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UNDECAPRENYL DIPHOSPHATE SYNTHASE REACTION WITH ARTIFICIAL SUBSTRATE HOMOLOGUES ------- NOVEL BEHAVIOR IN THE TERMINATION OF PRENYL CHAIN ELONGATION

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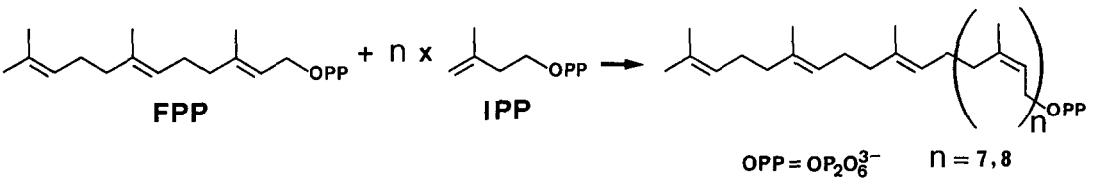
Abstract: (E)-3-Methyl-3-pentenyl diphosphate acted as an artificial homoallylic substrate in the reaction with several allylic diphosphates catalyzed by undecaprenyl diphosphate synthase of Bacillus subtilis. The synthase reaction with the artificial substrate proceeded in the same stereochemical manner as that with the natural homoallylic substrate, isopentenyl diphosphate, but it had a full stop at the stage where a single condensation of the C_6-homologue with an allylic primer is completed to form a chiral prenyl diphosphate with an extra methyl group at the 4-position. Allylic diphosphates that each have an extra methyl group at the 4-position were not accepted as substrates for this enzyme even when isopentenyl diphosphate was the homoallylic substrate.

INTRODUCTION

Prenyltransferases are the enzymes that catalyze the chain elongation by sequential condensation of IPP[1] with allylic diphosphates to give prenyl diphosphates with certain chain lengths and configurations. These enzymatic reactions are unique and attractive from both mechanistic and synthetic viewpoints because the polymerization of isoprene units proceeds stereospecifically and terminates precisely until the prenyl chains reach certain lengths depending on the specificities of enzymes.[2] Undecaprenyl diphosphate synthase [EC 2.5.1.31] catalyzes the oligomerization of eight isoprene units

with FPP as the priming substrate to give the C_{55} prenyl diphosphate with Z,E-mixed stereochemistry as shown in Scheme I.



Scheme I: Reaction catalyzed by undecaprenyl diphosphate synthase.

Studies on the substrate specificity of prenyltransferases with artificial substrate homologues have been developed extensively with respect to mammalian liver farnesyl diphosphate synthase. The structural requirement for allylic diphosphate is not very stringent, but as many as 40 allylic substrate homologues have been found to condense with IPP.[3] On the other hand, the requirement for the homoallylic substrate is so rigorous that only several artificial homologues of IPP have so far been known to act as substrates for farnesyl diphosphate synthase.[4] Some of the enzymatic reactions with the artificial homoallylic substrates, however, have been successfully applied to chiral syntheses of insect substances with biological activities.[5]

These results have prompted us to investigate the reactivities of artificial substrates in the reactions catalyzed by other prenyltransferases. It is of particular interest to study from these viewpoints undecaprenyl diphosphate synthase reaction, which is the C-C bond formation leading to (Z)-polyprenyl chains.

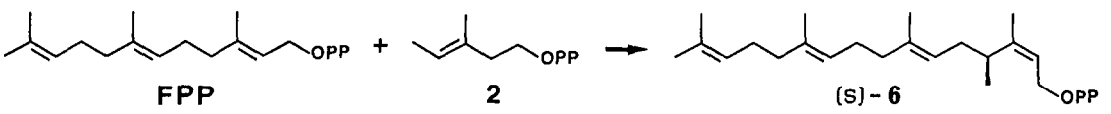
This paper describes the substrate specificity of undecaprenyl diphosphate synthase studied with artificial homoallylic diphosphates and artificial allylic diphosphates each having an extra methyl group at the 4-position. Part of the study with artificial homoallylic diphosphates has been reported as a preliminary communication.[6]

RESULTS

Reaction of homoallylic substrate homologues. The substrate specificity of undecaprenyl diphosphate synthase was examined at first with the artificial homoallylic diphosphates that have been shown to act as substrates for farnesyl diphosphate synthase.[4] Among the five homologues tested, 3-ethyl-3-butenyl- (**1**), (E)-3-methyl-3-pentenyl- (**2**), (Z)-3-methyl-3-pentenyl- (**3**), 4-methyl-4-pentenyl- (**4**) and 2-(cyclopenten-1-yl)ethyl diphosphate (**5**), only **2** was found to act as a homoallylic substrate instead of IPP to condense with (all-E)-FPP as the allylic priming substrate.

