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Substrate specificities of medium-prenylchain elongating enzymes, hexaprenyl- and heptaprenyl diphosphate synthases

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Abstract

In order to investigate substrate specificities of medium-prenylchain elongating enzymes, hexaprenyl diphosphate (HexPP) synthase from *Micrococcus luteus* B-P 26 and heptaprenyl diphosphate (HepPP) synthase from *Bacillus subtilis*, we examined the reactivities of 3-alkyl group homologs of allylic or homoallylic substrates for these enzymes. Only one molecule of but-3-enyl diphosphate (**2b**), which lacks a methyl group at the 3-position of isopentenyl diphosphate (**2a**) condensed with farnesyl diphosphate (**1**, FPP) to give *E*-norgeranylgeranyl diphosphate in the reaction catalyzed by either enzyme.

3-Ethylbut-3-enyl diphosphate (**2c**) reacted as a homoallylic substrate with FPP to give a mixture of (all-*E*)-3-ethyl-7,11,15-trimethylhexadeca-2,6,10,14-tetraenyl- and (all-*E*)-3,7-diethyl-11,15,19-trimethyleicosa-2,6,10,14,18-pentaenyl diphosphates by the condensation with one or two molecules of (**2c**), respectively, by the reaction of HexPP synthase. 3-Propylbut-3-enyl diphosphate (**2d**) also reacted with FPP to give a mixture of the corresponding single and double condensation products, respectively, by the action of the same synthase.

On the other hand, HepPP synthase reaction of (**2c**) or (**2d**) with FPP gave (all-*E*)-3-ethyl-7,11,15-trimethylhexadeca-2,6,10,14-tetraenyl diphosphate or (all-*E*)-3-propyl-7,11,15-trimethylhexadeca-2,6,10,14-tetraenyl diphosphate as a single product, respectively.

However, 3-butylbut-3-enyl diphosphate (**2e**), norfarnesyl diphosphate (**1a**) and norgeranylgeranyl diphosphate (**1b**) were never accepted as substrates by either of the enzymes at all.

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1. Introduction

Prenyl diphosphate synthases catalyze prenylchain elongation by consecutive condensation of isopentenyl diphosphate (IPP) with allylic primer substrate to afford variety of prenyl diphosphates as biosynthetic

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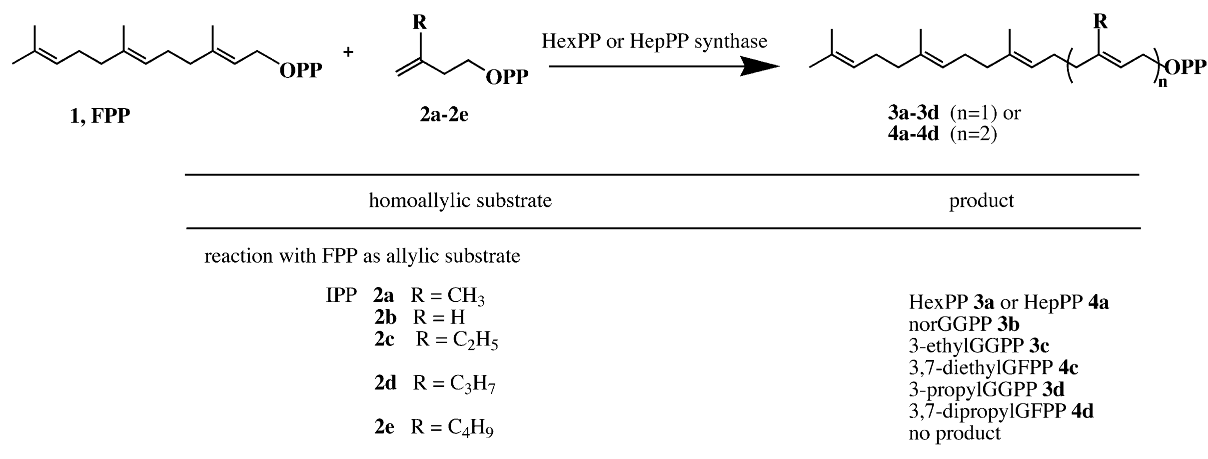


homoallylic substrate			product
reaction with FPP as allylic substrate			
IPP	**2a**	R = CH_3	HexPP **3a** or HepPP **4a**
	2b	R = H	norGGPP **3b**
	2c	R = C_2H_5	3-ethylGGPP **3c** 3,7-diethylGFPP **4c**
	2d	R = C_3H_7	3-propylGGPP **3d** 3,7-dipropylGFPP **4d**
	2e	R = C_4H_9	no product

