Biosynthesis of the labdane diterpene marrubiin in *Marrubium vulgare* via a non-mevalonate pathway

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The biosynthesis of the furanic labdane diterpene marrubiin has been studied in plantlets and shoot cultures of *Marrubium vulgare* (Lamiaceae). The use of [2-^{14}C]acetate, [2-^{14}C]pyruvate, [2-^{14}C]mevalonic acid and [U-^{14}C]glucose incorporation experiments showed that the labelling of sterols in etiolated shoot cultures of *M. vulgare* was in accordance with their biosynthesis via the acetate–mevalonate pathway. In contrast, the incorporation rates of these precursors into the diterpene marrubiin could not be explained by biosynthesis of this compound via the acetate–mevalonate pathway. Cultivation of etiolated shoot cultures of *M. vulgare* on medium containing [1-^{13}C]glucose and subsequent ^{13}C-NMR spectroscopy of marrubiin led to the conclusion that the biosynthesis of marrubiin follows a non-mevalonate pathway. All isoprenic units of ^{13}C-labelled marrubiin were enriched in those carbons that correspond to positions 1 and 5 of a putative precursor isopentenyl diphosphate. This labelling pattern from [1-^{13}C]glucose is consistent with an alternative pathway via trioses, which has already been shown to occur in Eubacteria and Gymnospermae. The labdane skeleton is a precursor of many other skeletal types of diterpenes. Therefore it becomes obvious that in connection with the few known examples of a non-mevalonate pathway to isoprenoids the formation of some isoprenoids in plants via a non-mevalonate pathway might be quite common.

INTRODUCTION

Isoprenoids are produced by all kinds of organisms. A large variety of skeletal types exists, although all isoprenoids are formed principally on the basis of isoprenic units [1,2]. The pioneering work on the biosynthesis of isoprenoids was done some decades ago, when the main pathway to the biosynthesis of cholesterol was established. Prenyl diphosphates are precursors of isoprenoids with a different number of carbon atoms. Their biogenesis starts with three molecules of acetyl-CoA and leads via mevalonic acid (MVA) to the biogenetically active units isopentenyl diphosphate (IPP) and dimethylallyl diphosphate. This biogenetic sequence is known as the acetate–mevalonate pathway. This pathway was accepted as being common for nearly all isoprenoids [3].

However, a detailed knowledge of isoprenoid biosynthesis in higher plants is restricted to only a few examples, whereas more and more compounds of this class with important biological activities are being found [4]. Therefore we established a system of differentiated and undifferentiated cultures to study the biosynthesis of furanic labdane diterpenes in *Marrubium vulgare* and also in *Leonurus cardiaca* [5,6]. Both plant species are members of the subfamily Lamioidae of the Lamiaceae [7]. The furanic labdane diterpene marrubiin is the bitter principle in extracts from the medicinal plant *M. vulgare*. The substance is formed from the authentic compound premarrubiin during extraction (for structures see Figure 1) [8]. The system has some features that make it suitable for biosynthetic studies: (1) marrubiin is accumulated in large amounts in the aerial parts of the plant as well as in differentiated shoot cultures, (2) the furanic moiety can be easily detected, (3) the compound is not volatile and (4) the labdane skeleton is derived from the direct cyclization of geranylgeranyl diphosphate (GGPP) without any rearrangements.

Since the acetate–mevalonate pathway was established as the principal route of isoprenoid biosynthesis, many experiments with plants have shown conflicting results [1]. There are many reports – and there may be even more unpublished data – stating low incorporation rates of MVA or selective inhibition of the biosynthesis of some isoprenoids by compounds that are known to inhibit the acetate–mevalonate pathway. Such results were often explained by compartmentation models [2,9]. A few years ago incorporation experiments showed an irregular labelling pattern of hopanes of the triterpenoid series in some Eubacteria [10]. This led to the discovery of an alternative pathway of isoprenoid formation, with triose phosphate units as the precursors [11,12]. There was a first report in 1994 on the biosynthesis of ginkgolides in *Ginkgo biloba*, which showed that there might exist a similar alternative pathway in the plant kingdom [13]. *G. biloba* is a gymnosperm and a species that is not quite representative of the plant kingdom. However, an alternative pathway to isoprenoids excluding MVA offers a possibility of explaining many conflicting results in isoprenoid research in plants.

