SOME STUDIES ON SHIKIMATE KINASE

Thesis submitted for the Degree of

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by

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TITLE: **SOME STUDIES ON SHIKIMATE KINASE**

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ABSTRACT

The mechanisms of the enzymes of the shikimate pathway, and the mechanisms of phosphoryl transfer by kinases in general are reviewed in chapter 1.

In chapter 2, the preparation of β,γ-[^{18}O]ATP, α,β-[^{18}O]ADP, α,β-[^{18}O]ATP, and α^1,β-[^{18}O]Ap_3A are reported. β,γ-[^{18}O]ATP is used to investigate the mechanism of phosphoryl transfer by shikimate kinase. In the presence of shikimate kinase and shikimate, randomisation of the β,γ-^{18}O label with the β-O_2 occurs as expected. No significant randomisation was observed in the presence of a novel fluoro shikimate analogue or in the absence of any cosubstrate. An associative SN2(P) mechanism of phosphoryl transfer is thereby assumed.

In chapter 3, the design, synthesis, and characterisation of analogues of shikimate are reported. An epoxy-shikimate analogue is observed to be a weak irreversible inhibitor of shikimate kinase. Substrate protection studies suggest that the modification is most likely at the ATP binding site. *Cis*-diol and *trans*-diol shikimate analogues, lacking the C-5 hydroxyl group, are observed to be substrates of the enzyme. The presence of the C-5 hydroxyl group is found to be important for specificity with a maximum contribution of 3.9 kcal mol^{-1} to binding energy. A fluoro shikimate analogue, also lacking the C-5 hydroxyl, is found to be a competitive inhibitor, with a K_i of 21 mM.

The design, synthesis and characterisation of shikimate analogues with altered hydroxyl stereochemistry at C-3 and C-4, prepared from (5*S*,6*R*)-5,6-dihydroxy-1-(trifluoromethyl)-cyclohexa-1,3-diene are reported in chapter 4. Each of the analogues demonstrates similar properties, showing weak non-competitive inhibition with shikimate kinase. The importance of the carboxylate group for the recognition of shikimate is discussed.

In chapter 5, shikimate kinase was observed to be inactivated by the reagents iodoacetamide, N-ethyl maleimide, iodoacetate, 2,3 butanedione and diethylpyrocarbonate at pH 7.0. N-ethyl maleimide was the most potent inhibitor, being 60- to 6000-times more potent than the other reagents. The presence of ATP was observed to give total protection against modification by iodoacetamide, N-ethyl pyrocarbonate, and diethylpyrocarbonate, suggesting modification at the active site of the enzyme. Shikimate was found also to partially protect against inactivation by each of these three reagents. A cysteine residue within the probable ATP binding site is tentatively assigned as the site of modification, and its possible roles discussed.

STATEMENT

The accompanying thesis submitted for the degree of Doctor of Philosophy entitled "Some studies on Shikimate kinase" is based on work in the Department of Chemistry at the University of Leicester during the period between October 1988 and November 1991.

The work has not been, and is not concurrently being presented for any other degree.

Signed: ..... Date: 19-11-93....

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ABBREVIATIONS AND SYMBOLS

General and Physical:

br	-	Broad
cm^{-1}	-	Wavenumber
δ	-	Chemical shift
d	-	Doublet
Enz/E	-	Enzyme
ΔG_{app}	-	Apparent binding energy
HRMS	-	High resolution mass spectrometry
IR	-	Infrared
J	-	Coupling constant
k_{cat}	-	Catalytic constant
K_i	-	Dissociation constant of enzyme-inhibitor complex
K_M	-	Michaelis constant
nmr	-	Nuclear magnetic resonance
●	-	^{18}O
PIX	-	Positional isotope exchange
ppm	-	Parts per million
q	-	Quartet
s	-	Singlet
S	-	Substrate
t	-	Triplet
t.l.c.	-	Thin-layer chromatography
V_{max}	-	Maximum rate of catalysis

ABBREVIATIONS AND SYMBOLS *(Cont.)*

Chemical:

AMP	-	Adenosine 5′ -Monophosphate
ADP	-	Adenosine 5′ -Diphosphate
ATP	-	Adenosine 5′ -Triphosphate
Ap_3A	-	Diadenosine 5′,5‴ -P^1,P^3- Triphosphate
Ap_5A	-	Diadenosine 5′,5‴ -P^1,P^5- Pentaphosphate
DMF	-	N,N-Dimethylformamide
DEAE	-	Diethylaminoethyl
EDTA	-	Ethylenediaminetetraacetic acid
Et	-	Ethyl
FMN	-	Flavin mononucleotide
LDH	-	Lactate dehydrogenase
mCPBA	-	*meta*-Chloroperoxybenzoic acid
Me	-	Methyl
NAD	-	Nicotinamide adenine dinucleotide
NADH	-	Nicotinamide adenine dinucleotide, reduced form
NADP	-	Nicotinamide adenine dinucleotide phosphate
NADPH	-	Nicotinamide adenine dinucleotide phosphate, reduced form
NMO	-	N-methylmorpholine-N-oxide
PEP	-	Phosphoenolpyruvate
Ph	-	Phenyl
Pi	-	Inorganic phosphate
Ⓟ	-	Phosphate moiety
PK	-	Pyruvate kinase
TEAB	-	Triethylammonium bicarbonate
THF	-	Tetrahydrofuran
TMS	-	Trimethylsilyl
Tris	-	Tris(hydroxymethyl)aminomethane

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CHAPTER 1

General Introduction

1.0 Introduction.

Shikimate kinase catalyses the phosphorylation of shikimic acid to yield shikimate-3-phosphate. This reaction is the fifth step of the early common pathway for the biosynthesis of aromatic amino acids, L-phenylalanine, L-tyrosine and L-tryptophan, in plants and microorganisms. Approximately 20% of the carbon fixed by green plants is routed through the pathway and the three aromatic amino acids are individually important precursors for numerous secondary metabolites including vitamins, lignins, alkaloids and phenolics.

(-)-Shikimic acid was first isolated from the plant *Illicium religiosum* (in Japanese, shikimi-no-ki), by Eykmann in 1885.[1,2] The complete structure and absolute stereochemistry of this carboxylic acid was determined during the 1930's by the studies of Fischer and Dangschat,[3-6] Freudenberg[7] and Karrer.[8] However it wasn't until the early 1950's that the significance of this intermediate to the biosynthesis of aromatic amino acids was fully recognised.[9] Furthermore being the first intermediate to be established led to the pathway being known as the shikimate pathway.

Over the next twenty years intensive effort by a number of workers notably Davis,[10] Sprinson[11] and Gibson[12] revealed a well defined series of eight metabolic intermediates leading from carbohydrate, in the form of glucose, to shikimic acid and then to chorismic acid. For the past two decades interest in the pathway has remained intense. The advent of molecular biology has led to the isolation and purification of the enzymes from the pathway from many sources, thus facilitating detailed molecular characterisation and mechanistic study. The shikimate pathway has been extensively reviewed elsewhere.[13-18]

It is the molecular characterisation and mechanistic study of the enzyme *Escherichia coli* shikimate kinase II that forms the central theme of this thesis.

1.1 The Common Pathway.

The main shikimate metabolic pathway, from carbohydrate to chorismic acid, is shown in figure 1.0.

1.1.1 3-Deoxyarabinoheptulosonate-7-phosphate Synthase.

Oxidation of glucose both by the pentose phosphate pathway and glycolysis affords D-erythrose-4-phosphate (E4P) *(1)* and phosphoenolpyruvate (PEP) *(2)* respectively. The condensation of E4P with PEP to give 3-deoxyarabinoheptulosonate-7-phosphate (DAHP) is catalysed by 3-deoxyarabinoheptulosonate-7-phosphate synthase (DAHPS) and constitutes the first committed step of this pathway. DAHPS has been isolated from a number of microbial and plant sources,[18] which show a degree of similarity between them. Many of the enzymes are feedback regulated by one or more of the aromatic amino acids and demonstrate a requirement of a divalent metal cation, with Co^{2+} and Mn^{2+} being the most common. Most of the mechanistic enzymology on DAHPS has been performed using the tyrosine-sensitive isoenzyme of *E.coli*.

The mechanism of carbon-carbon formation has been much debated since the first proposal of a concerted aldol type condensation from Sprinson (scheme 1.0).[19] However experiments by DeLeo and Sprinson,[20] and Nagano and Zalkin[21] with [$H_2{}^{18}O$] indicated that no ^{18}O is incorporated into inorganic phosphate. Furthermore when E4P was reacted with PEP carrying an ^{18}O label specifically in the C-O-P oxygen, ^{18}O was recovered almost entirely in the inorganic phosphate released. Thus these results were consistent with cleavage of the C-O (rather than the P-O) bond of PEP during the enzymic reaction. A mechanism was therefore postulated by Sprinson[20] in which PEP is first transferred to a nucleophilic group (e.g. carboxyl) on the enzyme by acid catalysed addition to the double bond. Elimination of the phosphate restores the alkene which upon acyl-oxygen cleavage can initiate the aldol condensation (scheme 1.1). This mechanism is further supported by the observation of

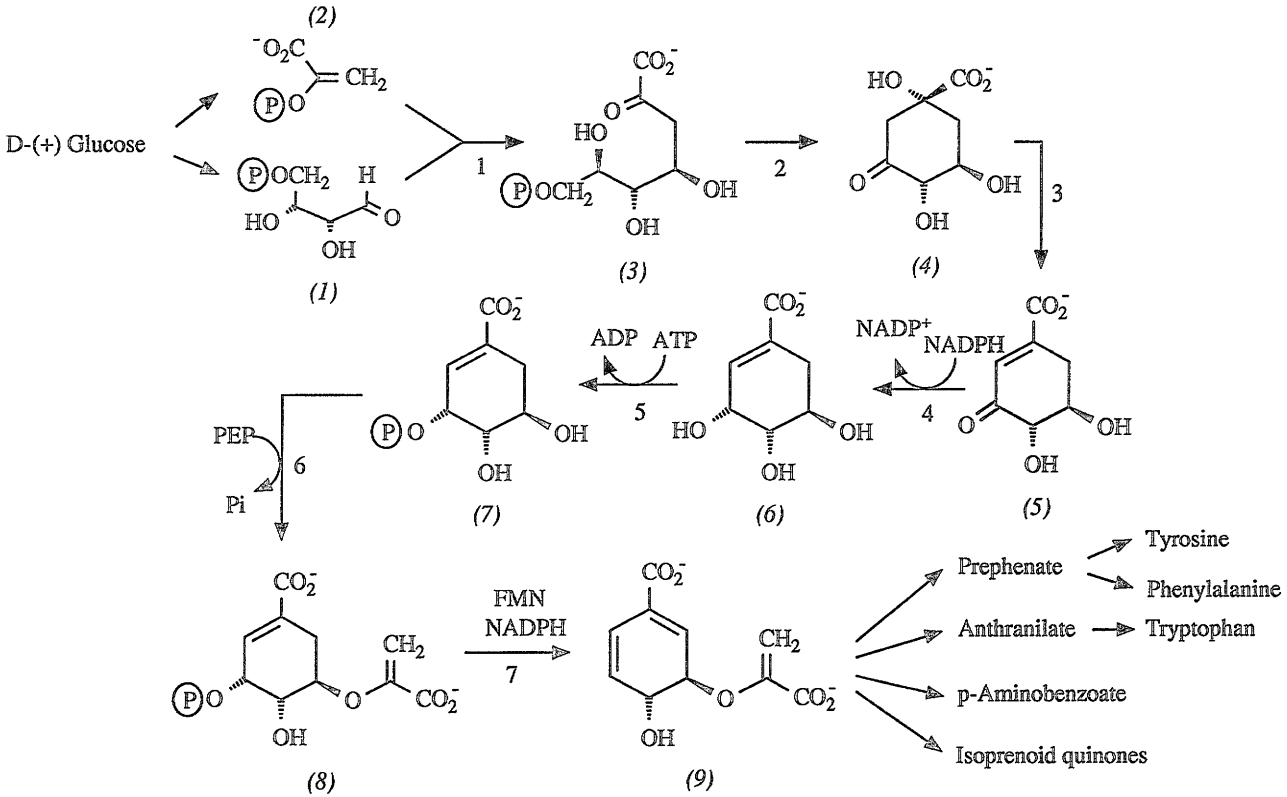
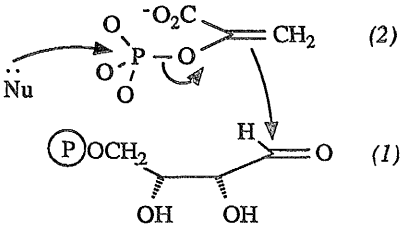
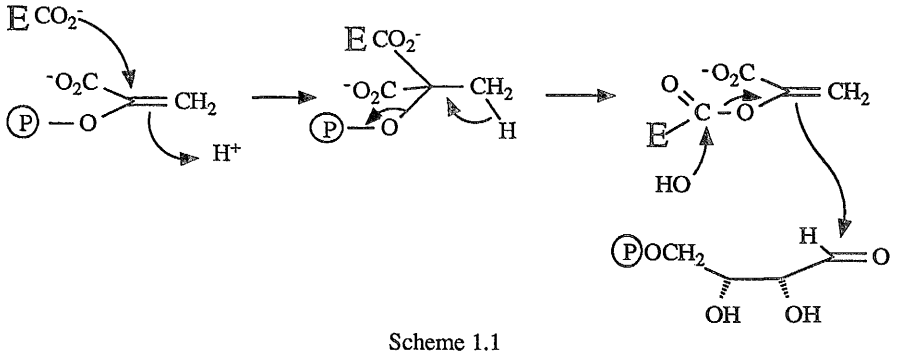


Figure 1.0. 1. 3-Deoxy-D-arabino-heptulosonic acid 7-phosphate synthase. 2. 3-Dehydroquinate synthase 3. dehydroquinase 4. Shikimate dehydrogenase 5. Shikimate kinase 6. 5-Enolpyruvylshikimate 3-phosphate synthase 7. Chorismate synthase.



Scheme 1.0

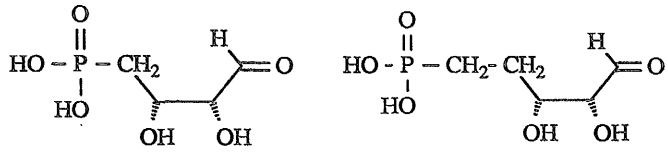


Scheme 1.1

tritium incorporation from medium ^3H_2O at C-3 of DAHP albeit at low level.[22]

Stereochemical studies by Floss *et al.*[23] using specifically monotritiated *E* and *Z*-phosphoenolpyruvates showed that the condensation was essentially stereospecific, suggesting the lack of free rotation of the methyl group. A mechanism in which the protonated intermediate is so short-lived that the same hydrogen is lost before rotation is consistent with the observation of low level take up from tritiated medium. Kinetic studies indicate that the *E.coli* (Tyr) DAHPS follows an ordered sequential mechanism,[24] PEP is the initial substrate to bind the enzyme and that inorganic phosphate leaves the enzyme-products complex first. DAHPS does not, however, catalyse the incorporation of ^3H from ^3H_2O into PEP in the absence of E4P[21] indicating that bound PEP is present as a carboxyvinyl rather than a pyruvyl intermediate.

The ability of the enzyme to handle phosphonate analogues of E4P was investigated by Le Maréchal *et al.*[25,26] (figure 1.1). Both compounds were found to be substrates generating the



non-isosteric phosphonate

isosteric homo phosphonate

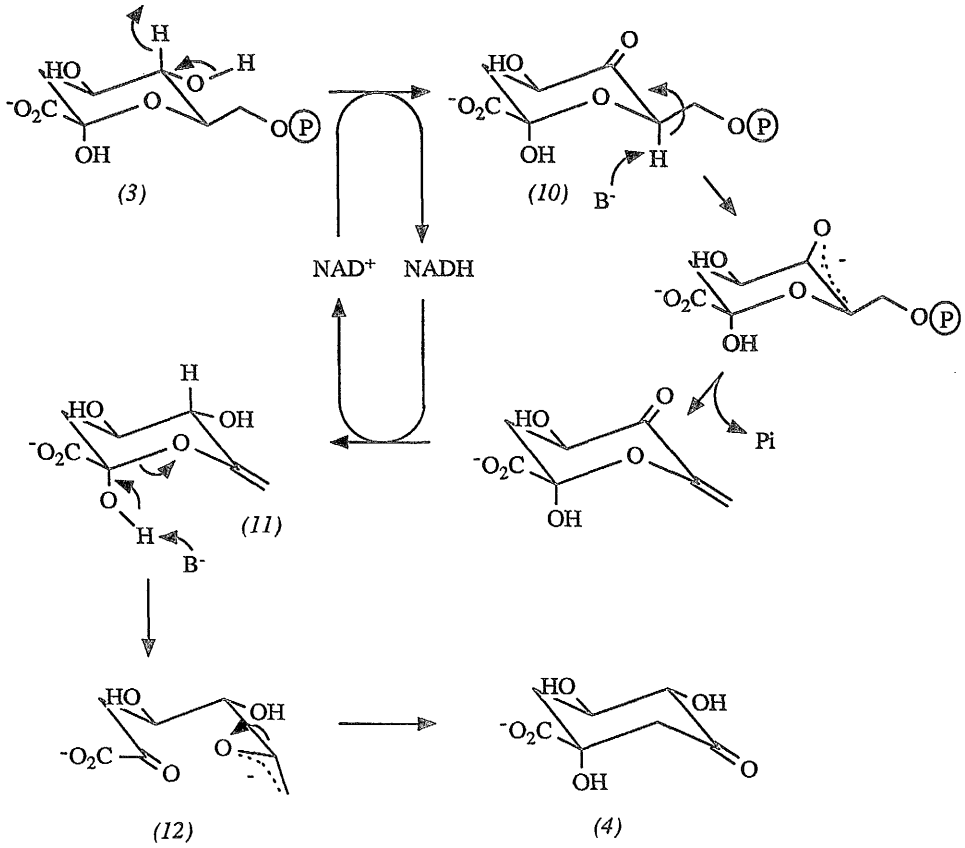
Figure 1.1

corresponding analogues of DAHP. The isosteric homophosphonate was found to be the better substrate with the kinetic data suggesting a K_M only slightly higher than E4P.

1.1.2 Dehydroquinate Synthase.

Dehydroquinate synthase (DHQS) catalyses the second step of the pathway, catalysing the ring closure of DAHP *(3)* to give 3-dehydroquinate (DHQ) *(4)*. The enzyme has also been isolated from microbial and plant sources. Enzymes from both sources demonstrate NAD^+ and Co^{2+} dependance.[18] As shown in scheme 1.2 the enzyme appears to catalyse an unusually complex series of chemical changes.[27,28] Firstly, the hydroxyl is oxidised at C-5 by bound NAD^+, the resulting carbonyl facilitating β-elimination of the bound phosphate. Reduction of the C-5 hydroxyl using the bound NADH, formed in the first step, followed by ring opening unmasks C-2 carbonyl and an enolate. Intramolecular aldol condensation of the carbonyl group and the enolate generates the cyclohexane DHQ.

In recent years the validity and stereochemistry of the proposed reaction has been tested by the synthesis of putative intermediates and related analogues. Knowles *et al.*[29] have probed the mechanism using 2-deoxy analogue *(13)* (figure 1.2). The analogue is processed by the enzyme resulting in the formation of inorganic phosphate and enol ether *(14)*. This result therefore substantiates the first three steps of the pathway and confirms that ring opening of DAHP does not occur until after the reduction step. The stereochemistry of the elimination



Scheme 1.2

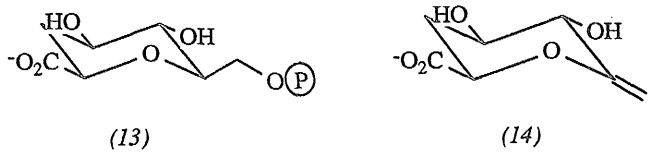
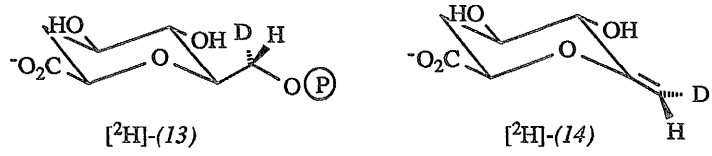


Figure 1.2

was further investigated by the synthesis of stereospecifically monodeuterated (7*S*)-[7-^{2}H]-*(13)*. Analysis of the resulting enol ether following incubation with the enzyme indicated the label was exclusively in the *E* position, therefore confirming the *syn* elimination of inorganic phosphate (figure 1.3). Since the overall reaction proceeds with

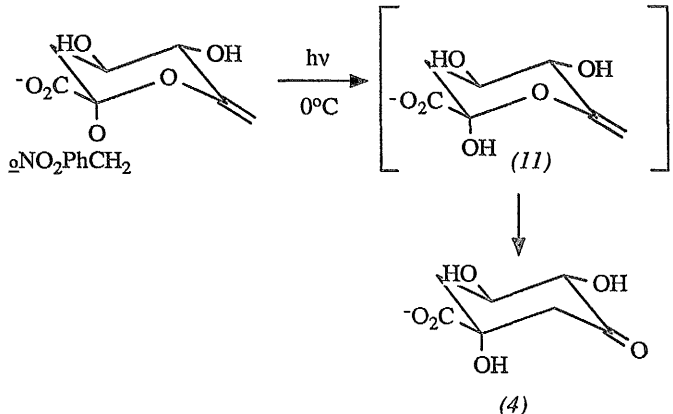


[^{2}H]-*(13)* [^{2}H]-*(14)*

Figure 1.3

inversion of configuration at C-7,[30] the geometry of the phosphate elimination suggests the transition state of the subsequent aldol condensation is most likely chair-like.

In 1988 the role of the enzyme in catalysing the whole sequence of reactions was put into question by the finding of Bartlett *et al.* that the non-enzymic synthesis of enolpyranose *(11)* led to the rapid formation of DHQ *(4)* in solution (figure 1.4).[31] Furthermore

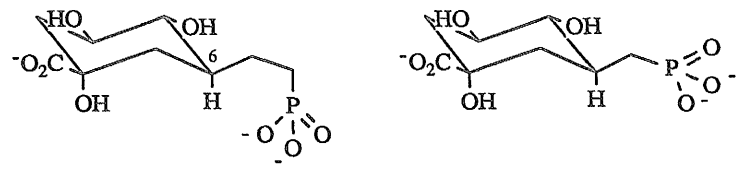


(11) *(4)*

Figure 1.4

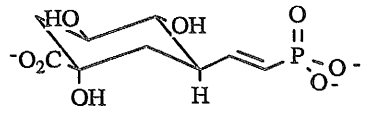
generation of monodeuterated (7Z)-[7-^{2}H]-*(11)* spontaneously rearranged to DHQ with identical stereochemistry to that observed in the biosynthetic transformation.

The contribution of the enzyme to the catalysis of the reaction was further questioned by Knowles *et al.* using a series of isosteric and nonisosteric analogues of DAHP (figure 1.5).[32] Analogue *(15)* was observed to be bound at the active site and underwent enzyme catalysed exchange of the C-6 proton in the presence of D_2O. Analogue *(16)* was found to be very strongly bound by the enzyme but the C-6 proton was not exchanged. The *E*-vinyl

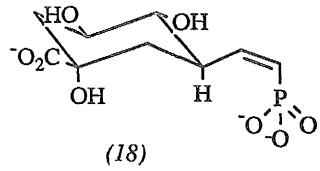


(15)

(16)



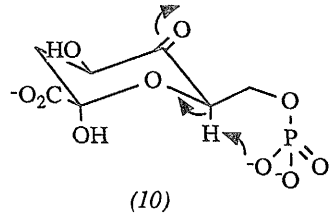
(17)



(18)

Figure 1.5

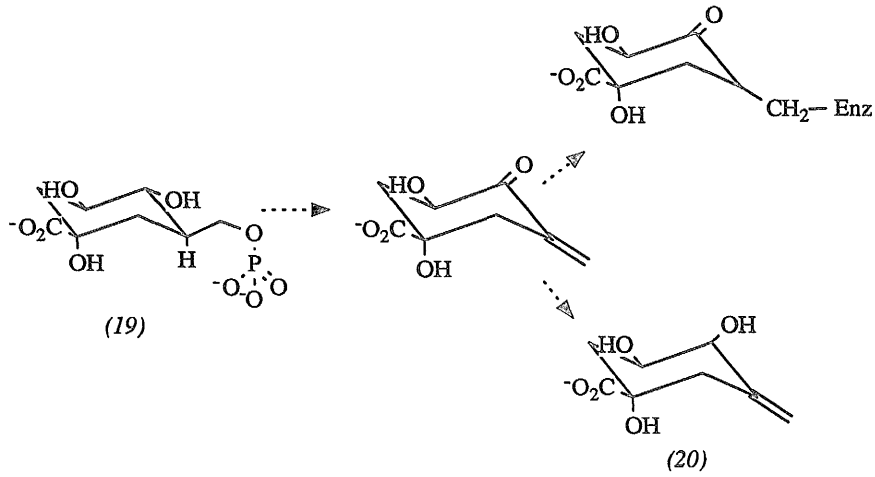
homophosphonate analogue *(17)* was only weakly bound by the enzyme and no proton exchange at C-6 was detectable. Only the Z-vinyl analogue *(18)* was observed to be both tightly bound and exchanges its C-6 proton with solvent. Knowles postulated that the base responsible for the removal of the proton at C-6 of intermediate *(10)* is a peripheral oxygen of the phosphate group of the substrate DAHP (figure 1.6).



(10)

Figure 1.6

The presence of an enzymic base responsible for the abstraction of the proton at C-6 was investigated by the research group of Ganem.[33] They prepared carbocyclic analogue *(19)* as a potential suicide inhibitor of the enzyme. It was reasoned that the enzyme might oxidise *(19)* and eliminate the C-6 proton to generate a more reactive enone which might then covalently bind to the local base (figure 1.7). However no inactivation of the enzyme was observed, instead *(19)* was processed to *(20)*. Despite the absence of suicide inhibition this

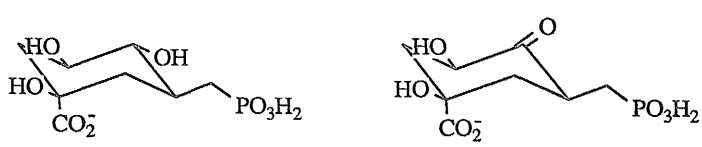


(19) *(20)*

Figure 1.7

result also substantiates the initial steps of the pathway and concurs with the proposal of Knowles.

Following the work of Le Maréchal *et al.*,[25,26] Frost *et al.* has also investigated the inhibitory effects of a variety of phosphonate and homophosphonate analogues with DHQS.[34-37] Consistent with the findings of Le Maréchal and Knowles *et al.*[32] nonisosteric phosphonate analogues were found to demonstrate significantly greater inhibitory effects than the isosteric homophosphonates. The importance and stereochemistry of the hydroxyl and carboxyl group attached to the anomeric carbon were also examined. Further to this work Frost has recently reported the inhibition of DAHPS with carbaphosphonate *(21)*[36] and with ketocarbaphosphonate *(22)*[37] (figure 1.8). Carbaphosphonate *(21)* was found to be



(21) *(22)*

Figure 1.8

a potent competitive inhibitor with a similar level of inhibition demonstrated by

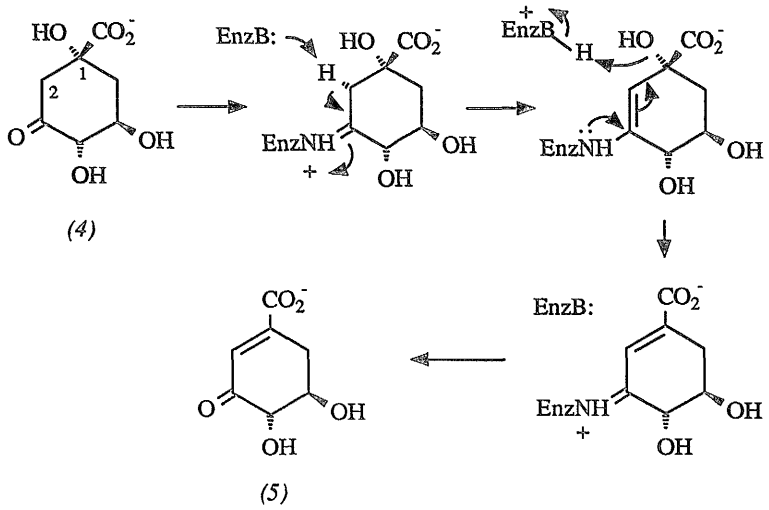
diastereomeric carbaphosphonate *(16)* prepared by Knowles *et al.*[32] Frost argues that the inhibition is derived from analogies between *(21)* and intermediate *(12)* (scheme 1.2). The interpretation also supported by computational molecular superimposition studies. This hypothesis contrasts to the picture presented by Bartlett *et al.*[31] and suggests the ring opening and subsequent aldol condensation take effect on, and are catalysed by the enzyme.

Ketocarbaphosphonate *(22)* is an analogue of intermediate *(10)* (scheme 1.2) and is reported to be an irreversible inhibitor of the enzyme. The mechanism of the inhibition is not clear however, and the inhibition is not protected but augmented by the presence of DAHP. The observation of *(22)* as an irreversible inhibitor is contrary to that observed by Knowles *et al.*,[32] since phosphonate *(16)* (which is the most potent competitive inhibitor to date) is reported to be readily oxidised at C-5 when bound to the enzyme and ketocarbaphosphonate *(22)* is thereby generated. Irreversible inhibition of the enzyme is not reported by Knowles.

1.1.3 Dehydroquinase.

Dehydroquinase catalyses the stereospecific *syn*-dehydration of DHQ *(4)* to dehydroshikimate (DHS) *(5)* and introduces the first double bond of the aromatic ring system. The enzyme has been isolated from microbial, fungal sources with the sequences demonstrating moderate homologies.[18] The *E.coli* enzyme is a dimer with a subunit molecular weight of 29,000.[38]

Early labeling experiments[39] established that the proton lost from the C-2 position of DHQ was *cis* to the hydroxyl at C-1, suggesting that the mechanism of the dehydration was not straightforward. Following the discovery of Schiff's base formation between a lysine residue and the ketone at C-3 of DHQ,[40] mechanisms were proposed involving conformational change of the substrate and subsequent abstraction of the proton at C-2 by a general base (scheme 1.3).[41,42] A base with pK of around 6, possibly histidine, is implicated from the pH/rate profile and inactivation using diethyl pyrocarbonate.[43] Recent covalent



Scheme 1.3

modification of the *E.coli* enzyme has confirmed the identification of essential histidine and methionine residues.[44,45]

The chemistry of the active site was also recently probed by Bugg *et al.*[46] A series of specifically designed affinity labels were found to irreversibly inhibit *E.coli* dehydroquinase. The proposed modes of action of the affinity labels were found to be consistent with the suggested mechanism, and are discussed as part of the introduction to chapter 3.

1.1.4 Shikimate Dehydrogenase.

Shikimate dehydrogenase (SDH) catalyses the NADPH-dependant reduction of DHS *(5)* to shikimate *(6)* and represents the fourth step of the pathway. The *E.coli* enzyme is a monomeric, monofunctional protein of molecular weight 32,000.[47] Yeast and fungal enzymes show moderate homology of amino acid sequence (27%) while the homology between fungal and bacterial enzymes is low (15%).[18] Bugg *et al.* have synthesised deoxy analogues of DHS allowing estimation of apparent binding energies (ΔG_{app}) between each

hydroxyl and the enzyme.[48] They found that the presence of the C-4 hydroxyl group was important for specificity with a ΔG_{app} of 7 kcal mol^{-1} for the 4-deoxy analogue consistent with the removal of a hydrogen bonding interaction between the substrate and a charged group at the active site. This was in agreement with the observation by Balinsky and Davies that the most effective aromatic inhibitors of the reverse reaction of *P.sativum* SDH possessed a *para*-hydroxyl group.[49] From examination of the pH/rate profile they suggested that this hydroxyl group forms a hydrogen bond to either cysteine or an α-NH_2 group on the enzyme.[50]

1.1.5 Shikimate Kinase.

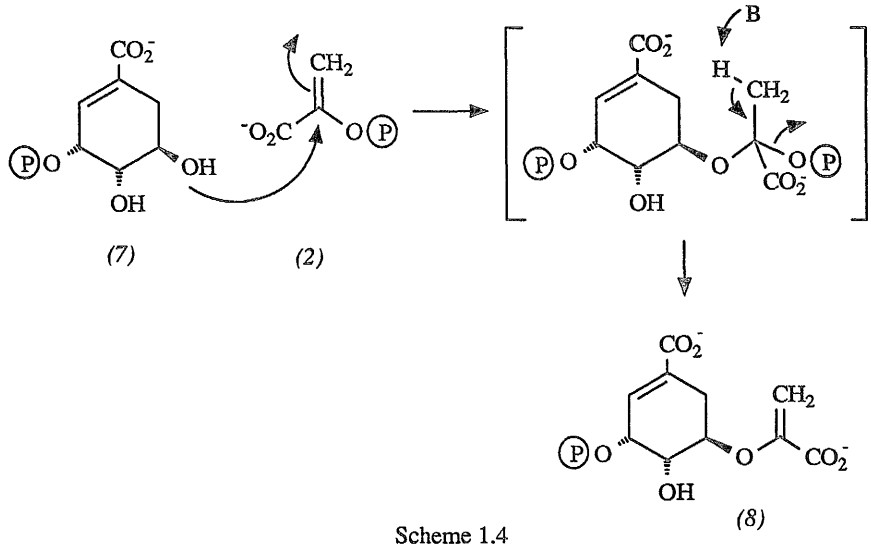
Shikimate kinase (SK) catalyses the phosphorylation of shikimic acid *(6)* to shikimate-3-phosphate (S3P) *(7)*. Both *S.typhimurium* and *E.coli* contain two isoenzymes, SK I and SK II.[51,52] Regulation of SK II by the aromatic amino acids has been demonstrated whereas SK I appears to be unaffected by these amino acids.[51] The reason for the existence of the two isoenzymes from *E.coli* is not known, but it has been suggested that shikimate may be a branch-point intermediate for the two distinct pathways.[15] SK I and SK II demonstrate significant differences in affinities for shikimate suggesting that SK I functions only when high intracellular levels of shikimate occur.[53] The *E.coli* enzyme has a molecular weight of 19,000[54] and consistent with other phosphotransferases has a requirement for a divalent metal ions, Mg^{2+} is observed to be the most effective.[53] The amino acid sequence of the *E.coli* enzyme contains a region homologous to other kinases and ATP-requiring enzymes.[54] A sequence motif common to DHQ and DHQS has also been identified.[55]

1.1.6 5-Enolpyruvylshikimate 3-phosphate Synthase (EPSPS).

5-Enolpyruvylshikimate 3-phosphate synthase (EPSPS) catalyses the transfer of the carboxyvinyl moiety of PEP to S3P *(7)*, yielding EPSP *(8)* and Pi, and is one of the most characterised enzymes of the pathway. The enzyme as isolated from plants and bacteria[56,57]

is a monomer with molecular weight 46,000.

Early work by Sprinson *et al.*[58] established that incubation of the enzyme with S3P and PEP in tritiated water gave tritium-labeled EPSP. This was seen as evidence for a novel reaction of PEP involving an addition-elimination mechanism, in which nucleophilic attack at C-2 with protonation at C-3, giving an enzyme bound intermediate from which phosphate is eliminated (scheme 1.4). Knowles *et al.*[59,60] confirmed that the C-3 of the enolpyruvyl

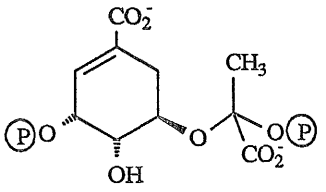


Scheme 1.4

group transiently becomes a methyl group by demonstrating exchange of C-3 protons with [3-^{2}H]PEP. Equal amounts of exchange with each of the protons is consistent with a methyl group that freely rotates. Knowles *et al.*[61] also showed that when (*Z*)-phospho[3-^{2}H,^{3}H]enolpyruvate was the substrate, significant amounts of the label are retained as a result of kinetic isotope effects in both the addition and elimination steps. Furthermore, analysis of the resulting EPSP demonstrated that the *Z* configuration was retained in the product, inferring that the addition and elimination steps have opposite stereochemistry.

Further isotope labeling and kinetic studies[62,63] were also in support of an addition-elimination mechanism. The detection and later isolation and characterisation of

the tetrahedral intermediate *(23)* (figure 1.9) by Anderson *et al.* offered final proof.[64,65] The

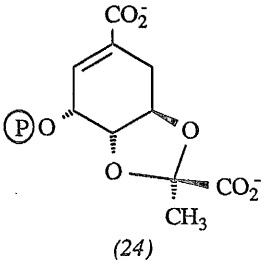


(23)

Figure 1.9

absolute configuration of the extracyclic stereocenter of *(23)* could not be determined. The kinetic parameters, determined by Anderson *et al.*, of each of the individual steps of the reaction demonstrated an ordered sequence of substrate binding and product release. S3P *(7)* binds prior to PEP *(2)* and, EPSP *(8)* follows inorganic phosphate release.[66,67]

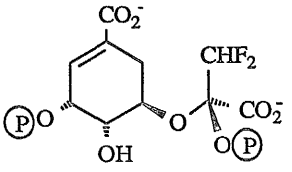
Bartlett *et al.* have synthesised tight binding analogues of the tetrahedral intermediate by replacing the phosphate with a more stable phosphonate.[68] Both the *R* and *S*-analogues were found to be competitive, with the *R*-diastereoisomer binding 60-fold more tightly than the *S*-inhibitor. The *R*-configuration of the side chain of intermediate *(23)* is therefore inferred. However, the *S*-configuration has also been suggested[69] from the stereochemistry of ketal *(24)* (figure 1.10), a decomposition product of *(23)* identified by Sammons *et al.*[70] A range



(24)

Figure 1.10

of analogues of EPSP and fluorinated analogues of intermediate *(23)* have recently been prepared and evaluated by Bartlett *et al.*[71] The most potent inhibitor was found to be the *R*-stereoisomer of the difluoromethyl derivative *(25)* (figure 1.11), however there was no clear stereochemical discrimination of the enzyme towards these analogues. Bartlett

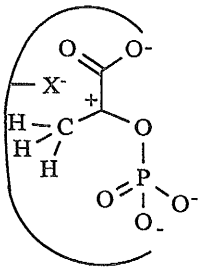


(25)

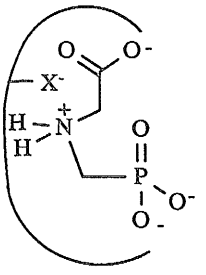
Figure 1.11

suggests that the phosphate and carboxylate moieties may interchange binding sites without undue penalty. Therefore it seems that only crystalisation of *(23)* will reveal the stereochemical identity of the extracyclic centre.

EPSPS is the target enzyme for glyphosate *(26)* or N-phosphonomethylglycine, the active ingredient in the broad spectrum herbicide 'Roundup'. Kinetic studies with glyphosate suggest a reversible interaction between glyphosate and the enzyme-S3P complex, in a reaction that is competitive versus PEP and uncompetitive with respect to S3P *(7)*.[72] It has been suggested and generally accepted that glyphosate inhibits EPSPS by acting as a transition state analogue for the PEP-derived carbocation (figure 1.12), with the positively



Enzyme bound oxocarbonium ion *(27)*



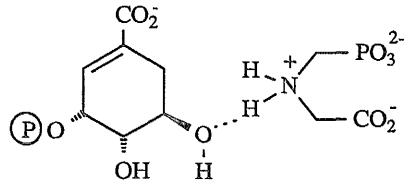
Enzyme bound glyphosate *(26)*

Figure 1.12

charged nitrogen of glyphosate mimicking the C-2 carbonium of PEP. The finding by Abeles *et al.*[73] and more recently by Pansegrau *et al.*[74] that slow deuterium exchange into the vinyl hydrogens of PEP occurs in the presence of dideoxy and deoxy analogues of S3P suggests that tetrahedral intermediate *(23)* is formed *via* the oxocarbonium ion *(27)*.[73]

However, unconventionally for transition state inhibitors, glyphosate binds to the enzyme relatively weakly (K_i = 0.5 μM, K_M = 15μM for PEP (*E.coli* enzyme)).[18] Additionally the isolation of highly glyphosate-tolerant EPSPS mutants without equivalent effects on PEP binding and catalysis also suggests glyphosate is only a poor transition state inhibitor.[75] Glyphosate is not a ground state PEP analogue since it does not inhibit other PEP utilizing enzymes. If glyphosate does, in the protonated state, mimic the protonated state it could be argued that N-methylglyphosate might have greater affinity. However, this and other N-substituted glyphosate analogues demonstrate only modest inhibition of the enzyme.[76]

These results as well as other considerations have led Anderson and Johnson to a different view about the mode of inhibition of glyphosate *(26)*.[76] They suggest that the S3P-glyphosate complex resembles the overall geometry of the tetrahedral intermediate *(23)* (figure 1.13) and that glyphosate interacts with the same active-site residues which



S3P-Glyphosate complex

Figure 1.13

catalyse the formation and breakdown of *(23)*. Anderson and Johnson attribute the herbicidal potency of glyphosate to the fact that glyphosate binds in the presence of S3P *(7)*,[77] and increasing concentrations of S3P resulting from the inhibition of the enzyme will augment, rather than overcome, the inhibition. Additionally, plant studies have shown that the transport of glyphosate into actively growing tissues contributes to its effectiveness.[78]

1.1.7 Chorismate Synthase.

Chorismate synthase (CS) catalyses the conversion of EPSP *(8)* to chorismic acid *(9)*

introducing the second double bond of the aromatic ring in an unusual *trans*-1,4-elimination of inorganic phosphate. The enzyme has a requirement for a reduced flavin cofactor although the reaction results in no net overall change in redox state. The majority of mechanistic studies have been conducted using CS from *N.crassa*,[79] since the *E.coli* enzyme is oxygen sensitive.

Early labeling experiments by the research groups of Hill[80] and Floss[81] demonstrated that the elimination proceeded with abstraction of the *pro-R* hydrogen at C-6. However, to date the mechanism is not known. Some of the postulated mechanisms as illustrated by Abell *et al.*[82] are shown in figure 1.14. Molecular orbital considerations and modelling experiments

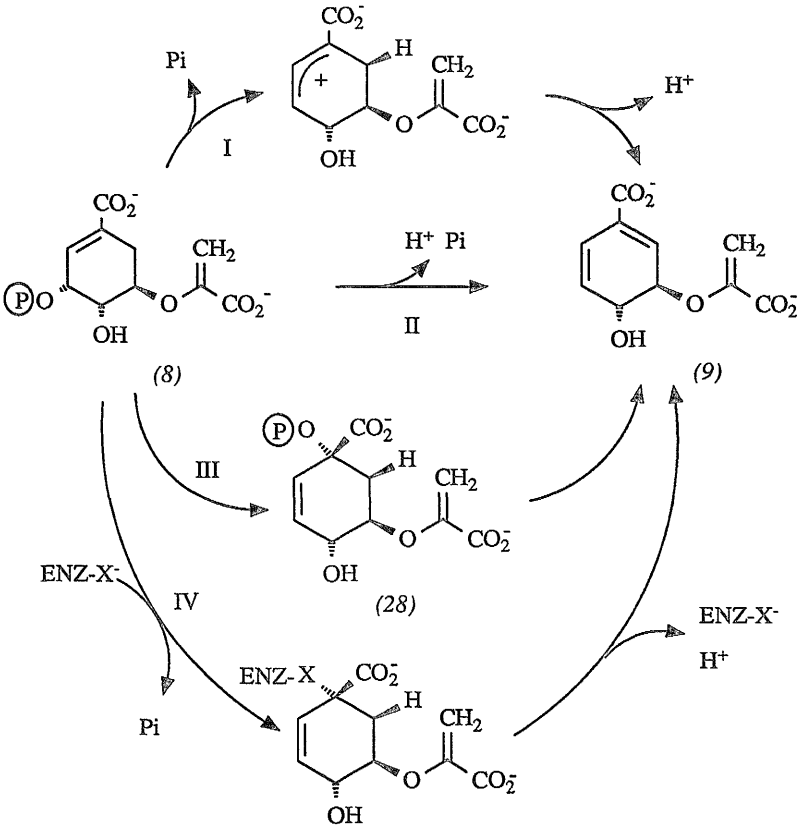


Figure 1.14

suggest that a concerted E2' elimination (mechanism II) is unlikely.[83,84] The rearrangement mechanism (mechanism III) first postulated by Ganem[14] also seems unlikely since putative intermediate *(28)* was found to a competitive inhibitor, rather than a substrate, of the enzyme.[85]

Hawkes and co-workers[86] have recently investigated mechanism IV, first suggested by Floss *et al.*[87] by observation of the pre-steady-state kinetics of phosphate loss. The absence of a detectable 'burst' of phosphate release suggests that phosphate is probably released concomitantly with the rate-limiting step. Hawkes *et al.* argue that the constraints imposed on mechanism IV, in the absence of phosphate burst, are too severe and that a mechanism involving a carbocation (mechanism I) better accommodates this result. The failure of 6*R* and 6*S*- fluoro analogues of EPSP to irreversibly inhibit the enzyme[82] also disfavours a mechanism involving a covalent enzyme intermediate with concomitant phosphate loss. Abell *et al.* has also investigated the possibility of a kinetic isotope effect at the *pro-R* hydrogen at C-6.[88] When this proton is replaced by deuterium there was a pronounced reduction in the rate of catalysis, suggesting that bond breaking at C-6 is rate limiting. The presence of a primary kinetic isotope effect therefore disfavours mechanism I involving a carbocation. Abell suggests that the absence of phosphate burst and the kinetic isotope effect can be best accommodated by a concerted mechanism despite being disfavoured on theoretical grounds.

