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Heterocyclic inhibitors of dihydrodipicolinate synthase are not competitive

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Abstract—A series of piperidine- and pyridine-2,6-dicarboxylate derivatives has been evaluated as potential inhibitors of dihydrodipicolinate synthase (DHDPS). The compounds were designed with oxygen functionality at the C-4 position in order to mimic the putative enzyme product HTHDP. Most compounds displayed weak–moderate inhibition of DHDPS. Additionally, the most potent inhibitors were shown not to be competitive, indicating they do not bind at the active site. Discrepancies between the two common assay systems—the imidazole assay and the coupled assay—were observed which are attributed to inherent problems in the imidazole assay, leading to artefactually low K_i measurements.

1. Introduction

The effectiveness of many life-saving antibiotics is being undermined by the emergence of drug resistant bacterial strains.[1–4] If medicine is to remain ahead of the evolution of these drug resistant bacteria then the development of new antibiotics is imperative. One target of antibacterial agents that has yet to be fully exploited is the biosynthesis of the amino acid lysine, and its immediate precursor *meso*-diaminopimelate (*meso*-DAP).[5–7] The lysine biosynthetic pathway in plants and bacteria yields the de novo synthesis of lysine for protein utilization. More importantly, lysine and *meso*-DAP are vital constituents of the bacterial peptidoglycan cell wall.[8,9] Hence, blockage of lysine (and *meso*-DAP) biosynthesis would inhibit bacterial growth via two mechanisms, and such inhibitors may provide a new class of antibacterial agents.[5–7] Additionally, mammals lack the ability to biosynthesize lysine and hence it is one of the essential amino acids that must be provided through a dietary source. The occurrence of the lysine biosynthetic pathway in microorganisms and plants but not in mammals suggests that specific inhibitors of this biosynthetic pathway may display novel antibacterial and/or herbicidal activity, with low mammalian toxicity.

2. Proposed mechanism of dihydrodipicolinate synthase

The first unique step in lysine biosynthesis involves the condensation of pyruvate **1** and (*S*)-ASA **3** to give dihydrodipicolinate (DHDP, **6**), a reaction catalyzed by the enzyme dihydrodipicolinate synthase (DHDPS, Fig. 1).[10–15] The reaction is initiated by condensation of

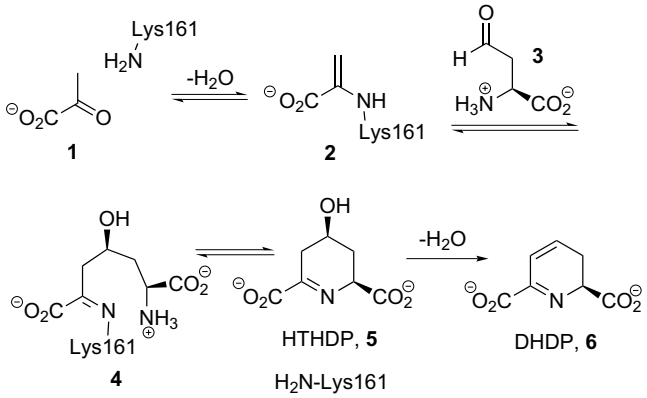

Figure 1. Production of DHDP **6** from ASA **3** and pyruvate **1**.

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pyruvate **1** with an active site lysine residue (Lys161 in *E. coli* DHDPS) forming a Schiff base. This has been confirmed by sodium borohydride trapping experiments[10,13,16] and the X-ray crystal structure of DHDPS with pyruvate bound.[16] Subsequent tautomerization gives the enamine **2**. Aldol-type reaction of the enamine **2** with (*S*)-ASA **3** then gives the acyclic enzyme-bound intermediate **4**. Transimination of the acyclic intermediate **4** gives the cyclic alcohol **5** with simultaneous release of the active site lysine residue. Loss of water then provides DHDP **6**. As the name suggests, it was originally believed that DHDP **6** was the enzymatic product formed in this reaction. However, recent work by Blickling et al.[16] has shown that the actual product is 4-hydroxytetrahydrodipicolinate (HTHDP, **5**), which was observed as a discrete species in solution. It was postulated that this species is the true product of the enzyme-catalyzed reaction, with dehydration to give DHDP **6** occurring spontaneously following release of the alcohol **5** into solution.[16]

To date, no potent inhibitors of DHDPS have been reported. Analogues of pyruvate such as phosphoenolpyruvate, phenylpyruvate and α-ketobutyrate are neither substrates nor inhibitors of DHDPS.[7] Similarly, analogues of (*S*)-ASA, including glutamate semialdehyde, acetylaspartate semialdehyde and homoserine lactone, are neither substrates nor competitive inhibitors of the enzyme.[17] These results suggest that the DHDPS enzyme has very specific binding sites for the substrates. Numerous heterocycles such as **7**–**13** (Fig. 2) have been shown to be weak to moderate inhibitors of DHDPS (ca. 0.2–5 mM), and were presumed to act as product analogues.[17,18] However, these inhibitors have been designed to mimic DHDP, which, in light of the findings of Blickling and co-workers, suggested that more potent inhibitors may yet be discovered by preparing analogues of the putative enzyme product HTHDP **5**.

