On The Mechanism Of *Dehydroquinate synthase*

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by

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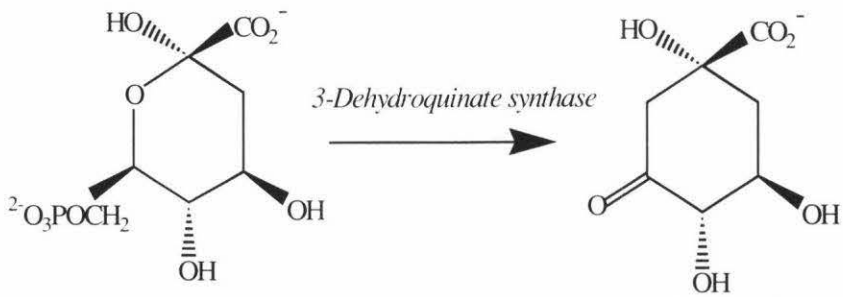
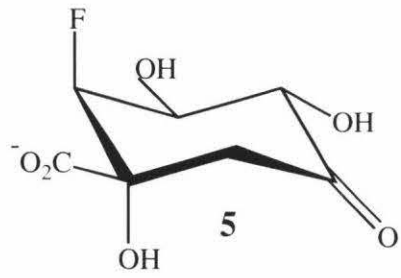
ABSTRACT

The aim of this thesis is to investigate the influence of fluorine substitution on the second reaction of the shikimate pathway catalysed by the enzyme 3-dehydroquinate synthase. The shikimate pathway is an essential pathway that is required for the synthesis of aromatic compounds in bacteria, microbial eukaryotes and plants. The enzyme, 3-dehydroquinate synthase, catalyses the second step of the shikimate pathway, the conversion of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) into 3-dehydroquinate (DHQ).

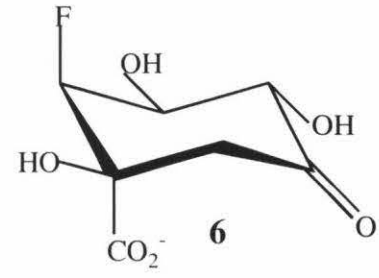


DAHP 3-dehydroquinate

It has been reported that when (3*S*)-3-fluoro DAHP (where fluorine occupies the C3 axial position) is treated with the enzyme 3-dehydroquinate synthase, two products, the expected (6*S*)-6-fluorodehydroquinate (**5**) and its C1 epimer, (6*S*)-6-fluoro-1-*epi*deydroquinate (**6**) are formed in a ratio of 2 : 1.



(6*S*)-6-fluorodehydroquinate



(6*S*)-6-fluoro-1-*epi*dehydroquinate

The C1 epimer of 3-dehydroquinate was reported to be formed from the natural substrate DAHP in a solution reaction, but not in the enzyme catalysed reaction. Therefore, it has been suggested that fluorine substitution at the axial position on C3 stabilises the fluoroenolpyranose intermediate allowing the intermediate to dissociate from the enzyme and cyclise to complete the formation of (6*S*)-6-fluoro-1-*epi*deydroquinate free in solution. The results reported in this thesis are from an investigation carried out to understand further the influence of fluorine orientation on the stereochemical outcome of the products in the dehydroquinate synthase reaction.

(3*S*)-3-Fluoro DAHP was synthesised in large amounts using both chemical and enzymatic synthesis. This was achieved by treating the isomers of 3-fluoro phosphoenolpyruvate and D-erythrose 4-phosphate with DAHP synthase, the first enzyme of the shikimate pathway. The erythrose 4-phosphate was prepared by lead tetraacetate oxidation of D-glucose 6-phosphate. The isomers of 3-fluoro phosphoenolpyruvate were prepared from 3-bromo, 3-fluoropyruvic acid by the Perkow reaction. Then (3*S*)-3-fluoro DAHP was purified by anion exchange chromatography. The chemical synthesis of erythrose 4-phosphate and the isomers of 3-fluoro phosphoenolpyruvate and the enzymatic synthesis of (3*S*)-3-fluoro DAHP and its purification are discussed in Chapter Two.

A recombinant *Escherichia coli strain* (pJB 14) was used to over-express the enzyme dehydroquinate synthase, and partial purification of the enzyme was achieved by anion exchange chromatography. Chapter Three describes the production and purification of the enzyme 3-dehydroquinate synthase.

Purified (3*S*)-3-fluoro DAHP was treated with the *E. coli* enzyme 3-dehydroquinate synthase. Formation of both (6*S*)-6-fluorodehydroquinate and its C1 epimer was observed. The reaction was followed at different pH and temperature values. The ratio of products produced in the enzyme-catalysed reaction was monitored by ^{19}F NMR spectroscopy. No significant change in the ratios was observed with the different conditions employed. The results from these experiments are discussed in Chapter Four. Our results are consistent with the hypothesis that the fluoroenolpyranose intermediate is released to the solution, where it cyclises without the constraint of an enzymatic template. To test this hypothesis unequivocally, further investigations are required and these are discussed in Future Directions.

Abbreviations

A5P	arabinose 5-phosphate
ADP/ATP	adenosine di/tri-phosphate
Bis-acrylamide	N,N'-methylene-bis-acrylamide
BTP	1,3-(tris(hydroxymethyl)-methylamino)propane
DAHP	3-deoxy-D-*arabino*-heptulosonate-7-phosphate
DAHP synthase(phe)	3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (phenylalanine sensitive)
DHQ	3-dehydroquinate
DHQ synthase	3-dehydroquinate synthase
E4P	D-erythrose 4-phosphate
HPLC	high performance liquid chromatography
IPTG	isopropyl-D-thiogalactoside
NAD^+	nicotinamide adenine dinucleotide
NAD(P)H	nicotinamide adenine dinucleotide(phosphate)(reduced form)
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PEP	phosphoenolpyruvate
ppm	parts per million
R5P	D-ribose 5-phosphate
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylene diamine
THF	tetrahydrofuran
Tris	tris(hydroxymethyl)aminomethane
tlc	thin layer chromatography
UV	ultra-violet
V_{max}	maximum velocity

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1 Introduction

1.1 The Shikimate Pathway

Organisms differ markedly in their ability to carry out the chemical reactions involved in the biosynthesis of the amino acids, which are the constituents of proteins and peptides. Amongst the amino acids, which cannot be produced by '*de novo*' synthesis in animals are the three aromatic amino acids L-phenylalanine, L-tyrosine and L-tryptophan. In plants and microorganisms the shikimate pathway (**Figure 1.1**) is responsible for the production of these aromatic amino acids, and other primary and secondary aromatic metabolites from ubiquinone to morphine. In a sequence of seven metabolic steps, phosphoenolpyruvate and erythrose 4-phosphate are converted to chorismate, the precursor of the aromatic amino acids and many aromatic metabolites such as anthranilate, *para*-hydroxybenzoate, and *para*-aminobenzoate.[1] This pathway is found in plants (where it can account for 20-30% of the carbon flux), fungi, protozoa, bacteria and recently been detected in several apicomplexan parasites.[21] This pathway is the target of the very successful broad-spectrum herbicide, glyphosate, the active ingredient of Roundup^{R} which inhibits 5-enolpyruvylshikimate-3-phosphate synthase, the sixth enzyme of the pathway.[10]

