

# The non-mevalonate pathway of isoprenoids: genes, enzymes and intermediates

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Although the mevalonate pathway had been considered for a long time as the unique source of biosynthetic isoprenoids, an alternative pathway has recently been discovered. The first intermediate, 1-deoxy-D-xylulose 5-phosphate, is assembled by condensation of glyceraldehyde 3-phosphate and pyruvate. A skeletal rearrangement coupled with a reduction step affords the branched-chain polyol, 2C-methyl-D-erythritol 4-phosphate, which is subsequently converted into a cyclic 2,4-diphosphate by the consecutive action of three enzymes via nucleotide diphosphate intermediates. The genes specifying these enzymes have been cloned from bacteria, plants and protozoa. Their expression in recombinant bacterial hosts has opened the way to the identification of several novel pathway intermediates.

## Addresses

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## Abbreviations

|       |                             |
|-------|-----------------------------|
| CDP   | cytidine 5'-triphosphate    |
| CTP   | cytidine 5'-diphosphate     |
| DMAPP | dimethylallyl pyrophosphate |
| IPP   | isopentenyl pyrophosphate   |

## Introduction

Terpenes are one of the largest groups of natural products. More than 30,000 representatives including medically important compounds such as vitamins, hormones and cytostatic agents have been described. They are all assembled from two precursors, dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP).

During a period of several decades, the mevalonate pathway was considered as the universal source of biosynthetic DMAPP and IPP. The existence of a second isoprenoid pathway was discovered relatively recently by the research groups of Rohmer and Arigoni in the course of stable isotope incorporation studies using various eubacteria and plants [1–3]. These data suggested that pyruvate and a triose phosphate can serve as precursors for the formation of IPP and DMAPP by an alternative pathway (for reviews, see [4–6]). Arigoni and co-workers also showed that 1-deoxy-D-xylulose, a known precursor of thiamine and pyridoxal [7–9], can be diverted very efficiently to terpenoids [2,6]. Subsequent work by several research groups identified 1-deoxy-D-xylulose 5-phosphate as the first intermediate of the alternative terpenoid pathway [10–12].

Enzymes specified by the *dxs* gene of *Escherichia coli* and its orthologs from various eubacteria and plants were shown to

catalyze the formation of 1-deoxy-D-xylulose 5-phosphate (3) from pyruvate (1) and glyceraldehyde 3-phosphate (2) as shown in Figure 1 [11–19]. In a second step, the enzyme product is converted into the branched-chain polyol 2C-methyl-D-erythritol 4-phosphate (4) by 2C-methyl-D-erythritol 4-phosphate synthases specified by the *ispC* genes of *E. coli* and its respective orthologs from *Mentha piperita*, *Arabidopsis thaliana*, *Synechocystis* sp. and *Plasmodium falciparum* (Figure 1) [20–25].

Recently, additional genes and cognate enzymes of this novel pathway have been discovered and the corresponding intermediates have been identified in rapid sequence [26–28]. They are described in the following sections.

## The orthologous *ispD* group

Initially, cell extract of *E. coli* was found to convert 2C-methyl-D-erythritol 4-phosphate (4) into a novel product in a cytidine 5'-triphosphate (CTP)-dependent reaction [26]. A database search for CDP (cytidine 5'-diphosphate) pyrophosphorylases retrieved the *ascI* gene of a serotype-specific DNA region [29] of *Haemophilus influenzae*. Subsequent similarity searches using that gene as a search motif retrieved the orthologous *ygbP* group (now designated *ispD*) represented in many eubacteria and in *A. thaliana*.

