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Bacterial Diaminopimelate Metabolism as a Target for Antibiotic Design

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Introduction

The search for antibiotic compounds has recently taken on a new urgency.[1,2] The world-wide increase in bacterial resistance to current antibiotics impacts both hospital and community based programs for previously treatable infections. A recent survey suggests that the direct cost to the US economy alone, of resistant bacterial infections is around $3 billion annually, with indirect costs about ten times this level.[3] The problem of resistance is promoted by a number of factors. Firstly, many current antimicrobials are derived from natural sources, such as bacteria and fungi, wherein resistance mechanisms are necessary to protect the producing organism. This resistance can spread by gene transfer and thereby disperse rapidly to new organisms. Secondly, resistance mechanisms often work against an entire class of compounds, for example β-lactams, rather than just a single compound so that a resistant micro-organism is often immune to treatment by many individual compounds within a class. Finally, research into novel antimicrobial compounds was not felt to be of primary importance during the 1970s and 1980s, and relatively few new compounds effective against resistant pathogens reached the market in the subsequent period.[2]

The above factors suggest that development of new classes of antimicrobial molecules, rather than generation of more examples of known classes, will result in more useful compounds. In addition, compounds which are not based on naturally occurring substances may circumvent the likelihood of encountering naturally occurring resistance mechanisms. Our current research is addressing these points by examining new enzyme targets from micro-organisms, with special emphasis on the metabolism of diaminopimelic acid (DAP).

Biosynthesis of bacterial cell wall components has long been accepted as a target for antibiotic action.[4,5] Penicillins, cephalosporins and glycopeptide drugs such as vancomycin[6] all act by inhibiting key steps in the assembly of the peptidoglycan layer of bacterial cell walls. Other antibiotics such as D-cycloserine act by inhibiting enzymes involved in the biosynthesis of the cell wall components themselves. Crucially most bacteria require either lysine, or its biosynthetic precursor, diaminopimelate (DAP),[7] as a component of the peptidoglycan layer of the cell wall. The biosynthesis of L-lysine via diaminopimelic acid appears not to be a target for naturally occurring antibiotics.[8–10] Since mammals do not make or use DAP and require L-lysine as a dietary component, inhibitors of the DAP biosynthetic pathway would not be expected to show mammalian toxicity. For these reasons we and others have concentrated research efforts into understanding the bacterial biosynthesis of L-lysine.[8–10]

Peptidoglycan

The peptidoglycan layer of the bacterial cell wall consists of chains of alternating *N*-acetyl glucosamine and

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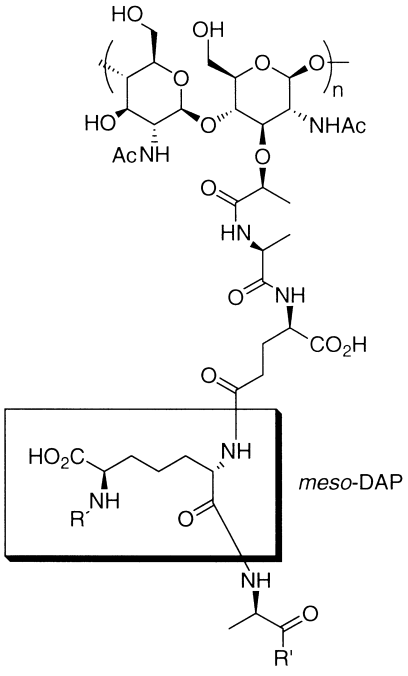
N-acetylmuramic acid, cross linked by short peptides **1a**.[11] Nearly all bacteria form these crosslinks using the diamines, lysine or DAP.[12–14] Formation of the cross links allows cell wall resistance to lysis by intracellular osmotic pressure.[15] Compounds which inhibit lysine or DAP biosynthesis could therefore be very effective antibiotics, if targeted towards cell wall biosynthesis, like other successful drugs such as β-lactams and glycopeptides. Many peptidoglycan monomers, including the potent toxin **1b** from *Bordetella pertussis* and *Neisseria gonorrhoeae*,[16] and similar DAP-containing peptides, possess a range of biological effects such as cytotoxicity,[17] anti-tumor activities[18] and angiotensin converting enzyme (ACE) inhibition.[19]



1a R = *D*-ala-*m*-DAP-*D*-glu-*L*-ala-(MurNAc-GlcNAc)$_n$, R'=*D*-ala, n = polymer, **1b** R = H, R' = OH, n = monomer

Early investigations into lysine biosynthesis have generally focused on the properties, mechanisms and modes of regulation of the enzymes.[20] However, more recently much more information has been gained from genetic studies. Using such an approach has ultimately resulted in both the cloning of the biosynthetic genes and the over-expression of most of the enzymes involved in lysine biosynthesis. This has led to the isolation and purification of substantial quantities of the proteins involved in lysine biosynthesis resulting in a more widespread study.[9,21]

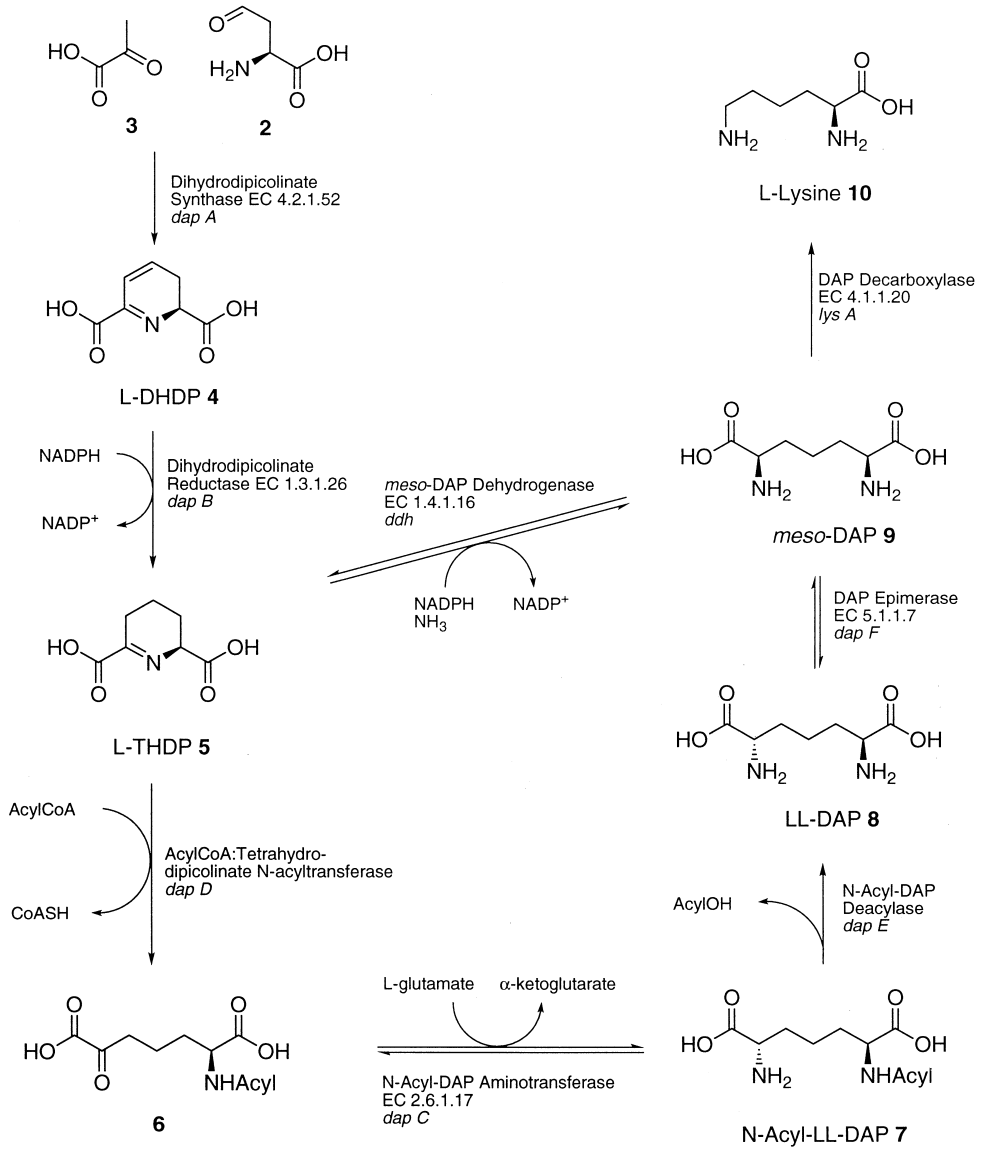
Enzymes of the Diaminopimelate Pathway

The first step of the diaminopimelate pathway which is common for both methionine and threonine biosynthesis,[22] involves formation of L-aspartate semialdehyde (**2**) by reduction of the primary metabolite aspartic acid in a NADPH dependent reaction. The first reaction unique to lysine biosynthesis (Scheme 1) is the L-DHDP synthase catalysed condensation of pyruvate **3** with L-aspartate semi-aldehyde (L-ASA) (**2**) to form L-1,2-dihydrodipicolinate (L-DHDP) (**4**).[23] Reduction of L-DHDP (**4**) is carried out by L-DHDP reductase to form L-1,2,3,4-tetrahydrodipicolinate (L-THDP) (**5**). The pathway splits after formation of L-THDP (**5**) into two main routes which have both been identified in different bacterial species.[24] The more prevalent of the two routes proceeds via acylation of L-THDP (**5**), producing an acyl-blocked α-amino-ε-ketopimelate (**6**). Succinate is the acyl group used for most bacterial species, including *Escherichia coli,* whereas in some species of *Bacillus* acetate is utilised.[12,25] The ketopimelate **6** undergoes transamination by a pyridoxal phosphate (PLP) dependent aminotransferase using glutamate as the amino-donor.[26] Deprotection[27] of the acyl-group from **7** then affords LL-DAP (**8**) which is then epimerised using *meso*-DAP epimerase to give the lysine precursor, *meso*-DAP (**9**).[28] In the less common pathway, *meso*-DAP **9** is produced directly from L-THDP (**5**) by *meso*-DAP dehydrogenase which is found in bacteria such as *Bacillus sphaericus*. In several bacterial species both pathways are found. For example the industrially important lysine producer, *Corynebacterium glutamicum* utilises both the succinyl-blocked and the dehydrogenase pathways.[29,30] In the final step of the pathway, the PLP dependent *meso*-DAP decarboxylase is used to catalyse the decarboxylation of *meso*-DAP **9** to give L-lysine **10**.[31]

The biosynthesis of lysine in plants is less understood. Both L-DHDP synthase and reductase enzymes as well as the *meso*-DAP epimerase and decarboxylase enzymes have been obtained from plant extracts. However, the presence in plants of the enzymes from the middle section of the pathway, such as *meso*-DAP dehydrogenase,[32] L-THDP acyltransferase or LL-*N*-acyl-DAP deacylase, is more controversial.[33] One suggestion is that direct transamination of L-THDP (**5**) may occur to afford LL-DAP (**8**).[34] Some results suggest that the biosynthesis of lysine in plants takes place within chloroplasts.[35] This coincides with further evidence which supports a bacterial origin for these organelles.[36]

Enzyme Structures and Mechanisms

The mechanisms of the DAP processing enzymes have generally been determined through careful and extensive kinetic investigations. Often these experiments have relied upon the use of enzymes purified in minute quantities from their natural producers. More recently, cloning and expression of the DAP pathway components has facilitated detailed investigations, and in some cases the structures of the enzymes have been determined by X-ray crystallography (Table 1). Often the data obtained by crystallography has reinforced the conclusions regarding mechanism obtained via kinetic methods, but sometimes the crystal data have contradicted inferences drawn from kinetics. Both kinetic and crystallographic aspects are discussed below.



Scheme 1. L-Lysine biosynthetic pathways in prokaryotes. Captions in italics denote genetic loci. Acyl = succinyl or acetyl.

Dihydrodipicolinate synthase

Kinetic studies involving the enzymes isolated from *E. coli*,[37] wheat[38] and maize (*Zea mays*)[39] suggests that pyruvate **3** binds to the enzyme active site followed by loss of water. Subsequent binding and reaction of L-ASA (**2**) (Scheme 2) then takes place. Several approaches have been used to investigate the enzyme active site. Initial studies showed that a reducible imine ($NaBH_4$ is inhibitory) is formed between pyruvate **3** and the ϵ-amino group of a lysine residue in the active site.[23] Further evidence for imine formation has recently been observed directly by electrospray mass spectrometry.[37] Formation of an enamine at the active site has been proven by enzyme catalysed reversible exchange of tritium between β-^{3}H pyruvate and water. Sequencing of tryptic digests of the reduced imino protein of the *E. coli* enzyme has identified lys-161 as the active site residue. This is the only conserved lysine in all known L-DHDP synthase sequences.[40]

A crystal structure of the *E. coli* enzyme at 2.5 Å resolution has been solved.[41] This has shown the active site lysine-161 lying at the bottom of a 10 Å deep by 30 Å long cleft. A series of five crystal structures of enzyme complexes with substrates, substrate analogues and inhibitors have been obtained at slightly lower resolution.[42] These include complexes with pyruvate, pyruvate with succinate β-semialdehyde, α-ketopimelic acid, dipicolinic acid, and L-lysine (Fig. 1). Together with NMR studies conducted by the same authors,[42] the results provide a detailed picture of the protein residues involved in catalysis and suggest the reaction mechanism depicted in Scheme 2. Interestingly, experiments with ^{13}C-labeled pyruvate suggest that the product released by the synthase may not be dihydrodipicolinate (**4**) but rather 4-hydroxy-2,3,4,5-tetrahydrodipicolinic acid (**11**), which may dehydrate in a subsequent non-enzymatic step. However, the relatively basic conditions (pH 9) used for the study may influence this step and account for the accumulation of the hydroxy intermediate.

Dihydrodipicolinate reductase

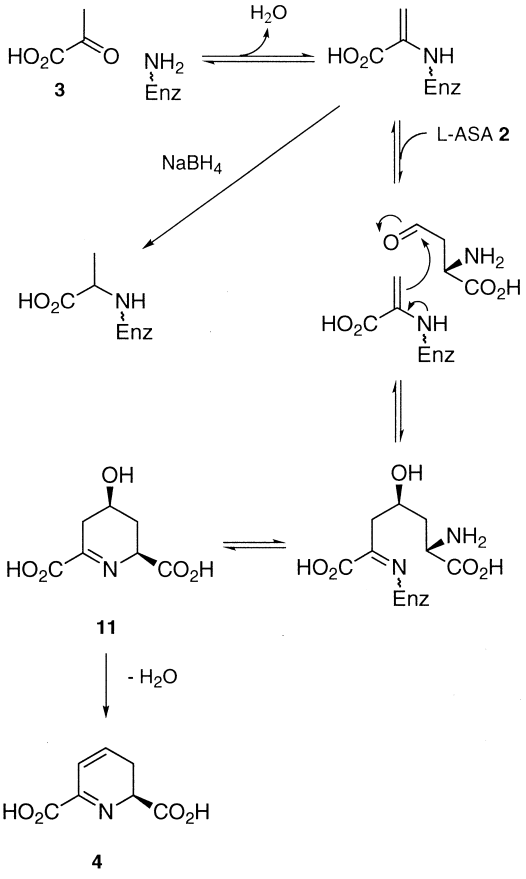
The L-DHDP reductase enzyme from *E. coli* is a homotetramer of approximate M_r 110,000–120,000. Gene sequencing has shown the subunit to consist of a polypeptide of 273 amino acids.[43] Direct cloning by PCR followed by overexpression in *E. coli* has been done.[44] The PCR product sequence predicts M_r 28757 which was confirmed by electrospray mass spectrometry. Further studies with the *dapB* gene from *Mycobacterium tuberculosis* suggests that two different classes of L-DHDP reductase enzyme exist in bacteria.[45] A smaller enzyme isolated from maize with a substrate specificity similar to that of the *E. coli* enzyme has been shown to have a M_r of approximately 80,000 for the tetramer.[46]

Table 1. X-ray structures of the enzymes of the DAP biosynthesis pathway

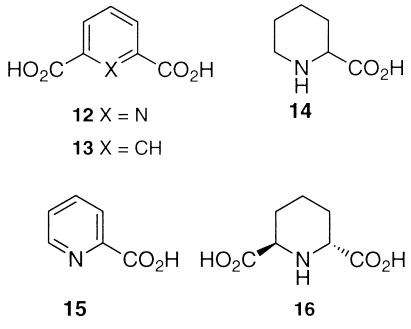
Enzyme	Organism	References
Dihydrodipicolinate synthase	*E. coli*	41,42
Dihydrodipicolinate reductase	*E. coli*	47,49,51
Tetrahydrodipicolinate *N*-succinyl transferase	*Mycobacterium bovis*	54,55
Aminotransferase		N/A
Desuccinylase		N/A
DAP epimerase	*Haemophilus influenzae*	82
DAP D-dehydrogenase	*Corynebacterium glutamicum*	84,86
DAP-decarboxylase		N/A

Overexpression of the protein in *E. coli* has allowed extensive mechanistic and crystallographic sudies.[47] Kinetic results show that the enzyme catalyses the transfer of the *pro-R* hydride from the cofactor to the substrate at its γ-position. Michaelis constants for the substrate, L-DHDP (**4**) (K_M 50 ± 12 μM) and the cofactor NADPH (K_M 8.0 ± 2.5 μM) have been determined. The enzyme binds the cofactor and the substrate sequentially before reaction with release of the product followed by the oxidised cofactor. Binding by dipicolinate **12**, a linear competitive inhibitor with respect to the substrate (K_i 26 ± 6 μM), suggests that the natural substrate **4** is bound in its cyclic state. Other substrate analogues such as *iso*-phthalic acid **13** (IC_{50} ~2 mM), and compounds with only one carboxylate, such as pipecolic **14** and picolinic **15** acids (IC_{50} > 20 mM), are also inhibitors although much less potent. Piperidine dicarboxylic acids **16** are not inhibitors of L-DHDP reductase.

