Journal of Molecular Catalysis B: Enzymatic 60 (2009) 186–190

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

# Substrate specificities of farnesyl diphosphate synthases from *Bacillus stearothermophilus* and porcine liver with cyclic substrate homologs

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ARTICLE INFO

*Article history:*
Received 27 January 2009
Received in revised form 22 April 2009
Accepted 8 May 2009
Available online 15 May 2009

*Keywords:*
Farnesyl diphosphate synthase
Substrate specificity
Cyclohexylideneethyl diphosphate
2-Cyclohexenylethyl diphosphate
*Bacillus stearothermophilus*

ABSTRACT

We investigated the substrate specificity of farnesyl diphosphate (FPP) synthase derived from *Bacillus stearothermophilus* and porcine liver by examining the reactivities of two cyclic substrate homologs, cyclohexylideneethyl diphosphate and cyclohexenylethyl diphosphate.

Reaction of geranyl diphosphate with 2-cyclohexenylethyl diphosphate using bacterial or porcine liver FPP synthase produced (*S*)-geranylcyclohexylideneethyl diphosphate, with relative yields of 13.6% for the bacterial enzyme and 42.2% for the porcine liver enzyme. Reaction of cyclohexylideneethyl diphosphate with isopentenyl diphosphate produced 10-cyclohexyliden-3,7-dimethyldeca-2,6-dien-1-ol as a double condensation product, with relative yields of 23.1% (bacterial enzyme) and 3.0% (porcine liver enzyme). Reaction of cyclohexylideneethyl diphosphate with 2-cyclohexenylethyl diphosphate using bacterial enzyme produced (cyclohexylideneethyl)-cyclohexylideneethyl diphosphate (0.8% yield).



## 1. Introduction

The cryptic stereochemistry involved in the enzyme-catalyzed C–C bond formation between prochiral molecules is unique and quite interesting, especially in the prenyltransferase-catalyzed isoprenoid syntheses. So far, we have been accumulating many prenyltransferase reactions with respect to artificial substrate homologs to examine the extent of the cryptic stereochemistry by the combination of precise organic syntheses and bioorganic analyses.

Prenyltransferase catalyzes the head-to-tail condensation between isopentenyl diphosphate (IPP, **1**) and an allylic prenyl diphosphate to produce several prenyl diphosphates, which are then converted into steroids, carotenoids, prenyl side-chains of quinones, and prenyl proteins (Scheme 1) [1–4]. Among prenyltransferases, farnesyl diphosphate (FPP) synthase [EC 2.5.1.10] plays an important role in early stage metabolic reactions in isoprenoid chemistry. FPP synthase exists almost universally in higher animals and bacteria, except for archaebacteria, and catalyzes the condensation of IPP and dimethylallyl diphosphate (**2**) *via* geranyl diphosphate (**3**) to farnesyl diphosphate (FPP, **4**) (Scheme 2).

Several studies have investigated the substrate specificities of FPP synthase with allylic and homoallylic substrate homologs [5–14]. For example, Koyama et al. reported that synthesis of chiral molecules by an enzymatic reaction is a useful method of building biologically active substances such as trail marker pheromones or juvenile hormones [15–20]. Our previous research on substrate specificity involving homoallylic substrate homologs such as 4-alkyl (methyl, ethyl, propyl, or butyl) group homologs of IPP indicated that FPP synthase is useful in the synthesis of chiral compounds. Farnesol homologs with an alkyl group at the 4-position with (*S*)- or (*R*)-configuration can be selectively prepared from (*E*)- or (*Z*)-4-alkylIPP, respectively [21]. Among the studies investigating cyclic substrate homologs using FPP synthase, Koyama et al. reported on the substrate specificity of FPP synthase with respect to cyclic compounds [12].

In order to get advanced insight into some artificial substrate homologs having cyclic structures, we will describe here some different properties of the substrate specificities between two FPP synthases from *Bacillus stearothermophilus* and from porcine liver.

