Aminoalkylphosphinate inhibitors of D-Ala-D-Ala adding enzyme

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Pseudo-tri- and -tetra-peptide aminoalkylphosphinic acids of general structure X-Lys-PO_2H-Gly-Ala have been synthesised as transition state analogues for D-Ala-D-Ala adding enzyme. The key synthetic step used to assemble the *C*-terminal dipeptide unit is a modified Arbusov reaction, coupling bromopropionyl-D-alanine methyl ester to a silylated aminoalkylphosphonite. Kinetic assays with the purified *E. coli* enzyme reveal that the phosphinate analogues act as reversible competitive inhibitors, with K_i values in the range 200–700 μM. Extended analogues mimicking the peptide chain of the UDPMurNAc-L-Ala-γ-D-Glu-*m*-DAP substrate show increased binding affinity for the enzyme active site. These are the first reported inhibitors for D-Ala-D-Ala adding enzyme.

Introduction

Enzymes involved in bacterial cell wall peptidoglycan biosynthesis represent good targets for development of new antibacterial agents, which are of increasing importance in the light of increasing bacterial antibiotic resistance.[1] While there are many inhibitors of the later extracellular enzymes (β-lactams, vancomycin, moenomycin), there are relatively few inhibitors of intracellular enzymes.[1]

The cytoplasmic peptidoglycan precursor UDPMurNAc-L-Ala-γ-D-Glu-*m*-DAP-D-Ala-D-Ala is synthesised from UDP-MurNAc by a series of ATP-dependent ligases.[1] The D-Ala-D-Ala dipeptide is synthesised by the ATP-dependent D-alanine:D-alanine ligase,[2] which has been shown to proceed mechanistically *via* an acyl phosphate intermediate.[3] Aminoalkylphosphinate analogues for the ensuing tetrahedral transition state have been synthesised,[4] and were found to act as potent slow-binding inhibitors for this enzyme, although they showed only limited antibacterial activity.[5] The D-Ala-D-Ala dipeptide is then condensed with UDPMurNAc-L-Ala-γ-D-Glu-*m*-DAP **1** by the ATP-dependent D-Ala-D-Ala adding enzyme.[2]

D-Ala-D-Ala adding enzyme has previously been purified to homogeneity from *E. coli* and found to be a 48 kDa monomeric enzyme with a turnover number of 784 min^{-1}.[6] The cloning and sequencing of the *E. coli murF* gene encoding D-Ala-D-Ala adding enzyme has allowed the overexpression of enzyme activity.[7–9] By analogy with D-alanine:D-alanine ligase and other amino acid adding enzymes, we anticipated that the mechanism for this enzymatic reaction would also proceed *via* an acyl phosphate intermediate **2** (Scheme 1). Attack on the acyl phosphate **2** by the amino group of D-Ala-D-Ala proceeds *via* tetrahedral transition state **3**, which would collapse with loss of phosphate to give the product UDPMurNAc-pentapeptide **4**.

We wished to examine whether aminoalkylphosphinate analogues of transition state **3** might act as potent and selective inhibitors for D-Ala-D-Ala adding enzyme. Our initial synthetic target was the tripeptide phosphinate Ac-Lys-PO_2H-D-Ala-D-Ala **5**. This enzyme is known to accept lysine in position 3 of the UDPMurNAc-tripeptide substrate in place of *meso*-diaminopimelic acid.[8] There are very few literature reports of the synthesis of tri- or tetra-peptide phosphinate analogues.[10] We report the development of methodology for the synthesis of a series of X-Lys-PO_2H-Gly-D-Ala analogues, and we report the properties of these analogues as inhibitors of *E. coli* D-Ala-D-Ala adding enzyme.

Results

Retrosynthetic disconnections

Three retrosynthetic disconnections were attempted for the synthesis of phosphinate target **5**, as shown in Scheme 2. Disconnection of the *C*-terminal D-alanine unit (disconnection *a*) leaves a Lys-PO_2H-Ala phosphinate, which could be assembled either *via* attack of phosphorus onto an imine (route *b*), or *via* attack of an aminophosphinic acid onto a methacrylate ester (route *c*). Alternatively, the *C*-terminal dipeptide unit could be coupled directly to phosphorus *via* route *d*.

Disconnection *b*—reaction of an imine with an organophosphorus P–H compound. 5-Phthalimidopentanal **6** was prepared by reaction of 5-aminopentan-1-ol with phthalic anhydride, followed by Swern oxidation,[11] in 90% overall yield. Reaction of 5-phthalimidopentanal with tritylamine in refluxing toluene over 4 Å molecular sieves for 4 days gave the trityl imine **7** in 100% crude yield. Phosphinic acid **8** was prepared using the general method of Boyd *et al.*,[12] by heating dry ammonium hypophosphite and hexamethyldisilazane at 100 °C, followed by treatment with methyl methacrylate at 0 °C, in 96% yield. The corresponding methyl ester **9** was prepared from acid **8** by treatment with diazomethane, and was obtained as a mixture of diastereoisomers (see Scheme 3).

Activation of either phosphinic acid **8** or methyl ester **9** with hexamethyldisilazane or trimethylsilyl chloride–triethylamine, followed by treatment with imine **7**, gave no reaction, contrary to literature reports.[13,14] No reaction was observed upon addition of Lewis acids, and no reaction was observed between ester **9** and imine **7** in the presence of sodium methoxide, which has also been used for activation of phosphinic esters.[4] The lack of reactivity in this case may be due to steric crowding.

Disconnection *c*—reaction of a 1-aminoalkylphosphinic acid with an α,β-unsaturated ester. Following the method of Baylis *et al.*,[15] the 1-aminoalkyl phosphinic acid **10** was prepared from 5-phthalimidopentanal **6** and (diphenylmethyl)amine, in 36% yield. In view of the insolubility of **10** in most organic solvents, it was converted into the benzyloxycarbonyl (Z) derivative **11** by deprotection in refluxing trifluoroacetic acid, followed by

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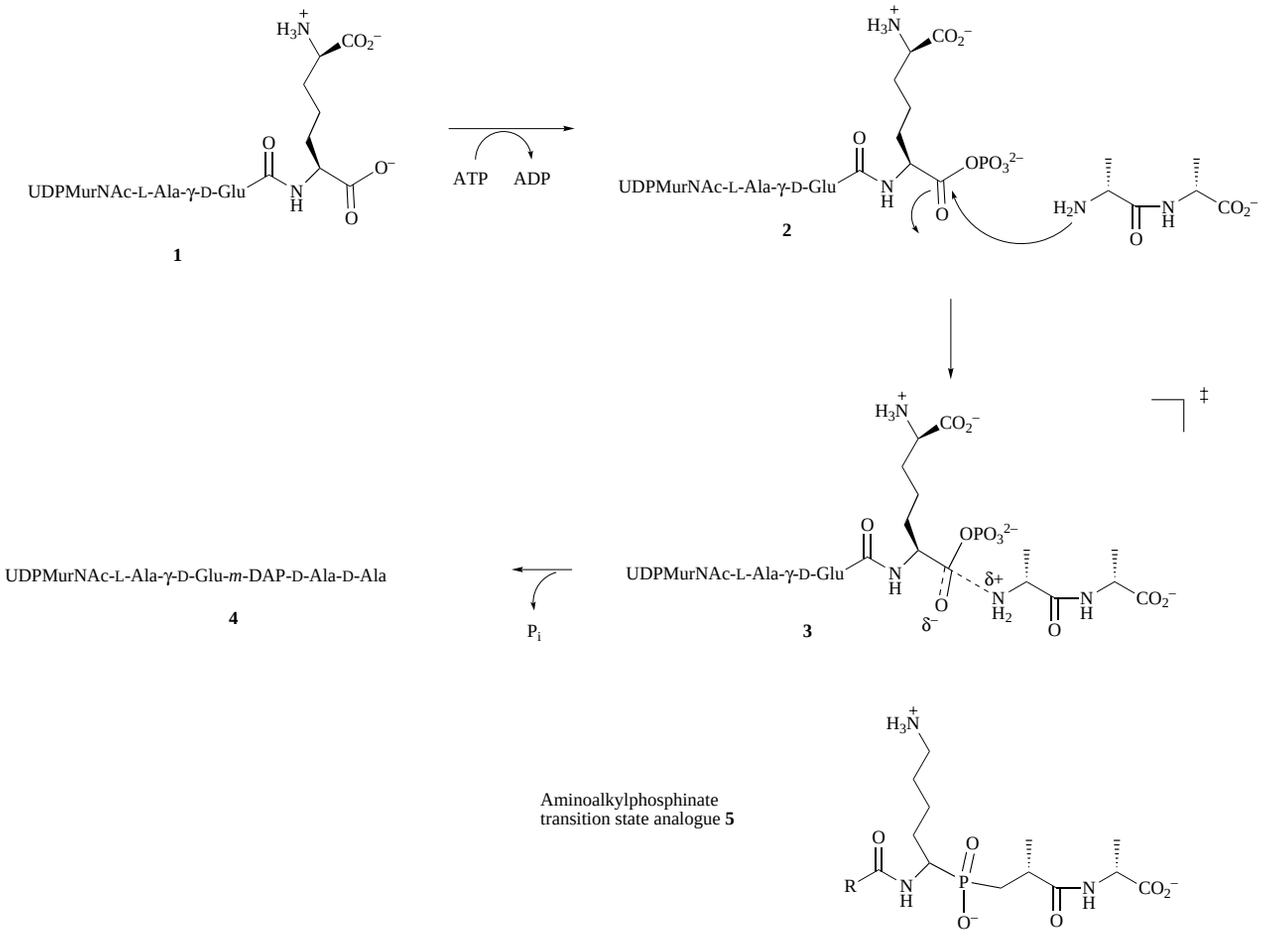
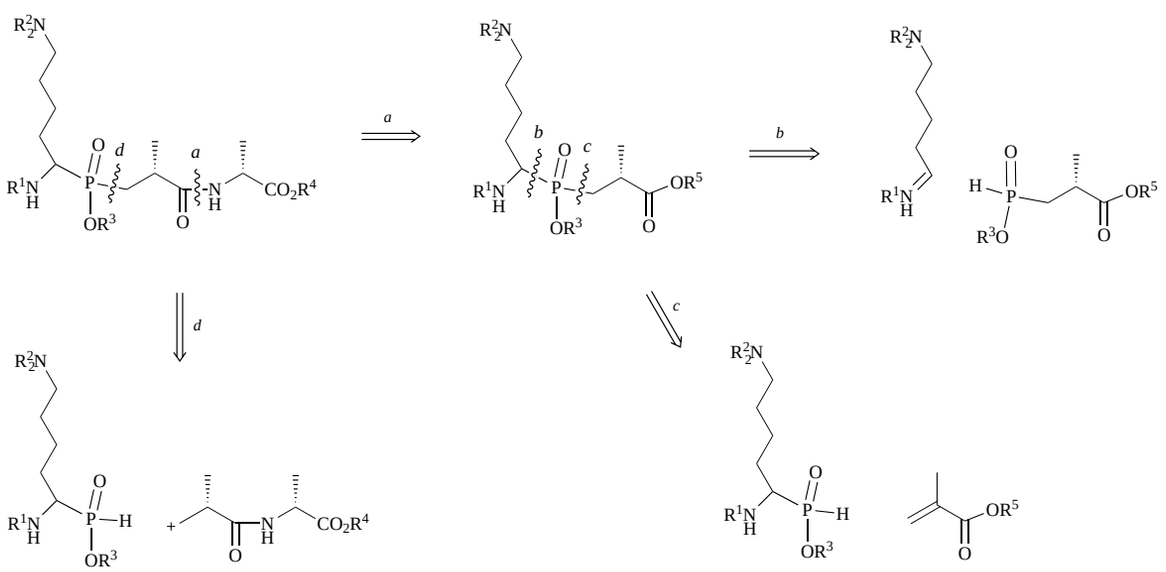


Scheme 1 Anticipated catalytic mechanism for D-Ala-D-Ala adding enzyme, showing acyl phosphate intermediate **2**, transition state **3**, and transition state analogue **5**



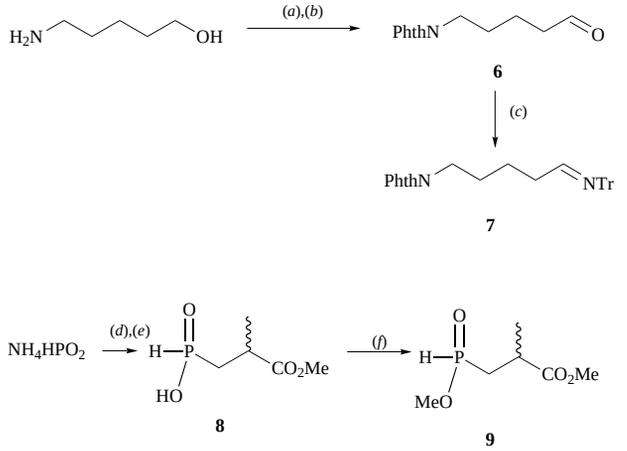
Scheme 2 Retrosynthetic strategies for the synthesis of aminoalkylphosphinate analogues

treatment with benzyl chloroformate, in 85% overall yield (see Scheme 4).

