# Biosynthesis of kelsoene and prespatane in cultured cells of the liverwort *Ptychanthus striatus*

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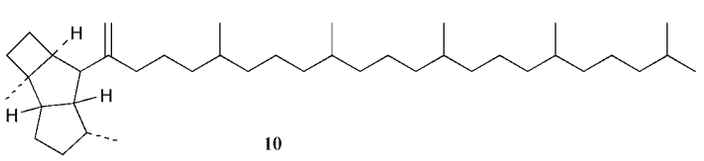
Biosynthesis of the unusual sesquiterpenes, (+)-kelsoene and (−)-prespatane, that accumulate in the cultured cells of the liverwort *Ptychanthus striatus* was investigated. A mixture of (+)- and (−)-alloaromadendrene was isolated from a variety of unidentified sesquiterpenes from cultured cells. However, the (−)-isomer, which is thought to be an immediate precursor to kelsoene, was present only as a minor component [a ratio of (−)-isomer to (+)-isomer of 1 : 25]. When the cells were incubated with a higher dosage (1.0 mmol) of mevalonate (MVA, 3,5-dihydroxy-3-methylvalerate) under heterotrophic conditions or with a normal dosage (0.5 mmol) under photoautotrophic conditions, the $^{2}$H- and $^{13}$C-labels from $^{2}$H- and $^{13}$C-MVA were incorporated into the sesquiterpenes at an extremely high level (30 to 40 and 46 atom% excess, respectively). Labelling patterns of the biosynthetically $^{2}$H- or $^{13}$C-labelled kelsoene and prespatane were determined by GLC-MS and $^{2}$H- and $^{13}$C-NMR analyses. The labelling pattern of the kelsoene demonstrates the randomization of the $^{2}$H- and $^{13}$C-labels between the isopropenylmethyl and the methylene groups, with loss of one H-1 proton of farnesyl diphosphate (FPP, farnesyl = 3,7,11-trimethyldodeca-2,6,10-trienyl), suggesting that kelsoene is biosynthesized from a germacradienyl cation with 7*R* configuration by means of a (−)-alloaromadendranyl cation. Randomization of the labels between the isopropenylmethyl and the methylene and loss of one H-1 proton of FPP were also observed in $^{2}$H- and $^{13}$C-labelled prespatane. Thus, prespatane is postulated to be biosynthesized from a (7*S*)-germacradienyl cation by means of a guaianyl cation. During the third cyclization of the guaianyl cation, one proton at the C-1 position of FPP was lost.

The uncommon tricyclic sesquiterpenes, (+)-kelsoene **1** (Scheme 1) and (−)-prespatane **2** (Scheme 2), were isolated for the first time from the tropical marine sponge, *Cymbastella hooperi*.[1] Shortly thereafter, compounds **1** and **2** were isolated from cultured cells of the liverworts, *Ptycanthus striatus*[2] and *Calypogeia muelleriana*,[3] which seemingly are not phylogenetically related to the marine sponge. They may represent a simple accumulation of the constituents from a dietary source such as marine algae. The tetraterpene poduran **10** with the same tricyclo[5.3.0.0$^{2,5}$]decane framework was isolated from the springtail *Podura aquatica*.[4] The relative configurations of **1** and **2** have been determined by König and Wright.[1] (+)-Kelsoene **1** was chemically synthesized by Mehta and Srinivas.[5] We[6] very recently established the absolute configurations of **1** and **2** to be (1*S*,2*R*,5*S*,6*S*,7*R*,8*R*)-2,8-dimethyl-6-(1-methylethenyl)tricyclo[5.3.0.0$^{2,5}$]decane and (1*R*,2*S*,5*R*,6*R*,7*R*,8*S*)-1,5-dimethyl-8-(1-methylethenyl)tricyclo[5.3.0.0$^{2,6}$]decane (IUPAC names),† respectively, by NMR analyses after derivatization of the olefins with a chiral reagent, 2′-methoxy-1,1′-binaphthalene-2-carbohydroximoyl chloride (MBCC).[7] Thus, the isopropenyl groups in kelsoene and prespatane are located on opposite planes of the tricyclic skeletons with 4- and 5-membered rings, suggesting that initial cyclization of farnesyl diphosphate (FPP) from the *Si*-face at C-10 to form the (7*R*)-germacradienyl cation [**5**, or its neutral intermediate, (+)-germacrene A] leads to kelsoene, while that from the *Re*-face leads to prespatane by means of the (7*S*)-germacradienyl cation [**7**, or (−)-germacrene A]. The absolute configurations of **5** and **7** also suggested the stereochemical course of cyclization of the germacradienyl cations **5** and **7** to kelsoene and prespatane (Schemes 1 and 2 indicate our numbering of the carbon atoms in each compound). The cation **5** is cyclized stereospecifically between C-1 and C-5 and between C-6 and C-11 to afford (−)-alloaromandendranyl cation **6**. Cleavage of the cyclopropane ring and ring closure between C-7 and C-6 with the loss of a proton yields kelsoene. We suggest that prespatane is formed from the germacradienyl cation **7** by means of a guaianyl cation **8** with ring closure between C-6 and C-10. Formation of both kelsoene and prespatane may involve protonation at C-4 of the cations **5** and **7** either by a proton from the medium or intramolecularly by the original C-1 proton of FPP. Preliminary examination of the biosynthetic sequence to form the kelsoene skeletone was conducted by incorporation of [2-$^{13}$C]MVA into kelsoene.[2] Randomized label incorporation between C-12 and C-13 of kelsoene was observed. However, the step that resulted in randomization remains unclear.

