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# Substrate Specificities of Several Prenyl Chain Elongating Enzymes with Respect to 4-Methyl-4-pentenyl Diphosphate

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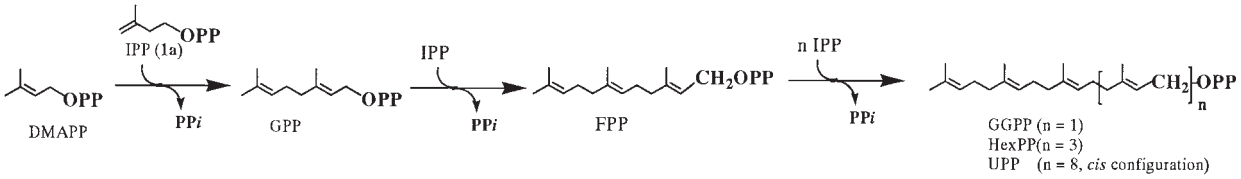
**In order to develop synthetic methods for biologically active homoallylic terpene sulfates, we examined the applicability and substrate specificities of several prenyl chain elongating enzymes with respect to 4-methyl-4-pentenyl diphosphate (homoIPP).**

**The reaction of dimethylallyl diphosphate with homoIPP by use of *Bacillus stearothermophilus* (all-*trans*)-farnesyl diphosphate synthase resulted in efficient yields of *cis*-(yield: 45.9%) and *trans*-4,8-dimethylnona-3,7-dien-1-ol (homoGOH, 25.5%), which has a carbon skeleton of 4,8-dimethylnona-3-en-1-sulfate, an antiproliferative compound from a marine organism (Aiello, A. *et al.*, *Tetrahedron*, 53, 11489–11492 (1997)). The homoIPP was found to be also active as a homoallylic substrate in place of isopentenyl diphosphate for *Sulfolobus acidocaldarius* geranylgeranyl diphosphate synthase to give diphosphate of *cis*- and *trans*-4,8,12-trimethyltrideca-3,7,11-trien-1-ol, for *Micrococcus luteus* B-P 26 hexaprenyl diphosphate synthase to give *cis*- and *trans*-4,8,12,16-tetramethylheptadeca-3,7,11,15-tetraen-1-ol (homoGGOH), and for *Micrococcus luteus* B-P 26 undecaprenyl diphosphate synthase to give *cis*-homoGGOH exclusively.**

**Key words:** prenyl chain elongating enzyme; farnesyl diphosphate synthase; geranylgeranyl diphosphate synthase; undecaprenyl diphosphate synthase; substrate specificity

The enzymatic prenyl chain elongating reaction proceeds with the condensation of an allylic primer substrate with isopentenyl diphosphate (IPP, **1a**) stereospecifically. The condensation terminates precisely when the elongation of the prenyl chain reaches a certain length according to the specificities of individual synthases, as shown in Scheme 1. These prenyl chain elongating enzymes can be classified into four classes according to mode of subunit composition as well as chain length and the stereochemistry of the final products.[1–3)]




**Scheme 1.** Prenyl Chain Elongating Enzymatic Reactions.

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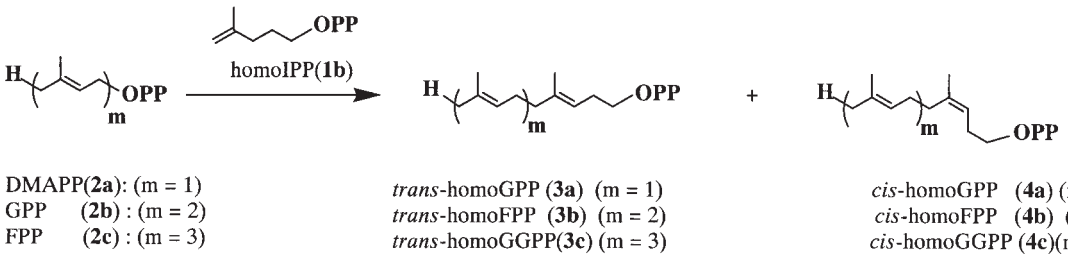
*Abbreviations*: FPP, farnesyl diphosphate; IPP, isopentenyl diphosphate; UPP, undecaprenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate

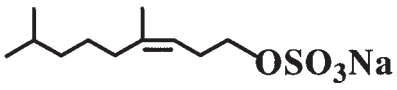



DMAPP(**2a**): (m = 1)
GPP (**2b**) : (m = 2)
FPP (**2c**) : (m = 3)

*trans*-homoGPP (**3a**) (m = 1)
*trans*-homoFPP (**3b**) (m = 2)
*trans*-homoGGPP(**3c**) (m = 3)

*cis*-homoGPP (**4a**) (m = 1)
*cis*-homoFPP (**4b**) (m = 2)
*cis*-homoGGPP (**4c**)(m = 3)

**Scheme 2.** Prenyl Chain Elongating Enzymatic Reactions between Allylic Substrate and HomolPP.
Porcine lever FPP synthase has been shown to produce **4a** or **4b** from the reaction with DMAPP or GPP, respectively.[7,8]




(**5**)

**Scheme 3.** Sodium *cis*-4,8-Dimethylnona-3-en-1-sulfate, an Antiproliferative Terpene from a Marine Organism.[9]

From the standpoint of application of the enzyme in organic syntheses, prenyl chain elongating enzymes have been shown to be useful in stereospecific syntheses of some kinds of biologically active compounds. Porcine liver farnesyl diphosphate (FPP) synthase reaction has been applied in the chiral syntheses of faranal[4] and 4-methyljuvenile hormone.[5] Undecaprenyl diphosphate (UPP) synthase from *Escherichia coli* has been applied to the one-pot synthesis of glycinoprenols.[6]

Ogura *et al.*[7,8] reported that 4-methyl-4-pentenyl diphosphate (homoIPP, **1b**) was accepted as a substrate in place of the homoallylic substrate, IPP, for a porcine liver FPP synthase, and that the product derived from the reaction of homoIPP with GPP (**2b**) or with DMAPP (**2a**) was exclusively *cis*-homofarnesyl-(**4b**) or *cis*-homogeranyl diphosphate (**4a**), respectively, as shown in Scheme 2.

Recently, antiproliferative terpene derivatives, which have homoallylic carbon skeletons such as *cis*-4,8-dimethylnona-3-en sodium sulfate (**5**), have been isolated from marine organisms such as the sea squirt,[9] as shown in Scheme 3.

In this study, in order to develop synthetic methods for this kind of biologically active homoterpene compounds, we examined the applicability and substrate specificities of several prenyl chain elongating enzymes with respect to an artificial IPP homolog, homoIPP.

## Materials and Methods

*Analysis.* Prenyl alcohols derived from the phosphatase treatment of products from prenyltransferase reactions were subjected to HPLC, a Hitachi type L-6000 equipped with Hitachi L-7420 (LaChrom) type UV–VIS detector with a Chromato Monitor (Nippon Filcon) and with a LichroCART (Merck) column with the eluents of solvent mixtures of hexane:2-propanol = 40:1 (A), or 80:1 (*v*/*v*) (B). Identification of the reaction products was carried out with a GC-MS, JMS-AM II 50 type GCG Mass spectrometer connected with a HP 5890 series II Gas chromatograph equipped with an Ultra-alloy-1 (S) column. 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. The relative yields of the prenyltransferase reactions, except for those of FPP synthase reactions, were calculated on the basis of the amount of recovered farnesol derived from the residual substrate FPP in the reaction mixture. The relative yields of FPP synthase reactions were those of the homologs of GPP and FPP derived from reactions with a set of substrates compared to the yield of FPP derived from the reaction with IPP and DMAPP or with IPP and GPP, respectively.

