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Biosynthesis of irregular diterpenes in *Anisotome lyallii* by head-to-head coupling of geranyl diphosphate†

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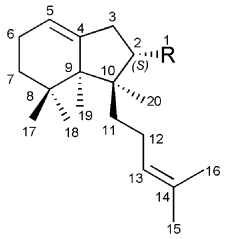
Anisotomenes, bicyclic irregular diterpenes found in the genus *Anisotome* (family Apiaceae), are shown to be products of a unique biosynthetic pathway, involving head-to-head coupling of two geranyl diphosphate units. ^{13}C labelling studies with [1-^{13}C]-glucose on plantlets of *A. lyallii* also revealed that the isoprene subunits were formed *via* the MEP pathway. The *in vitro*-cultured plant material also yielded a new irregular, linear diterpene alcohol, that shares the same biosynthetic pathway.

Introduction

Investigations into the predominantly New Zealand sub-alpine plant genus *Anisotome* (family Apiaceae) have yielded several diterpenes with an irregular skeleton (*e.g.* **1** and **2**).[1,2] The absolute stereochemistry of the anisotomenes has recently been assigned.[3]



1, R=COOH
2, R=CH_2OH
3, R=CH_2OAc

All of the diterpenes whose biosynthetic origins have been established are derived from geranylgeranyl diphosphate (GGPP).[4] However, the carbon skeleton of the anisotomenes seems incompatible with this mechanism, especially the occurrence of the two sets of geminal methyl groups and this suggests a biosynthesis involving a head-to-head coupling of two geranyl units.

Irregular diterpenes that appear to arise similarly have been reported from other plants of the Apiaceae, and we have proposed a unified biosynthetic pathway involving head-to-head linkage of two geranyl diphosphate (GPP) units.[5] Such head-to-head coupling is well established for farnesyl diphosphate (FPP), leading to the triterpenes and steroids,[6] and for GGPP to give the carotenoids.[7] Various examples of irregular sesquiterpenes arising from head-to-head condensation of terpene fractions have been found in the Asteraceae family.[8] Irregular monoterpenes have also been shown to arise by head-to-head linkage of isoprene units, as in the chrysanthemane and lavandulane-type compounds found in the plant families Asteraceae, Lamiaceae and Apiaceae.[9,10]

We now report the first direct evidence for this head-to-head linkage in diterpenes, from feeding [1-^{13}C]-glucose to *A. lyallii* Hook.f. seedlings *in vitro* and ^{13}C NMR analyses of the resultant anisotomenes. The cultured plants also yielded a new irregular diterpene **5**, closely related to the other acyclic products of the proposed pathway (Fig. 1).