Scheme 1. Medium-chain prenyl diphosphate synthase reactions of FPP with 3-alkyl homologs of IPP.

precursors of various isoprenoids, which are converted to steroids, carotenoids, prenylquinones, and prenylated proteins [1,2]. The condensation terminates precisely at a definite chain length for the final product depending on the product specificities of individual synthases. These enzymes can be classified into four groups. Short-chain (*E*)-prenyl diphosphate synthases such as farnesyl diphosphate (FPP) [3] and geranylgeranyl diphosphate (GGPP) synthases [4], require divalent ions (e.g. Mg^{2+} or Mn^{2+}) for their activities which are commonly required by all prenyl diphosphate synthases. Long-chain (*E*)-prenyl diphosphate synthases such as octaprenyl diphosphate- [5] and solanesyl diphosphate synthases [6] require carrier proteins for removing the hydrophobic polyprenyl products from their active sites and maintain the turnover of the catalysis. *Z*-Polyprenyl diphosphate synthases including undecaprenyl diphosphate (UPP) synthase [7] and natural rubber synthase [8] require phospholipids or detergents such as Triton X-100 for activity. Every enzyme of these three groups has a homodimeric structure tightly coupled with each other.

On the other hand, medium-chain (*E*)-prenyl diphosphate synthases consist of two dissociable heterodimeric components, neither of which has catalytic activity alone but the activity restores when the two components are bound together. This group includes hexaprenyl diphosphate (HexPP) synthase [9] which is composed of components A and -B, and heptaprenyl diphosphate (HepPP) synthase [10] composed of components I and -II, respectively. The HexPP synthase [EC 2.5.1.33] from *Micrococcus luteus* B-P 26 or the HepPP synthase [EC 2.5.1.30] from *Bacillus subtilis* catalyzes sequential condensation of three or four molecules of IPP with FPP as a primer to afford medium-chain prenyl diphosphate, HexPP (C_{30}) or HepPP (C_{35}) as shown in Scheme 1, respectively, which is involved in the biosynthesis of the side chain of menaquinones in the microorganisms.

Meanwhile, we have found that the FPP synthase reaction of dimethylallyl diphosphate (DMAPP) (or geranyl diphosphate (GPP)) with but-3-enyl diphosphate (**2b**) gave exclusively norgeranyl diphosphate (or norfarnesyl diphosphate) by a single condensation of **2b**, and *E*-but-2-enyl diphosphate was not accepted instead of DMAPP as a substrate at all [11]. This paper describes the reactivity of 3-alkyl homologs of IPP with respect to the medium-chain prenyl diphosphate synthases.

A part of this study on the reactivity of 3-alkyl homologs has already been reported as a preliminary communication [12].

2. Experimental

2.1. Analysis

The enzymatic products were analyzed by HPLC (Hitachi type L-6200) equipped with Hitachi type

L-4200 type UV-VIS detector and with Lichro-CART (Merck) column with the eluent of hexane: 2-propanol = 40:1 (v/v) in the similar manner as previously reported [11]. Identification of the reaction products was carried out by GC–MS, JMS-AM II 50 type GCG mass spectrometer connected with HP 5890 series II gas chromatograph equipped with Ultra-alloy-1 (S). The column temperature was programmed from 90 to 280 °C with a linear gradient of temperature at a rate of 15 °C/min and then held at 280 °C for 3 min. Relative yields of the enzymatic reactions were calculated on the basis of the amount of recovered farnesol derived from the residual FPP in the reaction mixture.

2.2. Chemicals

The 3-alkyl homologs of IPP, but-3-enyl- (**2b**); 3-ethylbut-3-enyl- (**2c**); 3-propylbut-3-enyl- (**2d**); and 3-butylbut-3-enyl diphosphates (**2e**), were synthesized according to the method reported previously [12–15].

The allylic substrate homolog, 7,11-dimethyldodeca-2,6,10-trienyl diphosphate (norFPP, **1a**) was synthesized by Horner–Emmons reaction essentially according to the method reported previously [12]. 7,11,15-Trimethylhexadeca-2,6,10,14-tetraenyl diphosphate (norGGPP, **1b**) was synthesized according to the method reported previously [12]. Potato acid phosphatase was purchased from Sigma.

2.3. Overproduction and purification of each component of HexPP- or HepPP synthases

For preparation of *B. subtilis* ISW1214 HepPP synthase components, each of the *Escherichia coli* transformant of Top10/pTrcHis A and Top10/pTrcHis B was used for overproduction of the components A and -B, respectively [16]. The overnight culture of the transformant in LB medium (100 ml) was inoculated into 50 ml of SOB medium containing 50 μg/ml ampicillin. The cells were grown at 37 °C to an approximate A_{600} value of 0.5, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and then the incubation was continued for additional 3 h at 30 °C. Overproduction of the proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Each of the His-tagged protein overproduced in *E. coli* cells was purified essentially according to the purification procedure reported by Zhang et al. [16].

