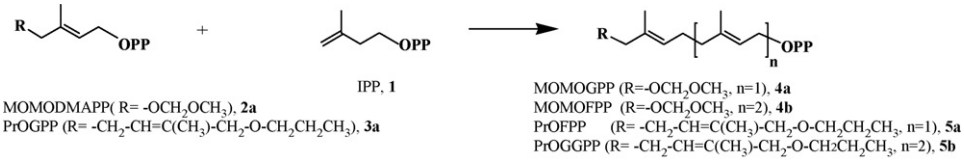
3. Results and discussion

To investigate the reactivities of MOMODMAPP and propoxyGPP, we examined substrate specificities of wild and mutated FPP synthases of *B. stearothermophilus*.

3.1. Reaction of MOMODMAPP (**2a**) by the mutated FPP synthases IPP (**1**) using wild FPP synthase or porcine liver FPP synthase

Reaction of MOMODMAPP (**2a**) with IPP (**1**) by use of the wild type of FPP synthase gave a product which was then hydrolyzed with alkaline phosphatase to the corresponding alcohol, the reactivity was 5% relative to that of FPP derived from **1** and DMAPP as shown in Scheme 4.

The mass spectrum of the alcohol with a retention time of 26.8 min on HPLC, showed a molecular ion at m/z 214 (rel. int. 0.95%), corresponding to $C_{12}H_{22}O_3$, and main fragment ions were observed at m/z 183 $[M-31]^+$ (15.9), 153 $[M-31-30]^+$ (3.3), 135



Scheme 4. Farnesyl diphosphate synthase reactions of MOMODMAPP (or PrOGPP) with IPP.

Table 1
Reactivity of allylic substrate homolog in the reaction with IPP catalyzed by *Bacillus stearothermophilus* FPS

DMAPP homolog	Product	Reactivity (%)			
		Porcine liver FPS	*B. stearothermophilus* FPS	Y81D FPS	Y81S FPS
Reaction with IPP					
DMAPP	FOH	100	100		
MOMODMAPP	MOMOGPP	1	5	n.d.	n.d.
	MOMOFPP	n.d.	n.d.	134	27
propoxyGPP	propoxyFPP	62	3	63	8
	propoxyGGPP	n.d.	n.d.	6	10

Incubation at 55 °C for 3 h. n.d.: not detected. Reactivity is expressed as the relative amount (%) of conversion of IPP into allylic substrate homolog. The products were enzymatically converted to the corresponding alcohols and analyzed by HPLC. Each value represents the mean of at least three determinations.
The relative yields of product alcohols derived from reactions by FPS were calculated as the relative (%) yield to that of FOH derived from the enzymatic reaction with IPP and DMAPP.

$[M-31-30-18]^+$ (4.1), and 91 (base peak), indicating that the product has a 8-methoxymethoxy-3,7-dimethylocta-2,6-dien-1-ol (8-methoxymethoxygeraniol, MOMOGOH) structure. It is reasonable to assign the product is MOMOGPP (**4a**) by considering the manner of the enzymatic reaction.

When we switched the enzyme to porcine liver FPP synthase, reaction of **2a** with **1**, the same product was obtained and the reactivity was 1% under the similar conditions for the bacterial enzyme except that the incubation temperature and pH were 37 °C and 7.5, respectively, as shown in Table 1.

3.2. Reaction of MOMODMAPP (*2a*) with IPP (*1*) with the mutated FPP synthases

Both of the alcohols derived from the reaction of **2a** with **1** by the mutant enzymes, Y81D or Y81S showed a peak on HPLC at a similar retention time of 28.3 min, which were then purified and subjected to GC–MS. The mass spectrum of the product showed a molecular ion $[M]^+$ at *m*/*z* 282 (rel. int. 0.1%), corresponding to $C_{17}H_{30}O_3$, and other fragment ions were observed at *m*/*z* 264 $[M-18]^+$ (0.5), 232 $[M-18-32]^+$ (2.3), 219 $[M-18-45]^+$ (4.1), 202 $[M-18-62]^+$ (64.8), 134 $[M-18-62-68]^+$ (21.1), and 79 (base peak), indicating that the product has a 12-methoxymethoxy-3,7,11-trimethyldodeca-2,6,10-trien-1-ol (methoxymethoxyfarnesol, MOMOFOH) structure. It is reasonable to assign the product to MOMOFPP, **4b** according to Poulter's mechanism of FPP synthase reaction [31]. These results suggest that the mutated FPP Y81D synthase reaction with **2a** and **1** stops at the stage of double condensation of **1**. The relative reactivity was 134% based on the product derived from the reaction between substrates **2** and **1**. The relative reactivity exceeded the basic reaction.

