Biosynthesis of (+)-cubenene and (+)-epicubenol by cell-free extracts of cultured cells of *Heteroscyphus planus* and cyclization of [^{2}H]farnesyl diphosphates

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The absolute stereochemistry of cubenene and epicubenol from cultured cells of *Heteroscyphus planus* was determined as both (+)-isomers by ^{1}H and ^{13}C NMR spectroscopy, GLC using a chiral capillary column, and optical rotations. Incubation of two geometrical isomers of deuteriated farnesyl diphosphate (FPP) with a cell-free extract from cultured cells indicated that both compounds were specifically formed from (2*E*,6*E*)-FPP. Gas–liquid chromatography–mass spectrometry (GLC–MS) and ^{2}H NMR analyses of (+)-cubenene and (+)-epicubenol generated from [1,1-$^2H_2$]- and [6-^{2}H]-FPP confirmed the presence of 1,2- and 1,3-hydride shifts in their formation.

Introduction

Sesquiterpenoids enantiomeric to the corresponding compounds from higher plants have been often isolated from liverworts. (+)-Epicubenol [**5**: (+)-*ent*-epicubenol[1]], which was first isolated from the microorganism, *Streptomyces* sp.,[2] is the enantiomer of (−)-epicubenol previously isolated from a number of higher plants.[3–5] (+)-Epicubenol was synthesized enzymically by incubation of differently ^{2}H- or ^{3}H-labelled farnesyl diphosphates (FPP) with a cell-free extract obtained from *Streptomyces* sp.[6] ^{2}H NMR analysis of (+)-epicubenol produced by conversion of [1,1-$^2H_2$]-FPP **1a** confirmed the presence of a 1,3-hydride shift in its formation (see Scheme 1).

Previously, we[7] reported the analysis of volatiles from cultured cells of the liverwort, *Heteroscyphus planus*, and the isolation of novel sesquiterpenes with a cadinane skeleton. Although the occurrence of epicubenol was confirmed by gas–liquid chromatography–mass spectrometry (GLC–MS) analysis, its absolute configuration has not yet been determined. We now report the identification of (+)-cubenene **6** and (+)-epicubenol **5** from the cultured cells of *H. planus*. Incubation of (2*E*,6*E*)-[1,1-$^2H_2$]- (**1a**) and (2*Z*,6*E*)-[1,1-$^2H_2$]-FPP (**1b**) with cell-free extracts from the cultured cells of *H. planus* indicated that both (+)-cubenene and (+)-epicubenol were specifically converted from the 2*E*,6*E*-isomer of FPP. GLC–MS and ^{2}H NMR analyses of (+)-cubenene and (+)-epicubenol converted from (2*E*,6*E*)-[1,1-$^2H_2$]- and [6-^{2}H]-FPP verified 1,2- and 1,3-hydride shifts in their formation. This report describes for the first time sesquiterpene cyclase activity from the liverwort.

Results and discussion

The relative configuration of epicubenol isolated from the cultured cells of *H. planus* was determined by GLC and GLC–MS using two achiral capillary columns, ^{1}H and ^{13}C NMR spectroscopy, and the direct comparison with those of an authentic sample of (−)-epicubenol obtained from *Pinus koraiensis*.[5] The absolute configuration of epicubenol was determined as the (+)-isomer by polarimetric comparison ($[\alpha]_D$; epicubenol from cultured cells: $+106.5 \times 10^{-1}$ deg cm^2 g^{-1}; lit.,[1] +111.6) and GLC using a chiral capillary column [retention times (t_Rs) on cyclodextrin B column; (+)-epicubenol: 66.7 min, (−)-isomer: 67.2 min]. (+)-Cubenene ($[\alpha]_D$: +24.4; lit.,[1] +22.3; ^{1}H NMR: see Table 1; and ^{13}C NMR; see Experimental section) and (−)-cubenene were chemically prepared by dehydration of the corresponding epicubenols by the method previously described by Connolly *et al.*[1] The occurrence of (+)-cubenene in the volatiles from cultured cells was confirmed by co-chromatography with chemically prepared (+)- and (−)-cubenene on GLC using two achiral and one chiral capillary columns [t_Rs: (+)-isomer: 53.4 min and (−)-isomer: 54.2 min] and MS comparison.