Preparation of each component of *M. luteus* B-P 26 HexPP synthase was carried out according to the purification procedure reported by Koike-Takeshita et al. [17]. The fractions of the proteins were analyzed for purity by SDS-PAGE with Coomassie Brilliant Blue staining.

2.4. Conditions of the enzymatic reaction

The incubation mixture for *M. luteus* B-P 26 HexPP synthase reaction contained, in a total volume of 1 ml, 50 mM of Tris–HCl buffer (pH 8.5), 1.0 mM of $MgCl_2$, 20 mM of β-mercaptoethanol, 50 mM of NH_4Cl, 10 ml of Triton X-100, 0.5 mM of an allylic substrate (FPP or **1a**–**1b**) to be examined, 1.5 mM of the homolog of IPP (**2a**–**2e**) to be examined, and the recombinant HexPP synthase (76 and 90 μg of components A and -B, respectively). *B. subtilis* HepPP synthase reaction was executed under the similar conditions as described for the HexPP synthase except that 2.0 mM of the homolog of IPP (or FPP) to be examined, and the recombinant HepPP synthase (124 and 74 μg of components I and -II, respectively) were employed. The mixture was incubated at 37 °C for 6 h, then another same amount of the HexPP- or the HepPP synthase was added and the incubation was continued for another 6 h. After incubation, the reaction mixture was extracted with 1-butanol and the products were hydrolyzed with acid phosphatase at 37 °C for 12 h. The hydrolysates were extracted with pentane and analyzed by HPLC and GC–MS [12,15].

2.5. Estimation of the optimized structures and heat of formation by MOPAC PM3 method

The heat of formation together with optimized structures of farnesol (FOH), 7,11-dimethyldodeca-2,6,10-trienol (norFOH), and their allylic cations were calculated by MOPAC PM3 method.

3. Results and discussion

In order to investigate substrate specificity of *M. luteus* B-P 26 HexPP synthase (or *B. subtilis* HepPP

synthase) toward IPP homologs having various alkyl chain at the 3-position, we examined a series of homologs **2a**–**2e** in the condensation with FPP as the allylic substrate. As a result, the HexPP (or HepPP) synthase reaction of FPP with **2b** (**2c**, or **2d**) were found to proceed but the prenylchain elongation stopped only at the first or second stages of condensation as shown in Scheme 1.

3.1. Reaction of but-3-enyl diphosphate (2a) with FPP (1) by use of HexPP (or HepPP) synthase

The alcohol derived from the product of HepPP synthase reaction of **2b** with FPP gave a single peak on HPLC at 16.0 min. The MS spectrum of the alcohol showed a molecular ion at *m/z* 276 (rel. int. 0.3%), corresponding to $C_{19}H_{32}O$, together with fragment ions at *m/z* 258 $[M-18]^+$ (0.2), 189 $[M-18-69]^+$ (1.3), 121 $[M-18-69-68]^+$ (5.5), and 69 (base peak), indicating the alcohol has norgeranylgeranyl structure lacking a methyl group at the 3-position. The alcohol derived from the product of **2b** with FPP by use of HexPP synthase showed similar fragment ions on GC–MS. As a result, the reaction of **2b** with FPP using either HexPP- or HepPP synthase gave norgeranylgeranyl diphosphate as a single product, which was reasonably assignable to be *E*-norgeranylgeraniol (**3b**–OH) [12] by considering the stereochemical manner of the (*E*)-prenylchain elongating enzymes.

3.2. Reactions of 3-alkylbut-3-enyl diphosphates (2c–2e) with FPP (1) by use of HexPP (or HepPP) synthase

3.2.1. Reaction of 3-ethylbut-3-enyl diphosphate (2c) with FPP

The alcohol derived from the product of HepPP synthase reaction of **2c** with FPP gave a peak at 14.2 min, which was purified by HPLC and subjected to GC–MS. The MS spectrum of the alcohol showed a molecular ion at *m/z* 304 (rel. int. 0.1%), corresponding to $C_{21}H_{36}O$ and other fragment ions at *m/z* 286 $[M-18]^+$ (0.1), 257 $[M-18-29]^+$ (0.5), 217 $[M-18-69]^+$ (0.5), 149 $[M-18-69-68]^+$ (14.7), 81 $[M-18-69-68-68]^+$ (46.0), and 69 (base peak), indicating that the product has 3-ethyl-7,11,15-trimethylhexadeca-2,6,10,14-tetraen-1-ol structure. Hence, it is reasonable to assign that the enzymatic product is (all-*E*)-3-ethyl-7,11,15-trimethylhexadeca-2,6,10,14-tetraenyl diphosphate (**3c**) [12].

