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Synthesis of new peptide inhibitors of the *meso*-diaminopimelate-adding enzyme

P Le Roux**, G Auger, J van Heijenoort, D Blanot*

Laboratoire des Enveloppes Bactériennes et des Peptides, CNRS, URA 1131, Bâtiment 432, Université de Paris-Sud, 91405 Orsay, France

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Summary — In order to obtain inhibitors of the *meso*-diaminopimelate-adding enzyme, which catalyzes an early reaction in the biosynthesis of bacterial peptidoglycan, several new peptide derivatives of general formula N^{α}-propionyl-L-alanyl-D(or *ambo*)-Xaa were synthesized: four transition-state analogs (Xaa = phosphinothricin, homocysteic acid, buthionine sulfoximine, buthionine sulfoximine phosphate), three analogs of γ-D-glutamyl phosphate (Xaa = 3-phosphonoacetamido-alanine, 3-phosphonomethylamino-aspartic acid, 2-amino-6-phosphonohexanoic acid), and one derivative with Xaa = ornithine. After preincubation with partially purified *meso*-diaminopimelate-adding enzyme from *Escherichia coli*, peptide derivatives with Xaa = buthionine sulfoximine, 3-phosphonoacetamido-alanine and 2-amino-6-phosphonohexanoic acid appeared to be among the best inhibitors obtained up to date, with I_{50} values between 0.6 and 1.2 mM. When the two first compounds were tested for *in vitro* antibacterial activity, they gave negative results.

meso-diaminopimelate-adding enzyme / synthetic inhibitors / peptide derivatives / transition-state analogs / γ-D-glutamyl phosphate analogs

Introduction

Peptidoglycan (murein) is an essential constituent of the cell wall of eubacteria [1]; therefore, the enzymes responsible for its biosynthesis are potential targets for antibacterial agents [2–4]. Recently, we purified three enzymes involved in the assembly of the peptide part of peptidoglycan and studied their properties [5–9]. In particular, a program of synthesis of inhibitors of the *meso*-A_2pm-adding enzyme was undertaken [5, 10, 11]. To date, the following conclusions can be drawn: i) owing to the high specificity of the enzyme, efficient competitive inhibitors should respect the integrity of the structure of the nucleotide substrate UDP-MurNAc-dipeptide [5]; and ii) on the other hand, inhibitors with simpler structures can be obtained on the basis of the mechanism of action of the enzyme (*eg* transition-state analogs or affinity labelers) [11].

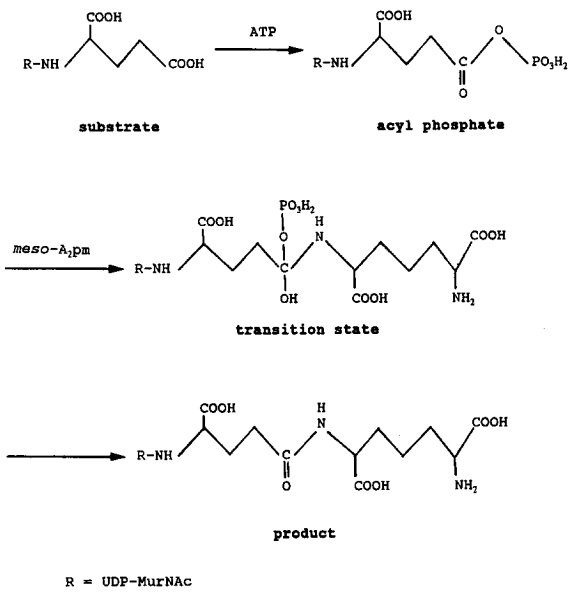
In this paper, we wish to report on the synthesis and the inhibitory properties of several new peptide derivatives, including four transition-state analogs for the *meso*-A_2pm-adding enzyme and three analogs of its acyl phosphate intermediate.

Results

A few years ago, we postulated that the mechanism of action of the *meso*-A_2pm-adding enzyme (scheme 1) was similar to that of glutamine and γ-glutamyl-cysteine synthetases [10, 11]: the γ-carboxyl group of glutamic acid is activated by ATP to give an acyl phosphate intermediate (γ-glutamyl phosphate), which undergoes nucleophilic attack of ammonia or cysteine to yield a tetrahedral transition state [12, 13]. Such an analogy has also been used by Patchett *et al* for the design of inhibitors of another enzyme of peptido-

*Correspondence and reprints.
**Present address:* Abbott Diagnostic, 94518 Rungis, France.

Abbreviations: Abbreviations for amino acids, peptides and their derivatives are those recommended by the IUPAC-IUB (*Eur J Biochem* 1984, 138, 9–37). In particular, the prefix *ambo* is used instead of the symbol DL in diastereoisomeric peptides whose ratio may not be unity. Other abbreviations: A_2pm, 2,6-diaminopimelic acid; Boc, *tert*-butyloxycarbonyl; Bu^t, *tert*-butyl; Bzl, benzyl; DCCI, *N,N'*-dicyclohexylcarbodiimide; DCHA, dicyclohexylamine; DMF, dimethylformamide; Et_3N, triethylamine; MurNAc, *N*-acetylmuramyl; Pr, propionyl; Z, benzyloxycarbonyl.

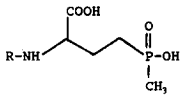
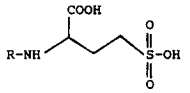
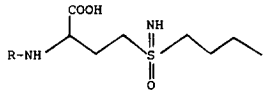
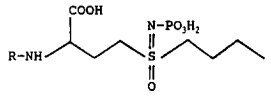
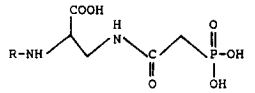
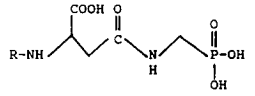
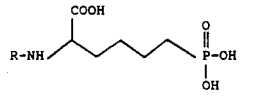
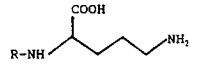


Scheme 1.

glycan biosynthesis, the D-alanine: D-alanine ligase [14, 15]. In previous work, we chose certain transition-state analogs of the two aforementioned synthetases: methionine *S*-oxide, methionine *S,S*-dioxide, methionine sulfoximine, methioninesulfoximine phosphate and 2-amino-4-phosphonobutyric acid. Their combination, in the D or *ambo* configuration, with Pr-L-Ala, a moiety that partly mimics UDP-MurNAc-L-Ala, provided moderate inhibition of the *meso*-A_2pm-adding enzyme from *Escherichia coli* [11].

