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REVIEW

From Molecular Fossils of Bacterial Hopanoids to the Formation of Isoprene Units: Discovery and Elucidation of the Methylerythritol Phosphate Pathway

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Abstract Investigations on the biosynthesis of bacterial triterpenoids of the hopane series led to the unexpected discovery of an alternative mevalonate independent pathway for the formation of isoprene units. Methylerythritol phosphate, already presenting the C_5 branched isoprene skeleton, is the key intermediate. This pathway was independently characterized in ginkgo embryos for the formation of diterpenoids. It is present in most bacteria and in the plastids of all organisms belonging to phototrophic phyla. The key steps of the discovery and elucidation of this metabolic route are presented in this review.

Keywords Deoxyxylulose phosphate · Dimethylallyl diphosphate · Isopentenyl diphosphate · Isoprenoid biosynthesis · Methylerythritol phosphate · Mevalonate

Abbreviations

DMAPP	Dimethylallyl diphosphate
DX	1-Deoxy-D-xylulose
DXP	1-Deoxy-D-xylulose 5-phosphate
HMBPP	(*E*)-4-Hydroxy-3-methylbut-2-enyl diphosphate
IPP	Isopentenyl diphosphate
ME	2-*C*-methyl-D-erythritol
MEcPP	2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate
MEP	2-*C*-methyl-D-erythritol 4-phosphate
MVA	Mevalonic acid

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Introduction

It was a great honor for me to be selected as a recipient of the George Schroepfer Jr. Award. I met George Schroepfer only once. This was in Japan in August 1996 at an International Symposium on Isoprenoid Biochemistry at Zao, in the mountains close to Sendai. I remember well the discussion we had on the use of silver ion high-performance-liquid chromatography for sterol separation that he pioneered with spectacular results [1]. We had previously corresponded regularly concerning topics of mutual interest. When I started my PhD thesis in 1970, I was working with Guy Ourisson and Pierre Benveniste on sterol **1** (Fig. 1) biosynthesis in maize [2], in the non-photosynthetic euglenoid *Astasia longa* [3] and in parasitic non-phototrophic higher plants such as broomrape and dodder [4]. At that time, George's work in the field of sterol isolation, identification and biosynthesis was quite familiar to me. Since this early work, I kept my interest in this field during my post-doctoral work with Carl Djerassi on sterols from marine organisms [5–7] and later in Mulhouse, my first professor position, when we were working on the poorly investigated sterol biosynthesis in lower eukaryotes, such as the soil amoebae *Acanthamoeba* and *Naegleria* [8, 9] or in the obligate downy mildew wine parasite *Plasmopara viticola* [10]. All knowledge earned in the field of sterol biosynthesis proved fruitful when I switched to investigations of other isoprenoid series, particularly to the chemistry and biochemistry of bacterial triterpenoids of the hopane series. I did not know that I would come back to plant isoprenoids, including sterols, about 20 years later when we discovered a novel pathway for the formation of isoprene units.

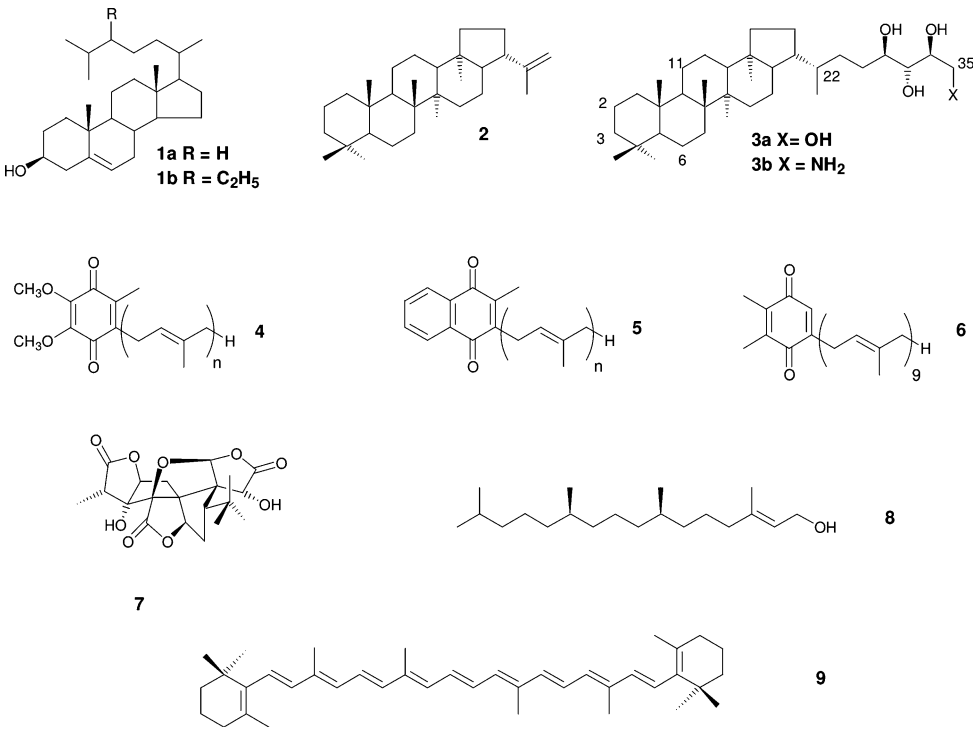

Fig. 1 Isoprenoids. **1a** cholesterol, **1b** sitosterol, **2** diploptene, **3a** bacteriohopanetetrol, **3b** aminobacteriohopanetriol, **4** ubiquinone, **5** menaquinone, **6** plastoquinone, **7** ginkgolide, **8** phytol, **9** β-carotene

