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Deoxyxylulose phosphate pathway of isoprenoid biosynthesis. Discovery and function of *ispDEFGH* genes and their cognate enzymes*

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Abstract: Isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) serve as the universal precursors for the biosynthesis of terpenes. Besides the well-known mevalonate pathway, a second biosynthetic pathway conducive to IPP and DMAPP via 1-deoxy-D-xylulose-5-phosphate and 2*C*-methyl-D-erythritol-4-phosphate has been discovered recently in plants and certain eubacteria. 2*C*-Methyl-D-erythritol-4-phosphate, the first committed intermediate of the deoxyxylulose phosphate pathway, is converted into 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate by the catalytic action of three enzymes specified by the *ispDEF* genes. The cyclic diphosphate is reductively opened by the IspG protein affording 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate. This compound can be converted into IPP as well as DMAPP by the catalytic action of IspH protein. The enzymes of this pathway are potential targets for novel antibacterial, antimalarial, and herbicide agents.

INTRODUCTION

Terpenes constitute a large group of natural products comprising more than 30 000 known compounds of tremendous structural diversity. A number of terpenes have medical relevance as vitamins, hormones, and cytostatic agents [1].

All terpenes are assembled from two simple precursors, isopentenyl diphosphate (IPP) (**1**) and dimethylallyl diphosphate (DMAPP) (**2**). Their biosynthesis from acetyl-CoA (**11**) via mevalonate (**14**) has been studied extensively in yeast and animal cells (Scheme 1) [2–5]. These pioneering studies were conducive to the development of the statins, inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase, which are among the most important drugs for prevention and therapy of cardiovascular disease.

In the decades following these classical studies, it became an established dogma that all terpenes are invariably biosynthesized via **14**, and occasional conflicting evidence was steadfastly ignored for a period of several decades [6].

About 10 years ago, independent work in the research groups of D. Arigoni and M. Rohmer showed that the biosynthesis of certain bacterial and plant terpenoids could in fact not be explained by the mevalonate pathway [7–9]. A second pathway was shown to start with the condensation of pyruvate

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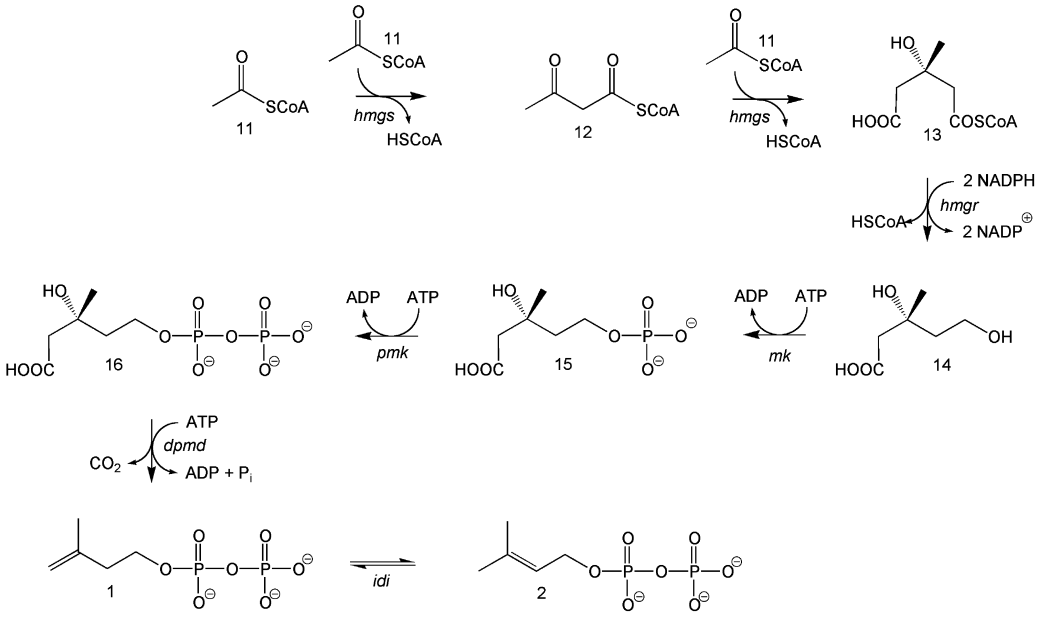


Scheme 1 Biosynthesis of IPP and DMAPP via mevalonate. **1**, IPP; **2**, DMAPP; **11**, acetyl-CoA; **12**, acetoacetyl-CoA; **13**, 3-hydroxy-3-methylglutaryl-CoA; **14**, mevalonate; **15**, phosphomevalonate; **16**, diphosphomevalonate.

