TITLE SYNTHETIC AND ENZYMATIC STUDIES RELATED TO THE BIOSYNTHESIS OF PENICILLIC ACID AND ACETOIN

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by

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CONTENTS

LIST OF FIGURES

63 ^{19}F NMR (84.67 MHz) spectra of enantiomeric mixture of the ester (S)-(-)-α-(trifluoromethyl)phenyl acetate of acetoin.

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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 1984 and August 1989 and has not been submitted previously for a degree at any institution.

SUMMARY

The stereochemistry of the loss of one enantiotopic C-2 hydrogen atom of malonyl-coenzyme A units during the transformations leading to penicillic acid was undertaken. Aspartic acid stereospecifically deuterated at C-3 *via* the formation of N-benzyl aspartic acid was attempted. The addition of benzylamine in ^2H_2O or dioxan across the double bond of maleic or fumaric acids was revealed to be non stereospecific and these results have been tentatively explained. (2S,3R)-[3^2H_1]- and (2S,3S)-[2,3-$^2H_2$]-aspartic acids were derivatized to the corresponding deuterated diethyl N-p-toluenesulfonyl aspartates for vibrational infra red circular dichroism measurements. This technique enabled the determination of the absolute configuration at C-2 of [2^2H_2]-aspartic acids. These stereospecifically deuterated aspartic acids were used for biological tracer experiments but no significant deuteration of penicillic acid was achieved. Malate was the next possible precursor to be used. The preparation of stereospecifically deuterated L-malic acid was undertaken *via* the reduction of cis- and trans-2,3-epoxysuccinate derivatives which had to be prepared enantiomerically pure. New chemical routes were devised for the preparation of diethyl trans- and cis-2,3-epoxysuccinate in high yield. The key intermediates were diethyl 2-tosyl tartrate for the synthesis of cis- and trans- isomers and its tert-butyldimethylsilyl ether for the preparation of diethyl cis-epoxysuccinate. The enzymatic kinetic resolution of diethyl cis- and trans-2,3-epoxysuccinates were attempted by ester hydrolysis but proved to be difficult due to their decomposition in aqueous medium. The transesterification of diethyl trans-2,3-epoxysuccinate with 1-heptanol was catalyzed by lipases. Diheptyl (2S,3S)- and diethyl (2R,3R)-epoxysuccinates were obtained with an ee ≥ 97%. Diethyl cis-2,3-epoxysuccinate did not undergo transesterification with any of the enzymes tested. Nevertheless, ethyl isobutyl cis-(2,3)-epoxysuccinate was prepared indirectly in enantiomerically pure form. An enzymatic method involved a regioselective and/or stereoselective hydrolysis of racemic or enantiomerically pure diethyl 2-tosyl-tartrate by α-chymotrypsin. The resulting epoxide had an ee of ≥ 97%. Also, a chemical regioselective transesterification of enantiomerically pure diethyl 2-tosyl tartrate with isobutyl alcohol was achieved, leading to epoxides with ee values ranging from 71% to 94%. Thus, it was undoubtedly possible to obtain from these enantiomerically pure epoxides L-malic acid stereospecifically deuterated at C-3.

In an attempt to understand the mode of action of pyruvate decarboxylase, an analytical method for estimating the enantiomeric composition of enzymatically produced samples of acetoin was required. Formation of the (S)-(-)-α-methoxy-α-(trifluoromethyl) phenyl acetate of acetoin was achieved using different coupling agents. The diastereomeric ratio could then be determined by HPLC, ^1H and ^{19}F NMR, but racemization was found to accompany ester formation.

ABBREVIATIONS

Me	Methyl
Et	Ethyl
iBu	isobutyl
Hept	Heptyl
Ar	Aryl
Tos	Toluene-4-sulfonyl
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCC	Dicyclohexylcarbodiimide
TFAE	Trifluoro-1-(9-anthryl)-ethanol
HOBT	1-Hydroxybenzotriazole hydrate
THF	Tetrahydrofuran
TPP	Thiamine pyrophosphate
NADPH	Dihydronicotinamide adenine dinucleotide phosphate
FMN	Flavine mononucleotide
CoA	Coenzyme A
NMR	Nuclear magnetic resonance
s	Singlet
d	Doublet
t	Triplet
q	Quartet
m	multiplet
br	Broad
J	Coupling constant
ppm	Parts per million
TMS	Tetramethylsilane

IR	Infra-red
sh	Shoulder
s	Strong
MS	Mass spectrum
EI	Electron ionisation
CI	Chemical ionisation
M	Parent molecular ion
VCD	Vibrational circular dichroism
[α]	Specific rotation
c	Concentration (g/100 ml)
ee	Enantiomeric excess
E	Enantiomeric ratio
GLPC	Gas liquid phase chromatography
R_T	Retention time
HPLC	High pressure liquid chromatography
TLC	Thin-layer chromatography
mp	Melting point
bp	Boiling point
DPM	Disintegrations per minutes
LF-AP 15	Lipase from Rhizopus javanicus
OCL	Lipase from Candida cylindracea
PPL	Pig pancreatic lipase
LP	Lipase from Pseudomonas fluorescens
LAP 6	Lipase from Aspergillus niger
PLE	Pig liver esterase
eu	Enzyme unit

CHAPTER 1

INTRODUCTION

1.1 ENZYMES : A VALUABLE TOOL FOR CHEMISTS

Until quite recently, interest in biological catalysts has been directed mainly towards the elucidation of the stereochemical and mechanistic nature of the chemical reactions they catalyse[1]. However, a new field of research designated "BIOTRANSFORMATIONS" has emerged strongly. It refers to the application of enzymes as chiral catalysts in organic synthesis[2]. Despite chemists' initial reluctance to acknowledge the biological system, they have recognised its inestimable value for asymmetric synthesis and the appearance of numerous articles in the literature has supported this fact[2,3,4]. Indeed, enzymes have remarkable properties which deserve to be mentioned, albeit briefly, in order that their potential may be appreciated.

1. Reaction specificity

Enzymes are extremely versatile and although each enzyme is generally specialized in one type of reaction, enzymes can be found to carry out the whole spectrum of organic reactions. This reaction specificity is the basis of the International Union of Biochemistry Classification[5] which is as follows:

Group 1 : Oxidoreductases

Group 2 : Transferases

Group 3 : Hydrolases

Group 4 : Lyases

Group 5 : Isomerases

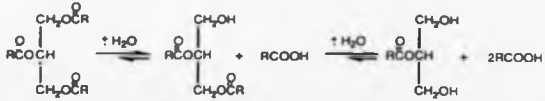
Group 6 : Ligases

The major sources of enzymes being principally mammalian and microbial, it is not surprising that enzymatic reactions take place generally in aqueous solution at neutral pH and close to room temperature. Despite these mild conditions, which overcome problems such as isomerization, racemization, epimerization, etc., which occur by the use of many organic reagents, enzymes can achieve amazing rate enhancements (up to 10^{12}) compared with corresponding non-enzymatic reactions.