Bacterial Biohopanoids

Geohopanoids probably represent the most abundant natural products on earth. This pentacyclic triterpenoid series is found in the organic matter of all sediments, independent of their age, origin or nature [11]. They represent the molecular fossils of a long overlooked family of bacterial metabolites. Triterpenes of the hopane series are rarely found in plants. They are more common in lichens, mosses and especially ferns. In contrast to the higher plant hopanoids, which are derived from the cyclization of oxidosqualene and possess an oxygenated function at C-3, hopanoids from lower eukaryotes are mostly derived from the direct cyclization of squalene and are accordingly devoid of such an oxygen function. Diploptene **2** (Fig. 1), a simple C_{30} hopanoid, was the first triterpene found in bacteria in the early 1970s. The major triterpenoids in all bacteria producing hopanoids were, however, always the C_{35} bacteriohopane derivatives (e.g. **3a**, **3b**, Fig. 1) [12]. Their discovery was rather fortuitous, by looking at the compounds responsible for the alignment of the cellulose microfibrils secreted by '*Acetobacter xylinum*' [13, 14]. This family of natural products, proved to be first precursors for the ubiquitous geohopanoids, presented an unique feature in natural product chemistry: an additional polyfunctionalized C_5 *n*-alkyl side-chain is linked by a carbon/carbon bond to one of the methyl group of isopropyl group of the hopane skeleton [15]. A huge structural diversity characterizes the bacterial biohopanoids. Modifications of the triterpene hopane skeleton include the introduction of double-bonds at C-6 and/or C-11 [16, 17], of an additional methyl group at C2β, C-2α or C-3β [18–21] or the presence of the two diastereomers at C-22 [15]. The side chains may differ by the number of the hydroxy groups, the replacement by the terminal C-35 hydroxy group by an amino group, the presence of polar moieties linked to the terminal hydroxy group (hexose derivatives linked via a glycosidic bond or carbapseudopentose linked via an ether bond) or to the terminal amino group (amino-acids or fatty acids via a peptide bond) [22]. Usually hopanoids are present in eubacterial cells in concentrations similar to those found for sterols in eukaryotes [12] and suitable for ^{13}C-NMR biosynthetic studies.

Like cholesterol **1a** (Fig. 1), biohopanoids are amphiphilic molecules with a flat, rigid skeleton due to the all *trans* ring junctions of the pentacyclic triterpene ring system and a length corresponding to the half of the thickness of a phospholipid bilayer. Such structural similarities suggested similar physiological roles. Indeed, in membrane models, hopanoids behave much like sterols, modulating the fluidity and the permeability of phospholipid mono and bilayers [23, 24]. This interpretation is corroborated by in vivo data. The hopanoid concentration increases with temperature in the thermoacidophile *Alicyclobacillus acidocaldarius*, counterbalancing the destabilizing effect of temperature [25]. In *Zymomonas mobilis*, a bacterium that can tolerate high ethanol concentrations up to 13% in its culture medium [26], the extremely high hopanoid concentrations (30 mg/g,

dry weight) has been proposed as being involved in the membrane stabilization in the presence of the solvent. Other roles may be assigned to hopanoids. In the cyst cells of the nitrogen-fixing *Frankia* sp., the high hopanoid concentrations may protect the sensitive nitrogenase from degradation by atmospheric oxygen [27]. In *Streptomyces coelicolor*, hopanoid biosynthesis is strongly linked to aerial growth and sporulation, these triterpenoids being nearly undetectable under vegetative growth conditions in submersed cultures [28].

The chemistry and biochemistry of the bacterial hopanoids turned out to be a rich topic. Hopanoid biosynthesis is a mine of yet undisclosed enzyme reactions involved in the linkage of the side-chain to the triterpene moiety and in the methylation of the A ring. The most surprising consequence was however, the non-programmed and non-programmable discovery of a novel pathway for the formation of the isoprene units.

The biosynthesis of isopentenyl diphosphate (IPP **18**, Fig. 2) and dimethylallyl diphosphate (DMAPP **19**), the universal precursors of isoprene units had already been elucidated in the 1950s, essentially using systems prepared from liver and from yeast, and resulted in the description of the mevalonate (MVA **15**) pathway (Fig. 2b). This pathway is also involved in the formation of plant triterpenoids, including sterols. MVA was believed to be the universal precursor of isoprene units despite many contradictory observations in the field of the biosynthesis of the isoprenoids from bacteria and especially plants (e.g. the biosynthesis of mono and diterpenes, carotenoids) [29].

Hopanoid Biosynthesis: First ^{13}C-Labeling Experiments and Discovery of the Methylerythritol Phosphate (MEP) Pathway

The first incorporations of ^{13}C-labeled precursors into bacterial hopanoids were designed in order to determine the origin of the C_5 side-chain linked to the triterpenes moiety [30]. For this purpose, selected bacteria were grown on a synthetic mineral medium with acetate as the sole carbon and energy source. These growth conditions differed from those of most former experiments where the labeled carbon source was usually administered in a complex medium in the presence of many other sources of carbon. Under such growth conditions, there is no competition for the utilization of different carbon sources. The cells are obliged to use the labeled substrate via known probable metabolic routes. Starting from the ^{13}C-labeled position and their isotope abundance, a retro-biosynthetic scheme, we expected to be able to deduce the enzyme reactions involved in the metabolism of the carbon source. Such experiments were first made on bacteria capable of utilizing acetate as the sole carbon and energy source: *Rhodopseudomonas palustris*, which is characterized by very simple hopanoid content, synthesizing only aminobacteriohopanetriol **3b** (Fig. 1), and *Methylobacterium organophilum*, which produces bacteriohopanetetrol **3a** (Fig. 1) derivatives and has a better versatility in the utilization of carbon sources, a feature which later proved interesting.

