Synthesis and *in vitro* enzyme activity of an oxa analogue of azi-DAP

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The synthesis of a novel epoxide inhibitor **6** of *meso*-DAP epimerase was achieved. A new and convenient assay for *meso*-DAP epimerase was devised which is a considerable improvement on previously reported assay methods. This assay was used to determine the inhibitory properties of **6**. The epoxide **6** shows limited competitive inhibition *vs.* *meso*-DAP epimerase, but clear time dependent inhibition indicative of the expected covalent attachment at the active site. Epoxide **6**, although an effective inhibitor of *meso*-DAP epimerase, was unstable at pH 7.3 in aqueous buffer, being hydrolysed to the corresponding diol.

Introduction

The development of specific inhibitors of bacterial L-lysine biosynthesis, *via* the diaminopimelate (DAP) pathway, is of current interest due to the potential antimicrobial properties of such compounds.[1–4] These compounds could overcome patterns of bacterial resistance to current commercial antibiotics. Recently significant progress has been made in the use of X-ray crystallography for the structural elucidation of these bacterial enzymes. For example, two enzymes from the pathway, *meso*-DAP dehydrogenase[5] and L-tetrahydrodipicolinate succinyltransferase[6] have been crystallised in the presence of substrate analogues or inhibitors. The structural data elucidated in these cases enabled the precise mapping of the active sites. This kind of data will be crucial for the future design of superior inhibitory compounds. X-Ray structures for crystals of other enzymes on the pathway have also been determined, but the absence of bound substrate or inhibitor has hampered attempts to understand the geometry of their active sites and the mechanism of the enzymes. A case in point is that of *meso*-DAP epimerase. X-Ray crystal structural data has been determined for this enzyme from *Haemophilus influenzae*.[7] In agreement with kinetic data it appears that two active site thiols (Cys-73 and Cys-217) may act as base and acid during the epimerisation of *meso*-DAP **1** to LL-DAP **2** (Scheme 1). This process seemingly utilises very weak thiol or thiolate bases, in water, to remove the α-proton of DAP—a proton with a comparatively high pK_a.[8] In very recent studies Vederas and co-workers have shown that Cys-73 of *H. influenzae* DAP epimerase removes protons from L-configured centres and Cys-217 removes protons from D-configured centres.[9] The active site structure and mechanism of DAP epimerase is thus of great interest. For further crystallographic work with *meso*-DAP epimerase, we required compounds which could specifically interact with the active site of the enzyme prior to structure determination.

A number of inhibitors of DAP epimerase are known. However, compounds such as β-chloro-DAP[10] **3** and β-fluoro-DAP[11] **4** are *reversible* inhibitors of the enzyme; that is, they do not form (lasting) covalent bonds with the enzyme. For our studies we required compounds which could covalently label the active site of *meso*-DAP epimerase. Such active site labels for *meso*-DAP epimerase are already known. Azi-DAP **5** is a potent inhibitor of the enzyme which forms a covalent attachment to one of the active site thiols, *via* nucleophilic attack at the aziridine (Scheme 1).[12] This compound was unavailable to us. However, we reasoned that similar properties could arise from the analogous epoxide **6** which could be accessed relatively simply from available materials. The epoxide should be able to irreversibly form a covalent linkage to one of the active site thiols of *meso*-DAP epimerase and give us the active-site label we desired. Although only one of the four possible stereochemical isomers of **6** was expected to be active, we did not know *a priori* which epoxide configuration would be preferred. The enzyme would presumably prefer the L-configuration at the distal centre. Although it is, in principle, possible to control the stereochemistry at each stereogenic centre, we chose to synthesise a racemic mixture so that DAP epimerase could select its preferred inhibitor *in vitro*.

Results and discussion

Synthesis of epoxyaminopimelate

The use of the carbonyl ene reaction is a convenient method for the formation of the DAP-skeleton. We have already extensively explored this route for the production of substrates and inhibitors of another DAP processing enzyme, *N*-succinyl-LL-DAP aminotransferase (DAP-AT).[13,14] Thus *N*-Cbz allylglycine methyl ester **7** can be reacted with methyl glyoxylate **8** in the presence of $SnCl_4$ to produce the unsaturated alcohol **9** (Scheme 2). Simple catalytic hydrogenation in the presence of chloroform then affords the saturated amino-alcohol **10** which is stable as the hydrochloride salt. This compound can be selectively *N*-acylated using a wide variety of acylating groups and methods.[4,13] For the synthesis of epoxyaminopimelate we required a protecting group which could be removed under the basic conditions used to cleave the methyl esters at the end of the synthesis. Thus reaction between the amino-alcohol hydrochloride **10** and methyl chloroformate smoothly afforded the protected amine **11** (Scheme 3). Alternatively ene reaction between the appropriately protected allylglycine **12** and methyl glyoxylate **8** followed by catalytic hydrogenation gave the same compound. Oxidation of the secondary alcohol to the corresponding α-ketoester **14** was effected with the Dess–Martin

