Studies on the nonmevalonate terpene biosynthetic pathway: Metabolic role of IspH (LytB) protein

Felix Rohdich*[†], Stefan Hecht*, Katrin Gärtner*, Petra Adam*, Cornelia Krieger*, Sabine Amslinger*, Duilio Arigoni[‡], Adelbert Bacher*, and Wolfgang Eisenreich*[†]

*Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstraβe 4, D-85747 Garching, Germany; and [‡]Laboratorium für Organische Chemie, Eidgenössische Technische Hochschule-Hönggerberg Chemisches Institut, H307, CH-8093 Zürich, Switzerland

Isopentenyl diphosphate and dimethylallyl diphosphate serve as the universal precursors for the biosynthesis of terpenes. Although their biosynthesis by means of mevalonate has been studied in detail, a second biosynthetic pathway for their formation by means of 1-deoxy-D-xylulose 5-phosphate has been discovered only recently in plants and certain eubacteria. Earlier *in vivo* experiments with recombinant *Escherichia coli* strains showed that exogenous 1-deoxy-D-xylulose can be converted into 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate by the consecutive action of enzymes specified by the *xylB* and *ispCDEFG* genes. This article describes the transformation of exogenous [U-^{13}C$_5$]1-deoxy-D-xylulose into a 5:1 mixture of [U-^{13}C$_5$]isopentenyl diphosphate and [U-^{13}C$_5$]dimethylallyl diphosphate by an *E. coli* strain engineered for the expression of the *ispH (lytB)* gene in addition to recombinant *xylB* and *ispCDEFG* genes.

biosynthesis of isoprenoids | 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate | isopentenyl diphosphate | dimethylallyl diphosphate

Terpenes are one of the largest groups of natural products comprising numerous medically relevant compounds (e.g., vitamins, hormones, and antitumor agents such as Taxol) (1). Bloch, Lynen, Cornforth, and their coworkers showed that the universal terpenoid precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), are biosynthesized by means of the mevalonate pathway in yeasts and animals (for review see refs. 2–5). These studies served as the basis for the development of metabolic inhibitors that are widely used for the treatment of hypercholesterolemia.

For a period of several decades, these milestone achievements completely eclipsed the existence of a second terpenoid pathway for the biosynthesis of IPP and DMAPP. Recently, however, knowledge on that pathway has been unfolding rapidly on the basis of seminal discoveries by the research groups of Arigoni and Rohmer (refs. 6–8, for review see refs. 9–12). Briefly, the mevalonate independent pathway starts from 1-deoxy-D-xylulose 5-phosphate, which is assembled from pyruvate and D-glyceraldehyde 3-phosphate (13, 14) and was already known to serve as a biosynthetic precursor of vitamins B$_1$ (thiamine) and B$_6$ (pyridoxal) (Fig. 1) (15–17). 1-Deoxy-D-xylulose 5-phosphate is converted into 2*C*-methyl-D-erythritol 4-phosphate, the first committed intermediate of the nonmevalonate pathway, by isomerization followed by a two-electron reduction step catalyzed by 2*C*-methyl-D-erythritol 4-phosphate synthase specified by the *ispC* gene (formerly designated *yaeM* and then *dxr*) (18) (Fig. 1).

In the next step, 2*C*-methyl-D-erythritol 4-phosphate is converted into the 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate by the sequential action of three enzymes specified by the *ispD*, *ispE*, and *ispF* genes (19–24). The last known step of the sequence, the reductive transformation of the cyclic diphosphate into 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate has been identified recently in work with a recombinant *Escherichia coli* strain engineered for hyperexpression of the *ispG* gene (previously designated *gcpE*) (25). Comparative genomics suggest that the protein specified by the *lytB* gene is involved in the conversion of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate into IPP and DMAPP (26). Mutagenesis studies

confirmed this suggestion (27–29), but no direct evidence for the specific role of the LytB protein has been reported up to now.

The nonmevalonate pathway is present in many eubacteria (for review see refs. 9–12). In higher plants, both pathways are operative; while triterpenes including the sterol precursor, cycloartenol, are known to be produced by means of the mevalonate pathway in the cytoplasmic compartment of plant cells, a large number of other terpenes (e.g., mono- and diterpenes) are produced in the plastid compartment by means of the deoxyxylulose phosphate pathway. The picture is complicated by the exchange of certain terpene precursors between the two compartments; because of this crosstalk between the two pathways, terpenes synthesized in plastids can be derived in part from the cytoplasmically located mevalonate pathway and vice versa.

For numerous microbial pathogens such as *Enterobacteria* and *Mycobacterium tuberculosis,* the nonmevalonate pathway is the exclusive source of terpenoids. The alternative terpenoid pathway has also been shown to be essential in *Plasmodium spp.*, which is responsible for more than a million deaths per year (30).

Because the enzymes of the nonmevalonate pathway have no orthologs in mammalian hosts, they are attractive targets for the development of novel antibiotic and/or antiprotozoal agents. Thus, the further elucidation of the nonmevalonate pathway enzymes appears urgent to provide molecular information for drug design. This article describes the role of the *ispH* (formerly designated *lytB*) gene product in the conversion of 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate into IPP and DMAPP.

Experimental Procedures

Materials. [U-^{13}C$_5$]1-deoxy-D-xylulose 5-phosphate was prepared by published procedures (31). Oligonucleotides were custom-synthesized by MWG Biotec, Ebersberg, Germany. IPP and DMAPP were purchased from Echelon, Salt Lake City, UT. Diaminobenzidine and alkaline phosphatase from bovine intestinal mucosa were purchased from Sigma. A rabbit polyclonal antiserum directed against recombinant IspH (LytB) protein was prepared by Eurogentec, Seraing, Belgium. Anti-rabbit horseradish peroxidase conjugated was purchased from Promega.

