Supplementary Material (ESI) for Molecular BioSystems
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Probing Phosphorylation by Non-mammalian Isoprenoid Biosynthetic Enzymes Using ^{1}H-^{31}P-^{31}P-Correlation Spectroscopy

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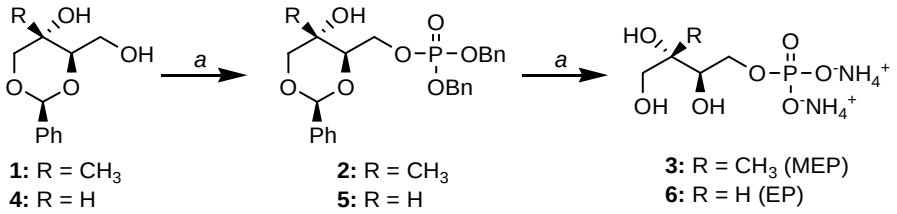
Scheme S1. Synthesis of IspD substrates methylerythritol phosphate (MEP) and erythritol phosphate (EP).



1: R = CH_3
4: R = H

2: R = CH_3
5: R = H

3: R = CH_3 (MEP)
6: R = H (EP)

Reaction conditions: *a) i.* $iPr_2NP(OBn)_2$, tetrazole, CH_3CN, r.t., 2 h, *ii. m*CPBA, 0°C, 1 h; *b) i.* $Pd(OH)_2/C$, EtOH, r.t., 40 psi, overnight, *ii*. $NH_{3(g)}$

*Synthesis of 2-C-Methyl-D-erythritol 4-Phosphate (MEP), Ammonium Salt (**3**).* The IspD substrate, MEP, was prepared from **1** (Scheme 2). Briefly, 0.45 M tetrazole in CH_3CN (2.00 mL, 0.90 mmol) was added to a solution of dibenzyl diisopropylphosphoramidite (148 μL, 0.45 mmol) in anhydrous CH_3CN (1.0 mL) under an atmosphere of argon. The cloudy mixture was left to stir at room temperature for 30 minutes. The resulting solution was added dropwise over 2 minutes to **1** (100 mg, 0.45 mmol) dissolved in anhydrous CH_3CN (1.5 mL). The reaction mixture was stirred at room temperature for 45 minutes. The reaction mixture was cooled to 0 °C, and *m*CPBA (234 mg, 1.35 mmol) was added in one portion. The icebath was removed and stirring was continued for 30 minutes. Solvents were then removed in vacuo and the resulting oily crude material was dissolved in 10 mL CH_2Cl_2 and washed with saturated $NaHCO_3$ (1 × 3 mL). The layers were separated and the organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Purification of the crude product by silica gel flash chromatography (30% to 50% EtOAc in hexanes) afforded **2** as a colorless oil (167 mg, 77%). Spectral data for compound **2** was in agreement with previously reported data [1]. ^{1}H NMR (acetone-d_6) δ 1.33 (s, 3H), d 3.73 and 3.80 (ABq, 2H, J = 10.6 Hz), δ 4.00 (dd, 1H, J = 8.3, 1.5 Hz), δ 4.13 (dt, 1H, J = 11.0, 8.1 Hz), δ 4.46 (ddd, 1H, J = 11.2, 6.8, 1.8 Hz), δ 5.04 (m, 4H), 5.59 (s, 1H), δ 7.33 (m, 13H), δ 7.51 (m, 2H); ^{31}P NMR (acetone-d_6) δ - 25.23.

Dibenzyl phosphate **2** (78mg, 0.16 mmol) was dissolved in EtOH (1.8 mL) and added to a test tube containing $Pd(OH)_2/C$ (37 mg). Hydrogenolysis was accomplished at 30-50 psi of H_2 overnight. The suspension was filtered, washed with MeOH (8 mL) and cooled to 0 °C. A stream

of $NH_{3(g)}$ was bubbled through the mixture for ~ 1 minute. The mixture was concentrated under reduced pressure to give **3** as a white solid (34 mg, 85%). Spectral data for compound **3** was in agreement with previously reported data [1]. ^{1}H NMR (D_2O) δ 3.59 (dd, 1H, *J* = 11.5, 5.9 Hz), δ 3.71 (m, 3H), δ 3.90 (m, 2H); ^{31}P NMR (D_2O) δ – 22.59.