The *E. coli* enzyme can accept either NADPH or NADH as cofactor. Studies have shown that the V_{max} value for NADH is only half the value for NADPH.[48] Due to a much lower K_M, NADH is the better substrate.[44] Further evidence has been gained by the X-ray



Scheme 2. Proposed mechanism of L-DHDP synthase and inhibition by $NaBH_4$.



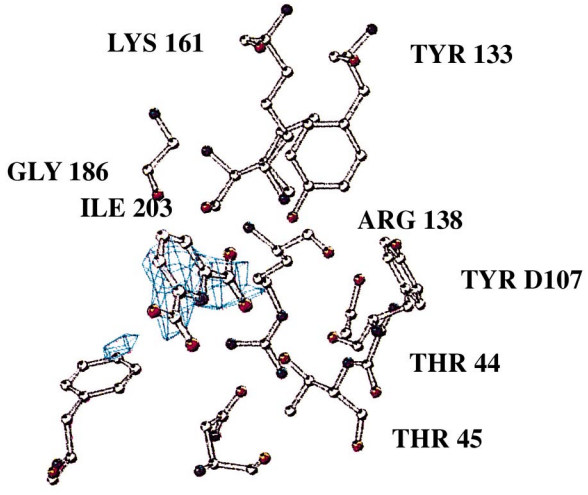


Figure 1. Defocused plot of the different densities in the active site in *E. coli* DHDP synthase. Soak with dipicolinic acid, density contoured at 5.0 σ. Reprinted with permission from *Biochemistry* **1997**, *36*, 24. Copyright 1997 American Chemical Society.

crystal structure obtained at 2.2 Å resolution. The crystal structure of the enzyme subunit consists of both a cofactor and a substrate binding domain. NADPH is bound in the crystal, and the proposed binding site of the substrate would juxtapose the substrate and cofactor in the correct orientation for reaction, although in the absence of substrate it would appear to be about 12 Å too far away.[47] More recently *E. coli* dihydrodipicolinate reductase has been crystallised in the presence of the two pyridine nucleotide cofactors.[49] Combined with detailed thermodynamic measurements using isothermal titration calorimetry, it has been shown that entropic factors play a significant role in the binding (overall $\Delta G = -RT\ln K_d = \Delta H - T\Delta S$) of the non-phosphorylated cofactors. The phosphate group of NADPH makes a specific charge interaction with Arg-39 of the *E. coli* enzyme, whereas this interaction is replaced with hydrogen bonding between Glu-38 and the ribose hydroxyl groups of NADH (Fig. 2).

The NADH binding domain of *E. coli* DHDP reductase is distinct from the substrate binding domain. The crystal structure suggested that these two domains could move such that the enzyme could take up 'open' and 'closed' conformations, moving the NADH closer to the bound substrate as required for catalysis. Recent work to measure proton/deuteron exchange rates between solvent and peptide NH protons has been coupled with the use of HPLC–ESMS to provide a powerful technique for analysing domain movement.[50] Binding by both NADH and substrate reduce the rates of H/D exchange in segments of the digested protein which either are involved in substrate binding or are part of the putative hinge region. Slowing of the H/D exchange rate is indicative of decreased solvent accessibility, consistent with closure of the protein upon cofactor and substrate binding. More recently crystals have been obtained of the protein binding both NADH and a substrate mimic, the reversible inhibitor dipicolinate **12**.[51] In this structure the NADH is situated at 3.5 Å from the substrate mimic (Scheme 3). The structure confirms that the *pro-R* hydride of NADH is transferred and that the reduction occurs *trans* across the double bond. The solvent derived proton at C3 is most likely derived from a water molecule hydrogen bonded to the conserved His-159. Conserved His-160 participates in substrate binding, interacting with the adjacent carboxylate of the substrate. The intermediate negatively charged species is stabilised by electron donation towards the substrate nitrogen atom which carries a partial positive charge due to interaction with the conserved Lys-163. The participation of Lys-163 and His-159 in the catalytic mechanism has been confirmed by site directed mutagenesis. Mutants displayed significantly lower specificity (k_{cat}/K_M) for the substrate due to both decrease in k_{cat} and increase in K_M.

Tetrahydrodipicolinate *N*-succinyl transferase

A stereochemical model for the mechanism of the succinyltransferase has been proposed and indicates that the enzyme binds L-THDP **5** in its cyclic form, then catalyses the addition of water to the *re* face of the imine (Scheme 4).[52] The *trans*-piperidine dicarboxylate intermediate **17** then reacts with succinyl-CoA and the ring is opened. Acyclic substrates and inhibitors must therefore bind in a ring-like manner in which the carboxyl groups are disposed in the same *trans* conformation as in intermediate **17**. This conformation accounts for both the apparently good substrate activities of **18** and the inhibition by D-α-aminopimelate (**19b**) and DL-α-hydroxytetrahydropyran-α,ε-dicarboxylate (HTHP) (**20**).

The crystal structure of a succinyltransferase with 94% identity to the *E. coli* transferase, possibly from *Mycobacterium bovis* has been determined.[53,54] The enzyme is a homotrimer. Initial work focused upon the determination of possible substrate binding sites through co-crystallisation with known inhibitors, such as cobalt and *p*-(chloromercuri)benzenesulfonic acid. Much more detailed information has been gained through co-crystallisation with CoA and substrates such as DHDP **4** and L-α-aminopimelate (**19a**).[55] The substrate binding

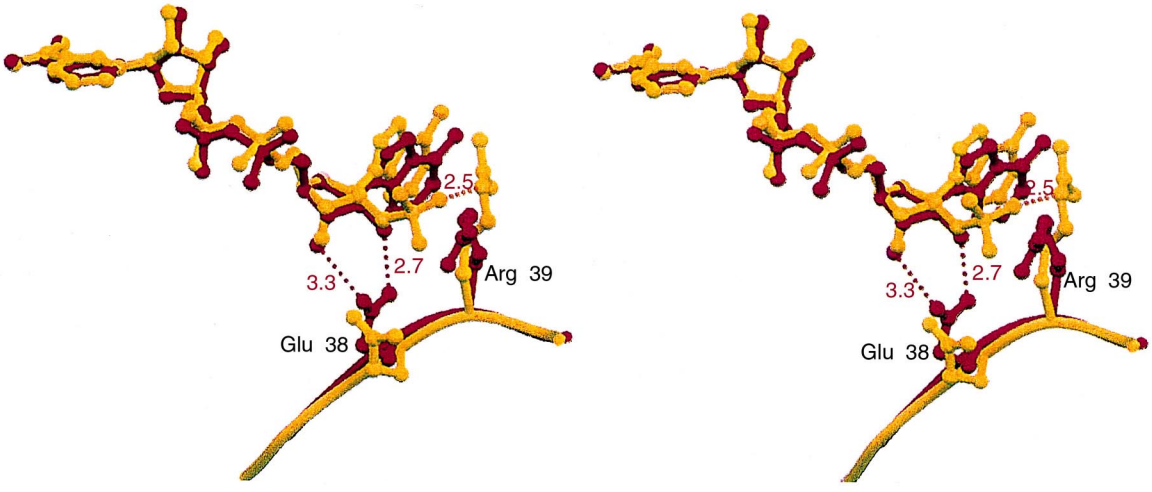
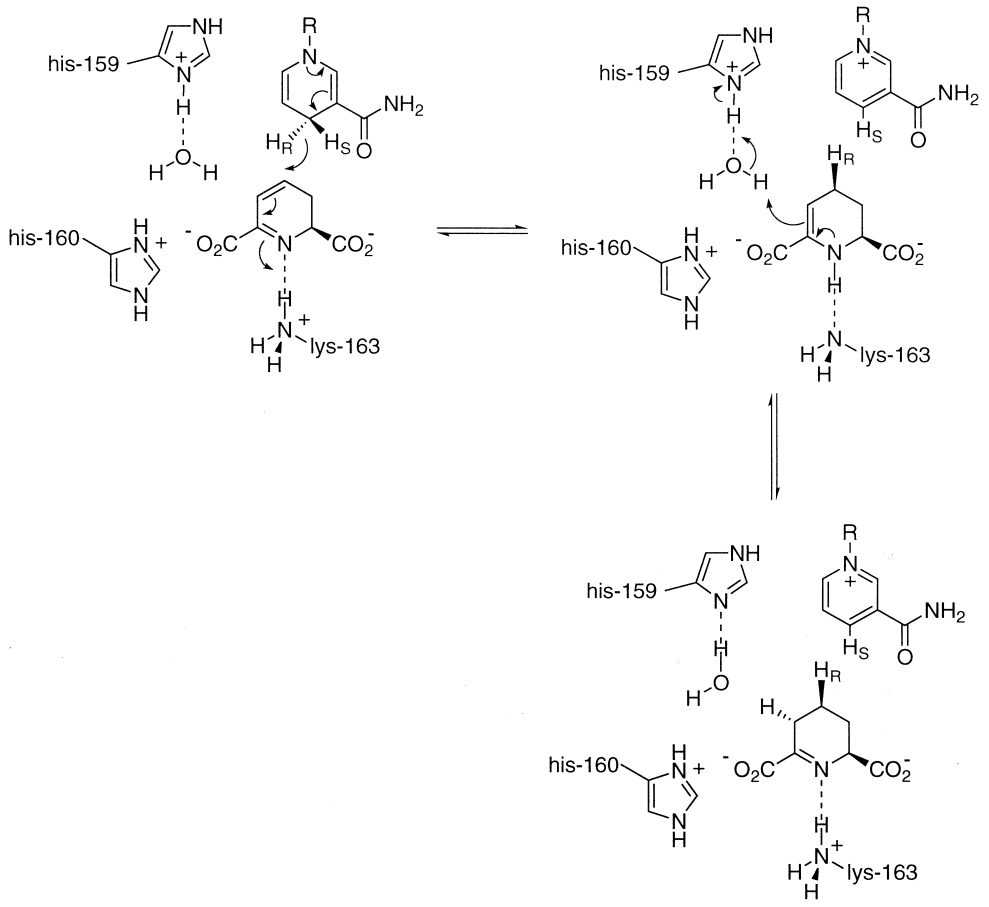
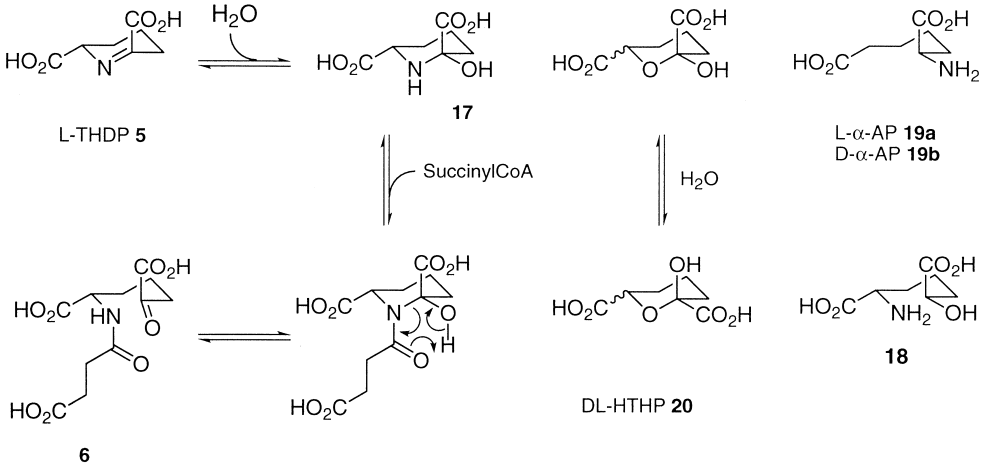


Figure 2. Superposition of the bound nucleotides, and the positions of the side chains of Arg39 and Glu38, observed in the DHPR-NADPH (yellow) and DHPR-NADH (red) complexes. Reprinted with permission from *Biochemistry* **1996**, *35*, 13294. Copyright 1996 American Chemical Society.



Scheme 3. Deduced mechanism of L-DHDP reductase.



Scheme 4. Stereochemical model of mechanism of succinyl-CoA:L-tetrahydrodipicolinate succinyltransferase.

sites are located between the monomers of the trimeric protein. The enzyme undergoes a conformational change upon substrate binding, such that the substrates are substantially buried and protected from the solvent. The CoA is bound in a 'hairpin' conformation, similarly to CoA binding in other acyl transferase enzymes. Most interestingly the substrate is bound in a *linear* form, quite differently than the cyclic conformation predicted from the earlier kinetic and inhibition studies (Fig. 3). L-THDP is bound as the linear *keto*-form, and it is unclear whether the enzyme first hydrolyses the cyclic imine, then binds the product, or whether the enzyme binds the ring opened compound which may have been generated spontaneously in the medium through aqueous hydrolysis. The nucleophilic amino group is located close to the terminal thiol of the phosphopantetheine, and is suitably positioned for nucleophilic attack on the succinyl group. The reaction may possibly be facilitated by

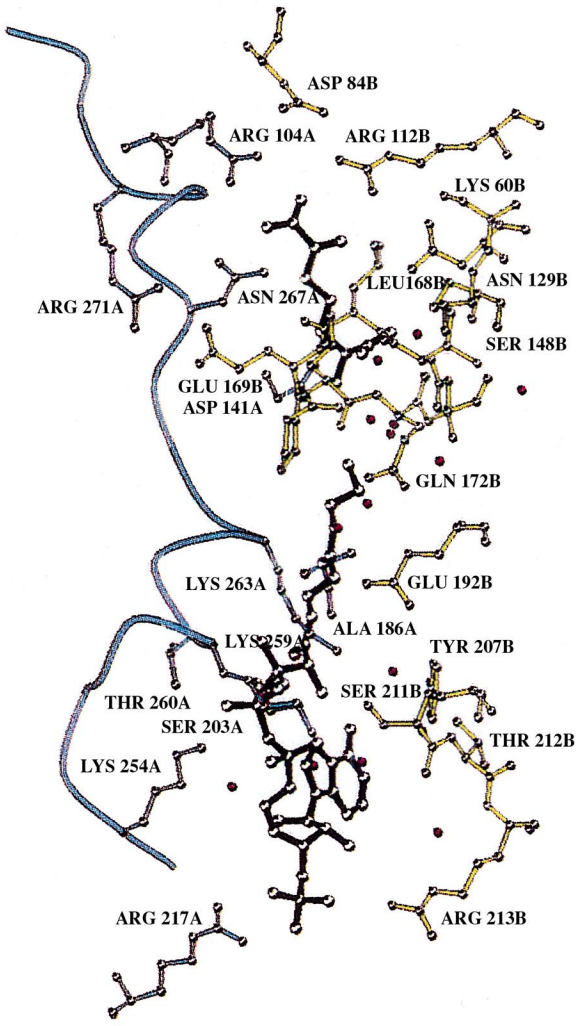


Figure 3. View of the THDP succinyltransferase active site. Residues from the A subunit (blue) and B subunit (yellow) are depicted as well as the substrates (bold). The polypeptide chain path of the C-terminal 18 residues (257A-274A) is depicted as a rope. Reprinted with permission from *Biochemistry* **1998**, *37*, 10363. Copyright 1998 American Chemical Society.

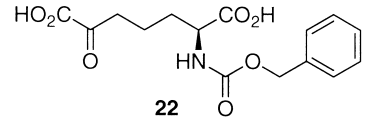
hydrogen bonding between the protonated amine of the substrate and Asp-141 of the enzyme.

Aminotransferase

Until very recently, investigations of DAP aminotransferase have used wild-type enzyme purified from *E. coli*.[56] Kinetic parameters for the natural substrates have been measured: for L-glutamate (**21**) K_M 1.21 mM; for L-*N*-succinyl-α-amino-ε-ketopimelate (**6**) K_M 0.18 mM, k_{cat} 86 s^{-1}. A sequential reaction mechanism has been proposed in which L-glutamate (**21**) reacts with the pyridoxal phosphate form of the enzyme, donating its amino group via aldimine, quinonoid and ketimine intermediates (Scheme 5). L-*N*-succinyl-α-amino-ε-ketopimelate (**6**) then reacts in the reverse direction, regenerating the PLP form of the enzyme with transfer of the amino group onto the product **7**. This mechanism is consistent with those of the 'model' system of aspartate amino transferase (EC 2.6.1.1).[57]

Surprisingly, very recent work indicates that at least in *E. coli* the *dapC* gene presumed to code for the aminotransferase enzyme is identical to *argC*, the gene that codes for the well-known *N*-acetyl-L-ornithine ε-aminotransferase.[58] The cloned enzyme accepts both *N*-succinyl-L-α-amino-ε-ketopimelate and *N*-acetyl-L-ornithine as substrates, although its utilization of the *N*-acetyl-L-α-amino-ε-ketopimelate proceeds with only 4% of the efficiency of the corresponding *N*-succinyl analogue.[56] In addition, preliminary studies suggest that *N*-succinyl derivatives of ornithine are poor substrates for this enzyme. This suggests that the specific recognition sites for the *N*-acyl group may be different or result in different substrate binding modes. It is uncertain whether the identity of *dapC* and *argC* proteins observed in *E. coli* will be seen in other organisms.

