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Artificial Substrates of Medium-Chain Elongating Enzymes, Hexaprenyl- and Heptaprenyl Diphosphate Synthases

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Abstract—We examined the reactivity of 3-alkyl group homologues of farnesyl diphosphate or isopentenyl diphosphate for medium-chain prenyl diphosphate synthases, hexaprenyl diphosphate- or heptaprenyl diphosphate synthase. But-3-enyl diphosphate, which lacks the methyl group at the 3-position of isopentenyl diphosphate, condensed only once with farnesyl diphosphate to give *E*-norgeranylgeranyl diphosphate by the action of either enzyme. However, norfarnesyl diphosphate was never accepted as an allylic substrate at all. 3-Ethylbut-3-enyl diphosphate also reacted with farnesyl diphosphate giving 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-trimethylicosa-2,6,10,14,18-pentaenyl diphosphates by hexaprenyl diphosphate synthase. On the other hand, heptaprenyl diphosphate synthase reaction of 3-ethylbut-3-enyl diphosphate with farnesyl diphosphate gave only (all-*E*)-3-ethyl-7,11,15-trimethylhexadeca-2,6,10,14-tetraenyl diphosphate.

Prenyl diphosphate synthases catalyze the head-to-tail condensations of isopentenyl diphosphate (IPP, C_5) with allylic prenyl diphosphates. The elongation terminates precisely at a settled chain length according to the specificities of individual enzymes. These prenyltransferases can be classified into four groups according to the mode of subunit composition as well as chain-length and geometry of the prenyl chain products.[1–3] Short-chain prenyl diphosphate synthases, which occur not only in plants and bacteria but also in mammals, include (all-*E*)-farnesyl diphosphate- (FPP, C_{15}) and geranylgeranyl diphosphate- (GGPP, C_{20}) synthases. Medium-chain prenyl diphosphate synthases which consist of two different protein components, include hexaprenyl diphosphate- (HexPP, C_{30}) and heptaprenyl diphosphate- (HepPP, C_{35}) synthases. Long-chain prenyl diphosphate synthases including solanesyl diphosphate- (C_{45}) and (all-*E*)-decaprenyl diphosphate- (C_{50}) synthases, require polyprenyl carrier proteins for removal of the long-chain product from the active site of the enzyme. These enzymes catalyze the formation of *E*-prenyl chain elongation. *Z*-Polyprenyl diphosphate synthases including undecaprenyl diphosphate- (UPP, C_{55}), dehydrodolichyl diphosphate- (C_{80}) and natural rubber synthases are the other groups.

Hexaprenyl diphosphate synthase (HexPS)[4,5] [EC 2.5.1.33] or heptaprenyl diphosphate synthase (HepPS)[6,7] [EC 2.5.1.30] catalyze the condensations of three or four molecules of IPP with FPP as a primer to yield HexPP or HepPP which are the precursors of menaquinone-6 or menaquinone-7, respectively, as shown in Scheme 1. HexPS has been shown to consist with two components A and B.[8] Similarly, HepPS is composed of two dissociated heteromeric subunits, components I and II.[9] Each component has no catalytic activity as the prenyl chain elongating enzyme unless they are combined.

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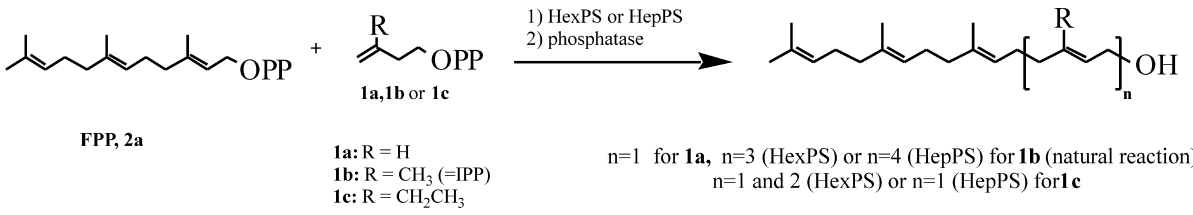


Scheme 1. Medium-chain prenyl diphosphate synthases reaction.