Despite their reaction specificity, enzymes are valuable catalysts for asymmetric synthesis because of their unusual selectivity concerning the structure and, most importantly, the stereochemistry of the substance used and therefore of the product formed.

2. Constitutional (or structural) specificity

Dimensions, shape and structure of the active site of each enzyme predetermine the type of structural range of substrate that it can accept. As a direct consequence, enzymes may be regiospecific to a certain extent, a quality which has been widely exploited in the past to achieve selective reactions[6]. The hydrolysis of acylglycerol substrates by lipases which exhibit a 1,3-regiospecificity provide a good example of this kind[7] (Scheme 1:1).



1,2(2,3) diacylglycerol

R:Hydrocarbon chain of the fatty acid moiety

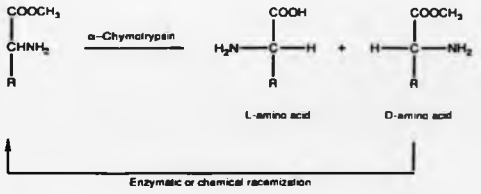
(Scheme1:1)

3. Stereospecificity

However, the most striking feature of enzymes for asymmetric synthesis comes from their generally high stereospecific control, attributed to the differences in the interactions of different stereoisomers with the chiral active sites of enzymes. Production of optically pure compounds may result from their catalysed specific reaction on racemates, prochiral substances and meso compounds as briefly reviewed below.

Enantiomeric differentiation

The use of hydrolytic enzymes in kinetic resolution of racemates has been the main application. The maximum theoretical yield of the desired enantiomer is 50%. Therefore, it is advantageous[3] to recycle the non hydrolyzed substrate after a racemization process (Scheme 1:2).



(Scheme1:2)

Prochiral distinctions

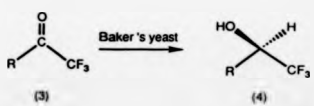
In this case, production of optically pure compounds from prochiral substrates is achieved with a theoretical yield of 100%. Thus prochiral stereospecificity is applicable to stereoheterotopic and homomorphic atoms or groups as well as stereoheterotopic faces of planar molecules. Two examples have been selected to illustrate these points.

The enantiotopic homomorphic group in the prochiral glycerol derivative (1) has been resolved successfully by the use of lipoprotein lipase[8]. The (R)-enantiomer (2) has been obtained in 91% ee (scheme 1:3).

(1) (2)

(Scheme1:3)

Baker's yeast[9] is able to reduce cyclohexyltrifluoromethylketone (3). The enantiomerically pure (R)-cyclohexyl-trifluoroethanol (4) with ee > 99% has been produced as the result of this enzymatic differentiation between the enantioheterotopic faces of the ketone (3) (Scheme 1:4).



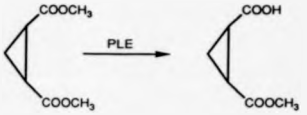
(3) (4)

R:Cyclohexyl

(Scheme1:4)

4. Stereospecific transformations of meso compounds

One of the most noteworthy stereospecificities of enzymes is their ability to distinguish between centres of opposite configuration in *meso* compounds. Consequently, production of enantiomerically pure compounds are possible again with yields of up to 100%. An example is provided by the hydrolysis of dimethyl *meso*-cyclopropanedioate (5), using pig liver esterase (PLE) as the biocatalyst[10], which led to the monoester (6) with a 97% ee (Scheme 1:5).



(5) (6)

(Scheme 1:5)

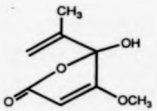
This quick glance at the properties of enzymes highlights the extent of their potential as chiral catalysts. Moreover, the collaborating work between biochemists, chemists and biologists has so far overcome most of the perceived limitations of their utility in organic synthesis[11,12]. For example, the major discovery that enzymes can work in low water systems has extended[13,14] their substrate acceptibility. Compounds, soluble only in organic solvents, become potential substrates. The catalytic repertoire of enzymes has been expanded : esterification, transesterification and interesterification reactions are possible. Also, their fundamental properties may be altered e.g. substrate specificity and thermostability.

In conclusion, the field of biotransformations is assuming enormous proportions owing to the different facets of enzymatic catalytic power. The research reported in this thesis has exploited some of these aspects and outlines the advantages of using enzymes as chiral catalysts in asymmetric synthesis compared with chemical catalysts.

1.2 AIM

This thesis deals with the investigations undertaken in an attempt to elucidate the stereochemical course of two unrelated enzymatic reactions.

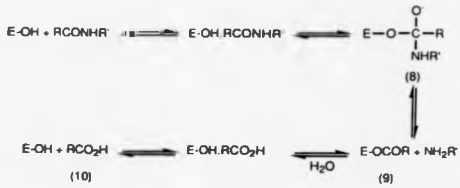
Chapters 2, 3 and 4 report the strategies used to explore the mechanism of the intermediate steps in the formation of penicillic acid (7), a secondary metabolite produced by a strain of Penicillium cyclopium.



(7)

Production of stereospecifically labelled precursors was one of the requirements for carrying out this study. This was accomplished by the use of organic synthesis and/or biotransformation reactions. Pig liver esterase (PLE), α-chymotrypsin and lipases from different origins, which are all members of the hydrolase group, have been used in these biotransformations. They present the advantages of being commercially available, stable, and inexpensive. Further, they do not require a cofactor to be catalytically active and they act upon a broad range of substrates while retaining their stereospecificity. Enigmas concerning the mechanism of action of lipases and esterases still remain partly because of the problems encountered when attempting to obtain homogeneous enzyme preparations[15]. However, a mechanism comparable to the one

established for the serine protease α-chymotrypsin[1,4] seems the most probable. Briefly, it involves a nucleophilic attack on the substrate by the hydroxyl group of a serine residue located at the active site of the enzyme. The result is the formation of a tetrahedral intermediate (8) which then collapses to the acyl enzyme (9). Following the hydrolysis of (9), the product (10) is obtained (Scheme 1:6).



(Scheme1:6)

Chapters 5 and 6 deal with the research of an analytical technique to evaluate the enantiomeric ratio of a chiral molecule, acetoin (11). The enzyme pyruvate decarboxylase from yeast is unusual in that it produces acetoin as a mixture of enantiomers rather than as an optically pure substance.

