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

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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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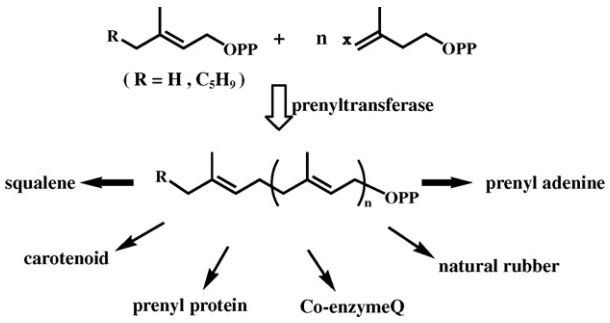


Scheme 1. The role prenyltransferase in isoprenoid metabolism.

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 δ 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 δ 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).

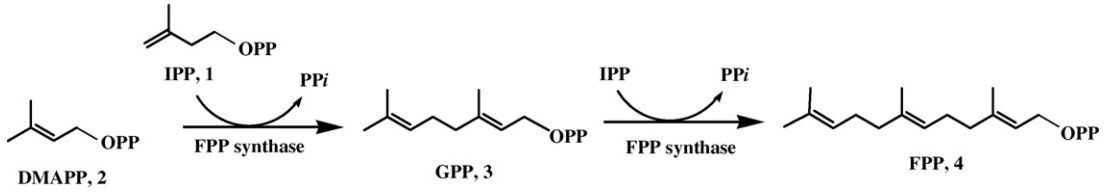
The MS spectrum of methyl cyclohexylideneacetate showed a molecular ion at *m*/*z* 154 (rel. int. 100%), corresponding to $C_9H_{14}O_2$, with fragment ions at *m*/*z* 139 $[M-15]^+$ (6.7%), 123 $[M-31]^+$ (34.6%), and 95 $[M-59]^+$ (66.1%). The ^1H NMR ($CDCl_3$, TMS) was δ 1.55–1.76 (6H, m), 2.01 (4H,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