Fig. 1 shows the segment of GLC–MS chromatograms, monitored at *m/z* 204, 205 and 206, of sesquiterpenes formed from (2*E*,6*E*)-[1,1-$^2H_2$]-FPP. The chromatograms clearly indicated that only (+)-cubenene and (+)-epicubenol were deuteriated among a variety of cyclic sesquiterpenes, while neither compound was generated from the 2*Z*,6*E*-isomer. Deuteriated (+)-cubenene was always observed at a roughly equivalent ratio to (+)-epicubenol under various reaction conditions. Under the optimal conditions (pH 7.5; 30 μmol dm^{-3} FPP; 20 mmol dm^{-3} Mg^{2+}), the ratio of deuteriated cubenene and epicubenol was 6.5:1.

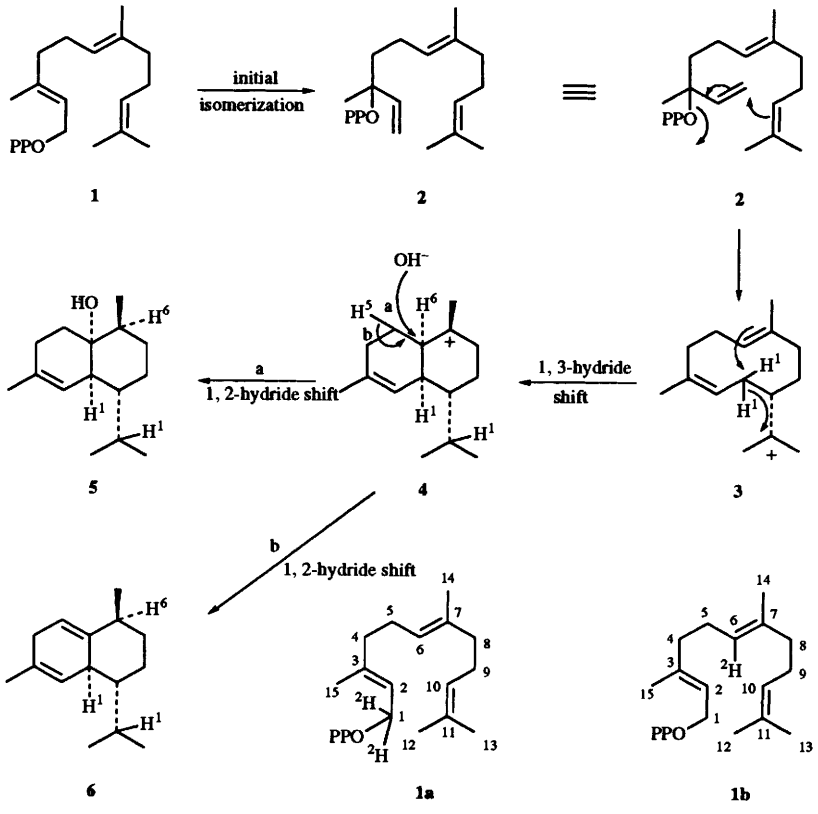
Table 1 and Fig. 2 summarize the mass ions of (+)-epicubenol **5** and (+)-cubenene **6** generated from [1,1-$^2H_2$]- and [6-^{2}H]-FPP. Both fragment ions **a** and **b** are formed with elimination of an isopropyl group. The ion **c** (a base ion) is due to cleavage of both the C-9/C-8 and C-6/C-5 bonds and a subsequent aromatization with elimination of hydrogen at C-2. An $[M - H_2O]^+$ ion of (+)-epicubenol generated from [1,1-$^2H_2$]-FPP and an M^+ ion of (+)-cubenene shift by two mass units (MUs), indicating that two ^{2}H atoms at C-1 in FPP were retained during cyclization, whereas shifts of the ions **a** and **b** together with the ion **c** by one MU indicated that one of two ^{2}H atoms was attached to isopropyl groups in both compounds, confirming the expected 1,3-hydride shift. The shifts of $(M^+ - H_2O)^+$ and the ions **b** and **c** from (+)-epicubenol generated from [6-^{2}H]FPP and those of M^+ and the ions **a**–**c** from (+)-cubenene by one MU confirmed that the ^{2}H atom at C-6 in FPP was retained during cyclization, thereby providing indirect evidence for a 1,2-hydride shift. The direct evidence of the 1,2- and 1,3-hydride shifts in formation of cubenene was provided by ^{2}H NMR analysis.

