Regioselective Hydrogen Elimination from the 10-Methyl Group of Geranyl Diphosphate in the Biological Formation of the 8(9)-Double Bond of Limonene

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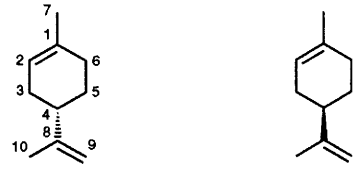
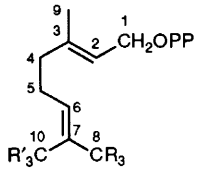
The biological formation of the 8(9)-double bond of both (4*S*)-(−)- and (4*R*)-(+)-limonenes in *Mentha spicata* and *Citrus unshiu* is found to be stereospecifically controlled by the regioselective elimination of a hydrogen atom from the 10-methyl group, *i.e.* the *Z*-methyl group, of geranyl diphosphate.

Mentha spicata (spearmint) and *Citrus unshiu* (unshiu orange) produce biospecifically (4*S*)-(−)- and (4*R*)-(+)-limonenes, respectively. In this connection, it was found that (4*S*)-(−)- and (4*R*)-(+)-limonenes **1a** and **1b** are biosynthesized from non-chiral geranyl diphosphate (GPP) **2a** with the limonene synthase preparation from *M. spicata* and *C. unshiu*, respectively and the cation intermediate being in an *anti-endo* conformation is involved in the biosynthesis of **1a** and **1b**.[1,2] However, little is known as to whether the 8(9)-double bond of **1a** and **1b** is formed by the regioselective or random elimination of a hydrogen atom from 8- and/or 10-methyl groups of **2a**. We could establish that the 8(9)-double bond is biologically formed by the regioselective hydrogen elimination from the 10-methyl group of **2a**.

[8,8,8-$^2H_3$]GPP and [10,10,10-$^2H_3$]GPP were suited to this purpose, since they should yield limonene deuteriated at the 8- or 10-position. Following the reported methods,[3–6] [8,8,8-$^2H_3$]GPP (93.1% deuteriated) and [10,10,10-$^2H_3$]GPP (99.0% deuteriated) were chemically synthesized from geranyl benzyl ether. Following our previous reports,[1,2] the enzyme system responsible for the cyclization of geranyl diphosphate to limonene was partially purified from the leaves of *M. spicata* and *C. unshiu*. To the limonene synthase preparation (*ca.* 270 μg protein ml^{-1}) dissolved in 50 mmol dm^{-3} TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer (pH 7.0; 3.0 ml), a solution of the substrate (15 μmol), *i.e.* [8,8,8-$^2H_3$]GPP **2b** or [10,10,10-$^2H_3$]GPP **2c**, magnesium chloride (40 μmol) and potassium fluoride (40 μmol) in the same buffer (1 ml) was added. The mixture was incubated at 30 °C for 20 h under stirring and N_2. The incubation was repeated eleven times in the case of *M. spicata* and eight times in the case of *C. unshiu*, and the



(4*S*)-(−)-Limonene **1a** (4*R*)-(+)-Limonene **1b**



GPP **2a**; R = R′ = H

[8,8,8-$^2H_3$]GPP **2b**; R = ^2H, R′ = H

[10,10,10-$^2H_3$]GPP **2c**; R = H, R′ = ^2H

Table 1 Mass spectra and intensity of the limonenes biosynthesized from [8,8,8-$^2H_3$]GPP **2b** and [10,10,10-$^2H_3$]GPP **2c** with the limonene synthase preparation of *M. spicata*

	m/z (rel. intensity, %)[a]
Limonene from **2b**	139 ([M$^+$], 17.5), 138[b] ([M$^+$], 0.9) 124 ([M − CH_3]$^+$, 16.7), 121 ([M − CD_3]$^+$, 11.6), 123[b] ([M − CH_3]$^+$, 4.9) 68 ([C_5H_8]$^+$, 100), 71 ([$C_5H_5D_3$]$^+$, 85.7)
Limonene from **2c**	138 ([M$^+$], 13.0), 139[b] ([M$^+$], 5.8) 123 ([M − CH_3]$^+$, 20.6), 124[b] ([M − CH_3]$^+$, 6.5), 121[b] ([M − CD_3]$^+$, 5.8) 68 ([C_5H_8]$^+$, 100), 70 ([$C_2H_5D_2$]$^+$, 80.2)

[a] Authentic sample of limonene exhibited fragments (rel. intensity, %) as follows: 136 ([M$^+$], 10.2), 121 ([M − CH_3]$^+$, 14.0) and 68 ([C_5H_8]$^+$, 100). [b] Fragment ion due to the deuteriated limonene arising from scrambling of the label.