*Chemicals.*

*Synthesis of IPP-homolog.* 4-Methyl-4-pentenyl diphosphate[7,10] (homoIPP) was synthesized by diphosphorylation[11] of 4-methyl-4-pentene-1-ol (yield: 47.0%), which was prepared by reduction of 4-methyl-4-pentenoic acid[12] with $LiAlH_4$.

*Chemical synthesis of an authentic sample of 4,8,12, 16-tetramethylheptadeca-3,7,11,15-tetraen-1-ol (homoGGOH).* Cyanation with sodium cyanide of geranylgeranyl bromide, which was derived from bromination of geranylgeraniol by the use of phosphorus bromide, gave 4,8,12,16-tetramethylheptadeca-3,7,11,15-tetraenonitrile (homogeranylgeranyl cyanide) (yield: 74.0%). 4,8,12, 16-tetramethylheptadeca-3,7,11,15-tetraenoic acid (homogeranylgeranoic acid), which was derived from hydrolysis of homogeranylgeranyl cyanide, was reduced by the use of lithium aluminum hydride to give a 3-*cis* and 3-*trans* mixture of 4,8,12,16-tetramethylheptadeca-3,7,11,15-tetraen-1-ol (homoGGOH, yield: 16.1%). The $^1$H NMR (400 MHz, $CDCl_3$, TMS) of the *cis*- and *trans* mixture of homoGGOH was constituted as follows: $\delta$: 1.64 (16H, s.), 2.06 (12H, dt.), 2.29 (2H, dt.), 3.62 (2H,t), 5.10 (4H, s). The $^{13}$C NMR (100 MHz, $CDCl_3$, TMS) of the *cis*- and *trans* mixture of homoGGOH was constituted as follows: $\delta$: 15.9, 16.0, 17.7, 23.4, 25.7, 26.5, 26.6, 26.7, 27.5, 27.6, 31.9, 32.4, 39.7, 39.9, 63.8, 119.8, 123.6, 124.2, 124.4, 124.9, 129.0, 131.3, 135.0, 138.9. And the IR $\nu_{max}$ (KBr) cm$^{-1}$ of homoGGOH was

constituted as follows: 3352 (OH), 1048 (C–O). It has been shown for these homoprenyl alcohols that under the GC conditions of these homologs, the *cis*-isomers have a shorter retention time than that of the corresponding *trans*-isomer.[13] The retention times of the *cis*- and *trans*-homoGGOH on GC were 12.2 min and 12.3 min respectively, and the area ratio of these peaks was 3:7. The MS spectral data of *cis*-homoGGOH were as follows: *m/z*: 304 ($M^+$) (rel. int. 0.4%), 286 ($M^+ - 18$) (1.7), 217 ($M^+ - 18 - 69$) (2.4), 149 ($M^+ - 18 - 69 - 68$) (8.8), 81 ($M^+ - 18 - 69 - 68$) (54.1), 69 (base peak). And the MS spectral data of *trans*-homoGGOH were as follows: *m/z*: 304 ($M^+$) (rel. int. 0.7%), 286 ($M^+ - 18$) (1.9), 217 ($M^+ - 18 - 69$) (2.5), 149 ($M^+ - 18 - 69 - 68$) (9.2), 81 ($M^+ - 18 - 69 - 68$) (54.2), 69 (base peak).

Other homologs such as homogeraniol were synthesized similarly according to the synthetic method reported.[8]

Potato acid phosphatase was purchased from Boehringer Mannheim (Roche, grade II, catalog no. 108 227).

*Purification of prenyl chain elongating enzymes.*

*Purification of S. acidocaldarius GGPP synthase.* *E. coli* DH5α cells were transformed with pUC119 plasmid that contained an insert of the *S. acidocaldarius* GGPP synthase gene.[14] The colonies were used to inoculate LB medium containing ampicillin and incubated at 37 °C overnight. The cells were harvested and disrupted by sonication. The homogenate was heated at 55 °C for 60 min and then centrifuged at 10,000 × *g*. The supernatant fraction of *E. coli* DH5α/pGGPS3 was precipitated with a 30–60% saturation of $(NH_4)_2SO_4$. The precipitated protein fraction was dialyzed and purified by two chromatographies (Butyl-Toyopearl and MonoQ).[14–16] The purities of the GGPP synthase fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after staining with Coomassie Brilliant Blue.