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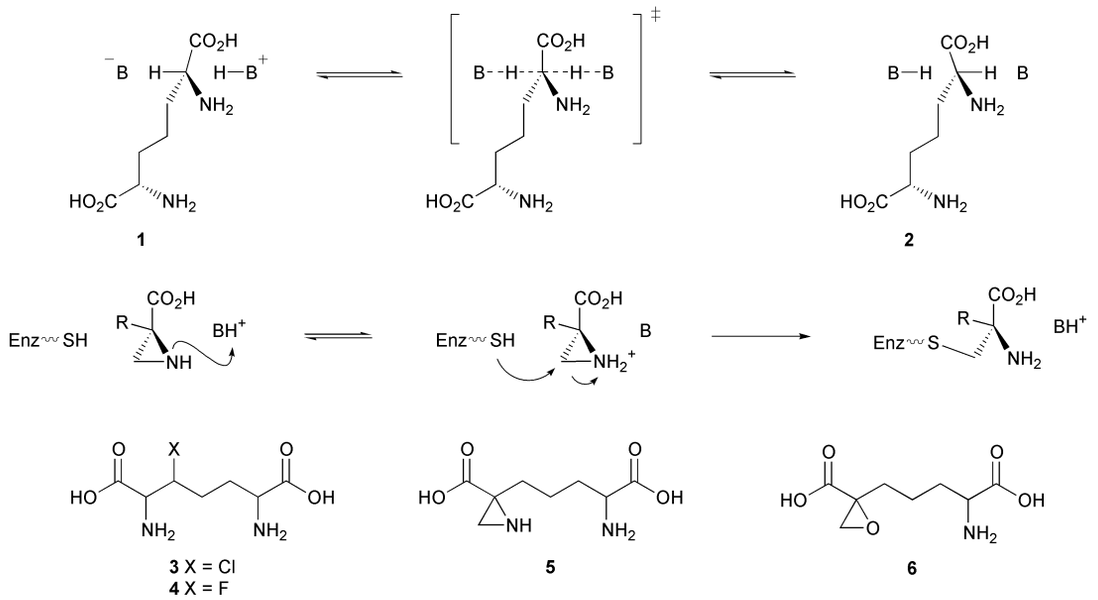
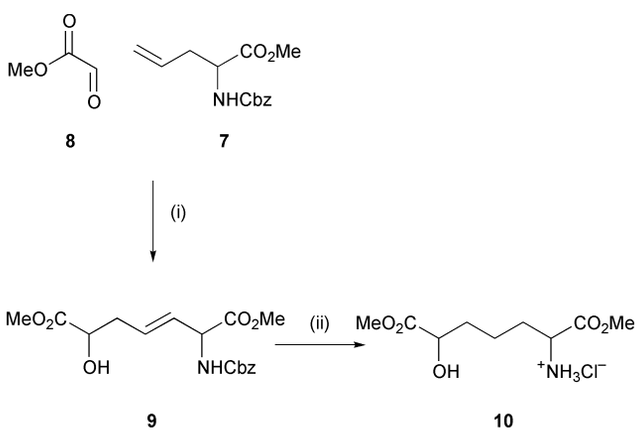
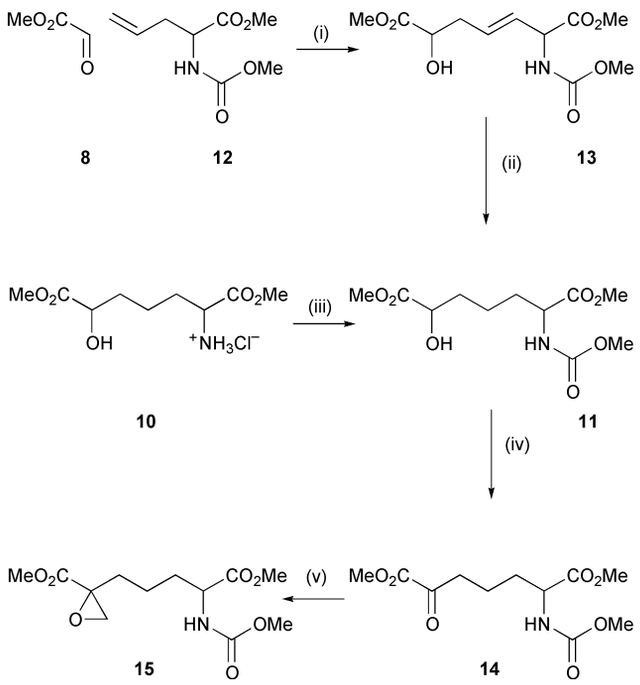


Scheme 1 Reactions catalysed by *meso*-DAP epimerase, known inhibitors **3**–**5** and putative inhibitor **6**.



Scheme 2 Synthesis of hydroxyaminopimelate **10**. *Reagents and conditions*: (i) CH_2Cl_2, $FeCl_3$, 0 °C to RT, 12 h, 65%; (ii) H_2/Pd/C, MeOH–$CHCl_3$, RT, 100%.

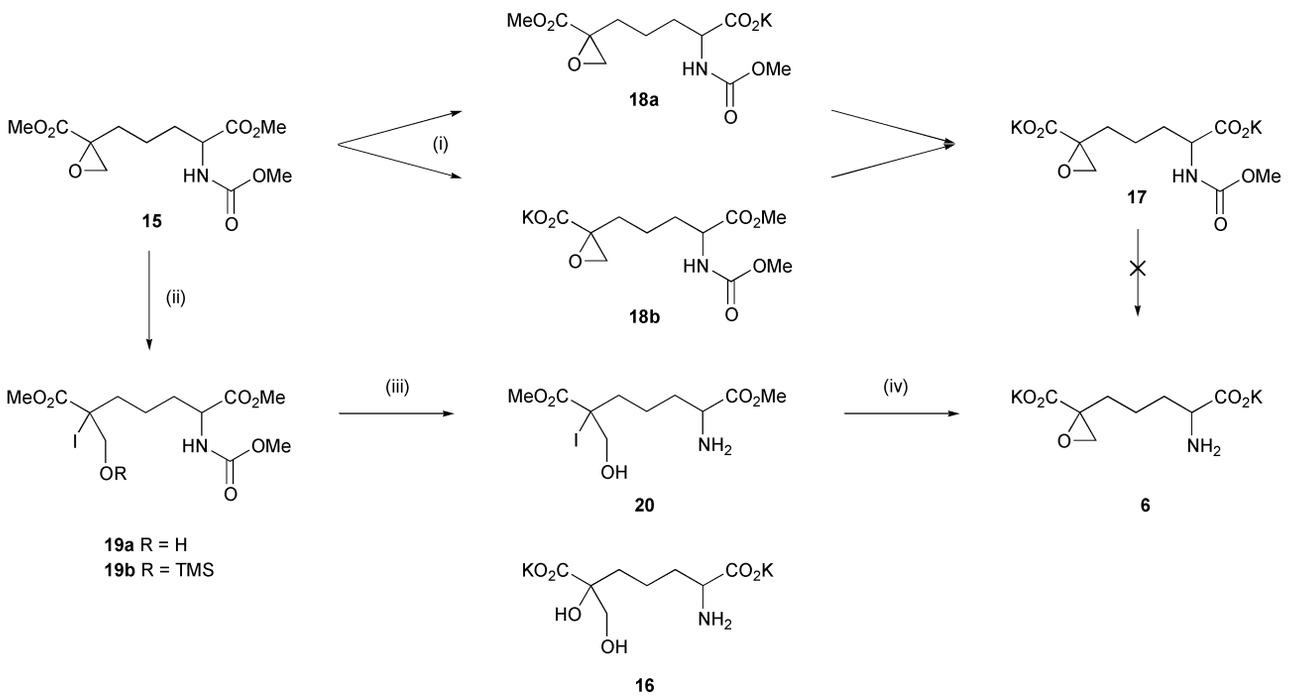


Scheme 3 Synthesis of fully protected epoxide **15**. *Reagents and conditions*: (i) CH_2Cl_2, $FeCl_3$, 0 °C to RT, 12 h, 31%; (ii) H_2/Pd/C, MeOH–$CHCl_3$, RT, 95%; (iii) MeOCOCl, Et_3N, CH_2Cl_2, RT, 58%; (iv) Dess–Martin periodinane, CH_2Cl_2, RT, 85%; (v) CH_2N_2, Et_2O, 0 °C, 60 min, 91%.

periodinane in good yield.[15] At this stage we had a choice of methods available for the formation of the epoxide. In principal Corey–Chaykovsky type epoxidation utilising a sulfur ylide such as dimethylsulfonium methanide[16] could have been utilised, or Wittig olefination followed by treatment with a peracid such as MCPBA. We decided to utilise diazomethane for this transformation and we were pleased to observe a quantitative conversion of the α-ketoester **14** to the corresponding epoxide **15** by the simple expedient of dissolving the ketone in a solution of diazomethane in diethyl ether at 0 °C.

