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Investigating the role of the hydroxyl groups of substrate erythrose 4-phosphate in the reaction catalysed by the first enzyme of the shikimate pathway

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ABSTRACT

3-Deoxy-D-*arabino*-heptulosonate 7-phosphate (DAH7P) synthase catalyses the first step of the shikimate pathway, which is responsible for the biosynthesis of aromatic amino acids in microorganisms and plants. This enzyme catalyses an aldol reaction between phosphoenolpyruvate and D-erythrose 4-phosphate to generate DAH7P. Both 2-deoxyerythrose 4-phosphate and 3-deoxyerythrose 4-phosphate were synthesised and tested as alternative substrates for the enzyme. Both compounds were found to be substrates for the DAH7P synthases from *Escherichia coli*, *Pyrococcus furiosus* and *Mycobacterium tuberculosis*, consistent with an acyclic mechanism for the enzyme for which neither C2 nor C3 hydroxyl groups are required for catalysis. The enzymes all showed greater tolerance for the loss of the C2 hydroxyl group than the C3 hydroxyl group.

3-Deoxy-D-*arabino*-heptulosonate phosphate (DAH7P, **3**) synthase catalyses the stereospecific aldol-like condensation reaction between phosphoenolpyruvate (PEP, **1**) and D-erythrose 4-phosphate (E4P, **2**) to generate DAH7P (Fig. 1). This is the first enzyme-catalysed reaction of the shikimate pathway. The shikimate pathway is responsible for biosynthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan as well a range of important aromatic metabolites.[1] The pathway is present in plants and microorganisms yet absent in higher organisms. Thus, as the biosynthesis of aromatic compounds is required for a number of significant pathogens DAH7P synthase has been identified as a target for antimicrobial drug design.[2,3]

Two distinct types of DAH7P synthases have been reported and although these types share very little sequence identity there is close correspondence of their structures and active site architecture.[4,5] Type I enzymes are found in both bacteria and fungi. The type I enzymes can be further divided into two groups on the basis of sequence, denoted types Iα and Iβ; these two groups share approximately 30% identity. The type II enzymes were originally found in plants but are now known to be present in a number of bacteria including significant human pathogens *Mycobacterium tuberculosis*, *Helicobacter pylori* and *Pseudomonas aeruginosa*. All types and subtypes share a catalytic $(\beta/\alpha)_8$ barrel, although there are quite distinct differences in quaternary structure and extra-barrel extensions associated with different mechanisms of allosteric inhibition of the enzyme.[6–10]

Many key features of the reaction mechanism catalysed by DAH7P synthase have been elucidated.[7–9,11,12] It is known that the reaction proceeds with cleavage of the C—O bond of PEP. Labelling experiments have clearly shown that the *si*-face of PEP attacks the *re*-face of E4P to generate the new C—C bond of product DAH7P stereospecifically.[11–13] All DAH7P synthases require a divalent metal ion for catalytic activity. It is likely that this metal ion is responsible for coordinating the carbonyl functionality of E4P and the reaction is thought to be initiated by attack of C3 of PEP on the activated C1 of E4P (Fig. 2). The reaction can potentially take two alternative pathways following this C—C bond formation. The carbonium intermediate could be attacked by water to give a linear tetrahedral intermediate, which, following elimination of phosphate, is released from the enzyme as the acyclic form of DAH7P. Alternatively, the cation could be captured by the C3 hydroxyl group of E4P giving a cyclic tetrahedral intermediate and yielding the cyclic pyranose form of DAH7P directly. The α-pyranose form of DAH7P is the most predominant form of this compound in solution.[14] Current evidence favours the formation of the linear intermediate; this is based on the structure of the active site, which

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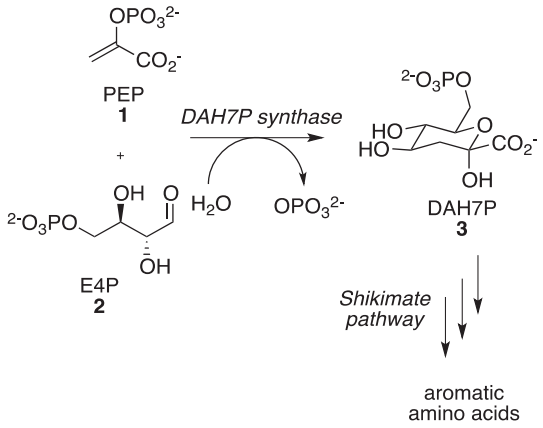


Figure 1. Reaction catalysed by DAH7P synthase.

would seem to support a linear intermediate, and on recent inhibition studies.[7,8,15] However, the role of a cyclic intermediate, even as part of product release or phosphate elimination has not been ruled out.

