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One-pot syntheses of the sex pheromone homologs of a codling moth, *Laspeyresia promonella* L.

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Abstract

In order to carry out one-pot syntheses of the sex pheromone homologs of a codling moth, *Laspeyresia promonella* L. by use of a thermophilic enzyme, we have examined the reactivities of 3-methylhex-2-enyl diphosphates as an allylic substrate and of some 3-alkyl homologs of isopentenyl diphosphate in the reaction catalyzed by farnesyl diphosphate (FPP) synthase of *Bacillus stearothermophilus*.

3-methylhex-2-enyl diphosphate (**1a**) was accepted as a substrate with 3-ethylbut-3-enyl diphosphate (**2b**) to give a mixture of (2*E*,6*E*)-3-ethyl-7-methyldeca-2,6-dienyl- (**3b**) and (2*E*, 6*E*, 10*E*)-3,7-diethyl-11-methyltetradeca-2,6,10-trienyl diphosphate (**4b**). **1a** was also reacted with 3-propylbut-3-enyl diphosphate (**2c**) to give exclusively (2*E*,6*E*)-7-methyl-3 propyldeca-2,6-dienyl diphosphate (**3c**). On the other hand, *Z*-3-methylhex-2-enyl diphosphate (**1b**) was accepted as a substrate with **2b** to give a mixture of (2*E*,6*Z*)-3-ethyl-7-methyldeca-2,6-dienyl- (**3′b**) and (2*E*,6*E*,10*Z*)-3,7-diethyl-11-methyltetradeca-2,6,10-trienyl diphosphate (**4′b**). The *Z*-isomer (**1b**) was reacted with **2c** to give (2*E*,6*Z*)-7-methyl-3 propyldeca-2,6-dienyl diphosphate (**3′c**).

On the basis of these reactivities, one-pot enzymatic syntheses of the sex pheromone homologs of the codling moth, **3c**-OH and **3′c**-OH, were carried out by the action of the FPP synthase, followed by an alkaline phosphatase, with yields of 9.6% and 0.6%, respectively.

Keywords: Thermostable farnesyl diphosphate synthase; Substrate specificity; Enzymatic synthesis; Homologs of isopentenyl diphosphate; Prenyltransferase

1. Introduction

In 1972, McDonough et al. [1] have determined the structure of a sex pheromone of a codling moth, *Laspeyresia promonella* L., to be (2*Z*,6*E*)-7-methyl-3-propyldeca-2,6-dien-1-ol. However, Cooke [2,3] has corrected the structure to be the (2*Z*,6*Z*)-isomer as the natural pheromone by chemical syntheses of every isomer of the sex pheromone, which required more than four steps. We thought that these isomers

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could be synthesized promptly by the action of a farnesyl diphosphate synthase. So, we planned one-pot synthesis, which was expected to make such kind of tedious works attainable with ease.

Farnesyl diphosphate synthase [EC 2.5.1.10] is one of the short-chain *E*-prenyl diphosphate synthases which is classified as a member of prenyltransferase I [4,5]. It catalyzes the ''head-to-tail'' condensation of isopentenyl diphosphate (IPP) with dimethylallyl diphosphate (DMAPP) or with geranyl diphosphate (GPP) to give (all-*E*)-farnesyl diphosphate (FPP) as shown in Scheme 1.

Porcine FPP synthase has been successfully applied to the stereospecific syntheses of several bioactive substances, such as faranal, a trail pheromone of Pharaoh's ant [6,7] and 4-methyl-juvenile hormone I of a tobacco hornworm [8]. Meanwhile, Koyama et al. [9] have carried out the gene cloning and efficient overproduction and purification of the FPP synthase of a thermophilic bacterium, *Bacillus stearothermophilus* in *Escherichia coli* cells. We have found that the specificity of the thermostable FPP synthase is more tolerant than that of the porcine liver enzyme, and that even 3-propylbut-3-enyl diphosphate (**2c**), which was not a good substrate for the liver enzyme, is accepted as a substrate for the thermostable FPP synthase [10]. Thus, from a standpoint of organic synthesis, application of this thermostable FPP synthase will be widely useful and convenient for its specificity and stability. In order to carry out the one-pot syntheses of several sex pheromone homologs of the codling moth using the FPP synthase, we examined the reactivities of *E*- or *Z*-3-methylhex-2-enyl diphosphate (**1a** and **1b**) as an allylic substrate and with some 3-alkyl group homologs of IPP as homoallylic substrate.

