New insight into the mechanism of methyl transfer during the biosynthesis of fosfomycin†

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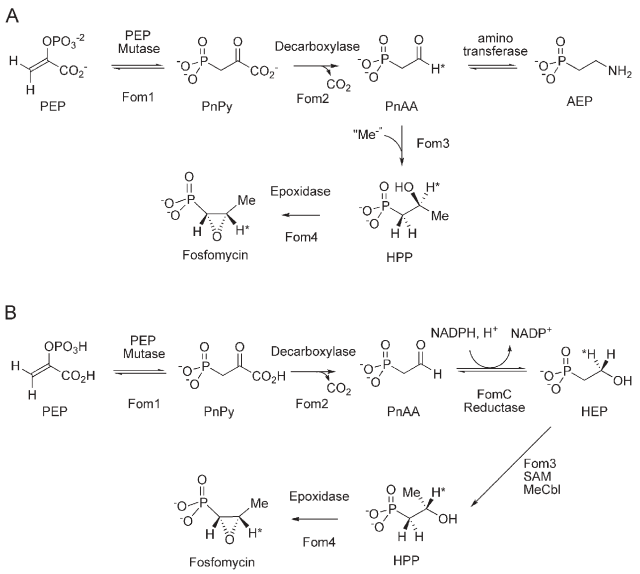
Hydroxyethylphosphonate is a required intermediate in fosfomycin biosynthesis.

(1*R*,2*S*)-Epoxypropylphosphonic acid, also known as fosfomycin, is a clinically utilized, highly effective antibiotic that has low toxicity and is effective against methicillin and vancomycin resistant pathogens.[1–3] Seminal pioneering studies by Seto and Hammerschmidt using genetic techniques complemented by feeding studies with isotopically labeled precursors suggested the biosynthetic pathway for fosfomycin shown in Scheme 1A.[4–11] Yet, the mechanism of an unprecedented methyl transfer step in the biosynthesis of fosfomycin has remained unresolved.[5,11] We recently identified the complete biosynthetic cluster for this natural product from *Streptomyces fradiae* and achieved heterologous production in *S. lividans*.[12] Here we use genetic and chemical complementation studies to show that *fomC*, a gene with a previously unknown role in fosfomycin biosynthesis, is absolutely required for heterologous production. These results in combination with bioinformatics analysis lead to a radically different mechanism for the methyltransferase encoded by *fom3*.

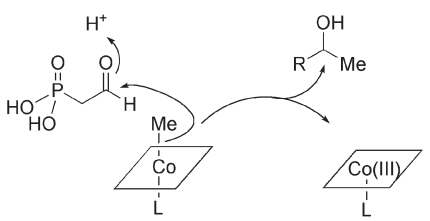


Scheme 1 Comparison of previously proposed biosynthetic pathway (A) and revised pathway (B).

In the currently accepted biosynthetic pathway for fosfomycin (Scheme 1A), PEP is converted to phosphonopyruvate (PnPy) by PEP mutase (Fom1),[13] followed by decarboxylation by Fom2 to produce phosphonoacetaldehyde (PnAA). In the subsequent step, Fom3 has been proposed to catalyze transfer of a methyl anion from methylcobalamin (MeCbl) to PnAA to form 2-hydroxypropylphosphonate (HPP) (Scheme 2).[5,11,14] In the final step, HPP is oxidized by Fom4 to form the epoxide of fosfomycin.[15–23] The proposed mechanism of the methyl transfer reaction catalyzed by Fom3 is fundamentally different from the mechanisms for all known MeCbl dependent methyltransferases in which methylation takes place *via nucleophilic attack by the substrate* at the methyl group of MeCbl.[24]

Intrigued by the apparent mechanistic paradox in how Fom3 would utilize B_{12} for methyl transfer, we initiated a reinvestigation of the pathway. Several genes besides *fom1–4* have been suggested to be part of the biosynthetic cluster and were labeled *fomA–F*.[4] FomA and FomB provide resistance to fosfomycin *via* phosphorylation.[25,26] We recently proposed a function for *fomD* and determined that *fomE*,*F* are not involved directly in fosfomycin production.[12] No function has yet been proposed for *fomC*.

