

## Research Article



## Exploration of Lysine Pathway for Developing Newer Anti-Microbial Analogs through Enzyme Inhibition Approach

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### ABSTRACT

Recently, a renewed interest towards development of new antibacterial agents has been created due to emergence of newer pathogenic bacterial strains showing high resistance to such agents. There has also been a decline in research by medical and pharmaceutical companies in last decade which has caused a shortfall in developing newer agents to fight present threat of drug resistance. Hence, there is continuous need to develop newer antibiotics that interact with essential mechanisms in bacteria. Recently, the enzymes responsible for biosynthesis of the essential amino acid lysine in plants, bacteria and fungi have been targeted and it has augmented interest to develop novel antibiotic compounds and to enhance lysine yields in over-producing organisms. Diaminopimelate (*DAP*) pathway in bacteria for biosynthesis of lysine and its immediate precursor meso-*DAP* have been represented as novel targets, both of which play a major role in cross-linking of peptidoglycan layer of microbial cell wall. Lysine is a constituent in gram-positive bacteria while gram-negative bacteria contain meso-*DAP*. Substrate-based inhibitors of enzymes in the *DAP* pathway have been reviewed and inhibitors that allow better understanding of the enzymology of the targets and provide insight for the design of new inhibitors have been discussed in this article. Synthetic enzyme inhibitors of the *DAP* pathway with appropriate substrate-based analog have been found to be more effective against resistant bacterial strains and less toxic to mammals. The enzymes involved in this pathway may be viable targets and shall be supportive to develop novel antimicrobial drugs.

**Keywords:** Bacterial resistance, Diaminopimelic acid, Diaminopimelate decarboxylase, Enzyme analogue, L-lysine.

### INTRODUCTION

Antibiotics are the compounds that are literally 'against life' and are typically antibacterial drugs, interfering with structure or process that is essential for growth or survival of microorganisms with least harm to the mammals. As we live in an era when antibiotic resistance to microorganisms has spread at an alarming rate, there is a constant need to develop newer antibacterials which shall combat with the bacterial survival strategies. However, clinically significant resistance develops in periods of few months to years. For penicillin, the resistance began to be noted within two years of its introduction in the mid 1940s.<sup>1-3</sup> The recent emergence of mutated bacterial strains that are resistant to currently available antibiotics has resulted in renewed interest in the search for novel antibacterial compounds. Such compounds should be targeted toward proteins that are essential for bacterial viability but are not present in mammals.<sup>4,5</sup> The diaminopimelic acid and lysine biosynthesis meets both of these criteria, presenting multiple targets for novel antimicrobial agents<sup>6,7</sup>. Lysine has been known to be an essential amino acid required in protein synthesis and a constituent of the peptidoglycan layer of cell walls in gram positive bacteria. The lysine biosynthesis also produces D,L-diaminopimelic acid (*meso-DAP*), which is also a component of the peptidoglycan layer of gram negative bacteria and mycobacterial cell walls. This review describes several recent advancements in structure-based drug discovery in the antimicrobial drugs which shall be useful to generate new promising future drug candidates.<sup>8-10</sup> Peptidoglycan

layer consists of a beta-1,4-linked polysaccharide of alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) sugar units as building blocks of cell wall. Attached to the lactyl side chain of NAM unit is a pentapeptide (muramyl residues) side chain of general structure L-Ala-g-D-Glu-X-D-Ala-D-Ala, where X is either L-Lysine or meso-*DAP*.<sup>11-13</sup> Formation of the cross links makes the bacterial cell wall resistant to lysis. Compounds which inhibit lysine or *DAP* biosynthesis could therefore be very effective antibiotics, if targeted towards cell wall biosynthesis. The enzymes which catalyze the synthesis of L-lysine in plants and bacteria have attracted interest from two directions; firstly from those interested in inhibiting lysine biosynthesis as a strategy for the development of novel antibiotic or herbicidal compounds and secondly to enhance lysine yields in over-producing organisms.<sup>14,15</sup> Many peptidoglycan monomers, including the potent toxin from *B. pertussis* and *N. gonorrhoeae*, and similar *DAP* containing peptides, possess a range of biological effects such as cytotoxicity, antitumor activities, angiotensin converting enzyme (ACE) inhibitor, immunostimulant, and sleep-inducing biological activities.<sup>16-18</sup>

### Lysine biosynthetic pathway in fungi

This is called as  $\alpha$ -aminoadeptate (AAA) pathway is a biochemical pathway for the synthesis of the amino acid L-lysine. In the eukaryotes, this pathway is unique to the higher fungi (containing chitin in cell walls) and euglenoids.<sup>19-22</sup> It has also been reported in bacteria of the genus *thermus*, like *T. thermophilus*, *T. aquaticus*



contain *lysJ*, genes that is essential for lysine biosynthesis.<sup>23</sup> L-lysine is the only the essential amino acid that has two distinct biosynthetic pathways; the DAP pathway in plants and bacteria and the AAA pathway in euglenoids and higher fungi.<sup>24</sup> The AAA pathway is unique to fungi and is thus can be a potential target for the rational design of antifungal drugs.<sup>25,26</sup>

