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Studies on Isopentenyl Pyrophosphate Isomerase with Artificial Substrates: *Z–E* Isomerization of *Z*-3-Methyl-3-pentenyl Pyrophosphate

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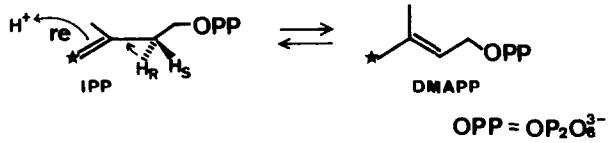
The reaction of isopentenyl pyrophosphate isomerase of pig liver in deuterated water was examined with five artificial substrate homologs, 3-ethyl-3-butenyl- (**1**), *E*-3-methyl-3-pentenyl- (**2**), *Z*-3-methyl-2-pentenyl- (**3**), *E*-3-methyl-2-pentenyl- (**4**), and *Z*-3-methyl-3-pentenyl pyrophosphate (**5**). The course of deuterium incorporation into the products was monitored by coupled gas chromatographic–mass spectrometric analysis using selected ion monitoring. Two new isomerization reactions for the artificial homologs were discovered in addition to those reported previously by us [*J. Biol. Chem.* **248**, 8043 (1973)]: The artificial homolog **5** is apparently isomerized irreversibly to the *E*-isomer **2** via **4** as an intermediate. The conversion of **2** to **1** was confirmed showing that the isomerization between **1** and **2** is reversible even though the equilibrium is heavily in favor of **2**.

INTRODUCTION

In the biosynthesis of isoprenoid compounds, isopentenyl pyrophosphate isomerase (EC 5.3.3.2) catalyzes the isomerization of isopentenyl pyrophosphate (IPP) to the allylic isomer, dimethylallyl pyrophosphate (DMAPP) prior to the chain-elongation of isoprene units catalyzed by prenyltransferase.

The stereochemical course of the reaction catalyzed by isopentenyl pyrophosphate isomerase has been demonstrated as shown in Scheme 1: The *pro-R* hydrogen atom at 2 position of IPP is eliminated (*1*). The new methyl group that is formed on DMAPP during the reaction is *trans* to the carbon atom 1 (*2, 3*). A proton adds to the *re* face of the double bond of IPP to form the new methyl group (*4, 5*). The same stereochemical course is followed in the reverse process from DMAPP to IPP.

We have reported (*3*) that a homoisopentenyl pyrophosphate, 3-ethyl-3-butenyl pyrophosphate (**1**) undergoes abnormal isomerization by the action of pig liver



SCHEME 1

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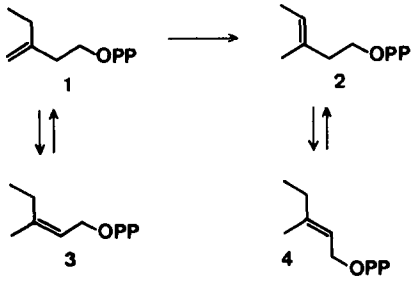


SCHEME 2

isopentenyl pyrophosphate isomerase to produce a mixture of *E*-3-methyl-3-pentenyl- (**2**), *Z*- (**3**) and *E*-3-methyl-2-pentenyl pyrophosphate (**4**), the homoallylic isomer **2** being the major product (Scheme 2). These data have led to the proposal that the enzyme has at least two binding sites (P site and M site)—one for the pyrophosphate moiety and the other for holding the terminal methyl group (*3*). In other words, the enzyme has at least two catalytic groups (X and Y) that are used for the donation and abstraction of protons during the reaction.

Since the homoisopentenyl pyrophosphate **1** has been thought to be a possible precursor of insect juvenile hormones (*6–11*) which have homoterpenoid structures, the unexpected results of the isomerization of **1** by the enzyme from pig liver led us to examine the detailed reaction mechanism of the enzyme by use of a new homologous artificial substrate. This paper describes another abnormal isomerization of a new homolog of isopentenyl pyrophosphate.

MATERIALS AND METHODS

Deuterium oxide (99.75% purity) for the reaction medium was a product of Merck. Alkaline phosphatase from calf intestine was purchased from Boehringer Mannheim. Isopentenyl pyrophosphate (IPP), 3-ethyl-3-butenyl- (**1**), *E*-3-methyl-3-pentenyl- (**2**), *Z*-3-methyl-2-pentenyl- (**3**), and *E*-3-methyl-2-pentenyl pyrophosphate (**4**) were the same preparations as used in a previous study (*3*). *Z*-3-Methyl-3-pentenyl pyrophosphate (**5**) was prepared by the phosphorylation of the corresponding alcohol **5-OH**[1] which was used in a previous study (*3*).

