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Isoprenoid biosynthesis in *Escherichia coli* via the methylerythritol phosphate pathway: enzymatic conversion of methylerythritol cyclodiphosphate into a phosphorylated derivative of (*E*)-2-methylbut-2-ene-1,4-diol

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Abstract—A crude cell-free system from an *Escherichia coli* strain overexpressing the cluster containing the three genes *yfgA*, *yfgB*, and *gcpE* converted 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (**1**) into a phosphorylated derivative of (*E*)-2-methylbut-2-ene-1,4-diol (**6**), which most probably represents a novel intermediate in the methylerythritol phosphate pathway for isoprenoid biosynthesis. The free diol **6** was accumulated by phosphatase treatment of the crude enzyme preparation and was identified by comparison with a synthetic reference.

The 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway for isoprenoid biosynthesis is present in most bacteria, in green algae, and in the chloroplasts of all phototrophic organisms, including the higher plants. The early steps starting from glyceraldehyde phosphate and from pyruvate via 1-deoxyxylulose 5-phosphate (DXP) and a series of 2-*C*-methyl-D-erythritol (ME) derivatives including MEP, 4-diphosphocytidyl ME, 4-diphosphocytidyl ME 2-phosphate, and ME 2,4-cyclodiphosphate **1** are now fairly well investigated.[1]

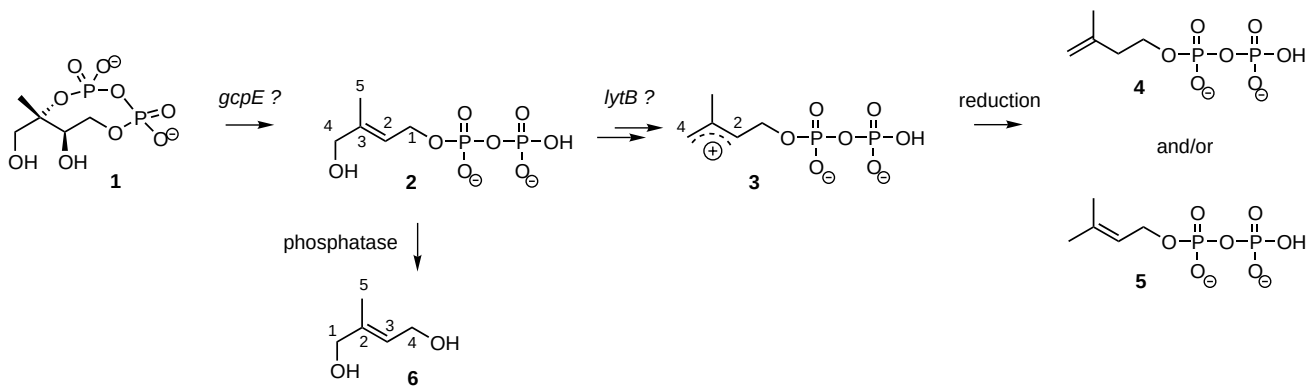

Figure 1. Conversion of methylerythritol 2,4-cyclodiphosphate (**1**) into a (*E*)-2-methylbut-2-ene-1,4-diol derivatives (**2**, **6**) by a cell-free system from *E. coli*.

Keywords: biosynthesis; 2-*C*-methyl-D-erythritol 4-phosphate; 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate; (*E*)-2-methylbut-2-ene-1,4-diol; *gcpE*; isoprenoids; *lytB*.

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ME 2,4-cyclodiphosphate **1** is the last identified intermediate, and very little is known concerning the steps downstream of this metabolite. The pathway is known to possess, at least in *Escherichia coli*, two branches, separately leading to isopentenyl diphosphate **4** (IPP) and dimethylallyl diphosphate **5** (DMAPP),[2] and two additional genes, *gcpE* and *lyt*B have been recognized as essential in this metabolic route.[3] In order to gain additional information on the MEP pathway, an *E. coli* mutant capable of utilizing exogenous MVA was constructed. For this purpose, the genes encoding mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, and IPP isomerase were introduced in its genome.[4] Disruption of the *gcpE* gene in this mutant showed that it was located on the trunk line or directly on the branching of this pathway.[3a] Feeding this mutant with [1-^{3}H]ME resulted in the accumulation of a single major metabolite, which was identified as ME 2,4-cyclodiphosphate **1**, suggesting that this cyclodiphosphate was the putative substrate of the *gcpE* gene product.[5] In this contribution, we describe the conversion of ME cyclodiphosphate **1** into a novel metabolite by a cell-free system prepared from an *E. coli* strain overexpressing the *yfgA*/*yfgB*/*gcpE* gene cluster (Fig. 1).[6]

