Supporting Information

Stereospecific Radical-mediated B_{12}-dependent Methyl Transfer by the Fosfomycin Biosynthesis Enzyme Fom3

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Contents

Materials and Methods

Materials. DNA primers were purchased from Integrated DNA Technologies (Coralville, IA). Cloning materials were obtained from New England Biolabs (Ipswich, MA). The plasmids pBAD1030A and pSUMO, and the *E. coli* SAM synthetase overproducing strain DM22pK8 were kind gifts of Prof. J. E. Cronan, Prof. C. Cameron, and Prof. V. Bandarian, respectively. Ethanolamine was obtained from Acros Organics via Thermo Fisher Scientific (Waltham, MA). Hydroxocobalamin hydrochloride (HOCbl), fosfomycin disodium salt, adenosine triphosphate disodium salt (ATP), cytidine triphosphate disodium salt (CTP), chloramphenicol (Cam), *E. coli* inorganic pyrophosphatase (PP_iase), 2-mercaptoethanol (βME), sodium borohydride ($NaBH_4$), and methyl-d_3 iodide (CD_3I) were obtained from Sigma-Aldrich (St. Louis, MO). Methylcobalamin (MeCbl) was purchased from Alfa Aesar (Ward Hill, MA). Isopropyl β-d-thiogalactopyranoside (IPTG), ampicillin sodium salt (Amp), kanamycin sulfate salt (Kan), and lysozyme were purchased from Gold Biotechnology (St. Louis, MO). Phenylmethanesulfonyl fluoride (PMSF) was obtained from Fluka Chemical Corp (Ronkonkoma, NY), and DL-dithiothreitol (DTT) was from Promega (Madison, WI); N-(2-hydroxyethyl)piperazine-N′-ethanesulfonic acid (HEPES), tris(hydroxymethyl)aminomethane (Tris), and Bradford reagent ("Coomassie Plus Protein Assay Reagent") were from Thermo Fisher Scientific (Waltham, MA). Tryptone was purchased from Dot Scientific (Burton, MI), yeast extract from IBI Scientific (Dubuque, IA), and sodium chloride (NaCl) from Santa Cruz Biotechnology (Dallas, TX). All other chemicals were reagent grade or higher.

Manipulation of oxygen- and light-sensitive materials. Handling of Fom3 and SUMO-Fom3 and synthesis of [methyl-$^2H_3$]methylcobalamin (CD_3Cbl) were performed in a Coy vinyl anaerobic chamber with an atmosphere of 96% N_2/4% H_2 in which the oxygen level was kept below 5 ppm. SUMO-Fom3

and methylcobalamin were handled under red LED light (619-628 nm, Sunshine Lighting) except for brief periods of white light illumination used for visual identification of SUMO-Fom3 during the chromatographic steps.

Generation of *E. coli* B_{12} uptake system expression constructs. *E. coli* DH5α genes *btuF* and *btuB* were amplified by PCR using Q5 polymerase and the following primers: *btuF*, btuFB G1/G4; *btuB*, btuFB G3/G6 (Table S1). The pBAD1030A vector[1] was amplified by PCR with complementary overhangs using the primer pair btuFB G2/G5. The *btuF* and *btuB* genes were inserted into the pBAD1030A backbone via three-piece Gibson assembly to form btuFB-pBAD1030A. Following successful assembly, the entire *btuFB* insert was amplified using the primers btu-pBAD G1 and btuFB-pBAD G6; simultaneously, the *btuCED E. coli* genomic fragment was amplified using the primers btuCED-pBAD G1 and btu-pBAD G2, and the pBAD1030A backbone was amplified using the primers btuCED-pBAD G2 and btuFB-pBAD G5. The *btuCED* and *btuFB* fragments were inserted into the pBAD1030A backbone via three-piece Gibson assembly, yielding a plasmid with the btuCEDFB genes from DH5α. Naturally occurring point mutations in DH5α encoding for BtuD M88T/Y99H and BtuB Q58* were removed by three-piece Gibson assembly using the btu stitch G1-G6 primers (Table S1), and the intergenic regions were modified to match the published construct pBAD42-btuCEDFB[2] (Figure S3) by four-piece Gibson assembly using the primers btu-pBAD2 G1-G8. Finally, the vector's *bla* ampicillin resistance gene was replaced with the *cat* chloramphenicol resistance gene from pACYCDuet (Novagen) by Gibson assembly using the primers btu-pBAD1030C-2 G1-G4, resulting in btu-pBAD1030C-2, the construct used for expression in this study.

Generation of Fom3 and SUMO-Fom3 expression constructs. An *E. coli* codon-optimized gene encoding *S. wedmorensis* Fom3 was synthesized by overlap extension PCR: Forty oligonucleotides of alternating directionality, each one 60 nucleotides long and overlapping its neighbors on either side by

20 nucleotides, were mixed at 50 nM each and incubated in a thermocycler for amplification. The thermocycler protocol included an initial denaturing step of 30 s at 98 °C; 40 cycles of 10 s at 98 °C, 30 s at 50 °C, and 1 min at 72 °C; and a final elongation step of 8 min at 72 °C. Forward and reverse outer primers incorporating a 5′ NdeI site and a 3′ NotI site were then added, and the full-length codon-optimized gene (coFom3) was amplified using the same thermocycler protocol with 20 amplification cycles and an annealing temperature of 55 °C. The codon-optimized gene was ligated into pET28a after digestion with NdeI and NotI, yielding a construct encoding N-terminally His_6-tagged Fom3. Subcloning into the pSUMO vector was performed by Gibson assembly using the coFom3-pSUMO G1/G4 primers for amplification of the insert and the coFom3-pSUMO G2/G3 primers for amplification of the vector, resulting in a construct encoding Fom3 with an N-terminal SUMO tag (itself N-terminally His_6-tagged) and no linker between the C-terminal GG motif of SUMO and the initiator methionine of Fom3.

Generation of Fom1 cytidylyltransferase domain (CyT), FomD, and Fom4 expression constructs. Residues 1-132 of Fom1 (the CyT domain) were amplified from *S. wedmorensis* genomic DNA using the CyT-pET28a G1/G4 primers; the pET28a backbone was simultaneously amplified using the CyT-pET28a G2/G3 primers (Table S1). The CyT coding sequence was then inserted between the NdeI and HindIII restriction sites by Gibson assembly, resulting in a construct encoding CyT with an N-terminal thrombin-cleavable His_6 tag. The coding sequences of the *fomD* and *fom4* genes were amplified from *S. wedmorensis* genomic DNA and inserted into vectors encoding N-terminal His_6 tags; *fomD* was ligated into pET15b using the NdeI and XhoI restriction sites, and *fom4* was ligated into pET28a using the NdeI and HindIII sites.

Expression and purification of CyT. Expression and purification procedures were adapted from Eguchi *et al.*[3] and Kuzuyama *et al.*[4] CyT-pET28a was used to transform *E. coli* Rosetta 2 cells, and 20 mL of overnight starter culture was inoculated into each of 4 × 6-L flasks containing 2 L of LB + Kan

(50 μg/mL) + Cam (34 μg/mL). The cultures were shaken at 37 °C and 200 rpm. At an optical density (OD_{600}) of 0.6, protein expression was induced with 250 μM IPTG for 12-16 h at 22 °C. Cells were harvested at 10,000 × *g* and the pellet from each 2-L culture was resuspended in 50 mL of lysis buffer (50 mM Tris pH 8.0, 0-600 mM NaCl, 20 mM imidazole, 10% (v/v) glycerol; NaCl concentration did not dramatically affect yield) at ambient temperature supplemented with 0.2 mg/mL lysozyme and ½ of an EDTA-free protease inhibitor tablet (Roche cOmplete). Cells were lysed by sonication and centrifuged at 38,000 × g and 18 °C for 40 min. Clarified lysate was loaded onto a column containing 3 mL of His-Pur Ni-NTA Superflow resin (GE Healthcare) and washed with 40 mL of lysis buffer followed by 50 mL of wash buffer (lysis buffer with 40 mM imidazole). Protein was eluted with 13 mL of elution buffer (lysis buffer with 200 mM imidazole and 20 mM $MgCl_2$), concentrated to <2.5 mL, exchanged into storage buffer (25 mM Tris pH 8.0, 0-600 mM NaCl, 20 mM $MgCl_2$, 10% [v/v] glycerol) using a PD-10 column (GE Healthcare), concentrated to <1.5 mL, and stored in liquid nitrogen.

