Biosynthetic Generation of the Species-specific Chirality of Limonene in *Mentha spicata* and *Citrus unshiu*

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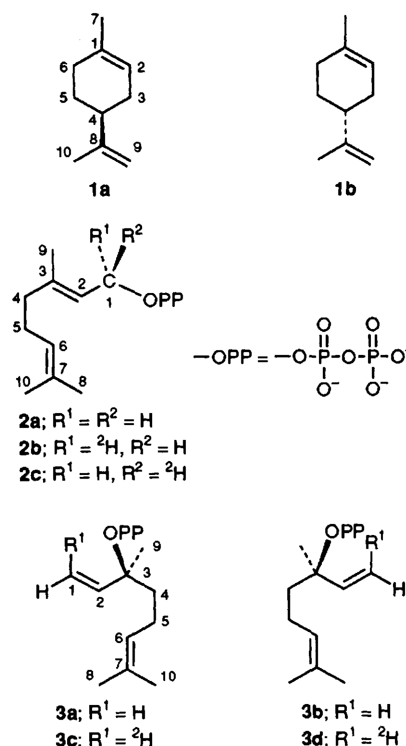
The biosynthetic generation of the species-specific chirality at C-4 of (4*S*)-(−)- and (4*R*)-(+)-limonenes in *Mentha spicata* and *Citrus unshiu*, respectively, is ascribed to the enantiomeric *endo*-spatial arrangement of linalyl cation intermediates which are both formed by the (2*Re*,3*Si*)-face elimination of the diphosphoryl group with respect to the 2(3)-double bond of geranyl diphosphate.

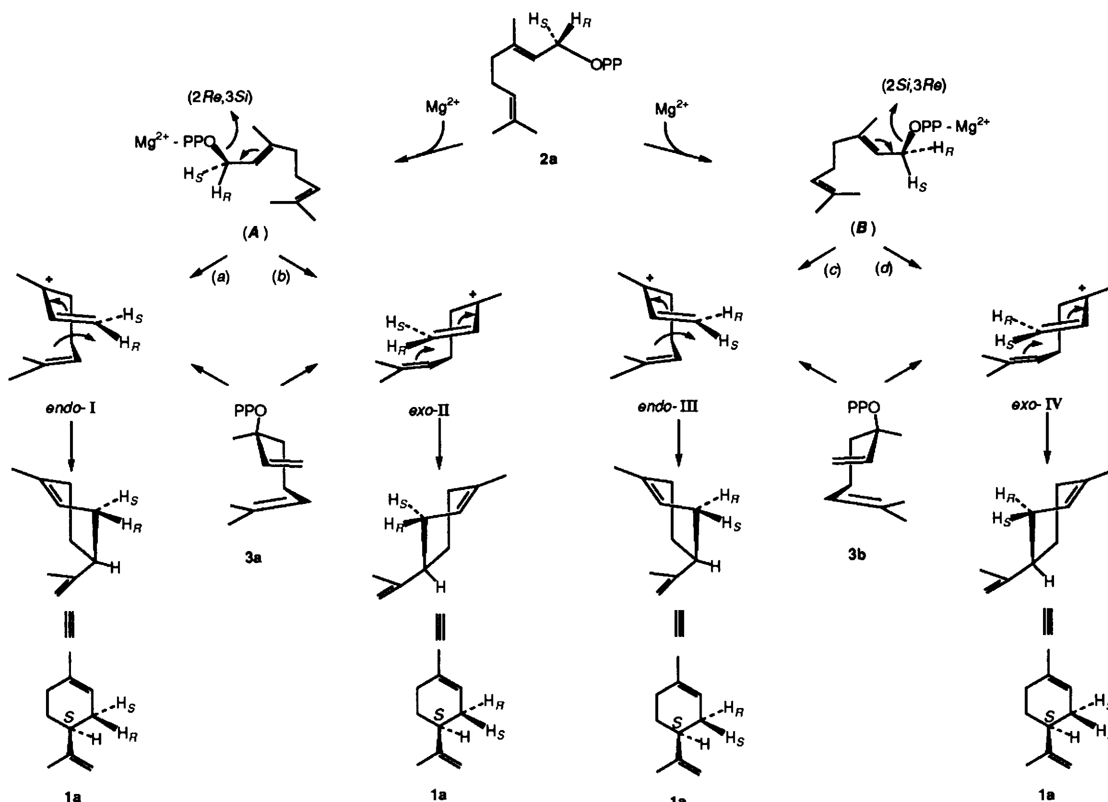
(4*S*)-(−)- and (4*R*)-(+)-Limonenes **1a** and **1b** are species specific to the essential oil of *Mentha spicata* (spearmint) and *Citrus unshiu* (unshiu orange), respectively. The limonene synthases of *M. spicata* and *C. unshiu* require divalent metal cations, such as Mn^{2+} and Mg^{2+}, for them to show activity.[1–3] The divalent metal ion binds to the diphosphoryl group of geranyl diphosphate (GPP) **2a** to form a GPP–metal chelate, and the chelation weakens the C–OP bond of **2a**, facilitating the elimination of the diphosphoryl group.[4] This report clarifies the stereochemical process governing the biosynthetic generation of the species-specific chirality at C-4 of **1a** and **1b**.

