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Stereospecific Radical-Mediated B_{12}-Dependent Methyl Transfer by the Fosfomycin Biosynthesis Enzyme Fom3

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Abstract

Fom3, the antepenultimate enzyme in the fosfomycin biosynthetic pathway in *Streptomyces* spp., is a class B cobalamin-dependent radical SAM methyltransferase that catalyzes methylation of (5′-cytidylyl)-2-hydroxyethylphosphonate (2-HEP-CMP) to form (5′-cytidylyl)-2-hydroxypropylphosphonate (2-HPP-CMP). Previously, the reaction of Fom3 with 2-HEP-CMP produced 2-HPP-CMP with mixed stereochemistry at C2. Mechanistic characterization has been challenging because of the insoluble expression and poor cobalamin (B_{12}) incorporation in *Escherichia coli*. Recently, soluble *E. coli* expression and incorporation of cobalamin into Fom3 were achieved by overexpression of the BtuCEDFB cobalamin uptake system. Herein, we use this new method to obtain Fom3 from *Streptomyces wedmorensis*. We show that the initiator 5′-deoxyadenosyl radical stereospecifically abstracts the pro-*R* hydrogen atom from the C2 position of 2-HEP-CMP and use the downstream enzymes FomD and Fom4 to demonstrate that our preparation of Fom3 produces only (2*S*)-2-HPP-CMP. Additionally, we show that the added methyl group originates from SAM under multiple-turnover conditions, but the first turnover uses a methyl donor already present on the enzyme; furthermore, cobalamin isolated from Fom3 reaction mixtures contains methyl groups derived from SAM. These results are consistent with a model in which Fom3 catalyzes methyl transfer from SAM to cobalamin and the resulting methylcobalamin (MeCbl) is the ultimate methyl source for the reaction.

Fosfomycin is a broad-spectrum phosphonate antibiotic that disrupts peptidoglycan biosynthesis by irreversibly inactivating UDP-*N*-acetylglucosamine enolpyruvyltransferase.[1,2] Under the name Monurol, fosfomycin is used for uncomplicated urinary tract infections and cystitis.[3,4] The compound is produced by strains of *Streptomyces* and *Pseudomonas*, but the two genera use different biosynthetic pathways that share only the first and last steps.[5–11]

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Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.8b00616. Materials, methods, Figures S1–S9, and Table S1 (PDF)

Both pathways involve conversion of phosphoenolpyruvate (PEP) to phosphonopyruvate by the PEP mutase Fom1 (Scheme 1).[12,13] In *Streptomyces*, subsequent decarboxylation by Fom2 produces phosphonoacetaldehyde (PnAA).[14] FomC then catalyzes the NADPH-dependent reduction of PnAA to 2-hydroxyethylphosphonate,[7,15] which undergoes cytidylylation by a cytidylyltransferase domain (CyT) on Fom1 to form (5′-cytidylyl)-2-hydroxyethylphosphonate (2-HEP-CMP).[10] Methylation of 2-HEP-CMP by the cobalamin (B_{12})-dependent class B radical SAM methyltransferase[16] Fom3 yields (5′-cytidylyl)-2-hydroxypropylphosphonate (2-HPP-CMP), which is proposed to undergo hydrolysis catalyzed by FomD to form CMP and 2-hydroxypropylphosphonate (2-HPP).[11] In the last step, the epoxidase Fom4 converts exclusively the *S* enantiomer of 2-HPP to fosfomycin.[6,17,18] The stereospecificity of this reaction has been demonstrated both by feeding stereospecifically ^{2}H-labeled 2-HPP to fosfomycin-producing *Streptomyces fradiae* and by *in vitro* enzyme assays,[6,18–20] which have also shown that Fom4 converts (*R*)-2-HPP to 2-oxopropylphosphonate (2-OPP).[18,20]

Previously, Fom3 was believed to catalyze the methylation of 2-HEP, a hypothesis consistent with isotope labeling and genetic studies.[7,8] Seminal work by Kuzuyama et al. recently identified the correct substrate by the observation that fosfomycin production in a *fom1* knockout strain could not be rescued by supplementation with the ostensibly downstream metabolite 2-HEP.[10] Subsequently, a cytidylyltransferase domain of Fom1 that had no existing assigned function was found to catalyze cytidylylation of 2-HEP to form 2-HEP-CMP.[10] Fom3 converted 2-HEP-CMP to 2-HPP-CMP *in vitro*, but curiously, the transformation was not stereospecific and produced comparable amounts of (2*R*)- and (2*S*)-2-HPP-CMP. This finding was unexpected given that (*R*)-2-HPP cannot be converted to fosfomycin by Fom4.

