A Facile Cell Free Synthesis of Isotopically Labeled (*E*)-4-Hydroxy-3-methylbut-2-enyl Diphosphate, a Precursor of the Plant Hormone Zeatin

by **Maja Raschke**, **Monika Fellermeier**, and **Meinhart H. Zenk***

Biozentrum, Martin-Luther-Universität Halle-Wittenberg, Weinbergweg 22, D-06120 Halle (Saale)
(phone: +49(0)3455525064; fax: +49(0)3455527301; e-mail: zenk@biozentrum.uni-halle.de)

Dedicated to Professor *Rolf Huisgen*, on the occasion of his 85th birthday, in gratefulness

(*E*)-4-Hydroxy-3-methylbut-2-enyl diphosphate (**1**) is a key intermediate of the deoxyxylulose phosphate pathway of isoprenoid biosynthesis and a precursor of the plant hormone zeatin. The availability of this intermediate with various labeling patterns is pivotal for its use in biosynthetic studies. The number of positions, however, that can be easily labeled by chemical synthesis is limited, and the synthesis by means of recombinant enzymes is laborious and time consuming. We demonstrated that chromoplasts from *Capsicum annuum,* whose enzyme activity was impaired by freeze-thawing, accumulate **1**. This observation built the basis for the development of a cell-free system allowing the synthesis of this intermediate with labels in various positions. With 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate (**5**) as substrate, yields were in the range of 50%.

Introduction. – (*E*)-4-Hydroxy-3-methylbut-2-enyl diphosphate (HMBDP; **1**), which is formed by the enzyme specified by the *ispG* gene, is a potent immunomodulatory agent and has been firmly established as intermediate of the recently discovered deoxyxylulose phosphate pathway in certain eubacteria and in the plastid compartment of higher plants [1][2], leading to isopentenyl diphosphate (IDP; **2**) and dimethylallyl diphosphate (DMADP; **3**), the universal building blocks of terpenoids (*Scheme*). Biosynthesis of cytokinins, a group of plant hormones, has been considered to proceed *via* the conjugation of DMADP (**3**) to adenosine 5′-monophosphate (AMP), followed by a hydroxylation of the dimethylallyl moiety to yield zeatin-type cytokinins. Recently, two cytochrome P450 monooxygenases (CYP735A1 and CYP735A2) from *Arabidopsis thaliana* have been cloned, which catalyze this hydroxylation of the dimethylallyl side chain [3]. Based on *in vivo* ^{2}H-labeling experiments with *Arabidopsis thaliana*, however, an alternative pathway for the formation of zeatin was proposed, involving the direct incorporation of a hydroxylated isoprenoid precursor [4]. Later, *Krall et al.* demonstrated that TZS, an enzyme isolated from the plant pathogen *Agrobacterium tumefaciens*, is capable of transferring **1** onto AMP to generate *trans*-zeatin riboside 5′-monophosphate (tZMP, **4**) *in vitro* (*Scheme*) [5]. In addition to its involvement in the formation of (*E*)-zeatin, **1** most likely plays a role in other transfer reactions as well, since this C_5 unit is possibly part of numerous plant products [6]. To gain further insight into the biosynthesis of these plant metabolites and to investigate its pharmacological properties, there is an obvious need for a facile synthesis of **1** preferably in labeled form. Though several chemical syntheses of HMBDP (**1**) have been published [6–10], these methods allow labeling of only a few

positions of this molecule, for instance, insertion of carbon isotopes at position C(4) [9] or a ^{3}H-label at position C(4) [6][10a]. However, to successfully employ **1** in biosynthetic studies, derivatives carrying isotopic labels in other positions than those mentioned would be desirable.

Here, we describe a chromoplast system from red bell pepper fruits for the synthesis of reasonable quantities of **1** from 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate (*c*MEDP; **5**). Accumulation of **1** was achieved by using chromoplasts undergoing freeze-thawing, a treatment which obviously inactivated IDP/DMADP synthase (IDDS), a rather sensitive enzyme, while HMBDP synthase (HDS) as well as the other enzymes of the deoxyxylulose phosphate pathway preceding HDS were still highly active. Ion exchange chromatography was used for purification of **1**.