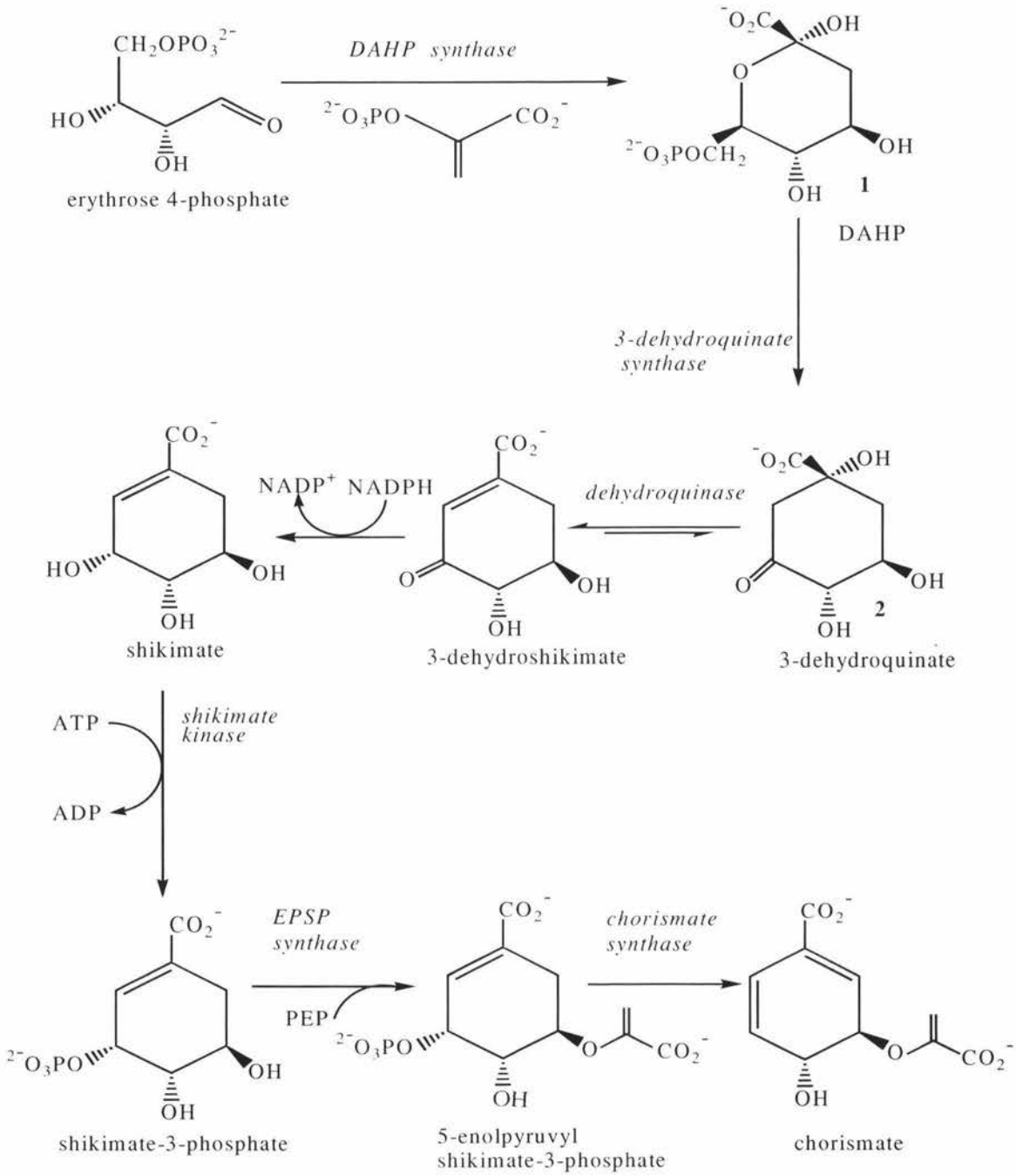


Figure 1.1 The Shikimate Pathway.[28]

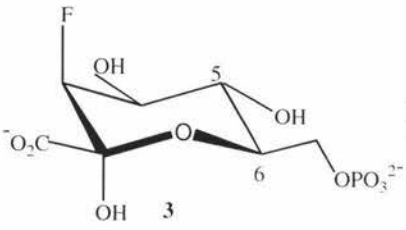
The second enzyme of the pathway, 3-dehydroquinate synthase (formerly named as 7-phospho-3-deoxy-D-*arabino*-heptulosonate phosphate lyase) catalyses the conversion of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (**1**, DAHP) into 3-dehydroquinate (**2**, DHQ), the first carbocyclic metabolite in the pathway (**Figure 1.1**).

As a key step in the shikimate pathway, DHQ synthase is a potential target for new antifungal and antibacterial drugs. The suitability of DHQ synthase as a target is supported by the finding that inactivation of DHQ synthase in *Salmonella typhimurium* results in a strain attenuated for virulence in BALB/c mice.[18] Similar effects on virulence may occur if appropriate chemical compounds could inhibit DHQ synthase. Inhibitors of DHQ synthase activity may therefore have therapeutic value. The rational development of DHQ synthase inhibitors demands a detailed characterisation of its catalytic mechanism. Detailed studies have already revealed much of the catalytic mechanism and are outlined in Section 1.3.

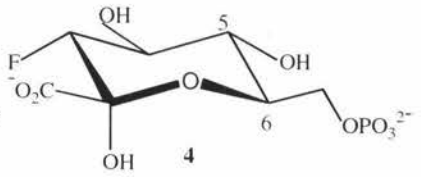
1.2 The aim of the studies

DAHP is the natural substrate for 3-dehydroquinate synthase. The isomers (3*S*)- and (3*R*)-3-fluoro DAHP (**3, 4**) have also been shown to act as substrates for *Escherichia coli* 3-dehydroquinate synthase.[28] It has been reported that the enzyme processes these isomers differently.[28] With the (3*R*)-isomer (**4**, fluorine in equatorial position), the expected product (6*R*)-6-fluorodehydroquinate (**7**) is formed, but with the (3*S*)-isomer (**3**, fluorine in axial position), two products, the expected (6*S*)-6-fluorodehydroquinate (**5**) and (6*S*)- 6-fluoro-1-*epi*dehydroquinate (**6**) are formed in a 2 : 1 ratio. This observation raises the question: why is there a difference in the outcome of the reaction of (3*S*)-3-fluoro DAHP and its (3*R*)-isomer in the reaction catalysed by DHQ synthase?

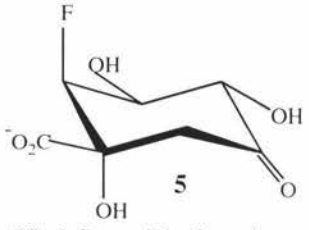
The objectives of the present study were to investigate the influence of the fluorine orientation on the stereochemical outcome of the process and determine the role of the enzyme DHQ synthase in guiding the formation of the correct epimeric product.