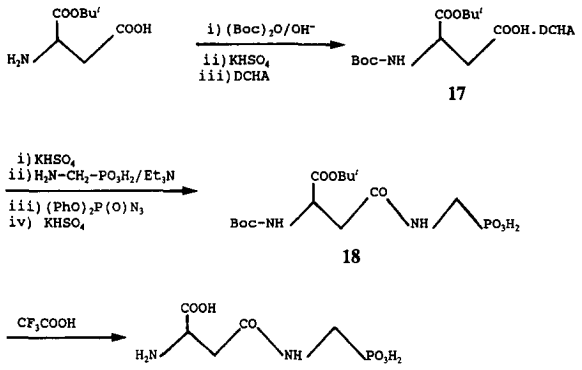
In the present work, we have synthesized eight new peptide derivatives of general formula Pr-L-Ala-Xaa (table I), in which Xaa (D or *ambo* configuration) represents: i) phosphinothricin **1**, a transition-state analog for glutamine synthetase [16]; ii) homocysteic acid **2**, buthionine sulfoximine **3** and buthionine sulfoximine phosphate **4**, all three being transition-state analogs for γ-glutamylcysteine synthetase [17–19]; iii) 3-phosphonoacetamido-alanine **5**, 3-phosphonomethylamino-aspartic acid **6** and 2-amino-6-phosphonohexanoic acid **7**, three analogs of γ-glutamyl phosphate. Compounds **5** (in the L form) is an efficient inhibitor of glutamine synthetase [20]; **6** and **7** (in the D form) are antagonists of excitatory amino acids responsible for neuronal depolarization [21, 22]; and iv) ornithine **8**, its δ-amino function being assumed to mimic the α-amino function of the second substrate, *meso*-A_2pm. This explanation has been proposed for the strong inhibition of folyl-polyglutamate synthetase, a similar enzyme, by analogs of folic acid, aminopterin or methotrexate containing ornithine in place of glutamic acid [23–25].

Table I. Formulae of compounds Xaa and peptide derivatives Pr-L-Ala-Xaa.

Formula	*Xaa (R=H)*	*Peptide derivative (R=Pr-L-Ala)*
	(DL)	1 9
	(DL)	2 10
	(DL,*RS*)	3 11
	(DL,*RS*)	4 12
	(DL)	5 13
	(D)	6 14
	(DL)	7 15
	(D)	8 16

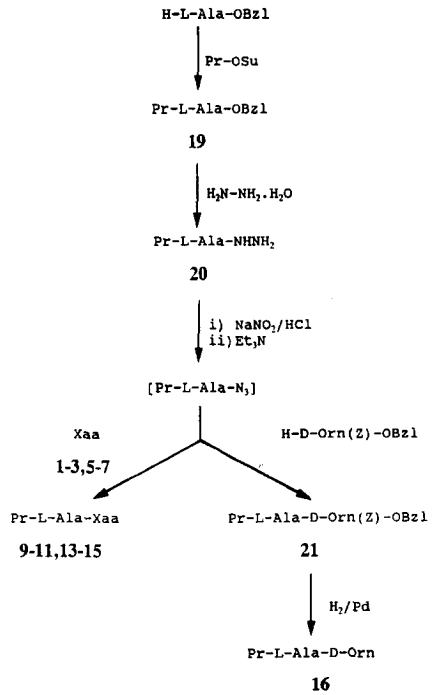
Synthesis

γ-Glutamyl phosphate analogs **5** and **6**, which were not commercially available, had to be synthesized. **5** was obtained from phosphonoacetylhydrazide and *rac*-2,3-diaminopropionic acid [20]. **6**, which is a β-aspartyl derivative, was prepared according to scheme 2. The problem was to protect the amino and α-carboxyl functions of D-aspartic acid, leaving the β-carboxyl function intact for coupling. For this purpose, Boc-D-Asp-OBu^t.DCHA (**17**) was synthesized from α-*tert*-butyl D-aspartate. Coupling with aminomethylphosphonate was carried out by utilizing diphenyl phosphoroazidate [26], giving protected **18** with a moderate yield. Removal of the protecting groups by trifluoroacetic acid afforded **6**.



Scheme 2.

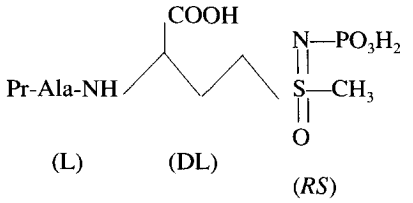
The synthesis of peptide derivatives **9–11** and **13–15** (scheme 3) was performed by coupling Pr-L-Ala-N_3 with Xaa (**1–3** and **5–7**). This strategy presents two characteristics: i) the terminal carboxyl group was left unprotected in order to by-pass the esterification step, which has been shown to give rise to important side reactions with amino acids containing a phosphonic or a sulfoximine function [11]; ii) the desired derivatives were obtained in one step from Xaa. This allowed the use of minimum amounts of these precious compounds. The azide method was used in order to avoid racemization of the alanyl residue during the coupling reaction. The purification was carried out by HPLC. Since **1**, **2**, **3**, **5** and **7** are racemic, mixtures of diastereoisomers were obtained for **9**, **10**, **11**, **13** and **15**. Owing to poor separations, no efforts were made to isolate them. Only in one case (**11**) was it possible to obtain an appreciable separation of the two isomers **11a** and **11b** (fig 1). Since buthionine sulfoximine **3** contains two chiral centers, **11a** and **11b** are themselves mixtures of two isomers. However, owing to the difficult availability of the different isomers of DL-buthionine-(*S,R*)-sulfoximine, it was not possible to assess the identity of **11a** and **11b**.



Scheme 3.

For the synthesis of **16** (scheme 3), we used an ornithine derivative in which the α-amino and the carboxyl functions were protected, N^{α}-*Z*-ornithine benzyl ester. This amino acid ester can be easily prepared [27]. After coupling by the azide method, protected dipeptide **21** was purified by silica gel column chromatography and the protecting groups were removed by catalytic hydrogenolysis.

Compound **12** was synthesized by phosphorylation of **11** according to scheme 4. This strategy had already been used for the synthesis of methionine sulfoximine phosphate [28] and of a peptide derivative thereof [11]. Two improvements were made: i) the condensation of methyl ester **22** with cyanoethyl phosphate in the presence of DCCI was carried out in the cold instead of at 30°C; ii) final purification was achieved by HPLC. The desired product was identified by amino acid and phosphate analyses (fig 2). The overall yield of synthesis of **12** from **22** was 32%. The same scheme of synthesis, applied to Pr-L-Ala-*ambo*-methionine sulfoximine, afforded derivative **23** with a 30% yield from the corresponding methyl ester, instead of 10% in our original preparation [11].