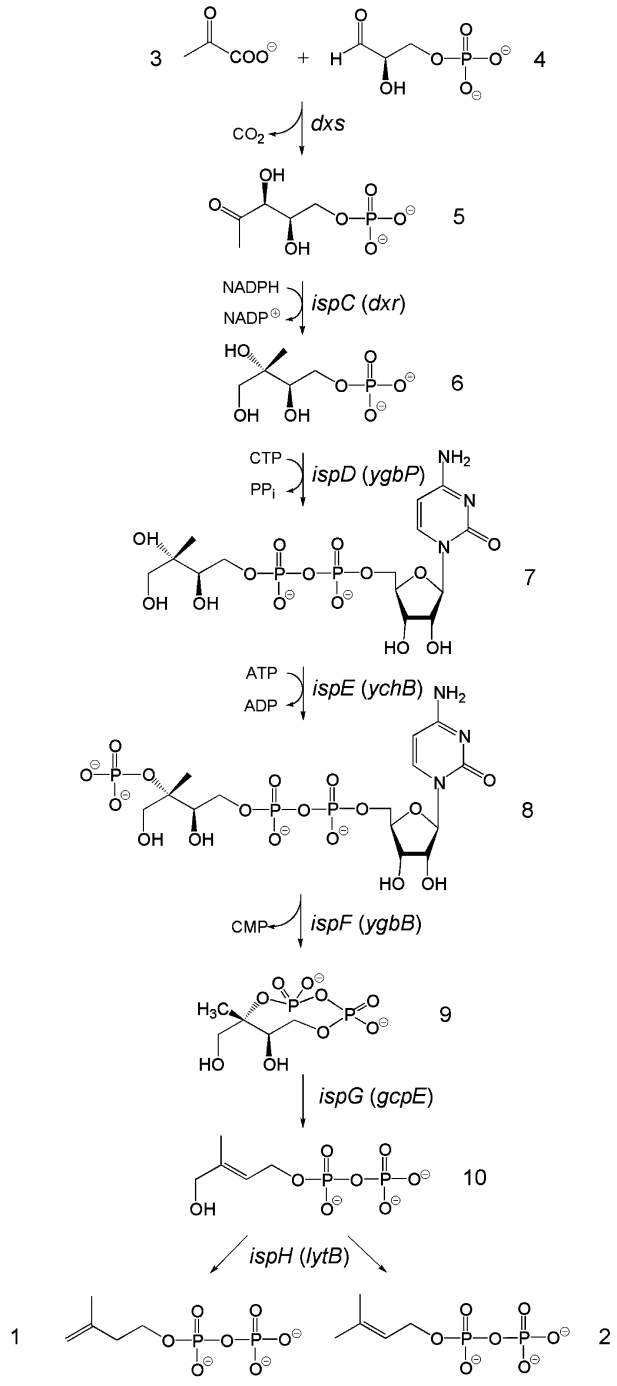
(**3**) and D-glyceraldehyde-3-phosphate (**4**) affording 1-deoxy-D-xylulose-phosphate (**5**), a known precursor for thiamine and pyridoxal [10–15] (Scheme 2). The reaction is catalyzed by 1-deoxy-D-xylulose-5-phosphate-synthase specified by the *dxs* gene [14,15]. The enzyme is similar to transketolases and requires thiamine diphosphate for activity. The early work on the deoxyxylulose phosphate pathway has been reviewed repeatedly, and the reader is directed to these articles for an in-depth discussion [6,16–19].

A skeletal rearrangement of **5** followed by reduction of the resulting, branched aldose affords 2*C*-methyl-D-erythritol-4-phosphate (**6**) (Scheme 2). This step is catalyzed by 2*C*-methyl-D-erythritol-4-phosphate-synthase specified by the *ispC* (*dxr*) gene [20] which requires reduced nicotinamide adenine dinucleotide phosphate (NADPH) as cosubstrate and magnesium ions as cofactor [18,19]. Studies of the stereochemical features revealed that the H_{Si} from C4 of NADPH is transferred to the H_{Re} position at C1 of **6** [21,22].

IspDEFGH GENES

The distribution of *dxs* and *ispC* genes in different microbial taxons is orthogonal to the distribution of mevalonate biosynthesis genes (Table 1). The search for additional genes mimicking the distribution of the known deoxyxylulose phosphate pathway genes played an important role in the further elucidation of the pathway.

