Supporting Information

Methylcobalamin-dependent Radical SAM *C*-Methyltransferase Fom3 Recognizes Cytidylyl-2-hydroxyethylphosphonate and Catalyzes the Non-stereoselective *C*-Methylation in Fosfomycin Biosynthesis

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List of Supporting Information

Experimental

Expression and purification of Fom3

The *fom3* gene was amplified by PCR with primers *fom3*-F (5′-CatATGACGATCGGTTCTCTGGGC-3′) and *fom3*-R (5′-aAGcTTTCAGTACTGGTTTGCGTTCCAC-3′) using the cosmid pFBG51 that contains a part of fosfomycin biosynthesis gene cluster derived from *Streptomyces wedomorensis* 144-91[1] as a template DNA. PCR reaction with PrimeSTAR GXL polymerase (Takara) was performed under the following conditions: 30 cycles of 98°C for 10 s, 60°C for 15s, and 68°C for 1min 40 s for extension of DNA. The manufacturer's protocol was used to amplify the target DNA. PCR product was treated with Takara Ex Taq DNA polymerase reaction and then sub-cloned into the pMD19 T-vector (TAkara) to obtain pMD19-*fom3* using *Escherichia coli* DH5α. After confirmation of the sequence (Fasmac), the *Nde*I-*Hin*dIII fragment of pMD19-*fom3* was inserted into pET28a (Novagen) vector to obtain pET28-*fom3*. The pET28-*fom3* plasmid and pRKSUF017[2] were introduced into *E. coli* BL21(DE3) (Novagen) in the presence of 30 μg/mL of kanamycin and 5 μg/mL of tetracycline. *E. coli* BL21(DE3) carrying pET28-*fom3* and pRKSUF017 was grown in 500 mL baffled flasks of LB medium (200 mL) containing 30 μg/mL of kanamycin and 5 μg/mL of tetracycline at 37°C until $OD_{590} \geq 0.8$ with 200 rpm agitation. The expression was induced by addition of 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG), 200 μM $FeSO_4(NH_4)_2SO_4$, and 200 μM L-cysteine. The cultivation was continued at 15°C for 20−24 h with 80 rpm agitation. The cells were harvested by centrifugation at 6000 rpm for 10 min, washed with buffer A (50 mM HEPES−NaOH (pH 8.0), 300 mM NaCl, 10% glycerol), and stored at −30°C until use. The wet cells were placed in a glovebox (UNICO, $[O_2] \leq 5$ ppm) and suspended in buffer A, which was degassed with a freeze−thaw procedure under reduced pressure, followed by bubbling with a mixture of argon and hydrogen gas purified through a reduced Cu catalyst. The suspension of cells on an aluminum beads bath that was kept at cooled temperature was disrupted by sonication (QSONICA) for 5 s with a 5 s interval (total 2 min) in the glovebox. Cell debris was removed by centrifugation at 10,000 rpm for 5 min at 4 °C. The supernatant was loaded onto a TALON resin (Clontech) column (15 × 15 mm), which had been previously equilibrated with buffer A. The column was washed with 100 mL of buffer A plus 15 mM imidazole to remove unbound proteins. Fom3 was eluted using buffer A plus 200 mM imidazole. The protein solution was collected and desalted with a PD-10 desalting column (GE Healthcare) equilibrated with buffer A. The purified Fom3 was treated with 5 mM DTT at 5˚C for 15 min. $FeSO_4(NH_4)_2SO_4$ (0.25 mM), $FeCl_3$ (0.25 mM), and Na_2S (0.5 mM) were then added, and the mixture was incubated at 5˚C for 2 h. The Fom3 sample was then desalted by passing through a PD-10 desalting column equilibrated with buffer A. The concentration of Fom3 was determined on the basis of UV absorption at 280 nm using an extinction coefficient (ε_{280nm} = 81.5 mM^{-1} cm^{-1}) determined by Edelhoch's method.[3] UV-visible absorption spectrums of Fom3 were determined by using a Shimadzu UV-2450 spectrophotometer. Enzymes were stored at -80˚C until use.

Enzymatic reaction of Fom3

The reconstituted Fom3 (2 μM) was anaerobically incubated with 0.5 mM HEP-CMP (obtained described below), 4 mM SAM (Sigma-Aldrich) and 0.1 mM MeCbl in buffer A in the presence of 1 mM MV, 4 mM NADH, and 10 mM DTT at

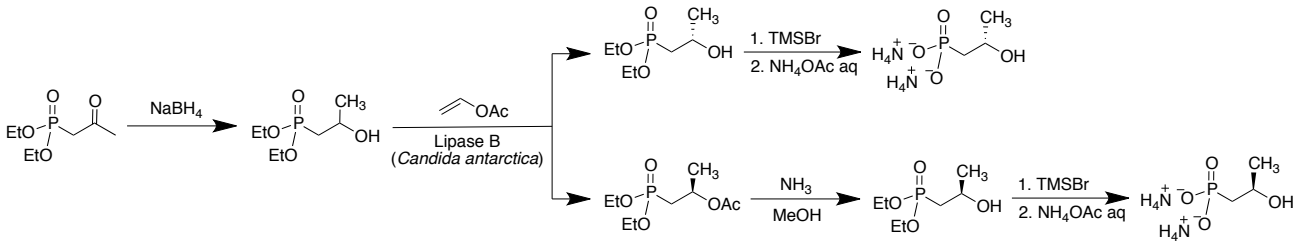
28˚C in a shaded tube for 48 h. A 10 µL of reaction solution was injected into a HPLC (Senshu SSC- 3215 degasser, Hitachi LaChrom ELITE pump L-2130, LaChrom ELITE Diode Array Detector L-2455, Senshu SSC-3215 column oven) equipped with an InertSustain® AQ-C18 column (5 µm, 4.6 × 250 mm, GL Sciences Inc.) equilibrated solvent A (20 mM phosphate buffer, pH 2.3). The elution was made with a flow rate of 1.0 mL/min at 40˚C using solvent A and solvent B (80% methanol, 20% 20 mM phosphate buffer, pH 2.3): 0% B for 15 min, 0-20% B for 15-30 min, 20% B for 30-35 min. Chromatography was monitored at 254 nm and 280 nm.

Isolation of the Fom3 reaction product

Two µM Fom3, 4 mM SAM, 0.1 mM MeCbl, 1 mM MV, 4 mM NADH, and 10 mM DTT were mixed with 0.5 mM of HEP-CMP in 250 µL of buffer A (total 4 tubes) and left at 28˚C for ≥ 60 h. This reaction was repeated a total of three times. A total 3 mL of reaction solution was loaded on a DOWEX AG1-X8 column ($HCOO^-$ form, 15 × 35 mm) and then eluted with a linear gradient from 0-1 M of ammonium formate (pH 7.5 adjusted by aqueous ammonia) to obtain a crude of HPP-CMP. The fractions containing HPP-CMP confirmed by HPLC using an InertSustain® AQ-C18 column (5 µm, 4.6 × 250 mm, GL Sciences Inc.) with an isocratic 20 mM phosphate buffer (pH 2.3) elution at 1.0 mL/min was lyophilized, resuspended in 200 µL of D_2O, and then its ^{1}H-NMR spectrum was recorded with a JEOL ECS-400 spectrometer. To purify HPP-CMP, a 10 µL of the crude solution was injected into a HPLC (Hitachi L-6250 Intelligent Pump, L-7300 Column Oven, Waters 996 Photodiode Array Detector) equipped with an InertSustain® AQ-C18 column (5 µm, 4.6 × 250 mm, GL Sciences Inc.) and eluted with an isocratic 20 mM phosphate buffer (pH 2.3) at a flow late of 1.0 mL/min at 40˚C. The peak of HPP-CMP was fractionated. This preparative HPLC was repeated a total of twenty times. The collecting solution was neutralized by 2 M NaOH and then lyophilized to obtain 10 mg of phosphate salt containing HPP-CMP. This white solid was suspended in D_2O and used for ^{1}H-NMR and LC-ESI-MS analysis.