(L) (DL) (*RS*)

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The purity and the identity of all peptide derivatives were checked by TLC, HPLC, amino acid analysis, ^{1}H-NMR and ^{31}P-NMR.

Inhibitory assays

The putative inhibitory effect of compounds **9–16** and **23** was tested on the partially purified *meso*-A_2pm-

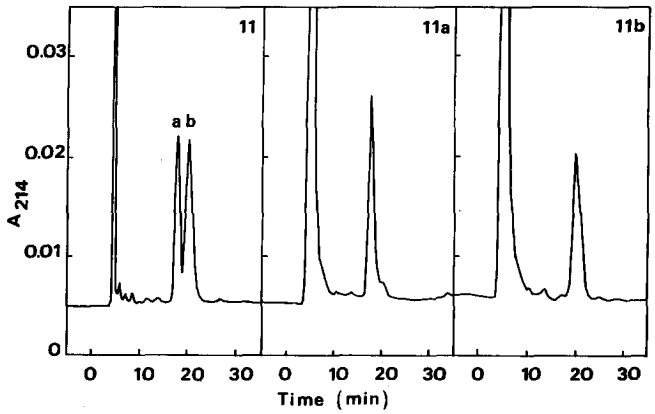
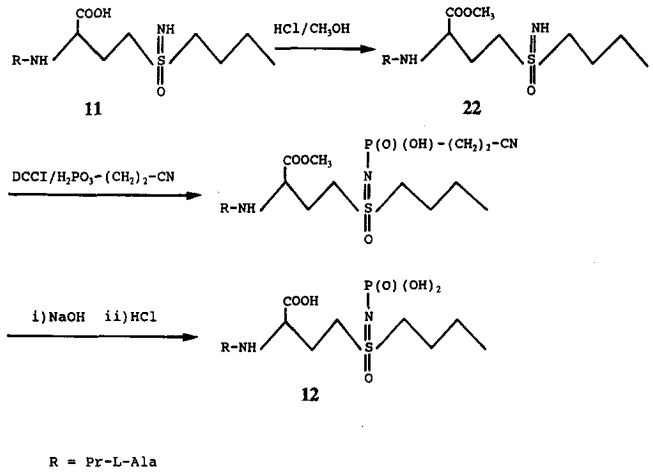
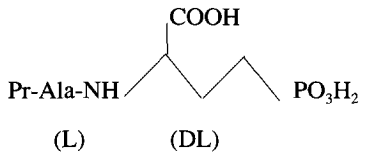


Fig 1. Analytical HPLC (condition A_3) of purified **11**, **11a** and **11b**. The peak at 5 min is due to acetic acid, the compounds being dissolved in dilute acetic acid.



Scheme 4.

adding enzyme from *E coli* [8]. A previously synthesized compound, Pr-L-Ala-*ambo*-2-amino-4-phosphonobutyric acid **24** [11] was also tested for comparison:



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Two types of assays were carried out. In the direct assay, the substrates, the putative inhibitor and the other constituents were mixed, and the reaction was started by the addition of the enzyme. In the other assay, the enzyme was preincubated with the putative inhibitor for 15 min in the presence of ATP and Mg^{2+} prior to the addition of the substrates. The results are shown in table II. Under the conditions of the direct assay, most compounds inhibited the enzyme very weakly, if at all. Their efficiency was in general much improved after the 15-min preincubation.

The case of sulfoximine phosphate derivatives **12** and **23** deserves special mention. Previously, **23** had been found to greatly activate the *meso*-A_2pm-adding enzyme of a crude *E coli* extract [11]. This had been explained by the hydrolysis of **23** by phosphatase activity present in the extract, yielding inorganic phosphate, an activator of the enzyme [8]. In the present work, we have checked by HPLC that **12** and **23** are totally stable in the conditions of incubation with partially purified *meso*-A_2pm-adding enzyme; in particular, no release of the corresponding sulfoximine derivative (**11** and Pr-L-Ala-*ambo*-methionine sulfoximine, respectively), *ie* no splitting of the N-P bond, could be detected (data not shown). Therefore, our enzymatic preparation contains no phosphatase activity, and the values obtained by the inhibition assays (table II) represent the actual inhibitory properties of these compounds, without any interference by an activating effect due to enzymatically released phosphate.

Antibacterial testing

Two peptide derivatives, **11** and **13**, were tested for *in vitro* antibacterial activity against several organisms (see list in *Experimental protocols*). No inhibition of growth was observed up to 256 μg/ml. Presumably, this can be in part explained by the lack of affinity of the dipeptide transport system for N^{α}-acyl dipeptides [29].

Discussion

This work is part of a program of rational search for efficient inhibitors of the *meso*-A_2pm-adding enzyme. Preliminary results obtained with simple transition-state analogs [11] encouraged us to synthesize more elaborated compounds. From the results of the enzymatic experiments, it appears that some of them (**11a**, **11b**, **13**, **15**) are among the best inhibitors obtained up to date. The comparison of present and previous [11] data allows us to draw some conclusions regarding the interactions of the inhibitors with the enzyme.

i) A phosphonic function in position γ (as in **24**) of the side chain of Xaa enhances the inhibitory effect, contrary to a phosphinic (**9**) or sulfonic (**10**) function. A beneficial effect of a phosphonic function in

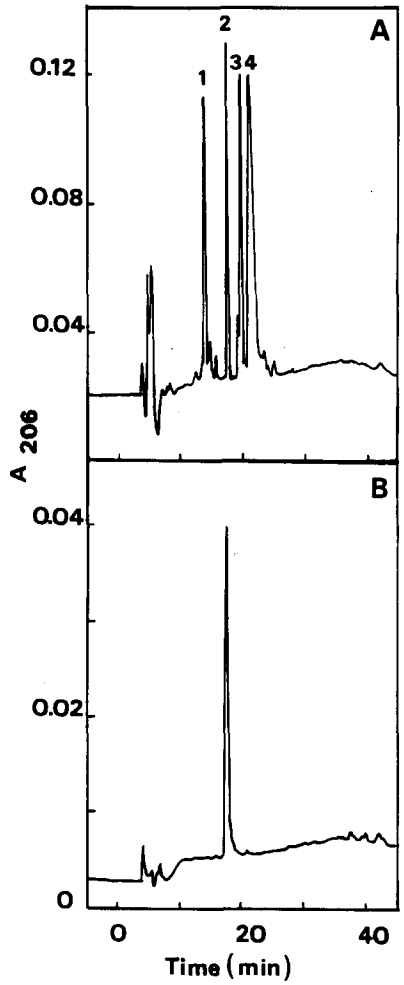

Fig 2. A. Analytical HPLC (condition A_1) of crude **12**. The four compounds were collected and analyzed for amino acids and phosphate. Peak 4 was starting material **11**. Peak 3 had the expected composition, but was unstable, yielding several peaks after reinjection. Peak 2 was the desired compound **12**. Peak 1 had two phosphates for one alanine and one buthionine sulfoximine; it decomposed partly into **12** on storage. **B.** Analytical HPLC (condition A_1) of **12** purified by semi-preparative HPLC in condition P_1.