Isopentenyl pyrophosphate isomerase. This enzyme was purified from pig liver essentially according to the method reported by Banthorpe *et al.* (*12*).

Pig liver (1.0 kg) was homogenized in 2 liters of 50 m*M* Tris–HCl buffer, pH 6.8, with a Waring blender for 90 sec. The homogenate was centrifuged at 23,000*g* for 30 min and then fractionated with ammonium sulfate. The fraction precipitating between 40 and 80% saturation was collected and dissolved in a minimum volume of 50 m*M* Tris–HCl buffer, pH 6.8. The resulting solution was desalted by passage through a Sephadex G-25 column equilibrated with the same buffer as used for the homogenization, and was then applied to a DE-52 column (4.5 × 90 cm), which was

[1] **5-OH**, **2-OH**, etc. refers to the free alcohol from pyrophosphate **5**, **2**, etc.

eluted with the same buffer. Protein in the enzymatically active fraction was precipitated with ammonium sulfate (80% saturation) and was collected by centrifugation (23,000*g*, 30 min). The precipitated protein was dissolved in a minimum volume of the starting buffer, and then about a quarter of the protein solution was applied to a Sephadex G-200 column (4.0 × 200 cm) which had been equilibrated with the starting buffer and was eluted with the same buffer. The enzyme fraction was collected and concentrated by ultrafiltration with an Amicon UM-10 membrane. The concentrated enzyme solution (usually 9.0 mg/ml protein) was used for the reaction.

Enzymatic reactions of artificial substrate homologs in deuterium oxide. At first, a solution which contained 20 μmol of $MgCl_2$, 20 μmol of KF, 40 μmol of Tris–HCl buffer, pH 6.8, and 4.5 mg of isopentenyl pyrophosphate isomerase fraction purified from pig liver was lyophilized to dryness. To the resulting residue was added deuterium oxide solutions of 10 μmol of dithiothreitol and 500 nmol of an artificial substrate to be examined. Then more deuterium oxide was added to a final volume of 2.0 ml. This mixture was incubated at 37°C for an appropriate time. After the incubation, 7 units of alkaline phosphatase and 500 μmol of Tris–HCl buffer, pH 9.0, were added and the mixture was incubated again at 37°C for more than 3 hr to hydrolyze pyrophosphate esters thoroughly. To the mixture was added 2 ml of saturated NaCl solution and 0.5 ml of 1 N NaOH, and the product alcohols were extracted several times with diethyl ether. The extracts were combined and washed with a saturated NaCl solution and subjected to gas chromatographic–mass spectrometric (GC–MS) analysis.

A Shimadzu-LKB gas chromatograph–mass spectrometer Type 9000 equipped with a multiple ion detector (MID) was used for the analysis. The gas chromatographic analysis was carried out under conditions A, at 130°C on a 3-m 20% carbowax 20 M column; or B, at 110°C on a 5-m 20% carbowax 20 M column. Helium carrier gas was used at a flow rate of 30 ml/min. The potential of the ionizing electron beam was 20 eV. These conditions were used throughout this study unless otherwise stated.

RESULTS

Enzymatic Isomerization of Isopentenyl Pyrophosphate in Deuterium Oxide

Using purified isopentenyl pyrophosphate isomerase, we have already studied the enzymatic isomerization of the natural substrate IPP, in deuterium oxide to demonstrate that the exo-methylene group of IPP becomes the methyl group *trans* to the $CH_2OP_2O_6^{3-}$ group in DMAPP (*3*).

This time the extent of isomerization of IPP in deuterium oxide was examined. The time course of the reaction (Fig. 1) showed that the isomerase reaction reached an equilibrium in about an hour when the relative content of DMAPP to IPP was about 75 : 25. This equilibrium composition is not in good agreement with previously reported values of about 85 : 15 (*12–14*). This is probably due to the presence in the substrate preparation of isopentenyl monophosphate which also gives isopentenol.

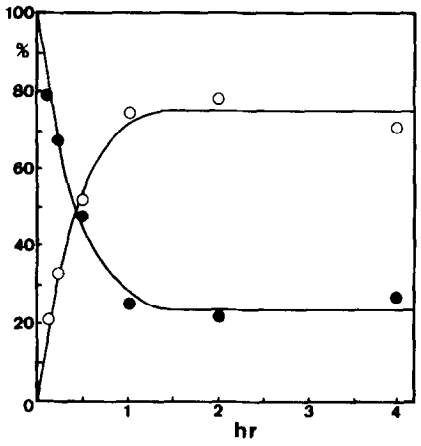


FIG. 1. Time course of enzymatic isomerization between IPP and DMAPP. IPP (500 nmol) was incubated in a similar reaction medium to that described under Materials and Methods. After treatment with alkaline phosphatase the relative content of IPP and DMAPP in the reaction mixture was calculated from the relative intensity of the peaks corresponding to isopentenol and dimethylallyl alcohol in the gas chromatograms of the reaction products. ○, DMAPP; ●, IPP.

