PERGAMON

Phytochemistry 51 (1999) 389–394

PHYTOCHEMISTRY

Biotransformation of cubenene to 7-hydroxycalamenene in cultured cells of the liverwort, *Heteroscyphus planus*

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Received 27 May 1998; received in revised form 22 September 1998

Abstract

The biosynthesis of 7-hydroxycalamenene using suspension cultured cells of the liverwort, *Heteroscyphus planus*, is described. The biotransformation of cubenene, but not calamenene, to 7-hydroxycalamenene suggests that it is formed via hydroxylation of the methylene carbon between its unconjugated double bonds. Thus followed by oxidation, thereby explaining the formation of 7-hydroxycalamenene without invoking an NIH shift. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Heteroscyphus planus*; Jungemanniales; Cultured cells; Liverwort; 7-Hydroxycalamenene; Calamenene; Cubenene; Deuterium label; Biotransformation; NIH shift

1. Introduction

Calamenenes and the less saturated sesquiterpenes, cadalenes, which are hydroxylated at the aromatic rings, occur in terrestrial plants and aquatic invertebrates (Bowden, Coll, Engelhardt, Topiolas, & White, 1986). In vascular plants, calamenene and cadalene derivatives, which are substituted at the aromatic ring are widely distributed in liverworts of Jungemanniales (Nabeta, Katayama, Nakagawara, & Katoh, 1993; Asakawa, 1995; Nabeta, 1995). In paticular, hydroxylated calamenenes and cadalenes are frequently found in liverworts (Asakawa, 1995) and in the leaves and cotyledons of cotton during the hypersensitive response to bacterial pathogens (Essenberg, & Pierce, 1995). Phytoalexins of the hydroxylated cadalene-type were isolated from the leaves and the cotyledons of cotton (*Gossypium* spp.). Evidence supporting the intermediacy of 6*E*-farnesyl diphosphate (FPP, **1**) in the biosynthesis of gossypol was obtained in two studies (Maciadri, Angst, & Arigoni, 1985; Stipanovic et al., 1986). Davis, and Essenberg, 1995 recently provided evidence that (+)-δ-cadinene is an enzymatic intermediate in the biosynthesis of cotton phytoalexins. However, the 'missing links' in the biosynthesis of oxygenated calamenenes and cadalenes from hydrocarbons of the cadinane type or corresponding carbocations in infected cotton plants remain to be identified.

Cultured cells of the liverwort *Heteroscyphus planus* produce 7-hydroxycalamenene (**2**) together with 7-methoxycalamenene (**3**), 7-hydroxy-1,2-dihydrocadalene (**4**), 7-methoxy-1,2-dihydrocadalene (**5**) and 7-methoxycadalene (**6**). 1, 2- and 1, 3-hydride shifts in their formation were verified by distribution of ^{2}H and ^{13}C atoms in the biosynthetically labeled compounds incorporating various ^{2}H- and ^{13}C-labeled mevalonates (Nabeta, Mototani, Tazaki, & Okuyama, 1994; Nabeta, Ishikawa, Kawae, & Okuyama, 1994) (Fig. 1). The formation of 7-hydroxycalamenene (**2**) may be the first step in biosynthesis of hydroxylated derivatives. However, the mode of aromatization and introduction of a hydroxy group in the aromatic ring still remain to be established. No NIH shift was observed at the 7 and 8 positions in the biosynthesis of **2** with feeding of deuterated mevalonic acid (Nabeta et al., 1994). Thus, it is unlikely that compound **2** is directly formed from

