Stereochemistry of the methyl→methylene elimination in the enzyme-catalysed cyclization of geranyl diphosphate to (4*S*)-limonene

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The cyclization of (*R*)-[9-$^2H_1$,$^3H_1$]geranyl diphosphate catalysed by a recombinant form of (4*S*)-limonene synthase from spearmint leaf (*Mentha spicata*) is terminated predominantly by *re*-facial, *anti* proton elimination at the *cis* methyl group, producing (4*S*, 8*E*)-[8-$^2H_1$,$^3H_1$]limonene.

(4*S*)-Limonene synthase from *Mentha* species catalyses the coupled isomerization–cyclization of geranyl diphosphate **1** (GPP) to (4*S*)-limonene **3** by way of the enzyme-bound intermediate, (3*S*)-linalyl diphosphate **2** (LPP) (Scheme 1).[1] (4*R*)-Limonene synthase in *Citrus* species is responsible for producing the enantiomeric component of peel oil.[2] (4*S*)-Limonene synthase has been purified from both peppermint (*M. piperita*) and spearmint (*M. spicata*) and characterized.[3]

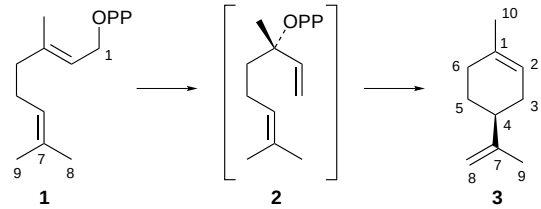
The mechanism of action and structural features of both (4*S*)- and (4*R*)-limonene synthases have been investigated using enzymes from *Mentha*, *Citrus* and other plant species.[4–6] The reaction leading to (4*S*)-limonene proceeds *via* stepwise, suprafacial isomerization of **1** to **2** followed by *anti*, *endo* S_N' cyclization with overall retention of configuration at C(1)[4] and terminating proton transfer from the *cis* methyl group [C(9)].[4,5] Here we report evidence that establishes the stereochemistry of the final $CH_3 \rightarrow CH_2$ elimination.

A protein-based cloning strategy was employed to isolate a cDNA encoding (4*S*)-limonene synthase from a spearmint leaf cDNA library.[7] The 1800 nucleotide open-reading frame translates a 500 amino acid protein bearing a putative plastidial targeting peptide. The cDNA isolate was functionally expressed in *E. coli* to give catalytically active protein that affords (*S*)-limonene and its minor co-products [2% each of myrcene, (−)-α-pinene and (−)-β-pinene]. The recent development of a high level heterologous expression system[8] provided sufficient quantities of the crude recombinant protein for product analyses by ^3H NMR spectroscopy.

Substrate bearing a chiral methyl group of (*R*) configuration at the *cis* C(9) position (**5c**) was synthesized as outlined in Scheme 2. Z-Selective condensation of (*E*)-6-benzyloxy-4-methylhex-4-enal with ethyl 2-(diethoxyphosphoryl)propionate followed by AlD_3 reduction afforded dideuterio alcohol **4a**. Catalytic ruthenate oxidation and asymmetric reduction of the labile *Z*-aldehyde with (*S*)-Alpine-Borane[9] provided **4b**, the expected *R* configuration and enantiomeric purity (≥97%) of which were confirmed by ^1H NMR analysis of its (1′*S*)-camphanate derivative.[10]† Evidence that the methanesulfonate (MsO) of **4b** undergoes nucleophilic substitution with inversion was obtained by its conversion to the *S* enantiomer of **4b** (≥97% *S*) with Bu_4NOAc in Et_2O followed by $LiAlH_4$ cleavage and ^1H NMR analysis of the (1′*S*)-camphanate.