Very recently Abell and coworkers have reported the direct involvement of the reduced flavin ($FMNH_2$) in the catalytic cycle of recombinant *E.coli* CS.[89] Furthermore upon binding of 6*R*-fluoro EPSP they report the formation of a stable N-5 flavin radical. Although this is not the same intermediate observed in the presence of EPSP, they suggest a free radical could be an intermediate in the enzyme reaction in which the rapid removal of a hydrogen radical from C-6 is possible. The involvement of a radical might therefore eliminate the unfavourable molecular orbital considerations of a classical concerted reaction.

1.1.8 The Post-Chorismate Pathway.

As shown in figure 1.15, chorismic acid *(9)* is converted by five separate enzymes to prephenate *(29)*, anthranilate *(30)*, *p*-aminobenzoate *(31)*, isochorismate *(32)* and *p*-hydroxybenzoate *(33)*. The enzymes, particularly chorismate mutase have attracted much attention[90-97] and the reader is referred to the recent authoritative review by Walsh *et al.*[98]

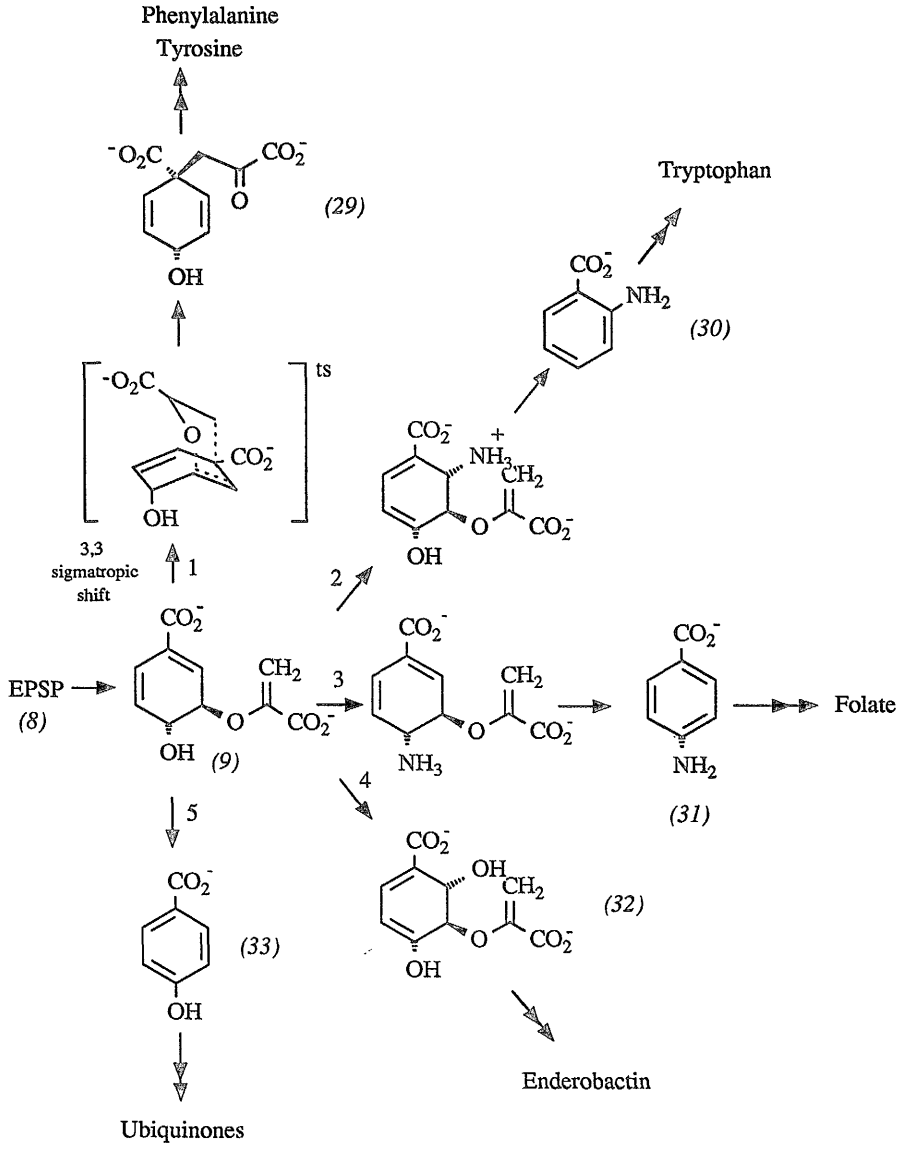


Figure 1.15

The Post-Chorismate pathway. 1. Chorismate Mutase 2. Anthranilate Synthase 3. *p*-Aminobenzoate Synthase 4. Isochorismate Synthase 5. Chorismate Lyase

1.2 Mechanistic Study of Phosphoryl Transfer.

1.2.1 Introduction.

The interest in mechanisms of phosphoryl transfer reactions stems from their extensive and central roles in metabolic pathways. It is the hydrolysis of phosphates, particularly adenosine 5'-triphosphate, that supplies the driving force for many key processes such as muscle action, biosynthesis, active transport, nerve action and gene replication.[99]

The three classes of enzymes that catalyse the transfer of the phosphoryl group ($-PO_3^{2-}$) are shown in figure 1.16. The phosphokinases, of which shikimate kinase is an example,

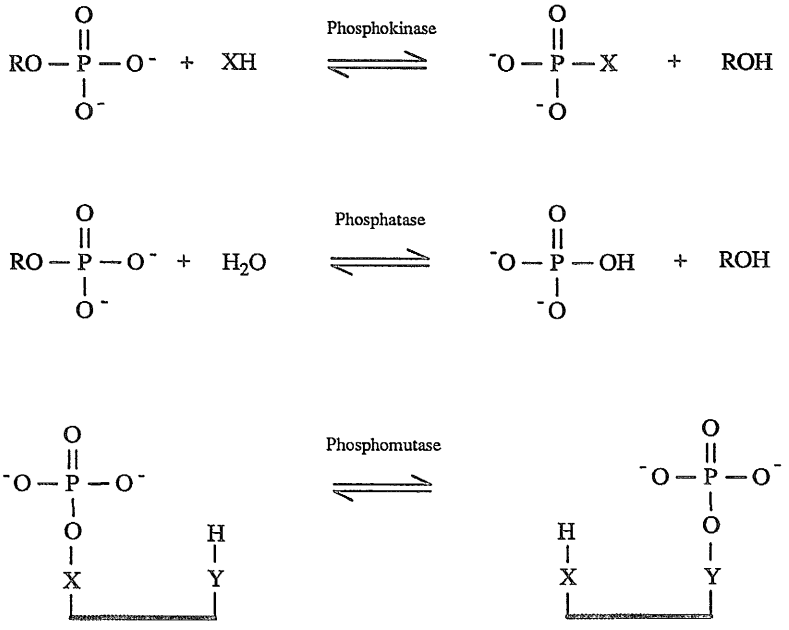


Figure 1.16

catalyses the transfer of the γ-phosphoryl group of a nucleoside triphosphate to a nucleophilic centre other than water. The nucleophilic centre may be a hydroxyl group e.g.

hexokinase (and shikimate kinase), a nitrogen nucleophile e.g. arginine kinase, a phosphate group e.g. nucleoside diphosphate kinase or a carboxyl group e.g. acetate kinase. The phosphatases catalyse the transfer of the phosphoryl group to a water molecule. The function of these enzymes may be merely hydrolytic e.g. alkaline phosphatase or couple the energy derived from the hydrolysis to some other metabolic function e.g. myosin ATPase. The phosphomutases catalyse the transfer of the phosphoryl group to another functional group on the same molecule. Enzyme catalysed phosphoryl transfer has been the subject of several reviews.[100-103]

1.2.2 Chemical Basis of Phosphoryl Transfer.

The fundamental mechanisms for nucleophilic displacement at phosphate monoesters may be classified into four mechanistic extremes;

(1) The dissociative mechanism (figure 1.17) is analogous to the S_N1 mechanism of carbon chemistry and involves the initial rate limiting elimination of a leaving group to liberate a monomeric metaphosphate anion. The process is completed by a nucleophilic addition, at either face, to the highly reactive metaphosphate intermediate.

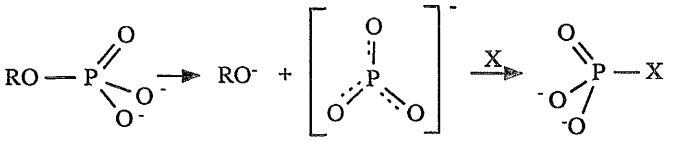


Figure 1.17

(2) The associative mechanism (figure 1.18) is analogous to the S_N2 mechanism of carbon chemistry and involves initial nucleophilic attack at the phosphorus atom to generate a pentacoordinate transition state. The attacking and leaving groups of the concerted reaction are apical and therefore results in the inversion of configuration at phosphorus.

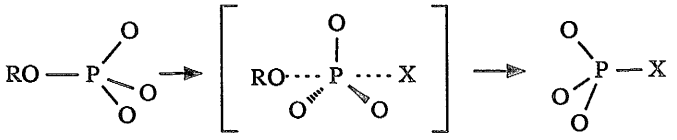


Figure 1.18

(3) The in-line addition-elimination mechanism (figure 1.19) is similar to the associative mechanism, but differs in that the pentacoordinate species is a discrete intermediate. As with (2) the mechanism results in the inversion of configuration at phosphorus.

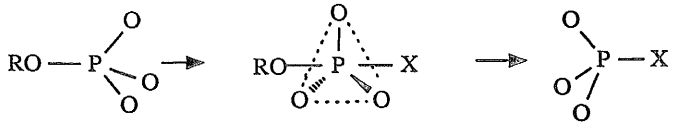


Figure 1.19

(4) The adjacent addition-elimination mechanism (figure 1.20) also involves a pentacoordinate intermediate. However, the approach of the nucleophile is adjacent i.e. on the same side of the leaving group. The intermediate then pseudorotates and the leaving group is lost along an apical coordinate. The process results in the retention of configuration at phosphorus.

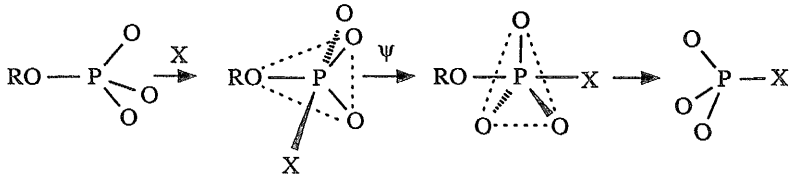


Figure 1.20

A wide variety of evidence exists to support the feasibility of phosphoryl transfer by each of these mechanisms. However, the majority of the data suggests that monosubstituted phosphate esters solvolyse via dissociative pathways in both aprotic and protic media. Evidence in support of a unimolecular reaction include; positive entropies of activation,[104] significant kinetic isotope effects,[105] negligible solvent deuterium isotope effects,[106] non-selective phosphorylation of solvent mixtures,[107] the Conant-Swan fragmentation reaction,[108] the three phase test,[109] and positional exchange studies.[110] However despite the

collective evidence of this and other work, it does not provide conclusive proof to the existence of a free long-lived monomeric metaphosphate intermediate in solution, a requisite for a truly dissociative pathway. Furthermore in contrast to the above work some thermodynamic,[111] kinetic[112,113] and positional isotope exchange studies[114] are indicative of an associative process in aqueous media.

The existence of monomeric metaphosphate intermediate in protic and aprotic solvent has been the focus of recent stereochemical studies, since a freely liberated intermediate should have equal probability of nucleophilic attack at each face of the planar species. Therefore, if the phosphate is made chiral (using stable isotopes of oxygen), reaction via a truly dissociative pathway would lead to racemisation (figure 1.21).

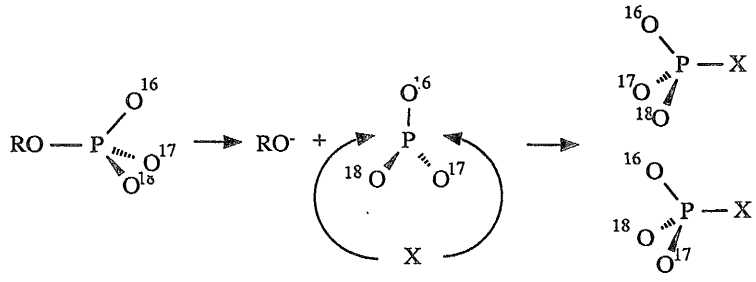


Figure 1.21

The stereochemical courses of the solvolysis of phenyl-[(R_p)-^{16}O,^{17}O,^{18}O] phosphate monoanion,[115] 2,4-dinitrophenyl[(R_p)-^{16}O,^{17}O,^{18}O] phosphate dianion[115] and N'-[(S_p)-^{16}O,^{17}O,^{18}O] phosphocreatine[116] in aqueous methanol were all found to proceed with inversion at phosphorus. The mechanisms of these reactions therefore cannot involve a truly free intermediate as previously thought. The apparent contradiction of these results was reconciled in terms of a preassociative mechanism formalized by Jencks.[117] The nucleophile does not provide kinetic assistance but must be preassociated with the phosphate before any bond breaking occurs. The mechanism (figure 1.22) which can be concerted or stepwise therefore accommodates the dissociative character suggested by many of the kinetic and thermodynamic studies and the associative character demonstrated

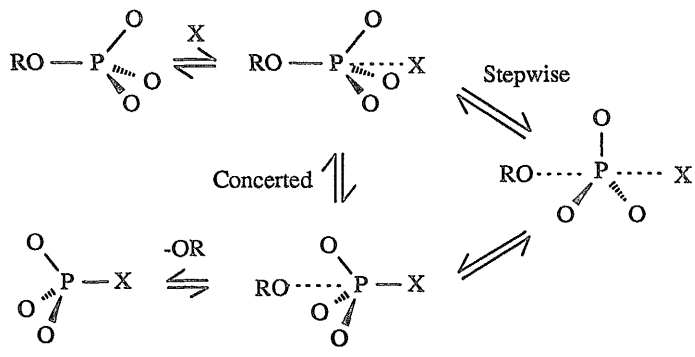


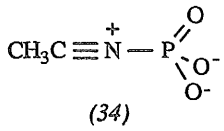
Figure 1.22

by the stereochemical studies.

Further evidence for a preassociative stepwise mechanism in hydroxylic media was recently offered by the work of Lightcap and Frey.[118] They observed the cleavage of μ-monothiopyrophosphate (MTP) by tris(hydroxymethyl)aminomethane (Tris) in competition with water. Tris was found to compete with water producing both *N*-phosphoryl-Tris and *O*-phosphoryl-Tris in comparable amounts (i.e. dissociative pathway character). Furthermore variation of the concentration of Tris did not alter the rate at which MTP is cleaved. However Tris was implicated in participating in the reaction since increased concentrations of Tris resulted in a higher proportion of phosphoryl-Tris to phosphate.

The first reported stereochemical investigation of phosphoryl transfer in aprotic solvent was of the Conant-Swan fragmentation reaction,[108,119] a reaction that was thought to be dissociative in character that had been extensively used to generate and study the properties of monomeric metaphosphate. The phosphoryl group was transferred to a secondary alcohol, in chloroform, and was found to proceed with inversion at phosphorus. The result was also rationalised in terms of a preassociative mechanism. A free intermediate in aprotic solvent was indicated however by significant racemisation observed for phosphoryl transfer from chiral phenyl phosphate to *tert*-butanol in acetonitrile.[120] Nevertheless the possibility

of the phospho group being transferred to solvent acetonitrile to give an acetonitrile-metaphosphate adduct *(34)* (figure 1.23) and successive phosphoryl transfer to



(34)

Figure 1.23

other solvent molecules cannot be eliminated as a source of the racemisation. The racemisation observed during the phosphoryl transfer from adenosine 5'-[β-(S)-^{16}O,^{17}O,^{18}O] diphosphate to a primary alcohol in acetonitrile is similarly inconclusive.[121] The finding by Cullis *et al.*[122] that phosphoryl transfer from a P^1,P^1-disubstituted pyrophosphate to a primary alcohol in dichloromethane results in ~70% racemisation must therefore represent the strongest evidence for a metaphosphate intermediate with effective lifetime in aprotic solvent.

1.2.3 Enzyme Catalysed Phosphoryl Transfer.

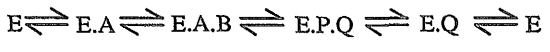
As with the study of chemical phosphoryl transfer, enzyme catalysed transfer has similarly been addressed by a variety of kinetic, isotopic and stereochemical methods. The following methods will be discussed with emphasis on kinase catalysed phosphoryl transfer.

(i) Kinetic methods.

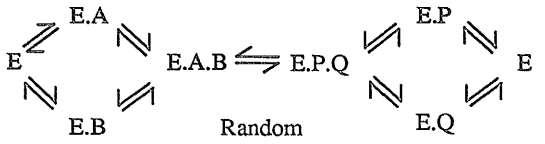
Kinetic studies have been used to address the 'kinetic mechanism' of the enzyme reaction (the sequence and relative rates of substrate binding and product release), and the 'chemical mechanism' of the enzyme reaction (the details of the chemical reaction e.g. are there any covalent intermediates). The application of kinetic studies to the determination of enzyme chemical mechanism is the subject of a comprehensive review by Cleland.[123] The kinetic mechanism of an enzyme can be classified broadly into two types, the sequential mechanism and the ping-pong mechanism. Sequential and ping-pong kinetic schemes for a

two substrate/two product reaction are shown in figure 1.24 where E is the free enzyme and F is the phosphorylated form of the enzyme.

Sequential mechanisms



Ordered



Ping-pong mechanism

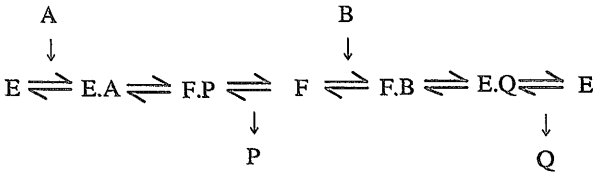


Figure 1.24

In a sequential mechanism the presence of both substrates at the active site of the enzyme (the ternary complex) is necessary for catalysis to occur. Sequential mechanisms may be further classified into two types, ordered and random. In an ordered mechanism the substrates combine and the products release according to an obligate and systematic pathway. In a random mechanism the formation of, or the release from the ternary complex is not predetermined. The ping-pong mechanism involves one substrate binding to the enzyme, covalently modifying the active site, and the product leaving. The second substrate is then bound, reacted with the phospho-enzyme intermediate and released.

Steady state kinetics can be used to distinguish between the two mechanisms. Sequential mechanisms are characterised by intersecting double-reciprocal plots whereas ping-pong mechanisms are characterised by parallel line plots. A number of kinases (See Cullis[103] and

references sited therein) have been investigated in this way and it appears that the majority of kinases exhibit intersecting plots implicating the sequential mechanism. Determination of whether the sequential mechanism follows an ordered or random pathway has been addressed by equilibrium isotope exchange kinetic studies.[124] Kinases were apparently observed to exhibit random sequential pathway kinetics, however there may be a preferred order of binding as in the case of hexokinase.[125]

The notable exception to the common random sequential mechanism is that of nucleoside diphosphate kinase[126] which demonstrates a ping-pong mechanism with a covalent intermediate that has been directly isolated and characterised. The participation of a transient covalent intermediate (figure 1.25) has also been implicated (by isolation) for the

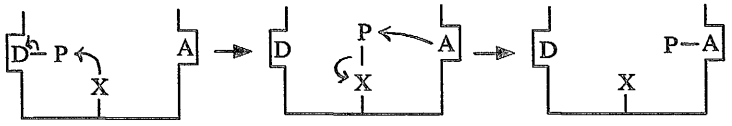


Figure 1.25

enzymes acetate kinase[127] and hexokinase.[128] However, the role of such an intermediate in the normal mechanistic pathway of these examples is questionable. The phosphoenzyme intermediate implicated for hexokinase is observed to be mechanistically incompetent since it fails to phosphorylate glucose in the normal way. While acetate kinase intermediate appears to be mechanistically competent, the kinetic competence of the intermediate is in doubt because the rate of the reactions involving the intermediate are relatively very slow in comparison to the rate of overall reaction.[129]

(ii) Isotope exchange methods.

Isotope exchange studies have been used to infer the existence of phospho-enzyme intermediates, by the demonstration of isotope exchange between substrate-product pairs in the absence of the other substrate. Such exchange is illustrated in figure 1.26. Many kinases have been shown to exhibit such ATP/ADP exchange,[103] however in the majority of cases

$$ADP^{*} + ATP \rightleftharpoons ATP^{*} + ADP$$

Figure 1.26

the rate is very slow with respect to the normal transfer reaction. A number of reasons have been suggested to account for the slow rate of the partial exchange reaction. 'Substrate synergism'[130] suggests that the second non-participating substrate is required (e.g. to induce a conformational change) to achieve the maximal rate for that partial reaction. Other suggested reasons for the slow rates include; exchange by a contaminating enzyme,[131] contamination by non-participating substrate allowing the enzyme to complete the full reaction pathway[132] or the possibility that the exchange is merely a side-reaction not relevant to the mechanism for the full reaction.[102]

A requirement of the intermolecular exchange reaction is that ADP can dissociate from the {phosphorylated-enzyme • ADP} complex, however, it would seem reasonable that the ADP might be tightly bound. The methods of positional isotope exchange (PIX)[133] overcome this limitation since the technique can detect the transient ADPO-PO_3 bond cleavage of bound ATP. Cleavage of this bond in the absence of the co-substrate would be indicative of either a transient phosphoenzyme or of an enzyme catalysed dissociative reaction. However, of those enzymes that have been subjected to isotope exchange analysis no significant exchange has been observed in the absence of co-substrate even in the case of ATPase[134] which involves a phosphoenzyme intermediate. The principles and application of PIX are discussed later in chapter 2.

(iii) Stereochemical methods.

As with the investigations of phosphoryl transfer in solution, knowledge of the stereochemical course of a reaction at the active site of the enzyme has been the focus of a large number of mechanistic studies for enzymes catalysing phosphoryl transfer (See Cullis[103] for comprehensive list). The availability of 3 stable isotopes of oxygen has been

used to synthesise isotopically chiral substrates for a wide range of enzymes.[135] For phosphatases a fourth substitution is required to render the product inorganic phosphate chiral. In this latter case sulphur substitutes for one of the oxygens such that chiral [^{16}O,^{17}O,^{18}O] thiophosphate is the final product and can be analysed to determine the stereochemical course. There are two significant general methods for the synthesis of chiral [^{16}O,^{17}O,^{18}O] phosphates.[136,137] Independant methods for determining the absolute configurations initially used mass spectrometry,[136] however the use of high field ^{31}P nmr following chemical or enzymic derivatisation has proved more convenient.[138,139]

The stereochemical course of phospho group transfer has now been determined for over 15 kinases[103] and in all but two exceptions [140,141] inversion of configuration at phosphorus is observed (figure 1.27). The simplest interpretation of this result is that phosphoryl transfer

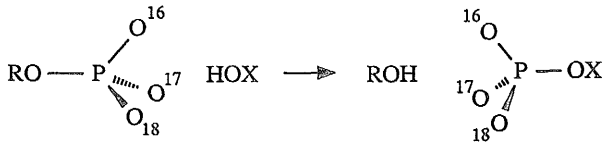


Figure 1.27

proceeds by a single in-line transfer between the two substrates in the ternary complex. Mechanisms involving adjacent displacement and double in-line displacement are therefore unlikely. Mechanisms involving any odd number of displacements can account for the observed inversion of configuration, however experimental evidence to support such mechanisms is scarce. The observed results of retention of configuration of nucleoside diphosphate kinase[140] and nucleoside phosphotransferase[141] is consistent with their kinetic and isotope exchange characteristics. Both enzymes are observed to follow clean ping-pong kinetic pathways involving a competent phosphoenzyme intermediate. Since the reactions are symmetric it has been suggested that the substrates and products share a single nucleotide binding site.

To summarise, kinetic, isotopic and stereochemical studies have permitted the detailed investigation into the mechanism of enzyme catalysed phosphoryl transfer. Clearly, the majority of kinases follow sequential pathways with the phosphoryl group being transferred between substrates in an 'in-line' mechanism resulting in inversion of configuration at phosphorus. Model studies provide evidence that the transition state for nonenzymatic phosphoryl transfer is metaphosphate-like, with a preassociative mechanism dominating in hydroxylic media. However, the kinetic, isotopic and stereochemical studies have failed to define whether enzymes change the metaphosphate-like transition state of the chemical reaction to a more associative mechanism.

The simplest approach to the question of dissociative/associative phosphoryl transfer is to ask how the enzyme could achieve the observed catalytic rate enhancement. If the transfer follows a dissociative pathway then the major kinetic barrier in the mechanism is the expulsion of the leaving group by the electron density on the phosphoryl oxygens. A catalytic site would therefore be required such that the electron density on the oxygen atoms of the phosphoryl group is destabilised to give an increase in bond order. Destabilisation may take effect by the presence of negatively charged residues or simply by the desolvation of the phosphoryl group. The presence of a general acid to assist the leaving group can also be envisaged. A postulated active site for a dissociative 'in-line' transfer is shown in figure 1.28.

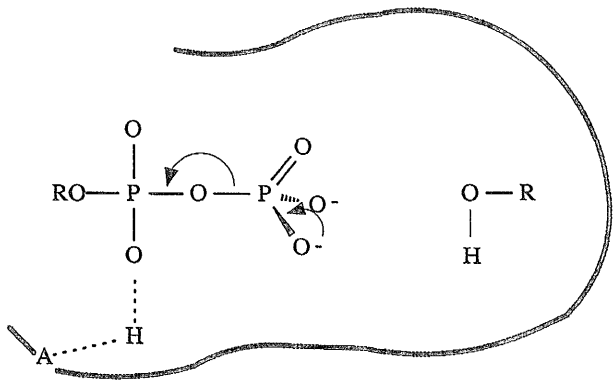


Figure 1.28

In contrast, if the transfer follows an associative pathway then the major kinetic barrier is the stabilisation of electron density on the peripheral oxygens to facilitate nucleophilic attact at phosphorus. Metal co-ordination, cationic (e.g. Lys or Arg) or hydrogen bond donating residues to these oxygens is therefore inferred. As with dissociative transfer, a general acid to protonate the β phosphoryl group, the leaving group, can be similarly envisaged. The presence of a general base in a position to abstract a proton and therefore increase the nucleophilicity of the acceptor is also expected. A postulated active site for an 'in-line' associative reaction, as described by Knowles[102] is shown in figure 1.29.

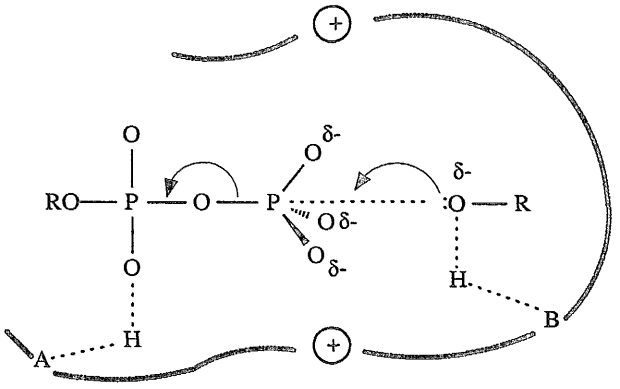


Figure 1.29

Thus identification of the nature and positioning of crucial active site functionalities, including the divalent metal ion, should provide evidence for the mechanism of phosphoryl transfer. It has been suggested that the presence of a general base to abstract a proton from the substrate is consistent with an associative process.[142] Indeed, for a number of kinases, bases such as histidine and aspartate have been implicated by pH rate study, X-ray and nmr data.[142] However, the presence a base may also be required to accept a proton from the product in a dissociative transition state.

All of the ATP-dependent enzymes demonstrate a requirement for a divalent metal ion

(commonly Mg^{2+}) for catalysis. Studies into the role of the metal ion have concentrated on finding its location at the catalytic site, since this might provide strong evidence for the mechanism of phosphoryl transfer. Metal coordination to the γ-phosphoryl group would localise electron density on the phosphoryl oxygens thus probably disfavouring a dissociative process. Complexation would also act in shielding, by neutralisation, the charge from the entering acceptor group in an associative transfer. Substitution inert complexes, the use of phosphorothioate ATP analogues and magnetic resonance methods are all in general agreement indicating co-ordination to the β- and γ-phosphoryl groups of ATP. However, it is unlikely that the ligation pattern remains the same throughout the catalytic process, since β,γ coordination in the transition state would probably be more likely to catalyse the reverse reaction. After phosphorylation the metal coordination may change to an α,β-bidentate ADP.[143] Furthermore recent EPR studies have shown that the Mn^{2+} of pyruvate kinase is chelated by oxygen atoms of the γ-phosphoryl group of ATP and the oxalate ion, an analogue of enolpyruvate anion.[144] Co-ordination of the metal ion to the γ-phosphoryl group and the co-substrate nucleophile is supported by recent model studies.[145]

Identification of the individual functional groups at the active site is hindered by the consequence of the mechanism of kinase binding and catalysis. X-ray structure analyses on a variety of phosphotransferases[103] indicate a general tendancy for structures comprising of two distinct lobes with the active site located in the cleft. Evidence, principally from hexokinase[146] and more recently adenylate kinase,[147] suggest that induced-fit mechanisms are in operation. Essentially, the binding of the substrates induces conformational changes in the protein such that the catalytic groups are so arranged to catalyse the phosphoryl transfer. Thus important functional groups, such as arginine residues to coordinate to the γ-phosphoryl group, may be distant from the catalytic site in the absence of the tertiary complex. The process therefore minimises the risk of adventitious phosphoryl transfer (i.e. ATPase activity) in the absence of the desired acceptor molecule.

The princpal of the induced-fit mechanism is illustrated by the recent high-resolution X-ray structural and molecular superimpositional studies of homologous adenylate kinases by Schulz *et al.* (figure 1.30).[148] The enzymes are made up of approximatly 210 residues (*Mr* ~ 23 000) and are characterised by two domains comprising of 30 and 38 residues. Comparison of the structure of adenylate kinase (from porcine muscle) (AK1) to that of AMP-bound adenylate kinase (from bovine mitochondrial matrix) (AK3) suggests that the binding of AMP induces the 30 residue domain to move by up to 8 Å. The effect of binding the second substrate (ATP) is inferred from the crystal structure of *E.coli* adenylate kinase (AKeco) with $MgAp_5A$. This suggests that the binding of ATP to AMP-bound adenylate kinase also induces a movement in the 30 residue domain of up to 7.5 Å and a movement in the 38 residue domain of up to 32 Å.

The difficulty in locating functional groups at the active site stems from the fact that X-ray and nmr data of the true tertiary structure are unavailable. Thus, as is illustrated with adenylate kinase above, structural analyses of enzymes with substrate analogues that are not turned over (providing the disruptions resulting from the change of substrate to analogue are negligible) provide the best view of the protein at the transition state. It will be interesting to see if similiar investigations perhaps through application of n.m.r. will be able to define absolutely the kinase active site.

1.3 Properties of Shikimate Kinase II.

Shikimate kinase II (SK) is the product of the *aro L*-gene from *Escherichia coli* K12 and cloned and overexpressed by the research groups of Pittard and DeFeyter (University of Melbourne, Australia) and Coggins (University of Glasgow, Scotland, U.K.).[51,53,54,149-151]

The enzyme is a relatively small monomeric enzyme with a molecular weight of 18937 and consisting of 173 amino acid residues. The sequence of amino acid residues as determined by Coggins *et al.*[54] is shown in chapter 5. The apparent K_M of SK for shikimate is 200 μM at

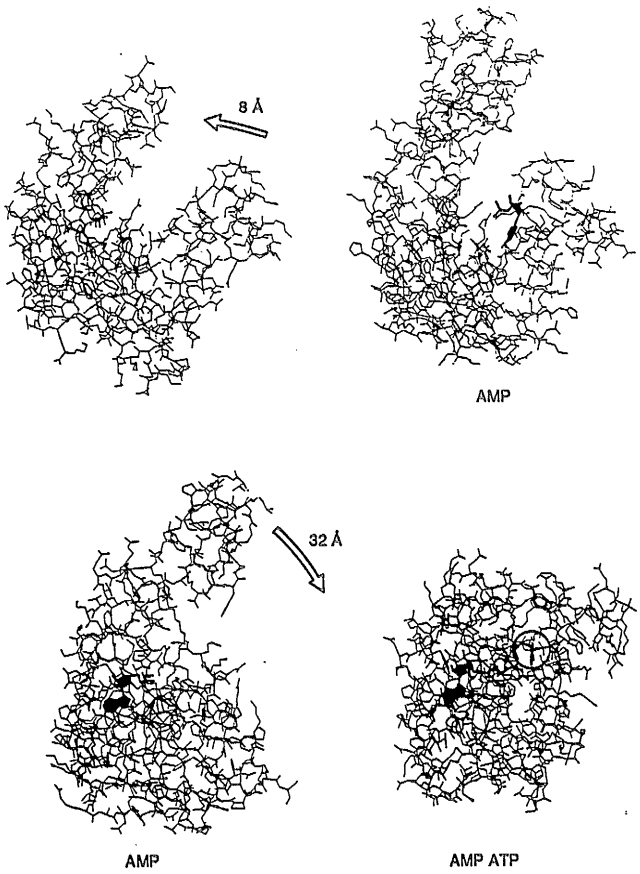


Figure 1.30 Top right: substrate free AK1, top left: AMP-bound AK3
bottom left: AMP-bound AK3, rotated through 90° around the verticle axis,
bottom right: Ap_5A bound AKeco.

5 mM ATP, and for ATP 160 μM at 1 mM shikimate.[151] The activity of the enzyme is decreased approximately 7-fold on increase of shikimate concentration (1 → 10 mM), suggesting an inhibition by higher levels of substrate.[53] The enzyme activity is dependent on the presence of a divalent cation as a cofactor. Mg^{2+} is the most effective, but significant activity is obtained with Fe^{2+},Ca^{2+},Mn^{2+}, or Co^{2+}.[151] SK is not significantly inhibited by 1 mM tyrosine, phenylalanine or tryptophan.[151]

1.4 Aims.

Shikimate kinase catalyses the fifth step of the shikimate pathway and is of interest for a number of reasons.

(1) The industrial importance of this metabolic pathway is highlighted by the commercial success of the herbicide glyphosate.

(2) The recent interest in highly functionalised cyclohexanes (e.g. inositol) as secondary messengers in cells demonstrates the importance in the understanding of the molecular recognition of such structures.

(3) The mechanism of phosphoryl transfer in kinases is uncertain.

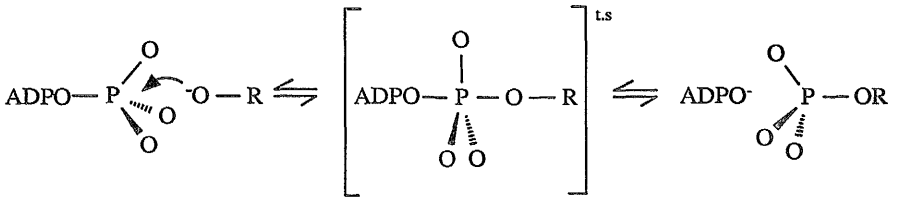
The relatively small size, availability and source of the enzyme makes it applicable to study by a wide range of techniques (e.g. high field ^1H nmr, site directed mutagenesis). This thesis aims to begin the process of molecular and mechanistic characterisation of the enzyme.

CHAPTER 2

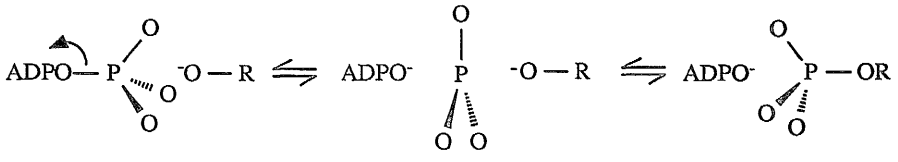
Phosphoryl Transfer: Associative vs. Dissociative. Positional Isotope Exchange Studies

2.0 Introduction.

Kinetic and mechanistic studies of enzyme-catalysed phosphoryl transfer of a number of kinases have been used to examine the nature of substrate binding (sequential or ping pong; random or ordered) and whether the process involves a phosphoenzyme intermediate.[102,103] As a result it is now clear than the vast majority of kinases follow sequential pathways, the phospho group being transferred between bound substrates in ternary complexes with the enzyme. Furthermore, in all but two cases[140,141] for phosphokinases studied to date, the transfer proceeds with inversion of configuration at phosphorus.[103] It is therefore not unreasonable to presume that the phospho group transfers with 'in-line' geometry directly from one enzyme-bound substrate to the other. The nature of the phosphoryl transfer may be probed further, however, if we ask the question; is the transfer associative or dissociative? (figure 2.1).



'in-line' associative



'in-line' dissociative

Figure 2.1

Such a question has been addressed in a number of kinases using *positional isotope exchange* (PIX), which was first described by Midelfort and Rose[133,152] for glutamine synthetase. The principle of PIX analysis is summarised in figure 2.2. ATP is uniquely

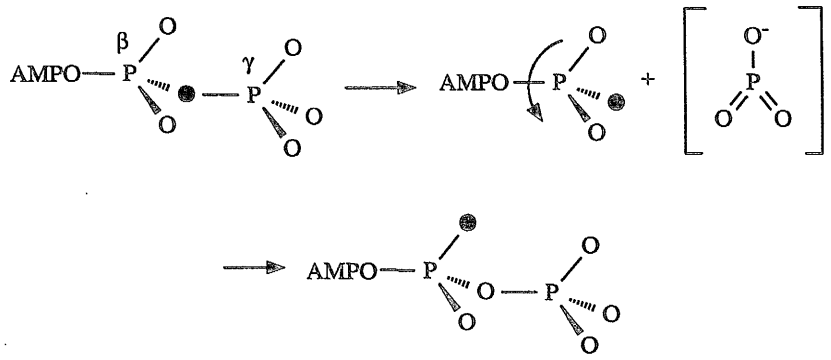
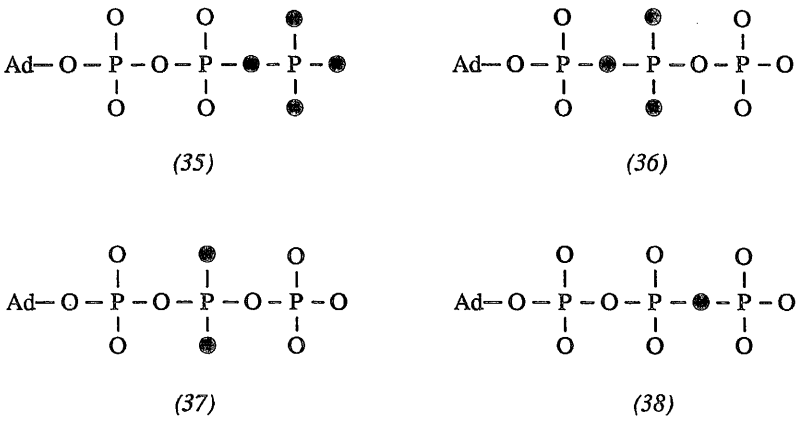


Figure 2.2

labelled with ^{18}O at the β,γ-bridge oxygen of the triphosphate chain. Thus, if on binding to the enzyme, the ATP reversibly dissociates to ADP and transiently bound metaphosphate and, if rotation of the β-phospho group of the bound ADP about the Pβ-OPα bond is unrestricted, then the reformed ATP would have the β,γ-bridging and the β-non-bridging positions exchanged. Cleavage of the Pγ-OPβ bond in the absence of the co-substrate or a suitable acceptor molecule would be suggestive of a dissociative mechanism.

A number of appropriately labelled ATP syntheses have been reported ([β,γ^{18}O, γ$^{18}O_3$]ATP[133] *(35)*, [α,β^{18}O, β$^{18}O_2$]ATP[153] *(36)*, [β^{18}O]ATP[154] *(37)*), including ATP specifically labelled at the Pγ-OPβ bridging position ([β,γ-^{18}O]ATP)[155,156] *(38)* (figure 2.3). The distribution of ^{18}O in the labelled ATP can be determined by mass-spectromeric analysis[133,152] or by high field ^{31}P nmr.[153,156]

In 1978 Lowe and Sproat[153,157] used [α,β-^{18}O, β-$^{18}O_2$]ATP *(36)* to examine the mechanism of phosphoryl transfer by pyruvate kinase. They found that in the presence of pyruvate kinase and pyruvate randomisation of the β-$^{18}O_2$ labels with the β,γ-O bridge does occur, moreover, they also reported that randomisation occurred in the presence of oxalate (an



(35) *(36)*

(37) *(38)*

Figure 2.3

analogue of enol pyruvate) and in the absence of pyruvate or a pyruvate analogue. They concluded that phosphoryl transfer in pyruvate kinase occurs by a dissociative $S_N1(P)$ mechanism. However, Knowles *et al.*[131] showed that the observed PIX varied with protein purity and they concluded that the majority of the PIX was due to a contaminant protein. Furthermore, in the absence of pyruvate PIX was virtually nonexistent. It was argued that this and other data were more consistent with an associative $S_N2(P)$ mechanism. PIX analysis of hexokinase by Rose[158] was in accord with Knowles findings for pyruvate kinase in that PIX was observed in the presence of glucose, but did not occur in the absence of cosubstrate or the substrate analogue lyxose. Similarly, in the PIX analysis of creatine kinase[159] scrambling was observed in the presence of creatine but not in its absence or the presence of poor substrates. The PIX investigation of the mechanism of phosphoryl transfer in the enzyme acetate kinase used an alternative approach.[160] The ^{18}O label was incorporated into the bridging acylphosphate oxygen of acetyl phosphate, a product of the reaction catalysed by the enzyme. No positional isotope exchange was observed between the bridging acylphosphate oxygen and the carbonyl group in the absence of ADP (figure 2.4).

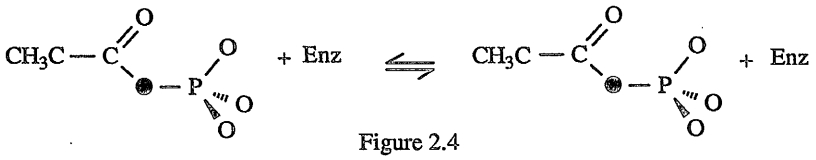


Figure 2.4

The failure of these experiments to observe a transiently bound metaphosphate intermediate in the absence of co-substrate provide evidence against the dissociative mechanism for phosphoryl transfer. However, the limitations of the experiment mean that a dissociative mechanism might still be occurring but not observed if:

(a) The enzyme prevents rotation of the β-phosphoryl group of the enzyme-bound ADP (e.g., via a bridging metal ion). The fact that the observed PIX is negligible in myosin ATPase,[134] an enzyme known to involve a phosphoenzyme intermediate, suggests that this might be so.