Expression and purification of FomD. FomD-pET15b was transformed into *E. coli* Rosetta 2 cells, and 20 mL of overnight culture was inoculated into 2 L of LB + Amp (100 μg/mL) + Cam shaken at 200 rpm and 37 °C. At an OD_{600} of 1.1, protein expression was induced with 200 μM IPTG for 20-24 h at 15 °C and 80 rpm. Cells were harvested at 10,000 × *g* and resuspended in 50 mL of lysis buffer (25 mM Tris pH 8.0, 500 mM NaCl, 20 mM imidazole, 10% [v/v] glycerol) containing 0.2 mg/mL lysozyme and ¼ of a Roche EDTA-free protease inhibitor tablet. Cells were lysed by sonication and centrifuged at 38,000 × g and 4 °C for 30 min. Clarified lysate was loaded onto a column containing 4 mL of HisPur Ni-NTA Superflow resin and washed with 50 mL of lysis buffer followed by 45 mL of wash buffer (lysis buffer with 40 mM imidazole). Protein was eluted with 12 mL of elution buffer (lysis buffer with 200 mM imidazole), concentrated to <2.5 mL, exchanged into storage buffer (25 mM Tris pH 8.0, 150 mM NaCl, 10% [v/v] glycerol) using a PD-10 column, concentrated to <1 mL, flash frozen, and stored at -80°C.

Expression and purification of Fom4. Expression and purification were adapted from published procedures for Psf4.[5] Fom4-pET28a was used to transform *E. coli* Rosetta 2 cells, and 20 mL of overnight culture was inoculated into 2 L of LB + Kan + Cam (concentrations see above) supplemented with 1 mM ferrous ammonium sulfate. The cultures were shaken at 200 rpm and 37 °C; at an OD_{600} of 0.6, protein expression was induced with 200 μM IPTG for 18 h at 18 °C and 200 rpm. Cells were harvested at 10,000 × *g* and resuspended in 60 mL of ice-cold lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10 mM imidazole, 10% [v/v] glycerol) containing 0.4 mg/mL lysozyme and 1 mM PMSF. Cells were lysed by sonication and centrifuged at 38,000 × g and 4 °C for 60 min; clarified lysate was loaded onto a column containing 3 mL of HisPur Ni-NTA Superflow resin equilibrated in lysis buffer. The column was washed with 50 mL of wash buffer (lysis buffer with 20 mM imidazole). Protein was eluted with 10 mL of elution buffer (lysis buffer with 250 mM imidazole), concentrated to <2.5 mL, exchanged into storage buffer (20 mM Tris pH 8.0, 20 mM NaCl, 10% [v/v] glycerol) using a PD-10 column, concentrated to <500 μL, flash frozen, and stored at -80 °C. Before addition to in vitro reactions, Fom4 was reconstituted with Fe(II) in an anaerobic chamber by mixing equal volumes of freshly thawed protein and an equimolar solution of $Fe(NH_4)_2(SO_4)_2$ in storage buffer. The mixture was placed on ice for 10-15 min before use.[6]

Expression of Fom3 without the *E. coli* B_{12} uptake system. Expression and purification procedures were adapted from the method of Eguchi.[3] coFom3-pET28a was used to transform *E. coli* BL21(DE3) cells harboring the plasmid suf-pACYCDuet (pPH151),[7] which contains the Fe-S cluster assembly genes of the *E. coli suf* operon under the T7-*lac* promoter. Overnight culture (4 × 20 mL) was inoculated into 4 × 3 L of LB + Kan + Cam incubating at 37 °C and 200 rpm. At an OD_{600} of 0.9-1.0, cultures were supplemented with 200 μM $Fe(NH_4)_2(SO_4)_2$ and 200 μM cysteine, and protein expression was induced with 200 μM IPTG for 30 h at 15 °C and 80 rpm. Cells were harvested at 10,000 × *g* and either stored in liquid nitrogen or used immediately for purification.

Purification of Fom3 expressed without the *E. coli* B_{12} uptake system. Cells were thawed if necessary and resuspended on ice in 75 mL of lysis buffer (50 mM HEPES pH 8.0, 300 mM NaCl, 15 mM imidazole, 10% [v/v] glycerol) containing 0.2 mg/mL lysozyme and ½ of a Roche EDTA-free protease inhibitor tablet. After stirring for 1 h, cells were lysed by sonication while stirring on ice using a QSonica Q55 sonicator at 70% amplitude for 10 × 1 min; cell debris was removed via aerobic centrifugation in tubes sealed with electrical tape at 38,000 × *g* at 0 °C for 30 min. Clarified lysate was loaded onto a 1.5-cm column containing 2.5 mL of cobalt TALON resin (Clontech), washed with 100 mL of ice-cold lysis buffer, and eluted with 8 mL of ice-cold elution buffer (lysis buffer with 200 mM imidazole). Protein was concentrated aerobically in a 30-kDa centrifugal filter (EMD Millipore) sealed with electrical tape and exchanged into storage buffer (lysis buffer without imidazole) using a PD-10 desalting column. The resulting pale green protein was supplemented with 5 mM DTT and incubated on ice for 30 min before reconstituting by slow addition of 250 μM $Fe(NH_4)_2(SO_4)_2$, 250 μM $FeCl_3$, and 500 μM Na_2S. After addition of sulfide, protein turned dark brown and was incubated on ice for 2 h. Excess iron and sulfide were removed using a PD-10 column equilibrated in storage buffer; the reconstituted protein was concentrated to <500 μL, flash frozen, and stored in liquid nitrogen.

Coexpression of SUMO-Fom3 with btu-pBAD1030C. Expression and purification procedures were adapted from the method of Booker.[2] coFom3-pSUMO was used to transform *E. coli* BL21(DE3) cells containing btu-pBAD1030C-2 and pDB1282, an Fe-S cluster assembly plasmid containing the genes of the *Azotobacter vinelandii isc* operon.[8] Overnight culture (4 × 40 mL) was inoculated into 4 × 4 L of M9-ethanolamine medium[9] + Kan + Amp + Cam (25 μg/mL) + 1.5 μM HOCbl in which the trace metals mix was replaced by the metal mix of Studier.[10] Cultures were shaken at 37 °C and 180 rpm. At OD_{600} = 0.3, expression of Fe-S assembly and B_{12} uptake genes was induced by addition of 0.2% (w/v) solid arabinose, and the medium was supplemented with 25 μM $FeCl_3$ and 150 μM cysteine. At OD_{600} = 0.6, expression of SUMO-Fom3 was induced with 250 μM IPTG, and the medium was supplemented

with an additional 25 μM $FeCl_3$ and 150 μM cysteine. After 18 h of expression at 18 °C and 180 rpm, cells were harvested at 10,000 × *g* and used immediately for purification.

Purification of SUMO-Fom3 coexpressed with btu-pBAD1030C. Freshly pelleted cells (36 g) were resuspended in 75 mL of ice-cold lysis buffer (50 mM HEPES pH 7.5, 300 mM KCl, 20 mM imidazole, 5% (v/v) glycerol, 10 mM βME) supplemented with 1 mM PMSF, 1 mg/mL lysozyme, 100 U/mL DNase I (EMD Millipore), and 200 μM HOCbl. Cells were lysed while stirring on ice using a QSonica Q55 sonicator at 70% amplitude for 8 × 1 min; cell debris was removed by aerobic centrifugation in tubes sealed with electrical tape at 35,000 × *g* and 4 °C for 45 min. Clarified lysate was loaded onto a 1.5-cm column containing 5 mL of HisPur Ni-NTA Superflow resin (Thermo Fisher), washed with 100 mL of ice-cold lysis buffer, and eluted with 10 mL of ice-cold elution buffer (lysis buffer with 300 mM imidazole and 20% (v/v) glycerol). Protein was concentrated aerobically in a sealed centrifugal filter device (EMD Millipore), exchanged into gel filtration buffer (50 mM HEPES pH 7.5, 300 mM KCl, 15% (v/v) glycerol, 5 mM DTT) using a PD-10 desalting column (GE Healthcare), and concentrated aerobically again to <500 μL. Further purification was performed by gel filtration on 100 mL of Superdex 200 prep grade resin (GE Healthcare) in a 1.6-cm jacketed column chilled with ice water. The column was equilibrated in gel filtration buffer and SUMO-Fom3 was chromatographed at 0.6 mL/min. Fractions containing SUMO-Fom3 were collected and analyzed by SDS-PAGE; fractions of acceptable purity were pooled and concentrated to 150 μL before immediate use in activity assays or storage in liquid nitrogen.

