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Monoterpenoid Essential Oils are Not of Mevalonoid Origin

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ABSTRACT: Incorporation of [1-^{13}C]- and [U-$^{13}C_6$]glucose shows that monoterpenoid essential oils (geraniol, menthone, pulegone, thymol) are biosynthesised in plants by a pathway which is different from the established mevalonic acid route. © 1997 Elsevier Science Ltd.

Monoterpenes are a major class of secondary compounds of considerable economic value occurring primarily in higher plants but also in some animals and microorganisms. Condensation of isopentenyl pyrophosphate (IPP) with dimethylallyl pyrophosphate (DMAPP) furnishes geranyl pyrophosphate which, by action of monoterpene cyclases, leads to various C_{10}-skeletal types.[1] It is generally assumed that plant-derived monoterpenoids are of mevalonoid origin, however, labelled mevalonic acid is only poorly incorporated into the target terpenoids.[2] This led to considerable doubts as to the relevance of the mevalonoid pathway for the formation of monoterpenoids in higher plants.[2] Recently, Arigoni, Schwarz and their coworkers[3] demonstrated that feeding of ^{13}C-labelled glucose to *Ginkgo biloba* embryos led to a labelling pattern in the diterpene ginkgolide A, which is incompatible with the mevalonoid origin of this terpenoid. Feeding of [U-$^{13}C_6$]- and [1-^{13}C]glucose to a cell culture of *Taxus chinensis* demonstrated that the diterpene taxuyunnanine C is also not of mevalonoid origin.[4] Recent studies on chlorophyll and carotenoids using *Lemna gibba*, *Hordeum vulgare*, and *Daucus carota* showed similar results.[5]

In this contribution we extend our feeding experiments to monoterpenes using [U-$^{13}C_6$]- and [1-^{13}C]glucose. Sixty rooted shoots (approximate length, 5 cm) of young developing peppermints (*Mentha* x *piperita*) were supplied with a solution of 0.5 % [1-^{13}C]glucose. In a second experiment, sixty rooted shoots were supplied with a solution containing 9.5 g of glucose and 0.5 g of [U-$^{13}C_6$]glucose per liter. The plants were allowed to take up the labelled aqueous solution (about 500 ml during the incubation period) under constant illumination with ventilation to enhance transpiration. After an incubation time of 14 days, the plants were steam-distilled, and the volatile fraction was subjected to TLC (toluene:ethyl acetate = 93:7; menthone, R_f 0.68).

^{1}H decoupled ^{13}C NMR spectra of biosynthetic samples and of samples with natural ^{13}C abundance (1.10 % ^{13}C) were measured under identical conditions (125 MHz; 20 °C; 30,000 scans; repetition rate, 3.13 s; 40° pulse angle; 64 k data size zero-filled to 128 k prior to Fourier transformation, 0.7 Hz line broadening prior to Fourier transformation). Relative ^{13}C abundance of individual carbon atoms was then calculated from the

integrals of biosynthetic samples by comparison with the natural abundance sample (% ^{13}C in Table I). The values were referenced to 1.10 % for the carbon with the lowest ^{13}C enrichment. The fraction of multiply labelled isotopomers in the biosynthetic sample from [U-$^{13}C_6$]glucose was calculated from the ^{1}H decoupled ^{13}C NMR spectra as the fraction of $^{13}C^{13}C$ coupled satellites relative to the integral of the entire ^{13}C signal intensity of the respective carbon atom (% $^{13}C^{13}C$ in Table I).[6]

Table I: ^{13}C NMR analysis of menthone from *Mentha* x *piperita* after feeding of [1-^{13}C]glucose or [U-$^{13}C_6$]glucose. Values indicative for IPP formation via the triose phosphate/pyruvate pathway are shown in bold type.

