Available online at www.sciencedirect.com
SCIENCE DIRECT®
PERGAMON
Phytochemistry 64 (2003) 199–207
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Early steps of deoxyxylulose phosphate pathway in chromoplasts of higher plants

Monika Fellermeier[a], Silvia Sagner[a], Peter Spiteller[b], Michael Spiteller[c], Meinhart H. Zenk[a,*]

[a]*Biozentrum der Universität Halle, Weinbergweg 22, D-06120 Halle, Germany*
[b]*Institut für Organische Chemie, Universität München, Butenandtstrasse 5-13, D-81377 München, Germany*
[c]*Institut für Umweltforschung, Universität Dortmund, Otto-Hahn-Strasse 6, D-44221 Dortmund, Germany*

Received 13 January 2003; received in revised form 10 February 2003

Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

1-Deoxy-D-xylulose 5-phosphate and 2*C*-methyl-D-erythritol 4-phosphate have been shown as intermediates of the deoxyxylulose phosphate pathway used for terpenoid biosynthesis in plants and many microorganisms. In plants this non-mevalonate pathway is located in plastids. In order to investigate the formation of five carbon intermediates, chromoplasts from *Capsicum annuum* and *Narcissus pseudonarcissus* were incubated with isotope-labeled 1-deoxy-D-xylulose 5-phosphate or 2*C*-methyl-D-erythritol 4-phosphate. The downstream metabolites were detected and separated by reversed-phase ion-pair radio-HPLC and their structures elucidated by mass spectroscopy. Here we report the isolation and structural identification of 4-diphosphocytidyl-2*C*-methyl-D-erythritol and 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate from chromoplasts; the genes of the corresponding enzymes had been previously identified from *Escherichia coli* and *Arabidopsis*.

Keywords: *Capsicum annuum*; Solanaceae; *Narcissus pseudonarcissus*; Amaryllidaceae; Chromoplast; Isoprenoid biosynthesis; Deoxyxylulose phosphate pathway; New intermediates

1. Introduction

In plants two different pathways are utilized in parallel for the biosynthesis of isopentenyl diphosphate and dimethylallyl diphosphate, the universal precursors of all terpenoids. The classical acetate-mevalonate pathway operates in the cytosol, whereas the recently discovered deoxyxylulose phosphate (**3**) (DXP) pathway is present in the plastids of plant cells. While the enzymology and regulation of the acetate-mevalonate pathway have been well studied, particularly in mammalian systems, the DXP pathway, which is present in various eubacteria, cyanobacteria, *Streptomyces*, algae, liverworts and higher plants, is only beginning to be understood in its reactions (for review, see Eisenreich et al., 1998, 2001; Lichtenthaler, 1999; Rohmer, 1999; Schwarz and Arigoni, 1999). The DXP pathway, however, is of fundamental importance for plants, since its products play numerous functional roles as hormones (e.g. abscisic acid, zeatin) (Milborrow and Lee, 1998; Krall et al., 2002), photosynthetic pigments (phytol side chain of chlorophyll, carotenoids) (Arigoni et al., 1997; Lichtenthaler et al., 1997) and electron carriers (side chain of plastoquinone) (Disch et al., 1998). Moreover many of the terpenoid secondary metabolites, synthesized *via* the DXP pathway, are defense compounds (Piel et al., 1998) or of commercial interest, such as paclitaxel (Eisenreich et al., 1996), steviol (Totté et al., 2000), hyperforin (Adam et al., 2002), the bitter acids humulone and lupulone from hops (Goese et al., 1999) as well as essential oils (Eisenreich et al., 1997) and many others.

The initial product, 1-deoxy-D-xylulose 5-phosphate (**3**), which is also utilized for both thiamine (vitamin B_1) (White, 1978) and pydridoxal (vitamin B_6) (Hill et al., 1996; Cane et al., 1999, 2000) biosynthesis, is formed by the condensation of pyruvate (**1**) with glyceraldehyde 3-phosphate (**2**)

* Corresponding author. Tel.: +49-345-5525-064; fax: +49-345-5527-301.
E-mail address: zenk@biozentrum.uni-halle.de (M.H. Zenk).

0031-9422/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0031-9422(03)00197-3

(Sprenger et al., 1997; Bouvier et al., 1998; Lange et al., 1998; Lois et al., 1998; Kuzuyama et al., 2000b). Subsequently DXP (**3**) is transformed by the action of a specific reductoisomerase to 2*C*-methyl-D-erythritol 4-phosphate (**4**) (MEP) (Takahashi et al., 1998; Lange and Croteau, 1999; Jomaa et al., 1999; Schwender et al., 1999) (Fig. 1). The availability of both early intermediates in isotope-labeled form (Hecht et al., 2001a,b) made it possible to investigate the further metabolism of these precursors in the chromoplast compartment. Chromoplasts from *Capsicum* and *Narcissus* have been chosen as experimental tools, since they have been previously shown to be an excellent organelle to study the biosynthesis of carotenoids (Camara, 1985, 1993; Kleinig and Beyer, 1985). It could be demonstrated that chromoplasts of *C. annuum* and *N. pseudonarcissus* contain the enzymes of the DXP pathway to transform the first intermediate DXP (**3**) to 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate (cMEPP) (**7**) (Fellermeier et al., 2001) with an additional three intermediates being passed through. All these enzymes are soluble and localized exclusively in the stroma of the chromoplasts of these plants. The main intermediate between MEP (**4**) and cMEPP (**7**) was identified as 4-diphosphocytidyl-2C-methyl-D-erythritol (CDP-ME) (**5**) by mass spectroscopy.

2. Results and discussion

2.1. Transformation of radio-labeled DXP (3) and MEP (4) to carotenoids by chromoplasts from C. annuum

As it had been shown previously (Fellermeier et al., 1999), chromoplasts are capable of transforming radio-labeled DXP (**3**) and MEP (**4**) efficiently into phytoene, β-carotene and geranyl geraniol, which are the predominant terpenoids being synthesized from IPP (**9**) in *Narcissus* and *Capsicum* chromoplasts (Kleinig and Beyer, 1985; Camara et al., 1983). This transformation reaction was clearly ATP dependent and omission of ATP significantly reduced the conversion of both labeled substrates, DXP (**3**) and MEP (**4**), into terpenoids. In order to investigate the nucleotide dependence of this reaction in more detail, [1,2-$^{14}C_2$]DXP (**3**) was incubated with chromoplasts from *C. annuum* in the presence of Mg^{2+} and Mn^{2+} with various nucleoside triphosphates and also in combination with each other. While ATP and CTP supported the incorporation of labeled DXP (**3**) reasonably well into the carotene fraction, GTP, ITP, TTP and UTP enhanced the synthesis of carotenoids only very little, if at all (Fig. 2). Moreover, the simultaneous inclusion of ATP and CTP in the incubation mixture resulted in a synergistic effect on the synthesis of carotenoids and their precursors. As demonstrated already previously (Fellermeier et al., 1999), a direct involvement