## 2. Experimental

### 2.1. Analysis

HPLC was used to measure the prenyl alcohols produced on treating the enzymatic reaction products with alkaline phos-

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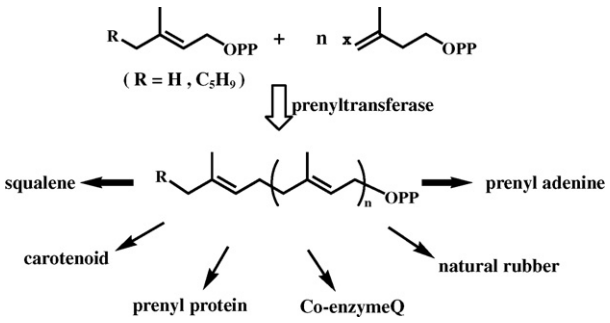
doi:10.1016/j.molcatb.2009.05.002




**Scheme 1.** The role prenyltransferase in isoprenoid metabolism.

phatase. Materials were similar to those used in previous experiments [21–23]: HPLC machine was a Hitachi L-6000 (Hitachi, Tokyo, Japan) equipped with a Hitachi LaChrom L-7420 UV-VIS detector and a ChromatoDAQ II (ULVAC, Inc., Chigasaki, Japan). We also used a LichroCART column (Merck-Japan, Tokyo, Japan) with a hexane:2-propanol (80:1 [A] and 40:1 [B]) eluent. We separated *R*- and *S*-derivatives of the prenyl alcohols by HPLC using a Chiralpak AD-H column (250 mm × 4.6 mm ID; Daicel Chemical Industries, Ltd., Osaka, Japan) with a hexane:ethanol (99:1) eluent.

Reaction products were identified by GC–MS analysis using a JMS-AM II 50 GCG mass spectrometer (JEOL, Tokyo, Japan) connected to an HP 5890 series II gas chromatograph (Hewlett-Packard Company, Palo Alto, CA, USA) equipped with an Ultra ALLOY-1 (HT) capillary column (S). Column temperature was programmed from 90 °C to 280 °C, with a linear gradient temperature increase rate of 15 °C/min, and held at 280 °C for 3 min.

IR spectra were obtained using a Hitachi 260-10 spectrometer (Hitachi) and Bio-Rad FTS-30 spectrometer (Bio-Rad, Hercules, CA, USA). NMR spectra were obtained using a JEOL JNMGX 270 FT (JEOL) and JEOL JNM-ECA 500 FT NMR spectrometer (JEOL) using tetramethylsilane as an internal standard and $CDCl_3$ as a solvent. Optical rotation was measured with a Horiba SEPA-300 high sensitive polarimeter (Horiba, Kyoto, Japan).

Reaction products were identified by LC–MS analysis using the Hitachi NanoFrontier LD. HPLC analyses were performed on a LaChrom ELITE HPLC system (Hitachi High Technologies, Nishi-Shinbashi, Tokyo, Japan) equipped with an L-2100 pumping system (Hitachi), a column oven (L-2300) (Hitachi), and a UV-VIS detector (Hitachi) coupled with EZChrom Elite software for Windows XP (Microsoft Corporation, Redmond, WA, USA).

Samples were eluted on an ODS column (Inertsil ODS-3, 33 mm × 2.1 mm; GL Science, Tokyo, Japan). HPLC analysis used acetonitrile as an eluent in a 1% formic acid solution at a flow rate of 0.2 ml/min. Gradient elution started at 70% acetonitrile and reached 100% acetonitrile in 12 min. The LC effluent was introduced into the electrospray ionization (ESI) source, and mass spectra were acquired using the Hitachi NanoFrontier LD spectrometer with an ESI source. Nitrogen was used as the sheath and a mixture of auxiliary gas and helium was used as the collision gas. ESI MS spectra were acquired in positive ion modes. Spectra were recorded in the range of *m*/*z* 100–2000 for a full scan MS analysis.

### 2.2. Chemicals

#### 2.2.1. Synthesis of cyclohexenylethyl diphosphate (**5**) and cyclohexylideneethyl diphosphate (**7**)

The dehydration reaction between methyl 1-hydroxycyclohexylacetate (23.2 mmol), obtained from the Reformatsky reaction of cyclohexanone with methyl bromoacetate, and diphosphorus pentaoxide produced two esters (26.5 mmol, 87.6% yield), methyl cyclohex-1-enylacetate and methyl cyclohexylideneacetate, which eluted in two peaks and separated at 45.9 min (0.56 g, 3.7 mmol) and 48.2 min (0.98 g, 6.34 mmol) on HPLC (eluent B), respectively.