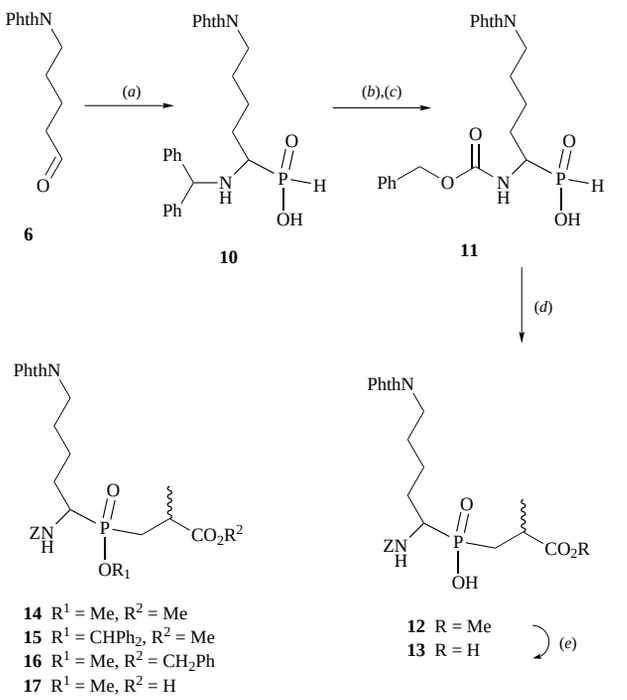
Coupling of phosphinic acid **11** with methyl methacrylate was accomplished using the general method of Yiotakis *et al.*[10] After drying *in vacuo* over phosphorus pentoxide, acid **11** was pyrolysed at 110 °C with hexamethyldisilazane, and the fused silyl phosphonite intermediate was treated with methyl methacrylate. This successfully gave the 1-aminoalkyl phosphinic acid **12** as a mixture of diastereoisomers in 94% yield.

Hydrolysis of the methyl ester **12** could be achieved under alkaline conditions, however the opening of the phthalimide protecting group also occurred rapidly under these conditions. Following treatment of **12** with aqueous potassium hydroxide, it was found that the phthalimide could be re-closed by treatment with 1,1′-carbonyldiimidazole, to give the diacid **13** in 86% yield.

Having assembled the pseudo-dipeptide phosphinic acid **13**, it was anticipated that the *C*-terminal D-alanine residue could be coupled *via* standard methods. However, attempts to couple acid **13** onto D-alanine methyl ester using a range of peptide



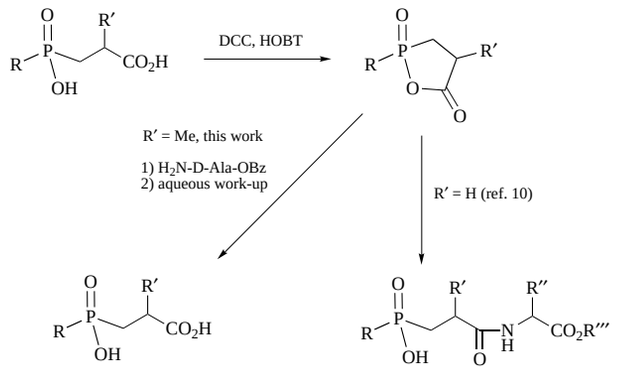
Scheme 3 Synthesis of imine **7** and phosphinic ester **9**. *Reagents and conditions:* (*a*), phthalic anhydride, toluene, heat, 99%; (*b*), i, Me_2SO, $(COCl)_2$, −78 °C; ii, NEt_3, 91%; (*c*), Ph_3CNH_2, toluene, heat, 100%; (*d*), $HN(SiMe_3)_2$, 110 °C; (*e*), $H_2C{=}C(CH_3)CO_2Me$, 96% overall; (*f*), CH_2N_2, Et_2O, 100%.



Scheme 4 Synthesis of pseudo-dipeptide phosphinate **13**. *Reagents and conditions:* (*a*), H_3PO_2, Ph_2CHNH_2, H_2O–EtOH, 80 °C, 36%; (*b*), CF_3CO_2H, heat, 85%; (*c*) $PhCH_2OCOCl$, H_2O, pH 9–9.5, <5 °C, 100%; (*d*), i, $HN(SiMe_3)_2$, 110 °C; ii, $H_2C{=}C(CH_3)CO_2Me$, 94%; (*e*), i, KOH, H_2O; ii, 1,1′-carbonyldiimidazole, CH_2Cl_2, 86%.

coupling reagents [dicyclohexylcarbodiimide–hydroxybenzotriazole (DCC–HOBT); 1,1′-carbonyldiimidazole; isobutyl chloroformate; benzotriazolyloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP)–HOBT; $SOCl_2$] gave only recovered starting materials. It was observed that an attempted DCC coupling gave rise to the expected dicyclohexylurea (DCU) by-product, indicating that acid activation had taken place. We hypothesise that the failure of this reaction is due to the formation of a sterically hindered cyclic anhydride intermediate, which hydrolyses upon work-up, as shown in Scheme 5. Yiotakis *et al.* report the successful coupling of a pseudo-dipeptide phosphinate lacking an α-substituent (Scheme 5, R′ = H),[10] thus it appears that the α-methyl group in **13** prevents attack of an amine nucleophile with such an intermediate.

Selective protection of the phosphinic acid functional group of **13** also proved problematical. The methyl phosphinate ester **14** and diphenylmethyl phosphinate ester **15** were both pre-



Scheme 5 Hypothetical intramolecular cyclisation of diacid **13** upon attempted DCC–HOBT coupling with D-alanine methyl ester

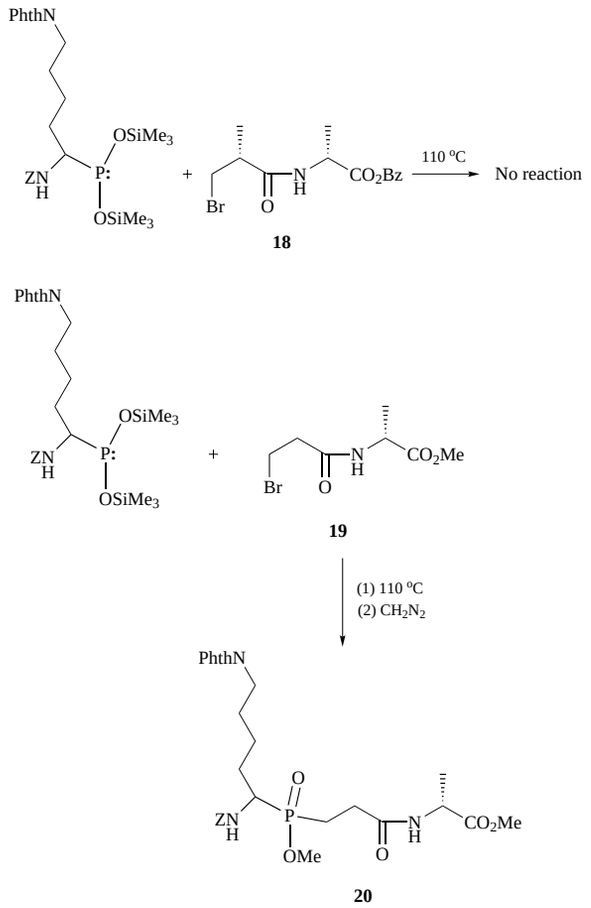
pared from **12**, using diazomethane and diphenyldiazomethane respectively. However, alkaline hydrolysis rapidly hydrolysed the phosphinic ester in each case. Accordingly, the benzyl carboxylic ester **16** was prepared by use of benzyl methacrylate in a Michael addition reaction, followed by methylation using trimethylsilyldiazomethane, in 75% yield. The only successful hydrogenation catalyst found for **16** was palladium black and cyclohexa-1,4-diene as hydrogen donor, which gave the desired carboxylic acid **17** in low yield. However, attempts to couple **17** with D-alanine methyl ester gave only the demethylated diacid **13**! The observed demethylation reaction could be rationalised by an intramolecular reaction similar to that shown in Scheme 5.

Disconnection *d*—reaction of a 1-aminoalkylphosphinic acid with an electrophilic dipeptide equivalent. In view of the extraordinary difficulty of coupling the *C*-terminal D-alanine residue onto a pseudo-dipeptide phosphinate, attempts were made to couple a dipeptide equivalent directly onto a silylated phosphonite, using an Arbusov reaction.[16] (2*R*)-3-Bromo-2-methylpropionyl-D-alanine benzyl ester **18** and 3-bromopropionyl-D-alanine methyl ester **19** were both synthesised using standard coupling methods. Silylation of 1-aminoalkylphosphinic acid **11** was carried out by heating with hexamethyldisilazane at 110 °C, at which point the appropriate bromide was added. Bromide **18** gave no reaction, however the less hindered bromide **19** was found to react, and after methylation of the crude product using diazomethane gave the pseudo-tripeptide phosphinate **20** in 46% yield (see Scheme 6).

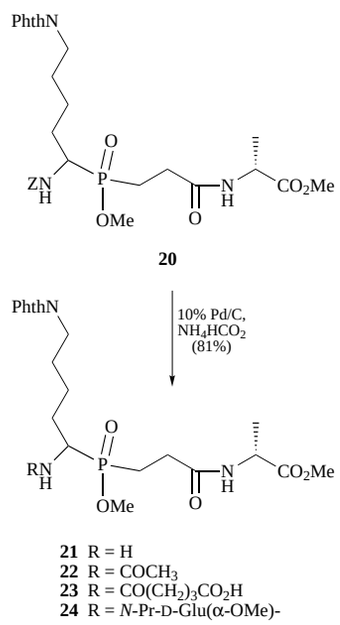
The behaviour of bromides **18** and **19** in this reaction indicates that this Arbusov-type reaction is sensitive to steric crowding, presumably since the attacking silylated phosphonite is very bulky. Although the pseudo-tripeptide phosphinate **20** is lacking the methyl group of the central D-alanine residue of the original target, it is known that D-Ala-D-Ala adding enzyme will accept the dipeptide Gly-D-Ala as a substrate in place of D-Ala-D-Ala, and in general that the substrate specificity for this enzyme is more strict for the *C*-terminal amino acid site.[17] Therefore it was decided to elaborate phosphinate **20** to give a series of X-Lys-PO_2H-Gly-D-Ala analogues which could be tested as enzyme inhibitors.

Elaboration of the amino terminus of pseudo-tripeptide phosphinate 20. Removal of the *N*-terminal Z protecting group by catalytic transfer hydrogenation using 10% palladium/charcoal and ammonium formate gave the free amine **21** in 81% yield. This free amino terminus was then acylated with groups of varying length, in order to mimic more closely the UDPMurNAc-L-Ala-γ-D-Glu-m-DAP substrate, as shown in Scheme 7.