Preparation of 1-Deoxy-D-Xylulose. Reaction mixtures containing 150 mM Tris·HCl (pH 9.5), 30 mM MgCl$_2$, 32 mM [U-^{13}C$_5$]1-deoxy-D-xylulose 5-phosphate, and 130 μg/ml^{-1} alkaline phosphatase were incubated at 37°C for 16 h. The reaction mixtures were monitored by NMR spectroscopy and used for feeding experiments without further treatment.
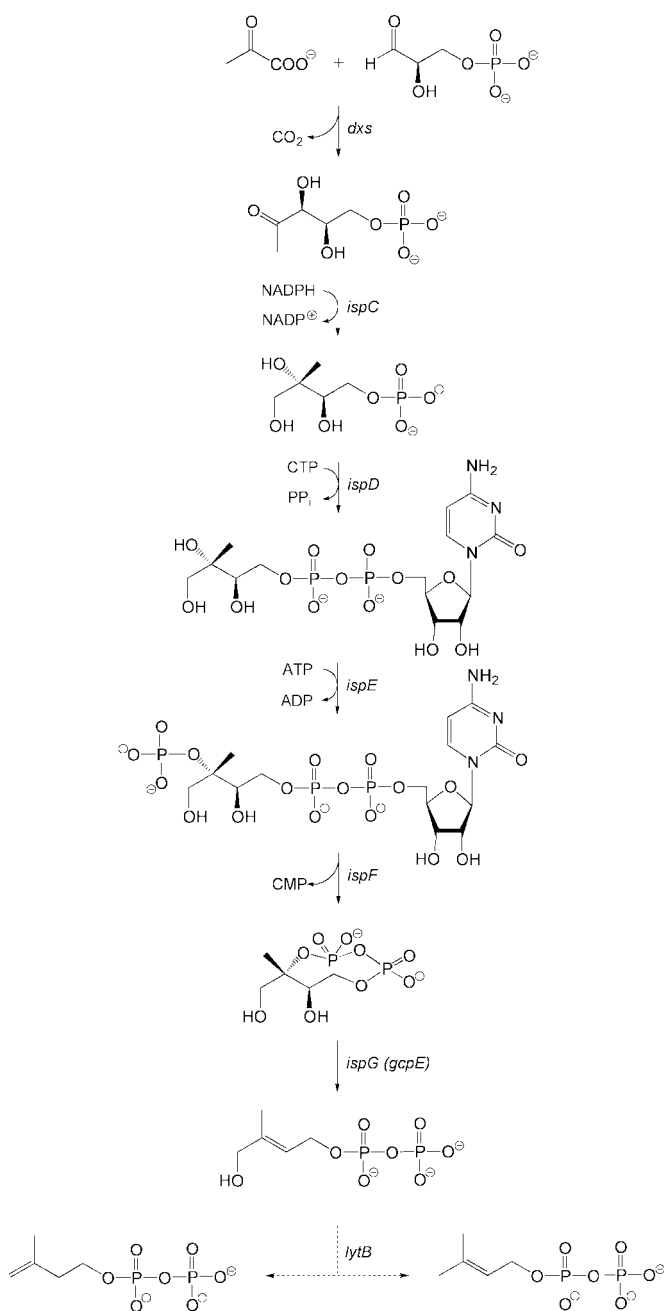
Fig. 1. The nonmevalonate pathway of isoprenoid biosynthesis.

Bacterial Strains and Plasmids. Bacterial strains and plasmids used in this study are summarized in Table 1.

DNA Sequence Determination. DNA was sequenced by the automated dideoxynucleotide method (32) using a 377 Prism sequencer from Perkin–Elmer.

Protein Sequencing. Automated Edman degradation was performed by using a PE BIO systems model 492 from Perkin–Elmer.

Cloning and Expression of the *ispH* Gene. The *ispH* gene of *E. coli* (formerly designated *lytB;* GenBank accession no. AE000113) was amplified from bp position 5618 to 6568 by PCR using chromosomal *E. coli* DNA as template and the oligonucleotides lytBvo and lytBhi as primers (Table 2). The amplificate was purified, treated

with the restriction enzymes *Bam*HI and *Pst*I, and ligated into the expression vector pQE30 (Qiagen, Hilden, Germany), which had been treated with the same enzymes. The resulting plasmid pQElytB was electrotransformed into *E. coli* strains XL1-Blue (Stratagene) (33) and M15(pREP4) (34), affording the recombinant strains XL1-pQElytB and M15-pQElytB, respectively.

Construction of a Synthetic Operon. Vector constructs for the *in vivo* utilization of 1-deoxy-D-xylulose into recombinant *E. coli* strains were synthesized in consecutive cloning steps as follows. The *ispH* gene of *E. coli* was amplified by PCR using chromosomal *E. coli* DNA as template and the oligonucleotides lytB*Sal*Ivo and lytB*E-ag*Ihi as primers (Table 2). The amplificate was purified, treated with *Sal*I and *Eag*I, and ligated into the plasmid vector pACYC184 (35), which had been treated with the same restriction enzymes. The resulting plasmid pACYClytB was electrotransformed into *E. coli* strain XL1-Blue, yielding the recombinant strain XL1-pACYClytB.

The *ispG* gene of *E. coli* (GenBank accession no. AE000338) was amplified from bp position 372 to 1204 by PCR using chromosomal *E. coli* DNA as template and the oligonucleotides gcpE*Bam*HIvo and gcpE*Sal*Ihi as primers (Table 2). The amplificate was purified, treated with *Bam*HI and *Sal*I, and ligated into the plasmid vector pACYClytB, which had been treated with the same restriction enzymes. The resulting plasmid pACYClytBgcpE was electrotransformed into *E. coli* strain XL1-Blue, yielding the recombinant strain XL1-pACYClytBgcpE.