(b) In the absence of substrate the lifetime of transiently bound metaphosphate is so fleeting that the intermediate reverts to substrate faster than it can rotate.

(c) The binding of the co-substrate is required to activate the phospho group for transfer, the phenomenon of substrate synergism.[130] Activation might be via conformational change and/or electrostatic interaction. Indeed, phosphotransferases proceeding *via* dissociative mechanisms would have to sequester metaphosphate away from water to prevent adventitious hydrolysis. It could achieve this by ensuring that the correct catalytic array is not assembled until the co-substrate is in place. X-ray crystallographic evidence shows large conformational changes induced by substrate binding (e.g. hexokinase,[161] adenylate kinase[147]) are consistent with such a model.

The nature of phospho transfer has also been implicated by the measurement of the intersubstrate distance on an enzyme using techniques such as NMR and X-ray

crystallography. Mildvan[142,162] reasoned that if the phosphoryl group to be transferred is ≤ 3.3 Å from the acceptor nucleophile i.e. within van der Waals contact, then the reaction is to be assumed associative. Similarly, a distance of ≥ 4.9 Å would indicate a dissociative mechanism. A molecular contact distance have been measured using NMR for a number of kinases[142] (adenylate kinase ≥3 Å, hexokinase ≥3.3 Å, creatine kinase ≥4 Å, phosphofructokinase 3.7 Å and pyruvate kinase 3 ± 1 Å) suggesting an associative mechanisms in each case. In contrast, distances of 5.3 ± 0.7 Å and ≥5 Å for protein kinase and alkaline phosphatase respectively are suggestive of dissociative mechanisms. Such measurements should be treated cautiously however since they do not provide distances in the transition state. Moreover in contrast to the NMR studies, X-ray data for hexokinase[142] gives a molecular contact distance of ≥6 Å therefore questioning the accuracy of the measurements.

Positive evidence in support of a dissociative mechanism is provided by the recent measurement of secondary ^{18}O kinetic isotope effects in alkaline phosphatase[163] and hexokinase[164] by Cleland and co-workers. In each case the three nonbridge oxygens on the phosphoryl group to be transferred are substituted by ^{18}O. The ^{18}O isotope effects are measured by the remote label method. In the case of Hexokinase these experiments employ the use of [γ-$^{18}O_3$,6-^{15}N]ATP where the enzyme is exposed to a known mixture of heavy- and light-labelled (6-^{14}N) ATP. The resulting ADP and ATP are then isolated and the ^{15}N content is then precisely determined for each sample by derivatisation and isotope ratio mass spectrometry. Analysis under different conditions suggested that the enzyme transferred the $\gamma P^{18}O_3$ slightly faster than the $\gamma P^{16}O_3$ thus inferring that the transition state involves an increase in bond order: a requisite of a dissociative type transfer. In the case of alkaline phosphatase the slightly faster hydrolysis of [γ-$^{18}O_3$, 1-^{13}C] glucose 6-phosphate than unlabelled substrate is similarly concluded.

2.1 Aims.

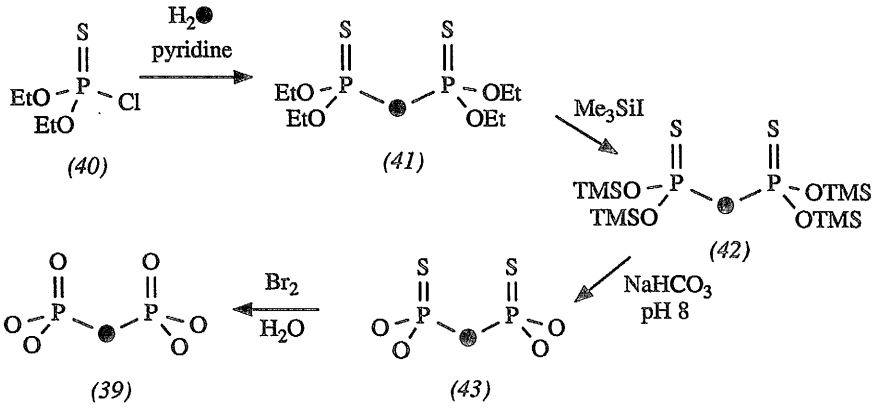
As is apparent the mechanism(s) of phosphoryl transfer of kinases are still ambiguous. This study intends to investigate the nature of phosphoryl transfer of shikimate kinase using the positional isotope exchange methods described. This study will aim to synthesise suitably labelled ATP and a novel non-phosphorylatable analogue of the co-substrate shikimate. The synthesis of other specifically labelled nucleoside polyphosphates for use in PIX studies will also be explored. The successful synthesis of suitably labelled ATP and the co-substrate analogue will then permit the analysis of shikimate kinase by PIX in the presence and absence of co-substrate, and in the presence of a substrate analogue to which the enzyme is unable to transfer the phospho group.

2.2 Results.

2.2.1 Preparation of Bridge-Oxygen-Labelled Pyrophosphate *(39).*

Early positional isotope exchange experiments used multi-labelled nucleoside polyphosphates (figure 2.3) with the oxygen isotope residing in both the bridge and non-bridging positions. However, the synthesis of bridge-^{18}O-labeled inorganic pyrophosphate *(39)* first reported by Cullis[155] has permitted the preparation of a range of specifically labelled nucleoside polyphosphates. The analysis (mass-spectral or ^{31}P nmr) of the positional exchange is thus simplified and the single label also facilitates the measurement of heavy-atom kinetic isotope effects.

The synthesis of *(39)* is outlined in scheme 2.1. Tetraethyl dithiopyrophosphate *(41)* was



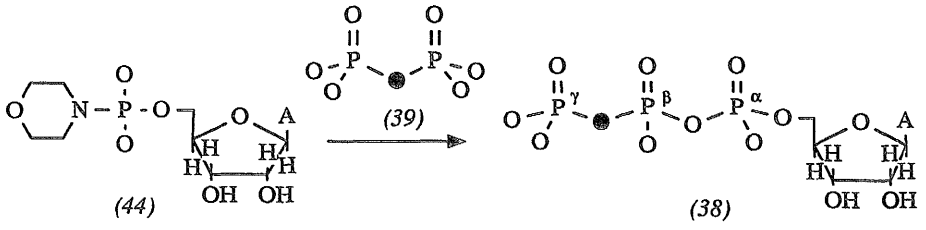
Scheme 2.1

prepared by the reaction of diethyl thiophosphosphoryl chloride *(40)* and 0.5 equivalent of water ($H_2{}^{18}O$, 97 atom %) in the presence of pyridine.[165] The ethyl groups were cleaved by prolonged treatment with trimethylsilyl iodide at elevated temperature and pressure to give tetrakis[trimethylsilyl] dithiopyrophosphate *(42)*.[166] Hydrolysis of *(42)* with aqueous sodium bicarbonate buffer removed the silyl groups to yield the symmetrical

dithiopyrophosphate *(43)* which was purified by ion exchange chromatography on DEAE sephadex. The rapid treatment of *(43)* with excess bromine in water[167] and purification as before gave bridge-^{18}O-labelled pyrophosphate *(39)* in a yield of 44% based on $H_2{}^{18}O$.

2.2.2 Preparation of Adenosine 5′[β,γ-^{18}O] Triphosphate *(38).*

The preparation of [β,γ-^{18}O]ATP *(38)* from [α,β-^{18}O] pyrophosphate *(39)* and adenosine 5′-phosphomorpholidate *(44)* by the method of Moffatt *et al.*[168] is shown in scheme 2.2.



Scheme 2.2

Prolonged treatment of *(44)* with excess pyrophosphate *(39)* in a concentrated solution of dry DMSO gave [β,γ-^{18}O]ATP *(38)* as observed by ^{31}P nmr. [β,γ-^{18}O]ATP *(38)* was isolated by ion exchange chromatography on DEAE sephadex in 70% yield based on the morpholidate *(44)*. The high field ^{31}P nmr of [β,γ-^{18}O]ATP is shown in figure 2.5. The spectrum is essentially identical with that of authentic ATP. The effect of the ^{18}O is to shift the Pβ and Pγ resonances upfield *ca.* 2.0 and 2.5 Hz respectively, with no perturbation of the Pα resonance. The small signals corresponding to unlabelled ATP are observed as a result of the residual ^{16}O in the $H_2{}^{18}O$ together with any dilutions that may have occurred during synthesis. The ratio of the peak heights of the upfield-shifted (^{18}O-perturbed) resonances for Pγ doublet (or the Pβ triplet) to the unshifted resonances indicate that the sample contains ~6% [β,γ-^{16}O]ATP.

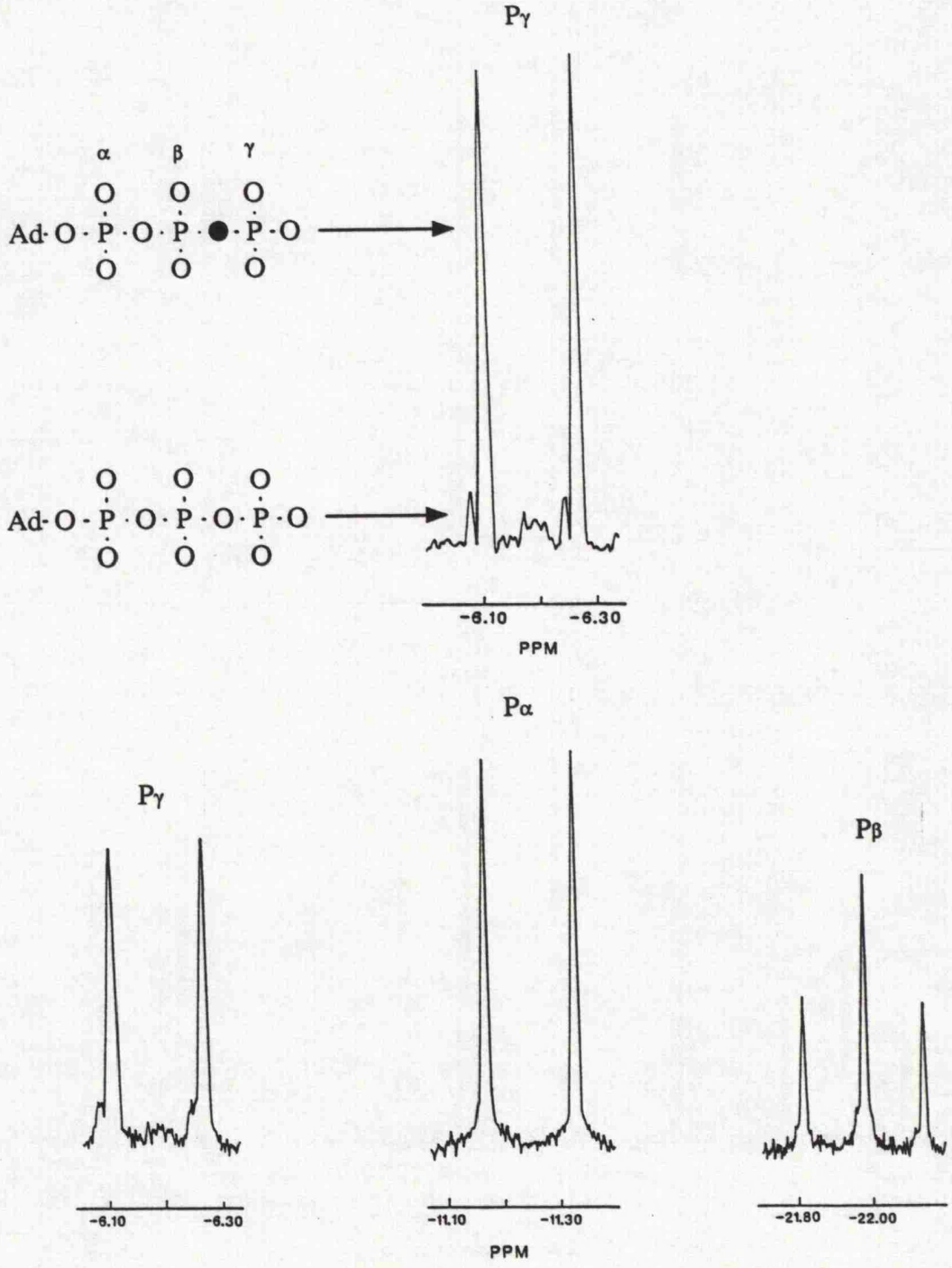


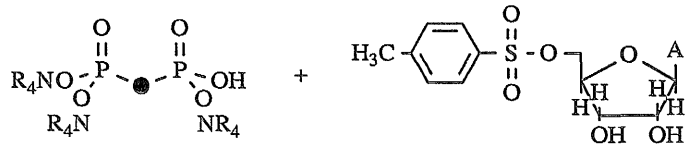
Figure 2.5

^{31}P n.m.r. spectra (121.5 MHz) of [β,γ-^{18}O]ATP *(38)* with expansion of Pγ resonance in 250 mM CAPS, 30 mM EDTA, 20% D_2O, pH 9.6.
Gaussian multiplication:
Gaussian broadening, 0.0 Hz; line broadening 0.0 Hz, full spectra
Gaussian broadening, 0.15 Hz; line broadening -1.0 Hz, expansion

2.2.3 Preparation of Adenosine 5′-[α,β-^{18}O] Diphosphate *(45)* and Adenosine 5′-[α,β-^{18}O] Triphosphate *(46)*.

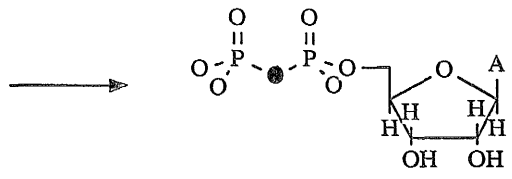
The availability of bridge-oxygen-labelled pyrophosphate *(39)* has facilitated the synthesis of a number of singly labelled polyphosphates applicable to use in PIX studies. To this end [α,β-^{18}O]ADP *(45)* has been used successfully to examine the possible role of monomeric metaphosphate in hydroxylic solutions.[110]

[α,β-^{18}O]ADP *(45)* was prepared from the reaction of pyrophosphate *(39)* and 5′-tosyl adenosine *(47)* by the method of Poulter *et al.*[169] Excess tris(tetra-n-butyl ammonium) [^{18}O] pyrophosphate *(39)* was added to *(47)* and solvent removed to give a highly concentrated viscous solution which was stored in a dry atmosphere at room temperature for 4 days to give [α,β-^{18}O]ADP *(45)* (scheme 2.3) as observed by ^{31}P nmr. Repeated attempts to isolate



(39) *(47)*

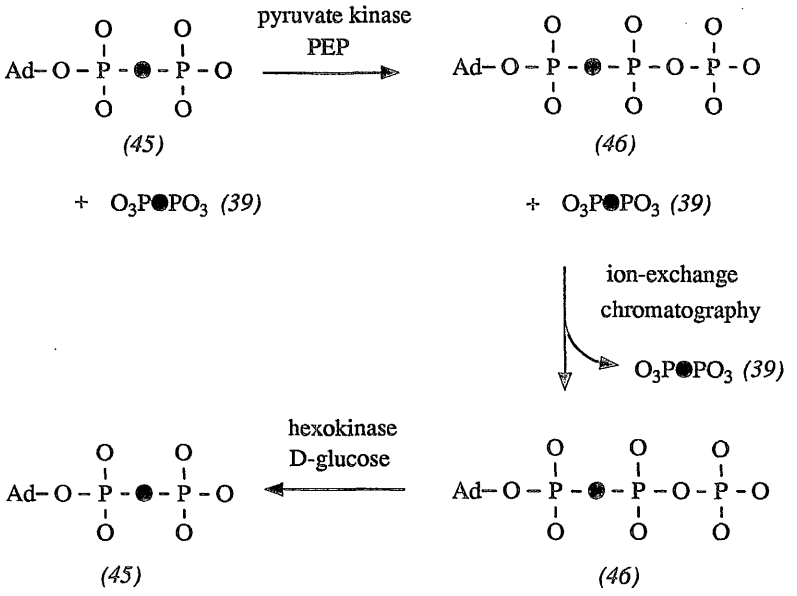
R = $((CH_2)_3CH_3)_4$



(45)

Scheme 2.3

(45) from the remaining pyrophosphate *(39)* by ion-exchange chromatography with various eluent concentration gradients and at various eluent pH failed to resolve the mixture. Separation was achieved by the procedure outlined in scheme 2.4. The mixture of ADP and



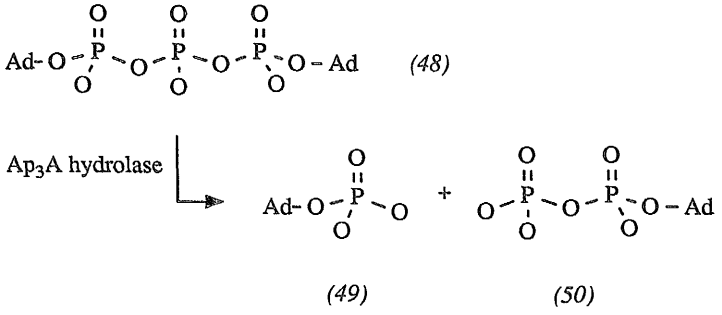
Scheme 2.4

pyrophosphate was added to a solution of pyruvate kinase and phospho(enol)pyruvate in tris(hydroxymethyl)amino methane buffer pH 7.6, containing 120 mM KCl and 60 mM $MgCl_2$. The resulting [α,β-^{18}O]ATP *(46)* was separated from *(39)* by ion exchange chromatography using DEAE-Sephadex in near quantitative yield. The enzymic dephosphorylation was achieved by treatment with hexokinase and D-glucose in buffer as before. [α,β-^{18}O]ADP *(45)* was isolated from the enzymic mixture by ion exchange chromatography in 93% yield. High field ^{31}P nmr (data not shown) confirmed the location of the isotope label in the phosphate bridge with small downfield resonances corresponding to the presence of ~5% [α,β-^{16}O]ADP.

2.2.4 Preparation of Diadenosine 5′,5‴-P^1,P^3-[α^1,β^2-^{18}O] Triphosphate *(48).*

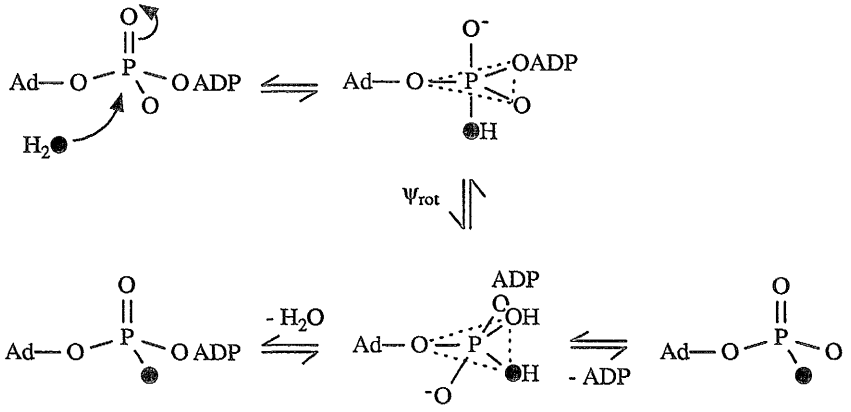
Positional isotope exchange also finds application in the mechanistic analysis of the enzyme diadenosine 5′,5‴ -P^1,P^3- triphosphatase (Ap_3A hydrolase). Ap_3A hydrolase is separated

and purified from yellow lupin seed extracts[170] along with an Ap_4A hydrolase, and a third enzyme of which Ap_3A and Ap_4A are both substrates. The role of Ap_3A in the cell has not been precisely defined but is believed to serve, in conjunction with related nucleotides, as an alarmone *i.e.* a substance whose intracellular concentration increases dramatically in response to stress.[171] The enzyme cleaves Ap_3A *(48)* at P^1 (≡ P^3) to give AMP *(49)* and ADP *(50)* (scheme 2.5).



Scheme 2.5

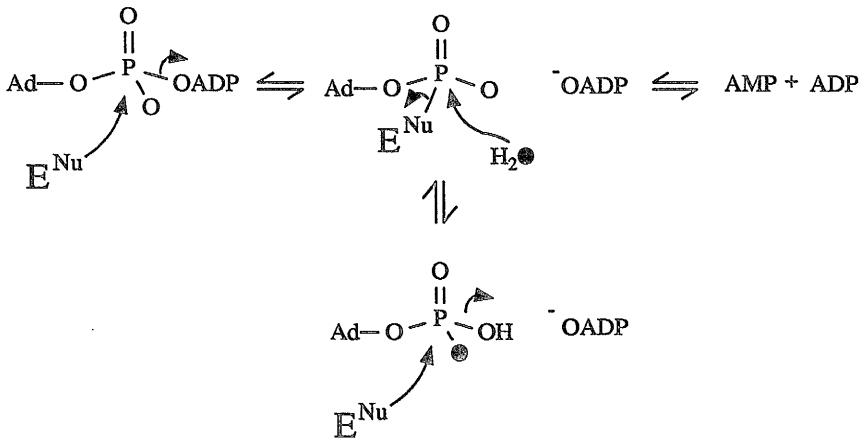
G. M. Blackburn and co-workers (unpublished results) have found that the reaction catalysed in the presence of oxygen labelled water ($H_2{}^{18}O$) yields AMP with the label present in a non-bridging position. Detectable levels of doubly labelled AMP were also



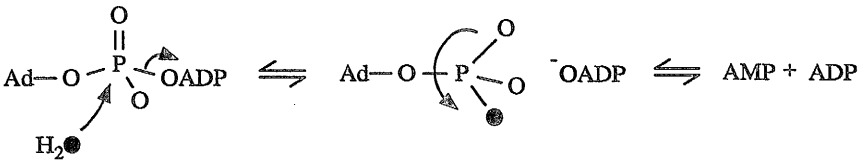
Scheme 2.6

observed. These results can be accommodated by a mechanism in which the Ap_3A undergoes nucleophilic attack by $H_2{}^{18}O$ at P^1 to generate a pentaco-ordinate intermediate (scheme 2.6). Pseudo rotation following proton shift and subsequent loss of ADP would yield singly labelled AMP. Alternatively, loss of $H_2{}^{16}O$, to regenerate $[^{18}O]Ap_3A$ followed by attack by a second molecule of $H_2{}^{18}O$ could generate doubly labelled AMP.

However, there is little evidence in favour of the pseudo rotation pathway in other phosphatases.[103] The observation might also be explained by reversible and multiple P^1-O-P^2 cleavage providing P^1 is torsionally unrestricted at the active site and, bound and bulk water are exchangeable. A reversible hydrolysis mechanism could involve a phosphoenzyme intermediate (scheme 2.7) or, alternatively, by direct attack of water (scheme 2.8). It was envisaged that the mechanism of the enzyme might be determined by

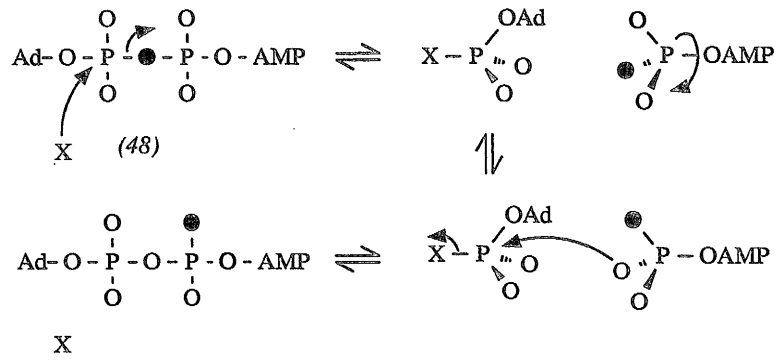


Scheme 2.7



Scheme 2.8

application of PIX. If the mechanistic pathway involves reversible and multiple P^1-O-P^2 cleavage then a ^{18}O label in the P^1-O-P^2 bridging position would become scrambled out of the bridge, providing that the phosphoryl group containing the label upon hydrolysis has rotational freedom (scheme 2.9).



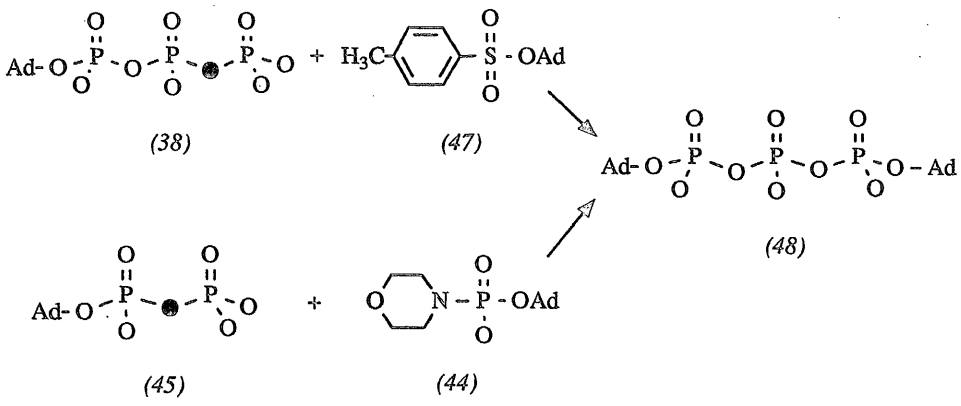
Scheme 2.9 X = H_2O or E.Nu

The potential of two routes to the synthesis of [α^1,β-^{18}O]Ap_3A *(48)* from [β,γ-^{18}O]ATP *(38)* (or [α,β-^{18}O]ATP *(46)*) and from [α,β-^{18}O]ADP *(45)* were ascertained (scheme 2.10).

Reaction of ATP with excess adenosine-5′-tosylate by the method of Poulter *et al.*[169] (analogous to the preparation of [α,β-^{18}O]ADP), was followed by ^{31}P nmr until the reaction mixture remained unchanged. Ap_3A, as identified by ^{31}P nmr, was isolated from the mixture by ion exchange chromatography in 21% yield based on ATP.

ADP was reacted with excess AMP morpholidate by the method of Moffatt *et al.*[168] (analogous to the preparation of [α,β-^{18}O]ATP). The reaction was monitored as before and Ap_3A, identical to the previous preparation, isolated by ion exchange chromatography in 41% yield.

Thus [α^1,β-^{18}O]Ap_3A was prepared from [α,β-^{18}O]ADP and AMP morpholidate by the

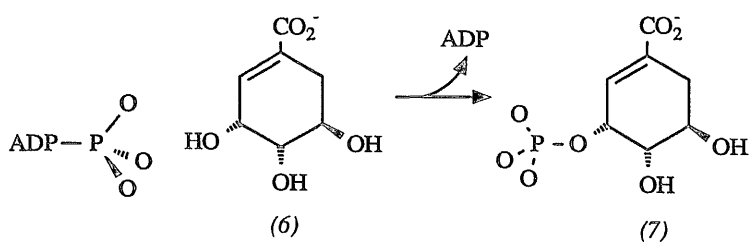


Scheme 2.10

latter method in equivalent yield. The absolute position of the ^{18}O label in [α^1,β-^{18}O]Ap_3A is yet to be confirmed, but can assumed with reasonable confidence based on the absence of bridge to non-bridge scrambling during similar synthetic manipulations for the preparation of [α,β-^{18}O]ADP and [α,β-^{18}O]ATP. [α^1,β-^{18}O]Ap_3A is currently awaiting PIX analysis with Ap_3A hydrolase in the research group of G.M. Blackburn.

2.2.5 Target Fluoro Analogue of Shikimic Acid.

Shikimate kinase catalyses phosphoryl transfer between shikimate and ATP to give shikimate-3-phosphate and ADP and has a requirement for a divalent cation (scheme 2.11).[151] It was envisaged that replacement of the 3-hydroxyl group with fluorine would



Scheme 2.11

give an analogue of shikimate that might bind effectively to the catalytic site, but be unable to be phosphorylated. The replacement of C-OH with C-F has been used widely in carbohydrate and inositol chemistry by reason of similarities of electronegativity, Van der Waals radii and that the fluorine can also accept a hydrogen bond.[172] Such an analogue therefore addresses two of the possible limitations associated with PIX. It is envisaged that the binding of a good substrate analogue will induce the correct conformational changes and/or electrostatic interactions to promote the dissociative pathway leading to metaphosphate, if this is indeed the mechanism. The lifetime of a transiently bound metaphosphate intermediate might also be increased. Fluoro-deoxy shikimate analogue *(51)* was considered a suitable analogue for this study. The molecule also lacks the 5-hydroxyl group, which eliminates the possibility of phosphorylation (or transient phosphorylation) at this position if the molecule were to bind to the catalytic site the opposite way round (Figure 2.6).

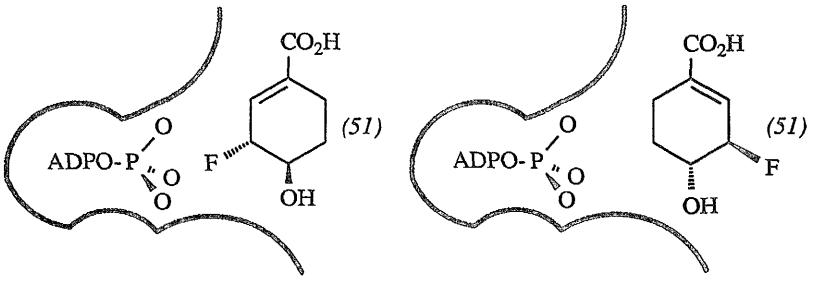
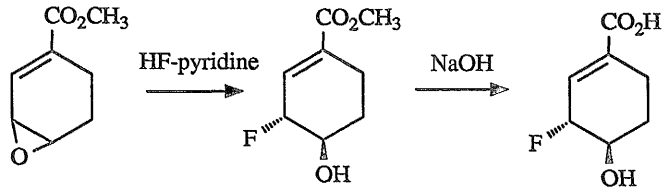


Figure 2.6 *Possible binding modes of analogue (51).*

2.2.6 Preparation and Inhibitor Properties of Fluoro-Shikimate Analogue *(51).*

Fluoro-deoxy shikimate *(51)* was prepared from epoxy ester *(52)* as described in chapter 3 and was isolated as a mixture of enantiomers (scheme 2.12). Analogue *(51)* was found to be a competitive inhibitor of shikimate with an observed inhibitor constant (K_i) of 21 mM. It should be noted that this value is derived from to a mixture of enantiomers, and it is expected that one enantiomer will be a more effective inhibitor than the other. At the



(52) *(53)* *(51)*

Scheme 2.12

extreme, if one enantiomer is totally ineffective as an inhibitor, then the real K_i for the other enantiomer is half of that observed for the mixture.

2.2.7 Positional Isotope Exchange by Shikimate Kinase.

It was planned to investigate the degree of scrambling, if any, of the bridge ^{18}O to non-bridge in [β,γ-^{18}O]ATP catalysed by shikimate kinase in the presence and absence of co-substrate shikimate. PIX in the presence of the shikimate analogue *(51)* will also be investigated. The shikimate kinase used for these experiments was a gift of John R. Coggins (Department of Biochemistry, University of Glasgow) and was purified by his research group.[54] SDS gel electrophoresis (data not shown) indicated that the sample contained one protein species only. All the PIX experiments were performed in (final concentrations) 50 mM-triethanolamine hydrochloride buffer, pH 7.0 containing 50 mM-KCl and 5 mM-$MgCl_2$.

Adenosine 5'[β,γ-^{18}O] triphosphate *(38)* was incubated with *E.coli* shikimate kinase in the presence of shikimate and sufficient Ap_5A to inhibit possible traces of adenylate kinase.[173] The reaction was allowed to progress to approximately 40%. Isolation of the ATP and ADP indicated that 42% of the ATP had been hydrolysed. The ^{31}P nmr spectrum of the isolated [β,γ-^{18}O]ATP (figure 2.7) shows the two P_γ doublets separated by approximately 2.3 Hz. The high field doublet corresponds to unscrambled [β,γ-^{18}O]ATP and the slightly larger low

field doublet corresponds to scrambled [β-^{18}O]ATP. The fraction F, of the ATP that has undergone positional isotope exchange is defined as $[(^{18}O/^{16}O)_t-(^{18}O/^{16}O)_{t=0}]/[(^{18}O/^{16}O)_{t=\infty}-(^{18}O/^{16}O)_{t=0}]$,[131] where the ratio (^{18}O/^{16}O) is determined from the ratio of the peak height of the downfield-shifted (^{18}O-perturbed) resonance for the γ-phospho group to the sum of the peak heights of the shifted and unshifted resonances for the γ-phospho group. The rate constant for the exchange process was then calculated from $k = \{-\ln(1-F)\}[ATP]/[E]t$,[152] where [E] is the concentration of the enzyme in subunits. Values of F and k are given in table 2.1.

In the second experiment [β,γ-^{18}O]ATP *(38)* was incubated with shikimate kinase in the absence of any shikimic acid or analogue. Ap_5A was added as before. After 18 hours isolation of nucleotide revealed the degree of hydrolysis of ATP to be <1%. The ^{31}P nmr spectrum of the isolated ATP is shown in figure 2.7. Values of F and k are given in table 2.1.

The third experiment was a replica of the second except for the presence of the fluoro-analogue *(51)* (25 mM). A higher concentration of *(51)* would have been desirable (ca. > 5 x K_i), however the concentration used was limited by supply. Again, isolation of ATP (and ADP) indicated that <1% hydrolysis of the ATP had occurred. The ^{31}P nmr spectrum of the isolated ATP is shown in figure 2.7. Values of F and k are given in table 2.1.

As is shown in table 2.1 shikimate kinase does, in the presence of the intended co-substrate shikimic acid, catalyse the exchange of ^{18}O from the β-γ bridge position to the Pβ non-bridge location of ATP. Furthermore, experiment 2 suggests that PIX also occurs (at a reduced rate) in the absence of substrate and is therefore suggestive that the mechanism of phosphoryl transfer might be dissociative. However, incubation of the enzyme with the fluoro analogue *(51)* over the same time period (*ca.* 108 half lives) greatly reduces the

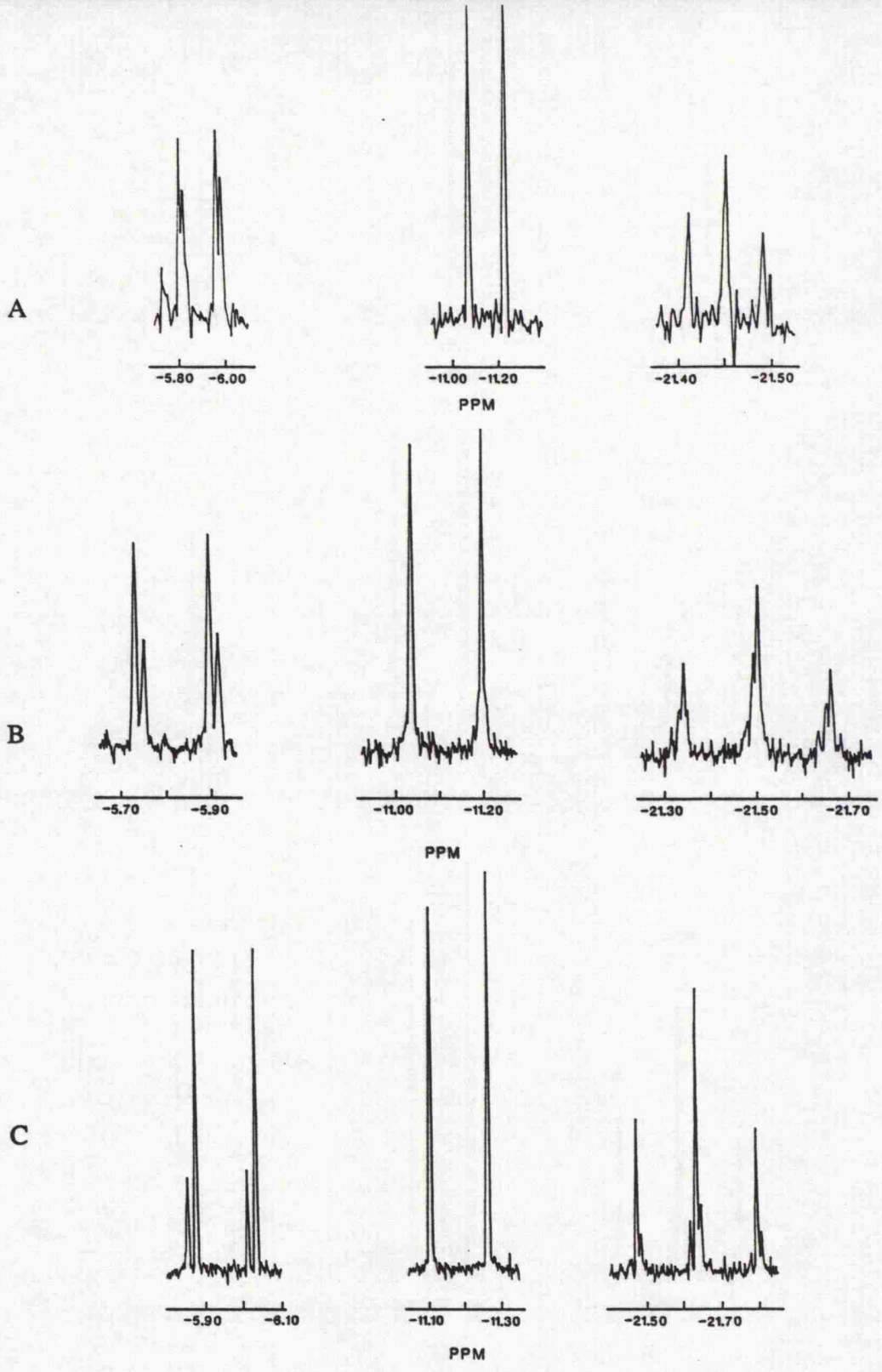


Figure 2.7

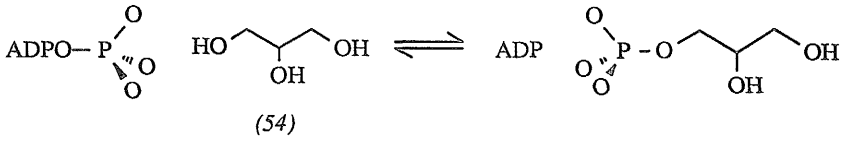
^{31}P n.m.r. spectra (121.5 MHz) of [β,γ-^{18}O]ATP *(38)* in 250 mM CAPS, 30 mM EDTA, 20% D_2O, pH 9.6.

Spectra A, Exp. No. 1, + shikimate, 42% ATP hydrolysis, (GB 0.15 Hz, LB -1.5 Hz)
Spectra B, Exp. No. 2, - shikimate, 18 hours, (GB 0.0 Hz, LB 0.0 Hz)
Spectra C, Exp. No. 3, + fluoro-shikimate, 18 hours, (GB 0.5 Hz, LB -0.5 Hz)

Experiment No.	Co-substrate	Co-substrate conc. (mM)	*F*	*k* min^{-1}
1	shikimate	0.5	0.84	2090
2	none	-	0.97	123
3	fluoro analogue *(51)*	25	0.27	11

Table 2.1

degree of observed exchange. The reduction of PIX by *(51)*, which is a competitive inhibitor of shikimate kinase, suggests that the observed PIX is indeed a property of shikimate kinase. It is possible that the PIX arises from a contaminating co-substrate. Possible identities of this contaminating co-substrate include an active site amino acid residue or a water molecule. However, it was considered that the triol glycerol *(54)*, which is present in the buffer in which the enzyme is stored, might also be a likely candidate due to the array of hydroxyl groups that might mimic shikimic acid (Scheme 2.13).



(54)

Scheme 2.13 *Possible role of glycerol as a substrate.*

This possibility was investigated by two further experiments where the enzyme had been dialysed against buffer lacking glycerol (2 x 500 ml at 4°C over 8 hours) prior to the incubation. In one experiment enzyme was incubated with [β,γ-^{18}O]ATP *(38)* in the absence of any other substrate as in experiment 2. The second experiment was also a replica of experiment 2 except for that an equivalent quantity of glycerol that had been removed by dialysis was reintroduced into the incubation.

Isolation of the ATP in each experiment revealed the degree of hydrolysis to be <0.2%. The

^{31}P nmr spectra of the isolated ATP is shown in figure 2.8. Values of F and k are given in table 2.2

Experiment No.	Co-substrate	Co-substrate conc. (mM)	F	k min^{-1}
4	none	-	0.40	18
5	added glycerol	6.3×10^{-3}	0.38	17

Table 2.2

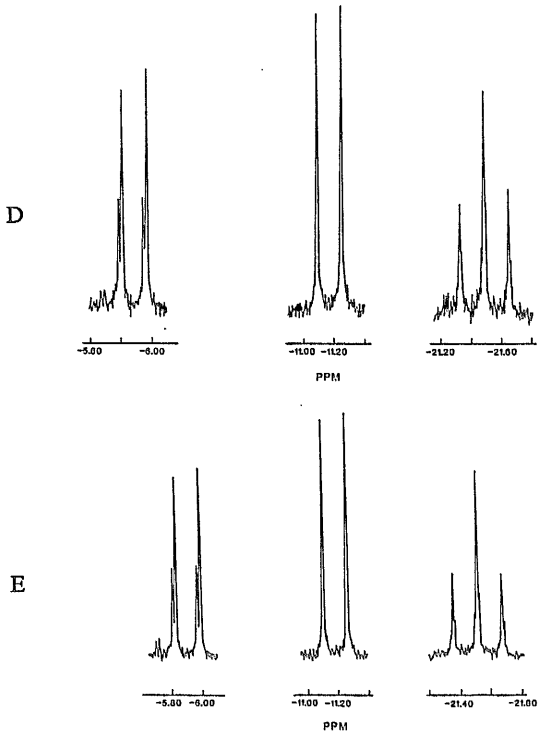


Figure 2.8

^{31}P n.m.r. spectra (121.5 MHz) of [β,γ-^{18}O]ATP *(38)* in 250 mM CAPS, 30 mM EDTA, 20% D_2O, pH 9.6.
Spectra D, Exp. No. 4, dialysed, (GB 0.0 Hz, LB 0.0 Hz)
Spectra E, Exp. No. 4, dialysed + glycerol, (GB 0.0 Hz, LB 0.0 Hz)

2.3 Discussion.

From the results shown in table 2.2 it is clear that glycerol is not acting as weak substrate, however, dialysis of the enzyme prior to the incubation reduces the observed PIX substantially. The indentity of the contaminant, removed by dialysis, is therefore most likely to be a small amount of shikimic acid. The low levels of rate of PIX observed in experiments 4 and 5 cannot unambiguously be associated dissociative heterolysis of bound ATP as it is conceivable that some contaminant shikimate may still be present even after dialysis. Furthermore, the possibility of scrambling by the presence of a unknown trace highly efficient enzyme impurity other than adenylate kinase cannot be ruled out.

Experiments 1, 2 and 3 all contained this trace presumed to be contaminant shikimate, which accounts for the total scrambling observed in the absence of substrate or substrate analogue (exp. 2). However, in the presence of the fluoro-shikimate analogue *(51)* the degree of observed PIX is greatly reduced. Thus the analogue, which is a competitive inhibitor, is preventing the binding of the contaminant shikimic acid and the subsequent scrambling of the bridge-labelled ATP. It is possible to conclude therefore, that the majority of the observed PIX is a property of shikimate kinase and not caused by the presence of contaminant protein.

The rate of positional isotope exchange catalysed by shikimate kinase in the presence of shikimic acid is slow in comparison to the overall rate of the reaction (k_{CAT} = 6.2 x 10^3 min^{-1}). Positional isotope exchange is dependant on the rotation of the β-phosphoryl group of the enzyme bound ADP and while this rotation is favoured on thermodynamic grounds[133] the constraints imposed by the enzyme's active site and/or by the complexation with the divalent metal may account for the slow rate if a dissociative process is occurring. Assuming that the rotational freedom of the $-\beta PO_3$ is not limiting it must be assumed that

the observed PIX arises from the return of the ternary products complex, E•ADP•Shik-3-P, to ATP and shikimate.

