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Biosynthesis of anthecotuloide, an irregular sesquiterpene lactone from *Anthemis cotula* L. (Asteraceae) *via* a non-farnesyl diphosphate route

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Retrobiosynthetic analysis of the allergenic sesquiterpene lactone, anthecotuloide, suggested that this natural product could be formed either by head to head condensation of geranyl diphosphate with dimethylallyl diphosphate, or from farnesyl diphosphate (FPP), the accepted regular sesquiterpene precursor *via* the rearrangement of a germacranolide precursor. Isotopic labelling of anthecotuloide has now been achieved by feeding [1-^{13}C]-glucose, [U-$^{13}C_6$]-glucose and [6,6-$^{2}H_2$]-glucose to aseptically grown plantlets of *Anthemis cotula*(family Asteraceae). Analysis of labelling patterns and absolute ^{13}C abundances using quantitative ^{13}C NMR spectroscopy showed that the isoprene building blocks of this sesquiterpene are formed exclusively *via* the MEP terpene biosynthetic pathway. This was supported by results from an experiment using [U-$^{13}C_6$]-glucose. A deuterium labelling experiment using [6,6-$^{2}H_2$]-glucose supported the original proposal and showed that anthecotuloide is formed from a non FPP precursor. Isotope ratio mass spectrometry suggested that there were two pathways for sesquiterpene biosynthesis in *A. cotula*.

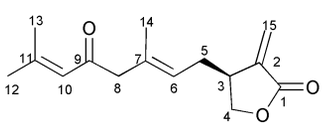
Introduction

The long-held view of sesquiterpene biosynthesis is that all the various structural types are derived from farnesyl diphosphate (FPP), which is derived from the head-to-tail coupling of geranyl diphosphate (GPP) and isopentenyl diphosphate (IPP). GPP itself is formed from the head-to-tail coupling of the isoprene precursors dimethylallyl diphosphate (DMAPP) and IPP. For decades DMAPP and IPP were believed to be exclusively derived from mevalonic acid (MVA). In the late 1980s an alternative to the MVA pathway was identified and later characterised. This new non-mevalonate pathway is operative in the plastids and is known by several names including the methylerythritol phosphate (MEP) pathway, the Rohmer pathway and the deoxyxylulose pathway.[1–3] In addition it has been shown that the formation of IPP and DMAPP occurs in independent rather than in sequential steps.[4] Only recently has the complete reaction sequence of this pathway been established, describing the following steps: the reductive transformation of methylerythritol cyclodiphosphate into 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate;[5] and the subsequent conversion of 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate into IPP and DMAPP.[6]

The discovery of the MEP pathway has contributed new and significant understandings to isoprenoid biosynthesis and metabolism in general. It seems that vertebrates and archaebacteria use only the MVA pathway for the build up of isoprenoids, while various eubacteria have been found to use either the MVA or the MEP pathway. Green algae (excluding unicellular organisms), bryophytes and higher plants use the MVA pathway for the biosynthesis of most sesquiterpenoids and triterpenoids including steroids; and the MEP pathway for the formation of hemiterpenoids, monoterpenoids, diterpenoids and carotenoids.[1–3] The MVA pathway appears to be operative in the cytoplasm compartment of plant cells, whereas the MEP pathway seems to be located in the plastids. Segregation of the two pathways, however, is not necessarily complete, and exchange of metabolites between the two pools has been observed in plants and plant cell cultures.[7–11]

Despite a significant amount of recent research in this area, our knowledge on the contribution of both pathways to the biosynthesis of the different plant terpenoid classes is still limited since there are relatively few pathways that have been fully characterised. Thus the underlying regulatory processes contributing to the formation of many specific plant secondary metabolites are unknown; this scientific restraint has been acknowledged in several literature reviews.[12,13]

The limited understanding of the details of specific isoprenoid biochemical pathways includes the irregular sesquiterpenes where the isoprene units are not clearly resolved. One such compound is the allergenic lactone anthecotuloide **1** found in *Anthemis cotula* L. and other members of the Asteraceae family.[14,15] *A. cotula* is one of approximately 130 species that are found predominantly in the Mediterranean region, and also South-west Asia and South Africa.[16] The genus has been chemosystematically examined for polyacetylenes, flavonoids and sesquiterpene lactones.[17] In addition to the irregular sesquiterpene anthecotuloide **1**, three types of regular sesquiterpene lactones were reported: the germacranolides, eudesmolides and guaianolides.



anthecotuloide 1

The carbon skeleton of anthecotuloide **1** seems incompatible with the conventional mechanism for sesquiterpene biosynthesis as the configuration of the furan ring carbons with the geranyl side-chain is not readily reconciled in terms of a farnesyl diphosphate precursor. The examination of biosynthetic mechanisms for other sesquiterpene lactones[18] suggested

