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How Streptococci Make Isoprenoids

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Abstract

Isoprenoids are the set of ~ 25,000 unique compounds based on the ubiquitous C_5 donor isopentenyl diphosphate (IPP), including quinones, steroid hormones, bile acids, protein membrane anchors and secondary metabolites. Streptococci and other gram-positive bacteria produce IPP *via* the mevalonate pathway, whose function is required for the respiratory pathogen *Streptococcus pneumoniae* to survive in lung and serum. With the discovery of potent selective feedback inhibition by the metabolite diphosphomevalonate (DPM), our laboratory has positioned the pneumococcal mevalonate pathway as a novel target for clinical intervention against an organism that claims the lives of over 4000 people daily. Our studies have revealed unique features of each of each of the three GHMP family kinases that comprise the pathway, including potent allosteric inhibition, a catalytic switch, and a concerted elimination mechanism-informing the design of antibiotics that can simultaneously inhibit multiple steps in a single pathway.

Introduction

Streptococcus pneumoniae kills over 1 million people each year worldwide, mostly children and the elderly, and is the primary bacterial cause of pneumonia, meningitis and otitis media [1, 2]. Antibiotic resistance remains a major problem in treating infections, and multiple-drug resistance rates as high as 95% are seen in some countries [3]. Despite the successful introduction in 2000 of a vaccine covering seven of the most prevalent and infectious of the

> 100 subtypes of pneumcoccus [5], nonvaccinated subtypes are rapidly filling the biological niche created by the vaccine and becoming more virulent [6]. There is an unequivocal need for new strategies to fight this pernicious bacterium.

Isoprenoid biosynthesis has recently emerged as a new target for antibiotic development. The isoprenoids are a class of ~ 25,000 unique compounds composed of a single building-block, isopentenyl diphosphate (IPP). The C_5 isoprene units of IPP are concatenated and then converted either to universal essential cofactors, vitamins, steroids and a host of secondary metabolites, or attached to other biomolecules, such tRNAs or proteins-facilitating association of the latter with membranes. Most eubacteria synthesize IPP starting from glyceraldehyde-3-phosphate and pyruvate using the methylerythritol phosphate pathway [7]. Gram-positive bacteria, including *S. pneumoniae*, archaebacteria and eukaryotes make IPP starting from acetyl-CoA *via* the mevalonate pathway, which converts mevalonate to IPP in three steps catalyzed by GHMP family kinases: mevalonate kinase (MK), phosphomevalonate kinase (PMK) and diphosphomevalonate decarboxylase (DPM-DC) (Fig. 1). A functional mevalonate pathway is essential for survival of *S. pneumoniae* in lung and serum [8].

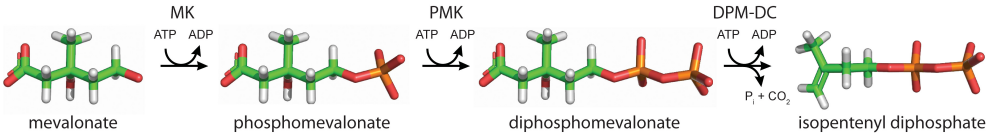

Figure 1. *The Mevalonate Pathway.* Mevalonate kinase (MK), phosphomevalonate kinase (PMK) and diphospho-mevalonate decarboxylase (DPM-DC).

Mevalonate Kinase

Feedback inhibition is a hallmark of metabolic pathways. Human mevalonate kinase is potently inhibited by end-products farnesyl diphosphate (C_{15}) and geranylgeranyl diphosphate (C_{20}) [9, 10]. We were therefore surprised to discover that the the MK homologue in *S. pneumoniae* is subject to inhibition, not by end-products, but by the intermediate DPM. The MK and PMK reactions each produce ADP, which can be stoichiometrically coupled to the oxidation of NADH by a pyruvate kinase/lactate dehydrogenase coupling system-thus providing a continuous optical assay for enzyme activity (Fig. 2). When MK and PMK are added in successive steps to an assay mixture containing mevalonate, one equivalent of DPM is produced. However, when MK and PMK are added simultaneously, very little product is formed. This result suggested that the DPM produced during the initial stages of the reaction was inhibiting the first enzyme, MK. Incubation of MK in the presence of DPM strongly inhibited turnover, confirming this hypothesis. Titration of DPM against MK showed that its potency was quite strong (IC_{50} ~ 400 nM). Tests of human MK yielded no DPM inhibition at levels up to 25 μM, confirming that *S. pneumoniae* MK could be specifically targeted [11].

