On The Mechanism Of *Dehydroquinate synthase*

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by

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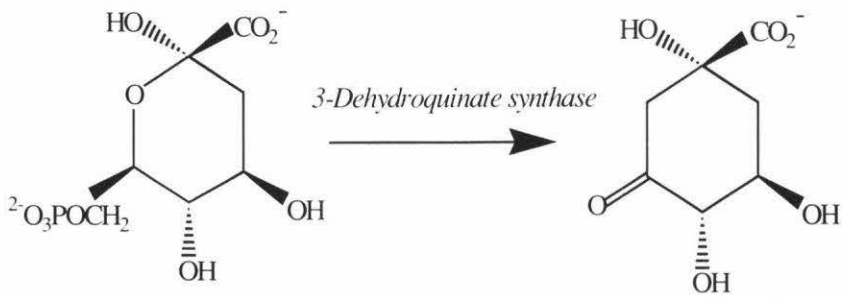
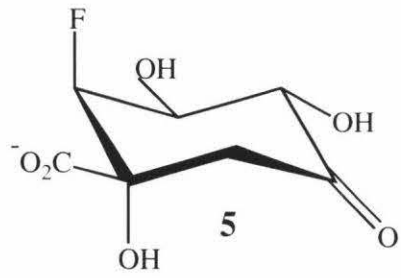
ABSTRACT

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).

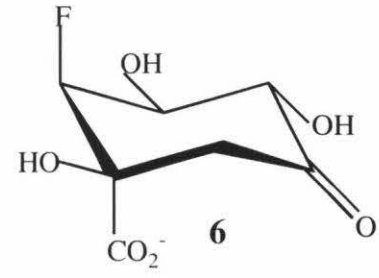


DAHP 3-dehydroquinate

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]

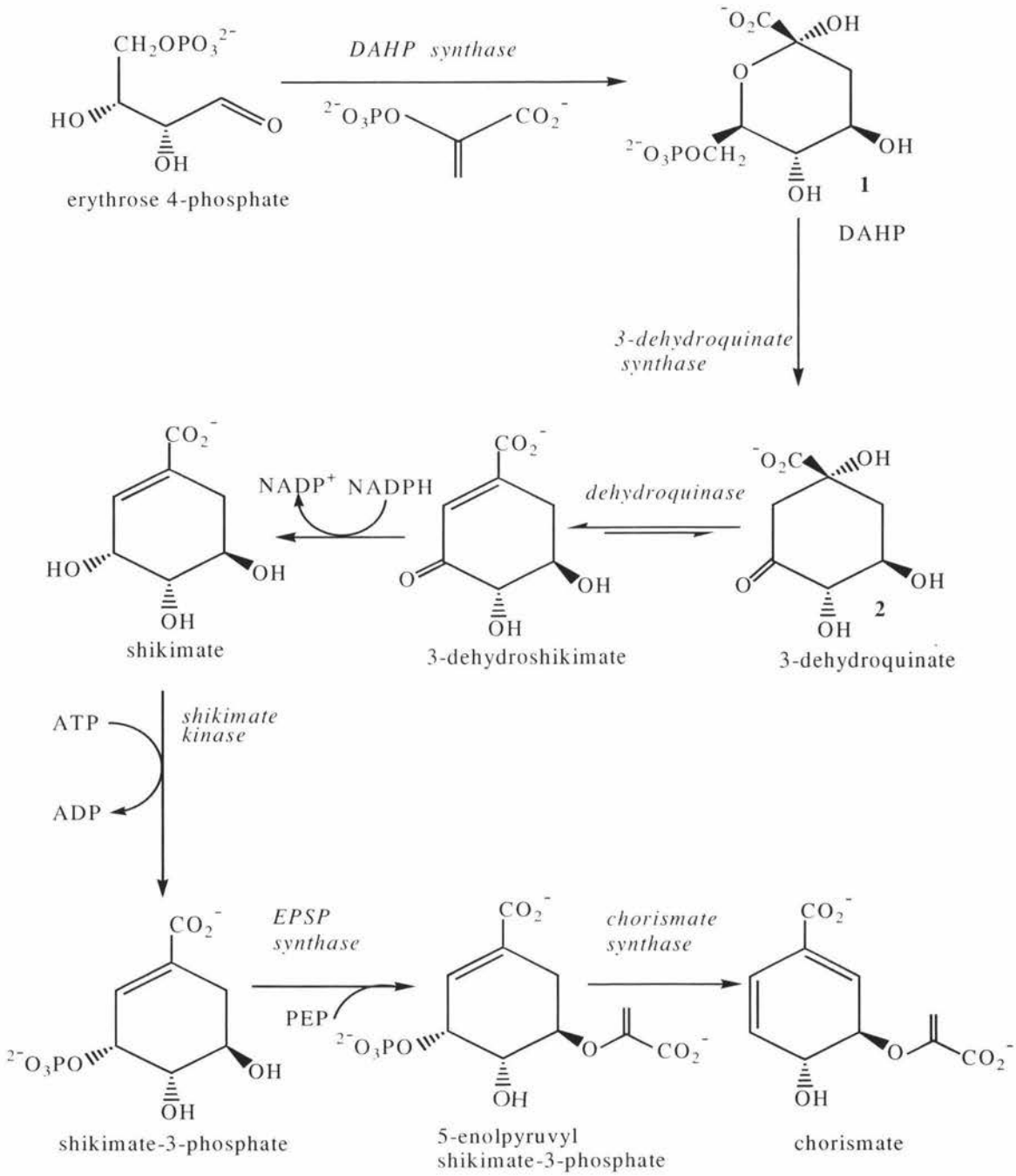


Figure 1.1 The Shikimate Pathway.[28]

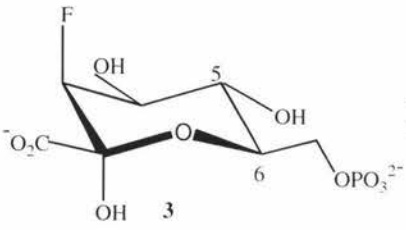
The second enzyme of the pathway, 3-dehydroquinate synthase (formerly named as 7-phospho-3-deoxy-D-*arabino*-heptulosonate phosphate lyase) catalyses the conversion of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (**1**, DAHP) into 3-dehydroquinate (**2**, DHQ), the first carbocyclic metabolite in the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

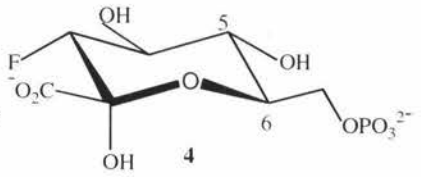
1.2 The aim of the studies

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?

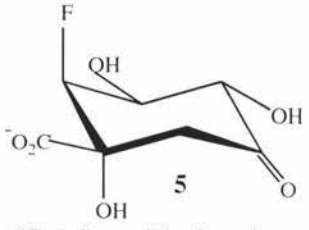
The objectives of the present study were to investigate the influence of the fluorine orientation on the stereochemical outcome of the process and determine the role of the enzyme DHQ synthase in guiding the formation of the correct epimeric product.



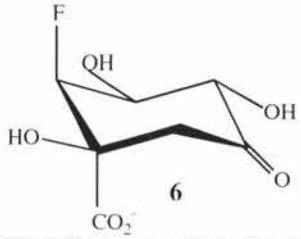
(3*S*)-3-fluoroDAHP



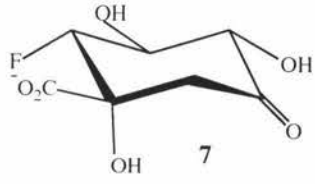
(3*R*)-3-fluoroDAHP



(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]

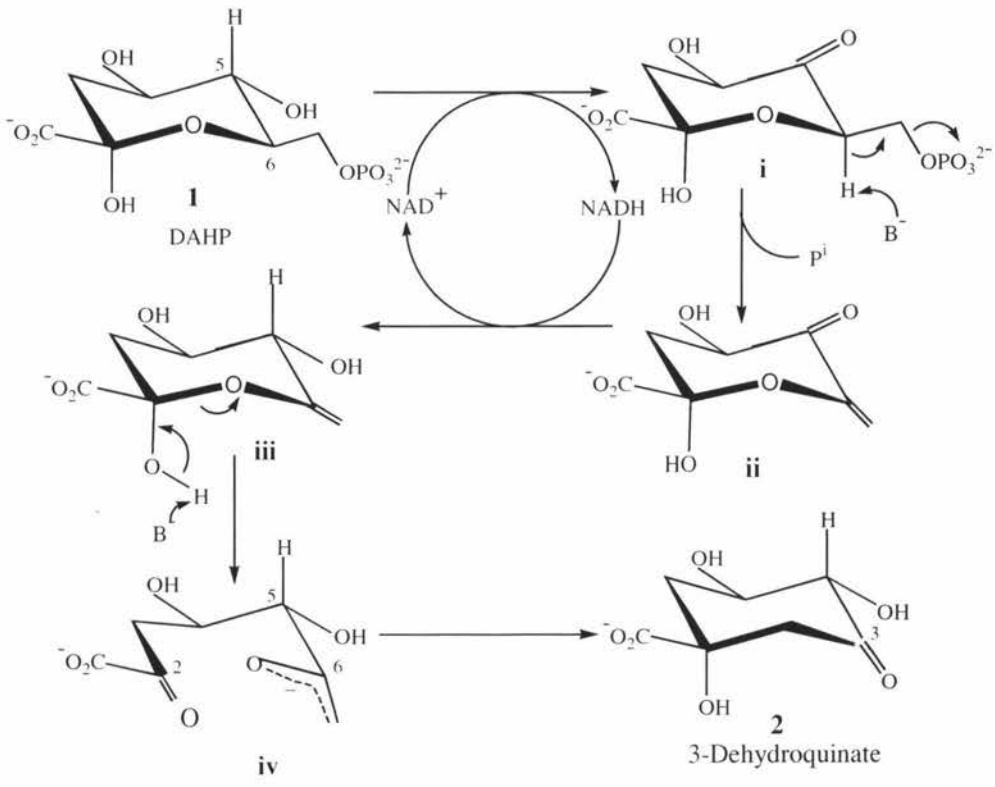


Figure 1.2 Proposed mechanism for the conversion of DAHP into 3-dehydroquinate.

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.

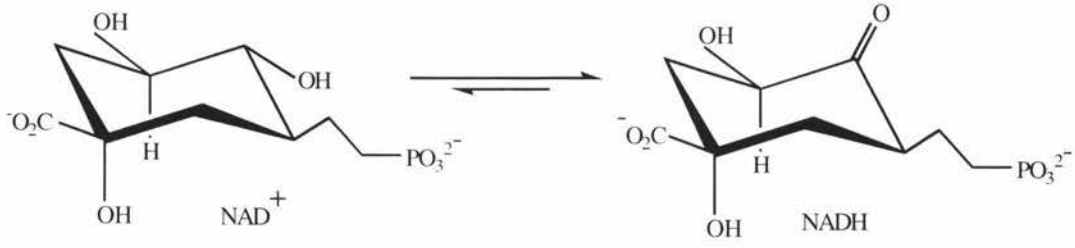


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Figure 1.3 Action of DHQ synthase on carbahomophosphonate.

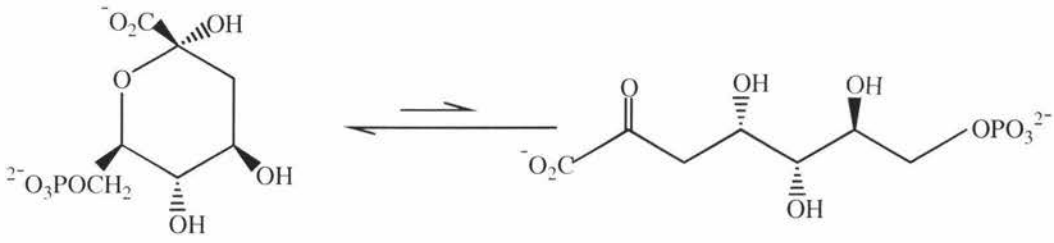


Figure 1.4 The cyclic and acyclic forms of DAHP.

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.

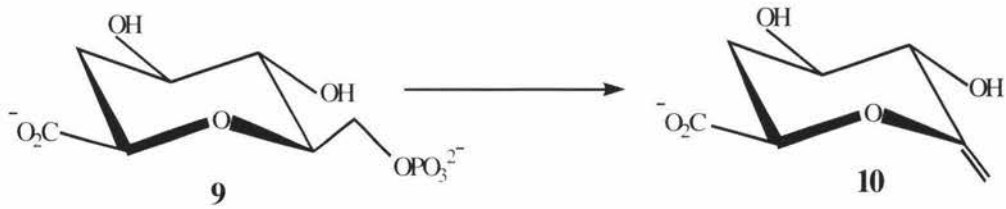
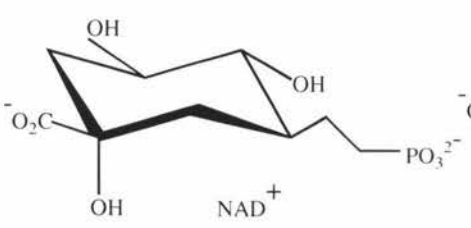


Figure 1.5 Action of DHQ synthase on 2-deoxy DAHP.

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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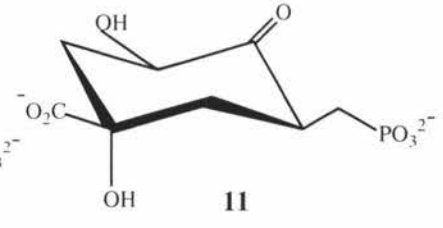

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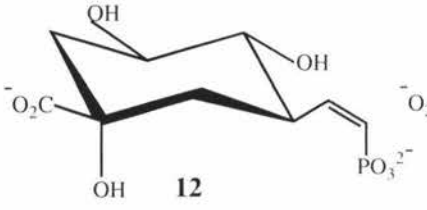

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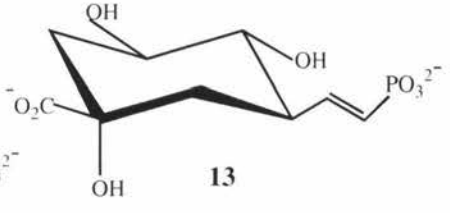

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From the studies with these substrate analogues it was established that the true substrate for the enzyme is apparently the pyranose or cyclic form of the DAHP and the β-elimination proceeds with *syn* stereochemistry from the cyclic α-pyranose form 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.**

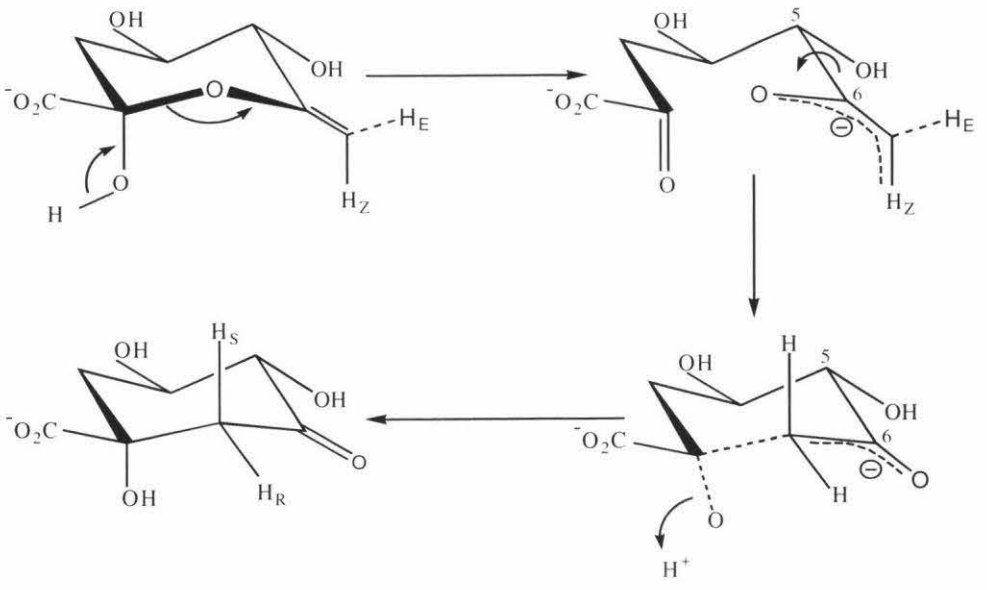


Figure 1.6 The overall picture for the final two steps of the DHQ synthase reaction.

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.

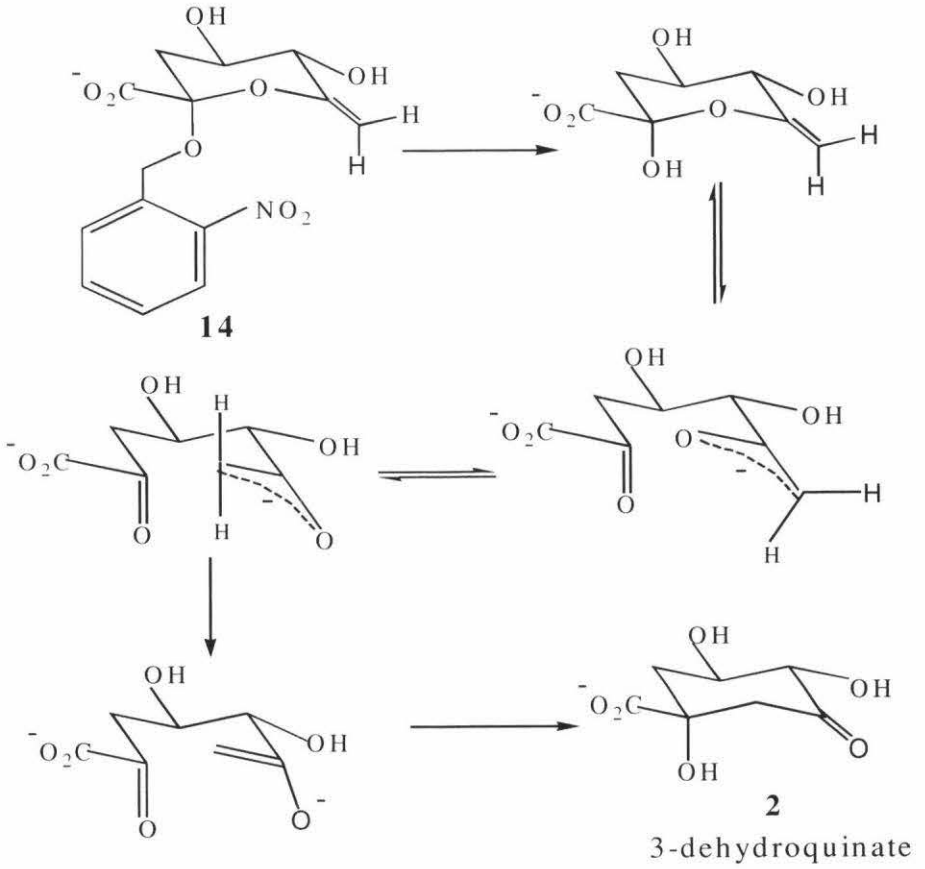
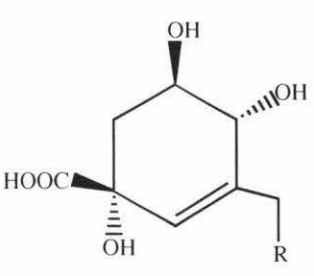


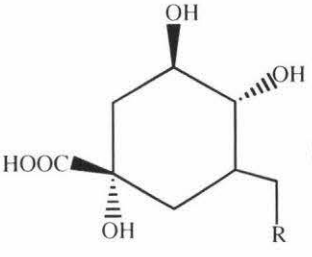
Figure 1.7 Formation of 3-dehydroquinate from the *o*-nitrobenzyl protected enolpyranose.

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

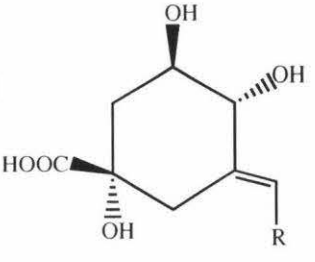
Montchamp and Frost[26] synthesised different cyclohexenyl (**15, 16, 17** and **18**) and cyclohexylidine (**23, 24**) inhibitors of DHQ synthase and measured their inhibition constants. When these values were compared with the inhibition constants of the corresponding cyclohexyl analogues (**19, 20, 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.

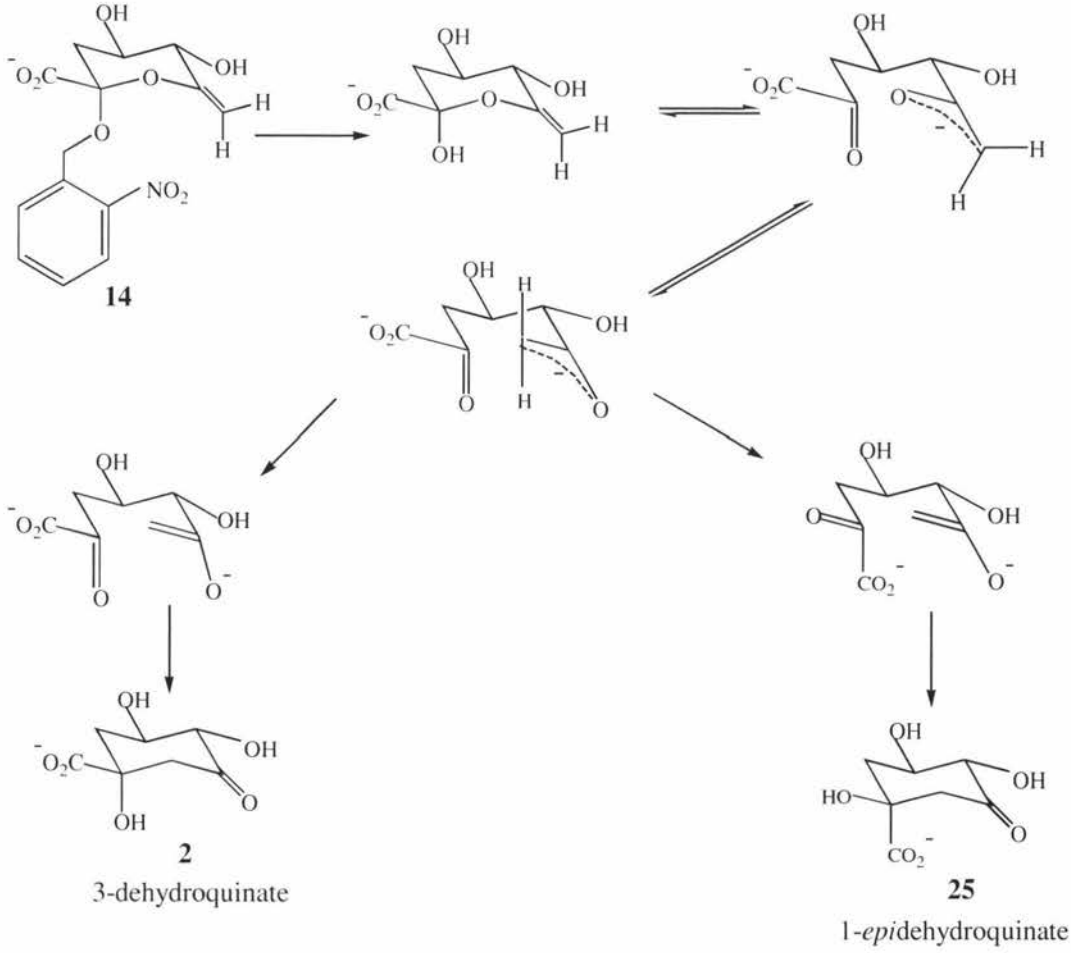


Figure 1.8 Formation of 3-dehydroquinate and 1-*epi*dehydroquinate from the *o*-nitrobenzyl protected enolpyranose.

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.