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Table 1 ^{1}H and ^{13}C NMR data[a] and ^{13}C abundances of isotopically-labelled anthecotuloide **1**

Position	^{1}H	^{13}C[b]	^{13}C Enrichment[c]	J_{CC} (coupled C)[d]
1	—	170.69	1.52	8 (3[e])
2	—	137.84	1.55	75 (15)
3	3.13	38.60	1.40	32 (4), 8 (1[e])
4	3.99, dd, *J*=5,9 4.41, dd, *J*=8,9	70.47	**2.06**	32 (3)
5	2.32, ddd, *J*=7.0, 7.5, 8.0 2.42, ddd, *J*=6.5, 7.0, 8.5	32.08	**2.02**	45 (6), 4.5 (8[e])
6	5.20, td, *J*=7, 1	123.80	1.46	45 (5)
7	—	133.63	1.51	42 (14)
8	3.06, s	54.95	1.57	4.5 (5)
9	—	198.37	**2.06**	54 (10), 5 (12[e])
10	6.06, dd, *J*=1, 1.5	122.79	1.46	54 (9)
11	—	156.36	1.57	39 (13)
12	1.87, d, *J*=1	27.67	1.55	nr
13	2.12, d, *J*=1.5	20.69	**1.92**	39 (11)
14	1.63, (s)	16.89	**1.98**	42 (7)
15	5.65, (d) *J*=2.5 6.25, (d), *J*=3	122.23	**1.98**	75 (2)

[a] Sample run in $CDCl_3$ at 25 °C. [b] Confirmed by HSQC and HMBC 2D experiments. [c] **Bold type**: ^{13}C-enriched positions from [1-^{13}C]-glucose experiment, ^{13}C abundances referenced to C-8 from the ^{13}C satellite analysis of H-8. [d] Values in Hz, from [U-$^{13}C_6$]-glucose experiment. [e] $^{2}J_{cc}$ long-range coupling. [f] $^{3}J_{cc}$ long-range coupling; nr = not resolved.

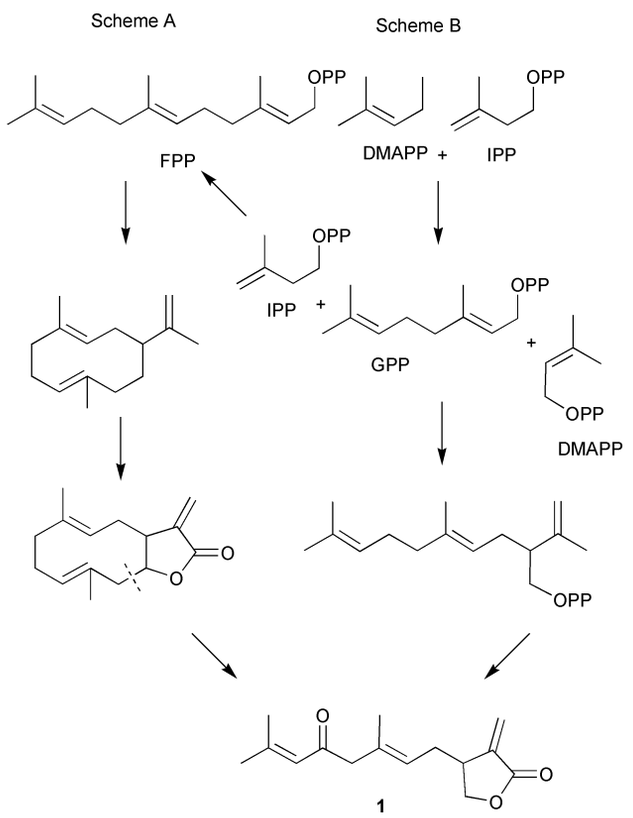


Fig. 1 Biosynthetic routes to anthecotuloide **1**.

that anthecotuloide could be formed from the fragmentation of a germacranolide (Fig. 1, Scheme A). The Asteraceae family is a rich source of such compounds and those with inuloide or eudesmanolide skeletons.[19] It has been speculated that the biosynthesis of other irregular sesquiterpene lactones from *Anthemis pseudocotula* could arise *via* this mechanism.[20] An earlier hypothesis proposed by Bohlmann *et al.* suggested that anthecotuloide **1** is formed from the condensation of geranyl diphosphate with dimethylallyl diphosphate (Fig. 1, Scheme B).[21] Neither of these two proposed pathway options has been proved experimentally. In this paper, we present results of a study on the incorporation of various isotopically-labelled glucose precursors into *A. cotula* terpenoids using *in vitro* plant cultures.

