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Dynamic pathway allocation in early terpenoid biosynthesis of stress-induced lima bean leaves

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Dedicated to Professor Rod Croteau on the occasion of his 60th birthday

Abstract

Two independent pathways contribute in higher plants to the formation of isopenteny1 diphosphate (IDP), the central building block of isoprenoids. In general, the cytosolic mevalonate pathway (MVA) provides the precursors for sesquiterpenes and sterols, whereas the plastidial methylerythritol pathway (MEP) furnishes the monoterpene-, diterpene- and carotenoids. Administration of deuterium labeled 1-deoxy-D-xylulose and mevalolactone to lima beans (*Phaseolus lunatus*), followed by gas chromatographic separation and mass spectrometric analysis of de novo produced volatiles revealed that the strict separation of both pathways does not exist. This could be confirmed by blocking the pathways individually with cerivastatin® (MVA) and fosmidomycin (MEP), respectively. Isotopic ratio mass spectrometry (IRMS) at natural abundance levels demonstrated independently and without the need for labeled precursors a dynamic allocation of the MVA- or the MEP-pathway in the biosynthesis of the nerolidol-derived homoterpene 4,8-dimethy1-nona-1,3,7-triene (DMNT). Insect-feeding upregulated predominantly the MVA-pathway, while the fungal elicitor alamethicin stimulated the biosynthesis of DMNT via the MEP-pathway.

Keywords: *Phaseolus lunatus*; Léguminosae; Lima bean; Isoprenoid biosynthesis; Cross talk; 1-Deoxy-D-xylulose; Mevalonate; Terpenoids; Volatiles; Isotopically labeled; Inhibitors; IRMS; Isotopic discrimination

1. Introduction

Terpenes are frequently used by the producing organism in interactions with its environment, as protection against enemies, parasites and pathogens as well as attractants for pollinators and seed-dispersing animals (Harborne, 1995). With more than 25,000 known compounds terpenes and terpenoids form one of the largest and perhaps most structurally diverse groups of secondary metabolites (Connolly and Hill, 1992).

Starting with isopentenyl diphosphate (IDP) which is transformed by IDP-isomerase to dimethylallyl diphoshate (DMADP), IDP and DMADP are condensed by prenyltransferases to geranyl diphosphate (GDP). A consecutive condensation of GDP with IDP yields farnesyl diphosphate (FDP) and a third generates geranylgeranyl diphosphate (GGDP). GDP is the precursor of all monoterpenes (C10), FDP furnishes the sesquiterpenes (C15) and GGDP the diterpenes (C20).

The synthesis of the terpenoid natural products is continued by terpene synthases and terpene cyclases. All of these later enzymatic reactions proceed through allylcation intermediates followed by isomerizations, cyclizations, and hydride- or methyl shifts. The reactivity of the cations along with their tendency to rearrange is one of driving forces generating the enormous structural diversity of terpenoids. Besides the loss of a proton yielding olefinic products, the

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cation-intermediate can react with nucleophiles leading to functionalized terpenoids. Finally, the skeleton can be substituted, oxidized, reduced or conjugated (e.g. with sugars) by a number of different tailoring enzymes.

1.1. The origin of IDP (The two pathways)

1.1.1. The MVA pathway

The MVA-pathway starts with a Claisen-condensation of two acetate units followed by an aldol-addition of a third, catalyzed by thiolase and the HMG-CoA synthase, to give 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) (see Scheme 1).

After reduction of the HMG-CoA by the membrane bound HMG-CoA reductase, the resulting mevalonic acid is transformed to IDP by two sequential phosphorylations and a co-elimination of the tertiary phosphate and the carboxyl group (Spurgeon and Porter, 1981). The HMG-CoA reductase plays a key role in the regulation of the isoprenoid biosynthesis in plants and animals (Bach et al., 1999).

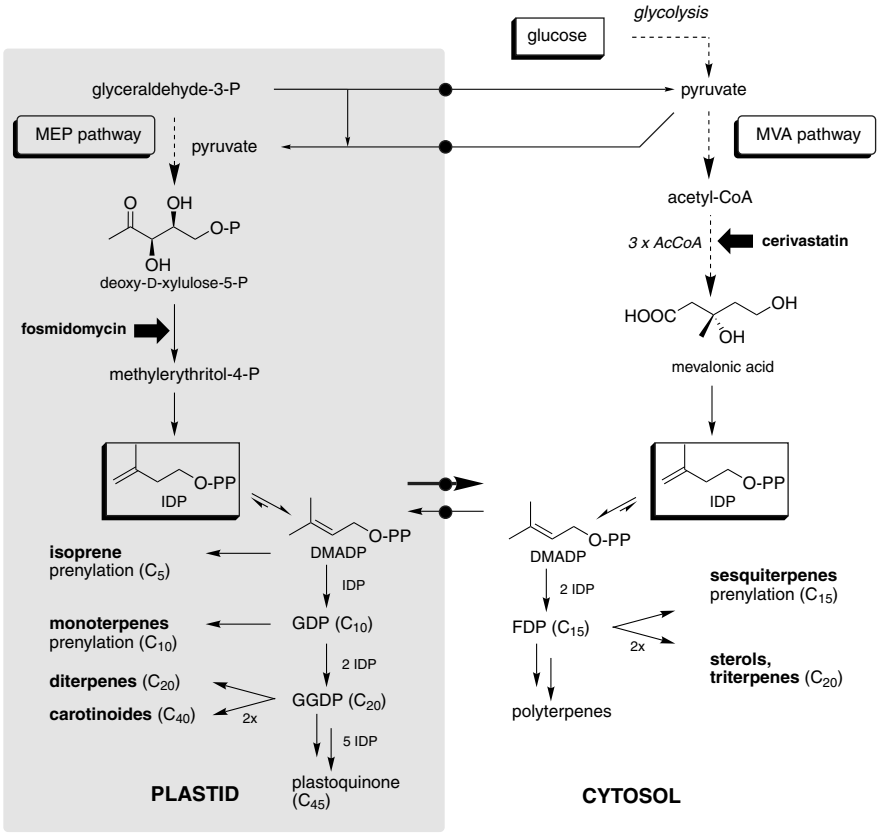
1.1.2. The MEP pathway

Administration experiments with ^{13}C-acetate and bacteria (Flesch and Rohmer, 1988) or ^{13}C-glucose and gingko leaves (*Gingko biloba*) (Schwarz, 1994) exhibited in the isolated terpenoids labeling patterns that were not consistent with an MVA-origin. This led to the discovery of a mevalonate-independent pathway starting by condensation of pyruvate and glyceraldehydephosphate to 1-deoxy-D-xylulose-5-phosphate (DOX) (Rohmer et al., 1993, 1996). The latter is rearranged and reduced to the key intermediate 2-*C*-methyl-D-erythritol 4-phosphate (MEP) (Takahashi et al., 1998). Transformation of MEP to IDP proceeds via reductive elimination of a cyclic diphosphate intermediate (Rohdich et al., 2000) for reviews, see e.g. Eisenreich et al. (2004), Rodriguez-Concepcion and Boronat (2002), Lichtenthaler (1999).

1.2. Crosstalk between the MEP- and MVA-pathway

In higher plants, the biosynthesis of the different terpenoid classes is located in different subcellular compartments. Whereas mono- and diterpenes are synthesized in the plastids, the production of sesquiterpenes and sterols is located in the cytosol (McGarvey and Croteau, 1995). Accordingly, the enzymes of the MVA- or MEP-pathway are also located in different compartments. Enzymes of the MVA-pathway have been isolated from the cytosol, whereas genes coding for enzymes of the MEP-pathway possess typical plastid-targeting sequences (Lange et al., 1998; Lange and Croteau, 1999).