For the convenience of detection, conversion of ME cyclodiphosphate **1** was tested using a ^{14}C labeled substrate. For this, [2-^{14}C]DXP was synthesized in a one-pot procedure from [2-^{14}C]pyruvate and non-labeled glyceraldehyde phosphate using the DXP synthase and directly converted into [2-^{14}C]MEP using the DXP isomeroreductase (D. Tritsch, unpublished results). [2-^{14}C]MEP was further converted into [2-^{14}C]ME cyclodiphosphate using a one-pot procedure similar to a recently described methodology involving a mixture of the *ygbP*, *ychB*, and *ygbB* gene products and the corresponding cofactors.[7] Incubation of [2-^{14}C]ME cyclodiphosphate **1** with a crude cell-free system from an *E. coli* strain overexpressing the *gcpE*, *yfgA*, and *yfgB* gene cluster[8] resulted in the formation of several radioactive products. Next to remaining starting material **1**, a most probably non-phosphorylated unknown compound as well as a compound, which might correspond to a diphosphate ester, were detected on TLC plates.[9] The two latter compounds most likely represented novel metabolites of [2-^{14}C]ME cyclodiphosphate. Noteworthy was the non-sensitivity of [2-^{14}C]ME cyclodiphosphate **1** towards the tested alkaline phosphatase, which hydrolyzed acyclic mono- and diphosphate esters, as tested on MEP and IPP. Formation of a non-phosphorylated compound was not surprising as the enzyme test was performed with a crude cell-free system containing endogenous phosphatases. Addition of an exogenous phosphatase to the incubation medium did not affect the substrate, but significantly increased the concentration of the above-mentioned least polar novel metabolite. This concentration increase was concomitantly accompanied by the disappearance of the most polar unknown metabolite, suggesting that it corresponded to the phosphorylated form of the former one.

Direct identification was therefore attempted. A large-scale incubation of non-labeled ME cyclodiphosphate (17.5 mg) prepared from *Corynebacterium ammoniagenes* treated with benzylviologen[10] was performed. To the resulting reaction mixture was added a small-scale incubation of [2-^{14}C]ME cyclodiphosphate, which allowed the detection of the unknown metabolite owing to its radioactive labeling. From the acetylated crude extract of the enzyme test, repeated fractionation by column and thin-layer chromatography afforded a single radioactive compound (1 mg), which was identified as (*E*)-2-methylbut-2-ene-1,4-diol (**6**) as a diacetylated derivative by TLC coelution and by comparison of its ^{1}H and ^{13}C NMR spectra with those of the *E* and *Z* stereomers of the corresponding diol obtained by chemical synthesis.[11]

(*E*)-2-Methylbut-2-ene-1,4-diol (**6**) was certainly not the primary reaction product obtained from the crude enzyme test. The free diol was most likely released from the corresponding diphosphate **2** by the action of either endogenous phosphatase from *E. coli* or of the exogenous phosphatase added to the incubation buffer in order to improve the conversion of [2-^{14}C]ME cyclodiphosphate **1** into the diol **6**. The diol was only obtained from the enzymatic conversion of [2-^{14}C]ME cyclodiphosphate: it was not observed when the incubation was performed with an enzyme preparation inactivated by boiling. As a crude cell-free system was utilized, it however cannot be excluded that the diol derivative resulted from the consecutive action of several enzymes, including the products of the *gcpE*, *yfgA*, and *yfgB* genes. Whether these proteins were effectively required for the conversion of [2-^{14}C]ME cyclodiphosphate **1** is still a matter of investigations. (*E*)-4-Hydroxy-3-methylbut-2-enyl diphosphate (**2**) was recently identified in *E. coli* as a major activator for human γδ T cells.[12] It is accumulated in cells defective in the *lytB* gene, whereas it is absent in cells defective in the *gcpE* gene. All available data are in accordance with ME cyclodiphosphate **1** as a substrate of the *gcpE* gene product and with diphosphate of **6** as reaction product of the *gcpE* gene product and substrate of the *lytB* gene product. The conversion of [2-^{14}C]ME cyclodiphosphate **1** into a diol **6** derivative formally requires a reduction and an elimination step. Accordingly, a cofactor has to be involved in the reduction step. The addition of any reducing cofactor such as NADPH, NADH, FMN or FAD did not influence however, the conversion yield. Because of the small amounts of the reaction product, which were synthesized de novo, the endogenous intracellular cofactor pools were most likely largely sufficient.

Finally, elimination of water or of a better leaving group such as phosphate from C-4 of diphosphate **2** (corresponding to C-4 of IPP **4** and DMAPP **5**) would produce an allylic cation **3** in the active site (Fig. 1). Such cations are often encountered in isoprenoid biosynthesis (see for instance the reactions catalyzed by the IPP isomerase, the prenyl transferases, the squalene synthase or the terpene synthases). A single reduction at C-1 or C-3 of such a carbocationic intermediate **3** would yield DMAPP **5** or IPP **4**, respectively.