Table 2 ^{13}C[a] abundances of phytol (**2**)[b]

Carbon atom[c]	δ_C	% ^{13}C[d]
13	24.78	**2.48**
14	39.35	1.54
15	27.95	1.72
16	22.60	1.79
17	22.69	**2.25**
9	24.45	**2.35**
10	37.41	1.67[e]
11	32.77	1.71
12	37.28	1.67[e]
18	19.69	**2.31**[f]
5	25.12	**2.16**
6	36.65	1.56
7	32.67	1.71
8	37.35	1.67[e]
19	19.72	**2.31**[f]
1	59.39	**2.32**
2	123.09	1.44
3	140.23	1.52
4	39.85	1.56
20	16.14	**2.30**

[a] Sample run in $CDCl_3$ at 25 °C. [b] Assignments based on that reported by Arigoni *et al.* see Ref. 8. [c] **Bold type**: ^{13}C-enriched positions from [1-^{13}C]-glucose experiment. [d] ^{13}C abundances referenced to C-20 from the ^{13}C satellite analysis of H-20. [e] Averaged values due to signal overlapping. [f] Averaged values due to signal overlapping.

Results and discussion

Isotopically-labelled glucose precursors were fed to *in vitro* cultures of *A. cotula* for 4 to 6 weeks. The sesquiterpene anthecotuloide **1** and the diterpene *trans*-phytol **2** were isolated using standard chromatographic procedures. To interpret biosynthetic ^{13}C data it was mandatory to assign unequivocally all ^{1}H and ^{13}C NMR signals of the target molecules. Assignments of the ^{13}C NMR signals of the purified compounds **1** and **2** were by standard two-dimensional (2D) correlation NMR experiments (HSQC and HMBC) on purified reference compounds that were isolated prior to labelling experiments. Assignments for **2** (Table 2) matched those reported in the literature.[8,10,22] However our NMR assignments for **1** (Table 1) revised the previously reported ^{13}C data,[23] with the assignments for C-12 and C-13 reversed. In addition we were able to resolve the ambiguity for the assignments of C-6 and C-10.

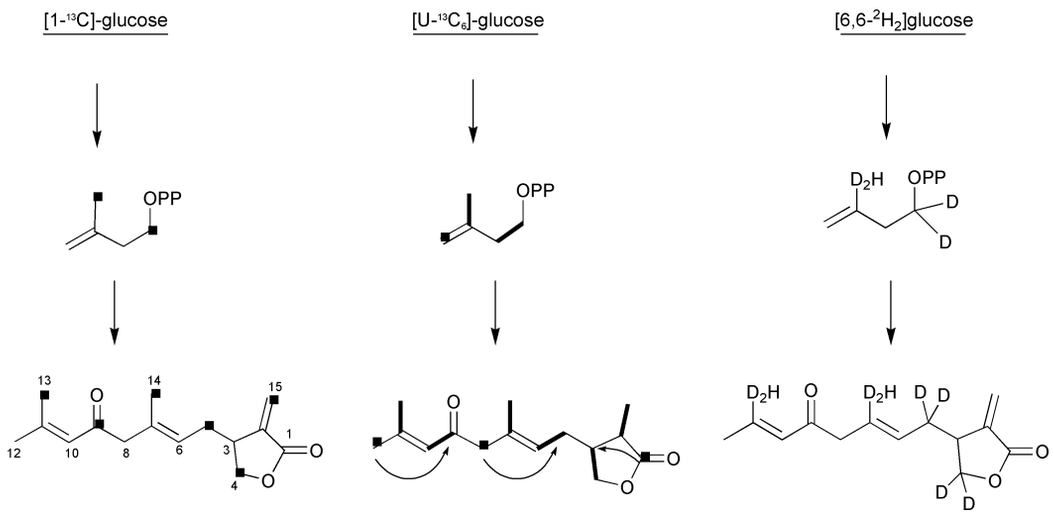


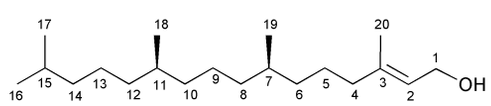
Fig. 2 Labelling patterns from isotope incorporation into anthecotuloide **1**.

Initial labelling of compounds **1** and **2** was achieved by raising sterile plantlets (grown from sterile rooted stem sections) on agar medium containing [1-^{13}C]-glucose. 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.[24] The spectra were run using an inverse-gated decoupling pulse sequence. For anthecotuloide **1** the absolute enrichment has been calculated from the integrals of ^{13}C NMR signals on the basis of ^{13}C satellite analysis of the well-separated signals of H-8 in the ^{1}H NMR spectrum. Based on the respective ^{12}C–^{13}C-proton signal ratios, ^{13}C abundances of the remaining carbon atoms were calculated from the integrals of their ^{13}C NMR signals (Table 1).

