In vitro biosynthesis of cadinanes by cell-free extracts of cultured cells of *Heteroscyphus planus*

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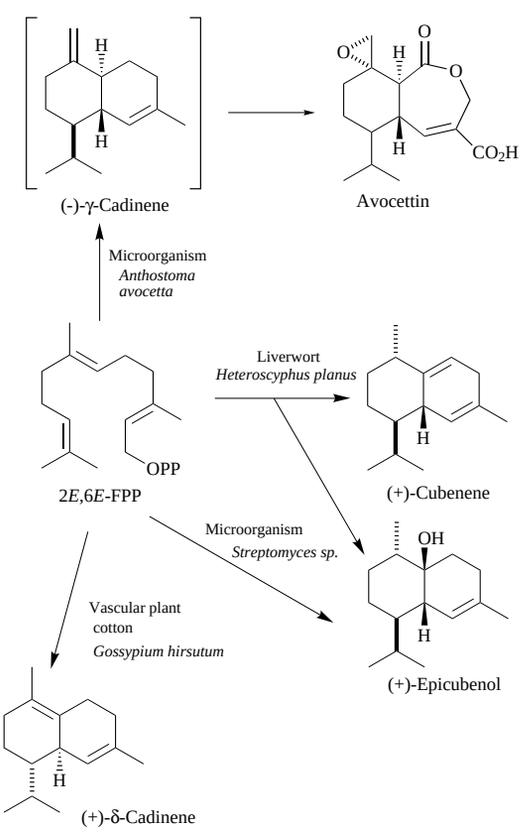
A cell-free extract from the calli of the liverwort *Heteroscyphus planus* catalyzes the divalent metal ion-dependent conversion of (2*Z*, 6*E*)-farnesyl diphosphate (FPP) into (−)-γ-cadinene and (+)-germacrene D, while it specifically converts (2*E*, 6*E*)-FPP into (+)-cubenene and (+)-epicubenol. The 1,3-hydride shift in the formation of (−)-γ-cadinene has been determined by conversion of (2*Z*, 6*E*)-[1,1-$^2H_2$]-FPP into (−)-γ-cadinene which was shown by GLC–MS and ^2H NMR spectroscopy to be labelled at the C-11 position. These findings suggest that (−)-γ-cadinene is directly formed from 2*Z*, 6*E*-FPP by intramolecular electrophilic attack of the primary carbocation on the C-10 position of FPP. (+)-Cubenene synthase and (−)-γ-cadinene synthase has been purified by fractionation with ammonium sulfate, gel filtration on Sephacryl S-200 HR and anion exchange chromatography on DEAE-Sepharose CL-6B. Separation of (−)-γ-cadinene synthase from (+)-cubenene synthase is facilitated by a change in the elution behaviour of enzymes during anion exchange chromatography. All the evidence strongly suggests that the activities of (+)-cubenene synthase and (+)-epicubenol synthase are dual functions of the same enzyme.

Introduction

The mechanism of cyclization of the acyclic diphosphates, geranyl diphosphate (GPP) and farnesyl diphosphate (FPP) in the biogenesis of cyclic monoterpenes and sesquiterpenes was initially formulated by Ruzicka.[1] In his hypothesis the *cis* (neryl) allyl cation undergoes cyclization to the monocyclic monoterpene cation, the terpinyl cation, which gives rise to the parent cations with a variety of structures by a series of further intramolecular additions, hydride shifts and rearrangement and intramolecular electrophilic attack. In contrast, the formation of six-membered rings from the *trans* allyl diphosphate precursors, GPP and 2*E*-FPP was supposed to be geometrically forbidden, since the π-orbitals of the double bond in these diphosphates cannot be properly aligned against the generated *trans* cation and the resultant cyclohexane derivative must contain a *cis* double bond. A similar constraint applies to the formation of 10- and 11-membered rings, which were produced by intramolecular attack by the carbon bearing the diphosphate on the distal double bond of FPP. In practice, however, many monoterpene and sesquiterpene cyclases specifically converted *trans* isomers of the allylic diphosphates into the corresponding cyclic terpenes but not the *cis* isomers.[2–4] Thus, it is generally accepted that such specific cyclization involves the enzyme-catalyzed allylic rearrangement of the primary diphosphate esters to the tertiary esters which are further converted into cyclic terpenes *via* S_N2' processes.[2–5]

