Enzymic Condensation of 3-Methyl-2-alkenyl Pyrophosphates with Isopentenyl Pyrophosphate

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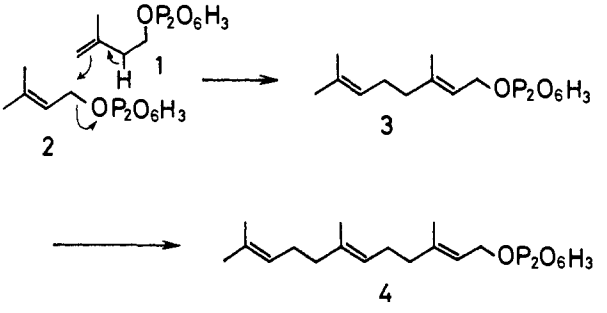
Abstract: The substrate specificity of farnesyl pyrophosphate synthetase of pumpkin fruit was studied by using synthetic 3-methyl-2-alkenyl pyrophosphates. *cis*-3-Methyl-2-hexenyl pyrophosphate (**19**) reacted enzymically with isopentenyl pyrophosphate (**1**) as well as the *trans* isomer **12**, and even *cis*-3-methyl-2-heptenyl pyrophosphate (**20**) was reactive, though far less reactive than its *trans* isomer **13**. However, *cis*-3-methyl-2-octenyl pyrophosphate (**21**) was no longer reactive. On the other hand, *trans*-3-methyl-2-octenyl (**14**), *trans*-3-methyl-2-nonenyl (**15**), and *trans*-3-methyl-2-decenyl pyrophosphate (**16**) were almost as highly reactive as geranyl pyrophosphate (**3**). Chromatographic analysis suggested that the products from these compounds, **14**, **15**, and **16**, were homologs not of farnesyl pyrophosphate (**4**) but of geranyl pyrophosphate (**3**), and that the products from **12**, **13**, **19**, and **20** consisted of homologs of **3** and **4**. The product of the longest chain that the enzyme could produce was trishomofarnesyl pyrophosphate derived from **13** or **20**.

Prenyltransferase which catalyzes the "head-to-tail" condensation of C_5 units in polyisoprenoid biosynthesis has been purified from various organisms. It is well established that a purified preparation of farnesyl pyrophosphate synthetase, one of the prenyltransferases, catalyzes condensation of isopentenyl pyrophosphate (**1**) both with dimethylallyl pyrophosphate (**2**) and with geranyl pyrophosphate (**3**) to give farnesyl pyrophosphate (**4**).[1-5]

Scheme I



Popják, *et al.*, showed recently that *trans*- (**11**) and *cis*-3-methyl-2-pentenyl pyrophosphate (**18**), the mixture of *trans*- and *cis*-3-methyl-2-hexenyl pyrophosphates (**12** plus **19**), and 3-ethyl-2-pentenyl pyrophosphate could be artificial substrates instead of **2** for pig liver farnesyl pyrophosphate synthetase.[6] We have studied independently the substrate specificity of farnesyl pyrophosphate synthetase of pumpkin fruit and obtained results similar to those of Popják, *et al.*, in that the compounds **11** and **12** were enzymically active.[7] It was, however, uncertain whether the *cis*-3-methyl-2-hexenyl pyrophosphate (**19**) was reactive or not. It is of interest how the specificity of the allylic pyrophosphate depends on the *trans* and *cis* structures since the enzyme, which discriminates stringently between **3** and its *cis* isomer, neryl pyrophosphate,[8,9] accepts both *trans* and *cis* isomers of 3-methyl-2-pentenyl pyrophosphate. Furthermore, the longer limit of the chain length of active allylic pyrophosphate remained to be defined.

In order to study these points, we have examined systematically the reactivities of 3-methyl-2-alkenyl pyrophosphates and observed an obvious difference between the *trans* and *cis* isomers. We defined also the longest chain of the substrate analog. This paper discusses the relationship between the structures of 3-methyl-2-alkenyl pyrophosphates and their reactivities with isopentenyl pyrophosphate catalyzed by farnesyl pyrophosphate synthetase.

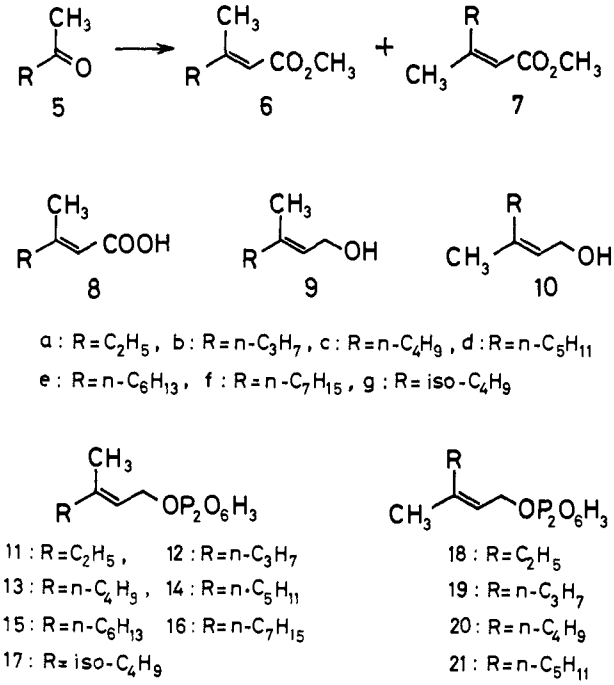
Synthesis of 3-Methyl-2-alkenyl Pyrophosphates

