Incorporation of 1-deoxy-D-xylulose into isoprene and phytol by higher plants and algae

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Abstract In further substantiating the novel mevalonate-independent pathway for isoprenoid biosynthesis, which generates isopentenyl diphosphate (IPP) via 1-deoxy-D-xylulose-5-phosphate, labeling experiments with 1-[$^2H_1$]deoxy-D-xylulose were performed with various higher plants and algae: efficient incorporation was observed into isoprene emitted by *Populus*, *Chelidonium*, and *Salix*, into the phytol moiety of chlorophylls in a red alga (*Cyanidium*), in two green algae (*Scenedesmus*, *Chlamydomonas*), and a higher plant (*Lemna*). By contrast, ^{13}C-mevalonate applied was incorporated into isoprene and phytol to a much lower extent or not at all. This demonstrates that this '1-deoxy-D-xylulose-5-phosphate pathway' for biosynthesis of plastidic isoprenoids is widely distributed in photosynthetic organisms.

Key words: Deoxy-D-xylulose; Isopentenyl diphosphate; Isoprene; Isoprenoid biosynthesis; Mevalonate; Phytol; Sterol

1. Introduction

Recent observations demonstrated that in bacteria, higher plants and algae there exist two alternative biosynthesis pathways for the formation of isopentenyl diphosphate (IPP), the common C_5 precursor of all natural isoprenoids, i.e. the classical mevalonate pathway of IPP formation (Fig. 1a, see e.g. [1]), and an alternative mevalonate independent biosynthetic route unravelled by ^{13}C-incorporation studies in bacteria [2], green algae [3–8], and higher plants [6,8–13]. In fact, in higher plants and green algae the biosynthesis of all plastidic isoprenoids such as the phytyl side chain of chlorophylls, the carotenoids and the nonaprenyl side chain of plastoquinone-9 [3–5,12] appears to follow this alternative route, of which the elucidation of the distinct biosynthetic steps is just at the beginning.

The first biosynthetic step of this alternative pathway appears to be the addition of a pyruvate-derived hydroxyethyl thiamine (by decarboxylation) onto D-glyceraldehyde-3-phosphate (GAP) in a transketolase-like manner [2,3,14] to form a 1-deoxy-D-pentulose-5-phosphate, which then undergoes a rearrangement of the linear carbon chain to the branched carbon skeleton of IPP [2,3,14] (Fig. 1b). In fact, 1-deoxy-D-xylulose (1-deoxy-D-*threo*-pent-2-ulose; dXu), a precursor of thiamine pyrophosphate and pyridoxol [15], was efficiently incorporated into the isoprenic side chain of ubiquinone in *Escherichia coli* [16]. Similarly, leaves of *Populus nigra* L., *Chelidonium majus* L., and *Salix viminalis* L. emitted deuterium-labeled isoprene after insertion of methyl-[1-$^2H_1$]-1-deoxy-D-xyluloside (Me-[1-$^2H_1$]-dXu, Fig. 1c), from which the free sugar ([1-$^2H_1$]-dXu) is released in vivo by plant glucosidases, thereby establishing dXu to be a biosynthetic precursor of isoprene [13]. Since 1-deoxy-D-xylulose-5-phosphate is the first intermediate, this alternative biosynthetic route to IPP can be termed the '1-deoxy-xylulose phosphate pathway' (Fig. 1b), inasmuch as the first enzyme of this pathway, the 1-deoxy-xylulose-5-phosphate synthase, has recently been characterized in *E. coli* [17].

In this account, we present evidence for the specific incorporation of [1-$^2H_1$]-dXu into isoprene emitted by poplar leaves and into phytol (side chain of chlorophyll) of a red alga. We also show that the free 1-deoxy-D-xylulose, when applied in its deuterated form to poplar leaves, is as efficiently incorporated into the isoprene as its methyl glycoside. In addition, a comparative evaluation of the incorporation of [2-^{13}C]mevalonate and of Me-[1-$^2H_1$]-dXu into phytol (diterpenic side chain of chlorophylls) and the sterols of three algae and a higher plant is reported.