The artificial *gcpE, lytB* operon of the plasmid pACYClytBgcpE was amplified by PCR and the oligonucleotides gcpE*Sac*IIvo and lytB*Not*I as primers (Table 2). The amplificate was purified, treated with *Sac*II and *Not*I, and ligated into the plasmid construct pBScyclo expressing the *xylB* and the *ispCDEF* genes (25), which had been treated with the same restriction enzymes. The resulting plasmid pBSxispC-H was electrotransformed into *E. coli* strain XL1-Blue, affording the recombinant strain XL1-pBSxispC-H.

Isotope Incorporation Studies. LB medium (0.2 l containing 36 mg ampicillin) was inoculated with recombinant *E. coli* XL1-Blue cells carrying plasmids overexpressing *xylB* in conjunction with genes of the nonmevalonate pathway (Table 1). The cells were grown with shaking at 37°C overnight. At an OD (600 nm) of 1.4, 24 ml of a solution containing 1.1 M lithium lactate and 6.7 mM [U-$^{13}C_5$]1-deoxy-D-xylulose, pH 7.0, was added continuously within 2 h. Aliquots of 40 ml were retrieved at intervals of 30 min. The cells were harvested, centrifuged for 10 min at 5,000 rpm and 4°C, washed with 0.9% NaCl, and centrifuged. The cells were resuspended in 600 μl of 10 mM NaF/D$_2$O in 50% (vol/vol) methanol-d$_4$, cooled on ice, and subjected to ultrasonic treatment. The suspension was centrifuged at 15,000 rpm for 15 min. To avoid degradation during work-up, the supernatants were subjected to NMR analysis without further purification.

NMR Spectroscopy. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded by using an AVANCE DRX 500 spectrometer from Bruker Instruments, Karlsruhe, Germany. Two-dimensional heteronuclear multiple quantum correlation (HMQC) and HMQC- total correlation spectroscopy (TOCSY) experiments were performed by using standard Bruker software (XWINNMR 3.0). The duration of TOCSY transfer was 60 ms in HMQC-TOCSY experiments.

Purification of Recombinant IspH (LytB) Protein. The *E. coli* strain M15-pQElytB was grown in LB broth containing 180 mg ampicillin and 50 mg kanamycin sulfate per liter. Cultures were incubated at 37°C with shaking. At an OD of 0.7 (600 nm), isopropyl β-D-thiogalactoside was added to a final concentration of 2 mM, and the culture was incubated for 5 h. The cells were harvested by centrifugation, washed with 0.9% (g/vol) sodium chloride, and stored at −20°C.

Frozen cell mass (8 g) was thawed in 80 ml of 50 mM potassium

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristic	Ref. or source
E. coli		
XL1-Blue	*RecA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac,* [F', *proAB, lacI^qZ*ΔM15, Tn10 *(tet^r)*]	33, Stratagene
M15(pREP4)	*Lac, ara, gal, mtl, recA^+, uvr^+,* [pREP4, *lacI, kan^r*]	34
Plasmids		
pQE30	High copy His-Tag expression vector	Qiagen
pQElytB	Expression of *ispH* (*lytB*) from *E. coli*	This study
pACYC184	Low copy cloning vector	35, NEB
pACYClytB	Expression of *ispH* from *E. coli*	This study
pACYClytBgcpE	Expression of *ispG* and *ispH* from *E. coli*	This study
pBluescript SKII⁻	High copy cloning vector	Stratagene
pBScyclo	Expression of *xylB, ispC, ispD, ispE,* and *ispF* from *E. coli*	25
pBSxispC-H	Expression of *xylB, ispC, ispD, ispE, ispF, ispG,* and *ispH* from *E. coli*	This study

phosphate, pH 7.0, containing 0.5 M sodium chloride and 20 mM imidazole hydrochloride. The suspension was subjected to ultrasonic treatment and centrifuged. The supernatant was applied to a column of Ni-chelating Sepharose FF (2.0 × 5 cm, Amersham Pharmacia), which had been equilibrated with 50 mM potassium phosphate, pH 7.0, containing 0.5 M sodium chloride and 20 mM imidazole (flow rate, 2 ml·min⁻¹). The column was washed with 150 ml of 50 mM potassium phosphate, pH 7.0, containing 0.5 M sodium chloride and 20 mM imidazole, and was then developed with a gradient of 20–300 mM imidazole in 240 ml of 20 mM potassium phosphate, pH 7.0. Fractions were combined and dialyzed overnight against 50 mM potassium phosphate, pH 7.0. The solution was concentrated by ultrafiltration. The protein solution was loaded on top of a Superdex 200 column (2.6 × 60), which had been equilibrated with 50 mM potassium phosphate, pH 7.0 at a flow rate of 3 ml·min⁻¹. The retention volume of IspH protein was 238 ml. Fractions were combined and stored at −20°C.

PAGE. SDS/PAGE was performed with the SE 250 Mighty small II electrophoresis system from Amersham Pharmacia at a constant current of 20 mA per gel, using 5% acrylamide stacking gels and 15% acrylamide separating gels (36). Gels were stained with 0.25% (wt/vol) Coomassie R-250 in 50% methanol/acetic acid/water (46:10:46; vol/vol/vol) and destained in methanol/acetic acid/water (30:10:70; vol/vol/vol).