This paper reports our examination of the biosynthetic pathways of kelsoene and prespatane by administration of [2-$^{2}H_2$]-, [5-$^{2}H_2$]- and [2-$^{13}$C]MVAs to suspension cell cultures of *P. striatus* under both heterotrophic and photoautotrophic conditions, as monitored by GLC-MS and NMR spectroscopy.

† The numbering system adopted in the Schemes and discussion is based on those of the proposed intermediates germacrene and bicyclogermacrene, rather than on that of the IUPAC name.

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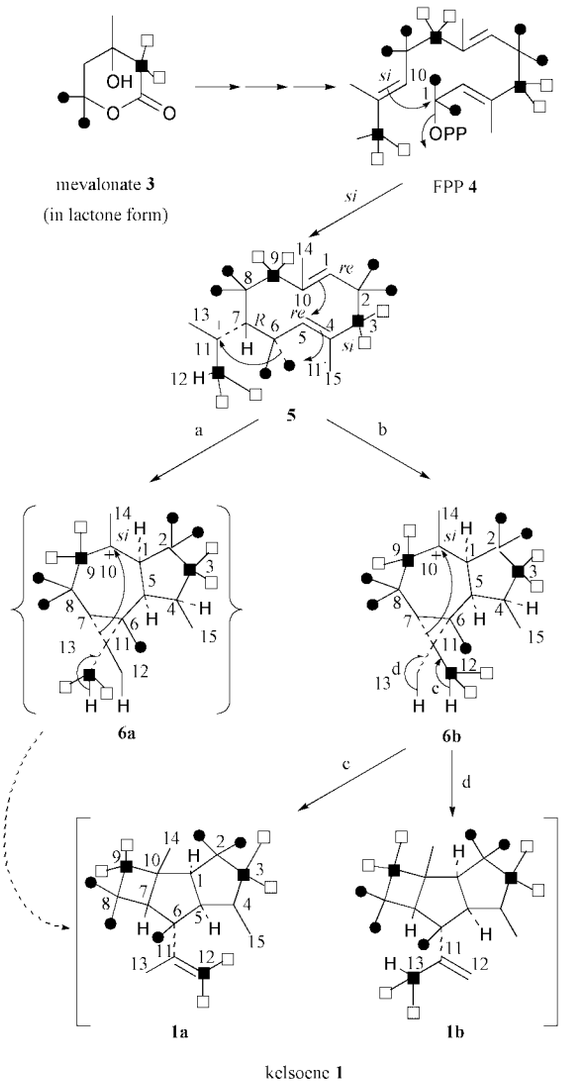



**Scheme 1** Biosynthetic pathway and labelling patterns of kelsoene from cultured cells of *Ptychanthus striatus*.

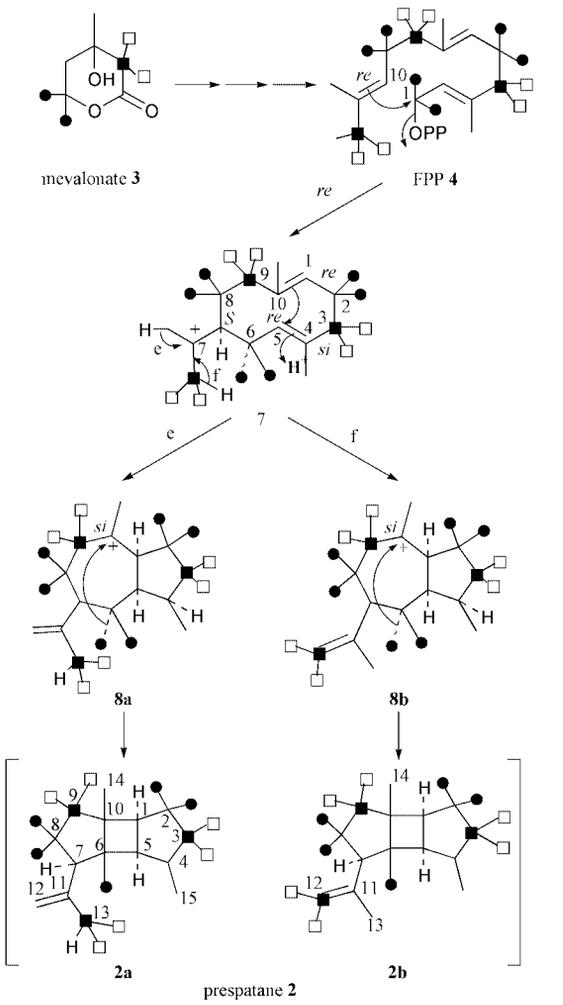
**Table 1** $^{1}$H (270 MHz) and $^{13}$C data (67.8 MHz) for (+)-alloaromadendrene from cultured cells of *P. striatus* in $CDCl_3$

| Position | $\delta_H$ (no. of H, multiplicity, *J*/Hz) | $\delta_C$ |
|---|---|---|
| 1 | 2.67 (1 H, br q, 9.3) | 50.8 |
| 2 | 1.73 (1 H, m) 1.88 (1 H, m) | 28.2 |
| 3 | 1.32 (1 H, m) 1.73 (1 H, m) | 31.2 |
| 4 | 2.06 (1 H, m) | 37.8 |
| 5 | 1.86 (1 H, m) | 42.2 |
| 6 | 0.24 (1 H, dd, 10.0 and 10.1) | 23.6 |
| 7 | 0.54 (1 H, m) | 24.8 |
| 8 | 1.24 (1 H, m) 1.84 (1 H, m) | 22.2 |
| 9 | 2.28 (1 H, m) 2.34 (1 H, m) | 35.7 |
| 10 | — | 152.6 |
| 11 | — | 17.2 |
| 12 | 0.95 (3 H, s) | 15.9 |
| 13 | 1.00 (3 H, s) | 28.6 |
| 14 | 4.71 (1 H, s), 4.73 (1 H, s) | 109.6 |
| 15 | 0.87 (3 H, d, 7.3) | 16.4 |

