

# Effect of analogues of diaminopimelic acid on the *meso*-diaminopimelate-adding enzyme from *Escherichia coli*

Geneviève Auger<sup>a</sup>, Jean van Heijenoort<sup>a</sup>, John C. Vederas<sup>b</sup>, Didier Blanot<sup>a,\*</sup>

<sup>a</sup>URA 1131 du CNRS, Biochimie Moléculaire et Cellulaire, Bâtiment 432, Université de Paris-Sud, 91405 Orsay, France

<sup>b</sup>Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada

Received 23 April 1996; revised version received 25 May 1996

**Abstract** Several analogues of diaminopimelic acid (A<sub>2</sub>pm) were tested as substrates or inhibitors of the *meso*-diaminopimelate-adding enzyme from *Escherichia coli*. They included lanthionine derivatives, a phosphonic analogue, heterocyclic compounds, 3-fluoro-A<sub>2</sub>pm, 4-methylene-A<sub>2</sub>pm and *N*-hydroxy-A<sub>2</sub>pm. The best substrates were, in decreasing order of specific enzyme activity, (2*S*,3*R*,6*S*)-3-fluoro-A<sub>2</sub>pm, *meso*-lanthionine sulfoxide and *N*-hydroxy-A<sub>2</sub>pm (mixture of stereoisomers). In those cases where all the stereoisomers were available, the specificity could be described as *meso* >> DD ≈ LL. *N*-Hydroxy-A<sub>2</sub>pm (mixture of stereoisomers) strongly inhibited the addition of radioactive *meso*-A<sub>2</sub>pm to UDP-*N*-acetylmuramoyl-dipeptide.

**Key words:** *meso*-Diaminopimelate-adding enzyme; Diaminopimelic acid analog; MurE; Peptidoglycan biosynthesis

## 1. Introduction

Diaminopimelic acid (A<sub>2</sub>pm, compound 1; Fig. 1) is involved in two essential anabolic pathways of bacteria: protein and peptidoglycan biosyntheses (Fig. 2). In all eubacteria and in higher plants the LL isomer of A<sub>2</sub>pm is synthesized from aspartic acid in several steps. It is then converted by epimerase DapF into the *meso* isomer, which is decarboxylated into L-lysine by decarboxylase LysA [1]. In some bacterial species a dehydrogenase directly converts tetrahydrodipicolinic acid into *meso*-A<sub>2</sub>pm (Fig. 2). A<sub>2</sub>pm is introduced into the peptidoglycan pathway by its addition to UDP-MurNac-dipeptide to yield UDP-MurNac-tripeptide. This step is catalyzed by the A<sub>2</sub>pm-adding enzyme MurE [2].

Since mammals lack the A<sub>2</sub>pm pathway, specific inhibitors of the enzymes along this route are potential antibacterial agents. There has been growing interest in the design and synthesis of A<sub>2</sub>pm analogues, some of which have been assayed either for antibacterial activity (see [3] for a recent example) or for a specific effect on the epimerase [4–9], the decarboxylase [9–11], the dehydrogenase [5,8,9] or less frequently the A<sub>2</sub>pm-adding enzyme [12–15]. Herein we take

advantage of the availability of several previously synthesized A<sub>2</sub>pm analogues (Fig. 1) to assay them as substrates or inhibitors of MurE from *Escherichia coli*.

## 2. Materials and methods

### 2.1. Chemicals

UDP-MurNac-L-Ala-D-Glu [16], UDP-MurNac-L-Ala-D-[<sup>14</sup>C]Glu [17] and *meso*-A<sub>2</sub>pm [18] were obtained according to published procedures. *meso*-[<sup>14</sup>C]A<sub>2</sub>pm (11.6 TBq mol<sup>−1</sup>) was purchased from CEA (Saclay, France). The synthesis of A<sub>2</sub>pm analogues 2–8 and 10 has already been described [5,6,8,9,11]; analogue 9 was synthesized according to the procedure of Girodeau et al. [10]. The MurE activity was partially purified from *E. coli* JM83(pHE5) as described previously [13].

