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Inhibition Studies on Enzymes Involved in Isoprenoid Biosynthesis: Focus on Two Potential Drug Targets: DXR and IDI-2 Enzymes

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Abstract

Isoprenoid compounds constitute an immensely diverse group of acyclic, monocyclic and polycyclic compounds that play important roles in all living organisms. Despite the diversity of their structures, this plethora of natural products arises from only two 5-carbon precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). This review will discuss the enzymes in the mevalonate (MVA) and methylerythritol phosphate (MEP) biosynthetic pathways leading to IPP and DMAPP with a particular focus on MEP synthase (DXR) and IPP isomerase (IDI), which are potential targets for the development of antibiotic compounds. DXR is the second enzyme in the MEP pathway and the only one for which inhibitors with antimicrobial activity at pharmaceutically relevant concentrations are known. All of the published DXR inhibitors are fosmidomycin analogues, except for a few bisphosphonates with moderate inhibitory activity. These far, there are no other candidates that target DXR. IDI was first identified and characterised over 40 years ago (IDI-1) and a second convergently evolved isoform (IDI-2) was discovered in 2001. IDI-1 is a metalloprotein found in Eukarya and many species of Bacteria. Its mechanism has been extensively studied. In contrast, IDI-2 requires reduced flavin mononucleotide as a cofactor. The mechanism of action for IDI-2 is less well defined. This review will describe how lead inhibitors are being improved by structure-based drug design and enzymatic assays against DXR to lead to new drug families and how mechanistic probes are being used to address questions about the mechanisms of the isomerases.

Keywords

DXR; IDI; isomerase; isopentenyl; isoprenoid; MEP; mevalonate; MVA; reductoisomerase

1. INTRODUCTION

Isoprenoid compounds constitute an immensely diverse group of acyclic, monocyclic and polycyclic compounds that play important roles in all living organisms [1]. They function as regulators of gene expression, constituents of membranes, vitamins, antimicrobial agents, mating pheromones, reproductive hormones, components of signal transduction pathways, and constituents of electron transport and photo-synthetic machinery [2].

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Despite the diversity of their structures, this plethora of natural products arises from only two 5-carbon precursors, isopentenyl diphosphate (IPP, (**1**)) and dimethylallyl diphosphate (DMAPP, (**2**)) (Fig. 1) [3].

2. THE MEVALONATE PATHWAY

Following the discovery of the mevalonate pathway (MEV pathway) in the 1960s [4], it was widely accepted that it was the sole route to IPP and DMAPP in all living organisms. The first step is the formation of the acetoacetyl-CoA by the condensation of two molecules of acetyl-CoA catalyzed by a thiolase. In the following reaction, a third molecule of acetyl-CoA condenses with the aceto-acetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA (**4**)); the reaction is catalyzed by the HMG-CoA synthase (HMGS). Then, HMG-CoA is reduced to 3R-mevalonate (**5**) during a reaction, which consumes two molecules of NADPH. This third stage of the pathway is catalysed by HMG-CoA reductase (HMGR). A series of three reactions then converts mevalonate into IPP with the consumption of one molecule of ATP. The first two steps are catalyzed by mevalonate kinase (MK) and phosphomevalonate kinase (PMK), which converts the primary hydroxyl group in 3R-mevalonate to a diphosphate ester. The third reaction, catalysed by the diphosphomevalonate decarboxylase (DPMD), phosphorylates the tertiary hydroxyl group in (**7**) and is followed by a fragmentation to give IPP (Fig. 2). The mevalonate pathway and its inhibition were studied [5, 6], especially HMGR as a target for the statins class of antihypercholesterolemia drugs but also targeting Alzheimer's disease and Parkinson's disease [7].

In 2006, Grochowski *et al*. reported that the translated protein from open reading frame MJ0044 in *Methanocal-dococcus jannaschii* (MJ) catalyzes the ATP-dependent phosphorylation of isopentenyl phosphate (IP) rather than mevalonate or mevalonate phosphate, and the protein was assigned as an IP kinase (IPK) [8]. The *M. jannaschii* protein and homologous proteins from *Thermoplasma acidophilum* and *Methanothermobacter thermautotrophicus* have high catalytic efficiencies for phosphorylation of IP ($\sim 10^6$ $M^{-1}s^{-1}$) [9] similar to those of established enzymes in the isoprenoid pathway. Grochowski *et al*. suggested an alternative mevalonate route in Archaea where decarboxylation of mevalonate 5-phosphate (**6**) produces isopentenyl 5-phosphate (IP (**8**)) the substrate for the isopentenyl 5-phosphate kinase (IPK), to give IPP (Fig. 2). In 2010, Mabanglo and coworkers described the first crystal structure [10] of this enzyme, which is now under investigation [9, 11].

3. THE MEP PATHWAY

3.1. General Description of the Pathway

The MEP pathway [12, 13] starts with the condensation of glyceraldehyde phosphate (**9**) and pyruvate (**10**) to afford 1-deoxyxylulose 5-phosphate (DXP) (**11**) in a reaction catalysed by 1-deoxyxylulose 5-phosphate synthase (DXS) [14, 15]. A sigmatropic rearrangement of 1-deoxyxylulose 5-phosphate followed by reduction of the intermediate aldehyde, catalysed by 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), gives the branched polyol, 2-*C*-methyl-D-erythritol 4-phosphate (MEP) (**12**) [16, 17]. This enzyme and its inhibition will be further developed in section 3.2. The 2-*C*-methyl-D-erythritol 4-phosphate is converted into cyclic diphosphate (**15**) by a series of three enzyme-catalysed reaction steps. Specifically, IspD converts the branched polyol and CTP into diphosphocytidyl derivative (CDP-ME) (**13**) [18]. Then, the IspE phosphorylates the tertiary hydroxyl group in CDP-ME to produce 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2- phosphate (CDP-ME2P) (**14**) [19], which is then converted into 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (MECDP) (**15**) with release of CMP by the catalytic action of the IspF protein [20, 21]. Cyclic diphosphate (**15**)

contains the 5-carbon branched motif required for IPP and DMAPP but is in a more highly oxidised state. This problem is addressed by the consecutive action of two structurally and mechanistically unique iron–sulphur enzymes specified by the ispG and ispH genes. More specifically, the IspG protein catalyses the reductive opening (two-electron reduction) of the eight-membered ring of **15**, which yields 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (HMBDP) (**16**) [22–25]. The residual primary hydroxyl group of **16** is then reductively removed by IspH protein [26] to give IPP and DMAPP (Fig. 3).

It is now firmly established that the non-mevalonate pathway is the single source of terpenoids in a large number of eubacteria [27]. Notably, Gram-positive cocci, *Coxiella burnetii* and *Borrelia burgdorferi* are the only major bacterial pathogens using the mevalonate pathway.

Moreover, the enzymes of the non-mevalonate isoprenoid pathway are essential in malaria parasites and in numerous pathogenic bacteria, which cause a wide variety of infectious diseases including tuberculosis that is estimated to cause around a million fatalities per year (Table 1) [28]. Due to the absence of the non-mevalonate pathway in humans, any anti-infective drugs designed to interrupt isoprenoid biosynthesis in the respective pathogens should be exempt from target-related toxicity, and understanding the structures and mechanisms of the pathway enzymes is important for inhibitor design.

At this time, there is no report of inhibitors of enzymes of the MEP pathway, other than DXR, with proven antimicrobial activity at pharmaceutically relevant concentrations.

3.2. Enzyme Inhibition in Drug Design: The Case of DXR

Identification of the *Escherichia coli* dxr gene and successful expression of the recombinant 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR, IspC, EC 1.1.1.86) were first reported in 1998 [29]. DXR is a well-studied 42–45 kDa enzyme with NADPH and a divalent cation (Mg^{2+}, Mn^{2+} or Co^{2+}) as cofactors. The enzyme typically has a pH optimum in the range 7–8 (for more details [30]). Table 2 summarizes the kinetic data available for DXR through mid-2011.

Two mechanisms have been proposed for the DXR-catalysed reaction, an α-ketol rearrangement (Fig. 4 path A) or a retroaldol/aldol rearrangement (Fig. 4 path B). Despite much effort, the catalytic mechanism of DXR remains elusive [37–44].

3.2.1. First Inhibition Studies to Design New Antimalarial Drugs—Fosmidomycin (FR-31564; 3-(N-formyl-N-hydroxy-amino)propyl phosphonic acid (Fig. 5, **1**)) was identified as a natural antibiotic from *Streptomyces lavendulae* [45]. Shortly after the discovery of DXR, it was established that this antibacterial compound is a mixed (competitive and non-competitive) inhibitor of *E. coli* DXR (*ec*-DXR) with a K_i of 38 nM [46]. When fosmidomycin was tested as an inhibitor of the *Z. Mobilis* DXR (*zm*-DXR), it was determined to be a competitive inhibitor with a K_i of 600 nM [31]. A related natural product, FR900098 (Fig. 5, **2**), is also an effective inhibitor of DXR and has shown greater antimalarial activity than fosmidomycin in a mouse model [47]. Subsequent work has shown that fosmidomycin is a slow, tight-binding inhibitor of DXR, with an initial phase of inhibition that is competitive with the substrate (DXP) and a second phase of inhibition that is non-competitive [33]. A conformational change in the enzyme has been proposed for this second phase and, in 2005, Mac Sweeney, *et al*. solved the X-ray crystal structure of the *ec*-DXR in a ternary complex with fosmidomycin and NADPH representing this closed conformation of the enzyme [48].

In 2006, Proteau and co-workers synthesised and evaluated fosmidomycin analogues as inhibitors of *Synechocystis sp*. PCC6803 DXR (*spcc*-DXR). The broad range of inhibition potencies of these analogues (go through and replace w/analogues) provided information about structural requirements for the design of more potent inhibitors [49]. Singh and coworkers published a review that summarizes the developments in DXR inhibition to 2007 [50]. Although fosmidomycin was shown to be effective in several clinical trials, high doses of the compound are required to achieve the desired results. This seems in part to be due to the highly polar nature of this molecule [51]. Therefore, Ortmann *et al*. addressed the question by synthesising more lipophilic and larger acyl residues and evaluating them against *ec*-DXR [52] (Fig. 5, Table 3). Unfortunately, these derivatives/analogues invariably have considerably reduced DXR inhibitory activity and exhibit no antibacterial activity. In addition, high-throughput screening of 32.000 compounds only yielded 30 hits with $IC_{50}<$ 20 μM. However, the structures of these hits were not disclosed and the active compounds therefore cannot be confirmed [53].

In order to expand the structure-activity relationships of non-hydrolysable phosphate mimics, linear modifications of the fosmidomycin structure was carried out by Link and coworkers in 2008 [54]. A set of phosphonic acids with inhibitory activity in the range from 0.1 to 20 μM against *ec*-DXR is presented in Fig. 6 and Table 3.

