Two complete syntheses of *(S)*-aspartate semi-aldehyde and demonstration that Δ^2-tetrahydroisophthalic acid is a non-competitive inhibitor of dihydrodipicolinate synthase

Sarah J. Roberts,[a] Jonathan C. Morris,*[b,c] Renwick C.J. Dobson,[a] Chris L. Baxter,[a] and Juliet A. Gerrard *[a]

[a] *School of Biological Sciences, University of Canterbury, Christchurch, NZ*
[b] *Department of Chemistry, University of Canterbury, Christchurch, NZ*
[c] *University of Adelaide, Adelaide, SA, Australia*
E-mail: jonathan.morris@adelaide.edu.au; juliet.gerrard@canterbury.ac.nz

Dedicated to Rod Rickards, with fond memories of mysterious faxes with lashings of sake, and Vauxhalls

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Abstract

We report, in full, two 3-step syntheses of *(S)*-aspartate semi-aldehyde, an important synthetic and biosynthetic precursor, from diprotected aspartic acid. The first synthesis proceeds via a thioester, the second via a Weinreb amide. Each route yields pure *(S)*-aspartate semi-aldehyde in excellent yield. The utility of *(S)*-aspartate semi-aldehyde prepared in this manner was demonstrated with an inhibition study of dihydrodipicolinate synthase, wherein Δ^2-tetrahydroisophthalic acid is shown to be a non-competitive inhibitor with respect to both substrates.

Keywords: Aspartate semi-aldehyde, lysine biosynthesis, DHDPS, inhibition kinetics

Introduction

The lysine biosynthesis pathway has remained a target for therapeutic agents for many years, although no commercial product has yet been shown to inhibit this pathway.[1,2] The enzyme that catalyses the branchpoint of the diaminopimelate pathway to lysine is dihydrodipicolinate synthase (DHDPS). Despite intense scrutiny over many years, no potent inhibitor of this pathway has yet been found. DHDPS catalyses the condensation of *(S)*-aspartate semi-aldehyde (ASA, **1**) and pyruvate (**2**) to form an unstable heterocycle, formally thought to be dihydrodipicolinate, but now believed to be 4-hydroxytetrahydrodipicolinate (**3**) (Scheme 1).[2] (*S*)-ASA is drawn here as the aldehyde, but is actually thought to exist predominantly in the hydrated form.[3,4]

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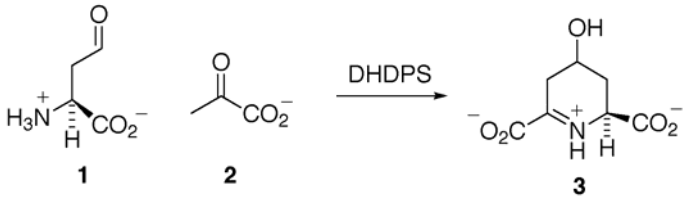


Scheme 1. The condensation of pyruvate and *(S)*-ASA to form 4-hydroxytetrahydrodipicolinate, catalyzed by DHDPS.

For detailed biochemical analysis of this reaction, a convenient source of pure *(S)*-ASA is required.[1] In particular, for screening of inhibitors of potential therapeutic use, the ASA employed must be free of impurities that may inhibit the enzyme and confuse the results. Enantiomerically pure ASA derivatives are also increasingly important synthetic intermediates, as the aldehyde moiety can be functionalized to yield more complex structures. The potential access to a variety of polyfunctional non-proteinogenic and unnatural amino acids using ASA and its derivatives has already been demonstrated.[2] ASA manipulation has also proved to be important in the synthesis of pharmaceuticals, aroma and flavour chemicals, pesticides and herbicides, dyes and pigments.[5]

ASA is difficult to synthesize and characterize since, like many amino aldehydes, it has a marked tendency to polymerize and is only stable in aqueous strong acid.[3-5] There are three methods for synthesizing *(S)*-ASA that are commonly used for biochemical studies.[3,6,7] The later methods[3,7]are derived from the original method of Black and Wright[6] which requires the ozonolysis of *(S)*-allylglycine. Other methods have also been reported in the literature, which yield either free ASA or diprotected ASA, but they are often complex multi-step procedures and/or are extremely low yielding procedures and have not been widely adopted.[4,8,9]