### 2.2. Assays of analogues as substrates

The analogues of A<sub>2</sub>pm were tested for their addition to UDP-MurNac-L-Ala-D-[<sup>14</sup>C]Glu in a mixture (final volume: 50 μl) containing 0.1 M Tris-HCl, pH 8.6, 0.1 M MgCl<sub>2</sub>, 5 mM ATP, 0.1 mM UDP-MurNac-L-Ala-D-[<sup>14</sup>C]Glu (0.9 kBq), 10 mM *meso*-A<sub>2</sub>pm or analogue, and enzyme (0.79–6.3 μg of protein dissolved in 15 μl of 20 mM potassium phosphate, 1 mM 2-mercaptoethanol, 0.1 mM MgCl<sub>2</sub>, pH 7.0). After 30 or 60 min at 37°C, the reaction was stopped by addition of 10 μl of glacial acetic acid. The amounts of product and unreacted UDP-MurNac-dipeptide were then determined by high-voltage electrophoresis (pH 1.9, 40 V cm<sup>−1</sup>, 2 h), autoradiography and scintillation counting. For K<sub>m</sub> determinations, the concentration of UDP-MurNac-dipeptide was 0.2 mM and those of the amino acids were 1–15 mM for 3a, 0.2–7.5 mM for 8 and 0.5–10 mM for 10; values ± standard deviations at 95% of confidence were calculated from the fitted regression equations [19] using the *v* vs. *v*/[S] plot.

### 2.3. Assays of analogues as inhibitors

The analogues of A<sub>2</sub>pm were tested for their ability to inhibit the addition of *meso*-[<sup>14</sup>C]A<sub>2</sub>pm to UDP-MurNac-dipeptide in a mixture (final volume: 50 μl) containing 0.1 M Tris-HCl, pH 8.6, 0.1 M MgCl<sub>2</sub>, 5 mM ATP, 1 mM UDP-MurNac-dipeptide, 10 μM *meso*-[<sup>14</sup>C]A<sub>2</sub>pm (0.9 kBq), 5 mM analogue, and enzyme (0.63 μg of protein dissolved in 15 μl of the same buffer as in Section 2.2). After 20 min at 37°C, the reaction was stopped by addition of 10 μl of glacial acetic acid. The mixture was lyophilized and taken up in the HPLC elution buffer. The radioactive substrate and product were separated by reverse-phase HPLC with a Nucleosil 5C<sub>18</sub> column (150 × 4.6 mm) as stationary phase, and isocratic elution at a flow rate of 0.5 ml min<sup>−1</sup> with 50 mM ammonium formate buffer, pH 3.9. Detection and quantification were performed with an LB 506 C-1 HPLC radioactivity monitor (Berthold, Bald Wildbald, Germany) using the Quickszint Flow 2 scintillator (Zinsser Analytic, Maidenhead, UK) at 0.5 ml min<sup>−1</sup>. For IC<sub>50</sub> determinations, the concentrations of the amino acids were 0.8–5 mM for 8 and 0.5–3 mM for 10; values ± standard deviations at 95% of confidence were calculated from the fitted regression equations [19] using the logit-log plot.

## 3. Results

The A<sub>2</sub>pm analogues 2–10 studied in this work are listed in Fig. 1. It is noteworthy that in all cases but 9 and 10, the stereochemistry of the asymmetric carbon atoms was defined.

\*Corresponding author. Fax: (33) (1) 69 85 37 15.  
E-mail: didier.blanot@ebp.u-psud.fr

**Abbreviations:** A<sub>2</sub>pm, 2,6-diaminopimelic acid; DapF, LL-2,6-diaminopimelate 2-epimerase; LysA, *meso*-2,6-diaminopimelate carboxylase; MurE, uridine-diphosphate-*N*-acetylmuramoyl-L-alanyl-D-glutamate:*meso*-2,6-diaminopimelate ligase (ADP-forming); MurNac, *N*-acetylmuramoyl