Synthesis of (*S*)-HPP and (*R*)-HPP

(*S*)-2-Hydroxypropyl phosphonate ((*S*)-HPP) and (*R*)-2-hydroxypropyl phosphonate ((*R*)-HPP) were synthesized from diethyl (2-oxopropyl)phosphonate according to a published procedure,[4,5] as shown in the scheme below. The stereochemistry of the obtained diethyl 2-hydroxypropyl phosphonates was confirmed according to literature.[6,7]



NMR spectra for ammonium (*S*)-2-hydroxypropyl phosphonate: ^{1}H (400 MHz, D_2O) δ 1.13 (3H, d, J = 6.0 Hz, 3-H), 1.70 (2H, m, 1-H), 3.97 (1H, m); ^{13}C (100 MHz, D_2O) δ 23.13 (d, J = 7.2 Hz, 3-C), 37.57 (d, J = 130 Hz, 1-C), 64.43 (s, 2-C); ^{31}P (D_2O) δ 22.05 (s)

NMR spectra for ammonium (*R*)-2-hydroxypropyl phosphonate: ^{1}H (400 MHz, D_2O) δ 1.13 (3H, d, *J* = 6.5 Hz, 3-H), 1.71 (2H, m, 1-H), 3.97 (1H, m, 2-H); ^{13}C (100 MHz, D_2O) δ 23.24 (d, *J* = 8.3 Hz, 3-C), 37.57 (d, *J* = 130 Hz, 1-C), 64.42 (s, 2-C); ^{31}P (D_2O) δ 22.05 (s)

Expression and purification of Fom1 CyTase domain

The pHis8_NtFom1CyTase plasmid[8] was introduced into *E. coli* BL21(DE3) in the presence of 30 μg/mL of kanamycin. *E. coli* BL21(DE3) carrying pHis8_NtFom1CyTase was grown in 500 mL baffled flasks of LB medium (200 mL) containing 30 μg/mL of kanamycin at 37 °C until OD_{590} 0.6 with 200 rpm agitation. The expression was induced by addition of 0.2 mM IPTG. The cultivation was continued at 15 °C for 20−24 h with 200 rpm agitation. The cells were harvested by centrifugation at 6000 rpm for 10 min, washed with buffer B (50 mM HEPES−NaOH, pH 8.0), and stored at −30°C until use. The wet cells were suspended in buffer B. The suspension of cells on ice bath was disrupted by sonication (QSONICA) for 5 s with a 5 s interval (total 2 min). Cell debris was removed by centrifugation at 10 000 rpm for 5 min at 4 °C. The supernatant was loaded onto a Ni super flow resin (Clontech) column (15 × 25 mm), which had been previously equilibrated with buffer B. The column was washed with 80 mL of buffer B plus 5 mM imidazole to remove unbound proteins. Fom1 CyTase domain was eluted using buffer B plus 200 mM imidazole. The protein solution was collected and desalted with a PD-10 desalting column (GE Healthcare) equilibrated with buffer B. The obtained Fom1(N) was concentrated with an Amicon Ultra-15 mL device (Ultracel-3K, Millipore, 3000 MWCO) at 4500 ×g at 4 °C. The concentration of Fom1 CyTase domain was determined on the basis of UV absorption at 280 nm using an extinction coefficient (ε_{280nm} = 17.8 mM^{-1} cm^{-1}) determined by Edelhoch's method.[3] Enzymes were stored at -80˚C until use.

Enzymatic synthesis of HEP-CMP and (*R*)-HPP-CMP

Ten μM Fom1 CyTase domain, 3 mM CTP (Sigma-Aldrich) and 1 mM $MgCl_2$ were mixed with 1 mM of HEP or (*R*)-HPP in 24 × 500 μL of buffer B and left at 28˚C for 4 h. The reaction solution was loaded on a DOWEX AG1-X8 column ($HCOO^-$ form, 15 × 40 mm) and then eluted with a linear gradient from 0-1 M of ammonium formate (pH 7.5 adjusted by aqueous ammonia). The fractions containing cytidylylated compound confirmed by UV-visible absorption at 280 nm was lyophilized, resuspended in 500 μL of H_2O, and then desalted with a Sephadex™ G-10 column (10 × 880 mm). Pure HEP-CMP or (*R*)-HPP-CMP was lyophilized to obtain as ammonium salt. The NMR spectra were shown in **Table S1** and **Figure S11-14**.

Enzymatic synthesis of (*S*)-HPP-CMP

Forty three μM Fom1(N), 3 mM CTP and 1 mM $MgCl_2$ were mixed with 1 mM of (*S*)-HPP in 30 × 500 μL of buffer B and left at 28˚C for 24 h. The formed (*S*)-HPP-CMP was purified according to the purification method as mentioned above. The NMR spectra were shown in **Table S1** and **Figure S6-10**.

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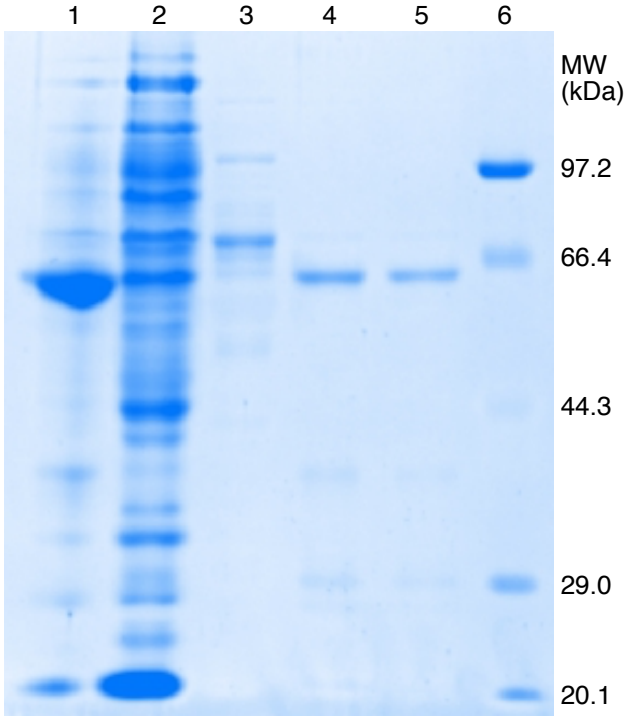
Table S1. Summary of NMR data for (*S*)-HPP-CMP, (*R*)-HPP-CMP, and HEP-CMP

	(*S*)-HPP-CMP	(*R*)-HPP-CMP	HEP-CMP
^{1}H-NMR (δ, multiplicity, *J* in Hz)			
HPP moiety			
H-1a	1.90 (m)	1.94 (m)	1.98 (dt, 18.7, 7.6)
H-2	4.05 (m)	4.09 (m)	3.72 (q, 7.6)
H-3	1.17 (d, 6.0)	1.21 (d, 6.0)	–
CMP moiety			
H-5	6.11 (d, 7.2)	6.19 (d, 7.6)	6.03 (d, 7.5)
H-6	7.99 (d, 7.2)	8.09 (d, 7.6)	7.88 (d, 7.5)
H-1′	5.84 (d, 3.2)	5.87 (d, 2.8)	5.87 (d, 4.0)
H-2′	4.21 (dd, 3.2, 5.2)	4.27 (m)	4.20 (dd, 4.0, 5.0)
H-3′	4.23 (dd, 5.2, 5.2)	4.27 (m)	4.23 (dd, 5.0, 5.0)
H-4′	4.17 (m)	4.22 (m)	4.15 (m)
H-5′a	4.15 (m)	4.18 (m)	4.15 (m)
H-5′b	4.07 (m)	4.11 (m)	4.08 (m)
^{13}C-NMR (δ, multiplicity, *J* in Hz)			
HPP moiety			
C-1	37.7 (d, 131.5)	31.7 (d, 135.3)	31.5 (d, 133.5)
C-2	64.2	64.1	57.1
C-3	23.1 (d, 8.6)	23.0 (d, 8.3)	–
CMP moiety			
C-2	149.7	152.0	156.5
C-4	162.5	161.8	165.3
C-5	95.9	95.8	96.4
C-6	142.9	143.1	141.7
C-1′	89.5	89.5	89.3
C-2′	74.3	74.3	74.2
C-3′	69.1	69.1	69.2
C-4′	83.0 (d, 9.6)	83.0 (d, 9.2)	82.7 (d, 9.2)
C-5′	64.3 (d, 4.8)	64.2 (d, 5.2)	64.4 (d, 5.2)
^{31}P-NMR (δ, multiplicity)			
	15.1 (m)	15.1 (m)	14.6 (d, 25.3)
	-10.7 (m)	-10.7 (m)	-11.8 (d, 25.3)

