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Two new irreversible inhibitors of dihydrodipicolinate synthase: diethyl (*E*,*E*)-4-oxo-2,5-heptadienedioate and diethyl (*E*)-4-oxo-2-heptenedioate

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Abstract—Dihydrodipicolinate synthase (DHDPS) is a key enzyme in lysine biosynthesis and an important antibiotic target. The enzyme catalyses the condensation of (*S*)-aspartate semialdehyde (ASA) and pyruvate to form dihydrodipicolinate. Two new irreversible inhibitors of dihydrodipicolinate synthase are reported, designed to mimic the acyclic enzyme-bound condensation product of ASA and pyruvate. These compounds represent an important new lead in the design of potent inhibitors for this enzyme.

The lysine biosynthesis pathway is essential for plants and micro-organisms, but is not present in mammals. As such, it remains a target for therapeutic agents, although no commercial product has yet been shown to inhibit this pathway.[1] The enzyme that catalyses the branchpoint of the diaminopimelate pathway to lysine is dihydrodipicolinate synthase (DHDPS). Remarkably, no potent inhibitor of this enzyme has yet been found.

DHDPS catalyses the condensation of (*S*)-aspartate semialdehyde (ASA, **2**)[2] and pyruvate (**1**) to form an unstable heterocycle, formerly thought to be dihydrodipicolinate (**4**), but now believed to be 4-hydroxytetrahydrodipicolinate (**3**),[3] with spontaneous dehydration to give **4** following release from the enzyme active site (Fig. 1).

The DHDPS-catalysed reaction is initiated by condensation of 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,[4] and the X-ray crystal structure of DHDPS

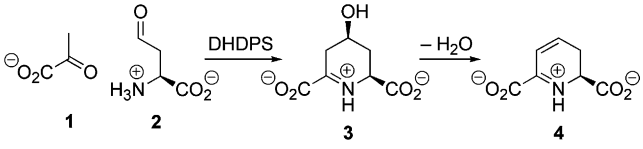

Figure 1. The condensation of pyruvate **1** and (*S*)-ASA **2** to form 4-hydroxytetrahydrodipicolinate **3**, catalysed by DHDPS.

with pyruvate bound.[3] Subsequent tautomerisation gives the enamine **5**. Aldol-type reaction of **5** with (*S*)-ASA **2** then gives the acyclic enzyme-bound intermediate **6** (Fig. 2). Transimination of the acyclic intermediate **6** is thought to yield the cyclic alcohol **3**, with simultaneous release of the active site lysine residue.

Analogues of pyruvate, such as phosphoenolpyruvate, phenylpyruvate and α-ketobutyrate are neither substrates nor inhibitors of DHDPS.[5] Similarly, analogues of (*S*)-ASA, including glutamate semialdehyde, acetylaspartate semialdehyde and homoserine lactone, are neither substrates nor competitive inhibitors of the enzyme.[6] The majority of inhibitors reported to date have been based on DHDP **4**. A variety of heterocyclic product analogues have been shown to be weak to moderate inhibitors of DHDPS (ca. 0.2–5 mM).[6,7] However, to date, none has shown potent inhibition.

Keywords: Dihydrodipicolinate synthase; DHDPS; Irreversible inhibitor.
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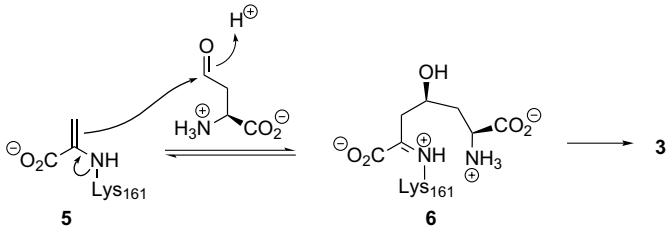

Figure 2. The condensation of pyruvate **1** and (*S*)-ASA **2** to give **3** proceeds through enamine **5** and enzyme-bound condensation product **6**.