The relative contents of deuterated species of IPP and DMAPP (Figs. 2A and B, respectively) were calculated on the basis of the data in Fig. 1. The content of nonlabeled isopentenol still remained at a level of 55% at an incubation time of 4 hr, indicating that about 13% of the substrate was isopentenyl monophosphate. Even after the reaction reached an apparent equilibrium in about 1 hr, the trideuterated species of DMAPP gradually increased to become the major reaction

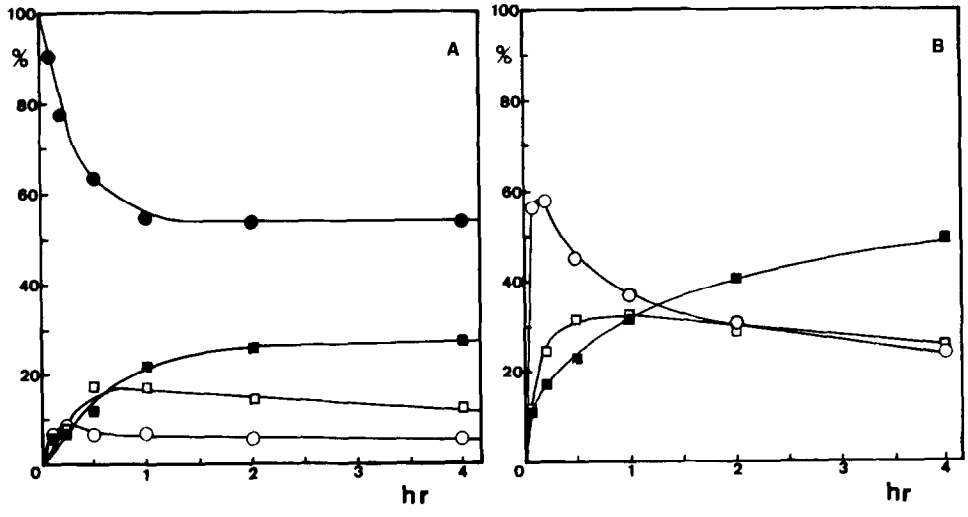


FIG. 2. Time course of deuterium incorporation into IPP (A) and DMAPP (B) during the reaction. The relative contents of deuterated species were calculated from the intensities of the molecular ions in the mass spectra taken by the GC–MS analyses of the same product alcohols as described in Fig. 1. ●, nonlabeled (*m/z* 86); ○, mono- (*m/z* 87); □, di- (*m/z* 88); and ■, trideuterated species (*m/z* 89).

product. This fact indicates that the enzyme is still active after 1 hr (Fig. 2B). Under these reaction conditions it took more than 4 hr for the enzymatic reaction to reach equilibrium with respect to relative contents of the deuterated species.

Reaction of 3-Ethyl-3-butenyl Pyrophosphate (**6**)

The unusual isomerization of **1** to the isomers **2**, **3**, and **4** by the action of pig liver isopentenyl pyrophosphate isomerase was further examined in deuterium oxide. Figure 3 is the time course of the isomerization of **1** in deuterated water. The reaction reached equilibrium in about 8 hr. The relative contents of the four isomers, **1**, **2**, **3**, and **4** in the reaction mixture after 16 hr were 11.8, 73.1, 2.9, and 12.2%, respectively. The formation of the nonallylic isomer **2** became predominant at the early stage of incubation (less than 1 hr).

Figure 4 shows the GC–MID chromatograms of the products obtained by the reaction of **1** in deuterated water. In the MID chromatograms for *m/z* 103 the peaks at retention times of 69 and 88 min which correspond to **2-OH** and **4-OH**, respectively, gradually become predominant as the incubation goes on.

No formation of *Z*-3-methyl-3-pentenyl pyrophosphate (**5**) was observed during the enzymatic reaction.

