Substrate Specificity of Farnesylpyrophosphate Synthetase. Application to Asymmetric Synthesis

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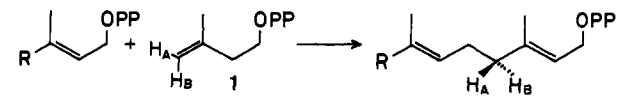
Abstract: Nine analogues of isopentenyl pyrophosphate (**1**) have been studied as substrates for pig liver farnesylpyrophosphate synthetase. (*E*)-3-Methylpent-3-enyl pyrophosphate (**12**) and its *Z* isomer **13** react enzymatically with geranyl pyrophosphate (**3**) to give (*S*)- (**14**) and (*R*)-4-methylfarnesyl pyrophosphate (**15**), respectively. **12** and **13** also react with dimethylallyl pyrophosphate (**2**) to give the corresponding enantiomers of 4-methylgeranyl pyrophosphate (**17** or **19**) and 4,8-dimethylfarnesyl pyrophosphate (**18** or **20**). 3,4-Dimethylpent-3-enyl (**21**), (*E*)-3-ethylpent-3-enyl (**22**), (*Z*)-3-ethylpent-3-enyl (**23**), and 2-(cyclohexen-1-yl)ethyl pyrophosphate (**25**) condense with **3**, but they cannot react with **2**. 2-(Cyclopenten-1-yl)ethyl pyrophosphate (**24**) is as highly reactive as **12** or **13**, reacting with both **2** and **3** to give optically active products. 2-Methylprop-2-enyl (**46**) and 3-methylpent-4-enyl pyrophosphate (**47**) are inactive.

Farnesylpyrophosphate synthetase catalyzes the stereospecific condensation of isopentenyl pyrophosphate (**1**) and dimethylallyl pyrophosphate (**2**) or geranyl pyrophosphate (**3**) to give (*E,E*)-farnesyl pyrophosphate (**4**). The stereochemical details have been established as shown in Scheme I by the elegant works of Cornforth, Popják, and their colleagues.[1] The structural requirement for an allylic pyrophosphate to react with **1** is not very stringent, but about 30 allylic substrate homologues have been found to condense with **1**.[2] On the other hand, the specificity for the nonallylic substrate is more rigorous and only a few homologues of **1** have so far been known. However, the structural modification of the isopentenyl pyrophosphate molecule has revealed an interesting site of behavior on this enzyme where 4-methylpent-4-enyl pyrophosphate (**5**) reacts with **2** and **3** in a cis fashion to give homoneryl (**6**) and (*Z,E*)-homofarnesyl pyrophosphate (**7**),[3] respectively, whereas 3-ethylbut-3-enyl pyrophosphate (**8**) reacts with **2** and **3** in a trans manner to afford predicted homologues with all *E* configuration, **9**, **10**, and **11**[4] (Scheme II).

These results have prompted us to explore other homologues of **1**. Introduction of a substituent at C-4 of **1** is particularly interesting because a homologue resulting from such modification is expected to afford a chiral molecule if it is accepted as the substrate in place of **1**. This paper describes mainly the enzymatic reaction of C-4-substituted isopentenyl pyrophosphates leading to chiral products. Part of the results with two of the nine isopentenyl pyrophosphate homologues described here has been reported as a preliminary communication.[5]

(*E*)-3-Methylpent-3-enyl pyrophosphate (**12**) and its *Z* isomer **13** synthesized by the phosphorylation of the corresponding alcohols were first examined, and they were found to react with **3** with almost equal facility. Enzymatic reaction was carried out on various scales with pig liver farnesylpyrophosphate synthetase under the usual conditions. After 1-h incubation of **3** and **12** with enzyme, the reaction mixture was treated with alkaline phosphatase, and the hydrolysates were extracted with petroleum ether and analyzed by GLC-mass spectrometry. As shown in Figure 1, a peak appeared at a retention time of 21.2 min (retention volume relative to that for (*E,E*)-farnesol, 1.02), and the mass spectrum corresponding