The mixture of methyl *trans*- and *cis*-3-methyl-2-alkenoates, obtained in a good yield by the reaction of methyl alkyl ketones and diethyl methoxycarbonylmethyl phosphonate in the presence of sodium methoxide, was treated with alkali to give a mixture of the free acids, from which the *trans* acid was purified by recrystallization. The filtrate after removal of the *trans* isomer was treated with diazomethane, and the mixture which had been enriched with the *cis* isomer was chromatographed on silica gel. Pure *cis* isomer was obtained in the earlier fractions. The esters and the acids thus obtained were reduced with $LiAlH_4$ to give the corresponding alcohols. The geometric isomers of the esters or alcohols were distinguished by the nmr spectra and by glpc. The chemical shift for the 3-methyl group of methyl *cis*-3-methyl-2-alkenoates appeared in the region of 1.86–1.90 ppm, and that of the *trans* isomer appeared at 2.12–2.15 ppm. All 3-methyl-2-alkenols synthesized showed a signal for the 3-methyl group in the region of 1.70–1.72 ppm for the *cis* isomers, and 1.62–1.63 ppm for the *trans* isomers. These values are in good accord

(1) F. Lynen, B. W. Agranoff, H. Eggerer, U. Henning, and E. M. Möslein, *Angew. Chem.*, 71, 657 (1959).
(2) C. R. Benedict, J. Kett, and J. W. Porter, *Arch. Biochem. Biophys.*, 110, 611 (1965).
(3) J. K. Dorsey, J. A. Dorsey, and J. W. Porter, *J. Biol. Chem.*, 241, 5353 (1966).
(4) P. W. Holloway and G. Popják, *Biochem. J.*, 104, 57 (1967).
(5) K. Ogura, T. Nishino, and S. Seto, *J. Biochem. (Tokyo)*, 64, 197 (1968).
(6) G. Popják, J. L. Rabinowitz, and J. M. Baron, *Biochem. J.*, 113, 861 (1969).
(7) K. Ogura, T. Nishino, T. Koyama, and S. Seto, Abstracts, 13th Symposium on the Chemistry of Natural Products, Sapporo, Japan, Sept 1969, p 242.
(8) G. Popják, P. W. Holloway, R. P. M. Bond, and M. Roberts, *Biochem. J.*, 111, 333 (1969).
(9) K. Ogura, T. Koyama, T. Shibuya, T. Nishino, and S. Seto, *J. Biochem. (Tokyo)*, 66, 117 (1969).

with those in the literature for the isomers of farnesol, geraniol, and related compounds.[10-12] Gas–liquid chromatography on columns packed with silicone oil on base-washed firebrick resolved the isomeric alcohols as well as the isomeric esters and also gave a criterion for the distinction; the *trans* isomers always had a larger retention volume than the *cis* isomers, relative retention volumes of the *trans* to the *cis* isomers being 1.21–1.33 and 1.13–1.17 for the esters and the alcohols, respectively. The allylic alcohols were phosphorylated and the pyrophosphate esters were obtained according to the procedure reported[13,14] for the preparation of dimethylallyl and geranyl pyrophosphate. Eleven allylic pyrophosphates were thus obtained, which are summarized in Scheme II.

Scheme II



Enzymic Reaction

The enzymic reaction of artificial substrates with isopentenyl pyrophosphate was assayed essentially by the same procedure as reported[5] for natural substrates, by determining radioactivity in acid-labile allylic pyrophosphates into which ^{14}C-isopentenyl pyrophosphate was incorporated by the condensation. An incubation mixture contained ^{14}C-isopentenyl pyrophosphate, an allylic pyrophosphate to be examined, magnesium chloride, and farnesyl pyrophosphate synthetase purified from pumpkin fruit[5] in phosphate buffer. After the reaction the mixture was treated with dilute acid to hydrolyze the allylic pyrophosphates, and the mixture was extracted with light petroleum. The radioactivity in the extracts corresponded to the amount of ^{14}C-isopentenyl pyrophosphate condensed with the partner, since the enzyme preparation was almost free of isopentenyl pyrophosphate isomerase. Initial velocities of enzymic condensations of 3-methyl-2-alkenyl pyrophosphates with isopentenyl pyrophosphate were examined at various concentrations (Figures 1 and 2). As shown in the figures, both the *cis*-3-methyl-2-hexenyl pyrophosphate (**19**) and the *trans* isomer **12** were enzymically active. However, *cis*-3-methyl-2-heptenyl pyrophosphate (**20**) showed only negligible activity as compared with the *trans* isomer **13** which was almost as reactive as *trans*-3-methyl-2-pentenyl pyrophosphate (**11**); *cis*-3-methyl-2-octenyl pyrophosphate (**21**) was