Treatment of amine **21** with acetic anhydride, triethylamine and dimethylaminopyridine gave the *N*-acetyl pseudo-tripeptide phosphinate **22** in 91% yield. Treatment of **21** with glutaric anhydride gave the *N*-glutaryl pseudo-tripeptide phosphinate **23** (ES^+ 582 [MH^+]), in which the *N*-glutaryl moiety mimics the α-carboxy group of D-glutamic acid. Finally, amine



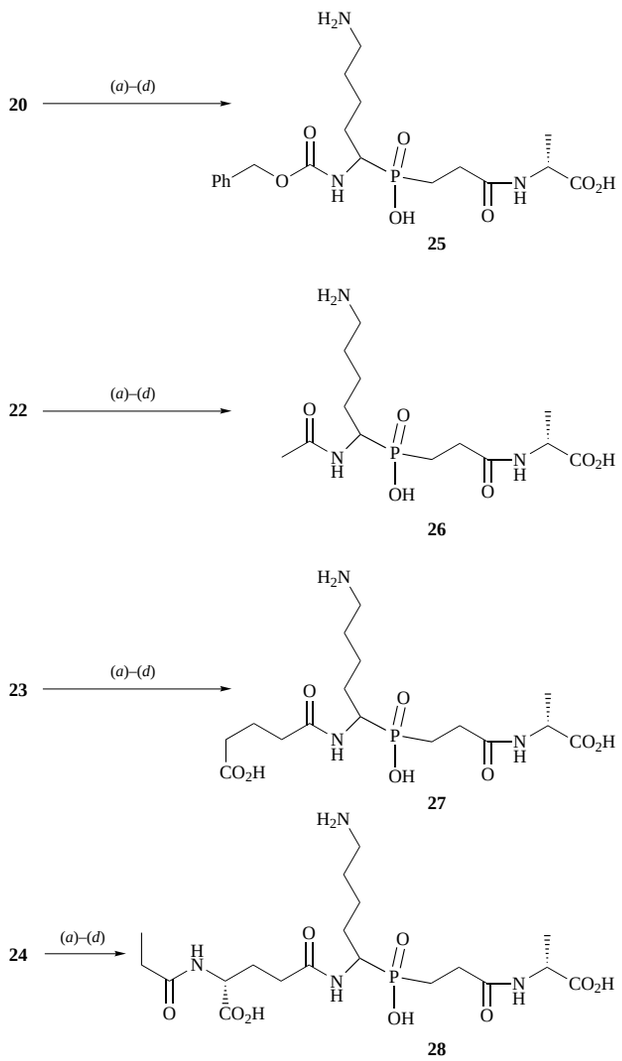
Scheme 6 Reactions of silylated phosphonite derivative of phosphinic acid **11** with bromides **18** and **19**. Pseudo-tripeptide phosphinate **20** isolated in 46% overall yield.



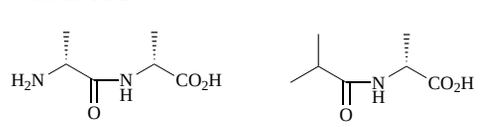
Scheme 7 Elaboration of pseudo-tripeptide phosphinate **20** to give protected phosphinate analogues. Conversion of **21** to **22**, **23** and **24** described in text and in Experimental section.

21 was coupled to *N*-propionyl-D-Glu-(α-OMe)-OH to give a pseudo-tetrapeptide phosphinate **24** (ES^+689 [MNa^+]).

Deprotection of the phthaloyl group by hydrazinolysis was found to form the *C*-terminal acid hydrazide ($RCONHNH_2$). Therefore the *C*-terminal methyl ester of the protected phosphinates was first hydrolysed using tributyltin oxide (5 equiv.).[18] Removal of the phthaloyl group was then achieved by treatment of the crude acid with sodium borohydride in 6:1 propan-2-ol–water, followed by treatment with acetic acid.[19] Deprotection of the phosphinate methyl ester was achieved by alkaline hydrolysis, and each of the deprotected phosphinic acids was purified by reversed phase HPLC. The yields of HPLC purified material obtained for the *N*-acetyl pseudo-tripeptide phosphinic acid **26**, *N*-glutaryl pseudo-tripeptide phosphinic acid **27** and pseudo-tetrapeptide phosphinic acid **28** are 12, 16 and 2.6% respectively, from the respective fully protected precursors. The low yield of **28** is thought to be due to inefficient peptide coupling of amine **21**. The *N*-Z-protected pseudo-tripeptide **20** was also similarly deprotected to give the *N*-Z-pseudo-tripeptide phosphinic acid **25** in 26% yield after HPLC purification. The structures of the final phosphinates (depicted in the protonated form) are shown in Scheme 8.



Structure for **29**:



D-Ala-D-Ala Isobutyryl-D-Ala **29**

Scheme 8 Deprotection of phosphinate target analogues. *Reagents and conditions:* (*a*), $(Bu_3Sn)_2O$, THF, heat; (*b*), $NaBH_4$, Pr^iOH/H_2O (6:1); (*c*), AcOH, heat; (*d*), NaOH, H_2O. Overall yields after HPLC purification: **25**, 26%; **26**, 12%; **27**, 16%; **28**, 2.6%.

Purification of *Escherichia coli* D-Ala-D-Ala adding enzyme

The *murF* gene encoding D-Ala-D-Ala adding enzyme in *Escherichia coli* has been identified amongst a cluster of genes

Table 1 Purification of *Escherichia coli* D-Ala-D-Ala adding enzyme[a] from JM105/pTB3

Step	Volume (ml)	Protein[b] (mg/ml)	Total protein (mg)	Activity[c] (units/ml)	Sp. activity (units/mg)	Yield (%)	Purification (x-fold)
Crude extract	42.0	29.0	1218	33.0	1.14	100	1.0
$(NH_4)_2SO_4$ ppt.	13.2	45.0	594	94.4	2.10	90	1.84
AcA54 gel filtration	60.0	1.87	112	14.2	7.6	61	6.7
Q Sepharose FPLC	60.0	0.35	21.0	7.2	20.6	31	18.0

[a] One unit of enzyme activity is defined as the activity required to convert 1 μmole of substrate per minute. [b] Protein concentration determined by the method of Bradford.[20] [c] Enzyme activity determined by P_i release assay.[6]

involved in peptidoglycan biosynthesis at minute 2 of the *E. coli* chromosome.[7] The *murF* gene has been overexpressed in plasmid pTB3 under the control of the *tac* promoter to approximately 6% of soluble cell protein.[9]

D-Ala-D-Ala adding enzyme was purified from extracts of JM105/pTB3 using ammonium sulfate precipitation, gel filtration chromatography and Q Sepharose FPLC anion exchange chromatography (see Table 1). The specific activity of the purified enzyme was 20.6 units m^{-1}, and the protein appeared as a band at 48 kDa by SDS-PAGE.

Steady state kinetic assays of purified enzyme were carried out using a phosphate release stopped assay.[6] In this assay a mixture of enzyme, UDPMurNAc-tripeptide, D-Ala-D-Ala, ATP and $MgCl_2$ in 50 mM Tris pH 8.6 is incubated at 20 °C. At selected time intervals aliquots are removed, and the release of phosphate quantitated using the method of Lanzetta *et al.*[21] Using this assay the amount of phosphate release *versus* time was measured over a 10 min assay, and initial rates deduced. In our hands we were unable to obtain consistent rate measurements using the reported continuous coupled assay involving pyruvate kinase and lactate dehydrogenase,[6,8] although this coupled assay works well for the preceding enzyme D-alanine : D-alanine ligase.[5]

Using the phosphate release assay, steady state kinetic parameters were measured for the purified enzyme. The enzyme was found to obey Michaelis–Menten kinetics, and a K_M value of 60 (±5) μM was measured for D-Ala-D-Ala under pseudo-first order conditions (100 μM UDPMurNAc-tripeptide, 1 mM ATP). This value is lower than reported K_M values of 220 μM[6] and 208 μM[8] determined using the continuous coupled assay, but was verified by several independent analyses. A K_M value of 220 (±10) μM was measured for co-substrate ATP. The sensitivity of the phosphate release assay (1–10 nmol P_i) was insufficient to measure the K_M for UDPMurNAc-tripeptide, implying that $K_M < 20$ μM (reported value 11 μM, determined by radiochemical assay[22]).

Inhibition of D-Ala-D-Ala adding enzyme by phosphinates 25–28

A series of preliminary enzyme assays was carried out at 100 μM D-Ala-D-Ala, in the presence of 1 mM concentrations of phosphinates **25**–**28**. Under these conditions the *N*-Z-phosphinate **25** showed no inhibition, however >50% inhibition was observed for phosphinates **26**–**28**.

K_i values for phosphinates **26** and **27** were determined by measuring apparent $K_{M'}$ values for D-Ala-D-Ala in the presence of fixed concentrations of inhibitor. Kinetic analysis using Eadie/Hofstee plots revealed that the observed inhibition was reversible and competitive with respect to D-Ala-D-Ala. Values of K_i were then calculated using the equation $K_i = [I]/[(K_{M'}/K_M) - 1]$, giving values of 700 (±50) μM and 200 (±20) μM respectively for the *N*-acyl phosphinate **26** and the *N*-glutaryl phosphinate **27**. Inhibition by the pseudo-tetrapeptide phosphinate **28** was complicated by nanomolar background levels of inorganic phosphate in the purified inhibitor, leading to increased scatter at low substrate concentrations. Therefore, assays were carried out at a fixed substrate concentration (100 μM D-Ala-D-Ala), and variable inhibitor concentrations (0, 200, 500 μM), and a K_i value of 200 (±20) μM deduced using a Dixon plot. These data are summarised in Table 2.

Table 2 Kinetic data for D-Ala-D-Ala adding enzyme substrates and inhibitors. Kinetic assays were carried out as described in the Experimental section. K_i values for phosphinates **26**–**28** refer to concentrations of diastereomeric mixture of phosphinate in solution (1 : 1 mixture of two diastereoisomers).

Substrate/inhibitor	K_M/μM	K_i/μM
D-Ala-D-Ala	60	—
Isobutyryl-D-Ala **29**	—	16 800
N-Z-Lys-PO_2H-Gly-D-Ala **25**	—	—
N-Ac-Lys-PO_2H-Gly-D-Ala **26**	—	700
N-glutaryl-Lys-PO_2H-Gly-D-Ala **27**	—	200
N-Pr-γ-D-Glu-Lys-PO_2H-Gly-D-Ala **28**	—	200

The measured K_i values are somewhat larger than the K_M value for D-Ala-D-Ala, which contains the α-amino group absent from the phosphinate inhibitors. In order to assess the importance of the α-amino group of D-Ala-D-Ala for substrate binding, the analogue isobutyryl-D-alanine **29** containing a methyl group in place of the α-amino group was synthesised and tested as an inhibitor. Analogue **29** was found to act as a reversible competitive inhibitor, with a K_i value of 16.8 mM. Thus, the α-amino group does appear to form a significant binding interaction with the enzyme active site. However, introduction of the Lys-PO_2H-phosphinyl moiety has given >20-fold tighter binding, relative to **29**.

In contrast to the phosphinate inhibitors of D-alanine : D-alanine ligase,[5] pre-incubation of enzyme and ATP with phosphinates **26**–**28** gave no increase in inhibition. Thus, these analogues are not time-dependent inhibitors. Attempts to improve the potency of inhibition by co-addition of 1 mM UDP or UDPGlcNAc, in order to occupy the UDPMurNAc-tripeptide binding site, also had no effect on enzyme inhibition. The antibacterial action of phosphinates **26**–**28** was tested with cultures of *Escherichia coli* K12 and *Bacillus subtilis* W23, and no retardation of growth was observed at 100 μg ml^{-1} final concentration.

Discussion

The aminoalkylphosphinate analogues described in this paper represent the first synthetic inhibitors for D-Ala-D-Ala adding enzyme, a peptidoglycan biosynthetic enzyme found in all bacteria. Pseudo-dipeptide phosphinate inhibitors have previously been synthesised for D-alanine : D-alanine ligase[4] and the D-Glu adding enzyme,[24] however there are very few literature reports of pseudo-tri- or -tetra-peptide phosphinate analogues.[10]

Our synthetic work has confirmed that the use of silylated phosphonites for Michael addition or Arbusov-type displacement reactions, after the work of Regan and co-workers[12,13,16] and Yiotakis *et al.*,[10] is a powerful method for the assembly of complex phosphinate skeletons. However, these reactions proceed only under harsh reaction conditions, and appear to be sensitive to steric factors.