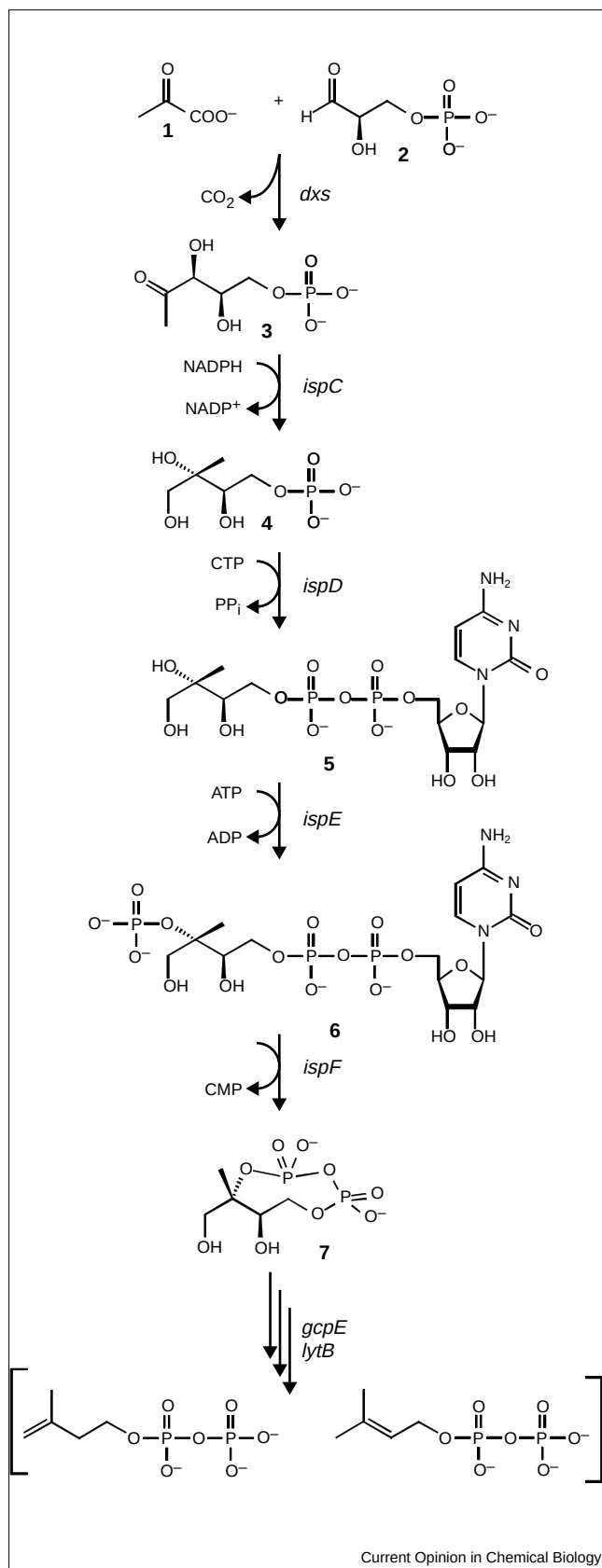
The *ispD* gene of *E. coli* was hyperexpressed in a recombinant *E. coli* strain [26]. The recombinant protein was shown to catalyze the formation of 4-diphosphocytidyl-2C-methyl-D-erythritol (5) from 2C-methyl-D-erythritol 4-phosphate and CTP (Figure 1; Table 1) [26,30]. The chromosomal *A. thaliana* gene comprises 11 introns and specifies a putative plastid-targeting sequence preceding the catalytic domain sequence [31]. 4-Diphosphocytidyl-2C-methyl-D-erythritol synthases require divalent cations, preferably  $Mg^{2+}$ , and could use several nucleotide triphosphates as substrates.

The product of the *IspD* proteins, 4-diphosphocytidyl-2C-methyl-D-erythritol, is incorporated efficiently into carotenoids of red pepper [26]. More recently, studies with an *ispD* deletion mutant of *E. coli*, engineered for utilization of a part of the mevalonate pathway, confirmed that the enzyme is required for IPP biosynthesis [32]. The phenotype of this strain is conditional lethal, and the recombinant bacteria can be rescued by addition of exogenous mevalonate.

## The orthologous *ispE* group

A search for orthologous groups with the same distribution pattern as *dxs*, *ispC* and *ispD* genes retrieved the *yhbB* (*ispE*) and *ygbB* (*ispF*) genes as potential members of the

Figure 1



The non-mevalonate pathway of isoprenoid biosynthesis.

non-mevalonate pathway (Table 2) [26,27]. The tomato ortholog of *ispE* had been shown earlier to be involved in fruit ripening [33]. In retrospect, it is clear that the phenotype of the tomato mutant was caused by the deficiency of carotenoid precursors caused by the mutation.