Scheme I

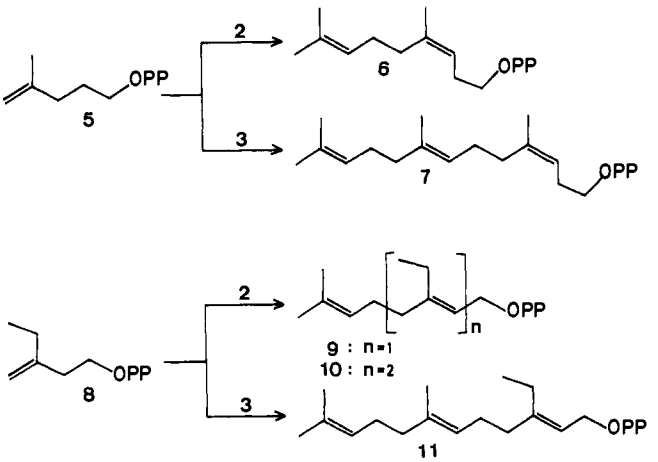


2: R = CH_3
3: R = $(CH_3)_2C{=}CHCH_2CH_2$-

3: R = CH_3
4: R = $(CH_3)_2C{=}CHCH_2CH_2$-

OPP = $OP_2O_6^{3-}$

Scheme II



to this peak revealed that this material was 4-methylfarnesol. The peak at 9 min was that of geraniol recovered from **3**. Compound **13** also reacted with **3** at a similar reaction rate, affording a product whose alcohol showed a completely identical GLC-mass spectral pattern with that of the product from **3** and **12**. The NMR spectrum showed that the product was (*E,E*)-4-methylfarnesol (Figure 2). The methyl signals appearing at δ 1.62 (s, 9 H) and 1.68 ppm (s, 3 H) support the all-trans structure. The methylfarnesol formed from **3** and **12** showed a negative ORD curve ($[\alpha]_D$ $-10.7 \pm 2.1°$), and that from **3** and **13** showed the opposite curve ($[\alpha]_D$ $+10.0 \pm 8.3°$), indicating that these two alcohols were enantiomeric with each other (Figure 3). When these two alcohols were oxidized to the corresponding aldehydes, the ORD signs were both reversed.

To determine the absolute configuration, the 4-methylfarnesol derived from **12** was subjected to ozonolysis followed by hypohalite oxidation. The methylsuccinic acid thus obtained was levorotatory. Since the configuration of (−)-methylsuccinic acid has been established to be *S* (**16**),[6] the product derived from **12** has been assigned the *S* structure (**14**); consequently, the product derived from **13** must be the *R* isomer (**15**).

Compound **12** or **13** reacted with dimethylallyl pyrophosphate (**2**) as well as geranyl pyrophosphate (**3**) to give 4,8-dimethylfarnesyl pyrophosphate (**18** or **20**) via 4-methylgeranyl pyrophosphate (**17** or **19**). This indicates that the enzymatic reaction of **12** or **13** proceeds in a trans manner. If the condensation were cis, the reaction should stop at the C_{11} stage, and 4,8-dimethylfarnesyl pyrophosphate could not be formed, because neryl pyrophosphate and its analogues are known to

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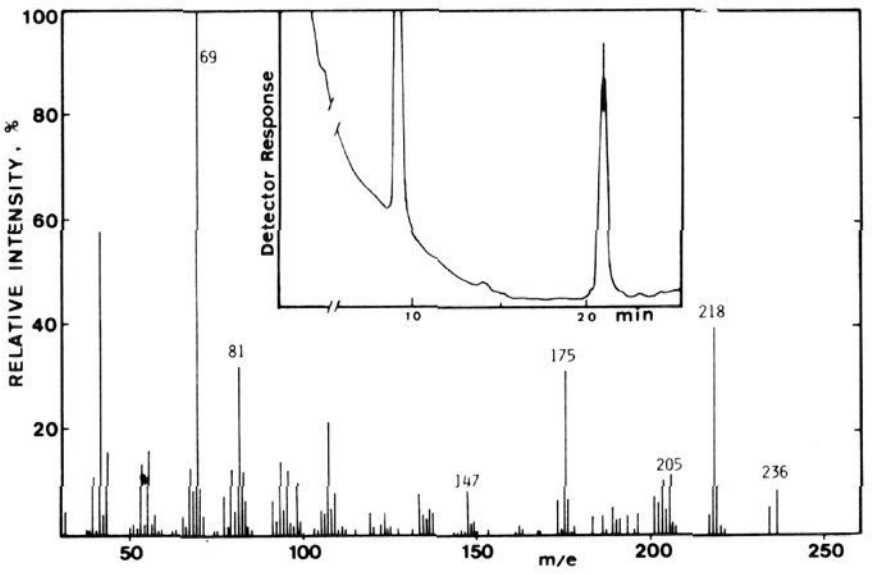


Figure 1. GLC–mass spectrum of the alcohol derived from the product (**14**) of the enzymatic reaction of **3** and **12.** The chromatography was carried out at a linear programmed temperature at a rate of 4 °C/min from 100 to 200 °C on a 1-m 5% poly(ethylene glycol) column with He gas at 25 mL/min. The inset shows the chromatogram. The perpendicular thin line on the peak indicates the position where the mass spectrum was measured.