The transformation of **6** into **1** resp. **2** via the removal of three hydroxy groups requires three 2-electron reduction steps and the transformation of the phosphomonoester motif into a pyrophosphate motif. Initial studies with *Escherichia coli* cell extracts showed a requirement of cytidine triphosphate for the enzymatic conversion of **6** into a hitherto unknown product [23]. Comparative genome analysis showed that the previously unassigned *ygbP* gene (subsequently designated *ispD*) of *Haemophilus influenzae* is similar to the *acs1* gene specifying a cytidyl phosphate transferase which converts ribitol 5-phosphate into 5-diphosphocytidyl-ribitol. Moreover, genomic comparison showed that the distribution of the *ispD* gene in microbial genomes mimicks that of the *dxs* and *ispC* genes (Table 1).



Scheme 2 Biosynthesis of IPP and DMAPP via 1-deoxy-D-xylulose-5-phosphate. **3**, pyruvate; **4**, D-glyceraldehyde-3-phosphate; **5**, 1-deoxy-D-xylulose-5-phosphate; **6**, 2*C*-methyl-D-erythritol-4-phosphate; **7**, 4-diphosphocytidyl-2*C*-methyl-D-erythritol; **8**, 4-diphosphocytidyl-2*C*-methyl-D-erythritol-2-phosphate; **9**, 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate; **10**, 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate.

Table 1 IPP and DMAPP biosynthesis genes in completely sequenced organisms.

Organism	Deoxyxylulose phosphate pathway							Mevalonate pathway						
	dxs	*ispC*	*ispD*	*ispE*	*ispF*	*ispG*	*ispH*	*hmgs*	*hmgr*	*mk*	*pmk*	*dpmd*	*idiI*	*idiII*
Bacteria														
Aquifales (*Aquifex aeolicus*)	+	+	+	+	+	+	+	−	−	−	−	−	−	−
Chlamydia group (*Chlamydophila pneumoniae*)	+	+	+	+	+	+	+	−	−	−	−	−	−	−
Cyanobacteria (*Synechocystis* sp.)	+	+	+	+	+	+	+	−	−	−	−	−	−	+
Deinococcus group (*Deinococcus radiodurans*)	+	+	+	+	+	+	+	−	−	−	−	−	−	+
Firmicutes (*Bacillus subtilis*)	+	+	+	+	+	+	+	−	−	−	−	−	−	+
(*Mycoplasma genitalium*)	−	−	−	−	−	−	−	−	−	−	−	−	−	−
(*Staphyloccus aureus*)	−	−	−	−	−	−	−	+	+	+	+	+	−	+
(*Streptomyces coelicolor*)	+	+	+	+	+	+	+	−	−	−	−	−	+	−
Proteobacteria (*Escherichia coli*)	+	+	+	+	+	+	+	−	−	−	−	−	+	−
(*Rickettsia prowazeckii*)	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Spirochaetales (*Treponema pallidum*)	+	+	+	+	+	+	+	−	−	−	−	−	−	−
(*Borrelia burgdorferi*)	−	−	−	−	−	−	−	+	+	+	+	+	−	+
Thermotogales (*Thermotoga maritima*)	+	+	+	+	+	+	+	−	−	−	−	−	−	−
Archaea														
Crenarchaeota *(Aeropyrum pernix)*	−	−	−	−	−	−	−	+	+	+	−	−	−	+
Euryarchaeota (*Archaeoglobus fulgidus*)	−	−	−	−	−	−	−	+	+	+	−	−	−	+
Eukaryotes														
Animals (*Homo sapiens*)	−	−	−	−	−	−	−	+	+	+	+	+	+	−
Plants (*Arabidopsis thaliana*)	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Protozoa (*Plasmodium falciparum*)	+	+	+	+	+	+	+	−	−	−	−	−	−	−
Yeasts (*Saccharomyces cerevisiae*)	−	−	−	−	−	−	−	+	+	+	+	+	+	−

Abbreviations: *dxs*, 1-deoxy-D-xylulose-5-phosphate-synthase; *ispC*, 2*C*-methyl-D-erythritol-4-phosphate-synthase; *ispD*, 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase; *ispE*, 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase; *ispF*, 2*C*-methyl-D-erythritol-2,4-cyclodiphosphate-synthase; *ispG*, 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate-synthase; *ispH*, 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate reductase; *hmgs*, 3-hydroxy-3-methylglutaryl-CoA synthase; *hmgr*, 3-hydroxy-3-methylglutaryl-CoA reductase; *mk*, mevalonate kinase; *pmk*, phospomevalonate kinase; *dpmd*, diphospomevalonat decarboxylase; *idiI/II*, isopentyldiphosphate isomerase type I/II.