Seto and coworkers showed that a mutant strain with a block in the vitamin B_{12} biosynthetic pathway could not produce fosfomycin, but did convert HPP to fosfomycin.[15] In addition ^{14}C-labeled MeCbl fed to this blocked mutant resulted in ^{14}C-labeled fosfomycin.[14] Based on these results, it was proposed that MeCbl was the methyl donor. However, no intermediates could be isolated from the mutants in which either the B_{12} pathway[15] or the putative methyltransferase Fom3[4] was disrupted and neither PnAA nor aminoethylphosphonate (AEP) could be converted to fosfomycin in *S. lividans* expressing Fom3 and Fom4, whereas HPP was successfully converted.[4] Thus, although the pathway in



Scheme 2 Proposed mechanism of B_{12}-dependent methyl transfer to PnAA.[5,11] The corrin ligand ring system is schematically represented by a square. L may be the benzimidazole ligand of B_{12} or an amino acid ligand from the protein.

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Scheme 1A is consistent with these findings, they do not provide insights into whether PnAA is indeed transformed directly into HPP by Fom3. With our heterologous production system, we set out to study the genes involved in conversion of PnAA to HPP with the goal of providing new insights into the methylation step.

A conserved domain search[27] of the Fom3 sequence showed it has two conserved domains. The N-terminal domain was identified as a B_{12}-like binding domain, whereas the C-terminal domain shows homology to the radical-SAM protein family,[28] containing three conserved Cys residues that serve as ligands to a [4Fe–4S] cluster. The FomC sequence has conserved domains similar to the class III Fe^{2+}-dependent ADH family.[29] A sequence alignment of FomC from *S. fradiae* with structurally characterized family members reveals several conserved residues, including His257, His271, and Asp189 (FomC numbering), which are ligands to the required Fe^{2+} in these homologs.[30] Typically, another His serves as the final enzyme ligand to the metal, however, in this place Gln193 is found in FomC.

In order to discern the order and nature of events between the formation of PnAA and the formation of HPP, four gene disrupted fosmids containing the fosfomycin biosynthetic cluster[12] were obtained by transposon insertion (Fig. 1A). Two of these gene disruptions, *fomC1*::mini-MuAE5 and *fomC2*::mini-MuAE5, were in *fomC*, a positive control disruption (*orf12*::mini-MuAE5) was ~ 12 kb downstream of the cluster in *orf12* (known not to be involved in fosfomycin production),[12] and the final gene disruption (*fom3*::mini-MuAE5) was in the putative methyltransferase *fom3*. These gene disrupted fosmids were introduced into *S. lividans* and checked for bioactivity (Fig. 1B). As expected, a large growth inhibition zone of a sensitive *E. coli* strain was observed for the positive control. This inhibition has been shown to be due to fosfomycin production.[12] The lack of inhibition zones for the other three strains suggests that *fomC* and *fom3* are both absolutely required for fosfomycin production. The requirement of Fom3 is consistent with previous genetic complementation studies,[4] and the importance of FomC was suggested by our recent determination of the minimal gene cluster for fosfomycin production.[12] The requirement of FomC is puzzling since it is not involved in the biosynthetic pathway in Scheme 1A. Its gene *fomC* appears to be transcriptionally coupled to *fom1* and *fom2* (Fig. 1A), and therefore its activity is likely linked to these two genes. Thus, PnAA, the product of the enzymes Fom1 and Fom2, was hypothesized to be the substrate for the dehydrogenase FomC, reducing it to hydroxyethylphosphonate (HEP).