Later, by using coordination chemistry combined with a structure based approach, Deng *et al*. obtained a large series of compounds that were tested against *ec*-DXR (Fig. 7, Table 3) [55]. Amongst them, they identified for the first time, a strong, lipophilic DXR inhibitor (compound **14**), which has a structure that is distinctly different than fosmidomycin.

Nevertheless, fosmidomycin remained the preferred scaffold for the design of new inhibitors of DXR. Additional structural variations around the phosphonate anchor and the spacer of this compound gave new whose structures are given in Fig. 8 and IC_{50} are presented in Table 3 [56, 57]. Indeed, a new highly active and selective *pf*-DXR inhibitor with a unique chemical structure (compound **28**) was developed by Behrendt *et al*. [57]. Molecule **28** is an α-phenyl phosphonic acid derivative of **17**. Data are not available for the corresponding α-phenyl derivative of fosmidomycin (**26**).

A series of phosphonated *N*-heteroarylcarboxamides have also been synthesised and evaluated against *ec*-DXR but all are poor inhibitors. The most active compounds are presented in Fig. 9 and Table 3 (**29–31**) [58].

More recently a new class of pyridine/quinolone containing phosphonates that inhibits DXR (Fig. 9, **32–36** Table 3) with IC_{50} as low as 0.84 μM were identified. These compounds provide a new scaffold for further inhibitor development. Moreover, they can be used to identify potential hydrophobic pockets in DXR [59, 60].

3.2.2. Structural Basis for Future Design and Development of Clinically Effective Inhibitors—Reuter and co-workers published the first structure of DXR [61]. This structure provided an initial look at the general fold of the protein. The enzyme is a homodimer with three distinct domains: a N-terminal domain presenting a typical Rossmann folded NADPH binding site, a central connecting domain, and a C-terminal helical domain. The V-shaped central domain contains most of the active site residues, as well as a flexible loop region that appears to function as a lid over the active site. Due to this intrinsic flexibility, it has been proposed that binding of cation, substrate/inhibitor could induce a conformational change [62]. From 2002 till 2005, five other articles have been published reporting crystal structures of the *Z. mobilis* DXR [63] and *E. coli* DXR [48, 62, 64, 65].

In 2005, a fragment-based approach was performed by Mercklé *et al*. [66] to understand the inhibition of DXR. None of the inhibitors they tested showed time-dependent inhibition of DXR suggesting that these compounds are not able to take up a conformation that is capable of inducing full-loop closure [48, 67]. Moreover, a close structural analogue of fosmidomycin ((S)-*N*-hydroxy-4-(phosphoryloxy) methyloxazolidin-2-one) showed neither cooperative nor time dependent inhibition of DXR, despite the fact that modelling and docking studies suggested it should be able to bind to the enzyme. These results highlighted two significant problems with simulated-docking strategies with this enzyme. The first is that standard force-field implementations are inadequate for simulating metal binding, and secondly the large changes in flexible loop conformation that accompany inhibitor binding can be difficult to simulate.

Nevertheless, in the absence of crystallographic structures from pathogenic microorganisms such as *P. falciparum* or *M. tuberculosis* during this period, molecular modelling continued to be used. This allowed several research groups to gain insight into the structure and function of the enzyme and also facilitated structure-based inhibitor design. In 2007, the first model of *mt*-DXR was built and evaluated on the basis of its ability to explain several site-directed mutagenesis data [68]. Furthermore, a comparison of this homology model with the X-ray structure published later [69, 70] shows excellent agreement and validates the theoretical approach. Similarly, a 3-D model of *pf*-DXR was published in 2010. This model was validated using structure-checking programs and protein docking studies and subjected to structure-function analysis of its active site and to ligand docking studies. It was also used to develop an efficient screening method to identify potential lead compounds for use in the rational design of novel DXR inhibitors, where IC_{50}s could be estimated [71]. A further validation of this model will be possible when a full account of a preliminary X-ray crystallographic study of *pf*-DXR is published [72]. The recent crystal structure of the hyperthermophile *T. maritima* DXR (*tm*-DXR) reveals a new extra space available for potential drug design. Moreover, the structure adopted the closed form by rigid domain rotation [48, 69] but without the flexible loop over the active site, to give a novel conformation for this enzyme [37].

Very recently, with further improvements to the NMR experimental procedure and sample conditions, it has been possible to solve a part of the flexible loop of *ec*-DXR and further experiments might provide information about the conformational changes in the protein during substrate and cofactor binding. The results published by Englebert *et al*. represent one of the relatively few NMR reports of a large ternary homodimeric complex. In particular, the experimental procedures reported will provide helpful clues for NMR-based drug-design for large flexible molecular targets [73].

In conclusion, biochemical approaches (e.g. [74]), together with computational approaches and high-throughput screening assays (e.g. [75]), should provide additional inhibitors of DXR. While much remains to be learned, considerable progress has been made in recent years and the prospects of obtaining clinically effective DXR inhibitors seems attainable.

4. ISOMERISATION AND ELONGATION OF THE POLYPRENYL CHAIN

DMAPP and IPP are both required as building blocks for the biosynthesis of terpenes. Since the primary product of the mevalonate pathway is IPP, an IPP isomerase is absolutely required for the formation of DMAPP as a prerequisite for elaboration of the downstream terpene pathways. On the other hand, the non-mevalonate pathway can function even in the absence of an isomerase since DMAPP and IPP can both be produced by IspH enzyme [26].

The five-carbon compounds IPP and DMAPP are condensed to form the 10-carbon geranyl diphosphate (GPP) (**17**). GPP serves as the precursor for the synthesis of all monoterpenes. The addition of another IPP unit to GPP yields the 15-carbon farnesyl diphosphate (FPP) (**18**). The enzyme FPP synthase catalyzes the synthesis of both GPP and FPP in mammals [76]. FPP sits at the branch-point between sterol and longer-chain nonsterol synthesis. The enzyme squalene synthase catalyzes the head-to-head condensation of two FPP molecules to form the sterol precursor squalene [77]. Subsequent cyclization steps lead to sterol synthesis. Geranylgeranyl diphosphate synthase catalyzes the addition of IPP to FPP to form GGPP (**19**) [78] (Fig. 10).

Both farnesyl and geranylgeranyl residues appended posttranslationally to large family of proteins in Eukaryotes. The hydrophobic groups enhance membrane association essential for protein function. These postransational modifications are extensively studied especially in the case of cancer treatment [79] and bone resorption [80].

In this section of the review, we will focus on isopentenyl diphosphate isomerase (IDI) and inhibition studies to help understand the mechanism of the isomerisation of IPP to DMAPP.

4.1. Isopentenyl Diphosphate Isomerase

Isopentenyl diphosphate:dimethylallyl diphosphate (IPP: DMAPP) isomerase (IDI; EC 5.3.3.2) is a key enzyme involved in the biosynthesis of isoprenoids and catalyses the isomerisation of IPP into DMAPP (Fig. 11).

Two types of IDIs are reported. They show no sequence similarity but catalyse the same reaction. Type I IDI (IDI-1) is a metalloprotein containing divalent cations, Zn^{2+} and Mg^{2+}, as cofactors [81–83]. Crystal structures of free and metal-bound *Escherichia coli* IDI-1 show that Zn^{2+} is involved in the active conformation folding, with the metal occupying a coordination site composed of three histidines and two glutamates [84]. Several lines of evidence have established the mechanism for isomerisation catalysed by IDI-1. In particular, studies with IPP analogues have provided extensive support for substrate protonation to generate a transient carbocationic intermediate. Epoxide and diene analogues (3,4-epoxy-3-methylbutyl diphosphate (eIPP) and 3-methylene-4-penten-1-yl diphosphate (vIPP), respectively) were shown to irreversibly inhibit the enzyme through formation of covalent adducts with active site cysteine and glutamate residues [85, 86]. In both cases, protonation serves to activate the analogue for attack by an active site nucleophile. N, N-dimethyl-2-amino-1-ethyl diphosphate (nIPP), a reactive intermediate analogue with a positively charged ammonium group, binds to the type I enzyme with subnanomolar affinity [87]. In addition, IPP and DMAPP analogues substituted with strong electron-withdrawing fluorine groups are poor substrates for isomerisation [87, 88]. These studies provide evidence for a mechanism that involves protonation at the double bond of IPP, followed by deprotonation of the carbocationic intermediate to generate DMAPP as the product [89–93].

It is important to note that type 2 IPP isomerases are essential enzymes in several classes of microorganisms using exclusively the mevalonate pathway, including *Streptococci*, *Staphylococci*, and *Enterococci* (Table 1). The occurrence of methicillin-resistant *Staphylococci* (MRSA) and vancomycin-resistant *Enterococci* (VRE) is increasing rapidly [94–96], and novel chemotherapeutic strategies are urgently needed. IPP isomerase, and more precisely IDI-2, inhibitors could thus possibly serve as selective drugs for infections by drug-resistant strains. In contrast to IDI-1, IDI-2 was discovered recently and its mechanism is still under investigation. This section will highlight the recent advances made on this enzyme.

4.1.1. Identification and Characterisation of IDI-2—In 2001, Kaneda *et al*. isolated an open reading frame (ORF), *orfD*, encoding an unknown protein. The recombinant product of *orfD* was purified as a soluble 37 kDa protein. The enzyme catalysed the isomerisation of IPP into DMAPP in the presence of both FMN and NADPH in bacteria and was classified as Type 2 IDI for the FMN- and NAD(P)H-dependent enzyme [97]. Since then different research groups focused their attention on this new interesting enzyme. They all concluded that IDI-2 requires reduced flavin mononucleotide and $MgCl_2$ as cofactors. Table 4 summarises the kinetic and crystal data available in the literature until 2011. A crystallographic state of the art of the enzymes involved in isoprenoid biosynthesis was published in 2008 [98].

Steinbacher and co-workers reported the first structure of IDI-2 from *B. subtilis (bs-IDI2*) at 1.9 Å resolution (PDB ID: 1P0K) in 2003 [99]. The monomer of IDI-2 consists of a regular TIM barrel (α8β8 barrel) domain. The N-terminal region forms an irregular structure and an anti-parallel two-stranded β-sheet that function as a support at one side of the barrel, a structural feature commonly observed for TIM barrels. The FMN cofactor is located in the standard phosphate binding (SPB) region of the TIM barrel as described for several FMN-dependent proteins [111]. However, due to the conformational flexibility of the enzyme, electron density was not detected for several amino acid residues at the active site of *bs-IDI2*. In 2008, we solved the first complete structure of IDI-2 from *T. thermophilus* (*tt*-IDI2) in complex with inorganic pyrophosphate (PDB ID: 3DH7) [107] confirming the theoretical model obtained in 2005 [112].

