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INTERCONVERSION AND CYCLIZATION OF ACYCLIC ALLYLIC PYROPHOSPHATES IN THE BIOSYNTHESIS OF CYCLIC MONOTERPENOIDS IN HIGHER PLANTS*

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Key Word Index—Biosynthesis; interconversion; cyclization; acyclic allylic pyrophosphates; cyclic monoterpenoids; higher plants.

Abstract—The biosynthesis of cyclic monoterpenoids has been investigated in both intact plants and cell-free extracts of several higher plants. The participation of a non-redox process in the biosynthesis of the cyclic monoterpenoids was indicated by the retention of all the tritium labels originating from [2-^{14}C,5-$^{3}H_2$]mevalonic acid and [1-^{14}C,1-$^{3}H_2$]geranyl, neryl and linalyl pyrophosphates. The cell-free extract catalysed the non-redox interconversions of geranyl, neryl and linalyl pyrophosphates to each other. By contrast, in both intact plants and cell-free extracts, the incorporation of linalyl pyrophosphate into the cyclic monoterpenoids occurred preferentially to the incorporation of neryl and geranyl pyrophosphates. These observations suggest the involvement of a tertiary allylic compound and/or its equivalent as a key intermediate, not only in the interconversion of the acyclic allylic pyrophosphates, but also in the formation of the cyclic monoterpenoids.

INTRODUCTION

Since the proposals for the biosynthesis of cyclic monoterpenoids were first presented by Ruzicka's group [2], neryl pyrophosphate (NPP) rather than geranyl pyrophosphate (GPP) has been generally accepted as the more likely direct precursor for the biosynthesis of cyclic monoterpenoids [3–8]. A probable mechanism for the biological formation of NPP is thought to involve either the direct *cis*-condensation of isopentenyl pyrophosphate (IPP) with 3,3-dimethylallyl pyrophosphate (DMAPP) [6] or the initial formation of GPP, followed by its isomerization to NPP [9–11]. For the isomerization of GPP to NPP, a redox interconversion via a corresponding aldehyde intermediate is proposed [5, 7]. Evidence of the redox *E*–*Z*-isomerization between geraniol (**1**) and nerol (**2**) or between (2*E*,6*E*)-farnesol and its (2*Z*)-isomer is described by several workers [12–16]. In contrast to this redox process, the non-redox *E*–*Z*-isomerization via a tertiary allylic pyrophosphate is now proposed for the biosynthesis of monoterpenoids and it has also been suggested for sesquiterpenoid synthesis [17, 18].

Recently, it was reported that GPP rather than NPP is the preferred substrate for the cyclization in the biosynthesis of cyclic monoterpenoids [19]. On the other hand, the possible intermediacy of linalyl pyrophosphate (LPP) in the biosynthesis of cyclic monoterpenoids was suggested on the basis of the seasonal fluctuation of linalool (**3**) and cyclic monoterpenoids in higher plants [20–22] and the incorporation of ^{14}C-labelled linalool into cyclic monoterpenoids [23–26]. The preferential incorporation of LPP rather than NPP and GPP into cyclic monoterpenoids was also observed in our previous studies on the isomerization–cyclization of acyclic allylic intermediates in higher plants [27]. Recently, an enzyme preparation from *Citrus limonum* was found to utilize LPP more efficiently than GPP and NPP as a substrate for the biosynthesis of cyclic monoterpenoids and it was considered that LPP is a good substrate analogue [28]. The participation of a tertiary allylic compound, such as LPP, is inferred by analogy with the proposal that (2*E*,6*E*)-farnesyl pyrophosphate is biologically cyclized to cyclonerodiol via nerolidyl pyrophosphate [18, 29]. However, it has been reported that the relative efficiency of LPP as precursor for cyclic monoterpenoids may not be net, as inhibition of endogenous phosphate-cleaving enzymes led to the reverse sequence [30].

We have investigated the initial stages in the biosynthesis of cyclic monoterpenoids, especially the interconversion and cyclization of acyclic allylic pyrophosphates [27, 31–33]. We describe now investigations on the following points: (1) the stereochemistry of the hydrogen elimination from C-4 of mevalonic acid (MVA) during the biological formation of cyclic monoterpenoids from MVA; (2) the involvement of the non-redox interconversion of the acyclic allylic compounds in the biosynthetic process of the cyclic monoterpenoids; (3) the most efficient substrate for the biological formation of the cyclic monoterpenoids in the higher plants.