The MS spectrum of methyl cyclohex-1-enylacetate showed a molecular ion at *m*/*z* 154 (rel. int. 24.0%), corresponding to $C_9H_{14}O_2$, with fragment ions at *m*/*z* 122 $[M-32]^+$ (13.4%), 94 $[M-60]^+$ (69.8%), and 80 (base peak). The $^1H$ NMR ($CDCl_3$, TMS) was $\delta$ 1.53 (2H, q $J$ = 5.7 Hz), 1.61 (2H, q $J$ = 5.7 Hz), 1.98 (4H, dt $J$ = 7.5, 14.9 Hz), 2.95 (2H, s), 3.68 (3H, s), and 5.56 (1H, m), and the $^{13}C$ NMR (DEPT) was $\delta$ 22.0 ($CH_2$), 22.7 ($CH_2$), 25.3 ($CH_2$), 28.4 ($CH_2$), 45.3 ($CH_2$), 51.7 ($CH_3$), 125.7 (CH), 131.1 (C), and 175.2 (C).

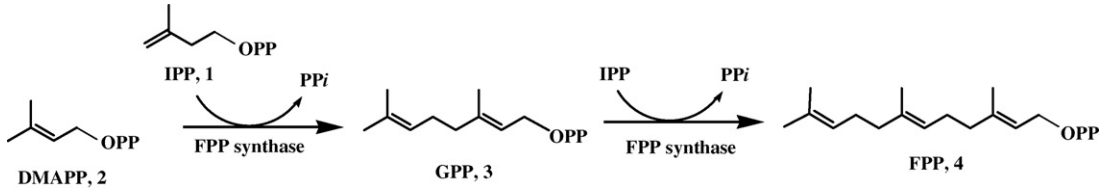
The MS spectrum of methyl cyclohexylideneacetate showed a molecular ion at *m*/*z* 154 (rel. int. 100%), corresponding to $C_9H_{14}O_2$, with fragment ions at *m*/*z* 139 $[M-15]^+$ (6.7%), 123 $[M-31]^+$ (34.6%), and 95 $[M-59]^+$ (66.1%). The $^1H$ NMR ($CDCl_3$, TMS) was $\delta$ 1.55–1.76 (6H, m), 2.01 (4H, m), 3.67 (3H, s), and 5.56 (1H, s).

Cyclohex-1-enylethyl tosylate (yield: 0.30 mmol) was prepared from 2-cyclohex-1-enylethanol (0.99 mmol), which was obtained by $LiAlH_4$ reduction of methyl cyclohex-1-enylacetate, by tosylation with tosyl chloride in pyridine. The MS spectrum of the tosylate showed a molecular ion at *m*/*z* 280 (rel. int. 0.01%), corresponding to $C_{15}H_{20}O_3S$, with fragment ions at *m*/*z* 108 $[M-172]^+$ (31.1%), 93 $[M-172-15]^+$ (73.2%), and 79 (base peak). The $^1H$ NMR ($CDCl_3$, TMS) was $\delta$ 1.48–1.58 (4H, m), 1.81–1.93 (4H, m), 2.26 (2H, t $J$ = 7.0 Hz), 2.45 (3H, s), 4.07 (2H, t $J$ = 7.0 Hz), 5.40 (1H, sept $J$ = 1.6 Hz), 7.33 (2H, d $J$ = 8.3 Hz), and 7.77 (2H, d $J$ = 8.3 Hz). The tosylate was diphosphorylated by Davisson's method to yield cyclohexenylethyl diphosphate [24].