Determination of protein concentration. SUMO-Fom3 concentration was determined by Bradford assay using bovine serum albumin as a standard and applying the correction factor (1.18) determined for untagged Fom3 by Booker *et al.*[2] Concentrations of other proteins were estimated by A_{280} using extinction coefficients calculated by ExPASy (web.expasy.org/protparam).

Determination of SUMO-Fom3 cobalamin content. SUMO-Fom3 was diluted to 40 μM in 50 mM NaOH, mixed with an equal volume of 0.2 M KCN in 10 mM NaOH, and incubated at 95 °C for 30 min. The resulting dicyanocobalamin was quantified by UV spectrophotometry (ε_{367} = 30800 M^{-1} cm^{-1})[11] in comparison to a standard curve of HOCbl treated in the same manner. For quantification of HOCbl and MeCbl, SUMO-Fom3 was denatured by mixing with an equal volume of 6 M guanidine hydrochloride and then diluted in 1 mM ammonium acetate pH 4.2 to 36 μM, with 100 μM tryptophan added as an internal standard. SUMO-Fom3 samples and standard curves of HOCbl and MeCbl (1-50 μM with 100 μM tryptophan) were analyzed by LC-MS using an Agilent 1200 Series single-quadrupole LC/MSD SL system equipped with an electrospray ionization (ESI) source. Chromatography was performed on a Synergi Fusion-RP column (Phenomenex, 4.6 x 150 mm, 4 μm particle size) at a flow rate of 1 mL/min, using 1 mM ammonium acetate pH 4.2 and 50% (v/v) acetonitrile + 1 mM ammonium acetate pH 4.2 as solvents A and B, respectively. Compounds were separated by a 2-min isocratic hold of 0% B followed by a gradient of 0-95% B over 7 min; under these conditions, tryptophan, HOCbl, and MeCbl were retained for 7.4, 9.1, and 10.2 min, respectively. HOCbl was quantified by the area under the extracted ion chromatogram (EIC) for m/z 664.8 relative to the area under the m/z 205.1 EIC; MeCbl was quantified by the area under the m/z 672.8 EIC relative to the 205.1 EIC.

Synthesis of [methyl-$^2H_3$]-methylcobalamin (CD_3Cbl). HOCbl (110 mg, 80 μmol) was dissolved in 5.9 mL H_2O while stirring in a round-bottomed flask. $CoCl_2$ (100 μL of a 250 mM stock solution, 25 μmol, 0.3 equiv) was added, followed by dropwise addition of $NaBH_4$ (2.0 mL of a 1.06 M stock solution, 2.1 mmol, 25 equiv) until the solution turned dark purple to gray in color. The flask was then sealed with a septum and CD_3I (3.0 mL, 48 mmol, 600 equiv) was added via syringe, causing an immediate color change to red. All subsequent procedures were performed under red light. Excess $NaBH_4$ was quenched by addition of 10% (v/v) glacial acetic acid until bubbling stopped, followed by further addition of 100 μL 5 M HCl and excess 2 M ammonium acetate, pH 4, to neutralize alkali. Solvent was

removed by rotary evaporation, the resulting dark red solids were dissolved in 13 mL of 40 mM ammonium acetate, pH 4, and the pH was adjusted to 4-5 using sodium bicarbonate. CD_3Cbl was purified from the solution by semi-preparative HPLC using a Waters XBridge C18 column (10 × 250 mm, 5 μm) connected to a Shimadzu Prominence LC-20AP system with a diode array detector. Chromatography was performed at a flow rate of 7 mL/min using 1 mM acetic acid, adjusted to pH 4.0 with NH_4OH, as Solvent A and 50% acetonitrile + 1 mM acetic acid, pH 4.2 with NH_4OH, as Solvent B. Optimum separation was achieved using the following method: 10% B for 2 min; ramp to 59% B over 2 min; gradient from 59-62% B over 4 min; ramp to 100% B over 2 min. The column was washed in 100% B for 2.5 min and equilibrated in 10% B for 3 min before every 6-mL injection. Under these conditions, CD_3Cbl eluted at 9.0-9.3 min; fractions were collected by monitoring absorbance at 535 nm, pooled, lyophilized, and redissolved anaerobically in 20 mM HEPES pH 7.5 + 20% (v/v) DMSO. Concentration was determined by conversion to dicyanocobalamin (see method above). Purified CD_3Cbl was stored in liquid nitrogen.

Enzymatic synthesis of SAM and CD_3-SAM. Procedures were adapted from Bandarian *et al.*[12] *E. coli* strain DM22pK8[13] was grown to stationary phase at 37 °C and 180 rpm in 4 × 4 L of LB + tetracycline (10 μg/mL). Cells were harvested at 10,000 × *g*, resuspended in 50 mL of ice-cold TE buffer (100 mM Tris-HCl, 1 mM EDTA, pH 8.0), lysed by sonication, and centrifuged at 38,000 × *g* for 30 min. Lysate (60 mL) was filtered and dialyzed at 4 °C against 3 × 4 L of TE buffer for a total of 48 h; 30 mL of lysate was further dialyzed against 3 × 4 L of 50 mM sodium acetate/50 mM $NaHCO_3$, pH 8.0, before flash freezing and storage at -80 °C. A typical SAM synthetase reaction contained 50 mM sodium acetate, 50 mM $NaHCO_3$, 50 mM KCl, 26 mM $MgCl_2$, 10 mM L-methionine (or [methyl-$^2H_3$]methionine), 13 mM ATP, 20% (v/v) acetonitrile, 0.5 U/mL *E. coli* inorganic phosphatase (PP_iase), and 15% (v/v) of DM22pK8 lysate in a final volume of 100 mL. The reaction mixture was adjusted to pH 8.0 before addition of acetonitrile, PP_iase, and lysate; upon initiation, the reaction proceeded at 25-

28 °C for 5 h before quenching to pH 5.0 with glacial acetic acid, twofold dilution in H_2O, and filtration to remove precipitate. The filtrate was loaded onto 75 mL of CM-Sephadex C-25 resin (GE Healthcare) equilibrated in 2 mM sodium acetate, pH 5.0. After washing with 1 L of 2 mM sodium acetate, pH 5.0, SAM was eluted with 800 mL of 40 mM H_2SO_4. Fractions with significant absorbance at 260 nm were pooled, neutralized to pH 4-5 with saturated barium hydroxide, filtered to remove barium sulfate precipitate, concentrated by rotary evaporation, redissolved in 20% acetonitrile, and lyophilized overnight. The resulting white solid was dissolved in H_2O under anaerobic conditions; SAM was quantified by UV spectrophotometry (ε_{260} = 15,400 M^{-1} cm^{-1}), flash frozen, and stored in liquid nitrogen.

Enzymatic synthesis of 2-HEP-CMP and isotopologues. 2-Hydroxyethylphosphonate (2-HEP) and [2-$^2H_2$]2-HEP were synthesized from triethyl phosphonoacetate as described previously.[6] (*R*)- and (*S*)-[2-$^2H_1$]2-HEP were synthesized from 2-butyne-1,4-diol as described previously.[14, 15] In order to produce 2-HEP-CMP (and isotopologues thereof), 5 mM 2-HEP (3.8 mM for (*R*)-[2-$^2H_1$]2-HEP and 4.5 mM for (*S*)-[2-$^2H_1$]2-HEP) was reacted in a final volume of 2-25 mL with 20 μM CyT, 5 mM CTP, and 5 mM $CoCl_2$ in 50 mM Tris pH 8.0. After initiation with enzyme, the reaction was incubated at 30 °C for 1-2 days; an additional 20 μM CyT was added and the reaction was continued for another 1-2 days until no more conversion was visible by ^{31}P NMR spectroscopy. The reaction mixture was then digested with 2-4 U/mL of *E. coli* PP_iase at room temperature for 1-2 h, filtered to remove CyT precipitate, and loaded onto at least 1 reaction volume of AG1-X8 resin (Bio-Rad) equilibrated in 100 mM ammonium formate pH 7.5. The column was washed with 2 column volumes of 200 mM ammonium formate, pH 7.5, and 1 column volume each of [225, 250, 275… 550] mM ammonium formate, pH 7.5. Fractions of 0.4-2 mL were collected and characterized by ^{31}P NMR spectroscopy based on absorbance at 271 nm; those containing exclusively two doublet signals at 14 and -11 ppm were pooled, lyophilized, redissolved in H_2O, and chromatographed at 0.2-0.5 mL/min on a 1.5 × 170 cm column containing LH-20 resin (GE Healthcare) equilibrated in H_2O at 4 °C. Then 2-mL fractions were pooled based on absorb-

ance at 271 nm and lack thereof at <240 nm. Fractions were lyophilized and redissolved in D_2O, and bound metals were removed by agitation with Chelex-100 resin for 15-30 min; the resin was removed by filtration and the solution characterized by ^{1}H and ^{31}P NMR spectroscopy before lyophilizing and redissolving anaerobically. Concentration was measured by UV absorbance using the CTP extinction coefficient $\varepsilon_{271} = 9100\ M^{-1}\ cm^{-1}$; stock solutions were stored in liquid nitrogen.