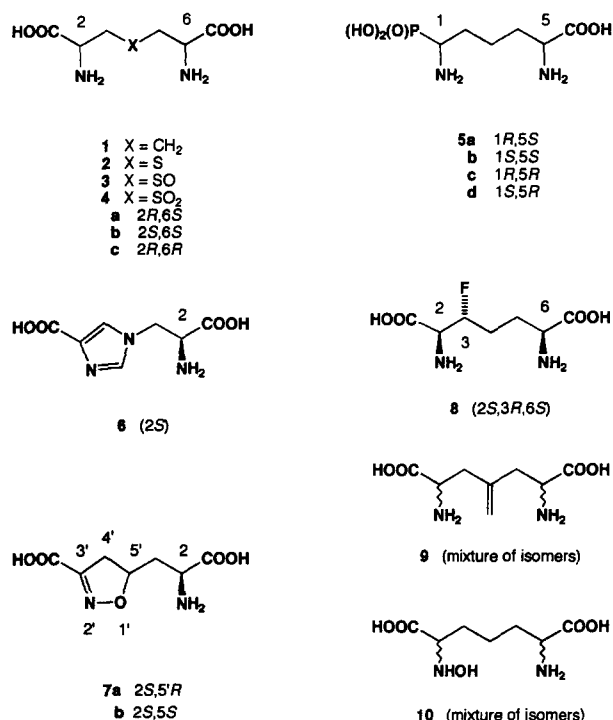


Fig. 1. Formulae of diaminopimelic acid and of its analogues.

Moreover, for lanthionine derivatives 2–4 and phosphonic analogue 5, all the possible stereoisomers were available (except for *meso*-lanthionine sulfoxide 3a, which was presumably an optically inactive mixture of two isomers at the sulfur atom [11]).

We first determined whether these compounds were substrates, at 10 mM, for the *meso*-A<sub>2</sub>pm-adding enzyme from *E. coli* (Table 1). UDP-MurNAc-L-Ala-D-[<sup>14</sup>C]Glu was used for this purpose. Owing to the difference in isoelectric point between the radioactive substrate and the putative product, high-voltage electrophoresis was used for their separation.

In a previous work [15], we had shown that the *meso* (2a) and LL (2c) isomers of lanthionine were substrates, the former being a much better one than the latter. (Because of priority rules in the *R/S* nomenclature system, LL-lanthionine has in fact a 2*R*,6*R* configuration.) Here, it appeared that the DD isomer 2b was also a substrate; the specific enzyme activity observed (1.0%) was approximately the same as with the LL isomer (1.3%). The addition of one oxygen atom to the sulfur (lanthionine sulfoxide 3) moderately reduced the specific activity, whereas that of two oxygen atoms (lanthionine sulfone 4) decreased it to a great extent. In each case, the *meso* isomer (3a, 4a) was a much better substrate than the LL (3c, 4c) or DD (3b, 4b) isomer.

No stereoisomers of the phosphonic analogue 5 were substrates, even at high enzyme concentration. This agreed with the result previously obtained with the unresolved mixture of the four isomers [14].

Heterocyclic, conformationally constrained analogues 6 (imidazole) and 7 (isoxazoline) were poor substrates; only with the 2*S*,5'*R* isomer 7a was non-negligible activity observed (5.2%).

Fluorinated analogue 8 [(2*S*,3*R*,6*S*)-3-fluoro-A<sub>2</sub>pm] was the

best substrate found in this study (spec. act. 55%). 4-Methylene-A<sub>2</sub>pm 9 and *N*-hydroxy-A<sub>2</sub>pm 10 were also substrates with lower specific activities (1.4 and 16%, respectively); since these compounds were mixtures of 3 and 4 stereoisomers, respectively, it is conceivable that one stereoisomer was a better substrate than the others.

The *K<sub>m</sub>* values of MurE for the best substrates were determined: 3.5 ± 0.8 and 9.2 ± 1.2 mM for 8 and 10, respectively. These values are to be compared with those previously found for *meso*-A<sub>2</sub>pm 1a, LL-A<sub>2</sub>pm 1b and *meso*-lanthionine 2a: 0.04, 36 and 1.5 mM, respectively [15,17]. MurE did not follow simple kinetics with the fairly good substrate 3a: *v* vs. log[S] plots were bell-shaped with the top near 10 mM (not shown). This kind of curve is obtained in the case of excess substrate inhibition [20,21]. Owing to the marked effect of this phenomenon, no *K<sub>m</sub>* value could be calculated.