The final stage of the synthesis required selective methyl ester and carbamate hydrolysis in the presence of the sensitive epoxide functionality. We had previously observed very clean selective methyl ester hydrolysis in the presence of other sensitive groups such as thiolesters when using $LiOH·H_2O$ in a mixture of H_2O and CH_3CN.[17] These conditions caused significant epoxide opening and formation of the diol, and also led to the formation of dimeric material as observed by electrospray mass spectrometry (ESMS). Treatment of **15** with three equivalents of aqueous KOH was no more successful; ESMS analysis indicated significant contamination with diol **16**.

We also tried the use of 3.3 equivalents of potassium trimethylsilanolate (TMSOK) in CH_3CN, THF or DMF, followed by removal of solvent and lyophillisation from deionised water. This process afforded material still containing appreciable amounts of *N*-methyl carbamate (*e.g.* **17**) by ESMS analysis. LCMS analysis over the course of these reactions showed that, after addition of two equivalents of TMSOK, fairly rapid cleavage of the methyl esters occurred. After 20 minutes the mixture contained potassium di-acid derivative **17**, isomeric mono-esters **18a**,**b** and di-ester **15**. After 90 min the majority of the mixture was potassium di-acid derivative **17** and after 2 h the reaction had reached completion. At this point addition of another equivalent of TMSOK caused no further change in composition of the reaction mixture (Scheme 4). Addition of stoichiometric, or excess, quantities of water to form KOH *in situ* (from the excess of TMSOK) was also ineffective, causing no cleavage of the carbamate and observable formation of the corresponding diol. We then tried to add a more potent reagent to cleave the carbamate after the removal of the two esters. TMSI has been used for this purpose and we reasoned that addition of three equivalents could form the bis-TMS ester and cause fragmentation of the carbamate. However, due to



Scheme 4 Deprotection of epoxide **15**. *Reagents and conditions*: (i) THF, 3.3 eq. TMSOK, RT; (ii) 1.0 eq. TMSI, CH_2Cl_2, 0 °C; (iii) 3.3 eq. TMSI, 15 °C, 2 h then 1.0 eq. TMSI, 40 °C, 15 min; (iv) 2 M KOH (aq.), 2 h, RT.

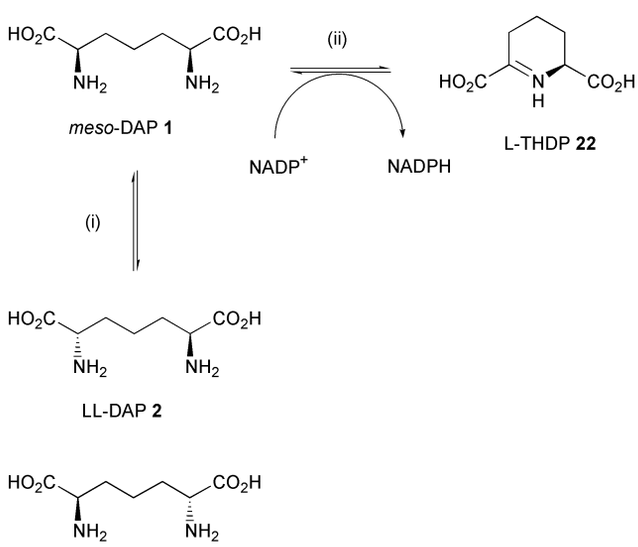
the low solubility of the dipotassium salt **17**, we observed no reaction.

Finally we attempted the careful use of TMSI alone. Addition of one equivalent of TMSI to **15** rapidly opened the epoxide to give the iodo alcohol **19a** and the iodo TMS-ether **19b** as determined by LCMS analysis (Fig 1A, Scheme 4). Addition of further equivalents of TMSI caused no reaction, but warming from 0 °C to 40 °C caused carbamate cleavage to give the *N*-deprotected iodo alcohol **20** as shown by LCMS analysis (Fig 1B, Scheme 4). Even prolonged heating at 60 °C did not cause cleavage of the esters. We reasoned that a basic work-up could cause hydrolysis of the esters and concomitantly reclose the epoxide *via* intramolecular S_N2 reaction. Indeed when the reaction mixture was quenched with 2 M aqueous KOH, and stirred at RT for 2 h an appreciable amount of the expected deprotected epoxide **6** with *m*/*z* 203 (MH^+ 204) was formed (Fig 1C). Attempts to isolate **6** from this solution were unsuccessful and the solution was used without further preparation for inhibition assays.

Assay of *meso*-DAP epimerase

Previous assays for DAP epimerase have either relied on radioactive methods (release of ^3H from α-^3H DAP) or on the use of pure LL-DAP **2** in conjunction with DAP-dehydrogenase. Pure samples of LL-DAP **2** were unavailable to us and so an assay was devised to utilise a commercially obtained mixture of DAP isomers (Scheme 5). Thus initial incubation of a mixture of DAP isomers (0.1 mM) was performed in the presence of *meso*-DAP dehydrogenase alone, in phosphate buffer (pH 7.3) lacking ammonium ions and containing an excess of $NADP^+$ (1.0 mM). Under these conditions the *meso*-DAP **1** was effectively consumed. The concentration of the NADPH formed was measured at 340 nm (Fig. 2A). The equilibrium constant for this reaction has been reported,[18] and calculation of the extent of reaction indicated that >98% of the *meso*-DAP would be consumed under these conditions at pH 7.3. This reaction then leaves a mixture of LL-DAP **2** and DD-DAP **21** isomers. DD-DAP **21** is not a known substrate or inhibitor for either enzyme.

Addition of the epimerase then causes conversion of the remaining LL-DAP **2** to *meso*-DAP **1** which is rapidly converted to L-THDP **22**, concomitantly converting more $NADP^+$ to



Scheme 5 Assay of *meso*-DAP epimerase. *Reagents and conditions*: (i) *meso*-DAP epimerase; (ii) *meso*-DAP dehydrogenase.