The failure to observe any significant positional isotope exchange in the absence of co-substrate or the presence of the non-phosphorylatable analogue of shikimic acid is consistent with the findings for creatine kinase,[159] hexokinase,[158] and pyruvate kinase.[131] However, as discussed earlier evidence does exist in favour of the dissociative mechanism for enzymic phosphoryl transfer; the intersubstrate distances of >5 Å for protein kinase and alkaline phosphatase;[142] the secondary ^{18}O isotope effects for hexokinase[164] and alkaline phosphatase,[163] despite the lack of PIX in the absence of co-substrate or lyxose[158] in the case of hexokinase.

Nevertheless, in the absence of any evidence to support a dissociative pathway for shikimate kinase postulation about the mechanism of phosphoryl transfer must be based on the majority of the evidence presented for phosphokinases studied thus far; inversion of configuration of the transferred phosphoryl group; the general chelation of the metal ion to the γ-phosphoryl residue; the lack of observed PIX in the absence of phosphoryl acceptor; the obligate presence of a nucleophile. The presumed transition state for an 'in-line' associative phosphoryl transfer as described by Knowles[102] is shown in figure 2.9.

Stabilisation of the associative transition state necessitates the presence of a general acid to assist the leaving group, a general base to increase the nucleophilicity of the acceptor, and up to three positively charged species to stabilise the negative charge that develops on the equatorial oxygens. The divalent metal ion may fulfil this role as well as that of a general acid. Consistent with such a model, it is worthy to note the conserved nature of positively charged amino acid residues in a region of the primary sequence of shikimate kinase and other proteins thought to be responsible for binding ATP[54] (see chapter 5).

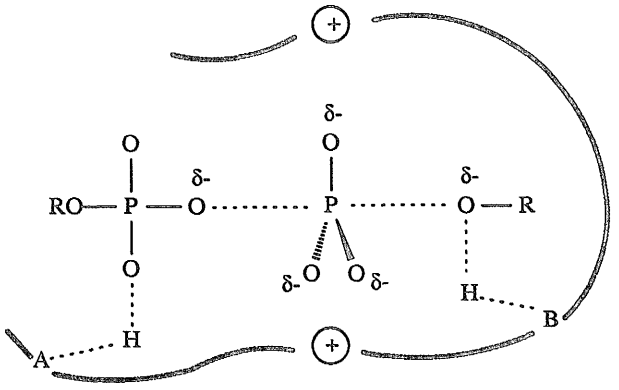


Figure 2.9

2.4 Summary.

Pyrophosphate labelled with ^{18}O in the bridging position has been used to prepare a number of specific and singly labelled nucleoside polyphosphates applicable to positional isotope exchange studies. The synthesis of [β,γ-^{18}O]ATP, [α,β-^{18}O]ADP, [α,β-^{18}O]ATP and [α^1,β-^{18}O]Ap_3A are reported. In order to address some of the constraints of PIX analysis, a non-phosphorylatable fluoro analogue of shikimic acid was prepared. Significant PIX was observed in the presence shikimic acid, but not in its absence. The rate of PIX was not enhanced by the presence of the non-phosphorylatable fluoro analogue. These results were consistent with previous PIX analysis of phosphoryl transfer in kinases, and associative transfer is therefore implicated.

CHAPTER 3

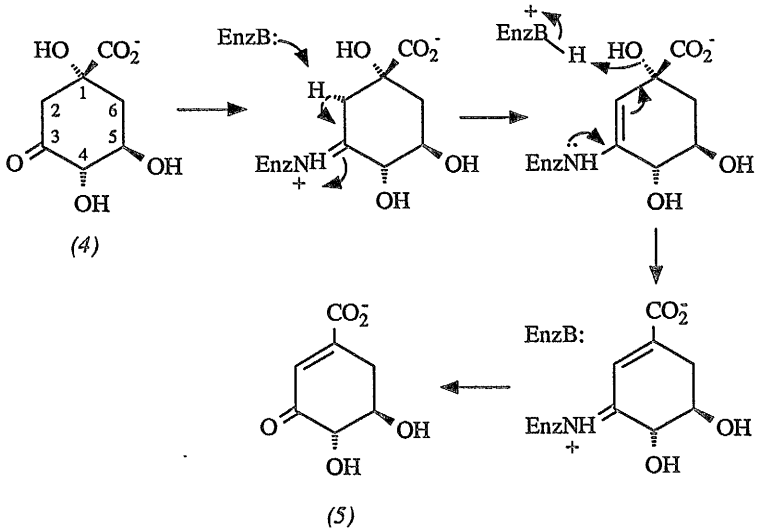
Synthesis and Specificity of Shikimic acid Analogues.

Strategy 1

3.0 Introduction.

The synthesis of analogues of the natural substrates of enzymes to generate inhibitors and alternative substrates has been widely used to provide insight to the functional requirements of binding sites and catalysis. Active-site-directed irreversible inhibitors, or affinity labels, are particularly useful in this regard and have been used to identify catalytically important residues. Affinity labels are designed such that they exploit the specificity of enzymes, which, through resemblance to the substrate (or product) enhances their reactivity with active site nucleophiles. Many hundreds of affinity labels have been synthesised, the majority based on halomethyl ketones or epoxides. Their application is nicely illustrated by their use to explore the active site of *E.coli* dehydroquinase.

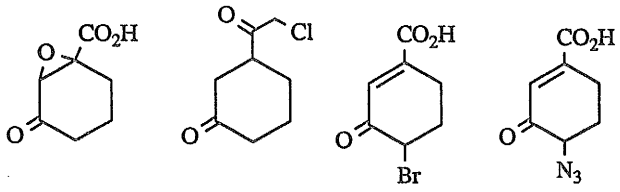
Dehydroquinase catalyses the stereospecific *cis*-dehydration of 3-dehydroquinic acid *(4)* to 3-dehydroshikimate *(5)* and represents the third step in the shikimate pathway. The



Scheme 3.1

mechanism of the enzyme is thought to proceed via the abstraction of a proton at C-2 following the formation of a Schiff's base between an active site lysine residue and the

carbonyl at C-3 (scheme 3.1). Evidence from the pH/rate profile and inactivation using the reagent diethylpyrocarbonate has been used to infer that the general base responsible for the abstraction may be a histidine residue. Abell *et al.*[174] synthesised four analogues of *(4)* incorporating chemical species which are known to be reactive towards nucleophilic amino acid residues (figure 3.1). In each case the electrophilic species are strategically positioned

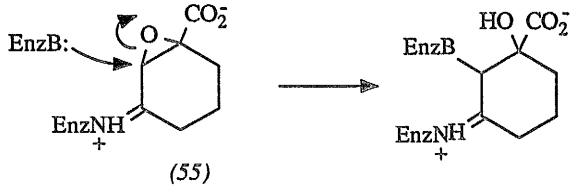


(55) *(56)* *(57)* *(58)*

Figure 3.1

such that they might be expected to react and modify nucleophilic residues involved in binding and/or catalysis. Values of K_i refer to the concentration of racemic material required for half-maximal rate of inactivation.

Epoxy analogue *(55)* was found to be the most potent irreversible inhibitor (K_i = 400 μM compared to K_M for *(4)* = 16 μM) and a possible mechanism involving nucleophilic attack of the active site general base (EnzB:) was envisaged (scheme 3.2). The ability of the



(55)

Scheme 3.2

natural substrate *(4)* to protect against inhibition confirmed that the alkylation of the enzyme was occurring at the active site.

The chloromethyl ketone analogue *(56)* was also found to be an active site irreversible

inhibitor (K_i = 680 μM). It was believed to be reacting with a putative lysine residue, thought to be present at the carboxylate binding sites of other enzymes in the shikimate pathway.[175]

The bromo analogue *(57)* was found not to be an inhibitor of the enzyme and thus consistent with the lack of nucleophilic residues in this region of the active site. Covalent modification at the active site of the enzyme was achieved by the introduction of an azide group at C-4 to give analogue *(58)* which is a photo-affinity label. The compound is stable in absence of light, but is photolyzed to give a highly reactive nitrene. Broad band illumination of *(58)*, in the presence of the enzyme, inactivated the protein with K_i = 1.1 mM. Presumably modification occurs at residues responsible for hydrogen bonding to the C-4 hydroxyl substituent which has been shown to be an important hydrogen bond for enzyme specificity.[176]

As observed, the use of active-site-directed inhibitors is most effectively applied to the identification of nucleophilic residues directly involved in catalysis. The relative contribution to binding energy of residues involved in weaker interactions, such as a neutral hydrogen bond between protein and ligand, has been probed by the kinetic study of the effect of mutating the protein,[177,178] altering the substrate[176,179-183] and in some cases both.[184] Site-directed mutagenesis is used to alter or more commonly delete specific amino acid side chains. The most prominent example of its use is that of tyrosyl-tRNA synthetase.[177,178] Fersht and co-workers have used the technique extensively to estimate the contribution of hydrogen bonding interactions to the stabilisation of both ground-state and transition-state complexes. They were able to identify the precise roles for individual amino acids in catalysis. The technique, however, has certain limitations. Firstly, extensive knowledge of the protein structure is required. Ideally a complete 3 dimensional X-ray structure of the protein is needed in order to identify active site residues that are involved in direct hydrogen bonds to the ligand. Secondly, this approach relies on the principle that the

amino acid substitutions do not disrupt the protein structure. Although crystallographic evidence does exist that suggests that this is usually the case,[185] it can not be assumed that this is always so and in most cases direct evidence from high field ^{1}H nmr or X-ray crystallography is required. Thirdly, the possible changes of the amino acid side chains are currently limited to those of the 20 naturally occurring amino acids. In contrast varying the substrate structure permits a wide range of chemically defined alterations to the enzyme/ligand interaction. Also any changes to the structure of the substrate are relatively easy to characterise.

The quantitative analysis of individual interactions between enzyme and ligand is achieved by a direct comparison of dissociation or specificity constants for the modified and unmodified complexes.[186] Such a comparison yields an apparent binding energy (ΔG_{app}) associated with the interaction that has been modified. The apparent binding energy for an enzyme-substrate interaction is given by eq. 3.1;

$$\Delta G_{app} = R\,T \ln [(k_{cat} / K_M)' / (k_{cat} / K_M)] \quad (3.1)$$

where (k_{cat} / K_M) and $(k_{cat} / K_M)'$ are the specificity constants for the unmodified and modified complexes determined from the Michaelis-Menten equation. The specificity constants of the enzymic catalysis are used in favour to the dissociation constants (K_M) as the former avoids complications from non-productive interaction of the enzyme and substrate. ΔG_{app} can be related to the energies of the specific bonds in the complex that have been altered. Consider the case of a base (B), on an enzyme (E), interacting with a hydrogen bond donor (XH), on a substrate (S). The binding of B and XH is an exchange reaction in which B and XH exchange interactions with water with each other (eq. 3.2).

$$\text{E-B}\bullet\text{HOH} + \text{S-XH}\bullet\text{OH}_2 \rightleftharpoons [\text{E-B}\bullet\text{HX-S}] + \text{H}_2\text{O}\bullet\text{HOH} \quad (3.2)$$

Since there are two hydrogen bonds on each side of the equilibrium (the types of hydrogen bond are also conserved) the energy expressed therefore results partially from enthalpic

differences due to different H-bond geometries, but mainly from the increase in entropy associated with release of water from the active site and substrate into bulk water. If the hydrogen bond donor is modified (S-XH → S-Y) the binding of the modified substrate is expressed by eq. 3.3.

$$E\text{-}B\bullet HOH + S\text{-}Y/OH_2 \rightleftharpoons [E\text{-}B/Y\text{-}S] + H_2O\bullet HOH \quad (3.3)$$

Removal of the hydrogen-bonding group from the substrate (S) need not lead to the loss of the full energy of the H-bond since no H-bond will exist between the substrate analogue (S-Y) and water before binding. Therefore, any energetic difference between the two systems will not equal the full H-bond energy but the difference in strengths of H-bonds formed with the enzyme and water. The magnitude of the difference could, however, be significant when comparing a H-bond in a transition state enzyme-substrate complex with that between a relatively mobile water molecule and a protein residue.

ΔG_{app} can be related to the energies of the specific bonds that have been modified according to a simple thermodynamic cycle as discussed by Fersht[186] to give eq. 3.4

$$\Delta G_{app} = (G_{S\text{-}Y/W} - G_{S\text{-}XH\bullet W}) - (G_{E\text{-}B/Y\text{-}S} - G_{E\text{-}B\bullet HX\text{-}S}) + \Delta G_{reorg} \quad (3.4)$$

where $G_{S\text{-}Y/W}$ is the energy of interaction between the modified substrate and water, $G_{S\text{-}XH\bullet W}$ is the energy of interaction of substrate and water, $G_{E\text{-}B/Y\text{-}S}$ is the dissociation energy of the modified complex, $G_{E\text{-}B\bullet HX\text{-}S}$ is the dissociation energy of the unmodified complex, and ΔG_{reorg} is a reorganisational energy term that includes any conformational changes, preturbations to the binding of the rest of the substrate to the enzyme, or rearrangement of the solvent as a result of the modified substrate. Thus the magnitude of ΔG_{reorg} depends on several factors associated with the precise mutation and is therefore unknown. However, using evidence from making many series of mutations to tyrosyl-tRNA

synthetase, Fersht suggests that ΔG_{reorg} is probably less than 0.5 Kcal mol^{-1} where gross structural changes are unlikely.

The ΔG_{app} (based upon k_{cat}/K_M) determined by the removal of side chains of tyrosyl-tRNA synthetase involved in hydrogen bonding interactions with the substrate displayed a range of values which were essentially categorised into two types.[186] Deleting a hydrogen bond to an uncharged hydrogen bond donor/acceptor resulted in a loss of affinity 0.5 to 1.8 Kcal mol^{-1}. However, deletion of a bond to a charged donor/acceptor weakens binding by a much greater amount, some 3 - 6 Kcal mol^{-1} for either one or both partners being charged.

The complementary approach to that of Fersht involving deletion of hydrogen bonding groups from a substrate also gives values of similar magnitude. Withers and coworkers measured ΔG_{app} of hydrogen bonds for the hydroxyl substituents of a number carbohydrate-enzyme complexes. Glycogen phosphorylase[179] catalyses the reversible phosphorolysis of glycogen to produce glucose 1-phosphate. It is an allosteric enzyme whose inactive (T-state) conformation is stabilised by glucose and whose active (R-state) conformation by substrates and activators. The X-ray crystal structure of the enzyme-glucose complex indicates that glucose binds at the active site of the enzyme. Withers synthesised a series of deoxy and deoxyfluoro analogues of glucose and tested their ability to act as competitive inhibitors enabling estimation (from K_i) of the relative strengths of hydrogen bonds to the carbohydrate in the T-state. Comparison of the values with the X-ray crystal structure showed good correlation with the values observed by Fersht.[186] Specifically they found that deletion of a hydrogen bond partner of a neutral group (in this case Gly, Leu, Asn, or Tyr) weakens binding energy by about 1.5 Kcal mol^{-1}, whilst deletion of a partner of a charged group (in this case Glu or His) reduced affinity by about 3 Kcal mol^{-1}. They also noted that the deletion of a hydroxyl observed to be hydrogen bonded to two neutral partners in the X-ray structure resulted in the loss of affinity by 3 Kcal mol^{-1} consistent with the above.

In a separate study[180] Withers used a series of deoxy and deoxyfluoro analogues of the substrate glucose 1-phosphate and tested their ability to act as substrates. Apparent binding energies (from k_{cat}/K_M) of hydrogen bond interactions at the transition-state were thus calculated. Withers found that a similar set of hydrogen bond partners existed the transition-state to those observed in the ground-state complex, but that the bonds were approximately twice the strength in the transition state. Withers concluded that the hydrogen bonds strengths of about 6 Kcal mol^{-1} were probably consistent a with relatively a strong hydrogen bond involving charged partners and values about 3 Kcal mol^{-1} might indicate a single strong or several weak bonds in the transition state complex. The observation of stronger interactions in the transition state complex is consistent with the theory that an enzyme catalyses a reaction by binding to the transition state preferentially.[99]

Withers also used the relative values of the dissociation constants (K_M) of the glucose 1-phosphate analogues to calculate H-bond interactions in the ground-state complex. Despite the interference of any nonproductive binding which can not be quantified values of H-bond interactions in the ground state were found to be broadly comparable to those determined from the enzyme-inhibitor complexes.

Similarly by the use of deoxy and deoxyfluoro substrate analogues and inhibitor analogues of glucose 1-phosphate Withers and coworkers were able to estimate hydrogen bonding interactions for ground and transition states of the enzyme phosphoglucomutase.[182] Values in the range 0.3 - 2.8 Kcal mol^{-1} for ground-state interactions and 2.7 - 4.5 Kcal mol^{-1} for the transition state were determined. The difference in ΔG_{app} (the differential binding energy $\Delta\Delta G_{app}$) between the ground and transition state complexes is observed to be considerably less than that observed for glycogen phosphorylase. The difference is in accord with the mechanisms for the two enzymes where no distortion of the sugar ring occurs for phosphoglucomutase, whereas considerable distortion is expected for glycogen

phosphorylase.

The apparent contributions of hydroxyl substituents to the binding of ligands in the transition-state have been estimated for a number of enzymes. A recent review[181] of hydroxyl groups making large contributions to enzyme affinities in the transition-state finds that the loss in binding energy may be as much as 10 Kcal mol^{-1} or more in the case of cytidine deaminase and adenosine deaminase. However, the majority of 'strongly contributing OH groups' fall into the range of 5 - 7.5 Kcal mol^{-1}. In each case values represent the deletion mutation (OH → H) on the ligand.

In order to start to map the active site of *E.coli* shikimate kinase and consider the design of substrate analogues and mechanism based inhibitors the likely mechanism of the enzyme must be considered. A putative model for the shikimate kinase active site is shown in Figure 3.2. Shikimic acid is shown in the half chair conformation which is the conformation

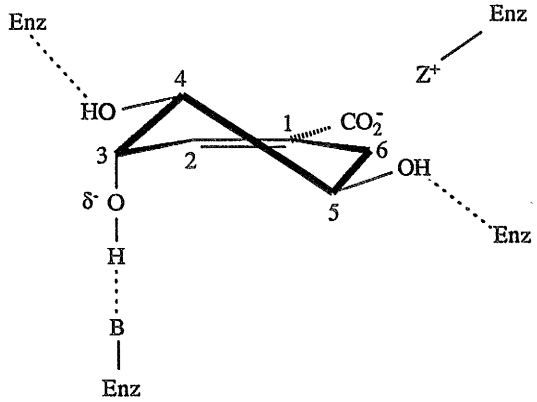


Figure 3.2

adopted in free solution determined by application of the Karplus equation to ^1H nmr data[187] and also is the conformation adopted by crystals of the sodium salt.[188] Similar measurements on a range of other shikimic acid derivatives containing the cyclohexene ring has shown that in almost all cases these adopt analogous half-chair conformations.[13]

Another conserved feature of the majority of metabolites in the shikimate pathway is the carboxylate at C-1. As in the other enzymes of the pathway a positively-charged active site residue to bind the carboxylate is considered likely. However, shikimate kinase has an absolute requirement for Mg^{2+}, presumably to chelate to the phosphoryl oxygens of ATP as in other kinases, but a possible role in coordination to the carboxylate cannot be ruled out.

As discussed in chapter 2 the mechanism of phosphoryl transfer of the vast majority of kinases is believed to proceed by nucleophilic attack of the second substrate, in this case shikimate, on the γ-phosphoryl group of ATP, a mechanism analogous to the SN2 type of carbon chemistry. A general base is envisaged to abstract or hydrogen bond to the C-3 hydroxyl proton thus increasing the nucleophilicity of the C-3 hydroxyl oxygen. It is also likely that there are several residues involved in hydrogen bonding interactions with C-4 and C-5 hydroxyl groups.

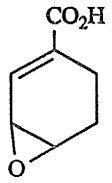
3.1 Aims.

This chapter aims to investigate the enzyme-substrate interactions described by the synthesis of analogues of shikimic acid and assaying their ability to act as substrates and/or inhibitors. The design, preparation, assaying and interpretation of kinetic data of such analogues will be described.

3.2 Results and Discussion.

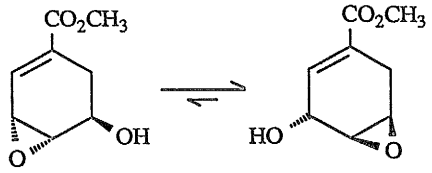
3.2.1 Target Analogue *(59)*: An Active-Site-Directed Irreversible Inhibitor.

To investigate the possible identity and role of the proposed general base in the putative model, an inhibitor was designed that was envisaged to be recognised by the enzyme such that it binds specifically to the active site and forms a covalent bond with a protein residue, hopefully the general base. Analogue *(59)* was considered a potentially a good target for



(59)

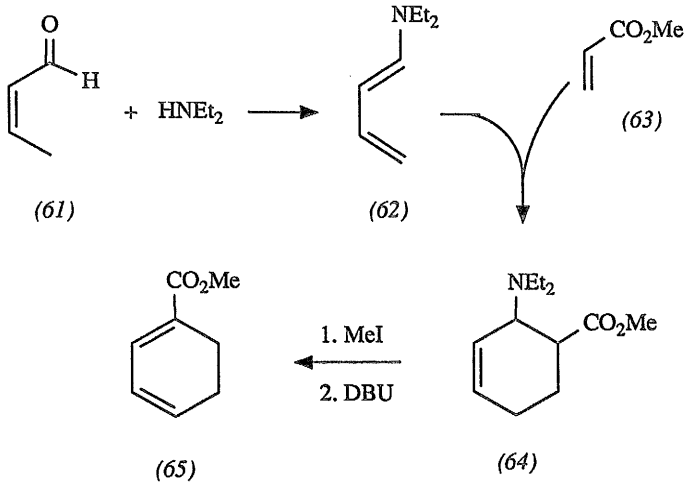
several reasons: The analogue maintains the cyclohexene-carboxylate skeleton which was considered to be the minimal structural requirement necessary for recognition and binding at the active site. The analogue contains an epoxide between C-3 and C-4. The strain of the three-membered ether ring makes epoxides highly reactive. It was thought that the epoxide might be opened by nucleophilic attack of the putative active site general base at C-3; the C-3 being more electrophilic than C-4 by reason of conjugation. The use of an epoxide may also aid recognition of the analogue as it maintains oxygen functionality at C-3 and C-4. Analogue *(59)* also differs from the native molecule by lacking a hydroxyl group at C-5. The lack of the hydroxyl group at C-5 eliminates the possibility of phosphorylation by the enzyme at this position in the absence of the C-3 hydroxyl. Furthermore, Payne rearrangement[189] as demonstrated by Ganem[190] with (+)-methyl 3,4 anhydroshikimate *(60)* is also avoided (figure 3.3).



(60)

Figure 3.3

3.2.2 Preparation of Epoxy-Shikimate Analogue *(59)*.



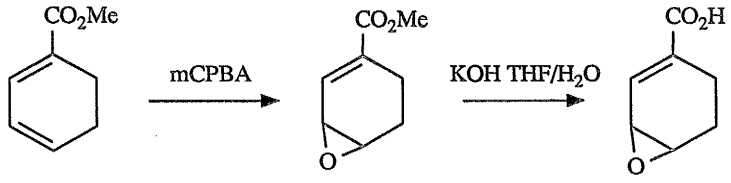
(61) *(62)* *(63)* *(65)* *(64)*

Scheme 3.3

Diene *(65)* was prepared according to the literature procedure of Kahanek[191] and is outlined in scheme 3.3. Treatment of freshly distilled crotonaldehyde *(61)* with diethylamine gave the butadiene *(62)*. Diels Alder reaction of *(62)* with methyl acrylate *(63)* gave the cyclohexene *(64)* as a mixture of diastereoisomers as indicated by the ^{13}C nmr spectrum which displayed 20 resonances. The ratio of diastereoisomers being 56:44 (as determined from the ^{13}C nmr) presumably in favour of the *endo* stereoisomers due to these being the adduct of a reaction in which the π electrons of the carbonyl are aligned to permit secondary orbital interactions with the dienophile according to the Alder rule. The observed

regioselectivity is consistent with that expected and observed from the reaction of an electron-rich diene and an electron-deficient dienophile.[192] Quarternisation of *(64)* with methyl iodide followed by elimination with base generated the diene *(65)* in an overall yield of 35% based on crotonaldehyde *(61)*.

The oxidation of *(65)* with 1 equiv. of m-CPBA to give the epoxy-ester *(66)* in quantitative yield was achieved by the method described by Berchtold *et al.*[193] (scheme 3.4). The



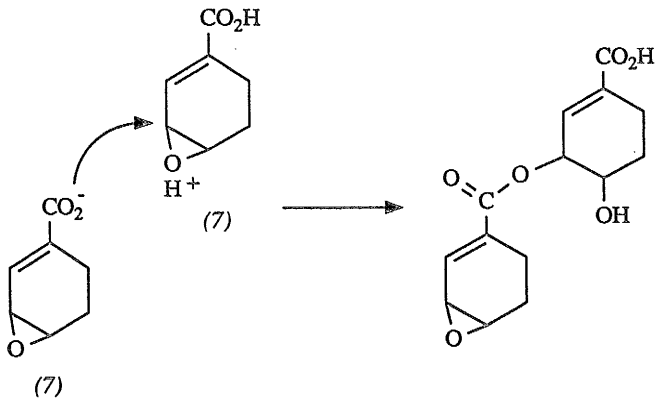
(65) *(52)* *(59)*

Scheme 3.4

epoxidation is regioselective at the C-3, C-4 olefin rather than the C-1, C-2 olefin. Such selectivity is as expected since the electron-withdrawing carboxylate group substantially reduces the nucleophilicity of this double bond. Steric interference between the C-1 substituent and the approaching reagent may also aid selectivity.

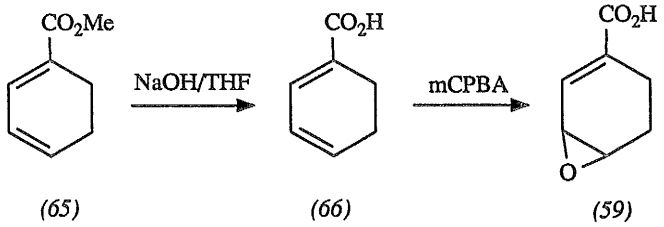
Saponification of ester *(52)* followed by careful acidification and extraction with ether gave a mixture of products. Repeated attempts to isolate the products by flash chromatography were unsuccessful. T.l.c evidence suggested that the product/s were possibly unstable. The instability is likely to result from the acid-catalysed ring opening of the epoxide by weak nucleophiles such as H_2O, MeOH, or possibly *(59)* itself where CO_2^- is the nucleophile (Scheme 3.5).

In an attempt to avoid the acidic conditions encountered during the saponification of the ester in the presence of the epoxide an alternative strategy was considered (Scheme 3.6).



Scheme 3.5

Diene *(65)* was saponified to give the corresponding acid *(66)*. Oxidation of *(66)* with m-CPBA proceeded to apparently give the desired product as observed by t.l.c. However, repeated attempts to isolate any product from m-chlorobenzoic acid by flash chromatography were also unsuccessful. T.l.c. evidence also suggested that the product was susceptible to ring opening under these conditions.



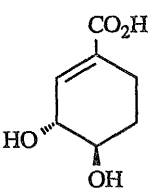
Scheme 3.6

The t.l.c. evidence of the attempts to prepare *(59)* by the two routes discussed suggested that the desired product might have been formed, but was unstable at pH < 7. However, literature examples of water soluble epoxyacids are known[194] (e.g. epoxide *(55)*, figure 3.1). The observed increased instability of the epoxide *(59)* is likely to result from its conjugation thus stabilising any cationic charge that may develop at C-3. No further attempts to isolate and characterise *(59)* were undertaken. However, some basic enzymic studies on the

inhibitor properties of *(59)* were preformed using the crude reaction mixture of *(59)*. Ester *(52)* was treated with base by the method described and the pH adjusted to 7.5 with tris/HCl buffer.

3.2.3 Inhibitor Properties of Reaction Mixture Containing Epoxy-Shikimate Analogue *(59)*.

When assayed against shikimate kinase, *(59)* was found not to be a substrate (apparent activity of enzyme was zero in the presence of approximately 10 units of shikimate kinase activity, 100 mM *(59)* under standard assay conditions) . This suggests that the epoxide ring has remained intact during the saponification as the *trans*-diol *(67)* has been demonstrated to be a substrate for the enzyme under similiar assay conditions (this chapter). The inhibitor



(67)

properties of *(59)* were investigated by incubating the inhibitor with the enzyme and determining the activity remaining by assay of the enzymic solution at time intervals. Incubations were carried out at concentrations of 100, 10, 0.75, and 0 mM (control). If inhibition is caused by the binding of a single molecule of an irreversible inhibitor per molecule of enzyme the reaction may be represented as eq. 3.5

$$E + I \xrightarrow{k} E\text{-}I \qquad (3.5)$$

thus the rate of inhibition will be given by the second-order equation 3.6. Assuming that the

$$\frac{-d[E]}{dt} = k\,[I]\,[E] \qquad (3.6)$$

inhibitor [I] is in great excess over the enzyme [E] then the equation simplifies to *pseudo*

first-order (eq. 3.7) which integrates to give eq. 3.8

$$\frac{-d[E]}{dt} = k' [E] \qquad (3.7)$$

$$\ln [E] = k' t \qquad (3.8)$$

where k' is the *pseudo* first-order rate constant. Thus a plot of ln[E] against t should give a straight line with gradient k'. Plots of ln[E] vs. t for the different inhibitor concentrations are shown in figure 3.4.

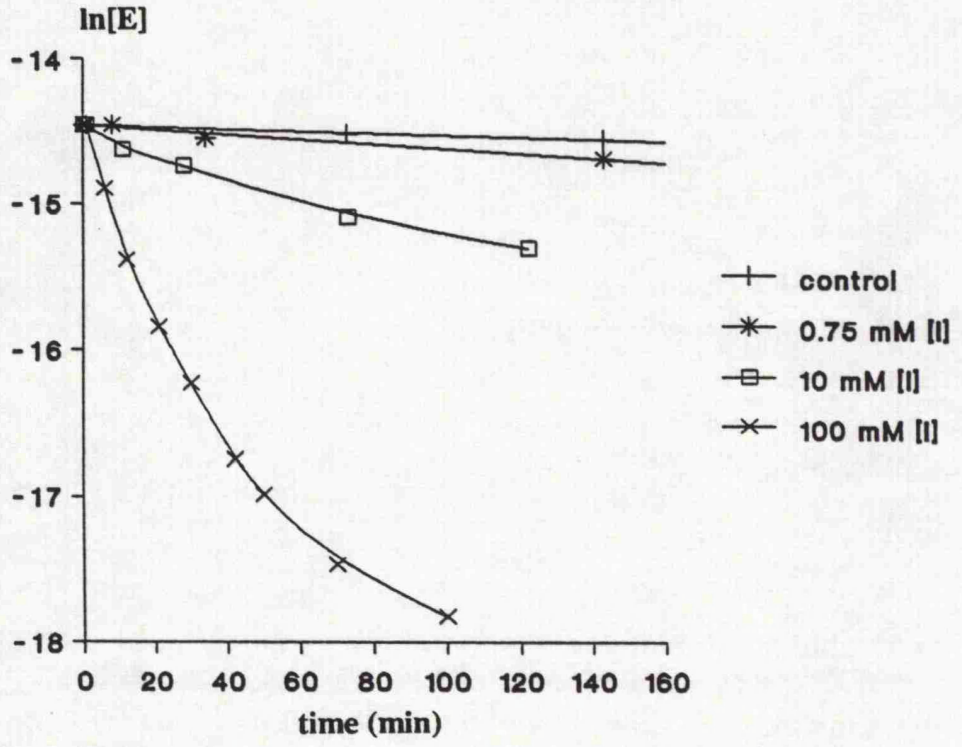


Figure 3.4 Inhibition with *epoxy-shikimate* analogue *(59)*.

The relatively high concentrations of *(59)* that are required to achieve detectable inactivation indicate that *(59)* is a relatively poor inhibitor as compared to other irreversible inhibitors of shikimate kinase (chapter 5). The semi-log plots at 10 and 100 mM *(59)* are not linear but curves suggesting that the inhibition is more complicated than exclusive reaction at a single site. The observed complications may result from one enantiomer of *(59)* being

more potent than the other, and/or be a result of the instability of the inhibitor in these conditions. However, non-linear first-order plots of this type have been shown to be characteristic of the situation where the modification of a residue at a second site leads to a partially active enzyme.[195] This type of system may be represented by figure 3.5, where X

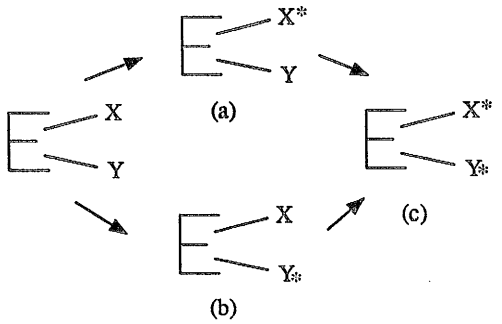


Figure 3.5

and Y are the residues that are modified, and * represents a modified residue. A non-linear first-order plot is observed if (a) has only a fraction of activity of the native enzyme, and possibly represents a modification distinct from the active site, whereas (b) and (c) are both inactive.

To investigate whether the modification is of an active site residue incubations were carried out as before in the presence of 100 mM inhibitor, but also in the presence of one of the substrates of the enzyme. The results for the incubation of the enzyme with the inhibitor in (i) the absence of substrate, (ii) the presence of 16 mM shikimic acid, and (iii) the presence of 25 mM ATP are shown in figure 3.6. It should be noted that the solution containing the inhibitor *(59)* had been stored for 24 hours at -20 °C prior to the incubations and this accounts for the loss in potency of the inhibitor as compared to the previous study.

As shown in figure 3.6, ATP offers total protection of the enzyme against inhibition by *(59)*. Assuming that ATP binds to the enzyme at one site only, this result indicates that *(59)* is

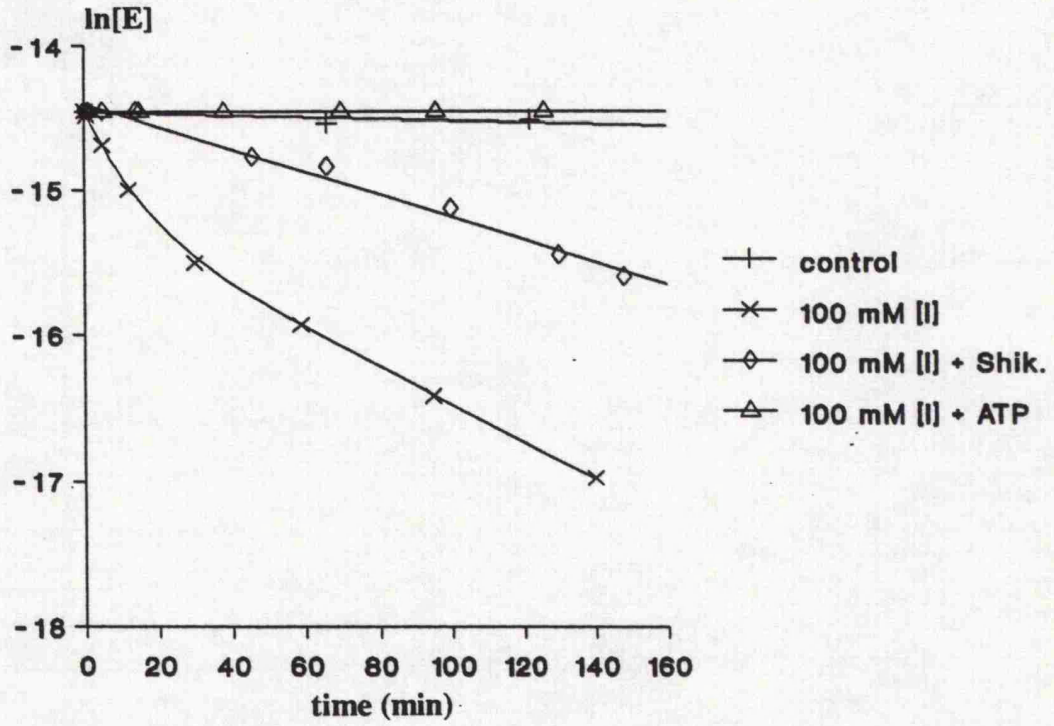


Figure 3.6 Substrate protection against inhibition by analogue *(59)*.

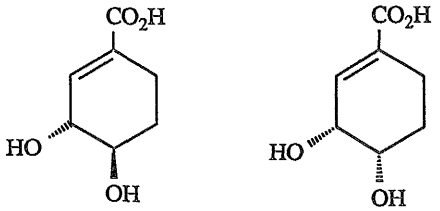
reacting at the active site and in the region of the protein responsible for the recognition of ATP. However, figure 3.6 also indicates that the substrate shikimate has the ability to protect against inhibition albeit less effectively. This may be a consequence of the mechanism of recognition and binding of shikimate if the enzyme is comparable to that of a number of phosphotransferases which demonstrate similar structural features.[103] Two of the most prominent examples of these are that of hexokinase[146,161] and adenylate kinase.[147] In the case of hexokinase, high resolution X-ray crystallographic studies of the protein as several different complexes has revealed that the enzyme has two distinct lobes flanking a marked cleft. Also these studies have shown that there is a substantial conformational change on binding glucose (in the cleft) where essentially one lobe moves towards the other to embrace the substrate. Thus if a similar mechanism also exists in the case of shikimate kinase, binding of shikimic acid might induce a conformational change restricting the access of the inhibitor to the active site residue that undergoes modification.

The recent study by Bugg and co-workers[55] of consensus sequences in enzymes of the shikimate pathway suggests that the sequence responsible for recognition of shikimate (res. 18-42) occurs immediately after the presumed ATP-binding site (res. 1-22, identified by sequence homologies between adenine-nucleotide binding proteins[54]) in *E.coli* shikimate kinase. This observation may suggest that the binding sites of the substrates are also found adjacent to each other in the tertiary structure of the enzyme. If this is the case it would seem reasonable that the covalent modification of an ATP-binding site residue might be restricted by shikimate binding in close proximity to that site.

Due to the instability and lack of pronounced potency of *(59)* no further studies were undertaken.

3.2.4 Target Shikimate Analogues *(67)* and *(68)*: Alternative Substrates.

In order to investigate the relative importance of stereochemistry of C-3 and C-4 hydroxyl substituents of shikimic acid, and to test the ability of the enzyme to tolerate the lack of hydroxyl functionality at C-5 analogues *(67)* and *(68)* were considered rational targets for this study (figure 3.7). Analogues were found to be accessible by the application of



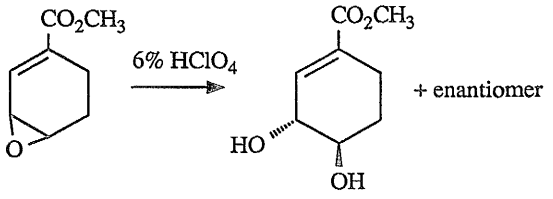
(67) *(68)*

Figure 3.7

precisely defined osmylation/epoxidation sequences to the diene *(65)* prepared in scheme 3.3.

3.2.5 Preparation of *Trans*-diol Shikimate Analogue *(67)*.

Diene *(65)* was oxidised with *m*-chloroperoxybenzoic acid (*m*-CPBA) in CH_2Cl_2 to give *(52)* in quantitative yield by the method described by Berchtold and Delany.[193] Hydrolysis of the epoxide with 6% perchloric acid appeared to proceed quantitatively by t.l.c., but the product proved difficult to isolate. Continuous extraction (48 hours) of the neutralised reaction mixture into ether followed by flash chromatography yielded at most 25% of product (Scheme 3.7). However, in a separate experiment attempting to ring-open the

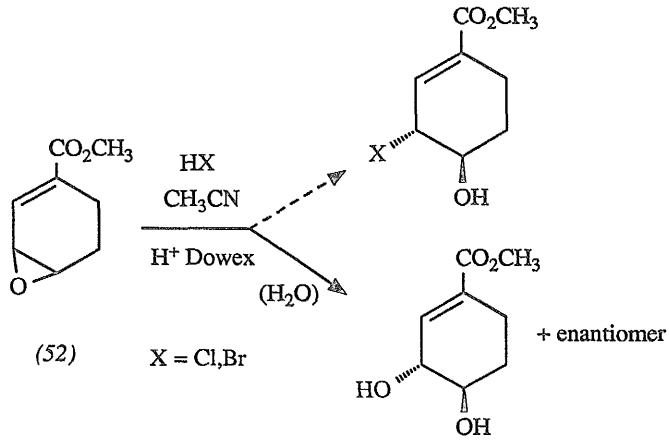


(52) *(69)*

Scheme 3.7

epoxide *(52)* to give a halohydrin using Dowex-50 (H^+ form) and sodium halide in acetonitrile,[196] the *trans*-diol *(69)* was tentatively observed by t.l.c. as a side product of the reaction presumably arising from contaminant water (scheme 3.8). Repetition of the experiment in the absence of the intended nucleophile (the salt) and in the presence of additional water gave the desired product *(69)* in almost quantitative yield. Isolation from the acid was simply achieved by filtration, evaporation of solvent and flash chromatography. The ^1H nmr spectrum of *(69)* is consistent with that of a twisted chair (see 3.2.9). Relatively large ^1H vicinal coupling constants (>7.5 Hz) between H-3 and H-4, and H-4 and H-5 indicate pseudodiaxial relationships between these protons in the preferred conformation. The hydroxyl substituents are thus assigned to pseudoequatorial positions and are consistent with their *trans*-geometry defined by the acid-catalysed ring opening of the epoxide. ^{13}C nmr and HRMS data are also consistent with structure indicated.

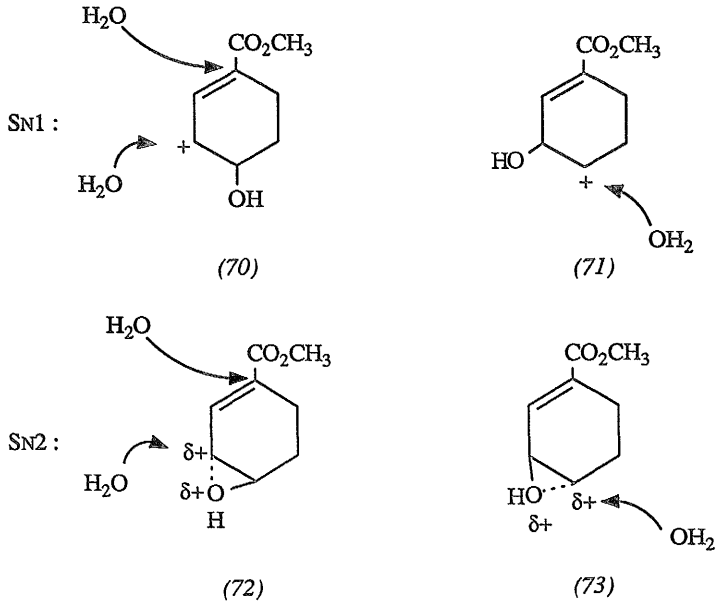
Whether the mechanism of the ring opening precedes by SN1 or SN2, attack of the



Scheme 3.8

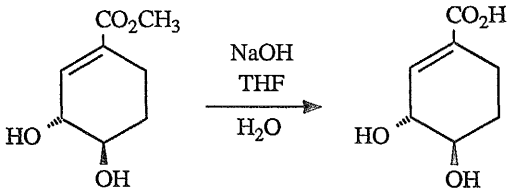
nucleophile (H_2O) can be envisaged at three different carbon centres: C-1, C-3 and C-4 (figure 3.8). However, no product derived from attack at C-1 was observed. The absence of this product might be a result of steric interference of the ester substituent, or from exclusive reaction at C-4 i.e. the lack of cationic charge at C-3. The former is likely to be correct as species *(71)* and *(73)* do not enjoy the resonance stabilisation of *(70)* and *(72)*, and therefore are less favourable. Hence the hydroxyl at C-3 is likely to be the one that is derived from bulk water.

