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PEPTIDE INHIBITORS OF N-SUCCINYL DIAMINOPIMELIC ACID AMINOTRANSFERASE (DAP-AT) : A NOVEL CLASS OF ANTIMICROBIAL COMPOUNDS.

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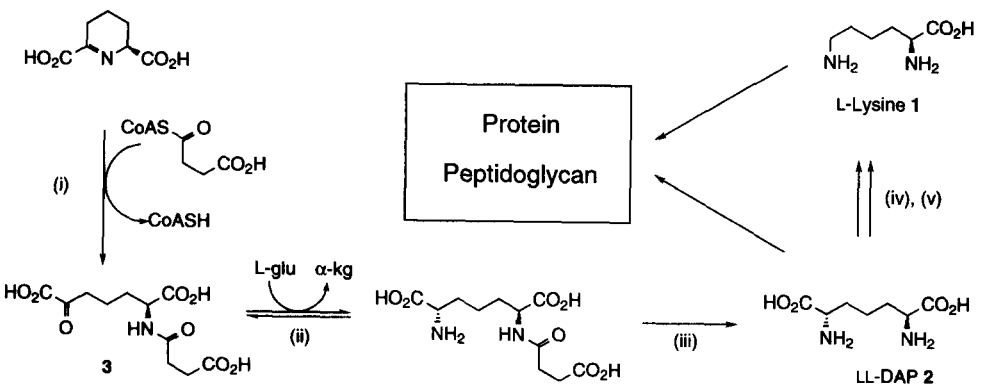
Abstract. Dipeptide substrates of *N*-Succinyl Diaminopimelic Acid Aminotransferase (DAP-AT) were converted to hydrazines by treatment with hydrazine and cyanoborohydride. These compounds were tested *in vitro* as inhibitors of DAP-AT from *E. coli* and *in vivo* as antibiotics. The hydrazinodipeptides showed potent slow binding inhibition of DAP-AT as well as antimicrobial activity.

The biosynthesis of L-lysine **1** by bacteria has been extensively studied because lysine, being required for protein synthesis, is essential to the growth and development of these organisms. Additionally lysine, or its precursor diaminopimelic acid (DAP, **2**) is utilised as an essential cross-linker in the peptidoglycan layer of the bacterial cell wall. Genetic experiments show that organisms in which lysine pathway genes have been deleted are not viable in the absence of exogenous DAP or lysine.[1] As humans do not biosynthesize lysine, inhibitors of lysine biosynthesis could be good antibiotics with low human side-effects.[2] Many of the enzymes from the bacterial lysine pathway (Scheme 1) have been investigated with the aim of developing potent and specific inhibitors, but in the main the results have been unimpressive, with inhibition constants (IC_{50}, K_i etc.) in the mM to μM range.[3]



Scheme 1. Part of the DAP pathway to L-lysine. Enzymes and corresponding genetic loci: (i) AcylCoA:Tetrahydrodipicolinate *N*-acyltransferase *dapD* (ii) *N*-Acyl-LL-DAP Aminotransferase (DAP-AT) EC 2.6.1.17 *dapC* (iii) *N*-Acyl-LL-DAP Deacylase EC 3.5.1.18 *dapE* (iv) *meso*-DAP Epimerase EC 5.1.1.7 *dapF* (v) *meso*-DAP Decarboxylase EC 4.1.1.20 *lysA*. Abbreviations: L-glu, L-glutamic acid; α-kg, α-ketoglutaric acid.

