1 PERKIN

Synthesis and *in vitro* enzyme activity of peptide derivatives of bacterial cell wall biosynthesis inhibitors

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Received (in Cambridge, UK) 5th April 2000, Accepted 3rd May 2000
Published on the Web 9th June 2000

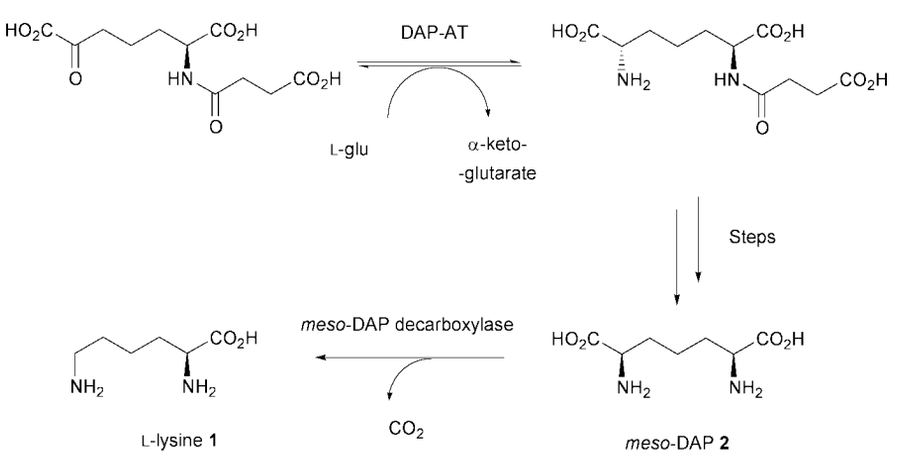
The enzyme diaminopimelate aminotransferase (DAP-AT) is a good potential target for the design of novel antibacterial agents. We have synthesised a series of peptide hydrazines based on the structure of the natural substrate of DAP-AT. These compounds show varied inhibition properties *in vitro vs.* DAP-AT from *E. coli* as well as moderate antimicrobial activity *vs. E. coli*. Examination of the kinetics of inhibition reveals that hydrazine, as well as the substituted hydrazino-peptides, shows two-phase slow-binding inhibition. Possible mechanisms for inhibition are discussed.

Introduction

Interest in novel antimicrobial compounds has increased recently as the problem of antibiotic-resistant pathogens has become more prevalent.[1] Resistance to almost all commercially available antibacterial drugs has been observed in both 'wild type' and laboratory strains of disease-causing bacteria. Worryingly, resistance is building up in bacteria which can cause major human epidemics, such as *Mycobacterium tuberculosis*, the causative agent of TB.[2] Resistance has emerged for a number of reasons. Many antimicrobial drugs are, or are closely related to, natural products. Many of these compounds are produced through fermentation of strains of bacteria and fungi. In order that these antibiotic producing organisms do not kill themselves they utilise a variety of mechanisms to ameliorate the action of the antibiotics. These resistance mechanisms are genetically encoded and under appropriate conditions resistance genes can propagate through the environment. The spread of resistance mechanisms often negates treatment by entire classes of antimicrobial compounds. Under these circumstances the development of novel classes of antimicrobial compounds is required.

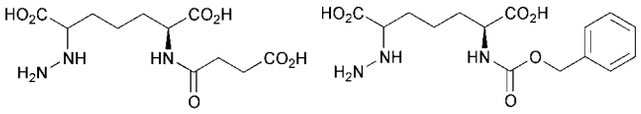
We have been studying specific enzymes involved in bacterial cell wall biosynthesis as potential targets for new classes of antimicrobial compounds. In particular the biosynthesis of L-lysine **1** in bacteria (Scheme 1) has interested us because of the central role of L-lysine and its precursors, *meso*- and LL-diaminopimelic acid (DAP, **2**), as key cross-linking elements in the strength-bearing peptidoglycan layer of the prokaryote cell wall.[3–5] The biosynthesis of the peptidoglycan structure is the target for successful antimicrobial drug classes including the penicillins and other β-lactams and the vancomycins and other glycopeptides.[6] Of course, L-lysine itself is also crucial to bacterial growth and development because of its requirement for protein synthesis. The biosynthesis of L-lysine, however, does not appear to be a target for existing naturally occurring compounds and resistance mechanisms may be absent. An additional attractive feature of this pathway is that it is absent from mammals (where L-lysine is obtained solely through the diet) and specific enzyme inhibitors could avoid mammalian side effects.

We have developed a series of compounds designed to inhibit a key enzyme in the bacterial L-lysine biosynthetic pathway. The hydrazines **3** and **4** are very potent, slow-binding inhibitors of the enzyme LL-*N*-succinyldiaminopimelate aminotransferase (DAP-AT) from *E. coli* (Scheme 1).[7,8] The most potent of them, **3**, possesses a K_i^* of 22 nM and is an extremely effective *in vitro* inhibitor of L-lysine biosynthesis. Other related compounds, where the *N*-succinyl group has been replaced by, for example, *N*-Cbz (*e.g.* **4**), are also potent inhibitors of DAP-AT. On complex growth media (which contain L-lysine and DAP isomers) **3** shows very little activity *vs. E. coli*, but on minimal growth



Scheme 1 Later steps during the biosynthesis of L-lysine by *E. coli*.

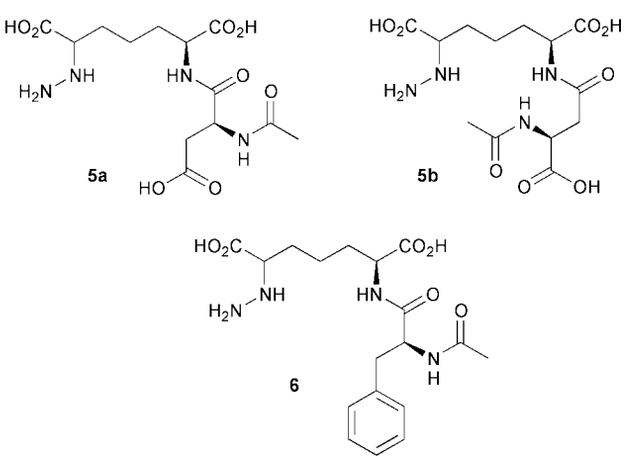
DOI: 10.1039/b002701o



3 4

media (containing only glucose and salts) the antibiotic activity is more evident. Despite their efficacy *vs.* lysine biosynthesis *in vitro*, these compounds do not show particularly effective antibiotic properties when compared with commercial antibiotics such as carbenicillin or tetracycline. This difference between *in vitro* and *in vivo* potency could be due to poor transport of the compounds through the bacterial cell wall.

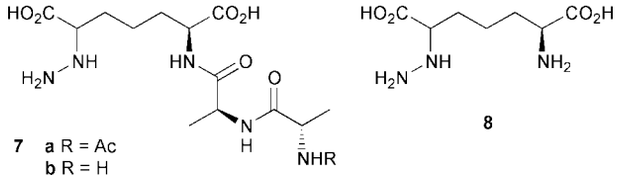
Many bacteria possess general peptide transport systems embedded into their cell walls.[9,10] These have sometimes been exploited in drug design as potential entry routes into the cell. For example, the alanine racemase (and thus peptidoglycan biosynthesis) inhibitors β-chloroalanine[11] and (1-aminoethyl)-phosphonic acid[12] are both much more effective antimicrobial agents when coupled to other amino acids to form peptides. These peptides are efficiently imported into bacterial cells and then cleaved by peptidases to reveal the active compounds. In order to attempt to overcome possible transport problems of the DAP-AT inhibitors we decided to exploit the apparently lax substrate specificity of DAP-AT for the *N*-acyl side-chain.[7] We therefore set out to make analogues of **3** and **4** bearing peptidic side-chains. Most similar to the natural *N*-succinyl group is the amino acid aspartic acid and we decided to synthesise both possible (*i.e.* α-linked **5a** and β-linked **5b**) isomers. As aromatic side-chains are also tolerated by DAP-AT we also undertook to examine the phenylalanyl dipeptide **6**.



5a 5b 6

The use of alanylalanyl dipeptides as transport agents has also been reported. For example, alanylalanyl dipeptides of sulfanilic acid are up to 207 times more potent than sulfanilic acid itself as antimicrobial agents. Alanylalanyl peptides of 6-aminopenicillanic acid have also been shown to be up to 100 times more potent than the free β-lactam *vs. Bacillus subtilis* and 10 times more potent *vs. E. coli*.[13] In the case of L-lysine biosynthesis, alanylalanine peptides have also proven successful. The weak L-THDP (L-tetrahydrodipicolinate) succinyl transferase inhibitor L-α-aminopimelate shows no antibacterial activity, but when it was included in alanyl and alanylalanyl dipeptides and depsipeptides, good antibacterial activity was observed with minimum inhibitory concentrations (MICs) of 1–16 μg ml^{-1} against a range of Gram-negative bacteria.[14] We therefore also set out to examine the alanylalanyl tripeptides **7**.

All of these compounds have the potential to be potent *in vivo* inhibitors of DAP-AT. However, in other systems, notably that of (1-aminoethyl)phosphonic acid, cleavage of the peptide occurs after penetration into the cell. For the hydrazino peptides **5**–**7** described here, this process would release *N*-amino-



7 **a** R = Ac
b R = H

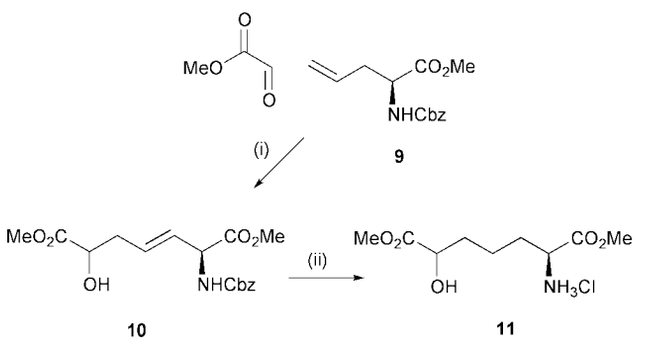
8

DAP **8**, a known inhibitor of the final L-lysine pathway enzyme *meso*-DAP decarboxylase.[15] Thus the peptide hydrazines described here have the potential to block lysine biosynthesis at two points in the pathway.

Results and discussion

Synthesis

The carbonyl ene reaction is a convenient method for the production of the C_7 DAP skeleton (Scheme 2).[16] The reac-



9 10 11

Scheme 2 *Reagents and conditions*: i, $SnCl_4$, CH_2Cl_2, −78 ⟶ 0 °C; ii, H_2, Pd/C, 10% $CHCl_3$–MeOH.

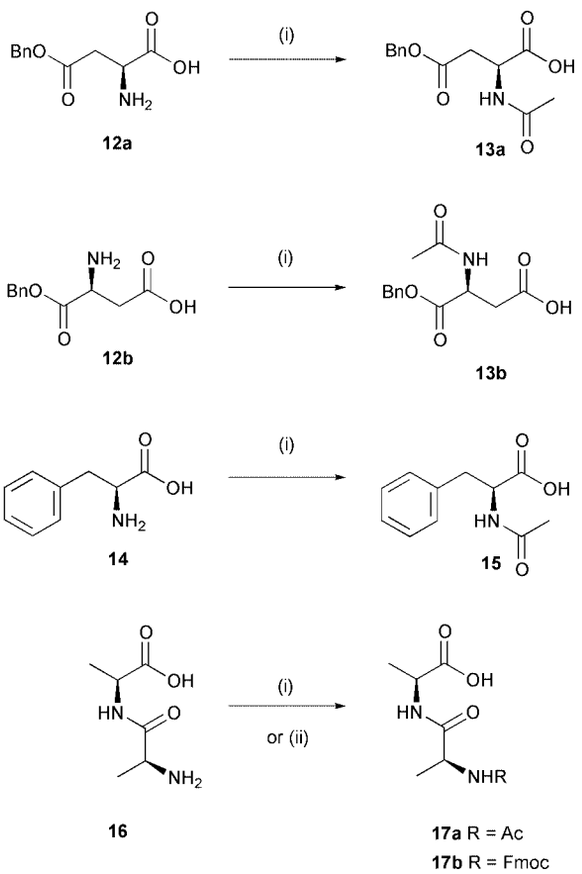
tion between enantiomerically pure protected L-allylglycine **9** and methyl glyoxylate, in the presence of $SnCl_4$, conveniently gives the protected aminopimelate skeleton **10**. When the N-protecting group is Cbz, hydrogenation then affords the fully saturated amino alcohol **11** bearing an L-configured α-amino ester. This compound is a key precursor to the DAP-AT substrates. We have already shown that the amino alcohol **11** can be selectively *N*-acylated by appropriate acyl chlorides.[7]

For the synthesis of the peptides **5**–**7** we required protected phenylalanine, aspartates and alanylalanine. Both regioisomers of L-aspartic acid benzyl ester **12a** and **12b** are commercially available, as is L-ala-L-ala **16**. *N*-Acetylation was easily achieved for all of the precursors, by treatment with acetic anhydride, although isolation and purification of *N*-acetylalanylalanine **17a** was complicated by its high water solubility. We also synthesised Fmoc-alanylalanine **17b** which was much less water soluble (Scheme 3).[17]

We initially concentrated on the phenylalanyl dipeptide series (Scheme 4). Selective *N*-coupling with the pimelate skeleton **11** was achieved using standard peptide-coupling methodology (DCC, HOBt) in good yield to give **18**. Oxidation of the ε-alcohol was achieved using the Dess–Martin periodinane,[18,19] in good yield, and the fully protected peptide ketone **19** was readily purified. Final deprotection of **19** was easily achieved using exactly two equivalents of $LiOH·H_2O$ in water–acetonitrile to afford the analytically pure dilithium salt **20** (Scheme 4).

The α- and β-linked aspartate skeletons, **21a** and **21b** respectively, were also assembled using peptide-coupling reagents (Scheme 5). Dess–Martin oxidation of **21a** smoothly gave the protected ketone **22a**. In an attempted deprotection, this α-linked aspartate was then subjected to hydrogenation, followed by LiOH hydrolysis using exactly 3.0 equivalents of $LiOH·H_2O$. This procedure did afford the trilithium salt as expected, but also caused evident ε-ketone reduction affording the alcohol **23a** (Scheme 5). In order to avoid using hydrogen-

ation conditions for final deprotection, the benzyl esters of the aspartyl peptide alcohols, **21a** and **21b**, were exchanged for methyl esters prior to Dess–Martin oxidation. Thus hydrogenation, followed by treatment with an excess of diazomethane, exchanged the esters in high yield giving trimethyl esters **24a** and **24b**. Following oxidation of the ε-alcohols to ε-ketones, **25a** and **25b**, full deprotection was achieved with exactly 3.0 equivalents of $LiOH·H_2O$ to afford the trilithium salts **26a** and **26b**.

