$b = 6.9912(7)$, $c = 12.3635(12)$ Å, $\alpha = 84.686(2)$, $\beta = 78.684(2)$, $\gamma = 68.044(2)^\circ$, $V = 486.16(8)$ Å^3, $Z = 1$, $\rho_{calcd} = 1.852$ Mg m^{-3}, $T = 133$ K, 5719 measured, 2771 crystallographically unique, and 2771 reflections with $I > 2\sigma(I)$, $Mo_{K\alpha}$, $\lambda = 0.71069$ Å, $2\theta_{max} = 30^\circ$, empirical absorption correction (SADABS)[16] ($\mu = 1.390$ mm^{-1}), $R(F_o) = 0.0320$, $wR(F^2) = 0.0785$ (all data), 153 parameters, anisotropic thermal parameters, H-atoms isotropic. Crystallographic data (excluding structure factors) for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication nos. CCDC-156869 – CCDC-156874. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: (+44) 1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).

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Classification of Terpenoids according to the Methylerythritolphosphate or the Mevalonate Pathway with Natural $^{12}C/^{13}C$ Isotope Ratios: Dynamic Allocation of Resources in Induced Plants**

Andreas Jux, Gerd Gleixner, and Wilhelm Boland*

Dedicated to Professor Ernst-G. Jäger on the occasion of his 65th birthday

Plant volatiles, whose biosynthesis is induced by feeding insects, may serve as markers to allow the parasites of the herbivores to find their hosts.[1, 2] Thus, they benefit the plant indirectly, and have been called the "plant's cry for help".[3] Major constituents of such induced volatile blends include terpenoids, aromatic compounds, and degradation and transformation products derived from fatty acids. Mono- and diterpenoids are produced in the plastids from isopentenyl diphosphate (IDP) formed through the methylerythritol phosphate pathway (MEP pathway), while the IDP for sesquiterpenoids is assembled by the well-known mevalonic acid pathway (MVA pathway) in the cytosol (Scheme 1).[4–6] However, this classical allocation of terpenoids to pathways is not completely strict. While administration of deuterium-labeled 5-deoxy-D-xylulose, an early precursor of IDP in the MEP pathway, in principle confirmed the normal allocation of pathways in herbivore-induced biosynthesis, the $^1H/^2H$ isotope signature of 4,8-dimethylnona-1,3,7-triene (DMNT, **4**; Figure 1) demonstrated a significant contribution from both pathways.[7] In another example, the incorporation of ^{13}C-labeled glucose into sesquiterpenes from chamomile resulted in an isotope signature that required contributions from both pathways.[8] Moreover, careful analysis of sitosterol (isolated from cell cultures of *Catharanthus roseus*), which is assembled from two units of farnesyl diphosphate, clearly demonstrated a contribution (approximately 6%) from the mevalonate-independent MEP pathway.[9] This classification of pathways, based on externally added precursors, is open to criticism, as administration may affect the natural balance of the cellular intermediates and result in shifted mass fluxes though the complex interacting pathways. Even the use of physiologically neutral precursors, such as glucose, has an impact on the metabolic network. Phototrophic organisms need to be kept under heterotrophic conditions to avoid dissipation of this valuable precursor into unwanted anabolic activities.[5] An alternative to precursor-based approaches is provided by analysis of the isotope ratio of relevant compounds, at natural abundance level, using isotope-ratio mass spectrometry (IRMS).[10] By linking an isotope-ratio mass spectrometer to a gas chromatograph, with online combustion of the eluting compounds to CO_2 und H_2O (GC-C-IRMS), analysis of the isotopic signature of individual compounds of complex mixtures is possible.[11]

Owing to thermodynamic and kinetic isotope effects, the natural abundance of carbon isotopes exhibits minor local and temporal shifts from a global average value. In the biosphere these shifts are related to the isotope effects of the reactions catalyzed by enzymes involved in the physiological processes. Prominent examples exhibiting very high discrimination of carbon isotopes were reported for the different modes of CO_2 fixation by C_3 and C_4 plants, which utilize ribulose diphosphate carboxylase or phosphoenolpyruvate carboxylase.[12] Further metabolism may additionally affect the isotope ratio, but in general these effects are less pronounced. Here we report that the different groups of terpenoid volatiles also exhibit significant differences in their $^{12}C/^{13}C$ ratio, which depend upon whether the universal building block IDP is produced predominantly from the MVA or MEP pathway (Scheme 1).