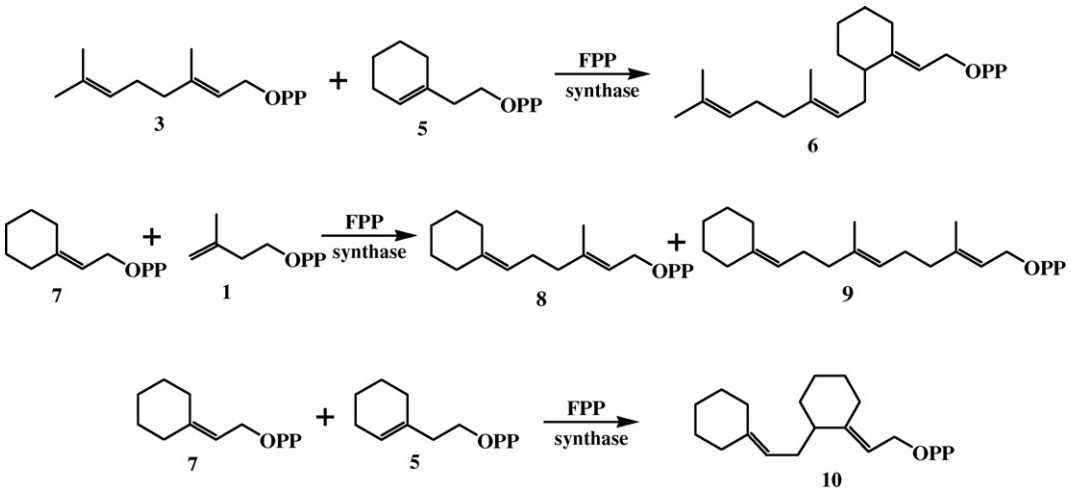
Cyclohexylideneethyl chloride was prepared from 2-cyclohexylideneethanol, which was obtained by $LiAlH_4$ reduction of methyl 2-cyclohexylideneacetate, by chlorination with N-chlorosuccinimide in dimethyl sulfide by the reported method [25,26]. The chloride was then converted by Davisson's method to cyclohexylideneethyl diphosphate [24].

#### 2.2.2. Synthesis of authentic geranylcyclohexylidene ethanol (**6-OH**)

Preparation of 2-geranylcyclohexanone was achieved by decarboxylation of 2-carboethoxy-2-geranylcyclohexanone (2.0 g, 6.6 mmol), which was obtained by reaction of 2-carboethoxycyclohexanone with geranyl bromide, with sodium chloride (0.48 g, 8.2 mmol) and water (0.44 g, 24 mmol) in dimethyl sulfoxide (6.0 ml) at 180 °C for 8 h, resulted in a yield of 0.48 g (31%). The




**Scheme 2.** FPP synthase reactions of dimethylallyl diphosphate with isopentenyl diphosphate.




**Scheme 3.** FPP synthase reactions between allylic and homoallylic substrate homologs.

$^1$H NMR ($CDCl_3$, TMS) of 2-geranylcyclohexanone was $\delta$ 1.59 (6H, s), 1.65 (2H, m), 1.67 (3H, s), 1.85 (2H, m), 2.01(2H, m), 2.03 (2H, m), 2.06 (2H, m), 2.14 (1H, m), 2.29 (2H, m), 2.46 (2H, m), 5.07 (1H, t $J$ = 7.0 Hz), and 5.09 (1H, t $J$ = 7.0 Hz). The $^{13}$C NMR was $\delta$ 16.0, 17.6, 25.0, 25.7, 26.6, 27.7, 28.0, 33.2, 39.8, 42.0, 51.1, 121.9, 124.3, and 213.0. The IR $\nu_{max}$ (KBr) was 1730 and 1672 cm$^{-1}$.

The Horner–Emmons reaction of 2-geranylcyclohexanone (0.37 g, 16 mmol) using diethyl ethoxycarbonylmethylphosphonate (0.53 g, 2.4 mmol) and sodium hydride (0.11 g, 2.8 mmol) in dry tetrahydrofuran gave ethyl 2-geranylcyclohexylideneacetate. Purification by silica gel flash column chromatography (eluent: 10/1 mixture of hexane/ethyl acetate) resulted in a yield of 0.13 g (27%). The $^1$H NMR ($CDCl_3$, TMS) of ethyl 2-geranylcyclohexylideneacetate was $\delta$ 1.28 (3H, t $J$ = 7.0 Hz), 1.37 (2H, m), 1.48 (2H, m), 1.52 (2H, m), 1.60 (6H, s), 1.67 (3H, s), 2.02 (2H, m), 2.06 (2H, m), 2.13 (2H, m), 2.19 (2H, m), 2.64 (1H, m), 4.13 (2H, q $J$ = 7.0 Hz), 5.07 (1H, t $J$ = 7.0 Hz), 5.08 (1H, t $J$ = 7.0 Hz) and 5.58 (1H, s). The $^{13}$C NMR was $\delta$ 14.3, 16.1, 17.6, 24.0, 25.6, 28.4, 28.5, 30.6, 33.6, 39.7, 46.0, 59.4, 111.9, 122.4, 124.2, 131.3, 136.3, 166.3, and 167.0. The IR $\nu_{max}$ (KBr) was 1714, 1642, and 1156 cm$^{-1}$.