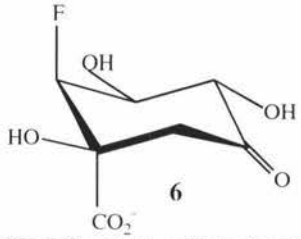
(3*S*)-3-fluoroDAHP



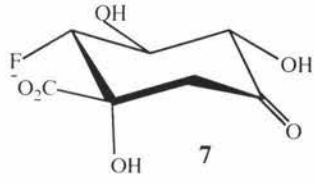
(3*R*)-3-fluoroDAHP



(6*S*)-6-fluorodehydroquinate



(6*S*)-6-fluoro-1-*epi*dehydroquinate



(6*R*)-6-fluorodehydroquinate

1.3 The mechanism of 3-dehydroquinate synthase

1.3.1 Proposed mechanism for the conversion of DAHP into 3-dehydroquinate

E. coli DHQ synthase is a monomeric protein of 362 amino acid residues that requires, for catalytic activity, the presence of both a divalent metal cation and NAD^+, a redox cofactor.[22] Although the enzyme requires NAD^+ for catalytic activity no overall oxidation occurs during the conversion. Sprinson[33] and colleagues proposed an interesting and ingenious mechanistic pathway for the conversion of DAHP to 3-dehydroquinate (**Figure 1.2**). A similar mechanism was later found for the 2-deoxy-scyllo-inosose synthase of *Streptomyces fradiae*, an enzyme involved in antibiotic biosynthesis.[36]

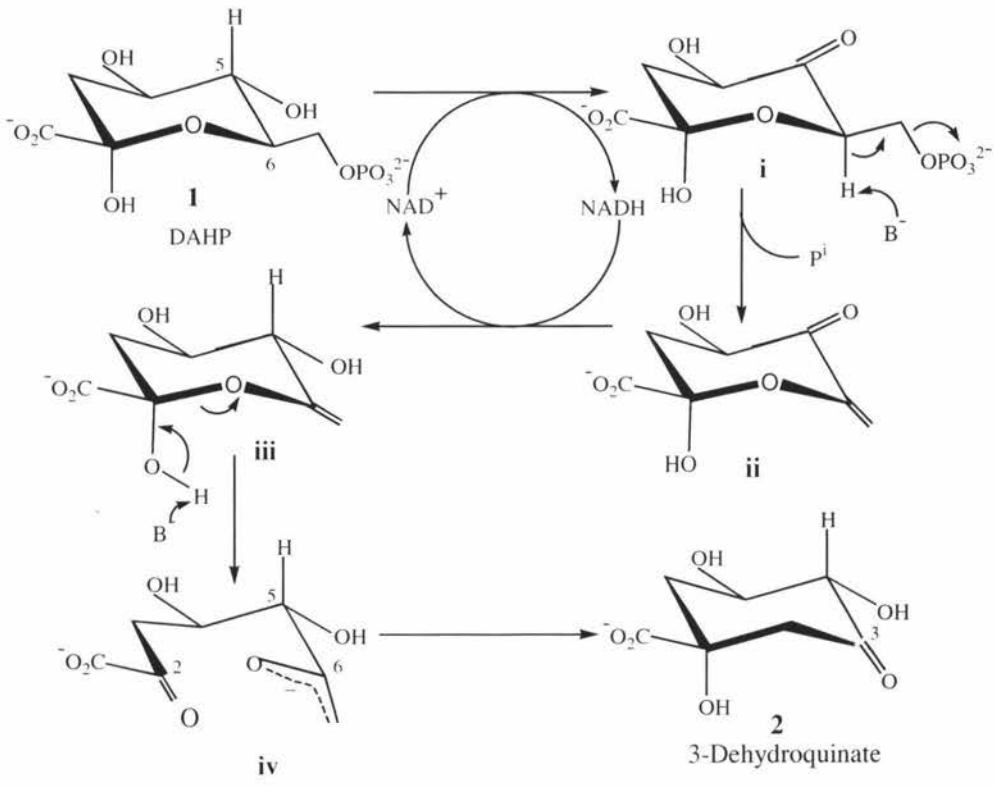


Figure 1.2 Proposed mechanism for the conversion of DAHP into 3-dehydroquinate.

In the first step of the mechanism, the substrate is oxidised at C5 of DAHP and NAD^+ is reduced. This involves hydride transfer from C5 of DAHP to the NAD^+ nicotinamide moiety. This increases the acidity of C6 hydrogen of DAHP, which facilitates the β-elimination of the phosphate group. The second step is β-elimination of the phosphate group. During phosphate elimination, a proton is removed from C6 of DAHP. In the third step, the C5 ketone of intermediate (**ii**) is reduced by NADH. This is the reversal of the first step, with hydride ion from NADH being transferred back to C5 of the substrate, generating the enolpyranose (**iii**) with unchanged stereochemistry of the C5 hydroxyl group. This enolpyranose is a key intermediate in the overall transformation. The final two steps of the reaction are ring opening followed by an intramolecular aldol condensation. The ring opening is followed by a rotation about the C5 – C6 bond and an intramolecular aldol condensation to form the carbocycle 3-dehydroquinate.

The complexity of the mechanism has raised the question of how oxidation and reduction, phosphate cleavage, ring opening and aldol ring closure reactions could be catalysed by a simple monomeric enzyme. Results of studies by Knowles[22] and Bender *et al.*[4, 5] and Bartlett *et al.*[2, 3] however, validated the mechanism and appeared to reduce the role of the enzyme to little more than that of an oxidoreductase.

1.3.2 Previous work done on the validation of the proposed mechanism

The difficulty in studying the mechanism of DHQ synthase with the natural substrate is that the reaction appears to be irreversible, and the enzyme-bound intermediates cannot be examined under equilibrium conditions. However substrate analogues have long been considered as valuable tools for probing enzyme mechanisms and enzyme substrate interactions. Knowles[4, 5, 22] and his group synthesised different substrate analogues of DAHP that can partially undergo the various stages along the reaction pathway illustrated in **Figure 1.2**. Evidence for the involvement of NAD^+ in the catalytic pathway comes from the production of a species absorbing at 340 nm (where NADH absorbs) when the carbahomophosphonate analogue of DAHP (**8, Figure 1.3**) was incubated with the enzyme.[4] This analogue cannot undergo phosphate elimination and therefore can only undergo the first oxidation step of the catalytic mechanism.

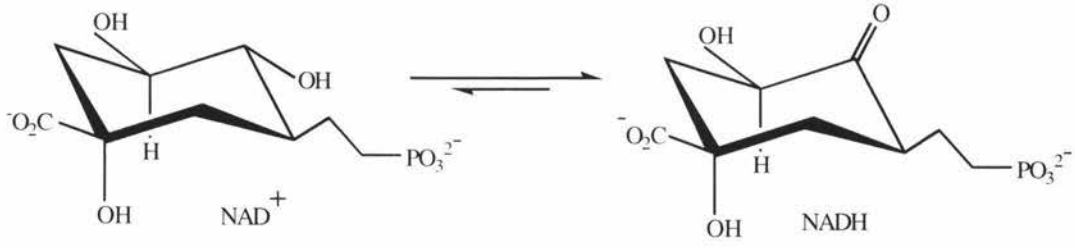


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Figure 1.3 Action of DHQ synthase on carbahomophosphonate.