In the original synthesis of *(S)*-ASA, the aldehyde moiety was obtained by oxidative cleavage of the double bond of *(S)*-allylglycine by ozonolysis.[6] The reaction is carried out in a 1 M aqueous hydrochloric acid solution at 0°C, with ozone bubbled through the solution. The reported yield of the desired product is 90-100%, as determined by an enzymatic assay following the conversion of ASA into homoserine by homoserine dehydrogenase.[6] However, *(S)*-ASA produced by this method has been found to have variable purity and no chemical characterization of the product has ever been reported.[3,4] This method is still commonly used today, presumably due to the one step procedure from allylglycine.[10-12] However, studies in our laboratory using the DHDPS/DHDPR coupled enzymatic assay[7] have suggested that the ozonolysis product is contaminated by material that has an inhibitory effect on the DHDPS enzyme.[13]

To address the deficiencies of the Black and Wright method, Tudor and coworkers have investigated the ozonolysis of *(S)*-N-*tert*-butoxycarbonyl allyglycine *p*-methoxybenzyl ester.[3] Removal of the protecting groups with TFA affords the hydrate of ASA as a trifluoroacetate salt **4**, which can be stored for many months at 0 °C as a stable solid. This method has since been modified so that a Lemieux-Johnson oxidation is used in place of the ozonolysis.[7] In our hands,

the overall yield of the latter method for the preparation of *(S)*-ASA was 59%, an increase on previously reported values of 42% and 43%.[3] However, the route is multi-step, involves several purifications, and is particularly tedious for those focusing on biochemical studies of DHDPS.

We report herein, in full detail, two convenient syntheses of *(S)*-ASA from commercially available α-*tert*-butyl *(S)*-N-*tert*-butoxycarbonyl aspartate (**5**).[14] Literature procedures were employed to activate the aspartic acid side chain, firstly, to a thio-ester[15] and secondly, to a Weinreb amide.[16] We also demonstrate the utility of the synthesis in a kinetic analysis of inhibition by Δ^2-tetrahydroisophthalic acid.

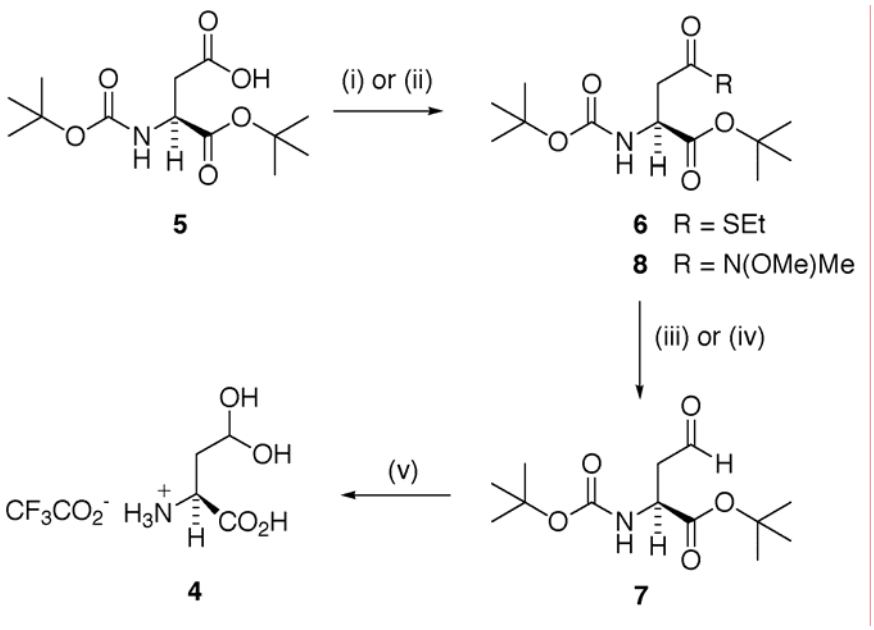
Results and Discussion

Synthesis of (S)-ASA

In the first synthesis, α-*tert*-butyl *(S)*-N-*tert*-butoxycarbonyl aspartate (**5**) in dichloromethane was reacted with DCC, ethanethiol and DMAP at room temperature. Purification was achieved by flash chromatography yielding the thioester **6** as a clear oil in 94% yield. Reduction of the thioester to the aldehyde **7** was achieved in 84% yield using triethylsilane/10% Pd/C. The method of Tudor and coworkers[3] was used to deprotect the aldehyde **7** by stirring in trifluoroacetic acid in dry dichloromethane, with *(S)*-ASA being isolated as the hydrated trifluoroacetate salt **4**, as a pale yellow solid, in 96% yield.

This procedure involves only three steps and was relatively simple, cutting out the need to use osmium tetroxide (not previously mentioned). The overall reaction yield was 75% and the purity of the ASA produced was also of a very high standard, as determined by the DHDPS/DHDPR coupled enzymatic assay (99%). The only drawback of this method is that there are two purification steps required.