A key step in biosynthesis of lysine AAA biosynthetic pathway in fungus like *Saccharomyces cerevisiae* is enzymatic reduction of R-aminoadipate to the semialdehyde which requires two gene products, *Lys2* and *Lys5*. Here, the gene *Lys5* is a specific posttranslational modification catalyst. The AAA pathway utilizes  $\alpha$ -ketoglutarate and acetyl CoA (AcCoA) serving as the precursor for L-lysine. Extensive genetic, enzymatic and regulatory studies of the lysine biosynthesis are being carried out in this fungal species.<sup>27</sup> Seven enzymes and more than twelve non-linked genes are responsible for the biosynthesis of lysine in *S. cerevisiae*. Steps comprising the first half of the pathway leading to  $\alpha$ -aminoadipate take place in the mitochondria with the exception being the synthesis of homocitrate in the nucleus and second half of pathway converting  $\alpha$ -aminoadipate to L-lysine are carried out in the cytoplasm. The first half of the AAA pathway shares many similarities with the tricarboxylic acid (TCA) cycle. The pathway (Figure 2). is initiated by the homocitrate synthase (HCS) enzyme catalyzed condensation of acetyl CoA and  $\alpha$ -ketoglutarate to give the enzyme bound intermediate homocitryl CoA, which undergoes hydrolysis by the same enzyme to give homocitrate as the product first enzymatic reaction. Homoaconitase (HAc) catalyzes the inter conversion of homocitrate and homoisocitrate through the intermediate homoaconitate.<sup>28</sup> The homoisocitrate is then oxidatively decarboxylated by the pyridine nucleotide-linked homoisocitrate dehydrogenase (HIDH) to give  $\alpha$ -ketoacidipate. The next step is carried out by pyridoxal 5'-phosphate (PLP)-dependent aminotransferase (AAT) by using L-glutamate as the amino donor to form  $\alpha$ -Aminoadipate. the conversion of  $\alpha$ -aminoadipate (AAA) to lysine, in *T. thermophilus* is similar to the latter portion of arginine biosynthesis. As the first half of the pathway takes place in the mitochondrion, the aminotransferase is being present in both mitochondrion and cytoplasm. In the cytoplasm, AAA is then reduced to  $\alpha$ -aminoadipate- $\delta$ -semialdehyde (AAS) via AAA reductase (AAR). This enzyme has to be activated by a phosphopantetheinyl transferase (PPT). Once the  $\alpha$ -aminoadipate- $\delta$ -semialdehyde (AAS) is formed, it is combined with glutamate and by this the imine is reduced by NADPH to L-saccharopine, reaction being catalyzed by saccharopine reductase (SR). Finally, saccharopine dehydrogenase (SDH) catalyzes the oxidative deamination of saccharopine to give L-lysine.<sup>29,30</sup>

AAA pathway in fungi is of significance as many fungal alkaloids synthesize lysine as a structural element or biosynthetic precursor. In addition, several AAA pathway

intermediates are used to generate secondary metabolites such as AAA as an essential precursor for the synthesis of ACV  $\delta$ -(L- $\alpha$ -aminoadipoyl)-L-cysteiny-L-valine) tripeptide which is further utilized as the biological precursor of penicillin. Systemic fungal infections are the most difficult infectious diseases in mammals to treat and are life-threatening for individuals who are immune-suppressed, including AIDS, autoimmune diseases, and chemotherapy and transplant surgery. Selective inhibition of the enzymes of this pathway by appropriate substrate analog to develop new antifungal drugs that are more effective and less toxic and the enzymes involved in this pathway may be viable targets for selective antifungal agents.<sup>31</sup>

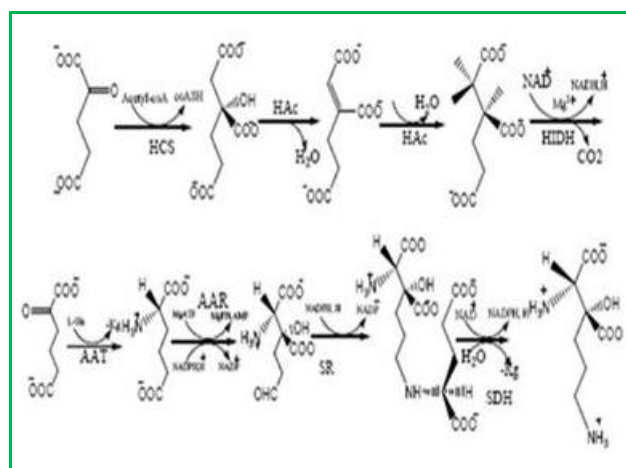


Figure 1: Lysine AAA biosynthetic pathway in fungi

#### Lysine biosynthetic pathway in bacteria

The synthesis of *meso*-DAP and lysine begins with the phosphorylation of L-aspartate to form L-aspartylphosphate catalyzed by *aspartate kinase*. Both *E.coli* and *B.subtilis* genomes encoding show three aspartokinase isozymes, required for different biosynthetic pathways starting from aspartate. *E.coli* has two bifunctional aspartokinase/homo-serine dehydrogenases, *ThrA* and *MetL*, and one monofunctional aspartokinase *LysC*, which are involved in the threonine, methionine and lysine synthesis. Transcription of the aspartokinase genes in *E.coli* is regulated by appropriate concentrations of the corresponding amino acids. In addition, *ThrA* and *LysC* are feedback inhibited by threonine and lysine; respectively.<sup>32,33</sup> Aspartate semialdehyde dehydrogenase converts the L-aspartylphosphate to aspartate semialdehyde. The first two steps of the DAP pathway is catalyzed by *aspartokinase* and aspartate semialdehyde dehydrogenase (ASADH) are common for the biosynthesis of amino acids of the aspartate family, like lysine, threonine and methionine.<sup>34</sup> Dihydrodipicolinate synthase catalyses the condensation of pyruvate (PYR) and aspartate semialdehyde (ASA) to form 4-hydroxy-2,3,4,5-tetrahydro-L,L-dipicolinic acid (HTPA). This enzyme belongs to the family of lyases, specifically the hydrolyases, which cleaves carbon-oxygen bonds.<sup>35,36</sup> The studies using <sup>13</sup>C-labelled pyruvate demonstrate that the