position ε of acyl phosphate analogs is also observed (**13** or **15**), depending on the structure of the side chain (compare with **14**).

ii) The buthionine sulfoximine derivative **11** is a better inhibitor than its methionine sulfoximine counterpart [11]. If one assumes, as demonstrated for glutamine synthetase [30], that the alkyl substituent of the sulfoximine compounds interacts with the binding site of the amino function of the second substrate, it is likely that the butyl moiety mimics, although imperfectly, *meso*-A_2pm and thus favors the binding of the inhibitor to the enzyme. It is curious to notice

Table II. Inhibition of *meso*-A_2pm-adding enzyme by peptide derivatives.

Compound	I_{50} *(mM)* [a]	
	Direct assay	*Assay with preincubation*
9	> 10 (13%)	> 10 (40%)
10	> 10 (26%)	> 10 (45%)
11a	9.6	1.1
11b	3.9	0.6
12	NI	> 10 (7%)
13	4.5	0.9
14	> 10 (39%)	4.2
15	8.0	1.2
16	> 10 (23%)	9.0
23	NI	8.2
24	5.8	1.1

[a]When I_{50} > 10 mM, the percentage of inhibition at 10 mM is given in parentheses. NI, no inhibition at 10 mM.

that both isolated pairs of diastereoisomers appreciably inhibit the enzyme, **11b** being twice as potent as **11a**. Since **11** contains three chiral centers, four isomers must be considered: LL*R*, LL*S*, LD*R* and LD*S*. Previously, we showed that compounds with Xaa in the L configuration inhibited the enzyme poorly or not at all [11]. Therefore, an explanation for the good inhibitory potencies of **11a** and **11b** might be a separation based only on the stereochemistry of the sulfur atom.

iii) Analog **16** is a very weak inhibitor of the enzyme. Therefore, the δ-amino function of ornithine is not sufficient as a structural element of the second substrate, contrary to the case of folylpolyglutamate synthetase [23–25].

iv) The sulfoximine phosphate compounds are in theory the closest analogs of the phosphorylated transition state and should, therefore, be the best inhibitors. However, even though one takes into account that they are mixtures of four isomers, compounds **12** and **23** appear to be devoid of inhibitory properties. We have no satisfactory explanation for these results.

The good inhibitory effects obtained with some compounds described here should not conceal their weak affinity for the enzyme: their I_{50} values in the direct assay are in the millimolar range, whereas the K_m for the nucleotide substrate is 76 μM [8]. This shows that these compounds very poorly mimic the reaction intermediates of the *meso*-A_2pm-adding enzyme. It seems obvious that a strong affinity necessitates the synthesis of compounds R-L-Ala-Xaa, in which Xaa (**3**, **5** or **7**) is combined to moieties (R) more complex than propionyl. An alternative might be

the synthesis of derivatives Pr-L-Ala-Yaa, in which Yaa would represent transition-state analogs comprising the nucleophile specific to the enzyme, *meso*-A_2pm. Another possibility in the search for inhibitors of this activity consists in analogs of the second substrate, *meso*-A_2pm [8, 31].

Experimental protocols

Melting points were taken on a Tottoli apparatus (Büchi, Switzerland) in open capillary tubes and were uncorrected. Optical rotations were determined with a Perkin–Elmer 141 polarimeter. Elemental analyses were performed by the Service Central d'Analyses du CNRS (Vernaison, France). Phosphorus was assayed as orthophosphate according to Chen *et al* [32] as modified by Ames and Dubin [33]. The amino acid composition of acid hydrolysates (6 M HCl, 95°C, 16 h) was determined with a Biotronik LC2000 analyzer (Biotronik, Frankfurt/Main, Germany); amino acid standards were hydrolyzed in the same conditions. ^{1}H-NMR spectra at 400 MHz and ^{31}P-NMR spectra at 200 MHz were recorded in D_2O at the Service de RMN of the ICSN (Gif-surYvette, France) on Bruker WM400 and SY200 apparatuses; chemical shifts (δ, ppm) were calculated relative to sodium 3-trimethylsilylpropionate-2,2,3,3-d_4 for ^{1}H or orthophosphoric acid for ^{31}P.

Ascending thin-layer chromatography (S_1-S_4) was performed on precoated plates of silica gel 60 (Merck, Darmstadt, Germany) using the following solvent systems (v/v): S_1, 1-butanol/acetic acid/water 3:1:1; S_2, ethyl acetate; S_3, 1-propanol/water/28% ammonium hydroxide 6:1:3; S_4, 1-propanol-water 2:1. Compounds were detected with iodine as a general reagent, ninhydrin for free amino groups, chlorine/*N,N,N'N'*-tetramethyl-4,4'-diaminodiphenylmethane [34] for amino acid derivatives and peptides, and Folin-Ciocalteu reagent [35] for hydrazide compounds.

Column chromatography was performed with silica gel 60, particle size: 0.2–0.5 mm (Merck). Analytical HPLC experiments (A_1–A_3) were carried out in the following conditions: A_1, 250 x 4-mm ODS-Hypersil column (particle size: 5 µm; Bischoff, Leonberg, Germany), solvent A (0.5% trifluoroacetic acid), solvent B (0.425% trifluoroacetic acid in water/acetonitrile 25:75 (v/v)), gradient from 2 to 98% B in 40 min, flow rate 0.5 ml/min; A_2, same column, solvents and flow rate, 0% B from 0 to 15 min, 0–20% B from 15 to 30 min, 20–98% B from 30 to 40 min; A_3, 250 x 4.6-mm Vydac 218TP (particle size: 10 µm; The Separations Group, Hesperia, CA), 20 mM triethylammonium phosphate (pH 4)/acetonitrile 94:6 (v/v), flow rate 0.83 ml/min. Compounds were detected either at 206 nm or at 214 nm. Semi-preparative HPLC experiments (P_1–P_6) were performed with a 300 x 7.8-mm µBondapak C_{18} column (Waters, Milford, MA) in 20 mM triethylammonium formate buffers, flow rate 2.5 ml/min, at the following pH values and buffer/acetonitrile proportions (v/v): P_1, pH 3.55, 92:8; P_2, pH 4.0, 100:0; P_3; pH 4.0, 99.5:0.5 ; P_4, pH 4.0, 99:1. Eluents for conditions P_5 and P_6 were 20 mM ammonium formate (pH 5.7)/acetonitrile 99:1, and 0.2% trifluoroacetic acid, respectively.