NADPH. The increase in NADPH concentration was again observed at 340 nm. A typical UV trace is shown in Fig. 2A. This procedure was repeated for total DAP concentrations between 0.02 mM and 0.2 mM. The concentration of NADPH yielded after DAP-dehydrogenase incubation was plotted *vs.* initial total DAP concentration, as was the final NADPH concentration after addition of the epimerase and incubation until equilibrium was reached (Fig. 2B). Comparison of the slopes of these two lines indicates the final equilibrium position (in which >98% of *meso*-DAP and LL-DAP has been consumed) to be consistently 33.3% higher than the position before consumption of LL-DAP. Using this data the ratio of isomers present in the commercial DAP may be calculated as 60 : 20 : 20 of *meso* : LL : DD. The initial rate (first 5% of reaction) of conversion of LL-DAP to *meso*-DAP catalysed by the epimerase was then plotted against LL-DAP concentration (20% of total DAP concentration) and is shown in Fig. 2C. This too gave a straight line, as expected when substrate concentration is well

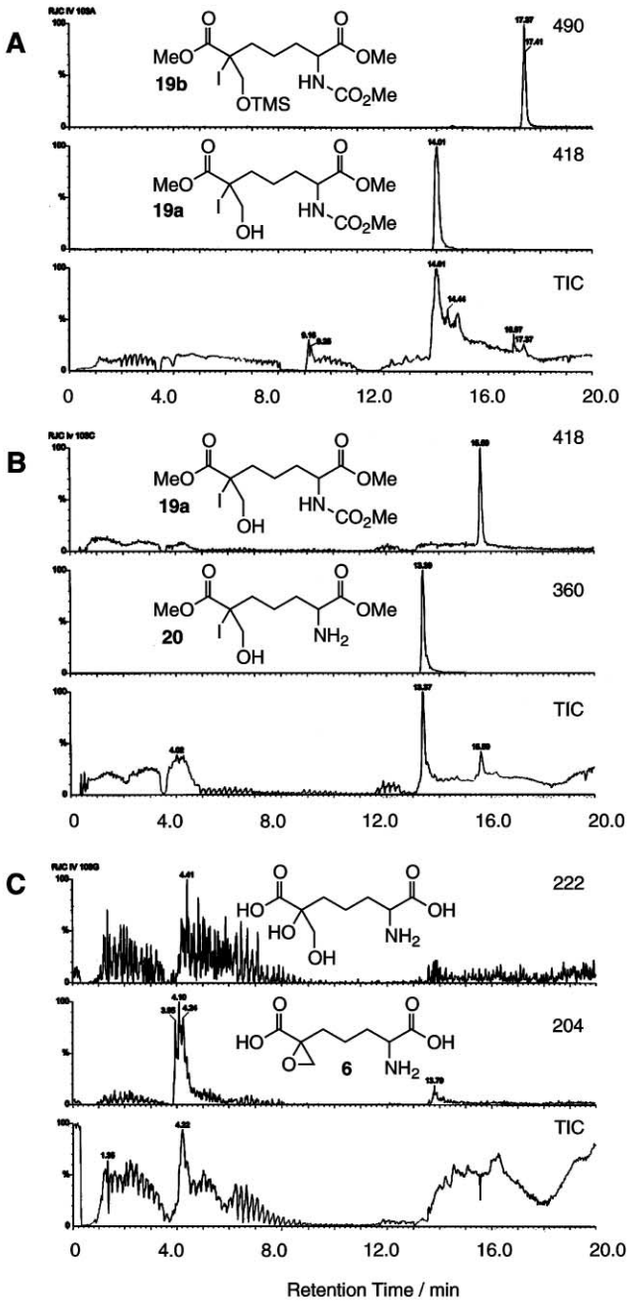


Fig. 1 LCMS analysis of TMSI deprotection of **15**. Upper two traces show selective ion trace at indicated *m*/*z*, lower trace shows TIC (total ion current for *m*/*z* between 150 and 600). **A**: Treatment of **15** with 3.3 eq. of TMSI at 0 °C for 15 min showing rapid cleavage of epoxide. **B**: After warming to 40 °C for 15 min (discrepancy in retention time caused by faulty pump). **C**: After quench with 2 M aqueous KOH—note no detection of diol at *m*/*z* 222.

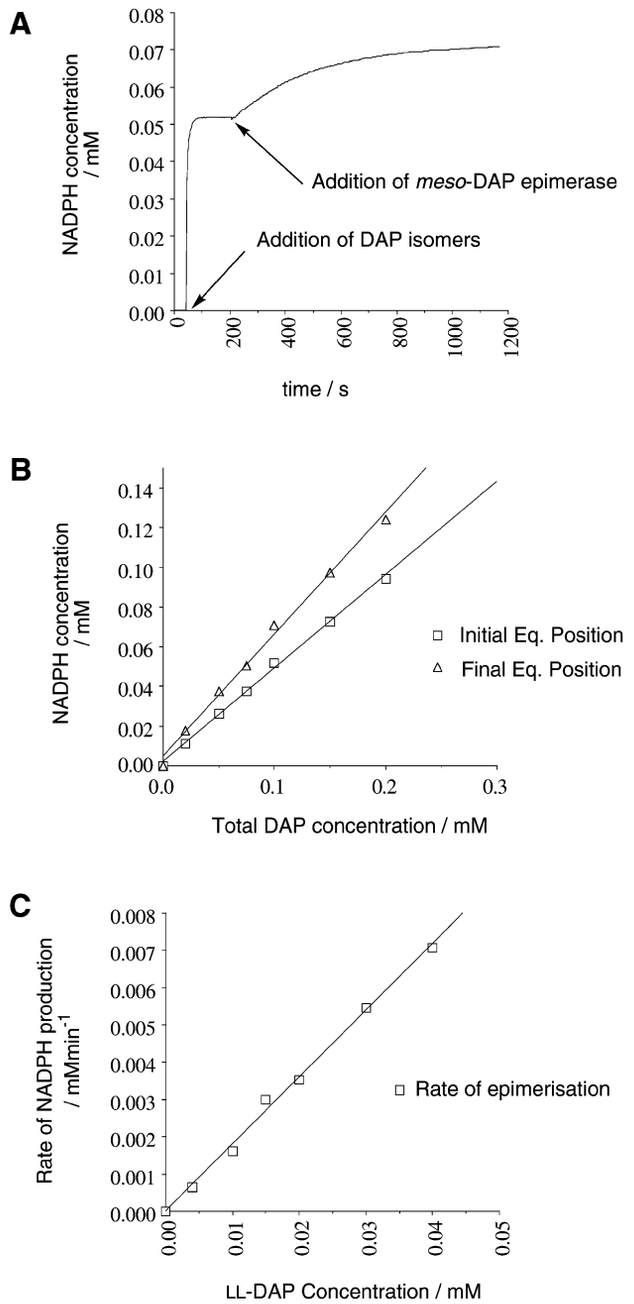


Fig. 2 Assay of DAP epimerase using continuous spectrophotometric procedure. **A**: Raw trace showing how NADPH concentration changes after addition of DAP isomers to *meso*-DAP dehydrogenase and $NADP^+$ at pH 7.3, followed by addition of *meso*-DAP epimerase. **B**: Variation of final equilibrium NADPH concentration (triangles) and initial equilibrium NADPH concentration (squares) with total DAP concentration. **C**: Rate of *meso*-DAP catalysed reaction *vs*. LL-DAP concentration.

below K_M (0.7 mM for LL-DAP has been reported).[8] This assay is a great improvement on previous methods as it is convenient, reproducible, cheap and continuous.