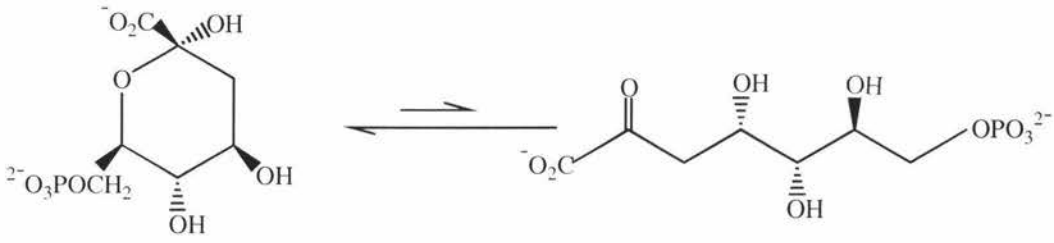


Figure 1.4 The cyclic and acyclic forms of DAHP.

It has been noted that, DAHP exists in equilibrium between cyclic and acyclic forms (**Figure 1.4**) and the cyclic form is found to be predominant.[28] The cyclic form of DAHP was proposed to be the substrate for DHQ synthase, when it was shown that the cyclic 2-deoxy analogue of DAHP (**9**, **Figure 1.5**) was accepted as a substrate by dehydroquinate synthase, but the acyclic 2-deoxy-substrate analogue was not.[4] Although **9** was accepted as a substrate for DHQ synthase, it cannot undergo the whole sequence of reactions illustrated in **Figure 1.2**. Without the hydroxyl group at C2, this analogue cannot undergo the ring opening from pyranose to open-chain ketose. When the cyclic 2-deoxy substrate analogue **9** was incubated with the enzyme, inorganic phosphate and the enol ether **10** were produced catalytically. This result shows that the enzyme will release intermediate analogues into solution, even those that have not undergone the last two suggested steps in the mechanism (**Figure 1.2**) and establishes the fact that ring opening does not occur until after the reduction step.

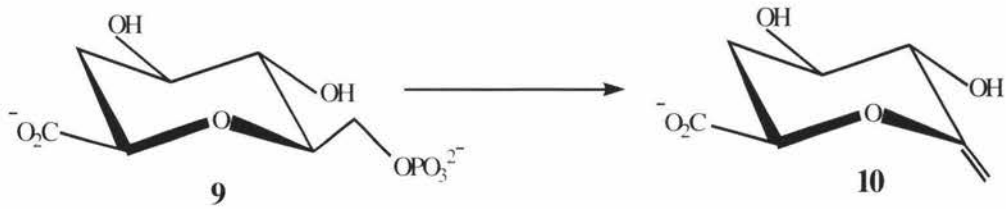
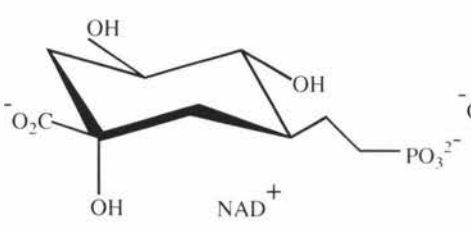


Figure 1.5 Action of DHQ synthase on 2-deoxy DAHP.

Stereospecifically labelled [7*S* - ^{2}H]-2-deoxy DAHP was used to show that the elimination proceeds with *syn* stereochemistry.[37] It was proposed that *syn* elimination of phosphate was most likely to proceed through an enolate intermediate by a stepwise E1cb mechanism. To probe this the substrate analogues **8, 11, 12** and **13,** were incubated with the enzyme in D_2O to examine if there was exchange of the C6 proton. Very slow exchange was observed for the carbahomophosphonate (**8**), but not the phosphonate (**11**) and similarly slow exchange was observed for the *cis*-vinyl homophosphonate (**12**), but not the *trans*-vinyl homophosphonate (**13**). This finding led to the suggestion that the phosphate dianion of the substrate can act as its own base to abstract the C6 proton.



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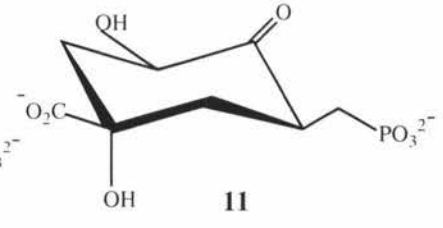

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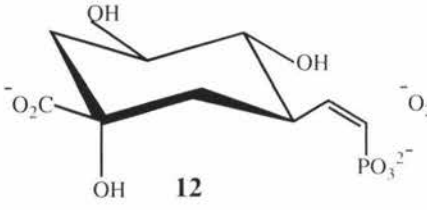

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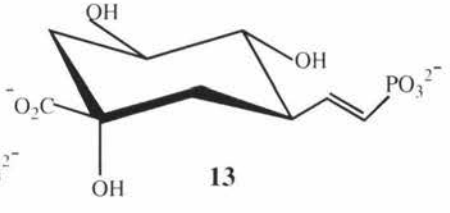

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From the studies with these substrate analogues it was established that the true substrate for the enzyme is apparently the pyranose or cyclic form of the DAHP and the β-elimination proceeds with *syn* stereochemistry from the cyclic α-pyranose form of the substrate. More information concerning the possible transition state for the aldol condensation was derived by considering the overall stereochemistry for the *syn* elimination. As already noted by Srinivasan *et al.*[33] during conversion of DAHP to DHQ, the C7 configuration of DAHP undergoes inversion. The two possible transition states that satisfy both these observations (*syn* elimination, and overall inversion) are a *chair*-like transition state and a *boat*-like transition state. However with the consideration of steric effects and of minimal motion the most favoured transition state is a *chair*-like transition state for the ring closure reaction.

It can be obtained from the α pyranose form of DAHP by a 180° rotation about the bond between C5 and C6. This also seems likely since the preferred conformation for DHQ is a *chair* structure. The C6 to C7 double bond adds to the *re* face of the carbonyl bond at C2 from above. The overall picture for the final two steps of the mechanism is shown in **Figure 1.6.**