The *ispE* gene of *E. coli* was expressed in a recombinant *E. coli* strain where it directed the synthesis of a 30 kDa peptide. The purified recombinant enzyme from *E. coli* was shown to catalyze the ATP-dependent phosphorylation of 4-diphosphocytidyl-2C-methyl-D-erythritol (5) at the C-2 hydroxy group yielding 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate (6) [27]. Again, the use of  $^{13}\text{C}$ -labeled enzyme substrates was crucial for the elucidation of the enzyme product by NMR spectroscopy. More recently, these data were confirmed by other authors [34].

Orthologous *ispE* genes were also cloned from tomato and peppermint [35,36]. A gene cassette specifying the putative catalytic domain of the tomato 4-diphosphocytidyl-2C-methyl-D-erythritol kinase was engineered for high expression in enzymatically active form in a recombinant strain of *E. coli* [35]. Some properties are summarized in Table 1. Whereas the IspE orthologs of tomato, peppermint and *Arabidopsis* are highly similar over their entire length (about 74% identity), their similarity with the microbial enzyme is much lower (30% identity). The plant enzymes carry putative plastid-targeting sequences.

The IspE proteins of *E. coli* and peppermint have been reported to convert isopentenyl monophosphate into isopentenyl pyrophosphate [36], but the reported rates ( $178 \pm 81$  and  $1.43 \text{ pmol g}^{-1} \text{ s}^{-1}$  for the *E. coli* and peppermint enzymes, respectively) were so low that any physiological relevance appears doubtful.

### The orthologous *ispF* group

The members of the *ispF* (formerly *ygbB*) orthologous group follow the distribution pattern of the genes described above in completely sequenced genomes (Table 2). Moreover, *ispF* orthologs are closely linked to or fused with *ispD* orthologs in numerous microorganisms [26,28].

The *E. coli ispF* gene was expressed in a recombinant host where it directed the synthesis of a 16 kDa peptide. The recombinant protein was shown to catalyze the formation of 2C-methyl-D-erythritol 2,4-cyclodiphosphate (7) from 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate (6) [28,37]. At a much lower rate, the enzyme also catalyzes the formation of 2C-methyl-D-erythritol 3,4-cyclodiphosphate from 4-diphosphocytidyl-2C-methyl-D-erythritol (5) [28]. Orthologous *ispF* sequences from *A. thaliana* and *Catharanthus roseus* carry putative plastid-targeting sequences and are highly similar over their entire length (88% identity).

The amino-terminal segment of the IspF protein from *P. falciparum* is likely to act as an apicoplexan-targeting sequence. The similarity of the plant enzymes with the