In a bid to cut down the number of purification steps required, the procedure of Wernic and coworkers was investigated as an alternative to obtaining *(S)*-ASA (Scheme 2).[16] α-*tert*-Butyl *(S)*-N-*tert*-butoxycarbonyl aspartate (**5**) was converted to the corresponding Weinreb amide **8** in excellent yield (86%), using N,O-dimethylhydroxylamine hydrochloride, (benzotriazol-1-yloxy)tris(dimethylamino) phosphonium hexafluorophosphate (BOP.PF_6), and triethylamine. The resulting product **8** was reduced with diisobutyl aluminium hydride at –78 °C to give aldehyde **7** in 95% yield, which required no further purification. The method of Tudor[3] was again used to deprotect the aldehyde **7** to afford *(S)*-ASA as the hydrated trifluoroacetate salt **4** in 96% yield. This procedure was a much faster and easier method for obtaining *(S)*-ASA than the oxidation of diprotected *(R,S)*-allyglycine by osmium tetroxide/periodate or by ozonolysis.[6]

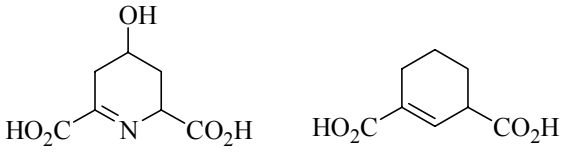


Scheme 2. Three step syntheses of (S)-ASA via either the thioester or Weinreb amide. (i) DCC, EtSH, DMAP, CH_2Cl_2 (94%); (ii) BOP.PF_6, Et_3N, CH_3ONHCH_3.HCl, CH_2Cl_2 (86%); (iii) Et_3SiH/10% Pd/C, CH_2Cl_2 (84%); (iv) DIBAL/THF –78°C (95%); (v) CF_3CO_2H (96%).

The overall yield of the reaction from the diprotected aldehyde **5** was also greatly increased (82%) when compared to other literature procedures.[3] The sequence has routinely been carried out on a 2 millimole scale. The above procedure is only three steps to the pure aldehyde and only one purification step is required. The purity of the ASA generated was also of a very high standard (99%) as checked by the coupled assay of DHDPS and DHDPR. No evidence of contaminating inhibitory compounds was found.

Inhibition of DHDPS by Δ^2-tetrahydroisophthalic acid

Δ^2-Tetrahydroisophthalic acid is a product mimic of the DHDPS reaction product (Scheme 3). In the literature, Δ^2-tetrahydroisophthalic acid has been reported to be a weak inhibitor of DHDPS.[7] To date, no detailed kinetic studies have been done to determine the type of inhibition.



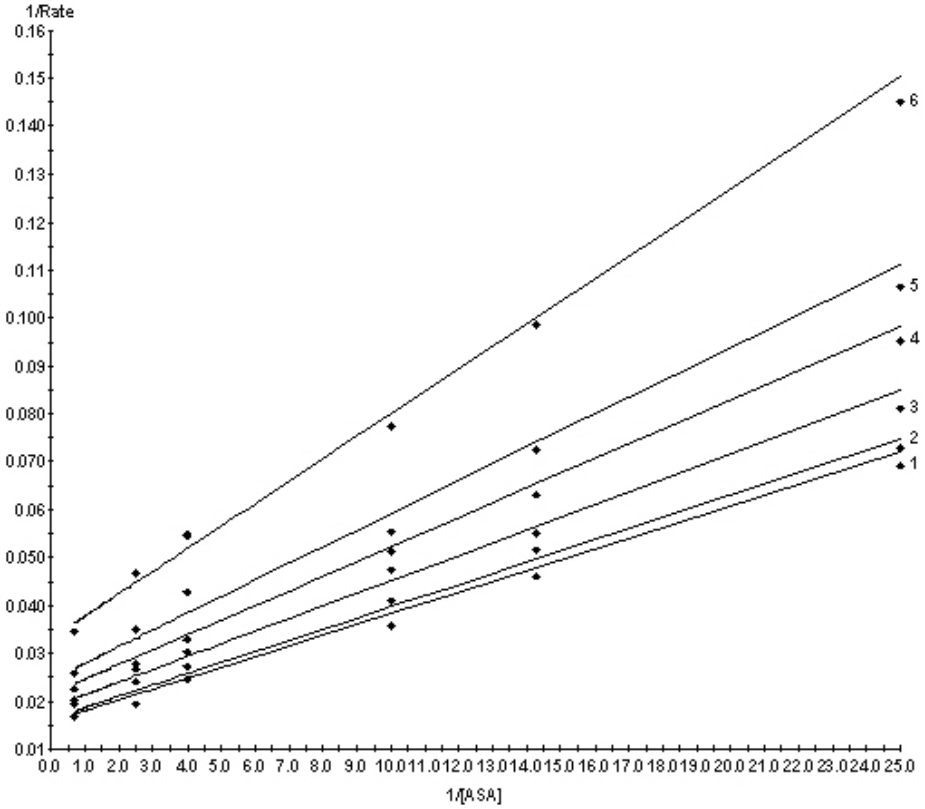
4-Hydroxy-2,3,4,5-tetrahydrodipicolinate