RESULTS AND DISCUSSION

The prochirality in the elimination of the C-4 hydrogen atoms of MVA during the biosynthesis of cyclic monoterpenoids from MVA

We first examined the stereochemistry of the hydrogen elimination from C-4 of MVA in the generation of an acyclic intermediate at the initial stage of the bio-

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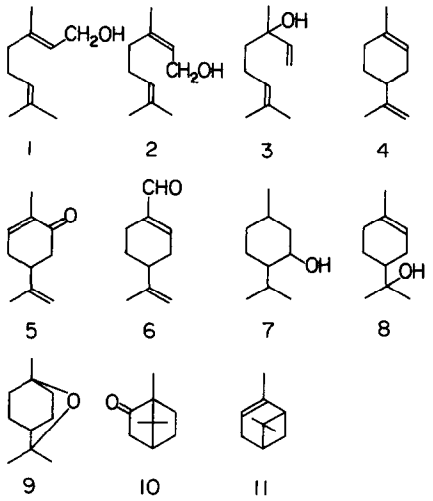
synthesis of cyclic monoterpenoids. (4*R*)- and (4*S*)-[2-^{14}C,4-^{3}H]MVA were administered separately to the peel of *Citrus natsudaidai* Hayata and to the leaves of *Mentha spicata* L., *Perilla frutescens* Britton and *Mentha piperita* L. After uptake of the double labelled MVA, the radioactive cyclic monoterpenoids, such as limonene (**4**), carvone (**5**), perillaldehyde (**6**), menthol (**7**) and α-terpineol (**8**), were isolated from these plant materials. Their radioactivities and ^{3}H:^{14}C ratios are shown in



Expts 1 and 2 of Table 1. The atomic ratios observed for the cyclic monoterpenoids **4**–**8** were consistent with those expected for the elimination of the *pro*-4*S* hydrogen atom of MVA in both cases during their biosynthesis from (4*R*)- and (4*S*)-[2-^{14}C,4-^{3}H]MVA. Following Cornforth's biosynthetic principle [34, 35], the present results seem to rule out the occurrence of direct *cis*-condensation of IPP and DMAPP. However, the occurrence of the direct *cis*-condensation may not be ruled out for the biosynthesis of monoterpenoids in higher plants, because the C-4 hydrogen elimination opposed to Cornforth's principle has been found in the biological formation of *Z*-prenyl units of polyprenols in higher plants [36, 37]. Some other examples that break the principle are also observed in the biosynthesis of nerol [5–8, 38, 39] and (2*Z*,6*E*)-farnesol [6] with several higher plants. The biological formation of the *Z*-prenyl units without loss of the 4*R*-hydrogen atom of MVA may be explained by assuming an *E*–*Z*-isomerization of *E*-prenyl units initially generated by the *trans*-condensation of IPP with an allylic residue.

The involvement of a non-redox process in the interconversion of geranyl pyrophosphate to neryl and linalyl pyrophosphates and in the formation of the cyclic monoterpenoids

[2-^{14}C,5-^{3}H$_2$]MVA was administered to the peel of *C. natsudaidai* and to the leaves of *M. spicata*, *Perilla*

Table 1. Isotope ratios in the cyclic monoterpenoids biosynthesized from (4*R*)- and (4*S*)-[2-^{14}C,4-^{3}H]MVA and [2-^{14}C,5-^{3}H$_2$]MVA

Expt No.: MVA used (^{3}H:^{14}C ratio)	Compounds*	Observed ^{3}H (dpm)	Observed ^{14}C (dpm)	Observed ^{3}H:^{14}C ratio	Atomic ratio ^{3}H:^{14}C	Expected atomic ratios: Route A ^{3}H:^{14}C	Expected atomic ratios: Route B ^{3}H:^{14}C
Expt 1: (4*R*)-[2-^{14}C,4-^{3}H]MVA							
(7.56)	Limonene (**4**)	1484	211	7.03	1.86:2	1:2†	2:2†
(4.28)	Carvone (**5**)	547	132	4.14	1.93:2	1:2	2:2
(4.28)	Perillaldehyde (**6**)	1801	383	4.70	2.20:2	1:2	2:2
(7.56)	Menthol (**7**)	5302	632	8.39	2.22:2	1:2	2:2
Expt 2: (4*S*)-[2-^{14}C,4-^{3}H]MVA							
(6.00)	Carvone (**5**)	790	1210	0.65	0.22:2	1:2†	0:2†
(6.21)	α-Terpineol (**8**)	1540	3370	0.46	0.15:2	1:2	0:2
Expt 3: [2-^{14}C,5-^{3}H$_2$]MVA							
(3.31)	Limonene (**4**)	1616	478	3.38	4.08:2	3:2‡	4:2‡
(8.76)	Carvone (**5**)	718	83	8.65	3.95:2	3:2	4:2
(3.31)	Perillaldehyde (**6**)	3633	1090	3.33	4.02:2	3:2	4:2
(8.76)	1,8-Cineol (**9**)	1045	133	7.84	3.61:2	3:2	4:2
(8.76)	Camphor (**10**)	2793	319	8.65	3.98:2	3:2	4:2
(3.31)	Camphor (**10**)§	2720	860	3.16	3.81:2	3:2	4:2
(8.76)	α-Pinene (**11**)	4980	574	8.62	3.94:2	3:2	4:2

*These compounds were obtained from the following plants: **4** from *C. natsudaidai*, **5** and **8** from *M. spicata*, **6** from *P. frutescens*, **7** from *M. piperita*, **9** and **10** from *A. annua* and **11** from *P. thunbergii*.

