INVESTIGATIONS RELATED TO BRANCHED-CHAIN AMINO ACID METABOLISM

by

EDWARD ROBERT LEE (B.Sc.)

Submitted for the degree of Doctor of Philosophy

UNIVERSITY OF WARWICK

DEPARTMENT OF CHEMISTRY

April 1989

To my parents.

Why should any phenomenon be impossible?
It looks like the earth is a great thought
and not a simple machine.

CONTENTS

CHAPTER 8: Experimental

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to thank Professor D.H.G. Crout for the guidance and encouragement which he has provided throughout the course of this work.

I would also like to thank Dr. D. Pearson (I.C.I. Agrochemicals) for his advice during the three years of research.

Thanks are due to Novo Industri A/S, Dr. J.V. Schloss (Du Pont Company) and Dr. T. Hawkes (I.C.I. Agrochemicals) for the enzymes used during the course of this work.

I must also thank Dr. O. Howarth and Dr. A. Harrison for their assistance with the nmr studies and Dr. N. Alcock and G. Pike for the x-ray crystal structures.

I would like to thank my colleagues in the laboratory, especially M. Christen and J.M. Ricca for their help and advice.

The financial support of the S.E.R.C. and I.C.I. Agrochemicals is gratefully acknowledged.

DECLARATION

The work described in this thesis is the original work of the author except where acknowledgement is made to work and ideas previously described. It was carried out in the Department of Chemistry, University of Warwick, between October 1985 and September 1988 and has not been submitted previously for a degree at any institution.

SUMMARY

A novel synthetic route to heterogeneous acyloins was developed using benzothiazole/thiazolium species. The synthetic route mimics the biological action of thiamine pyrophosphate (TPP) in acetohydroxyacid synthase, the first enzyme of the valine-isoleucine biosynthetic pathway. The synthetic intermediates were examined by x-ray crystallography.

Racemic [3,4-$^{13}C_2$]-α-acetolactate was synthesised. Treatment of the ^{13}C-labelled α-acetolactate with acetolactate decarboxylase and analysis of the subsequent reactions by ^{1}Hnmr showed that the enzyme rapidly decarboxylated the S-isomer to give [1,2-$^{13}C_2$]-3-hydroxybutan-2-one and the R-isomer underwent an enzyme catalysed tertiary ketol rearrangement and then decarboxylation to yield [3,4-$^{13}C_2$]-3-hydroxybutan-2-one.

The stereochemistry of a base-catalysed tertiary ketol rearrangement was investigated. R-α-acetohydroxybutyrate was treated with alkali and the resulting products were analysed by reaction with acetolactate decarboxylase. It was found that there is a preference for a syn-conformation of the C-O bonds during the carboxylate ion migration.

A novel sixteen step synthesis of methyl α-acetolactate with a chiral methyl group at the α-position was developed.

Chemical syntheses of intermediates of the valine-isoleucine biosynthetic pathway were developed, including the attempted synthesis of trifluoromethyl-analogues.

High field ^{1}Hnmr techniques were used to investigate directly the reactions catalysed by AHAS isoenzyme 2 (Salmonella typhimurium) and AHAS isolated from pea plants. The ^{1}Hnmr investigations permitted the analogous reactions catalysed by AHAS to be studied and gave an insight into the nature of the reactions occurring at the enzyme active site.

ABBREVIATIONS

TPP	-	thiamine pyrophosphate
FAD	-	flavin adenine dinucleotide
AHAS	-	acetohydroxyacid synthase
ADC	-	acetolactate decarboxylase
PLE	-	pig liver esterase
nmr	-	nuclear magnetic resonance
δ	-	chemical shift in ppm
ppm	-	parts per million
J	-	coupling constant
s, S	-	singlet
d	-	doublet
t	-	triplet
q	-	quartet
b	-	broad
arom	-	aromatic
m	-	multiplet
FAB	-	fast atom bombardment
GLY	-	glycerol
M.S.	-	mass spectrum
E.I.	-	electron ionisation
C.I.	-	chemical ionisation
M	-	parent molecular ion
I.R.	-	infra-red
TMS	-	tetramethylsilane
DABCO	-	1,4-diazabicyclo[2.2.2]octane
DMF	-	N,N-dimethylformamide
THF	-	tetrahydrofuran

DME	-	1,2-dimethoxyethane
m.p.	-	melting point
b.p.	-	boiling point

CHAPTER 1

Introduction

The biosynthesis of the branched chain amino acids valine and isoleucine in plants and microorganisms occurs by a series of equivalent chemical transformations which are catalysed by four separate enzymes (Fig. 1.1). Interest in the enzymes and the intermediates of the biological pathway has been stimulated by the discovery of two new powerful families of herbicides, the sulphonyl ureas (E.I. du Pont de Nemours and Co.) and the imidazolinones (American Cyanamid Co.). The herbicides inhibit acetohydroxyacid synthase the first enzyme of the valine-isoeucine pathway.

The isoleucine-valine biosynthetic pathway[1,2] consists of four enzymatic steps, each enzyme has the ability to transform two separate substrates. The first reaction in the biosynthesis of valine and isoleucine is catalysed by the enzyme acetohydroxyacid synthase and involves the homologous condensation of two pyruvate molecules or the heterologous condensation of one pyruvate molecule with an α-keto-butyrate molecule to give the corresponding α-hydroxy-β-keto acids, α-acetolactate and α-acetohydroxybutyrate.[3] The subsequent rearrangement, α-acetolactate undergoing a methyl migration and α-acetohydroxybutyrate an ethyl migration and reduction to yield an α, β-dihydroxyacid are accomplished by a single enzyme, acetohydroxyacid reductoisomerase.[4,5,6] The third enzyme of the biosynthetic pathway dihydroxyacid dehydratase[4] converts the α, β -dihydroxy acids into the corresponding α-keto acids which are then converted by an aminotransferase[7] enzyme into the L-amino acids valine and isoleucine.[8]

Fig. 1.1 The isoleucine and valine biosynthetic pathway.

Initial studies on the biosynthetic pathway were performed using cellular extracts from Saccharomyces cerevisiae[4], Neurospora

crassa[9] and H.E. Umbarger showed that in wild type Escherichia coli[5] labelled α-acetolactate-3-C^{14} and α-acetohydroxybutyrate-3-C^{14} were incorporated into valine and isoleucine respectively.

Detailed information about acetohydroxyacid synthase, the first enzyme of the valine-isoleucine biosynthetic pathway has come from work on bacterial enzymes. Studies have shown that the L-amino acids produced inhibit acetohydroxyacid synthase regulating the biosynthetic pathway by feedback control.[10,11,12] Investigations have also shown that bacteria express multiple forms of acetohydroxyacid synthase,[13] for example Salmonella typhimurium (wild type) expresses two forms of acetohydroxyacid synthase,[14,15] one form is inhibited by valine, the other form is resistant to valine inhibition. Genetic analysis in Escherichia coli (K-12)[16] indicates the existence of structural genes coding for three acetohydroxyacid synthase activities.[17,18] Wild type Escherichia coli expresses only two of the acetohydroxyacid synthase isoenzymes (I,III) both of which are subject to inhibition by valine. The isoenzyme (II) which is insensitive to valine inhibition is not expressed in E. coli due to a cryptic structural gene. The expression of different acetohydroxyacid synthase isoenzymes which differ in their feedback regulation, levels of expression and substrate specificities allows a fine control of the amino acid intracellular pool.[19]

Acetohydroxyacid synthase has been purified to homogeneity in two cases, isoenzyme I from Escherichia coli (Grimminger and Umbarger;[20] Eoyang and Silverman[21]) and isoenzyme II from Salmonella typhimurium (Schloss *et al*[22]). In both cases the enzyme was found to be an $\alpha_2\beta_2$ tetramer of subunits with molecular weights of 60,000 and 9,500.

Acetohydroxyacid synthase has not been purified from plants

due to the lability and low concentrations of the enzyme found in plant tissues. The specific activity of purified acetohydroxyacid synthase isozyme II from Salmonella typhimurium was reported by Schloss to be 15 μmol of acetolactate formed/min/mg at 37°C,[22], whereas the activity of a crude extract from pea plants has been reported to be 6-10 nmol of acetolactate formed/min/ml at 37°C.[24]

Investigations by Davies[25] and Miflin[26,27] using pea and barley plants have showed that partially purified acetohydroxyacid synthase from the plants produces α-acetolactate and α-acetohydroxybutyrate, the initial intermediates in the biosynthesis of valine and isoleucine, from pyruvate and α-ketobutyrate. The acetohydroxyacid synthase activity is found to be regulated by individual and cooperative feedback by valine, leucine and isoleucine.

Early studies have shown that acetohydroxyacid synthase from plants and microorganisms has an absolute requirement for magnesium, thiamine pyrophosphate and flavin adenine dinucleotide.[18,20,24] The role of enzyme bound TPP is shown in fig. 1.2.

Enzyme bound thiamine pyrophosphate at the first active site of acetohydroxyacid synthase reacts with the α-keto group of a pyruvate molecule, the product of which then undergoes decarboxylation to yield a hydroxyethyl-TPP intermediate ("active acetaldehyde").[28,29] The hydroxyethyl-TPP intermediate acts as a nucleophile condensing with a pyruvate or α-ketobutyrate molecule bound at the second binding site of the enzyme to give α-acetolactate or α-aceohydroxybutyrate and the TPP-anion.

The hydroxyethyl-TPP intermediate has been isolated and characterised by Schloss[28] and Cinkranik using chemical quenching experiments to trap the reaction intermediates of the acetohydroxyacid synthase reaction. The relative preferences of pyruvate and

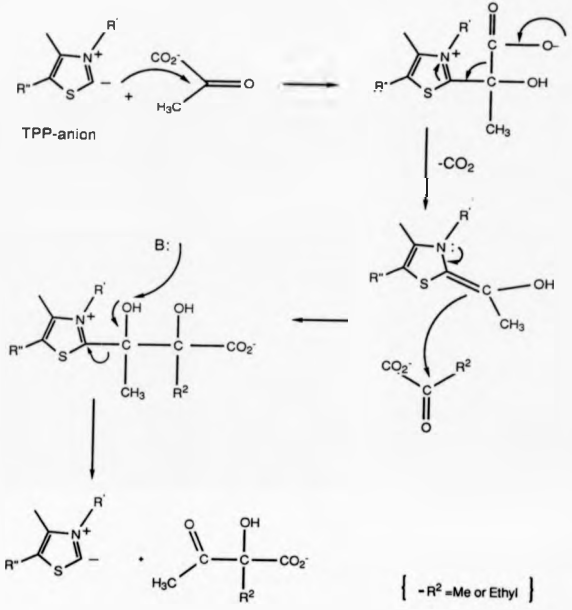


Fig. 1.2 The biological mode of action of thiamine pyrophosphate

α-ketobutyrate for the two binding sites of acetohydroxyacid synthase has been investigated using the bacterial isoenzyme II from Salmonella

typhimurium. Isotope mass ratio spectroscopy[21] of the release of enzyme-derived CO_2 from an equimolar mixture of α-ketobutyrate and ^{13}C-carboxyl-labelled pyruvate indicates that pyruvate is preferred over α-ketobutyrate in the first binding site by a factor of 19 to 1 and the converse is true at the second binding site. This mechanism minimises the non-productive decarboxylation of α-ketobutyrate.

The steady state kinetics of acetohydroxyacid synthase have been described by Schloss.[22] Saturation by pyruvate shows hyperbolic and not sigmoidal[23] kinetics, which is believed to be due to the tight binding of a pyruvate molecule at the first active site and the kinetic irreversibility of decarboxylation of the enzyme bound pyruvate.[22] The Km for pyruvate is approx. 8mM[22] for acetohydroxyacid synthase isoenzyme II from Salmonella typhimurium and 1.6mM[24] for the enzyme from peas.

The regulation and activity of acetohydroxyacid synthase from tobacco plants (Nicotiana tabacum L.) has been studied using mutagenic techniques developed by Bourgin.[25] It was found that mutant tobacco plants could be grown which exhibited altered sensitivity to amino acid feedback control. Valine suppresses plant growth by inhibiting acetohydroxyacid synthase. It has been found that mutant valine-resistant plants contain an altered form of acetohydroxyacid synthase which can be transmitted in sexual crosses.[24] The development of mutant plant lines[25,26] has permitted investigations of the valine-isoleucine biosynthetic pathway and the same techniques can be used to create herbicide resistant plants.[27]

Interest in the isoleucine and valine biosynthetic pathway was greatly stimulated by the discovery that the pathway was the target of two new families of herbicide, the sulphonyl ureas and the imidazolinones.

The sulphonyl ureas (E.I. du Pont de Nemours and Co.) and the imidazolinones (American Cyanamid Co.) were both discovered by random screening tests[34,35] and though structurally unrelated kill plants in an identical and distinctive fashion. Both families of herbicide are characterised by an unprecedented activity with application rates of grams per hectare. The herbicides also have low soil half lives of generally less than two months and a low mammalian toxicity making the herbicides enviromentally attractive. The structures and uses of some of the herbicides are shown in fig. 1.3.

Early studies on the mode of action of the sulphonyl ureas showed them to be potent inhibitors of plant growth.[40] Growth inhibition was observed in corn seedlings within 2 hours of foliar treatment with 28nM chlorsulfuron. The basis for growth inhibition was found to be inhibition of plant cell division. Initially plant photosynthesis, respiration, RNA and protein synthesis were unaffected. DNA synthesis was affected but was shown to be a secondary effect of the herbicide.[41]

Chlorsulfuron is absorbed by both roots and foliage and is readily translocated, the first signs of plant death appear in the meristematic tissue. Death of treated plants is generally slow and is accompanied by chlorosis, necrosis, terminal bud death, vein discolouration and complete inhibition of plant growth.

The physiological effects[42] of the imidazolinones have been found to be similar to the sulfonyl ureas, with a rapid inhibition of plant growth.[43]

The biochemical site of action of the herbicide sulfometuron methyl was originally investigated by La Rossa and Schloss.[44,45] It was found that sulfometuron methyl inhibited the bacterial growth of Salmonella typhimurium in a minimal medium containing valine, and that

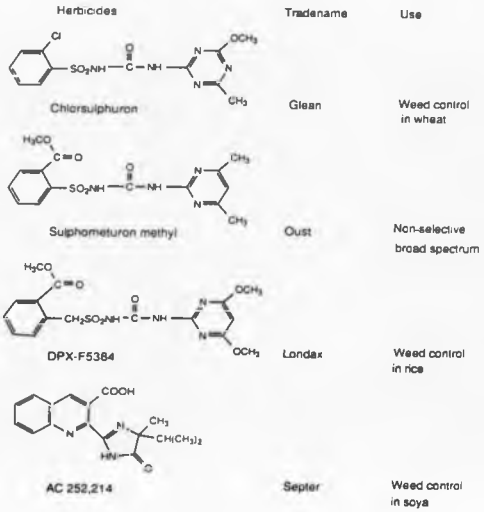


Fig. 1.3. Examples of sulfonyl urea and imidazolinone herbicides.

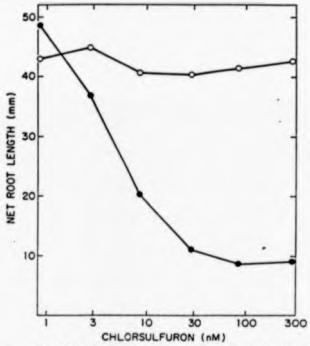
the inhibition could be reversed by the addition of isoleucine. The results indicated that the branched-chain amino acid biosynthetic pathway of Salmonella typhimurium was being blocked at some stage by

sulfometuron methyl. The acetohydroxyacid synthase isoenzyme (II) from Salmonella typhimurium which is resistant to inhibition by valine, was shown to be potently inhibited by sulfometuron methyl. Mutants of Salmonella typhimurium which were resistant to sulfometuron methyl showed a mutation mapped to the ilvG region of the genome, which normally codes for sulfometuron methyl sensitive acetohydroxyacid synthase isoenzyme II.

Following the investigations into the inhibitory effect of the sulfonyl ureas on acetohydroxyacid synthase isoenzymes in bacteria, herbicide mode of action studies in plants were undertaken. Ray[46] used excised pea root cultures as a sensitive bioassay for evaluating the effects of chlorsulfuron on plant growth. It was found that 2.8nM chlorsulfuron significantly inhibited root growth, whilst 28nM inhibited growth by 80%. Protection from the growth inhibitory effects of chlorsulfuron on the pea roots could only be achieved by supplementing the growth medium with both valine and isoleucine. (fig.1.4).

The results suggested that chlorsulfuron interferes with valine and isoleucine production in the pea plants. Acetohydroxyacid synthase the first common enzyme of the valine-isoleucine biosynthetic pathway was extracted from pea shoots and shown to be strongly inhibited by chlorsulfuron.

The imidazolinone herbicide AC 243,997[47] was tested using maize seedlings and showed similar growth inhibitory effects found with the sulfonyl urea herbicides. The primary toxic effect of the imidazolinone herbicide was on branched-chain amino acid synthesis and inhibition of acetohydroxyacid synthase. It was shown that AC 243,997 when applied to maize seedlings resulted in a decrease in the pool size of valine, leucine and isoleucine which produced a decrease



The effect of 100 μM each of Val and Ile on the growth of pea roots in the presence of various concentrations of chlorsulfuron. Each point is the average net length of 10 roots. Control without amino acid supplement (●); with 100 μM valine and isoleucine (○).

Fig. 1.4 The activity of pea acetohydroxyacid synthase in the presence of chlorsulphuron (Ray T.[48]).

in protein synthesis and, in turn, caused a reduction in the rate of cell division, and eventually death of the cells. The initial effects of the herbicide are found in the meristematic tissue since mature tissue has larger pools of amino acids and protein reserves that can be catabolised for amino acids.[48]

A considerable number of genetic studies have been carried out using plants and microorganisms in an effort to confirm that the site of action of the sulfonyl ureas and the imidazolinones is the enzyme acetohydroxyacid synthase. Tobacco mutants[46,50] resistant to sulfonyl urea herbicides have been isolated from cell cultures, by selecting for cell lines which could grow in the presence of

sulfometuron methyl or chlorsulfuron. Plants regenerated from these selected cell lines retained their resistance. Acetohydroxyacid synthase from the sulphonyl urea resistant cell lines is less sensitive to inhibition by the herbicides than the enzyme from the wild-type sensitive cell lines. It was shown that the mutant gene coding for the resistant form of acetohydroxyacid synthase could be sexually transmitted. Sulfonyl urea resistant mutants of Salmonella typhimurium, Escherichia coli[51] and Saccharomyces cerevisiae[52] have also been isolated. The sulphonyl urea resistance, in all cases, has been mapped to a gene coding for a resistant form of acetohydroxyacid synthase. Sequence analysis of the mutant genes has shown that a single nucleotide change results in a single amino acid substitution in the acetohydroxyacid synthase enzyme conferring resistance to the sulfonyl ureas.[53]

The physiological, biochemical and genetic studies confirm that the sulfonyl ureas and imidazolinones are potent inhibitors of acetohydroxyacid synthase, preventing the biosynthesis of valine, leucine and isoleucine.

The sulfonyl ureas and the imidazolinones that have been developed control weeds in a wide range of crops. The selectivity of the herbicides arises from the ability of certain crops to rapidly metabolise the herbicides to non-toxic products. Studies[54] on plants tolerant to chlorsulfuron for example, wheat, oats and barley show that the plants metabolise chlorsulfuron by hydroxylation of the phenyl ring of chlorsulfuron, followed by conjugation with a carbohydrate moiety. The carbohydrate metabolite does not inhibit acetohydroxyacid synthase.

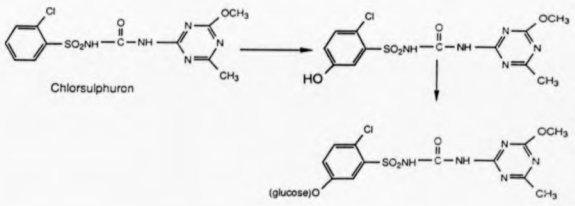


Fig. 1.5 The metabolism of chlorsulphuron by wheat.

Investigations with a considerable number of plants[35,36,42] have shown that the selective action of the sulfonyl ureas and the imidazolinones results from the various metabolic pathways that exist in resistant plants, which are able to convert the herbicides into inactive compounds, whereas sensitive plants lack the ability to metabolise the herbicides.

The interaction of the sulfonyl urea and imidazolinone herbicides with the enzyme acetohydroxyacid synthase is an area of great interest. The acetohydroxyacid synthase enzyme has an absolute requirement for magnesium, TPP and FAD. The requirement for FAD is unusual since the reaction catalysed by acetohydroxyacid synthase involves no net redox change. It has been suggested by Walsh that FAD[67] plays a structural or allosteric role. Experiments by Schloss

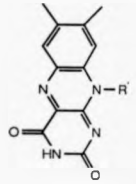
with acetohydroxyacid synthase isoenzyme II from Salmonella typhimurium showed that the absorbance of enzyme bound FAD between 370 and 530 nm decreases upon initiation of the enzymic reaction in the presence of TPP, magnesium and pyruvate.[88] It has been suggested that this loss of absorbance is due to the isoalloxazine ring of FAD acting as an electrophile and stabilising the "active acetaldehyde" carbanionic intermediate. This intermediate is formed at the first binding site by decarboxylation of the initial TPP-pyruvate complex.

The effects of sulfometuron methyl (sulfonyl urea) and Scepter (imidazolinone) on acetohydroxyacid synthase isoenzyme II from Salmonella typhimurium have both been studied.[44] Inhibition of acetohydroxyacid synthase II by sulfometuron methyl develops slowly with time and can be modelled as a biphasic process, involving the rapid formation of an initial relatively weak enzyme-inhibitor (EI) complex that slowly isomerises to a more tightly bound form (EI*). The slow-binding inhibition of acetohydroxyacid synthase II by sulfometuron methyl gives an initial K_i of 1.7 μM, and a final steady state K_i of 82nM.[88] The tightly bound enzyme-inhibitor complex only forms in the presence of all the cofactors and pyruvate. Evidence indicates that addition of pyruvate to TPP at the first binding site gives the intermediate form of the enzyme, to which sulfometuron methyl binds most tightly. The binding site of sulfometuron methyl overlaps with the second enzyme binding site, resulting in inhibition by sulfometuron methyl which is competitive with respect to pyruvate. The imidazolinone Scepter also shows slow-binding inhibition of acetohydroxyacid synthase II with an initial K_i of 0.8mM and a final steady state K_i of 20 μM.[88] Scepter also binds most tightly to the initial pyruvate-TPP enzyme complex; however, Scepter does not overlap with the second enzyme binding site of acetohydroxyacid

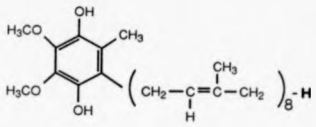
synthase II, resulting in uncompetitive inhibition with respect to pyruvate. The inhibitory mode of action of Scepter and sulfometuron methyl therefore appears to be remarkably similar.

The mode of inhibition of acetohydroxyacid synthase II by Scepter and sulfometuron methyl, indicates that they are binding to a previously unrecognised binding site of the enzyme.[65] A partial understanding of the nature of the herbicide binding site has come from the determination of the sequence for the poxB gene, that encodes for the pyruvate oxidase of Escherichia coli.[66] This sequence displays substantial homology with ilvB,[61,62] ilvG,[63] and ilvI[64] the genes that code for the three acetohydroxyacid synthase isoenzymes. Pyruvate oxidase utilises FAD for standard redox chemistry unlike acetohydroxyacid synthase isoenzyme II, where FAD serves no redox rôle. After oxidation of pyruvate, the reduced FAD in pyruvate oxidase is oxidised *in vivo* by ubiquinone-40.[66] Ubiquinone appears to bind to an equivalent region of the active site of the enzyme with respect to FAD, as does sulfometuron methyl in acetohydroxyacid synthase isoenzyme II. Ubiquinone binding in pyruvate oxidase is affected by the substrate, pyruvate,[66,67] analogous to the effect of pyruvate on sulfometuron methyl binding in acetohydroxyacid synthase II.

It appears that the ubiquinone binding domain of pyruvate oxidase, and the herbicide binding domain of acetohydroxyacid synthase share a common evolutionary heritage.[65] The reason why this site is maintained in acetohydroxyacid synthase is as yet unclear, and the full nature of the herbicide binding site has yet to be discovered.



FAD



Ubiquinone-40
reduced form

It has been found that the sulfonyl urea[43] and imidazolinone[44] herbicides inhibit acetohydroxyacid synthase from plants in a similar manner to the enzyme from bacteria. Experiments with corn[43] and pea[44] acetohydroxyacid synthase showed that both types of herbicide were potent inhibitors of the enzyme, and the inhibition was found to slowly increase with time.

The sensitivity of certain crops to the sulfonyl urea and imidazolinone herbicides precludes their use , for example a combination of sulfonyl urea herbicides would provide control of weeds endemic to tobacco fields, but the sensitivity of tobacco to sulfonyl ureas prevents their use. Recent studies have shown that mutants of tobacco plants[45] resistant to sulfometuron methyl have an altered form of acetohydroxyacid synthase. The mutant

acetohydroxyacid synthase gene has been cloned from these plants and can be used to introduce herbicide resistance into other crop plants.[66] The future use of genetic techniques to modify the response of plants to the new families of herbicides will allow a wider spectrum of herbicide applicability.[66]

CHAPTER 2

The development of a synthetic pathway which produces heterogeneous acyloins using thiazole-thiazolium compounds

Thiamine pyrophosphate(1) is the cofactor in a number of important enzymatic reactions, including the decarboxylation of pyruvic acid to acetaldehyde, the conversion of pyruvic acid into acetoin and the transketolase reaction. As previously described in Chapter 1, the first enzyme of the valine-isoleucine biological pathway, acetohydroxyacid synthase, which catalyses the homologous and heterologous condensations of pyruvate and α-ketobutyrate, also has an absolute requirement for thiamine pyrophosphate. The reactions all have one common feature in that they can be considered to involve the formation of an intermediate acyl carbanion. The catalytic role of thiamine pyrophosphate has been investigated by Breslow.[76] It was found that a number of thiazolium species, for example 3,4-dimethylthiazolium bromide, possess an acidic C-2 hydrogen atom which can be readily exchanged with a deuterium atom in neutral aqueous solution. It was also found that certain thiazolium species would catalyse the benzoin condensation. These observations provide a rationale for the biochemical mechanism in thiamine pyrophosphate containing systems. The thiamine pyrophosphate thiazolium C-2 carbanion condenses with a reactive carbonyl species, subsequent decarboxylation yields a stabilised acyl carbanion equivalent which reacts further depending on the type of enzymatic system involved. The mode of action of thiamine pyrophosphate in acetohydroxyacid synthase and pyruvate decarboxylase is shown in figures 1.2 and 2.1 respectively.

Thiazolium salts have been widely used to catalyse acyloin and benzoin condensations. For example Stetter[74] successfully used 3-

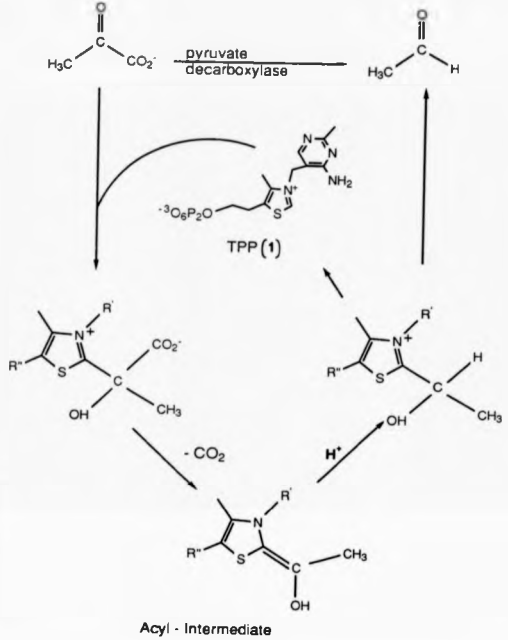
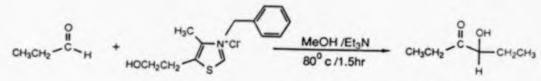


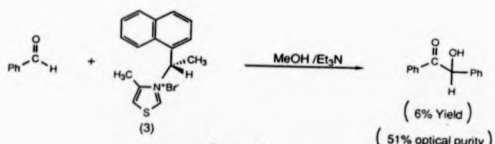
Fig. 2.1 The mode of action of thiamine pyrophosphate in pyruvate decarboxylase



Scheme 1

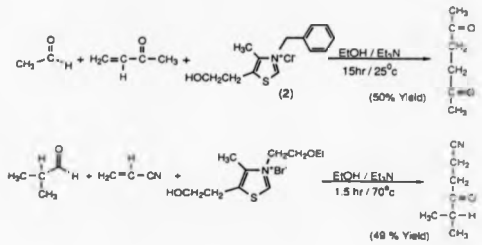
benzyl-5-(2-hydroxyethyl)-4-methyl-1,3-thiazolium chloride (2) to catalyse a wide range of acyloin condensations in refluxing ethanol and triethylamine.

Sheehan and Hara[72] have studied the benzoin condensation using optically active thiazolium salts, which have a bulky chiral centre bonded directly to the quarternary nitrogen atom of the thiazolium catalyst. They found a substantial degree of asymmetric induction was obtained when (S)-(+)-4-methyl-3- α -(1-naphthyl)ethylthiazolium bromide (3) was employed as a catalyst, benzoin was formed with an optical purity of greater than 50%.



Scheme 2

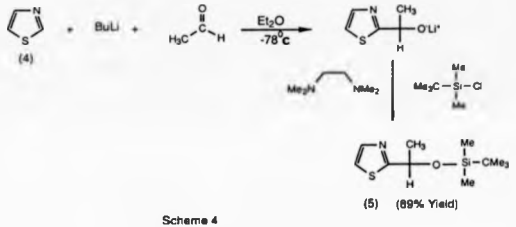
Recently Breslow[73] has observed rate enhancements in the benzoin condensation by attachment of the heterocycle to a cyclodextrin receptor. Stetter and co-workers have shown that a variety of thiazolium species can be used to catalyse the condensation of various aldehydes with electrophiles,[74] such as α,β-unsaturated nitriles,[75] esters[76] and ketones.[77] (Scheme 3).



Scheme 3

This chapter describes the development of a novel heterogeneous acyloin condensation using thiazolium compounds. The generation of hydroxy-ketones using thiazolium species provides a model system for the biological mode of action of thiamine pyrophosphate. The intermediates of the synthetic pathway are hypothesised as inhibitors of acetohydroxyacid synthase, the first enzyme of the valine-isoleucine pathway.

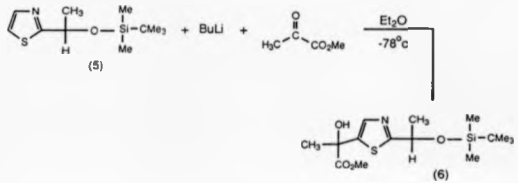
The initial investigations into thiazolium-type chemistry involved the use of thiazole(4) itself. 1-(2-Thiazolyl)-1-tert-butyldimethylsiloxy ethane (5) was prepared by treating thiazole with butyl lithium, the stable carbanion formed at the C-2 position of the thiazole ring condensed with acetaldehyde. The alkoxide thus formed was protected by the addition of tert-butyldimethylsilyl chloride. The alkoxide would only react with tert-butyldimethylsilylchloride in the presence of tetramethylethylenediamine. Tetramethylethylenediamine coordinates to the lithium ions present in the reaction greatly increasing the nucleophilicity of the alkoxide.



Scheme 4

The attempted removal of the C_{α}-hydrogen of 1-(2-thiazolyl)-1-tert-butyldimethylsiloxyethane(5) using butyl lithium and subsequent

reaction of the carbanion formed with methyl pyruvate was unsuccessful. The C-5 hydrogen atom of the thiazole ring was preferentially removed , and the 5-lithio species formed, condensed with the α-carbonyl group of methyl pyruvate. The ^{1}Hnmr spectrum of the product (6) isolated from the reaction exhibited a single aromatic peak at δ7.65. The ^{13}Cnmr spectrum showed a strong resonance at δ138.9 and two weak resonances at δ178.3 and δ 141.3, characteristic of a C-2 and C-5 substituted thiazole ring.



Scheme 5

Metzger[7] has described the reactivity of the thiazole C-5 position towards organolithium reagents.