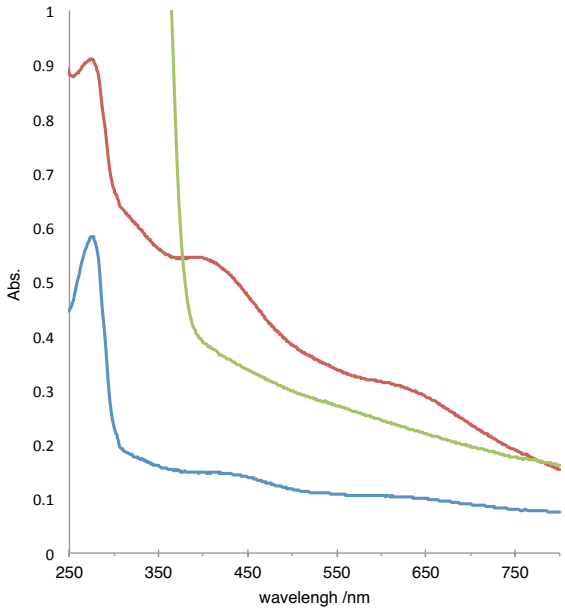
The ^{31}P chemical shifts are relative to phosphoric acid as external standard at 0 ppm.

Figure S1. SDS-PAGE of purified Fom3



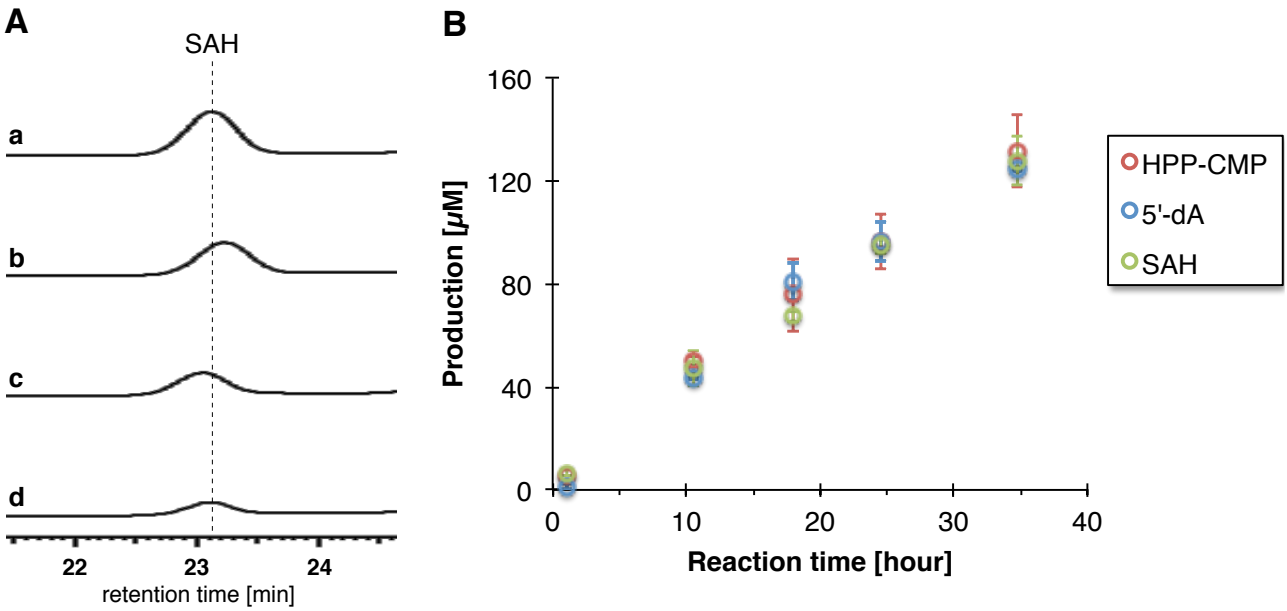
1: precipitate, 2: supernatant, 3: washed with 15 mM imidazole buffer, 4: purified Fom3 before reconstitution, 5: purified Fom3 after reconstitution, 6: low molecular weight marker. The calculated molecular weight of N-terminal hexahistidine tagged Fom3 is 62.5 kDa.

Figure S2. UV-vis spectra of purified Fom3



Blue: purified Fom3 before reconstitution, red: reconstituted Fom3, green: reconstituted Fom3 treated with 5 mM sodium dithionite.

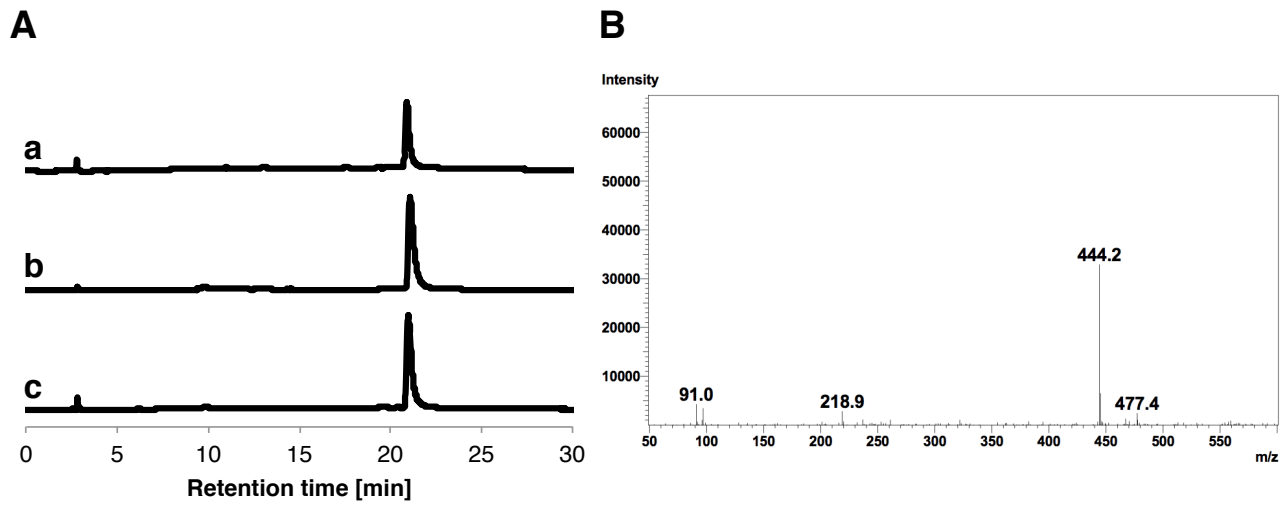
Figure S3. SAH formation upon the *C*-methylation catalyzed by Fom3



A, HPLC traces showing the production of SAH: (a) authentic SAH, (b) Fom3 reaction, (c) without Fom3, (d) without DTT.

B, Stoichiometry of HPP-CMP (red), 5′-dA (blue) and SAH (green). The standard curves were generated with the authentic cytidine monophosphate so as to correlate peak area with the amount of HPP-CMP. The standard curves were generated with the authentic 5′-dA so as to correlate peak area with the amount of 5′-dA and SAH. Control reaction without Fom3 were used to assess the quantity of non-enzymatically produced SAH and this amount was subtracted from the enzyme assay result.

Figure S4. LC-ESI-MS analysis of the Fom3 reaction product



A. LC charts monitored at 280 nm; (a) the isolated Fom3 reaction product, (b) authentic (*S*)-HPP-CMP, (c) authentic (*R*)-HPP-CMP. **B.** MS spectrum of peaks at 21 min in the negative mode. A 5 μL of the aqueous solutions were injected into an LC-ESI-MS instrument (Shimadzu LCMS-2010EV mass spectrometer equipped with LC-20AD pump, CTO-20A column oven, and SPD-M20AUV detector). A TKS-GEL Amide-80 column (3 μm, 2.10 × 150 mm, TOSOH) pre-equilibrated in 90% CH_3CN and 10% H_2O containing 10 mM of $HCOONH_4$ (pH 3.2) was used for separation. Elution was performed with a linear gradient from 10-50% H_2O containing 10 mM of $HCOONH_4$ (pH 3.2) over 30 min at a flow rate of 0.2 mL/min at 40 °C.