DL-Buthionine-*(S,R)*-sulfoximine, *rac*-homocysteic acid, *rac*-2-amino-6-phosphonohexanoic acid and aminomethylphosphonic acid were obtained from Sigma (St Louis, MO). *rac*-Phosphinothricin [36, 37] was a gift from Hoechst Pflanzenschutzforschung-Biologie (Frankfurt, Germany). 2-Cyanoethylphosphate was prepared from its barium salt (Sigma). Di-*tert*-butyldicarbonate and diphenyl phosphoroazidate were purchased from Fluka (Buchs, Switzerland) and Aldrich (Steinheim, Germany), respectively. *meso*-A_2pm was prepared according to van Heijenoort and Bricas [38]. *meso*-[^{14}C]A_2pm (11.6 TBq/mol) was provided by CEA (Saclay, France). UDP-MurNAc-L-Ala-D-Glu was prepared according to Flouret *et al* [39]. Partially purified *meso*-A_2pm-adding enzyme was obtained from *E coli* JM83 (pHE5) as already described [8].

Boc-D-Asp-OBu^t.DCHA ***17***

α-*tert*-Butyl D-aspartate (946 mg, 5 mmol) [40] was dissolved in water (13 ml) containing sodium hydroxide (7.5 mmol). Di-*tert*-butyl dicarbonate (1.31 g, 6 mmol) in dioxane (7 ml) was added dropwise. After stirring for 24 h at room temperature, the mixture was evaporated. The residue was taken up in ethyl acetate (70 ml) and water (50 ml). After acidification with 1 M $KHSO_4$, the aqueous phase, which separated, was extracted again with ethyl acetate (3 x 70 ml). The pooled organic phases were washed with water, dried over $MgSO_4$ and evaporated. The product was crystallized in ether as dicyclohexylammonium salt (Tomasz [41] described the synthesis of the free acid of the L-isomer by a different route). Yield 1.65 g (70%). mp 142°C. $[\alpha]_D^{20}$ + 6.8° (*c* 1.3, methanol). TLC: $R_f(S_1)$ 0.92 and 0.77 (DCHA). Anal $C_{25}H_{46}N_2O_4$ (C, H, N).

Boc-D-Asp(NH-CH_2-PO_3H_2)-OBu^t ***18***

Compound **17** (2.55 g, 5.41 mmol) was converted into free acid according to Spangenberg *et al* [42]. Boc-D-Asp-OBu^t was dissolved in DMF (6 ml). Aminomethylphosphonic acid (600 mg, 5.41 mmol) and Et_3N (1.6 ml, 11.4 mmol) were added. The solution was cooled to 0°C, and diphenyl phosphoroazidate (1.3 ml, 5.95 mmol) was added dropwise. The reaction mixture was stirred for 2 h at 0°C, then for 4 days at 4°C. On the third day, more diphenyl phosphoroazidate (1.3 ml, 5.95 mmol) was added. The solvent was evaporated and the mixture was taken up in ethyl acetate (60 ml) and water (50 ml). After acidification with 1 M $KHSO_4$, the organic phase, which separated, was extracted again with water (50 ml). The pooled aqueous phases were lyophilized. The lyophilisate was extracted with ethyl acetate/isopropanol 1:1 (v/v; 2 x 30 ml). The product obtained after evaporation was purified by silica gel column chromatography in chloroform/methanol/formic acid 60:40:1 (v/v). Yield 1.24 g (60%) . TLC: $R_f(S_1)$ 0.51. Amino acid analysis: Asp 1.00; aminomethylphosphonic acid 1.05.

3-Phosphonomethylamino-D-aspartic acid ***6***

Compound **18** (1.15 g, 3 mmol) was dissolved in trifluoroacetic acid (10 ml) and anisole (2 ml). The solution was left for 1 h at room temperature. After evaporation of the acid, ether (20 ml) and water (20 ml) were added. The organic phase, which separated, was extracted with water (20 ml). The pooled aqueous phases were washed with ether (10 ml) and lyophilized. Yield 887 mg (87%) . TLC: $R_f(S_3)$ 0.17. Amino acid analysis: Asp 1.00; aminomethylphosphonic acid 1.10. ^{1}H-NMR: 2.7–3.0 (m, 2H, ND-CH_2-P); 3.2–3.4 (m, 2H, CH_2-CO); 3.96 (dd, 1H, CH^α). ^{31}P-NMR: 11.15 (s).

Pr-L-Ala-OBzl ***19***

L-Alanine benzyl ester, toluene-4-sulfonate (2.11 g, 6 mmol) [43] was dissolved in DMF (50 ml). Et_3N was added until pH 8.5–9 (moist indicating paper). The solution was cooled to 0°C, and *N*-hydroxysuccinimidyl propionate (1.03 g, 6 mmol) [5] in DMF (18 ml) was added dropwise. After overnight stirring at room temperature, the solvent was evaporated and the residue

was taken up in ethyl acetate (50 ml). The organic solution was washed with 0.5 M $KHSO_4$ and brine, dried over $MgSO_4$ and evaporated. The oily residue was dried under vacuum. Yield 1.47 g (>100%). TLC: $R_f(S_1)$ 0.89.

Pr-L-Ala-NHNH$_2$ ***20***
The preceding oily residue **19** was dissolved in absolute ethanol (5 ml). After addition of hydrazine hydrate (2 ml), the mixture was stirred for 4 h at room temperature. After evaporation of the solvent and trituration in petroleum ether, the crude product obtained was crystallized from methanol/ethyl acetate. Yield 793 mg (83%). mp 151°C. $[\alpha]_D^{20}$ –61.8° (*c* 1, H_2O). TLC: $R_f(S_1)$ 0.40. The product was further characterized as its benzylidene derivative: recrystallization from 20% ethanol, mp 182–184°C. $[\alpha]_D^{20}$ +42.4° (*c* 1, ethanol), anal $C_{13}H_{17}N_3O_2$ (C, H, N).