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PII: S0031-9422(98)00737-7

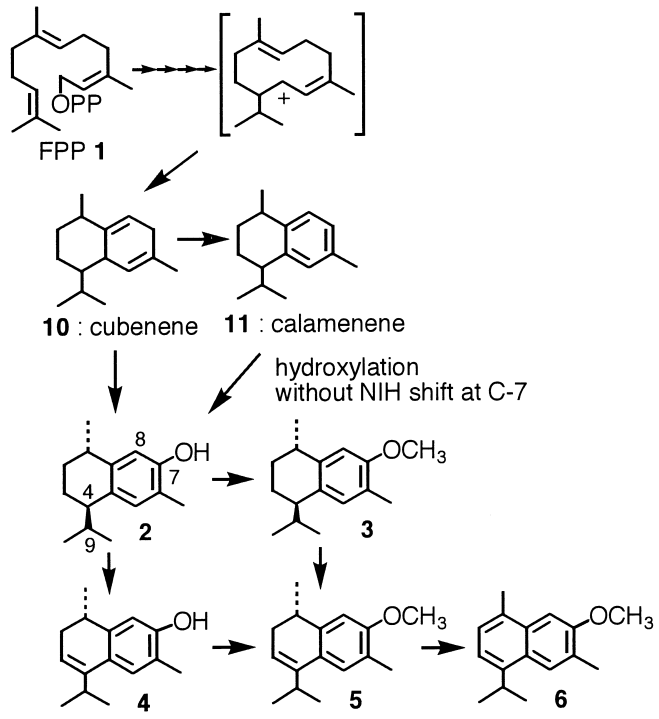

Fig. 1. Proposed biosynthesis of hydroxylated calamenenes and cadalenes from FPP in cultured cells of *Heteroschypnus planus*.

calamenene via hydroxylation with an NIH shift. An unconjugated diene sesquiterpene, cubenene **10**, which is a cometabolite in cultured cells of *H. planus*, and is specifically produced from 2*E*, 6*E*-FPP by a cell-free extract of cultured cells (Nabeta et al., 1995, 1997), is the most likely precursor for **2**, since **2** may be formed from cubenene via hydroxylation and then oxidation without the NIH shift. In the present study, tests of the conversion of cubenene **10** and calamenene **11** to 7-hydroxycalamenene **2** in suspension cultured cells of *H. planus* provided direct evidence for biotransformation of cubenene **10**, but not calamenene **11**, to form 7-hydroxycalamenene **2**.

2. Results and discussion

2.1. Preparation of precursors

Chemical conversion of (−)-α-cubebene (**7**) was carried out using a slight modification of the methods of Ohta, Ohara, and Hirose, 1968 and Anderson, Syrdal, and Graham (1972) for the preparation of cubenene (**10**) and calamenene (**11**) (Fig. 2). A mixture of three dienes, (−)-cadina-4,6(1)-diene (**8**), (+)-δ-cadinene (**9**) and (−)-cubenene (**10**) were prepared from equimolar amounts of (−)-α-cubebene **2** and TFA in a ratio of **8**:**9**:**10** of 4:1:2, avoiding the formation of hydroxylated sesquiterpenes such as epicubenol (Ohta et al., 1968).

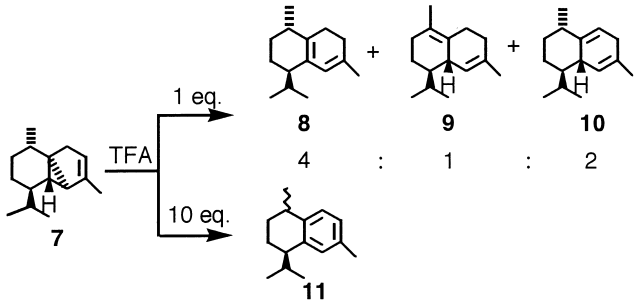

Fig. 2. Chemical conversion of (−)-α-cubebene **2** with TFA.

Treatment afforded compound **7** with a 10-fold molar excess of TFA produced a mixture of *cis*- and *trans*-calamenene (**11**) in a ratio of 1:1, which was determined by ^{1}H NMR (Heymes, Plattier, & Teisseire, 1974; Ladwa, Joshi, & Kulkarni, 1978) (Fig. 2). No deuterium incorporation into the products was observed when the reactions were carried out with deuterated TFA.

[$^2H_6$]-Calamenene (**13**) was prepared from [$^2H_7$]-4′-methyl acetophenone (**12**) by the method reported by Condon, and West, 1980. Birch reduction of compound **13** did not produce deuterated cubenene (**10**), and gave predominately the unconjugated diene **14** (Fig. 3). [$^2H_2$]-Cubenene (**17**) was obtained instead by the dehydration (Connolly, Phillips, & Haneck, 1982) of [$^2H_2$]-epicubenol (**16**), which was prepared by a slight modification of the method reported by Cane, and Tandon, 1994a, 1994b. Pyridinium chlorochromate was used for oxidation of compound **15**, instead of the Dess–Martin reaction (Fig. 4).