**Table 1****Recombinant enzymes of the non-mevalonate pathway.**

| Enzyme<br>Organism                                   | Characteristics                                       |          |                                     | References |
|------------------------------------------------------|-------------------------------------------------------|----------|-------------------------------------|------------|
|                                                      | $v_{\max}$<br>$\mu\text{mol min}^{-1} \text{mg}^{-1}$ | Cofactor | Metal ion                           |            |
| 1-Deoxy-D-xylulose 5-phosphate synthase              |                                                       |          |                                     |            |
| <i>Arabidopsis thaliana</i>                          |                                                       | TPP      | Mg <sup>2+</sup>                    | [18]       |
| <i>Bacillus subtilis</i>                             |                                                       | TPP      | Mg <sup>2+</sup>                    | [16]       |
| <i>Capsicum annuum</i>                               | 500                                                   | TPP      | Mg <sup>2+</sup>                    | [14]       |
| <i>Escherichia coli</i>                              | 300                                                   | TPP      | Mg <sup>2+</sup>                    | [11,12]    |
| <i>Lycopersicon esculentum</i>                       |                                                       | TPP      | Mg <sup>2+</sup>                    | [17]       |
| <i>Mentha piperita</i>                               |                                                       | TPP      | Mg <sup>2+</sup>                    | [13]       |
| <i>Streptomyces</i> sp.                              | 370                                                   | TPP      | Mg <sup>2+</sup>                    | [19]       |
| <i>Synechococcus leopoliensis</i>                    |                                                       | TPP      | Mg <sup>2+</sup>                    | [15]       |
| <i>Synechocystis</i> sp.                             |                                                       | TPP      | Mg <sup>2+</sup>                    | [16]       |
| 2C-methyl-D-erythritol 4-phosphate synthase          |                                                       |          |                                     |            |
| <i>Arabidopsis thaliana</i>                          |                                                       | NADPH    | Mn <sup>2+</sup> , Mg <sup>2+</sup> | [22]       |
| <i>Escherichia coli</i>                              | 11.8                                                  | NADPH    | Mn <sup>2+</sup> , Mg <sup>2+</sup> | [20]       |
| <i>Mentha piperita</i>                               |                                                       | NADPH    | Mn <sup>2+</sup> , Mg <sup>2+</sup> | [21]       |
| <i>Plasmodium falciparum</i>                         |                                                       | NADPH    | Mn <sup>2+</sup> , Mg <sup>2+</sup> | [25]       |
| <i>Synechocystis</i> sp.                             |                                                       | NADPH    | Mn <sup>2+</sup> , Mg <sup>2+</sup> | [23]       |
| <i>Synechococcus leopoliensis</i>                    |                                                       | NADPH    | Mn <sup>2+</sup> , Mg <sup>2+</sup> | [26]       |
| 4-Diphosphocytidyl-2C-methyl-D-erythritol synthase   |                                                       |          |                                     |            |
| <i>Escherichia coli</i>                              | 23                                                    | CTP      | Mg <sup>2+</sup> , Mn <sup>2+</sup> | [26,32]    |
| <i>Arabidopsis thaliana</i>                          | 67                                                    | CTP      | Mg <sup>2+</sup> , Ni <sup>2+</sup> | [31]       |
| 4-Diphosphocytidyl-2C-methyl-D-erythritol kinase     |                                                       |          |                                     |            |
| <i>Escherichia coli</i>                              | 34                                                    | ATP      | Mg <sup>2+</sup>                    | [27,34]    |
| <i>Lycopersicon esculentum</i>                       | 33                                                    | ATP      | Mg <sup>2+</sup>                    | [35]       |
| 2C-Methyl-D-erythritol 2,4-cyclodiphosphate synthase |                                                       |          |                                     |            |
| <i>Escherichia coli</i>                              |                                                       |          | Mg <sup>2+</sup> , Mn <sup>2+</sup> | [28,37]    |
| <i>Plasmodium falciparum</i>                         | 4.3                                                   |          | Mg <sup>2+</sup> , Mn <sup>2+</sup> | [38]       |

microbial and/or protozoal enzymes is low (45% and 38% identity, respectively).

The pseudo-mature recombinant *Plasmodium* IspF protein was shown to catalyze the same reactions as described above for the *E. coli* IspF protein [38]. The enzyme also catalyzed the formation of 2-phospho-2C-methyl-D-erythritol 3,4-cyclodiphosphate from 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate, albeit at a relatively low rate [38]. Kinetic properties of the enzymes from *E. coli* and *Plasmodium* are summarized in Table 1.

2C-methyl-D-erythritol 2,4-cyclodiphosphate (**7**) was shown to be transformed into carotenoids by isolated chromoplasts from *Capsicum annuum* [28]. This finding confirms that the cyclic diphosphate is a committed intermediate in the non-mevalonate pathway of terpenoid biosynthesis. In earlier reports, **7** had been reported to be formed in certain microorganisms under stress conditions [39,40].

### The orthologous *lytB* and *gcpE* groups

The distribution patterns of the orthologous *lytB* and *gcpE* groups are identical with those of the other orthologous groups of the non-mevalonate pathway genes (Table 2). Insertions into the coding region of *lytB* were lethal for

*Synechocystis* sp. Supplementation with isopentenol and dimethylallyl alcohol completely alleviated this growth impairment [41]. Studies with an *E. coli* *gcpE* deletion mutant that was engineered for utilization of a partial mevalonate pathway confirmed that the enzyme is required for IPP biosynthesis in *E. coli* [42]. The phenotype of this strain is conditional lethal; the mutant bacteria can be rescued by addition of exogenous mevalonate. The reactions catalyzed by *LytB* and *GcpE* proteins are still unknown.