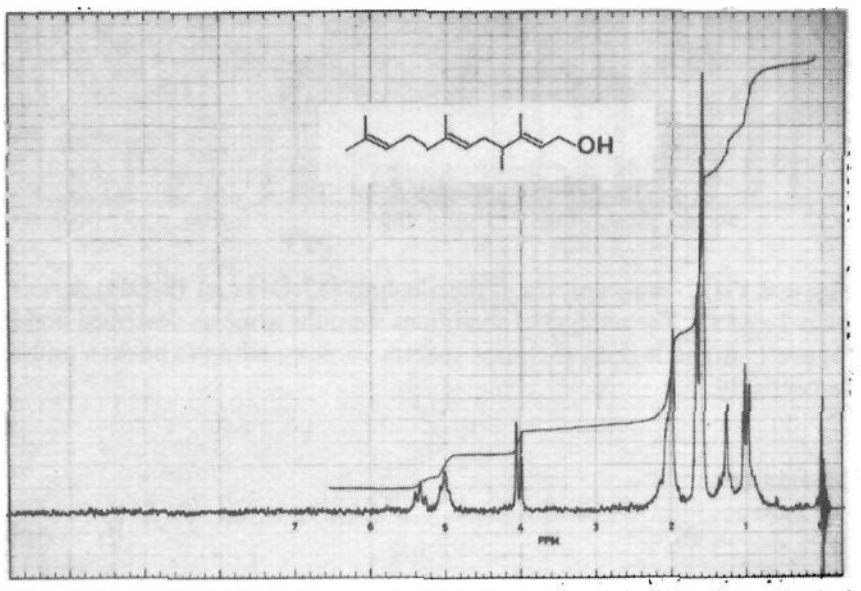
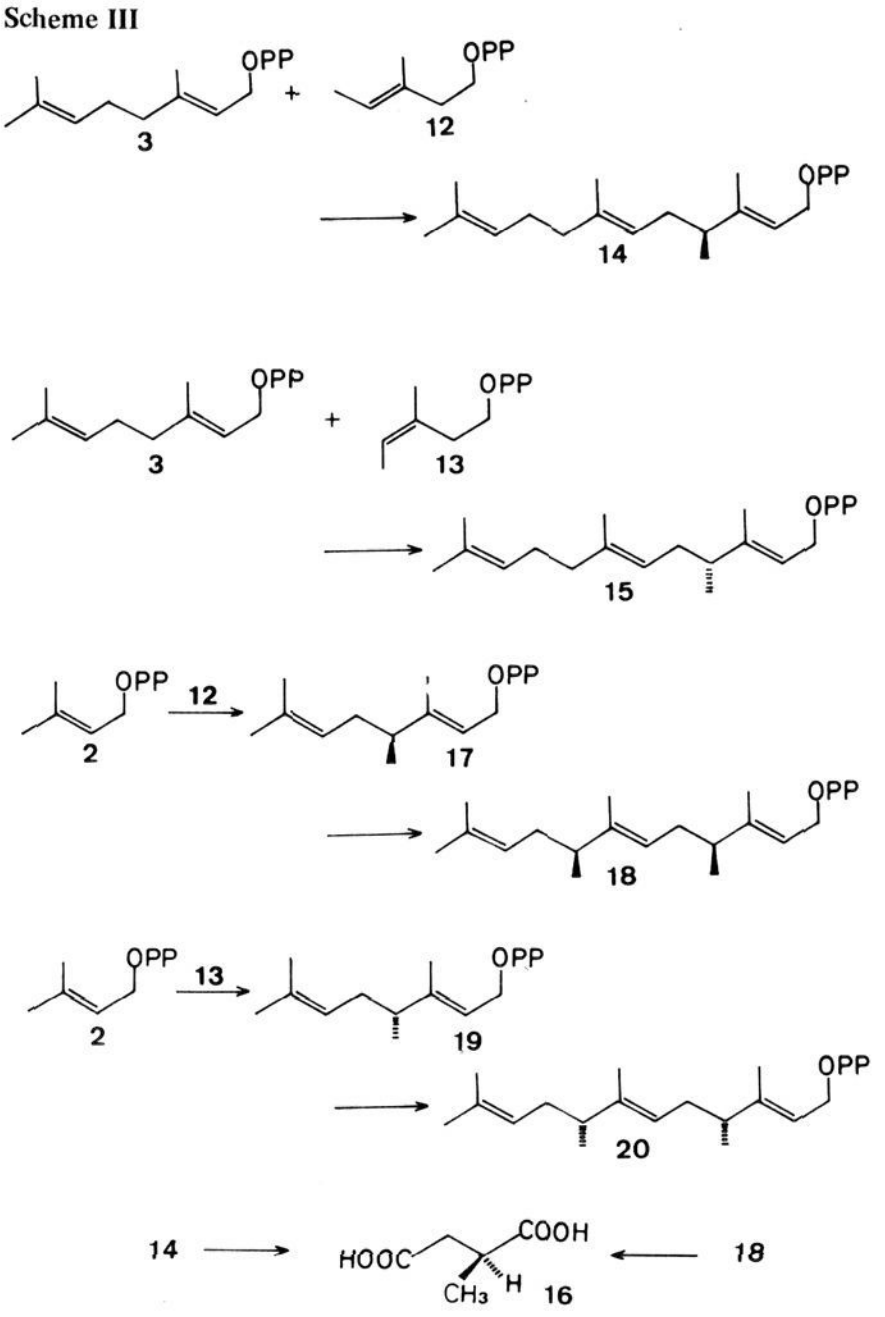