*Purification of other enzymes.* *B. stearothermophilus* or porcine liver FPP synthase was purified according to the method reported previously.[13,17,18] HexPP- and UPP synthases from *M. luteus* B-P 26 were purified in the similar manner, as reported previously.[19,20]

*Conditions of the enzyme-catalyzed reaction.*

*Thermostable enzyme-catalyzed reactions.* The incubation mixture for the *S. acidocaldarius* GGPP synthase reaction contained, in a total volume of 1.0 ml, 100 mM of phosphate buffer (pH 5.8), 2.0 mM of $MgCl_2$, 10 mM of β-mercaptoethanol, 10 mM of $NH_4Cl$, 1.0 mM of an allylic substrate (GPP or FPP), 1.0 mM of IPP or homoIPP, and the recombinant GGPP synthase solution (200 μl). The *B. stearothermophilus* FPP synthase reaction was executed under conditions similar to those described for the GGPP synthase, except that 200 mM of Tris–HCl buffer (pH 8.5), 1.0 mM of IPP or homoIPP, 1.0 mM of an allylic substrate (DMAPP or GPP), and FPP synthase solution (25 μg, 100 μl) was used. After incubation at 55 °C for 6 h, the reaction mixture was treated with alkaline phosphatase for 5 h, and the reaction products were extracted with pentane and analyzed by HPLC and GC-MS.[13]

*Mesophilic enzymatic reactions.* The incubation mixture for *M. luteus* B-P 26 HexPP synthase reaction contained, in a total volume of 1.0 ml, 50 mM of Tris–HCl buffer (pH 8.5), 1.0 mM of $MgCl_2$, 20 mM of β-mercaptoethanol, 50 mM of $NH_4Cl$, 1.0 mM of FPP, 1.0 mM of IPP or homoIPP, and the recombinant HexPP synthase (76 and 90 μg of components A and -B, respectively).[20] HexPP synthase consists of two dissociable heterodimeric components A and -B, each of which lacks catalytic activity but is activated when combined with the other.[19] The *M. luteus* B-P 26 UPP synthase reaction was executed under similar conditions, as described previously.[21] The mixture was incubated at 37 °C for 6 h. After incubation, the reaction mixture was treated 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.

## Results and Discussion

In order to develop synthetic methods for biologically active terpenes such as antiproliferative compounds, we examined the substrate specificities with respect to homoIPP of several prenyl chain elongating enzymes.

*Reaction of DMAPP or GPP with homoIPP by use of FPP synthase*

*Reaction of DMAPP (**2a**) with homoIPP (**1b**) by use of FPP synthase.* The hydrolysates derived from the product of the *B. stearothermophilus* FPP synthase reaction of **1b** with DMAPP as substrate gave two peaks on HPLC (eluent B) at 29.3 and 31.1 min, which were then subjected to GC-MS. The MS spectrum of the former alcohol showed a molecular ion at *m/z* 168 (rel. int. 2.3%), corresponding to $C_{11}H_{20}O$, together with main fragment ions at *m/z* 150 ($M^+ - 18$) (4.6), 81 ($M^+ - 18 - 69$) (40.9), and 69 (base peak), indicating that the alcohol has a 4,8-dimethylnona-3,7-dien-1-ol (homogeraniol) structure. On the other hand, the spectrum of the latter alcohol showed the same molecular ion as well as similar fragment ions with some differences in relative intensity, which also indicates a homogeraniol structure.

In order to determine the geometry of the double bond at the 3-position, authentic *cis*- and *trans*-homogeraniol were synthesized from geranyl bromide *via* corresponding 4,8-dimethylnona-3,7-dienoic acid by lithium aluminum hydride reduction.

