

Isoprenoid biosynthesis via the methylerythritol phosphate pathway. *(E)*-4-Hydroxy-3-methylbut-2-enyl diphosphate: chemical synthesis and formation from methylerythritol cyclodiphosphate by a cell-free system from *Escherichia coli*

Murielle Wolff,^{a,†} Myriam Seemann,^{a,†} Catherine Grosdemange-Billiard,^a Denis Tritsch,^a Narciso Campos,^b Manuel Rodríguez-Concepción,^b Albert Boronat^b and Michel Rohmer^{a,*}

^aUniversité Louis Pasteur/CNRS, Institut Le Bel, 4 rue Blaise Pascal, F-67070 Strasbourg Cedex, France

^bDepartament de Bioquímica i Biologia Molecular, Facultat de Química, Universitat de Barcelona, Martí i Franquès, E-08028 Barcelona, Spain

Abstract—2-*C*-Methyl-D-erythritol cyclodiphosphate is converted into *(E)*-4-hydroxy-3-methylbut-2-enyl diphosphate by a cell-free system from an *Escherichia coli* strain overexpressing the *gcpE* gene. The latter diphosphate, representing probably the last intermediate in the MEP pathway for isoprenoid biosynthesis, was identified by comparison with reference material obtained by chemical synthesis.

In most bacteria as well as in the plant plastids, isoprenoids are synthesized via the mevalonate-independent methylerythritol phosphate 4 (MEP) pathway (Fig. 1).¹ If the first steps leading from pyruvate 1 and glyceraldehyde 3-phosphate 2 to methylerythritol (ME) 2,4-cyclodiphosphate 5 are rather well known,¹ the last steps leading from ME cyclodiphosphate 5 to the ubiquitous precursors for all isoprenoids, isopentenyl diphosphate 8 (IPP) and dimethylallyl diphosphate 9 (DMAPP), are still under investigation. The further metabolism of ME cyclodiphosphate 5 involves the *gcpE* and *lytB* gene products. Indeed, *gcpE*² and *lytB*³ were identified by genetic methods as essential genes of the MEP pathway in *Escherichia coli*. Deletion of the *gcpE* gene in an *E. coli* strain capable of utilizing exogenous MVA for isoprenoid biosynthesis after introduction of the genes encoding MVA kinase, phosphomevalonate kinase and diphosphomevalonate decarboxylase resulted in the accumulation of tritium labeled ME cyclodiphosphate after feeding of [1-³H]ME, suggesting that ME cyclodiphosphate 5 might be the substrate of the GcpE protein.⁴ In addition, [2-¹⁴C]ME cyclodiphosphate 5 was converted into a

phosphorylated derivative of *(E)*-2-methylbut-2-ene-1,4-diol 10.⁵ The cofactors required for this conversion were not identified using such a crude cell-free system. ME cyclodiphosphate 5 was formed from ¹³C-labeled 1-deoxy-D-xylulose (DX) by an *E. coli* strain overexpressing the gene of the xylulose kinase, which is capable of phosphorylating DX, as well as the genes of the enzymes catalyzing the first four steps of the MEP pathway following the reaction catalyzed by the DX phosphate 3 synthase (i.e. *dxr*, *ygbP*, *ygbB* and *ygbB*). Overexpression in addition of the *gcpE* gene is followed by the formation of the diphosphate 6 of the former diol 10.⁶ The latter compound was also found as a highly immunogenic compound in an *E. coli* *lytB*-deficient mutant, whereas it was not detected in *gcpE*-deficient mutants.⁷ In this contribution, a fast chemical synthesis of *(E)*-4-hydroxy-3-methylbut-2-enyl diphosphate is described, as well as its formation from ¹⁴C-labeled ME cyclodiphosphate by a cell-free system from *E. coli* overexpressing the GcpE protein. Sequence comparisons of the GcpE proteins indicated also that GcpE belongs to the proteins with an iron/sulfur cluster.

1. Synthesis of *(E)*-4-hydroxy-3-methylbut-2-enyl diphosphate 6

(E)-4-Hydroxy-3-methylbut-2-enyl diphosphate 6 was obtained via a four-step synthesis starting from the

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* Corresponding author. Fax: +33 (0)3 90 24 13 45; e-mail: mirohmer@chimie.u-strasbg.fr

† M.W. and M.S. contributed equally to this work.