Enzymatic synthesis of (2*R*)- and (2*S*)-2-HPP-CMP. (*R*)- and (*S*)-2-hydroxypropylphosphonate (2-HPP) were synthesized from dimethyl 2-oxopropylphosphonate as described previously.[16] Reactions contained 50 mM Tris pH 8.0, 5 mM $CoCl_2$, 5 mM CTP, 2 U/mL PP_iase, 3 mM (*R*)-2-HPP or 5 mM (*S*)-2-HPP, and 160 or 270 μM CyT for (*R*)- and (*S*)-2-HPP, respectively, in a final volume of 10 mL. After 30 h at 30 °C, an additional 2 U/mL PP_iase was added to both reactions, and another 160 μM of CyT was added to the (*S*)-2-HPP reaction. By 50 h, the (*R*)-2-HPP reaction was >90% complete; the (*S*)-2-HPP reaction was left for 5 days before no additional conversion was visible by ^{31}P NMR spectroscopy. (2*R*)- and (2*S*)-2-HPP-CMP were purified from reaction mixtures using the same procedure as for 2-HEP-CMP (see above) and stored at -80 °C.

SUMO-Fom3 activity assays. Unless otherwise specified, all reactions contained, in a final volume of 50 μL: 100 mM HEPES, pH 8.0; 20 μM SUMO-Fom3; 4 mM SAM; 4 mM NADH; 1 mM methyl viologen; 10 mM DTT; 1 mM 2-HEP-CMP. Reactions were assembled under red light in opaque tubes in an anaerobic chamber, initiated by addition of 2-HEP-CMP, and allowed to proceed for 8-12 h at ambient temperature (26-28 °C) before quenching with 200 μL of 0.3% (v/v) formic acid. After removal of protein by centrifugal concentration, reactions were further diluted tenfold in 0.1% (v/v) formic acid and analyzed by LC-MS/MS using an UltiMate 3000 HPLC system coupled to a Q Exactive Hybrid Quadrupole Orbitrap mass spectrometer (Thermo Scientific). Chromatography was performed on a Synergi Fusion-RP column (Phenomenex, 4.6 × 150 mm, 4 μm particle size) at a flow rate of 1 mL/min using

0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid in acetonitrile as solvents A and B, respectively. Separation was effected by isocratic flow of 0% B for 5 min, followed by a linear gradient of 0-95% B over 15 min; under these conditions, 2-HEP-CMP and 2-HPP-CMP eluted at 2.28 and 2.55 min, respectively. Full MS scans (m/z 50-1000) were collected in both positive and negative mode, and MS/MS product ion scans were collected for the most abundant precursor ions in each MS scan using a data-dependent method.

LC-MS analysis of MeCbl from SUMO-Fom3 reactions. Reactions contained 50 μM SUMO-Fom3, 50 μM 2-HEP-CMP, and 250 μM HOCbl, and were otherwise identical to SUMO-Fom3 activity assays described in the previous section. After 2 h of reaction at 26-28 °C, reactions were quenched in an equal volume of 6 M guanidine hydrochloride, diluted 1:6 in water, and analyzed by LC-MS using an Acquity UPLC system coupled to a Waters Synapt Q-TOF mass spectrometer. Chromatography was performed on a Jupiter C18 column (Phenomenex, 1 × 150 mm, 5 μm particle size) at a flow rate of 0.1 mL/min using 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid in acetonitrile as solvents A and B, respectively. The LC method consisted of isocratic flow of 5% B for 2 min, followed by a linear gradient of 5-95% B over 10 min; MeCbl eluted at approximately 6.5 min under these conditions. MS was performed with a capillary voltage of 2.35 kV, a sample cone voltage of 21 V, and an extraction cone voltage of 4 V; source and desolvation temperatures were 120 and 300 °C, respectively. Scans were collected in positive mode for m/z 50-2000 at intervals of 1 s.

Determination of 2-HPP-CMP stereochemistry. A 500-μL reaction containing 100 μM SUMO-Fom3, 4 mM SAM, 4 mM NADH, 1 mM methyl viologen, 10 mM DTT, and 1 mM 2-HEP-CMP in 100 mM HEPES (pH 8.0) was allowed to proceed at ambient temperature (26-28 °C) in an anaerobic chamber for 12 h. To this assay mixture, and to solutions of (2*R*)- and (2*S*)-2-HPP-CMP standards, was added FomD (1 μM) with $MgCl_2$ (1 mM); the reactions were incubated at 26-28 °C for 4 h, followed by addi-

tion of NADH (5 mM), FMN (2 mM), and Fom4 (100 μM) freshly reconstituted with 1 equivalent of ferrous ammonium sulfate.[6] Fom4 reactions were allowed to proceed overnight at 26-28 °C in a sealed plastic bag that was evacuated and refilled with O_2 (estimated O_2 content >80%). Protein was removed from the samples by centrifugal concentration and metals were removed by 15 min of agitation with Chelex-100 resin (sodium form, Sigma) before lyophilization and redissolving in D_2O for ^{31}P NMR spectroscopy. Samples were spiked with authentic standards of fosfomycin and 2-oxopropylphosphonate (2-OPP, synthesized as described previously[5]) to verify assignments.

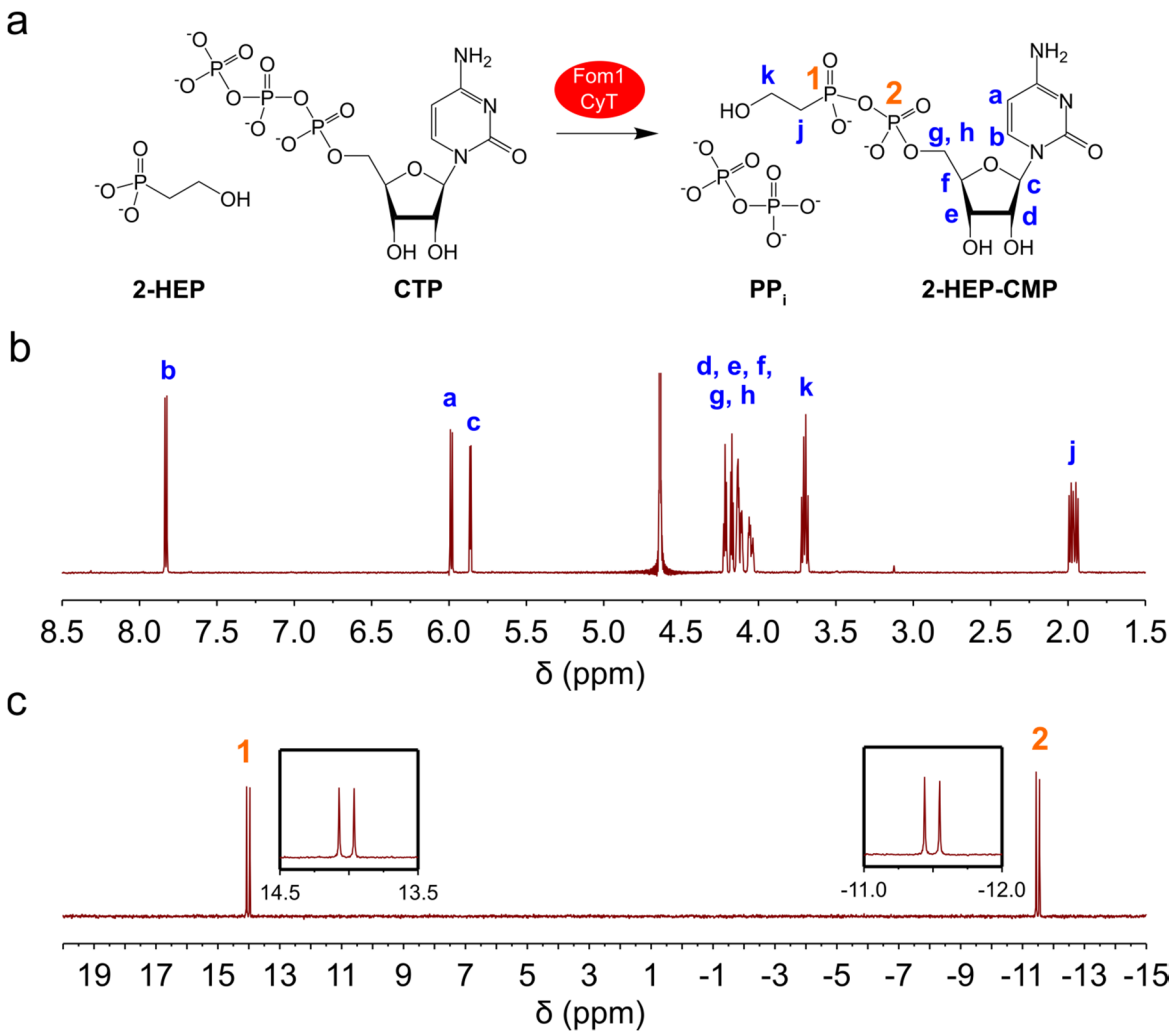


Figure S1. Enzymatic synthesis of 2-HEP-CMP. (a) The reaction catalyzed by the Fom1 cytidylyltransferase domain (CyT). (b) ^{1}H NMR spectrum (600 MHz, D_2O) of purified 2-HEP-CMP. (c) ^{31}P NMR spectrum (600 MHz, D_2O) of purified 2-HEP-CMP.