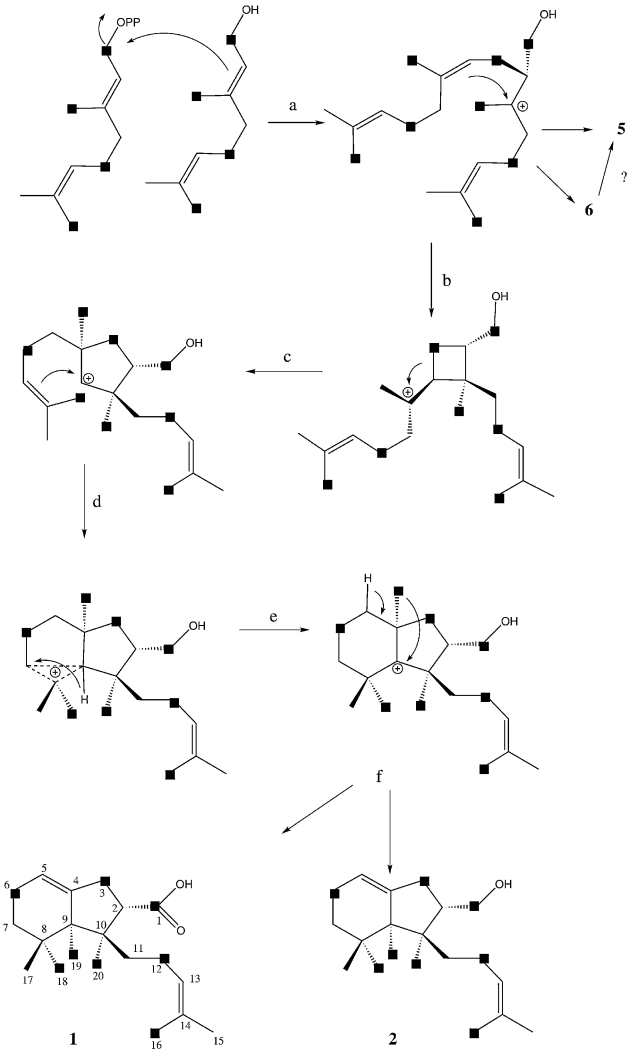


Fig. 1 Proposed biosynthesis of anisotomenoic acid **1** and anisotomenol **2** and labelling patterns from [1-^{13}C]-glucose feeding to *in vitro* cultures of *A. lyallii*.

Feeding of [1-^{13}C]-glucose is the prime method for distinguishing the mevalonic acid (MVA) and the non-mevalonate or methyl erithritol phosphate (MEP), pathways to isoprenoid biosynthesis.[11,12] The MVA pathway occurs in the cytoplasm

† Electronic supplementary information (ESI) available: tables of ^{13}C abundances of labelled compounds **1** and **4**. See http://www.rsc.org/suppdata/ob/b4/b416391e/

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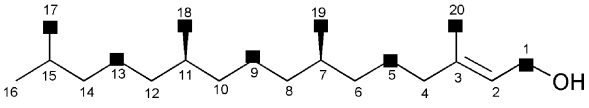
compartment of plant cells and generally leads to sesquiterpenes and triterpenes. The MEP pathway seems to be confined to the plastids and generally leads to monoterpenes, diterpenes and carotenoids.[11] We now report that the irregular *Anisotome* diterpenes are products of the MEP pathway.

Results and discussion

[1-^{13}C]-Glucose was fed to *in vitro* cultures of *A. lyallii* plants for up to 16 weeks. The plants grew slowly on a standard modified Gamborg[13] medium that we have used successfully for similar labelling studies.[8] The plants were dried and exhaustively extracted with chloroform, followed by chromatography to yield anisotomenoic acid **1** and anisotomenol **2**, as expected from this species,[2] plus *trans*-phytol **4** and a new compound, the linear diterpene alcohol **5**.



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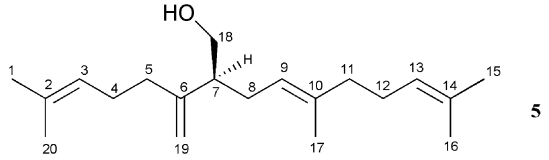

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Labelling patterns and absolute ^{13}C enrichments of the compounds were determined by quantitative ^{13}C NMR spectroscopy using Cr(III)-acetylacetonate as a relaxation reagent for the carbon nuclei.[14] For the anisotomenes, the quantitative ^{13}C NMR experiments were run at 45 °C to reduce peak broadening due to conformational flexibility.[1,5] *trans*-Phytol **4**, which is synthesised in the chloroplasts by the MEP pathway,[11] was isolated to check for specificity of the labelling. The carbon atoms expected to be specifically enriched (C-1, -5, -9, -13, -17, -18, -19 and -20)[8] had a ^{13}C average abundance of 2.30%, significantly greater ($P < 0.05$) than the remaining carbon atoms, which had an average abundance of 1.66% (see Supplementary Data). This increase in general labelling can be explained by complex metabolic turnover of [1-^{13}C]-glucose, including reversibility of reactions during carbohydrate metabolism and recycling during photosynthesis.[15]

In the sample of anisotomenoic acid **1** from plant material grown on the [1-^{13}C]-glucose-enriched media, eight carbon atoms appeared enriched, supporting the MEP origin of the four isoprene units (the MVA pathway gives three labelled atoms per isoprene unit[11,12]). We could not establish the absolute abundances of the individual carbon atoms since there were no signals in the ^{1}H NMR spectrum that were resolved sufficiently to be used as an accurate reference. The specifically-enriched carbons of **1** were C-1, -3, -6, -12, -16, -18, 19 and -20 (Fig. 1), and showed a relative abundance of 97% compared to the 'unlabelled' carbons 78% (based on C-1 as 100%, see Supplementary Data†).

