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Synthesis of a Key Intermediate in the Diaminopimelate Pathway to L-Lysine: 2,3,4,5-Tetrahydrodipicolinic Acid

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Abstract: 2,3,4,5-Tetrahydrodipicolinic acid (**3**) is a key intermediate in the diaminopimelate (DAP) pathway to L-lysine (**7**). It was synthesized as the stable racemic potassium salt (**25**) from dipicolinic acid (**14**) by esterification, hydrogenation to the *cis*-diester (**17**), followed by elimination of *p*-toluenesulfinic acid from the *N*-toluenesulfonyl derivative (**24**) of dimethyl *cis*-piperidine-2,6-dicarboxylate with concomitant cleavage of the ester groups. (±)-2,3,4,5-Tetrahydrodipicolinic acid is unstable in neutral or acidic solution, and in basic solution it exists in equilibrium with the corresponding enamine (**27**) and 2-oxo-6-aminoadipate (**26**).

INTRODUCTION

The biosynthesis of L-lysine (**7**) occurs only in micro-organisms and plants. The absence of lysine biosynthesis in animals has stimulated interest in a more detailed understanding of lysine biosynthesis which could result in the development of new antibiotics and herbicides. There are two main pathways to L-lysine (**7**). Bacteria and higher plants make L-lysine (**7**) via the diaminopimelate (DAP) pathway (Scheme 1),[1] whereas yeast and fungi utilize the α-aminoadipate pathway.[2] The first specific step in the DAP pathway involves the condensation of L-aspartic acid β-semialdehyde (**1**) with pyruvate to form L-2,3-dihydrodipicolinic acid (**2**).[3] In order to study this step in more detail we devised a synthetic route to aspartic acid β-semialdehyde as the trifluoroacetate salt which could be isolated in a stable solid form.[4] This encouraged us to study the enzyme for this condensation step, dihydrodipicolinate synthase. This enzyme was purified and crystallized by Laber *et al.*[5] and we confirmed that the first step in the mechanism catalyzed by this enzyme involves imine formation between the enzyme and pyruvate.[6] The next step in the DAP pathway involves a reductase which catalyses the formation of L-2,3,4,5-tetrahydrodipicolinic acid (THDPA) (**3**). The synthase and reductase enzymes have been isolated from *Escherichia coli*[7] and from plants.[8] However little chemical or spectroscopic evidence existed for the product (**3**) and no literature method was available for the synthesis of **3** in a form which could be isolated and characterized. A stable form of THDPA (**3**) was urgently required in order to set up an assay for the reductase enzyme and as a substrate for a study of the succinylase step in the DAP pathway leading to the

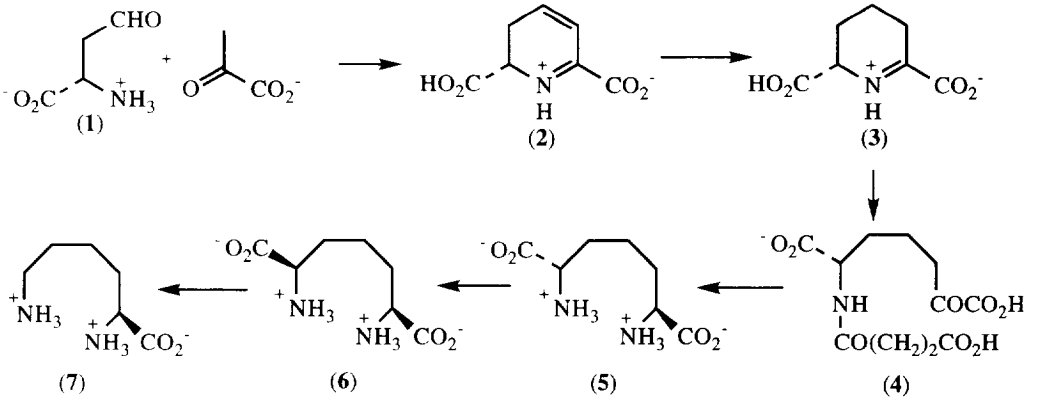
succinamide derivative (**4**).[9] The final steps in the DAP pathway involve hydrolysis of the succinamide (**4**) to yield LL-DAP (**5**), followed by epimerization to give DL-DAP (**6**), and decarboxylation to afford L-lysine (**7**). We now describe the work leading up to the synthesis of the solid, stable, racemic potassium salt of THDPA (**3**).

RESULTS AND DISCUSSION

Gilvarg and co-workers reported the synthesis of THDPA (**3**) from *meso*-DAP (**6**) using *meso*-DAP dehydrogenase (EC 1.4.1.16) in the presence of $NADP^+$ at pH 10.5.[10] This enzyme is used by some Gram positive bacteria in the reverse direction, converting THDPA (**3**) into *meso*-DAP (**6**) using NADPH, in a shortened route to L-lysine (**7**).[11] Formation of THDPA from (**6**) was monitored by us by the absorbance at 280 nm and it was purified by gel filtration chromatography. Although we could get evidence for the formation of THDPA by this method we were unable to isolate suffficient material for characterization.