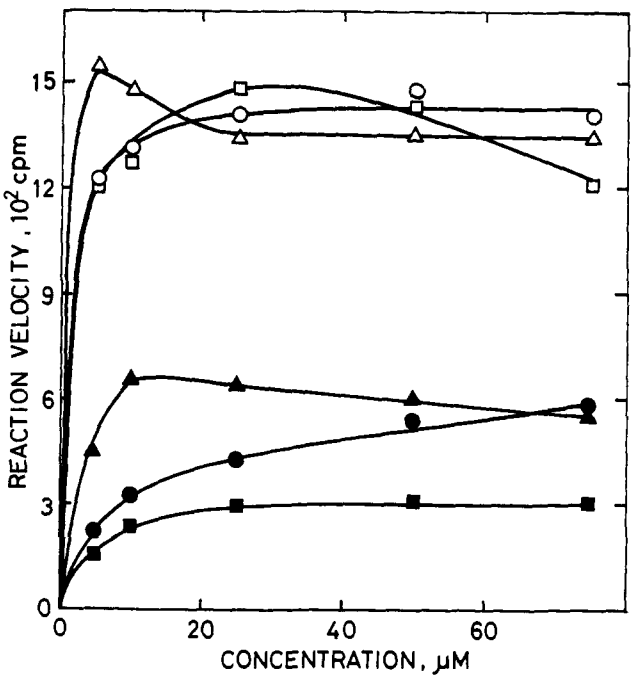


Figure 1. Dependence of reaction velocity of *trans*-3-methyl-2-alkenyl pyrophosphates on concentration: ●—●, *trans*-3-methyl-2-pentenyl (**11**); ■—■, *trans*-3-methyl-2-hexenyl (**12**); ▲—▲, *trans*-3-methyl-2-heptenyl (**13**); ○—○, *trans*-3-methyl-2-octenyl (**14**); □—□, *trans*-3-methyl-2-nonenyl (**15**); △—△, *trans*-3-methyl-2-decenyl pyrophosphate (**16**).

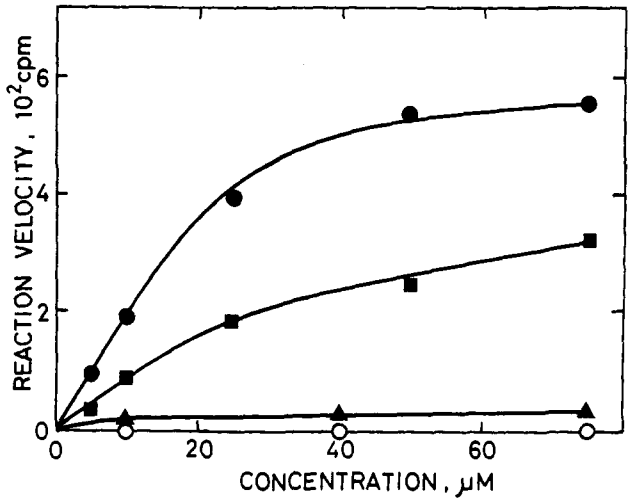


Figure 2. Dependence of reaction velocity of *cis*-3-methyl-2-alkenyl pyrophosphates on concentration: ●—●, *cis*-3-methyl-2-pentenyl (**18**); ■—■, *cis*-3-methyl-2-hexenyl (**19**); ▲—▲, *cis*-3-methyl-2-heptenyl (**20**); ○—○, *cis*-3-methyl-2-octenyl pyrophosphate (**21**).

(10) R. B. Bates, D. M. Gale, and B. J. Gruner, *J. Org. Chem.*, **28**, 1086 (1963).
(11) J. W. K. Burrell, R. F. Garwood, L. M. Jackman, E. Oskay, and B. C. L. Weedon, *J. Chem. Soc. C*, 2144 (1966).
(12) G. Popják, P. W. Holloway, and J. M. Baron, *Biochem. J.*, **111**, 325 (1969).
(13) F. Cramer and W. Böhm, *Angew. Chem.*, 71, 775 (1959).
(14) A. A. Kandutsch, H. Poulus, E. Levin, and K. Bloch, *J. Biol. Chem.*, **239**, 2507 (1964).

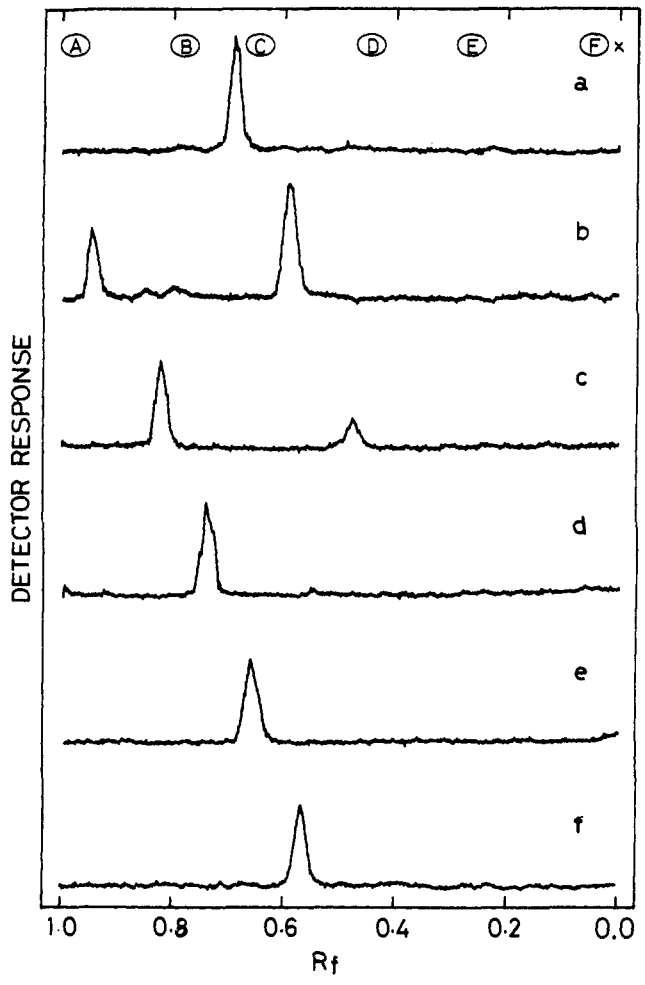


Figure 3. Thin-layer radiochromatograms of products derived from *trans*-3-methyl-2-alkenyl pyrophosphates. Starting substrates are **11** (a), **12** (b), **13** (c), **14** (d), **15** (e), and **16** (f). Spots of reference alcohols: A, geraniol; B, farnesol; C, nerolidol; D, geranylgeraniol; E, geranyllinalool; F, phytol.