Enzymatic reactions with the artificial substrates were examined under the usual conditions using an undecaprenyl diphosphate synthase fraction prepared from Bacillus subtilis cells.[7] After incubation of **2** and [1-^{14}C]-(all-E)-FPP with the enzyme, the reaction product was treated with acid phosphatase as usual,[8] and the hydrolysate was chromatographed on silica gel TLC. The radio-TLC showed two radioactivity peaks attributable to a new product and the farnesol recovered from the primer substrate. The Rf value of the new product (0.50) was larger than that of (all E)-geranylgeraniol (0.36) but smaller than that of (all-E)-decaprenol (0.53). The mass spectrum of this alcohol exhibited peaks at m/z 304 (M, $C_{21}H_{36}O$), 286 (M-18), 217 (M-18-69), 149 (M-18-69-68), 81 and 69 (base peak), indicating that the alcohol was a 4-methyl derivative of geranylgeraniol. In order to determine the geometry of the newly formed double bond at the 2-position, we synthesized (all-E)- and (2Z,6E,10E)-4-methylgeranylgeraniols from 1-methyl-1-(all-E)-farnesyl-2-propanone. The alcohol derived from the enzymatic reaction cochromatographed (Rf, 0.50) exactly with the 2Z,6E,10E-isomer, which migrated faster (Rf, 0.50) than its all-E isomer (Rf, 0.43). For determination of the absolute configuration at the 4-position, the alcohol obtained by incubation of preparative scale was subjected to ozonolysis. The 3-methyllevulinic acid showed a negative Cotton curve with $[\theta]_{280} = -3600 \pm 700^{\circ}$. Authentic specimens of both (R)- and (S)-3-methyllevulinic acid were prepared by ozonolysis of (S)- and (R)-4-methylfarnesol, respectively, synthesized by the farnesyl diphosphate synthase reaction with **2**.[9] The authentic (R)-3-methyllevulinic acid showed a CD spectrum with $[\theta]_{280} = -3200 \pm 200^{\circ}$, which coincided with

the value of the acid derived from the product of undecaprenyl diphosphate synthase reaction. As we have demonstrated that both (S)- and (R)-4-methylfarnesols synthesized by the farnesyl diphosphate synthase method are of 100% enantiomeric purity,[10] the results described above indicate that the product of the undecaprenyl diphosphate synthase reaction of **2** with (all-E)-FPP is (S)-(2Z,6E,10E)-4-methylgeranylgeranyl diphosphate (**6**) with an enantiomeric excess of 100% within experimental error (Scheme II). The yield of **6** based on the allylic substrate, [1-^{14}C]-(all-E)-FPP was 11.4%. No product other than **6** was detected.



Scheme II: Undecaprenyl diphosphate synthase reaction with **2** and (all-E)-FPP as substrates.

Reactivity of **2** with various allylic substrates. It is of particular interest that the introduction of a methyl group at the 4 position of IPP causes the undecaprenyl diphosphate synthase reaction to have a full stop at the stage where a single condensation of the C_6-homologue **2** with (all-E)-FPP is completed to form a chiral C_{21} compound. In order to see whether the reaction of **2** always results in such a single condensation regardless of the structures of allylic primers, enzymatic reactions of tritium labelled **2** with various allylic substrates were examined. Among the allylic diphosphates tested, (all-E)-GGPP condensed with **2** most actively (Table I). It is notable that (2Z,6E,10E)-GGPP, which reacts with IPP as actively as (all-E)-FPP or (all-E)-GGPP, showed only a poor activity in the condensation with the artificial substrate **2**.

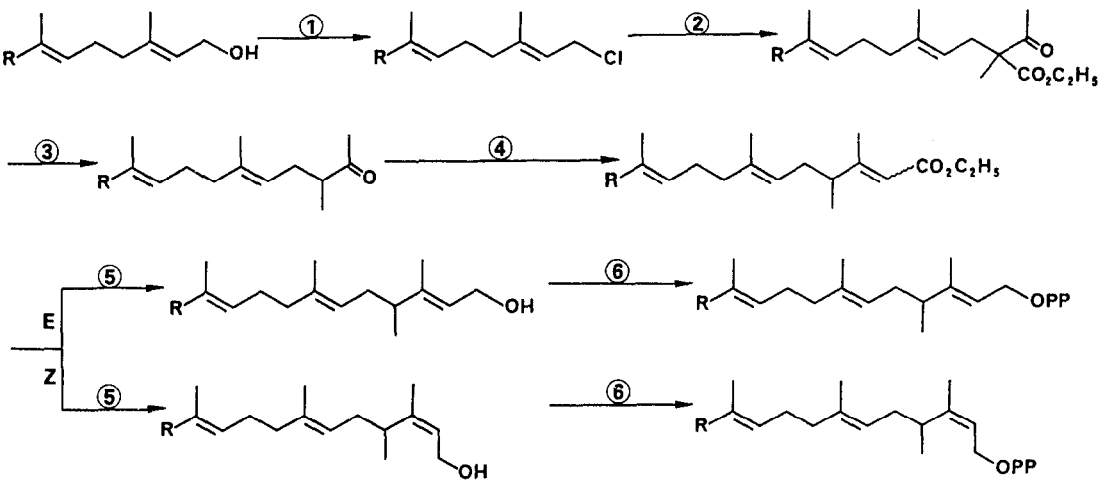
Table I: Reactivities of **2** with various allylic substrates. The enzymatic reactions were carried out as described in EXPERIMENTAL.