These first experiments showed that the bacteriohopane side-chain is indeed a D-pentose, derived from the non-oxidative pentose phosphate pathway and linked via its C-5 carbon atom to the hopane isopropyl group [30, 31]. The most striking result was, however, found on the triterpene moiety. The labeling pattern of the hopane isoprene units (Fig. 3) was not in accordance with the expected one from the MVA pathway. At the time of these early investigations, there was no reason to reject the universally accepted MVA pathway. Results were interpreted in the frame of this metabolic route, expecting that MVA had to be formed from two distinct acetyl-CoA pools, although a completely different pathway could not be excluded [30].

Incorporation of ^{13}C-Labeled Glucose Isotopomers into the Hopanoids of *Zymomonas mobilis*: The Origin of the Carbon Atoms of Isoprene Units in the MEP Pathway

Zymomonas mobilis is a good hopanoid producer [32] and possesses minimal enzymatic equipment, utilizing only hexoses (mainly glucose) as a carbon and energy source and having no complete tricarboxylic acid cycle. These properties made this bacterium an interesting target for the further investigations that we performed in collaboration with the group of Hermann Sahm (Forschungszentrum Jülich, Germany). Incorporation of ^{13}C-labeled D-glucose isotopomers into the hopanoids of the bacterium *Zymomonas mobilis* (with labeling either at C-1, C-2, C-3, C-5 or C-6) gave the first insights into an alternative metabolic route for the formation of isoprene units [33]. The carbon atoms of IPP **18** (for IPP skeleton numbering, cf. Fig. 2c) could be divided into two subgroups. C-3 and C-5 had a dual origin, being respectively equally derived from C-2 or C-5 of glucose for the former and C-3 and C-6 of glucose for the second one, whereas C-1, C-2 and C-4 of IPP had a single origin, being respectively derived from C-6, C-5 and C-4 of glucose. This labeling pattern characterizes the glucose catabolism via the Entner–Doudoroff pathway in *Z. mobilis*. The first subgroup corresponds to the C-2 and C-3 carbon atoms of pyruvate **11**, and the second one to the complete carbon skeleton of D-glyceraldehyde-3-phosphate **10** (Fig. 2). This interpretation requires a rearrangement allowing the insertion of the two-carbon subunit derived from pyruvate (by decarboxylation) between the carbon atoms from D-glyceraldehyde-3-phosphate derived from