To examine the mechanism of the Fom3 reaction, we used the cytidylyltransferase domain of Fom1 (UniProtKB P96074) to enzymatically synthesize 2-HEP-CMP (Figure S1) and standards of (2*R*)- and (2*S*)-2-HPP-CMP.[11] Coexpression of N-terminally His_6-tagged Fom3 from *Streptomyces wedmorensis* (UniProtKB Q56184) with the *Escherichia coli suf* iron–sulfur cluster assembly system yielded Fom3 that lacked cobalamin but exhibited methylation activity in reactions supplemented with methylcobalamin (MeCbl) or hydroxocobalamin (HOCbl) (Figure S2). We hypothesized that incorporation of B_{12} during overexpression might improve both the yield and the activity and attempted to promote incorporation of cobalamin into Fom3 by coexpression with the *E. coli* B_{12} uptake system, composed of the outer membrane permease BtuB, solute binding protein BtuF, and ATP-dependent inner membrane transporter BtuCD, along with the functionally uncharacterized protein BtuE.[21–23] Our expression construct placed the *btu* genes under an arabinose promoter and incorporated an artificial ribosome binding site (RBS) in front of *btuF*, *btuB*, and *btuCED*, which was cloned as an intact genomic fragment (Figure S3a). Coexpression of this construct with N-terminally MBP-tagged Fom3 did not improve the protein yield or activity.

Rather than troubleshoot these findings, we adopted the method recently reported by Booker and co-workers using a similar strategy that was successful with Fom3.[24] These authors constructed a different plasmid expressing BtuCEDFB (Figure S3b) and overexpressed

SUMO-tagged Fom3 in *E. coli* grown in M9-ethanolamine medium. This procedure resulted in Fom3 containing 0.86 equiv of B_{12} (82% MeCbl and 18% HOCbl). We mutated the *btuCEDFB* region of our plasmid to match the published design, which used a different artificial RBS sequence, included an additional RBS between *btuC* and *btuE*, removed the intergenic regions, and generated a 1 bp overlap between the stop codon of one gene and the start codon of the next for *btuDFB* (Figure S3c). Expression and purification using this construct yielded SUMO-tagged Fom3 containing 0.7 equiv of B_{12} [30% MeCbl and 70% HOCbl (Figure S4)]. The activity of this protein was much greater than that of our previous preparation, and addition of exogenous MeCbl or HOCbl was not necessary for catalysis (Figure S5). SUMO-Fom3 prepared by this method was used for all subsequent experiments.

The previously observed formation of both diastereomers of 2-HPP-CMP could be caused either by abstraction of a hydrogen atom from both prochiral positions on C2 of 2-HEP-CMP or by addition of a methyl group to both faces of the resulting radical. To distinguish between these two possibilities, we enzymatically cytidylylated 2-HEP isotopologues deuterated at the pro-*R* position, the pro-*S* position, and both positions on C2.[25] The products of SUMO-Fom3 reactions with these substrates were analyzed by liquid chromatography–mass spectrometry (LC–MS). Reactions with (2*S*)-[2-$^2H_1$]-2-HEP-CMP showed label retention in the 2-HPP-CMP product, (2*R*)-[2-$^2H_1$]-2-HEP-CMP caused transfer of the deuterium label to 5′-deoxyadenosine (5′-dA), and [2-$^2H_2$]-2-HEP-CMP retained one label and transferred one to 5′-dA (Figure 1). These results complement previous studies by Hammerschmidt et al. showing that feeding (*S*)-[2-$^2H_1$]-2-HEP to *S. fradiae* results in the incorporation of deuterium into fosfomycin while feeding (*R*)-[2-$^2H_1$]-2-HEP does not[19] and are consistent with stereospecific abstraction of the pro-*R* hydrogen atom at C2 of 2-HEP-CMP by a 5′-deoxyadenosyl radical.