### Conclusions

The occurrence of the two isoprenoid pathways in different species can be gleaned from recent isotope incorporation studies in conjunction with comparative genomics [4,5,27,43]. Archaea, fungi and animals use exclusively the mevalonate pathway. Plants invariably utilize the mevalonate pathway in the cytosolic compartment and the non-mevalonate pathway in plastids. The translocation into plastids was demonstrated for the tomato 1-deoxy-D-xylulose 5-phosphate synthase [17]. The majority of eubacteria and certain algae use the non-mevalonate pathway. Most human pathogens are in the non-mevalonate pathway group, and the pathway enzymes are attractive targets for the development of novel antibiotics. *Streptomyces* and possibly Gram-positive cocci may use

Table 2

## Terpenoid genes in completely sequenced organisms.

| Organism                          | Non-mevalonate pathway |      |      |      |      |      |      | Mevalonate pathway |      |       |       |      |      |       |
|-----------------------------------|------------------------|------|------|------|------|------|------|--------------------|------|-------|-------|------|------|-------|
|                                   | dxs                    | ispC | ispD | ispE | ispF | lytB | gcpE | mvaS               | mvaA | mvaK1 | mvaK2 | mvaD | ipil | ipill |
| <b>Bacteria</b>                   |                        |      |      |      |      |      |      |                    |      |       |       |      |      |       |
| <i>Aquifex aeolicus</i>           | +                      | +    | +    | +    | +    | +    | +    |                    |      |       |       |      |      |       |
| <i>Bacillus subtilis</i>          | +                      | +    | +    | +    | +    | +    | +    |                    |      |       |       |      |      | +     |
| <i>Buchnera</i> sp.               | +                      | +    | +    | +    | +    | +    | +    |                    |      |       |       |      |      |       |
| <i>Campylobacter jejuni</i>       | +                      | +    | +    | +    | +    | +    | +    |                    |      |       |       |      |      |       |
| <i>Chlamydia</i> sp.              | +                      | +    | +    | +    | +    | +    | +    |                    |      |       |       |      |      |       |
| <i>Deinococcus radiodurans</i>    | +                      | +    | +    | +    | +    | +    | +    |                    |      |       |       |      |      |       |
| <i>Escherichia coli</i>           | +                      | +    | +    | +    | +    | +    | +    |                    |      |       |       |      | +    |       |
| <i>Haemophilus influenzae</i>     | +                      | +    | +    | +    | +    | +    | +    |                    |      |       |       |      |      |       |
| <i>Helicobacter pylori</i>        | +                      | +    | +    | +    | +    | +    | +    |                    |      |       |       |      |      |       |
| <i>Mycobacterium tuberculosis</i> | +                      | +    | +    | +    | +    | +    | +    |                    |      |       |       |      | +    |       |
| <i>Neisseria meningitidis</i>     | +                      | +    | +    | +    | +    | +    | +    |                    |      |       |       |      |      |       |
| <i>Pseudomonas aeruginosa</i>     | +                      | +    | +    | +    | +    | +    | +    |                    |      |       |       |      |      |       |
| <i>Synechocystis</i> sp.          | +                      | +    | +    | +    | +    | +    | +    |                    |      |       |       |      |      | +     |
| <i>Thermotoga maritima</i>        | +                      | +    | +    | +    | +    | +    | +    |                    |      |       |       |      |      |       |
| <i>Trepomema pallidum</i>         | +                      | +    | +    | +    | +    | +    | +    |                    |      |       |       |      |      |       |
| <i>Vibrio cholerae</i>            | +                      | +    | +    | +    | +    | +    | +    |                    | +    |       |       |      |      |       |
| <i>Xylella fastidiosa</i>         | +                      | +    | +    | +    | +    | +    | +    |                    |      |       |       |      |      |       |
| <i>Borrelia burgdorferi</i>       |                        |      |      |      |      |      |      | +                  | +    | +     | +     | +    |      | +     |
| <b>Archaea</b>                    |                        |      |      |      |      |      |      |                    |      |       |       |      |      |       |
| <i>Aeropyrum pernix</i>           |                        |      |      |      |      |      |      | +                  | +    | +     |       |      |      | +     |
| <i>Archeoglobus fulgidus</i>      |                        |      |      |      |      |      |      | +                  | +    | +     |       |      |      | +     |
| <i>Halobacterium</i> sp.          |                        |      |      |      |      |      |      | +                  | +    | +     |       |      |      | +     |
| <i>M. thermoautotrophicum</i>     |                        |      |      |      |      |      |      | +                  | +    | +     |       |      |      | +     |
| <i>Methanococcus jannaschii</i>   |                        |      |      |      |      |      |      | +                  | +    | +     |       |      |      | +     |
| <i>Pyrococcus abyssi</i>          |                        |      |      |      |      |      |      | +                  | +    | +     |       |      |      | +     |
| <i>Pyrococcus horikoshii</i>      |                        |      | +    |      |      |      |      | +                  | +    | +     |       |      |      | +     |
| <i>Thermoplasma acidophilum</i>   |                        |      |      |      |      |      |      | +                  | +    | +     |       |      |      | +     |
| <b>Eukarya</b>                    |                        |      |      |      |      |      |      |                    |      |       |       |      |      |       |
| <i>Arabidopsis thaliana</i>       | +                      | +    | +    | +    | +    | +    | +    | +                  | +    | +     | +     | +    | +    |       |
| <i>Caenorhabditis elegans</i>     |                        |      |      |      |      |      |      | +                  | +    | +     | +     | +    | +    |       |
| <i>Drosophila melanogaster</i>    |                        |      |      |      |      |      |      | +                  | +    | +     | +     | +    | +    |       |
| <i>Homo sapiens</i>               |                        |      |      |      |      |      |      | +                  | +    | +     | +     | +    | +    |       |
| <i>Saccharomyces cerevisiae</i>   |                        |      |      |      |      |      |      | +                  | +    | +     | +     | +    | +    |       |