Our aim in these studies was to examine the roles that both the 2- and 3-hydroxyl groups play in both substrate binding and catalysis for each of the different types of DAH7P synthase. In order to do this, 2- and 3-deoxyE4P substrate analogues were synthesized and tested as alternative substrates for DAH7P synthases from different organisms.

The synthesis of 2-deoxyE4P in both enantiopure and racemic forms has been previously described.[16,17] For the synthesis of

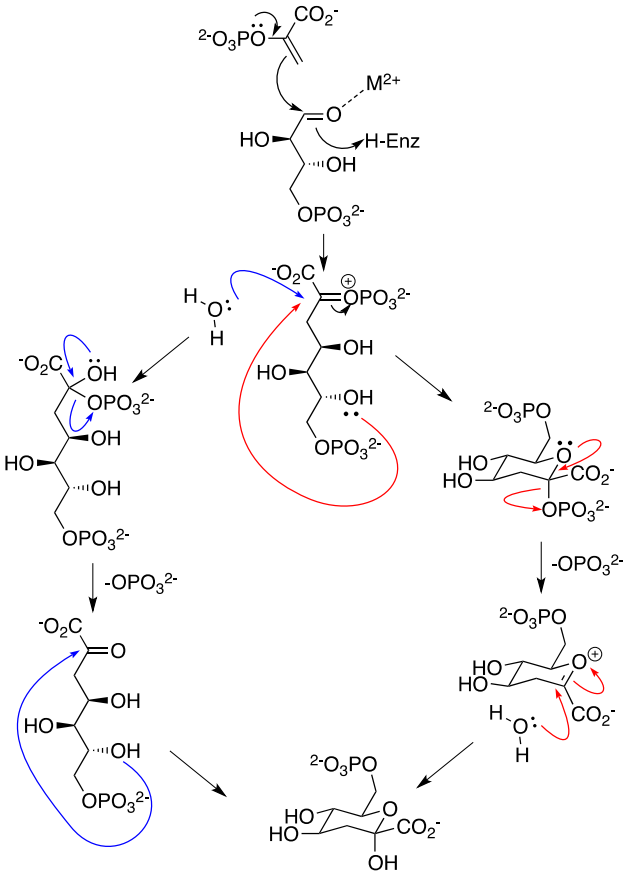
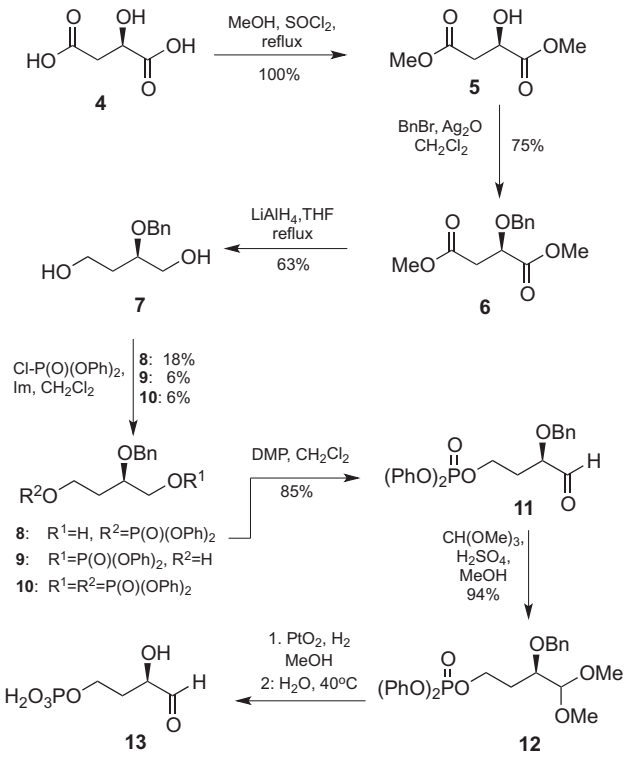


Figure 2. Acyclic (left) and cyclic (right) mechanisms for the formation of DAH7P.