Inhibition studies with epoxyaminopimelate 6

The preliminary experiments described above indicated that the rate of DAP epimerase could be easily measured and that the assay was suitable for further studies. Preliminary experiments attempted to determine whether the epoxide **6** could inhibit the epimerase. In competition with DAP isomers no more than 40% inhibition was caused by **6** at *ca*. 1 mM. However, in assays to observe the expected time dependence of inhibition in the absence of DAP isomers, the epoxide **6** appeared more effective at *ca*. 1 mM (Fig. 3). Using this assay we attempted to measure K_I for irreversible inhibition. However, consistently poor results led us to examine the stability of **6** in the near-neutral assay buffer (pH 7.3). LCMS analysis revealed addition of water to **6** at this pH, presumably *via* epoxide hydrolysis. We reasoned that in 2 M KOH, both carboxylates of **6** are ionised, and electrostatic repulsion between the carboxylates would prevent, or greatly decrease, the rate of epoxide opening catalysed by the α-carboxylate or direct hydroxide attack. At pH 7.3, this situation would be unlikely and it is possible that either water hydrolyses the epoxide of **6** directly, or with the assistance of the α-carboxylate. Either way, at pH 7.3 aqueous hydrolysis of **6**, coupled with non-specific denaturation of DAP epimerase over the course of the assay, prevents accurate determination of K_I.

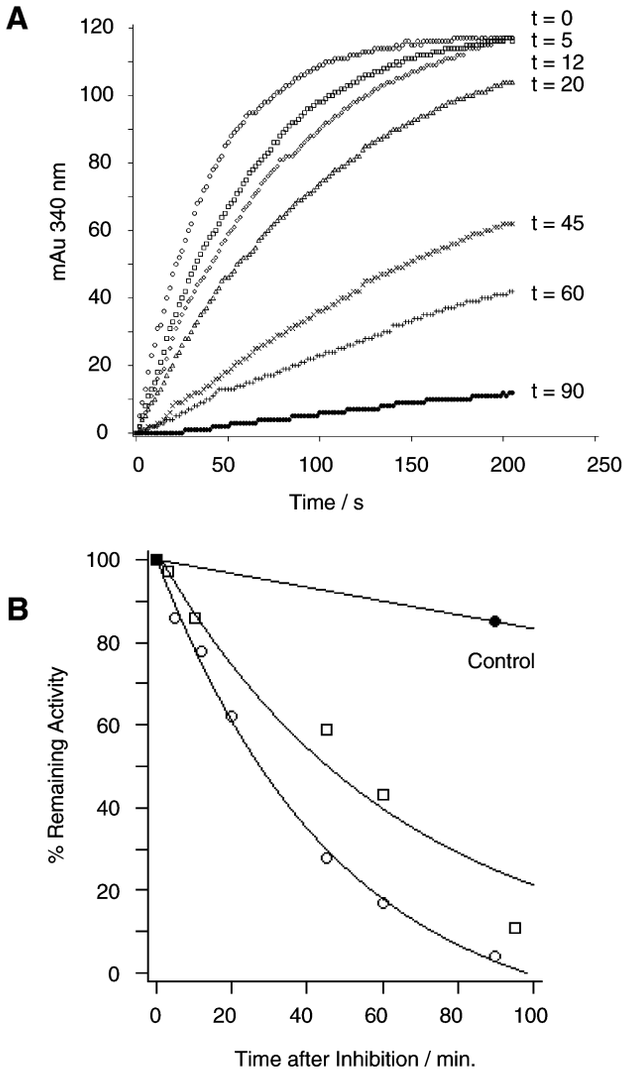


Fig. 3 Time dependent inhibition of *meso*-DAP epimerase by **6**. **A**: Decreasing concentration of active *meso*-DAP epimerase after treatment with **6** in the absence of DAP isomers after an indicated time (min). **B**: Replot of rate data. Circles at *ca*. 1 mM **6**, squares *ca*. 0.5 mM **6**—N.B. *meso*-DAP epimerase loses *ca*. 15% activity over the period of the inhibition assay due to non-specific denaturation.

Overall we have developed a new and convenient assay for DAP epimerase which is consistently reliable and suitable for inhibition studies. This assay procedure is a major improvement over the previous methods. We have also synthesised the epoxide **6** and shown that it indeed inhibits DAP epimerase as expected. However the epoxide **6** is unstable at neutral pH, in contrast to the aziridine **5**. Despite its instability, it may still be possible to obtain crystals of DAP epimerase containing bound **6** and our efforts are currently focussed on this task.

Experimental

All reagents and solvents were obtained from the Sigma-Aldrich chemical company and were of ACS grade and not further purified unless otherwise stated. All anhydrous solvents were purchased from Fluka and were transferred under dried N_2 gas. NMR spectra were obtained using JEOL Λ-300, Δ-270 and Δ-400 spectrometers operating at 300, 270 and 400 MHz (^{1}H) and 75.5, 67.9 and 100.7 MHz (^{13}C) respectively. Chemical shifts are quoted in ppm relative to TMS. Coupling constants (*J*) are quoted in Hz. IR spectra were obtained using a Perkin Elmer 1600 FTIR spectrometer, using KBr discs for solids and thin film between NaCl plates for oils. Mass spectra were obtained in the indicated mode using a VG analytical autospec instrument (EI, CI, FAB, accurate mass) or Fisons VG Quattro spectrometer (ESMS). Flash chromatography was performed according to the method of Still *et al*. [19] or using an improvised automatic system comprising a nitrogen constant pressure head, column packed with Merck silica gel 60 (0.040–0.063 mm), Gilson Holochrome UV detector set at 254 nm and an LKB fraction collector. TLC analysis was performed using Merck glass backed 0.2 mm silica plates (F254) developed with phosphomolybdic acid when necessary.

HPLC methods

HPLC was carried out using a Beckman System Gold 126 pump module equipped with a Beckman 507 autosampler and Beckman 168 diode array UV detector detecting at 218 and 254 nm. Solvents were: **A**, 0.05% trifluoroacetic acid in degassed deionised water; **B**, 0.045% trifluoroacetic acid in HPLC grade acetonitrile (Rathburn).

Method 1: 4.6 × 250 mm Rainin Dynamax 300 Å C_8 column equipped with C_8 guard column eluted at 1 mL min^{-1}; 0–5 min 0% **B**; 5–35 min 0–70% **B**; 35–37 min 70–100% **B**; 37–39 min 100% **B**; 39–41 min 100–0% **B**

Method 2: 4.6 × 250 mm Rainin Dynamax 60 Å C_{18} column equipped with C_{18} guard column eluted at 1 mL min^{-1}; 0–5 min 0% **B**; 5–35 min 0–70% **B**; 35–37 min 70–100% **B**; 37–39 min 100% **B**; 39–41 min 100–0% **B**

LCMS analysis. Solvents were the same as used for HPLC. A Waters 600 pump module, autosampler and UV diode array detector, detecting between 200 and 300 nm, were coupled to a Micromass Platform mass spectrometer operating in positive ion electrospray mode. HPLC method: 4.6 × 250 mm Phenomenex 5 μ C_8 column at 1 mL min^{-1}; 0–13 min 0–100% **B**; 13–17 min 100% **B**; 17–18 min 100–0% **B**; 18–20 min 0% **B**. Approximately 15% of the flow was diverted to the MS for on-line mass analysis (*m*/*z* 150–600).