Saponification of ester *(69)* with NaOH in a mixture of THF and water proceeded quantitatively to give the desired acid *(67)* (Scheme 3.9). The ^{1}H, ^{13}C nmr and HRMS data confirmed the loss of the methoxy group and was fully consistent with the desired product. The conformation (pseudo-axial or pseudo-equatorial) of the hydroxyl substituents in solution were analogous to those determined for the corresponding ester *(69)*. Detailed analysis of the solution conformation of *(67)* and other analogues is given in 3.2.9.



(70) (71) (72) (73)

Figure 3.8

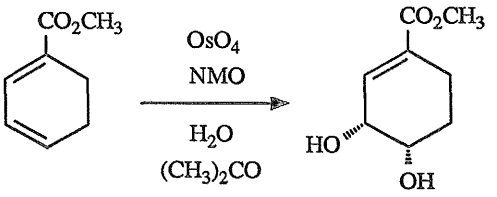


(69) (67)

Scheme 3.9

3.2.6 Preparation of *Cis*-diol Shikimate Analogue *(68).*

Oxidation of diene *(65)* with a catalytic amount of osmium tetroxide in the presence of N-methylmorpholine-N-oxide (NMO) in acetone and water[197] gave *cis*-diol *(75)* in 54% yield after work up (Scheme 3.10). Again the ^1H nmr spectra is consistent with the cyclohexene adopting a twisted chair structure in solution (see 3.2.9). Examination of the



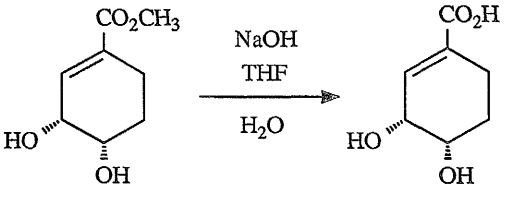
(65) *(74)*

Scheme 3.10

^1H vicinal coupling constants as in 3.2.5 indicate that the hydroxyl substituent at C-3 is pseudoequatorial and at C-4 is pseudoaxial in the preferred conformation. The pseudoequatorial conformation of the hydroxyl group at C-3 inferred by the nmr data is contrary to that observed in shikimic acid[187] despite the similar *cis* relationship of the hydroxyl funtionality in each case. ^{13}C nmr and HRMS data were also consistent with the structure indicated. The melting point of crystalline *cis*-diol *(74)* was found to be ~18°C lower than that of the *trans*-diol *(69)*.

Once again the regioselectivity of osmylation can be rationalised as for the epoxidation (3.2.2), on the grounds of the electron-withdrawing properties of the C-1 carboxylate group resulting in substantially reduced nucleophilicity of the C-1, C-2 olefin.

Saponification of *(74)* as in 3.2.5 was effective in the removal of the methoxy group (scheme 3.11), and ^1H, ^{13}C nmr and HRMS data confirmed the maintenance of



(74) *(68)*

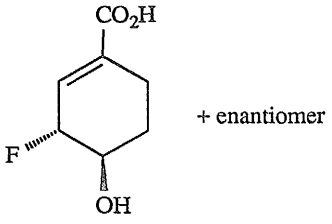
Scheme 3.11

conformation described. Detailed analysis of the solution conformation of *(68)* is given in

3.2.9.

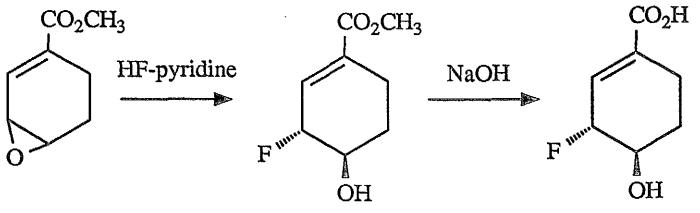
3.2.7 Target Shikimate Analogue *(51)*: A Competitive Inhibitor.

As described in chapter 2 the fluorinated shikimate analogue *(51)* was selected as a rational target as a potential competitive inhibitor for the use in positional isotope exchange studies.



(51)

3.2.8 Preparation of Fluoro Shikimate Analogue *(51)*.



(52) *(53)* *(51)*

Scheme 3.12

Reaction of the epoxide *(52)* with HF-pyridine reagent[198] in ether gave the fluorohydrin *(53)* as a single racemic diastereoisomer (scheme 3.12). The acid catalysed ring-opening occurred >85% regioselectively as determined by a single major peak in the decoupled ^{19}F nmr. The product *(53)* was purified by flash chromatography (yield 74%). The regioselectivity of the ring-opening of the epoxide at C-3 was confirmed by the large ^{2}J ^{19}F-^{1}H and ^{1}J ^{19}F-^{13}C coupling constants in the ^{1}H, and ^{13}C nmr. The reasons for the

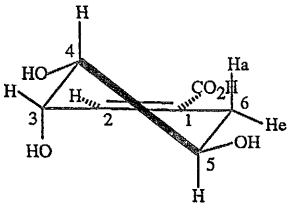
observed regioselectivity are analogous to those already described with respect to the hydrolysis of the epoxide *(52)* (3.2.5). The atomic composition was confirmed by high resolution mass spectrometry.

Extremely careful saponification by slow titration with 0.1M NaOH in an aqueous mixture of the ester *(53)*, followed by careful acidification, extraction into ether and purification *via* flash chromatography (yield 58%) gave the analogue *(51)* which was characterised by ^{19}F, ^{1}H and ^{13}C nmr and high resolution mass spectra. As in the case of the *trans*-diol *(67)* the ^{1}H nmr data indicates that the fluoro group adopts a pseudo-equatorial position in the preferred conformation (see 3.2.9).

3.2.9 ^{1}H Nmr of Shikimate Analogues *(67)*, *(68)* and *(51)*.

The half-chair conformation is that preferred by the majority of cyclohexenes.[13] Hall[187] used the ^{1}H nmr spectrum of (-)-shikimic acid to determine the preferred conformation in aqueous solution. Similarly the preferred conformations were determined for analogues *(67)*, *(68)* and *(51)*. The orientation of hydroxyl and fluoro functionalities, either pseudoaxial or pseudoequatorial, are determined on the basis of identification of diaxial relationships between vicinal protons. Application of the Karplus equation indicates that large vicinal coupling constants (7-13 Hz) correspond to a dihedral angle between the protons of ~180° i.e. approximately diaxial.

Thus, in the case of (-)-shikimic acid[187] (figure 3.9) the data ($J_{4,5}$= 8.4 Hz) indicates such a pseudodiaxial relationship between protons at C-4 and C-5. The deduction that the proton at C-4 is pseudoaxial assigns the hydroxyl group at this carbon in a pseudoequatorial position. The *cis* relationship between the hydroxyl groups at C-3 and C-4 assigns the hydroxyl group at C-3 to a pseudoaxial position. The smaller vicinal coupling constant (3.9 Hz) between the pseudoaxial and pseudoequatorial protons at C-3 and C-4 is consistent with this assignment.



J values (Hz)

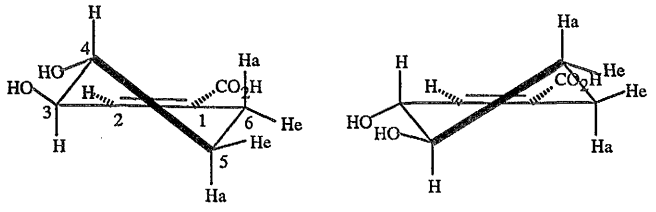
$J_{2,3}$	$J_{2,6}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6a}$	$J_{5,6e}$	$J_{6a,6e}$
4.0	1.8	3.9	8.4	6.2	5.0	18.5

Figure 3.9

Furthermore it would seem reasonable that the conformation shown would be the favoured one as only one hydroxyl group adopts a pseudoaxial position thus minimising 1,3 diaxial interactions. Ring-flipping the shown conformation results in two of the three hydroxyl groups adopting pseudoaxial positions.

Similarly assignments of the position of protons and subsequently hydroxyl groups can obtained from the ^{1}H nmr spectra of shikimic acid analogues *(67)*, *(68)* and *(51)*. The preferred conformation of both enantiomers of *trans*-diol *(67)* is shown in figure 3.10. The large vicinal coupling constant (J=10.8) between protons at C-4 and C-5 indicate a diaxial relationship. Thus as with the analysis of shikimic acid, the assignment of the proton at C-4 as pseudoaxial infers the hydroxyl at this carbon assumes a pseudoequatorial position. The *trans* geometry of the diol, achieved by acid catalysed ring opening of an epoxide, thus assigns the hydroxyl group at C-3 to be also pseudoequatorial. This assignment is consistent by the relatively large vicinal (J= 7.4 Hz) between C-3 and C-4 protons indicating a pseudodiaxial relationship.

In contrast to the *trans*-diol *(67)*, the *cis*-diol *(68)* displays vicinal coupling constants (J= 3



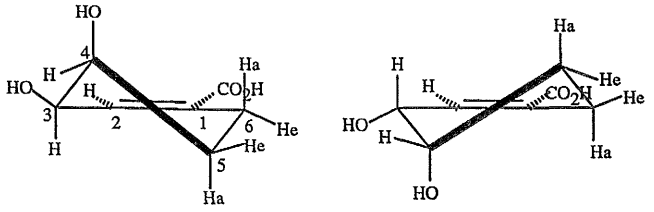
J values (Hz)

$J_{2,3}$	$J_{2,6}$	$J_{3,4}$	$J_{4,5a}$	$J_{4,5e}$
2.7	1.5	7.4	10.8	3.8

$J_{5a,6a}$	$J_{5a,6e}$	$J_{5a,5e}$	$J_{5e,6}$	$J_{5e,6}$
9.2	6.5	13.1	5.3	3.8

Figure 3.10

and 6 Hz) between protons at C-4 and C-5 that indicate that the proton at C-4 is pseudoequatorial and thus the hydroxyl at this carbon occupies the pseudoaxial position. The *cis* geometry of the diol, achieved by oxidation with osmium tetroxide, assigns the hydroxyl group at C-3 in pseudoequatorial position. A vicinal coupling constant of 3 Hz



J values (Hz)

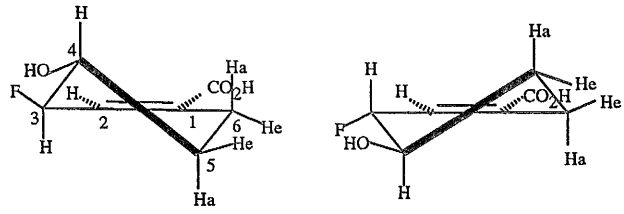
$J_{2,3}$	$J_{2,6}$	$J_{3,4}$	$J_{4,5a}$	$J_{4,5e}$	$J_{5a,6a}$
3.6	1.8	3	3	6	6.3

$J_{5a,6e}$	$J_{5a,5e}$	$J_{5e,6a}$	$J_{5e,6e}$	$J_{6a,6e}$
5.5	14	6.3	7.5	18

Figure 3.11

between protons at C-3 and C-4 corresponds to a diequatorial assignment. The preferred conformation of both enantiomers of *(68)* are given in figure 3.11.

^{1}H nmr data of *trans*-fluoro hydrin *(51)* is comparable to that of the *trans*-diol *(67)* with very similar or equivalent vicinal coupling constants (figure 3.12). Assignment of the favoured conformation is therefore equivalent with the fluoro group at C-3 and the hydroxyl at C-4 both being assigned to pseudoequatorial positions.



J values (Hz)

$J_{2,3}$	$J_{2,6}$	$J_{3,4}$	$J_{4,5a}$	$J_{4,5e}$	$J_{5a,6a}$
2.6	2.6	7.0	11.0	4.0	9.5
$J_{5a,6e}$	$J_{5a,5e}$	$J_{5e,6a}$*	$J_{5e,6e}$*	$J_{6a,6e}$*	
6.3	13.4	8	6	18	

Figure 3.12 * data taken from corresponding ester *(53)*

To summarise it is apparent that the analogues *(67)*, *(68)* and *(51)* all have favoured conformations in which the functional group (either hydroxyl or fluoro) at C-3 occupies a pseudoequatorial position. This is in contrast to shikimic acid where the hydroxyl at C-3, which is phosphorylated in the enzymic reaction, occupies a pseudoaxial position. Despite *cis*-diol analogue *(68)* having the same geometry of functional groups about C-3 and C-4 as the native molecule a different favoured conformation is apparent. Thus, assuming the conformation of shikimic acid determined in solution is the one that is bound at the active site of the enzyme, it might be reasonable to suggest that the C-5 hydroxyl might be of

greater importance for catalysis than simply the contribution of a single hydrogen bond for binding at the active site.

3.2.10 Substrate Properties of Analogues *(67)* and *(68)*.

Analogues *(67)* and *(68)* were assessed for their ability to act as substrates for shikimate kinase. The rate of catalysis V was observed, at fixed concentration of enzyme and ATP, with *trans*-diol analogue *(67)* in the range 0.5 - 10 mM and with *cis*-diol analogue *(68)* in the range 0.2 - 10 mM. The results are expressed as plots of $\frac{1}{V}$ against $\frac{1}{[S]}$ (Lineweaver-Burk plots)[199] (figures 3.13 and 3.14). The straight lines observed are

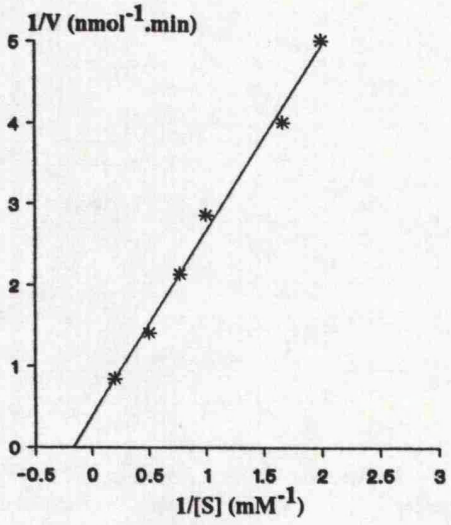


Figure 3.13 Subtrate properties of *trans*-diol analogue *(67)*

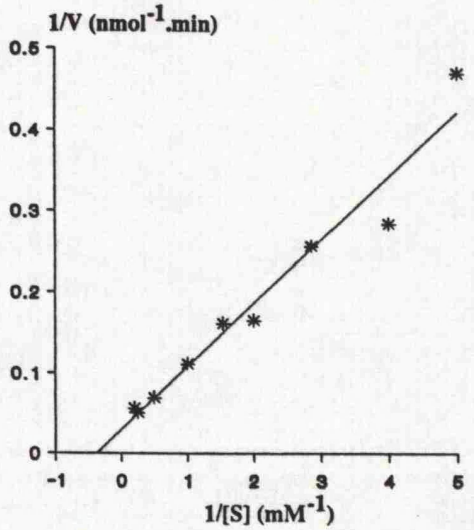
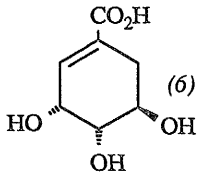
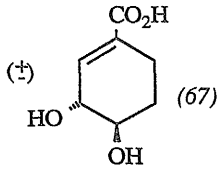
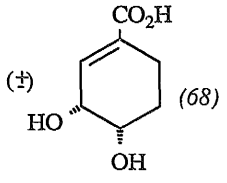


Figure 3.14 Substrate properties of *cis*-diol analogue *(68)*

characteristic for a reaction obeying Michaelis-Menten (or saturation) kinetics. The intercept on the y axis gives $\frac{1}{V_{max}}$ and the intercept on the x axis gives $\frac{-1}{K_M}$. The slope of the line is $\frac{K_M}{V_{max}}$. k_{cat} was calculated from $k_{cat}= \frac{V_{max}}{[E]_o}$. The parameters determined were compared directly to those determined for the natural substrate, shikimic acid *(6)*, under similar conditions which are comparable to those determined by Coggins *et al.*[54] and

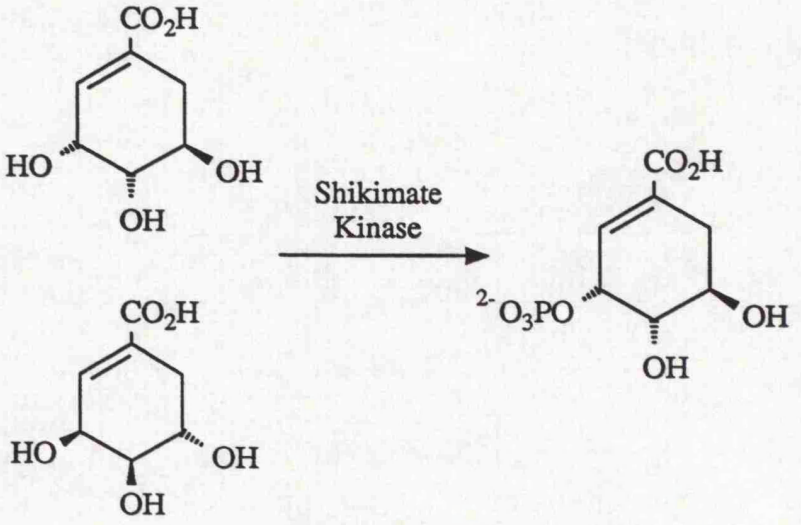
Defeyter *et al.*[151] Values of K_M and k_{cat} are given in table 3.1.

Substrate	K_M mM	V_{max} μmol.min^{-1}	k_{cat} s^{-1}	k_{cat} / K_M M^{-1}s^{-1}
 *(6)*	0.19	0.35	103	5.4 x 10^5
 *(67)*	5.7 (2.9)	2.5 x 10^{-3}	0.07	1.2 x 10^1 (2.4 x 10^1)
 *(68)*	2.3 (1.2)	28.9 x 10^{-3}	0.9	3.9 x 10^2 (7.5 x 10^2)

Values in parentheses are corrected for enantiomeric specificity.

Table 3.1

Analogues *(67)* and *(68)* are both observed to be substrates for shikimate kinase, although in each case with significant loss in specificity. As the analogues are enantiomeric mixtures it was of interest to investigate whether the enzyme was enantioselective for one enantiomer in favour of the other to such an extent that the observed rate of catalysis approximates to the rate of catalysis of one enantiomer only. The use of *E.coli* shikimate kinase by Abell and Balasubramanian[200] to resolve racemic shikimate to give homoshikimate phosphate (scheme 3.13) suggested that this might be likely. The possibility that shikimate kinase was



Scheme 3.13

enantioselective towards analogues *(67)* and *(68)* was investigated by conducting single assays in which a quantitative amount of analogue was added that would theoretically result in the reduction of the absorbance of NADH at 340 nm by 1 AU assuming that both enantiomers were substrates and 0.5 AU if only one enantiomer is catalysed (figure 3.15).

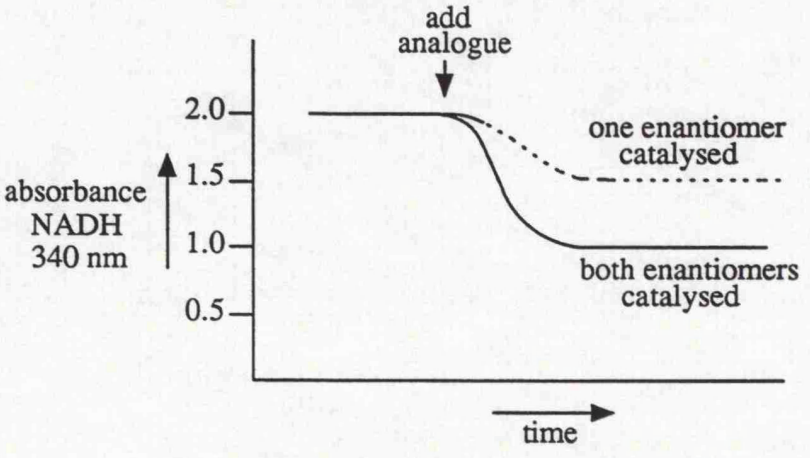


Figure 3.15

In the case of *cis*-diol *(68)*, the standard assay mixture contained approximately 15 units of shikimate kinase activity and traces were corrected for background turnover of ATP in the absence of cosubstrate. The observed turnover for a series of five additions was found to equate to approximately 48% of the analogue added. This suggested that only one

enantiomer is phosphorylated significantly, presumably that enantiomer in which the hydroxyl substituents are in the α position (as in (-)-shikimate). Analysis of the *trans*-diol analogue *(67)* proved impossible due its poor ability to act as substrate combined with the observation that at very high concentrations of enzyme (>30 units), background turnover of ATP comparable to the slow turnover of analogue *(67)*. The source of this background activity might be shikimate kinase acting as a weak ATPase or arise from trace contaminant protein. The identity of the activity was not investigated further.

The observed enantioselectivity is assumed to be in favour of that enantiomer in which the C-3 hydroxyl substituent is in the α position as it is in the native substrate. However, it could be argued that in the case of *trans*-diol *(67)* the C-3(βOH) analogue (where C-4 OH is α) would be preferred since the interconversion of pseudoaxial and pseudoequatorial substituents are of greater significance at the C-4, rather than the C-3 positions of a cyclohexene ring. That is the effect of changing the C-4 hydroxyl from α→β is greater than the effect of changing the C-3 hydroxyl from α→β and thus the C-3(βOH), C-4(αOH) enantiomer of analogue *(67)* most closely resembles the native substrate (-)-shikimic acid. These arguments are further complicated by the possibility that the conformation of the substrate (and/or analogues) on binding to the active site of the enzyme.

As discussed in the introduction to this chapter the measurement of apparent second order rate constants (k_{cat}/K_M) with natural and analogues of the substrate allows the estimation of the loss in apparent binding energy (ΔG_{app}).[186] The ΔG_{app} for the C-5 hydroxyl group of shikimic acid can thus be calculated. The ΔG_{app} determined from analysis of *cis*-diol analogue *(68)* is 3.9 kcal mol^{-1}. This value is consistent with values determined by Fersht,[186] Withers,[179,180,182] and others[181] for hydrogen bond interactions at the transition-state. Comparison to these values suggests that the C-5 hydroxyl group partakes in a single strong or several weak bonds in the transition-state complex. The magnitude of ΔG_{app} is such that the involvement of charged partners can not be eliminated.

Similarly ΔG_{app} for *trans*-diol *(67)* can be determined (5.9 kcal mol^{-1}). Assuming that the enzyme is enantioselective towards this analogue and the enzyme preferentially binds to the enantiomer with C-3(αOH) then the difference between this value and ΔG_{app} for *(68)* will represent the loss or gain in specificity resulting from a change in stereochemistry of the C-4 hydroxyl substituent of α to β (down to up). The loss of 2 Kcal mol^{-1} resulting from this change might have resulted from the absence of a relatively weak hydrogen bond.

Withers has determined ΔG_{app} for ground state interactions for the enzyme Glycogen Phosphorylase[179,180] by the comparison of dissociation constants (K_i and K_M) of non-catalysable substrate analogues that bind to the ground-state and also by comparison of dissociation constants (K_M' and K_M) of substrate analogues that are catalysed. Despite the unknown complications that might interfere with the latter determination Withers found that the values were broadly comparable.

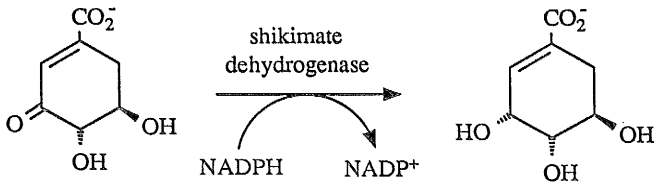
Determination of ground-state interactions of the C-5 hydroxyl group *via* comparison of dissociation constants of analogues and substrate gives ΔG_{app} = 1.1 Kcal mol^{-1} (from analogue *(68)*) and ΔG_{app} = 1.6 Kcal mol^{-1} (from analogue *(67)*). Thus it might be concluded that the C-5 hydroxyl group is bound by a neutral hydrogen bond in the ground state, but the strength or number of bonds increases as the transition state is achieved. This, however tentative, is consistent with the theory that an enzyme catalyses a reaction by binding to the transition state preferentially.[99]

As discussed in the introduction to this chapter, ΔG_{app} is a composite of energies of specific bonds that have been modified and includes the term ΔG_{reorg} which is assumed to be relatively small. However, the ^1H nmr analysis (3.2.9) of analogues *(67)* and *(68)* as compared to similar analysis of shikimic acid indicates that the removal of the C-5 hydroxyl substituent has a significant effects the preferred conformation of the hydroxyl substituents.

This may result in the weakening of interaction between hydroxyl substituents at C-3 and C-4, and/or increase in the contribution of ΔG_{reorg}. Thus ΔG_{app} probably overestimates the contribution of the C-5 hydroxyl group.

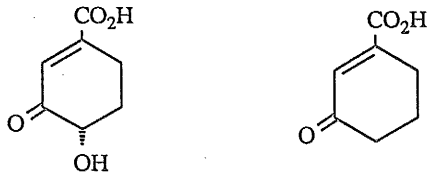
However, the preferred conformation of shikimic acid is not necessarily the conformation that binds shikimate kinase in the ground-state or transition-state. Indeed distortion of the ring via more favourable interactions to achieve the transition-state has been observed for a number of enzymes e.g. shikimate dehydrogenase.[41] If was the case ΔG_{app} might underestimate the contribution assigned to a particular substituent since ΔG_{reorg} might be lower for an analogue with desired transition-state conformation than the native substrate which has to undergo a distortion in conformation to achieve the transtition-state. In the absence of experimental evidence suggesting that the transition-state involves such a distortion ΔG_{app} must be considered an overestimate of the strength of the hydrogen bond deleted.

The observation of an amino acid motif amongst enzymes of the shikimate pathway[55] implies that the binding and/or mechanism of the intermediates of the pathway may also hold common similarities. The contribution of hydroxyl substituents of 3-dehydroshikimic acid *(5)* towards its catalysis by *E.coli* shikimate dehydrogenase have been determined by Abell *et al.*[176] Shikimate dehydrogenase catalyses the reduction of *(5)* to shikimic acid *(6)* (figure 3.16). Abell synthesised deoxy analogues *(75)* and *(76)* and determined their apparent binding energies from k_{cat}/K_M. The C-5 hydroxyl was found to contribute 0.4 kcal mol^{-1} (ΔG_{app} for analogue *(75)*), whereas the C-4 hydroxyl group ΔG_{app} = 7 kcal mol^{-1} (ΔG_{app} for *(76)* - ΔG_{app} for *(75)*). Abell concluded that the C-5 hydroxyl substituent contributed little to specificity, while the C-4 hydroxyl group was very significant and probably involved in a hydrogen bond with a charged residue at the active site. Thus despite the possible presence of common binding motifs in shikimate kinase and shikimate dehydrogenase it would seem that the C-5 hydroxyl group is of greater importance to



(5) *(6)*

Substrate analogues:



(75) *(76)*

Figure 3.16

shikimate kinase than dehydrogenase.

To summarise, shikimate kinase has been shown to phosphorylate deoxy analogues of shikimic acid and in at least in one case enantiospecifically. Analysis of Michaelis-Menten parameters has indicated a maximum energetic contribution of the C-5 hydroxyl substituent of shikimic acid. Evidence is presented that suggests that the hydroxyl substituents not involved directly in the phosphorylation reaction contribute significantly to binding and catalysis. In addition to providing a site for hydrogen bonding the C-4 and C-5 hydroxyl substituents profoundly influence the preferred conformations of the ligands. The influence this has on binding has been discussed. The relative energetic contribution of the C-5 hydroxyl group has been discussed and its importance compared with similar interactions in shikimate dehydrogenase observed by Abell and coworkers.

3.2.11 Inhibition Properties of Fluoro Analogue *(51)*.

Fluoro analogue *(51)* was designed and synthesised to be a competitive inhibitor of shikimate kinase for the use in positional isotope exchange studies (see chapter 2). A brief investigation (data not shown) using the standard assay procedure confirmed that *(51)* was not a substrate for the enzyme. To determine whether analogue *(51)* was an inhibitor of the enzyme, and if so the type of inhibition, Michaelis-Menten parameters were determined for shikimic acid in the normal way but in the presence of fluoro analogue *(51)*. The rate of catalysis *V* was observed at fixed concentrations of enzyme and ATP with shikimic acid in the range 0.07-0.5 mM at concentrations of analogue *(51)* of 0, 5, 10 and 30 mM. The results were expressed by Lineweaver-Burk plots[199] (figure 3.17).

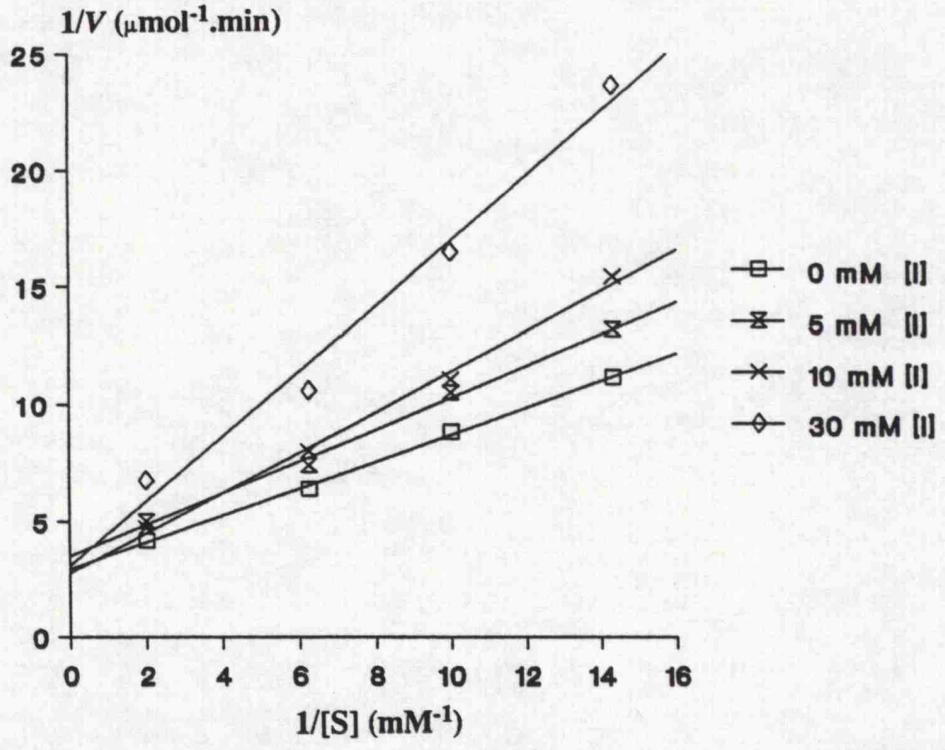


Figure 3.17 Inhibition with *fluoro* analogue *(51)*

The Lineweaver-Burk plots describe a pattern that are characteristic of a fully competitive reversible substrate inhibitor;

(i) V_{max} given by the intercept on the y axis, remains constant at different concentrations of inhibitor i.e. it is possible to drive all the inhibitor from the enzyme converting it to enzyme substrate complex by addition of sufficient substrate.

(ii) The apparent K_M increases with increasing concentrations of inhibitor because as [I] increases more substrate is required to reach V_{max} and hence more is required to reach half V_{max} i.e. K_M.

The simple Michaelis-Menten kinetics describing such an inhibitor are given by figure 3.18

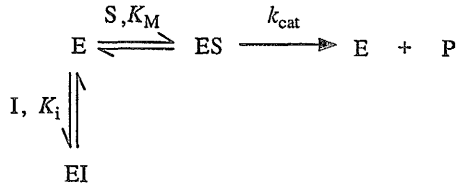


Figure 3.18

where K_i is the dissociation of the enzyme-inhibitor complex. K_i was determined graphically[195] by a plot of the slope (V/[S]) of individual Lineweaver-Burk plots against [I], the intercept on the x axis yielding K_i = 21 mM (figure 3.19). Thus fluoro-analogue *(51)* appears to be a relatively poor competitive inhibitor, however with respect to the evidence suggesting that shikimate kinase is selective towards enantiomers with the C-3 hydroxyl substituent in the α position it might be reasonable to suggest that the enzyme exhibits a degree of enantioselectivity towards analogue *(51)*. In the extreme case where the enzyme does not demonstrate any affinity towards one enantiomer the K_i would be half that observed.

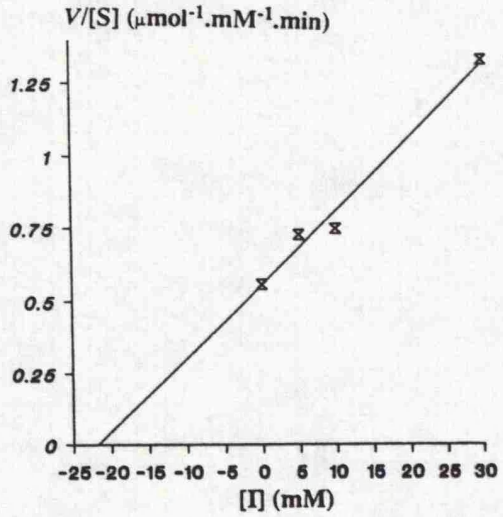


Figure 3.19 Dermination of K_i of *fluoro*-analogue *(51)*

The reduction in affinity resulting from the replacement of a hydroxyl group with fluorine has been measured for a number of enzyme/ligand complexes.[179,180,182,184] These observations suggest that the loss in affinity varies considerably and in some cases a 60-fold reduction is observed. Comparison of the K_i of *(51)* to the K_M' of analogue *(67)* since this represents a single mutation of OH→F indicates only an about 4 fold loss in affinity. A quantitative measure of the loss of affinity may be very roughly estimated by determination of ΔG_{app} using the apparent dissociation constant (K_M') of *(67)* and inhibitor constant (K_i) of *(51)*. Assuming that the enantiospecificity of the enzyme is equivalent for each analogue, then the apparent loss of affinity is 0.8 kcal mol^{-1} suggesting a relatively weak hydrogen bond between the C-3 hydroxyl group and the enzyme in the ground-state complex.

3.3 Summary.

In order to probe the active site of shikimate kinase, analogues of shikimic acid have been synthesised and their ability to act as substrates or inhibitors tested. An epoxy-shikimate analogue was found to be a weak active-site irreversible inhibitor. Substrate protection experiments indicated that ATP active site was modified. Deoxy-shikimate analogues were found to be substrates of the enzyme but with markedly different turnover rates. The C-5

hydroxyl group of shikimic acid was found to be significant for hydrogen bond interactions with the protein, and in determining the preferred conformation of the molecule. The substitution of the C-3 hydroxyl substituent with a fluorine atom generated a competitive inhibitor of the enzyme. The loss of affinity resulting from this mutation was observed to be low.

CHAPTER 4

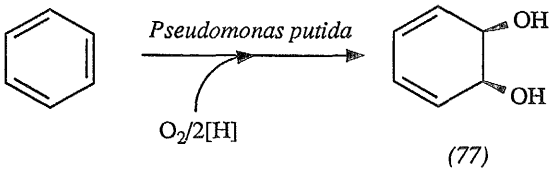
Synthesis and Specificity of Shikimic acid Analogues.

Strategy 2

4.0 Introduction.

In the previous chapter, analogues of shikimic acid were prepared and assayed to measure the relative importance of hydroxyl substituents for the specificity of the substrate. The presence of the C-5 hydroxyl group was found to be important for catalysis, and for the determination of the preferred conformation of the cyclohexene ring in solution. The enzyme was also observed to exhibit enantioselectivity for the racemic substrate analogues. It is the purpose of this chapter to examine precisely the stereochemical requirements of hydroxyl substituents at C-3 and C-4 for catalysis.

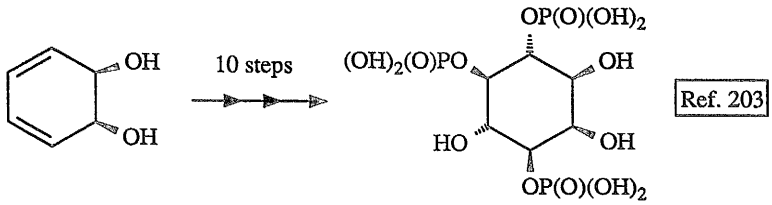
In 1968 Gibson and coworkers reported the microbial oxidation of benzene to *cis*-3,5-cyclohexadiene-1,2-diol *(77)* in large quantities using *Pseudomonas putida*[201] (scheme 4.0). The reaction proceeds under extremely mild conditions and has been shown



(77)

Scheme 4.0

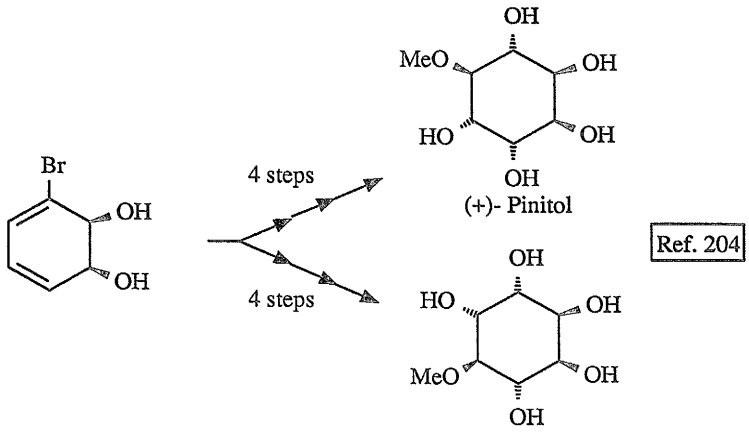
to tolerate a variety of functional groups attached to the benzene ring. When the plane of symmetry in the molecule is destroyed by the presence of a substituent, optically active products are obtained.[202] The significance of the biotransformation derives from the fact that it has made available, to the synthetic chemist, a range of chiral building blocks currently unavailable from synthetic chemistry alone. The diene diols have found to be well suited to subsequent functionalisation and several research groups have exploited their availability to synthesise a diverse range of highly functionalised chiral products with biological activity (figure 4.0).



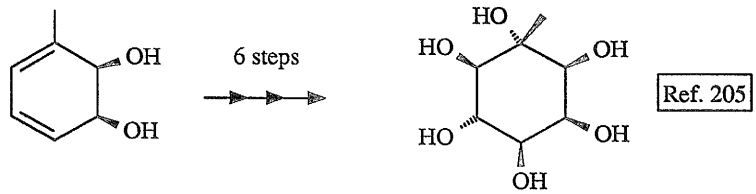
Ref. 203

D-*myo*-inositol 1,4,5 -triphosphate

(IP_3)



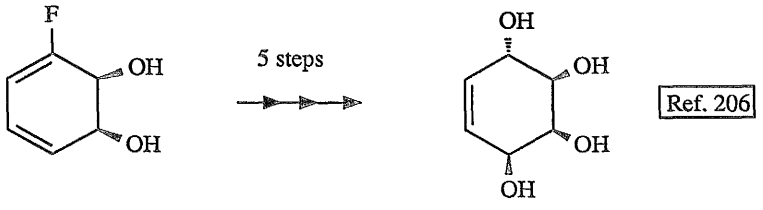
Ref. 204



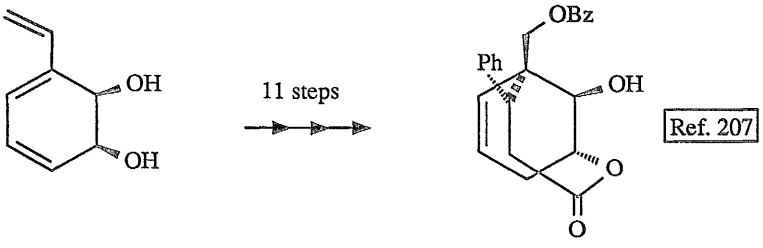
Ref. 205

(-)- lamitol

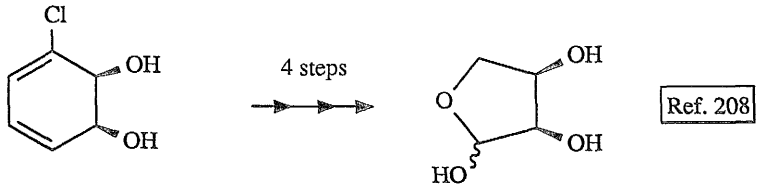
Figure 4.0



(+)- Conduritol C



(-)- Zeylena



2,3 -O-isopropylidene D-erythrose

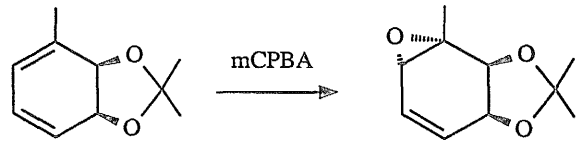
Figure 4.0 cont.

The versatility of diene diols such as those shown results from several characteristics conferred by these molecules:

(1) As is demonstrated by the examples shown, many natural product cyclitols contain the *cis*-diol moiety which is established in the biotransformation step.

(2) The diene permits straightforward functionalisation by employing precisely defined osmylation and epoxidation sequences.

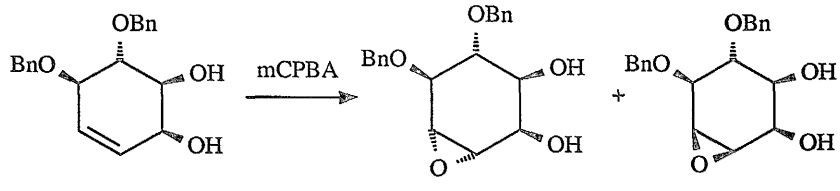
(3) Functionality at C-1 allows for differentiation of the two double bonds by approaching reagents. Regioselectivity results from the difference in the nucleophilicity of the conjugated double bonds induced by the C-1 functionality. Steric interference between the substituent at C-1 and the approaching reagent may also aid selectivity. Epoxidation of diene *(78)* with mCPBA is highly regioselective (and stereoselective) for the trisubstituted double bond[205] (scheme 4.1). Here the greater nucleophilicity of the trisubstituted double bond resulting from the electron donating methyl group determines the regioselectivity of the epoxidation.



(78)

Scheme 4.1

(4) The diol can be used to achieve stereoselectivity by complexing to subsequent reagents, for example treatment of diol *(79)* with mCPBA gives the epoxides *(80)* and *(81)* in a 1:9 ratio (scheme 4.2).[203] Complexation between the reagent and the



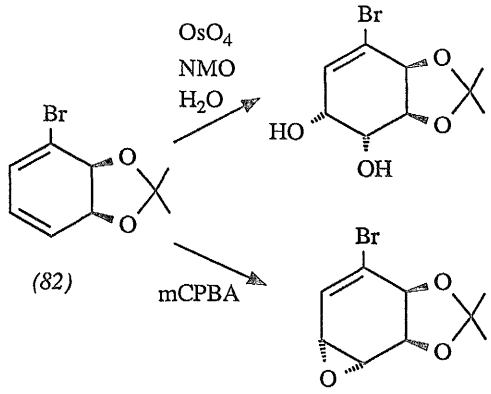
(79) *(80)* *(81)*

Scheme 4.2

hydroxyl substituents directs the peroxycarboxylic acid to the upper face of the

molecule.

(5) Protection of the *cis*-diol with a rigid protection group such as acetonide confers facial selectivity during subsequent transformation. Osmylation or epoxidation of the acetonide *(82)* occurs with complete facial selectivity from the lower face[204] (scheme 4.3). The acetonide group prevents attack from the upper side by sterically blocking the path of the reagent to this face. Complete regioselectivity is also observed.