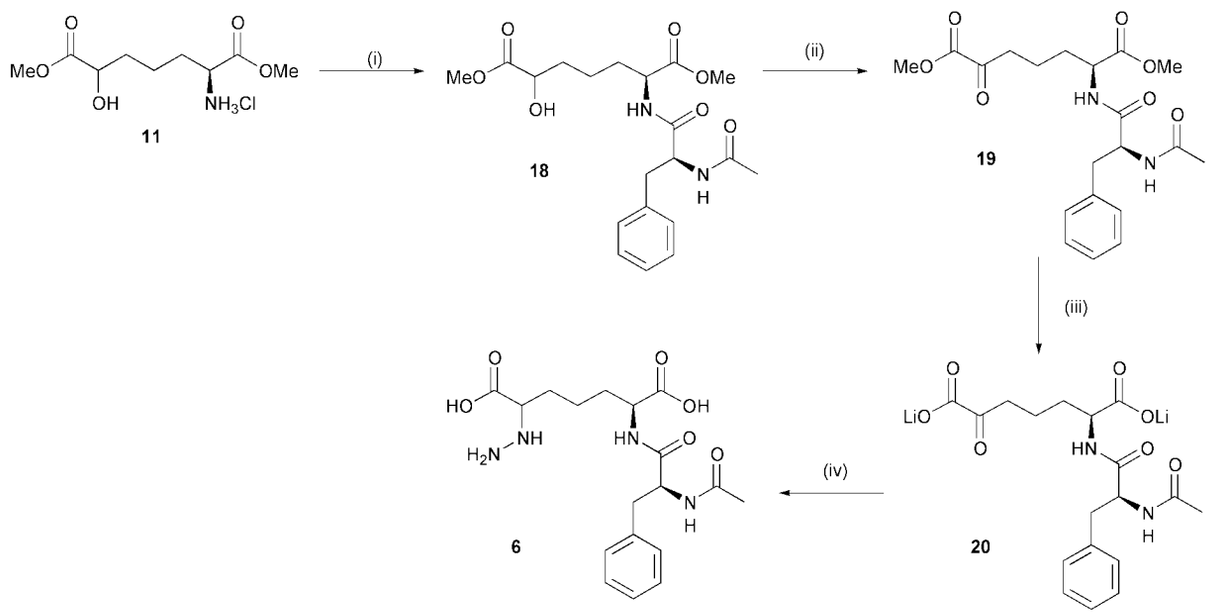


Scheme 3 *Reagents and conditions*: i, Ac_2O, $NaHCO_3$ (aq); ii, Fmoc-OSu, $(CH_3)_2CO$, $NaHCO_3$.

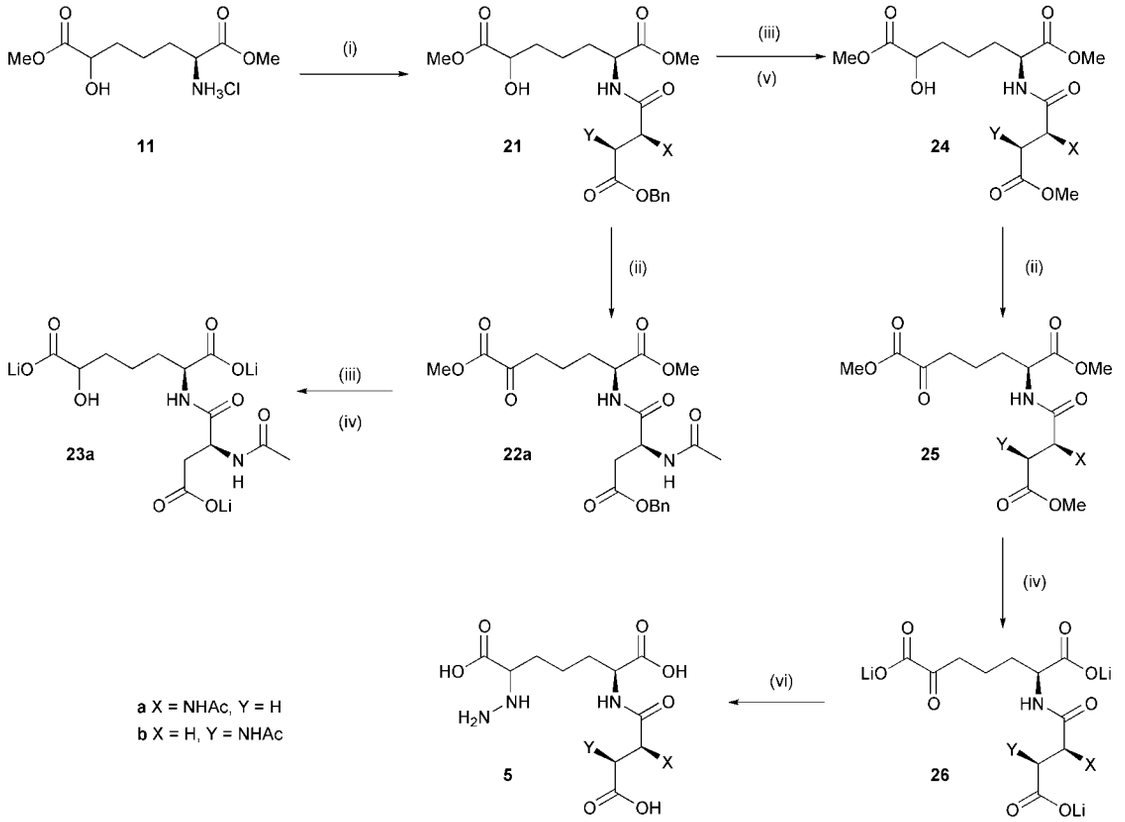
The protected dipeptides described above were relatively simple to purify, but in the case of the *N*-acetylalanylalanyl tripeptide **27a**, high water solubility and difficulties with column chromatography drastically reduced the yield of purified product and the synthesis was abandoned at this stage. The Fmoc-protected tripeptide **27b** was obtained in high yield after column chromatography, however, presumably because of its increased hydrophobicity. Conversion of **27b** to the ketone **28** was also straightforward (Scheme 6). The previously successful LiOH deprotection strategy was then applied to the Fmoc-protected tripeptide **28**. Initial treatment of **28** with exactly 2.0 equivalents of $LiOH·H_2O$ left some residual methyl esters as judged by the ^1H NMR spectrum. Analysis of the hydrolysis product by HPLC and electrospray mass spectroscopy (ESMS) indicated some Fmoc cleavage under the basic conditions. Sufficient $LiOH·H_2O$ was therefore added to the deprotection reaction to cause full methyl ester hydrolysis. Removal of solvent afforded a mixture of peptides **29a** and **29b** with and without Fmoc protection.

The use of piperidine to fully cleave the Fmoc protection was then attempted. Analysis by HPLC indicated this to be very rapid, with complete substrate consumption in less than 15 min. However, after removal of solvent and excess piperidine, analysis of the product indicated formation of a majority of the piperidinyl enamine **31** in addition to the desired ketone **30**. In principle enamine **31** should be easily hydrolysed in aqueous acid, but we found that even prolonged treatment in aq. trifluoroacetic acid (TFA) caused little hydrolysis to **30**. In order to avoid enamine formation we utilised the non-nucleophilic *N*-methylmorpholine as the basic deprotection agent. Deprotection of the mixture of **29a** and **29b** was significantly slower than with piperidine, with reaction in 50% aq. *N*-methylmorpholine requiring at least 24 h for complete deprotection. However, after removal of reagents and solvent, and purification by semi-preparative HPLC, the tripeptide **30** was obtained in pure form.

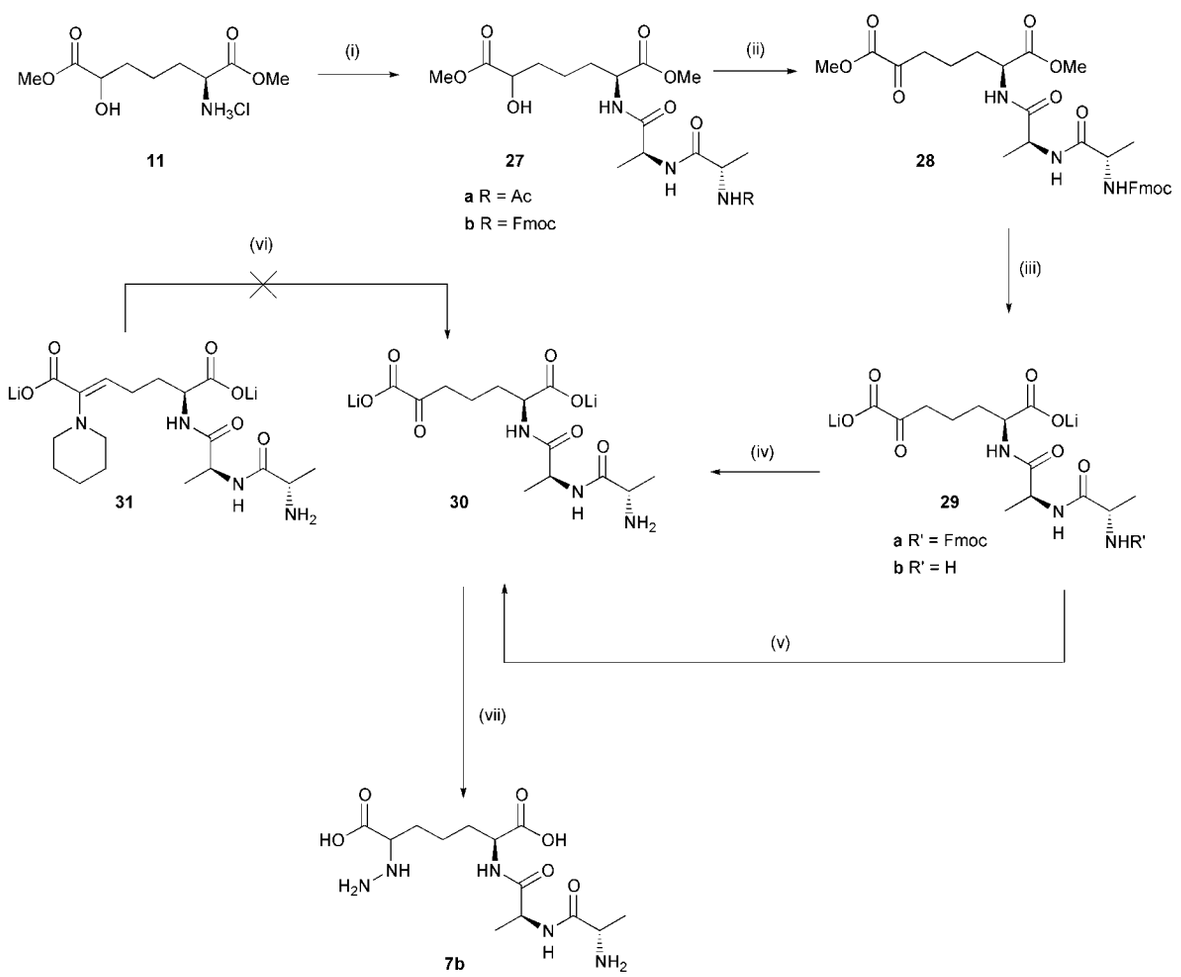
The hydrazines **5**–**7** were simply obtained from their corresponding ketones by treatment with an excess of hydrazine in the presence of $NaCNBH_3$. At pH 5.0 this reaction is selective for hydrazone reduction and causes little reduction of ketones. Purification of the resulting hydrazino peptides was achieved by ion-exchange chromatography. Characterisation of the hygroscopic products was difficult due to their production in small (≈10 mg) quantities. However, an extremely useful characteris-



Scheme 4 *Reagents and conditions*: i, **15**, DCC, HOBt, CH_2Cl_2; ii, Dess–Martin periodinane, CH_2Cl_2; iii, 2.0 equiv. $LiOH·H_2O$, aq. CH_3CN; iv, NH_2NH_2, MeOH, CF_3CO_2H (to pH 5.0), $NaCNBH_3$.

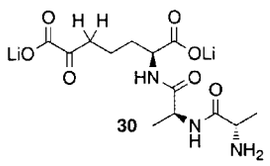


Scheme 5 *Reagents and conditions*: i, **13a** or **13b**, EDCI, HOBt, CH_2Cl_2; ii Dess–Martin periodinane, CH_2Cl_2; iii, H_2, Pd/C, MeOH; iv, 3.0 equiv. $LiOH{\cdot}H_2O$, aq. CH_3CN; v, excess of ethereal CH_2N_2; vi, NH_2NH_2, MeOH, CF_3CO_2H (to pH 5.0), $NaCNBH_3$.



Scheme 6 *Reagents and conditions*: i, **17**, EDCI, HOBt, CH_2Cl_3; ii, Dess–Martin periodinane, CH_2Cl_2; iii, ≈3.0 equiv. $LiOH{\cdot}H_2O$, aq. CH_3CN; iv, piperidine, DMF; v, *N*-methylmorpholine, water; vi, 10% aq. CF_3CO_2H; vii, NH_2NH_2, MeOH, CF_3CO_2H (to pH 5.5), $NaCNBH_3$.

ation technique is ESMS. Not only can this analytical method detect microgram quantities of the peptides, but dissolution of the sample in a mixture of H_2O and D_2O allows statistical deuterium exchange into NH, OH and activated CH positions. Since the deuteriated compounds can be resolved and counted this technique gives a useful double-check of the expected structure. For example, in negative-ion mode, the ES spectrum of a sample of peptide ketone **30** dissolved in 30% D_2O in H_2O (Fig. 1) clearly shows the expected envelope of masses for exchange of up to 4 of the available 5 labile protons above the parent anion. The pattern is mirrored for lithium-exchanged anions at 6 and 12 *m*/*z* units lower.