Figure 2. ^{1}H NMR spectrum (100 MHz) of a CCl_4 solution of the alcohol derived from the product (**14**) of the enzymatic reaction between **3** and **12.**

be inactive as substrates.[7] The NMR spectrum of the alcohol derived from **18** also supported the all-trans structure, showing peaks at δ 0.97 (d, 3 H), 1.01 (d, 3 H), 1.60 (s, 3 H), 1.64 (s, 6 H), 1.69 (s, 3 H), 2.05 (br s, 6 H), 4.06 (d, 2 H), 5.04 (m, 2 H), and 5.37 ppm (t, 1 H). The 4,8-dimethylfarnesol derived from **12** also showed a negative ORD curve ($[\alpha]_D$ −11.4 ± 5.1°), and the methylsuccinic acid obtained from this alcohol was also levorotatory. Therefore, the enzymatic reactions of these 4-methyl derivatives of **1** are expressed as shown in Scheme III. The formation of a geranyl pyrophosphate homologue (**17** or **19**) was confirmed by GLC–mass spectrometry. The amounts of these intermediates (**17** and **19**) formed relative to those of the final products (**18** and **20**) were almost equal. Peaks a and b in Figure 4 are attributed to 4-methylgeraniol and 4,8-dimethylfarnesol, respectively.

It is not always reasonable to predict the stereochemical course of the reaction of artificial substrates on the basis of that of the natural substrate, since we have observed abnormal reactions of **5.** However, the evidence described above shows that these two isopentenyl pyrophosphate homologues react in the same stereochemical fashion as that of the natural substrate. This means that the enzyme catalyzes the C–C bond formation with the recognition of the plane-trigonal face at C-3 irrespective of the position of the substituent at C-4. This is consistent with our hypothetical model of the catalytic site of this enzyme consisting of the binding sites for the pyrophosphoryl and methyl groups of **1.**[4]

Scheme III



It is of interest from the standpoint of the application of enzyme to organic synthesis that an enzyme can produce artificially the *S* or *R* enantiomer depending on whether it is supplied with the *E* or *Z* isomer as substrate.

The fact that both **12** and **13** are good substrates for this enzyme suggested that the 4,4-dimethyl derivative (**21**) of **1** might also be accepted as a substrate. Although the reactivity was much lower than that of **12** or **13,** this homologue also condensed with **3** to give the corresponding product from which 4,4-dimethylfarnesol [**26**-OH *m/e* 250 (M^+), 232 (M − 18), 219 (M − 31), 69 (base)] was liberated on phosphatase treatment. (*E*)-**22** and (*Z*)-3-ethylpent-3-enyl pyrophosphate (**23**) were also active as substrates to react with **3.** The GLC–mass spectra of the alcohol products derived from **22** and **23** showed the same pattern indicating that both were bishomofarnesols [**27**-OH or **28**-OH *m/e* 250 (M^+), 232 (M − 18), 219 (M − 31), 69 (base)], but the yield was too low for the ORD to be measured. The reactivities of **21, 22,** and **23** were only about 1% of that of **12.** Furthermore, none of these three substrates condensed with **2.**

In contrast, **24** (having a cyclic structure) condensed with **3** as efficiently as **12** or **13,** and afforded the corresponding product, from which an alcohol showing a mass spectrum compatible with that of geranylcyclopentylideneethanol was obtained (Figure 5). Compound **24** also reacted with **2,** but the reaction stopped to give the corresponding C_{12} compound, which did not react with **24** any longer. This is not surprising but rather predictable from our knowledge of the substrate specificity of this enzyme with respect to the allylic substrate. The alcohol product obtained by the reaction between **3** and **24** showed a strong negative ORD curve ($[\alpha]_D$ −30.6 ± 2.5°) as shown in Figure 6. Although direct demonstration for the absolute configuration has not yet been obtained, the structures