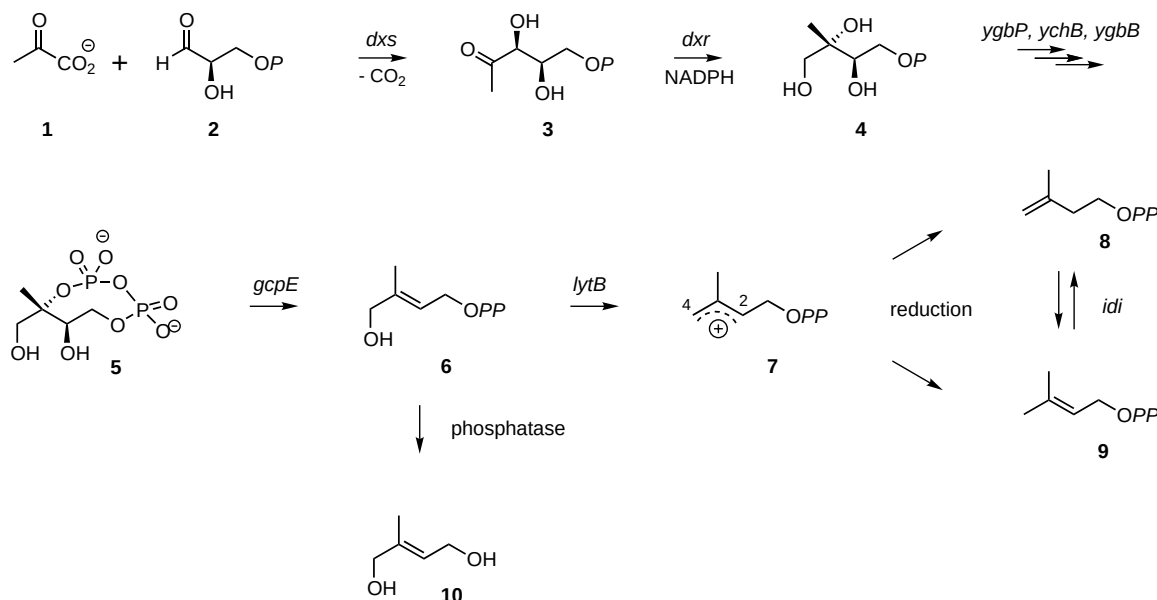


Figure 1. Methylerythritol phosphate (MEP) pathway for isoprenoid biosynthesis in *Escherichia coli*.

commercially available methyl 2-bromopropionate **11** (Fig. 2).⁸ The synthetic strategy involved the separate formation of the two oxygenated functional groups at C-1 and C-4, allowing the selective introduction of the diphosphate group in the desired position. The carbon framework was obtained by a Wittig reaction. The stabilized Wittig reagent derived from methyl 2-bromopropionate was condensed with glyoxylic acid monohydrate in CH_3CN to give only the acid **12** with the *E* configuration (73% yield).⁹ Selective reduction of the acid was performed with a 1 M solution of the borane–tetrahydrofuran complex,¹⁰ yielding compound **13** (77% yield). Bromination of the allylic alcohol **13** with phosphorus tribromide in CCl_4 afforded **14** (67% yield).¹¹ The ester **14** was reduced with diisobutyl aluminum hydride in toluene.¹² The resulting bromoalcohol was not isolated, and directly converted into the diphosphate **6** by substitution with tris(tetra-*n*-butylammonium) hydrogen diphosphate (24% overall yield).¹³ Confirmation of the *E* configuration of the double bond was obtained by nuclear Overhauser effect correlations. The vinylic proton at C-2 and the C-4 methylene protons on the one hand, and the methyl protons and the C-1 methylene protons on the other hand showed cross-peaks indicating they were in *cis* position and confirming the *E* configuration of the double bond in diphosphate **6**. NMR data of our synthetic compound **6** are in full agreement with those of the corresponding immunogenic compound **6** isolated from *E. coli*.⁷ No intermediate product had to be purified between **11** and **6**. This was the main advantage of this synthesis. According to the NMR spectra, the purity of all compounds was satisfactory. Only tetra-*n*-butylammonium ions of diphosphate **6** were exchanged for ammonium ions by ion exchange chromatography, and the end product **6** of the whole reaction sequence was purified by cellulose chromatography.