General procedure for the synthesis of Pr-L-Ala-Xaa ***9–11, 13–15***
Compound **20** (80 mg, 0.5 mmol) was dissolved in DMF (2.5 ml) . The solution was cooled to –10°C, and concentrated HCl (0.125 ml, 1.5 mmol) was added. Then, a solution of sodium nitrite (35 mg, 0.5 mmol) in water (0.125 ml) and DMF (0.625 ml) was added dropwise. The reaction mixture was neutralized with Et_3N (0.140 ml, 1 mmol), and a solution of Xaa **1–3** or **5–7** (0.5 mmol) and Et_3N (70 µl, 0.5 mmol) in DMF (0.75 ml) and water (0.75 ml) was added dropwise. The mixture was stirred for 4 days at 4°C. The progress of the reaction was followed by TLC and, when necessary, more Pr-L-Ala-N_3 was added. The solvents were evaporated and the residue was taken up in ethyl acetate (40 ml) and water (40 ml). After acidification to pH 2 with 5% $KHSO_4$, the aqueous phase, which separated, was washed with ethyl acetate (2 x 40 ml). The aqueous phase was lyophilized, and the lyophilizate was extracted with a mixture of organic solvents (3 x 50 ml) [11]. After evaporation, the product was purified by column chromatography or/and semi-preparative HPLC. Extraction mixtures and chromatography conditions are described below, together with analytical data .

Pr-L-Ala-ambo-phosphinothricin ***9***
Extraction: ethyl acetate/isopropanol 1:1 (v/v). Purification: semi-preparative HPLC in condition P_4. Yield 80%. $[\alpha]_D^{20}$ –42.0° (*c* 0.39, H_2O). TLC: $R_f(S_3)$ 0.33. Analytical HPLC: $R_t(A_1)$ 12.9 min (split peak); $R_t(A_2)$ 29.9 and 30.4 min. Amino acid analysis: Ala 1.00; phosphinothricin 1.00. ^{1}H-NMR: 1.06 (dt, 3H, C*H*$_3$-CH$_2$-CO); 1.23 (q, *ca* 26H, Et_3N); 1.27 (CH$_3$-P, partly covered by Et_3N); 1.34 (dd, 3H, CH-C*H*$_3$); 1.43 (m, 2H, CH$_2$-P); 1.79 (m, 1H, C*H*$_2$-CH$_2$-P); 1.94 (m, 1H, C*H*$_2$-CH$_2$-P); 2.24 (m, 2H, CH$_3$-C*H*$_2$-CO); 3.16 (q, *ca* 17H, Et_3N); 4.14 (m, 1H, CH$^\alpha$ phosphinothricin); 4.26 (q, 1H, CH$^\alpha$ Ala). ^{31}P-NMR: 43.51 (d).

Pr-L-Ala-ambo-homocysteic acid ***10***
Extraction: ethyl acetate/isopropanol 1:1 (v/v). Purification: ion-exchange chromatography on AG 1-X2 resin (OH^-; elution with 1.5 M formic acid) and semi-preparative HPLC in condition P_4. Yield 40%. $[\alpha]_D^{20}$ –40.4° (*c* 0.10, H_2O). TLC: $R_f(S_1)$ 0.32. Analytical HPLC: $R_t(A_1)$ 7.2 min; $R_t(A_2)$ 9.9 min (split peak). Amino acid analysis: Ala 1.00; homocysteic acid 0.93. ^{1}H-NMR: 1.06 (dt, 3H, C*H*$_3$-CH$_2$-CO); 1.24 (t, *ca* 12H, Et_3N); 1.34 (dd, 3H, CH-C*H*$_3$); 2.06 (m, 1H, C*H*$_2$-CH$_2$-S); 2.24 (m, 3H, C*H*$_2$-CH$_2$-S + CH$_3$-C*H*$_2$-CO); 2.84 (m, 2H, CH$_2$-S); 3.16 (q, *ca* 8H, Et_3N); 4.26 (m, 2H, 2CH$^\alpha$).

Pr-L-Ala-ambo-buthionine sulfoximine ***11***
Extraction: ethyl acetate/butanol-1 1:1 (v/v). Purification: silica gel column chromatography in chloroform/methanol/formic acid/water 50:50:1:2 (v/v). Yield 35%. $[\alpha]_D^{20}$ –42.6° (*c* 0.5, H_2O). TLC: $R_f(A)$ 0.47. Analytical HPLC: $R_t(A_1)$ 21.5 min; $R_t(A_3)$ 17.5 and 20.0 min. Amino acid analysis: Ala 1.00; buthionine sulfoximine 0.92. ^{1}H-NMR: 0.95 (br t, 3H, C*H*$_3$-CH$_2$-CH$_2$); 1.12 (br t, 3H, C*H*$_3$-CH$_2$-CO); 1.40 (br d, 3H, CH-C*H*$_3$); 1.49 (m, 2H, CH$_3$-C*H*$_2$-CH$_2$); 1.78 (m, 2H, CH$_3$-CH$_2$-C*H*$_2$); 2.17 (m, 1H, CH-C*H*$_2$); 2.25–2.45 (m, 3H, CH$_3$-C*H*$_2$-CO + CH-C*H*$_2$); 3.1–3.4 (m, 4H, 2S-CH$_2$); 4.25–4.40 (m, 2H, 2CH$^\alpha$).
By semi-preparative HPLC in condition P_5, two groups of diastereoisomers were separated: i) **11a**. Analytical HPLC: $R_t(A_1)$ 21.5 min; $R_t(A_3)$ 17.5 min (trace of **11b** at 20.0 min; see fig 1). Amino acid analysis: Ala 1.00, buthionine sulfoximine 1.02. ^{1}H-NMR: 0.91 (t, 3H, C*H*$_3$-CH$_2$-CH$_2$); 1.08 (t, 3H, C*H*$_3$-CH$_2$-CO); 1.36 (d, 3H, CH-C*H*$_3$); 1.45 (m, 2H, CH$_3$-C*H*$_2$-CH$_2$); 1.74 (m, 2H, CH$_3$-CH$_2$-C*H*$_2$); 2.12 (m, 1H, CH-C*H*$_2$); 2.26 (q, 2H, CH$_3$-C*H*$_2$-CO); 2.31 (m, 1H, CH-C*H*$_2$); 3.1–3.3 (m, 4H, 2S-CH$_2$); 4.25–4.40 (m, 4H, 2CH$^\alpha$); ii) **11b**. Analytical HPLC: $R_t(A_1)$ 21.5 min; $R_t(A_3)$ 20.0 min (trace of **11a** at 17.5 min; see fig 1). Amino acid analysis: Ala 1.00, buthionine sulfoximine 0.98. ^{1}H-NMR: 0.96 (t, 3H, C*H*$_3$-CH$_2$-CH$_2$); 1.14 (t, 3H, C*H*$_3$-CH$_2$-CO); 1.42 (d, 3H, CH-C*H*$_3$); 1.50 (m, 2H, CH$_3$-C*H*$_2$-CH$_2$); 1.79 (m, 2H, CH$_3$-CH$_2$-C*H*$_2$); 2.18 (m, 1H, CH-C*H*$_2$); 2.33 (q, 2H, CH$_3$-C*H*$_2$-CO); 2.37 (m, 1H, CH-C*H*$_2$); 3.1–3.3 (m, 4H, 2S-CH$_2$); 4.25–4.40 (m, 2H, 2CH$^\alpha$).