product is the unstable heterocycle HTPA. Rapid decomposition of the  $^{13}\text{C}$ -NMR signals of HTPA following its production indicates that formation of L-dihydrodipicolinate (DHDP) occurs via a nonenzymatic step. Dihydrodipicolinate reductase (DHDPR) catalyses the pyridine nucleotide-dependent reduction of DHDP to form L-2,3,4,5,-tetrahydrodipicolinate (THDP). THDP and DHDP is synthesized from aspartate semialdehyde by the products of the *dapA* and *dapB* genes (Figure 3). However, the metabolic pathway then diverges into four sub-pathways depending on the species, namely the succinylase, acetylase, dehydrogenase and aminotransferase pathways from tetrahydrodipicolinate are known in bacteria. The presence of multiple biosynthetic pathways is probably a result of the importance of DAP and lysine to bacterial survival<sup>37</sup>. The most common of the alternative metabolic routes is the succinylase pathway, which is inherent to many bacterial species including *E.coli*. This sub-pathway begins with the conversion of tetrahydrodipicolinate to *N*-succinyl-L-2-amino-6-ketopimelate (NSAKP) catalyzed by 2,3,4,5-tetrahydropyridine-2-carboxylate *N*-succinyltransferase (*dapD*, THPC-NST),<sup>1</sup> NSAKP is then converted to *N*-succinyl-L,L-2,6,-diaminopimelate (NSDAP) by *N*-succinyldiaminopimelate aminotransferase (*DapC*, NSDAP-AT). Subsequently it is desuccinylated by succinyldiaminopimelate desuccinylase (*dapE*,SDAP-DS)

to form L,L-2,6-diaminopimelate (LL-DAP). As for the succinylase pathway, the acetylase pathway involves four enzymatic steps, but incorporates *N*-acetyl groups rather than *N*-succinyl moieties. This pathway is common to several *Bacillus* species, including *B. subtilis* and *B. anthracis*.<sup>38</sup> The succinylase pathway is utilized by all gram negative and many gram positive bacteria, while the acetylase pathway appears to be limited to certain *Bacillus* species. There are one additional sub-pathways that are less common to bacteria.<sup>39</sup> Accordingly after the formation of L,L-DAP, which is common in these pathway, the enzyme L,L-Diaminopimelate epimerase (DAPE, *dapF*) catalyzes the epimerization of L,L-DAP to form *meso*-DAP. The dehydrogenase pathway, Diaminopimelate dehydrogenase (DAPDH) catalyzes the direct conversion of tetrahydrodipicolinate to *meso*-DAP in the dehydrogenase pathway of lysine biosynthesis. All these alternative pathways then converge to utilize the same enzyme for the final step of lysine biosynthesis, namely diaminopimelate decarboxylase (DAPDC, *LysA*) which catalyses the decarboxylation of *meso*-DAP to yield lysine and carbon dioxide. This step is important for the overall regulation of the lysine biosynthesis. Comparative analysis of genes and regulatory elements Identify the lysine-specific RNA element, named the LYS element, in the regulatory regions of bacterial genes involved in biosynthesis and transport of lysine.