The report of Couper et al.[18] suggests that planar compounds, and those with 2,6-substituents in a *cis*-disposition, are more effective inhibitors of DHDPS than the corresponding *trans*-disposed compounds (i.e., **8b** and **13b** are more potent than **12b**), presumably as they more effectively mimic the geometry of the near-planar product. The results also suggest that the diesters are equipotent to, or considerably more potent than, the corresponding diacids (Fig. 2). The reasons for the poor inhibition of DHDPS by the piperidine-2,6-dicarboxylic acids **12a** and **13a** compared with the corresponding diesters **12b** and **13b** were not clear, and cast doubt on their proposed mode of inhibition as product mimics. Although several compounds have been screened as DHDPS inhibitors, very few have had their mode of inhibition determined. Karsten[15] has reported dipicolinic acid **8a** to be an uncompetitive inhibitor of DHDPS with respect to pyruvate ($K_i = 11$ mM) and a competitive inhibitor with respect to ASA ($K_i = 18$ mM) (compared with an IC_{50} of 0.4 mM reported by Couper et al.[18]). The dinitrile **7** is reported by Couper et al. to have an IC_{50} value of 0.3 mM, being noncompetitive with respect to pyruvate ($K_i = 0.34$ mM) and ASA ($K_i = 1.25$ mM).[18]

3. Design of novel dihydrodipicolinate synthase inhibitors

Unlike DHDP, HTHDP **5** contains a hydroxyl group at the C-4 position. This prompted the design of several novel analogues containing oxygen functionality at the C-4 position. As compounds incorporating oxygen at the C-4 position mimic the putative product HTHDP **5** more closely than compounds designed to mimic DHDP **6**, there are potentially more binding interactions that could occur at the enzyme active site, thereby providing a lead in the development of more potent inhibitors of DHDPS. Therefore, compounds **14**–**17** (Fig. 3) were designed as potential inhibitors, all with oxygen functionality at the C-4 position. The cyclic alcohol **14** was designed to mimic the putative enzyme product HTHDP **5**. It closely resembles the *cis*-piperidine diester **13b** but also contains a C-4 hydroxyl group to

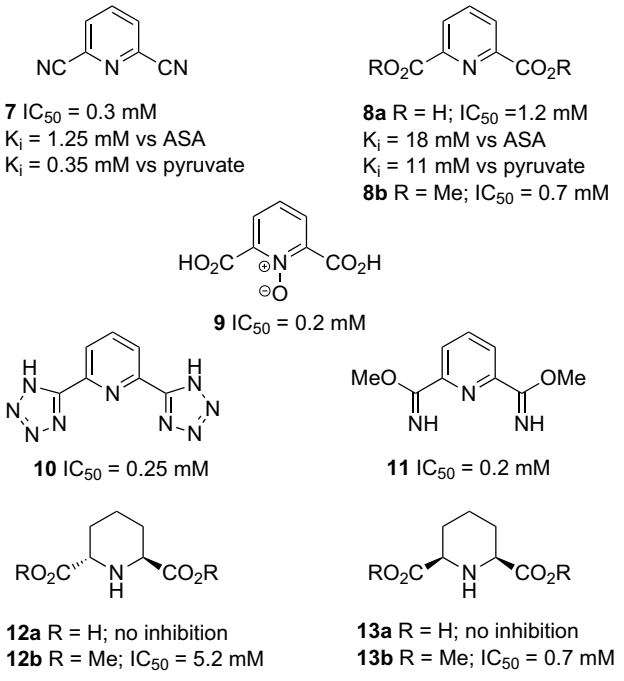

Figure 2. Heterocyclic inhibitors of DHDPS.

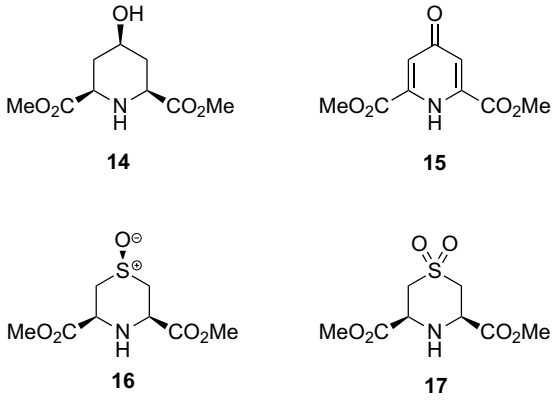

Figure 3. Proposed DHDPS inhibitors.

mimic HTHDP **5**. Compound **14** was designed with a combination of 4(*S*)-stereochemistry to match that reported by Blickling et al.[16] and 2(*S*)-stereochemistry to match that derived from (*S*)-ASA, along with dimethyl ester groups in the favorable *cis*-2,6 relationship delineated from the work of Couper et al.,[18] to generate the all *syn*-compound **14**. The logical extension of the design of the hydroxypiperidine inhibitor **14** was the corresponding 4-hydroxydipicolinate, which exists as the pyridone tautomer **15** and is commonly known as chelidamic acid.

