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Conformationally constrained diketopimelic acid analogues as inhibitors of dihydrodipicolinate synthase

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Abstract—Dihydrodipicolinate synthase (DHDPS) is a key enzyme in lysine biosynthesis and a potential antibiotic target. The enzyme catalyses the condensation of (*S*)-aspartate semi-aldehyde (ASA) and pyruvate to form dihydrodipicolinate. Constrained diketopimelic acid derivatives have been designed as mimics of the acyclic enzyme-bound condensation product of ASA and pyruvate. Several of the compounds are shown to be active, slow-binding inhibitors with improved inhibition of DHDPS.
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The biosynthesis of lysine in plants and micro-organisms proceeds via the diaminopimelate (DAP) pathway, which is not present in mammals. In addition to the de novo synthesis of lysine for incorporation into proteins, lysine, and its immediate precursor, *meso*-DAP, are important constituents of the bacterial peptidoglycan cell wall. As such, enzymes in the DAP pathway have been investigated as targets for therapeutic agents.[1–4]

The enzyme that catalyses the first committed step towards lysine in the DAP pathway is dihydrodipicolinate synthase (DHDPS). DHDPS catalyses the condensation of (*S*)-aspartate semi-aldehyde (ASA, **2**) and pyruvate (**1**) to form an unstable heterocycle, 4-hydroxytetrahydrodipicolinate (HTPA, **3**), with spontaneous dehydration to give dihydrodipicolinate (DHDP, **4**) following release from the enzyme active site (Fig. 1).[5]

The DHDPS-catalysed reaction is initiated by condensation of pyruvate **1** with an active site lysine residue (lys161 in *Escherichia coli* DHDPS) forming a Schiff base. This has been confirmed by sodium borohydride trapping experiments,[6] and by X-ray crystallographic analysis.[5] 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.

Many analogues of (*S*)-ASA, including glutamate semi-aldehyde, acetylaspartate semi-aldehyde and homoserine lactone, are neither substrates nor competitive inhib-

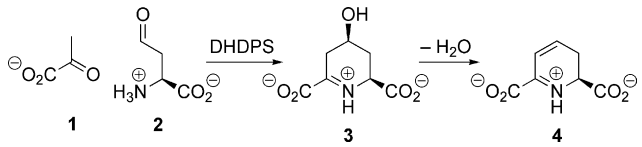

Figure 1. Condensation of pyruvate **1** and (*S*)-ASA **2** to form HTPA **3**, catalysed by DHDPS, then dehydration to give DHDP **4**.

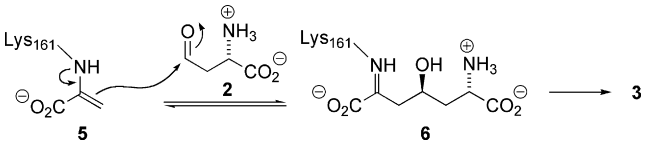

Figure 2. Condensation of pyruvate **2** and (*S*)-ASA **1** to give **3** proceeds through enamine **5** and enzyme-bound condensation product **6**.

Keywords: Dihydrodipicolinate synthase; DHDPS; Enzyme inhibitors; Lysine biosynthesis.
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itors of the enzyme.[7] Succinic semi-aldehyde—related to ASA but lacking the amino group—is a competitive inhibitor of DHDPS with respect to ASA (K_i = 0.3 mM).[5] Analogues of pyruvate are not substrates,[2] however analogues such as α-ketobutyrate, α-ketoglutarate, glyoxylate and fluoropyruvate have been shown to be competitive inhibitors of DHDPS with respect to pyruvate.[8] The majority of inhibitors reported to date have been based on DHDP **4**[7–9] or HTPA **3**,[10] but in general show only weak to moderate inhibitory activity. Dipicolinic acid and chelidamic acid show K_i = 11 mM[8] and 14 mM[10], respectively, with respect to pyruvate (Fig. 3). The reported sub-mM inhibition by related heterocyclic compounds[9] is likely due to flaws in the assay methods used.[10]

The failure of a large number of substrate and product analogues to display potent competitive inhibition led us to explore a new class of inhibitors, 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,[5] suggested that this might be a valid approach. The crystal structures of DHDPS with either α-ketopimelic acid or the adduct of pyruvate and succinic semi-aldehyde bound at the active site show that the pimelic acid moiety is positioned in a largely extended conformation, with torsion angles from C2 to C6 typically 141°–179°.[5] The bis(keto-acid) **8** was therefore designed as a conformationally constrained analogue of the acyclic intermediate **6**, with the ketoacid group able to condense with the active site lysine residue in the same manner as ketopimelic acid, and the aromatic ring serving to constrain the torsion angles of the central C–C bonds to 180°. Accordingly, compound **8** and various analogues **9**–**15** were synthesised and assayed for inhibition of DHDPS activity.