Compounds were also assayed, at 5 mM, for the inhibition of the addition of *meso*-[<sup>14</sup>C]A<sub>2</sub>pm to UDP-MurNAc-dipeptide (Table 1). The radioactive substrate and product were separated by reverse-phase HPLC and quantitated by on-line scintillation counting, as already described for the L-alanine-adding and D-glutamate-adding enzymes [22,23]. Generally, the greatest extents of inhibition were observed with the best substrates: this presumably reflected the fact that these compounds behaved as competitive substrates. IC<sub>50</sub> values for the most potent inhibitors were determined: 2.3 ± 0.2 and 0.56 ± 0.06 mM for 8 and 10, respectively. The stereoisomers of phosphonic analogue 5, which were not substrates, displayed moderate inhibition (approx. 35% for 5c and 5d). Here again, this was consistent with our previous result obtained with the mixture of the four stereoisomers (50% inhibition at 10 mM) [14].

Table 1  
Effects of the A<sub>2</sub>pm analogues on the *meso*-A<sub>2</sub>pm-adding activity

Compound	Specific activity <sup>a</sup> (%)	Inhibition <sup>b</sup> (%)
2a	52 <sup>c</sup>	n.d. <sup>d</sup>
2b	1.0 ± 0.1	20 ± 4
2c	1.3 ± 0.1	n.i. <sup>e</sup>
3a	34 ± 1	14 ± 4
3b	0.36 ± 0.20	n.i.
3c	0.36 ± 0.03	n.i.
4a	1.12 ± 0.01	n.i.
4b	0.20 ± 0.02	n.i.
4c	0.20 ± 0.05	n.i.
5a	n.p. <sup>f</sup>	9 ± 3
5b	n.p.	20 ± 5
5c	n.p.	34 ± 2
5d	n.p.	35 ± 2
6	0.10 ± 0.02	n.i.
7a	5.2 ± 1.0	n.i.
7b	1.0 ± 0.3	n.i.
8	55 ± 3	68 ± 1
9	1.4 ± 0.1	10 ± 2
10	16 ± 1	90 ± 1

<sup>a</sup>Substrate activity at 10 mM, calculated relative to that obtained with *meso*-A<sub>2</sub>pm at the same concentration [100% = 33 ± 1 nmol min<sup>-1</sup> (mg protein)<sup>-1</sup>]. Mean ± S.D. of two determinations.

<sup>b</sup>100(1 - *v<sub>i</sub>*/*v<sub>0</sub>*), where *v<sub>0</sub>* is the uninhibited velocity and *v<sub>i</sub>* the velocity at 5 mM compound. Mean ± S.D. of three determinations.

<sup>c</sup>Taken from [15].

<sup>d</sup>n.d., not determined.

<sup>e</sup>n.i., no inhibition at 5 mM compound.

<sup>f</sup>n.p., no product formation after 60 min with 6.3 μg of protein.