Although the crystal structure of the aminotransferase has not been reported, the substrate specificity has been investigated. Early work of Gilvarg[59] showed the aminotransferase to be present in *E. coli*. It was distinct from a number of transaminases known at the time, and was claimed to be distinct from *N*-acetyl ornithine aminotransferase.[60] Our more recent work has indicated that the best substrates are those which preserve the amide linkage.[56] Surprisingly aromatic groups in the side chain show nearly as good substrate activity (k_{cat}/K_M) as the natural substrate **6**. Cbz and cinnamoyl protected substrates, **22** and **23** showed the best activity with 25 and 75% k_{cat}/K_M of **6** respectively. Dipeptides are also substrates; the α and β aspartyl DAP analogues as well as the phenylalanyl dipeptide all showed turnover in enzyme assays.[61]



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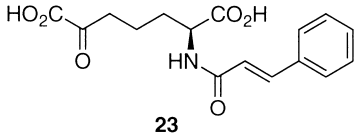
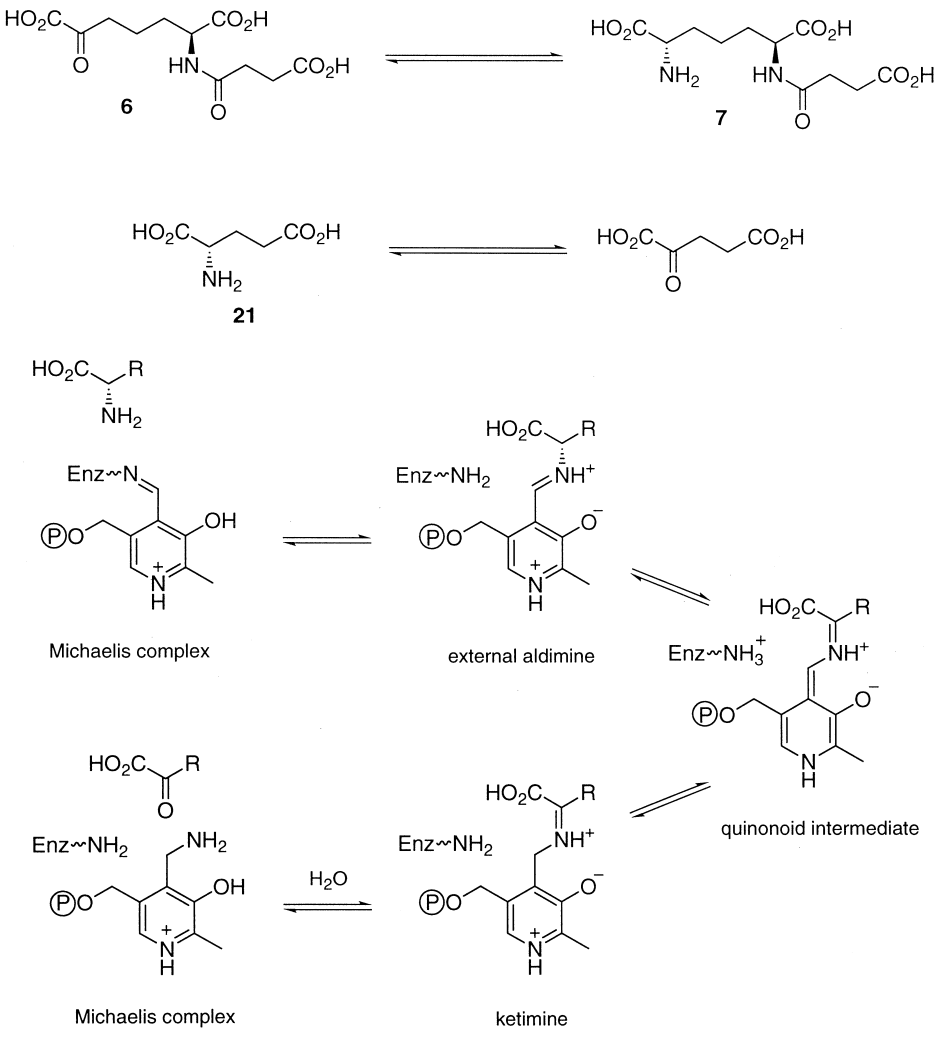


23

Desuccinylase

DAP desuccinylase from *E. coli* has been purified to homogeneity,[62] and DAP deacylase activity has been detected in numerous bacterial species.[63] Nucleotide sequencing of the enzymes from *E. coli*[64] and *Corynebacterium glutamicum*[65] both indicate a subunit M_r of approximately 40,000. Recently the *dapE* gene in *Helicobacter pylori*, which colonizes human gastric mucosa and causes gastritis or ulceration, has also been identified and sequenced.[66]

The *E. coli* enzyme utilises a metal ion, ideally cobalt II (K_M 4.0 μM), but zinc (K_M 1.2 μM), iron III, nickel II and manganese II ions are also effective. The enzyme is similar in function to other carboxypeptidases,[67] and shows significant sequence similarity with both the



Scheme 5. Mechanism of *N*-succinyl-LL-DAP aminotransferase.

cobalt II dependent acetylornithine deacetylase (EC 3.5.1.16) from *E. coli* and the *Pseudomonas* sp G2-carboxypeptidase.[68]

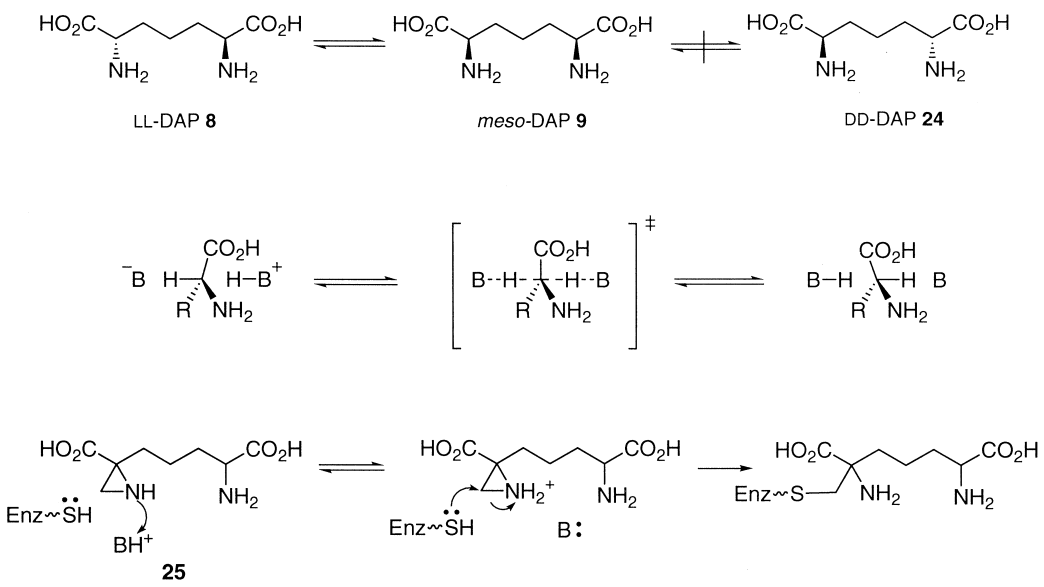
Detailed kinetic investigation of the *Haemophilus influenzae* enzyme has recently been reported.[69] Results of these experiments suggest that there are two metal binding sites per monomer, one of high affinity and the other of lower affinity. The high affinity site is usually occupied by zinc, while the low affinity site can be occupied by zinc or cobalt. The proposed mechanism is similar to that for carboxypeptidase G2 in which zinc activated water acts as a nucleophile, a tetrahedral intermediate is transiently stabilised, which then collapses with protonation of the departing amine. Notwithstanding these results, significant mechanistic insight into the desuccinylase will inevitably have to await the determination of a high resolution crystal structure of the enzyme.

DAP epimerase

The *dapF*-encoded diaminopimelate epimerase catalyses the interconversion of LL-DAP (**8**) and *meso*-DAP (**9**).[70] DD-DAP (**24**) is not a substrate and, therefore, the stereochemistry of the non-reacting α-carbon is critical for substrate recognition by the enzyme (Scheme 6). Kinetic parameters for LL-DAP (**8**) (K_M 160 μM, k_{cat} 84 s^{-1}, k_{cat}/K_M 525,000 K^{-1} S^{-1}) and *meso*-DAP (**9**) (K_M 360 μM, k_{cat} 67 s^{-1}, k_{cat}/K_M 186,111 M^{-1} S^{-1}) have been determined. HPLC analysis of the equilibrium constant produces a value of 2 (reflecting the statistical distribution of LL and *meso* isomers).[70]

Initial investigations of DAP epimerase showed that the enzyme is PLP independent and requires neither metals nor nicotinamide or flavin cofactors for catalysis. The enzyme is not inhibited by hydrazine or hydroxylamine and an imine is not an intermediate as sodium borohydride is not inhibitory. For the enzyme to remain active dithiothreitol must usually be present. Time dependent inhibition by iodoacetamide is observed with one equivalent of inhibitor bound per enzyme. These results suggest that *meso*-DAP epimerase has at least one reduced cysteine residue in the active site and operates via base catalysed α-proton abstraction.

Tritium at the substrate α-position exchanges rapidly with the solvent.[70] Exchange occurs by the loss of an



Scheme 6. *meso*-DAP Epimerase; its reaction, proposed mechanism and inhibition by azi-DAP (**25**).

α-proton to solvent, and a solvent derived proton is preferentially delivered to the substrate. The results are consistent with a mechanism in which two bases act on the substrate, the first base removes the α-hydrogen from one face and the protonated form of the second base delivers hydrogen from the opposite face (Scheme 6). *meso*-DAP epimerase resembles a number of related bacterial amino acid epimerases in this respect, including proline racemase,[71,72] glutamate racemase,[73] aspartate racemase[74,75] and hydroxyproline epimerase.[76] In each case, a relatively non-acidic α-hydrogen is removed from an amino acid without the use of metals or additional cofactors such as pyridoxal phosphate. Such a reaction is not easily accomplished in vitro because the pK_a of the hydrogen is relatively high for the zwitterionic or anionic amino acid. The α-hydrogen of the fully protonated "ammonium" acid will have a much lower pK_a, but kinetic deprotonation occurs at the carboxyl oxygen. Presumably the enzyme fixes the locations of the proton donors and bases such that both the carboxyl and the amino groups of the substrate are kept fully protonated while the α-hydrogen is being removed. For DAP epimerase, generation of anionic character at the α-carbon has been demonstrated by elimination of β-fluoride from 3-fluoro DAP isomers (see below).

Kinetic analysis suggests that the *meso*-DAP epimerase bases are thiols. This concurs with the observation that the irreversible inhibitor azi-DAP (**25**), which is generated in situ from the β-fluoromethyl precursor, specifically covalently labels Cys-73 of *meso*-DAP epimerase (Scheme 6).[77] Careful examination of pH dependence and solvent kinetic isotope effects supports a model in which proton isomerization after catalysis and substrate dissociation is kinetically significant.[78] A single solvent 'overshoot' is observed when LL-DAP is incubated with the epimerase in D_2O; however, an unprecedented double overshoot is observed when DL-DAP is incubated with the enzyme in D_2O. Other enzymes operating by a 'two base' mechanism such as mandelate racemase (EC 5.1.2.2) have also been extensively studied.[79] The concept of short, strong hydrogen bonds[80] between an active site residue and the substrate carboxylate in the transition state (or intermediate) has been examined in this context.[81] Such 'low barrier' hydrogen bonds may stabilise the transition state significantly, thus influencing the pK_a of the α-proton, and thereby enhancing the ability of such epimerases to remove substrate α-protons.

The recent crystal structure of the *Haemophilus influenzae* DAP epimerase supports the inferences derived via kinetic and inhibition studies.[82] The enzyme forms a novel fold in which C and N-terminal domains are structurally homologous (Fig. 4). Cys-73, previously shown to be one of the active site bases, forms a disulfide linkage with Cys-217 in the other domain. It is suggested that in the active reduced form of the enzyme these two conserved amino acids provide the two thiol bases required for activity. DAP must bind in the cleft between the N-terminal and C-terminal domains, but a detailed picture awaits the determination of a structure containing DAP or an analogue such as azi-DAP (**25**).

DAP D-dehydrogenase

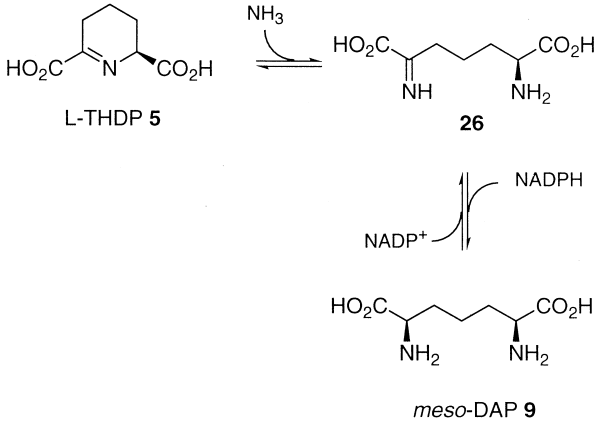
Detailed kinetic analyses of reactions catalysed by the *B. sphaericus* DAP dehydrogenase enzyme have revealed that the reaction is sequentially ordered,[83] like the classical glutamate dehydrogenase mechanism. For the forward reaction NADPH binds first, followed by L-THDP (**5**) and then ammonia. After the reaction is complete, *meso*-DAP (**9**) is released, followed by $NADP^+$.

The forward reaction proceeds via ring opening of L-THDP (**5**) by ammonia, forming a planar imine intermediate **26** (Scheme 7) which is reduced stereospecifically by the 4-*pro-S* hydrogen of NADPH to

Figure 4. Ribbon diagram on *H. influenzae* DAP epimerase. The N- and C-termini are labeled, as are the secondary structural elements and the two conserved cysteines. Domain I (residues 1–117 and 263–274) is shown in yellow, Domain II (residues 118–262) is shown in magenta and the disulfide connecting the two domains is shown in green. Reprinted with permission from *Biochemistry* **1998**, *37*, 16452. Copyright 1998 American Chemical Society.

generate the D-centre of *meso*-DAP (**9**). Neither DD- **24** nor LL-DAP (**8**) are substrates for this enzyme, which like DAP epimerase shows that the stereochemistry of the non-reacting α-carbon is crucial for substrate recognition.

The crystal structure of the enzyme from *Corynebacterium glutamicum* has been determined.[84] The enzyme structurally resembles other amino acid dehydrogenases and would appear to be related to DHDP reductase, the enzyme preceding the dehydrogenase in this variant of the pathway. The enzyme has three main domains, one binding substrate, one binding NADPH and the third forming a 'dimerization' domain. The C-4 position of NADPH is located near to the proposed substrate binding site. In the absence of DAP, the crystals are obtained with an excess of acetate in the crystallisation buffer. Two bound acetates, separated by about 5 Å, are seen in the structure and it is proposed that these two molecules delineate the ends of the putative DAP binding site. When *meso*-DAP is modeled into this site it is clear that the C4 position of $NADP^+$ is located close enough to the substrate to accept hydride (Fig. 5). Comparison with the structures of other amino acid dehydrogenases shows that *meso*-DAP dehydrogenase from *Corynebacterium glutamicum* binds NADPH in an anti conformation, presenting the *pro-R* hydrogen towards the substrate (in contrast to the *Bacillus* enzyme). Of course the orientation of the bound substrate is also reversed. The crystallographic studies indicate that conformational reorganization occurs upon binding of substrates, and electrospray mass spectrometry has been employed to further examine these changes using hydrogen/deuterium exchange.[85] NADPH and DAP binding both reduce the extent of deuterium exchange in the dehydrogenase, suggesting that certain domains 'close' to a catalytic form upon substrate binding.

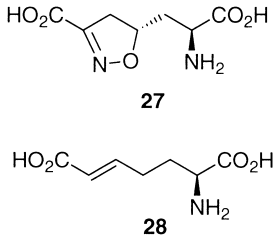


Scheme 7. Proposed stepwise reaction catalysed by *meso*-DAP dehydrogenase.