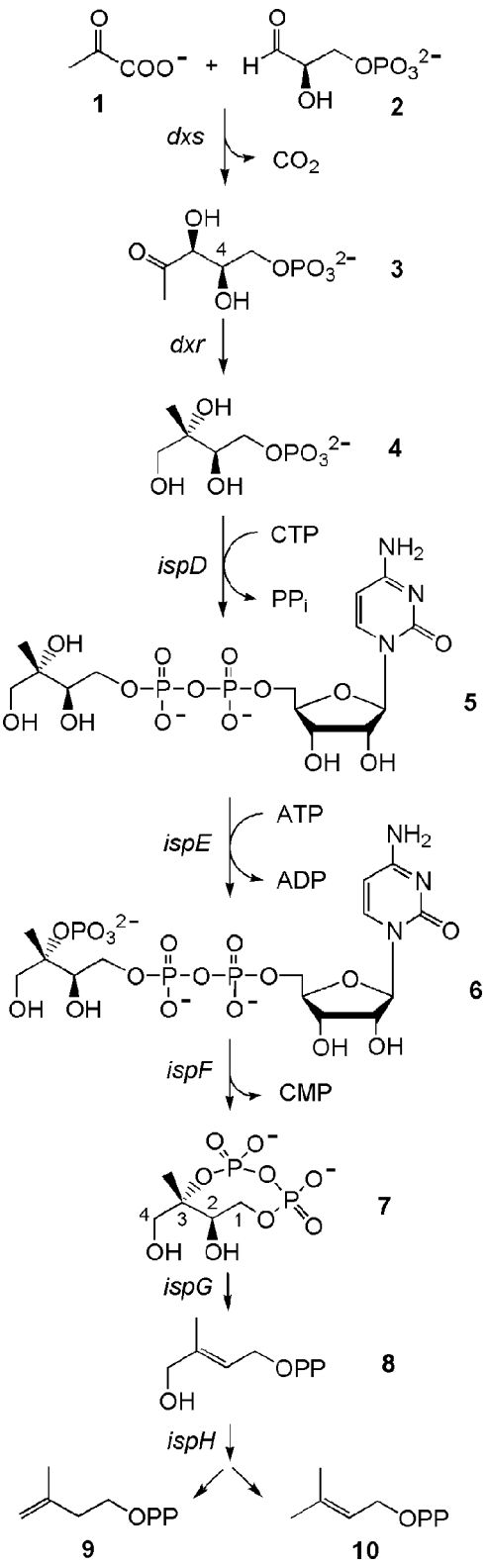

Fig. 1. The deoxyxylulose phosphate pathway of isoprenoid biosynthesis. **1**, Pyruvate; **2**, D-Glyceraldehyde 3-phosphate; **3**, 1-Deoxy-D-xylulose 5-phosphate; **4**, 2*C*-Methyl-D-erythritol 4-phosphate; **5**, 4-Diphosphocytidyl-2*C*-methyl-D-erythritol; **6**, 4-Diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate; **7**, 2*C*-Methyl-D-erythritol 2,4-cyclodiphosphate; **8**, (*E*)-4-Hydroxy-3-methylbut-2-enyl diphosphate; **9**, Isopentenyl diphosphate; **10**, Dimethylallyl diphosphate.

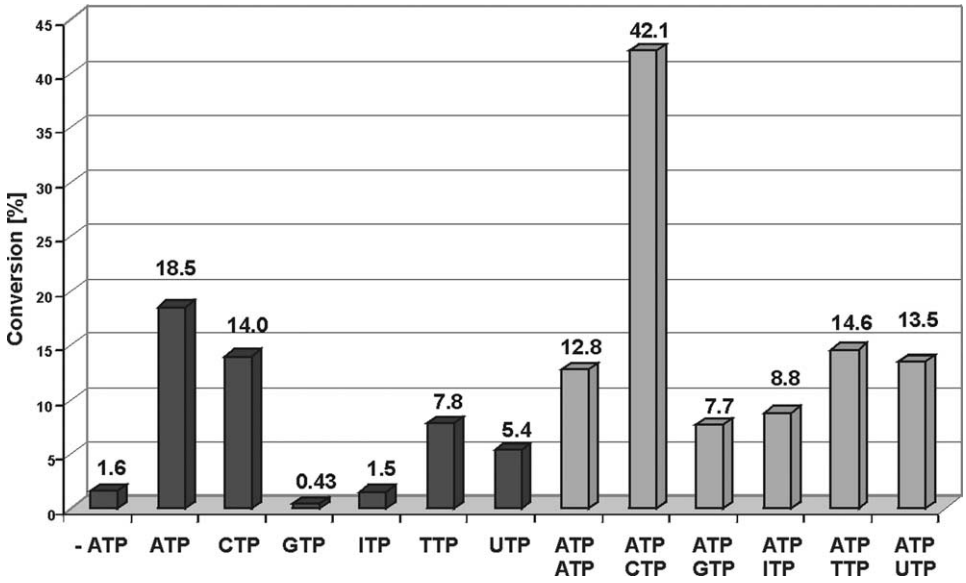

Fig. 2. Incorporation of [1,2-$^{14}C_2$]DXP into the carotene fraction by chromoplasts of *C. annuum* in the presence of various nucleoside triphosphates. Incubations were performed as described under Experimental. Assay mixtures containing 0.1 μCi [1,2-$^{14}C_2$]DXP (62.5 mCi mmol^{-1}), 6 mM of a nucleoside triphosphate (as indicated) or 6 mM of ATP together with 3 mM of a nucleoside triphosphate (as indicated) were incubated for 12 h.

of the cofactors NADP, NADPH and FAD in the enzyme reactions could not be established. Since NADPH is known to be a cofactor for the conversion of DXP (**3**) to MEP (**4**), by the reductoisomerase, this pyridine nucleotide is most likely available in the chromoplast suspension or stroma fraction at saturating concentration or is generated from NADP by enzymes of the oxidative pentose phosphate pathway (Camara et al., 1995).

2.2. Search for intermediates of the DXP pathway

In order to search for downstream intermediates of DXP (**3**) and MEP (**4**) carrying phosphate ester groups, a reversed-phase (C8) ion-pair radio-HPLC method as previously described (Beyer et al., 1985; McCaskill and Croteau, 1993) was developed. Enzyme assays with chromoplasts containing [^{14}C]- or [^{3}H]-labeled substrates and various cofactors as well as ATP were vigorously extracted with ethyl acetate after an incubation period of 4 h to remove any unphosphorylated labeled terpenoid products, while phosphate esters were expected to remain in the aqueous phase and thus should be detected and monitored by radio-HPLC.

The metabolism of both substrates, [1,2-$^{14}C_2$]DXP (**3**) and [1-^{3}H]MEP (**4**), by the chromoplast systems of *C. annuum* (Fig. 3A, B) and *N. pseudonarcissus* (Fig. 3C, D) yielded in the presence of ATP two unknown compounds, coded M38 and M59, which were strongly labeled.