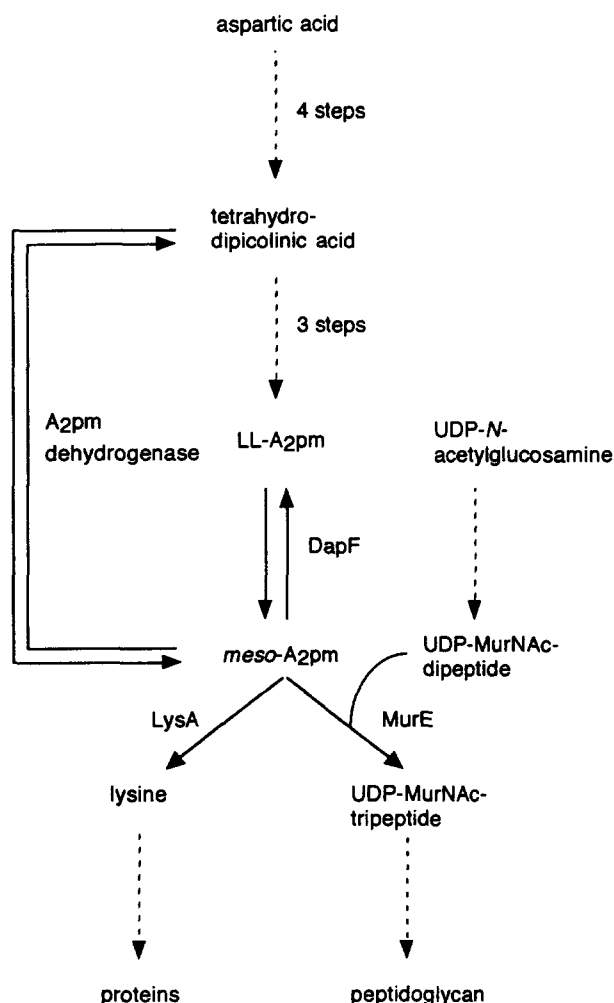


Fig. 2. The diaminopimelate pathway.

#### 4. Discussion

The results obtained with lanthionine and derivatives thereof show that the specificity of MurE towards these substrates varies in the order: lanthionine > lanthionine sulfoxide >> lanthionine sulfone. Moreover, for each compound, the specificity for the stereoisomers can be described as: *meso* >> *DD* ≈ *LL*. As far as the *DD* and *LL* isomers are concerned, this result is somewhat different from that found with A<sub>2</sub>pm, where the specific activity with the *DD* isomer **1c** is 2.6-fold higher than with the *LL* isomer **1b** [15]. This would mean a stricter stereospecificity towards the distal recognition site of A<sub>2</sub>pm (the one which carries the nonreacting amino group) than towards the proximal recognition site (the one which participates in the formation of the peptide bond). When considering the substrate assays only, this rule does not seem to apply to lanthionine and its derivatives. However, the fact that *DD*-lanthionine **3b** inhibits the incorporation of *meso*-[<sup>14</sup>C]A<sub>2</sub>pm whereas *LL*-lanthionine **3c** does not suggests that the affinity of the enzyme for **3b** is greater than that for **3c**.

It is noteworthy that the order lanthionine > lanthionine sulfoxide > lanthionine sulfone, on the one hand, and the order *meso* > *LL* ≈ *DD*, on the other, were also found when considering the specific activities of A<sub>2</sub>pm dehydrogenase

from *Bacillus sphaericus* and DapF from *E. coli* [5]. However, these in vitro effects were not translated into conspicuous antibacterial effects: none of the isomers of **3** or **4** caused any inhibition of growth when tested against a series of organisms on defined media on plates; only **4c** inhibited slightly *B. subtilis* growth at high doses (≥350 μg per test disc) [11]. On the other hand, it was shown that A<sub>2</sub>pm auxotrophs could grow, in the presence of lysine, with exogenous lanthionine ([15], and references therein), the *meso* isomer of the sulfur amino acid being incorporated into peptidoglycan [15]. Such a result illustrates the fact that certain A<sub>2</sub>pm analogues can totally replace A<sub>2</sub>pm in peptidoglycan without affecting cell viability.

Substrate analogues in which a phosphonic acid moiety replaces a carboxyl group have often proven to be good enzyme ligands. This does not hold true for MurE, since monophosphonate compounds **5** are not substrates and are only weak inhibitors; moreover, the biphosphonate analogue (1,5-diaminopentane-1,5-diyl)diphosphonic acid (mixture of isomers) does not inhibit the enzyme at all [14]. Nevertheless, one can notice that, from a stereochemical point of view, the poorest inhibitor **5a** corresponds to *LL*-A<sub>2</sub>pm (**5d** corresponding to *DD*-A<sub>2</sub>pm, and **5b** and **5c** to *meso*-A<sub>2</sub>pm): this may be another indication of the stricter stereochemical requirements of the distal recognition site. We previously showed [9] that the isomers of **5** were generally weak competitive inhibitors (millimolar range) of A<sub>2</sub>pm decarboxylase from wheat germ *Triticum vulgaris* (which has the same properties as bacterial LysA), A<sub>2</sub>pm dehydrogenase from *B. sphaericus*, and DapF from *E. coli*. Compound **5b** inhibited the growth of *Salmonella typhimurium* LT-2 (MIC, 1 μg ml<sup>-1</sup>), and a tripeptide containing **5a** was active against *E. coli* and *Citrobacter freundii* (MIC range, 4–32 μg ml<sup>-1</sup>). These effects were reversed by *LL*-A<sub>2</sub>pm or *meso*-A<sub>2</sub>pm, indicating that the target was A<sub>2</sub>pm metabolism. However, owing to the very weak inhibitory potency of **5b** and **5a** towards MurE, the involvement of MurE inhibition in the antibacterial effects is unlikely.