The sample of anisotomenol **2** was acetylated to compound **3**, to introduce unlabelled ^{13}C atoms to allow absolute measurement of ^{13}C abundance. NMR experiments were run in C_6D_6 since this gave better dispersion of the proton signals and 2D NMR techniques were used to confirm the assignments of this new compound. The specifically labelled ^{13}C signals had an average abundance of 2.17% ± 0.10% (Table 1, Fig. 1). The remaining 12 carbon atoms also display an increased ^{13}C abundance (1.73%, ± 0.13%) compared to the expected natural abundance of 1.11%.

The results above show that the MEP pathway is responsible for the build-up of the precursor GPP. In addition to the number of specifically labelled carbons, their location at C-12 and C-16 of the obvious isoprene unit in **2** and **3** (Table 1, Fig. 1) rules out the MVA pathway. Fig. 1 shows the labelling of GPP units derived from [1-^{13}C]-glucose by the MEP pathway, and their coupling and proposed rearrangements to give anisotomenoic acid **1** and anisotomenol **2**.

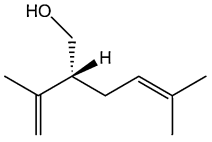
Table 1 ^{13}C abundances of labelled anisotomene acetate **3**[a]

Carbon atom	δ_C	%^{13}C
1	68.9	**2.28**
2	43.7	1.82
3	35.0	**2.30**
4	145.7	1.63
5	117.6	1.84
6	22.9	**2.16**
7	37.2	1.86[b]
8	37.4	1.65[b]
9	51.1	1.56
10	51.6	1.55
11	42.6	1.89
12	24.5	**2.08**
13	126.3	1.84[b]
14	131.2	1.86[d]
15	26.3	1.59[b]
16	18.2	**2.15**
17	28.9	1.74
18	26.6	**2.20**[b]
19	21.7	**2.10**
20	16.9	**2.01**
COO (acetyl)	170.4	1.18
CH_3 (acetyl)	21.3	1.11[c]

[a] Data recorded at 500 MHz in C_6D_6 at 45 °C. [b] Integration resolved by deconvolution (line fitting) of close signals. [c] Natural abundance standard set to 1.11%. [d] Accuracy limited due to signals being close to solvent residual signal. **Bold type**: specifically labelled positions.

We attempted to elucidate further details and confirm the proposed pathway by growing cultures of *A. lyallii in vitro* using a [U-$^{13}C_6$]-glucose labelling experiment to examine the ring formations and migrations (steps c to f, Fig. 1). However, due to slow culture growth (resulting in poor yield of secondary metabolites), low incorporation and increased background labelling, any ^{13}C–^{13}C coupling of the carbon atoms in the anisotomenes was not able to be resolved. Although this proposed biosynthesis is not proven, it does explain the location of the specifically labelled atoms in **1** and **2** (Fig. 1) and the formation of other irregular diterpenes and sesquiterpenes in the Apiaceae.[5]

In addition to the bicyclic diterpenes, we isolated another diterpene from the *in vitro*-cultured plant material. The linear diterpene alcohol **5** was isolated from a VLC fraction of the extract obtained from the [1-^{13}C]-glucose labelling experiment, though there was insufficient material for quantitative ^{13}C NMR analysis to show the location of specific labelling. HRMS supported the molecular formula of $C_{20}H_{34}O$, and the ^{13}C NMR spectrum (Table 2) showed four carbon–carbon double bonds, so the molecule is a linear (*i.e.* not cyclic) diterpene. Analysis of 2D NMR data (Table 2) showed the presence of two almost equivalent $(CH_3)_2C{=}CH{-}CH_2$-isoprene units, with chemical shifts very close to C-12 and C-16 of the anisotomenes (Table 1). NMR correlations also showed that the other two isoprene units were a lavandulol **6** substructure, confirmed by ^{13}C NMR shift similarities.[16] Therefore the proposed structure is **5**, which is a novel compound, analogous to lavandulol **6** from head-to-head coupling of two dimethyl allyl diphosphate (DMAPP) units and to peplusol **7** from two FPP units.[17]