Arigoni[6] extensively studied the biosynthesis of a group of cadinane and related sesquiterpenes and found that avocettin, an oxidized metabolite of (−)-γ-cadinene, is formed from the initially formed germacryl cation from 2*E*,6*E*-FPP by a 1,3 hydride shift (see Scheme 1). Recently Cane *et al.*[7] proved that (+)-epicubenol was specifically formed from 2*E*,6*E*-FPP *via* 1,3- and 1,2-hydride shifts using a cell-free extract from *Streptomyces* sp. We[8] also reported that (+)-epicubenol and (+)-cubenene were specifically formed from 2*E*,6*E*-FPP *via* 1,2- and 1,3-hydride shifts by a cell-free extract from the calli of the liverworts, *Heteroscyphus planus*. (+)-δ-Cadinene is also specifically generated from 2*E*,6*E*-FPP.[9] Taken together, these find-



Scheme 1 Biosyntheses of cadinanes from 2*E*,6*E*-farnesyl diphosphate

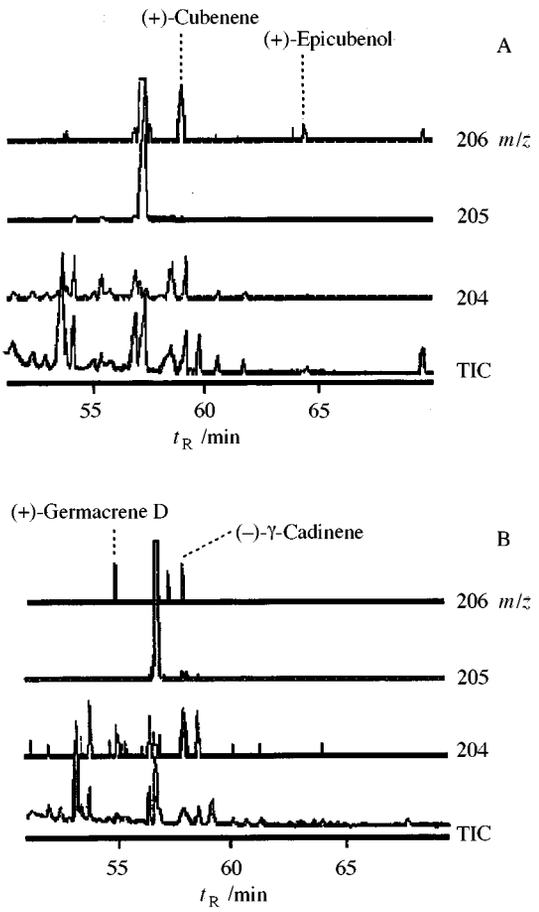


Fig. 1 SIM-mass chromatograms of sesquiterpenes generated from (A): (2*E*,6*E*)-[1,1-$^2H_2$]-FPP (ref. 9) and (B): (2*Z*,6*E*)-isomer by incubation with a cell-free extract of *H. planus* cells. TIC: total ion current

ings can be completely accounted for by a cyclization mechanism involving initial allyl rearrangement of FPP to nerolidyl diphosphate, followed by ionization and electrophilic cyclization of the distal double bond.

In the calli of *H. planus*, (−)-γ-cadinene co-occurs with (+)-cubenene and (+)-epicubenol as a cadinane sesquiterpene. We examined the formation of (−)-γ-cadinene from 2*Z*,6*E*-FPP and separated (−)-γ-cadinene synthase activity from (+)-cubenene synthase activity by anion exchange chromatography. We also describe the formation of (−)-γ-cadinane involving a 1,3-hydride shift.

