Inhibitors of Lysine Biosynthesis as Antibacterial Agents

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Abstract: Bacterial biosynthesis of lysine has come under increased scrutiny as a target for novel antibacterial agents as it provides both lysine for protein synthesis and *meso*-diaminopimelate for construction of the bacterial peptidoglycan cell wall. Recent studies of the enzymes of the lysine biosynthetic pathway, development of inhibitors and investigations of their antibacterial properties are discussed.

keywords: Lysine, DAP pathway, diaminopimelate, antibiotics, enzyme inhibitors.

INTRODUCTION

Much recent scientific and media attention has focussed on the emerging resistance of bacteria to even our most powerful 'antibiotics-of-last-resort', such as vancomycin [1-3]. Although vancomycin had been used for about 30 years without bacterial resistance, the rapidly increasing reports of resistant strains, such as vancomycin-resistant enterococci (VRE), highlight the need for continual development of new antibacterial agents, ideally with a novel mode of action.

One target of antibacterial agents that has yet to be fully exploited is the biosynthesis of the amino acid lysine (**10**), and its immediate precursor *meso*-diaminopimelate (*meso*-DAP, **9**). The lysine biosynthetic pathway in plants and bacteria yields the *de novo* synthesis of lysine for utilisation in protein synthesis. More importantly, lysine (**10**) and *meso*-DAP (**9**) are vital constituents of the bacterial peptidoglycan cell wall [4,5]. Hence, blockage of lysine (and *meso*-DAP) biosynthesis would inhibit bacterial growth *via* two mechanisms, and such inhibitors may provide a new class of antibacterial agents. Additionally, mammals lack the ability to biosynthesise lysine and hence it is one of the essential amino acids which must be provided through a dietary source. The occurrence of the lysine biosynthetic pathway in microorganisms and plants but not in mammals

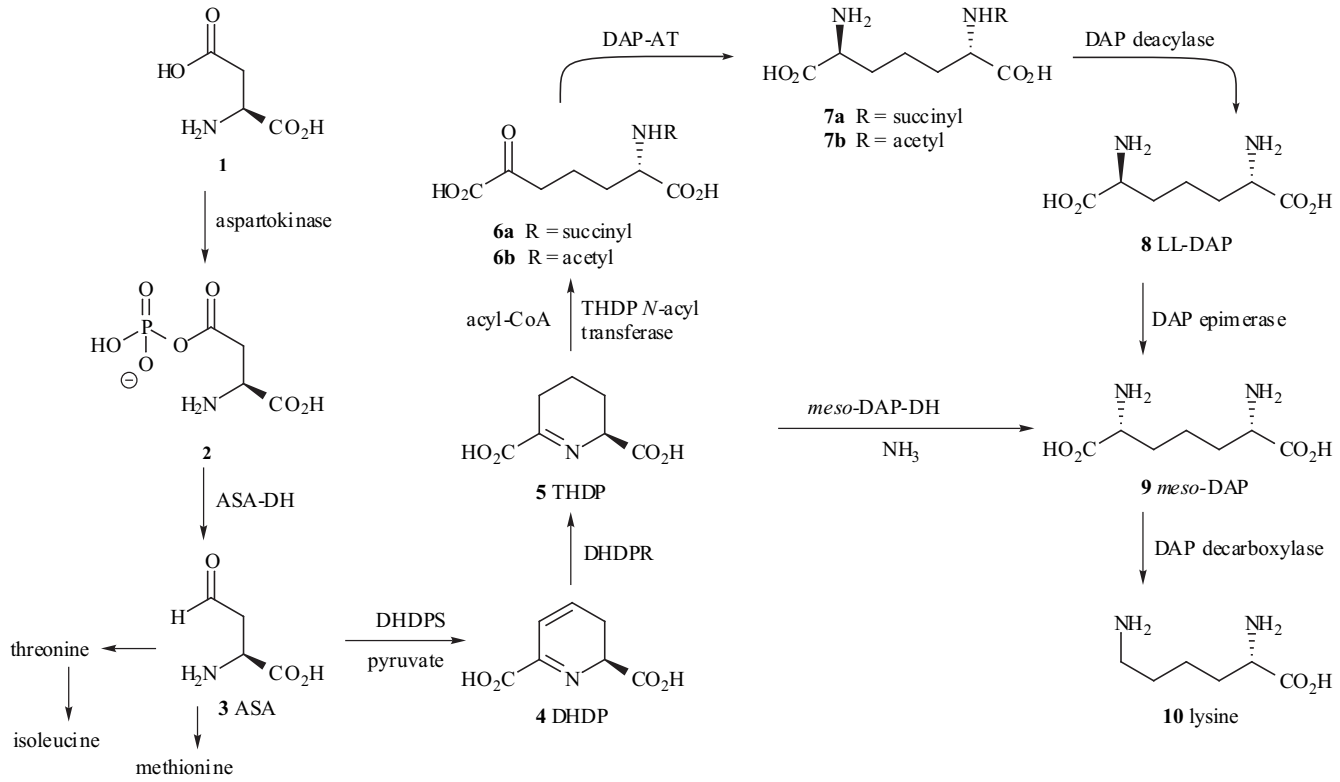

Fig. (1). Lysine biosynthetic pathway.

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suggests that specific inhibitors of this biosynthetic pathway may display novel antibacterial activity with low mammalian toxicity.

The biosynthetic pathway leading to *meso*-DAP and lysine production in bacteria is known as the diaminopimelate pathway (Fig. **1**). The initial steps toward the diaminopimelate pathway involve the conversion of aspartate (**1**) to aspartyl phosphate (**2**), with subsequent reduction to aspartate semi-aldehyde (ASA, **3**). These steps are common to lysine, threonine, isoleucine and methionine biosynthesis [6,7]. The first committed step towards lysine biosynthesis is the conversion of ASA and pyruvate to dihydrodipicolinate (DHDP, **4**), catalysed by DHDP synthase (DHDPS). Reduction of DHDP to tetrahydrodipicolinate (THDP, **5**) by DHDP reductase (DHDPR) then occurs. The majority of bacterial species then convert THDP to *N*-succinyl-2-amino-6-ketopimelate (**6a**), a process catalysed by an *N*-succinyltransferase. Some species, including *Bacillus*, incorporate an *N*-acetyl group rather than an *N*-succinyl group. Yet another small group of species (again including some *Bacillus* species) possess the enzyme *meso*-DAP dehydrogenase which catalyses the direct conversion of THDP (**5**) to *meso*-DAP (**9**), thereby shortcutting the predominant route by several steps. However, most species utilise the succinyl pathway in which *N*-succinyl-2-amino-6-ketopimelate (**6a**) is converted to *N*-succinyl-L,L-DAP (**7a**) by an aminotransferase, with subsequent de-acylation and epimerisation then providing *meso*-DAP. Finally, DAP decarboxylase catalyses the conversion of *meso*-DAP to lysine.

Kinetic and mechanistic investigations of all of the enzymes of the DAP pathway have been conducted, and the structures of several have been determined by X-ray crystallography. The information obtained from these investigations has been used in the design of inhibitors of many of the enzymes. Enzymes of the lysine biosynthetic area have previously been reviewed [8–11]. Recent investigations of DAP-pathway enzymes and progress towards the development of potent inhibitors of these enzymes will be discussed in this review.