The authentic *cis*- and *trans*-homogeraniol eluted on HPLC (eluent A) had retention times of 16.2 and 17.4 min, respectively. These results indicate that the former, derived from the enzymatic reaction product, is

assignable to *cis*-homogeraniol (**4a-OH**), and the latter to the *trans*-isomer (**3a-OH**). The relative yields of **4a** and **3a** were 45.9% and 25.5% of that of GPP derived from the reaction with DMAPP and IPP.

Our re-experiment on the action of porcine liver FPP synthase-catalyzed reaction with IPP homolog (**1b**) confirmed exclusive formation of the *cis*-isomer, although enzyme activity was as low as 1% compared with that of the thermostable enzyme.

In 1997, Aiello *et al.* reported the isolation of an antiproliferative terpene sulfate from a Mediterranean ascidian.[9] This biologically active terpene has the same carbon skeleton to that of *cis*-homogeraniol. From the viewpoint of the possibility of synthetic application of FPP synthase to obtain the carbon skeleton of the antiproliferative terpene, the *B. stearothermophilus* FPP synthase appears to be more useful because of the large yield compared to that of the liver enzyme.

*Reaction of GPP with homoIPP by use of FPP synthase.* The alcohols derived from the reaction of the thermostable FPP synthase with homoIPP (**1b**) and GPP (**2b**) as substrates eluted on HPLC as two peaks at 35.5 (relative yield: 2.9%) and 36.9 min (relative yield: 2.6%), which were then purified and subjected to GC-MS. In the MS spectrum of the former product, the molecular ion was observed at $m/z$ 236 (rel. int.: 5.0%), corresponding to $C_{16}H_{28}O$, with other fragment ions at $m/z$ 218 ($M^+ - 18$) (5.4), 149 ($M^+ - 18 - 69$) (8.9), 81 ($M^+ - 18 - 69 - 68$) (62.4), and 69 (base peak), indicating that the alcohol has a 4,8,12-trimethyltrideca-3,7,11-trien-1-ol (homofarnesol) structure. The latter product also showed a molecular ion at $m/z$ 236 (rel. int.: 2.7%), and other fragment ions similar to those of the former product, at $m/z$ 218, 149, 81, and 69, for example, suggesting that the product has a homofarnesol structure.

As mentioned above, *cis*-homogeraniol eluted on the HPLC with a shorter retention time than that of the corresponding *trans*-isomer. The former product was assigned to *cis*-homofarnesol (**4b-OH**) and the latter to the *trans*-isomer (**3b-OH**).

When porcine liver FPP synthase was used, the *cis*-isomer (**4b-OH**, relative yield: 7%) was obtained exclusively, as reported previously.[7]

*Reactions of GPP or FPP with homoIPP by use of GGPP synthase*

*Reaction of GPP (**2b**) with homoIPP (**1b**) by use of S. acidocaldarius GGPP synthase.* The hydrolysate derived from acid phosphatase treatment of the product of the *S. acidocaldarius* GGPP synthase reaction of **1b** with GPP gave two peaks at 29.4 (relative yield: 17.6%) and 30.0 min (relative yield: 28.2%) on HPLC (eluent B). These were subjected to GC-MS analysis. The two alcohols showed similar MS spectra to those of the homofarnesols, **3b-OH** and **4b-OH**, indicating that the former is the *cis*-homofarnesol (**4b-OH**) and the latter the *trans*-isomer (**3b-OH**).

*Reaction of FPP (**2c**) with homoIPP (**1b**) by use of S. acidocaldarius GGPP synthase.* The alcohols derived from phosphatase treatment of the products of the *S. acidocaldarius* GGPP synthase reaction of **1b** with FPP gave two peaks on GC at 12.1 (relative yield: 6.3%) and 12.2 min (relative yield: 11.2%). These two products were found to be identical with those of authentic *cis*- and *trans*-homogeranylgeraniol respectively. Hence the former was identified as *cis*-(**4c-OH**) and the latter as *trans*-homogeranylgeraniol (**3c-OH**).