This differential allocation of the early pathways to certain terpenoid classes was also observed for stress-induced isoprenoids after wounding or treatment of plants with elicitors. For example, in leaves of the lima bean, administration of deuterium labeled mevalonic acid resulted in a



Scheme 1.

high incorporation of the isotopomer in the nerolidol-derived homoterpene 4,8-dimethylnona-1,3,7-triene (DMNT) (**1**), but not in the monoterpenes of the emitted volatile blend. On the other hand, monoterpenes and sesquiterpenes, especially DMNT, also showed a high degree of labeling after administration of deuterium labeled deoxy-D-xylulose (DOX). By simultaneous blocking of the MVA-pathway with cerivastatin®, the administration of deuterated DOX resulted in an even higher degree of labeling of DMNT. This observation suggests that the plant may be able to use both pathways in a dynamic fashion to overcome limiting conditions (Piel et al., 1998). Other exceptions from the classical assignment of the different terpenoid classes to the MVA- and MEP-pathway have been reported for chamomile (Adam and Zapp, 1998), *Catharanthus roseus* (Arigoni et al., 1997) and volatiles emitted from snapdragon flowers (*Antirrhinum majus*) (Dudareva et al., 2005) and other plants (Piel et al., 1998). The use of ^{13}C-labeled glucose in combination with ^{13}C NMR spectroscopy has provided in recent years a very detailed view into the metabolic networks funneled by glucose-derived building blocks (Eisenreich et al., 1996). This technique also allowed to quantify the contribution of the MVA- and MEP-pathway to the biosynthesis of individual terpenoids (Eisenreich et al., 2004).

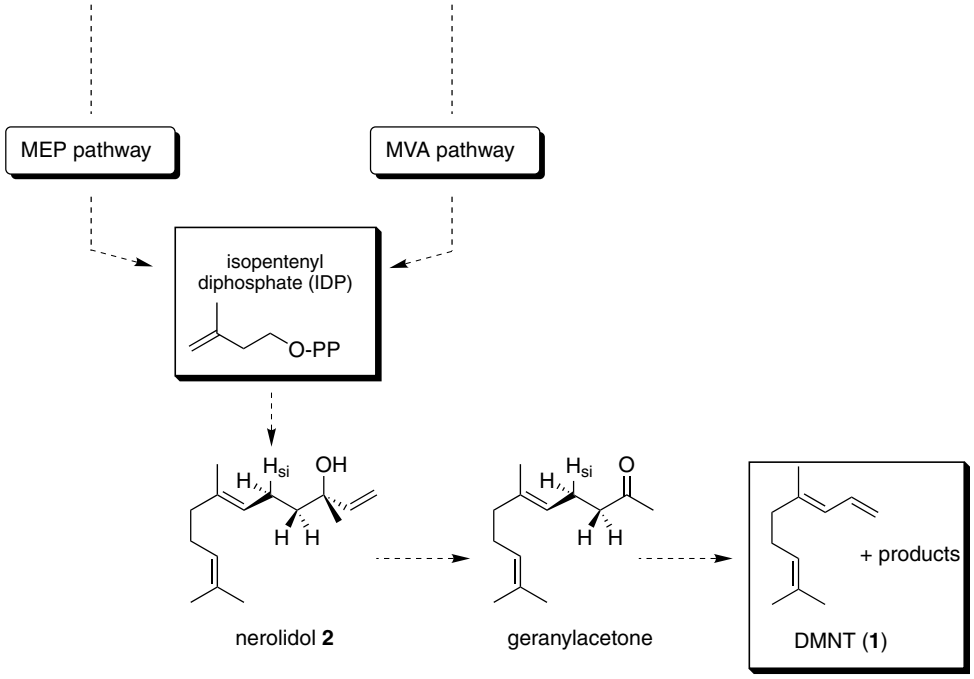
Studies on the cross-talk between the pathways showed in *Arabidopsis thaliana* no altered expression of the relevant genes suggesting that posttranscriptional factors control the early terpenoid biosynthesis (Laule et al., 2003). Moreover, isolated chloroplasts and proteoliposomes from solubilized proteins of the plastidial envelope membranes were recently shown to be capable of an unidirectional export of IDP and GDP. The process was found to be controlled by Ca^{2+} and could be stimulated by pH- and membrane potential gradients. Farnesyl diphosphate and DMAP were transported at lower rates, MVA and GGDP were not actively transported (Bick and Lange, 2003).

The biosynthesis of DMNT in lima bean leaves provides unique advantages as a model, to study a dynamic pathway allocation in response to stress factors and elicitors. The plant does not store terpenoids, but synthesizes DMNT and other volatile terpenoids de novo after elicitation. Moreover, as outlined in Scheme 2, DMNT (**1**) results from the sesquiterpene alcohol, nerolidol **2**, by oxidative degradation. Owing to the identity of the late steps, any differences in the incorporation of isotopes from labeled precursors or changes in the natural isotopic composition of DMNT can be exclusively attributed to the early steps (MVA and/or MEP) of the nerolidol biosynthesis (Boland et al., 1998). This is a major difference to complex sesquiterpenoid blends from other plants in which the formation of different skeletons may cover minor effects (on the natural isotopic composition, vide infra) owing to thermodynamic and kinetic effects of the late transformations. Here we report on the dynamic pathway allocation in the lima bean (*Phaseolus lunatus*) during early terpenoid biosynthesis focusing on the biosynthesis of DMNT as a model system.

2. Results and discussion

2.1. Labeling studies

After insect wounding or induction by jasmonic acid (JA), lima bean leaves start to emit a blend of volatiles.



Scheme 2.

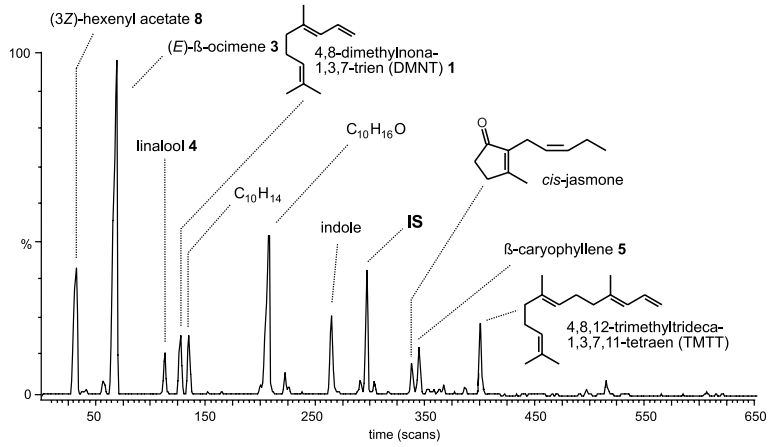
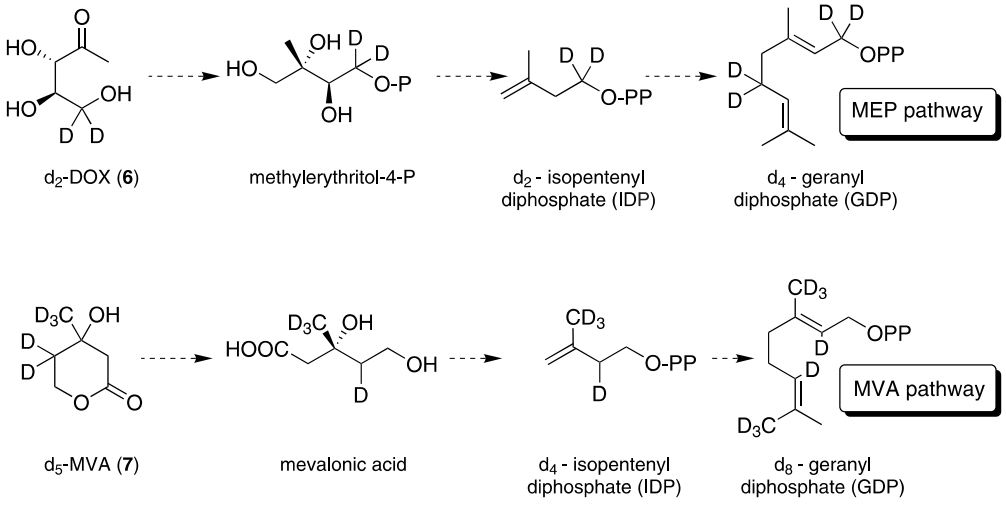


