*Pure Appl. Chem.*, Vol. 71, No. 12, pp. 2279–2284, 1999.
Printed in Great Britain.


# The mevalonate-independent methylerythritol 4-phosphate (MEP) pathway for isoprenoid biosynthesis, including carotenoids*

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*Abstract:* A mevalonate-independent route to isopentenyl diphosphate (IPP), the universal precursor of isoprenoids, is present in many bacteria, in some unicellular green algae and in the plant plastids. All essential isoprenoids related to photosynthesis, including the carotenoids, are synthesized via this alternative metabolic route. IPP is formed from pyruvate and glyceraldehyde 3-phosphate via 1-deoxy-D-xylulose 5-phosphate and 2-*C*-methyl-D-erythritol 4-phosphate. Later steps are still poorly known, although extensive data on the origin of the hydrogen atoms of IPP are now available.

## INTRODUCTION

Isoprenoids are present in all living organisms. The first investigations on their biosynthesis were mainly performed on sterols, with liver tissues, yeast and several plant systems [1–3]. The $C_5$ isoprenic skeleton was shown to be derived from the well-known sequence found in all textbooks (Fig. 1, Scheme **A**), starting from acetyl-CoA **4** and leading to isopentenyl diphosphate **7** (IPP) via mevalonate **6** (MVA). The committed step of this biosynthetic route is the reduction of hydroxymethylglutaryl CoA **5** into MVA catalyzed by the HMGCoA reductase. Mevalonate **6** was unanimously accepted as the universal isoprenoid precursor in all living organisms, despite some contradictory results mainly obtained when working on isoprenoid biosynthesis from plant chloroplasts and from bacteria. Feeding experiments using $^{13}$C-labeled carbon sources allowed the detection in bacteria and the partial elucidation of an alternative route towards IPP (Fig. 1, Scheme **B**), starting from triose phosphate derivatives, in which 2-*C*-methyl-D-erythritol 4-phosphate **10** (MEP) is the first intermediate presenting the branched isoprenic skeleton. IPP **7** remains, however, a common intermediate to both routes and must therefore be considered as the universal isoprenoid precursor. This brief contribution is not intended as an extensive survey of the topic. It summarizes some of the important steps of the discovery and the elucidation of this novel metabolic route. More details are found in the accompanying references.

## ON THE ORIGIN OF THE CARBON ATOMS

The first conclusive feeding experiments concerning an alternative metabolic route towards IPP were performed with hopanoid producing bacteria. The $C_{35}$ triterpenoids of the bacteriohopane series **11** (Fig. 2) are characterized by an unusual polyhydroxylated $C_5$ side chain linked by a carbon–carbon bond to the triterpenic hopane skeleton [4]. In order to decipher the origin of this unusual feature, labeling experiments were performed with [1-$^{13}$C]- and [2-$^{13}$C]-acetate [5]. All feeding experiments were made using a minimal medium, only containing mineral salts and a single $^{13}$C-labeled carbon source. The bacteria were thus forced to utilize this labeled carbon source. The metabolic pathways were usually well known, and it was accordingly possible to deduce from the observed labeling pattern the origin of the

*Lecture presented at the 12th International Symposium on Carotenoids, Cairns, Australia, 18–23 July 1999, pp. 2205–2302.

