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Artificial substrates for undecaprenyl diphosphate synthase from *Micrococcus luteus* B-P 26

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Abstract

Substrate specificity of undecaprenyl diphosphate synthase of *Micrococcus luteus* B-P 26 was investigated with respect to some alkyl- and bromo-group homologs of isopentenyl diphosphate. Among the homologs relating to the 3-methyl group, but-3-enyl diphosphate (**2b**) and 3-ethylbut-3-enyl diphosphate (**3b**) were accepted as substrates, with (all-*E*)-farnesyl diphosphate (FPP) to give 7,11,15-trimethylhexadeca-2,6,10,14-tetraenyl diphosphate, and a mixture of 3-ethyl-7,11,15-trimethylhexadeca-2,6,10,14-tetraenyl- and 3,7-diethyleicosa-2,6,10,14,18-pentaenyl diphosphates, respectively. With respect to the homologs modified at the 4 position of isopentenyl diphosphate, (4*E*)-3-methylpent-3-enyl diphosphate (**2f**) was accepted as a substrate to give (4*S*)-(2*Z*,6*E*,10*E*,14*E*)-4-methylgeranylgeranyl- and (4*S*,8*S*)-(2*Z*,6*Z*,10*E*,14*E*,18*E*)-4,8-dimethylgeranylfarnesyl diphosphates. Neither (4*Z*)-3-methylpent-3-enyl diphosphate nor 4-bromo-3-methylbut-3-enyl diphosphates was accepted as a substrate at all.

Keywords: Undecaprenyl diphosphate synthase; Substrate specificity; Homologs of isopentenyl diphosphate; Prenyltransferase; Chiral synthesis

1. Introduction

Prenyltransferase reaction proceeds the condensation of an allylic diphosphate with isopentenyl diphosphate (IPP) stereospecifically and the condensation terminates precisely until the elongation of prenyl chain reaches certain lengths depending on specificities of the enzymes. These enzymes can be classified into two main types of *E*- and *Z*-prenyl chain elongating reactions [1–3].

Undecaprenyl diphosphate (UPP) synthase [EC 2.5.1.31] which belongs to *Z*-type, catalyzes the sequential oligomerizations of eight molecules of IPP with (all-*E*)-farnesyl diphosphate (FPP) as a priming allylic substrate to give (all-*E*)-farnesyl-(all-*Z*)-octaprenyl diphosphate, UPP as shown in Scheme 1 [4,5].

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In bacteria, UPP is utilized as the precursor of a glycosyl carrier lipid in the biosynthesis of cell wall components such as peptidoglycan [6]. Allen et al. [4] first isolated and characterized UPP synthase from a bacterium, *Lactobacillus plantarum*. Then, Itoh et al. [7] determined the absolute stereochemistry with respect to the double bond of IPP during the *Z*-prenyl chain elongation catalyzed by the UPP synthase from *Bacillus subtilis* by using [4-^{2}H]IPP. They have also reported that (*E*)-3-methyl-3-pentenyl diphosphate (**2f**) reacts with FPP by the action of UPP synthase from *B. subtilis* to exclusively give (4*S*)-(2*Z*,6*E*,10*E*)-4-methylgeranylgeraniol, but its (*Z*)-isomer **2g** does not react at all [9–11]. UPP synthase reaction has been applied to the syntheses of glycinoprenols [12].

Recently, Shimizu et al. [8] have first cloned the gene for the UPP synthase of *Micrococcus luteus* B-P 26 among *Z*-prenyl elongating enzymes, constructed an overproducing system of the enzyme, and purified it in large amounts.

These reports have prompted us to study further on substrate specificity of the purified recombinant enzyme, which can be easily available for a synthetic use of such a *Z*-prenyl chain elongating enzyme for some fine chemicals.

We have examined the substrate specificity of a thermostable FPP synthase as *E*-prenyl chain elongating enzyme of *B. stearothermophilus* with some 3-alkyl group homologs of IPP [13,14]. It is also interesting from the standpoint of a mechanistic enzymology of the *Z*-condensation, as well as of comparative biochemistry between *E*- and *Z*-prenyl chain elongating enzymes.