*Reaction of FPP with homoIPP by use of HexPP synthase or UPP synthase*

*Reaction of FPP (**2c**) with homoIPP (**1b**) by use of M. luteus B-P 26 HexPP synthase.* The alcohols derived from the products of the *M. luteus* B-P 26 HexPP synthase reaction of **1b** with FPP gave two peaks on GC at 12.2 (relative yield: 3.5%) and 12.3 min (relative yield: 5.2%). Both of the MS spectra of these products exactly coincided with the authentic *cis*-(**4c-OH**) and *trans*-homogeranylgeraniol (**3c-OH**) respectively.

*Reaction of FPP (**2c**) with homoIPP (**1b**) by use of M. luteus B-P 26 UPP synthase.* The hydrolysate derived from acid phosphatase treatment of the product of the *M. luteus* B-P 26 UPP synthase reaction of **1b** with FPP gave a peak on GC at 12.2 min (relative yield: 2.0%). The GC-MS spectrum of the product gave a similar MS spectrum to that of *cis*-homogeranylgeraniol (**4c-OH**), indicating that the enzymatic product was *cis*-geranylgeranyl diphosphate (**4c**).

## Conclusion

Scheme 2 illustrates the prenyl chain elongating reactions with respect to homoIPP as the non-allylic substrate catalyzed by the prenyltransferases examined in this study. The relative yields of the homoallylic alcohols obtained by phosphatase treatment of each prenyltransferase reaction product are shown in Table 1.

By obtaining 3-*cis*-4,8-dimethylnona-3,7-dien-1-ol (**4a-OH**) derived from a large-scale enzymatic reaction by thermostable FPP synthase, it could be possible to synthesize the antiproliferative homoterpene sulfate by Sharpless epoxidation[22] of the corresponding THP ether obtained by the use of PPTS[23] for the protection of the double bond at C-3 and by hydrogenation at C-7, and then reduction of epoxide to olefine *via* iodohydrin,[24,25] followed by sulfuric esterification.[26]

## Acknowledgment

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**Table 1.** Relative Yields of the Homoallylic Alcohols Derived from Prenyltransferase Reactions with HomoIPP as Non-allylic Substrate

| Prenyltransferase | Allylic substrate | Product homoallylic alcohols derived from phosphatase treatment of each enzymatic product (%)* | | | | | |
|---|---|---|---|---|---|---|---|
| | | **3aOH** | **3bOH** | **3cOH** | **4aOH** | **4bOH** | **4cOH** |
| FPP synthase (porcine liver) | DMAPP | n.d. | — | — | 0.1 | — | — |
| | GPP | — | n.d. | — | — | 7.4 | — |
| FPP synthase (*B. stearothermophilus*) | DMAPP | 25.5 | — | — | 45.9 | — | — |
| | GPP | — | 2.6 | — | — | 2.9 | — |
| GGPP synthase (*S. acidocaldarius*) | GPP | — | 1.7 | — | — | 1.1 | — |
| | FPP | — | — | 11.2 | — | — | 6.3 |
| HexPP synthase (*M. luteus* B-P 26) | FPP | — | — | 5.2 | — | — | 3.5 |
| UPP synthase (*M. luteus* B-P 26) | FPP | — | — | n.d. | — | — | 2.0 |

*The relative yields of product alcohols derived from reactions by FPP synthase were relative to the yields of FOH derived from the corresponding enzymatic reactions with IPP and DMAPP (or GPP). The relative yields of product alcohols derived from reactions by GGPP synthase were relative to the yields of GGOH derived from the corresponding enzymatic reactions with IPP and FPP. The relative yields of product alcohols derived from reactions by HexPP synthase or UPP synthase were calculated on the basis of the amount of recovered farnesol derived from the residual substrate FPP in the reaction mixture.
n.d., not detected.



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