2. Materials and methods

2.1. ^2H- and ^{13}C-labeled precursors

Methyl-[1-$^2H_1$]-1-deoxy-α/β-D-xyluloside (Me-[1-$^2H_1$]-dXu) was prepared by methanolysis of [1-$^2H_1$]-1-deoxy-2,3-*o*-isopropylidene-β-D-xylulofuranose as described previously [13], the ^2H-labeled dXu correspondingly by acid hydrolysis in water (20 µl 2 M HCl in 1 ml of water+50 mg of isopropylidene-dXu, 50°C, 8 h) followed by a careful neutralization with 5% $NaHCO_3$ [13]. The solution was used directly for incubation experiments. dl-[2-^{13}C]Mevalolactone was purchased from IC Chemikalien, D-85737 Ismaning, Germany.

2.2. Labeling of isoprene emitted by leaves of Populus nigra L.

Branches and leaves of poplar (*Populus nigra* L.) were taken from trees on the campus of the University of Karlsruhe.

Headspace GC-MS experiments were conducted as described before [13]. Five mg of [1-$^2H_1$]-dXu, Me-[1-$^2H_1$]-dXu, or [2-^{13}C]-mevalolactone were dissolved in water and fed through the freshly cut petiole. For better uptake the transpiration was reinforced by a flow of air at 25°C. Isoprene production was induced by illumination of the cut branch with a slide projector (up to 2000 µmol photons m^{-2} min^{-1}) at a temperature of ≃30°C.

For NMR examination, comparatively large amounts of labeled isoprene are required. Therefore a cold trapping apparatus was assembled: charcoal-filtered ambient air was pumped with a flow rate of

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Abbreviations: dXu, 1-deoxy-D-xylulose; GAP, D-glyceraldehyde-3-phosphate; GC-MS, gas chromatography-mass spectrometry; IPP, isopentenyl diphosphate; Me-[1-$^2H_1$]-dXu, methyl-[1-$^2H_1$]-1-deoxy-α/β-D-xyluloside; MVA, mevalonic acid; NMR, nuclear magnetic resonance; TLC, thin layer chromatography

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1.4 l h^{-1} through a transparent plastic chamber illuminated by two slide projectors containing a poplar branch with 15 green leaves. The flow of air was subsequently passed through two cold traps cooled with dry ice and one drying tube filled with $Mg(ClO_4)_2$ for removal of the high amounts of transpiration water. In the ensuing third cold trap (dry ice) with charcoal (100 mg activated charcoal 20/40, Supelco), the isoprene was trapped. The stripping apparatus was flushed with air for 8 h a day under illumination of 1200 μmol photons m^{-2} min^{-1} at a temperature of 30°C inside the plastic chamber. The poplar branch was placed in a solution of 30 mg of methyl-[1-^{2}H]-1-deoxy-D-xyluloside in 30 ml of water. Further 30 mg of Me-[1-$^2H_1$]-dXu were added later in two portions through a Teflon tube which was fitted airtight into the chamber. From time to time additional water was added through the tube to prevent the plant from drying up. After 3 days the activated carbon from the last trap was eluted with 1 ml of $CDCl_3$ in three portions and analyzed by ^{1}H-NMR (500 MHz) on a Bruker DRX 500 spectrometer. In addition, phytol and the sterols of the poplar branch were extracted and investigated by GC-MS as given below for *Lemna*.

2.3. Growth of Cyanidium caldarium on Me-[1-$^2H_1$]-dXu

The unicellular red alga *Cyanidium caldarium* (strain CPD, obtained from S. Beale, Brown Univ., Providence, RI, USA) was grown autotrophically (irradiance of 100 μmol photons m^{-2} s^{-1}, Osram L. fluorescent lamps, aeration 20 l h^{-1}) on 2 l of a mineral medium with addition of 0.025% (w/v) Me-[1-$^2H_1$]-dXu. The mineral medium of Beale et al. [18] was applied with modifications: $(NH_4)_2SO_4$ 2 mM, NH_4Cl 6 mM, KH_2PO_4 5 mM, Mg SO_4 2.5 mM, $CaCl_2$ 0.1 mM, pH 4.5. After 14 days the cells (1 g dry weight) were harvested by centrifugation.

2.4. Growth of duckweed and algae on Me-[1-$^2H_1$]-dXu and [2-^{13}C]MVA

Lemna gibba (*Lemna gibba* L.) was grown on a medium as described in [12] with glucose (0.1% w/v) at an irradiance of 100 μmol photons m^{-2} s^{-1} (Osram L. fluorescent lamps). Either Me-[1-$^2H_1$]-dXu (0.05% w/v) or [2-^{13}C]mevalonic acid lactone (0.02% w/v) were filter-sterilized and added to the culture medium. After 6 days, about 1 g fresh weight was harvested from a 50 ml culture.