The inability to couple a further amino acid onto the carboxy terminus of a pseudo-dipeptide phosphinic acid **13** represents a

severe limitation in the synthesis of larger phosphinates, and should be considered by other workers in this area. In this case we have circumvented this problem by attaching a *C*-terminal dipeptide unit as bromopropionyl-D-alanine methyl ester, using an Arbusov reaction. However, this approach only allows for the introduction of a glycine analogue immediately adjacent to the phosphinate. During the completion of this work Yiotakis *et al.* have published a method for the protection of a phosphinic acid group using an adamantyl protecting group, which provides an alternative route for the assembly of higher phosphinates.[23]

Kinetic assays of these analogues has revealed that they act as inhibitors of *E. coli* D-Ala-D-Ala adding enzyme, with K_i values in the range 200–700 μM. Since these K_i values are comparable with, but not lower than, the K_M value for D-Ala-D-Ala (60 μM), it seems that these analogues have not fully realised the target of resembling the transition state for the enzymatic reaction. However, comparison with the carbon analogue isobutyryl-D-Ala (K_i 16.8 mM) reveals that the presence of the phosphinic acid unit has increased the binding efficiency by >20-fold.

It is also noteworthy that increasing the length of the amino-terminal peptide chain significantly increases the effectiveness of inhibition, presumably by mimicking the UDPMurNAc-tripeptide chain. The *N*-glutaryl phosphinate **27** and the pseudo-tetrapeptide **28** have 3-fold lower K_i values than the *N*-acetyl phosphinate **26**, suggesting that the α-carboxylate of the D-Glu residue forms a favourable binding interaction with the enzyme, although the presence of the L-Ala-D-Glu amide bond in **28** gives no further increase in binding affinity. It has been found recently that the kinetic mechanism of this enzyme proceeds *via* initial binding of the UDPMurNAc-tripeptide substrate.[8] Therefore, it seems likely that in order to achieve submicromolar levels of inhibition *via* this approach, a significant amount of the UDPMurNAc-tripeptide skeleton would need to be included in the inhibitor design. Similarly, Tanner *et al.* have shown that most effective inhibition of the *E. coli* D-Glu adding enzyme was achieved using a pseudo-dipeptide phosphinate analogue covalently linked to uridine monophosphate.[24]

An alternative explanation for the decreased activity of these phosphinate analogues compared with the phosphinate inhibitors of D-alanine:D-alanine ligase[4,5] is that the latter enzyme is strongly inhibited by the product D-Ala-D-Ala,[3] and therefore binds dipeptides tightly. In contrast, D-Ala-D-Ala adding enzyme is not inhibited by its product UDPMurNAc-pentapeptide.[6,22] The lack of antibacterial activity of phosphinates **26**–**28** could either be due to inefficient transport into the bacterial cell, or the inability to accumulate inhibitor concentrations inside the cell up to the observed values of K_i. Therefore, the potential of D-Ala-D-Ala adding enzyme as an antibacterial target remains to be confirmed.

Experimental

General

Nuclear magnetic resonance spectra were recorded on either a Bruker AM 300 Fourier transform spectrometer (300 MHz) or a Bruker AM 360 Fourier transform spectrometer. *J* Values are given in Hz. Ultraviolet–visible spectra were recorded on a Cary-1 UV-visible spectrophotometer. Infrared spectra were recorded on a 1600 series Perkin-Elmer FTIR spectrometer. Mass spectra were recorded on a VG-70-250 mass spectrometer in Electron Impact (EI), Chemical Ionisation (CI) or Fast Atom Bombardment (FAB) mode or a VG Platform Quadrupole Electrospray Ionisation mass spectrometer (ES).

All chemicals were supplied by Sigma-Aldrich Chemical Co. Tritylamine (triphenylmethylamine) was prepared by the method of Soroka and Sygmunt.[25] D-Alanine methyl ester was prepared by the method of Miller *et al.*,[26] (2*R*)-3-Bromo-2-methylpropionic acid was prepared by the method of Salomon *et al.*[18] and *N*-butoxycarbonyl-D-glutamic acid α-methyl ester γ-benzyl ester‡ was prepared by the method of Manesis and Goodman.[27]

5-Phthalimidopentanal **6** was prepared by reaction of 5-aminopentan-1-ol with phthalic anhydride,[28] to give 5-phthalimidopentan-1-ol in 99% yield, followed by oxidation using the method of Swern *et al.* in 91% yield[11] {ν_{max}(liquid film)/cm^{-1} 2724 (w), 1771 (s), 1710 (s); δ_H(300 MHz, $CDCl_3$) 1.60–1.80 (4H, m), 2.25 (2H, t, *J* 7), 3.62 (2H, t, *J* 7), 7.60–7.90 (4H, m, C_6H_4), 9.73 (1H, s); δ_C(75 MHz, $CDCl_3$) 19.09, 27.83, 37.32, 43.07, 123.1, 133.9, 131.9, 168.3, 201.9; *m*/*z* (ES^+) 463 (71%, $[2M + H]^+$), 485 (100, $[2M + Na]^+$)}. 5-Phthalimido-*N*-tritylpentanimine **7** was prepared by reaction of 5-phthalimidopentanal with tritylamine in refluxing toluene over 4 Å molecular sieves for 4 days, in quantitative yield.[25]

2-Methoxycarbonylpropylphosphinic acid **8** was prepared from ammonium hypophosphite and methyl methacrylate using the method of Boyd *et al.*,[12] in 96% yield {ν_{max}(liquid film)/cm^{-1} 3403 (br, m), 2340 (w), 1731 (s); δ_H(300 MHz, $CDCl_3$) 1.32 (3H, d, *J* 6.5), 1.83–2.27 (2H, m), 2.85–3.16 (1H, m), 3.70 (3H, s), 7.19 (1H, d, J_{H-P} 560, PH); δ_C(75 MHz, $CDCl_3$) 19.10, 33.02 (d, J_{C-P} 93, PCH_2), 33.83, 52.33, 175.7; δ_P(121 MHz, $CDCl_3$) 34.7 ppm downfield from 85% H_3PO_4; *m*/*z* (ES^-) 165 (10%, M^-)}.

5-Phthalimido-1-[(diphenylmethyl)amino]pentylphosphinic acid **10** was prepared from 5-phthalimidopentanal **6** by the method of Baylis *et al.*[15] (mp 134–135 °C, lit.,[15] 134–135 °C). *N*-Isobutyryl-D-alanine benzyl ester was prepared by the coupling of isobutyric acid and D-Ala-benzyl ester using hydroxybenzotriazole and dicyclohexylcarbodiimide as coupling reagents in dichloromethane. Debenzylation was carried out by hydrogenation (stirring overnight in ethanol with 10% Pd/charcoal under a hydrogen atmosphere for 16 h at 25 °C). After filtration through Celite and removal of solvent under reduced pressure, the deprotected compound was recrystallised from ethyl acetate–hexane {δ_H(500 MHz, D_2O) 1.06 [6H, d, $(CH_3)_2CH$], 1.37 (3H, d, *J* 8, CH_3CH), 2.51 [1H, septet, *J* 7, $(CH_3)_2CH$], 4.28 (1H, q, *J* 8, NHCH); *m*/*z* (FAB) 160 [M + H]; HRMS (FAB) MH^+, 160.0974. $C_7H_{14}NO_3$ requires 160.0970}.

2-Methoxycarbonylpropylphosphinic acid methyl ester 9

To a stirred solution of phosphinic acid **8** (1.02 g, 6.14 mmol) was added a solution of diazomethane in diethyl ether until evolution of nitrogen ceased and the yellow colour of diazomethane remained permanent. The solution was stirred overnight at room temperature to allow excess diazomethane to evaporate and the solvent was removed under reduced pressure to give the title compound as a colourless oil (1.10 g, 100%); ν_{max}(liquid film)/cm^{-1} 2338 (w), 1735 (s); δ_H(300 MHz, $CDCl_3$) 1.29 and 1.30 (3H, 2 d, *J* 7), 1.75–1.90 and 2.10–2.30 (2H, m, PCH_2), 2.88 (1H, tq, *J* 7 and 14, CH_2CHCH_3), 3.68 (3H, s, CO_2CH_3), 3.73 and 3.77 (3H, 2 s, $POCH_3$), 7.13 and 7.14 (1H, 2 d, J_{H-P} 550, PH); δ_C(75 MHz, $CDCl_3$) 18.98, 19.20, 32.29 (d, J_{C-P} 93, PCH_2), 33.53, 33.89, 52.23, 52.34, 52.93, 53.07, 175.4; δ_P(121 MHz, $CDCl_3$) 38.9 and 39.1 (downfield from 85% H_3PO_4); *m*/*z* (ES^+) 181 (100%, $[M + H]^+$).

5-Phthalimido-1-aminopentylphosphinic acid

A stirred solution of 5-phthalimido-1-(diphenylmethyl)aminopentylphosphinic acid[15] **10** (1.38 g, 2.98 mmol) in trifluoroacetic acid (15 ml) was heated under reflux under an atmosphere of dry nitrogen for 1 h. After cooling to room temperature, trifluoroacetic acid was removed under reduced pressure and the

‡ In this name α-methyl ester corresponds to esterification of the carboxy group attached to the α-carbon, and γ-benzyl ester corresponds to esterification of the carboxy group attached to the γ-carbon. The IUPAC name for this compound is *N*-butoxycarbonyl-D-glutamic acid 1-methyl ester 5-benzyl ester.

residue partitioned between ethyl acetate (50 ml) and water (50 ml). The separated aqueous layer was further extracted with ethyl acetate (2 × 50 ml) and then evaporated *in vacuo*. The title compound was obtained as a white crystalline solid after recrystallisation from water–acetone (0.752 g, 85%), mp 208–210 °C; ν_{max}(KBr disc)/cm^{-1} 3300 (s, br), 2346 (m), 1773 (s), 1710 (s); δ_H(300 MHz, D_2O) 1.28–1.90 (6H, m), 3.04 (1H, m, H_2NCHP), 3.56 (2H, t, *J* 7, PhthNCH_2), 6.91 (1H, d, J_{H-P} 530, PH), 7.69 (4H, br s, C_6H_4); δ_C(75 MHz, D_2O) 25.06 (d, J_{C-P} 8.5, PCHCH_2), 28.25, 29.81, 39.70, 52.71 (d, J_{C-P} 92.5, H_2NCHP), 125.6, 137.0, 133.6, 173.0; δ_P(121 MHz, D_2O) 20.4 (downfield from 85% H_3PO_4); *m*/*z* (FAB) 297 (40%, [M + H]$^+$), 319 (22, [M + Na]$^+$).

5-Phthalimido-1-(benzyloxycarbonylamino)pentylphosphinic acid 11

To a stirred solution of 5-phthalimido-1-aminopentylphosphinic acid (3.87 g, 13.1 mmol) in water (50 ml) at 0 °C was added 4 M sodium hydroxide solution to pH 9.5. A solution of benzyl chloroformate (2.46 g, 14.4 mmol) in dioxane (50 ml) was then added dropwise over 10 min whilst maintaining the solution in the range pH 9–9.5. The solution was then stirred at 0 °C for a further 4 h, maintaining the solution in the range pH 9–9.5. After warming to room temperature, the solution was extracted with diethyl ether (3 × 100 ml) and the separated aqueous layer was acidified to pH 1 by dropwise addition of 2 M hydrochloric acid. The resulting white precipitate was extracted with ethyl acetate (3 × 100 ml) and the combined organic extracts were washed with brine (300 ml). The organic solution was then dried ($MgSO_4$), filtered and evaporated to give the title compound as a white foam (5.68 g, 100%); ν_{max}(CH_2Cl_2 solution)/cm^{-1} 2354 (w), 1771 (w), 1712 (s); δ_H(300 MHz, $CDCl_3$) 1.31–1.91 (6H, m), 3.63 (2H, t, *J* 7, PhthNCH_2), 3.85–3.95 (1H, m, NHC*H*P), 5.04 (2H, s, $C_6H_5CH_2$), 5.62 (1H, d, *J* 9, N*H*CHP), 6.96 (1H, d, J_{H-P} 567, PH), 7.29 (5H, br s, $C_6H_5CH_2$), 7.63–7.78 (4H, m, C_6H_4); δ_C(75 MHz, $CDCl_3$) 22.70, 26.15, 27.85, 37.22, 49.97 (d, J_{C-P} 100, NHCHP), 67.03, 123.1, 133.9, 125.7, 128.0, 128.4, 134.0, 136.0, 156.7, 168.4; δ_P(121 MHz, $CDCl_3$) 32.6 (downfield from 85% H_3PO_4); *m*/*z* (ES$^+$) 431 (100%, [M + H]$^+$), 453 (34, [M + Na]$^+$); HRMS (FAB) MH$^+$, 431.1373. $C_{21}H_{24}N_2O_6P$ requires 431.1372.