This paper describes the one-pot syntheses of the sex pheromone homologs of the codling moth by use of the reactivities of artificial allylic diphosphate with an alkyl group homolog of IPP, **2c**, with FPP synthase.

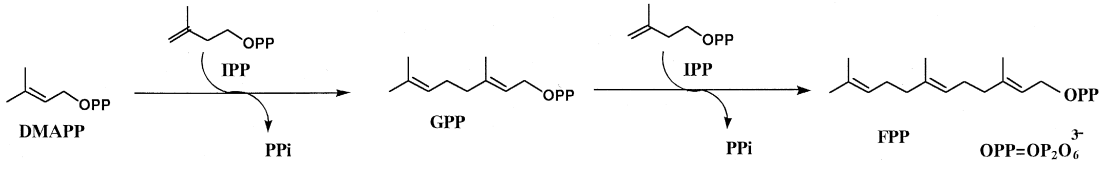
2. Experimental

2.1. Chemicals

The allylic substrate homologs, *E*-(**1a**) and *Z*-3-methylhex-2-enyl diphosphate (**1b**) were synthesized according to the methods reported previously [11], and with Ando's reagent [12]. The IPP homologs (**2a**–**2c**) were the same preparations as used in the previous report [10]. Diphosphorylation of the alcohols were carried out by the method of Davisson et al. [13]. Alkaline phosphatase (calf intestine) was purchased from Boehringer Mannheim.

2.2. Purification of thermophilic FPP synthase

The cells of *E. coli* JM105 harboring pEX11 [9], which had been incubated to overproduce the *B. stearothermophilus* FPP synthase, were disrupted by sonication, and the extracts were collected by ultracentrifugation ($100{,}000 \times g$). Heat treatment (at 60°C for 1 h) of the extract was effective, because most of all the protein derived from the host cell could be denatured and removed without loss of activity of the thermostable FPP synthase. The protein fraction



Scheme 1. Reaction catalyzed by FPP synthase.

precipitated by 30–60% saturation of ammonium sulfate was purified by two (Butyl-Toyopeal and MonoQ) chromatographic procedures. The purity of the FPP synthase fraction after the gel filtration was more than 90% as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie Brilliant Blue staining.

2.3. Conditions of the enzymatic reaction

The incubation mixture for the FPP synthase reaction contained, in a final volume of 2.0 ml, 200 μmol of Tris–HCl buffer (pH 8.5), 20 μmol of $MgCl_2$, 10 μmol of KCl, 100 μmol of β-mercaptoethanol, 1.0 μmol of an allylic substrate (DMAPP, **1a** or **1b**), 1.0 μmol of an IPP-homolog (**2a**–**2c**), and 25 μg of the FPP synthase protein of *B. stearothermophilus*.

After incubation at 55°C for 3 h, the reaction was terminated by chilling the mixture with an ice bath. Then, the reaction mixture was treated with alkaline phosphatase at 37°C for 12 h. The hydrolysate were extracted with *n*-pentane and analyzed by HPLC and GC-MS. In the case of *Z*-3-methylhex-2-enyl diphosphate (**1b**), incubation was carried out at 55°C for 24 h, another 25 μg of the FPP synthase was added and the incubation was continued for another 24 h.

2.4. Product analysis

The prenyl alcohols which derived by alkaline phosphatase treatment of the products obtained from FPP synthase reaction were subjected to HPLC analysis. The conditions of HPLC (Hitachi, type L-6200) were similar as described in our previous paper [10]. The relative amount of all the products and recovered substrate were obtained by the integrations of the corresponding alcohols measured by the HPLC. Then, the relative yields were calculated on the basis of the yield of FPP derived from the reaction with IPP and DMAPP.

Identification of the prenyl alcohols derived from the enzymatic reaction products was performed using GC-MS (JMS-AM II 50 type GCG Mass spectrometer and HP 5890 series II gas chromatograph). The conditions of GC-MS were the same as previously reported [14].