Exact Mass (M) : 343.15

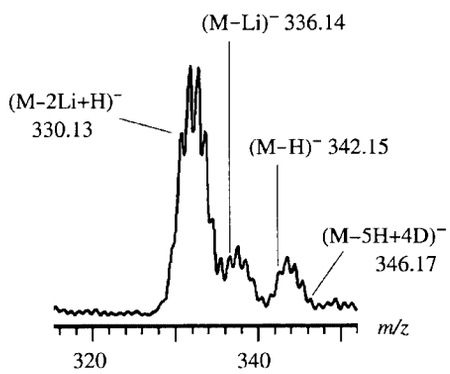
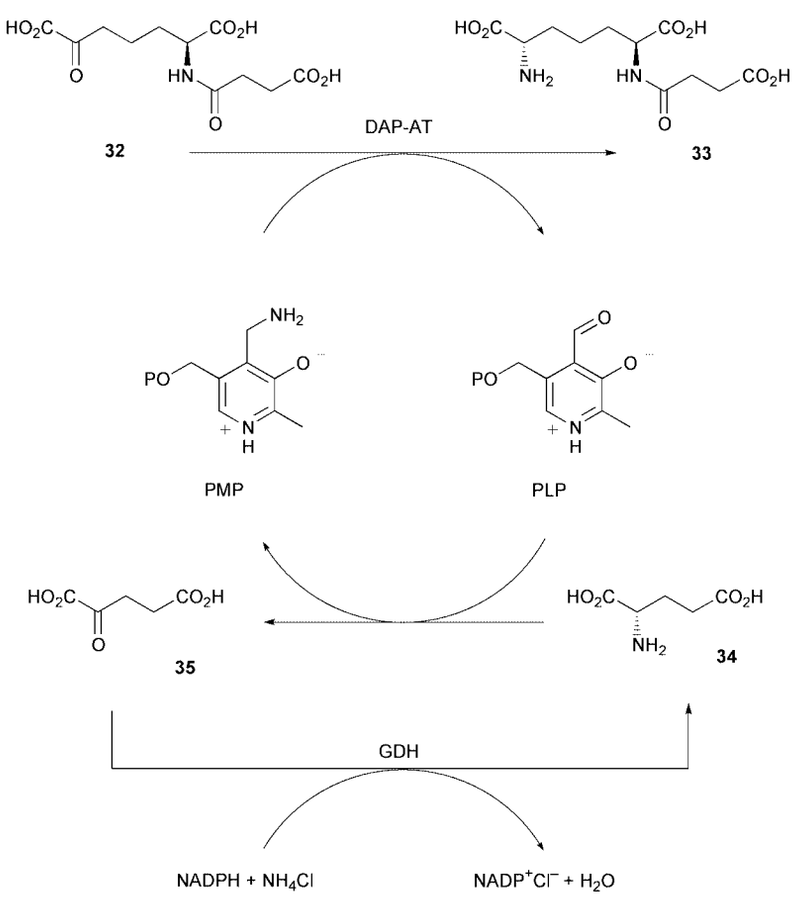


Fig. 1 The electrospray ionisation mass spectrum of **30** after incubation in 30% D_2O in H_2O, (negative-ion mode).

Substrate activity

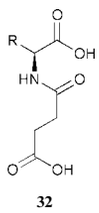
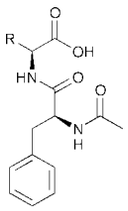
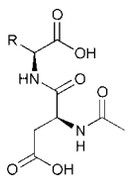
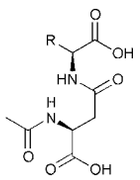
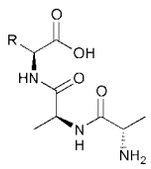
DAP-AT is a pyridoxal phosphate (PLP) dependent enzyme. DAP-AT was isolated from *E. coli* DH5α by a procedure involving rapid sonication of whole cells, cation and anion chromatography and ultrafiltration. The enzyme fraction obtained showed high activity with the natural substrate **32** with no detectable background activity in the absence of substrate. In the standard assay DAP-AT, in its pyridoxamine phosphate (PMP) form, converts the natural substrate **32** (≈1 mM) to the ε-L-amine **33** (Scheme 7). This generates the PLP form of the enzyme which then reacts in the reverse direction with an excess of L-glutamate **34** (10.0 mM) to generate α-ketoglutarate **35**. A coupling enzyme, glutamate dehydrogenase (GDH), then converts the α-ketoglutarate **35** rapidly back to L-glutamate **34** with the consumption of ammonium ions and NADPH. The fall in NADPH concentration is conveniently monitored at 340 nm in order to obtain rate data.

In the standard activity assay all of the synthetic peptide ketones were turned over by the enzyme. Apparent Michaelis–Menten kinetic parameters were measured for each substrate by measuring the rate of NADPH consumption with increasing substrate concentration (Table 1). When compared with the natural substrate **32**, all of the peptides are clearly somewhat poorer substrates of DAP-AT in terms of their turnover number (k_{cat}). Interestingly, K_M-values for the substrate analogues are remarkably similar to that for the natural substrate, in the range 1–5 mM, the major difference in activity being the maximal rate of reaction. In terms of specificity (k_{cat}/K_M), it is clear that the bulky alanylalanyl peptide **30** is the poorest substrate, with the phenylalanyl peptide also being a very poor substrate. Of the two aspartyl peptides the α-linked dipeptide appears



Scheme 7 Assay system for DAP-AT. GDH = glutamate dehydrogenase.

Table 1 Michaelis–Menten kinetic values for DAP-AT substrates

Substrate R = $(CH_2)_3COCO_2H$	 **32**				
K_M^{app}/mM [a,b]	2.25	< 1	1.91	4.69	2.63
k_{cat}/s^{-1} [b]	164	< 2.1	20.3	43.2	1.5
k_{cat}/K_M^{app}/s^{-1} mM^{-1}	72.9	< 2.1	10.6	9.2	0.53
% Natural substrate	100	< 0.8	14.5	12.6	0.008

[a] At 10 mM L-glutamate. [b] ± 10%.

marginally the better of the two. The dependence of substrate specificity on side-chain bulk has previously been observed; for example, Boc-protected DAP analogues are also very poor substrates of DAP-AT.[7]

Inhibition activity

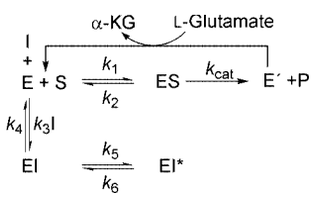
The succinyl-DAP hydrazine **3** is a very potent slow binding inhibitor of DAP-AT.[7] Previous kinetic studies have shown that it appears to inhibit *via* a two-step mechanism involving an initial binding event followed by a second event which, although reversible, is very slow in the reverse direction. Although not proven, it is likely that the hydrazine nucleophile of the inhibitor forms a hydrazone with the PLP form of the cofactor in the enzyme active site. It is not clear whether the 'slow reverse' second step of the inhibition process is an 'opening' of the active site or simply the slow, enzyme-catalysed hydrolysis of the hydrazone.

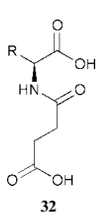
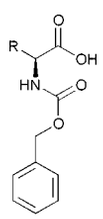
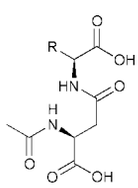
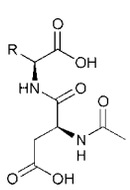
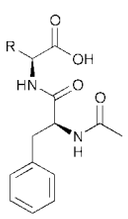
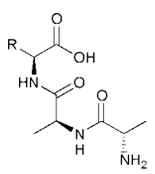
In either event the hydrazino peptides synthesized in this study were also expected to act as slow-binding inhibitors of DAP-AT. Slow-binding inhibition is characterised by time-dependent, but reversible inhibition. The process has been extensively studied and described by Morrison and Walsh.[20] For the two-step process observed for the hydrazine **3** (Table 2) this manifests itself as a decrease in rate for the initial reaction in the first seconds to minutes of reaction. This could easily be interpreted as simple reversible competitive inhibition were it not for a second, slower process, manifesting itself over minutes to hours, leading to much more substantial inhibition.

In order to test for slow-binding inhibition the peptide hydrazines were added to reactions containing all assay components including the substrate, and the effect of the inhibitor was monitored over time. Initially little inhibition was observed, but over time the effect became more pronounced until equilibrium was reached. This process was repeated for increasing inhibitor concentrations for each of **5a**, **5b**, **6** and **7**. Data analysis for this type of inhibition is relatively complex, but analysis of multiple progress of inhibition curves (*e.g.* Fig. 2A) does allow estimation of a number of kinetic parameters. Each progress-of-inhibition data-set was directly fitted to the integrated rate equation described by Morrison and Walsh (*e.g.* Fig. 2A for inhibition by **5b**).[21,22] This gave precise figures for the initial rates of reaction as well as the final equilibrium rates of reaction at each inhibitor concentration tested. Plotting the reciprocal of the rate *vs.* the inhibitor concentration (*e.g.* Fig. 2B for inhibition by **5b**) then allowed estimation of K_I (*i.e.* the inhibition constant for the initial process)† as well as the overall inhibition constant K_I^* in the usual way (Table 2).[23]

† K_I and K_I^* are *concentration* terms. When the concentration of inhibitor reaches K_I, the measured K_M of the substrate is doubled, *i.e.* at K_I half of the active sites are filled with inhibitor. K_I is thus a measure of affinity between the enzyme and the inhibitor – the lower K_I the higher the affinity.

Table 2 Inhibition kinetic values obtained for peptide hydrazines



Inhibitor R = $(CH_2)_3CH(NHNH_2)$-CO_2H	 **32**					NH_2NH_2	
	Succ **3** (ref. 7)	Cbz **4** (ref. 7)	Ac-βAsp **5b**	Ac-αAsp **5a**	Ac-Phe **6**	Hydrazine	Ala-Ala **7**
K_I/μM [a,b]	5.7	4.5	18.0	17.8	52.0	60.0	265
k_5/s^{-1}	29.3×10^{-3}	20.6×10^{-3}	7.7×10^{-3}	17.3×10^{-3}	13.5×10^{-3}	5.4×10^{-3}	$>15.6 \times 10^{-3}$
k_6/s^{-1}	0.15×10^{-3}	0.26×10^{-3}	0.38×10^{-3}	1.1×10^{-3}	1.9×10^{-3}	0.79×10^{-3}	$>2.1 \times 10^{-3}$
$K_{5/6} = k_5/k_6$	195	79.2	20.3	15.7	7.1	6.8	7.4
K_I^*/nM	29	55	834	1100	6400	7700	31500

[a] At 10 mM L-glutamate. [b] ± 10%.

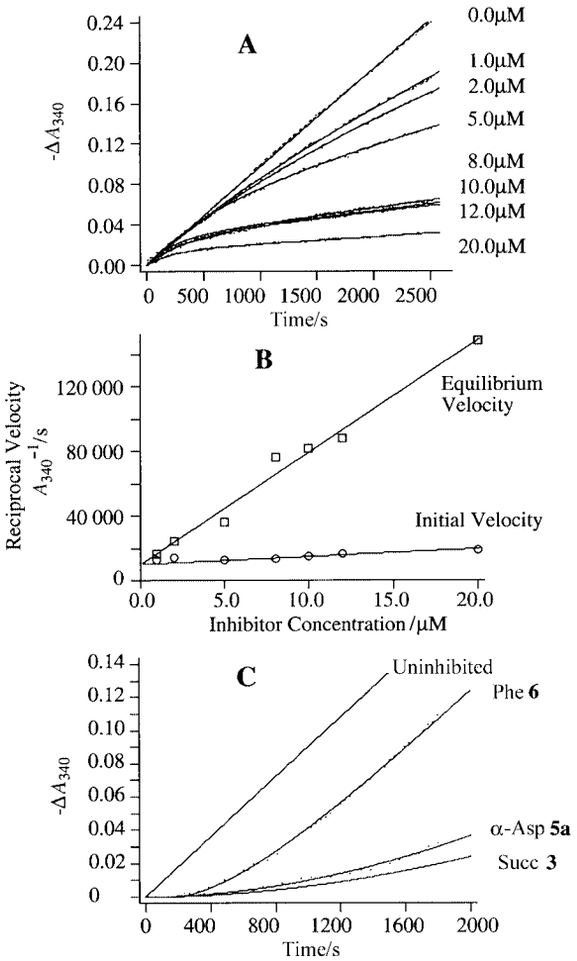


Fig. 2 **A**, Experimental progress-of-inhibition data obtained for inhibition of DAP-AT by **5b** at the indicated concentrations (dots) and best-fit curve to the integrated rate equation of Morrison and Walsh (curve);[20] **B**, Replots of the reciprocal of the 'initial' (*i.e.* first few seconds) and equilibrium (*i.e.* after *ca.* 30 min) rates *vs.* **5b** concentration from panel **A**;[20] **C**, Regeneration of activity of fully inhibited (by the indicated compounds) DAP-AT upon 100-fold dilution.[20]

In further experiments DAP-AT was incubated with an excess of each potential inhibitor in the absence of substrates. This inhibited enzyme was then added to a standard assay solution and the rate at which reaction proceeded was monitored (Fig. 2C for data for **3**, **5a** and **6**). In these experiments very low or zero initial enzyme activity was observed, indicative of full enzyme inhibition, but over time the rate of enzyme reaction increased as the inhibitors diffused out of the active site. Conditions were chosen such that the final inhibitor concentration in the activity assay was low enough not to cause significant inhibition. Under these conditions the reaction is essentially irreversible and the rate constant k_6 can be easily estimated. Already knowing K_I and K_I^*, the rate constant k_5 can then be also calculated (Table 2).[20]

All of the peptide hydrazines synthesised here show inhibitory activity *vs.* DAP-AT. However, the most potent of them, the β-aspartyl peptide **5b**, shows inhibitory activity around 30-fold lower than the *N*-succinyl-DAP hydrazine **3**. The α-aspartyl inhibitor **5a** is somewhat worse at 1/40th the potency of **3**, while the phenylalanyl **6** and alanylalanyl **7** peptides show poor inhibition in the μM range. Hydrazine itself also inhibits DAP-AT, but with an overall K_I^* of 7.7 μM. All of the inhibitors, including hydrazine, showed two-phase inhibition kinetics, indicative of slow-binding inhibition. However, close examination of the individual kinetic constants for the inhibition processes shows why the succinyl- and Cbz-based inhibitors are most potent. It is clear that for these two compounds the initial inhibition (specific inhibition constant K_I) favours formation of the presumed EI complex when compared to the other compounds. This may be indicative of favourable molecular recognition events and binding interactions during the early phase of inhibition for **3** and **4**, compared with poorer binding and/or disfavourable steric interactions for **5**–**7**. For example, **7**, with the bulkiest *N*-acyl side-chain, shows the poorest inhibition constant.

During the second phase of inhibition the succinyl **3** and Cbz **4** compounds again show the most favourable kinetic behaviour. Consideration of the equilibrium constants for this process shows that for **3** and **4** the EI* state is highly favoured (*e.g.* $K_{5/6} = k_5/k_6 = 195$ for **3**), while for the other compounds this value falls to ≈10. Hydrazine itself shows a very similar value.