As mentioned above, ATP and CTP increased the incorporation of [1,2-$^{14}C_2$]DXP (**3**) into carotenoids synergistically. Accordingly the influence of these nucleotides on the formation of the phosphate esters was studied, using a stroma fraction of *Capsicum* chromoplasts depleted of cofactors. While in the presence of ATP only M38 and M59 were formed (Fig. 4A), the addition of CTP led to the formation of a new compound with a retention time shorter than M38, designated M35 (Fig. 4B). The simultaneous addition of ATP and CTP produced high levels of M38 (Fig. 4C). M59 was not formed in the presence of CTP (Fig. 4B and 4C) and none of the other nucleotides (GTP, ITP, TTP or UTP) yielded M35 either (data not shown).

In order to ascertain, that M35, M38 and M59 were true intermediates of the DXP pathway, they were isolated from enzyme assays containing either 50 μCi [1-^{3}H]MEP (**4**) and 0.04 mM CTP (in case of M35), 50 μCi [1-^{3}H]MEP (**4**) and 6 mM ATP (in case of M38) and stromal extracts of *Capsicum* chromoplasts, equivalent to 1 mg of protein or 120 μCi [1-^{3}H]MEP (**4**) and 6 mM ATP (in case of M59) and stromal extracts of *Capsicum* chromoplasts, equivalent to 2.4 mg of protein. After an incubation period of 4 h isolation of the various metabolites was performed by paper chromatography and subsequently their purity checked by reversed-phase ion-pair radio-HPLC. Through application of the metabolites M35, M38 and M59 to intact chromoplasts of *C. annuum* incorporation into carotenoids should be demonstrated. M35 could be isolated in 33%, M38 in 37% and M59 in 10.8% total yield. The specific activity of the individual unknown metabolites was assumed to be that of the substrate [1-^{3}H]MEP (**4**): 751 mCi mmol^{-1} for M35 and M38 and 31.9 mCi mmol^{-1} for M59. Incubation of the isolated metabolites (each 5 μCi) with chromoplasts of *C. annuum* for 12 h showed that both M35 and M38 were converted into carotenoids with a yield of 11% and 10% respectively, while the conversion of M59 was, if at

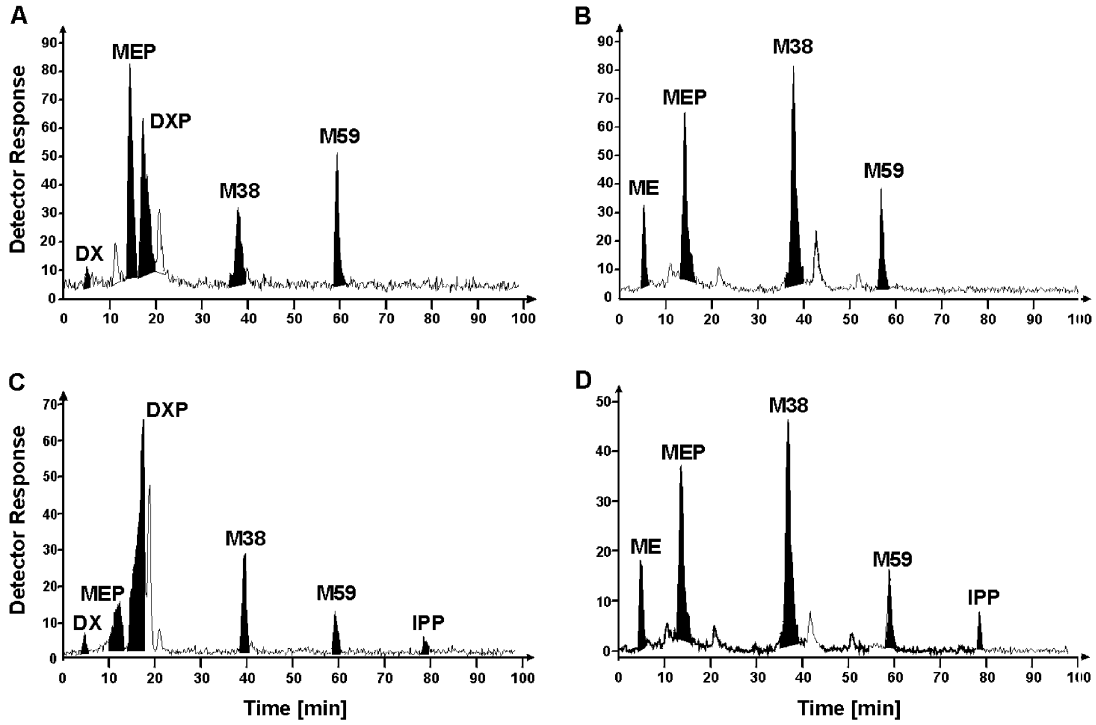

Fig. 3. Analysis by radio-HPLC of water-soluble metabolites derived from 0.1 μCi [1,2-$^{14}C_2$]DXP (62.5 mCi mmol^{-1}) (A, C) and 5 μCi [1-^{3}H]MEP (751 mCi mmol^{-1}) (B, D) after incubation with chromoplasts of *Capsicum annuum* (A, B) or *Narcissus pseudonarcissus* (C, D) for 4 h. Buffer and cofactors were as described in the Experimental section. The peaks are DX, 1-deoxy-D-xylulose; DXP, 1-deoxy-D-xylulose 5-phosphate; ME, 2*C*-methyl-D-erythritol; MEP, 2*C*-methyl-D-erythritol 4-phosphate; M38/cMEPP, 2*C*-Methyl-D-erythritol 2,4-cyclodiphosphate; M59/ADP-ME, 4-Diphosphoadenyl-2*C*-methyl-D-erythritol; IPP, isopentenyl diphosphate.

all, below 1%. Analysis of the aqueous phases by reversed-phase ion-pair radio-HPLC showed, that M35 had been quantitatively converted to M38, the amount of the substrate M38 decreased compared to that at time zero of the experiment; M59, however, was not transformed at all. The non-incorporation of M59 into carotenoids and other metabolites made it very likely that M59 is a dead-end product. It was shown later by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy after application of [^{13}C]-labeled MEP, that this compound (M59) is 4-diphosphoadenyl-2*C*-methyl-D-erythritol (ADP-ME) (Fellermeier, 2000). The efficient incorporation of both M35 and M38 into carotenoids, however, and the ATP-dependent conversion of M35 to M38 in the chromoplast as well as the stroma preparation, provided evidence that these two compounds are possible intermediates of the non-mevalonate pathway.

2.3. Structure elucidation of M35

In order to obtain structural information about M35, incubations with CTP, radio-labeled in different molecular positions, were performed. Enzyme assays with a stroma preparation of *Capsicum* chromoplasts in the presence of unlabeled **4** and [γ-^{32}P]CTP surprisingly yielded no radioactivity in M35 upon separation with radio-HPLC. Addition of [α-^{32}P]CTP as well as [5-^{3}H]CTP, however, resulted in strong labeling of M35. This suggested that at least the entire CMP moiety of the proffered CTP was incorporated into M35. To unequivocally determine the structure of this new metabolite, the product obtained by incubation of MEP (**4**) and CTP with a stroma preparation was isolated chromatographically and analyzed by mass spectrometry.