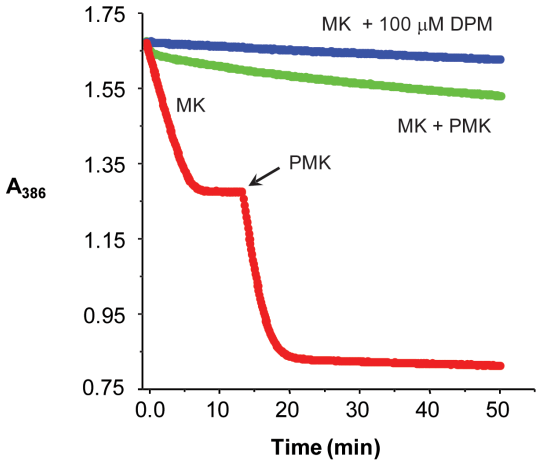

Figure 2. *Discovery of MK Inhibition by DPM.* Reaction progress curves for reactions in which enzymes were added sequentially or simultaneously. ADP produced by MK or PMK is stoichiometrically coupled to oxidation of NADH by a pyruvate kinase/lactate dehydrogenase coupling system, resulting in a decrease in absorbance at 386 nm.

An important consideration when developing an antibiotic that targets a metabolic pathway is the mechanism of inhibition. An allosteric mechanism of pathway inhibition – in which the inhibitory ligand binds distal to active site – has an advantage over simple occlusion of the active site, in that inhibition cannot be overcome by the thermodynamic push that accompanies a buildup of the metabolite (i. e., the substrate) just upstream of the inhibited step. MK inhibition by DPM was investigated using initial-rate experiments that simulate this condition and can thus distinguish between mechanisms: the maximal reaction velocity at (theoretically) infinite substrate concentration was determined as a function of inhibitor concentration. If DPM binds to the active site, its inhibitory effects (at any concentration of inhibitor) will be completely irrelevant at infinite substrate concentration and have no impact on the maximal velocity. We observed that DPM reduced this maximal velocity – with equal potency *versus* both mevalonate and ATP – indicating that DPM must bind to an allosteric site. This result was confirmed by the observation that DPM had an identical affinity for MK both in the presence and absence of saturating concentrations of mevalonate and AMPPNP (a non-hydrolyzable ATP analogue) in an equilibrium binding study. If DPM bound at the active site, its apparent affinity should have been altered by the presence of competing ligands. The stoichiometry of DPM binding to the MK dimer was shown to be 1:1, suggesting that the allosteric site was symmetrically disposed to both subunits, and was perhaps located at the dimer interface.

The X-ray crystal structure of MK in the presence of mevalonate, AMPPNP and DPM showed that two molecules of DPM are bound, one to each subunit, in the mevalonate binding pocket (Fig. 3A) [12]. This result is perhaps not surprising, given that DPM is a

partial bisubstrate analogue whose pyrophosphoryl moiety could take the place of the beta- and gamma-phosphates of ATP; however, the result stands at odds with the functional data described above and may represent a crystallographic artifact. The structure also revealed a pore at the dimer interface having excellent charge- and shape-complementarity to DPM; this pore could be the allosteric site. (Fig. 3B). We are currently pursuing the solution-phase structure of the fully-liganded MK (bound to mevalonate, AMPPNP and DPM) by NMR, to identify this site and study structural changes to the enzyme that occur upon allosteric binding.

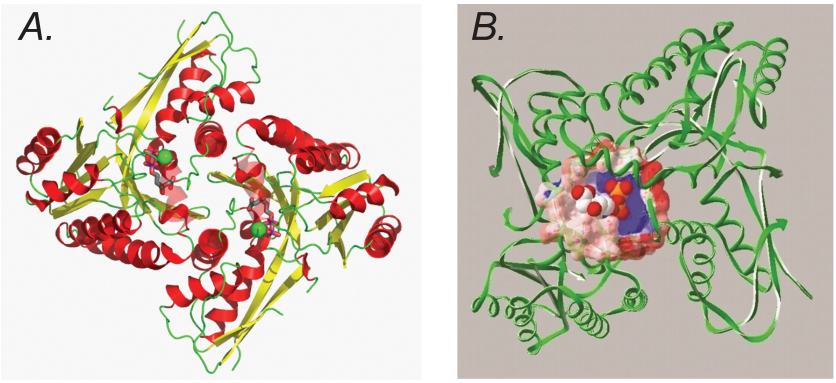

Figure 3. *Locating the DPM Allosteric Site.* (A) Crystal structure of *S. pneumoniae* MK with DPM (magenta sticks) and Mg^{2+} (green spheres) bound (PDB: 2OI2). (B) MK model with DPM positioned at a pore in the subunit interface. Vacuum electrostatics near the pore are displayed as colored surfaces (blue, positive; red, negative).