2.2. Biotransformation of cubenene and calamenene

Biotransformation of cubenene (**10**, 50 μg) and calamenene (**11**, 50 and 500 μg) was carried out in 1 ml of 28-day-old cultures (0.04 g fr. wt.) of *H. planus*. The cultures were further incubated at 25°C with stirring at 110 rpm under continuous light for 12 h. The resulting suspension was then centrifuged at 3000*g*, and the precipitated cells were washed with distilled water and ether to remove possible precursors adhering to the

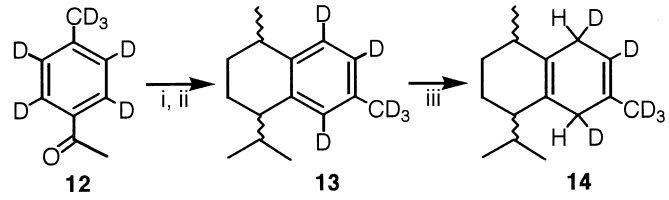

Fig. 3. Synthesis of [$^2H_6$]-calamenene (**13**) and its Birch reduction product (**14**): (i) 1-bromo-4-methyl pentane, Mg, 0°C (36%); (ii) P_2O_5, 120°C (54%); (iii) Li, ethylenediamine, EtOH, THF, 0°C (22%).

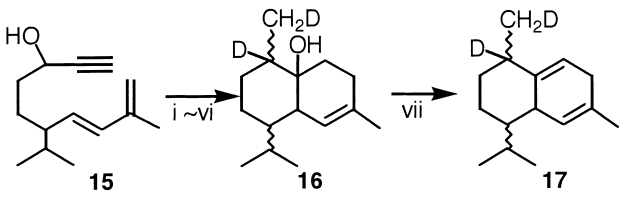

Fig. 4. Synthesis of [$^2H_2$]-cubenene (**17**): (i) Pyridinium chlorochromate, CH_2Cl_2, room temp. (41%); (iii) *m*-chloroperbenzoic acid, CH_2Cl_2, room temp. (41%); (iv) HF-pyridine, THF, 0°C (79%); (v) methyltriphenylphosphonium bromide, NaH, DMSO, 0°C (66%); (vi) $NaBD_4$, $CoCl_2{\cdot}6H_2O$, CD_3OD, 0°C (38%); (vii) $SOCl_2$, pyridine, 0°C, then 10% $NaHCO_3$ (56%).

cells. The harvested cells were extracted with MeOH at 4°C, and the MeOH soln was partitioned with *n*-pentane. To determine if cubenene-, calamenene- and 7-hydroxycalamenene were either taken into cells, or biotransformed within them, the cells were extracted immediately after inoculation (indicated as 0 h in Tables 1 and 2). The *n*-pentane soln was concentrated and then analyzed by GC and GC–MS analyses. No significant increase (>0.1 mg/0.04 g fr. wt.) in amounts of cubenene, calamenene and 7-hydroxycalamenene was observed during even a 12 h incubation without precoursors being added (Table 1). When cultured cells were incubated with 50 μg/ml cubenene, however, the amounts of 7-hydroxycalamenene and calamenene increased approximately two- and threefold, respectively (Table 1). In contrast, the amount of 7-hydroxycalamenene did not increase when cultured cells were incubated with calamenene at 50 or 500 μg/ml (Table 2). Moreover, a higher amount of calamenene detected in cultured cells harvested immediately after incubation with feeding calamenene at 500 μg/ml may not simply be ascribed to uptake of calamenene, but may indicate that calamenene was easily absorbed onto the cell surface of *H. planus* with the adhering calamenene not being easily removed with Ether. Thus, 7-hydroxycalamenene (**2**) apparently was formed from cubenene, but not from calamenene.

Direct evidence for conversion of exogenously supplied cubenene, but not calamenene, to 7-hydroxycalamenene was provided administering [$^2H_2$]-cubenene (**17**) and [$^2H_6$]-calamenene (**13**). The MS spectra of 7-hydroxycalamenene incorporating [$^2H_2$]-cubenene, [$^2H_6$]-calamenene and no exogenous substrate are shown in Fig. 5. Thus the 7-hydroxycalamenene incorporating [$^2H_2$]-cubenene (**17**) gave a base ion peak at *m/z* 177 (relative intensity to unlabelled product (M+) ion at *m/z* 175: 13.7%), which was formed by cleavage is the C-4/C-9 bond, indicative of two ^2H atoms attached to a dihydronaphthalene ring. Moreover, the MS ion peak monitored at *m/z* 177 was only observed in the MS chromatogram of 7-hydroxycalamenene incorporating the [$^2H_2$]-cubenene (Fig. 6). On the other hand the MS spectrum of 7-hydroxycalamenene from the cells fed [$^2H_6$]-calamenene (**13**) did not give a base ion peak at *m/z* 180, again supporting the previous finding that [$^2H_6$]-calamenene was not converted into 7-hydroxycalamenene.