Reaction of E-3-methyl-3-pentenyl Pyrophosphate (**2**)

According to the mechanism that we proposed previously (*3*), **2** undergoes reversible isomerization to give the allylic isomer **4** in the same stereochemical manner as that of the natural substrate, IPP. Thus, the reaction in D_2O would produce monodeuterated **2** and monodeuterated **4** as shown in Scheme 3. The

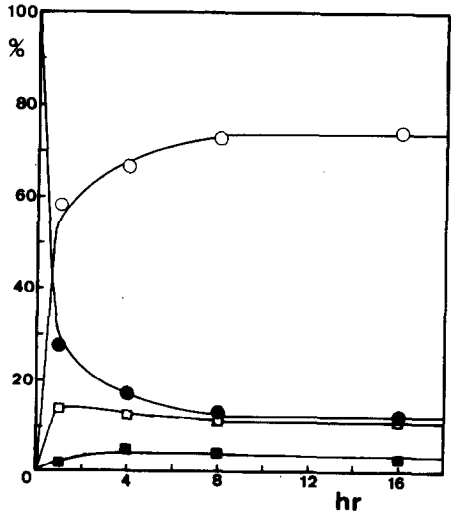


FIG. 3. Time course of isomerization of **1** in deuterium oxide. The relative contents of the isomers were calculated from the relative intensities of the peaks in the TIM chromatograms of the corresponding alcohols of the products in Fig. 4. ●, **1**; ○, **2**; ■, **3**; □, **4**.

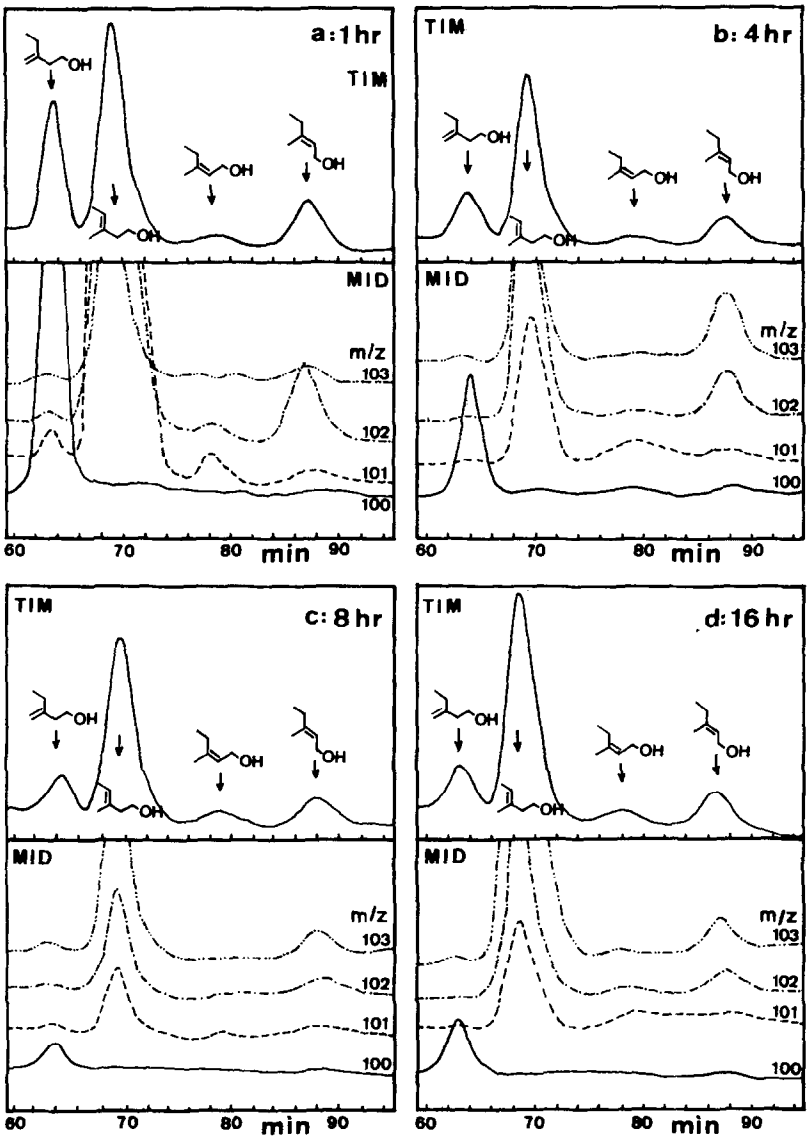
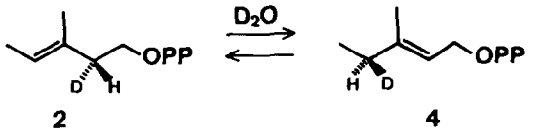


FIG. 4. GC–MID chromatograms of the products obtained by the isomerization of **1** in deuterium oxide. Enzymatic isomerization of **1** was carried out for various incubation times as described under Materials and Methods. Incubation times; a, 1; b, 4; c, 8; and d, 16 hr. The MID chromatograms (lower) were obtained by monitoring the fragments ions at *m/z* 100 (—), 101 (-----), 102 (-·-·-), and 103 (-···-). Conditions of GLC: B.