The recombinant expression of the *ispD* gene afforded a protein catalyzing the transformation of **6** into 4-diphosphocytidyl-2*C*-methyl-D-erythritol (**7**) [23] (Scheme 2). The protein requires Mg^{2+} for activity. Besides cytidine 5′-triphosphate (CTP), the enzyme accepts various other nucleotide triphosphates as substrates, although at a significantly lower rate [23]. IspD protein from *Arabidopsis thaliana* has been characterized in some detail [18,19,24]. The three-dimensional structure of the *E. coli* enzyme has been determined by X-ray crystallography [25,26].

Systematic analysis of ortholog families following the distribution of the *dxs* and *ispCD* gene families allowed the functional assignment of the genes *ychB, ygbB, gcpE,* and *lytB* which were subsequently renamed *ispEFGH* [27,28] (Table 1). These recent developments are discussed below.

The recombinant expression of the *ispE* (*ychB*) gene of *E. coli* afforded a protein which catalyzes the phosphorylation of the C2 hydroxy group of **7** affording 4-diphosphocytidyl-2*C*-methyl-D-erythritol-2-phosphate (**8**) as demonstrated by NMR spectroscopy using ^{13}C-labeled substrates [28] (Scheme 2). The enzyme requires magnesium as cofactor [18,19]. An ortholog from tomato was cloned and characterized in some detail [29].

By extension of the experimental approach described above, we could further show that the product **8** of IspE protein serves as substrate for recombinant IspF protein [30]. More specifically, the IspF proteins of *E. coli* and *Plasmodium falciparum* were both shown by NMR studies to convert **8** into 2*C*-methyl-D-erythrityl-2,4-cyclodiphosphate (**9**) under release of cytidine monophosphate [30,31] (Scheme 2). The enzymes require magnesium ions as cofactor [18,19]. Recently, the threedimensional structure of the *E. coli* protein was solved [32–34]. The three topologically equivalent active sites of the homotrimeric protein contain zinc ions coordinated by two conserved histidine and one aspartate residue [32]. The structures of several substrate and product complexes indicate that the zinc ion helps to position the substrate at the active site and facilitates the nucleophilic attack of the 2-phosphate group [32]. The magnesium ion in the active site coordinates the α- and β-phosphate groups of the CDP moiety.

The transformation of the cyclic diphosphate **9** into **1** resp. **2** requires the reductive opening of the 8-membered ring, a mechanistically nontrivial reaction. As mentioned above, genomic analysis identified *gcpE* and *lytB* as candidate genes which were subsequently renamed *ispG* and *ispH* [28].

Initially, we used an in vivo system to elucidate the functions of the proteins specified by these genes. The recombinant expression of the *xylB* gene specifying D-xylulokinase [35] in conjunction with the *ispCDEF* genes enabled an engineered *E. coli* strain to transform exogenous ^{13}C-labeled 1-deoxy-D-xylulose (**17**) into copious amounts of intracellular ^{13}C-labeled **9** [36] (Fig. 1). The additional imple-

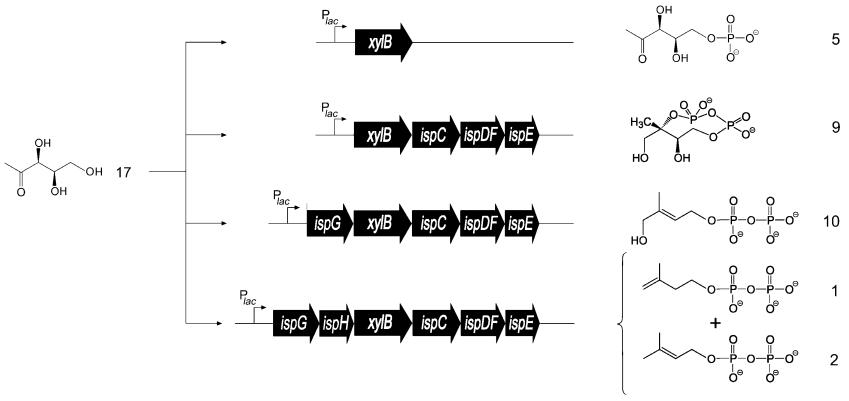


Fig. 1 ^{13}C-labeled compounds detected in cell extracts obtained from recombinant *E. coli* cells. The bacterial strains were engineered for overexpression of the *xylB* gene in conjunction with deoxyxylulose phosphate pathway genes. **17**, 1-deoxy-D-xylulose.