Assay mixtures containing 20 μmol of **4** (200 μCi [1-^{3}H]MEP), 1 mM CTP and 8 mg of stromal protein were incubated for 4 h. Using solid phase extraction with a strong basic anion exchange matrix, as described in the Experimental section, careful washing and elution with NH_4HCO_3 solution allowed an enrichment of the nucleotide bound compound M35. Further purification was achieved by paper chromatography yielding 3.8 μmol of M35 (19%). M35 (250 nmol) was hydrolyzed by immobilized alkaline phosphatase, the product treated with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) (20 μl, 40 °C for 60 min) after lyophilization and subsequently analyzed by combined capillary GC-MS. It could be demonstrated that it possesses the identical retention time (14.35 min in case of 3-fold silylation and 15.54 min in case of 4-fold silylation) and mass spectrum as an authentic sample of silylated 2*C*-methyl-D-erythritol. The 4-fold silylated compound showed the following EIMS 70 eV, *m*/*z* (rel. int.): 321

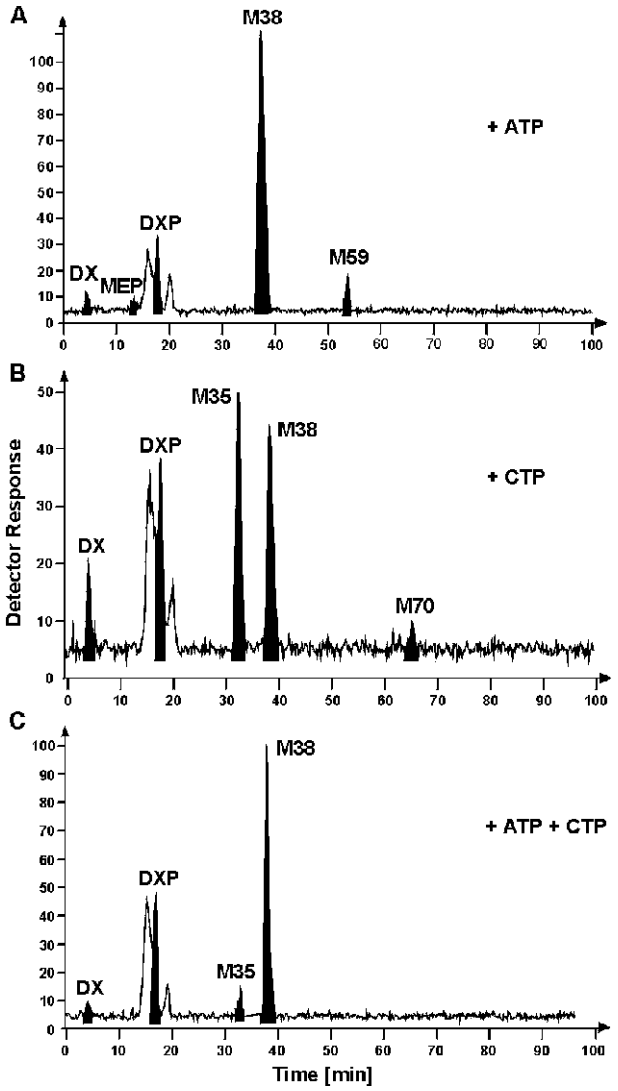

Fig. 4. Radio-HPLC separation of water-soluble metabolites after incubation of a stromal extract of *Capsicum chromoplasts* with 0.1 μCi [1,2-$^{14}C_2$]DXP (62.5 mCi mmol^{-1}), various nucleoside triphosphates (concentration each 6 mM) and NADPH (1 mM) for 4 h. Trace A: ATP, trace B: CTP, trace C: ATP + CTP. The other cofactors were as described under Experimental. The peaks are DX, 1-deoxy-D-xylulose; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2*C*-methyl-D-erythritol 4-phosphate; M35/CDP-ME, 4-Diphosphocytidyl-2*C*-methyl-D-erythritol; M38/cMEPP, 2*C*-Methyl-D-erythritol 2,4-cyclodiphosphate; M59/ADP-ME, 4-Diphosphoadenyl-2*C*-methyl-D-erythritol; M70/CDP-MEP, 4-Diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate.

(13) $[M-CH_2OSiMe_3]^+$, 262 (3) $[M-Me_3SiOSiMe_3]^+$, 219 (100) $[(Me_3SiOCH_2)C(Me)(OSiMe_3)]^+$, 189 (4) $[M-Me_3SiOSiMe_3-SiMe_3]^+$, 147 (16) $[Me_2SiOSiMe_3]^+$, 129 (6) $[219-HOSiMe_3]^+$, 117 (12), 103 (2) $[CH_2OSiMe_3]^+$, 73 (12) $[SiMe_3]^+$. Clearly, the compound M35 contains a 2C-methyl-D-erythritol moiety. The purified compound was also subjected to electrospray ionisation mass spectrometry (ESI-MS). The negative ESI-MS afforded for M35 a $[M-H]^-$ ion at *m/z* 520 corresponding to a molecular mass of 521. The MS/MS spectrum for the $[M-H]^-$ ion of M35 showed significant fragment ions at *m/z* 384 $[M-H-136\ (C_5H_{12}O_4)]^-$, *m/z* 277 $[M-H-243\ (C_9H_{13}N_3O_5)]^-$ and *m/z* 197 $[M-H-323\ (C_9H_{14}N_3O_8P)]^-$ and an additional signal at *m/z* 322 which was derived from $[CMP-H]^-$. On the basis of these data, the structure of M35 was assigned as 4-diphosphocytidyl-2*C*-methyl-D-erythritol (CDP-ME) (**5**) (see Fig. 1). This biosynthetic transformation was also shown to occur in extracts of *E. coli* and the corresponding gene (*ispD*) was cloned and the product characterized (Rohdich et al., 1999). It was this synergistic effect of CTP on the ATP dependent conversion of DXP (**3**) to cMEPP (**7**), shown above, and the transient accumulation of the new intermediate M35 (Fig. 4), which led colleagues at the Technical University Munich, to search for the underlying gene (*ispD*) catalyzing this CTP dependent reaction (Rohdich et al., 1999).

2.4. Structure elucidation of M38

Large scale incubations (30 ml) with [U-$^{13}C_5$]MEP (**4**) (18 μmol; 300 μCi [1-^{3}H]MEP) in the presence of ATP (3 mM), CTP (0.5 mM) and a soluble stroma protein fraction of chromoplasts (12 mg of protein) led to the formation of 13.7 μmol (76.1%) of the unknown metabolite M38 (incubation period 12 h). This compound was isolated by solid phase extraction using a strong basic anion exchange matrix and subsequent paper chromatography as described for M35, and identified by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy as cMEPP (**7**) (Fellermeier et al., 2001; Fellermeier, 2000).