Methyl 3-[(1-benzyloxycarbonylamino-5-phthalimidopentyl)-(hydroxy)phosphonyl]-2-methylpropionate 12

A stirred mixture of phosphinic acid **11** (480 mg, 1.12 mmol) and hexamethyldisilazane (0.35 ml, 1.68 mmol) was heated at 110 °C under an atmosphere of dry nitrogen for 40 min. After cooling the fused mixture to 90 °C, methyl methacrylate (0.15 ml, 1.34 mmol) was added dropwise over 5 min and the mixture stirred at 98 °C for 25 min. The mixture was then allowed to cool to 70 °C and ethanol (10 ml) was added. The resulting solution was stirred at this temperature for 2 min and then allowed to slowly cool to room temperature. The solvent was removed under reduced pressure and the residue redissolved in 10% aqueous sodium hydrogen carbonate (15 ml). This solution was washed with diethyl ether (3 × 15 ml) and the separated aqueous layer was acidified to pH 1 with 2 M aqueous hydrochloric acid. The resulting white precipitate was extracted with ethyl acetate (4 × 40 ml) and the combined organic extracts were washed with brine (200 ml). The organic solution was then dried ($MgSO_4$), filtered and evaporated to give the title compound as a white foam (560 mg, 94%); ν_{max}(KBr disc)/cm^{-1} 3335 (m, br), 1772 (s), 1707 (s, br), 1527 (s); δ_H(300 MHz, $CDCl_3$) 1.24 (3H, d, *J* 6.5, CH_3CH), 1.40–2.18 (8H, m), 2.83–2.93 (1H, m, CH_3CH), 3.60–3.65 (2H, m, PhthNCH_2), 3.65 (3H, s, CO_2CH_3), 3.87–3.98 (1H, m, NHC*H*P), 5.06 (2H, ABq, *J* 12.5, $C_6H_5CH_2$), 5.48 (1H, d, *J* 9.5, N*H*CHP), 7.31 (5H, br s, $C_6H_5CH_2$), 7.63–7.78 (4H, m, C_6H_4); δ_C(75 MHz, $CDCl_3$) 18.70, 18.82, 22.77 (d, J_{C-P} 11.5, PCHCH_2), 26.89, 27.77, 29.87 (d, J_{C-P} 91.5, PCH_2), 33.45, 37.21, 49.98 (d, J_{C-P} 105, NHCHP), 52.06, 67.04, 123.1, 127.9, 128.1, 128.4, 132.0, 133.8, 136.2, 156.3, 168.3, 175.8; δ_P(121 MHz, $CDCl_3$) 53.67 (downfield from 85% H_3PO_4); *m*/*z* (NH_3, CI$^+$) 531 (8%, [M + H]$^+$, 513 (12, [M + H − H_2O]$^+$).

Methyl 3-[(1-benzyloxycarbonylamino-5-phthalimidopentyl)-(methoxy)phosphonyl]-2-methylpropionate 14

To a stirred solution of pseudo-dipeptide phosphinic acid **12** (516 mg, 0.974 mmol) in chloroform (20 ml) was added dropwise a solution of diazomethane in diethyl ether until the evolution of nitrogen ceased and the yellow colour of diazomethane was no longer discharged. The solution was then left to stand for 16 h to allow excess diazomethane to evaporate. The solvent was removed under reduced pressure to give the title compound as a colourless oil (530 mg, 100%); ν_{max}($CHCl_3$ solution)/cm^{-1} 3227 (m), 1771 (s), 1711 (s, br), 1529 (m); δ_H(300 MHz, $CDCl_3$) 1.20 and 1.25 (3H, 2d, *J* 8, CH_3CH), 1.36–2.35 (8H, m), 2.87 (1H, ddd, *J* 7.5, 13.5, 24.5, CH_3CHCH_2), 3.66–4.03 (9H, m), 4.97–5.53 (3H, m), 7.32 (5H, br s, $C_6H_5CH_2$), 7.65–7.81 (4H, m, C_6H_4); δ_C(75 MHz, $CDCl_3$) 18.60, 18.71, 18.91, 19.03, 27.73 (d, J_{C-P} 12, PCHCH_2), 27.20, 27.74, 29.00 (d, J_{C-P} 88.5, PCH_2CH), 33.60, 37.06, 49.51, 49.75 (d, J_{C-P} 106, NHCHP), 51.95, 123.0, 127.8, 127.9, 128.0, 128.1, 128.4, 131.9, 133.8, 136.1, 156.2, 168.3, 175.7; δ_P(121 MHz, $CDCl_3$) 52.65, 52.93, 52.98, 53.73 (downfield from 85% H_3PO_4).

Benzyl 3-[(1-benzyloxycarbonylamino-5-phthalimidopentyl)-(methoxy)phosphonyl]-2-methylpropionate 16

A stirred mixture of phosphinic acid **11** (5 g, 11.6 mmol) and hexamethyldisilazane (3.70 ml, 17.4 mmol) was heated under an atmosphere of dry nitrogen at 110 °C for 40 min. After cooling the fused mixture to 85 °C, benzyl methacrylate (2.36 ml, 14.0 mmol) was added dropwise over 10 min and the resulting mixture stirred for 1 h at 85 °C. After cooling to 70 °C, ethanol (60 ml) was added and the resulting solution stirred whilst slowly cooling to room temperature. The solvent was removed under reduced pressure and the residue redissolved in 8% aqueous sodium hydrogen carbonate (80 ml). This solution was washed with diethyl ether (3 × 80 ml) and then acidified to pH 1 by dropwise addition of 10% aqueous hydrochloric acid. The resulting white precipitate was extracted into ethyl acetate (3 × 100 ml) and the pooled organic extracts were washed with brine (300 ml). The organic solution was then dried (Na_2SO_4), filtered and evaporated to give a white foam. This was redissolved in 1:1 (v/v) acetonitrile–methanol (100 ml) and to this stirred solution under a dry nitrogen atmosphere was added trimethylsilyldiazomethane (2 M, solution in hexanes) until evolution of nitrogen ceased and the yellow colour of trimethylsilyldiazomethane was permanent. The solution was then stirred for 2 h at room temperature. The solvent was removed under reduced pressure and the residue purified by flash chromatography on silica gel, eluting with a solvent gradient [from 100% dichloromethane to 24:1 (v/v) dichloromethane–methanol] to give the title compound as a white foam (5.37 g, 75%); ν_{max}(KBr disc)/cm^{-1} 3400 (w), 1772 (m), 1712 (s, br), 1508 (m); δ_H(500 MHz, $CDCl_3$) 1.27 and 1.32 (3H, 2d, *J* 7.5, CH_3CH), 1.40–2.00 (7H, m), 2.32 (1H, m, CH_3CHCH_2), 2.85 (1H, m), 3.60–4.12 (6H, m), 4.80–5.20 (5H, m), 7.32–7.36 (10H, m), 7.69–7.83 (4H, m); δ_C(75 MHz, $CDCl_3$) 18.86, 18.97, 19.24, 19.36, 22.98 (d, J_{C-P} 11.5, PCHCH_2), 27.50, 28.02, 28.65, 29.82, 34.06, 37.33, 50.05, 50.27 (d, J_{C-P} 105, NHCHP), 51.95, 52.19, 66.81, 67.23, 123.3, 128.1, 128.2, 128.3, 128.4, 128.7, 132.2, 134.0, 136.0, 136.4, 156.5, 168.6, 175.3, 175.4; δ_P(200 MHz, $CDCl_3$) 48.76, 48.86, 50.04 and 50.73 (downfield from 85% H_3PO_4); *m*/*z* (FAB$^+$) 621 (30%, [M + H]$^+$), 643 (10, [M + Na]$^+$).

Methyl 3-[(1-benzyloxycarbonylamino-5-phthalimidopentyl)-(benzyloxy)phosphonyl]-2-methylpropionate 15

To a stirred mixture of benzophenone hydrazone (393 mg, 2.00

mmol) and anhydrous magnesium sulfate (200 mg) in dichloromethane (10 ml) was added manganese dioxide (869 mg, 10.0 mmol) and the mixture stirred for 1.5 h at room temperature. The solution was filtered to give a solution of diphenyldiazomethane. This solution was added dropwise to a stirred solution of pseudo-dipeptide phosphinic acid **12** (530 mg, 1.00 mmol) in dichloromethane (5 ml). The resulting solution was then heated under reflux for 2 h. The solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel [eluting with 1:1 (v/v) ethyl acetate–light petroleum] to give the title compound as a colourless oil (335 mg, 48%); δ_H(300 MHz, $CDCl_3$) 1.01–2.20 (10H, m), 2.23–2.39 (1H, m), 2.56–2.80 (1H, m, CH_3CH), 3.49–3.61 (5H, m), 3.86–4.06 (1H, m, NHC*H*P), 4.92–5.11 and 5.28–5.29 (3H, m, $C_6H_5CH_2$ and N*H*CHP), 6.53 and 6.57 [1H, 2s, $(C_6H_5)_2CH$], 7.32–7.40 (15H, m, $3 \times C_6H_5$), 7.67–7.83 (4H, m, C_6H_4); δ_C(75 MHz, $CDCl_3$) 18.96, 19.07, 19.35, 19.47, 23.00 (d, J_{C-P} 12, PCH*C*H_2), 27.38, 27.97, 30.61 (d, J_{C-P} 86.5, P*C*H_2CH), 33.84, 37.45, 50.50, 50.86 (2d, J_{C-P} 105, NHCHP), 52.15, 67.23, 76.12, 78.01, 123.33, 126.7, 127.0, 127.3, 127.5, 127.7, 128.1, 128.2, 128.3, 128.4, 128.5, 128.7, 128.8, 129.0, 132.3, 134.0, 136.5, 140.8, 144.4, 156.5, 168.5, 175.7, 175.9; δ_P(121 MHz, $CDCl_3$) 53.29, 53.45, 53.55, 53.68 (downfield from 85% H_3PO_4).

3-[(1-Benzyloxycarbonylamino-5-phthalimidopentyl)(hydroxy)-phosphonyl]-2-methylpropionic acid 13

A solution of pseudo-dipeptide phosphinic acid methyl ester **12** (2.50 g, 4.72 mmol) in 1 M aqueous potassium hydroxide (45 ml) was stirred for 1 h at room temperature. The solution was acidified to pH 1 by dropwise addition of 2 M hydrochloric acid and the resulting white precipitate was extracted with ethyl acetate (3×50 ml). The combined organic extracts were dried ($MgSO_4$), filtered and evaporated to give the ring-opened phthalamic acid as a white foam (2.36 g, 94%).

To a stirred solution of the phthalamic acid (1.90 g, 3.56 mmol) in dry tetrahydrofuran (20 ml) was added 1,1′-carbonyldiimidazole (1.73 g, 10.7 mmol) and the solution stirred at room temperature for 1 h. The solvent was removed under reduced pressure and the residue redissolved in 10% aqueous sodium hydrogen carbonate (20 ml). This solution was washed with diethyl ether (3×20 ml) and then acidified to pH 1 by dropwise addition of 2 M hydrochloric acid. The resulting white precipitate was extracted with ethyl acetate (3×40 ml) and the combined organic extracts were washed with brine (120 ml). The organic solution was then dried ($MgSO_4$), filtered and evaporated to give the title compound as a white foam (1.58 g, 86%); ν_{max}/cm^{-1} 3322 (s, br), 1770 (s), 1713 (s, br), 1530; δ_H(300 MHz, $CDCl_3$) 1.18 (3H, d, *J* 6, CH_3CH), 1.33–2.32 (8H, m), 2.73–2.95 (1H, m, CH_3CH), 3.61 (2H, t, *J* 7, PhthNCH_2), 3.85 (1H, m, NHC*H*P), 4.97–5.13 (2H, m, $C_6H_5CH_2$), 5.80–5.98 (1H, br, N*H*CHP), 7.23–7.31 (5H, br s, C_6H_5), 7.61–7.76 (4H, m, C_6H_4); δ_C(75 MHz, $CDCl_3$) 19.23, 23.11, 26.93, 27.99, 30.00 (d, J_{C-P} 88, PCH*C*H_2), 34.12, 37.55, 50.30 (d, J_{C-P} 105, NHCHP), 67.26, 123.3, 128.0, 128.1, 128.6, 132.2, 134.0, 136.4, 156.9, 168.6, 178.7; δ_P(121 MHz, $CDCl_3$) 52.01 (downfield from 85% H_3PO_4); *m*/*z* (ES^+) 517 (100%, $[M + H]^+$).