not active at all. On the other hand, *trans*-3-methyl-2-octenyl (**14**), *trans*-3-methyl-2-nonenyl (**15**), and *trans*-3-methyl-2-decenyl pyrophosphate (**16**) showed almost as high reactivity as the natural substrates. These three compounds appear not only to have larger maximum reaction velocity but also to have stronger affinity of binding to the enzyme than the compounds of shorter chain. Although more detailed kinetic study is required for the comparison of reactivities of these artificial substrates, reaction velocities of the substrates relative to geranyl pyrophosphate (**3**) at the concentration of 25 μM give a rough comparison as follows: the velocities of *trans* isomers **11**, **12**, **13**, **14**, **15**, and **16** are 0.28, 0.20, 0.43, 0.96, 1.02, and 0.92, respectively, and those of *cis* isomers **18**, **19**, and **20** are 0.26, 0.12, and 0.02, respectively. The velocity of dimethylallyl pyrophosphate (**2**) relative to **3** was 0.73. It is noteworthy that compound **17** which has a branched structure is inactive. Although it is difficult to decide from the data given in Figure 2 whether the *cis* compound **20** was reactive or not, the analysis of the products obtained from a long incubation with an increased amount of the enzyme proved its undoubted reactivity as described in a later section.

Products of Enzymic Reactions

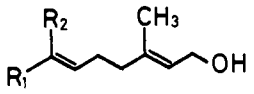
The data given in Figures 1 and 2 showed only that certain artificial substrates were enzymically active. However, since farnesyl pyrophosphate synthetase catalyzes two consecutive condensations (**1** + **2** → **3** and **1** + **3** → **4**), it was important to see whether the product was a homolog of geranyl pyrophosphate (**3**) or of farnesyl pyrophosphate (**4**), probably depending on the chain length of the substrates used.

Popják, *et al.*,[6] showed that both 3-methyl-2-pentenyl pyrophosphate and 3-methyl-2-hexenyl pyrophosphate gave a homolog of **4** and that 3-ethyl-2-pentenyl pyrophosphate gave not only a homolog of **4** but also a homolog of **3**. The analysis of our products is expected to answer the following questions. (1) What is the product of the longest chain derived from a 3-methyl-2-alkenyl pyrophosphate? (2) By what structural factor is the product controlled to be a homolog of **3** or a homolog of **4**? (3) Is there any difference between the chain lengths of the products derived from *cis* and *trans* isomers?

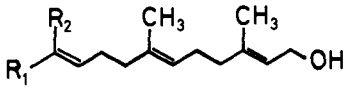
For analysis of the products, the radioactive alcohols liberated by alkaline phosphatase hydrolysis were applied to a reverse phase tlc in which the moving of prenols decreased sensitively and orderly with increase of the chain length.[15] The comparison of the radiochromatograms reasonably revealed whether the product was a homolog of **3** or of **4**. As shown in Figure 3, the product derived from **11** (C_6 compound) showed only a single radioactive peak which moved slower than farnesol, as expected for homofarnesol. The products of **12** (C_7 compound), however, showed two peaks, the slower moving peak (R_f 0.59) probably being due to bishomofarnesol **31** and the faster moving one (R_f 0.95) to bishomogeraniol **22**. Two radioactive peaks were observed also in the chromatogram of the products from **13** (C_8 compound), but the faster moving one (R_f 0.82), probably due to trishomogeraniol **24**, was predominant. The other peak appeared with low intensity in the expected region for trishomofarnesol. Thus farnesol and its homologs appeared as a series with R_f values of 0.78, 0.69, 0.59, and 0.47 according to the chain length. In the chromatogram of the product of **14** (C_9 compound), a peak in the predicted region for tetrahomofarnesol did not appear, but a single peak was found at R_f 0.73, forming a geraniol homolog series in the chromatograms. The products from **15** and **16** (C_{10} and C_{11} compound) also gave, as expected, only peaks which belonged to the geraniol homolog series. Thus, it is reasonable to assume that homologs of geraniol, **22**, **24**, **26**, **27**, and **28**, form a series with R_f values of 0.95, 0.82, 0.73, 0.66, and 0.57. In the chromatograms of the products derived from the *cis* isomers, **18**, **19**, and **20** (Figure 4), the radioactive peaks appearing at R_f 0.70, 0.59, and 0.46 can undoubtedly be attributed to homologs of farnesol, **30**, **32**, and **34**, respectively. The formation of bishomogeraniol **23** from **19** was also suggested by the radioactive peak at an R_f of 0.92 (Figure 4h). However, the formation of trishomogeraniol **25**, which was expected to be derived from **20**, was obscure in the chromatogram (Figure 4i) because of the presence of ^{14}C-farnesol due to the contamination of the preparation of farnesyl pyrophosphate synthetase with iso-

(15) G. P. McSweeney, *J. Chromatogr.*, 17, 183 (1965).