Using the wild type FPP synthase, the reaction of **2a** with **1** gave **4a**, showing that the prenyl chain elongation stopped only at the first stage of condensation. This means that FPP product type resulting from condensation of two molecules of **1** was detected.

Furthermore, when the similar reactions were examined using the mutated FPP synthases, the FOH type, **4b** was obtained.

However, when the optimized structures of MOMOFOH, **4b**–**OH** and GGOH were calculated by MM2 method, the length of a MOMO group is almost equivalent to that of one isoprene unit as shown in Scheme 5. Therefore, it is reasonable to interpret that **4b**–**OH** is a GGOH type product from the total length of a molecule.

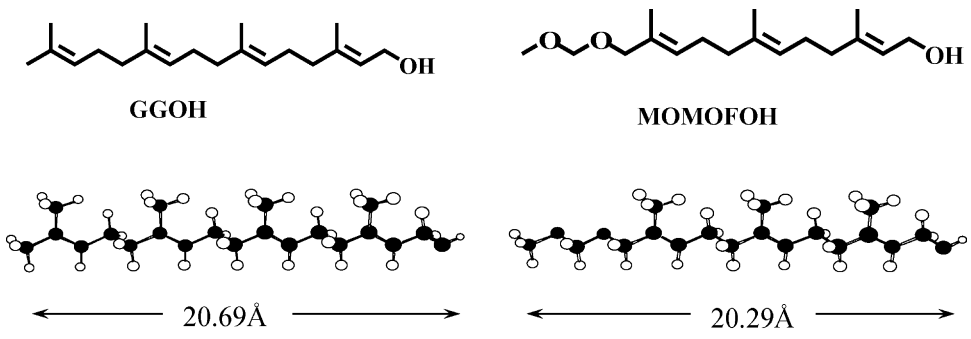
3.3. Reactions of propoxyGPP (*3a*) with IPP (*1*) using FPP synthases

The alcohols derived from the products of the porcine liver FPP synthase-catalyzed reaction of propoxyGPP (**3a**) with **1** gave a peak with a retention time of 42.1 min on HPLC. The mass spectrum of the product showed a molecular ion at *m*/*z* 280 (rel. int. 0.3%), corresponding to $C_{18}H_{32}O$, having main fragment ions at *m*/*z* 262 $[M-18]^+$ (4.8), 202 $[M-18-60]^+$ (26.4), 134 $[M-8-60-68]^+$ (14.3), and 93 (base peak), indicating that the product has a 12-propoxy-3,7,11-trimethyldodeca-2,6,10-trien-1-ol (propoxyfarnesol, propoxyFOH) structure. The prenylalcohol was also reasonably assigned to (all-*E*)-propoxyFOH, **5a**–**OH**. The relative reactivity was 62% based on the product derived from the reaction between natural substrates **2** and **1**.