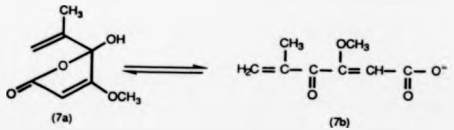
(11)

CHAPTER 2

BIOSYNTHETIC STUDIES RELATED TO THE TETRAKETIDE PENICILLIC ACID

2.1 INTRODUCTION

Penicillic acid (7), a secondary metabolite produced by different moulds such as Penicillium and Aspergillus species, which are contaminants of foods[16,17], exhibits antibiotic acitvity[18] but also has proved to be toxic to mammals, carcinogenic to rats and is consequently classified as a mycotoxin[19]. At neutral pH it exists in the pseudo acid form (7a) and at alkaline pH in an open structure (7b)[20,21] as indicated in scheme (2:1)



(7a) (7b)

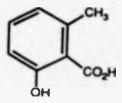
(Scheme 2:1)

Penicillic acid is a typical polyketide, more specifically a tetraketide. Polyketides are found mostly in plants[22] and microorganisms[23] and can be defined mainly as natural secondary metabolites of vast diversity e.g. : tetracyclines, macrolides, but which are

related to each other by an overall similarity in biosynthetic origin. Principally they are all formed from C_2 units derived from acetate involving condensation reactions[24].

In fungal systems such as Penicillium, the biosynthesis of acetate-derived phenols[25] is catalyzed by multienzyme complexes. The reaction involves condensation of (generally) acetyl-coenzyme A as the "starter" unit, with the appropriate number (n) of malonyl-coenzyme A units to form a poly-β-ketomethylene chain, $-[CH_2CO]_n-$. This ketomethylene system may subsequently undergo varying degrees of transformations to a specific phenol.

Some analogy has been established between the formation of a typical phenol polyketide, 6-methylsalicylic acid (12) produced by Penicillium patulum and the formation of fatty acids in yeasts[22,23,24,25]. Fatty acids, principally primary metabolites, are considered to be a sub-category of polyketides despite major differences in their biosynthethic pathways.



(12)

2.2 ORIGIN OF ACETYL- AND MALONYL-COENZYME A

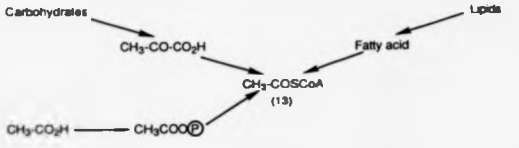
Secondary metabolism has been suggested to be a regulatory system whereby microorganisms "eliminate" from their metabolic pool the excess of acyl-coenzyme A species e.g. acetyl-coenzyme A in fungi. The accumulation of "acyl-coenzyme A" units occurs when protein synthesis ceases as the result of unfavourable conditions of growth possibly due to a depletion of nitrogen in the culture medium.

The main sources of acetyl-coenzyme A (13) are : (Scheme 2.2)

- the metabolism of carbohydrates to pyruvic acid, followed by the action of pyruvate dehydrogenase, an enzymatic complex containing thiamine pyrophosphate and lipoic acid as coenzymes, to produce acetyl-coenzyme A.

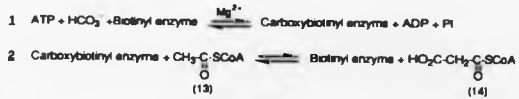
- the catabolism of fatty acids by the β-oxidation pathway.

- the ATP-dependent activation of exogenous acetate by acetyl-coenzyme A synthetase. Consequently, acetate labelled with ^{13}C, ^{14}C and ^{3}H has been used successfully for biosynthetic studies[26]. However, it must be kept in mind that its eventual entry into the tricarboxylic acid cycle causes randomization and dilution of the label.



(Scheme 2:2)

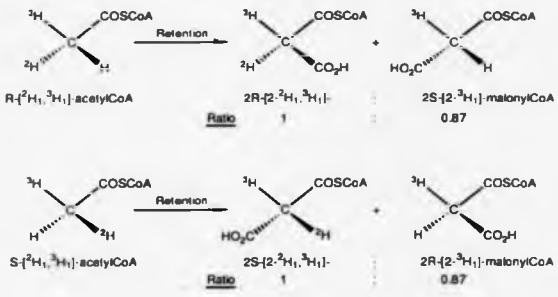
On the other hand, malonyl-coenzyme A (14) originates from acetyl-coenzyme A (13) by carboxylation with a CO_2-biotinyl enzyme : acetyl-coenzyme A carboxylase. The reaction involves the formation of a carboxy-biotinyl enzyme complex, followed by transfer of CO_2 from this complex to acetyl coenzyme A (Scheme 2:3).



(Scheme 2:3)

Cornforth and Sedgwick[27,28] have investigated, by the use of chiral acetate, the stereospecificity of the acetyl-coenzyme A carboxylase purified from chicken liver. As the result of their experiments, they have concluded that carboxylation of acetyl-coenzyme A occurs with retention of configuration at C-2, but the small

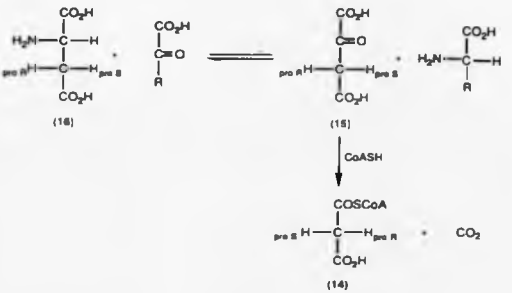
intramolecular isotope effect led to almost equal populations of S and R tritiated malonyl-coenzyme A, starting from R-[$^2H_1$, $^3H_1$]- or S-[$^2H_1$, $^3H_1$]-acetyl coenzyme A (Scheme 2:4).



(Scheme 2:4)

An alternative path to malonyl-coenzyme A is via oxaloacetate (15), an intermediate in the tricarboxylic acid cycle. Subsequently, aspartic acid (16) labelled with tritium at C_3 was used as a precursor for biosynthetic studies[29]. The production of malonyl-coenzyme A (14) from aspartic acid (16) involves a transamination reaction followed by oxidative decarboxylation (Scheme 2:5).

The main advantage of using aspartic acid is that the methylene protons are not readily exchangeable in aqueous media, prior to uptake by the microorganism, as in the case of malonic acid[28,37].



(Scheme 2:5)

2.3 YEAST FATTY ACID SYNTHETASE

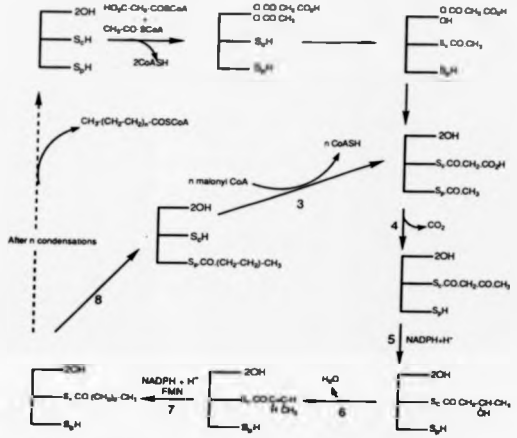
Lynen et al[25] were the first to show that baker's yeast fatty acid synthetase is a tightly bound trimeric multienzyme complex. It is able to produce palmitoyl-coenzyme A ($C_{16:0}$) and stearoyl-coenzyme A($C_{18:0}$) in presence of acetyl-coenzyme A as a "starter" unit, malonyl-coenzyme A, NADPH and FMN, without release of intermediates during the course of the synthesis.