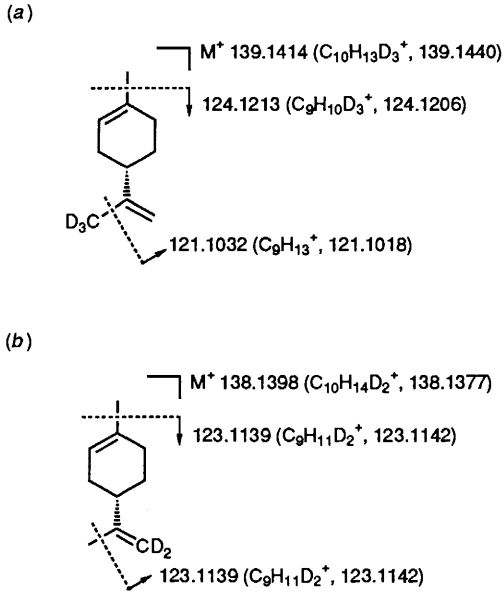
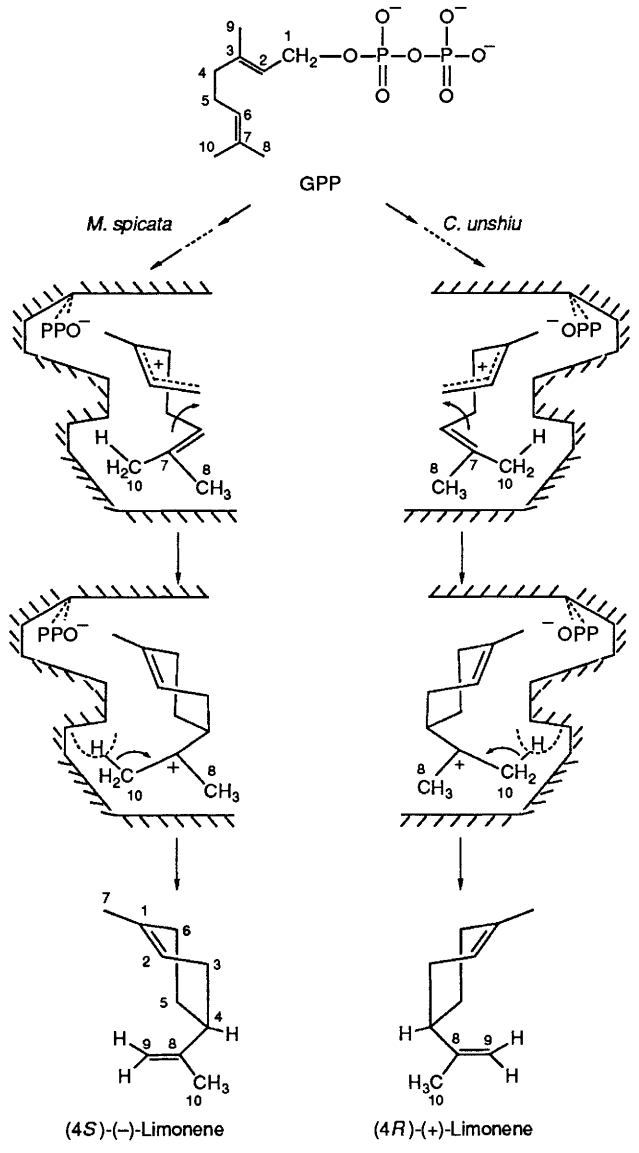


Fig. 1 High-resolution mass data of the molecular and fragment ions of (*a*) trideuteriated and (*b*) dideuteriated limonenes biosynthesized from [8,8,8-$^2H_3$]GPP and [10,10,10-$^2H_3$]GPP, respectively, with the limonene synthase preparation of *M. spicata*. The figures shown outside the parentheses indicate *m/z* observed for the molecular and fragment ions and those shown inside the parentheses are *m/z* calculated for the formulae of the ions.