Results and Discussion

Formation of cadinanes from deuteriated FPPs

A cell-free extract of *H. planus* was prepared from the calli by the same method as we previously reported[9] except for the use of the enzyme solution at pH 8.0. Fig. 1 shows the segments of GLC-MS chromatograms, monitored at *m/z* 204, 205 and 206, of sesquiterpenes from A: (2*E*,6*E*)-[1,1-$^2H_2$]-FPP and B: (2*Z*,6*E*)-[1,1-$^2H_2$]-FPP under the optimal conditions {pH 7.5 for 2*E*,6*E*-FPP (30 μmol dm^{-3}) and 20 mmol dm^{-3} of Mg^{2+}, and pH 8.0 for its 2*Z*,6*E*-isomer}. As we previously reported, (+)-cubenene and (+)-epicubenol were selectively formed from 2*E*,6*E*-FPP, while two sesquiterpene hydrocarbons, germacrene-D [retention times (t_Rs): 53.1 min on DB-1 (methyl silicone type, 60 m × 0.25 mm) and 38.4 min on HR-20 M (Carbowax 20 M type, 50 × 0.25 mm)], and γ-cadinene (t_Rs: 56.2 min and 41.2 min, respectively], were specifically generated from the (2*Z*,6*E*)-isomer. These two compounds were identified by GLC and GLC-MS, and co-chromatography with authentic samples of (−)-germacrene D (−180.0 deg cm^2 g^{-1}) and (+)-γ-cadinene (+32.8 deg cm^2 g^{-1}) which were isolated from citronella oil (an essential oil from *Cymbopogen winterianus*).[10] The absolute configurations of germacrene D and γ-cadinene from *H. planus* calli were determined as (+)-germacrene D and (−)-γ-cadinene, respectively, by comparing the GLC retention times of germacrene-D [t_Rs: (+)-isomer from *H. planus* calli, 49.05 min and (−)-isomer, from *C. winterianus*, 49.67 min] and γ-cadinene [t_Rs: (−)-isomer from *H. planus* calli, 50.60 min and (+)-isomer from *C. winterianus*, 51.41 min] on a chiral column (CP-Cyclodextrin B, 50 m × 0.25 mm). Retention times of (+)-germacrene D and (−)-γ-cadinene which were generated from non-labelled and [1,1-$^2H_2$]-(2*Z*,6*E*)-FPPs, were identical with those from *H. planus* calli but not identical with those of authentic samples of (−)-germacrene D and (+)-γ-cadinene from *C. winterianus*. Unexpectedly, (+)-germacrene-D and (−)-γ-cadinene were not formed from 2*E*,6*E*-FPP under various reaction conditions (temperature, pH, concentrations of substrates and Mg^{2+} and Mn^{2+}) at various purification stages (ammonium sulfate fractionation, gel filtration and anion exchange chromatography).

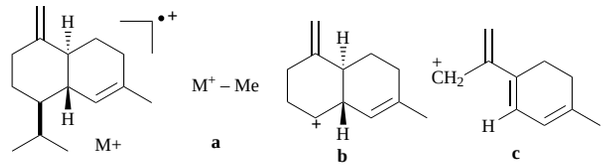


Fig. 2 Mass fragment ions from (−)-γ-cadinene

Table 1 Mass fragment ions from non-labelled and deuteriated (−)-γ-cadinene

	Ions (*m/z*)[a]			
	M^+	**a**	**b**	**c**
(−)-γ-Cadinene generated from non-labelled FPP	204	189	161	133
(−)-γ-Cadinene generated from [1,1-$^2H_2$]-FPP	206	191	162	134

[a] Ions shown in Fig. 2.

Table 1 and Fig. 2 show the mass ions of (−)-γ-cadinene derived from non-labelled and [1,1-$^2H_2$]-(2*Z*,6*E*)-FPP. A base fragment ion **b** is formed with elimination of an isopropyl group. The ion **c** arises from cleavage of both the C-5/C-6 and C-7/C-8 bonds. A molecular ion ([M]$^+$) and an [M-methyl] ion (ion **a**) from deuteriated FPP shift by two mass units (MUs), while fragment ions **b** and **c** shift by one MU, indicating that two ^2H atoms of [1,1-$^2H_2$]-FPP were retained during the formation of (−)-γ-cadinene and one of two ^2H atoms was attached to the isopropyl group; this confirms the expected 1,3-hydride shift in the formation of (−)-γ-cadinene (Fig. 4).

Direct evidence of a 1,3-hydride shift was provided by ^2H-NMR analysis of (−)-γ-cadinene derived from (2*Z*,6*E*)-[1,1-$^2H_2$]-FPP. The methine proton at C-11 (δ 2.22, Table 2) was clearly resolved, while the signal for the methine proton at C-5 overlapped with that of the 8-β proton. Unambiguous assignment of ^1H NMR resonances for γ-cadinene was made according to previously reported ^1H NMR and ^{13}C NMR data for (+)-γ-cadinene[11,12] and analysis of a ^1H-^1H 2D homonuclear chemical-shift correlation (COSY), a ^{13}C-^1H 2D heteronuclear COSY and nuclear Overhauser and exchange spectroscopy (NOESY, Fig. 3). Table 2 summarizes the ^1H and ^2H NMR data for unlabelled (+)-γ-cadinene and deuteriated (−)-γ-cadinene. A broad singlet signal at δ(^2H) 2.24 indeed confirmed the occurrence of a 1,3-hydride shift during enzymatic cyclization of FPP to (−)-γ-cadinene.