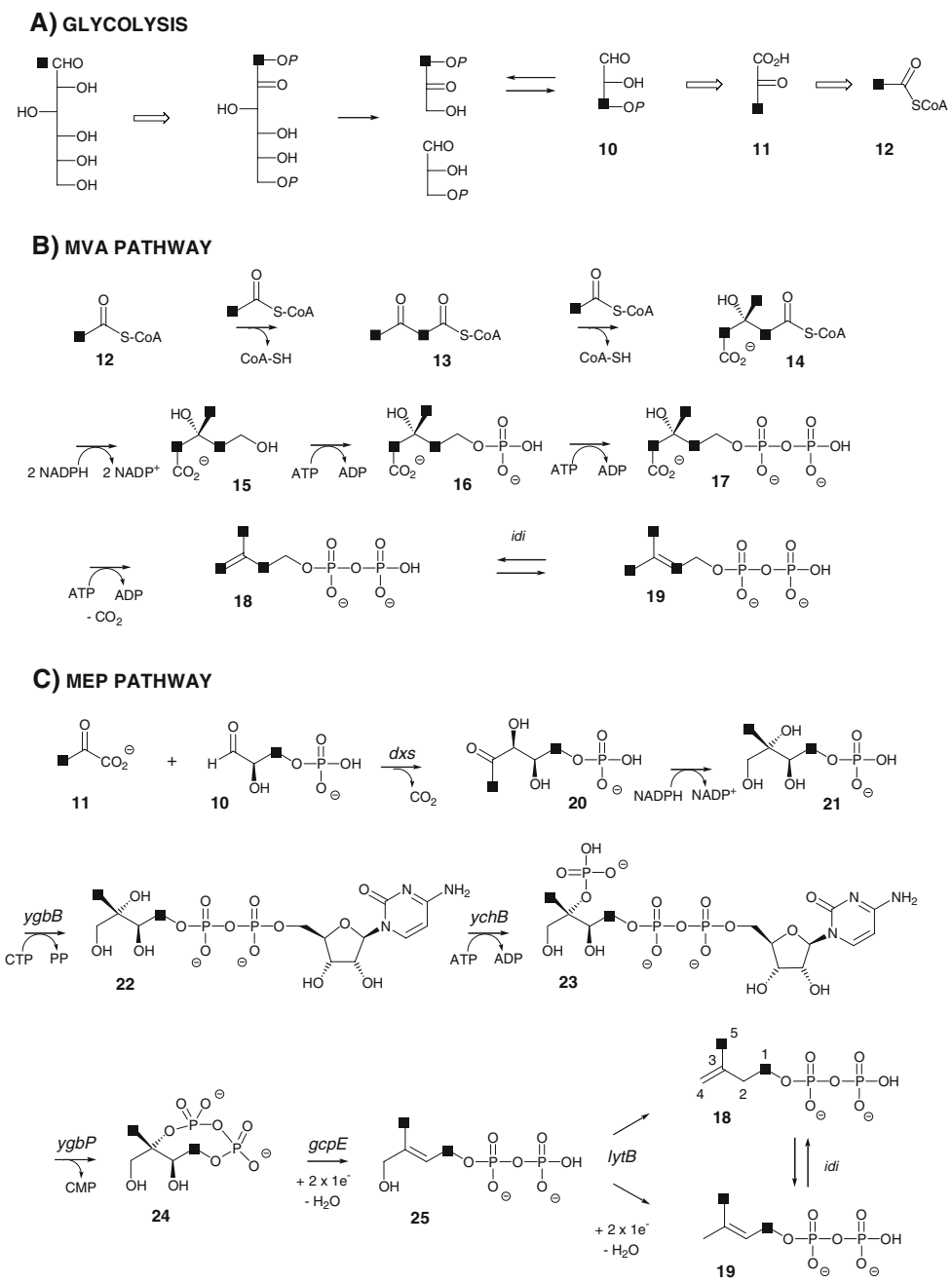

Fig. 2 Biosynthesis of isoprene units. **a** Labeling from [1-^{13}C] glucose of glyceraldehyde phosphate **10** and pyruvate **11**, the precursors of the methylerythritol phosphate (MEP) pathway, and of acetyl-CoA **12**, the precursor of the mevalonate (MVA) pathway. **b** Mevalonate pathway. **12** acetyl-CoA, **13** acetoacetyl-CoA, **14** hydroxymethylglutaryl-CoA, **15** MVA, **16** phosphoMVA, **17** diphosphoMVA, **18** IPP, **19** DMAPP. **c** Methylerythritol phosphate pathway. **10** D-glyceraldehyde 3-phosphate, **11** pyruvate, **20** 1-deoxy-D-xylulose 5-phosphate, **21** 2-*C*-methyl-D-erythritol 4-phosphate, **22** 4-diphosphocytidyl-2-*C*-methyl-D-erythritol, **23** 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate, **24** 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate, **25** (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate, **18** IPP, **19** DMAPP. Adapted from reference [117]

C-4 and C-5 of glucose. Incorporation of doubly labeled [4,5-$^{13}C_2$] glucose showed in the isoprene units of the bacteriohopanetetrol derivatives and of 2β-methyldiplopterol characteristic $^{2}J^{13}C/^{13}C$ coupling constants, indicating that C-4 and C-5 from glucose are introduced into the isoprene units via a single precursor molecule and representing the signature of the previously described rearrangement. Confirmation of the role of pyruvate and D-glyceraldehyde-3-phosphate was obtained after incorporation of uniformly labeled [U-$^{13}C_6$] glucose into the hopanoids of *Z. mobilis* and the incorporation of [1-^{13}C] glucose into the prenyl chain of ubiquinone in *E. coli* mutants, each lacking an enzyme of the triose phosphate metabolism inter converting glycerol and pyruvate [34]. Evidence for an alternative biosynthetic route was later obtained for other bacterial isoprenoid series. In *Escherichia coli*, which do not synthesize hopanoids, the same labeling pattern was obtained in the isoprene units of the prenyl chain of ubiquinone upon feeding with ^{13}C-labeled acetate as in the hopanoids from *R. palustris* and *M. organophilum*. Upon feeding with [1-^{13}C] glucose, the labeling distribution could be analyzed in the same way as

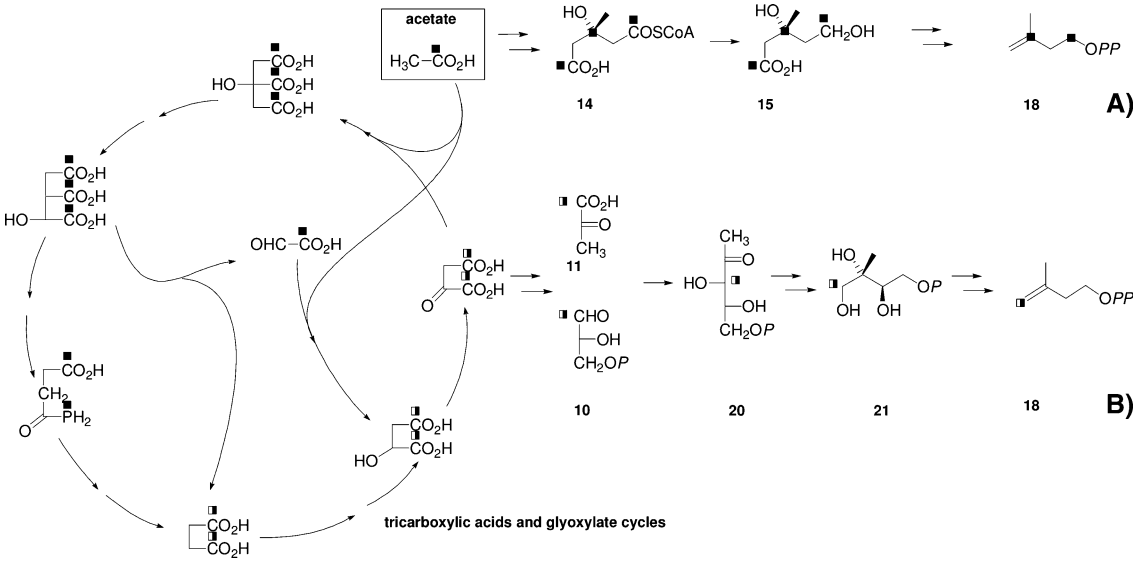

Fig. 3 Incorporation of [1-^{13}C] acetate for isoprene unit biosynthesis into the MVA pathway (***A***) or via the tricarboxylic acids and the glyoxylate cycles into the MEP pathway (***B***). For the sake of simplicity, cofactors have been omitted in the tricarboxylic acids and glyoxylate cycles. Only the carbon skeletons of the metabolites have been represented

those obtained for the formation of isoprene units in *Z. mobilis*, but in the frame of the glycolysis, which is utilized by *E. coli* for glucose catabolism [33].