2 **4**

SCHEME 3

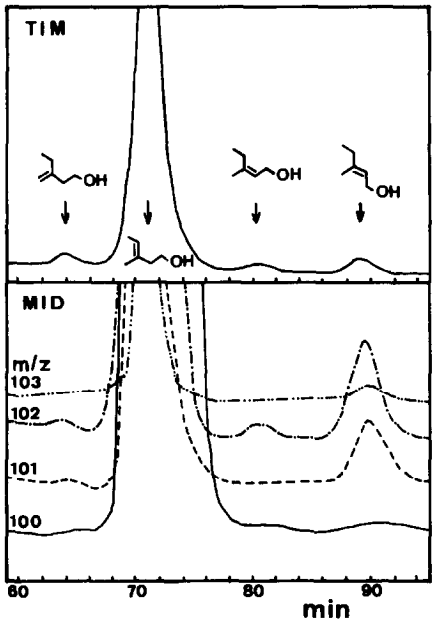


FIG. 5. GC–MID chromatogram of the products obtained by the enzymatic isomerization of **2** in deuterium oxide. Enzymatic reaction of **2** was carried out for 1 hr as described under Materials and Methods. The MID chromatogram (lower) was obtained by monitoring the fragment ions at *m/z* 100 (—), 101 (-----), 102 (–·–·–), and 103 (–···–···). Condition of GLC : B.

incorporation of deuterium at **4** position must also be stereospecific because there is no formation of **5**. Figure 5 depicts the GC–MID chromatogram of the products resulting from 1-hr incubation of **2** as the substrate. The appearance of not only mono- (*m/z* 101) but also di- (*m/z* 102) and trideuterated products (*m/z* 103) indicates that there must be an isomerization in addition to that proposed between **2** and **4** in Scheme 3. Labeling with more than one deuterium is most likely attributable to the reversible reaction between **2** and **1**. If the reaction between **1** and **2** is reversible, isomerization between **2** and **1** occurs in addition to the interconversion of **2** and **4**, and then the number of deuterium atoms incorporating into the products becomes more than two. The isomerization between **1** and **2** must be very rapid with the equilibrium of the reaction leaning heavily to **2**, because only a small amount of **1** was detected during the reaction.

*Reaction of Z-3-Methyl-2-pentenyl Pyrophosphate (**3**)*

As shown in Fig. 6 the allylic isomer **3** is apparently less reactive than the homoallylic isomers **1** or **2**. In the TIM chromatogram the major peak appeared at a retention time of 28.5 min which corresponded to **3-OH** derived from the starting material. Although the MID chromatogram at *m/z* 100 indicates that the majority of **3-OH** is derived from the starting material **3** which did not react, a small amount of the dideuterated alcohol was also detected in the chromatogram at *m/z* 102. This product must result from more than two processes of enzymatic isomeriza-

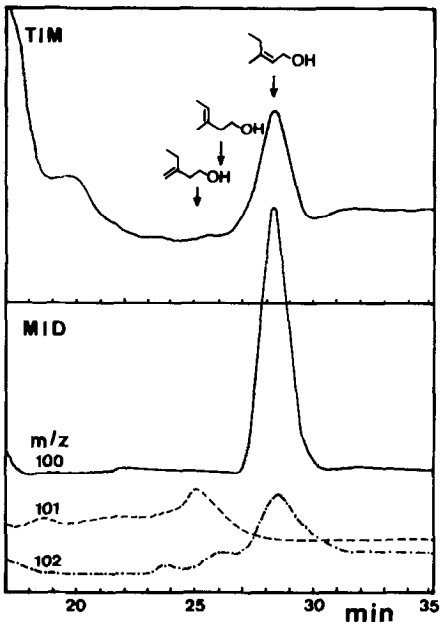


FIG. 6. GC–MID chromatogram of the products obtained by the enzymatic isomerization of **3** in deuterium oxide. Enzymatic reaction of **3** was carried out for 8 hr as described under Materials and Methods. The MID chromatogram (lower) was obtained by monitoring the fragment ions at *m/z* 100 (—), 101 (-----), and 102 (–·–). Conditions of GLC: A.

tion. As monodeuterated **1-OH** is found in the MID chromatogram at *m/z* 101, the product of the first isomerization of **3** must be **1**. Although the isomerization between **1** and **3** is reversible, the reaction seems too slow to produce trideuterated species.