After ruling out nonstereospecificity in H atom abstraction to explain the formation of two product diastereomers, we attempted to confirm production of (2*R*)- and (2*S*)-2-HPP-CMP by SUMO-Fom3 using ^1H and ^{31}P nuclear magnetic resonance (NMR) spectroscopy. Unfortunately, the C3 proton signals of a mixture of (2*R*)- and (2*S*)-2-HPP-CMP standards could not be resolved (Figure 2a) and neither could the ^{31}P signals (Figure S6). We therefore developed another method for distinguishing the two diastereomers using the differential activity of Fom4 with respect to (*R*)- and (*S*)-2-HPP. To convert 2-HPP-CMP to 2-HPP, we expressed N-terminally His_6-tagged FomD from *S. wedmorensis* (UniProtKB O83033), the previously suggested candidate for catalysis of the hydrolysis step.[10,11] FomD indeed hydrolyzed both the putative native substrate (2*S*)-2-HPP-CMP and the 2*R* diastereomer (Figure S7). This substrate tolerance enabled stereochemical analysis of 2-HPP-CMP by the two-step enzymatic conversion of (2*R*)- and (2*S*)-2-HPP-CMP to 2-OPP and fosfomycin, respectively (Scheme 2).

Thus, the SUMO-Fom3 reaction product and an equimolar mixture of (2*R*)- and (2*S*)-2-HPP-CMP standards were each treated sequentially with FomD and Fom4, and the resulting products were characterized by ^{31}P NMR spectroscopy (Figure 2b and Figure S8). Fosfomycin was the dominant phosphonate species formed from the SUMO-Fom3 reaction product, indicating the generation of (2*S*)-2-HPP-CMP and not (2*R*)-2-HPP-CMP under our assay conditions.

To determine the ultimate source of the methyl group, SUMO-Fom3 was reacted with excess 2-HEP-CMP in the presence of [*methyl*-$^2H_3$]SAM (CD_3-SAM) and MeCbl and conversely with unlabeled SAM and [*methyl*-$^2H_3$]MeCbl (CD_3Cbl). Minimal methyl transfer from MeCbl or CD_3Cbl was observed in the product, even with concentrations of MeCbl or CD_3Cbl that were 10-fold higher than that of SAM (Figure 3a), implicating SAM as the methyl donor for steady state catalysis. However, under single-turnover conditions with CD_3-SAM and no added MeCbl, a significant amount of unlabeled product was formed, indicating the presence of transferable methyl groups on SUMO-Fom3 (Figure 3b). Furthermore, reaction mixtures containing excess CD_3-SAM and HOCbl produced CD_3Cbl (Figure S9). Together, these results suggest methyl transfer from SAM to 2-HEP-CMP via enzyme-bound cobalamin.

In summary, we show that abstraction of a hydrogen atom from 2-HEP-CMP by SUMO-Fom3 is stereospecific and that the hydrogen is transferred to 5′-deoxyadenosine. The transfer of methyl to the resulting proposed substrate radical is also stereospecific, causing inversion of configuration at C2 and forming (2*S*)-2-HPP-CMP. Added methyl- or hydroxocobalamin is not necessary for Fom3 activity, and during steady state turnover, the methyl group is provided by SAM, which was confirmed by using deuterium-labeled SAM. This result is consistent with observations for other class B radical SAM methyltransferases.[26–29] However, single-turnover studies demonstrate that the methyl group is likely transferred from SAM to cobalamin and then to the substrate. This observation is consistent with the observed net retention of stereochemistry in the methyl group of fosfomycin when methionine with a chiral methyl group was fed to the producing organism.[30] Collectively, these observations are consistent with the proposed mechanism depicted in Scheme 3.[7]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

2-HEP 2-hydroxyethylphosphonate

2-HEP-CMP (5′-cytidylyl)-2-hydroxyethylphosphonate

2-HPP 2-hydroxypropyl-phosphonate

2-HPP-CMP (5′-cytidylyl)-2-hydroxypropyl-phosphonate

2-OPP	2-oxopropylphosphonate
5′-dA	5′-deoxyadenosine
CyT	cytidylyltransferase
HOCbl	hydroxocobalamin
LC–MS	liquid chromatography–mass spectrometry
MBP	maltose binding protein
MeCbl	methylcobalamin
PEP	phosphoenolpyruvate
PnAA	phosphonoacetaldehyde
RBS	ribosome binding site
SAM	*S*-adenosyl-L-methionine
SUMO	small ubiquitin-like modifier