Figure S5. ^{1}H-^{1}H COSY (500 MHz, D_2O) of the Fom3 reaction product from HEP-CMP

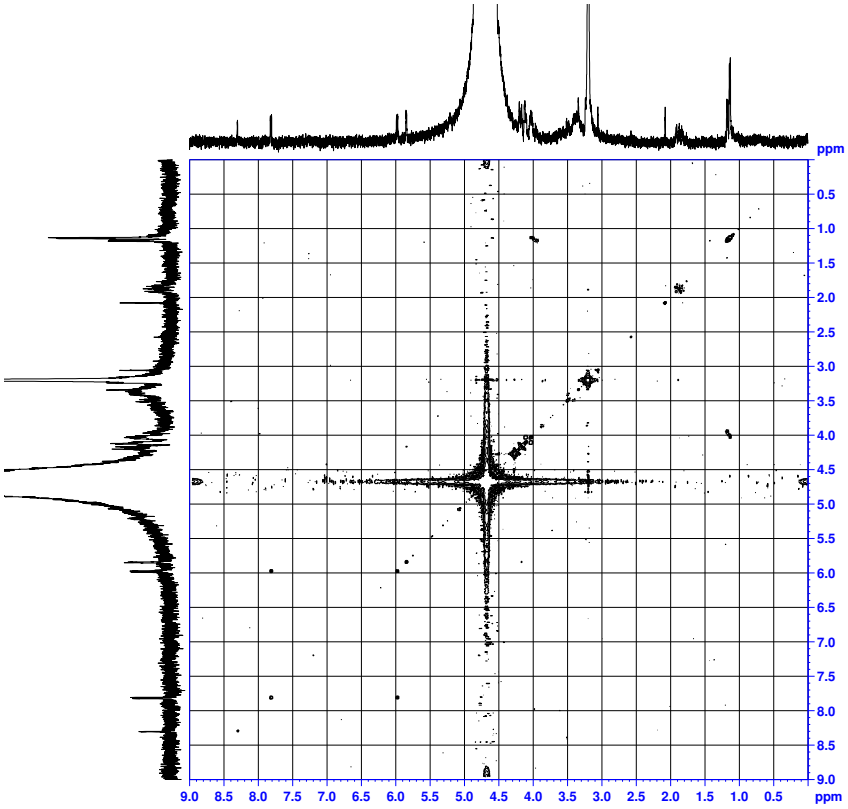


Figure S6. ^{1}H-NMR (400 MHz, D_2O) of (*S*)-HPP-CMP

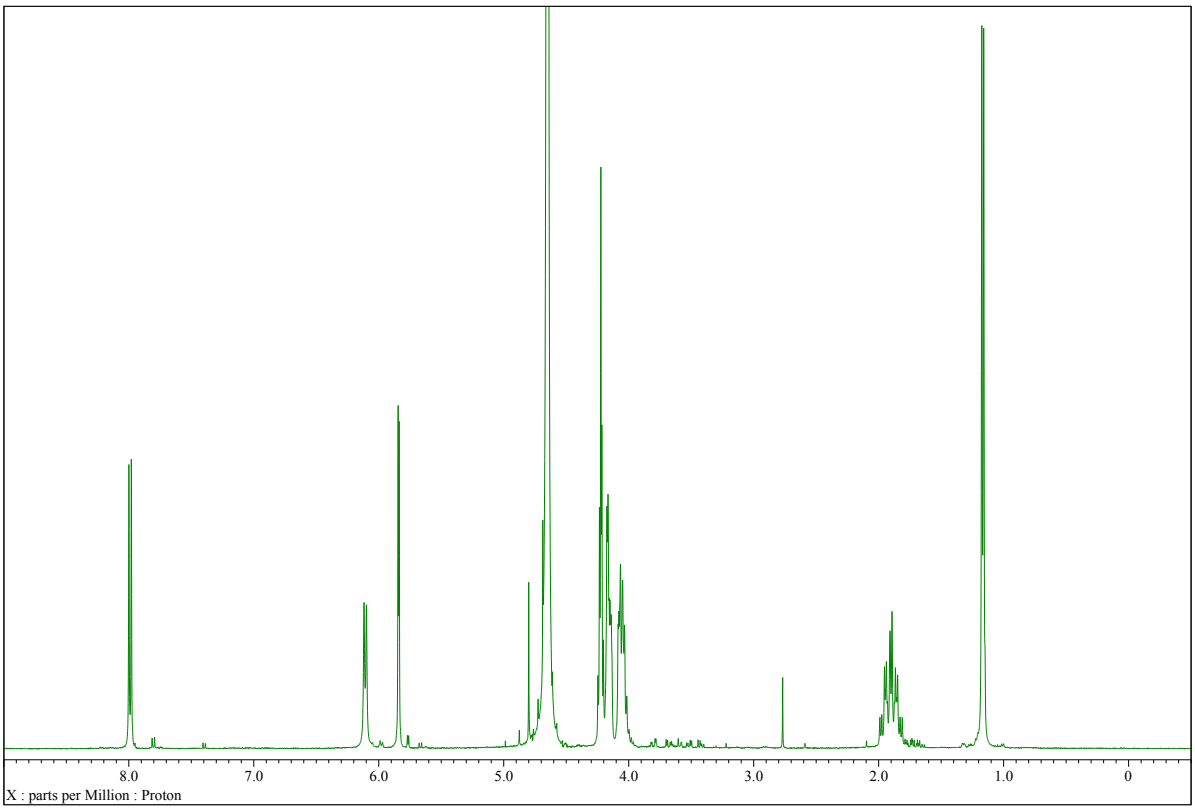


Figure S7. ^{13}C-NMR (100 MHz, D_2O) of (*S*)-HPP-CMP