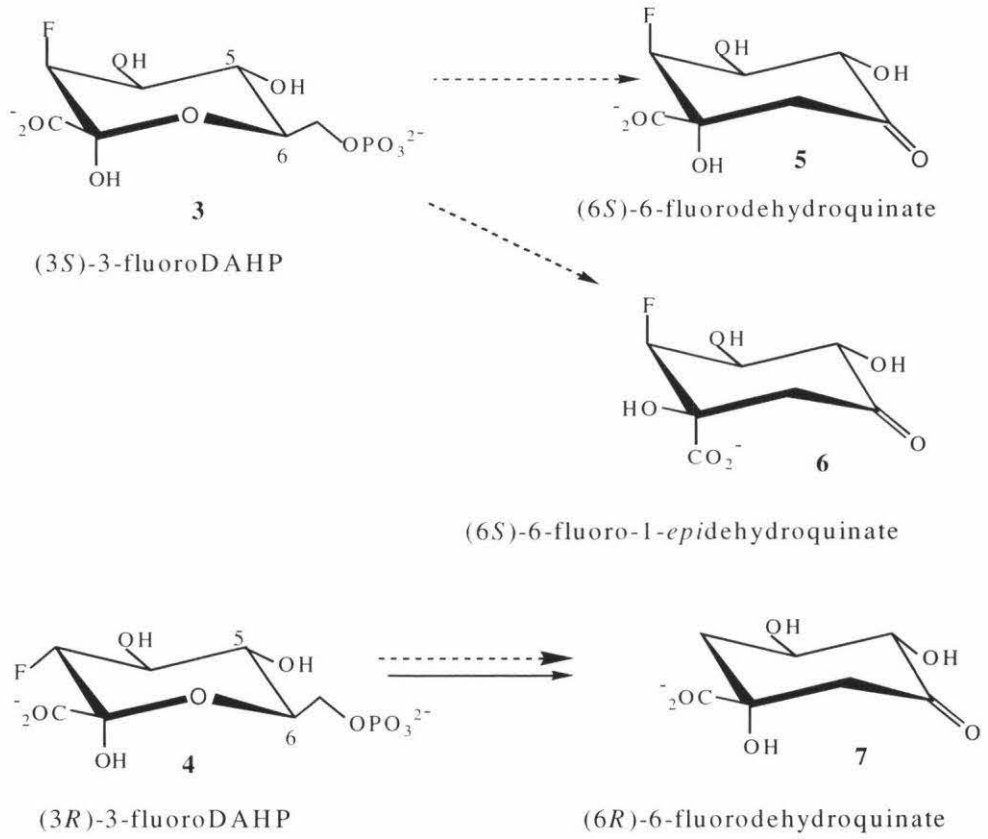


Figure 1.9 Treatment of 3-fluoro DAHP with DHQ synthase.

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.

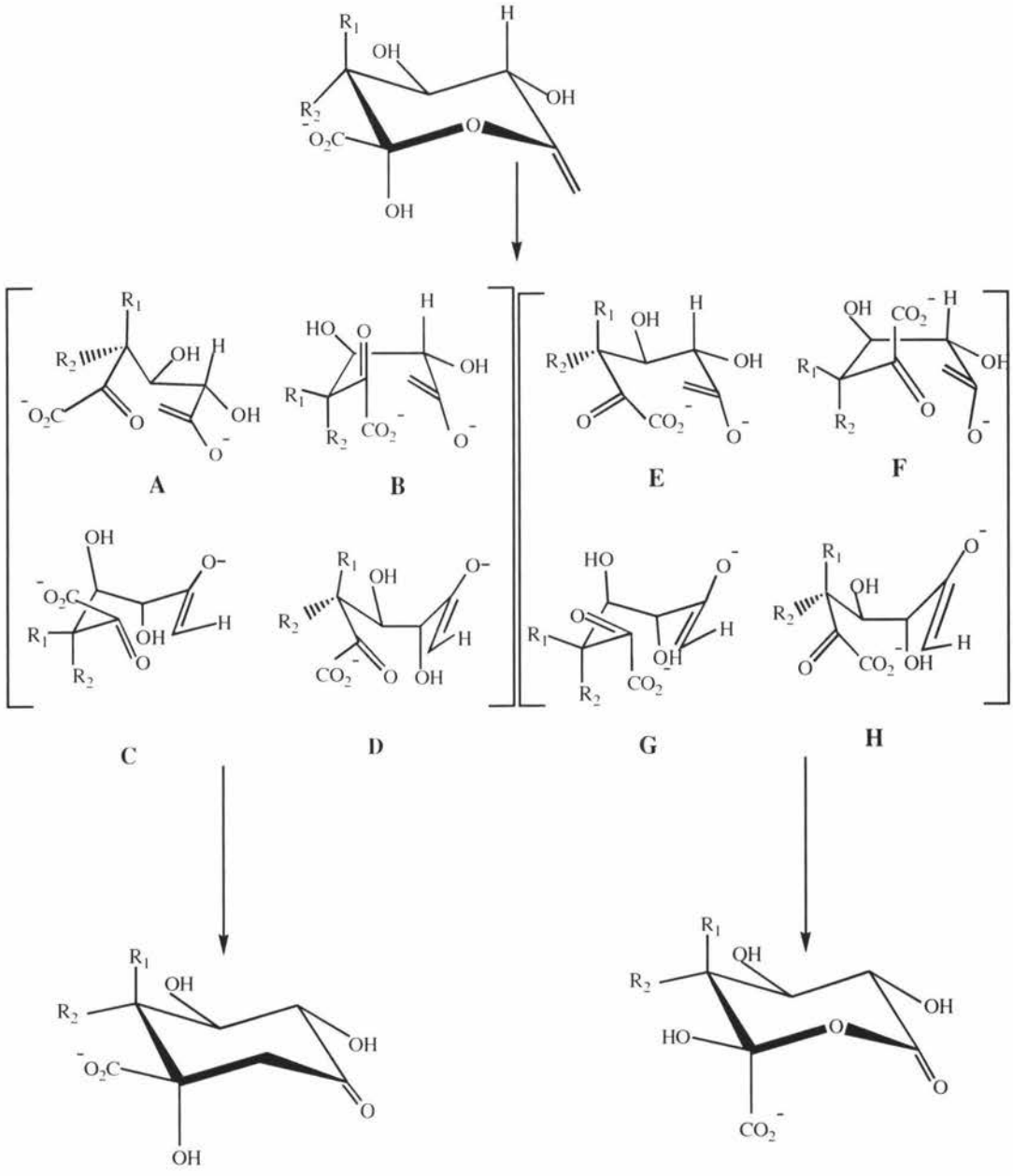
There are two *chair* and two *boat* transition states for the reaction of the enolate to 3-dehydroquinate and correspondingly four transition states for reaction to the C1 epimer of dehydroquinate (**Figure 1.10**). As outlined in section 1.3.2, the *si* face of the enolate attacks the *re* face of 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.

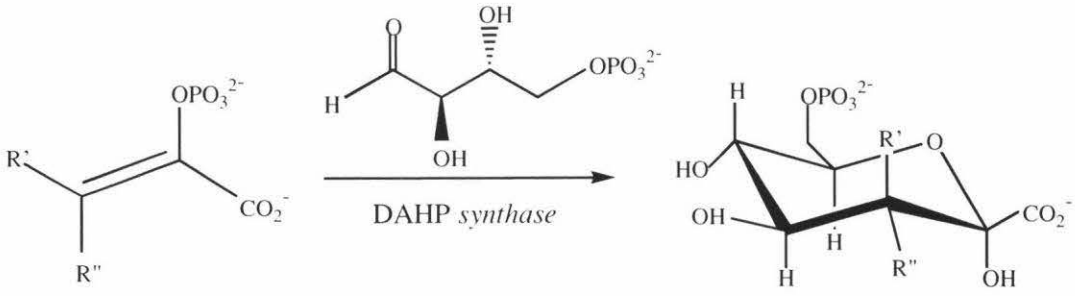
2 Synthesis of 3-fluoro DAHP

2.1 Introduction to the preparation of 3-fluoro DAHP, 3-fluoro phosphoenolpyruvate and erythrose 4-phosphate.

In order to investigate the influence of fluorine substitution on the DHQ synthase reaction, large quantities of 3-fluoro DAHP were required. 3-Fluoro DAHP can be prepared in procedures similar to those that have been used to synthesise DAHP. Many different chemical and microbial preparations of DAHP have been reported. Hermann *et al.*[19] synthesised DAHP by a one step chemical synthesis through condensation of oxaloacetate with erythrose 4-phosphate. This synthesis requires metal ion catalysis and is not stereospecific with respect to the configuration of the new stereocentre in the product. Mehdi *et al.*[24] obtained DAHP by isolation from a mutant *E. coli* strain, JB5, which has the phenotype *aro B*$^-$ tyr R$^-$. This strain lacks DHQ synthase and the repressor protein that controls the transcription of two of the three DAHP synthases, and therefore accumulates reasonable quantities of DAHP. However, the simplest preparation is the aldol condensation between C3 of phosphoenolpyruvate and C1 of D-erythrose 4- phosphate catalysed by the first enzyme of the shikimate pathway, DAHP synthase. This reaction is dependent on the presence of a metal ion, and proceeds at room temperature and neutral pH.

The (*Z*)- and (*E*)-3-fluoro PEP are substrates for *E. coli* DAHP synthase (phe) and the products (3*S*)- and (3*R*)-3-fluoro DAHP have been isolated and characterised.[28] The kinetic parameters for these reactions have also been reported.[28] (3*R*)-3-Fluoro

DAHP was formed from the (*E*)-isomer and (3*S*)-3-fluoro DAHP was formed from the (*Z*)-isomer. This is in agreement with the facial selectivity of DAHP synthase for its normal substrates D-erythrose 4-phosphate and PEP. DAHP synthase catalyses the *si* face addition of the C3 of PEP to the *re* face of C1 of the erythrose 4-phosphate.



R', R" = H PEP
R' = H, R" = F (*E*)-3-fluoro PEP
R' = F, R" = H (*Z*)-3-fluoro PEP

R', R" = H DAHP
R' = H, R" = F (3*R*)-3-fluoro DAHP
R' = F, R" = H (3*S*)-3-fluoro DAHP

Figure 2.1 DAHP synthase catalysed reaction.

The most convenient method to synthesise 3-fluoro DAHP is using the DAHP synthase catalysed reaction of (*Z*)- and (*E*)-3-fluoro PEP with erythrose 4-phosphate (**Figure 2.1**). Pilch *et al.*[30] synthesised 3-fluoro DAHP by two different methods. They chemically synthesised from fluorooxaloacetate and erythrose 4-phosphate. They also obtained 3-fluoro DAHP chemo-enzymatically. In the chemo-enzymatic method 3-fluoro PEP was synthesised by the Perkow reaction and erythrose 4-phosphate was synthesised by lead tetraacetate oxidation of glucose 6-phosphate. Then these two were condensed together with the first enzyme DAHP synthase to give 3-fluoro DAHP. Parker[28] synthesised 3-fluoro DAHP by reacting chemically prepared 3-fluoro PEP and erythrose 4-phosphate (generated *via* the transketolase reaction) with DAHP synthase in solution (**Figure 2.2**). Both these preparations have used the first enzyme to catalyse the reaction, but the source of erythrose 4-phosphate is different in the two methods. In the present study both these chemo-enzymatic approaches were investigated.

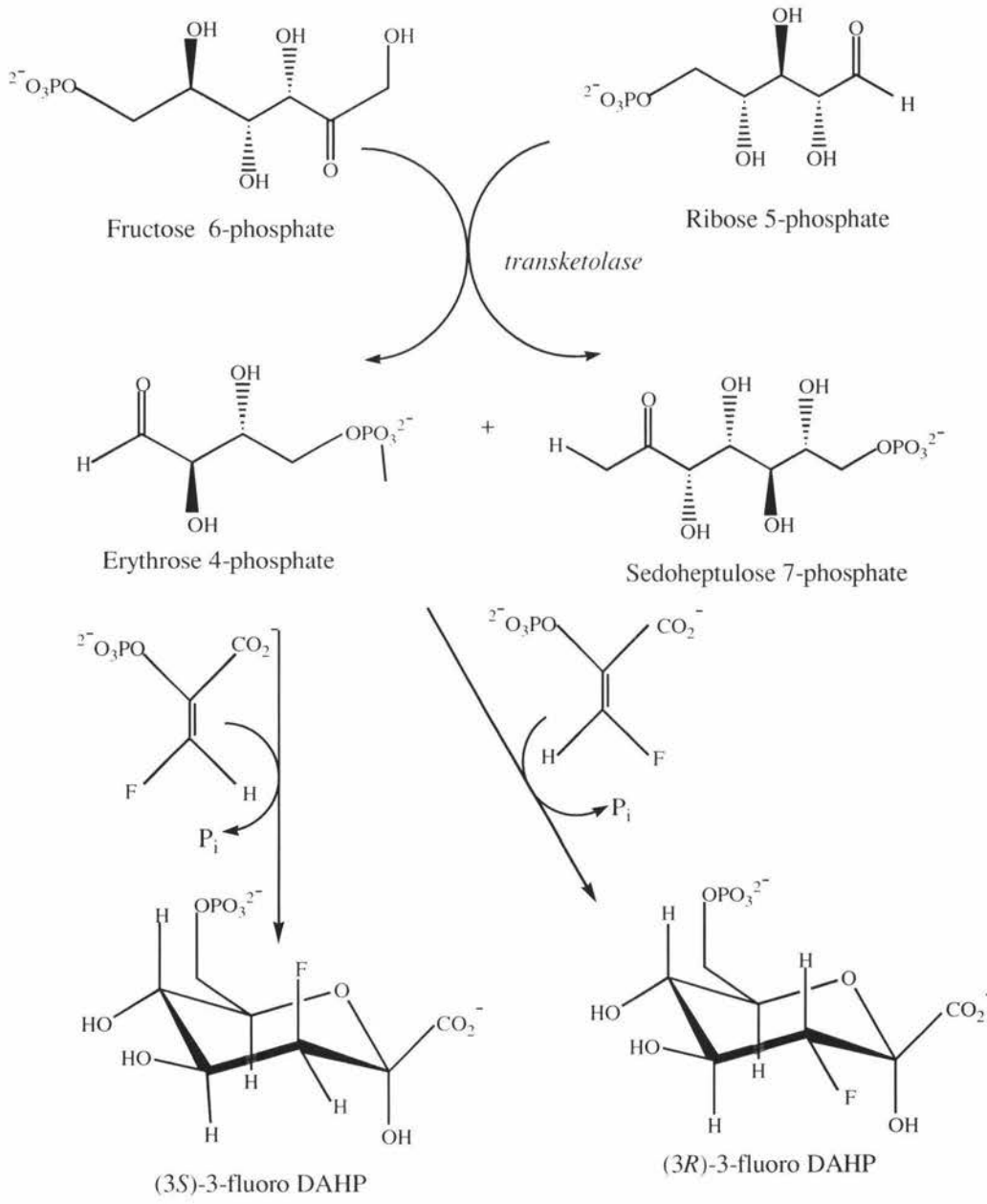
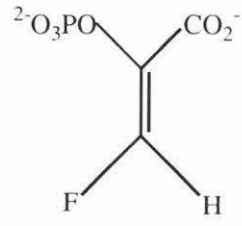
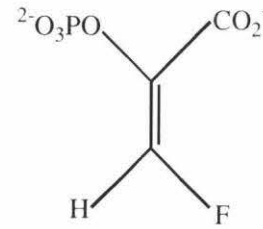


Figure 2.2 Generation of (3*S*)- and (3*R*)-3-fluoro DAHP in solution using transketolase and DAHP synthase.

In order to synthesise the isomers of 3-fluoro DAHP, a mixture of (*Z*) and (*E*)-3-fluoro PEP was required.



(*Z*)-3-fluoro PEP



(*E*)-3-fluoro PEP

Figure 2.3 Isomers of 3-fluoro PEP

(*Z*)- and (*E*)-3-Fluoro PEP can be synthesised by Perkow reaction. This involves a common step modelled on the facile synthesis of PEP from bromopyruvic acid. This method involves the production of the enol phosphate ester by the Perkow reaction from a brominated pyruvic acid derivative and trimethylphosphite. Subsequent hydrolysis of the phosphate ester yields the enol phosphate.[12, 13] 3-Fluoro PEP can also be synthesised by *E. coli* enzyme phosphoenolpyruvate synthetase, which catalyses the reaction with fluoropyruvate and adenosine triphosphate. PEP synthetase has been over expressed and purified by Jakeman and Evans.[20] This enzyme allows the one-step synthesis of stereochemically pure (*Z*)-3-fluoro PEP and does not involve either toxic chemicals or many synthetic steps. In the present study (*Z*)- and (*E*)-3-fluoro PEP was synthesised by Perkow reaction and this is described in section 2.2.1.

Erythrose 4-phosphate can also be synthesised in different ways. It can be prepared by the chemical oxidation of glucose 6-phosphate with Pb(IV) or by the action of the enzyme transketolase on fructose 6-phosphate and ribose 5-phosphate to give erythrose 4-phosphate and sedoheptulose 7-phosphate (**Figure 2.4**). Erythrose 4-phosphate is very unstable in solution, and this causes difficulties in handling erythrose 4-phosphate solutions. It has been reported that erythrose 4-phosphate spontaneously dimerises in aqueous solutions and most of the dimerisation takes place during evaporation.[9] It forms three major dimeric species (**Figure 2.5**). The

dimers dissociate at low concentrations to form the monomer. The monomer exists as the hydrate in solution. The position of equilibrium between the dimer and monomer depends on the erythrose 4-phosphate concentration. Therefore, when handling erythrose 4-phosphate solutions for use in enzymatic reactions the solutions should be prepared and allowed to equilibrate for at least 2 hours. If not, a burst of DAHP synthase activity can be observed with the consumption of erythrose 4-phosphate monomer, followed by a slower rate, which is limited by the dissociation of the dimers to the monomeric form.

Another phenomenon encountered in the utilisation of erythrose 4-phosphate in buffers with a primary amine is Schiff base formation. The aldehyde of erythrose 4-phosphate forms a Schiff base with the amine of Tris or carbinolamine. The preparation of erythrose 4-phosphate for use in these studies is described in section 2.2.3.

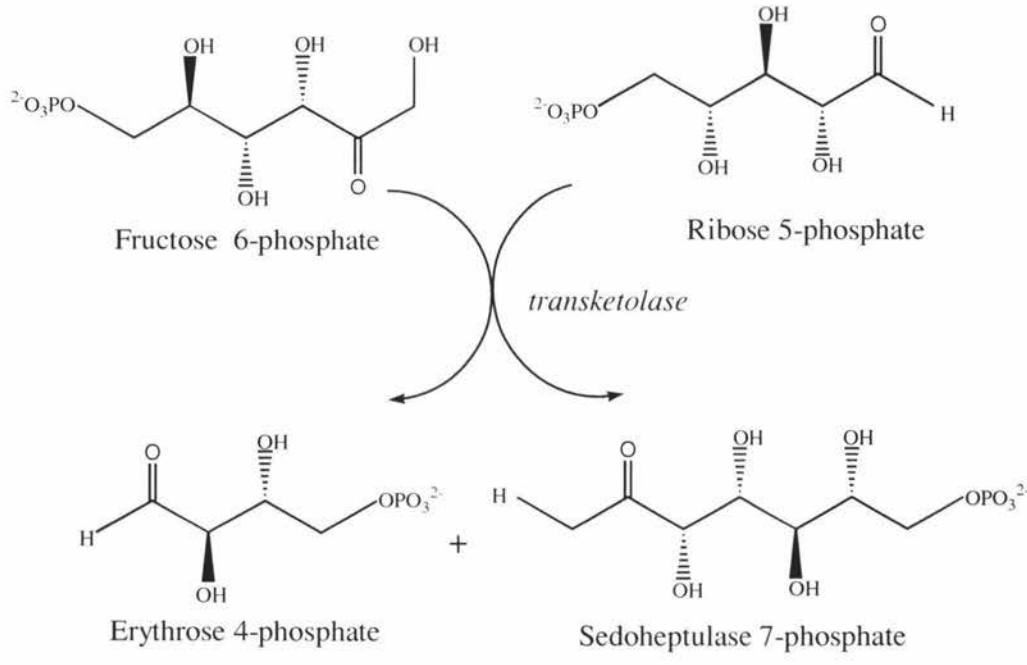


Figure 2.4 Enzyme catalysed synthesis of erythrose-4-phosphate.

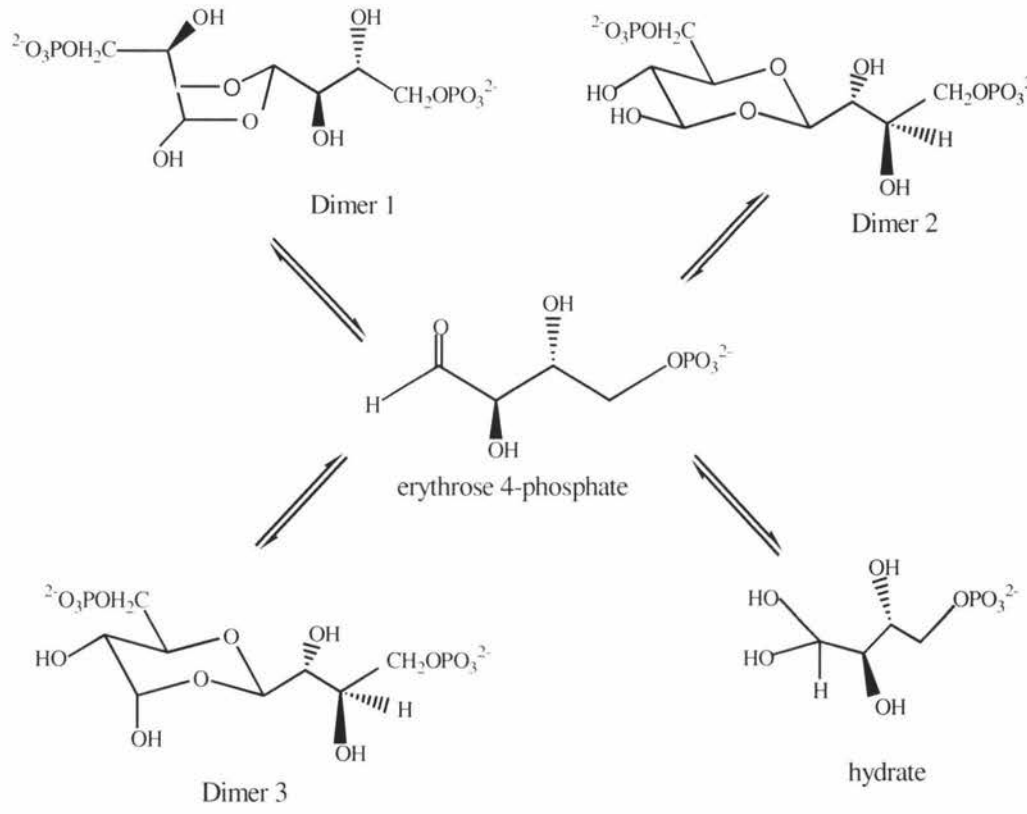


Figure 2.5 Equilibration of erythrose 4-phosphate.

2.2 Results and Discussion

2.2.1 Preparation of 3-fluoro PEP

To synthesise 3-fluoro PEP using the Perkow reaction, bromofluoropyruvic acid was first prepared in a three-step procedure starting with diethyl oxalate and ethyl fluoroacetate. Freshly prepared sodium ethoxide was used in the reaction. Direct bromination of the isolated enolate gave diethyl bromofluorooxaloacetate. This was found to be unstable at room temperature and was stored at 4 °C. Hydrolysis and decarboxylation of the diethyl bromofluorooxaloacetate gave bromofluoropyruvic acid, which was purified by base extraction from ether into water, then acidification and extraction into ethyl acetate. The schematic diagram of the synthesis of 3-fluoro PEP from diethyl oxalate and ethyl fluoroacetate is shown in **Figure 2.6**. For

the hydrolysis and decarboxylation much more vigorous reaction conditions (temperature of 105 °C and hydrolysis time of 6 hrs) were required than those reported by Stubbe and Kenyon[34] (temperature of 80 °C and hydrolysis time of 2 hours).

Dimethyl 3-fluoro PEP was prepared by the reaction of the bromofluoropyruvic acid with trimethylphosphite in THF. This dimethyl ester was hydrolysed by dissolving it in water and stirring at room temperature for 48 hours. Cyclohexylamine was added and recrystallisation of the cyclohexylammonium salt from methanol/ether gave 3-fluoro PEP. Sundaram *et al.*[35] have recently reported that addition of cyclohexylamine followed by stirring, results in complete product decomposition. But, during this preparation, the cyclohexylamine was added and left with stirring for 15 minutes and no product decomposition was observed.