The failure of a large number of product analogues of the reaction to display potent competitive inhibition led us to explore a new class of inhibitor, based on the acyclic enzyme-bound intermediate **6**. A previous report that α-ketopimelic acid is an irreversible inhibitor of DHDPS, with a K_i of 0.17 mM,[3] suggested that this might be a valid approach. Diethyl (*E*)-4-oxo-2-heptenedioate **11** and diethyl (*E*,*E*)-4-oxo-2,5-heptadienedioate **9** (Fig. 3) were therefore synthesised and tested as potential irreversible DHDPS inhibitors. These compounds were designed to resemble the carbon framework and functionality of the acyclic intermediate **6**, while also possessing an electrophilic Michael-acceptor to react with an active site nucleophile.

Diethyl (*E*,*E*)-4-oxo-2,5-heptadienedioate **9** was synthesised from diethyl 4-oxopimelate **7** by the method of Lemaire-Audoire and Vogel,[8] in excellent yield. Diethyl (*E*)-4-oxo-2-heptenedioate **11** was synthesised using a modification of this method, using only 1 equiv of bromine to give the monobromide intermediate **10** (Fig. 3).[9]

Each compound was tested for inhibition of DHDPS using the coupled assay,[6,10] in which the NADPH-dependent reduction of DHDP **4**, by the subsequent enzyme in the pathway dihydrodipicolinate reductase, is followed by the absorption at 340 nm. Assay methods were modified to include a pre-incubation step of the inhibitor with DHDPS, in order to monitor the effects of an irreversible inhibitor.

Pre-incubation studies with DHDPS and alkene **11** in the assay buffer showed **11** to be a weak inhibitor of DHDPS. It can be seen clearly in Figure 4 that the activity was only 50% that of native DHDPS at 50 mM after 15 min and that by 30 min the activity was only 10% that of native DHDPS. To confirm that the inhibition was irreversible, DHDPS was incubated with alkene **11** in buffer for 30 min followed by dialysis for 16 h against Tris buffer (20 mM, pH 8.0 at 4 °C) did not show a return in activity when compared to controls, indicating that the alkene **11** does indeed act as an irreversible inhibitor.

Pre-incubation studies with DHDPS and diene **9** in the assay buffer confirmed that **9** was a good inhibitor of DHDPS. After 4 min, the activity was diminished by 50% at a concentration of 0.5 mM and by 15 min the activity was only 15% of native DHDPS (Fig. 5). Following incubation of DHDPS with **9** for 30 min and dialysis for 16 h against Tris buffer (20 mM, pH 8.0 at 4 °C), no recovery of activity was observed. Controls in which DHDPS was incubated and dialysed, in the absence of inhibitor showed DHDPS retained its activity under these conditions. Thus diene **9** was shown to be an irreversible inhibitor of DHDPS.

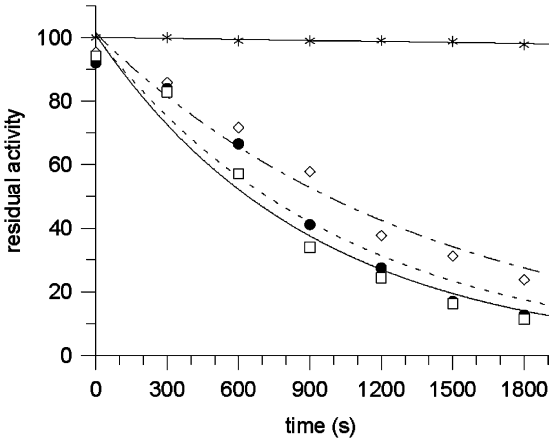

Figure 4. Effect of **11** on the activity of DHDPS. ∗ control, no inhibitor; ◇ 20 mM; ● 50 mM; □ 100 mM.