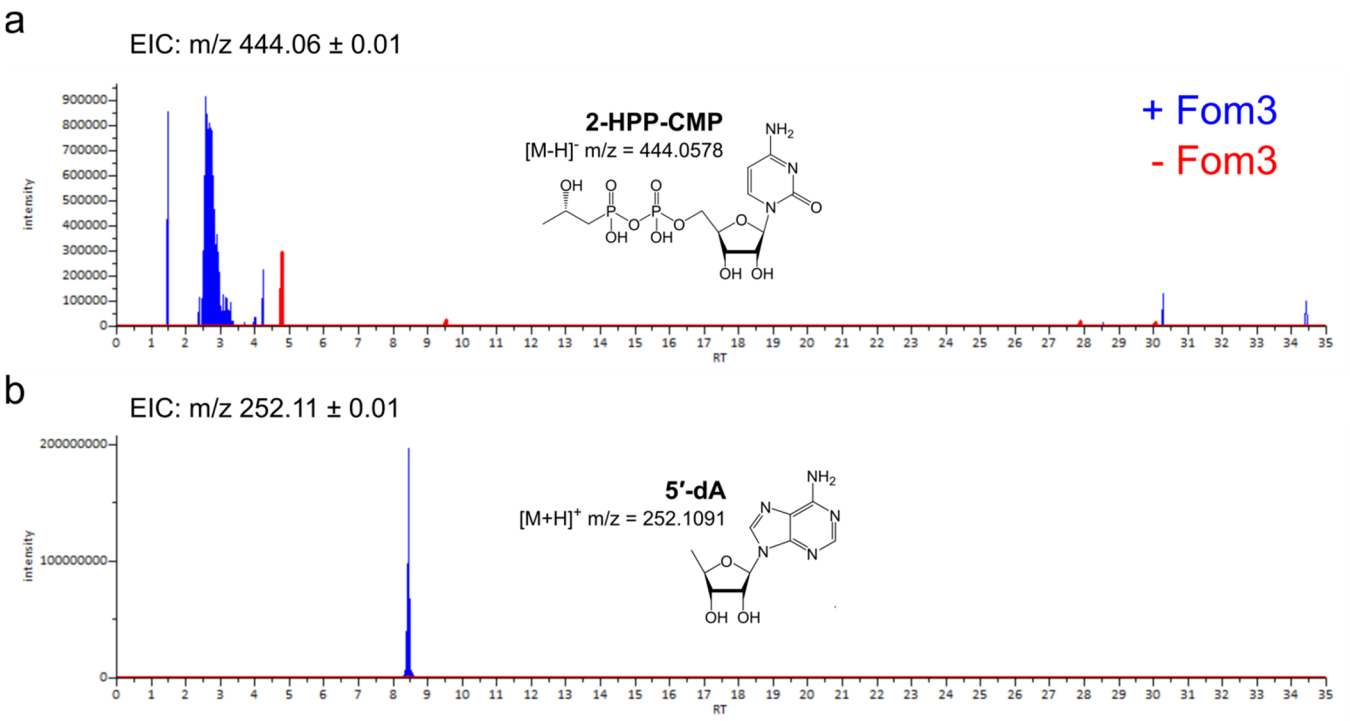
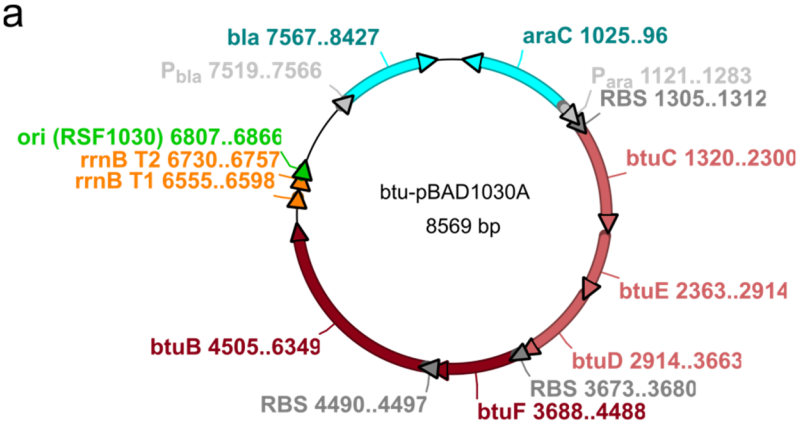


Figure S2. Activity of Fom3 expressed without the *E. coli* B_{12} uptake system. (a) LC-MS analysis of a Fom3 reaction supplemented with MeCbl (blue) and a no-enzyme control (red), showing the extracted ion chromatogram (EIC) for the product, 2-HPP-CMP (m/z 444.06). Reactions were performed at 30 °C for 48 h and contained 20 μM Fom3, 1 mM 2-HEP-CMP, 250 μM MeCbl, 4 mM SAM, 4 mM NADH, 10 mM DTT, and 1 mM methyl viologen in 100 mM HEPES pH 8.0. (b) LC-MS analysis of the same reactions, showing the EIC for the co-product 5′-dA (m/z 252.11).

a



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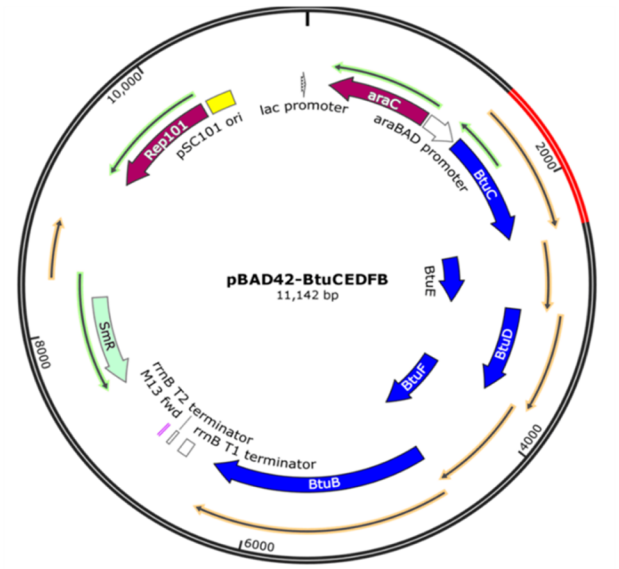
b



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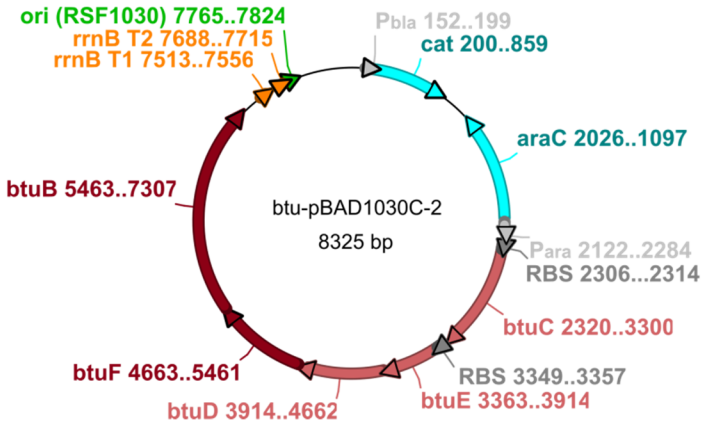
ACTGACCACTAAGGAGGTAAACGATG-btuE-TAAT

G-btuD-TGATG-btuF-TGATG-btuB-TGA...

c



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ACTGACCACTAAGGAGGTAAACGATG-btuE-TAAT

G-btuD-TGATG-btuF-TGATG-btuB-TGATCTA

GATTT...