N-Acyl-LL-diaminopimelate aminotransferase (DAP-AT, EC 2.6.1.17, Scheme 1) is a pyridoxal phosphate (PLP) dependent enzyme from the bacterial lysine pathway. In recent work this enzyme has been

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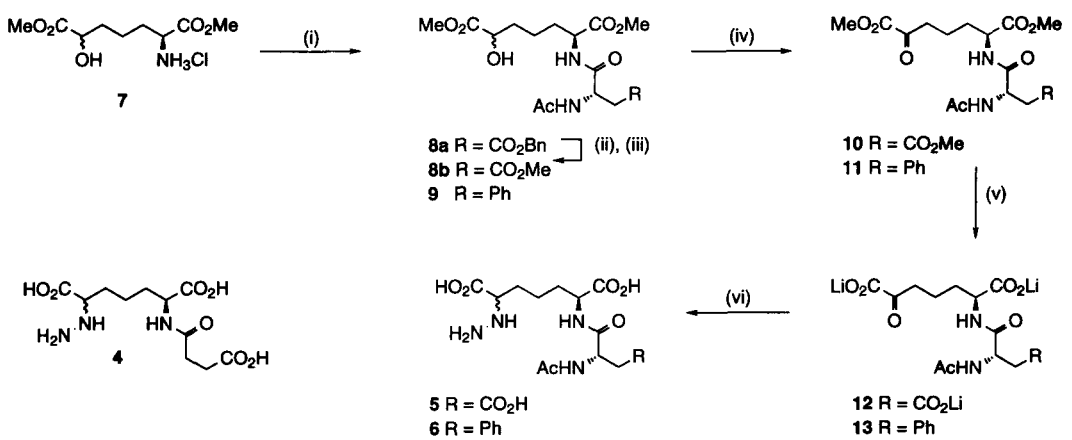
isolated and purified from *E. coli*.[3] We have developed a flexible synthesis of the natural succinyl substrate **3** and a number of substrate analogues. Measurement of k_{cat}/K_m values for these substrates allowed an assessment of substrate specificity for the *E. coli* enzyme.[3] As part of these studies hydrazino product analogues were examined as potential inhibitors of DAP-AT from *E. coli*. The results showed that compounds such as **4** (Scheme 2) were potent slow tight-binding inhibitors of DAP-AT with K_i^* values in the nM range. They also indicated a relaxed specificity of *E. coli* DAP-AT for substrate acyl side-chains; a range of acyl groups were tolerated, those with aromatic or acidic groups being preferred. We reasoned that di-peptide substrates could also be accepted by the enzyme. Peptidic inhibitors of lysine pathway enzymes have previously been shown to posses improved antimicrobial properties over their non-peptidic congeners, possibly due to improved membrane transport properties.[4] Indeed improvement of membrane transport properties of other cell wall biosynthesis inhibitors, such as L-1-aminoethylphosphonic acid, results in significant improvement in antimicrobial activity.[5] Initial targets for potential antimicrobial compounds were therefore the aspartyl **5** and phenylalanyl **6** product analogues in which the L-α-aminopimelate skeleton required for enzyme binding and recognition was coupled with modified peptidic side-chains, and in which an ε-keto or ε-hydrazino group were incorporated. In this *letter* we report the synthesis of dipeptide hydrazines, their properties as inhibitors of *E. coli* DAP-AT and their antimicrobial activities.

Synthesis

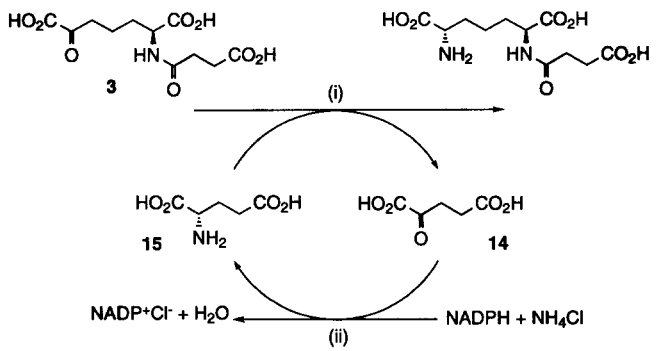
The L-amino-alcohol hydrochloride **7** was synthesized as previously reported, using metal mediated 'ene' methodology with protected L-allyl glycine.[3,6] The amino-alcohol was coupled with *N*-acetyl protected L-phenylalanine and *N*-acetyl-γ-benzyl protected L-aspartate using standard peptide coupling conditions. The resulting peptide alcohols **8b** and **9** were oxidised to the corresponding ketones **10** and **11** using the Dess-Martin periodinane after protecting group manipulation as necessary.[7,8] The methyl esters were hydrolysed with LiOH in water and acetonitrile, and final selective reductive hydrazination proceeded smoothly to afford the desired hydrazino-dipeptides **5** and **6** (Scheme 2). Hydrazino product analogue **4** was synthesized by similar methodology.[3,9]