Early work on the biosynthesis of marrubiin was done in the 1960s [14]. From the results of incorporation experiments with different ^{14}C-labelled precursors, e.g. acetate, pyruvate, oxoglutarate and succinate, the authors concluded that marrubiin is an isoprenoid and that this compound is biosynthesized via the acetate–mevalonate pathway.

Preliminary incorporation experiments in our laboratory with shoot cultures of *M. vulgare* were made with [2-^{14}C]MVA to confirm the work reported in the literature. However, results were very puzzling. Incorporation of radioactivity from MVA

Abbreviations used: GGPP, geranylgeranyl diphosphate; IPP, isopentenyl diphosphate; MVA, mevalonic acid.

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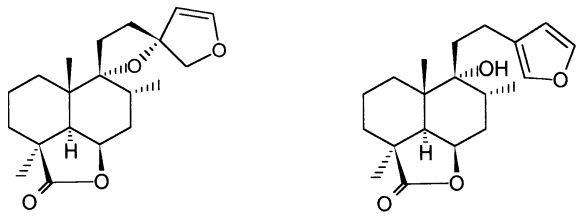


Figure 1 Structures of premarrubiin and marrubiin

into marrubiin was very slight. Here we present the results of new incorporation experiments with plantlets and shoot cultures of *M. vulgare*, showing that there is another interpretation that implies a non-mevalonate biosynthetic pathway.

EXPERIMENTAL

Plant material

Plants of *M. vulgare* L. (Lamiaceae) were grown in the botanical garden of the Institute of Pharmaceutical Biology (University of Bonn, Bonn, Germany). Plantlets of *M. vulgare* were grown from fruits that had been previously kept in the refrigerator for at least 24 h. Plantlets were grown under greenhouse conditions with a natural day–night regime.

Shoot cultures

Shoot cultures of *M. vulgare* were established from spontaneously regenerating callus cultures or from shoot apices of young plants, which were grown under aseptic conditions [5,6]. Shoot cultures were cultivated on a modified Murashige-Skoog medium [15] with half-concentrated salts and without phytohormones. Shoots were maintained on solid medium containing agar. Incorporation experiments were performed with shoot culture material grown in liquid medium. Short-term experiments (up to 24 h incubation with isotopically labelled precursors) were made with 7-day-old shoot cultures; long-term experiments were made by inoculating fresh culture medium with five to ten shoot apices (the upper pairs of leaves and the terminal bud).

Isotopically labelled compounds

[2-^{14}C]Acetate, [2-^{14}C]pyruvate, [2-^{14}C]MVA and U-[^{14}C]glucose were purchased from NEN–DuPont, Brussels, Belgium. [4,8,12,16-^{14}C]GGPP was prepared as described elsewhere [16]. [1-^{13}C]Glucose was purchased from Cambridge Isotope Laboratories.

Extraction and isolation of marrubiin and sterols

Shoot culture material was homogenized with 20 ml of acetone/g of fresh material with an Ultra-Turrax and extracted for 30 min at 4 °C. The solution was filtered, evaporated under vacuum and the more polar part of the residue redissolved in methanol/water (8:2, v/v). The solution was prefractionated by column chromatography on reverse-phase material (Bakerbond octadecyl SPE columns), which was preconditioned with methanol/water (8:2, v/v). Marrubiin was found in the flow-through fraction, while sterols were eluted with methanol and combined with the apolar part of the residue.

Marrubiin was purified by HPLC [reverse-phase C_{18} column, methanol/water (70:30, v/v), UV detection at 215 nm]. The total fraction of sterols was obtained by preparative TLC [silica gel, developed with chloroform/methanol (95:5, v/v); partly sprayed with vanillin/sulphuric acid (1% w/v); sterols at R_F 0.81; marrubiin at R_F 0.87].