4.1.2. Inhibition Studies to Further Understand the Enzymatic Mechanism—Most flavoproteins carry out reactions with net redox changes: including oxidations, reductions, monooxygenations, dioxygenations and electron transfers. However, there are a number of unusual flavoproteins that catalyze reactions with no net redox change. These fall in four groups: those that utilize two-electron flavin chemistry, others that involve free radical flavin chemistry, a number where the role of flavin is not yet clear and the remainder that intriguingly do not appear to involve the flavin directly in catalysis (for review [113]).

The first mechanism proposed for IDI-2 by Kaneda *et al*. in 2001 was based on this of IDI-1, which involved a carbocationic intermediate [106] (Fig. 12 (**2**)). In 2004, Hemmi and co-workers performed spectrometric analyses and enzyme assays under anaerobic conditions, proving that the reduced form of the flavin co-enzyme ($FMNH_2$) is sufficient for the enzyme to catalyse the isomerase reaction. Moreover, using 5-deaza FMN as cofactor, *S. shibatae* IDI-2 (*ss*-IDI2) was nearly devoid of IPP isomerase suggesting the possibility of a redox role of FMN. Based on these results, they proposed a reaction mechanism, similar to the one reported for UDP-galactopyranose mutase [114], in which a radical intermediate is generated [115].

Indeed, detection of trace amounts of isolated neutral flavin semiquinone in reaction mixtures by electron paramagnetic resonance (EPR) provided initial evidence for a radical mechanism (Fig. 13). However, no coupled substrate radical was observed. Subsequent attempts to detect intermediates in stopped-flow studies under single turnover conditions failed and several radical clock mechanistic probes also failed to reveal the existence of radical substrates intermediates [108, 116].

In 2006, Eguchi and co-workers studied the inhibition of *M. jannaschii* IDI-2 (*mj*-IDI2) by eIPP, a mechanism-based inhibitor of IDI-1. The mechanism for inhibition of wild type *E. coli* IDI-1 by eIPP involves the protonation of the epoxide ring in eIPP, which alkylates the nucleophilic thiolate moiety of a cysteine (Cys-67) in the active site [89] Eguchi and co-workers assumed that if protonation of the double bond in IPP was the first step in the

reaction catalysed by IDI-2, as in the case of IDI-1, eIPP would inhibit the enzyme by alkylation of a near-by nucleophilic group of the IDI-2 active site (Fig. 14a). Upon incubation of racemic eIPP with *mj*-IDI2, they observed a time- and concentration-dependent decrease of the enzymatic activity (K_I = 56.6 mM, k_{inact} = 0.10 s^{-1}, k_{inact}/K_I = 1.76 s^{-1} M^{-1}) suggesting that the IDI-2 reaction proceeded *via* a carbocation-type intermediate as in the case of IDI-1. During the isomerisation, the aromatic ring of the reduced flavin should stabilise the carbocation intermediate [109] (Fig. 14b).

To further probe the mechanism of the IDI-2 reaction, additional epoxy and new cyclopropyl substrate analogues (oIPP, cIPP and cDMAPP), designed as mechanism-based irreversible inhibitors, were synthesised and evaluated against IDI-2 from *T. thermophilus* (*tt*-IDI2) by Walker and coworkers [117, 118]. They observed by ^{1}H NMR that cIPP isomerized to cDMAPP and that a similar experiment starting with cDMAPP resulted in the transient production of cIPP, with the unstable cDMAPP analogue undergoing non-enzymatic solvolysis. They concluded that these diphosphates were alternative substrates that do not irreversibly inhibit IDI-2. In contrast, incubation of reduced IDI-2 with oIPP resulted in a rapid first-order irreversible loss of activity of the enzyme (K_I = 1.4 μM, k_{inact} = 0.37 min^{-1}). Combined with mass spectrometry and UV spectroscopy, those results showed that the epoxide ring is activated by protonation at oxygen rather than by hydrogen atom addition to the double bond of oIPP, followed by trapping of a radical intermediate by flavin semiquinone [117, 118]. This mechanism is consistent with the previous one suggested by Hoshino [109] and similar to the one adopted for IDI-1. So additional IDI-1 irreversible inhibitors (Fig. 15), epoxy, diene and fluorinated substrate analogues, were analysed as mechanistic probes for IDI-2 by Poulter and coworkers [119].

eIPP (K_I = 48.6 μM, k_{inact} = 0.041 min^{-1}), vIPP (K_I = 8.0 μM, k_{inact} = 1.2 min^{-1}) and 3-(fluoromethyl)-3-buten-1-yl diphosphate (fmIPP) (K_I = 7.4 μM, k_{inact} = 0.044 min^{-1}) all inactivate *tt*-IDI2 through formation of covalent adducts with the reduced flavin. At this point, UV-visible spectra of the inactivated complexes were consistent with modification of the isoalloxazine ring at N5. Two other alkyne (3-butyn-1-yl diphosphate (1-OPP), K_I = 48 μM) and allene (2,3-butadien-1-yl diphosphate (2-OPP), K_I = 36 μM) substrate analogues for the enzyme were synthesised to distinguish between the mechanisms initiated by proton transfer and hydrogen atom transfer to the double bond in IPP and suggested a protonation-deprotonation mechanism for the enzyme-catalysed isomerisation of IPP and DMAPP [120]. Consistent with a carbocationic mechanism, the alkyne and allene analogues were not substrates. Inhibition constants are summarised in Table 4.

Based on the latest results, Poulter and Hemmi's groups performed UV, structural and mutagenic studies and demonstrated that reduced FMN, not amino acid residues, acts as a general acid-base catalyst in the protonation-deprotonation mechanism [101, 119]. They proposed a mechanism where the N5 nitrogen of FMN seems the most plausible candidate for the catalyst and that this mechanism should be distinct from the established one for human type 1 IDI because of their antarafacial and assumed suprafacial nature, IDI-1 and IDI-2 respectively (Fig. 16).

Additional studies performed on IDI-2 from *Staphilococcus aureus* IDI-2 (*sa*-IDI2) by the Liu research group are consistent with the growing body of experimental evidence suggesting that the flavin coenzyme of IDI-2 serves a novel function as an acid-base catalyst [105, 116, 121].

5. CONCLUSION

The structural diversity of isoprenoids is immense, including acyclic, monocyclic and polycyclic compounds. Terpenoids play important roles in all living organisms. They function as regulators of gene expression, constituents of membranes, vitamins, antimicrobial agents, mating pheromones, reproductive hormones, components of signal transduction pathways, and constituents of electron transport and photosynthetic machinery. In light of the many diverse functions of terpenes, it is not surprising that their biosynthesis has been subject of intense investigation for more than four decades.

The first pathway starting from mevalonate and leading to the building blocks of large isoprenoids was discovered in 1950s. Only recently, an alternative route was discovered requiring decarboxylation of mevalonate 5-phosphate to produce the substrate for the isopentenyl 5-phosphate kinase (IPK), which would catalyze formation of IPP.

The second pathway starting from 1-deoxy-xylulose 5-phosphate is present in some human pathogens and plants and totally absent from human species. This is why the enzymes of this pathway were extensively characterised in order to design new anti-infectious agents or herbicides. The most representative example is the alternative treatment of malaria and tuberculosis. Indeed, multi-drug resistant strains of pathogens have recently forced many countries to change national treatment protocols.

This review summarises the latest improvements made on a better comprehension of two key enzymes (DXR and IDI) involved in the biosynthesis of isoprenoids by pathogenic microorganisms.

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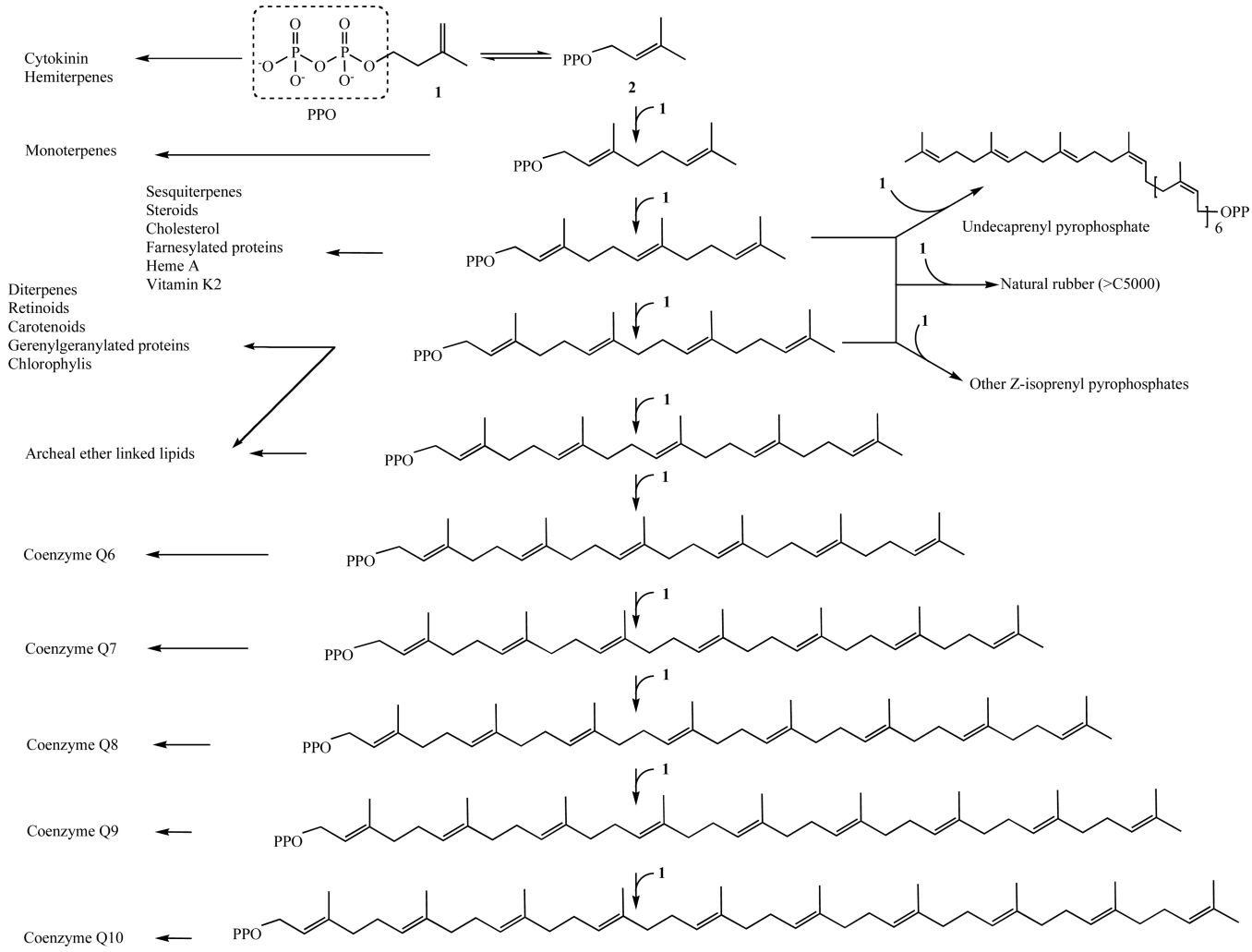

Fig. 1.