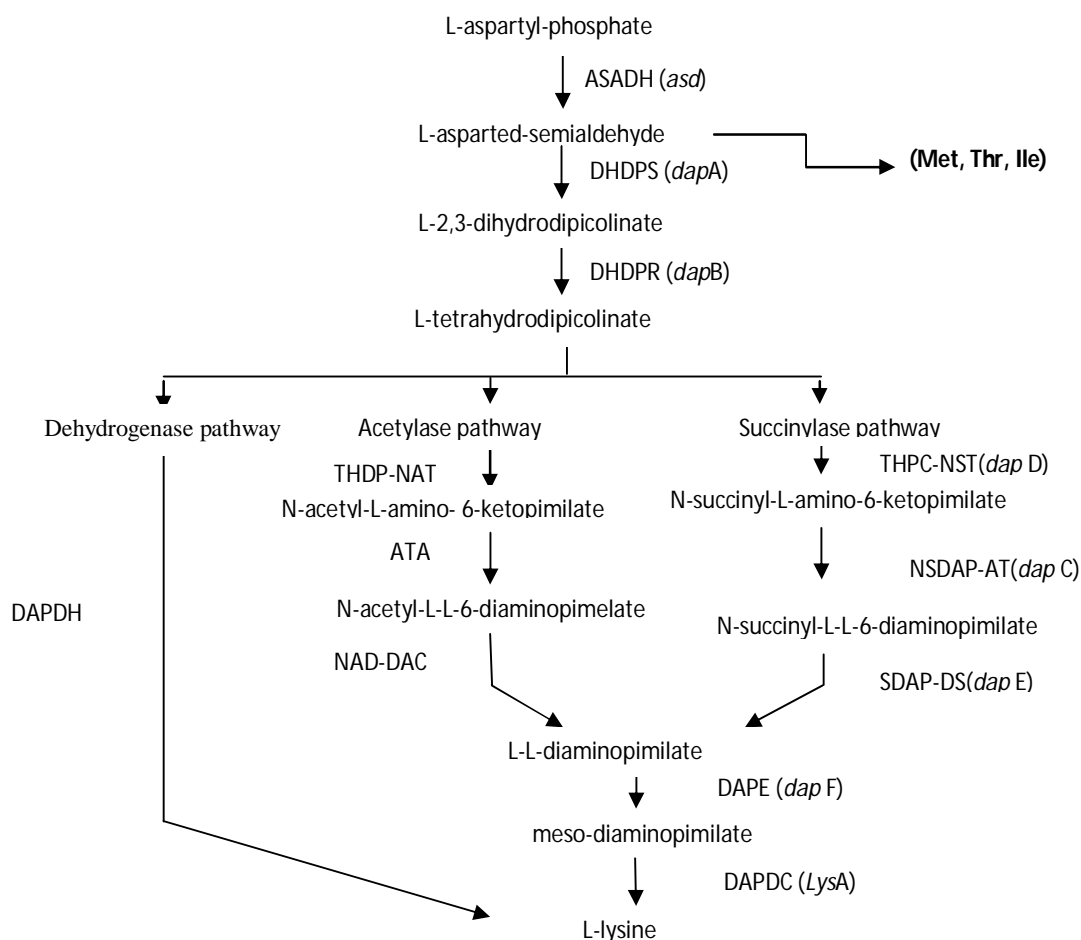


Figure 2: Enzymes of the lysine biosynthetic pathway in bacteria

The LYS element includes regions of lysine-constitutive mutations previously identified in *E.coli* and *B. subtilis*. The lysine biosynthetic pathway has a special interest for pharmacology, since the absence of DAP in mammalian cells allows for the use of the lysine biosynthetic genes as a bacteria specific drug target.<sup>38</sup>

### Enzymes Involved In Biosynthetic Pathway of Lysine

This review describes the essential details of the key enzymes functioning in the lysine biosynthetic pathway that are the products of essential bacterial genes that are not expressed in humans. The pathway is of interest to antibiotic discovery research. Accordingly, it also gives the current status of rational drug design initiatives targeting essential enzymes of the lysine biosynthesis pathway in pathogenic bacteria. More recently, cloning and expression of the DAP pathway components has facilitated detailed investigations and structures of the enzymes have been determined by X-ray crystallography.<sup>40</sup>

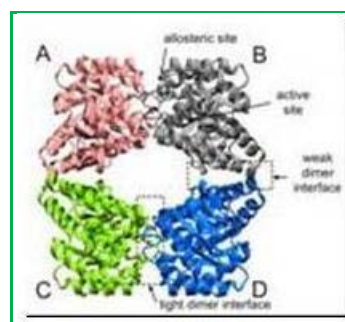
### Dihydrodipicolinate synthase dihydrodipicolinate synthase (DHDPS, EC 4.2.1.52, *dapA*)

DHDPS was first purified in 1965 from *E.coli* extracts and isolated from the same bacteria, wheat, maize (*Zea mays*) and higher plants. Kinetic studies suggest that pyruvate (PYR) binds to the enzyme active site followed by loss of water. The enzyme is the product of the *dapA* gene, which has been shown to be essential in several bacterial species.<sup>41</sup> The currently accepted mechanism of DHDPS is in which the structure of ASA is presumed to be the hydrate (figure 4). In the first step of the mechanism, the active site lysine (Lys161 in *E.coli* DHDPS) forms a Schiff base with pyruvate, subsequent binding of the second substrate ASA is followed by dehydration and cyclisation to form the product.<sup>42,43</sup> Based on the X-ray crystal structure of the *E.coli* enzyme, sequence homologies with DHDPS from other sources and site directed mutagenesis studies, it is proposed that a catalytic triad of three residues, tyrosine 133, threonine 44, and tyrosine 107 (*E.coli* numbering),<sup>44</sup> act as a proton-relay to transfer protons to and from the active site via a water-filled channel leading to the lysine binding site and bulk solvent. Subsequent binding and reaction of ASA then takes place. Several approaches have been used to investigate the enzyme active site. Initial studies showed that a reducible imine (NaBH<sub>4</sub> is inhibitory) is formed between pyruvate and the  $\alpha$ -amino group of a lysine residue in the active site is confirmed by electrospray mass spectrometry. Formation of an enamine at the active site has been proven by enzyme catalysed reversible exchange of tritium between  $\beta$ -H pyruvate and water.<sup>45</sup> In some organisms, the activity of DHDPS is regulated allosterically by lysine via a classical feedback inhibition process. Lysine feedback inhibition of DHDPS has been investigated in several plants, gram-negative and gram-positive bacterial species to date.<sup>46,47</sup> In contrast, DHDPS from bacteria are significantly less

sensitive to lysine inhibition than their plant counterparts.<sup>48</sup>

### Structure of DHDPS

The subunit and quaternary structure of DHDPS of *E. coli*, *M. tuberculosis*, *N. meningitides*, MRSA and several other species is a homotetramer in both crystal structure and solution (Figure 3).<sup>49</sup> In *E.coli*, the monomer is 292 amino acids in length and is composed of two domains. Each monomer contains an N-terminal ( $\beta/\alpha$ ) 8-barrel (residues 1-224) with the active site located within the centre of the barrel responsible for catalysis and regulation. The C-terminal domain (residues 225-292) consists of three  $\alpha$ -helices and contains several key residues that mediate tetramerisation.<sup>50</sup> The association of the four monomers leaves a large water-filled cavity in the centre of the tetramer, The tetramer can also be described as a dimer of dimers, with strong interactions between the monomers A & B and C & D at the so-called tight dimer interface and weaker interactions between the dimers A-B and C-D at the weak dimer interface. All point mutations resulted in destabilization of the 'dimer of dimers' tetrameric structure. A tetrameric structure is not essential for activity, confirming the importance of the dimeric unit as the minimal functional assembly for efficient lysine binding.<sup>51</sup>



**Figure 3:** The active sites, allosteric sites, dimerisation interface (tight dimer interface) and tetramerisation interface of *E. coli* DHDPS structure (PDB: 1YXC).