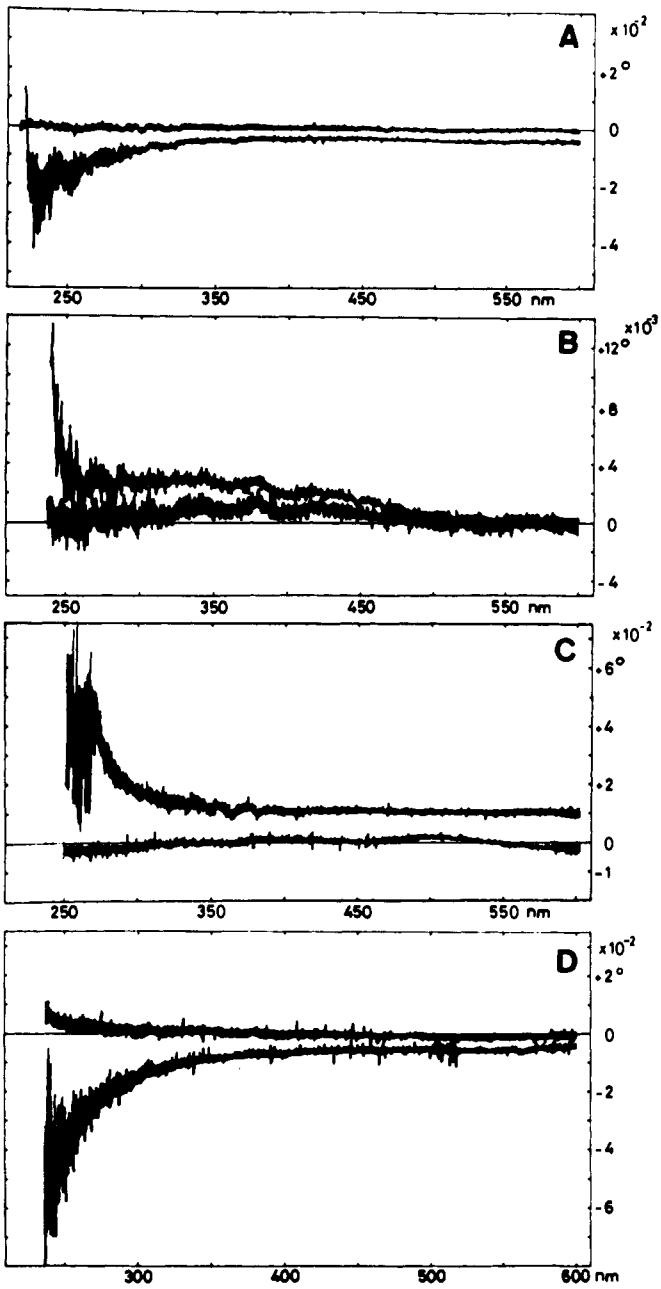


Figure 3. ORD curves of products of enzymatic reaction in hexane with a light path of 1 mm: (A) 4-methylfarnesol derived from **3** and **12** (3.5 mg/0.1 mL); (B) 4-methylfarnesol derived from **3** and **13** (0.6 mg/0.1 mL); (C) 4-methylfarnesal derived from sample A (1.7 mg/0.1 mL); (D) 4,8-dimethylfarnesol derived from **2** and **12** (3.7 mg/mL).

of the products derived from **24** are expected to be **29** and **30** (Scheme IV). Compound **25** with a six-membered ring was also active when **3** was the cosubstrate, affording a similar product, but the reactivity was negligible compared with that of **24**. The alcohol product showed a mass spectrum reasonable for geranylcyclohexylideneethanol [**31**-OH *m/e* 262 (M^+), 244 (M − 18), 231 (M − 31), 81 (base), 69]. The reactivity of **25** was too poor to detect when **2** was the cosubstrate.

The excellent reactivity of **24** led us also to examine its condensation with artificial allylic substrates, namely the enzymatic reactions between artificial substrates. Seven allylic pyrophosphates (**32–38**) were found to condense with **24** to give the corresponding products as shown in Scheme V. The structures of the products were confirmed by the GLC-mass spectrometric analysis of the resulting alcohols [**39**-OH *m/e* 208 (M^+), 190 (M − 18), 177 (M − 31), 55 (base); **40**-OH *m/e* 194 (M^+), 176 (M − 18), 163 (M − 31), 55 (base);

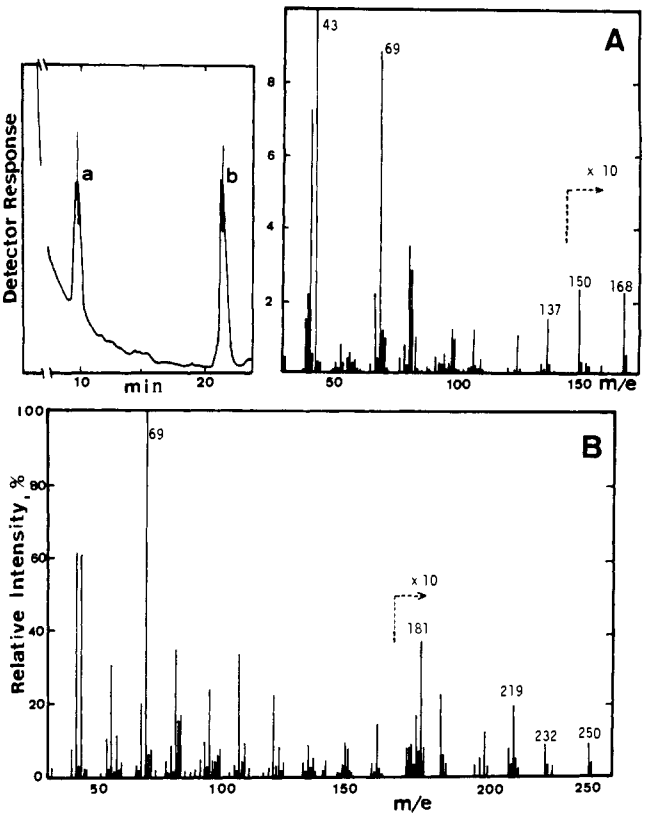
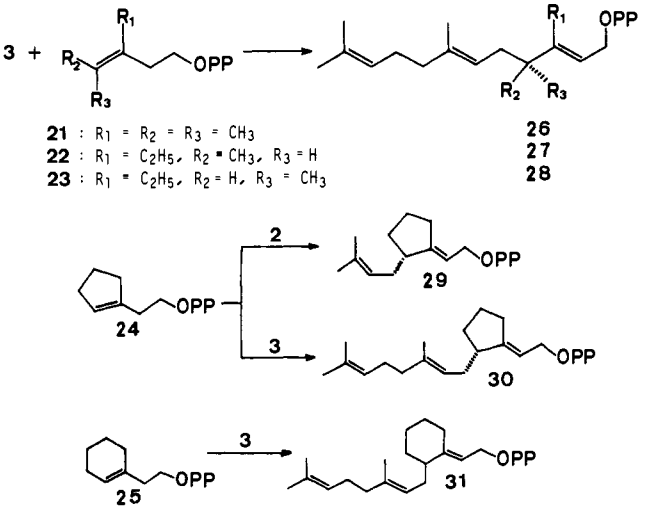
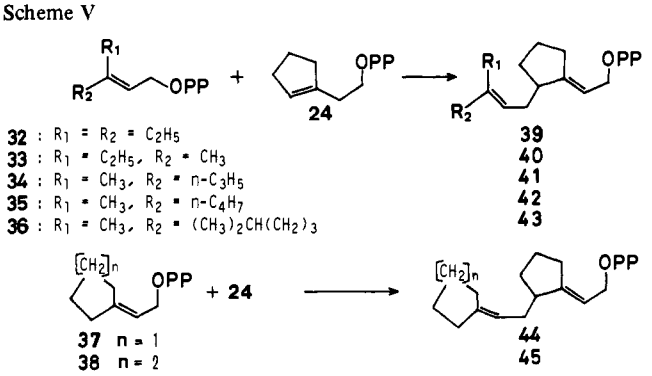