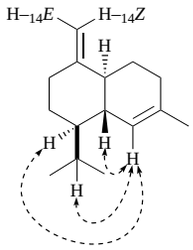


Fig. 3 Important NOEs in (−)-γ-cadinene

Partial purification of (−)-γ-cadinene and (+)-cubenene synthases

A summary of the protocol used in the partial purification of the (+)-cubenene, (+)-epicubenol and (−)-γ-cadinene synthases from the calli of *H. planus* is shown in Table 3. Preliminary ammonium sulfate fractionation of the 40 000 g supernatant showed that the bulk of both the (+)-cubenene (51%) and (−)-γ-cadinene synthases (63%) precipitated between 15 and 55% saturation. (+)-Cubenene synthase was co-purified with (−)-γ-cadinene synthase through gel filtration on a Sephacryl S-200 HR column (70.5 cm). However, these enzymes could be separated by anion exchange chromatography on a DEAE-Sepharose CL-6B column (14 cm) by stepwise elution with NaCl (0, 0.1, 0.2, 0.3 and 0.4 mol dm^{-3}) in 50 mmol dm^{-3} 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES)-NaOH buffer (pH 7.5) containing 10% D-sorbitol, 1 mmol dm^{-3} dithiothreitol (DTT), 20 mM $MgCl_2$. (−)-γ-Cadinene synthase was eluted in the HEPES buffer containing 0 (total activity: 4.9 nmol h^{-1})–0.1 mol dm^{-3} NaCl (15.0 nmol h^{-1}), while both (+)-cubenene synthase and (+)-epicubenol synthase were eluted in the HEPES buffer containing 0.2 [total activity of (+)-cubenene synthase and that of (+)-epicubenol synthase: 27.2 nmol h^{-1} and 21.3 nmol h^{-1}, respectively]–0.3 mol dm^{-3} NaCl (8.5 nmol h^{-1} and 1.4 nmol h^{-1}). These results clearly indicate that (−)-γ-cadinene synthase is a distinctly different enzyme from (+)-cubenene synthase and (+)-epicubenol synthase. The procedure at this step of purification resulted in a 205 and 247-fold increase in (+)-cubenene and (+)-epicubenol activity, respectively. Because of the small quantity of (−)-γ-cadinene synthase, the specific activity of (−)-γ-cadinene synthase was not determined at this step, and was not further separated by anion exchange chromatography on the DEAE-Sepharose CL-6B column (14 cm) eluted by a NaCl linear gradient. (+)-Cubenene and (+)-epicubenol synthases were always co-chromatographed by gel filtration, anion exchange chromatography by the NaCl stepwise elution and the NaCl gradient elution. Although the purification of the two active principals to homogeneity would provide the only conclusive proof of identity, the consistency of all lines of evidence strongly suggest that (+)-cubenene synthase and (+)-epicubenol synthase are dual functions of the same enzyme.

Two structurally similar sesquiterpenes, (+)-cadinene and (−)-γ-cadinene were synthesized by two distinctly different enzymes which require different geometric isomers of FPP as substrates, namely, the 2*E*,6*E*-isomer for (+)-cubenene synthesis and the 2*Z*,6*E*-isomer for (−)-γ-cadinene. In sesquiterpene biosynthesis, the tertiary diphosphate, nerolidyl diphosphate is of potential importance as a key intermediate.[2–5] The formation of six- or seven-membered rings by an intramolecular attack of the C-1 carbon bearing the pyrophosphate on the central double bond requires prior isomerization of the *trans*-2,3-bond to the tertiary pyrophosphate to form sterically permitted *cis*-cyclohexane or cycloheptene. A similar constraint applies to formation of 10- and 11-membered rings which are formed by internal attack by the C-1 carbon in the 2,3-*trans* FPP on the distal double bond. In the formation of humulene-derived metabolites, the humulyl (11-membered ring) cation is considered to be also generated from the 2,3-*trans*-FPP by direct electrophilic attack of C-1 on C-11.[13] Among the sesquiterpenes formally derivable from a germacradienyl intermediate, cadinane sesquiterpenes such as avocettin,[5] (+)-δ-cadinene,[7] (+)-epicubenol[8,9] and (+)-cubenene[9] (see Fig. 1 and Fig. 4) investigated to date are specifically formed from 2*E*-FPP. Strong evidence for the intermediacy of nerolidyl diphosphate has been obtained from biosynthetic studies on these compounds. However, (−)-γ-cadinane synthases reported herein markedly differs from other cadinane synthases in that it requires the 2*Z*-FPP. Consideration of the geometric requirements of the conformer of the cation indicated in Fig. 4 leads to the prediction that the initial intramolecular electrophilic attack of the carbocation at C-1 in FPP on C-11 must take place from the *re*-face. However, the possibility of the initial isomerization of *cis*-FPP to nerolidyl diphosphate followed by the cyclization *via* an S_N2' process may not be excluded. After all cyclase-active site topology must be investigated to fully understand the genuine cyclization mechanisms at the active site.