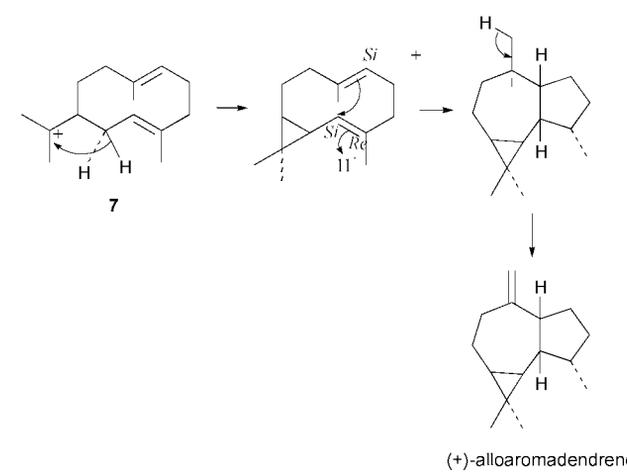
## Results

### Co-metabolites of kelsoene and prespatane in cultured cells of *P. striatus*

Components of sesquiterpene hydrocarbons were examined to provide evidence for the hypothetical biosynthetic pathway of kelsoene and prespatane. Alloaromadendrene was isolated by chromatography on a $AgNO_3$–silica gel column. The data




**Scheme 2** Biosynthetic pathway and labelling patterns of prespatane from cultured cells of *Ptychanthus striatus*.




**Scheme 3** Biotransformation of a (7*S*)-germacradienyl cation to (+)-alloaromadendrene.

obtained from the $^{1}$H- and $^{13}$C-NMR spectra (Table 1) of alloaromadendrene (Scheme 3) isolated from the cultured cells of *P. striatus* were completely identical to those of commercially available (−)-alloaromadendrene and those previously reported for (−)-alloaromadendrene.[8] The optical composition of alloaromadendrene from *P. striatus* was determined by comparison of its GLC retention time with that of (−)-alloaromadendrene ($t_R$ 52.82 min) and by co-chromatography with the (−)-isomer on a chiral capillary column (CP-Cyclodextrin B, 50 m × 0.25 mm). The major optical isomer was identified as (+)-alloaromadendrene ($t_R$ 53.25 min).

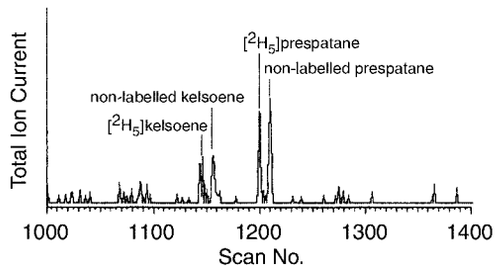



**Fig. 1** Segment of the total ion monitored GC chromatogram of volatile components from cultured cells of *P. striatus*.

However, (−)-alloaromadendrene was present at an extremely low level [the ratio of the (−)-isomer to the (+)-isomer was *ca.* 1 : 25].

### Incorporation of MVA into kelsoene and prespatane under heterotrophic and photoautotrophic conditions

The extent of $^{13}$C-enrichment of kelsoene[2] and striatol[9] from incorporation of 0.5 mmol [2-$^{13}$C]MVA in 100 cm$^3$ of culture medium under heterotrophic conditions (40 g dm$^{-3}$ glucose under 3000 lux of continuous light) has been previously determined to be low (an average of 3.3 and 1.2 atom% excess, respectively) as determined from the $^{13}$C-NMR spectra of the biosynthetically $^{13}$C-labelled compounds. To improve the degree of $^2$H- or $^{13}$C-enrichment, cells were fed [2-$^{13}$C]- and [2-$^2H_2$]MVA at a higher concentration (1.0 mmol per 100 cm$^3$) under heterotrophic conditions, or were grown in the presence of [5-$^2H_2$]MVA at the same concentration (0.5 mmol per 100 cm$^3$) under photoautotrophic conditions. The cultures were aerated with 1% carbon dioxide. The biosynthetically labelled terpenes were separated on day 21 after inoculation, and then analyzed by GLC and GLC-MS. The extent of $^2$H- and $^{13}$C-enrichment was determined from the relative areas of total ion monitored peaks in the mass chromatograms or from the relative peak intensities of the MS ions in the mass spectra.[10,11]