Thus, in the biosynthesis of hydroxylated calamenenes and cadalenes in suspension cultured cells of *H. planus*, was shown to be an intermediate for 7-hydroxycalamenene. Thus, in turn, is presumed to be an intermediate in the biosynthesis of cadalene **6** via compounds **3** or via the sequential formation of **4** and **5** (Fig. 1). The hypothetical biosynthetic pathway is clearly in contrast to an intermediacy of δ-cadinene in the biosynthesis of cadalenes in infected cotton plants (Essenberg, & Pierce, 1995). The position of the hydroxy group may be directed by the positions of the double bonds (Fig. 7). In preliminary experiments, compound **14**, a geometrical isomer of cubenene, was also converted to monohydroxylated calamenene.

3. Experimental

3.1. General

(−)-α-Cubebene (98%) was purchased from Fluka Chemie (Buchs, Switzerland). GC and GC–MS were performed on a Shimadzu GC-17A and a Hitachi M-80B spectrometer equipped with an FS/WCOT column (Ulbon H-1, Shinwa Chemical Industries; 50 m × 0.25 mm, temp. was elevated from 150 to 220°C at 2°C

Table 1
Biotransformation of cubenene (50 μg) in the suspension cultured cells of *H. planus*. Values are expressed as μg per μl culture medium (0.04 g fr. wt.) and represent the mean ± S.D. ($n=2$)

Incubation time (h)	Cubenene (μg/μl)	7-Hydroxycalamenene (μg/μl)	Calamenene (μg/μl)
0	0.46 ± 0.09	0.12 ± 0.02	0.68 ± 0.05
12	2.20 ± 0.20	0.35 ± 0.08	1.20 ± 0.10
(12–0)	1.74	0.23	0.52
12[a] (without substrate)	0.40 ± 0.02	0.14 ± 0.01	0.60 ± 0.03

[a] Significant increase (>0.1 μg/0.04 g fr.wt.) in amounts of cubenene, calamenene and 7-hydroxycalamenene was not observed during 12-h incubation without precursors.

Table 2
Biotransformation of calamenene (50 and 500 μg) in the suspension cultured cells of *H. planus*. Values are expressed as μg per μl culture medium (0.04 g fr. wt) and represent the mean ± S.D. ($n=2$)

Amount (μg/μl)	Incubation time (h)	Calamenene (μg/μl)	7-Hydroxycalamenene (μg/μl)
50	0	0.80 ± 0.05	0.011 ± 0.003
	12	5.20 ± 0.15	0.010 ± 0.002
	(12–0)	4.40	–
500	0	9.15 ± 0.64	0.010 ± 0.003
	12	25.08 ± 5.05	0.006 ± 0.002
	(12—0)	15.93	–

min^{-1}). To estimate concentrations of sesquiterpenes, the calibration curves for **10**, **11** and **2** were determined. ^1H NMR spectra were recorded on a JEOL GX-270 spectrometer with $CDCl_3$ as internal standard. Reversed-phase HPLC was performed by a JASCO system equipped with a Finepak SIL C18T-5 column (JASCO, 250 × 4.6 mm, 5 mm particles) at a flow rate of 1 ml min^{-1} {CH_3CN–H_2O (3:1)}. Sesquiterpenes were detected with a UV spectrophotometer (UVIDEC-100-V, JASCO) at 215 nm. The fractions containing sesquiterpenes were diluted with an equal volume of H_2O and then extracted with *n*-pentane, followed by salting out with NaCl.