Pr-L-Ala-phosphonoacetamido-ambo-alanine ***13***
Extraction: ethyl acetate/isopropanol 1:1 (v/v). Purification: ion-exchange chromatography on AG1-X2 resin (OH^-; elution with 1.5 M formic acid) and semi-preparative HPLC in condition P_3. Yield 35%. $[\alpha]_D^{20}$ –4.6° (*c* 0.22, H_2O). TLC: $R_f(S_3)$ 0.27. Analytical HPLC: $R_t(A_1)$ 7.9 min (split peak); $R_t(A_2)$ 12.3 and 13.1 min. Amino acid analysis: Ala 1.00; diaminopropionic acid 1.08. ^{1}H-NMR: 1.04 (dt, 3H, C*H*$_3$-CH$_2$-CO); 1.21 (t, *ca* 16H, Et_3N); 1.29 (dd, 3H, CH-C*H*$_3$); 2.24 (q, 2H, CH$_3$-C*H*$_2$-CO); 2.65 (d, 2H, *J* = 20 Hz, CO-CH$_2$-P); 3.14 (q, *ca* 10H, Et_3N); 3.40 (m, 1H, CH$_2$-ND); 3.60 (m, 1H, CH$_2$-ND); 4.26 (m, 2H, 2CH$^\alpha$). ^{31}P-NMR: 14.61 (d).

Pr-L-Ala-phosphonomethylamino-D-aspartic acid ***14***
Extraction: ethyl acetate/isopropanol 1:1 (v/v). Purification ion-exchange chromatography on AG1-X2 resin (OH^-; elution with 1.5 M formic acid) and semi-preparative HPLC in condition P_4. Yield 30%. $[\alpha]_D^{20}$ –14.6° (*c* 0.24, H_2O). TLC: $R_f(S_1)$ 0.18. Analytical HPLC: $R_t(A_1)$ 7.2 min; $R_t(A_2)$ 11.2 min. Amino acid analysis: Ala 1.00; Asp 1.20; aminomethylphosphonic acid 0.95. ^{1}H-NMR: 1.04 (t, 3H C*H*$_3$-CH$_2$-CO); 1.21 (t *ca* 14H, Et_3N); 1.28 (dd, 3H, CH-C*H*$_3$); 2.23 (q, 2H, CH$_3$-C*H*$_2$-CO); 2.5–2.8 (m, 2H, ND-CH$_2$-P); 3.14 (q, *ca* 9H, Et_3N); 3.33 (m, 2H, CH-C*H*$_2$-CO); 4.25 (dq, 1H, CH$^\alpha$ Ala); 4.42 (m, 1H, CH$^\alpha$ Asp). ^{31}P-NMR: 15.82 (s).

Pr-L-Ala-ambo-2-amino-6-phosphonohexanoic acid ***15***
Extraction: ethyl acetate/methanol 1:1 (v/v). Purification: semi-preparative HPLC in condition P_2. Yield 29%. $[\alpha]_D^{20}$ –37.0° (*c* 0.28, H_2O). TLC: $R_f(S_3)$ 0.55. Analytical HPLC: $R_t(A_1)$ 13.2 and 13.7 min; $R_t(A_2)$ 31.3 and 32.1 min. Amino acid analysis: Ala 1.00; 2-amino-6-phosphonohexanoic acid 0.92. ^{1}H-NMR: 1.14 (br t, 3H, C*H*$_3$-CH$_2$-CO); 1.31 (t, *ca* 12H, Et_3N); 1.41 (br, 5H, CH-C*H*$_3$ + CH$_2$-P); 1.58 (br, 4H, CH-C*H*$_2$-C*H*$_2$); 1.74 (m, 1H, CH-C*H*$_2$); 1.86 (m, 1H, CH-C*H*$_2$); 2.32 (br, 2H, CH$_3$-C*H*$_2$-CO); 3.24 (q, *ca* 8H, Et_3N); 4.23 (br, 1H, CH$^\alpha$ Ala); 4.35 (m, 1H, CH$^\alpha$ 2-amino-6-phosphonohexanoic acid). ^{31}P-NMR: 23.75 (s).

Pr-L-Ala-D-Orn(Z)-OBzl ***21***
This compound was prepared by the general procedure described above, except that N^{δ}-*Z*-D-ornithine benzyl ester, toluene-4-sulfonate [27] was dissolved in pure DMF. The residue resulting from the evaporation of the reaction mixture was taken up in ethyl acetate. After washing with 5% $KHSO_4$, 1 M $NaHCO_3$ and brine, the organic solution was dried over $MgSO_4$ and concentrated. The product was purified by silica gel column chromatography in benzene/acetonitrile 1:1 (v/v). It crystallized upon trituration in cold petroleum ether. Yield 60%. mp 133°C. $[\alpha]_D^{20}$ –38.4° (*c* 1, chloroform). TLC: $R_f(S_2)$ 0.40. Anal $C_{26}H_{33}N_3O_6$ (C, H, N).

Pr-L-Ala-D-Orn ***16***
Compound **21** (238 mg, 0.5 mmol) was dissolved in methanol (4 ml), acetic acid (2 ml) and water (2 ml). 5% Pd/C was added, and the mixture was hydrogenated for 45 min. After removal of the catalyst by filtration, the solvents were evaporated. The residue was taken up in water, and the solution was filtered on Millipore HAWP filter (0.45 µm) and lyophilized. Yield 103 mg (80%). $[\alpha]_D^{20}$ –66.7° (*c* 0.2, H_2O). TLC: $R_f(S_3)$ 0.35. Analytical HPLC: $R_t(A_1)$ 13.3 min; $R_t(A_2)$ 30.0 min. Amino acid analysis: Ala 1.00; Orn 0.94. ^{1}H-NMR: 1.05 (t, 3H, CH_3-CH_2-CO); 1.33 (d, 3H, CH-CH_3); 1.56 (m, 2H, CH-CH_2-CH_2); 1.66 (m, 2H, CH-CH_2-CH_2); 2.24 (q, 2H, CH_3-CH_2-CO); 2.93 (t, 2H, CH_2-ND_2); 4.15 (t, 1H, CH^{α} Orn); 4.21 (q, 1H, CH^{α} Ala).