Scheme III



22 : $R_1 = n\text{-}C_3H_7$, $R_2 = CH_3$
23 : $R_1 = CH_3$, $R_2 = n\text{-}C_3H_7$
24 : $R_1 = n\text{-}C_4H_9$, $R_2 = CH_3$
25 : $R_1 = CH_3$, $R_2 = n\text{-}C_4H_9$
26 : $R_1 = n\text{-}C_5H_{11}$, $R_2 = CH_3$
27 : $R_1 = n\text{-}C_6H_{13}$, $R_2 = CH_3$
28 : $R_1 = n\text{-}C_7H_{15}$, $R_2 = CH_3$



29 : $R_1 = C_2H_5$, $R_2 = CH_3$
30 : $R_1 = CH_3$, $R_2 = C_2H_5$
31 : $R_1 = n\text{-}C_3H_7$, $R_2 = CH_3$
32 : $R_1 = CH_3$, $R_2 = n\text{-}C_3H_7$
33 : $R_1 = n\text{-}C_4H_9$, $R_2 = CH_3$
34 : $R_1 = CH_3$, $R_2 = n\text{-}C_4H_9$

pentenyl pyrophosphate isomerase. The contamination with isopentenyl pyrophosphate isomerase was negligible for the incubations of substrates of sufficient reactivity, so that radioactive farnesol was not detected in the chromatograms shown in Figure 3. However, in the case of *cis*-3-methyl-2-heptenyl pyrophosphate (**20**) the isomerase activity, even though weak, cannot be neglected; particularly because of the poor reactivity of **20** a large amount of the enzyme had to be used in the incubation. Since even in the incubation with **19** a small amount of radioactive farnesol was observed (Figure 4h), it must be assumed that a much larger portion of the radioactivity in the products of **20** was associated with farnesol. Accordingly, the radioactivity in the peak around R_f 0.8 cannot be attributed wholly to trishomogeraniol **25**, but at least partly to farnesol.[16]

From these facts it is concluded that the product of the longest chain that can be synthesized by the pumpkin prenyltransferase is trishomofarnesyl pyrophosphate (C_{18} compound) derived from either **13** or **20**. This fact indicates indirectly also that trishomogeranyl pyrophosphate (C_{13} compound), regardless of the geometry with respect to the double bond at the 6 position, is the reactive homolog of the longest chain, and that tetrahomogeranyl pyrophosphate (C_{14} compound) derived from **14** can no longer react with another molecule of isopentenyl pyrophosphate (**1**). The shortest homolog that accumulates as an intermediate is bishomogeranyl pyrophosphate (C_{12} compound).[17]

(16) The control incubation of the enzyme with ^{14}C-isopentenyl pyrophosphate without allylic pyrophosphate gave ^{14}C-farnesol in an amount corresponding to one-third of the radioactivity in the product of **20**, but this value would not be necessarily true in the incubation with **20** because the effect of **20** on the isopentenyl pyrophosphate isomerase and prenyltransferase reactions with the natural substrates is unknown.

(17) In the experiment by Popják, *et al.*,[6] with liver enzyme, accumulation of bishomogeranyl pyrophosphate was not observed in the reaction of the 3-methyl-2-hexenyl pyrophosphates.

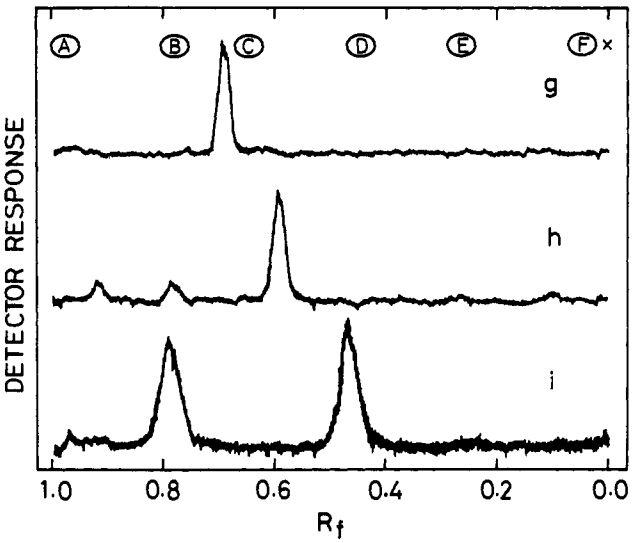


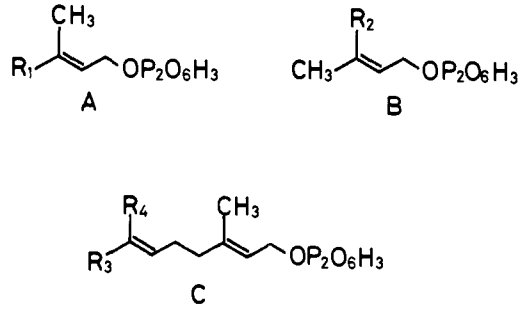
Figure 4. Thin-layer radiochromatograms of products derived from *cis*-3-methyl-2-alkenyl pyrophosphates. Starting substrates are **18** (g), **19** (h), and **20** (i). Spots of reference alcohols: A, geraniol; B, farnesol; C, nerolidol; D, geranylgeraniol; E, geranyllinalool; F, phytol.

Comparison of the chromatograms (Figures 3b and 4h) indicates that accumulation of bishomogeranyl pyrophosphate in the reaction of *trans*-3-methyl-2-hexenyl pyrophosphate (**12**) is more conspicuous than a similar product derived from the *cis* isomer **19**. In the products derived from *trans*-3-methyl-2-heptenyl pyrophosphate (**13**), trishomogeranyl pyrophosphate was the major product and trishomofarnesyl pyrophosphate was a minor product, whereas the main product from the *cis* isomer **20** was trishomofarnesyl pyrophosphate (Figures 3c and 4i). In intermediates such as bishomogeranyl and trishomogeranyl pyrophosphate that are formed with difficulty from the *cis* substrates, **19** and **20**, the *cis* structure is far removed from the pyrophosphate moiety, but they have the *trans* geometry at the 2 position. The enzyme might prefer these homologs of geranyl pyrophosphate to the starting *cis* substrates to give rise to the homologs of farnesyl pyrophosphate as a major product. Homogeranyl pyrophosphate (C_{11} compound), which is thought to be the intermediate in the reaction of **11** or **18**, is converted into homofarnesyl pyrophosphate without accumulation. The probable structures of the homologs of geraniol and farnesol derived from nine 3-methyl-2-alkenyl pyrophosphates are summarized in Scheme III.