The green algae *Scenedesmus obliquus* (strain 276-3d, Algensammlung Göttingen, Germany), *Chlamydomonas reinhardtii* (strain 11-31, Algensammlung Göttingen) and the red alga *Cyanidium caldarium* were grown in Erlenmeyer flasks on a rotary shaker at an irradiance of 100 μmol photons m^{-2} s^{-1} (Osram L. fluorescent lamps) in 50 ml of the following media: a mineral medium as described in [3], containing 0.1% (w/v) glucose for *S. obliquus*, a mineral medium as described in [19] for *C. reinhardtii* and the same medium as described above (Section 2.3) for *Cyanidium caldarium*. In each case, either Me-

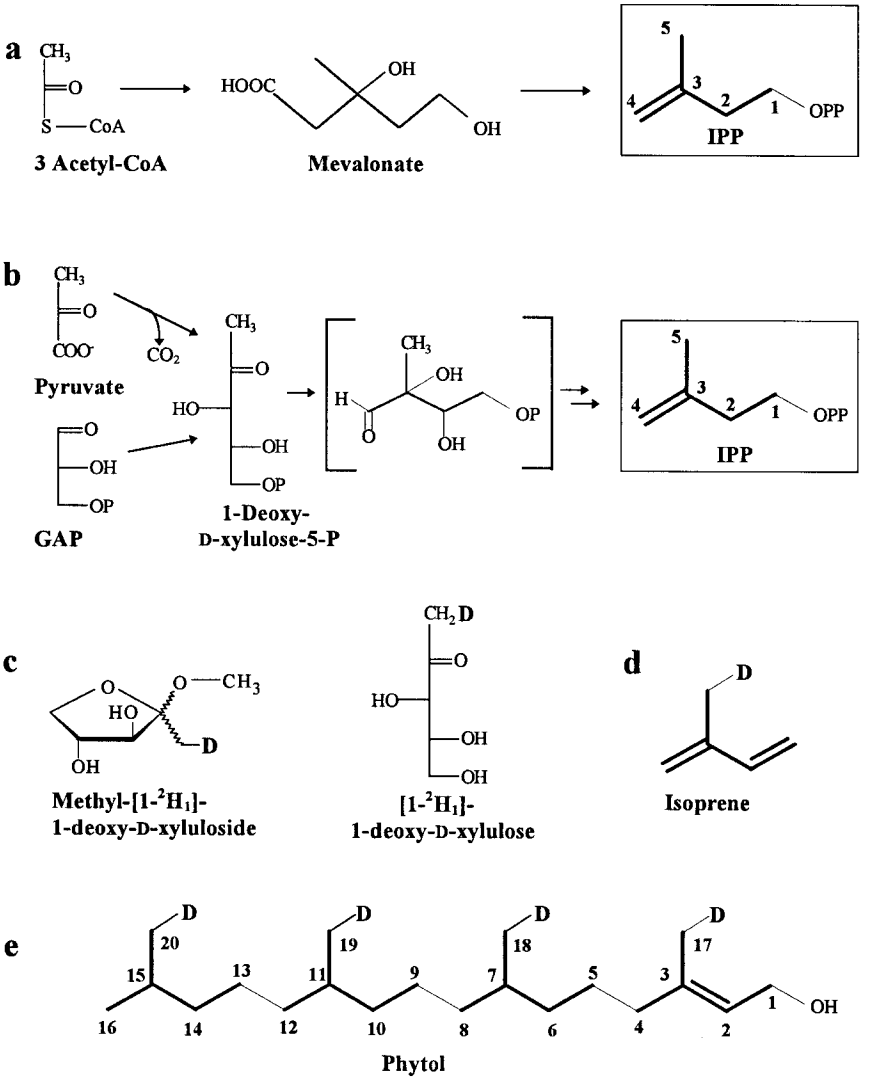


Fig. 1. Formation of isopentenyl pyrophosphate (IPP) via the classical acetate/mevalonate biosynthesis (a) and via the alternative 1-deoxy-xylulose-5-phosphate pathway (b). (c) Structures of the deuterium-labeled precursors which have been applied to higher plants or algae; the label (D) was found n the methyl group of the isoprene emitted (d) and in methyl groups of phytol (e).

[1-$^2H_1$]-dXu (0.05% w/v) or [2-^{13}C]mevalonic acid lactone (0.02% w/v) were filter-sterilized and added to the culture medium.