This paper describes the substrate specificity of UPP synthase as *Z*-prenyl chain elongating enzyme of *M. luteus* B-P 26 with respect to several artificial homologs of the homoallylic substrate IPP.

2. Experimental

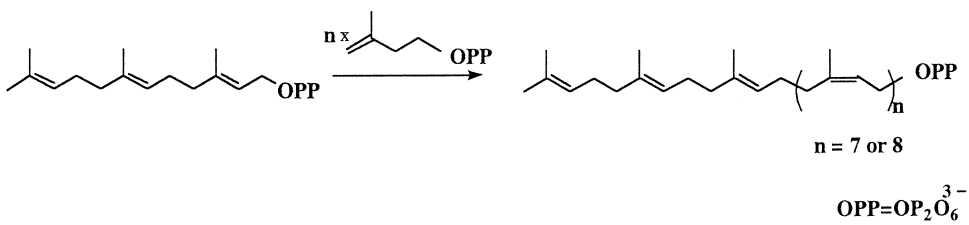
2.1. Analysis

HPLC (Hitachi type L-6200) of the prenyl alcohols that was obtained by acid phosphatase treatment of the enzymatic reaction product was performed with LichroCART (Merck) column using a mixture of hexane:2-propanol = 40:1 (v/v) as the eluent in the similar manner as previously reported [13].

Identification of the enzymatic reaction products was carried out using GC-MS (JMS-AM II 50 type GCG Mass spectrometer and 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 constant at 280°C for 3 min.

2.2. Chemicals

The 3- and 4-alkyl homologs of IPP, but-3-enyl diphosphate, **2b**; 3-ethylbut-3-enyl diphosphate, **2c**; 3-propylbut-3-enyl diphosphate, **2d**; 3-butylbut-3-enyl diphosphate, **2e**; *E*- and *Z*-methylpent-3-enyl diphosphates, **2f** and **2g**; and *E*- and *Z*-4-bromobut-3-enyl diphosphates, **2h** and **2i**; were synthesized according to the



Scheme 1. UPP synthase reaction.

method reported previously [15,16]. Diphosphorylation of the corresponding alcohols were carried out by the method of Davisson et al. [17]. Potato acid phosphatase was purchased from Sigma.

2.3. Purification of UPP synthase

Escherichia coli cells harboring pET22bMLU [8], which had been incubated and induced by isopropyl-β-D-thiogalactopyranoside treatment to overproduce *M. luteus* B-P 26 UPP synthase, were disrupted by sonication, and the extracts were collected by ultracentrifugation (100 000 $\times g$). The protein fraction precipitated by 30–60% ammonium sulfate was applied to a MonoQ HR-, followed by Superdex 200 chromatographies. The purity of the UPP synthase fraction after the gel filtration was more than 90% as judged by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) with Coomassie Brilliant Blue staining.

2.4. Conditions of the enzymatic reaction

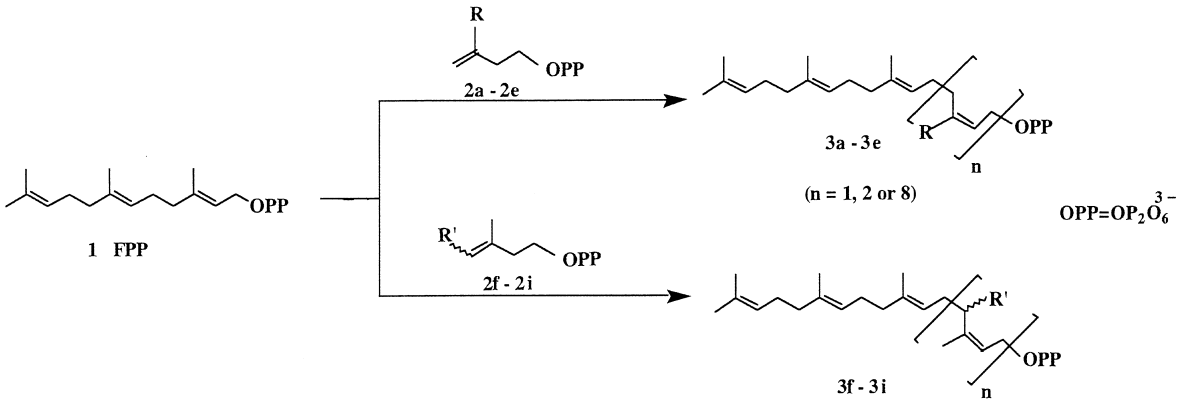
The incubation mixture for the UPP synthase reaction contained, in a final volume of 2.0 ml, 200 μmol of Tris–HCl buffer (pH 7.5), 1.0 μmol of $MgCl_2$, 100 μmol of KCl, 0.05% of Triton X-100, 1.0 μmol of FPP, 1.0 μmol of an IPP-homolog to be examined, and 72 μg of the recombinant *M. luteus* B-P 26 UPP synthase.