†The expected atomic ratios were calculated by assuming that the monoterpenoids **4**–**8** are biosynthesized by cyclization of the acyclic intermediate, which is formed through either the *cis*-condensation of IPP and DMAPP (Route A) or the *trans*-condensation (Route B) following Cornforth's principle.

‡The expected atomic ratios were calculated by assuming that the monoterpenoids are formed by the cyclization via the aldehyde intermediate (Route A), or via an alternative intermediate which retains both of the C-1 hydrogen atoms (Route B).

§Obtained from *C. camphora*.

frutescens, *Artemisia annua* L., *Cinnamomum camphora* Sieb. and *Pinus thunbergii* Parl. From these plant materials, double labelled cyclic monoterpenoids, such as limonene (**4**), carvone (**5**), perillaldehyde (**6**), 1,8-cineol (**9**), camphor (**10**) and α-pinene (**11**), were isolated, respectively, and their radioactivities were determined, as given in Expt 3 of Table 1. The original $^3H:^{14}C$ ratios of the MVA were retained in all these cyclic monoterpenoids. If the biosynthetic process involves the proposed redox isomerization of GPP via an aldehyde intermediate as a prerequisite to the cyclization [7, 12], the $^3H:^{14}C$ atomic ratios in the cyclic monoterpenoids should be 3:2 as a result of loss of a tritium atom at C-1 in GPP generated from the double labelled MVA. If the isomerization proceeds without loss of the tritium atoms at C-1, on the other hand, the $^3H:^{14}C$ atomic ratios in the cyclic monoterpenoids should be 4:2. The observed atomic ratios were consistent with the ratios expected for the latter case, as given in Expt 3 of Table 1. This fact shows unambiguously that the cyclic monoterpenoids were biosynthesized through a non-oxidative process involving the retention of the C-1 hydrogen atoms of GPP, and that an aldehyde intermediate was not involved in the process of the biological formation of the cyclic monoterpenoids in the higher plants.

The occurrence of the non-redox interconversion was further indicated by experiments with a cell-free extract, which was prepared from the growing shoots of *M. spicata*. The incubations of the [1-^{14}C,1-$^3H_2$]-labelled GPP, NPP and LPP with this cell-free extract gave radioactive products, such as geraniol (**1**), nerol (**2**), linalool (**3**) and α-terpinol (**8**). The $^3H:^{14}C$ ratios of the products isolated are given in Table 2. These results show that the allylic pyrophosphates are not only interconverted, but they also cyclize to α-terpineol (**8**) by the cell-free extract. The original $^3H:^{14}C$ ratios of the substrates used were retained in all of the products; no loss of the C-1 hydrogen atoms of the acyclic allylic pyrophosphates occurred during the *E–Z* interconversion and the cyclization. This observation completely ruled out the occurrence of the redox interconversion via the aldehyde intermediate [7, 12] in the process of the biological formation of the cyclic monoterpenoids from the acyclic allylic pyrophosphates. Similar observations have been reported on the biosynthesis of several monoterpenoids by use of the cell-free extract of *Salvia officinalis* [40].

Thus, the non-redox process was demonstrated to be involved in the interconversion and the cyclization of the acyclic allylic pyrophosphates in both the intact plants and the cell-free system. The process is postulated to involve a tertiary-allylic compound or its equivalent as a key intermediate for the interconversion and the cyclization mechanisms.

The preferential incorporations of linalool and linalyl pyrophosphate into the cyclic monoterpenoids

The seasonal variation in the content of linalool (**3**) and carvone (**5**) was followed by examining the essential oil of *M. spicata*. The content of linalool (**3**) decreased while there was an increase in that of carvone (**5**) during April to June. This observation is analogous to the seasonal fluctuation of linalool (**3**) and limonene (**4**) in the essential oil of *Citrus* species [22]. Such a seasonal fluctuation seems to indicate a significant role for linalool (**3**) in the biosynthesis of carvone (**5**) and limonene (**4**).