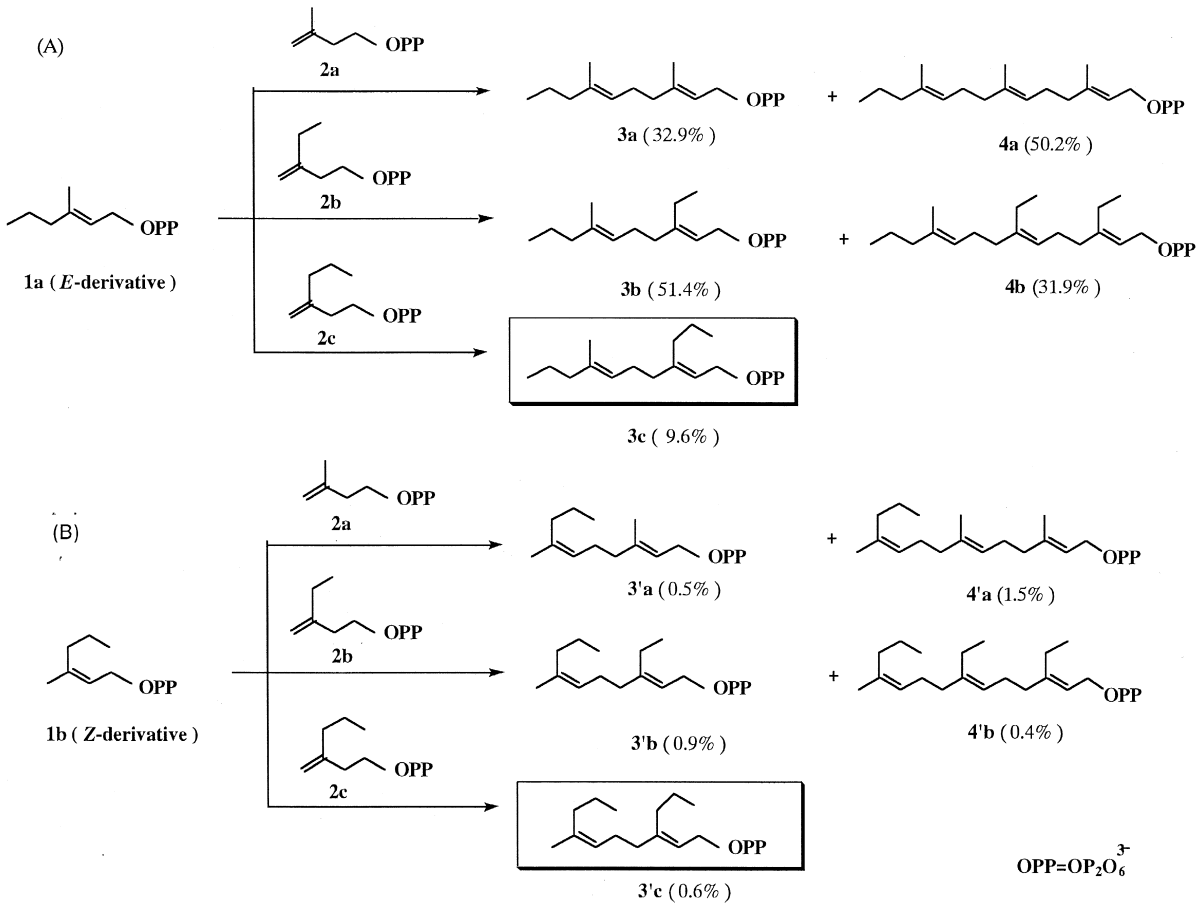
3. Results and discussion

3.1. Reaction of E-3-methylhex-2-enyl diphosphate (1a) with IPP-homologs (2a–2c)

We have reported that homologs of IPP (**2a**, **2b** and **2c**), which are hardly acceptable as a substrate for the porcine liver FPP synthase, are acceptable as substrates by the FPP synthase from *B. stearothermophilus* [10]. On the other hand, Ogura et al. [11] and Nishino et al. [15] have reported that IPP reacts with *E*-3-methylhex-2-enyl diphosphate (**1a**) by the action of FPP synthases from either porcine liver or pumpkin fruit to give (2*E*,6*E*)-3,7-dimethyldeca-2,6-dienyl- (**3a**) and (2*E*,6*E*,10*E*)-3,7,11-trimethyltetradeca-2,6,10-trienyl diphosphate (**4a**), respectively. These findings prompted us to carry out one-pot synthesis of some of the sex pheromone homologs using the thermostable FPP synthase.

In order to achieve one-pot syntheses of some homologs of the sex pheromone of a codling moth [*L. promonella* L] (Scheme 2), we first examined reactivities of an allylic diphosphate **1a** and homoallylic homologs, **2b**, **2c**, using the thermostable FPP synthase of *B. stearothermophilus*.