MVA and MEP pathways can be distinguished on the basis of different labelling patterns of the isoprene units resulting after incorporation of [1-^{13}C]-glucose into terpenoids.[1–3] In the isolated sample of anthecotuloide **1** there were two distinct groups of signals with different ^{13}C abundances. Six carbon atoms had an average ^{13}C abundance of 2.00 ± 0.05%. This and the location of these specifically-labelled carbons indicated the exclusive MEP origin of the isoprene units (Table 1, Fig. 2). The remaining nine carbon atoms also displayed an increased ^{13}C abundance (1.51 ± 0.06%) compared to the expected natural abundance of 1.11%. This increase in the background labelling can be explained by complex metabolic turnover of [1-^{13}C]-glucose, including recycling *via* photosynthesis of the $^{13}CO_2$ from glucose catabolism. In addition, reversibility of reactions during carbohydrate metabolism has an observable effect on the distribution of labelled atoms in the intermediates.[25] The NMR spectrum of the [1-^{13}C]-glucose-derived anthecotuloide **1** showed that carbon 15 in the "*trans*" position was enriched rather than the expected enrichment of carbon 1 in the "*cis*" position. This may be due to the isomerization that would occur if the oxidation process involved singlet oxygen attacking the exocyclic methylene, though it is also possible that the *cis* methyl group of DMAPP could undergo deprotonation prior to union with GPP rather than the *trans* methyl as originally proposed. If anthecotuloide **1** were indeed formed from FPP *via* such a skeleton, this would result in the labelling pattern observed in the present study, since in the formation of germacrene A,[26] it is the *cis* methyl group of FPP that undergoes deprotonation and therefore the exocyclic methylene of the lactone ring would be labelled.

The observed MEP labelling pattern for anthecotuloide **1** was unexpected since most sesquiterpenes are thought to originate from the classical MVA pathway.[27] However, there have been a few reported exceptions including that recently reported by Steliopoulos *et al.* that showed exclusive MEP origin of germacrene D from *Solidago canadensis*.[28] An earlier report by Adam and Zapp showed the isoprene units of chamomile sesquiterpenes were of mixed (MEP and MVA) biosynthetic origin.[9] Interestingly these studies both involved Asteraceae plants, so there may be a unique set of plastidial enzymes in this plant family. In addition, recent results from labelling studies of an irregular sesquiterpene from a New Zealand liverwort have also found an exclusively MEP-derived sesquiterpene.[29] Studies by Jux *et al.* have shown that terpenoid classification, according to the MEP or the MVA pathways, is a dynamic process that can be influenced by external stimuli.[30] This suggests that further studies are necessary to determine the extent and contribution of these influences to pathway allocation.

Phytol is synthesised in the chloroplasts by the MEP pathway, so as a reference point, it was also isolated from the culture with [1-^{13}C]-glucose. The labelling pattern of the isolated sample of *trans*-phytol **2** from *A. cotula* proved the formation of the isoprene units *via* the MEP pathway with an average abundance of ^{13}C in specifically enriched carbon atoms of 2.3 ± 0.1% (Table 2). Again, an increased ^{13}C background labelling was observed (1.6 ± 0.1%, Table 2).



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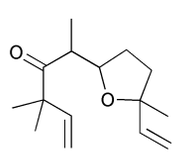

3

There were several sesquiterpene hydrocarbons (M^+ = 204) identified by GC-MS in the crude extracts of *A. cotula*. One of these was isolated from a bulk sample of fresh *A. cotula* and identified by 2D NMR studies to be germacrene D. Unfortunately there was insufficient plant material from the *A. cotula* cultures grown on isotope-labelled glucose to isolate enough for quantitative ^{13}C NMR analysis. This was compounded by the instability of the germacrenes which readily rearrange. However, there are other indirect methods for determining probable pathway allocation, such as the analysis of the isotopic signature of metabolites. Owing to kinetic isotope effects during an enzymatic transformation the ^{13}C–^{12}C-ratio of a product may be different from that of the educts. In isotopomer-sensitive reactions, bonds between the lighter isotopes (*e.g.* ^{12}C) are preferentially cleaved leading to products in which the heavier isotope (*e.g.* ^{13}C) is depleted. The effects

accumulate along a pathway and become especially obvious if alternative pathways with different enzymatic transformations exist. Such differences have been reported for the terpenoid pathways where compounds derived from mevalonate are more ^{13}C-depleted than those from the MEP pathway.[30] This is thought to be in particular due to the pyruvate dehydrogenase preferring light isotopomers.[31] Three molecules of the resulting ^{13}C-depleted acetyl-CoA are then joined to give mevalonic acid, the ultimate precursor of isopentenyl diphosphate. The generation of isopentenyl diphosphate along the MEP-pathway comprises only one mechanistically related step (DXP-synthase).