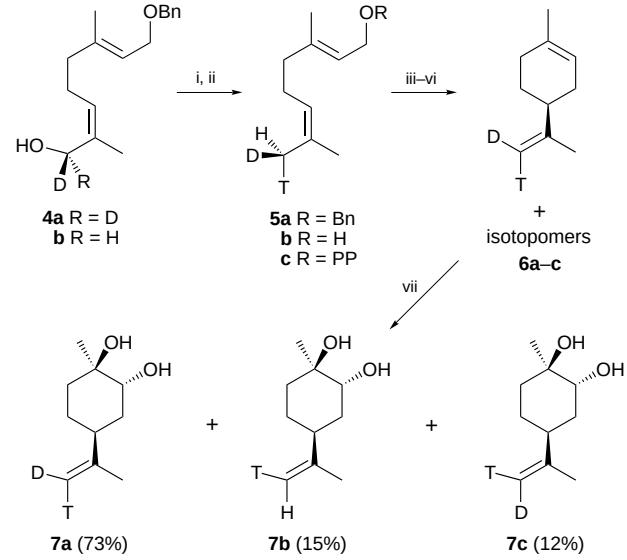
Reduction of **4b**–OMs with $LiBEt_3T$[11] (THF, 0 °C) was assumed to proceed with inversion, creating an *R* chiral methyl group in **5a** (1.0 Ci, *ca*. 6.7 Ci mmol^{-1}). Conversion to (*R*)-[9-$^2H_1$,$^3H_1$]GPP **5c** (23 mCi, 66%) was accomplished by literature procedures[12] adapted for radiochemical operations. The radiochemical purity of (*R*)-[9-$^2H_1$,$^3H_1$]geraniol **5b** (114 m Ci, 88%) was established by radio TLC, ^3H NMR spectroscopy and isotopic dilution analysis as the 3,5-dinitrobenzoate derivative.†

Incubation of **5c** with the crude recombinant (*S*)-limonene synthase[8]‡ afforded, after dilution with 1 mg of cold carrier and filtration over silica gel, [8-^2H,^3H]limonenes **6a**–**c**, (4.1 m Ci, 21%). Regioselective epoxidation followed by acid-catalysed hydrolysis and chromatographic purification gave [8-^2H,^3H]-menth-7-ene-1,2-diols **7a**–**7c** (1.0 m Ci, 24%), judged to be of high radiochemical purity by radio TLC and dilution analysis. Assignments of the isopropenyl vinyl proton signals at δ 4.81 and 4.77 in the 500 MHz ^1H NMR spectrum (C_6D_6) of unlabelled diol to H_Z and H_E, respectively, were secured by



1 **2** **3**

Scheme 1



4a R = D
b R = H

5a R = Bn
b R = H
c R = PP

6a–c

7a (73%) **7b** (15%) **7c** (12%)

Scheme 2 *Reagents and conditions:* i, Pr_4NRuO_4, *N*-methylmorpholine *N*-oxide, MeCN; (*S*)-*B*-isopinocampheyl-9-borabicyclo[3.3.1]nonane, THF; ii, $MeSO_2Cl$, NEt_3, Et_2O, 0 °C; $LiBEt_3T$, THF, 0 °C; iii, Li, NH_3, THF; iv, $MeSO_2Cl$, Et_4NCl, 2,4,6-collidine, CH_2Cl_2, 0 °C; v, $(Bu_4N)_3HP_2O_7$, MeCN; vi, (*S*)-limonene synthase lyophilisate, 50 mM $MgCl_2$, Tris buffer, pH 7, 31 °C (see footnote ‡); vii, MCPBA, $NaHCO_3$, 0 °C; $HClO_4$, aq. THF

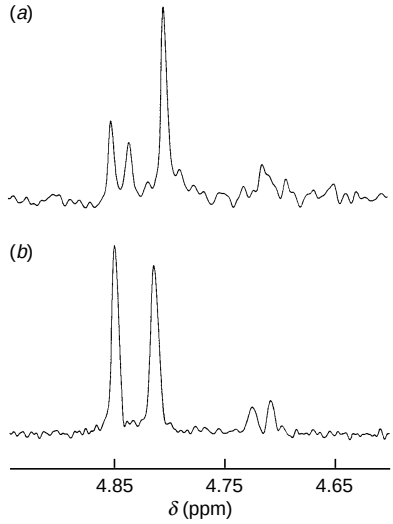


Fig. 1 (*a*) Vinyl proton region of the 640 MHz $^3H\{^1H\}$ NMR spectrum of [8-^{3}H]menth-7-ene-1,2-diols (**7b**, **7c** and **7a**) in C_6D_6. (*b*) The same region of the 600 MHz ^{1}H NMR spectrum of the unlabelled carrier diol for comparison. A slight impurity appears in the ^{1}H NMR spectrum at δ 4.71 and 4.73.†

NOE measurements. The magnitude of the intramolecular primary kinetic isotope effect for the $CH_3 \rightarrow CH_2$ elimination (k_H/k_D = 4.6 ± 0.5) was independently determined by GC–MS analysis of [8-^{2}H]limonene biosynthesized similarly using [9-$^2H_2$]-**1** as substrate.