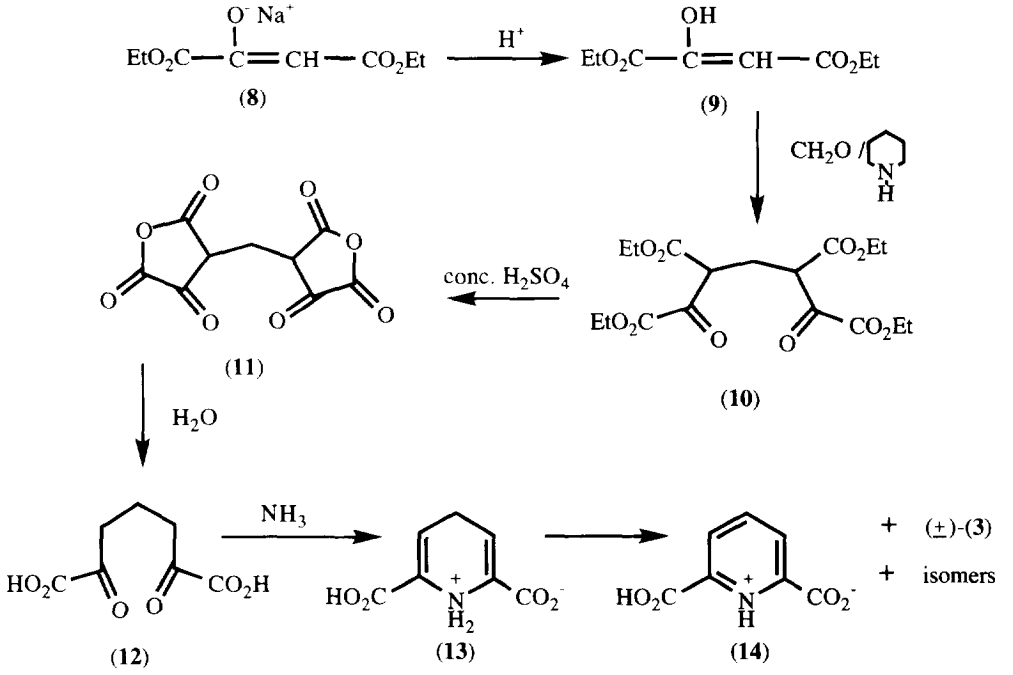
Another enzymic method for the formation of THDPA was reported by Shapshak.[12] This involved treatment of DL-DAP (**6**) with the L-amino acid oxidase purified from *Neurospora crassa,* although no chemical or spectroscopic evidence was provided for the formation of 2-oxo-6-aminopimelic acid and its cyclization to THDPA (**3**). We separated DL-DAP from a commercial mixture of DD-, LL-, and DL-isomers by crystallisation from water and ethanol.[13] When DL-DAP (**6**) was treated with L-amino acid oxidase (EC 1.4.3.2) or D-amino acid oxidase (EC 1.4.3.3) in D_2O at pD 6 at 18 °C or 40 °C no reaction was observed after 2 weeks according to ^1H NMR spectroscopy.

Next we turned to chemical methods of transamination. Ohto and Okamoto found that the imine formed between benzylamine and isonicotinaldehyde was converted into benzaldehyde in quantitative yield by treatment with DBU followed by acidic hydrolysis.[14] A mixture of DL-DAP (**6**) with isonicotinaldehyde in DMF with DBU as base did not afford any THDPA (**3**).



Scheme 1

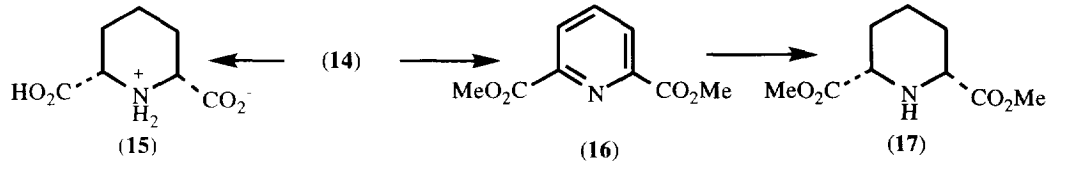
Treatment of αα'-dioxopimelic acid (**12**) with ammonia was reported by Kimura and Sasakawa[15] to yield dipicolinic acid (**14**) and (±)-THDPA (**3**) (Scheme 2). Since oxygen had no effect on the rate of reaction, it was assumed that the initial reaction product, 1,4-dihydrodipicolinic acid (**13**), disproportionated to dipicolinic acid (**14**) and (±)-THDPA (**3**). The evidence for the formation of **14** was provided by UV absorption and by colour reactions with ninhydrin and *o*-aminobenzaldehyde for (±)-THDPA (**3**). We prepared αα'-dioxopimelic acid by a modification of the route of Cope and Fournier.[16] Condensation of two moles of diethyl oxaloacetate (**9**) [prepared from the commercially available sodium salt (**8**)] with one of formaldehyde gave the pimelate derivative (**10**) which was converted into the bisanhydride (**11**) and hydrolysis and decarboxylation afforded αα'-dioxopimelic acid (**12**). When αα'-dioxopimelic acid was left with liquid ammonia in a sealed tube overnight a mixture of products was obtained. Examination of the residue by ^{1}H and ^{13}C NMR spectroscopy indicated that dipicolinic acid (**14**) was present as its diammonium salt together with another major component which could have been (±)-THDPA (**3**). There was also evidence of a number of partially reduced forms, but the mixture could not be separated and individual components could not be identified.