This cyclic diphosphate had been found in parallel investigations in bacteria to be a prominent metabolite of the DXP pathway as the product of the IspF protein in *E. coli* (Herz et al., 2000). Moreover this compound had been known to occur in several microorganisms and was claimed to be a product accumulating under "oxidative stress conditions" (Ostrovsky et al., 1992, 1998).

Lüttgen et al. (2000) demonstrated, and this was later corroborated by Kuzuyama et al. (2000a), that there is another enzyme specified by the *ispE* gene, catalyzing the phosphorylation of the tertiary hydroxyl group of CDP-ME (**5**), yielding 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate (CDP-MEP) (**6**) (see Fig. 1). This intermediate could also be detected by reversed-phase ion-pair radio-HPLC, when incubating [1,2-^{14}C]DXP (**3**) or [1-^{3}H]MEP (**4**) with chromoplasts, but attempts to isolate this intermediate failed, because the amounts were too low (see Fig. 4B, M70).

In detailed separate studies, [^{13}C]- and [^{14}C]-labeled 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate, cMEPP (**7**), was shown to be incorporated efficiently (up to 90%) into phytoene (Fellermeier et al., 2001). Thus this metabolite is clearly an intermediate of the DXP pathway in higher plants as well.

A further downstream intermediate from the cyclic diphosphate cMEPP (**7**) to IPP/DMAPP (**9**/**10**), has

recently been described for the chromoplast system of *Narcissus* and *Capsicum* (Gao et al., 2002). (*E*)-4-Hydroxy-3-methylbut-2-enyl diphosphate (**8**) is a transient intermediate, directly derived from cMEPP (**7**), which is subsequently further dehydroxylated to yield IPP/DMAPP (**9/10**). Thus, the deoxyxylulose phosphate pathway in higher plants seems to be completed at the level of intermediates (Fig. 1).

Since this biosynthetic pathway is present in many eubacteria and higher plants, but absent in mammalian systems, it represents a novel target for antibacterial drugs and herbicides. Inhibitors of the reductoisomerase have been shown recently to be potent herbicides (Zeidler et al., 1998) as well as antimalarial drugs (Jomaa et al., 1999). The characterization of this pathway at the molecular level provides new possibilities for the bioengineering of terpenoid formation in plants (Mahmoud and Croteau, 2001).

3. Experimental

3.1. Plant material

Fruits of red pepper, *C. annuum* and flowers of *N. pseudonarcissus* were purchased at local markets.

3.2. Chemicals

2*C*-Methyl-D-erythritol 4-phosphate was prepared as described by Kis et al. (2000). The preparation of [1,2-$^{14}C_2$]1-deoxy-D-xylulose 5-phosphate (sp. act. 62.5 mCi mmol^{-1}), [1-^{3}H]2*C*-methyl-D-erythritol 4-phosphate (sp. act. 751 mCi mmol^{-1}) and [U-$^{13}C_5$] 2*C*-methyl-D-erythritol 4-phosphate has been described (Hecht et al., 2001a,b).

[5-^{3}H]Cytidine 5′-triphosphate, ammonium salt (sp. act. 24.0 Ci mmol^{-1}) was purchased from Amersham Pharmacia Biotech, [α-^{32}P]cytidine 5′-triphosphate, tetra-triethylammonium salt (sp. act. >3,000 Ci mmol^{-1}) was from ICN and [γ-^{32}P]adenosine 5′-triphosphate (sp. act. 7,000 Ci mmol^{-1}) was purchased from NEN.

Preparation of [γ-^{32}P]CTP. A reaction mixture containing 50 mM Tris HCl, pH 7.6, 10 mM $MgCl_2$, 0.5 mM cytidine 5′-diphosphate, 0.07 μM [γ-^{32}P]adenosine 5′-triphosphate (sp. act. 7,000 Ci mmol^{-1}) and 1 unit of nucleoside 5′-diphosphate kinase was incubated at 25 °C. After 1 h the reaction was completed and the enzyme removed by ultrafiltration with centrifugal filter tubes with a 30 kDa cut-off (Eppendorf).

3.3. Enzymes

Nucleoside 5′-diphosphate kinase and alkaline phosphatase from bovine intestinal mucosa, attached to beaded agarose, were from Sigma.

3.4. Preparation of chromoplasts and stroma fraction

Isolation of chromoplasts from *Capsicum annuum*. Chromoplasts were isolated according to the method of Camara with slight modifications (1985, 1993). Pericarp of red pepper (500 g) was homogenized at 4 °C in 600 ml of isolation medium (50 mM Hepes, pH 8.0, containing 1 mM DTE, 1 mM EDTA and 0.4 M sucrose). The suspension was filtered through four layers of nylon cloth (50 μm) and centrifuged (10 min, 4,500 rpm, GSA rotor) to obtain a pellet of crude chromoplasts, which was resuspended in 400 ml of isolation medium and centrifuged again (10 min, 4,500 rpm, GSA rotor). The supernatant was discarded. The pellet of chromoplasts was homogenized and resuspended in 3 ml of 50 mM Hepes, pH 7.6, containing 1 mM DTE. The suspension was filtered through one layer of nylon cloth (50 μm).

Preparation of stroma subfraction from *Capsicum* chromoplasts. A suspension of washed chromoplasts (about 5 ml, protein concentration 10–15 mg ml^{-1}) was diluted with 50 mM Hepes, pH 7.6, containing 1 mM DTE to a final volume of 40 ml. The mixture was kept for 10 min at 4 °C and then centrifuged (60 min, 35,000 rpm, Ti 50 rotor). The supernatant was applied to PD-10 columns (Sephadex G 25, Pharmacia), which had been equilibrated with 50 mM Hepes, pH 7.6, containing 1 mM DTE. The columns were developed with the same buffer. Fractions were combined and concentrated using a Centriprep-10 concentrator (Amicon). The final protein concentration was about 1–2 mg ml^{-1}.

Isolation of chromoplasts from *Narcissus pseudonarcissus*. The isolation followed a procedure described by Kleinig and Beyer (1985). Inner coronae of *N. pseudonarcissus* (80 g) were homogenized in 250 ml of 67 mM Tris HCl, pH 7.5, containing 5 mM $MgCl_2$, 1 mM DTE, 1 mM EDTA, 0.2% (w/v) polyvinylpyrrolidone K90 and 0.74 M sucrose. The suspension was filtered (3 layers of nylon cloth, 50 μm) and centrifuged (5 min, 3,000 rpm, GSA rotor). The supernatant was centrifuged again (20 min, 12,500 rpm, GSA rotor) to obtain a pellet of crude chromoplasts, which was resuspended in 2 ml of 67 mM Tris HCl, pH 7.5, containing 5 mM $MgCl_2$, 1 mM DTE and 50% (w/v) sucrose. The suspension was filtered through one layer of nylon cloth (50 μm). Aliquots (2 ml) of the chromoplast suspension were transferred to centrifuge tubes and overlaid with equal volumes of 40, 30 and 15% (w/v) sucrose in 67 mM Tris HCl, pH 7.5, containing 5 mM $MgCl_2$ and 1 mM DTE. Subsequent to centrifugation (60 min, 19,000 rpm, SW28 rotor) the fractions of intact chromoplasts at the 40/30% interphase were collected and diluted with 67 mM Tris HCl, pH 7.5, containing 5 mM $MgCl_2$ and 1 mM DTE to a final sucrose concentration of 15% (w/v). The suspension was centrifuged (20 min, 14,500 rpm, SS34 rotor) and the pellet was suspended in 2 ml of 67 mM Tris HCl, pH 7.5, containing 5 mM $MgCl_2$ and 1 mM DTE.