It is possible to speculate as to the physical meanings of the two processes occurring during inhibition (Scheme 8). It is obvious that an initial step must be recognition and binding of the inhibitor by DAP-AT. This fits well with the properties of the various inhibitors as discussed above. The second phase of inhibition presumably involves hydrazone formation, the reverse process being hydrazone hydrolysis. In other PLP-dependent aminotransferases significant closure of the active site has been observed during reaction and it may be that the second equilibrium process is the formation of a tightly bound inhibitor in a 'closed' active site.[24] The $K_{5/6}$ value for hydrazine inhibition is remarkably similar to that of the other 'poor' inhibitors. As hydrazine is likely to form a hydrazone with PLP in the enzyme active site, without specific interactions with other parts of the active site, it may be that the observed $K_{5/6}$ equilibrium constant of ≈10 represents simple hydrazone formation for all of the poorer inhibitors. A further active-site-closure event could then account for the enhanced binding of the better inhibitors. Overall this model would give an equilibrium constant for hydrazone formation of ≈7–10, with a further contribution of a factor of ≈10–20 for full active-site closure. The closure of the active site then affects the off rate (k_6), significantly impeding hydrazone hydrolysis for the better inhibitors **3** and **4**. The α- and β-aspartyl peptides, **5a** and **5b**, show intermediate behaviour, perhaps representing cases involving partial active-site closure.

Antimicrobial activity

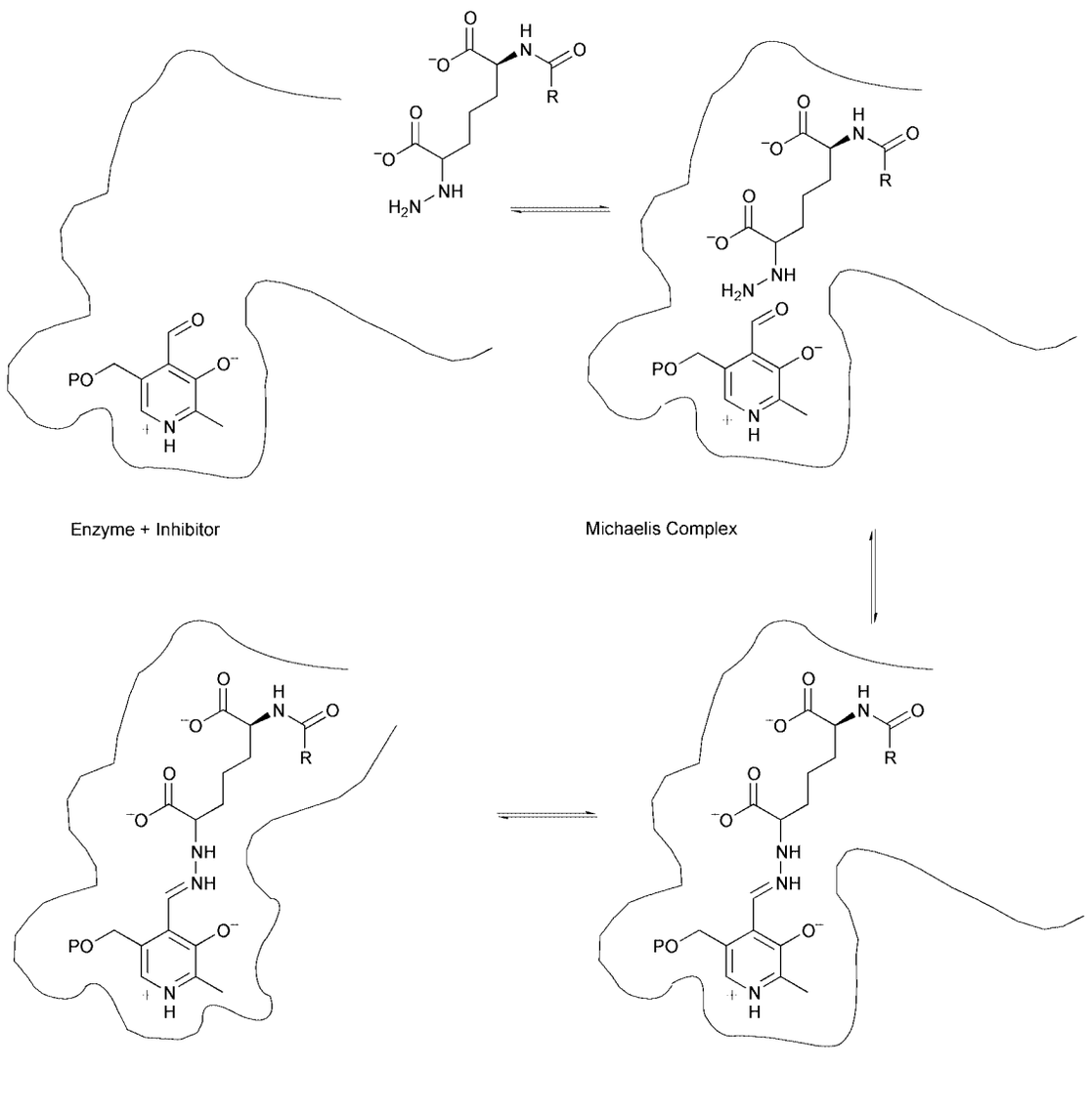
In order to test the antimicrobial activity of the hydrazino peptides a simple assay system was devised in which small disks of filter paper soaked in varying concentrations of the inhibitors was placed on an agar surface on which *E. coli* cells were growing. After 24 h the radius of the inhibition zone around the filter paper disk was measured. This system was tested with a number of commercial antibiotics as well as with the synthetic ketones, the synthetic hydrazines, and hydrazine itself. The results are shown in Table 3. As expected the commercial antibiotics show significant growth-inhibition zones on both nutrient and minimal media. The peptide ketones show minimal growth inhibition. The peptide hydrazines with the best *in vitro* inhibition characteristics with respect to DAP-AT also show marked antimicrobial activity, but only on minimal media. The L-Agar nutrient media used contains hydrolysed protein extract, and presumably contains significant L-lysine which overcomes the effects of DAP-AT inhibition. Hydrazine itself shows very good antimicrobial activity, because it presumably inhibits all PLP-dependent enzymes. It should be remembered, however, that due to its low molecular mass, the hydrazine molar concentration in these assays is around 10-fold higher than the peptides at equivalent mass loadings.

Our initial idea that poor cell-penetration properties could be the cause of the low *in vivo* potency of these DAP-AT inhibitors would not appear to be supported by the results with the ala-ala-tripeptide hydrazine **7**. This compound would be

Table 3 Antimicrobial activity of potential DAP-AT inhibitors *vs. E. coli* DH5α

	Radius of inhibition zone/mm[a]							
	Inhibitor per disk/ug L-Agar				Inhibitor per disk/ug M9-Minimal Agar			
Inhibitor	300	30	3	0.3	300	30	3	0.3
*N*Ac-α-Asp-AP-$NHNH_2$ **5a**	1	0	0	0	10	5	0	0
*N*Ac-β-Asp-AP-$NHNH_2$ **5b**	0	0	0	0	9	2.5	0	0
*N*Ac-Phe-AP-$NHNH_2$ **6**	1	0	0	0	6	0	0	0
Succ-AP-$NHNH_2$ **3**	1	0	0	0	13	8	0	0
Ala-Ala-AP-$NHNH_2$ **7**	0	0	0	0	0	0	0	0
NH_2NH_2					15	10	0	0
*N*Ac-α-Asp-AP-O **26a**	0	0	0	0	1	0	0	0
*N*Ac-β-Asp-AP-O **26b**	0	0	0	0	1	0	0	0
*N*Ac-Phe-AP-O **20**	0	0	0	0	1	0	0	0
Ala-Ala-AP-O **30**	0	0	0	0	1	0	0	0
Tetracycline	13	10	7	2.5	17	10	8	2
Chloroamphenicol	13	10	3.5	0	17	12	6	3
Carbenicillin	13	8	4.5	0	12	7	4	2
Water	0	0	0	0	0	0	0	0

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



Scheme 8 A possible role for active-site closure and hydrazone formation during inhibition of DAP-AT by hydrazines **3**–**7**.

expected to be more efficiently transported than the other DAP-AT inhibitors described here. However, no significant growth inhibition of *E. coli* could be observed for this compound. It is conceivable that the low *in vivo* potency of this compound is simply due to its poor inhibition of DAP-AT. We also hoped that enzyme-catalysed hydrolysis of the synthetic peptide

hydrazines could lead to the generation of the known *meso*-DAP decarboxylase inhibitor *N*-amino-DAP *in vivo*.[15] However, it would appear that even if this compound is being formed it is not in sufficient concentration to cause lethal inhibition of *meso*-DAP-decarboxylase. Recently Blanchard and Ledwidge have determined that the *arg*D encoded α-*N*-acetylornithine aminotransferase (NAcO-AT) can also process the DAP-AT substrate **32**.[25] It may be that the poor general *in vivo* potency of DAP-AT inhibitors is due to NAcO-AT activity substituting for DAP-AT.

It is clear from the results presented here that *N*-acyl side-chain bulk has a detrimental effect on both *in vitro* and *in vivo* inhibition of DAP-AT by hydrazino peptides. Further work on the design and synthesis of potential irreversible inhibitors of DAP-AT and the *in vivo* effects of NAcO-AT will be reported in the near future.

Experimental

General

All reagents and solvents were obtained from the Sigma-Aldrich chemical company and were of ACS grade and not further purified unless otherwise stated. All anhydrous solvents were purchased from Fluka and were transferred under dried N_2 gas. NMR spectra were obtained using JEOL Λ-300, Δ-270 and Δ-400 spectrometers operating at 300, 270 and 400 MHz (^{1}H) and 75.5, 67.9 and 100.7 MHz (^{13}C) respectively. Chemical shifts are quoted in ppm relative to TMS. Coupling constants (*J*) are quoted in Hz. IR spectra were obtained using a Perkin-Elmer 1600 FTIR spectrometer, using KBr discs for solids and thin films between NaCl plates for oils. Mps were obtained using a Reichert hot-stage apparatus equipped with microscope and Comark digital thermometer, and are uncorrected. Mass spectra were obtained in the indicated mode using a VG analytical autospec instrument (EI, CI, FAB, accurate mass) or Fisons VG Quattro spectrometer (ESMS). Optical rotations were obtained using a Perkin-Elmer 141 polarimeter using a 1 dm cell of 1 ml capacity. $[\alpha]_D$-Values are given in units of 10^{-1} deg cm^2 g^{-1}. Flash chromatography was performed according to the method of Still[26] or using an improvised automatic system comprising a nitrogen constant pressure head, column packed with Merck silica gel 60 (0.040–0.063 mm), Gilson Holochrome UV detector set at 254 nm, and an LKB fraction collector. TLC analysis was performed using Merck glass-backed 0.2 mm silica plates (F254) developed with phosphomolybdic acid when necessary. DAP-AT was purified from *E. coli* DH5α by previously described methods.[7,8] Enzyme-assay methods have been reported elsewhere.[7,8] For enzyme assays the UV spectrophotometer used was a Pharmacia LKB ultrospec III, equipped with a water-heated cell holder. All assays were performed at 37 °C.

Peptide nomenclature

All residues are L-configured unless otherwise stated; sequence runs from N to C termini; amino acids are denoted by standard three letter abbreviations, AP = α-aminopimelic acid; bracketed carboxylic group protection is at non-alpha position. The AP skeleton is labelled α to ε, the α-carbon bearing the amido group and the ε-carbon bearing the oxygen functionality.

HPLC Methods

All HPLC was carried out using a Beckman System Gold 126 pump module equipped with a Beckman 507 autosampler and Beckman 168 diode array UV spectrophotometer detector detecting at 218 and 254 nm. Solvents were: **A**, 0.05% trifluoroacetic acid (TFA) in degassed, deionised water; **B**, 0.045% TFA in HPLC-grade acetonitrile (Rathburn). Method 1: 4.6 × 250 mm Rainin Dynamax 60 Å C_{18} column equipped with C_{18} guard eluted at 1 ml min^{-1}, 0–5 min 0% B, 5–35 min 0–70% B, 35–37 min 70–100% B, 37–39 min 100% B, 39–41 min 100% B to 0% B; method 2: 10 × 250 mm Chromapak spherisorb C_{18} column eluted at 4 ml min^{-1}, 0–5 min 0% B, 5–15 min 0–100% B, 15–17 min 100% B, 17–19 min 100–0% B. For semi-preparative scale purifications fractions were collected manually.

Ac-Phe-OH 15[27]

A stirred solution of L-phenylalanine **14** (2.0 g, 12.1 mmol) in aq. KOH (10 M; 10 ml) was treated with acetic anhydride (2.47 g, 24.2 mmol) in portions over a period of 30 min. The solution was acidified to pH 2 by addition of conc. HCl. The mixture was diluted with water (50 ml) and extracted into EtOAc (3 × 100 ml). The combined organic extracts were evaporated *in vacuo*, and the solid residue was recrystallised from EtOAc to afford the product as colourless crystals (820 mg, 32%): mp 168.0–169.5 °C (lit.,[28] 170–171 °C); δ_H(300.40 MHz; $CDCl_3$) 7.19–7.18 (5H, m, Ph), 5.77 (1H, d, *J* 6.1, NH), 4.86–4.70 (1H, m, αH), 3.18 (1H, dd, *J* 5.7, 14.1, βCH), 3.07 (1H, dd, *J* 14.1, 6.4, βCH), 1.93 (3H, s, CH_3); *m*/*z* (CI) 207 (M^+, 17%), 208 (MH^+, 100).

Ac-Asp(OBn)-OH 13a[29]

To a stirred solution of H-Asp(OBn)OH **12a** (NovaBiochem, 1.0 g, 4.48 mmol) in saturated aq. $NaHCO_3$ (20 ml), was added acetic anhydride (548 mg, 550 µmol). After 2 h a second portion of acetic anhydride (23 mg, 225 µmol) was added and the mixture was stirred for 60 min. The mixture was acidified to pH 2 with conc. aq. HCl and extracted into EtOAc (3 × 20 ml). The solvent was removed *in vacuo* to afford the product as colourless crystals (1.1 g, 93%); δ_H(300.4 MHz; $CDCl_3$) 7.39–7.30 (5H, m, Ph), 6.77 (1H, d, *J* 7.90, NH), 5.13 (2H, s, OCH_2), 4.93–4.84 (1H, m, αCH), 3.11 (1H, dd, *J* 17.4, 4.4, βCH), 2.92 (1H, dd, *J* 17.4, 4.6, βCH), 2.02 (3H, s, CH_3); *m*/*z* (EI) 265 (M^+, 52%); *m*/*z* (CI) 266 (MH^+, 71%) (Found: $[MH]^+$, 266.103 22. $C_{13}H_{16}NO_5$ requires *m*/*z*, 266.102 85) (Calc. for $C_{13}H_{15}NO_5$ requires C, 58.86; H, 5.70; N, 5.28. Found: C, 58.65; H, 5.52; N, 5.31%).