Scheme 1 Mechanistic interpretation of the regioselective formation of the 8(9)-double bond from non-chiral geranyl diphosphate in an active site of limonene synthase in the biosynthesis of (4*S*)-(−)- and (4*R*)-(+)-limonenes by *M. spicata* and *C. unshiu*

incubation mixture was extracted with dimethyl ether. Identification of biosynthesized limonene in the dimethyl ether extract was done by GLC and co-GLC analyses. The deuteriation of the limonene was determined by GC–MS analysis in each case. Separation of the deuteriated limonene from the combined dimethyl ether extract was done by silica gel column chromatography with pentane as solvent, and the limonene separated was subjected to high-resolution mass spectrometry and ^2H NMR measurement to determine the deuterium labelling positions.†

In the case of *M. spicata*, deuterium enrichments of the C-10 trideuteriated and C-9 dideuteriated limonenes were 99.3 and 99.1%, respectively, on the basis of the intensity of the molecular ion peak.[7] The limonene biosynthesized from **2b** was found to consist of 95.4 ± 4.3% of C-10 trideuteriated limonene and 4.6 ± 4.3% of C-9 dideuteriated limonene on the basis of the relative intensity of molecular ion and M − CD_3/CH_3 fragment ion peaks given in Table 1.[7,8] The high-resolution mass data of the molecular and fragment ions

† Attempts, after the ^2H NMR measurements, to obtain the ^1H NMR spectrum of the deuteriated sample failed, since the sample had been lost during evaporation of the solvent ($CHCl_3$).

were as shown in Fig. 1. The deuterium labelling positions in the limonenes were established not only by the mass fragmentation patterns, but also by the deuterium NMR signals at δ 1.65 assigned to the 10-methyl group. These results indicated that the hydrogen atom at C-10 of **2b** was regioselectively eliminated. On the other hand, the deuteriated limonene biosynthesized from **2c** was found to consist of 69.7 ± 15.2% of C-9 dideuteriated limonene and 30.3 ± 15.2% of C-10 trideuteriated limonene in the same manner as above. The low ratio of C-9 dideuteriated limonene to C-10 trideuteriated limonene should be ascribed to the $^{1}H/^{2}H$ isotope effect in the elimination of deuterium atom from C-10 of **2c**.[5,9] Thus, the 9-methylene of (4*S*)-(−)-limonene **1a** was shown to arise specifically from the 10-methyl group of GPP **2a** in the biosynthesis of **1a** by *M. spicata*.

In the same manner as in the case of *M. spicata*, incubation of the trideuteriated substrates **2b** and **2c** with the limonene synthase preparation from *C. unshiu* yielded (4*R*)-(+)-limonenes. Their GC–MS and ^{2}H NMR spectra showed the same patterns as those described in the case of *M. spicata*. The results indicated that the 10-methyl group of GPP **2a** also participates in the biological formation of 9-methylene of (4*R*)-(+)-limonene **1b** by *C. unshiu*.

Thus, it was established that the biological formation of the 8(9)-double bond of both (4*S*)-(−)- and (4*R*)-(+)-limonenes in *M. spicata* and *C. unshiu* is stereospecifically controlled by the regioselective hydrogen elimination from the 10-methyl group, *i.e.* the *Z*-methyl group, of GPP. When considering the cyclization of GPP *via* the stereochemically specified intermediate situated in the *anti-endo* spatial arrangement,[2] it is fascinating to note that the 8(9)-double bond of both (4*S*)-(−)- and (4*R*)-(+)-limonenes might be formed by the regioselective elimination of a hydrogen atom from the C-10 methyl group of the enantiomeric carbocation intermediate with restricted rotation in an active site of the enzyme, as shown in Scheme 1.

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