Trimethylphosphite is very reactive towards moisture and produces phosphorous acid. Extreme care was taken in this reaction to exclude moisture from the reactants. Trimethylphosphite was pre-treated with sodium wire. Then it was freshly distilled under argon, and used immediately. All glassware and syringes were oven dried. Bromofluoropyruvic acid was dried under high vacuum for several hours prior to reaction with trimethylphosphite. If trimethylphosphite is present in excess it will be hydrolysed in the next step to phosphorous acid. This was reduced by delivering the exact amount of trimethylphosphite (1 : 1 mol ratio) and when evaporating the solvent from dimethyl-3-fluoro PEP *in vacuo*, evaporation was continued a further 10 minutes, in order to remove any unreacted phosphite. Even after all these procedures and recrystallisation, the sample contained bromofluoropyruvic acid and phosphorous acid in a ratio of approximately 3-fluoro PEP : phosphorous acid : bromofluoropyruvic acid = 50 : 30 : 20 . The ^{19}F NMR spectrum of the crude sample contained three major signals. As reported earlier by Parker the major signal at -132.5 ppm was assigned as the (*Z*)-isomer and the signal at -136.5 ppm was assigned as the (*E*)-isomer. The signal at -150 ppm was due to the unreacted bromofluoropyruvic acid. The ratio of *Z : E*

isomers of 3-fluoro PEP were 9 : 1. The exact amount of 3-fluoro PEP produced was calculated by measuring the depletion of 3-fluoro PEP by DAHP synthase in the presence of excess erythrose 4-phosphate. For these enzymatic assays commercially available erythrose 4-phosphate was used.

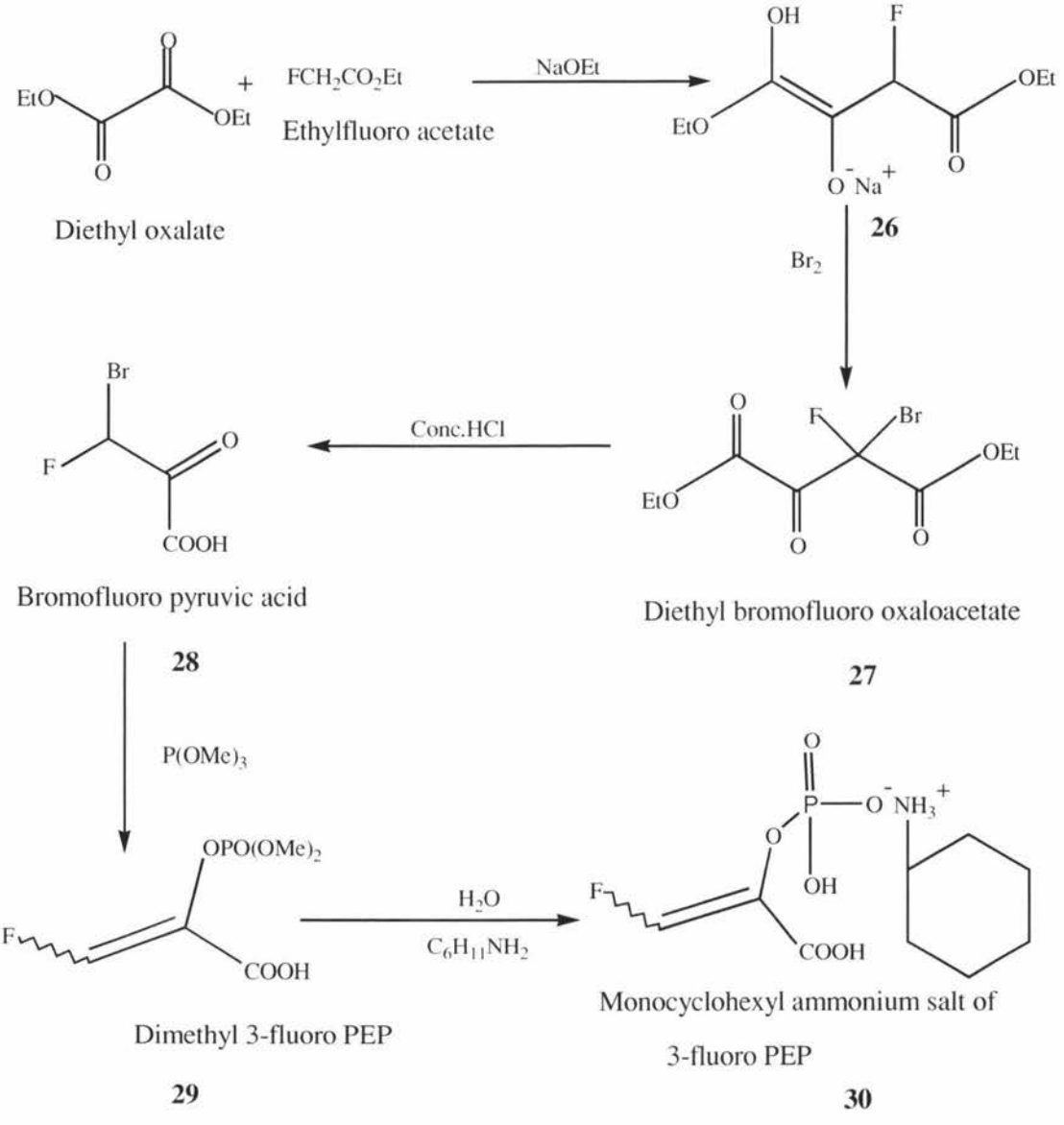


Figure 2.6 Synthesis of 3-fluoro PEP.

The crude 3-fluoro PEP was purified by anion exchange chromatography. The 3-fluoro PEP formed was separated on an Amberlite (IRA 400, NH_4^+ form) column using an Econo pump system. The 3-fluoro PEP was eluted with a binary gradient

of 0 - 1 M ammonium bicarbonate. The 3-fluoro PEP was found to elute at the end of the gradient with 1 M ammonium bicarbonate. The eluted 3-fluoro PEP was treated with Dowex (50W-X8, H^+) to remove the NH_4^+ and freeze-dried. From the ^{19}F NMR spectrum and from the ^1H NMR spectrum (**Figure 2.8, Figure 2.7**), it appeared that clean separation was achieved. However, the 3-fluoro PEP separated in the way appeared to contain a considerable amount of ammonium salt. This caused problem in the subsequent synthesis of 3-fluoro DAHP as explained in Section 2.4.4. Therefore, this purification procedure was omitted and the crude 3-fluoro PEP was used directly in the synthesis of 3-fluoro DAHP.

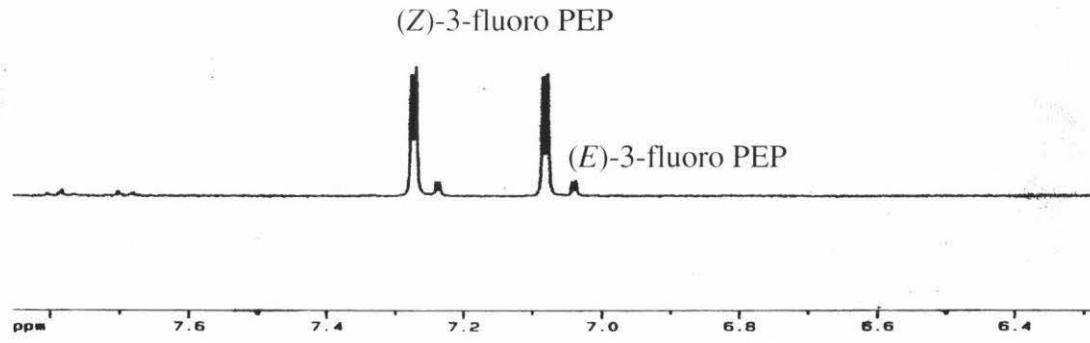


Figure 2.7 ^1H NMR spectrum of purified 3-fluoro PEP.

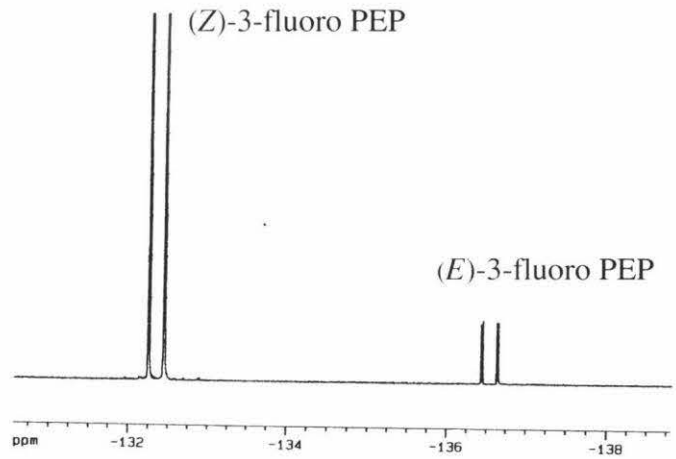


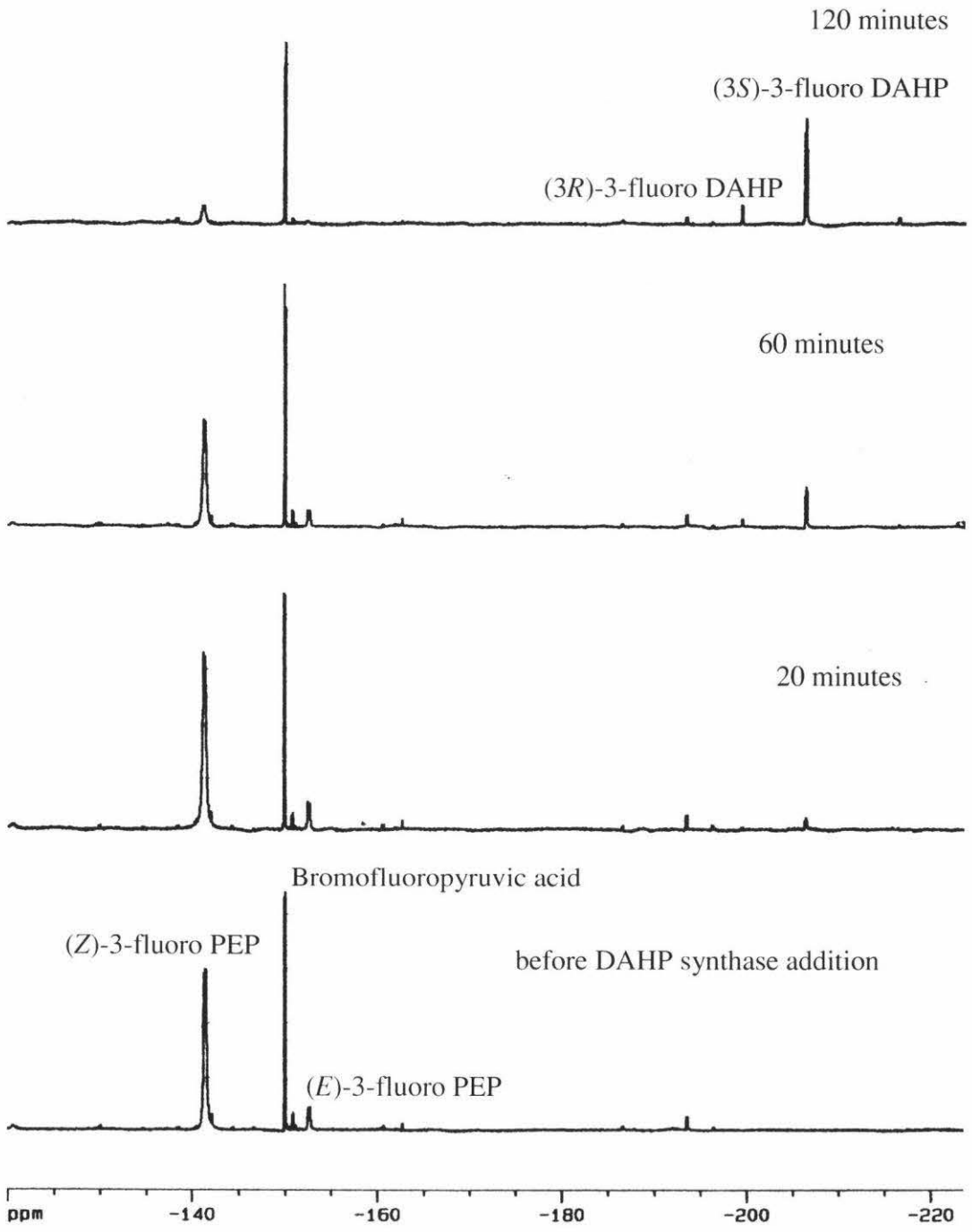
Figure 2.8 ^{19}F NMR spectrum of (*Z*)-and (*E*)-3-fluoro PEP.

2.2.2 Preliminary investigations on the preparation of 3-fluoro DAHP

Before attempting a large-scale synthesis of 3-fluoro DAHP, it was decided to carry out a small-scale synthesis of 3-fluoro DAHP in a NMR tube. The progress of the reaction was followed by ^{19}F NMR spectroscopy. The results are shown in **Figure 2.9**. Crude 3-fluoro PEP was used in the experiment, and therefore some contaminant peaks are obvious from the spectra. The most prominent peak at -150 ppm is due to contaminating bromofluoropyruvic acid. However, when the enzyme was added two new peaks were observed and the peak intensities of these increased with time. At the same time the signal intensities of the isomers of 3-fluoro PEP decreased. The peak due to bromofluoropyruvic acid did not change during the course of the reaction.

The major new peak at -206 ppm is due to the α - pyranose form of (3*S*)-3-fluoro DAHP and the peak at -198 ppm is due to the (3*R*)-3-fluoro DAHP. The other minor peak formed at ~ 215 ppm is due to the β-pyranose form of (3*S*)-3-fluoro DAHP. These assignments were consistent with the earlier assignments of Parker.[28] From the spectra it is clear that the enzyme DAHP synthase catalyses the formation of both the isomers of 3-fluoro DAHP at a similar rate, which is in agreement with the earlier results of Parker.[28]

For the small-scale synthesis, commercially available erythrose 4-phosphate was used. However it is very expensive. Therefore, for large-scale synthesis it was decided to generate erythrose 4-phosphate *via* the transketolase reaction of fructose 6-phosphate and ribose 5-phosphate to give erythrose 4-phosphate and sedoheptulose 7-phosphate according to the method of Parker.[28] The erythrose 4-phosphate generated is then reacted with the 3-fluoro PEP in the DAHP synthase catalysed reaction to give 3-fluoro DAHP.



[3-fluoro PEP] = 45 mM; [Mn^{2+}] = 80 μM; [E4P] = 40 mM; buffer = 250 mM BTP, pH 7.0; temperature 25 °C

Figure 2.9 ^{19}F NMR spectra for conversion of a mixture of (*Z*)- and (*E*)-3-fluoro PEP into (3*S*)- and (3*R*)-3-fluoro DAHP.

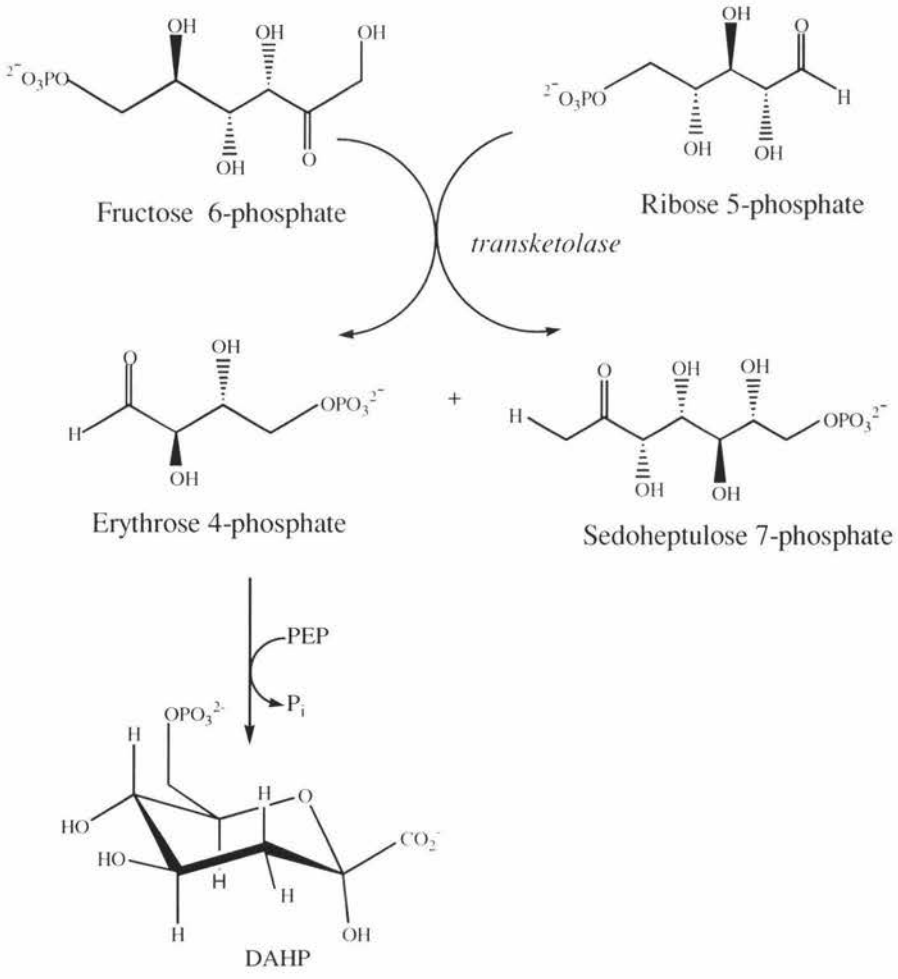


Figure 2.10 Formation of DAHP in solution using transketolase and DAHP synthase.

In the transketolase reaction the formation of erythrose 4-phosphate is reversible, but it is forced irreversibly to product by the condensation with 3-fluoro PEP in the DAHP synthase reaction. It was decided to investigate the preparation of DAHP by this method before it was used in the preparation of 3-fluoro DAHP (**Figure 2.10**).

Calculated amounts of fructose 6-phosphate, ribose 5-phosphate, PEP and cocarboxylase were dissolved in water. The pH of the solution was adjusted to 6.8. Manganese(II)sulphate (50 μM) was added. The enzymes transketolase and DAHP synthase were added and the progress of the reaction was monitored by the

disappearance of PEP at λ = 270 nm. After 12 hours, no more change in absorbance was observed. The amount of DAHP formed was determined by a coupled assay using DHQ synthase and dehydroquinase. It was found that, approximately 20 % of the original PEP had been converted to DAHP. To determine, which reactant was limiting the reaction progress, small amounts of the reactants, fructose 6-phosphate, ribose 5-phosphate, PEP and cocarboxylase were added. No further reaction was observed. More DAHP synthase was added and no progress was observed. It was found that transketolase was limiting, and unfortunately no more transketolase was available to carry out the reaction.

Due to the unavailability of the transketolase, an alternative method was used to prepare DAHP and 3-fluoro DAHP. Erythrose 4-phosphate, prepared by the chemical oxidation of glucose 6-phosphate with Pb(IV) acetate, was reacted with PEP and (*Z*)- and (*E*)-3-fluoro PEP in the presence of DAHP synthase to give DAHP and (3*S*)- and (3*R*)-3-fluoro DAHP, respectively.

2.2.3 Preparation of erythrose 4-phosphate

Erythrose 4-phosphate was prepared by the method of Simpson *et al.*[32] In their method, oxidation of 1 equivalent of D-glucose 6-phosphate with 1.85 equivalents of Pb (IV) acetate mixed with sulphuric acid yields a preparation containing 0.77 equivalents of D-erythrose 4-phosphate and 0.20 equivalents of unoxidised glucose 6-phosphate. About 0.03 equivalents of erythrose 4-phosphate were reported to over oxidise to glyceraldehyde 3-phosphate. If 2 molar equivalents of lead tetraacetate were used in the oxidation, it was reported that the divalent lead formed precipitates some of the glucose 6-phosphate starting material. This affects the stoichiometry of the oxidation and the excess lead tetraacetate in the reaction mixture promotes over oxidation of erythrose 4-phosphate to glyceraldehyde 3-phosphate. It was reported that this over oxidation could be reduced by

1 Using less than the theoretical amount of lead tetraacetate,

2 Using sulphuric acid to precipitate the divalent lead as it is formed during the reaction and adding the sulphuric acid together with lead tetraacetate very slowly to the reaction mixture, so that Pb (IV) is never present in excess.

Erythrose 4-phosphate formed in this way by Simpson *et al.*[32] was then separated from the D-glucose 6-phosphate by chromatography on Dowex 1-formate, and recovered as the barium salt of the hydrazone in 50 % yield. For the studies described in this thesis erythrose 4-phosphate was prepared by a similar procedure, but the amount of reactants was doubled and the purification procedure was omitted.

The chemically prepared erythrose 4-phosphate was assayed with excess of PEP using the enzyme DAHP synthase. No change in the absorbance was observed. The pH of the erythrose 4-phosphate solution was measured and it was found to be 1.5. Since the DAHP synthase catalysed reaction between erythrose 4-phosphate and PEP proceeds at neutral pH, the erythrose 4-phosphate solution was pH adjusted with 2M NaOH. A considerable amount of NaOH (1:1 ratio) was required to adjust the pH to neutral. This is because a large volume of acetic acid was used in the preparation of erythrose 4-phosphate and most of the acetic acid remained in the erythrose 4-phosphate solution. Adjusting the pH with NaOH, not only resulted in an increase in the volume of erythrose 4-phosphate solution but also resulted in the formation of sodium acetate.

From the work done by Simpson *et al.*[32], it is clear that the presence of considerable amounts of acid or sodium acetate in the erythrose 4-phosphate solution causes large losses of erythrose 4-phosphate. In the presence of 0.2 N acetic acid only 41 % of erythrose 4-phosphate was recovered and in the presence of 0.2 M sodium acetate only 36 % of erythrose 4-phosphate was recovered after concentration.[32] The presence of sodium acetate also caused difficulties later in the purification of (3*S*)-3-fluoro DAHP by anion exchange chromatography as explained in the next section. Evaporating or freeze drying the erythrose 4-phosphate solution to remove

acetic acid dephosphorylates the erythrose 4-phosphate and therefore a more effective method of removing acetic acid from the crude erythrose 4-phosphate solution was required.

Subsequently most of the acetic acid was removed by continuous extraction with ether for 15 - 20 hours, and the aqueous solution containing erythrose 4-phosphate was treated with Chelex. The solution was then concentrated on a rotatory evaporator. Then the pH of the erythrose 4-phosphate solution was adjusted with 1 M NaOH and reacted with PEP and 3-fluoro PEP to give DAHP and 3-fluoro DAHP respectively. This time the amount of NaOH needed to adjust the pH was reduced. The amount of erythrose 4-phosphate and glyceraldehyde 3-phosphate produced was calculated from a coupled assay system using the enzymes transaldolase, triose phosphate isomerase and glycerol 3-phosphate dehydrogenase. These analyses are explained in section 5.2 (**Figure 5.1**). When compared to glucose 6-phosphate 85 % of erythrose 4-phosphate yield was observed, however the amount of erythrose 4-phosphate decreased with storage. This loss may have been prevented by purifying the erythrose 4-phosphate solution as described by Simpson *et al.*[32] But this purification procedure was not attempted, as it is necessary to purify 3-fluoro DAHP later and 3-fluoro DAHP is less sensitive than erythrose 4-phosphate. As was reviewed in section 2.1, difficulties were encountered with the handling of erythrose 4-phosphate solution. Therefore freshly prepared crude erythrose 4-phosphate was used for the synthesis of DAHP and 3-fluoro DAHP.