3.2. Precursor preparation

3.2.1. (−)-Cadina-4,6(1)-diene (*8*), (+)-δ-cadinene (*9*) and (−)-cubenene (*10*)

(−)-α-Cubebene (**7**, 21.6 mg, 110 μmol) in *n*-pentane (500 μl) was treated with equimolar of TFA (8.1 μl, 110 μmol) at 0°C. The reaction mixture was stirred at 0°C for 0.5 h and quenched with satd $NaHCO_3$ (500 μl). The organic layer was sepd, dried over dry Na_2SO_4, filtrated, and conc. with N_2 flow to yield a

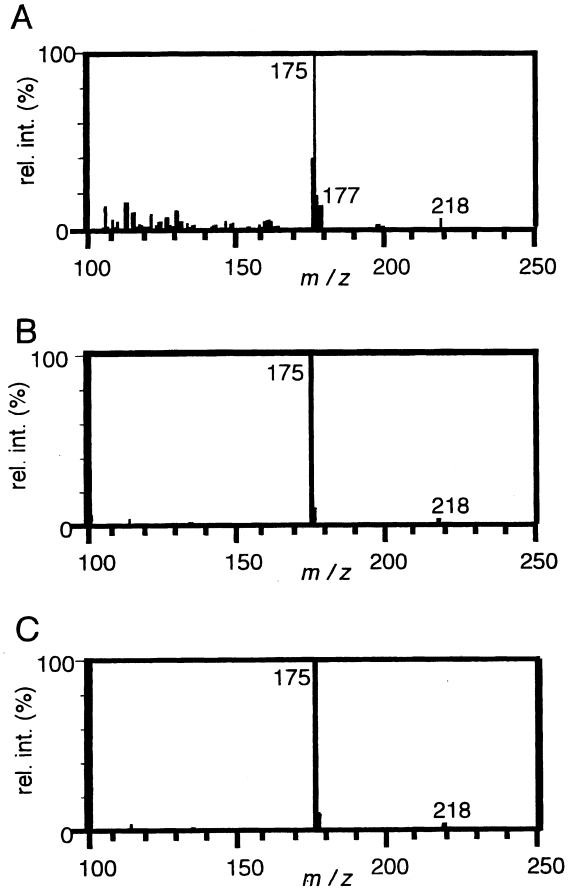

Fig. 5. Mass spectra of 7-hydroxycalamenene **2** synthesized in suspension cultured cells of *H. planus* fed with [$^2H_2$]-cubenene **17** (a), [$^2H_6$]-calamenene **13** (b) and without precursor (c).

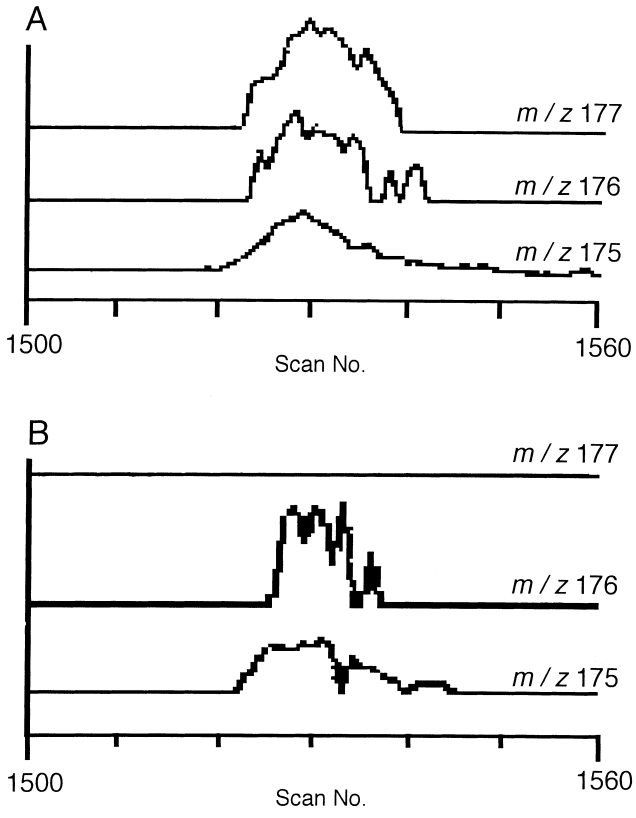

Fig. 6. Segments of GC–MS ion chromatograms of 7-hydroxycalamenene (*m/z* 175–177) incorporating [$^2H_2$]-cubenene **17** (a) and without cubenene **17** (b).