Futher information has been gained from crystals containing *meso*-DAP dehydrogenase and a planar isoxazoline inhibitor **27**.[86] The DAP binding site has been fully revealed by these studies. These crystals confirmed the extended all-*trans* binding conformation and explained the unique selectivity for the *meso* isomer. The distal amino acid binding site is specific for the L-configured centre, while the reacting centre may only bind as the D-configuration. The α-proton of this centre is presented to the $NADP^+$ cofactor. Intriguingly the planar inhibitor **27**, designed to mimic the intermediate imine at the reacting centre, binds to the substrate binding site in the opposite orientation to that expected (Fig. 6). The isoxazoline moiety binds in the distal L-binding pocket, thus presenting the L-amino acid to the cofactor at the reaction pocket. In its bound conformation the α-proton of the L-centre is now held away from the $NADP^+$ and reaction cannot proceed. Similar binding may be observed for the unsaturated L-α-aminopimelic acid (**28**) which is also a non-competitive inhibitor of this D-dehydrogenase.[87]

DAP decarboxylase

DAP decarboxylases from various sources are unique among PLP-dependent amino acid decarboxylases in that they catalyse reaction at a D-centre. This is reflected by protein sequence studies which suggest that DAP-decarboxylases are closely related as a group, but seem to be unrelated to most other PLP dependent



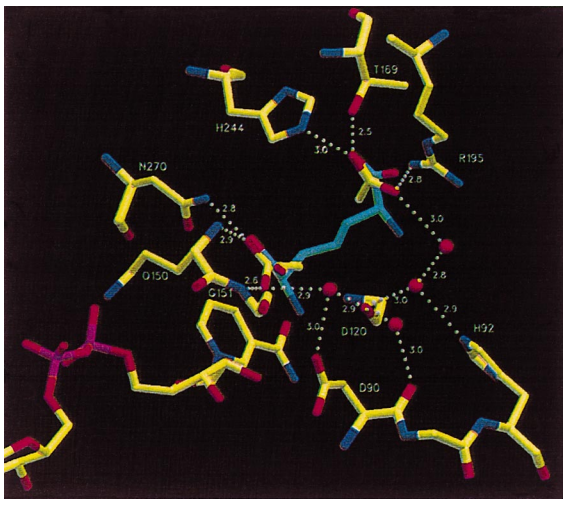


Figure 5. Close-up of the binding site of the acetate molecules in DAP dehydrogenase. A molecule of DAP (carbon atoms are in blue) has been overlaid onto the two acetates. Reprinted with permission from *Biochemistry* **1996**, *35*, 13540. Copyright 1996 American Chemical Society.

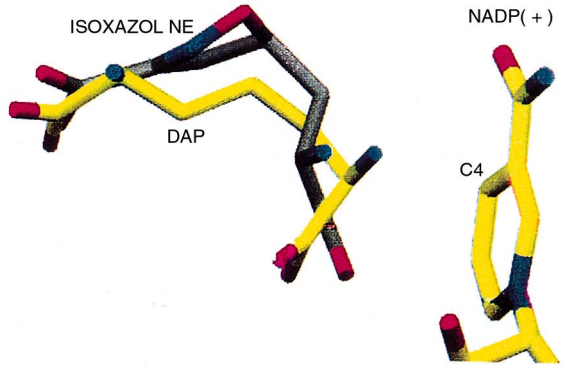


Figure 6. Overlay of DAP and the inhibitor in the diaminopimelate dehydrogenase substrate binding site. Reprinted with permission from *Biochemistry* **1998**, *37*, 3278. Copyright 1998 American Chemical Society.

enzymes.[88–91] Unlike other PLP dependent decarboxylases, where the reaction is accompanied by retention of stereochemistry, investigation of the DAP decarboxylase from *B. sphaericus*[92] and wheat enzymes[93] has shown that in both cases decarboxylation occurs with inversion of stereochemistry. Two mechanisms could account for the overall geometrical outcome. A 'swinging door' mechanism would involve rotation of the substrate-PLP complex after departure of CO_2 to allow protonation from the same side as CO_2 loss. However, this model would involve a severe conformational change of the enzyme–substrate complex. An alternative hypothesis, similar to other PLP dependent decarboxylase mechanisms involves formation of a common quinonoid intermediate **29** in the enzyme active site (Scheme 8). Protonation of this quinonoid intermediate **29** from the *re* face at the α-carbon in each case would achieve the observed stereochemical outcome (i.e., inversion at D-centres by DAP decarboxylases and retention at L-centres in other PLP dependent decarboxylases).

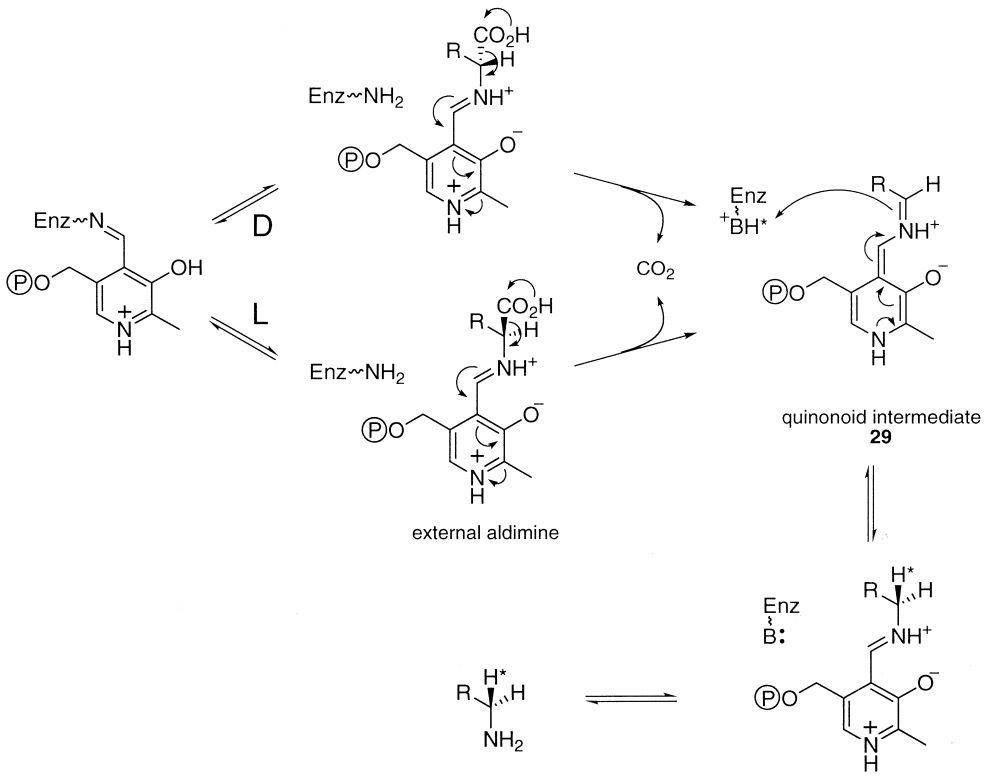
Synthetic Methods

The structures of the substrates and inhibitors of the DAP processing enzymes are diverse, and the variety of chemistry used to access these compounds reflects this. A chief synthetic problem has been access to *meso*-DAP analogues bearing useful protective groups such that the L- or D-amino groups (or respective carboxylates) can be selectively unmasked without transforming the corresponding group at the other end of the molecule. Attempts to utilise commercially available DAP (a statistical mixture of stereoisomers) as a starting material are usually disappointing because of the great difficulty in separating the diasteromers which result upon attempts at protection. Even the biochemical production of pure DAP isomers[94,95] or separation of the statistical mixture of unprotected DAP stereoisomers[96–98] is quite tedious. Early synthetic methods involving Kolbe coupling of amino acids suffer similar lack of selectivity and are inadequate for the production of uncontaminated DAP isomers. A more recent study using Kolbe decarboxylative coupling of mixtures of optically pure *N*- and α-carboxyl protected glutamic and aspartic acids gives pure stereoisomers of DAP with selective protection of all functional groups, but the yields are only 10–13% (Scheme 9).[99] However, advantages of the method are that the starting materials are readily available and the transformation is a single step process.

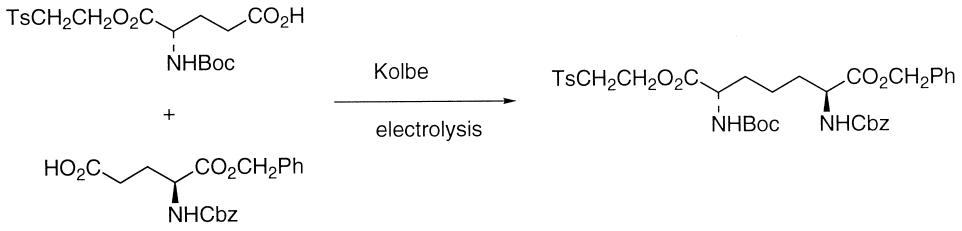
Several modern methods afford access to only one specific isomer (*meso* or LL) of DAP or its derivatives via symmetrical intermediates. Arakawa and co-workers employed a Diels–Alder reaction of azodibenzoyl with 1,3-cycloheptadiene to produce a bicyclic adduct **30** in 93% yield (Scheme 10).[100] Oxidative cleavage of the double bond to **31** followed by hydrogenolysis gives *meso*-DAP. In a different approach, palladium-catalysed coupling of the organozinc reagent derived from the benzyl ester of *N*-Boc-L-3-iodoalanine and carbon monoxide affords fully protected (2*S*,6*S*)-4-oxo-2,6-diaminopimelic acid.[101] Presumably the 4-oxo group could be reductively removed to afford dibenzyl bis(*N*-Boc)-LL-DAP, although this was not reported.

One way of achieving synthesis of all individual DAP stereoisomers is to build up the DAP skeleton from enantiomerically pure starting materials bearing orthogonal protecting groups. Early work,[102] exploited by us, involves the use of ene methodology for coupling an enantiomerically pure protected allyl glycine unit **32** with methyl glyoxylate (Scheme 11).[56] Enantiomerically pure allyglycine is readily available via resolution of the racemic *N*-acetates. This approach has the advantage of yielding stereochemically pure amino esters such as **33** in protected form, but the newly formed ε-alcohol is racemic. Of course, this is not a problem for synthesis of the DAP-AT substrate **6**, but is a handicap for a route to enantiomerically pure DAP isomers.

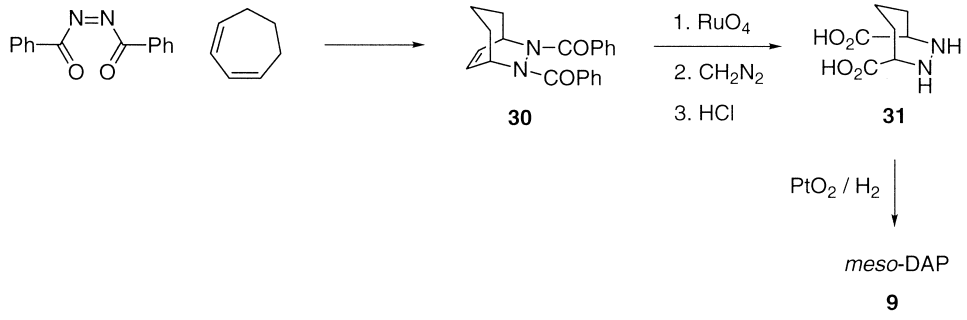
Stereoselective reduction of ketone **34** could provide access to optically pure compounds which could then be converted to DAP. The stereoselective reduction



Scheme 8. Reactions catalysed by PLP dependent L-amino acid decarboxylases (path L) and *meso*-DAP decarboxylases (path D).

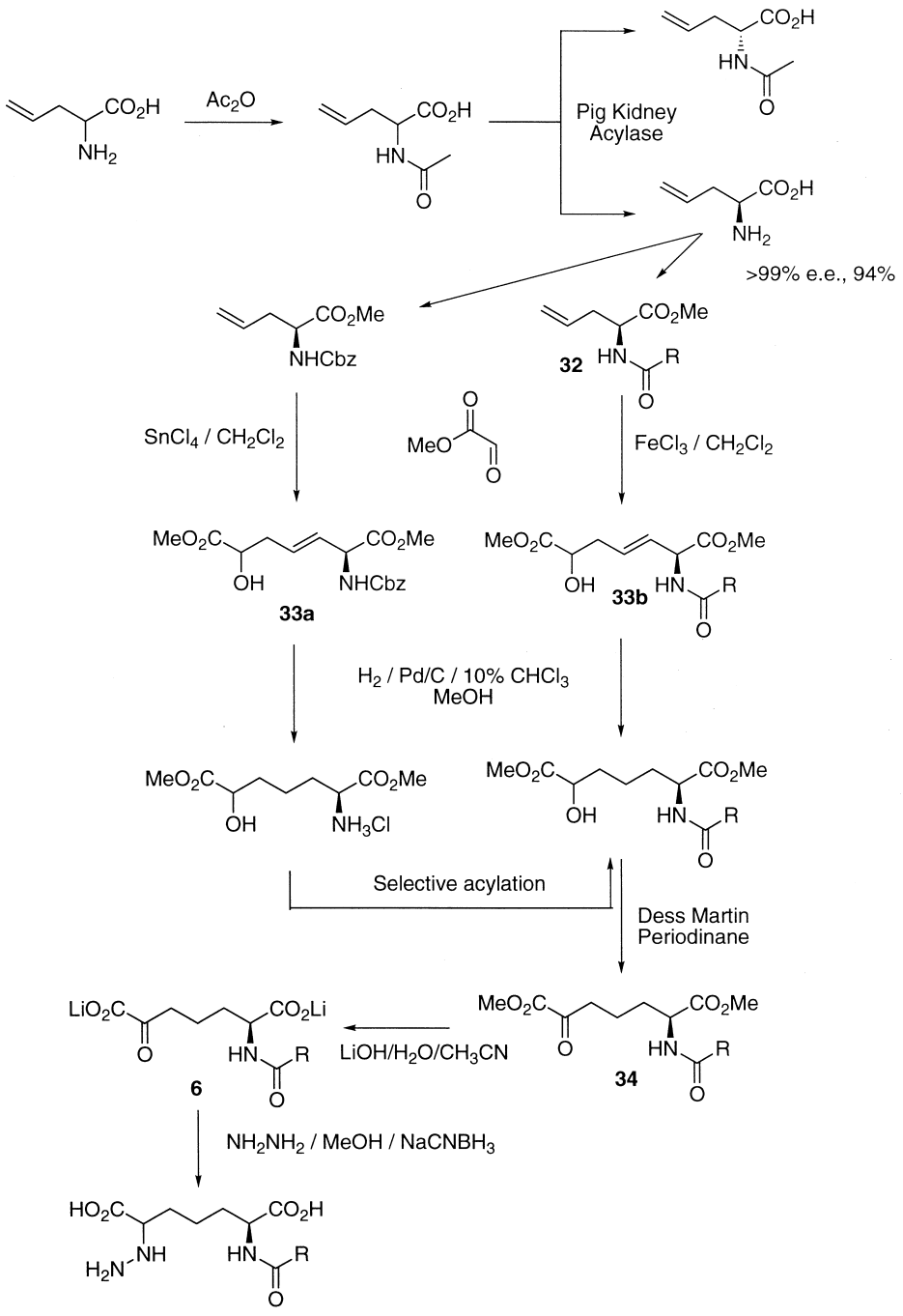


Scheme 9. Kolbe electrolysis to produce selectively protected DAP.



Scheme 10. Diels–Alder approach to DAP synthesis.

conditions of Noyori were examined for synthesis of enantiomerically pure alcohols (Scheme 12).[103] Disappointingly enantiomeric excesses of only 58% (isomer ratio 79:21) could be obtained under optimized conditions. The use of chiral glyoxylates for ene reactions has been thoroughly investigated by Whitesell.[104] Application of this methodology to the synthesis of DAP was more successful, generating 70% e.e. (85:15 mixture of diastereomers) at the newly formed alcohol bearing ϵ-carbon when phenylcyclohexyl esters of glyoxylate were used.[103] Conversion to DAP via mesylation, azide displacement and reduction generates mixtures of DAP isomers containing no DD-DAP, thereby indicating no racemisation at the α-carbon during ene reaction. The

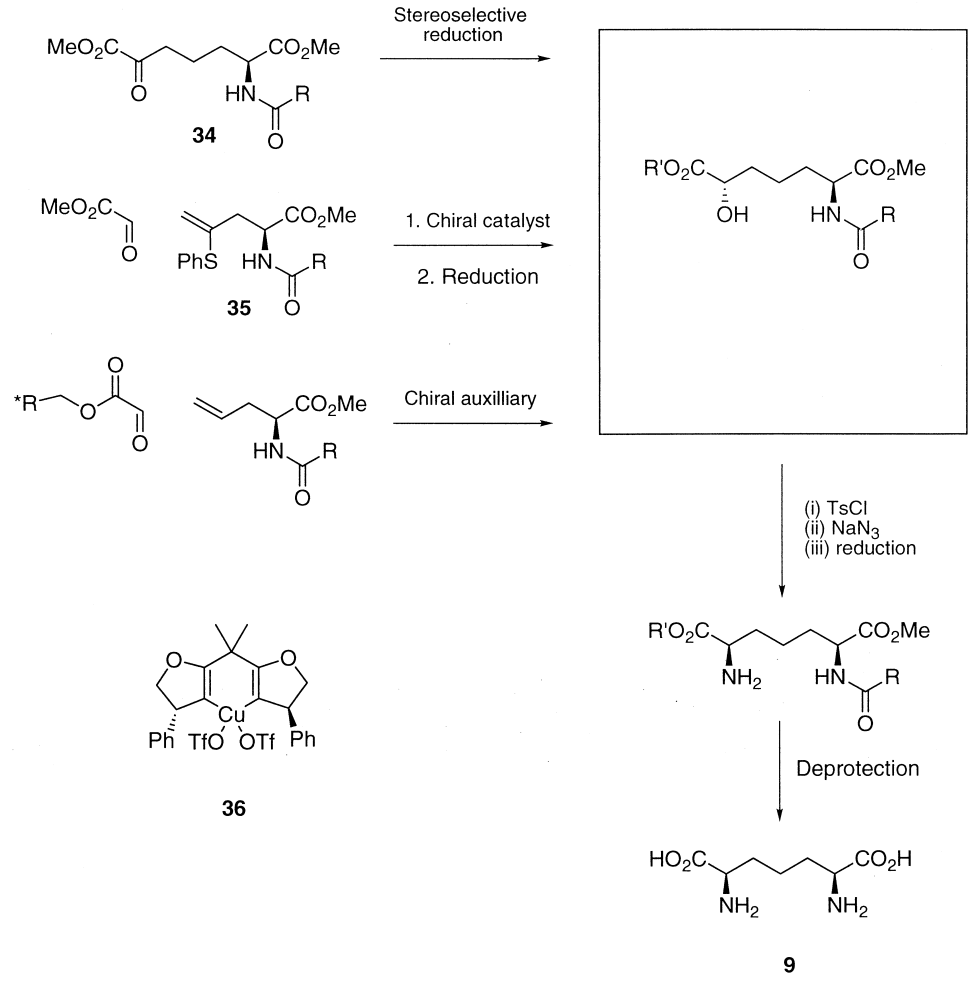


Scheme 11. Synthesis of DAP derivatives using ene methodology.