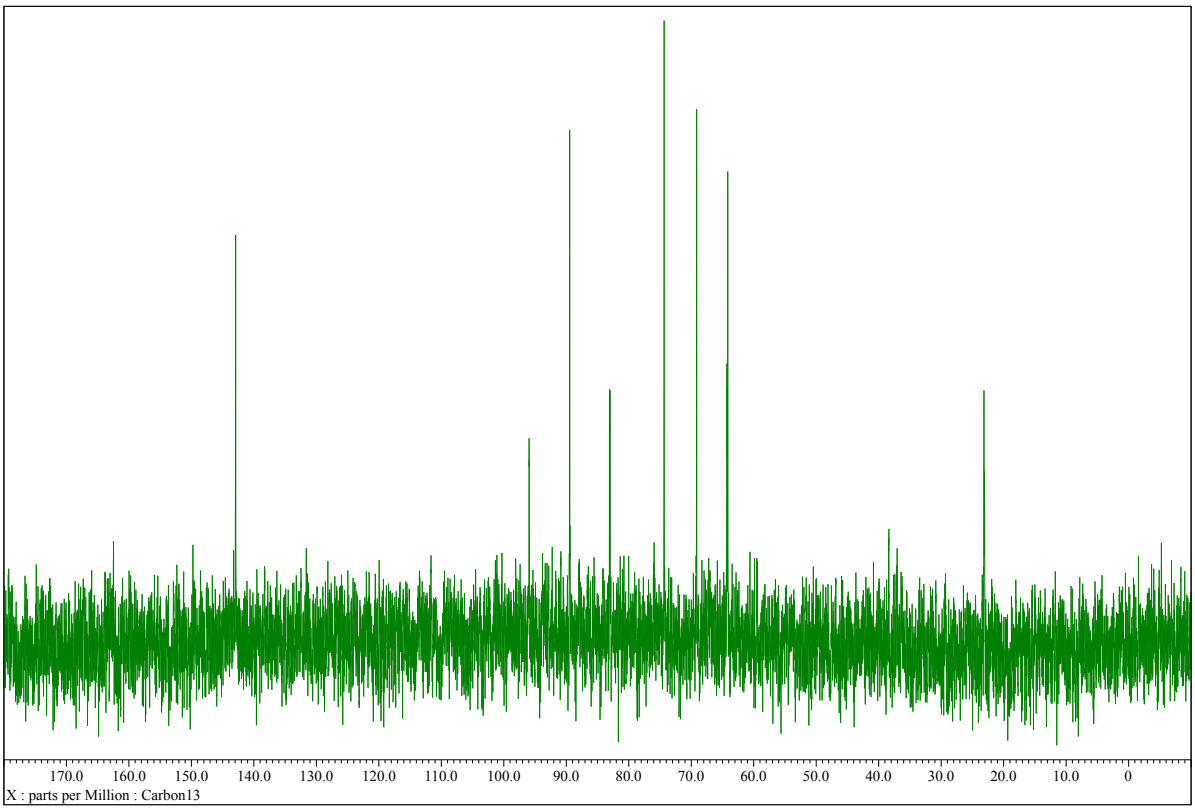


Figure S8. ^{31}P-NMR (161 MHz, D_2O) of (*S*)-HPP-CMP

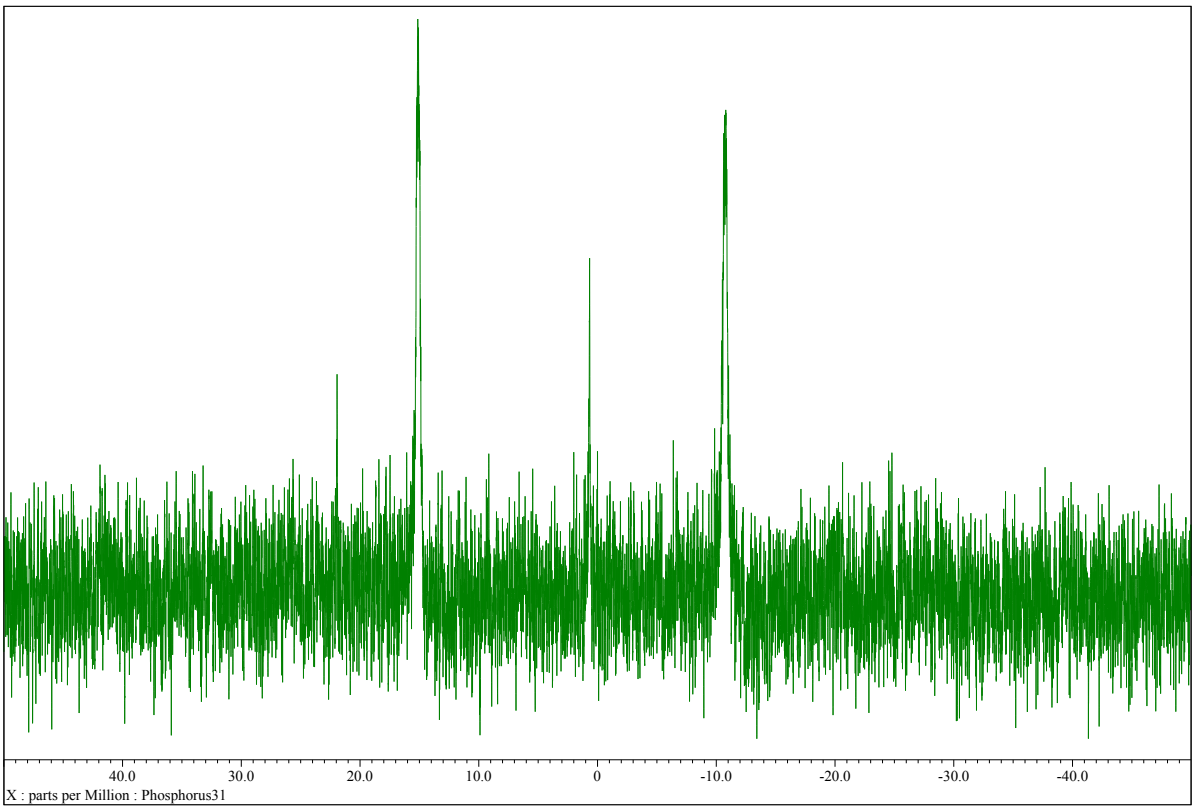


Figure S9. ^{1}H-^{1}H COSY (400 MHz, D_2O) of (*S*)-HPP-CMP

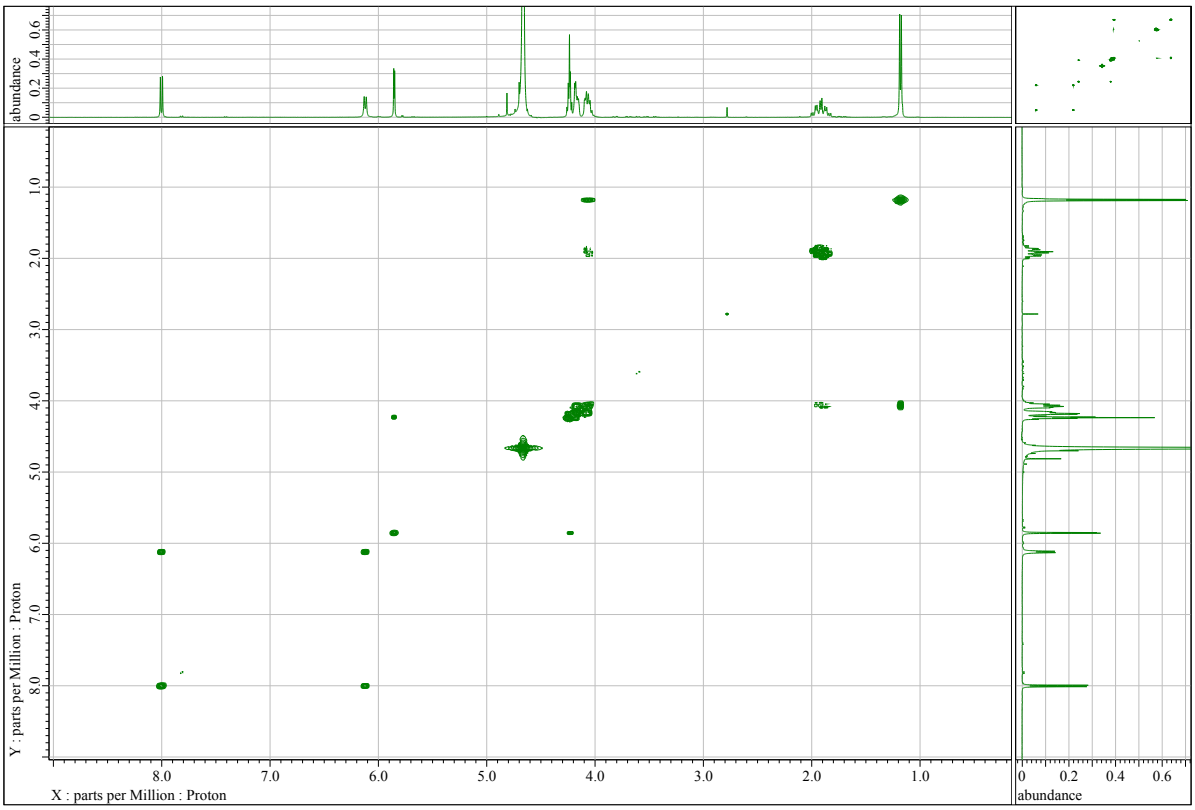