Since transition state analogues have frequently been proven to be very potent inhibitors of enzymes as they are very tightly bound by the enzyme in the active site,[19,20] the sulfoxide **16** (Fig. 3) was also proposed as a potential inhibitor of DHDPS. The sulfoxide **16** is closely related to the hydroxypiperidine **14**, with the secondary alcohol group replaced by a sulfoxide group. Sulfoxides have an inherent charge separation, with a partial negative charge on the oxygen and a partial positive charge on the sulfur atom. This charge separation mimics the developing negative charge on the (*S*)-ASA oxygen in the transition state of the DHDPS reaction, as the nucleophilic enamine **2** attacks, and accordingly the sulfoxide **16** was designed to mimic this transition state.

Finally, the sulfone **17** was designed to mimic the hydrate of (*S*)-ASA since it possesses two oxygens at the C-4 position. The hydrate is one of the potentially biologically relevant forms of (*S*)-ASA[21,22] and thus the sulfone **17** was designed to mimic that arrangement.

4. Results and discussion

4.1. Choice of assay

There are three assays available for studying DHDPS activity: the *o*-aminobenzaldehyde assay, the imidazole buffer assay, and the coupled assay.[30] The aminobenzaldehyde assay is used extensively in the purification of DHDPS, but is not used for kinetic studies. The imidazole buffer assay monitors the rise in absorption at 270 nm of dipicolinic acid (formed by the autoxidation of DHDP) upon incubation of DHDPS and its substrates in imidazole buffer.[13] However, a lag phase of more than 10 s is present before the absorbance at 270 nm increases, and the kinetics of the oxidation step have not been determined, thereby making the initial rates provided by this assay somewhat questionable. The advantage of this assay is that it is easy to perform, and thus it continues to be used by many research groups.[8,12,18] The coupled assay involves the determination of DHDPS activity by monitoring the conversion of DHDP **6** to THDP **18** in the presence of excess DHDPR, with the utilization of NADPH by DHDPR detected at 340 nm (Fig. 4). This assay is able to measure DHDPS kinetics if DHDPR is present in excess, and DHDPR kinetics if DHDPS is present in excess. This assay was therefore used to investigate the nature of inhibition of the compounds synthesized. Although the

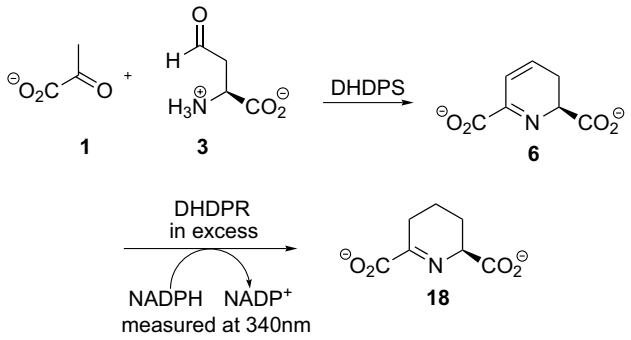

Figure 4. Coupled assay to test for DHDPS activity.

coupled assay was developed in 1965 by Yugari and Gilvarg[30] it was used rarely until the early 1990s, probably due to a lack of availability of DHDPR. As new procedures become available for obtaining sufficient quantities of both DHDPS and DHDPR, with the advent of recombinant DNA technology, the coupled assay is being used more frequently by numerous groups. [15,17,31]

4.2. Preliminary inhibition screening

A preliminary screen of designed and known DHDPS inhibitors yielded some interesting results. All compounds were initially screened at 50 mM, then at various concentrations to determine IC_{50} values. Dipicolinic acid **8a** is a known inhibitor of DHDPS with Couper et al.[18] reporting an IC_{50} value of 0.4 mM (imidazole assay) whilst Karsten[15] reports a K_i of 11 mM versus pyruvate and a K_i of 18 mM versus ASA (coupled assay). The results obtained herein (Fig. 5) gave an IC_{50} value of 20 mM, in close agreement with the results reported by Karsten.[15] The large discrepancy between the inhibition constants measured using the imidazole and

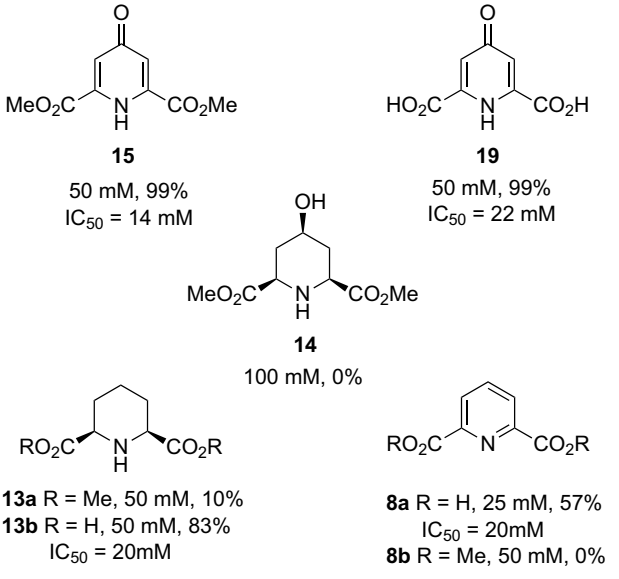

Figure 5. Nitrogen containing heterocyclic inhibitors of DHDPS: percent inhibition of DHDPS activity at stated concentration, [pyruvate] = 1.0 mM and [ASA] = 1.0 mM.

coupled assays suggests one of these assays is flawed. From these and other results (described below) we believe that it is the imidazole assay that is inaccurate. Not only does the imidazole assay contain a lag phase due to the requisite autoxidation of DHDP to give the chromophore, dipicolinic acid, but dipicolinic acid is itself a weak inhibitor of DHDPS. Hence the imidazole assay gives rise to artefactually low K_i measurements.