Fig. 1. Spider mite (*Tetranychus urticae*) induced volatiles from the lima bean (*P. lunatus*). IS: internal standard (*n*-bromodecane).



Scheme 3.

Major compounds are terpenoids such as (*E*)-β-ocimene (**3**), linalool **4**, β-caryophyllene (**5**), and DMNT (**1**), together with compounds from fatty acid metabolism and the shikimate pathway (see Fig. 1). To investigate the pathway allocation in the induced terpenoids, labeled precursors for the MVA- or MEP-pathway, namely d_2-DOX (**6**) and ds-MVA (**7**) were administered to freshly detached lima bean leaves and the incorporation of the deuterium atoms into the terpenoids was determined by gas chromatography mass spectroscopy (GC–MS). However, since metabolic studies with externally added precursors alter their local concentration and may redirect mass fluxes of metabolites, this approach could result in an unnatural preference of one pathway over the other. To minimize this impact, the incorporation of the different precursors into the lima bean terpenoids was followed in a concentration dependent manner.

Thus, primary leaves of the lima bean (12–14 days-old) were incubated with different concentrations of [5,5-$^2H_2$]-1-deoxy-D-xylulose (d_2-DOX) (**6**) or racemic [4,4,6,6,6-$^2H_5$]-mevalolactone (d_5-MVA) (**7**), and treated with JA to induce the terpenoid biosynthesis. Then, the emitted volatiles were collected and analyzed by GC-MS.

2.1.1. Monoterpenes

Depending on the labeled precursor, the molecular masses of the terpenes could shift by two or four mass units per isoprene unit after administration of d_2-DOX (**6**) or d_5-MVA (**7**), respectively (see Scheme 3).

In the case of the monoterpenes (*E*)-β-ocimene (**3**) and linalool **4**, the molecular masses shifted by 2 or 4 units depending on incorporation of one or two molecules of d_2-DOX (**6**). The same terpenoids displayed a shift of 4 or 8 mass units after administration of d_5-MVA (**7**). The number of deuterium isotopes per molecule was determined by integration of the corresponding ion traces and the degree of labeling was calculated as follows:

$$M_{\mathrm{DOX}}(\%) = \frac{\frac{\text{peak area}(M^+ + 2)}{2} + \text{peak area}(M^+ + 4)}{\text{peak area}(M^+) + \text{peak area}(M^+ + 2) + \text{peak area}(M^+ + 4)},$$
$$M_{\mathrm{MVA}}(\%) = \frac{\frac{\text{peak area}(M^+ + 4)}{4} + \text{peak area}(M^+ + 8)}{\text{peak area}(M^+) + \text{peak area}(M^+ + 4) + \text{peak area}(M^+ + 8)} \quad (1)$$

where M_{DOX}, M_{MVA} are the degree of labeling derived from d_2-DOX (**6**) and d_5-MVA (**7**) respectively; M^+ the molecular mass of the unlabeled terpene. In case of (*E*)-β-ocimene (**3**) and linalool **4**, the incorporation of d_2-DOX (**6**) led to a maximum degree of labeling between 80% and 90%, which is in good agreement with previous data (Piel et al., 1998). As shown in Fig. 2 the degree of labeling initially increased almost linearly with the concentration of the externally added d_2-DOX (**6**) reaching a maximum of ca. 90% labeling at a d_2-DOX-level of 2 mg ml^{-1}. Further increase had no effect on the degree of labeling. In contrast, significant incorporation of d_5-MVA (**7**) was observed only at concentrations equal or above 3 mg ml^{-1} with a maximum degree of labeling not exceeding 15–20%.

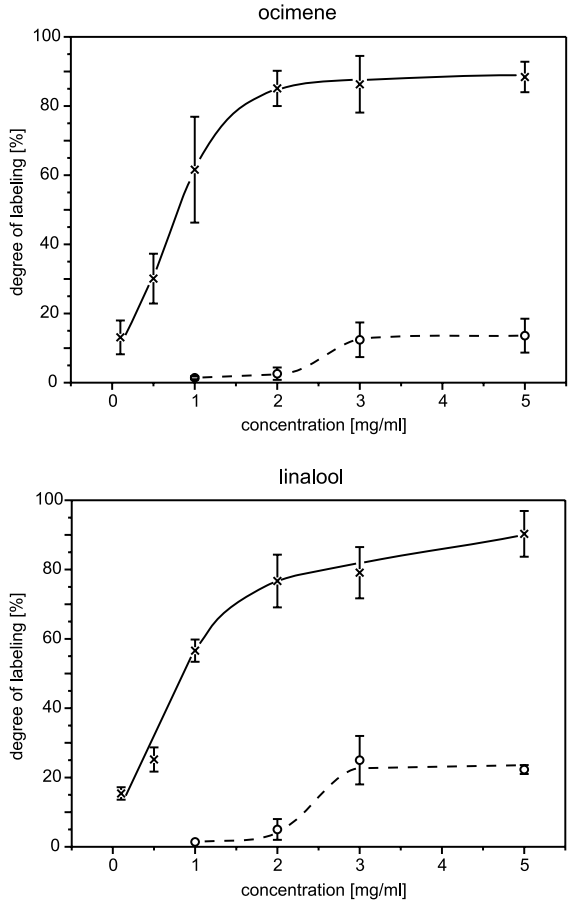
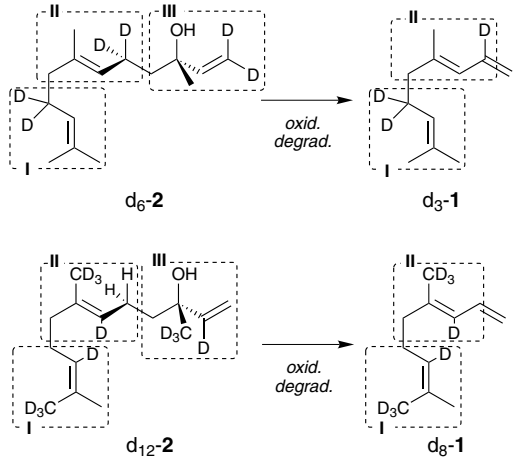


Fig. 2. Concentration-dependent degree of labeling of the monoterpenes ocimene **3** and linalool **4**. The degree of labeling of the collected volatiles was calculated according to Eq. (1). Labeled precursors: × = d_2-DOX (**6**), ○ = d_5-MVA (**7**). The concentration of **7** corresponds to the *R*-enantiomer in the administered racemate. Average values and standard deviations from three independent feeding experiments are given.