Substrate	Enzymatic activity (dpm)
(all-E)-FPP	8049
(2Z,6E)-FPP	0
(all-E)-GGPP	30378
(2Z,6E,10E)-GGPP	2250
(all-E)-FGPP	5583
(2Z,6E,10E,14E)-FGPP	7935
(2Z,6Z,10E,14E)-FGPP	13365

A single radioactivity peak was observed in the radio-TLC of the alcohol obtained by acid phosphatase treatment of the enzymatic product derived from (all-E)-GGPP and [1-^{3}H]-**2**. The mass spectrum of the product alcohol exhibited peaks at m/z 372 (M, $C_{26}H_{44}O$), 354 (M-18), 285 (M-18-69), 217 (M-18-69-68), 149 (M-18-69-2x68), 81 and 69 (base peak). Thus, the alcohol was proved to be a 4-methyl derivative of C_{25}-prenol. This indicates that the undecaprenyl diphosphate synthase reaction starting with (all-E)-GGPP also stops at the stage of single condensation of the C_6-homologue. Moreover, on the radio-TLC (reversed phase KC-18; Acetone/MeOH, 9/1), the alcohols derived from the products formed by the synthase reactions of [1-^{3}H]-**2** with (2Z,6E,10E,14E)-FGPP and with (2Z,6Z,10E,14E)-FGPP showed single radioactivity peaks with Rf values of 0.69 and 0.68, respectively, the latter being identical with that of authentic (2Z,6Z,10Z,14E, 18E)-hexaprenol (betulaprenol-C_{30}). These facts indicate that the synthase reaction of **2** always stops at the stage where a single condensation of **2** with an allylic diphosphate is completed without regard to the chain length of priming allylic substrates.

Reactivity of various allylic diphosphates each having an extra methyl group at the 4-position. In order to find the reason why the synthase reaction of **2** always stops at a single condensation, we examined the reactivities of various allylic diphosphates that

each have an extra methyl group at the 4-position. (RS)-4-Methylgeranyl- (**7**), (RS)-(all-E)-4-methylfarnesyl- (**8**), (RS)-(2Z,6E)-4-methylfarnesyl- (**9**), (RS)-(all-E)-4-methylgeranylgeranyl- (**10**) and (RS)-(2Z,6E,10E)-4-methylgeranylgeranyl diphosphate (**6**) were synthesized according to Scheme III, and their reactivities were examined with



Scheme III: Synthesis of 4-methyl-prenyl diphosphates.

Key: ①, N-chlorosuccinimide; ②, ethyl 2-methylacetoacetate, sodium ethoxide; ③, 1)KOH, 2)HCl; ④, 1)diethyl ethoxycarbonylmethylphosphonate, NaH 2)HPLC; ⑤, $LiAlH_4$; ⑥, tris(tetrabutylammonium)hydrogen pyrophosphate.

[1-^{14}C]IPP as usual. As shown in Table II, the artificial homologues that have methyl groups at the 4-position showed little activities. In particular, neither **6** nor **9**, which has a Z-double bond at the 2-position, is active, whereas **8** or **10**, which has an E-double bond at the 2-position, shows a low but significant reactivity.

Table II: Reactivities of various allylic diphosphates as substrates for undecaprenyl diphosphate synthase. The incubations were carried out as described in EXPERIMENTAL.

Substrate	Enzymatic Activity (dpm)
Geranyl-PP[a]	1629
4-Methylgeranyl-PP (**7**)	0
(all-E)-FPP	10278
(2Z,6E)-FPP	6243
(all-E)-4-Methyl-FPP (**8**)	933
(2Z,6E)-4-Methyl-FPP (**9**)	78
(all-E)-GGPP	16200
(2Z,6E,10E)-GGPP	12825
(all-E)-4-Methyl-GGPP (**10**)	345
(2Z,6E,10E)-4-Methyl-GGPP (**6**)	0

a, PP stands for diphosphate

DISCUSSION

The formation of (S)-(2Z,6E,10E)-4-methylgeranylgeranyl diphosphate (**6**) indicates that the enzymatic condensation between **2** and FPP proceeds in the same stereochemical manner as that between the natural substrates, IPP and (all-E)-FPP.[11] The mode of termination of the chain elongation, however, is quite different from that between the natural substrates. The undecaprenyl diphosphate synthase reaction of **2** with FPP has a full stop at the stage where a single condensation of the C_6-unit with the allylic primer is completed to form a chiral C_{21} product. This mode of condensation of **2** seems to be common to all cases regardless of the chain length of allylic priming substrates. The reaction of **2** with (all-E)-GGPP gave a 4-methyl derivative of C_{25}-prenyl diphosphate. Moreover, the enzymatic reaction of **2** with either (2Z,6E,10E,14E)- or (2Z,6Z,10E,14E)-FGPP resulted in the formation of a prenyl diphosphate whose hydrolysate showed an Rf value similar to that of C_{30}-betulaprenol on reversed phase TLC. These facts indicate that the introduction of a methyl group at the 4-position of IPP always

causes the undecaprenyl diphosphate synthase reaction to have a full stop at the stage where a single condensation of the C_6-homologue with any allylic priming substrates is completed to form a chiral prenyl diphosphate having an extra methyl group at the 4-position. This catalytic function may be synthetically useful as shown previously in the case of FPP synthase.[5] Although all of the absolute configurations of the products formed by the condensation between **2** and the allylic diphosphates examined have not been determined, it is plausible that they all have the S-2Z-structure as demonstrated for **6**.

