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Synthesis and evaluation of a mechanism-based inhibitor of a 3-deoxy-D-*arabino* heptulosonate 7-phosphate synthase

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Abstract—The first mechanism-based inhibitor of a 3-deoxy-D-*arabino* heptulosonate 7-phosphate (DAH7P) synthase has been synthesised in 12 steps from D-arabinose, and has been found to be a very slow binding inhibitor of *Escherichia coli* DAH7P synthase.

The shikimate pathway is responsible for the biosynthesis of a number of biologically important compounds, including the three aromatic amino acids, tyrosine, phenylalanine and tryptophan.[1] This pathway is present in plants and microorganisms, but absent in mammals, and consequently the pathway has received considerable interest as a potential target for herbicidal and antimicrobial agents.[2–4]

The first enzyme of this pathway, 3-deoxy-D-*arabino* heptulosonate 7-phosphate (DAH7P) synthase, catalyses the stereospecific aldol-like condensation between phosphoenolpyruvate **1** (PEP) and D-erythrose 4-phosphate **2** (E4P) giving rise to the seven carbon sugar DAH7P **5**, and inorganic phosphate. A number of labelling,[5] structural[6–12] and alternative substrate studies[13–15] have helped to elucidate the key mechanistic features of this reaction. These studies have shown that the reaction proceeds via cleavage of the phosphate C–O bond of PEP. This unusual bond cleavage of PEP is thought to occur by elimination of phosphate from the hemiketal phosphate intermediate **4**, which could be formed via a transient oxocarbenium ion **3** (Fig. 1).[9,12,15] This ion results from the nucleophilic attack of PEP C3 on the aldehyde carbonyl of E4P, which has been activated towards nucleophilic attack by coordination to the enzyme-bound metal co-factor. This mechanism is supported by modelling studies, which have demonstrated that the carbonyl of E4P can be appropriately placed for metal coordination.[12] However, there has been some debate over the nature of the enzyme mechanism, and an alternative hypothesis is that the reaction is initiated by attack of a metal-generated hydroxide ion at C2 of PEP, followed by attack of C3 of PEP at C1 of E4P, leading to the hemiketal phosphate **4** without the involvement of the oxocarbenium ion **3**.[12]

A similar aldol-like reaction is catalysed by the cell wall biosynthesis enzyme 3-deoxy-D-*manno* octulosonate 8-phosphate (KDO8P) synthase, and a number of structural,[16,17] biochemical[18–21] and bioinformatics[22,23] studies have shed light on the close relationship between these two enzymes. Both reactions involve the stereospecific attack of the *si* face of PEP on the *re* face of the

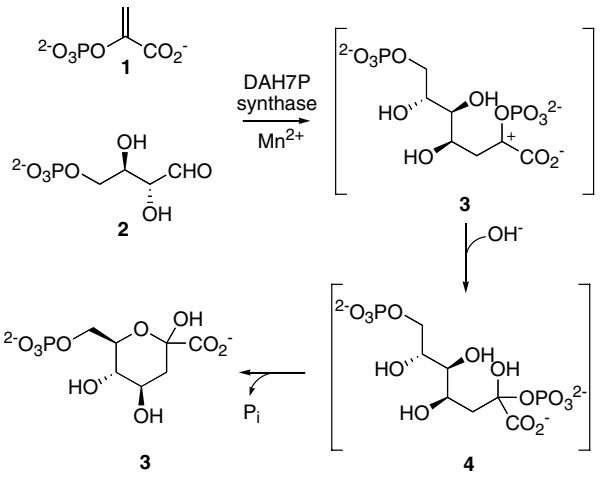

Figure 1. Reaction catalyzed by DAH7P synthase.

Keywords: DAHP; Shikimate; DAH7P; Transition-state inhibitor.
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aldehyde of an aldose phosphate (E4P for DAH7P synthase, arabinose 5-phosphate (A5P) for KDO8P synthase), and both reactions result in the cleavage of the C–O bond of the PEP phosphate group.[21,24,25] However, despite these similarities, there are apparent differences in the enzyme mechanisms. All known DAH7P synthases require a divalent metal ion as a co-factor, while both metallo- and nonmetallo-KDO8P synthases have been isolated and characterised.[26] Furthermore, the metallo-KDO8P synthases can be converted to their non-metalloenzyme counterparts by a single point mutation, while the corresponding point mutation in DAH7P synthase fails to produce active nonmetallo-DAH7P synthase.[15,27,28] In addition, KDO8P and DAH7P synthases differ in their response to the alternative substrates (*E*)- and (*Z*)-3-fluoroPEP, while DAH7P synthase processes both isomers equally well, KDO8P synthase displays a strong preference for the (*E*)-isomer.[29] Alternative substrate studies have also shown KDO8P synthase has little tolerance for change in stereochemistry at the C2 position of A5P, while DAH7P synthase is more accommodating of alternative stereochemistry at C2 of E4P.[15,20] These disparate findings have recently been explained by a new model for the reactivity of DAH7P and KDO8P synthases, which suggests while DAH7P synthases utilise Lewis acid activation of the E4P carbonyl (via metal–carbonyl coordination), KDO8P synthases employ protic acid catalysis (via protonation of the carbonyl of A5P).[15] So while DAH7P synthase and KDO8P synthase utilise different tactics for the activation of their aldose substrates, both enzyme mechanisms appear to produce an oxocarbenium ion.