Ethyl 2-geranylcyclohexylideneacetate (0.13 g, 0.43 mmol) was then reduced with diisobutylaluminum hydride (1.8 ml, 1.7 mmol) in dry dichloromethane and hexane at −40 °C for 1 h to produce 2-geranylcyclohexylideneethanol (68 mg, 60% yield), in accordance with a similar previously reported method [27]. The mass spectrum of 2-geranylcyclohexylideneethanol showed a molecular ion at $m/z$ 262 (rel. int. 0.35%), corresponding to $C_{18}H_{30}O$, with fragment ions at $m/z$ 244 [M−18]$^+$ (26.9%), 175 [M−69]$^+$ (13.0%), 137 [M−69−107]$^+$ (2.5%), 107 [M−69−68]$^+$ (28.1%), and 69 (base peak). The $^1$H NMR ($CDCl_3$, TMS) was $\delta$ 1.31 (2H, m), 1.48 (2H, m), 1.50 (2H, m), 1.60 (6H, s), 1.64 (3H, s), 2.04 (2H, m), 2.06 (2H, m), 2.08 (2H, m), 2.19 (2H, m), 2.35 (1H, m), 4.17 (2H, m), 5.08 (1H, t $J$ = 7.0 Hz), 5.09 (1H, t $J$ = 7.0 Hz) and 5.35 (1H, t $J$ = 7.0 Hz). The $^{13}$C NMR was $\delta$ 16.1, 17.7, 24.4, 25.7, 26.7, 27.8, 28.3, 30.6, 33.2, 39.8, 44.6, 58.7, 119.1, 123.2, 124.3, 131.2, 135.6, and 147.1. The IR $\nu_{max}$ (KBr) was 3312 cm$^{-1}$.

The 2-geranylcyclohexylideneethanol showed a peak at 12.6 min on HPLC analysis using a normal phase column with eluent A. Subsequent LC–MS analysis showed a main peak at $m/z$ 285.2159 [M+Na] (calculated for $C_{18}H_{30}ONa$: 285.2194). A further two peaks were observed at 21.2 (50.9%) and 27.1 (49.1%) min on an HPLC equipped with a chiral column. After preparative HPLC, the products were analyzed for specific optical rotation, showing a rotation of $[\alpha]_D^{20} + 2.2$ ($c$ 0.01, hexane) for the first peak and $[\alpha]_D^{20} - 0.3$ ($c$ 0.01, hexane) for the second.

### 2.3. Purification of *B. stearothermophilus* FPP synthase

The purification of FPP synthase derived from *B. stearothermophilus* was carried out according to our previously reported method [14,21–23,27].

### 2.4. Enzymatic reaction conditions

The incubation mixture for the *B. stearothermophilus* FPP synthase reaction contained 100 μmol of Tris–HCl buffer (pH 8.5), 10 μmol of $MgCl_2$, 50 μmol of β-mercaptoethanol, 5 μmol of KCl, 0.5 μmol of the allylic substrate being examined (GPP or 2-geranylcyclohexylideneethyl diphosphate), 0.5 μmol of a homoallylic substrate (IPP or cyclohexenylethyl diphosphate), and FPP synthase (approximately 25 mg) in a total volume of 1 ml. After 3 h incubation at 55 °C, the reaction mixture was treated with alkaline phosphatase for 5 h, extracted with pentane, and analyzed by HPLC and GC–MS. The porcine liver FPP synthase reaction was conducted under the same conditions with an incubation temperature of 37 °C and a pH of 7.0.