Phosphomevalonate Kinase

The X-ray structure of phosphomevalonate kinase presents a classic GHMP kinase scaffold. Comparison of the apo and ternary-complex forms of the enzyme (Fig. 4) reveals that four regions undergo significant conformational changes as a result of ligand binding [13, 14].

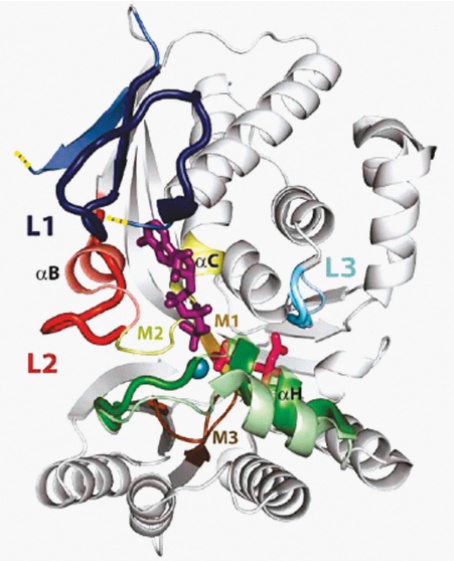

Figure 4. *Comparison of the Apo and Ternary-Complex Structure of PMK from S. pneumoniae*. Regions that do not change noticeably upon ligand binding are grey, the four responsive elements (L1, L2, L3 and αH) are colored in blue, red, cyan and green, respectively – the more intense colors are associated with the ternary complex.

These regions (L 1, L 2 L 3 and αH) are color-coded, and the more intense colors are associated with the ternary complex. L 1 (blue) is disordered in the absence of ligand, and reorganizes upon binding of nucleotide (AMPPNP, purple) with the result that residues that would otherwise obstruct binding are withdrawn from the binding pocket, and hydrogen bonds to the adenine ring are established. L 2 (red) undergoes a considerable structural change in which a small helical element unravels to deliver the ammonium group of Lys101 into direct contact with the β,γ-bridging atom of AMPPNP (Fig. 5), where it will stabilize the negative charge expected to develop at this position during bond cleavage in a dissociative reaction. In the apo structure, Lys101 forms a salt bridge to the carboxylate of Glu98, and is positioned to obstruct the binding of nucleotide. Upon binding, the salt bridge is broken, the helix unwinds, ‘‘swinging’’ Lys101 past Lys100, which hydrogen bonds to the amide backbone of Lys208. The structural change resemble a ‘‘lysine switch’’ that when thrown, activates catalysis. It is interesting to note that while other GHMP kinases also feature a lysine switch, the catalytic lysine has migrated to a different position in the active site.

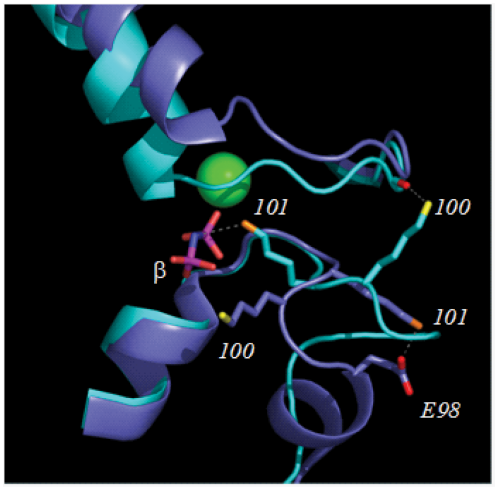

Figure 5. *A Di-lysine Catalytic Switch*. The L2 regions of the apo- (purple) and ternary-complex (cyan) forms of PMK are overlain. The β- and γ- phosphoryl groups of AMPPNP are shown.