2.1.2. Sesquiterpenes

An opposite preference for d_2-DOX and d_5-MVA was observed in the biosynthesis of the sesquiterpenes, especially DMNT. The conjugated diene (**1**) is formed by an elimination of the C(5)-H_{Si} along with a formal C_4-segment from the polar head (Schemes 2 and 4) of (3*S*)-nerolidol (**2**) (Degenhardt and Gershenzon, 2000; Donath and Boland, 1995). As demonstrated in Scheme 4, this fragmentation not only removes four carbons of the terminal isoprene unit but also one deuterium atom from the central C_5-block (d_2-DOX as precursor) of d_6-**2**. Accordingly, the molecular mass of DMNT (**1**) can increase only by 3 mass units. If only a single IDP-unit is incorporated, its position directly follows from the molecular mass of labeled DMNT (**1**). d_2-IDP as the starting unit of the growing terpene chain results in a shift of 2 mass units, while at the second position only a single deuterium atom remains due to the fragmentation reaction. The labeling pattern of the first two C_5-segments after incorporation of d_5-MVA (**7**) is not affected by fragmentation and leads to the maximum possible increase of eight mass units (see Scheme 4). In this case, the position of a single labeled C_5-unit within the DMNT molecule **1** can be deduced from the characteristic fragmentation pattern of the mass spectrum (see Fig. 4).

Cyclization of farnesyl diphosphate to caryophyllene **5** proceeds with loss of a hydrogen atom after the cyclization. If three d_5-MVA derived d_4-IDP units are incorporated, the molecular ion will be shifted from *m/z* 204 to *m/z*



Scheme 4.

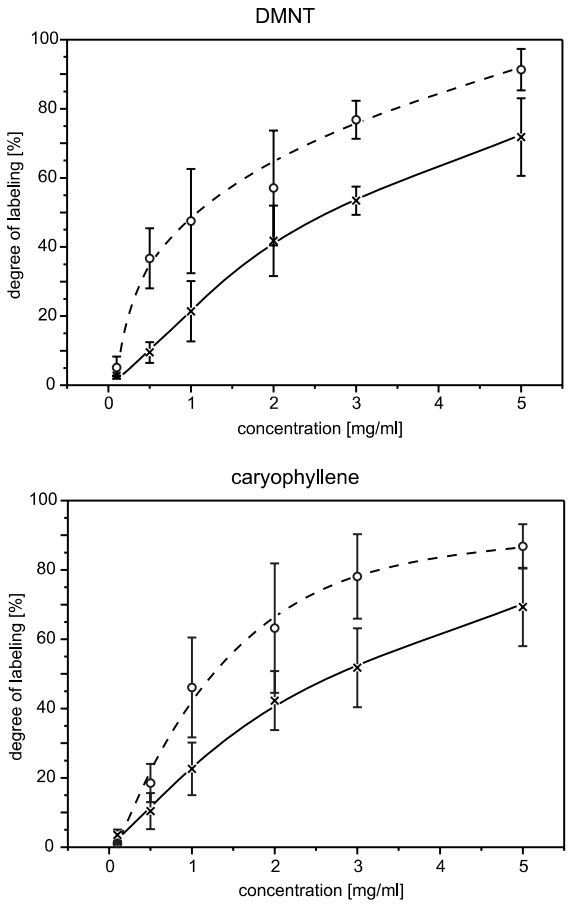


Fig. 3. Concentration-dependent degree of labeling of the sesquiterpenes DMNT (**1**) and caryophyllene **5**. The degree of labeling was calculated according to Eq. (1). Labeled precursors: × = d_2-DOX (**6**), ○ = d_5-MVA (**7**). The concentration of **7** corresponds to the *R*-enantiomer in the administered racemate. Average values and standard deviations from three independent feeding experiments are given.

215. Incorporation of only one or two d_4-IDP units leads to mass shifts of 3 and 4, or 7 and 8 mass units. As expected, the biosynthesis of both sesquiterpenes DMNT (**1**) and caryophyllene **5** predominantly followed the MVA-route (Fig. 3), which is reflected by the higher concentration-dependent degree of labeling in both molecules (70–90%) after administration of d_5-MVA (**7**). However, administration of d_2-DOX (**6**) also led to a concentration-dependent high degree of labeling in DMNT (**1**) and caryophyllene **5** (ca. 60%).

The enzymes of the MEP-pathway are located in the chloroplast, whereas the biosynthesis of the sesquiterpenes takes place in the cytosol. Since the MEP-pathway contributes much more to the sesquiterpene biosynthesis (Fig. 3) than the MVA-pathway to the biosynthesis of monoterpenes (Fig. 2), an export of precursors such as IDP (vide infra) from the plastid to the cytosol is the preferred direction. The initial linear increase of the extent of labeling of the monoterpenes indicates that the export of the intermediates from the chloroplast to the cytosol depends on the concentration of plastidial d_2-DOX (**6**) and the according concentration of d_2-IDP. This is further supported by inhibitor experiments. Blocking the MVA-pathway by cerivastatin® led to an increased incorporation of d_2-DOX (**6**) into DMNT (**1**) (Piel et al., 1998) consistent with a dynamic pathway allocation presumably driven by the relative concentration differences of IDP in the cytosol or the plastid, respectively.

2.1.3. Simultaneous application of labeled precursors

More information on the exchange of precursors (IDP or GDP) between the compartments was obtained from a simultaneous administration of d_5-MVA (**7**) and d_2-DOX (**6**). The analysis of the complex isotopomer mixture profits from the fact that the boiling point of the labeled compounds is inversely correlated with their deuterium content. This allowed a rather efficient separation of the individual isotopomers by gas chromatography.

The analysis of the mass spectra in the peak areas **A**–**D** (Fig. 4) supported the presence of four different isotopomers of DMNT (**1**). Peak **A** represents unlabelled, natural DMNT (**1**) (*m*/*z* 150). The molecular mass (*m*/*z* 156) of the isotopomer **C** (d_6-DMNT) is consistent with a simultaneous incorporation of one d_4-IDP from d_5-MVA (**7**) and one d_2-IDP from d_2-DOX (**6**). The molecular ion (*m*/*z* 158) of peak **D** results from the consecutive incorporation of two d_4-IDPs derived from d_5-MVA (**7**). Considering a random incorporation of plastid-derived d_2-IDP (D or d) or cytosolic d_4-IDP (M or m), as well as unlabeled IDP (U or u), into the molecule of DMNT (**1**), in total nine different isotopomers can be envisaged (Table 1).

From a careful analysis of their fragmentation patterns along with the molecular ions of the isotopomers the origin (d_2-IDP and d_4-IDP) and sequence of the incorporation of the first two C_5-units of DMNT (**1**) can be determined. The labeling of the third isoprene unit is lost (Scheme 4) during the oxidative degradation of (3*S*)-nerolidol (**2**) (Donath and Boland, 1995). Even a discrimination between chromatographic overlapping isotopomers of **1** (**Um**, **Mu**, Table 1) with an identical molecular mass at *m*/*z* 154 was possible based on their different fragmentation patterns. The ionization of the terminal dimethyl allyl group leads to a loss of a methyl group (see inset in spectrum **D**, Fig. 4). In the case of **Um** only a single intense fragment at *m*/*z* 139 resulting from the loss of an unlabeled methyl group is observed, while in the case of **Mu** two fragments at *m*/*z* 139 (158-CH_3) and *m*/*z* 136 (158-CD_3) were present. In a similar manner the structures, i.e. the position of the d_2- and d_4-IDP units, of all other isotopomers could be unequivocally determined. However, the probability was not identical for all possible combinations. As to be taken from Fig. 5, two unlabeled or two d_5-MVA-derived d_4-IDPs were preferably used for the biosynthesis of the DMNT precursor (3*S*)-nerolidol (**2**). Such a finding would be consistent with a preferential condensation of two cytosolic IDPs (**Uu** or **Mm**, Table 1) to give GDP followed by another C_5-elongation with an IDP imported from the plastid. However, this sequence is not strict since also other positional combinations were observed albeit at lower abundance.