2. Conversion of ME cyclodiphosphate into (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate **6** and biogenetic implications

We recently described a cell-free system prepared from an *E. coli* strain overexpressing the *gcpE* gene.⁵ Incubation of $[2\text{-}^{14}\text{C}]$ ME cyclodiphosphate with this cell-free system and in the presence of a phosphatase resulted in the formation of the free diol **10**, which was identified by NMR spectroscopy and comparison with a synthetic reference. This diol resulted from the hydrolysis of a phosphorylated metabolite, most likely the diphosphate **6**, which could neither be isolated for direct identification by NMR spectroscopy, nor be identified by radiochemical methods. Addition of the now available non-labeled synthetic carrier to the cell-free system after incubation of $[2\text{-}^{14}\text{C}]$ ME cyclodiphosphate in the absence of a phosphatase, permitted the identification of the diphosphate **6** among the remaining unidentified radiolabeled compounds.¹⁴ Indeed, a radioactive compound coeluted by TLC with the synthetic carrier **6**. Upon alkaline phosphatase hydrolysis, it released the

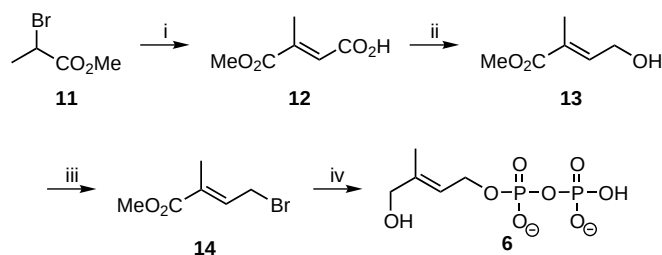


Figure 2. Synthesis of (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate **6**. *Reagents and conditions* (i) (a) PPh_3 , CH_3CN , 65°C , overnight, (b) $\text{OHC-CO}_2\text{H}$ monohydrate, CH_3CN , 0°C for 2 h, rt overnight; (ii) BH_3 , THF, -10°C to rt, overnight; (iii) PBr_3 , CCl_4 , 0°C to rt, 1 h; (iv) (a) DIBAL, toluene, -78°C , 1 h, (b) $(n\text{-Bu}_4\text{N})_3\text{HP}_2\text{O}_7$, CH_3CN , rt, overnight.

diol **10**, which was identified by TLC coelution of the free diol and of the corresponding diacetate.¹⁴ This incubation proved thus for the first time the direct precursor to product relationship between ME cyclophosphate **5** and (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate **6**.

Incorporation of [4-²H]DX and [3-²H]ME into the prenyl chain of ubiquinone and menaquinone of *E. coli* pointed out different labeling patterns of the isoprene units, with deuterium retention in the DMAPP derived starter unit and deuterium loss in those derived from IPP.¹⁵ In addition, the IPP isomerase (IdI) activity is very low in *E. coli*, and deletion of the *idi* gene encoding IdI is not lethal.¹⁶ All these data suggested the presence of a branching in the MEP pathway, separately leading to IPP **8** and DMAPP **9** from an intermediate to be identified. Definitive proof for such a branching was obtained by a genetic approach.¹⁷ An *E. coli* strain engineered for the utilization of exogenous MVA grew after disruption of the essential *dxr* gene either on exogenous MVA, or on ME. Growth on

MVA produces, however, only IPP, which has to be isomerized into DMAPP by the IdI. After additional disruption of the *idi* gene, growth on MVA was fully blocked whereas growth on ME was normal. This strategy clearly showed that there is no other way for interconverting IPP and DMAPP than the reaction catalyzed by the protein encoded by the *idi* gene and that IPP **8** and DMAPP **9** are separately synthesized in the MEP pathway by two different ways from the same intermediate (Fig. 1). LytB is most likely the last enzyme in the MEP pathway. After deletion of the essential *lytB* gene in *E. coli* genetically engineered for utilizing exogenous MVA, growth was only possible after supplementation with MVA or complementation of the cells with an episomal copy of *lytB*.^{3b} This is in agreement with a position of *lytB* in the trunk line of the MEP pathway. Diphosphate **6** is most likely the substrate of the LytB protein. Indeed, the accumulation of [U-¹³C₅]diol diphosphate **6** after feeding with [U-¹³C₅]DX an *E. coli* strain overexpressing the xylulose kinase gene and all MEP pathway genes from *dxr* downstream up to *gcpE* was replaced by the accumula-