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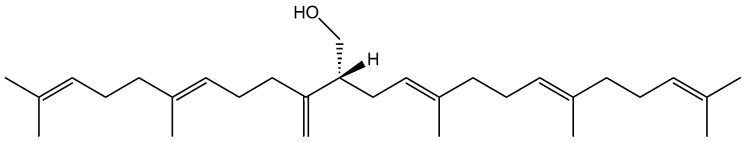
The discovery of the linear diterpene alcohol **5** was initially surprising since there was no evidence of this compound in our

Table 2 NMR data for linear diterpene alcohol **5**[a]

Position	^{1}H	Coupling	^{13}C	DEPT	CIGAR
1	1.91	*d* (1)	26.46[b]	CH_3	C2, C3, C20[b]
2	—	—	131.89[b]	C	—
3	5.43	*m*	125.39[b]	CH	C1, C20[b]
4	2.40	*m*	27.49	CH_2	C2, C3,
5	2.25	*t* (7)	35.73	CH_2	C3, C4, C6, C7, C19
6	—	—	150.79	C	—
7	2.45	*m*	49.86	CH	C5, C6, C8, C9, C19, C18
8	2.34	*m*	30.12[b]	CH_2	C6, C7, C9, C10, C18[b]
9	5.47	*m*	123.82	CH	C11, C17
10	—	—	136.75	C	—
11	2.30	*t* (7)	40.87	CH_2	C9, C10, C13, C17
12	2.45	*m*	30.08[b]	CH_2	C10, C11[b]
13	5.45	*m*	125.52[b]	CH	C15, C16[b]
14	—	—	132.22[b]	C	—
15	1.93	*d* (1)	26.51[b]	CH_3	C13, C14, C16[b]
16	1.80[b]	*br s*	18.40[b]	CH_3	C13, C14, C15[b]
17	1.83	*d* (0.5)	16.86	CH_3	C9, C10, C11
18	3.66	*m*	65.06	CH_2	C6, C7, C8
19 *Z*	5.08	*br m*	111.46	CH_2	C5, C6, C7
19 *E*	5.16	*dd* (1.5, 3)	—	—	C5, C6, C7
20	1.79[b]	*br s*	18.40[b]	CH_3	C1, C2, C3[b]

[a] Data recorded at 500 MHz in C_6D_6 at 25 °C. [b] Interchangeable within column.



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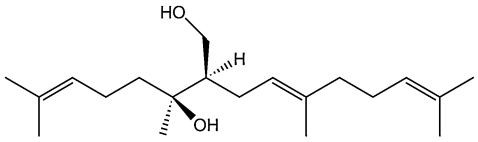
earlier studies on *Anisotome*.[1,2,5] Furthermore, a detailed study on the constituents of *A. lyallii* did not identify this compound.[18] The *A. lyallii* plants grew slowly *in vitro* compared with both greenhouse-grown plants and other plants cultured *in vitro* under similar conditions, and it is likely that this influenced secondary metabolism and the accumulation of **5**.

Compound **5** could be formed by proton loss from the carbocation intermediate formed in the initial coupling step of the proposed anisotomene biosynthesis (a, Fig. 1). Based on this, and the absolute stereochemistry of the anisotomenes,[3] we propose that diterpene **5** has the absolute stereochemistry shown. Alternatively, this first carbocation could be trapped by a nucleophile leading to **8**, with subsequent dehydration to yield **5**. Compound **8**, from *Peucedanum oreoselinum* (Apiaceae),[19] has been synthesized and its absolute stereochemistry determined.[20] The configuration at C7 of **5** and **8** is the same, though the C5 OH of **8** changes the assignment at C7 to *R*. It would be interesting to examine the minor constituents of other members of the Apiaceae that produce irregular diterpenes[5] to see whether these produce compound **5**.