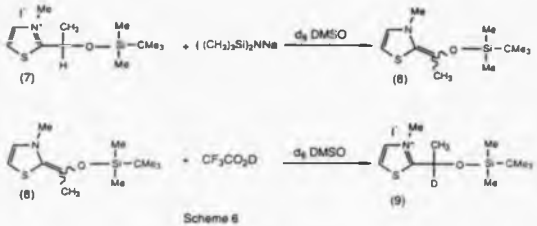
In order to increase the acidity of the C_α-hydrogen atom, the nitrogen atom of the heterocyclic ring was quaternised. Quaternization of the heterocyclic ring was found to be extremely

difficult. Only after refluxing the thiazole (5) in tetrahydrofuran for seven days, with a large excess of methyl iodide was the N-methyl thiazolium iodide (7) obtained. The problems encountered when attempting to quaternise a thiazole ring with a substituent at the C-2 position have been discussed by White and Ingraham.[76]

Using the experimental conditions described by Jordan, Kudzin and Rios[19] the N-methyl thiazolium salt (7) was dissolved in d_6-DMSO and the strong, non-nucleophilic base sodium bis-trimethylsilylamide was added. The ^{1}Hnmr spectrum of the sample was then recorded. The spectra obtained, indicated that the N-methyl thiazolium species (7) on treatment with sodium bis-trimethylsilylamide gave two new sets of resonances (table 1). The new resonances could be assigned to the formation of cis and trans isomers of an enamine intermediate (8), which was formed by deprotonation of the C_α -hydrogen atom. The deprotonation of the C_α-hydrogen atom was reversed, by neutralisation of the basic reaction mixture with deutero-trifluoroacetic acid. The ^{1}Hnmr spectra showed that on neutralisation the enamine intermediate was deuterated at the C_α -position, and the original aromatic resonances at δ 8.3 and 8.45 were observed to reappear. (Table 1).

The reversible formation of an intermediate enamine models the key intermediate formed in thiamine pyrophosphate-dependent enzymatic pathways.

The successful production of a stable enamine (8) using a synthetic thiazolium salt (7), was followed by numerous attempts to react the deprotonated thiazolium species with various reagents including methyl iodide, acetyl chloride and methyl pyruvate. All efforts to condense the enamine intermediate with reagents other than those producing simple protonation failed. The N-methyl thiazolium iodide (7) was insoluble in most organic solvents apart from DMSO. In



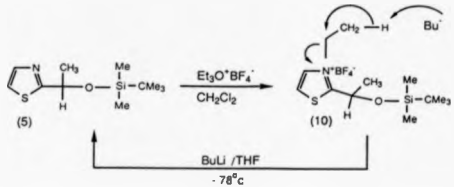
Scheme 6

Table 1 ^{1}Hnmr Chemical Shifts

Compound	N-CH_3	CH_3-C	C-H	H aromatic	
7	4.15(s)	1.55 (d)	5.7(q)	8.3 (d)	8.45 (d)
8	3.0 (s)	2.0 (s)	-	6.2 (d)	5.62 (d)
8	3.1 (s)	1.7 (s)	-	6.15(d)	5.59 (d)
9	4.15(s)	1.5 (s)	-	8.3 (d)	8.45 (d)

order to produce a thiazolium compound with a greater solubility and versatility, the Meerwein[88] salt of compound (5) was synthesized. The N-ethyl thiazolium tetrafluoroborate salt (10) was found to be soluble in a range of organic solvents. However, treatment with various

bases, including butyl lithium, resulted in regeneration of the non-quaternised thiazole compound. The β-hydrogen atom of the N-ethyl alkyl chain is more acidic than the (C-2) α-hydrogen atom, a property which leads to the loss of the N-ethyl group.

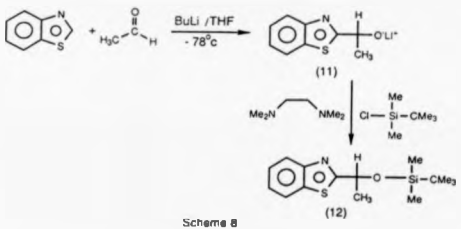


Scheme 7

It was decided that benzothiazole rather than thiazole might be a better system. Benzothiazole possesses an acidic C-2 hydrogen atom, the C-4 and C-5 positions of the thiazole ring are masked by the benzene ring and a (C-2)- α -carbanion would be expected to be reasonably stable. Chikashita[11] *et al* have recently described the formation of α-lithio-2-methylbenzothiazole and its use in synthesis.

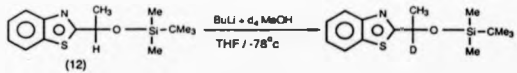
Benzothiazole was treated with butyl lithium in tetrahydrofuran at -78°C and the (C-2)- α -carbanion formed was condensed with acetaldehyde. The resulting alkoxide (11) was protected by reaction with tert-butyldimethylsilylchloride in the presence of tetramethylethylenediamine.

1-(2-Benzothiazolyl)-1-tert-butyldimethylsiloxyethane (12) was dissolved in tetrahydrofuran, cooled to -78°C and then treated with butyl lithium. It was noted that the initial yellow solution turned



Scheme 8

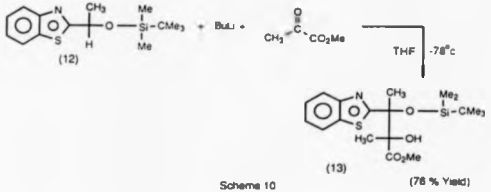
blood-red on addition of the butyl lithium, and that the red colour was lost when the reaction was quenched with (d_4)-methanol. The ^{1}Hnmr spectrum of the product isolated from the reaction showed that the doublet at δ 1.67 (J=7.2Hz) associated with the α-methyl group of compound (12) had been replaced by a single broad peak, characteristic of the incorporation of a deuterium atom adjacent to the thiazole ring.



Scheme 9

A solution of 1-(2-benzothiazolyl)-1-tert-butyldimethyl-

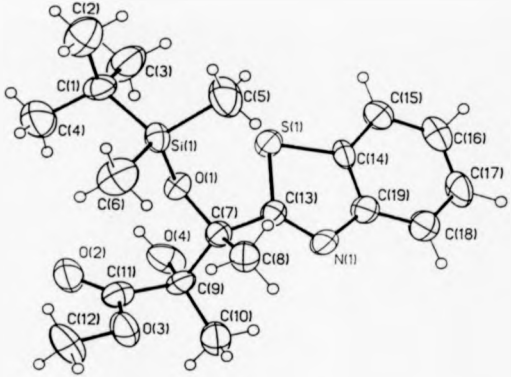
siloxyethane (12) in tetrahydrofuran was cooled to -78°C, and reacted with one equivalent of butyl lithium. The α-carbanion thus formed was reacted with methyl pyruvate. The α-carbanion attacked the α-carbonyl group of methyl pyruvate producing methyl 2-hydroxy-2-methyl-3-tert-butyldimethylsiloxy-3-(2-benzothiazolyl)-butanoate (13).



Scheme 10

Methyl 2-hydroxy-2-methyl-3-tert-butyldimethylsiloxy-3-(2-benzothiazolyl)-butanoate (13) was obtained from the reaction as a diastereomeric mixture inseparable by chromatography. However, one diastereomeric pair preferentially crystallised from the crude mother liquor. Characterisation of the crystals using x-ray crystallography made it possible to assign to the compound the absolute configuration 2R,3R and 2S,3S. The crystal structure is shown in figure 2.2

The next stage of the reaction pathway involved the removal of the protecting group. Treatment of (2R,3R) and (2S,3S) methyl 2-hydroxy-2-methyl-3-tert-butyldimethylsiloxy-3-(2-benzothiazolyl)-butanoate with tetrabutylammonium fluoride[22] in tetrahydrofuran resulted in loss of the tert-butyldimethylsilyl protecting group. However, the stereochemical integrity of the compound was lost, and a



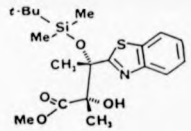


Fig. 2.2. X-ray crystal structure of methyl 2-hydroxy-2-methyl-3-tert-butyldimethylsilcxy-3-(2-benzothiazolyl)-butanoate.

Table. 2. Bond angles and lenths.

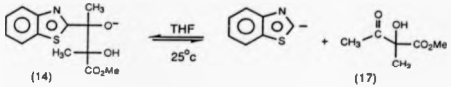
Bond angles (°)

C(14)-S(1)-C(13)	88.3(4)	C(1)-Si(1)-O(1)	104.4(3)
C(5)-Si(1)-O(1)	111.3(4)	C(5)-Si(1)-C(1)	110.8(5)
C(6)-Si(1)-O(1)	109.7(4)	C(6)-Si(1)-C(1)	110.8(4)
C(6)-Si(1)-C(5)	109.7(5)	C(7)-O(1)-Si(1)	137.1(5)
C(12)-O(3)-C(11)	116.7(7)	C(19)-N(1)-C(13)	110.6(7)
C(2)-C(1)-Si(1)	110.0(7)	C(3)-C(1)-Si(1)	112.6(6)
C(3)-C(1)-C(2)	109.0(8)	C(4)-C(1)-Si(1)	111.0(7)
C(4)-C(1)-C(2)	107.4(9)	C(4)-C(1)-C(3)	106.7(9)
C(8)-C(7)-O(1)	114.8(7)	C(9)-C(7)-O(1)	105.1(6)
C(9)-C(7)-C(8)	113.0(7)	C(13)-C(7)-O(1)	107.1(6)
C(13)-C(7)-C(8)	108.3(7)	C(13)-C(7)-C(9)	108.3(7)
C(7)-C(9)-O(4)	107.2(6)	C(10)-C(9)-O(4)	106.7(7)
C(10)-C(9)-C(7)	115.1(7)	C(11)-C(9)-O(4)	106.6(7)
C(11)-C(9)-C(7)	109.3(7)	C(11)-C(9)-C(10)	111.5(7)
O(3)-C(11)-O(2)	123.9(9)	C(9)-C(11)-O(2)	123.7(9)
C(9)-C(11)-O(3)	112.4(7)	N(1)-C(13)-S(1)	116.3(6)
C(7)-C(13)-S(1)	118.2(6)	C(7)-C(13)-N(1)	125.5(7)
C(15)-C(14)-S(1)	126.9(7)	C(19)-C(14)-S(1)	109.8(6)
C(19)-C(14)-C(15)	123.3(8)	C(16)-C(15)-C(14)	115.7(9)
C(17)-C(16)-C(15)	120.3(9)	C(18)-C(17)-C(16)	121.2(9)
C(19)-C(18)-C(17)	120.6(10)	C(14)-C(19)-N(1)	115.0(7)
C(18)-C(19)-N(1)	126.8(9)	C(18)-C(19)-C(14)	118.1(8)

Bond lengths (A)

S(1)-C(13)	1.753 (8)	S(1)-C(14)	1.741 (8)
Si(1)-O(1)	1.667 (5)	Si(1)-C(1)	1.871 (9)
Si(1)-C(5)	1.848 (9)	Si(1)-C(6)	1.855 (9)
O(1)-C(7)	1.399 (9)	O(2)-C(11)	1.186 (10)
O(3)-C(11)	1.331 (10)	O(3)-C(12)	1.432 (10)
O(4)-C(9)	1.416 (10)	N(1)-C(13)	1.277 (10)
N(1)-C(19)	1.401 (10)	C(1)-C(2)	1.512 (13)
C(1)-C(3)	1.531 (13)	C(1)-C(4)	1.522 (13)
C(7)-C(8)	1.518 (12)	C(7)-C(9)	1.588 (12)
C(7)-C(13)	1.527 (11)	C(9)-C(10)	1.499 (11)
C(9)-C(11)	1.530 (13)	C(14)-C(15)	1.397 (11)
C(14)-C(19)	1.377 (12)	C(15)-C(16)	1.388 (11)
C(16)-C(17)	1.388 (13)	C(17)-C(18)	1.358 (12)
C(18)-C(19)	1.376 (11)		

mixture of all four possible diastereomers of methyl 2,3-dihydroxy-2-methyl-3-(2-benzothiazolyl)-butanoate was obtained. Racemisation of the deprotected compound probably occurs by rapid loss of the benzothiazole ring from the alkoxide (14), produced as a result of removal of the silicon protecting group, and subsequent recombination with the α-carbonyl centre results in racemisation.

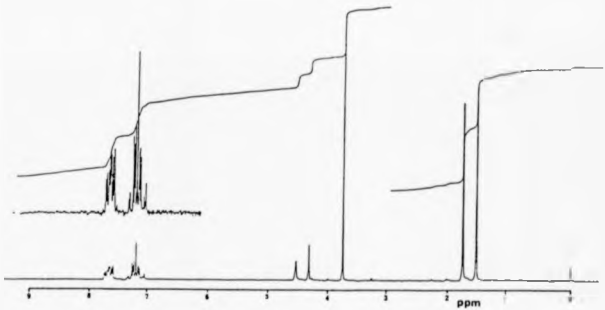


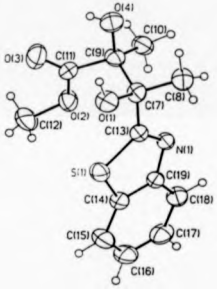
(14) (17)

Scheme 11

In an effort to retain the stereochemical integrity of the protected material, the removal of the silyl-protecting group was attempted under acidic conditions (HOAC, H_2O, THF).[88] The silylated material was incredibly resistant to acid catalysed cleavage and decomposition of the compound occurred before the loss of the protecting group.

As a result of the racemisation observed when removing the silyl-protecting group the non-crystalline diastereomeric mixture of methyl 2-hydroxy-2-methyl-3-tert-butyldimethylsiloxy-3-(2-benzothiazolyl)-butanoate (13) was treated with tetrabutylammonium fluoride under anhydrous conditions. The resulting (2R3R, 2S3S) and (2S3R, 2R3S) diastereomeric pairs of methyl 2,3-dihydroxy-2-methyl-3-(2-benzothiazolyl)-butanoate (15) were separated using flash chromatography and after separation, one of the diastereomeric pairs





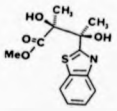


Fig. 2.3. X-ray crystal structure and ^{1}Hnmr spectrum of methyl 2,3-dihydroxy-2-methyl-3-(2-benzothiazolyl)-butanoate (15).

Table 3. Bond angles and lengths.

Bond angles (°)

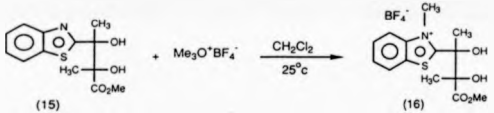
C(13)-S(1)-C(14)	88.6(2)	C(11)-O(2)-C(12)	115.9(3)
C(13)-N(1)-C(19)	111.0(3)	O(1)-C(7)-C(8)	110.0(3)
O(1)-C(7)-C(9)	108.2(2)	C(8)-C(7)-C(9)	111.7(3)
O(1)-C(7)-C(13)	103.7(3)	C(8)-C(7)-C(13)	110.9(3)
C(9)-C(7)-C(13)	112.0(3)	O(4)-C(9)-C(7)	105.8(3)
O(4)-C(9)-C(10)	108.6(3)	C(7)-C(9)-C(10)	113.9(3)
O(4)-C(9)-C(11)	108.4(3)	C(7)-C(9)-C(11)	109.9(3)
C(10)-C(9)-C(11)	110.0(3)	O(2)-C(11)-O(3)	123.4(4)
O(2)-C(11)-C(9)	112.8(3)	O(3)-C(11)-C(9)	123.8(4)
S(1)-C(13)-N(1)	115.9(3)	S(1)-C(13)-C(7)	118.5(2)
N(1)-C(13)-C(7)	125.6(3)	S(1)-C(14)-C(15)	129.6(3)
S(1)-C(14)-C(19)	110.1(3)	C(15)-C(14)-C(19)	120.3(3)
C(14)-C(15)-C(16)	117.4(4)	C(15)-C(16)-C(17)	122.2(4)
C(16)-C(17)-C(18)	120.9(4)	C(17)-C(18)-C(19)	118.4(3)
N(1)-C(19)-C(14)	114.3(3)	N(1)-C(19)-C(18)	124.8(3)
C(14)-C(19)-C(18)	120.8(3)		

Bond lengths (Å)

S(1)-C(13)	1.746 (4)	S(1)-C(14)	1.717 (4)
O(1)-C(7)	1.423 (4)	O(2)-C(11)	1.324 (5)
O(2)-C(12)	1.447 (6)	O(3)-C(11)	1.198 (5)
O(4)-C(9)	1.412 (5)	N(1)-C(13)	1.281 (4)
N(1)-C(19)	1.386 (5)	C(7)-C(8)	1.518 (5)
C(7)-C(9)	1.570 (4)	C(7)-C(13)	1.507 (5)
C(9)-C(10)	1.533 (5)	C(9)-C(11)	1.521 (5)
C(14)-C(15)	1.405 (6)	C(14)-C(19)	1.393 (5)
C(15)-C(16)	1.375 (6)	C(16)-C(17)	1.377 (6)
C(17)-C(18)	1.373 (5)	C(18)-C(19)	1.390 (5)

crystallised. Assignment of the relative configuration of the crystals was made by x-ray crystallography. The crystals obtained were found to be composed of molecules with either the 2S,3R or 2R,3S configuration. The crystal structure and corresponding ^{1}Hnmr spectrum are shown in figure 2.3.

The final stage of the reaction pathway, designed to produce methyl α-acetolactate (17), involved two consecutive steps. The (2S,3S),(2R,3R) and (2S,3R),(2R,3S) diastereomers of methyl 2,3-dihydroxy-2-methyl-3-(2-benzothiazolyl)-butanoate (15) were each treated with trimethyloxonium tetrafluoroborate in order to quaternise the nitrogen atom of the heterocyclic aromatic ring. The quaternisation of the benzothiazole ring was followed by ^{1}Hnmr, the growth of a quaternary nitrogen methyl peak at δ 3.3 and a shift in the aromatic hydrogen resonances from δ 7.5 and 8.0 to δ 7.85 and 8.15 were used to study the progress of the reaction.



(15) (16)

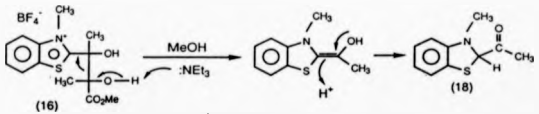
Scheme 12

The N-methyl benzothiazolium salts (16) were each used immediately on formation, in the next reaction, which involved refluxing the salts in a methanol-triethylamine mixture. The products obtained from the reactions were separated by flash-chromatography.

It was found that each of the quaternised diastereomeric diols gave two major products, and one minor product. (The yields of the products formed, are shown in Table 4.)

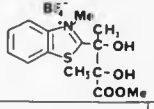
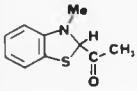
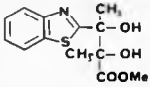
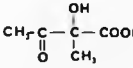
Methyl α-acetolactate (17) was produced from both diastereomeric diols. The ^{1}Hnmr spectrum of methyl α-acetolactate exhibited three characteristic methyl peaks at δ 3.82, 2.3 and 1.6, and the ^{13}Cnmr spectrum showed six carbon resonances at δ 204.7, 171.6, 80.9, 53.1, 23.9 and 21.7. Each diastereomeric diol pair gave a high percentage recovery of unreacted diol, after treatment with trimethyloxonium tetrafluoroborate and refluxing in methanol and triethylamine. It was also found that 2-acetyl-3-methylbenzo-dihydro-thiazole (18) was produced from both diastereomeric diols (15). The ^{1}Hnmr spectrum of 2-acetyl-3-methylbenzodihydrothiazole (18) exhibited a characteristic N-methyl peak at δ 2.95, an acetyl peak at δ 2.3 and a single proton resonance at δ 5.3 associated with a proton at the C-2 position of the dihydrothiazole ring.

2-Acetyl-3-methylbenzo-dihydrothiazole is formed by a reverse aldol condensation, resulting from deprotonation of the hydroxyl group adjacent to the ester group.



Scheme 13

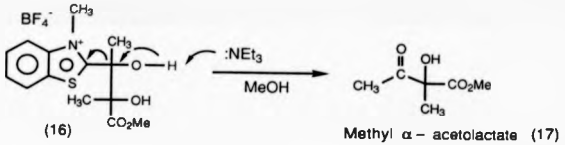
Table 4. The formation of methyl α-acetolactate (17).

PRODUCTS :	 % YIELD (19)	
	(2R,3S),(2S,3R)	(2R,3R),(2S,3S)
 (18)	9·8	9·4
 (19)	27·0	37·2
 (17)	14·8	26·5

All efforts to protect selectively the (C-2)-hydroxyl group, and prevent a reverse-aldol process were unsuccessful. The attempted addition of various protecting groups to methyl 2-hydroxy-2-methyl-3-tert-butyldimethylsiloxy-3-(2'-benzothiazolyl)-butanoate (13), including acyl and tetrahydropyran, failed owing to the highly hindered nature of the tertiary hydroxyl group.

The recovery of a significant proportion of the benzothiazole diols after quaternisation and treatment with methanol and triethylamine, may be due to incomplete quaternisation of the thiazole ring (the trimethyloxonium tetrafluoroborate began to decompose over the long period of time required for N-methylation) or displacement of the N-methyl group during the reaction. From the data obtained, the precise nature of the benzothiazole-diol recovery is unclear.

Methyl α-acetolactate was isolated from the reaction formed by the displacement of the quaternised benzothiazole fragment.



(16) Methyl α – acetolactate (17)

Scheme 14

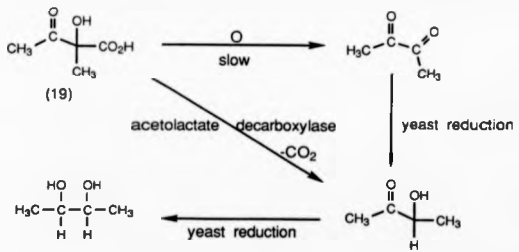
Both diastereomeric pairs of methyl 2,3-dihydroxy-2-methyl-3-(2-benzothiazolyl)-butanoate gave methyl α-acetolactate. However the (2R3R, 2S3S) enantiomeric pair appeared to give a significantly higher proportion of methyl α-acetolactate (17).

The synthetic pathway developed provides a novel route to heterogeneous acyloins using thiazole-thiazolium species in a non-catalytic process. The use of thiazole-thiazolium compounds allows the formation of stable carbanionic species of the type R-$\overset{\ominus}{C}$=O, which can be reacted in principle with any suitable acceptor compound. The thiazole-thiazolium species can be removed under mild conditions and therefore provide a useful synthetic tool. The synthetic pathway provides a model syatem for the action of thiamine pyrophosphate in acetohydroxyacid synthase, the first enzyme of the valine-isoleucine pathway. The synthetic intermediates isolated are directly comparable with the biological intermediates found in thiamine pyrophosphate containing systems.

CHAPTER 3

Investigation into the mode of action of acetolactate decarboxylase

α-Acetolactate (19) and α-acetohydroxybutyrate, the biological precursors of valine and isoleucine respectively, are the natural substrates of the enzyme acetolactate decarboxylase. The enzymic decarboxylation of α-acetolactate has potential use in the brewing industry.[63] During the maturing process of beer, α-acetolactate formed by yeast fermentation undergoes non-enzymatic oxidative decarboxylation to give butan-2,3-dione. The concentration of butan-2,3-dione produced during maturation of beer plays a major role in determining the flavour of the beer; a concentration of greater than 0.1ppm results in an objectionable taste. Butan-2,3-dione produced during maturation is converted by enzymic reduction to 2,3-butandiol.



Scheme 15

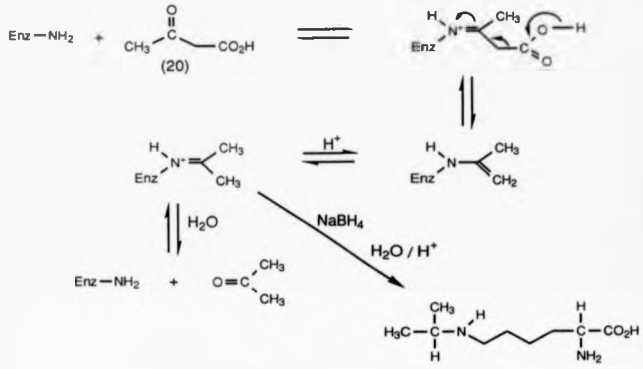
The addition of acetolactate decarboxylase to maturing beer by-passes the rate limiting production of butan-2,3-dione, and allows the direct conversion of α-acetolactate into acetoin. The addition of acetolactate decarboxylase therefore permits a shortening of the maturation period, and a fine control of the concentrations of α-acetolactate and butan-2,3-dione.

The mechanism of enzyme catalysed β-keto acid decarboxylation has been studied by Westheimer[84,85] *et al.* The enzyme acetoacetate decarboxylase (EC4.1.1.4) was investigated. It was found that at the active site of the enzyme, the ε-amine group of a lysine residue forms a protonated Schiff's base with the β-keto group of acetoacetate(20). The formation of a Schiff's base facilitates the loss of carbon dioxide and subsequent hydrolysis of the Schiff's base results in the formation of the decarboxylated product (acetone). The Schiff's base intermediate was trapped by incubating the enzyme and radio-labelled acetoacetate in the presence of sodium borohydride[86] (scheme 16).

Juni[87] investigated acetolactate decarboxylase isolated from *Aerobacter aerogenes*. It has been found[88] that the (S)-isomers of α-acetolactate and α-acetohydroxybutyrate are the preferred substrates for the enzyme. The (R)-isomers are also decarboxylated by the enzyme, but at a rate 1/20th of that associated with the (S)-isomers. Stereochemical studies showed that (S)-(+)- α-acetolactate was decarboxylated by acetolactate decarboxylase, to yield (R)-(-)-acetoin. It has been postulated that the reaction proceeds via an enzyme bound enediol or Schiff's base intermediate (scheme 17).

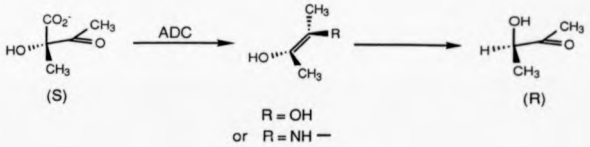
Crout and co-workers[89] established from studies of acetolactate decarboxylase isolated from *Klebsiella aerogenes* that protonation of the postulated intermediate occurred at the carbon atom

to which the carboxyl group was originally attached, with overall inversion of configuration.



Scheme 16

Investigations by Rathbone and Crout[84] using acetolactate decarboxylase isolated from *Bacillus brevis*, showed that racemic α-acetohydroxybutyrate was decarboxylated rapidly to give 3-hydroxypentan-2-one, up to 50% reaction. This was followed by a



Scheme 17

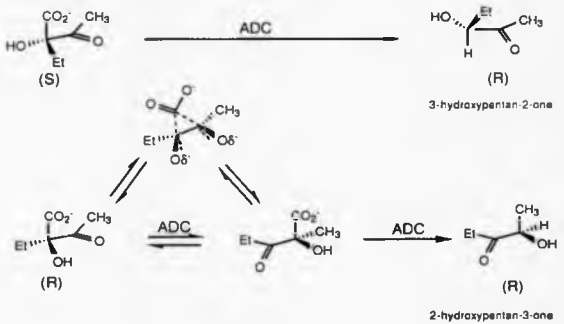
slower, consecutive reaction yielding 2-hydroxypentan-3-one. Chiral analysis showed that the two products were formed with high optical purities (>93% ee).

It was proposed that the (S)-isomer of α-acetohydroxybutyrate is rapidly decarboxylated with inversion of configuration to give (R)-3-hydroxypentan-2-one. However, (R)- α -acetohydroxybutyrate undergoes an enzyme catalysed, intramolecular carboxylate group migration, to give (S)-2-hydroxy-2-methyl-3-oxopentanoate, which is then decarboxylated to yield (R)-2-hydroxypentan-3-one (scheme 18).

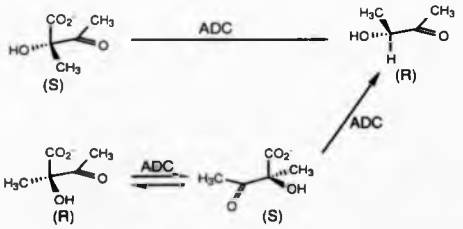
It was also found that racemic α-acetolactate when treated with acetolactate decarboxylase gave exclusively (R)-(-)-acetoin (>99% ee).[89] It was postulated that (S) α-acetolactate was rapidly decarboxylated to yield (R)-acetoin and that (R) α-acetolactate was converted into (R)-acetoin via prior rearrangement into (S) α-acetolactate and subsequent decarboxylation (scheme 19).

The base-catalysed[90] racemisation of optically pure α-acetolactate provides a model system for the hypothesised enzymic carboxylate migration. It has been shown using isotopically labelled α-acetolactate[91] that at a pH greater than 12.9,[48] α-acetolactate

undergoes an intramolecular tertiary ketol rearrangement with migration of the carboxylate group. This rearrangement is accompanied by racemisation.



Scheme 18



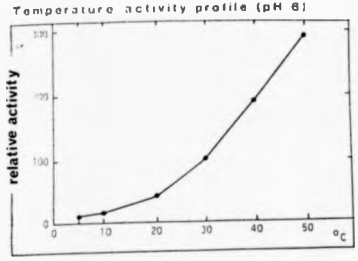
Scheme 19

The decarboxylation of racemic α-acetolactate by acetolactate decarboxylase has been shown to produce a single enantiomer of acetoin. The aim of the work described in this section was to synthesise [3,4-$^{13}C_2$]- α -acetolactate in an attempt to study in greater detail the mode of action of acetolactate decarboxylase.

Acetolactate decarboxylase utilised in these studies was supplied by Novo Industri A/S. The enzyme, isolated from *Bacillus brevis*, was used in a partially purified form. The enzyme has been purified by Ottesen and co-workers[83], and was found to exist as a dimer molecular weight 70,000. The purified enzyme had a specific activity of 900U/mg (1U=1 micromol. of acetoin produced per min. at 30°C). The temperature and pH activity profiles of the purified enzyme are shown in figure 3.1. The enzyme was found to be stable up to 40°C. However, at higher temperatures rapid non-enzymatic decarboxylation of the substrate occurred. The pH optimum of the enzyme was found to be 6.

Ethyl[3,4-$^{13}C_2$]-tiglate (21) was synthesised using phosphorus ylide chemistry developed by Wadsworth and Emmons.[92] Triethyl-2-phosphonopropionate was dissolved in 1,2-dimethoxyethane and treated with one equivalent of sodium hydride. The resulting phosphorus ylide was reacted with [1,2-$^{13}C_2$]-acetaldehyde (98%).

High resolution ^{1}Hnmr (400MHz) of the isolated material indicated the formation of a cis-trans isomeric mixture of ethyl(3,4-$^{13}C_2$]-tiglate. Complex multiplets at δ 6.0and 6.8 were characteristic of a trans olefinic hydrogen atom, and a cis olefinic hydrogen atom, respectively. The ratio of the cis-isomer to the trans-isomer was 1:13. The preferential formation of trans ethyl[3,4-$^{13}C_2$]-tiglate results from the formation of the thermodynamically preferred betaine intermediate (fig. 3.2).



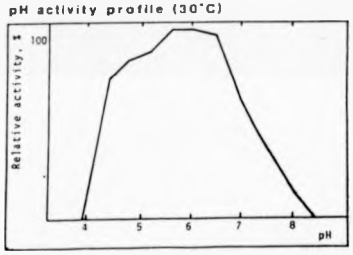
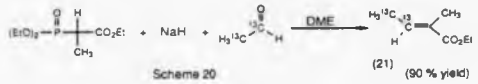


Fig. 3.1. Temperature and pH activity profiles of acetolactate decarboxylase.

Characterisation of the product by ^{1}Hnmr showed that the two ^{13}C-atoms incorporated into the molecule gave complex nmr splitting patterns, resulting from 1,2 and 3-bond carbon-hydrogen coupling.



Scheme 20

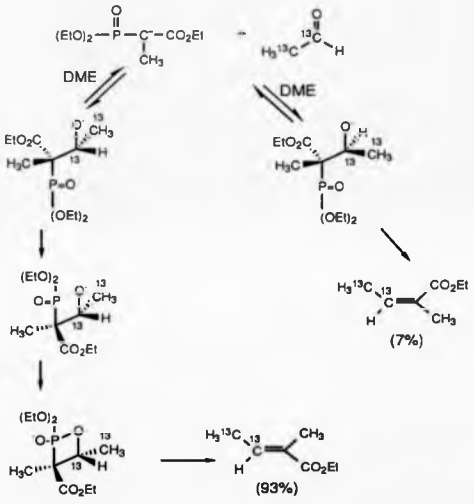
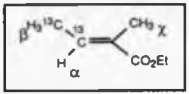


Fig. 3.2 Phosphorus ylide chemistry.