As dipicolinic acid **8a** is also a competitive inhibitor of DHDPR,[31] increased levels of DHDPR were added during coupled assay measurements to ensure that the DHDPS-catalyzed reaction remained the rate limiting step. However, even with additional DHDPR, this inhibitor could not be screened at high concentrations as biphasic kinetics were observed, consistent with inhibition of both enzymes. The corresponding methyl ester **8b** did not inhibit DHDPS at any of the concentrations tested, in contrast with the IC_{50} value of 0.7 mM obtained by Couper et al.,[18] again revealing inconsistencies between the coupled and the imidazole assays.

The piperidine dimethyl ester **13b** was reported by Couper et al.[18] to have an IC_{50} of 0.7 mM using the imidazole buffer assay. However, when **13b** was screened for inhibition using the coupled assay it showed very little activity; at 50 mM concentration only 10% inhibition of DHDPS was observed. Additionally, the corresponding acid **13a**—which was reported by Couper et al.[18] not to inhibit DHDPS—showed 83% inhibition at 50 mM, yielding an IC_{50} value of 20 mM. Again, this highlights major discrepancies between the coupled and imidazole assays.

Dimethyl chelidamate **15** was found to inhibit DHDPS by 99% at a concentration of 50 mM and an IC_{50} value of 14 mM was obtained. The corresponding diacid **19** was also screened and at 50 mM, 99% inhibition was observed. An IC_{50} value of 22 mM was obtained, indicating that the acid **19** had similar activity to the ester **15**.

Interestingly, the HTHDP mimic **14** showed no inhibition, even at 100 mM concentration. This was an intriguing result as **14** is the closest mimic of HTHDP and suggests that product mimicry is not an effective route to the design of DHDPS inhibitors.

Numerous sulfur-containing heterocyclic compounds were also comprehensively screened as potential DHDPS inhibitors (Fig. 6). The *cis*-substituted diester **20a** is very similar in structure to **13a** and also possessed similar inhibitory activity (36% inhibition at 50 mM). Surprisingly, the diacid **20b** showed no inhibition at all, whereas **13b** was a significantly better inhibitor than the ester **13a**. The *trans*-substituted compounds **21a** and **21b** exhibited similar inhibitory activity to **20a** and **20b**, in contrast to the reported result that *cis*-compounds are better inhibitors of DHDPS.[18]

The introduction of a C-4 oxygen substituent led to increased inhibition in only one case, the all *syn*-sulfoxide **16**, with 48% inhibition occurring at 9 mM. The epimeric *anti*-sulfoxide **24a** showed only 18% inhibition at

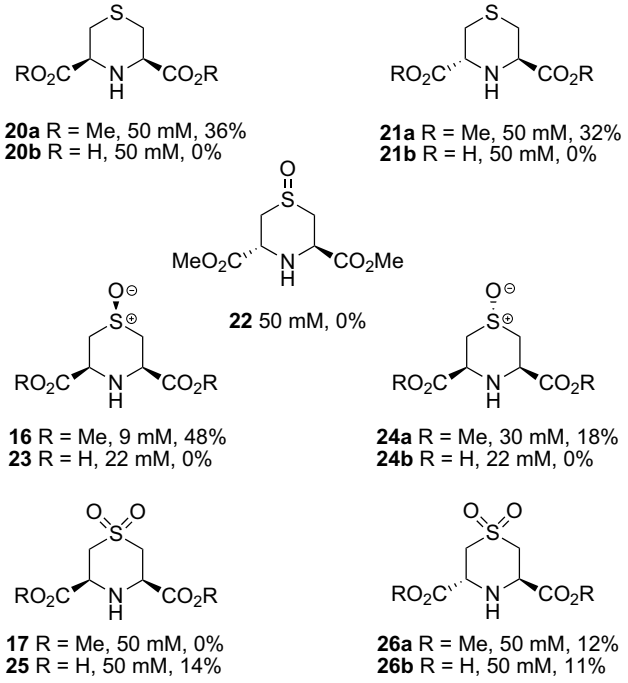

Figure 6. Sulfur containing heterocyclic inhibitors of DHDPS: percent inhibition of DHDPS activity at stated concentration, [pyruvate] = 1.0 mM and [ASA] = 1.0 mM.

30 mM. The corresponding acids **23** and **24b** did not display any inhibition.

The *trans*-sulfoxide **22** did not inhibit DHDPS, even at high concentrations (50 mM). This result is not surprising, as the X-ray crystal structure of **22**[28] shows that both the C-4 oxygen and one of the methoxycarbonyl groups occupy axial positions, whereas the product HTHDP **5** is expected to possess pseudo-equatorial substituents at these positions.