2.2.4 Large scale synthesis of DAHP, (3*S*)-3-fluoro DAHP and (3*R*)-3-fluoro DAHP

As a first attempt the purified 3-fluoro PEP was reacted with the erythrose 4-phosphate prepared by the lead tetraacetate oxidation to produce 3-fluoro DAHP. The progress of the reaction was monitored spectrophotometrically at $\lambda = 270$. The reaction progressed for 3 hours and stopped. Adding more erythrose 4-phosphate or DAHP synthase resulted in no further reaction. This may have been due to

ammonium salts present in the 3-fluoro PEP or due to higher concentrations of acetic acid present in the erythrose 4-phosphate even though the pH was adjusted to neutral. As discussed in Section 2.2.1, 3-fluoro PEP was eluted with 1M ammonium bicarbonate during its purification. The presence of ammonium salts in erythrose 4-phosphate solution causes problems in handling erythrose 4-phosphate solutions and considerable difficulty was encountered with the removal of acetic acid from the erythrose 4-phosphate solution, as discussed in the previous section.
To know the cause for the difficulty encountered with the preparation of 3-fluoro DAHP it was decided to prepare DAHP by reacting commercially available PEP with erythrose 4-phosphate prepared by chemical oxidation, in the DAHP synthase catalysed reaction. Only a very small amount of PEP was converted to DAHP. It appears that the reason for the poor yields in these preparations was the acetic acid present in the erythrose 4-phosphate solution.

Therefore for the successful synthesis of DAHP and 3-fluoro DAHP, care was taken in the synthesis of erythrose 4-phosphate to make sure to remove most of the acetic acid from the erythrose 4-phosphate solution. The DAHP produced was freeze-dried and left in the -70 °C freezer and later purified by anion exchange chromatography.

Although a reasonable amount of 3-fluoro DAHP (40 mg) was produced, the percentage yield from 3-fluoro PEP was low. As evidenced from the trace from the purification by anion exchange chromatography (**Figure 2.12**), a huge peak at about 100 minutes was, due to unreacted 3-fluoro PEP. Adding more erythrose 4-phosphate may have allowed the reaction to achieve completion. However, after 48 hours the reaction was stopped by removing the enzyme from the reaction mixture using centricon concentrators with molecular weight cut-off 10. The filtrate was freeze dried and left in the -70 °C freezer. Later, it was purified by anion exchange chromatography.

2.2.5 The purification of (3*S*)-3-fluoro DAHP

A method of purifying (3*S*)-3-fluoro DAHP from the (3*R*)-3-fluoro DAHP was required. In the earlier studies of Parker,[28] (3*S*)-3-fluoro DAHP was separated from the other products with Pharmacia MonoQ 10/10 column, eluting with 0 - 1 M ammonium bicarbonate gradient.

UnoQ S6 and MonoQ (5/5) anion exchange columns were investigated for the purification. With the UnoQ S6 column it was observed that the DAHP or (3*S*)-3-fluoro DAHP did not bind to the column. Using the MonoQ (5/5) column produced better results, and considerable separation was achieved. The DAHP and 3-fluoro DAHP used in these investigations were synthesised from the earlier preparation of erythrose 4-phosphate, which contained considerable amount of acetic acid. As explained in the previous section these preparations contained a large amount of sodium acetate that caused difficulties in the purification by anion exchange chromatography.

However with the arrival of a new MonoQ (10/10) anion exchange column a more satisfactory purification was achieved than with the UnoQ S6 or the smaller MonoQ (5/5) columns. Fractions containing DAHP and (3*S*)-3-fluoro DAHP were detected using a coupled assay system that used the enzymes DHQ synthase and dehydroquinase and the production of dehydroshikimate was observed at λ = 234 nm. The conditions employed for this assay is in the experimental section. The (3*S*)-3-fluoro DAHP was found to elute between 30 – 40 % of 1 M ammonium bicarbonate solution. The fraction containing DAHP and (3*S*)-3-fluoro DAHP were then pooled and freeze-dried. From the ^{19}F NMR spectrum it appeared that the (3*R*) isomer was separated from the (3*S*) isomer. ^{19}F NMR spectrum of purified (3*S*)-3-fluoro DAHP is given in **Figure 2.11**. From the fluorine signal at -206.5 ppm (dd , J = 49.2, 30.2 Hz) it was established that the C3 fluorine is coupled to C3 proton as well as C4 proton. The three bond coupling constant value of J = 30.2 Hz observed between C4 proton (axial) and the C3 fluorine is consistent with a transdiaxial orientation of both the atoms in the product. This result is in agreement

with the earlier reported observation and supports the facial selectivity of the DAHP synthase catalysed reaction.[28] The purified (3*S*)-3-fluoro DAHP was used in the investigation with DHQ synthase and is described in Chapter 4. The DAHP synthesised was also purified by the same procedure and was used in the investigation of the effects of temperature and pH on the catalytic activity of DHQ synthase. These experiments are discussed in Chapter 4.

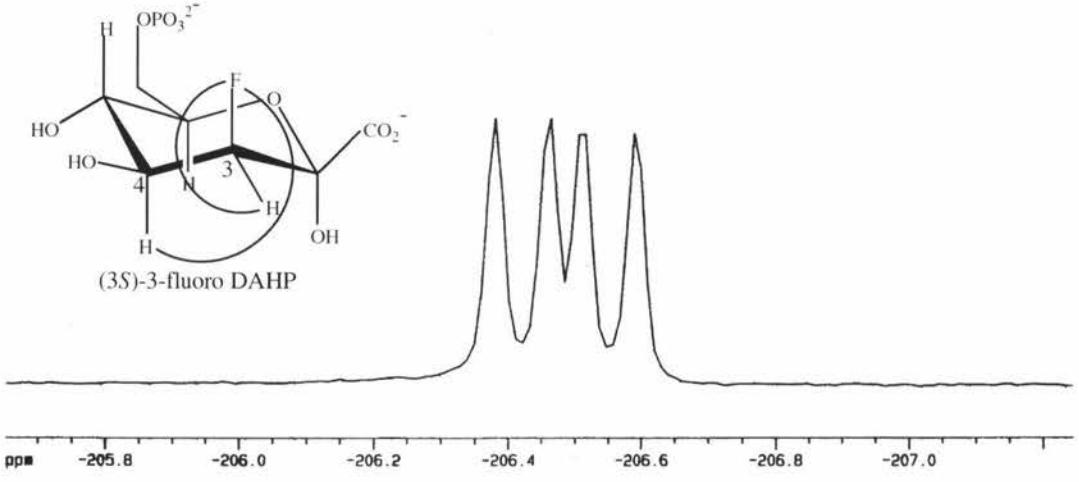


Figure 2.11 ^{19}F NMR spectrum of (3*S*)-3-fluoro DAHP.

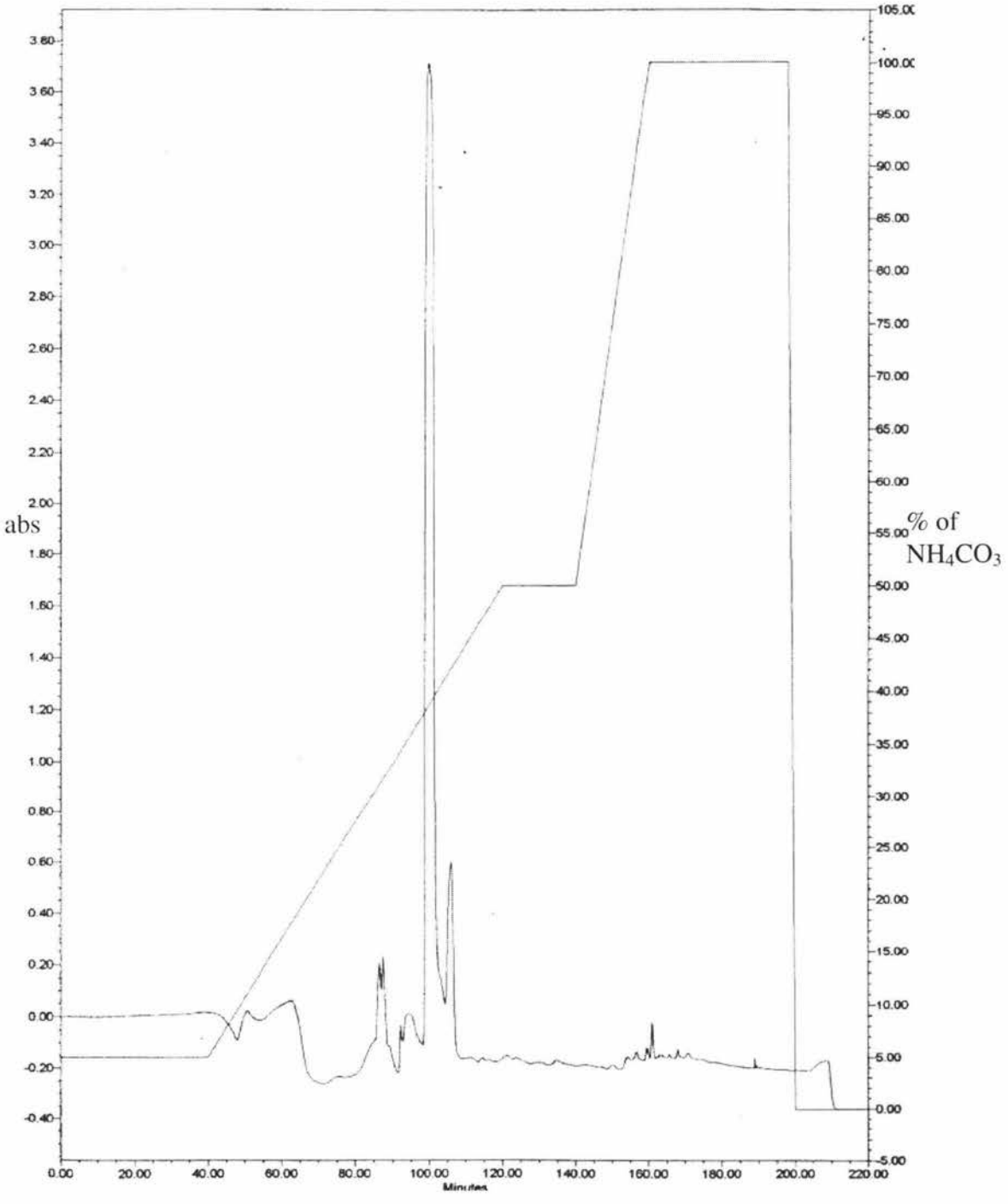


Figure 2.12 A_{230} trace of separation of (3*S*)-3-fluoro DAHP with a binary gradient of 50 – 1000 mM NH_4CO_3.

2.3 Studies of *E. coli* DAHP synthase (phe) with 3-fluoro PEP and five-carbon analogues.

E. coli DAHP synthase (phe) catalyses the condensation of D-erythrose 4-phosphate with phosphoenolpyruvate to give DAHP. It has been reported that *E. coli* DAHP synthase (phe) also 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 to give 3-deoxy-D-*manno*-octulosonate 8-phosphate, 3-deoxy-D-*altro*-octulosonate 8-phosphate, and 3,5-dideoxy-D-*gluco(manno)*-octulosonate 8-phosphate, respectively. The kinetic parameters were also reported and are summarised below.[31]

Monosaccharide	K_M^{PEP} (μM)	$K_M^{monosaccharide}$ (μM)	V_{max} (μmol/mg/min)
E4P	5.3	141	16.3
R5P	10	6000	1.13
A5P	5	30	0.085
2-deoxy-R5P	15	6800	0.73

Recently Sundaram and Woodard[35] reported the products formed between these five carbon monosaccharide analogues with (*Z*)- and (*E*)-[3-^{2}H]-phosphoenolpyruvate in the presence of *E. coli* DAHP synthase (phe). The (*Z*)-isomer gave the corresponding (3*S*)-monosaccharides and the (*E*)-isomer gave the corresponding (3*R*)-monosaccharides, which is in complete agreement with the facial selectivity of DAHP synthase for its normal substrates PEP and D-erythrose 4-phosphate.

As 3-fluoro PEP was synthesised for the production of (3*S*)-3-fluoro DAHP, it was decided to utilise 3-fluoro PEP with the five carbon analogues D-arabinose 5-phosphate, and D-ribose 5-phosphate in the presence of *E. coli* DAHP synthase (phe). 3-Fluoro PEP and the five carbon monosaccharides were incubated in a cuvette with purified DAHP synthase in the presence of Mn^{2+} and at neutral pH.

The progress of the condensation of 3-fluoro PEP with D-arabinose 5-phosphate, and D-ribose 5-phosphate was monitored by UV-spectrophotometry by the disappearance of the absorption at λ = 232 nm. The conditions used in the experiment are summarised in the experimental section. It was observed that the absorbance decreased with time. The rate of change of absorbance for D-arabinose 5-phosphate was observed to be very much slower than the absorbance change for D-ribose 5-phosphate. This is consistent with the observation of Sheflyan *et al.*[31] who reported that, the rate of turnover for D-arabinose 5-phosphate with PEP is 200 times slower than the rate of turnover of E4P with PEP, while the rate of turnover of D-ribose 5-phosphate was 14 times slower. Therefore it was decided to perform some experiments with D-ribose 5-phosphate as a substrate in order to verify, whether this change in absorbance observed is due to the depletion of 3-fluoro PEP by D-ribose 5-phosphate.

It was decided to attempt to follow the reaction between 3-fluoro PEP and D-ribose 5-phosphate by ^{19}F NMR spectroscopy. This would prove, whether the slow rate observed with D-ribose 5-phosphate is indeed due to the turnover of D-ribose 5-phosphate with 3-fluoro PEP and also provide information about the product formed between 3-fluoro PEP and D-ribose 5-phosphate. Several attempts were made to follow the reaction by ^{19}F NMR spectroscopy. However, the rate of turnover was very slow and little progress was achieved. The reaction was carried out at 37 °C and followed by ^{19}F NMR spectroscopy. Increasing the temperature did not improve the results. Varying the concentrations of the reactants or the enzyme did not appear to make much difference. Some new peaks were observed in the ^{19}F NMR spectra, but these were small and did not increase substantially with time. From the earlier work of Kohen *et al.*[23] with 3-fluoro PEP and KDO8P synthase, one would expect peaks in the region between -180 ppm to -215 ppm. Two small new peaks were observed in the region between -225 and -235 ppm. Another peak was observed at -196 ppm. However none of the peaks were a clear doublet. In all the attempts a new singlet peak at -120 ppm was observed and it

might be due to decomposition of 3-fluoro PEP or bromofluoropyruvic acid, which was a contaminant in the 3-fluoro PEP.

The kinetic measurements reported by both Parker[28] and Sheflyan *et al.*[31] have shown that the rate of turnover of 3-fluoro PEP with E4P is 100 times slower than the rate of turnover of PEP with E4P, and the rate of turnover of D-ribose 5-phosphate with PEP is 14 times slower than the rate of turnover of E4P with PEP. Therefore the rate of turnover of D-ribose 5-phosphate with 3-fluoro PEP would be expected to be about 1400 times slower than that with PEP and E4P. From our preliminary experiments it appears that the relative rate of D-ribose 5-phosphate with 3-fluoro PEP we are observing is in the same order of magnitude with this expected rate.

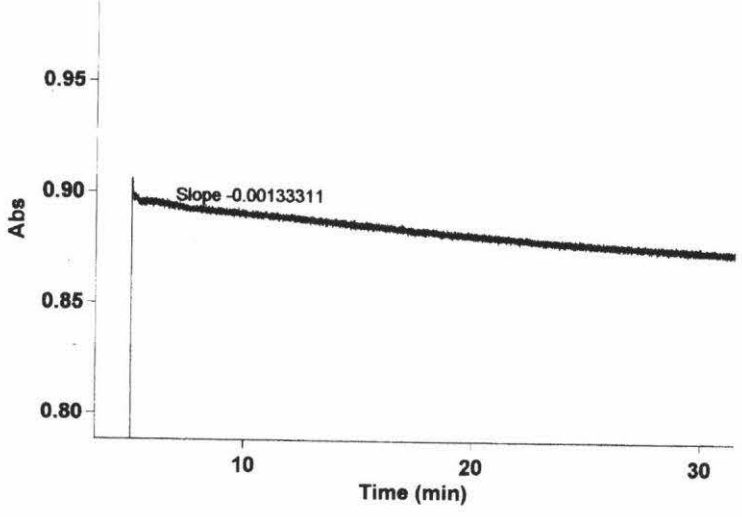


Figure 2.13 The progress of the reaction between D-ribose 5-phosphate and 3-fluoro PEP by spectrophotometrically at $\lambda = 232$ nm. The absorbance change was dependent on the concentration of ribose 5-phosphate and on the amount of enzyme.

It was also noted with these experiments, as would be expected that the rate was dependent on D-ribose 5-phosphate concentration. As the rates observed were so

slow a full K_M calculation was however not done. Assays with 3-fluoro PEP and D-ribose 5-phosphate were difficult because of the high enzyme concentration required and the presence of PEP in the enzyme preparations. From the earlier work it has been shown that, the enzyme DAHP synthase is unstable in the absence of PEP.[28] Therefore all the DAHP synthase enzyme preparations were stored in a buffer containing small amount of PEP. It was observed that, when the enzyme was added to the D-ribose 5-phosphate, 3-fluoro PEP solution, a sudden fall in absorbance, followed by a slower change in absorbance. This is due to the depletion of PEP by D-ribose 5-phosphate, followed by the depletion of 3-fluoro PEP by D-ribose 5-phosphate. To minimise the concentration of PEP the enzyme would need to be substantially concentrated. This was not done in these preliminary experiments.

These observations are consistent with DAHP synthase also catalysing the condensation between 3-fluoro PEP with D-arabinose 5-phosphate, and D-ribose 5-phosphate but not surprisingly with a very slow turnover rate. To prove that they form the expected eight carbon sugars further experiments are needed in the future. The most useful one would be the periodate -thiobarbituric acid assay to detect the presence of a 2-deoxymonosaccharide.[24]

3 Dehydroquinate synthase : Enzyme preparation

3.1 Introduction

To investigate the interaction of the enzyme 3-dehydroquinate synthase with (3*S*)-3-fluoro DAHP, a considerable amount of 3-dehydroquinate synthase had to be prepared and purified. Several workers[16, 24] have purified the monofunctional 3-dehydroquinate synthase from *E. coli* to homogeneity. The *E. coli* enzyme was first studied by Sprinson and his colleagues[33], who observed that the enzyme required a catalytic amount of NAD^+ (*i.e.* stoichiometric with the enzyme) for activity. Frost *et al.*[15] obtained a 9000-fold purification from wild type *E. coli* K12, but the preparation was not homogeneous. Using recombinant DNA technology, they produced a strain (*E. coli* pLC 29-47) with a 20-fold increase in DHQ synthase activity in crude lysates. A 550-fold purification of 19 g of cells from this strain yielded nearly 2 mg of almost homogeneous enzyme. Later they obtained larger quantities of the pure enzyme by subcloning the gene for DHQ synthase (*aroB*) from plasmid pLC 29-47 of the Carbon Clarke library of the *E. coli* K12 genome. This engineered strain overproduces DHQ synthase to the extent that the enzyme constitutes about 5 % of the soluble protein.

The primary structure of *E. coli* enzyme was deduced by Millar and Coggins.[25] The molecular weight calculated from the 362 amino acids sequence is 38,880 and the enzyme is a monomer, which contains one tightly bound Co^{2+} and one NAD^+ per

one mole of the polypeptide chain. Zn^{2+} was also bound at a second lower affinity inhibitory site. On the basis of the greater availability of Zn^{2+}, it seems likely that DHQ synthase is naturally a Zn^{2+}- metalloenzyme, yet for *in vitro* studies, Co^{2+} offers several practical advantages.

Bender *et al.*[6] studied the role of metal cations and of NAD^+ in catalysis and found that on incubation with EDTA the enzyme lost activity rapidly, giving rise to a stable, but inactive, apoenzyme. The addition of excess Co^{2+} restored catalytic activity completely, but Zn^{2+} restored only 15 % of the catalytic activity. They also noted that NAD^+ dissociated from the NAD^+- enzyme complex with a half-life of about 10 hours. The dissociation of NAD^+ from the holoenzyme is a complex process and is accelerated 40-fold under turnover conditions with saturating levels of the substrate. Even in the presence of DAHP, NAD^+ analogs can bind to the enzyme with high affinity. Unless high purity NAD^+ is used, catalytic activity may be lost as a result of the presence of unidentified impurities that function as inhibitors.[24] This is an important practical consideration when using and purifying this enzyme.

3.2 Results and Discussion

The cell growth was carried out in LB media, similar to the procedure used by Frost *et al.*[16] The *E. coli* strain (pJB14) bearing an expression vector containing the *aro B* gene was initially grown on LB plates. Cultures were grown using minimal media in a large 2 L flask kept on a shaker at 37 °C. At midlogarithmic phase IPTG was added to induce the expression of DHQ synthase. The cells were harvested by centrifugation and the cell pellets were stored at -20 °C. Cells destined for long periods of storage were made into cell popcorns and stored at -70 °C.

Initially the production of DHQ synthase was confirmed by lysing the cells and assaying the crude cell extract for activity by coupling to dehydroquinase and following the increase in absorbance at 234 nm due to the production of 3-

dehydroshikimate. This was necessary because neither the substrate nor the products of the reaction of DHQ synthase absorb significant UV light. For this experiment some cell pellets (stored at -20 °C) were suspended in 50 mM BTP buffer rather than in a Co^{2+}, NAD^+ and glycerophosphate buffer which Frost *et al.*[16] used, and sonicated with the thin probe at a power setting of 3 and centrifuged to remove the cell debris. The supernatant was tested for activity and kept at 4 °C for purification. But after a week it was observed that most of the activity had been lost. This loss of activity is likely to be due to the absence of Co^{2+} and NAD^+ from the lysis buffer as DHQ synthase has a requirement for Co^{2+} and NAD^+ for catalytic activity as well as for structural stability of the protein.[22] Therefore all buffers were made up with the presence of Co^{2+} and NAD^+. It was observed that the stability of DHQ synthase is improved by the presence of $CoCl_2$ and of NAD^+ and the activity of the crude lysate was retained for at least a week at 4 °C.

Problems were also encountered with lysing cells. Sonication had to be carried out for longer periods than reported earlier.[15] Some cells were suspended in β-glycerophosphate buffer containing Co^{2+} and NAD^+, and sonicated for longer periods and when the cells were centrifuged a brownish yellow precipitate was observed. This precipitation may be due to NAD^+ analogues binding with the enzyme. According to the procedure of Parker the cells were suspended in phosphate buffer with 100 mM NaCl, DTT, sucrose and spermidine to remove DNA and lysed with BRIJ and lysozyme.[28] This procedure was omitted in our preparation and the cells were ruptured by sonication. This may also be the cause for the longer pulses required for sonication and the formation of yellowish brown precipitate from the crude lysate after centrifugation.

Therefore some cells were suspended in the same buffer and lysed by a French press cell. Better activity was observed and it appears that lysing cells by a French press cell was more effective in breaking the cells. This crude lysate was purified on a hydroxyapatite (biogel) column according to the procedure of Parker.[28] The crude lysate was diluted five times with the starting buffer. It was loaded on to the

column, washed with five column volumes of the starting buffer and eluted with a gradient of 0 - 75 mM phosphate in 10 mM β-glycerophosphate buffer (pH 6.6) containing Co^{2+} and NAD^+. The purification was left running overnight and the fractions were tested for activity on the following day. A single peak was observed in the chromatogram at about 40 - 45 % phosphate concentration. When those fractions were tested for enzyme activity no activity was observed. The exact reason for this inactivity is unclear. The reason for this inactivity may be due to the removal of Co^{2+} by hydroxyapatite from the eluting buffer. This left the protein in a diluted form in the buffer without Co^{2+} for a considerable length of time, or the protein eluted may not be the DHQ synthase fraction. But, when these fractions were left in air the colour of the solution turned yellow, which suggested, these fractions may contain NAD^+, as NADH solutions left in the air turn yellow. The hydroxyapatite packing material must be discarded after use as the leading portion of the column becomes loaded with Co^{2+}. For this reason, it was decided to try anion exchange chromatography.

MonoQ and SourceQ anion exchange columns were both examined to determine which achieved the best purification. The protein was first purified on a small packed SourceQ (5/5) column. The crude lysate was washed and desalted by concentration and dilution using Amicon concentrator, and loaded on to the column. This step was done in order to remove NAD^+ and Co^{2+} from the lysis buffer, which might affect the performance of the anion exchange column. The protein was eluted with a gradient of 0 - 1 M NaCl in BTP buffer (10 mM, pH 6.6) over 100 ml. The protein was found to elute between 12 - 15 % of 120 - 150 mM NaCl salt concentrations. Two-ml fractions were collected. The fractions were tested for DHQ synthase activity, and the active fractions were 3 to 4 times repeatedly diluted with 2 ml of β-glycerophosphate buffer containing Co^{2+} and NAD^+, and concentrated to 0.5 to 1 ml. Immediately, the active fractions were snap frozen in liquid nitrogen and stored at -70 °C. This was done in order to avoid any loss of activity during storage. The active fractions were run on a 12 %

polyacrylamide SDS gel and are shown in **Figure 3.1**. The conditions for the preparation of SDS gel are explained in the experimental section.