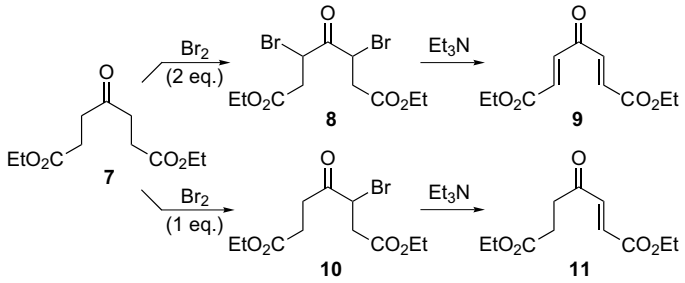

Figure 3. Synthesis of diethyl (*E*,*E*)-4-oxo-2,5-heptadienedioate **9** and diethyl (*E*)-4-oxo-2-heptenedioate **11**.

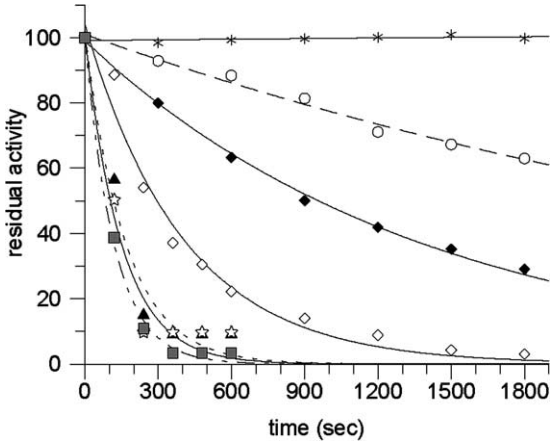

Figure 5. Effect of **9** on the activity of DHDPS. ✳ control, no inhibitor; ○ 125 μM; ◆ 250 μM; ◇ 0.5 mM; ▲ 1.2 mM; ☆ 1.5 mM; ■ 2.0 mM.

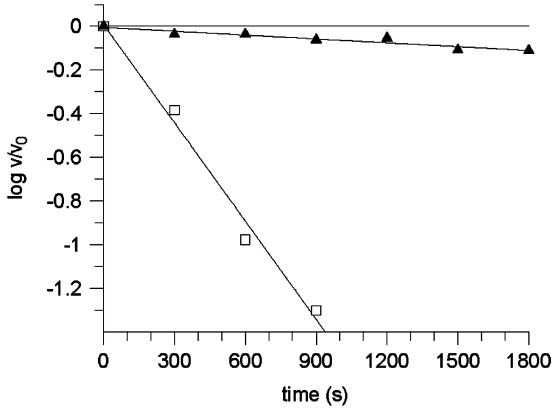

Figure 7. Substrate protection of enzyme inactivation. ▲ no pyruvate; □ 10 mM pyruvate.

An analysis of the data according to the method of Tipton[11] indicates that although the alkene **11** is only a weak inhibitor, with a second order rate constant for enzyme inactivation of 0.11 M^{-1} s^{-1}, the diene **9** is much more potent, with a second order rate constant for enzyme inactivation of 5.4 M^{-1} s^{-1}. This is a very promising result, which provides a new lead compound for the design of DHDPS inhibitors.

It is proposed that the unsaturated ketones **9** and **11** undergo a Michael-type addition with a nucleophile in the enzyme active site, such as the ε-amino group of lysine-161. This would result in covalent attachment to this key active site residue of *E.coli* DHDPS, and inactivate the enzyme (Fig. 6). The initial adduct **12** formed from the diene **9** could potentially undergo a subsequent, intramolecular Michael-type addition to give the piperidinone adduct **13**. Further work is underway to explore this possibility.

In accordance with the postulated mechanism, the alkene **11** and diene **9** were shown to exhibit time-dependent irreversible inhibition. Additionally, the saturated analogue **7** did not inhibit DHDPS at all.