Scheme 4.3

4.1 Aims.

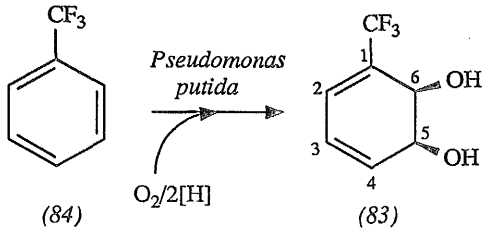
This chapter aims to exploit the versatility of diene diols to develop enantiospecific syntheses of shikimic acid analogues with altered stereochemistry of the C-3 and C-4 hydroxyl groups. In a similar manner to that described in the previous chapter, kinetic data derived from the catalysis or inhibition demonstrated by these analogues will allow the quantitative determination of the stereochemical importance of the hydroxyl substituents. In addition, the availability of various substituted diene diols will also enable the investigation of the ability of the enzyme to tolerate an additional hydroxyl group at C-6 and the substitution of the carboxylate group for that of a trifluoromethyl group. The

trifluoromethyl group was also of special interest because it would provide an extremely sensitive nmr probe. ^{19}F nmr chemical shifts have been shown to be particularly sensitive to local environment e.g. ref. 209. Furthermore Hammond[210] has shown that the ^{19}F-^{1}H through-space interactions can provide information about the nature and indentity of the amino acid side chains in close proximity to the ^{19}F probe. The design preparation, assaying and interpretation of kinetic data of such analogues will be described.

4.2 Results and Discussion.

4.2.1 Target Shikimate Analogues from *Cis*-Dihydrodiol *(83)*.

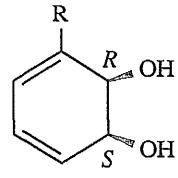
Compound *(83)* is available in multigram quantities from the microbial oxidation of trifluoromethylbenzene *(84)* (scheme 4.4). Compound *(83)* was a gift of Shell UK Ltd. Diol



(84) *(83)*

Scheme 4.4

(83) has been used as part of a series of substituted cyclohexadiene-1,2-*cis*-diols to investigate various cycloaddition reactions,[211-213] however subsequent functionalisation to introduce further oxygen functionality has not been reported heretofore. The enantiomeric excess of *(83)* has not been determined, but can be assumed by analogy. The importance of the substituent in determining the regio- and stereoselectivity of the bacterial oxidation of aromatic hydrocarbons by *P. Pudita* has been studied extensively.[202,214-216] In all cases where the absolute stereochemistry of the oxidation of monosubstituted benzenes (figure 4.1) has been determined the hydroxylated carbons were found to be (*R*) *ortho* to the



R = CH_3, F, Cl, Br, CH_2CH_3, C_6H_5

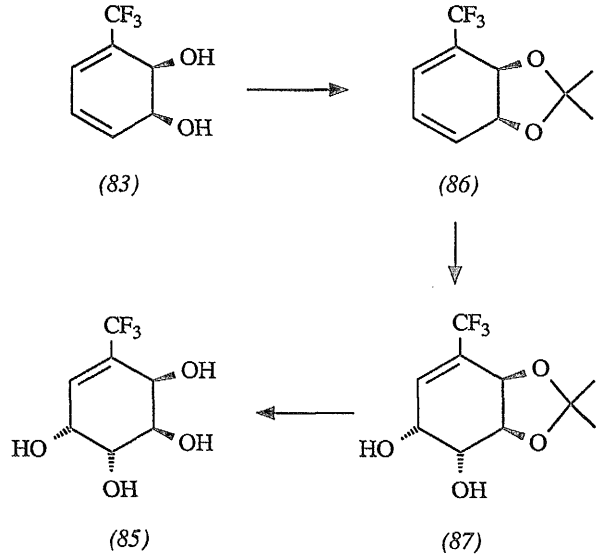
Figure 4.1

substituent and (*S*) *meta*. Furthermore, the enantiomeric excess determined for each

biotransformation is found to be ≥ 98%.

It was envisaged that the application of precisely defined osmylation/epoxidation procedures to the *cis*-dihydrodiol *(83)* will permit regioselective functionalisation of the C-3, C-4 double bond. Furthermore it was also envisaged that protection or non-protection of the *cis*-diol also will permit stereoselective functionalisation of this double bond leading to a series of tetrols of high enantiopurity with stereochemistries at C-3 and C-4 either the same as shikimic acid or inverted by appropriate choice of hydroxylation sequence. Since the tetrols would also differ from shikimic acid by mutations at C-1 and C-6 the initial target will be synthesis of an analogue with equivalent stereochemistry to that observed in shikimic acid. Thus such an analogue will provide a standard by which to measure the effect changing the stereochemistry of the hydroxyl groups.

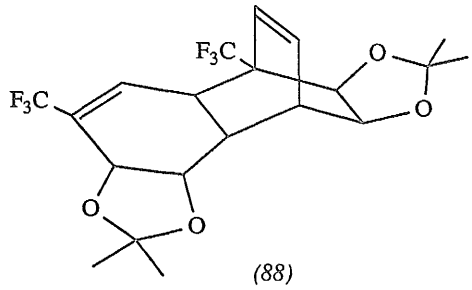
4.2.2 Preparation of *RRRR* analogue *(85).*



(83) *(86)* *(85)* *(87)*

Scheme 4.5

Treatment of *cis*-dihydrodiol *(83)* with excess dimethoxypropane in the presence of *p*-toluene sulphonic acid gave the acetonide *(86)* (scheme 4.5). *(86)* was purified by flash chromatography in 87% yield and was characterised by ^{1}H and ^{13}C nmr spectroscopy. As compared to the starting material *(83)* the ^{1}H nmr spectrum contains two additional 3H singlets (δ 1.43 and 1.42 ppm) corresponding to the acetonide methyl groups and the ^{13}C nmr spectrum contains three additional resonances (δ 106.4, 26.6 and 25.1 ppm) corresponding to the $C(CH_3)_2$ and $C(CH_3)_2$ carbon atoms respectfully. Repeated attempts to obtain observe a molecular ion corresponding to *(83)* in the HRMS failed, however the MH^+ at 441.15 was found to be consistent with the presence of dimer *(88)* (figure 4.2).



(88)

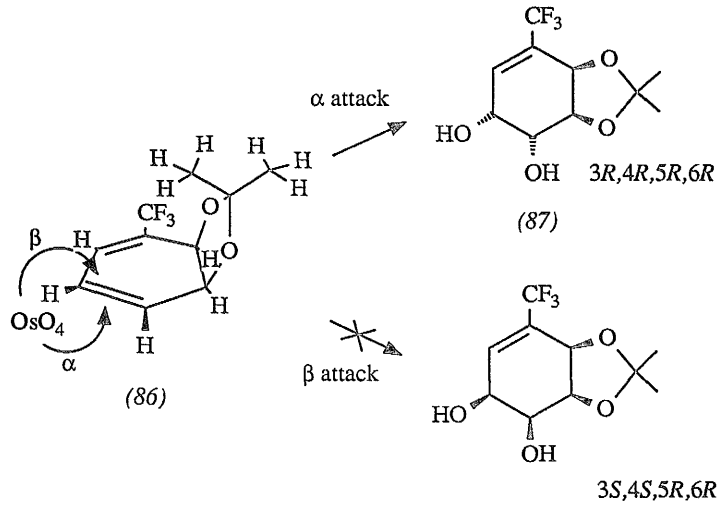
Figure 4.2

Dimerisation of the similar diene acetonide *(82)* has been reported and indentified by X-ray chromatography.[217] As in this case dimerisation was retarded by storage in a dilute solution at -20°C.

Oxidation of the diene *(86)* was achieved with a catalytic amount of osmium tetroxide in the presence of N-methylmorpholine-N-oxide (NMO) in acetone and water.[197] Purification by flash chromatography and recrystallisation gave the *cis* diol *(87)* in 64% yield and was characterised by ^{1}H nmr, ^{13}C nmr spectroscopy and HRMS. The regioselective functionalisation of the C-3, C-4 double bond was identified by the observation of one olefinic proton (2-H) coupled to each of the three fluorine atoms (^{4}J = 1.3 Hz) in the ^{1}H nmr. Signals at δ 4.46 and 4.20 ppm were consistent with protons sited geminal to hydroxyl

substituents. A broad singlet at δ 3.60 ppm corresponded to the presence of two hydroxyl groups. The ^{13}C spectrum was also consistent with this assignment displaying a quartet at δ 133.4 ppm ($J_{C,F}$ = 5.2 Hz) corresponding to the sp^2 carbon C-2 and signals at δ 75.3, 70.0, 69.0 and 65.3 ppm corresponding to sp^3 carbons attached to oxygen atoms. The regioselective functionalisation of the C-3, C-4 double bond is consistent with that observed in the previous chapter (3.2.5 and 3.2.6) where the C-1 substituent of the cyclohexadiene ring is also an electron withdrawing group. As discussed in the previous chapter the selectivity is due to the greater nucleophilicity of the C-3, C-4 double bond. Steric interference between the CF_3 substituent and the approaching osmium tetroxide may also aid selectivity.

The observation of only one set of signals in both the ^1H and ^{13}C nmr indicated that the reaction had proceeded stereoselectively. Reaction from both the lower and upper face of the molecule would have led to a mixture of diastereoisomers (scheme 4.6) and thus distinguishable in the nmr spectra. The stereoselectivity demonstrated by osmium tetroxide

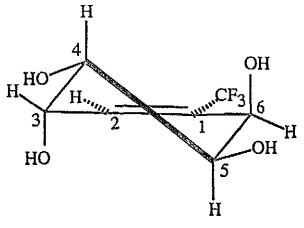


Scheme 4.6

is well documented and occurs from the less hindered side of the double bond.[204,218,219]

Simple molecular models suggest that the bulky acetonide group is sited well over the upper (β) face of the cyclohexadiene ring thus impeding approach from the reagent at this face. The identity of the diastereoisomer is therefore that resulting from attack of the reagent from the lower α face i.e. *cis* diol *(87)*.

Prolonged treatment of the acetonide *(87)* with Dowex (H^+ form) in a solution of acetonitrile and water gave the tetrol *(85)* in quantitative yield. Deprotection was confirmed by ^1H nmr, ^{13}C nmr and HRMS. Comparison of vicinal coupling constants in the ^1H nmr with those obtained for shikimic acid,[187] indicate that the molecule correspondingly adopts a twisted chair with the hydroxyl substituents adopting the positions shown (figure 4.3) in the preferred conformation. The relatively large vicinal coupling constant between protons



J values (Hz)

$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$
3.9	4.1	9.3	3.8
(4.0)	(3.9)	(8.4)	(5.0)

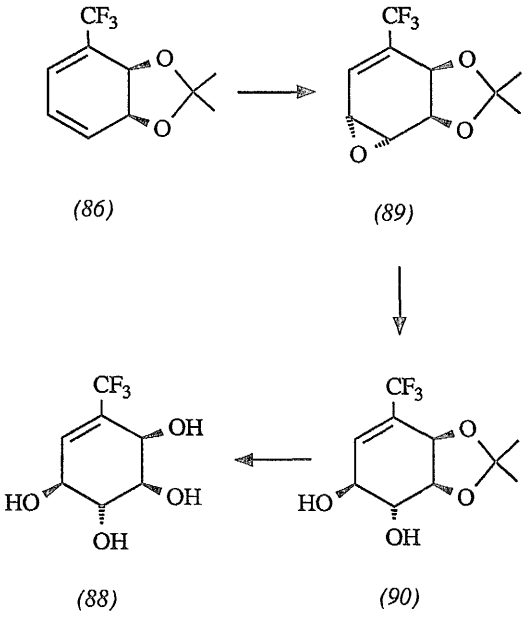
Figure 4.3

Values in parentheses are the corresponding vicinal coupling constants of shikimic acid as recorded by Hall[187]

at C-4 and C-5 ($^3J_{4,5}$ = 9.3 Hz) indicate a dihedral angle approaching 180° i.e. pseudodiaxial. The hydroxyl substituents at these carbon atoms are therefore assigned to pseudoequatorial positions. This assignment thus confirms the facial selectivity of the

oxidation reagent in the osmylation reaction as being for the lower face. Smaller vicinal coupling constants ($^3J_{3,4}$ and $^3J_{5,6} \approx 4$ Hz) are consistent with equatorial-equatorial and equatorial-axial relationships, and thus assign protons at C-3 and C-6 to pseudoequatorial positions in the preferred conformation. Consequently the hydroxyl substituents at these carbon atoms are assigned to pseudoaxial positions. Thus the positions of the hydroxyl substituents (pseudoaxial or pseudoequatorial) in the preferred conformation are equivalent to those observed for shikimic acid by the same analysis.

4.2.3 Preparation of *SRRR* analogue *(88).*



(86) *(89)* *(88)* *(90)*

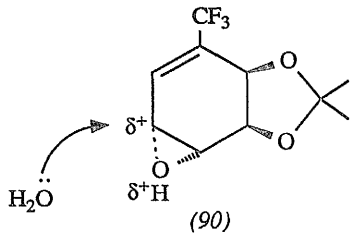
Scheme 4.7

Treatment of acetonide *(86)* with mCPBA gave the epoxide *(89)* in 74% yield after purification as determined by ^{1}H nmr and ^{13}C nmr. The assignments of proton signals in the nmr were determined by irradiation experiments and confirmed by a ^{1}H-^{1}H cosy

experiment. The ^{1}H and ^{13}C nmr indicate that the epoxidation is regioselective for the C-3, C-4 double bond. The observed regiospecificity is consistent with that observed in the osmylation of diene *(86)*, being more reactive to this double bond because of its greater nucleophilicity. The nmr spectra also indicates that the functionalisation is stereoselective giving a single diastereoisomer (and enantiomer). The absence of stereoselectivity would have resulted in a mixture of diastereoisomers and thus observable in the nmr spectra. The stereochemistry of epoxidation with peroxycarboxylic acids has been well studied.[220] In a similar manner to that observed by Hudlickly,[204] Ley[217] and in scheme 4.6 attack and addition of oxygen occurs preferentially from the less hindered side of the molecule. Therefore the indentity of the diastereoisomer will be that resulting from attack of the mCPBA from the lower α face.

Treatment of an aqueous solution of epoxide *(89)* with Dowex resin (H^+ form) at room temperature for 6 hours gave mixture of two products as observed by t.l.c. However, prolonged treatment (4.5 days) resulted in the formation of single product as observed by t.l.c. The product was indentified as tetrol *(88)* by ^{1}H nmr, ^{13}C nmr and HRMS and was isolated in 89% yield. Assignments in the ^{1}H nmr were confirmed by irradiation experiments. The second less polar product observed in the early stages of the reaction was believed to be diol *(90)* and was not isolated. The presence of only one diastereoisomer in the nmr of *(88)* indicated that the ring had been opened regioselectively. The regioselectivity of ring-opening of analogous epoxides is well documented[193,204,206] and the ring-opening mechanism of the similar epoxide *(52)* was examined in chapter 3 (3.2.5). Correspondingly, the ring-opening of *(89)* occurs by attack of H_2O at C-3 by reason that the development of cationic charge at this carbon is resonance stabilised (figure 4.4)

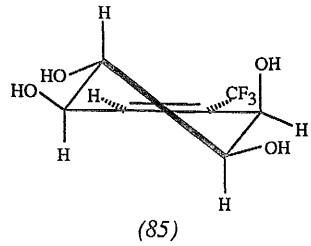
Comparison of vicinal coupling constants in the ^{1}H nmr with those of shikimic acid[187] and tetrol *(85)* indicate that the molecule also adopts a twisted chair in the preferred conformation. The relatively large vicinal coupling constants between protons C-3 and C-4,



(90)

Figure 4.4

and C-4 and C-5 ($^3J_{3,4}$ = 7.8 Hz and $^3J_{4,5}$ = 10.5 Hz) indicate pseudodiaxial relationships and thus assign the hydroxyl substituents at these carbon atoms to pseudoequatorial positions (figure 4.5). Consequently the *cis* geometry of diol introduced in the



(85)

J values (Hz)

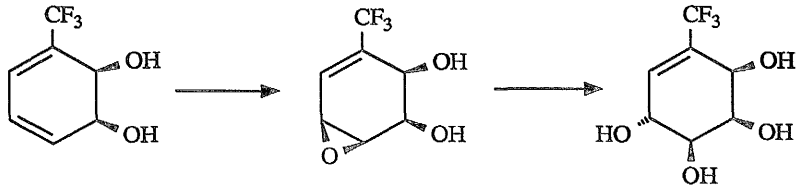
$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$
2.9	7.8	10.5	3.9

Figure 4.5

biotransformation step assigns the hydroxyl substituent at C-6 as pseudoaxial. Thus the C-4, C-5, and C-6 hydroxyl substituents are observed to adopt equivalent positions to those of *RRRR* analogue *(85)*. Tetrol *(88)* may be therefore considered to represent an analogue differing by a single mutation (C-3 hydroxyl, β → α) from analogue *(85)*.

4.2.4 Preparation of *RSRR* analogue *(91)*.

Treatment of *cis*-dihydrodiol *(83)* with mCPBA gave the epoxide *(92)* in 71% yield after



(83) *(92)* *(91)*

Scheme 4.8

purification and was characterised by ^{1}H nmr, ^{13}C nmr spectroscopy and HRMS. The ^{1}H and ^{13}C nmr indicate the regioselective functionalisation of the C-3, C-4 double bond. The functionalisation is consistent with that observed for the osmylation and epoxidation of diene *(86)*. Also consistent with the previous reactions, the nmr spectra demonstrates that the epoxidation is stereoselective. The absence of stereoselectivity would have resulted in a mixture of diastereoisomers and therefore observable in the nmr spectra. The stereochemistry of epoxidation with peroxycarboxylic acids has been well studied. As observed with the epoxidation of diene *(86)* attack and addition of oxygen normally occurs preferentially from the less hindered side of the molecule.[204,217,220] However exceptions for the preference of the least hindered side of the molecule when the molecule contains polar substituents, particularly hydroxyl groups are well documented.[203,205,206,221-223] The hydroxyl groups apparently complex with the reagent, so that the likely identity of the diastereoisomer is that with the epoxide on the upper (β) face of the ring. A representation of the proposed transition state is shown in figure 4.6.

Prolonged treatment of the epoxide *(92)* with Dowex resin (H^{+} form) in a solution of acetonitrile and water gave the tetrol *(91)* in near quantitative yield as determined by ^{1}H nmr, ^{13}C nmr and HRMS. The presence of only one diastereoisomer in the nmr indicated that the ring had been opened regioselectively. The ring-opening of *(92)* is likely to occur by attack of H_2O at C-3 by reason that development of cationic charge at this carbon is resonance stabilised as in the ring-opening of epoxide *(89)*. The vicinal coupling constants

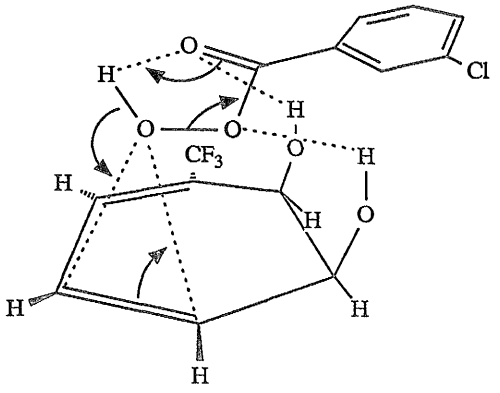
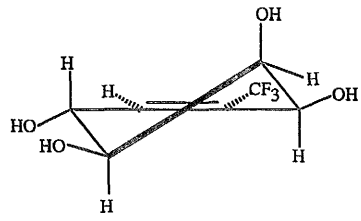


Figure 4.6

displayed in the ^1H nmr of tetrol *(88)* suggest that the preferred conformation of the molecule is distorted somewhat from that of the twisted chair conformation observed with tetrols *(85)* and *(88)*. The distortions in the conformation are likely to be a result of steric interaction between the three hydroxyl substituents located at C-4, C-5 and C-6, since these are all located on the same face of the ring. However comparison of the vicinal coupling constants between C-3 and C-4 with those for shikimic acid[187] and other similar cyclohexene rings[187] (figure 4.7) indicates that a $J_{3,4}$ of approximately 7 Hz is consistent with pseudodiaxial hydrogens. In contrast a $J_{4,5}$ of approximately 7 Hz in a cyclohexene ring is more consistent with equatorial-axial and equatorial-equatorial interactions. Hence the hydroxyl substituents at C-3, C-4 and C-6 are assigned to pseudoequatorial positions, whilst the hydroxyl substituent at C-5 is assigned to a pseudoaxial position in the preferred conformation. The preferred conformation of C-3, C-5 and C-6 hydroxyl groups are thus observed to be different from that of the 'standard' *RRRR* analogue *(85)* although they have equivalent stereochemistry in each molecule. The effect of this difference to the binding and/or catalysis of this analogue may be negligible but should be considered when interpreting any data relating to such.



(91)

	J values (Hz)			
	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$
RSRR analogue *(91)*	2.7	6.9	6.9	3.7
Shikimic acid[187]	4.0	3.9	8.4	5.0
RRRR analogue *(85)*	3.9	4.1	9.3	3.8
SRRR analogue *(88)*	2.9	7.8	10.5	3.9

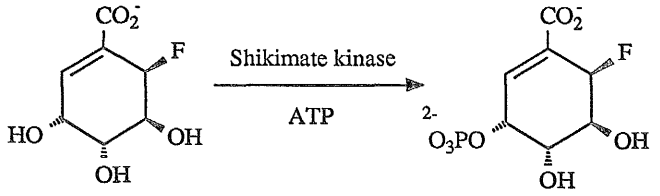
Figure 4.7

4.2.5 Substrate/Inhibitor Properties of Analogues *(85)*, *(88)*, and *(91)*.

Analogues *(85)*, *(88)*, and *(91)* were assayed for their ability to act as substrates for shikimate kinase. The rate of catalysis *V* was observed, at a fixed concentration of ATP (2.5 mM), with each of the tetrol analogues in the range 0.5-10 mM. Enzyme concentration was varied in the range 0.05 - 10.0 U/ml. The rates of catalysis were corrected for background turnover of ATP at high enzyme concentration. In each case no turnover corresponding to the phosphorylation of the tetrol was detected.

The use of shikimate kinase to phosphorylate *(6R)*-6-fluoroshikimate *(93)* by Abell and co-workers[82] (scheme 4.9) suggests that the additional hydroxyl group at C-6 in each of the

analogues would not be expected to preclude any catalysis, although the hydroxyl group would have a greater steric demand than the fluoro substituent. The lack of the ability of the



(93)

Scheme 4.9

analogues to act as substrates is most likely to result from the absence of carboxylate at C-1. The trifluoromethyl group was considered a reasonable substitution for several reasons:

(1) Like carboxylate the CF_3 substituent is also an electron withdrawing group (electronegativity 3.35),[224] and it is this characteristic that allows regioselective functionalisation of the C-3, C-4 double bond.

(2) Fluorine and oxygen are similar in size, Van der Waals radii for O = 1.52 Å and F = 1.47 Å.

(3) Fluorine also has the potential to act as a hydrogen-bond acceptor.

It was also considered that the trifluoromethyl group would be less solvated than the corresponding carboxylate. As discussed in the previous chapter the desolvation of the substrate contributes to the apparent binding energy of the substrate. Therefore it would seem reasonable to suggest that the loss of negative charge might be offset by the easier desolvation of the trifluoromethyl group.

Thus despite the physical similarities between the trifluoromethyl and carboxylate groups, and the favourable reduced solvation of the trifluoromethyl group, catalysis does not occur. This suggests that the charge carried by the carboxylate is of importance and therefore implicates the presence of a positively-charged active site residue to complex with it. The

failure of the enzyme to phosphorylate the methyl ester of analogue *(68)* (chapter 3) (data not shown) would also suggest this, however the factor of size is likely to be of greater influence in this case.

Examination of the consensus sequence of *E.coli* shikimate kinase believed to be responsible for binding shikimic acid[55] reveals two arginine (R) residues in the centre of the sequence (res. 27 and 28).[54] The absence of any other potentially positively charged residues in this region suggests that these are responsible for the proposed 'ion-pair' binding of carboxylate. However, these residues do not form part of the conserved residues, that identify the motif, observed in other shikimate pathway enzymes that also bind substrates with the carboxylate substituent (18 enzymes in total indentified with the consensus sequence). If the comparison is limited to shikimate kinase enzymes from different sources however a pattern of similarity is observed (figure 4.8). In each of the enzymes shown at

Consenesus sequence	- - A L - - A L - - - F V D L D - - L E T L - -
N. carassa qa-1S	I M A - S T A M K R K I V D L E S E F H H L T G
A. nidulans arom	G N W V S K A L N R P F V D L D T E L E T V E G
S. cerevisae arom	S K W C A S A L G Y K L V D L D E L F E Q Q H N
E. coli aroL	G M A L A D S L N R R F V D T D Q W L Q S Q L N

Figure 4.8

Boxed residues are those indentified by Bugg *et al.*[55] as repeat residues that contribute to thebinding motif.

least one arginine (R) or lysine (K) is observed in the two positions immediately prior the strongly conserved F/L.V.D.L.D/E pentapeptide sequence. The occurrence of two arginine residues occupying these sites in *E.coli* shikimate kinase might implicate the importance of the carboxylate interaction with the protein in this case.

To investigate if the presence of the trifluoromethyl group was preventing the binding of the

substrate or just the catalysis of the substrate, each of the tetrol analogues were assayed for their ability to act as competitive inhibitors. If this was the case it would therefore indicate that the analogues were being bound at the active site. Furthermore it would possibly allow characterisation of the ground-state interactions between protein and ligand by the methods disscussed in the previous chapter.

Competition experiments were preformed in which shikimate kinase was assayed at fixed concentrations of ATP (2.5 mM) and with shikimic acid in the range 0.07-0.5 mM in the presence of a range of concentrations (0 mM, 1 mM and 10 mM) of *(85)*, *(88)* and *(91)*. Figure 4.9 shows Lineweaver Burk plots[199] for each analogue. The plots indicate that each

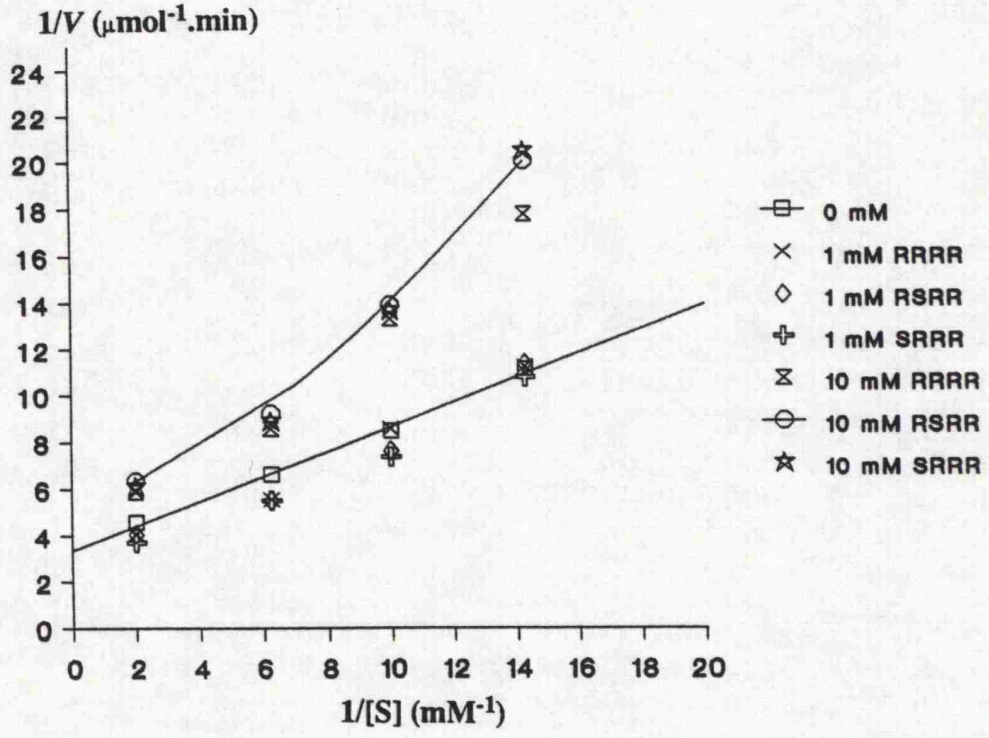


Figure 4.9 Inhibition of shikimate kinase by tetrol analogues *(85)*, *(88)* and *(91)*

of the tetrol analogues appears to be similarly mildly inhibitive at high concentration. The curvature in the graphs suggests that the mode of inhibition is complicated and cannot be classified in the simple terms. A further single experiment with 25 mM *RSRR* analogue *(91)*

preformed as before also yielded a pronounced curve (figure 4.10). Dixon and Webb[195]

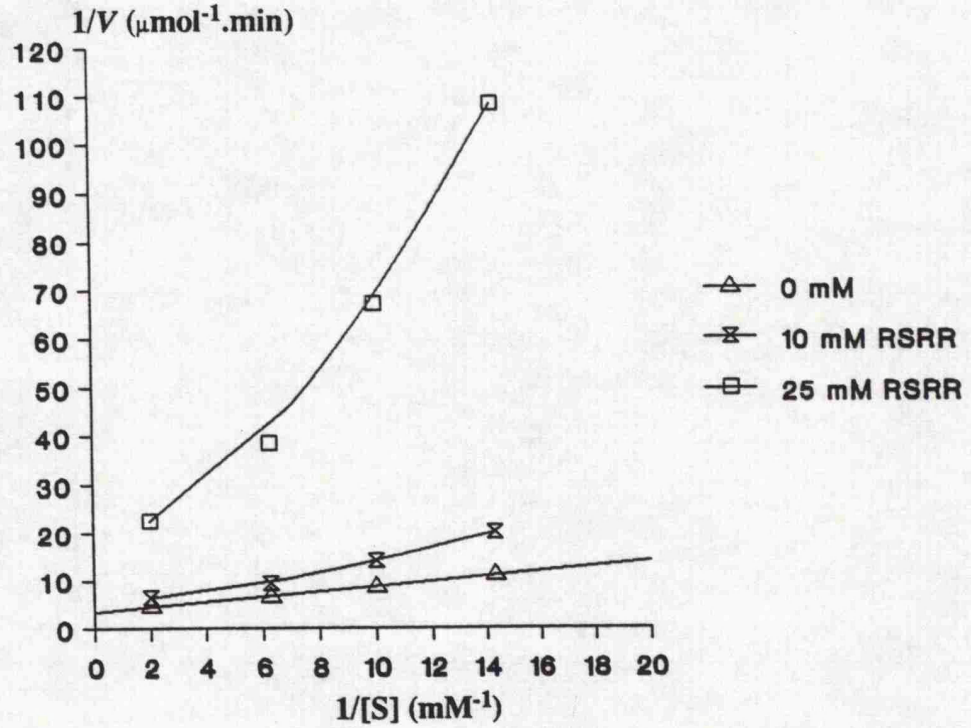


Figure 4.10 Inhibition of shikimate kinase with 25 mM analogue *(91)*

suggest that such a non-linear double-reciprocal plot might be observed for a non-competitive inhibitor. This proposal would also seem reasonable in view of the observation by DeFeyter[53] and observed during this research that shikimate kinase is inhibited by high concentrations of shikimic acid (>10 x K_M) (figure 4.11). The tetrol analogues although non-substrates may be able to act in the same manner as shikimate at high concentrations possibly forming non-productive complexes that prevent access of substrates to the active site.

4.3 Summary.

In an attempt to probe the active site interactions of *E.coli* shikimate kinase, three single enantiomer diastereomeric analogues of shikimic acid have been synthesised *via* short, efficient stereocontrolled routes from *(93)*. Their ability to act as substrates or inhibitors of the enzyme was investigated. Each diastereoisomer was found not to be a substrate of the enzyme. The absence of catalysis has been discussed and the importance of C-1 carboxylate identified. Each analogue was found to similarly inhibitive. The mode of inhibition was observed to be most likely non-competitive.

CHAPTER 5

Chemical Modification of Shikimate Kinase

5.0 Introduction.

Chemical modification of proteins has been widely used to explore their structure and mechanism.[225-228] In particular, chemical modification has been used to identify active site residues, attach spectroscopic probes at specific positions, and in the preparation of protein derivatives containing heavy atoms for X-ray crystallographic studies. Each protein reacts in a unique way with a given reagent because of the particular reactivity conferred on the amino acid side-chains by their three-dimensional structure.

The principal chemically reactive groups in proteins are nucleophiles. The nucleophilicity of proteins arises from the reactivity of the side-chains of the sulphur-containing amino acids (cysteine and methionine), the basic amino acids (lysine, histidine and arginine), the acidic amino acids (aspartic and glutamic acids) and the activated aromatic amino acids (tyrosine and tryptophan). Approximate orders of reactivity in modification reactions[225] are given in table 5.1 The hydroxylic side-chains of serine and threonine are generally

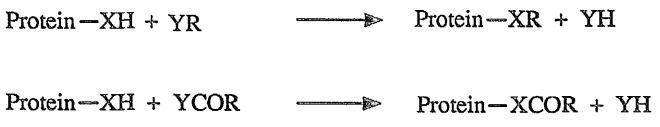
Alkylation	Acylation	Arylation
Methionine	-	-
Cysteine	Cysteine	Cysteine
Imidazole	Phenol	Amine
Amine	Imidazole	Phenol
Phenol	Amine	Imidazole
Carboxyl	Carboxyl	Carboxyl

Groups are listed in order of decreasing reactivity

Table 5.1

unreactive towards modification with the notable exception of the serine proteases in which the hydroxyl group has a direct role in catalysis. The nucleophilicity of the amino acid side-chains are strongly pH dependant. The reactive species are the unprotonated forms of the various amino acids (neutral of basic amino acids and methionine, and the anionic forms of cysteine, tyrosine and the acidic amino acids) hence the pH dependence.

The majority of reagents that are used to modify proteins are electrophiles, usually acylating or alkylating agents such as acyl- or alkyl halides (figure 5.1). Alkylation may also take



(X = S, NH or O; Y = halogen)

Figure 5.1

place through nucleophilic conjugate addition to an α,β-unsaturated carbonyl compound (Michael addition) e.g the reaction of N-ethylmaleimide with a cysteine residue (figure 5.2).

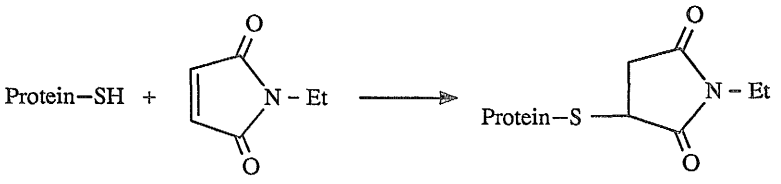


Figure 5.2

The stoichiometry of the reaction may be observed by using a reagent which is labelled either radioactively, or with a chromophoric substituent and measuring the radioactivity or amount of chromophore incorporated. If the modified amino acid is stable to 6M HCl, amino acid analysis can be used to identify those residues that are modified.

Specific reaction at the active site of an enzyme can arise in a number of ways. The enzyme active site may have an increased affinity for the reagent by in some way resembling the natural substrate. In addition, the active site residues are often more accessible than deeply buried groups. Furthermore the amino acid side-chains directly involved in catalysis are often the most reactive by reason of being activated to effect catalysis. Specificity for residues may also be achieved by choice of reagent used. There are a number of modifying

reagents which have a tendency to react with one type of side-chain more than another, and are termed group-specific modifying reagents. Such reagents can be used to make tentative identification of the key catalytic groups.

As disscussed in chapter 1, possible identities of the key catalytic groups at the active site of shikimate kinase can be considered. In accordance with an 'in-line' associative phosphoryl transfer mechanism for kinases as described by Knowles *et al.*[131] (figure 2.11) it has been suggested that the presence of a general base to abstract a proton from the substrate might be likely. Indeed, for a number of kinases,[142] bases such as histidine and aspartate have been implicated. Stabilisation of the charge on the peripheral oxygens on the phosphoryl group would also be in accordance with an associative mechanism. Metal co-ordination, cationic (e.g. Lys or Arg) or hydrogen bond donating residues to these oxygens are therefore inferred. An appropriately placed general acid to protonate the β-phosphoryl group, thereby assisting the leaving group, can be similarly envisaged. In chapter 4 the importance of the carboxylate of shikimate for the recognition of the substrate was demonstrated. Consequently positively charged residues, such as arginine or lysine, to form charge-charge interactions were implicated.

There are many literature examples of group specific modification, and subsequent identification of catalytic residues. The sulfhydryl groups of cysteine are the most reactive amino acid side chains and are easily alkylated, acylated, arylated and oxidised. The binding of N-ethylmaleimide (NEM) to cysteine residues is observed to be quite specific.[229-234] Other N-alkylmaleimides are also sulfhydryl-directed reagents and this specificity has been exploited for the attachment of fluorescent probes to thiol groups of proteins.[235-236] As observed in figure 5.2 it is the double bond of the maleimide that undergoes addition. Other sulfhydryl-directed reagents include the arylating and alkylating reagents 5,5'-dithiobis(2-nitrobenzoic acid),[231,237,238] 2,4,6-trinitrobenzene-1-sulphonate,[237] *p*-hydroxymercurybenzoate,[230] O-iodosobenzoate,[231,232]

2,2'- dithiodipyridine,[230] iodoacetate,[234,239,240] and iodoacetamide.[241,242]

The ϵ-amino group of lysine is the second most reactive nucleophile after cysteine, and there are a number of regents that tend to be specific towards amino groups. Acetylation with acetic anhydride[243] is probably the most frequently used procedure for the modification of protein amino groups. Succinic anhydride and maleic anhydride have been observed to have a tendency for lysine modification.[244,245] Diethyl pyrocarbonate (DEP) has also been shown to be reactive for lysine[246] but more usually specificity towards histidine is observed.[237,247-253] Specific modification of the guanidino group of arginine has been demonstrated by the dicarbonyls 2,3-butanedione[254,255] and cyclohexane-1,2-dione.[256,258]

The δ- and ϵ- carboxyl groups of aspartic acid and glutamic acids, respectively are the principal anionic groups in the proteins. The carboxyl groups have been shown to be specifically converted to amides by the use of water soluble carbodiimides.[248,259]

The indole side-chain of tryptophan residues are relatively reactive but they are often protected from reaction by being buried in the interior of the protein along with other hydrophobic side-chains. Specificity is best obtained with tryptophan using the reagents N-bromosuccinimide[230,257,260,261] and 2-hydroxy-5-nitrobenzylbromide.[262]

The phenolic group of tyrosine residues are susceptible to modification by many acylating and alkylating agents. The aromatic ring is also susceptible to modification and substitution by a number of electrophilic reagents. Specific modification has been demonstrated by N-acetylimidazole[257,263,264] and tetranitromethane.[248,265]

The aliphatic hydroxyl groups of serine and threonine are relatively resistant towards modification. However the reagent di-isopropyl fluorophosphate (DFP) and other such compounds such as α-phenylmethylsulfonyl fluoride (PMSF) has been used to selectively

modify and identify a single catalytically important active site serine residue common to a number of esterases and proteinases.[195,266] The selectivity observed is believed to be derived partly from these reagents acting as pseudosubstrates. In the case of DFP, the stable di-isopropyl phosphoryl enzyme, resulting from inactivation, is similar in some respects to the acyl enzyme intermediates formed in the normal hydrolytic reactions of these enzymes.

5.1 Aims.

This chapter aims to probe the nature of catalytically important amino acids of shikimate kinase using a range of modifying reagents, including group specific reagents. The chapter will focus on the modification of active site residues, at pH 7.0. Location of the amino acids at the active site will be explored by substrate protection experiments. It is envisaged that the ability of a substrate to protect against inactivation might enable the identification of residues important for the binding and/or catalysis of that substrate.

5.2 Results.

5.2.1 Modification by Iodoacetamide *(94)*.

(94)

Figure 5.3

The selective alkylation of sulfhydryl groups with iodoacetamide (figure 5.3) has been observed for a number of enzymes.[241,242] Shikimate kinase was rapidly inactivated at pH 7.0 with iodoacetamide *(94)*. The reaction mixture (1 ml) consisted of 1.6 μM of shikimate kinase, 10 mM iodoacetamide, and 50 mM triethanolamine hydrochloride buffer, pH 7.0, 50 mM KCl, 5 mM $MgCl_2$. Aliquots (50 μl) were withdrawn at appropriate time intervals and the enzymic activity was determined by the standard assay. Controls containing no

modifying reagents were conducted in parallel and the activity of the modified enzyme at any given time was calculated relative to the control.

If inhibition is predominately caused by the irreversible binding of the inhibitor to a single amino acid residue and the inhibitor is in large excess over the enzyme then the kinetic analysis applied to the inhibition of the enzyme with the active site directed inhibitor *(59)* (3.2.3) also applies. Thus the reaction may be represented as

$$E + I \xrightarrow{k} E\text{-}I$$

and the rate of inhibition k will be given by the second-order equation

$$\frac{-d[E]}{dt} = k\,[I]\,[E]$$

As the inhibitor [I] is in great excess over the enzyme [E] then the equation simplifies to *pseudo*-first-order

$$\frac{-d[E]}{dt} = k'\,[E]$$

which integrates to give

$$\ln\,[E] = k'\,t$$

Thus the *pseudo*-first order rate constant k' is given by a plot of ln[E] against t. The second order rate constant k for the inactivation can then determined since $k' = k\,[I]$. A plot of ln[E] versus t is shown in figure 5.4. Second order rate constants for the inactivation of shikimate kinase at pH 7.0 are given in table 5.2.

5.2.2 Modification by N-Ethylmaleimide (NEM) *(95)*.

NEM *(95)* shows high specificity for reaction with more exposed protein sulfhydryl groups[229-234] at pH values near neutrality, and has been used to determine the number of sulfhydryl groups in a number of proteins.[226] Shikimate kinase was extremely rapidly inactivated with NEM *(95)*. The modification of the enzyme was performed by an analogous method to the inactivation with iodoacetamide, with aliquots of the reaction

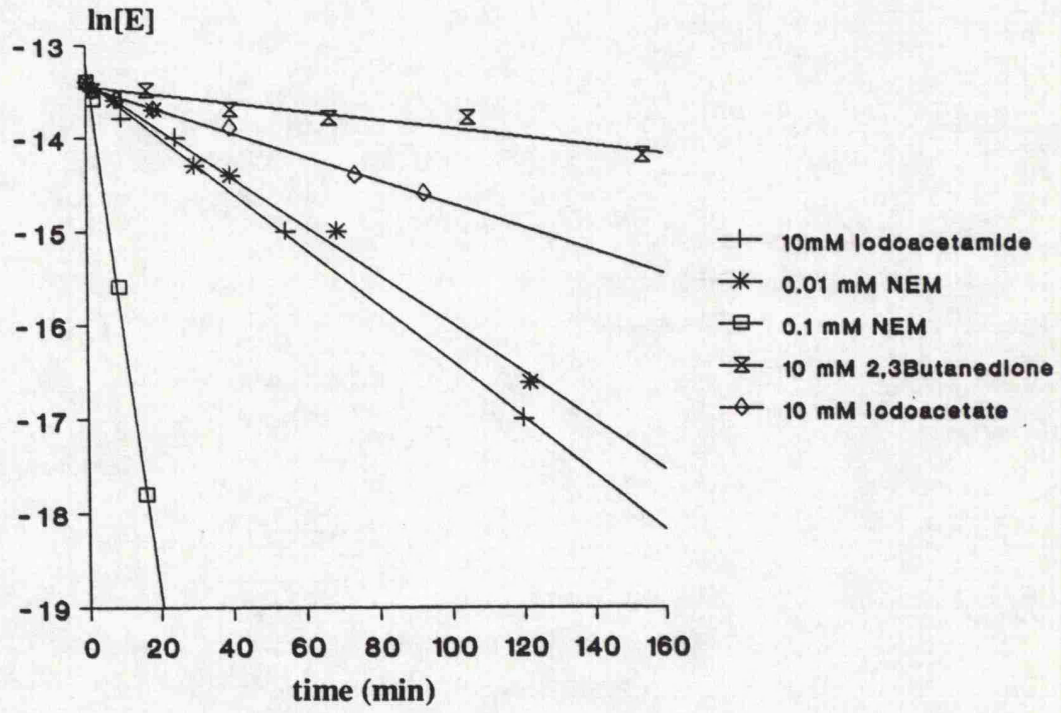


Figure 5.4 Semi-logrithmic plots of free enzyme concentration as a function of time for the inactivation of shikimate kinase by the reagents iodoacetamide, N-ethylmaleimide, 2,3-butanedione and iodoacetate.

mixture being assayed for enzyme activity at appropriate time intervals. Shikimate kinase (1.6 μM) was observed to be completely inactivated in the presence of 10 mM and 1 mM NEM within two minutes. Inactivation by 0.1 mM and 0.01 mM NEM appeared to be *pseudo*-first order and the kinetics of inactivation are given in figure 5.4. Second order rate constants for the inactivation are shown in table 5.2.