The MonoQ column was also tested for purification with the same procedure used for the SourceQ purification. Using this column the protein appeared to bind more tightly and was found to elute between 20 - 25 % of salt concentrations. The active fractions separated by the Mono Q column and some other active fractions separated with the Source Q column were run together on a 12 % polyacrylamide SDS gel to observe whether there are any differences between these columns (**Figure 3.2**). The difference between these columns is that, in the MonoQ column, the band is narrower and the protein is found mainly in three fractions. But in the SourceQ column the band is slightly wider and the protein is spread in more fractions. It is also interesting to note that, in all the active protein fractions, another impurity band at ~ 45 KDa was observed, in addition to ~ 40 KDa DHQ synthase band. The exact identity of this band was unknown. However from the SDS gel (**Figure 3.2**) it appeared that there is not significant benefits in using the more expensive MonoQ column. Therefore, as we were concerned about loading such a crude sample, it was decided to use cheaper SourceQ column.

The SourceQ column was used again for the purification with a slower flow rate of 0.5 ml/min and with slower gradient. The salt gradient was applied for 150 minutes. For the first 120 minutes 0 - 400 mM salt gradient was applied and then the next 30 minutes a steeper 400 - 1000 mM salt gradient was applied. The protein was found to elute between 10 - 15 % salt concentrations. The active fractions were run on 12 % polyacrylamide SDS gel and are shown in **Figure 3.3**. Fraction 18 (lane 6) was the most pure fraction. The most pure fraction purified by this manner had 5.4 units/ml of activity.

Although the activity of the enzyme was maintained throughout the purification, an extremely large peak was eluted with 1 M salt concentration. When these fractions were left for a day at 4 °C, the colour of the solution turned yellow. To determine

whether, this peak contains proteineous material, a 20 µl sample from this yellow solution was run on a protein gel and are shown in Figure 3.2, lane 10. From the protein gel (**Figure 3.2, lane 10**), it is obvious that the yellow solution eluted with 1 M NaCl also has the impurity band at ~ 45 KDa. Since the solution turned yellow and the high absorbance observed suggest that this peak also had NAD^+ in it.

Bender *et al.*[6] reported, that the use of NAD^+ that are not of the highest purity results in the exponential loss of catalytic activity to a new steady state in which much of the enzyme bound to some unidentified inhibitor. In this studies 95 % NAD^+ from Sigma Chemical Company was used. The NAD^+ may also be the reason for the difficulty encountered with the purification of DHQ synthase by hydroxyapatite column.

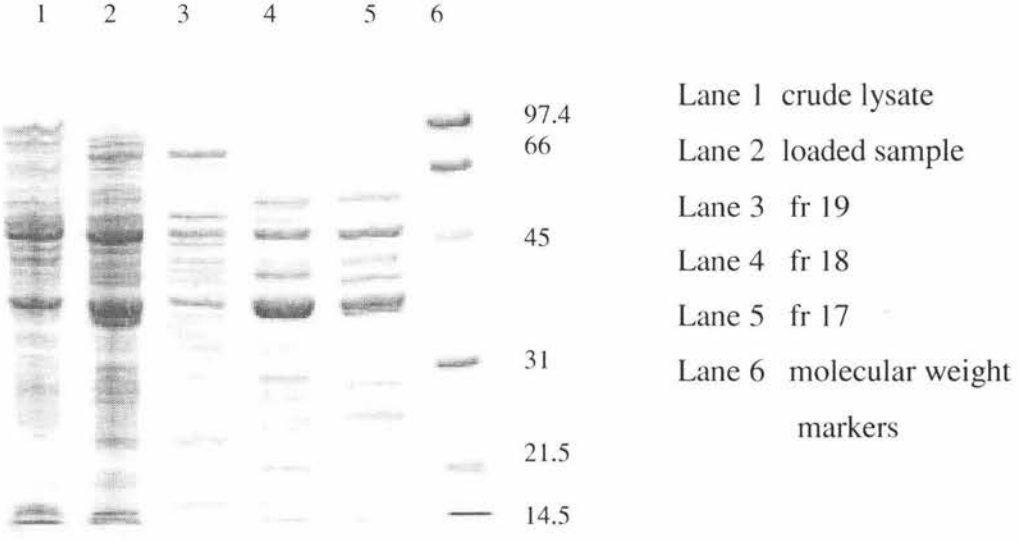


Figure 3.1 SDS PAGE of active fractions from SourceQ separation.

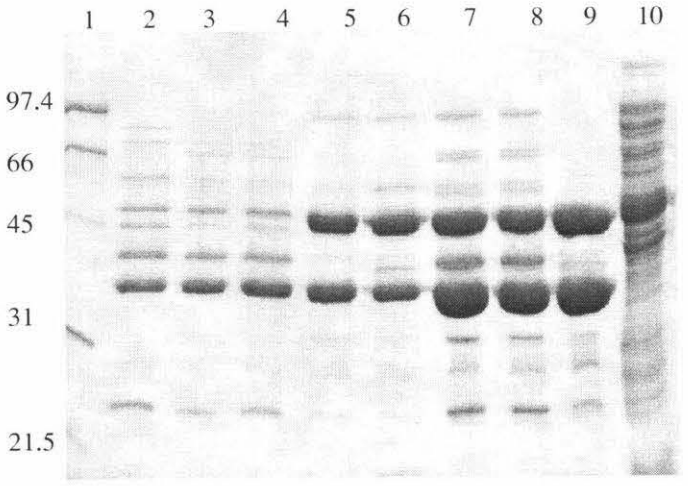


Figure 3.2 SDS PAGE of active fractions.

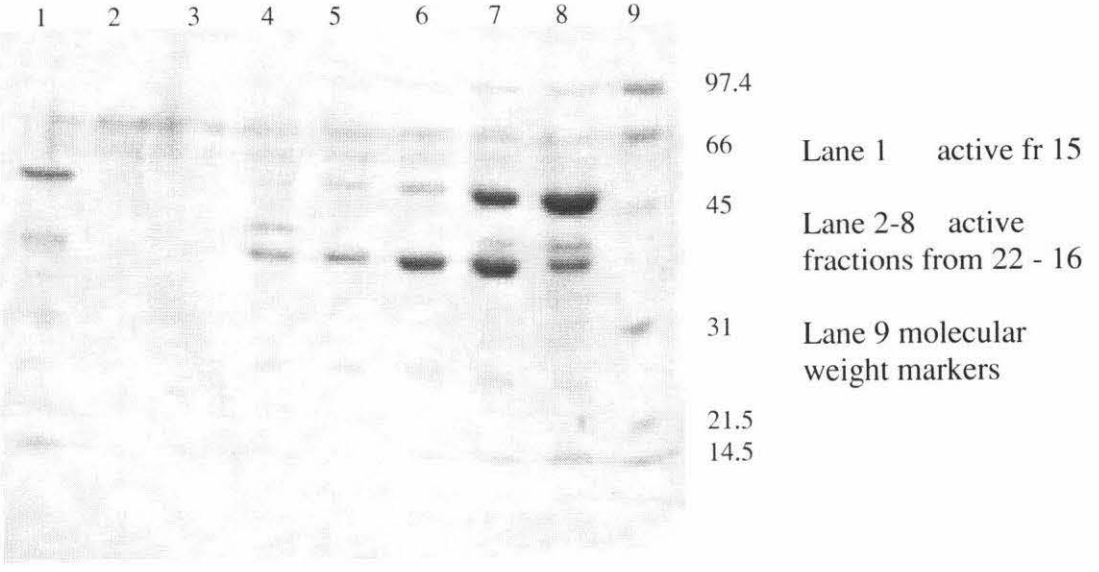
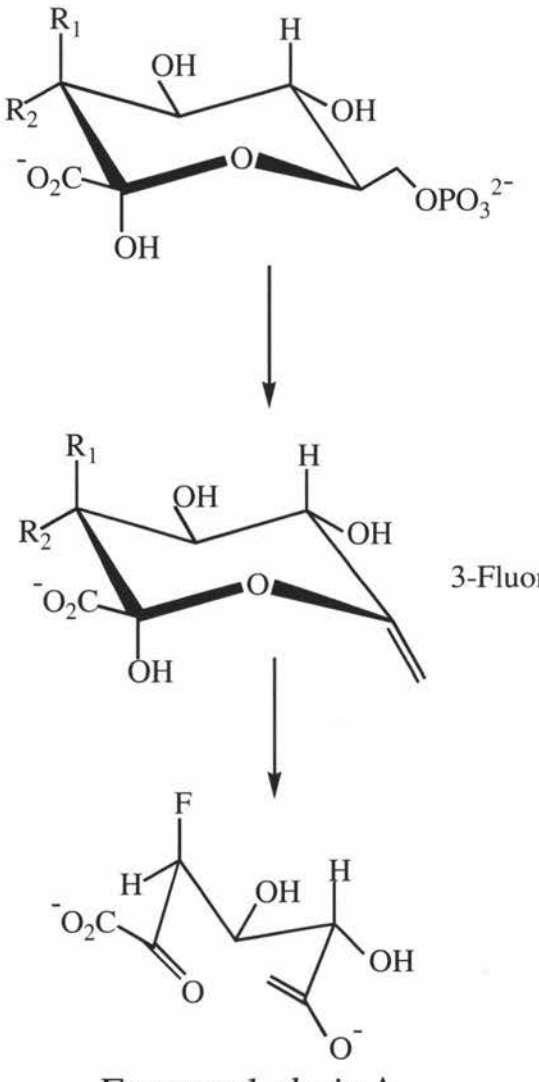


Figure 3.3 SDS PAGE of fractions separated by SourceQ column with slower gradient.

The active fractions separated from all purifications were combined and again separated with the SourceQ column using the same gradient to determine whether further separation could be achieved. Better separation was achieved however repeated purification caused protein loss and was time consuming. Therefore, the remaining crude lysate was purified on a SourceQ column with the same slower gradient. Although it is desirable to use highly purified enzyme, due to the difficulty faced with the hydroxyapatite column and due to the loss of protein by repeated purification by anion exchange chromatography, partially purified enzyme was used in preliminary experiments to test the interaction with (3*S*)-3-fluoro DAHP. From this studies, it is significant that anion exchange chromatography can also be used for the purification of DHQ synthase. Although complete purification was not achieved, a reasonable purification was achieved using anion exchange chromatography as the first step.



3-Fluoroenolpyranose intermediate

Favoured *chair* **A**

R_1 = F , R_2 = H (3*S*)-3-fluoro DAHP

R_1 = H , R_2 = F (3*R*)-3-fluoro DAHP

4 Interaction of (3*S*)-3-fluoro DAHP with 3-dehydroquinate synthase

4.1 Introduction

The results from the treatment of (3*S*)-3-fluoro DAHP with partially purified enzyme DHQ synthase at different temperature and pH values are discussed in this Chapter. As reviewed in Chapter 1, (3*S*)-3-fluoro DAHP has been shown to give rise to two reaction products (6*S*)-6-fluorodehydroquinate and (6*S*)-6-fluoro-1-*epi*dehydroquinate in a 2:1 ratio when reacted with DHQ synthase. It has been suggested that, due to the stereoelectronic influence of fluorine substitution on (3*S*)-isomer, the fluoroenolpyranose intermediate dissociates from the enzyme and cyclises free in solution.[27] This is because, in the (3*S*)- isomer the axial position of fluorine disfavours the favoured *chair* A transition state for the formation of 3-dehydroquinate. As explained in the introduction, it is not known, whether both (6*S*)-6-fluorodehydroquinate and its C1 epimer are formed entirely due to non-enzymatic reaction of the enzyme-generated fluoroenolpyranose or the expected (6*S*)-6-fluorodehydroquinate is formed on the enzyme and the C1 epimer is formed off the enzyme.

To understand this result further, (3*S*)-3-fluoro DAHP was treated with partially purified enzyme DHQ synthase at different reaction conditions and the reaction

progress was monitored by ^{19}F NMR spectroscopy to verify whether there are any differences between the ratio of (6*S*)-6-fluorodehydroquinate and (6*S*)-6-fluoro-1-*epi*dehydroquinate formed. If (6*S*)-6-fluorodehydroquinate is formed on the enzyme and its epimer is formed off the enzyme, adjusting the reaction conditions may alter the ratio of these products. If the two products were formed entirely due to non-enzymatic cyclisation under the same influence in solution, then changing the pH or temperature might not be expected to significantly alter the ratio.

Changes in temperature and pH might be expected to have different effects on on-enzyme and off-enzyme reactions and so might contribute to the product distribution. If the (6*S*)-6-fluorodehydroquinate and its epimer are formed by on-enzyme and solution cyclisation respectively, then at physiological pH and at optimal temperature on-enzyme product would be more favoured than the off-enzyme product. Therefore, it was decided to carry out the experiments at different temperature and pH values as an initial probe for understanding the influence of the fluorine substitution on the DHQ synthase reaction.

A series of experiments was carried out initially with purified DAHP and the partially purified enzyme DHQ synthase in order to determine the influence of pH and temperature changes on the catalytic activity of the enzyme. In the presence of Co^{2+} and NAD^+, the catalytic activity of DHQ synthase with DAHP was monitored against temperature and pH values. To determine the effect of pH, the appropriate pH BTP buffer was prepared and the sample pH was adjusted to the correct pH with the appropriate buffer. For temperature experiments, the sample was equilibrated to the correct temperature and the enzymes were added. The change in absorbance was observed with the coupled enzyme assay using dehydroquinase and DHQ synthase at A_{234}. The rate was obtained from the first 10 s of the progress curve and the relative rate was plotted against the temperature and pH values and are shown in **Figure 4.1**.

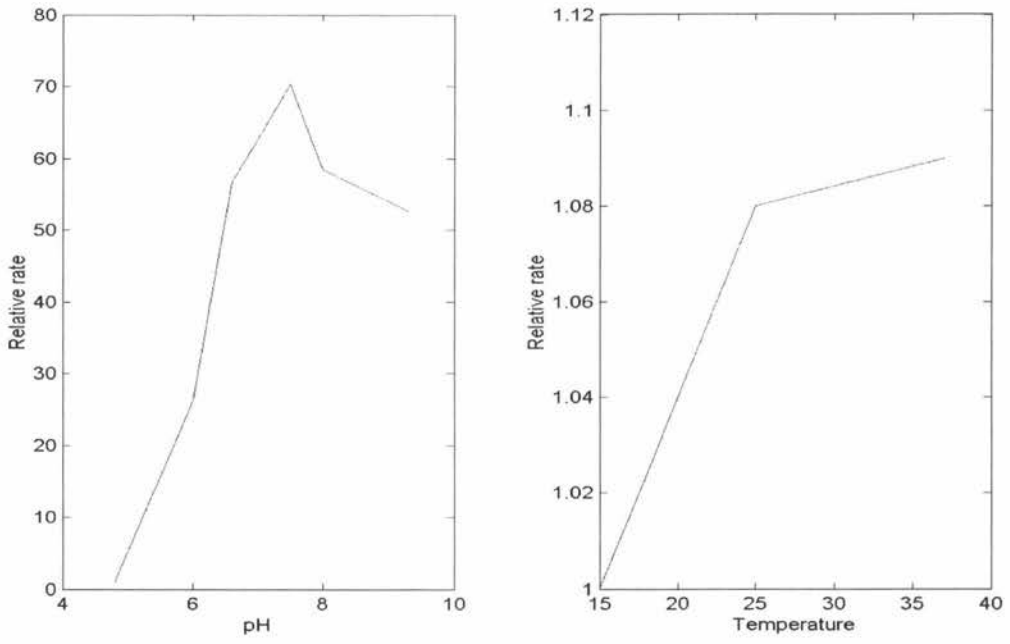


Figure 4.1 The effect of temperature and pH on the activity of DHQ synthase.

These results place limitations on the extremes of pH and temperature that can be employed. From these results, it is obvious that the most suitable pH for this reaction is between 7 and 8 and the most suitable temperature is 25 °C. Based on these results it was decided to carry out the subsequent experiments at pH 6 and 8 and at temperatures of 15 °C and 37 °C to get reasonable rate of reaction. A reasonable rate of reaction was necessary in order to follow the reaction by ^{19}F NMR spectroscopy.

4.2 Results and Discussion

The results of the effect of pH and temperature on the DHQ synthase reaction with (3*S*)-3-fluoro DAHP are detailed below. A calculated amount of pure sample of (3*S*)-3-fluoro DAHP was weighed and dissolved in D_2O. This solution was divided into two portions. NAD^+ and Co^{2+} were added to each portion, and the required pH (pH 6 and pH 8) was adjusted with 100 mM BTP buffer to a final volume of 1 ml. Partially purified DHQ synthase was added and the ^{19}F NMR spectra obtained are

given in **Figure 4.4**. The (3*S*)-3-fluoro DAHP was observed to be completely converted to the products within two hours. The spectra were obtained with 256 scans and the base lines were difficult to correct because of the small concentration of fluorine. Therefore, for the next experiment it was decided to increase the amount of (3*S*)-3-fluoro DAHP used.

The second experiment was performed at a pH of 6.8 and a temperature of 15 °C. The sample was incubated in a cuvette and the temperature of the spectrophotometer was set at 15 °C. When the sample had equilibrated to the correct temperature the same amount of DHQ synthase was added. In this experiment also (3*S*)-3-fluoro DAHP was completely converted to products in two hours time. The ^{19}F NMR spectrum obtained is given in **Figure 4.6**. By increasing the amount of (3*S*)-3-fluoro DAHP by 4 mg, some improvements in signal to noise ratio was observed. However, the amount of (3*S*)-3-fluoro DAHP was further increased by 3 mg in the next experiment to improve the results.

The third experiment at 37 °C was carried out in a temperature-controlled room. The sample was pH adjusted to 6.8 and, when the sample had equilibrated to 37 °C the same amount of DHQ synthase was added. The ^{19}F NMR spectrum obtained is given in **Figure 4.5**. This reaction was not completed in two hours. All the other reactions were completed within two hours. This may be due to the higher concentration of 3-fluoro DAHP used in the reaction.

It was decided to carry out the fourth reaction at pH 7.3 with the remaining 5 mg of (3*S*)-3-fluoro DAHP. This experiment was performed at room temperature. The same amount of enzyme was added. The ^{19}F NMR spectrum obtained is given in **Figure 4.3**.

When the spectra were obtained for these experiments, in addition to the expected peaks of (6*S*)-6-fluorodehydroquinate (-210 ppm, dd, *J* 25.5 Hz, 41 Hz) and (6*S*)-6-fluoro-1-*epi*dehydroquinate (-216 ppm, dd, *J* 27, 43 Hz) another peak was observed

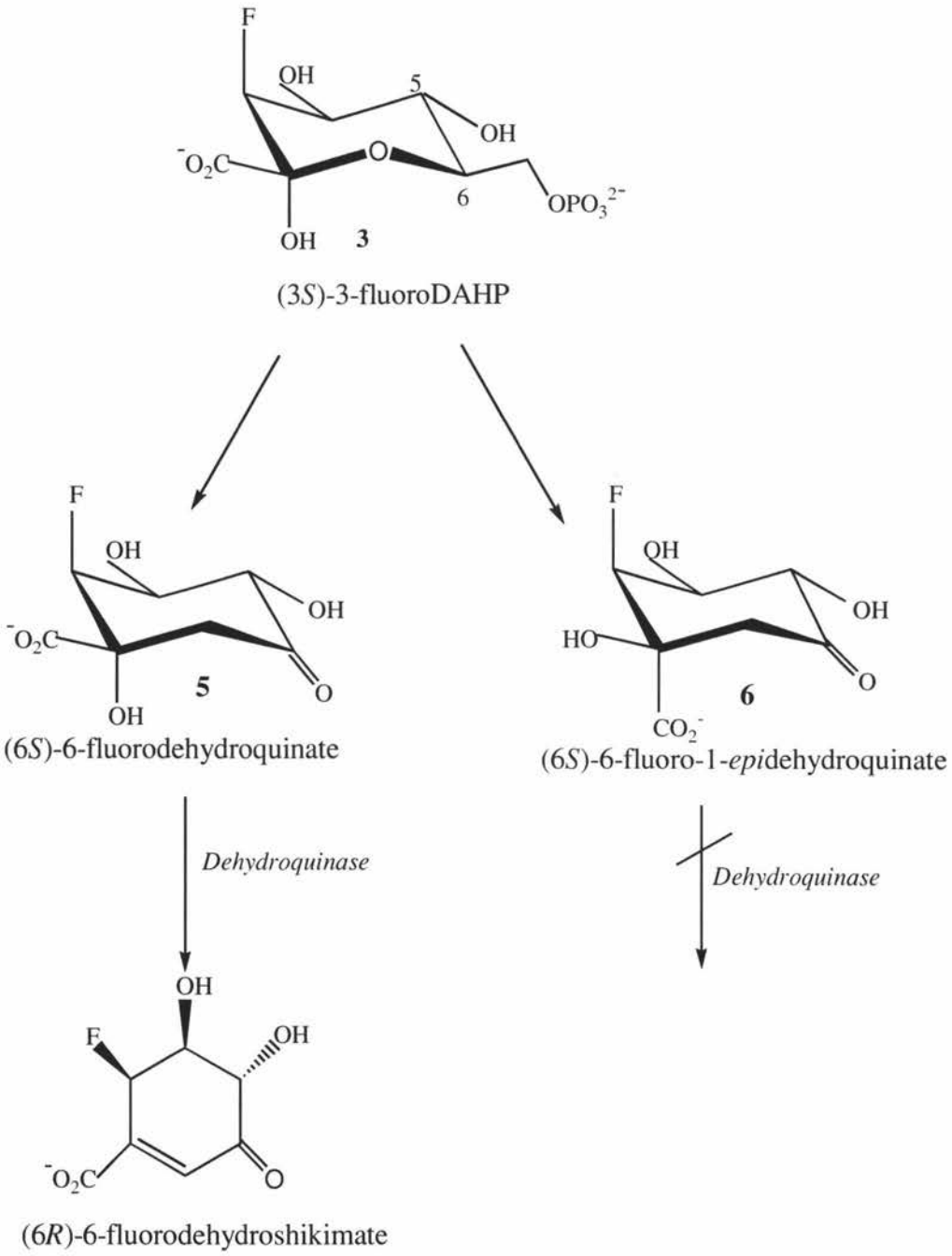
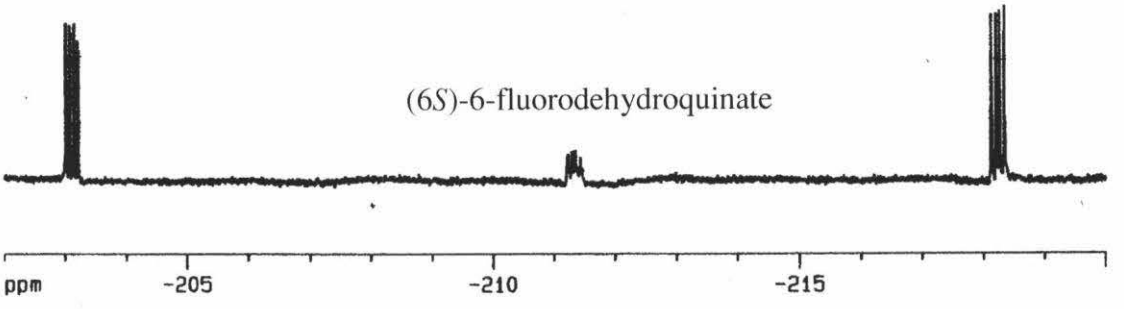


Figure 4.2 The formation of (6*R*)-6-fluorodehydroshikimate from (6*S*)-6-fluorodehydroquinate.