The mechanism of this synthesis, as shown in Scheme 2:6, involves the acceptance of acetyl-coenzyme A on the multienzyme complex by an acetyl transferase through an ester bond with the hydroxyl function of a serine residue. The acetyl moiety is then successively transferred to "central" and "peripheral" thiol groups. The "central" thiol group has been identified as 4'-phosphopantetheine linked to a specific protein and the "peripheral" thiol site as belonging to a cysteine residue of the condensing enzyme. Malonyl-coenzyme A follows the same fate as acetyl-coenzyme A by the action of a malonyl-coenzyme A transferase, but becomes attached to the "central" thiol group. At this stage, a condensation occurs between the two acyl residues with concomittant release of CO_2. The resulting acetoacetyl group bound to the "central" thiol group is reduced and dehydrated leading to the trans unsaturated product and finally reduced to give a butyryl moiety which is transferred to the "peripheral" site.

Steps 3 to 8 are repeated until formation of a palmitoyl- or stearoyl-group fixed to the "central" thiol site, which is then removed from the multienzyme complex as palmitoyl- or stearoyl-coenzyme A.

The stereochemical course followed during the biosynthesis of fatty acid by the synthetase extracted from baker's yeast and chicken liver has been investigated by Cornforth et al[27,28]. Using chiral acetate[27], the proportion of tritium retained in palmitic acid was slightly higher when using S-(2-^{14}C,^{2}H$_1$,^{3}H$_1$)-acetyl-coenzyme A than using R-(2-^{14}C,^{2}H$_1$,^{3}H$_1$)-acetyl-coenzyme A . This result has been confirmed by the use of stereospecifically tritiated malonyl thiolesters[28] which gave a much higher discrimination in the retention of tritium from 2S-[U-^{14}C, 2-^{3}H$_1$]-malonyl thiol ester than from 2R-[U-^{14}C, 2-^{3}H$_1$]-malonyl thiol ester in palmitic acid produced by yeast synthetase.

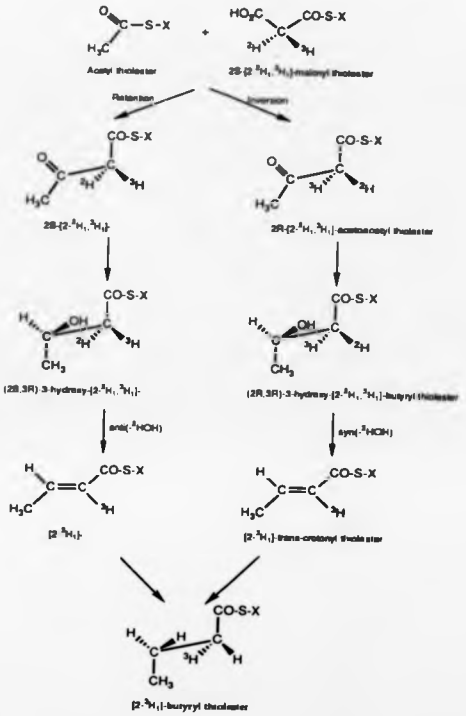
According to the results of these two experiments and taking into account the intramolecular isotope effect and partial exchange of isotope occuring with the synthetase after the formation of malonyl-coenzyme A, it was concluded that the dehydration step involved the removal of pro-R hydrogen atoms and reduced to two the stereochemical possibilities for the synthetase (Scheme 2:7).



-S_1H:4'-phosphopantetheine
-S_2H group of condensing enzyme
-2OH:malonyl- and acetyl- transferases

Synthesis of fatty acids by yeast synthetase

(Schema 2:6)


Acetyl thioester
2S-[2-$^2H_1$, $^3H_1$]-malonyl thioester
Retention
Inversion
2S-[2-$^2H_1$, $^3H_1$]-
2R-[2-$^2H_1$, $^3H_1$]- acetoacetyl thioester
(2S,3R)-3-hydroxy-[2-$^2H_1$, $^3H_1$]-
(2R,3R)-3-hydroxy-[2-$^2H_1$, $^3H_1$]-butyryl thioester
anti(-^{2}HOH)
syn(-^{2}HOH)
[2-$^2H_1$]-
[2-$^2H_1$]-trans-crotonyl thioester
[2-$^2H_1$, $^3H_1$]-butyryl thioester

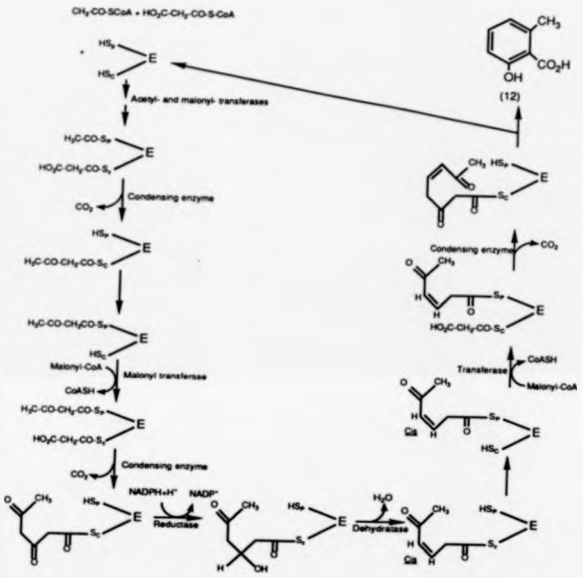
(Schema 2:7)

2.4 6-METHYL SALICYLATE SYNTHETASE

This enzymatic complex is the only one which has been extensively purified from Penicillium patulum[23,25], and the studies carried out on it have provided a model for the biosynthesis of all the aromatic polyketides. Evidence has shown that as with the fatty acid synthetase, the enzyme contains acyltransferases which bring the acyl groups successively to two thiol groups, one at the centre of the enzyme -S_CH identified as 4'-phosphopantetheine and the other at the periphery of the enzyme -SpH. This complex is able to produce 6-methylsalicylic acid (12) from acetyl-coenzyme A, malonyl-coenzyme A and NADPH by a series of reactions which do not involve the release of any intermediate (Scheme 2:8).

One of the main differences to the fatty acid synthetase resides in the absence of reduction after each condensation of malonyl units. The single reduction step seems to occur at the C_6-stage, followed by dehydration to give a 5-keto-3-cis-hexenoyl derivative. This unsaturation is retained until completion of the process and is thought, together with other factors such as chelation, to confer on the poly-β-ketomethylene chain the conformation needed for the formation of 6-methylsalicylic acid[30].

6-Methylsalicylate synthetase has been separated from the fatty acid synthetase during the course of purification and has been shown to have half the molecular weight of the latter[31]. In addition, this enzyme has been proved to be a constitutive enzyme of the microorganism concerned, but is not expressed during favourable conditions of growth. Consequently some authors[22] have advanced the idea that the 6-methylsalicylate synthetase may be derived by a modification of fatty acid synthetase, modifications of which occur as a consequence of metabolic changes.