Discussion

The structural requirement for the substrate of the pumpkin prenyltransferase is that the substituent R_1, with reference to formula A, can be as large as $n\text{-}C_7H_{15}$ but that R_2 in **B**, however, can never be larger than $n\text{-}C_4H_9$. When homologs of geranyl pyrophosphate as intermediate are taken into account, either R_3 or R_4 in formula C cannot be larger than $n\text{-}C_4H_9$. Popják, *et al.*,[6] suggested that the substrates of liver prenyltransferase fit into a narrow groove in the enzyme by showing that neither 3-*t*-butyl-2-butenyl nor 3-

Scheme IV



phenyl-2-butenyl pyrophosphates were substrates or inhibitors. This idea is supported also by our observation that even *trans*-3,5-dimethyl-2-hexenyl pyrophosphate (**17**) was not accepted, in contrast to the reactivity of the nonbranched isomer, *trans*-3-methyl-2-heptenyl pyrophosphate (**13**).

The reactive 3-methyl-2-alkenyl pyrophosphates can be placed in three categories according to their behaviors; one is the DPP type acting in place of dimethylallyl pyrophosphate (**2**) to condense with two molecules of isopentenyl pyrophosphate (**1**); the second is the GPP type which condenses with one molecule of **1**; and the third is the mixed type which belongs to both types giving two products. An analog of the GPP type was not found in the *cis* series. The reaction velocity of *cis* isomers decreases with the increase of the chain length, while that of *trans* isomers, however, shows a quite different tendency; the C_7 compound appeared to show the lowest reactivity, and C_9, C_{10}, and C_{11}, which are analogs of the GPP type, are highly reactive.

Table I. Relation between Starting Substrates and the Products

Carbon no. of Starting substrate	Product	Type of analog
trans series		
C_6	$\rightarrow (C_{11})^a \rightarrow C_{16}$	DPP
C_7	$\rightarrow C_{12} \rightarrow C_{17}$	Mixed
C_8	$\rightarrow C_{13} \rightarrow C_{18}$	Mixed
C_9	$\rightarrow C_{14}$	GPP
C_{10}	$\rightarrow C_{15}$	GPP
C_{11}	$\rightarrow C_{16}$	GPP
cis series		
C_6	$\rightarrow (C_{11}) \rightarrow C_{16}$	DPP
C_7	$\rightarrow C_{12} \rightarrow C_{17}$	Mixed
C_8	$\rightarrow C_{13} \rightarrow C_{18}$	Mixed

[a] Parentheses indicate an unisolated intermediate.

There might be two separate sites for dimethylallyl and geranyl transferring activities in farnesyl pyrophosphate synthetase, and C_6 compounds might fit to the former site, C_9, C_{10}, and C_{11} compounds to the latter site, and C_7 and C_8 compounds, though with difficulty, to both sites.

Previous observations on selective inactivation of dimethylallyl transferring activity[18] and differential inhibitions of the two activities of farnesyl pyrophosphate synthetase of pumpkin by substrate analogs[9] are not inconsistent with this assumption that two sites are involved in the enzyme.

(18) K. Ogura, T. Koyama, and S. Seto, *Biochem. Biophys. Res. Commun.*, **35**, 875 (1969).

Experimental Section

Materials. Farnesyl pyrophosphate synthetase was obtained from pumpkin fruit by the method previously reported.[5] ^{14}C-Isopentenyl pyrophosphate (specific activity, 1.2 μCi/μmol), dimethylallyl pyrophosphate, and geranyl pyrophosphate were the same preparations as in the previous study.[5] Methyl alkyl ketones were the products of Tokyo Chemical Industry Co., Ltd., except methyl *n*-hexyl ketone, which was obtained by chromic acid oxidation of commercially available 2-heptanol.

General Procedures of Synthesis. Methyl 3-Methyl-2-alkenoates. Diethyl methoxycarbonylmethyl phosphonate (0.1 mol) was added to a solution of sodium methoxide (0.1 g-atom of sodium in 20 ml of absolute methanol), and then methyl alkyl ketone (0.1 mol) was added dropwise over 30 min, while the mixture was stirred and occasionally cooled to avoid violent reaction. After the complete addition the mixture was stirred for 1 hr at room temperature and *ca.* 50 ml of water was added. The mixture was then extracted three times with ethyl ether, and the combined extracts were dried over sodium sulfate overnight. After removal of the solvent the residue was distilled to afford a mixture of *trans* and *cis* isomers of methyl 3-methyl-2-alkenoate[19] (ratio of *trans* to *cis* ester, *ca.* 3:1) in 80–90% yield. The mixture of the esters was stirred in 1.2 equiv of 1 *N* sodium hydroxide and 5–10 ml of methanol at 70° until solution was complete (*ca.* 4 hr). The resulting solution was washed with ethyl ether, acidified with dilute hydrochloric acid, and then extracted with ethyl ether or petroleum ether. Evaporation of the solvent left colorless crystals on cooling, which were collected and recrystallized from suitable solvents to afford pure *trans* acid. 3-Methyl-2-pentenoic (**8a**) and 3-methyl-2-hexenoic acid (**8b**) were recrystallized from petroleum ether (bp 40–50°), 3-methyl-2-