3.5. Enzyme assays

Enzyme assays with chromoplasts. The standard assay mixtures contained in a total volume of 500 μl (unless otherwise stated): 100 mM Hepes, pH 7.6, 2 mM $MnCl_2$, 10 mM $MgCl_2$, 5 mM NaF, 2 mM NADP, 1 mM NADPH, 6 mM ATP, 20 μM FAD and chromoplasts equivalent to 1 or 2 mg of protein. 0.1 μCi of [^{14}C]-labeled or 5 μCi of [^{3}H]-labeled substrates were added. The mixtures were incubated at 30 °C for various periods of time. The reaction was terminated by ethyl acetate extraction. The lipid extract was evaporated to dryness, the residue dissolved in a definite volume of ethyl acetate and an aliquot used for determination of the incorporated radioactivity by liquid scintillation counting (LS 6500, Beckmann). TLC of the lipid extract assured, that radioactivity resides entirely in the carotenoid fraction (Fellermeier et al., 1999). The remaining aqueous phase was analyzed by reversed-phase ion-pair radio-HPLC as described in Section 3.7.

Enzyme assays with stromal extracts from *Capsicum* chromoplasts. Assay mixtures contained in a total volume of 500 μl (unless otherwise stated): 50 mM Hepes, pH 7.6, 2 mM $MnCl_2$, 10 mM $MgCl_2$, 5 mM NaF and 200 μg of stromal protein. Incubations were performed at 30 °C.

Hydrolysis of unknown metabolites with alkaline phosphatase. Assay mixtures contained in a total volume of 100 μl: 10 mM NH_4HCO_3, pH 9.0, 15.7 U of alkaline phosphatase from bovine intestinal mucosa (attached to beaded agarose, suspended in 10 mM NH_4HCO_3, pH 9.0) and 250 nmol of the unknown metabolite. After an incubation period of 4 h at room temperature the agarose beads were removed by centrifugation, the supernatant transferred to a new tube and lyophilized.

3.6. Isolation and purification of intermediates

The reaction mixtures were applied to CHROMABOND® SB anionic exchange columns (500 mg, Macherey-Nagel) preconditioned with hexane, methanol and water. The columns were washed with water and developed with 0.1 M H_4HCO_3. The H_4HCO_3 eluates were passed through a column of DOWEX 50WX8 (100–200 mesh, 1.7 meq/ml, Bio-Rad). The eluate was lyophilized and the residue was dissolved in a small amount of water. The sample was applied to a sheet of paper (2043 b, 0.22 mm, 580×600 mm, Schleicher and Schüll) and developed with a mixture of *tert*-butanol-formic acid-water (50:13:20, v/v/v). Radioactivity was monitored with a radio scanner (Automatic TLC-Linear Analyser Tracemaster 20, Berthold). M35 and M59 had an R_f-value of 0.20 and M38 of 0.38. The radio-labeled metabolites were eluted with water and the eluate was lyophilized.

3.7. Reversed-phase ion-pair radio-HPLC

Reversed-phase ion-pair radio-HPLC analysis was performed according to a method of Beyer et al. (1985) and McCaskill and Croteau (1993) with a Luna C8 column (4×250 mm, 5 μm particle size and a guard column cartridge of the same material, 4×3 mm, Phenomenex); solvents: A, 10 mM tetrabutylammonium hydrogensulfate (pH 6); B, 10 mM tetrabutylammonium hydrogensulfate in 70% (v/v) aqueous methanol (pH 6); gradient: 100% A (hold for 20 min), gradient to 40% A/60% B (80 min); flow rate: 0.75 ml min^{-1}; radio detector: Ramona 2000 (Raytest) equipped with Software Winnie 32, vers. 2.0.

3.8. Mass spectroscopy

Electrospray ionisation mass spectroscopy was performed on a Finnigan TSQ 7000 mass spectrometer, Finnigan API ion source interface, negative ESI mode: ionisation 4.5 kV, capillary temperature 200 °C, mass range 50–800 mu, multiplier 1000 V (scan mode); MS/MS: argon collision gas 1.5 mTorr, sheath gas (N_2) 4 psi, multiplier 1400 V, collision energy automatically rotated at −20, −30, −40 eV.

GC-MS analysis was performed on a Finnigan MAT 90 double focusing mass spectrometer, equipped with an EI ion source operated at 70 eV. For sample separation a Varian G 3400 gas chromatograph with a fused silica DB-5ms (Macherey-Nagel) capillary column (30 m×0.25 mm, coated with a 0.25 μm layer of liquid phase) and helium as carrier gas was used. The injector temperature was kept at 300 °C. Temperature program: 2 min isothermal at 50 °C, then 10 K/min up to 300 °C, finally 15 min isothermal at 300 °C.

Acknowledgements

This research was supported by the Deutsche Forschungsgemeinschaft (SFB 369), Bonn and the Fonds der Chemischen Industrie, Frankfurt a.M. We thank Wolfgang Roedl, Melinda Kisz, Maja Raschke and Juraithip Wungsintaweekul for technical assistance.

References

Adam, P., Arigoni, D., Bacher, A., Eisenreich, W., 2002. Biosynthesis of hyperforin in *Hypericum perforatum*. J. Med. Chem. 45, 4786–4793.

Arigoni, D., Sagner, S., Latzel, C., Eisenreich, W., Bacher, A., Zenk, M.H., 1997. Terpene biosynthesis from 1-deoxy-D-xylulose in higher plants by intramolecular skeletal rearrangement. Proc. Natl. Acad. Sci. USA 94, 10600–10605.

Beyer, P., Kreuz, K., Kleinig, H., 1985. Separation of mevalonate phosphates and isopentenyl pyrophosphate by thin-layer chroma-

tography and of short-chain prenyl phosphates by ion-pair chromatography on a high-performance liquid chromatography column. In: Law, J.H., Rilling, H.C. (Eds.), Methods in Enzymology, Vol. 111. Academic Press, London, pp. 248–252.

Bouvier, F., d'Harlingue, A., Suire, C., Backhaus, R.A., Camara, B., 1998. Dedicated roles of plastid transketolases during the early onset of isoprenoid biogenesis in pepper fruits. Plant Physiol 117, 1423–1431.