Scheme 2. Synthesis of Hydrazino-Dipeptides. Reagents and Conditions: (i) L-*N*-acetyl amino acid, HOBt, EDCI, CH_2Cl_2, 66-75%; (ii) H_2, 10% Pd/C, MeOH; (iii) Et_2O/CH_2N_2 95% (two steps); (iv) Dess-Martin periodinane, CH_2Cl_2, 75-80%; (v) $LiOH.H_2O$, CH_3CN/H_2O, quant.; (vi) NH_2NH_2, CF_3CO_2H, $NaCNBH_3$, MeOH, then HCl (aq), cation exchange chromatography, 65%.

Enzyme Assays

DAP-AT was purified from *E. coli* DH5α, by a combination of precipitation and anion and cation exchange chromatography.[3,10] Synthetic substrate **3** was used for activity assays during purification. In reactions catalysed by DAP-AT, substrate consumption is accompanied by concomitant production of α-ketoglutarate **14** from L-glutamate **15**.[11] The reaction is conveniently observed spectrophotometrically at 340nm by allowing α-ketoglutarate **14** to be reductively aminated by L-glutamate dehydrogenase with one equivalent of NADPH and NH_4^+ ions (Scheme 3).



Scheme 3. Assay for DAP-AT activity (i) DAP-AT; (ii) L-Glutamate dehydrogenase.

Initial results showed the two peptide ketones **12** and **13** to be substrates of the enzyme (Table 1). The *N*-acetyl aspartyl substrate analogue **12** differs from the natural substrate **3** only in the presence of an α-*N*HAc moiety. As expected this compound is turned over effectively by the enzyme. The *N*-acetyl phenylalanyl dipeptide **13** is a significantly worse substrate however. Turnover could be detected in our standard activity assays, although at a level too low to accurately determine kinetic parameters. These results clearly indicate that the dipeptide ketones are recognised and turned-over by DAP-AT. The phenylalanyl peptide substrate **13** also behaves as a weak competitive inhibitor of DAP-AT, but with a mM range IC_{50} value.

Table 1. Kinetic Parameters for DAP-AT substrates and inhibitors.[a]

Compound	K_m^{app} mM	k_{cat}[b] s^{-1}	k_{cat}/K_m^{app} $mM^{-1}s^{-1}$	%	IC_{50} μM	$t_{1/2}$ regen. s	K_i* nM
3	0.60	164	273	100	-	-	-
12	1.57	40	25.4	9.3	-[c]	-	-
13	<1	<2.1	<2.1	<0.8	>50 000	-	-
4	-	-	-	-	0.5	4716	22
5	-	-	-	-	0.8	2937	169
6	-	-	-	-	20	364	556

[a] ± 10%; [b]Concentration of stock DAP-AT solution calculated as 50μM. [c] Overall rate of substrate **3** consumption increases at 20mM **12**.

