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BIOSYNTHESIS OF CYCLIC MONOTERPENOIDS. THE PIVOTAL ACYCLIC PRECURSOR FOR THE CYCLIZATION LEADING TO THE FORMATION OF α-TERPINEOL AND LIMONENE IN MENTHA SPICATA AND CITRUS NATSUDAIDAI[1)]

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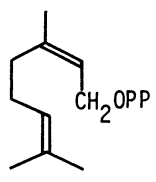
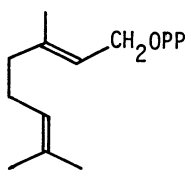
As a result of making a direct comparison among the incorporations of the pairs of ^{14}C- and ^{3}H-labeled linalyl, geranyl, and neryl pyrophosphates into α-terpineol and limonene in the cell-free extract of Mentha spicata and Citrus natsudaidai, respectively, the linalyl pyrophosphate was found to be the most pivotal, immediate precursor for the cyclization leading to the formation of the cyclic monoterpenoids.

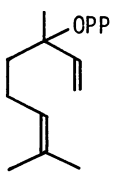
Since it was proposed that all cyclic monoterpenoids arise from nerol by its cyclization,[2,3)] neryl pyrophospate (NPP) has been generally accepted as a precursor for the biosynthesis of cyclic monoterpenoids.[4)] However, it was suggested that geranyl pyrophosphate (GPP) rather than NPP might be a preferred substrate for the cyclization on the basis of a comparison of V_{max}/K_m values in the cyclization of GPP and NPP,[5)] while it was proposed that linalool might be a precursor for the biosynthesis of cyclic monoterpenoids on the basis of the seasonal fluctuations of the acyclic and cyclic monoterpenoids.[6-9)] Before it was reported that linalyl pyrophosphate (LPP) rather than NPP was more effective as a substrate for the enzymatic synthesis of cyclic monoterpenoids,[12)] we had observed the preferential incorporation of linalool rather than nerol and gerniol into cyclic monoterpenoids by administrating separately the ^{14}C-labeled acyclic allylic pyrophosphates.[1,13)] We have now made a direct comparison among the incorporations of LPP, NPP, and GPP into α-terpineol (**1**) and limonene (**2**) by incubating the pairs of these ^{3}H- and ^{14}C-labeled allylic substrates with the cell-free extract of Mentha spicata and Citrus natsudaidai, respectively, and here wish to communicate promptly the results.



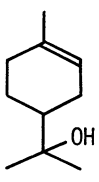
NPP



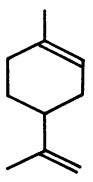
GPP



LPP



1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The ^{3}H- and ^{14}C-labeled acyclic allylic pyrophosphates were prepared by phosphorylation[14] of the ^{3}H- and ^{14}C-labeled linalool, geraniol, and nerol, and purified by preparative TLC on a silica gel-H plate with 1-propanol—aqueous ammonia—water (6:3:1, v/v). The homogeneity of the allylic pyrophosphates was confirmed by the experimental result that each of the allylic pyrophosphates on enzymatic hydrolysis with Potato apyrase[15] gave only the corresponding acyclic allylic terpenoid when the hydrolyzate was assayed by radio-GLC (Aloka RGC-212; 10% DEGS column at 120°C; Sensitivity, >150 dpm for ^{14}C and >200 dpm for ^{3}H at 300 ml/min of carrier gas). The specific radioactivity of the allylic pyrophosphates was as follows: (±)-[1-$^{3}H_2$]LPP, 7.5 mCi/mmol ; [1-$^{3}H_2$]GPP, 7.5 mCi/mmol ; [1-$^{3}H_2$]-NPP, 7.5 mCi/mmol ; (±)-[1-^{14}C]LPP, 1.1 mCi/mmol ; [1-^{14}C]-GPP, 1.1 mCi/mmol ; [1-^{14}C]NPP, 1.1 mCi/mmol . A pair of the ^{3}H- and ^{14}C-labeled acyclic allylic pyrophosphates was prepared by mixing the ^{3}H-labeled substrate with the ^{14}C-labeled substrate in the $^{3}H/^{14}C$ ratio as shown in Tables 1 and 2 and then adjusting the amount of each substrate in the mixture to equimolecular amounts with addition of the corresponding non-radioactive substrate. Following the method described in the reference,[16] the crude cell-free extract was prepared from the leaves of M. spicata and the peel of C. natsudaidai, and the cell-free extract used in this investigation was the supernatant which had been obtained by centrifugation at 30000 g for 20 min. With this cell-free extract the pairs of the ^{3}H- and ^{14}C-labeled allylic pyrophosphates was incubated at 30°C for 90 min. After incubation, the reaction mixture was treated with Potato apyrase in a manner similar to that described in the reference[15] and then worked up in a usual manner to give the cyclic monoterpenoids. Incubation of the allylic pyrophosphates with the active cell-free extract of M. spicata and C. natsudaidai gave radioactive α-terpineol (1) and limonene (2), respectively, but incubation of the allylic pyrophosphates with the boiled-enzyme control gave only the pair of the acyclic allylic terpenoids corresponding to the allylic pyrophosphates administered. The $^{3}H/^{14}C$ ratios in 1 and 2 are given in Tables 1 and 2.