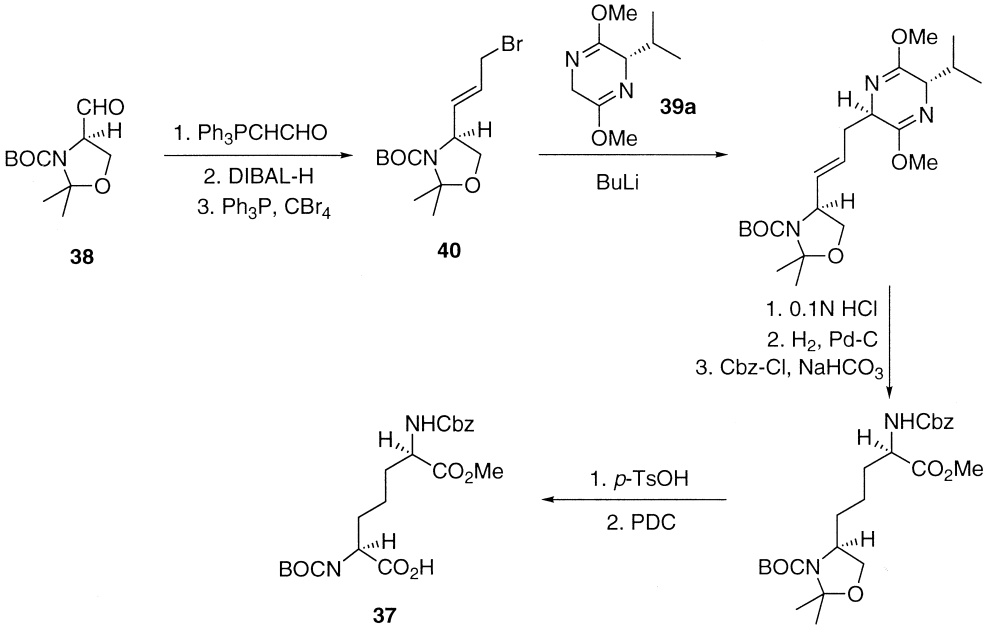
use of chiral catalysts for modified stereoselective ene reactions has also been investigated. Most common catalysts are insufficiently active to catalyse reaction between glyoxylate and unreactive terminal olefins. Phenylthioalkenes are more reactive however, and the reaction between the alkene **35** and methyl glyoxylate catalysed by the bis(oxazoline)copper compound **36** gives a 42% yield of the corresponding alcohol.[103] Conversion to DAP affords material of 88% d.e. (94:6 *meso*:LL).

Jurgens has prepared orthogonally protected *meso*-2,6-diaminopimelic acid **37** by linking a Garner oxazolidine **38** with the L-valine derived Schöllkopf bislactim ether **39a** using a C-2 linker (Scheme 13).[105] A two carbon homologation of the Garner aldehyde **38** via a Wittig reaction gave solely the *trans*-α,β-unsaturated aldehyde which was converted to the bromide **40** in two steps. Alkylation of the bislactim ether in nearly quantitative yield gave the *meso*-DAP skeleton. Hydrolysis of the bislactim ether, hydrogenation of the double bond followed by protecting group interconversion and finally oxidation gave orthogonally protected *meso*-DAP **37** as a single diastereomer.

In a different strategy DAP, and its homologues, can be viewed as two independent glycines joined by a C_3 linker



Scheme 12. Chiral ene reactions and chiral reductions to generate DAP precursors.



Scheme 13. Jurgen's synthesis of orthogonally protected *meso*-DAP.

(Scheme 14). Williams has exploited his chiral glycine synthon in this way for the synthesis of differently protected DAP isomers and 2,3-cyclopropyl-DAP analogues (Scheme 15).[106,107] Thus commercially available antipodes of the diphenyloxazinones were utilised as chiral protected sources of glycine. In a typical procedure the *cis* diphenyloxazine (**41**) was treated with LHMDS and homoallyl iodide to give the terminal olefin **42**. Ozonolysis smoothly provided the aldehyde **43a** which could be reacted with the dibutylboron enolate of diphenyloxazine (**44**) to give the differently protected **45** with excellent diastereoselectivity favoring the anti product. Barton deoxygenation affords the bis diphenyloxazine (**46**). Reduction then selectively gives the *meso*-DAP skeleton **47** in which the L stereocentre is protected with BOC. Overall this procedure provides DAP in very high e.e. (>99%). The procedure allows the flexible interchange of starting diphenyloxazinones which are available with either BOC or Cbz protecting groups, giving ultimately any of the isomers of DAP selectively protected. Simple variation in the procedure gives the unsaturated analogue **48** which can be further manipulated to give the cyclopropane **49** and its deprotected product **50**.

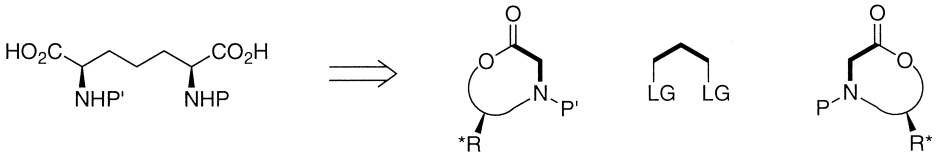
Again using diphenyloxazinones as chiral glycine equivalents, the research groups of both Williams[108] and Baldwin[109] have reported the stereoselective synthesis of 2,6-diamino-6-(hydroxymethyl)pimelic acid (**51**), a constituent of a natural antibiotic isolated from *Micromonospora chalcea*.[110] Williams and co-workers accomplished their synthesis in 8 steps using an aldol reaction and a Barton de-oxygenation as the key steps. Baldwin and co-workers were able to link the chiral oxazinones by alkylation in the presence of 15-crown-6 to eventually give (2*S*,6*S*)-α-hydroxymethyl-DAP (**51**) in 6 steps with an overall yield of 32% (Scheme 16).

Similarly, the Schöllkopf bislactim ether methodology has been applied by Bold in the synthesis of DAP and substituted analogues (Scheme 17).[111] In a straight-forward synthesis of DAP, *R*-bislactim ether (**39b**) was treated with base and the C_3 linker 1,3-dibromopropane to give a 69% yield of the desired coupling product **52a**. Acid treatment then liberated LL-DAP (**8**) in high yield and >98% e.e. A recent synthesis of bis(α-methyl) DAP employs a similar alkylation of two Schöllkopf bislactim ethers.[112] Introduction of selectively positioned protecting groups using this strategy is more difficult than with the method of Williams. However, the use of protected and functionalised amino acids as one of the DAP termini allows the coupling of a protected chiral glycine equivalent and the introduction of differently protected amino acid termini. A good example of this is the synthesis of β-hydroxy and β-fluoro-DAP diastereoisomers.

Initial attempts to synthesise fluorinated DAP isomers utilised the protected aldehyde **53** derived from L-glutamate (Scheme 18).[113] Condensation between this and the antipodes of the Seebach chiral glycine synthon **54** gave good *syn* diastereoselectivity to give the two alcohols **55a** and **55b** in greater than 95% d.e. Facile acid catalysed deprotection then gave the amino alcohols **56a** and **56b**, but SF_4/HF treatment gave only the γ-fluoro isomers **57**, perhaps via fluoride attack at a γ-carbocation. DAST treatment of the fully protected compounds **55** was also unsuccessful. In a complementary approach (Scheme 19) the protected aldehyde **53** was condensed with the Schöllkopf bislactim ether **39b**. Fortuitously diastereoselectivity in these reactions was low, giving 55:45 ratio of products, in favour of the *syn* product, when the 3-*R* bis-lactim ether was used and 83:17 when the 3-*S* bislactim ether was used. The products **58** were not separated, but treated with DAST to give low yields of the desired fluorine containing compounds **59** arising from inversion at the β-carbon. These fluorinated compounds could be separated by chromatography and acid catalysed aqueous deprotection then gave all four of the desired diastereomers **60**. Very recently this stereoselective synthesis of β-fluoro DAP has been improved by condensation of the anion of the Schöllkopf bislactim ether with glutamate semialdehyde methyl ester bearing two *N*-Boc protecting groups.[114] Interestingly, DAST reaction of the resulting alcohol permits isolation and characterization of the intermediate alkoxy-*N*,*N*-dialkylaminodifluorosulfurane.

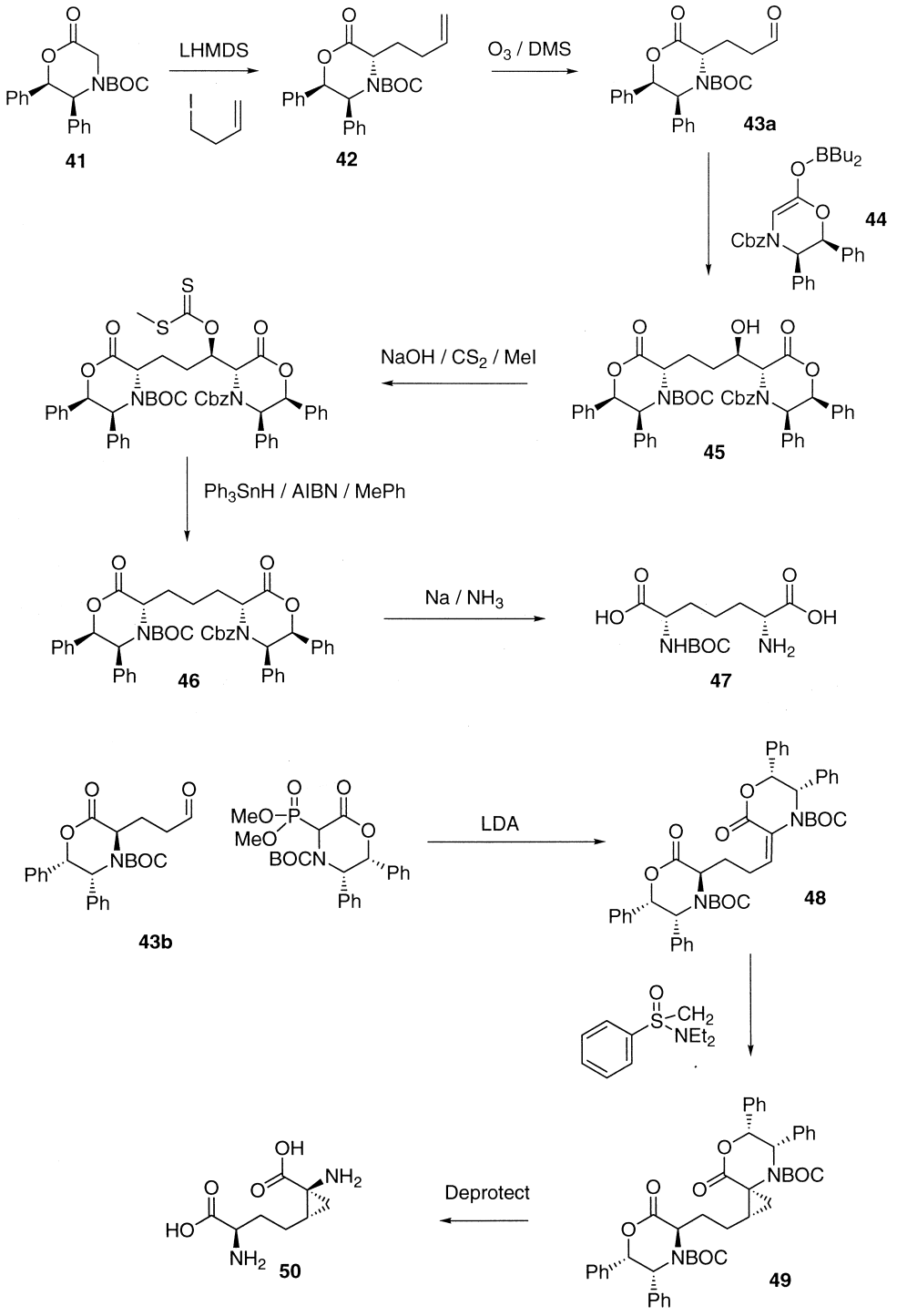
(2*R*,3*S*)-β-Hydroxy DAP (**56b**) has also been prepared by Bold and co-workers who have used a novel titanium–carbohydrate complex to facilitate a stereoselective aldol reaction in the key step.[115]

Phosphonic acid analogues of DAP have been synthesised as mixtures of all possible stereoisomers.[116] More recently a stereoselective synthesis of phosphono-DAP analogues has been developed (Scheme 20).[117] Dibromopropane was extended first with the Seebach chiral glycine synthon **54a**, and then with the (−)-camphor imine of diethyl aminomethylphosphonate (**61**). Diastereoselectivity in the second condensation favoured formation of the LL configured compound **62a** over the LD configuration **62b** in a 4:1 ratio. These diastereomers



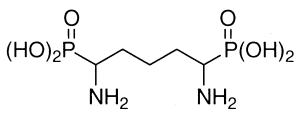
P, P' : Orthogonal protecting groups
LG : Nucleofuge
R* : Chirality

Scheme 14. Strategy for DAP synthesis.



Scheme 15. Williams' synthesis of DAP isomers.

could be separated and the deprotected compounds **63a** and **63b** were formed upon two-stage acid catalysed hydrolysis. Beginning with the opposite Seebach enantiomer gave the other two diastereomers, although in a 3:2 ratio. The racemic diphosphonate **64** was synthesised by similar methodology using a C_3 linker and two of the protected phospho-glycine units.



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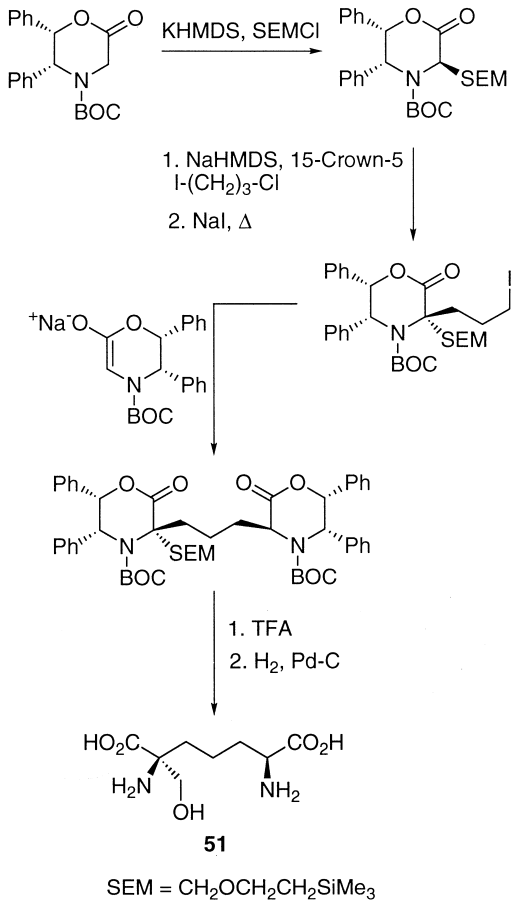


51

Scheme 16. Baldwin's synthesis of (2*S*,6*S*)-α-hydroxymethyl-DAP.