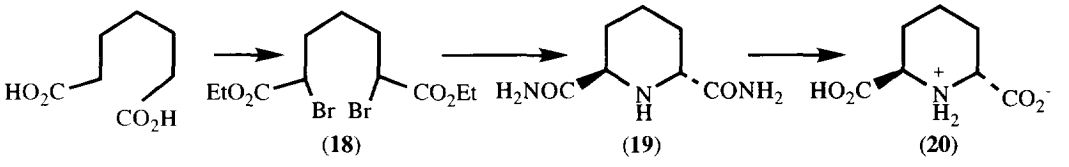
Scheme 2

A number of methods are known for the oxidation of amines to the corresponding imines. Leete and Kim oxidized hygrine by heating it in dilute acetic acid in the presence of mercuric acetate to generate the corresponding imine.[17] This procedure was carried out with *cis*-piperidine-2,6-dicarboxylic acid (**15**) prepared by the hydrogenation of dipicolinic acid but starting material was recovered. The corresponding diester (**17**)

was most conveniently made by esterification of dipicolinic acid (**14**) followed by catalytic hydrogenation (Scheme 3). This material was not oxidized to the imine on warming in mercuric acetate and dilute acetic acid but instead was hydrolyzed to **15**. The *trans*-diacid (**20**) was prepared by α-bromination of pimelic acid leading to the dibromodiester (**18**). Cyclization of **18** using liquid ammonia gave a mixture of the *cis*- and *trans*-diamides, from which the racemic *trans*-isomer (**19**) could be isolated after removal of the less water soluble *cis*-diamide (Scheme 4).[18] Hydrolysis of **19** gave the racemic *trans*-diacid. This diacid (**20**) also failed to react on treatment with mercuric acetate in acetic acid. Reaction of these compounds may be prevented because of the formation of a mercury complex with the amino diacid system.



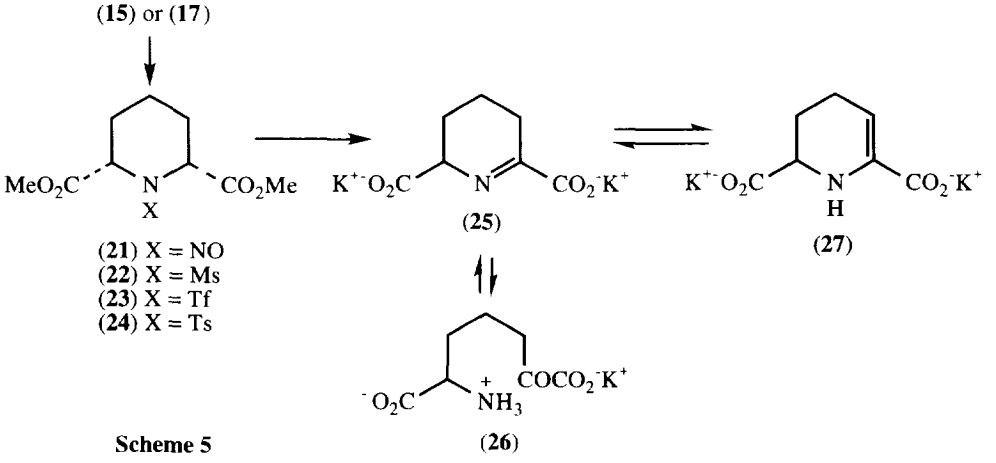
Scheme 3



Scheme 4

Amines have been converted into imines by reaction with electrophilic reagents such as *N*-bromosuccinimide, followed by elimination of HBr from the *N*-haloamines on treatment with base. When dimethyl *cis*-piperidine-2,6-dicarboxylate (**17**) was subjected to these conditions, a mixture of products was formed as judged by the ^{13}C NMR spectra. Other leaving groups on nitrogen were tried. The elimination of HNO from nitrosoamines on treatment with base is known to yield imines.[19] The *N*-nitroso derivative (**21**) of the *cis*-diester (**17**) was prepared using nitrous acid, but elimination of HNO could not be achieved with a wide variety of bases. The mesylate (**22**), triflate (**23**) and *p*-toluenesulfonate (**24**) derivatives of the diester (**17**) were also prepared but could not be induced to form the racemic imine (**3**) using DBU, DBN, NaH or KH although sulfonamides are known to undergo elimination under the influence of strong bases.[20] When the *p*-toluenesulfonamide (**24**) was treated with KH or $KN(TMS)_2$ in DMSO and the organic extracts were acidified, *p*-toluenesulfinic acid was obtained and the aqueous solution was freeze dried to give a mixture of products. When the *cis*-diester (**24**) was treated with KO^tBu, an exothermic reaction took place and a yellow solid precipitated after several hours. Evidence for the formation of an imine by elimination of *p*-toluenesulfinic acid was obtained from ^1H and ^{13}C NMR data which also indicated that cleavage of the ester groups had taken place. Attempted purification of this material using ion exchange chromatography under neutral or acidic

conditions gave complex mixtures. Purification of a solution of the yellow solid was finally achieved with a weak anion exchanger in the hydroxide form. An ion of *m/z* 171 present in the FAB MS of this solid could be due to the imine (**3**). The ^{13}C NMR spectrum taken in D_2O was consistent with the presence of the potassium salt (**25**) of (±)-THDPA together with (±)-2-oxo-6-aminopimelic acid (**26**) in *ca.* a 1:1 ratio *e.g.* two methine signals were present at δ 67.2 and 63.8 and there was a ketone carbonyl at δ 216.7. Further detailed ^1H NMR spectroscopic examination at 270 MHz in D_2O provided evidence for the presence of three racemates in solution, namely THDPA (**25**), the open chain form (**26**), and the enamine (**27**) in a 2.8:1:3.7 ratio, *e.g.* the enamine showed a dd at δ 5.20 due to the olefinic proton and the three separate signals for the proton α to an amino acid in the three racemic compounds **25**, **26** and **27** integrated in total for one proton. Samples of the mixture (**25**)-(**27**) that had been left for some time also showed formation of dipicolinic acid (δ 8.34 in the ^1H NMR spectrum) in small amounts. The effect of pH on the NMR spectra of the mixture was studied. At neutral or acidic pH the spectra changed rapidly indicating decomposition of the material and formation of a number of other compounds.



Scheme 5

Hydrogenation of the mixture of racemates (**25**)-(**27**) in water using PtO_2 as catalyst gave a single product, *cis*-piperidine-2,6-dicarboxylic acid (**20**), in 95% yield. This was identified by ^1H, ^{13}C NMR, m.p., and mixed m.p. with authentic material. This is strong supporting evidence for the presence of racemates (**25**)-(**27**) in equilibrium.