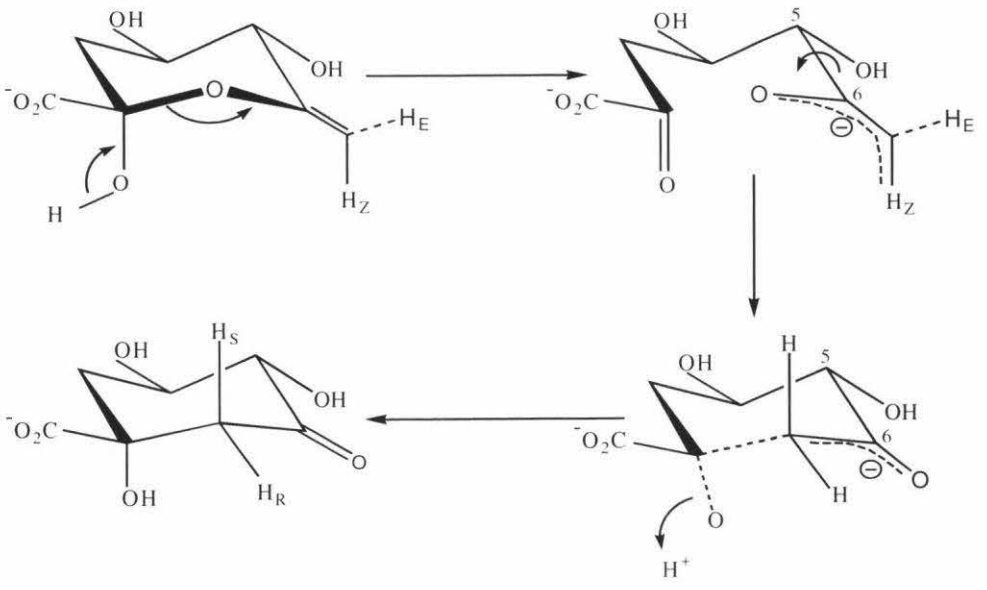


Figure 1.6 The overall picture for the final two steps of the DHQ synthase reaction.

To study the involvement of the enzyme in the final two steps of the reaction, Bartlett *et al.*[3] synthesised the *o*-nitrobenzyl protected enolpyranose intermediate **14** and showed that following the photochemical removal of the *o*-nitrobenzyl protecting group from **14**, the enolpyranose was completely converted into 3-dehydroquinate (**2**) in the absence of the enzyme (**Figure 1.7**). Studies with a deuterium-labelled form of precursor **14** were consistent with the cyclisation proceeding through the same *chair*-like transition state as had been suggested for the enzyme-catalysed aldol step.[3] These findings were taken to imply that the enzyme does not play any role in the ring opening and cyclisation steps and that enolpyranose is the true product of the DHQ synthase reaction. This result is also

consistent with the Knowles[4] earlier studies which showed that the enzyme will release intermediate analogues into solution, even those that have not undergone the last two suggested steps in the mechanism. With the consideration of Bartlett' s work on the final two steps of the proposed mechanism and his own work with the substrate analogues, Knowles described this enzyme as a "banal dehydrogenase" responsible only for the formation of the enolpyranose and stated that the enzyme is only actively involved in the oxidation and reduction steps and other steps take place spontaneously.[22] However, later studies have revealed the involvement of the enzyme in some of the other steps of the mechanism as described in the next section.

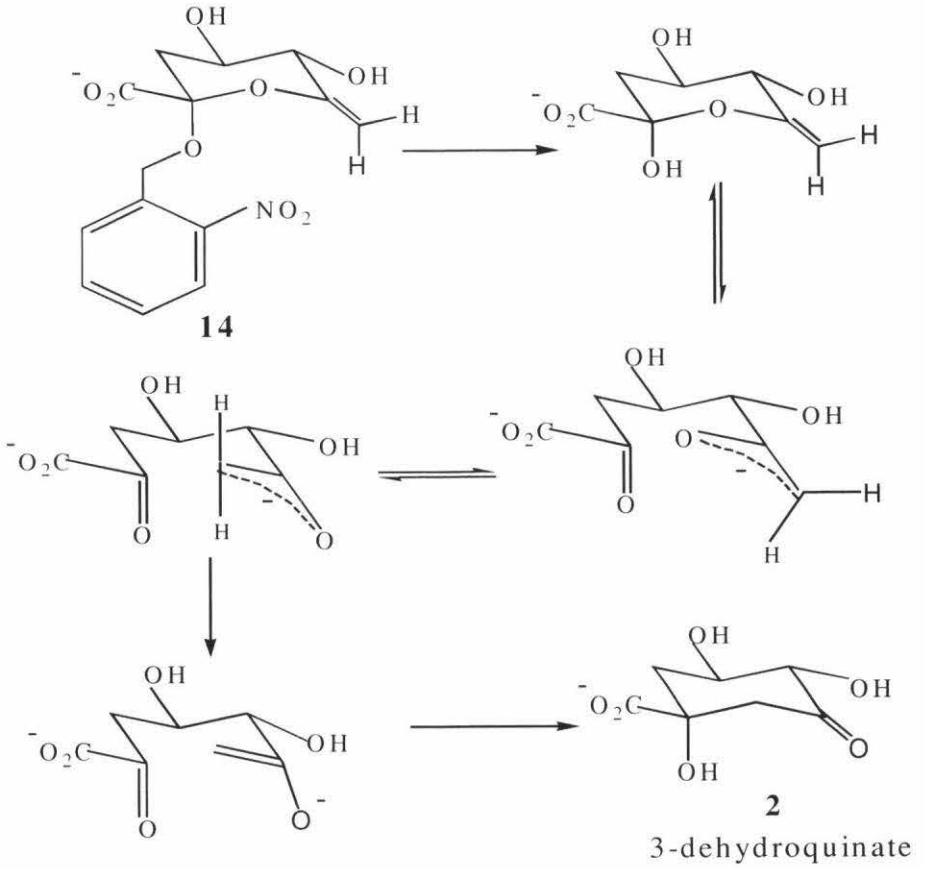
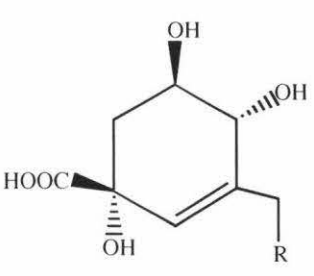


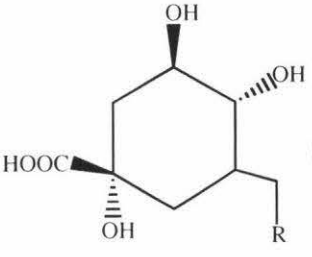
Figure 1.7 Formation of 3-dehydroquinate from the *o*-nitrobenzyl protected enolpyranose.

1.3.3 The studies that support the involvement of the enzyme in the phosphate elimination step and the final two steps of the mechanism

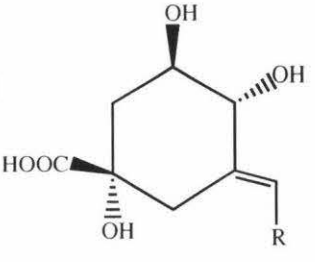
Montchamp and Frost[26] synthesised different cyclohexenyl (**15, 16, 17** and **18**) and cyclohexylidine (**23, 24**) inhibitors of DHQ synthase and measured their inhibition constants. When these values were compared with the inhibition constants of the corresponding cyclohexyl analogues (**19, 20, 21** and **22**), an increased inhibition for cyclohexenyl and cyclohexylidine analogues relative to the corresponding cyclohexyl analogues was observed with one exception. From the impact of strategically placed olefinic residues in inhibitors on active site binding, they suggested that the active site of DHQ synthase might not merely be a spectator during elimination of inorganic phosphate from intermediate **i** (**Figure 1.2**). It has been proposed that the active site might accelerate the elimination of inorganic phosphate by restricting the conformational flexibility of the phosphorylmethyl group of intermediate **i** or might stabilise the E1cb intermediate or E1cb-like transition state.[26]