Table 2 ^1H and ^2H NMR assignments for γ-cadinene

H	δ_H (mult)	*J* (Hz)	$\delta_{^2H}$ (2,*Z*,6*E*)-[1,1-$^2H_2$]-FPP
1			
	1.9–2.1 (m)		
2			
4	5.49 (broad s)		
5	1.21 (m)		1.23
6	1.70 (m)		
7	2.02 (m)		
	1.76 (m)		
8	α 2.37 (broad d)		
	β 1.14 (m)		
10	1.60 (m)		
11	2.22 (m)		2.24
12			
	0.78 (d)	6.9	
	0.94 (d)	6.9	
13			
14	*E* 4.60 (broad s)		
	Z 4.49 (broad s)		
15	1.67 (s)		

Assignments were based on DEPT, ^1H-^1H COSY, ^{13}C-^1H COSY, NOESY, difference NOE and COLOC experiments.

Experimental

Materials

(2*E*,6*E*)- and (2*Z*,6*E*)-[1,1-$^2H_2$]-FPP were prepared as previously reported.[9,14–16] Authentic samples of both (+)-γ-cadinene {$[\alpha]_D$ +32.8 (*c* 0.25, $CHCl_3$)}† and (−)-germacrene-D {$[\alpha]_D$-180.0 (*c* 0.38, $CHCl_3$)} were isolated from commercially available citronella oil (an essential oil from *Cymbopogon winterianus*), manufactured in China, by combinations of LC over silica gel and or $AgNO_3$-silica gel columns. The assignment of ^1H and ^{13}C atoms in (+)-γ-cadinene and (−)-germacrene D was achieved by DEPT, ^1H-^1H COSY, ^{13}C-^1H COSY, NOESY and COLOC NMR measurements.

(+)-γ-Cadinene and (−)-germacrene D

(+)-γ-Cadinene δ_H(270 MHz, in $CDCl_3$): see Table 3; δ_C(67.8 MHz, in $CDCl_3$) 15.4 (C-12 or C-13), 21.9 (C-13 or C-12), 24.9 (C-15), 25.9 (C-9), 26.4 (C-11), 26.6 (C-3), 30.7 (C-8), 36.5 (C-2), 44.3 (C-4), 45.1 (C-10), 47.1 (C-5), 103.9 (C-14), 122.8 (C-6), 134.4 (C-1) and 152.6 (C-7).

(−)-Germacrene D δ_H(270 MHz, in $CDCl_3$) 0.81 (3H, d, *J* 6.9, 13-Me), 0.86 (3H, d, *J* 6.9, 12-Me), 1.44 (1H, m, H-11), 1.45 (2H, m, 2 × H-9), 1.51 (3H, br s, 14-Me), 2.08 (1H, m, H-2), 2.10 (1H, m, H-3), 2.2 (1H, m, 7-H), 2.27 (2H, m, 2 × H-8), 2.34 (2H, m, H-2 and H-3), 4.74 (1H, dd, *J* 11.5 and 1, H-15), 4.79