Interestingly the *cis*-sulfone diester **17** showed no inhibition, but the diacid **25** showed 14% inhibition at 50 mM. However, the *trans*-sulfone diester **26a** and acid **26b**, both showed a small degree of inhibition: 12% for **26a** and 11% for **26b** at 50 mM.

From the preliminary screening results shown in Figures 5 and 6 no clear inhibitory trends could be ascertained. No trends at all were apparent for the thiamorpholine-based compounds (Fig. 6). The pyridine- and piperidine-based diacids (Fig. 5) appear to be better inhibitors than the corresponding esters, as would be expected based on analogy to the reported product, but in contrast to the results of Couper et al.[18] However, the chelidamic ester **15** proved to be a better inhibitor then the corresponding acid **19**. Chelidamic acid **19** possesses approximately the same IC_{50} value as dipicolinic acid **8a**, indicating that the introduction of functionality at the C-4 position does not result in increased inhibitory activity. The most striking result from the preliminary screen was that compound **14**, possessing 4-OH functionality in the piperidine ring, showed no inhibition of DHDPS whatsoever. Even though we determined

the corresponding compound lacking the 4-OH group (**13b**) to be only a very weak inhibitor, inhibition was measurable. These results raise some interesting questions. Firstly, if HTHDP is the true product of the enzyme-catalyzed reaction, why does introduction of oxygen functionality at the C-4 position not affect, or diminish, the potency of the inhibition? Secondly, if these product analogues are binding at the active site, why are diesters often more potent than the corresponding diacids? These questions prompted a detailed investigation as to the mode of inhibition of these compounds, in order to establish whether they were binding at the active site.

4.3. Detailed kinetic analyses of compounds 15 and 19

In order to determine whether **15** or **19** act as competitive inhibitors of DHDPS, further detailed kinetic analyzes were necessary. Inhibition kinetics of dimethyl chelidamate hydrochloride **15** with respect to pyruvate and ASA were run at a range of inhibitor and substrate concentrations. Where possible, the inhibitor concentrations were varied by 0.1 and 10 times the IC_{50} value obtained for the individual inhibitor. Dimethyl chelidamate hydrochloride **15** concentrations were varied between 0–7.5 mM with respect to pyruvate, and 0–15 mM with respect to ASA. At very high concentrations of inhibitor, significant absorbance problems and deviations from linear rates were observed, especially with respect to pyruvate. Again, this precluded the acquisition of accurate initial rates at these high inhibitor concentrations. As such, the data obtained must be interpreted carefully, as a wide range of inhibitor concentrations above the IC_{50} value were not taken, so the behavior of the inhibitor at high concentrations remains unknown. The concentration of (*S*)-ASA was varied between 0.04 and 1.5 mM, while pyruvate concentrations were varied between 0.05 and 2.0 mM. The kinetic data were fitted to mathematical models using the Enzfitter computer program that simulated competitive, noncompetitive, uncompetitive, and mixed inhibition patterns in order to determine the model of best fit and subsequently the inhibition constant K_i. The inhibition was found to be noncompetitive with respect to both substrates, pyruvate and ASA (Fig. 7). The K_i with respect to pyruvate was found to be 6.90±0.82 mM ($R^2 = 0.97$), while the K_i with respect to ASA was found to be 14.0±1.43 mM ($R^2 = 0.96$). The result that dimethyl chelidamate hydrochloride **15** is a noncompetitive inhibitor with respect to both substrates is very significant, in that it demonstrates that the inhibitory effects observed are due to allosteric binding rather than binding at the active site.

Inhibition kinetics of chelidamic acid **19** were also performed with respect to pyruvate and ASA, at a range of inhibitor and substrate concentrations. Chelidamic acid **19** concentrations were varied between 0 and 15 mM with respect to pyruvate and 0 and 20 mM with respect to ASA. With respect to pyruvate when inhibitor concentrations higher than 15 mM were used, deviations from linear rates were observed. Again, this precluded the acquisition of accurate initial rates at high inhibitor concentrations. This situation is analogous to that observed in the case of dimethyl chelidamate hydrochloride **15**. The concentration of pyruvate was varied between 0.05 and 2.0 mM, while ASA concentrations were varied between 0.04 and 1.5 mM. The kinetic data were fitted to mathematical models using the Enzfitter computer program that simulated competitive, noncompetitive, uncompetitive, and mixed inhibition patterns in order to determine the model of best fit and subsequently the inhibition constant K_i.

The inhibition was found to be uncompetitive with respect to both substrates, pyruvate and ASA (Fig. 8). The K_i with respect to pyruvate was found to be 22.0±1.8 mM ($R^2 = 0.98$), while the K_i with respect to ASA was found to be 24.8±1.9 mM ($R^2 = 0.99$). The result that chelidamic acid **19** is an uncompetitive