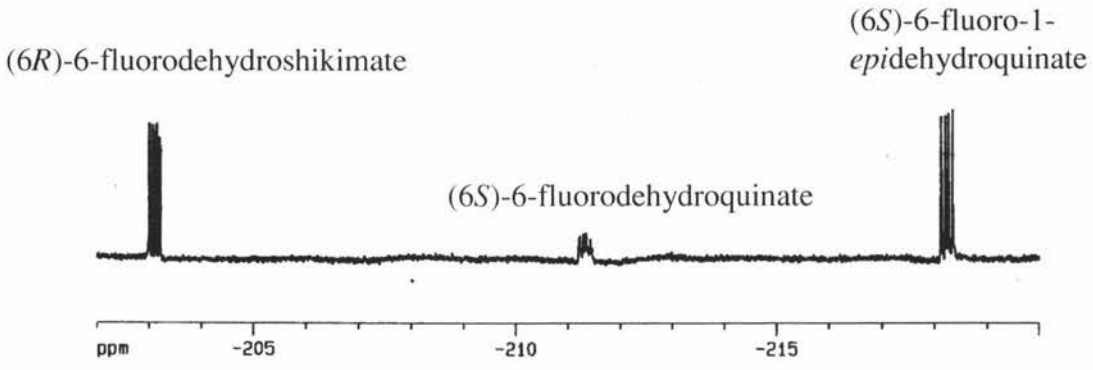
at -203 ppm (dd, *J* 20, 40 Hz). The chemical shift value corresponded with the earlier reported chemical shift value of (6*R*)-6-fluorodehydroshikimate.[28] This indicated that the enzyme DHQ synthase contained the next enzyme dehydroquinase also. To know the reason for this contamination some cell popcorns stored in the -70 °C freezer were suspended in BTP buffer and sonicated and tested for dehydroquinase activity with dehydroquinate stored in the freezer. Although very little dehydroquinase activity was observed, it appears that the enzymes have been mixed prior to addition to the NMR tube. Therefore the (6*S*)-6-fluorodehydroquinate formed in the reaction is converted into (6*R*)-6-fluorodehydroshikimate. There is equilibrium between these two products. The priority of the substituents on the C6 change on reaction to 6-fluorodehydroshikimate, leading the formation of (6*R*)-6-fluorodehydroshikimate from (6*S*)-6-fluorodehydroquinate (**Figure 4.2**). It is obvious from the spectra that (6*S*)-6-fluorodehydroquinate is a substrate for the next enzyme dehydroquinase, but its C1 epimer is not.



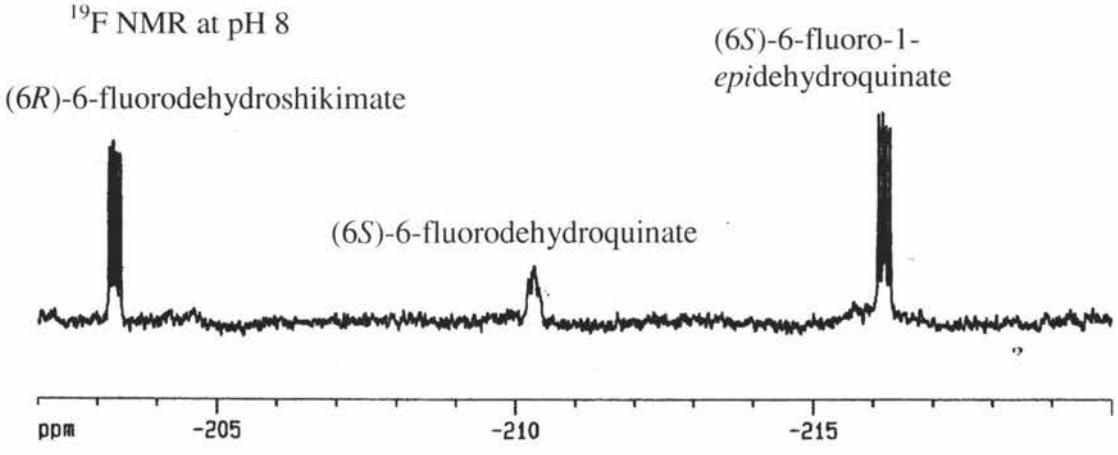
[(3*S*)-3-fluoro DAHP] = 18 mM, [NAD^+] = 30 μM, [Co^{2+}] = 50 μM, pH = 7.3, Temperature = 25 °C, [dehydroquinate synthase] = 8 units, 20 % D_2O

Figure 4.3 ^{19}F NMR spectra at pH 7.3.

^{19}F NMR at pH 6

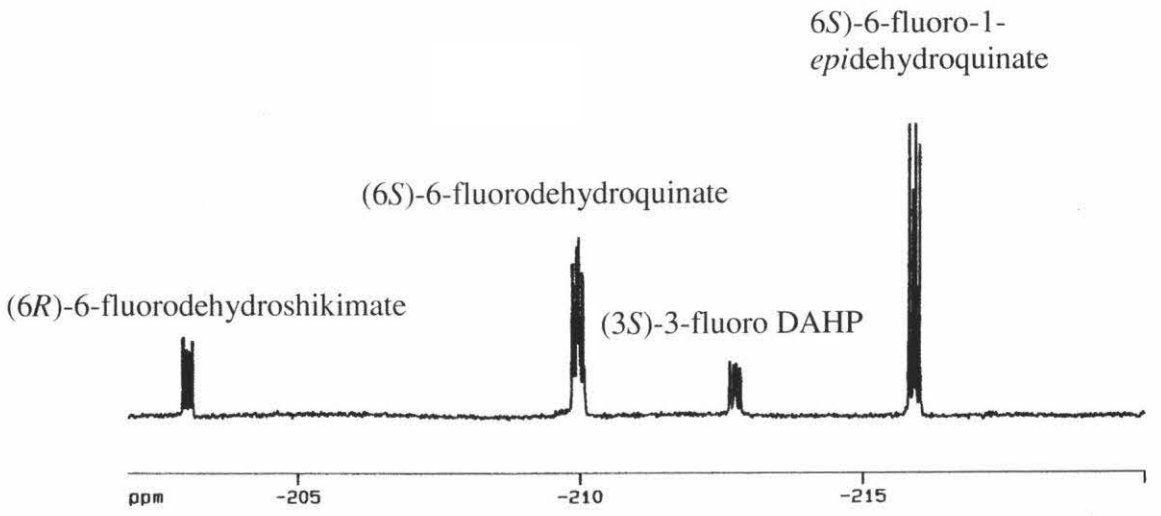


[(3*S*)-3-fluoro DAHP] = 20 mM, [NAD^+] = 30 μM, [Co^{2+}] = 50 μM, pH = 6, Temperature = 25 °C, [dehydroquinate synthase] = 8 units, 20 % D_2O.



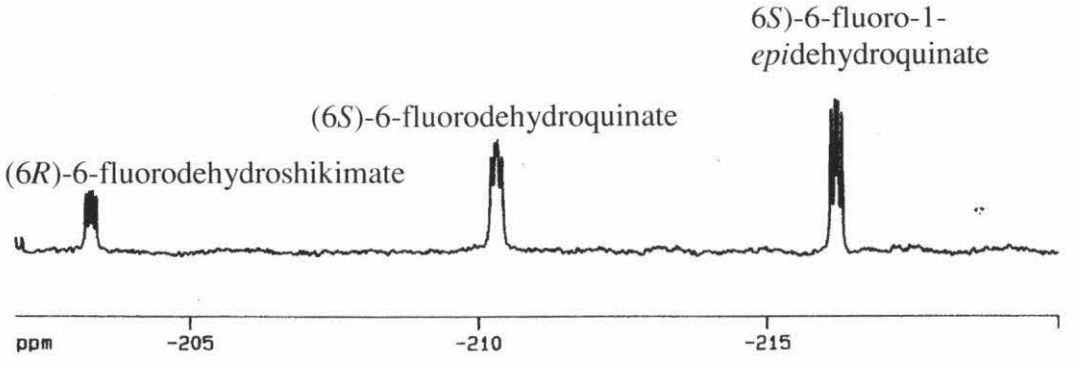
[(3*S*)-3-fluoro DAHP] = 20 mM, [NAD^+] = 30 μM, [Co^{2+}] = 50 μM, pH = 8, Temperature = 25 °C, [dehydroquinate synthase] = 8 units, 20 % D_2O

Figure 4.4 ^{19}F NMR spectra at pH 6 and at pH 8.



[(3*S*)-3-fluoro DAHP] = 43 mM, [NAD^+] = 30 μM, [Co^{2+}] = 50 μM, pH = 6.8, Temperature = 37 °C, [dehydroquinate synthase] = 8 units, 20 % D_2O

Figure 4.5 ^{19}F NMR at 37 ºC



[(3*S*)-3-fluoro DAHP] = 33 mM, [NAD^+] = 30 μM, [Co^{2+}] = 50 μM, pH = 6.8, Temperature = 15 °C, [dehydroquinate synthase] = 8 units, 20 % D_2O

Figure 4.6 ^{19}F NMR at 15 ºC

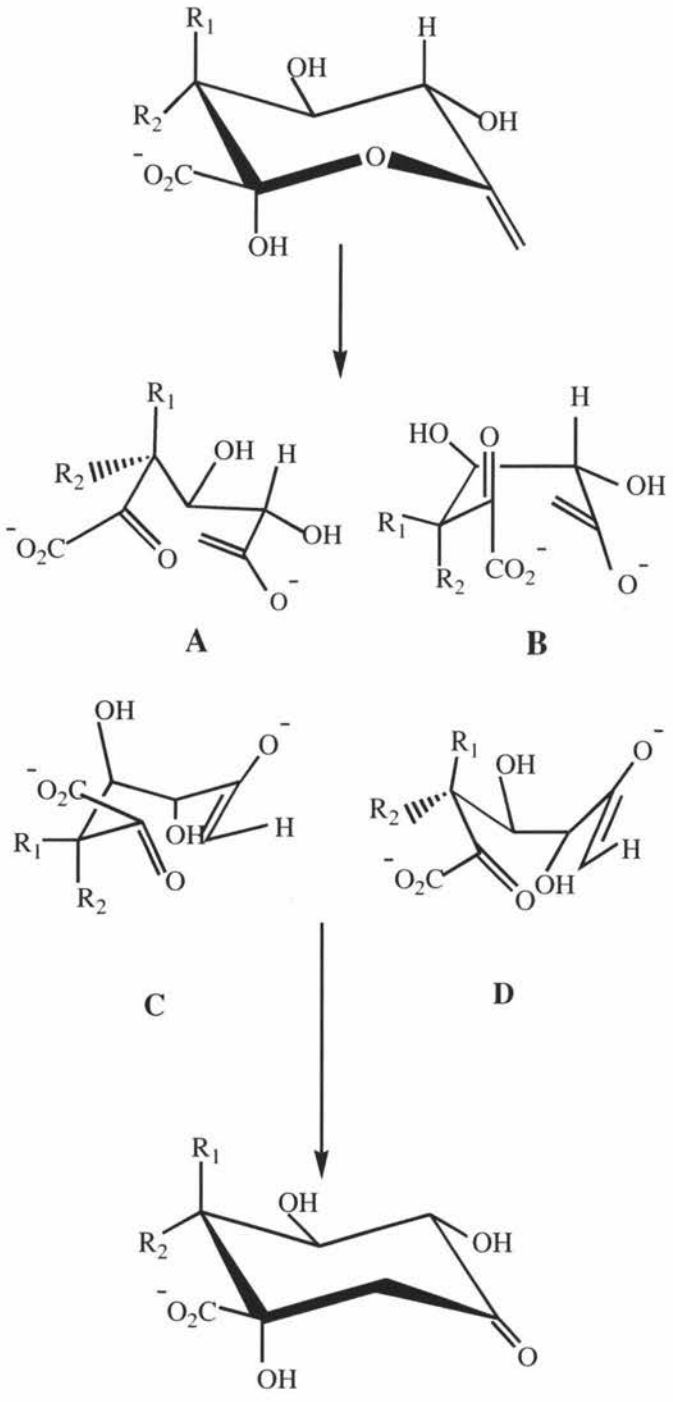
The ^{19}F NMR signals of the spectra obtained at different temperature and pH values were integrated. Since the (6*S*)-6-fluorodehydroquinate formed was in equilibrium with (6*R*)-6-fluorodehydroshikimate, the final integration ratio of (6*S*)-6-fluorodehydroquinate was considered as the sum of the ratios of the two compounds. The ratio of the C1 epimer was obtained by the direct integration of the (6*S*)-6-fluoro-1-*epi*dehydroquinate signal. The integration values are summarised in Tables 1 and 2.

Table 1 Product ratios at pH 6.8, as a function of temperature.

Temperature	(6*R*)-6-fluoro-dehydroshikimate	(6*S*)-6-fluoro-dehydroquinate	(6*S*)-6-fluoro-1-*epi*-dehydroquinate	Ratio of fluoro DHQ to C1 epimer
15 °C	1	2.2	2.3	1.4 : 1
37 °C	1	2.7	3.0	1.2 : 1

Table 2 Product ratios at temperature, as a function of pH.

pH	(6*R*)-6-fluoro-dehydroshikimate	(6*S*)-6-fluoro-dehydroquinate	(6*S*)-6-fluoro-1-*epi*-dehydroquinate	Ratio of fluoro DHQ to C1 epimer
6	1	0.9	1.7	1.1 : 1
7.3	1	0.5	1.0	1.5 : 1
8	1	0.4	1.2	1.1 : 1



$R_1 = R_2 = H$, dehydroquinate

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

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

Smaller concentrations of 3-fluoro DAHP was used in order to allow the reaction to achieve completion. Therefore, all these spectra were obtained with 256 scans. This resulted in difficulties with the integration of ^{19}F NMR spectra. The base lines were difficult to correct. The spectra were obtained in a range from -120 to -230 ppm. This was the reason for obtaining spectra with poor base lines. This should have been avoided by choosing smaller sweep width.

However the results suggest that there is not a significant difference between the ratio of products formed. The ratios appear to be the same within the considered experimental error limits. Therefore, it appears that both products have been formed through a similar process or that the change in conditions has altered both processes in the same way. As it is unlikely that both products are formed on the enzyme, the most likely explanation is that both products are formed off the enzyme. This is possible if the axial fluorine at C3 stabilises the enolpyranose intermediate and allows the dissociation of the intermediate from the enzyme, or decreases the further reaction of the enolpyranose intermediate on the enzyme, or both.

As explained in the introduction, with the natural substrate DAHP, 3-dehydroquinate is only detected in the enzymatic reaction, and both 3-dehydroquinate and its C1 epimer are formed in the solution reaction. Two *chair* transition states and two *boat* transition states are possible for the formation of each of 3-dehydroquinate and 1-*epi*dehydroquinate. The two *chair* transition states differ from each other by which face of the enolate, is involved in the cyclisation reaction. Consistent with labeling studies Knowles *et al.*[22] has suggested that the *chair* A transition state is involved in the enzymatic cyclisation to produce 3-dehydroquinate. From the labeling studies with the *o*-nitrobenzyl protected enolpyranose intermediate, Bartlett *et al.*[3] proposed that the same *chair* A transition state is involved in the solution reaction to produce 3-dehydroquinate. In the *chair* A transition state the *re* face of the carbonyl, is attacked by the *si* face of the enolate. No labeling studies have been carried out to determine the transition state involved in the formation of 1-*epi*dehydroquinate in the solution reaction.

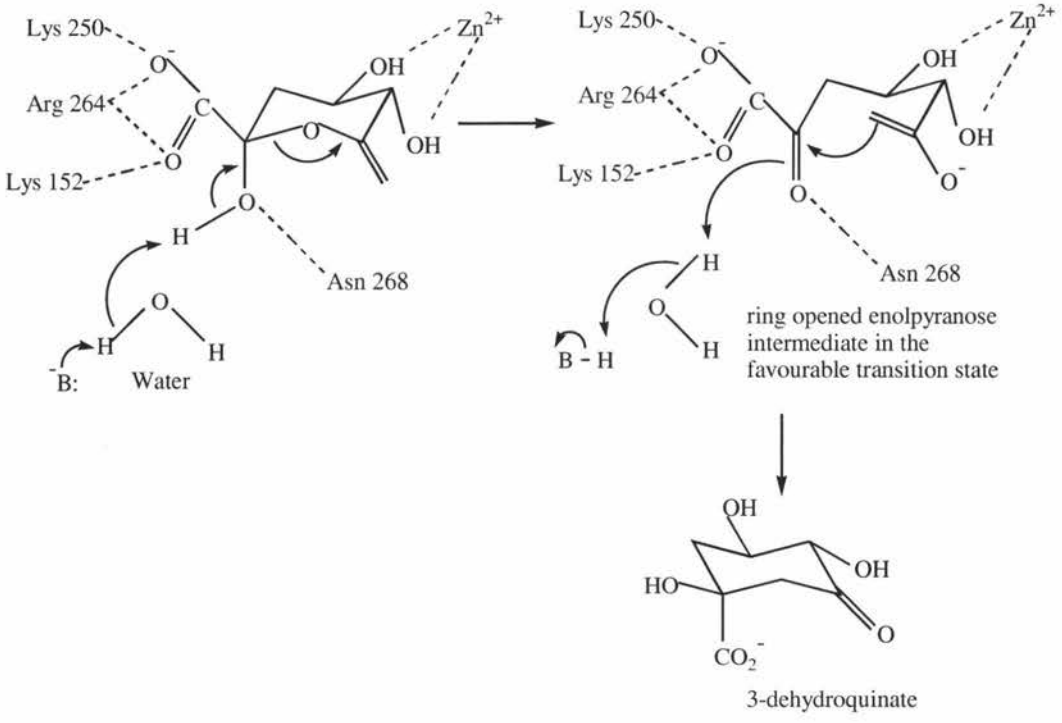


Figure 4.7 The active site interactions in the final two steps of the DHQ synthase reaction.

From the crystal structure of *A. nidulans* DHQ synthase with the inhibitor carbaphosphonate (**Figure 4.9**) Carpenter *et al.*[11] have suggested that the carboxylate group is strongly held in position by the active sites lys 152, lys 250 and arg 264. Therefore they have suggested that it is impossible for the enolate to attack the other face of the carbonyl to produce the C1 epimer in the enzymatic reaction, supporting the proposal of Bartlett *et al.*[2] that the enzyme acts as a template to prevent the formation of other products.

The crystal structure of *E. coli* DHQ synthase has not yet been determined. A comparison between the sequences of the *E. coli* DHQ synthase and *A. nidulans* DHQ synthase is shown in **Figure 4.8**. This comparison reveals that the key catalytic residues are the same in both sequences. Therefore the active site of these enzymes may be expected to be very similar. Using the structure of *A. nidulans* DHQ synthase we have carried out some modelling experiments with the transition state predicted for the formation of 3-dehydroquinate from DAHP (**Figure 4.10**).

The modelling was carried out by putting the carboxylate group of the ring opened enolpyranose intermediate into the binding pocket of the carboxylate group of carbaphosphonate inhibitor. The active site interactions seen for the final two steps of the DHQ synthase reaction from this modelling are shown diagrammatically in **Figure 4.7**. In our modelled structure the enolpyranose intermediate is oriented correctly for the attack of the enolate to produce 3-dehydroquinate. There are interactions between the carboxylic acid group of the enolpyranose intermediate and the active sites lys 152, lys 250, and arg 264. There are also interactions between the hydroxyl with asn 268 and a water molecule. Therefore these interactions would be expected to prevent the other face of the keto group being exposed to the attacking enolate. Therefore it appears that epimerisation at C1 is not possible on the enzyme, consistent with the observations of Bartlett *et al.*[2]

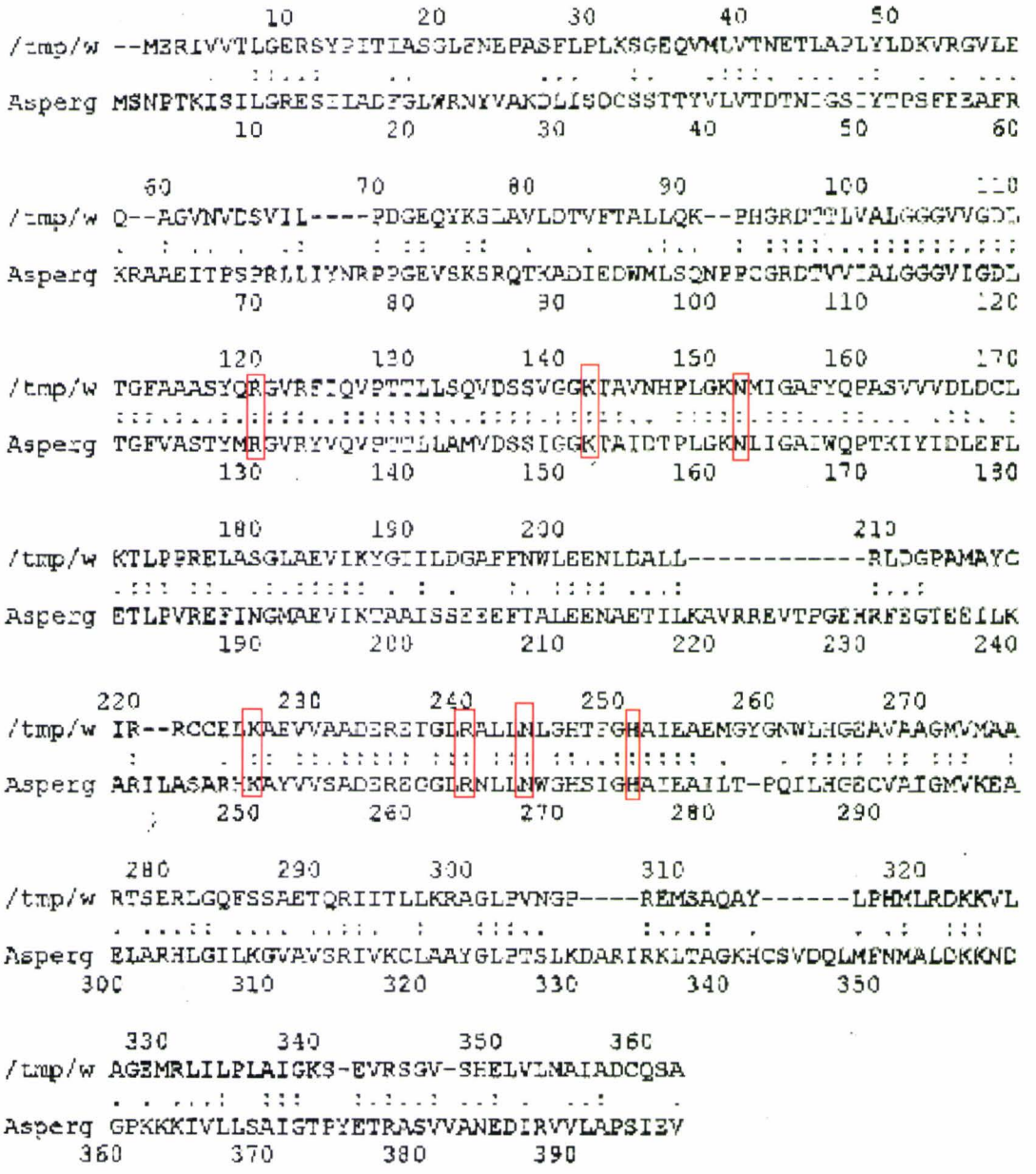


Figure 4.8 Sequence alignment between *E.coli* DHQ synthase and *A. nidulans* DHQ synthase. Key active site residues are highlighted.