In order to demonstrate the role of linalool (**3**) in the biosynthesis of cyclic monoterpenoids, the incorporation of linalool (**3**) was directly compared with those of geraniol (**1**) and nerol (**2**) by administering simultaneously pairs of ^3H- and ^{14}C-labelled substrates (for example [1-$^3H_2$]nerol and [1-^{14}C]linalool mixture or [1-$^3H_2$]geraniol and [1-^{14}C]linalool mixture) to the leaves of *M. spicata*. The $^3H:^{14}C$ ratios in carvone (**5**) were smaller than the initial ratios in the pairs of the ^3H- and ^{14}C-labelled substrates (Table 3, Expts 1 and 2). This clearly indicates that the incorporation of ^{14}C-labelled linalool into carvone was higher than the incorporations of ^3H-labelled geraniol and nerol. The preferential incorporations of linalool and its pyrophosphate into limonene and perillaldehyde was also observed in feeding experiments with the peel of *C. hassaku* and the leaves of *P. frutescens* (Table 4).

All these facts with the intact plants indicates that linalool (**3**) and its pyrophosphate were preferentially incorporated into the cyclic monoterpenoids rather than geraniol (**1**) and nerol (**2**) and their pyrophosphates.

Direct comparisons of the incorporations of the acyclic allylic pyrophosphates were next made by incubating mixtures of [1-$^3H_2$]LPP and [1-^{14}C]GPP, [1-$^3H_2$]LPP and [1-^{14}C]NPP, or [1-$^3H_2$]NPP and [1-^{14}C]GPP with the cell-free extracts of *C. natsudaidai* and *M. spicata*. The incubations gave limonene (**4**) and α-terpineol (**8**) in the case of *C. natsudaidai* and *M. spicata*, respectively. As shown in Table 3 (Expts 3–5), the $^3H:^{14}C$ ratios in limonene (**4**) arising from the mixtures of [1-$^3H_2$]LPP and [1-^{14}C]GPP, [1-$^3H_2$]LPP and [1-^{14}C]NPP, and [1-$^3H_2$]NPP and [1-^{14}C]GPP, respectively, were larger in comparison to the initial ratios of the substrate mixtures. The $^3H:^{14}C$ ratios in α-terpineol (**8**) arising from the analogous pairs of the ^3H- and ^{14}C-labelled substrates were also larger than the initial ratios in the pairs of these substrates (Table 3, Expts 9–11). These incorporation experiments with the cell-free extracts indicate the preferential conversion of LPP into limonene (**4**) and α-terpineol (**8**) rather than GPP and NPP.

However, such differences in the incorporations might be caused by an isotope effect due to the ^3H and ^{14}C labels.

Table 2. The $^3H:^{14}C$ ratios in several monoterpenoids biosynthesized from the [1-^{14}C,1-$^3H_2$]-labelled GPP, NPP and LPP in the cell-free system of *Mentha spicata*

Substrate	$^3H:^{14}C$ ratio in substrate	$^3H:^{14}C$ ratios in products			
		Geraniol (**1**)	Nerol (**2**)	Linalool (**3**)	α-Terpineol (**8**)
[1-^{14}C,1-^{3}H]GPP	4.53	4.59	4.42	4.25	4.00
[1-^{14}C,1-^{3}H]NPP	5.32	5.28	5.42	4.95	5.30
[1-^{14}C,1-^{3}H]LPP	10.7	10.9	9.70	10.7	11.6

Table 3. The ^3H:^{14}C ratios in limonene (4), carvone (5) and α-terpineol (8) biosynthesized from the mixtures of the ^3H- and ^{14}C-labelled geraniol, nerol, linalool, GPP, NPP and LPP

	Substrates			Products				Incorp. (%)	
Expt	The pairs*	μCi†	^3H:^{14}C ratio	Compounds‡	^3H (dpm)	^{14}C (dpm)	^3H:^{14}C ratio	^3H	^{14}C
1	^3H-G + ^{14}C-L	0.8	23.1	Carvone (5)	2.78×10^3	1.13×10^3	2.46	0.007	0.064
2	^3H-N + ^{14}C-L	0.9	19.6	Carvone (5)	9.73×10^3	7.65×10^2	12.7	0.025	0.038
3	^3H-LPP + ^{14}C-GPP	1.5	10.0	Limonene (4)	4.95×10^5	2.16×10^4	22.9	1.44	0.65
4	^3H-LPP + ^{14}C-NPP	1.5	8.10	Limonene (4)	2.22×10^5	1.85×10^4	12.0	0.82	0.56
5	^3H-NPP + ^{14}C-GPP	1.5	7.01	Limonene (4)	3.55×10^5	1.16×10^4	30.6	1.52	0.35
6	^3H-GPP + ^{14}C-LPP	1.0	9.24	Limonene (4)	5.50×10^4	1.52×10^4	3.62	0.27	0.68
7	^3H-NPP + ^{14}C-LPP	1.0	11.7	Limonene (4)	1.50×10^5	3.49×10^4	4.30	0.58	1.57
8	^3H-GPP + ^{14}C-NPP	1.5	7.78	Limonene (4)	1.24×10^5	3.22×10^4	3.85	0.48	0.97
9	^3H-LPP + ^{14}C-GPP	0.1	7.72	α-Terpineol (8)	1.73×10^4	8.60×10^2	20.1	1.01	0.39
10	^3H-LPP + ^{14}C-NPP	0.1	9.52	α-Terpineol (8)	1.24×10^4	1.12×10^3	11.1	0.59	0.50
11	^3H-NPP + ^{14}C-GPP	0.2	12.6	α-Terpineol (8)	3.64×10^4	1.54×10^3	23.6	0.65	0.35
12	^3H-GPP + ^{14}C-LPP	0.4	9.75	α-Terpineol (8)	1.57×10^4	9.47×10^3	1.66	0.18	1.07
13	^3H-NPP + ^{14}C-LPP	0.4	13.3	α-Terpineol (8)	3.38×10^4	7.26×10^3	4.66	0.29	0.82
14	^3H-GPP + ^{14}C-NPP	0.4	14.5	α-Terpineol (8)	1.16×10^4	8.49×10^3	1.37	0.09	0.96