Fig. 1 shows a segment of the total ion monitored (TIM) GLC-MS chromatogram of the volatile components from incorporation of [5-$^2H_2$]MVA under photoautotrophic conditions. Deuterated kelsoene ([$^2H_5$], $t_R$ 54.56 min) and prespatane ([$^2H_5$], $t_R$ 55.13 min) were resolved from the corresponding non-labelled compounds (54.73 min and 55.29 min, respectively) using a chemically bonded capillary column (methylsilicone-type) by means of the $^2$H isotopic effect.[11,12] The $^2$H-enrichments of both compounds were roughly estimated from $^2$H enrichment (%) = $PA_{2H}/(PA_{\text{non-labelled}} + PA_{2H}) \times 100$, where $PA_{2H}$ and $PA_{\text{non-labelled}}$ are relative peak areas of penta-deuterated and non-labelled compounds, respectively. Thus, $^2$H-enrichment of kelsoene was estimated to be 46%. The levels of $^2$H-enrichment of the sesquiterpenes were extremely high, when compared with those found in cultured cells of *P. striatus* grown under heterotrophic conditions and fed with 0.5 mmol MVA. Partially labelled compounds such as [$^2H_4$]-, [$^2H_3$]-, [$^2H_2$]- or [$^2H_1$]- were not observed in the chromatograms (Fig. 1). Thus, when the cultured cells were grown under photoautotrophic conditions, the exogenously supplied MVA was chiefly used for sesquiterpene biosynthesis without dilution with the endogenously formed MVA.

Tables 2 and 3 summarise the fragment ions formed from kelsoene and prespatane incorporating [2-$^{13}$C]MVA. A remarkable rise in the $^{13}$C-enrichments (30–40%) was estimated from the relative peak intensities of the isotopic ions, when the concentration of MVA added was increased twofold. Stress produced by a higher dosage of MVA may enhance enzymatic activity in the synthesis of sesquiterpenes.

### Labelling patterns determined by GLC-MS

MS spectra of both deuterated kelsoene and prespatane are reproduced in Fig. 2(C) and (D), respectively, together with those of the non-labelled compounds [(A) and (B)]. No molecular ion peaks [M$^+$] were observed in the spectra of either kelsoene or prespatane. A predominant fragment ion **A** at *m/z* 176 (Fig. 2) in non-labelled kelsoene, which is formed *via* elimination of ethylene from the cyclobutane ring, is prone to further elimination of an isopropenyl moiety to give an ion **B** at *m/z* 122. The fragment ions **A** and **B** shift by 3 mass units (amu) in kelsoene incorporating [5-$^2H_2$]MVA, indicating that if kelsoene had been labelled with five $^2$H atoms, three of the five $^2$H atoms were incorporated into a bicyclo[3.3.0]octane ring and the other two were incorporated into a $CH_2$–$CH_2$ moiety in the cyclobutane ring. This conclusion was supported by $^2$H-NMR analysis (Table 4). On the other hand, the fragment ions **A** and **B** from kelsoene incorporating [2-$^2H_2$]MVA shift by 4 and 2 amu, respectively, demonstrating that two $^2$H atoms are retained at the isopropenyl group, while two other $^2$H atoms become attached to the bicyclooctane ring.

**Table 2** Fragment ions **A** and **B** from kelsoene incorporating [2-$^{13}$C]-MVA

| | Ions *m/z* (Relative intensity, %) | |
|---|---|---|
| | **A** | **B** |
| F[a] | 176 (8.60) | 135 (18.17) |
| F + 1 | 177 (4.06) | 136 (16.34) |
| F + 2 | 178 (3.57) | |
| $^{13}$C enrichment[b] | 32% | 42% |

[a] F, F + 1, F + 2; fragment ions and their isotopic ions. [b] $^{13}$C-Enrichments were roughly estimated as $^{13}$C-enrichment of **A** (%) = $100 \times \{2 \times (PI_{F+2} - PI_F \times NA_{F+2}) + (PI_{F+1} - PI_F \times NA_{F+1})\}/2(PI_{F+2} + PI_{F+1} + PI_F)$ and $^{13}$C enrichment of **B** (%) = $100 \times (PI_{F+1} - PI_F \times NA_{F+1})/(PI_{F+1} + PI_F)$, where $PI_F$, $PI_{F+1}$ and $PI_{F+2}$ are the relative peak intensities of fragment ions and their isotopic ions, and $NA_{F+1}$ and $NA_{F+2}$ are the natural abundances of the isotopic ions.

**Table 3** Fragment ions **C** and **D** from prespatane incorporating [2-$^{13}$C]-MVA

| | Ions *m/z* (Relative intensity, %) | |
|---|---|---|
| | **C** | **D** |
| F[a] | 122 (6.49) | 81 (8.77) |
| F + 1 | 123 (8.30) | 82 (5.54) |
| F + 2 | 124 (1.61) | |
| $^{13}$C enrichment[b] | 33% | 36% |

[a] F, F + 1, F + 2; fragment ions and their isotopic ions. [b] $^{13}$C-Enrichments were roughly estimated as indicated in the footnote of Table 2.

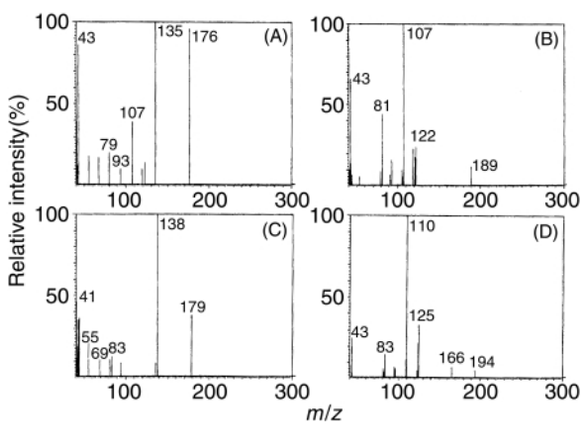



**Fig. 2** Mass spectra of non-labelled (A) and [$^2H_5$]kelsoene from [5-$^2H_2$]MVA (C), and non-labelled (B) and [$^2H_5$]prespatane from [5-$^2H_2$]MVA.