Figure S3 (previous page). Constructs for expression of the *E. coli* B_{12} uptake system (left) and the sequences of their multiple cloning sites (right). (a) Initial design with intergenic regions. (b) Construct published by Booker *et al.* (adapted from Figure 4 of ref. 2). (c) Adaptation of (a) used in this study.

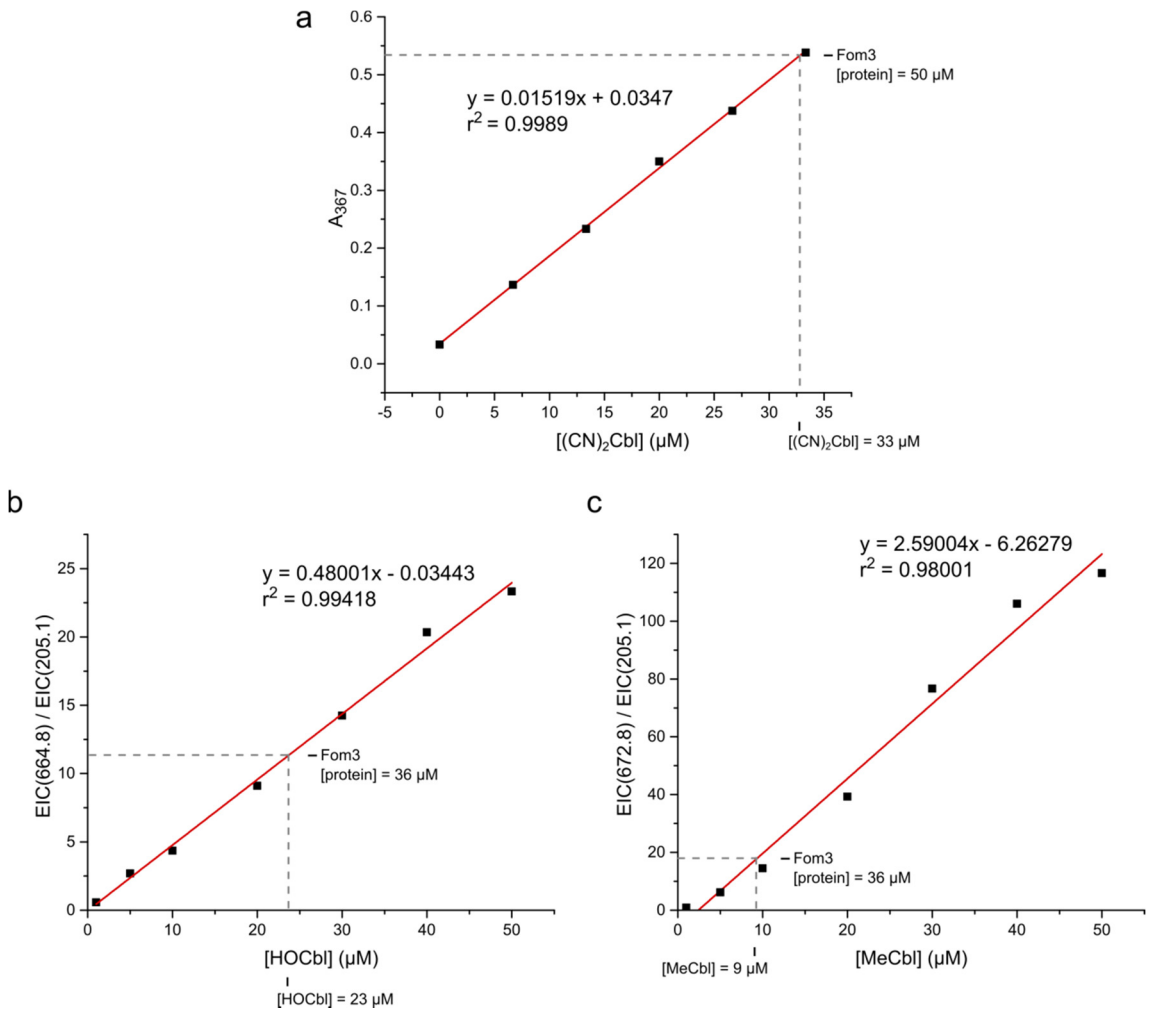

Figure S4. Determination of SUMO-Fom3 cobalamin content. (a) Dicyanocobalamin standard curve and Fom3 content determined by absorbance at 367 nm after boiling a 50 μM sample of Fom3 in 100 mM KCN. (b) HOCbl determined by LC-MS of a 36 μM sample of SUMO-Fom3. (c) MeCbl content of Fom3 determined by LC-MS of a 36 μM sample of SUMO-Fom3. Data points show standard curve measurements with red best-fit lines; dotted lines are sample measurements and concentration estimates.

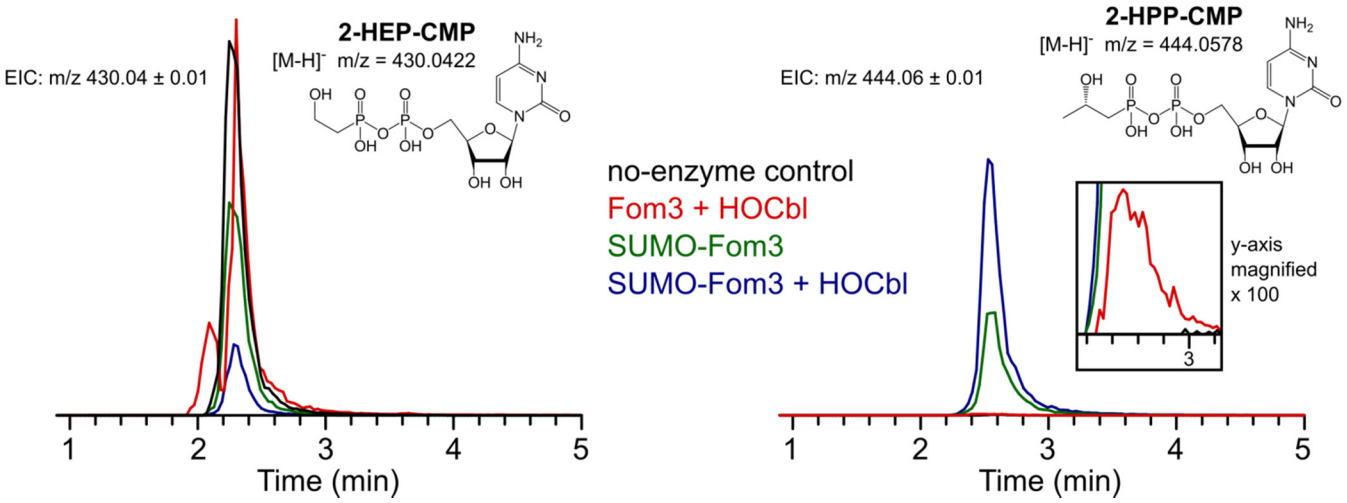

Figure S5. Activity improvement of Fom3 when expressed with the *E. coli* B_{12} uptake system. Pictured are EICs of substrate (left) and product (right) from LC-MS analysis of reactions containing: no enzyme (black); Fom3 expressed without btu-pBAD1030C-2 and supplemented with HOCbl (red); SUMO-Fom3 expressed with btu-pBAD1030C-2 with no added cobalamin (green); and SUMO-Fom3 expressed with btu-pBAD1030C-2 and supplemented with HOCbl (blue). SUMO-Fom3 coexpressed with btu-pBAD1030C-2, even without supplemental HOCbl in the reaction mixture, displays improved conversion over Fom3 expressed without the B_{12} uptake system. All reactions were carried out at 26-28 °C overnight and contained 20 μM Fom3, 1 mM 2-HEP-CMP, 0 or 250 μM HOCbl, 4 mM SAM, 4 mM NADH, 10 mM DTT, and 1 mM methyl viologen in 100 mM HEPES pH 8.0.