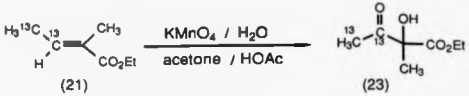


Hydrogen atoms	Chemical shift / ppm	Coupling constants / Hz
α H-^{13}C=	6.8(m)	J C-3,Hα =156.9
		JC-3,Hβ = 7.0
β ^{13}CH$_3$	1.74(dddq)	JC-3,Hχ = 5.6
		JHχ,Hα =1.6
χ CH$_3$	1.8(m)	JC-4,Hβ = 126.8
		JHβ,Hα = 7.0
		JHχ,Hβ = 1.2

The conversion of alkenes into hydroxy-ketones using potassium permanganate under acidic conditions has been reported by Srinivasan and Lee.[83,84] The reaction procedure was extended to include $\alpha\beta$-unsaturated esters, hence, ethyl[3,4-$^{13}C_2$]-tiglate was dissolved in aqueous acetone, containing a small quantity of acetic acid (2%). The reaction mixture was cooled to -10°C and potassium permanganate was added, the subsequent reaction produced a 70% yield of ethyl [3,4-^{13}-C_2]- α-acetolactate (23).

The ^{1}Hnmr spectrum of the product (fig. 3.3) exhibited a doublet δ 1.57 ($J^{13}C(3)H$=4Hz) due to the α-methyl protons and a doublet of doublets δ 2.25 ($J^{13}C(4)H$=129Hz,$J^{13}C(3)H$=6Hz) resulting from the ^{13}C-methyl group. The ^{13}Cnmr spectrum of the product exhibited two intense doublets at δ 24 and δ 204.7, corresponding to the carbon resonances of the ^{13}C-methyl group and the ^{13}C-carbonyl

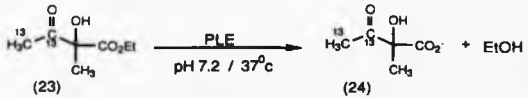
group respectively. The observed coupling to give two doublets J=42.7Hz, arises from ^{13}C-^{13}C coupling.



(21) (23)

Scheme 21

In order to study the mode of action of acetolactate decarboxylase, ethyl [3,4-$^{13}C_2$]- α -acetolactate was converted into the free acid by treatment with pig liver esterase. Mild enzymic conditions were used for the hydrolysis reaction, because there is the possibility of β-keto ester cleavage under aqueous alkali conditions. The hydrolysis reaction was followed by ^{1}Hnmr (220MHz c.w.). The ^{1}Hnmr spectrum of the hydrolysed material (24) showed a doublet of doublets δ 2.2 resulting from the ^{13}C-methyl protons (J=129.5Hz, J=6.3Hz) and a doublet δ 1.38 (J=4.8Hz) resulting from the α-methyl protons.



(23) (24)

Scheme 22

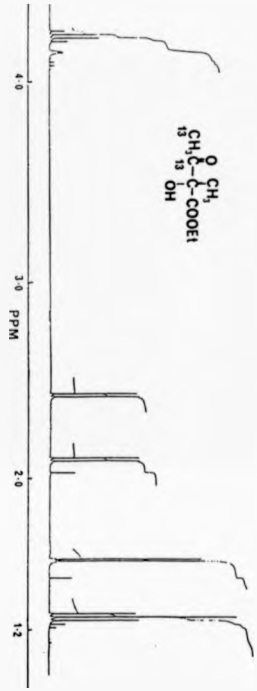
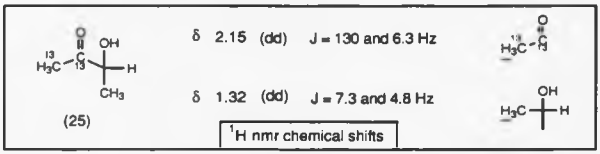


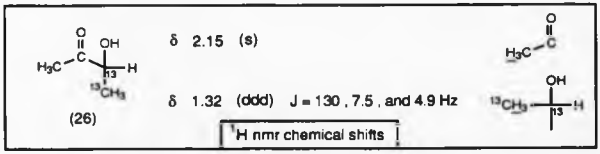
Fig. 3.3 ^{1}Hnmr spectrum of ethyl[3,4-$^{13}C_2$]- α-acetolactate

On completion of the hydrolysis reaction, acetolactate decarboxylase was added to the reaction mixture, and the subsequent decarboxylation reaction was studied by ^{1}Hnmr. It was found that there was an initial fast reaction producing resonances attributable to [1,2-$^{13}C_2$]-3-hydroxybutan-2-one (25), up to 50% reaction.



Scheme 23

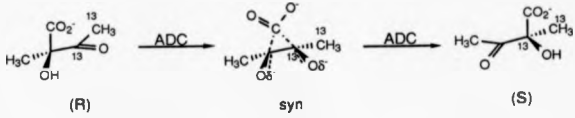
There was also an appreciably slower reaction producing nmr signals attributable to [3,4-$^{13}C_2$]-3-hydroxybutan-2-one (26). The rate of formation of [3,4-$^{13}C_2$]-3-hydroxybutan-2-one only became significant after the production of [1,2-$^{13}C_2$]-3-hydroxybutan-2-one had virtually ceased. (fig. 3.4).



Scheme 24

^{1}Hnmr analysis of the reaction showed that racemic [3,4-$^{13}C_2$]-α-acetolactate on treatment with acetolactate decarboxylase gave sequentially, two different forms of ^{13}C-labelled 3-hydroxybutan-2-one

(acetoin). Experimental studies by Rathbone[88] showed that racemic α-acetolactate gave exclusively (R)-(-)-acetoin. It is proposed that the (S)-isomer of [3,4-$^{13}C_2$]- α-acetolactate is preferentially bound to the active site of the enzyme, and undergoes decarboxylation with inversion of configuration, to produce [1,2-$^{13}C_2$]-3-hydroxybutan-2-one (fig. 3.5). The (R)-isomer of [3,4-$^{13}C_2$]- α-acetolactate undergoes an enzyme bound, intramolecular, carboxylate ion migration. If the carboxylate ion migration occurs with a syn arrangement of the hydroxyl and keto oxygen atoms (R)-[3,4-$^{13}C_2$]- α-acetolactate is converted into the corresponding (S)-isomer (scheme 25).



Scheme 25

The enzyme catalysed rearrangement results in an apparent shift of the two ^{13}C-atoms. The decarboxylation of the rearranged α-acetolactate occurs with inversion of configuration and protonation now occurs at the ^{13}C-α position, to produce (R)-[3,4$^{13}C_2$]-3-hydroxybutan-2-one. (fig. 3.5).

The experiments with [3,4-$^{13}C_2$]- α-acetolactate indicate that acetolactate decarboxylase has the ability to convert both the S and R isomers of α-acetolactate into acetoin. The less-favoured R-isomer undergoes an enzyme catalysed rearrangement which is then followed by decarboxylation.

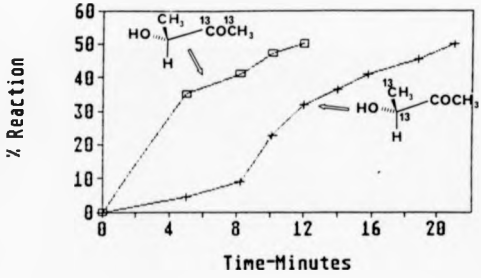


Fig. 3.4 The decarboxylation of [3,4-$^{13}C_2$]- α -acetolactate catalysed by acetolactate decarboxylase

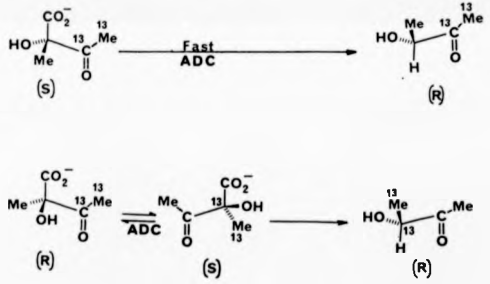
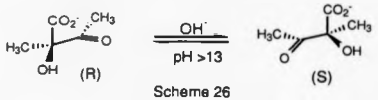


Fig. 3.5 The enzyme catalysed decarboxylation of [3,4-$^{13}C_2$]-α-acetolactate.

CHAPTER 4

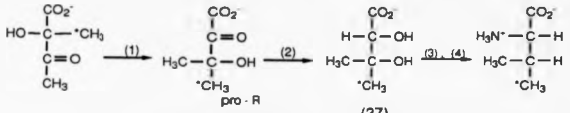
Stereochemistry of the base catalysed rearrangement of α-acetolactate (19)

The base-catalysed racemisation of α-acetolactate (2-hydroxy-2-methyl-3-oxobutanoic acid) has been shown to proceed by a reversible, intramolecular, tertiary ketol rearrangement with migration of the carboxylate ion.[81] The carboxylate ion migration only occurs at a pH greater than 13.[81]. (Scheme 26).



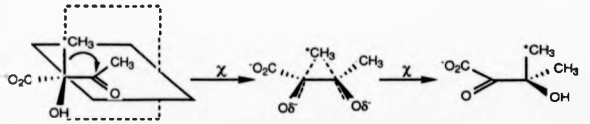
Scheme 26

As described in Chapter 1 (fig. 1.1) α-acetolactate is a biological precursor of valine. α-Acetolactate is converted into valine by three consecutive enzymatic reactions. Acetohydroxyacid reductoisomerase catalyses two reactions in which α-acetolactate undergoes a tertiary ketol rearrangement with migration of the α-methyl group to produce 3-hydroxy-3-methyl-2-oxobutanoate, (step (i), scheme 27); subsequent reduction produces 2,3-dihydroxy-3-methylbutanoate (step (ii), scheme 27). The diol-intermediate is then converted into L-valine by sequential enzymatic reactions catalysed by α,β-dihydroxyacid dehydratase and transaminase B. (steps (iii) and (iv), scheme 27).



Scheme 27

The tertiary ketol rearrangement catalysed by acetohydroxyacid reductoisomerase has been investigated.[98] It was found that the methyl group migrating during the reaction becomes the pro-R methyl group of the intermediate diol (27). The conformation of α-acetolactate at the active site of the enzyme must therefore be as shown in scheme 28. It follows that during the rearrangement the methyl group is delivered to the *re* face of the trigonal acetyl system. The conformation adopted by the molecule permits maximum overlap between the orbitals of the migrating C-methyl σ-bond and the π-system of the carbonyl group.



χ - acetohydroxyacid reductoisomerase

Scheme 28

The stereochemical result indicates that the substrate conformation during the reaction requires a *syn* arrangement of the C-O bonds at the C-2 and C-3 positions.

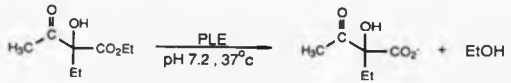
The alkali-catalysed rearrangement of α-acetolactate (scheme 26) results in racemisation of optically pure starting material, hence the transition state must be partly, if not wholly, represented by a *syn* arrangement of the C-O bonds at the C-2 and C-3 positions. If an anti-arrangement of the C-O bonds was to pertain exclusively in the transition state, migration of the carboxylate ion would lead to the formation of a new chiral centre of identical configuration compared with that of the starting material.

The migration of the carboxylate group in preference to the methyl group under aqueous alkaline conditions is attributable to the greater migratory aptitude of the carboxylate group. This conclusion is supported by parallel investigations into the benzilic acid rearrangement of 2,3-dioxobutanoate, where the same preferential migration of the carboxylate group was observed.[84]

The aims of the work described were to examine whether the alkali-catalysed carboxylate ion migration occurs under stereochemical control, whereby the R-isomer of α-acetolactate would be converted exclusively into the S-isomer. From such an investigation it would be possible to determine whether a conformation is assumed by the substrate similar to that proposed for the enzymatic methyl migration, but with the carboxylate group rather than the methyl group in the plane perpendicular to the trigonal acetyl system.

The stereochemical course of the tertiary ketol rearrangement was investigated by the following procedure. α-Acetohydroxybutyrate (28) was used in preference to α-acetolactate for the stereochemical investigations since the α-ethyl group of α-acetohydroxybutyrate

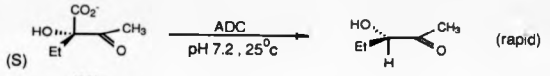
permitted the ^{1}Hnmr interpretation of the reactions studied. The ester group of racemic ethyl α-acetohydroxybutyrate was hydrolysed by treatment with pig liver esterase (PLE). (Scheme 29).



(28)

Scheme 29

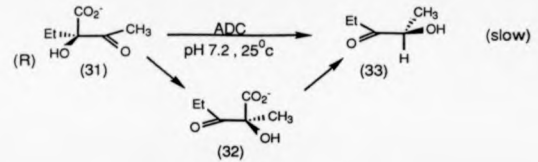
Subsequent treatment of racemic α-acetohydroxybutyrate with acetolactate decarboxylase separated the R-enantiomer of α-acetohydroxybutyrate from the S-enantiomer. Acetolactate decarboxylase rapidly decarboxylates the S-enantiomer of α-acetohydroxybutyrate (29) producing 3-hydroxypentan-2-one (30) (scheme 30).



(29) (30)

Scheme 30

The R-enantiomer of α-acetohydroxybutyrate (31) undergoes slow decarboxylation.[88] An enzyme-catalysed rearrangement to 2-hydroxy-2-methyl-3-oxopentanoate (32) precedes decarboxylation to yield 2-hydroxypentan-3-one (33) (scheme 31).



Scheme 31

The rate of decarboxylation of the R-enantiomer of α-acetohydroxybutyrate only becomes significant after decarboxylation of (S)-α-acetohydroxybutyrate is complete.

Thus treatment of racemic α-acetohydroxybutyrate with acetolactate decarboxylase resulted in the rapid decarboxylation of (S)-α-acetohydroxybutyrate up to 50% reaction. Subsequent quenching of the now slow enzymic decarboxylation of (R)-α-acetohydroxybutyrate by removal of the enzyme material (via centrifugation through a micro-pore filter) allowed the isolation of (R)-α-acetohydroxybutyrate. The volatile decarboxylation products, 3-hydroxypentan-2-one and traces of 2-hydroxypentan-3-one were removed by freeze drying.

The enzyme-catalysed ester hydrolysis and decarboxylation reactions of racemic ethyl α-acetohydroxybutyrate were investigated by ^{1}Hnmr.

The hydrolysis of ethyl α-acetohydroxybutyrate catalysed by pig liver esterase gave nmr peaks attributable to ethanol and α-acetohydroxybutyrate (fig. 4.1A). α-Acetohydroxybutyrate exhibited an α-acetyl singlet at δ 2.27, the methyl protons of the α-ethyl group gave a triplet at δ 0.85, and the diastereotopic methylene protons of the α-ethyl group gave multiplets at δ 1.8 and 2.0.

Addition of acetolactate decarboxylase to the solution

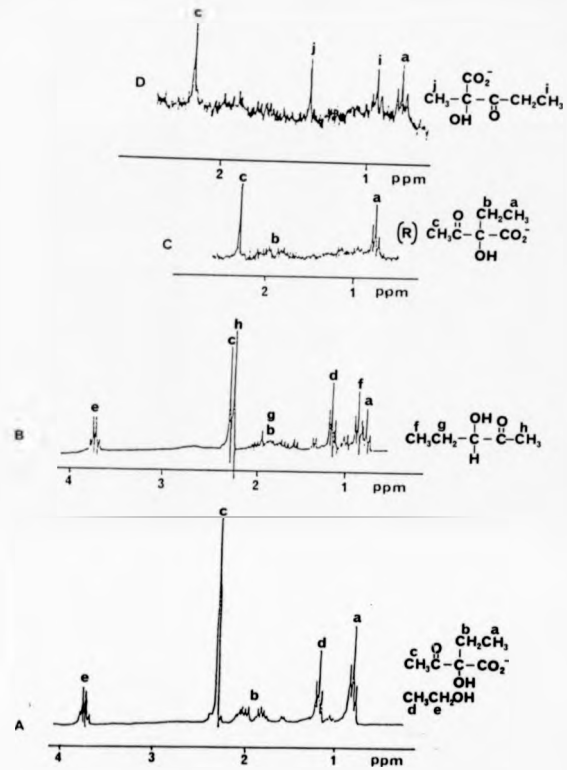


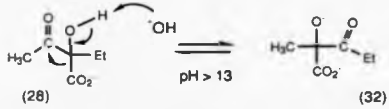
Fig. 4.1 ^{1}Hnmr spectra of the enzymic resolution of racemic α -acetchydroxybutyrate (28).

containing racemic α-acetohydroxybutyrate resulted in the rapid formation of a new set of ^{1}Hnmr peaks, including a new acetyl singlet at δ 2.1 (fig. 4.1.B). A corresponding loss in the size of the initial α-acetohydroxybutyrate ^{1}Hnmr peaks was also observed. The new ^{1}Hnmr peaks resulted from the formation of 3-hydroxypentan-2-one (30), which was produced by decarboxylation of the S-enantiomer of α-acetohydroxybutyrate. The decarboxylation reaction was quenched when the growth of ^{1}Hnmr peaks associated with 3-hydroxypentan-2-one had ceased, and a new doublet at δ 1.3 began to appear indicating formation of 2-hydroxypentan-3-one (33) from decarboxylation of (R)-α-acetohydroxybutyrate.

^{1}Hnmr showed that after quenching of the decarboxylation reaction and subsequent freeze-drying only resonances attributable to (R)-α-acetohydroxybutyrate remained. (fig. 4.1.C).

(R)-α-acetohydroxybutyrate isolated from the enzyme-catalysed decarboxylation reaction was used to probe the stereochemistry of the base-catalysed tertiary ketol rearrangement.

(R)-α-acetohydroxybutyrate was dissolved in aqueous sodium hydroxide and the subsequent reaction was investigated by ^{1}Hnmr. The characteristic α-acetyl singlet (δ 2.3) of α-acetohydroxybutyrate (28) was observed to decrease in size whilst new peaks associated with the formation of 2-hydroxy-2-methyl-3-oxopentanoate (32) were seen to increase in size with time. (fig. 4.1.D). 2-Hydroxy-2-methyl-3-oxopentanoate (32) is produced by the carboxylate group of (R)-α-acetohydroxybutyrate migrating to the adjacent carbonyl centre.

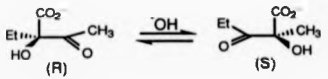


Scheme 32

The base-catalysed rearrangement was allowed to proceed until the ^{1}Hnmr spectra indicated a reaction mixture composition of 70% α-acetohydroxybutyrate (28) and 30% 2-hydroxy-2-methyl-3-oxopentanoate (32). The rearrangement was quenched by acidification of the reaction mixture to pH 8.

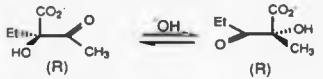
The base-catalysed tertiary ketol rearrangement of α-acetohydroxybutyrate might occur with or without stereochemical control. There are three stereochemical cases to be considered for the rearrangement reaction:

(i) If a syn arrangement of the C-2 and C-3 carbon-oxygen bonds pertained exclusively during the carboxylate ion migration, (R)-α-acetohydroxybutyrate would be in equilibrium with (S)-2-hydroxy-2-methyl-3-oxopentanoate (scheme 33).



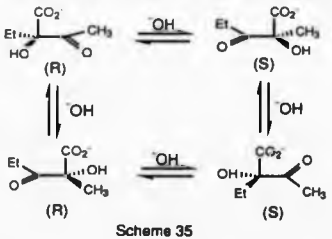
Scheme 33

(ii) If an anti arrangement of the C-2 and C-3 carbon-oxygen bonds pertained exclusively, (R)- α -acetohydroxybutyrate would be in equilibrium with (R)-2-hydroxy-2-methyl-3-oxopentanoate. (Scheme 34).



Scheme 34

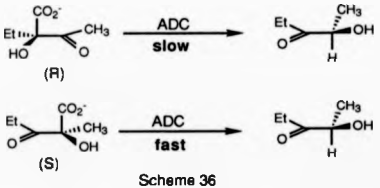
(iii) If the carboxylate ion migration occurred with a syn and anti arrangement of the C-2 and C-3 carbon-oxygen bonds, a mixture of (R,S)- α -acetohydroxybutyrate and (R,S)-2-hydroxy-2-methyl-3-oxopentanoate would be produced. (Scheme 35).



Scheme 35

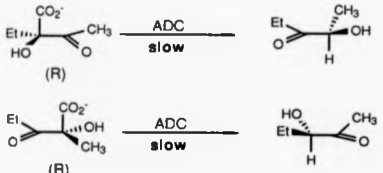
The stereochemistry of the carboxylate ion migration was investigated by reacting the products obtained from the rearrangement reaction with acetolactate decarboxylase. Subsequent analysis of the decarboxylation products by ^{1}Hnmr allowed the stereochemical composition of the rearrangement products to be determined. There are three sets of reactions to be considered:

(i) If a syn conformation of the C-2 and C-3 carbon-oxygen bonds were to pertain during the carboxylate ion migration, (R)-α-acetohydroxybutyrate and (S)-2-hydroxy-2-methyl-3-oxopentanoate would result from the rearrangement reaction. Treatment with acetolactate decarboxylase will then result in the slow decarboxylation of (R)-α-acetohydroxybutyrate to yield 2-hydroxypentan-3-one and rapid decarboxylation of (S)-2-hydroxy-2-methyl-3-oxopentanoate to yield 2-hydroxypentan-3-one. (Scheme 36).



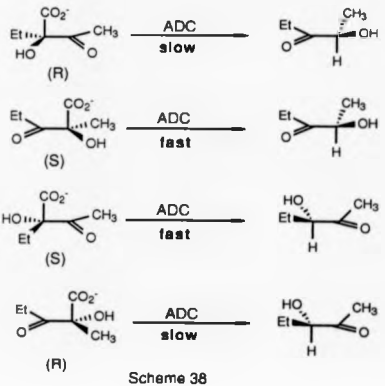
Scheme 36

(ii) If an anti conformation pertained during the carboxylate ion migration, (R)-α-acetohydroxybutyrate and (R)-2-hydroxy-2-methyl-3-oxopentanoate would result from the base-catalysed rearrangement. Treatment with acetolactate decarboxylase will then result in the slow decarboxylation of (R)-α-acetohydroxybutyrate to produce 2-hydroxypentan-3-one and slow decarboxylation of (R)-2-hydroxy-2-methyl-3-oxopentanoate to yield 3-hydroxypentan-2-one. (Scheme 37).



Scheme 37

(iii) If there was no stereochemical control (R,S)-α-acetohydroxybutyrate and (R,S)-2-hydroxy-2-methyl-3-oxopentanoate would result from the rearrangement reaction, and hence on treatment with acetolactate decarboxylase, (R)-α-acetohydroxybutyrate and (S)-2-hydroxy-2-methyl-3-oxopentanoate would be decarboxylated, at different rates, to yield 2-hydroxypentan-3-one. However (S)-α-acetohydroxybutyrate and (R)-2-hydroxy-2-methyl-3-oxopentanoate would be decarboxylated to yield 3-hydroxypentan-2-one. (Scheme 38).

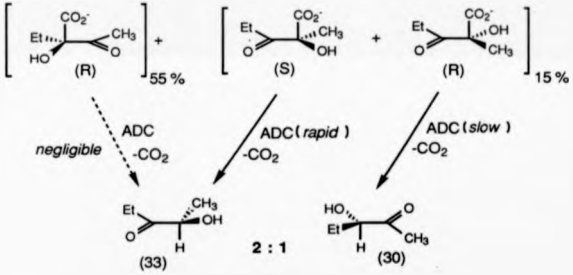


Scheme 38

The products resulting from the tertiary ketol rearrangement, α-acetohydroxybutyrate and 2-hydroxy-2-methyl-3-oxopentanoate, were treated with acetolactate decarboxylase and the ensuing reactions were

studied by ^{1}Hnmr (400 MHz, with water suppression and continuous data acquisition). It was found that the α-methyl singlet at δ 1.42 associated with 2-hydroxy-2-methyl-3-oxopentanoate rapidly decreased in size. The size of the acetyl singlet at δ 2.23 associated with α-acetohydroxybutyrate marginally decreased. It was also observed that a new acetyl singlet at δ 2.18 slowly increased in size, attributable to the formation of 3-hydroxypentan-2-one and a new doublet at δ 1.32 associated with the production of 2-hydroxypentan-3-one rapidly grew in size. (fig. 4.2).

An interpretation of the ^{1}Hnmr observations is shown in scheme 39.



Scheme 39

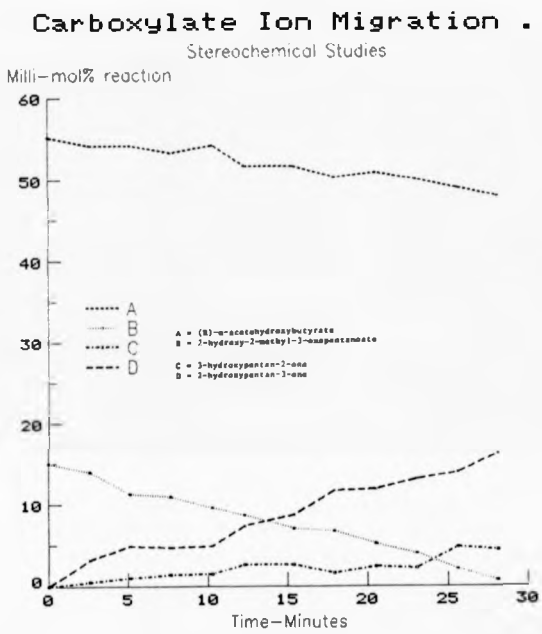


Fig. 4.2 Graph of the decarboxylation reactions catalysed by ADC, after the base-catalysed rearrangement of α - acetohydroxybutyrate.

The small decrease in the α-acetohydroxybutyrate nmr signals is attributable to the slow decarboxylation of the (R)-enantiomer of α-acetohydroxybutyrate producing 2-hydroxypentan-3-one (33). The base-catalysed rearrangement of (R)- α-acetohydroxybutyrate was not permitted to proceed to equilibrium. It is therefore suggested that only a small percentage of the (S)-enantiomer of α-acetohydroxybutyrate would be expected to be formed. The decarboxylation of 2-hydroxy-2-methyl-3-oxopentanoate, and corresponding loss in the nmr signals associated with 2-hydroxy-2-methyl-3-oxopentanoate, is accompanied by the formation of 3-hydroxypentan-2-one (30) and 2-hydroxypentan-3-one (33). The formation of 3-hydroxypentan-2-one and 2-hydroxypentan-3-one results from the decarboxylation of the (R) and (S)-enantiomers of 2-hydroxy-2-methyl-3-oxopentanoate respectively.

The formation of both hydroxy-ketones, at markedly different rates, excludes the possibility of strict anti or syn conformations during the tertiary ketol rearrangement. However analysis of the percentage compositions of the products resulting from reaction with acetolactate decarboxylase indicated that the ratio of 2-hydroxypentan-3-one (33) to 3-hydroxypentan-2-one (30), produced by the decarboxylation of 2-hydroxy-2-methyl-3-oxopentanoate, was 2 to 1.

The predominant formation of 2-hydroxypentan-3-one resulting from the decarboxylation of (S)-2-hydroxy-2-methyl-3-oxopentanoate (Scheme 39), suggests that during the base-catalysed rearrangement of (R)- α-acetohydroxybutyrate to 2-hydroxy-2-methyl-3-oxopentanoate, the S-enantiomer of 2-hydroxy-2-methyl-3-oxopentanoate is preferentially formed with respect to the R-enantiomer. Hence, a syn arrangement of the C-2 and C-3 carbon-oxygen bonds can be assumed to be the preferred conformation during the tertiary ketol rearrangement.

The base-catalysed rearrangement of (R)-α-acetohydroxybutyrate into 2-hydroxy-2-methyl-3-oxopentanoate was allowed to proceed to 30% conversion. The amount of back-reaction was therefore assumed to be small, and hence the proportion of (S)-α-acetohydroxybutyrate present in the mixture was treated as a minimum. However, if (S)-α-acetohydroxybutyrate was present in the rearrangement mixture, enzyme catalysed decarboxylation of (S)-α-acetohydroxybutyrate would give 3-hydroxypentan-2-one and hence the proportion of 3-hydroxypentan-2-one resulting from the decarboxylation of (R)-2-hydroxy-2-methyl-3-oxopentanoate would be less than that observed and therefore the preference for a syn-arrangement during the tertiary ketol rearrangement is possibly greater than the previously suggested 2:1 value.

An additional experiment involved the brief treatment of (R)-α-acetohydroxybutyrate with aqueous sodium hydroxide, and the products obtained were then treated with excess acetolactate decarboxylase. ^{1}Hnmr (400MHz) analysis of the initial and final concentrations of the substrates and decarboxylation products again indicated that a greater proportion of 2-hydroxypentan-3-one was formed by the decarboxylation of (S)-2-hydroxy-2-methyl-3-oxopentanoate relative to 3-hydroxypentan-2-one formed by the decarboxylation of (R)-2-hydroxy-2-methyl-3-oxopentanoate (fig. 4.3). The results obtained confirm the preferential formation of a syn conformation during the carboxylate ion migration.

The stereochemical studies suggest that the base-catalysed tertiary ketol rearrangement occurs with a degree of stereochemical control. During the rearrangement there is a preference for a syn conformation of the C-2 and C-3 carbon-oxygen bonds. The rearrangement does not occur with absolute stereochemical control, since in

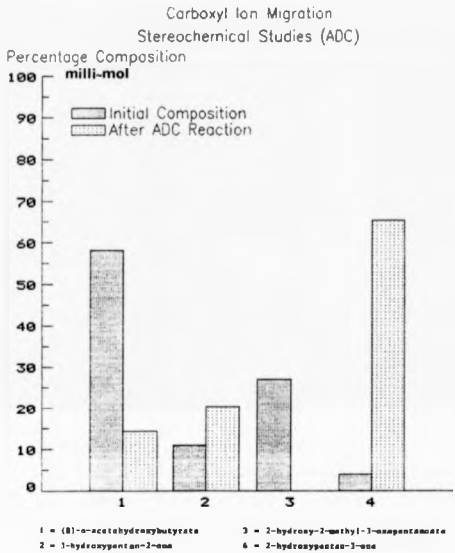


Fig. 4.3 Graph of the decarboxylation reactions catalysed by excess ADC, after the base-catalysed rearrangement of α-acetohydroxybutyrate.

aqueous solution there is the possibility of rotation about the (C-2)-(C-3) bond of α-acetohydroxybutyrate and the energy difference between conformers would be expected to be small. The formation of a preferred syn conformation is directly analogous to the syn conformation adopted by α-acetohydroxybutyrate during the ethyl migration catalysed by acetohydroxyacid reductoisomerase.

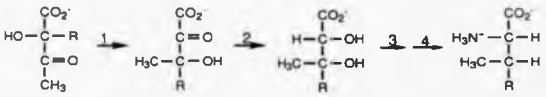
The lack of strict stereochemical control observed during the base-catalysed rearrangement, and the formation of syn and anti conformations during the carboxylate ion migration, suggests that the preferred syn conformation found in the reactions catalysed by acetohydroxyacid reductoisomerase and acetohydroxyacid decarboxylase results not from a strong stereo-electronic effect but from the nature of the enzyme active site.

CHAPTER 5

A novel chemical synthesis of

Methyl 2-hydroxy-2([^{2}H,^{3}H]methyl)-3-oxobutanoate

The biological conversion of pyruvate into valine and α-ketobutyrate into isoleucine involves four consecutive enzyme-catalysed reactions (fig. 1.1). The second enzyme of the biological pathway acetohydroxyacid reductoisomerase catalyses two reactions: an alkyl migration and the subsequent reduction of a carbonyl moiety. (Scheme 40).

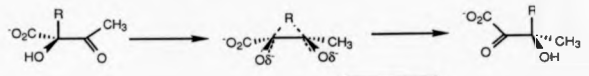


1 and 2 acetohydroxyacid reductoisomerase

Scheme 40

R = Me ⇒ Valine
or R = Et ⇒ Isoleucine

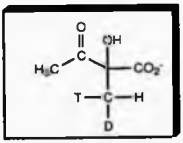
Stereochemical investigations[9,8] have shown that during the enzyme-catalysed rearrangement of 2-ethyl-2-hydroxy-3-oxobutanoate to 3-hydroxy-3-methyl-2-oxopentanoate and 2-hydroxy-2-methyl-3-oxobutanoate to 3-hydroxy-3-methyl-2-oxobutanoate the migrating alkyl group is transferred to the re-face of the trigonal centre at C-3. (Scheme 41).



Scheme 41

Further stereochemical studies[97] showed that the ethyl migration associated with 2-ethyl-2-hydroxy-3-oxobutanoate, and catalysed by acetohydroxyacid reductoisomerase, proceeds with retention of configuration at the migrating centre. Retention of configuration at the migrating centre during the tertiary ketol rearrangement has been predicted on theoretical grounds.[98]

In order to investigate whether the methyl migration associated with the enzymatic conversion of α-acetolactate (2-hydroxy-2-methyl-3-oxobutanoate) to 3-hydroxy-3-methyl-2-oxobutanoate occurs with retention or inversion of configuration at the migrating centre, it was proposed to synthesise 2-hydroxy-2-methyl-3-oxobutanoate with a chiral-methyl group at the 2-position.