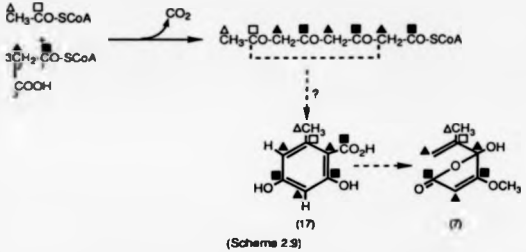

CH3-CO-SCoA + HO2C-CH2-CO-S-CoA
Acetyl- and malonyl- transferases
Condensing enzyme
Malonyl-CoA
CoASH
Malonyl transferase
Condensing enzyme
NADPH+H+
NADP+
Reductase
Dehydratase
H2O
Transferase
CoASH
Malonyl-CoA
Condensing enzyme
CO2
(12)

(Scheme 2:2)

2.5 SYNTHESIS OF PENICILLIC ACID

2.5.1 Formation of orsellinic acid

Investigations using ^{14}C and ^{18}O labelled acetate[32,33,34] and ^{14}C malonate[33] in whole cell feeding experiments, supported by ^{3}H NMR[35,36] and ^{13}C NMR[37] studies, have led to the conclusion that orsellinic acid (17) is synthesized by the condensation of one acetyl-coenzyme A as the starter unit with chain extension by three malonyl-coenzyme A. The resulting C_8 polyacetate chain cyclized to orsellinic acid by aldol condensation without undergoing reduction or any other modification (Scheme 2.9). Few studies have been undertaken at the enzymatic level owing mainly to the instability of orsellinic acid synthase, which has only been partially purified[40]. Furthermore, labelled ^{14}C orsellinic acid[34,39] has been used in feeding experiments with Penicillium species and consequently been identified as a key intermediate in the biosynthesis of penicillic acid (Scheme 2.9).



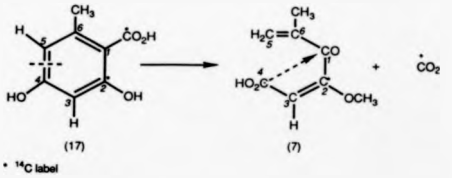
(Scheme 2.9)

As seen in Scheme 2.9, the formation of orsellinic acid (17) results in the loss of one of the enantiotopic C-2 hydrogen atoms of malonyl-coenzyme A which might be, as Packter[25] speculated, the result of enolisation of the keto group to confer the needed conformational rigidity on the polyacetate chain bound to the enzyme, to lead to orsellinic acid. Nevertheless, the stereochemistry of this hydrogen loss has been the object of one study involving the use of doubly labelled aspartate in the

production of penicillic acid in Penicillium cyclopium[29]. The retention of tritium was higher when using (2S, 3S)-[3-^{3}H, 3-^{14}C]-aspartic acid than with (2S, 3R)-(3-^{3}H, 3^{14}C)-aspartic acid. Consequently, it has been suggested that the same stereochemical course might be followed by each C-2 hydrogen atom of malonyl thiol ester leading to the preferential retention of the pro-R hydrogen (pro-S hydrogen in aspartic acid), a result which differs from the preferential retention of the pro-S hydrogen atom of the malonyl thiol ester in the dehydration step of fatty acid.

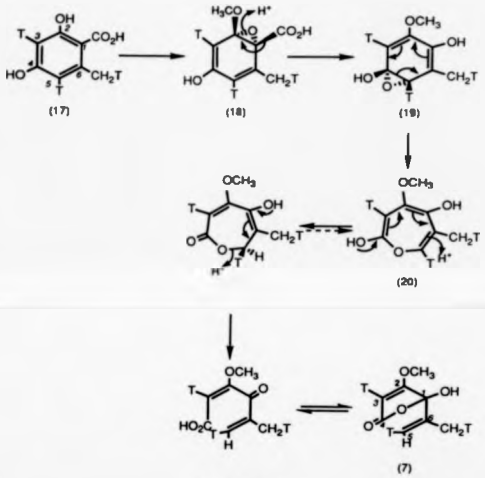
2.5.2 Biotransformation of orsellinic acid into penicillic acid

The subsequent transformations of orsellinic acid (17) into penicillic acid (7) have received much attention. It has been suggested that orsellinic acid was converted by oxidative cleavage into penicillic acid. Consequently, one of the first concerns was to establish the site of the ring scission of orsellinic acid. The answer has been given by Mosbach[39], who suggested a scission between C_4 and C_5 on the basis of feeding experiment with 2- and carboxy-^{14}C orsellinic acid (Scheme 2:10). This result was later unambiguously confirmed by Seto[37] using NMR ^{13}C-^{13}C coupling as well as by Thomas et al[35,36] using ^{3}H NMR.



(Scheme 2:10)

From feeding experiments using [^{3}H] acetate as a precursor and ^{3}H NMR as a tool for analysis, Thomas et al[35,36] have proposed a mechanism for the formation of penicillic acid. It involves a methylation of the hydroxyl group at C-2 of orsellinic acid (17) followed by a decarboxylation of the hypothetical intermediate epoxide (18), which leads to the arene oxide (19). This latter is then transformed to the oxepin (20) which consecutively tautomerized, ring opened and lactonized to give penicillic acid (Scheme 2:11).

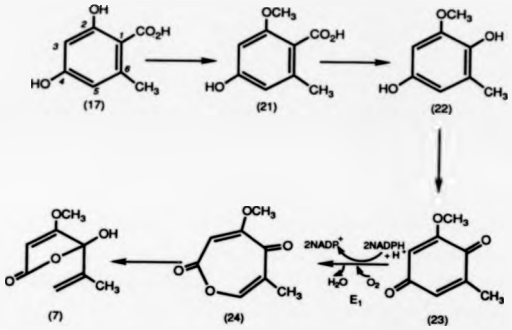


(Scheme 2:11)

Furthermore, the tritium at the C-5 methylene group was located mainly in the position trans to the C-4 methyl group, which certainly reflects the stereospecificity of

the enzyme involved.

However, Axberg and Gatenbeck[38,41], following incorporation studies with putative intermediates labelled with ^{14}C into penicillic acid produced by a strain of Penicillium cyclopium, have proposed a second mechanism. 2-O-Methylorsellinic acid (21) obtained by methylation hydroxyl group at C-2 of orsellinic acid, undergoes oxidative decarboxylation to give the hydroquinone (22) which is then oxidized to the quinone (23). The action of a monooxygenase (E_1), which has been isolated and partially purified from Penicillium cyclopium, yields the quinoid intermediate (24) which then after rearrangement and reduction leads to penicillic acid (7) (Scheme 2:12). The monooxygenase seems to catalyze a Baeyer-Villiger type oxidation, the oxygen being activated by a NADPH-FMN-Fe^{2+} system which is analogous to that of the monooxygenase involved in the oxidation of D-(+)-camphor[42].