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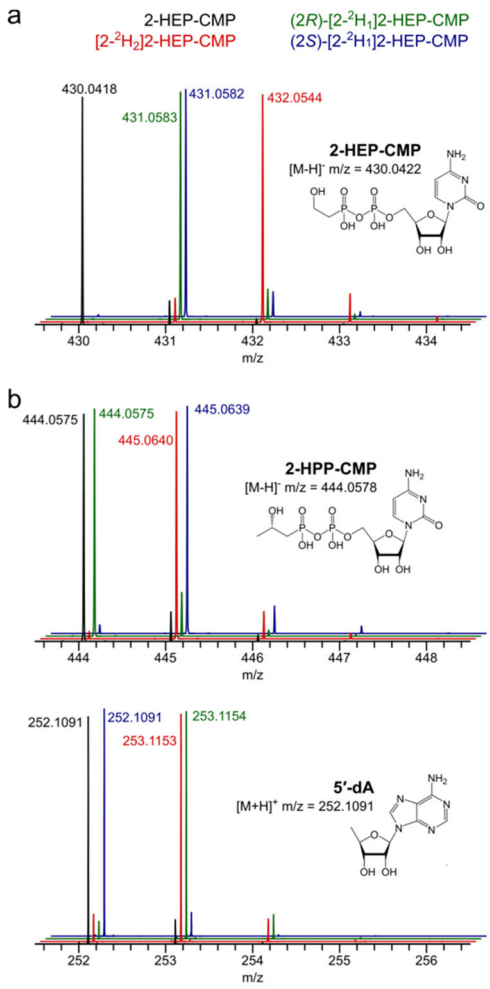


Figure 1.

(a) Liquid chromatography–mass spectrometry (LC–MS) analysis of 2-HEP-CMP (black), [2-$^2H_2$]-2-HEP-CMP (red), (2*R*)-[2-$^2H_1$]-2-HEP-CMP (green), and (2*S*)-[2-$^2H_1$]-2-HEP-CMP (blue). (b) LC–MS analysis of 2-HPP-CMP (left) and 5′-dA (right) produced from SUMO-Fom3 reactions with 2-HEP-CMP isotopologues.

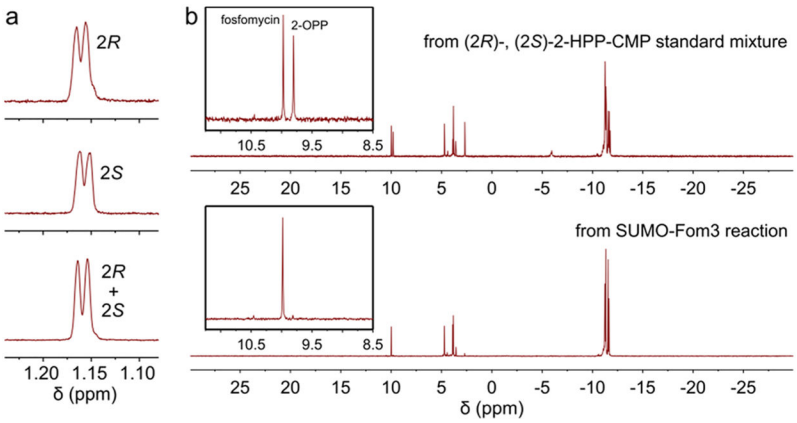


Figure 2.

Stereochemistry determination of the Fom3 product. (a) Methyl signals in ^{1}H NMR spectra (D_2O, 600 MHz) of standards of (2*R*)-2-HPP-CMP, (2*S*)-2-HPP-CMP, and a mixture of (2*R*)- and (2*S*)-2-HPP-CMP. (b) ^{31}P NMR spectra (D_2O, 600 MHz) of the products after reaction of FomD and Fom4 with a mixture of (2*R*)- and (2*S*)-2-HPP-CMP standards (top) and with 2-HPP-CMP produced by Fom3 (bottom), demonstrating that only fosfomycin is formed from the Fom3 product and not 2-OPP.