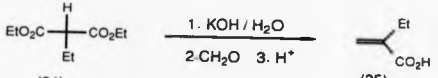


The synthesis of chiral-methyl α-acetolactate would allow stereochemical investigation of the methyl migration associated with

the valine biosynthetic pathway. Chiral analysis[99] of valine produced from chiral methyl α-acetolactate would permit the determination of whether the methyl migration catalysed by acetohydroxyacid reductoisomerase occurs with retention or inversion of configuration at the migrating methyl centre. Methyl group migration with retention of configuration has been demonstrated in 1,2-carbonium ion migrations occurring during steroid biosynthesis.[100]

The work described in this chapter reports a novel synthetic pathway to chiral methyl α-acetolactate.

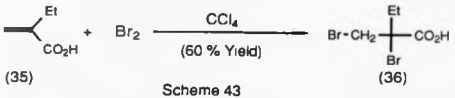
Diethyl ethyl malonate (34) was treated with aqueous alkali and the α-carbanion formed was reacted with formaldehyde. Subsequent treatment of the adduct formed with acid resulted in the formation of 2-ethyl-2-propenoic acid (35).[101,102]



(34) (35) (26 % Yield)

Scheme 42

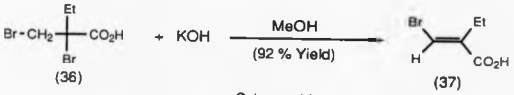
Bromination of 2-ethyl-2-propenoic acid in carbon tetrachloride yielded 2,3-dibromo-2-ethylpropanoic acid (36). The diastereotopic hydrogen atoms at the C-3 position of the dibromo-compound (36) exhibited an AB coupling system at δ 4.0 (J=10.7Hz) in the ^{1}Hnmr of the product. The dibromo-compound (36) was dehydrobrominated[103] by treatment with methanolic potassium



(35) (36)

Scheme 43

hydroxide.[104] Elimination of hydrogen bromide gave exclusively the E-product (37).

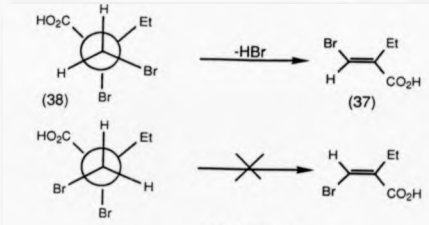


(36) (37)

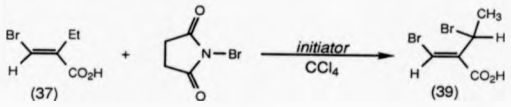
Scheme 44

The ^{1}Hnmr of 3-bromo-2-ethyl-2-propenoic acid exhibited a single olefinic peak at δ 7.7 attributable to the formation of the E-isomer only. (Anti-elimination[105] from the preferred conformation (38) would give the expected E-product, scheme 45.) Treatment of E-3-bromo-2-ethyl-2-propenoic acid (37) with N-bromosuccinimide[106] and a radical initiator[107] 2,2'-azo-bis(2-methylpropionitrile) resulted in allylic bromination and the formation of 3-bromo-2-(1-bromoethyl)-2-propenoic acid (39).

The ^{1}Hnmr of the allylic brominated compound (39) exhibited a single olefinic peak at δ 7.81 and the 1-bromoethyl side chain gave a quartet δ 5.36 (1H,J=7.2Hz) and a doublet δ2.0(3H,J=7.2Hz). The presence of two bromine atoms in the molecule (m.w.=258) was evident



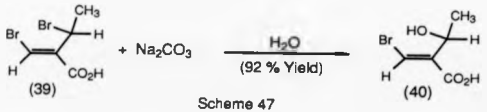
Scheme 45



Scheme 46 (90 % Yield)

from the ammonia chemical ionisation mass spectrum in which the $(MNH_4)^+$ ion gave peaks at 274/276/278 in the ratio 1:2:1 associated with the natural abundance of the isotopes of bromine.

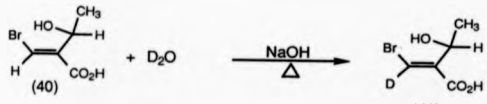
Treatment of 3-bromo-2-(1-bromoethyl)-2-propenoic acid (39) with aqueous sodium carbonate resulted in the replacement of the allylic bromine atom with a hydroxyl group.



Scheme 47

It was found that during the base-catalysed hydrolysis of the allylic bromine a small amount of trans-cis isomerisation occurred. The ^{1}Hnmr of the product (40) exhibited a major olefinic peak at δ 7.7 and also a minor olefinic peak at δ 7.0. The compound was purified by forming the dicyclohexylammonium salt of the trans-cis isomeric mixture of product, and subsequent recrystallisation and removal of the dicyclohexylammonium using ion exchange resin (Dowex-50W-X8-H) yielded E-3-bromo-2-(1-hydroxyethyl)-2-propenoic acid (40).

Reaction of E-3-bromo-2-(1-hydroxyethyl)-2-propenoic acid with sodium hydroxide and D_2O (deuterated water) resulted in the incorporation of a deuterium atom adjacent to the bromine atom at the C-3 position.



Scheme 48

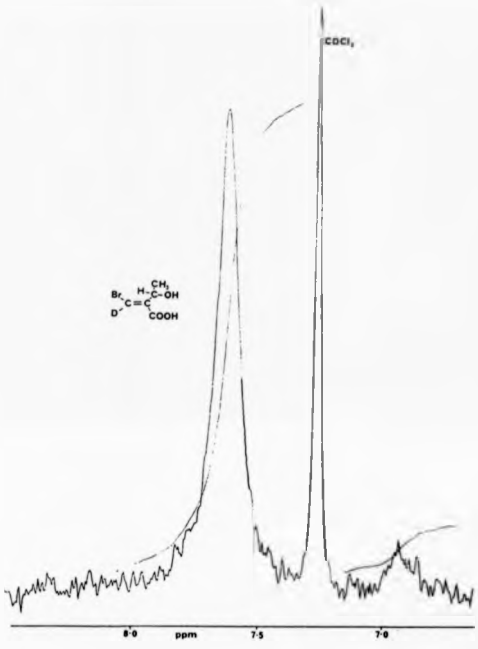
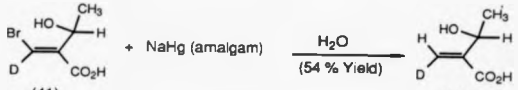


Fig. 5.1 ^{1}Hmmr spectrum of [3-^{2}H]-3-bromo-2-(1-hydroxyethyl)-2-propanoic acid

The ^{1}Hnmr of the product (41) exhibited a broad peak at δ 7.61 (fig. 5.1) associated with an olefinic deuterium atom.

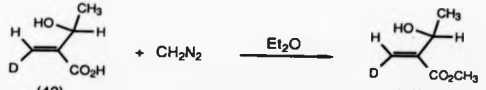
Sodium amalgam reduction[100] of the 3-bromopropenoic acid (41) in the presence of water resulted in the reduction of the bromine atom at the C-3 position with retention of the stereochemistry of the double-bond.



(41) (42)

Scheme 49

A minor disadvantage of the sodium amalgam reduction employed, was that over-reduction to the corresponding propionate occurred, to the extent of 12-15%. Purification of the product via its dicyclohexylammonium salt facilitated the isolation of the desired material (42). The purified acid was then converted into the methyl ester by treatment with diazomethane to yield methyl [3-^{2}H]-2-(1-hydroxyethyl)-2-propenoate (43).



(42) (43)

Scheme 50

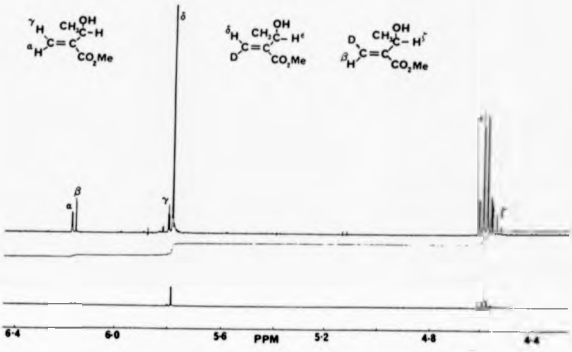
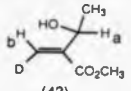


Fig. 5.2 ^{1}Hnmr (400MHz) spectrum of methyl [3-^{2}H]-2-(1-hydroxyethyl)-2-propenoate (43).

High field ^{1}Hnmr (400MHz) of the methyl ester (43) permitted the accurate determination of the stereochemical integrity of the product (fig. 5.2), (88.2%-Z isomer).

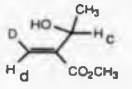


(43)

(88.2 %)

δHb = 5.79 (d)

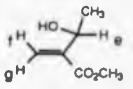
J HaHb = 1.28 Hz



(6.5 %)

δHd = 6.16 (d)

J HcHd = 0.65 Hz



(5.3 %)

δHf = 5.8 (dd)

J HeHf = 1.1 Hz

J HfHg = 1.1 Hz

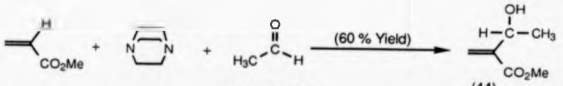
δHg = 6.17 (dd)

J HeHg = 0.65 Hz

J HgHf = 1.0 Hz

Scheme 51

In order to evaluate the proposed synthetic route to chiral-methyl α-acetolactate, methyl 2-(1-hydroxyethyl)-2-propenoate (44) was synthesised in an attempt to optimise the subsequent reactions without using valuable labelled material. Methyl 2-(1-hydroxyethyl)-2-propenoate was synthesised by the condensation of ethanal with methyl acrylate in the presence of 1,4-diazabicyclo[2.2.2] octane (DABCO).[109,110]

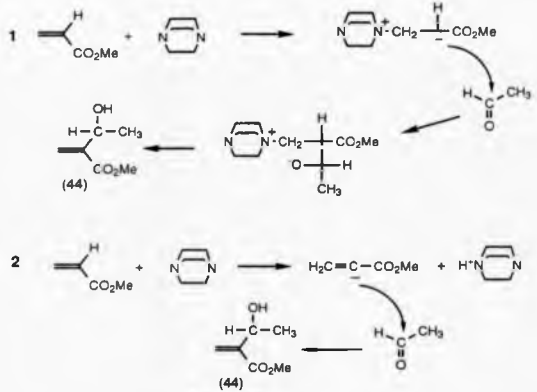


Scheme 52

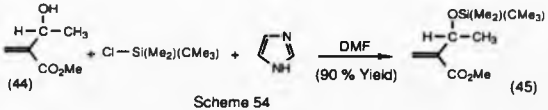
The aldol condensation catalysed by DABCO can be described by two possible mechanisms (Scheme 53). The amine might act as a nucleophile attacking the C-3 position of the acrylate system. Alternatively it might act as a base leading to the formation of a vinyl carbanion. Experimental studies have not elucidated which mechanism is favoured.[111]

The hydroxyl group of methyl 2-(1-hydroxyethyl)-2-propenoate (44) was protected as a tert-butyldimethylsilyl derivative. The tert-butyldimethylsilyl protecting group was used because of its stability over a wide range of conditions and facile cleavage by tetra-n-butylammonium fluoride.[82] Reaction of methyl 2-(1-hydroxyethyl)-2-propenoate with tert-butyldimethylsilylchloride in the presence of imidazole gave methyl 2-(1-tert-butyldimethylsiloxyethyl)-2-propenoate (45).

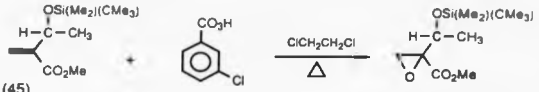
The attempted epoxidation of the silyl-protected compound (45) using 3-chloroperoxybenzoic acid at room temperature was unsuccessful. However, treatment of compound (45) with 3-chloroperoxybenzoic acid at 83°C in the presence of a radical inhibitor to prevent thermal decomposition[112] of the peracid yielded methyl 2,3-epoxy-2-(1-tert-butyldimethylsiloxyethyl)-propanoate (46).



Scheme 53



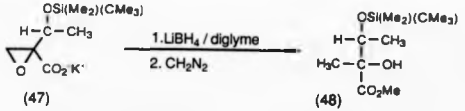
Scheme 54



Scheme 55

The ^{1}Hnmr of the product (46) indicated a diastereomeric mixture in the ratio 3:4, the protons at the C-3 position associated with the oxirane ring gave two AB-systems centred on δ 3.0. The ^{13}Cnmr spectrum of the epoxide (46), (fig. 5.3) exhibited two sets of peaks associated with the diastereomeric composition of the product.

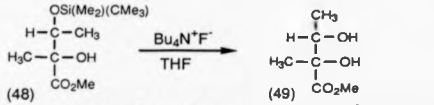
The methyl ester of the epoxide (46) was hydrolysed by treatment with potassium carbonate dissolved in methanol and water. The resulting potassium salt (47) was dissolved in diglyme and reacted with lithium borohydride at 100°C. Reduction of the epoxide and subsequent reaction with diazomethane yielded methyl 2-hydroxy-2-methyl-3-tert-butyldimethylsiloxybutanoate (48).



Scheme 56

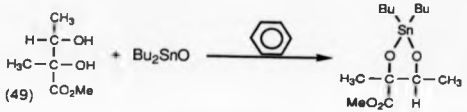
The silicon protecting group of compound (48) was cleaved by reaction with tetrabutylammonium fluoride under anhydrous conditions.

Fig. 5.3 ^{13}Cnmr spectrum of methyl 2,3-epoxy-2-(1-tert-butyldimethylsiloxyethyl)-propanoate.



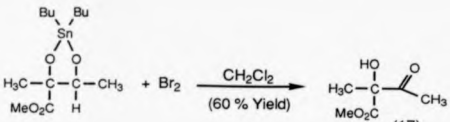
Scheme 57

Methyl 2,3-dihydroxy-2-methylbutanoate (49) was oxidised to a hydroxyketone by the brominolysis of a stannylene derivative.[113] The diol (49) was reacted with dibutyltin oxide in benzene suspension with azeotropic removal of water.



Scheme 58

The cyclic stannylene derivative was oxidised by treatment with bromine in a dichloromethane solution to yield methyl 2-hydroxy-2-methyl-3-oxobutanoate (17).

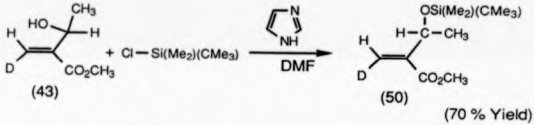


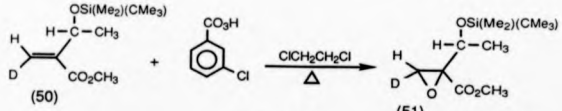
(17)

Scheme 59

The ^{1}Hnmr of methyl 2-hydroxy-2-methyl-3-oxobutanoate exhibited three methyl peaks at δ 3.85, 2.31 and 1.62 associated with the ester, C-3 and C-2 methyl groups respectively.

After investigation of the synthetic route with unlabelled compounds and characterisation of the intermediates of the synthetic pathway, chiral-methyl α-acetolactate was synthesised.

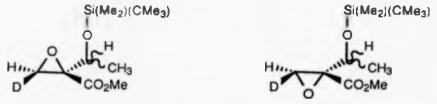




Scheme 60

Z-Methyl [3-^{2}H]-2-(1-hydroxyethyl)-2-propenoate (49) was protected as a tert-butyldimethylsilyl derivative (50) and high temperature epoxidation using 3-chloroperoxybenzoic acid gave methyl [3-^{2}H]-2,3-epoxy-2-(1-tert-butyldimethylsiloxyethyl)-propanoate (51).

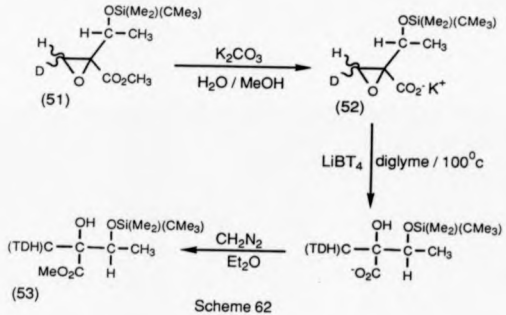
The deuterated epoxide was formed as a diastereomeric mixture, epoxidation occurring on both faces of the double-bond. The ^{1}Hnmr of the product exhibited two peaks at δ 3.01 and 2.85 associated with the C-3 hydrogen atom of the diastereomeric mixture.



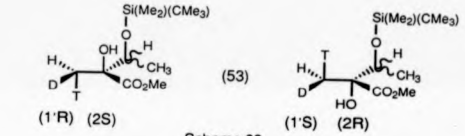
Scheme 61

The ester group of epoxide (51) was hydrolysed by reaction with potassium carbonate dissolved in methanol and water. The potassium salt (52) obtained was reduced by reaction with $LiBT_4$ (100mCi) at 100°C, and after esterification with diazomethane, methyl 2-hydroxy-2-([^{2}H,^{3}H]methyl)-3-tert-butyldimethylsiloxybutanoate (53) was isolated.

The reduction of the epoxide gave a diastereomeric mixture 2S,1R,(3,R and S), and 2R,1S,(3,R and S) of the desired product (53).

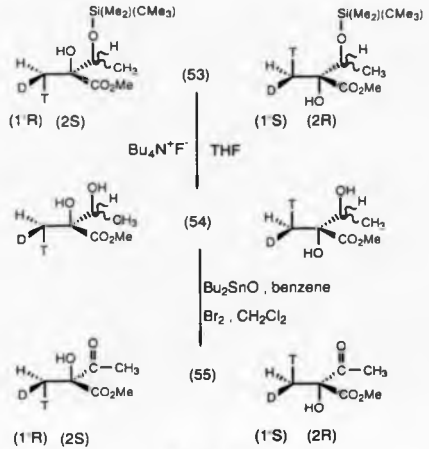


Scheme 62



Scheme 63

Subsequent removal of the silyl-protecting group using tetra-butylammonium fluoride and oxidation of the resulting diol(54) via its stannylene derivative yielded (2R,1'S) and (2S,1'R) methyl 2-hydroxy-2-([^{1}H,^{3}H]methyl)-3-oxobutanoate (55), (scheme 64).



Scheme 64

The work described reports the first sixteen step synthesis of methyl α-acetolactate with a chiral methyl group at the α-position.

The synthetic pathway developed produces a (2S,1R) and (2R,1S) diastereomeric mixture of the product. However, proposed investigation of the enzyme reductioisomerase does not require resolution of the labelled material since the enzyme utilises only (2S) 2-hydroxy-2-methyl-3-oxobutanoate.[88]

CHAPTER 6

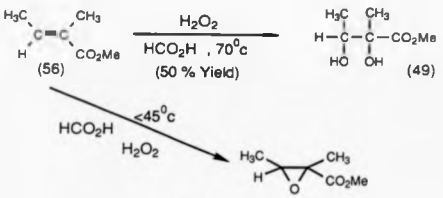
The synthesis of methyl α-acetolactate and analogous compounds

The biosynthesis of valine and isoleucine occurs by a series of equivalent chemical transformations which are catalysed by four enzymes. In order to investigate the biosynthetic pathway there is a need for simple and efficient syntheses of the intermediates of the pathway. The synthetic routes developed to produce intermediates of the biological pathway enable the rational design of analogues of the natural substrates which are possible enzyme inhibitors.

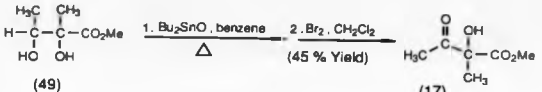
The synthetic studies reported in this chapter describe the formation of methyl α-acetolactate; the first intermediate in the biosynthesis of valine resulting from the acetohydroxyacid synthase catalysed condensation of two pyruvate molecules. Also reported is the attempted formation of a trifluoromethyl analogue of α-acetolactate, which if successfully produced would provide a useful chemical probe of the biological pathway.

Treatment of methyl tiglate (56) with hydrogen peroxide[114] and formic acid at 70°C led to the formation of methyl 2,3-dihydroxy-2-methylbutanoate (49). It was found that a reaction temperature below 45°C resulted in the formation of methyl 2,3-epoxy-2-methylbutanoate (57).

Reaction of the diol (49) with dibutyl tin oxide gave a stannylene[115] derivative which on treatment with bromine yielded methyl α-acetolactate (17). The ^{1}Hnmr spectrum of methyl α-acetolactate gave three methyl peaks at δ 3.8, 2.3 and 1.6



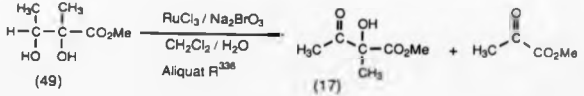
Scheme 65



Scheme 66

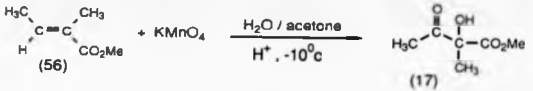
The diol (49) was also oxidised by a novel procedure reported by Suzuki and Noro-oka.[118] Treatment of the diol with sodium bromate in the presence of a catalytic quantity of ruthenium trichloride hydrate, in a biphasic solution of chloroform and water gave a 45% yield of methyl α-acetolactate. However, oxidation of the secondary

hydroxyl group was accompanied by cleavage of the diol to give methyl pyruvate.



Scheme 67

The most efficient synthesis of methyl α-acetolactate was carried out by a one step oxidation of methyl tiglate employing potassium permanganate under acidic conditions.[44,116]

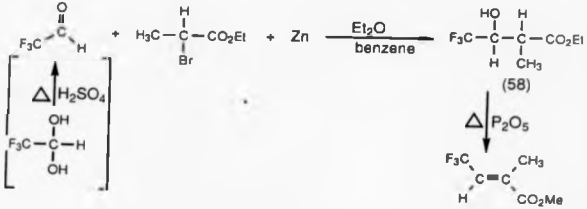


Scheme 68

The synthetic routes to methyl α-acetolactate can be readily extended to produce a wide range of analogous compounds. The ester group of methyl α-acetolactate is easily cleaved by treatment with pig liver esterase, at pH 7.2 to yield α-acetolactate.

It was decided to attempt to produce a trifluoromethyl analogue of methyl α-acetolactate. Trifluoroacetaldehyde hydrate was

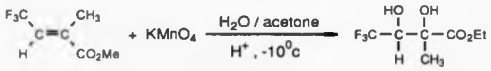
used as a source of trifluoroacetaldehyde.[117] It was found that the simplest and most efficient method of dehydrating trifluoroacetaldehyde hydrate was to heat the hydrate in the presence of concentrated sulphuric acid. Trifluoroacetaldehyde was condensed with ethyl 2-bromopropionate in the presence of zinc to yield ethyl 2-methyl-3-hydroxy-4,4,4-trifluorobutanoate (58).[118]



Scheme 69

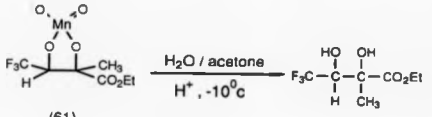
Dehydration of the condensation product with phosphorus pentoxide gave E-ethyl 4,4,4-trifluoro-2-methylbut-2-enoate (59). The ^{1}Hnmr of the product exhibited an olefinic quartet of quartets at δ 6.68 (J_{HF}=8.3Hz, J_{HH}=1.6Hz), characteristic of the formation of the E-product.

Subsequent oxidation of the E-trifluoromethyl alkene with potassium permanganate under acidic conditions did not give the expected 2-hydroxy-3-keto species but instead gave a 2,3-diol. (60).



(59) Scheme 70 (60)

The formation of a diol indicates that the strongly electron withdrawing trifluoromethyl group results in the cyclic hypomanganate species (61) undergoing hydrolysis to yield a cis-diol in preference to further oxidation to the ketonic compound.

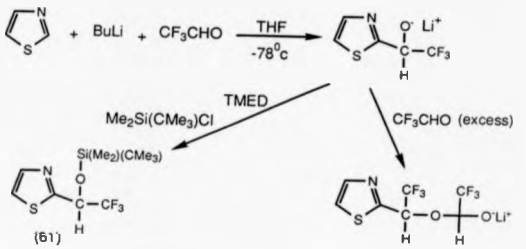


(61) Scheme 71 (60)

All efforts to oxidise the trifluoromethyl diol to the ketonic species using the procedures previously described and numerous other oxidative reactions were unsuccessful.

The thiazole chemistry described in Chapter 2 was also used in an attempt to synthesise a trifluoromethyl analogue of α-acetolactate. Trifluoroacetaldehyde was condensed with the C-2 carbanionic thiazole species, formed by reaction of thiazole with butyl lithium. The resulting alkoxide was protected by reaction with

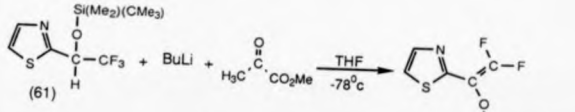
tert-butyldimethylsilylchloride . It was noted that if there was excess trifluoroacetaldehyde present in the reaction mixture, additional condensation occurred between the alkoxide and excess trifluoroacetaldehyde (scheme 72).



Scheme 72

The trifluoromethyl thiazole compound (61) was treated with butyl lithium in an attempt to deprotonate the hydrogen atom adjacent to the thiazole ring and react the resulting carbanion with methyl pyruvate. However, treatment of the thiazole compound (61) with butyl lithium resulted in the loss of hydrogen fluoride and the formation of an alkene (62).

The ^{19}Fnmr of the difluoro-alkene gave two doublets at δ -104.9 and -95.7, with a coupling constant of 42Hz between the two fluorine atoms.

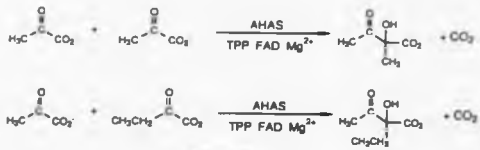

Si(Me2)(CMe3)
N
S
O
C
CF3
H
(61)
+ BuLi +
O
C
H3C
CO2Me
THF
-78°c
N
S
F
C
F
C
O
Si(Me2)(CMe3)
(62)

Scheme 73

CHAPTER 7

^{1}Hnmr Investigations of Acetohydroxyacid Synthase

The first common step in the biosynthesis of valine and isoleucine in bacteria, yeast and higher plants is catalysed by acetohydroxyacid synthase. Acetohydroxyacid synthase catalyses two analogous reactions forming α-acetolactate and α-acetohydroxybutyrate (scheme 74).



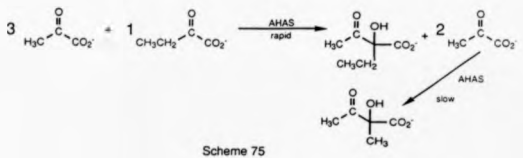
Scheme 74

Investigations of the reactions catalysed by acetohydroxyacid synthase have employed three main analytical techniques: continuous spectrophotometric monitoring of the loss of absorbance of pyruvate or α-ketobutyrate at 333nm, acid catalysed decarboxylation of α-acetolactate or α-acetohydroxybutyrate formed during the reaction and determination of the decarboxylation products by the method of Westerfeld,[119] isotope mass spectroscopy of the release of enzyme derived carbon dioxide from mixtures of ^{13}C-carboxyl-labelled pyruvate and α-ketobutyrate.[81]

The analytical techniques previously employed to study the enzymatic reactions are limited by the lack of a simple and efficient method of differentiating between the homologous substrates and homologous products.[120] The work presented in this chapter describes the use of high resolution ^{1}Hnmr in an attempt to study the reactions catalysed by acetohydroxyacid synthase. The initial studies were performed using bacterial acetohydroxyacid synthase isoenzyme II (*Salmonella typhimurium*), expressed, grown and isolated from *Escherichia coli* K-12. The reactions catalysed by partially purified acetohydroxyacid synthase isolated from pea plants were also investigated.

Initial studies were performed using acetohydroxyacid synthase isoenzyme II. Treatment of pyruvate (0.2M) with acetohydroxyacid synthase resulted in the growth of peaks at δ 2.15 and 1.35 associated with the formation of α-acetolactate (graph 1). Treatment of an equimolar mixture of pyruvate (0.1M) and α-ketobutyrate (0.1M) with acetohydroxyacid synthase resulted in peaks attributable to the formation of α-acetohydroxybutyrate at δ 2.2, 1.82 and 0.75, (graph 1). It was noted that the initial rate of formation of α-acetolactate (2.0 μmol min^{-1}) was slower than that of α-acetohydroxybutyrate (3.0 μmol min^{-1}).

Acetohydroxyacid synthase when added to a solution containing pyruvate and α-ketobutyrate in the ratio 3:1 resulted in the rapid production of α-acetohydroxybutyrate (initial rate, 1.9 μmol min^{-1}), when the reaction producing α-acetohydroxybutyrate was 95% complete the formation of α-acetolactate was observed (initial rate, 0.9 μmol min^{-1}), (graph 2).

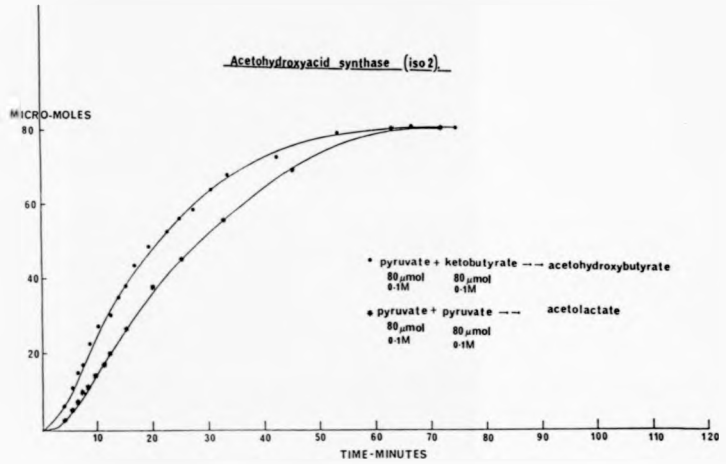


Scheme 75

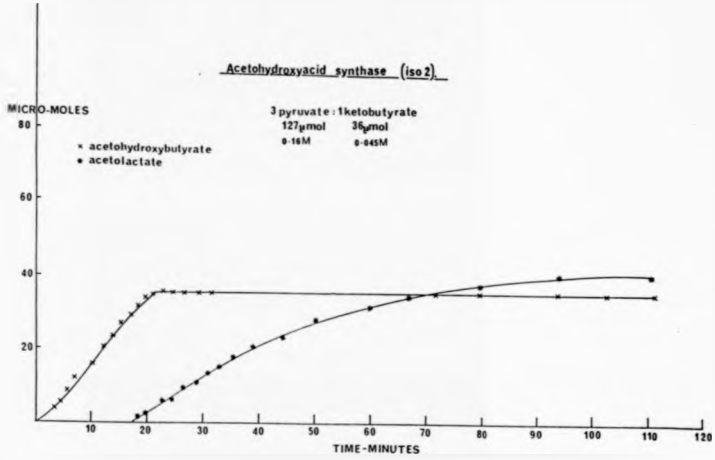
The overall concentration of α-ketobutyrate was reduced further such that the enzyme was added to a solution containing pyruvate and α-ketobutyrate in the ratio of 5:1. The ^{1}Hnmr spectra recorded showed the rapid production of α-acetohydroxybutyrate, the formation of α-acetolactate resulting from the homologous condensation of two pyruvate molecules was only observed after completion of the heterogeneous condensation of pyruvate and α-ketobutyrate (graph 3).

Additional studies of acetohydroxyacid synthase isoenzyme II using low substrate concentrations and employing high field ^{1}Hnmr (400MHz) were undertaken. Initial investigations indicated that α-acetolactate concentrations as low as 80nmol per ml of water could be detected using the 400MHz nmr spectrometer.