Biosynthetic pathways catalyzed by isoprenyl diphosphate synthases and the final reaction products. Adapted from Wang and Ohnuma [3].

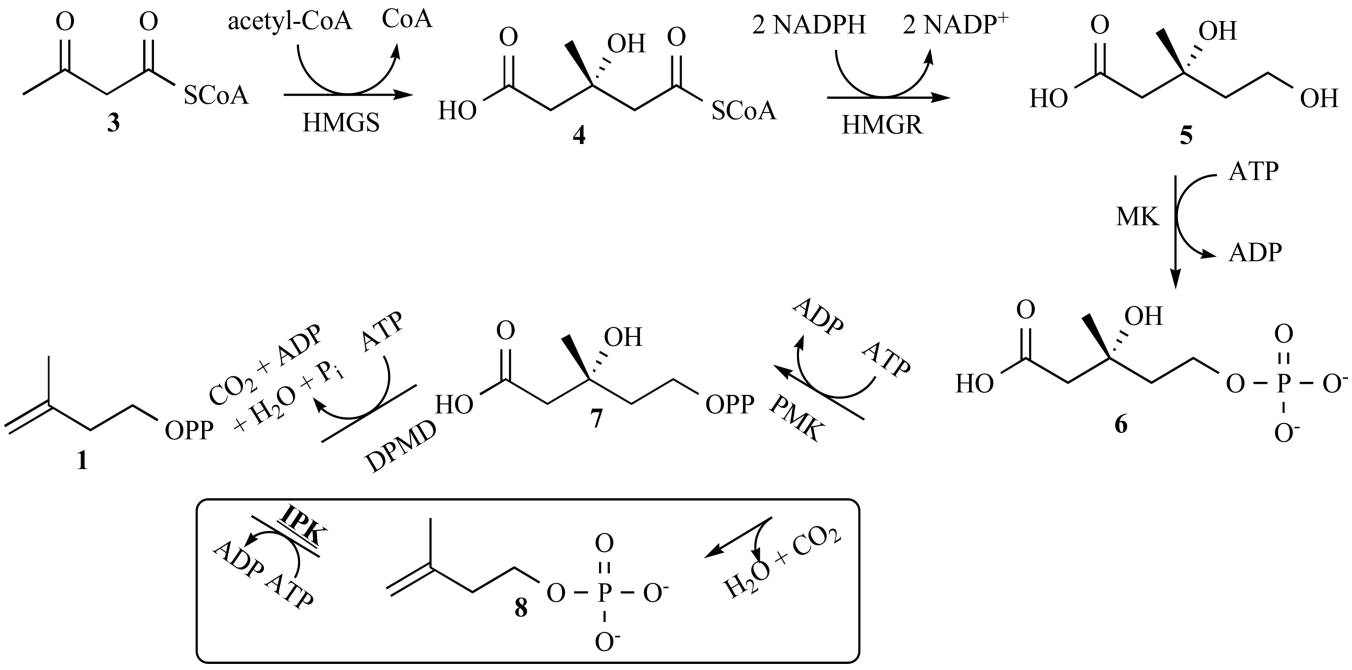

Fig. 2.

Overview of the well-described mevalonate pathway. The alternative route described by Grochowski *et al*. in Archae is represented in the box.

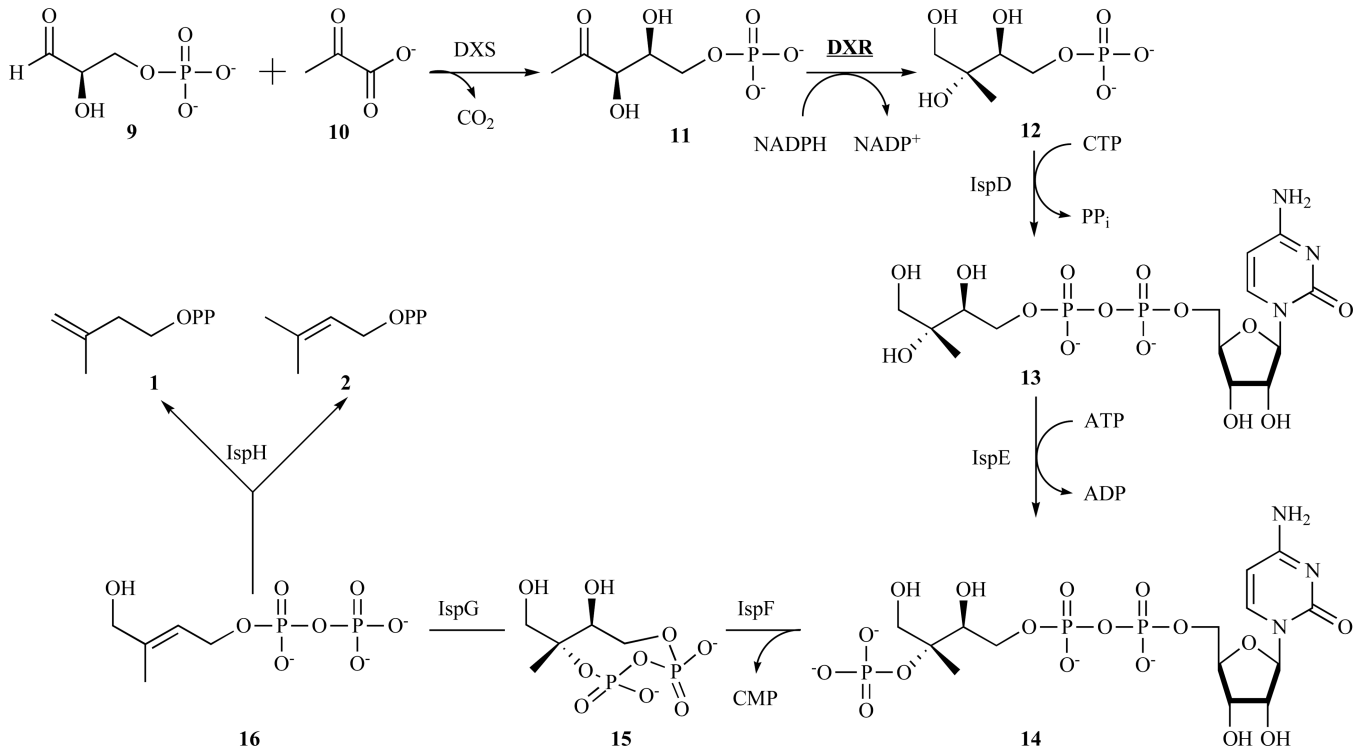

Fig. 3.

Overview of the mevalonate-independent pathway. Interestingly, IPP (**1**) and DMAPP (**2**) are obtained in a kinetically controlled reaction.

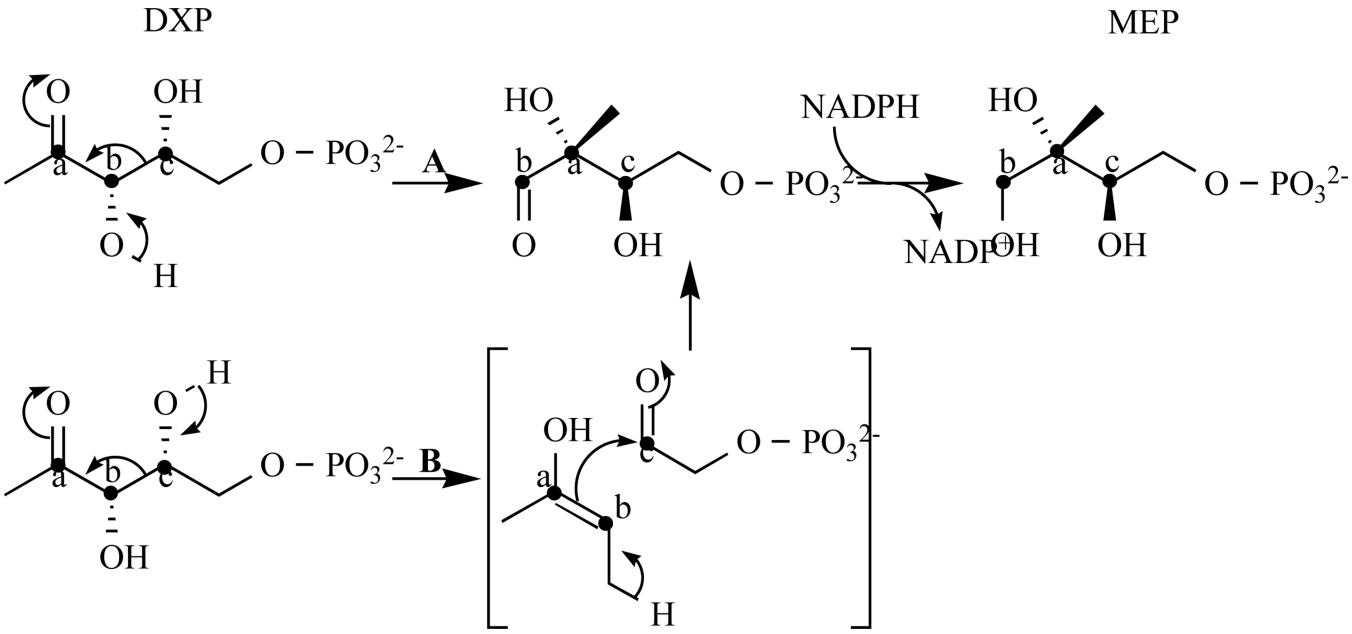

Fig. 4.
Representation of path A: α-ketol or path B retroaldol/aldol rearrangement mechanisms. This latter is preferred by Munos *et al*. [44].