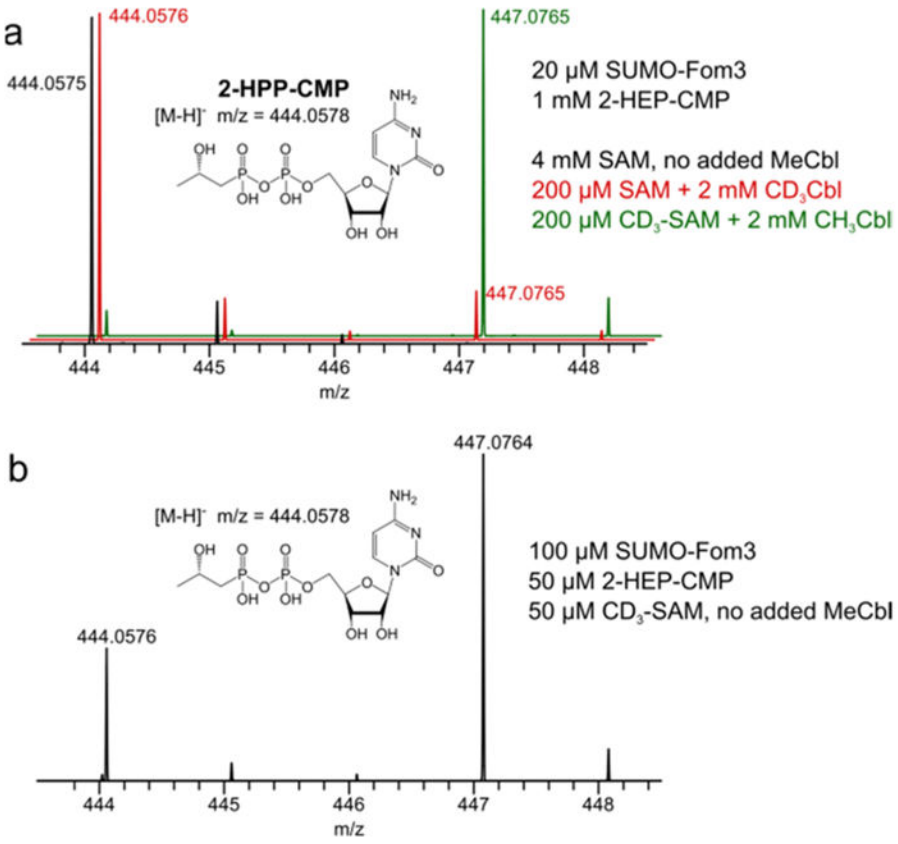
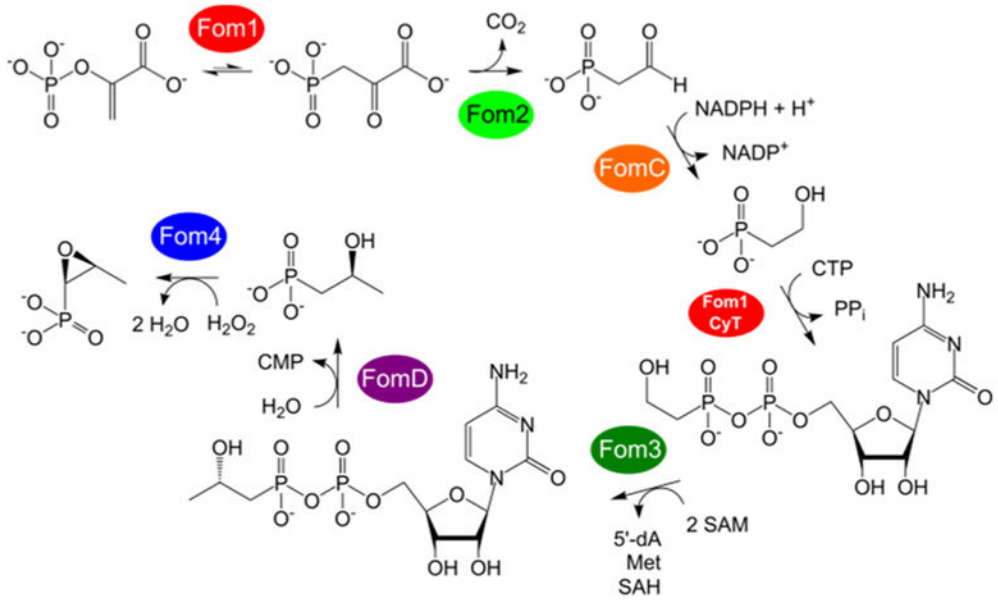
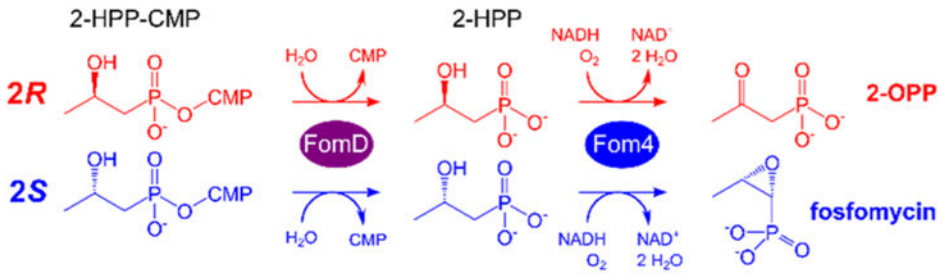