8

Various mechanisms for head-to-head condensations have been proposed for the formation of irregular lower terpenoids similar to the mechanisms established for the biosynthesis of squalene and phytoene.[9] Most of these have been reported from the Asteraceae family and are based on a 1′–2–3 condensation involving the formation of a cyclopropyl-carbinyl precursor, with subsequent rearrangements.[9] However, *R*-lavandulol **6**, which has been reported in the Lamiaceae, Apiaceae and Asteraceae families,[21] appears to be formed *via* a 1′–2 condensation of two molecules of DMAPP.[22] A comparable 1′–2 coupling mechanism, involving two molecules of GPP, is proposed in the biosynthesis of irregular diterpenes in *Anisotome* (Fig. 1). A detailed deuterium labelling experiment could provide further support for this mechanism and subsequent rearrangements.[23] Such rearrangements are likely to be driven by the formation of more stable carbocations, assisted through enzyme active site stabilisation, leading to the bicyclic anisotomane skeleton. Recently the non-head-to-tail chrysanthemyl diphosphate synthase gene has been isolated and the enzyme characterized,[24] so it would seem worthwhile probing *Anisotome* for similar genes and comparing their homology.

Experimental

Spectroscopy and spectrometry

^{1}H NMR (500 MHz) and^{13}C NMR (125 MHz) spectra were recorded on a Bruker DRX 500 and/or or a Varian Inova 500 spectrometer in either: $CDCl_3$ relative to the residual solvent peaks at δ_H 7.24 and δ_C 77.0; or C_6D_6 relative to the residual solvent peaks at δ_H 7.40 and δ_C 128.7. ^{13}C multiplicities were determined using the DEPT pulse sequence. 2D spectra were recorded as COSY, NOESY, HSQC, and HMBC and/or CIGAR experiments. Quantitative ^{13}C NMR measurements were recorded with an inverse gated decoupling pulse sequence (with a 3 second delay) in the presence of 0.1 M $Cr(acac)_3$. For integration of **3** and **4**, the signal-to-noise ratio of the^{13}C-signals was at least 20 : 1. Samples for infrared (IR) spectra were prepared as thin films (oils) on KBr disks and spectra were recorded using a Perkin-Elmer 1600 Fourier transform spectrophotometer. Optical rotations were measured as $CHCl_3$ solutions at listed wavelengths using a Jasco DIP-370 digital polarimeter. High resolution mass spectra were recorded on a Kratos MS80 (electron impact, 70 eV) mass spectrometer.

Reagents

[1-^{13}C]-glucose and [U-$^{13}C_6$]-glucose were purchased from Euriso-top (Saint-Aubin, France). All solvents were distilled before use. Removal of solvents from chromatography fractions was by rotary evaporation at temperatures up to 40 °C. Vacuum

liquid chromatography (VLC) was performed with Merck Kieselgel 60 H silica (15 μm).

Plant material

Plants were raised from *A. lyallii* seed collected in Dunedin, New Zealand. Seeds were surface disinfected in 3.8% NaOCl solution containing 0.5% Tween 80, washed in sterile water and transferred to 250 mL conical flasks containing 100 mL of modified Gamborg B5 medium[13] containing 10 g L^{-1} glucose, solidified with 9 g L^{-1} agar. After germination, seedlings were transferred to a medium containing isotope-enriched glucose. Plants were grown under continuous incandescent light (60 μmol photons m^{-2} s^{-1} photosynthetically active radiation) at 20 °C. For the feeding experiments some of the unlabelled glucose in the medium was substituted with either 20% [1-^{13}C]-glucose or 5% [U-$^{13}C_6$]-glucose. After 10 weeks' growth the foliage was trimmed (dried and stored at −20 °C prior to extraction) and the plantlets transferred to new reagent tubes (35 × 160 mm) with 20 mL of the same fresh medium for a further 6 weeks. Then the entire plants were harvested and combined with the earlier harvested material.