(2*R*)-2-(3-Bromo-2-methylpropionylamino)propionic acid benzyl ester 18

To a stirred solution of (2*R*)-3-bromo-2-methylpropionic acid (91 mg, 0.545 mmol) and *N*-methylmorpholine (60 ml, 0.545 mmol) in dry tetrahydrofuran (3 ml) at −15 °C under a dry nitrogen atmosphere was added isobutyl chloroformate (71 ml, 0.545 mmol) and the solution stirred for 15 min. To the resulting solution was added a solution of (*R*)-alanine benzyl ester *N*-toluene-*p*-sulfonate (192 mg, 0.545 mmol) and *N*-methylmorpholine (60 ml, 0.545 mmol) in dry *N*,*N*-dimethylformamide (2 ml). This solution was then stirred whilst slowly warming to room temperature. The solvent was removed *in vacuo* and the residue partitioned between water (5 ml) and ethyl acetate (10 ml). The separated organic layer was washed with saturated aqueous sodium hydrogen carbonate (10 ml), 5% hydrochloric acid (10 ml) and brine (10 ml). The organic layer was then dried ($MgSO_4$), filtered and evaporated. The residue was purified by flash chromatography on silica gel [eluting with 2:1 (v/v) light petroleum–ethyl acetate] to give the title compound as a white crystalline solid which was recrystallised from ethyl acetate–hexane (123 mg, 70%), mp 73–74 °C; ν_{max}(Nujol mull)/cm^{-1} 3325 (s), 1726 (s), 1655, 1531; δ_H(500 MHz, $CDCl_3$) 1.27 (3H, d, *J* 5), 1.47 (3H, d, *J* 7.5), 2.65 (1H, m), 3.38 (1H, dd, *J* 5.5, 10), 3.61 (1H, dd, *J* 8.5, 10), 4.69 (1H, quintet, *J* 7.5), 5.20 (2H, ABq, *J* 12), 6.14 (1H, d, *J* 6.5), 7.38 (5H, m); *m*/*z* (FAB) 329 (100%, $[M + H]^+$, 248 (24, $[M - Br]^+$).

3-Bromopropionyl-D-alanine methyl ester 19

To a stirred solution of 3-bromopropionic acid (3.30 g, 21.6 mmol) in dry tetrahydrofuran (30 ml) under an atmosphere of dry nitrogen was added *N*-methylmorpholine (2.36 ml, 21.6 mmol) and the solution cooled to −15 °C. Isobutyl chloroformate (2.80 ml, 21.6 mmol) was then added and the solution stirred for 15 min. To this solution was added a solution of (*R*)-alanine methyl ester hydrochloride (3.00 g, 21.6 mmol) and *N*-methylmorpholine (2.36 ml, 21.6 mmol) in dry *N*,*N*-dimethylformamide (30 ml) plus washings *via* a cannula. The solution was stirred for a further 15 min at −15 °C and then slowly allowed to warm to room temperature. The solution was filtered and the solvent removed *in vacuo*. The residue was partitioned between ethyl acetate (50 ml) and water (50 ml). The separated organic layer was washed with saturated aqueous sodium hydrogen carbonate (50 ml), 2 M hydrochloric acid (50 ml) and brine (50 ml). The organic layer was then dried ($MgSO_4$), filtered and evaporated to give a yellow oil that was purified by flash chromatography on silica gel [eluting with 1:1 (v/v) ethyl acetate–petroleum ether] to give the title compound as a white crystalline solid that was recrystallised from ethyl acetate–hexane (2.29 g, 45%), mp 70–72 °C (lit.,[29] 54–56 °C); ν_{max}(CH_2Cl_2 solution)/cm^{-1} 3417 (w), 1740 (s), 1674 (s), 1515 (m); δ_H(300 MHz, $CDCl_3$) 1.43 (3H, d, *J* 7), 2.72–2.88 (2H, m), 3.57–3.69 (2H, m), 3.76 (3H, s), 4.63 (1H, quintet, *J* 7), 6.28 (1H, br); δ_C(75 MHz, $CDCl_3$) 18.65, 27.21, 39.60, 48.31, 52.73, 169.3, 173.5; *m*/*z* (ES^+) 238/240 (12%, $[M + H]^+$), 260/262 (70, $[M + Na]^+$).

Methyl (2*R*)-2-{3-[(1-benzyloxycarbonylamino-5-phthalimidopentyl)(methoxy)phosphonyl]propionylamino}propionate 20

A stirred mixture of phosphinic acid **11** (430 mg, 1.00 mmol) and hexamethyldisilazane (316 μl, 1.50 mmol) was heated at 110 °C for 40 min under an atmosphere of dry nitrogen. After cooling to 90 °C, 3-bromopropionyl-D-alanine methyl ester **19** (238 mg, 1.00 mmol) was added and the resulting mixture stirred at 90 °C under a dry nitrogen atmosphere for 1 h. The mixture was cooled to 70 °C and ethanol (10 ml) was added. The solution was allowed to cool to room temperature and the solvent removed under reduced pressure. The residue was redissolved in 10% aqueous sodium hydrogen carbonate and washed with diethyl ether (3×10 ml). The separated aqueous solution was acidified to pH 1 by dropwise addition of 2 M hydrochloric acid and the resulting white precipitate extracted into ethyl acetate (3×20 ml). The pooled organic extracts were washed with brine (60 ml) then dried ($MgSO_4$), filtered and evaporated under reduced pressure to give a white foam. This compound was dissolved in dichloromethane (40 ml) and treated with a solution of diazomethane in diethyl ether until the evolution of nitrogen ceased and the yellow colour of diazomethane remained permanent. The solution was allowed to stand for 16 h, treated dropwise with acetic acid, and the solvent removed under reduced pressure. The crude product was purified by flash chromatography on silica gel [eluting with 49:1 (v/v) chloroform–methanol] to give the title compound as a white foam (274 mg, 46%); ν_{max}(Nujol mull)/cm^{-1} 3225 (m), 1772 (m),

1708 (s), 1743 (s), 1655 (s), 1537 (s); δ_H(300 MHz, $CDCl_3$) 1.33–1.37 (3H, m, CH_3CH), 1.38–1.96 [6H, m, $NCH_2(CH_2)_3$], 2.04–2.19 (2H, m, PCH_2), 2.43–2.62 (2H, m, PCH_2CH_2), 3.62–3.71 (8H, m), 3.99 (1H, q, *J* 10, NHC*H*P), 4.52 (1H, quintet, *J* 7, C*H*CH_3), 4.97–5.08 (2H, m, PhCH_2), 5.42 and 5.97 (1H, 2d, *J* 7, N*H*CHP), 6.95–7.08 (1H, m, N*H*$CHCH_3$), 7.30 (5H, br s, C_6H_5), 7.64–7.80 (4H, m, C_6H_4); δ_C(75 MHz, $CDCl_3$) 18.01, 19.95, 20.19, 21.14, 21.36, 21.57 (m, PCH_2), 22.93, 23.10, 23.26, 27.35, 27.75, 27.93, 37.29, 37.46, 48.18, 48.77, 49.50, 50.11 (multiplet, NHCHP), 51.91, 51.99, 52.11, 52.20, 52.40, 67.06, 67.11, 67.17, 67.23, 123.26, 132.19, 127.96, 128.00, 128.10, 128.16, 128.27, 128.59, 134.02, 136.25, 156.66, 168.55, 171.09, 173.59; δ_P(121 MHz, $CDCl_3$) 54.85, 55.03, 55.31 and 55.37 (downfield from 85% H_3PO_4); *m*/*z* (ES^+) 602 (9%, $[M + H]^+$), 624 (100, $[M + Na]^+$); HRMS (FAB) MH^+, 602.2304. $C_{29}H_{37}N_3O_9P$ requires 602.2267.

Methyl (2*R*)-2-{3-[(1-amino-5-phthalimidopentyl)(methoxy)-phosphonyl]propionylamino}propionate 21

To a stirred solution of Z-protected pseudo-tripeptide phosphinate **20** (234 mg, 0.389 mmol) in 5% ammonium formate solution in methanol (10 ml) was added 10% palladium on activated charcoal (200 mg, 0.188 mmol Pd) and the mixture stirred at room temperature for 2 h. The catalyst was removed by filtration through a short pad of Celite and the solvent removed *in vacuo*. The residue was purified by flash chromatography on silica gel [eluting with 19:1 (v/v) chloroform–methanol] to give the title compound as a colourless oil (147 mg, 81%); ν_{max}(liquid film)/cm^{-1} 3260 (m, br), 1766 (m), 1708 (s), 1743 (s), 1666 (s), 1549 (m); δ_H(300 MHz, $CDCl_3$) 1.37 (3H, d, *J* 7, CH_3CH), 1.38–1.88 [6H, m, $NCH_2(CH_2)_3$], 1.92 (2H, br, NH_2), 1.94–2.25 (2H, m, PCH_2), 2.40–2.67 (2H, m, PCH_2CH_2), 2.80–2.95 (1H, m, H_2NCHP), 3.65–3.72 (8H, m), 4.51 (1H, quintet, *J* 7, CH_3CH), 7.08–7.23 (1H, m, CH_3CHNH), 7.68–7.83 (4H, m, C_6H_4); δ_C(75 MHz, $CDCl_3$) 17.97, 19.39, 19.79 (2d, J_{C-P} 86, PCH_2), 22.25, 23.32, 23.49, 28.11, 29.91, 37.43, 48.06, 49.99 (d, J_{C-P} 100, H_2NCHP), 51.67, 52.29, 123.1, 132.0, 133.9, 168.3, 171.1, 173.4; δ_P(121 MHz, $CDCl_3$) 57.96, 58.02, 58.23, 58.32 (downfield from 85% H_3PO_4); *m*/*z* (ES^+) 468 (12%, $[M + H]^+$), 490 (30, $[M + Na]^+$).

Methyl (2*R*)-2-{3-[(1-acetylamino-5-phthalimidopentyl)-(methoxy)phosphonyl]propionylamino}propionate 22

To a stirred solution of α-amino pseudo-tripeptide phosphinate **21** (371 mg, 0.794 mmol) in dichloromethane (5 ml) at 0 °C was added triethylamine (188 μl, 1.35 mmol), followed by acetic anhydride (105 μl, 1.11 mmol) and 4-dimethylaminopyridine (small crystal) and the solution stirred for 5 min. The reaction was quenched by the addition of a drop of acetic acid and the solvent removed under reduced pressure. The residue was redissolved in water (10 ml) and extracted with dichloromethane (3 × 10 ml). The pooled organic extracts were washed with brine (30 ml) then dried ($MgSO_4$), filtered and evaporated. The residue was purified by flash chromatography on silica gel [eluting with 19:1 (v/v) chloroform–methanol] to give the title compound as a white foam (367 mg, 91%); ν_{max}(CH_2Cl_2 solution)/cm^{-1} 3050 (m), 1768 (w), 1707 (s), 1740 (m), 1674 (m), 1543 (m), 1515 (m); δ_H(300 MHz, $CDCl_3$), 1.38 (3H, 2d, *J* 7, CH_3CH), 2.02 (3H, 3s, CH_3CONH), 1.59–2.70 (10H, m), 3.64–3.75 (8H, m), 4.24–4.39 (1H, m, N*H*CHP), 4.47–4.58 (1H, m, CH_3CH), 6.33–6.36 (1H, m, N*H*), 6.96–7.22 (1H, m, NH), 7.68–7.84 (4H, m, C_6H_4); δ_C(75 MHz, $CDCl_3$) 17.92, 18.09, 20.99 (d, J_{C-P} 87, PCH_2), 22.96, 23.10, 26.66, 26.87, 27.39, 27.86, 27.99, 28.61, 37.42, 37.09, 46.46, 48.24, 50.63, 52.30, 123.3, 132.2, 134.1, 168.5, 168.6, 171.2, 173.6; δ_P(121 MHz, $CDCl_3$) 50.68, 50.76, 55.17, 55.29, 55.85, 55.90 (downfield from 85% H_3PO_4); *m*/*z* (ES^+) 510 (16% $[M + H]^+$), 532 (100, $[M + Na]^+$); HRMS (FAB) MH^+, 510.2015. $C_{23}H_{33}N_3O_8P$ requires 510.2005.