The assignment of the methine and methylene protons in (+)-cubenene **6** was ambiguous in the previous work.[1] Therefore the ^{1}H assignment of (+)-cubenene was achieved by analysis of a ^{1}H–^{1}H 2D homonuclear chemical-shift correlation (COSY)

Table 1 Fragment ions of (+)-cubenene **6** and (+)-epicubenol **5** generated from non- and ^{2}H-labelled farnesyl diphosphates

Fragment ions		(+)-Cubenenes and (+)-epicubenols (*m/z*)		
(+)-Cubenene	(+)-Epicubenol	non-labelled	from [1,1-$^2H_2$]FPP[a]	from [6-^{2}H]FPP[a]
M^+	($M^+ - H_2O$)	204	206	205
	a	179	180	180
b	**b**	161	162	162
c	**c**	119	120	120
d	**d**	105	106	106

[a] 2*E*,6*E*-Isomer.



Scheme 1 Biosynthetic pathway to (+)-cubenene **6** and (+)-epicubenol **5**

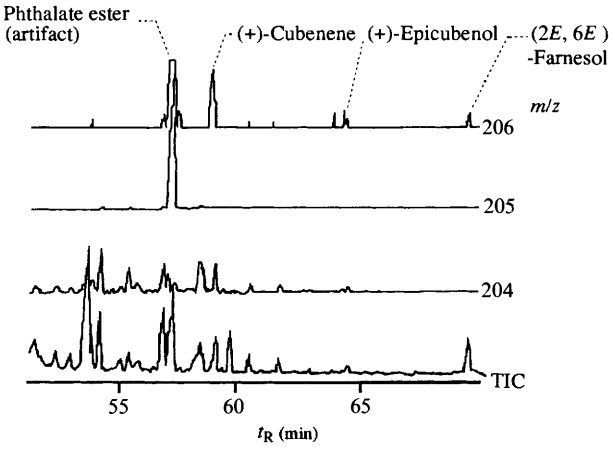


Fig. 1 SIM-mass chromatograms of sesquiterpenes generated from (2*E*,6*E*)-[1,1-$^2H_2$]FPP by incubation with a cell-free extract of *H. planus* cells. TIC: total ion current.

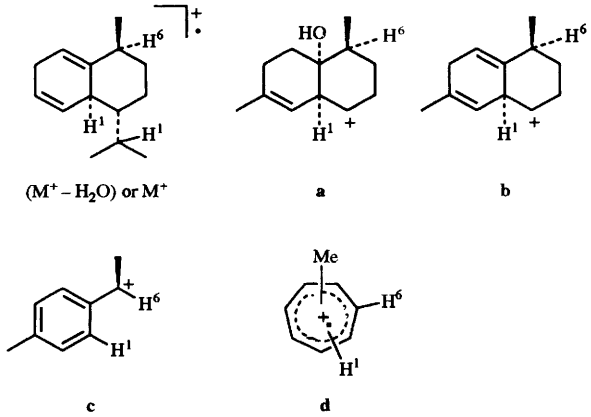


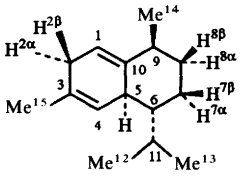
Fig. 2 Mass fragment ions from non-labelled and deuteriated (+)-cubenene **6** and (+)-epicubenol **5**. H^n in fragment ions; '*n*' represents carbon number in FPP.

spectrum which showed that 5-H at δ_H 2.32, 9-H at δ 1.90 and 11-H at δ 2.09 gave cross-peaks with vinylic 4-H, Me-14 and Me-12 and Me-13 respectively. Table 2 summarizes the ^{1}H and ^{2}H NMR data of non-labelled and ^{2}H-labelled (+)-cubenene. The ^{2}H NMR spectrum of (+)-cubenene obtained from (2*E*,6*E*)-