ASPARTATE SEMI-ALDEHYDE DEHYDROGENASE (ASA-DH)

Aspartate semi-aldehyde (ASA, **3**) is produced by the reduction of aspartyl phosphate in a reaction catalysed by the NADPH-dependent aspartate semi-aldehyde dehydrogenase (ASA-DH) [12]. The structure of the *E. coli* enzyme complexed with NADP and a substrate analogue has recently been determined [13]. Comparison of this ternary structure with a previously determined structure of the apo-enzyme [14] has shown that upon binding NADP a domain-closure occurs, with the N-terminal domain rotating approximately 6° with repect to the C-terminal domain. This closure results in the cleft between the two domains closing around the bound NADP, and also an ordering of a 12-residue loop in the C-terminal domain. Once NADP has bound, the conformational change creates a binding pocket for the substrate (ASA or aspartyl phosphate). The enzyme was crystallised in the presence of *S*-methylcysteine sulfoxide (SMCS, **11**), an analogue of ASA in which the sulfur atom of SMCS corresponds to the aldehydic carbon of ASA. However, no electron density corresponding to the *S*-methyl or *S*-oxygen groups was detected, and the substrate analogue was modelled as a cysteine residue covalently bound to the active-site cysteine through a disulfide linkage (i.e., formation of a cystine moiety). Mass spectrometric analysis provided further evidence for the cystine linkage. The mechanism of attack and subsequent reaction between the active site cysteine and SMCS has not been established, though it seems highly unlikely that such a reductive demethylation could occur under these conditions, and it may be that a trace contaminant in the SMCS sample (such as cysteine) is in fact the species covalently bound to the active site.

The first reported inhibitor of ASA-DH, **12**, a difluoromethylene analogue of aspartyl phosphate, has recently been prepared (Fig. **2**) [15]. The difluoromethylene unit effectively mimics an oxygen atom and the pK_a of difluoromethlyene phosphonates closely matches that of the analogous phosphates. Additionally, the electron-withdrawing difluoromethylene group renders the adjacent carbonyl group an excellent electrophile and likely target for thiol attachment. Pre-incubation of **12** with ASA-DH showed time-dependent inhibition, with a calculated $K_i = 95$ μM. The inhibition was shown to be slowly reversible, consistent with reversible covalent attachment of the inhibitor to the active site Cys135 residue. The further development of potent inhibitors of ASA-DH would be extremely useful as this enzyme is common to the biosynthesis of methionine, threonine, isoleucine and lysine and would therefore cause significant disruption to protein biosynthesis.

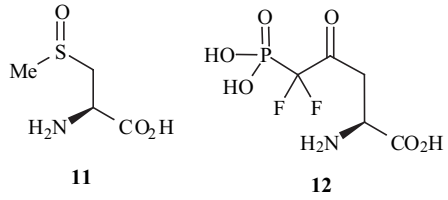


Fig. (2). Structure of ASA analogue SMCS (**11**) and ASA-DH inhibitor **13**.

DIHYDRODIPICOLINATE SYNTHASE (DHDPS)

The first unique step in lysine biosynthesis involves the condensation of pyruvate and ASA to give dihydrodipicolinate (DHDP, **4**), a reaction catalysed by the enzyme dihydrodipicolinate synthase (DHDPS) [16–21]. The reaction is initiated by condensation of an active site lysine residue with pyruvate to give the corresponding enamine **13** (Fig. **3**). Aldol-type reaction of the enamine **13** with ASA then gives the acyclic enzyme bound intermediate **14**. Transimination of the acyclic intermediate gives the cyclic alcohol **15** with simultaneous dissociation of the active site lysine residue. Loss of water then provides DHDP **4**. As the name suggests, it was originally believed that DHDP was the enzymatic product formed in this reaction, however Blickling and co-workers [22] observed 4-

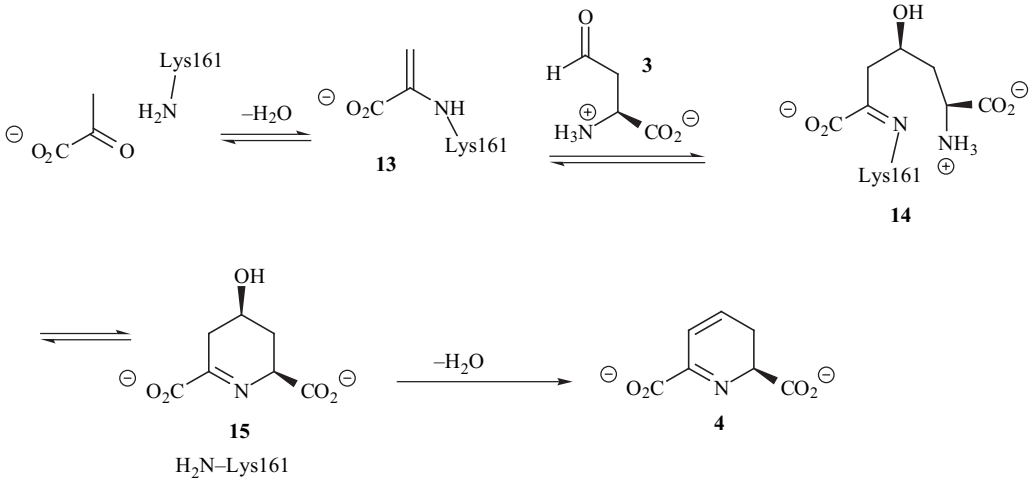


Fig. (3). Production of DHDP from ASA and pyruvate.

hydroxytetrahydrodipicolinate (HTHDP, **15**) as a discrete species in solution and postulated that this species is the true product of the enzyme-catalysed reaction, with dehydration to give DHDP occuring spontaneously following release of the alcohol **15** into solution.

The proposed mechanism of DHDPS involves attack of the enamine on ASA to generate the corresponding enzyme-bound alkoxide, which must then be protonated. X-Ray crystallographic analysis of the *E. coli* enzyme with various inhibitors and substrate analogues bound in the active site shows that the C4-oxygen is situated close to the backbone carbonyl oxygen of Gly186 and the Lys161-derived imine/enamine nitrogen [22]. Hence, protonation of the alkoxide could presumably only come from the protonated imine. Although this group is within hydrogen-bonding distance (2.8 Å), geometric and stereoelectronic factors preclude a concerted reaction in which proton transfer occurs simultaneously with C–C bond formation. Proton transfer from the protonated imine to the naked alkoxide could only occur subsequent to C–C bond formation, though it is noted that in the reported crystal structure the imine proton is directed away from the C-4 oxygen atom.

Studies of the solution structure of ASA have revealed that ASA exists in solution predominantly as the hydrate **16**, with only minor amounts of the aldehyde **3** (Fig. **4**) [23,24]. The cyclic lactol **17** has been proposed as another possible form of ASA in solution [24]. Analogues of the different forms of ASA have been prepared and their inhibition of DHDPS analysed in an attempt to define the form of ASA that binds to DHDPS (Fig. **4**) [24]. Homoserine lactone (**18**), (*S*)-3-aminopyrrolidin-2-one (**19**) and 2-aminocyclopentanone (**20**) were prepared as analogues of the cyclic lactol form of ASA (**17**). Homoserine lactone (**18**) was found to be a non-competitive inhibitor of DHDPS with respect to both ASA and pyruvate. Pyrrolidinone **19** failed to inhibit DHDPS, whereas ketone **20** showed reversible non-competitive inhibition with respect to ASA. These results suggest that the biologically relevant form of

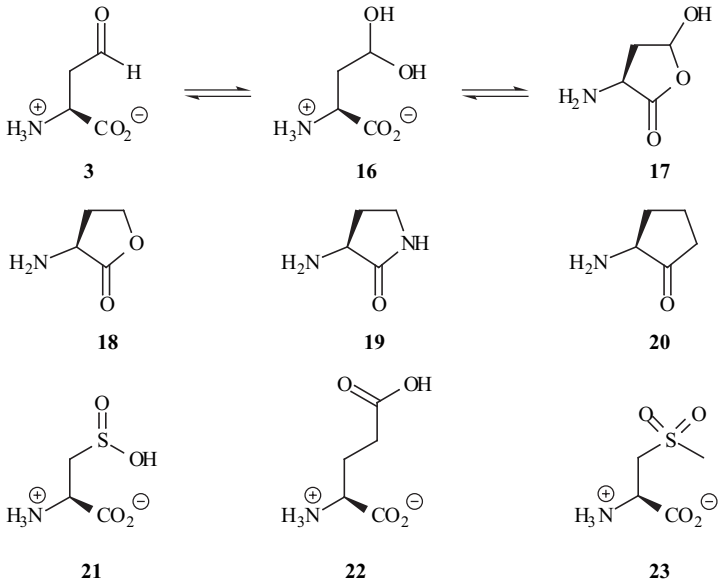


Fig. (4). Substrate-based inhibitors of DHDPS.