Scheme 1. Synthesis of 3-deoxyE4P.

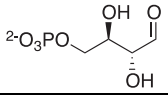
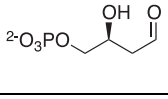
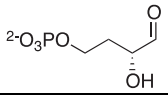
3-deoxy E4P (Scheme 1), (*R*)-malic acid **4** was first esterified to give its dimethyl ester **5**. The secondary hydroxy group was then protected as the benzyl ether, and the ester functionalities were reduced using $LiAlH_4$ to give diol **7**. Phosphorylation of this diol produced the isomeric monophosphorylated products **8** and **9** and the diphosphorylated compound **10** in a 3:1:1 ratio. A significant amount of unreacted starting material was also recovered (24%). The best yields of the desired monophosphorylated product **8** were obtained using 0.9 equiv of the phosphorylating reagent. Oxidation of phosphate **8** using Dess–Martin periodinane (DMP) gave aldehyde **11**. The aldehyde functionality was protected as its dimethyl acetal prior to hydrogenolysis. Final deprotection gave (*R*)-3-deoxyE4P **13**. Racemic (*RS*)-3-deoxyE4P was also synthesised using the same route starting with DL-malic acid.

Both 2-deoxyE4P and 3-deoxyE4P were tested as alternative substrates for DAH7P synthases representing each of the three different types and subtypes. Specifically, DAH7P synthases from *Escherichia coli* (type Iα), the hyperthermophilic archeon *Pyrococcus furiosus* (type Iβ), and the organism responsible for tuberculosis, *M. tuberculosis* (type II) were employed. All these enzymes have been both structurally and functionally characterised.[9,18–20] Both 2- and 3-deoxyE4P analogues were substrates for all three enzymes (Table 1).

Apart from (*S*)-2-deoxyE4P, which intriguingly was a considerably better substrate for the *P. furiosus* enzyme than the natural substrate E4P, the deoxyE4P analogues were generally found to be poorer substrates than E4P. Catalysis by *M. tuberculosis* DAH7P synthase was only marginally impaired when 2-deoxyE4P was used, but the rate of reaction catalysed by the *E. coli* enzyme was quite significantly impaired, substantially due to a significantly higher K_M value for this alternative substrate.

(*R*)-3-DeoxyE4P was a substrate for all the enzymes, ruling out an essential role for the 3-hydroxyl group in the mechanism of this enzyme, and an obligate cyclic mechanism for the DAH7P synthase (Fig. 2). This result is entirely consistent with the active-site

Table 1
Kinetic parameters of E4P, 2-deoxy and 3-deoxyE4P with DAH7P synthases from different organisms, representing each of the different types and subtypes of DAH7P synthase

DAH7P synthase source (and type)[a]		E4P 	(*S*)-2-DeoxyE4P 	(*R*)-3-DeoxyE4P 
E. coli (type Iα)	K_M (μM)	39 ± 4	410 ± 40	2700 ± 140
	k_{cat} (s^{-1})	26 ± 2	25 ± 3	4.5 ± 0.1
	k_{cat}/K_M^{E4P} ($s^{-1}\mu M^{-1}$)	0.67	0.06	0.002
P. furiosus (type Iβ)	K_M (μM)	9 ± 1	6 ± 1	200 ± 30
	k_{cat} (s^{-1})	1.4 ± 0.1	3.0 ± 0.1	2.1 ± 0.1
	k_{cat}/K_M^{E4P} ($s^{-1}\mu M^{-1}$)	0.16	0.5	0.011
M. tuberculosis (type II)	K_M (μM)	37 ± 2	46 ± 5	77 ± 10
	k_{cat} (s^{-1})	5.4 ± 0.1	4.9 ± 0.2	2.4 ± 0.1
	k_{cat}/K_M^{E4P} ($s^{-1}\mu M^{-1}$)	0.15	0.11	0.031

[a] DAH7P synthases from all sources were assayed in accordance with previously reported procedures as described in the Supplementary data the presence of Mn^{2+}. The *E. coli* enzyme used in these studies is the phenylalanine-sensitive isozyme.[21]

architecture of the enzyme and very recent studies indicating that a linear tetrahedral intermediate is an efficient inhibitor of the *M. tuberculosis* enzyme.[15]

Whereas (*R*)-3-deoxyE4P was a substrate for all three enzymes, this compound was found to be a substantially poorer substrate than both (*S*)-2-deoxyE4P and D-E4P in all cases. This was particularly apparent for the type Iα *E. coli* DAH7P synthase, for which (*R*)-3-deoxyE4P is a very poor substrate exhibiting a high Michaelis constant and a specificity constant over 300 times smaller than that determined for the natural substrate, E4P. On the other hand for *M. tuberculosis* DAH7P synthase, a corresponding diminution in specificity constant of only five-fold was observed.