Furthermore when the racemic potassium salt (**25**) of THDPA was incubated with *meso*-DAP dehydrogenase, NADPH was used up indicating that one enantiomer of (**25**) is a substrate for this enzyme.

(±)-THDPA is stable if kept as the potassium salt (**25**). In neutral or acid solution it rapidly decomposes. This could explain why methods which employ neutral or acidic conditions have failed to generate (±)-(**3**). (±)-THDPA is now available to study the reductase step in the lysine pathway and to act as a substrate for the succinylase step.

EXPERIMENTAL

All melting points were measured with a Kofler hot-stage apparatus and are uncorrected. IR spectra were obtained on a Perkin Elmer 580 spectrophotometer as KBr discs unless otherwise stated. UV spectra were recorded with a Pye-Unicam SP-100 spectrophotometer. NMR spectra were recorded with a Perkin Elmer R 32 spectrometer operating at 90 MHz (δ_H); with a Bruker WP-200 SY spectrometer operating at 200 MHz (δ_H) or 50 MHz (δ_C); or with a JEOL GSX270 spectrometer operating at 270 MHz (δ_H). Mass spectra were determined with AEI MS 12 or 902 spectrometers. FAB MS were obtained with a JEOL DX303 spectrometer. TLC was carried out on Kieselgel 60 F_{254} plastic sheets of 0.25 mm thickness.

THF was distilled from KOH and then from sodium-benzophenone under nitrogen prior to use. DMSO and dichloromethane were distilled from CaH_2. Organic solvents were dried with sodium sulfate. All enzymes were purchased from Sigma.

Separation of meso-*Diaminopimelic Acid (**6**) from DD- and DL-Diaminopimelic Acid.*[13] Diaminopimelic acid (5.0 g, 26.3 mmol), (available from Sigma as a mixture of isomers) was dissolved in boiling water (125 ml). The solution was allowed to cool to room temperature and ethanol (100 ml) was added, giving a turbid solution. This solution was left overnight and the crystals were filtered off to give *meso*-diaminopimelic acid (**6**) (2.7 g, 14.2 mmol, 54%) m.p. > 300 °C (lit.,[13] > 300 °C); ν_{max} 3040, 2500, 1630, 1600 and 1530 cm^{-1}; δ_H (90 MHz) (D_2O + DCl) 1.89 (2H, m), 2.19 (4H, m) and 4.20 (2H, m); *m/z* 190 (M^+, 1%), 128 (23%) and 56 (100%).

Attempted Enzymic Transamination of meso-*Diaminopimelic Acid (**6**).* *meso*-Diaminopimelic acid (**6**) (70 mg) was dissolved in D_2O and DCl. The pD was adjusted to 6 using NaOD. D-Amino acid oxidase enzyme (EC 1.4.3.3.) (6 mg, 1.62 units) was added. The reaction was followed by ^1H NMR spectroscopy, but after 7 d no change had taken place. The reaction was repeated using L-amino acid oxidase (EC 1.4.3.2.) but no reaction took place.

Attempted Transamination of Diaminopimelic Acid. Isonicotinaldehyde (0.38 g, 3.5 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (5.49 g, 9 mmol) were added to a suspension of diaminopimelic acid (0.57 g, 3 mmol) in DMF (20 ml). The suspension was stirred for 18 h at room temperature. The solid was filtered off (0.57 g) and ^1H NMR spectroscopy indicated that this was starting material.

Diethyl Oxaloacetate. A mixture of the sodium salt (**8**) of diethyl oxalacetate (100 g, 0.54 mol) in 2.5M H_2SO_4 (200 ml) and diethyl ether (150 ml) was stirred for 1 h at room temperature. The two layers were separated and the aqueous layer was extracted with diethyl ether (2 x 50 ml). The combined ether extracts were dried, filtered, and concentrated to afford an oil. Distillation gave diethyl oxaloacetate as a clear oil, (50 g, 57%), b.p. 110 °C (2.6 mmHg), R_F ($CHCl_3$) 0.42; ν_{max} (liq. film) 2990, 1740, 1650, and 1250 cm^{-1}; δ_H (90 MHz) ($CDCl_3$) 1.20 (12H, t), 3.70 (1H, t), 4.20 (8H, q) and 5.90 (1H, s); *m/z* 188 (M^+, 100%), and 115 (48%) (Found: C, 44.66; H, 6.38. $C_8H_{12}O_5$ requires C, 44.68; H, 6.38%).

*Methylene Bis(diethyl oxaloacetate) (**10**).* A mixture of distilled diethyl oxaloacetate (50 g, 0.26 mmol), 30% formaldehyde solution (10.6 g, 0.13 mmol), piperidine (0.1 ml), and ethanol (5 ml) was stirred for 48 h at room temperature. The white solid was filtered off and dried to give methylene bis(diethyl oxaloacetate) (35 g, 70%), m.p. 80 - 82 °C (lit.,[16] 80 - 84 °C); R_F ($CHCl_3$) 0.15; ν_{max} 2990, 1740, 1640, and 1210 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.42 (12H, t), 2.80 (2H, m), 3.40 (2H, m), and 4.30 (8H, q); δ_C (50 MHz) ($CDCl_3$) 13.72 (q), 17.91 (t), 45.20 (d), 61.09 (t), 62.75 (t), 94.78 (s), 168.32 (s), and 169.21 (s); *m/z* 84 (100%) (Found: C, 52.57; H, 6.23. $C_{17}H_{24}O_{10}$ requires C, 52.53; H, 6.22%).