**Table 4** $^{13}C$- and $^{2}H$-Enrichment of carbons and protons in kelsoene (**1**) incorporating [2-$^{13}C$]-, [2-$^{2}H_2$]- and [5-$^{2}H_2$]mevalonate. Figures in parentheses show $^{13}C$-enrichment relative to C-12

| Position | $\delta_{^{13}C}$ Non-labelled prespatane | $\delta_{^{13}C}$ [2-$^{13}C$] | $\delta_{^{1}H}$ | $\delta_{^{2}H}$ [2-$^{2}H_2$]$^{d}$ | $\delta_{^{2}H}$ [5-$^{2}H_2$]$^{d}$ |
|---|---|---|---|---|---|
| 1 | 57.8 | | 2.08 | | |
| 2 | 25.9 | | α 1.37 | | 1.40 |
| | | | β 1.49 | | 1.50$^{c}$ |
| 3 | 33.2 | 32.7 (2.47) | α 1.34 | 1.29 | |
| | | | β 1.78 | 1.60$^{a}$ | |
| 4 | 36.3 | | 2.27 | | |
| 5 | 49.9 | | 2.86 | | |
| 6 | 48.1 | | 2.37 | | 2.40 |
| 7 | 47.4 | | 2.40 | | |
| 8 | 14.6 | | α 1.52 | | 1.50$^{c}$ |
| | | | β 1.75 | | 1.80 |
| 9 | 33.0 | 32.5 (2.47) | α 1.68 | 1.60$^{a}$ | |
| | | | β 1.66 | 1.60$^{a}$ | |
| 10 | 45.7 | | | | |
| 11 | 145.6 | | | | |
| 12 | 109.8 | 109.3 (1) | Ha 4.80 | 4.87$^{b}$ | |
| | | | Hb 4.87 | 4.87$^{b}$ | |
| 13 | 24.2 | 23.7 (1.23) | 1.66 | 1.60$^{a}$ | |
| 14 | 23.5 | | 1.16 | | |
| 15 | 17.7 | | 0.88 | | |

The $^{13}C$-enrichments relative to C-12 are estimated as relative peak areas of $^{13}C$-labelled carbons to that of C-12 or those in the non-labelled compound. $^{a}$ Unresolved peaks of 3β-$^{2}H$, 9α-$^{2}H$, 9β-$^{2}H$ and 13-methyl $^{2}H$. $^{b}$ Unresolved peaks of 12-$^{2}Ha$ and 12-$^{2}Hb$. $^{c}$ Unresolved peaks of 2β-$^{2}H$ and 8α-$^{2}H$. $^{d}$ Relative $^{2}H$ peak areas observed at $\delta$ 1.29, 1.60 and 4.87 ppm were 2.76 : 7.62 : 1, while those at 1.40, 1.50, 1.80 and 2.40 ppm were 1.1 : 2.0 : 1.0 : 1.1. Assignments of $^{1}H$ and $^{13}C$ data were accomplished by DEPT, $^{1}H$–$^{1}H$ and $^{1}H$–$^{13}C$ COSY, HMBC, HMQC, NOESY and NOEDS experiments.

Non-labelled prespatane gave an $[M^+ - CH_3]$ ion at *m*/*z* 189, and intense fragment ions **C**, **D** and **E** at *m*/*z* 125, 83 and 110, respectively. The $[M^+ - CH_3]$ ion shifts by 5 amu in the labelled prespatane, confirming that a total of five $^{2}H$ atoms was retained in the deuterated prespatane from [5-$^{2}H_2$]MVA. The ions **C** and **D** were simultaneously formed by cleavage of the cyclobutane ring with the elimination of one hydrogen. Elimination of a methyl group of the ion **C** gave ion **E** at *m*/*z* 110. The shift of 3 amu for the **C** and **E** ions of prespatane incorporating [5-$^{2}H_2$]MVA demonstrates that three $^{2}H$ atoms were incorporated into the cyclopentane ring attached to an isopropenyl group, while the shift of 2 amu of ion **D** indicates that two $^{2}H$ atoms became attached to the other cyclopentane ring. In contrast, fragment **C** from the prespatane incorporating [2-$^{2}H_2$]MVA shifts by 4 amu, while fragment ion **E** shifts by 4 and 2 amu. The fragment ion at *m*/*z* 109 is formed from the isopropenyl moiety of ion **C** by elimination of a $C^{2}H_2{}^{1}H$ radical, while that at *m*/*z* 111 is formed by elimination of a $C^{1}H_3$ radical.

These labelling pattern suggest that the $^{2}H$ atoms of [2-$^{2}H_2$]-MVA are randomly incorporated into both the methyl and methylene groups of the isopropenyl group. This random labelling pattern was also confirmed by $^{2}H$-NMR spectroscopic analysis (Table 5). Incorporation of five $^{2}H$ atoms of [5-$^{2}H_2$]MVA into the endocyclic positions of both kelsoene and prespatane indeed suggests protonation at C-4 of the germacradienyl cations **5** and **7** by a proton from the medium with loss of the original C-1 proton of FPP.