On the other hand, the phosphatase hydrolysates of the HexPP synthase reaction products of **2c** with FPP gave two alcohols, showing retention times at 14.1 (major) and 13.2 min (minor). The major product gave a similar spectrum to that of the HepPP synthase reaction product, which is reasonably assigned to (all-*E*)- 3-ethyl-7,11,15-trimethylhexadeca-2,6,10,14-tetraen-1-ol (**3c**–OH). The spectrum of the minor product showed a molecular ion at *m/z* 386, corresponding to $C_{27}H_{46}O$, with fragment ions at *m/z* 368, 299, 231, 163, 81, and 69 (base peak), indicating the alcohol has 3,7-diethyl-11,15,19-trimethyleicosa-2,6,10,14,18-pentaen-1-ol structure. It is reasonable to assign to (all-*E*)-3,7-diethyl-11,15,19-trimethyleicosa-2,6,10,14,18-pentaen-1-ol (**4c**–OH).

3.2.2. Reaction of 3-propylbut-3-enyl diphosphate (2d) (or 3-butylbut-3-enyl diphosphate (2e)) with FPP

The alcohols derived from the HexPP synthase reaction of **2d** with FPP eluted on HPLC as two peaks at 13.2 min (major) and 12.8 min (minor), which were then purified by HPLC and subjected to GC–MS. In the MS spectrum of the major compound, the molecular ion was ambiguous, but the dehydration ion $[M-18]^+$ was observed distinctly at *m/z* 300 (rel. int. 0.9%), together with fragment ions at *m/z* 257 $[M-18-43]^+$ (1.0), 231 $[M-18-69]^+$ (1.0), 163 $[M-18-69-68]^+$ (2.2), 95 $[M-18-69-68-68]^+$ (16.0), and 69 (base peak), indicating that the alcohol has 3-propyl-7,11,15-trimethylhexadeca-2,6,10,14-tetraen-1-ol structure. In the spectrum of the minor compound, the molecular ion was also obscure, but the dehydration ion $[M-18]^+$ was observed at *m/z* 396 $[M-18]^+$ (rel. int. 0.4%), with other fragment ions at *m/z* 327 $[M-18-69]^+$ (0.4), 191 $[M-18-69-68]^+$ (5.1), 148 $[M-18-69-68-68-43]^+$ (1.9), and 69 (base peak), indicating 11,15,19-trimethyl-3,7-dipropyleicosa-2,6,10,14,18-pentaen-1-ol structure. It is also reasonable to assign the former product to (all-*E*)-3-propyl-7,11,15-trimethylhexadeca-2,6,10,14-tetraen-1-ol (**3d**–OH), and the latter to (all-*E*)-11,15,19-trimethyl-3,7-dipropylhexadeca-2,6,10,14,18-pentaen-1-ol (**4d**–OH), respectively, by considering the stereochemical manner of the enzymatic reaction. On the other hand, the

HepPP synthase reaction of **2d** with FPP also gave a product, which was hydrolyzed with phosphatase to the corresponding alcohol, showing a retention time on HPLC at 13.7 min. The MS spectrum of the alcohol showed a molecular ion at *m/z* 318 (rel. int. 0.3%), corresponding to $C_{22}H_{38}O$, and other main fragment ions were observed at *m/z* 300, 257, 163, and 69 (base peak). Hence, the enzymatic product is reasonably assigned to (all-*E*)-3-propyl-7,11,15-trimethylhexadeca-2,6,10,14-tetraenyl diphosphate (**3d**), suggesting that the chain elongation stopped after the condensation of one molecule of **2d**. However, **2e** was hardly accepted as a substrate for either of the HexPP synthase or the HepPP synthase.