Quantitative analysis of marrubiin and sterols

Purified fractions of marrubiin and sterols were separated by TLC. Plates were sprayed with vanillin/sulphuric acid and heated at 110 °C for 5 min. For quantitative analysis of marrubiin the absorption at 527 nm was determined after 24 h and the amount of marrubiin calculated from a standard graph with authentic marrubiin (Extrasynthese, Genay, France) in the range 0.4–2.0 μg per sample. For analysis of sterols the absorption at 527 nm was measured after 2 h and the total amount of non-esterified sterols calculated from a standard graph with β-sitosterol.

The radioactivity of samples of marrubiin or total sterol was measured by liquid-scintillation counting after purification.

Evaluation of ^{13}C-enrichment in marrubiin

The ^{13}C-NMR spectra of marrubiin obtained from an incorporation experiment with [1-^{13}C]glucose and a reference sample from field-grown plants were recorded in $CDCl_3$ under identical conditions with a Bruker DRX 500 spectrometer. Because there is no signal from marrubiin that could be used as internal standard, the evaluation of ^{13}C enrichment was made by comparison of both spectra as reported for ubiquinones [11].

RESULTS AND DISCUSSION

Uptake of [2-^{14}C]MVA and [^{14}C]GGPP by plantlets and shoot cultures of *M. vulgare*

MVA was taken up by differentiated tissues of *M. vulgare*. The stems of *M. vulgare* plantlets approx. 10 weeks old or light-grown shoot culture material were placed in small tubes containing 37.5 kBq of [2-^{14}C]MVA in 1 ml of water. After 24 h the plant material was extracted with acetone and approx. 85% of the applied radioactivity was found in the extract. By processing the different parts of a plantlet separately, i.e. each leaf pair, stem and bud, it was shown that the radioactivity was equally distributed over all parts of the plantlets with respect to the fresh weight. However, marrubiin from neither source was significantly labelled. On the basis of these experiments the incorporation rate was lower than 0.005%. Similar results were obtained when light-grown shoot culture material was shaken for 24 h in liquid medium containing 37.5 kBq of [2-^{14}C]MVA.

Breccia and Badiello [14] also reported a low incorporation, but stated a value of 0.84% incorporation rate after 24 h when field-grown plants were fed with 937.5 kBq of [2-^{14}C]MVA. They described how the maximum incorporation of MVA into marrubiin was within the first 24 h and concluded that marrubiin was rapidly metabolized.

A rapid turnover is very unusual for a typical secondary metabolite and was in contrast with our observations on the accumulation of furanic labdane diterpenes in plantlets and shoot cultures. The accumulation of marrubiin was fully in parallel with growth and fresh weight increase.

Shoot cultures of *M. vulgare* were able to incorporate radioactivity from [^{14}C]GGPP into marrubiin in detectable amounts

Table 1 Labelling of marrubiin (a) and sterols (b) in etiolated shoot cultures of *M. vulgare* from ^{14}C-labelled precursors

Shoot cultures of *M. vulgare* were cultivated for 7 days on modified Murashige-Skoog medium containing glucose instead of sucrose (without light, 25 °C, 80 rev./min, 100 ml Erlenmeyer flasks with 30 ml medium). Marrubiin and the sterol fractions were purified by means of TLC. Subsequently the quantity was estimated and the radioactivity measured in a liquid-scintillation counter. Data represent the means of three measurements.