(2*R*)-2-{3-[(1-Benzyloxycarbonylamino-5-phthalimidopentyl)(hydroxy)phosphonyl]propionylamino}propionic acid 25

To a stirred solution of Z-protected pseudo-tripeptide phosphinate **20** (200 mg, 0.333 mmol) in dry tetrahydrofuran (10 ml) was added bis(tributyltin) oxide (706 μl, 1.33 mmol) and the solution heated under reflux for 48 h. After cooling to room temperature, the solvent was removed under reduced pressure and the residue redissolved in ethyl acetate (10 ml). This solution was extracted with 10% aqueous sodium hydrogen carbonate solution (3 × 5 ml) and the pooled aqueous extracts were washed with *n*-pentane (3 × 10 ml). The separated aqueous layer was acidified to pH 1 by dropwise addition of 2 M hydrochloric acid and the resulting white precipitate extracted with ethyl acetate (3 × 15 ml). The pooled organic extracts were washed with brine (45 ml) then dried ($MgSO_4$), filtered and evaporated. The residue was redissolved in 6:1 (v/v) propan-2-ol–water (2 ml) and to this stirred solution was added sodium borohydride (31 mg, 0.810 mmol). The solution was stirred for 16 h and then acetic acid (0.2 ml) was added. After effervescence had subsided, the flask was stoppered and then heated to 80 °C for 2 h. After cooling to room temperature the solution was diluted to ~5 ml by addition of water and washed with diethyl ether (3 × 5 ml). The water was removed *in vacuo* and the residue redissolved in 1 M potassium hydroxide solution (2 ml). This solution was stirred at room temperature for 4 h. The solution was neutralised by dropwise addition of 2 M hydrochloric acid and the water removed by freeze drying. The crude product was purified by reversed phase HPLC (25 cm × 16 mm Zorbax ODS column, eluting with a gradient from 0.1% trifluoroacetic acid–water to 0.1% trifluoroacetic acid–acetonitrile over 40 min, flow rate 4 ml min^{-1}, detecting at 254 nm) to give the title compound as a white fluffy solid (39 mg, 26%); HPLC retention time t_R (conditions as above) 18 min, mp 116–120 °C; ν_{max}(KBr disc)/cm^{-1} 3404 (m, br), 1691 (s), 1539 (m); δ_H(300 MHz, D_2O) 1.37 and 1.39 (3H, 2d, *J* 7.5, CH_3CH), 1.44–1.92 [8H, m, $H_2NCH_2(CH_2)_3$ and PCH_2CH_2], 2.33–2.51 (2H, m, PCH_2CH_2), 2.83–2.98 [2H, m, $H_2NCH_2(CH_2)_3$], 3.71–3.79 (1H, m, CH_3CH), 4.29 (1H, dt, *J* 7.5, 13, NHC*H*P), 5.11–5.12 (2H, m, PhCH_2), 7.39 (5H, br s, C_6H_5); δ_C(75 MHz, D_2O), 18.68, 24.26, 24.73, 24.88, 25.46, 28.65, 29.01, 30.25 (d, PCH_2), 41.81, 51.34, 52.02 (d, *J* 102, NHCHP), 69.84, 130.0, 130.15, 131.0, 131.4, 139.1, 160.7, 177.1, 179.2; δ_P(121 MHz, D_2O) 47.30 (downfield from 85% H_3PO_4); *m*/*z* (ES^+) 444 (100%, $[M + H]^+$), 466 (9, $[M + Na]^+$); HRMS (FAB) MH^+, 444.1912. $C_{19}H_{30}N_3O_7P$ requires 444.1900.

(2*R*)-2-{3-[(1-Acetylamino-5-aminopentyl)(hydroxy)-phosphonyl]propionylamino}propionic acid 26

To a stirred solution of *N*-acetyl pseudo-tripeptide phosphinate methyl ester **22** (276 mg, 0.542 mmol) in dry tetrahydrofuran (20 ml) was added bis(tributyltin) oxide (1.11 ml, 2.17 mmol) and the solution heated under reflux for 16 h. After cooling to room temperature the solvent was removed under reduced pressure and the residue redissolved in ethyl acetate (10 ml). This solution was extracted with 10% aqueous sodium hydrogen carbonate (3 × 5 ml) and the pooled aqueous extracts were washed with *n*-pentane (3 × 10 ml). The aqueous solution was acidified to pH 1 by dropwise addition of 2 M hydrochloric acid and the water removed *in vacuo*. The residue was redissolved in 6:1 (v/v) propan-2-ol–water (2 ml) and sodium borohydride (48 mg, 1.26 mmol) was added. This solution was stirred for 16 h at room temperature and then acetic acid (0.2 ml) was added. When effervescence had subsided, the flask was stoppered and heated at 80 °C for 2 h. After cooling to room temperature the solvent was removed *in vacuo* and the residue redissolved in water (10 ml). This solution was washed with diethyl ether (3 × 10 ml) and the solvent removed by freeze drying. The crude product was purified by reversed phase HPLC (25 cm × 16 mm Zorbax ODS column, eluting with a gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in

acetonitrile over 40 min, flow rate 4 ml min^{-1}, detecting at 220 nm) to give the title compound as a white fluffy solid (31 mg, 12%); HPLC t_R (conditions as above) 9 min, mp 148–152 °C (decomp.); ν_{max}(KBr disc)/cm^{-1} 3421 (s, br), 1640 (s), 1547 (m); δ_H(360 MHz, D_2O) 1.08 and 1.39 (3H, 2d, *J* 7, CH_3CH), 1.43–1.87 (8H, m), 2.03 (3H, s, CH_3CO), 2.36–2.53 (2H, m, PCH_2CH_2), 2.96 [2H, t, *J* 7.5, $H_2NCH_2(CH_2)_3$], 3.96–4.04 (1H, m, NHC*H*P), 4.31 (1H, q, *J* 7.5, CH_3CH); δ_C(90 MHz, D_2O) 18.32, 18.69, 24.52, 25.12 (d, J_{C-P} 16, PCH*C*H_2), 26.32, 28.11, 29.07, 31.04 (d, J_{C-P} 35, P*C*HCH$_2$), 41.81, 50.97 (d, J_{C-P} 77, NHCHP), 51.54, 176.3, 177.8, 180.0; δ_P(121 MHz, D_2O) 42.16 (downfield from 85% H_3PO_4); *m*/*z* (ES^+) 352 (8% $[M + H]^+$).

2-(3-{[1-(4-Carboxybutanoylamino)-5-aminopentyl](hydroxy)-phosphoryl}propanoylamino)propanoic acid 27

To a stirred solution of α-amino pseudo-tripeptide phosphinate methyl ester **21** (68 mg, 0.146 mmol) in dichloromethane (2 ml) at 0 °C was added triethylamine (40 µl, 0.293 mmol) followed by glutaric anhydride (25 mg, 0.220 mol) and 4-dimethylaminopyridine (small crystal) and the solution stirred for 5 min. The reaction was quenched by the addition of a drop of acetic acid and the solvent removed under reduced pressure. The residue was redissolved in 10% aqueous sodium hydrogen carbonate (5 ml) and washed with diethyl ether (3 × 5 ml). The aqueous solution was then acidified to pH 1 by dropwise addition of 2 M hydrochloric acid and extracted with ethyl acetate (3 × 10 ml). The pooled organic extracts were washed with brine (30 ml) then dried ($MgSO_4$), filtered and evaporated, to give the *N*-glutaryl pseudo-tripeptide phosphinate **23** (ES^+ 582 $[MH^+]$).

The crude solid **23** was redissolved in dry tetrahydrofuran (5 ml) and bis(tributyltin) oxide (175 ml, 0.344 mmol) was added. This stirred solution was heated under reflux for 48 h and after cooling to room temperature the solvent was removed under reduced pressure. The residue was triturated with *n*-pentane (3 × 5 ml) and then redissolved in 6:1 (v/v) propan-2-ol–water (2 ml). To this solution was added sodium borohydride (16 mg, 0.430 mmol) and the solution stirred for 16 h at room temperature. Acetic acid (0.2 ml) was added and after effervescence had subsided, the flask was stoppered and heated at 80 °C for 2 h. After cooling to room temperature the solvent was removed *in vacuo* and the residue redissolved in 1 M potassium hydroxide solution (1 ml). This solution was stirred for 1 h at room temperature then neutralised by the dropwise addition of 2 M hydrochloride acid. After isolation by freeze drying, the crude product was purified by reversed phase HPLC (25 cm × 16 mm Zorbax ODS column, eluting with a gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in acetonitrile over 40 min, flow rate 4 ml min^{-1}, detecting at 220 nm) to give the title compound as a white fluffy solid (10 mg, 16%); HPLC t_R (conditions as above) 10.5 min, mp 132–140 °C (decomp.); ν_{max}(KBr disc)/cm^{-1} 3429 (s, br), 1649 (s), 1539 (m); δ_H(300 MHz, D_2O) 1.38 (3H, d, *J* 7.5, CH_3CH), 1.43–1.92 (10H, m), 2.33–2.51 (6H, m), 2.96 [2H, t, *J* 8, $H_2NCH_2(CH_2)_3$], 3.99 (1H, ddd, *J* 3, 9, 12, NHC*H*CH_2), 4.30 (1H, q, *J* 7.5, NHC*H*CH_3); δ_C(67.5 MHz, D_2O) 16.79, 20.29, 21.38, 23.10, 24.47, 26.86, 28.81, 33.65, 35.45, 39.97, 48.00, 49.52, 175.8, 176.3, 178.5; δ_P(121 MHz, D_2O) 42.85 (downfield from 85% H_3PO_4); *m*/*z* (ES^+) 424 (100%, $[M + H]^+$), 446 (15, $[M + Na]^+$); HRMS (FAB) MH^+, 424.1797. $C_{16}H_{31}N_3O_8P$ requires 424.1849.

N-Propionyl-D-glutamic acid γ-benzyl ester α-methyl ester

A solution of *N*-butoxycarbonyl-D-glutamic acid γ-benzyl ester α-methyl ester[27] (2.04 g, 5.82 mmol) in 25% trifluoroacetic acid in dichloromethane (30 ml) was stirred at room temperature for 1 h. The solvent was removed under reduced pressure and the residue redissolved in water (20 ml). This solution was extracted with diethyl ether (3 × 20 ml) and the water removed from the separated aqueous layer by freeze drying. This gave the intermediate D-glutamic acid γ-benzyl ester α-methyl ester trifluoroacetate salt as a white solid (1.65 g, 78%).

To a stirred solution of D-glutamic acid γ-benzyl ester α-methyl ester trifluoroacetate salt (808 mg, 2.21 mmol) in dichloromethane (10 ml) at 0 °C was added triethylamine (617 µl, 4.43 mmol) followed by propionic anhydride (426 µl, 3.32 mmol). This solution was stirred for 5 min and then the reaction was quenched by the addition of a drop of acetic acid. The solvent was removed under reduced pressure and the residue redissolved in ethyl acetate (50 ml). This solution was washed with 10% aqueous citric acid (50 ml), water (50 ml) and brine (50 ml). The separated organic solution was then dried ($MgSO_4$), filtered and evaporated. The residue was purified by flash chromatography on silica gel [eluting with 1:1 (v/v) light petroleum–ethyl acetate] to give the title compound as a colourless oil which solidified upon standing (586 mg, 86%), mp 55–56 °C; ν_{max}(CH_2Cl_2 solution)/cm^{-1} 3407 (w), 1740 (s), 1680 (s), 1661 (s), 1514 (m); δ_H(300 MHz, $CDCl_3$) 1.14 (3H, t, *J* 7.5, CH_3CH_2), 1.96–2.55 (4H, m), 2.22 (2H, q, *J* 7.5, CH_3CH_2), 3.73 (3H, s, CO_2CH_3), 4.64 (1H, dt, *J* 5, 7.5, NHC*H*CH_2), 5.12 (2H, s, PhCH_2), 6.21 (1H, d, *J* 7.5, NH), 7.36 (5H, br s, C_6H_5); δ_C(75 MHz, $CDCl_3$) 9.52, 27.23, 29.36, 30.28, 51.48, 52.47, 66.74, 128.2, 128.3, 128.5, 135.6, 172.5, 172.7, 173.7; *m*/*z* (ES^+) 308 (100%, $[M + H]^+$), 330 (28, $[M + Na]^+$).