Western Blotting. Protein samples dissolved in 3% SDS containing 3% mercaptoethanol and 0.1% bromophenol blue were subjected to SDS/PAGE as described above. The proteins were electrotransferred to Immobilon-P transfer membranes by using the Transblot SD from Bio-Rad with a constant current of 2 mA·cm⁻² gel for 1 h (37). A rabbit polyclonal antiserum directed against IspH (LytB) protein from *E. coli* (diluted 1:8,000) was used as the first antibody. Anti-rabbit IgG (Fc) conjugated to horseradish peroxidase was used as second antibody. Blots were developed with diaminobenzidine and H_2O_2 (38).

Radial Immunodiffusion. Experiments were performed by the procedure of Mancini *et al.* (39). Gels contained 50 mM Tris·HCl (pH 7.6), 0.7% agarose (Serva), 0.03% sodium azide, and 90 µl/ml anti-IspH serum containing 150 mM sodium chloride. Wells with diameters of 4 mm were loaded with samples of 20 µl. The plates were incubated in a humid chamber at room temperature. Recombinant IspH protein was used as standard.

Results

The *ispH* (*lytB*) gene of *E. coli* was placed under the control of a T_5 promoter and *lac* operator in the hyperexpression plasmid pQE30. The ORF was preceded by a synthetic DNA segment specifying the amino acid motif MRGSHHHHHHGS to expedite the purification of the recombinant protein by metal chelating affinity chromatography. A recombinant *E. coli* strain carrying that plasmid directed the synthesis of a peptide with a relative mass of ≈37 kDa as judged from SDS/PAGE. The recombinant protein accounted for ≈5.6% of soluble cell protein as judged by single radial immunodiffusion.

The recombinant protein was purified to apparent homogeneity by affinity chromatography on a Ni-chelating column. The N-terminal sequence obtained by partial Edman degradation matched the prediction based on the DNA sequence of the expression plasmid. Electrospray MS indicated a relative mass of 36,161 ± 40 Da in agreement with the predicted mass of 36,173 Da. The recombinant IspH (LytB) protein was used to prepare a rabbit antiserum, which was subsequently used to monitor IspH (LytB) protein in recombinant strains designed for hyperexpression of isoprenoid biosynthesis genes.

We have shown earlier that the hyperexpression of D-xylulokinase specified by the *xylB* gene enables *E. coli* to convert exogenous 1-deoxy-D-xylulose into 1-deoxy-D-xylulose 5-phosphate with high efficacy (25) and that strains expressing the *xylB* gene in conjunction with *ispCDEFG* genes convert exogenous 1-deoxy-D-xylulose into a mixture of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate and 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (25) (Fig. 1).

Table 2. Oligonucleotides used in this study

| Designation	5'-Sequence-3'
lytBvo	TGGAGGGGATCCATGCAGATCCTGTTGGCC
lytBhi	GCATTTCTGCAGAACTTAGGC
lytBSalIvo	GCTTGCGTCGACGAGGAGAAATTAACCATGCAGATCCTGTTGGCCACC
lytBEagIhi	GCTGCTCGGCCGTTAATCGACTTCACGAATATCG
gcpEBamHIvo	CGTACCGGATCCGAGGAGAAATTAACCATGCATAACCAGGCTCCAATTC
gcpESalIhi	CCCATCGTCGACTTATTTTTCAACCTGCTGAACGTC
gcpESacIvo	GCGGGAGACCGCGGGAGGAGAAATTACCATGCATAACCAGGCTCCAATTCAACG
gcpENotIhi	AGGCTGGCGGCCGCTTAATCGACTTCACGAATATCG
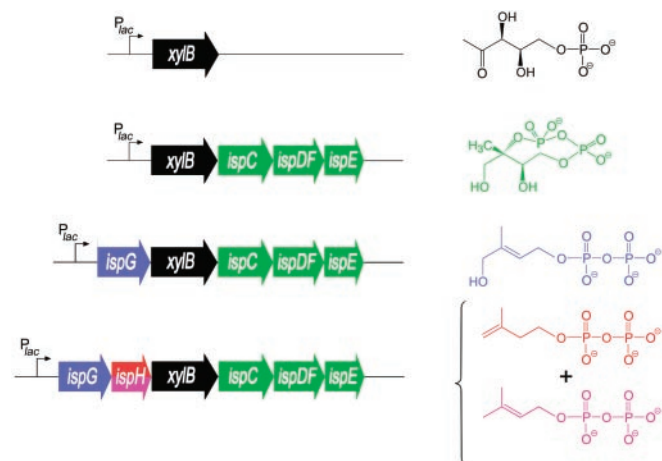
Fig. 2. Expression vectors comprising synthetic operons of *xylB* together with genes of the nonmevalonate pathway used for the *in vivo* production of intermediates in the nonmevalonate pathway. ^{13}C-labeled compounds detected in cell extracts obtained from *E. coli* cells overexpressing the indexed genes and supplied with [U-[13]C$_5$]1-deoxy-D-xylulose are indicated. Genes and their respective products are shown in the same colors.

Elaborating on this strategy, we have now constructed a synthetic operon directing the expression of the *ispH* gene (formerly designated *lytB*) together with the *xylB* and *ispCDEFG* genes (Fig. 2). The synthetic operon was placed under the control of a lac promoter and operator in the plasmid pBSXispC-H (Table 2). Cell extracts of an *E. coli* strain harboring that plasmid were shown to contain ≈1.5% of IspH protein based on total cell protein as shown by immunoanalysis. The levels of IspH protein in *E. coli* strains expressing the *xylB* and *ispCDEFG* genes (25) (Fig. 2) were below the detection level (<80 ng = 0.5% based on cell protein).

Recombinant *E. coli* strains carrying one of the plasmids shown in Fig. 2 were supplemented with [U-[13]C$_5$]1-deoxy-D-xylulose and cultivated as described under *Experimental Procedures*. The cells were harvested and disrupted by ultrasonic treatment. The crude supernatants were analyzed by ^{13}C NMR spectroscopy without any pretreatment.