In the biosynthesis of **1a**, the diphosphoryl group may eliminate either at the (2*Re*,3*Si*)- or the (2*Si*,3*Re*)-face with respect to the plane of the 2(3)-double bond of **2a**, as shown in (***A***) and (***B***) of Scheme 1. The elimination of the diphosphoryl group at the (2*Re*,3*Si*)-face (***A***) from C-1 of **2a** results in the generation of a geranyl cation followed by the isomerization to a cyclizable linalyl cation situated in an *endo*-spatial arrangement **I** or an *exo*-arrangement **II**, while the elimination at the (2*Si*,3*Re*)-face (***B***) gives the linalyl cation situated in an *endo*-spatial arrangement **III** or an *exo*-arrangement **IV**. These cation intermediates **I–IV** lead to **1a** by cyclization.

(1*S*)-[1-^{2}H]GPP **2b**† and (1*R*)-[1-^{2}H]GPP **2c**† were used as substrates to determine the elimination face of the diphosphoryl group from **2a** and the spatial arrangement of the linalyl cation intermediate in the biosyntheses of **1a** and **1b**, since they should give limonene stereospecifically deuteriated



† Compounds **2b** and **2c** were prepared[7] from (1*S*)-[1-^{2}H]geraniol[8] (98% deuteriated, 99% e.e., 99.9% on GLC) and (1*R*)-[1-^{2}H]geraniol[9] (98% deuteriated, 89% e.e., 99.9% on GLC), respectively (e.e. = enantiomeric excess). Enantiomeric purities were retained after phosphorylation, as confirmed by ^{1}H NMR analyses of the (*R*)-(+)-α-methoxy-α-trifluoromethylphenylacetates (MTPA) of the geraniols obtained from **2b** and **2c** on hydrolysis with alkaline phosphatase.



Scheme 1 The pathways expected for the biosynthesis of **1a** from **2a** by the limonene synthase of *M. spicata* and the expected location of the *pro*-1*S* hydrogen atom of **2a** in **1a**. In *C. unshiu*, the cyclization of **2a** *via* mirror images of these intermediates forms **1b**. $^{-}$OPP–Mg^{2+} denotes the diphosphoric acid–magnesium ion chelate.[4] *Endo* and *exo* stand for the *endo*- and *exo*-spatial arrangements of the linalyl cation, respectively. The configuration of the linalyl cation is described as *endo* and *exo* rather than boat and chair, respectively.[5,6]

at the 3-position. Following the reported method,[2,3] limonene synthase systems were prepared from the leaves of *M. spicata* and *C. unshiu* and partially purified about 54-fold. To the enzyme system dissolved in TES [*N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid] buffer (pH 7.0), **2a** (7.5 mmol dm^{-3}) was added and the solution was incubated at 30 °C for 20 h in the presence of Mg^{2+} (10 mmol dm^{-3}) and KF (10 mmol dm^{-3}). The biosynthesized limonene was purified by silica gel chromatography and identified by co-GLC and by comparison of the mass spectrum with that of authentic limonene.

The transformation of **2a** into **1a** by limonene synthase system prepared from *M. spicata* was 1.2% and **1a** was laevorotatory {$[\alpha]_D^{25}$ −93 (*c* 0.13, hexane)}; this optical property is similar to that of **1a** present in the intact plant of *M. spicata*. The deuteriated **1a** biosynthesized from **2b** exhibited mass spectral ions at *m/z* ([ion], rel. intensity) 137 ([M^+], 5), 122 ([M − CH_3]$^+$, 9) and 69 ([C_5H_7D]$^+$, 100), while those of the authentic sample of **1a** were at *m/z* 136 ([M^+], 10), 121 ([M − CH_3]$^+$, 14) and 68 ([C_5H_8]$^+$, 100). The deuterium enrichment of the deuteriated **1a** was 98% on the basis of the intensity of the molecular ion peak.[10] The deuterium introduced to C-3 was *cis* to 4-H. This was established by the appearance of the deuterium NMR signal and the disappearance of the proton NMR signal at δ 2.07. In the deuteriated **1a** biosynthesized from **2c**, the deuterium enrichment was 98% and the deuterium atom at C-3 was introduced to the position *trans* to 4-H (δ 1.90 in the ^{2}H NMR spectrum). These findings indicate that the biosynthesis of (3*S*)-[3-^{2}H]-**1a** from **2b** in *M. spicata* proceeds either by (2*Re*,3*Si*)-face elimination of the diphosphoryl group with the formation of a linalyl cation situated in an *endo*-spatial arrangement **I** or by (2*Si*,3*Re*)-face elimination giving an *exo*-arranged cation **IV**, followed by cyclization of the cations **I** and **IV**, as shown in Scheme 1.