After 5 days (in the case of *Cyanidium* 14 days) the algal cells (about 50 mg dry weight) were harvested by centrifugation.

2.5. Extraction and purification of phytol and sterols of plants and algae

The plant tissue or the algal cells were extracted several times with methanol. For saponification methanolic KOH was added to a final concentration of 3% KOH. In the case of the large scale culture of *Cyanidium* (cf. Section 2.3) the lipids were fractionated by TLC (silica gel, CH_2Cl_2, two migrations) and only one fraction containing chlorophyll and sterols (R_f 0–0.2) was saponified. Isolation of phytol- and sterol fractions and acetylation was performed as described in [3].

2.6. Analysis of phytyl- and steryl-acetates

The fractions containing the acetates of phytol and sterols were analyzed by GC-MS (Hewlett-Packard 5890 Series II, crosslinked methylsilicone 20 m×0.32 mm, coupled with a Hewlett Packard 5971A Mass Selective Detector). Temperature programming was 80°C for 3 min, heating to 280°C (20°C/min), and 20 min at 280°C. The incorporation of ^{13}C and ^{2}H into phytol and different sterols was determined from the mass spectra which were obtained by GC-MS. The ions phytyl$^+$ (*m/e* = 278.3) and in the case of the sterylacetates e.g. sitosteryl$^+$ at (*m/e* = 396), are accompanied by a cluster of isotopomers which arise from the natural ^{13}C-abundance, and from the additional isotopes when labeled substrates have been incorporated. For a given ion cluster the distribution of the isotope peak intensities can be calculated by combining two binomial distributions: one by considering the natural abundance of ^{13}C (n carbon atoms, probability of 0.011 for occurrence of ^{13}C), and the other relying on the distinct number of C- or H-positions in the molecule prone to incorporation of [2-^{13}C]mevalonic acid or Me-[1-$^2H_1$]-dXu. Comparing the calculated isotope distribution pattern with the one measured from the MS allows to determine the incorporation rates in the range of 2 to 100% for the isoprenoids phytol and several sterols.

Phytylacetate of the 2 l culture of *Cyanidium caldarium* (cf. Section 2.3) was subjected to ^{1}H-NMR analysis on a Bruker AM300 spectrometer in $CDCl_3$ as solvent (cf. Fig. 3).

3. Results and discussion

3.1. Labeling of isoprene

After the deuterium-labeled free sugar [1-$^2H_1$]-dXu was fed to poplar leaves, GC-MS analysis [13] revealed that [1-$^2H_1$]-dXu was incorporated into isoprene to similar degrees of