Several experiments with short-chain prenyl diphosphate synthases have shown that the methyl group at the 3-position of an allylic substrate for prenyl elongating enzymes is very important for the enzymatic reactions.[10–13] We have reported that the reactions of but-3-enyl diphosphate (**1a**) with DMAPP or GPP by use of a thermostable farnesyl diphosphate synthase (FPS) gave exclusively *E*-norgeranyl diphosphate or *E*-norfarnesyl diphosphate, respectively. However, the counterpart allylic homologue, but-2-enyl diphosphate was not accepted as a substrate at all.[13]

Recently, we have shown that the reaction of **1a** with FPP by use of a *Z*-polyprenyl diphosphate synthase, undecaprenyl diphosphate synthase (UPS) afforded *Z*-norgeranylgeranyl diphosphate as the single enzymatic product.[14]

This paper describes the reactivities of 3-alkyl-IPP homologues with respect to medium-chain prenyl diphosphate synthases, *Micrococcus luteus* B-P 26 HexPS or *Bacillus subtilis* HepPS to give a condensation product with only one molecule of **1a**, showing that the methyl group at the 3-position of the allylic substrate is also important for medium-chain prenyl diphosphate synthases.

3-Alkyl group homologues of IPP were synthesized according to our method reported previously.[10,13] Norfarnesyl diphosphate as an allylic homologue lacking a methyl group at the 3-position was synthesized from 5,9-dimethyldeca-4,8-dien-1-al via the corresponding ethyl 7,11-dimethyldodeca-2,6,10-trienoate by Horner–Emmons reaction.[15]

The incubation mixture for *M. luteus* B-P 26 HexPS reaction contained, in a final 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 μL of Triton X-100, 0.5 mM of FPP (or IPP), 1.5 mM of the homologue of IPP (or FPP) to be examined, and recombinant HexPS (76 and 90 μg of components A and B, respectively). *B. subtilis* HepPS reaction was carried out under similar conditions as described for HexPS except that 2.0 mM of the homologue of IPP (FPP) to be examined, and recombinant HepPS (124 and 71 μg of components I and II, respectively) were employed. The mixture was incubated at 37 °C for 6 h, then another same amount of HexPS or HepPS was added and the incubation was continued for another 6 h. After the incubation, the reaction mixture was extracted with 1-butanol and the products were hydrolyzed with acid phosphatase at 37 °C for 12 h.[14] The hydrolysates were extracted with pentane and analyzed by HPLC and GC–MS.

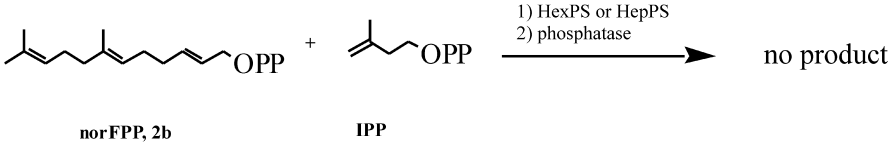
The alcohol derived from the products of HexPS reaction of but-3-enyl diphosphate (**1a**) with FPP gave a peak on HPLC at 15.9 min. After preparative HPLC, this product was subjected to GC–MS. The spectrum of the product showed a molecular ion at *m/z* 276 (rel. int. 0.04%), corresponding to $C_{19}H_{32}O$, and fragment ions were observed at *m/z* 258 $[M-18]^+$ (0.1), 205 $[M-18-53]^+$ (3.4), 189 $[M-18-69]^+$ (1.2), 136 $[M-18-53-69]^+$ (4.0), 121 $[M-18-69-68]^+$ (14.2), and 69 $[C_5H_9]^+$ (base peak), indicating that the alcohol has a norgeranylgeraniol structure, which lacks a methyl group at the 3-position.

The alcohol derived from the reaction of **1a** with FPP by use of HepPS showed a single peak on HPLC at 16.0 min, which showed similar results on GC–MS to those of the norgeranylgeraniol above described.