*^3H-G, ^{14}C-L, ^3H-N, ^3H-GPP, ^3H-NPP, ^3H-LPP, ^{14}C-GPP, ^{14}C-NPP and ^{14}C-LPP denote [1-$^3H_2$]geraniol, [1-^{14}C]linalool, [1-$^3H_2$]nerol, [1-$^3H_2$]GPP, [1-$^3H_2$]NPP, [1-$^3H_2$]LPP, [1-^{14}C]GPP, [1-^{14}C]NPP and [1-^{14}C]LPP, respectively.

†The radioactivities are expressed with respect to only ^{14}C.

‡Compounds 4, 5 and 8 were obtained from the cell-free extract of *C. natsudaidai*, the leaves of *M. spicata* and the cell-free extract of *M. spicata*, respectively.

Table 4. Incorporation of [1-$^3H_2$]-labelled geraniol, nerol, linalool, GPP, NPP and LPP into the cyclic monoterpenoids in *Citrus hassaku* and *Perilla frutescens*

Substrates		Products		
Compounds	Radioactivity (μCi)	Compounds*	Radioactivity (dpm)	Incorporation (%)
[1-$^3H_2$]Geraniol	1.28	Limonene (4)	3.52×10^3	0.12
[1-$^3H_2$]Nerol	0.97	Limonene (4)	1.30×10^3	0.07
[1-$^3H_2$]Linalool	0.29	Limonene (4)	3.55×10^3	0.55
[1-$^3H_2$]Geraniol	5.90	Perillaldehyde (6)	2.91×10^3	0.022
[1-$^3H_2$]Nerol	1.98	Perillaldehyde (6)	1.16×10^3	0.026
[1-$^3H_2$]Linalool	2.99	Perillaldehyde (6)	4.68×10^3	0.071
[1-$^3H_2$]GPP	1.93	Limonene (4)	1.80×10^2	0.004
[1-$^3H_2$]NPP	1.20	Limonene (4)	1.87×10^2	0.007
[1-$^3H_2$]LPP	0.98	Limonene (4)	1.40×10^2	0.128

*Compounds 4 and 6 were obtained from the peels of *C. hassaku* and the leaves of *P. frutescens*, respectively.

A comparison of the incorporations was then made by use of mixtures in which the labelling of the substrates was reversed, such as [1-$^3H_2$]GPP and [1-^{14}C]LPP, [1-$^3H_2$]NPP and [1-^{14}C]LPP, or [1-$^3H_2$]GPP and [1-^{14}C]NPP. As shown for Expts 6–8 and 12–14 of Table 3, the ^3H:^{14}C ratios in the cyclic monoterpenoids were smaller than the initial ratios in the pairs of substrates; this confirms that the incorporation of these allylic substrates into the cyclic monoterpenoids is in the order LPP > NPP > GPP. The most preferential incorporation of LPP into the cyclic monoterpenoids occurred even when the racemic LPP was used as a substrate. If only one enantiomer of the racemic mixture of LPP participates in the biological formation of the cyclic monoterpenoids, the incorporation of LPP would be twice that of the racemic LPP, and the ^3H:^{14}C ratios in limonene (4) and α-terpineol (8) would be twice and one-half the ratios given in Expts 3, 4, 9 and 10 and Expts 6, 7, 12 and 13 of Table 3, respectively. It was thus confirmed that, during the biological formation of limonene (4) and α-terpineol (8) in the cell-free extract, the acyclic allylic pyrophosphates were incorporated into these cyclic monoterpenoids in the following order: LPP > NPP > GPP.