Δ^2-Tetrahydroisophthalic acid

Scheme 3. Structure of Δ^2-tetrahydroisophthalic acid compared to 4-hydroxy-2,3,4,5-tetrahydrodipicolinate.

Inhibition kinetics of Δ^2-tetrahydroisophthalic acid with respect to ASA and pyruvate were carried out according to previously described methods,[13] with Δ^2-tetrahydroisophthalic concentrations being varied between 1 and 30 mM. The kinetic data were fitted to mathematical models using the Enzfitter computer program that simulated competitive, noncompetitive, uncompetitive and mixed inhibition patterns to determine the best fit and inhibition constant K_i. Lineweaver-Burk and Eadie-Hofstee plots were used to show the quality of the generated data.

The inhibition was found to be noncompetitive with respect to both substrates, ASA and pyruvate. The K_i for ASA was found to be in the range of (24 – 31) mM, while the K_i of pyruvate was found to be in the range of (22 – 28) mM (Figures 4-7). These data suggest that Δ^2-tetrahydroisophthalic acid does not bind at the active site, which explains the weak inhibition observed. The high quality of the data generated in all cases confirms the utility of *(S)*-ASA generated by these methods for kinetic study.



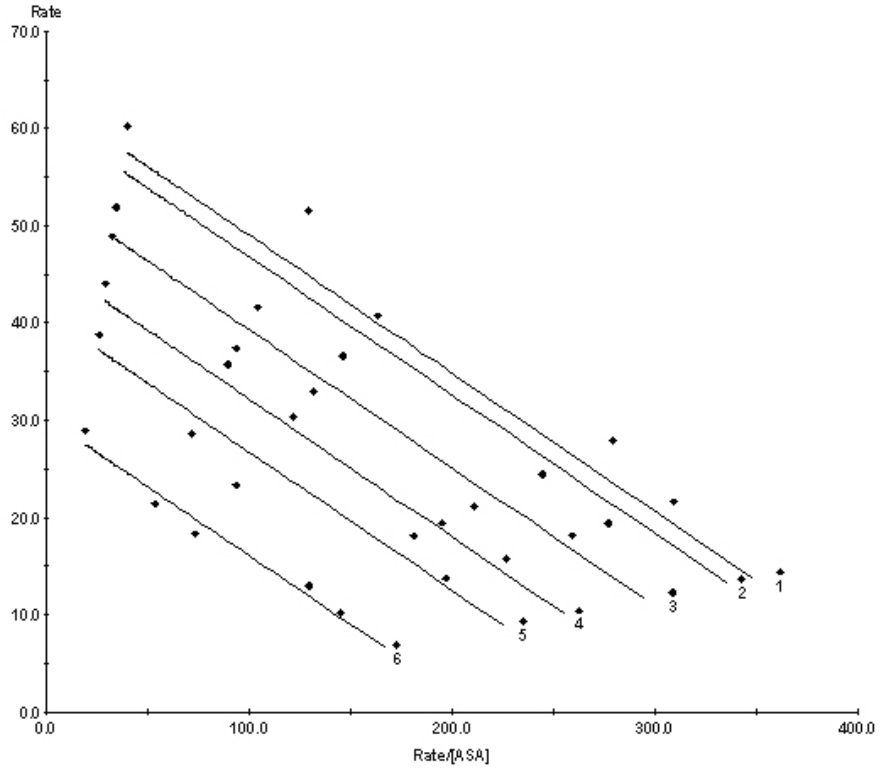
Legend

1. [Delta 2- tetrahydroisophthalic acid] = 0.0 mM **2. [Delta 2- tetrahydroisophthalic acid] = 1.0 mM**

3. [Delta 2- tetrahydroisophthalic acid] = 5.0 mM **4. [Delta 2- tetrahydroisophthalic acid] = 10 mM**

5. [Delta 2- tetrahydroisophthalic acid] = 15 mM **6. [Delta 2- tetrahydroisophthalic acid] = 30 mM**