Reaction of E-3-Methyl-2-pentenyl Pyrophosphate (4)

As reported previously (*3*), the allylic isomer **4** was rapidly isomerized to **2**. In the TIM chromatogram in Fig. 7 a distinct peak appears at a retention time of 26 min which corresponds to **2-OH**. The formation of both monodeuterated **2-OH** and monodeuterated **4-OH** indicates that the reversible isomerization between **4** and **2** occurs in a stereochemical manner similar to that of the natural substrates. The occurrence of dideuterated **2-OH**, however, seems not to be the result of direct isomerization between **4** and **2**. This may be attributable to a reversible isomerization between **2** and **1** in addition to the reaction between **4** and **2**.

Reaction of Z-3-Methyl-3-pentenyl Pyrophosphate (5)

As shown in Figs. 4–7, **5**, a geometric isomer of **2**, could not be detected as a reaction product from any of the other isomers **1–4**. Nevertheless, TIM chromatography of the products derived from **5** clearly indicates that **5** can be a substrate of the isomerase to undergo isomerization to **2** and other isomers (Fig. 8). The

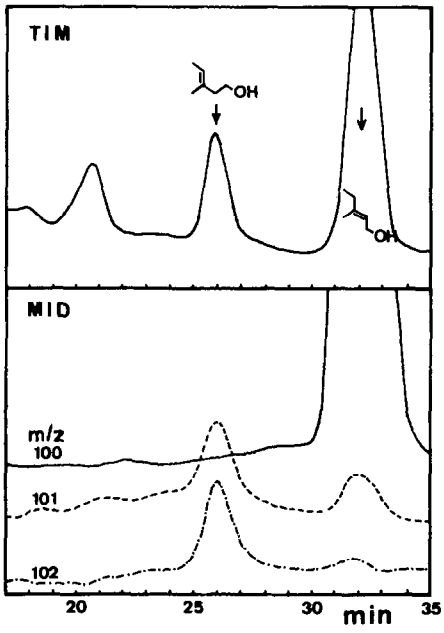


FIG. 7. GC–MID chromatogram of the products obtained by the enzymatic isomerization of **4** in deuterium oxide. Enzymatic reaction of **4** was carried out for 8 hr as described under Materials and Methods. The MID chromatogram (lower) was obtained by monitoring the fragment ions at *m/z* 100 (—), 101 (-----), and 102 (-·-). Conditions of GLC: A.

formation of **2** appears to predominate throughout the reaction. The MID chromatograms show distinctly that the major product **2** consists of di- and trideuterated species and that no monodeuterated species of **2** is present in the reaction mixture. This fact indicates that the homoallylic substrate **5** is isomerized indirectly to the *E* isomer **2** *via* an intermediate isomer. A possible candidate for the intermediate between **5** and **2** must be the allylic isomer **4**, because the MID chromatogram at *m/z* 101 shows a small peak at a retention time of 89 min which corresponds to **4-OH**. Although the MID chromatogram at *m/z* 101 also has a more distinct peak at 73 min which corresponds to **5-OH**, this peak is attributable to the M + 1 peak due to the natural abundance of ^{13}C. Since peaks corresponding to **2-OH** and **4-OH** are also detected in the MID chromatograms at *m/z* 103, trideuterated species of **2** and **4** are produced during the reaction. This fact also indicates that the isomerization between **1** and **2** is reversible. Consequently, the isomerization of **5** by the action of pig liver isopentenyl pyrophosphate isomerase can be depicted as in Scheme 4: At first **5** is isomerized irreversibly to **4**, followed by the reversible isomerization between **4**, **2**, and **1**.