A sample of *A. cotula* from greenhouse-grown plants was analysed by gas chromatography–combustion–isotope ratio mass spectrometry (GC-C-IRMS). The δ ^{13}C value of $-33.4 \pm 0.3\%$ found for anthecotuloide **1** is significantly less negative than that of $-37.4 \pm 0.4\%$ ($n = 9$) found for germacrene D ($M^+ = 204$). This lends further support to the MEP-origin of anthecotuloide **1**. From the absolute ^{13}C–^{12}C-ratio alone it is not possible to make reliable conclusions since the individual values for the MVA-derived compounds in *A. cotula* at this stage are unknown. Further experiments using inhibitors of the two pathways and measurement of individual terpenoids for their characteristic ^{13}C–^{12}C values would enable unequivocal pathway assignment for the regular sesquiterpene hydrocarbons.

The exclusive MEP origin of anthecotuloide **1** was also confirmed from the results of a [U-$^{13}C_6$]-glucose-labelling experiment. In this experiment [U-$^{13}C_6$]-glucose was administered to the *in vitro* cultures at a ratio of 20 : 1 unlabelled : labelled glucose. After 5 weeks growth the plants were harvested, extracted and pure compounds isolated as above. Incorporation of the labelled atoms into the plant secondary metabolites results in the formation of a complex mixture of isotopomers.[32] ^{1}H-decoupled ^{13}C NMR analysis revealed that all ^{13}C signals of anthecotuloide **1** had satellites indicative of ^{13}C–^{13}C couplings (Table 1). The relative levels of ^{13}C–^{13}C coupled satellite signals of most of the atoms were between 20 and 30%, far above the natural abundance background level of 0.01%. The NMR spectra showed three $^{13}C_3$-labelled isotopomers consistent with an MEP origin, *i.e.* these fragments are derived from the same [U-$^{13}C_3$]-glyceraldehyde 3-phosphate unit. In the linear portion of anthecotuloide, strong $^3J_{cc}$ long range coupling was observed, while in the cyclic portion of the molecule it was the $^2J_{cc}$ that dominated. This coupling pattern is attributed to the differences in structure and electronic environments (see reference 33, for more detailed explanation) since in the linear portion of the compound the isoprenes are DMAPP-like (central double bond), while there is no double bond in the ring portion of the molecule, which is also complicated by the ether linkage. These data (Table 1) showed there was no methyl migration or other disassociation–reassociation process in the formation of the anthecotuloide ring that could have resulted from the rearrangement of a linear terpenoid precursor. However, this information does not discount the possibility of a skeletal rearrangement involving a fragmentation process (Fig. 1, Scheme A). The labelling patterns observed from experiments using either [1-^{13}C]-glucose or [U-$^{13}C_6$]-glucose could not be used to distinguish the two pathways since they would be identical.

A [6,6-$^2H_2$]-glucose feeding experiment was undertaken to try to differentiate between the two pathways (Fig. 1). After culture and isolation, anthecotuloide was analysed by ^{1}H NMR, which showed that the level of incorporation of deuterium into anthecotuloide was quite low since the spectrum was indistinguishable from that of unlabelled anthecotuloide. This was primarily due to the level of endogenous carbohydrates present in the sterile stem sections when they were transferred to the culture. Two other factors contributed to the low incorporation rate: deuterium loss in the reversible reactions of glucose metabolism (*e.g.* the interconversion of pyruvate–phosphoenol pyruvate); and isotope dilution from non-labelled glucose derived from photosynthesis. Therefore a deuterium NMR experiment was undertaken, which clearly showed that carbon positions 4 and 5 both had two deuteriums attached (see Figs 2 and 3). In addition positions 13, 14 (methyls) and 15 (methylene) showed deuterium enrichment, as expected for a labelling experiment with [6,6-$^2H_2$]-glucose. As expected, no deuterium labelling was seen in positions 3, 6, 8 or 10. A deuterium signal was observed at the same chemical shift as that for position 12 and may be explained by some scrambling between the methyl groups in the reaction catalysed by IPP isomerase.