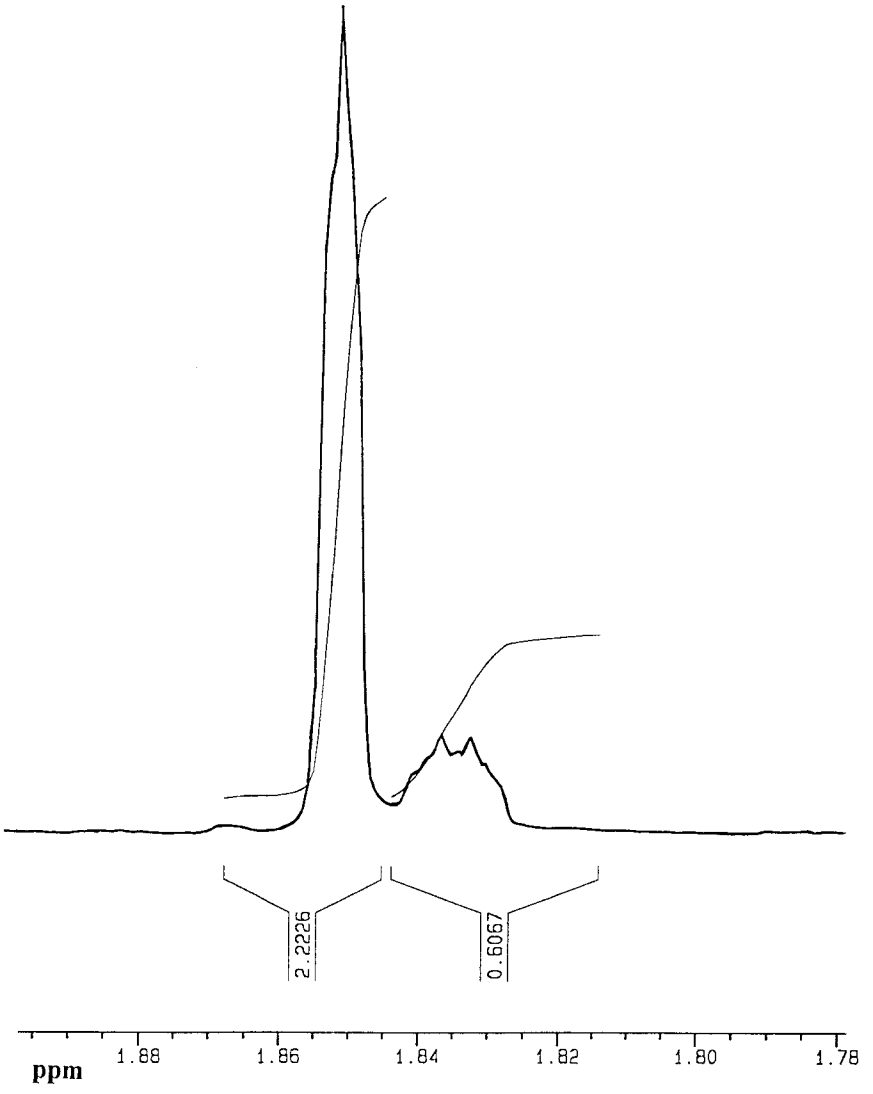
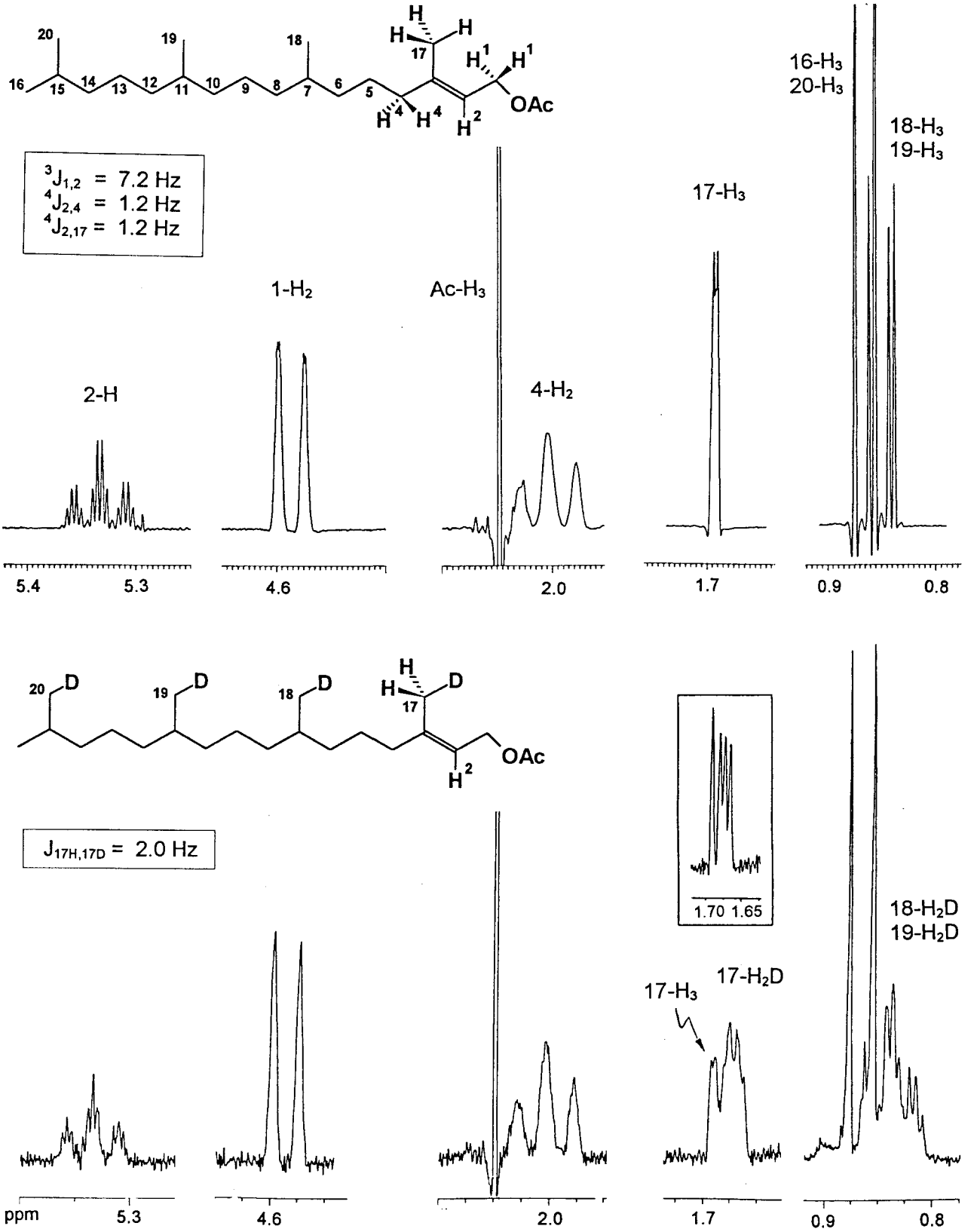