Figure 4. GLC-mass spectra of the alcohols (**17**-OH and **18**-OH) derived from **2** and **12**. The analytical conditions were the same as described under Figure 1. A and B show the mass spectra corresponding to peaks a and b, respectively.

Scheme IV



Scheme V



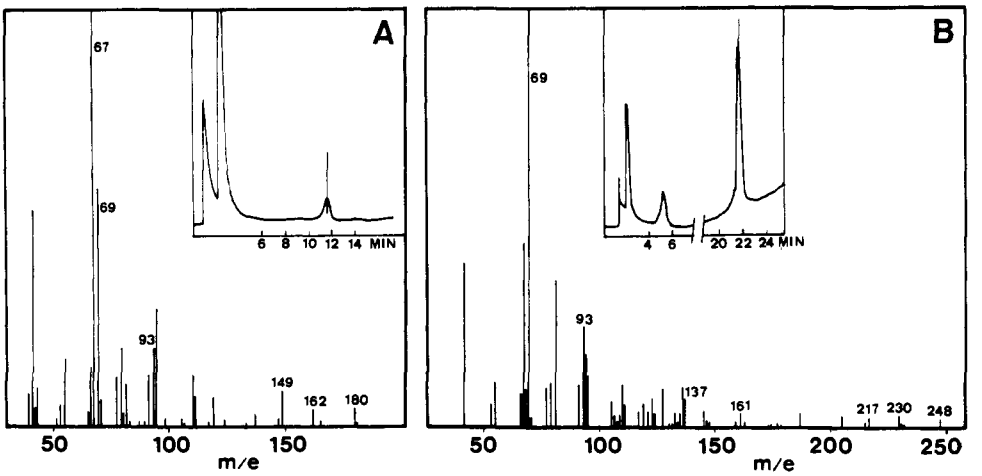
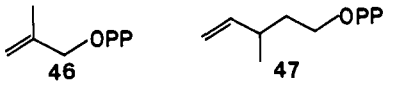
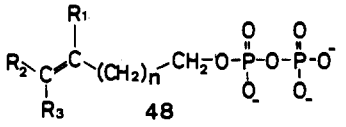


Figure 5. GLC-mass spectra of the alcohols derived from the products of enzymatic reaction of **24** with **2** (A) and with **3** (B).

41-OH *m/e* 208 (M^+), 190 (M − 18), 177 (M − 31), 55 (base); **42**-OH *m/e* 222 (M^+), 204 (M − 18), 191 (M − 31), 69 (base); **43**-OH *m/e* 250 (M^+), 232 (M − 18), 219 (M − 31), 83 (base); **44**-OH *m/e* 206 (M^+), 188 (M − 18), 175 (M − 31), 95 (base); **45**-OH *m/e* 220 (M^+), 202 (M − 18), 189 (M − 31), 67 (base)]. However, none of these artificial allylic substrates gave a better yield of product than **3**.

The fact that 4-methylpent-4-enyl pyrophosphate (**5**) can be a substrate whereas but-3-enyl pyrophosphate is inactive[4a] led us to examine 2-methylprop-2-enyl (**46**) and 3-methylpent-4-enyl pyrophosphate (**47**). However, neither of them was active with **2** or **3** at all.