Table 1. A highly conserved amino acid region of the GcpE proteins from various organisms that may indicate the presence of a Fe-S cluster

Aquifex aeolicus	VQEKLSGVKTPCLKVAVMGCVVNAIGEAREADIGLACGR-G
Bacillus halodurans	MEEYISKIRAPIKVAVLGCAVNGPGEAREADIGIAGAR-G
Bacillus subtilis	VEEYISKIKAPIKVAVLGCAVNGPGEAREADIGIAGAR-G
Buchnera aphidicola	LEKNLEDISTPIDVSIIGCVVNGIGESKIATLGLAGSHKK
Chlamydia muridarum	IKERTKHLPGGLKIAVMGCI VNGPGEMADADFGYVGSKPG
Chlamydia pneumoniae	IRKRTQHLPG-LKIAIMGCI VNGPGEMADADFGFVGSKTG
Chlamydia trachomatis	IRERTQHLPGGLKIAVMGCI VNGPGEMADADFGYVGSKPG
Escherichia coli	LEQRLEDIITPMDVSIIGCVVNGPGEALVSTLGVTTGNKK
Haemophilus influenzae	LEQRLEDIITPMDVSIIGCVVNGPGEALVSTLGVTTGNKK
Helicobacter pylori	VEKRLSHIKTPLDISVMGCVVNALGEAKHADMAIAFGN-R
Mycobacterium leprae	VSAGLDGLEVPLRIAVMGCVVNGPGEARDADLGVASGN-G
Mycobacterium tuberculosis	VTAGLDGLDVPLRVAVMGCVVNGPGEAREADLGVASGN-G
Pasteurella multocida	LEQRLEDIITPMDVSIIGCVVNGPGEALVSDLGVTGNKK
Providencia stuartii	LEQRLEDIITPMDVSIIGCVVNGPGEAEVSTLGVAGAKTK
Streptomyces coelicolor	LR-----VAVMGCVVNGPGEAREADLGVASGNKG
Synechocystis sp.	LD-----IAVMGCI VNGPGEMADADYGYVGKQAG
Thermotoga maritima	LK-----IAVMGCVVNGIGEGKDADLGVAGLRDG
Treponema pallidum	IT-----VAVMGCVVNGPGEKHADLGISGAEDS
Deinococcus radiodurans	EWKAKYPGVEDMQVAVMGCVVNGPGESKHANIGISLPGTG
Campylobacter jejuni	LN-----ISVMGCVVNALGEAKGADVAIAFGKNQ
Vibrio cholerae	PMD-----VSIIGCVVNGPGEAEVSHLGLAGSNKK
Neisseria meningitis	IWRTLYPGVESLNVAVMGCVVNGPGESKLADIGISLPGTG
Caulobacter crescentus	MS-----LSIIGCVVNGPGEALMTDIGFTGGGAG
Clostridium acetobutylicum	IK-----VAVMGCVVNGPGEAREADIGIAGGKGE
Clostridium perfringens	IK-----VAVMGCVVNGPGEAREADIGIAGGKGE
Rhizobium loti	VWREKYPGVENLKVAVMGCIVNGPGESKHADIGISLPGTG
Rhizobium meliloti	VWREKYPGVEALKVAVMGCIVNGPGESKHADIGISLPGTG
Agrobacterium tumefaciens	IWREKYPGVEALNVAVMGCIVNGPGESKHADIGISLPGTG
Brucella melitensis	LWREKYPGVEALSVAVMGCIVNGPGESKHADIGISLPGTG
Salmonella typhimurium	MD-----VSIIGCVVNGPGEALVSTLGVTTGNKK
Salmonella enterica	MD-----VSIIGCVVNGPGEALVSTLGVTTGNKK
Plasmodium falciparum	K-----IAVMGCI VNGIGEMADAHFGYVGSAPK
Arabidopsis thaliana	S-----IAIMGCI VNGPGEMADADFGYVGGSPG