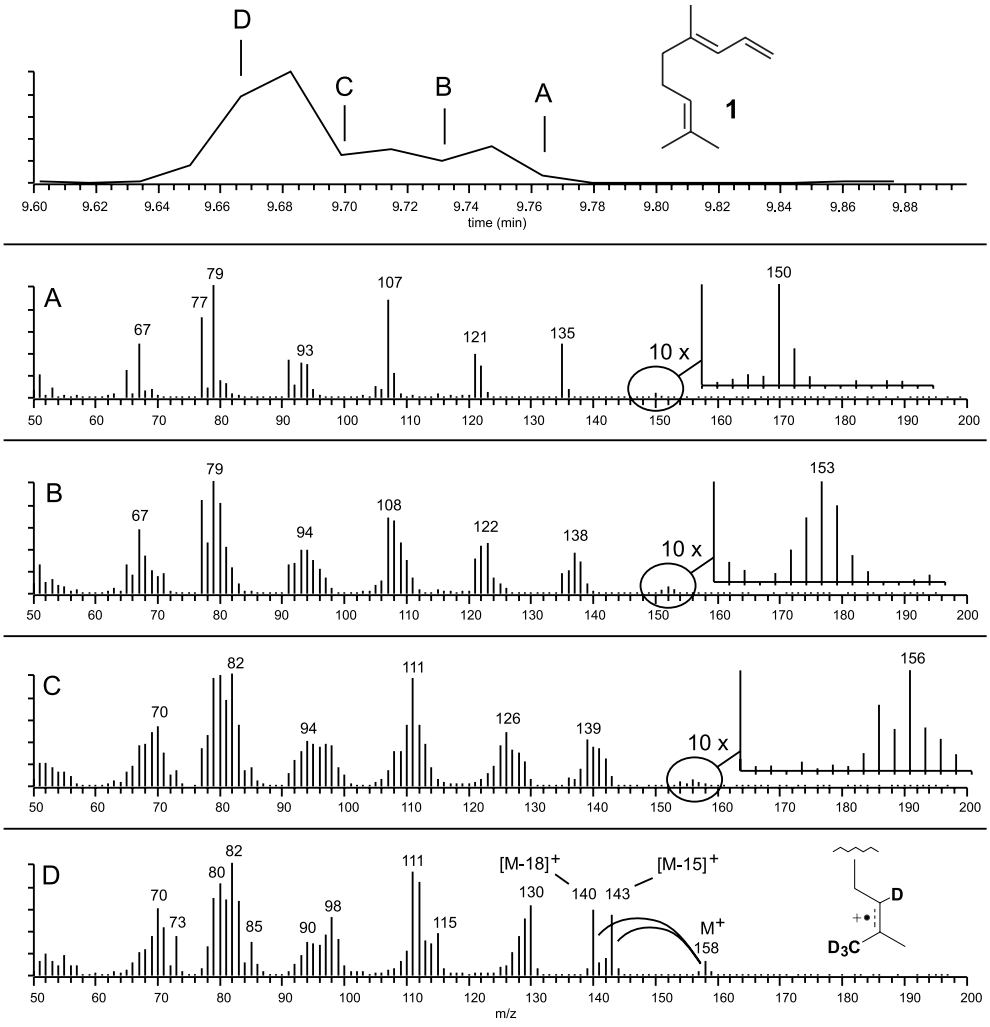


Fig. 4. Chromatographic separation (top) and identification of DMNT (**1**) isotopomers after simultaneous administration of d_2-DOX (**6**) and d_5-MVA (**7**). (A) No incorporation of labeled precursors, (B) exclusive incorporation of d_2-DOX (**6**), (C) simultaneous incorporation of d_2-DOX (**6**) and d_5-MVA (**7**), (D) exclusive incorporation of d_5-MVA (**7**) (inset: fragmentation of the molecular ion by cleavage of the allylic methyl groups).

Interestingly, and in contrast to the linear correlation of the labeling rate of DMNT (**1**) with the concentration of d_2-DOX (**6**) in the medium, the simultaneous administration of d_2-DOX (**6**) and d_5-MVA (**7**) shows only an initial correlation (ca. 20% labeling) with the concentration of d_2-DOX (**6**) (up to ca. 0.5 mg ml^{-1}). Higher concentrations of d_2-DOX (**6**) even led to a reduction of the degree of labeling (ca. 15% at 3 mg ml^{-1}), while higher concentrations of d_5-MVA (**7**) raised the labeling (up to ca. 70% at 3 mg ml^{-1}) in a clear linear correlation.

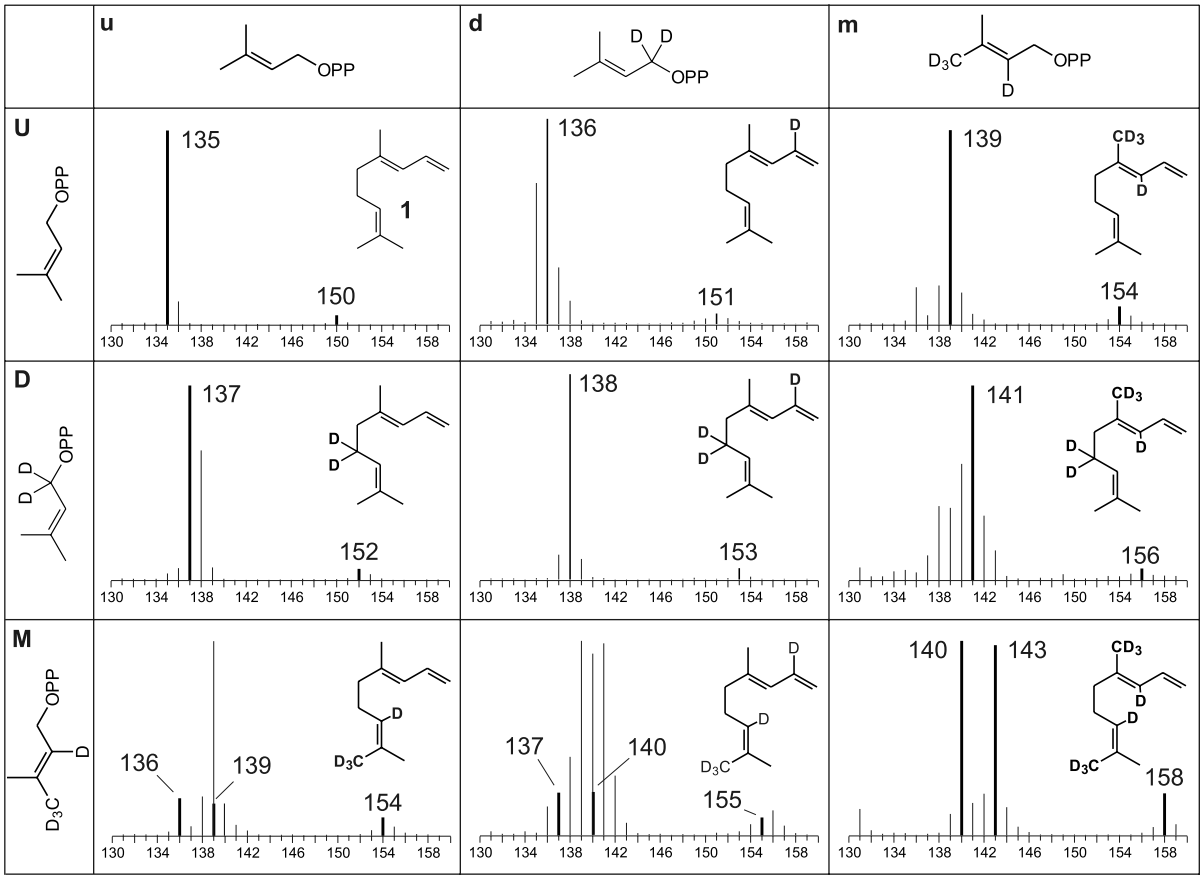
Such a finding is, again, consistent with a picture in which IDP from the plastid is imported into the cytosol only if the level of plastidial IDP (MEP-derived) is high and that of the cytosolic IDP (MVA-derived) is low. Since a charged molecule like IDP cannot pass membranes, the recently described transporters balance the gradient between the compartments (Bick and Lange, 2003). Thus, increasing concentrations of d_4-IDP in the cytosol prevent the import of d_2-IDP from the cytosol, consistent with the data of Fig. 6.