Figure S10. HMQC (400 MHz, D_2O) of (*S*)-HPP-CMP

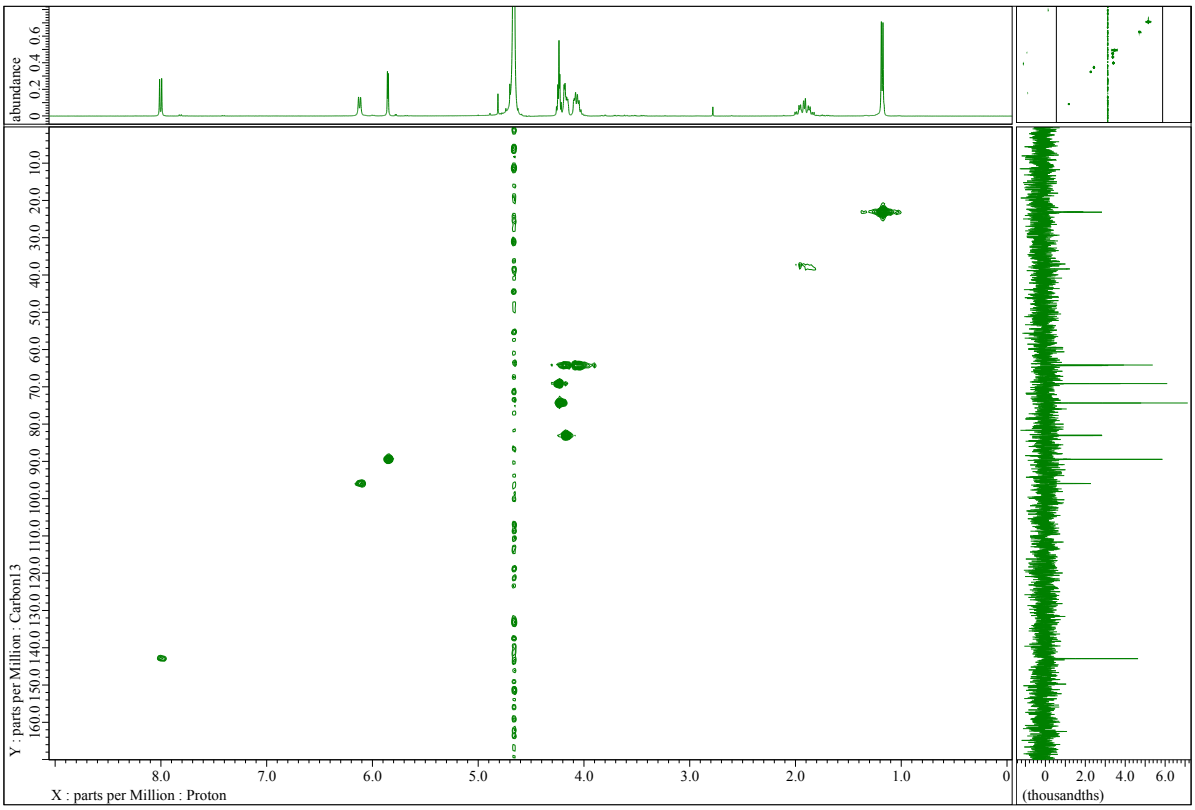


Figure S11. ^{1}H-NMR (400 MHz, D_2O) of (*R*)-HPP-CMP

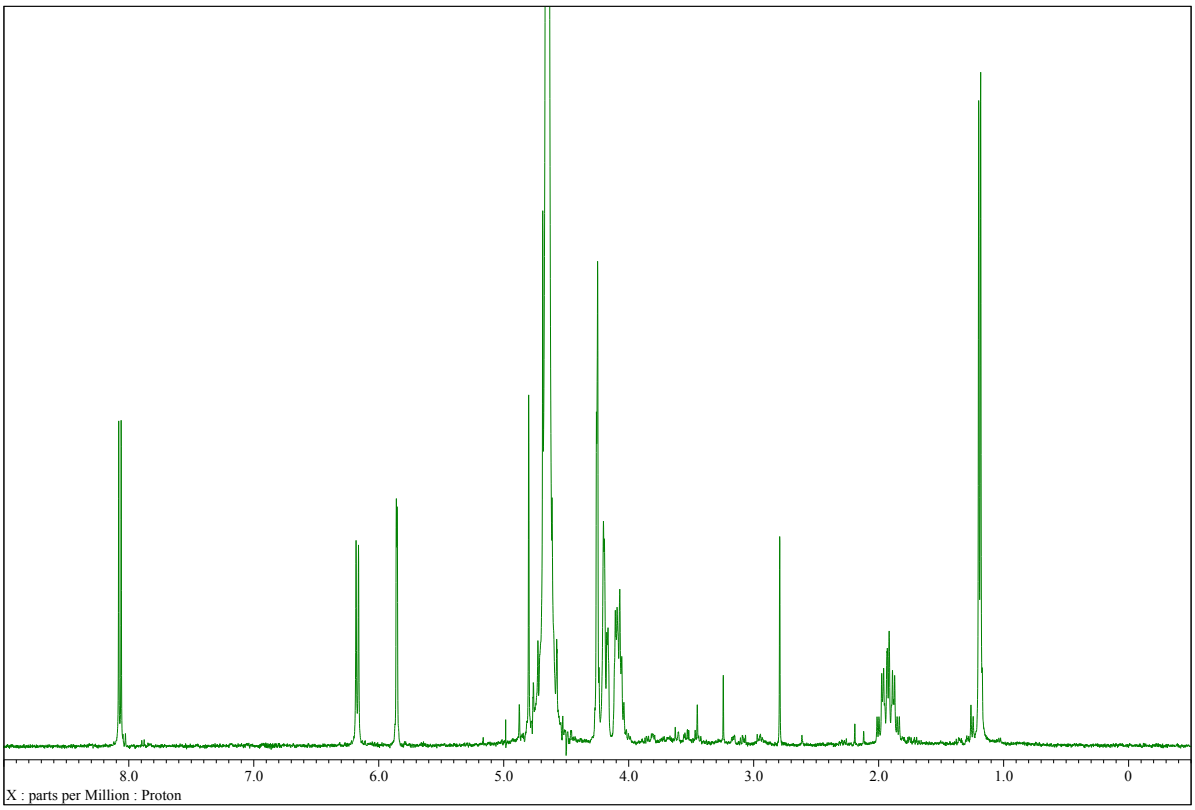


Figure S12. ^{13}C-NMR (125 MHz, D_2O) of (*R*)-HPP-CMP

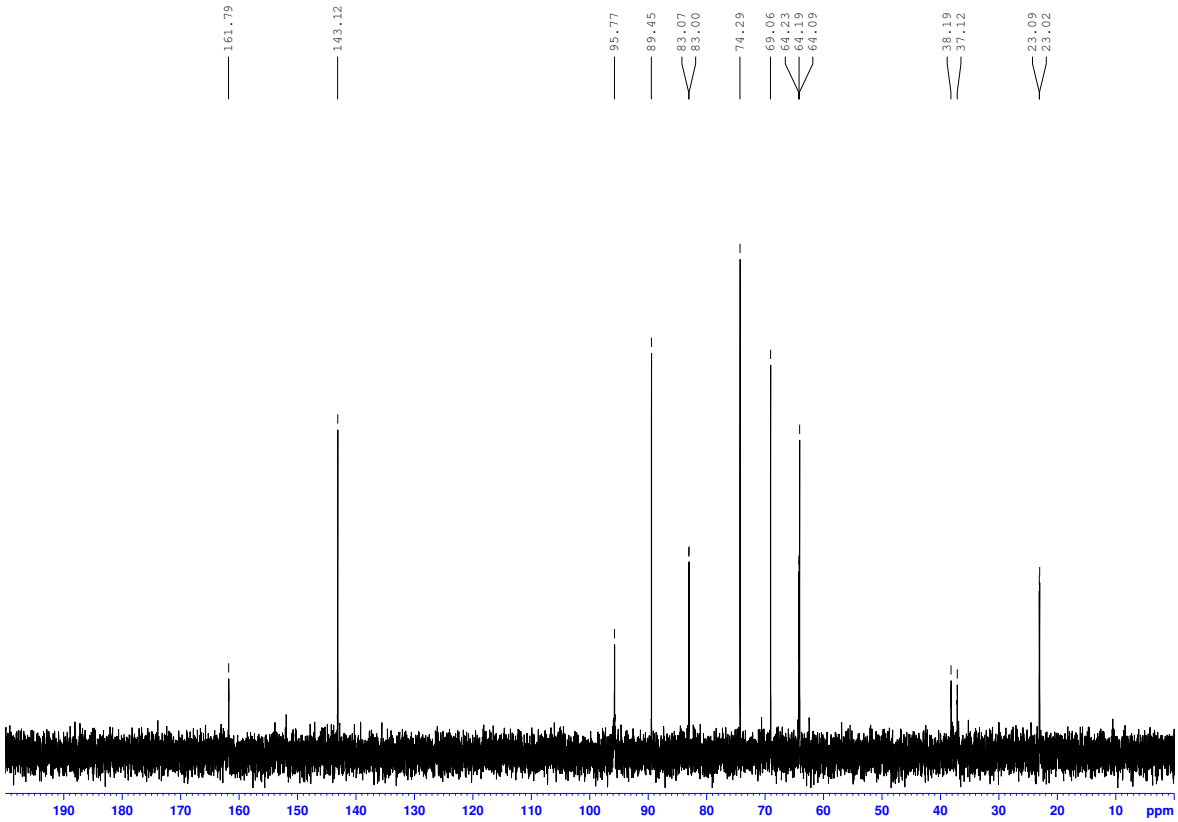