Results and Discussion. – Chromoplasts prepared from the fruits of *Capsicum annuum*, which are commercially available year-round, are an excellent source to study isoprenoid biosynthesis [11]. The complete set of the enzymes of the deoxyxylulose phosphate pathway is present in these organelles [12–14] as well as the enzymes transforming IDP (**2**) and DMADP (**3**) into carotenoids [15]. Application experiments with labeled DXP (**6**) or cMEDP (**5**), and chromoplasts in the presence of various cofactors led to the formation of labeled phytoene and β-carotene as well as more complex carotenoids (*Scheme*). However, analysis of the phosphorylated intermediates of the deoxyxylulose phosphate pathway by an HPLC method described in [16] showed that these intermediates are present only in trace amounts during the transformation process.

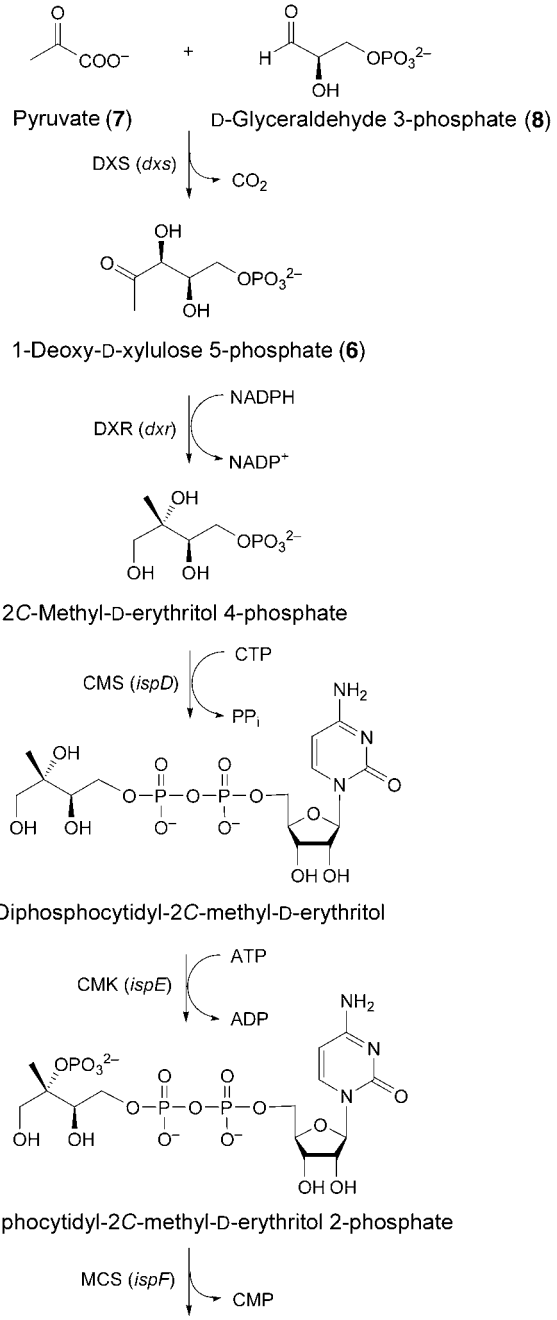
By chance, we observed that incubation of chromoplasts, which had undergone freezing to $-80°$ for 1 to 22 h, with cMEDP (**5**) or DXP (**6**) leads to accumulation of **1**. Though the degree of ripeness of the pepper fruits had a slight influence on the yield of **1**, its amount was at least doubled when previously frozen chromoplasts were used in the enzyme assays compared to freshly isolated chromoplasts.

Since yields of **1** were considerably higher when cMEDP (**5**) was employed as substrate, we made use of the *in vitro* system based on spinach chloroplast stroma, which we had described earlier [17]. It allows the production of radioactive as well as heavy-isotope-labeled **5** in reasonable quantities from DXP (**6**). Compound **6** can be synthesized from pyruvate (**7**) and D-glyceraldehyde 3-phosphate (GAP; **8**), which can both either be generated in labeled form from labeled glucose [18] or are commercially available with various isotopic labels. Thus, cMEDP (**5**) is accessible with isotopic labels in almost any position.