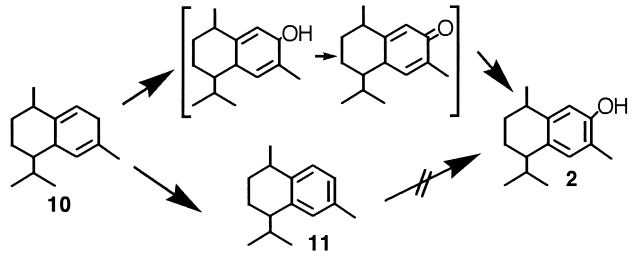

Fig. 7. Postulated biosynthesis of 7-hydroxycalamenene from cubenene **10** in the suspension cultured cells of *H. planus*.

colorless oil (15.2 mg, 70%). The ratio of compounds **8**, **9** and **10** in the reaction mixture was estimated by GC–MS analysis to be 4:1:2. Further purification was performed with reversed-phase HPLC to obtain pure **8** (4.2 mg, 19%), **9** (1.0 mg, 5%) and **10** (2.5 mg, 12%). **8**: $[\alpha]_D$ −70.1° (*c* 0.3, $CHCl_3$, lit. Ohta et al., 1968: −67.8°), EIMS 70 eV, *m/z* (rel. int): 204 $[M]^+$ (100), 189 (39), 161 (91), 119 (24), 105 (27), ^{1}H NMR (270 MHz, $CDCl_3$); δ 0.85 (3H, d, J=6.3 Hz, -CH(CH_3)$_2$), 0.90 (3H, d, J=6.3 Hz, -CH(CH_3)$_2$), 1.03 (3H, d, J=6.3 Hz, >CHCH_3), 1.10 (1H, m, CH), 1.25 (2H, m, CH_2), 1.60 (3H, s, -C(CH_3)=CH-), 1.70 (2H, m, CH_2), 1.92 (1H, m, CH), 2.02 (1H, m, CH), 2.15 (2H, m, CH_2), 2.20 (2H, m, CH_2), 5.42 (1H, s, =C–CH=C(CH_3)-), **9**: $[\alpha]_D$ +81.8° (*c* 0.4, $CHCl_3$, lit. Ohta et al., 1968: +84.7°), EIMS: 204 $[M]^+$ (75), 189 (18), 161 (100), 134 (45), 119 (30), 105 (27), ^{1}H NMR; δ 1.78 (3H, s, -CH–CH=C(CH_3)), 5.30 (1H, s, -CH–CH=C(CH_3)-), **10**: $[\alpha]_D$ −20.2° (*c* 0.2, $CHCl_3$, lit. Ohta et al., 1968: −23.2°), EIMS: 204 $[M]^+$ (51), 161 (64), 119 (100), 105 (55), ^{1}H NMR; δ 1.68 {3H, s, -CH–CH=(C(CH_3)}, 5.25 (1H, br s, CH_2C*H*=C<), 5.48 {1H, br s, -CH–C*H*=C(CH_3)}.

3.2.2. Calamenene (*11*)

Compound **7** (10.4 mg, 51.0 μmol) in *n*-pentane (150 μl) was treated with 10 molar equivalents of TFA (39 μl, 506 μmol) at 0°C. The reaction mixture was stirred at 0°C for 0.5 h and quenched with satd $NaHCO_3$ soln (500 μl). The organic layer was extracted with *n*-pentane (350 μl), dried over dry Na_2SO_4, concentrated under a N_2 atmosphere, and purified by silica gel column chomatography {*n*-hexane–Et_2O (9:1)} to yield **11** (6.2 mg, 60%). EIMS 70 eV, *m/z* (rel. int): 202 $[M]^+$ (9), 159 (100), 145 (5), ^{1}H NMR (270 MHz, $CDCl_3$); δ 0.69 (*trans*), 0.74 (*cis*) (total 3H, d, J=7 Hz, -CH(CH_3)$_2$), 0.98 (*trans*), 1.01 (*cis*) (total 3H, d, J=7 Hz, -CH(CH_3)$_2$), 1.21 (3H, d, J=7 Hz, >CHCH_3), 2.29 (3H, s, CH_3Ar), 6.94 (1H, d, J=7 Hz, ArH), 7.06 (1H, s, ArH), 7.12 (1H, d, J=7 Hz, ArH).

3.2.3. [$^2H_6$]-Calamenene (*13*)

A mixture of *cis*- (40%) and *trans*- (60%) [$^2H_6$]-calamenene was prepared from [$^2H_7$]-4′-methyl acetophenone (**12**), as described in Heymes et al. (1974) and Ladwa et al. (1978). EIMS 70 eV, *m/z* (rel. int): 208 $[M]^+$ (4), 206 (9), 165 (100), 164 (95), ^{1}H NMR (270 MHz, $CDCl_3$); δ 0.69 (*trans*), 0.74 (*cis*) {total 3H, d, J=6.9 Hz, -CH(CH_3)$_2$}, 0.98 (*trans*), 1.01 (*cis*) {total 3H, d, J=6.9 Hz, -CH(CH_3)$_2$}, 1.21 (3H, d, J=6.9 Hz, >CHCH_3), 1.60 (4H, m, CH_2×2), 1.85 (1H, m), 2.30 (1H, m), 2.80 (1H, m).