None of the allylic diphosphates each having a Z-double bond at the 2-position and an extra methyl group at the 4-position was accepted as substrate for undecaprenyl diphosphate synthase even when IPP was the homoallylic substrate. Consequently, it should be thought that the 4-methyl group of the allylic diphosphate rather than that of the homoallylic diphosphate is responsible for the full stop of the synthase reaction of **2**. Kinetic studies revealed that the allylic homologue **6** is a potent competitive inhibitor against (2Z,6E,10E)-GGPP with a Ki value comparable to the Km value of the natural substrate. This fact indicates that the presence of a methyl group at the 4-position of 2Z-allylic substrate makes it impossible for the compound to fit the binding site for the allylic substrate suitably enough to lead to a productive enzyme-substrate complex, though the compound has a strong affinity for the same site. The topology of the binding cavity of undecaprenyl diphosphate synthase will be discussed elsewhere on the basis of detailed kinetic studies with these artificial substrates.

EXPERIMENTAL

Materials. [1-^{14}C]IPP was purchased from Amersham. Geranyl diphosphate, (all-E)-FPP, (2Z,6E)-FPP, (all-E)-GGPP, (2Z,6E,10E)-GGPP, (all-E)-FGPP, (2Z,6E,10E,14E)-FGPP, (2Z,6Z,10E,14E)-FGPP, 3-ethyl-3-butenyl- (**1**),(E)-3-methyl-3-pentenyl- (**2**), [1-^{3}H]-(**2**), (Z)-3-methyl-3-pentenyl- (**3**), 4-methyl-4-pentenyl- (**4**) and 2-(cyclopenten-1-yl)ethyl diphosphate (**5**) were the same preparations as used previously.[7,9] [1-^{14}C]-(all-E)-FPP was prepared from [1-^{14}C]IPP and dimethylallyl diphosphate by the action of pig liver FPP synthase.

Cells of Bacillus subtilis were obtained according to the procedure described by Fujii et al.[12] Acid phosphatase was a product of Boehringer Mannheim. Dowex AG 50W-X8 cation-exchange resin (100-200 mesh) was purchased from Muromachi Chemicals Co. Ltd.

Reagent grade anhydrous diethyl ether was distilled from $LiAlH_4$. Anhydrous ethanol and benzene were distilled from Mg and Na, respectively. Other solvents were of reagent grade. Dimethylallylbromide, ethyl 2-methylacetoacetate, diethyl ethoxycarbonylmethylphosphonate, dimethyl sulfide and tetrabutylammonium hydroxide were purchased from Tokyo Kasei Kogyo, Co Ltd. Sodium ethoxide, N-chlorosuccinimide and disodium dihydrogen pyrophosphate were obtained from Nacalai Tesque Inc. Pre-coated silica gel TLC plates and $LiAlH_4$ were purchased from Merck. Sodium hydride was a product of Aldrich. Cellulose powder (CF 11) was obtained from Whatman Inc. Undecaprenyl diphosphate synthase was partially purified from Bacillus subtilis cells according to the procedure of Takahashi et al.[7] and it was confirmed that the synthase fraction was free of any other prenyltransferases.

Ethyl 2-acetyl-2,5-dimethyl-4-hexenoate. A solution of dimethylallyl bromide (6.73 g, 45 mmol) in 30 ml of absolute ethanol was added dropwise to a solution of the sodium compound of ethyl 2-methylacetoacetate [ethyl 2-methylacetoacetate, 7.81 g (54 mmol), sodium ethoxide, 4.90 g, (72 mmol) and absolute ethanol, 170 ml] under a nitrogen atmosphere at 0°C. After stirring at room temperature for 12 h, water was added and the mixture was extracted with hexane. The hexane extract was washed in turn with saturated solutions of NH_4Cl and of NaCl, and then dried over $MgSO_4$; yield, 9.61g, 45mmol (100%); ^{1}H-NMR ($CDCl_3$), δ (ppm) 1.26 (3H, t, $J_{H,H}$= 7.0 Hz), 1.31 (3H, s), 1.61 (3H, s), 1.68 (3H, s), 2.14 (3H, s), 2.54 (2H, d, $J_{H,H}$= 7.4 Hz), 4.19 (2H, q, $J_{H,H}$= 7.0 Hz), 4.95 (1H, t, $J_{H,H}$= 7.4 Hz).

3,6-Dimethyl-5-hepten-2-one. Ethyl 2-acetyl-2,5-dimethyl-4-hexenoate (5.68 g, 26.8 mmol) was added to a solution of NaCl (3.13 g, 53 mmol), dimethylsulfoxide (60 ml) and H_2O (1.42 ml, 78 mmol), and the mixture was stirred at 170°C for 5 h. The resulting mixture was extracted with hexane and the organic layer was washed with saturated NaCl solution and dried over $MgSO_4$. After removal of the solvent, the residue was purified by silica gel chromatography to give 3,6-dimethyl-5-hepten-2-one (527.6 mg, 3.8 mmol) and the recovered ester (2.9 g, 13.7 mmol).