### Labelling patterns determined by $^{2}H$- and $^{13}C$-NMR

Tables 4 and 5 summarise the $^{2}H$- and $^{13}C$-enrichments of kelsoene and prespatane incorporating [2-$^{2}H_2$]-, [5-$^{2}H_2$]- and [2-$^{13}C$]MVA. Because of low yields (200 μg from 2 cultures) and higher enrichment (30–40 atom% excess, see Tables 2 and 3), the $^{13}C$ peaks of each carbon (except for the biosynthetically $^{13}C$-enriched signals) were not observed by $^{13}C$-NMR analysis. Thus, only relative $^{13}C$-enrichments of C-12 carbons in kelsoene and prespatane are indicated in Tables 4 and 5. Although the extent of $^{13}C$-enrichment (30 to 40%) was high, the $^{13}C$-labelling positions in kelsoene were the same as reported previously.[2] The random labelling pattern between C-12 and C-13 observed in kelsoene was also confirmed in prespatane that incorporated [2-$^{13}C$]MVA. The remaining two of the three $^{13}C$ atoms originating from C-2 of MVA were incorporated into C-3 and C-9.

The $^{2}H$-NMR spectrum of kelsoene incorporating [5-$^{2}H_2$]-MVA demonstrated that H-6 was labelled with $^{2}H$ ($\delta$ $^{2}H$ 2.40 ppm), while the signal at $\delta$ 2.27 ppm showed H-4 was not deuterated. This labelling pattern supports protonation at C-4 of cations **5** by a proton from the medium. Because of overlapping of the signals due to $^{2}H$-3β, $^{2}H$-9α, $^{2}H$-9β and $^{2}H$-13 at $\delta$ $2_H$ 1.6 ppm in the $^{2}H$ NMR spectrum of kelsoene incorporating [2-$^{2}H_2$]MVA, it is difficult to assign each $^{2}H$ peak. However, the relative intensity of these overlapping peaks to those for $^{2}H$-3α at 1.29 ppm and $^{2}H$-12 at 4.87 ppm (observed; 7.62 : 2.76 : 1) may support the randomization of labels in the isopropyl group (without randomization, the ratio would be roughly calculated to be 5 : 1 : 0 or 3 : 1 : 2). In a similar manner, the relative $^{2}H$-12 signal at 4.8 ppm in prespatane incorporating [2-$^{2}H_2$]MVA was less intense than the calculated peak. This may also indicate the randomization of the label between C-12 and C-13 in prespatane.

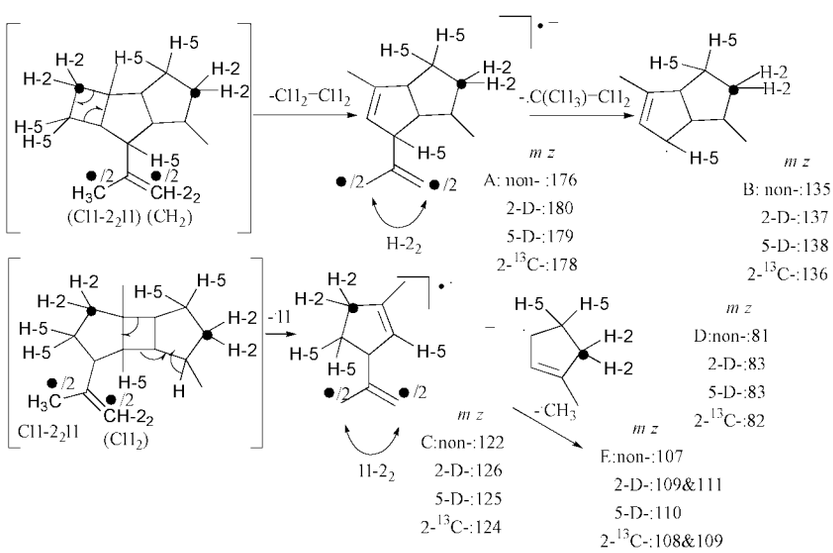
## Discussion

These labelling results can be accounted for by a cyclization of FPP by electrophilic attack[12] of C-1 of the FPP precursor on C-10 to give an enantiomeric pair of germacradienyl cations **5** and **7** in the biosynthesis of kelsoene and prespatane. The absolute configurations of the bridgehead carbons, C-1 and C-5, and of the methyl at C-4 in kelsoene are identical to those of C-1, C-5, and C-4 in prespatane. The absolute sense of the folding of the germacradienyl cation **5** with the 7*S* configuration to form the (−)-alloaromadendranyl cation **6** was the same as that of cation **7** with the 7*R* configuration to form the guaianyl cation **8**. Thus, both germacradienyl cations **5** and **7** may cyclize to cations **6** or **8**, respectively, at the same active site of the same enzyme or at different active sites of different