All these features are inconsistent with the MVA pathway and a novel biosynthetic route had to be imagined [33, 34]. This was proposed by analogy with known enzymatic and chemical reactions. The pyruvate-derived two-carbon subunit is obtained from pyruvate decarboxylation by a thiamine diphosphate enzyme, much like the reactions catalyzed by pyruvate decarboxylase or pyruvate dehydrogenase, yielding (hydroxyethylidene) thiamine diphosphate. The later intermediate is a nucleophile that can be added onto the carbonyl group of glyceraldehyde-3-phosphate in a reaction resembling the reaction catalyzed by a transketolase and yielding a 1-deoxypentulose phosphate identified as 1-deoxy-D-xylulose 5-phosphate (DXP, **20**, Fig. 2c). A further step is the intramolecular rearrangement mentioned above, followed by the concomitant reduction of the resulting aldehyde intermediate. It was thought to be an acid-catalyzed rearrangement of a α-ketol resembling the rearrangement involved in the formation of the carbon skeleton of the branched amino-acids, but was later characterized by analysis of the isotope effects induced by the presence of a deuterium atom either at C-3 or at C-4 in the substrate as a retro-aldol/aldol reaction [35]. The reaction product is 2-*C*-methyl-D-erythritol 4-phosphate (MEP, **22**, Fig. 2C), a tetrol already presenting the branched isoprene skeleton. In contrast with DXP, which is also in *E. coli* a precursor of thiamine diphosphate and pyridoxal phosphate, no other function than that of an isoprenoid precursor is known for MEP. Accordingly, the pathway was proposed to be named after this intermediate.

The first two candidates for the C_5 precursors of this novel MVA-independent pathway are the phosphates of already known natural products. 1-Deoxy-D-xylulose (DX, **26**, Fig. 4) was previously isolated from the fermentation broth of a *Streptomyces* sp. and was known as a precursor of pyridoxal phosphate thiamine diphosphate in *E. coli*, and 2-*C*-methyl-D-erythritol (ME, **27**, Fig. 4) is accumulated in many plants, often in stress conditions [29, 36]. Incorporation of deuterium-labeled isotopomers of 1-deoxy-D-xylulose by the group of Duilio Arigoni [37] and of 2-*C*-methyl-D-erythritol [38–40] by our group into the prenyl chain of ubiquinone **4** and menaquinone **5** (Fig. 1) from *Escherichia coli* confirmed that these compounds are involved in an isoprenoid biosynthetic pathway.

The odd labeling patterns obtained with the first ^{13}C-labeled acetate incorporations [30] can now be easily interpreted. One has only to find out how glyceraldehyde-3-phosphate **10** and pyruvate **11** are synthesized from acetate via the tricarboxylic acid and the glyoxylate cycles, which are utilized by bacteria when acetate is the only carbon and energy source (Fig. 3).

Isoprenoid Biosynthesis in Plant Plastids

While we were carrying out our investigations on the biosynthesis of bacterial hopanoids, the group of Duilio Arigoni (ETH, Zürich, Switzerland) was investigating

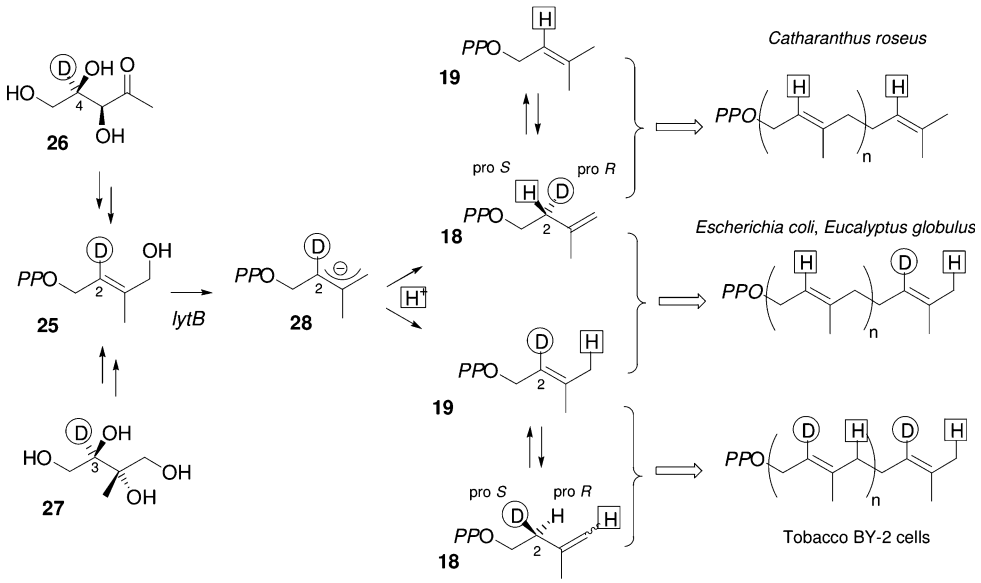

Fig. 4 IPP **18** and DMAPP **19** as precursors of isoprene units in the MEP pathway. Tracing the origin of the isoprene units by incubation of [4-^{2}H]DX **26** or [3-^{2}H]ME **27**. Adapted from reference [98] and reproduced with permission

independently and nearly simultaneously the biosynthesis of diterpenoids of the ginkgolide series **7** (Fig. 1) in *Ginkgo biloba* embryos [41, 42]. In this study, and again in contrast with former work on plant isoprenoid biosynthesis where ^{13}C-labeled acetate was utilized, labeling experiments were performed with ^{13}C-labeled glucose. Results were straightforward. Labeling patterns determined in the diterpenoid isoprene units did not correspond to those expected from the MVA pathway. They corresponded to those described for the bacterial MEP pathway. Isoprene units were synthesized from pyruvate and GAP, both derived from labeled glucose via glycolysis [41].