† $[\alpha]_D$ Values given as 10^{-1} deg cm^2 g^{-1}

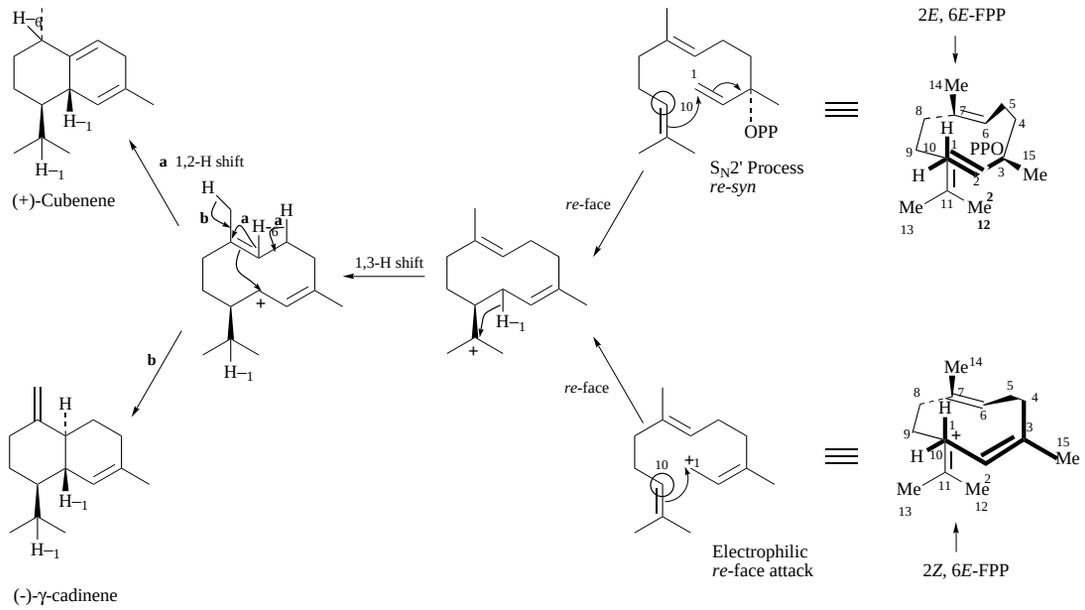


Fig. 4 Possible mechanisms for the conversion of 2*E*,6*E*-FPP into (+)-cubenene in the presence of 1,2- and 1,3-hydride shifts and that of 2*Z*,6*E*-FPP into (−)-γ-cadinene in the presence of a 1,3-hydride shift. H_{-n}: *n* represents carbon number in FPP.

Table 3 Purification procedure of the (+)-cubenene, (+)-epicubenol and (−)-γ-cadinene syntheses from *H. planus* calli

Step	Protein (mg)	Total activity (nmol h^{-1})	Specific activity (nmol h^{-1} mg^{-1} protein)	Purification factor
(+)-Cubenene synthase				
Supernatant (40 000 g)	109.4	530.7	4.85	1
Salt out {$(NH_4)_2SO_4$ 15–55%}	3.81	271.3	71.2	14.7
Sephacryl S-200 HR	2.09	213.1	102	21.2
DEAE-Sepharose CL-6B[a]	0.125	124.2	993	205
(+)-Epicubenol synthase				
Supernatant (40 000 g)	109.4	143.7	1.31	1
Salt out {$(NH_4)_2SO_4$ 15–55%}	3.81	102.8	27.0	20.6
Sephacryl S-200 HR	2.09	61.52	29.4	22.4
DEAE-Sepharose CL-6B[a]	0.125	40.52	326	247
(−)-γ-Cadinene synthase				
Supernatant (40 000 g)	109.4	85.79	0.78	1
Salt out {$(NH_4)_2SO_4$ 15–55%}	3.81	54.42	14.3	18.3
Sephacryl S-200 HR	2.09	33.64	16.1	20.6
DEAE-Sepharose CL-6B[a]	—	—	—	—

(—): not determined. [a] NaCl stepwise elution.

(1H, dd, *J* 11.5 and 1, H-15), 5.14 (1H, dd, *J* 10.1 and <2, H-1), 5.24 (1H, dd, *J* 15.4 and 9.9, H-6) and 5.78 (1H, d, *J* 15.4, H-5); δ_C(67.8 MHz, in $CDCl_3$) 15.9 (C-14), 19.3 (C-13), 20.8 (C-12), 29.2 (C-2), 32.7 (C-11), 34.5 (C-3), 40.7 (C-9), 52.9 (C-7), 109.0 (C-4), 133.5 (C-1), 134.0 (C-10), 135.5 (C-5) and 148.9 (C-15). The ^{1}H and ^{13}C NMR data for the two compounds were identical with the results reported previously.[11,12,17]

GLC and GLC-MS analyses

To estimate the total concentrations of (−)-γ-cadinene, (+)-cubenene and (+)-epicubenol, and to identify (−)-γ-cadinene and (+)-germacrene D, the ether extracts and isolated compounds were submitted to GLC using achiral columns, DB-1 (J & W Scientific, 0, 25 mm × 60 m) R_t: γ-cadinene = 55.2 min, germacrene D = 53.1 min, cubenene = 56.2 min and epicubenol = 66.4 min) and Ulbon HR-20M (Shinwa Chemical Industries, γ-cadinene = 41.2 min, germacrene D = 38.4 min, cubenene = 42.7 min and epicubenol = 66.4 min) and chiral column, CP-Cyclodextrin B (GL Science, 50 × 0.25 mm i.d.). Column temperature: initial temperature at 60 °C was kept for 5 min and then elevated at 2 °C min^{-1} to 220 °C, with a flow of He at 1.4 cm^3 min^{-1}. GLC-MS analyses were carried out under the same conditions as those for GLC analyses except for a flow of He of 1.13 cm^3 min^{-1} with HR-1 (ionizing voltage at 70 eV). Total ion and ions at *m/z* 204, 205 and 206 were monitored for mass chromatography. Concentrations of γ-cadinene, cubenene and epicubenol were determined on the basis of the relative GLC peak areas of the terpenes to the known amount of tridecane. Concentrations of deuteriated γ-cadinene, cubenene and epicubenol derived from [1,1-$^2H_2$]-FPPs were determined on the basis of the SIM peak areas monitored at *m/z* 206 and 204 and the estimated concentrations of cubenene and epicubenol.