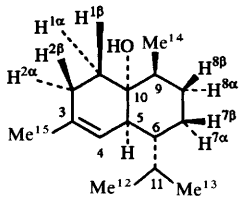
Table 2 ^1H and ^2H NMR assignments for (+)-cubenene **6**

H	δ_H (multi.)	J(Hz)	^1H-shift correlated	$\delta(^2H)$ [1,1-$^2H_2$]-FPP[a]	$\delta(^2H)$ [6-^2H]-FPP[a]
1	5.27 (m)		2α,2β		
2α	2.54 (m)	unresolved peaks	1,4,5,15 (Me)		
2β	2.54 (m)				
4	5.47 (m)		2α,2β,5,15 (Me)		
5	2.32 (m)		2α,2β,4,6,15 (Me)	2.31	
6	1.15 (m)		5,7β		
7α	0.98 (m)[b]		7β		
7β	1.63 (m)[b]		6,7α,8α,8β		
8α	1.82 (m)[c]		7α,7β,8β,9		
8β	1.22 (m)[c]		7β,8α		
9	1.90 (m)		14 (Me)		1.90
11	2.09 (m)		12 (Me), 13 (Me)	2.08	
12 (Me)	0.85 (d)	6.9	11		
13 (Me)	0.90 (d)	6.9	11		
14 (Me)	1.03 (d)	6.4	9		
15 (Me)	1.69 (s)		2α,2β,4,5		

[a] 2*E*,6*E*- Isomer. [b,c] Assignments may be interchanged.



(+)-Cubenene **6**



(+)-Epicubenol **5**

[1,1-$^2H_2$]FPP showed broad singlet peaks at $\delta(^2H)$ 2.08 (11-H) and 2.31 (5-H), while that from [6-^2H]FPP showed one broad singlet, at $\delta(^2H)$ 1.90 (9-H). These ^2H signals confirmed 1,3- and 1,2- hydride migration during enzymic cyclization of FPP to form (+)-cubenene.

Cyclization of FPP with (+)-epicubenol synthase from a microbial source, *Streptomyces* sp., was first reported by Cane *et al.*[6] Judging from the enzymic activities and the labelling results, the (+)-epicubenol synthase from cultured cells of *H. planus* can be seen to resemble that from *Streptomyces* sp. The labelling results of (+)-epicubenol obtained by incubation of a cell-free extract of *S.* sp. by using ^2H- and ^3H-labelled FPP[6] were explained by a cyclization mechanism involving initial isomerization of all-*trans*-FPP to nerolidyl diphosphate **2**,[8-10] followed by ionization and electrophilic attack at C-10 to form a *cis*-germacradienyl cation **3** which is prone to further conversion into the cadinanyl cation **4** with a 1,3-hydride shift (Scheme 1). This mechanism is completely consistent with a specific conversion of (2*E*,6*E*)-FPP and 1,3-hydride shift in the formation of (+)-epicubenol by a cell-free extract of *H. planus* cultured cells. Indirect evidence for the predicted 1,2-hydride shift was provided by the MS data of (+)-epicubenol generated from [6-^2H]-FPP.

The present study describes for the first time the (+)-cubenene synthase reaction. The labelling results gained by ^2H NMR and GLC–MS analysis verified conclusively the occurrence of 1,2- and 1,3-hydride shifts in its formation. (+)-Cubenene must be also derived through the intermediacy of the cadinanyl cation with elimination of 1-H. Although the structurally related cyclic olefins were often synthesized as co-products of the sesquiterpene alcohol synthases,[11] whether (+)-cubenene is synthesized as a co-product of (+)-epicubenol synthase remains inconclusive

Experimental

General procedure

GLC was performed on a Shimadzu GC-14 B model and detection was with a single field ionization detector. GLC–MS was performed on a Hitachi M-80B spectrometer. ^1H NMR (500 MHz and 270 MHz) spectra were recorded on a Bruker M-500 and a JEOL EX-270 MHz spectrometer, respectively, for solutions in D_2O with sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) as internal standard (δ_H 0) or in $CDCl_3$ [or $CDCl_3$–CCl_4 (1:4)] with $CHCl_3$ as internal standard (δ 7.26); ^{13}C NMR spectra were recorded at 67.8 MHz on a JEOL-EX-270 spectrometer with $^{13}CDCl_3$ as internal standard (δ_C 77.0). ^2H NMR spectra (41.3 MHz; JEOL EX-270) were recorded for $CHCl_3$–CCl_4 (1:4) solutions with C^2HCl_3 (δ_D 7.26) as internal standard. *J*-Values are given in Hz.