mentation of the recombinant *ispG* (*gcpE*) gene was then conducive to the formation of a novel metabolite which was identified as 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate (**10**) by direct NMR analysis of crude cell extract without purification [36]. The same compound was also isolated from an *ispH* chromosomal mutant of *E. coli* [37]. A crude cell extract of a recombinant *E. coli* strain expressing the *ispG*, *yfgA*, and *yfgB* genes, which are closely linked to *ispG* on the chromosomes of certain bacteria, was reported to catalyze the in vitro conversion of **9** into **10** [38].

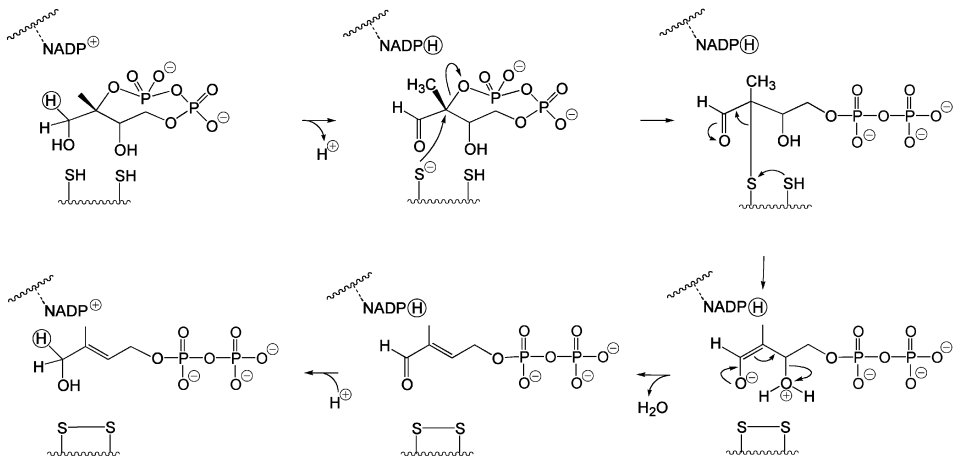
The recombinant expression of the *ispH* (*lytB*) gene in conjunction with the *xylB* and *ispDEFG* genes enabled an engineered strain to transform exogenous **17** beyond the level of **10**, thus affording a 5:1 mixture of **1** and **2** [39] (Fig. 1). Purified IspH protein required the addition of crude cell extract for catalytic activity thus suggesting that one or several additional proteins serve as electron shuttles [40]. reduced nicotinamide adenine dinucleotide (NADH) and flavine adenine dinucleotide (FAD) are required as cofactors.

REACTION MECHANISMS OF THE IspG AND IspH PROTEINS

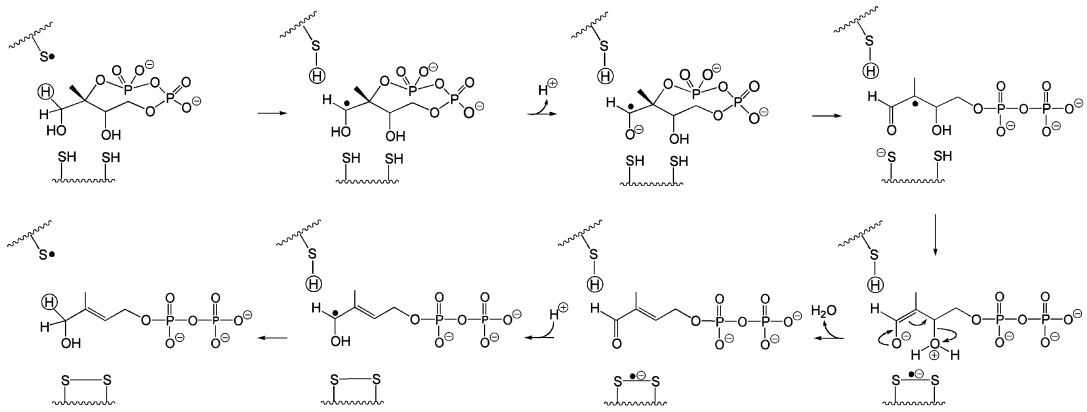
The overall reaction catalyzed by the IspG protein is a two-electron reduction involving the cleavage of two carbon–oxygen bonds. Earlier work on the biosynthesis of terpenes in *E. coli* had shown that all the carbon-bonded hydrogen atoms of the cyclodiphosphate precursor are preserved in **1** and **2** [41–43]. This puts severe limitations on the choice of a plausible reaction mechanism for the unusual biochemical transformation. Two hypothetical reaction sequences are summarized in Schemes 3 and 4, both of which are critically dependent on the presence of at least two thiol groups; notably, *ispG* genes invariably specify three absolutely conserved cysteine residues [36].