Isolation

Dried plant material (24.7 g) from the [1-^{13}C]-glucose feeding was ground and extracted in a Soxhlet apparatus using $CHCl_3$ (1.5 L) After removal of the solvent, the crude extract (200 mg) was chromatographed by silica gel (15 g) VLC employing an *n*-hexane–EtOAc gradient. The anisotomenes eluted with 95% *n*-hexane–EtOAc: anisotomenol **2** (12 mg, Si gel R_f = 0.47, *n*-hexane–EtOAc, 4 : 1, purple with 1% vanillin–H_2SO_4) and anisotomenoic acid **1** (5 mg, Si gel R_f = 0.44, *n*-hexane–EtOAc, 4 : 1, blue/green with 1% vanillin–H_2SO_4). The anisotomenes were identified by previously published NMR data.[1,5] Anisotomenol **2** (10 mg) was acetylated using acetic anhydride (1 mL) in pyridine (1 mL) overnight. The reaction mixture was washed with dilute H_2SO_4 and extracted with EtOAc. The organic phase was washed with sat. Na_2CO_3 and dried over anhyd. Na_2SO_4 to yield **3** (8 mg) a colourless oil: Si gel TLC R_f = 0.55 (9 : 1 hex–EtOAc, purple-blue with 1% vanillin); $[\alpha]_{589}^{23}$ = −23° ($CHCl_3$, 0.15%); IR(film) ν_{max} cm^{-1} 2924 (C–H), 1741 (C=O), 1233 (C–O); ^{1}H NMR (C_6D_6, 25 °C) δ 1.06 (3H, s, H-19), 1.12 (3H, s, H-17), 1.23 (3H, s, H-20), 1.25 (3H, s, H-18), 1.44 (2H, m, H-7), 1.66 (1H, ddd, J = 5, 12, 6 Hz, H-11), 1.84 (3H, br s, H-16), 1.89 (3H, br s, H-15), 1.96, (3H, s, Ac–Me), 2.00 (1H, m overlapped, H-11), 2.05 (1H, m, H-6), 2.25 (1H, m overlapped, H-6), 2.33 (2H, m overlapped, H-12), 2.37 (1H, m overlapped, H-3), 2.42 (1H, m overlapped, H-2), 2.48 (1H, m overlapped, H-3), 4.28 (1H, dd, J = 8, 11 Hz, H-1), 4.49 (1H, dd, J = 6,11 Hz, H-1), 5.45 (1H, br t, J = 7 Hz, H-13), 5.61 (1H, br d, J = 6 Hz, H-5); ^{13}C NMR see Table 1; HR-EIMS (70 eV) m/z 332.27144 ([M]$^+$, 1.3%, $C_{22}H_{36}O_2$ req. 332.27153) 257 (13), 189 (43), 173 (27), 133 (100), 121 (26), 119 (22), 105 (22), 93 (25), 83 (36), 71 (52), 69 (70), 57 (70), 55 (46).

The chlorophyll-containing fractions were saponified by reaction overnight at room temperature with 6% (*w*/*v*) KOH in methanol (10 mL). Water (10 mL) was added to the solution, which was then extracted with *n*-hexane (3 × 20 mL). The hexane extract was subjected to preparative TLC (20 × 20 mm plate, Merck Kieselgel 60 F_{254}, 1 mm, *n*-hexane–EtOAc, 1 : 1), yielding *trans*-phytol (**4**, 4 mg). A fraction (18 mg) that eluted from the Si-gel VLC column in 95 : 5 hexane–EtOAc, prior to the anisotomenes **1** and **2**, was further purified by HPLC (Waters M-45) using a Si-gel column (8 × 250 mm, Knauer) and refractive index (RI) detection (Bischoff 8110). Repeated manual injections (50 μL) and collection of a peak that eluted at ~6.5 minutes with isocratic 95 : 5 hexane–EtOAc (flow rate of 5 mL min^{-1}) led to the isolation of compound **5** (<1 mg, >95% purity by HPLC, Si gel TLC R_f 0.55, *n*-hexane–EtOAc, 4 : 1, pink with vanillin–H_2SO_4); HR EIMS (70 eV) m/z (rel. int.) 290.26097 ([M]$^+$, 2%, calcd. for $C_{20}H_{34}O$ 290.260965), 259 (3), 221 (5), 203 (4), 109 (19), 69 (100). NMR data – see Table 2.

Acknowledgements

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