5.2.3 Modification by Iodoacetic Acid *(96).*

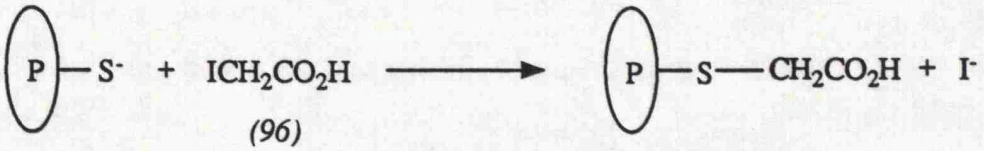


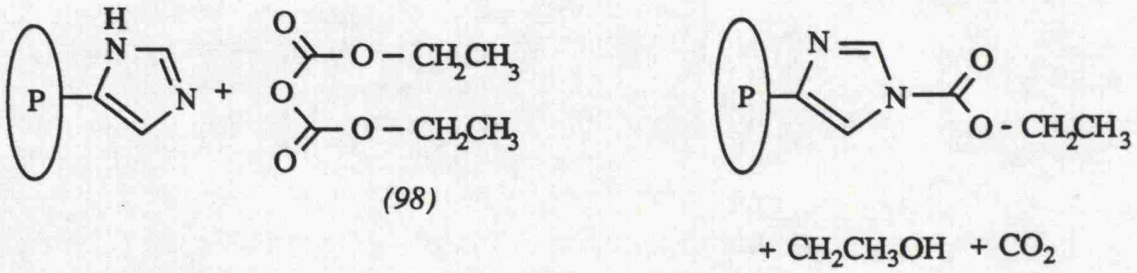
Figure 5.5

The reactivity of sulfhydryl groups with haloacetates far exceeds that of other protein groups under almost all conditions, with optimal pH between approximately 6 and 8.5.[226] The selective carboxymethylation of thiol has been observed for a number of proteins.[234,239,240] Following the rapid inactivation of shikimate kinase by iodoacetamide, it was of interest to also investigate the inactivation of the protein with iodoacetate, since the presence of the negatively charged group might enhance specificity for the shikimic acid active site. The modification of the enzyme was also performed by an analogous method to the inactivation with iodoacetamide. Correspondingly, incubation of shikimate kinase (1.6 μM) with 10 mM iodoacetate at pH 7.0 also resulted in the rapid inactivation of the protein. The kinetic analysis (figure 5.4) also indicates that the alkylation follows pseudo-first order kinetics. The second order rate constants for the inactivation is shown in table 5.2.

5.2.4 Modification by 2,3-Butanedione *(97)*.

2,3-Butanedione *(97)* has been shown to reactive towards basic side chains with a degree of specificity towards the guanidino functional group of arginine.[254-256] Since arginine residues appear to be widely used in a range of enzymes to bind phosphate esters it was of interest to determine the sensitivity of shikimate kinase to such reagents. Shikimate kinase (1.6 μM) was incubated with 10 mM 2,3-butanedione *(97)* at pH 7.0. The rate of modification of the enzyme was determined in an equivalent manner to modification with iodoacetamide. The kinetic analysis (figure 5.4) of the inactivation by the dicarbonyl follows pseudo-first order kinetics. The second order rate constant for the inactivation is shown in table 5.2.

5.2.5 Modification by Diethylpyrocarbonate (DEP) *(98).*



(98)

Figure 5.6

The reagent diethylpyrocarbonate *(98)* has been widely used in enzyme modification studies, primarily for the elucidation of functional roles of histidine residues (figure 5.8).[247-253] Ethoxyformylation of shikimate kinase with DEP was carried out by the method of Berger[267] since DEP is unstable in aqueous solutions. Principally, shikimate kinase was incubated in the presence of DEP at various concentrations (0.5 - 10 mM) at pH 7.0. Aliquots (50 μl) were removed from the reaction mixture (1 ml) and initial pseudo-first order rates (k') determined (within 10 min.) for each concentration of DEP. The second order rate constant for the inactivation was then determined from the plot of the initial rates against concentration of DEP (figure 5.7) and is given in table 5.2.

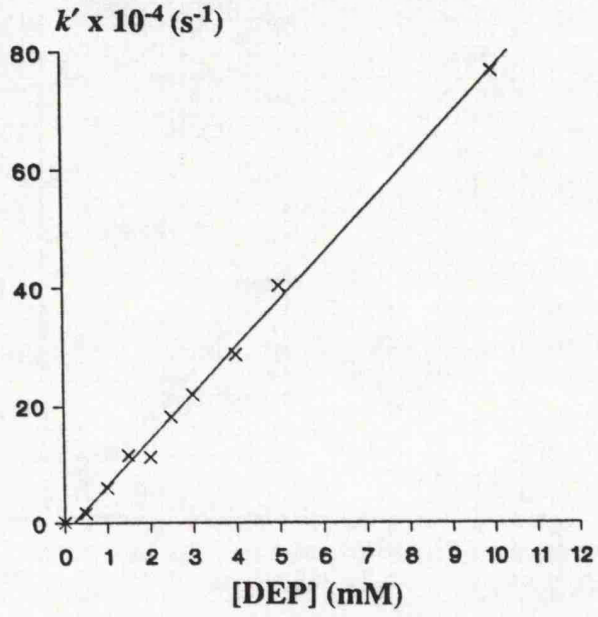


Figure 5.7 Plot of initial first order rates as a function of reagent concentration for the inactivation of shikimate kinase with diethylpyrocarbonate.

Reagent	k $M^{-1}s^{-1}$
Iodoacetamide *(94)*	0.044
N-Ethylmaleimide *(95)*	44.5
Iodoacetic acid *(96)*	0.022
2,3-Butanedione *(97)*	0.0074
Diethyl pyrocarbonate *(98)*	0.759

Table 5.2
Second order rate constants for the inactivation of shikimate kinase at pH 7.0

5.2.6. Substrate Protection Studies.

Pseudo-first order kinetic data were also observed for the inactivation of shikimate kinase by iodoacetamide *(94)*, N-ethylmaleimide *(95)* and diethyl pyrocarbonate *(98)* in the presence of the substrates. Reaction with each of the reagents was carried out as before in the presence of either saturating (>10 x K_M) shikimic acid or ATP. Figure 5.8 shows the level of protection offered by each of the substrates to the modification of the enzyme. The apparent similarity in the level of protection offered by each substrate towards modification by each of the reagents suggests reaction at a common amino acid residue. In each case near maximal protection is observed with ATP. These results indicate that each of the three reagents binds to the active site responsible for binding ATP. Partial protection is also observed with shikimic acid. The decrease in enzymic inactivation rate resulting from shikimate protection demonstrates either that shikimic acid also binds at the site of reagent modification or induces a protein conformation change which decreases the reactivity of the reagent sensitive amino acid.

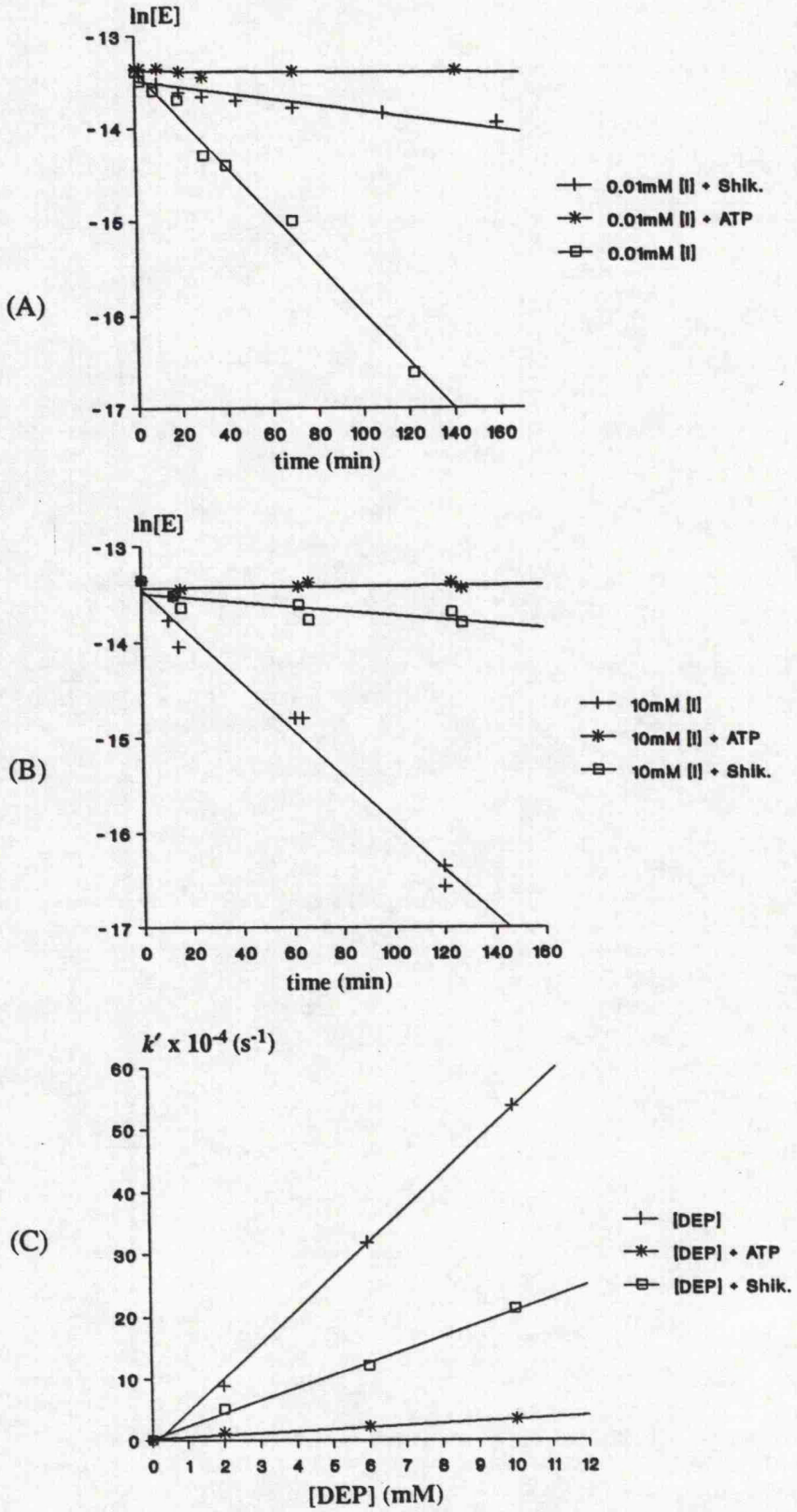


Figure 5.8 Substrate protection form inactivation of shikimate kinase by (A) N-Ethylmaleimide, (B) Iodoacetamide and (C) Diethyl pyrocarbonate.

5.3 Discussion.

These results demonstrate that shikimate kinase is rapidly inactivated by a variety of reagents at pH 7.0. In each case, the kinetics of inactivation is observed to be *pseudo*-first order and therefore indicates modification of a single amino acid side chain or a single class of equivalent side chains. Of the reagents used, NEM *(95)* and DEP *(98)* are observed to be particularly reactive under these conditions. NEM and DEP have been used extensively for protein modification studies, and they have been observed to express a high degree of selectivity for reactive sulfhydryl and histidine residues respectively. Presumably the difference in reactivity of the reagent NEM and the reagents iodoacetamide and iodoacetic acid, which also demonstrate specificity towards sulfhydryl groups, is a consequence of different mechanistic reactions which lead to modification.

The inactivation of the protein by NEM, DEP, and iodoacetamide was observed to be reduced significantly by the presence of both ATP and shikimic acid, the substrates of the enzyme. The near maximal protection offered by ATP in each case is strongly indicative that the reagents are responsible for the modification of residues involved in binding ATP. Furthermore, the pattern of protection with each reagent, near total protection with ATP and similar partial protection with shikimic acid, suggests modification at a common residue. In support of this, such a pattern of protection was also observed for the modification of the protein with affinity label *(59)*. A residue which is observed to show greatly magnified reactivities towards certain reagents and occasionally towards all reagents tested is termed superreactive (such as the active serine of serine proteases and the active thiol in cysteine proteases) and often, although not necessarily, has an important role in catalysis.

Comparison of the complete amino acid sequence of *E.coli* shikimate kinase (figure 5.9) to other adenine-nucleotide-binding proteins[55] and amongst enzymes of the shikimate

T Q P L F L I G P R G C G K T T V G M A L A D S L N R R F
V D T D Q W L Q S Q L N M T V A E I V E R E E W A G F R A
R E T A A L E A V T A P S T V I A T G G G I I L T E F N R
H F M Q N N G I V V Y L C A P V S V L V N R L Q A A P E E
D L R P T L T G K P L S E E V Q E V L E E R D A L Y R E V
A H I I I D A T N E P S Q V I S E I R S A L A Q T I N C

Figure 5.9

Complete amino acid sequence of *E.coli* shikimate kinase. Residues strongly contributing to the adenine-nucleotide-binding motif are square boxed. Residues strongly contributing to the shikimate binding motif are round boxed .

pathway[54] has identified strongly conserved sequences therefore suggesting appropriate binding sites for each substrate. In view of the relative reactivity and selectivity of the modifying reagents used, examination of the ATP binding sequence for potential sites for modification reveals Cys-12 and Lys-14 as the most likely sites of modification. While both NEM and DEP show a high level of specificity towards sulfhydryl and imidazole groups respectively, both reagents are also known to react with amino groups under certain conditions.[226] DEP has also been observed to react with -OH, -SH, and indolyl groups.[268-270] The observed very high reactivity of NEM with shikimate kinase strongly suggests that Cys-12 the site of modification.

What then is the role of a reactive sulhydryl group at the active site? The sulfhydryl group could act as a nucleophile towards γ-phosphate of ATP, forming a transient phosphoenzyme intermediate in the presence of cosubstrate. However there is no evidence to support the occurence of such an intermediate in kinases generally. Alternatively, the sulfhydryl might coordinate to the magnesium ion with an important role in binding and/or catalysis. Despite the apparent reactivity of the residue, the sulfhydryl might have a purely structural role, forming strong hydrogen bonds with other residues. Examination of corresponding adenine-nucleotide-binding motifs (figure 5.10) shows that the presence of cysteine at this

Protein	Residues	Sequences
Adenylate kinase	8-29	S K I I F V V G G P G S G K G T Q C E K I V
*rec*A protein	59-80	G R I V E I Y G P E S S G K T T L T L Q V I
rho protein	127-193	G Q R G L I V A P P K A G K T M L L Q N I A
DNA B protein	223-224	S D L I I V A A R P S M G K T T F A M N L V
EF-Tu	11-32	H V N V G T I G H V D H G K T T L T A A I T
DNA helicase II	22-43	R S N L L V L A G A G S G K T R V L V H R I
Shikimate kinase	1-22	T Q P L F L I G A R G C G K T T V G M A L A

Figure 5.10

Sequence homologies between adenine-nucleotide-binding protein and *E.coli* shikimate. Strongly conserved regions are boxed. All proteins are *E.coli* except adenylate kinase (porcine).

position in the motif is exclusive (to those compared). However the position, immediately prior to the strongly conserved Gly-Lys-Thr sequence is observed to have preference for serine. The similar catalytic roles of serine and cysteine are comprehensively demonstrated by the cysteine and serine proteinases.[195] Of the enzymes to which shikimate acid can be compared, the structure and related function of adenylate kinase from many sources has been studied extensively.[147,271-274] In this protein and others the conserved gly,lys,thr motif is observed to form a Glycine-rich loop. X-ray, NMR, site-directed mutagenesis studies, and more recently X-ray studies on co-crystals of *E.coli* adenylate kinase with $MgAp_5A$, an inhibitor of the enzyme, have suggested that the main function of the loop (with the exception of the highly conserved lysine residue) is in substrate binding. The crystal structures indicate that the β- and α- phosphates of ATP are held partly by polar contacts between all the peptide nitrogens of the residues in the conserved Gly-X-Gly-Lys-Thr-X sequence (figure 5.11). This suggests that the Gly-loop forms an anionic hole, and the function of the amino acid side chains in this sequence is essentially structural. The ϵ-amino group of the highly conserved lysine is however involved in polar contacts with the β- and γ- phosphate groups, and is believed to play a key role in the stabilisation of the γ-

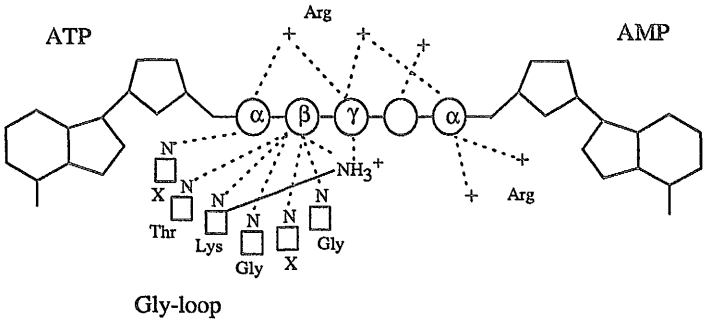


Figure 5.11. Sketch of main polar contacts between the five phosphates of Ap_5A in *E.coli* adenylate kinase as determined by Schulz.[147]

phosphate at the transition state. Thus, in view of the current understanding of the role of the Gly-loop in NTP-binding proteins, a possible catalytic role for the reactive cysteine of shikimic kinase is not apparent. However, the structural/functional studies of adenylate kinase does place this amino acid in such a position that important catalytic function cannot be excluded.

In addition to protection of modification by ATP, the rate modification is also significantly reduced by the presence of the other substrate of the enzyme, shikimic acid. The partial protection may be a result of the proximity of active sites in the enzyme since, in the case of an associative mechanism, the γ- phosphoryl group of ATP and the C-3 hydroxyl of shikimate would be expected to be within van der Waals contact (≤ 3.3 Å).[142] Alternatively, the partial protection observed in the presence of shikimate may be a consequence of the mechanism of the enzyme. As discussed in chapter 1, X-ray structure analyses on a variety of phosphotransferases[103] indicate a degree of generality defined by two distinct lobes with the active site located in the cleft. Evidence, principally from hexokinase[146] and adenylate kinase,[147] suggest that induced-fit mechanisms are in operation. Essentially, the binding of individual substrates induces movements of the lobes to embrace the substrate. Thus the active site groups are not arranged in the correct catalytic conformation until both substrates

are bound. If shikimate kinase also adopts a similar mechanism the polypeptide movement resulting from the binding of shikimate might restrict access to, or change the nucleophilicity of, the amino acid undergoing modification.

5.4 Summary.

Shikimate kinase has been shown to be rapidly inactivated by the presence of a number of electrophiles. The presence of the ATP was shown to offer near maximal protection against inactivation of the enzyme, indicating reaction at the active site responsible for the binding of ATP. Shikimic acid was also shown to protect against inhibition albeit at a reduced level. The similarity of the degree of protection offered by each substrate for the three most effective reagents and the affinity label *(59)* (3.3.3) is strong evidence for modification at a single site. Possible sites for the modification are discussed and the role of a likely site (Cys-12) is considered by examination of the analogous ATP binding fold in adenylate kinase. The possible reasons for the observed partial protection of shikimate kinase modification, by shikimate, are also examined.

CHAPTER 6

Experimental

6.0 General Comments:

(i) Materials.

Unless otherwise stated all solvents, chemicals, and enzymes were obtained from:- Aldrich Chemical Company Ltd, Sigma Chemical Ltd or Fluka Chemicals Ltd.

(ii) Solvents.

Solvents were purified following the methods of Perrin and Armarego,[275] as follows:
Methanol and ethanol were dried and distilled from their respective magnesium alkoxides prepared *in situ*. Tetrahydrofuran (THF) and dioxan were purified by passing through an alumina column followed by distillation from sodium and benzophenone; the dried solvents were stored under nitrogen. Acetonitrile, tert-butanol, dichloromethane and ethyl acetate were distilled from calcium hydride and then stored over 4Å molecular sieves. Sodium dried benzene was used throughout. Diethyl ether (ether) was dried over sodium wire and then purified by reflux then distilling from lithium aluminium hydride.

(iii) Methods

Flash chromatography was carried out according to the method of Still *et al.*[276] using silica (Merck and Co., Kiesel 60, 230-400 mesh). Thin layer chromatography (t.l.c.) was conducted on precoated aluminium sheets (60F-254) with a 0.2 mm layer thickness manufactured by Merck and Co. Triethylammonium bicarbonate (TEAB) buffer was prepared by bubbling gaseous CO_2 through the appropriate triethylamine and water mixture until the solution pH was 7.0-7.5.

(iv) Instrumentation.

Melting points were determined using a Kofler hot stage apparatus and are reported uncorrected. The pHs of aqueous solutions were measured with a single glass electrode. Infra-Red IR Spectra were recorded with a Perkin-Elmer 298 and Perkin-Elmer 1605 FTIR spectrometers. Ultraviolet and visible spectophotomeric measurements and assays were

preformed on a Shimadzu UV-240 Graphicord spectrophotometer with a Shimadzu PR-1 graphic printer, the UV cell jackets were temperature equilibrated by a Lauda thermocoupled water bath. Low resolution mass spectra were recorded on a V. G. Micromass 16B instrument operating at 70 eV. High resolution, chemical ionisation (CI) and electronic ionisation (EI) mass spectrometery were carried out by the SERC Mass spectrometery Centre at University College Swansea. Routine ^{1}H n.m.r spectra were recorded with a Varian EM-390 (90MHz) spectrometer. Routine ^{31}P n.m.r. spectra (^{1}H-decoupled) were recorded on a Joel FX60 spectrometer at 24.15 MHz or with a Joel JNM-FX90Q spectrometer at 36.21 MHz, positive shifts are downfield from the external standard tetrahydroxyphosphonium ion $[P(OH)_4]^+$.[277] Highfield n.m.r. spectra were recorded with a Bruker AM300 n.m.r. spectrometer (^{1}H at 300MHz, ^{13}C at 75MHz, ^{31}P at 121.5MHz, ^{19}F 282.4MHz). Chemical shifts are reported as positive when downfield of the reference, and negative when upfield.

(v) Shikimate kinase and enzyme assay.

Shikimate kinase was a kind gift of John R. Coggins (Department of Biochemistry, University of Glasgow) and was purified to homogeniety by his research group.[54] The properties and characteristics of the enzyme are catalogued in chapter 1 (section 1.3). Shikimate kinase activity was assayed at 25°C by coupling the release of ADP to the pyruvate kinase (PK) and lactate dehydrogenase (LDH) reactions[54] (figure 6.1).

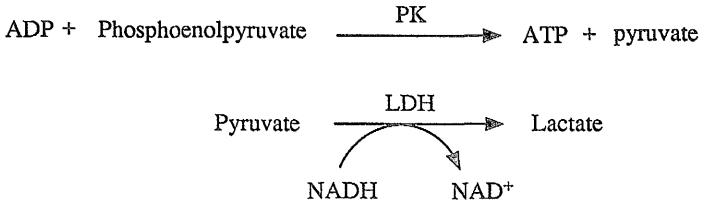


Figure 6.1

Shikimate-dependant oxidation of NADH was monitored at 340 nm (ϵ = 6180 $M^{-1}cm^{-1}$). The assay mixture (3 ml volume) contained (final concentrations) 50 mM-triethanolamine hydrochloride/KOH buffer, pH 7.0, 50 mM-KCl, 5 mM-$MgCl_2$, 1.6 mM-shikimate acid, 2.5

mM-ATP, 1 mM-phosphoenolpyruvate, 0.1 mM-NADH, 2 units of pyruvate kinase/ml (from BLC Boeringer) and 2 units of lactate dehydrogenase/ml (from BLC Boeringer). One unit of enzyme activity is defined as the amount of enzyme catalysing the conversation of 1 μmol of substrate/min. Shikimate kinase was stored in a 50% (v/v) glycerol/buffer (50 mM-Tris/HCl, pH 7.5, containing 50 mM-KCl, 5 mM $MgCl_2$ and 0.4 mM dithiothreitol) and sealed in a Wheaton micro vial in an argon atmosphere at -20 °C.

(vi) New compounds

All new compounds were characterised by high field ^{1}H, ^{13}C, and ^{31}P (where appropriate) n.m.r and by high resolution chemical ionisation, or electronic ionisation, mass spectrometry.

6.1 Chapter 2, Synthesis.

6.1.1 Preparation of [^{18}O]-Tetraethyl Dithiopyrophosphate *(41)*.[165]

A mixture of water (97% atom ^{18}O) (250 µl, 13.9 mmol) and pyridine (2.1 ml, 26.5 mmol) was added slowly, with stirring, to diethyl thiophosphoryl chloride *(40)* (3.9 ml, 25.0 mmol). The temperature of addition was maintained at 38-40°C by periodic cooling. Upon completion of the addition (45 min) periodic cooling was continued until no further rise in temperature was observed (10 min). The stirring was continued without external cooling until the temperature had dropped to 32°C (80 min). The reaction mixture was heated to 38-40°C for two hours and left to stand at room temperature overnight. The thick white paste obtained was dissolved in ether (20 ml) and washed with water (3 x 15 ml). The organic layer was dried ($MgSO_4$), filtered, and concentrated *in vacuo*. Remaining volatile components were removed by heating rapidly (10 min) to 80°C under high vacuum. The product *(41)* was isolated as a clear liquid (4.18 g, 76%).

$\delta(^{31}P)$ (24.15 MHz, neat) δ 52.44 (s)

6.1.2 Preparation of [^{18}O]-Tetrakis(trimethylsilyl) Dithionopyrophosphate *(42)*.[166]

(41) (1.01 g, 2.56 mmol) was treated with trimethylsilyl iodide (1.82 ml, 12.79 mmol) in a sealed vial at 108°C for 18 hours to give a dark brown mixture. The product was not isolated, but directly hydrolysed as described below.

$\delta(^{31}P)$ (24.15 MHz, reaction mixture) δ 25.82 (s)

6.1.3 Preparation of [^{18}O]- Dithiopyrophosphate *(43)*.[166]

The reaction mixture of *(42)* was added, with stirring, to sodium bicarbonate (1.2 g, 14.29 mmol) dissolved in water (10 ml). The pH of the decolourised solution was adjusted to pH 8 with sodium bicarbonate. The reaction mixture was diluted with water (20 ml) and washed with ether (3 x 10 ml). The aqueous solution was further diluted with water (150 ml) and applied to an ion-exchange column (130 ml) of DEAE-Sephadex A-25 resin. The column was eluted with a linear gradient of TEAB (200 mM - 600 mM), pH 7.6, over 30 hours (total volume of elutant used 3400 ml). The appropriate fractions (20 ml) (judged by the use of the Briggs test[278]) were combined and concentrated. TEAB was removed by coevaporation with methanol (4 x 50 ml). The product *(43)* (tris- triethylammonium salt) eluted at approximate buffer concentration of 350 - 470 mM, and was isolated as a clear yellow gum (0.98 g, 75%).

$\delta(^{31}P)$ (24.15 MHz,CH_3OH) δ 39.33 (s)

6.1.4 Preparation of Bridge -^{18}O -Labelled Pyrophosphate *(39)*.[167]

Bromine (464 µl, 11.96 mmol) was quickly added to a rapidly stirring solution of *(43)* (0.98 g, 1.91 mmol) in water (6 ml). After 5 min excess bromine was quenched by addition of a solution of sodium bicarbonate (1.28 g, 15.28 mmol) and mercaptoethanol (40 µl, 0.77 µmol) in water (20 ml) with rapid stirring. The reaction mixture was diluted with water (150 ml) and applied to an ion-exchange column (130 ml) of DEAE-Sephadex resin. The column was eluted with a linear gradient of TEAB (250 mM - 600 mM), pH 7.6, over 26 hours (total volume of elutant used 3000ml). The pyrophosphate-containing fractions (20 ml) (determined by the Briggs phosphate test[278]) were combined and concentrated. TEAB was removed as before. The product *(39)* (tris-triethylammonium salt) eluted at approximate buffer concentration 420 - 480 mM, and was isolated as a clear gum (0.64 g, 77%).

δ[^{31}P] (24.15 MHz, CH_3OH) -9.08 (s).

6.1.5 Preparation of Adenosine 5′[β,γ-^{18}O] Triphosphate *(38)*.[168]

Bridge-^{18}O-labeled pyrophosphate *(39)* (600 μmol) was dissolved in water (5 ml) and applied to a column of Dowex 50-W resin (pyridinium form, 40 ml). The resin was eluted with water (150 ml), and the total filtrate evaporated *in vacuo* to a volume of approximately 10 ml. Pyridine (20 ml) was added followed by tri-n-butylamine (0.43 ml, 1.8 mmol). The solution was evaporated to a semi-solid and rendered anhydrous by coevaporation with pyridine (3 x 5 ml).

Adenosine 5′-phosphomorpholidate *(44)* (0.18 g, 250 μmol) was rendered anhydrous by coevaporation with pyridine (2 x 5 ml). The pyrophosphate *(39)* was dissolved in DMSO (6 ml) and added to *(44)*. The solution was left to stand at room temperature under an atmosphere of dry nitrogen for 120 hours. The reaction mixture was then diluted with water (100 ml) and applied to an ion-exchange column (130 ml) of DEAE-Sephadex resin. The column was eluted with a linear gradient of TEAB (100 mM - 700 mM), pH 7.6, over 26 hours (total volume of elutant used 3000 ml). The eluent from the column was continuously monitored by a UV detector at λ 260 nm. The appropriate fractions (18 ml) were combined, concentrated and the TEAB was removed as before. The product *(38)* (tris-triethylammonium salt) eluted at approximate buffer concentration 460 - 500 mM, and was isolated as a clear gum (421 μmol, 70%).

δ[^{31}P] (24.15 MHz, CH_3OH) -9.48 (d, *J* 19 Hz, Pγ), -11.39 (d, *J* 24 Hz, Pα), -22.99 (t, *J* 19 and 24 Hz, Pβ).

Enzymic assay of [β,γ-^{18}O]ATP with shikimate kinase in the presence of shikimic acid, under standard assay conditions, gave equivalent rates of catalysis as for unlabelled ATP.

6.1.6 Preparation of Adenosine 5′-[α,β-^{18}O] Diphosphate *(45)*[169] and Adenosine 5′-[α,β-^{18}O] Triphosphate *(46)*.

Bridge-^{18}O-labeled pyrophosphate *(39)* (400 μmol) was dissolved in water (5 ml) and passed through a column of Dowex 50-W resin (H^+ form, 20 ml) into a flask containing tetra-n-butylammonium hydroxide (0.78 ml, 1.2 mmol). The resin was washed with water (100 ml) and the total filtrate evaporated to dryness. The pyrophosphate *(39)* was rendered anhydrous by coevaporation with acetonitrile (5 x 2 ml). 5′-Tosyladenosine *(47)* (78.8 mg, 187 μmol) was similarly dried, added to the pyrophosphate, and solvent removed *in vacuo*. The resulting gum was maintained in an atmosphere of dry nitrogen at 27°C for 4 days. The reaction was diluted with water (100 ml) and applied to an ion-exchange column (160 ml) using DEAE-Sephadex resin. The column was eluted using a linear gradient of TEAB (100 mM - 600 mM), pH 7.6, over 20 hours (total volume of elutant used 3000 ml). The eluent from the column was continuously monitored by a UV detector at λ 260 nm. The appropriate fractions (20 ml) were combined, concentrated and TEAB was removed as before. The product *(45)* (triethylammonium salt) (129 μmol, 69%) coeluted with the excess pyrophosphate (triethylammonium salt) at approximate buffer concentration 420 - 480 mM.

The mixture of ADP *(45)* and pyrophosphate *(39)* was dissolved in tris(hydroxymethyl)amino methane buffer (5 ml), pH 7.6, containing 120 mM-potassium chloride, and 60 mM-magnesium chloride. Phospho(enol)pyruvate (80 mg, 300 μmol) and pyruvate kinase (0.5 mg, ~250 units, salt free powder from rabbit muscle) were added. The mixture was stirred at room temperature for 2 hours and then diluted with water (50 ml) and applied to an ion-exchange column (80 ml) of DEAE-Sephadex resin. The column was eluted with a linear gradient of TEAB (100 mM - 700 mM), pH 7.6, over 20 hours (total volume of elutant used 1800 ml). The eluent from the column was continuously monitored at λ 260 nm. The appropriate fractions were combined, concentrated and TEAB was removed as before. Adenosine 5′-[α,β-^{18}O]triphosphate *(46)* eluted at approximate buffer concentration 470 - 550 mM, and was isolated as a clear gum (123 μmol, 95%).

$\underline{\delta(^{31}P)}$ (24.15 MHz, CH_3OH/Et_3N) -8.3 (d, *J* 19 Hz, P_γ), -11.3 (d, *J* 24 Hz, P_α), -22.4 (t, *J* 19 and 24 Hz, P_β).

[α,β-^{18}O]ATP *(46)* was dissolved in buffer (as before) (5 ml), and D-glucose (200 mg, 1.1 mmol) and hexokinase (1 mg, ~ 150 units, lyophilised powder from bakers yeast) added. The reaction mixture was stirred for 2 hours at room temperature. The mixture was diluted with water and applied to an ion exchange column of DEAE-Sephadex resin. The column was eluted with a linear gradient of TEAB (50 mM - 450 mM), pH 7.6, over 20 hours (total volume of elutant used 1800 ml). The eluent from the column was continuously monitored at λ 260 nm. The appropriate fractions were combined, concentrated and TEAB was removed as before. Adenosine 5′-[α,β-^{18}O]diphosphate *(45)* eluted at approximate buffer concentration 310 - 360 mM, and isolated as a clear gum (114 μmol, 93%).

$\underline{\delta(^{31}P)}$ (24.15 MHz, CH_3OH/Et_3N) -6.1 (d, *J* 24 Hz, P_β) -10.6 (d, *J* 24 Hz, P_α).

6.1.7 Preparation of Diadenosine 5′,5‴-P^1,P^3-Triphosphate (Ap_3A) *(48)* from ATP and 5′-Tosyladenosine *(47)*.[169]

Adenosine 5′triphosphate (55.1 mg, 100 μmol) was dissolved in water (2 ml) and passed through a column of Dowex 50-W (pyridinium form, 10 ml) directly into a 40% solution of tetrabutylammonium hydroxide (0.195 ml, 300 μmol). The resin was washed with water (50 ml) and the total filtrate concentrated *in vacuo* to a clear gum. The ATP salt was rendered anhydrous by coevaporation with dry acetonitrile. 5′-tosyladenosine *(47)* (84.3 mg, 200 μmol) was similarly dried, added to the ATP, and solvent removed *in vacuo*. The reaction mixture was maintained in an atmosphere of dry nitrogen at 27°C for 4 days. The reaction mixture was diluted with water (80 ml) and applied to an ion-exchange column (70 ml) of DEAE-Sephadex resin. The column was eluted with a linear gradient of TEAB (100 mM - 800 mM), pH 7.6, over 14 hours (total volume of elutant used 1000 ml). The elutant from

the column was monitored continuously at λ 260 nm. The appropriate fractions (8 ml) were combined and concentrated. TEAB was removed by coevaporation with methanol (4 x 20 ml). Ap_3A *(48)* (triethylammonium salt) eluted at approximate buffer concentration 355 - 400 mM, and was isolated as a clear gum (21 μmol, 21%).

δ[^{31}P] (24.15 MHz, $CH_3OH/_nBu_3N$) -12.10 (d, *J* 19 Hz, Pα), -23.19 (t, *J* 19 Hz, Pβ).

6.1.8 Preparation of Diadenosine 5′,5‴-P^1,P^3-[α,β-^{18}O] Triphosphate ([α,β-^{18}O]Ap_3A) *(48)* from [α,β -^{18}O]ADP *(45)* and AMP-morpholidate *(44)*.[168]

[α,β-^{18}O] ADP *(45)* (55 μmol) was dissolved in water and passed through a column of Dowex-50W resin (H^+ form, 5 ml) directly into a flask containing tri-n-butylamine (27.3 μl, 115 μmol). The resin was washed with water (70 ml) and the total filtrate evaporated to dryness. The resulting gum was rendered anhydrous by coevaporation with pyridine (2 x 5 ml). Adenosine 5′phosphomorpholidate *(44)* was similarly dried. *(45)* was added to *(44)* with the aid of three 0.5 ml portions of dry DMSO, and the reaction mixture stored in an atmosphere of dry nitrogen at 27°C. After 3 days solvent was removed *in vacuo*, and the reaction mixture diluted with water (80 ml). The solution was applied to an ion-exchange coloumn of DEAE-Sephadex resin (70 ml). The column was eluted with a linear gradient (100 mM - 650 mM), pH 7.6, over 16 hours (total volume of elutant used 1000 ml). The eluent from the column was continuously monitored at λ 260 nm. The appropriate fractions were combined and concentrated. TEAB was removed as before. Diadenosine 5′,5‴-P^1,P^3-[α,β-^{18}O]triphosphate (triethylammonium salt) *(48)* eluted at approximate buffer concentration 355 - 395 mM, and was isolated as a clear gum (24 μmol, 44%).

δ[^{31}P] (24.15 MHz, CH_3OH) -11.5 (d, *J* 19 Hz, Pα), -22.6 (t, *J* 19 Hz, Pβ).

6.2 Chapter 2, Positional Isotope Exchange.

6.2.1 Experiment 1 - 40% Reaction.

The following incubation was set up at 25°C containing (final concentrations);

50 mM-triethanolamine hydrochloride/KOH buffer, pH 7.0, containing 50 mM-KCl and 5 mM-$MgCl_2$ (buffer A)(30 ml)

1 mM [β,γ-^{18}O]ATP (triethylammonium salt) *(38)*

0.5 mM shikimate acid

5 units shikimate kinase (from solution in storage buffer)

1.8 μM Ap_5A

After 10 min ~40% turnover of ATP was observed (assay of a 100 μl aliquot using NADH, PEP, pyruvate kinase and lactate dehydrogenase as in the standard shikimate kinase assay). The reaction solution was then diluted with water (70 ml) and applied directly to an ion-exchange column (15 ml) using DEAE-Sephadex resin. The column was eluted with a linear gradient of TEAB (200 mM - 800 mM), pH 7.6, over 10 hours (total volume of elutant used 600 ml). The eluent from the column was continuously monitored at λ 260 nm. The appropriate fractions (5 ml) were combined and concentrated. TEAB was removed by coevaporation with methanol (3 x 10 ml). 17 μmol of ATP and 13 μmol of ADP (triethylammonium salts) were isolated indicating 43% ATP hydrolysis. The reisolated ATP was analysed using ^{31}P-NMR; 121.5 MHz, in 250 mM-3-cyclohexylamino-1-propanesulphonic acid (CAPS), 30 mM EDTA, 20% D_2O, pH 9.6 (figure 2.7, A)

6.2.2 Experiment 2 - Control Incubation.

The following incubation was set up at 25°C containing (final concentrations);

Buffer A (10 ml)

2 mM [β,γ-^{18}O]ATP (triethylammonium salt) *(38)*

1 unit shikimate kinase (from solution in storage buffer)

1.8 μM Ap_5A

After 18 hours the mixture was diluted with water (80 ml) and the ATP (16 μmol) was isolated and analysed by ^{31}P nmr as before (figure 2.7, B)

6.2.3 Experiment 3 - Incubation with Fluoro Analogue *(51)*.

The following incubation was set up at 25°C containing (final concentrations);

Buffer A (10 ml)

2 mM [β,γ-^{18}O]ATP (triethylammonium salt) *(38)*

25 mM *trans*-3-fluoro-4-hydroxycyclohex-1-ene-1-carboxylic acid *(18)*

1 unit shikimate kinase (from solution in storage buffer)

1.8 μM Ap_5A

After 18 hours the mixture was diluted with water (80 ml) and the ATP (16 μmol) was isolated by ion-exchange chromatography as in experiment 1. Analysis was also as in experiment 1 (for spectra see figure 2.7, C).

6.2.4 Experiment 4 - Controlled Incubation Minus Glycerol.

The following incubation was set up at 25°C containing (final concentrations);

Buffer A (10 ml)

2 mM [β,γ-^{18}O]ATP (triethylammonium salt) *(38)*

1 unit shikimate kinase (from 0.2 ml solution dialysed against storage buffer lacking glycerol 2 x 500 ml at 4°C over 8 hours)

1.8 μM Ap_5A

After 18 hours ATP (15 μmol) was isolated and analysed as before (for spectra see figure 2.8, D).

6.2.5 Experiment 5 - Controlled Incubation Plus Glycerol.

The following incubation was set up at 25°C containing (final concentrations);

Buffer A (10 ml)

2 mM [β,γ-^{18}O]ATP (triethylammonium salt) *(38)*

1 unit shikimate kinase (from 0.2 ml solution dialysed against storage buffer lacking glycerol 2 x 500 ml at 4°C over 8 hours)

5 μl glycerol

1.8 μM Ap_5A

After 18 hours ATP (16 μmol) was isolated and analysed as before (for spectra see figure 2.8, E).

6.3 Chapter 3.

6.3.1 Preparation of 3-(Diethylamino)-carbomethoxycyclohexene *(64)*.[191]

To a stirred mixture of diethylamine (131.6g, 1.80 mol) and anhydrous potassium carbonate (30 g) cooled to -10°C was added dropwise, during 30 min, a solution of crotonaldehyde *(61)* (freshly distilled under nitrogen) (63.1 g, 0.90 mol) in dry benzene (90 ml). The mixture was allowed to warm to 0°C over a period of 1 hour with gentle swirling at intervals. The mixture was then allowed to warm to room temperature and left to stand for 4 hours, filtered and hydroquinone (0.5 g) added. Excess diethylamine and solvent were removed *in vacuo*. Vacuum distillation at 62-64°C, 0.2 mmHg afforded diethylaminobutadiene *(62)* as a yellow oil (65.3g, 58%). To a solution of the freshly distilled butadiene *(62)* in dry benzene (75 ml) was added freshly distilled methyl acrylate *(63)* (71.7 g, 0.83 mol) and the mixture allowed to stand at room temperature in the dark for 6 days. The reaction mixture was shaken with ether (100 ml) and the adduct extracted with 2M HCl (400 + 100 ml). The combined aqueous layers were then basified by the slow addition of NaOH pellets at 0°C, and extracted with ether (200 + 100 ml). The combined extracts were dried ($MgSO_4$), filtered, and concentrated *in vacuo*. Vacuum distillation (101 - 104°C, 0.2 mmHg) gave 53.0 g (48%) of 3-(diethylamino)-carbomethoxycyclohexene *(64)* as a colourless oil (mixture of diastereoisomers); R_f 0.45 (4% methanol/dichloromethane).

IR $\nu_{max}(CH_2Cl_2)$: 3020w, 2970m, 2930m, 2840w,1735s cm^{-1}.

$\delta(^1H)$ (300 MHz, $CDCl_3$): 5.95-5.87 (0.5H, m), 5.82-5.62 (1.5H, m), 3.70-3.62 (0.5H, m) overlapping with 3.67 (1.5H, s) and 3.66 (1.5H, s), 3.62-3.55, (0.5H, m), 2.75-2.36 (5H, m), 2.25-1.71, (4H, m), 0.99 (3H, t, *J* 7.1 Hz), 0.95 (3H, t, *J* 7.1 Hz).

$\delta(^{13}C)$ (75 MHz,$CDCl_3$): 175.0 and 173.1 (CO_2CH_3), 129.0, 127.8, 127.0 and 125.0 (3-C and 4-C), 58.1 and 54.8 (2-C), 50.3 and 49.9 (CO_2CH_3), 45.4 and 44.2 (1-C), 45.2 and 43.7 (5-C), 25.4, 24.1, 23.5 and 19.5 (6-C and CH_2CH_3), 14.1 and 13.3

(CH_2CH_3).