*Synthesis of D-erythritol 4-Phosphate (EP), Ammonium Salt (**6**).* Compound **6** [2] was prepared according to the procedure outlined for compound **3** (MEP) above (27 mg, 89%). ^{1}H NMR (D_2O) δ 3.59 (dd, 1H, *J* = 11.5, 5.9 Hz), δ 3.71 (m, 3H), δ 3.90 (m, 2H); ^{31}P NMR (D_2O) δ – 22.59; Calcd. For $C_4H_{11}O_7P$ (free acid): *m/z* 202.0 $[M+H]^+$; Found (positive ion ESI MS *m/z)*: 203.0 $[M+H]^+$.

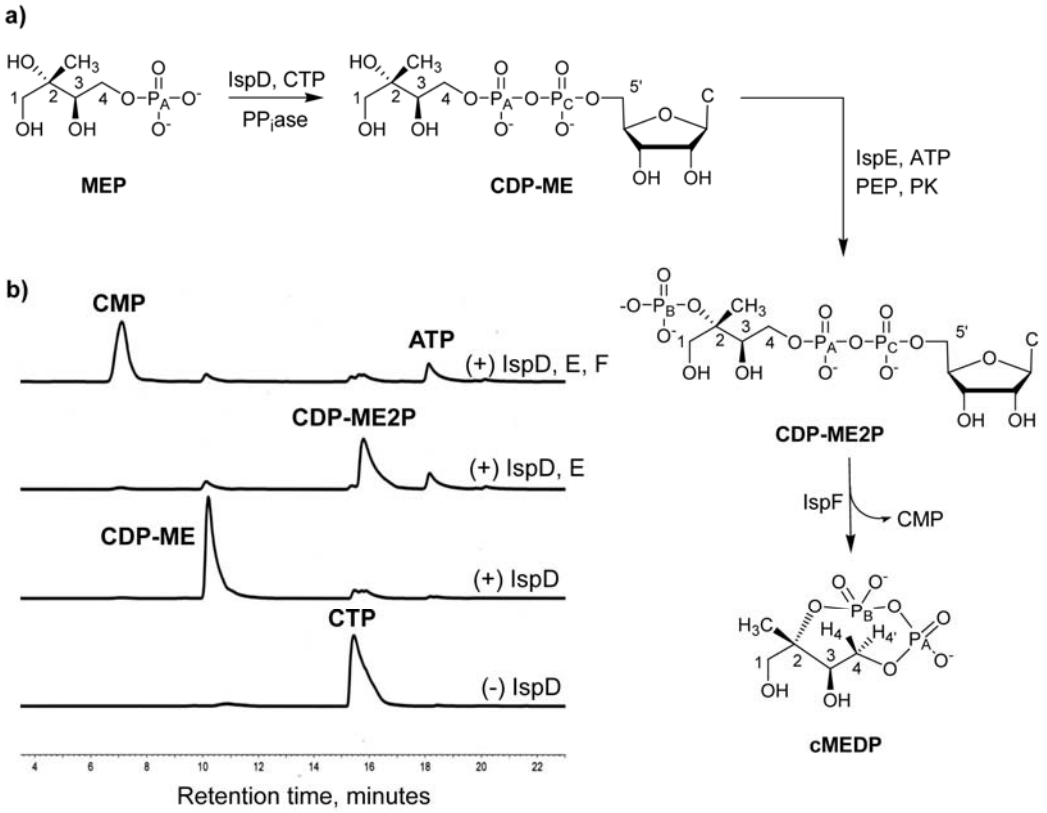


Figure S1. HPLC analysis of tandem enzyme reactions to monitor formation of cMEDP from MEP. **a)** IspD, E, F tandem enzyme sequence. **b)** HPLC stackplot showing conversion of MEP to cMEDP via CDP-ME. The tandem enzyme sequence was carried out at 37 °C and monitored by HPLC using the following procedure: 25 μL aliquots were quenched in 25 μL cold methanol. The quenched mixtures were incubated at 4 °C for 20 minutes, and the supernatants were analyzed by HPLC [gradient: buffer A to 70:30 buffer A/CH_3CN over 20 minutes; buffer A = 100 mM NH_4OAc/5 mM tetrabutylammonium bisulfate, pH 6.0]. Under these reaction conditions, the disappearance of CTP (R_T = 15.3 min) to form CDP-ME (R_T = 6.8 min) in the presence of IspD was complete within 10 minutes. Complete conversion of CDP-ME (R_T = 6.8 min) to CDP-ME2P (R_T = 15.8 min) in the presence of IspE was observed within 15 minutes. The product of IspE was converted in tandem to cMEDP and CMP by the addition of IspF. Disappearance of CDP-ME2P (R_T = 15.8 min) to form CMP (R_T = 4.9 min) was complete within 30 minutes. Unnatural enzyme reactions exhibited similar reaction profiles by HPLC (data not shown).