Methyl (2*RS*)-2-[*N*-(methyloxycarbonyl)amino]pent-4-enoate 12

Racemic allylglycine (Sigma, 5.0 g, 43.5 mmol) was dissolved in aqueous KOH (10 M, 25 ml) and methyl chloroformate (6.0 g, 63.5 mmol) was added dropwise with stirring over a period of 1 h at RT. The pH was maintained at *ca*. 10 by the addition of further KOH solution as required. The reaction mixture was acidified with concentrated aqueous HCl, extracted into ethyl acetate (3 × 25 ml) and the extracts combined, dried ($MgSO_4$) and evaporated to afford a colourless oil which was treated with an excess of a diethyl ether solution of diazomethane.[20] Destruction of the excess diazomethane was achieved by the addition of glacial acetic acid. Removal of solvent gave *methyl (2RS)-2-[N-(methyloxycarbonyl)amino]pent-4-enoate* **12** as a colourless oil (7.1 g, 37.8 mmol, 87%). ν_{max} (thin film)/cm^{-1} 3372, 3083, 2956, 2841, 1643; δ_H(300 MHz, $CDCl_3$) 5.68 (1H, m, δH), 5.16 (3H, m, δH, γH, NH), 4.45 (1H, m, αH), 3.76 (3H, s, OMe), 3.68 (3H, s, OMe), 2.54 (2H, m, βCH_2); δ_C(75.5 MHz, $CDCl_3$) 172.0 (CO_2), 156.6 (CONH), 132.0 (γCH), 119.4 (δCH_2), 54.0 (αCH), 52.4 (2 × OCH_3), 36.7 (βCH_2); *m*/*z* (CI, CH_4) 188 $(MH)^+$ 20%, 156 $(M - MeO)^+$ 19%. Calcd for $C_8H_{14}NO_4$ $(MH)^+$ 188.09228, found 188.09175.

Dimethyl (*E*)-(2*RS*,6*RS*)-2-[*N*-(methyloxycarbonyl)amino]-6-hydroxyhept-3-en-1,7-dioate 13

A solution of freshly distilled methyl glyoxylate **8** (2.95 g, 33.5 mmol) in anhydrous CH_2Cl_2 (55 ml) was stirred under dry nitrogen at 0 °C using a magnetic stirrer and a large 'rugby ball' Teflon coated stirrer bar. Anhydrous $FeCl_3$ (10.9 g, 67.1 mmol) was added and the black suspension was stirred for 30 min at 0 °C. A solution of alkene ester **12** (2.09 g, 11.2 mmol) in anhydrous CH_2Cl_2 (35 ml) was added over 10 min. The reaction mixture was allowed to warm to RT and stirred for 24 h. The mixture was poured onto crushed ice (100 ml) in a separatory

funnel and then extracted with CH_2Cl_2 (4 × 100 ml). The combined organic extracts were dried ($MgSO_4$), evaporated and purified by flash chromatography (60% EtOAc in Hexane, R_f 0.3) to afford *dimethyl (E)-(2RS,6RS)-2-(N-(methyloxycarbonyl)amino)-6-hydroxyhept-3-en-1,7-dioate* **13** as a colourless oil (0.95 g, 3.45 mmol, 31%). ν_{max} (liquid film)/cm^{-1} 3415, 2956, 2845, 1705; δ_H(300 MHz, $CDCl_3$) 5.80 (1H, m, γH), 5.63 (1H, dd, $^3J_{\beta\gamma}$ 16.1, $^3J_{\beta\alpha}$ 5.2, βH), 5.37 (1H, br s, NH), 4.86 (1H, m, αH), 4.27 (1H, m, εH), 3.78 (3H, s, OMe), 3.77 (3H, s, OMe), 3.70 (3H, s, OMe), 2.84 (1H, d, ^3J 6.0, OH), 2.50 (2H, m, δCH_2); δ_C(75.5 MHz, $CDCl_3$) 174.5 (CO_2), 171.0 (CO_2), 156.1 (CONH), 128.3 (CH), 127.7 (CH), 76.6 (εCH), 55.3 (αCH), 52.2 (OCH_3), 52.1 (OCH_3), 52.0 (OCH_3), 36.7 (βCH_2); *m/z* (CI, NH_3) 276 (MH^+) 100%, 244 (M − MeO) 20%. Calcd for $C_{11}H_{18}NO_7$ $(MH)^+$ 276.10833, found 276.10795.

Dimethyl (2*RS*,6*RS*)-2-[*N*-(methyloxycarbonyl)amino]-6-hydroxypimelate 11

Method A. Dimethyl (2*RS*, 6*RS*)-2-amino-6-hydroxypimelate hydrochloride[4,13] **10** (1.24 g, 4.85 mmol) was dissolved in anhydrous CH_2Cl_2 (20 mL) and stirred in an atmosphere of dry N_2 at RT. Methyl chloroformate (690 mg, 844 μL, 1.5 eq.) was added dropwise *via* syringe, followed by the addition of triethylamine (1.47 g, 1.07 mL, 3.0 eq.). The reaction mixture became warm and after 1 h water (30 mL) was added and the organic phase separated. The organic phase was washed with dilute aqueous HCl (30 mL), dried ($MgSO_4$) and evaporated *in vacuo* to afford an orange oil. The oil was purified by flash chromatography (70% ethyl acetate in hexane, R_f 0.32) to afford *dimethyl (2RS,6RS)-2-(N-(methyloxycarbonyl)amino)-6-hydroxypimelate* **11** as a colourless oil (782.9 mg, 58%).

Method B. Dimethyl (*E*)-(2*RS*,6*RS*)-2-[*N*-(methyloxycarbonyl)amino]-6-hydroxyhept-3-en-1,7-dioate **13** (375 mg, 1.35 mmol) was dissolved in anhydrous MeOH (10 mL) and stirred under H_2 (1 atm) for 12 h at RT in the presence of 10% Pd/C (25 mg). Removal of catalyst by filtration through a bed of Celite and removal of solvent *in vacuo* afforded *dimethyl (2RS,6RS)-2-[N-(methyloxycarbonyl)amino]-6-hydroxypimelate* **11** as a colourless oil (357 mg, 1.28 mmol, 95%). ν_{max} (thin film)/cm^{-1} 3480, 3372, 2956, 1738, 1536, 1440; δ_H(300 MHz, $CDCl_3$) 5.24 (1H, d, ^3J 11.5, NH), 4.35 (1H, m, αH), 4.15 (1H, m, εH), 3.69 (3H, s, OMe), 3.67 (3H, s, OMe), 3.64 (3H. s, OMe), 2.65 (1H, br s, OH), 1.80 (2H, m, CH_2), 1.60 (2H, m, CH_2), 1.45 (2H, m, CH_2); δ_C(75.5 MHz, $CDCl_3$, *two diastereomers*) 173.3 (CO), 170.6 (CO), 156.5 (CON), 70.6 and 70.5 (εCH), 54.2 and 54.1 (αCH), 53.8, 53.0, 52.8, 52.7, 52.6 and 52.5 (OMe), 34.2 and 34.1 (δCH_2), 32.8 and 32.7 (βCH_2), 21.2 and 21.1 (γCH_2); *m/z* (CI, CH_4) 278 $(MH)^+$ 20%, 246 $(M - MeO)^+$ 19%. Calcd for $C_{11}H_{20}NO_7$ $(MH)^+$ 278.12398, found 278.12462.