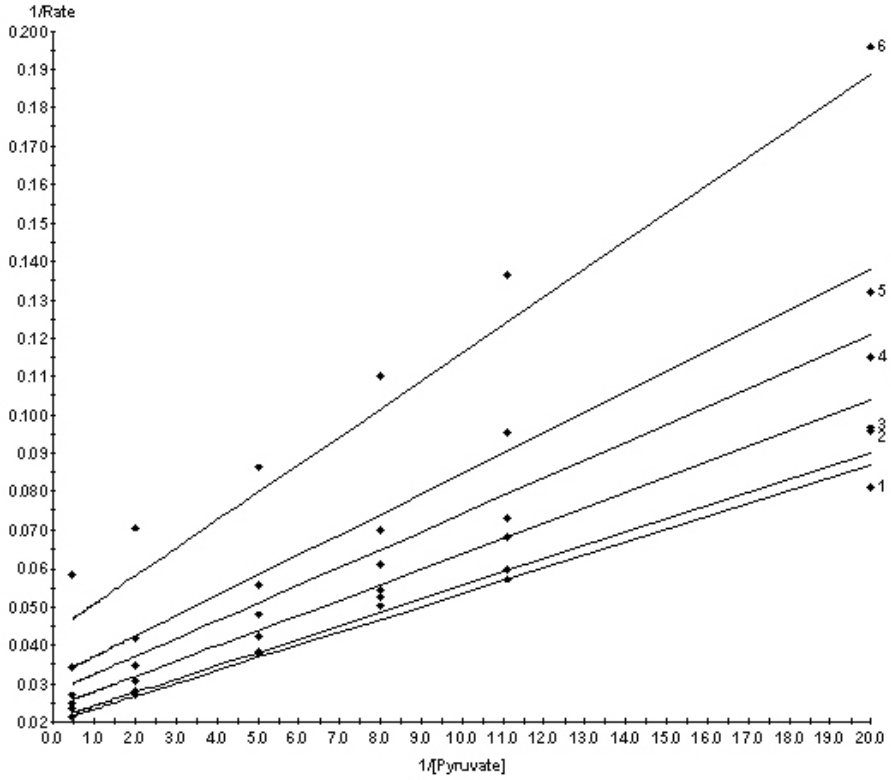
Figure 4. Lineweaver-Burk plot of DHDPS with respect to ASA at different Δ^2-tetrahydroisophthalic acid concentrations. ($R^2 = 0.985$, $P < 0.05$).



Legend

1. **[Delta 2- tetrahydroisophthalic acid] = 0.0 mM**
2. **[Delta 2- tetrahydroisophthalic acid] = 1.0 mM**
3. **[Delta 2- tetrahydroisophthalic acid] = 5.0 mM**
4. **[Delta 2- tetrahydroisophthalic acid] = 10 mM**
5. **[Delta 2- tetrahydroisophthalic acid] = 15 mM**
6. **[Delta 2- tetrahydroisophthalic acid] = 30 mM**

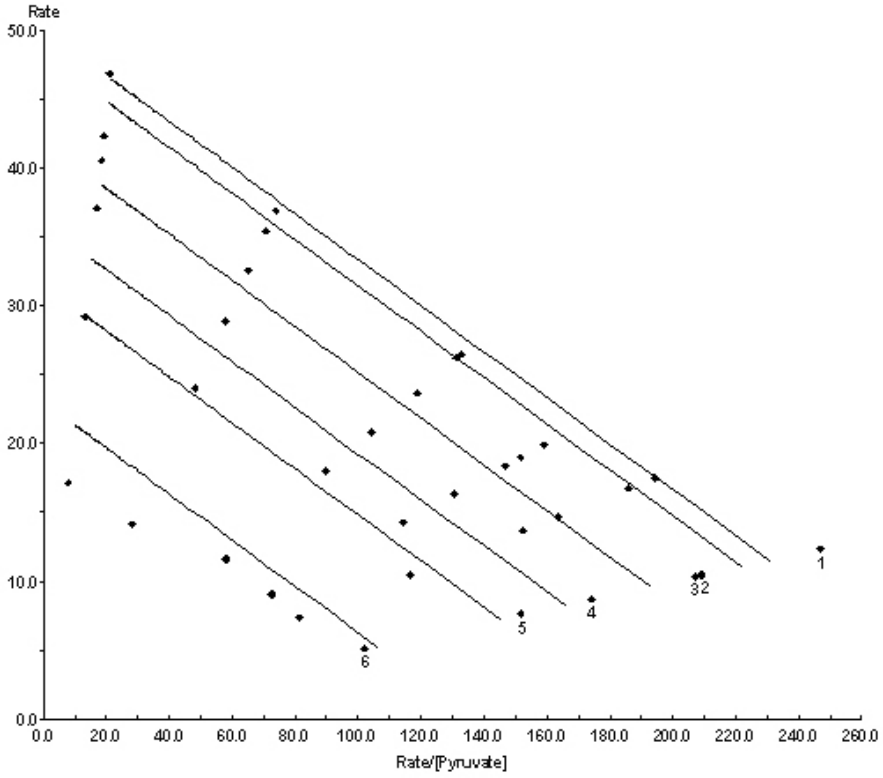
Figure 5. Eadie-Hofstee plot of DHDPS with respect to ASA at different Δ^2-tetrahydroisophthalic acid concentrations. ($R^2 = 0.985$, $P < 0.05$).