The mechanism illustrated in Scheme 3 is patterned after the mode of action of vitamin K epoxyquinone reductase [44]. The aldehyde group generated in the first step using $NADP^+$ as a putative cofactor is expected to accelerate the subsequent nucleophilic displacement of the pyrophosphate group at C2 by a thiolate ion and provides the necessary electron sink in the step which leads to the formation of a disulfide bond. Following β-elimination of water from the resulting enolate, the original oxidation state of C1 is then restored by back-transfer from the cofactor of the hydride ion removed in the first step.

The alternative presented in Scheme 4 is a radical process akin to the one exploited by ribonucleotide reductase [45], in which the role of the immediate initiator is assigned, by analogy, to a thiyl radical derived from the third conserved cysteine residue of the enzyme. Participation of such a radical without or with negligible exchange of the resulting thiol group with solvent protons is well precedented [45].



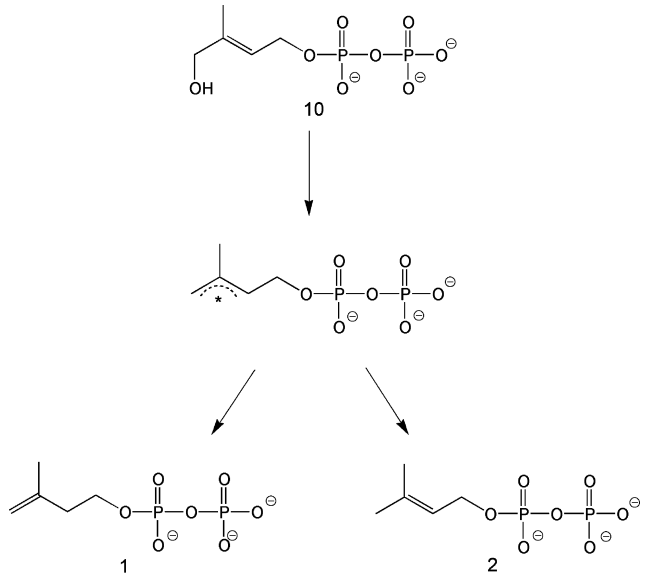
Scheme 3 Hypothetical mechanism of the IspG protein-mediated reaction patterned after the mode of action of vitamin K epoxyquinone reductase [44].



Scheme 4 An alternative hypothetical mechanism of the IspG protein-mediated reaction patterned after the mode of action of ribonucleotide reductase [45].

In both schemes, reductive cleavage of the disulfide bond is necessary for multiple turnover. No clues are as yet available concerning the nature of the reducing agent. An unusual sensitivity of the latter may explain why the cyclic diphosphate **9** is accumulated in certain bacteria under conditions of oxidative stress [46].

Similar to IspG protein, IspH protein also comprises three absolutely conserved cysteine residues which are believed to be relevant for catalysis [39]. One can assume the formation of bidentate reactive intermediates of the type illustrated in Scheme 5, in which the symbol * is a reference to the unknown number of electrons (two, three, or four) in the delocalized allyl system. The reaction could proceed via an anionic, radical or cationic allyl system. Both **1** and **2** can be obtained by the transfer of H^+, $H^\bullet$ or H^- to either the 1 or 3 position of the hypothetical intermediate, thus explaining the branching in the deoxyxylulose phosphate pathway, which is discussed in more detail below.



Scheme 5 A generalized structure for the hypothetical intermediate(s) at the branching point of the IspH-mediated reaction. The symbol * is related to the unknown number of electrons (two, three, or four) in the delocalized allyl system.

BRANCHING IN THE DEOXYXYLULOSE PHOSPHATE PATHWAY

In the mevalonate pathway, **1** is generated from **2** by an isomerase that exploits a protonation-deprotonation mechanism (Scheme 1) [47–49]. In the deoxyxylulose phosphate pathway, sequential formation of the two compounds **1** and **2** seems unlikely because the deletion of the *idi* gene specifying IPP isomerase does not affect the viability and growth rates for *E. coli* [50].