All spectra in Fig. 3 display a set of five multiplets shown in green, which is characteristic of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate, the product of the IspF protein (Fig. 2). A second set of five signals shown in blue in Fig. 3 *B* and *C* represents the product of IspG protein, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate, which has been described in our previous study (25).

In addition to these features, the spectrum in Fig. 3*C* shows a series of novel multiplets, which are displayed in more detail in Figs. 4 and 5. The complex [13]C[13]C coupling patterns indicate that these novel metabolites are universally ^{13}C-labeled and must therefore have been derived from the proffered [U-[13]C$_5$]1-deoxy-D-xylulose. On the basis of [13]C[13]C coupling constants, [1]H[13]C correlation experiments and chemical shift comparison with reference compounds, these novel compounds can be identified as IPP (metabolite A, shown in red), isopentenol (metabolite B, shown in light blue), and DMAPP (metabolite C, shown in magenta). A detailed description of the NMR data follows.

Fig. 4 shows the expanded signals of metabolites A and B. Three ^{13}C NMR signals (21.6, 63.0, and 111.2 ppm) of metabolite A are doublets, indicating that each of the three carbon atoms is connected to a single ^{13}C-labeled neighbor. One signal (37.4 ppm) displays a pseudotriplet signature, indicating a ^{13}C atom with two adjacent ^{13}C atoms, and one signal (142.9 ppm) is a doublet of pseudotriplets, indicating a ^{13}C atom with three directly connected ^{13}C atoms. The large coupling constant of 71 Hz observed for the signals at 111.2 and 142.9 ppm is typical for olefinic carbon atoms.
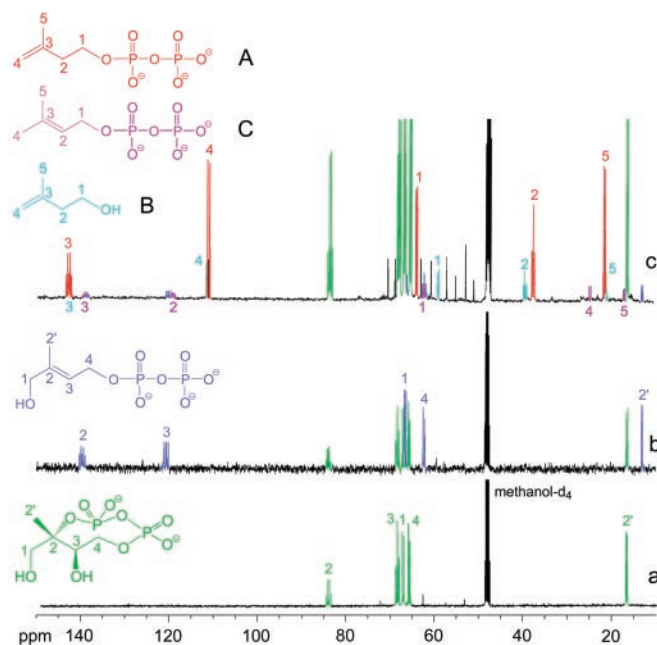

Fig. 3. ^{13}C NMR spectra of crude extracts obtained from the feeding of [U-[13]C$_5$]1-deoxy-D-xylulose to recombinant cells of *E. coli* overexpressing (*a*) the *xylB, ispC-F* genes, (*b*) the *xylB, ispC-ispG* genes, and (*c*) the *xylB, ispC-ispH* genes.

Long-range [13]C[13]C coupling is responsible for fine splitting or apparent signal broadening for the signals at 37.4, 63.0, and 111.2 ppm of the universally ^{13}C-labeled metabolite A.

HMQC and HMQC-TOCSY experiments revealed the ^{1}H NMR chemical shifts as well as the complete ^{13}C and ^{1}H spin systems of metabolite A. Specifically, the ^{13}C NMR signal at 111.2 ppm is correlated to a ^{1}H NMR signal at 4.73 ppm. The signal at 142.9 ppm gave no [1]H[13]C correlation and represents a quaternary carbon. The ^{13}C signals at 63.0, 37.4, and 21.6 ppm show correlations to ^{1}H signals at 4.00, 2.31, and 1.68 ppm, respectively. As shown by
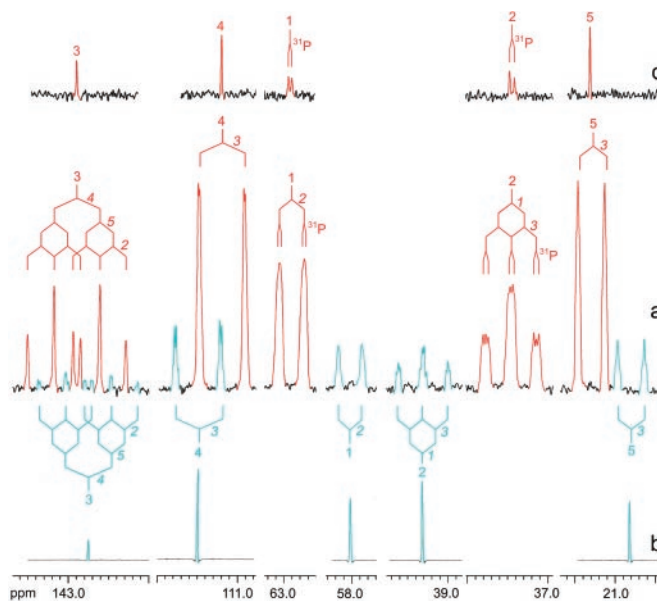

Fig. 4. ^{13}C NMR signals of IPP (in red) and isopentenol (in light blue) (*a*) of the crude extract obtained from the feeding of [U-[13]C$_5$]1-deoxy-D-xylulose to recombinant cells of *E. coli* overexpressing the *xylB, ispC-H* genes (cf. Fig. 3) (*b* and *c*) of reference samples with natural ^{13}C abundances.