Legend

1. **[Delta 2- tetrahydroisophthalic acid] = 0.0 mM**
2. **[Delta 2- tetrahydroisophthalic acid] = 1.0 mM**
3. **[Delta 2- tetrahydroisophthalic acid] = 5.0 mM**
4. **[Delta 2- tetrahydroisophthalic acid] = 10 mM**
5. **[Delta 2- tetrahydroisophthalic acid] = 15 mM**
6. **[Delta 2- tetrahydroisophthalic acid] = 30 mM**

Figure 6. Lineweaver-Burk plot of DHDPS with respect to pyruvate at different Δ^2-tetrahydroisophthalic acid concentrations. ($R^2 = 0.983$, $P < 0.05$).



Legend

1. **[Delta 2- tetrahydroisophthalic acid] = 0.0 mM**
2. **[Delta 2- tetrahydroisophthalic acid] = 1.0 mM**
3. **[Delta 2- tetrahydroisophthalic acid] = 5.0 mM**
4. **[Delta 2- tetrahydroisophthalic acid] = 10 mM**
5. **[Delta 2- tetrahydroisophthalic acid] = 15 mM**
6. **[Delta 2- tetrahydroisophthalic acid] = 30 mM**

Figure 7. Eadie-Hofstee plot of DHDPS with respect to pyruvate at different Δ^2-tetrahydroisophthalic acid concentrations. ($R^2 = 0.983$, $P < 0.05$).

In conclusion, *(S)*-ASA has been synthesized from commercially available α-*tert*-butyl *(S)*-N-*tert*-butoxycarbonyl aspartate (**5**) in three steps via side-chain activation with an overall yield of 82%. This represents a significant advance over previously published routes to *(S)*-ASA and is the preferred synthetic route to *(S)*-ASA for future biochemical investigations.[1,13,20] The utility of (S)-aspartate semi-aldehyde prepared in this manner was demonstrated with an inhibition study of dihydrodipicolinate synthase, wherein Δ^2-tetrahydroisophthalic acid is shown to be a non-competitive inhibitor with respect to both substrates.

Experimental Section

General Procedures. Starting materials were obtained from Aldrich Chemicals, (Sigma Chemical) Company Ltd (Castle Hill, Australia.) Unless otherwise stated, all synthetic reactions were performed in dry glassware under an atmosphere of oxygen-free nitrogen or argon. All organic extracts were washed with brine and dried over anhydrous magnesium sulfate. After filtration of solutions to remove drying agents, the solvents were removed under reduced pressure.

^{1}H NMR spectra were recorded on either a Varian Unity 300 or Varian Inova 500 spectrometer, operating at 300 and 500 MHz respectively. ^{13}C NMR spectra were obtained on either a Varian Unity 300 or Varian Inova 500 spectrometer, operating at 75 and 126 MHz respectively. For ^{1}H NMR, all chemical shifts are reported relative to tetramethylsilane (TMS), if run in deuterated chloroform ($CDCl_3$). For samples run in deuterium oxide (D_2O) the spectra were referenced to the residual protonated solvent at 4.70 ppm, and for samples run in deuterated methanol (CD_3OD) the spectra were referenced to the residual protonated solvent peak at 3.30 ppm. For ^{13}C spectra run in deuterated chloroform ($CDCl_3$) the spectra were referenced to the residual protonated solvent at 77.0 ppm, and for samples run in deuterated methanol (CD_3OD) the spectra was reference to the residual protonated solvent at 49.3 ppm. Melting points were determined using an electrothermal melting point apparatus and are uncorrected.