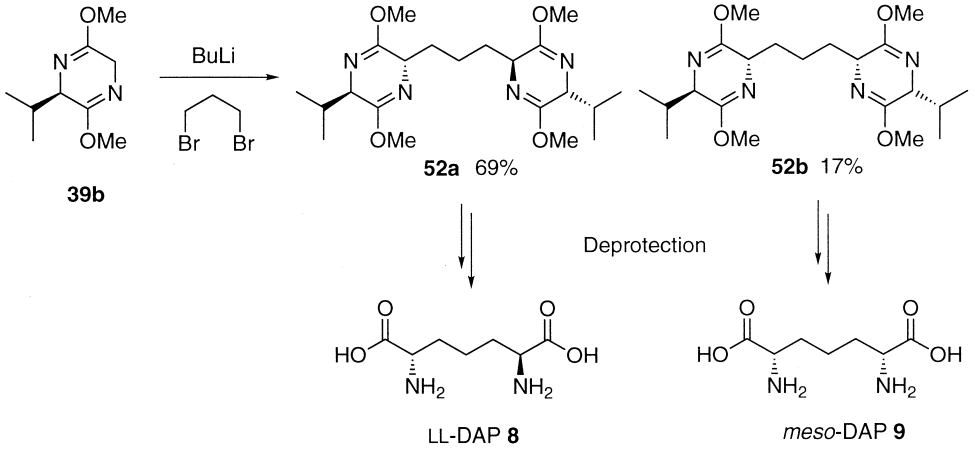
The protected glutamate synthon **53** has also been used by Holcomb et al. for a DAP synthesis relying on asymmetric olefin reduction to furnish the second stereocentre. In this strategy (Scheme 21)[118] **53** is coupled with the potassium anion of aminophosphonate **65** to give the unsaturated DAP skeleton **66** as as mixture of isomers which were readily separated. The major (*Z*) isomer (6.4:1) was subjected to asymmetric reduction using the rhodium catalyst *S*,*S*-chiraphos $Rh(NBD)_2ClO_4$ to give a 3:1 ratio of diastereomers with the major isomer possessing D configuration at the newly formed stereocentre of **67**. TMS-ethyl ester **68** was then formed and the diastereomers separated.

Recent novel advances in metathesis chemistry have provided new routes to DAP related diamines. Independently, we and the group of Williams have investigated Grubbs catalyst for the stereochemically controlled synthesis of differently protected diaminosuberic and diaminopimelic acids (Scheme 22).[103,119,120] Typically a short diol linker is utilised as a scaffold to tether two enantiomerically pure orthogonally protected amino acids bearing terminal olefins. Employment of Grubbs catalyst, followed by catalytic olefin reduction then efficiently gives the required carbon framework **69** and simple ester hydrolysis affords the protected diamines **70** in high yields and optical purity. Although this methodology has proved successful for the production of diaminosuberic acid **70**, synthesis of DAP requires the use of unstable vinyl glycine analogues and difficulties in preventing unwanted isomerisation of the olefin **71** to **72** have so-far hampered attempts at the synthesis of DAP isomers.

Inhibitors

Reversible competitive inhibitors

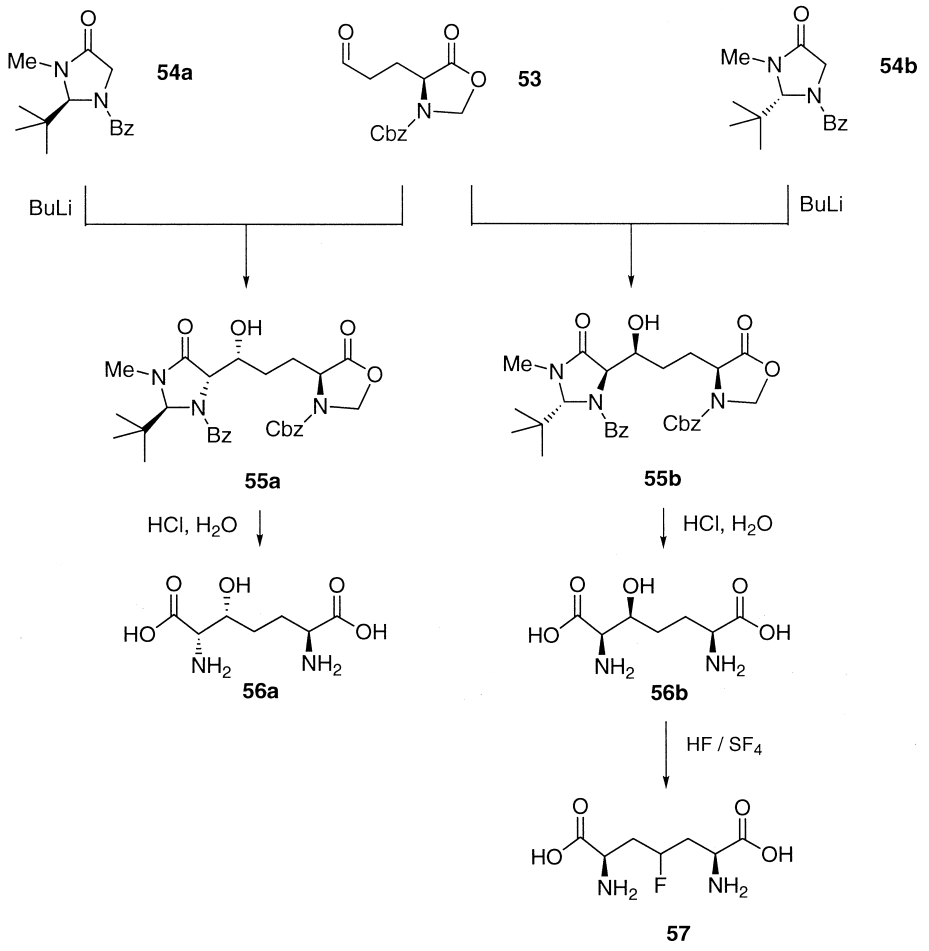
While the acyclic compound L-α-aminopimelate (L-α-AP) (**19a**) has been used as an alternative substrate for L-THDP syccinyl transferase (K_M 1 mM, k_{cat}/K_M 1.3% that of L-THDP (**5**)),[52] the enantiomer, D-α-aminopimelate (**19b**), is a competitive inhibitor (K_i 0.76 mM against L-THDP (**5**), 0.31 mM against L-α-AP (**19a**)) (Scheme 4). An investigation of a number of acyclic substrate analogues found that the enzyme was specific for diacids with a carbon chain length of seven. For example a statistical mixture of isomers **73a–d** was succinylated 21% faster than L-α-AP (**19a**). The conformationally restricted (ring-like) compound 2*E*,5*E*-γ-ketoheptadienedioic acid **74** is also an inhibitor (K_i^{app} 0.53 mM against L-α-AP (**19a**)).



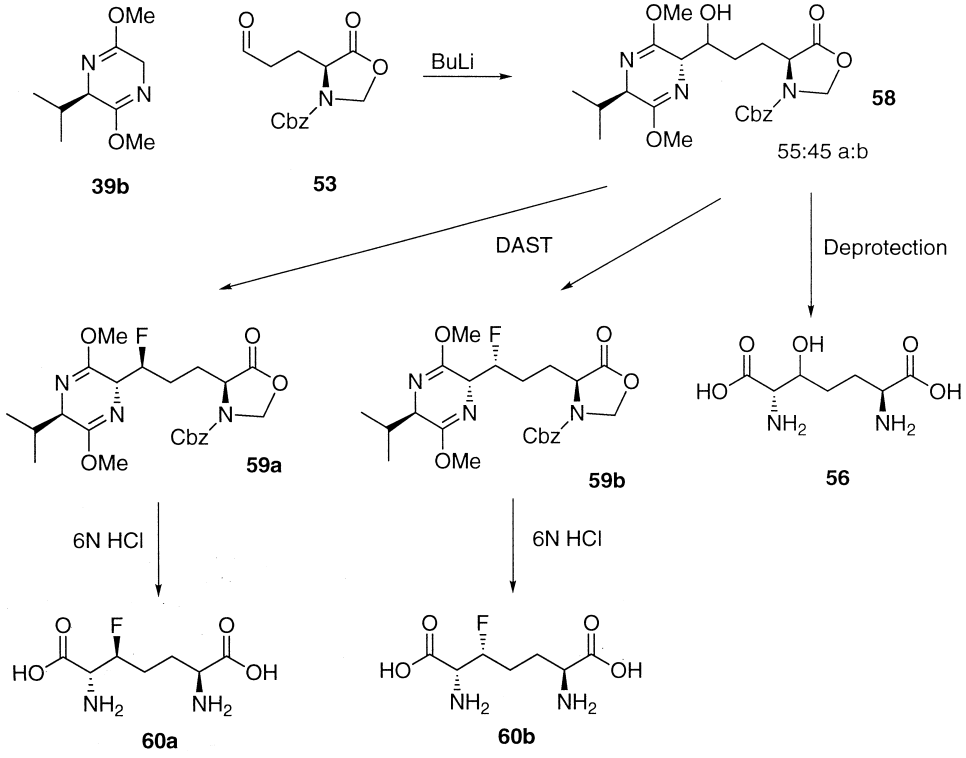
39b **52a** 69% **52b** 17%

LL-DAP **8** *meso*-DAP **9**

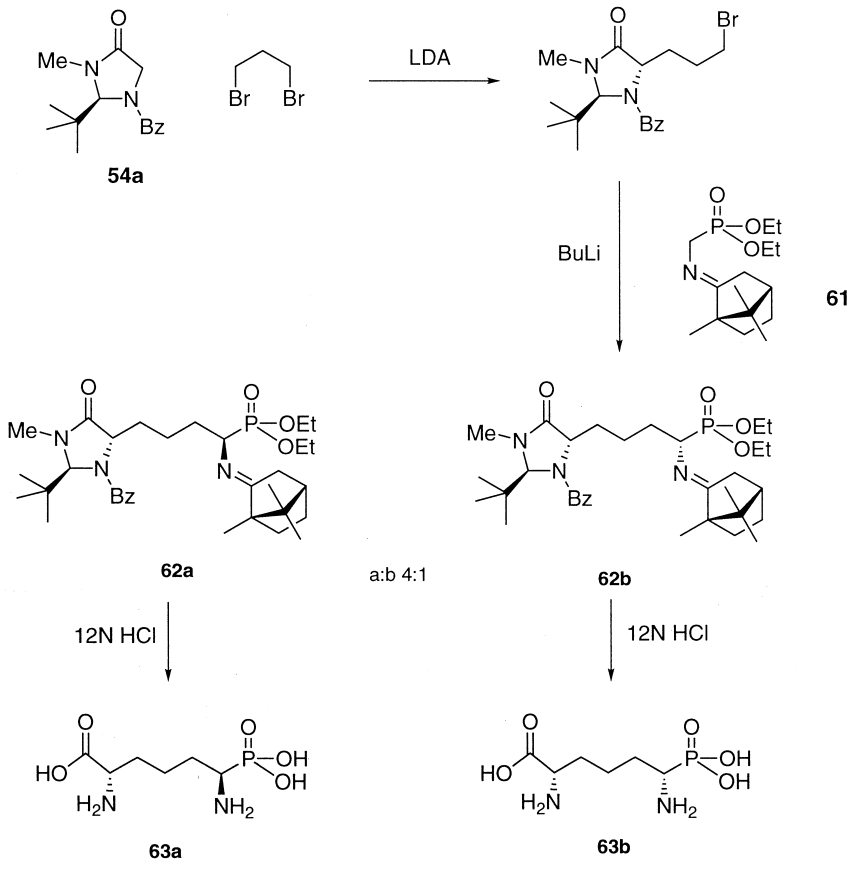
Scheme 17. Bold's synthesis of DAP isomers.



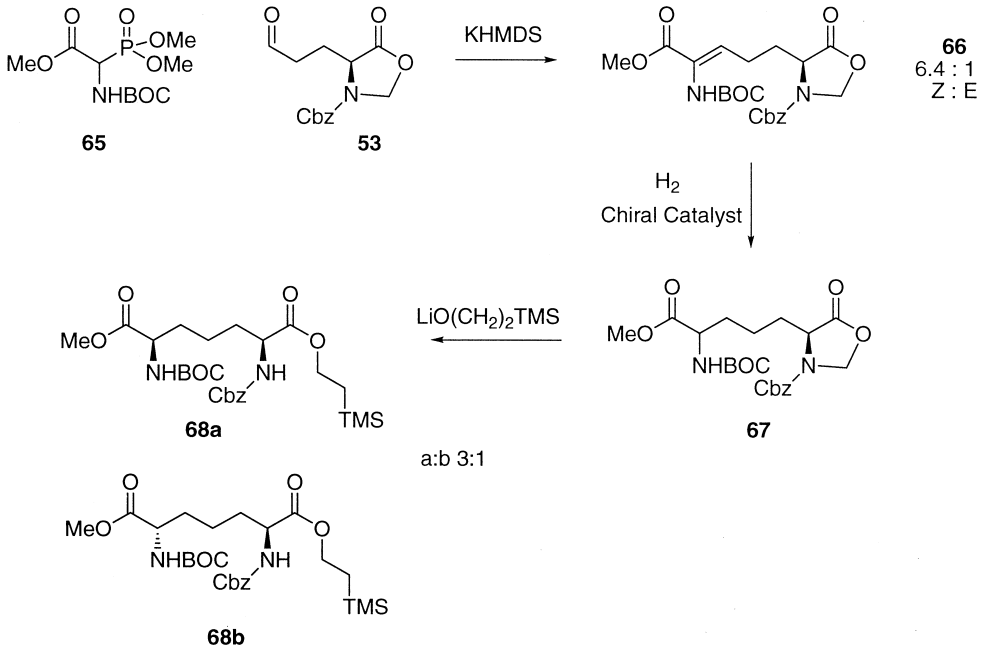
Scheme 18. Attempted synthesis of β-hydroxy and β-fluoro-DAP stereoisomers using Seebach technology.



Scheme 19. Synthesis of β-hydroxy and β-fluoro-DAP stereoisomers using Schöllkopf technology.

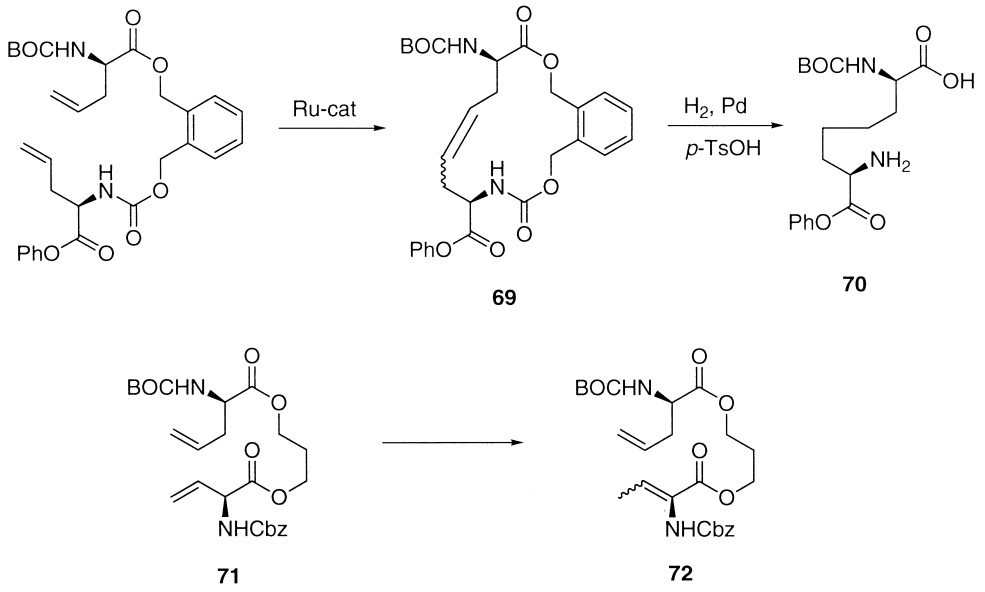


Scheme 20. Synthesis of phosphono-DAP isomers.



Scheme 21. Holcomb's synthesis of differently protected DAP diastereomers.

Cyclic analogues of L-THDP have been tested against the succinyltransferase. 3,4-Dihydro-2H-1,4-thiazine-3,5-dicarboxylic acid (DHT) (**75**) was found to be a substrate (K_M^{app} 2 mM, k_{cat}/K_M^{app} 0.5% that of L-THDP (**5**)) while other compounds tested were generally poor inhibitors (Fig. 7). It was also observed that unsaturated compounds bearing *trans* carboxyl groups are better inhibitors than those with *cis* carboxylates. One com-

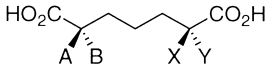


Scheme 22. Metathesis approach to DAP isomers and their homologues.

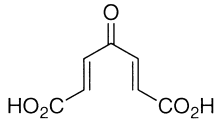
pound, DL-α-hydroxytetrahydropyran-α-ϵ-dicarboxylate (HTHP) (**20**), is a very potent competitive inhibitor (K_i 58 nM against L-α-AP (**19a**)), presumably because it can take up a conformation similar to that proposed for the bound substrate (Scheme 4).