*α,α′-Dioxopimelic Acid (**12**).* Methylene bis(diethyl oxaloacetate) (**10**) (12.10 g, 31 mmol) in conc. H_2SO_4 (13 ml) was stirred at room temperature for 7 d. The solid was filtered off, washed with toluene (20 ml), and dried to give the anhydride (**11**) as white crystals (5.0 g) which were used immediately. The anhydride (**11**) (5.0 g) in water (15 ml) was heated at reflux for 30 min. The solution was concentrated under reduced pressure and dried over P_2O_5 to give α,α′-dioxopimelic acid (**12**) as a white solid (2.6 g, 45%), m.p. 125 - 127 °C (lit.,[16] 127 °C); R_F ($CHCl_3$ - MeOH - AcOH, 74 : 24 :1) 0.42; ν_{max} 3400, 2990, 1740, 1720, and 1100 cm^{-1}; δ_H (200 MHz) (d_6-DMSO) 1.72 (2H, m), and 2.95 (4H, t); δ_C (50 MHz) (d_6-DMSO) 16.39 (t), 27.74 (t), 162.75 (s), and 196.34 (s); *m/z* 188 (100%), and 142 (42%) (Found: M^+, 188.0296. $C_7H_8O_6$ requires M^+, 188.0321).

*Cyclization of α,α′-Dioxopimelic Acid (**12**).* α,α′-Dioxopimelic acid (1.00 g, 5.32 mmol) was kept in a sealed tube with liquid ammonia at room temperature for 18 h. The tube was opened at -78 °C and allowed to warm to room temperature, λ_{max} (H_2O) 273 (ε 78,600); ν_{max} 3300, 1600, 1580 and 1250 cm^{-1}; δ_H (200 MHz) (D_2O) for the reduced form of dipicolinic acid, 1.50 - 2.00 (6H, m) and 3.40 (1H, m); δ_H (200 MHz) (D_2O) for dipicolinic acid, 7.79 (1H, s); δ_C (50 MHz) (D_2O), for the reduced form of dipicolinic acid, 18.79 (t), 22.22 (t), 25.91 (t), 41.77 (d), 45.32 (d), 171.65 (s), 171.65 (s) and 180.03 (s); δ_H (50 MHz) (D_2O) for dipicolinic acid, 126.18 (d), 139.64 (d), 153.44 (s) and 178.91 (s); *m/z* too involatile to measure.

cis-*2,6-Piperidinedicarboxylic Acid (**15**).* Dipicolinic acid (**14**) (1.50 g, 8.7 mmol) in glacial acetic acid (50 ml) was hydrogenated at atmospheric pressure and room temperature for 30 h using PtO_2 (0.15 g) as catalyst. The catalyst was removed by filtration through Celite, and the filtrate was acidified with conc. HCl to precipitate a white solid. Crystallisation from water gave *cis*-2,6-piperidinedicarboxylic acid (**15**) as a white powder (1.51 g, 96%), m.p. 290 - 295 °C, (lit.,[18] 290 - 295 °C); ν_{max} 3400, 2990 and 1750 cm^{-1}; δ_H (200 MHz) (D_2O) 1.80 - 1.85 (4H, m), 2.31 (2H, m) and 3.80 (2H, m); δ_C (50 MHz) (D_2O) 23.05 (t), 26.27 (t), 58.05 (d) and 172.36 (s); *m/z* 173 (M^+, 31%), 128 (26%) and 84 (100%) (Found: C, 39.98; H, 5.72; N, 6.65. $C_7H_{11}O_4N$ requires C, 40.10; H, 5.77; N, 6.68%).

*Attempted Synthesis of (±)-Tetrahydrodipicolinic Acid (**3**).* A mixture of *cis*-2,6-piperidinedicarboxylic acid (**15**) (0.60 g, 3.4 mmol) in 5% acetic acid (15 ml) with mercuric acetate (4.02 g, 12 mmol) was heated at reflux for 4 h. The mixture was cooled and the mercury salts were filtered off. The filtrate was saturated with H_2S gas and the black solid that formed was filtered off through Celite. This treatment with H_2S gas was repeated twice. The filtrate was acidified with conc. HCl and concentrated to afford a white solid (0.31 g, 53%). From ^1H and ^{13}C NMR spectroscopy this was starting material.

*Diethyl α,α′-Dibromopimelate (**18**).* Pimelic acid (5.40 g, 34 mmol) in thionyl chloride (6.09 ml) was heated at 40 °C for 18 h. Iodine (0.10 g) and bromine (4.02 ml, 75 mmol) were added and the mixture was heated at 80 °C for 6 h. After cooling the solution was added to ethanol (30 ml). Water (75 ml) was added and the aqueous solution was extracted with diethyl ether (3 x 25 ml). The combined ether extracts were washed with 10% sodium thiosulfate solution (5 x 20 ml). The ether layer was dried, filtered, and concentrated to an oil. Distillation gave diethyl α,α′-dibromopimelate (**18**) as a clear oil (60% yield), b.p. 165 - 170 °C (4 mmHg); R_F (hexane) 0.68; ν_{max} (liq. film) 2980, 1740, 1425, 1230 and 1100 cm^{-1}; δ_H (90 MHz) ($CDCl_3$) 1.30 (6H, t), 1.62 (2H, m), 2.10 (4H, m), 4.00 (2H, m), and 4.20 (4H, q); *m/z* 222 (62%); 166 (70%) and 73 (100%) (Found: C, 35.82; H, 5.07. $C_{11}H_{18}O_4Br_2$ requires C, 35.51; H, 5.05%).