15 R = OPO_3H_2
16 R = $CH(COOH)_2$
17 R = PO_3H_2
18 R = $CH_2PO_3H_2$



19 R = OPO_3H_2
20 R = $CH(COOH)_2$
21 R = PO_3H_2
22 R = $CH_2PO_3H_2$



23 R = PO_3H_2
24 R = $CH_2PO_3H_2$

Bartlett[2] reinvestigated the non-enzymatic generation of 3-dehydroquinate from *o*-nitrobenzyl protected enolpyranose intermediate **14**. From this reinvestigation, it was found that the reaction of the enolpyranose was not entirely stereospecific with between 2.5 and 4 % of 1-*epi*dehydroquinate **25** also being formed (**Figure 1.8**). By contrast the enzymatic reaction is entirely specific. Addition of excess enzyme did not effect the ratio of 3-dehydroquinate to 1-*epi*dehydroquinate formed, suggesting

that the rate of spontaneous rearrangement of the enolpyranose is faster than the uptake by the enzyme. Bartlett *et al.*[2] proposed that the 1-*epi*dehydroquinate was formed from attack of the enolate on the *si* face of the carbonyl in a solution reaction that passes through a similar *chair*-like transition state as that proposed in the reaction to form 3-dehydroquinate. 1-*Epi*dehydroquinate is not observed in the enzymatic reaction. Therefore, Bartlett *et al.*[2] stated that the enzyme is acting as a template in guiding the conversion of enolpyranose to 3-dehydroquinate with the correct stereochemistry.

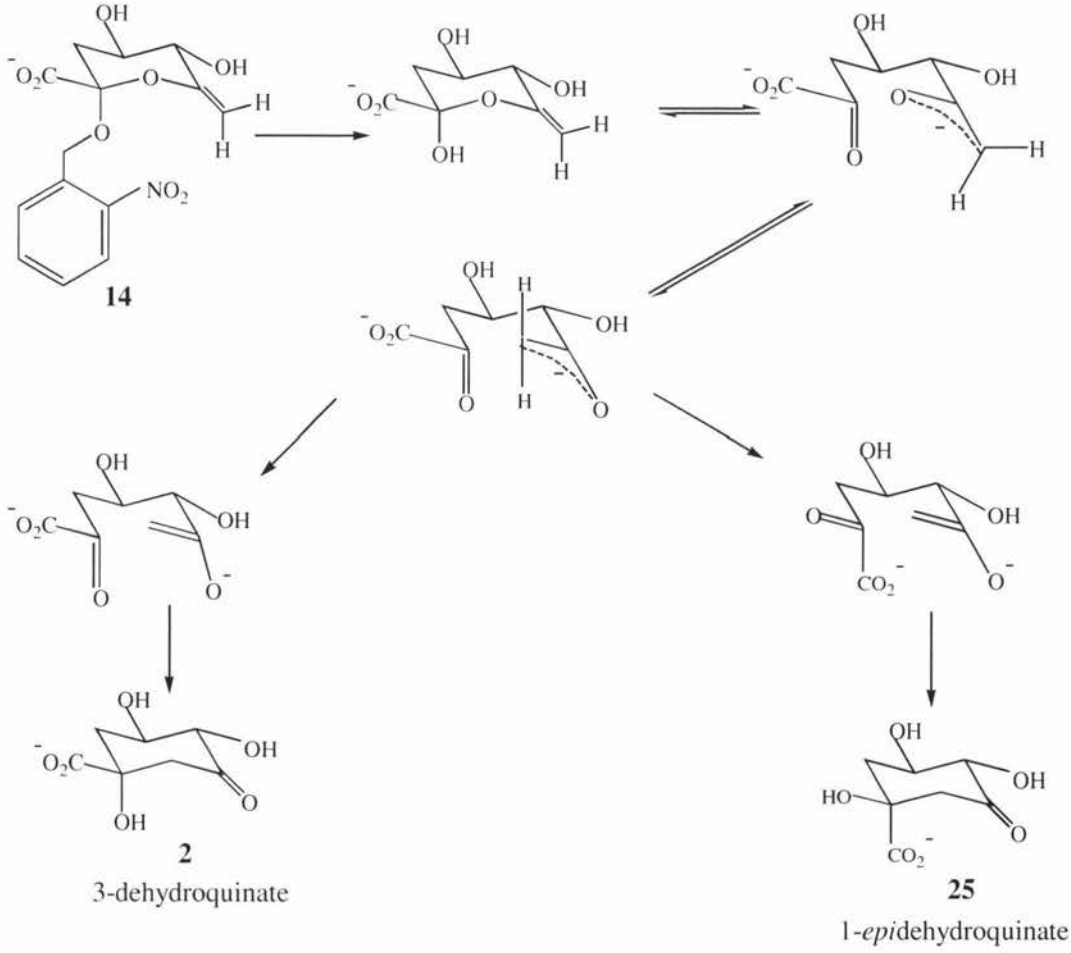


Figure 1.8 Formation of 3-dehydroquinate and 1-*epi*dehydroquinate from the *o*-nitrobenzyl protected enolpyranose.

The crystal structure of the DHQ synthase domain of the AROM protein from *Aspergillus nidulans* reveals that the enzyme may catalyse the phosphate elimination by providing a phosphate binding pocket, thus forcing the phosphate oxygens into a position where it can remove the proton from C6.[11] It also reveals, that the carboxylate group of DAHP is held by the active sites of the enzyme and it is not possible for the enolate to attack the other face of the keto group for the formation of the C1 epimer of 3-dehydroquinate on the enzyme and supports the finding of Bartlett *et al.*[2] that the enzyme is acting as a template for the formation of the correct product.[11] On the basis of computational molecular superimposition studies with carbaphosphonate Piehler *et al.*[29] also suggest the existence of an enzyme active side residue, which enables DHQ synthase to play an active catalytic role during intramolecular aldol condensation. The question still remains, however, whether or not the enzyme is actively catalysing the conversion of DAHP into 3-dehydroquinate.

1.4 Studies with the isomers of 3-fluoro DAHP

The isomers of 3-fluoro DAHP were synthesised by Parker[28] as part of the studies to prepare 6-fluoroshikimates enzymatically to study their antibiotic properties. During these studies it was observed that when (3*R*)-3-fluoro DAHP (**4**) generated by DAHP synthase was treated with the enzyme DHQ synthase the (3*R*)-isomer was rapidly converted to the expected (6*R*)-6-fluorodehydroquinate (**7**, **Figure 1.9**). However the (3*S*)-3-fluoro DAHP (**3**) was slowly converted to a mixture of (6*S*)-6-fluorodehydroquinate (**5**) and (6*S*)-6-fluoro-1-*epi*dehydroquinate (**6**). This was the first time the formation of epimer had been observed during enzymatic reaction. In (3*R*)-3-fluoro DAHP the fluorine atom is in the down or equatorial position whereas in (3*S*)-3-fluoro DAHP the fluorine occupies the up or axial position. These compounds differ from the natural substrate only by the substitution of hydrogen for fluorine and from each other solely by the orientation of the fluorine.