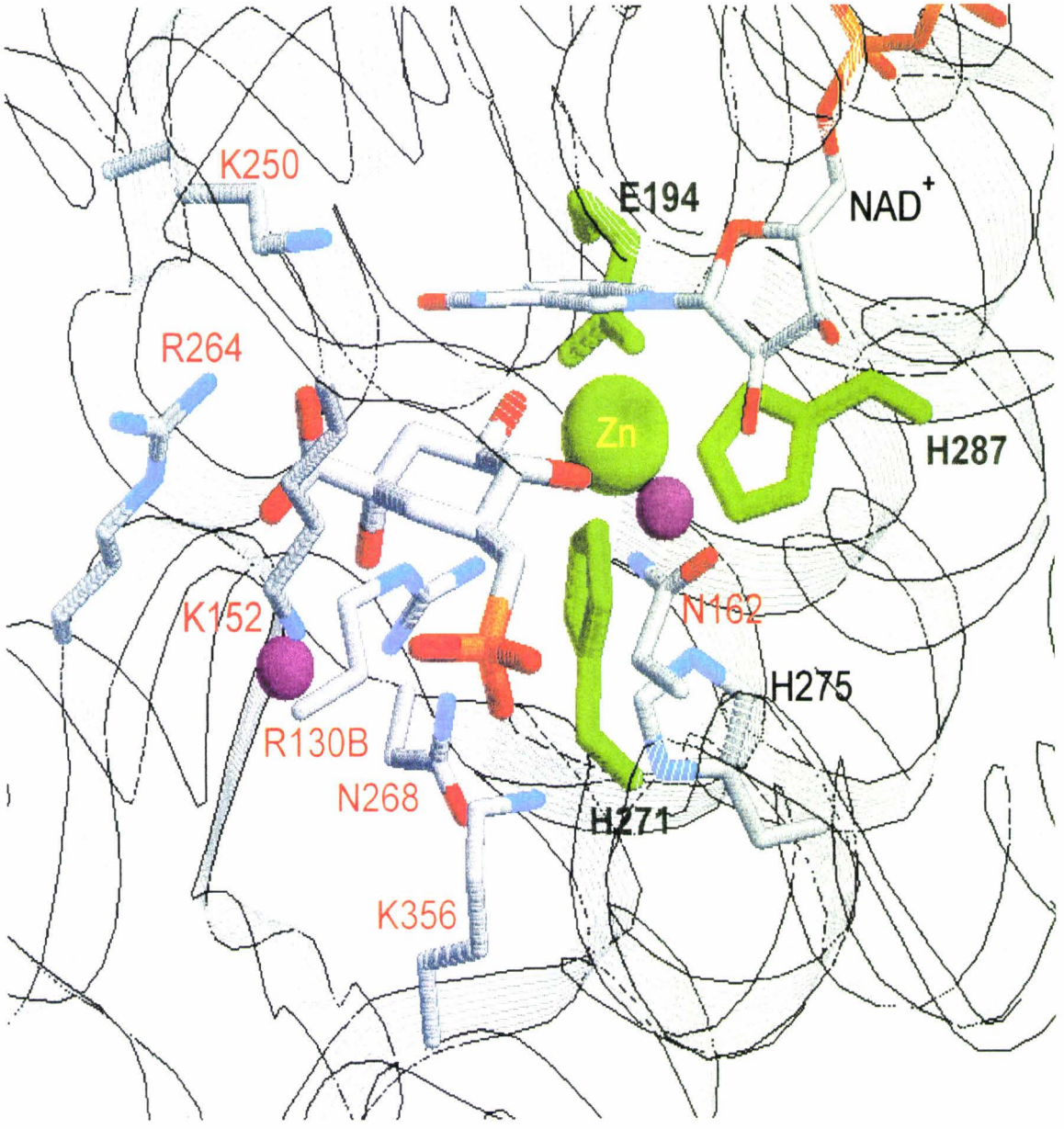


Figure 4.9 The crystal structure of *A. nidulans* DHQ synthase with the inhibitor carbaphosphonate.[11] The nitrogen atoms are coloured blue, oxygen atoms are coloured red, carbon atoms are coloured grey and phosphorous atoms are coloured orange. The water molecules are coloured purple and the ligands to zinc are coloured green.

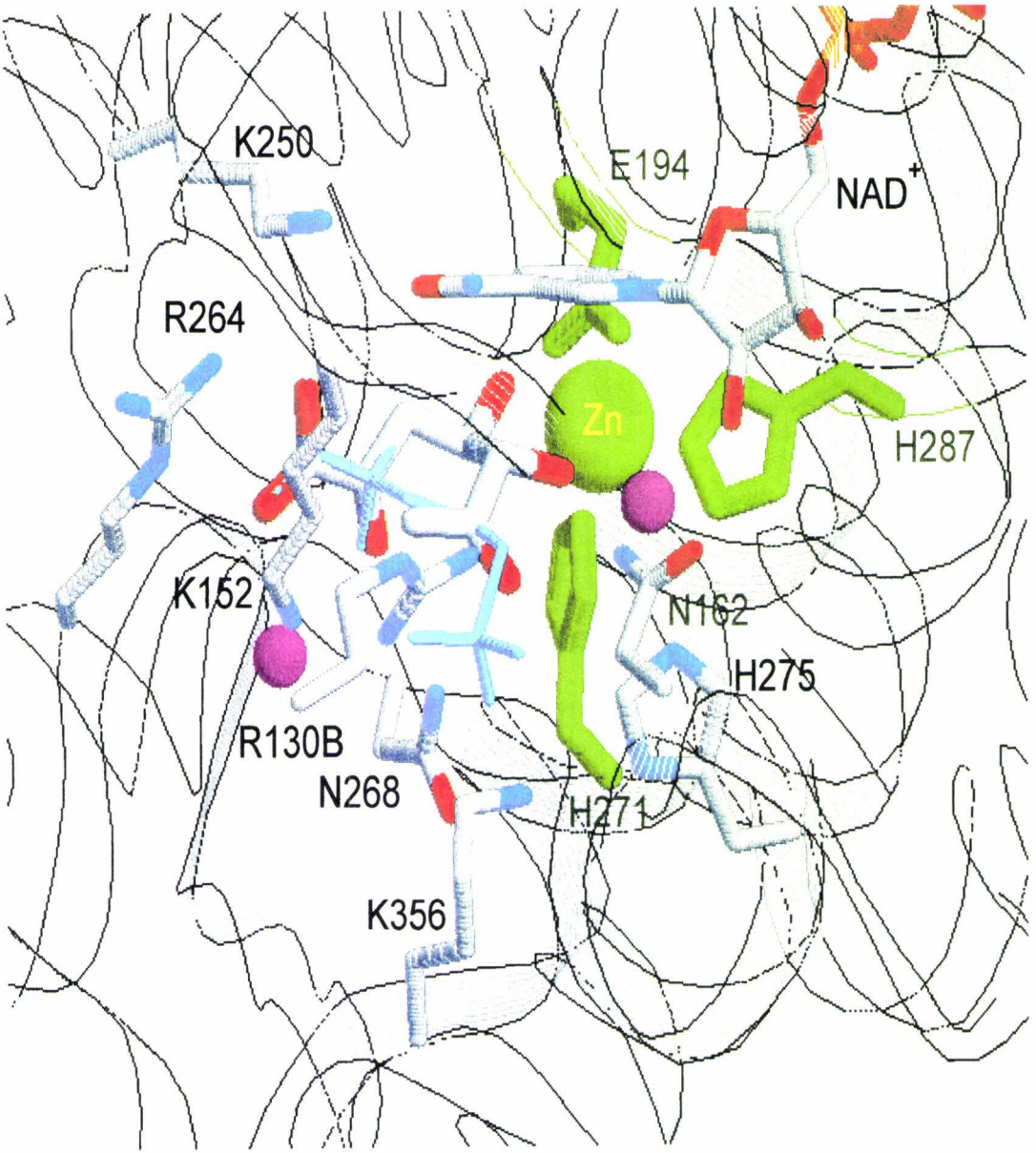


Figure 4.10 The model structure of *A. nidulans* DHQ synthase with the inhibitor carbaphosphonate (coloured light blue) and the enolpyranose intermediate. The nitrogen atoms are coloured blue, oxygen atoms are coloured red, carbon atoms are coloured grey and phosphorous atoms are coloured orange. The water molecules are coloured purple and the ligands to zinc are coloured green.

Modelling studies support the fact that epimerisation at C1 of 3-dehydroquinate is not possible in the enzymatic reaction. This leads to the conclusion that the epimer is formed off the enzyme. ^{19}F NMR studies at different temperature and pH values are consistent that both products have been formed through similar process. Therefore, it appears likely that the fluoroenolpyranose intermediate is released completely into the solution, where it cyclises to give both the expected and unexpected products. If this is the case, the reason for the release of the fluoroenolpyranose from the enzyme needs to be addressed.

It is possible that the fluorine substitution may provide stability to the fluoroenolpyranose intermediate. When there are fluorine atoms adjacent to hemiketals and hydrates, they are more stable than the corresponding ketones and[26] this thermodynamic stability may allow the intermediate to dissociate from the enzyme completely or partially and cyclise free in solution without the constraint of the enzyme template.

If the greater stability of the fluoroenolpyranose due to the adjacent fluorine, gives rise to the dissociation of the (3*S*)-fluoroenolpyranose intermediate, then the fluoroenolpyranose intermediate arising from the (3*R*) isomer would also be expected to dissociate from the enzyme. When (3*R*)-3-fluoro DAHP was treated with the enzyme, (6*R*)-6-fluorodehydroquinate was observed, and no epimer has been observed and so it is not known, whether this fluoroenolpyranose intermediate is released from the enzyme or not. If (6*R*)-6-fluorodehydroquinate is the only favoured product in the solution cyclisation reaction, then it is also possible that the fluoroenolpyranose intermediate arising from the (3*R*) isomer is released from the enzyme, but only the expected product is formed.

If the fluoroenolpyranose is released from the enzyme, the cyclisation reaction can occur with less constraint on conformation. If we consider all the possible transition states involved in the formation of fluorodehydroquinate, for the (3*R*)- isomer the equatorial fluorine is orthogonal to the carbonyl bond and at 180° to the direction of

attack by the enolate in the favoured *chair* A transition state for the enzymatic reaction. Therefore, for the (3*R*)-isomer the *chair* A transition state is favoured by Felkin-Anh model.[14] However for the (3*S*)-isomer, the *chair* transition state A is disfavoured, according to the Felkin-Anh model[14] as the electronegative fluorine points towards the direction of attack by the nucleophilic enolate.

As the likely *chair* A transition state, for the enzymatic cyclisation is disfavoured by the presence of axial fluorine, it is possible that this destabilisation confers kinetic stability on the (3*S*)-fluoroenolpyranose intermediate, allowing it to dissociate from the enzyme and cyclise unrestricted by the enzyme template to give two reaction products.

With the consideration of these results and the arguments presented in this thesis it appears reasonable to propose that the fluoroenolpyranose intermediate is released into the solution where it cyclises free to give two products. However these results are not sufficient to prove that the enzyme is not involved in the final two steps of the reaction. Further investigations are warranted and are discussed in Future directions.

4.3 Future directions

There are a number of experiments that may clarify the role of the enzyme in the cyclisation step when 3-fluoro DAHP is a substrate. It has already been shown that the (3*S*)-3-fluoro DAHP gives two reaction products, while the (3*R*)-isomer gives only one product.[27] The experiments carried out with the (3*S*)-isomer at different temperature and pH values suggest that both products have been formed through a similar process. Modelling studies of enolpyranose intermediate with *A. nidulans* DHQ synthase suggest that epimerisation at C1 is not possible in the enzyme catalysed reaction. The amino acid residues of *A. nidulans* DHQ synthase, which have interactions with the substrate, are all conceived in the *aro B* of *E. coli* DHQ synthase. Therefore, it appears that both products are formed by non-enzymatic reaction.

To get further evidence to support this, the *o*-nitro benzyl protected enolpyranose with fluorine substitution at 3 axial position (**Figure 4.11**) needs to be synthesised. This intermediate plays a key role in the transformation of (3*S*)-3-fluoro DAHP into two products. By photochemically removing the *o*-nitro benzyl protected group and observing the ratio of reaction products formed would confirm whether both these products are formed through non-enzymatic reaction. Bartlett *et al.*[3] synthesised the unfluorinated *o*-nitro benzyl protected enolpyranose intermediate in a series of 10-step reactions from DAH. They photochemically removed the *o*-nitro benzyl protecting group and observed the ratio of dehydroquinate and 1-*epi*dehydroquinate formed in the solution reaction. However no labelling studies have been done to identify the transition state involved in the formation of 1-*epi*dehydroquinate in the solution reaction. According to the Felkin-Anh model[14] the favoured transition states for the formation of (6*S*)-6-fluorodehydroquinate and its C1 epimer are shown in Figure 4.10. Labelling studies would provide direct evidence, whether these transition states are involved in the cyclisation step. Stereospecifically labeling one of the C7 hydrogens of the fluoroenolpyranose with deuterium, the transition state involved in the formation of (6*S*)-6-fluorodehydroquinate and its C1 epimer can be limited to one *boat* and one *chair*.

The fluoro PEP synthesised could be used to synthesise more 3-fluoro DAHP. More E4P needs to be synthesised. The synthetic strategy used by Bartlett *et al.*[3] to synthesise *o*-nitro benzyl protected enolpyranose from DAH could be used to synthesise o-nitro benzyl protected enolpyranose with 3-axial fluorine from (3*S*)-3-fluoro DAH. Modelling of the transition states discussed in the introduction and calculating their relative energies may also help to understand the effect of fluorine substitution on the reaction. Modelling studies of the *E. coli* DHQ synthase with the substrate (3*S*)-3-fluoro DAHP may also provide information on the effect of fluorine substitution. For this the crystal structure of *E. coli* DHQ synthase need to be determined.

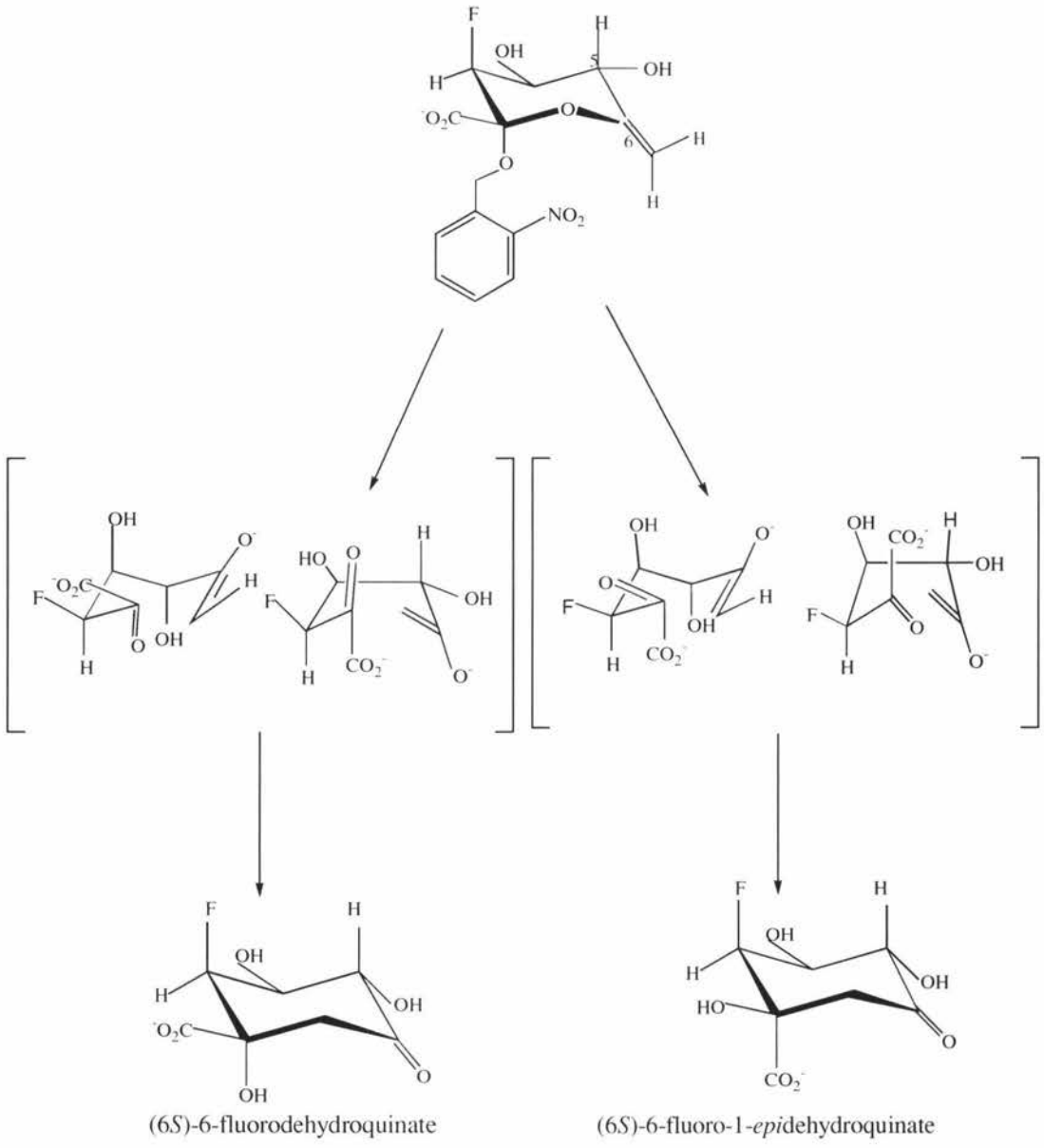


Figure 4.11 Felkin-Anh model[14] predicted favoured transition states for the formation of (6*S*)-6-fluorodehydroquinate and (6*S*)-6-fluoro-1-*epi*dehydroquinate.

The cell popcorns of DHQ synthase produced need to be purified. Although considerable purification was achieved with the purification of dehydroquinate synthase, by anion exchange chromatography, more work is required to find a protocol that can be used to produce higher purity enzyme.

The next enzyme of the shikimate pathway, dehydroquinase further processes (6*S*)-6-fluorodehydroquinate, however its C1 epimer is not further processed. This result shows how vital is stereochemical control in enzymatic reactions. Understanding the influence of fluorine on this cyclisation reaction may help us understand how enzymes control stereochemistry with the precision that they do.

5 Experimental

5.1 General procedures

NMR Spectroscopy

^{1}H NMR and ^{19}F NMR spectra were recorded on a Bruker 400 MHz spectrometer. Chemical shifts are reported in parts per million (ppm). Samples were dissolved in solvents as stated. For ^{1}H NMR, when the sample was dissolved in D_2O, spectra were referenced to HOD at 4.67 ppm and when the sample was dissolved in $CDCl_3$, the spectra were referenced to $CHCl_3$ at 7.24 ppm. ^{19}F NMR spectra were referenced to CF_3COOH at -78.9 ppm.

UV/visible spectrophotometry

UV spectrophotometry was performed on a Varian Carry 1 UV/Visible spectrophotometer at 25 °C, using 1cm quartz cells unless otherwise stated. Initial rates were calculated using the least square gradient facility over the first 10 % of the progress curve.

Solvents and Reagents

Solvents were laboratory grade. Other chemicals were of the highest grade available.

HPLC

HPLC columns were run on a Waters 600 liquid chromatograph system. The peaks were detected at 230 nm and 214 nm.

Freeze-drying

Samples to be freeze-dried were frozen in liquid nitrogen and placed for 1 - 2 days on a Vir Tris freeze mobile 12 freeze-drier.

TLC

TLC was performed on Merck silica gel Aluminium sheets 60 F_{254}, and spots were visualised with $KMnO_4$.

FPLC

Anion exchange chromatography was carried out on a Waters 650E advanced purification system. Detection was by a Waters 441 detection system and fraction collection was by a Hitachi L-5200 fraction collector.

Centrifugation

For harvesting of cells, a Sorvall superspeed RC2-β centrifuge was used with a GSA rotor. Other cell and protein samples were centrifuged in a Sorvall RT 7 centrifuge with a swinging bucket RTH-750 MPC or a fixed angle SL 50 RT rotor.

Sonication

Cells lysis was performed using a Virsonic Digital 475 cell disrupter equipped with a thin (1/8 inch) and thick (1/2 inch) probe or French pressed using a WABASH French press cell at ~ 7000 p.s.i.

pH measurement

pH measurements were made using a Denver model 20 pH/conductivity meter.

Gel preparation

The resolving gel contained distilled water (3.5 ml), 1.5 M Tris-HCl (pH 8.8, 2.5 ml), acrylamide / Bis (30 % stock, 4.0 ml degassed for 40 minutes at room temperature) and was polymerised with 10 % ammonium persulfate (100 µl), and TEMED (5 µl).

The stacking gel contained distilled water (6.1 ml), 0.5 M Tris-HCl (pH 6.8 2.5 ml), 10 % (w/v) SDS (100 µl), acrylamide/Bis (30% stock, 1.3 ml degassed for 15 minutes at room temperature) and was polymerised with 10 % ammonium per sulfate (100 µl), and TEMED (10 µl).

The electrophoresis tank buffer contained 51 mM Tris base, 384 mM glycine and 0.1 % (w/v) SDS. The protein samples were treated at 100 °C for 2 minutes in an equal volume of loading buffer. Gels were electrophoresed at 50 V (Bio-Rad 200/02 - power supply) until the samples had migrated to the interface between the stacking and resolving gels. Molecular markers were from Sigma Chemical Company supplied in a kit form.

Enzymes

The enzyme DAHP synthase was produced and purified by Esther Bulloch and Andrew Baldwin in our laboratory. The enzyme dehydroquinase was produced by Esther Bulloch in our laboratory and used without further purification.

Assay used to determine DAHP and 3-fluoro DAHP concentrations

Unknown DAHP or 3-fluoro DAHP

NAD^+ 25 µM

Co^{2+} 50 µM

dehydroquinase (type1, 5 µl) was added and the sample was equilibrated and DHQ synthase 0.2 – 0.5 units was added and the progress was monitored at A_{234} (ε = 1.2 x 10^4 $M^{-1}cm^{-1}$). The measurements were determined at 25 °C in 1 ml, 1 cm pathlength, quartz cuvettes. NAD^+ is from Sigma with approximate purity of 95 %.

3-Dehydroquinate synthase

The following enzyme assay was used for enzyme activity.

PEP 150 µM

E4P 350 µM

$MnSO_4$ 50 µM

DAHP synthase 5 µl was added and when the transformation was complete NAD^+ (25 µM), Co^{2+} (50 µM) and dehydroquinase (type1, 5 µl), and appropriate amounts of DHQ synthase were added and the activity of dehydroquinate synthase was monitored at A_{234} ($\varepsilon = 1.2 \times 10^4\ M^{-1}cm^{-1}$). All measurements were determined at 25 ^oC in 1 ml, 1 cm pathlenth, quartz cuvettes. NAD^+ was from Sigma with approximate purity of 95 %.

5.2 Experimental for chapter 2

Diethylfluorooxaloacetate (sodium salt): Diethyloxalate (6 ml, 6.4 g, 44 mmol) was added dropwise over 10 minutes with stirring to freshly prepared sodium ethoxide (3 g, 44 mmol) in dry benzene (100 ml) under nitrogen at 0 °C. Ethylfluoro acetate (4.2 ml, 4.6 g, 44 mmol) was then added and the resulting solution was stirred at room temperature. After 24 hrs, the mixture was filtered and the solid washed with dry ether until the filtrate was colourless. The white solid was dried *in vacuo* giving 5.1 g, (50.8 %) of the sodium enolate salt of diethylfluorooxaloacetate.

δ_H (400 MHz, D_2O) = 4.12 (2H, q, *J* 7.2 Hz, $-CH_2CH_3$), 4.08 (2H, q, *J* 7.1 Hz, $-CH_2CH_3$), 1.10 (3H, t, *J* 7.1 Hz, $-CH_2CH_3$), 1.06 (3H, t, *J* 7.2 Hz, $-CH_2CH_3$).
δ_F (400 MHz, D_2O) = -183.86 (s)

Diethyl bromofluorooxaloacetate: Bromine was added dropwise with stirring to diethyl fluorooxaloacetate (5.1 g, 22 mmol), suspended in 100 ml of dry benzene, maintaining the temperature of the mixture below 50 °C. When the colour of the bromine persisted, the solution was washed with water and saturated sodium

sulphite solution. The combined aqueous extracts were washed with toluene. The organic extracts were combined and dried with $MgSO_4$. The solvent was removed *in vacuo* to give diethyl bromofluoro oxaloacetate as a pale yellow oil (6 g, 96 %).

δ_H (400 MHz, $CDCl_3$) = 4.34 (4H, 2 overlapping quartets, $-CH_2CH_3$), 1.31 (6H, 2 overlapping triplets, $-CH_2CH_3$).
δ_F (400 MHz, $CDCl_3$) = -127.40, -129.00.

Bromofluoropyruvic acid : Diethylbromofluorooxaloacetate (6 g, 21.1 mmol) was refluxed (105 °C) for 6 hrs in 100 ml of conc HCl. After the removal of the excess HCl *in vacuo*, the resulting black oil was extracted from ether into 0.1 M sodium hydroxide. The aqueous layer was acidified to pH 1 with conc HCl and extracted five times with ethylacetate. Removal of the solvent *in vacuo* pressure yielded of bromofluoropyruvic acid (2.3 g, 59 %) as a white solid.