(2*E*,6*E*)- and (2*Z*,6*E*)-[1,1-$^2H_2$]FPP

(6*E*)-[1,1-$^2H_2$]Farnesoate was prepared from nonlabelled geranylacetone by the method reported by Popjak *et al.*[12] A mixture of (2*E*,6*E*)- and (2*Z*,6*E*)-[1,1-$^2H_2$]farnesol was prepared by the reduction of ethyl (6*E*)-farnesoate (3.53 g, 13.4 mmol) with LiAl[$^2H_4$] (1.63 g, 47.0 mmol; 99.4% enrichment) and chromatographed on $AgNO_3$ (15% w/w)-silica gel with ethyl acetate–benzene (9:1) to yield (2*E*,6*E*)-farnesol (327 g) and (2*Z*,6*E*)-isomer (426 mg). (2*E*,6*E*)-FPP was synthesized from the corresponding farnesol by the method previously reported by Dixit *et al.*,[13] while (2*Z*,6*E*)-FPP was prepared by a slightly different method. Diisopropylamine was added to avoid cyclization by a trace of acid(s) during the preparation of (2*Z*,6*E*)-farnesyl chloride. Farnesyl diphosphates were purified separately by the method described by Davisson *et al.*[14] (2*E*,6*E*)-[1,1-$^2H_2$]FPP: δ_H(500 MHz; D_2O) 1.60 (6 H, s, Me-13 and Me-14), 1.66 (3 H, s, Me-12), 1.70 (3 H, s, Me-15), 2.01 (2 H, t, *J* 7.2), 2.09 (4 H, m), 2.14 (2 H, t, *J* 7.2), 5.17 (1 H, t, *J* 6.9), 5.20 (1 H, t, *J* 6.9) and 5.44 (1 H, s, 2-H); and (2*Z*,6*E*)-FPP: δ_H 1.60 (6 H, s, Me-13 and Me-14), 1.67 (3 H, s, Me-12), 1.71 (3 H, s, Me-15), 2.01 (2 H, t, *J* 7.4), 2.12 (6 H, m), 5.19 (2 H, m, 6- and 10-H) and 5.45 (1 H, s, 2-H).

[4-^2H]Geranylacetone

To a stirred suspension of Zn (6.19 g, 0.095 mol) in anhydrous benzene (150 cm^3) was added dropwise a mixture of ethyl bromo-[2,2-$^2H_2$]acetate (10 g, 0.059 mol; 99.4 atom%) and 6-methylhept-5-en-2-one (10.46 g, 0.083 mol) in anhydrous benzene (47 cm^3) over a period of 3 h. The mixture was refluxed

for 2.5 h, cooled to room temp., and quenched by the addition of 10% H_2SO_4. The benzene layer was separated and the resulting aq. layer was extracted with diethyl ether. The organic layers were combined, washed successively with water, saturated aq. $NaHCO_3$, and brine, and evaporated to yield crude ethyl 3-hydroxy-3,7-dimethyl-[2,2-$^2H_2$]octa-2,6-dienoate (35.1 g).

This ester was dissolved in anhydrous pyridine (330 cm^3) containing phosphoryl trichloride (50 cm^3) and the solution was left for 72 h at room temp. The reaction was quenched by the addition of water at 0 °C. The whole mixture was extracted with diethyl ether. The extracts were washed successively with 0.5 mol dm^{-3} HCl, saturated aq. Na_2CO_3, and brine, dried (Na_2SO_4), and evaporated. Chromatography of the residue on silica gel with hexane–diethyl ether (8:2) yielded ethyl 3,7-dimethyl-[2-^2H]octa-2,6-dienoate (15.85 g).

