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A CONVENIENT METHOD OF STEREOCHEMICAL ANALYSIS OF PRENYLTRANSFERASE REACTION AND ITS APPLICATION

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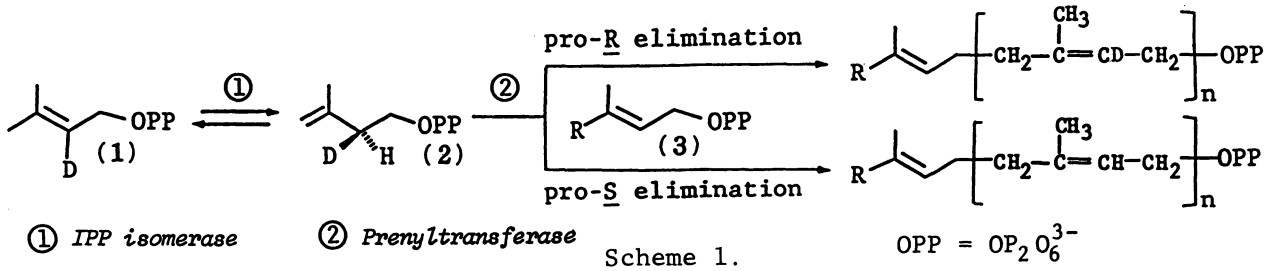
A convenient method based on the utilization of isopentenyl pyrophosphate (IPP) isomerase was developed to facilitate the discrimination of the prochirality of the hydrogen eliminated from IPP during prenyltransferase reaction. *E*,*E*-Farnesyl pyrophosphate synthetase of pumpkin was shown to catalyze the addition of allylic moiety to the *si* face of the double bond of IPP with loss of the 2-pro-*R* hydrogen.

It was once generally accepted that prenyl chains which retain the 4-pro-*R* hydrogen of mevalonic acid (MVA) are biogenetically *E* whereas those which retain the 4-pro-*S* hydrogen are biogenetically *Z*.[1-3] However, such a stereochemical correlation has no longer been considered as a general rule, since subsequent studies revealed that some *Z*-prenyl moieties were formed in higher plants with retention of the 4-pro-*R* hydrogen of MVA.[4-7]

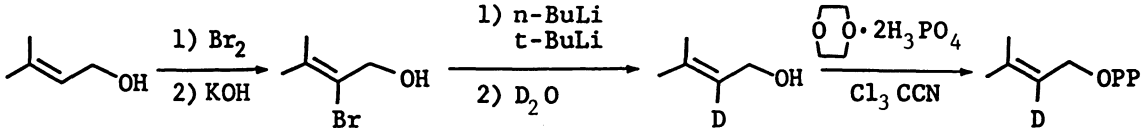
It is now necessary to refine the stereochemical aspect of prenyltransferase reaction by more extensive investigation with enzymes from many different sources. The stereochemistry of the hydrogen elimination of isopentenyl pyrophosphate (IPP) has so far been investigated by the conventional method[8] using ^{14}C- and ^{3}H-mixed labelled specimens of MVA which have to be synthesized by troublesome procedures including radioisotope manipulation. In order to facilitate the stereochemical analysis, we examined a convenient method based on the utilization of dimethylallyl pyrophosphate (DMAPP) labelled with deuterium and the stereospecificity of IPP isomerase reaction. This paper is concerned with this method and its application.

Pig liver IPP isomerase [EC 5.3.3.2] is known to catalyze reversible isomerization between IPP and DMAPP by stereospecific proton addition and elimination, i.e., the conversion of IPP into DMAPP is accompanied by the loss of the 2-pro-*R* hydrogen, and in the reverse reaction a proton is added at the *re* face of C-2 of DMAPP, becoming the 2-pro-*R* hydrogen of IPP.[8,9] Therefore the strategy was that the reaction of [2-^{2}H]DMAPP (**1**) with the isomerase would give a mixture of **1** and (*S*)[2-^{2}H]IPP (**2**), which could be used as a direct substrate for stereochemical diagnosis of prenyltransferase reaction as shown in Scheme 1. It is not necessary to add an allylic substrate (**3**) when the prenyltransferase to be examined can use **1** as the primer as shown in the case of farnesyl pyrophosphate synthetase.