Fig. 2. Methyl group region of the ^{1}H-NMR spectrum of isoprene emitted by poplar leaves after application of Me-[1-$^2H_1$]-dXu via the shoot: the undeuterated CH_3 signal appears at 1.85 ppm as a not fully resolved triplet, whereas the CH_2D signal is slightly shifted to higher field (multiplet centered around 1.83 ppm). From the signal intensities an incorporation rate of 35% of the deuterium labeled precursor into isoprene was estimated.



about 40% as the methyl glycoside, supporting our assumption that applied Me-[1-$^2H_1$]-dXu, which unlike the free dXu is a stable compound, is hydrolyzed to the free sugar before incorporation into isoprene.

After feeding Me-[1-$^2H_1$]-dXu to a cut-off poplar branch, ^{1}H-NMR [20] and GC-MS analysis showed that the volatiles emitted and trapped from the poplar shoot in 3 days consisted of isoprene to at least 99%, confirming the early findings of Sanadze et al. concerning isoprene emission in poplar [21]. Our NMR data showed some 35% of the methyl groups of

Fig. 3. Relevant signals from the ^{1}H-NMR spectrum (300 MHz in $CDCl_3$) of authentic phytyl acetate (top) and the *Cyanidium*-derived sample deuterated upon application of [1-$^2H_1$]-1-deoxy-D-xylulose. The olefinic proton (2-H), through monodeuteration at C-17 (→17-H_2D), changes from a triplet of sextets-multiplicity to a triplet of quintets. In the 17-methyl group (top, δ_H = 1.69, 1.2-Hz doublet) monodeuteration not only entails a shift of the CH_2D-signal to lower δ_H-values (deuterium isotope shift) but an additional 1:1:1-splitting by H-C-D coupling ($^2J_{H,D}$) of 2 Hz – a fact that is nicely corroborated such that the only partly resolved 17-H_2D-multiplet, upon irradiation with the resonance frequency of 2-H, is decoupled to a 1:1:1 triplet, that of the 17-H_3 to a singlet (cf. inset). The degree of monodeuteration at C-17 amounts to 80%, based on the intensities of the 17-H_3 doublet (δ = 1.69) and the 17-H_2D multiplet at δ = 1.67 (1:2.75 ratio of signals, corresponding to 1:4.1 when considering the number of protons). Analogous inferences are derivable from the methyl resonances in the δ = 0.8–0.9 region: the two protons, 7.2-Hz doublet for the magnetically equivalent terminal methyl groups (16-H_3, 20-H_3) appears in the deuterated sample (bottom) with substantially reduced intensity, indicating one of these CH_3 to be extensively monodeuterated (→20-CH_2D), consequently shifted to lower δ_H values and further split by the $^2J_{H,D}$ coupling of 2 Hz. The same holds for the two 7.2-Hz doublets for the 18- and 19-H_3 (top), giving in the deuterated sample (bottom) only partially resolved multiplets, shifted to lower δ_H values. Although the quantitative intensity analysis is less exact here due to signal overlap, an 80% monodeuteration at the 18-, 19-, and 20-methyl groups can be inferred.
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the isoprene emitted to contain one deuterium atom whereas all other carbon atoms were free of deuterium (Fig. 2). A cross-check with GC-MS showed that about 35% of the isoprene molecules were heavier by one mass unit. Both findings indicate that all deuterium-label incorporated from Me-[1-$^2H_1$]-dXu into isoprene is positioned in its methyl group (Fig. 1d) as expected from the biosynthetic scheme (cf. Fig. 1b). As compared to isoprene, a significant labeling of phytol or sterols of the poplar branch from Me-[1-$^2H_1$]-dXu was not detected, indicating that a substantial de novo synthesis of these substances had not occurred during the 3-day incubation period.