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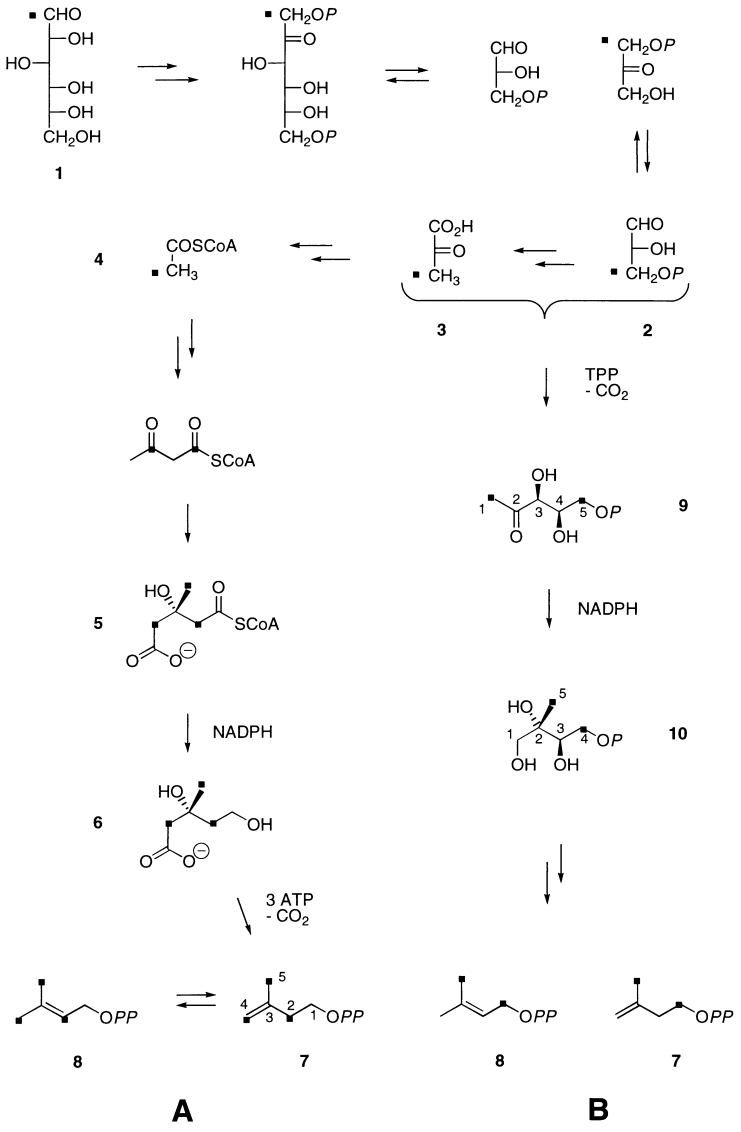


**Fig. 1** Incorporation of [1-$^{13}$C]-glucose into isoprenoids via the MVA pathway (**A**) or via the MEP pathway (**B**).

carbon atoms by a retrobiosynthetic analysis. Such labeling conditions were later extended to most of the labeling experiments performed, and were quite different from previous labeling studies, which were made with bacteria grown on complex media. The additional carbon atoms of the bacteriohopanepolyol side chain were derived from a D-pentose (according to the stereochemistry, most probably from a D-ribose derivative) linked via its C-5 carbon to the hopane isopropyl group. The most interesting problem was, however, found in the triterpenic moiety. The observed labeling pattern found in the hopane isoprene units did not fit with that expected from the MVA pathway. The first attempts to interpret these results in the frame of the MVA route [5] could not be confirmed by further incorporations of $^{13}$C-labeled glucose isotopomers into the hopanoids **11** from the bacteria *Zymomonas mobilis*, *Methylobacterium fujisawaense* and *Alicyclobacillus acidoterrestris* and the prenyl side chain of ubiquinone **12** from *Escherichia coli* [6].

Isoprenic units were formed in the new metabolic route from two subunits: the first was derived from the C-2 and C-3 carbon atoms of pyruvate **3**, and the other was D-glyceraldehyde phosphate **2** (GAP) [6,7]. All labeling patterns resulting from the above-described labeling experiments were consistent with the formation of pyruvate and GAP from glucose either via the glycolysis or via the Entner–Doudoroff pathway or from acetate via the tricarboxylic acid and the glyoxylate cycles [5,6]. Incorporation of doubly