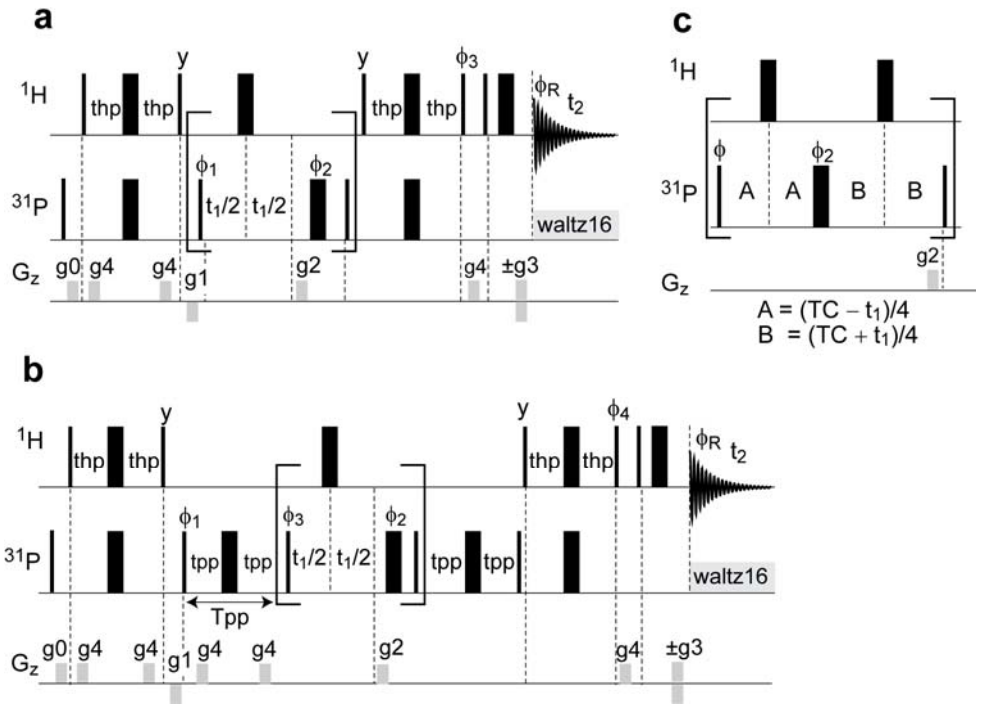


Figure S2. Pulse sequences for **(a)** ^{1}H- ^{31}P HSQC and **(b)** ^{1}H- ^{31}P -^{31}P COSY experiments. For the constant-time [3, 4] version of these experiments, the segments enclosed in brackets are replaced by element shown in **(c)**. Narrow and broad lines represent 90 and 180^0 pulses, respectively. Phases are *x*, except where specified. Data were acquired on a Varian INOVA spectrometer operating at a ^{1}H frequency of 500 MHz (11.7 T), and a pentaprobe (^{1}H, ^{13}C, ^{15}N, ^{31}P, ^{2}H) equipped with actively shielded *z*-gradient coils. The ^{1}H carrier was placed at the H_2O resonance frequency (4.82 ppm @ 19 ^{0}C) and the ^{31}P carrier was adjusted within the range –40 to –20 ppm (referenced w.r.t. TPPO) in the different reaction products. High power ^{1}H and ^{31}P pulses were applied with 900 pulse-widths of 7–9 μs and 20 μs respectively. WALTZ-16 [5] ^{31}P decoupling during acquisition (t_2) was carried out using a field strength of 1.6 kHz. Rectangular pulsed-field gradients (g0 – g4) were applied at ~ 15 G/cm (20% of the maximum strength), with durations of 1.0 ms (g0,g1,g2), 0.5 ms (g4) and 0.4 ms (g3). g2 and g3 were coherence selection gradients. Quadrature detection in the ^{31}P dimension was achieved by inverting the sign of the gradient g3 between the two FIDs obtained for each t_1 increment and subsequently processing the data in an echo-antiecho manner [6]. For dilute samples, or samples with intense solvent peaks, weak presaturation of the solvent signal (50 Hz rf field) was applied during the inter scan delay. The 90^0–g4–90 *z*-filter preceding the g3 readout gradient was used to minimize phase distortions. Pulse-sequence specific phase cycles are as follows: **(a)** $\phi_1 = x,-x$, $\phi_2 = x,x,y,y$, $\phi_3 = 4(x), 4(-x)$, $\phi_R = x,-x,-x,x,-x,x,x,-x$ **(b)** $\phi_1 = x,-x$, $\phi_2 = x,x,y,y$, $\phi_3 = 8(y),8(-y)$, $\phi_4 = 4(x), 4(-x)$, $\phi_R = x,-x,-x,x,-x,x,x,-x$ **(c)** $\phi = \phi_1$ for CT-^{1}H-^{31}P HSQC and $\phi = \phi_3$ for CT-^{1}H- ^{31}P -^{31}P COSY, $\phi_2 = x,x,y,y$. Delays thp, tpp, TC are listed in Tables S1-S4 below.