Cell-free extracts of calli

Cell-free extracts of the calli of *H. planus* were prepared from the calli of 30-day cultures. Harvested calli were suspended in 50 mmol dm^{-3} 2-[4-(2-hydroxyethyl)-piperazin-1-yl]ethanesulfonic acid (HEPES) buffer [pH 8.0 for 2*Z*,6*E*-FPP as a substrate and pH 7.5 for 2*E*,6*E*-isomer,[9] 5 times volumes (v/w)] containing 10% D-sorbitol, 1 mM dithiothreitol (DTT), 20 mM

$MgCl_2$ and polyvinylpolypyrolidinone (PVPP) [10% (w/w) of fresh weight of calli] and mechanically ground at 0–4 °C. After centrifugation (2000 g) of the broken-cell suspension at 0–4 °C for 10 min, the resulting supernatant was recentrifuged at 40 000 g at the same temp. for 10 min. After XAD-4 polystyrene resin (equal weight to cells) had been added to the mixture it was stirred at the same temp. for 60 min and filtered; the 40 000 g supernatant (2.21 mg protein g^{-1} calli[9]) were incubated at 30 °C for 60 min with 30 μmol l^{-1} of FPPs. The cell-free extracts from the calli displayed maximal (−)-γ-cadinene synthase activity at pH 8.0 [pH 7.5 for (+)-cubenene and (+)-epicubenol synthase activities].

(−)-γ-Cadinene synthase activity and protein determination in cell-free extracts

Cyclase activity was measured by incubating the enzyme preparation [2.56 mg protein/1.2 g calli (fresh wt.)] in 6 cm^3 HEPES buffers (at pH 7.5 for 2*E*,6*E*-$^2H_2$-FPP and at pH 8.0 for 2*Z*,6*E*-isomer) with 30 μmol dm^{-3} of non-labelled and ^2H labelled FPPs (>99.4 atom % excess) at 30 °C for 60 min, followed by chilling in an ice-bath. The reaction mixture was treated with tridecane (6.0 μg) in diethyl ether (6 cm^{-3}) and centrifuged at 2000 g for 10 min. The diethyl ether layer was separated, dried (Na_2SO_4) and concentrated by hand warming.[18] The concentrate was subsequently analyzed by GLC and GLC-MS. The (−)-γ-cadinene activity was determined by its relative peak area to tridecane in the GLC chromatogram and the peak areas of the selected ion-monitored (SIM) mass chromatogram at *m/z* 206 and 204 as described previously.[9] Protein was determined by the Coomasic brilliant blue (CBB) methods[19] (the modified dye binding method)

In the presence of a saturating concentration of 2*Z*,6*E*-FPP (30 μmol dm^{-3}), (−)-γ-cadinene activity increased with increasing concentrations of both magnesium chloride and manganese chloride up to 20 mmol dm^{-3}: 0.223 and 0.056 nmol h^{-1} mg^{-1} protein, respectively, but further increases in the concentrations of both Mg^{2+} and Mn^{2+} decreased the (−)-γ-cadinane synthase activity. Removal of DTT for the preparation by dialysis resulted in a complete loss of (−)-γ-cadinene synthase activity, and readdition of the reagent (1.0 mmol dm^{-3}) led to partial recovery of the activity (49%). (−)-γ-Cadinane was not formed from 2*E*,6*E*-FPP under all conditions tested.

Cyclization of 2*Z*,6*E*-[2,2-$^2H_2$]-FPP to (−)-γ-cadinene

The crude cell-free extract (600 cm^3) from *H. planus* calli (125 g) was incubated for 180 min at 30 °C with 40 μmol dm^{-3} of 2*Z*,6*E*-[2,2-$^2H_2$]-FPP in the presence of 20 mmol dm^{-3} $MgCl_2$. The reaction was halted by chilling in ice. The reaction mixture was extracted with an equal volume of diethyl ether (× 3). The ether extracts were dried (Na_2SO_4) and evaporated *in vacuo* to *ca.* 1 cm^3 and further concentrated by hand warming. The residue was separated on a silica gel column (5 g) with pentane (20 cm^3) to obtain sesquiterpene hydrocarbons including (−)-γ-cadinene.