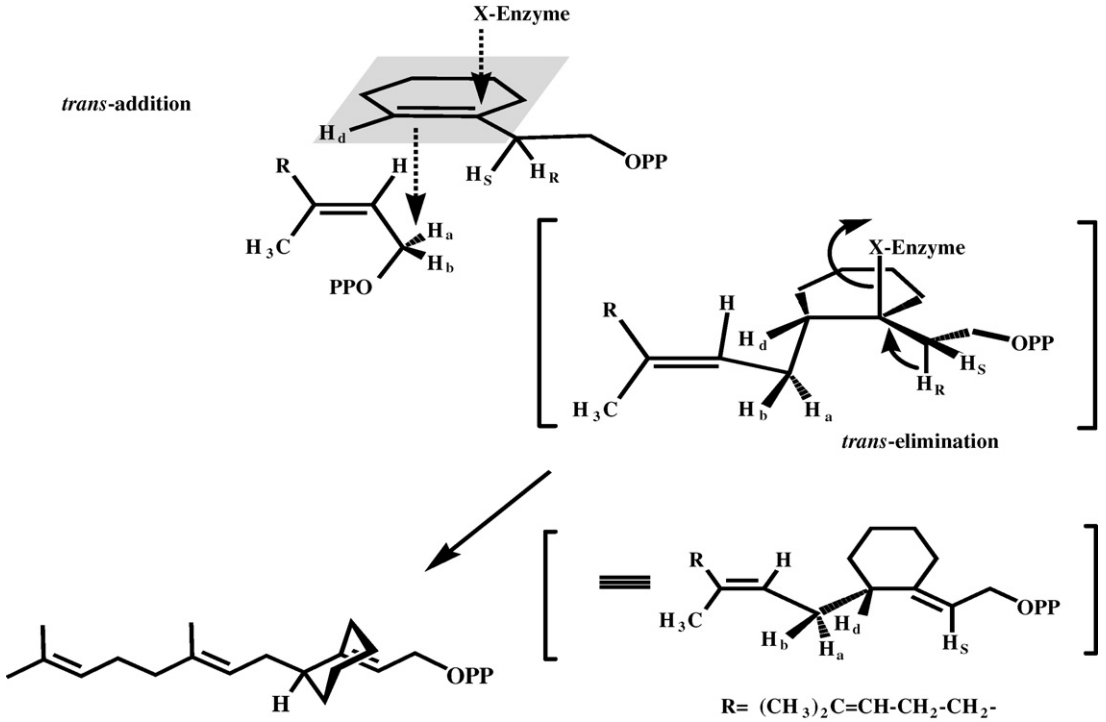
Relative reactivity was based on the product (100%) which was hydrolyzed the reaction between natural substrates, **1** and **2** [14].

## 3. Results and discussion

To investigate the reactivities of cyclic allyl and homoallyl substrate homologs, we examined substrate specificities of FPP synthases derived from *B. stearothermophilus* and porcine liver.

### 3.1. Reaction of geranyl diphosphate (*3*) with cyclohexenylethyl diphosphate (*5*) by FPP synthase

As shown in Scheme 3, the hydrolysate derived from the product of the *B. stearothermophilus* FPP synthase reaction of **3** with **5** gave a peak on HPLC (eluent B) at 15.9 min, and was then analyzed by LC–MS and GC–MS. LC–MS showed a main peak at $m/z$ 295.2615 (calculated for $C_{19}H_{35}O_2$: 295.2637), and GC–MS was ambiguous, but the dehydration ion [M−18]$^+$ was distinctly observed at $m/z$ 244 (rel. int. 10.5%) with other fragment ions at $m/z$ 175 [M−18−69]$^+$




**Scheme 4.** Reaction mechanism of GPP with cyclic substrate homolog according to Cornforth's theory.

(5.3%), 107 [M−18−69−68]$^+$ (16.3%), and 91 (base peak), indicating that the product had a 2-geranylcyclohexylideneethanol structure. Relative reactivity was determined by comparison of the total amounts of the products that was obtained by the enzymatic hydrolysis of the reaction products to that of the standard prenyltransferase reaction (100%) between the natural substrates, **1** and **2** [14].

To determine the configuration of the product using $^1$H NMR spectra, we examined the reaction at a 20-fold scale and purified the product by preparative HPLC. $^1$H NMR ($CDCl_3$, TMS) was $\delta$ 1.30–1.40 (6H, m), 1.58 (3H, s), 1.60 (6H, s), 1.98–2.09 (8H, m), 2.35 (1H, m), 4.18 (2H, m), 5.10 (1H, t $J$ = 7.0 Hz), 5.08 (1H, t $J$ = 7.0 Hz) and 5.35 (1H, t $J$ = 7.0 Hz). On comparing the $^1$H NMR and HPLC (retention time) data with that from an authentic sample, we determined the product to be (*E*)-2-geranylcyclohexylideneethanol (**6-OH**) [28]. A similar product was obtained on reaction of **3** with **5** using porcine liver FPP synthase under similar conditions with an incubation temperature of 37 °C and a pH of 7.5. The relative reactivity was 42.2%.