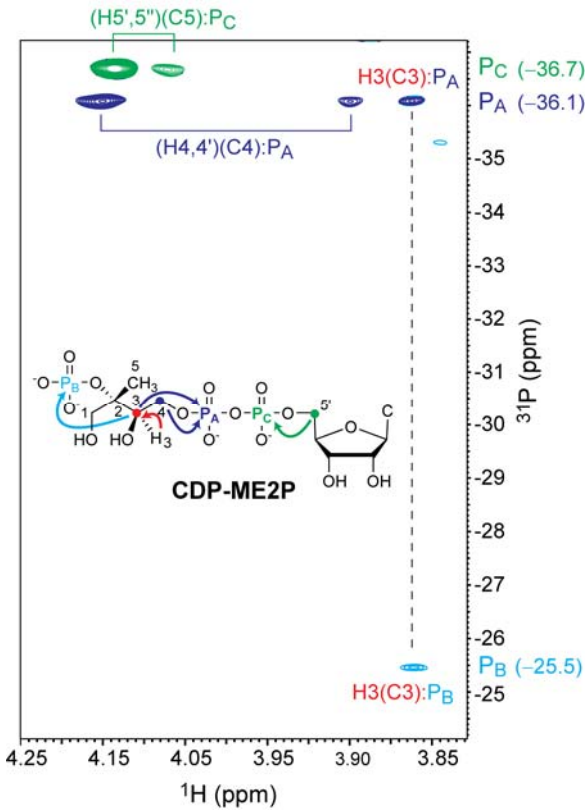


Figure S3. ^{1}H-(^{13}C)-^{31}P correlation spectrum of CDE-ME2P acquired using the pulse sequence in Figure S2. $^{1}H \rightarrow {}^{13}C \rightarrow {}^{31}P$ magnetization transfer pathways are indicated by arrows on the molecular structure of CDE-ME2P in the Figure. ^{1}H:^{31}P correlations are labeled H(C)P to indicate that they are not obtained via direct $^{1}H \rightarrow {}^{31}P$ magnetization transfer. The two correlations $H_3(C_3)P_B$ and $H_3(C_4)P_A$ at the H_3 proton frequency (connected by a dashed line) demonstrate that P_B and P_A are indeed part of the same molecule. Data acquisition parameters are listed in the legend to Figure S2.

Table S1. Data Acquisition Parameters for ^{1}H-^{31}P and ^{1}H-^{31}P-^{31}P COSY Experiments (Figures 1 and 2). Inter scan (relaxation) delay was 1.2 s for all experiments.