The active site is located in cavities formed by the two monomers of the dimer. A long solvent-accessible catalytic crevice with a depth of 10 Å is formed between  $\beta$ -strands 4 and 5 of the barrel.<sup>52,53</sup> Lys161, involved in Schiff-base formation is situated in the  $\beta$ -barrel near the catalytic triad of three residues, namely Tyr133, Thr44 and Tyr107, which act as a proton shuttle. Thr44 is hydrogen bonded to both Tyr133 and Tyr107<sup>54</sup> and its position in the hydrogen-bonding network may play a role in Schiff base formation and cyclisation. The dihedral angles of Tyr107 fall in the disallowed region of the Ramachandran plot. It involved in shuttling protons between the active site and solvent. In contrast, Tyr133 plays an important role in substrate binding, donating a proton to the Schiff base hydroxyl. It is also thought to coordinate the attacking amino group of ASA, which requires the loss of a proton subsequent to cyclisation (Figure 4). A marked reduction in activity is observed in



single substitution mutants, highlighting the importance of this catalytic triad.<sup>55</sup>

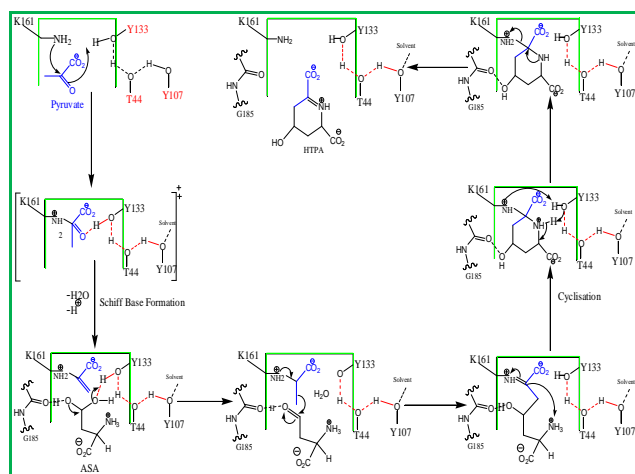


Figure 4: Proposed mechanism of DHDPS

### Inhibitors of DHDPS

The design of inhibitors of DHDPS has traditionally been based upon substrate and product analogy (figure 6). Non-heterocyclic Analogues of pyruvate have shown competitive inhibition with respect to pyruvate, and these include 3-fluoropyruvate, ketobutyrate, ketovalerate, glyoxylate and diketopimelic acid. Diketopimelic acid has been reported to be an irreversible inhibitor of DHDPS. The diene inhibitors, 4a and 4b, are more potent than the corresponding mono-enes, 5a and 5b, with the diene diester 4a being the best inhibitor of DHDPS in this series. Mass spectrometric analyses have further explored the enzyme–inhibitor interaction and determined the sites of enzyme alkylation.<sup>56</sup> The specificity of a range of heterocyclic product analogues displayed clear differentiation in inhibition of DHDPS enzymes from different pathogenic species like *B.anthraxis*, *M.tuberculosis* and *MRSA*. This suggests that the development of species-specific inhibitors of DHDPS as potential targeted to specific pathogens.<sup>57</sup> Product analogues have been designed to mimic the heterocycles DHDP and HTPA. A series of piperidine and pyridine-2,6-dicarboxylate derivatives has been evaluated as potential inhibitors of DHDPS. Amongst the heterocyclic analogues the piperidine diester 6b exhibited the most potent inhibition of *E.coli* DHDPS. Chelidamic acid 8a displayed high levels of inhibition of both DHDPS activity and bacterial growth the planar compounds with 2,6-substituents in a cis disposition, are more effective inhibitors of DHDPS than the corresponding transdisposed compounds (i.e., 7b and 10b are more potent than 15b), Unlike DHDP, HTHDP an immediate precursor of DHDP contains a hydroxyl group at the C-4 position. Therefore, compounds (Figure 6) were designed as potential inhibitors containing oxygen functionality at the C-4 position, thereby providing a lead in the development of more potent inhibitors of DHDPS.<sup>58</sup> Some novel constrained bis(ketoacid) and bis(oximino-acid) derivatives have been display time-dependent inhibition

and undergo condensation with the enzyme, generating an enzyme-bound adduct that closely resembles the enzymatic intermediate. Inclusion of hydroxyl functionality in phenols 18b and 19b in order to better mimic the enzymatic intermediate results in a switch of enzyme inhibition mode to a slow-tight binding model.