Ethyl (RS)-(E)- and (RS)-(Z)-3,4,7-trimethyl-2,6-octadienoate. To a suspention of NaH (1.7 g, 71 mmol) in dry benzene, diethyl ethoxycarbonylmethylphosphonate (13.3 g, 58 mmol) was added. Then 3,6-dimethyl-5-hepten-2-one (1.66 g, 11.8 mmol) was added, and the mixture was stirred at room temperature for 1 h and finally refluxed at 65°C overnight. Water was added and the mixture was extracted with hexane. After removal of the solvent of the extract by evaporation, the residue was distilled under reduced pressure to give a mixture of ethyl (RS)-3,4,7-trimethyl-2,6-octadienoate; yield, 2.15 g. The E and Z isomers (E/Z=10/1) of the ester were separated from each other by silica gel chromatography with a solvent system of hexane/ethyl acetate, 25/1: ^{1}H-NMR; Ethyl (RS)-(E)-3,4,7-trimethyl-2,6-octadienoate δ(ppm) 1.02 (3H, d, $J_{H,H}$= 7.0 Hz), 1.26

(3H, t, $J_{H,H}$= 7.20 Hz), 1.58 (3H, s), 1.66 (3H, s), 2.0 (3H, m), 2.10 (3H, d, $J_{H,H}$= 2.0 Hz), 4.15 (2H, q, $J_{H,H}$= 7.2 Hz), 5.0 (1H, m), 5.64 (1H, d, $J_{H,H}$= 2.0 Hz); Ethyl (RS)-(Z)-3,4,7,-trimethyl-2,6-octadienoate δ(ppm) 1.02 (3H, d, $J_{H,H}$= 7.0 Hz), 1.26 (3H, t, $J_{H,H}$= 7.20 Hz), 1.58 (3H, s), 1.66 (3H, s), 1.76 (3H, d, $J_{H,H}$= 2.0 Hz), 2.0(3H, m), 4.15 (2H, q, $J_{H,H}$= 7.2 Hz), 5.0 (1H, m), 5.64 (1H, d, $J_{H,H}$= 2.0 Hz).

(RS)-(E)-4-Methylgeraniol. Ethyl (RS)-(E)-3,4,7-trimethyl-2,6-octadienoate (520 mg, 2.47 mmol) was added under nitrogen atmosphere to a suspension of $LiAlH_4$ (212 mg, 5.6 mmol) in diethyl ether (50 ml), and the mixture was stirred at 0°C for 1 h. The reaction mixture was worked up routinely, and the product was purified by silica gel chromatography with hexane/ethyl acetate, 5/1; yield, 323 mg, 1.92 mmol (78%); ^{1}H-NMR ($CDCl_3$), δ(ppm) 0.99 (3H, d, $J_{H,H}$= 7.0 Hz), 1.6 (9H, m), 2.0 (3H, m), 4.16 (2H, d, $J_{H,H}$= 7.0 Hz), 5.0 (1H, m), 5.41 (1H, t, $J_{H,H}$= 7.0 Hz).

(RS)-(E)-4-Methylgeranyl diphosphate (7). Diphosphorylation of (RS)-(E)-4-methylgeraniol was carried out essentially according to the method of Davisson *et al.*[13] except that silica gel flash chromatography with a solvent system of propanol / NH_4OH / H_2O (6/3/1) was employed prior to the chromatography on cellulose for purification of the diphosphate. Detection of phosphate esters on silica gel TLC plates was carried out with Zinzadze reagent[14]: yield, 291 mg, 0.77 mmol (90%).

Ethyl 2-geranyl-2-methyl-3-oxobutanoate. A solution of geranyl chloride (5.0 g, 28.9 mmol) in 30 ml of absolute ethanol was added dropwise to a solution of the sodium compound of ethyl 2-methylacetoacetate (ethyl 2-methylacetoacetate, 4.59 g, 31.0 mmol; sodium ethoxide, 2.95 g, 43.4 mmol; absolute ethanol, 170 ml) under nitrogen atmosphere at 0°C, and the mixture was stirred at room temperature for 12 h. After addition of a small volume of water, the mixture was treated with hexane. The extracts were washed in turn with saturated solutions of NH_4Cl and of NaCl, and then dried over $MgSO_4$. The solvent was removed by evaporation and the residue was chromatographed on a column of silica gel (2 x 25 cm) with a mixture of hexane/ethyl acetate (10/1) to give 6.75 g (83.4%) of ethyl 2-geranyl-2-methyl-3-oxobutanoate; ^{1}H-NMR ($CDCl_3$), δ(ppm) 1.26 (3H, t, $J_{H,H}$= 7.2 Hz), 1.30 (3H, s), 1.6 (9H, m), 2.0 (4H, m), 2.14 (3H, s), 2.54 (2H, d, $J_{H,H}$= 7.5 Hz), 4.19 (2H, q, $J_{H,H}$= 7.2 Hz), 5.0 (2H, m).

3-Geranyl-2-butanone. After a mixture of ethyl 2-geranyl-2-methyl-3-oxobutanoate (6.54 g, 23.2 mmol), 2 mol dm^{-3} KOH (150 ml) and ethanol (25 ml) was stirred at room temperature overnight, the mixture was acidified with HCl and then extracted with diethyl ether. The ether extracts were worked up routinely to give 3-geranyl-2-butanone (4.82 g, 99%); ^{1}H-NMR ($CDCl_3$), δ(ppm) 1.08 (3H, d, $J_{H,H}$= 6.8 Hz), 1.6 (9H, m), 2.0 (6H, m), 2.14 (3H, s), 2.56 (1H, q, $J_{H,H}$= 6.8 Hz), 5.0 (2H, m).