ASA is not the cyclic lactol **17**. Compounds **1** and **21**–**23** were designed and assayed as analogues of ASA hydrate (**16**). Both cysteine sulfinic acid (**21**) and glutamic acid (**22**) were found to be non-competitive inhibitors of DHDPS with respect to ASA, and aspartic acid (**1**) was a mixed inhibitor, indicating that these compounds also act at an allosteric site rather than the active site of the enzyme. Another analogue of the hydrate, *S*-methylcysteine sulfone (**23**), did not inhibit DHDPS. *S*-Methylcysteine sulfoxide (SMCS, **11**), an analogue of the aldehyde form of ASA, did not inhibit DHDPS either, so no conclusive information about the form of ASA recognised by DHDPS could be ascertained from these results.

Determination of the form of ASA that binds to DHDPS remains essential to the rigorous elucidation of the mechanism of the reaction. As the hydrate **16** is the predominant species in solution, a modified mechanism incorporating this substrate should be considered (Fig. **5**). Direct displacement of one of the hydroxyl groups from the hydrate is highly unlikely, with a more plausible mechanism being a two-step process, with initial loss of water generating the protonated aldehyde **24**. The Tyr133 residue is positioned to donate a proton to the leaving hydroxyl group, thereby generating water, with the Gly186 backbone carbonyl group accepting a hydrogen bond from the other hydroxyl group of the hydrate. The Thr44 residue has been shown to be essential for full activity of DHDPS, with Tyr133, Thr44 and Tyr107 proposed to be involved in a proton shuffle between the active site and solvent [25]. An alternative interpretation is that the extensive hydrogen-bonding network of these residues stabilises the Tyr133 anion. The second step of this process then involves attack of the enamine onto the protonated ASA **24** to form the acyclic intermediate **14**. The Tyr133 anion (or proton shuffle network) then abstracts the proton from the ASA-derived ammonium group to generate a neutral amine, which effects transimination/cyclisation. The Tyr133 residue is also

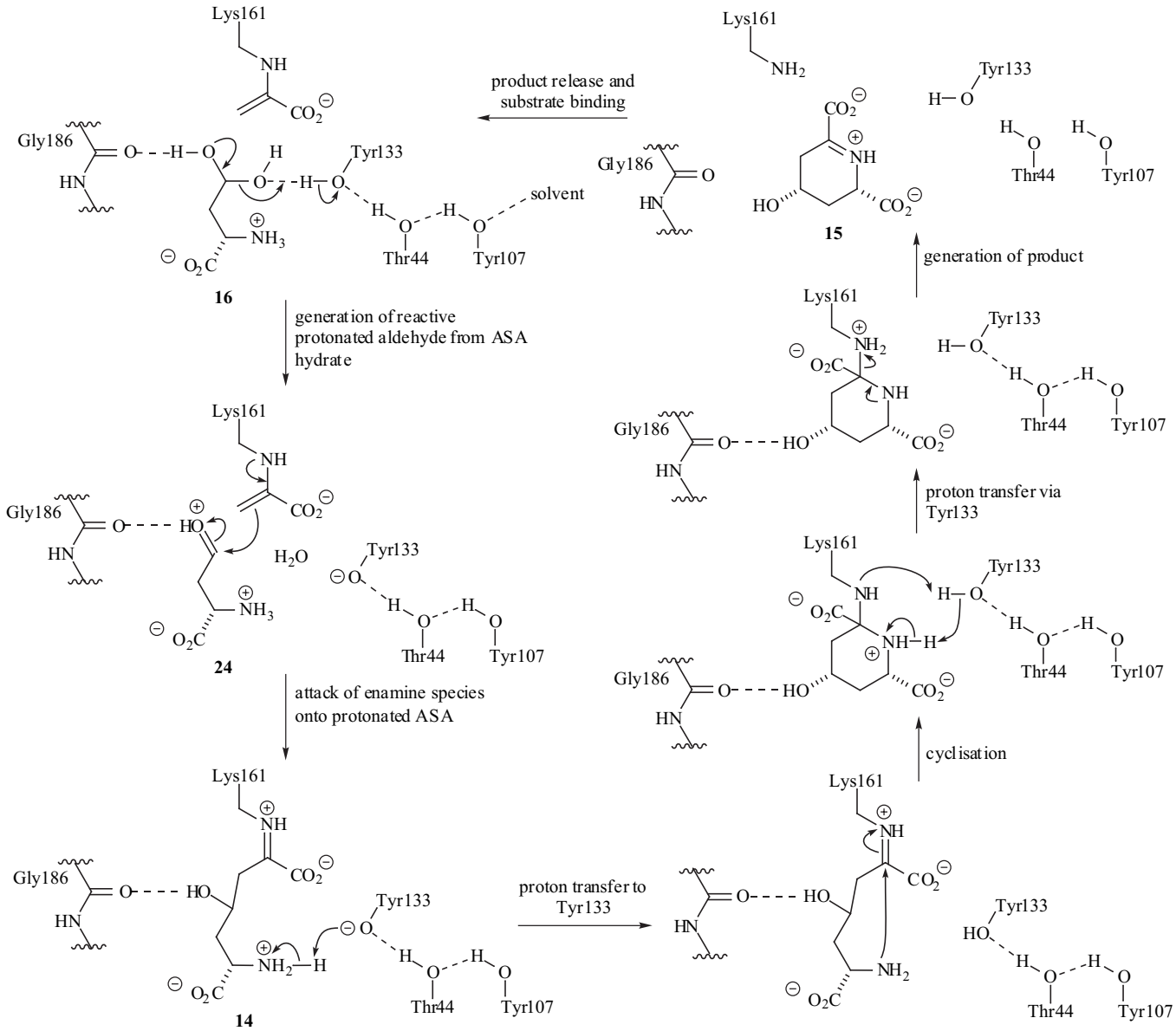


Fig. (5). Proposed DHDPS mechanism.

implicated in further proton transfers between the two nitrogens.

Several heterocyclic compounds (**25**–**33**) have been synthesised as potential inhibitors of DHDPS [26,27], based on their analogy to DHDP (Fig. **6**). Planar compounds and those with 2,6-substituents in a *cis*-disposition are more effective than the corresponding *trans*-disposed compounds, presumably as they more effectively mimic the geometry of the near-planar product. Reasons for the poor inhibition of DHDPS by the piperidine-2,6-dicarboxylates **28** and **30** compared with the corresponding diesters **29** and **31** are not immediately obvious.

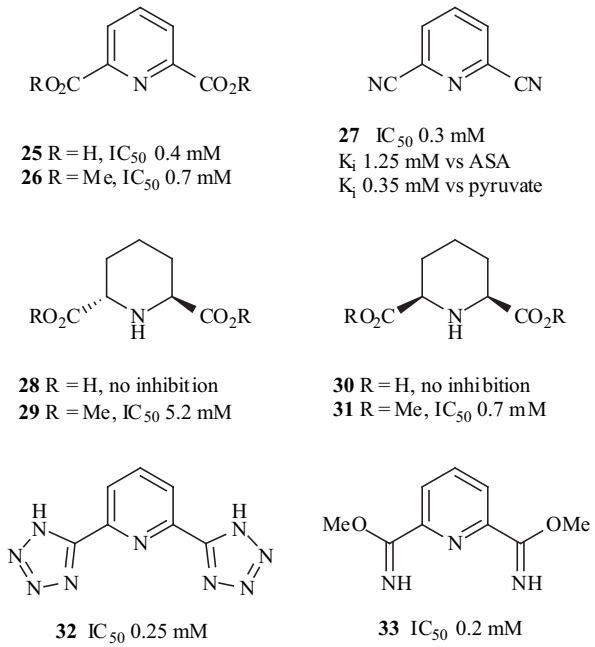


Fig. (6). DHDPS inhibitors.