To a stirred suspension of $LiAlH_4$ (4.47 g, 0.118 mol) in anhydrous THF (75 cm^3) was added a solution of ethyl 3,7-dimethyl-[2-^2H]octa-2,6-dienoate in dry THF (8 cm^3) dropwise at −30 °C for a period of 2 h. The mixture was stirred for 5 h at −30 °C, the reaction was quenched by the addition of water–THF (1:1, v/v). The whole solution was acidified with 1 mol dm^{-3} HCl and extracted with diethyl ether. The extracts were washed successively with saturated aq. $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated. Chromatography of the resultant residue on $AgNO_3$–silica gel (15%, w/w) column with benzene–ethyl acetate (1:9) yielded purified [2-^2H]geraniol (655 mg) and partially purified [2-^2H]nerol (264 mg). Identities of nerol and geraniol were confirmed by GLC. [4-^2H]geranylacetone was synthesized from [2-^2H]geraniol by the method outlined by Ruzicka.[15] [4-^2H]Geranylacetone; *m/z*: 195 (M^+), 152 (M^+ − 43), 126, 108 and 69; δ_H(270 MHz; $CDCl_3$) 1.59 (3 H, s), 1.60 (3 H, s), 1.67 (3 H, s), 1.97 (2 H, m), 2.04 (2 H, m), 2.13 (3 H, s), 2.25 (2 H, m), 2.45 (2 H, m) and 5.03 (1 H, t).

(2*E*,6*E*)-[6-^{2}H]FPP

(2*E*,6*E*)-[6-^2H]FPP was prepared from [4-^2H]geranylacetone by a method similar to that for [1,1-$^2H_2$]FPP. (2*E*,6*E*)-[6-^2H]-FPP showed δ_H(270 MHz; D_2O) 1.60 (6 H, s, Me-13 and Me-14), 1.66 (3 H, s, Me-12), 1.70 (3 H, s, Me-15), 2.00–2.11 (8 H, m), 4.44 (2 H, 2 × br d, 2 × 1-H), 5.19 (1 H, m, 10-H) and 5.44 (1 H, t, 2-H).

Cell cultures of *H. planus*

Callus and suspension cell cultures of *H. planus*,[7] grown on MSK-4 medium[16] for 28 days and 21 days, respectively, were previously reported.

Isolation of (+)-epicubenol

Calli (210 g) of *H. planus* grown on MSK-4 medium were extracted with MeOH at room temp. The combined MeOH extracts were partitioned with pentane. The combined pentane phases were concentrated (2.2 g), and fractionated by a silica gel column (100 g) with successive elution of pentane (200 cm^3) and pentane–diethyl ether (9:1, 4:1, 7:3 and 3:2; each 200 cm^3). The pentane–diethyl ether (7:3) eluates were rechromatographed by column chromatography on silica gel (40 g) with chloroform–ethyl acetate (19:1), and then by repeated HPLC on a ODS column (30 cm × 1.5 cm, i.d.) with acetonitrile at 3 cm^3 min^{-1} and then on a silica gel column (24 cm × 1 cm, i.d.) with chloroform–ethyl acetate (19:1) at 1 cm^3 min^{-1} to yield (+)-epicubenol (13.4 mg) as an oil with $[\alpha]_D$ +106.5 (*c* 0.27, $CHCl_3$; lit.,[1] +111.6); *m/z* 222 (M^+), 204 ($M^+ - H_2O$), 179, 161, 119 (100%), 105 and 82; δ_H(270 MHz; $CDCl_3$) 0.80 (3 H, d, *J* 7.0, Me-12), 0.87 (3 H, d, *J* 7.0, Me-13), 0.96 (3 H, d, *J* 6.7, Me-14), 1.05 (1 H, m, 7-H$^\beta$), 1.10 (1 H, m, 8-H$^\alpha$), 1.17 (1 H, m, 6-H), 1.54 (1 H, m, 8-H$^\beta$), 1.57 (1 H, m, 7-H$^\alpha$), 1.61 (1 H, m, 9-H), 1.63 (2 H, m, 1-H_2), 1.68 (1 H, m, 5-H), 1.70 (3 H, s, Me-15), 1.97 (1 H, m, 11-H), 2.05 (1 H, m, 2-H$^\alpha$), 2.08 (1 H, m, 2-H$^\beta$) and 5.45 (1 H, m, 4-H). The relative stereochemistry of (+)-epicubenol was finally confirmed by the direct comparison of ^1H NMR, GLC–MS and GLC data with those of an authentic sample of (−)-epicubenol.[5]