### Dihydrodipicolinate reductase (DHDPR, EC 1.3.1.26, *dapB*)

DHDPR was first purified and isolated from *E. coli* in 1965. Since further, the enzyme has been characterised from several species including *Bacillus spaericus*, *MRSA*, *M. tuberculosis*, *S.aureus*, and many other bacteria.<sup>59</sup> In *MRSA*, DHDPR is the product of the *dapB* gene. Analyses of the *dapB* proteins from different bacterial species suggest that two different classes of DHPR enzymes may exist in bacteria. Like most dehydrogenases, DHDPR from various bacterial species exhibits dual nucleotide specificity. *E. coli* DHDPR and *M. tuberculosis* DHDPR have been shown to have a preference for NADH over NADPH.<sup>60</sup> *S.aureus* DHDPR structure reveals different conformational states of this enzyme even in the absence of a substrate or nucleotide cofactor. The structure from *S. aureus* provides a rationale-Lys35 compensates for the co-factor site mutation. These observations are significant for biligand inhibitor design that relies on ligand-induced conformational changes as well as co-factor specificity for this important drug target.<sup>61</sup>

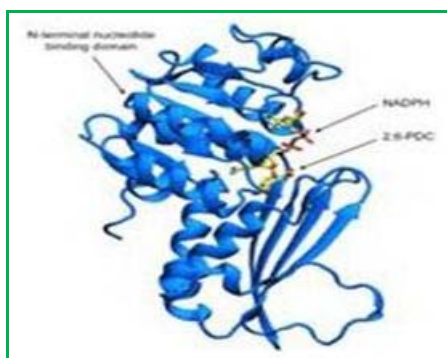
### Structure of DHDPR

The three-dimensional structure of DHDPR has been elucidated by X-ray crystallography from different bacterial species including, *E. coli*, *M. tuberculosis* and *S. aureus*. DHDPR from *E. coli* was the first DHDPR enzyme to be extensively studied in terms of structure and function. The open reading frame codes for a 240 amino acid protein with a monomeric molecular weight of 26,662 Da. DHDPR is a homotetramer with each monomer unit consisting of an N-terminal nucleotide binding (cofactor) domain and a C-terminal substrate binding (tetramerization) domain linked by a flexible loop. The active site is located at the interface between the nucleotide binding and substrate binding domains (figure 5). Hydrogen exchange experiments and the crystal structures of DHDPR bound to nucleotides and substrate analogues suggest that the hydride transfer reaction requires the movement of N-terminal domain towards the C-terminal domain.<sup>61</sup> The four monomeric subunits interact with each other by forming a tetramerisation interface consisting of a sixteen stranded central  $\beta$ -barrel comprising 4  $\beta$ -strands from each monomer.

The consensus sequence, E(L/A)HHXXKDAPSGTA is found in the substrate binding domain of all known bacterial DHDPR enzymes. Molecular modelling studies, using the apo form (enzyme in the absence of substrate) of *E. coli* DHDPR as a structural template, suggest a cluster of five basic residues are the key catalytic site residues, namely His159, His160, Arg161, His162 and Lys163 all contained within the consensus sequence.

These residues are located in the loop connecting  $\beta$ -strand B7 to  $\alpha$ -helix A5. Structural studies of *E. coli* DHDPR in complex with NADH and the substrate analogue and inhibitor, 2,6-pyridinedicarboxylate (2,6-PDC), show that 2,6-PDC is bound to the substrate binding domain of DHDPR, in a spherical cavity bordered by residues from both the nucleotide binding and substrate binding domains (Figure 5). The bound inhibitor makes several hydrogen bonding interactions with the atoms of the conserved E(L/A)HHXXKDAPSGTA motif. Similar interactions are observed between 2,6-PDC and DHDPR from *M. tuberculosis*.<sup>61</sup>

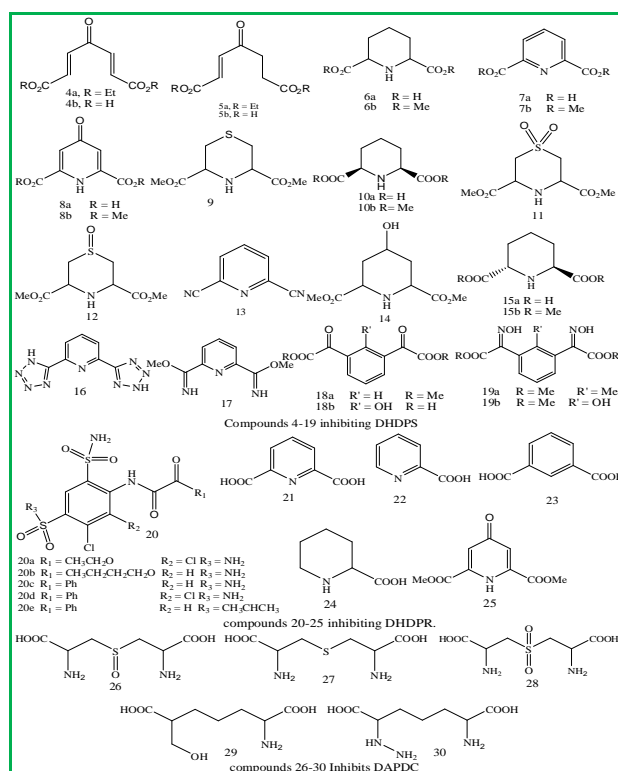
The nucleotide binding domain of DHDPR adopts a Rossmann fold, which is typical of nucleotide-dependent dehydrogenases (Figure 5). The consensus sequence (V/I)(A/G)(V/I)-XGXXGXXG located within this domain, is conserved in all NAD(P)H-dependent dehydrogenases, including DHDPR. Structural analyses of *E. coli* DHDPR show that this motif extends from the C-terminal end of  $\beta$ -strand B1 to the loop that connects B1 to  $\alpha$ -helix A1. From the structures of DHDPR bound to NAD(P)H from *E. coli* and *M. tuberculosis* it has been demonstrated that the conserved nucleotide binding motif (GXXGXXG) and the acidic residue (Glu38 in *E. coli* DHDPR) located 19-20 residues downstream of the glycine-rich region is important in binding the cofactor.<sup>62</sup> The two hydroxyl groups from the adenine ribose are known to interact with the side chain of Glu38 and also the backbone atoms of the glycine rich motif GXXGXXG. Several hydrophobic interactions exist between the adenine ring of NADH and the residues Arg39, Gly84 and His88. The pyrophosphate group of NADH is located over the  $\alpha$ -helix A1 and interacts with residues contained within the loop connecting  $\beta$ -strand B1 and  $\alpha$ -helix A1. MRSA-DHDPR exhibits a unique nucleotide specificity utilizing NADPH as a cofactor more effectively than NADH. Isothermal titration calorimetry (ITC) studies reveal that MRSA-DHDPR has 20-fold greater binding affinity for NADPH relative to NADH. Enzyme follows a compulsory-order ternary complex catalytic mechanism and cofactor preference of MRSA-DHDPR and provides insight into rational approaches to inhibiting this valid antimicrobial target.<sup>62</sup>