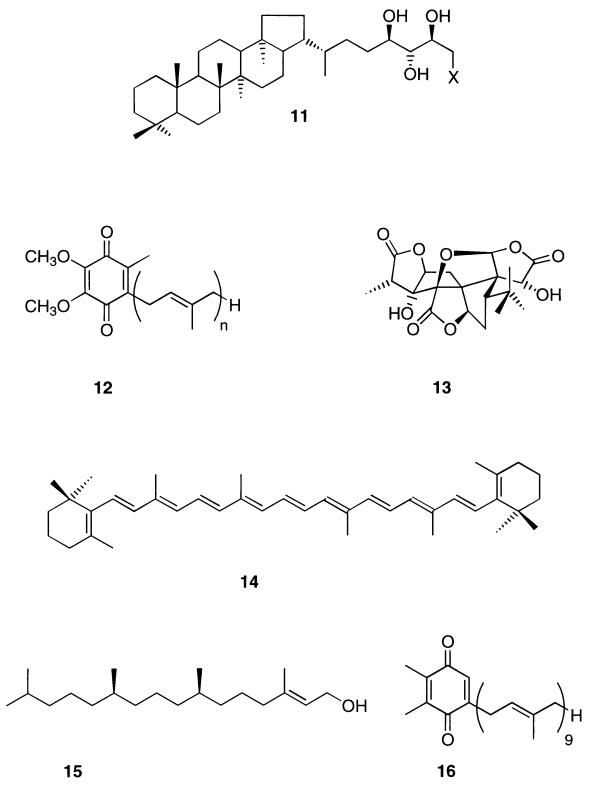


**Fig. 2** Key isoprenoids for the identification of the MEP pathway: hopanoids (**11**), ubiquinone (**12**), ginkgolides (**13**), carotenoids (**14**), phytol (**15**) and plastoquinone (**16**).

labeled [4,5-$^{13}C_2$]-glucose into the hopanoids and ubiquinone from *Methylobacterium fujisawaense* showed, in addition, from the long-range $^{13}C/^{13}C$ $^{2}J$ coupling constants that a rearrangement allowed the insertion of the $C_2$ subunit derived from pyruvate decarboxylation between the C-2 and C-3 carbon atoms from GAP [6]. The presence of such a rearrangement was corroborated by the incorporation of [U-$^{13}C_6$]-glucose into the hopanoids of *Zymomonas mobilis* [7]. From these data, a hypothetical biogenetic scheme, which proved later to be verified, was proposed. Similar experiments were performed by Schwarz and Arigoni on the biosynthesis of diterpenoids of the ginkgolide **13** series in *Ginkgo biloba* embryos and led to identical conclusions [8,9].

## MEVALONATE PATHWAY VERSUS METHYLERYTHRITOL PHOSPHATE PATHWAY

Labeling experiments using $^{13}C$-labeled carbon sources allow a clear differentiation to be made between the MVA and the MEP pathways [6,8,10]. In an organism utilizing glucose via glycolysis, one has to determine how the first isoprenoid precursors (acetyl-CoA **4** for the MVA route, pyruvate **3** and GAP **2** for the MEP route) are obtained from [1-$^{13}C$]-glucose **1** for instance (Fig. 1). The resulting labeling patterns are quite distinct and characteristic for each biosynthetic pathway (Fig. 1, Schemes **A** and **B**). Many plant isoprenoids, such as carotenoids **14**, the phytyl chain **15** of chlorophylls, and mono- and diterpenes, are usually poorly labeled from [$^{14}C$]-MVA [11]. Furthermore, mevinolin, a potent inhibitor of the HMGCoA reductase, efficiently blocks sterol biosynthesis in plant systems, whereas it does not affect carotenoid biosynthesis in chloroplasts. These results have usually been interpreted within the framework of the MVA pathway in terms of a lack of permeability of the plastid membrane towards MVA and mevinolin [11] and of a cytoplasm-independent IPP biosynthesis in the chloroplasts via the MVA route, even if a completely different biosynthetic route cannot be excluded. In contrast, carotenoids for instance are easily labeled from [$^{14}C$]-pyruvate or from carbon dioxide. All these results suggest the presence of an alternative biosynthetic route for the plastid isoprenoids. The approach utilizing $^{13}C$-labeled glucose in the place of acetate, which proved useful for the elucidation of the biosynthesis of bacterial isoprenoids,

was applied to labeling experiments performed with phototrophic organisms. A clear dichotomy was found in higher plants. The MVA route occurred in the cytoplasm and was responsible, as expected, for the formation of the triterpenoids, including the sterols, whereas the MEP pathway afforded in the chloroplasts the essential isoprenoids required for the photosynthetic apparatus (carotenoids **14**, phytol **15** and plastoquinone **16**) [10], as well as the whole series of plastid-related secondary metabolites (isoprene, mono- and diterpenes) [12,13].