Ac-Asp(OH)-OBn 13b[29]

To a stirred solution of H-Asp(OH)-OBn·HCl **12b** (NovaBiochem) (1.5 g, 6.7 mmol) in saturated aq. $NaHCO_3$ (15 ml) was added acetic anhydride (7.0 ml, 1.2 equiv.). After 45 min the reaction mixture was acidified (conc. aq. HCl) and extracted into EtOAc (2 × 50 ml) and CH_2Cl_2 (2 × 50 ml). The combined dried ($MgSO_4$) solvents were removed *in vacuo*, to yield **13b** as a clear oil (1.54 g, 86%); ν_{max}(KBr)/cm^{-1} 3436, 2928, 1740, 1664, 1460; δ_H(300.4 MHz; $CDCl_3$) 7.36 (5H, m, Ph), 6.61 (1H, d, *J* 8.1, NH), 5.20 (2H, s, CH_2), 4.90 (1H, m, αH), 3.22 (1H, br s, CO_2H), 3.09 (1H, dd, *J* 8.1, 4.4, βH), 2.93 (1H, dd, *J* 8.1, 4.4, βH), 2.05 (3H, s, CH_3); δ_C(75.45 MHz; $CDCl_3$) 174.1 (CO_2H), 170.6 (CO_2Bn), 170.3 (CONH), 135.0 (Ph), 128.7 (Ph), 128.6 (Ph), 128.3 (Ph), 67.8 (OCH_2), 48.6 (αCH), 35.9 (βCH_2), 23.0 (CH_3); *m*/*z* (CI) 266 (MH^+) (Found: $[MH]^+$, 266.102 70. $C_{13}H_{16}NO_5$ requires *m*/*z*, 266.102 85).

Ac-Ala-Ala-OH hemihydrate 17a[30]

To a stirred solution of H-Ala-Ala-OH **16** (Sigma, 1.028 g, 5.1 mmol) in saturated aq. $NaHCO_3$ (9 ml) was added acetic anhydride (730 µl, 1.2 equiv., 6.12 mmol) dropwise over a period of 60 min. After a further 60 min more acetic anhydride (365 µl, 0.6 equiv., 3.06 mmol) was added dropwise over a period of 30 min. After a further 60 min the reaction mixture was neutralised (dil. aq. HCl) and applied to the H^+-form of a column of Dowex AG50 WX8 cation-exchange resin. The acidic eluent was concentrated *in vacuo*, and the white solid product dissolved in acetone and the solution filtered. Evaporation of

the filtrate afforded the target compound as a hemihydrate; ν_{max}(KBr)/cm^{-1} 3387, 3350, 3307, 3279, 3068, 2996, 1705, 1655; δ_H(300 MHz; D_2O) 4.70 (1H, q, *J* 7.3, C*H*CH$_3$), 4.63 (1H, q, *J* 7.3, C*H*CH$_3$), 2.36 (3H, s, *J* 7.3, CH$_3$CO), 1.77 (3H, d, *J* 7.4, CH$_3$), 1.72 (3H, d, *J* 7.3, CH$_3$); *m*/*z* (ES$^-$) 201 (M$^-$); *m*/*z* (ES$^+$, H_2O–D_2O) 203 [(M)H$^+$, 10], 204 [(M − H + D)H$^+$, 18], 205 [(M − 2H + 2D)H$^+$, 30], 206 [(M − 3H + 3D)H$^+$, 25], 207 [(M − 3H + 3D)D$^+$, 20], 225 [(M)Na$^+$, 40], 226 [(M − H + D)Na$^+$, 95], 227 [(M − 2H + 2D)Na$^+$, 100], 228 [(M − 3H + 3D)Na$^+$, 80] [Calc. for $(C_8H_{14}N_2O_4)_2$·H_2O: C, 45.49; H, 7.16; N, 13.26. Found: C, 45.56; H, 7.09; N, 13.06%]; HPLC (Method 1) t_R 10.05 min.

Fmoc-Ala-Ala-OH 17b

H-Ala-Ala-OH **16** (Sigma, 0.75 g, 4.68 mmol) and NaHCO$_3$ (0.39 g, 4.68 mmol) were dissolved in a mixture of acetone (9 ml) and water (9 ml) with stirring at RT. After 5 min fluoren-9-ylmethyl succinimidyl carbonate (1.74 g, 5.15 mmol) was added and the suspension stirred for 14 h during which time a white precipitate formed. The mixture was diluted with water (50 ml), acidified (2 M aq. HCl) and extracted with EtOAc (3 × 50 ml). The organic extracts were combined, dried (MgSO$_4$) and evaporated *in vacuo* to afford the title compound **17b** as a colourless solid (1.72 g, 96%); mp >210 °C (from CH$_3$OH–CHCl$_3$); $[\alpha]_D^{24}$ −14.6 (*c* 1.0, CH$_3$OH); ν_{max} (KBr)/cm^{-1} 3300, 3062, 2977, 2933, 1691, 1647, 1600, 1540, 1450, 1311, 1262, 1234; δ_H(270 MHz; CD_3OD) 7.35 (8H, m, ArH), 4.25 (2H, m, 2 × αCH), 1.39 (3H, d, *J* 3.0, CH$_3$), 1.33 (3H, d, *J* 3.0, CH$_3$); δ_C(75.45 MHz; DMSO-d_6) 174.3 (CO_2H), 171.3 (CONH), 155.6 (CO_2NH), 143.7 (Ph), 140.6 (Ph), 127.6 (Ph), 127.1 (Ph), 125.3 (Ph), 120.0 (Ph), 65.6 (Fmoc CH), 50.1 (αCH), 48.9 (αCH), 46.6 (OCH$_2$), 18.4 (CH$_3$), 18.1 (CH$_3$); *m*/*z* (ES$^+$, D_2O–H_2O) 383 [(MH)$^+$, 1%], 383 [(MD)$^+$, 6], 384 [(M − H + D)D$^+$, 15], 385 [(M − 2H + 2D)D$^+$, 12], 386 [(M − 3H + 3D)D$^+$, 5], 405 [(M)Na$^+$, 20], 406 [(M − H + D)Na$^+$, 55], 407 [(M − 2H + 2D)Na$^+$, 55], 408 [(M − 3H + 3D)Na$^+$, 25], 421 [(M)K$^+$, 5], 422 [(M − H + D)K$^+$, 10], 423 [(M − 2H + 2D)K$^+$, 100], 424 [(M − 3H + 3D)K$^+$, 40]; HPLC (method 1) t_R 26 min.

Ac-Phe-(ε-DL-hydroxy)AP(OCH$_3$)-OCH$_3$ 18

A solution of H-(ε-DL-hydroxy)AP(OCH$_3$)-OCH$_3$·HCl **11** (49.6 mg, 194 μmol), Ac-Phe-OH **15** (60.8 mg, 294 μmol), DCC (44 mg, 213 μmol), HOBt (2.62 mg, 19.4 μmol) and pyridine (17.2 μl, 213 μmol) in dry CH_2Cl_2 (3 ml) was stirred for 3 h. The mixture was diluted with CH_2Cl_2 (20 ml) and then washed with dil. HCl (2 × 30 ml), followed by saturated aq. NaHCO$_3$ (2 × 30 ml). The organic extract was dried (MgSO$_4$) and evaporated *in vacuo* to give a white solid, which was purified by flash chromatography (10% CH$_3$CN in EtOAc, R_f 0.11) to afford **18** (52.1 mg, 66%); $[\alpha]_D^{24}$ −2.17 (*c* 4.60, CH_2Cl_2); ν_{max}(KBr)/cm^{-1} 3287, 3064, 2953, 2862, 1743, 1650, 1547, 1439, 1375; δ_H(300.4 MHz; $CDCl_3$) 7.24 (5H, m, Ph), 6.43 (1H, d, *J* 7.7, NH), 6.20 (1H, d, *J* 7.30, NH), 4.65 (1H, m, Phe αCH), 4.48 (1H, m, AP αCH), 4.08 (1H, m, εC*H*OH), 3.69 (3H, s, OCH$_3$), 3.63 (3H, s, OCH$_3$), 3.35 (1H, br s, OH), 2.98 (2H, m, Phe βCH$_2$), 1.89 (3H, s, CH$_3$), 1.75 (4H, m, βCH$_2$ + δCH$_2$), 1.35 (2H, m, γCH$_2$); δ_C(75.45 MHz; $CDCl_3$) 175.0 (CO_2), 172.0 (CO_2), 171.0 (CONH), 170.0 (CONH), 137.0 (Ph), 129.2 (Ph), 128.6 (Ph), 127.0 (Ph), 70.0 (εCH), 54.5 (OCH$_3$), 52.4 (OCH$_3$), 52.3 (αCH), 49.2 (αCH), 38.0 (*C*H$_2$Ph), 33.9 (CH$_2$), 25.6 (CH$_2$), 24.9 (CH$_2$), 23.1 (CH$_3$); *m*/*z* (EI) 408 (M$^+$, 48%); *m*/*z* (CI) 409 (MH$^+$, 84%) (Calc. for $C_{20}H_{28}N_2O_7$: C, 58.81; H, 6.91; N, 6.86. Found: C, 58.64; H, 7.09; N, 6.77%).

Ac-Asp(OBn)-(ε-DL-hydroxy)AP(OCH$_3$)-OCH$_3$ 21a

A solution of H-(ε-DL-hydroxy)AP(OCH$_3$)-OCH$_3$·HCl **11** (143 mg, 559.8 μmol), Ac-Asp(OBn)-OH **13a** (222.5 mg, 839.6 μmol), EDCI (117.8 mg, 615.7 μmol), HOBt (7.65 mg, 55.9 μmol) and pyridine (49.7 μl, 615.7 μmol) in dry CH_2Cl_2 (15 ml) was stirred for 60 min. The mixture was diluted with CH_2Cl_2 (30 ml) and was washed with dil. aq. HCl (2 × 30 ml), followed by saturated aq. NaHCO$_3$ (2 × 30 ml). The organic extract was dried (MgSO$_4$) and evaporated *in vacuo*. The residue was purified by flash chromatography (10% CH$_3$CN in EtOAc, R_f 0.19) to afford **21a** (93 mg, 36%) as a viscous yellow oil; $[\alpha]_D^{24}$ −1.22 (*c* 2.30, CH_2Cl_2); ν_{max}/cm^{-1} 3305.4, 3065.7, 3007.0, 2956.4, 1739.6, 1657.3, 1536.8, 1455.4, 1438.4, 1412.3, 1376.4, 1261.0, 1214.4, 1172.9, 1106.2, 1016.4; δ_H(300.4 MHz; $CDCl_3$) 7.31–7.24 (5H, m, Ph), 7.03 (1H, d, *J* 7.8, NH), 6.86–6.70 (1H, m, NH), 5.15–5.02 (2H, m, OCH$_2$), 4.84–4.77 (1H, m, αCH), 4.50–4.40 (1H, m, αCH), 4.14–4.07 (1H, m, αCH), 3.71 (3H, s, OCH$_3$), 3.64 (3H, s, OCH$_3$), 2.99–2.86 (1H, m, Asp βCH), 2.70–2.58 (1H, m, Asp βCH), 1.96 (3H, s, CH$_3$), 1.25–1.80 (6H, m, 3 × CH$_2$); δ_C(75.45 MHz; $CDCl_3$) 175.4, 172.1, 172.0, 171.0, 170.3, 128.6, 128.4, 128.31, 128.29, 70.0, 67.0, 52.2, 52.1, 49.2, 35.8, 33.5, 31.5, 23.1, 20.6, 20.4; *m*/*z* (EI) 466.1940 (M$^+$) (Calc. for $C_{22}H_{30}N_2O_9$: *M*, 466.1951) (Calc. for $C_{22}H_{30}N_2O_9$: C, 56.65; H, 6.48; N, 6.01. Found: C, 56.98; H, 6.14; N, 6.08%).

Ac-Asp(OCH$_3$)-(ε-DL-hydroxy)AP(OCH$_3$)-OCH$_3$ 24a

Ac-Asp(OBn)-(ε-DL-hydroxy)AP(OCH$_3$)-OCH$_3$ **21a** (800 mg, 1.71 mmol) was dissolved in HPLC-grade methanol (15 ml) and stirred under H_2 (1 atm) at RT in the presence of 10% Pd/C (100 mg) for 16 h. After this time TLC analysis indicated complete consumption of the benzyl ester. The catalyst was removed by filtration through a bed of Celite and methanol was removed *in vacuo* to afford the carboxylic acid as a colourless solid (601 mg, 95%).

The solid was treated with an excess of an ethereal solution of diazomethane.[31] Excess of diazomethane was destroyed by addition of glacial acetic acid, and solvent was removed *in vacuo*. Flash chromatography (10% CH$_3$CN–90% EtOAc, R_f 0.15) yielded the trimethyl ester as a colourless solid (480 mg, 71.9%); mp 97–100 °C; δ_H(300 MHz; $CDCl_3$) 7.07 (1H, d, *J* 7.9, NH), 6.91 (1H, d, *J* 8.0, NH), 4.85 (1H, m, αCH), 4.55 (1H, ddd, *J* 5.1, 8.0, 13.2, αCH), 4.18 (1H, m, εCH), 3.79 (3H, s, OCH$_3$), 3.74 (6H, s, 2 × OCH$_3$), 2.99 (1H, dd, *J* 4.1, 17.3, Asp βCH), 2.65 (1H, dd, *J* 6.6, 17.1, Asp βCH), 2.07 (3H, s, CH$_3$), 2.0–1.5 (6H, m, 3 × CH$_2$); δ_C(75.45 MHz; $CDCl_3$) 175.4 (CO_2), 172.8 (CO_2), 172.2 (CO_2), 170.5 (CONH), 170.3 (CONH), 77.2 (Asp βCH$_2$), 70.0 (εCH), 53.4 (αCH), 52.6 (αCH), 52.5 (OCH$_3$), 52.3 (OCH$_3$), 52.2 (OCH$_3$), 49.2 (CH$_2$), 35.5 (CH$_2$), 33.5 (*C*H$_3$CO), 23.2 (CH$_2$); ν_{max}(KBr)/cm^{-1} 3423, 2957, 1732, 1652, 1547, 1440, 1374; *m*/*z* (EI) 390 (M$^+$, 30%); *m*/*z* (CI) 391 (MH$^+$, 32%) (Calc. for $C_{16}H_{26}N_2O_9$: C, 49.23; H, 6.71; N, 7.18. Found: C, 48.85; H, 6.68; N, 6.98%).