(+)- and (−)-Cubenene

(+)-Cubenene was prepared from (+)-epicubenol by the method reported by Connolly *et al.*[1] To a stirred solution of (+)-epicubenol (1.6 mg) in anhydrous pyridine (0.03 cm^3) was added a solution of thionyl dichloride (15 mg) in pyridine (0.013 cm^3) at 0 °C. After 30 min, the reaction was quenched by the addition of saturated aq. $NaHCO_3$ (15 cm^3) and the mixture was extracted with diethyl ether. The extracts were washed successively with 10% aq. H_2SO_4, water, saturated aq. $NaHCO_3$, and water, and were evaporated to yield (+)-cubenene **6** (purity: ~95%) as an oil (1.4 mg) with $[\alpha]_D$ +24.4 (*c* 0.05, $CHCl_3$; lit.,[1] +22.3), *m/z* 204 (M^+), 179, 161, 119 (100%), 105 and 82; δ_H[270 MHz; $CDCl_3$–CCl_4 (1:4)]: see Table 2; ^{13}C NMR; δ_C[67.8 MHz; $CDCl_3$–CCl_4 (1:4)] 15.4 (Me-12), 18.5 (Me-13), 21.8 (Me-14), 23.7 (Me-15), 24.8 (C-7), 26.7 (C-11), 31.7 (C-8), 36.9 (C-2), 37.7 (C-6), 42.0 (C-9), 51.2 (C-5), 112.8 (C-1), 121.4 (C-4), 131.1 (C-3) and 142.8 (C-10). The MS spectrum and ^1H NMR data of Me protons and vinyl protons and the ^{13}C NMR data of chemically prepared (+)-cubenene were identical with those reported previously.[1] The assignments of aliphatic methine and methylene protons were achieved by a ^1H–^1H COSY experiment. (−)-Cubenene containing ~10% (+)-isomer was prepared from (+)-epicubenol ($[\alpha]_D$ −82.5[5]) isolated from needles of *Pinus koraiensis* in a manner similar to that for the (+)-isomer. (−)-Cubenene was an oil with $[\alpha]_D$ − 15.0 (*c* 0.17, $CHCl_3$); its mass and ^1H NMR spectra were identical with those of the (+)-isomer.

Cell-free extracts of cultured cells

Cell-free extracts of cultured cells of *H. planus* were prepared from the calli of 30-day cultures or the cells from 21-day suspension cultures.[7] The harvested calli or suspension cells, after being washed with doubly distilled water and patted dry, were suspended in 50 mmol dm^{-3} 2-[4-(2-hydroxyethyl)-piperazin-1-yl]ethanesulfonic acid (HEPES) buffer [pH 7.5, 5 times volumes (v/w) for the calli and 10 times for suspension cells] containing 10% D-sorbitol, 1 mmol dm^{-3} dithiothreitol (DTT), 20 mmol dm^{-3} magnesium chloride and polyvinylpolypyrrolidinone (PVPP) [10% (w/w) of fresh weight of cells] and ground in a motor at 0–4 °C. After centrifugation (2000 ***g***) of the broken-cell suspension at 0–4 °C for 30 min, the resulting supernatants were recentrifuged at 40 000 ***g*** at the same temp. for 10 min. After XAD-4 polystyrene resin (equal weight to cells) had been added and the mixture stirred at the same temp. for 60 min and filtered, the 40 000 ***g*** supernatants (2.21 mg protein g^{-1} calli and 6.72 mg protein g^{-1} suspension cells) were incubated at 30 °C for 60 min with 30 μmol dm^{-3} of FPPs {(2*E*,6*E*)- and (2*Z*,6*E*)-[1,1-$^2H_2$]-, and (2*E*,6*E*)-[6-^2H]-}. Protein was determined by the modified dye binding method of Bradfold[17] using bovine serum albumin as reference standard. The (+)-cubenene and (+)-epicubenol synthase activity was determined by the peak areas of the selected-ion-monitored (SIM) mass chromatogram at *m/z* 206 ($[M - H_2O]^+$) of (+)-epicubenol converted from [1,1-$^2H_2$]FPP. The cell-free extracts from the calli displayed maximal (+)-cubenene and (+)-epicubenol synthase activity at pH 7.5 and the activity showed an absolute dependence for Mg^{2+}. Maximal stimulation occurred at 20 mmol dm^{-3} Mg^{2+} [(+)-cubenene and (+)-epicubenol: 0.936 and 0.144 nmol h^{-1} mg^{-1} protein, respectively]. Between 0.05 and 0.5 mmol dm^{-3}, the manganese dication slightly stimulated the activity (epicubenol: 0.057 nmol