Camara, B., Bardat, F., Dogbo, O., Brangeon, J., Monéger, R., 1983. Terpenoid metabolism in plastids: Isolation and biochemical characteristics of *Capsicum annuum* chromoplasts. Plant Physiol. 73, 94–99.

Camara, B., 1985. Carotene synthesis in *Capsicum* chromoplasts. In: Law, J.H., Rilling, H.C. (Eds.), Methods in Enzymology, Vol. 110. Academic Press, London, pp. 244–253.

Camara, B., 1993. Plant phytoene synthase complex: Component enzymes, immunology, and biogenesis. In: Packer, L. (Ed.), Methods in Enzymology, Vol. 214. Academic Press, London, pp. 352–365.

Camara, B., Hugueney, P., Bouvier, F., Kuntz, M., Monéger, R., 1995. Biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants. Int. Rev. Cytol. 163, 175–247.

Cane, D.E., Du, S., Robinson, J.K., Hsiung, Y., Spenser, I.D., 1999. Biosynthesis of vitamin B_6: Enzymatic conversion of 1-deoxy-D-xylulose-5-phosphate to pyridoxol phosphate. J. Am. Chem. Soc. 121, 7722–7723.

Cane, D.E., Du, S., Spenser, I.D., 2000. Biosynthesis of vitamin B_6: Origin of the oxygen atoms of pyridoxol phosphate. J. Am. Chem. Soc. 122, 4213–4214. and references quoted therein.

Disch, A., Hemmerlin, A., Bach, T.J., Rohmer, M., 1998. Mevalonate-derived isopentenyl diphosphate is the biosynthetic precursor of ubiquinone prenyl side chain in tobacco BY-2 cells. Biochem. J. 331, 615–621.

Eisenreich, W., Menhard, B., Hylands, P.J., Zenk, M.H., Bacher, A., 1996. Studies on the biosynthesis of taxol: The taxane carbonskeleton is not of mevalonoid origin. Proc. Natl. Acad. Sci. USA 93, 6431–6436.

Eisenreich, W., Sagner, S., Zenk, M.H., Bacher, A., 1997. Monoterpenoid essential oils are not of mevalonoid origin. Tetrahedron Lett. 38, 3889–3892.

Eisenreich, W., Schwarz, M., Cartayrade, A., Arigoni, D., Zenk, M.H., Bacher, A., 1998. The deoxyxylulose phosphate pathway of terpenoid biosynthesis in plants and microorganisms. Chem. Biol. 5, R221–R233.

Eisenreich, W., Rohdich, F., Bacher, A., 2001. Deoxyxylulose phosphate pathway to terpenoids. Trends Plant Sci. 6, 78–84.

Fellermeier, M., Kis, K., Sagner, S., Maier, U., Bacher, A., Zenk, M.H., 1999. Cell-free conversion of 1-deoxy-D-xylulose 5-phosphate and 2-C-methyl-D-erythritol 4-phosphate into β-carotene in higher plants and its inhibition by fosmidomycin. Tetrahedron Lett. 40, 2743–2746.

Fellermeier, M., 2000. Untersuchungen zum Alternativen Terpenbiosyntheseweg in Pflanzen. Ph.D. Thesis, Ludwig-Maximilians Universität München.

Fellermeier, M., Raschke, M., Sagner, S., Wungsinatweekul, I., Schuhr, C.A., Hecht, S., Kis, K., Radykewicz, T., Adam, P., Rohdich, F., Eisenreich, W., Bacher, A., Arigoni, D., Zenk, M.H., 2001. Studies on the nonmevalonate pathway of terpene biosynthesis: The role of *2C-methyl-D-erythritol 2,4-cyclodiphosphate* in plants. Eur. J. Biochem. 268, 6302–6310.

Gao, W., Loeser, W., Raschke, M., Dessoy, M.A., Fulhorst, M., Alpermann, H., Wessjohann, L.A., Zenk, M.H., 2002. (E)-4-*Hydroxy-3-methylbut-2-enyl diphosphate*: an intermediate in the formation of terpenoids in plant chromoplasts. Angew. Chem. Int. Ed 41, 2604–2607.

Goese, M., Kammhuber, K., Bacher, A., Zenk, M.H., Eisenreich, W., 1999. Biosynthesis of bitter acids in hops: A ^{13}C NMR and ^{2}H NMR study on the building blocks of humulone. Eur. J. Biochem. 263, 447–454.

Hecht, S., Kis, K., Eisenreich, W., Amslinger, S., Wungsintaweekul, J., Herz, S., Rohdich, F., Bacher, A., 2001a. Enzyme-assisted preparation of isotope-labeled 1-deoxy-D-xylulose 5-phosphate. J. Org. Chem. 66, 3948–3952.

Hecht, S., Wungsintaweekul, I., Rohdich, F., Kis, K., Radykewicz, T., Schuhr, C.A., Eisenreich, W., Richter, G., Bacher, A.J., 2001b. Biosynthesis of terpenoids: Efficient multistep biotransformation procedures affording isotope-labeled *2C-methyl-D-erythritol 4-phosphate* using recombinant 2C-methyl-D-erythritol 4-phosphate synthase. J. Org. Chem. 66, 7770–7775.

Herz, S., Wungsintaweekul, J., Schuhr, C.A., Hecht, S., Lüttgen, H., Sagner, S., Fellermeier, M., Eisenreich, W., Zenk, M.H., Bacher, A., Rohdich, F., 2000. YgbB protein converts 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate to *2C-methyl-D-erythritol 2,4-cyclodiphosphate*. Proc. Natl. Acad. Sci. USA 97, 2486–2490.

Hill, R.E., Himmeldirk, K., Kennedy, I.A., Panloski, R.M., Sayer, B.G., Wolf, E., Spenser, I.D., 1996. The biogenetic anatomy of vitamin B_6. A ^{13}C NMR investigation of the biosynthesis of pyridoxol in *Escherichia coli*. J. Biol. Chem. 271, 30426–30435.

Jomaa, H., Wiesner, J., Sanderbrand, S., Altinicicek, B., Weidmeyer, C., Hintz, M., Türbachova, I., Eberl, M., Zeidler, J., Lichtenthaler, H.K., Soldati, D., Becker, E., 1999. Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. Science 285, 1573–1576.

Kis, K., Wungsintaweekul, J., Eisenreich, W., Zenk, M.H., Bacher, A., 2000. An efficient preparation of *2C-methyl-D-erythritol 4-phosphoric acid* and its derivatives. J. Org. Chem. 65, 587–592.

Kleinig, H., Beyer, P., 1985. Carotene synthesis in spinach (*Spinacia oleracea* L.) chloroplasts and Daffodil (*Narcissus pseudonarcissus* L.) chromoplasts. In: Law, J.H., Rilling, H.C. (Eds.), Methods in Enzymology, Vol. 110. Academic Press, London, pp. 267–273.

Krall, L., Raschke, M., Zenk, M.H., Baron, C., 2002. The Tzs protein from *Agrobacterium tumefaciens* C58 produces zeatin riboside 5′-phosphate from *4-hydroxy-3-methyl-2-(E)-butenyl diphosphate* and AMP. FEBS Lett. 527, 315–318.