As shown in Exp. A of Table 1, the $^{3}H/^{14}C$ ratios in α-terpineol (1) arising from the pairs of [1-$^{3}H_2$]LPP and [1-^{14}C]GPP and [1-$^{3}H_2$]LPP and [1-^{14}C]NPP were larger in comparison with the initial ratios in the pairs of these substrates. This indicates that the ^{3}H-labeled LPP was incorporated into α-terpineol (1) in preference to the ^{14}C-labeled GPP and NPP. The $^{3}H/^{14}C$ ratios in limonene (2) arising from the pairs of [1-$^{3}H_2$]LPP and [1-^{14}C]GPP, [1-$^{3}H_2$]LPP and [1-^{14}C]NPP, and [1-$^{3}H_2$]NPP and [1-^{14}C]GPP were also larger than the initial ratios in the pairs of these substrates, as shown in Exp. A of Table 2. This indicates that not only the ^{3}H-labeled LPP was incorporated into α-terpineol (1) in preference to the ^{14}C-labeled GPP and NPP, but also the ^{3}H-labeled NPP was done so in preference to the ^{14}C-labeled GPP. As mentioned above, the most preferential incorporation of LPP into the cyclic monoterpenoids occurred even when the racemic LPP was used as a substrate. If

Table 1. The $^{3}H/^{14}C$ ratios in α-terpineol (1) biosynthesized from the pairs of the ^{3}H- and ^{14}C-labeled LPP, GPP, and NPP in the cell-free extract of Mentha spicata

Exp.	Precursor			α-Terpineol (1)[c]				
	Pair of substrates[a]	μCi[b]	$^{3}H/^{14}C$ ratio	^{3}H dpm×10^{-3}	^{14}C dpm×10^{-2}	$^{3}H/^{14}C$ ratio	Incorp. / % ^{3}H	Incorp. / % ^{14}C
A	^{3}H-LPP + ^{14}C-GPP	0.1	7.72	2.15	1.07	20.1	1.01	0.39
	^{3}H-LPP + ^{14}C-NPP	0.1	9.52	1.75	1.57	11.1	0.59	0.50
B	^{3}H-GPP + ^{14}C-LPP	0.4	9.75	2.04	12.3	1.66	0.18	1.07
	^{3}H-NPP + ^{14}C-LPP	0.4	13.3	3.19	6.85	4.66	0.29	0.82

a) ^{3}H-LPP, ^{3}H-GPP, ^{3}H-NPP, ^{14}C-GPP, ^{14}C-NPP, and ^{14}C-LPP denote [1-$^{3}H_2$]LPP, [1-$^{3}H_2$]GPP, [1-$^{3}H_2$]NPP, [1-^{14}C]GPP, [1-^{14}C]NPP, and [1-^{14}C]LPP, respectively.
b) The radioactivities are expressed with respect to only ^{14}C.
c) The radioactivities cannot be compared among different batches, since different quantities of the carrier and/or the tracer are used.

Table 2. The $^{3}H/^{14}C$ ratios in limonene (2) biosynthesized from the pairs of the ^{3}H- and ^{14}C-labeled LPP, GPP, and NPP in the cell-free extract of Citrus natsudaidai

Exp.	Precursor			Limonene (2)[c]				
	Pair of substrates[a]	μCi[b]	$^{3}H/^{14}C$ ratio	^{3}H dpm×10^{-4}	^{14}C dpm×10^{-3}	$^{3}H/^{14}C$ ratio	Incorp. / % ^{3}H	Incorp. / % ^{14}C
A	^{3}H-LPP + ^{14}C-GPP	1.5	10.0	10.3	4.50	22.9	1.44	0.65
	^{3}H-LPP + ^{14}C-NPP	1.5	8.10	3.57	2.97	12.0	0.82	0.56
	^{3}H-NPP + ^{14}C-GPP	1.5	7.01	4.43	1.45	30.6	1.52	0.35
B	^{3}H-GPP + ^{14}C-LPP	1.0	9.24	0.59	1.63	3.62	0.27	0.68
	^{3}H-NPP + ^{14}C-LPP	1.0	11.7	1.79	4.16	4.30	0.58	1.57
	^{3}H-GPP + ^{14}C-NPP	1.5	7.78	3.66	9.51	3.85	0.48	0.97

a)—c) Refer to a)—c) in Table 1, respectively.

the only one enantiomer of the racemic mixture of LPP participates in the formation of the cyclic monoterpenoids, the incorporation of LPP would be twice as many as that of the racemic LPP, and the $^{3}H/^{14}C$ ratios in 1 and 2 would be twice and one-half the ratios shown in Exp. A and Exp. B of Tables 1 and 2, respectively. It was thus indicated that, in the formation of α-terpineol (1) and limonene (2) with the cell-free extract, the acyclic allylic pyrophosphates were incorporated into these cyclic monoterpenoids in the following order: LPP > NPP > GPP.

In these cases, such a difference in the incorporation might be caused by the isotope effect due to the ^{3}H and ^{14}C labels. Then a comparison among the incorporations was therefore made by use of the pairs of reversely labeled substrates, such as [1-$^{3}H_2$]GPP and [1-^{14}C]LPP, [1-$^{3}H_2$]NPP and

[1-^{14}C]LPP, and [1-$^{3}H_2$]GPP and [1-^{14}C]NPP. As shown in Exp. B of Tables 1 and 2, the $^{3}H/^{14}C$ ratios in the cyclic monoterpenoids were smaller than the initial ratios in the pairs of substrates; this confirms that these allylic substrates were transformed into the cyclic monoterpenoids in the order of LPP > NPP > GPP as described above.

It was thus demonstrated that the pivotal, immediate precursor for the cyclization leading to the formation of the cyclic monoterpenoids is linalyl pyrophosphate rather than geranyl and neryl pyrophosphates. Further investigations including kinetic studies are now in progress for giving a coherent picture of the cyclization.

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