Thus, it was demonstrated that the conversion of LPP into cyclic monoterpenoids in both intact plants and cell-free extracts of the higher plants predominates over the

conversions of GPP and NPP. This implies that LPP or its tertiary-allylic equivalent is a prerequisite intermediate for the cyclization leading to the formation of the cyclic monoterpenoids. Quite recently, the two centre energy between C-1 and C-6 of linalool in the favourable conformation for cyclization was found to be higher than those of geraniol and nerol in the MO calculation on these allylic alcohols [41]. This observation also suggests that the tertiary-allylic compound is a prerequisite for the formation of cyclic monoterpenoids. The cyclization may be explained by the participation of the tertiary-allylic intermediate, which is the carbocation with the nucleophile such as a pyrophosphate anion [18, 42] or a nucleophilic group of the enzyme [38].

EXPERIMENTAL

Preparative TLC was on silica gel (Merck GF_{254}, 0.75 mm thick). TLC radiochromatographic measurements were made by an Aloka radiochromatogram scanner (JTC-203) using silica gel plates (Merck GF_{254}, 0.25 mm thick). GC-RC was performed on an Aloka radio-gaschromatograph system (RGC-212) equipped with a glass column (3 mm × 2 m) packed with 15% DEGS on Chromosorb AW-DMCS (80–100 mesh), an oxidation–reduction furnace, and a gas-flow proportional radioactivity counter (sensitivity, > 150 dpm for ^{14}C and > 200 dpm for ^{3}H at 300 ml/min of methane gas). Radioactivities of ^{3}H- and ^{14}C-labelled samples were measured on a Packard Tri-Carb liquid scintillation spectrometer (Model 3330) with counting efficiencies of 45 and 75%, respectively, in Bray's scintillant [43]. The counting error was about 3% for ^{3}H and about 4% for ^{14}C. Radioactivities of [^{3}H,^{14}C]-labelled samples were assayed with the same spectrometer in the same scintillant. Counting efficiencies of ^{3}H and ^{14}C were 35 and 57%, respectively, and the maximum overlap of ^{14}C into ^{3}H channel was about 10%.

(4*R*)-[4-^{3}H]MVA (1.8 mCi/mmol), DL-[5-$^{3}H_2$]MVA (6.7 mCi/mmol) and DL-[2-^{14}C]MVA (13 mCi/mmol) were products of the Radiochemical Centre, Amersham. $LiAl^{3}H_4$ (171.4 mCi/mmol) and [1-^{14}C]bromoacetic acid (6.29 mCi/mmol) were products of New England Nuclear, Boston, MA. (4*S*)-[4-^{3}H]MVA (307 mCi/mmol) was prepared as described in our previous paper [1].

Preparation of [1-^{14}C]*-labelled geraniol, nerol and linalool.* Methyl bromo[1-^{14}C]acetate (177 mg, 1.0 mCi), which was prepared from bromo[1-^{14}C]acetic acid with CH_2N_2, was heated with triethyl phosphite (190 mg) at 130° for 5 hr. Removal of excess phosphite under red. pres. (60°, 5 mmHg) gave methyl diethylphosphono[1-^{14}C]acetate (200 mg). 6-Methyl-5-hepten-2-one (118 mg) was added to a soln of the phosphonoacetate (200 mg) and NaH (43 mg; 66% in oil) in DMF (3 ml) under N_2. The mixture was allowed to stand for 3 days at 30° and then extracted with Et_2O after addition of H_2O. The Et_2O extract was subjected to preparative TLC with hexane–Et_2O (49:1) to give methyl nerate (24 mg) and methyl geranate (71 mg), followed by the $LiAlH_4$ reduction to yield [1-^{14}C]nerol (15 mg, 0.14 mCi) and [1-^{14}C]geraniol (43 mg, 0.40 mCi), respectively.

A soln of [1-^{14}C]geraniol (8 mg, 0.074 mCi) and 1 M HCl (0.1 ml) in Me_2CO (0.16 ml) was stirred at 40° for 5 hr. The reaction mixture, after neutralization with 0.5 M Na_2CO_3, was extracted with Et_2O. The Et_2O soln was washed with H_2O, dried over Na_2SO_4 and concd. The crude product obtained was subjected to preparative TLC with EtOAc–hexane (1:4) to give [1-^{14}C]linalool (4 mg, 0.037 mCi) and unchanged geraniol (2.5 mg).

Preparation of [1-$^{3}H_2$]*-labelled geraniol, nerol and linalool.* [1-$^{3}H_2$]Geraniol and [1-$^{3}H_2$]nerol were prepared by reduction of methyl geranate and methyl nerate with $LiAl^{3}H_4$, respectively. [1-$^{3}H_2$]Linalool was prepared from [1-$^{3}H_2$]geraniol by the acid-catalysed rearrangement [44] as described for the preparation of [1-^{14}C]linalool.