Apart from the label of isoprene from Me-[1-$^2H_1$]-dXu, also [2-^{13}C]mevalolactone was applied and incorporated to some extent into isoprene of poplar leaves. A quantitative comparison of incorporation rates between Me-[1-$^2H_1$]-dXu and [^{13}C]MVA is not possible with the leaf incubation system. Since the acetate/mevalonate pathway is localized in the cytosolic compartment of higher plants, a bypass of cytosolic IPP into the plastidic compartment, where the isoprene synthase is located [22], could have occurred. This observation needs therefore further investigation.

3.2. *Specific labeling of phytol in Cyanidium caldarium*

In *Cyanidium*, phytol was labeled to a very high degree (80%) upon application of Me-[1-$^2H_1$]-dXu, as evidenced by MS and ^{1}H-NMR data. In the mass spectrum, the phytyl$^+$ ion had an M^+ higher by four mass units than the unlabeled phytol-derived M^+, attributable to incorporation of four molecules of [1-$^2H_1$]-dXu into the phytol, i.e. into each of the four isoprene units. That these four deuterium atoms reside in the four branched methyl groups of the phytol chain (i.e. 17-, 18-, 19- and 20-CH_2D, Fig. 1e), is unequivocally derived from a comparison of the coupling patterns of the authentic phytyl acetate and of the *Cyanidium*-derived, deuterium-labeled species (Fig. 3). As detailed in the legend of Fig. 3, the extent of deuteration in the branched methyl groups can readily be inferred from the intensities of the respective CH_2D signals, and amounts to a sizable 80% in each of the four positions. Thus the isoprenic units of phytol are labeled by specific incorporation of [1-$^2H_1$]-dXu as it was shown for isoprene (Section 3.1).

3.3. *Incorporation of Me-[1-$^2H_1$]-dXu and [2-^{13}C]MVA into phytol and sterols*

By small scale feeding experiments and GC-MS analysis, phytol of *Cyanidium* again was highly labeled from Me-[1-$^2H_1$]-dXu. In comparison, [2-^{13}C]MVA was almost not incorporated into phytol although it labeled ergosterol at a high degree (Fig. 4, Table 1). This demonstrates that dXu, but not MVA, is a precursor of phytol in *Cyanidium*. In a similar way, phytol of *Lemna gibba* was labeled from Me-[1-$^2H_1$]-dXu (although only to 10%), but not from [2-^{13}C]MVA (Table 1). The latter labeled, however, the *Lemna* sterols at good rates (Table 1).

According to these results it is evident that in *Lemna* and in *Cyanidium* the phytyl side chain of chlorophylls is formed via the deoxy-xylulose phosphate pathway, whereas the sterols are formed via the mevalonate pathway. Since in plants chlorophylls are plastid-bound and sterols are formed in the cytosol/ER compartment [8], the IPP biosynthesis via deoxy-xylulose-5-phosphate seems to be localized in the plastids and thus segregated from the cytosolic IPP biosynthesis (via mevalo-