Scheme 1.

[2-^{2}H]DMAPP (^{2}H content, 86%) was synthesized by pyrophosphorylation of the corresponding alcohol obtained as shown below.



In order to confirm the validity of this method, it was applied to bacterial farnesyl pyrophosphate synthetase [EC 2.5.1.10] and undecaprenyl pyrophosphate synthetase [EC 2.5.1.31] reactions which are known to proceed with elimination of the 2-pro-*R* hydrogen and the 2-pro-*S* hydrogen, respectively.

For farnesyl pyrophosphate synthetase reaction, the incubation mixture contained, in a final volume of 8 ml, 800 μmol of Tris-HCl buffer (pH 8.5), 80 μmol of $MgCl_2$, 80 μmol of 2-mercaptoethanol, 40 mg of Triton X-100, 4 μmol of **1**, 30 units (one unit of enzyme converts 1 nmol of IPP per min) of pig liver IPP isomerase[9] and farnesyl pyrophosphate synthetase partially purified from 2.5 g (wet weight) of *Bacillus subtilis*.[10] After being incubated at 37 °C for 10 h, the mixture was treated with alkaline phosphatase, and the farnesol thus liberated was extracted and subjected to GC-MS analysis. The mass spectrum (Fig. 1) clearly indicated that the product was [2,6,10-$^2H_3$]farnesol, being consistent with the fact that the 2-pro-*R* hydrogen is eliminated.[10]

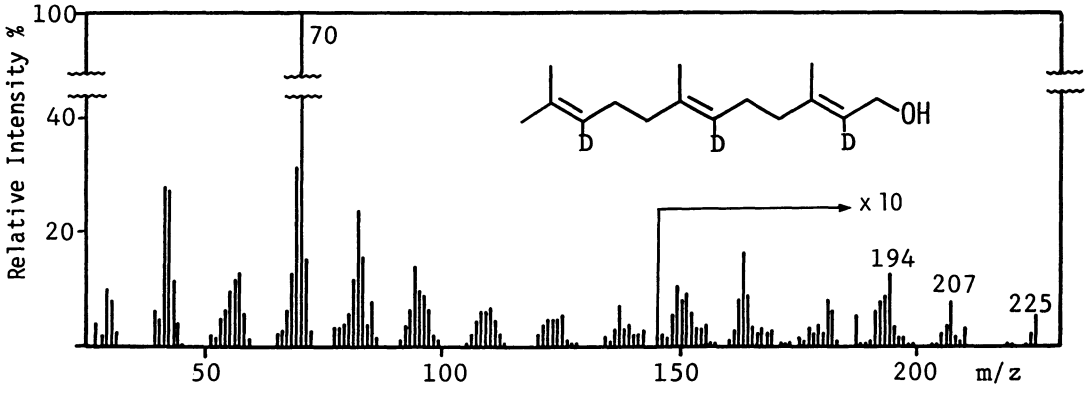


Fig. 1. Mass spectrum of [2,6,10-$^2H_3$]farnesol derived from [2-^{2}H]DMAPP (**1**) by the reaction of farnesyl pyrophosphate synthetase coupled with IPP isomerase. The sample was analyzed by GC-MS with a Shimadzu-LKB 9000. The gas chromatography was carried out at 180 °C on a 1 m column of 5% PEG 20M with helium gas at a flow rate of 30 ml/min. The mass spectrum was taken at an ionizing potential of 70 eV.