The stereochemistry of the enzymatic product, **6-OH** is (*S*)-configuration as shown in Scheme 4, when the reaction mechanism of Cornforth and researches of Ogura and Koyama, etc. are considered comprehensively [12,16,21,28,29]. As compared with the configuration of the product of GPP and 3-methylpent-3-enyl diphosphate (*E*-4-methylIPP) which Koyama et al have reported, it is clear that it is *S*-derivative [12,16,21]. In addition, **6-OH** showed plus sign of rotation.

### 3.2. Reaction of cyclohexylideneethyl diphosphate (*7*) with isopentenyl diphosphate (*1*) by FPP synthase

The alcohols obtained by the phosphatase treatment of the products of the FPP synthase reaction of **7** with **1** gave a peak on HPLC at 16.5 min. After preparative HPLC, the product was analyzed by GC–MS, where it eluted at 12.0 min. Mass spectrum analysis showed a molecular ion [M]$^+$ at $m/z$ 262 (rel. int. 0.04%), corresponding to $C_{18}H_{30}O$, and fragment ions at $m/z$ 244 [M−18]$^+$ (22.2%), 109 (49.8%), 135 [M−18−109]$^+$ (7.6%), 67 [M−18−109−68]$^+$ (base peak). These data indicated that the alcohol had a 10-cyclohexyliden-3,7-dimethyldeca-2,6-dien-1-ol structure (**9-OH**), suggesting that chain elongation stopped at the second stage of condensation. The relative reactivity was 23.1%.

In the enzymatic reaction of **7** and **1**, the single condensation product was detected at the retention time of 8.8 min on GC but not by HPLC analysis. On the GC–MS analysis, the molecular ion was not detected but the dehydration ion [M−18]$^+$ was observed at $m/z$ 176 (rel. int. 73.3%) with other fragment ions at $m/z$ 109 (38.4%), 67 [M−18−109]$^+$ (48.9%), and 78 (base peak), indicating that the structure was 6-cyclohexyliden-3-methylhex-2-en-1-ol (**8-OH**).

Similar reaction of **7** with **1** using porcine liver FPP synthase resulted in a condensation product (**9-OH**, relative reactivity 3%) with two molecules of **1**.

### 3.3. Reaction of cyclohexylideneethyl diphosphate (*7*) with 2-cyclohexenylethyl diphosphate (*5*) by FPP synthase

The product of the FPP synthase reaction of **7** with **5** gave an alcohol after hydrolysis with alkaline phosphatase. The alcohol showed a peak on HPLC at 17.1 min. On GC–MS, the MS showed a molecular ion [M]$^+$ at $m/z$ 234 (rel. int. 0.02%), corresponding to $C_{16}H_{26}O$, with main fragment ions at $m/z$ 216 [M−18]$^+$ (14.8%), 109 [M−18−107]$^+$ (17.3%), 107 [M−18−109]$^+$ (7.6%), and 91 (base peak), indicating that the product's structure was 2-(cyclohexlidenethyl)-cyclohexylideneethanol (**10-OH**). The relative reactivity was 0.8% based on the product derived from the reaction between natural substrates **2** and **1**. Although the relative product yields were low, these cyclic substrate homologs were accepted as substrates for bacterial FPP synthase.

## 4. Conclusion

To investigate substrate specificities of FPP synthases derived from *B. stearothermophilus* and porcine liver, we examined the reactivities of cyclic substrate homologs **5** and **7**. Reaction of **3** with **5** produced (*S*)-**6** with relative reactivities of 13.6% for bacterial and

42.2% for porcine liver synthase. In contrast, reaction of **7** with **1** using bacterial FPP synthase gave a double-condensation product, **9** (yield: 23.1%), as the main product, with only a trace of **8**. Reaction of **7** with **5** using bacterial FPP synthase produced **10** (0.8% yield) exclusively. In conclusion, both **5** and **7** were found to be acceptable substrates for FPP synthases. These findings are interesting with regard to models of chiral synthesis in organic chemistry, and may aid future researchers in successfully synthesizing biologically active chiral substances.

## Acknowledgments

This work was supported by a Grant for Science Research (to M.N.) from the Nikkol Group (Cosmos Technical Center Co. Ltd., Tokyo, Japan).

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