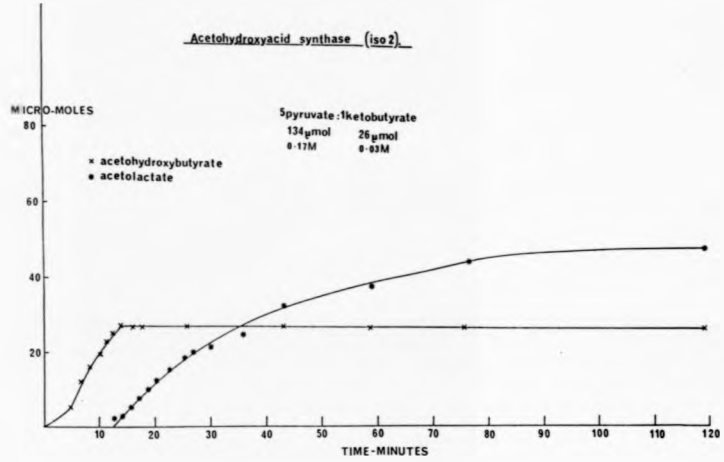
The reaction of pyruvate (80mM) with acetohydroxyacid synthase is shown in fig. 6.1. The growth of two methyl singlets at δ 2.25 and 1.46 attributable to the formation of α-acetolactate were observed with an associated loss in the size of the pyruvate peak at δ 2.38. The heterogeneous condensation between pyruvate (40mM) and


Acetohydroxyacid synthase (iso 2).
MICRO-MOLES
80
60
40
20
10
20
30
40
50
60
70
80
90
100
110
120
TIME-MINUTES
• pyruvate + ketobutyrate →→ acetohydroxybutyrate
80 μmol 0·1M
80 μmol 0·1M
◆ pyruvate + pyruvate →→ acetolactate
80 μmol 0·1M
80 μmol 0·1M

Graph 1


Acetohydroxyacid synthase (iso 2).
MICRO-MOLES
3 pyruvate : 1 ketobutyrate
127μmol 36μmol
0·16M 0·045M
× acetohydroxybutyrate
• acetolactate
80
60
40
20
10
20
30
40
50
60
70
80
90
100
110
120
TIME-MINUTES

Graph 2


Acetohydroxyacid synthase (iso 2)
MICRO-MOLES
80
60
40
20
5pyruvate : 1ketobutyrate
134µmol 26µmol
0·17M 0·03M
× acetohydroxybutyrate
• acetolactate
10
20
30
40
50
60
70
80
90
100
110
120
TIME-MINUTES

Graph 3

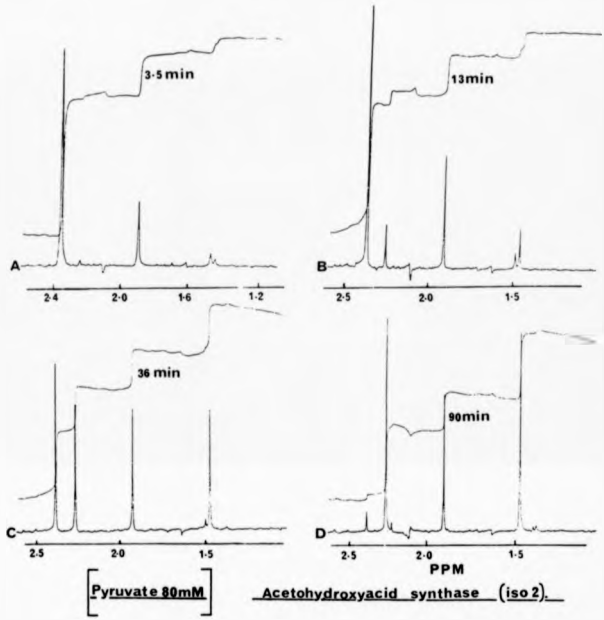
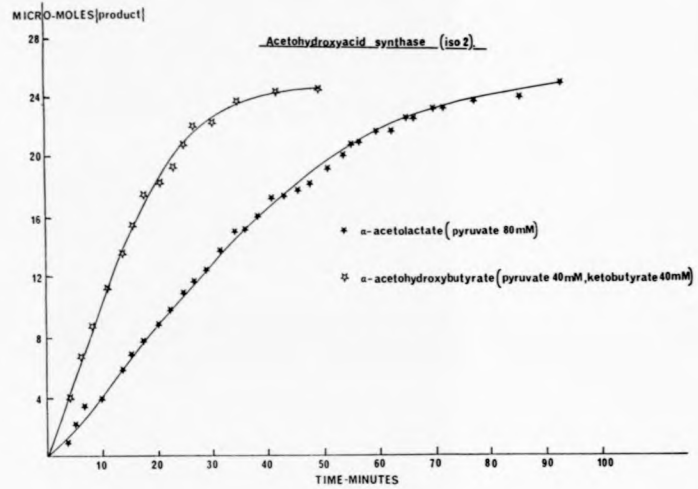


Fig. 6.1 ^{1}Rmr spectra which show the homologous condensation of pyruvate, catalysed by acetohydroxyacid synthase isoenzyme II,(referenced to sodium acetate at δ 1.82).

α-ketobutyrate (40mM) to yield α-acetohydroxybutyrate was also observed using ^{1}Hnmr. It was found that the initial rate of formation of α-acetohydroxybutyrate (1.0 μmolmin^{-1}) was faster than that associated with the production of α-acetolactate (0.43 μmolmin^{-1}), (graph 4).

The reaction of pyruvate (60mM) and α-ketobutyrate (20mM) with acetohydroxyacid synthase was also investigated. It was found that initially only peaks associated with the rapid formation of α-acetohydroxybutyrate were observed (δ 2.25(3H,S,CH_3-C=O), 0.81(3H,t,CH_2CH_3-)), (fig. 6.2, A, B, C and D). The formation of α-acetolactate (δ 2.25(3H,S,CH_3C=O), 1.46 (3H,S,CH_3-C)) was observed only after the reaction producing α-acetohydroxybutyrate was essentially complete. (fig. 6.2, E and F), (graph 5). A similar experiment was performed with a low concentration of α-ketobutyrate (12.8mM) relative to pyruvate (67.2mM), again the rapid formation of α-acetohydroxybutyrate was followed by the slower formation of α-acetolactate (graph 5).

The ^{1}Hnmr studies show that acetohydroxyacid synthase (isoenzyme II) when presented with a mixture of pyruvate and α-ketobutyrate preferentially catalyses the heterogeneous condensation between pyruvate and α-ketobutyrate to yield α-acetohydroxybutyrate, and the formation of α-acetolactate resulting from the homologous condensation of two pyruvate molecules is essentially only observed in the absence of α-ketobutyrate. This observation is consistent with the mechanism described in Chapter 1 (fig. 1.2) in which pyruvate is bound to the first binding site of the enzyme and undergoes decarboxylation to yield a thiamine pyrophosphate "active acetaldehyde" intermediate. Pyruvate and α-ketobutyrate compete for the second binding site of the enzyme and subsequent reaction with the


MICRO-MOLES|product|
Acetohydroxyacid synthase (iso 2)
28
24
20
16
12
8
4
10
20
30
40
50
60
70
80
90
100
TIME-MINUTES
α-acetolactate (pyruvate 80mM)
α-acetohydroxybutyrate (pyruvate 40mM, ketobutyrate 40mM)

Graph 4

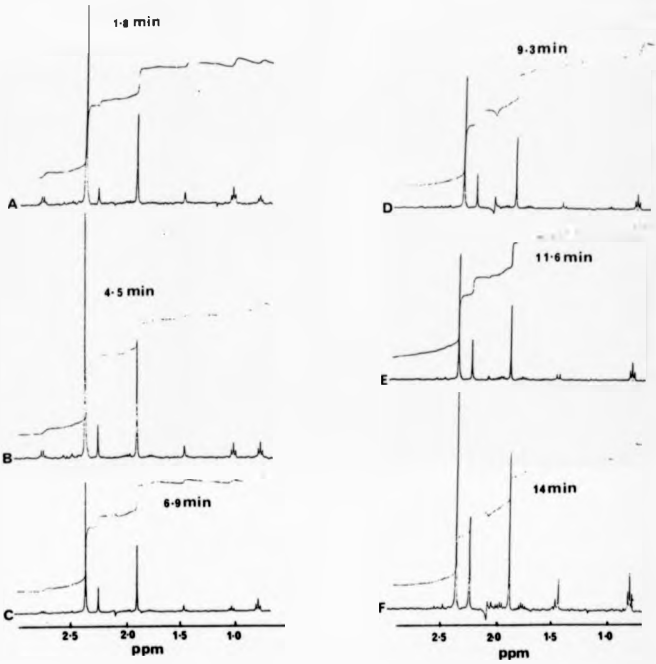


Fig. 6.2 ^{1}Hnmr spectra (400 MHz) which shows the reactions catalysed by AHAS in the presence of pyruvate and α-ketobutyrate in the ratio 3:1.

PAGINATION ERROR

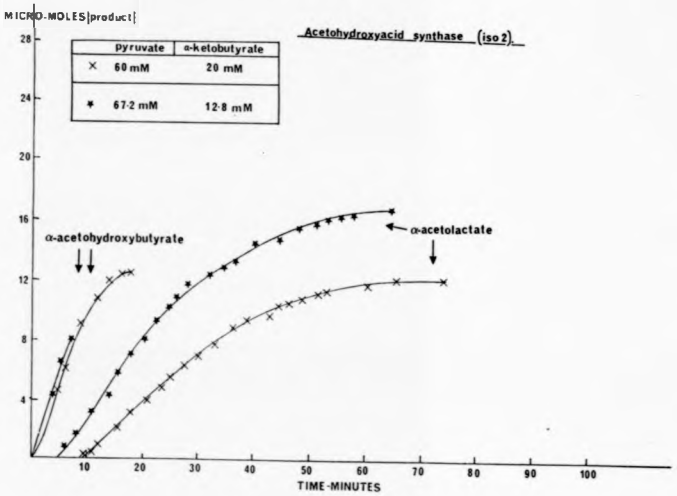
TABLE 5

"active acetaldehyde" intermediate. The observed preferential formation of α-acetohydroxybutyrate clearly shows that α-ketobutyrate is bound in preference to pyruvate at the second binding position of acetohydroxyacid synthase isoenzyme II. The enzyme mechanism proposed minimises the non-productive decarboxylation of α-ketobutyrate. It is interesting to note however, that the enzyme would catalyse the homologous condensation of α-ketobutyrate but at an extremely slow rate.

The ^{1}Hnmr investigations also indicate that the initial rate of formation of α-acetohydroxybutyrate catalysed by acetohydroxyacid synthase is approximately twice as fast as the initial rate of formation of α-acetolactate (table 6).

TABLE 6 AHAS		Conc. mM		Rate μmolmin^{-1}	
Field MHz	Enzyme	Pyruvate	α-ketobutyrate	α-acetohydroxybutyrate	α-acetolactate
220	0·5	160	40	1·8	0·95
220	0·5	170	30	3·0	1·5
400	0·25	80	—	—	0·43
400	0·25	40	40	1·0	—
400	0·25	60	20	1·1	0·45
400	0·25	67·2	12·8	1·1	0·5

In order to explain the apparent difference in the rates of the two analogous reactions catalysed by acetohydroxyacid synthase, a possible model for the mode of action of the enzyme is shown in fig. 6.3. Initially the enzyme binds with pyruvate. The enzyme-bound pyruvate then undergoes essentially irreversible decarboxylation to yield an "active acetaldehyde" moiety. The second substrate pyruvate or α-ketobutyrate binds to the enzyme and the catalytic reaction


MICRO-MOLES [product]
Acetohydroxyacid synthase (iso 2)
Pyruvate
α-ketobutyrate
× 60 mM 20 mM
* 67·2 mM 12·8 mM
α-acetohydroxybutyrate
α-acetolactate
28
24
20
16
12
8
4
10
20
30
40
50
60
70
80
90
100
TIME-MINUTES

Graph 5

occurs to give an enzyme-bound product, which is then released. An expression for Vmax can be derived:

$$Vmax = \frac{K_3 K_7 K_8 [Eo]}{K_7 K_8 + K_3 (K_7 + K_8 + K_9)}$$

If the enzymatic decarboxylation of pyruvate is rate limiting, that is K_3 is small; ($Vmax = K_3 [Eo]$), the overall rate of reaction will be independent of subsequent steps and hence the initial rate of formation of α-acetolactate and α-acetohydroxybutyrate will be expected to be the same. However, the ^{1}Hnmr experiments indicate a difference in the initial rate of formation of the analogous products. If the overall rate of the enzymatic reaction is independent of the rate of decarboxylation of pyruvate, that is K_3 is large;

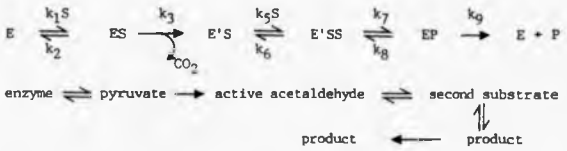
$$Vmax = \frac{K_7 K_8 [Eo]}{(K_7 + K_8 + K_9)}$$

Therefore, if K_3 is not rate limiting the observed rates of reaction are dependent upon the catalytic reaction of each substrate at the second binding site and release of the products. The observed difference in the rates of formation of α-acetolactate and α-acetohydroxybutyrate indicates that the enzymatic reaction occurring after the binding of the second substrate and release of the product is dependent upon which of the two substrates is bound at the second binding site.

^{1}Hnmr investigations were carried out to test the effect of a sulphonyl urea herbicide on the activity of bacterial acetohydroxyacid synthase isoenzyme II. The rate of production of α-acetolactate was monitored in the presence of varying concentrations of chlorsulphuron. The results obtained are shown in graph 6. It was found that a concentration of 3 μM of chlorsulphuron was sufficient to suppress enzyme activity from 2 μmolmin^{-1} to 0.2 μmolmin^{-1}. The concentration of chlorsulphuron which inhibits acetohydroxyacid synthase activity by 50% (I_{50}) is in the range of 0.3 to 3 μM.

Fig. 6.3. Kinetic model for acetohydroxyacid synthase.

KINETIC MODEL FOR ACETOHYDROXYACID SYNTHASE



Using steady state approximations :

$k_1[S][E] = (k_2 + k_3)[ES]$ - (1)

$k_3[ES] + k_6[E'SS] = k_5[S][E'S]$ - (2)

$k_5[S][E'S] + k_8[EP] = [E'SS](k_6 + k_7)$ - (3)

$k_7[E'SS] = [EP](k_8 + k_9)$ - (4)

from (4)

$$\Rightarrow [E'SS] = \frac{[EP](k_8 + k_9)}{k_7}$$

from (3)

$$\Rightarrow [E'S] = \frac{[E'SS](k_6 + k_7) - k_8[EP]}{k_5[S]}$$

$$\Rightarrow [E'S] = [EP]\left[\frac{(k_8 + k_9)(k_6 + k_7) - k_7k_8}{k_7k_5[S]}\right]$$

from (2)

$$\Rightarrow k_3[ES] = k_5[S][E'S] - k_6[E'SS]$$

$$\Rightarrow k_3[ES] = k_5[S][EP]\left[\frac{(k_8 + k_9)(k_6 + k_7) - k_8k_7}{k_7k_5[S]}\right] - \frac{k_6[EP](k_8 + k_9)}{k_7}$$

$$\Rightarrow k_3[ES] = \frac{[EP]}{k_7}(k_8k_6 + k_8k_7 + k_9k_6 + k_9k_7 - k_8k_7 - k_6k_8 - k_6k_9)$$

$$\Rightarrow k_3[ES] = [EP]k_9$$

$$\Rightarrow \quad [ES] = [EP]\frac{k_9}{k_3}$$

from (1)

$$\Rightarrow \quad [E] = \frac{(k_2 + k_3)[ES]}{k_1[S]}$$

$$\Rightarrow \quad [E] = \frac{k_9(k_2 + k_3)[EP]}{k_3k_1[S]}$$

since $\quad [E_o] = [E] + [ES] + [E'S] + [E'SS] + [EP]$

$$[E_o] = [EP]\left[\frac{k_9(k_2 + k_3)}{k_3k_1[S]} + \frac{k_9}{k_3} + \frac{(k_8 + k_9)(k_6 + k_7) - k_8k_7}{k_5k_7[S]} + \frac{(k_8 + k_9)}{k_7} + 1\right]$$

since $\quad V = k_9[EP]$

$$\frac{[E_o]}{V} = \frac{(k_2 + k_3)}{k_1k_3[S]} + \frac{1}{k_3} + \frac{(k_8 + k_9)(k_6 + k_7)}{k_5k_7k_9[S]} - \frac{k_8}{k_9k_5[S]} + \frac{(k_8 + k_9)}{k_7k_9} + \frac{1}{k9}$$

$$\frac{[E_o]}{V} = \frac{1}{k_3} + \frac{1}{k_9} + \frac{(k_8 + k_9)}{k_7k_9} + \frac{1}{[S]}\left[\frac{(k_2 + k_3)}{k_1k_3} + \frac{(k_6 + k_7)(k_8 + k_9)}{k_5k_7k_9} - \frac{k_8}{k_5k_9}\right]$$

- (5)

since $\quad V = \dfrac{k_{cat}[E_o][S]}{K_m + [S]}$

$$\Rightarrow \quad \frac{E_o}{V} = \frac{K_m + [S]}{k_{cat}[S]}$$

$$\Rightarrow \quad \frac{E_o}{V} = \frac{K_m}{k_{cat}[S]} + \frac{1}{k_{cat}} \qquad - (6)$$

if [S] is large, from equation (5) :

$$\Rightarrow \quad \frac{1}{k_3} + \frac{1}{k_9} + \frac{(k_8 + k_9)}{k_7k_9} = \frac{1}{k_{cat}}$$

$$\Rightarrow \quad k_{cat} = \frac{k_3k_7k_9}{k_7k_9 + k_3k_7 + k_3(k_8 + k_9)} \qquad - (7)$$

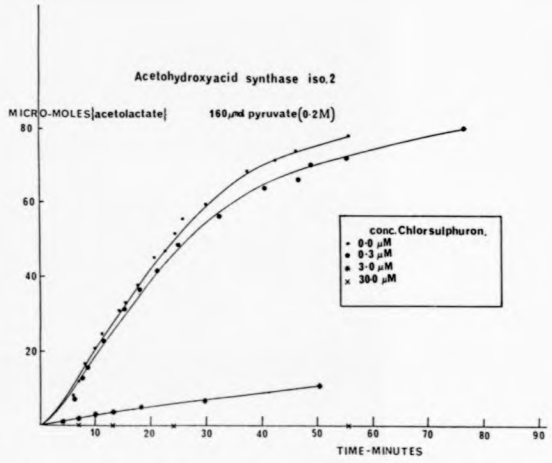
comparing terms of 1/[S] in equations (5) and (6) :

$$K_m = \frac{k_3k_7k_9}{k_3k_7 + k_7k_9 + k_3(k_8 + k_9)}\left[\frac{(k_2 + k_3)}{k_1k_3} + \frac{(k_8 + k_9)(k_6 + k_7)}{k_5k_7k_9} - \frac{k_8}{k_5k_9}\right]$$

from (7)

$$\Rightarrow \quad V_{max} = \frac{k_3k_7k_9[E_o]}{k_3k_7 + k_7k_9 + k_3(k_8 + k_9)}$$

$$\Rightarrow \quad V_{max} = \frac{k_3k_7k_9}{k_7k_9 + k_3(k_7 + k_8 + k_9)}[E_o] \qquad - (8)$$


Acetohydroxyacid synthase iso.2
MICRO-MOLES acetolactate
160 µM pyruvate (0·2M)
80
60
40
20
10
20
30
40
50
60
70
80
90
TIME-MINUTES
conc. Chlorsulphuron.
0·0 µM
0·3 µM
3·0 µM
30·0 µM

Graph 6

Inhibition of acetohydroxyacid synthase isoenzyme II by chlorsulphuron has been shown to be a biphasic process involving the formation of an initial, weak enzyme-inhibitor complex which isomerises to a more tightly bound form. Therefore, if the nmr experiments were repeated with pre-incubation of the enzyme with the inhibitor a greater inhibitory effect of chlorsulphuron would be expected. The ^{1}Hnmr experiments show that chlorsulphuron is an extremely potent inhibitor of acetohydroxyacid synthase isoenzyme II. It has also been found that chlorsulphuron[48] is a potent inhibitor of plant acetohydroxyacid synthase with I_{50} values as low as 18.5nM.

The initial studies of bacterial acetohydroxyacid synthase were followed by an investigation of acetohydroxyacid obtained from pea plants. Acetohydroxyacid synthase has not been purified from plants; the difficulty has been that plant tissue contains so little of the enzyme. High field ^{1}Hnmr (400MHz) enabled the formation of α-acetolactate and α-acetohydroxybutyrate catalysed by acetohydroxyacid synthase, isolated in a partially purified form from pea plants, to be observed (fig. 6.4). High field nmr enabled the analogous reactions to be studied at a micro-molar level, independant of the impurities present in the crude enzyme mixture. The homogeneous condensation of pyruvate to yield α-acetolactate occurred at an initial rate of 2.75×10^{-3} μmolmin^{-1}, the heterogeneous condensation of pyruvate and α-ketobutyrate to give α-acetohydroxybutyrate occurred at an initial rate of 2.2×10^{-3} μmolmin^{-1} (graph 7).

The investigations performed show that by using nmr techniques the reactions catalysed by acetohydroxyacid synthase can be investigated, simply and at low concentrations of material and without problems of differentiation between the analogous reactions previously encountered using other analytical techniques.

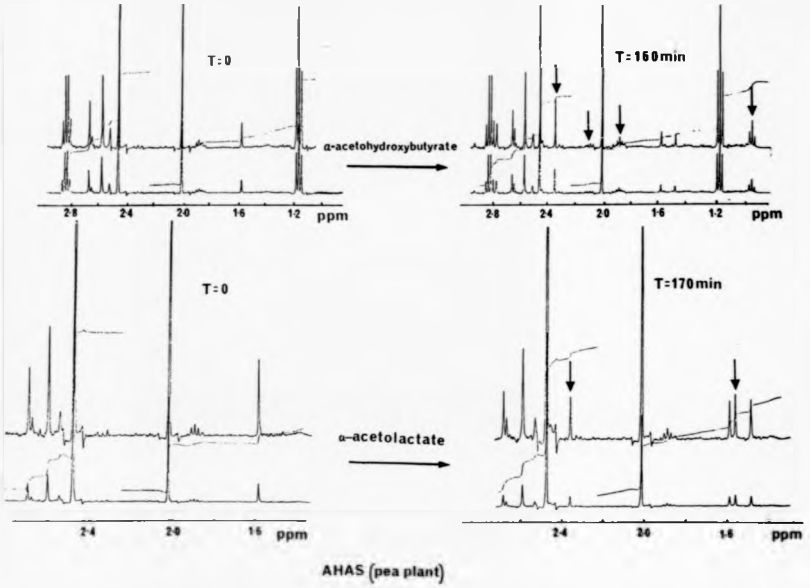
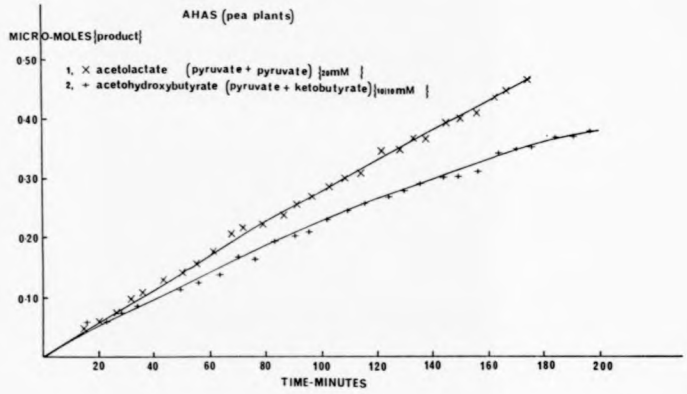


Fig. 6.4 ^{1}Hnmr spectra of the reactions catalysed by acetohydroxyacid synthase isolated from pea plants.


AHAS (pea plants)
MICRO-MOLES [product]
1, × acetolactate (pyruvate + pyruvate) [2mM]
2, + acetohydroxybutyrate (pyruvate + ketobutyrate) [10mM]
0·50
0·40
0·30
0·20
0·10
20
40
60
80
100
120
140
160
180
200
TIME-MINUTES

Graph 7

CHAPTER 8

8.1 Introduction to Experimental Chapters

i) ^{1}Hnmr spectra were recorded at 220MHz on a Perkin-Elmer R34 spectrometer or at 400MHz on a Bruker WH400 spectrometer, with tetramethylsilane (TMS) as an internal standard.

^{19}Fnmr spectra were recorded at 84.67MHz on a Bruker WH90 spectrometer, with fluorotrichloromethane ($CFCl_3$) as an internal standard.

^{13}Cnmr spectra were recorded at 100.62MHz on a Bruker WH400 spectrometer, with TMS as an external reference.

ii) Mass spectra were recorded using a Kratos MS80 instrument fitted with a DS55 data system.

iii) Microanalyses were carried out by Butterworth Laboratories Ltd., Teddington, Middlesex, U.K.

iv) Infra red spectra were recorded using a Perkin-Elmer 580-B spectrophotometer, typically a 3% sample/chloroform liquid cell.

v) Melting points were determined using a Gallenkamp apparatus and are quoted uncorrected.

vi) Flash chromatography was performed on Merck Kieselgel 60 silica gel (230-400 mesh). Approximately 100g of silica gel was used per gram of compound.

8.2 Experimental to Chapter 2

1-(2'-Thiazolyl)-1-tert-butyldimethylsiloxy ethane (5).

Thiazole (1g, 0.85ml, 0.012 mol) was dissolved in dry diethyl ether (40ml) under a nitrogen atmosphere, and the solution was coolad to -78°C. Into the reaction mixture was injected butyl lithium (4.65ml, 2.4M solution in hexane), whilst maintaining the reaction at -78°C. The dark red solution formed on addition of the butyl lithium was stirred for 45 minutes and then acetaldehyde (0.66ml, 1 equiv) was added slowly over a 5 minute period. After a further 30 minutes, tetramethylethylenediamine (1.8ml, 0.012mol) and tert-butyldimethylsilyl chloride (1.8g, 0.012mol) were added to the reaction mixture. The reaction was maintained at -78°C for 60 minutes, and then allowed to warm up to room temperature over a 12 hour period. The reaction mixture was then poured onto water (50ml) and extracted with diethyl ether (3 x 50ml). The diethyl ether extracts were combined, dried with magnesium sulphate and evaporated under reduced pressure to give a crude yellow oil. The oil was purified by flash chromatography (100g, 230:400 mesh silica, dichloromethane 1:1 cyclohexane), tubes 37 to 56 contained material giving one spot by t.l.c. Further purification by preparative t.l.c. (diethyl ether 1:20 cyclohexane) gave two bands Rf 0.1 and 0.7, the lower band contained the desired compound (900mg, 33% yield).

^{1}H-nmr ($CDCl_3$) δ 7.72 (1H,d,J=3.6Hz, H-C(S)=C), 7.28 (1H,d,J=3.6Hz, H-C(N)=C), 5.16 (1H,q,J=7.2Hz,H-C(CH_3)-O), 1.56 (3H,d,J=7.2Hz, CH_3-C(H)-O), 0.95 (9H,S,(CH_3)$_3$C-), 0.11 (3H,S,CH_3-Si), 0.09 (3H,S,CH_3 -Si).

M.S. (E.I.), 228 $(M-Me)^+$, 186 $(M-CMe_3)^+$, 112 $(M-OSi(Me_2)CMe_3)^+$. M.S. (+Ve C.I. ammonia) 244 $(MH)^+$.

I.R. (liquid cell, 3% $CHCl_3$), 2850, 2950, 2920(-C-H,m), 1515 (thiazole ring,w)cm^{-1}.

2- (1-(1-tert-Butyldimethylsiloxyethyl))-5-(2-(methyl-2-hydroxy-propanoate)) thiazole(6).

The thiazole (5), (110mg, 0.45mmol) was dissolved in dry diethyl ether (5ml); under a nitrogen atmosphere; and cooled to -78°C. To the cooled reaction was added butyl lithium (2.4M, 0.2ml) whilst maintaining the temperature at -78°C. After stirring for one hour tetramethylethylenediamine (0.07ml, 1 equiv.) and methyl pyruvate (0.04ml, 1 equiv.) were injected into the reaction mixture. The reaction was left stirring at -78°C for 2 hours and was then quenched by the addition of aqueous ammonium chloride (5ml, 0.1M). The reaction mixture was allowed to warm up to room temperature over a six hour period. It was then poured onto water (20ml) and the mixture was extracted with diethyl ether (4x50ml). The combined ether extracts were dried with magnesium sulphate and evaporated under reduced pressure to give a crude yellow oil. The crude oil was purified by preparative t.l.c. (dichloromethane 1:1 cyclohexane). Two bands were obtained, the upper band was found to be starting material (30mg) and the lower band was isolated and purified by further preparative t.l.c. (diethyl ether 1:1 cyclohexane) giving a band Rf 0.32 which was found to be the condensation product. (25mg, 16% yield).

^{1}Hnmr ($CDCl_3$) δ 7.65 (1H,S,H-C(N)=C), 5.1 (1H,q,J=7.2Hz,H-C(CH_3)-), 3.85 (3H,S,CH_3-O) 1.85 (3H,s,CH_3-C(OH)-) 1.55 (3H,d,J=7.2Hz, CH_3-C(N)-OSi), 0.95 (9H,S,-C(CH_3)$_3$), 0.11 (3H,S,CH_3-Si), 0.09(3H,S,CH_3-Si)

^{13}Cnmr (CDCl$_3$) δ 178.3(s,-C(N)=S), 174.7(S,C=O) 141.3(S,-C(S)=C), 138.9 (S,H-C(N)=C), 73(S,-C-OH), 69.4 (S,-C-OSi), 53.6(S,CH$_3$-O), 29.6 (S,C(CH$_3$)$_3$), 27.9 (2xs, fine diastereomeric splitting, CH$_3$-C(OH)-), 25.0 (2xs, fine diastereomeric splitting, CH$_3$-C (OSi)-), 25.6 (S,-C(CH$_3$)$_3$), -4.7 (S,CH$_3$-Si), -5.2(s,CH$_3$-Si)

M.S. (N.I.), 330 (M-Me)$^+$, 288(M-CMe$_3$)$^+$, 214(M-OSi(Me$_2$)CMe$_3$)$^+$. M.S. (+VeC.I. ammonia), 346 (MH)$^+$.

1-(2'-N-methyl thiazolium iodide)-1-tert-butyldimethylsiloxy ethane (7)

The thiazole (5), (176mg, 0.72 mmol) was added to dry tetrahydrofuran (0.5ml) under an argon atmosphere. To this mixture was added methyl iodide (1ml). The reaction mixture was heated at 50°C for five days. An additional quantity of methyl iodide (0.5ml) was added after three days. After five days a yellow precipitate was formed. The crystalline material was filtered off and triturated with hot diethyl ether. The crystals were dried to give 198mg of product (70% yield). An analytical sample was obtained by recrystallising a small quantity of the crystalline material from ethanol : m.p. 182-183°C.

^{1}Hnmr ($CDCl_3$) δ 8.45(1H,d,J=4.4Hz, H-C(N)=C), 8.3(1H,d,J=4.4Hz, H-C(S)=C), 5.7 (1H,q,J=7.2Hz, H-C(CH_3)-), 4.17 (3H,S,CH_3-$\overset{+}{N}$), 1.55(3H,d,J=7.2Hz, CH_3-C(N)-), 0.95 (9H,S,$(CH_3)_3$C-), 0.12(3H,S,CH_3-Si), 0.2(3H,S,CH_3-Si).

I.R. (liquid cell, 4% in $CHCl_3$), 2950 (S,-CH_3), 1565(w,thiazole ring)cm^{-1}.

FAB M.S. (glycerol-H_2O matrix) : +Ve, 258 (M-I)$^+$, 644 (2M-I+H)$^+$: -Ve, 127(I)$^-$, 219 (GLY + I)$^-$, 512(M + I)$^-$.

Analysis: Calculated for $C_{12}H_{24}NSOSiI$: C,37.4; H,6.23; N,3.63; S,8.31. Found: C,37.1; H,6.33; N,3.52; S,8.22.

1-(2-Benzothiazolyl)-1-tert-butyldimethylsiloxy ethane (12).

Benzothiazole (3.24ml, 0.03mol); freshly distilled, was dissolved in dry tetrahydrofuran (50ml) under an argon atmosphere. The reaction mixture was cooled to -78°C, and butyl lithium (2.4M, 12ml) added dropwise over a two hour period. Acetaldehyde (1.64ml, 1 equiv.) was then added and the reaction mixture stirred for one hour at -78°C. To the reaction mixture, tetramethylethylenediamine (4.48ml) and tert-butyldimethylsilyl chloride (4.48g) were then added and the reaction mixture left to warm up to room temperature overnight. The reaction mixture was then taken up in diethyl ether (100ml) and then washed successively with dilute hydrochloric acid (1N), aqueous sodium hydrogen carbonate and water. The ethereal solution was then dried with magnesium sulphate and evaporated under reduced pressure to give a crude oil. The crude product was purified by flash chromatography (230-400 mesh silica 200g, dichloromethane 1:1 cyclohexane), tubes (25ml) 11 to 25 contained material giving one spot by t.l.c. (Rf.0.25). On evaporation of the solvent 6.94g, (89% yield) of the desired compound was obtained. An analytical sample was obtained by Kugelrohr distillation (220°C, 1mmHg).

^{1}Hnmr ($CDCl_3$): δ 8.05 (2H,m,Harom.), 7.52(2H,m,Harom.), 5.32 (1H,q,J=7Hz, H-C(CH_3)-), 1.67 (3H,d,J=7Hz, CH_3-C(H)-), 1.0 (9H,S,(CH_3)$_3$C-), 0.15 (3H,S,CH_3-Si-), 0.12 (3H,S,CH_3-Si-)

I.R. : 2950(m,) bands, -CH_3), 1555 and 735 (m, aromatic vibrations)cm^{-1}.