(a)

Substrate	Substrate specific radioactivity (kBq/μmol)	Radioactivity in substrate (kBq)	Radioactivity in marrubiin (Bq)	Incorporation into marrubiin (%)	Specific radioactivity of marrubiin (kBq/μmol)	Specific incorporation (%)
[2-^{14}C]MVA	2180	187.5	38.8	0.021	0.22	0.0019
[2-^{14}C]Pyruvate	854	187.5	98.3	0.054	0.26	0.0046
[2-^{14}C]Acetate	296	187.5	106.0	0.058	0.79	0.0266
[U-^{14}C]Glucose	0.44	468.8	111.7	0.024	0.51	35.1

(b)

Substrate	Substrate specific radioactivity (kBq/μmol)	Radioactivity in substrate (kBq)	Radioactivity in sterols (Bq)	Incorporation into sterols (%)	Specific radioactivity of sterols (kBq/μmol)	Specific incorporation (%)
[2-^{14}C]MVA	2180	187.5	1518	0.828	21.9	0.134
[2-^{14}C]Pyruvate	854	187.5	167	0.091	1.7	0.020
[2-^{14}C]Acetate	296	187.5	1121	0.611	16.2	0.377
[U-^{14}C]Glucose	0.44	468.8	50	0.011	0.6	27.8

within 24 h. Approx. 5–6 % of the applied radioactivity was found in the acetone extract and 0.15 % of the applied radioactivity was detected in marrubiin isolated and purified from shoot culture material. Besides the general differences with the experiments presented by Breccia and Badiello [14], these results show that the amphipolar molecule GGPP must be transported to the site where the formation of the labdane skeleton is occurring. In contrast, in our system the more common isoprenoid precursor MVA is totally excluded from this pathway.

Incorporation of [2-^{14}C]acetate, [2-^{14}C]pyruvate, [2-^{14}C]MVA and [U-^{14}C]glucose into marrubiin and sterols into etiolated shoot cultures of *M. vulgare*

Diterpenes are assumed to be biosynthesized in plastids [2,9]. Therefore incorporation experiments with [2-^{14}C]acetate, [2-^{14}C]pyruvate, [2-^{14}C]MVA and [U-^{14}C]glucose were performed to obtain information on the early steps of isoprenoid biosynthesis. The incorporation into the plastid-derived diterpenes and into sterols that are biosynthesized in the cytosol was compared.

Etiolated shoot cultures of *M. vulgare* were used as the experimental system because they grow heterotrophically. Their metabolism is based on the carbon sources supplied by the medium. There is no additional dilution of precursors by the products of carbon fixation. Dark-grown shoot cultures of *M. vulgare* produce premarrubiin in the same amounts as in light-grown shoot cultures. They were incubated with radiolabelled precursors for 7 days.

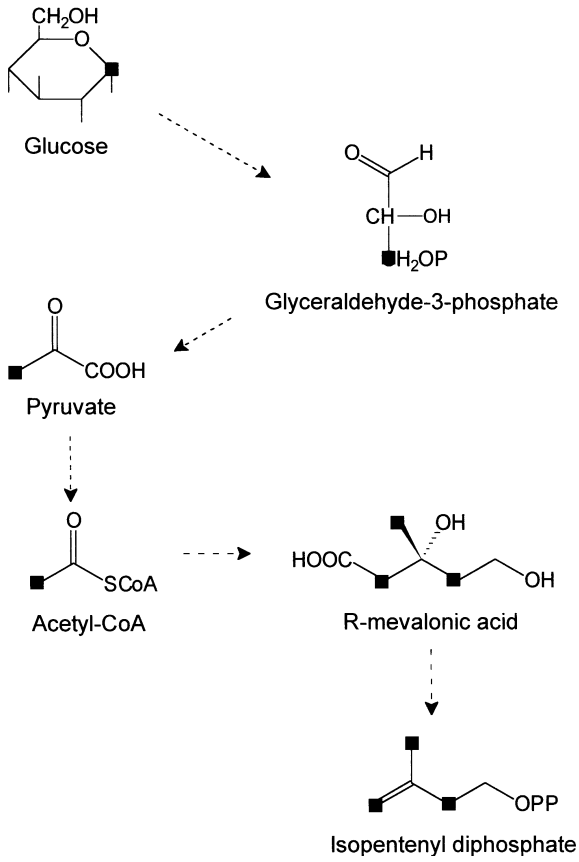
The results showed (Table 1) that acetate and MVA could be regarded as precursors of sterols. Both precursors were incorporated into sterols at reasonable rates of 0.83 % for MVA and 0.61 % for acetate. The incorporation of pyruvate and glucose was much lower. These results confirm the assumption that sterols are biosynthesized via the acetate–mevalonate pathway. The more specific substrates showed a higher incorporation, also resulting in a higher specific activity of the sterols.