Fig. 5. ^{13}C NMR signals of DMAPP (in magenta) (*b*) of the crude extract obtained from the feeding of [U-[13]C$_5$]1-deoxy-D-xylulose to recombinant cells of *E. coli* overexpressing the *xylB, ispC-H* genes (cf. Fig. 3) (*a*) of a reference sample with natural ^{13}C abundance.

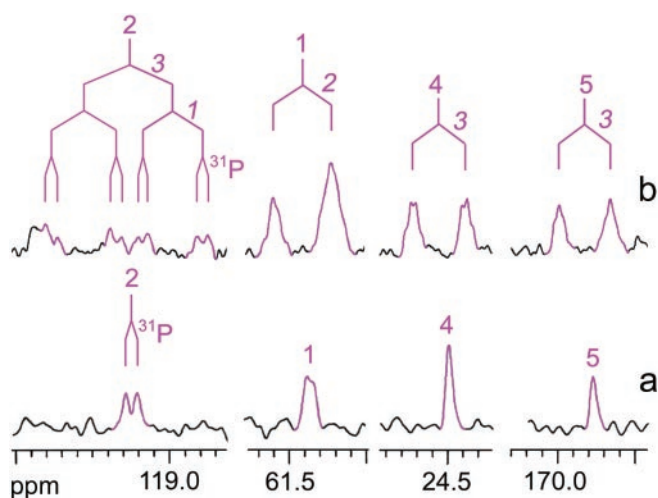HMQC-TOCSY experiments, the proton signals at 2.31 and 4.00 were [1]H-coupled, whereas the signals at 4.73 and 1.68 ppm appeared as singlets in the HMQC-TOCSY experiment. All of these features identify metabolite A as an isopentene derivative.

The ^{13}C and ^{1}H chemical shifts of metabolite A agree with those of authentic, unlabeled IPP. Carbon atoms 1 and 2 of biosynthetic IPP as well as the synthetic reference sample show [13]C[31]P coupling. The assignment of metabolite A as IPP is also well in line with ^{31}P NMR spectra, indicating the presence of an organic diphosphate derivative in significant amounts (Table 3).

The coupling and correlation patterns of metabolite B as gleaned from one- and two-dimensional (HMQC and HMQC-TOCSY) NMR spectra are closely similar to those of metabolite A (IPP). The [13]C[13]C and [13]C[1]H connectivity patterns of metabolite B and IPP are identical, and the ^{1}H and ^{13}C chemical shift values of metabolite B are identical with those of isopentenol. The absence of [13]C[31]P

coupling agrees with the identification of metabolite B as [U-[13]C$_5$]isopentenol, which may have been formed by dephosphorylation of IPP.

The structure of metabolite C (Figs. 3 and 5) was assigned by the same approach. The ^{13}C coupling patterns (three doublets, one double doublet, one multiplet) suggested that the compound is an isopentane derivative. The chemical shifts observed for the double-doublet (119.2 ppm) and the multiplet (139.0 ppm) show that a carbon–carbon double bond connects C-2 (coupled to two adjacent ^{13}C atoms) and C-3 (coupled to three adjacent ^{13}C atoms) of the molecule. The signal at 119.2 ppm showed fine splitting caused by [13]C[31]P coupling. The signals at 61.4, 24.5, and 16.7 ppm were broadened because of long-range [13]C[13]C couplings. The ^{1}H NMR chemical shifts revealed by HMQC and HMQC-TOCSY experiments show two singlets at 1.75 and 1.71 ppm and a spin system comprising signals at 5.43 and 4.45 ppm. In conjunction with the chemical shifts, this correlation pattern shows that metabolite C is a dimethylallyl derivative.

The ^{13}C and ^{1}H NMR chemical shifts of an authentic sample of DMAPP were identical to the chemical shifts of the signals attributed to metabolite C (Fig. 5).

By evaluation of the NMR signal integrals, it can be estimated that the concentrations of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate, IPP, and DMAPP are ≈25, 1, 5, and 1 mM, respectively. The concentration of isopentenol increased during the period of feeding and NMR experiments probably caused by degradation of IPP.

Discussion

Earlier *in vivo* studies had shown that IspG protein catalyzes the reductive ring opening of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate to 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (25). We have now extended this approach to include a recombinant IspH protein and were able to detect the formation of a 5:1 mixture of IPP and DMAPP upon feeding of ^{13}C-labeled 1-deoxy-D-xylulose to an *E. coli* strain engineered for the overexpression of the *ispH* (*lytB*) gene in addition to recombinant *xylB* and *ispCDEFG* genes.