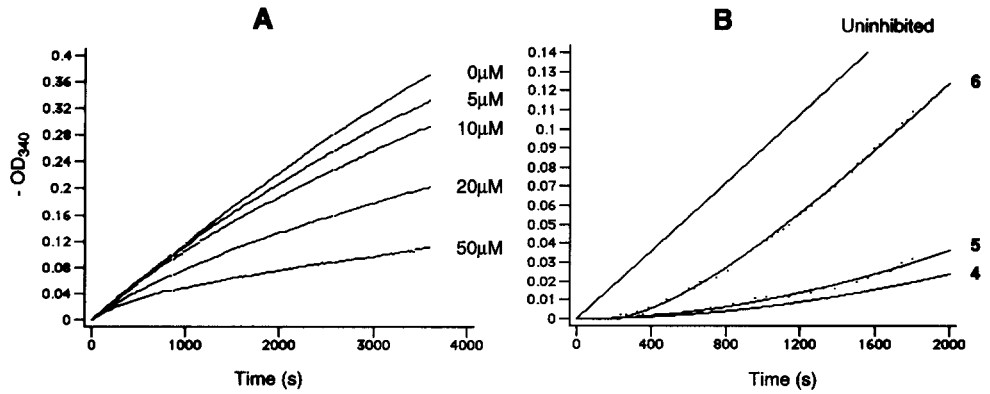


Fig 1. Kinetic plots of inhibition by hydrazino-peptides. **A** Progress of DAP-AT Inhibition by **6** at the indicated concentrations. **B** Regeneration of DAP-AT activity upon 100-fold dilution after complete inhibition by **4**, **5** and **6**.

Table 2. Antimicrobial properties of DAP-AT inhibitors vs. *E. coli* DH5α.

	Radius of Inhibition Zone, mm[a]							
	μg Inhibitor per disk L - Agar				μg Inhibitor per disk M9 - Minimal Agar			
Inhibitor	300	30	3	0.3	300	30	3	0.3
*N*Ac-Asp-AP-$NHNH_2$ **5**	1	0	0	0	10	5	0	0
*N*Ac-Phe-AP-$NHNH_2$ **6**	1	0	0	0	6	0	0	0
Succ-AP-$NHNH_2$ **4**	1	0	0	0	13	8	0	0
*N*Ac-Asp-AP-O **12**	0	0	0	0	1	0	0	0
*N*Ac-Phe-AP-O **13**	0	0	0	0	1	0	0	0
Tetracycline	13	10	7	2.5	17	10	8	2
Chloramphenicol	13	10	3.5	0	17	12	6	3
Carbenicillin	13	8	4.5	0	12	7	4	2
H_2O	0	0	0	0	0	0	0	0

[a] Radius of inhibition zone minus radius of filter disc (2.5mm). The results are averages of four separate experiments.

The synthetic dipeptide hydrazines **5** and **6** were also tested with *E. coli* DAP-AT. When added to aminotransferase reactions containing the natural substrate **3**, the hydrazines initially caused little inhibition. However, over time, inhibition of turnover became more pronounced, eventually reaching equilibrium (Figure 1A). This behaviour is typical of slow-binding reversible inhibition and is consistent with our previous results where we showed that **4** was a potent slow binding inhibitor of DAP-AT. In a separate experiment DAP-AT which had been treated with inhibitors **4**, **5** and **6** under pre-incubation conditions which would cause full inhibition, were diluted 100-fold into a standard activity assay. Enzyme activity was seen to slowly regenerate under these conditions, eventually giving 100% enzyme activity (Fig 1B).

These experiments proved the reversible nature of the slow binding inhibition. Full kinetic analysis of the inhibition progress curves, according to the methods of Morrison and Walsh,[12] allowed the elucidation of inhibition constants for **4**, **5** and **6** (Table 1). The inhibition constants mirror the trend seen in K_m^{app} and k_{cat} values for the corresponding ketone substrates - increasing side-chain bulk seems to weaken binding interactions.