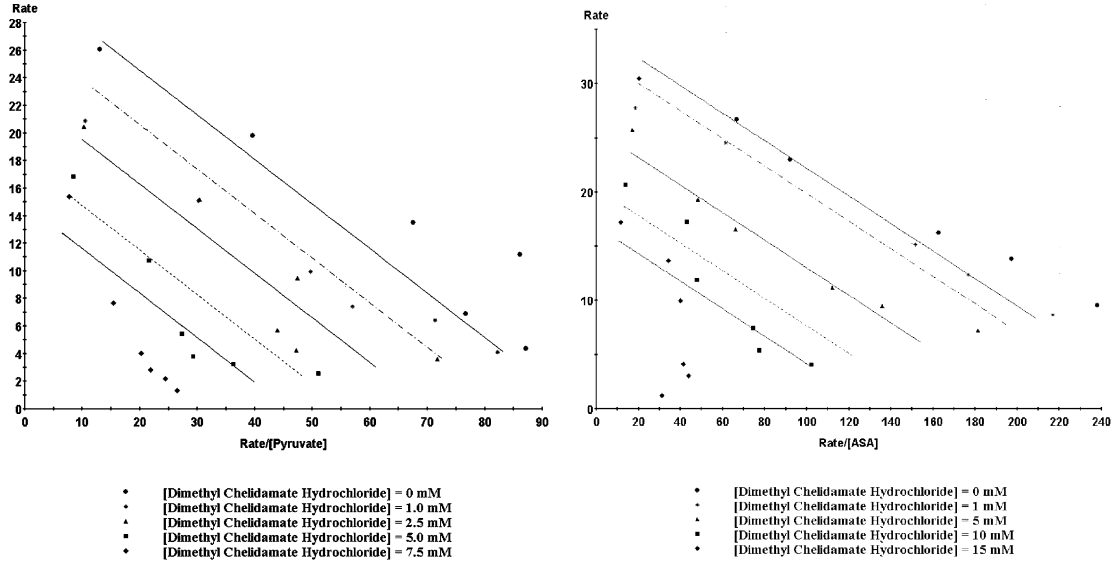

Figure 7. Eadie–Hofstee plots of DHDPS inhibition by dimethyl chelidamate hydrochloride, with respect to pyruvate and (*S*)-ASA.

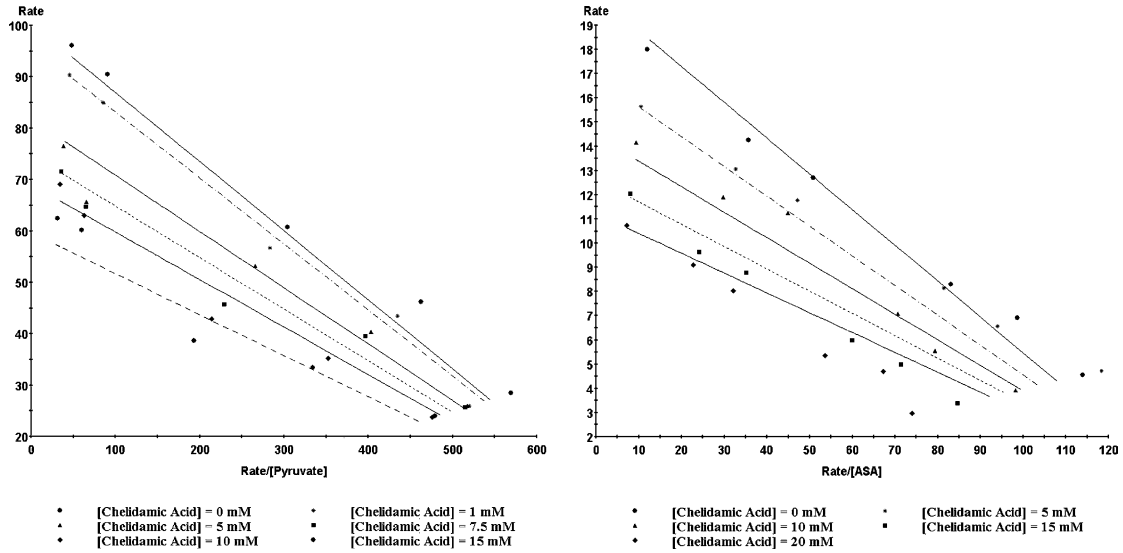

Figure 8. Eadie–Hofstee plots of DHDPS inhibition by chelidamic acid, with respect to pyruvate and (*S*)-ASA.

inhibitor with respect to both substrates implies the inhibitory effects observed are due to allosteric binding.

5. Conclusion and perspective

The results obtained from the inhibitor studies showed that none of the designed compounds possessing oxygen functionality at the C-4 position was a potent inhibitor, with the 4-hydroxypiperidine **14** showing no inhibition of DHDPS whatsoever. Additionally, the most potent of the compounds that did show inhibition of DHDPS were shown not to be competitive inhibitors, belying their design as product analogues. Major discrepancies have been observed between the two common assay systems used to analyze DHDPS activity—the imidazole assay and the coupled assay. The imidazole assay typically yields inhibitory values ~50-fold more potent than the coupled assay, although several compounds (such as the piperidine diacids) show no inhibition in the imidazole assay whereas they do in the coupled assay. Both assay systems employ a second transformation which is analyzed to monitor the progress of the DHDPS-catalyzed reaction. In the coupled assay, the second transformation is the reduction catalyzed by DHDPR, and the assay can be set up to ensure that the DHDPS reaction is the rate-determining step. In the imidazole assay, the second transformation is presumed to be the autoxidation of DHDP to dipicolinic acid. A significant lag time has been observed in this assay. The kinetics of this process have not been determined, and they are likely to vary depending on the concentration of dissolved oxygen in the assay buffer. Also, dipicolinic acid is a weak inhibitor of DHDPS, leading to artefactually low K_i measurements when the imidazole buffer is used. These observations suggest that inhibitory activities calculated using the imidazole assay are likely to be flawed.