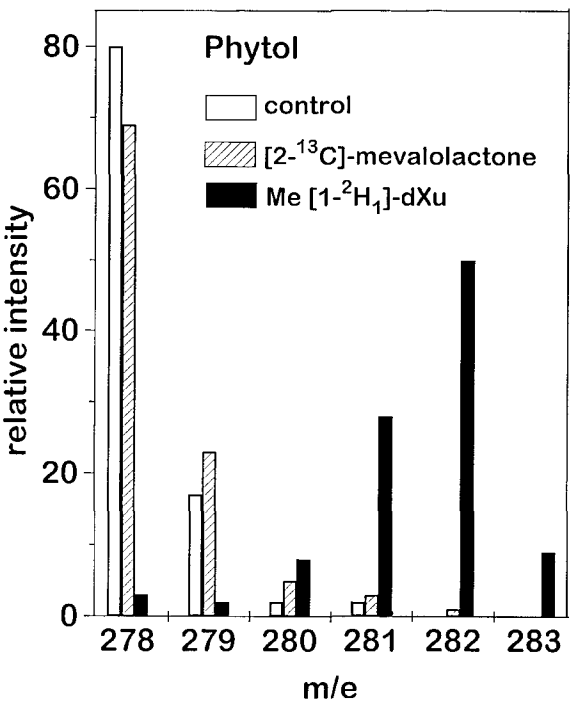

Fig. 4. Labeling of phytol of the red alga *Cyanidium caldarium* from [2-^{13}C]mevalolactone and Me-[1-$^2H_1$]-dXu as shown by a section of the mass spectra of phytylacetate. When grown on [2-^{13}C]-mevalolactone the isotope peaks 279.3, 280.3 and 281.3 are significantly enhanced (hatched bars) as compared to the unlabeled control (white bars). In contrast, after feeding Me-[1-$^2H_1$]-dXu, a very strong shift of the mass peaks of phytol by four mass units was observed with the highest intensity at mass peak 282.3 (black bars). This indicates that phytol is highly labeled (80%) and that four deuterium from the applied precursor have been incorporated into the phytol molecules.

Table 1
Percentage incorporation of the applied deuterium and [^{13}C]-labeled precursors Me-[1-$^{2}H_1$]-1-deoxy-D-xyluloside and [2-^{13}C]mevalolactone into the plastidic isoprenoid phytol and the cytosolic sterols

	Percentage incorporation of the applied	
	Me-[1-$^{2}H_1$]-dXu	[2-^{13}C]mevalolactone
Cyanidium caldarium		
Phytol	**80–85**	3–5
Ergosterol	**40–60**	**65–95**
Lemna gibba		
Phytol	**8–10**	0–1
Sitosterol	0–1	**5–10**
Stigmasterol	0–1	**5–10**
Scenedesmus obliquus		
Phytol, Chondrillasterol,	**8–12**	0
Ergost-7-enol	**8–12**	0
Chlamydomonas reinhardtii		
Phytol, Ergosterol,	**5–10**	0
7-Dehydroporiferasterol	**5–10**	0

The incorporation rates were estimated from the relative intensities of isotope peaks in the mass spectra (see Section 2 and compare Fig. 4).

nate). The same was shown in earlier incorporation studies: after growth of *Lemna* on [1-^{13}C]glucose, the plastidic isoprenoids (phytol, β-carotene, lutein, plastoquinone-9) exhibited a ^{13}C-labeling pattern which is characteristic of the alternative IPP pathway while the cytosolic sterols were labeled according to the mevalonate pathway [12]. The same difference in labeling pattern has recently been shown also for phytol and ergosterol of *Cyanidium* grown on [1-^{13}C]glucose [23].

Whether an exchange of IPP between the plastidic and the cytosolic compartments is possible, as revealed by the high labeling degree of ergosterol from dXu in *Cyanidium* (40–60%), which is, however lower than that of phytol (Table 1), is a matter of further research.

Unlike *Lemna* and *Cyanidium*, in the green algae *Scenedesmus* and *Chlamydomonas* the applied Me-[1-$^{2}H_1$]-dXu labeled both phytol and sterols to about the same degree in the range of 5–12% (Table 1). By administration of [2-^{13}C]MVA, in turn, no incorporation into phytol or into sterols was found (Table 1). Thus, in both green algae, dXu appears to be the IPP precursor instead of MVA. This is in agreement with earlier results: plastidic isoprenoids of *Scenedesmus* (phytol, β-carotene, lutein, plastoquinone-9) as well as the cytosolic sterols have been labeled from [1-^{13}C]glucose according to the alternative IPP pathway [3]. So far no evidence for a cytosolic mevalonate pathway in *Scenedesmus* or *Chlamydomonas* has been found.

While *Populus* and *Cyanidium* exhibited good incorporation of Me-[1-$^{2}H_1$]-dXu into isoprenoids (35% into isoprene and 80% into phytol, respectively), *Lemna*, *Scenedesmus* und *Chlamydomonas* did obviously not incorporate dXu at such high degrees. This may be due to the ability of these organisms to transform Me-dXu to dXu and finally dXu to dXu-5-phosphate, the actual intermediate of the alternative IPP pathway.

To summarize, the utilization of 1-deoxy-D-xylulose in isoprenoid biosynthesis, as demonstrated here for higher plants (*Populus*, *Lemna*), a red alga (*Cyanidium*) and two green algae (*Scenedesmus*, *Chlamydomonas*), provides ample evidence that this pentulose is a real precursor of IPP (rather than a precursor-related metabolite), which in turn proves this mevalonate-independent *1-deoxy-xylulose-5-phosphate pathway* to be widely distributed among photosynthetic organisms as a fully established biosynthetic route complementary – and conceivably even in competition – with the classical acetate/mevalonate elaboration of IPP.

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