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Biosynthesis of Cyclic Monoterpenoids. Involvement of Stereochemically Specified Linalyl Cation in the Cyclization of C_{10}-Prenyl Chain[1)]

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Incubation of geranyl diphosphate (GPP) and the enantiomers and racemate of linalyl diphosphate (LPP) with the limonene synthetase isolated from Mentha spicata resulted in the conversion of GPP and (S)-LPP into (S)-limonene; this can be taken as evidence that linalyl cation having not only the (S)-LPP-like configuration but also C-1 and C-6 double bonds situated in the anti spatial arrangement is involved in the process of the cyclization of GPP to (S)-limonene. The intermediacy of the linalyl cation was supported by the evaluation of the potential energy of the cation with the MO calculation.

Geranyl diphosphate (GPP) was generally known to be the parent precursor for the biosynthesis of monoterpenoids,[2)] whereas, in the biosynthesis of cyclic monoterpenoids, neryl diphosphate (NPP) rather than GPP has been widely accepted as the more likely precursor.[3-5)] Recently, the possible intermediacy of linalyl diphosphate (LPP) or its tertiary allylic equivalent was suggested by the incorporation of LPP into cyclic monoterpenoids.[6-10)] On the other hand, it was recently demonstrated that GPP is the preferred substrate for the enzymatic cyclization in the cases of no interconversion of these allylic diphosphates and the absence of the competing phosphatase.[11-13)] However, little is known of what is a prerequisite intermediate in the process of the enzymatic cyclization of C_{10}-prenyl chain. We have investigated the intermediate for the enzymatic cyclization leading to the formation of p-menthane derivatives from GPP in Mentha spicata.[14)]

The stereochemistry in the enzymatic cyclization of C_{10}-prenyl chain to limonene was investigated by incubating GPP and the enantiomers and racemate of LPP with the enzyme system of M. spicata. The enzyme system responsible for the cyclization of acyclic allylic diphosphates was partially purified from the leaves of M. spicata, as follows. The leaves of M. spicata were homogenized in 0.1 M TES buffer (pH 7.0) in the presence of polyvinylpolypyrrolidone and dithiothreitol. The resulting paste was filtered through two layers of cheesecloth and the filtrate was centrifuged at 10000g and then 100000g. After treatment of the supernatant with 80% saturated ammonium sulfate and then Sephadex G-25, a

crude enzyme solution was fractionated by the column chromatography on Sephadex G-200 and then DEAE-cellulose to give limonene synthetase.[15] To the limonene synthetase (about 270 μg protein) in 0.1M TES buffer (pH 7.0; 3 cm^3), a solution of the substrates (15 μmol), such as GPP, (S)-(+)-, (R)-(-)-, and (RS)-LPPs,[16] and magnesium chloride (1 mM) in the same buffer (1 cm^3) were added. The mixture was incubated at 30 °C for 6 h, because the incorporation of GPP into limonene became constant after a 4-h incubation. The reaction mixture was extracted with ether and then subjected to GLC and GC-MS. Large scale experiments were also carried out by the similar procedure, and limonene produced was purified by HPLC on a Finepak-Sil column with hexane/ether (19:1, v/v). The incorporation of these substrates into limonene and the optical rotation of limonene obtained are shown in Table 1.

Table 1. Incorporation of LPP and GPP into limonene with the limonene synthetase isolated from M. spicata

Substrate		Limonene produced		
Compd.	$[\alpha]_D^{25}$(c, H_2O)	Incorp.(%)[a]	$[\alpha]_D^{25}$(c, hexane)	C-4 configuration
GPP	—	1.2 ± 0.1	-93° (0.13)	S
(S)-LPP	+5.1° (0.2)	1.4 ± 0.2	-95° (0.06)	S
(R)-LPP	-5.0° (0.2)	0.2 ± 0.2	—	—
(RS)-LPP	0.0° (0.3)	0.8 ± 0.3	-90° (0.04)	S

a) In the control experiments with the heat-inactivated enzyme system, the incorporation of these substrates was below 0.1%.

The incorporation of GPP into limonene was 1.2% and the optical rotation of the limonene produced was levorotatory as shown in Table 1; this optical property is characteristic of limonene in the intact plant of M. spicata. This observation indicates the involvement of the cyclization process from GPP to (S)-limonene in M. spicata. In order to predict a prerequisite intermediate in the cyclization process, the reaction path from GPP to limonene was evaluated on the basis of the MO calculation.[19] The energy barrier in the isomerization of geranyl cation to linalyl cation is 1.31×10^4 J mol^{-1}, whereas the barrier in that of geranyl cation to neryl cation is 2.61×10^4 J mol^{-1}. The barrier from neryl cation to limonene is 1.72×10^4 J mol^{-1} and that from linalyl cation to limonene is only 1.22×10^4 J mol^{-1}. These calculations suggest that linalyl cation is involved in the cyclization process of the C_{10}-prenyl chain. The intermediacy of linalyl cation in the cyclization process was supported by the incorporation experiments of the enantiomers of LPP which may be a potential source for linalyl cation. The incorporation of (S)-LPP was comparable to that of GPP and twice for that of (RS)-LPP, while (R)-LPP was scarcely incorporated. These observations indicate that a stereochemically specified linalyl cation having a configuration similar to (S)-LPP participates in the biological formation of limonene from GPP.