The proposed mechanisms for *meso*-DAP epimerase have all indicated that negative charge should be concentrated at the α-carbon during reaction. This has led to the design of possible *meso*-DAP epimerase inhibitors unstable to elimination (i.e., β or N-substituted), or compounds which could mimic the putative planar transition state (Scheme 6). A mixture of stereoisomers of β-chloro-DAP (**76**) (Scheme 23) have been synthesised and found to potently inhibit the epimerase (K_i 200 nM).[121] Inhibition is reversible and competitive with the substrate. However, at low inhibitor concentrations, a time dependent decrease of inhibition was observed, suggesting inhibitor turnover. Reduction of the product of this reaction by *meso*-DAP dehydrogenase gave L-THDP (**5**). The epimerase likely catalyses the elimination of HCl from β-chloro-DAP (**76**), forming the intermediate **77** (Scheme 23). Intermediate **77** is planar at the reacting α-carbon and is therefore a mimic of the postulated transition state (Scheme 6). As *meso*-DAP dehydrogenase is specific for L-THDP (**5**), intermediate **77** for the epimerase must have possessed L-configuration at the distal (non-reacting) end.

The four stereoisomers of β-fluoro-DAP **60a–d** are good inhibitors of the epimerase (IC_{50} values: **60a** 4 μM; **60b** 10 μM; **60c** 25 μM; **60d** 8 μM).[113] Like the β-chloro-DAP analogues **76**, elimination (in this case HF) is catalysed by the epimerase, and the eventual product is L-THDP (**5**). The rates of HF elimination, however, vary, and epimerisation at the reacting α-carbon have been independently determined.[113] For one pair of isomers **60a,d**, the elimination is slow, but epimerisation is fast and the two isomers are in rapid equilibrium. For the other pair **60b,c**, only fast elimination is observed. These results suggest that the position of the charged groups and the bulky substituent R are fixed in the enzyme active site (Scheme 23). Fast elimination to give *E*-**77** is expected when H and F are fixed either *syn* or *anti*-coplanar in the enzyme active site as in stereoisomers **60b,c**.[122] For isomers **60a,d** where H and F are fixed *gauche* epimerisation occurs rapidly, and HF elimination is slow.



73a B = OH, X = NH_2, A = Y = H
73b A = OH, X = NH_2, B = Y = H
73c B = OH, Y = NH_2, A = X = H
73d A = OH, Y = NH_2, B = X = H



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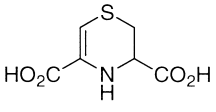
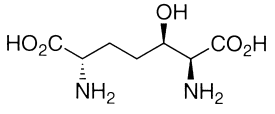
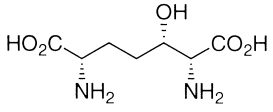


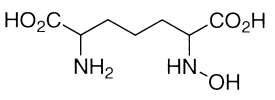
75 DHT



56a



56b



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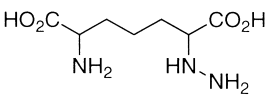

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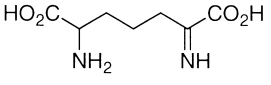

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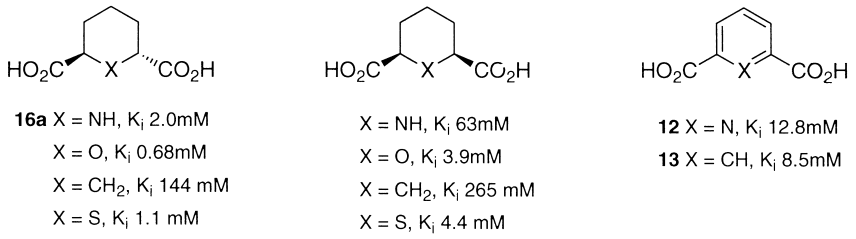
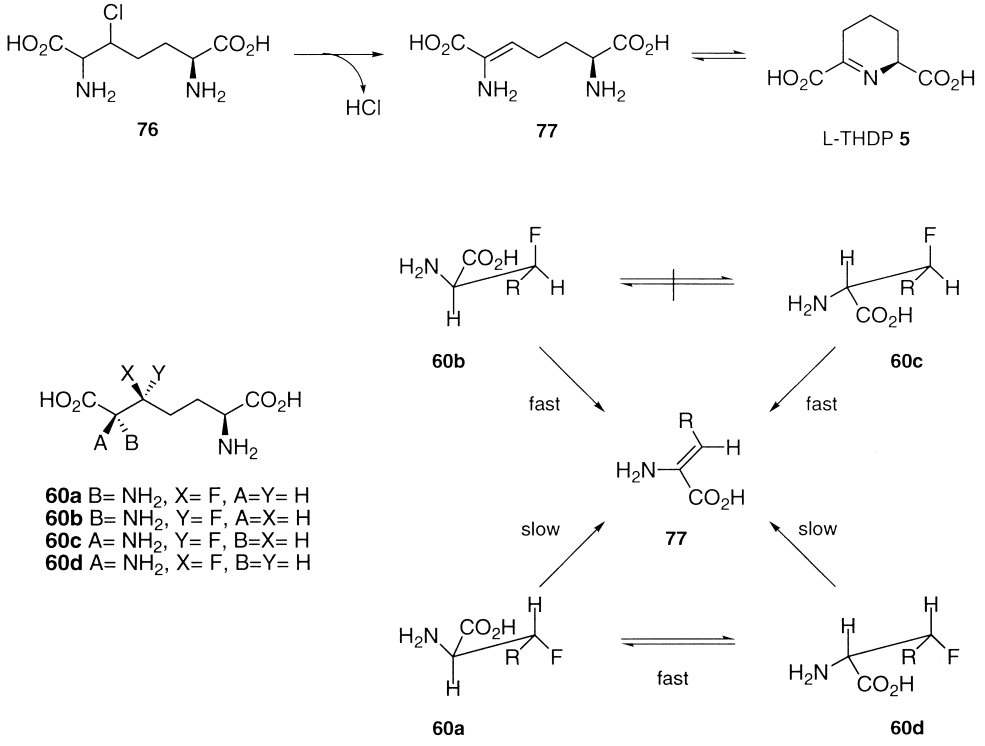


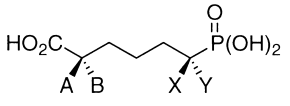
Figure 7. Inhibitors of succinylCoA:L-tetrahydrodipicolinate succinyltransferase.



Scheme 23. Reaction of β-chloro-DAP (**76**) catalysed by *meso*-DAP epimerase and stereochemical rationalisation of reactions of β-fluoro-DAP stereoisomers catalysed by *meso*-DAP epimerase.

The β-hydroxy DAP isomers **56** are poor inhibitors of the epimerase (IC_{50} 2.5 and 4 mM respectively) since H_2O is not eliminated. Compound **56a** is epimerised at the α-centre distal from the hydroxyl while **56b** is not epimerised at all.[113,121,123] A mixture of stereoisomers of *N*-hydroxy-DAP (**78**) reversibly and competitively inhibited the epimerase (K_i 56 μM).[124] The corresponding *N*-amino-DAP (**79**) was a much poorer inhibitor (K_i 2.9 mM). Elimination of water from **78** would lead to the planar imine **80** which could either tightly bind to the epimerase or reversibly react with one of the active site bases.

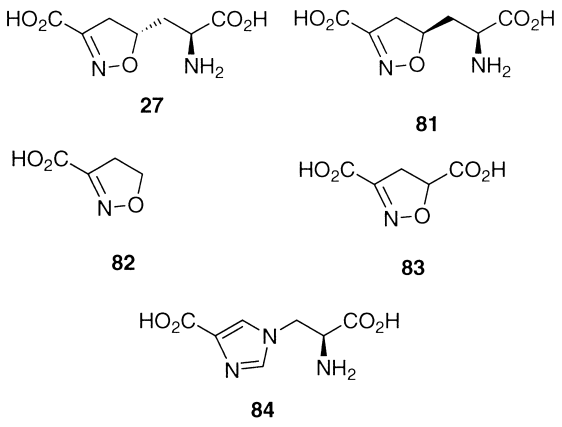
Substitution of carboxylate by phosphonate would allow the design of possible inhibitors of DAP-epimerase with a different α-proton pK_a, as is observed in the potent slow-binding inhibition of alanine racemase (EC 5.1.1.1) by phosphono analogues of alanine.[125–127] Therefore, the four stereochemically pure phosphono-DAP isomers **63a–d** were tested against DAP epimerase.[117] However, they were found to be relatively poor competitive inhibitors (K_i 3.9–7.2 mM).



63a B = X = NH_2, A = Y = H
63b B = Y = NH_2, A = X = H
63c A = X = NH_2, B = Y = H
63d A = Y = NH_2, B = X = H

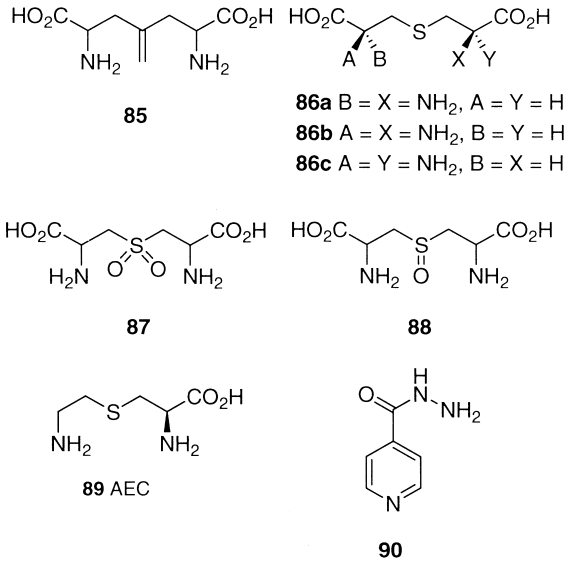
Heterocyclic compounds **27**, **81**, **82**, **83** and **84** in which a planar configuration about the α-carbon is rigidly held have been synthesised as possible inhibitors of the epimerase.[128] Compounds of a similar nature have been shown to inhibit 'two-base' epimerases such as proline racemase.[129] However, due to either the steric bulk or the rigidity of the ring none of these compounds showed significant inhibition.

Other compounds such as methylene DAP (**85**)[130] (K_i 0.95 mM)[124] and the sulfur containing DAP analogue *meso*-lanthionine (**86b**) (K_i 0.18 mM) have both been



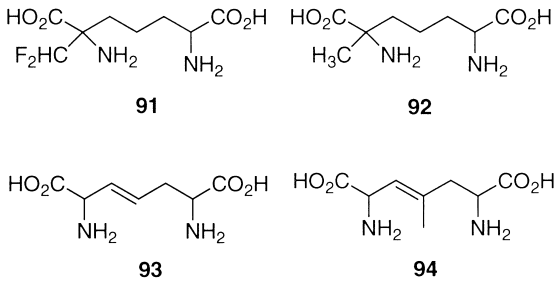
tested against the epimerase, although showing only modest inhibition in both cases. While the *meso*-lanthionine **86b** was found to be a mixed competitive inhibitor, its LL isomer **86a** inhibits competitively (K_i 0.42 mM). The DD analogue **86c** shows no inhibition. Oxidation of these compounds to a sulfone **87** or sulfoxide **88** produced siginificantly poorer inhibitors (K_i 11 and 21 mM respectively for *meso* isomers).[124]

Phosphono-DAP analogues **63a–d** are weak inhibitors of DAP-dehydrogenase.[117] Inhibition may occur as the amine adjacent to the phosphono group is probably unable to undergo oxidation, or to cyclise onto the α-imino carbon at the reacting end. The two *meso*-isomers **63b** (K_i 7.4 mM) and **63c** (K_i 4.3 mM) bind more tightly than either LL **63a** (K_i 12 mM) or DD **63d** (K_i 26 mM) analogues. DAP isomers themselves have been shown to be competitive inhibitors of the forward reaction of DAP dehydrogenase, with K_i values of 3.1, 4.0 and 4.2 mM for LL- **8**, DD- **24** and *meso*-DAP **9** respectively.[83]



Due to the tight substrate specificity of DAP-decarboxylase very few inhibitors are known. Neither LL- **8** nor DD-DAP (**24**) inhibit the reaction,[28] but L-lysine (**10**) and its analogues, such as AEC **89**, are generally weak competitive inhibitors of DAP decarboxylases from many plant sources, with IC_{50} values >20 mM.[131,132] The compound *meso*-lanthionine **86b** is a modest inhibitor of the enzymes from *B. sphaericus* (IC_{50} 10 mM) and wheat germ (IC_{50} 14 mM). The lanthionine sulfoxides **88** are slightly better inhibitors with IC_{50} values of ~1 mM. Sulfone analogues **87** showed poorer inhibition (IC_{50} ~10 mM).[133] As expected the enzyme is inhibited by 'carbonyl' reagents such as hydroxylamine and isonicotinic acid hydrazide **90** (isoniazid),[28,134] but detailed investigations have not been carried out. DAP analogues of these compounds such as *N*-amino-DAP (**79**) and *N*-hydroxy-DAP (**78**), were found merely to be effective competitive inhibitors of the enzymes from *B. sphaericus* (K_i 100 μM and K_i 84 μM respectively) and wheat germ (K_i 910 μM and K_i 710 μM respectively).

Amino acids which contain an α-difluoromethyl group are known inhibitors of PLP dependent enzymes. These compounds can undergo a series of enzyme catalysed elimination reactions in the active site, resulting in irreversible enzyme inactivation.[135] However, the α-difluoromethyl DAP analogue (**91**) was a weak competitive inhibitor (IC_{50} ~10 mM) of the DAP-decarboxylases from wheat germ and *B. sphaericus*. Unsurprisingly α-methyl-DAP isomers **92** were also poor inhibitors.[130] These results again show the tight substrate specificity of *meso*-DAP decarboxylase, as many other PLP dependent decarboxylases can accept α-methyl or α-difluoromethyl substrate analogues into their active sites.[136] Unsaturated substrate analogues such as the mixture of stereoisomers **93** (K_i 180 μM), have been shown to be moderate inhibitors of DAP decarboxylase from *E. coli*. However, structural modification to the γ-methyl analogue **94** as well as isomers of γ-methylene-DAP (**85**) are uniformly poor inhibitors.[130]



Reversible noncompetitive and uncompetitive inhibitors

The inhibition of *E. coli* L-DHDP synthase by dipicolinate (**12**) (IC_{50} 1.2 mM)[38] has led to the investigation of a number of heterocycles as potential inhibitors.[137] Various pyridines and piperidines **95–98** (Fig. 8) were found to be moderate inhibitors (IC_{50} values <1 mM). Kinetic analysis of compounds **12**, **99**, **16b**, **100**, **101** and **102** showed that esters inhibit more strongly than the corresponding acids and a planar geometry of substituents is preferred.

In *Bacillus spp*. dipicolinate (**12**) is produced by oxidation of L-DHDP (**4**).[138] This compound is a good inhibitor of L-DHDP reductase (K_i 85 μM for *B. cereus*), but inhibits non-competitively versus L-DHDP (**4**), suggesting a regulatory role. Another class of L-DHDP reductase has been isolated from sporulating *B. sub-*

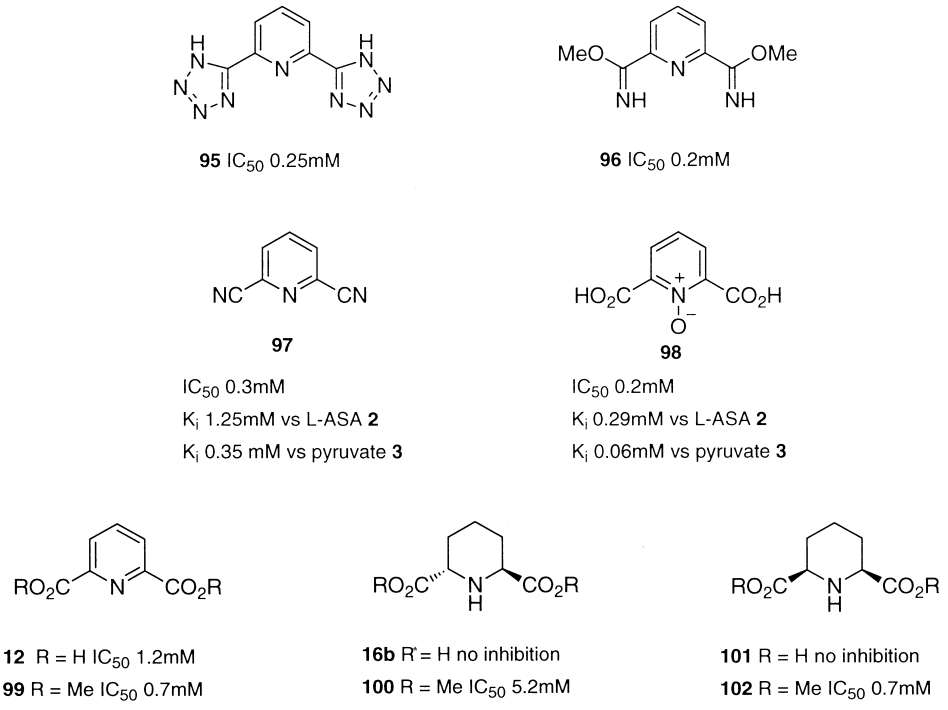
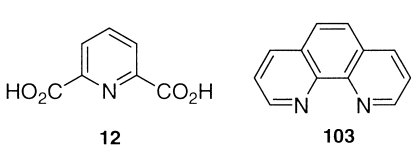


Figure 8. Heterocyclic inhibitors of L-DHDP synthase.

tilis,[139] and differs markedly from the other L-DHDP reductases in containing flavin mononucleotide (FMN). Dipicolinate (**12**) is again inhibitory (IC_{50} ~0.4 mM) and inhibits non/uncompetitively with respect to L-DHDP (**4**), but competitively against NAD(P)H.[140] Inhibition of this enzyme with *o*-phenanthroline **103** (IC_{50} ~70 μM) and the reduction of various synthetic dyes, suggests it may not be a dedicated L-DHDP reductase.