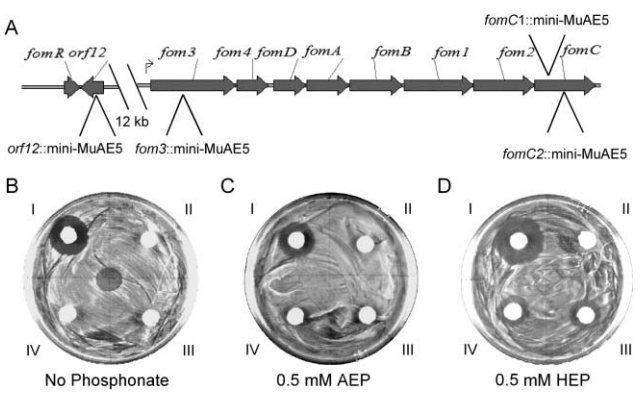
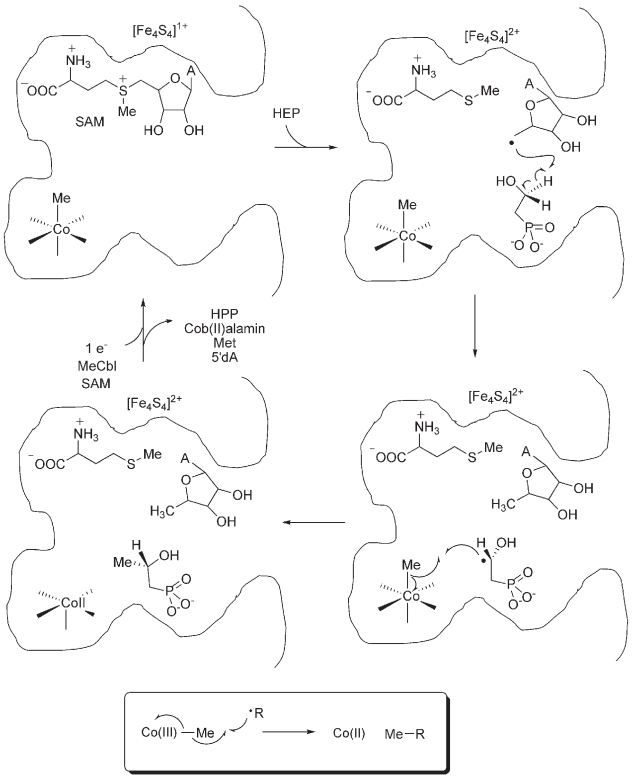


Fig. 1 Chemical complementation. A) Transposon disruptions in the fosmid of *orf12* (positive control), *fom3* (negative control), and *fomC* were reintroduced into *S. lividans*. B) Gene disruption of *fom3* and *fomC* abolishes fosfomycin heterologous production as evidenced by a bioassay against a sensitive *E. coli* strain.[12] C) Feeding *S. lividans* gene disrupted strains 0.5 mM AEP did not complement fosfomycin production. D) Feeding *S. lividans* gene disrupted strains 0.5mM HEP resulted in small inhibition zones for *fomC* disrupted clones, but no complementation for *fom3* was observed. I = *orf12*::mini-MuAE5, II = *fom3*::mini-MuAE5, III = *fomC1*::mini-MuAE5, IV = *fomC2*::mini-MuAE5.

The *S. lividans* clones containing the gene-disrupted clusters were subsequently grown in the presence of 0.5 mM AEP (Fig. 1C). Addition of AEP did not successfully complement the gene disruptions of either *fomC* or *fom3*, whereas it has been shown to complement *fom2* loss of function mutants in *S. wedmorensis*[4] (Scheme 1A). Previous studies have also shown that *fom3* loss of function mutations can be complemented by HPP.[4] Therefore, it can be concluded that both *fom3* and *fomC* are involved in the conversion of PnAA into HPP. Importantly, production of fosfomycin was successfully complemented in the *fomC* disrupted clones by the addition of 0.5 mM HEP to the media (Fig. 1D), whereas this did not complement fosfomycin production in the *fom3* disrupted clone. These data strongly support the hypothesis that HEP is a productive intermediate in the fosfomycin pathway as the product of FomC. This result would also imply that HEP is the substrate of *fom3*, resulting in a revised route for fosfomycin biosynthesis (Scheme 1B).