Figure	Experiment	^{1}H Dimension			^{31}P Dimension						
		scans	spectral width (kHz/ppm)	complex data points	^{31}P carrier (ppm)	spectral width (kHz/ppm)	complex data points (increments)	Total Time (h:m)	thp (ms)	TC (ms)	tpp (ms)
Purified cMEDP											
1a	HSQC	8	8/16	1000	-38.3	0.31/1.5	50	0:20	100		
1b	CT-HSQC	8	8/16	1000	-38.3	0.31/1.5	50	0:20	100	41	
1c	CT-HPP-COSY	16	8/16	1000	-38.3	0.31/1.5	12	0:10	100	41	5
1d	CT-HPP-COSY	16	8/16	1000	-38.3	0.31/1.5	12	0:10	100	41	10
MEP											
2a	HSQC	2	6/12	600	-32.4	1.3/6.5	196	0:20	40		
CDP-ME											
2b	CT-HSQC	8	8/16	1000	-29.5	1.2/5.9	58	0:24	41	49	
2c	CT-HPP-COSY	8	8/16	1000	-29.5	1.2/5.9	58	0:24	41	49	12
CDP-ME2P											
2d	CT-HSQC (upper panel)	32	8/16	800	-36.3	0.6/3	28	0:45	37	48	
2e	CT-HPP-COSY (upper panel)	32	8/16	800	-36.3	0.6/3	28	0:45	37	48	11
2d	CT-HSQC (lower panel)	48	8/16	800	-25.7	0.6/3	28	1:05	125	48	
2e	CT-HPP-COSY (lower panel)	48	8/16	800	-25.7	0.6/3	28	1:05	125	48	11
cMEDP											
2f	CT-HSQC	2	6/12	600	-31.3	4.65/23	209	0:20	86	45	
2g	CT-HPP-COSY	8	6/12	720	-31.3	4.65/23	209	1:25	86	45	5.5

Table S2. Data acquisition parameters for ^{1}H-^{31}P and ^{1}H-^{31}P-^{31}P COSY spectra acquired on the erythritol-containing unnatural IspD, E and F products shown in Figure 3. Inter scan (relaxation) delay was 1.2 s for all experiments.

Figure	Experiment	^{1}H Dimension			^{31}P Dimension						
		scans	spectral width (kHz/ppm)	complex data points	^{31}P carrier (ppm)	spectral width (kHz/ppm)	complex data points (increments)	Total Time (h:m)	thp (ms)	TC (ms)	tpp (ms)
CDP-E											
3a	CT-HSQC	16	8/16	1000	-36.4	0.35/1.7	18	0:15	33	49	
	CT-HPP-COSY	16	8/16	1000	-36.4	0.35/1.7	18	0:15	33	49	12
CDP-E2P											
3b	CT-HSQC (upper panel)	8	8/16	960	-36.4	0.3/1.5	15	0:07	39	53	
	CT-HSQC (lower panel)	8	8/16	960	-22.2	0.3/1.5	16	0:07	15	53	
	CT-HPP-COSY	16	8/16	960	-36.4	0.3/1.5	15	0:12	39	49	12
cEDP											
3c	CT-HSQC	16	8/16	960	-35.5	0.22/1.1	10	0:08	7	42	
	CT-HPP-COSY	16	8/16	960	-35.5	0.22/1.1	10	0:08	7	42	10

Table S3. Data acquisition parameters for ^{1}H-^{31}P and ^{1}H-^{31}P-^{31}P COSY spectra acquired on the 3-diphosphocytidyl-glycerol and 5-diphosphocytidyl-D-ribose products and time course snapshots of the reaction of reaction of glycerol-3-phosphate with CTP in the presence of IspD shown in Figure 4 . Inter scan (relaxation) delay was 1.2 s for Figures 4(a)-(c) and 1.1 s for Figure 4(d). In each spectrum, all relevant information was available in less than half the total acquisition time.

Figure	Experiment	^{1}H Dimension			^{31}P Dimension						
		scans	spectral width (kHz/ppm)	complex data points	^{31}P carrier (ppm)	spectral width (kHz/ppm)	complex data points (increments)	Total Time (h:m)	thp (ms)	TC (ms)	tpp (ms)
3-diphosphocytidyl-glycerol product (765 μM substrate concentration)											
4a	CT-HSQC	16	6/12	600	-29.0	1.15/5.7	30	0:24	39	48	
	CT-HPP-COSY	32	6/12	600	-29.0	1.15/5.7	30	0:51	39	48	14
3-diphosphocytidyl-glycerol product (200 μM substrate concentration)											
4b	CT-HSQC	32	6/12	600	-29.0	1.15/5.7	30	0:48	39	48	
	CT-HPP-COSY	48	6/12	600	-29.0	1.15/5.7	30	1:10	39	48	14
5-diphosphocytidyl-D-ribose product											
4c	CT-HSQC	16	6/12	600	-29.0	1.15/5.7	50	0:41	42	50	
	CT-HPP-COSY	48	6/12	600	-29.0	0.3/1.5	50	2:07	42	50	12.5
3-diphosphocytidyl-glycerol product time course											
4d	CT-HSQC	8	6/12	600	-29.0	1.15/5.7	16	0:06	39	48	

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