Figure S13. ^{31}P-NMR (161 MHz, D_2O) of (*R*)-HPP-CMP

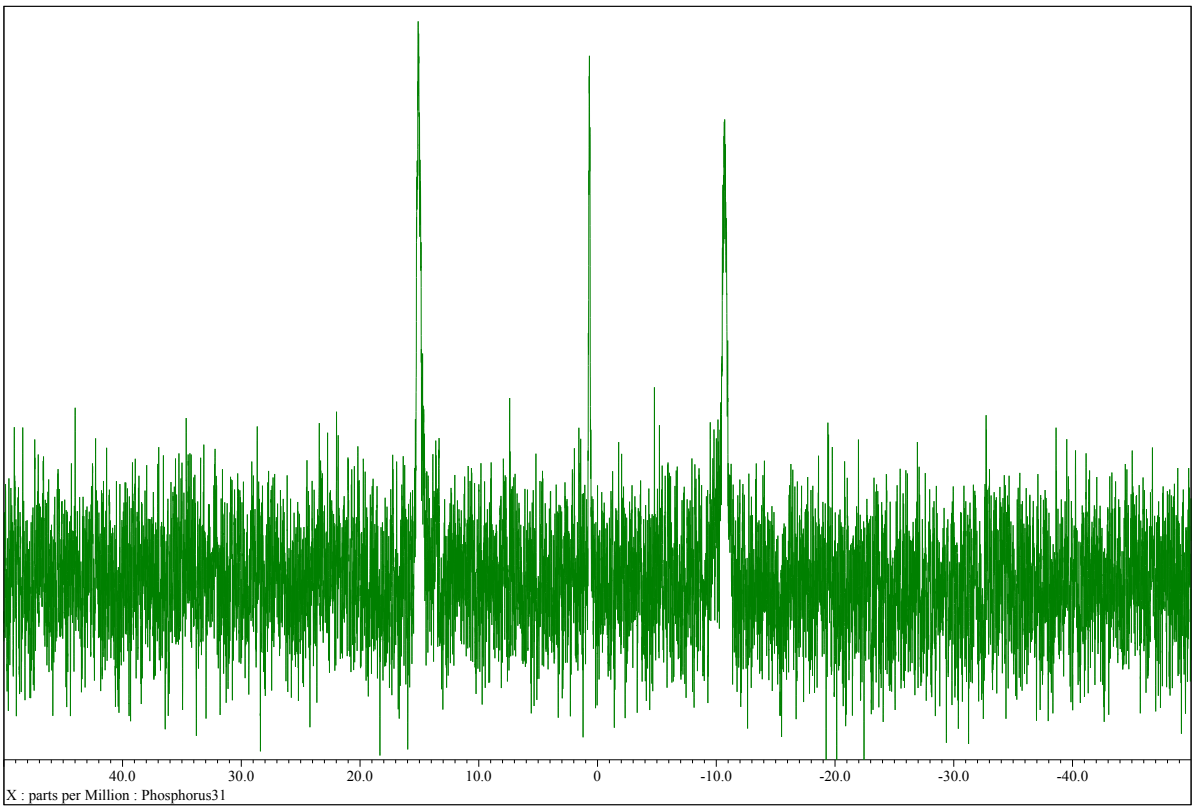


Figure S14. ^{1}H-^{1}H COSY (400 MHz, D_2O) of (*R*)-HPP-CMP

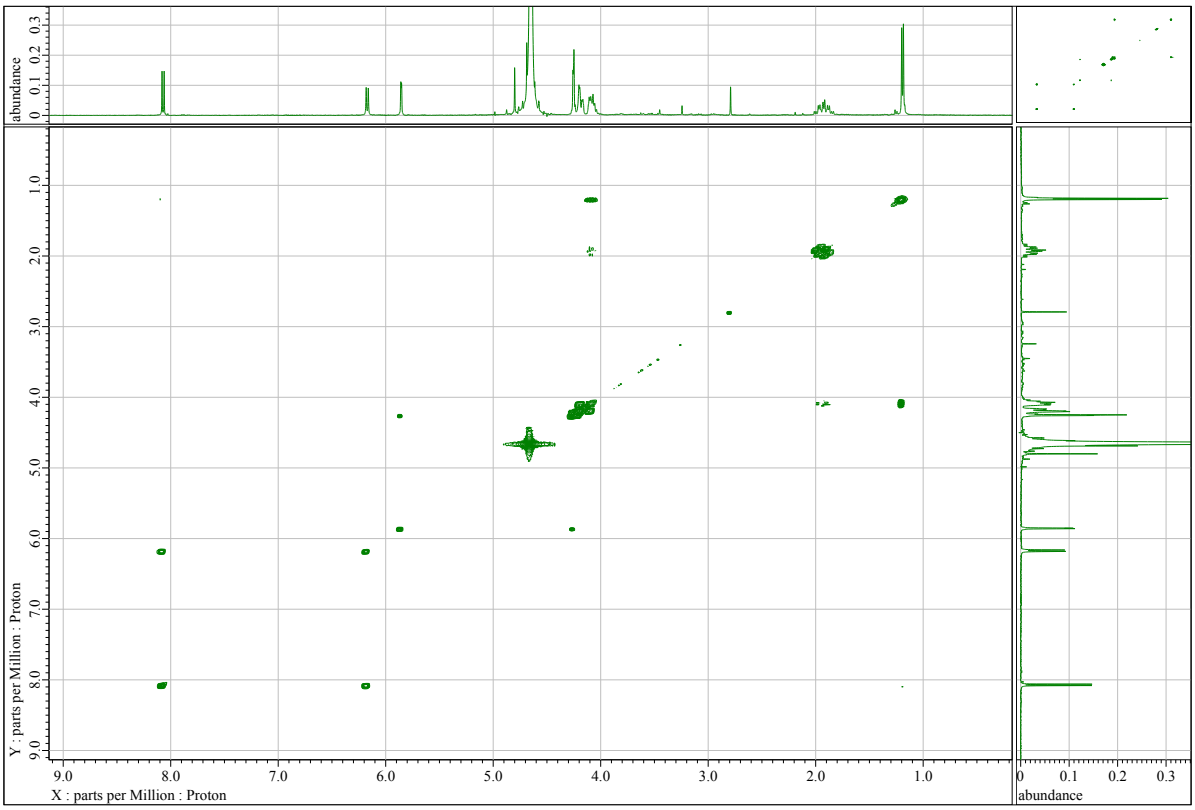
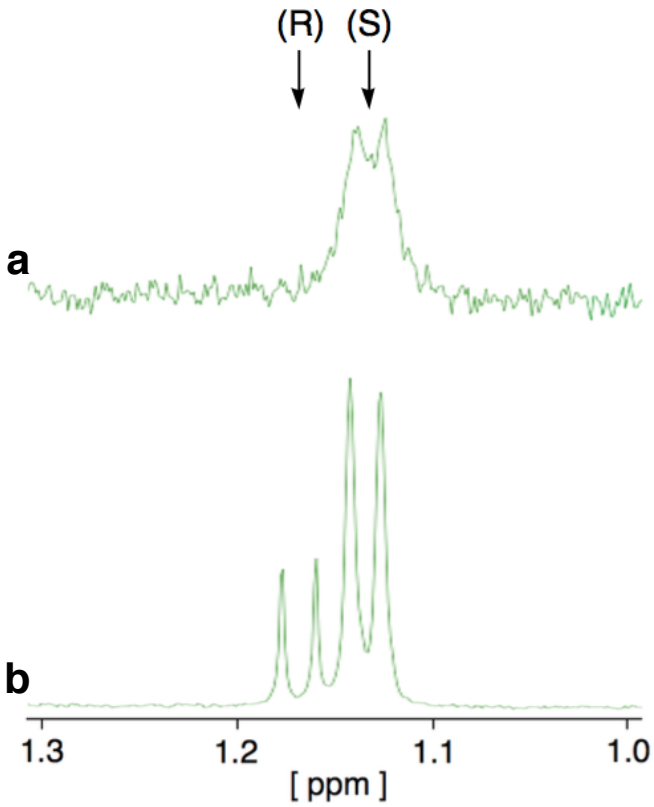
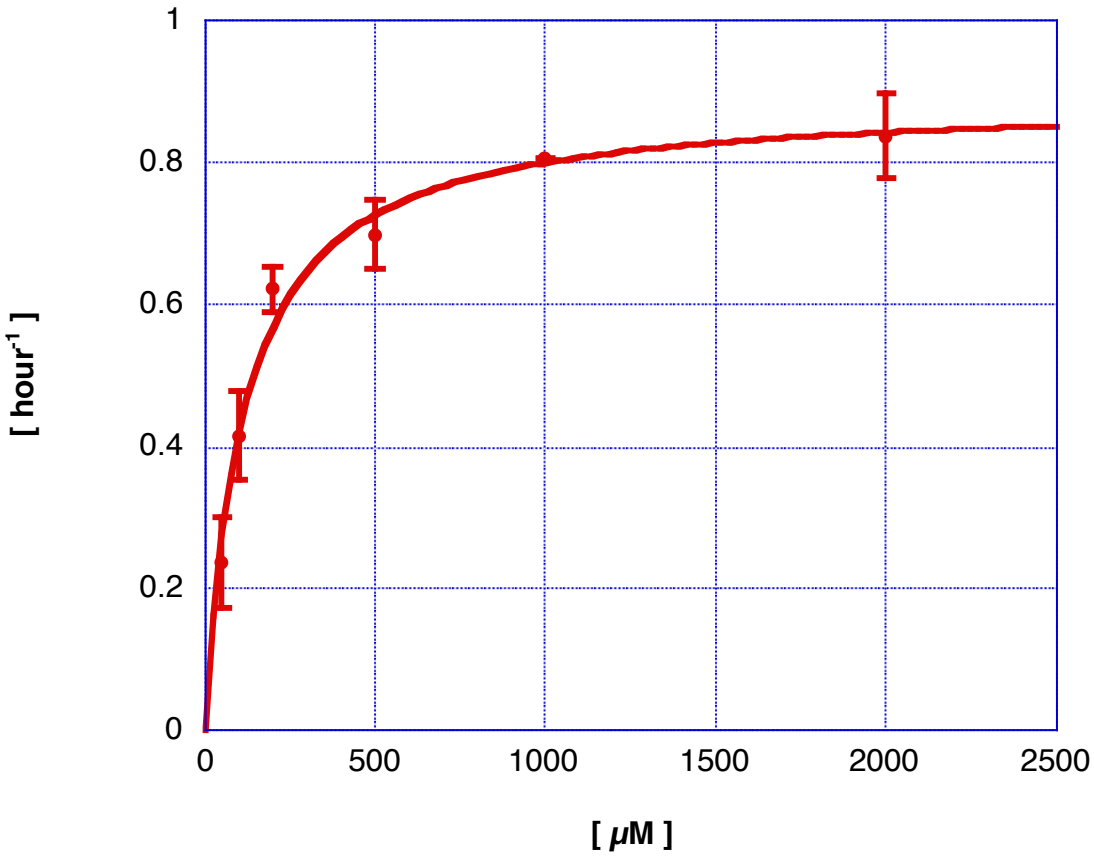


