COMMUNICATION

Is *N*-acetylornithine aminotransferase the real *N*-succinyl-LL-diaminopimelate aminotransferase in *Escherichia coli* and *Mycobacterium smegmatis*?

Russell J. Cox * and Paul S. H. Wang

School of Chemistry, University of Bristol, Cantock's Close, Clifton, Bristol, UK BS8 1TS. E-mail: r.j.cox@bris.ac.uk

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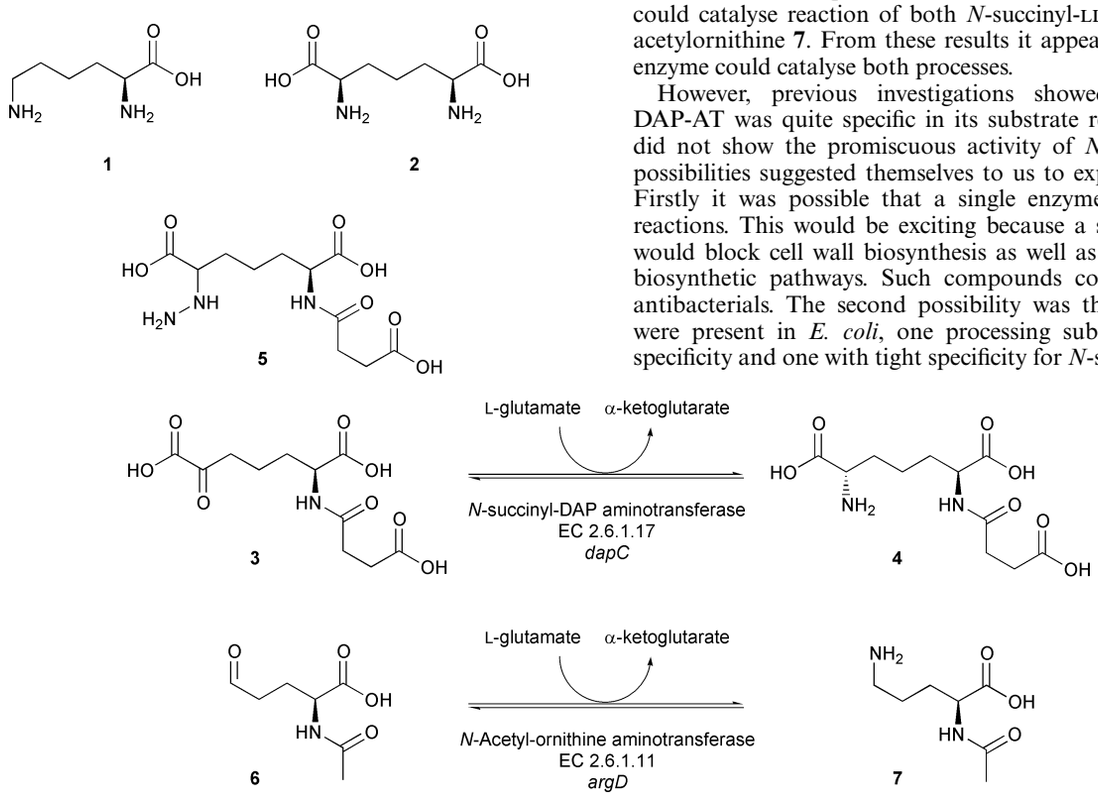
We examined the similarities and differences between the activities of *N*-succinyl-LL-diaminopimelate aminotransferase (DAP-AT) and *N*-acetylornithine aminotransferase (*N*AcO-AT) from *E. coli* and *M. smegmatis* in order to investigate recent claims that the two enzymes are one and the same. The results do not support the hypothesis that the two activities are catalysed by a single enzyme.

The biosynthesis of cell wall components in bacteria is an attractive target for *de novo* antibiotic design. In particular the biosynthetic pathway leading to L-lysine **1** is a potentially attractive target because **1** and its biosynthetic precursor (diaminopimelic acid **2**) are key components of the peptidoglycan layer of the bacterial cell wall.[1] We, and others, have focused attention on devising selective inhibitors of enzymes on this pathway.[2] Many of the enzymes on the pathway display very high substrate specificities and it has been difficult to devise compounds which are both selective and potent. However, *N*-succinyl-LL-diaminopimelate aminotransferase (DAP-AT), a key enzyme from the mid-section of the pathway, appears to be more suitable for inhibitor design.