**Figure 5:** Structure of the *E. coli* DHDPR monomer bound to NADH and the substrate analogue, 2,6-PDC (PDB: 1ARZ)

## Inhibitors of DHDPR

DHDPR was screened for novel inhibitors by a molecular modeling approach which used available crystal structure of the enzyme with an inhibitor bound at active site shown to be very effective in identifying novel inhibitors of the enzyme as well as conventional screening proved beneficial in identifying compounds with greater structural diversity. Molecular modeling and X-ray crystal structure of *E. coli* DHDPR reductase with 2,6-PDC inhibitor bound in active site was used with FLOG system for database searching and docking to identify enzyme inhibitors. Assignment of polar hydrogens, hydrogen bonds and appropriate tautomeric states of imidazoles from three dimensional structures typically shows that each inhibitor candidate constraint 5 and 25 conformations, with heavy atoms represented by seven atom types (hydrogen bond donors, hydrogen bond acceptors, polar). A number of sulfonamide compounds were found to be among most potent inhibitors discovered through the FLOG-based molecular modeling search. Heterocyclic and aromatic inhibitors of DHDPR identified from general screening. All compounds are competitive with respect to DHDPR including The substrate analogue, 2,6-PDC(21).<sup>63</sup> Other substrate analogues such as picolinic acid (22), isophthalic acid (23), pipercolic acid (24) and dimethyl chelidamate (25), are much weaker inhibitors. A vinyllogous amide that acts as a competitive inhibitor of DHDPR has been one of the most potent inhibitors of it. Molecular modeling with conventional drug screening strategies has identified novel inhibitors, including sulfones and sulfonamides (Figure 6).



**Figure 6:** Substrate based analogue of enzyme involve in lysine biosynthesis.

**Diaminopimelate dehydrogenase (DAPDH, EC 1.4.1.16)** is a NADPH dependant enzyme that catalyses the reductive amination of L-2-amino-6-ketopimelate (AKP), the acyclic form of L-2,3,4,5,-tetrahydrodipicolinate (THDP), to produce *meso*-DAP (figure 2). It is assumed that the reaction occurs via an imine intermediate as a result of amination of AKP. Reduction of the imine by hydride transfer from NADPH generates *meso*-DAP. Only a small group of Gram-positive and Gram-negative bacteria posses DAPDH activity. These include *B. sphaericus*, *Brevibacterium sp*, *C. glutamicum*, *Cl. thermocellum* and *P. vulgaris*.<sup>64</sup> Characterised DAPDH enzymes are comprised of approximately 320 residues and share greater than 27% sequence identity across the species. Some bacterial species possessing DAPDH activity use multiple pathways to synthesise lysine. For example, *C. glutamicum* can synthesise lysine by either the dehydrogenase or succinylase pathway, whilst *Bacillus macerans* can employ enzymes of the dehydrogenase or acetylase pathways.<sup>65</sup>

### Structure of DAPDH

DAPDH from *C. glutamicum* forms a homodimer of approximately 70 kDa (Figure 7).<sup>65</sup> The DAPDH monomer subunit is comprised of three domain, a dinucleotide binding domain, that is similar but not identical to classical Rossmann fold, a dimerisation domain, and a C-terminal domain (Figure 8). Monomer subunits interact via two  $\alpha$ -helices and three-stranded antiparallel  $\beta$ -sheet to form dimer. Crystal structure of *C. glutamicum* DAPDH in complex with ligand shows that oxidised cofactor, NADP<sup>+</sup>, is bound within each of the dinucleotide binding domains. Each monomer domains exhibit open and closed conformations thought to represent binding and active states of DAPDH. In closed conformation the NADP<sup>+</sup> pyrophosphate forms seven additional noncovalent contacts. Subsequent studies demonstrate the product, *meso*-DAP, binds within an elongated cavity formed at interface of the dimerisation and dinucleotide binding domains.<sup>66</sup> In open conformer the dinucleotide is accessible to solvent, while in closed conformer both NADPH and DAP are protected from solvent. DAP binds in an elongated cavity with hydrogen bond donors and acceptors situated such that only D-amino acid center can bind near the oxidized nucleotide. The carboxylate groups of amino acid centers face cavity, bordered by protein atoms, while amino groups are exposed to water.