Modelling of E4P into the active site of DAH7P synthase provides some insight into why the C3 hydroxyl of E4P is more important to substrate binding and catalysis than the C2 hydroxyl group (Fig. 3). The essential divalent metal found in the active site of DAH7P synthase is coordinated by four residues, Cys, Glu, His and Asp. These residues are absolutely conserved in all DAH7P synthases types. Modelling of E4P into the active site of the enzyme indicates that the metal-coordinating Asp residue is also predicted to hydrogen bond to the C3 hydroxyl. Removal therefore of this hydroxyl group not only removes a key binding contact of the aldehydic substrate but also would be expected to alter the reactivity of the metal centre, and hence the activation of the carbonyl group.

In *P. furiosus* and *E. coli* DAH7PS the C2 hydroxyl appears to make a hydrogen bond to an active-site Arg in the PEP binding site (Arg115 *P. furiosus* DAH7PS numbering). In the *E. coli* DAH7P synthase there is also a likely hydrogen-bonding contact between the C2 hydroxyl group and the main-chain carbonyl oxygen of the conserved proline in the E4P binding pocket, whereas in the *P. furiosus* DAH7PS structure this proline (in the absence of E4P) is observed to be disordered. The absence of this interaction may account for the observation that (*S*)-2-deoxyE4P is a relatively poor substrate for the *E. coli* enzyme.

The racemic forms of the deoxy substrate analogues were also tested on the enzymes. Both the *E. coli* and *M. tuberculosis* enzymes utilised only half of the total concentration of the racemic 2-deoxyE4P mixture, indicating that only (3*S*)-2-deoxyE4P, with the correct C3 stereochemistry, was indeed a substrate for these enzymes. In marked contrast, the *P. furiosus* enzyme was far more promiscuous, with both enantiomers of 2-deoxyE4P acting as alternative substrates. However, the rates of reaction were not identical, and clear kinetic resolution of the mixture was observed through reaction with the enzyme, as indicated by the initial fast use of the (3*S*)-2-deoxyE4P enantiomer, followed by significantly slower consumption of (3*R*)-2-deoxyE4P (Fig. 4).

In contrast to the promiscuity of the *P. furiosus* DAH7P synthase towards 2-deoxyE4P substrates, racemic 3-deoxyE4P was not a substrate for the enzyme, consistent with significant inhibition of this enzyme and nonproductive binding with (*S*)-3-deoxyE4P preventing productive binding by (*R*)-3-deoxyE4P. This result was unexpected given the tolerance of this enzyme to the loss of the C2 hydroxyl group. Even more surprisingly, we observed that *E. coli* DAH7P synthase was able to tolerate variation in the configuration at C3 with the racemic 3-deoxyE4P substrate. Indeed, 100% utilisation of the racemic mixture was observed and the K_M determined