The parent bis(keto-acid) **8** was available in one step (98% yield) from commercially available 1,3-diacetyl benzene **7**, by oxidation with selenium dioxide (Fig. 4). Esterification in acidified methanol provided a mixture of the corresponding diester **9** in variable yield (45–97%) with the dimethyl ketal **10** being the major byproduct. The byproduct **10** could be recycled by hydrolysis to the bis(keto-acid) **8** in quantitative yield.

The bis(keto-acid) **8** was also converted to the corresponding bis-oxime **11** by treatment with hydroxylamine hydrochloride in the presence of sodium carbonate, giving the product in quantitative yield as a single isomer (Fig. 5). Similar treatment of bis(keto-ester) **9** with hydroxylamine hydrochloride and pyridine gave the bis-oxime **12** in 64% yield as a 3:2 ratio of (*Z*,*Z*)- and (*E*,*Z*)-isomers (Fig. 6).

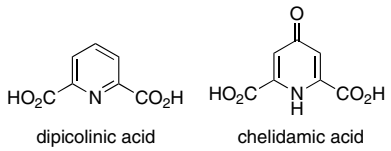

Figure 3. Heterocyclic inhibitors of DHDPS.

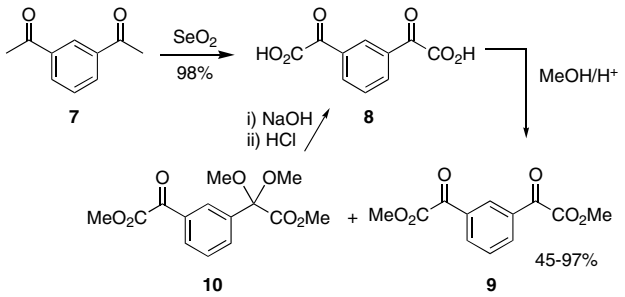

Figure 4. Synthesis of bis-ketoester **9**.

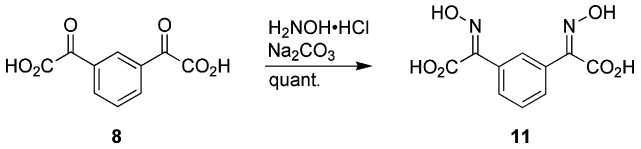

Figure 5. Synthesis of bis(oxime-acid) **11**.

The keto- and oxime-esters **9** and **12** were reduced to the corresponding diol **14** and diamine **13**. Treatment of the bis(keto-ester) **9** with sodium borohydride gave the diol **14** as a mixture of stereoisomers. Reduction of the oxime ester **12** with zinc/formic acid gave the bis-amine **13** in good yield (92%). The diol diester **14** was hydrolysed to the corresponding diacid **15** in quantitative yield by treatment with lithium hydroxide (Fig. 6).

All compounds prepared were tested for inhibition of DHDPS activity using the coupled assay, in which the NADPH-dependent reduction of DHDP **4** by the subsequent enzyme in the pathway, dihydrodipicolinate reductase (DHDPR), is followed by the absorption at 340 nm.[8,11] With the possibility of condensation of the ketone or oxime functional groups present in **8**–**12** with the active site lysine residue, these inhibitors were also tested for time-dependent inhibition. Assay conditions

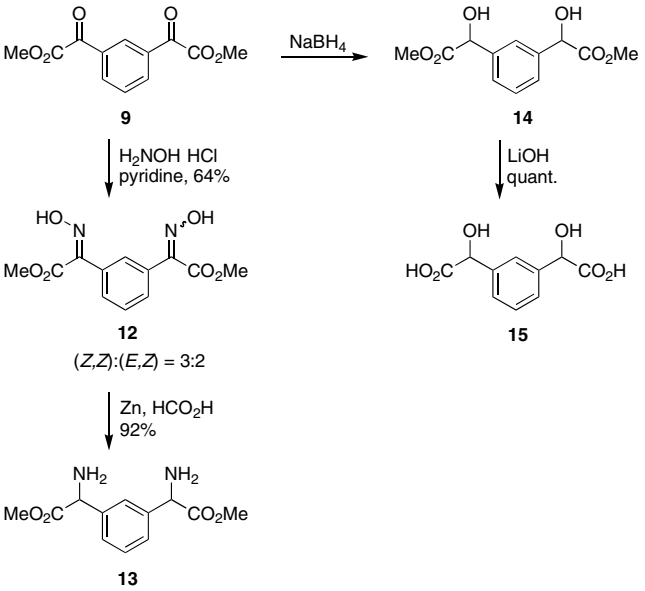

Figure 6. Synthesis of further derivatives **12**–**15**.