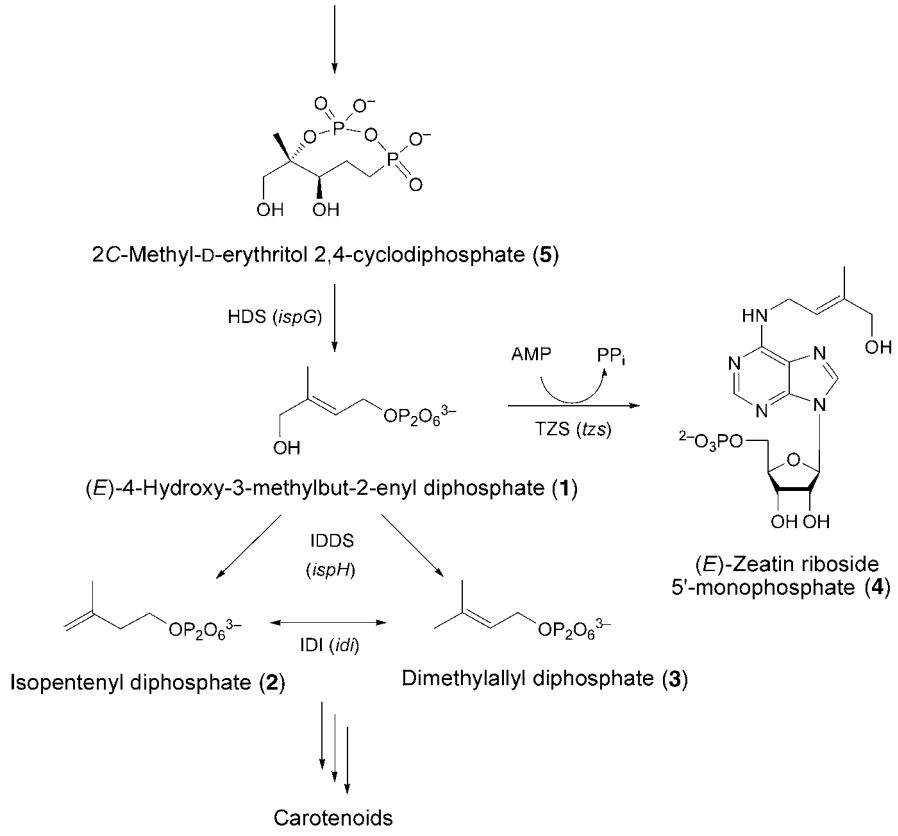
Isolated chromoplasts of *C. annuum* equivalent to 2 mg of protein, which had been frozen for 2 h at $-80°$, were incubated with 0.4 mM 2-[*methyl*-$^{13}C_1$][2-$^{13}C_1$]-**5** for 8 h at 30° as described in the *Exp. Part*. Trace amounts of ^{14}C-labeled **5** were added to monitor the transformation of the substrate as well as the purification of the product by radio-HPLC (*Fig.*, *a* and *b*). In the presence of 2 mg of chromoplast protein, the concentration of the applied cMEDP (**5**) up to 2 mM had no significant influence on the rate of enzymatic conversion. Incubation for more than 8 h did not improve the product formation. On average, 50% of **5** was accumulated in **1**.

HMBDP (**1**) was purified by anion-exchange chromatography on a *DEAE-Sephadex* column with a continuous ammonium formate gradient as described in [17].

Scheme. *The Deoxyxylulose Phosphate Pathway of Isoprenoid Biosynthesis and Its Postulated Interaction with the Biosynthesis of Zeatin-Type Cytokinins.* Enzymes (corresponding genes in parentheses): DXS: DXP synthase; DXR: DXP reductoisomerase; CMS: CDP-ME synthase; CMK: CDP-ME kinase; MCS: cMEDP synthase; HDS: HMBDP synthase; IDDS: IDP/DMADP synthase; IDI: IDP/DMADP isomerase; TZS: *trans*-zeatin synthesizing protein.



Scheme (cont.)



There were no radioactive impurities detectable by radio-HPLC (*Fig., b*). The yield was *ca.* 37% based on the applied amount of substrate.

Subsequent MS analysis unequivocally revealed the product to be HMBDP (**1**), as evidenced by the two ^{13}C-atoms from the applied 2-[*methyl*-$^{13}C_1$][2-$^{13}C_1$]-**5**. It shared exact fragmentation characteristics with an authentic standard of **1**. The negative-ion electrospray ionization mass spectrum afforded for 2-[*methyl*-$^{13}C_1$][2-$^{13}C_1$]-**1** a $[M - H]^-$ ion at *m*/*z* 263 corresponding to a molecular mass of 264. The MS/MS spectrum for the $[M - H]^-$ ion of **1** showed significant fragment ions at *m*/*z* (rel. int. [%]): 245 ($[M - H - H_2O]^-$, 6), 165 ($[M - H - H_3PO_4]^-$, 1), 159 ($[HP_2O_6]^-$, 29), 79 $[PO_3]^-$, 100).