Heterocyclic analogues **6** and **7** are poor substrates for MurE. They were shown to be devoid of inhibitory activity towards DapF or A<sub>2</sub>pm dehydrogenase, except isoxazoline **7b**, which was a good inhibitor of the latter enzyme [8]. Its antibacterial effect against *B. sphaericus* [8] can therefore be ascribed to the inhibition of A<sub>2</sub>pm dehydrogenase, this organism being dependent on this activity for the synthesis of *meso*-A<sub>2</sub>pm.

The fact that fluorinated analogue **8** is a good substrate for the enzyme is not surprising, since it stereochemically corresponds to *meso*-A<sub>2</sub>pm. Unfortunately, the other isomers of 3-fluoro-A<sub>2</sub>pm [6] could not be tested, having decomposed upon storage. Compound **8** was shown to be a good competitive inhibitor of DapF and to undergo a rapid epimerase-catalyzed elimination of hydrogen fluoride; however, it was devoid of antibacterial activity, except against *B. megaterium* (50% inhibition at 100 μg ml<sup>-1</sup>) [6].

*N*-Hydroxy-A<sub>2</sub>pm **10** is the most interesting compound of this study since it is active on the four enzymes MurE, DapF, LysA and A<sub>2</sub>pm dehydrogenase. It functions as an alternative substrate for MurE and strongly inhibits the incorporation of *meso*-[<sup>14</sup>C]A<sub>2</sub>pm. Assuming that the inhibition is competitive, the IC<sub>50</sub> value of 0.56 mM would correspond to a *K<sub>i</sub>* value of 0.4 mM according to the Cheng-Prusoff equation [24]. Since the *K<sub>m</sub>* value found is 9.2 mM, one can suppose that certain

stereoisomers are good inhibitors without being substrates. In this regard, the synthesis of stereochemically defined *N*-hydroxy analogues would be of great interest. Previously, we showed that the mixture of isomers **10** was an alternative substrate for A<sub>2</sub>pm dehydrogenase from *B. sphaericus* (22% velocity relative to *meso*-A<sub>2</sub>pm), a moderate inhibitor of LysA from *B. sphaericus* ( $K_i = 0.91$  mM) and a very potent inhibitor of DapF from *E. coli* ( $K_i = 5.6$   $\mu$ M); it inhibited by 75% the growth of *B. megaterium* at 20  $\mu$ g ml<sup>-1</sup>, and totally that of *B. subtilis* at 500  $\mu$ g ml<sup>-1</sup> [5,11].

The case of 4-methylene-A<sub>2</sub>pm **9** deserves special comment. First synthesized by the Delalande group [10], and then by ourselves [5], it was shown to be a weak inhibitor of *E. coli* LysA (30% inhibition at 10 mM) and a noncompetitive inhibitor of *E. coli* DapF ( $K_i = 0.95$  mM) [5,10]. Interestingly, it displayed substantial antibacterial activity against Gram-negative species in defined minimal media [10], but not in complex media [5]. Moreover, it was capable of restoring the growth of an A<sub>2</sub>pm and lysine auxotroph in the presence of lysine [10]. Although a poor substrate for MurE, **9** is therefore incorporated into UDP-MurNAc-tripeptide and peptidoglycan in vivo; this is another example of the replacement of A<sub>2</sub>pm by an analogue in peptidoglycan. The antibacterial effect observed in minimal media probably reflects a depletion of intracellular lysine, as indicated by its reversal upon L-lysine supplementation [10].

**Acknowledgements:** This work was supported by grants from the Centre National de la Recherche Scientifique (URA 1131) and the National Sciences and Engineering Council of Canada. We thank Dr. D. Mengin-Lecreux for helpful discussions.

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