DISCUSSION

The experimental data reported here demonstrate the discovery of two new

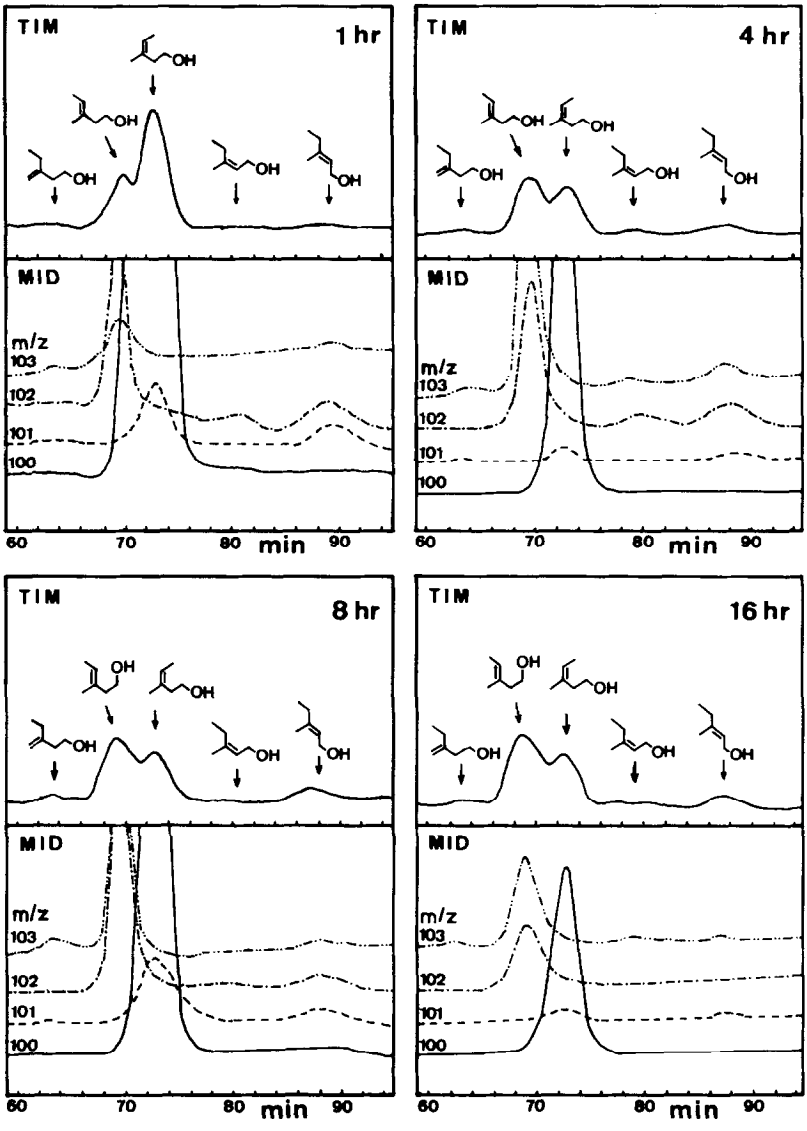
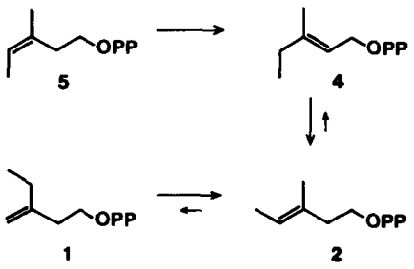


FIG. 8. GC–MID chromatograms of the products obtained by the enzymatic isomerization of **5** in deuterium oxide. Enzymatic reaction of **5** was carried out for various incubation times as described under Materials and Methods. The MID chromatograms (lower) were obtained by monitoring the fragment ions at *m/z* 100 (—), 101 (-----), 102 (–·–·–), and 103 (–···–···). Conditions of GLC: B.

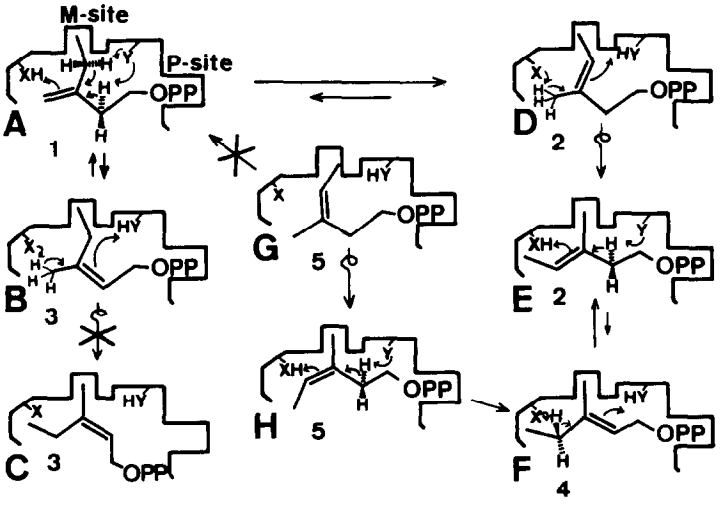
isomerization reactions of artificial homoisopentenyl pyrophosphates in addition to those reported previously (*3*): The homoallylic substrate **5** is isomerized irreversibly to the *E* isomer **2** via **4** as an intermediate. In a strict sense, the isomerization between **1** and **2** is reversible, though the equilibrium leans heavily to the formation of **2**.



SCHEME 4

These observations are reasonably explained by our proposal that isopentenyl pyrophosphate isomerase has at least two binding sites, the P and M sites, for the pyrophosphate moiety and for the methyl group, respectively, and at least two catalytic groups (X and Y) for donating and abstracting protons (*3*). One of the features of our model is that the M site appears to poorly accomodate homologs of IPP where ethyl replaces methyl. In fact, alternate conformations of certain ethyl-containing homologs manipulated to place methyl in the M site give good correlations with our data.