Interestingly, this study on ginkgo embryos showed for the first time the dichotomy characterizing isoprenoid biosynthesis in plants: sitosterol **1b** (Fig. 1) is synthesized in the cytoplasm as expected via the MVA pathway whereas diterpenoids, which are plastid isoprenoids, are derived from the alternative MEP route [41, 42].

We later extended this observation in collaboration with the group of Hartmut K. Lichtenthaler (University of Karlsruhe, Germany) to algae [43–46] and to other plants and to the normal isoprenoid constituents of the photosynthetic apparatus. Incubation of barley seedlings, an axenic duckweed culture or a carrot tissue culture with [1-^{13}C] glucose all showed the same dichotomy: the cytoplasmic phytosterols are synthesized via the MVA pathway and phytol **8** (Fig. 1) from chlorophyll, carotenoids **9** and the prenyl chain of plastoquinone **6** via the MEP route [47]. Confirmation of such a compartmentation was obtained for many other systems. The key to the success for all these experiments was the utilization of ^{13}C-labeled glucose for in vivo incubation in the place of labeled acetate. Hemiterpenes (such as isoprene and 2-methylbut-3-en-2-ol) [48, 49], monoterpenes [50, 51], diterpenes [52–54] and carotenoids [47, 55], are mainly synthesized in the plastids and derived from MEP pathway. This intracellular localization of the MEP pathway was later corroborated by the presence of a plastid targeting sequence for all enzymes involved in this metabolic route [56, 57].

Molecular Biology: The Key for the Full Elucidation of the MEP Pathway

When the MEP pathway was discovered, no bacterial genome was completely sequenced. The formation of DXP was thought to be catalyzed by a thiamine diphosphate enzyme with a transketolase resembling mechanism. Indeed, deoxyxylulose phosphate synthase shares sequence similarities with transketolases and was thus rapidly identified in the genomes of *E. coli* [58, 59] and peppermint [60]. Once ME was shown to be incorporated into the isoprenoids of *E. coli*, the search for auxotrophic mutants requiring this tetrol for growth led to the identification of the DXP reductoisomerase [61, 62], an enzyme which catalyzes as expected the reversible rearrangement of DXP into 2-*C*-methyl-D-erythrose phosphate [63] and the concomitant NADPH-dependent reduction of the latter aldehyde into MEP.

In the meantime, more and more bacterial genomes were sequenced facilitating the search for the MEP pathway genes among unannotated genes. The next genes were detected by genome mining. Incubation of ^{3}H-labeled MEP with crude cell-free extracts from *E. coli* led to the isolation of a radioactive compound when the reaction mixture contained ATP. Yields were improved in the presence of CTP, and radioactivity was incorporated from [α-^{32}P]CTP,

but not from [γ-^{32}P]CTP, suggesting that the detected product is a MEP adduct to a nucleoside derivative. This compound could not be further purified and characterized by chemical methods. The search for the gene of an enzyme catalyzing the coupling of a polyol phosphate to a nucleotide triphosphate resulted in the finding of the recently described *acs1* gene from *Haemophilus influenzae* encoding a bifunctional enzyme, which catalyzes the conversion of ribulose 5-phosphate into ribitol 5-phosphate and the further coupling of ribitol 1-phosphate to CTP yielding the CDP adduct of ribitol [64]. This *acs1 H. influenzae* gene presents homologies with the unannotated *ygbP* gene from *E. coli*, and the cognate protein catalyzes the conversion of MEP into 4-diphosphocytidyl-2-*C*-methyl-D-erythritol **22** (Fig. 2c) in the presence of CTP [65]. The next two genes belong, with the former one, to a small cluster in the *E. coli* genome. They were consecutively identified, and the corresponding enzymes characterized. The unannotated *ychB* and *ygbB* genes of *E. coli* were revealed by bacterial genomics. Their distribution in bacterial genomes was identical with that of the already known genes of the MEP pathway, and the corresponding recombinant proteins catalyzed the next steps: the phosphorylation of the tertiary hydroxy group yielding 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate **23** [66], followed by the elimination of UMP yielding 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP) **24** [67] (Fig. 2c). The two latter compounds were incorporated into carotenoids by isolated *Capsicum annuum* chromoplast and were thus established as intermediates of the MEP pathway. Sequence homologies led to the identification of the corresponding genes in *Arabidopsis thaliana* and tomato [68, 69].

Isoprenoid biosynthesis is an essential metabolic pathway. Any deletion of a MEP pathway gene is therefore lethal. Another approach for the identification of MEP pathway genes was the deletion of genes in an *E. coli* construct capable of utilizing exogenous MVA as isoprenoid precursor after the insertion of all genes required for the conversion of MVA into IPP (MVA kinase, phosphoMVA kinase and diphosphoMVA decarboxylase, Fig. 2a) [70, 71]. Growth after deletion of a MEP pathway gene is thus restored by addition of exogenous MVA to the culture medium. This approach was independently utilized for the characterization of the genes encoding the three enzymes responsible for the conversion of MEP into MEcPP **24** [70, 72, 73].

The last steps correspond to the reduction of a tetrol derivative, MEcPP **24**, into a primary allylic (DMAPP, **19**) and/or homoallylic (IPP, **18**) alcohol diphosphate (Fig. 2c). This implies unprecedented elimination as well as reduction steps, i.e. the involvement of an associated reduction system. The genes corresponding to these last steps were found by bacterial genome mining. The search for the genes accompanying the already known MEP pathway genes led to the identification of two unannotated genes in *E. coli gcpE* and *lytB*. Their involvement in isoprenoid biosynthesis was shown using the *E. coli* mutants capable of utilizing MVA described above: the lethal deletion of *gcpE* or *lytB* was rescued by the addition of exogenous MVA [74, 75]. In vivo incorporation of [U-$^{13}C_6$]DX by *E. coli* constructs overexpressing MEP pathway genes followed by ^{13}C-NMR analysis of the resulting crude cell extracts afforded interesting clues about the respective role of the GcpE and LytB proteins [76, 77]. Overexpression of all genes upstream of GcpE was followed by the accumulation of MEcPP **24** (Fig. 2c). Additional overexpression of *gcpE* resulted in the accumulation of (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP, **25**, Fig. 2), and of *gcpE* and *lytB* in the accumulation of IPP **18** as well as DMAPP **19** in a ca. 5:1 ratio, identifying MEcPP and HMBPP as substrates of respectively GcpE and LytB, and HMBPP and IPP as well as DMAPP as the reaction products of the same enzymes.