Similar observations were made for the biosynthesis of β-caryophyllene (**5**). The differently labeled d_2-IDP (from d_2-DOX (**6**)) and d_4-IDP (from d_5-MVA (**7**)) were also found in different combinations in **5**, but the amount of emitted caryophyllene **5** was too low for a detailed analysis. As to be expected, the biosynthesis of the monoterpenes ocimene **3** and linalool **4** profited only to a very limited extent from the MVA-pathway. Isotopomers indicating a simultaneous incorporation of d_2-DOX (**6**) derived d_2-IDP and d_5-MVA (**7**) derived d_4-IDP were observed, but only at rather high concentrations of d_5-MVA (**7**) (2 mg ml^{-1}). Moreover, the amount of the accordingly labeled isotopomers was not sufficient for a detailed analysis.

2.1.4. Inhibitor studies

Additional information on the dynamic allocation of the different routes towards IDP could be obtained by selective blocking of the pathways with specific inhibitors. To block the MEP-pathway, fosmidomycin (Okuhara et al., 1980),

Table 1
Possible combinations of incorporation of unlabeled IDP (U, u) , d_2-DOX (**6**) derived IDP (D, d) and d_5-MVA (**7**) derived IDP (M, m) into DMNT (**1**)



Majuscules (U, D, M) indicate the unit as the first and minuscules (u, d, m), as the second building block in the sesquiterpene biosynthesis of the DMNT precursor nerolidol **2**. Mass spectra show the region of the molecular ions of the different isotopomeres. The mass peaks for identification are highlighted. In cells **Mu** and **Md** mass 139 is overlapped by the fragmentation of isotopomer **Um**.

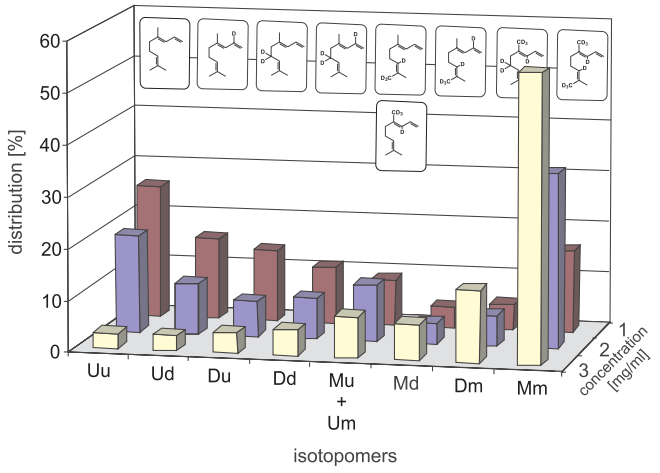


Fig. 5. Distribution of DMNT-isotopomers (%) at different concentrations of administered labeled precursors (d_2-DOX (**6**), *R*-d_5-MVA (**7**)). For each isotopomer the average value of three administration experiments is given. For abbreviations of the isotopomers, see Table 1.

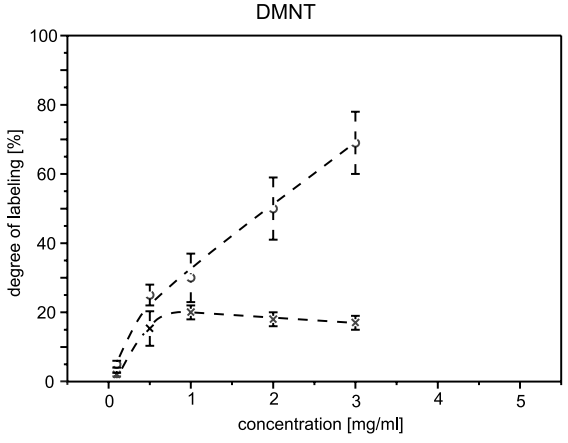


Fig. 6. Degree of labeling of DMNT (**1**) after simultaneous administration of the labeled precursors (× = d_2-DOX (**6**), ○ = d_5-MVA (**7**), for details see Fig. 2). Average value and standard deviation of five administration experiments are given. Administration of both labeled precursors at concentration of about 5 mg ml^{-1} led to severe necrosis of the leaves; no volatile production was observed.

an inhibitor of the DXP-reducto isomerase (Jomaa et al., 1999) was used. Cerivastatin® served as an efficient inhibitor of the HMG-CoA reductase (Kuhlmann et al., 1998) of the MVA-pathway (see Scheme 1).

The chromatograms (Fig. 7) illustrate the impact of the different inhibitors on the induced volatile emission. Pretreatment of the lima bean leaves with fosmidomycin com-