Ethyl (RS)-(all-E)- and ethyl (RS)-(2Z,6E)-4-methylfarnesate. To a suspension of NaH (1.06 g, 44 mmol) in dry benzene was added diethyl ethoxycarbonylmethylphosphonate (5.26 g, 23 mmol). Then 3-geranyl-2-butanone (4.42 g, 21.3 mmol) was added to the solution. After the mixture was stirred at room temperature for 1 h and then at 65°C overnight, small amounts of water and hexane were added, and the mixture was shaken. The organic layer was treated as usual to give an E/Z mixture (22/3) of ethyl 4-methylfarnesate (1.50 g, 24%). The E and Z isomers were separated from each other by HPLC on silica gel with hexane/ethyl acetate, 200/1: ^{1}H-NMR; Ethyl (RS)-(all-E)-4-methylfarnesate δ (ppm) 1.03 (3H, d, $J_{H,H}$= 6.1 Hz), 1.26 (3H, t, $J_{H,H}$= 7.0 Hz), 1.6 (9H, m), 2.0 (7H, m), 2.11 (3H, d, $J_{H,H}$= 2.0 Hz), 4.12 (2H, q, $J_{H,H}$= 7.0 Hz), 5.0 (2H, m), 5.65 (1H, d, $J_{H,H}$= 2.0 Hz); Ethyl (RS)-(2Z,6E)-4-methylfarnesate δ (ppm) 1.03 (3H, d, $J_{H,H}$= 6.1 Hz), 1.26 (3H, t, $J_{H,H}$= 7.0 Hz), 1.6 (9H, m), 1.79 (3H, d, $J_{H,H}$= 2.0 Hz), 2.0 (7H, m), 4.12 (2H, q, $J_{H,H}$= 7.0 Hz), 5.0 (2H, m), 5.62 (1H, d, $J_{H,H}$= 2.0 Hz).

(RS)-(all-E)-4-Methylfarnesol. Ethyl (RS)-(all-E)-4-Methylfarnesate (194.2 mg, 0.7 mmol) was added under nitrogen atmosphere to a suspension of $LiAlH_4$ (55.7 mg, 1.4 mmol) in diethyl ether (10 ml), and the mixture was stirred at 0°C for 1 h. The reaction mixture was worked up routinely, and the product was purified by silica gel chromatography with hexane/ethyl acetate, 5/1; yield, 152.7 mg (93%): ^{1}H-NMR ($CDCl_3$) δ (ppm) 0.99 (3H, d, $J_{H,H}$= 6.3 Hz) 1.6 (12H, m), 2.0 (7H, m), 4.15 (2H, d, $J_{H,H}$= 6.8 Hz), 5.1 (2H, m), 5.40 (1H, t, $J_{H,H}$= 6.8 Hz); MS m/z 236 (1.5), 218 (1.5), 205 (3.5), 175 (2), 149 (5), 136 (6), 121 (6), 107 (10), 93 (12), 81 (38), 69 (100).

(RS)-(all-E)-4-Methylfarnesyl diphosphate (**8**). Diphosphorylation of (RS)-(all-E)-4-methylfarnesol was carried out essentially according to the procedure described above for the synthesis of **7**; yield, 450 mg, 1.01 mmol (90%).

(RS)-(2Z,6E)-4-Methylfarnesol. Ethyl (RS)-(2Z,6E)-4-methylfarnesate (104 mg, 0.37 mmol) was reduced in the same way as described above for the preparation of the 2E-isomer; yield, 75.7 mg, 0.32 mmol, (86%); ^{1}H-NMR ($CDCl_3$) δ (ppm) 1.00 (3H, d, $J_{H,H}$= 6.8 Hz), 1.6 (12H, m), 2.0 (7H, m), 4.08 (2H, d, $J_{H,H}$= 6.8 Hz), 5.1 (2H, m), 5.42 (1H, t, $J_{H,H}$= 6.8 Hz); MS m/z 236 (4), 218 (8), 205 (7), 175 (8), 149 (12), 136 (18), 121 (16), 107 (40), 93 (43), 81 (98), 69 (100).

(RS)-(2Z,6E)-4-Methylfarnesyl diphosphate (**9**). Diphosphorylation of the corresponding alcohol was carried out essentially by the method described above for the synthesis of **7**; yield, 24.0 mg, 0.05 mmol (17%).

Ethyl 2-farnesyl-2-methyl-3-oxobutanoate. A solution of farnesyl chloride (3.7 g, 15.4 mmol) in 30 ml of absolute ethanol was added dropwise to a solution of the sodium compound of ethyl 2-methylacetoacetate (ethyl 2-methylacetoacetate, 2.66 g, 18.5 mmol, sodium ethoxide, 2 g, 29 mmol, and absolute ethanol, 120 ml) under nitrogen atmosphere

at 0°C. After stirring at room temperature for 12 h, the reaction mixture was worked up routinely; yield, 4.64 g (87%); ^{1}H-NMR ($CDCl_3$) δ (ppm) 1.27 (3H, t, $J_{H,H}$= 7.2 Hz), 1.32 (3H, s), 1.6 (12H, m), 2.0 (8H, m), 2.15 (3H, s), 2.55 (2H, d, $J_{H,H}$= 7.5 Hz), 4.19 (3H, q, $J_{H,H}$= 7.2 Hz), 5.1 (3H, m).