In the mevalonate pathway IPP and DMAPP are generated sequentially, the latter arising from the former in an equilibration reaction catalyzed by an isomerase that exploits a protonation-deprotonation mechanism (40–42). In the present case sequential

Table 3. NMR data of IPP and DMAPP

Position	Chemical shifts, ppm			Coupling constants, Hz					Correlation pattern	
	[1]H*	[13]C*	[31]P†	J$_{PC}$	J$_{HH}$	J$_{PP}$	J$_{PH}$	J$_{CC}$‡	HMQC‡	HMQC-TOCSY‡
IPP (metabolite A)										
1	4.00	63.0		4.9	6.6		6.6	34	1	1, 2
2	2.31	37.4		8.0	6.7			40, 40	2	2, 1
3		142.9						71, 41, 41		
4	4.73	111.2						71	4	
5	1.68	21.6						41	5	
P			−7.8			nd				
P			−11.9			19.5				
DMAPP (metabolite C)										
1	4.45	61.4		3.6	6.6		6.6	47	1	1, 2
2	5.43	119.2		9.0	7.2			75, 48	2	2, 1
3		139.0						nd		
4	1.75	24.5						42	4	
5	1.71	16.7						41	5	
P			−9.1			21.7				
P			−6.4			21.5				

nd, not determined.
*Referenced to external trimethylsilylpropane sulfonate.
†Referenced to external 85% orthophosphoric acid.
‡Observed with [U-[13]C$_5$]DMAPP.

formation of the two compounds seems unlikely for a variety of reasons. (*i*) The IspH protein does not display isomerase activity (27, 43). (*ii*) The *idi* gene of *E. coli* encodes an IPP isomerase (44). Its catalytic activity, however, is 20-fold lower than the one of the yeast enzyme (45) and does not seem to contribute significantly to terpene biosynthesis; moreover, the *idi* gene has been shown to be dispensable in this bacterium (44). (*iii*) In contrast to the *ispH* gene the *idi* gene is not present in the genome of many bacteria operating on the mevalonate independent pathway (12, 26, 27). It seems therefore more realistic to conclude that the IspH protein is capable of generating both IPP and DMAPP along parallel ways starting from 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate as a single precursor. Accordingly, this protein can be identified as the responsible agent for the branching in the nonmevalonate pathway, which had been postulated earlier to account for the anomalous results observed in feeding experiments with deuterated substrates both with *E. coli* (46, 47) and *Eucalyptus globulus* (48). Independent genetic evidence for the existence of such a branching has been provided for the machinery of *E. coli* (43). Elucidation of the role of the protein encoded by the *ispH* gene fills the last gap in the sequence of enzymatic steps of the pathway that leads from pyruvate and D-glyceraldehyde 3-phosphate to IPP and DMAPP.

Because the experiments reported in this article were carried out *in vivo* the cofactors of the reaction catalyzed by the IspH protein remain unidentified and detailed reaction mechanisms can be proposed only with qualification. The simplest general explanation for the observed branching is provided by assuming the formation of a bidentate reactive intermediate of the type illustrated in Fig. 6, in which ∗ is a reference to the unknown number of electrons (2 to 4) in the delocalized allyl system. Results from the Rohmer group
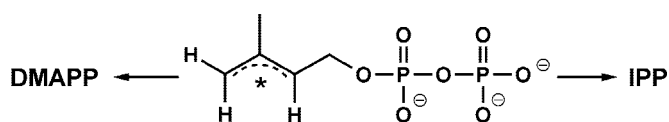

Fig. 6. A generalized structure for the hypothetical intermediate(s) at the branching point of the IspH-mediated reaction. ∗ is related to the unknown number of electrons (2 to 4) in the delocalized allyl system.

on the mevalonate-independent biosynthesis of bacteriohopans in *Zymomonas mobilis* (49) suggest the involvement of NADPH as a reducing agent in the last step of the reaction sequence that leads to IPP, and this, in turn, could be taken to imply the formation of a cationic intermediate in the reaction catalyzed by the IspH protein. The available evidence, however, does not conclusively rule out alternative pathways involving radical or anionic intermediates and the development of an appropriate *in vitro* system is clearly needed to settle the remaining mechanistic issues.

Note Added in Proof. In a paper published after submission of this manuscript (50) M. Hintz *et al.* have reported a significant accumulation in *lytB*- (now *ispH*-) deficient mutants of *E. coli* of a compound subsequently identified as 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate.