were thus modified to include a pre-incubation of enzyme with inhibitor with residual activity determined over time.[11]

Initial screening of compounds **8**–**15** identified the bis(keto-acid) **8** and bis(oxime-ester) **12** as exhibiting significant inhibitory activity. The dimethyl ketal **10**, bis(oxime-acid) **11**, bis(α-hydroxyester) **14** and bis(α-hydroxyacid) **15** showed weak inhibitory activity, whereas the bis(keto-ester) **9** and bis(α-amino-ester) **13** displayed virtually no inhibitory activity (Table 1).

Further analysis of the most active compounds was performed to determine the type of inhibition. Residual DHDPS activity in the presence of 0.5–25 mM of bis(keto-acid) **8** was determined after pre-incubations of 0.5–60 min (Fig. 7). Kinetic analysis demonstrated that bis(keto-acid) **8** was inhibiting DHDPS in a time-dependent manner consistent with the slow-binding model of inhibition described by Copeland,[12,13] with $K_i^{app} = 2.96$ mM ($R^2 = 0.99$) (Fig. 9).

The bis(oxime-ester) **12** was shown to be the most potent inhibitor of DHDPS of the compounds analysed, exhibiting time-dependent inhibition with a $K_i^{app} = 0.33$ mM ($R^2 = 0.96$), with kinetic analysis also corresponding to a slow-binding model of inhibition (Figs. 8 and 9).

Table 1. DHDPS inhibitory activity of compounds **8**–**15**

Compound	Inhibition at 5 mM, $t = 1$ min	Time-dependent inhibition	Inhibition type
8	49%	$K_i^{app} = 2.96$ mM	Slow
9	2%	—	—
10	10%	—	—
11	23%	34% at 50 mM, $t = 60$ min	—
12	15% (at 1 mM)[a]	$K_i^{app} = 0.33$ mM	Slow
13	0%	—	—
14	12%	—	—
15	12%	—	—

[a] Initial screen of **12** was conducted at 1 mM due to high absorbance at 340 nm.

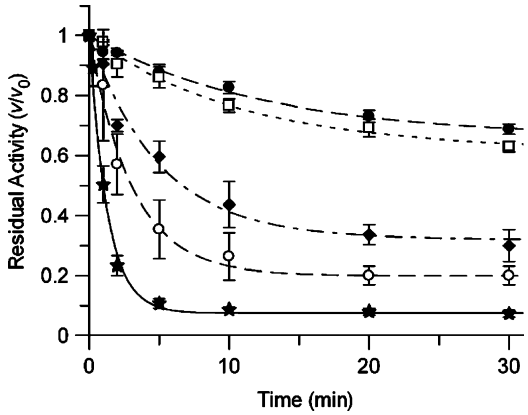

Figure 7. Time-dependent inhibition of DHDPS by bis(keto-acid) **8**; ● = 0.5 mM, □ = 1.0 mM, ♦ = 2.5 mM, ○ = 10.0 mM, ★ = 25.0 mM.

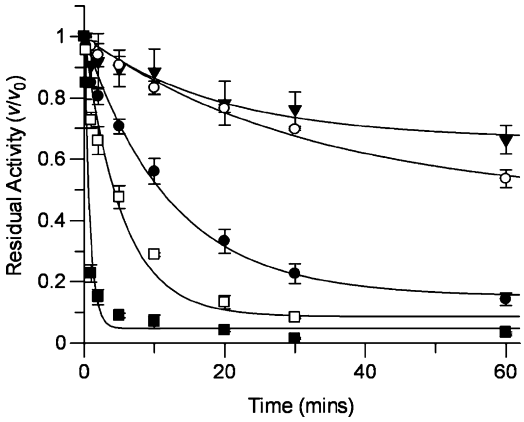

Figure 8. Time-dependent inhibition of DHDPS by bis(oxime-ester) **12**; ▼ = 0.05 mM, ○ = 0.1 mM, ● = 0.5 mM, □ = 1.0 mM, ■ = 2.5 mM.