After the incubation at 37°C for 6 h, the reaction was terminated by chilling the mixture with an ice bath. Then, the reaction mixture was treated with *n*-butanol and the extracted prenyl products were hydrolyzed with acid phosphatase at 37°C for 12 h [18]. The hydrolysate prenylalcohols were extracted with *n*-pentane and analyzed by HPLC and GC-MS.

3. Results and discussion

3.1. Reaction of 3-alkylbut-3-enyl diphosphate (2a–2e) with FPP (1)

In order to investigate substrate specificity of the UPP synthase of *M. luteus* B-P 26 toward IPP homologs having various alkyl chain at 3-position, we examined a series of homologs **2b** to **2e** in the condensation with FPP as the allylic substrate. As a result, the UPP synthase reactions of FPP with but-3-enyl diphosphate



Scheme 2. UPP synthase reactions with **2a–2i** and FPP as substrates.

(**2b**) and with 3-ethylbut-3-enyl diphosphate (**2c**) were found to proceed but the prenyl chain elongation stopped only at the first or second stages of condensation (Scheme 2).

The alcohol derived from the reaction product of **2b** with FPP was isolated by HPLC (retention time at 14.7 min) and subjected to GC-MS analysis. Though the molecular ion was obscure, the dehydration ion, $[M-18]^+$ was observed distinctly at m/z 258 (rel. int. 2.0%), and other fragment ions were observed at m/z 205 $[M-18-C_4H_5]^+$ (1.0), 189 $[M-18-69]^+$ (1.6), 136 $[M-18-C_4H_5-69]^+$ (6.8), 121 $[M-18-69-68]^+$ (15.4), and 69 $[C_5H_9]^+$ (base peak), indicating that the alcohol has the 7,11,15-trimethylhexadeca-2,6,10,14-tetraen-1-ol (**3b-OH**) structure. This result indicates that **2b** is accepted as a substrate for the UPP synthase but the chain elongation stops at the first condensation of **2b**, yielding **3b** ($n=1$), although the yield is less than 0.3% as shown in Table 1. On the other hand, we reported that the yield of *E*-7,11-dimethyldodeca-2,6,10-trienyl diphosphate by the action of the *B. stearothermophilus* FPP synthase from **2b** with dimethylallyl diphosphate (DMAPP) was 8% [13]. The presence of a methyl group at the 3-position of a homoallylic substrate IPP is not essential for both of *E*- and *Z*-type prenyltransferases, but the methyl group of IPP is more important for substrate recognition for the *Z*-prenyl chain elongating enzyme rather than for the *E*-chain elongating enzyme.

Table 1
Relative yield of the products derived from IPP homologs (**2a–2i**) with FPP in the enzymatic reaction catalyzed by UPP synthase *M. luteus* B-P 26

IPP homolog	Product[a]	Relative yield (%)[b]
IPP	UPP ($n=8$)	100
2b	**3b** ($n=1$)	0.3
2c	**3c** ($n=1$)	9.3
	3c ($n=2$)	7.5
2d	(**3d**)	n.d.[c]
2e	(**3e**)	n.d.
E-**2f**	**3f** ($n=1$)	44
	3f ($n=2$)	0.2
E-**2g**	(**3g**)	n.d.
E-**2h**	(**3h**)	n.d.
Z-**2i**	(**3i**)	n.d.