The structure of the PMK ternary complex suggested that the substrates are positioned in a non-reactive orientation. The phosphoryl-group of phosphomevalonate is nearly orthogonal to what is expected for in-line nucleophillic attack at the γ-phosphate of ATP (Fig. 6A). Comparison of the PMK arrangement to that of six other GHMP-kinase ternary complexes revealed that of the seven total structures, three exhibited the non-reactive arrangement, three exhibited what appeared to be an excellent positioning for in-line attack (e. g., Fig. 5B), and one, erythritol kinase, exhibited both conformations. Clearly, these active sites can bind substrates in either conformation, suggesting that these forms might interconvert during the catalytic cycle. To assess the likelihood that the PMK active site might accommodate interconversion, the substrate configurations were aligned by superposing the C_α-traces of the ternary complex of PMK with that of Gal-NAc kinase – a good example of a reactive complex. The alignment suggested that the only large-scale movement was the migration of the divalent cation between "walls" of the active site, and that the active site was indeed capacious enough to accommodate such migration.

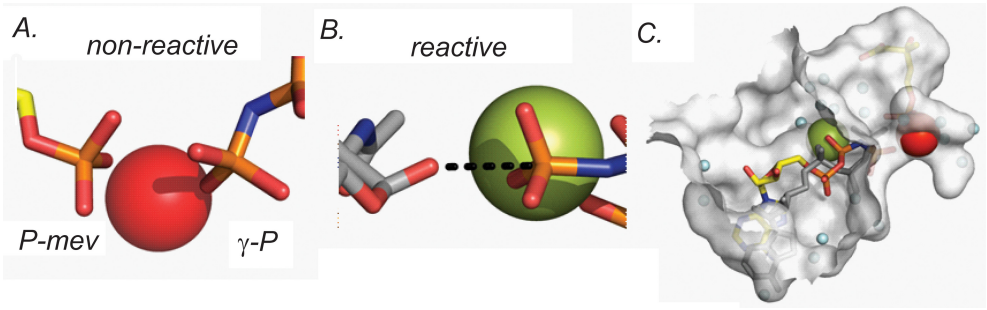

Figure 6. *Reactive and Non-Reactive Positioning of Substrates in the GHMP-Kinase Family.* (A) Positioning in the PMK ternary complex. (B) Positioning in the Gal-Nac kinase complex. (C) Superposition of the reactive and non reactive structures in the PMK active site. Color scheme: *reactive* position – grey nucleotide, green Mg^{+2}; *non-reactive* position – atom-colored nucleotide, red Mg^{2+}; aqua spheres – water.

The PMK active site harbors a water pentamer – five molecule of water arranged with an oxygen at each apex. Five of the ten pentamer-water hydrogens help form the ring (each shared by two oxygen atoms); the remaining five are distributed radially. The geometry of the pentamer allows the ring-hydrogen bonds to form with little if any strain [15], resulting in strong ring-hydrogen bonds and acidification of the radial protons. Four of the pentamer positions are occupied well in the apo-form of the enzyme, the fifth appears to be stabilized binding of ligands. The radial protons of the pentamer form hydrogen bonds to the negatively charged aspects of the substrates and appear well positioned to participate in chemistry (Fig. 7). Indeed, the reactive moieties of the substrates are interlaced in a large, complex water structure that remarkably manages to foster chemistry without short-circuiting it by hydrolyzing the γ-phosphate of ATP.

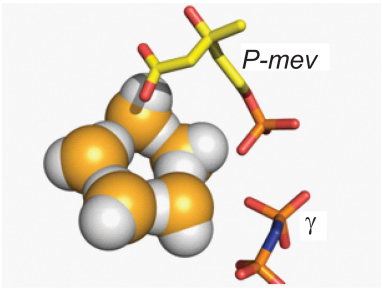

Figure 7. *Substrate Interacts with an Active-Site Water Pentamer.* The β- and γ-phosphates of AMPPNP are shown. Pentamer color scheme: orange – oxygen; white – protons.