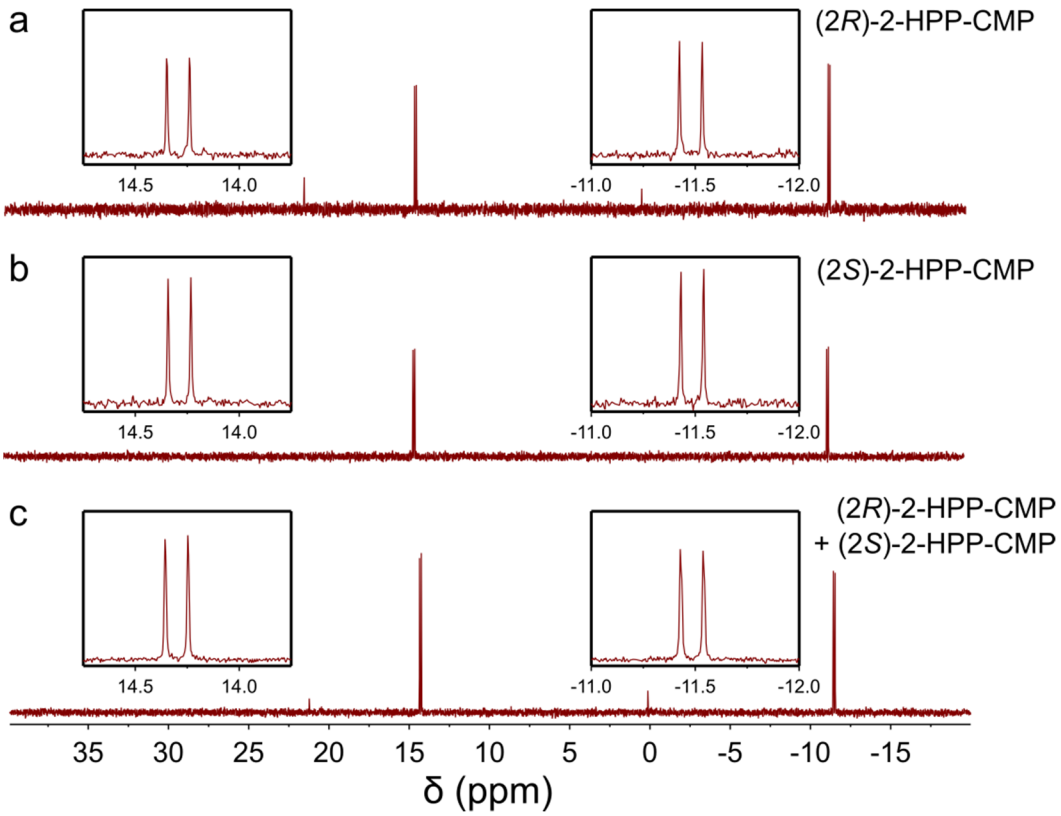

Figure S6. ^{31}P NMR spectra of (2*R*)- and (2*S*)-2-HPP-CMP. (a) ^{31}P NMR spectrum (600 MHz, D_2O) of 250 µM (2*R*)-2-HPP-CMP. (b) ^{31}P NMR spectrum (600 MHz, D_2O) of 250 µM (2*S*)-2-HPP-CMP. (c) ^{31}P NMR spectrum (600 MHz, D_2O) of 125 µM (2*R*)-2-HPP-CMP + 125 µM (2*S*)-2-HPP-CMP.

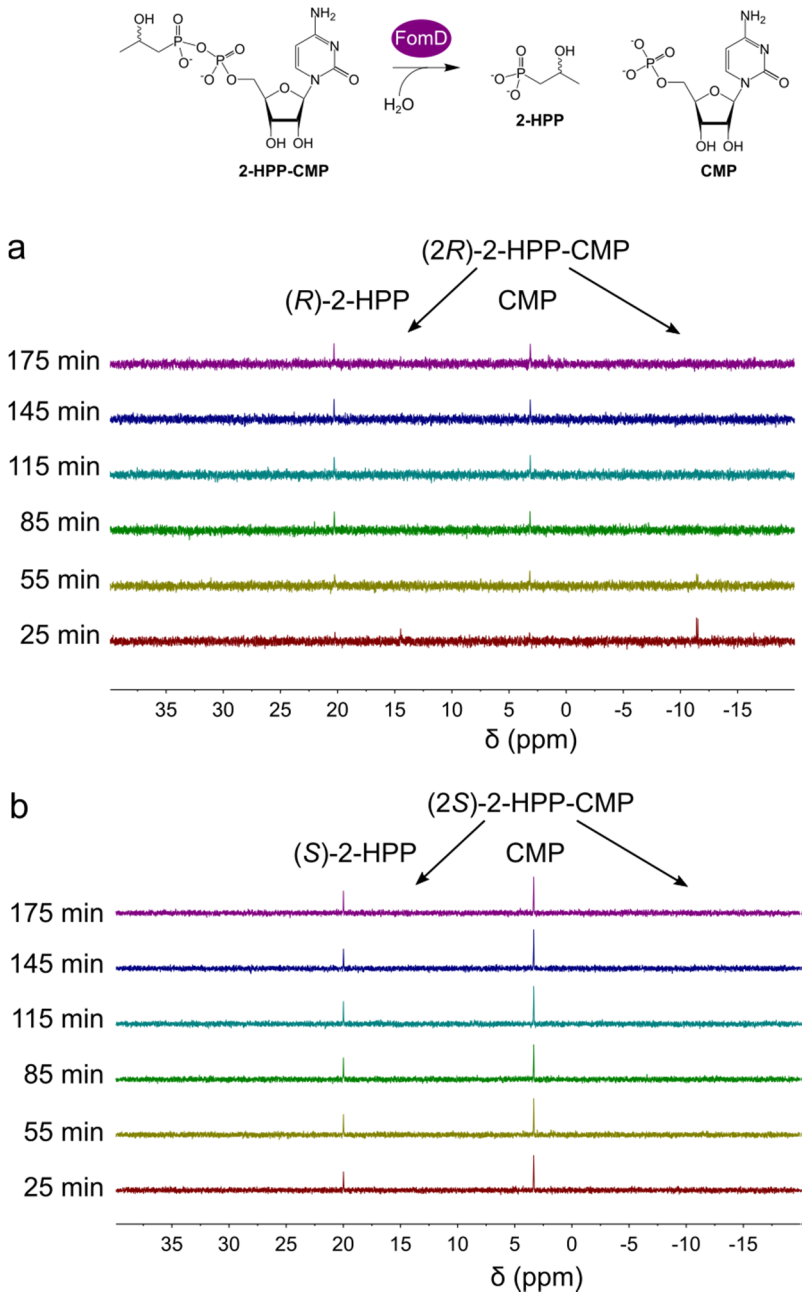

Figure S7. Hydrolysis of (2*R*)- and (2*S*)-2-HPP-CMP by FomD. (a) ^{31}P NMR time course (600 MHz, D_2O) of 1 µM FomD with 250 µM (2*R*)-2-HPP-CMP in 10 mM Tris/D_2O pD 8.0 + 2 mM $MgCl_2$. (b) ^{31}P NMR time course (600 MHz, D_2O) of 1 µM FomD with 250 µM (2*S*)-2-HPP-CMP in 10 mM Tris/D_2O pD 8.0 + 2 mM $MgCl_2$. (2*S*)-2-HPP-CMP is completely hydrolyzed within 25 min under these conditions.