The formation of marrubiin via essentially the same pathway should give a similar sequence. Instead, MVA gave the lowest incorporation rate and the lowest specific activity for marrubiin. The incorporation rates of pyruvate and acetate were in the same range and 2–3-fold higher than that of MVA.

These results demonstrate that in *M. vulgare* isoprenic units of sterols and the diterpene marrubiin are biosynthesized by different systems. The specific radioactivity of marrubiin obtained from these experiments was too low to perform degradation experiments, which could show the sites of incorporation of radioactivity. Therefore it is not shown whether these results are due to a strict channelling of biosynthesis and compartmentation of metabolites or whether marrubiin is built by a completely different pathway.

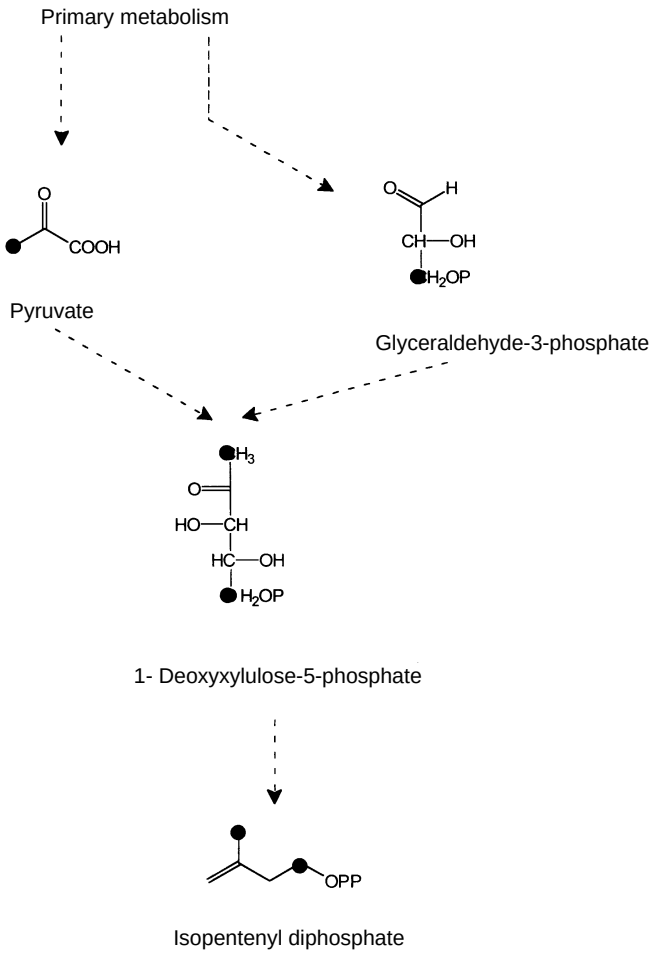
Labelling of isoprenoids from [1-^{13}C]glucose

Labelling experiments with [1-^{13}C]glucose were performed to obtain information on the biogenetic pathway. Glucose is degraded by glycolysis via pyruvate and subsequently acetate arises from oxidative decarboxylation of pyruvate. The ^{13}C label from C-1 in glucose is then found at C-2 of acetate. IPP, which is formed via the acetate–mevalonate pathway, is labelled in positions C-2, C-4 and C-5 (Scheme 1). The alternative pathway as proposed by Rohmer et al. for Eubacteria [11,12] starts with pyruvate and glyceraldehyde 3-phosphate from glycolysis and leads via a hypothetical linear condensation product to IPP labelled at positions C-1 and C-5 (Scheme 2). Because each glucose molecule is degraded into two trioses the label from [1-^{13}C]glucose is diluted in a ratio 1:1. Etiolated shoot cultures of *M. vulgare* were a suitable system for investigating the incorporation of [1-^{13}C]glucose into marrubiin for the following reasons. (1) The shoots grow and produce premarrubiin on a medium in which sucrose is totally supplemented by glucose. Premarrubiin is the main compound of the furanic labdane diterpenes. It is completely converted into marrubiin during extraction and purification. (2) Because the etiolated shoots grow heterotrophically, glucose is the only carbon source and the main precursor of all biogenetic pathways. (3) Premarrubiin is formed constitutively. As far as our results show, there is no rapid turnover of this diterpene. Incubations can be made for a longer period. (4) By using apices to inoculate shoot cultures there is