Diphosphomevalonate Decarboxylase

The final step in the mevalonate pathway is carried out by diphosphomevalonate decarboxylase (DPM-DC), which phosphorylates the C_3-hydroxyl of DPM and subsequently eliminates phosphate and CO_2. This reaction is thought to proceed in a stepwise manner, with phosphate departing first, resulting in the formation of a carbocation. Abeles and coworkers provided evidence that a carbocation transiently forms by showing that substitution of the C_3-methyl group with a hydrogen or a fluoromethyl group causes the reaction to halt after phosphorylation, implying that the electron-donating methyl group is required to stabilize the carbocation inductively [16]. We sought to exploit carbocation formation to inactivate DPM-DC through use of DPM analogues in which the C_3-methyl group was replaced by substituents that were able, by resonance with the carbocation, to generate strongly electrophilic species that could become covalently bound to the enzyme (Fig. 8) [3]. One of these analogues contained a cyclopropyl substituent that was proposed to undergo ring opening upon ionization, forming a homoallyl cation that is stabilized by the loss of 27 kcal/mol of ring strain [17, 18]. If this rearrangement occurred, we anticipated that a nucleophile on the enzyme surface could attack the carbocationic intermediate, forming a covalent bond and inactivating the enzyme. However, inactivation did not occur over the course of > 1000 turnovers, suggesting that no such adduct had formed. We therefore monitored the fate of the analogue during turnover using the unique NMR signal of the cyclopropyl protons. If the ring opened and formed a primary alcohol (by quenching with water), the proton chemical shifts associated with the ring would move significantly downfield [19]. Instead, we observed only a very small change (0.3 ppm) in the cyclopropyl proton chemical shifts that was consistent with an intact cyclopropyl group adjacent to a carbon-carbon double bond; this structure was subsequently confirmed by 2-D NMR. These data rule out a ring-opened product, and suggest that the analogue undergoes a decarboxylation that resembles that of the native substrate, DPM.

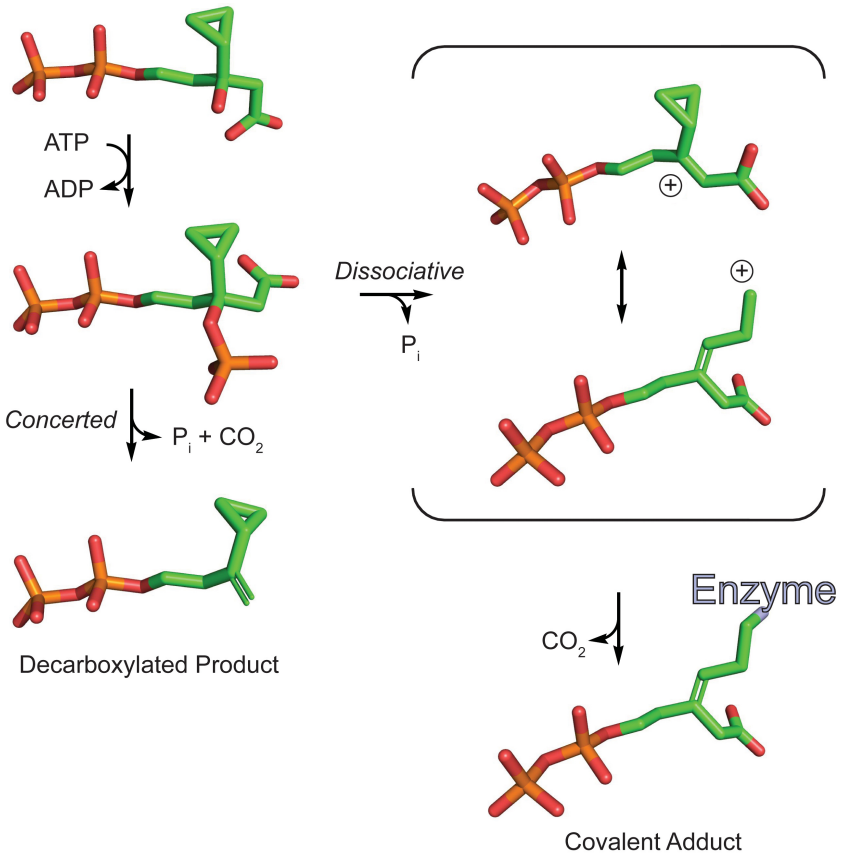

Figure 8. *Probing the Mechanism of DPM-DC with Cyclopropyl-DPM*. An analogue of DPM with a cyclopropyl substitution at C_3 is phosphorylated by the enzyme. If a carbocation forms during the reaction (*Dissociative* branch), it can be delocalized into the cyclopropyl ring, causing the ring to open, forming a strong electrophile. This ring-opened carbocation can be quenched by a nucleophile on the enzyme surface or water. Alternatively, a concerted mechanism produces only decarboxylated products.