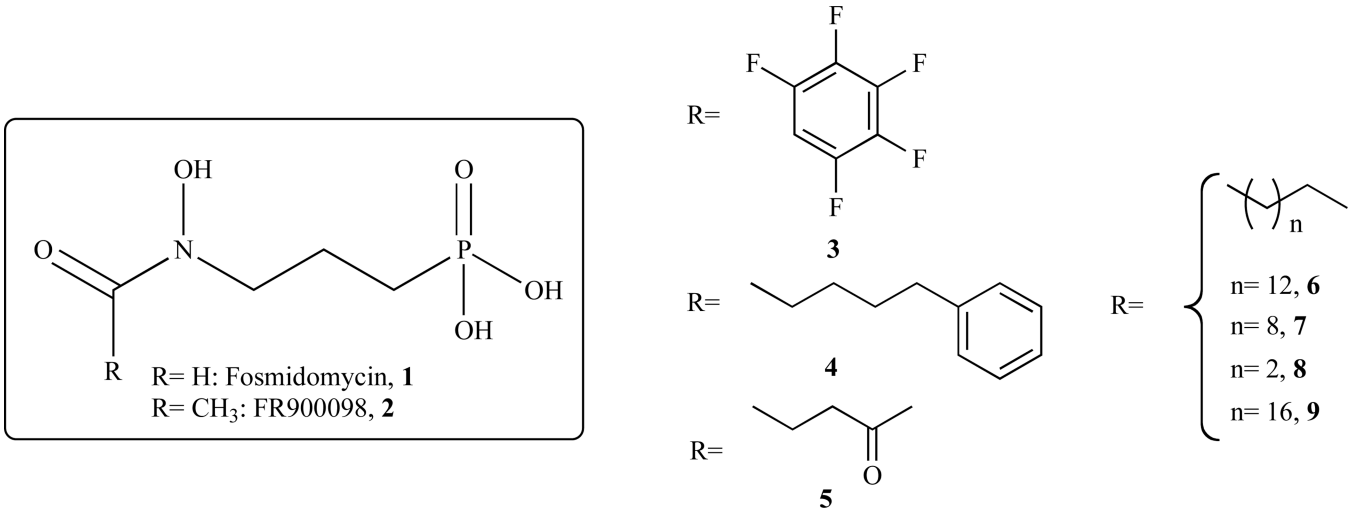

Fig. 5.

Chemical structures of selected compounds synthesized and evaluated by Ortman *et al*. Chemical structures of the reference inhibitors are represented in the box.

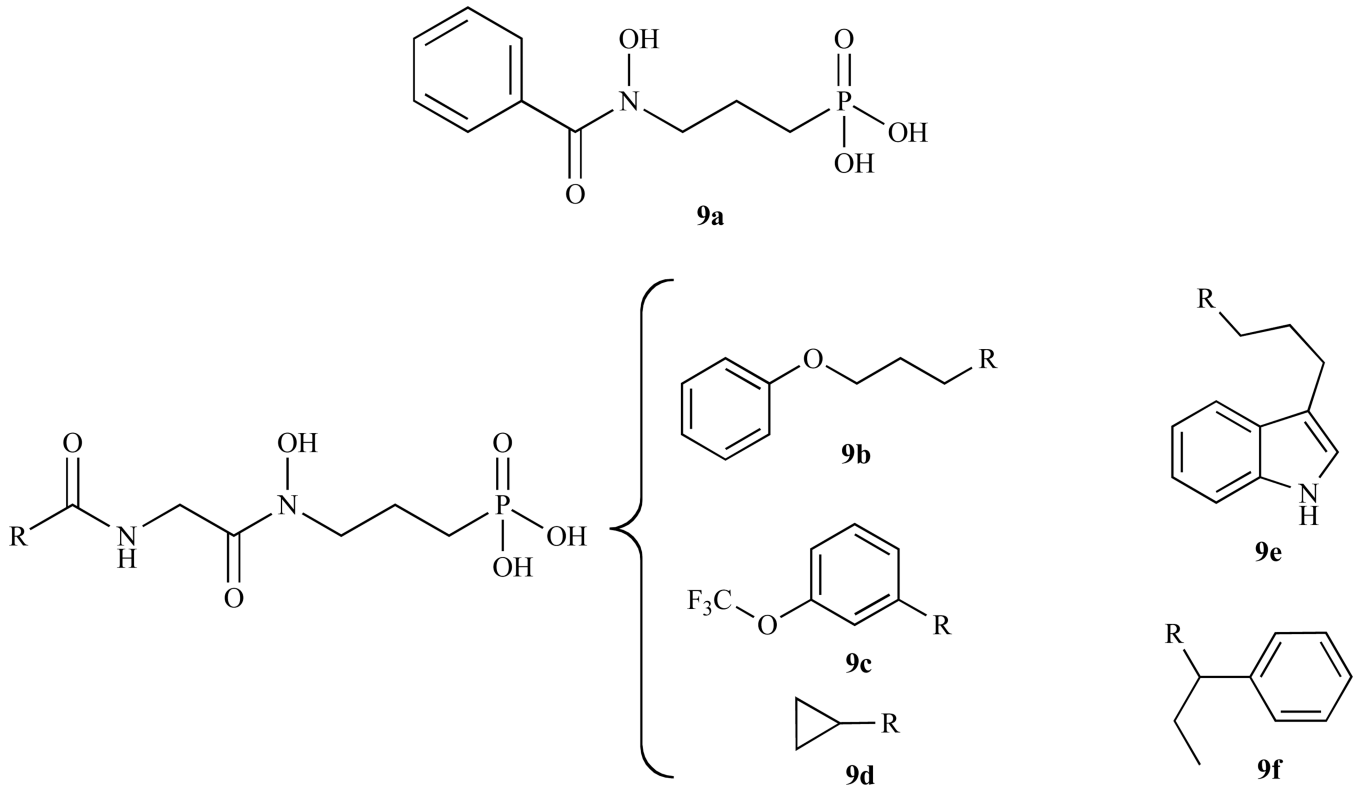

Fig. 6.
Chemical structures of selected compounds synthesized and evaluated by Link *et al*.

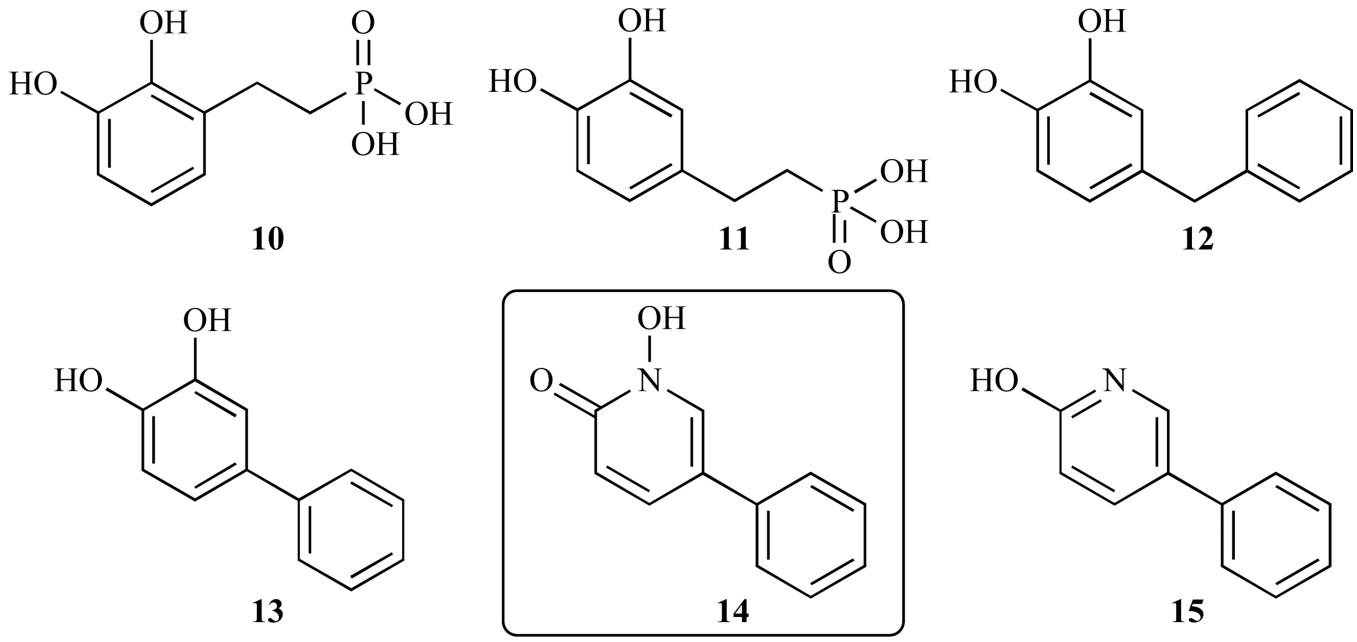

Fig. 7.
Chemical structures of selected compounds synthesized and evaluated by Deng *et al*. Compound **14** is strong, lipophilic inhibitor and has a distinct structure from fosmidomycin.

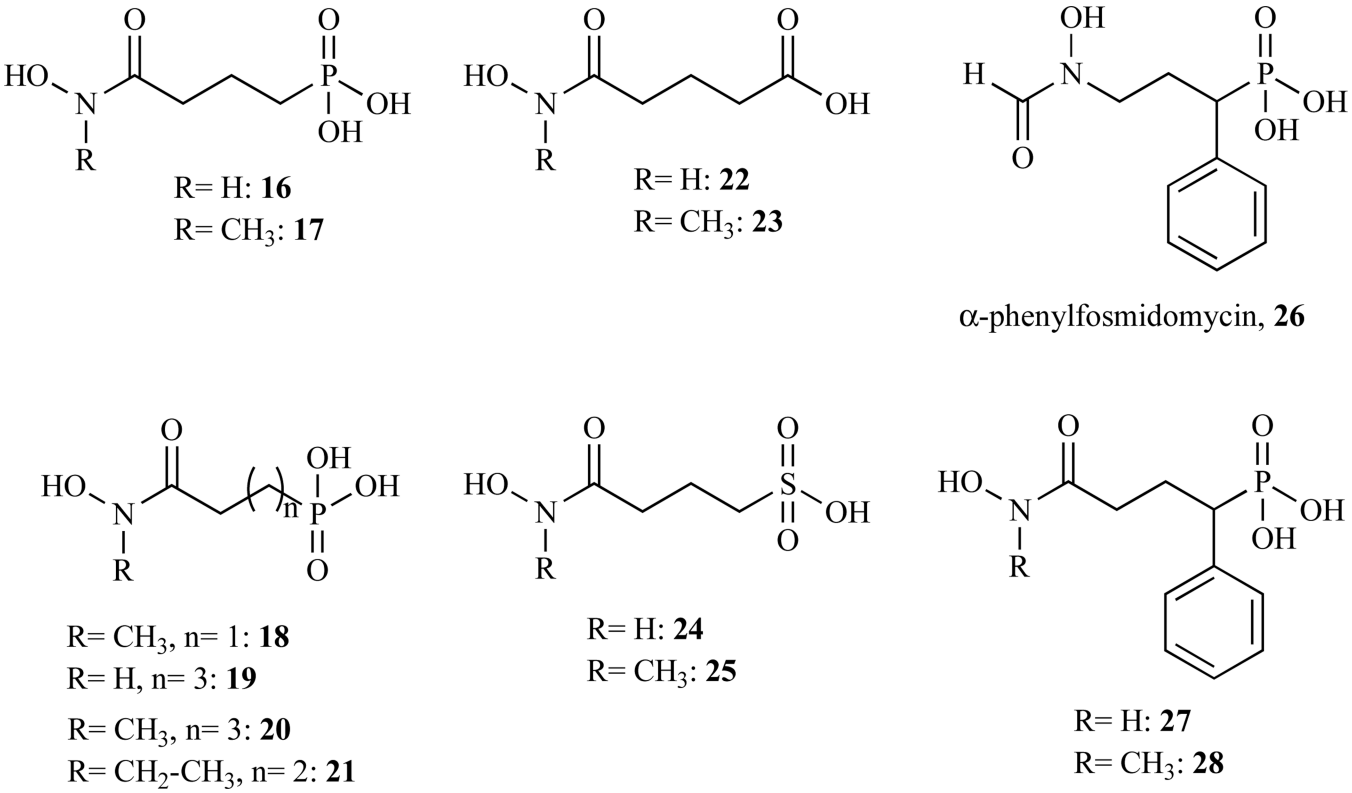

Fig. 8.