The prenyl alcohols derived from the reaction products of **2b** and **1a** with FPP synthase, appeared on HPLC as two peaks, a major (retention time at 16.0 min) and a minor peak (14.7 min). These alcohols were purified by HPLC and subjected to GC-MS analysis. In the MS spectrum of the major one, the molecular ion was obscure, but the dehydration ion $[M-18]^+$ was observed at m/z 178 (rel. int. 4.2%), together with fragment ions at m/z 149 $[M-18-C_2H_5]^+$ (4.4), 135 $[M-18-C_3H_7]^+$ (9.5),



Scheme 2. Farnesyl diphosphate synthase reactions with artificial substrates. The relative yields of all the products, as shown in parentheses, were calculated by the amount of the corresponding alcohols relative to that of FPP derived from the reaction with IPP and DMAPP. Each value represents the mean of at least three determinations.

and 107 (base peak), indicating a 3-ethyl-7-methyldeca-2,6-dien-1-ol structure. It is reasonable to assign the alcohol to (2*E*,6*E*)-3-ethyl-7-methyldeca-2,6-dien-1-ol (**3b-OH**) by considering the similar stereochemical reaction of *E*-type prenyltransferases [4,5]. The spectrum of the minor alcohol showed a molecular ion at *m/z* 278 (rel. int. 0.4%), corresponding to $C_{19}H_{34}O$, and other fragment ions were observed at *m/z* 260 $[M-18]^+$ (1.1), 231 $[M-18-C_2H_5]^+$ (2.6), 217 $[M-18-C_3H_7]^+$ (0.9) and 69 (base peak), which represents an 3,7-diethyl-11-methyl-tetradeca-2,6,10-trien-1-ol structure. It is also assignable to (2*E*,6*E*,10*E*)-3,7-diethyl-11-methyl-tetradeca-2,6,10 trien-1-ol (**4b-OH**). These results indicate that the thermostable FPP synthase reaction of **1a** with **2b** proceeds in a similar manner to that with natural substrate IPP to give products corresponding to either single or double condensation as shown in Scheme 2 (A).

The alcohol derived from the product of the FPP synthase reaction with **1a** and **2c** showed a peak at 17.9 min on HPLC. The MS spectrum of the product showed a molecular ion at *m/z* 210 (rel. int. 4.6%), corresponding to $C_{14}H_{26}O$, and fragment ions were observed at *m/z* 192 $[M-18]^+$ (5.8), 149 $[M-18-C_3H_7]^+$ (5.0), and 100 (base peak), which showed a 7-methyl-3-propyldeca-2,6-dien-1-ol structure. This fact

indicates that the enzymatic chain elongation stopped at the single condensation stage of **2c**. It is also reasonable to assign the alcohol to (2*E*,6*E*)-7-methyl-3-propyldeca-2,6-dien-1-ol (**3c-OH**) by considering the similar stereochemical manner of *E*-prenyl elongating enzymes. This compound is one of the double bond isomers of the sex pheromone of the codling moth, *L. promonella* L. [1–3]

*3.2. Reaction of Z-3-methylhex-2-enyl diphosphate, **1b** with IPP homologs (**2b** to **2c**)*

According to the reports on the substrate specificity of porcine liver FPP synthase [15] (*Z*)-3-methylhex-2-enyl diphosphate (**1b**) was not a good substrate. Hence, to test the specificity of the thermostable FPP synthase, conditions of the enzymatic reactions of the *Z*-isomer **1b** with IPP were more prolonged incubation time and increased enzyme amounts than the standard conditions.

The result was that the bacterial FPP synthase barely accepted **1b** as an allylic substrate. The alcohols derived from the products of the condensation of **1b** with IPP gave two peaks at 17.3 and 19.0 min on HPLC. The MS spectrum of the former showed a molecular ion at *m/z* 250 (rel. int.0.2%), corresponding to $C_{17}H_{30}O$, with other fragment ions at *m/z* 232 $[M-18]^+$ (4.8), 189 $[M-18-C_3H_7]^+$ (4.0), 163 $[M-18-69]^+$ (1.0), and 86 (base peak), which is similar to the fragment pattern of (2*E*,6*E*,10*E*)-3,7,11-trimethyltetradeca-2,6,10-trien-1-ol (**4a-OH**). The spectrum of the latter showed a molecular ion at *m/z* 182 (rel. int. 0.02%), corresponding to $C_{12}H_{22}O$, and fragment ions at *m/z* 164 $[M-18]^+$ (25.1), and 121 $[M-18-C_3H_7]^+$ (base peak), which indicates a structure of 3,7-dimethyldeca-2,6-dien-1-ol. This compound is reasonably assigned to (2*E*,6*Z*)-3,7-dimethyldeca-2,6-dien-1-ol (**3′a-OH**), as the product derived from the single condensation of **1b** with IPP as shown in Scheme 2 (B).