Table II. Characteristics of Compounds

Compd R	δ (ppm) in nmr[a] CH_3—C=	H—C=	Retention time in glpc, min[b]	Mp, °C[c]
trans-R(CH_3)C=$CHCO_2CH_3$				
C_2H_5 (**6a**)	2.14	5.61	3.8	
n-C_3H_7 (**6b**)	2.13	5.65	5.8	
n-C_4H_9 (**6c**)	2.12	5.65	9.0	
n-C_5H_{11} (**6d**)	2.13	5.61	14.9	
n-C_6H_{13} (**6e**)	2.14	5.63	24.0	
n-C_7H_{15} (**6f**)	2.15	5.65	41.6	
i-C_4H_9 (**6g**)	2.13	5.63	6.8	
cis-R(CH_3)C=$CHCO_2CH_3$				
C_2H_5 (**7a**)	1.89	5.61	3.1	
n-C_3H_7 (**7b**)	1.90	5.65	4.8	
n-C_4H_9 (**7c**)	1.86	5.65	7.2	
n-C_5H_{11} (**7d**)	1.88	5.61	11.6	
n-C_6H_{13} (**7e**)	1.86	5.63	18.1	
n-C_7H_{15} (**7f**)	1.86	5.61	31.4	
i-C_4H_9 (**7g**)	1.86	5.63	5.9	
trans-R(CH_3)C=$CHCO_2H$				
C_2H_5 (**8a**)	2.21	5.71		46.0–48.0[d]
n-C_3H_7 (**8b**)	2.17	5.70		33.0–34.0
n-C_4H_9 (**8c**)	2.17	5.69		31.0–32.0
n-C_5H_{11} (**8d**)	2.18	5.70		9.0–10.0
n-C_6H_{13} (**8e**)	2.18	5.68		6.0–7.0
n-C_7H_{15} (**8f**)	2.17	5.68		24.0–25.0
i-C_4H_9 (**8g**)	2.12	5.62		39.5–40.0

[a] The nmr spectra were measured with a JNM-C-60HL nmr instrument (Japan Electron Optics Laboratory Co., Ltd.) on samples in carbon tetrachloride with tetramethylsilane as internal standard. [b] The glpc was carried out with a Hitachi gas chromatograph Model KGL-2A fitted with a 2-m column packed with 11% silicone oil DC 705 on base-washed 30–50 mesh firebrick with helium gas at a flow rate of 25.0 cc/min. The column temperature was 153.0°. [c] Melting points were not corrected. [d] Mp 48–49° in literature (G. A. R. Kon, R. P. Linstead, and J. M. Wright, *J. Chem. Soc.*, 599 (1934); J. W. Cornforth, R. H. Cornforth, G. Popják, and L. Yengoyan, *J. Biol. Chem.*, **241**, 3970 (1966).

(19) A mixture of **6b** and **7b** was obtained by Popják, *et al.*,[6] by the Reformatsky reaction followed by dehydration. They reported that the separation of either **6b** and **7b** or the corresponding alcohols, **9b** and **10b**, was not successful.

Table III. Characteristics of Compounds

Compd R	δ (ppm) in nmr[a] $CH_3—C=$	$H—C=$	$—CH_2—O$	$—CH_2—C=$	$CH_3—CH_2—$	Retention time in glpc,[b] min
trans-$R(CH_3)C=CHCH_2OH$						
C_2H_5 (**9a**)	1.63	5.30	3.98	2.00	1.00	2.6
n-C_3H_7 (**9b**)	1.63	5.40	4.06	2.02	0.90	4.0
n-C_4H_9 (**9c**)	1.62	5.30	4.00	1.98	0.90	6.3
n-C_5H_{11} (**9d**)	1.63	5.37	4.06	2.01	0.88	10.8
n-C_6H_{13} (**9e**)	1.63	5.36	4.05	2.00	0.89	17.0
n-C_7H_{15} (**9f**)	1.63	5.37	4.06	2.00	0.89	28.1
i-C_4H_9 (**9g**)	1.63	5.36	4.07	1.87		4.5
cis-$R(CH_3)C=CHCH_2OH$						
C_2H_5 (**10a**)	1.70	5.30	4.00	2.06	0.98	2.3
n-C_3H_7 (**10b**)	1.72	5.40	4.05	2.06	0.90	3.5
n-C_4H_9 (**10c**)	1.70	5.30	3.98	2.02	0.91	5.4
n-C_5H_{11} (**10d**)	1.72	5.35	4.02	2.06	0.90	9.3

[a] The nmr spectra were measured with a JNM-C-60HL nmr instrument (Japan Electron Optics Laboratory Co., Ltd.) on samples in carbon tetrachloride with tetramethylsilane as internal standard. [b] The glpc was carried out with a Hitachi gas chromatograph Model KGL-2A fitted with a 2-m column packed with 11% silicone oil DC 705 on base-washed 30–50 mesh firebrick with helium gas at a flow rate of 25.0 cc/min. The column temperature was 153.0°.