Ac-Asp([ε-DL-hydroxy]AP[OCH$_3$]-OCH$_3$)-OCH$_3$ 24b

To a stirred solution of Ac-Asp(OH)-OBn **13b** (2.76 g, 10.4 mmol, 1.2 equiv.) in 30 ml dry THF under nitrogen were added EDCI (2.0 g, 10.4 mmol, 1.2 equiv.) in DMF (14 ml), HOBt (1.4 g, 10.4 mmol, 1.2 equiv.) and pyridine (0.77 ml, 10.0 mmol, 1.1 equiv.). After 5 min a solution of amino alcohol **11** (2.215 g, 8.7 mmol) in dry CH_2Cl_2 (15 ml) was added. After 2.5 h EtOAc (100 ml) was added and the reaction mixture washed successively with water (2 × 50 ml), aq. dilute HCl (1 × 50 ml) and then saturated aq. NaHCO$_3$ (1 × 50 ml). The combined organic solvents were dried (MgSO$_4$) and evaporated *in vacuo* to yield **21b** as a slightly yellow gel (4.2 g, 87%); δ_H(270 MHz; $CDCl_3$) 8.30 (1H, m, NH), 8.12 (1H, m, NH), 7.23 (5H, s, Ph), 5.24 (2H, m, OCH$_2$), 4.93 (1H, m, αCH), 4.57 (1H, m, αCH), 4.26 (1H, m, αCH), 3.80 (3H, s, OCH$_3$), 3.70 (3H, s, OCH$_3$), 3.01 (2H, m, Asp βCH$_2$), 2.15 (3H, s, CH$_3$), 1.86 (2H, m, CH$_2$), 1.72 (2H, m, CH$_2$), 1.54 (2H, m, CH$_2$).

A solution of benzyl-protected ester **21b** (4.2 g, 9.0 mmol) in HPLC-grade CH$_3$OH (150 ml) and $CHCl_3$ (10 ml) was stirred under an atmosphere of hydrogen gas with 10% Pd/C (100 mg).

After 36 h the reaction mixture was filtered through Celite and the solvent removed *in vacuo* to yield the carboxylic acid as a slightly yellow oil. This was dissolved in a 50 : 50 mix of CH_2Cl_2–diethyl ether (10 ml), and methylated using freshly prepared diazomethane.[31] Residual diazomethane was removed by adding a drop of acetic acid, and removal of solvent *in vacuo* yielded crude **24b** as a yellow oil. Purification by flash chromatography (15% CH_3CN–85% EtOAc, R_f 0.10) yielded **24b** as a slightly yellow oil (0.960 g, 27%); δ_H(300 MHz; $CDCl_3$) 7.00 (1H, d, *J* 7.7, NH), 6.72 (1H, m, NH), 4.83 (1H, m, αCH), 4.52 (1H, m, αCH), 4.18 (1H, m, αCH), 3.79 (3H, s, OCH_3), 3.77 (3H, s, OCH_3), 3.75 (3H, s, OCH_3), 3.17 (1H, br s, OH), 2.97 (1H, dd, *J* 15.6, *J* 4.4, Asp βCH), 2.79 (1H, dd, *J* 15.6, 4.4, Asp βCH), 2.05 (3H, s, CH_3), 1.84 (2H, m, CH_2), 1.68 (2H, m, CH_2), 1.47 (2H, m, CH_2); δ_C(75.45 MHz; $CDCl_3$), 175.2 (CO_2), 172.6 (CO_2), 171.5 (CO_2), 170.4 (CONH), 170.2 (CONH), 70.0 (εCH), 52.7 (OCH_3), 52.5 (OCH_3), 52.0 (OCH_3), 52.0 (αCH), 49.0 (αCH), 37.3 (CH_2CO), 33.4 (CH_2), 31.6 (CH_2), 23.0 (CH_3CO), 20.8 (γCH_2); ν_{max}(KBr)/cm^{-1} 3362, 3066.4, 2955.8, 2866.9, 1736.2, 1660.1; *m*/*z* (EI) 391.2 (MH^+, 28%); *m*/*z* (CI) 391 (MH^+) [Calc. for $C_{16}H_{27}N_2O_9$ (MH^+), 391.1717. Found: *m*/*z*, 391.1724].

Fmoc-Ala-Ala-(ε-DL-hydroxy)AP(OCH_3)-OCH_3 27b

Fmoc-Ala-Ala-OH **17b** (1.19 g, 3.12 mmol), EDCI (660 mg, 3.43 mmol) and HOBt (452 mg, 3.43 mmol) were dried under high vacuum for 90 min before being dissolved in anhydrous THF (20 ml). Pyridine (280 µl, 3.43 mmol) was added to the solution, which was stirred at RT for 15 min during which time a white suspension formed. The amino alcohol hydrochloride **11** (400 mg, 1.46 mmol) was added as a solution in anhydrous CH_2Cl_2 (10 ml) and the reaction mixture stirred at RT for a further 16 h. Solvent was removed *in vacuo* and water (150 ml) was added. The mixture was extracted with EtOAc (3 × 50 ml) and CH_2Cl_2 (2 × 50 ml). The combined organic extracts were dried ($MgSO_4$), and evaporated *in vacuo*. The crude product was purified by flash chromatography (10% CH_3CN–90% EtOAc, R_f 0.38) which yielded **27b** as a fluffy colourless solid (350 mg, 39%); ν_{max}(KBr)/cm^{-1} 3065, 2953, 1735, 1650, 1531; δ_H(270 MHz; $CDCl_3$, *two* diastereomers) 7.4 (8H, m, Ph), 6.2 (1H, d, *J* 6.2, NH), 5.9 (2H, m, OCH_2), 4.55 (2H, m, 2 × C*H*CH_3), 4.13 (2H, m, αCH + εCH), 3.68 (3H, s, OCH_3), 3.66 (3H, s, OCH_3), 1.70 (2H, m, CH_2), 1.38 (6H, m, 2 × CHC*H*$_3$), 1.45 (2H, m, CH_2), 1.36 (2H, m, CH_2); δ_C(75.45 MHz; $CDCl_3$) 175.1 (CO_2CH_3), 172.5 (CO_2CH_3), 172.4 (CONH), 172.3 (CONH), 156.2 (OCONH), 143.7 (Ph), 141.1 (Ph), 127.6 (Ph), 126.9 (Ph), 125.0 (Ph), 119.8 (Ph), 70.1 (OCH_2), 66.9 (Fmoc CH), 52.2 (OCH_3), 52.0 (OCH_3), 50.4 (εCH), 48.8 (αCH), 46.9 (2 × Ala αCH), 33.4 (δCH_2), 31.4 (βCH_2), 20.6 (γCH_2), 18.8 (Ala CH_3), 17.9 (Ala CH_3); *m*/*z* (ES^+) 622 [$(M + K)^+$, 15%], 606 [$(M + Na)^+$,70], 584 [$(MH)^+$, 51].

Ac-Phe-(ε-keto)AP(OCH_3)-OCH_3 19

To a stirred solution of α-hydroxy ester **18** (70 mg, 170 µmol) in dry CH_2Cl_2 (2 ml) was added Dess–Martin periodinane (72 mg, 170 µmol). After 90 min the solution was added to saturated aq. $NaHCO_3$ (5 ml) containing $Na_2S_2O_3$ (0.5 g). The mixture was stirred vigorously for 5 min and then extracted into CH_2Cl_2 (3 × 15 ml). The dried ($MgSO_4$) extracts were evaporated *in vacuo*, and the residue was purified by flash chromatography (10% CH_3CN in EtOAc, R_f 0.28) to afford **19** as an oil (60 mg, 87%): $[\alpha]_D^{24}$ +1.48 (*c* 6.10, CH_2Cl_2); ν_{max}(KBr)/cm^{-1} 3583.1, 3282.5, 3061.7, 2362.2, 1732.3, 1648.7, 1542.5, 1437.6, 1373.4, 1261.0, 1042.6, 746.1; δ_H(300.40 MHz; $CDCl_3$) 7.25–7.13 (5H, m, Ph), 6.40 (1H, d, *J* 7.7, NH), 6.10 (1H, d, *J* 7.9, NH), 4.70 (1H, m, αCH), 4.48 (1H, m, αCH), 3.79 (3H, s, OCH_3), 3.64 (3H, s, OCH_3), 3.06 (2H, d, *J* 7.0, Phe βCH_2), 2.85 (2H, t, *J* 7.1, δCH_2), 1.92 (3H, s), 1.84 (1H, m, βCH), 1.66 (1H, m, βCH), 1.60 (2H, m, γCH_2); δ_C(75.45 MHz; $CDCl_3$), 194.0 (CO), 173.0 (CO_2), 171.9 (CO_2), 171.0 (CONH), 152.0, 136.4 (Ph), 129.4 (Ph), 128.8 (Ph), 127.2 (Ph), 54.5 (OCH_3), 53.1 (OCH_3), 52.6 (αCH), 52.0 (αCH), 38.6 (δCH_2), 38.2 (*C*H_2Ph), 31.4 (CH_2), 23.3 (CH_2), 18.5 (γCH_2); *m*/*z* (EI) 406 (M^+, 5%); (CI) 407 (MH^+, 90%) (Calc. for $C_{20}H_{26}N_2O_7$: C, 59.10; H, 6.45; N, 6.89. Found: C, 58.73; H, 6.68; N, 6.64%).

Ac-Asp(OBn)-(ε-keto)AP(OCH_3)-OCH_3 22a

To a stirred solution of Ac-Asp(OBn)-(ε-DL-hydroxy)-AP(OCH_3)-OCH_3 **21a** (99.1 mg, 212.7 µmol) in dry CH_2Cl_2 (3 ml) was added Dess–Martin periodinane (90.2 mg, 212.7 µmol). After 3 h the solution was added to saturated aq. $NaHCO_3$ (5 ml) containing $Na_2S_2O_3$ (0.5 g). The mixture was stirred vigorously for 5 min and then extracted into CH_2Cl_2 (3 × 20 ml). The dried ($MgSO_4$) organic extracts were evaporated *in vacuo*, and the residue was purified by flash chromatography (10% CH_3CN in EtOAc, R_f 0.33) to afford compound **22a** as an oil (31 mg, 67%); $[\alpha]_D^{24}$ −1.36 (*c* 2.95, CH_2Cl_2); ν_{max}(KBr)/cm^{-1} 3307, 3064, 2954, 1731, 1658, 1537, 1437, 1377, 1262, 1173, 1047; δ_H(300.40 MHz; $CDCl_3$) 7.38 (5H, m, Ph), 7.13 (1H, d, *J* 8.10, NH), 6.82 (1H, d, *J* 7.9, NH), 5.18 (1H, d, *J* 12.3, Bn CH), 5.12 (1H, d, *J* 12.3, Bn CH), 4.87 (1H, m, αCH), 4.53 (1H, m, αCH), 3.87 (3H, s, OCH_3), 3.73 (3H, s, OCH_3), 3.02 (1H, dd, *J* 4.3, 17.1, Asp βCH), 2.87 (2H, t, *J* 7.2, δCH_2), 2.69 (1H, dd, *J* 6.8, 17.0, Asp βCH), 2.04 (3H, s, CH_3), 1.88 (2H, m, CH_2), 1.65 (4H, m, 2 × CH_2); δ_C(75.45 MHz; $CDCl_3$) 193.3 (CO), 172.0 (CO_2), 171.9 (CO_2), 170.4 (CONH), 170.3 (CONH), 161.2 (CO_2), 135.3 (Ph), 128.6 (Ph), 128.4 (Ph), 128.2 (Ph), 67.0 (OCH_2), 54.0 (OCH_3), 53.0 (OCH_3), 52.0 (αCH), 49.0 (αCH), 38.4 (CH_2CO), 35.7 (CH_2CO), 31.0 (βCH_2), 23.1 (γCH_2), 18.4 (CH_3); *m*/*z* (EI) 464 (MH^+, 78%).

Ac-Asp(OCH_3)-(ε-keto)AP(OCH_3)-OCH_3 25a

A solution of Ac-Asp(OCH_3)-(ε-DL-hydroxy)AP(OCH_3)-OCH_3 **24a** (316.3 mg, 811 µmol) in anhydrous CH_2Cl_2 (10 ml) was stirred at RT under dry N_2. Dess–Martin periodinane (413 mg, 1.2 equiv.) was added and the reaction mixture stirred for 1 h. A further 50 mg of periodinane was added and stirring was continued for 20 min. After this time the mixture was poured into a vigorously stirred solution of $Na_2S_2O_3$ in 1 M aq. $NaHCO_3$ (20 ml). After 10 min the mixture was extracted into CH_2Cl_2 (4 × 25 ml), and the organic extracts were combined, dried ($MgSO_4$) and evaporated. The residue was purified by flash chromatography (15% CH_3CN in EtOAc, R_f 0.65), yielding compound **25a** as a colourless solid (132.2 mg, 42%); mp 93–102 °C; $[\alpha]_D^{24}$ −4.2 (*c* 2.1, CH_2Cl_2); δ_H(300 MHz; $CDCl_3$) 7.17 (1H, d, *J* 8.1, AP NH), 6.91 (1H, d, *J* 8.1, Asp NH), 4.86 (1H, ddd, *J* 4.2, 6.8, 11.0, Asp αCH), 4.54 (1H, m, AP αCH), 3.87 (3H, s, OCH_3), 3.74 (3H, s, OCH_3), 3.72 (3H, s, OCH_3), 2.98 (1H, dd, *J* 4.0, 17.0, Asp βCH), 2.89 (2H, t, *J* 6.8, CH_2CO), 2.65 (1H, dd, *J* 6.8, 17.1, Asp βCH), 2.25 (1H, m, AP βCH), 2.07 (3H, s, CH_3), 1.90 (1H, m, AP βCH), 1.68 (2H, m, γCH_2); δ_C(75.45 MHz; $CDCl_3$) 193.4 (CO), 172.6 (CO_2), 171.9 (CO_2), 170.5 (CO_2), 170.4 (CONH), 161.2 (CONH), 53.0 (OCH_3), 52.6 (OCH_3), 52.2 (OCH_3), 52.0 (αCH), 49.2 (αCH), 38.5 (CH_2), 35.5 (CH_2), 31.1 (CH_2), 23.2 (CH_3CO), 18.5 (CH_2); ν_{max} (KBr)/cm^{-1} 3387, 2960, 1731, 1656, 1536, 1440, 1374, 1280; *m*/*z* (EI) 389 (MH^+, 0.2%), 388 (M^+, 0.2); *m*/*z* (CI) 389 (MH^+, 50%), 357 ($MH^+ - CH_3OH$, 22) (Calc. for $C_{16}H_{24}N_2O_9$: *M*, 388.1482. Found: M^+, 388.1494) (Calc. for $C_{16}H_{24}N_2O_9$: C, 49.48; H, 6.23; N, 7.21. Found: C, 48.96; H, 6.11; N, 7.03%).