Chemical structures of selected compounds synthesized and evaluated by Zinglé *et al*. (**16–25**) and by Behrendt *et al*. (**26–28**).

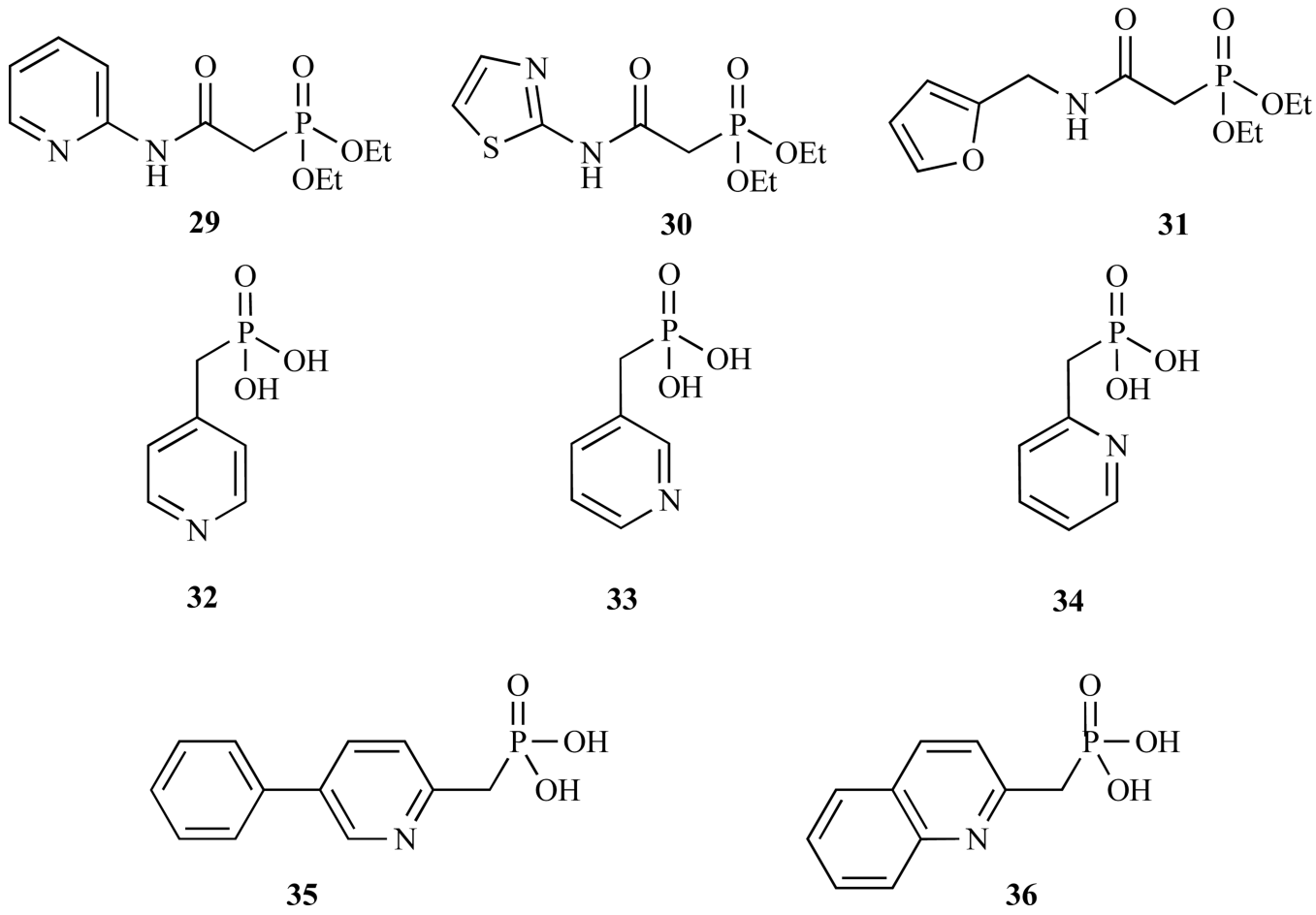

Fig. 9.
Most recent chemical structures of selected compounds synthesized and evaluated against DXR.

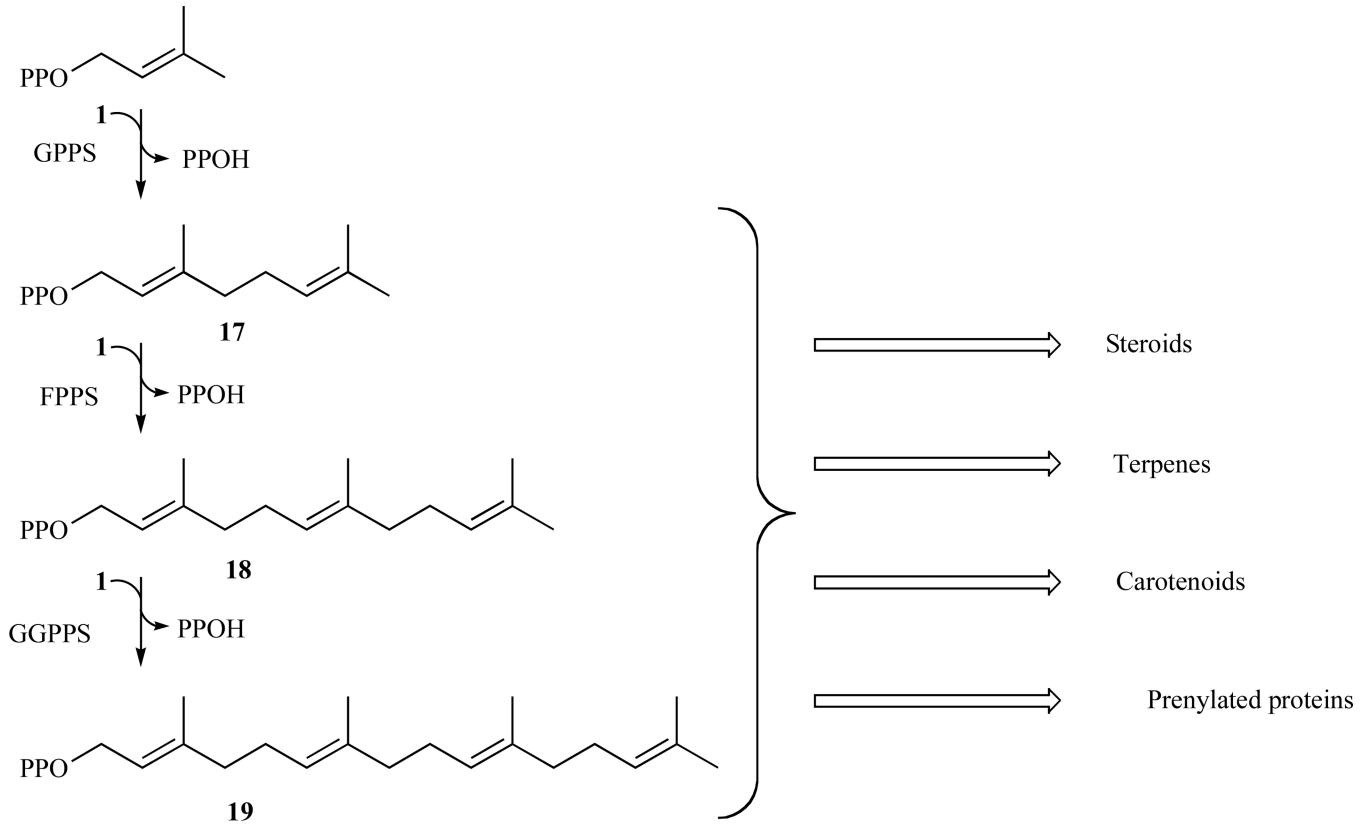

Fig. 10.
Elongation of isoprenoids in order to obtain complex and diverse essential molecules.

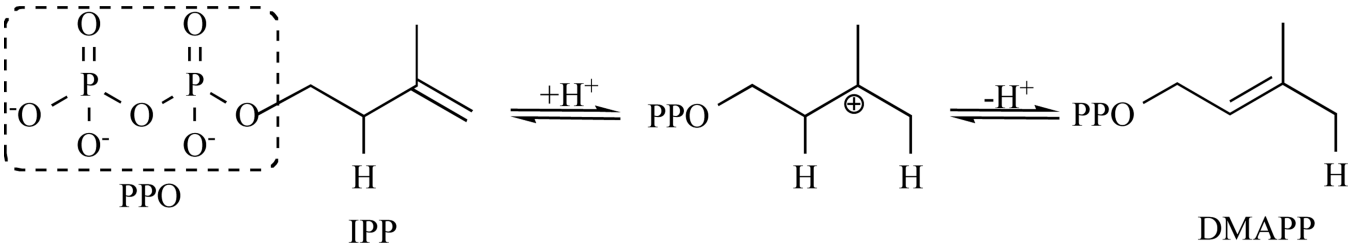

Fig. 11.
Representation of the catalytic mechanism of IDI-1 *via* protonation of the IPP and deprotonation of the carbocationic transition state.