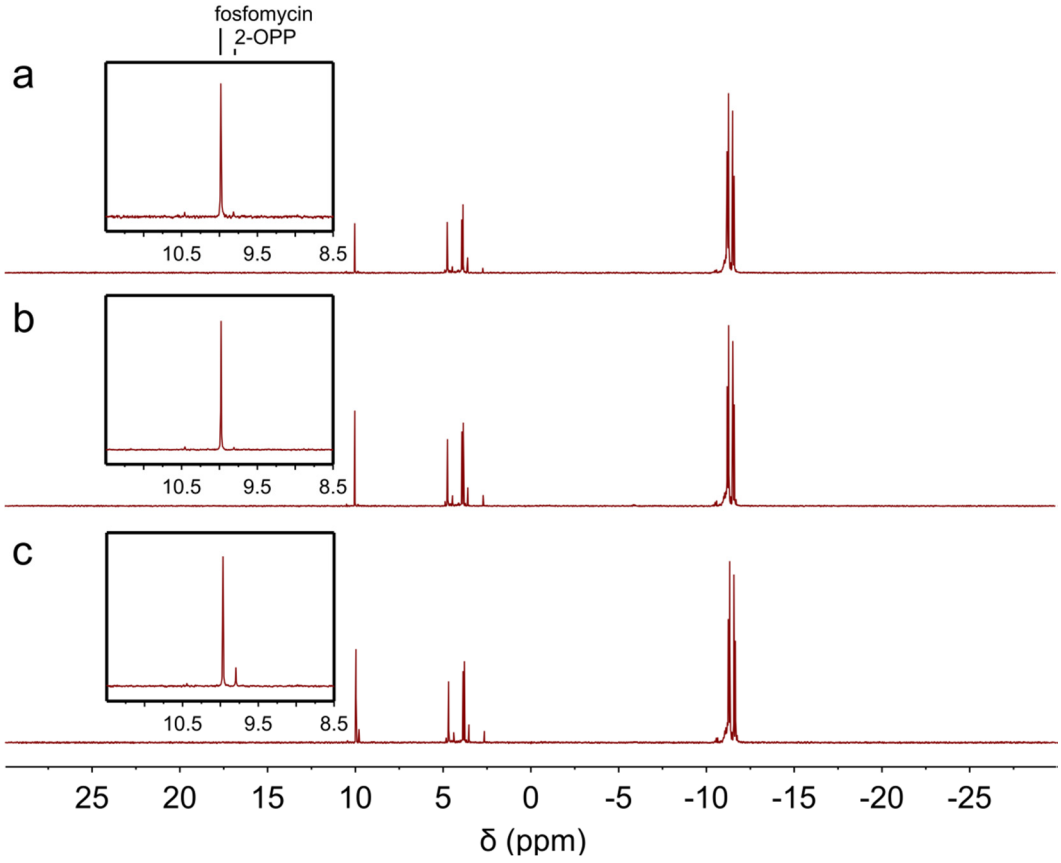

Figure S8. Confirmation of Fom3 product stereochemistry. (a) ^{31}P NMR spectrum (600 MHz, D_2O) of a Fom3 reaction mixture after further reaction with FomD and Fom4. (b) The sample in (a), spiked with a fosfomycin standard. (c) The sample in (b), spiked with a 2-OPP standard.

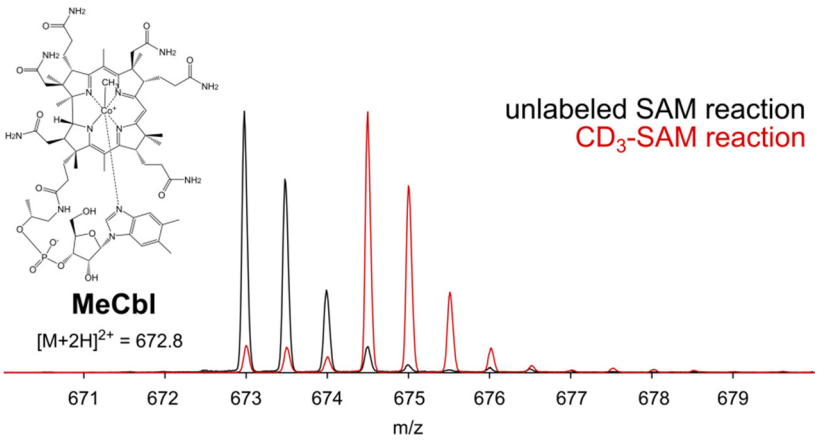

Figure S9. LC-MS analysis of methylcobalamin isolated from SUMO-Fom3 reactions containing unlabeled SAM (black) and CD_3-SAM (red), illustrating the transfer of methyl groups from SAM to cobalamin. Reactions were conducted at 26-28°C for 2 h and contained 50 µM SUMO-Fom3, 50 µM 2-HEP-CMP, 250 µM HOCbl, 4 mM SAM, 4 mM NADH, 10 mM DTT, and 1 mM methyl viologen in 100 mM HEPES pH 8.0.

Table S1. Primer sequences.

Primer	Sequence (5′-3′)
BtuCED-pBAD G1	GTTTTTTTGGGCTAGGAAGGAGAATTCACCatgctgacacttgcccgccaacaacagc
BtuCED-pBAD G2	GCAACTCTCTACTGTTTCTCCATACCCGTTTTTTTGGGCTAGGAAGGAGAATTCACC
BtuCED-pBAD G3	gatttcgaccatctgaGGTACCCGGGGATCCTCTAGAGTCGACCTGC
BtuCED-pBAD G4	GATCCCCGGGTACCtcagatggtcgaaatcagcattctgtgaccttcg
BtuFB-pBADG1	TTTTTGGGCTAGGaaGgaGAAGGTACCatggctaagtcactgttcagggcgctgg
BtuFB-pBAD G2	catGGTACCTTCtcCttCCTAGCCCAAAAAAACGGGTATGGAGAAACAGTAGAGAGTTGC
BtuFB-pBAD G3	agattagtgaaggagactttacaatgattaaaaaagcttcgctgctgacggcg
BtuFB-pBAD G4	ttaatcattgtaaagtctccttcactaatctacctgtgaaagcgcattacagagc
BtuFB-pBAD G5	gctacaccttctgaTCTAGATTTGGCGGATGAGAGAAGATTTTCAGC
BtuFB-pBAD G6	ATCCGCCAAATCTAGAtcagaaggtgtagctgccagacaaggtgtattcc
btu-pBAD G1	gatttcgaccatctgaTTGGGCTAGGaaGgaGAAGGTACCatggc
btu-pBAD G2	tcCttCCTAGCCCAAtcagatggtcgaaatcagcattctgtgacc
btu stitch G1	ccggtctggcactacctgacactgcatcaATacgataaaacgCgtaccgaactactg
btu stitch G2	TtgatgcagtgtcaggtagtgccagaccggcAttgcgaacggcggcgtctgc
btu stitch G3	tcgaccgctggCagtcgacctcggtcaatgatgtgctgcgccgtcttccg
btu stitch G4	cattgaccgaggtcgactGccagcggtcgatatcctgacgggtcacaacg
btu stitch G5	CAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGG
btu stitch G6	TAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACC
btu-pBAD2 G1	TTTTGGGCTAttaaggaggtAATTCatgctgacacttgcccgccaac
btu-pBAD2 G2	agcatGAATTacctccttaaTAGCCCAAAAAAACGGGTATGGAGAAACAGTAG
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btu-pBAD2 G7	ttcacaggtagattgatgattaaaaaagcttcgctgctgacgg
btu-pBAD2 G8	agctttttaatcatcaatctacctgtgaaagcgcattacagagc
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btu-pBAD1030C-2 G2	GATTTTTTTCTCCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGG
btu-pBAD1030C-2 G3	GCGGGGCaTAACTGTCAGACCAAGTTTACTCATATATACTTTAG
btu-pBAD1030C-2 G4	AACTTGGTCTGACAGTTAtGCCCCGCCCTGCC
CyT-pET28a G1	ccgcgTggTagccatatgcagcggccgatcgtctacgtc
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CyT-pET28a G3	gacccgcctctgaAGCTTgGggccgcactcgagcaccac
CyT-pET28a G4	gagtgcggccCcAAGCTtcagaggcgggtcgaggagatgccc
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coFom3-pSUMO G2	TCCCAATAGTCATACCTCCAATCTGTTCGCGGTGAGCCTC
coFom3-pSUMO G3	AAACCAGTATTAAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAG
coFom3-pSUMO G4	AGCAGCCGGATCTTAATACTGGTTTGCATTCCACTGTTTTTGCGGCG

Sequence of codon-optimized Fom3:

5′-

ATGACTATTGGGAGCTTGGGTTCTACAGAATTTGCGTTGCATGGAAAACCGGCCATCCGC

TGGGGTGATCTGCCGCAACGTGTAGGGAAACCGGAAACCCGTCGTTATCAAAAAGTGCTG

TTACTTAATCCGAGTGCTACGCTGTTCCGCCATGATCTCCCGCGTTGTACTTACCCGCTT

GGCCTGGGGTACATTGCTGCGGTTCTGGAGAAGTATGGCTATGAAGTTAAAATTCTGGAT

GTATTTGCGGAGGGGTATTATAACGCACAGCCTGTGGACGGCGATGATCAATTCCTTCGT

TACGGCCTCAGCGATGATGACATTGTTAAAGTTATGAAGGAATTTGGCCCTGACGTCGTT

GGTATTAGCAGTATTTTTAGCAACCAGGCTGACAACGTGCACCATTTGTTAAAGCTGGCG

GATTTGGTTACGCCGGAAGCTGTGACCGCGATCGGTGGCGCGCATGCCCGCTACTTCCCG

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CGCATGGATGAAGAGCTGTTAGATCTCATGGCCGAAAGCGGCGCCTATCAGGTAACCTTC

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GGTGAGGAGATCGCAGAGTTAGCCGCCAAATTCAATCGTGATTTTAATGCACGCCGTGAT

CGCGCTTATACGCCGCAAAAACAGTGGAATGCAAACCAGTATTAA

-3′

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