3.2.4. Birch reduction of *13*

[$^2H_6$]-Calamenene (**13**) (40.0 mg, 0.20 mmol) and lithium (7.0 mg, 1.01 mmol) were suspended in THF (0.15 ml). Ethylenediamine (200 mg, 3.33 mmol) in EtOH (0.15 ml) was added to the suspension at 0°C. The reaction mixture was stirred at 0°C for 4 h, quenched with 1 N HCl, and extracted with *n*-hexane. The organic layer was washed with satd $NaHCO_3$ and brine, dried over dry Na_2SO_4, filtrated, concd under a N_2 atmosphere and purified using reversed phase HPLC to yield **14** (8.7 mg, 22 %). EIMS 70 eV, *m/z* (rel. int): 210 $[M]^+$ (5), 208 (10), 206 (12), 167 (100), 166 (98), 165 (90), ^{1}H NMR (270 MHz, $CDCl_3$); δ 0.68, 0.72 {total 3H, d, J=6.3 Hz, -CH(CH_3)$_2$}, 0.94 (3H, d, J=6.3 Hz, >CHCH_3), 0.97, 1.0 {total 3H, d, J=6.3 Hz, -CH(CH_3)$_2$}, 1.25 (1H, m), 1.50 (2H, m), 1.70 (1H, m), 1.90 (2H, m), 2.10 (1H, m), 2.5 (1H, m), 2.10 (1H, m).

3.2.5. [$^2H_2$]-Cubenene (*17*)

[$^2H_2$]-Epicubenol was prepared by a slight modification of the method reported by Cane, and Tandon, 1994a, 1994b. Pyridinium chlorochomate was used for oxidation of **15**, instead of the Dess–Martin reaction. A mixture of *cis*- (50%) and *trans*- (50%) [$^2H_2$]-cubenene was prepared from **16** by the published procedure (Connolly et al., 1982). EIMS 70 eV, *m/z* (rel. int): 206 $[M]^+$ (8), 205 (18), 163 (60), 162 (40), 121 (100), 120 (85), ^{1}H NMR (270 MHz, $CDCl_3$); δ 0.80, 0.85 (total 3H, d, J=6.3 Hz, -CH(CH_3)$_2$), 0.90, 0.94 (total 3H, d, J=6.3 Hz, -CH(CH_3)$_2$), 1.0 (2H, m, >CDCH_2D), 1.25 (1H, m), 1.50 (2H, m), 1.65 (3H, m), 1.70 (1H, m), 1.90 (2H, m), 2.10 (1H, m,), 2.10 (1H, m,), 5.15 (1H, br s), 5.38 (1H, br s).

3.2.6. Feeding experiment

The origins of *H. planus*, as well as the medium and conditions for suspension culture have been described earlier (Takeda, & Katoh, 1981; Nabeta et al., 1993). One ml of 28 day-old culture was incubated without or with precursors (50 μg for **10**, and 50 or 500 μg for **11**) in *n*-pentane (10 μl). The liquid suspensions were agitated continuously at 110 rpm at 25°C under continuous light of 2000 lux for 0 or 12 h. Duplicate experiments were carried out to determine amounts of products by GC.

3.2.7. Extraction

The cell culture suspension was centrifuged at 3000*g* for 10 min and the supernatant was decanted. The ppt. cells were washed with H_2O and then Et_2O, and extracted with MeOH (1 ml) at 4°C for 12 h. The MeOH extract was partitioned with *n*-pentane (1 ml). The *n*-pentane soln was conc. to 10 μl under N_2 flow and then subjected to GC and GC–MS analyses.

Acknowledgements

We thank Dr. Noriyuki Nakajima (Toyama Prefectural University) for discussion of substrate synthesis. This work was supported by Hokkaido Foundation for the Promotion of Scientific and Industrial Technology and a Grant-in-Aid for Scientific Research (A, No. 0836021 and C, No. 08660125) from the Ministry of Education, Science and Culture, Japan.

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