To date most inhibitors of DHDPS have been developed by analogy to the substrates—pyruvate and ASA—or the 'product' DHDP [26,27]. The report [22] that HTHDP (**15**) is the true DHDPS-catalysed reaction product prompted our group to design potential DHDPS inhibitors based on analogy to HTHDP (**15**). Several inhibitors have been designed incorporating oxygen functionality at the 4-position of various heterocycles (Fig. **7**) [28]. The inhibitory activities of these compounds were investigated using a coupled assay employing DHDP reductase (DHDPR). There are three assays available for the studying DHDPS activity; the aminobenzaldehyde assay, the imidazole buffer assay, and the coupled assay [16]. The aminobenzaldehyde assay is used extensively in the purification of DHDPS, but is not used for kinetic studies. The imidazole buffer assay monitors the rise in absorption at 270nm on incubation of DHDPS and its substrates in imidazole buffer. The chromophore detected is dipicolinic acid (**25**), which forms upon autoxidation of DHDP. However, a lag phase is present before the absorbance at 270nm increases, and no studies of the kinetics of HTHDP dehydration or DHDP oxidation have been conducted. Thus, the accuracy of the kinetic results provided by this assay is questionable. The advantage of this assay is that it is easy to perform and thus it continues to be used by many research groups [17,19,26]. The coupled assay involves determination of DHDPS activity by monitoring the conversion of DHDP to THDP in the presence of excess DHDPR, with the utilisation of NADPH by DHDPR detected at 340nm. This assay is able to measure DHDPS kinetics, if DHDPR is present in excess, and DHDPR kinetics, if DHDPS is present in excess [21,29], and we therefore used this assay to investigate the activities of compounds **34**–**39**.

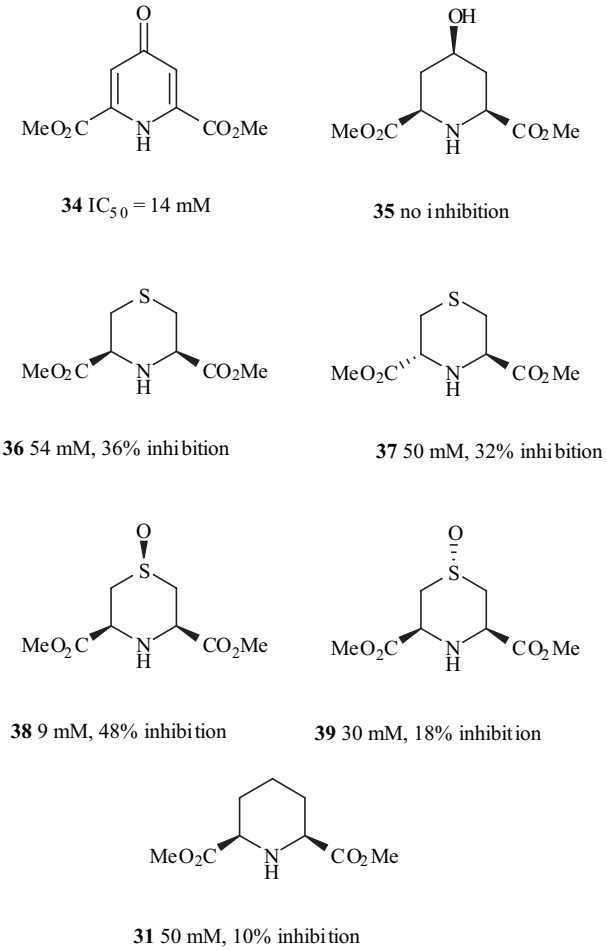


Fig. (7). DHDPS inhibitors.

The initial experiments employing the coupled assay provided a mixture of expected and unusual results. The sulfoxide **38** is more potent than the corresponding sulfide **36**, providing credence for the inclusion of the 4-oxygen substituent in the inhibitor design. Also, the *syn*-sulfoxide **38**, possessing the same stereochemistry as **15** at the C-2 and C-4 positions, is more active than the epimeric sulfoxide **39**. However, inhibition of DHDPS by *cis*-piperidine-2,6-dicarboxylate dimethyl ester (**31**) was found to be vastly different using the coupled assay (only 10% inhibition at 50 mM) compared to the reported value determined using the imidazole assay (IC_{50} = 0.7 mM) [26]. Similar discrepancies have also been observed for dipicolinic acid (**25**), which was determined to have a K_i = 11 mM (*vs* pyruvate), 18 mM (*vs* ASA) using the coupled assay [21], compared with an IC_{50} = 0.4 mM determined using the imidazole assay [26]. It is believed that these variations highlight the inaccuracy of the imidazole assay.

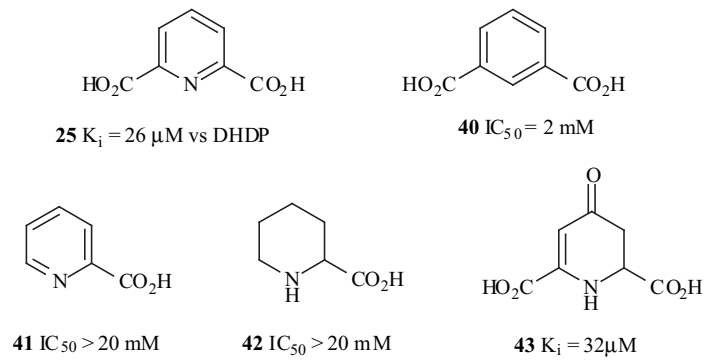


Fig. (8). DHDPR inhibitors.

Finally, the 4-hydroxypiperidine derivative **35**, which closely resembles HTHDP (**15**) but lacks the imine functionality, did not show any inhibitory activity. A rationale for this result has yet to be established and work in this area continues.

DIHYDRODIPICOLINATE REDUCTASE (DHDPR)

The second step in the biosynthesis of lysine is the reduction of dihydrodipicolinate (**4**) to tetrahydrodipicolinate (THDP, **5**), a process catalysed by the NADPH dependent enzyme dihydrodipicolinate reductase (DHDPR). The reaction is initiated by hydride transfer from the 4-*pro-R* position of NADPH to the C4-position of DHDP, with the resultant intermediate enamine then undergoing tautomerization to yield the product THDP [30,31]. Dipicolinic acid (**25**) is a linear competitive inhibitor of DHDPR with respect to the substrate DHDP (K_i = 26 μM), but inhibits uncompetitively against NADPH (K_i = 330 μM) [32]. Much weaker inhibition has been observed for other substrate analogues such as *iso*-pthalic acid (**40**, IC_{50} = 2 mM) [31] and picolinic (**41**) and pipecolic (**42**) acids (IC_{50} > 20 mM) [32]. The vinylogous amide **43** has been shown to be a competitive inhibitor of DHDPR, with a K_i = 32 μM with respect to DHDP [33]. Thus, **43** is one of the most potent inhibitors of DHDPR reported, with a similar activity to that of dipicolinate (**25**) (Fig. **8**).

Molecular modelling combined with conventional drug screening strategies has recently been employed to identify novel inhibitors of DHDPR [34]. Through this work a variety of inhibitors were found with K_i values ranging from 7–90 μM, including the sulfonamides **44** and **45** and the sulfone **46** (Fig. **9**). The six-fold increase in potency for the sulfone **46** has been rationalised using molecular modelling, which suggests that the isopropyl group of **46** interacts favourably with Val217, Phe243 and the methylene carbons of the Arg240 side chain. Although the sulfonamides **44** and **45** were shown to be good inhibitors of DHDPR they lacked good antimicrobial activity. This is perhaps not surprising considering sulfonamide-based drugs have been in clinical use for numerous years and thus many microbes may have already aquired resistance to them.