(Scheme 2:12)

2.6 CONCLUSION

Orsellinic acid synthase presents some analogies with 6-methyl salicylate- and fatty acid -synthetases. It also uses acetyl-coenzyme A as the starter unit and

malonyl-coenzyme A (3 units) in successive condensation reactions with concomitant decarboxylation to produce a poly-ß-ketomethylene chain without the release of intermediates. No reduction occurs during the process and, following an internal aldol condensation, leads to the phenol orsellinic acid.

This "all or nothing" character has caused many difficulties for the detailed understanding of polyketide biosynthesis and compounds the problems involved in following the stereochemical fate of the two hydrogen atoms of each malonyl coenzyme A during conversion into phenols such as orsellinic acid. In addition, owing to the instability of orsellinic acid synthase, it has been impossible so far to work at the enzymatic level and a whole microorganism must be used. This means that generally, induced enzymes[43] are present which transform orsellinic acid into more complex substances such as penicillic acid. Fortunately, penicillic acid retains the hydrogen atoms of orsellinic acid at C-3 and C-5 (Scheme 2:11) but this further complicates the stereochemical analysis.

Following the encouraging results obtained with the incorporation of ^{3}H, ^{14}C aspartate into penicillic acid produced by Penicillium cyclopium[29], it was decided to investigate further the stereochemistry of hydrogen loss from each malonyl unit in a similar manner. The project involved the synthesis of aspartic acid labelled stereospecifically at C-3 with deuterium instead of tritium. This means that aspartic acid will be produced with greater deuterium enrichment (up to 100%) compared to the incorporation of tritium in previous samples and that no radioactive hazard will be involved. It was intended that penicillic acid obtained from feeding experiments would then be analysed by deuterium NMR, to give directly the position of deuterium into penicillic acid, distinguish between the non equivalent hydrogen atoms at C-5 and consequently avoiding the long and tedious chemical degradation of penicillic acid for the localization of the label.

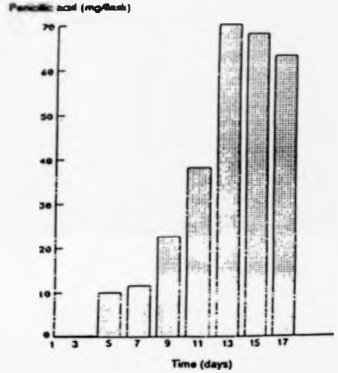
CHAPTER 3

PENICILLIC ACID BIOSYNTHETIC STUDIES INVOLVING DEUTERATED ASPARTIC ACID AS PRECURSOR

3.1 OPTIMIZATION OF THE CONDITIONS FOR PRECURSOR INCORPORATION INTO PENICILLIC ACID

The use of labelled precursor in whole organisms for the investigation of polyketide biosynthesis is a widespread practice[24,25]. However, due to the complexity of biological systems such an approach has several drawbacks. For example, the perturbations of the normal "functioning" of the biological system by addition of large amounts of tracer is to be avoided. The over-feeding often encountered when using heavy-isotope labelled precursors[24,45] may result in metabolic changes e.g. stimulation and/or inhibition of "normal" or "abnormal" pathways. Cases have been reported where the production of the secondary metabolites has been suppressed under over-feeding conditions. As a result, trial fermentation experiments using Penicillium cyclopium as the microorganism of choice to produce penicillic acid in high yield[44] were undertaken to determine the optimal conditions for the incorporation of the labelled precursor (aspartic acid) into penicillic acid. As the production of secondary metabolites is known to start at the end of the exponential phase of the growth cycle[46], addition of the labelled precursor to the culture media must coincide with the beginning of penicillic acid formation. This information could be obtained by

monitoring the production of penicillic acid with time. Therefore, 8 identical cultures of Penicillium cyclopium were grown and every 2 days the total contents of one flask was analyzed for penicillic acid as described in the experimental section (7.2.1.3). The profile is shown in figure (3:1), and from it one can see that addition of the precursor should be made on the 5th day after inoculation of the culture medium. Furthermore, the yield of penicillic acid reached a maximum of 70 mg per flask and then declined slowly; this is perhaps due to its decomposition or further metabolic transformation. This showed the importance of the harvesting time, which is best done on the 13th day of growth.



Figure(3:1)

Finally, before producing L-aspartic deuterated stereospecifically at the prochiral position, that incorporation of aspartic acid in high yield into penicillic acid takes place must be checked. This trial incorporation experiment was carried out with a sample of L-(3R)-[3 ^{3}H, 3 ^{14}C]-aspartic acid obtained during previous research[29]. The use of doubly radiolabelled aspartic acid presented two main advantages.

(1) Because detection and measurement of radioactive tracers by scintillation counting is an extremely sensitive detection method, the amount of labelled precursor used can be correspondingly small, avoiding possible over-feeding complications.

(2) No loss of ^{14}C label is known to occur during biosynthesis, in contrast with tritium, which was a good probe for this incorporation assay. Consequently, tritium could be measured more accurately by reference to the ^{14}C label.

A sterile aqueous solution of L-(3R)-[3 ^{3}H, 3 ^{14}C]-aspartic acid (0.644 μCi ^{14}C, 6.5 μCi ^{3}H) was distributed between 6 cultures of Penicillium cyclopium on the 5th day of growth. The total sample of crude penicillic acid (286.5 mg) was collected on the 13th day. After two crystallizations, the specific activity of the penicillic acid was essentially constant (Table 3:1).

Precursor		L-(3R)-[3 ^{3}H, 3 ^{14}C]-aspartic acid
Weight of penicillic acid harvested		286.5
Activity after 1st crystallization	^{3}H (DPM mg^{-1})	131.9
	^{14}C (DPM mg^{-1})	291.0
Activity after 2nd crystallization	^{3}H (DPM mg^{-1})	122.0
	^{14}C (DPM mg^{-1})	310.0
Final ^{3}H : ^{14}C ratio		0.39

Analysis of the radiolabelled penicillic acid produced by Penicillium cyclopium, with L-(3R)-[3 ^{3}H, 3 ^{14}C]-aspartic acid as precursor.

(Table 3:1)

The ^{14}C incorporation and ^{3}H retention into penicillic acid could be deduced as follows:

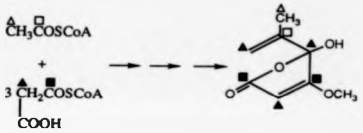
$$\% \text{ of } ^{14}C \text{ incorporation} = \frac{\text{activity (Dpm mg}^{-1}) \times \text{weight of crude penicillic acid}}{^{14}C \text{ activity fed}} \times 100$$

$$^{3}H \text{ retention} = \frac{\text{Final } ^{3}H : ^{14}C \text{ ratio}}{\text{Initial } ^{3}H : ^{14}C \text{ ratio}} \times 100$$

$$\Rightarrow \% \text{ of } ^{14}C \text{ incorporation} = \frac{3100 \times 286.5}{1429680} \times 100 = 6.2\%$$

$$^{3}H \text{ retention} = \frac{0.39}{10.10} \times 100 = 3.86\%$$

Taking into account that a maximum of 2/3 tritium label could be retained in penicillic acid (Scheme 3:1), the ^{3}H retention after correction was deduced to be of 5.79%.