According to this model, the artificial substrate **1** binds in a distorted way so that position 2 of the substrate is situated too far from the group Y which is responsible for the abstraction of the *pro-R* hydrogen at position 2 and that the methylene of the ethyl group occupies the position suitable for proton abstraction by Y (formula A in Scheme 5). As a result, formation of the homoallylic isomer **2** is dominant over the allylic isomer **3**. Since the *Z*-homoallylic isomer **5** is not formed, one of the hydrogens, probably the *pro-S* hydrogen on the methylene of the ethyl group, is removed. The homoallylic pyrophosphate **2** thus produced



SCHEME 5

turns upside-down to fit the enzyme in a more favorable way, with the methyl group and the pyrophosphate moiety occupying the M and P sites of the enzyme (Formula E) like the normal substrate, and, consequently, the substrate undergoes an addition–abstraction of protons in the normal stereochemical manner.

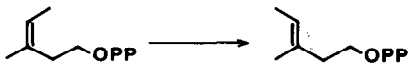
When the homoallylic isomer **2** still remains in the cavity in the same conformation as that when it is produced from **1** (Formula D), in other words, before **2** can rearrange to the more stable form as depicted in Formula E, **2** will undergo reverse isomerization to produce **1**. However, the equilibrium of the isomerization between **1** and **2** leans so heavily toward **2** that the reaction is almost irreversible. On the other hand, the other homoallylic isomer **5** cannot be isomerized directly to **1** probably because the binding state as shown in formula G is not suitable for the catalytic sites. If the isomer **5** turns upside-down to bind to both of the M and P sites of the enzyme in a more suitable way (Formula H), it can be isomerized to **4** in such a normal way that a proton is added by HX– to C-4 from the *re* side with elimination of the *pro-R* hydrogen from C-2. The ethyl group of the isomer **4** thus produced from **5** turns round as shown in Formula F, and then the subtraction by X– of the other hydrogen than that donated from HX– results in the isomerization to the homoallylic isomer **2**.

The equilibrium in the enzymatic isomerization among the C_6– artificial homologs is heavily in favor of the formation of the homoallylic isomer **2**. Thus, such an apparently peculiar isomerization of **5** to **2** occurs as shown in Scheme 6.

We have demonstrated that all of the four homologs of farnesyl pyrophosphate whose carbon skeletons are the same as those of insect juvenile hormones discovered so far can be synthesized enzymatically from four sets of three out of the four substrates, **3**, **1**, DMAPP, and IPP by the action of pig liver farnesyl pyrophosphate synthetase (*6*, *15*). The occurrence of both farnesyl pyrophosphate synthetase and isopentenyl pyrophosphate isomerase in an insect (*Bombyx mori*) has been demonstrated by experiments with cell-free extracts (*15*). In addition to these results, biosynthetic studies using corpora allata of *Manduca sexta* (*7*, *9*–*11*) or of *Hyalophora cecropia* (*8*) indicate that the biosynthesis of juvenile hormones involves isomerization of homoisopentenyl pyrophosphate (**1**) to the allylic isomer **3**. Recently, Baker *et al.* (*16*) have reported that crude homogenates from adult female *M. sexta* corpora cardiaca–corpora allata gland complexes isomerize **1** to **3** and that no homoallylic isomer **2** is formed during the reaction, in contrast to the results obtained in this work using mammalian enzyme.

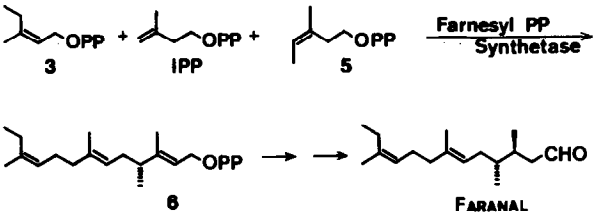
On the other hand, the absolute configuration of faranal, a trail pheromone of the Pharaoh's ant, *Monomorium pharaonis* (*17*), implies that the biosynthesis of the pheromone includes the enzymatic synthesis of the carbon skeleton from **3**, IPP, and **5** by the action of farnesyl pyrophosphate synthetase (Scheme 7).

Thus, the substrate specificity of the isopentenyl pyrophosphate isomerase of



5 **2**

SCHEME 6



SCHEME 7

insects with respect to homoisopentenyl pyrophosphates might be different from that of the enzyme from pig liver. Studies on the specificity of insect isopentenyl pyrophosphate isomerase are now in progress.

ACKNOWLEDGMENTS

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