cis- *and* trans-*2,6-Piperidinedicarboxamide (**19**).* Diethyl α,α′-dibromopimelate (**18**) (1.0 g, 2.9 mmol) in liquid ammonia in a sealed tube was left standing at room temperature for 3 d. The tube was opened at -78 °C and allowed to warm to room temperature. Ice water (5 ml) was added to the solid residue. The remaining solid was filtered off and dried to give *cis*-2,6-piperidinedicarboxamide (0.15 g, 30%), m.p. 226 - 228 °C (lit.,[18] m.p. 226 - 228 °C); ν_{max} 3300, 2980, 1680, 1630 and 1400 cm^{-1}; δ_H (200 MHz) (D_2O) 1.48 - 1.53 (4H, m), 1.81 (2H, m), and 4.14 (2H, m); δ_C (50 MHz) (D_2O) 17.96 (t), 25.64 (t), 54.35 (d), and 171.64 (s); *m/z* 171 (M^+, 20%), 128 (42%) and 84 (100%) (Found: M^+, 171.0769. $C_7H_{11}O_4N$ requires M^+, 171.1110). Concentration of the filtrate gave crystals of *(±)-trans*-2,6-piperidinedicarboxamide (**19**) (0.10 g, 20%), m.p. 264 - 268 °C (lit.,[18] 265 - 269 °C); ν_{max} 3300, 2980, 1680, 1630 and 1400 cm^{-1}; δ_H (D_2O) (200 MHz) 1.26 - 1.60 (4H, m), 1.85 (2H, m), and 3.72 (1H, m); δ_C (50 MHz) (D_2O) 21.85 (t), 25.93, (t), 57.26 (d), and 171.50 (s); *m/z* 171 (M^+, 26%), 128 (39%) and 84 (100%) (Found: M^+, 171.1009. $C_7H_{13}O_2N_3$ requires M^+, 171.1008).

cis-*2,6-Piperidinedicarboxylic Acid (**15**).* *cis*-2,6-Piperidinedicarboxamide (0.40 g, 2.3 mmol) in 10% barium hydroxide solution was heated at reflux for 2 h. Carbon dioxide was added to the solution and the solid barium carbonate was filtered off. The filtrate was acidified with conc. HCl and concentrated to leave *cis*-2,6-piperidinedicarboxylic acid (**15**) hydrochloride as a white solid (0.32 g, 79%), m.p. 292 - 295 °C (lit.,[21] 290 - 295 °C); ν_{max} 3300, 2990 and 1750 cm^{-1}; δ_H (200 MHz) (D_2O) 1.30 - 1.84 (4H, m), 2.31 (2H, m), and 3.80 (2H, m); δ_C (50 MHz) (D_2O) 23.09 (t), 26.24 (t), 58.10 (d) and 172.40 (s); *m/z* 173 (M^+, 24%), 128 (62%), and 84 (100%) (Found: M^+, 173.0686. $C_7H_{11}O_4N$ requires M^+ 173.0688).

(±)-trans-*2,6-Piperidinedicarboxylic Acid (**20**).* The above hydrolysis procedure was repeated using *(±)-trans*-2,6-piperidinedicarboxamide (**19**) in 10% barium hydroxide solution to give *(±)-trans*-2,6-piperidinedicarboxylic acid hydrochloride as a white powder (14% yield), m.p. 276 - 279 °C (lit.,[18] 275 - 278 °C); ν_{max} 3300, 2950, 1750 and 1150 cm^{-1}; δ_H (200 MHz) (D_2O) 1.29 - 1.83 (4H, m), 2.28 (2H, m), and 4.30 (1H, m); δ_C (50 MHz) (D_2O) 22.44 (t), 26.06 (t), 58.49 (d), and 172.48 (s); *m/z* 173 (M^+, 12%), 128 (81%), and 84 (100%) (Found: M^+, 173.0791. $C_7H_{11}O_4N$ requires M^+, 173.0688). *(±)-trans*-2,6-Piperidinedicarboxylate (**20**) was treated with mercuric acetate as in the above procedure and also gave starting material.

Dimethyl Dipicolinate (16). A solution of dipicolinic acid (**14**) (4.00 g, 24 mmol) in methanol (50 ml) and conc. sulfuric acid (10 ml) was heated for 18 h. Water (30 ml) was added and the aqueous solution was neutralised with sodium carbonate. The solution was acidified with conc. HCl and extracted with chloroform (4 x 25 ml). The combined extracts were dried, filtered and concentrated to leave a white solid. Crystallisation from chloroform gave dimethyl dipicolinate (**16**) as a white powder (2.87 g, 96%), m.p. 117 - 119 °C; R_F ($CHCl_3$ - conc. NH_3, 99:1) 0.78; ν_{max} 2990, 1740, 1590, 1250 and 1110 cm^{-1}; δ_H (270 MHz) ($CDCl_3$) 4.00 (6H, s), 8.05 (1H, t) and 8.34 (2H, d); *m/z* 195 (M^+, 24%), 87 (62%) and 79 (100%) (Found: C, 55.45; H, 4.60; N, 7.18. $C_9H_9O_4N$ requires C, 55.43; H, 4.65; N, 7.18%).

Dimethyl cis-*2,6-Piperidinedicarboxylate (17).* A solution of dimethyl dipicolinate (**16**) (1.0 g, 5.1 mmol) in chloroform (20 ml) was hydrogenated at atmospheric pressure and room temperature for 24 h with PtO_2 (0.10 g) as catalyst. The catalyst was removed by filtration through Celite. The filtrate was concentrated to afford a white solid. Crystallisation from methanol gave dimethyl *cis*-2,6-piperidinedicarboxylate (**17**) as a white powder (0.94 g, 94%), m.p. 210 - 212 °C; ν_{max} 2990, 1740, 1320 and 1150 cm^{-1}; δ_H (270 MHz) ($CDCl_3$) 1.38 - 1.83 (4H, m), 2.40 (2H, m) 3.80 (6H, s) and 4.20 (2H, m); *m/z* 201 (M^+, 28%), 147 (42%) and 84 (100%) (Found: C, 53.78; H, 7.53; N, 6.96. $C_9H_{15}O_4N$ requires C, 53.77; H, 7.52; N, 6.96%).