::: ** . ** :

Consensus line.*=identical or conserved residues in all sequences in the alignment

: =indicates conserved substitutions

. =indicates semi-conserved substitutions.

tion of [U-¹³C₅]IPP and [U-¹³C₅]DMAPP, when in addition *lytB* was overexpressed.¹⁸ These data strongly suggest that *lytB* corresponds to the branching point of the MEP pathway, and that both IPP and DMAPP are the reaction products of the LytB protein. The conversion of diphosphate **6** into IPP or DMAPP formally implies a reduction step. This is best finalized by the reduction of an allylic (most likely cationic or radical) intermediate **7** characterized by electron delocalization either on the carbon atom corresponding to C-2 or C-4 of IPP or DMAPP (Fig. 1).^{5,6}

3. GcpE, an iron–sulfur protein

The (I,V,L)(A,S)(V,I)(M,I,L)GCXVN(G,A)XGEXXX-(A,S,T)XX(G,A) motif is conserved in the GcpE protein family (Table 1). The corresponding amino acid sequence of each organism was analyzed in a PRINTS/PROSITE search using the bioinformatic site of the School of Biochemistry and Molecular Biology of the University of Leeds (<http://bmsbsgil1.leeds.ac.uk/>). It appeared that these sequences either matched a 4Fe–4S ferredoxin signature (maximum score 47%, *E. coli*), or an aconitase signature (maximum score 43%, *T. pallidum*), or both. The latter enzymes are characterized by an active [4Fe–4S] cluster that mediates electron transfer. The only exception was the *Buchnera aphidicola* sequence, which displayed a flavoprotein pyridine nucleotide cytochrome reductase signature that has also been found in several other organisms. During this search, analogies of some sequences with those of FAD/NAD(P)H reductases were noticed. In addition, Fe–S clusters are usually oxygen sensitive. This was also observed for the GcpE assay, which was best performed in degassed buffers and under an argon atmosphere. In addition, the orthologous *gcpE* group is characterized by three conserved cysteines,⁶ a characteristic that is again compatible with the presence of an iron–sulfur cluster in GcpE. We suggest therefore that GcpE contains a Fe–S cluster and may use FAD and/or NADPH as cofactor(s). This feature would be in agreement with a mechanism for the GcpE enzyme-catalyzed conversion of ME cyclodiphosphate **5** into the diol diphosphate **6** resembling those of enzymes implied in the biosynthesis of deoxysugars such as 2-deoxyribose or ascarylose.¹⁹

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- 2-Methylmonomethylfumarate 12.** To a solution of methyl 2-bromopropionate **11** (2.00 g, 11.97 mmol, 1 equiv.) in dry CH₃CN (30 mL) was added triphenylphosphine (2.83 g, 10.78 mmol, 0.9 equiv.). After stirring at 65°C overnight, diisopropylethylamine (1.88 mL, 0.78 mmol, 0.9 equiv.) and glyoxylic acid monohydrate (0.99 g, 0.78 mmol, 0.9 equiv.) dissolved in dry CH₃CN (5 mL) were added to the reaction mixture at 0°C. The solution was further stirred at 0°C for 2 h and at room temperature overnight. Half of the solvent was removed under reduced pressure, and ethyl acetate (10 mL) was added. The resulting solution was washed with saturated aqueous NaHCO₃ (3×30 mL). The combined aqueous layers were extracted with ethyl acetate (2×10 mL), acidified (pH 1–2) at 0°C with concentrated HCl (5 mL) and extracted with ethyl acetate (3×30 mL). The combined organic layers were evaporated to dryness, yielding a pale yellow solid (1.14 g, 73%, *R*_f=0.30, ethyl acetate/hexane, 7:3), which was used for the next reaction without further purification. ¹H NMR (200 MHz, CDCl₃): δ=2.31 (3H, d, *J*_{3,5}=1.5 Hz, 5-H), 3.82 (3H, s, CO₂CH₃), 6.80 (1H, q, *J*_{3,5}=1.5 Hz, 3-H). ¹³C NMR (50 MHz, CDCl₃): δ=14.6 (C-5, CH₃), 52.8 (CO₂CH₃), 126.0 (C-3), 146.0 (C-2), 167.4 (CO), 171.0 (CO).
- Methyl (E)-4-hydroxy-2-methylbut-2-enoate 13.** To a solution of **12** (1.00 g, 6.94 mmol, 1 equiv.) in dry THF (40 mL) was added dropwise a 1 M solution of borane–tetrahydrofuran complex (6.9 mL, 6.94 mmol, 1 equiv.) at –10°C. The stirred reaction mixture was gradually allowed to warm up to room temperature overnight. The reaction was quenched at room temperature by slow dropwise addition of 50% aqueous acetic acid (3 mL). After removal of the solvent, a saturated aqueous NaHCO₃ (40 mL) was dropwise added at 0°C. The aqueous layer was extracted with ethyl acetate (1×40 mL, 2×15 mL). The extracts were washed with saturated aqueous NaHCO₃ (25 mL) and the combined aqueous layer was again extracted with ethyl acetate (2×25 mL). The organic layers were combined and concentrated to afford a pale yellow oil (695 mg, 77%, *R*_f=0.41, ethyl