Figure S15. ^{1}H-NMR analysis (400 MHz, D_2O) of the Fom3 reaction with (*S*)-HPP-CMP



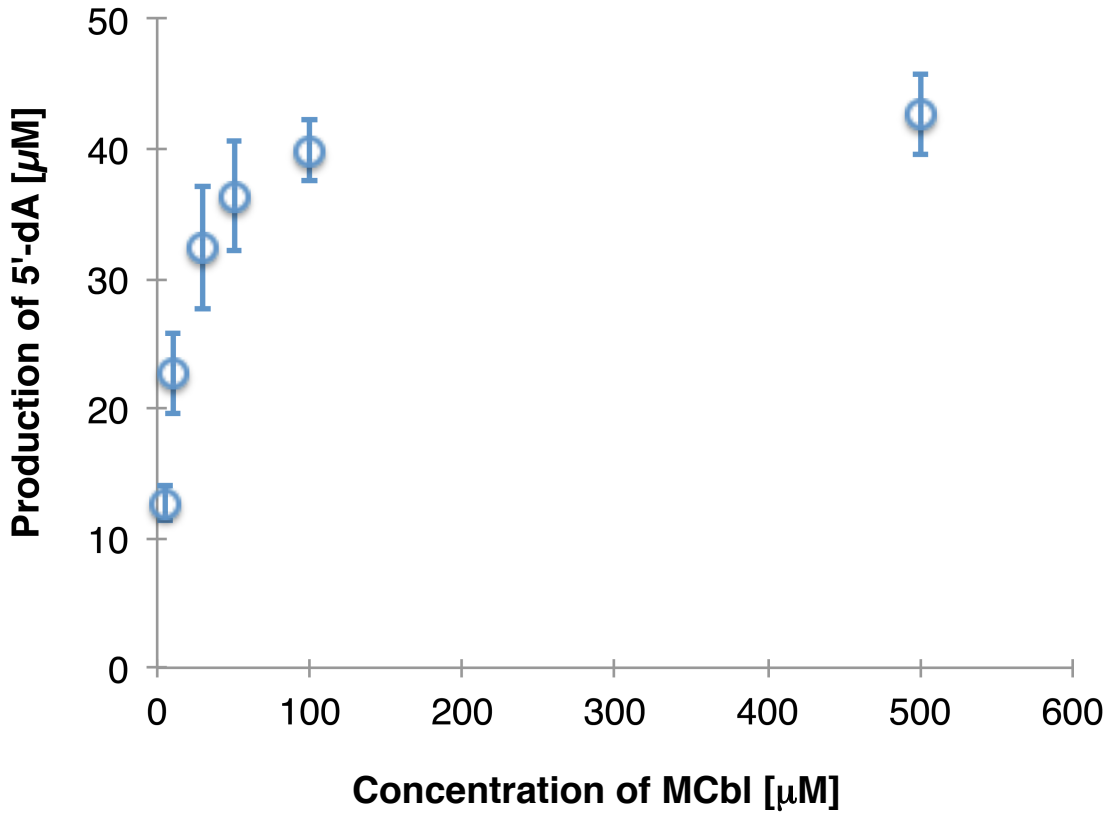
(a) An enzymatic reaction solution incubated in the presence of 4 mM (*S*)-HPP-CMP, 0.1 mM MeCbl, 4 mM SAM, 1 mM MV, 4 mM NADH, and 10 mM DTT with 2 μM Fom3 in a shaded tube at 28˚C for 48 hours, and (b) a 2:1 mixture of authentic (*S*)- and (*R*)-HPP-CMP. The enzymatic activity of Fom3 in this experiment was separately confirmed by HPLC analysis of the HPP-CMP production from HEP-CMP and also the 5′-dA production from SAM.

Figure S16. Kinetic analysis of the Fom3 reaction by detecting 5′-dA



Each point represents an average of two replicates, and error bars are the standard deviations among the replicates. Solid lines represent non-linear fits to the Michaelis-Menten equation using KaleidaGraph software (Synergy Software, Reading, PA). 50, 100, 200, 500, 1000 and 2000 μM of HEP-CMP was incubated in the presence of 4 mM SAM, 0.1 mM MeCbl, 1 mM MV, 4 mM NADH, and 10 mM DTT with 2 μM Fom3 in a shaded tube at 28˚C. The K_M value for HEP-CMP was estimated to be 113 ± 15 μM. The k_{cat} value was estimated to be 0.89 ± 0.03 hour^{-1}.

Figure S17. MeCbl concentration dependency for the Fom3 reaction.



Each point represents an average of two replicates, and error bars are the standard deviations among the replicates. 5, 10, 30, 50, 100 and 500 μM of MeCbl was incubated in the presence of 4 mM SAM, 0.5 mM HEP-CMP, 1 mM MV, 4 mM NADH, and 10 mM DTT with 2 μM Fom3 in a shaded tube at 28˚C for 18 hours.