Preparation of ^{3}H- *and* ^{14}C*-labelled LPP, GPP and NPP.* Each of ^{3}H- and ^{14}C-labelled linalool, geraniol and nerol prepared as above was converted to the corresponding pyrophosphate ester with a dioxane diphosphate complex following the method reported previously [45, 46]. The homogeneity of these allylic pyrophosphates was confirmed by the observation that only the corresponding acyclic allylic alcohol was yielded from each of the pyrophosphate on enzymatic hydrolysis with potato apyrase [47]; this was checked by the radio-GC analysis.

Seasonal variation of the contents of linalool (3) *and carvone* (5) *in* M. spicata. The fresh sprigs (10 cm in length and about 30 g in total wt) of *M. spicata* were collected in April to September at intervals of 15 days. The sprigs were frozen with liquid N_2, ground in a mortar and extracted with Et_2O. The Et_2O soln was dried over Na_2SO_4 and concd to give an essential oil, which was subjected to GLC with a 15% DEGS column at 120°. The contents of linalool (3) and carvone (5) were determined from the GLC trace by use of a standard curve prepared with an authentic sample of carvone.

Feeding of the labelled substrates to the peel of Citrus *species.* Each of the labelled substrates dissolved in a phosphate buffer (pH 7.3) was fed to the cut-peel of *C. natsudaidai* and *C. hassaku.* After uptake of the substrate, the phosphate buffer was supplemented over 24 hr. The peel was ground with Na_2SO_4 in a mortar and extracted with Et_2O (5 × 100 ml). An essential oil obtained from the Et_2O soln was subjected to CC over silica gel with hexane to give limonene (4), which was converted to the tetrabromide derivative (mp 103–104°) with bromine and/or the 1,2-dihydroxy derivative (mp 71–72°) [48] by hydrolysis of the 1,2-oxide obtained from the limonene by epoxidation with *m*-chloroperbenzoic acid. These derivatives were purified to a constant specific radioactivity by recrystallization (Tables 1 and 4).

Feeding of the labelled substrates to the leaves of the higher plants. A soln of each of the labelled substrates in a phosphate buffer (pH 7.3) was fed to the leaves of the higher plants, such as *M. spicata, M. piperita, P. frutescens, C. camphora, A. annua* and *P. thunbergii,* through their cut-stems and then the phosphate buffer was soaked up into the leaves over 24 hr. The plant materials were ground with Na_2SO_4 in a mortar and extracted with hexane (5 × 30 ml). The hexane extract was subjected to preparative TLC with EtOAc–hexane (1:9) to give the respective products. After addition of a carrier, each of the products was converted to a crystalline derivative, which was purified to a constant specific radioactivity by TLC and/or recrystallization. Carvone (5) and menthol (7) obtained from *M. spicata* and *M. piperita* were converted to the semicarbazone derivative (mp 142–143°) and the 3,5-dinitrobenzoate derivative (mp 152–153°), respectively. Perillaldehyde (6) and camphor (10) from *P. frutescens* and *C. camphora* were converted to perillyl alcohol and isoborneol, respectively, by the $LiAlH_4$ reduction (Tables 1 and 4).

Feeding of the paired substrates [1-$^{3}H_2$]*nerol and* [1-^{14}C]*linalool or* [1-$^{3}H_2$]*geraniol and* [1-^{14}C]*linalool to the leaves of* M. spicata. Each of the mixtures of [1-$^{3}H_2$]nerol (2.7 mCi/mmol) and [1-^{14}C]linalool (0.14 mCi/mmol) or [1-$^{3}H_2$]geraniol (3.2 mCi/mmol) and [1-^{14}C]linalool (0.14 mCi/mmol) was dissolved in 5% EtOH (5 ml) and fed to the leaves of *M. spicata* (30 g). After uptake of the substrate, additional H_2O was taken up into the leaves over 72 hr. The plant materials were cut into small pieces, ground with Na_2SO_4 in a mortar and extracted with Et_2O (3 × 100 ml). The extract was

subjected to preparative TLC with EtOAc–hexane (1:9) to give carvone (5) (about 60 mg), which was converted to the semicarbazone derivative (mp 141–142°). Its radioactivity was as given in Table 3.

Preparation of the cell-free extracts of M. spicata *and* C. natsudaidai. Following the described procedure [49], cell-free extracts were prepared from the leaves of *M. spicata* and the peel of *C. natsudaidai*. All of the operations were carried out at 4°. The fresh tissues (15 g) were frozen with liquid N_2, ground in a mortar and slurried with insoluble polyvinylpyrrolidone (20 g) in 0.1 M K-Pi buffer (pH 7.3, 110 ml) containing 2 mM 2-mercaptoethanol. The resulting paste was filtered through two layers of cheesecloth. The filtrate was centrifuged at 3000 *g* for 5 min and then 30 000 *g* for 20 min. The 30 000 *g* supernatant was used as the cell-free extract for incubations.