The revised pathway is consistent with all previously reported studies. In fact, this pathway explains why neither PnAA nor AEP could be converted to fosfomycin by *S. lividans* expressing *fom3* and *fom4* alone, whereas HPP was converted;[4] HEP complementation was not reported in this context. Interestingly, it has been shown that HEP can be converted to fosfomycin by wild type producing organisms,[11,15] but without compelling evidence HEP was believed to be an off-pathway intermediate. The revised pathway is also consistent with the elegant labeling studies by Hammerschmidt and coworkers.[8,10,11] When [2,2-$^2H_2$]-HEP was fed to *S. fradiae*, 34% of the fosfomycin produced contained label.[8] Feeding (*S*)-[2-$^2H_1$]-HEP and (*R*)-[2-$^2H_1$]-HEP resulted in 32% labeled fosfomycin from the (*S*)-labeled compound and no labeled fosfomycin from the (*R*)-labeled compound.[10] Moreover, when ^{18}O-HEP was fed to *S. fradiae*, 50% of the produced fosfomycin was ^{18}O-labeled.[11] These studies revealed for the first time the unusual nature of the epoxidation step, but as pointed out by Hammerschmidt,[11] the retention of the label requires remarkably fast utilization of labeled PnAA since the half-life for exchange of oxygen in acetaldehyde is about 1 min at pH 7.[31] The pathway in Scheme 1B, however, does not require any kinetic limitations since the label would never be in an exchangeable position.

The revised pathway argues against the methyl anion mechanism proposed[5,11] for Fom3 in Scheme 2. Considering the bioinformatics data and the observed complementation of *fomC* disrupted mutants with HEP, it is likely that HEP is the substrate for Fom3. Then a chemically more pleasing mechanism can be drawn (Scheme 3) in which SAM and a reduced [4Fe–4S] cluster form a 5′-deoxyadenosyl radical, as would be typical of radical-SAM protein family members.[32–34] The adenosyl radical then abstracts the *pro-R* hydrogen (directly or through an enzyme radical intermediate[35]) from the C2 position of HEP. Such hydrogen atom abstractions by a 5′-deoxyadenosyl radical are well



Scheme 3 Proposed methyltransferase mechanism. One electron is transferred from the reduced iron–sulfur cluster to SAM to form an adenosyl radical and methionine. The adenosyl radical abstracts the *pro-R* hydrogen atom from C2 of HEP, and the resulting substrate radical reacts with MeCbl yielding HPP and cob(II)alamin. The enzyme is then returned to the active state by reduction of the 4Fe4S cluster back to the +1 state and binding of SAM and MeCbl. Alternatively, cob(II)alamin might be reduced to cob(I)alamin and methylated while bound to the enzyme similar to the reductive methylation of Met synthase.[36] No information is currently available regarding this question. Box: Organic radicals have been shown to react with methylcobalamin to provide one-carbon homologated products.[37]

precedented in adenosylcobalamin dependent enzymes.[24] The resulting organic free radical can then react with the methyl group of MeCbl, yielding the desired HPP and cob(II)alamin. This mechanism is consistent with all prior labeling studies, as well as the bioinformatics and molecular genetic studies presented here. Furthermore, this mechanism finds support in model studies by Kräutler and Montforts and their coworkers who showed that organic radicals react with MeCbl to form homologated products (Inset Scheme 3).[37,38] The mechanism in Scheme 3 can also be applied to the related methyltransferases present in the phosphinothricin tripeptide,[39] fortimicin,[40] and clorobiocin[41] biosynthetic pathways, whereas the methyl anion mechanism does not fit without major modification in each case. The results presented here thus suggest a new and radically different strategy for methylation reactions in secondary metabolism. This strategy requires stoichiometric use of both SAM and MeCbl to achieve methylation of non-activated carbon centers.

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