Dimethyl (2*RS*)-2-[*N*-(methyloxycarbonyl)amino]-6-oxopimelate 14

To a solution of dimethyl (2*RS*,6*RS*)-2-[*N*-(methyloxycarbonyl)amino]-6-hydroxypimelate **11** (369 mg, 1.33 mmol) in anhydrous CH_2Cl_2 (10 mL) was added the Dess–Martin periodinane[21] (677 mg, 1.2 eq.). The mixture was stirred at RT for 1 h, then added to a vigorously mixed solution of $Na_2S_2O_3$ (5 g) in saturated aqueous $NaHCO_3$ (20 mL). The mixture was extracted with CH_2Cl_2 (3 × 20 mL). The combined organic extracts were dried ($MgSO_4$) and evaporated *in vacuo* to afford an oil which was purified by column chromatography (50% EtOAc in hexane, R_f 0.47) to give *dimethyl (2RS)-2-[N-(methyloxycarbonyl)amino])-6-oxopimelate* **14** as a colourless oil (309.5 mg, 1.125 mmol, 85%). ν_{max} (thin film)/cm^{-1} 3377, 2956, 1732, 1526, 1454, 1401; δ_H(300 MHz, $CDCl_3$) 5.29 (1H, d, ^3J 8.2, NH), 4.30 (1H, m, αCH), 3.79 (3H, s, OMe), 3.68 (3H, s, OMe), 3.61 (3H, s, OMe), 2.83 (2H, t, ^3J 6.6, δCH_2), 1.80 (1H, m, βCH), 1.60 (3H, m, βCH + γCH_2); δ_C(75.5 MHz, $CDCl_3$) 193.8 (εCO), 173.0 (CO), 170.4 (CO), 157.0 (CONH), 58.38 (αCH), 53.4 (OMe), 52.9 (OMe), 52.8 (OMe), 38.9 (δCH_2), 32.1 (βCH_2), 19.1 (γCH_2); *m/z* (CI, CH_4) 276 $(MH)^+$ 8%, 244 (M − MeO)$^+$ 22%; calcd for $C_{10}H_{14}NO_6$ $(M - OMe)^+$ 244.08208, found 244.08239.

Dimethyl (2*RS*,6*RS*)-2-[*N*-(methyloxycarbonyl)amino]-6,6-(oxymethylene)pimelate 15

Dimethyl (2*RS*)-2-[*N*-(methyloxycarbonyl)amino]-6-oxopimelate **14** (300 mg, 1.04 mmol) was treated with a solution of CH_2N_2 in diethyl ether at 0 °C for 1 h.[20] Solvent was removed *in vacuo* and the residue purified by flash chromatography (50% EtOAc in hexane, R_f 0.5; 10% CH_3CN in CH_2Cl_2 was also effective, R_f 0.27) to give *dimethyl (2RS,6RS)-2-[N-(methyloxycarbonyl)amino]-6,6-(oxymethylene)pimelate* **15** as a colourless oil (273.4 mg, 946 μmol, 91%). ν_{max} (thin film)/cm^{-1} 3605, 3510, 3369, 2999, 2955, 1736, 1530, 1440; δ_H(300 MHz, $CDCl_3$, *two diastereomers*) 5.45 (1H, d, ^3J 7.9, NH), 4.35 (1H, m, αCH), 3.76, 3.75, 3.74, 3.72, 3.71, 3.68 (9H, 3 × OMe), 3.05 (1H, m, ϕCH), 2.80 (1H, m, ϕCH), 2.17 (1H, m, δCH), 1.88 (1H, m, βCH), 1.70 (2H, m, βCH + γCH), 1.55 (2H, m, γCH + δCH); δ_C(75.5 MHz, $CDCl_3$, *two diastereomers*) 172.9, 171.8, 170.6, 170.0, 156.7, 156.6, 58.0, 57.9, 56.63, 56.60, 53.7, 53.4, 52.6, 52.4, 52.3, 52.1, 46.1, 45.6, 32.3, 30.8, 30.62, 29.4, 21.4, 20.8; *m/z* (CI, CH_4) 290 $(MH)^+$ 5%, 258 $(M - MeO)^+$ 10%, calcd for $C_{12}H_{20}NO_7$ $(MH)^+$ 290.12398, found 290.12367.

Deprotection of dimethyl (2*RS*,6*RS*)-2-[*N*-(methyloxycarbonyl)amino]-6,6-(oxymethylene)pimelate 15. Dimethyl (2*RS*, 6*RS*)-2-[*N*-(methyloxycarbonyl)amino]-6-epoxymethanopimelate **15** (60.3 mg, 208.6 μmol) was dissolved in anhydrous CH_2Cl_2 (2 ml) in a septum sealed Wheaton reaction vial. The solution was stirred under an atmosphere of dry nitrogen at 0 °C and TMSI was added dropwise by syringe (138 mg, 98 μl, 3.3 eq.) to give a pale lemon yellow solution. The reaction mixture was warmed to 15 °C over the period of 2 h and a further aliquot of TMSI was added (33 μl). The reaction was warmed to 40 °C for 15 min, then quenched by the addition of 2 M KOH (1 ml). The mixture became colourless immediately, and was stirred at RT for a further 2 h. The CH_2Cl_2 layer was removed by careful pipetting and discarded. The aqueous solution of **6** was analysed by LCMS and stored at −20 °C. Immediately prior to enzyme assays the solution was neutralised by the careful adition of *ca*. 2 M aqueous HCl. LCMS method 1: R_t 4.2 min; *m/z* ($ESMS^+$) 204 (MH^+) 100%, 242 (MK^+) 25%; *m/z* ($ESMS^-$) 202 $(M - H)^-$ 100%.