To summarize, the structural requirements for the nonallylic pyrophosphate (gross structure **48**) to condense with an allylic pyrophosphate, in the reaction catalyzed by pig liver farnesylpyrophosphate synthetase, are: (1) R_1 should be CH_3 or C_2H_5; (2) R_2 and R_3 can be H, CH_3, or C_2H_5; (3) cyclization between R_1 and R_2 forming a five- or six-membered ring is acceptable; and (4) *n* should be 1 or 2.

In order to see the reactivity in more detail, kinetic properties of **12** were compared with those of **1** and **8** using radiolabeled substrates. Figure 7 shows time courses of the reaction of these substrates compared at an initial concentration of 50 μM. The reaction rate of **12** is about 30% that of **1**, but more than 3 times as much as **8**. Figure 8 shows the relative amount of formation of 4-methylgeranyl pyrophosphate (**17**) and 4,8-dimethylfarnesyl pyrophosphate (**18**) from **2** and **12** as a function of the incubation time. The K_m value for **12** was estimated by the Lineweaver-Burk method to be 18 μM, which is 10 times larger than that of **1** but about one-half of that for **8**.

These results indicate that the introduction of a methyl at the C-4 position of **1** results in a decrease of reactivity to a lesser extent than the replacement of the 3-methyl of **1** by an ethyl group. There is also a marked difference between the two homologues, **12** and **8**, in that the former reacts with **2** and **3** with equal facility, whereas the latter reacts with **2** and **3** at relative rates of 1:4. The order of reactivity of the isopentenyl pyrophosphate analogues is roughly estimated to be **12** ≈ **13** ≈ **24** ≫ **23** > **22** > **21** ≒ **25**.

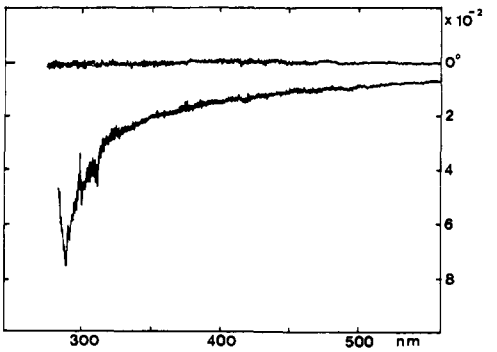


Figure 6. ORD curve of the alcohol derived from **3** and **24** taken at a concentration of 2.1 mg/0.1 mL of hexane.

Experimental Section

Materials. Farnesylpyrophosphate synthetase was obtained from pig liver essentially according to the method of Holloway and Popják.[8] Dimethylallyl pyrophosphate, geranyl pyrophosphate, and other allylic pyrophosphates were prepared as described previously.[2c-g] New homologues of isopentenyl pyrophosphate were synthesized by the phosphorylation of the corresponding alcohols obtained as described below. (*E*)- and (*Z*)-3-methylpent-3-en-1-ol were synthesized by the method previously reported.[9]

3,4-Dimethylpent-3-en-1-ol. One-half of a solution of 3-methylbutanone-2 (12.9 g) and methyl bromoacetate (23.1 g) in 60 mL of dry benzene was refluxed with activated granular zinc (10.8 g) to start the Reformatsky reaction. When the mixture became turbid, the rest of the solution was added dropwise over 2 h, and the mixture was kept under reflux for an additional 0.5 h. After being cooled, the mixture was poured into ice-cooled 10% H_2SO_4 (200 mL) with vigorous stirring. The benzene layer was separated and the aqueous layer was neutralized with $NaHCO_3$ and extracted with ether. The organic layers were combined and dried over anhydrous Na_2SO_4 and the solvents were removed. The residual ester was then distilled under reduced pressure. The hydroxy ester (16 g) was collected at 49–50 °C (3 mm). The ester was dissolved in 100 mL of benzene, P_2O_5 (12 g) was added slowly to this solution with vigorous stirring, and the mixture was refluxed for 2 h. The solution was decanted and washed successively with saturated $NaHCO_3$ and NaCl solutions and dried over Na_2SO_4. The solution was then distilled to give a mixture of dehydrated products. The mixture (2.3 g) was dissolved in ether (20 mL) and treated with $LiAlH_4$ (0.5 g) in the usual way. GLC-mass spectrometric analysis indicated that the product contained 3,4-dimethylpent-3-en-1-ol as a major component. The alcohol was purified by preparative GLC with a 4-m column of 20% poly(ethylene glycol) 20M on Chromosorb WAW.

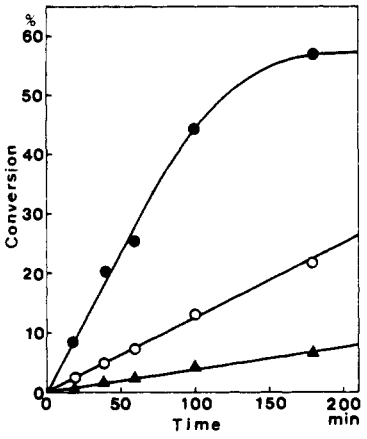


Figure 7. Time course of the conversion of isopentenyl pyrophosphate and its homologues into allylic products. The substrates were: (●) **1** and **2**; (▲) **8** and **2**; (○) **12** and **2**.