The isotope ratios of monoterpenes as $\delta(^{13}C)$ values (see Experimental Section for definition) have already been determined by others, mainly to evaluate the origin and authenticity of compounds, and were observed within the range -26 to -30‰.[13, 14] The value for ocimene (**2**; Figure 1), which is released from leaves of the lima bean (*Phaseolus lunatus*, used here as a model plant), was measured by us to be -29.0‰, a value that fits perfectly into the expected range (Figure 2). In contrast, the value of -37.4‰ for DMNT (an oxidative degradation product of the sesquiterpenoid ner-

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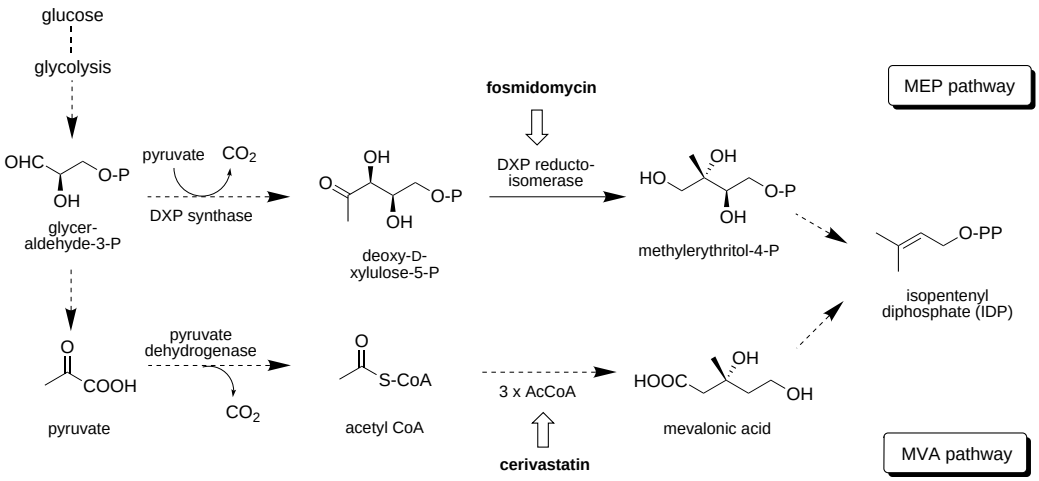

Scheme 1. Alternative biosyntheses of isopentenyl diphosphate (IDP) by the methylerythritol phosphate (MEP) pathway and the acetyl CoA/mevalonic acid (MVA) pathway.