Scheme 1 Labelling of IPP from [1-^{13}C]glucose via the acetate–mevalonate pathway

^{13}C atoms are marked with black rectangles.



Scheme 2 Labelling of IPP from [1-^{13}C]glucose via a non-MVA pathway, according to Rohmer et al. [12]

^{13}C atoms are marked with black circles.

only a small amount of endogenous storage compounds that can be used for biosynthetic pathways.

Starting with 3 g fresh weight of apices of etiolated shoot cultures we obtained 23 g fresh weight of shoot material after 14 days of cultivation in medium containing glucose and [1-^{13}C]glucose in a 9:1 ratio. The starting material contained approx. 200 μg of marrubiin; after 14 days of incubation we obtained approx. 2 mg of purified [^{13}C]marrubiin. More than 80 % of this marrubiin was biosynthesized during the cultivation. The enrichment of carbon atoms in [^{13}C]marrubiin was investigated by means of ^{13}C-NMR spectroscopy [11]. A reference spectrum was recorded from 15 mg of unlabelled marrubiin, which was purified from leaves of field-grown plants.

Assignment of ^{13}C-NMR data of marrubiin

The assignment of chemical shifts of ^{13}C-NMR spectra to the carbons of marrubiin has already been reported [17]. First interpretations of the enrichment of ^{13}C in marrubiin showed that there were irregularities in the labelling pattern of the four isoprenic units of marrubiin. On the assumption that marrubiin is built from isoprenic units and that these units should show a similar labelling pattern, we concluded that there might be some errors in the assignment and reinvestigated the assignment with actual NMR techniques.

Generally, the isoprenic units of marrubiin according to the ^{13}C-NMR data from the literature were enriched in ^{13}C in positions 1 and 5 of IPP. The only exceptions were C-1 and C-11 of marrubiin. C-11 of marrubiin (position 1 of IPP) was not labelled, whereas C-1 (position 4 of IPP) was enriched in ^{13}C.

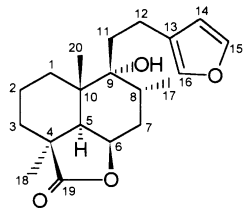
By means of distortionless enhancement by polarization transfer (DEPT), ^{1}H,^{1}H-COSY, heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) spectra it was shown that the assignment of C-1, C-3 and C-11 in the literature was wrong. C-1 must be assigned the signal at 28.7 p.p.m., C-3 the signal at 28.4 p.p.m. and C-11 the signal at 35.2 p.p.m. (Table 2). The new assignment is in accordance with data recently reported for furanic labdane diterpenes from *Leonurus persicus* [18].

MVA is not a precursor of the diterpene marrubiin

The carbons of marrubiin that were enriched in ^{13}C were calculated by dividing the isotopic relative intensities of each atom from the spectrum of [^{13}C]marrubiin by the intensities measured in the reference spectrum of unlabelled marrubiin. The proportions of relative intensities within a spectrum without enrichment are nearly constant. Therefore the quotient of ^{13}C-enriched atoms is higher. The calculation of ratios (Table 2) showed that eight carbon atoms were significantly labelled. The

Table 2 Analysis of ^{13}C enrichment in marrubiin

Chemical shifts of signals from carbons in ^{13}C-NMR spectra of marrubiin according to the literature [17] and the new assignment. Relative intensities of ^{13}C-NMR spectra from 2 mg of marrubiin were obtained from a labelling experiment with etiolated shoot cultures of *M. vulgare* cultivated on medium with [1-^{13}C]glucose and from a reference spectrum from 15 mg of marrubiin isolated from field-grown plants. Labelled positions (see diagram below) in isoprenic units expected from the acetate–mevalonate pathway (AMP) are compared with the experimental results.