***α-tert*-Butyl β-*S*-ethyl *(S)*-*N*-(*tert*-butoxycarbonyl) thioaspartate (6).**[15] To a solution of α-*tert*-butyl β-(S)-ethyl *(S)*-N-(*tert*-butoxycarbonyl)aspartate (**5**) (135 mg, 0.467 mmol) in dichloromethane (1.4 mL) was added DCC (116 mg, 0.564 mmol), ethanethiol (108 µl, mmol) and DMAP (5.4 mg, 0.046 mmol). The reaction mixture was stirred at RT for 3 h, then filtered to remove the precipitate. The filtrate was evaporated and the residue was purified by flash chromatography on silica, using 10% ethyl acetate/petroleum ether as the eluant, to give the title compound (**6**) as a clear oil (165 mg, 94%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.24 (3H, t, J = 7.2 Hz, EtS), 1.44 (9H, s, $C(CH_3)_3$), 1.45 (9H, s, $C(CH_3)_3$), 2.95-2.8 (2H, m, EtS), 3.02 (1H, dd, J = 5, 16 Hz, CHH'), 3.18 (1H, dd, J = 4.8, 16.5 Hz, CHH'), 4.41 (1H, m, CH), 5.39 (1H, m, NH).

***N*-tert-BOC-*(S)*-Aspartic acid 1-(*tert*-butyl ester) *N*-methoxy-*N*-methylamide (8).**[16] (Benzotriazol-1-yloxy)tris(dimethylamino) phosphonium hexafluorophosphate (BOP.PF_6) (163 mg, 0.380 mmol) was added to a stirred solution of N-*tert*-BOC-*(S)*-aspartic acid 1-(*tert*-butyl ester) (**5**) (100 mg, 0.346 mmol) and triethylamine (54 µL, 0.385 mmol) in dichloromethane (3.5 mL) at room temperature. After 5 minutes of stirring, O,N-dimethylhydroxyamine hydrochloride (40 mg, 0.397 mmol) was added, followed by triethylamine (54 µL, 0.385 mmol). All solid material dissolved within 10 minutes and the mixture was stirred for 2 h at RT. The reaction mixture was washed with 1 M aqueous HCl solution (3 x 1 mL), H_2O (1 x 1 mL), 1 M aqueous $NaHCO_3$ solution (2 x 1 mL) and H_2O (2 x 1 mL). The solvent was removed *in vacuo* to give a clear yellow oily product, which was purified by flash chromatography on silica, using 30% ethyl acetate/petroleum ether as the eluant, to give the title compound (**8**)[16] as a colourless oil

(100 mg, 86%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.44 (9H, s, -C(CH_3)$_3$), 1.46 (9H, s, -C(CH_3)$_3$), 2.89 (2H, m, -CH_2-), 3.17 (3H, s, -CH_3), 3.69 (3H, s, -OCH_3), 4.45 (1H, m, -CHNH-), 5.66 (1H, m, -NH-). ^{13}C NMR (75 MHz, $CDCl_3$) δ 27.88, 28.33, 29.64, 31.91, 50.35, 61.19, 79.49, 83.39, 155.74, 169.78, 192.13.

***tert*-Butyl *(S)*-2-[*N*-(*tert*-butoxycarbonyl)amino]-4-oxobutanoate (7).**[15] *Method A:* Triethylsilane (238 μL, 1.485 mmol) was quickly added to a solution of α-*tert*-butyl β-S-ethyl *(S)*-N-(*tert*-butoxycarbonyl) thioaspartate (6) (165 mg, 0.495 mmol) and 10% Pd/C (10.6 mg, 0.0099 mmol) in dichloromethane (2 mL) cooled in an ice bath to maintain the internal temperature between 15-20 °C. The reaction was monitored for 20 minutes, after which the temperature no longer increased. The reaction mixture was then filtered through celite and concentrated *in vacuo*. Flash chromatography on silica, using 15% ethyl acetate/petroleum ether as the eluant, gave *tert*-butyl *(S)*-2-[N-(tert-butoxycarbonyl)amino]-4-oxobutanoate (7)[15] as a clear oil (114 mg, 84%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.44 (9H, s, -C(CH_3)$_3$), 1.47 (9H, s, -C(CH_3)$_3$), 2.96 (2H, m, -CH_2-), 4.47 (1H, m, -CHNH-), 5.38 (1H, m, -NH-), 9.74 (1H, s, -COH). ^{13}C NMR (75 MHz, $CDCl_3$) δ 27.79, 28.23, 46.31, 49.23, 80.02, 82.67, 155.33, 169.88, 199.40.