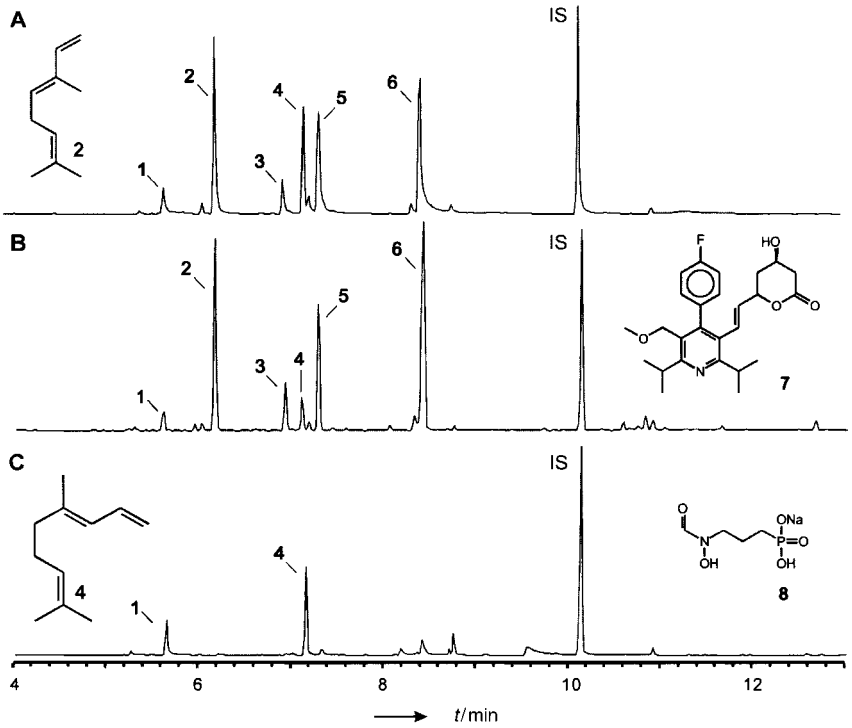

Figure 1. Chromatographic separation of volatiles produced by *Phaseolus lunatus* after induction with jasmonic acid (JA). A) JA-induced blend of volatiles without inhibitor pretreatment. B) JA-induced blend of volatiles after inhibition of the MVA pathway by cerivastatin (**7**). C) JA-induced blend of volatiles after inhibition of the MEP pathway by fosmidomycin (**8**). Compounds: (3*Z*)-hex-3-enyl acetate (**1**), ocimene (**2**), linalool (**3**), 4,8-dimethylnona-1,3,7-triene (DMNT, **4**), $C_{10}H_{14}$ (**5**), $C_{10}H_{16}O$ (**6**); IS = internal standard (1-bromodecan). Linalool coelutes with a contaminant, which prevents exact determination of its isotope ratio.

olidol),[15] displays a significant depletion of the ^{13}C isotope. As the sesquiterpenoids are produced by the MVA pathway, while monoterpenoids originate from the MEP pathway, the difference in the isotopic signatures of ocimene and DMNT is explained by the different modes of the biosynthesis of their common intermediate IDP. A pronounced discrimination of isotopes has been reported for the production of acetyl CoA from pyruvate by decarboxylation (MVA pathway, Scheme 1). The thiamine-dependent pyruvate dehydrogenase prefers the lighter isotopomers, producing acetyl CoA that is ^{13}C depleted.[16] Although a thiamine-dependent decarboxylation of pyruvate is also involved in the biosynthesis of 5-deoxy-D-xylulose-5-phosphate (DXP) along the MEP pathway,[17] the assembly of the carbon skeleton of DXP requires only a single decarboxylation of pyruvate. In contrast, mevalonic acid originates from three molecules of acetyl CoA, which therefore eventually contribute to the biosynthesis of IDP. Owing to this exclusive use of acetyl CoA, the MVA pathway should produce sesquiterpenoids that are significantly depleted in ^{13}C isotopomers. Since the carbonyl carbon atoms of glyceraldehyde and pyruvate possess, due to their origin from glucose, a higher level of ^{13}C isotopomers,[18] the incorporation of intact glyceraldehyde-3-P into DXP additionally enhances the difference in the isotopic ratio of compounds produced along either of the two pathways.

The $\delta(^{13}C)$ values for ocimene (MEP pathway) and DMNT (MVA pathway; Figure 2) clearly confirm the expected trend. The $\delta(^{13}C)$ value of -37.4‰ for DMNT is close to that of -40.2‰ observed for hexenyl acetate. The latter should display a low ^{13}C level due to its exclusive origin from acetyl CoA. A comparable discrimination of carbon isotopes in fatty acids and products of the MEP pathway was reported by Schouten et al. for algal lipids from marine or fresh water environments. The $\delta(^{13}C)$ value of palmitic acid was about 2–5‰ lower than that of phytol (MEP pathway).[19]

Previous findings from precursor studies with deuterium-labeled 5-deoxy-D-xylulose have to be considered to estimate the significance of the $\delta(^{13}C)$ value of DMNT.[7] These data clearly showed the potential for mixed origin. Accordingly, the $\delta(^{13}C)$ value of DMNT (Figure 2) should vary, if one of the two pathways is blocked by inhibitors. This was demonstrated by pretreatment of the lima bean leaves, prior to induction of volatile biosynthesis, with cerivastatin (**7**), a highly efficient inhibitor of HMGCoA reductase in the MVA pathway.[20] The MEP pathway is blocked by fosmidomycin (**8**), which inhibits the DXP

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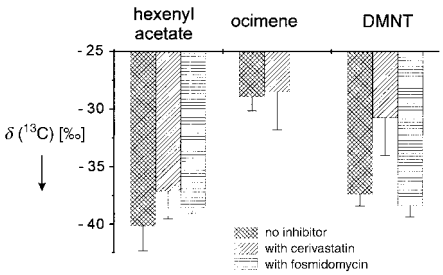