C atom	Assignment (p.p.m.)		Relative intensity		Ratio B/A	Labelled positions	IPP atom no.	Expected labelling pattern	
	Literature	This study	Marrubiin (A)	[^{13}C]Marrubiin (B)				AMP	Alternative pathway
1	35.2	28.7	5.56	1.51	0.27		4	×	
2	18.2	18.2	5.75	3.18	0.55	×	1		×
3	28.7	28.4	5.34	1.37	0.26		2	×	
4	43.8	43.8	3.42	0.86	0.25		3		
5	44.9	44.9	4.33	1.45	0.33		2	×	
6	76.1	76.1	5.72	3.14	0.55	×	1		×
7	31.5	31.5	4.63	1.66	0.36		4	×	
8	32.4	32.4	4.30	1.45	0.34		3		
9	75.8	75.8	2.75	0.69	0.25		2	×	
10	39.8	39.8	2.71	0.85	0.31		3		
11	28.4	35.2	4.45	3.17	0.71	×	1		×
12	21.0	21.0	4.62	1.55	0.34		4	×	
13	125.0	125.0	1.82	0.55	0.30		3		
14	110.7	110.7	4.66	1.21	0.26		2	×	
15	143.1	143.1	4.34	3.29	0.76	×	1		×
16	138.6	138.6	4.95	3.78	0.76	×	5	×	×
17	16.6	16.6	5.01	4.31	0.86	×	5	×	×
18	23.0	23.0	5.40	1.54	0.29		4	×	
19	183.8	183.8	1.61	1.28	0.80	×	5	×	×
20	22.3	22.3	5.04	4.24	0.84	×	5	×	×

ratio (0.55:0.86) was 2–3-fold that of unlabelled carbons (0.25:0.34). The value of unlabelled carbons corresponded to the natural isotopic abundance of ^{13}C (1.1 %). Therefore the isotopic abundance of ^{13}C-enriched carbons was approx. 2–3 %.

The analysis of the positions of ^{13}C-labelled atoms showed that marrubiin was not biosynthesized via the acetate–mevalonate pathway. The isoprenic units of marrubiin were not labelled in positions C-2, C-4 and C-5 of a putative IPP, but this unit was labelled in positions C-1 and C-5 (Table 2 and Scheme 3). These results indicate that marrubiin might be biosynthesized by a similar pathway to that proposed for some isoprenoids in bacteria by Rohmer et al. [12].

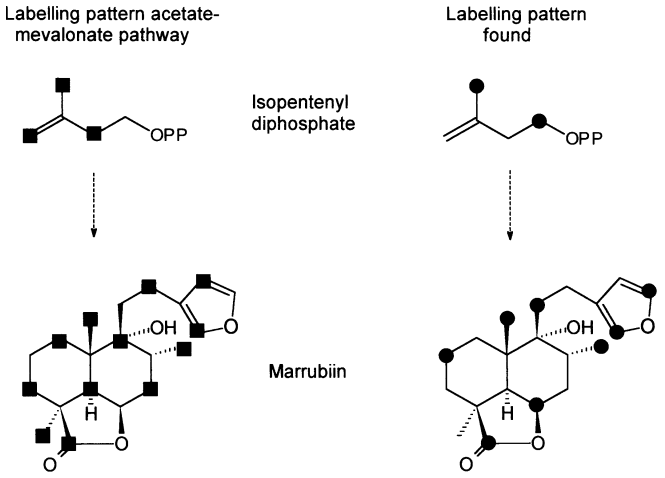
The enrichment of ^{13}C in C-2 and C-6 of marrubiin was significant, but lower than that of the other carbons. This could either be because these two carbons are those with the highest relative intensity in the reference spectrum, or it could indicate that there might be additional differences in biosynthetic pathways that are yet to be investigated.