Analysis: Calculated for $C_{15}H_{23}NSOSi$: C, 61.4; H,7.8; N,4.8; S,10.9. Found : C,61.14; H,7.99; N,4.74; S,11.62.

1-Deutero-1-(2-benzothiazoloyl)-1-tert-butyldimethylsiloxy ethane.

The benzothiazole (12), (32mg, 0.1mmol.) was dissolved in dry tetrahydrofuran (2.5ml), under an argon atmosphere, and cooled to -78°C. Butyl lithium (2.4M, 43.8 μl) was injected into the reaction mixture forming a red solution. The reaction mixture was stirred at -78°C for 30 minutes and then quenched by the addition of d_4-methanol (0.2ml). The reaction mixture was then stirred for 30 minutes at -78°C and then allowed to warm up to room temperature over a two hour period. The reaction mixture was poured onto water (10ml) and extracted with diethyl ether (3 x 50ml). The combined ether extracts were dried with magnesium sulphate and evaporated under reduced pressure to give an oil which was purified by preparative thin layer chromatography (dichloromethane 1:1 cyclohexane). Characterisation of the main band showed successful deuterium incorporation.

^{1}Hnmr ($CDCl_3$): δ 8.0 (2H,m,Harom.), 7.5 (2H,m,Harom.), 1.68 (3H,s,CH_3-C(D)), 1.0 (9H,S,CH_3)$_3$C-), 0.15 (3H,S CH_3-Si-), 0.12 (3H,S,CH_3-Si-).

rac.Methyl 2-hydroxy-2-methyl-3-tert-butyldimethylsiloxy-3-(2-benzothiazolyl)-butanoate (13).

The benzothiazole (12), (3.35g, 11.4mmol) was dissolved in dry tetrahydrofuran (30ml); under an argon atmosphere; and the solution was cooled to -78°C. Butyl lithium (2.4M, 4.55ml) was added dropwise, over a forty five minute period, to the reaction mixture, maintaining a temperature of -78°C. Methyl pyruvate (2.08ml, 1 equiv.) was then added to the reaction mixture dropwise, over a twenty minute period. After stirring for thirty minutes the reaction mixture was warmed up to -20°C and ammonium chloride solution (1M, 13ml) was added to quench the reaction. The reaction mixture was then taken up in diethyl ether (100ml), washed with water (2 x 40ml) and then the ether mixture was dried with magnesium sulphate and evaporated under reduced pressure to give a crude oil. The oil was purified by flash chromatography (200g, 230-400 mesh silica, ether 1:9 petrol). Two compounds were obtained with Rfs 0.35 and 0.17. The compound with Rf 0.35 was found to be starting material (0.95g, 29%), the material Rf.0.17 was characterised as a diastereomeric mixture (A+B) of the desired product (2.19g, 50% yield, diastereomeric ratio A/B=3/2).

^{1}Hnmr ($CDCl_3$): δ_A 8.0 (2H,m,Harom.), 7.5 (2H,m,Harom.), 3.68 (3H,S,-CO_2CH_3), 1.98 (3H,S,CH_3-C(OH)CO_2CH_3), 1.52 (3H,S,CH_3-C-OSi-), 1.05 (9H,S,$(CH_3)_3C$-), 0.2 (3H,S,CH_3-Si-), 0.1 (3H,S,CH_3-Si-).

δ_B 8.0 (2H,m,Harom.), 7.5 (2H,m,Harom.), 3.57 (3H,S,-CO_2CH_3), 1.97 (3H,S,CH_3-C(OH)-CO_2CH_3), 1.6 (3H,S,CH_3-C-OSi-), 1.05 (9H,S,$(CH_3)_3C$-), 0.2 (3H,S,CH_3-Si-), 0.1 (3H,S,CH_3-Si-).

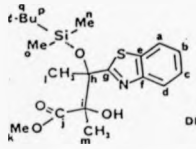
M.S.: (C.I. ammonia) : 396 (MH)$^+$

(E.I.) 338 (M-$C(CH_3)_3$)$^+$, 292(M-$CH_2C(OH)CO_2Me$)$^+$, 235 (M-$CH_2C(OH)CO_2Me$-$C(CH_3)_3$)$^+$, 135 (benzothiazole)$^+$

I.R.: (liquid cell - 3% chloroform) : 2950 (m, 3 bands, $-CH_3$), 1740 (S,C=O), 1500 (w, aromatic C-H)cm^{-1}.

Analysis : Calculated for $C_{19}H_{25}NSO_4Si$: C,57.7; H,7.34; N,3.54. Found : C,57.44; H,7.53; N,3.42.

^{13}Cnmr (Table 5).



DIASTEREOMERIC

LABELS	^{13}C CHEMICAL SHIFTS/mm			
a,b,c,d	125·7	125·6	124·8	124·7
	123·6	122·9	121·4	121·3
e, j	135·2	135·1		
f	153·4	152·8		
g	178·1	177·5		
h	80·4	80·1		
i	82·7	81·7		
j	174·2	174·0		
k	52·1	52·2		
l, m	20·7	19·3	23·4	23·2
n, o	-1·8	-2·2	-2·5	
p	18·6	18·5		
q	25·9	25·8		

Table 5. ^{13}Cnmr chemical shifts.

It was found that the purified oil from the flash column began to crystallise after 2 months. Isolation of the crystalline material and subsequent recrystallisation from pentane and ethyl acetate yielded crystals that were characterised as one enantiomeric pair.

^{1}Hnmr ($CDCl_3$) :δ8.0 (2H,m,Harom.), 7.5 (2H,m,Harom.), 4.5 (1H,S,-OH), 3.68 (3H,S,-CO_2CH_3), 1.95 (3H,S,CH_3-C(OH)-), 1.52 (3H,S,CH_3-C(OSi)-), 1.05 (9H,S,$(CH_3)_3C$-), 0.2 (3H,S,CH_3-Si) 0.1 (3H,S,CH_3-Si).

Melting point : 80-81°C.

Analysis : Calculated for $C_{14}H_{24}NSO_4Si$: C,57.7; H,7.34; N,3.54. Found : C,57.69; H,7.43; N,3.56.

Crystal Structure: The crystals were characterised as the SS, RR enantiomeric pair.

Methyl 2,3-dihydroxy-2-methyl-3-(2'-benzothiazolyl)-butanoate (15).

The benzothiazole (13), (1.589g, 4 mmol) was dissolved in dry tetrahydrofuran (50ml) and the mixture was stirred at room temperature, under a nitrogen atmosphere. To the solution was then injected tetrabutylammonium fluoride (1.0M in THF, 4ml) and the reaction mixture was stirred for one hour. The reaction mixture was taken up in diethyl ether (150ml) and washed successively with hydrochloric acid (0.25M), aqueous sodium hydrogen carbonate and water. The ether mixture was then dried with magnesium sulphate and evaporated under reduced pressure to give a crude oil. The crude oil was purified by flash-chromatography (80g, flash silica-230:400 mesh. petrol 4:1 ethyl acetate). Two compounds were isolated Rfs' 0.15 (521.2mg) and 0.08 (336.5mg). The compounds were characterised as the diastereomeric dihydroxy-benzothiazole compounds (76% yield).

Characterisation of Rf 0.15 material:

^{1}Hnmr ($CDCl_3$):δ 7.95 (2H,m,Harom.), 7.48 (2H,m,Harom.), 5.75 (1H,S,-OH), 5.2 (1H,S,-OH), 3.64 (3H,S,-CO_2CH_3), 1.71 (3H,S,CH_3-C(OH)-CO_2CH_3), 1.63 (3H,S,CH_3-C(OH)- thiazole ring).

^{13}Cnmr ($CDCl_3$): δ 180.8 (S,C-C(N)=S), 177.9 (S,C=O), 152.2 (S,C=C(C)=N), 134.6 (S,C=C(C)=S), 126.1, 125.1, 122.7, 121.7 (4xS, Caromatic), 78.9, 78.1 (2xS,C-OH,C-OH), 23.1, 19.9 (2xS, CH_3-C(OH)-, CH_3-C(OH)-), 52.68 (S,-CO_2CH_3).

M.S. E.I.: 178(M-$CH_3C(OH)CO_2CH_3$)$^+$, 136(M-$CH_3C(OH)CO_2CH_3$-C_2H_2O)$^+$

Accurate Mass: C.I. (ammonia) : (MH)$^+$ $C_{11}H_{14}NO_4S$ (282.0810)

I.R. (liquid cell, 3% chloroform): 3460 (S,broad-OH), 1740 (S,C=O), 1520, 740 (M, C-H aromatic) cm^{-1}.

Characterisation of Rf 0.08 material:

^{1}Hnmr:δ 8.0 (2H,m,Harom.), 7.5 (2H,m,Harom.), 4.48 (1H,S,-OH),

4.65 (1H,S,-OH), 3.85 (3H,S,-$CO_2\underline{CH_3}$), 1.8 (3H,S,$\underline{CH_3}$-C(OH)-CO_2CH_3), 1.55 (3H,S,CH_3-C(OH)-thiazole ring).

^{13}Cnmr ($CDCl_3$): δ 176.6 (S,C-$\underline{C}$(N)=S), 175.1 (S,$\underline{C}$=O) 152.6 (S,C=$\underline{C}$(C)=N), 134.9(S,C=$\underline{C}$(C)=S), 125.9, 125.0, 122.9, 121.6 (4xs Caromatic), 79.2, 78.4 (2xs,-$\underline{C}$-OH,-$\underline{C}$-OH), 24.7, 21.2 (2xs,$\underline{CH_3}$-C(OH)-, $\underline{CH_3}$-C(OH)-), 52.9 (S,-$CO_2\underline{CH_3}$).

M.S. E.I.: 178 (M-$CH_3C(OH)CO_2CH_3$)$^+$, 136(M-$CH_3C(OH)CO_2CH_3$-C_2H_2O)$^+$.

Accurate Mass: C.I. (ammonia) : (MH)$^+$ $C_{13}H_{14}NO_4S$ (282.0798).

I.R. (liquid cell, 3% chloroform): 3500 (S,broad-OH), 1740(S,C=O), 1525, 720(m, C-H aromatic) cm^{-1}.

(2R3S, 2S3R) Methyl 2,3-dihydroxy-2-methyl-3-(2-benzothiazolyl)-butanoate (15).

Benzothiazole (3.24ml, 0.03mol) was dissolved in dry tetrahydrofuran (50ml), under an argon atmosphere, and the reaction mixture was cooled to -78°C. Butyl lithium (2.4M, 12ml) was then added to the reaction mixture dropwise over a 2 hour period. To the reaction mixture was then added methyl- α-acetolactate , (2.19g, 0.015mol) maintaining the temperature at -78°C. The reaction mixture was stirred at -78°C for one hour and was then warmed up to -20°C; gradually over a further period of one hour. A solution of ammonium chloride (1M,30ml) was added to quench the reaction. The reaction mixture was then diluted with diethyl ether and washed with water (3x50ml). The ether mixture was dried with magnesium sulphate and evaporated under reduced pressure to give a crude oil. The oil was purified by flash chromatography (ethyl acetate 1:5 petrol). Two fractions were obtained (1.01g and 1.11g) and characterised as the benzothiazole dihydroxy diastereomers (overall yield 50.4%). The lower running fraction crystallised and was recrystallised from ethyl acetate and hexane. A sample for x-ray crystallography was prepared using a slow diffusion recrystallisation from ethyl acetate and pentane.

Melting point of crystals: 94.5 to 94.8°C.

^{1}Hnmr ($CDCl_3$): δ 8.0 (2H,m,Harom.), 7.5 (2H,m,Harom.), 4.48 (1H,S,-OH), 4.65 (1H,S,-OH), 3.85 (3H,S,$-CO_2CH_3$), 1.8 (3H,S,CH_3-C(OH)-CO_2CH_3), 1.55 (3H,S,CH_3-C(OH)-thiazole ring).

Analysis : Calculated for $C_{12}H_{13}NO_4S$: C,55.52; H,5.34; N,4.98; S,11.39. Found: C,55.43; H,5.39; N,4.93; S,11.13.

Methyl α-acetolactate (17).

(2R,3S and 2S,3R) Methyl 2,3-dihydroxy-2-methyl-3-(2-benzothiazolyl)-butanoate (15), (223mg, 0.79mmol) was dissolved in dry dichloromethane (5ml) and trimethyloxonium tetrafluoroborate (117.5mg, 0.79mmol) was added. The progress of the reaction was followed by removing aliquots and recording the proton nmr. The growth of a quaternary nitrogen methyl signal at δ 3.3ppm and the shift of the aromatic hydrogen atoms from δ 7.5 and 8.0 to 7.85 and 8.15 ppm, were used to follow the reaction. After stirring for four days under an argon atmosphere the dichloromethane was removed on a rotary evaporator; to give an off-yellow solid; traces of solvent were removed on a high vacuum pump. The solid material was placed under an argon atmosphere and dry methanol (10ml) and triethylamine (112 μL) were added, the mixture was then heated at reflux for forty-eight hours. After cooling the reaction mixture to room temperature the volume of methanol was reduced to approx. 0.5ml on a rotary evaporator. The residue was then purified by flash chromatography (2% methanol : chloroform). A number of benzothiazole containing compounds, with Rfs' of 0.9 to 0.75, were isolated, the major component of which was characterised as: 2-acetyl-3-methyl benzo-dihydrothiazole (15mg, 9.8% yield).

^{1}Hnmr ($CDCl_3$): δ 7.1, 6.8, 6.5 (4H,m,Harom.), 5.3 (1H,S,N-C(S)-H), 2.95 (3H,S,CH_3-N), 2.3 (3H,S,CH_3-C=O).

^{13}Cnmr ($CDCl_3$) : δ 168.5 (S,CH_3-C=O), 162.7 (S,N-C-S), 140.3 (S,C=C(C)-N), 135 (S,C=C(C)-S), 129.5, 129.3, 128.8, 128.5 (4xS, C aromatic), 33.3 (S,CH_3-C=O), 29 (S,N-CH_3).

M.S: C.I. (ammonia) : 194 $(MH)^+$, 150 $(M-CH_2CO)^+$.

I.R. (liquid film): 1720 (S,C=O), 1580, 740 (m,C-H aromatic) cm^{-1}.

A material Rf of 0.38 was isolated and found to be the desired product methyl-α-acetolactate (17.2mg, 14.8%).

^{1}Hnmr (CDCl$_3$): δ 3.82 (3H,S,-CO$_2$C<u>H$_3$</u>), 2.3 (3H,S,C<u>H$_3$</u>C=O), 1.6 (3H,S,C<u>H$_3$</u>-C(OH)-).

Accurate mass: E.I: (MH)$^+$, $C_6O_4H_{11}$, 147.0668.

M.S.: E.I.: 147 (MH)$^+$, 104 (M-C_2H_2O)$^+$, 59 (CH_3CO_2)$^+$ 43(CH_3CO)$^+$

^{13}Cnmr (CDCl$_3$): δ 204.7 (S,C<u>H$_3$</u>C=O), 171.6 (S,-<u>C</u>O$_2$CH$_3$), 80.9 (S,-<u>C</u>(OH)-CH$_3$), 53.1 (S,-CO$_2$<u>C</u>H$_3$), 23.9 (S,<u>C</u>H$_3$-C=O), 21.7 (S, <u>C</u>H$_3$-C(OH)-).

I.R. (liquid cell, 3% chloroform): 3500 (m, broad, -OH), 1720 and a shoulder at 1740 (S, overlapping C=O) cm^{-1}.

A final component from the flash column Rf 0.33 was isolated and characterised as the starting dihydroxy material (60.5mg, 27%).

Methyl α-acetolactate (17).

(2R,3R and 2S,3S) Methyl 2,3-dihydroxy-2-methyl-3-(2-benzothiazolyl)-butanoate (15), (322.5mg, 1.1mmol) was dissolved in dry dichloromethane (5ml) and trimethyloxonium tetrafluoroborate (170mg, 1.1mmol) was added. The reaction mixture was stirred; under a nitrogen atmosphere, at room temperature. The reaction was also kept in the dark. After stirring for four days the dichloromethane was removed under reduced pressure and traces of solvent were removed using a high vacuum, yielding a yellow thiazolium salt. The salt was placed under an argon atmosphere and dry methanol (10ml) and triethylamine (164 μL) were added. The mixture was then boiled under reflux for forty-eight hours. After cooling, the reaction solvent was reduced to a minimum on a rotary evaporator and the residue was purified by flash chromatography (2% methanol: chloroform). A number of fast-running benzothiazole containing compounds were isolated, the major component was again found to be 2-acetyl-3-methyl benzo-dihydrothiazole, (20mg 9.4% yield).

Material with an Rf 0.42 was isolated and identified as the starting dihydroxy compound (120mg, 37.2%). The final fraction Rf 0.37 from the column was identified as methyl α- acetolactate (42.6mg, 26.5% yield).

^{1}Hnmr ($CDCl_3$): δ 3.82 (3H,S,-CO_2CH_3), 2.3 (3H,S,CH_3-C=O), 1.6 (3H,S,CH_3-C(OH)-).

An analytical sample was obtained by Kugelrohr distillation (150°C/15mmHg).

Analysis: Calculated for $C_6H_{10}O_4$: C,49.31; H,6.85.

Found: C,49.29; H,7.13.

8.3 Experimental to Chapter 3

Ethyl [3,4-$^{13}C_2$]-tiglate(21).

Sodium hydride (80%, 0.34g, 11.3mmol) was dissolved in dry 1,2-dimethoxyethane (4ml) and stirred at 0°C under a nitrogen atmosphere. To the reaction mixture was then added dropwise, a solution of triethyl-2-phosphonopropionate (2.7g, 11.3mmol) in 1,2-dimethoxyethane (4ml). After the evolution of hydrogen gas had ceased, the reaction mixture was allowed to warm up to room temperature. [1,2-$^{13}C_2$]-Acetaldehyde (98% $^{13}C_2$, supplied by M.S.D. isotopes, 0.63ml, 11.3mmol) was dissolved in 1,2-dimethoxyethane (4ml) and injected into the reaction mixture, forming instantly a yellow jelly-like precipitate. The reaction mixture was stirred for thirty minutes and then poured onto water (25ml). The mixture was extracted with diethyl ether (3x30ml). The ether extracts were combined, dried with magnesium sulphate and the solvent evaporated under reduced pressure. Residual 1,2-dimethoxyethane was removed by dissolving the oily product in pentane (100ml) and washing with water (2x50ml). The pentane fraction was dried with magnesium sulphate and evaporated under reduced pressure to yield a crude oil (1.33g, 10.2mmol, 90% yield).

^{1}Hnmr ($CDCl_3$): δ 6.8 (1H,m,H-^{13}C=), 4.14 (2H,q,J=7.2Hz,-O-CH_2CH_3), 1.8 (3H,m,CH_3-C=), 1.74 (3H,m,$^{13}CH_3$-^{13}C=), 1.25 (3H,t,J=7.2Hz, -OCH_2CH_3).

Accurate Mass: E.I. (M)$^+$ $C_5{}^{13}C_2O_4H_{12}$ (130.0902)

I.R. (liquid cell, 3% chloroform): 1700 (S,C=O, - unsaturated ester), 1625 (W,C=C), 908 (S,=C-H) cm^{-1}.

M.S., E.I.: 130(M)$^+$, 114 (M-$^{13}CH_3$)$^+$, 102 (M-C_2H_4)$^+$, 85 (M-OCH_2CH_3)$^+$, 57 (M-C_2H_4-CO_2H)$^+$.

Ethyl [3,4-$^{13}C_2$]-α-acetolactate (23).

Ethyl [3,4-$^{13}C_2$]-tiglate (21), (1.16g, 8.9mmol) was dissolved in acetone (150ml), water (40ml) and acetic acid (3ml). The combined mixture was cooled to -10°C. Potassium permanganate (2.44g, 15.4mmol) was then added in 0.5g portions, maintaining a reaction temperature of -10°C. The reaction mixture was stirred at -10°C for one hour and then filtered through a layer of celite removing manganese (IV) oxide formed during the reaction. The filtrate was evaporated under reduced pressure in order to remove the acetone component of the reaction, and then the aqueous fraction was extracted with dichloromethane (3x100ml). The dichloromethane extracts were combined, dried with magnesium sulphate and evaporated under reduced pressure to yield a colourless oil. The crude oil was purified by Kugelrohr distillation (115°C, 10mmHg), (1.0g, 6mmol 70% yield).

^{1}Hnmr ($CDCl_3$): δ 4.24 (2H,q,J=7Hz, -OC$\underline{H}_2$CH$_3$), 2.25 (3H,dd,$J^{13}C_{(3)}H$=6Hz, $J^{13}C_{(4)}H$=129Hz, -$^{13}C\underline{H}_3$), 1.57 (3H,d,$J^{13}C_{(3)}H$=4Hz,-C$\underline{H}_3$), 1.28 (3H,t,J=7Hz,-OCH$_2$C$\underline{H}_3$).

^{13}Cnmr ($CDCl_3$): δ 204.7 (d,$Jc_{(3)}c_{(4)}$=42.7Hz, -$^{13}\underline{C}$(=O)^{13}CH$_3$), 171.2 (S,-$\underline{C}$O$_2$CH$_2$CH$_3$), 81 (dd,$Jc_{(2)}c_{(3)}$ =39Hz, $Jc_{(2)}c_{(4)}$=12Hz, -$\underline{C}$(OH)), 62.5 (S,-O$\underline{C}$H$_2$CH$_3$), 24 (d,$Jc_{(3)}c_{(4)}$=42.7Hz, -^{13}C(=O)$^{13}\underline{C}$H$_3$), 21.6 (S,-C(OH)-$\underline{C}$H$_3$), 13.9 (S,-OCH$_2\underline{C}$H$_3$).

I.R. (liquid cell, 4% chloroform): 3500 (m, broad, -OH), 1720 (S,C=O, ester)cm^{-1}.

Accurate Mass: E.I.: (MH)$^+$ $C_6{}^{13}C_2O_4H_{13}$ (163.0886).

The decarboxylation of racemic[3,4-$^{13}C_2$]- α -acetolactate catalysed by the enzyme acetolactate decarboxylase.

Information pertaining to the enzymes used:

Pig liver esterase (PLE) EC 3.1.1.1:

Supplier: Boehringer Mannheim.

The enzyme was supplied as a suspension in ammonium sulphate solution (30mg of protein in 3ml of suspension). The enzyme was reported to have a specific activity of 130 units per mg (25°C, butyric acid ethyl ester as substrate.

Acetolactate decarboxylase (ADC):

Supplier: Novo Industri A/S

The enzyme was supplied in a crude form isolated from selected strains of *Bacillus brevis*, approximate activity 13 units per mg.

Enzyme studies: To a precision nmr tube was added ethyl [3,4-$^{13}C_2$]-α-acetolactate (23), (36mg, 0.22mmol), phosphate buffer (0.6ml, 1M, pH 7.2) and pig liver esterase (0.4mg, 52U). The solution was then incubated at 37°C and the ester hydrolysis was followed by ^{1}Hnmr (220MHz, C.W.). After complete hydrolysis of the ester group (one hour), acetolactate decarboxylase (1mg) was added and the subsequent reaction was followed by ^{1}Hnmr.

8.4 Experimental to Chapter 4

Resolution of racemic α-acetohydroxybutyrate (28) and analysis of the tertiary ketol rearrangement.

Ethyl α-acetohydroxybutyrate (60mg, 0.34mmol) was dissolved in phosphate buffer (dipotassium hydrogen orthophosphate and potassium dihydrogen orthophosphate, 0.5M, pH7.2, 3ml) and the solution was warmed to a constant 37°C. To the solution was then added pig liver esterase (100 μL, 130 units). The ester-hydrolysis was followed directly by ^{1}Hnmr (220MHz, c.w.); ^{1}Hnmr spectra were recorded at 20 minute intervals; after 2 hours the hydrolysis was complete. The ^{1}Hnmr exhibited resonances attributable to ethanol and racemic α-acetohydroxybutyrate

^{1}Hnmr: α-acetohydroxybutyrate : (phosphate buffer, ref. water)δ 2.29 (3H,S,CH_3(C=O)-), 1.9 (2H,m,CH_2CH_2-), 0.85 (3H,t,J=7.2Hz, CH_2CH_3-).

:ethanol: δ 3.68 (2H,q,J=7Hz), 1.2 (3H,t,J=7Hz).

To the buffer solution containing the hydrolysed material was then added acetolactate decarboxylase (1mg, 15 units). The decarboxylation reaction was continually monitored by ^{1}Hnmr. The ^{1}Hnmr exhibited resonances attributable to α-acetohydroxybutyrate and also 3-hydroxypentan-2-one.

^{1}Hnmr : 3-hydroxypentan-2-one: δ 2.1 (3H,S,CH_2C(=O)-), 1.8 (2H,m,CH_2CH_2-), 0.9 (3H,t,J=7.3Hz, CH_2CH_3-).

After 45 minutes the ^{1}Hnmr spectra indicated that the decarboxylation of (S)- α -acetohydroxybutyrate to yield 3-hydroxypentan-2-one was complete and that resonances attributable to 2-hydroxypentan-3-one began to appear at δ 1.3 (d,J=7.2Hz, CH_3-C(H)-). After 45 minutes the reaction mixture was centrifuged, at -10°C, through a 10,000 MW cutoff micro-pore filter (Centricon-10 at 4500g)

in order to remove the enzyme material. The filtered solution was then freeze-dried. If the enzyme material was not removed prior to freeze-drying, the desired product decomposed during the freeze-drying process. The white solid obtained from freeze-drying was then dissolved in sodium hydroxide (1.5M, 3ml) and filtered through glass wool until a clear solution remained. ^{1}Hnmr spectra of the alkali solution were then recorded at 15 minutes intervals, after 1 hour resonances attributable to α-acetohydroxybutyrate and 2-hydroxy-2-methyl-3-oxopentanoate were observed.

^{1}Hnmr (ref.H_2O): 2-hydroxy-2-methyl-3-oxopentanoate : δ 1.4 (3H,S,CH_3-C(OH)-), 0.95 (3H,t,J=7.2Hz, CH_3CH_2-C(=O)-).

^{1}Hnmr (ref. H_2O δ 4.85 at 37^{0}C) .

The alkali catalysed rearrangement was allowed to proceed until a 70:30 mixture of α-acetohydroxybutyrate and 2-hydroxy-2-methyl-3-oxopentanoate resulted from the rearrangement reaction. The alkali solution was then treated with hydrochloric acid (1.2M) until a pH of 8.4 was achieved. The solution was then freeze-dried. The white solid material obtained after freeze-drying was dissolved in water (0.9ml), filtered and then acetolactate decarboxylase (0.5mg, 7 units) was added and the subsequent reaction was studied at 25°C by ^{1}Hnmr (400MHz, water suppression techniques were used and the data acquisition was set to produce a spectrum every 3 minutes).

8.5 Experimental to Chapter 5

2-Ethyl-2-propenoic acid (35).

Diethyl ethyl malonate (150g, 0.8mol) was added slowly to potassium hydroxide (129g, 2.26mol) in water (140ml), after complete addition the mixture was heated to reflux for 24 hours. The solution was allowed to cool to room temperature and then formaldehyde solution (40%, 270ml) was added to the reaction mixture. The reaction mixture was then stirred for ten days at room temperature. The mixture was acidified with concentrated hydrochloric acid (540ml) and heated to reflux for 12 hours. The cold solution was extracted with diethyl ether containing hydroquinone (4x250ml). The ethereal extracts were combined and dried with magnesium sulphate. The solvent was removed under reduced pressure and the residue obtained was distilled to yield a colourless oil. (b.p. 76-80°C/15mmHg), (20.76g, 26% yield).

^{1}Hnmr ($CDCl_3$, ref. TMS) δ 6.32 (1H,S,Hcis), 5.69 (1H,S,Htrans), 2.35 (2H,q,J=7.2Hz,-CH_2), 1.11 (3H,t,J=7.2Hz, -CH_3)

2,3-Dibromo-2-ethylpropanoic acid (36).

2-Ethyl-2-propenoic acid (21.12g, 0.21mol) was dissolved in carbon tetrachloride (100ml) and the solution was cooled to 0°C. A solution of bromine (15ml, 1.1equiv.) dissolved in carbon tetrachloride (50ml) was then added gradually over a two hour time period. The reaction mixture was stirred for fifteen hours at room temperature and then heated at reflux for 2 hours. The reaction mixture was cooled to room temperature and the solvent evaporated under reduced pressure. The crude orange solid obtained was recrystallised from pentane to yield off-white crystals (33.5g, 61% yield), (m.p. 75.2-75.4°C).

^{1}Hnmr ($CDCl_3$, Ref. TMS) δ 11.4 (1H,broad s, $-CO_2\underline{H}$), 4.0 (2H, ABq, J=10.7Hz, $-C\underline{H}_2Br$), 2.3 (2H, broad q, J=7.3Hz, $-C\underline{H}_2CH_3$), 1.1 (3H,t,J=7.3Hz, $-CH_2C\underline{H}_3$).

^{13}Cnmr ($CDCl_3$) δ 174.9 (s,$\underline{C}O_2H$), 61.7 (S,$\underline{C}$-Br), 33.8 (S,-$\underline{C}H_2Br$), 29.1 (S,-$\underline{C}H_2CH_3$), 8.7 (S,-$CH_2\underline{C}H_3$).

I.R. ($CHCl_3$, liquid film) : 2500-3200 (-OH), 1715 (C=O) cm^{-1}

M.S. (E.I.). 179/181 (1:1) (M-Br)$^+$, 99 (M-Br-HBr)$^+$

Analysis calculated for $C_5H_8Br_2O_2$: C,23.08;H,3.08; Br,69.23. Found : C,23.25; H,3.09; Br,58.63.

E-3-Bromo-2-ethyl-2-propenoic acid (37)

2,3-Dibromo-2-ethylpropanoic acid (33.48g, 0.13mol) was dissolved in dry methanol (100ml) and potassium carbonate (10.5g) was added to the reaction mixture. A solution consisting of potassium hydroxide (43.5g, 0.76mol) dissolved in methanol (150 ml) was then added gradually (maintaining a reaction temperature below 40°C) to the reaction mixture. The reaction mixture was heated at reflux for 2.5 hours. To the cooled solution was added solid carbon dioxide, in order to convert the remaining potassium hydroxide into potassium carbonate. The reaction mixture was then reheated for 15 minutes, cooled to room temperature and filtered. The precipitate was washed with hot methanol (80ml). The combined methanol fractions were evaporated under reduced pressure. The white crystalline material obtained was dissolved in water (25ml) and diethyl ether (200ml) was added to the solution. The solution was stirred and acidified to pH 1 by the addition of concentrated hydrochloric acid. The aqueous phase was separated and extracted with diethyl ether (2x50ml). The ethereal extracts were combined, dried with magnesium sulphate and the solvent was removed under reduced pressure to yield a crystalline material which was recrystallised from pentane. (21.15g, 92% yield), (m.p. 47.2-48°C).

^{1}Hnmr ($CDCl_3$, Ref.TMS) δ 7.7 (1H,S,H-C=C), 2.51 (2H,q,J=7.3Hz, $-CH_2CH_3$), 1.09 (3H,t,J=7.3Hz, $-CH_2CH_3$)

I.R. ($CHCl_3$, liquid cell) : 3500 (broad-OH), 1690 (C=O), 1600 (C=C) cm^{-1}.

M.S. (E.I.): 178/180 (1:1),$(M)^+$, 99$(M-Br)^+$

M.S. (C.I., ammonia): 196/198 (1:1),$(MNH_4)^+$

Analysis calculated for $C_5H_7BrO_2$: C,33.52; H,3.91; Br,44.69. Found: C,33.77; H, 3.93; Br,43.37.

3-Bromo-2-(1-bromoethyl)-2-propenoic acid. (39)

E-3-Bromo-2-ethyl-2-propenoic acid (13.51g, 7.5mmol) was dissolved in carbontetrachloride (120ml) and to the reaction mixture was added 2,2'-azo-bis(2-methylpropionitrile), (10mg), and N-bromosuccinimide (15.4g). The reaction mixture was heated at reflux for 12 hours. The reaction mixture was then cooled to room temperature and filtered. The filtrate was evaporated under reduced pressure to yield a crystalline product, which was recrystallised from pentane (17.5g, 90% yield).

^{1}Hnmr ($CDCl_3$, Ref. TMS) δ 7.81 (1H,S,H-C=C), 5.36 (1H,q,J=7.2Hz, H-C(CH_3)Br), 2.01 (3H,d,J=7.2Hz, H-C(CH_3)Br)

I.R. (liquid film) 3000 (broad-OH), 1700 (C=O), 1585 (C=C) cm^{-1}

M.S. (E.I.): 179/177 (1:1), (M-Br)$^+$, 97 (M-Br-HBr)$^+$

M.S. (C.I.): 274/276/278 (1:2:1), (MNH_4)$^+$

Analysis calculated for $C_5H_6Br_2O_2$: C,21.25; H,2.32. Found: C,23.22; H,2.42.