As a result, the reaction of **1a** with FPP by HexPS or HepPS produced norgeranylgeranyl diphosphate as the single product, which was reasonably assigned to be *E*-norgeranylgeraniol (each relative yield,[16] 0.1%).

However, norfarnesyl diphosphate, **2b**, as an allylic homologue was never accepted as a substrate for both HexPS and HepPS at all, as shown in Scheme 2. This fact has been reasonably explained that the prenyltransferases tested never accept the allylic substrates which lack the alkyl (methyl) group at the 3-position.

These findings suggest that a methyl group at the 3-position of homoallylic substrate is not essential for



Scheme 2. Medium-chain prenyl diphosphate synthases reaction of **2b** with **IPP**.

prenyltransferase reactions but that a 3-methyl group of allylic substrate is strictly essential for these reactions.

On the other hand, the alcohols derived from the products of the HexPS reaction of 3-ethylbut-3-enyl diphosphate (**1c**) with FPP eluted on HPLC as two peaks at 14.1 (major, relative yield: 13.8%) and 13.2 min (minor, relative yield: 0.8%), which were purified by HPLC and subjected to GC–MS. The spectrum of the former (major compound) showed a molecular ion at *m*/*z* 304 (rel. int. 0.1%), corresponding to $C_{21}H_{36}O$ and fragment ions were observed at *m*/*z* 286 $[M-18]^+$ (1.8), 257 $[M-18-29]^+$ (1.4), 217 $[M-18-69]^+$ (1.4), 149 $[M-18-69-68]^+$ (2.9), 81 $[M-18-69-68-68]^+$ (49.0), and 69 $[C_5H_9]^+$ (base peak), indicating that the alcohol has a 3-ethyl-7,11,15-trimethylhexadeca-2,6,10,14-tetraen-1-ol structure. The spectrum of the minor product showed a molecular ion at *m*/*z* 386 (rel. int. 0.1%), corresponding to $C_{27}H_{46}O$, together with fragment ions at *m*/*z* 368 $[M-18]^+$ (0.3), 299 $[M-18-69]^+$ (0.3), 231 $[M-18-69-68]^+$ (3.5), 163 $[M-18-69-68-68]^+$ (2.0), 81$[M-18-69-68-68-130]^+$ (65.2), and 69 $[C_5H_9]^+$ (base peak), indicating that the alcohol has a 3,7-diethyl-11,15,19-trimethyleicosa-2,6,10,14,18-pentaen-1-ol structure. It is reasonable to assign the former to (all-*E*)-3-ethyl-7,11,15-trimethylhexadeca-2,6,10,14-tetraen-1-ol and the latter to (all-*E*)-3,7-diethyl-11,15,19-trimethyleicosa-2,6,10,14,18-pentaen-1-ol, respectively, by considering the stereochemical manner of the enzymatic reaction.

On the other hand, the HepPS reaction of **1c** with FPP also gave a product, which was hydrolyzed with phosphatase to the corresponding alcohol showing a retention time on HPLC at 14.1 min. This product gave a similar spectrum for that of the HexPS reaction product, showing a molecular ion at *m*/*z* 304, corresponding to $C_{21}H_{36}O$, with main fragment ions at *m*/*z* 286 $[M-18]^+$, 217 $[M-18-69]^+$, 149 $[M-18-69-68]^+$, 81 $[M-18-69-68-68]^+$, and 69 $[C_5H_9]^+$. The product is reasonably assigned to (all-*E*)-3-ethyl-7,11,15-trimethylhexadeca-2,6,10,14-tetraen-1-ol (relative yield: 38.3%), suggesting that the chain elongation stopped after the condensation of one molecule of **1c**.

The authors have already found that the UPS reaction of **1c** with FPP gave two products as the reaction products of single and double condensations.[14]

In conclusion, the methyl group at the 3-position of the homoallylic substrate is not essential, but the methyl group at the 3-position of the allylic substrate is strictly essential for most of all prenyl diphosphate synthases including short-, mediun-, and long (*E*)-prenyl chain elongating enzymes as well as (*Z*)-prenyl chain elongating enzymes.