Antimicrobial Assays

The dipeptide ketones and hydrazines were tested against *E. coli* on solid media.[13] Peptide ketones **12** and **13** show no inhibitory effects against *E. coli* on L-agar at the concentrations tested (Table 2). On M-9 minimal agar (lacking lysine and DAP), however, very weak growth inhibition is observed at the highest concentration. The results of tests with the dipeptide-hydrazines **5** and **6** were significantly better. These compounds show good antimicrobial activity against *E. coli* on minimal media. At higher loadings the effectiveness of inhibition is similar to that caused by commercial antibiotics. However at lower loadings the activity falls away. The succinyl hydrazine inhibitor **4** is around 8-fold more effective than the aspartyl hydrazine **5** and more than 20-fold more effective than the phenylalanyl hydrazine **6** in the *in vitro* inhibition assays in terms of K_i^*. The order of effectiveness is retained in the *in vivo* assays, although the marginal difference in potency between **4** and **5** may suggest that enhanced cell penetration of the peptide inhibitors results in a higher *in vivo* concentration, compensating for lower efficacy of inhibition. The antimicrobial properties of the hydrazino peptides and the peptide-ketones are severely attenuated on complex media, presumably because the availability of lysine and/or DAP in this media is able to compensate for the lack of *in vivo* synthesis.

Conclusions

DAP-AT has been shown to be a suitable target for the development of novel antimicrobial compounds. Hydrazino-peptide product analogues are potent slow-binding inhibitors of DAP-AT. *In vitro* activity against DAP-AT from *E. coli* is mirrored by *in vivo* activity against the whole cells. The demonstration of biological activity of these synthetic peptides is the first step in a longer term strategy to develop peptide libraries and peptide-mimetics. Our current work in this area is focussed on the development of new classes of DAP-AT inhibitors and the elucidation of the inhibition mechanism of the dipeptide hydrazines, as well as the assessment of antimicrobial potency against a range of Gram positive and Gram negative organisms.

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9. Previous results[3] have shown that there is little, if any, stereoselectivity during reductive hydrazination in these systems; the hydrazine bearing ε-carbon should be assumed to be racemic. No evidence for epimerisation was observed at the other two stereocentres by ^{1}H-nmr spectroscopy or HPLC analysis. All synthetic compounds gave satisfactory analytical data.

10. Stock DAP-AT solution was adjusted to a final DAP-AT concentration of 50μM (2mg/ml); 25μL of this solution was used in standard 1ml rate assays giving Δ-OD_{340} of 62 $mAUmin^{-1}$ with 1mM substrate **3** and 25μM NADPH.

11. Stock Solution (100 mM Tris buffer pH 8.0 containing EDTA tetra-sodium salt (0.1 mM), NaN_3 (5 mM), Bovine Serum Albumin (1.0 mg/mL) and NH_4Cl (100 mM)) was used to prepare the Assay Solution (Pyridoxal Phosphate (PLP) 3.0 mg and β-NADPH 6.0 mg made up to 40.0 mL with Stock Solution). The solutions were made using ACS grade reagents and Milli-Q water. Assays were performed at 37°C and contained sufficient DAP-AT to give Δ-OD_{340} of 20-100 mAU_{340}/min, 0-20 mM substrate, 10 mM L-glutamate, 10 units of L-glutamate dehydrogenase (EC 1.4.1.4, Sigma) and Assay Solution to give a final volume of 1000 μL. Inhibition assays also contained inhibitor at concentrations of 1.0-50 μM. The decrease in β-NADPH concentration was observed at 340 nm over 300 seconds for activity assays and over 3600 seconds for inhibition testing. A Pharmacia-LKB Ultrospec III Spectrophotometer equipped with a cuvette having a heated-water jacket was used for all assays.

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13. L and M9 minimal media agar were prepared according to literature procedures,[14] sterilised by autoclaving for 25 min at 120°C and poured into sterile 9 cm petri plates. *E. coli* DH5α was grown in liquid L media overnight at 37°C. The cells were precipitated and resuspended in M9 minimal liquid media. The washing process was repeated and 50 μl of the resulting cell suspension was evenly spread onto the surface of each sterile agar plate. Sterile filter disks (5mm diameter, whatman number 1 paper) were soaked with 3μl of a solution containing the appropriate amount of antibiotic dissolved in sterile deionised water. The filter disks were placed on the surface of the agar and the plates were incubated at 37°C for 16h (L-plates) or 36h (M9 minimal plates). The radius of the inhibition zone was measured and the radius of the filter disk (2.5mm) subtracted.

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