These results call into question the extent of carbocation formation in the DPM-DC transition state. If a full carbocation formed, we expected to observe rearrangement of the cyclopropyl group. It is conceivable that a carbocation could form but not react, provided that no nucleophile is in close proximity to the carbocation (the protein surface or water); however, the presence of four crystallographic water molecules in the large pocket adjacent to C_3 and the proximity of the Asp276, Lys18, Ser185, and Met189 side chains and the Tyr19 carbonyl as potential nucleophiles argue against this possibility (Fig. 9). Alternatively, carbocation formation may be minimal or absent, in which case elimination of the carboxylate and the phosphate is concerted rather than dissociative. Abeles' work on the effects of altered electron induction at C_3 with the mammalian enzyme shows clearly that DPM-DC chemistry is sensitive to such changes and supports the development of a positive charge at

C_3 in the transition state [16]. Further, by replacing C_3 with a positively charged tertiary amine, he created an analogue that mimicked the structure and charge characteristics of a dissociative transition state. The affinity of this analogue (0.75 μM) was only 20-fold higher than that of the substrate [20], which, while supportive of a dissociative character in the transition state, is perhaps more consistent with development of partial rather than complete positive charge at C_3. While studies that correlate the extent of positive charge formation with degree of ring opening in cyclopropyl ring systems do not yet exist, our results, which demonstrate no detectible ring opening, are consistent with only slight positive charge formation in the transition state. Using kinetic isotope effects it may be possible to directly assess the extent of positive charge development on C_3 at the transition state.

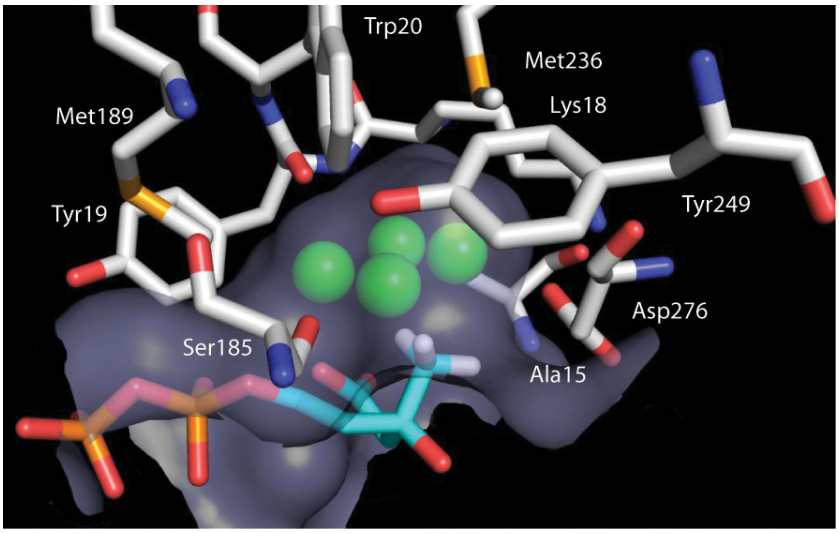

Figure 9. *DPM-DC Substrate Binding Pocket with Modeled DPM*. A homology model of the *S. pneumoniae* DPM-DC based on the 70 % identical *Streptococcus pyogenes* DPM-DC (white, PDB: 2GS8) is shown with manually positioned DPM (cyan) (for full description, see [3]. The DPM C3-methyl points into a large water-filled cavity composed of nine conserved residues. The surface (light blue) represents the Van der Waals contact surface of the protein model.

Outlook for Mevalonate Pathway Inhibition

Diphosphomevalonate interacts with each of enzymes in the *S. pneumoniae* mevalonate pathway – as an allosteric inhibitor of MK, a reaction product of PMK and a substrate for DPM-DC. Thus, non-reactive analogues of DPM have the potential to inhibit all three of the enzymes in this pathway. A challenge for moving DPM-based antibiotics into the clinic will be getting a highly charged (4-) molecule across the bacterial membrane. It should be possible to overcome this barrier using a prodrug strategy in which uncharged mevalono-lactone or mevalonate analogue esters are taken up by the cell, saponified and fed into the endogenous mevalonate pathway, which converts them to the active diphosphorylated spe-

cies *in situ* [21, 22]. It must also be considered that, while DPM selectively inhibits pneumococcal MK, DPM analogues may cross-react with the human homologues of PMK and DPM-DC. However, inhibitors of the human mevalonate pathway, such as the widely-used cholesterol-lowering statins and anti-proliferative bisphosphonates, are generally very well tolerated and have shown the potential to have a clinical impact on asthma [23, 24], wound-healing [25], hepatitis [26, 27] and HIV [28], suggesting that even nonselective inhibitors have potential therapeutic benefits.

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