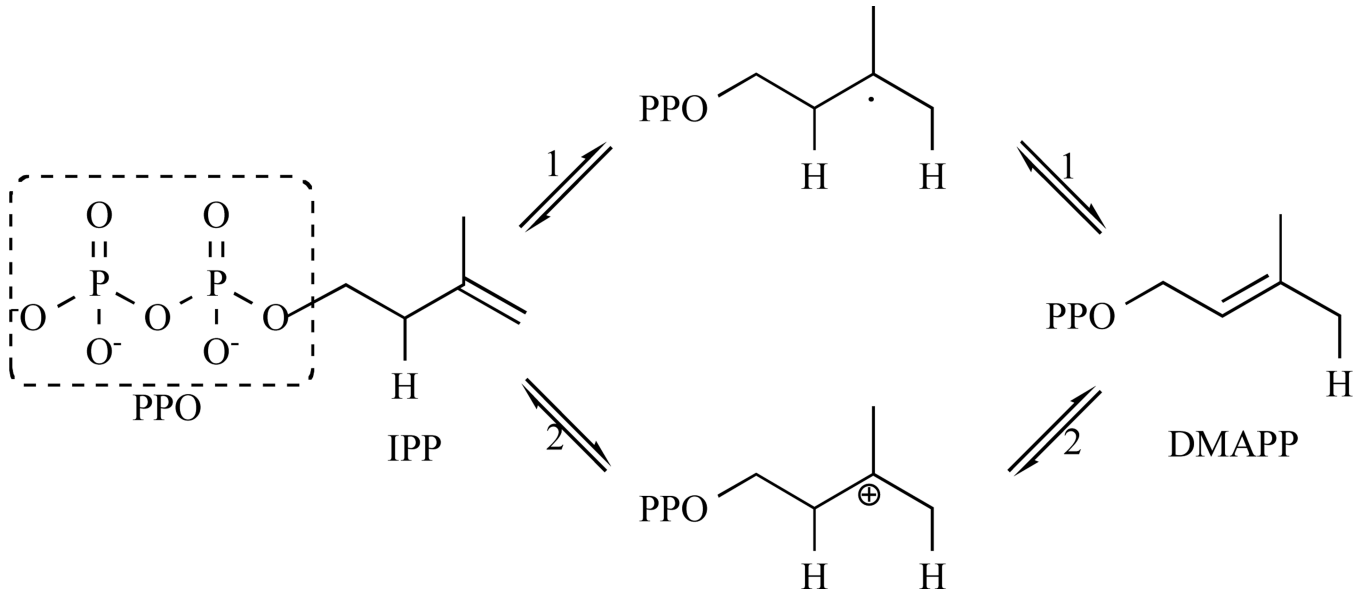

Fig. 12.

Representation of the two putative IDI-2 mechanisms through a radical rearrangement (1) or through a protonation/deprotonation mechanism similar to IDI-1 (2).

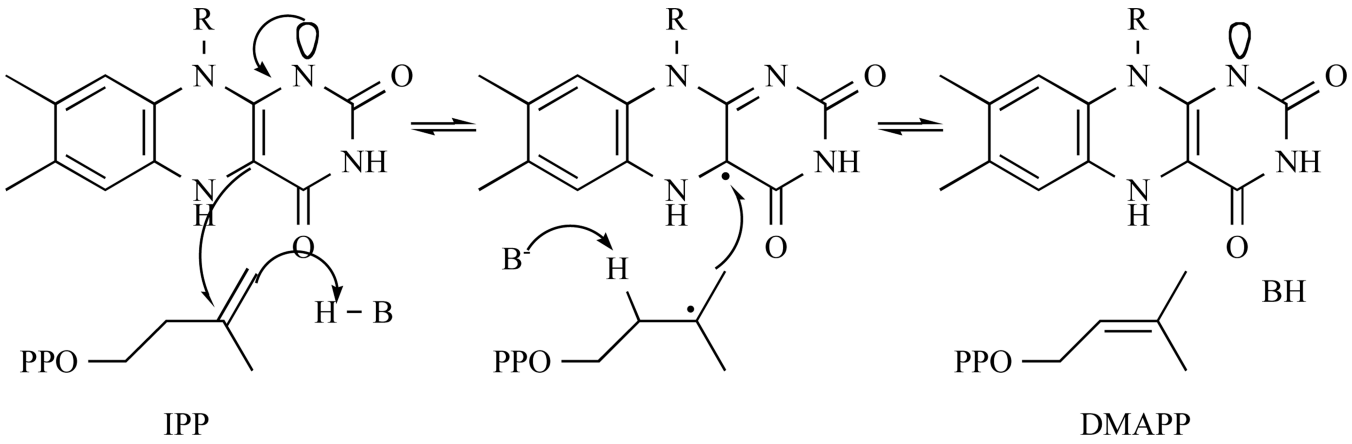

Fig. 13.
Representation of the radical mechanism suggested by Hemmi *et al*. and similar to the UDP-galactopyranose mutase mechanism.

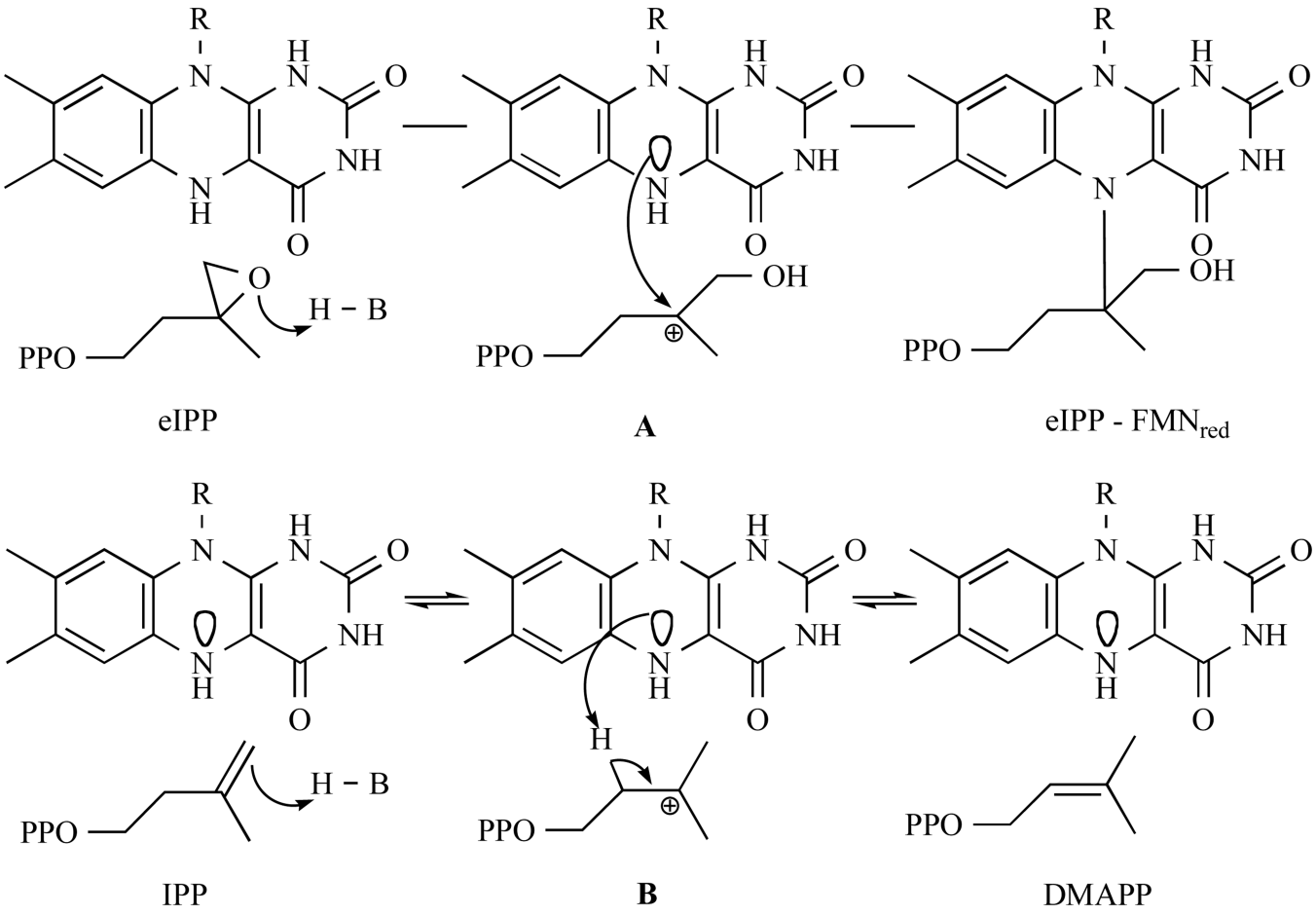

Fig. 14.

a. Representation of the inhibition mechanism of eIPP against IDI-2. First epoxide is activated by protonation and then an attack of a near-by nucleophilic group of the IDI-2 active centre form covalent bond. **b**. IDI-2 reaction is similar to IDI-1, which proceeds *via* carbocation-type intermediate. The $FMNH_2$ is acting as a nucleophilic group.