In conclusion, the development of this biosynthetic method based on chromoplasts allows the synthesis of HMBDP (**1**) in good quantities, and with various radioactive and heavy isotopic labels, thus providing a suitable tool to further investigate its involvement in the biosynthesis of plant products and its role as an immunomodulatory agent.

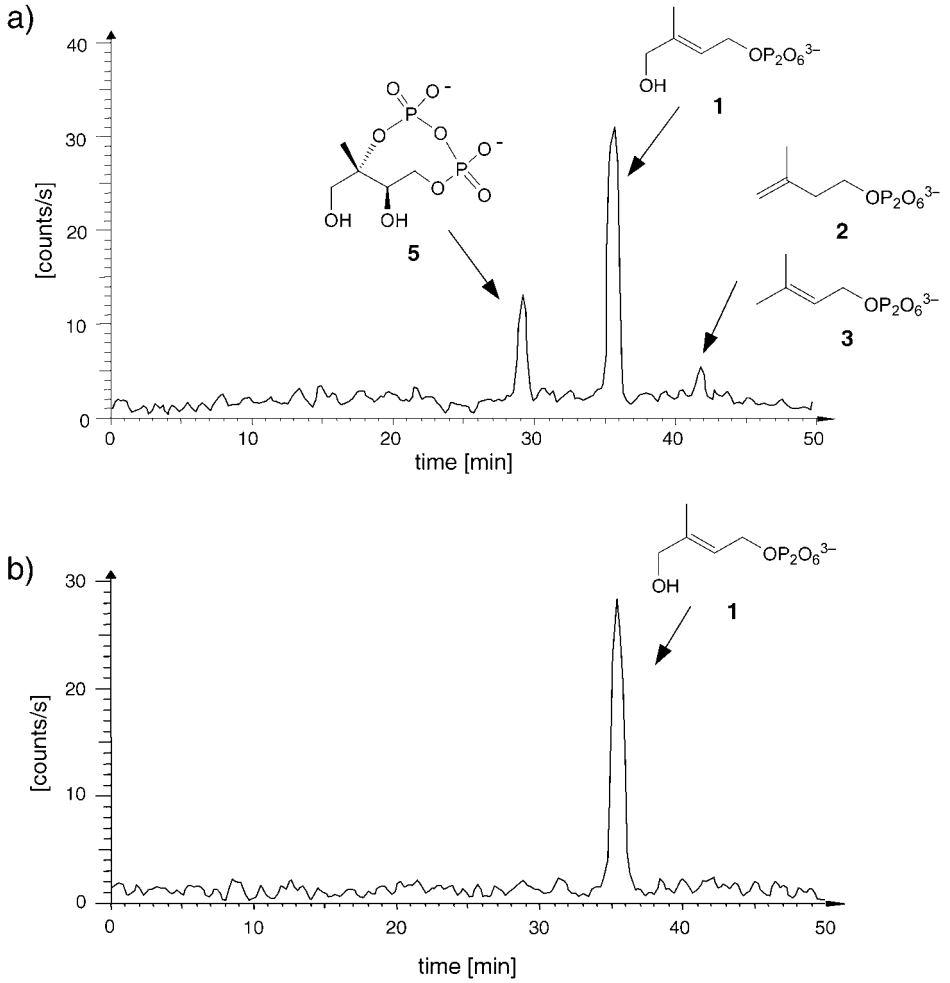


Figure. *Radio-HPLC analysis of the aqueous phase of an enzyme assay with chromoplasts* (2 mg of protein) *from* C. annuum *that had been frozen for 2 h at* $-80°$ *prior to incubation and 0.4 m*M *2-[*methyl-$^{13}C_1$*][2-*$^{13}C_1$*]-***5** *in the presence of* Hepes-*KOH* (pH 7.6; 100 mM), *$MnCl_2$* (2 mM), *$MgCl_2$* (10 mM), *NaF* (5 mM), *$NADP^+$* (2 mM), *NADPH* (1 mM), *and ATP* (6 mM) *in a total volume of 500 µl*. 0.2 µCi [1,3,4-$^{14}C_3$]-**5** was added to monitor the effluent. *a*) After 8 h of incubation at 30°; *b*) after 8 h of incubation and purification on a *DEAE-Sephadex* column.

Experimental Part

Plant Material. Fruits of *Capsicum annuum* were purchased at local markets.

Buffers. Buffer *A* used for preparation of chromoplasts contained 50 mM *Hepes*-KOH (pH 8.0), 0.4M sucrose, 1 mM DTE, and 1 mM EDTA. Buffer *B* used for preparation of chromoplasts contained 50 mM *Hepes*-KOH (pH 7.6) and 1 mM DTE.