## 1-DEOXY-D-XYLULOSE 5-PHOSPHATE AND 2-*C*-METHYL-D-ERYTHRITOL 4-PHOSPHATE AS IPP AND DMAPP PRECURSORS

Condensation of (hydroxyethyl)thiamine diphosphate and GAP yields 1-deoxy-D-xylulose 5-phosphate **9** (DXP) (Fig. 1, Scheme **B**). The first evidence for DXP as an isoprenoid precursor was given by Broers and Arigoni *et al*. [15]. Deuterium-labeled free 1-deoxy-D-xylulose (DX) was efficiently incorporated into the prenyl [14] chain of ubiquinone and menaquinone from a wild-type *E. coli* strain [14]. Similar experiments were later performed with several plant systems and confirmed the role of DXP as an isoprenoid precursor. Incorporation of [2,3,4,5-$^{13}C_4$]-DX into phytol and lutein from *Catharanthus roseus* cell cultures [15] and of [2,3-$^{13}C_2$]- or [2,4-$^{13}C_2$]-DX into the prenyl chain of the ubiquinone from *E. coli* [16] showed that DX in a plant and in a bacterium was incorporated without any previous degradation and that the branched isoprenic skeleton resulted from an intramolecular rearrangement.

2-*C*-Methyl-D-erythritol (ME) or the corresponding lactone was repeatedly reported from higher plants [11]. The cyclodiphosphate of this tetrol is found in normal growth conditions in the bacterium *Desulfovibrio desulfuricans* or under oxidative stress induced by benzylviologen in several Gram-positive bacteria [11]. Formally, ME directly results from DX via an acid-catalyzed α-ketol rearrangement followed by a reduction [7]. It was therefore tempting to check whether ME or one of its derivatives was involved in the alternative route for isoprenoid biosynthesis. Several $^{13}$C-labeled glucose isotopomers were incorporated into the prenyl chain of the dihydromenaquinones from *Corynebacterium ammoniagenes* and into the ME cyclodiphosphate. The $C_5$ carbon skeletons of the isoprenic units, as well as that of the branched tetrol, showed identical labeling patterns, indicating that both resulted from the same reaction sequence of the MVA-independent route [17]. Deuterium-labeled ME was incorporated, although in low yield, into the prenyl chain of ubiquinone and menaquinone from a wild-type *E. coli*, indicating that ME is an isoprenoid precursor [18]. $^{13}$C-labeled DX was, in addition, incorporated into ME by the leaves of *Liriodendron tulipifera* [19].

The gene of the DXP synthase was cloned from *E. coli* and from peppermint and overexpressed in *E. coli* [20–22], and that of the DXP isomero-reductase from *E. coli* and later from peppermint and *Arabidopsis thaliana* [23–25]. The latter enzyme utilizes only DXP as substrate (and not free DX). It catalyzes the rearrangement of the pentulose phosphate and the concomitant nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction of the resulting methylerythrose phosphate yielding MEP **10**. The identification of these genes allowed the construction of *E. coli* mutants with disrupted DXP synthase and/or isomero-reductase genes. Such mutants required free ME for their growth, suggesting that a kinase capable of converting ME into MEP was present and proved useful to confirm the role of MEP in isoprenoid biosynthesis [26].