Method B: A solution of diisobutylaluminum hydride (DIBAL) in hexane (1 M, 0.55 mL, 0.55 mmol) was added dropwise over 20 minutes to a stirred solution of N-t-BOC-*(S)*-aspartic acid 1-(*tert*-butyl ester) N-methoxy-N-methylamide (**8**) (110 mg, 0.36 mmol) in anhydrous THF (1.8 mL) at –78 °C. The mixture was allowed to stir at –78 °C for 2 h. The reaction mixture was partitioned between 0.35 M aqueous $NaHSO_4$ solution (3.6 mL) and diethyl ether (5.5 mL). After separation, the aqueous layer was extracted with diethyl ether (3 x 2 mL). The combined ethereal layers were washed with 1 M HCl solution (3 x 1mL), 1 M aqueous $NaHCO_3$ solution (3 x 1 mL), and brine (3 x 1 mL). Concentration *in vacuo* gave 1-*tert*-butyl *(S)*-2-[N-(*tert*-butoxycarbonyl)amino]-4-oxobutanoate as a colourless oil (92 mg, 95%), which solidified on standing at room temperature.

***(S)* Aspartic acid β-semi-aldehyde hydrate trifluoroacetate. (4).**[7] *tert*-Butyl *(S)*-2-[N-(*tert*-butoxycarbonyl)amino]-4-oxobutanoate (681 mg, 2.5 mmol) was dissolved in dry dichloromethane (15 mL). The reaction was stirred under argon and trifluoroacetic acid (15 mL) was added via a syringe. The reaction was stirred for 2 h at room temperature and developed a deep red colour. The solvent was then removed *in vacuo* to give a brown oily residue, which was partitioned between water (50 mL) and ethyl acetate (50 mL). Upon separation, the aqueous layer was washed with ethyl acetate (2 x 50 mL). The aqueous layer was then concentrated (using a freeze-drier) to yield *(S)*-aspartate-β-semi-aldehyde hydrate trifluoroacetate (**4**) as a pale yellow solid (598 mg, 96%). Purity was determined to be 99% using the coupled assay of DHDPS and DHDPR.[7] ^{1}H NMR (500 MHz, D_2O) δ 1.99 (2H, m, -C**H**$_2$CHNH$_2$), 3.98 (1H, m, -C**H**NH$_2$), 5.21 (1H, m, -C**H**(OH)$_2$). ^{13}C NMR (75 MHz, D_2O) δ 36.52, 50.33, 88.04, 108.59, 172.05.

Methyl Δ^2-tetrahydroisophthalate.[17,18] Thionyl chloride (32.5 mL, 0.44 mol) was added dropwise to a stirred solution of dimethyl cyclohexanol 2,6–dicarboxylate[17,19] (1 g, 4.63 mmol) in pyridine (17 mL, 0.203 mol) at 0 °C. The reaction was stirred for 30 minutes while slowly

warming to room temperature. The reaction was then stirred for 1 h, before being re-cooled to 0 °C. The reaction was quenched by the slow addition of water (45 mL). The reaction mixture was extracted with diethyl ether (3 x 70 mL). The combined ethereal layers were dried over $MgSO_4$ and the solvent removed *in vacuo* to yield a colorless oil. Purification by column chromatography on silica, using 10% ethyl acetate/petroleum ether as the eluant gave methyl Δ^2-tetrahydroisophthalate as a colourless oil (495 mg, 54%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.56 – 1.63 (2H, m, -$CH_2CH_2CH_2$-), 1.78 – 1.80 (2H, m, -CH_2CHCH=), 2.24 – 2.3 (2H, m, -CH_2C=), 3.2 – 3.26 (1H, m, -CHCH=), 3.75 – 3.76 (6H, m, -OCH_3), 7.02 (1H, bs, =CH-).

Δ^2-Tetrahydroisophthalic acid.[17,18] Methyl Δ^2-tetrahydroisophthalate (200 mg, 1.01 mmol) was dissolved in 20% aqueous KOH solution (15 mL) and the mixture refluxed until the starting material disappeared (as monitored by TLC - approx. 3 h). The solution was acidified to pH 3 with 2 M HCl solution and extracted with ethyl acetate (3 x 30 mL). The solvent was removed *in vacuo* to yield Δ^2-tetrahydroisophthalic acid as a white solid (127 mg, 73%) which needed no further purification. ^{1}H NMR (300 MHz, CD_3OD) δ 1.52 – 1.60 (2H, m, -$CH_2CH_2CH_2$-), 1.62 – 1.80 (2H, m, -CH_2CHC=), 2.15 – 2.20 (2H, m, -CH_2C=), 3.19 – 3.23 (1H, m, -CH–CH=), 7.02 – 7.03 (1H, bs, -CH=). ^{13}C NMR (75 MHz, CD_3OD) δ 22.1, 25.4, 26.0, 43.4, 133.4, 138.0, 171.0, 177.0.

Kinetic assays: All kinetic assays for DHDPS were carried out as described in reference 13.

Acknowledgements

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