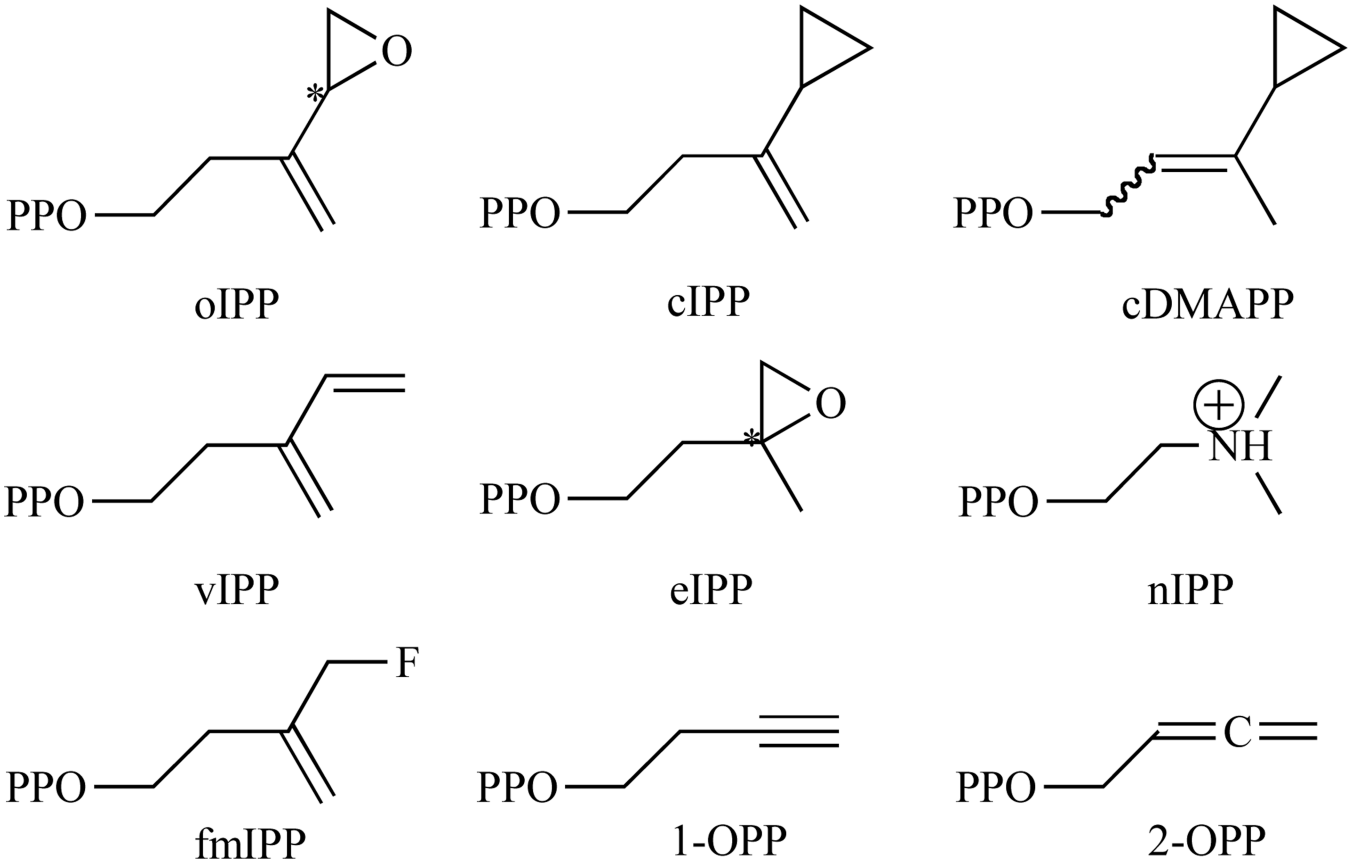

Fig. 15.
Chemical structures of the studied IDI-2 inhibitors (substrate or product analogues).

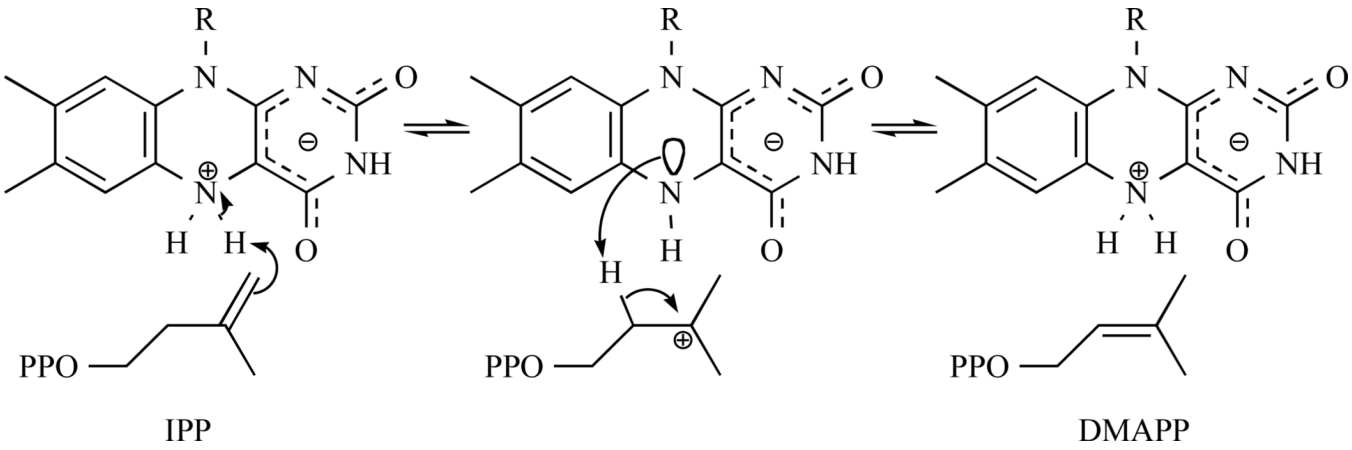

Fig. 16.
Latest suggested IDI-2 mechanism where the N5 nitrogen of FMN seems the most plausible candidate for the catalyst.

Table 1

Distribution of Isoprenoid Biosynthetic Enzymes in Major Human Bacterial Pathogens. As for Reference Human Utilizes the MEV Pathway and Type I IPP Isomerase to Produce Isoprenoid Compounds

Microorganism	MEP pathway	MEV pathway	IPP isomerase	Examples of diseases
Gram-positive cocci				
Staphylococcus aureus	−	+	Type II	Impetigo follicularis, furunculosis
Staphylococcus epidermidis	−	+	Type II	Wound infections
Streptococcus pyogenes	−	+	Type II	Scarler fever, toxic-shock syndrome
Streptococcus agalactiae	−	+	Type II	Neonatal septicaemia
Streptococcus viridans	−	+	Type II	Endocarditis lenta
Streptococcus pneumoniae	−	+	Type II	Pneumonia
Enterococcus faecalis	−	+	Type II	Endocarditis, Bacteraemia
Gram-negative cocci				
Neisseria meningitis	+	−	−	Meningitis
Neisseria gonorrhoea	+	−	−	Gonorrhoea
Gram-positive rods				
Listeria monocytogenes	+	+	Type II	Listeriosis
Bacillus anthracis	+	−	Type II	Anthrax
Clostridium botulinum	+	−	−	Botulism
Clostridium tetani	+	−	−	Tetanus
Gram-negative rods				
E. coli	+	−	Type I	Enterocolitis
Salmonella typhi	+	−	Type I	Typhus
Salmonella paratyphi	+	−	Type I	Bacteraemia
Shigella sonnei	+	−	Type I	Typhus
Gram-negative/spiral-shaped bacteria				
Vibrio cholerae	+	−	Type I	Cholera
Helicobacter pylori	+	−	-	Gastritis Type B
Camphylobacter jejuni	+	−	-	Enterocolitis
Spirochaetal bacteria				
Borrelia burgdorferi	−	+	Type II	Lyme disease
Acid-fast rods				
Mycobacterium tuberculosis	+	−	Type I	Tuberculosis
Mycobacterium leprae	+	−	Type I	Leprosy
Obligate intracellular bacteria				
Rickettsia prowazecki	−	−	Type II	Typhus
Rickettsia rickettsii	−	−	Type II	Rocky mountain spotted fever
Coxiella burnetii	−	+	Type I	Q fever

Table 2

Enzymatic Characteristics of DXR form Several Microorganisms. Temperature is the Temperature at which Enzymatic Assays were Conducted

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	Temperature (°C)	Reference
Z. mobilis	300	14	0.05	40	[31]
S. coelicolor	190	19.2	0.10	25	[32]
E. coli	720	21.7	0.03	37	
	115	116	0.99	37	[33]
Synechocystis sp.PCC6803	134	5	0.04	37	[34]
M. tuberculosis	42	2.1	0.05	25	[35]
F. tularensis	104	2	0.02	22	[36]
T. maritima	110	5.5	0.05	85	[37]
	40	0.29	0.01	50	

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Table 3

Inhibition Power of Selected Among the Huge Amount of Synthesized Compounds. N.d. Stands for Not Determined

		Studied microorganism		IC50 (jiM)		Reference
	1	*E.coli*	*P.falciparum*	0.22	0.14	[57]
	2			0.13	0.015	
2007	**3**	*E.coli*		1.3		[52]
	4			5.1		
	5			5.4		
	6			5.6		
	7			9.0		
	8			10.0		
	9			< 30		
2008	**9a**	*E.coli*	*P.falciparum*	0.13	0.061	[54]
	9b			1.0	0.40	
	9c			5.1	2.9	
	9d			7.1	3.3	
	9e			17	6.6	
	9f			20	9.3	
2009	**10**	*E.coli*		24.8		[55]
	11			4.5		
	12			44.7		
	13			22.4		
	14			1.4		
	15			75		
2010	**16**	*E. coli*		0.17		[56]
	17			0.049		
	18			19		
	19			0.27		
	20			0.11		

		Studied microorganism	IC50 (μM)	Reference
	21		6.5	
	22		720	
	23		25	
	24		1000	
	25		48	
	26	*E. coli*	n.d.	[57]
	27	*E.coli*	*P.falciparum*	0.59	0.012
	28			0.24	0.003
2011	**29**	*E.coli*		472	[58]
	30			730	
	31			408	
	32			7.1	[59]
	33			10.0	
	34			4.6	
	35			0.84	
	36			15.9	

Table 4

Available Kinetic and Crystal Data for IDI-2 Until 2011. N.d. Stands for Not Determined.

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)	Optimal temperature (°C)	Crystal Structure(s)	Reference
B. subtilis	670	25	3.7×10^4	37	1P0K, 1P0N [99]	[100]
	n.d.	0.4	n.d.	n.d.		[27]
S. shibatae	63	0.2	3.2×10^3	60	2ZR(U–Z) [101]	[102]
Synechocystis sp.6803	52	0.2	4.4×10^3	37	n.d.	[103]
M. thermoautotrophicus	64	1.6	2.5×10^4	70	n.d.	[104]
S. aureus	16.8	0.7	4.1×10^4	n.d.	n.d.	[105]
	19	1.3	6.8×10^4	n.d.	n.d.	[106]
Streptomyces sp. CL190	450	0.7	1.6×10^3	35	n.d.	
T. thermophilus	5.6	0.2	3.2×10^4	n.d.	3DH7 [107]	[108]
M. jannashii	15.3×10^3	191	1.3×10^4	85–95	n.d.	[109]
T. kodakaraensis	84	76	9.0×10^5	80	n.d.	[110]

Table 4

Inhibition Parameters of the Studied Inhibitors. N.d. Stands for Not Determined

		Protein studied	K_I (μM)	k_{inact} (min^{-1})	k_{inact}/K_I ($s^{-1}.mM^{-1}$)	References
Inhibitors	eIPP	*mj*-IDI2	56.5 (mM)	0.10 (s^{-1})	0.002	[109]
		tt-IDI2	48.6 ± 8.2	0.041 ± 0.01	0.014	[117]
	oIPP	*tt*-IDI2	1.4 ± 0.3	0.37 ± 0.07	4.4	[119]
	vIPP	*tt*-IDI2	8.0 ± 2.0	1.2 ± 0.1	2.5	
	fmIPP	*tt*-IDI2	7.4 ± 0.9	0.044 ± 0.002	0.1	
	nIPP	*tt*-IDI2	5.1 ± 0.5	n.d.	n.d.	[108]
	1-OPP	*tt*-IDI2	48 ± 6	n.d.	n.d.	[120]
	2-OPP	*tt*-IDI2	36 ± 5	n.d.	n.d.	