*Substrate Synthesis. 2-[*Methyl-$^{13}C_1$*][2-*$^{13}C_1$*]-2-methyl-*D*-erythritol 2,4-cyclodiphosphate* (2-[*Methyl*-$^{13}C_1$]-[2-$^{13}C_1$]-**5**) was synthesized from [U-$^{13}C_3$]pyruvate according to the method described in [17].

Preparation of Chromoplasts. Chromoplasts from the pericarp of red pepper were isolated as described in [12]. In brief, fruits (400 g) were cut in small pieces and homogenized in a *Braun* kitchen blender (step II) in

500 ml of ice-cold buffer *A*. After filtration through four layers of *Nylon* gauze (50 μm), the homogenate was centrifuged at 3.300 × *g* for 10 min at 4°. The resulting pellet of chromoplasts was carefully resuspended in buffer *A* and centrifuged as described above. After discarding the supernatant, the pellet was resuspended in 1 to 1.5 ml of ice-cold buffer *B* and filtered through one layer of *Nylon* gauze (50 μm). The protein concentration of the chromoplast suspension (4 to 5 ml) was *ca.* 23 to 25 mg/ml, measured as described in [19].

*Biosynthesis of (*E*)-4-Hydroxy-3-methylbut-2-enyl Diphosphate* (HMBDP; **1**) *from cMEDP.* Prior to incubation, isolated chromoplasts were frozen for 2 h at −80°. The reaction mixture contained 100 mM *Hepes*-KOH (pH 7.6), 2 mM $MnCl_2$, 10 mM $MgCl_2$, 5 mM NaF, 2 mM $NADP^+$, 1 mM NADPH, 6 mM ATP, and chromoplasts equivalent to 2 mg of protein in a total volume of 500 μl. 2-[methyl-$^{13}C_1$][2-$^{13}C_1$]-**5** at a concentration of 0.4 mM was added as substrate. Ten assays with this composition were prepared and incubated for 8 h at 30°. To monitor the purification process by scintillation counting and radio-HPLC, 0.2 μCi [1,3,4-$^{14}C_3$]-**5** (spec. act. 155.5 μCi/μmol) was added to one of the 10 assays. The reaction was terminated by AcOEt extraction. The remaining aq. phase was immediately frozen in liquid N_2, lyophilized, and purified on a *DEAE Sephadex* column as described below. The yield of **1** was *ca.* 50%.

Purification of **1**. The residue of the aq. phase obtained after lyophilization was dissolved in 500 μl of H_2O, transferred to a small glass vial, heated briefly to 70°, and centrifuged (3.000 × *g*, 30 min). The supernatant was applied to a *DEAE-Sephadex* column (7.0 × 0.8 cm) and developed with a continuous ammonium formate gradient (0.06 to 0.5 M, pH 8.0) at a flow rate of 1 ml/min as described in [17]. The effluent was monitored by scintillation counting. The retention volumes of cMEDP (**5**) and HMBDP (**1**) were 96 and 120 ml, resp. IDP (**2**) and DMADP (**3**), which were formed only in trace amounts, eluted both with a retention volume of 48 ml. Fractions containing **1** were combined and lyophilized. The overall yield was *ca.* 37%. The residue was taken up in 200 μl of 80% (*v*/*v*) MeOH and analyzed by electrospray ionization mass spectroscopy (ESI-MS).

HPLC System. The HPLC system used was a *Merck-Hitachi LaChrom*® interfaced with a radiodetector, *Ramona 2000* (Raytest), for the measurement of radioactivity. The detector signal was recorded and integrated by a personal computer and a chromatography software program (Winnie 32, Vers. 2.0). The HPLC method was a slight modification of that described in [16]. Reversed-phase ion-pair HPLC separations were performed with a *Luna C8 (2)* column (5 μm, 250 × 4 mm) equipped with a guard column of the same material (4 × 3 mm, *Phenomenex*), and a binary solvent system consisting of 10 mM tetrabutylammonium hydrogen sulfate (TBAS) in H_2O, pH 6.0 (solvent *A*) and 10 mM TBAS in 70% (*v*/*v*) MeOH (solvent *B*). The column was developed with 100% solvent *A* for 10 min, followed by a linear gradient to 60% solvent *B* over the next 25 min at a flow rate of 0.75 ml/min. The column was regenerated by a switch from 60% solvent *B* to 100% solvent *A* within 5 min, followed by isocratic elution with 100% solvent *A* for another 10 min.