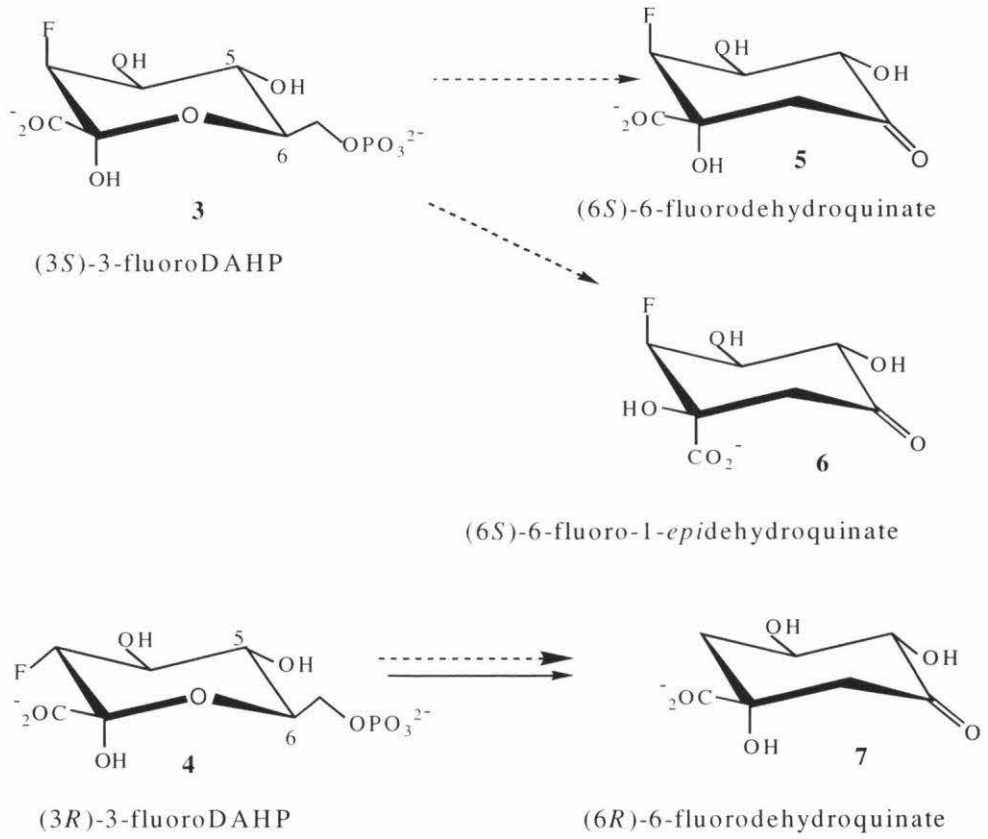


Figure 1.9 Treatment of 3-fluoro DAHP with DHQ synthase.

Two products can be formed from the DHQ synthase reaction due to attack of the enolate on the two different faces of the carbonyl in the final cyclisation step (**Figure 1.10).** When the non fluorinated enolpyranose was generated in solution formation of both 3-dehydroquinate and the epimer was observed, while in the enzymatic reaction formation of 3-dehydroquinate only is detected. The generation of two products, the expected (6*S*)-6-fluorodehydroquinate and the unexpected (6*S*)-6-fluoro-1*epi*dehydroquinate, when the substrate for DHQ synthase is (3*S*)-3-fluoro DAHP, suggests that the aldol ring closure may occur non-enzymatically. Already Bartlett *et al.*[2] had shown that the enzyme is acting as a template for the formation of the product with correct stereochemistry. The crystal structure of the DHQ synthase from *A. nidulans* also suggests that there is interaction between the active sites of the enzyme and the carboxylate group of DAHP, in a manner that

would hold the keto functionality so that attack on it would give rise to only one product. Therefore it seems unlikely that the epimer is produced on the enzyme.

There are two possible scenarios that could account for the formation of two products from (3*S*)-3-fluoro DAHP. It is possible that the fluoroenolpyranose intermediate is released into the solution completely or that it is released partially. If the fluoroenolpyranose intermediate is released completely into solution it may cyclise giving both the correct expected product and the unexpected epimer. In the solution reaction the ring opened fluoroenolpyranose intermediate has the conformational freedom to produce the unexpected epimer. The other possibility is that fluoroenolpyranose intermediate may leak partially into solution and the conformational freedom of the intermediate can lead to the exclusive formation of the unexpected epimer. Therefore, it is possible that the expected product is formed on the enzyme, and the unexpected epimer is generated off the enzyme. To clarify these results, an understanding of the transition states involved in the final cyclisation is necessary.

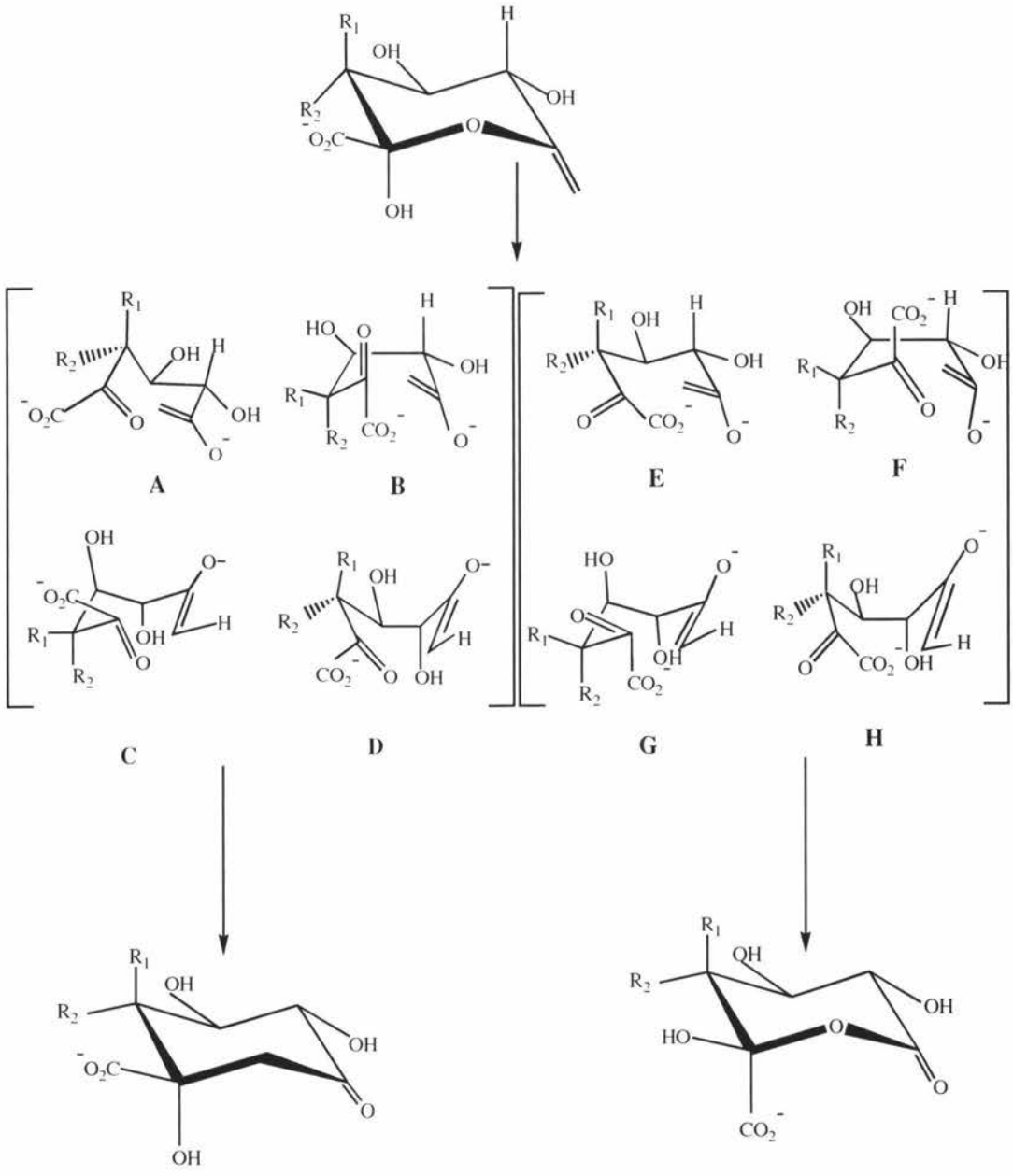
There are two *chair* and two *boat* transition states for the reaction of the enolate to 3-dehydroquinate and correspondingly four transition states for reaction to the C1 epimer of dehydroquinate (**Figure 1.10**). As outlined in section 1.3.2, the *si* face of the enolate attacks the *re* face of carbonyl in the enzymatic cyclisation giving 3-dehydroquinate. Only the *chair* A and *boat* B transition states are consistent with this, however the *chair* transition state has been considered to be the most likely and the *boat* transition state has been paid little attention. For non-enzymatic generation of 3-dehydroquinate, labelling studies have also shown that either *chair* A or *boat* B transition state is involved.[3] For formation of 1-*epi*dehydroquinate Bartlett *et al.*[2] suggested that a *chair* transition state is involved in which the *si* face of enolate attacks the *si* face of the carbonyl. However no labelling studies have been carried out to support this suggestion, so the face of the enolate involved in the formation of 1-*epi*dehydroquinate is unknown.