Characterization of the corresponding enzymatic activities was rather tricky. The first hints of the role of GcpE were obtained in vivo with an *E. coli* strain with a deleted *dxr* gene, engineered for the utilization of exogenous MVA and overexpressing *gcpE*: in the presence of a cocktail of cofactors (including those required for reduction reactions), ^{3}H-labeled ME was converted into MEcPP **24** (Fig. 2c), suggesting that the cyclodiphosphate is the substrate of GcpE [78]. Further, a crude cell free-system from an *E. coli* strain overexpressing *gcpE* converted MEcPP into (*E*)-methylbut-2-ene-1,4-diol in the presence of a phosphatase [79] or into HMBPP **25** (Fig. 2c) in the presence of fluoride as phosphatase inhibitor [80]. In contrast, in a MVA utilizing and *lytB*-deficient *E. coli* strain HMBPP **25** was accumulated in sufficient amounts for direct spectroscopic characterization, indicating that this allylic diol diphosphate is most probably the substrate of LytB [81].

Improvements in the enzyme assay methods were only possible once the real nature of the GcpE and LytB proteins was recognized. GcpE and LytB share with [4Fe–4S] cluster enzymes three conserved cysteins serving as anchors for such a cluster [79, 80, 82]. Most of these enzymes are quite oxygen sensitive, losing their prosthetic group and consequently their activity in the presence of air. They usually require handling under an inert atmosphere in a glove box as well as reconstitution of their prosthetic group in the presence of Fe^{3+}, sulfide and dithiothreitol. This observation opened up new possibilities for the investigations on the last two steps of the MEP pathway. When bacterial [4Fe–4S] cluster enzymes are involved in a reducing process, they are associated in vivo to flavodoxin/flavodoxin reductase/NADPH for the regeneration of the

reduced state of the prosthetic group. The former biological system can be replaced in an enzyme assay by the semiquinone radical obtained by photoreduction of deazaflavin. This approach proved fruitful. Reconstituted recombinant GcpE enzyme from *E. coli* [83] or the native as-isolated protein [84] converted in the presence of the biological or of the chemical reducing system MEcPP **24** into HMBPP **25** (Fig. 2c). Even dithionite was an effective reducing agent with the *Thermus thermophilus* GcpE [85]. Binding of intermediates to the cluster was suggested by EPR for the GcpE from *E. coli* [86].

Similarly, the last enzyme encoded by the *lytB* gene was also found to be a [4Fe–4S] enzyme converting HMBPP **25** (Fig. 4) into either IPP **18** or DMAPP **19** in a ca. 1:5 ratio [82] in the presence of the same associated biological reducing systems for the *E. coli* enzyme [87], and even in the presence of dithionite in the case of the *Aquifex aeolicus* enzyme [88]. Protonation of an allylic anion intermediate **28** (Fig. 4) is the most likely final step of the LytB catalyzed reaction (Fig. 4) [89].

Characterization of the cluster was performed by EPR spectroscopy of the LytB enzyme from *E. coli* with the cluster in its reduced $[4Fe–4S]^{1+}$ form from *E. coli* obtained upon dithionite reduction [87]. The as-isolated enzyme presented the signal of a $[3Fe–4S]^{2+}$ cluster, which represented about 10% of the protein and was most likely derived from the degradation by oxygen [87]. The same signal was also reported from the spectrum of LytB isolated under an inert atmosphere [90]. Mössbauer spectroscopy allowed the characterization of the GcpE/IspG from *E. coli* and *Arabidopsis thaliana* in the oxidized $[4Fe–4S]^{2+}$ form upon reconstitution with ^{57}Fe [91].

In plants and other organisms phylogenetically related to phototrophic phyla, the MEP pathway is localized in the plastids where flavodoxin is absent. GcpE interacts with ferredoxin in the cyanobacterium *Thermosynechococcus elongatus* [92] and in the malaria parasite *Plasmodium falciparum* [93], and activity was characterized in the presence of the associated reducing system ferredoxin/ferredoxin reductase/NADPH. In higher plants, the electrons required for the reduction converting MEcPP **24** into HMBPP **25** are derived also from ferredoxin. Indeed, a preparation containing *Arabidopsis thaliana* GcpE and purified spinach thylakoids afforded upon illumination, and in the absence of any reducing cofactor, HMBPP **25** from MEcPP **24** (Fig. 2c) [94], showing that the electron flow derived from the photo-oxidation of water can be directly diverted via photosystems I and II towards isoprenoid biosynthesis. In the dark (or in roots), the electron flow must come from catabolic processes, and ferredoxin is associated with the NADPH-dependent ferredoxin reductase under these conditions [94].

Conclusion: Further Developments

The MEP pathway for the biosynthesis of isoprene units in bacteria and plant plastids is now completely elucidated. All genes and enzymes are known. Many structural features of the enzymes as well as mechanistic aspects of the catalyzed reactions have, however, still to be deciphered. The discovery of this new metabolic pathway opened additional unexplored fields in plant and bacterial metabolism.