While little has been published on the inhibition of DAH7P synthase,[30] several recent studies have identified inhibitors of KDO8P synthase enzymes. Among these, the inhibitors **5**[31] and **6**[32] are unique in that they have been designed to inhibit the enzyme based on their mimicry of the putative KDO8P synthase oxocarbenium ion **7** (Fig. 2).

The success of **6** and **7** as inhibitors for KDO8P synthase prompted us to design an analogous compound **8** for DAH7P synthase, in order to illuminate the mechanistic differences and similarities of these two important biosynthetic enzymes.

In order to synthesise **8**, we decided on a multi-step synthesis utilising D-arabinose as the source of chirality in **8**. This multi-step approach should allow the ability to synthesise a variety of analogues of **8** bearing differing functionality (Scheme 1).

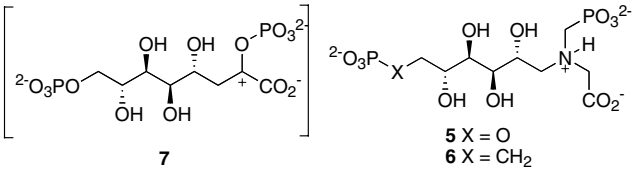


Figure 2. KDO8P synthase inhibitors **5** and **6** and putative transition-state **7** for the KDO8P synthase catalysed reaction.

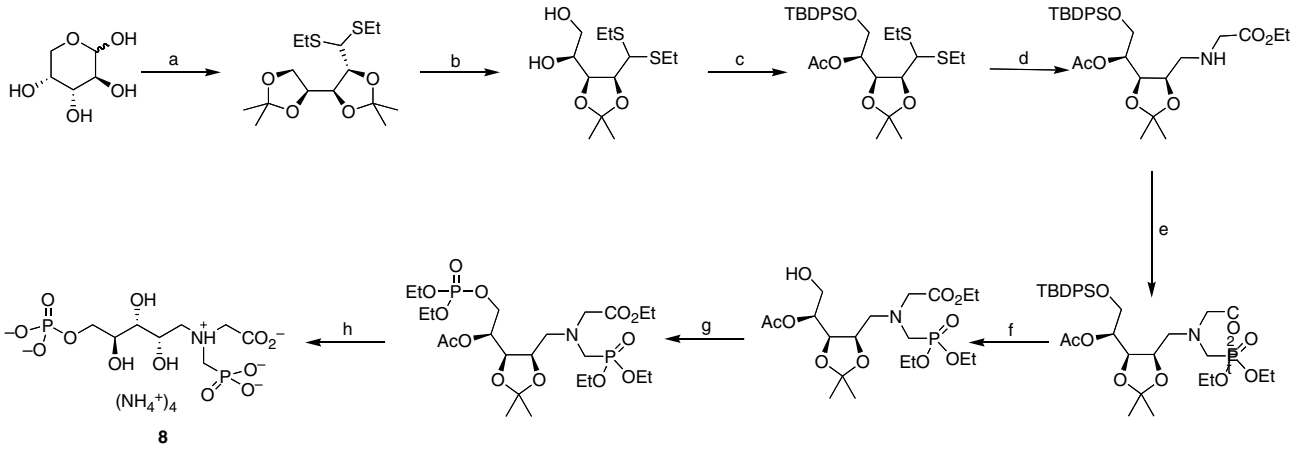
Treatment of D-arabinose with ethanethiol in 6 M hydrochloric acid gave the corresponding diethyl dithioacetal,[33] and treatment with acetone under conditions previously used for the corresponding trimethylene dithioacetal[34] selectively gave the kinetic 2,3:4,5-diisopropylidene acetal **10** in high yield. Cleavage of the terminal 4,5-isopropylidene acetal using conditions reported for the L-enantiomer[35] gave the diol **11**, which was protected at the 4 and 5 positions with acetyl and *tert*-butyldiphenylsilyl (TBDPS) protecting groups, respectively, in a high yielding one-pot procedure to provide **12**. The dithioacetal group was removed by treatment with mercuric chloride and mercuric oxide, and the resulting aldehyde was reductively aminated with ethyl glycinate (generated from the hydrochloride in situ) to give the secondary amine **13** in acceptable yield. Alkylation with diethyl phosphonomethyl triflate[36] gave the amino phosphonate **14** in 52% yield. Treatment of **14** with tetrabutylammonium fluoride produced alcohol **15** in high yield. This was phosphorylated with diethyl iodophosphate[37] to give the fully protected precursor **16**. Precursor **16** was deprotected to **8** by treatment with trimethylsilyl bromide and triethylamine, the resulting silyl esters hydrolysed with water and KOH and purified by anion-exchange chromatography to give **8** in 35% yield, an overall yield of 0.8% over 12 steps.