**Table 5** $^{13}$C- and $^{2}$H-Enrichment of carbons and protons in prespatane (**2**) incorporating [2-$^{13}$C]-, [2-$^{2}H_2$]- and [5-$^{2}H_2$]mevalonate. Figures in parentheses show $^{13}$C-enrichment relative to C = 12

| Position | $\delta_{^{13}C}$ Non-labelled prespatane | [2-$^{13}$C] | $\delta_{^{1}H}$ | $\delta_{^{2}H}$ [2-$^{2}H_2$]$^{f}$ | [5-$^{2}H_2$]$^{f}$ |
|---|---|---|---|---|---|
| 1 | 45.3 | | 2.03 | | |
| 2 | 28.1 | | α 1.66 | | 1.66$^{d}$ |
| | | | β 1.42 | | 1.46 |
| 3 | 34.7 | 34.7 (2.45) | α 1.28 | 1.25–1.42$^{a}$ | |
| | | | β 1.72 | 1.48–1.75$^{b}$ | |
| 4 | 36.8 | | 1.76 | | |
| 5 | 38.5 | | 1.99 | | |
| 6 | 43.8 | | 1.93 | | 1.93$^{e}$ |
| 7 | 50.3 | | 2.40 | | |
| 8 | 28.2 | | α 1.97 | | 1.93$^{e}$ |
| | | | β 1.69 | | 1.66$^{d}$ |
| 9 | 41.7 | 41.7 (2.64) | 1.41 | 1.25–1.42$^{a}$ | |
| | | | β 1.57 | 1.48–1.75$^{b}$ | |
| 10 | 42.1 | | | | |
| 11 | 145.3 | | | | |
| 12 | 109.2 | 109.2 (1) | Ha: 4.72 | 4.8$^{c}$ | |
| | | | Hb: 4.79 | 4.8$^{c}$ | |
| 13 | 23.4 | 23.4 (1.28) | 1.66 | 1.48–1.78$^{b}$ | |
| 14 | 20.3 | | 0.94 | | |
| 15 | 14.3 | | 0.88 | | |

The $^{13}$C-enrichments relative to C-12 were estimated as the relative peak areas of $^{13}$C-labelled carbons to that of C-12 or those in the non-labelled compound. $^{a}$ Unresolved peaks of 3α-$^{2}$H and 9α-$^{2}$H. $^{b}$ Unresolved peaks of 3β-$^{2}$H, 9β-$^{2}$H and 13-methyl-$^{2}$H. $^{c}$ Unresolved peaks of 12-$^{2}$Ha and 12-$^{2}$Hb. $^{d}$ Unresolved peaks of 2α-$^{2}$H and 8β-$^{2}$H. $^{e}$ Unresolved peaks of 6-$^{2}$H and 8α-$^{2}$H. $^{f}$ Relative $^{2}$H peak areas of peak$^{a}$ : peak$^{b}$ : peak$^{c}$ observed were 4.2 : 7.9 : 1, while those at 1.46, 1.66 and 1.93 ppm were 1 : 2.75 : 3.75. Assignments of $^{1}$H and $^{13}$C data were accomplished by DEPT, $^{1}$H–$^{1}$H and $^{1}$H–$^{13}$C COSY, HMBC, HMQC, NOESY and NOEDS experiments.




**Scheme 4** Mass fragmentation process of $^{2}$H- and $^{13}$C-labelled kelsoene and prespatane. 2-D, 5-D and 2-$^{13}$C represent fragment ions derived from [2-$^{2}H_2$]-, [5-$^{2}H_2$]- and [2-$^{13}$C]MVA.

enzymes that closely resemble each other. The germacradienyl cation **5** with a symmetrical isopropyl cation rotating freely around the 7,11 single bond is further cyclized to a cyclopropane ring with removal of a proton at C-6 of **5**. At this cyclization step, labels at the *gem*-dimethyls of the cyclopropane ring in bicyclogermacrene [or the (−)-alloaromadendranyl cation **6**] incorporating [2-$^{2}H_2$]- and [2-$^{13}$C]MVA may become scrambled, although there is no evidence to indicate that the biosynthetic sequence from **5** to **6** is accomplished by means of bicyclogermacrene. Cleavage of the cyclopropane ring between C-7 and C-11 of **6** and recyclization between C-7 and C-10 with elimination of a proton from the *gem*-dimethyls of the cyclopropane ring yield kelsoene **1a** and **1b**. Scrambling of the label at the isopropenyl group of kelsoene could be also caused by equivalent loss of a proton from the *gem*-dimethyls at this stage. Scrambling of the label was also observed at the isopropenyl group of prespatane fed with [2-$^{2}H_2$]- and [2-$^{13}$C]MVA. Equivalent removal of protons from the *gem*-dimethyls of a symmetrical isopropanyl cation of **7** is likely to cause label scrambling. Poduran **10**, an unusual tetraterpene from springtail, contains a kelsoene moiety whose relative configuration is identical to that of kelsoene. Thus, the stereochemical course of cyclization of an acyclic $C_{40}$ precursor to form **10** may proceed in the same manner as for kelsoene.

We propose that (−)-alloaromadendrane (or its cation) is the "missing link" in kelsoene biosynthesis as indicated in Scheme 1. It is interesting to note that cultured cells of *P. striatus* accumulate predominantly (+)-alloaromadendrane (**9**), but the (−)-isomer only as a minor component. (+)-Alloaromadendrene may be formed from the germacradienyl cation **7** with an *S* configuration at C-7 by a different enzyme(s) for kelsoene or prespatane (Scheme 2), since the absolute configurations of

the bridgehead carbons in the 5- to 7-membered ring systems and a secondary methyl group at C-4 are opposite to those of kelsoene and prespatane. Further investigations of the mechanism and stereochemical course of kelsoene and prespatane in cell-free systems are in progress.