Figure 3.

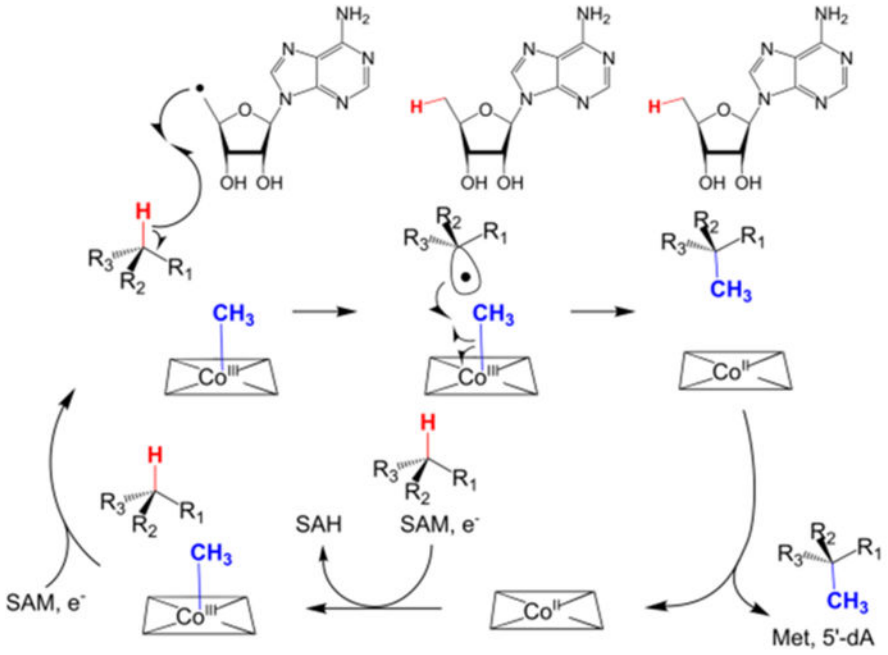
LC–MS analysis of 2-HPP-CMP from SUMO-Fom3 reactions with CD_3-SAM and/or CD_3Cbl under (a) multiple-turnover conditions (20 μM SUMO-Fom3 and 1 mM 2-HEP-CMP) and (b) single-turnover conditions (100 μM SUMO-Fom3 and 50 μM 2-HEP-CMP). Formation of the unlabeled product in single-turnover reactions with CD_3-SAM and no added MeCbl demonstrates that as-isolated SUMO-Fom3 contains transferable methyl groups.



Scheme 1.
Biosynthesis of Fosfomycin in *Streptomyces* spp.



Scheme 2.
Differentiation of (2*R*)- and (2*S*)-2-HPP-CMP



Scheme 3.
Proposed Mechanism for Fom3