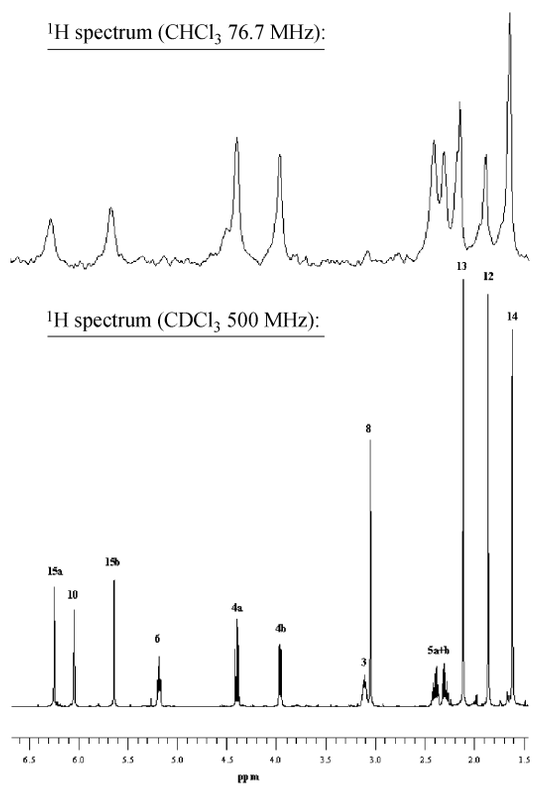

Fig. 3 ^{1}H and ^{2}H NMR spectra of anthecotuloide **1**.

The data obtained from the deuterium labelling experiment provides the key argument in favour of biosynthesis of **1** *via* a non-FPP derived C15 isoprenoid. Specifically, this information strongly discounts the possibility of anthecotuloide **1** being formed from an FPP germacrane-derived skeleton since at least one deuterium would be lost from C-4 (Fig. 1, Scheme A). This lends support to the original scheme proposed by Bohlmann,[21] *i.e* that anthecotuloide **1** is not biosynthesised *via* FPP, but *via* "head-to-head" linkage of GPP to DMAPP (Fig. 1, Scheme B).

There are few reports on biosynthesis of non-farnesyl derived sesquiterpenes. One of the most notable is that of the C15 plant hormone abscisic acid (ABA). It is now thought that ABA is formed from the degradation of a carotenoid precursor and is therefore not surprising that ABA is derived from glyeraldehyde 3-phosphate and pyruvate *via* the MEP pathway.[34] Of particular interest to the present study is artemone **3** from *Artemisia pallens* which has been shown to be derived from the condensation of IPP with dimethylvinyl carbinylpyrophosphate (DMVCPP).[35] Again this example is from an Asteraceae species and it now seems likely that artemone **3** may also be biosynthesised *via* the MEP pathway.

Conclusions

In conclusion, this retrobiosynthetic approach combined with a quantitative study of isotope-labelling patterns has provided

a powerful tool for examining the biogenesis of an irregular sesquiterpene natural product *in vivo*. These results, plus other recent reports seem to suggest that the commonly-held view that plant sesquiterpenes are synthesized exclusively in the cytoplasm needs to be re-evaluated. Also the discovery of a non-FPP derived sesquiterpene in another Asteraceae suggests that FPP is not the universal sesquiterpene precursor in this plant family. Further experiments with other irregular terpene-containing plants are necessary to assess abundance and distribution of these anomalies as they occur in plants and other organisms. Apart from the sterol and carotenoid pathways, little is known about the head-to-head linkage of isoprenoids at the genetic or at the enzyme level.[36] Further research in this area, combined with the rapid advances in the technology of genomics and the discovery of new biochemical and genetic functions will offer further insight into the evolution and regulation of terpenoid metabolism.

Experimental

Spectroscopy and spectrometry

Standard NMR spectra were recorded in $CDCl_3$ (^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz)) relative to $CDCl_3$ at δ_H 7.24; δ_C 77.0. ^{13}C multiplicities were determined using the DEPT pulse sequence. 2D Spectra were recorded as COSY, HSQC and HMBC 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 $Cr(acac)_3$.[24] For integration, the signal-to-noise ratio of the ^{13}C-signals was at least 40 : 1. ^{2}H NMR spectra were recorded at 76.7 MHz in $CHCl_3$ at 35 °C. GC-MS was performed with a Hewlett Packard G1800A GCD system using helium (60 Kpa, 1 ml min^{-1}) as the carrier gas. Samples were injected (250 °C) and analysed on a fused silica HP-5 column (15 m × 0.25 mm ID, 0.25 μm film). The temperature was programmed from 60 °C to 250 °C at a ramp rate of 15 °C min^{-1}. The isotope ratio analysis was performed on a Micromass IsoPrime spectrometer, linked to an Agilent 6890 GC system equipped with a fused silica DB-5 capillary column (30 m × 0.25 mm, film thickness: 0.25 μm). The temperature was programmed from 60 °C (1 min isothermal) to 185 °C at 15 °C min^{-1}, followed by 5 °C min^{-1} to 250 °C (3 min isothermal). The interface was held at 350 °C and the eluting compounds were combusted online at 850 °C with a CuO catalyst. The resulting CO_2 was transferred into the isotope-ratio mass spectrometer for analysis. An aliquot of the injected sample (10%) was split after separation and transferred into a GCT mass spectrometer for on-line identification of the eluting compounds. All isotope ratios are given as δ ^{13}C values: $\delta\ ^{13}C\ [‰] = [(R_{sample}/R_{standard}) - 1] \times 10^3$. R corresponds to the ^{13}C–^{12}C ratio of the sample and the standard (Vienna Pee Dee Belemnite). δ ^{13}C-Values were calculated from several repeated runs ($n = 9$).