3.3. Reactions of 7,11-dimethyldodeca-2,6,10-trienyl diphosphate (1a) or 7,11,15-trimethylhexadeca-2,6,10,14-tetraenyl diphosphate (1b) with IPP by use of HexPP or HepPP synthase

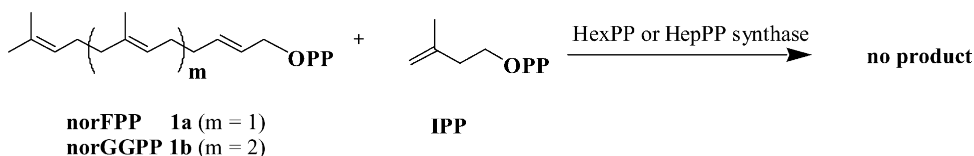
As described in the Section 3.1, the HexPP- or HepPP synthase reaction between FPP and **2d**, which has no alkyl group at the 3-position, gave exclusively 3-desmethylgeranylgeranyl diphosphate (**3b**) as the product, indicating that the chain elongation stopped at the condensation of one molecule of IPP. As the product **3b** lacks the methyl group at the 3-position of GGPP, we examined the reactivities of the allylic substrate homologs which have no alkyl group at the 3-position, norfarnesyl- (**1a**) and norgeranylgeranyl diphosphate (**1b**). As a result the nor-allylic diphosphates, **1a** and **1b** were never accepted as a substrate for either HexPP- or HepPP synthases at all (Scheme 2). From these results, it is concluded that the alkyl group at the 3-position of the allylic substrate is indispensable for the allylic substrate of the medium-chain prenyl diphosphate synthase reaction. This fact coincides with the results of FPP- and UPP synthases reported previously [11,15]. Accordingly, the alkyl (methyl) group at the 3-position of the allylic substrate is essential for most of prenylchain elongating enzymes.

3.4. Calculation of heat formation of prenyl cations from the prenyl alcohols

Poulter et al. [18] have proposed the “ionization–condensation–elimination” mechanism for the head-to-tail coupling reaction catalyzed by prenylchain elongating enzymes. If the medium-chain prenyl diphosphate synthase exactly follow the mechanism, the formation of the prenyl cation will be very important to start the prenyltransferase reaction. Thus, we calculated the energy difference of the cationic formation for both FOH and norFOH by the MOPAC as shown in Table 1. The energy for the farnesyl cation formation from FOH was calculated to be 243.4 kcal mol^{-1}, while the energy difference between norFOH (**1a**–OH) and 7,11-dimethyldodeca-2,6,10-trienyl cation was 250.6 kcal mol^{-1}. These values clearly indicate that norFOH which has no alkyl group at the 3-position, needs more energy than FOH for their cation formation. This means that the major reason for the strict insusceptibility as the substrate for the homologs having no alkyl group at the 3-position is because of the energetic difficulties for their conversion to the corresponding cations in the active site of prenyltransferases.

We have already reported that 2-butenyl diphosphate was not accepted as a substrate at all for the thermostable FPP synthase [11]. According to the value of crotyl cation formation listed in Table 1, it is reasonable to attribute the insusceptibility to the relative difficulty of the cation formation.

On the other hand, 3-desmethylgeranyl diphosphate was found to be a potent competitive inhibitor (K_i = 11.4 μM) for FPP synthase (data not shown). This means that the 3-desmethyl allylic homologs can bind to the proper binding site for allylic diphosphate but these homologs cannot be converted to the corresponding cation to start the prenyltransferase reaction.



Scheme 2. HexPP or HepPP synthase reaction of IPP with norFPP (**1a**) or norGGPP (**1b**).

Table 1
Comparison of the heat of formation of several prenyl alcohols and their cations

Formation energy ($kcal\,mol^{-1}$)		Difference (Δ, $kcal\,mol^{-1}$)
FOH -58.8	**farnesyl cation 184.7**	243.5
norFOH -50.4	**norfarnesyl cation 199.8**	250.2
GOH -54.7	**geranyl cation 189.1**	243.8
norGOH -45.0	**norgeranyl cation 232.9**	277.9
DMAOH -50.2	**dimethylallyl cation 225.8**	276.0
crotylalcohol -41.4	**crotyl cation 237.6**	279.0

GOH: geraniol; norGOH: norgeraniol; DMAOH: dimethylallyl alcohol.

4. Conclusion

As the substrate for medium prenylchain elongating enzymes, HexPP- and HepPP synthases, the reactivities of the homologs whose 3-methyl group is replaced with various alkyl group decrease sharply in proportion to the bulkiness of the alkyl group. The IPP homolog having a 3-butyl group was not accepted as a substrate at all.

The reaction of 3-ethyl- (**2c**) or 3-propyl-IPP (**2d**) with FPP by the HexPP synthase gave the products of single and double condensations, respectively. However, similarly the reaction of 3-ethyl- or 3-propyl-IPP with FPP by the HepPP synthase gave exclusively 3-ethyl- or 3-propylgeranylgerany diphosphate, respectively, which is the product by the single condensation. Furthermore, the reaction product of nor-IPP (**2b**) with FPP by either of HexPP- or HepPP synthase, norgeranylgeranyl diphosphate (**1b**), is never accepted as the allylic substrate for both of the enzymes.

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