The decay curves observed for inhibition of DHDPS by compounds **8** (Fig. 7), **11** (data not shown) and **12** (Fig. 8) are indicative of either slow or slow-tight binding, where there is slow-onset or time-dependent inhibition. Morrison and Walsh have defined the nature of slow or slow-tight-binding inhibitors and determined by their very nature that they act as substrate analogues, binding competitively within the active site of the enzyme.[14] Binding at the active site was confirmed by substrate protection through co-incubation of DHDPS with inhibitor (**8** or **12**) and pyruvate, with little loss in DHDPS activity observed over time (Fig. 10).

The results demonstrate that the ketoacid **8** mimics the enzyme substrate pyruvate and binds to active site lysine. The introduction of the ester **9** removes all charge from the inhibitor and eliminates almost all activity. Interestingly, conversion of the ketone to an oxime substantially increased the inhibitory activity, even with the observed negative influence of ester groups in **12**. This may be due to the higher potential of the oxime to become protonated on the nitrogen, thereby activating the oxime to nucleophilic attack by the active site lysine. However, the oxime acid **11** is not highly active. This may be due to the carboxylate and adjacent oxime groups chelating sodium ions—a known phenomenon in co-ordination chemistry[15]—thereby dramatically altering the binding properties of the compound.

In summary, new constrained inhibitors of DHDPS have been identified and tested. Both the ketone **8** and oxime **12** act in a similar manner to pyruvate; binding with the active site lysine residue, as demonstrated by both competition studies with substrate and their mode of inhibition. Discovery of the bis-oxime ester **12** as a relatively potent inhibitor of DHDPS (compared with reported inhibitors) has validated the constrained acyclic-intermediate model as a potential inhibitor lead, in which the functionalities present and enforced extended conformation closely resemble the acyclic enzyme-bound intermediate. Further modifications of the aromatic ring are possible, which may result in improvements in activity and work continues in this area.

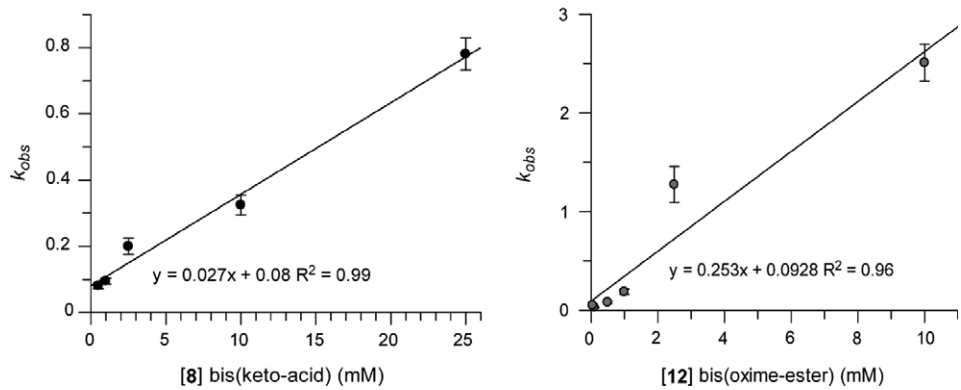

Figure 9. Plots of k_{obs} versus (**8**) and k_{obs} versus (**12**).

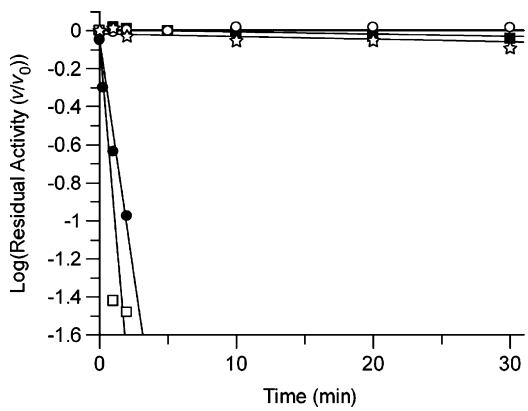

Figure 10. Substrate protection of DHDPS inactivation. ○ = 10 mM pyruvate; ■ = 25 mM **8** + 10 mM pyruvate; ☆ = 10 mM **12** + 10 mM pyruvate; ● = 25 mM **8**; □ = 10 mM **12**.

Acknowledgments

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Slow binding	$k_{obs} = k_4(1 + [I]/K_i^{app})$
Slow-tight binding	$k_{obs} = k_{6+}(k_5[I]/(K_i^{app} + [I]))$
Irreversible	$k_{obs} = k_5[I]/(K_i^{app} + [I])$

Plots of k_{obs} versus [I] were derived and the appropriate model of binding and K_i^{app} determined from the slope of the plot.
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