DAP-AT catalyses the reversible interconversion of **3** and **4**, using L-glutamic acid as the amine donor (Scheme 1).[3] It is a typical pyridoxal phosphate (PLP) dependent enzyme. We have recently reported a series of highly potent inhibitors of DAP-AT (*e.g.* **5**) which are *N*-amino product analogues. Their potency stems from likely covalent bond formation between the hydrazine and PLP at the active site.[4]

Until recently all of the genes coding for the enzymes from the DAP pathway had been identified and cloned except for the DAP-AT encoding *dapC*. We, and others, had used purified enzyme preparations from wild type *E. coli* for *in vitro* investigations, but the cloning of *dapC* could mean access to large amounts of over-expressed protein for crystallographic investigations. This appeared to be a possibility when Blanchard reported the partial peptide sequencing of DAP-AT from *E. coli*.[5] This partial sequence was used to identify *dapC* from the *E. coli* genome.[6] The identified open reading frame (ORF) corresponded to a PLP dependent enzyme which was already well known. It appeared that *dapC* was in fact *argD*, coding for the enzyme *N*-acetylornithine aminotransferase (*N*AcO-AT).[7] This enzyme performs a transamination step on the biosynthetic pathway to arginine (Scheme 1). *E. coli argD* was then cloned and expressed and it was shown that the protein could catalyse reaction of both *N*-succinyl-LL-DAP **4** and *N*-acetylornithine **7**. From these results it appeared that a single enzyme could catalyse both processes.

However, previous investigations showed that purified DAP-AT was quite specific in its substrate requirements and did not show the promiscuous activity of *N*AcO-AT.[8,9] Two possibilities suggested themselves to us to explain these facts. Firstly it was possible that a single enzyme catalysed both reactions. This would be exciting because a specific inhibitor would block cell wall biosynthesis as well as two amino acid biosynthetic pathways. Such compounds could be excellent antibacterials. The second possibility was that two enzymes were present in *E. coli*, one processing substrates with low specificity and one with tight specificity for *N*-succinyl-LL-DAP.



Scheme 1 DAP-AT and *N*AcO-AT catalysed reactions.

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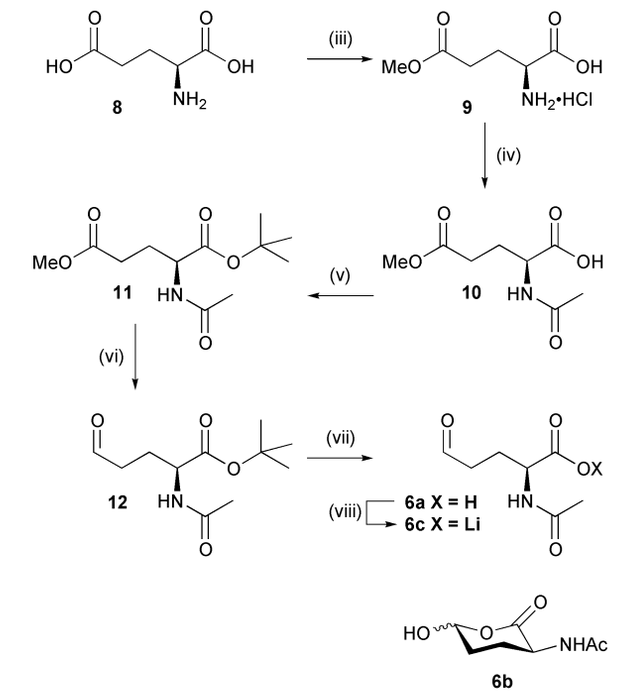
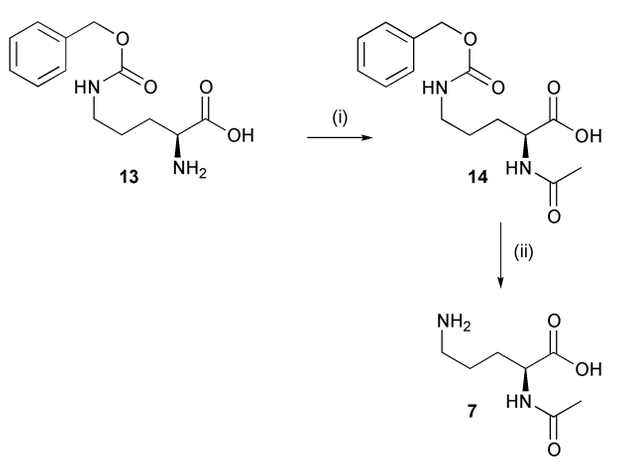
The substrate for DAP-AT, **3**, has been previously synthesised.[3] In order to access the aldehyde **6** we started from L-glutamic acid **8** which was mono-δ-methylated using standard procedures (Scheme 2).[10] Acetylation of **9** with acetic anhydride