General structure screening identified a number of compounds not detected in the molecular modelling approach, including the relatively simple compounds **47** and

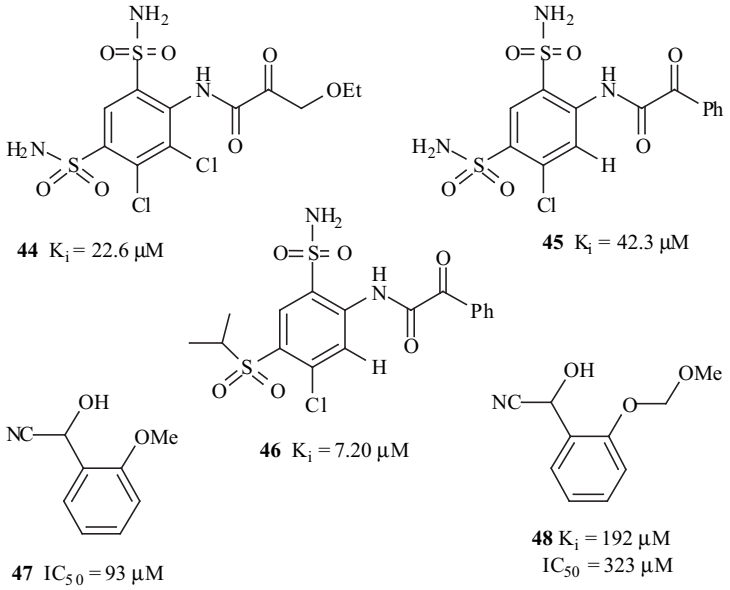


Fig. (9). DHDPR inhibitors.

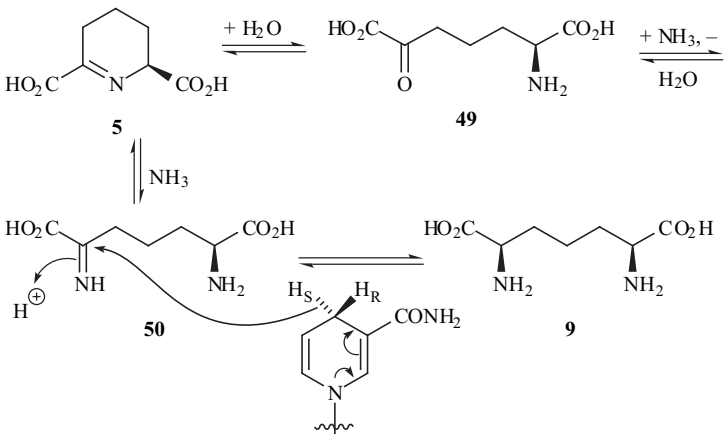


Fig. (10). Mechanism of DAP-DH.

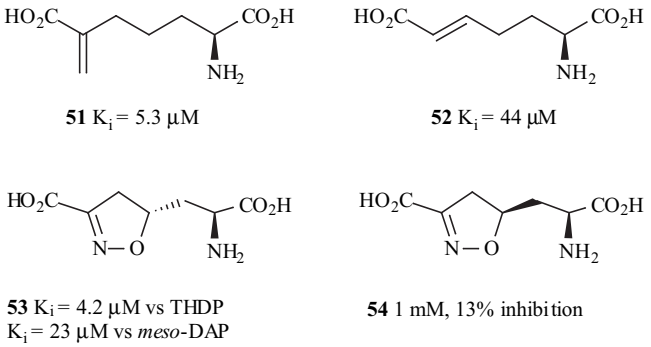
48. Modelling of these two compounds in the active site of the enzyme suggested they bind in the same position as dipicolinic acid (**25**).

MESO-DAP DEHYDROGENASE

In the limited bacterial species possessing *meso*-DAP dehydrogenase (*Corynebacterium glutamicum*, *Bacillus sphaericus* and a *Brevibacterium* and *Pseudomonas* species) THDP (**5**) is converted directly to *meso*-DAP (**9**) [35–39]. No comprehensive mechanistic studies have been performed, though mechanisms have been proposed based on analogy to other amino acid dehydrogenases. It has been proposed that the reaction proceeds via hydrolysis of THDP to give the corresponding keto-acid **49**, followed by condensation with ammonia to form an imine intermediate **50**, with subsequent reduction by NADPH yielding *meso*-DAP (Fig. **10**) [10]. However, the direct ring opening of THDP by ammonia to provide the imine **50** remains a viable alternative mechanism [8,40]. *meso*-DAP dehydrogenase (DAP-DH) has a similar domain structure to known dehydrogenases, but is more closely related structurally to DHDPR. This result is not surprising given that both enzymes employ similar substrates (THDP *vs* DHDP) and co-factor (NADPH) [41].

The structure of a ternary complex of the *Corynebacterium glutamicus* DAP-DH enzyme with NADPH and an inhibitor (**51**) has recently been determined by X-ray crystallography [42]. The 6-methylene-DAP inhibitor **51** mimics the imine intermediate and is one of the most potent inhibitors of the *Bacillus sphaerius* DAP-DH enzyme reported [40]. The structure of the enzyme/co-factor/inhibitor complex provides useful new information regarding the binding interactions of the imine with the enzyme. Previously, the structure of DAP-DH with isoxazoline inhibitor **53** bound in the active site was determined [43], but it was found that the isoxazoline **53** was bound in the reverse direction to that expected, with the L-amino acid centre positioned where the D-amino acid centre of *meso*-DAP would be located.

The isoxazoline **53** shows potent inhibitory activity against *B. sphaericus* DAP-DH, being an inhibitor of both the forward (K_i = 4.2 μM vs THDP) and reverse (K_i = 23 μM vs *meso*-DAP) reactions [44]. Kinetic analysis showed that for the reverse reaction the isoxazoline **53** inhibits non-competitively versus *meso*-DAP and uncompetitively against NADP (K_i = 9.2 μM). These results suggest that the isoxazoline **53** competes only for the THDP binding site, implying separate binding sites for THDP and *meso*-DAP. These seemingly unusual results are explained upon recognising that the active enzyme is a dimer. The oxazoline **53** binds to one subunit of the dimer and induces a conformational change of the protein which renders the other subunit inactive [40]. The unsaturated DAP analogue **52** is also a non-competitive inhibitor with respect to *meso*-DAP (K_i = 44 μM), and it has been suggested that **52** may bind in same non-productive manner as the isoxazoline **53** [40].



51 K_i = 5.3 μM

52 K_i = 44 μM

53 K_i = 4.2 μM vs THDP
K_i = 23 μM vs *meso*-DAP

54 1 mM, 13% inhibition

Fig. (11). DAP-DH inhibitors.

A novel preparation of L-THDP has recently been reported [45]. While racemic THDP has previously been prepared [46,47], this represents the first enantiopure preparation of THDP. The solution structure of L-THDP was studied, and was shown to consist of an equilibrating mixture of imine and acyclic compounds (**5** and **49**, respectively) and the corresponding enamine, in accord with previous reports [46].

Although many compounds have been investigated for inhibitory activity of DAP-DH [33,40,44,48,49] the application of these of these compounds as antibacterial agents would be of limited use because DAP-DH is only found in limited species.

THDP *N*-SUCCINYLTRANSFERASE

The great majority of bacterial species produce *meso*-DAP (**9**) from THDP (**5**) by an *N*-acylation/deacylation process. The great majority of these employ the succinyl pathway, with only some *Bacillus* species reported to employ the analogous acetyl pathway [50]. The succinyltransferase enzyme catalyses the formation of *N*-succinyl-2-amino-6-ketopimelate (**6a**) from THDP (**5**) and succinyl-CoA, thus stabilising the acyclic form of THDP.