One of the last bottlenecks in the elucidation of the MEP pathway is now being solved by the discovery of

this [2-^{14}C]ME cyclodiphosphate metabolite. It remains to isolate now the native compound, most likely the diphosphate **2**,[14] which released the diol **6** upon phosphatase treatment. IPP **4** and DMAPP **5** are synthesized in *E. coli* from an unknown common intermediate derived from ME cyclodiphosphate **1** via two distinct branches.[2] It also remains to check how the diphosphate **2** is converted into IPP and/or DMAP and whether it belongs to the main trunk or to the IPP or the DMAPP branch of the MEP pathway.

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6. Because of the genetic links *yfgA*, *yfgB*, and *gcpE*, which are on the same operon of the *E. coli* genome, an expression plasmid containing the genomic region covering *yfgB*, *yfgA*, and *gcpE* was constructed and stably transformed into *E. coli* creating the strain BL21(DE3)pLys [PET-T7-yfgA-yfgB-gcpE].
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8. Enzyme assays. In a typical experiment, the above mentioned *E. coli* strain was grown for about 4 h at 30°C in LB medium (50 mL) containing chloramphenicol (34 μg mL^{-1}) and ampicillin (100 mg mL^{-1}) until reaching a 0.65 OD (600 nm). Induction was performed with IPTG (0.5 mM) for 6 h. Cells were harvested by centrifugation (7000 g, 10 min), resuspended in degassed buffer (4 mL, 50 mM Tris–HCl, pH 8, 1 mM PMSF, 1 mM DTT, and 5 mM $MgCl_2$), and broken by sonication (2×20 s, with 1 min cooling). Cell debris was removed by centrifugation (16000 g, 10 min). The resulting crude cell-free material (130 μL) was completed with buffer (20 μL) and used for enzyme assays at 37°C for 7 and 20 h with [2-^{14}C]-2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate solution (1.1×10^5 cpm, 16 Ci mol^{-1}). When required, an *E. coli* Type-III S alkaline phosphatase (0.25 U) was added to the reaction mixture.
9. Isolation and identification of **6**. Direct TLC of aliquots of the reaction medium revealed several radioactive spots including **6** (R_f=0.85), free ME (R_f=0.70), and **1** (R_f=0.52). Additional TLC of **6** ($CHCl_3$–CH_3OH, R_f=0.56) indicated that the compound was not phosphorylated: its polarity was comprised between those of isopentenol (R_f=0.56) and ME (R_f=0.22). To fully characterize **6**, a large-scale incubation from a 1 L culture yielding cell-free system (40 mL) diluted with additional buffer (20 mL) was performed with unlabeled **1** (17.5 mg, 58 μMol). A small-scale incubation was made as described above, but with larger amounts of [2-^{14}C]-**1** (10^6 cpm). The two assays were mixed and lyophilized. The residue was acetylated (Ac_2O–pyridine, 1:1, 10 mL) overnight at room temperature. After removal of the reagents, the residue was resuspended in $CHCl_3$ (12 mL), and the insoluble material removed by filtration. After concentration to dryness, the filtrate (1 g, 836000 cpm) was separated on a silica column (8 g) eluted with hexane–EtOAc (3:1). Fractions (5 mL) were collected, and an aliquot (4 μL) analyzed by TLC. Radioactivity was monitored with a PhosphorImager, and labeled fractions with the same R_f were pooled together. Three radioactive fractions were detected: a first one containing the diacetate of **6** (R_f=0.40, 200 mg, 3×10^5 cpm), a second one containing an unidentified compound (R_f=0.25, 100 mg, 3.2×10^4 cpm), and a third one containing ME triacetate resulting from the chemical degradation of **1** during the work-up (R_f=0.20, 20 mg, 4.2×10^5 cpm). The least polar fraction was further purified on a silica column (9 g) eluted first with CH_2Cl_2 in order to remove nearly all impurities, and then with EtOAc yielding the radioactive diacetate of **6** (1 mg, 1.8×10^5 cpm). Diacetate of **6**. ^{1}H NMR spectrum (400 MHz, $CDCl_3$): δ (ppm)=1.75 (3H, broad s, 5-H), 2.09 (3H, s; **CH$_3$**COO-), 2.11 (3H, s, **CH$_3$**COO-), 4.51 (2H, s, 1-H), 4.65 (2H, d, $J_{3,4}$=7 Hz, 4-H), 5.63 (1H, tq, $J_{3,4}$=7 Hz, $J_{3,5}$=1.5 Hz, 3-H). ^{13}C NMR (500 MHz, $CDCl_3$) spectrum: δ (ppm)=14.2 (C-5), 20.9 (**CH$_3$**COO-), 21.0 (**CH$_3$**COO-), 60.6 (C-4), 68.6 (C-1), 121.7 (C-3), 135.8 (C-2), 170.7 (CH$_3$**CO**O-), 171.0 (CH$_3$**CO**O-).
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