3-Bromo-2-(1-hydroxyethyl)-2-propenoic acid. (40).

3-Bromo-2-(1-bromoethyl)-2-propenoic acid (17.49g, 67.8mmol) was dissolved in water (50ml) and sodium carbonate (3.6g) was added to the solution. The reaction mixture was stirred at room temperature for 4 hours. The solution was extracted with diethyl ether (50ml) and then acidified to pH 1 with hydrochloric acid (2M). The acidified solution was extracted with ethyl acetate (4x50ml). The ethyl acetate fractions were combined, dried with magnesium sulphate and the solvent was evaporated under reduced pressure to yield a viscous oil. (12.2g, 92% yield). Spectroscopic analysis indicated a 86:14/trans:cis mixture of the desired product.

^{1}Hnmr ($CDCl_3$, Ref. TMS) δ 7.7 (1H,S,H-C=C), 5.02 (1H,q,J=7.3Hz, H-C(OH)), 1.5 (3H,d,J=7.3Hz, H-C-CH_3).

cis-isomer: δ 7.01 (1H,S,H-C=C), 4.7 (1H,q,J=7.3Hz, H-C(OH)), 1.45 (3H,d,J=7.3Hz, H-C-CH_3).

^{13}Cnmr ($CDCl_3$) δ 167.3 (CO_2H), 137.84 (C=C-CO_2H), 124.38 (Br-C=C), 67.39 (-C-OH), 21.54 (-CH_3)

cis-isomer: δ 169.54, 140.11, 115.6, 69.44, 22.09.

I.R. ($CHCl_3$, liquid cell, 3%): 3500 (broad-OH), 1690 (C=O), 1600 (C=C) cm^{-1}.

M.S.(E.I.): 179/181 (1:1),$(M-CH_3)^+$, 161/163 (1:1),$(M-H_2O-CH_3)^+$, 97 $(M-H_2O-Br)^+$.

E-3-Bromo-2-(1-hydroxyethyl)-2-propenoic acid was isolated by dissolving the cis/trans mixture of 3-bromo-2-(1-hydroxyethyl)-2-propenoic acid (885.8mg, 4.54mmol) in methanol (20ml) and dicyclohexylamine (822mg, 0.9ml) was added to the reaction mixture. After stirring at room temperature for 6 hours the methanol was removed under reduced pressure. The crystalline dicyclohexylamine

salt obtained was then recrystallised from ethyl acetate and pentane (three times) yielding a white crystalline material (280mg). Spectroscopic analysis of the dicyclohexylamine salt indicated isolation of the trans-isomer. (m.p.134-135°C).

^{1}Hnmr (D_2O Ref. T.S.S.) δ 7.25 (1H,S,H-C=C), 5.0 (1H,q,H-C-CH_3), 1.55 (3H,d,H-C-CH_3), and dicyclohexylamine resonances.

^{13}Cnmr ($CDCl_3$) δ 169.4 (-CO_2), 144.3 (C=C-CO_2), 115.6 (Br-C=C), 67.6 (-C-OH), 23.0 (-CH_3), 52.8, 29.0, 25.0, 24.6 (dicyclohexylamine).

Analysis: Calculated for $C_{17}H_{30}NO_3Br$: C,54.2; H,7.98; N,3.72. Found: C,54.46; H,8.02; N,3.66.

[3-^{2}H]-3-Bromo-2-(1-hydroxyethyl)-2-propenoic acid (41).

3-Bromo-2-(1-hydroxyethyl)-2-propenoic acid (11.77g, 60mmol) was dissolved in D_2O (30ml) and cooled to 5°C. A solution of sodium hydroxide (5g, 0.12mol) dissolved in D_2O (15ml) was added to the reaction mixture. The reaction mixture was warmed to room temperature and then heated at reflux for 5 hours. The incorporation of a deuterium atom into the starting material was followed by ^{1}Hnmr. After 5 hours the olefinic resonance (H-(Br)C=C) δ 7.2 was observed to have diminished totally in size. After cooling the reaction mixture was acidified to pH 1 and extracted with diethyl ether (3x75ml). The combined ethereal extracts were dried with magnesium sulphate and the solvent was evaporated under reduced pressure to yield a crude oil (8.04g, 68% yield).

^{1}Hnmr ($CDCl_3$, Ref. TMS) δ 5.05 (1H,q,J=7.2Hz, H-C(OH)-CH_3), 1.5 (3H,d,J=7.2Hz, H-C(OH)-CH_3)

^{2}Hnmr ($CHCl_3$, Ref. $CDCl_3$) δ 7.61 (S,Br(D)C=C)

I.R. (liquid film): 2800-3500 (broad-OH), 1700 (C=O), 1590 (C=C) cm^{-1}

M.S. (C.I. ammonia): 213/215 (1:1), $(MNH_4)^+$, 196/198 (1:1), $(MH)^+$

M.S. (E.I.): 180/182 (1:1), $(M-CH_3)^+$, 162/164 (1:1), $(M-CH_3-H_2O)^+$, 98$(M-Br-H_2O)^+$.

[3-^{2}H]-2-(1-hydroxyethyl)-2-propenoic acid (42).

[3-^{2}H]-3-Bromo-2-(1-hydroxyethyl)-2-propenoic acid (6.75g, 34mmol) and sodium carbonate (1.82g) were dissolved in water (20ml) and the solution was cooled to 0°C. Sodium-mercury amalgam (2.5% Na/Hg, 1.58g Na/61.62g Hg) was added to the reaction mixture gradually, over one hour, and then the reaction mixture was stirred for 18 hours. The aqueous phase was decanted from the mercury residue and the mercury residue was washed with water (2x30ml). The combined aqueous fractions were washed with diethyl ether (30ml). The aqueous fraction was then acidified to pH 1 and extracted with diethyl ether (4x50ml). The ethereal extracts were dried with magnesium sulphate and the solvent was evaporated under reduced pressure to yield a crude oil. The oil obtained was dissolved in diethyl ether (20ml) and dicyclohexylamine (6.3g, 35mmol) was added to the ethereal solution. The diethyl ether was removed under reduced pressure and the residue was dissolved in water (10ml). The aqueous fraction was then washed with diethyl ether (5ml) and the water was removed under reduced pressure to yield a white solid. The solid obtained was recrystallised from ethyl acetate to yield white crystals (5.501g, 54% yield).

m.p. 124-125°C.

^{1}Hnmr ($CDCl_3$, Ref. TMS) δ 5.31 (1H,s,H-(D)C=C), 4.5 (1H,q,J=7.3Hz, H-C(OH)-CH_3), 1.37 (3H,d,J=7.3Hz, H-C(OH)-CH_3), 1-3 (dicyclohexylamine peaks).

M.S. (FAB, glycerol, D_2O matrix) : (-Ve) : 235 (M_2H)$^-$, 117 (M)$^-$: (+Ve) : 184 (X)$^+$.

Analysis: Calculated for $C_{17}H_{32}DO_2N$: C,68.46; H,10.74; N,4.7. Found: C,68.24; H,10.73; N,4.53.

The dicyclohexylamine salt of [3-^{2}H]-2-(1-hydroxyethyl)-2-propenoic acid (4.02g, 13.5mmol) was dissolved in water (100ml) and Dowex 50W-X8(H), (16-40 mesh, 28g) was added to the solution. The reaction mixture was then stirred for 8 hours at room temperature. The solution was filtered and the resin was washed with water (100ml). The aqueous filtrate was extracted with ethyl acetate (4x100ml). The ethyl acetate fractions were combined and dried with magnesium sulphate. The solvent was evaporated under reduced pressure to yield a colourless oil. (1.49g, 95% yield).

^{1}Hnmr ($CDCl_3$, Ref. TMS) δ 8.2 (S,broad,-OH), 5.98 (1H,S,H-(D)C=C), 4.7 (1H,q,J=7.3Hz, H-(OH)C-CH_3), 1.4 (3H,d,J=7.3Hz, H-(OH)C-CH_3)

Methyl [3-^{2}H]-2-(1-hydroxyethyl)-2-propenoate (43).

[3-^{2}H]-2-(1-hydroxyethyl)-2-propenoic acid (1.49g, 12.8mmol) was dissolved in diethyl ether (25ml) and a solution of diazomethane in diethyl ether (1mmol CH_2N_2/2.4ml Et_2O) was added to the reaction mixture until a yellow colour remained and the evolution of nitrogen had ceased (approx. 30ml of reagent). Acetic acid was added to the reaction mixture until the yellow colour of the solution was no longer present. The reaction mixture was then added to ethyl acetate (150ml). The ethyl acetate fraction was washed with aqueous sodium bicarbonate and then water. The ethyl acetate component was dried with magnesium sulphate and the solvent was evaporated under reduced pressure to yield a crude oil. The oil obtained was purified by flash-chromatography (150g silica (230/400mesh), 1:4 ethyl acetate to petrol). Chromatography gave a colourless oil (1.51g, 90% yield).

^{1}Hnmr(400MHz), ($CDCl_3$, Ref. TMS) δ 5.79 (1H,d,J=1.28Hz, H-(D)C=C), 4.59 (1H,dq,J=6.47Hz and 1.28Hz, H-(OH)C-CH_3), 3.74 (3H,S,-CO_2Me), 1.34 (3H,d,J=6.4Hz, H-(OH)C-CH_3).

^{13}Cnmr ($CDCl_3$) δ 166.8 (S,CO_2Me), 143.4 (S,=C-CO_2), 123.5 (1:1:1 triplet, J_{CD}=24.9Hz, (D)-C=C), 66.5 (S,C-OH), 51.7 (S, CO_2CH_3), 22.0 (C-CH_3)

I.R. (liquid cell, chloroform, 1%): 1740 (C=O, ester) cm^{-1}

^{2}Hnmr ($CHCl_3$, Ref. $CDCl_3$) δ 6.21 (S,(D)-C=C)

Methyl 2-(1-hydroxyethyl)-2-propenoate (44)

Ethanal (65g, 1.48mol), methyl acrylate (82.56g, 0.96mol) and 1,4-diazabicyclo[2.2.2]octane (5.48g, 50mmol) were combined in a sealed flask and stirred at room temperature for 10 days. The reaction mixture was poured into ethyl acetate (400ml) and then the ethyl acetate mixture was washed successively with hydrochloric acid (6M), sodium hydroxide (2M) and saturated sodium chloride solution. The ethyl acetate component was dried with magnesium sulphate and the solvent was evaporated under reduced pressure to yield a crude oil. The oil was purified by distillation under reduced pressure. (b.p. 79-80°C/15mmHg , 52.6g, 55% yield).

^{1}Hnmr ($CDCl_3$, Ref. TMS) δ 6.25 (1H,S,H-C=C), 5.8 (1H,S,(H)-C=C), 4.65 (1H,q,J=7Hz,H-C(OH)), 3.8 (3H,S,CO_2Me), 1.4 (3H,d,J=7Hz, H-C(CH_3)).

I.R. (liquid film): 3420 (broad-OH), 1725 (C=O), 1630 (C=C) cm^{-1}

M.S. (E.I.): 115 (M-CH_3)$^+$, 98(M-CH_3OH)$^+$, 87(M-CH_3CO)$^+$

Accurate mass (C.I. ammonia): (MH)$^+$, $C_6H_{11}O_3$, 131.0711 (MNH_4)$^+$, $C_6H_{14}NO_3$, 148.1015.

Analysis: Calculated for $C_6H_{10}O_3$: C, 55.38; H,7.69. Found: C,55.28; H,7.73.

Methyl 2-(1-tert-butyldimethylsiloxyethyl)-2-propenoate (45).

Methyl 2-(1-hydroxyethyl)-2-propenoate (5g, 38.5mmol) was dissolved in N,N-dimethylformamide (35ml). To the reaction mixture maintained under a nitrogen atmosphere, was added tert-butyldimethylsilylchloride (9.85g, 1.7 equiv.) and imidazole (8.9g, 3.4 equiv.). The reaction mixture was warmed to 45°C and stirred for 12 hours. Ethyl acetate (100ml) was added to the cooled reaction mixture. The ethyl acetate mixture was then washed with water (2x50ml) and the ethyl acetate fraction was dried with magnesium sulphate. The solvent was evaporated under reduced pressure and the crude oil obtained was purified using flash-chromatography (flash silica 230/400 mesh, 1:9 ethyl acetate to petrol) to yield a colourless oil (8.2g, 87% yield).

^{1}Hnmr ($CDCl_3$, Ref. TMS) δ 6.2 (1H,S,H-C=C), 5.95 (1H,S,(H)-C=C), 4.7 (1H,q,J=7Hz, H-C(CH_3)), 3.78 (3H,S,CO_2Me), 1.4 (3H,d,J=7Hz, H-C(CH_3)), 0.9 (9H,S,-CMe_3), 0.08 (3H,S,Si-Me), 0.02 (3H,S,Si-Me).

Methyl 2,3-epoxy-2-(1-tert-butyldimethylsiloxyethyl) propanoate (46).

Methyl 2-(1-tert-butyldimethylsiloxyethyl)-2-propenoate (4.15g, 17mmol), 3-chloroperoxybenzoic acid (3.83g, 1.3 equiv.) and 3-tert-butyl-4-hydroxy-5-methylphenyl sulphide (250mg) were dissolved in 1,2-dichloroethane (70ml) and the reaction mixture was heated to reflux. The reaction mixture was refluxed for 6.5 hours. The cooled reaction mixture was then poured into dichloromethane (150ml). The dichloromethane fraction was washed with saturated sodium bicarbonate solution (2x100ml) and then water (2x50ml). The dichloromethane component was dried with magnesium sulphate and the solvent was evaporated under reduced pressure to yield a crude oil. The oil obtained was purified using flash-chromatography (silica (150g) 230/400 mesh, 1:20 ethyl acetate to petrol). Chromatography yielded a colourless oil (2.7g, 61% yield).

^{1}Hnmr ($CDCl_3$, Ref. TMS) δ 4.46 (1H,m,H-C-CH_3), 3.79, 3.78 (3H, 2xs, CO_2Me), 3.0 (2H, 2xAB systems, $H_2C(O)$), 1.25, 1.30 (3H,2xd, J=6.5Hz, CH_3-C-H), 0.9 (9H,S,CMe_3), 0.1 (6H,S,$SiMe_2$)

Spectroscopic analysis indicated a diastereomeric mixture of the desired product in the ratio 3:4.

^{13}Cnmr ($CDCl_3$) δ 169.93, 169.87 (CO_2Me), 66.24, 65.28 (H-C-CH_3), 60.85, 59.9 (C-C(O)-CO_2Me), 52.38, 52.31 ($H_2C(O)$), 49.7, 47.72 (CO_2Me), 25.79, 25.74 (CMe_3), 19.79, 19.65 (H-C-CH_3), 18.11 (CMe_3), -4.66, -4.82, -4.94 ($Si-Me_2$).

M.S. (C.I. ammonia): 261 $(MH)^+$, 278 $(MNH_4)^+$.

M.S. (E.I.): 245 $(M-Me)^+$, 203 $(M-CMe_3)^+$, 173 $(M-CH_2O-CMe_3)^+$.

Potassium 2,3-epoxy-2-(1-tert-butyldimethylsiloxyethyl) propancate (47).

Methyl 2,3-epoxy-2-(1-tert-butyldimethylsiloxyethyl)-propanoate (2.4g, 9.2mmol) was dissolved in methanol (20ml) and a solution of potassium carbonate (1.27g) dissolved in water (20ml) was added to the reaction mixture. The reaction mixture was stirred for 20 hours at room temperature and then water (30ml) was added. The solution was extracted with diethyl ether and the aqueous fraction was freeze-dried to yield a white solid. (1.89g, 73% yield).

^{1}Hnmr (D_2O) δ 4.75 (1H,q,J=7Hz, H-C-CH_3), 2.9 (2H,m,$H_2C(O)$), 1.25, 1.1 (3H,2xd, J=7Hz, H-C-CH_3), 0.9 (9H,S,CMe_3), 0.14, 0.15 (6H,2xS,$SiMe_2$).

M.S. (FAB, glycerol-water matrix): +Ve : 323 $(MK)^+$, 607 $(M_2K)^+$: -Ve: 245 $(M-K)^-$, 529 $(M_2-K)^-$, (K=potassium).

Analysis: Calculated for $C_{11}H_{21}O_4SiK$: C,46.48; H,7.39. Found: C,44.72; H,7.6.

Methyl 2-hydroxy-2-methyl-3-tert-butyldimethylsiloxybutanoate (48)

Potassium 2,3-epoxy-2-(1-tert-butyldimethylsiloxyethyl)-propanoate (1.31g, 4.62mmol) was dissolved in diglyme (15ml) and a solution of lithium borohydride dissolved in diglyme (0.88M, 9.24ml) was added to the reaction mixture. The reaction mixture was heated at 100°C for 3 hours. The cooled reaction mixture was poured into diethyl ether (150ml) and the ethereal mixture was washed with hydrochloric acid (0.1M). The ethereal extract was dried with magnesium sulphate and the solvent was removed under reduced pressure to produce an oil. The crude oil containing residual diglyme was dissolved in diethyl ether (10ml) and a solution of diazomethane dissolved in diethyl ether (0.23M, approx. 20ml) was added to the reaction mixture. The diazomethane solution was added portion-wise until a yellow colour persisted in the reaction mixture. Acetic acid was then added to the reaction mixture until decolourisation occurred. The reaction mixture was dissolved in ethyl acetate (100ml) and the mixture was washed with saturated sodium bicarbonate solution and water. The ethyl acetate mixture was dried with magnesium sulphate and the solvent was evaporated under reduced pressure to yield a crude oil. The oil was purified using flash-chromatography (silica (230/400 mesh), 1:9 ethyl acetate to petrol). Chromatography separated the diastereomers of the desired product. Two fractions were isolated: Rf. 0.4 (360 mg) and Rf. 0.34 (345 mg): combined yield 61%.

(Rf0.4): ^{1}Hnmr ($CDCl_3$, Ref. TMS) δ 4.1 (1H,q,J=6.8Hz, H-C-CH_3), 3.75 (3H,S,CO_2Me), 3.11 (1H,S,-OH), 1.26 (3H,S,CH_3-C-OH), 1.2 (3H,d,J=6.8Hz, CH_3-C-H), 0.9 (9H,S,CMe_3), 0.06 (6H,S,$SiMe_2$).

(Rf 0.34):^{1}Hnmr ($CDCl_3$, Ref. TMS) δ 3.86 (1H,q,J=6.8Hz, H-C-CH_3), 3.78 (3H,S,CO_2Me), 3.15 (1H, S, -OH), 1.35 (3H,S, CH_3-C-OH), 1.2 (3H,d,J=6.8Hz, CH_3-C-H), 0.9 (9H,S,CMe_3), 0.05 (6H,S,$SiMe_2$).

^{13}Cnmr ($CDCl_3$) δ 174.93 ($\underline{C}O_2Me$), 77.95 (CH_3-$\underline{C}$-OH), 74.04 (CH_3-$\underline{C}$-OSi), 52.2 ($CO_2\underline{Me}$), 25.57 ($C\underline{Me}_3$), 21.49 ($\underline{C}H_3$-C-OH), 18.39 ($\underline{C}H_3$-C-OSi), 17.8 ($\underline{C}Me_3$), -5.08, -4.43 ($Si\underline{Me}_2$)

M.S.: (E.I.): Accurate mass: (MH)$^+$, found for $C_{18}H_{27}O_4Si$ 263.1676

M.S. (E.I.): 263 (MH)$^+$, 205 (M-CMe_3)$^+$, 187 (M-CMe_3-H_2O)$^+$, 145.

Methyl 2,3-dihydroxy-2-methylbutanoate (49).

Methyl 2-hydroxy-2-methyl-3-tert-butyldimethylsiloxybutanoate (228mg, 0.87 mmol) was dissolved in tetrahydrofuran (10ml) and tetrabutylammonium fluoride (1M, 2.6ml, solution in THF) was added. The reaction mixture was stirred at room temperature for 6 hours. The solvent was then evaporated under reduced pressure and the residue was dissolved in water (40ml) and extracted with diethyl ether using a continuous liquid-liquid extraction. The ethereal fraction was dried with magnesium sulphate and the solvent was removed under reduced pressure to yield a crude oil which was purified using flash-chromatography (silica 230/400 mesh, 20g, 1:9 petrol to diethyl ether). Chromatography yielded a colourless oil (102mg, 78% yield).

^{1}Hnmr ($CDCl_3$ Ref. TMS) δ 3.85 (3H,S,$CO_2\underline{Me}$), 3.85 (1H,q,H-C-$\underline{CH_3}$), 1.46 (3H,S,$\underline{CH_3}$-C-CO_2Me), 1.18 (3H,d,J=7.2Hz, $\underline{CH_3}$-C-H).

^{13}Cnmr ($CDCl_3$) δ 175.76 ($\underline{C}O_2$Me), 77.11($\underline{C}$-CO_2Me), 72.11 (CH_3-$\underline{C}$-OH), 52.71 ($CO_2\underline{Me}$), 22.13 ($\underline{C}H_3$-), 17.55 ($\underline{C}H_3$-).

M.S. (C.I. ammonia, accurate mass): Found: $(MH)^+$, $C_6H_{13}O_4$, 149.0829; $(MNH_4)^+$, $C_6H_{16}NO_4$, 166.1074.

M.S. (E.I.) : 104 $(M-C_3H_6O)^+$, 89, 43.

I.R. (liquid film): 3500 (broad-OH), 1745 (C=O) cm^{-1}

Analysis: Calculated for $C_6H_{12}O_4$: C,47.97; H,8.11. Found: C,48.15; H, 8.35.

Methyl 2-hydroxy-2-methyl-3-oxobutanoate (17).

Methyl 2,3-dihydroxy-2-methylbutanoate (55.6mg, 0.38mmol) was dissolved in benzene (40ml) and dibutyl tin oxide (93.5mg, 1 equiv.) was added to the reaction mixture. The reaction mixture was heated to reflux for 24 hours using a Dean-Stark apparatus. The reaction mixture was cooled to room temperature and the benzene was evaporated under reduced pressure. The white solid obtained was dissolved in dichloromethane (5ml) and a solution of bromine dissolved in dichloromethane (0.53ml, 0.78M) was added to the reaction mixture. The reaction was stirred for 48 hours, in the dark. The solvent was then evaporated under reduced pressure and the oil obtained was added to a silica column (60/120 mesh, 5g). Elution of the column with carbon tetrachloride (250ml) removed the tin residues. Subsequent elution with ethyl acetate (150ml), and removal of the solvent gave a crude oil, which was purified using flash-chromatography (silica (230/400 mesh), 3:7 ethyl acetate to petrol) to yield an oil (33mg, 60% yield).

^{1}Hnmr ($CDCl_3$, Ref. TMS) δ 4.26 (1H,S,-OH), 3.85 (3H,S,$CO_2\underline{Me}$), 2.31 (3H,S,-$\underline{CH_3}$), 1.62 (3H,S,-$\underline{CH_3}$).

M.S. (C.I. ammonia, accurate mass): Found $(MH)^+$; $C_5H_{11}O_4$, 147.0652; $(MNH_4)^+$; 164.0901

Analysis: Calculated for $C_5H_{10}O_4$: C,49.31; H,6.85. Found: C,49.42; H,6.96.

Methyl [3-^{2}H]-2-(1-tert-butyldimethylsiloxyethyl)-2-propenoate (50)

Methyl [3-^{2}H]-2-(1-hydroxyethyl)-2-propenoate (540 mg, 4.1mmol) was dissolved in N,N-dimethylformamide (15ml) and imidazole (0.96 g, 3.4 equiv.) and tert-butyldimethylsilylchloride (1.06 g, 1.7 equiv.) were added to the reaction mixture. The reaction mixture was stirred at 45°C for 5 hours. The cooled reaction mixture was poured into ethyl acetate (50ml) and the mixture was washed with water. The ethyl acetate fraction was dried with magnesium sulphate and the solvent was evaporated under reduced pressure. The oil obtained was purified using flash-chromatography (silica 230 400 mesh 140g, 1:9 ethyl acetate to petrol). (655mg, 70% yield).

^{1}Hnmr ($CDCl_3$, Ref. TMS) δ 6.0 (1H,S,H-C=C), 4.72 (1H,q,J=6.8Hz, H-C-OSi), 3.8 (3H,S,CO_2Me), 1.3 CH_3-C-OSi), 0.9 (9H,S,CMe_3), 0.04, 0.08 (6H,2xs,$SiMe_2$).

Methyl [3-^{2}H]-2,3-epoxy-2-(1-tert-butyldimethylsiloxyethyl)-propanoate (51).

Methyl [3-^{2}H]-2-(1-hydroxyethyl)-2-propenoate (412.9 mg, 1.68mmol), 3-chloroperoxybenzoic acid (410mg, 2.38mmol), and 3-tert-butyl-4-hydroxy-5-methylphenylsulphide (25mg) were dissolved in 1,2-dichloroethane (20ml), and the reaction mixture was heated to reflux for 6.5 hours. The cooled reaction mixture was diluted with dichloromethane (100ml) and the mixture was washed with saturated sodium bicarbonate solution (2x100ml) and water (2x50ml). The dichloromethane fraction was dried with magnesium sulphate and the solvent was evaporated under reduced pressure. The resulting crude oil was purified using flash-chromatography (silica (230 400 mesh), 1:20 ethyl acetate to petrol). Chromatography yielded a colourless oil (265.3mg, 60% yield).

^{1}Hnmr 400MHz ($CDCl_3$, Ref. TMS) δ 4.41 (1H, 2xq, J=6.2Hz, H-C-OSi), 3.74, 3.73 (3H, 2xs, CO_2Me, diastereomeric ratio 4:3), 3.01, 2.85 (1H, 2xs, H(D)C(O)), 1.25, 1.20 (3H, 2xd, J=6.4, 6.2Hz, CH_3-C-OSi), 0.85, 0.84 (9H, 2xs, CMe_3), 0.058, 0.054, 0.05, 0.028 (6H, 4xs, $SiMe_2$).

I.R. (liquid cell, chloroform): 1735 (C=O, ester)cm^{-1}

M.S: (E.I.): 204 $(M-CMe_3)^+$, 173$(M-CMe_3-CHDO)^+$

M.S: (C.I. ammonia, accurate mass): $(MH)^+$, $C_{12}H_{24}DO_4Si$, 262.1630.

Methyl 2-hydroxy-2-([^{2}H,^{3}H]methyl)-3-tert-butyldimethylsiloxybutanoate (53).

Methyl [3-^{3}H]-2,3-epoxy-2-(1-tert-butyldimethylsiloxyethyl)-propanoate (250mg, 0.96mmol) was dissolved in methanol (20ml) and a solution of potassium carbonate (132mg) dissolved in water (20ml) was added to the reaction mixture. The reaction mixture was stirred for 20 hours. Water (30ml) was then added to the reaction mixture and the solution was extracted with diethyl ether. The aqueous component was freeze-dried to yield a white solid (253.8mg, 0.89mol, 93% yield).

The potassium salt obtained was placed in a dry flask under an argon atmosphere. A solution of sodium borohydride (181.6mg) dissolved in diglyme (2.4ml) was prepared and an aliquot (2ml) was added to lithium bromide (152.8mg) and sodium boro[^{2}H] hydride (0.55mg, 100mCi, supplied by Amersham International). The diglyme mixture was stirred for 30 minutes and then left to settle. The clear supernatent, a solution of lithium borohydride (boro[^{2}H]hydride), (0.88M, 1.2ml) was added to the potassium salt. The reaction mixture was stirred for 3 hours at 100°C. The cooled reaction mixture was poured into ethyl acetate (50ml) and the ethyl acetate mixture was washed with hydrochloric acid (2M). The ethyl acetate fraction was dried with magnesium sulphate and the solvent was evaporated under reduced pressure.

The residue obtained was dissolved in diethyl ether and a solution (0.23M) of diazomethane dissolved in diethyl ether was added to the reaction mixture until a yellow colour persisted. Acetic acid was added to the ethereal mixture until decolourisation occurred. The ethereal mixture was then washed with sodium bicarbonate solution and water.

The ethereal fraction was dried with magnesium sulphate and

the solvent was evaporated under reduced pressure. The oil obtained was purified using flash-chromatography (silica 230,400 mesh, 80g, 1:9 ethyl acetate to petrol). Chromatography yielded a colourless oil the thin layer chromatograph of which was comparable to that of authentic unlabelled material. (40mg, 25% yield).

Specific activity: 2.6mCi/mmol.

(Scintillation counting was performed using a LKB 1215 RACKBETA liquid scintillation counter and LKB Optiphase-Safe scintillant cocktail).

Methyl 2,3-dihydroxy-2([^{2}H,^{3}H]methyl)-butanoate (54).

Methyl 2-hydroxy-2-([^{2}H,^{3}H]methyl)-3-tert-butyldimethylsilyloxybutanoate (40mg, 0.15mmol) was dissolved in tetrahydrofuran (5ml) and tetrabutylammonium fluoride (1M, 0.35ml) was added to the reaction mixture. The reaction mixture was stirred for 2 hours. The tetrahydrofuran was removed under reduced pressure and the residue obtained was purified using flash-chromatography (silica (230,400 mesh), 25g, 1:9 ethyl acetate to petrol). Chromatography yielded an oil (16.2mg, 72% yield).

Specific activity: 0.2mCi/mmol.

Methyl 2-hydroxy-2-([^{3}H,^{3}H]methyl)-3-oxobutanoate. (55)

Methyl 2,3-dihydroxy-2-([^{3}H,^{3}H]methyl)-butanoate (16.2mg, 0.1mmol) was dissolved in benzene (30ml) and dibutyl tin oxide (30mg) was added to the reaction mixture. The reaction mixture was heated to reflux in a Dean-Stark apparatus for 15 hours. The benzene was then removed under reduced pressure to yield a solid material (stannylene). The solid was dissolved in dichloromethane and a solution of bromine dissolved in dichloromethane (0.7M, 165 μL) was added to the reaction mixture. The reaction mixture was stirred for 48 hours. The solvent was then evaporated under reduced pressure to yield a crude oil. The oil was purified by addition to a silica column (60-120 mesh), elution with carbontetrachloride removed the tin residues, and subsequent elution with ethyl acetate and removal of the ethyl acetate gave a colourless oil. The oil obtained was further purified using flash-chromatography (silica 230,400 mesh, 3:7 ethyl acetate to petrol). Chromatography yielded a colourless oil (4mg, 25% yield). The thin layer chromatograph of the oil was found to be consistent with that of authentic unlabelled methyl 2-hydroxy-2-methyl-3-oxobutanoate.

Specific activity: 0.45μCi/mmol

8.6 Experimental to Chapter 6

Methyl 2-methyl-2,3-dihydroxybutanoate (49)

Methyl tiglate (3g, 26.3mmol) was dissolved in formic acid (98%, 7.2ml) and hydrogen peroxide (4ml, 30%) was added to the stirred reaction mixture. The reaction mixture was heated at 75°C for 13 hours. To the cooled solution was added toluene (50ml) and the reaction mixture was evaporated under reduced pressure. The residue was purified by flash chromatography (hexane 1:9 diethyl ether). The diol, Rf 0.21, was isolated as a colourless oil. (1.97g, 50% yield).

^{1}Hnmr ($CDCl_3$, Ref. TMS): δ 3.82 (3H,S,-OCH_3), 3.8 (1H,q,H-C(OH), 3.5 (1H,S,-OH), 2.35 (1H,S,-OH), 1.45 (3H,S,-CH_3), 1.25 (3H,d,J=7.2Hz, CH_3-C-H).

^{13}Cnmr ($CDCl_3$, Ref. TMS) : δ 175.8 (C=O), 75.7 (C-CO_2), 72.3 (C-H), 52.7 (CO_2CH_3), 22.2 (-CH_3), 17.7 (-CH_3).

I.R. (liquid film): 3500 (broad-OH), 1735 (C=O, ester)cm^{-1}

M.S. (ammonia C.I.): 149 $(MH)^+$, 166 $(MNH_4)^+$

Analysis calculated for $C_6H_{12}O_4$: C,48.65; H,8.11. Found: C,48.15; H,8.35.

Three synthetic routes to methyl α-acetolactate.

i) Methyl tiglate (1.0g, 8.8mmol) was dissolved in a solution consisting of water (10ml), acetone (50ml) and acetic acid (0.85ml). The solution was cooled to -10°C and a solution of potassium permanganate (3.03g) dissolved in acetone (50ml) and water (15ml) was added gradually, maintaining a reaction temperature below -5°C. The reaction mixture was stirred at -10°C for 1 hour and then the solution was filtered through celite. The filtrate was evaporated under reduced pressure in order to remove the acetone component, and then the aqueous residue was extracted with dichloromethane (3x40ml). The combined dichloromethane fractions were dried with magnesium sulphate and the solvent was removed under reduced pressure. The oil obtained was purified by distillation under reduced pressure. (b.p. 120-122°C/10mmHg, 0.89g, 69% yield).