(*Z*)- and (*E*)-3-Ethylpent-3-en-1-ol. Methyl 3-ethyl-3-hydroxypentanoate (2.0 g) obtained by the Reformatsky reaction of methyl bromoacetate and diethyl ketone was treated with P_2O_5 in a way similar to that described above, and the dehydration products were reduced to a mixture of the corresponding isomeric alcohols, from which the *Z* and *E* isomers of 3-ethylpent-3-en-1-ol were purified by preparative GLC. The ratio of formation of the *Z* and *E* isomers was 5:6: NMR *Z* isomer, δ 0.98 (t, 3 H), 1.60 (d, 3 H), 1.85–2.30 (m, 4 H), 3.53 (t, 2 H), and 5.18 ppm (q, 1 H); *E* isomer, δ 0.99 (d, 3 H), 1.61 (d, 3 H), 1.80–2.40 (m, 4 H), 3.53 (t, 2 H), and 5.30 (q, 1 H).

2-(Cyclopenten-1-yl)ethanol. Methyl 1-hydroxycyclopentaneacetate obtained by the Reformatsky reaction between cyclopentanone and methyl bromoacetate was treated similarly with P_2O_5, and the methyl 1-cyclopenteneacetate was purified from the mixture containing dehydration products by preparative GLC. The ester was reduced as usual with $LiAlH_4$ to give 2-(cyclopenten-1-yl)ethanol.

2-(Cyclohexen-1-yl)ethanol. This alcohol was obtained by a procedure similar to that for the synthesis of 2-(cyclopenten-1-yl)ethanol.

3-Methylpent-4-en-1-ol. 3-Methylpent-4-en-1-al[10] (2 g) was reduced with $LiAlH_4$ in ether to give the alcohol (1.6 g), bp 78–79 °C (60 mm).

Enzymatic Reaction and Product Analysis. The composition of the incubation for the enzymatic reaction was the following: 20 mM Tris-HCl buffer (pH 7.7), 5 mM $MgCl_2$, 100 μM dimethylallyl pyrophosphate or geranyl pyrophosphate, 100 μM isopentenyl pyrophosphate analogue, and farnesylpyrophosphate synthetase, 0.1 mg/mL (specific activity, 60 nmol of farnesyl pyrophosphate synthesized min^{-1} mg^{-1}). The incubation volume was usually 1 to 5 mL. After incubation at 37 °C for 1 h, the mixture was treated with alkaline phosphatase as usual, and was extracted with petroleum ether. The extracts were subjected to GLC–mass spectrometric analysis with a 1-m 5% poly(ethylene glycol) column. The mass spectra were taken at 70 eV of ionizing electron beam. To avoid missing very small amounts of products, mass fragmentography was also carried out at 20 eV by recording the ion intensity at *m/e* 69.

For kinetic experiments, [1-^{3}H]-3-ethylbut-3-enyl pyrophosphate (specific activity, 4.50 μCi/μmol) and (*E*)-[1-^{3}H]-3-methylpent-3-enyl pyrophosphate (specific activity, 5.73 μCi/μmol) were used, and the enzyme activity was assayed by determining the amount of conversion of the radioactive substrates into acid-labile allylic pyrophosphate products as usual.

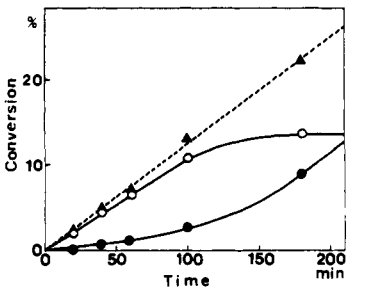


Figure 8. Time course of the conversion of **12** into **17** (○) and **18** (●). The triangles indicate the total conversion.

Oxidative Degradation of 4-Methylfarnesol and 4,8-Dimethylfarnesol. 4-Methylfarnesol (3.5 mg) obtained by a large scale (400 mL) incubation of **12** and **3** was dissolved in 2 mL of petroleum ether. To the solution, 20 mg of active MnO_2 was added portion by portion over 4 h with efficient stirring at room temperature. After removal of MnO_2, evaporation of the solvent left 4-methylfarnesal. The 4-methylfarnesal was ozonized in 2 mL of ethyl chloride at −70 °C by passing ozone in air for 10 min. After the solvent was evaporated at room temperature, the residue was treated with formic acid and H_2O_2 and then with I_2–KI and Na_2CO_3 according to the procedure of Cornforth et al.[1] for the degradation of farnesol to succinic acid. The formation of methylsuccinic acid was confirmed by the conversion of a part of the acid to the methyl ester and its GLC–mass spectrometric analysis. 4,8-Dimethylfarnesol prepared enzymatically from **12** and **2** was also degraded directly (not via the aldehyde) to methylsuccinic acid. Both specimens of the methylsuccinic acid showed negative ORD curves.

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References and Notes

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