At pH 7.8, the isoxazoline **27** is a potent inhibitor of both the forward (K_i 4.2 μM versus L-THDP (**5**)) and reverse reactions (K_i 23 μM with respect to *meso*-DAP (**9**)) of *meso*-DAP dehydrogenase from *B. sphaericus*.[128] Kinetic analysis as well as information from enzyme crystals containing the isoxazoline have shown the inhibitor competes only for the L-THDP (**5**) binding site and does not occupy the *meso*-DAP (**9**) binding site.[86] These results imply separate binding sites for the two substrates. The designated binding site of L-THDP (**5**) may contain ionisable residues since both inhibition by **27** and substrate activity of L-THDP (**5**) fall off at high pH.



Surprisingly, similar compounds to **27** with alternative side chains (**81**–**84**) were very poor inhibitors of DAP dehydrogenase. In particular the isoxazoline **81**, differing only in ring junction stereochemistry, showed a poor 13% inhibition at 1 mM.

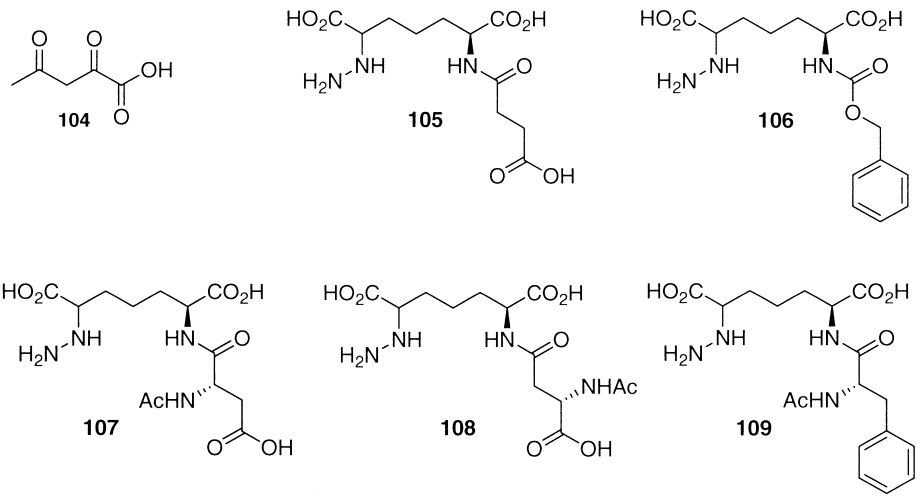
Reversible slow binding inhibitors

Below pH 8 acetopyruvate (**104**) is an effective slow-binding inhibitor (K_i 5 μM) of *E. coli* L-DHDP synthase.[141] At higher pH values, inhibition falls off, indicating that the protonated form of **104** is the active species.

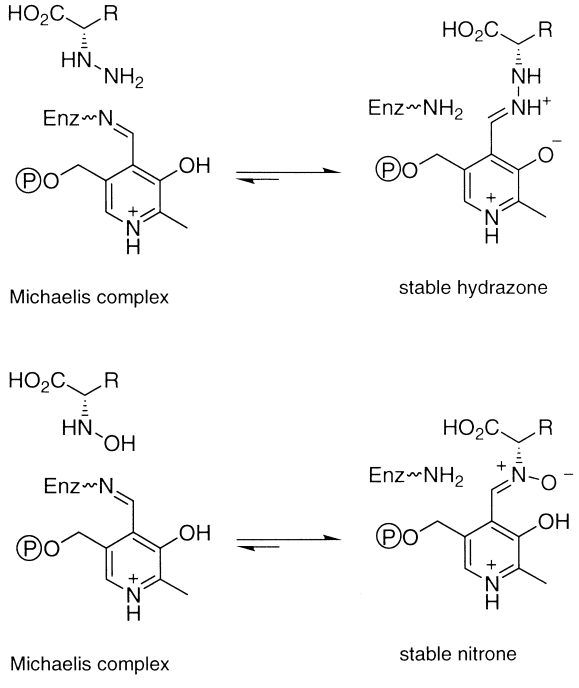
Like other PLP-dependent enzymes, DAP aminotransferase, is inhibited by hydroxylamine, and hydrazine. These compounds form a stable nitrone[142] or hydrazone[143] with pyridoxal phosphate at the active site (Scheme 24). Using this approach substituted hydrazino DAP analogues have been synthesised with the motifs required for good recognition, specifically a DAP skeleton with a succinyl **105** or Cbz **106** and more recently peptidic **107**, **108** and **109** substituted L-amines. Compounds **105** (K_i 22 nM) and **106** (K_i 54 nM) are especially potent, tight, slow-binding inhibitors of the enzyme.[56,61] Investigation of the PLP enzyme, aspartate aminotransferase, has shown that reaction takes place with a conformational closure of the active site promoting fast reaction and product release.[144] Kinetic analysis of these substituted hydrazines with *N*-succinyl-LL-DAP aminotransferase shows a similar but prolonged closure of the active site may be occurring.

Allosteric regulation

In plants and some bacterial organisms lysine **10**, is regulatory. For L-DHDP synthase from *E. coli* and wheat enzymes, lysine **10** is a non-competitive inhibitor with respect to pyruvate **3**, but inhibits competitively with respect to L-ASA (**2**). Most bacterial enzymes are not inhibited by lysine except for *Bacillus sphaericus*[145]



where lysine **10** is a weak inhibitor (K_i 0.6 M).[145,146] However, the plant enzymes are characterised by potent allosteric inhibition by lysine. For example wheat germ (IC_{50} 11 μM),[147] tobacco (*Nicotiana sylvestris*) (IC_{50} 15 μM),[148] spinach (*Spinacea oleracea*) (IC_{50} 20 μM),[149] maize (IC_{50} 23 μM),[39] and wheat (IC_{50} 51 μM)[38] are all significantly inhibited by lysine **10**. Analogues of lysine are somewhat less effective. Threo-β-hydroxy-L-lysine (THL) (**110**) and (2-aminoethyl)-L-cysteine (AEC) (**89**) are both modest inhibitors of wheat (IC_{50} 141 μM and 288 μM respectively).[38] AEC **9** also inhibits the tobacco synthase (IC_{50} 120 μM),[150] and the spinach homologue (IC_{50} 400 μM),[149] whereas the pea enzyme is inhibited by L-α-(2-aminoethoxyvinyl)-glycine (AVG) (**111**) (IC_{50} 155 μM). Interestingly, phosphono-DAP analogue mixture **63ab** and β-hydroxy-DAP isomer **56b** have been shown to be activators of the pea enzyme.[151] Heterologous expression of L-DHDP synthases from bacterial sources in canola, soybean[152] and tobacco chloroplasts[35] leads to increased levels of L-lysine synthesis in these plants. This evidence shows how lysine production is feedback controlled in the native plant.



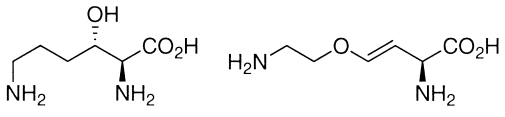
Scheme 24. Inhibition of PLP dependent enzymes by hydrazino and oxyamino acids.

Irreversible inhibitors

Apart from general alkylating agents such as iodoacetate, bromopyruvate and thiol specific agents such as *p*-nitrophenyldisulfide, irreversible inactivators of DAP processing enzymes have been difficult to develop. One notable exception is the irreversible inhibitor azi-DAP (**25**) ($K_i \geqslant 25$ μM) which specifically covalently labels cys-73 of *meso*-DAP epimerase (Scheme 6).[77] Other analogues of this compound such as **50** have been synthesised by Williams (Scheme 15) and tested against the DAP enzymes by researchers at Roche.[107] Although acting as substrates for the DAP-adding enzyme, these compounds did not show any activity against the epimerase.

Antibiotic Properties of DAP Pathway Inhibitors

Few naturally occurring inhibitors of the DAP pathway are known, suggesting the importance of products of this pathway to bacterial growth and development. However, an alanyl dipeptide **112** of α-hydroxymethyl-DAP isolated from *Micromonospora chalcea*, shows antibiotic activity against *E. coli*.[110]



Bioavailability of inhibitors of the DAP pathway is a common problem. In *E. coli*[153] and *Salmonella typhimurium*,[154] DAP is transported across the cell membrane via the cystine uptake mechanism.[155] Efficient cellular transport systems are also used to transport

DAP analogues as di- or tri-peptides. This approach is used by other peptidoglycan biosynthesis inhibitors. The peptidic antibiotic alaphosphin is cleaved in vivo to release the alanine racemase (EC 5.1.1.1) inhibitor phosphonoalanine.[156]

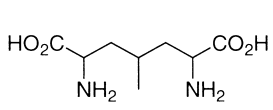
Although the succinyltransferase inhibitor, L-α-AP (**19a**) blocks DAP biosynthesis in cell-free protein extracts of *E. coli*, L-α-AP (**19a**) itself showed no antibacterial activity.[157] However, when L-α-AP (**19a**) was included in alanyl dipeptides, good antibacterial activity was observed. The alanyl dipeptide **113** was the most potent against a range of Gram-negative bacteria. These dipeptides have been shown to inhibit DAP biosynthesis in 'resting' *E. coli* cells at 2.4 mM while causing lysis of growing *Enterobacter cloacae* at 0.2 mM. Addition of LL-DAP (**8**) reversed these results showing that DAP biosynthesis was the likely target of action.

These results suggest that, if properly delivered even modest enzyme inhibitors can be effective antibiotics. This strategy has led to the synthesis of a depsipeptide analogue **114** of enantiomerically pure L-HTHP (**20**) as well as other potential transition state analogues of the succinyltransferase **115** and **116**.[158] This approach has also been adopted for other DAP inhibitors, such as phosphono-DAP analogues. However, these show little or no antibacterial activity, except for the *meso*-compound **63b** which inhibits the growth of *Salmonella tryphimurium* at 1 μg/mL. The tripeptide **117** is a more effective growth inhibitor, and is active against a wider range of bacteria such as *E. coli* and *Citrobacter freundii*.

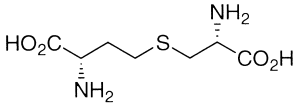
The *N*-amino-DAP analogues **105–109** which are very potent inhibitors of the aminotransferase appear to show limited antimicrobial activity on complex media against *E. coli*. However, their activity is dramatically improved when minimal agar is used which contains no lysine or DAP indicative of their intended action blocking the DAP pathway.[61]

Other compounds tested have been *N*-amino-DAP (**79**) and *N*-hydroxy-DAP (**78**), inhibitors of *meso*-DAP epimerase. These inhibit the growth of *Bacillus megaterium* at 20 μg/mL, while γ-methylene-DAP (**85**) inhibits the growth of *E. coli*. The potent dehydrogenase inhibitor, heterocycle **27** inhibits the growth of the dehydrogenase dependent *Bacillus sphaericus*. β-chloro-DAP (**76**), and the stereoisomers of β-fluoro-DAP **60**, are inactive or weak growth inhibitors of *E. coli*.[113]

In the absence of DAP, compounds such as β-hydroxy-DAP (**56**), can be incorporated into the peptidoglycan of *E. coli*. It has been proposed that the DAP-condensing enzyme which is involved in the synthesis of muramyl peptides is responsible. Lanthionine (**86**), γ-methyl-DAP (**118**) and cystathionine (**119**) may also be incorporated.[123,159] For *dapF* mutants lacking *meso*-DAP epimerase, LL-DAP (**8**) is incorporated into the peptidoglycan of *E. coli*.[160] In cases where *dapA* (coding for L-DHDP synthase) has been deleted, the DAP-condensing enzyme can overcome the absence of DAP by using alternative substrates. However, L-lysine must still be supplied to allow protein synthesis.[161]



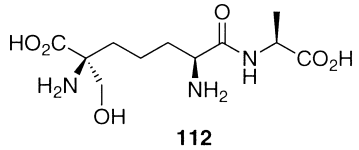
118



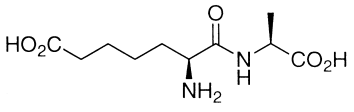
119

Conclusions

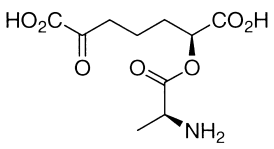
The appearance of pathogenic bacteria which are resistant to conventional antibiotics has led to increased urgency to find broad spectrum, antibacterial compounds. A possible means to combat this is the disruption of bacterial cell wall synthesis by inhibition of the biosynthesis of DAP, a key crosslinking constituent of the peptidoglycan layer. The recent cloning and overexpression of many of the enzymes in the DAP biosynthetic pathway has allowed much understanding of the molecular machinery responsible for generation of this critically important bacterial metabolite. Organic synthesis has also generated a library of DAP inhibitors which has helped elucidate the mechanisms of many of



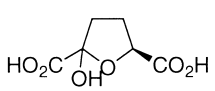
112



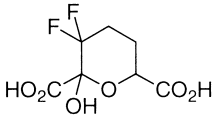
113



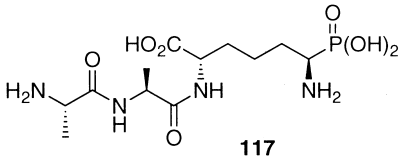
114



115



116



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the enzymes involved in lysine biosynthesis. While no potent broad spectrum antimicrobial compounds have yet emerged, further studies towards this goal are underway.

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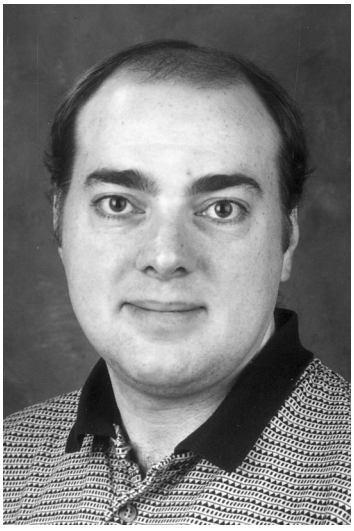
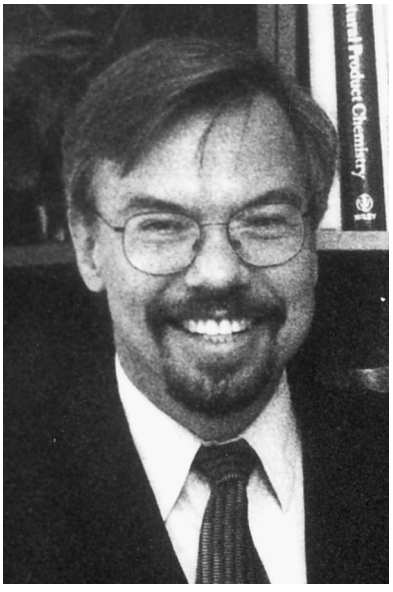
Biographies

Russell John Cox. Russell Cox was born in the New Forest in England in 1967. He studied for his first and second degrees in chemistry at the University of Durham, graduating in 1992. A two year period of post doctoral research with Professor John Vederas at the University of Alberta was followed by a year working with Professor Sir David Hopwood, FRS, at the John Innes Centre in Norwich, UK. He was appointed to a lectureship in organic chemistry in the School of Chemistry at the University of Bristol in 1996 where his interests include the enzymology of polyketide synthase and amino acid processing enzymes.



Andrew Sutherland. Andrew Sutherland was born in 1972 in Wick, in the far North of Scotland. After completing his Bachelor of Science degree with first class honours, at the University of Edinburgh in 1994, he moved to the University of Bristol where he undertook a Ph.D. under the guidance of Dr. Christine Willis on the chemoenzymatic synthesis of enantiomerically pure α-hydroxy and α-amino acids. After completion of his Ph.D in 1997, he joined the research group of Professor John Vederas at the University of Alberta where he is currently involved in studies of the biosynthesis of diaminopimelate.



John Christopher Vederas. John Vederas was born in 1947 in Detmold, Germany, to Lithuanian refugee parents. He emigrated as a preschooler to the United States, became a US citizen and completed high school in Cleveland, Ohio. After finishing a B.Sc. in Chemistry at Stanford University in 1969, he obtained his Ph.D. degree in 1973 doing organic synthesis with the late Professor George Büchi at the Massachusetts Institute of Technology. Postdoctoral work on biosynthesis of fungal metabolites with Professor Christoph Tamm in Basel, Switzerland, and on enzyme mechanisms with Professor Heinz Floss at Purdue University preceeded his appointment as an Assistant Professor at the University of Alberta in 1977. Currently he is professor and a Fellow of the Royal Society of Canada. His research interests in bioorganic chemistry are described in ca. 150 publications and include antimicrobial peptides (bacteriocins), enzyme mechanism and inhibition, biosynthesis of secondary metabolites, peptidomimetrics and new synthetic methods.