The alcohols derived from the reaction of **1b** with **2b** gave two peaks at 15.3 and 16.7 min on HPLC. The MS spectrum of the former was similar to that of **4b-OH** showing a molecular ion at *m/z* 278 (rel. int. 2.7%), corresponding to $C_{19}H_{34}O$. Fragment ions were observed at *m/z* 260 $[M-18]^+$ (4.5), 231 $[M-18-C_2H_5]^+$ (3.9), 217 $[M-18-C_3H_7]^+$ (0.6), and 105 (base peak), indicating the 3,7-diethyl-11-methyltetradeca-2,6,10-trien-1-ol structure. Thus, the product is reasonably assigned to (2*E*,6*E*,10*Z*)-derivative (**4′b-OH**). In a similar manner, the latter showed a molecular ion at *m/z* 196 (rel. int. 0.3%), corresponding to $C_{13}H_{24}O$, and fragment ions *m/z* 178 $[M-18]^+$ (5.4), 149 $[M-18-C_2H_5]^+$ (75.2), and 121 (base peak), showing similar cleavage pattern of **3b-OH**. Thus, the latter product is reasonably assigned to (2*E*,6*Z*)-3-ethyl-7-methyldeca-2,6-dien-1-ol (**3′b-OH**).

Then FPP synthase reaction of **1b** was applied to the synthesis of the sex pheromone homolog of a codling moth by employing the IPP homolog **2c** instead of IPP. The single reaction product after the phosphatase treatment showed a retention time at 17.2 min on HPLC. The MS spectrum showed a molecular ion at *m/z* 210 (rel. int. 4.5%), corresponding to $C_{14}H_{26}O$, with fragment ions at *m/z* 192 $[M-18]^+$ (10.2), 149 $[M-18-C_3H_7]^+$ (27.5), and 55 (base peak), which was similar to those of **3c-OH**. Thus, the product is reasonably assigned to (2*E*,6*Z*)-7-methyl-3-propyldeca-2,6-dien-1-ol (**3′c-OH**), which is the geometrical isomer of **3c-OH**, one of the homologs of the codling moth sex pheromone.

Using this enzyme, in the reaction with the natural substrates, DMAPP and IPP, all the enzymatic reaction proceeds to yield a double condensation product, FPP without giving GPP as an intermediary product. However, in the reaction with **1a** or **1b** as the allylic substrate, and with **2a** or **2b** as the homoallylic substrate, both of the single and double condensation products were produced. Moreover, when the homoallylic homolog, **2c** was employed only the single condensation product, **3c** or **3′c** was obtained. The propyl group at the 3-position of

the GPP homologs, **3c** or **3′c** seems too large to be acceptable for the enzyme as the allylic GPP homolog. This will be convenient from the standpoint of the pheromone synthesis, although the yields were not enough especially when **1b** was the allylic substrate.

4. Conclusions

In order to synthesize the homologs of the codling moth [*L. promonella* L] sex pheromone, we examined reactivities of *E*-and *Z*-3-methylhex-2-enyl diphosphates as allylic substrates and of an IPP homolog, 3-propylbut-3-enyl diphosphate **2c** using a thermophilic FPP synthase.

E-3-methylhex-2-enyl diphosphate **1a** was effectively accepted as a substrate with **2c** to give the sex pheromone homolog, **3c-OH** as a single product with a yield of 9.6%. Although *Z*-3-methylhex-2-enyl diphosphate (**1b**) was poorly accepted as a substrate under the standard reaction conditions, the yield of the (2*E*,6*Z*)-isomer (**3′b-OH**) of the pheromone could be improved by changing the conditions of the enzymatic reaction to 0.6% as shown in Scheme 2.

Thus, the thermostable FPP synthase reaction can be applicable to the one-pot syntheses of the sex pheromone homologs. It is interesting to test the biological activities of these homologs from the standpoint of structure–activity relationships.

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