1. Sacchettini, J. C. & Poulter, C. D. (1997) *Science* **277,** 1788–1789.
2. Qureshi, N. & Porter, J. W. (1981) in *Biosynthesis of Isoprenoid Compounds,* eds. Porter, J. W. & Spurgeon, S. L. (Wiley, New York) Vol. 1, pp. 47–94.
3. Bloch, K. (1992) *Steroids* **57,** 378–382.
4. Bach, T. J. (1995) *Lipids* **30,** 191–202.
5. Bochar, D. A., Friesen, J. A., Stauffacher, C. V. & Rodwell, V. W. (1999) in *Comprehensive Natural Product Chemistry*, ed. Cane, D. (Pergamon, Oxford) Vol. 2, pp. 15–44.
6. Rohmer, M., Knani, M., Simonin, P., Sutter, B. & Sahm, H. (1993) *Biochem. J.* **295,** 517–524.
7. Broers, S. T. J. (1994) Thesis (Eidgenössische Technische Hochschule, Zürich).
8. Schwarz, M. K. (1994) Thesis (Eidgenössische Technische Hochschule, Zürich).
9. Eisenreich, W., Schwarz, M., Cartayrade, A., Arigoni, D., Zenk, M. H. & Bacher A. (1998) *Chem. Biol.* **5,** R221–R233.
10. Rohmer, M. (1999) in *Comprehensive Natural Product Chemistry*, ed. Cane, D. (Pergamon, Oxford) Vol. 2, pp. 45–68.
11. Schwarz, M. & Arigoni, D. (1999) in *Comprehensive Natural Product Chemistry*, ed. Cane, D. (Pergamon, Oxford) Vol. 2, pp. 367–399.
12. Rohdich, F., Kis, K., Bacher, A. & Eisenreich, W. (2001) *Curr. Opin. Chem. Biol.* **5,** 535–540.
13. Sprenger, G. A., Schörken, U., Wiegert, T., Grolle, S., deGraaf, A. A., Taylor, S. V., Begley, T. P., Bringer-Meyer, S. & Sahm, H. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 12857–12862.
14. Lois, L. M., Campos, N., Putra, S. R., Danielsen, K., Rohmer, M. & Boronat, A. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 2105–2110.
15. White, R. H. (1978) *Biochemistry* **17,** 3833–3840.
16. David, S., Estramareix, B., Fischer, J.-C. & Thèrisod, M. (1981) *J. Am. Chem. Soc.* **103,** 7341–7342.
17. Hill, R. E., Sayer, B. G. & Spenser, J. D. (1989) *J. Am. Chem. Soc.* **111,** 1916–1917.
18. Takahashi, S., Kuzuyama, T., Watanabe, H. & Seto, H. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 9879–9884.
19. Rohdich, F. Wungsintaweekul, J., Fellermeier, M., Sagner, S., Herz, S., Kis, K., Eisenreich, W., Bacher, A. & Zenk, M. H. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 11758–11763.
20. Kuzuyama, T., Takagi, M., Kaneda, K., Dairi, T. & Seto, H. (2000) *Tetrahedron Lett.* **41,** 703–706.
21. 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) *Proc. Natl. Acad. Sci. USA* **97,** 1062–1067.
22. Kuzuyama, T., Takagi, M., Kaneda, K., Watanabe, H., Dairi, T. & Seto, H. (2000) *Tetrahderon Lett.* **41,** 2925–2928.
23. 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) *Proc. Natl. Acad. Sci. USA* **97,** 2486–2490.
24. Takagi, M., Kuzuyama, T., Kaneda, K., Watanabe, H., Dairi, T. & Seto, H. (2000) *Tetrahedron Lett.* **41,** 3395–3398.
25. Hecht, S., Eisenreich, W., Adam, P., Amslinger, S., Kis, K., Bacher, A., Arigoni, D. & Rohdich, F. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 14837–14842.
26. Adam, P., Eisenreich, W., Fellermeier, M., Hecht, S., Rohdich, F., Schuhr, C. A., Wungsintaweekul, J., Zenk, M. H. & Bacher, A. (2000) German Patent Appl. 10027821.3.
27. Cunningham, F. X., Jr., Lafond, T. P. & Grantt, E. (2001) *J. Bacteriol.* **182,** 5841–5848.
28. Altincicek, B., Kollas, A., Eberl, M., Wiesner, J., Sanderbrand, S., Hintz, M., Beck, F. & Jomaa, H. (2001) *FEBS Lett.* **499,** 37–40.
29. McAteer, S., Coulson, A., McLennan, N. & Master, M. (2001) *J. Bacteriol.* **183,** 7403–7407.
30. Jomaa, H., Wiesner, J., Sanderbrand, S., Altinicicek, B., Weidemeyer, C., Hintz, M., Türbachova, I., Eberl, M., Zeidler, J., Lichtenthaler, H. K., *et al.* (1999) *Science* **285,** 1573–1576.
31. Hecht, S., Kis, K., Eisenreich, W., Amslinger, S., Wungsintaweekul, J., Herz, S., Rohdich, F. & Bacher, A. (2001) *J. Org. Chem.* **66,** 3948–3952.
32. Sanger, F., Nicklen, S. & Coulson, A. R. (1992) *Biotechnology* **24,** 104–108.
33. Bullock, W. O., Fernandez, J. M. & Short, J. M. (1978) *BioTechniques* **5,** 376–379.
34. Zamenhof, P. J. & Villarejo, M. (1972) *J. Bacteriol.* **110,** 171–178.
35. Chang, A. C. Y. & Cohen, S. N. (1978) *J. Bacteriol.* **134,** 1141–1156.
36. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
37. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
38. Hinzpeter, M. (1993) in *Methods of Immunological Analysis*, eds. Massyeff, R. F., Albert, V. H. & Steines, N. A. (VCH, Weinheim, Germany), Vol. 1, pp. 529–535.
39. Mancini, G., Carbonara, A. O. & Heremans, J. F. (1965) *Immunochemistry* **2,** 235–254.
40. Reardon, J. E. & Abeles, R. H. (1986) *Biochemistry* **25,** 5609–5616.
41. Muehlbacher, M. & Poulter, C. D. (1988) *Biochemistry* **27,** 7315–7328.
42. Poulter, C. D., Muehlbacher, M. & Davis, D. R. (1989) *J. Am. Chem. Soc.* **101,** 3740–3742.
43. Rodriguez-Concepcion, M., Campos, N., Maria Lois, L., Maldonado, C., Hoeffler, J.-F., Grosdemange-Billiard, C., Rohmer, M. & Boronat, A. (2000) *FEBS Lett.* **473,** 328–332.
44. Hahn, F. M., Hurlburt, P. A. & Poulter, C. D. (1999) *J. Bacteriol.* **181,** 4499–4504.
45. Street, I. P. & Poulter, C. D. (1990) *Biochemistry* **29,** 7531–7538.
46. Giner, J.-L., Jaun, B. & Arigoni, D. (1998) *Chem. Commun.* **17,** 1857–1858.
47. Charon, L., Hoeffler, J. F., Pale-Grosdemange, C., Losi, L. M., Campos, N. Boronat, A. & Rohmer M. (2000) *Biochem J.* **346,** 737–742.
48. Rieder, C., Jaun, B. & Arigoni, D. (2000) *Helv. Chim. Acta* **83,** 2504–2513.
49. Charon, L., Pale-Grosdemange, C. & Rohmer, M. (1999) *Tetrahedron Lett.* **40,** 7231–7234.
50. Hintz, M., Reichenberg, A., Altincicek, B., Bahr, U. Gschwind, R. M., Kollas, A. K., Beck, E., Wiesner, J., Eberl, M. & Jomaa, H. (2001) *FEBS Lett.* **509,** 317–322.