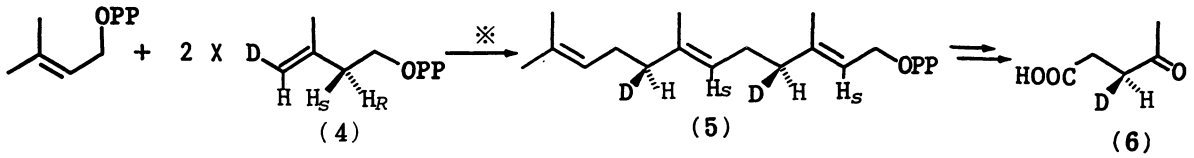
Undecaprenyl pyrophosphate synthetase of *B. subtilis* was examined as follows. This enzyme is known to catalyze the condensation of *E,E*-farnesyl pyrophosphate and eight molecules of IPP in a *Z* fashion with elimination of the 2-pro-*S* hydrogen.[11] Since DMAPP cannot act as substrate for this enzyme, *E,E*-farnesyl pyrophosphate must be added as the primer. Thus, the incubation mixture contained, in a final volume of 40 ml, 4 mmol of Tris-HCl buffer (pH 8.5), 20 μmol of $MgCl_2$, 200 mg of Triton X-100, 20 μmol of **1**, 3.3 units of pig liver IPP isomerase, 10 μmol of *E,E*-farnesyl pyrophosphate and undecaprenyl pyrophosphate synthetase partially purified from 5 g (wet weight) of *B. subtilis*.[11] After incubation at 37 °C for 24 h, the mixture was extracted with 1-butanol. The butanol extract was treated with acid phosphatase,[12] and the undecaprenol thus formed was purified by HPLC and subjected to mass spectrometry. The mass spectrum of the undecaprenol, which was formed from *E,E*-farnesyl pyrophosphate and (*S*)[2-^{2}H]IPP (**2**) derived from [2-^{2}H]DMAPP (**1**) *in situ*, was the same as that of authentic undecaprenol,[11] indicating that the deuterium of **1** had been lost as expected.

Thus, it was found that the prochirality of the hydrogen eliminated from C-2 of IPP could be determined by this method more easily than ever.

The stereochemistry of hydrogen elimination is of interest in relation to not only the geometry of the newly formed double bond but also the face of the double bond of IPP at which the C-C bond formation occurs. We studied farnesyl pyrophosphate synthetase of pumpkin in these respects in order to examine whether the stereochemical correlation determined by Cornforth *et al*[13] with pig liver enzyme hold widely for similar enzymes from higher plants.

The pumpkin synthetase was similarly incubated with **1** and IPP isomerase, and analyzed in the same manner as described above. The mass spectrum of the farnesol indicated that three deuteriums were incorporated, indicating that the 2-pro-*R* hydrogen was lost.

In order to determine the stereochemistry of C-C bond formation, the enzymatic reaction of *E*-[4-^{2}H]IPP (**4**) with DMAPP was investigated by the method we developed previously.[14] The incubation mixture contained, in a final volume of 2400 ml, 120 mmol of Tris-HCl buffer (pH 7.7), 12 mmol of $MgCl_2$, 24 mmol of 2-mercaptoethanol, 150 μmol of DMAPP, 150 μmol of **4**, and farnesyl pyrophosphate synthetase obtained from 1.9 kg of pumpkin.[15] The product was treated with alkaline phosphatase and the [4,8-$^2H_2$]farnesol liberated was subjected to ozonolysis. The [3-^{2}H]levulinic acid (**6**) thus obtained showed a positive CD curve, $[\theta]_{280} = +179 \pm 60°$. From these facts it was evidenced that the C-C bond formation took place at the *si* face of the double bond of IPP with concomitant elimination of the 2-pro-*R* hydrogen. Namely, the correlation is the same as that reported for pig liver farnesyl pyrophosphate synthetase.[13]



(4) (5) (6)

※ *Farnesyl PP Synthetase of pumpkin*

Scheme 2.

The method reported here has the following advantages: (1) The substrate is easy to synthesize. (2) Discrimination is clear because of direct analysis by mass spectrometry. (3) No radioisotope handling is needed. (4) No interpretation is necessary for factors due to other metabolic processes such as *Z*,*E*-isomerization, because purified prenyltransferase is used. Purification of enzyme sometimes may be laborious, but it would be outweighed by the merits described above. The sensitivity of this mass spectrometric method, however, is undoubtedly lower than that of the conventional method based on radioactivity counting.

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