Moreover, the genomes of many eubacteria depending on the deoxyxylulose phosphate pathway most probably do not specify an IPP isomerase (Table 1). Additionally, a branching in the deoxyxylulose phosphate pathway had been postulated earlier in order to account for the anomalous results observed in feeding experiments with deuterated substrates with *E. coli* [41,43], *Eucalyptus globulus* [51], and tobacco [52]; independent genetic evidence for the existence of such a branching has been provided for the machinery of *E. coli* [53]. Comparative genome analysis suggested that the IspH protein might be involved in the last steps of the deoxyxylulose phosphate pathway [28,54]. Indeed, in vivo NMR studies showed this protein is the responsible agent for the branching in the deoxyxylulose phosphate pathway, capable of generating both **1** and **2** along parallel ways starting from **10** (Scheme 2) [39].

SYNTHESIS OF INTERMEDIATES OF THE DEOXYXYLULOSE PHOSPHATE PATHWAY

Chemical and enzyme-assisted synthesis of intermediates with or without isotopic labels was essential in the elucidation of the deoxyxylulose phosphate pathway.

Chemical methods for the preparation of **5**–**7**, and **9** had been reported in the literature [55–65]. Seven methods for the preparation of **10** have been published in rapid sequence since the discovery of that compound [38,66–71]. Notably, this compound can now be obtained from 2-methyl-2-vinyl-oxirane in two reaction steps [71].

The recombinant pathway enzymes were used extensively for the preparation of isotope-labeled **5**–**7**, and **9** [72–75]. The multistep enzyme-assisted syntheses can be performed as convenient one-pot reaction starting from simple precursors such as glucose and pyruvate and afford an almost unlimited variety of isotopomers by parallel synthesis [75]. As an example, the one-pot synthesis of **9** is summarized in Scheme 6. Notably, that reaction sequence involves the cooperation of 15 enzymes.