(Scheme 3:1)

In conclusion, this high incorporation of ^{14}C (6.2%) justified further investigation and the synthesis of stereospecifically deuterated aspartic acid was undertaken. For the ^{3}H retention, no useful comparison could be drawn with previous experiments, which undoubtedly were conducted under different conditions e.g. differences in feeding and harvesting times.

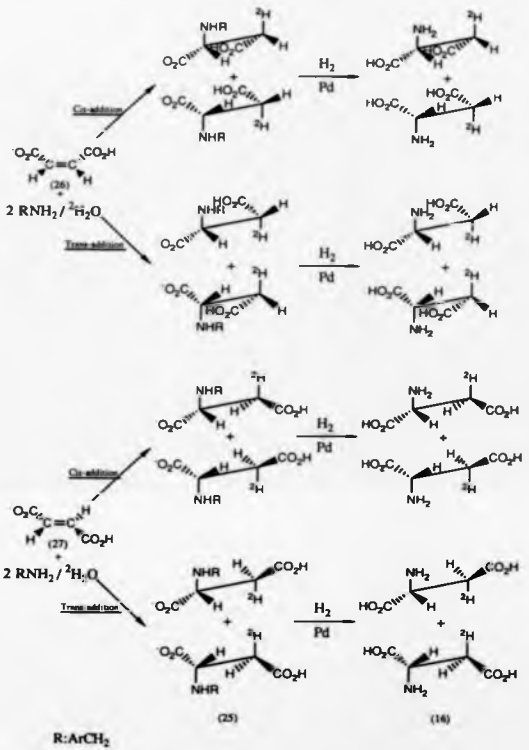
3.2 ATTEMPTED PREPARATION OF ASPARTIC ACID STEREOSPECIFICALLY DEUTERATED AT C-3 VIA N-BENZYLASPARTIC ACID

The addition of amines such as benzylamine and α-methyl benzylamine to fumaric and maleic acids and their derivatives have received attention in the past in attempts to synthesize enantiomerically pure aspartic acid and its derivatives[47,48]. However, no studies have been undertaken to determine the overall stereochemistry of these Michael type additions to determine whether the reaction afforded cis- and/or trans-addition products.

Therefore, the synthesis of aspartic acid labelled stereospecifically at C-3 with deuterium was envisaged as outlined in scheme (3:2). The benzylamine salt of N-benzyl aspartic acid (25) may be obtained by addition of benzylamine in ^2H_2O across the double bond of maleic (26) or fumaric (27) acids. If this reaction is stereoselective, hydrogenolysis of compound (25) would afford a racemate of aspartic acid stereospecifically labelled at C-3 (16). The production of enantiomerically pure aspartic acid may then be easily achieved by well-established enzymatic methods[49] or by the resolution of N-benzyl aspartic acid by fractional crystallization[50].

3.2.1 Preparation of non-labelled aspartic acid

Initially, in order to optimize the conditions of this reaction, water instead of deuterium oxide was used as the solvent. Refluxing 1 equivalent of maleic acid (26) with 2 equivalents of benzylamine in water afforded quantitatively the benzylamine salt of N-benzyl-D,L-aspartic acid (25). For purification, the procedure of Amiel et al[51] was applied to afford N-benzyl-D,L-aspartic acid. A poor yield of 43% was obtained, probably as a consequence of the difficulties encountered in its crystallization from the aqueous medium. Thus it was decided to use the crude benzylamine salt (25) obtained previously for subsequent experiments. The hydrogenation over palladium on charcoal of compound (25) gave a sample of aspartic acid which, by purification on a Dowex 50W-X8 (H^+ form) ionic exchange resin column, eluting with an ammonia solution, afforded ammonium aspartate in high yield. The 400 MHz ^1H NMR


Cis-addition
Trans-addition
2 RNH2 / 2H2O
(26)
(27)
H2
Pd
(25)
(16)
R:ArCH2

(Scheme 3:2)

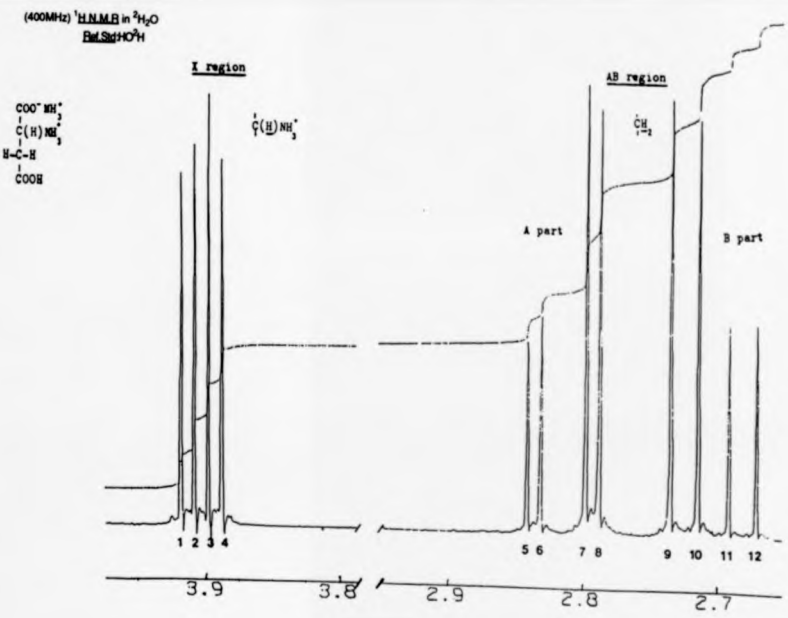
spectrum of this salt, recorded in ^2H_2O, is shown in figure (3:2). A very clear ABX spectrum is observed. Four lines in the "X region" and two interleaved AB-type quartets in the "AB region" characterize the methine and methylene protons respectively. From the values reported in Table (3:2) the spectrum can be analysed.

Peak No.	Frequency	ppm		
1	1568.029	3.9188		
2	1564.058	3.9089	X region	
3	1559.842	3.8983		
4	1555.873	3.8884		
5	1137.397	2.8426		
6	1133.400	2.8326	A part	
7	1119.906	2.7989		
8	1115.933	2.7889		AB region
9	1094.749	2.7360		
10	1086.562	2.7155	B part	
11	1077.267	2.6923		
12	1069.080	2.6718		

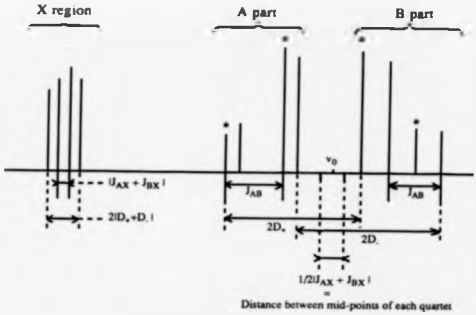
^1H NMR data for the ammonium salt of aspartic acid.