The inhibitory properties of **8** against *Escherichia coli* DAH7P synthase were evaluated by an assay, where the activity of the enzyme is followed by the decrease in absorbance at 232 nm, corresponding to the disappearance of the PEP enol phosphate moiety.[38] The inhibitor **8** was found to be a very slow binding inhibitor of *E. coli* DAH7P synthase. The rates of reaction of enzyme preincubated were found to go through a lag period before maximum velocity is reached. The same convex progress curve effect was observed by Du et al. in the inhibition of KDO8P synthase by **5**.[39] Both the length of lag time and the overall maximum rate reached were dependent on the length of time the inhibitor **8** had been preincubated with the enzyme (Fig. 3).

The slow rate of formation of the inhibitor–enzyme complex relative to substrate consumption precluded measurement of an inhibition constant by the method of Williams and Morrison,[40] as significant substrate consumption occurs before the effects of inhibition are seen. The slow binding behaviour of **8** is illustrated in Figure 3, which shows the progress curves for reactions initiated with enzyme preincubated with inhibitor **8**, and then diluted 500-fold into the assay cuvette after varying incubation times (10–80 min).

The time-dependent inhibition of *E. coli* DAH7P synthase by **8** was also found to be concentration-dependent with respect to **8** with an IC_{50} value of 6.6 μM.[41]

In summary, the first mechanism-based inhibitor of a DAH7P synthase has been designed and synthesised



Scheme 1. Synthesis of inhibitor **8**. Reagents and conditions: (a) 1—EtSH, aq HCl, (84%); 2—acetone, HCl (85%); (b) aq HCl, MeOH, 50 °C (55%); (c) 1—TBDPSCl, Im, MeCN; 2—Ac_2O, Py, DMAP, Δ, (79%, two steps); (d) 1—$HgCl_2$, HgO, aq acetone; 2—ethyl glycinate·HCl, NaOAc, 4 Å molecular sieves, $NaBH_3CN$, MeOH (31%, two steps); (e) diethyl phosphonomethyl triflate, K_2CO_3, MeCN (52%); (f) TBAF, THF (84%); (g) $P(OEt)_3$, I_2, CH_2Cl_2, then **15**, Py, CH_2Cl_2 (57%); (h) 1—TMSBr, NEt_3, MeCN, 4 °C; 2—H_2O, 75 °C; 3—aq KOH, 75 °C then anion-exchange chromatography (35%, three steps).

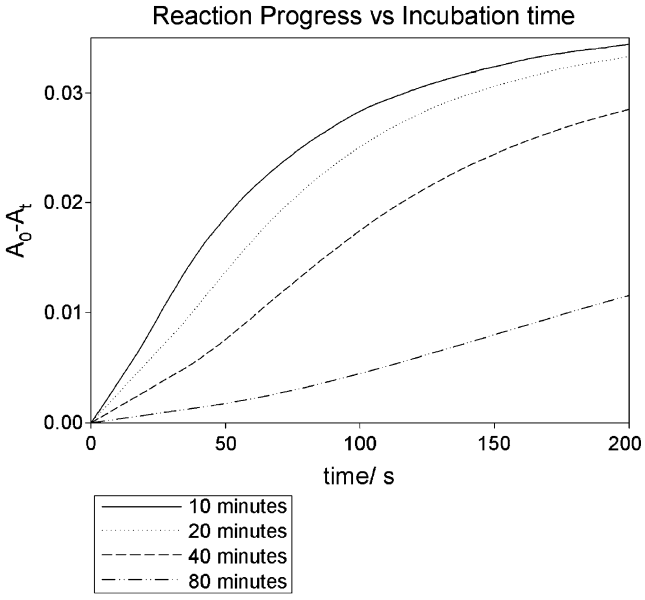


Figure 3. Inhibition of the *Escherichia coli* DAH7P synthase activity by **8**. A solution of enzyme and **8** (3.55 mM) was incubated for the length of time indicated, and then an aliquot was diluted 500-fold into assay mixture, containing E4P (8.0 μM), PEP (12.3 μM) and $MnSO_4$ (47.3 μM), in buffer (50 mM BTP, 10 μM EDTA, pH 6.8) and the absorbance was monitored at 232 nm.

in 12 steps from D-arabinose. This compound has been found to be a very slow binding inhibitor against *E. coli* DAH7P synthase. Additional studies are underway in our laboratory to characterise fully the interaction of **8** with DAH7P synthases from a variety of species.

Acknowledgment

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41. A solution of enzyme and varying concentrations of **8** (0–3.55 mM) was incubated for 40 min, and then an aliquot was diluted 500-fold into assay mixture, containing E4P (8.0 μM), PEP (12.3 μM) and $MnSO_4$ (47.3 μM), in buffer (50 mM BTP, 10 μM EDTA, pH 6.8) and the absorbance was monitored at 232 nm. The listed concentrations refer to inhibitor concentration in the final assay mixture. Initial linear rates were taken over the first 20 s and plotted to give IC_{50}.