Incubations of the [1-^{14}C,1-$^{3}H_2$]*-labelled NPP, GPP and LPP with the cell-free extract of* M. spicata. The [1-^{14}C,1-$^{3}H_2$]-labelled allylic pyrophosphates (1.0 μCi of ^{14}C) were prepared by mixing the [1-$^{3}H_2$]-labelled allylic pyrophosphate (7.5 mCi/mmol) and the [1-^{14}C]-labelled allylic pyrophosphate (1.1 mCi/mmol) to give the ^{3}H:^{14}C ratio shown in Table 2 and then adjusting the total amount to 5 μmol with addition of the corresponding non-radioactive allylic pyrophosphate. Each of the double labelled NPP, GPP and LPP was dissolved in 0.1 M K-Pi buffer containing $MgCl_2$ (4 mM). To this soln (1 ml) in a glass-stoppered tube, the cell-free extract (2 ml) was added. The mixture was incubated for 90 min at 30°. A control experiment was carried out in parallel by use of cell-free extract deactivated by heating at 85° for 3 min. After incubation the mixture was adjusted to pH 9 and incubated with potato apyrase [47] for 3 hr at 30°. The reaction mixture was extracted with Et_2O (3 × 2 ml). The Et_2O extract was analysed by GLC radio-chromatography (Fig. 1). After addition of a cold sample of nerol, geraniol, linalool and α-terpineol (each 10 mg), the mixture was subjected to preparative TLC with EtOAc–hexane (1:4) to give the respective compound, which was further purified by preparative TLC on 10% $AgNO_3$–silica gel plates with EtOAc–hexane (1:1). The radioactivities of geraniol (1), nerol (2), linalool (3) and α-terpineol (8) are given in Table 2.

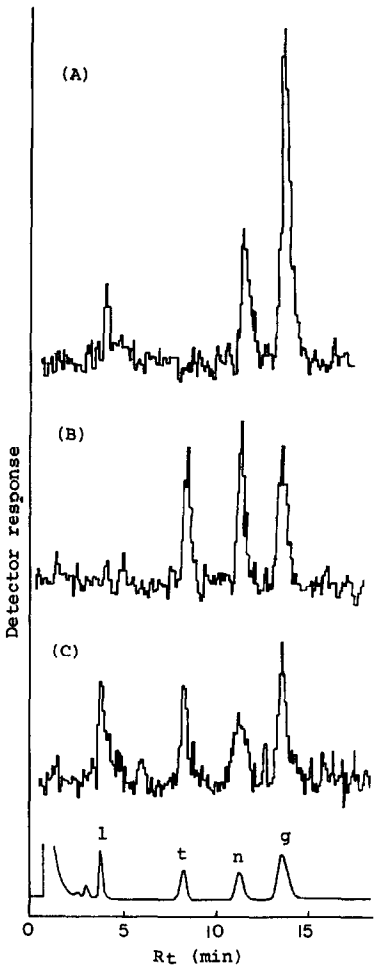


Fig. 1. GLC radiochromatograms of the monoterpenoids formed from [1-^{14}C,1-$^{3}H_2$]-labelled GPP (A), NPP (B) and LPP (C) by incubation in a cell-free extract of *Mentha spicata*. The bottom trace is the response of the flame ionization detector of the GLC to co-injected standards of linalool (l), α-terpineol (t), nerol (n) and geraniol (g).

Incubations of the paired substrates of ^{3}H- *and* ^{14}C*-labelled NPP, GPP and LPP with the cell-free extract of* C. natsudaidai *and* M. spicata. A mixture of the ^{3}H- and ^{14}C-labelled acyclic allylic pyrophosphates was prepared by mixing the ^{3}H-labelled substrate with the ^{14}C-labelled substrate to give the ^{3}H:^{14}C ratio shown in Table 3 and then adjusting the amounts with addition of the corresponding non-radioactive allylic pyrophosphate. The paired substrate was incubated with the cell-free extract of *C. natsudaidai* and *M. spicata* for 90 min at 30°. In the case of *C. natsudaidai*, limonene (50 mg) was added to the incubation mixture as carrier and then the radioactive limonene (4) was isolated and converted to the tetrabromide derivative (mp 103–104°). This derivative was purified to constant specific radioactivity by recrystallization. In the case of *M. spicata*, α-terpineol (50 mg) was added to the incubation mixture as carrier and then the radioactive α-terpineol (8) was isolated. The radioactivities of 4 and 8 are given in Table 3.

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