h^{-1} mg^{-1} protein at 0.1 mmol dm^{-3} Mn^{2+}). In common with all other terpenoid cyclases,[18] cubenene and epicubenol synthase exhibited the divalent-metal-cation requirement. Removal of DTT from the preparation by dialysis resulted in a complete loss of (+)-epicubenol synthase activity, and readdition of the reagent (1.0 mmol dm^{-3}) led to partial recovery of the activity to ~40%. The requirement for the thiol reagent in the enzyme reaction mixture was usually observed in mono- and sesquiterpene cyclase activities.[19–22] No detectable amounts of deuteriated (+)-cubenene and (+)-epicubenol were produced in incubations using the boiled control.

Enzyme assay

Cyclase activities were measured by incubating the enzyme preparation (calli: 0.4 mg protein and suspension cells: 0.2 mg) in HEPES buffer (2 cm^3) with 30 μmol dm^{-3} non- or ^2H-labelled FPP (> 99.4 atom% excess) at 30 °C for 60 min, followed by chilling in ice. The reaction mixture was treated with decane (2.2 μg) in diethyl ether (2 cm^3) and extracted with diethyl ether. The extract was dried, concentrated by hand warming,[23] and analysed by GLC and GLC–MS.

Cyclization of (2*E*,6*E*)-[1,1-$^2H_2$]- and (2*E*,6*E*)-[6-^2H] FPP

Aliquots (30 μmol dm^{-3}) of deuteriated FPPs were cyclized to (+)-cubenene by cell-free extracts (660 cm^3 for [1,1-$^2H_2$]-FPP and 460 cm^3 for [6-^2H]FPP) from calli for 120 min at 30 °C. The reaction mixture was extracted with diethyl ether. The extract was evaporated, and the residue was separated on a silica gel column (5 g) with pentane (20 cm^3) to remove epicubenol and farnesol. (+)-Cubenene was detected as the sole deuteriated compound in the pentane eluate by GLC–MS. Thus, after evaporation, the pentane eluate (2–4 mg) was directly analysed by ^2H NMR spectroscopy [41.34 MHz; $CHCl_3$–CCl_4 (1:4)].

GLC and GLC–MS analysis

To estimate the total concentrations of (+)-cubenene and (+)-epicubenol and to identify the above two compounds, the ether extracts and isolated compounds were submitted to GLC using both achiral columns, *viz.* Ulbon HR-1 (Shinwa Chemical Industries, 50 m × 0.25 mm i.d.; t_R cubenene: 59.2 min and epicubenol: 64.9 min) and Ulbon HR-20M (Shinwa Chemical Industries; t_R cubenene: 42.7 min and epicubenol: 66.4 min) and a chiral column *viz.* CP-Cyclodextrin B (GL Science, 50 m × 0.25 mm i.d.). Column temp.: initial temp. at 60 °C was kept for 5 min and then the temperature was elevated at 2 °C min^{-1} to 220 °C; He flow rate 1.4 cm^3 min^{-1}. GLC–MS analyses were carried out under the same conditions as those for GLC analyses except that the He flow rate was 1.13 cm^3 min^{-1} with HR-1 [ionizing voltage 70 eV]. Total ion and ions at *m/z* 204, 205 and 206 were monitored for GLC–MS. Concentrations of cubenene and epicubenol were determined on the basis of the relative GLC peak areas of terpenes to the known amount of decane by considering the corresponding multiplification factors (epicubenol: 1.07, cubenene: 0.97). Concentrations of deuteriated cubenene and epicubenol derived from [1,1-$^2H_2$]FPP 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.

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