Figure 2. $\delta(^{13}C)$ values of selected volatiles after induction with jasmonic acid. Average values from three samples are given.

reductoisomerase (Scheme 1).[21] As shown in Figure 1B and C a single inhibitor is not able to suppress the biosynthesis of DMNT.[7] The efficiency of the fosmidomycin treatment is demonstrated by the almost complete lack of the monoterpenes (compounds **2**, **3**, **5**, and **6**; Figure 1C), while application of cerivastatin reduces the biosynthesis of DMNT by approximately 70% relative to that of the noninhibited plant (Figure 1C). Only the simultaneous application of both inhibitors completely suppresses the production of DMNT.

After inhibition of the MVA pathway by cerivastatin, the emitted DMNT displays a $\delta(^{13}C)$ value of -30.8‰, which is close to that of ocimene (-28.5‰) produced along the MEP pathway (Figure 2). On the other hand, if the MEP pathway is inhibited, the $\delta(^{13}C)$ value of DMNT (-38.4‰) approaches the typical value of acetogenins such as hexenyl acetate. The $\delta(^{13}C)$ value of ocimene remains unchanged after inhibition of the MVA pathway. Since the late steps of the biosynthesis of DMNT are identical in both inhibitor experiments, the observed differences in the $^{12}C/^{13}C$ ratios can be unequivocally attributed to the early steps of the MVA or MEP pathway.

Interestingly, treatment of the lima bean with different elicitors may channel the biosynthesis of DMNT through the MEP or the MVA pathway. Stimulation of leaves with the fungal elicitor alamethicin provides such an example.[22, 23] With this pretreatment the $\delta(^{13}C)$ value for DMNT (-30.7‰) is close to that of ocimene and, hence, suggests the predominant utilization of the MEP pathway.

Similar shifts in the isotope ratios of DMNT are observed if the volatile biosynthesis is triggered by feeding insects (Figure 3). Again, application of the individual inhibitors does not completely suppress the biosynthesis of DMNT. As seen from the $\delta(^{13}C)$ values of DMNT (-39.0‰), herbivory preferentially stimulates the cytosolic MVA pathway (compare Figures 2 and 3).[23] If this pathway is blocked, by pretreatment with cerivastatin, the MEP pathway compensates and the $\delta(^{13}C)$ value for DMNT (-34.4‰) is raised. The

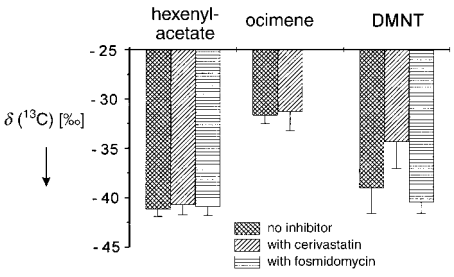

Figure 3. $\delta(^{13}C)$ values of selected volatiles after induction by feeding insects (*Spodoptera frugiperda*). Average values from three samples are given.

data suggest that natural induction processes may also benefit from a dynamic allocation of both pathways.[7]

Experimental Section

For induction of volatile biosynthesis, freshly cut stems of the lima bean (12–14 days old with two fully developed primary leaves) were placed in an aqueous solution of jasmonic acid (JA; 1 mM). After 12 h the plants were transferred into a closed system (desiccator, 3 L) and the emitted volatiles were collected over a period of 48 h on a small charcoal pad.[24] For selective inhibition of the MVA or MEP pathway, the excised plants were placed for 24 h into solutions of the inhibitors cerivastatin or fosmidomycin (3×10^{-5} M each), prior to the elicitation of volatile biosynthesis with JA (1 mM), alamethicin (10 μM), or herbivore feeding (four larvae of *Spodoptera frugiperda* per excised plant).

Analysis and separation of volatiles was achieved on a fused silica capillary column (DB-5-MS ITD, Alltech, dimensions: 30 m × 0.25 mm, film thickness: 0.25 μm; conditions: 50 °C (2 min isotherm), then temperature rise at 5 °C min^{-1} to 220 °C). The eluting compounds were combusted online at 940 °C with 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 and transferred into a normal mass spectrometer (GCQ, Thermoquest) for identification of the eluting compounds. All isotope ratios are given as $\delta(^{13}C)$ 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).

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