Ac-Asp([ε-keto]AP[OCH_3]-OCH_3)-OCH_3 25b

To a stirred solution of Ac-Asp([ε-DL-hydroxy]AP[OCH_3]-OCH_3)-OCH_3 **24b** (720 mg, 1.9 mmol) in dry CH_2Cl_2 (20 ml) was added Dess–Martin periodinane (1.22 g, 2.9 mmol, 1.6 equiv.). After one hour the reaction mixture was poured onto aq. sodium thiosulfate (10 g) in water (100 ml) and stirred

vigorously for 10 min, before extraction into CH_2Cl_2 (3 × 50 ml). The combined organic extracts were dried ($MgSO_4$) and the solvent removed *in vacuo* to yield crude **25b** (0.593 g) as a yellow oil. Purification by flash chromatography (15% CH_3CN–85% EtOAc, R_f 0.26) yielded compound **26b** as a slightly yellow oil (207 mg, 29%); δ_H(300 MHz; $CDCl_3$) 6.84 (1H, d, *J* 8.2, NH), 6.37 (1H, m, NH), 4.84 (1H, m, αCH), 4.55 (1H, m, αCH), 4.10 (3H, s, OCH_3), 3.93 (3H, s, OCH_3), 3.75 (3H, s, OCH_3), 2.87 (2H, m, Asp βCH_2), 2.68 (2H, m, $COCH_2$), 2.18 (3H, s, CH_3CO), 1.86 (2H, m, CH_2), 1.66 (2H, m, CH_2); δ_C(75.45 MHz; $CDCl_3$) 200.0 (CO), 175.3 (CO_2), 170.6 (CO_2), 168.3 (CO_2), 160.2 (CONH), 157.4 (CONH), 53.8 (CH_2CO), 52.8 (OCH_3), 52.6 (OCH_3), 52.4 (OCH_3), 51.2 (αCH), 49.6 (αCH), 46.2 (CH_2), 30.1 (CH_2), 25.3 (CH_2), 23.2 (CH_3); *m/z* (EI) 389 (MH^+, 15%), 329 ($M^+ - CO_2CH_3$, 12); *m/z* (CI) 389 (MH^+, 100%), 371 ($MH^+ - H_2O$), 405 ($M^+ + NH_3$) (Calc. for $C_{16}H_{25}N_2O_9$ *M*, 389.1560. Found: M, 389.1570).

Fmoc-Ala-Ala-(ε-keto)AP(OCH_3)-OCH_3 28

Dess–Martin periodinane (1.077 g, 2.54 mmol) was added slowly to a stirred solution of Fmoc-Ala-Ala-(ε-DL-hydroxy)-AP(OCH_3)-OCH_3 **27b** (271 mg, 465 μmol) in anhydrous CH_2Cl_2 (10 ml) at RT. After 40 min a further portion of oxidising agent (20 mg, 46 μmol) was added and the mixture was stirred for a further 20 min. The reaction mixture was added to aq. $NaHCO_3$ (150 ml) and extracted with CH_2Cl_2 (4 × 60 ml). The organic extracts were combined, dried ($MgSO_4$) and concentrated *in vacuo*. Purification by flash chromatography (5% CH_3CN–95% EtOAc, R_f 0.34) yielded compound **28** as a colourless foam (155 mg, 57.4%); mp 162.5–163.5 °C; $[\alpha]_D^{24}$ −2.45 (*c* 13.8, CH_2Cl_2); ν_{max}(KBr)/cm^{-1} 2956, 1734, 1647, 1531; δ_H(270 MHz; $CDCl_3$) 7.40 (8H, m, Ph), 6.70 (1H, d, *J* 6.7, NH), 6.55 (1H, d, *J* 6.2, NH), 5.25 (1H, br s, NH), 4.55 (1H, m, αCH), 4.45 (2H, m, OCH_2), 4.15 (2H, m, 2 × Ala αCH), 3.82 (3H, s, OCH_3), 3.73 (3H, s, OCH_3), 2.82 (2H, m, δCH_2), 1.85 (1H, m, βCH), 1.60 (3H, m, βCH + γCH_2), 1.40 (6H, m, 2 × Ala CH_3); δ_C(75.45 MHz; $CDCl_3$) 193.3 (CO), 172.5 (CO_2), 172.3 (CO_2), 161.1 (2 × CONH), 157.0 (OCONH), 143.8 (Ph), 141.2 (Ph), 127.7 (Ph), 127.1 (Ph), 125.1 (Ph), 120.0 (Ph), 67.1 (OCH_2), 53.1 (Fmoc CH), 52.9 (OCH_3), 52.5 (OCH_3), 51.8 (αCH), 50.5 (αCH), 48.9 (αCH), 47.0 (δCH_2), 38.5 (βCH_2), 31.2 (γCH_2), 19.1 (Ala CH_3), 18.5 (Ala CH_3); *m/z* (FAB) 582 (MH^+, 20%), 604 $(MNa)^+$, 35].

Di-[Ac-Phe-(ε-keto)AP(OLi)-OLi] pentahydrate 20

To a stirred solution of oxo diester **19** (31.7 mg, 78.1 μmol) in 1:1 CH_3CN–H_2O (1 ml) was added 2 equiv. LiOH·H_2O (6.55 mg, 156.2 μmol). After 60 min the solvent was removed *in vacuo*, and the residue was dissolved in water (1 ml). The solution was freeze-dried to afford the salt **20** as a yellow powder (28.2 mg, 93%); mp 230 °C (decomp.); ν_{max}(KBr)/cm^{-1} 3387, 2933, 1639, 1417, 1122, 1031; δ_H(300.4 MHz; D_2O) 7.35 (5H, m, Ph), 4.61 (1H, m, αCH), 4.13 (1H, m, αCH), 3.18–3.08 (2H, m, Phe βCH_2), 1.88 (3H, s, CH_3), 1.78 (4H, m, β + γCH_2); δ_C(75.45 MHz; D_2O) 178.9 (CO), 174.8 (CO_2), 173.1 (CO_2 + CONH), 171.0 (CONH), 137.5 (Ph), 130.0 (Ph), 129.5 (Ph), 127.9 (Ph), 55.8 (2 × αCH), 38.0 (m, δCD_2 solvent exchanged), 37.8 (Phe βCH_2), 32.0 (βCH_2), 22.5 (γCH_2), 20.0 (CH_3); *m/z* (FAB^+) 385 [(M − Li + H)H^+, 20%], 401 [(M − 2Li + 2H)Na^+, 40] (Calc. for $C_{36}H_{40}Li_4N_4O_{14}$·$5H_2O$: C, 49.67; H, 5.79; N, 6.44. Found: C, 49.50; H, 6.00; N, 6.17%).

Ac-Asp(OLi)-(ε-keto)AP(OLi)-OLi hexahydrate 26a

A solution of Ac-Asp(OCH_3)-(ε-keto)AP(OCH_3)-OCH_3 **25a** (132.2 mg, 355 μmol) in CH_3CN (1 ml) and H_2O (1 ml) was stirred at room temperature. Solid LiOH·H_2O (42.9 mg, 1.06 mmol, 3.0 equiv.) was added and allowed to dissolve slowly. After 90 min all traces of solid had disappeared and the solvent was removed *in vacuo*. The resulting yellow solid was dissolved in deionised water (2 ml) and freeze-dried to afford the title compound as a crisp yellow solid (105 mg, 64.8%); mp > 250 °C (decomp); $[\alpha]_D^{24}$ +4.7 (*c* 2.0, H_2O); δ_H(300 MHz; D_2O) 4.44 (1H, dd, *J* 4.5, 9.5, Asp αCH), 3.97 (1H, m, AP αCH), 2.65 (1H, m, Asp βCH), 2.48 (1H, m, Asp βCH), 1.45 (4H, m, AP βCH_2 + γCH_2); δ_C(75.45 MHz; D_2O) 208.0 (CO), 179.8 (CO_2), 178.7 (CO_2), 178.3 (CO_2), 174.2 (CONH), 173.1 (CONH), 56.0 (αCH), 52.9 (αCH), 39.1 (Asp βCH_2), 31.8 (AP βCH_2), 22.8 (γCH_2), 20.0 (CH_3); ν_{max}(KBr)/cm^{-1} 3421, 1603, 1413; *m/z* (FAB) 345 [(M − 3Li + 3H)$^+$, 1%], 389 [(M − 3Li + H + 2Na)$^+$, 1.5] (Calc. for $C_{13}H_{15}Li_3N_2O_8$·$6H_2O$: C, 34.23; H, 5.97; N, 6.14. Found: C, 34.23; H, 5.52; N, 5.85%).

Ac-Asp([ε-keto]AP[OLi]-OLi)-OLi tetrahydrate 26b

A solution of Ac-Asp([ε-keto]AP[OCH_3]-OCH_3)-OCH_3 **25b** (207.0 mg, 0.7 mmol) in 1:1 CH_3CN–water (4 ml) was stirred with 3.0 mol equiv. of LiOH·H_2O (88 mg, 2.1 mmol). After 90 min the solvent was removed *in vacuo* and the resulting product was re-dissolved in water (1 ml) and freeze dried, to yield trilithium salt **26b** as a crisp yellow solid (169 mg, 87%); mp > 220 °C (decomp.); ν_{max}(KBr)/cm^{-1} 3420, 2946, 1617, 1420; δ_H(300 MHz; D_2O–CD_3CN) 4.55 (1H, m, αCH), 4.26 (1H, m, αCH), 3.26 (2H, m, Asp βCH_2), 2.05 (3H, s, CH_3), 1.94 (2H, m, βCH_2), 1.69 (2H, m, γCH_2); δ_C (75.5 MHz; D_2O–CD_3CN) 198.5 (CO), 183.0 (CO_2), 181.4 (CO_2), 178.8 (CON), 176.5 (CON), 58.7 (αCH), 55.7 (αCH), 38.6 (Asp βCH_2), 34.7 (AP βCH_2), 24.3 (AP γCH_2), 22.2 (CH_3); *m/z* (ES^+, D_2O–H_2O) 347 [(M − 3Li + 3H)H^+, 5%], 348 [(M − 3Li + 2H + D)H^+, 8], 352 [(M − 2L + 2H)$^+$, 9], 353 [(M − 2Li + 2H)H^+, 45], 354 [(M − 2Li + H + D)H^+, 52], 355 [(M − 2Li + 2D)H^+, 40], 356 [(M − 2Li + 2D)D^+, 20], 359 [(M − Li + H)H^+, 38], 360 [(M − Li + D)H^+], 361 [(M − Li + D)D^+], 362 [(M − Li− − H + 2D)D^+], 364 [(M)$^+$, 80], 365 [(M)H^+, 100], 366 [(M)D^+, 50], 367 [(M − H + D)D^+, 45], 368 [(M − 2H + 2D)D^+, 22], 369 [(M − 3H + 3D)D^+, 20], 370 [(M − 4H + 4D)D^+, 22], 371 [(M)Li^+, 28], 372 [(M − H + D)Li^+, 34], 373 [(M − 2H + 2D)Li^+, 35], 374 [(M − 3H + 3D)Li^+, 15], 375 [(M − 4H + 4D)Li^+, 8] (Calc. for $C_{13}H_{15}Li_3N_2O_9$·$4H_2O$: C, 35.80; H, 5.33. Found: C, 35.88; H, 5.19%).

Ac-Asp(OLi)-(ε-DL-hydroxy)AP(OLi)-OLi 23a

A stirred suspension of **22a** (31 mg, 66.8 μmol), and 10% Pd/C (5 mg) in dry CH_3OH (1 ml) was stirred under 1 atm H_2. After 2.5 h 10% Pd/C (5 mg) was added and hydrogenation was continued. After a further 24 h 10% Pd/C (5 mg) and dry CH_3OH (1 ml) were added. After a further 24 h the mixture was filtered through methanol-washed Celite, and eluted with further CH_3OH. The filtrate was evaporated *in vacuo* to yield an oily residue (31 mg, 66.8 μmol).

To a stirred solution of the oily residue (31 mg, 66.8 mmol) in 1:1 CH_3CN–water (1 ml) was added LiOH·H_2O (8.4 mg, 200 μmol). After 60 min the solvent was removed *in vacuo*, and the residue was dissolved in water (1 ml). The solution was freeze-dried to afford a yellow powder (22.6 mg, 92.4%) which proved to be the ε-alcohol **23a**; mp 247–250 °C; ν_{max}(KBr)/cm^{-1} 3854, 3423, 2361, 1594, 1419, 1320, 1120, 668; δ_H(300.40 MHz; D_2O) 4.60 (1H, dd, *J* 9.7, 5.3, αCH), 4.14 (1H, dd, *J* 7.9, 4.8, αCH), 3.99 (1H, dd, *J* 7.7, 3.8, εCH), 2.73 (1H, dd, *J* 4.4, 16.0, Asp βCH), 2.50 (1H, dd, *J* 5.2, 16.0, Asp βCH), 2.13 (3H, s), 1.71 (4H, m, AP β- and δ-CH_2), 1.38 (2H, m, AP γCH_2); δ_C(75.5 MHz; D_2O) 182.4 (CO_2), 179.7 (CO_2), 178.7 (CO_2), 175.0 (CONH), 173.7 (CONH), 72.9 (εCH), 56.1 (αCH), 52.7 (αCH), 39.5 (Asp βCH_2), 34.6 (δCH_2), 32.7 (βCH_2), 22.6 (γCH_2), 22.0 (CH_3); *m/z* (ES^+, H_2O) 349 [(M − 3Li + 3H)H^+, 10%], 355 [(M − 2Li + 2H)H^+, 12] 361 (M − Li + H)H^+, 15], 367 [(M)H^+, 8], 371 [(M)Li^+, 28]; *m/z* (ES^-, H_2O) 347 [(M − 3L + 2H)$^-$, 100], 353 [(M − 2Li + H)$^-$, 50], 359 [(M − Li)$^-$, 12], 375 [(M − 2Li + Na)$^-$, 8].