3-Farnesyl-2-butanone. A mixture of ethyl 2-farnesyl-2-methyl-3-oxobutanoate (2.2 g, 6.3 mmol), 1.3 M KOH (75 ml) and ethanol (75 ml) was stirred at room temperature for 3 days and then refluxed for 2 h. The mixture was acidified by addition of HCl and shaken with diethyl ether. The ether extracts were worked up routinely to give 3-farnesyl-2-butanone quantitatively: ^{1}H-NMR ($CDCl_3$) δ (ppm) 1.07 (3H, d, $J_{H,H}$= 6.8 Hz), 1.6 (12H, m), 2.0 (10H, m), 2.13 (3H, s), 2.5 (1H, m), 5.1 (3H, m).

Ethyl (RS)-(all-E)- and ethyl (RS)-(2Z,6E,10E)-4-methylgeranylgeranate. After addition of diethylethoxycarbonylmethylphosphonate (4.7 g, 21 mmol) to a suspension of NaH (615 mg, 26 mmol) in dry benzene, was added dropwise 3-farnesyl-2-butanone (1.84 g, 6.7 mmol). The mixture was then stirred at room temperature for 1 h and then at 65°C overnight. After addition of a small volume of water, the mixture was extracted with hexane, and the solvent was removed from the extracts. The residue was then chromatographed on silica gel with hexane/ethyl acetate, 30/1 to give the (2E)-isomer (1667 mg, 4.8 mmol) and the (2Z)-isomer (201 mg, 0.58 mol): ^{1}H-NMR ($CDCl_3$); Ethyl (RS)-(all-E)-4-methylgeranylgeranate δ (ppm) 1.04 (3H, d, $J_{H,H}$= 6.34 Hz), 1.27 (3H, t, $J_{H,H}$= 7.20 Hz), 1.6 (12H, m), 2.0 (11H, m), 2.11 (3H, d, $J_{H,H}$= 1.3 Hz), 4.14 (2H, q, $J_{H,H}$= 7.20 Hz), 5.1 (3H, m), 5.66 (1H, d, $J_{H,H}$= 1.3 Hz); Ethyl (RS)-(2Z,6E,10E)-4-methylgeranylgeranate δ (ppm) 1.02 (3H, d, $J_{H,H}$= 6.7 Hz), 1.26 (3H, t, $J_{H,H}$= 7.2 Hz), 1.6 (12H, m), 1.77 (3H, d, $J_{H,H}$= 1.2 Hz), 2.0 (11H, m), 4.12 (2H, q, $J_{H,H}$= 7.2 Hz), 5.1 (3H, m), 5.62 (1H, d, $J_{H,H}$= 1.2 Hz).

(RS)-(all-E)-4-Methylgeranylgeraniol. Reduction of ethyl (RS)-(all-E)-4-methylgeranylgeranate (739 mg, 2.14 mmol) to the corresponding alcohol was carried out according to a procedure similar to that described above for the preparation of (all-E)-4-methylfarnesol; yield, 456 mg, 1.48 mmol (69%): ^{1}H-NMR ($CDCl_3$) δ (ppm) 1.00 (3H, d, $J_{H,H}$= 6.4 Hz), 1.6 (15H, m), 2.0 (11H, m), 4.16 (2H, d, $J_{H,H}$= 7.2 Hz), 5.1 (3H, m), 5.42 (1H, t, $J_{H,H}$= 7.2 Hz); MS m/z 304 (0.5), 286 (1), 273 (1.5), 217 (3), 149 (5), 137 (10), 121 (9), 107 (13), 95 (14), 93 (13), 81 (50), 69 (100).

(RS)-(all-E)-4-Methylgeranylgeranyl diphosphate (**10**). Diphosphorylation of the corresponding alcohol was carried out essentially by the method described above for the synthesis of **7**; yield, 705 mg, 1.37 mmol (92.4%).

(RS)-(2Z,6E,10E)-4-Methylgeranylgeraniol. Ethyl (RS)-(2Z,6E,10E)-4-methylgeranylgeranate (95.9 mg, 0.277 mmol) was reduced to the corresponding alcohol by

a method similar to that described above for the preparation of (all-E)-4-methylfarnesol; yield, 81.8 mg, 0.27 mmol (97%): ^{1}H-NMR ($CDCl_3$) δ(ppm) 1.00 (3H, d, $J_{H,H}$= 7.0 Hz), 1.6 (15H, m), 2.0 (11H, m), 4.10 (2H, m), 5.1 (3H, m), 5.4 (1H, m); MS m/z 304 (1), 286 (1.5), 273 (1), 217 (3), 149 (5), 137 (9), 121 (8), 107 (13), 95 (14), 93 (13), 81 (45), 69 (100).

(RS)-(2Z,6E,10E)-4-Methylgeranylgeranyl diphosphate (**6**). Diphosphorylation of the corresponding alcohol was carried out essentially by the method described above for the synthesis of **7**; yield, 111 mg, (80%).

Enzymatic reaction of [1-^{14}C]-(all-E)-FPP and artificial homoallylic diphosphates. The incubation mixture contained, in a final volume of 1.0 ml, 1 μmol of an artificial homoallylic diphosphate to be examined, 0.3 nmol of [1-^{14}C]FPP, 100 μmol of Tris-HCl buffer (pH 8.5), 0.5 μmol of $MgCl_2$, 5 mg of Triton X-100 and a suitable amount of undecaprenyl diphosphate synthase. After incubation at 37°C for 24 h, the mixture was shaken with butanol, and the extract was treated with acid phosphatase as usual.[8] The hydrolysate was extracted with pentane and the extracts were subjected to product analysis.