The fact that none of the product analogues were potent inhibitors of DHDPS, and that the most active compounds, **15** and **19**, did not show competitive inhibition, suggest that alternative designs not based on substrate/product mimicry will be required to generate potent, competitive inhibitors of DHDPS in the future.

6. Experimental

6.1. General

Chemicals were purchased from Sigma–Aldrich, Novabiochem or Amersham Biosciences. Protein concentration was measured by the method described by Bradford.[23] Enzymes were manipulated at 4 °C and were stored in Tris–HCl buffer (20 mM) at −20 °C. (*S*)-ASA was prepared according to the method of Roberts et al.[24] and the purity determined by ^{1}H NMR and the coupled assay (generally >80% purity) and stored at −80 °C. For kinetic studies, fresh solutions of (*S*)-ASA, NADPH and pyruvate were used. Kinetic data were collected in 1 mL cuvettes using a Hewlett Packard 8452A diode array spectrophotometer with a constant temperature of 30 °C maintained by attachment of a recirculating water bath.

6.2. Preparation of inhibitors

Compounds **14** and **15** were prepared according to the method of Hermann and Dreiding.[25] Compounds **8a** and **13a/b** were prepared according to the method of Chrystal et al.[26] Compounds **20a** and **21a** were prepared according to the method of Paradisi et al.[27] We have previously reported the synthesis of compounds **16**, **17**, **22**, **24a** and **26**.[28] The corresponding diacids **20b**, **21b**, **23**, **24b**, **25**, and **26b** were derived from the dimethyl esters as described below.

6.2.1. Representative procedure for preparation of diacids. To a solution of diester **20a** (518 mg, 2.36 mmol) in THF/water (2:1, 15 mL) was added lithium hydroxide (169 mg, 7.09 mmol, 3 equiv). The mixture was stirred at room temperature for 16 h. The organic solvent was

removed under reduced pressure and the concentrate freeze-dried to yield the dilithium salt of **20b** (492 mg, 103%, contaminated with lithium hydroxide) as a white solid; ^{1}H NMR (300 MHz, D_2O) δ 3.33 (2H, dd, J = 2.1, 11.2 Hz, H3/H5), 2.69 (2H, dd, J = 2.1, 13.5 Hz, $H2_{eq}/H6_{eq}$), 2.51 (2H, app t, J = 11.4 Hz, $H2_{ax}/H6_{ax}$); ^{13}C NMR (75 MHz, D_2O) δ 181.4, 64.3, 32.3; IR ν_{max} (KBr)/cm^{-1} 3295, 1607, 1452, 1391; MS m/z (ESI) −ve ion $[M-Li]^-$ 196 (100%); HRMS (ESI) −ve ion $[M^{2-}+H]^-$ 190.0193; $C_6H_8NO_4S$ requires 190.0182.

6.2.2. Data for dilithium salt of 21b. ^{1}H NMR (300 MHz, D_2O) δ 3.89 (2H, dd, J = 3.6, 7.0 Hz, H3/H5), 2.88 (2H, dd, J = 3.6, 14.1 Hz, H2/H6), 2.80 (2H, dd, J = 7.0, 14.1 Hz, H2/H6); ^{13}C NMR (75 MHz, D_2O) δ 175.6, 57.1, 28.0; IR ν_{max} (KBr)/cm^{-1} 3283, 1742, 1414, 1315; MS m/z (ESI) −ve ion, $[M-Li]^-$ 196 (100%); HRMS (ESI) −ve ion $[M^{2-}+H]^-$ 190.0185; $C_6H_8NO_4S$ requires 190.0180.

6.2.3. Data for dilithium salt of 23. ^{1}H NMR (400 MHz, D_2O) δ 3.64 (2H, d, J = 11.6 Hz, $H2_{eq}/H6_{eq}$), 3.26 (2H, d, J = 12.0 Hz, H3/H5), 2.37 (2H, app t, J = 11.9 Hz, $H2_{ax}/H6_{ax}$); ^{13}C NMR (100 MHz, D_2O) δ 178.5, 58.5, 54.4; IR ν_{max} (KBr)/cm^{-1} 3450, 1627, 1364, 1015; MS m/z (ESI) −ve ion, $[M-Li]^-$ 212 (100%); HRMS (ESI) −ve ion $[M^{2-}+H]^-$ 206.0124; $C_6H_8NO_5S$ requires 206.0128.

6.2.4. Data for dilithium salt of 24b. ^{1}H NMR (400 MHz, D_2O) δ 3.67 (2H, d, J = 12.0 Hz, $H2_{eq}/H6_{eq}$), 3.05 (2H, d, J = 14.5 Hz, H3/H5), 2.40 (2H, app t, J = 13.3 Hz, $H2_{ax}/H6_{ax}$); ^{13}C NMR (100 MHz, D_2O) δ 178.5, 51.1, 46.2; IR ν_{max} (KBr)/cm^{-1} 3486, 1745, 1229, 1043; MS m/z (ESI) −ve ion $[M-Li]^-$ 212 (100%); HRMS (ESI) −ve ion $[M^{2-}+H]^-$ 206.0131; $C_6H_8NO_5S$ requires 206.0128.