Crystallographic studies of the succinyltransferase revealed that L-2-amino-6-oxopimelate (**49**), the hydrated acyclic derivative of THDP, binds in a fully extended conformation within the active site [51]. The amino group is held within a hydrogen bonding triad and is oriented in close proximity and roughly co-linear to the mercaptoethylamine group of CoA. L-2-Aminopimelate was found to bind in essentially the same manner, and in total preference to the corresponding D-enantiomer. The same group has also very recently determined the ternary structures of the succinyltransferase in the presence of pimelate/succinyl-CoA, and L-2-aminopimelate/succinamide-CoA (a non-reactive succinyl-CoA co-factor analogue) [52]. By comparing superimposed structures it has been determined that the thioester carbonyl group of succinyl-CoA is in close proximity (2.9 Å) to the 2-amino group of the substrate **49**, suggesting that a direct attack of the 2-amino group on succinyl-CoA is indeed possible. Comparison of the primary sequence of the *E. coli N*-succinyltransferase with those of the *N*-acetyltransferase enzymes from *B. pumilis* and *B. subtilis* suggests a rationale for the specificity of these enzymes for their the succinyl-CoA and acetyl-CoA co-factors. Arg187A and Glu189B of the succinyltransferase make electrostatic and hydrogen-bonding contacts with the terminal carboxylate group of succinyl-CoA. In the acetyltransferase, these residues are replaced by Asn and Val, respectively. In addition, the absence of a side chain for Gly163B of the succinyltransferase allows for a large enough binding pocket for the succinyl group, whereas the acetyltransferases have a Val residue at this position.

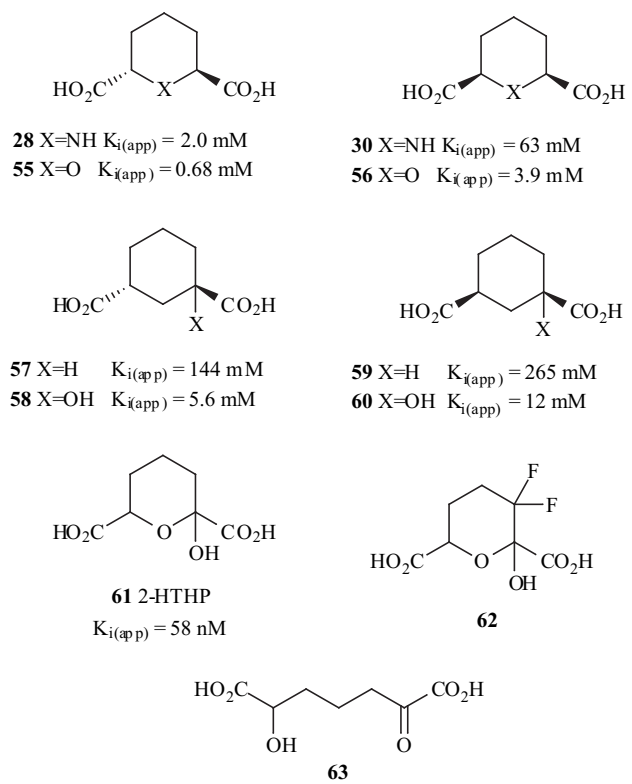


Fig. (12). Succinyltransferase inhibitors.

Previous work [53] showed that several THDP analogues (**28**, **30**, **55**–**61**, Fig. **12**) are good inhibitors of the *E. coli* succinyltransferase enzyme, with 2-hydroxytetrahydropyran-2,6-dicarboxylate (2-HTHP, **61**) being extremely potent ($K_{i(app)}$ = 58 nM). It was proposed that the potency of **61** is due to its analogy to the transition state of the enzyme-catalysed reaction. Based upon this data a mechanism was put forward for the conversion of THDP (**5**) to *N*-succinyl-2-amino-6-keto-L-pimelate (**6a**) in which THDP binding is followed by addition of water to give the carbinolamine **64**, which is *N*-succinylated to give **65**. Ring opening of **65** then gives the product **6a** (Fig. **13**, *path a*). However, the recent crystallographic studies [51,52] of the succinyltransferase suggest that the enzyme mechanism proceeds via *path b*, in which *N*-succinylation of the extended conformation of the open-chain substrate **49** occurs. Notwithstanding the recent improvements in our understanding of the mechanism of the succinyltransferase, their remains no conclusive evidence as to whether hydrolysis of the cyclic substrate (**5**) is enzyme-catalysed or occurs in solution. The hydroxypyran **61** exists in solution as an equilibrium mixture of stereoisomers and the open-chain form **63**, such that either the cyclic form **61** or the open chain form **63** could be the inhibitory species, mimicking **64** or **49**, respectively. Valuable information about the nature of the substrate of the succinyl transferase would be provided by investigations of the difluoro-analogue **62**, which has been synthesised and shown to exist only in the cyclic form [54]. However, no enzyme inhibition studies of **62** have been reported.

N-SUCCINYL-L,L-DAP AMINOTRANSFERASE (DAP-AT)

N-Succinyl-L,L-diaminopimelate aminotransferase (DAP-AT) catalyses the formation of *N*-succinyl-L,L-diaminopimelic acid (**7a**) from *N*-succinyl-L-2-amino-6-ketopimelic acid (**6a**) via the transfer of an amino group derived from L-glutamic acid. The DAP-AT enzyme was for a long time one of the least-characterised enzymes of the DAP-pathway, largely due to difficulties encountered in cloning the corresponding gene, *dapC*. Initial work by Peterkofsky and Gilvarg [55] lead to the 110-fold purification of the aminotransferase, but it was not until Vederas and co-workers [56] reported a 1500-fold purification of the wild-type *E. coli* enzyme some thirty-five years later that any further insight was gained. Major breakthroughs in our understanding of the DAP aminotransferase, however, were initiated upon the discovery by Ledwidge and Blanchard [57] that *N*-acetylornithine aminotransferase (NAcO-AT), an enzyme in the arginine biosynthetic pathway, can catalyse the conversion of keto-acid **6a** to the L,L-DAP derivative **7a**. It was suggested that

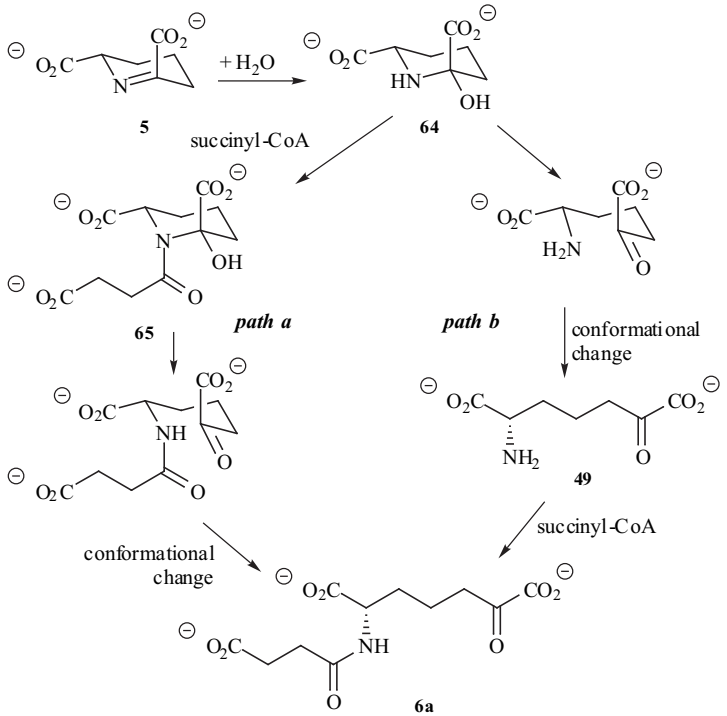
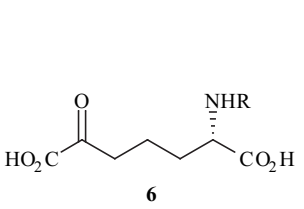


Fig. (13). Mechanism of succinyltransferase.