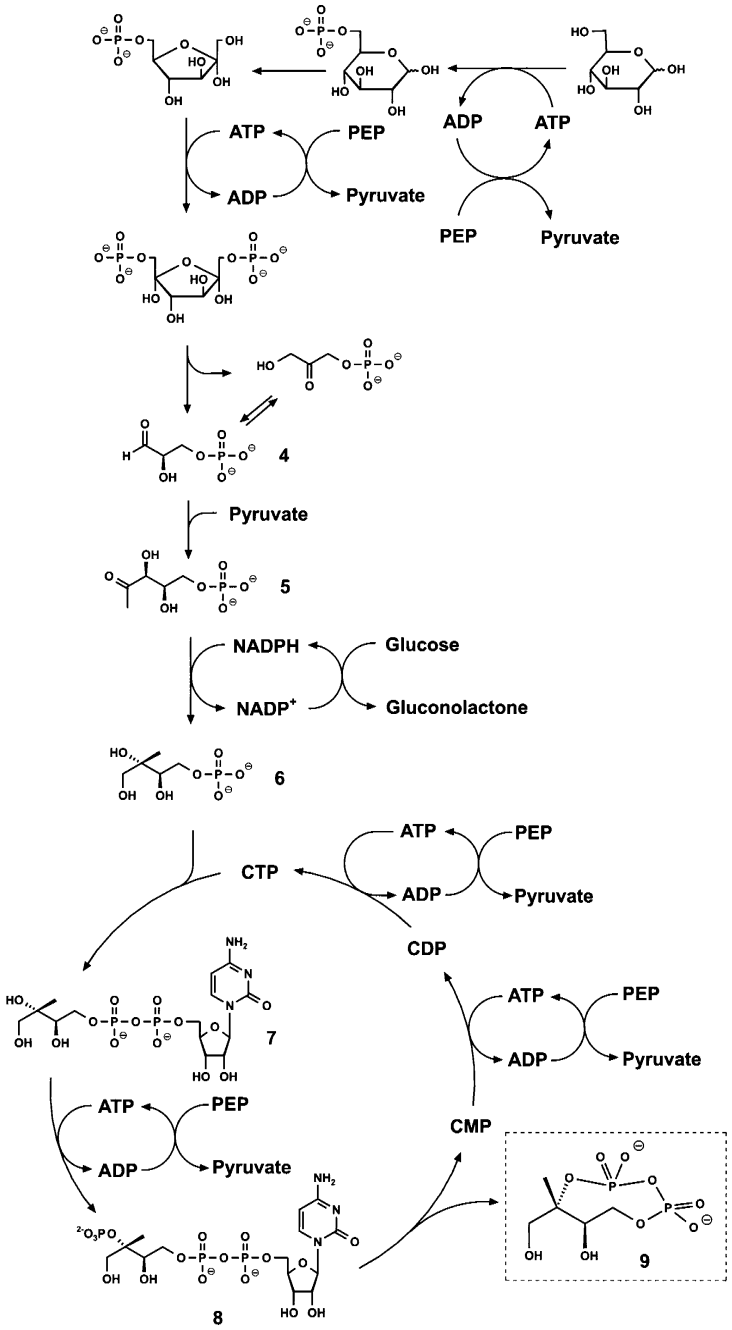
PRACTICAL ASPECTS

The recently acquired knowledge on the deoxyxylulose phosphate pathway has potential significance for medicine and agriculture. It is now clear that bacterial pathogens with the exception of gram-positive cocci such as *Streptococcus* spp. and *Staphylococcus* spp. use exclusively the deoxyxylulose phosphate isoprenoid pathway (Table 1). The same holds true for *Plasmodium* spp., the causative agents of malaria.

Since the genes and enzymes of the deoxyxylulose phosphate pathway have been shown to be essential in the pathogens and to be absent in eukaryotic hosts (Table 1), they are potential targets for the development of novel anti-infective agents. In fact, the antibiotic, fosmidomycin, a product of *Streptomyces lavendulae* [76], has been shown recently to act via inhibition of 2*C*-methyl-D-erythritol-4-phosphate-synthase [77] and to cure mice infected with *Plasmodium vinckii*, the causative agent of a mouse disease similar to malaria [78].

Although plants use both isoprenoid biosynthetic pathways, the genes of the deoxyxylulose phosphate pathway are believed to be essential. Hence, the enzymes of the pathway are potential herbicide targets.

It had been known for some time that **1** and structurally related compounds stimulate the proliferation of certain γδ T lymphocytes [79–82]. Recent studies show that the deoxyxylulose phosphate pathway intermediate **10** is a much more potent stimulator than **1** [83]. More specifically, the median EC_{50} for the growth proliferation of human γ9δ2 T cells is 70 pM [84], and the biosynthetic interme-



Scheme 6 Enzyme-assisted one-pot reaction for the synthesis of isotope-labeled 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate [75].

diate may qualify as a "pathogen-associated molecular pattern" used by the innate immune system for early detection of pathogens, in parallel with the detection of various other molecular markers of pathogens such as lipopolysaccharides, flagellin, and bacterial DNA by receptors of the innate immune system [85].

TAXONOMIC ASPECTS

Based on biochemical studies and on genomic comparison, the taxonomic distribution of the two isoprenoid pathways can now be described in some detail (Table 1). Archaebacteria, animals, and fungi use exclusively the mevalonate pathway. Plants use the mevalonate pathway for the biosynthesis of sterols and triterpenes in the cytoplasm and the deoxyxylulose phosphate pathway for the biosynthesis of carotenoids, phytol, and of a wide variety of secondary metabolites in the plastid compartment [16].

In plants, **1** and further unknown metabolites downstream from **1** can be exchanged to variable extent between the cytoplasmic resp. plastid compartments. This cross-talk was one of the factors contributing to the incorrect generalization of the mevalonate pathway as the unique source of terpenes [86].

The distribution of the two pathways in the eubacterial kingdom is complex. As of this writing, gram-negatives with the exception of *Borrelia burgdorferi* appear to use the deoxyxylulose phosphate pathway (Table 1). Certain gram-positive cocci including *Streptococcus* spp. and *Staphylococcus* spp. use the mevalonate pathway [87]. Streptomycetes are now believed to alternatively use the mevalonate pathway (e.g., *Streptomyces* sp. strain CL190) [88] or the deoxyxylulose phosphate pathway (e.g., *Streptomyces coelicolor*) (Table 1). In completely sequenced genomes of *Mycoplasma* and *Rickettsia* spp., no orthologs of either isoprenoid pathway have been found (Table 1). It remains open whether these symbiotic organisms obtain terpenes from their hosts.

Plants, fungi, animals, and certain eubacteria use the IPP isomerase type I specified by the *idiI* gene to convert **1** obtained via the mevalonate pathway into **2**. Archaea are invariably devoid of isomerase I but were recently shown to specify a structurally unrelated type II isomerase. A type II isomerase specified by the *idiII* gene was recently found in *Streptomyces* sp. strain CL190 [89], and putative orthologs are also present in gram-positive eubacteria including *Streptococcus* and *Staphylococcus* spp. (Table 1). This enzyme has been reported to use flavin mononucleotide (FMN) and NADPH as cofactors.

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