Pr-L-Ala-ambo-buthionine sulfoximine methyl ester ***22***
Anhydrous HCl was bubbled for 30 min through a solution of **11** (1.05 g, 3 mmol) in methanol (35 ml). After evaporation of the solvent, the residue was dried under vacuum over KOH. The product was purified by silica gel column chromatography in chloroform/methanol 20:1 (v/v). Yield 871 mg (80%). TLC: $R_f(S_3)$ 0.79. Amino acid analysis: Ala 1.00; buthionine sulfoximine 0.93.

Pr-L-Ala-ambo-buthionine sulfoximine phosphate ***12***
A solution of **22** (0.1 mmol) in pyridine (2 ml) was cooled to 0°C. 2-Cyanoethylphosphate (0.25 ml of 1 M solution in pyridine) and DCCI (0. 36 mmol) were added. After stirring for 3 days at 4°C, water (4 ml) was added. Dicyclohexylurea was removed by filtration and the solvents were evaporated. The residue was taken up in acetonitrile, and a second crop of dicyclohexylurea was removed. After evaporation of acetonitrile, the residue was dried under vacuum. It was dissolved in methanol (2 ml) and 1 M NaOH (2 ml). The mixture was stirred for 1 h at 40°C, then cooled in ice. The pH was adjusted to 4.5 with cold 1 M HCl and the solution was evaporated to dryness. The product was purified by semi-preparative HPLC in condition P_1. Yield 32%. TLC: $R_f(S_4)$ 0.60. Analytical HPLC: $R_t(A_1)$ 17.7 min; $R_t(A_2)$ 37.7 min. Ratio Ala/buthionine sulfoximine/phosphate: 1.00:1.10:1.10. ^{1}H-NMR: 0.97 (dt, 3H, CH_3-CH_2-CH_2); 1.15 (dt, 3H, CH_3-CH_2-CO); 1.31 (t, *ca* 13H, Et_3N); 1.43 (dd, 3H, CH-CH_3); 1.49 (m, 2H, CH_3-CH_2-CH_2); 1.81 (m, 2H, CH_3-CH_2-CH_2); 2.23 (m, 1H, CH-CH_2); 2.33 (dq, 2H, CH_3-CH_2-CO); 2.41 (m, 1H, CH-CH_2); 3.23 (q, *ca* 9H, Et_3N); 3.36 (m, 4H, 2S-CH_2); 4.37 (m, 2H, 2CH^{α}). ^{31}P: –2.53; –6.15.

Pr-L-Ala-ambo-methionine sulfoximine phosphate ***23***
This compound was synthesized from Pr-L-Ala-*ambo*-methionine sulfoximine methyl ester [10] by the procedure described for **12**. It was purified by semi-preparative HPLC in condition P_6. Yield 30%. TLC: $R_f(S_4)$ 0.54. Analytical HPLC: $R_t(A_1)$ 10.5 min; $R_t(A_2)$ 20.1 min. Ratio Ala/methionine sulfoximine/phosphate 1.00:1.05: 1.08. ^{1}H-NMR: 1.13 (dt, 3H, CH_3-CH_2-CO); 1.42 (d, 3H, CH-CH_3); 2.33 (m, 3H, CH_3-CH_2-CO + CH-CH_2); 2.53 (m, 1H, CH-CH_2); 3.31 (d, 3H, S-CH_3); 3.37–3.60 (m, 2H, CH_2-S); 4.32 (m, 1H, CH^{α} Ala): 4.59 (m, 1H, CH^{α} methionine sulfoximine). ^{31}P-NMR: –2.55; –5.42.

Enzymatic assays

The *meso*-A_2pm-adding activity was assayed by following the appearance of UDP-MurNAc-L-Ala-D-Glu(-*meso*-[^{14}C]A_2pm) in a mixture (final volume: 50 µl) containing 0.1 M Tris/HCl buffer (pH 8.6), 0.1 M $MgCl_2$, 5 mM ATP, 0.1 mM UDP-MurNAc-L-Ala-D-Glu, 0.1 mM *meso*-A_2pm, *meso*-[^{14}C]-A_2pm (0.8 kBq), and enzyme (15 µl in 20 mM potassium phosphate (pH 7.0) containing 1 mM 2-mercaptoethanol and 0.1 mM $MgCl_2$). The mixture was incubated for 30 min at 37°C, and the reaction was stopped by addition of 10 µl glacial acetic acid. The amounts of product and unreacted *meso*-A_2pm were then determined by high-voltage electrophoresis and scintillation counting according to Mengin-Lecreulx *et al* [44].

Direct assay of inhibition

The inhibitory effect of the compounds prepared was studied in a mixture similar to that described in the preceding paragraph, the reaction being initiated by addition of the enzyme. The I_{50} value for a compound (the concentration required to reduce the enzyme activity to 50% of the control value) was calculated using the Dixon equation [45]: $1/V_i = 1/V_0 + [I]/(V_0\ I_{50})$ where V_0 is the uninhibited rate and V_i the rate in the presence of inhibitor concentration [I].

Assay with preincubation

The enzyme was preincubated for 15 min at 37°C with the putative inhibitor dissolved in a mixture containing Tris/HCl, ATP and $MgCl_2$ (total volume: 40 µl). The reaction was initiated by addition of a mixture (10 µl) of the substrates. The remaining part of the assay was run as previously described.

Antibacterial testing

Organisms were clinical isolates obtained either from the Hôpital Saint-Joseph (Paris, France) or from the American Type Culture Collection (ATCC): *E coli* 58898, 59131, 59639, 61529 and 61670, *Klebsiella oxytoca* 59969, *K pneumoniae* 57189, 59674 and 60038, *Enterobacter cloacae* 58927 and 61466, *Citrobacter* 59894, *Pseudomonas aeruginosa* 31267 and 31270, *Bacillus cereus* ATCC 9634, *B pumilus* ATCC 14884, *B subtilis* ATCC 6633. Cells were grown on agar plates for periods of 24–48 h at 37°C [46]. M63 medium [47] supplemented by glucose (0.2%) was used. Peptides were tested at final concentrations ranging from 1 to 256 µg/ml, using serial two-fold dilutions.

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