acetate/hexane, 7:3), which was not further purified. ^1H NMR (200 MHz, CDCl_3): δ = 1.84 (3H, dt, $J_{3,5} = J_{4,5} = 1$ Hz, 5-H), 3.75 (3H, s, CO_2CH_3), 4.35 (2H, dq, $J_{4,5} = 1$ Hz, $J_{3,4} = 6.2$ Hz, 4-H), 6.82 (1H, tq, $J_{3,5} = 1$ Hz, $J_{3,4} = 6.2$ Hz, 3-H). ^{13}C NMR (50 MHz, CDCl_3): δ = 12.9 (C-5), 52.1 (CO_2CH_3), 59.9 (C-4), 128.7 (C-2), 140.3 (C-3), 168.3 (CO).

Methyl (E)-4-bromo-2-methylbut-2-enoate 14. To a solution of **13** (220 mg, 1.69 mmol, 1 equiv.) in CCl_4 (5 mL) was added at 0°C phosphorus tribromide (52 μL , 0.56 mmol, 0.33 equiv.) diluted in a minimum of CCl_4 (500 μL). After stirring at room temperature for 1 h, the reaction mixture was quenched by addition of saturated aqueous NaHCO_3 (500 μL). The organic layer was washed with water (500 μL) and the resulting aqueous layers were then extracted with pentane (2 $\times$ 2 mL). The combined organic layers were concentrated to a colorless liquid (220 mg, 67%, R_f = 0.63, ethyl acetate/hexane, 3:7), which was used for the last step without further purification. ^1H NMR (200 MHz, CDCl_3): δ = 1.92 (3H, d, $J_{3,5} = 1.5$ Hz, 5-H), 3.76 (3H, s, CO_2CH_3), 4.03 (2H, d, $J_{3,4} = 8.6$ Hz, 4-H), 6.92 (1H, tq, $J_{3,5} = 1.5$ Hz, $J_{3,4} = 8.6$ Hz, 3-H). ^{13}C NMR (50 MHz, CDCl_3): δ = 12.3 (C-5), 26.0 (C-4), 52.2 (CO_2CH_3), 132.0 (C-2), 135.2 (C-3), 167.8 (CO).