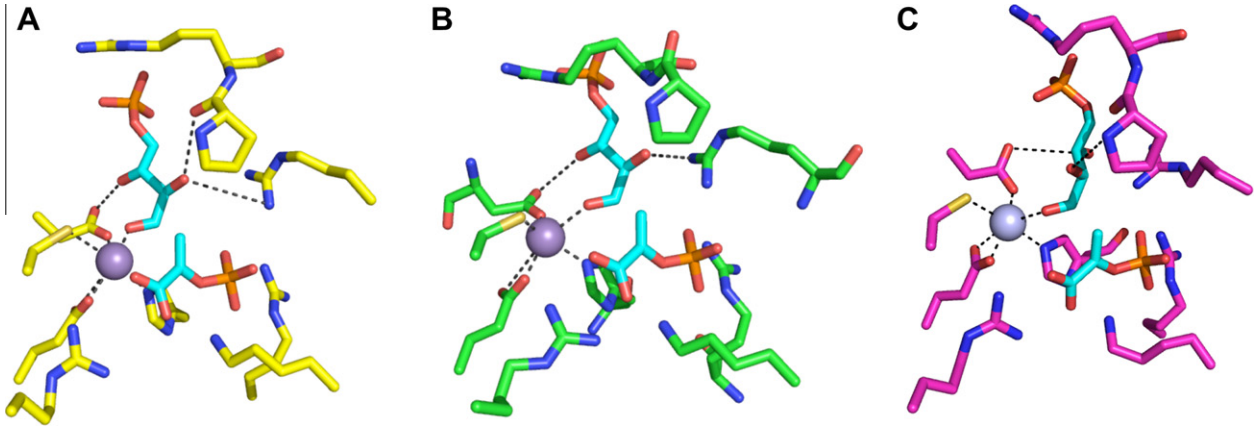


Figure 3. Active site of DAH7P synthase with E4P modelled into position so as to interact with the metal ion and expose the *re*-face of the aldehyde moiety to PEP. (A) *E. coli* DAH7P synthase, (B) *P. furiosus* DAH7P synthase and (C) *M. tuberculosis* DAH7P synthase.

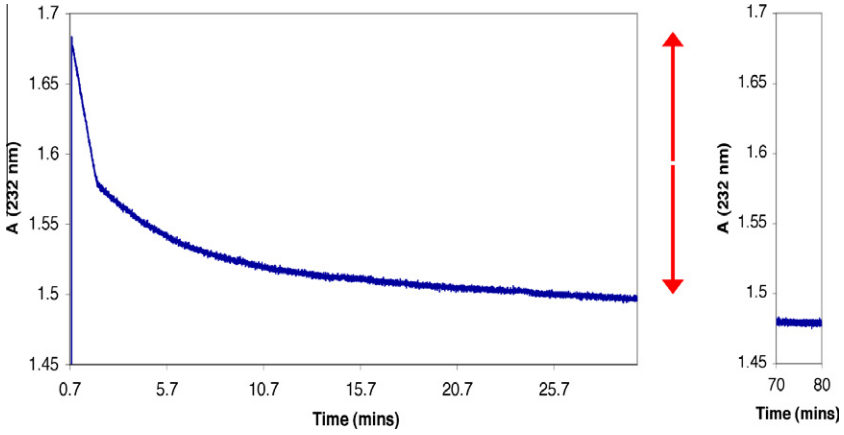


Figure 4. Reaction of racemic (*RS*)-2-deoxyE4P catalysed by *P. furiosus* DAH7P synthase, showing initial fast rate corresponding to the use of (3*S*)-2-deoxyE4P (equivalent to the rate of reaction in the presence of the enantiopure (3*S*)-2-deoxyE4P) followed by slow reaction rate due to consumption of the (3*R*)-2-deoxyE4P. The reaction progress is followed by the loss of PEP at 232 nm as described in the Supplementary data.

for this mixture was virtually unchanged (2600 ± 330 μM) – although, it should be noted that K_M for (*S*)-2-deoxyE4P and (*R*)-3-deoxyE4P are very much larger compared to E4P for the *E. coli* DAH7P synthase than for the *M. tuberculosis* and *P. furiosus* DAH7P synthases. In contrast the type II *M. tuberculosis* DAH7P synthase was intolerant to variation of configuration at C3, with the racemic 3-deoxyE4P substrate utilising only 50% of the racemic mixture, presumably the (*R*)-3-deoxyE4P based on the enantiopure results. The conformational flexibility that allows both enantiomers of 2-deoxyE4P to be substrates for *P. furiosus* DAH7PS allows the (*S*) enantiomer of 3-deoxyE4P to bind in a manner that precludes the binding and reaction observed for enantiopure (*R*)-3-deoxyE4P.

In summary, both 2- and 3-deoxy variants of E4P were found to be substrates for both types I and II DAH7P synthases. This indicates that whereas the hydroxyl groups, and in particular the C3 hydroxyl group of this aldehydic substrate support efficient reaction, they are not essential for DAH7P synthase catalysis. The observation that 3-deoxyE4P acts as an alternative substrate strongly supports an acyclic reaction mechanism for the generation of DAH7P by this enzyme.

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Supplementary data

Supplementary data associated (details of synthetic procedures for the preparation of 3-deoxyE4P and the kinetic assays) with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.09.017.

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