Analysis of the product obtained by enzymatic reaction between [1-^{14}C]-(all-E)-FPP and **2**. The radioactive alcohol derived from the product of the synthase reaction was chromatographed on a silica gel TLC plate with a solvent system of benzene/ethyl acetate (9/1). For mass spectrometry, the product derived from a 30 mL-incubation was purified by silica gel TLC developed with the same solvent system as above followed by HPLC on a Hitachi porous polymer gel # 3011 column with a solvent system of MeOH/hexane (4/1). The product thus purified was subjected to mass spectrometric analysis with a JEOL gas chromatography-mass spectrometry system type JMS-DX 300. The gas chromatography was carried out at a linear programmed temperature at a rate of 4 °C/min from 100 °C to 200 °C on a 1 m, 1% silicon OV-1 column. Helium gas was used at a flow rate of 30 mL/min. The potential of the ionizing electron beam was 70 eV.

Enzymatic reaction between [1-^{3}H]-**2** and various allylic diphosphates. The incubation mixture contained, in a final volume of 4.0 mL, 200 nmol of [1-^{3}H]-**2** (0.23 Ci/mol), 100 nmol of an allylic substrates to be examined, 200 μmol of Tris-HCl buffer (pH 8.5), 1 μmol of $MgCl_2$, 10 mg of Triton X-100, 20 μmol of 2-mercaptoethanol, 100 μmol of NH_4Cl and a suitable amount of undecaprenyl diphosphate synthase. After the mixture was incubated at 37°C for 20 h, the mixture was extracted with 5 mL of butanol, and an aliquot of the extract was counted for radioactivity. For analysis of the products, the butanol extracts were treated with acid phosphatase as usual.[8] The hydrolysate was extracted with pentane and the extracts were chromatographed on a reversed phase silica gel KC-18 TLC plate (Whatman Inc.) with a solvent system of acetone/MeOH, 9/1. Mass

spectrometric analysis of the products was carried out under the same conditions as described above.

Ozonolysis of 4-methylgeranylgeraniol derived from the enzymatic reaction between FPP and **2**. For determination of the absolute structure of the enzymatic product of the reaction between (all-*E*)-FPP and **2**, 425 μg of the 4-methylgeranylgeraniol was prepared by an 800 mL-incubation followed by acid phosphatase treatment as described above. The alcohol was ozonized in 2 mL of ethyl chloride at -78 ^{o}C with a stream of ozone in oxygen until a pale blue color persisted in the solution. Removal of the solvent at room temperature left the ozonide, to which 0.5 mL of 98% formic acid and 0.1 mL of 30% H_2O_2 were added. After the mixture had been allowed to stand for 4 h at room temperature, the peroxides were decomposed by gradual addition of powdered $FeSO_4$ until the evolution of gas ceased. A small volume of diethyl ether was added and the mixture was dried over anhydrous $MgSO_4$. Filtration of the mixture through a glass filter and evaporation of the solvent gave a mixture of 3-methyllevulinic acid and levulinic acid. The acid was subjected to CD spectrometry with a Jasco CD spectrograph, Type J-400 X; $[\theta]_{280}$= -3600±700^{o} (methanol, c= 0.17 mM)

Ozonolysis of (S)-4-Methylfarnesol. (*S*)-4-Methylfarnesol was obtained by the pig liver farnesyl diphosphate synthase method.[4d] The (*S*)-4-methylfarnesol (2 mg) was ozonized in a way similar to that described above to give a mixture of (*R*)-3-methyllevulinic acid and levulinic acid. The acid was subjected to CD spectrometry. (*R*)-3-methyllevulinic acid; $[\theta]_{280}$= -3200±200^{o} (methanol, c= 0.71 mM)

Enzymatic reaction of (all-*E*)-GGPP and **2**. The incubation mixture contained, in a final volume of 2L, 40 µmol of (all-*E*)-GGPP, 100 µmol of **2**, 0.5 mmol of $MgCl_2$, 100 mmol of Tris-HCl buffer (pH 8.5), 5 g of Triton X-100, 50 mmol of NH_4Cl, 10 mmol of 2-mercaptoethanol, 10 mmol of KF and an appropriate amount of undecaprenyl diphosphate synthase. After incubation at 37^{o}C for 16 h, the mixture was extracted with butanol, and the extract was concentrated on a rotary evaporator. The resulting residue was then treated with acid phosphatase as usual.[8] The product was purified on HPLC [Hitachi porous polymer #3011, hexane/methanol (4/1)], and was subjected to mass spectrometric analysis.

Enzymatic reaction between [1-^{14}C]IPP and artificial allylic substrates. The incubation mixture contained, in a final volume of 1.0 mL, 25 nmol of [1-^{14}C]IPP (1 Ci/mol), 25 nmol of allylic substrates to be examined, 50 µmol of Tris-HCl buffer (pH 8.5), 250 nmol of $MgCl_2$, 2.5 mg of Triton X-100, 5 µmol of 2-mercaptoethanol, 25 µmol of NH_4Cl, 5 µmol of KF and an appropriate amount of undecaprenyl diphosphate synthase. The reaction mixture was incubated at 37^{o}C for 2 h, and then extracted with 3.0 mL of butanol. An aliquot of the extract was counted for radioactivity.

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1. Abbreviations: IPP, isopentenyl diphosphate; FPP, Farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; FGPP, farnesylgeranyl diphosphate.
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