*dxs*, 1-deoxy-D-xylulose 5-phosphate synthase; *ipil*, isopentenyl pyrophosphate isomerase type I; *ipill*, Isopentenyl pyrophosphate isomerase type II; *ispC*, 2C-methyl-D-erythritol 4-phosphate synthase; *ispD*, 4-diphosphocytidyl-2C-methyl-D-erythritol synthase; *ispE*, 4-diphosphocytidyl-2C-methyl-D-erythritol kinase;

*ispF*, 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; *mvaA*, HMG-CoA reductase; *mvaD*, mevalonate diphosphate decarboxylase; *mvaK1*, mevalonate kinase; *mvaK2*, phosphomevalonate kinase; *mvaS*, HMG-CoA synthase.

both pathways [19,44]. *Mycoplasma*-type bacteria appear not to biosynthesize isoprenoids. It is unknown whether they can obtain required terpenoids from their hosts. The situation in protozoa is incompletely known. For *Leishmania* and *Trypanosoma* sp. there is strong evidence for the mevalonate pathway [45,46]. In *Plasmodium falciparum*, only enzymes of the non-mevalonate pathway have been found up to now [25].

The genes, enzymes and reactions involved in the conversion of 2C-methyl-D-erythritol 2,4-cyclodiphosphate (7) into IPP and DMAPP are still unknown. A ring-opening reaction, two dehydration steps and two reduction steps are required for transformation of 7 into IPP and DMAPP, which both appear to be obtained via a common precursor as opposed to isomerization [47]. Putative orthologs of the classical (type 1) isomerase or

of the recently discovered type II isomerase [48] are present in the genomes of some eubacteria using the non-mevalonate pathway (Table 2) but their metabolic role is unknown.

The non-mevalonate pathway is absent in vertebrates (Table 2). Isoprenoid biosynthesis is essential, and the blocking of genes or enzymes involved in an isoprenoid pathway is lethal. Therefore, the enzymes of the non-mevalonate pathway are attractive targets for the development of herbicides and antibiotics. The 2C-methyl-D-erythritol 4-phosphate synthase inhibitor fosmidomycin has been shown to have bactericidal, herbicidal and anti-malarial activities [25]. The detailed knowledge of the mechanisms and regulation processes of the non-mevalonate pathway may also benefit the biotechnological production of commercially interesting isoprenoids.

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