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References and Notes

1. Ogura, K.; Koyama, T. *Chem. Rev.* **1998**, *98*, 1263.
2. Koyama, T.; Ogura, K. In *Comprehensive Natural Product Chemistry*; Barton, D., Nakanishi, K., Eds.; Elsevier Science: Oxford, 1999; Vol. 2, pp 69–96.
3. Koyama, T. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 1671.
4. Yoshida, I.; Koyama, T.; Ogura, K. *Biochem. Biophys. Res. Commun.* **1989**, *160*, 448.
5. Shimizu, N.; Koyama, T.; Ogura, K. *J. Bacteriol.* **1998**, *180*, 1578.
6. Zhang, Y.; Koyama, T.; Marecak, D. M.; Prestwich, G. D.; Maki, Y.; Ogura, K. *Biochemistry* **1998**, *37*, 13411.
7. Takeshita, A.; Koyama, T.; Obata, S.; Ogura, K. *J. Biol. Chem.* **1995**, *270*, 18396.
8. Fujii, H.; Koyama, T.; Ogura, K. *J. Biol. Chem.* **1982**, *257*, 14610.
9. Fujii, H.; Koyama, T.; Ogura, K. *FEBS Lett.* **1983**, *161*, 257.
10. Ogura, K.; Koyama, T.; Seto, S. *J. Chem. Soc., Chem. Commun.* **1972**, 881.
11. Ogura, K.; Nishino, T.; Koyama, T.; Seto, S. *J. Am. Chem. Soc.* **1970**, *92*, 6036.
12. Koyama, T.; Saito, A.; Ogura, K.; Seto, S. *J. Am. Chem. Soc.* **1980**, *102*, 3614.
13. Nagaki, M.; Koyama, T.; Nishino, T.; Shimizu, K.; Maki, Y.; Ogura, K. *Chem. Lett.* **1997**, 497.
14. Nagaki, M.; Sato, S.; Maki, Y.; Nishino, T.; Koyama, T. *J. Mol. Catal. B, Enzymatic* **2000**, *9*, 33.
15. The Horner–Emmons reaction of 5,9-dimethyldeca-4,8-dien-1-al, which was reduced with diisobutylaluminium hydride (DIBAL) of 5,9-dimethyldeca-4,8-dienonitrile followed by dehydroxylation of 2-cyano-5,9-dimethyldeca-4,8-dienoic acid, with diethylethoxycarbonylmethyl-phosphonate gave ethyl 7,11-dimethyldodeca-2,6,10-trienoate. ^{1}H NMR of the ester (400 MHz, $CDCl_3$, *J*/Hz) δ 1.28 (3H, t, *J* = 7.4), 1.60 (6H, s), 1.68 (3H, s), 1.96–2.26 (8H, m), 4.20 (2H, dd, *J* = 7.1, 7.3), 5.82 (1H, dd, *J* = 1.5, 1.7, 15.6), 6.97 (1H, dt, *J* = 6.6, 7.5, 15.6). ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.23, 16.00, 17.62, 25.61, 26.44, 26.60, 32.41, 39.62, 60.05, 121.39, 122.73, 124.16, 131.35, 136.32, 148.87, 166.66. Then, the ester was reduced with DIBAL to give 7,11-dimethyldodeca-2,6,10-trien-1-ol. ^{1}H NMR of the alcohol (400 MHz, $CDCl_3$, *J*/Hz) δ 1.59 (6H, s), 1.68 (3H, s), 1.97–2.10 (8H, m), 4.08 (2H, d, *J* = 5.1), 5.61–5.70 (2H, m). ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.00, 17.63, 25.64, 25.65, 27.54, 32.39, 39.65, 63.74, 123.58, 124.26, 129.03, 131.30, 132.99, 135.51. After chlorination of the alcohol, 7,11-dimethyldodeca-2,6,10-trienyl chloride was converted to the norfarnesyl diphosphate according to the method of Davisson et al.[17]
16. Relative yield was calculated based on FPP recovered from the reaction.
17. Davisson, V. D.; Woodside, A. B.; Poulter, C. D. *Method Enzymol.* **1985**, *110*, 133.