heptenoic (**8c**), 3-methyl-2-octenoic (**8d**), 3-methyl-2-nonenoic (**8e**), and 3-methyl-2-decenoic acid (**8f**) from methanol, and 3,5-dimethyl-2-hexenoic acid (**8g**) from methanol–water. Individual melting points are given in Table II. The purity of each acid was confirmed by the glpc of its methyl ester obtained by treatment of a part of the specimen with diazomethane and by the nmr of the free acid and its ester. The yield of the *trans* acids was 1–2 g. For the preparation of **8a**, **8b**, and **8c**, ice cooling of the mixture caused crystallization, but the mixture was cooled in a Dry Ice–acetone bath for the crystallization of the other acids. Recrystallization was carried out in a cold room (−10°). To the combined filtrates after removal of the *trans* acid, a slight excess of ethereal diazomethane was added, and the resulting mixture of the esters (ratio of *trans* to *cis* isomer, *ca.* 1) was placed on a column (1.5 × 200 cm) of silica gel. The column was eluted with *n*-hexane. Pure *cis* isomer emerged in the earlier fractions. After evaporation of the solvent from the combined fraction, the residue was distilled under diminished pressure to give the *cis* ester. The purity was proved by glpc and nmr. Approximately 300–500 mg of pure specimens was obtained.

***trans*-3-Methyl-2-alkenyl Alcohols.** A solution of a *trans* acid (2–3 mmol) in 10 ml of dry ether was added dropwise into an ice-cooled suspension of $LiAlH_4$ (1.2 equiv) in ether with stirring. After the complete addition the mixture was stirred at room temperature for 2 hr. Crushed ice was added to the mixture to decompose the excess $LiAlH_4$, and then 50% sodium hydroxide (*ca.* 20 ml) was added. The resulting slurry mixture was extracted with ethyl ether, and the combined ethereal solution was centrifuged to remove precipitates. After drying the solution over sodium sulfate, the ether was distilled off, and the residue was then distilled under vacuum to afford the corresponding alcohol in 70–75% yield.

***cis*-3-Methyl-2-alkenyl Alcohols.** *cis*-Esters (300–500 mg) were treated with $LiAlH_4$, the products were worked up in the same way as described for the *trans* isomers, and the corresponding alcohols (200–300 mg) were obtained. For the preparation of *cis*-3-methyl-2-octenol (**10d**), the experiment was carried out in a half-scale, and *ca.* 100 mg of **10d** was obtained.

The purity was examined by glpc and nmr, the data of which are summarized in Tables II and III.

3-Methyl-2-alkenyl Pyrophosphates. The alcohols were phosphorylated and the pyrophosphate esters were obtained in 10–15% yield by the method of Cramer and Böhm[13] as modified by Kandutsch, *et al.*[14] The phosphorylations were carried out in a 1-mmol scale except that 0.3 mmol of *cis*-3-methyl-2-octenol (**10d**) was phosphorylated. Identification of the products was made on the basis of the comparison of the ir with those of authentic specimens of **2** and **3**.

Enzymic Reaction. In the experiments with the *trans* isomers (Figure 1), the reaction mixture contained, in a final volume of 1.0 ml, 40 μmol of phosphate buffer, pH 7.0, 5 μmol of magnesium chloride, 25 mμmol of ^{14}C-isopentenyl pyrophosphate (1.2 μCi/μmol), an allylic pyrophosphate to be examined in the amount indicated (5 mμmol–75 mμmol), and 0.1 mg (estimated from optical density at 280 mμ) of farnesyl pyrophosphate synthetase. After the mixture had been incubated at 37° for 20 min the reaction was stopped by the addition of 0.3 ml of 1 *N* hydrochloric acid, and the mixture was kept at 37° for 15 min to complete the hydrolysis of allylic pyrophosphates. The mixture was made alkaline with 2.5 ml of 0.2 *N* sodium hydroxide. The mixture was then extracted with 5 ml of *n*-hexane and the extracts were washed with water. The radioactivity in the *n*-hexane extracts was determined in a toluene scintillator with a Kobekogyo liquid scintillation counter Model GCL-111. In the experiments with the *cis* isomers (Figure 2) the incubations were carried out in 12 times scale in order to obtain sufficient radioactivity in the *n*-hexane extracts. For the sake of comparison the reaction velocity in Figure 2 was given by one-twelfth of the total radioactivity in the extracts.

Analysis of Products of Enzymic Reaction. For preparing the samples for tlc analysis, large scale incubations (four times as much as the standard incubation described in the preceding section) were carried out for 1 hr, unless otherwise stated. After the incubation the mixture was adjusted to pH 9.0 with Tris buffer, and intestinal alkaline phosphatase (10 μl, Boehringer, Grade II, 10 mg/ml) was added. After the mixture was incubated at 37° for 3 hr, it was allowed to stand at room temperature overnight. The mixture was then diluted by the addition of 2 ml of water, and extracted with petroleum ether (two 4-ml portions). The combined petroleum extracts which had been washed thoroughly with water were evaporated to a small volume, and portions of the concentrated solution were applied to a thin-layer plate. The samples thus obtained did not contain ^{14}C-isopentyl alcohol derived from the starting substrate. As an exception, in preparing a sample of the products derived from *cis*-3-methyl-2-heptenyl pyrophosphate (**20**), four times the amount of enzyme was used and the incubation was made for 3 hr.

Thin-layer chromatography was carried out, essentially according to the method of McSweeney,[15] on an Avicel SF plate (microcrystalline cellulose, Funakoshi Chemicals Co., Tokyo) impregnated with liquid paraffin in a system of acetone–water (65:35) saturated with liquid paraffin. Geraniol, farnesol, nerolidol, geranylgeraniol, geranyllinalool, and phytol were added to the sample for the reference. After development, radioactive regions on the plate were located with an Aloka thin-layer radiochromatogram scanner (Nippon Musen Co.). The reference alcohols were located by exposing the plate to iodine vapor.

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