At high concentrations of diene **9**, precipitation in the cuvette was observed, whereas no precipitation was observed with alkene **11**. As diene **9** can undergo two Michael-type additions it was postulated that protein cross-linking could be occurring. Incubating various concentrations of inhibitor **9** with DHDPS, followed by SDS-PAGE visualised by staining with Coomassie brilliant blue (data not shown), indicated protein cross-linking may be responsible for precipitation of insoluble protein at high concentrations of **9**. This suggested that part of the inhibition displayed may be due to nonspecific binding of the inhibitor to surface lysines. In order to determine whether the irreversible inhibitor **9** is acting specifically at the active site, pre-incubation studies in the presence of excess substrate (pyruvate) were performed. These studies showed that pyruvate almost completely protects the enzyme against inactivation by **9**, even at high concentrations of the inhibitor (20 mM) (Fig. 7), thereby confirming that the irreversible inhibitor **9** is acting specifically at the active site.

In summary, two new inhibitors of DHDPS have been identified, one of them active at micromolar concentrations. Further work is underway to enhance the potency of inhibition in second-generation compounds.

Acknowledgements

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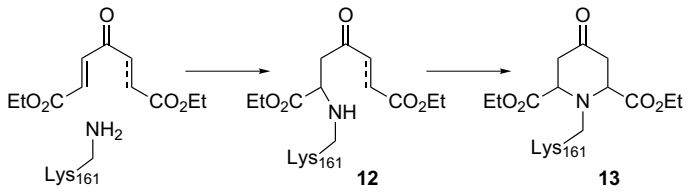

Figure 6. Proposed mode of action of irreversible inhibitors **9** and **11**.

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9. To a solution of diethyl 4-oxopimelate **7** (3.00 g, 13.0 mmol) in dry dichloromethane (20 mL) at 0 °C was added a solution of bromine (653 μL, 13.0 mmol) in dry dichloromethane (2 mL). The mixture was allowed to warm to room temperature and stirred for 30 min. The mixture was diluted with dichloromethane (20 mL), washed with $Na_2S_2O_3$ (3 × 40 mL), dried (Na_2SO_4) and concentrated in vacuo to give a pale yellow oil. Purification by chromatography on silica yielded the bromide **10** as a yellow oil (2.98, 74%). ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.69 (1H, t, J = 6.3 Hz), 4.15 (2H, q, J = 7.1 Hz), 4.14 (2H, q, J = 7.1 Hz), 3.34–3.18 (2H, m), 2.97–2.82 (2H, m), 2.64 (2H, t, J = 6.3 Hz), 1.27 (3H, t, J = 7.1 Hz), 1.25 (3H, t, J = 7.1 Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 201.6, 172.3, 170.1, 61.5, 60.8, 44.2, 38.3, 34.5, 28.2, 14.1(×2); HRMS m/z 308.0257; $C_{11}H_{17}{}^{79}BrO_5$ requires 308.0259. To a solution of **10** (2.50 g, 8.09 mmol) in dry dichloromethane (20 mL) at 0 °C was added triethylamine (1.13 mL, 8.09 mmol). The mixture was stirred at 0 °C for 30 min, then was diluted with water (20 mL), extracted with dichloromethane (3 × 15 mL), dried (Na_2SO_4) and concentrated in vacuo to yield the alkene **11** as a yellow oil (1.65 g, 89%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.08 (1H, d, J = 16.1 Hz), 6.71 (1H, d, J = 16.1 Hz), 4.26 (2H, q, J = 7.0 Hz), 4.14 (2H, q, J = 7.0 Hz), 2.96 (2H, t, J = 6.5 Hz), 2.68 (2H, t, J = 6.5 Hz), 1.34 (3H, t, J = 7.0 Hz), 1.27 (3H, t, J = 7.0 Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 197.7, 172.3, 165.4, 138.9, 131.2, 61.4, 60.8, 35.9, 27.8, 14.1, 14.0; HRMS m/z 228.1000; $C_{11}H_{16}O_5$ requires 228.0998.
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