H-Ala-Ala-(ε-keto)AP(OLi)-OLi 30

Fmoc-Ala-Ala-(ε-keto)AP(OCH_3)-OCH_3 **28** (230.4 mg, 395 μmol) was suspended in a mixture of THF (1.0 ml) and deionised water (1.0 ml). $LiOH{\cdot}H_2O$ (≈3 equiv.) was added in portions until HPLC analysis (method **1**) revealed full methyl ester deprotection (t_R dimethyl ester 30.9 min; t_R monomethyl esters 27.1 min and 28.3 min; t_R diacid 25.9 min). $ESMS^+$ analysis of the mixture indicated some additional Fmoc cleavage. Removal of solvent was achieved *in vacuo* and the residue was dissolved in a mixture of *N*-methylmorpholine (5 ml) and water (5 ml). After stirring of the mixture at RT for 24 h HPLC analysis (method 1) and $ESMS^+$ analysis revealed full Fmoc deprotection. Again, solvent was removed *in vacuo*. Final purification was carried out by HPLC (method 2). The title compound was eluted at 0–3 min. Product containing fractions were combined, concentrated *in vacuo* and lyophilised to afford a pale yellow solid (45 mg, 33%); mp > 210 °C; $[\alpha]_D^{24}$ −2.4 (*c* 1.0, H_2O); δ_H(300 MHz; D_2O) 4.64 (1H, m, αCH), 4.20 (1H, m, αCH), 3.90 (1H, m, αCH), 1.65 (4H, m, 2 × CH_2), 1.36 (3H, d, *J* 7.0, CH_2), 1.23 (3H, d, *J* 7.2, CH_2); δ_C(75.45 MHz; D_2O) 190.1 (CO), 176.4 (CO_2), 175.5 (CO_2), 171.3 (CONH), 164.1 (CONH), 53.6 (αCH), 50.6 (αCH), 49.7 (αCH), 30.7 (CH_2), 19.7 (CH_2), 17.3 (CH_3), 17.1 (CH_3); *m/z* (ES^-) 330 [$(M - 2Li + H)^-$, 20%], 336 [$(M - Li)^-$, 25], 352 [$(M - 2Li + Na)^-$, 60]; HPLC (method 1) t_R 4.0 min.

Piperidinyl enamine 31

The enamine was isolated after treatment of **29a** and **29b** with piperidine in water and acetonitrile by HPLC (method 2, t_R 10.0 min), selected data: δ_H(300.4 MHz; D_2O) 4.25 (2H, m, 2 × αCH), 3.94 (1H, dd, *J* 7.2, 13.8, αCH), 3.00 (4H, 2 × CH_2N), 1.62 (3H, m, βCH_2 + γCH), 1.51 (1H, m, γCH), 1.39 (6H, m, 2 × CH_3), 1.25 (6H, m, 3 × CH_2); *m/z* (ES^-) 419.6 [$(M - 2H + Na)^-$, 8%].

Ac-Phe-(ε-DL-hydrazino)AP(OH)-OH 6

To a solution of di-[Ac-Phe-(ε-keto)AP(OLi)-OLi] pentahydrate **20** (61.4 mg, 141 μmol) in HPLC-grade CH_3OH (2 ml) was added hydrazine hydrate (70 μl, 72.1 mg, 1.44 mmol). The solution was acidified to pH 5.0 by the careful addition of CF_3CO_2H and after 30 min $NaCNBH_3$ (89.4 mg, 1.42 mmol) was added. The mixture was stirred at RT overnight, then acidified by the addition of conc. aq. HCl. Solvent was removed *in vacuo* and the solid residue was dissolved in deionised water (1 ml). The solution was applied to the H^+-form of a column of Dowex AG50 WX-8 cation-exchange resin (2 ml bed volume) and eluted with deionised water until the washings were neutral. The desired product was eluted with 1 M NH_3 in deionised water. Removal of solvent afforded the product as a glassy semi-solid (52.1 mg, 93.8%); δ_H(300 MHz; D_2O) 7.25 (5H, m, Ph), 4.60 (1H, m, αCH), 4.15 (1H, m, αCH), 3.55 (1H, m, αCH), 3.20 (1H, m, Phe βCH), 2.95 (1H, m, Phe βCH), 2.20 (1H, m, AP βCH), 1.95 (3H, s, CH_3), 1.90–1.20 (5H, m, AP γ + δCH_2 + βCH); *m/z* (ES^+, H_2O–D_2O) 394 (M^+, 5%), 395 [$(M - H + D)^+$, 80], 396 [$(M - 2H + 2D)^+$, 75], 397 [$(M - 3H + 3D)^+$, 65], 398 [$(M - 4H + 4D)^+$, 33], 399 [$(M - 5H + 5D)^+$, 8].

Ac-Asp(OH)-(ε-DL-hydrazino)AP(OH)-OH 5a

Ac-Asp(OLi)-(ε-keto)AP(OLi)-OLi hexahydrate **26a** (35.8 mg, 83.3 μmol) was dissolved in HPLC-grade CH_3OH (1.5 ml). Hydrazine hydrate (820 μmol, 41 mg, 40 μl) was added and the mixture adjusted to pH 5 by the judicious addition of CF_3CO_2H. After 30 min $NaCNBH_3$ (64 mg, 1.0 mmol) was added and the mixture stirred overnight before being acidified by the addition of aq. HCl (1 M; 1.0 ml) to destroy excess of cyanoborohydride. Removal of solvent *in vacuo* afforded a white solid, which was dissolved in deionised water (1 ml). This was applied to the H^+-form of a column of Dowex AG50 WX-8 cation-exchange resin (2 ml bed volume) and eluted with deionised water until the washings were neutral. The desired product was eluted with 1 M NH_3 in deionised water. Removal of solvent afforded the title product as a glassy semi-solid (28.0 mg, 92.8%); δ_H(300 MHz; D_2O) 4.28 (1H, dd, *J* 4.2, 8.8, Asp αCH), 4.10 (1H, m, αCH), 3.48 (1H, m, αCH), 2.60 (2H, m, Asp βCH_2), 2.18 (1H, m, AP βCH), 2.00 (3H, s, CH_3), 1.85–1.35 (5H, m, γ- + δ-CH_2 + AP βCH); *m/z* (ES^+, H_2O–D_2O) 362 (M^+, 20%), 363 [$(M - H + D)^+$, 50], 364 [$(M - 2H + 2D)^+$, 85], 365 [$(M - 3H + 3D)^+$, 100], 366 [$(M - 4H + 4D)^+$, 84], 367 [$(M - 5H + 5D)^+$, 50], 368 [$(M - 6H + 6D)^+$, 25], 369 [$(M - 7H + 7D)^+$, 5].

Ac-Asp([ε-DL-hydrazino]AP[OH]-OH)-OH 5b

To a stirred solution of Ac-Asp([ε-keto]AP[OLi]-OLi)-OLi **26b** (75.2 mg, 210 μmol) in HPLC-grade CH_3OH (3 ml) was added hydrazine monohydrate (100 μl, 103 mg, 2.1 mmol, 10 equiv.). The pH was adjusted to pH 5.5 with TFA. CH_3OH (0.5 ml), CH_3CN (0.5 ml) and water (0.5 ml) were added to solubilise the hydrazone. After 15 min $NaCNBH_3$ was added (130 mg, 2.1 mmol, 10 equiv.). After 5 h the reaction mixture was acidified (conc. HCl) and passed down the H^+-form of a Dowex AG50 column. The column was washed with deionised water until neutral before addition of 1 M aq. ammonia. The basic fractions were collected and the solvent removed *in vacuo* to yield compound **5b** as a glassy semi-solid (43 mg, 55%); δ_H(400 MHz; D_2O–CD_3CN) 4.50 (1H, m, αCH), 4.18 (1H, m, αCH), 3.43 (1H, m, αCH), 2.83 (2H, m, Asp βCH_2), 2.10 (3H, s, CH_3), 1.85 (2H, m, CH_2), 2.75 (2H, m, CH_2), 1.64 (2H, m, CH_2); δ_C(100 MHz; D_2O–CD_3CN) 180.6 (CO_2H), 178.2 (CO_2H), 177.5 (CO_2H), 175.2 (CON), 173.8 (CON), 67.7 (αCH), 65.1 (αCH), 56.7 (εCH), 39.6 (CH_2), 24.9 (CH_2), 23.2 (CH_2), 20.1 (CH_2), 19.0 (CH_3); *m/z* (ES^+, H_2O–D_2O) 362 (M^+, 3.8%), 363 [$(M - H + D)^+$, 11.2], 364 [$(M - 2H + 2D)^+$, 15.7], 365 [$(M - 3H + 3D)^+$, 20.3], 366 [$(M - 4H + 4D)^+$, 20.0], 367 [$(M - 5H + 5D)^+$, 14.7], 368 [$(M - 6H + 6D)^+$, 9.6], 369 [$(M - 7H + 7D)^+$, 3.8], 370 [$(M - 8H + 8D)^+$, 1.5].

H-Ala-Ala-(ε-DL-hydrazino)AP(OH)-OH 7b

To a stirred solution of H-Ala-Ala-(ε-keto)AP(OLi)-OLi **30** (37.7 mg, 110 μmol) in a mixture of HPLC-grade CH_3OH (1 ml) and water (100 μl) was added hydrazine monohydrate (100 μl, 103 mg, 2.1 mmol). The pH was adjusted to pH 5.5 with TFA. After 15 min $NaCNBH_3$ was added (65 mg, 1.0 mmol). After 20 h the reaction mixture was acidified (conc. HCl) and passed down the H^+-form of a Dowex AG50 column. The column was washed with deionised water until neutral before addition of 1 M aq. ammonia. The basic fractions were collected and the solvent removed *in vacuo* to yield **7b** as a colourless foam (34.0 mg, 89.1%); mp >200°C; δ_H(300 MHz; D_2O) 4.25 (1H, m, αCH), 4.05 (1H, m, αCH), 3.85 (2H, m, 2 × αCH), 1.65 (4H, m, βCH_2 + δCH_2), 1.35 (8H, m, γCH_2 + 2 × CH_3); δ_C(75.45 MHz; D_2O) 179.4 (CO_2), 174.7 (CO_2), 174.6 (CONH), 174.6 (CONH), 55.76 (αCH), 50.6 (αCH), 50.52 (αCH), 50.0 (αCH), 32.1 (CH_2), 22.9 (CH_2), 18.6 (CH_2), 17.3 (CH_3), 17.2 (CH_3); *m/z* (ES^-, before ion exchange) 352.4 [$(M - 2H + Li)^-$, 30%], 368.5 [$(M - 2H + Na)^-$, 10], 384.5 [$(M - 2H + K)^-$, 2]; *m/z* (ES^+, before ion exchange) 366.5 [$(M - 2H + 2Li)Li^+$, 3%], 382.5 [$(M - 2H + 2Li)Na^+$, 5], 398.3 [$(M - 2H + 2Li)K^+$, 8]; *m/z* (ES^-, after ion exchange, H_2O–D_2O) 346 [$(M - H)^-$, 20%], 347 [$(M - 2H + D)^-$, 45], 348 [$(M - 3H + 2D)^-$, 40], 349 [$(M - 4H + 3D)^-$, 10], 368 [$(M - 2H + Na)^-$, 25], 369 [$(M - 3H + Na + D)^-$, 55], 370 [$(M - 4H + Na + 2D)^-$, 100], 371 [$(M - 5H + Na + 3D)^-$, 65], 372 [$(M - 6H + Na + 4D)^-$, 48], 373 [$(M - 7H + Na + 5D)^-$, 33]; *m/z* (ES^+ after ion exchange) 348 [$(M)H^+$, 35%], 349 [$(M - H + D)H^+$, 100], 350 [$(M - 2H + 2D)H^+$, 95], 351 [$(M - 3H + 3D)H^+$, 72], 352 [$(M - 4H + 4D)H^+$, 43], 353

[(M − 5H + 5D)H$^+$, 30], 354 [(M − 6H + 6D)H$^+$, 15], 355 [(M − 7H + 7D)H$^+$, 10], 370 [(M)Na$^+$, 22], 371 [(M − H + D)Na$^+$, 53], 372 [(M − 2H + 2D)Na$^+$, 62], 373 [(M − 3H + 3D)Na$^+$, 51], 374 [(M − 4H + 4D)Na$^+$, 40], 375 [(M − 5H + 5D)Na$^+$, 42], 376 [(M − 6H + 6D)Na$^+$, 25].

Enzyme assays

Stock assay solution [100 mM Tris buffer, pH 8.0, containing EDTA tetrasodium salt (0.1 mM), NaN_3 (5 mM), Bovine Serum Albumin (1.0 mg ml^{-1}) and NH_4Cl (100 mM)] was prepared, and used to prepare the working assay solution [pyridoxal phosphate (PLP) 3.0 mg and NADPH 6.0 mg made up to 40 ml with stock assay 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 ΔA_{340} of 20–100 mAU min^{-1}, using 0–20 mM substrate **32**, 10 mM L-glutamate **34**, 10 units 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 s for activity assays, 14 400 s for regeneration assays and over 3600 s for inhibition testing. The cuvette was incubated in a heated water jacket at 37 °C for all assays. Progress-of-inhibition assays contained no free PLP.

Data analysis

Data points (absorption at 340 nm, A_{340}) were collected every 2 s into an Excel database. Rates of reaction were calculated from the initial linear portions of the curves. Michaelis–Menten parameters were calculated from direct fits to the equation Rate = (k_{cat} × [DAP-AT] × [Substrate])/([Substrate] + K_M) using the program MacCurveFit. Absolute concentrations were calculated using the Beer–Lambert Law $A_{340} = 6220 \times$ [NADPH]. For inhibition, progress-of-inhibition curves were fitted directly to the integrated rate equation of Morrison and Walsh[20] using MacCurveFit and parameters were calculated by averaging 4–6 independent runs.

Antimicrobial tests

The dipeptide hydrazines **3, 6, 5a, 5b and 7b** were tested against *E. coli* on both L and M-9 minimal agar. The medium was prepared according to literature procedures,[34] sterilised by autoclaving (25 min, 120 °C) and 25 ml was poured into sterile 9 cm petri dishes. *E. coli* DH5α was grown overnight (37 °C) in L media (3 ml), precipitated and resuspended in minimal medium (3 ml). 50 ml of the resulting suspension was evenly spread onto the surface of each agar plate. Each plate was divided into quarters and in the centre of each quarter was placed a sterile filter disk (5 mm diameter, Whatman no. 1 paper) soaked in 3 µl of the appropriate amount of antibiotic dissolved in sterile, deionised water. The plates were incubated at 37 °C for 16 h (L medium) and 36 h (minimal medium). The inhibition zone was measured as the radius of inhibition minus the radius of the filter disk (2.5 mm).

Acknowledgements

R. J. C. thanks the School of Chemistry, University of Bristol, for financial assistance and the other authors who were undergraduate students at the time of this investigation. The Nuffield Foundation generously made available an Undergraduate Research Bursary (to J. A. S., NUF-URB97). Mr Paul Wang and Mr Simone Tortioli are gratefully thanked for technical assistance.

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