[a] Structures are in Scheme 2.
[b] Each value represents the mean of at least three determinations. Relative yield was calculated based on FPP consumed during the reaction. Structures are in Scheme 2.
[c] n.d., not detected.

The alcohols derived from the products of the UPP synthase reaction of **2c** with FPP gave two peaks on HPLC at 13.6 min (major) and 12.6 min (minor). After preparative HPLC, these products were subjected to GC-MS. The spectrum of the major product showed a molecular ion at m/z 304 (rel. int. 0.9%), corresponding to $C_{21}H_{36}O$, and fragment ions were observed at m/z 286 $[M-18]^+$ (2.7), 257 $[M-18-C_2H_5]^+$ (1.8), 217 $[M-18-69]^+$ (4.8), 149 $[M-18-69-68]^+$ (7.3), 81 $[M-18-69-68-68]^+$ (51.8), and 69 $[C_5H_9]^+$ (base peak), which are assignable to 3-ethyl-7,11,15-trimethylhexadeca-2,6,10,14-tetraen-1-ol (**3c-OH**, $n=1$). The mass spectrum of the minor one showed fragment ions at m/z 368 (rel. int. 1.7%), corresponding to $[M-18]^+$, 339 $[M-18-C_2H_5]^+$ (0.9), 299 $[M-18-69]^+$ (0.7), 231 $[M-18-69-68]^+$ (2.5), 163 $[M-18-69-68-68]^+$ (2.7), and 69 $[C_5H_9]^+$ (base peak), indicating a 3,7-diethyl-11,15,19-trimethyleicosa-2,6,10,14,18-pentaen-1-ol structure (**3c-OH**, $n=2$). These results indicate that the UPP synthase reaction with **2c** instead of IPP stops at the stage of single or double condensation of the C-6 homolog. However, both 3-propylbut-3-enyl diphosphate (**2d**) and 3-butylbut-3-enyl diphosphate (**2e**) were hardly accepted as substrate.

On the other hand, the FPP synthase from a thermophilic bacterium showed broader specificity toward 3-alkyl-IPP homologs [14]. The reaction of **2c** with DMAPP gives two condensation products of one or two molecules of **2c**. Even the 3-propyl-(**2d**) or 3-butyl-(**2e**) IPP homologs can be accepted as substrates instead of IPP [14]. The specificity of the *Z*-chain elongating enzyme with respect to the 3-alkyl group homologs of IPP seems more stringent than that of the *E*-chain elongation enzyme.

*3.2. UPP synthase reaction of E- and Z-3-methylpent-3-enyl diphosphates and E- and Z-4-bromobut-3-enyl diphosphates (**2f–2i**) with FPP*

In order to examine the specificity of the *M. luteus* UPP synthase toward IPP homologs with a methyl group or a bromine added to the 4-position, we examined the reactivities of the homologs, **2f** to **2i** with FPP.

As a result, *E*-3-methylpent-3-enyl diphosphate, **2f** was found to be reactive as a substrate for the UPP synthase, giving two reaction products. The two alcohols derived from the reaction products of **2f** with FPP, which showed retention times at 11.8 (major) and 10.3 min (minor) on HPLC, were purified and subjected to GC-MS. The former showed a molecular ion at m/z 304 (rel. int. 0.04%), corresponding to $C_{21}H_{36}O$, and fragment ions at m/z 286 $[M-18]^+$ (6.9), 271 $[M-18-15]^+$ (2.8), 217 $[M-18-69]^+$ (13.1), 149 $[M-18-69-68]^+$ (24.6), 81 $[M-18-69-68-68]^+$ (86.1), and 69 $[C_5H_9]^+$ (base peak), which was exactly coincided with the results obtained by Koyama et al. [9] and Ohnuma et al. [10]. Hence, it is reasonable to assign the product to (4*S*)-(2*Z*,6*Z*,10*Z*)-4-methylgeranylgeranyl diphosphate (**3f**, $n=1$) by considering the similar stereochemical manner of UPP synthase [7].