The linalyl cation intermediates, *endo*-**I** and *exo*-**IV**, can be directly generated by *anti*-elimination[5,6] of the diphosphoryl group from (3*S*)-(+)-LPP **3a**‡ and (3*R*)-(−)-LPP **3b**,‡ respectively. The energy barriers for the elimination of the diphosphoryl group from **2a** and the LPPs were evaluated on the basis of MO calculations[11] to be 369.9 and 330.2 kJ mol^{-1}, respectively. The energy barrier for the 1,3-suprafacial migration of the diphosphoryl group in **2a** to form the LPPs was estimated to be 405.4 kJ mol^{-1}. These energy values suggest that the LPPs rather than **2a** give more easily a linalyl cation by elimination of the diphosphoryl group.

The transformation of **3a** into **1a** by the above limonene synthase system was 1.4% and the optical rotation of **1a** was $[\alpha]_D^{25}$ −95 (*c* 0.06, hexane). On the other hand, **3b** was hardly transformed into **1a**. The deuteriated **1a** (74.2% deuterium enrichment) biosynthesized from (3*S*)-(+)-[1*Z*-^{2}H]LPP **3c**‡ had the 3-deuterium atom situated *cis* to 4-H (δ 2.07 in the ^{2}H NMR spectrum). These facts indicate that the linalyl cation situated in an *endo*-spatial arrangement **I** is an intermediate in the biosynthesis of **1a** from **3a** and that no conversion of *endo*-**I** into *exo*-**IV** by rotation of the 2(3)-single bond of *endo*-**I** takes place. Therefore, the biosynthesis of **1a** from **2a** involves the linalyl cation situated in an *endo*-spatial arrangement **I** which is formed by the elimination of the diphosphoryl group at the

‡ Compounds **3a** and **3b** were prepared[12] from (3*S*)-(+)-linalool {$[\alpha]_D^{25}$ +16.8 (neat), 83% e.e.} and (3*R*)-(−)-linalool {$[\alpha]_D^{25}$ −20.1 (neat), 99% e.e.}, respectively. Similarly, **3c** and **3d** were prepared from (3*S*)-(+)-[1*Z*-^{2}H]- and (3*R*)-(−)-[1*Z*-^{2}H]linalools[13] (74.2% deuteriated), respectively. The enantiomeric purities of **3a**, **3b**, **3c** and **3d** were determined to be 83, 99, 82 and 98% e.e, respectively, by GLC analyses of the corresponding linalools obtained from the diphosphates on hydrolysis with alkaline phosphatase.

(2*Re*,3*Si*)-face with respect to the plane of the 2(3)-double bond of the GPP-metal chelate [pathway (*a*) in Scheme 1].

Next, substrates, **2a**, **2b**, **2c**, **3a** and **3b**, were incubated with the limonene synthase system of *C. unshiu* in the same manner as in the case of *M. spicata*. Of these substrates, **2a** and **3b** were transformed into (4*R*)-(+)-limonene **1b** {$[\alpha]_D^{25}$ +93 (*c* 0.05, hexane), 0.47% yield; $[\alpha]_D^{25}$ +120 (*c* 0.10, hexane), 0.46% yield, respectively}, but **3a** was hardly transformed into **1b**. The GC-MS and ^2H NMR spectra of the deuteriated **1b** (98% deuterium enrichment) biosynthesized from **2b** and **2c** showed that the deuterium atom at C-3 is *cis* and *trans* to 4-H, respectively. In the deuteriated **1b** (74.2% deuterium enrichment) biosynthesized from (3*R*)-(−)-[1*Z*-^2H]LPP **3d**,‡ the deuterium atom at C-3 is *cis* to the 4-H (δ 2.07 in the ^2H NMR spectrum). These results indicate that the biosynthesis of **1b** from **2a** with the limonene synthase system of *C. unshiu* involves the intermediacy of a mirror image of the *endo*-linalyl cation **I** which is formed by the elimination of the diphosphoryl group at the (2*Re*,3*Si*)-face, *i.e.*, at the same face as in the case of *M. spicata* [mirror image of pathway (*a*) in Scheme 1].

In conclusion, the spatial arrangement of the linalyl cation intermediate was proved to hold the key in the generation of the species-specific chirality of limonene. The diphosphoryl group of **2a** is eliminated from the (2*Re*,3*Si*)-face by limonene synthase systems of both *M. spicata* and *C. unshiu*. However, the linalyl cations formed are mirror image related, indicating that the active site of these enzymes are enantiomeric. The species-specific chirality of limonene is thus ascribed to the enantiomeric *endo*-spatial arrangement of the linalyl cation itnermediate in the active site of the limonene synthase.

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