Mass Spectroscopy. ESI-MS was performed on a *Finnigan MAT TSQ 7000* mass spectrometer (*Thermo Finnigan*, San Jose, CA, USA); negative ESI mode: ionization 4.0 kV, capillary temp. 200°; collision-induced dissociation (CID): Ar collision gas 1.8×10^{-3} Torr, sheath gas N_2, collision energy 25 eV. Samples were loaded with a syringe pump (flow rate 25 μl/min).

We are grateful to Dr. *J. Schmidt*, Institute of Plant Biochemistry, D-Halle, for performing the MS measurements of (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (**1**). This work was supported by grants from the *Deutsche Forschungsgemeinschaft*, Bonn.

REFERENCES

[1] A. Hemmerlin, E. Gerber, J.-F. Feldtrauer, L. Wentzinger, M.-A. Hartmann, D. Tritsch, J.-F. Hoeffler, M. Rohmer, T. J. Bach, *Lipids* **2004**, *39*, 723.
[2] F. Rohdich, A. Bacher, W. Eisenreich, *Curr. Opin. Chem. Biol*. **2004**, *5*, 535.
[3] K. Takei, T. Yamaya, H. Sakakibara, *J. Biol. Chem.* **2004**, *279*, 41866.
[4] C. Åstot, K. Dolezal, A. Nordström, Q. Wang, T. Kunkel, T. Moritz, N.-H. Chua, G. Sandberg, *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 14778.
[5] L. Krall, M. Raschke, M. H. Zenk, C. Baron, *FEBS Lett.* **2002**, *527*, 315.
[6] W. Gao, R. Loeser, M. Raschke, M. A. Dessoy, M. Fulhorst, H. Alpermann, L. A. Wessjohann, M. H. Zenk, *Angew. Chem., Int. Ed.* **2002**, *41*, 2604.
[7] J. L. Ward, M. H. Beale, *J. Chem. Soc., Perkin Trans. 1* **2002**, 710.
[8] M. Wolff, M. Seemann, C. Grosdemange-Billiard, D. Tritsch, N. Campos, M. Rodríguez-Concepción, A. Boronat, M. Rohmer, *Tetrahedron Lett.* **2002**, *43*, 2555.

[9] J.-L. Giner, *Tetrahedron Lett.* **2002**, *43*, 5457.
[10] a) S. Hecht, S. Amslinger, J. Jauch, K. Kis, V. Trentinaglia, P. Adam, W. Eisenreich, A. Bacher, F. Rohdich, *Tetrahedron Lett.* **2002**, *43*, 8929; b) S. Amslinger, K. Kis, S. Hecht, P. Adam, F. Rohdich, D. Arigoni, A. Bacher, W. Eisenreich, *J. Org. Chem.* **2002**, *67*, 4590; c) D. T. Fox, C. D. Poulter, *J. Org. Chem.* **2002**, *67*, 5009.
[11] B. Camara, P. Hugueney, F. Bouvier, M. Kuntz, R. Monéger, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1995**, *46*, 521.
[12] M. Fellermeier, S. Sagner, P. Spiteller, M. Spiteller, M. H. Zenk, *Phytochemistry* **2003**, *64*, 199.
[13] M. Fellermeier, M. Raschke, S. Sagner, J. Wungsintaweekul, C. A. Schuhr, S. Hecht, K. Kis, T. Radykewicz, P. Adam, F. Rohdich, W. Eisenreich, A. Bacher, D. Arigoni, M. H. Zenk, *Eur. J. Biochem.* **2001**, *268*, 6302.
[14] P. Adam, S. Hecht, W. Eisenreich, J. Kaiser, T. Gräwert, D. Arigoni, A. Bacher, F. Rohdich, *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 12108.
[15] B. Camara, *Methods Enzymol.* **1993**, *214*, 352.
[16] M. Raschke, M. Fellermeier, M. H. Zenk, *Anal. Biochem.* **2004**, *335*, 235.
[17] W. Gao, M. Raschke, H. Alpermann, M. H. Zenk, *Helv. Chim. Acta* **2003**, *86*, 3568.
[18] C. A. Schuhr, S. Hecht, K. Kis, W. Eisenreich, J. Wungsintaweekul, A. Bacher, F. Rohdich, *Eur. J. Org. Chem.* **2001**, *17*, 3221.
[19] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248.

Received February 28, 2005