The other alcohol showed ambiguous molecular ion, with fragment ions at m/z 368 $[M-18]^+$ (rel. int. 2.3%), 299 $[M-18-69]^+$ (1.0), 231 $[M-18-69-68]^+$ (3.2), 163 $[M-18-69-68-68]^+$ (15.6), 133 (53.6), 107 (56.2), and 69 $[C_5H_9]^+$ (base peak), suggesting that the alcohol has a 4,8-dimethylgeranylfarnesol structure (**3f-OH**, $n=2$). This result indicates that the *M. luteus* UPP synthase reaction with **2f** can extend the chain length up to the stage where double condensation of the C_6-homolog with FPP is completed, although the yield of **3f** is low (Table 1). Ohnuma et al. have reported that the reaction of **2f** with FPP by *B. subtilis* UPP synthase proceeds in the same stereochemical manner as that with the natural homoallylic substrate, IPP, but it had a full stop at the stage where a single condensation of the C_6-homolog with an allylic primer is completed to form a chiral prenyl diphosphate, (4*S*)-(2*Z*,6*Z*,10*Z*)-4-methylgeranylgeranyl diphosphate [9,10]. It is reasonable to assign the double condensation product to (4*S*,8*S*)-(2*Z*,6*Z*,10*E*,14*E*)-4,8-diethyl-geranylfarnesyl diphosphate.

On the other hand, the *Z*-isomer, **2g** was scarcely accepted as a substrate for the UPP synthase. This result was similar to that by the *B. subtilis* enzyme [10,11]. Moreover, when we examined the 4-bromo-homologs, *E*- and *Z*-4-bromo-IPP (**2h** and **2i**), neither **2h** nor **2i** was acceptable as substrate for the UPP synthase at all.

The relative yield of **3f**, which is produced by single condensation of **2f** with FPP is 44% to that of the normal condensation of IPP with FPP (Table 1). This value is four times larger than that by the UPP synthase from *B. subtilis* [12], suggesting the usefulness of the *M. luteus* UPP synthase for application to organic synthesis like the case of *B. stearothermophilus* FPP synthase than the liver enzymes as reported earlier [19].

4. Conclusions

Substrate specificity of the UPP synthase of *M. luteus* B-P 26 was investigated with respect to some alkyl- and bromo-group homologs of IPP.

The product of UPP synthase reaction obtained by the condensation of **2b** with FPP gave a little amount of 7,11,15-trimethylhexadeca-2,6,10,14-tetraenyl diphosphate **3b** ($n=1$), indicating that the presence of a methyl group at the 3-position of IPP is not essential but more important for the substrate recognition by the *Z*-chain elongation enzyme than the *E*-chain enzyme, FPP synthase.

As shown in Table 1, *M. luteus* UPP synthase reaction of **2c** with FPP gave 3-ethyl-7,11,15-trimethylhexadeca-2,6,10,14-tetraenyl-

3c ($n = 1$) and 3,7-diethyl-11,15,19-trimethyleicosa-2,6,10,14,18-pentaenyl diphosphates **3c** ($n = 2$), indicating that the prenyl chain elongation stopped at the first and second stages of condensation, respectively. However, the homologs with *n*-propyl or *n*-butyl group at 3-position in place of the methyl group of IPP, were not accepted as a substrate at all.

E-3-Methylpent-3-enyl diphosphates **2f** reacted with FPP to give (*S*)-4-methylgeranylgeranyl diphosphate **3f** ($n = 1$) and (*S*,*S*)-4,8-dimethylgeranylfarnesyl diphosphate **3f** ($n = 2$) by condensations with one and two molecules of **2f**, respectively. But its (*Z*)-isomer, **2g** was not accepted as a substrate at all.

Neither (*E*)- nor (*Z*)-4-BrIPP was accepted as a substrate at all.

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