6.2.5. Data for dilithium salt of 25. ^{1}H NMR (400 MHz, D_2O) δ 3.52 (2H, dd, J = 2.0, 12.1 Hz, $H2_{eq}/H6_{eq}$), 3.38 (2H, br d, J = 12.9 Hz, H3/H5), 2.93 (2H, app t, J = 12.4 Hz, $H2_{ax}/H6_{ax}$); ^{13}C NMR (50 MHz, D_2O) δ 175.9, 58.2, 54.1; IR ν_{max} (KBr)/cm^{-1} 3312, 1638, 1403, 1281, 1127; MS m/z (ESI) −ve ion $[2M-Li]^-$ 463 (100%); $[M-Li]^-$ 228 (55%) HRMS (ESI) −ve ion $[M-Li]^-$ 228.0172; $C_6H_7LiNO_6S$ requires 228.0159.

6.2.6. Data for dilithium salt of 26b. ^{1}H NMR (400 MHz, D_2O) δ 3.93 (2H, t, J = 5.5 Hz, H3/H5), 3.28 (4H, d, J = 5.5 Hz, H2/H6); ^{13}C NMR (100 MHz, D_2O) δ 176.9, 56.0, 53.5; IR ν_{max} (KBr)/cm^{-1} 3312, 1630, 1410, 1283, 1134; MS m/z (ESI) −ve ion $[M-Li]^-$ 228 (100%); HRMS (ESI) −ve ion $[M-Li]^-$ 228.0172; $C_6H_7LiNO_6S$ requires 228.0159.

6.3. Over-expression and purification of DHDPS and DHDPR

Wild-type DHDPS was expressed from an *E. coli* XL-1 Blue cell line harbouring the plasmid pJG001, as described by Dobson et al.[29] DHDPS was purified according to the methods of Mirwaldt et al.[12] with the following modifications. DHDPS was freeze-thawed seven times rather than sonicated and the crude mixture was not heat shocked. Additionally the FPLC step was omitted since the enzyme was of sufficient purity for use in kinetic studies. Several different preparations of DHDPS were used in the inhibition studies; in each case the enzyme was judged homogenous by SDS-PAGE stained with Coomassie brilliant blue and yielded a specific activity of approximately 1.8 units/mg (one unit of enzyme activity is equal to the loss of 1 μmol of NADPH per second).

Wild-type DHDPR was expressed from an *E. coli* XL-1 Blue cell line harbouring the plasmid pJK001, as described by Coulter et al.[17] DHDPR was purified according to the method of Dobson et al.[29] The enzyme was judged homogenous by SDS-PAGE stained with Coomassie brilliant blue and yielded a specific activity of approximately 1.5 units/mg. To ensure the absence of contaminating DHDPS, control assays containing only DHDPR, NADPH, pyruvate and (*S*)-ASA were performed; these showed no activity.

6.4. Enzyme assays

DHDPS activity was measured using the coupled assay as reported[17,29] with some modifications. HEPES buffer (250 mM) was used to maintain a constant pH of 8.0 (this is because at lower buffer concentrations, the addition of dilithium salts caused the pH to rise to 14). The kinetic parameters obtained with respect to pyruvate and (*S*)-ASA were determined and found to be respectively, K_m of 0.38±0.02, K_m of 0.14±0.01. Extreme care was taken to ensure that DHDPR was present in excess especially in the case of known dual DHDPS/DHDPR inhibitors. Lack of DHDPR was often self-indicating as a biphasic initial rate was observed. Percentage inhibition readings were initially performed in order to screen the compounds rapidly for interesting inhibition properties; a number of known inhibitors of DHDPS were also tested as positive controls. Where possible, IC_{50} values were obtained. Compounds were tested in aqueous solutions at concentrations up to 100 mM, or to the limit of their solubility. However, a number of compounds tested did not dissolve well in aqueous solution and had considerably greater solubility in methanol or DMSO: in these cases, the assay was run in 10% methanol or DMSO. In the case of methanol, the initial rate decreased by approximately 5%, but inhibition could still be quantified by placing methanol in the control incubations for comparison. DMSO did not appear to affect the initial rate at all; however, it was placed in all control incubations, as an extra precaution.

6.5. Kinetic data collection and processing

Inhibition kinetics were run at a range of inhibitor and substrate concentrations. Inhibitor concentrations were varied in most cases by 0.1 and 10 times the IC_{50}. (*S*)-ASA concentrations ranged between 0.04 and 1.5 mM, while pyruvate concentrations ranged between 0.05 and 2.0 mM. All data points were collected in duplicate.

The kinetic data were fitted to mathematical models using the Enzfitter computer program from Biosoft (Cambridge, UK) that simulated competitive, noncompetitive, uncompetitive and mixed inhibition patterns, in order to determine the model of best fit and subsequently the inhibition constant K_i. Enzfitter was also used to generate the Eadie–Hoftsee (Rate vs Rate/[Substrate]) plots.

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