N-Propionyl-D-glutamic acid α-methyl ester

To a stirred solution of *N*-propionyl-D-glutamic acid δ-benzyl ester α-methyl ester (502 mg, 1.64 mmol) in ethanol (20 ml) was added 10% palladium on activated charcoal (87 mg, 81.8 mmol Pd). This mixture was stirred under an atmosphere of hydrogen for 1.5 h at room temperature. The catalyst was removed by filtration through a short pad of Celite and then the solvent was removed under reduced pressure. The product was recrystallised from ethyl acetate–hexane to give the title compound as a white crystalline solid (360 mg, 100%), mp 72–73 °C; ν_{max}(KBr disc)/cm^{-1} 3288 (s, br), 1818 (s), 1738 (s), 1650 (s), 1538 (s); δ_H(300 MHz, $CDCl_3$) 1.15 (3H, t, *J* 7.5, CH_3CH_2), 1.91–2.04 (1H, m), 2.15–2.22 (1H, m), 2.37–2.52 (2H, m), 2.28 (2H, q, *J* 7.5, CH_3CH_2), 3.75 (3H, s, CO_2CH_3), 4.66 (1H, dt, *J* 5, 8, NHC*H*), 6.50 (1H, d, *J* 8, NH); δ_C(75 MHz, $CDCl_3$) 9.57, 27.23, 29.37, 30.00, 51.45, 52.60, 172.6, 174.5, 176.8; *m*/*z* (ES^+) 218 (12%, $[M + H]^+$).

(2*R*)-2-(3-{[1-(4-Carboxy-4-propionylaminobutanoylamino)-5-aminopentyl](hydroxy)phosphonyl}propionylamino)propionic acid 28

To a stirred solution of *N*-propionyl-D-glutamic acid α-methyl ester (340 mg, 1.57 mmol) in dichloromethane (10 ml) at 0 °C was added *N*-hydroxysuccinimide (180 mg, 1.57 mmol) followed by dicyclohexylcarbodiimide (323 mg, 1.57 mmol) and the solution stirred at 0 °C for 1 h. The resulting precipitate was removed by filtration and the solvent removed under reduced pressure. The residue was redissolved in diethyl ether (10 ml) and filtered again to remove residual dicyclohexylurea. The solvent was then removed under reduced pressure and the crude activated ester redissolved in dichloromethane (2 ml).

To a stirred solution of α-amino pseudo-tripeptide phosphinate **21** (202 mg, 0.433 mmol) in dichloromethane (5 ml) at 0 °C was added triethylamine (218 ml, 1.57 mmol) followed by the solution of crude activated ester prepared earlier, plus washings. This solution was then stirred for 1 h at 0 °C and for 16 h at room temperature. The solvent was removed under reduced pressure and the residue redissolved in ethyl acetate (5 ml). This solution was washed with 10% aqueous citric acid (5 ml), saturated aqueous sodium hydrogen carbonate (5 ml) and brine (5 ml). The separated organic layer was then dried ($MgSO_4$), filtered and evaporated to give the protected pseudo-tetrapeptide phosphinate **24** (ES^+ 689 $[M + Na^+]$).

The residue was redissolved in dry tetrahydrofuran (5 ml)

and bis(tributyltin) oxide (594 μl, 1.17 mmol) and the stirred solution heated under reflux for 48 h. After cooling to room temperature the solvent was removed under reduced pressure and the residue triturated with *n*-pentane (3 × 5 ml). The residue was then redissolved in 6:1 (v/v) propan-2-ol–water (2 ml) and to this solution was added sodium borohydride (28 mg, 0.730 mmol). This solution was then stirred for 16 h at room temperature and acetic acid (0.2 ml) was added. After effervescence had subsided the flask was stoppered and heated to 80 °C for 2 h. The solution was allowed to cool to room temperature and the solvent removed *in vacuo*. The residue was redissolved in 1 M potassium hydroxide solution (1 ml) and this solution stirred for 1 h at room temperature. The solution was neutralised by dropwise addition of 2 M hydrochloric acid and the water removed by freeze drying. The crude product was purified by reversed phase HPLC (25 cm × 16 mm Zorbax ODS column, eluting with a gradient ranging from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in acetonitrile over 40 min, flow rate 4 ml min^{-1}, detecting at 220 nm) to give the title compound as a white powder (5.5 mg, 2.6%); HPLC t_R (conditions as above) 11 min; δ_H(360 MHz, D_2O, COSY analysis) 1.14 (3H, t, *J* 7.5, CH_3CH_2), 1.40 (3H, d, *J* 7.5, CH_3CH), 1.59–2.24 [8H, m, $H_2NCH_2(CH_2)_3$ and $HO_2CCHCH_2CH_2$], 2.29–2.48 (8H, m, PCH_2CH_2, $HO_2CCHCH_2CH_2$ and CH_3CH_2), 3.02 [2H, m, $H_2NCH_2(CH_2)_3$], 3.64 (1H, t, *J* 6.5, HO_2CCHCH_2), 4.19–4.34 (2H, m, NHCHP and CH_3CH); δ_P(121 MHz, D_2O) 40.91 (downfield from 85% H_3PO_4); *m/z* (FAB) 495 (MH^+); HRMS (FAB) MH^+, 495.2353. $C_{19}H_{36}N_4O_9P$ requires 495.2220.

Uridinediphospho-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-*m*-diaminopimelic acid (UDPMurNAc-tripeptide)

Cell Wall Synthesis Medium (CWSM) contains, per dm^3: Na_2HPO_4 0.26 g, NH_4Cl 2.0 g, KCl 4.0 g, $MgCl_2$ 4.0 g, Na_2SO_4 0.15 g, $FeSO_4$ 0.1 g, glucose 2.0 g, uracil 40 mg, L-glutamic acid 120 mg, L-lysine 500 mg, L,L-diaminopimelic acid 120 mg, L-alanine 50 mg, chloramphenicol 50 mg, ampicillin 50 mg and D-cycloserine 150 mg. CWSM was sterilised by autoclaving the mixture before addition of $FeSO_4$ and antibiotics. Solutions containing antibiotics and $FeSO_4$ were added through a 0.22 μm sterile filter immediately before inoculation.

Uridinediphospho-*N*-acetylmuramyl-L-Ala-γ-D-Glu-*m*-DAP was isolated from cultures of *Bacillus subtilis* W23 (NCIMB 11 824). *B. subtilis* was grown to stationary phase in 50 ml of PYP medium (bacteriological peptone 20 g dm^{-3}, yeast extract 1.5 g dm^{-3}, KH_2PO_4 5.5 g dm^{-3}) in a baffled flask at 37 °C with shaking at 240 rpm. This was used to inoculate at 2% 4 × 500 ml PYP cultures in 2 l baffled flasks and grown to OD_{660} 1.5. The cultures were chilled in an ice bath and cells were resuspended in 50 ml of CWSM. This was added (25 ml each) to 2 × 225 ml prewarmed CWSM cultures in 2 l baffled flasks and incubated at 37 °C with shaking at 240 rpm for 45 min before chilling in an ice bath. Cells were collected by centrifugation at 5000 *g* (10 min) and stored at −20 °C (5.8 g cells, wet weight). Thawed cells were re-suspended in ice cold 5% trichloroacetic acid (30 ml, 5 ml g^{-1} cells) and left on ice for 30 min. Precipitated material was removed by centrifugation at 5000 *g* (10 min). The pellet was extracted with ice-cold 5% trichloroacetic acid (2 × 15 ml). The supernatants were pooled and extracted with diethyl ether (3 × 60 ml). The aqueous phase was neutralised with 3 M NaOH and concentrated to ~1.5 ml *in vacuo*. This solution was clarified by microcentrifugation and applied to a Biogel P2 column (1.5 × 30 cm column, flow rate 0.9 ml min^{-1}) taking 2 ml fractions. Each fraction was tested for UV absorbance at 262 nm. Active fractions were analysed by reversed phase HPLC [4.6 mm × 25 cm Zorbax ODS column, eluting with 0.1 M $NH_4H_2PO_4$ (pH 4.6), flow rate 1 ml min^{-1}, detecting at 262 nm] using an authentic standard of the title compound to identify fractions containing UDPMurNAc-tripeptide. Fractions containing UDPMurNAc-tripeptide only were pooled and freeze-dried to give the title compound as a white powder (12.8 mg); HPLC t_R (conditions as above) 12.8 min; *m/z* (ES^-) 1050 (15%, $[M - H]^-$).

Purification of D-Ala-D-Ala adding enzyme from JM105/pTB3 (see Table 1)

All steps were performed at 4 °C unless otherwise specified. Enzyme activity was followed by the previously reported phosphate release assay.[6] Protein concentration was determined by the method of Bradford.[20] Buffer A consisted of 50 mM HEPES (pH 7.2), 5 mM $MgCl_2$ and 1 mM EDTA.

JM105/pTB3 was grown at 37 °C in 5 dm^3 Luria Broth containing 100 μg ml^{-1} ampicillin to an A_{595} of 0.6, whereupon IPTG was added to a final concentration of 1 mM, to induce the *tac* promoter. Cells were then grown for a further 5 h at 37 °C and harvested by centrifugation at 6000 *g* for 10 min. The cell pellet (13.9 g) was stored overnight on ice, and re-suspended in 40 ml of 100 mM HEPES (pH 7.2) containing 300 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA and 5 mM DTT, and was passed twice through a French press at 1000 psi. Cell debris was removed by centrifugation at 25 000 *g* for 30 min.

Powdered ammonium sulfate was gradually added to the supernatant to a a final concentration of 25% saturation, and the solution was stirred for 1 h. The solution was cleared by centrifugation at 12 000 *g* for 30 min, and ammonium sulfate was added to the supernatant to a concentration of 50% saturation. After the solution was stirred for a further 1 h, the precipitate was collected by centrifugation at 12 000 *g* for 30 min. The precipitate was re-suspended in 10 ml of buffer A, and was loaded onto an Ultrogel AcA54 gel filtration column (2.5 × 108 cm) and eluted at 60 ml h^{-1} with buffer A. Fractions containing D-Ala-D-Ala adding enzyme activity were pooled, and applied directly to a Q Sepharose 16/10 anion exchange column (Pharmacia). The enzyme was eluted at 4.0 ml min^{-1} using a gradient of 100–300 mM KCl in buffer A over 300 ml, activity eluting at 200 mM KCl. The purified enzyme appeared as a 48 kDa protein by SDS-PAGE, and was judged to be >95% homogeneous. Its specific activity was 20.6 u mg^{-1}.

Kinetic assays of D-Ala-D-Ala adding enzyme

K_M values for D-Ala-D-Ala and ATP, and K_i values for D-Ala-D-Ala analogues were determined using the P_i release assay previously described.[6] Assays (100 μl) contained 100 mM Tris (pH 8.6), 10 mM $MgCl_2$, 100 μM UDPMurNAc-tripeptide (10 nmol), 1 mM ATP, 5–200 μM D-Ala-D-Ala and enzyme (6×10^{-4} units), and were incubated for a fixed time period (5 or 10 min) at 37 °C prior to addition of colour reagent (800 μl) and sodium citrate (100 μl) and measurement of A_{660}. Assays were carried out in duplicate at varying concentrations of D-Ala-D-Ala (or ATP), with a reaction containing no D-Ala-D-Ala as a control, which showed no background ATPase activity. P_i release was quantitated using 1–10 nmol potassium phosphate standards, and K_M values were calculated by plots of $1/v$ *vs.* $1/[S]$. K_i values were measured by determination of the apparent K_M (K_M') for D-Ala-D-Ala in the presence of a fixed concentration of inhibitor, and K_i calculated using $K_M' = K_M(1 + [I]/K_i)$.

Assays using the HPLC-purified pseudo-tetrapeptide phosphinate **28** were complicated by background levels of P_i in the inhibitor which precluded assays at low concentrations of D-Ala-D-Ala. Therefore, assays were carried out at fixed substrate concentrations (100 μM D-Ala-D-Ala) and variable inhibitor concentrations (0–500 μM). K_i was determined by a Dixon plot ($1/v$ versus $[I]$),[30] giving a linear plot and a value of 200 (±20) μM for K_i.

Note added in proof

A recent paper published since the submission of this article provides several experimental observations which strongly support the hypothetical intramolecular mechanism proposed in Scheme 5 for reactions of phosphinic acid **13** (L. A. Reiter and B. P. Jones, *J. Org. Chem.*, 1997, **62**, 2808).

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