δ_H (400 MHz, D_2O) = 6.05 (d, J_{FCH} 49 Hz).
δ_F (400 MHz, D_2O) = -151.57 (d, J_{FCH} 49 Hz).

3-Fluorophosphoenolpyruvate : Freshly distilled trimethylphosphite (1.5 ml, 13.2 mmol) was added to bromofluoropyruvic acid (2.3 g, 13.2 mmol) dissolved in 20 ml of dry THF at 0 °C under argon. The resulting solution was stirred while warming to room temperature over 5 hrs. The solvent was removed *in vacuo*, and the evaporation was continued a further 10 minutes in order to remove unreacted phosphite. The resulting yellow oil was re-dissolved in 10 ml of water and left at room temperature with stirring overnight. Two millilitres of cyclohexylamine was added and the solvent was removed under high *vaccum* giving a pale yellow solid (2 g). It was recrystallised from methanol/ether. The solid was dissolved with gentle warming in 45 ml of methanol. While the methanolic solution was still warm an equal volume of ether was added and the solution was left at 0 °C overnight. Even after recrystallisation the sample contained bromofluoropyruvic acid and

phosphorous acid in a ratio approaximately F-PEP: phosphorous acid : bromofluoro pyruvic acid = 50 : 30 : 20 .

δ_H (400 MHz, D_2O) = 7.55 (1H, dd, *J* 73 and 2.8 Hz, (*Z*)-isomer), 7.37 (1H, dd, *J* 74.9 and 3.3 Hz, (*E*)-isomer).
δ_F (400 MHz, D_2O) = -132.4 (dd, *J* 73, 6,1 Hz, (*Z*)-isomer), –136.5 (dd, *J* 74.8, 7.8 Hz, (*E*)-isomer).

Erythrose-4 Phosphate: Glucose 6-phosphate (0.564 g, 2 mmol) in a 500 ml beaker was moistened with 2 ml of water, then treated with 5 ml of glacial acetic acid, and the suspension warmed for a few minutes at 50 - 60 °C to dissolve the salt. Sulfuric acid (6 N, 0.35 ml) was added while the solution was stirred, then 245 ml of glacial acetic acid was added. Just before use, a solution of lead tetraacetate (1.52 g, 3.4 mmol) in glacial acetic acid (~ 40 ml) was mixed with sulfuric acid (6 N, 0.6 ml) in a dropping funnel. This reagent was added drop-wise from the funnel into the rapidly stirred solution of glucose 6-phosphate at about 25 °C. It was delivered at a very slow rate, so that the reaction mixture never contained more than a slight excess of oxidant. The total time required was about 45 minutes.

The reaction mixture was then filtered through a thin layer of celite "filter-aid", and the filtrate concentrated *in vacuo* at 35 - 40 °C to a volume of 15 - 20 ml. The precipitate was washed with water, and the washings combined with the concentrate. The evaporation was continued until the volume was 15 - 20 ml, thus removing most of the acetic acid. The concentrate was diluted with water to a volume of 100 - 125 ml, and extracted continuously with ether for 15 - 20 hrs. The aqueous layer was then treated with Chelex (~2 g) and filtered to remove the cations. The filtrate and the washings were reduced in a rotary evaporator at 20 °C to 10 - 15 ml. The crude erythrose 4-phosphate prepared in this manner was used immediately to prepare 3-fluoro DAHP and DAHP. The concentrations of erythrose 4-phosphate and glyceraldehyde 3-phosphate were measured using the coupled assay system (**Figure 5.1**). This assay involved the oxidation of NADH (ε_{340} = 6.2 x

10^3 M^{-1} cm^{-1}) under the action of glycerol 3-phosphate dehydrogenase, converting erythrose 4-phosphate to glycerol 3-phosphate *via* glyceraldehyde 3-phosphate and dihydroxy acetone phosphate.

1 ml of E4P solution was diluted with 9 ml of BTP buffer (pH 6.8). From this solution 10 µl was pipetted into a cuvette and 300 µM fructose 6-phosphate, 1 mM EDTA, and ~120 µM NADH were added. The total volume of the solution was adjusted to 1 ml with BTP buffer (pH 6.8). Two units of triose phosphate isomerase and 0.1 units of glycerol 3-phosphate dehydrogenase were added and when all the glyceraldehyde 3-phosphate had been consumed two units of transaldolase were added. This assay was performed in duplicate. The amount of erythrose 4-phosphate formed was ~300 mg.

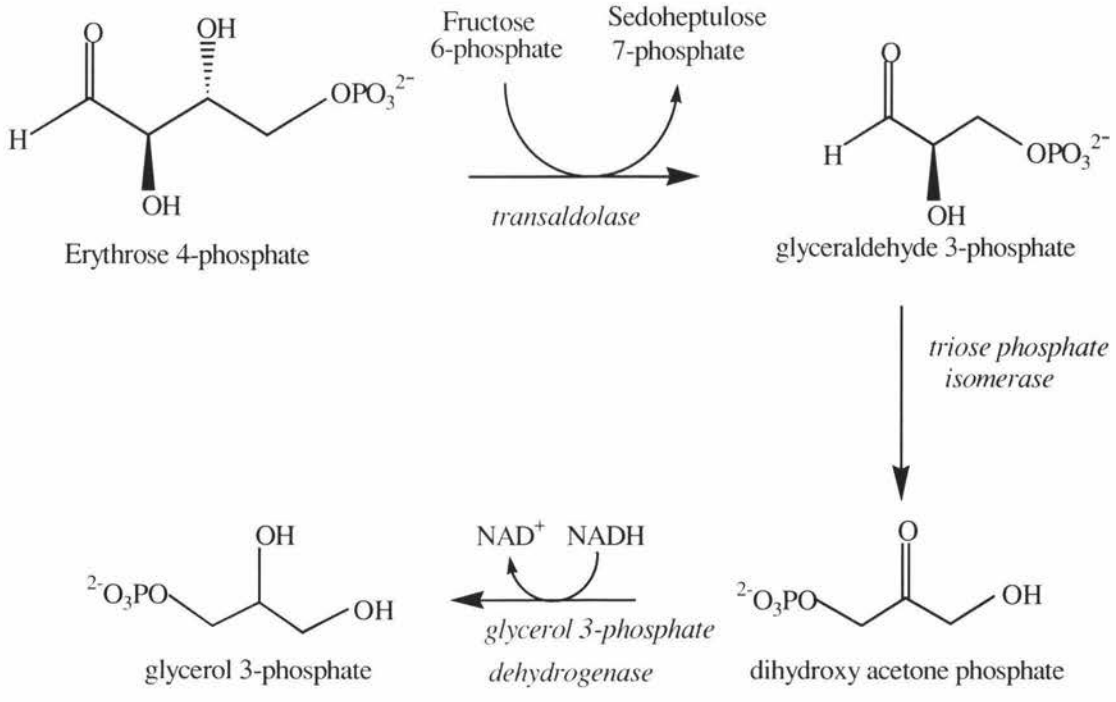


Figure 5.1 Assay for erythrose 4-phosphate using transaldolase, triose phosphate isomerase and glycerol 3-phosphate dehydrogenase.

Synthesis of DAHP : 25 mg of PEP, 25 mg of freshly prepared erythrose 4-phosphate, 10 μM Mn^{2+} in 1 ml BTP buffer (50 mM, pH 6.8) were incubated in a 1 ml cuvette and the reaction was initiated by the addition of DAHP synthase. The progress of the reaction was monitored by determining the decrease in PEP concentration indicated by the absorbance at 270 nm. The rate of PEP consumption declined as the reaction proceeded. After 8 hrs, the reaction was stopped by removing the enzyme in a centricon concentrator (molecular weight cut-off 10) and the filtrate was left in the -70 °C freezer and later purified on a Mono Q (10/10) anion exchange column and eluted with a linear gradient of 50 - 1000 mM NH_4HCO_3 over 200 ml. Fractions containing DAHP were found to elute at 200 – 250 mM NH_4HCO_3. Fractions containing DAHP were determined by the coupled assay system with DHQ synthase and dehydroquinase (0.4 and 1.4 units) at 234 nm. The fractions containing DAHP were freeze-dried and stored in a -70 °C freezer (12 mg).

Large scale preparation of fluoro DAHP : 75 mg of 3-fluoro PEP, 25 mg of E4P, 10 μM Mn^{2+} in 1 ml BTP buffer (50 mM, pH 6.8) were incubated in a 1 ml cuvette and the reaction was initiated by the addition of DAHP synthase. The reaction was carried out by a procedure similar to that of the synthesis of DAHP. After 48 hrs, the reaction was stopped by removing the enzyme in a centricon concentrator (molecular weight cut-off 10) and the filtrate was left in a -70 °C freezer. The filtrate was later purified on a Mono Q (10/10) anion exchange column and eluted with a linear gradient of 5 - 1000 mM NH_4HCO_3 over 200 ml. Fractions containing (3*S*)-3-fluoro DAHP were found to elute at 350 - 375 mM NH_4HCO_3. Fractions containing (3*S*)-3-fluoro DAHP were determined by the coupled assay system with DHQ synthase and dehydroquinase (0.4 and 1.4 units) at 234 nm. The fractions containing (3*S*)-3-fluoro DAHP were freeze dried and stored at -70 °C (40 mg).

The experimental assay for the studies with R5P as a substrate:

3-fluoro PEP (50 μM),

ribose 5-phosphate (800 μM)

Mn^{2+} (50 μM)

DAHP synthase 40 μl (five times more concentrated than the normal enzyme) was added and the progress of the reaction was monitored at A_{232}. Different concentrations of ribose 5-phosphate were employed.

The ^{19}F NMR experiments with ribose 5-phosphate and 3-fluoro PEP.

3-Fluoro PEP (10 mg), R5P (25 mg, ~100 mM) were dissolved in 200 μl of D_2O. The pH of the solution was adjusted to 6.8 with BTP buffer to a final volume of 1 ml. 20 μl of concentrated (~ 6 times) DAHP synthase was added. The progress of the reaction was followed by ^{19}F NMR spectroscopy. For the experiment at 37 °C, the sample was equilibrated at a temperature controlled room and the enzyme was added and the progress of the reaction was followed. A minimum amount of 10 mg of 3-fluoro PEP was needed to get a reasonable ^{19}F NMR spectrum. The amount of R5P was varied from 10 to 50 mg.

5.3 Experimental for chapter 3

Buffers:

A 10 mM β-glycerophosphate buffer containing 0.25 mM cobalt(II)chloride and 0.25 mM NAD^+, pH 6.6

B 10 mM BTP buffer, pH 6.6

C 10 mM BTP buffer containing 1 M NaCl, pH 6.6

Cell growth : *E. coli* cells (PJB14) were plated out on LB plates and grown overnight at 37 °C. These cells were used to grow four 60 ml pre cultures in LB medium consisting of bactotryptone (10 g), bacterial yeast extract (5 g), and sodium chloride (10 g) in 1 L (50 mg/L amphiciline was added to the medium). This was used to inoculate four 4 L shaker flasks, each containing LB medium (1 L). After growth for about an hour, when the OD_{550} was between 0.4 - 0.5, IPTG (0.238 g)

was added to each flask, and growth was continued for 8 hrs. Then the cells were harvested by centrifugation at 8000 rpm for 30 minutes in GSA tubes and at 4 ºC. The supernatant was quickly removed and half of the cell pellets were stored at -20 °C, and the rest of the cell pellets were made into cell popcorns and stored immediately at -70 °C for long term use. The cell pellets were suspended in buffer A (10 mM β-glycerophosphate buffer containing 0.25 mM cobalt(II)chloride and 0.25 mM NAD^+, pH 6.6). 10% Sucrose was added and the solution was dropped into liquid Nitrogen to form popcorns, which were immediately transferred to the -70° C freezer. The yield of wet cell paste was 20 g.

Enzyme purification: The cells stored at -20 °C were suspended in buffer A (40 ml) and sonicated with the thick probe at a power setting of 5 at 6 x 30 s periods with cooling in between each sonication. The lysed cells were centrifuged at 4000 rpm for about 30 minutes to remove cell debris. The cell extract was concentrated by using ym-10 Diaflo ultrafiltration membrane (Amicon). Again, the concentrated cell extract was suspended with buffer A (50 ml) and concentrated to 10 ml. Five ml of concentrated cell lysate was diluted 3 times with buffer B and loaded onto a packed SourceQ column. The protein was then eluted with a linear gradient of buffers B and C and fractions of 2 ml were collected. The protein was eluted at about 10 - 15 % C. Enzyme activity in the fractions was detected by coupling with the activity of DAHP synthase and dehydroquinase. Fractions containing the enzyme activity were transferred to a concentrator and diluted with 2 ml of buffer A and concentrated to 0.5 - 1 ml. This step was repeated three times and immediately the enzyme was snap frozen and kept in the -70 °C freezer. The same purification procedure was repeated with the rest of the lysate.

5.4 Experimental for Chapter 4

The assay used to study the effect of temperature and pH on the catalytic activity of dehydroquinate synthase.

DAHP ~ 2 μM

NAD^+	30 μM
Co^{2+}	50 μM

For the pH experiments 50 mM BTP buffer (pH = 4.8, 6, 6.6, 7.5, 8, 9.3) were prepared and the sample pH was adjusted to the appropriate pH, with the appropriate buffer to a final volume of 1 ml. For the temperature experiments, the spectrophotometer was set to the required temperature and the sample had equilibrated to the correct temperature and the enzymes were added. 0.5 Units of dehydroquinase were added and then 2 units of DHQ synthase were added and the progress of the reaction was measured at A_{234}. The rate was measured for the first 10 sec of the progress curve and the relative rate was plotted against the temperature and pH values.

^{19}F NMR experiments with (3*S*)-3-fluoro DAHP and 3-dehydroquinate synthase.

Experiments at pH 6 and 8.

3-Fluoro DAHP (12 mg) was dissolved in 500 μl of D_2O. It was divided into two portions. NAD^+ (30 μM) and Co^{2+} (50 μM) were added to each portion. The required pH was adjusted with the appropriate pH BTP buffer and 200 μl of DHQ synthase was added and the ^{19}F NMR spectra were obtained before and after the addition of the enzyme. Every thirty minutes intervals the progress of the reaction was followed by ^{19}F NMR spectroscopy.

Experiments at 15 °C and 37 °C.

3-Fluoro DAHP (10 mg) was dissolved in 250 μl of D_2O. NAD^+ (30 μM) and Co^{2+} (50 μM) were added and the pH was adjusted to 6.8 with BTP buffer to a final volume of 1 ml. The temperature of the spectrophotometer was set at 15 °C and when the sample had equilibrated the enzyme (200 μl) was added. The experiment at 37 °C was carried out in a temperature controlled room. For this experiment 13

mg of 3-fluoro DAHP was used and 200 µl of enzyme was added. The progress of the reaction was followed as for pH experiments.

Experiment at pH 7.3.

This experiment was carried out with the remaining 5 mg of 3-fluoro DAHP and at room temperature. The same amount of DHQ synthase was added and the progress of the reaction was followed as above.

Appendix

Amino acid codes

A	Ala	alanine
C	Cys	cysteine
D	Asp	aspartate
E	Glu	glutamate
F	Phe	phenylalanine
G	Gly	glycine
H	His	histidine
I	Ile	isoleucine
K	Lys	lysine
L	Leu	leucine
M	Met	methionone
N	Asn	asparagine
P	Pro	proline
Q	Gln	glutamine
R	Arg	arginine
S	Ser	serine
T	Thr	threonine
V	Val	valine
W	Trp	tryptophan
Y	Tyr	tyrosine

References

1. Abell C. "*Enzymology and molecular biology of the shikimate pathway*", Comprehensive Natural Product Chemistry, 1999, 573 - 607.
2. Bartlett P. A., Mc Laren K. L. and Marx M. A. "*Divergence between the enzyme-catalysed and noncatalysed synthesis of 3-dehydroquinate*", J. Org. Chem., v. 59, 1994, 2082 - 2085.
3. Bartlett P. A. and Satake K. "*Does dehydroquinate synthesise dehydroquinate*", J. Am. Chem. Soc., v. 110, 1988, 1628 - 1630.
4. Bender S. L., Widlanski T. and Knowles J. R. "*Dehydroquinate synthase:The use of substrate analogues to probe the early steps of the catalysed reaction*", Biochemistry, v.28, 1989, 7560 - 7572.
5. Bender S. L., Widlanski T. and Knowles J. R. "*Dehydroquinate synthase:The use of substrate analogues to probe the late steps of the catalysed reaction*", Biochemistry, v.28, 1989, 7572 - 7582.
6. Bender S. L., Mehdi S., Knowles J. R. "*Dehydroquinate synthase:The role of divalent metal cations and of nicotinamide adenine dinucleotide in catalysis*", Biochemistry, v.28, 1989, 7555 - 7560.
7. Bently R. "*The shikimate pathway - a metabolic tree with many branches*", Critical Reviews in Biochemistry and Molecular biology, v.25, 1990, 307 - 384.
8. Bergmann E. D. and Shahak I. J. "*Organic fluorine compounds-An ester of phosphoenol-fluoropyruvic acid and related compounds*", J. Chem.Soc., 1960, 462 - 463.
9. Blackmore P. F., Williams J. F. and Mac Leod J. K. "*Dimerisation of erythrose 4-phosphate*", FEBS Letters, v.64, 1, 1976, 222 - 226.
10. Boocock M. R. and Coggins J. R. "*Kinetics of 5-enolpyruvylshikimate-3-phosphate synthase inhibition by glyphosate*", FEBS Letters, v.154, 1983, 127 - 133.
11. Carpenter E. P., Hawkins A. R., Frost J. W. and Brown K. A. "*Structure of dehydroquinate synthase reveals an active site capable of multistep catalysis*", Nature, v.394, 16, 1998, 299 - 302.

12. Clark V. M. and Kirby A. J. "*Phosphoenol pyruvic acid (monocyclo hexyl ammonium salt)*", Biochemical Preparations, v.11, 1966, 101 - 104.

13. Clark V. M. and Kirby A. J. "*Neighbouring group participation in phosphate ester hydrolysis*", J. Am. Chem. Soc., v. 85, 1963, .3705 - 3706.

14. Eliel E.L., Wilen S.H. "*Stereochemistry of organic compounds*", John Wiley and Sons, New York, 1994, 872 - 880.

15. Bulloch E. "*Structure and substrate binding studies of DAHP synthase*", BSc(hons) research report, Massey University, 2000.

16. Frost J. W., Bender J. L., Kadonaga J. T. and Knowles J. R. "*Dehydroquinate synthase from Escherichia coli: Purification, cloning, and construction of overproducers of the enzyme*", Biochemistry, v. 23, 1984, 4470 - 4475.

17. Garner C. C., Hermann K. M. "*Structural analysis of 3-deoxy-D-arabino-heptulosonate 7-phosphate by ^{1}H and natural-abundance ^{13}C-n.m.r. spectroscopy*", Carbohydrate Research, v. 132, 1984, 317 - 322.

18. Günel-Özcan A., Brown K.A., Allen A.G. and Maskell D. J. "*Salmonella typhimurium aroB mutants are attenuated in BALB/c mice*", Microbial Pathogenesis, v. 23, 1997, 311 - 316.

19. Hermann K. M., and Poling M. D. "*The synthesis of 3-deoxyheptulosonic acid 7-phosphate*", Journal of Biological Chemistry, v. 230, 17, 1975, 6817 - 6821.

20. Jakeman D. L. and Evans J. N. S. "*Overexpression, purification, and use of phosphoenol pyruvate synthetase in the synthesis of PEP analogues*", Bioorganic Chemistry, v. 26, 1998, 245 - 253.

21. Keeling P. J., Palmer J. D., Donald R. G. K., Roos D. S., Waller R. F., Mc Fadden G. I. "*Shikimate pathway in apicomplexan parasites*", Nature, v. 397, 1999, 219 - 220.

22. Knowles J. K. "*Mechanistic ingenuity in enzyme catalysis : Dehydroquinate synthase*", Aldrichimica Acta, v. 22, 3, 1989, .59 - 67.

23. Kohen A., Berkovich R., Belakhov V., Bassov T. "*Stereochemistry of the KDO8P synthase. An efficient synthesis of the 3-fluoro analogues of KDO8P*", Bioorganic and medicinal chemistry letters, v. 3, 8, 1993, 1577 - 1582

24. Mehdi S., Frost J. W., Knowles J. R. "*Dehydroquinate synthase from Escherichia coli, and its substrate 3-Deoxy-D-arabino-heptulosonic Acid 7-phosphate*", Methods Enzymology, v. 39, 1987, 306 - 315.
25. Millar G., Coggins J.R. "*The complete amino acid sequence of 3-dehydroquinate synthase of Escherichia coli K 12*", FEBS Lett., v.200, 1986, 11 - 17.
26. Montchamp J. L. and Frost J. W. "*Cyclohexenyl and cyclohexylidene inhibitors of 3-dehydroquinate synthase:Active site interactions relevant to enzyme mechanisms and inhibitor design*", J. Am. Chem. Soc., v. 119, 1997, 7645 - 7653.
27. Parker E. J., Coggins J. R. and Abell C. "*Derailing dehydroquinate synthase by introducing a stabilizing stereoelectronic effect in a reaction intermediate*", J. Org. Chem., v. 62, 1997, 8582 - 8585.
28. Parker E. J. "*Mechanistic studies on shikimate pathway enzymes*", PhD Thesis, Cambridge University, England, 1996.
29. Piehler L.T., Montchamp J.L., Frost J.W., Manly C.J. "*Predicting inhibition of dehydroquinate synthase*" Tetrahedron, v.47, 14/15, 1991, 2423 - 2432.
30. Pilch F. T., Sommerville L. R. "*Fluorine-containing analogues of intermediate in the shikimate pathway*", Biochemistry, v. 15, 24, 1976, 5315 - 5320.
31. Sheflyan G.Y., Howe D.L., Wilson T.L., Woodard R.W. "*Enzymatic synthesis of 3-Deoxy-D-manno-octulosonate 8-phosphate, 3-Deoxy-D-altro-octulosonate 8-phosphate, 3,5-Dideoxy-D-gluco-octulosonate 8-phosphate by 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase*", J. Am. Chem. Soc., v. 120, 43, 1998, 11027 - 11031.
32. Simpson F. J., Perlin A. S. and Sieben A. S. "*Erythrose 4-Phosphate*", Methods Enzymology, v. 9, 1966, 35 - 38.
33. Srinivasan P. R., Rothschild J. and Sprinson D. B. "*The enzymatic conversion of 3-deoxy-D-arabino-heptulosinic acid 7- phosphate to 5-dehydroquinate*", J. Biol. Chem., v. 238, 1963, 3176 - 3182.

34. Stubbe J. A. and Kenyon G. L. "*Analogs of phosphoenolpyruvate, substrate specificities of enolase and pyruvate kinase from rabbit muscle*", Biochemistry, v. 11, 1972, 338 - 345.

35. Sundaram A.K., Woodard R.W. "*Probing the stereochemistry of E. coli 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase (phenylalanine-sensitive)-catalysed synthesis of KDO 8-P analogues*", *J. org. chem.*, v.65, 2000, 5891 - 5897.

36. Yamauchi N., Kakinuma K. J. "*Enzymatic carbocycle formation in microbial secondary metabolism-The mechanism of the 2-deoxy-scyllo-inosose synthase reaction as a crucial step in the 2-deoxystreptamine biosynthesis in streptomyces-fradiae*", *J. org. chem.*, v.60, 1995, 5614 - 5619.

37. Widlanski T., Bender S. L. and Knowles J. R. "*Stereochemical course of the cryptic elimination and cyclisation steps in the reaction catalysed by dehydroquinate synthase*", J. Am. Chem. Soc., v. 109, 1987, 1873 - 1875.

Errata sheet

Throughout the thesis the abbreviation for millilitre should be mL and not ml.

p. 34 last paragraph, line 3 : The unit for wavelength was nm.

p. 35 last paragraph, line 7 : molecular weight cut-off 10 KDa.

p. 43 last paragraph, line 1 : The primary structure of the *E. coli* enzyme dehydroquinate synthase was deduced by Millar and Coggins.

p. 78 paragraph 2 : The monosodium salt of glucose 6-phosphate was used in the preparation of erythrose-4 phosphate.

Throughout the thesis the activity of the enzyme is measured in units and a unit is defined as the consumption of one µmole of the enzyme substrate by the enzyme per minute at a temperature of 25 °C.