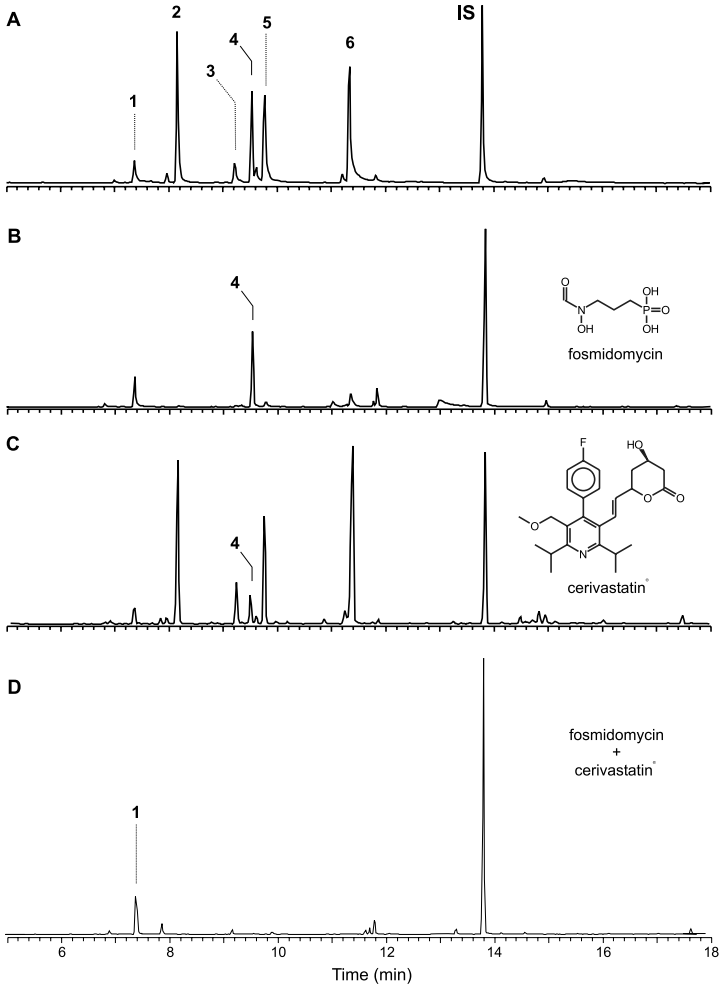


Fig. 7. Gas chromatographic separation of JA-induced volatiles from *Phaseolus lunatus*. (A) without inhibitor pre-treatment, (B) after inhibition of the MVA-pathway by cerivastatin®, (C) after inhibition of the MEP-pathway by fosmidomycin, (D) after simultaneous inhibition of both pathways by cerivastatin® and fosmidomycin. Compounds: (**1**): (3*Z*)-hex-3-enyl acetate, (**2**): ocimene, (**3**): linalool, (**4**): 4,8-dimethylnona-1,3,7-triene (DMNT), (**5**): (3*E*,5*E*)-2,6-dimethyl-octa-1,3,5,7-tetraene, (**6**): (3*E*,5*E*)-2,6-dimethyl-octa-3,5,7-trien-2-ol, IS: internal standard (1-bromodecane).

pletely suppressed the biosynthesis of the monoterpenes ocimene **3** and linalool **4**. The production of the fatty acid derived (3*Z*)-hexenyl acetate (**8**) remained unaffected. A pre-treatment of the leaves with cerivastatin® did not completely block the biosynthesis of DMNT (**1**), but reduced its production by ca. 70%. Simultaneous addition of both inhibitors fully suppressed the de novo biosynthesis of all terpenoids. Consistent with the results of the administration experiments (vide supra) the limited impact of the HMG-CoA reductase inhibition with cerivastatin® on the de novo synthesis of DMNT (**1**) independently confirms the importance of plastidial IDP for the cytosolic biosynthesis of sesquiterpenes. On the other hand, the cytosolic IDP did not support the plastidial biosynthesis of monoterpenes after blocking the MEP-pathway by fosmidomycin and reflects the unidirectional character of the recently suggested proton symport system in plastid membranes (Bick and Lange, 2003).

2.1.5. IRMS studies

Isotopic ratio mass spectroscopy (IRMS) of stable isotopes at natural abundance is an excellent tool for the reliable investigation of the dynamic pathway allocation in the biosynthesis of terpenoids (Jux et al., 2001). The major advantage of the method is the fact that no administration of labeled precursors is required. Depending on the active biosynthetic pathways and the different isotope effects of the involved enzyme-catalyzed reactions, the isotopic signatures of the metabolites differ from each other (Schmidt, 2003). This also holds for the two alternative routes to IDP. In the MVA-pathway, pyruvate is transformed to acetyl-CoA by the pyruvate dehydrogenase (see Scheme 1). This C–C bond cleaving reaction (elimination of CO_2) prefers the lighter isotopes (Melzer and Schmidt, 1987). In consequence, the resulting acetyl-CoA is depleted in ^{13}C and due to the fact that three acetyl-CoA units are required for the synthesis of IDP via the MVA-route, a

considerable depletion of ^{13}C is to be expected for all sesquiterpenoids resulting exclusively from this pathway.

In the MEP-route the ^{13}C-discriminating decarboxylation of pyruvate, catalyzed by the DXP-synthase, occurs only once. Moreover, the carbonyl carbons of glyceraldehyde and pyruvate are enriched in ^{13}C, since the precursor glucose is ^{13}C-enriched at C-3 and C-4 (Schmidt and Gleixner, 1998). In summary, these effects lead to a clear difference in the isotopic signature of isoprenoids derived from MEP- or MVA-pathway (Jux et al., 2001).

The degraded sesquiterpene DMNT (**1**) displays a $\delta^{13}C$-value of −37.4‰ while the monoterpene (*E*)-β-ocimene (**3**) shows a $\delta^{13}C$-value of −29.0‰ (Fig. 8). Thus, DMNT is considerable more ^{13}C-depleted than (*E*)-β-ocimene (**3**) which is exclusively assembled from MEP-derived IDP. The ^{13}C-signature of ocimene **3** corresponds to previously reported ^{13}C-signatures of other monoterpenes (−26‰ to −30‰) (Schmidt et al., 1998). The value of DMNT (**1**) is close to the value of (3*Z*)-hexenyl acetate (**8**) (−40.2‰), which, as a catabolite of linolenic acid, displays a strong depletion in ^{13}C; this also holds for the MVA-derived sesquiterpenes.

If a dynamic allocation of the two pathways exists for the biosynthesis of the cytosolic sesquiterpenes, one should expect varying isotopic signatures for DMNT (**1**) if the supply by the one or the other of the two pathways is blocked (Fig. 8). Thus, after inhibition of the MVA-route with cerivastatin® the $\delta^{13}C$-value of DMNT (**1**) shifted to −30.8‰, close to that of the monoterpene (*E*)-β-ocimene (**3**) (−29‰). On the other hand, a block of the MEP-pathway with fosmidomycin resulted in a $\delta^{13}C$-value of −38.4‰ for DMNT (**1**), close to that of the fatty acid derived (3*Z*)-hexenyl acetate (**8**) (−38.6‰). Consistent with the results of the feeding experiments (vide supra) the isotopic ratio of ocimene **3** is not affected by inhibition of the MVA-route.

2.1.6. Dynamic pathway allocation in stressed plants

The described shifts of the ^{13}C-signatures were also observed when the $\delta^{13}C$-values of terpenoids from stressed

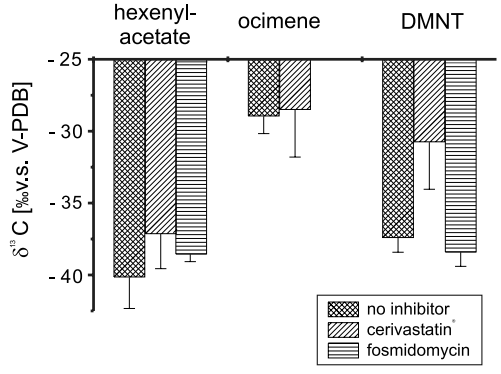


Fig. 8. $\delta^{13}C$-values of (3*Z*)-hexenyl acetate (**8**), ocimene **3** and DMNT (**1**) after induction with jasmonic acid and after blocking the individual pathways with cerivastatin® and fosmidomycin respectively. Average value and standard deviation of three samples are given.