Reagents

[1-^{13}C]-glucose, [U-$^{13}C_6$]-glucose and [6,6-$^2H_2$]-glucose were purchased from Euriso-top (Saint-Aubin, France). All other standard laboratory-grade chemicals were obtained from the university chemistry department supply.

Plant material

Plants were raised from *A. cotula* seed and were greenhouse-grown. Stem sections (3–4 cm) containing at least two nodes, were surface disinfected in 1% NaOCl solution containing a small amount of detergent for two minutes, washed repeatedly in sterile water and transferred to large reagent tubes (35 × 160 mm) containing 25 mL of modified Gamborg B5 media.[37] The media contained 10 g l^{-1} glucose, solidified with 9 g l^{-1} agar. For the feeding experiments some of the unlabelled glucose in the medium was substituted with either 20% [1-^{13}C]-glucose, 5% [U-$^{13}C_6$]-glucose or 100% [6,6-$^2H_2$]-glucose. Plants were grown under continuous incandescent light (60 μmol photons m^{-2} s^{-1} photosynthetically active radiation) at 20 °C for a period of 6, 5 and 4 weeks for the [1-^{13}C]-glucose, [U-$^{13}C_6$]-glucose and [6,6-$^2H_2$]-glucose-labelling experiments respectively.

Isolation

The extraction and isolation scheme followed the standard procedures used by our group.[38] Unlabelled reference compounds were first obtained from greenhouse-grown plants. A generalised procedure is outlined below for the isolation of anthecotuloide and phytol from the *in vitro* cultured *A. cotula* plants. Dried plant material (4 g) was ground with CH_2Cl_2 (200 mL) using a Soxhlet apparatus for at least 8 h. After removal of the solvent, the crude extract (280 mg) was chromatographed using silica gel (15 g, Merck TLC-Kieselgel 60H, 15 μm) vacuum liquid chromatography (VLC) employing an *n*-hexane–EtOAc gradient. The VLC fractions containing crude anthecotuloide (**1**, 60 mg, 25–50% EtOAc), also contained chlorophyll fractions and were further purified *via* preparative TLC (20 × 20 mm plate, Merck Kieselgel 60 F_{254}, 1 mm, *n*-hexane–EtOAc, 1 : 1), yielding anthecotuloide (**1**, 40 mg, $R_f = 0.60$) and chlorophyll-containing material (12 mg, $R_f = 0.78$). The chlorophyll-containing band was 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 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 (**2**, 4 mg).

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References

1 W. Eisenreich, M. Schwarz, A. Cartayrade, D. Arigoni, M. H. Zenk and A. Bacher, *Chem. Biol.*, 1998, **5**, R221–R233.
2 H. K. Lichtenthaler, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 1999, **50**, 47–65.
3 M. Rohmer, *Nat. Prod. Rep.*, 1999, **16**, 565–574.
4 C. Rieder, B. Bernhard Jaun and D. Arigoni, *Helv. Chim. Acta*, 2000, **83**, 2504–2513.
5 S. Hecht, W. Eisenreich, P. Adam, S. Amslinger, K. Kris, A. Bacher, D. Arigoni and F. Rohdich, *Proc. Natl. Acad. Sci.*, 2001, **98**, 14837–14842.
6 F. Rohdich, S. Hecht, K. Gärtner, P. Adam, C. Krieger, S. Amslinger, D. Arigoni, A. Bacher and W. Eisenreich, *Proc. Natl. Acad. Sci.*, 2002, **99**, 1158–1163.
7 K. Nabeta, T. Ishikawa, T. Kawae and H. Okuyama, *J. Chem. Soc., Chem. Commun.*, 1995, 681.
8 D. Arigoni, S. Sagner, C. Latzel, W. Eisenreich, A. Bacher and M. H. Zenk, *Proc. Natl. Acad. Sci.*, 1997, **94**, 10600–10605.
9 K. P. Adam and J. Zapp, *Phytochem.*, 1998, **48**, 953–959.
10 K. P. Adam, R. Thiel and J. Zapp, *Arch. Biochem. Biophys.*, 1999, **369**, 127–132.
11 J. Piel, J. Donath, K. Bandemer and W. Boland, *Angew. Chem., Int. Ed.*, 1998, **37**, 2478–2481.
12 D. V. Banthorpe, *Nat. Prod. Rep.*, 1994, 303–328.
13 K. Nakanishi, in *Comprehensive Natural Products Chemistry, Volume 2: Isoprenoids Including Carotenoids and Steroids*, ed. D. E. Cane, Elsevier Science Ltd., Oxford, pp. xxi–xxxviii.
14 B. M. Hausen, E. Busker and R. Carle, *Planta Med.*, 1984, **50**, 229–232.
15 R. N. Baruah, F. Bohlmann and R. M. King, *Planta Med.*, 1985, **51**, 531–532.