The 640 MHz ^{3}H NMR spectrum (Fig. 1) of [8-^{2}H,^{3}H]diols **7a**–**c** (C_6D_6) shows peaks at δ_T 4.849 (0.15 T), 4.833 (0.12 T), and 4.802 (0.73 T).† The most intense peak is assigned to the doubly labelled species **7a** generated by kinetically favored proton elimination. The signal at δ_T 4.849 is ascribed to the singly labelled species **7b** arising from deuteron elimination since its downfield position relative to **7a** ($\Delta\delta_T$ = +0.047) corresponds to the chemical shift difference between the vinyl protons ($\Delta\delta_H = \delta_{H_Z} - \delta_{H_E}$ = +0.037) plus a typical upfield deuterium isotope shift for **7a** of ($\Delta\delta_T$ = − 0.10).[13] The ratio of integrals (0.73/0.15 = 4.9) for the high and low field peaks is consistent with the primary deuterium isotope effect. The small centre peak is attributed to the minor doubly labelled form **7c** (isotope shift $\Delta\delta$ − 0.016 ppm from δ^{H_E} 4.849).

The results establish that the major doubly labelled product **6a** has arisen by proton elimination from the *R* chiral methyl group *anti* to the C(3)–C(4) bond formed in the cyclization step (Scheme 3). It is unclear whether the minor doubly labelled product **6c** is formed by competing *syn* elimination from **5c** or by *anti* elimination from a contaminant of the *S* chiral methyl enantiomer. The predominant *anti* S_E' stereochemistry of this cyclization–elimination sequence is the same as that observed for the bridging cyclization–elimination in the formation of (+)- and (−)-α-pinene catalysed by pinene cyclases I and II from sage.[14] A plausible explanation for the *anti* elimination producing limonene is the preservation of a through-space bonding interaction between C(4) and C(7) of the presumed (*S*)-terpinyl ion intermediate required for cyclization to the pinenes.

The identity and radiochemical purity of all [^{3}H] compounds were verified by radio TLC analyses and comparisons with the unlabelled substance. All compounds were characterized by ^{1}H and/or ^{3}H NMR spectroscopy and other appropriate data.†

This research was supported by NIH grants GM 13956 (RMC), GM 31354 (RBC), and NCRR P41 RR01237(PGW).

Footnotes and References

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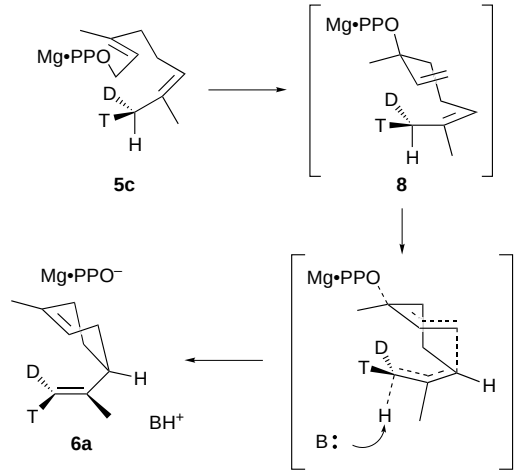
† *Selected data* for the (1′*S*)-camphanate of **4b**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.69 (s, 1 H, C*H*DOR). For the (1′*S*)- camphanate of *ent*-**4b**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.72 (s, 1 H, CD*H*OR). For **5a**: ^{3}H NMR (310 MHz, C_6D_6): δ 1.58 (d, *J* 15.6, CHDT). For **5b**: $^3H\{^1H\}$ NMR (310 MHz, C_6D_6): δ 1.51 (t, *J* 2.2, CHDT). For the 3,5-dinitrobenzoate of **5b** diluted with carrier: 236 μCi mmol^{-1}, mp 59–61 °C. For **5c**: radio TLC R_f 0.5 (silica gel, 1 : 2 : 1 MeCN–Pr^{i}OH–50 mM aq. $NaHCO_3$). For unlabelled **7**: mp 69 °C. For **7a**–**c** $^3H\{^1H\}$ NMR, see Fig. 1. The ^{1}H NMR spectrum was referenced to C_6D_5H and the ^{3}H NMR peak positions were computed using the average Larmor frequency ratio 1.066639739 (ref. 15).

‡ Preparative incubation procedure: **5c** (20 mCi; 3.6 μmol, 0.8 mM), $MgCl_2$ (50 mM), Tris buffer (pH 7), lyophilised enzyme (133 mg), 4.5 ml total volume with hexane overlay (1 ml), 31 °C, 12h.



Scheme 3 mechanism for the enzyme-catalysed conversion of (*R*)-[9-^{2}H,^{3}H]GPP **5c** through *anti* S_N' cyclization of (3*S*,9*R*)-[9-^{2}H,^{3}H]LPP **8** in the *endo* conformation showing cisoid, *anti* elimination to (4*S*,8*E*)-[8-^{2}H,^{3}H]limonene **6a**

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Received in Corvallis, OR, USA, 20th June 1997; 7/04370H