## ON THE ORIGIN OF THE HYDROGEN ATOMS IN ISOPRENIC UNITS

Incorporation experiments with $^{13}$C-labeled precursors allowed the identification of the first precursors of the MEP pathway. However, little is known about the further steps. Additional information was expected from the knowledge of the fate of the hydrogen atoms in this pathway. Incorporation of [5,5,5-$^2H_3$]-DX [27] and [3,5,5,5-$^2H_4$]-ME [26] into the ubiquinone prenyl side chain showed that all methyl hydrogen atoms were preserved in the isoprenoid biosynthetic pathway in *E. coli*. Feeding of *E. coli* with [4,4-$^2H_2$]- or [1,1,4,4-$^2H_4$]-ME [18,26] or of *Zymomonas mobilis* with [6,6-$^2H_2$]-glucose [28] also indicated that all these deuterium atoms were retained in the isoprenic units of ubiquinone. Incorporation of [2-$^{13}$C,4-$^2$H]-DX into phytol and lutein by cell cultures of *Catharanthus roseus* resulted in a complete loss of the deuterium in all isoprenic units [29]. In contrast, the deuterium from [4-$^2$H]-DX was retained in the DMAPP **8**-derived starter unit of the prenyl chain of the ubiquinone from *E. coli* and lost in all those

derived from IPP **7** [30]. This observation was corroborated by the incorporation of [3,5,5,5-$^2H_4$]-ME in an *E. coli* mutant lacking the DXP synthase gene, which solely synthesized its isoprenoid from the exogenous ME added to the culture medium. Whereas the ubiquinone methyl groups retained their three deuteriums of ME, the C-3 deuterium was only found in the DMAPP-derived unit and was integrally lost in those derived from IPP [26].

The conversion of MEP **10** into IPP **7** formally requires the elimination of three molecules of water, two reduction steps and one phosphorylation. Evidence for a first reduction was obtained by the discovery of the DXP isomero-reductase, which introduces a hydride from NADPH on the carbon atom corresponding to C-4 of IPP. Further evidence for a reduction was obtained by feeding *Zymomonas mobilis* with [1-$^2$H]-glucose as the only carbon source [31]. This bacterium utilizes glucose via the Entner–Doudoroff pathway, is unable to convert pyruvate into GAP and is a strong ethanol producer in anaerobic growth conditions. C-1 of glucose is completely lost as $CO_2$ by pyruvate decarboxylation and, accordingly, is not incorporated into the isoprenic units of the hopanoids. This bacterium has no tricarboxylic cycle. Under such growth conditions, deuterium-labeled NADPH pools are synthesized. In the triterpenoids of the hopane series, deuterium was found in all carbon atoms derived from C-4 of IPP or DMAPP, as expected from the DXP isomero-reductase, as well as on all carbon atoms corresponding to C-2 of DMAPP and IPP. The latter deuterium atom was the signature of an additional reduction step occurring at an unknown stage of the reaction sequence of the biosynthetic route. The results of this experiment on the biosynthesis of hopanoids from *Zymomonas mobilis* shed light on the equivalence of the isoprenic units, whether they were derived from IPP or from DMAPP, and are consistent with those obtained with the *Catharanthus roseus* cell culture [29].

The stereochemistry of the reaction catalyzed by an IPP isomerase [32] and a prenyl transferase [33] from *E. coli* has recently been determined. Both enyzmes eliminate the pro *R* hydrogen from IPP, like all other known equivalent enzymes. Furthermore, it was shown that the IPP isomerase is not essential in *E. coli*: its disruption does not prevent the growth of the bacterium [34]. These data suggest that, if no other IPP isomerase is present, two distinct routes occur in *E. coli*, yielding separately IPP **7** and DMAPP **8** from the same intermediate derived from MEP **10**.

## CONCLUSION

The discovery of a second route for isoprenoid biosynthesis has opened up new fields in isoprenoid biochemistry. The simultaneous presence of both the MVA and MEP pathways in plant cells and the exchange of intermediates (IPP, GP, FPP) between the cytoplasm and the plastids indicate the significance of this cross-talking [8,15,35,36]. An interesting interpretation is the regulation of the biosynthesis of volatile isoprenoids released in stress conditions [36].

The presence of the MEP route in bacteria, which are either opportunistic pathogens or closely related to pathogenic species, opens up a route towards the discovery of enzyme inhibitors, which should represent novel types of antibacterial agents [37,38]. Finally, the recent finding of the presence of the two known genes of the MEP pathway in *Plasmodium falciparum*, the microorganism responsible for malaria, as well as the growth inhibition of this parasite by fosmidomycin, an inhibitor of the DXP isomero-reductase, allows new hopes for curing this major disease [39].

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