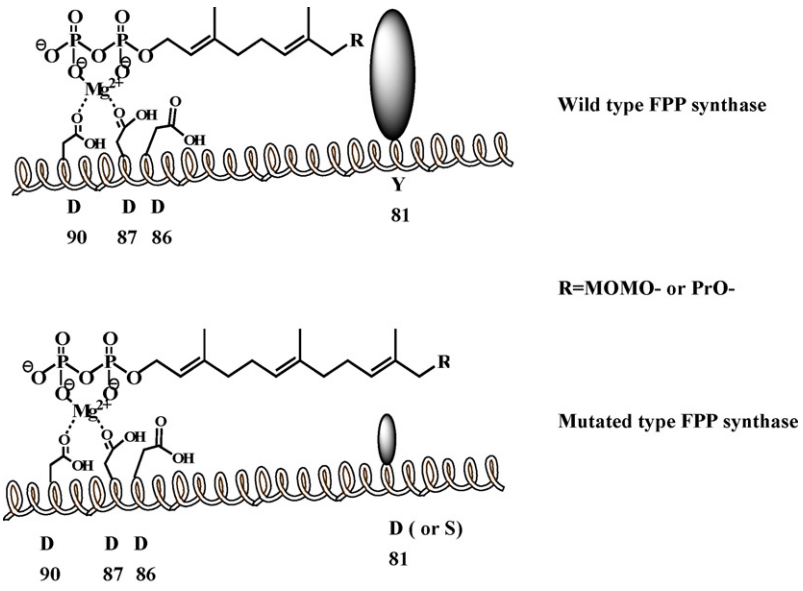
When the wild type FPP synthase reaction of **3a** with **1** was examined, the similar product, **5a**–**OH** was obtained and its reactivity was 3%, showing that the substrate specificity of bacterial enzyme is stricter.

3.4. Reactions of propoxyGPP (*3a*) with IPP (*1*) using mutated FPP synthases

The product of the mutated FPP synthase Y81D (or Y81S) reaction of **3a** with **1** were hydrolyzed with alkaline phosphatase to the corresponding alcohols, which showed retention times on HPLC at 42.1 (relative reactivity: 63%) and 38.1 min



Scheme 5. The molecular length of the enzymatic reaction products.



Scheme 6. Prenylchain elongating decision mechanism of FPP synthase.

(relative reactivity: 6%), respectively. The mass spectrum of the former showed a similar mass spectrum to that of propoxy-FOH (**5a**–**OH**), indicating that the major enzymatic product was propoxyfarnesyl diphosphate. In the mass spectrum of the latter product, the molecular ion was observed at *m*/*z* 348 (rel. int. 0.2%), corresponding to $C_{23}H_{40}O_2$, with fragment ions at *m*/*z* 330 $[M-18]^+$ (0.3), 270 $[M-18-60]^+$ (8.3), 202 $[M-18-60-68]^+$ (7.8), 134 $[M-18-60-68-68]^+$ (13.1), and 93 (base peak), indicating that the product has a 16-propoxy-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraen-1-ol (propoxyGGOH) structure. It is reasonable to assign to (all-*E*)-propoxyGGOH (**5b**–**OH**), suggesting that the chain elongation stopped after the condensation of two molecules of **1**.

A plausible mechanism for the chain length determination in the wild type FPP synthase is depicted in Scheme 6, in which a bulky group tyrosine-81 plays an important role to form a stopping barrier for the elongation.

When the mutated FPP synthase (Y81D or Y81S) with a smaller amino acid residues at position 81 was used, further chain elongation over the position 81 could occur to form the longer-chain product such as propoxyGGPP, **5b**.

On the other hand, in research of the avian FPS by Tarshis et al., it proved that the phenyl groups of F112 and/or F113 have played the role important for chain length determination. Furthermore, they have succeeded in obtaining the higher chain length isoprenoids diphosphates, such as C20 and C25, using the mutated enzyme (F112A and/or F113S).

By *B. stearothermophilus* FPS, since higher chain length isoprenoid diphosphates were obtained by Y81D or Y81S, we also have thought that Y81 is corresponding to F112 or F113 [23,24].

4. Conclusion

Allylic substrate homologs such as MOMODMAPP and propoxyGPP were examined for the reactivity as artificial substrates for farnesyl diphosphate synthase as illustrated in Scheme 4.

The wild type FPP synthase reaction of **2a** with **1** gave **4a** as a single condensation product. On the other hand, the reaction with the mutated FPP synthase (Y81D or Y81S) gave **4b** as a double condensation product. However, according to the optimized calculation of MOMOFOH, the molecular length of the optimized structure of **4b**–**OH** resembles to that of GGOH (Scheme 5).

At the FPP synthase reaction of **3a** and **1**, when the wild type enzyme was used, **5a** was obtained, and when the mutated enzyme Y81D was used, **5a** and **5b** were the products.

Acknowledgments

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