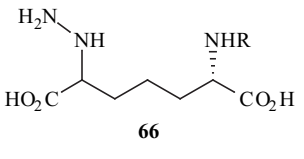
one dual-purpose enzyme could be responsible for both NAcO-AT and DAP-AT activities in the arginine and lysine biosynthetic pathways. However, Cox and Wang [58] recently confirmed that there are indeed two distinct enzymes with DAP-AT activity (in *E. coli* and *M. smegmatis*), only one of which possesses NAcO-AT activity. One *E. coli* enzyme selectively catalyses the conversion of keto-acid **6a** to DAP derivative **7a** (K_M = 2.4 mM), with no activity detected using the NAcO-AT substrate, *N*-acetylglutamate semialdehyde. The other *E. coli* enzyme catalyses the conversion of both substrates, indicating that while the *E. coli* DAP-AT enzyme (DapC) is specific for substrate **6a**, the NAcO-AT enzyme (ArgD) is able to catalyse the transamination of both *N*-acetylglutamate semialdehyde and **6a**. The discovery of two distinct enzymes with DAP-aminotransferase activity may explain the limited success of potent *in vitro* inhibitors of DAP-AT in antibacterial assays [59], as the bacteria can generate their *N*-succinyl-L,L-DAP requirement through two pathways. This finding makes DAP-AT an unlikely target for antibacteral compounds, as simultaneous inhibition of NAcO-AT is required to block *meso*-DAP and lysine biosynthesis.

The *dapC* gene from *B. pertussis* has recently been cloned and shown to indeed code for DAP-AT [60]. The *dapC* gene occurs as part of a locus with *dapD* and *dapE* genes closeby and has no similarity with the *argD* gene coding for NAcO-AT, providing further evidence for two distinct enzymes. The first cloning of a bacterial *dapC* gene should enable further characterisation of the DAP-AT enzyme in the near future.

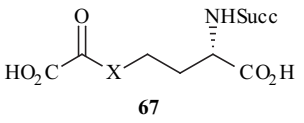
The substrate specificity of *E. coli* DAP-AT has been investigated [56,59,61] and the enzyme was found to tolerate moderate variation of the *N*-acyl group. While the *N*-acetyl (**6b**) and *N*-Boc (**6c**) compounds were not good substrates, the *N*-Cbz (**6d**) and *N*-cinnamoyl (**6e**) derivatives and α- and β-linked *N*-acetylaspartate derivatives (**6f**, **6g**) were moderate to good substrates (Fig. **14**).



	substrate activity (%)
a R = Succ	100
b R = Ac	4.3
c R = Boc	4.3
d R = Cbz	23
e R = cinnamoyl	70
f R = α-Asp	14.5
g R = β-Asp	12.6



	K_i* (nM)
a R = Succ	29
b R = Cbz	55
c R = α-Asp	1100
d R = β-Asp	834



	K_i (mM)
a X = S	no inhibition
b X = NH	26
c X = O	–

Fig. (14). Substrates and inhibitors of DAP-AT.

The corresponding hydrazine derivatives **66a**–**d** were found to be potent, reversible, slow-binding inhibitors of the aminotransferase enzyme (Fig. **14**) [59,61]. It was proposed that reaction between the terminal amino group of the hydrazine moiety and an active site PLP co-factor leads to the formation of a hydrazone complex between the inhibitor and pyridoxal within the enzyme active site. The hydrazine derivatives **66a**,**c** showed moderate antimicrobial activity,

but only on minimal media and at relatively high concentrations. The poor *in vivo* potency of these inhibitors may be due to NAcO-AT substituting for DAP-AT as described above.

The aza- , thia- and oxa-analogues (**67a**–**c**) of the DAP-AT substrate have been designed as mechanism-based irreversible inhibitors of the DAP-AT enzyme (Fig. **14**) [62]. The sulfur-containing substrate analogue **67a** showed no inhibition of DAP-AT, whereas the nitrogen-containing compound **67b** was a weak inhibitor (K_i = 2.6 mM). The extent of inhibition did increase with incubation time, but the residual activity was always >55%. The corresponding oxa-compound **67c** has yet to be prepared as cyclisation or elimination reactions accompany the final deprotection of the anticipated precursor to **67c**.

N-SUCCINYL-L,L-DAP DESUCCINYLASE

The recent work clarifying the role of the DAP-AT enzyme has probably rendered the *N*-succinyl-L,L-diaminopimelate desuccinylase enzyme the least-characterised of the lysine biosynthetic pathway. The *dapE*-encoded desuccinylase from *H. influenza* has recently been cloned and overexpressed [63], and sequence alignment with *Pseudomonas* carboxypeptidase G2 confirms that it is a metallo-amidase. The active site contains two metal ions, with zinc or cobalt required for maximal activity. One metal ion is tightly bound and is essential for activity, while the other is weakly bound and increases activity 2–7-fold. A mechanism for the desuccinylase has been proposed which is similar to those proposed for other dinuclear metallo-amidases [64] (Fig. **15**).

The desuccinylase is inhibited by the product of the reaction, L,L-DAP (K_{is} = 8 mM), and by *meso*-DAP (K_{is} = 12 mM), but D,D-DAP was a poor inhibitor of the enzyme (K_{is} = 90 mM).

DAP EPIMERASE

DAP epimerase catalyses the interconversion of L,L-diaminopimelic acid (**8**) and its stereoisomer, *meso*-DAP (**9**). Kinetic studies of the enzyme suggest that it operates via a two-base mechanism [65], common to other non-PLP-dependent racemases [66-69] (Fig. **16**).

The irreversible inhibitor azi-DAP (**68**) (Fig. **17**) has been shown to covalently attach via a sulfide bond to Cys73, suggesting this residue is one of the two residues implicated in the two-base mechanism [70,71]. Determination of the structure of the *H. influenzae* epimerase showed that Cys73 is linked through a disulfide bond to Cys217, and that these residues lie at the interface of two domains, implicating Cys217 as the second residue in the two-base mechanism [72,73]. Confirmation of this proposal has recently been provided through preparation of Cys→Ala and Cys→Ser mutants of DAP epimerase. Studies of the mutant enzymes in the presence of the known DAP epimerase inhibitors, fluoro-DAP analogues **70** and **71** [74], have been conducted [75]. The C73A and C217A mutants possessed no epimerase activity, but catalysed the elimination of HF from D,L-3-fluoro-DAP (**71**) and L,L-3-fluoro-DAP (**70**), respectively. The C73S and C217S mutants catalysed both the elimination of HF and epimerisation, with the C73S mutant able to catalyse elimination from **71** more readily than from **70**, and with the reverse trend seen for the C217S mutant. The double

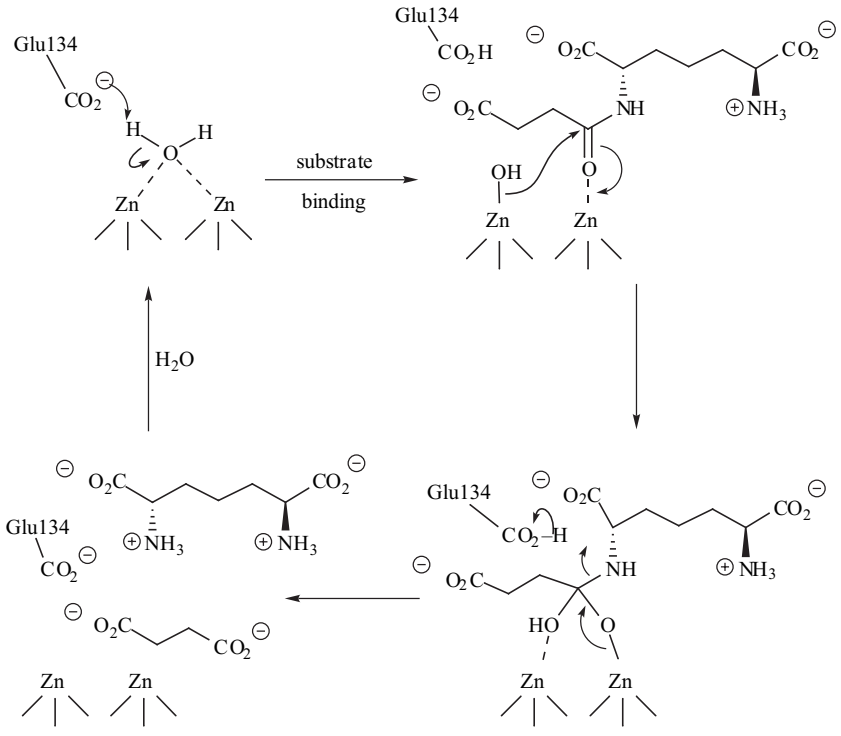


Fig. (15). Mechanism of desuccinylase.