For the Ag^+-TLC, a silica gel TLC plate was dipped into a 15% solution of $AgNO_3$ (v/w) in acetonitrile. The sesquiterpene hydrocarbon fraction was spotted with authentic (+)-γ-cadinene isolated from citronella oil on the same plate and developed with pentane–ether (9:1). The TLC zone (R_F 0.42) corresponding to (+)-γ-cadinene was scraped from each plate and extracted with pentane. The pentane extract was directly analyzed by ^2H NMR spectroscopy and GLC-MS.

Separation of (−)-γ-cadinene, (+)-cubenene and (+)-epicubenol synthases

Crude cell extracts and enzyme preparations were kept on ice or 4 °C throughout purification. Proteins in fractions obtained in this experiment were determined by the CBB method. Cyclase activities in each fraction were determined by addition of the 2*Z*,2*E*-FPP for (−)-γ-cadinene activity and by addition of the 2*E*,6*E*-isomer for the (+)-cubenene and (+)-epicubenol activities.[9] Adequate volumes of fractions were diluted to 2 cm^3 with 10% (w/v) D-sorbitol, 1.0 mmol dm^{-3} DTT, 20 mmol dm^{-3} $MgCl_2$, 50 mmol dm^{-3} HEPES–NaOH buffer [pH 7.5 for (+)-cubenene and (+)-epicubenol synthase activities and pH 8.0 for (−)-γ-cadinane synthase activity]. To the enzyme solution, 30 μmol dm^{-3} of 2*Z*,6*E*- and 2*E*,6*E*-FPPs were separately added and incubated at 30 °C for 60 min. For control, the enzyme solution was incubated without addition of FPP. After cooling in an ice-bath, the reaction mixture was treated with tridecane (2 mg) in diethyl ether (2 cm^3) and centrifuged at 2000 g for 10 min. The diethyl ether solution was separated, dried (Na_2SO_4), concentrated by hand warming and analyzed by GLC. Ammonium sulfate was added slowly to the stirred 40 000 g supernatant (190 g) to give 15% saturation. After the solution had been stirred for an additional 60 min, it was centrifuged for 30 min at 40 000 g and the pellet discarded. The supernatant was adjusted to 55% saturation with ammonium sulfate, and the precipitated proteins, which showed 63% of (−)-cadinene synthase activity and 51% of (+)-cubenene synthase activity, were pelletted by centrifugation as above. The pellet was resuspended in 10% (w/v) D-sorbitol (8 cm^3), 1.0 mmol dm^{-3} DTT, 20 mmol dm^{-3} $MgCl_2$, 50 mmol dm^{-3} HEPES-NaOH buffer (pH 7.5), and dialyzed against the same HEPES–NaOH buffer at 4 °C overnight. A solution (8 cm^3) which contained 0.48 mg cm^{-3} protein was loaded onto a Sephacryl S-200 HR column (1.5 × 75 cm) equibrated in the HEPES-NaOH buffer as above. The bound proteins were eluted with the same buffer at 14.4 cm^3 h^{-1} by UV monitoring at 280 nm and fractionated into 2 cm^3 aliquots. The eluted fractions having (−)-γ-cadinene, (+)-cubenene and (+)-epicubenol synthase activity (all activities were eluted into the same fractions 28 to 36) were combined and immediately loaded onto a DEAE-Sepharose CL-6B column (1.0 × 1.0 cm) equibrated with the HEPES-NaOH buffer. The proteins were eluted stepwise with the HEPES-NaOH buffers (40 cm^3 each) containing 0, 0.1, 0.2, 0.3 and 0.4 mol dm^{-3} NaCl and fractioned into 2 cm^3 aliquots. Predominant (−)-γ-cadinane activity was eluted with the HEPES–NaOH buffers containing 0 and 0.1 mol dm^{-3} NaCl, while both (+)-cubenene and (+)-epicubenol active fractions were eluted with those containing 0.2 and 0.3 mol dm^{-3} NaCl. Because of low activity of (−)-γ-cadinene synthase, it was not further purified. The combined fractions (fraction 40 to 50) containing (+)-cubenene and (+)-epicubenol synthase were dialyzed and loaded on a DEAE-Sepharose C:-6B column (1.0 × 9 cm). Elution was carried out with a linear concentration gradient of NaCl from 0.1 to 0.25 mol dm^{-3} (200 cm^3) at a flow rate of 13.3 cm^3 h^{-1}. (+)-Cubenene and (+)-epicubenol synthetase were co-purified in the same fractions (fractions 91 to 120, each a 2 cm^3 aliquot). Because of small quantity of the protein, the molecular weight of (+)-cubenene synthase was not determined at this stage.

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