Dimethyl N-*Nitroso*-cis-*2,6-piperidinedicarboxylate (21).* A mixture of *cis*-2,6-piperidinedicarboxylic acid (**15**) (1.0 g, 5.8 mmol) and sodium nitrite (0.67 g, 10 mmol) in 1M HCl (15 ml) was stirred for 1 h at room temperature. The aqueous solution was extracted with dichloromethane (3 x 20ml). The combined extracts were dried, filtered and concentrated to give a yellow solid. Addition of methanol and concentration of the solution with heating gave dimethyl *N*-nitroso-*cis*-2,6-piperidinedicarboxylate (**21**) as a yellow oil (0.8 g, 63%); R_F ($CHCl_3$) 0.38; ν_{max} (liq. film) 2990, 1740, 1480 and 1160 cm^{-1}; δ_H (270 MHz) ($CDCl_3$) 1.10 (4H, m), 1.52 (2H, m) and 6.05 (2H, m); δ_C (50 MHz) ($CDCl_3$) 17.21 (t), 24.89 (t), 26.03 (t), 48.46 (d), 52.09 (q), 54.27 (d), 168.72 (s), 169.33 (s); *m/z* 230 (M^+, 21%,) 172 (14%) 114 (100%) and 84 (32%); (Found: M^+, 230.0980. $C_9H_{14}O_5N_2$ requires M^+, 230.0903).

Dimethyl N-Toluenesulfonyl-cis-*2,6-piperidinedicarboxylate (24).* Dimethyl *cis*-2,6-piperidinedicarboxylate (**17**) (2.50 g, 12.5 mmol) in pyridine (25 ml) with *p*-toluenesulfonyl chloride (4.80 g, 24 mmol) was stirred at room temperature for 24 h. The reaction mixture was poured into water (25 ml) and the aqueous solution was extracted with dichloromethane (3 x 30 ml). The combined extracts were dried, filtered and concentrated to an oil. Crystallisation from methanol and water gave dimethyl *N*-toluenesulfonyl-*cis*-2,6-piperidinedicarboxylate (**24**) (2.90 g, 65%), m.p. 58 - 61 °C; R_F ($CHCl_3$) 0.61; ν_{max} 2930, 1730, 1360 and 1160 cm^{-1}; δ_H (270 MHz) ($CDCl_3$) 1.52 (3H, m), 1.80 (3H, m), 2.30 (3H, s), 3.46 (6H, s), 4.60 (2H, m), 7.30 and 7.70 (4H, AA'XX' system, *J* 8Hz); δ_C (50 MHz) ($CDCl_3$) 15.94 (t), 21.39 (t), 21.55 (t), 51.85 (q), 52.89 (d), 124.87 (d), 126.67 (d), 138.67 (s), 142.54 (s) and 170.73 (s); *m/z* 296 (M^+,21%), 236 (18%) and 84 (100%) (Found: C, 53.99; H, 5.96; N, 3.94. $C_{16}H_{19}O_6SN$ requires C, 54.00; H, 5.96; N, 3.94%).

Dimethyl N-*Methanesulfonyl*-cis-*2,6-piperidinedicarboxylate (22).* A mixture of dimethyl *cis*-2,6-piperidinedicarboxylate (**17**) (0.75 g, 3.75 mmol), methanesulfonyl chloride (0.38 ml, 5.6 mmol) and triethylamine (0.50 ml, 3.75 mmol) was stirred for 18 h at room temperature under nitrogen. Water (20 ml)

was added and the aqueous solution was extracted with dichloromethane (3 x 25 ml). The combined extracts were dried, filtered, and concentrated to an oil. Crystallisation from diethyl ether gave dimethyl *N*-methanesulfonyl*cis*-2,6-piperidinedicarboxylate (**17**) as white needles (0.54 g, 43%), m.p. 102 - 105 °C; ν_{max} 2970, 1730, 1310, and 1245 cm^{-1}; δ_H (270 MHz) ($CDCl_3$) 1.60 (4H, m), 2.05 (2H, m), 3.08 (3H, s), 3.62 (6H, s) and 4.75 (2H, m); *m/z* 279 (M^+, 6%), 221 (20%) 142 (60%) and 84 (100%) (Found: C, 43.07; H, 6.15; N, 5.02. $C_{10}H_{17}O_6SN$ requires C, 43.05; H, 6.14; N, 5.02%).

Dimethyl N-*Trifluoromethanenesulfonyl*-cis-*2,6-piperidinedicarboxylate (**23**).* A mixture of dimethyl *cis*-2,6-piperidinedicarboxylate (**17**) (1.00 g, 5 mmol) and trifluoromethanesulfonic anhydride (1.41 g, 5 mmol) in pyridine (30 ml) was stirred at 0 °C for 4 h. The reaction mixture was poured into ice water (30 ml) and the aqueous solution was extracted with dichloromethane (3 x 20 ml). The combined extracts were dried, filtered and concentrated to an oil. Purification was achieved by a silica column, eluting with hexane and adding increasing proportions of dichloromethane to give dimethyl *N*-trifluoromethanenesulfonyl-*cis*-2,6-piperidinedicarboxylate (**23**) as a yellow oil (1.20 g, 72%); R_F ($CHCl_3$) 0.48; ν_{max} (liq film) 2980, 1740, 1600, 1360, 1250 and 1100 cm^{-1}; δ_H (270 MHz) ($CDCl_3$) 1.24 (2H, m), 1.50 (2H, m), 1.83 (2H, m), 3.40 (6H, s) and 4.00 (2H, m); *m/z* 201 (6%), 142 (84%) and 84 (100%).