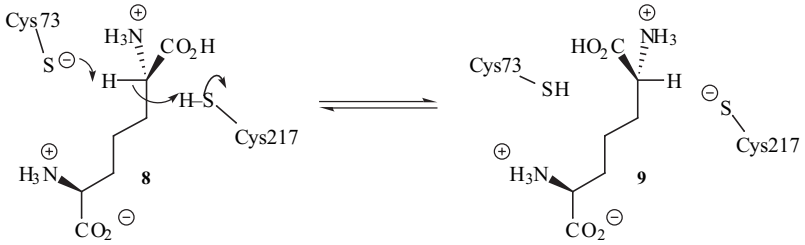


Fig. (16). Two-base mechanism of DAP epimerase.

mutant, C73S/C217S, did not act as an epimerase but was able to catalyse the elimination of HF from both **70** and **71** to a moderate degree. On the basis of these results Cys73 is implicated as the general base in the L,L→D,L direction (**8→9**) with Cys217 being the general base in the opposite direction (Fig. **16**).

Further studies of DAP epimerase will benefit from X-ray crystallographic structure determination of the enzyme with a substrate analogue or irreversible inhibitor bound in the active site. The oxygen analogue (**69**) of azi-DAP has recently been synthesised as a potential irreversible inhibitor of DAP epimerase (Fig. **17**) [76]. While this compound was shown to be an effective inhibitor of the epimerase, it is unstable at neutral pH, being hydrolysed to the corresponing diol. In this study [76] a new assay for DAP epimerase was also developed which utilises a commercially available mixture of DAP isomers rather than radio-labelled DAP or pure samples of L,L-DAP [44,65,74,77,78]. The DAP mixture is initially treated with *meso*-DAP dehydrogenase to convert *meso*-DAP to THDP, leaving a mixture of L,L- and D,D-DAP. Addition of DAP epimerase then catalyses conversion of L,L-DAP to *meso*-DAP, with activity of the epimerase determined by monitoring the conversion of *meso*-DAP to THDP by observing the conversion of NADP to NADPH at 340nm. The D,D-DAP isomer does not affect the assay as it is not a known substrate or inhibitor of the epimerase.

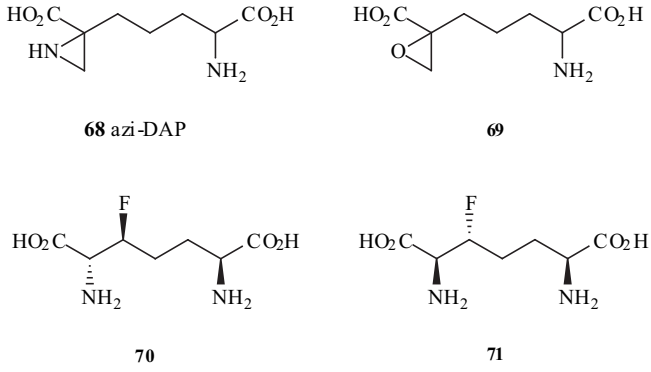


Fig. (17). Inhibitors of DAP epimerase.

DAP DECARBOXYLASE

The stereospecific conversion of *meso*-DAP (**9**) to L-lysine (**10**) is catalysed by *meso*-DAP decarboxylase [79]. This enzyme is the only known PLP-dependent decarboxylase to operate at the D-stereocentre of an amino acid. The *meso*-DAP decarboxylase enzymes from different organisms are closely related to each other, but appear to be unrelated to other amino acid decarboxylases, and have been shown to operate with an inversion of configuration in contrast to other PLP-dependent decarboxylases which operate with retention [80,81].

While a range of DAP analogues have been designed as potential inhibitors of DAP decarboxylase, few have shown potent inhibition. *N*-Amino-DAP (DAP hydrazine, **72**) exhibits 93% inhibition of the *B. sphaericus* decarboxylase at 0.4 mM [82]. The unsaturated DAP analogues **73** and **74** [83], and all stereoisomers of the phosphonate DAP analogue **75** [84] are reported to be poor inhibitors of the decarboxylases from *E. coli* and *T. vulgaris*, respectively (Fig. **18**). Compounds **73**–**75** also show weak inhibition of DAP epimerase and *meso*-DAP dehydrogenase due to their analogy to the common substrate of all these enzymes, *meso*-DAP. These results indicate that DAP decarboxylase has stringent substrate recognition requirements. 3,4-Dehydro-DAP **73** is the most potent of the unsaturated DAP derivatives but shows no antibacterial activity. Surprisingly, 4-methylene-DAP **74** shows moderate antibacterial activity, though it is a very weak inhibitor of the decarboxylase, indicating the problems associated with correlating *in vivo* antibacterial activity with *in vitro* enzyme inhibition.

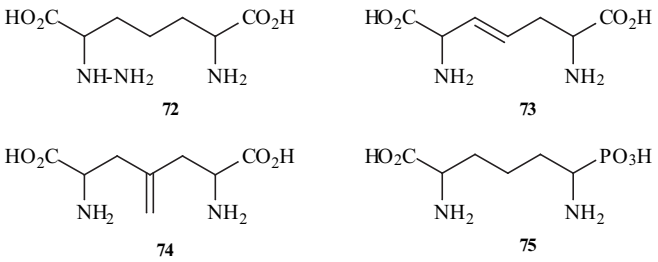


Fig. (18). Inhibitors of DAP decarboxylase.

Few investigations of DAP decarboxylase have been reported in recent years, and further studies have been hampered by the lack of crystallographic structure determination. However, a recent report [85] of the crystallisation of *E. coli* DAP decarboxylase suggests that structure determination of the enzyme may soon be accomplished, which will provide valuable information for the design of novel inhibitors.

SUMMARY

Significant advances in our understanding of the enzymes of the lysine biosynthetic pathway have occurred in recent years, particularly by the Blanchard, Cox and Vederas

groups and collaborators. Presumably these advances will allow for the design of potent inhibitors of a number of enzymes, with the potential to display antibacterial activity. However, at the present time there have been few potent (nanomolar) inhibitors of DAP-pathway enzymes developed, and fewer still that possess antibacterial properties. A possible rationale is that most inhibitors developed to date are analogues of the relatively small amino acid-derived enzyme substrates and products. These small molecules possess few groups available for specific interactions in the appropriate enzyme active sites. Additionally, those reasonably active inhibitors so far developed are often quite polar and suffer from lack of transport across the bacterial cell wall. Parallel synthesis and general screening approaches such as those intitiated by Merck Research Laboratories may uncover inhibitors with more 'drug-like' properties, though this will require the parallel development of suitable high-throughput assays. While no potent, broad-spectrum antibiotics which operate through inhibition of lysine biosynthesis have yet been developed, the improvements in our understanding of this pathway in recent years will no doubt improve our efforts toward this goal.

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