Scheme 2 Synthesis of *N*AcO-AT substrates. *Reagents and conditions:* (i) Ac_2O, aq. $NaHCO_3$, 56%; (ii) H_2 (1 atm), 10% Pd/C, MeOH, then recrystallisation, 29%; (iii) $SOCl_2$, MeOH, 94%; (iv) Ac_2O, aq. $NaHCO_3$, 85%; (v) isobutylene, CH_2Cl_2, H_2SO_4, RT, 80%; (vi) 2.5 eq. DIBAL-H, CH_2Cl_2, −78 °C, 59%; (vii) 50% TFA–CH_2Cl_2; (viii) 1.0 eq. $LiOH{\cdot}H_2O$, quant. (2 steps).

in mild aqueous base then gave the carboxylic acid **10**. Treatment with isobutylene in acidic solution then afforded the *tert*-butyl ester **11** in good yield. DIBAL-H reduction of **11** gave the required aldehyde **12** and deprotection of the *tert*-butyl ester using 50% TFA–CH_2Cl_2 cleanly afforded the acid **6a**.[11] By ^{13}C and ^{1}H NMR **6** exists as the expected[12,13] mixture of δ-epimers of the lactol **6b**. For ease of handling the acid **6** was treated with 1.0 eq. of $LiOH{\cdot}H_2O$ to afford the lithium salt **6c** in quantitative yield. *N*-Acetylornithine **7** was obtained by treating L-δ*N*-CBz ornithine **13** (NovaBiochem) with acetic anhydride. The product **14** was hydrogenated under standard conditions to give **7** after recrystallisation.

E. coli DH5α was grown in liquid culture (1 L LB medium) under standard conditions.[4] Cells were collected at mid log phase and lysed by sonication in 30 mM phosphate buffer pH 7.5. After removal of cell solids and nucleic acids the cell-free extract was adjusted to pH 7.0 and applied to a Q-sepharose

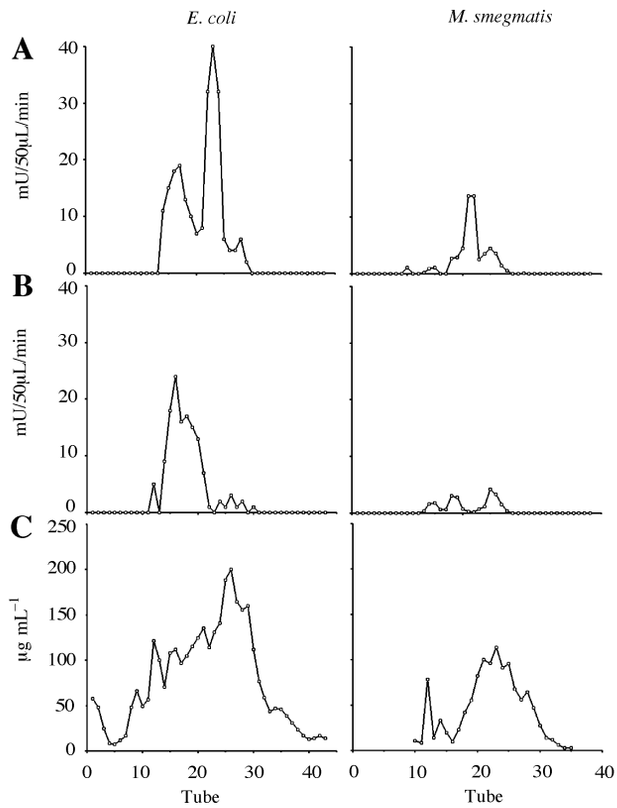
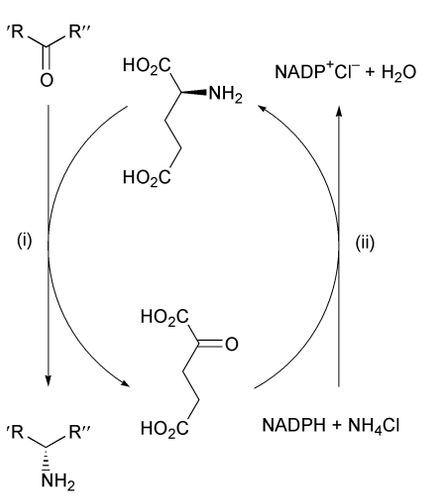