16 V. H. Heywood and C. J. Humphries, in *Biology and Chemistry of the Compositae*, eds. V. M. Heywood, J. B. Harborne and B. L. Turner, Academic Press, New York, 1977, pp. 851–898.
17 H. Greger, in *Biology and chemistry of the compositae*, eds. V. M. Heywood, J. B. Harborne and B. L. Turner, Academic Press, New York, 1977, pp. 899–941.
18 N. Fischer, *Recent Ad. Phytochem.*, 1990, **24**, 161–201.
19 C. Yang, Y-P. Shi and Z-J. Jia, *Planta Med.*, 2002, **68**, 626–630.
20 M. Abou El-Ela, J. Jakupovic, F. Bohlman, A. A. Ahmed, A. Seif El-Din, S. Khafgi, N. Sabri and M. El-Ghazouly, *Phytochem.*, 1990, **29**, 2704–2706.
21 F. Bohlmann, C. Zidero and M. Grenz, *Tetrahedron Lett.*, 1969, **28**, 2417–2418.
22 K. P. Adam, R. Thiel, J. Zapp and H. Becker, *Arch. Biochem. Biophys.*, 1998, **354**, 181–187.
23 H. Yamazaki, M. Miyakado and T. J. Mabry, *J. Nat. Prod.*, 1982, **45**, 508.
24 S. Braun, H. O. Kalinowski and S. Berger, 100 and more basic NMR experiments, VCH-Wiley, Weinheim, New York, 1996.
25 B. D. Follstad and G. Stephanopoulos, *Eur. J. Biochem.*, 1998, **252**, 360–371.
26 D. E. Cane, in *Comprehensive Natural Products Chemistry, Volume 2: Isoprenoids Including Carotenoids and Steroids*, ed. D. E. Cane, Elsevier Science Ltd., Oxford, pp. 1–14.
27 D. E. Cane, *Chem. Rev.*, 1990, **90**, 1089–1103.
28 P. Steliopoulos, M. Wust, K. P. Adam and A. Mosandl, *Phytochem.*, 2002, **60**, 13–20.
29 A. J. Barlow, S. D. Lorimer, E. R. Morgan and R. T. Weavers, *Phytochemistry*, 2003, **63**(1), 25–29.
30 A. Jux, G. Gleixner and W. Boland, *Angew. Chem., Int. Ed.*, 2001, **40**, 2091–2093.
31 E. Melzer and H. L. Schmidt, *J. Biol. Chem.*, 1987, **262**, 8159–8164.
32 P. Adam, D. Arigoni, A. Bacher and W. Eisenreich, *J. Med. Chem.*, 2002, **45**, 4786–4793.
33 J. L. Marshall, in *Carbon-carbon and carbon-proton nmr couplings: applications to organic stereochemistry and conformational analysis*, Verlag Chemie International, Deer Beach, Florida. 1983.
34 A. J. Cutler and J. E. Krochko, *Trends Plant Sci.*, 1999, **4**(12), 472–478.
35 A. Akhila, P. K. Sharma and R. S. Thakur, *Tetrahedron Lett.*, 1986, **27**, 5885–5888.
36 S. B. Rivera, B. D. Swedlund, G. J. King, R. N. Bell, C. E. Hussey, D. M. Shattuck-Eidens, W. M. Wrobel, G. D. Peiser and C. D. Poulter, *Proc. Natl. Acad. Sci.*, 2001, **98**, 4373–4378.
37 O. L. Gamborg, R. A. Miller and K. Ojima, *Exp. Cell Res.*, 1968, **50**, 151–158.
38 A. J. Barlow, H. Becker and K. P. Adam, *Phytochem.*, 2001, **57**, 7–14.