Position[a]	**δ [ppm]**[b]	**[1-^{13}C]glucose**	**[U-$^{13}C_6$]glucose**	
		^{13}C Abundance	Satellite signal	
		% ^{13}C	% $^{13}C^{13}C$	Jcc [Hz][c]
1	35.3	1.16	50.6	35.6(7)
2	50.5	1.19	46.7	37.6(3), **1.6(6)**
3	212.3	**1.55**	50.9	37.4(2), **1.3(6)**
4	55.5	1.10*	47.3	30.7(5)
5	27.4	**1.48**	50.7	30.7(4), **1.9(10)**
6	33.5	1.18	n.d.[d]	**1.6(2), 1.3(3)**
7	21.9	**1.59**	n.d.	n.d.
8	25.4	1.12	44.6	33.6(9)
9	18.3	**1.40**	50.7	35.4(8)
10	20.9	n.d.	n.d.	n.d.

[a]for signal assignments see Ref 7.

[b]referenced to solvent signal at 77.0 ppm.

[c]from one dimensional ^{13}C spectrum of the biosynthetic sample from the experiment with [U-$^{13}C_6$]glucose; coupling partners by analysis of the coupling constants and two-dimensional INADEQUATE experiments are in parentheses.

[d]not determined due to signal overlapping.

*signal with lowest ^{13}C enrichment referenced to 1.10 % ^{13}C.

Label from [1-^{13}C]glucose was diverted to C-3, C-5, C-7, and C-9 of menthone (1.50 + 0.08 % ^{13}C abundance, Table I, Fig. 1). The other carbon atoms of menthone were virtually not ^{13}C-enriched (1.15 + 0.04 % ^{13}C abundance).

When the shoots were supplied with a mixture of [U-$^{13}C_6$]glucose and unlabelled glucose at a ratio 1:20, the ^{13}C NMR spectrum of isolated menthone showed significant satellite signals by $^{13}C^{13}C$ coupling (Table I, Fig. 1). The coupling pattern shown in Fig. 1 was assigned by analysis of the coupling constants in the one-

dimensional ^{13}C NMR spectrum and by two-dimensional INADEQUATE experiments. Adjacent ^{13}C pairs were characterised by coupling constants of 30 - 40 Hz due to one-bond coupling. The satellite signals of menthone C-2, C-3, and C-5 and the center signal of C-6 showed fine splitting of 1-2 Hz due to ^{13}C coupling via two or three bonds (Table I).

A very similar pattern was observed when shoots of *Pelargonium graveolens, Thymus vulgaris*, and *Mentha pulegium* were fed with [1-^{13}C]glucose or with a mixture of [U-$^{13}C_6$]glucose and unlabelled glucose at a ratio of 1:4. Geraniol (R_f 0.27), thymol (R_f 0.55) and pulegone (R_f 0.52), respectively, were isolated from the individual species by steam distillation and TLC (as above). ^{13}C Enrichment and coupling patterns were measured by ^{13}C NMR spectroscopy as described above and are summarised in Fig. 1.

On basis of these data, the labelling patterns of IPP and DMAPP were retrobiosynthetically constructed assuming established mechanisms.[1] The labelling patterns of IPP and DMAPP were virtually identical within the limits of experimental accuracy due to a fast equilibrium between the two precursors by action of IPP-isomerase (Fig. 1).