Enzyme purification

His_6-*meso*-DAP epimerase. A recombinant form of the enzyme with an amino terminal polyhistidine affinity label was used in this study. To ensure the preparation of an active form of the enzyme, the enzyme was purified *via* conventional chromatography techniques in reducing conditions. The *E. coli* strain BL21 (DE3) was transformed with recombinant plasmid pET28-HiDapF in which the DapF gene is under control of a T7 promoter system. Cells were grown in Luria Bertani (LB)[14] media at 37 °C, supplemented with kanamycin to 35 μg ml^{-1} to maintain plasmid selection and were grown to an OD_{600} = 0.5 prior to the addition of IPTG to 0.5 mM to induce expression of the *dapF* gene. The cultures were grown for a further three hours before harvesting by centrifugation. Crude extracts were prepared by ultrasonication in a buffer comprised of 50 mM Hepes pH 8.0 and 5 mM dithiothreitol (DTT) (buffer A). After centrifugation at 30000 g to remove cellular debris, the crude extract was applied to a 25 ml Q-Sepharose HP (Amersham-Pharmacia Biotech) column equilibrated in buffer A and developed with a linear gradient of NaCl to 0.5 M over 20 column volumes. The DapF protein eluted as the major protein peak at approximately 200 mM NaCl. Active fractions

were pooled and diluted with buffer A to reduce the NaCl concentration to <50 mM. The protein was then purified to greater than 99% purity using a high resolution MonoQ10/10 anion exchange column (Amersham-Pharmacia Biotech) equilibrated and developed as described above. *m/z* (ESMS) 32293 ± 13 (calculated 32296 for His_6-*Haemophilus influenzae* DAP epimerase).

His_6-*meso*-DAP dehydrogenase. The gene encoding *Corynebacterium glutamicum* DAP dehydrogenase was cloned from chromosomal DNA preparations by PCR techniques and placed under the control of a T7 promoter system after insertion into the vector pET28 (Novagen Inc). Competent BL21 (DE3) cells were transformed with this recombinant plasmid which were then grown and induced in similar conditions as for the DapF producing cell line. After three hour induction DAP dehydrogense accumulated to in excess of 30% of total soluble protein in the cell. The protein was then purified as previously described [22] by anion exchange and red-sepharose column chromatography to in excess of 90% purity. *m/z* (ESMS) 35087 ± 24 (calculated 35099 for *C. glutamicum* His_6-DAP dehydrogenase).

Enzyme assays

Standard activity assay. Assays were conducted in a 10 mm path-length quartz cuvette at 37 °C in a water heated cuvette holder using a Pharmacia LKB ultrospec III spectrophotometer recording at 340 nM. Assay reactions initially contained a mixture of DAP isomers (Sigma, 0.01–0.1 mM), DAP dehydrogenase (200 μg) and $NADP^+$ (1 mM) in a total volume of 980 μL, made up with 100 mM phosphate buffer (pH 7.3) containing 1.0 mM DTT. When all of the available *meso*-DAP had been consumed (*ca*. 1 min) and a steady equilibrium position had been reached, *meso*-DAP epimerase (20 μL of a 5 mg mL^{-1} solution) was added and the rate of increase in absorption at 340 nm (A_{340}) was measured over the first 30 seconds of reaction. Reactions were allowed to reach equilibrium and the final A_{340} was also measured. NADPH concentration was calculated using the Beer–Lambert law $A_{340} = 6220 \times [NADPH]$.

Inhibition studies. Competetive inhibition studies were carried out as described above, except that assay reactions also contained the epoxide **6** (0.1–2 mM maximum). Time dependent studies were carried out by adding the epoxide **6** to a reaction mixture containing the epimerase in the presence of all other reaction components except DAP isomers, incubated at 37 °C. At time points 10 μL aliquots were removed from the reaction mixture and assayed for epimerase activity as described above. Residual epimerase activity (*i.e.* rate of NADPH production) was plotted *vs*. time of assay.

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References

1 R. J. Cox, *Nat. Prod. Rep.*, 1996, **13**(1), 29–44.
2 R. J. Cox, A. Sutherland and J. C. Vederas, *Bioorg. Med. Chem.*, 2000, **8**, 843–871.
3 G. Scapin and J. S. Blanchard, *Adv. Enzymol.*, 1998, **72**, 279–324.
4 R. J. Cox, H. Jenkins, J. A. Schouten, R. A. Stentiford and K. J. Wareing, *J. Chem. Soc., Perkin Trans. 1*, 2000, 2023–2036.
5 G. Scapin, S. G. Reddy and J. S. Blanchard, *Biochemistry*, 1996, **35**, 13540–13551; G. Scapin, M. Cirilli, S. G. Reddy, Y. Gao, J. C. Vederas and J. S. Blanchard, *Biochemistry*, 1998, **37**, 3278–3285.
6 T. W. Beaman, D. A. Binder, J. S. Blanchard and S. L. Roderick, *Biochemistry*, 1997, **36**, 489–494; T. W. Beaman, J. S. Blanchard and S. L. Roderick, *Biochemistry*, 1998, **37**, 10363–10369.
7 M. Cirilli, R. Zheng, G. Scapin and J. S. Blanchard, *Biochemistry*, 1998, **37**, 16452–16458.
8 C. W. Koo and J. S. Blanchard, *Biochemistry*, 1999, **38**, 4416–4422.
9 C. W. Koo, A. Sutherland, J. C. Vederas and J. S. Blanchard, *J. Am. Chem. Soc.*, 2000, **122**, 612–6123.
10 R. J. Baumann, E. H. Bohme, J. S. Wiseman, M. Vaal and J. S. Nichols, *Antimicrob. Agents Chemother.*, 1988, **32**(8), 1119–1123.
11 M. H. Gelb, Y. Lin, M. A. Pickard, Y. Song and J. C. Vederas, *J. Am. Chem. Soc.*, 1990, **112**, 4932–4942.
12 F. Gerhart, W. Higgins, C. Tardif and J.-B. Ducep, *J. Med. Chem.*, 1990, **33**, 2157–2162.
13 R. J. Cox, W. A. Sherwin, L. Lam and J. C. Vederas, *J. Am. Chem. Soc.*, 1996, **118**, 7449–7460.
14 R. J. Cox, J. A. Schouten, R. A. Stentiford and K. J. Wareing, *Bioorg. Med. Chem. Lett.*, 1998, **8**, 945–950.
15 D. B. Dess and J. C. Martin, *J. Org. Chem.*, 1983, **48**, 4156–4158; D. B. Dess and J. C. Martin, *J. Am. Chem. Soc.*, 1991, **113**, 7277–7287.
16 E. J. Corey and M. Chaykovsky, *J. Am. Chem. Soc.*, 1962, **84**, 3782–3783.
17 R. J. Cox and P. S. H. Wang, *J. Chem. Soc., Perkin Trans. 1.*, 2001, 2022–2034.
18 H. Misono and K. Soda, *Agric. Biol. Chem.*, 1980, **44(9)**, 2125–2128.
19 W. C. Still, M. Kahn and A. Mitra, *J. Org. Chem.*, 1978, **43**, 2923.
20 B. S. Furniss, A. J. Hannaford, P. W. G. Smith and A. R. Tatchell, *Vogel's Textbook of Organic Chemistry*, Longman, New York, 5th edn., 1989, pp. 430–433.
21 R. E. Ireland and L. Longbin, *J. Org. Chem.*, 1993, **58**, 2899.
22 S. G. Reddy, G. Scapin and J. S. Blanchard, *Proteins: Struct. Funct., Genet.*, 1996, **25**, 514–516.