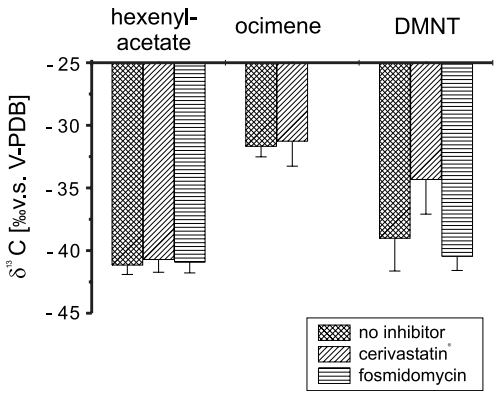


Fig. 9. Comparison of $\delta^{13}C$-values of DMNT (**1**) induced by feeding caterpillar (*Spodoptera frugiperda*), jasmonic acid and the fungal elicitor alamethicin.

lima beans were analyzed after herbivory (*Spodoptera frugiperda*), elicitation with jasmonic acid, or after treatment with the fungal elicitor alamethicin (Engelberth et al., 2001). All three treatments led to clearly different $\delta^{13}C$-values for DMNT (**1**), consistent with an either predominant contribution of the MVA-pathway or an almost exclusive supply of IDP by the MEP-pathway (Fig. 9).

The $\delta^{13}C$-values for DMNT (**1**) resulting from herbivory and elicitation by jasmonic acid are comparable (−36.1‰ to −39.0‰) with respect to the standard deviation. This result perfectly fits with the fact that feeding insects raise the level of jasmonate, which in turn acts as a master switch for the coordinate induction of defense responses. Interestingly, the fungal elicitor alamethicin triggers only the plastidial MEP-pathway which then delivers IDP to the cytosol. DMNT assembled from this plastidial IDP exhibits a $\delta^{13}C$-value of −30.7‰ which is close to that of the MEP-derived monoterpenes. Plasticity in the pathway allocation is also observed, if the lima bean is stressed by feeding herbivores, especially in combination with simultaneous application of inhibitors. DMNT emitted from herbivore-damaged leaves (no inhibitor present) displayed a $\delta^{13}C$-value of −39.0‰. If the MVA-pathway was blocked by cerivastatin®, the $\delta^{13}C$-value of herbivore-induced DMNT (**1**) raised to −34.4‰ , while the same administration experiment in the presence of fosmidomycin resulted in a more depleted $\delta^{13}C$-value of −40.4‰ for the same compound. Accordingly, the dynamic use of both pathways is an intrinsic feature of the lima bean that maintains the production of sesquiterpenoids even, if one of the two pathways is blocked.

3. Conclusions

Plant volatiles resulting from de novo biosynthesis after insect damage serve parasites of the herbivores as important markers to find their hosts (Arimura et al., 2005; van Poecke and Dicke, 2004). In general, volatile mono- and sesquiterpenes are the major constituents of such

induced blends. Owing to the outstanding importance of these compounds for the survival of the plant, a dynamic allocation of the two early pathways of terpenoid biosynthesis furnishes the plant with two different and independent resources to nourish their terpenoid biosynthesis. Salivary secretions of the feeding insects which could contain inhibitors will therefore not result in a complete suppression of terpenoid biosynthesis. The incorporation studies with labeled MVA **7** and labeled DOX **6** suggest that transport systems exist (Bick and Lange, 2003) that allow a unidirectional flux of IDP from the plastid to the cytosol. In the lima bean, the reverse direction is also possible, but has probably no significance since only in the case of rather high concentrations of cytosolic IDP this transfer was observed. Besides jasmonic acid, ethylene has also been recently found to control terpenoid biosynthesis in *Medicago truncatula* (Arimura et al., unpublished) by suppressing monoterpene- and enhancing the sesquiterpene production. In this context, the differential contribution of both pathways to the biosynthesis of the different groups of terpenoids may be relevant for a regulatory network controlling the plants response against microbial or insect attackers.

4. Experimental

4.1. Chemicals

General: Reactions were performed under Ar; solvents were dried according to standard procedures. Instrumentation: NMR spectrometer: Bruker Advance DRX 500 (Bruker, Karlsruhe, D); mass spectrometer: Micromass MassSpec 2 (Micromass/Waters, Manchester, UK).

Chemicals: [5,5-$^2H_2$]-Deoxy-D-xylulose (**6**) was obtained as described previously (Jux and Boland, 1999). Deuterated mevalolactone **7** was synthesized according to Schwarz (1994), or Jux (2001). Fosmidomycin was synthesized following a patent of the Fujisawa Pharmaceutical Company, Limited (Kamiya et al., 1980; Jux, 2001). Cerivastatin® was a gift from the Bayer AG; Alamethicin was purchased from Calbiochem.

4.2. Plant material

Seeds of *Phaseolus lunatus* Cultivar "Jackson Wonder Bush" were from Kellogs Seed Inc., USA. Two beans were grown in plastic pots (i.d. 5.5 cm) at 22–26 °C and 50–60% rel. humidity. The plants were illuminated with fluorescent tubes (270 μE m^2 s^{-1}) 14 h a day. The soil (Capriflor®, Germany) was sterilized by heating in a microwave (3 min, 900 W) prior to use.

4.3. Administration of precursors and volatile sampling

For induction of volatile biosynthesis, freshly cut plantlets of the lima bean (12–14-days-old with two fully developed primary leaves) were placed in an aqueous solution of jasmonic acid (1 mM). After 12 h, the induced plants were transferred into a closed system (desiccator, 3 L) and the emitted volatiles were collected over a period of 48 h on charcoal as described (Donath and Boland, 1995).

Labeling studies were carried out by placing the plantlets in aqueous solutions of the labeled precursors at appropriate concentrations (0.1, 0.5, 1.0, 2.0, 3.0, 5.0 mg ml^{-1} d_2-DOX (**6**)). Twice as high concentrations were administered in case of racemic d_5-MVA (**7**) to compensate for the presence of the wrong enantiomers. Precursors were applied 24 h prior to elicitation. After preincubation, the plantlets were transferred to solutions containing the same concentrations of the precursors and additionally jasmonic acid at 1 mM. Collection of volatiles was accomplished as described above.

For selective inhibition of the MVA- or the MEP-pathway, the plantlets were placed for 24 h into solutions of the inhibitors cerivastatin® or fosmidomycin (3×10^{-5} M, each), prior to elicitation of volatile biosynthesis with JA (1 mM), alamethicin (10 μM) or herbivore feeding (four larvae of *Spodoptera frugiperda* per excised plant).

4.4. GC-MS

Separation of volatiles was achieved on a fused silica capillary column (EC-5-MS, Alltech, 30 m × 0.25 mm, 0.25 μm) under programmed conditions: 50 °C (2 min isotherm), then at 5 °C min^{-1} to 220 °C. Mass spectrometric analysis was accomplished with a GCQ-mass spectrometer (Finnigan MAT, Bremen, Germany).

4.5. IRMS

Volatiles were separated on a fused silica capillary column (DB-5-MS ITD, Alltech, 30 m × 0.25 mm, 0.25 μm) under programmed conditions: 50 °C (2 min isotherm), then at 5 °C min^{-1} to 220 °C. The eluting compounds were combusted on-line (at 940 °C) using a catalyst system of CuO/NiO/PtO and the resulting CO_2 was transferred into an Delta^{plus} XL-Isotope-Ratio-Mass spectrometer (Thermoquest, Egelsbach, Germany). An aliquot of the injected sample (10%) was split after separation but before combustion and transferred into an organic mass spectrometer (GCQ, Thermoquest) for identification of the eluting compounds. All isotope ratios are given as δ^{13}C-values (‰):

$$\delta^{13}\mathrm{C}\ (‰) = [(R_{\mathrm{sample}}/R_{\mathrm{standard}}) - 1] \times 10^3,$$

where R corresponds to the $^{13}C/^{12}C$-ratio of the sample and the standard (Vienna Pee Dee Belemnite).

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