Biosynthesis of isoprenoids in higher plants

Marrubiin is an example of a diterpene of secondary metabolism from an angiosperm that is not built via the acetate–mevalonate pathway. The labdane skeleton is a precursor for many other diterpenes; consequently all diterpenes derived from the labdane skeleton might be also biosynthesized by an alternative pathway. By using the same experimental system for labelling experiments with ^{14}C precursors and for incorporation experiments with [1-^{13}C]glucose we showed that low incorporation of MVA might be explained not only by compartmentation but also by the existence of different pathways to produce isoprenic units. Many studies on isoprenoid biosynthesis in higher plants will have to be reinvestigated because many conclusions were made on the assumption that MVA was a precursor of the investigated pathway.

Until now there have been only a few published examples from the plant kingdom showing that isoprenic units are not formed via MVA: diterpenes from secondary metabolism are ginkgolides from *G. biloba* (Ginkgoaceae, Gymnospermae) [13], taxan derivatives from *Taxus chinensis* (Taxaceae, Gymnospermae) [19] and now marrubiin from *M. vulgare* (Lamiaceae, Angiospermae). This was also demonstrated for phytol, carotenoids and the prenyl moiety of plastoquinone in the green alga *Scenedesmus obliquus* [20] and similar results were obtained with angiosperms [21]. Although the number of examples is limited, they cover a wide range of the plant kingdom. Thus the possibility of forming isoprenic units by a non-MVA pathway seems to be common.

The known examples all refer to compounds that are considered to be of plastidic origin. This could be the reason that poor incorporation of MVA into certain classes of isoprenoids could often be partly explained by compartmentation. It will be interesting to know whether the biosynthetic pathway in plastids



Scheme 3 ^{13}C labelling of IPP and marrubiin from [1-^{13}C]glucose

Marrubiin was isolated from etiolated shoot cultures of *M. vulgare* grown on medium containing [1-^{13}C]glucose for 15 days. The labelling pattern of marrubiin and the deduced isoprenic unit IPP as expected from biosynthesis via the acetate–mevalonate pathway (black rectangles) is compared with the experimental results (black circles), which can be explained by the alternative pathway proposed by Rohmer et al. [12].

is completely identical with the pathway that was found in Eubacteria.

Regulation of isoprenoid biosynthesis

The non-MVA pathway to isoprenoids in higher plants is seemingly localized in plastids. It is not light-dependent because marrubiin was biosynthesized in shoot cultures in the dark. As well as detailed investigation of intermediates of the alternative pathway and a comparison of plants and eubacteria, the existence of parallel pathways to isoprenoids will lead to new concepts of regulation of isoprenoid metabolism. All systems that have been studied so far were grown under heterotrophic conditions, even if building phytol and carotenoids. The rapid incorporation of carbon dioxide into isoprenoids of plastidic origin [22] might reflect the fact that the alternative pathway is the main route of isoprenoid biosynthesis in plastids also under light conditions. But this still has to be proven.

There might be also a limited interchange between both pathways to isoprenoids, because incorporation experiments with larger amounts of MVA show little incorporation into diterpenes. Another reason could be the degradation of MVA, the so-called MVA shunt [1], and the incorporation of the degradation products. With regard to the cultivation time it has to be considered that there might be an overall turnover from precursors in different metabolic processes. Therefore the shoot cultures of *M. vulgare* are an advantageous system compared with the other systems that produce diterpenes of secondary metabolism. Whereas embryos of *G. biloba* were cultivated for 30 days [13] and cell-suspension cultures of *T. chinensis* for as much as 45 days [19], the results with shoot cultures from *M. vulgare* were obtained within 14 days, reducing the possibility of secondary additional incorporation. By increasing the amount of labelled precursor this time could even be shortened so that effects of metabolic regulators could be investigated.

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