6.3.2 Preparation of Methyl 2,3-Dihydrobenzoate *(65)*.[191]

To a solution of 3-(diethylamino)-carbomethoxycyclohexene *(64)* (53 g, 251 mmol) in dichloromethane (100 ml) was added methyl iodide (7.7 equiv, 273.6 g). The reaction mixture was stirred at room temperature for 21 hours. The reaction mixture was concentrated *in vacuo* and the residue was dissolved in methylene chloride (300 ml). The solution was cooled (4°C) and DBU (2.0 equiv, 76 g) added dropwise over 10 min. The reaction mixture was stirred at room temperature for 30 min. The solution was washed with 1 M HCl (300 ml). The organic layer was dried (Na_2SO_4), filtered, and concentrated *in vacuo* to give a brown liquid. Vacuum distillation (42-46°C, 0.2 mmHg) gave 24.6 g (72%) of methyl 2,3-dihydrobenzoate *(65)* as a colourless oil; R_f 0.72 (4% methanol/dichloromethane).

IR $\nu_{max}(CH_2Cl_2)$: 3030w and 2995w, 2950m, 2882m, 2838m, 1702s, 1638m cm^{-1}.

$\delta(^1H)$ (300 MHz, $CDCl_3$): 6.99 (1H, md, $J_{2,3}$ 5.3 Hz, 2-H), 6.12 (1H, dtd, $J_{4,3}$ 9.3, $J_{4,5}$ 4.2, $J_{4,2}$ 1.0 Hz, 4-H), 6.05 (1H, ddt, $J_{3,4}$ 9.3, $J_{3,2}$ 5.3, $J_{3,5}$ 1.8 Hz, 3-H), 3.75 (3H, s, OCH_3), 2.45 (2H, ddt, J_{gem} 10.8, $J_{6,5a}$ 9.2, J 1.6 Hz [=($J_{6,5e}$ + $J_{6,2}$)/2], 6-H), 2.26 (2H, m, 5-H).

$\delta(^{13}C)$ (75 MHz, $CDCl_3$): 167.7 (CO_2CH_3), 133.3, 133.0 and 123.8 (2-C, 3-C, and 4-C), 126.9 (1-C), 51.4 (CO_2CH_3), 22.7 and 20.6 (5-C and 6-C).

6.3.3 Preparation of Methyl-3,4-epoxy-cyclohex-1-ene-1-carboxylate *(52)* (Methyl-7-Oxabicyclo[4.1.0] hept-2-ene-3-carboxylate).[193]

A solution of the diene *(65)* (8.0 g, 58 mmol) in methylene chloride (200 ml) was cooled to -78°C. *m*-CPBA (1.1 equiv, 13.8 g) was added and the solution stirred at -78°C for 30 min. The solution was allowed to warm to room temperature and was stirred for 2 hours. The

solution was cooled to -78°C, filtered, and washed with saturated Na_2SO_3 (100 ml), H_2O and saturated $NaHCO_3$ (50 ml each). The organic layer was dried (Na_2SO_4) and concentrated *in vacuo* to give 8.5 g (95%) of methyl-3,4-epoxy-cyclohex-1-ene-1-carboxylate *(52)* as a light yellow oil; R_f 0.65 (ethylacetate).

IR $\nu_{max}(CH_2Cl_2)$: 3030w and 3000w, 2950m, 2850w, 1712s, 1642m cm^{-1}.

$\delta(^1H)$ (300 MHz, $CDCl_3$): 7.09 (1H, dd, $J_{2,3}$ 4.2, $J_{2,6a}$ 3.1 Hz, 2-H), 3.75 (3H, s, OCH_3), 3.59 (1H, ddt, $J_{4,3}$ 4.2, $J_{4,5e}$ 2.5, J 0.8 Hz [=($J_{4,5a}$ + $J_{4,6?}$)/2], 4-H), 3.39 (1H, t, J 4.2 Hz [=($J_{3,2}$ + $J_{3,4}$)/2], 3-H), 2.57 (1H, ddt, J_{gem} 16.9, $J_{6e,5a}$ 6.3, $J_{6e,5e}$ 1.5 Hz, 6e-H), 2.34 (1H, dddd, J_{gem} 14.5, $J_{5e,6a}$ 7.6, $J_{5e,4}$ 2.5, $J_{5e,6e}$ 1.5 Hz, 5e-H), 2.07 (1H, dddd, J_{gem} 16.9, $J_{6a,5a}$ 12.5, $J_{6a,5e}$ 7.6, $J_{6a,2}$ 3.1 Hz, 6a-H), 1.61 (1H, dddd, J_{gem} 14.5, $J_{5a,6a}$ 12.5, $J_{5a,6e}$ 6.3, $J_{5a,4}$ 0.8 Hz, 5a-H).

$\delta(^{13}C)$ (75 MHz, $CDCl_3$): 166.5 (CO_2CH_3), 134.1 (1-C), 133.7 (2-C), 55.5 and 46.3 (3-C and 4-C), 51.9 (CO_2CH_3), 21.0 and 19.5 (5-C and 6-C).

6.3.5 Preparation of 2,3-Dihydrobenzoic Acid *(66)*.

To a solution of freshly distilled ester *(65)* (8.4 g, 51 mmol) in THF (35 ml) was added 4M NaOH (40 ml). The reaction mixture was stirred and methanol (30 ml) added, during 30 min, to achieve homogeneity. The reaction mixture was stirred at room temperature for 3.5 hours and volatile components removed *in vacuo*. The aqueous residue was acidified with 1 M HCl to pH 1 and extracted with ethyl acetate (3 x 100 ml). The extracts were combined, dried ($MgSO_4$), filtered, and concentrated *in vacuo* to give a brown oil. Recrystallisation (H_2O) and desiccation over P_2O_5 gave 5.0 g (66%) of 2,3-dihydrobenzoic acid *(66)* as a white powder; R_f 0.34 (5% methanol/dichloromethane).

IR $\nu_{max}(CH_2Cl_2)$: 2950m (broad), 2620w (broad), 1688s, 1580m cm^{-1}.

$\delta(^1H)$ (300 MHz, $CDCl_3$): 11.51 (1H, br s, CO_2H), 7.12 (1H, dtd, $J_{2,3}$ 5.4, $J_{2,6}$ 1.6, $J_{2,4}$ 1.1 Hz, 2-H), 6.19 (1H, dtd, $J_{4,3}$ 9.4, $J_{4,5}$ 4.3, $J_{4,2}$ 1.1 Hz, 4-H), 6.10 (1H, ddt, $J_{3,4}$

9.4, $J_{3,2}$ 5.4, $J_{3,5}$ 1.8 Hz, 3-H), 2.45 (2H, ddt, J_{gem} 10.8, $J_{6,5a}$ 9.2, J 1.6 Hz [=($J_{6,5b}$ + $J_{6,2}$)/2], 6-H), 2.29 (2H, m, 5-H).

δ(^{13}C) (75 MHz, $CDCl_3$): 173.1 ($C0_2H$), 135.4, 134.6 and 126.3 (2-C, 3-C and 4-C), 126.3 (1-C), 22.8 and 20.2 (5-C and 6-C).

m/z (EI) 124 (M^+, 10%), 122 (25), 105 (35), 79 (24), 77 (40).

6.3.7 Preparation of Methyl-3,4-*trans*-dihydroxycyclohex -1-ene-1-carboxylate *(69)*.

To a solution of the epoxide *(52)* (122 mg, 0.79 mmol) in acetonitrile (2 ml) and water (1 ml) was added Dowex 50-W (H^+ form, 300 mg) and the mixture stirred for 15 min. The reaction mixture was filtered and acetonitrile and water were removed *in vacuo*. Flash chromatography (SiO_2; ethyl acetate) and recrystallisation (ethyl acetate-hexane) gave 119 mg (87%) of methyl-3,4-*trans*-dihydroxycyclohex-1-ene-1-carboxylate *(69)* as white crystals; m.p. 89.5-91.5°C; R_f 0.28 (ethyl acetate);

IR ν_{max}(nujol): 3500-3150m (broad), 2600w (broad), 1716s cm^{-1}.

δ(^{1}H) (300 MHz, $CDCl_3$): 6.69 (1H, br s, 2-H), 4.42 exchangeable with D_2O (1H, br s, OH), ~4.2 exchangeable with D_2O (1H,br s, OH) overlapping with 4.19 (1H, md, $J_{3,4}$ 7.8 Hz, 3-H), 3.74 (3H, s, OCH_3), 3.62 (1H, ddd, $J_{4,5a}$ 11.8, $J_{4,3}$ 7.8, $J_{4,5e}$ 3.8 Hz, 4-H), 2.47 (1H, md, J_{gem} 18 Hz, 6-H), 2.30 (1H, md, J_{gem} 18 Hz, 6-H), 1.99 (1H, md, J_{gem} 13 Hz, 5-H), 1.64 (1H, dtd, J_{gem} 12.9 Hz, J 11.6 [=($J_{5a,4}$ + $J_{5a,6a}$)/2], $J_{5a,6e}$ 6.1 Hz, 5a-H).

δ(^{13}C) (75 MHz, $CDCl_3$): 167.1 (CO_2CH_3), 138.8 (2-C), 131.2 (1-C), 73.0 and 72.4 (3-C and 4-C), 52.0 (CO_2CH_3), 28.4 and 24.1 (5-C and 6-C).

m/z (EI) 172 (M^+, 4%), 141 (20), 140 (92), 129 (23), 128 (26); HRMS calcd for $C_8H_{12}O_4$ (M^+) 172.0736, found 172.0735.

6.3.8 Preparation of 3,4-*Trans*-dihydroxycyclohex-1-ene-1-carboxylic Acid *(67)*.

To a solution of the ester *(69)* (72 mg, 0.42 mmol) in 1:1 (v/v) THF:H_2O (3 ml) was added 1 M NaOH (1 ml) and the solution was stirred for 1 hour. THF and water were removed *in vacuo*. The residue was redissolved in water (3 ml) and passed slowly through a column of Dowex 50-W (H^+ form, 25 ml). The resin was washed with water (200 ml) and the total filtrate concentrated *in vacuo* to give 66 mg (100%) of the 3,4-*trans*-dihydroxycyclohex-1-ene-1-carboxylic acid *(67)* as a white solid.

IR ν_{max}(nujol): 3500-3000m (broad), 2600 (broad), 1693s cm^{-1}.

$\delta(^1H)$ (300 MHz, D_2O): 6.66 (1H, dt, $J_{2,3}$ 2.7, J 1.5 Hz [=($J_{2,6a}$ + $J_{2,6e}$)/2], 2-H), 4.20 (1H, dq, $J_{3,4}$ 7.4, J 2.7 Hz [=($J_{3,2}$ + $J_{3,5}$ + $J_{3,6?}$)/3], 3-H), 3.67 (1H, ddd, $J_{4,5a}$ 10.8, $J_{4,3}$ 7.4, $J_{4,5e}$ 3.8 Hz, 4-H), 2.43-2.20 (2H, m, 2x 6-H), 1.98 (1H, ddt, J_{gem} 13.1, $J_{5e,6}$ 5.3, J 3.8 [=($J_{5e,4}$ + $J_{5e,6}$)/2], 5e-H), 1.72 (1H, dddd, J_{gem} 13.1, $J_{5a,4}$ 10.8, $J_{5a,6a}$ 9.2, $J_{5a,6e}$ 6.5 Hz, 5a-H).

$\delta(^{13}C)$ (75 MHz, D_2O): 173.1 (CO_2H), 141.5 (2-C), 134.3 (1-C), 73.94 and 73.90 (3-C and 4-C), 30.1 and 25.6 (5-C and 6-C).

m/z (EI) 158 (M^+, 3%), 140 (90), 122 (13%), 114 (67), 96 (>100); HRMS calcd for $C_7H_{10}O_4$ (M^+) 158.0579, found 158.0580.

6.3.9 Preparation of Methyl-3,4-*cis*-dihydroxycyclohex-1-ene-1-carboxylate *(74)*.

To a mixture of N-methylmorpholine-N-oxide (0.72 ml, 3.7 mmol, 60% wt sol. in water), acetone (1 ml) and osmium tetroxide (40 µl, ~ 6 µmol, 4% wt sol. in water) in *t*-butanol (0.5 ml) was added a solution of diene *(65)* (0.46 g, 3.4 mmol) in acetone (0.5 ml). The reaction mixture was stirred in the dark, under an atmosphere of dry nitrogen, at room temperature for 16 hours. To the purple solution was added a slurry of sodium bisulfite (50 mg), magnesium silicate (talc) (660 mg). Water (5 ml) was added and the mixture filtered and the filtrate neutralised to pH 7 with 0.2 M H_2SO_4. The acetone was removed *in vacuo*, and the

pH was further adjusted to pH 2. The solution was saturated with NaCl and extracted with ethyl acetate (15 ml). The aqueous phased was concentrated *in vacuo* and further extracted with ethyl acetate (2 x 10 ml). The combined extracts were dried (Na_2SO_4) and filtered. Flash chromatography (SiO_2; ethyl acetate) and recrystallisation (ethyl acetate-hexane) gave 298 mg (54%) of methyl-3,4-*cis*-dihydroxycyclohex-1-ene-1-carboxylate *(74)* as white crystals; m.p. 71.5-73°C; R_f 0.34 (ethyl acetate).

IR ν_{max}(nujol): 3310m (broad), 3270m (broad), 1706s cm^{-1}.

$\delta(^1H)$ (300 MHz, $CDCl_3$): 6.75 (1H, m, 2-H), 4.25 (1H, m, 3-H), 3.89 (1H, ~dt, $J_{4,5e}$ ~6, J ~3 Hz [=($J_{4,3}$ + $J_{4,5a}$)/2], 4-H) overlapping with 3.86 (2H, br s, exchanges with D_2O, 2x OH), 3.74 (3H, s, OCH_3), 2.45 (1H, md, J_{gem} 18 Hz, 6-H), 2.25 (1H, md, J_{gem} 18 Hz, 6-H), 1.93 (1H, ddt, J_{gem} 13.5, $J_{5e,6a}$ 7.7 J 5.7 Hz [=($J_{5e,4}$ + $J_{5e,6e}$)/2], 5e-H), 1.17 (1H, dddd, J_{gem} 13.5, J ~7 ($J_{5a,6e}$ and $J_{5a,6a}$), $J_{5a,4}$ 3 Hz, 5a-H).

$\delta(^{13}C)$ (75 MHz, $CDCl_3$): 167.4 (CO_2CH_3), 137.5 (2-C), 132.3 (1-C), 67.4 and 66.9 (3-C and 4-C), 52.0 (CO_2CH_3), 25.9 and 21.4 (5-C and 6-C).

m/z (EI) 140 (M^+ - 32, 32%), 128 (20), 112 (8), 96 (100);
(CI) 190 ({M + NH_4}$^+$, 100%), 172 (15), 155 (16), 139 (14); HRMS calcd for $C_8H_{16}O_4N$ {M + NH_4}$^+$ 190.1079, found 190.1080.

6.3.10 Preparation of 3,4-*Cis*-dihydroxycyclohex-1-ene-1-carboxylic Acid *(68)*.

To a solution of ester *(74)* (72 mg, 0.42 mmol) in 1:1 (v/v) THF:H_2O (3 ml) was added 1 M NaOH (1 ml, ~1 mmol). The reaction mixture was stirred for 1 hour and then concentrated *in vacuo*. The residue was redissolved in water (2 ml) and passed slowly through a column of Dowex 50-W (H^+ form, 25 ml). The column was washed with water (200 ml) and the total eluent concentrated *in vacuo* to give 66 mg (100%) of 3,4-*cis*-dihydroxycyclohex-1-ene-1-carboxylic acid *(68)* as a white solid.

IR ν_{max}(nujol): 3324m (broad), 3150m (broad), 1685s cm^{-1}.

δ(^{1}H) (300 MHz, $(CD_3)_2CO$): 6.69 (1H, dt, $J_{2,3}$ 3.6, $J_{2,6}$ 1.8 Hz, 2-H), 4.24 (1H, m, 3-H), 3.86 (1H, dt, $J_{4,5e}$ 6, J 3 Hz (=[$J_{4,3}$ + $J_{4,5a}$]/2), 4-H), 2.33 (1H, dddt, J_{gem} 18, $J_{6e,5e}$ 7.5, $J_{6e,5a}$ 5.5, J 2 Hz (=[$J_{6e,2}$ + $J_{6e,3?}$]/2), 6e-H), 2.15 (1H, dtt, J_{gem} 18, J 6.3 (=[$J_{6a,5a}$ + $J_{6a,5e}$]/2), J 2 Hz (=[$J_{6a,2}$ + $J_{6a,3?}$]/2), 6a-H), 1.86 (1H, ddt, J_{gem} 14 Hz, $J_{5e,6e}$ 7.5, J 6.3 Hz [=($J_{5e,4}$ + $J_{5e,6a}$)/2], 5e-H), 1.68 (1H, dddd, J_{gem} 14, $J_{5a,6a}$ 6.3, $J_{5a,6e}$ 5.5, $J_{5a,4}$ 3 Hz, 5a-H).

δ(^{13}C) (75 MHz, $(CD_3)_2CO$): 170.1 (CO_2H), 138.9 (2-C), 133.0 (1-C), 68.1 and 67.3 (3-C and 4-C), 26.1 and 22.2 (5-C and 6-C).

m/z (EI) 158 (M^+, 1%), 140 (63), 114 (40), 96 (>100); HRMS calcd for $C_7H_{10}O_4$ 158.0579, found 158.0580.

6.3.11 Preparation of Methyl-*trans*-3-fluoro-4-hydroxycyclohex-1-ene-1-carboxylate *(53).*

To a solution of epoxide *(52)* (223 mg, 1.45 mmol) in dry ether (2 ml) was added HF-pyridine (2 ml, ~70% HF, ~30% pyridine). The reaction mixture was stirred in an atmosphere of dry nitrogen, for 1 hour. The reaction was then quenched by addition of iced water (6 ml). The product was extracted with ether (20 + 10 ml). The aqueous layer was concentrated *in vacuo* and further extracted with ether (10 ml). The combined extracts were dried (Na_2SO_4), filtered, and concentrated *in vacuo*. Flash chromatography (SiO_2; ethyl acetate) gave 187 mg (74%) of methyl-*trans*-3-fluoro-4-hydroxycyclohex-1-ene-1-carboxylate *(53)* as a light yellow oil; R_f 0.52 (ethyl acetate).

IR ν_{max}(neat): 3420m (broad), 2950m, 1715s, 1655m cm^{-1}.

δ(^{1}H) (300 MHz, $CDCl_3$): 6.76 (1H, dtd, $J_{2,F}$ 12.2, J 2.6 [=($J_{2,3}$ + $J_{2,6}$)/2], $J_{2,6}$ 1.2 Hz, 2-H), 5.02 (1H, dddt, $J_{3,F}$ 49.5, $J_{3,4}$ 7.4, $J_{3,2}$ 2.6, $J_{3,6?}$ 2.2 Hz, 3-H), 3.95 (1H, dddd, $J_{4,F}$ 14.5, $J_{4,5a}$ 11.7, $J_{4,3}$ 7.4, $J_{4,5e}$ 4.2 Hz, 4-H), 3.77 (3H, s, OCH_3), 3.4-3.2 (1H, br s, OH), 2.6-2.3 (2H, m, 2 x 6H), 2.06 (1H, m, 5e-H), 1.72 (1H, ddddd, J_{gem} 12.4, $J_{5a,4}$ 11.7, $J_{5a,6a}$ 10.5, $J_{5a,6e}$ 6.2, $J_{5a,F}$ 1.1 Hz, 5a-H).

δ(^{13}C) (75 MHz, $CDCl_3$): 166.6 (CO_2CH_3), 133.7 (d, $J_{C,F}$ 9.1 Hz, 1-C), 133.6 (d, $J_{C,F}$ 22.2 Hz, 2-C), 92.6 (d, $J_{C,F}$ 169.3 Hz, 3-C), 69.7 (d, $J_{C,F}$ 19.9 Hz, 4-C), 52.1 (s, CO_2CH_3), 27.4 (d, $J_{C,F}$ 6.6 Hz, 5-C), 23.6 (d, $J_{C,F}$ 2.6 Hz, 6-C).

δ(^{19}F) (282.4 MHz, $CDCl_3$): -184.99.

m/z (EI) 174 (M^+, 13%), 154 (24%), 142 (100%), 131 (32%); HRMS calcd for $C_8H_{11}O_3F$ 174.0692, found 174.0692.

6.3.12 Preparation of *Trans*-3-fluoro-4-hydroxycyclohex-1-ene-1-carboxylic Acid *(51)*.

To the ester *(53)* (160 mg, 0.92 mmol) was added water (5 ml) and the pH adjusted to pH 8.0 with 0.1 M NaOH. The mixture was stirred and the pH monitored continuously using a single glass electrode. The pH was maintained at pH 8.0 by the manual addition of NaOH solution. After 6 hours the pH remained constant and the reaction acidified to pH 2.5 with dil. HCl. The reaction mixture was extracted with ethyl acetate (3 x 10 ml), concentrated *in vacuo* and further extracted (10 ml). The combined extracts were dried ($NaSO_4$), filtered, and concentrated *in vacuo*. Flash chromatography (SiO_2; ethyl acetate) gave 86 mg (58%) of *trans*-3-fluoro-4-hydroxycyclohex-1-ene-1-carboxylic acid *(51)* as white solid; R_f 0.2 (ethyl acetate).

δ(^{1}H) (300 MHz, CD_3CN): 6.68 (1H, dtd, $J_{2,F}$ 11.7, J 2.6 [=($J_{2,3}$ + $J_{2,6}$)/2], $J_{2,6}$ 1.3 Hz, 2-H), 4.96 (1H, ddq, $J_{3,F}$ 48.8, $J_{3,4}$ 7.0, J 2.5 Hz [=($J_{3,2}$ + (2x $J_{3,6?}$))/3], 3-H), 3.83 (1H, dddd, $J_{4,F}$ 14.9, $J_{4,5a}$ 11.0, $J_{4,3}$ 7.0, $J_{4,5e}$ 4.0 Hz, 4-H), 2.45-2.23 (2H, m, 2 x 6H), 1.95 (1H, m, 5e-H), 1.66 (1H, ddddd, J_{gem} 13.4, $J_{5a,6a}$ 9.5, $J_{5a,6e}$ 6.3, $J_{5a,F}$ 1.0, 5a-H).

δ(^{13}C) (75 MHz, CD_3CN): 167.4 (s, CO_2H), 134.9 (d, $J_{C,F}$ 10 Hz, 1-C), 134.6 (d, $J_{C,F}$ 21.6 Hz, 2-C), 92.7 (d, $J_{C,F}$ 168.4 Hz, 3-C), 69.6 (d, $J_{C,F}$ 19.2 Hz, 4-C), 28.5 (d, $J_{C,F}$ 6.5 Hz, 5-C), 23.9 (d, $J_{C,F}$ 2.8 Hz, 6-C).

δ(^{19}F) (282.4 MHz, CD_3CN): -181.75

m/z (EI) 160 (M^+, 1%), 142 (45), 140 (15), 122 (57), 105 (68); HRMS calcd for

$C_7H_9O_3F$ 160.0536, found 160.0540.

6.3.13 Inhibitor Properties of Epoxy Analogue *(59)*.

To the epoxy-ester *(52)* (253 mg, 1.45 mmol) was added a mixture of water/THF (1:1) (2 ml). 1 M NaOH (1 ml) was added and the mixture stirred to homogeneity (2 hours) at room temperature. THF was removed *in vacuo* and the pH adjusted to pH 7.5 with 50 mM-triethanolamine hydrochloride/KOH buffer, pH 7.0, containing 50 mM-KCl and 5 mM-$MgCl_2$ (buffer A) (14 ml).

Inhibitor properties of epoxy-analogue *(59)* were examined by incubating freshly prepared *(59)* (0.75, 10 and 100 mM) with shikimate kinase in buffer A, pH 7.0, at 25°C (total volume 1 ml). Aliquots (50 µl) were assayed for activity over a time period of up to 3 hours. The rate of inactivation of the enzyme, at a particular concentration of *(59)*, was determined from plots of ln[E] verses time. Appropriate control experiments in the absence of *(59)* were also conducted.

The chemical modification was further examined by incubations in the presence of either shikimate or ATP. In each case the concentration of substrate was saturating (>10 x K_M) and adjusted to deliver the correct quantity of substrate to the enzyme assay. Aliqots of the enzyme were assayed, and the kinetic parameters determined as before

6.3.14 Substrate Properties of 5-Deoxy Analogues *(67)* and *(68)*.

The rate of catalysis V (initial velocity) was observed at different concentrations of analogue (0.2 mM - 10 mM) in the absence of shikimate. Assays contained a variety of kinase concentrations dependant on the effectiveness of the analogue as a substrate, typically 1 - 10 units of activity were used. Kinetic parameters of the catalysis were determined from Lineweaver-Burk (double reciprocal) plots.

6.3.15 Inhibition Studies of Fluoro Shikimate Analogue *(51)*.

The rate of catalysis V (initial velocity) was observed at different concentrations of shikimate (0.07, 0.1, 0.16 and 0.5 mM) in the presence of fixed concentrations of analogue *(51)*, in the range 1 - 30 mM. Kinetic parameters of the inhibition and the type of inhibition were determined from Lineweaver-Burk plots.

6.4 Chapter 4.

6.4.1 Preparation of (5*S*,6*R*)-5,6-O,O-Isopropylidene-1-(trifluoromethyl)-cyclohexa-1,3-diene *(86)*.

To a solution of (5*S*,6*R*)-5,6-dihydroxy-1-(trifluoromethyl)- cyclohexa-1,3-diene *(83)* (0.58 g, 3.2 mmol) in acetone (6 ml) was added 2,2 dimethoxypropane (1.98 ml, 16.1 mmol) and *p*-toluene sulphonic acid (60 mg, 0.3 mmol). The reaction mixture was stirred for 15 min at room temperature. The reaction was then neutralised by addition of excess $NaHCO_3$. Volatile components were removed *in vacuo* and the residue was extracted with dichloromethane (3 x 20 ml). The organic fractions were combined, dried (Na_2SO_4), filtered, and concentrated *in vacuo*. Flash chromatography (SiO_2; ethyl acetate) gave 612 mg (87%) of (5*S*,6*R*)-5,6-O,O-isopropylidene-1 -(trifluoromethyl)-cyclohexa-1,3-diene *(86)* as a colourless oil; R_f 0.69 (ethyl acetate).

δ(^{1}H) (300 MHz, $CDCl_3$): 6.57 (1H, m, 2-H), 6.07 (2-H, m, 3-H and 4-H), 4.84 (1H, ddd, $J_{5,6}$ 8.4, $J_{5,4}$ 2.1, $J_{5,3}$ 1.0 Hz, 5-H), 4.71 (1H, dd, $J_{6,5}$ 8.4, $J_{6,2}$ 0.8 Hz, 6-H), 1.43 (3H, s, CH_3), 1.42 (3H, s, CH_3).

δ(^{13}C) (75 MHz, $CDCl_3$): 131.3 (3-C or 4-C), 126.9 (q, $J_{C,F}$ 6.0 Hz, 2-C), 125.2 (q, $J_{C,F}$ 29.1 Hz, 1-C), 123.6 (q, $J_{C,F}$ 272.0 Hz, CF_3), 120.8 (3-C or 4-C), 106.4 ($C(CH_3)_2$), 71.8 and 67.6 (5-C and 6-C), 26.6 and 25.1 (2 x CH_3).

δ(^{19}F) (282.4 MHz, $CDCl_3$): -66.48 ppm.

m/z (Dimer) (CI) 441 (MH^+, 4%), 425 (6) 383 (100); HRMS calcd for $C_{20}H_{23}O_4F_6$ (MH^+) 441.1501, found 441.1500.

6.4.2 Preparation of (3*R*,4*R*,5*R*,6*R*)-3,4-Dihydroxy-5,6-O,O-isopropylidene-1-trifluoromethyl-cyclohex-1-ene *(87)*.

To a mixture of N-methylmorpholine-N-oxide (0.33 ml, 1.68 mmol, 60% wt sol. in water), acetone (1 ml) and osmium tetroxide (20 μl, ~3 μmol, 4% wt sol. in water) in *t*-butanol (0.25 ml) was added a solution of diene *(86)* (336 mg, 1.53 mmol). The reaction mixture was stirred in the dark, under an atmosphere of dry nitrogen, at room temperature for 16 hours. Work up as for 6.3.9. Flash chromatography and recrystallisation (ethyl acetate-hexane) gave 249 mg (64%) of (3*R*,4*R*,5*R*,6*R*)-3,4-dihydroxy-5,6-O,O-isopropylidene-1-trifluoromethyl-cyclohex-1-ene *(87)* as white crystals; R_f 0.52 (ethyl acetate).

IR ν_{max}(nujol): 3600-3200m cm^{-1}.

$\delta(^1H)$ (300 MHz, $CDCl_3$): 6.37 (1H, dq, $J_{2,3}$ 3.9, $J_{2,F}$ 1.3 Hz, 2-H), 4.80 (1H, d, $J_{6,5}$ 5.4 Hz, 6-H), 4.46 (1H, m, 3-H) overlapping with 4.45 (1H, t, *J* 5.4 Hz [=($J_{5,4}$ + $J_{5,6}$)/2], 5-H), 4.20 (1H, br t, *J* ~4 Hz [=($J_{4,3}$ + $J_{4,5}$)/2], 4-H), 3.60 (2H, br s, 2 x OH), 1.40 (6H, s, 2 x CH_3).

$\delta(^{13}C)$ (75 MHz, $CDCl_3$): 133.4 (q, $J_{C,F}$ 5.2 Hz, 2-C), 128.5 (q, $J_{C,F}$ 30.0 Hz, 1-C), 122.6 (q, $J_{C,F}$ 273.6 Hz, CF_3), 110.5 ($C(CH_3)_2$), 75.3, 70.0, 69.0, 65.3 (3-C, 4-C, 5-C and 6-C), 27.4 and 25.9 (2 x CH_3).

$\delta(^{19}F)$ (282.4 MHz, $CDCl_3$): -66.29 ppm.

m/z (EI) 239 (M^+-15, 38%), 179 (10), 161 (11), 151 (14);
(CI) 255 (MH^+, 69%), 101 (100%); HRMS calcd for $C_{10}H_{14}O_4F_3$ (MH^+) 255.0844, found 255.0840.

6.4.3 Preparation of (3*R*,4*R*,5*R*,6*R*)-3,4,5,6-Tetrahydroxy-1-trifluoromethyl-cyclohex-1-ene *(85)*.

To a solution of diol *(87)* (75 mg, 0.30 mmol) in acetonitrile (3 ml) and water (1 ml) was added Dowex 50-W resin (H^+ form, 200 mg) and stirred at room temperature for 12.5 days.

The reaction mixture was filtered and volatile components removed *in vacuo* to give 63 mg (100%) of (3*R*,4*R*,5*R*,6*R*)-3,4,5,6-tetrahydroxy-1-trifluoromethyl-cyclohex-1-ene *(85)* as a white solid; R_f 0.13 (ethyl acetate).

δ(^{1}H) (300 MHz, $CDCl_3/CD_3OD$): 6.44 (1H, br d, $J_{2,3}$ 3.9 Hz, 2-H), 4.46 (1H, d, $J_{6,5}$ 3.8 Hz, 6-H), 4.42 (1H, m, 3-H), 3.98 (1H, dd, $J_{4,5}$ 9.3, $J_{4,3}$ 4.1 Hz, 4-H), 3.90 (1H, dd, $J_{5,4}$ 9.3, $J_{5,6}$ 3.8 Hz, 5-H).

δ(^{13}C) (75 MHz, $CDCl_3/CD_3OD$): 133.9 (q, $J_{C,F}$ 5.5 Hz, 2-C), 130.7 (q, $J_{C,F}$ 29.5 Hz, 1-C) 123.7 (q, $J_{C,F}$ 123.7 Hz, CF_3), 69.5, 68.9, 65.7, 65.1 (3-C, 4-C, 5-C and 6-C).

δ(^{19}F) (282.4 MHz, $CDCl_3/CD_3OD$): -66.41 ppm.

m/z (EI) 196 (M^+-18, 2%), 178 (4), 167 (17), 154 (>100);
(CI) 232 ({M + NH_4}$^+$, 100%), 214 (1); HRMS calcd for $C_7H_{13}O_4NF_3$ {M + NH_4}$^+$ 232.0797, found 232.0800.

6.4.4 Preparation of (3*R*,4*R*,5*S*,6*R*)-3,4-Epoxy-5,6-O,O-isopropylidene-1-trifluoromethyl-cyclohex-1-ene *(89)*.

A solution of diene *(86)* (202 mg, 0.92 mmol) in dichloromethane (3 ml) was cooled to -78°C. *m*-CPBA (232 mg, 1.35 mmol) was added and the solution stirred for 30 min. The solution was allowed to warm to room temperature and was stirred for 6 hours. The solution was cooled to -78°C, filtered, and washed with saturated Na_2SO_3 (20 ml), H_2O, and saturated $NaHCO_3$ (10 ml each). The organic layer was dried (Na_2SO_4), filtered, and concentrated *in vacuo*. Flash chromatography (SiO_2; dichloromethane) gave 160 mg (74%) of the (3*R*,4*R*,5*S*,6*R*)-3,4-epoxy-5,6-O,O-isopropylidene-1-trifluorometh yl-cyclohex-1-ene *(89)* as a white solid; R_f 0.73 (ethyl acetate).

δ(^{1}H) (300 MHz, $CDCl_3$): 6.69 (1H, dqd, $J_{2,3}$ 4.0, $J_{2,F}$ 1.4, $J_{2,6}$ ~1 Hz, 2-H), 4.84 (1H, dd, $J_{5,6}$ 6.8, $J_{5,4}$ 1.3, 5-H), 4.59 (1H, br d, $J_{6,5}$ 6.8 Hz, 6-H), 3.63 (1H, dd, $J_{4,3}$ 3.6, $J_{4,5}$ 1.5 Hz, 4-H), 3.46 (1H, t, $J_{3,2}$ 4.0, $J_{3,4}$ 3.6 Hz, 3-H), 1.43 (6H, s, 2 x CH_3).

Decoupling;

Irradiate	Effect
6.69 (2-H)	3.46 t → d
	4.59 brd → d
4.59(6-H)	6.69 dqd → dq
	4.84 dd → d
3.63(4-H)	4.84 dd → d
	3.36 t → d

δ(^{13}C) (75 MHz, $CDCl_3$): 131.7 (q, $J_{C,F}$ 30.1 Hz, 1-C), 128.4 (q, $J_{C,F}$ 5.7 Hz, 2-C), 122.6 (q, $J_{C,F}$ 273.8 Hz, CF_3), 111.8 ($C(CH_3)_2$), 71.1 and 68.4 (3-C and 4-C), 49.9 and 45.5 (5-C and 6-C), 27.4 and 25.5 (2 x CH_3).

δ(^{19}F) (282.4 MHz, $CDCl_3$): -64.86 ppm.

6.4.5 Preparation of (3*S*,4*R*,5*R*,6*R*)-3,4,5,6-Tetrahydroxy-1-trifluoromethyl-cyclohex-1-ene *(88)*.

To a solution of epoxide *(89)* (84 mg, 0.36 mmol) in acetonitrile (3 ml) and water (1.5 ml) was added Dowex 50-W resin (H^+ form, 400 mg). The reaction mixture was stirred for 4.5 days at room temperature. The mixture was then filtered and water/acetonitrile removed *in vacuo*. Flash chromatography (SiO_2; 5% MeOH/ethyl acetate) gave 68 mg (89 %) of (3*S*,4*R*,5*R*,6*R*)-3,4,5,6-tetrahydroxy-1-trifluoromethyl-cyclohex-1-ene *(88)* as a white solid; R_f 0.14 (5% MeOH/ethyl acetate).

IR ν_{max}(nujol): 3600-3000s cm^{-1}.

δ(^{1}H) (300 MHz, $CDCl_3/CD_3OD$): 6.36 (1H, dq, $J_{2,3}$ 2.9, $J_{2,F}$ 1.4 Hz, 2-H), 4.38 (1H, d, $J_{6,5}$ 3.9 Hz, 6-H), 4.06 (1H, ddq, $J_{3,4}$ 7.8, $J_{3,2}$ 2.9, $J_{3,F}$ 2.8 Hz, 3-H), 3.74 (1H, dd, $J_{4,5}$ 10.5, $J_{4,3}$ 7.8 Hz, 4-H), 3.45 (1H, dd, $J_{5,4}$ 10.5, $J_{5,6}$ 3.9 Hz, 5-H).

Decoupling;

Irradiate	Effect
6.36 (2-H)	4.06 ddq → dq

4.06 (3-H) 6.36 dq → br s

3.74 dd → d

δ(^{13}C) (75 MHz, $CDCl_3$ + CD_3OD): 136.6 (q, $J_{C,F}$ 5.4 Hz, 2-C), 129.3 (q, $J_{C,F}$ 29.9 Hz, 1-C), 123.6 (q, $J_{C,F}$ 273.2 Hz, CF_3), 72.5, 71.8, 71.6, 65.1 (3-C, 4-C, 5-C and 6-C).

δ(^{19}F) (282.4 MHz, $CDCl_3$ + CD_3OD): -66.5912 ppm, dd, $J_{F,3}$ 2.8, $J_{F,2}$ 1.4 Hz.

m/z (EI) 196 (M^+- 18, 1%), 178 (7), 167 (69), 154 (>100).

(CI) 232 ({M + NH_4}$^+$, 100%), 214 (3%). HRMS calcd for $C_7H_{13}O_4NF_3$ {M + NH_4}$^+$ 232.0797, found 232.0800.

6.4.6 Preparation of (3*S*,4*S*,5*R*,6*R*)-5,6-Dihydroxy-3,4-epoxy-1-trifluoromethyl-cyclohex-1-ene *(92)*.

A solution of diene *(83)* (311 mg, 1.73 mmol) in dichloromethane (5 ml) was cooled to -78°C. *m*-CPBA (437 mg, 2.53 mmol) was added and the solution stirred for 30 min. The solution was allowed to warm to room temperature and stirred for 2 hours. The reaction mixture was cooled to -78°C, filtered and concentrated *in vacuo*. Flash chromatography (SiO_2; ethyl acetate) gave 242 mg (71%) of (3*S*,4*S*,5*R*,6*R*)-5,6-dihydroxy-3,4-epoxy-1-trifluoromethyl-cyclohex-1-ene *(92)* as a colourless oil; R_f 0.67 (ethyl acetate).

IR ν_{max}(nujol): 3700-3010s cm^{-1}.

δ(^{1}H) (300 MHz, $CDCl_3$): 6.78 (1H, dqd, $J_{2,3}$ 4.1, $J_{2,F}$ 1.6, $J_{2,6}$ 0.5 Hz, 2-H), 4.29 (1H, br s, 5-H), 3.99 (1H, d, $J_{6,5}$ 4.5 Hz, 6-H), 3.76 (1H, dd, $J_{4,5}$ 2.4, $J_{4,3}$ 4.1 Hz, 4-H), 3.65 (1H, t, J 4.1 [=($J_{3,2}$ + $J_{3,4}$)/2], 3-H), 3.5 (1H, br s, OH), 2.6 (1H, br s, OH).

δ(^{13}C) (75 MHz, $CDCl_3$): 136.0 (q, $J_{C,F}$ 31.4 Hz, 1-C), 130.9 (q, $J_{C,F}$ 5.6 Hz, 2-C), 122.4 (q, $J_{C,F}$ 272.6 Hz, CF_3), 67.8, 64.6, 57.9 and 48.7 (3-C, 4-C, 5-C and 6-C).

δ(^{19}F) (282.4 MHz, $CDCl_3$): -68.70 ppm.

m/z (EI) 179 (M^+-17, 11%), 167 (26), 149 (56), 139 (56).

(CI) 214 ({M + NH_4}$^+$, 100%), 179 (8), 149 (95). HRMS calcd for $C_7H_{11}O_3NF_3$

$\{M + NH\}^+$ 214.0691, found 214.0690.

6.4.7 Preparation of (3*R*,4*S*,5*R*,6*R*)-3,4,5,6-Tetrahydroxy-1-trifluoromethyl-cyclohex-1-ene *(91)*.

To a solution of epoxide *(92)* (105 mg, 0.54 mmol) in acetonitrile (3 ml) and water (1.5 ml) was added Dowex-50W resin (400 mg). The reaction mixture was stirred for 27 hours at room temperature. The reaction was filtered, and acetonitrile/water removed *in vacuo* to give 114 mg (99%) of (3*R*,4*S*,5*R*,6*R*)-3,4,5,6-tetrahydroxy-1-trifluoromethylcyclohex-1-ene *(91)* as a white solid; R_f 0.18 (5% MeOH/ethyl acetate).

IR ν_{max}(nujol): 3600-3200s (OH) cm^{-1}.

$\delta(^1H)$ (300 MHz, CD_3OD): 6.29 (1H, dquint, $J_{2,3}$ 2.7, J 1.4 [=(($J_{2,F}$ x 3) + $J_{2,6}$)/4], 2-H), 4.36 (1H, ddq, $J_{3,4}$ 6.9, $J_{3,2}$ 2.7, $J_{3,F}$ 2.4 Hz, 3-H), 4.01 (1H, dd, $J_{6,5}$ 3.7, $J_{6,2}$ 1.4 Hz, 6-H), 3.65 (1H, t, J 6.9 [=($J_{4,3}$ + $J_{4,5}$)/2], 4-H), 3.60 (1H, dd, $J_{5,4}$ 6.9, $J_{5,6}$ 3.7 Hz, 5-H).

$\delta(^{13}C)$ (75 MHz, $(CD_3)_2CO$): 135.1 (q, $J_{C,F}$ 5.6 Hz, 2-C), 130.4 (q, $J_{C,F}$ 30.0 Hz, C-1), 124.4 (q, $J_{C,F}$ 272.7 Hz, CF_3), 75.3, 71.6, 69.5 and 66.9 (3-C, 4-C, 5-C and 6-C).

$\delta(^{19}F)$ (282.4 MHz, CD_3OD): -64.54 ppm.

m/z (EI) 196 (M^+-18, 1%), 178 (11), 167 (54), 154 (>100).

(CI) 232 ($\{M + NH_4\}^+$, 100%), 214 (3), 196 (4). HRMS calcd for $C_7H_{13}O_4NF_3$ $\{M + NH_4\}^+$ 232.0797, found 232.0797.

6.5 Chapter 5.

6.5.1 Chemical Modification of Shikimate Kinase.

(i) Analysis by time-based assay.

Freshly prepared reagent (2,3 Butanedione *(97)*, N-ethylmaleimide *(95)*, iodoacetamide *(94)* or iodoacetic acid *(96)*) in 50 mM-triethanolamine hydrochloride/KOH buffer, pH 7.0, containing 50 mM-KCl and 5 mM-$MgCl_2$ (buffer A) was added to shikimate kinase (1 unit) in buffer A at 25°C (total volume 1 ml). Aliquots (50 μl) were assayed for activity over a time period of up to 2 hours. The concentrations of the reagents used varied according to the ability of the reagent to inhibit and are reported in the text. The rate of inactivation of the enzyme, at a particular concentration of reagent, was determined from plots of ln[E] verses time. Appropriate control experiments in the absence of reagent were also conducted.

(ii) Analysis by concentration-based assay.

Freshly prepared reagent diethylpyrocarbonate (DEP) *(98)* in acetonitrile was added to shikimate kinase (1 unit) in buffer A, pH 7.0, at 25°C (total volume 1 ml) to give [DEP] in the range 0.5 - 10 mM. Aliquots 50 μl were removed and assayed for activity after 5 and 10 minutes. Initial first-order rates k' for each concentration were determined by plots of ln[E] versus time. The second order rate constant k for the inactivation was then determined from a plot of initial rates k' against concentration of DEP.

(iii) Substrate Protection.

The chemical modification of the most effective reagents, namely iodoacetamide *(94)*, NEM *(95)* and DEP *(98)*, was further examined by incubations in the presence of either shikimate or ATP. In each case the concentration of substrate was saturating (>10 x K_M)

and adjusted to deliver the correct quantity of substrate to the enzyme assay. Aliqots of the enzyme were assayed, and the kinetic parameters determined as before.

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