## Experimental

### General procedure and materials

GLC analyses were performed on a chiral CP Cyclodextrin-B column (GL Science, 0.25 mm × 50 m) to determine the absolute configuration of alloaromadendrene. GLC-MS measurements were made on a Hitachi M-80B spectrometer equipped with a J & W Scientific DB WAX column (0.25 mm × 30 m). All $^{13}C\{^1H\}$ NMR and $^2H\{^1H\}$ spectra of biosynthetically $^{13}C$- or $^2H$-labelled kelsoene and prespatane were recorded on a JEOL EX-270 NMR ($^{13}C$-NMR, 67.8 MHz; $^2H$-NMR, 41.3 MHz) or a Bruker M-500 ($^2H$ NMR, 76.8 MHz) spectrometer with natural abundance $^{13}CDCl_3$ (0.40 cm$^3$, $\delta_C$ 77.0 ppm) and $C^2HCl_3$ in $CHCl_3$ (0.40 cm$^3$, $\delta_{^2H}$: 7.26 ppm) as internal standards. [2-$^2H_2$]- (>99 atom%), [5-$^2H_2$]- (>99%) and [2-$^{13}$C]mevalonolactone (>99%) were prepared as described previously.[13–15] Authentic samples of (−)-alloaromadendrene ([$\alpha$]$_D$ −33) were purchased from Fluka Chemika-BioChemica.

### Feeding experiments

Suspensions of cultured cells were induced and subcultured as reported previously.[2,3]

**Heterotrophic conditions.** Two to four cultures, each 5.0 g fresh weight on average in 100 cm$^3$ MSK-4 medium[2] with 4% glucose and without 2,4-dichlorophenoxyacetic acid, were incubated with 0.5 mmol of MVA ([2-$^2H_2$]-, [5-$^2H_2$]- and [2-$^{13}$C]-) The liquid suspensions were agitated at 110 rpm at 25 ± 1 °C under continuous light of 3000 lux illuminance. Cells were harvested 21–28 days after inoculation to determine the $^{13}C$- and $^2H$-enrichment and the labelling positions by NMR and GLC-MS analyses.

**Photoautotrophic conditions.** Four cultures [each 5.0 g cells in 100 cm$^3$ MSK-4 medium with citric acid (0.04 mg cm$^{-3}$), malic acid (0.04 mg cm$^{-3}$), pyruvic acid (0.02 mg cm$^{-3}$), and fumaric acid (0.04 mg cm$^{-3}$) and without glucose] were incubated with 0.5 mmol of [2-$^2H_2$]MVA.[16] Air containing 1% (v/v) $CO_2$ as a carbon source was admitted to the flasks at a rate of 50 cm$^3$ h$^{-1}$. Cells were grown at 25 ± 1 °C under continuous light of 8000 lux illuminance for 21 days.

### GLC and GLC-MS analysis

GLC was carried out on the CP Cyclodextrin-B column under the following conditions. The initial temperature of 60 °C was maintained for 5 min and then raised at a rate of 2 °C min$^{-1}$ to 220 °C with a flow of He at 1.4 cm$^3$ min$^{-1}$. GLC-MS analyses were performed on a J & W DB WAX column under the following conditions. A flame ionization detector was used, and the initial temperature of 60 °C was maintained for 5 min and then raised at a rate of 2 °C min$^{-1}$ to 220 °C under a flow of He of 1.12 cm$^3$ min$^{-1}$. An ionizing voltage of 70 eV was employed. $^2H$-Enrichments of kelsoene and prespatane were estimated on the basis of the relative peak areas of the total ion monitored peaks of kelsoene ([$^2H_5$]-, $t_R$ 54.56 min and non-labelled, 54.73 min) and prespatane (55.13 min and 55.29 min).

### Isolation of (+)-alloaromadendrene from suspension-cultured cells

(+)-Alloaromadendrene was isolated from suspension-cultured cells of *P. striatus* (350 g, fresh weight). Cells were extracted with 5 vol. (v/w) of ethyl acetate (×2). The ethyl acetate extracts were combined, concentrated under reduced pressure below 4 °C and chromatographed on a silica gel column (100 g) with successive elution of pentane–diethyl ether [19:1, (v/v, 400 cm$^3$), 3:2 (400 cm$^3$)] and diethyl ether (400 cm$^3$). Fractions containing (+)-alloaromadendrene, which was eluted with pentane–diethyl ether (19:1), were combined and concentrated on a Widmar column. (+)-Alloaromadendrene (2.3 mg) was isolated by successive chromatography on a silica gel column (30 g) eluted with pentane and on a 15% $AgNO_3$–silica gel column (30 g), also eluted with pentane. $^1H$- and $^{13}C$-NMR spectra of (+)-alloaromadendrene were identical to those reported previously and to those of authentic samples of the (−)-isomer.

### Isolation of isotopically labelled kelsoene and prespatane

Fresh cells (12–27 g) were extracted with 5 vol. of ethyl acetate (v/w, ×3). The ethyl acetate extracts were combined, concentrated on a Widmar column and chromatographed on a silica gel column (30 g) with pentane. The fractions containing kelsoene and prespatane were combined, concentrated and chromatographed repeatedly on 15% (w/w) $AgNO_3$–silica gel columns (19 g) to yield kelsoene (*ca.* 20 μg g$^{-1}$ fresh weight of cells) and prespatane (15 μg g$^{-1}$).

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