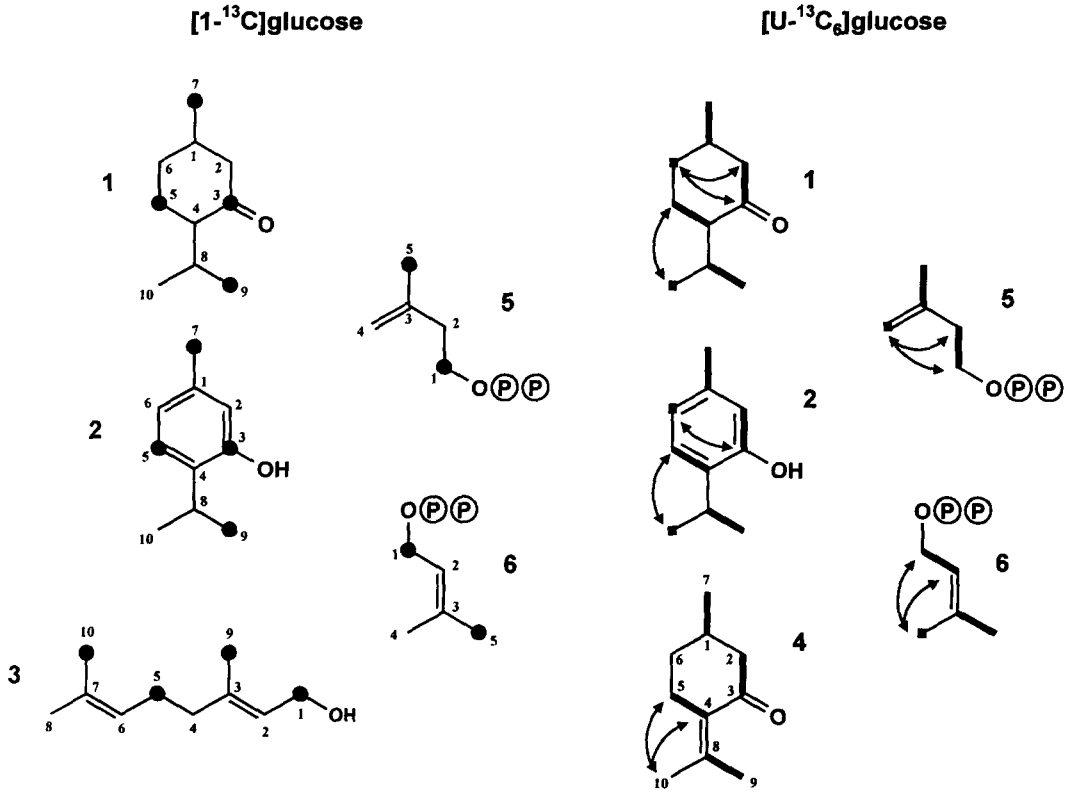

Fig. 1. ^{13}C Labelling patterns of menthone (**1**), thymol (**2**), geraniol (**3**), and pulegone (**4**) after feeding experiments with [1-^{13}C]glucose and [U-$^{13}C_6$]glucose. ^{13}C Enriched carbon atoms from [1-^{13}C]glucose are indicated by •. $^{13}C^{13}C$ Coupling after incorporation of [U-$^{13}C_6$]glucose is indicated by bold lines or arrows. The labelling patterns of IPP (**5**) and DMAPP (**6**) were reconstructed from the monoterpenes using established mechanisms.[1]

The labelling pattern of IPP/DMAPP was interpreted on the basis of the metabolism of glucose in plants.[4] Label from [1-^{13}C]glucose is diverted to C-3 of triose phosphate/pyruvate and C-2 of acetyl-CoA by glycolysis and by action of pyruvate dehydrogenase. IPP synthesis starting from three C-2 labelled acetyl-CoA molecules via the mevalonate pathway should result in labelling of C-2, C-4, and C-5 in IPP. On the other hand, IPP biosynthesis from C-3 labelled pyruvate and glyceraldehyde 3-phosphate via the alternative mechanism[3,4,5] should divert label to C-1 and C-5 of IPP. By comparison with the observed labelling pattern (Fig. 1) it is immediately obvious that the IPP/DMAPP units in menthone are not biosynthesised via the mevalonate pathway but by the alternative triose phosphate/pyruvate pathway.

The same conclusion can be drawn from the labelling pattern observed in the experiment with [U-$^{13}C_6$] glucose. A direct evidence for a [U-$^{13}C_3$]precursor (most probably glyceraldehyde 3-phosphate) separated into an adjacent ^{13}C labelled moiety and a single ^{13}C atom in IPP by a subsequent intramolecular rearrangement during biosynthesis[3,4,5] is the presence of long-range ^{13}C coupling as shown by arrows in Fig. 1.

As a result of these studies it is quite clear that in higher plants monoterpenes as well as diterpenes,[3,4] and the phytol side chain of chlorophyll[5] and carotinoids[5] are not formed in a major pathway via the classical acetate/mevalonate pathway but rather are formed by the alternative triosephosphate/pyruvate pathway[3,5] most likely involving a free or phosphorylated intermediate of 1-deoxyxylulose as shown by Broers and Arigoni[8] in the case of ubiquinone 8 and menaquinone 8, formed in the bacterium *Escherichia coli*.

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