Fig. 1 **A** Activity of **3** as a substrate by tube; **B** Activity of **6** as a substrate by tube; **C** Protein concentration (Bradford) by tube. 1 mU = $0.001\Delta OD_{340}$ = 161 nM NADPH.

column (Pharmacia) which was then eluted with a linear gradient of NaCl (0.0 to 0.5 M). The eluent was collected in ice cooled test tubes. Each tube was then assayed for DAP-AT and *N*AcO-AT activity (Fig. 1) and also assayed for total protein content (Bradford). In order to assay DAP-AT activity the natural substrate **3** (1 mM) was added to a reaction vial containing PLP, NH_4Cl, NADPH, glutamate dehydrogenase, L-glutamic acid and buffer (Scheme 3). Reaction was initiated by



3, **4** R′ = CO_2H, R″= $(CH_2)_3CH(NHSucc)CO_2H$
6, **7** R′ = H, R″ = $(CH_2)_2(NHAc)CO_2H$

Scheme 3 Assay procedure for DAP-AT and *N*AcO-AT. (i) DAP-AT or *N*AcO-AT; (ii) glutamate dehydrogenase (GDH).

addition of 50 μL of enzyme solution. The rate of NADPH consumption was then monitored at 340 nm. *N*AcO-AT activity was assayed in the same way by substituting **3** with *N*-acetylglutamate semi-aldehyde **6**.

DAP-AT activity was eluted as two bands. The first activity band eluted in tubes 14–20 peaking at tubes 16/17 (Fraction A), and the second DAP-AT band eluted between tubes 21 and 25, peaking at tube 23 (Fraction B). *N*AcO-AT activity was eluted as a single band between tubes 14 and 21, peaking at tube 16 corresponding to Fraction A. The DAP-AT band (Fraction B) was further purified by SP-sepharose chromatography. Fraction B was used to measure K_M^{app} (*i.e.* at constant L-glutamate concentration 10 mM) for **3** (2.41 ± 0.2 mM, lit.[4] 2.25 ± 0.2 mM), but no substrate activity was observed for **6**, even at elevated concentrations (20 mM). In Blanchard's original report the activity assay was run in reverse, using **7** as the substrate.[5] However, even at elevated **7** concentrations (20 mM), in the presence of $NADP^+$ and in the absence of NH_4Cl, no activity was detected when using Fraction B.

We also examined the situation in the Gram positive *Mycobacterium smegmatis*. A cell free extract was prepared, fractionated and assayed with **3** and **6** exactly as above. Although protein concentrations and enzyme activities were somewhat lower than measured for *E. coli*, a similar pattern was observed (Fig. 1). DAP-AT activity was eluted in two main fractions, the first of which showed no *N*AcO-AT activity and the second of which showed both DAP-AT and *N*AcO-AT activity (*i.e.* the opposite elution order to *E. coli*).

It is clear from these results that two bacterial enzymes can process **3**: the catholic *N*AcO-AT and the more selective DAP-AT. This makes the strategy of inhibiting L-lysine biosynthesis by targeting DAP-AT rather unlikely to be successful – inhibition of DAP-AT could be overcome by *N*AcO-AT activity. These results may explain why the potent DAP-AT inhibitors such as **5** show extremely limited *in vivo* potency despite their nM *in vitro* activity.[4] These results may also explain the recent results of Fuchs who has cloned a putative DAP-AT encoding gene from *Bordetella pertussis*.[14] The *B. pertussis dapC* gene sequence obtained is not highly homologous to *argD*, but instead shows similarities to *E. coli* 'unknown' ORFs encoding PLP dependent enzymes.

Acknowledgements

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11 **6b** *major diastereomer selected data*: ν_{max} (liq.)/cm^{-1} 3422, 1637, 1440; δ_H(300 MHz; CD_3CN) 7.32 (1H, d, *J* 7.3, NH), 5.35 (1H, m, δCH), 4.50 (1H, m, αCH), 2.32 (2H, m, γCH_2), 2.12 (2H, m, βCH_2), 2.00 (3H, s, CH_3); δ_C(74.5 MHz, CD_3CN) 172.5 (CO), 171.3 (CO), 89.5 (δCH), 57.4 (αCH), 37.2 (γCH_2), 28.2 (βCH_2), 21.3 (CH_3); *m/z* (ESMS)$^+$ 156 ([M − H_2O + H]$^+$, 100%), 174 ([MH]M$^+$, 25%), 196 ([M + Na]$^+$, 15%), 215 ([M − H + CH_3CN]$^+$, 20%).
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