The cyclization of the linalyl cation is predicted to proceed through an intermediate (a) or (b) which possibly are in conformers as depicted in Fig. 1. The formation of (S)-(-)-limonene should be expected in the cyclization via the intermediate (a), whereas (R)-(+)-limonene should be formed by the cyclization

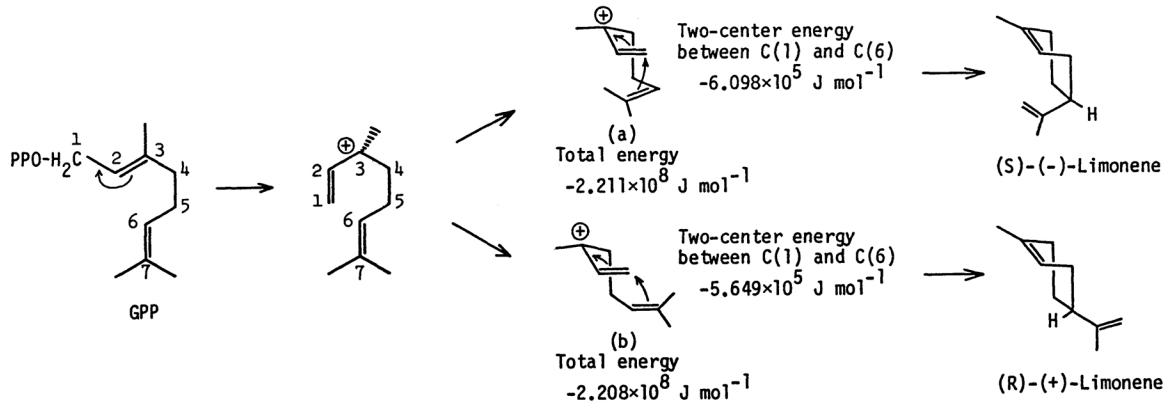

Fig. 1. Possible intermediates involved in the biosynthesis of limonene from geranyl diphosphate and the total and two-center energies of the intermediates.

via the intermediate (b). As shown in Table 1, the optical rotation of all the limonenes biosynthesized from GPP and (S)- and (RS)-LPPs by incubation with limonene synthetase was levorotatory. The formation of (S)-(-)-limonene in the enzymatic cyclization indicates that the cyclization proceeds through the intermediate (a). The total energy for the intermediate (a) was estimated to be lower than that of the intermediate (b) by 3.01×10^5 J mol^{-1} on the basis of the MO calculation,[19] as shown in Fig. 1. Furthermore, the two-center energy between C-1 and C-6 in the intermediate (a) is lower than that of the intermediate (b) by 4.49×10^4 J mol^{-1}. The MO calculations support that the intermediate (a) is the most likely one in the cyclization of linalyl cation.

Thus, it was established that (i) GPP is the parent precursor for the biosynthesis of (S)-limonene in M. spicata, (ii) the linalyl cation having the configuration similar to (S)-LPP is a prerequisite intermediate in the cyclization process, and (iii) the cyclization occurs via the intermediate (a) having the C-1 and C-6 double bonds situated in the anti spatial arrangement. The C-1 double bond of the intermediate (a) would be attacked from the backside of the counter anion, such as a diphosphate anion or a nucleophilic group of the enzyme, by the C-6 double bond to form stereospecifically (S)-limonene.

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14) The leaves of M. spicata, which had been grown on the campus of Hiroshima University, produced an essential oil containing up to 90% of p-menthane derivatives, such as carvone (71%) and limonene (22%), in August.
15) The limonene synthetase was purified about 54-fold against the ammonium sulfate fraction. The molecular weight was estimated to be about 100000. The presence of bivalent metal ions, especially magnesium and manganese ions, was essential for the enzymatic cyclization.
16) According to the reported procedure,[17] GPP was prepared from geraniol by chlorination, followed by phosphorylation. (S)-(+)- and (R)-(-)-LPPs were prepared from (+)- and (-)-linalools ($[\alpha]_D^{25}$ +19.1° and -20.0° (neat), respectively) by phosphorylation with a dioxane diphosphate complex.[18] (±)-LPP was prepared by mixing the same amount of (+)- and (-)-LPPs.
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19) The potential energies for the allylic cations were calculated on the CNDO/2 method.[20,21] The bond lengths and angles used for this calculation were the values reported in the Ab initio MO calculation of isopentenyl cations.[22] The standard geometries of the C_{10}-allyl cations were optimized by use of the MM2 calculation.[23] The CNDO/2 calculations for several geometries were done by changing the torsional angle, C(1)-C(2)—C(3)-C(4), at 30° increments from 0° to 360°.
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