IPP and/or DMAPP as Precursor of the Isoprene Units?

The origin of the hydrogen atoms found in the isoprene units derived from MEP pathway was followed by incorporation of ^{2}H-labeled DX or ME. If the hydrogen atoms corresponding to those located at C-1, C-4 and C-5 of MEP are integrally retained in the isoprene units, quite striking observations were made on the retention or the loss of the hydrogen atom at C-4 of DXP corresponding to the hydrogen atom at C-3 of MEP. Three labeling patterns were described for isoprene units upon feeding of DX **26** or ME **27** (Fig. 4) with ^{2}H labeling at C-4 or C-3 respectively: (i) quantitative loss in all isoprene units wherever they are derived from IPP or from DMAPP as found in the carotenoids and phytol from a plant cell culture of *Catharanthus roseus* [95], (ii) quantitative retention in the DMAPP derived unit and quantitative loss in those derived from IPP in the prenyl chains of ubiquinone and menaquinone from the bacterium *Escherichia coli* [40, 96] or significant retention in the DMAPP derived unit and nearly complete loss in that derived from IPP in the monoterpene cineol from *Eucalyptus globulus* [97] and finally (iii) retention of deuterium in all isoprene units derived either from IPP or from DMAPP as observed for phytoene and the prenyl chain of plastoquinone from a tissue culture of tobacco BY-2 [98] (Fig. 4). Such apparently contradictory observations may in fact be explained, keeping in mind that IspH/LytB, the last enzyme of the MEP pathway, affords two reaction products, IPP **18** or alternatively DMAPP **19**. Taking into account the stereoselectivity of the introduction of the proton on the allylic anionic intermediate and of the elimination of the pro-*R* proton of IPP by the IPP isomerase and the *trans*-prenyl transferase, each labeling pattern reflects the origin of the isoprene units, the presence of deuterium representing the signature of a DMAPP origin, and its absence the signature of an IPP origin [36, 98] (Fig. 4).

Cross-Talk Between Cytoplasmic and Plastidial Compartments in Plant Cells

In plant cells, two pathways contribute to isoprenoid biosynthesis: the MVA pathway localized in the cytoplasm and the MEP pathway found in the plastids. The origin of the isoprene unit precursors, IPP and/or DMAPP is, however, scrambled by exchanges of intermediates between these two cell compartments. This aspect is well illustrated by the labeling patterns observed after incorporation of [1-^{13}C] glucose into plant isoprenoids, and was already clearly documented in the first study on ginkgolide biosynthesis. From the labeling pattern observed in the diterpenes skeleton of ginkgolides, it was deduced that most of the geranylgeranyl diphosphate (ca. 98%) was made from isoprene units uniquely derived from the MEP pathway. A small but significant part of the C_{20} skeleton has a composite origin: a C_{15} farnesyl diphosphate chain synthesized from isoprene units derived from the MVA pathway is completed by a fourth unit solely derived from the MEP pathway [41]. This shows that an acyclic C_{15} precursor, most probably farnesyl diphosphate, is carried from the cytoplasm to the plastids. Similar observations describing a dual origin for isoprene units were later reported for many other systems from higher plants [41, 55, 99–101], liverwort [102] and hornwort [103], illustrating the possibility of exchanges of C_5, C_{10} and C_{15} intermediates between cytoplasm and plastids. A striking example is given by the tobacco BY-2 cell culture where MEP and MVA pathways can complement each other [104]. Upon inhibition of the MVA pathway by a sublethal dose of mevinolin, phytosterol in the cytoplasm are synthesized via the MEP pathway from exogenous DX. Similarly, upon inhibition of the MEP pathway by fosmidomycin, the prenyl chain of the plastidial plastoquinone is synthesized from MVA, like the isoprene units of sterols [104].

The simultaneous presence of two independent pathways opens new understanding of plant physiology at the level of the regulation of isoprenoid biosynthesis. For instance, the emission of volatile plant defense terpenoid is dependent on both pathways: sesquiterpenoids being preferentially derived from the MVA route whereas monoterpenoids are synthesized via the MEP pathway [105, 106]. In addition, a nycthemeral rhythm (corresponding to the succession of day and night) has been pointed out for the MEP pathway in snapdragon flowers. Emission of volatile terpenoid synthesized via the MEP pathway occurs essentially during the day and is controlled by the circadian clock. Both plastidial monoterpenes and the cytosolic sesquiterpene nerolidol are derived from MEP pathway, suggesting a unidirectional trafficking of precursor(s) from plastids towards the cytoplasm [107].

MEP Pathway Enzymes as Targets for Antibacterial Drugs

The MEP pathway is the major metabolic route for isoprenoid biosynthesis in eubacteria. It is present in most bacterial taxa, including many obligate pathogens as well as opportunistic pathogens responsible for hospital-acquired diseases [108, 109]. In addition, some eukaryotes phylogenetically related to photosynthetic phyla, possess the MEP pathway, among them the *Plasmodium* spp. responsible for malaria [110].

The MEP pathway is absent in humans and animals, and isoprenoid biosynthesis is an essential metabolic route in all living organisms. Inhibition of any enzyme of this pathway has lethal consequences. This means that all enzymes of the MEP represent unexplored targets for the design of antibacterial or antiparasitic drugs [109–111]. This aspect is particularly interesting to overcome the widespread resistance towards most current commercial antibiotics. The concept has already been validated. Indeed, despite poor pharmacokinetic properties, a rapid elimination in urine [112] and the fast appearance of bacterial resistance, fosmidomycin, a natural antibiotic and a potent inhibitor of the second enzyme of the MEP pathway, the DXP reducto-isomerase [113], is quite effective against bacterial growth [112, 114] or against simple *Plasmodium* infections in rodents and in man [110, 115, 116].

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