Kuzuyama, T., Takagi, M., Kaneda, K., Watanabe, H., Dairi, T., Seto, H., 2000a. Studies on the nonmevalonate pathway: conversion of 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol to its 2-phospho derivative by 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase. Tetrahedron Lett. 41, 2925–2928.

Kuzuyama, T., Takagi, M., Takahashi, S., Seto, H., 2000b. Cloning and characterization of 1-deoxy-D-xylulose 5-phosphate synthase from *Streptomyces* sp. strain CL190, which uses both the mevalonate and nonmevalonate pathways for isopentenyl diphosphate biosynthesis. J. Bacteriol. 182, 891–897.

Lange, B.M., Croteau, R., 1999. Isoprenoid biosynthesis via a mevalonate-independent pathway in plants: cloning and heterologous expression of 1-deoxy-D-xylulose-5-phosphate reductoisomerase from peppermint. Arch. Biochem. Biophys. 365, 170–174.

Lange, B.M., Wildung, M.R., McCaskill, D., Croteau, R., 1998. A family of transketolases that directs isoprenoid biosynthesis via a mevalonate-independent pathway. Proc. Natl. Acad. Sci. USA 95, 2100–2104.

Lichtenthaler, H.K., Schwender, I., Disch, A., Rohmer, M., 1997. Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate independent pathway. FEBS Lett. 400, 271–274.

Lichtenthaler, H.K., 1999. The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. Annu. Rev. Plant. Physiol. Plant Mol. Biol. 50, 47–65.

Lois, L.M., Campos, N., Putra, S.R., Danielsen, K., Rohmer, M., Boronat, A., 1998. Cloning and characterization of a gene from *Escherichia coli* encoding a transketolase-like enzyme that catalyzes the synthesis of D-1-deoxyxylulose 5-phosphate, a common pre-

cursor for isoprenoid, thiamin, and pyridoxol biosynthesis. Proc. Natl. Acad. Sci. USA 95, 2105–2110.

Lüttgen, H., Rohdich, F., Herz, S., Wungsintaweekul, J., Hecht, S., Schuhr, C.A., Fellermeier, M., Sagner, S., Zenk, M.H., Bacher, A., Eisenreich, W., 2000. Biosyntheis of terpenoids: YchB protein of Escherichia coli phosphorylates the 2-hydroxy group of *4-diphosphocytidyl-2C-methyl-D-erythritol*. Proc. Natl. Acad. Sci. USA 97, 1062–1067.

Mahmoud, S.S., Croteau, R., 2001. Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase. Proc. Natl. Acad. Sci. USA 98, 8915–8920.

McCaskill, D., Croteau, R., 1993. Procedure for the isolation and quantification of the intermediates of the mevalonic acid pathway. Anal. Biochem. 215, 142–149.

Milborrow, B.V., Lee, H.-S., 1998. Endogenous biosynthetic precursors of (+)-abscisic acid. VI*. Carotenoids and ABA are formed by the 'non-mevalonate' triose-pyruvate pathway in chloroplasts. Aust. J. Plant Physiol. 25, 507–512.

Ostrovsky, D., Diomina, G., Lysak, E., Matveeva, E., Ogrel, O., Trutko, S., 1998. Effect of oxydative stress on the biosynthesis of 2-C-methyl-D-erythritol-2,4-cyclopyrophosphate and isoprenoids by several bacterial strains. Arch. Microbiol. 171, 69–72.

Ostrovsky, D., Kharatian, E., Dubrovsky, T., Ogrel, O., Shipanova, I., Sibeldina, L., 1992. The ability of bacteria to synthesize a new cyclopyrophosphate correlates with their tolerance to redox-cycling drugs: on a crossroad of chemotherapy, environmental toxicology and immunobiochemical problems. Biofactors 4, 63–68.

Piel, J., Donath, J., Bandemer, K., Boland, W., 1998. Induzierte und konstitutiv emittierte Pflanzendüfte: mevalonat-unabhängige Biosynthese terpenoider Duftstoffe. Angew. Chem 110, 2622–2625.

Rohdich, F., Wungsintaweekul, J., Fellermeier, M., Sagner, S., Herz, S., Kis, K., Eisenreich, W., Bacher, A., Zenk, M.H., 1999. Cytidine 5′-triphosphate dependent biosynthesis of isoprenoids: YgbP protein of Escherichia coli catalyzes the formation of *4-diphosphocytidyl-2C-methylerythritol*. Proc. Natl. Acad. Sci. USA 96, 11758–11763.

Rohmer, M., 1999. The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. Nat. Prod. Rep. 16, 565–574.

Schwarz, M., Arigoni, D., 1999. Ginkgolide biosynthesis. In: Sir Barton, D.H.R., Nakanishi, K., Metu-Cohn, O. (Eds.), Comprehensive Natural Product Chemistry, Vol. 2. Pergamon, Oxford, pp. 367–399.

Schwender, J., Müller, C., Zeidler, J., Lichtenthaler, H.K., 1999. Cloning and heterologous expression of a cDNA encoding 1-deoxy-D-xylulose-5-phosphate reductoisomerase of *Arabidopsis thaliana*. FEBS Lett. 455, 140–144.

Sprenger, G.A., Schörken, U., Wiegert, T., Grolle, S., deGraaf, A.A., Taylor, S.V., Begley, T.P., Bringer-Meyer, S., Sahm, H., 1997. Identification of a thiamin-dependent synthase in *Escherichia coli* required for the formation of the 1-deoxy–xylulose 5-phosphate precursor to isoprenoids, thiamin, and pyridoxol. Proc. Natl. Acad. Sci. USA 94, 12857–12862.

Takahashi, S., Kuzuyama, T., Watanabe, H., Seto, H., 1998. A 1-deoxy-D-xylulose 5-phosphate reductoisomerase catalyzing the formation of 2-C-methyl-D-erythritol 4-phosphate in an alternative nonmevalonate pathway for terpenoid biosynthesis. Proc. Natl. Acad. Sci. USA 95, 9879–9884.

Totté, N., Charon, L., Rohmer, M., Compernolle, F., Baboef, I., Geuns, J.M.C., 2000. Biosynthesis of the diterpenoid steviol, an *ent*-kaurene derivative from *Stevia rebaudiana* Bertoni, via the methylerythritol phosphate pathway. Tetrahedron Lett. 41, 6407–6410.

White, R.H., 1978. Stable isotope studies on the biosynthesis of the thiazole moiety of thiamin in *Escherichia coli*. Biochem. 17, 1833–3840.

Zeidler, J., Schwender, J., Müller, C., Wiesner, J., Weidemeyer, C., Beck, E., Jomaa, H., Lichtenthaler, H., 1998. Inhibition of the non-mevalonate 1-deoxy-D-xylulose-5-phosphate pathway of plant isoprenoid biosynthesis by fosmidomycin. Z. Naturforsch 53c, 980–986.