**Figure 7:** Structure of dimeric *C. glutamicum* DAPDH in complex with NADPH and L-2-amino-6-methylpimelate.

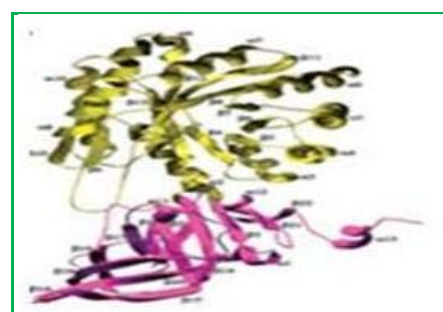
The dimerisation (orange), dinucleotide binding (blue), and C-terminal (green) domains are indicated. The cofactor NADPH (yellow) and inhibitor L-2-amino-6-methylene-pimelate (yellow) are bound by active site residues (pink) (PDB:1F06).

### Inhibitors of DADPH

Crystal structures of *C. glutamicum* DAPDH complexes with the inhibitors (2S,5S)-2-amino-3-(3-carboxy-2-isoxazolin-5-yl)-propanoic acid and L-2-amino-6-methylene-pimelate show that they form similar interactions with DAPDH as the product *meso*-DAP.<sup>67</sup> The structure of DAPDH with NADP<sup>+</sup> and an isoxazoline inhibitor bound was also solved. The inhibitor binds to the DAP site, but with its L-amino acid center at the position of the D-amino acid center of the substrate. This orients the C $\alpha$ -H bond away from the C4 position of the nicotinamide ring of NADP<sup>+</sup> and prevents catalysis.

### 6. Diaminopimelate Decarboxylase (DAPDC, EC 4. 1. 1. 20, lysA)

DAPDC encoded by the *lysA* gene in bacteria, which is an essential bacterial gene.<sup>68</sup> In *E.coli*, lysine specific gene *lysA* is transcriptionally controlled by the LysR regulator protein.<sup>69</sup> DAPDC is a vitamin B6-dependent enzyme that catalyzing non-reversible reaction in which DAPDC stereospecifically converts *meso*-DAP to L-lysine and carbon dioxide. Unlike other PLP-dependant decarboxylases that decarboxylate an L-stereocentre, DAPDC specifically cleaves the D-stereocentre carboxyl group. Thus, the enzyme possesses a means to differentiate between two stereocentres. DAPDC is classified as a type III class PLP enzyme, from the alanine racemase family. The study on DAPDC structure and function of this enzyme from *H.pylori*, *M.tuberculosis*, and *M.jannaschii*. Structure of DAPDC from *M. tuberculosis* have shown that the active site of DAPDC is located at the dimer interface, the dimer is the minimal catalytic requirement. In species such as *M. jannaschii* and *M. tuberculosis*, DAPDC is function of a homodimer and each subunits associate to form a head-to-tail quaternary architecture (Figure 9).<sup>70</sup>



**Figure 8:** *M. tuberculosis* DAPDC monomer - The N-terminal (yellow) and C-terminal (magenta) domains are indicated.

### Structure of DAPDC

The DAPDC monomer is composed of two domains, consisting of an N-terminal 8-fold  $\alpha/\beta$ -barrel domain and a C-terminal  $\beta$ -sheet domain (Figure 8). In *M. tuberculosis*



DAPDC, the N-terminal  $\alpha/\beta$ -barrel domain (residues 48-308) is comprised of  $\beta$ -strands  $\beta 4$ – $\beta 13$  and helices  $\alpha 2$ – $\alpha 10$ . The C-terminal domain (residues 2-47 and 309-446) is comprised of  $\beta$ -strands  $\beta 1$ – $\beta 3$ ,  $\beta 14$ – $\beta 21$  and helices  $\alpha 1$ ,  $\alpha 11$ – $\alpha 13$ . The active site is located at the interface between the  $\alpha/\beta$ -barrel domain of one subunit and  $\beta$ -sheet domain of both subunits (Figure 8). The X-ray structure of *H. pylori* DAPDC has allowed identification of key residues involved in substrate and cofactor recognition. The enzyme was crystallized in the presence of PLP and lysine. The *H. pylori* structure is very similar to that of *M. tuberculosis* DAPDC, forming a homodimer in a head-to-tail conformation. In this enzyme, PLP forms Schiff base linkages with Lys46 and lysine to produce a lysine-PLP external aldimine. This aldimine is believed to mimic the catalytic intermediate formed between meso-DAP and PLP.<sup>71,72</sup>

### Inhibitors of DAPDC

Diaminopimelic acid analogues (Figure 6) described in the section DAPE have been synthesised to study the inhibition of DAPDC from different bacteria. Mixtures of isomers of N-hydroxydiaminopimelate (29) and N-aminodiaminopimelate (30) are potent competitive inhibitors of DAPDC.<sup>73</sup> Lanthionine sulfoxides (26 & 28) are good competitive inhibitors with 50% inhibition at 1 mM. Weaker competitive inhibitors include the meso and LL-isomers of lanthionine sulfone and lanthionine, whereas the DD-isomers were less effective.<sup>74-76</sup>

### CONCLUSION

The review thus focuses on the development of newer chemical analogs acting as antimicrobials through inhibition of enzymes especially involved in lysine biosynthetic pathway. It imposes the need to develop these analogs to fight with the resistance imposed by the bacteria to the current antibiotics. Such compounds shall be able to specifically target and inhibit enzymes and proteins essential to the survival of bacteria. Selective inhibition of the enzymes of DAP pathway by appropriate substrate analogs might lead to newer drugs that are more effective and less toxic to mammals. The drug discovery towards protein and enzyme inhibition in this way shall generate newer avenues in treatment of diseases using antimicrobial therapy. Such drugs shall be able to stand in the market for a longer duration of time and also with a higher success rate.

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