^{1}Hnmr ($CDCl_3$, Ref. TMS): δ 4.3 (1H,S,-OH), 3.83 (3H,S,$CO_2\underline{Me}$), 2.31 (3H,S,$\underline{CH_3}C=O$), 1.62 (3H,S,$\underline{CH_3}$-C-OH).

^{13}Cnmr ($CDCl_3$, Ref. TMS): δ 204 ($CH_3\underline{C}=O$), 171.4 ($\underline{C}O_2Me$), 80.8 ($\underline{C}$-OH), 52.9 ($CO_2\underline{Me}$), 23.4 ($\underline{CH_3}C=O$), 21.4 ($\underline{CH_3}$-C).

I.R. (liquid film): 3500 (broad-OH), 1730 with shoulder 1750 (C=O) cm^{-1}.

M.S. (E.I.): 104 ($M-C_2H_2O$)$^+$

M.S. (C.I. ammonia): 147(MH)$^+$, 164 (MNH_4)$^+$.

Analysis calculated for $C_5H_8O_4$: C,49.31; H,6.85. Found: C,49.42; H,7.01.

ii) Methyl 2-methyl-2,3-dihydroxybutanoate (1.6g, 0.01 mol) was dissolved in benzene (60ml) and dibutyl tin oxide (2.68g) was added to the mixture and then the reaction mixture was heated at 80°C for 5

hours, in a Dean-Stark apparatus. The solvent was then removed by distillation, and traces of benzene were removed under reduced pressure. The solid material obtained was dissolved in dichloromethane (20ml) and bromine (0.5ml) was added to the reaction mixture. The reaction mixture was stirred for 15 hours and then the solvent was removed under reduced pressure. The residue was purified using a silica column, elution with carbontetrachloride removed the tin by-products, and subsequent elution with ethyl acetate and removal of the solvent under reduced pressure gave a colourless oil. The crude oil was purified by distillation under reduced pressure. (b.p. 119°C/10mmHg, 0.7g, 44% yield). Characterisation of the product was identical with that of methyl α-acetolactate produced by the permanganate oxidation of methyl tiglate.

iii) Methyl 2-methyl-2,3-dihydroxybutanoate (1.38g, 9.3mmol), sodium bromate (2.82g), disodium hydrogen orthophosphate (2g), ruthenium trichloride (15mg), Aliquat ^{R}336 (75mg), chloroform (50ml) and water (25ml) were combined and stirred in a sealed flask for 3 days. The reaction mixture was then poured into water (100ml) and the solution was extracted with dichloromethane (3x50ml). The combined dichloromethane fractions were dried with magnesium sulphate and the solvent was removed under reduced pressure. The crude oil was purified by preparative gas liquid chromatography (15% S.E.30 (10mm), 170°C, 150ml/min(N_2)). Two major components were isolated: methyl pyruvate and the desired product (600mg, 44% yield). Characterisation of the product was identical with that of methyl-α-acetolactate formed by permanganate oxidation of methyl tiglate.

Ethyl 2-methyl-3-hydroxy-4,4,4-trifluorobutanoate (58).

Concentrated sulphuric acid (75ml) was added to trifluoroacetaldehyde hydrate (37.62g, 0.32mol) and the reaction mixture was heated to 100°C. The trifluoroacetaldehyde gas produced was passed into a reaction vessel containing zinc powder (12g), diethyl ether (60ml) and benzene (60ml) maintained at -78°C. After the production of trifluoroacetaldehyde has ceased, the gas inlet was replaced with a dry-ice/acetone condenser and the reaction mixture allowed to warm to room temperature. Ethyl 2-bromopropionate (27.7g, 0.15mol) was then added dropwise to the reaction mixture and the reaction mixture was heated at 80°C for 1 hours. An additional quantity of zinc powder (3g) was added to the mixture and the reaction mixture was heated at 80°C for a further 3 hours. The cooled reaction mixture was poured onto ice (50g) and sulphuric acid (conc., 50ml). The solution was filtered and the aqueous fraction was separated and extracted with diethyl ether (100ml). The organic fractions were combined and washed with sodium bicarbonate solution and water. The organic fraction was then dried with magnesium sulphate and the solvent was removed under reduced pressure to yield an oil. The oil was purified by distillation under reduced pressure:

b.p. 49-52°C/10mmHg ethyl 2-bromopropionate.

b.p. 56-59°C/10mmHg desired product (8.5g, 28% yield).

^{1}Hnmr ($CDCl_3$, Ref. TMS): δ 4.58, 4.39 (1H,2xdq,J=3Hz,7Hz), 4.2, 4.15 (2H,2xq, J=7Hz), 2.9, 2.8 (1H,2xm), 1.35, 1.30 (3H,2xd,J=7Hz), 1.26, 1.25 (3H,2xt,J=7.1Hz).

G.L.C.: (3% S.E. 30, 120°C, N_2 flow 30ml/min): indicates diastereomeric mixture in the ratio 44.2% to 55.8%.

M.S.(C.I., ammonia): 201 $(MH)^+$, 218 $(MNH_4)^+$

M.S. (E.I.): 155 $(M-OEt)^+$, 127 $(M-OEt-CO)^+$

I.R. (liquid film): 3450 (broad-OH), 1725 (C=O, ester)cm^{-1}

E-Ethyl 2-methyl-4,4,4-trifluorobut-2-enoate (59).

Ethyl 2-methyl-2-hydroxy-4,4,4-trifluorobutanoate (2.8g, 14mmol) was added to phosphorus pentoxide (2g) and the reaction mixture was vigorously shaken. The dehydration product was then distilled from the reaction mixture:

b.p. 70-76°C/760mmHg, colourless oil, 1.07g, 42% yield.

^{1}Hnmr ($CDCl_3$, Ref. TMS): δ 6.68 (1H,qq,J_{HF}=8.3Hz,J_{HH}=1.6Hz, H-C=C), 4.25 (2H,q,J=7Hz, $-CH_2CH_3$), 2.09 (3H,dq, J_{HF}=2.4Hz, J_{HH}=2.4Hz, C=C-CH_3), 1.33 (3H,t,J=7.1Hz, $-CH_2CH_3$).

^{19}Fnmr (Bruker WH90, $CDCl_3$, Ref. $CFCl_3$): δ -59.8 (d,J_{HF}=8.3Hz, $-CF_3$).

I.R. (liquid film): 1810, 1740 (C=O, ester), 1680 (C=C, conjugated double bond)cm^{-1}.

M.S. (E.I.): 137 (M-OEt+)$^+$, 109 (M-OEt+-CO)$^+$, 69 (CF_3)$^+$.

Analysis: Calculated for $C_7H_9F_3O_2$: C,46.15; H,4.94. Found: C,46.22; H,4.87.

(2R,3R),(2S,3S) Ethyl 2-methyl-2,3-dihydroxy-4,4,4-trifluorobutanoate. (60).

E-Ethyl 2-methyl-4,4,4-trifluorobut-2-enoate (900mg, 4.9mmol) was dissolved in acetone (40ml) and water (10ml). Acetic acid (0.7ml) was added to the solution and the reaction mixture was cooled to -10°C. A solution of potassium permanganate (1.7g) dissolved in acetone (35ml) and water (13ml) was added gradually to the reaction mixture maintaining a temperature below -5°C. The reaction mixture was stirred at -10°C for one hour. The solution was then filtered through celite in order to remove manganese dioxide. The filtrate was evaporated under reduced pressure in order to remove the acetone component. The remaining aqueous fraction was extracted with dichloromethane (3x50ml). The dichloromethane extracts were combined and dried with magnesium sulphate, and the solvent was removed under reduced pressure. A white crystalline solid was obtained (522mg, 48% yield, recrystallised from acetone and heptane).

^{1}Hnmr ($CDCl_3$, Ref. TMS): δ 4.26 (2H,q,J=7.1Hz, $-\underline{CH_2}CH_3$), 4.16 (1H,q,$\underline{H}$-C-CF_3), 1.47 (3H,m,$-\underline{CH_3}$), 1.28 (3H,t,J=7.1Hz, $-CH_2\underline{CH_3}$).

^{13}Cnmr ($CDCl_3$, Ref. TMS): δ 174.1 (S,$\underline{C}$=O) 124 (q, J_{CF}=284Hz, $-\underline{C}F_3$), 74.9 (S,HO-$\underline{C}$-CH_3), 72.8 (q, $^2J_{CF}$=30.3Hz, $CF_3-\underline{C}$), 63 (S,O-$\underline{C}H_2CH_3$), 21.8 (S,$-\underline{C}H_3$), 13.7 (S,$-CH_2\underline{C}H_3$).

I.R. (liquid cell, 3% chloroform): 3450 (broad-OH), 1740 (C=O, ester)cm^{-1}.

M.S. (C.I., ammonia): (accurate mass): $(MH)^+$, $C_7H_{11}O_4F_3$/217.0698, $(MNH_4)^+$, $C_7H_{14}NO_4F_3$/234.0924.

Analysis: Calculated for $C_7H_{11}O_4F_3$: C,38.89; H,5.09. Found: C,39.12; H,5.12.

1-(2-thiazolyl)-1-tert-butyldimethylsiloxy-2,2,2-trifluoroethane (61).

Thiazole (200mg, 2.3mmol) was dissolved in diethyl ether (20ml) and the reaction mixture was cooled to -78°C and maintained under an argon atmosphere. Butyl lithium (2.4M, 0.94ml) was added to the reaction mixture. On addition of butyl lithium a yellow solution was formed. The reaction mixture was stirred at -78°C for one hour. In a separate reaction vessel trifluoroacetaldehyde hydrate (2.5g, 20mmol) was added to concentrated sulphuric acid (10ml) and the mixture was warmed to 45°C. The trifluoroacetaldehyde gas produced (b.p. -19°C) was condensed in a cold-finger maintained at -78°C. The cold-finger was gradually warmed and trifluoroacetaldehyde was distilled into the reaction mixture containing the thiazole carbanion. The reaction mixture was stirred at -78°C for one hour and then tetramethylethylenediamine (0.35ml) and tert-butyldimethysilylchloride (0.35g) were added to the mixture. The reaction mixture was allowed to warm to room temperature over a 15 hour period. The reaction mixture was then poured into diethyl ether (100ml) and the mixture was washed with water (3x50ml), the ethereal component was dried with magnesium sulphate and the solvent removed under reduced pressure. The crude oil obtained was purified by flash chromatography (diethyl ether 1:9 hexane) and preparative thin layer chromatography (dichloromethane 3:2 cyclohexane). A band Rf 0.28 was found to be the desired product (150mg, 25% yield).

^{1}Hnmr ($CDCl_3$, Ref. TMS): δ 7.85 (1H,d,J=2.4Hz, Harom.), 7.45 (1H,d,J=2.4Hz, Harom.), 5.4 (1H,q,J=6.0Hz, H-C-CF_3), 0.95 (9H,S,$SiCMe_3$), 0.16 (3H,S,SiMe), 0.06 (3H,S,SiMe).

^{19}Fnmr (Jeol FX90, $CDCl_3$, Ref. $CFCl_3$): δ -78.54 (d,J_{HF}=6.3Hz, $-C\underline{F}_3$).

M.S. (E.I.): 282 $(M-Me)^+$, 240 $(M-CMe_2)^+$, 144, 116.

M.S. (ammonia, +Ve C.I.): 298 $(MH)^+$.

1-(2-thiazolyl)-1-tert-butyldimethylsiloxy-2,2-difluoroethylene (62).

The trifluoroacetaldehyde-thiazole adduct (61), (105mg, 0.35mmol) was dissolved in tetrahydrofuran (5ml) and the reaction mixture was cooled to -78°C. Butyl lithium (1.0M, 0.19ml) was added dropwise to the cooled reaction mixture. The reaction mixture was stirred at -78°C for 30 minutes and then D_2O (0.5ml) was added to quench the reaction. The reaction mixture was allowed to warm to room temperature and the D_2O component was separated from the ethereal fraction. After the ethereal layer was dried with magnesium sulphate the solvent was removed under reduced pressure. The crude oil obtained was purified by preparative thin layer chromatography (dichloromethane 1:1 cyclohexane) and the major band was isolated (90mg, 92% yield).

^{1}Hnmr ($CDCl_3$, Ref. TMS): δ 7.4 (1H,d,J=6Hz, Harom.), 7.9 (1H,d,J=6Hz, Harom.), 1.0 (9H,S,$SiCMe_3$), 0.2 (6H,S,$SiMe_2$).

^{19}Fnmr ($CDCl_3$, Ref. $CFCl_3$, Bruker WH 90): δ -104.9 (d,J_{FF}=46Hz, (F)F-C=C), -95.7 (d,J_{FF}=46Hz,(F)F-C=C).

M.S. (E.I.): 258 (M-F)$^+$, 172.

8.7 Experimental to Chapter 7

^{1}Hnmr (220MHz) studies of acetohydroxyacid synthase (isoII)

Acetohydroxyacid synthase isoenzyme II (*Salmonella typhimurium*) was supplied by J.V. Schloss (E.I. DuPont de Nemours and Company, Wilmington). The enzyme was supplied as its light-sensitive FAD-enzyme complex (0.1ml, 149mg/ml) with a reported activity of 15 micromole of acetolactate formed/min/mg at 37°C. The enzyme was stored frozen, repeated freezing and thawing did not result in a loss of activity.

Four solutions were prepared containing varying proportions of pyruvate and α-ketobutyrate.

	Solutions (molarity)			
Compound	A	B	C	D
$MgCl_2$	60mM	60mM	60mM	60mM
TPP	5mM	5mM	5mM	5mM
FAD	5mM	5mM	5mM	5mM
Sodium acetate (Ref.)	60mM	60mM	60mM	60mM
sodium pyruvate	0.2M	0.1M	0.155M	0.17M
sodium α-ketobutyrate	-	0.1M	0.045M	0.03M
phosphate buffer (1M)	0.8ml	0.8ml	0.8ml	0.8ml
pH	7.2	7.2	7.2	7.2

The solutions were pre-incubated to 37°C and then acetohydroxyacid synthase (0.5 μL) was added to the solution contained in an nmr tube. The sample was then placed immediately in the nmr probe maintained at 37°C. ^{1}Hnmr spectra were then recorded at 3 minute intervals.

The following results were obtained:

Solution A (pyruvate), ^{1}Hnmr signals attributable to the formation of α-acetolactate were observed to grow in size with time, initial rate v=2 μmolmin^{-1}.

^{1}Hnmr, 220MHz, (H_2O, Ref. NaOAc, δ 1.82): δ 2.15 (3H,S), 1.35 (3H,S).

Solution B (pyruvate 1:1 α-ketobutyrate), signals due to the formation of α-acetohydroxybutyrate were observed to grow rapidly with time, initial rate v=3 μmolmin^{-1}.

^{1}Hnmr, 220MHz, (H_2O, Ref. NaOAc): δ 2.2 (3H,S), 1.82 (2H,m), 0.75 (3H,t).

Solution C (pyruvate 3.4:1 α-ketobutyrate), initially the rapid growth of signals attributable to the formation of α-acetohydroxybutyrate were observed (v=1.9 μmolmin^{-1}), signals attributable to α-acetolactate were only observed (v=0.95 μmolmin^{-1}) after complete reaction of the α-ketobutyrate.

Solution D (pyruvate 5.6:1 α-ketobutyrate), the growth of resonances attributable to the formation of α-acetohydroxybutyrate were observed initially (v=3 μmolmin^{-1}), and after complete reaction of α-ketobutyrate signals attributable to the formation of α-acetolactate were observed to steadily increase in size (v=1.5 μmolmin^{-1}).

^{1}Hnmr (400MHz) investigations of acetohydroxyacid synthase (isoII).

Four solutions were prepared each containing varying proportions of sodium pyruvate and α-ketobutyrate.

Compound	Solution Molarity A	B	C	D
$MgCl_2$	16mM	16mM	16mM	16mM
TPP	0.16mM	0.16mM	0.16mM	0.16mM
FAD	0.16mM	0.16mM	0.16mM	0.16mM
sodium acetate (Ref.)	16mM	16mM	16mM	16mM
sodium pyruvate	80mM	40mM	60mM	67.2mM
sodium α-ketobutyrate	-	40mM	20mM	12.8mM
Phosphate buffer (1M)	0.5ml	0.5ml	0.5ml	0.5ml
pH	7.2	7.2	7.2	7.2
D_2O	100μL	100μL	100μL	100μL

Each solution was pre-incubated to 37°C and in separate experiments acetohydroxyacid synthase was added to each solution contained in an nmr tube. ^{1}Hnmr spectra were then recorded sequentially. ^{1}Hnmr spectra were recorded using a Bruker 400MHz nmr instrument with the nmr probe maintained at 37°C. Spectra were recorded using water suppression and continuous data acquisition, typically 32 scans per spectrum. Spectra were recorded at 3 minute intervals.

The following results were obtained:

Solution A (pyruvate), ^{1}Hnmr signals attributable to the formation of α-acetolactate were observed to grow in size with time, initial rate $v=0.45\ \mu molmin^{-1}$.

^{1}Hnmr, 400MHz, (H_2O, Ref. NaOAc, δ 1.85): δ 2.25 (3H,S), 1.46 (3H,S).

Solution B (pyruvate 1:1 α-ketobutyrate), signals due to the rapid formation of α-acetohydroxybutyrate were observed to grow with time, initial rate 1.0 μmolmin^{-1}.

^{1}Hnmr, 400MHz, (H_2O, Ref. NaOAc): δ 2.25 (3H,S), 0.81 (3H,t).

Solution C (pyruvate 3:1 α-ketobutyrate), initially only the rapid growth of signals attributable to the formation of α-acetohydroxybutyrate were observed (v=1.1 μmolmin^{-1}), signals attributable to α-acetolactate were observed (V=0.4μmolmin^{-1}) after complete reaction of the α-ketobutyrate.

Solution D (pyruvate 5:1 α-ketobutyrate), the growth of resonances attributable to the formation of α-acetohydroxybutyrate were observed (v=1.1 μmolmin^{-1}), and after complete reaction of α-ketobutyrate the slower formation of signals attributable to α-acetolactate was observed (v=0.41 μmolmin^{-1}).

^{1}Hnmr (220MHz) studies of the inhibition of acetohydroxyacid synthase (isoII) by chlorsulphuron.

Four separate solutions were prepared each containing pyruvate (0.2M), magnesium chloride (0.15M), thiamine pyrophosphate (5mM), flavin adenonine dinucleotide (5mM), sodium acetate (64mM) and phosphate buffer (0.8ml, 1M, pH7.2). Each solution was prepared in an nmr tube and the samples were pre-incubated to 37°C. To each sample was then added a different concentration of chlorsulphuron, 0M, 0.1μM, 3.0 μM, and 30 μM. To each solution was then added acetohydroxyacid synthase isoenzyme II (0.5 μL) and ^{1}Hnmr spectra were recorded at three minute intervals. The rate of production of α-acetolactate characterised by the growth of peaks at δ 2.21 and 1.41 was investigated at the various concentrations of herbicide employed.

^{1}Hnmr (400MHz) investigations of acetohydroxyacid synthase isolated from pea plants.

Acetohydroxyacid synthase isolated, in a partially purified form, from pea plants was supplied by T. Hawkes (I.C.I. Agrochemicals, Jealotts Hill). The enzyme was supplied frozen (at -78°C) in tricine buffer (40mM, pH 8.0). The enzyme solution also contained EDTA (1mM), dithiothreitol (0.5mM), glycerol (10%) in order to stabilise the activity of the enzyme. The enzyme solution had a reported activity of 6-10nmol/min/ml of acetolactate formed at 37°C. Two solutions were prepared, one containing only pyruvate, the other an equimolar mixture of pyruvate and α-ketobutyrate.

Solution A: Sodium pyruvate (19.4mM), sodium acetate (6mM), TPP (1.6mM), FAD (1.6mM), 100 micro-litres D_2O, enzyme solution (0.42g).

Solution B: Sodium pyruvate (10mM), Sodium α-ketobutyrate (10mM), Sodium acetate (6mM), TPP (1.6mM), FAD (1.6mM), 100 micro-litres D_2O, enzyme solution (0.39g).

The solutions were pre-incubated to 37°C and then in separate experiments $MgCl_2$ (20mM) was added to the warmed solution, initiating the enzyme reaction. The solution contained in an nmr tube was rapidly placed in the nmr probe maintained at 37°C and nmr spectra were recorded at 3 minute intervals.

The following results were obtained:

Solution A: (pyruvate), signals due to the production of α-acetolactate could be observed at δ 2.37 and 1.57 (initial rate $V=2.7\times10^{-9}$ μmolmin^{-1}).

Solution B: (pyruvate and α-ketobutyrate), peaks due to the formation of α-acetohydroxybutyrate were observed at δ 2.37, 1.9 and 0.93 (initial rate $v=2.2\times10^{-8}$ μmolmin^{-1}).

REFERENCES

1 Krampitz L., *Arch. Biochem.*, 1948, 17, 81.

2 Umbarger H., *J. Am. Chem. Soc.*, 1957, 79, 2980.

3 Lewis K. and Weinhouse S., *J. Am. Chem. Soc.*, 1958, 80, 4913.

4 Weinhouse S., Strassman M. and Shatton J., *J. Biol. Chem.*, 1960, 235, 700.

5 Umbarger H., Brown B. and Eyring E., *J. Biol. Chem.*, 1960, 235, 1425.

6 Wagner R. and Armstrong F., *J. Biol. Chem.*, 1961, 236, 3252.

7 Radhakrishnan A. and Satyanarayana T., *Biochim. Biophys. Acta.*, 1962, 56, 197.

8 Umbarger H. and Leavitt R., *J. Biol. Chem.*, 1961, 236, 2486.

9 Wagner R., Bergquist A. and Forrest H., *J. Biol. Chem.*, 1959, 234, 99.

10 Magee P. and Szulmejster H., *Eur. J. Biochem.*, 1968, 3, 507.

11 Umbarger H. and Brown B., *J. Biol. Chem.*, 1958, 233, 1156.

12 Umbarger H. and Leavitt R., *J. Bacteriol.*, 1962, 83, 624.

13 Snell E. and Radhakrishnan A., *J. Biol. Chem.*, 1960, 235, 2316.

14 Freundlich M. and O'Neill J., *Biochem. Biophys. Res. Commun.*, 1972, 48, 437.

15 Umbarger H., Pledger W., *Biochem. Biophys. Res. Commun.*, 1972, 48, 444.

16 Felice M., Squires C. and Levinthal M., *Biochim. Biophys. Acta.*, 1978, 541, 9.

17 Felice M., *Recent Trends in Medical Genetics*, 1985, 195.

18 Felice M., *Anal. de Microbiol.*, 1982, 133A, 251.

19 Felice M., *Microbiol. Rev.*, 1979, 43, 42.

20 Grimminger H. and Umbarger H., *J. Bacteriol.*, 1979, 137, 846.

21 Koyang L. and Silverman P., *J. Bacteriol.*, 1984, 157, 184.

22 Schloss J., *Biochemistry*, 1985, 24, 4952.

23 Schloss J., personal communication. (E.I., du Pont).

34 Hawkes T., personal communication. (I.C.I., agrochemicals).

35 Davies M., *Plant Physiol.*, 1964, 39, 53.

36 Miflin B., *Arch. Biochem. Biophys.*, 1971, 146, 542.

37 Miflin B., *J. Exp. Bot.*, 1972, 23, 511.

38 Metzler D., *J. Biol. Chem.*, 1959, 234, 733.

39 Kohlhaw G. and Holzer H., *Biochem. Biophys. Res. Commun.*, 1961, 5, 452.

40 Schloss J. and Ciskanik L., *Biochemistry*, 1985, 24, 3357.

41 Abell L., O'Leary M. and Schloss J., *Biochemistry*, 1985, 24, 3357.

42 Schloss J., submitted for publication in *Methods in Enzymology*.

43 Bourgin J., *Mol. Gen. Genet.*, 1978, 161, 225.

44 Wallsgrove R., *Planta.*, 1986, 169, 46.

45 Wallsgrove R., *Plant Sci.*, 1986, 43, 109.

46 Wallsgrove R., *Plant Cell Rep.*, 1986, 5, 223.

47 Rank G., *Can. J. Genet. Cytol.*, 1986, 28, 852.

48 Savers R. and Levitt G., Pesticides through rational approach, *Am. Chem. Soc.*, 1984, 21.

49 Los M., Pesticides through rational approach, *Am. Chem. Soc.*, 1984, 29.

50 Ray T., *Pestic. Biochem. Physiol.*, 1982, 17, 10.

51 Ray T., *Pestic. Biochem. Physiol.*, 1982, 18, 262.

52 Shaner D. and Robson P., *Weed Sci.*, 1985, 33., 469.

53 Shaner D. and Reider M., *Pestic. Biochem. Physiol.*, 1986, 25, 248.

54 LaRossa R. and Schloss J., *J. Biol. Chem.*, 1984, 259, 8753.

55 Ray T., *Trends Biochem. Sci.*, 1986, 4, 180.

56 Ray T., *Plant Physiol.*, 1984, 75, 827.

57 Anderson P. and Hibberd K., *Weed Sci.*, 1985, 33, 479.

58 Shaner D., *Plant Physiol.*, 1984, 76, 545.

49 Ray T. and Chaleff R., *Science*, 1984, 223, 1148.

50 Ray T. and Chaleff R., *Science*, 1984, 224, 1443.

51 LaRossa R. and Smulski D., *J. Bacteriol.*, 1984, 160, 391.

52 Falco S. and Domas K., *Genetics*, 1985, 109, 21.

53 McDevitt R., Proc. Natl. Acad. Sci. U.S.A., 1986, 83, 4418.

54 Sweetser P., Schow G. and Hutchison J., *Pestic. Biochem. Physiol.*, 1982, 17, 18.

55 Sweetser P., Erbes D. and Takeda S., *Weed Res.*, Japan, 1986, 31, 157.

56 Sweetser P., Hutchison J. and Shapiro, *Pestic. Biochem. Physiol.*, 1984, 22, 243.

57 Walsh C., Enzymatic reaction mechanisms, W.H. Freeman and Co., 1979, 689.

58 Schloss J., Flavins and flavoproteins, Walter de Gruyter and Co., Berlin, 1984, 737.

59 Schloss J., unpublished data.

60 Grabau C. and Cronan J., *Nucleic Acids Res.*, 1986, 14, 5449.

61 Wek C., Hauser C. and Hatfield G., *Nucleic Acids Res.*, 1985, 13, 3995.

62 Friden P., Donegan J., Mullen J., Tsui P., Freundlich M., *Nucleic Acids Res.*, 1985, 13, 3979.

63 Lawther P., Calhoun D., Adams C., Hauser C., Gray J., Hatfield G., *Proc. Natl. Acad. Sci., U.S.A.*, 1981, 78, 992.

64 Squires C., Felice M. and Devereux J., *Nucleic Acids Res.*, 1983, 11, 5299.

65 Koland J., Miller J. and Gennis R., *Biochemistry*, 1984, 23, 445.

66 Kiuchi K. and Nager P., *Arch. Biochem. Biophys.*, 1984, 233, 776.

67 Carter K. and Gennis R., *J. Biol. Chem.*, 1985, 260, 10986.

68 Mazur B., *World Biotech. Rep.*, 1985, 2, 97.

69 Chaleff R., *Pure Appl. Chem.*, 1988, 60, 821.

70 Breslow R., *J. Am. Chem. Soc.*, 1985, 80, 3719.

71 Stetter H., Rämsch R. and Kuhlman H., *Synthesis*, 1976, 733.

72 Sheehan J. and Hara T., *J. Org. Chem.*, 1974, 39, 1196.

73 Breslow R. and Kool E., *Tetrahedron Lett.*, 1988, 1635.

74 Stetter H. and Kuhlman H., *Chem. Ber.*, 1976, 109, 2890.

75 Stetter H., Basse W. and Nienhaus J., *Chem. Ber.*, 1980, 113, 690.

76 Stetter H. and Kuhlmann H., *Tetrahedron Lett.*, 1974, 4505.

77 Metzger J., Heterocyclic Compounds, *Interscience.*, 1979, 14, 119.

78 White F. and Ingraham L., *J. Am. Chem. Soc.*, 1962, 85, 3109.

79 Jordan F., Kudzin Z. and Rios C., *J. Am. Chem. Soc.*, 1987, 109, 4415.

80 Lienhard G., Crossby J. and Stone R., *J. Am. Chem. Soc.*, 1970, 92, 2891.

81 Chikashita H., Ikegami S., Okumura T. and Itoh K., *Synthesis*, 1986, 375.

82 Corey E. and Venkateswarlu, *J. Am. Chem. Soc.*, 1972, 94, 6190.

83 Godtfredsen S. and Ottesen M., *Carlesberg Res. Commun.*, 1982, 47, 92.

84 Hamilton G. and Westheimer F., *J. Am. Chem. Soc.*, 1959, 81, 6332.

85 Fridovich I. and Westheimer F., *J. Am. Chem. Soc.*, 1962, 84, 3208.

86 Warren S., Zerner B. and Westheimer F., *Biochemistry*, 1966, 5, 817.

87 Juni E., *J. Biol. Chem.*, 1952, 195, 715.

88 Crout D., Littlechild J., Mitchell B. and Morrey S., *J. Chem. Soc. Perkin Trans.* 1, 1984, 2271.

89 Crout D. and Rathbone D., *J. Chem. Soc. Chem. Commun.*, 1988, 98.

90 Hill R. and Sawada S., *Bioorg. Chem.*, 1979, 8, 175.

91 Crout D. and Hedgecock J., *J. Chem. Soc. Perkin Trans.1*, 1979, 1982.

92 Wadsworth W. and Emmons W., *J. Am. Chem. Soc.*, 1961, 83, 1733.

93 Coleman J., Ricciuti C. and Swern D., *J. Am. Chem. Soc.*, 1956, 78, 5432.

94 Srinivasan N. and Lee D., *Synthesis*, 1979, 520.

95 Crout D. *et al.*, *Eur. J. Biochem.*, 1980, 110, 439.

96 Rodé-Gowal H. and Dahn H., *Helv. Chim. Acta.*, 1973, 56, 2070.

97 Crout D. *et al.*, *Eur. J. Biochem.*, 1980, 106, 97.

98 Alder R., *Tetrahedron Lett.*, 1971, 193.

99 Cornforth J., *et al.*, *Eur. J. Biochem.*, 1970, 14, 1.

100 Clifford K. and Phillips G., *J. Chem. Soc. Chem. Commun.*, 1975, 419.

101 Ghosh R., McOmie J. and Wilson J., *J. Am. Chem. Soc.*, 1945, 67, 705.

102 Miller R. and Smith B., *Synth. Commun.*, 1973, 3, 359.

103 Stalick W., Khorrami A. and Hatton K., *J. Org. Chem.*, 1986, 51, 3577.

104 Winstein, Pressman and Young, *J. Am. Chem. Soc.*, 1939, 61, 1645.

105 Kimura and Regen, *J. Org. Chem.*, 1983, 48, 195.

106 March J., Advanced Organic Chemistry, Wiley-Interscience, 1985, 624.

107 Dauben and McCoy, *J. Am. Chem. Soc.*, 1959, 81, 4863.

108 Crout D. and Corkill J., *Tetrahedron Lett.*, 1977, 4357.

109 Hofmann H. and Rabe J., *Angew. Chem. Int. Ed. Engl.*, 1983, 22, 795.

110 Hoffman H. and Rabe J., *J. Org. Chem.*, 1985, 50, 1849.

111 Drewes S. and Emslie N., *J. Chem. Soc. Perkin Trans.1.*, 1982, 2079.

112 Kishi Y., Aratani M., Tanino H., Fukuyama T. and Goto T., *J. Chem. Soc. Chem. Commun.*, 1972, 64.

113 David S. and Thieffry A., *J. Chem. Soc. Chem. Commun.*, 1979, 1568.

114 Swern D., *Org. React.*, 1953, 7, 378.

115 Suzuki H., Moro-oka Y. and Yamamoto Y., *Tetrahedron Lett.*, 1985, 26, 2107.

116 Crout D. and Rathbone D., *Synthesis*, 1989, 40.

117 Hooper D., Loucks L. and Liu H., *J. Chem. Educ.*, 1975, 52, 131.

118 McBee, Pierce O. and Smith D., *J. Am. Chem. Soc.*, 1954, 73, 3723.

119 Westerfeld W., *J. Biol. Chem.*, 1945, 161, 495.

120 Chipman D. and Barak Z., *J. Bacteriol.*, 1987. 169, 3750.

TITLE INVESTIGATIONS RELATED TO BRANCHED-CHAIN AMINO ACID METABOLISM

AUTHOR EDWARD ROBERT LEE

INSTITUTION and DATE UNIVERSITY OF WA. - CK 1987