(E)-4-Hydroxy-3-methylbut-2-enyl diphosphate 6. To a solution of **14** (220 mg, 1.14 mmol, 1 equiv.) in toluene (6 mL) was added dropwise a 1 M solution of DIBAL in hexane (2.8 mL, 2.85 mmol, 2.5 equiv.) at -78°C . After being stirred for 1 h, the reaction mixture was quenched with 50% aqueous acetic acid (300 μL). The resulting insoluble material was filtered through Celite and the precipitate was washed with acetone (5 $\times$ 30 mL). After removal of the solvent, the green oil was dissolved in CH_3CN (500 μL) and the solution was added to a solution of tris(tetra-*n*-butylammonium) hydrogen pyrophosphate trihydrate (1540 mg, 1.71 mmol, 1.5 equiv.) in CH_3CN (3 mL). The reaction mixture was stirred overnight and solvent was removed. The pale yellow oil dissolved in water (2 mL) was passed through a column of Dowex AG 50W-X8 (1 $\times$ 15 cm, 100–200 mesh, ammonium form) cation-exchange resin. The product was eluted with an ion-exchange buffer (100 mL, isopropanol/25 mM NH_4HCO_3 , 1:49). After evaporation of the solvent, the material was frozen and lyophilized. The resulting white solid (400 mg) was dissolved in a 50 mM NH_4HCO_3 solution (1 mL). Isopropanol/ CH_3CN (1:1, 4 mL) was added, and the mixture was mixed on a vortex and centrifuged for 5 min. at 700g. The combined supernatants were removed, and the pellet was suspended in a 50 mM NH_4HCO_3 solution (1 mL) and the process was repeated eightfold. After removal of the solvent and lyophilization, a white solid was obtained. This material dissolved in isopropanol/ CH_3CN /0.1 M NH_4HCO_3 (4.5:2.5:3, 1 mL) was loaded onto a cellulose column (2 $\times$ 17 cm). The column was eluted with the buffer and thirteen fractions were collected. After evaporation of the organic solvent under reduced pressure, the remaining water was removed by lyophilization to give a fluffy colorless solid (87 mg, 24%, R_f = 0.27, silica gel isopropanol/water/ethyl acetate, 6:3:1). ^1H NMR (400 MHz, D_2O): δ = 1.69 (3H, broad s, 5-H), 4.00 (2H, broad s, 4-H), 4.51 (2H, dd, $J_{1,P} = J_{1,2} = 6.9$ Hz, 1-H), 5.64 (1H, m, 2-H). ^{13}C NMR (100.8 MHz, D_2O): δ = 13.1 (C-5), 62.1 (C-1, d, $J = 4.8$ Hz), 66.5 (C-4),

121.0 (C-2, d, $J = 7$ Hz), 139.5 (C-3). ^{31}P NMR (162 MHz, D_2O): δ = -5.3 (d, $J = 21.7$ Hz), -9.0 (d, $J = 21.7$ Hz).

ESI MS (negative ion detection mode, H_2O): m/z = 261 ($\text{C}_5\text{H}_{11}\text{O}_8\text{P}_2$) $^-$, 130 ($\text{C}_5\text{H}_{10}\text{O}_8\text{P}_2$) $^{2-}$.

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14. *Conversion of ME cyclodiphosphate into (E)-4-hydroxy-3-methylbut-2-enyl diphosphate 6.* An aliquot (3 μL) of the cell-free enzymatic assay prepared as previously described⁵ was mixed with synthetic (E)-4-hydroxy-3-methylbut-2-enyl diphosphate and analyzed by TLC on silica gel plates (isopropanol/water/EtOAc, 6:3:1). Using a PhosphorImager, radioactivity was detected in the free diol (R_f = 0.80), in methylerythritol cyclodiphosphate (R_f = 0.57), in two unidentified compounds (R_f = 0.41, R_f = 0.37) and in a more polar compound (R_f = 0.23). The latter compound comigrated on TLC plates with (E)-4-hydroxy-3-methylbut-2-enyl diphosphate **6**. For structure confirmation, an aliquot of the GcpE assay was eluted on silica gel plates (isopropanol/water/ethyl acetate 6:3:1), and the silica containing the more polar compound (R_f = 0.23) coeluting with synthetic (E)-4-hydroxy-3-methylbut-2-enyl diphosphate was recovered and treated with alkaline phosphatase (50 mM Tris HCl buffer, pH 8, 5 mM MgCl_2 , 16 h, 25°C). After centrifugation to remove the silica, the supernatant was analyzed by TLC ($\text{CHCl}_3/\text{MeOH}$, 8:2). Radioactivity was monitored with a PhosphorImager and coeluted with synthetic diol **10** (R_f = 0.56). After lyophilization and acetylation a single radioactive spot was detected, coeluting with the diacetate of the diol **10** (ethyl acetate/hexane, 1:1, R_f = 0.48). A control incubation of [^{14}C]ME cyclodiphosphate with a boiled cell-free system was also performed. No radioactivity was detected in the (E)-4-hydroxy-3-methylbut-2-enyl diphosphate fraction indicating that this compound was derived from an enzymatic conversion of the incubated ME cyclodiphosphate.
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