For the reaction of (3*S*)-3-fluoro DAHP, and for the non-enzymatic generation of 1-*epi*dehydroquinate from DAHP, all the possible transition states need to be considered, as no labelling studies have been carried out to determine which face of the enolate is involved. According to the Felkin – Anh model,[14] transition states for the internal aldol reaction would be expected to be most favoured when the fluorine is orthogonal to the carbonyl bond and at 180° to the direction of attack by enolate. Applying this argument *boat* F and *chair* G would be expected to be favoured among the four transition states E to H for epimer formation. Of the four transition states leading to the formation of (6*S*)-6-fluorodehydroquinate, *boat* B and *chair* C would be expected to be more favoured. *Chair* A, the favoured transition state conformation for the enzymatic cyclisation, would be disfavoured.

Fluorine atoms adjacent to hemiketals and hydrates are more stable than the corresponding ketones.[27] Therefore fluorine substitution may provide stability on the fluoroenolpyranose intermediate. This may lead to the intermediate dissociating from the enzyme completely or partially and cyclising free in solution.

For the (3*R*)-isomer, it is difficult to predict, whether the fluoroenolpyranose intermediate comes off the enzyme or not. The fluorine occupies a position orthogonal to the carbonyl bond and *anti* to the direction of attack by the enolate in *chair* transition state A. Therefore, the reaction might be expected to go rapidly with exclusive formation of the correct expected product.

Therefore, it has been hypothesised that axial fluorine stabilises the enolpyranose intermediate long enough for it to dissociate from the enzyme completely or partially and once in solution the fluoroenolpyranose intermediate cyclises without the constraint of the enzymatic template. Off the enzyme, the influence of the fluorine gives rise to the unexpected epimer.[27]



$R_1 = R_2 = H$, dehydroquinate

$R_1 = F$, $R_2 = H$, (6*S*)-6-fluorodehydroquinate

$R_1 = H$, $R_2 = F$, (6*R*)-6-fluorodehydroquinate

$R_1 = R_2 = H$, 1-*epi*dehydroquinate

$R_1 = F$, $R_2 = H$, (6*S*)-6-fluoro-1-*epi*dehydroquinate

Figure 1.10 Possible transition states for the formation of epimers.

1.5 Outline of the project

To test this hypothesis and gain more information about the final two steps of the mechanism, as a preliminary investigation, it was decided to examine the interaction of (3*S*)-3-fluoro DAHP with DHQ synthase at different reaction conditions, such as temperature and pH by using ^{19}F NMR spectroscopy. A change in the reaction conditions may provide information as to whether the reaction is occurring on the enzyme or off the enzyme. If one product is formed on the enzyme and the other is formed off the enzyme, changing the reaction conditions may lead to significant changes in the ratio of products formed. Therefore it was decided to monitor the ratio of products formed. There are other approaches, which can also be used to test this hypothesis and are explained in future directions.

In these studies a considerable amount of (3*S*)-3-fluoro DAHP was synthesised by the DAHP synthase catalysed reaction of D-erythrose 4-phosphate and the isomers of 3-fluoro PEP. Purification of (3*S*)-3-fluoro DAHP was achieved by anion exchange chromatography. The preparation and purification of (3*S*)-3-fluoro DAHP are discussed in Chapter 2. Erythrose 4-phosphate was prepared by the method of Simpson *et al.*[32] (*Z*)- and (*E*)-3-Fluoro PEP was synthesised by the Perkow reaction. Their chemical syntheses are explained in Chapter Two. It has been recently reported that *E. coli* DAHP synthase (phe) catalyses the aldol type condensation of PEP with the five carbon analogues D-arabinose 5-phosphate, D-ribose 5-phosphate, and 2-deoxy-D-ribose 5-phosphate.[31] Since 3-fluoro PEP was synthesised for the production of (3*S*)-3-fluoro DAHP, it was decided to test, whether, *E. coli* DAHP synthase (phe) catalyses the aldol type condensation of 3-fluoro PEP with the five carbon analogues D-arabinose 5-phosphate, and D-ribose 5-phosphate. From these studies it appears that the enzyme also catalyses these reactions. The findings from this study are discussed in Chapter Two.

E. coli strain (pJB 14) was used in the expression of the enzyme DHQ synthase. The cell growth and purification of the enzyme DHQ synthase were carried out

according to Frost *et al.*[16], with some modifications to the purification protocol. The production and purification of the enzyme are discussed in Chapter Three.

Chapter Four discusses the interaction of the enzyme DHQ synthase with (3*S*)-3-fluoro DAHP at different temperature and pH values. The ratio of products produced in the enzyme-catalysed reaction was monitored by ^{19}F NMR spectroscopy. No significant change in the ratio of products formed was observed. These observations are consistent with the hypothesis that the fluoroenolpyranose intermediate is released to the solution, where it cyclises freely to give two products. To further test this hypothesis other investigations are necessary and these are discussed in the future directions.