General Procedure for Attempted Imine Formation using KH. To the *N*-substituted diesters (**21**)-(**24**) (2 mmol) in dry THF (25 ml) was added KH (0.30 g). The suspension was heated at reflux for 4 h, then cooled and poured into isopropanol (30 ml). Water (20 ml) was added and the solution was extracted with dichloromethane (3 x 15 ml). The combined extracts were dried, filtered, and concentrated. The residue was studied by ^1H NMR spectroscopy. The aqueous layer was freeze dried and the residue was examined by ^1H and ^{13}C NMR spectroscopy. A mixture of products was obtained.

General Procedure for Attempted Imine Formation using DBU or DBN. A solution of DBU or DBN (4 mmol) in toluene (2 ml) was added to a solution of the *N*-substituted diesters (**21**)-(**24**) (3 mmol) in toluene (8 ml). The mixture was heated at reflux for 72 h. The solution was cooled to room temperature and poured into ice water (15 ml) and the aqueous solution was extracted with dichloromethane (3 x 10ml). The combined extracts were dried, filtered and concentrated. The DBU or DBN was removed from the residue by flash chromatography on silica, eluting with dichloromethane. Elution with methanol gave starting material in all four cases.

General Procedure for Attempted Imine Formation using NaH. A mixture of *N*-substituted diesters (**21**)-(**24**) (2 mmol) and NaH (3 mmol) in DMSO (10 ml) was heated at reflux for 72 h. The reaction mixture was cooled and poured into isopropanol (30 ml). Water (20 ml) was added and the aqueous solution was acidified with 1M HCl and extracted with dichloromethane (3 x 15ml). The combined extracts were dried, filtered, and concentrated to give starting material in each case.

Attempted Imine Formation using Potassium Bis(trimethylsilyl)amide. A mixture of the *N*-substituted diesters (**21**)-(**24**) and potassium bis(trimethylsilyl)amide (0.5M solution in toluene, 6.5 ml) in DMSO (20 ml) was heated at 60 °C for 4 h under nitrogen. The solution was poured into water (15 ml) and acidified with 1M HCl.

The aqueous solution was extracted with dichloromethane (3 x 15 ml). The combined extracts were dried, filtered, and concentrated. The residue was examined by ^1H and ^{13}C NMR spectroscopy. The aqueous layer was freeze dried and the residue was examined by ^1H and ^{13}C NMR spectroscopy. Starting material was recovered in all four cases.

*Attempted Imine Formation on (**16**) using Potassium t-Butoxide.* The *N*-substituted diester (**21**) (4 mmol) in dry dichloromethane (20 ml) under nitrogen was treated with potassium t-butoxide (12 mmol). The solution was stirred for 24 h at room temperature, then the solution was washed with 1M HCl (2 x 15 ml). The organic layer was dried, filtered, and concentrated and the residue was shown by ^1H and ^{13}C NMR spectroscopy to be starting material.

*Potassium Salt of (±)-2,3,4,5-Tetrahydrodipicolinate (**3**).* A mixture of *N*-toluenesulfonyl-*cis*-2,6-piperidinedicarboxylate (**24**) (4.0 g, 13 mmol) and potassium *t*-butoxide (4.50 g, 40 mmol) in dry dichloromethane (25 ml) under nitrogen was stirred for 4 h. The solid was filtered off, dissolved in water (25 ml) and stirred with Amberlite 1R-45 anion exchange resin (HO^- form) (30 g) for 24 h. The resin was filtered off and the filtrate was concentrated to a solid. Crystallisation from methanol and ether afforded a yellow hygroscopic solid of the potassium salt of (±)-2,3,4,5-tetrahydrodipicolinate (**3**).(1.30 g, 41%). m.p. >300 °C; ν_{max} 3300, 1600 and 1400 cm^{-1}; δ_H (200 MHz) (D_20) 1.55 - 1.72 (8H, m), 2.25 (4H, m) and 4.30 (2H, m); δ_C (50 MHz) (D_20) 18.60 (t), 22,49 (t), 25.99 (t), 26.38 (t), 57.26 (d), 63.79(d), 171.86 (s), 175.42 (s), 182.74 (s) and 216.72 (s); *m/z* (FAB) 171 (M^+ $C_7H_9NO_4$).

cis-*2,6-Piperidinedicarboxylic Acid (**15**) from (±)-2,3,4,5-Tetrahydrodipicolinate (**3**).* The potassium salt of (±)-2,3,4,5-tetrahydrodipicolinic acid (**3**) (0.50 g, mmol) in water (10 ml) was hydrogenated at atmospheric pressure and room temperature for 12 h using PtO_2 (0.005 g) as catalyst. The catalyst was removed by filtration through Celite, and the filtrate was acidified with conc. HCl to precipitate a white solid. The solid was filtered off and dried over P_2O_5 to give *cis*-2,6-piperidinedicarboxylic acid (**15**) (0.49 g, 97%), m.p. and mixed m.p. 290 - 295 °C (lit.,[21] 290 - 295 °C); ^1H and ^{13}C NMR spectra were the same as an authentic sample of *cis*-2,6-piperidinedicarboxylic acid (**15**).

Enzymic Experiment with meso-*DAP Dehydrogenase.*[22] The enzyme was isolated from *Bacillus sphaericus* and partially purified.[11] The rate of decrease in absorption at 340 nm was measured as 2mM NADPH was consumed in 100 mM Tris buffer with 200 mM NH_4Cl containing 28.6 units of meso-DAP Dehydrogenase and 10 mM substrate (±)-(**3**).

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