Table (3:2)

All coupling constants and chemical shifts cannot be read directly from this non-first order spectrum and must be calculated[52]. This can be achieved by the use of parameters measured directly from the spectrum as indicated in figure (3.3)


(400MHz) ¹H.N.M.R in ²H₂O
Ref.Std:HO²H
X region
AB region
A part
B part
1 2 3 4
5 6 7 8 9 10 11 12
3.9
3.8
2.9
2.8
2.7

Figure(3:2)



Figure(3:3)

Substitution of these parameter values into equations 1-4, calculated from the transition energies and relative intensities expected from an ABX system[53], afforded $|J_{AB}|$, $|J_{BX}|$, $|J_{AX}|$ and ν_a, ν_b and ν_x.
These values are reported in Table (3:2).

$$(\nu_a - \nu_b) + \tfrac{1}{2}|J_{AX} - J_{BX}| = (4D_a^2 - J_{AB}^2)^{\frac{1}{2}} \qquad (1)$$

$$(\nu_a - \nu_b) - \tfrac{1}{2}|J_{AX} - J_{BX}| = (4D_b^2 - J_{AB}^2)^{\frac{1}{2}} \qquad (2)$$

$$\nu_a = \nu_o + \tfrac{1}{2}(\nu_a - \nu_b) \qquad (3)$$

and $$\nu_b = \nu_o - \tfrac{1}{2}(\nu_a - \nu_b) \qquad (4)$$

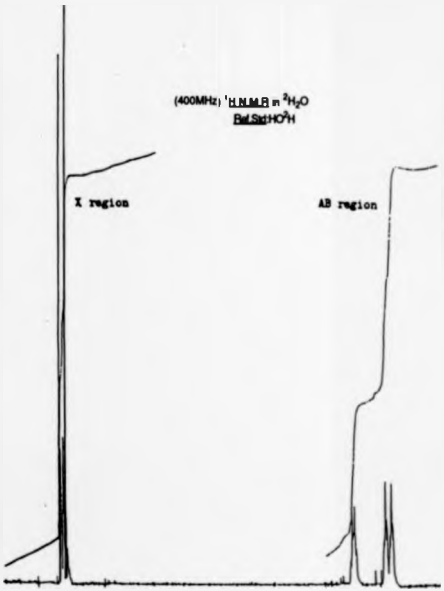
Chemical shifts	ν_a = 1124.88 Hz => 2.81 ppm
	ν_b = 1083.69 Hz => 2.70 ppm
	ν_x = 1561.95 Hz => 3.90 ppm
Coupling constants	$\lvert J_{AB} \rvert$ = 17.5 Hz
	$\lvert J_{AX} \rvert$ = 3.8 Hz
	$\lvert J_{BX} \rvert$ = 8.4 Hz

(Table 3:2)

The assignment of the ^1H NMR signals to each prochiral proton can be determined from Kainosho and Ajisaka's ^1H NMR studies[54]. They deduced from the ^1H NMR spectrum of L-(2S, 3R)-[3-^2H]-aspartic acid (AX system) that the 3-proS hydrogen was at higher field than the 3-proR hydrogen. Consequently, the B part and the A part of the AB region (see Figure 3:2) can be assigned to the 3-proS and 3-proR protons respectively for L-aspartic acid. In addition, from the ^1H NMR spectrum of unlabelled aspartic acid they concluded that the coupling constant between the α-proton and the pro-S β-proton is greater than that between the α-proton and the pro-R β-proton in L-aspartic acid. This result was confirmed by the ^1H NMR of the ammonium salt of aspartic acid, based on the knowledge that although the coupling constants vary with pH, they vary with different, non overlapping ranges[54,55].

3.2.2. Preparation of deuterated aspartic acid

The same experiments were carried out using ^2H_2O as the solvent for the reaction between maleic acid and benzylamine, leading to a sample of the ammonium salt of deuteriated aspartic acid. Its 400 MHz ^1H NMR is shown in Figure (3:4a). At this stage, for clarity of the discussion, it must be assumed that only L-aspartate was obtained. Indeed, a mixture of enantiomers gives the same spectrum, but the pro-R and pro-S methylene protons cannot be assigned unless reference is made to either L- or D-aspartate. The reason for this is that the pro-S proton for



33

4a

Figure(3+4)

L-aspartate for example will exhibit the same resonance as that of the pro-R proton for D-aspartate. The spectrum shown, however, is simpler than the one obtained for the unlabelled material, due to the absence of the AB proton coupling. The presence of two sets of signals for the methylene protons clearly shows that this approach did not lead to stereospecifically labelled aspartic acid. This result was confirmed by ^{2}H NMR Figure (3:4b) which would have given only one signal in the case of a stereospecific reaction.

The incorporation of deuterium and the percentage of deuterium in the pro-R and pro-S positions of L-aspartate can be calculated from both of these spectra as indicated below.

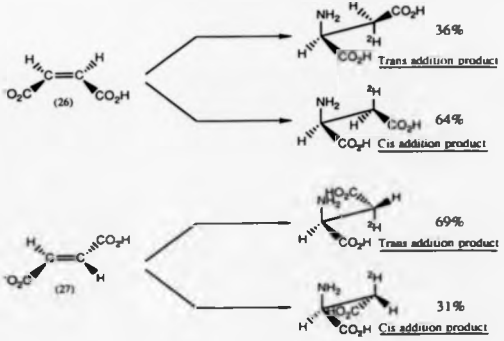
From the ^{2}H NMR spectrum, the integration values indicate the presence of 64% and 36% deuterium at the pro-R and pro-S positions respectively. (Similar results were obtained using fumaric acid instead of maleic acid : 69% and 31% for pro-R and pro-S positions).

From the ^{1}H NMR spectrum the integrations are:

X part	125 mm corresponding to one proton
AB part	133 mm

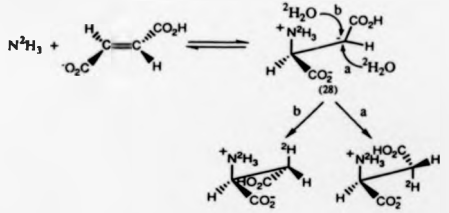
If 100% of the aspartate was monodeuterated at C-3, an integration value of 125 mm was expected. Instead, a value of 133 mm was obtained leading to the conclusion that 94% of the aspartate is monodeuterated. These values were cross-checked by mass spectrometry. The mass spectrum showed that less than 1.5% of the aspartate was dideuterated (calculated as indicated in section (3:3)); thus neither the exchange reaction of the aspartate methylene protons in $^{2}H_{2}O$[56] nor the possible reversibility of the reaction were significant under the experimental conditions.

In summary, the addition of benzylamine to maleic and fumaric acids led to an excess of cis-addition and trans-addition products respectively (Scheme 3:3).



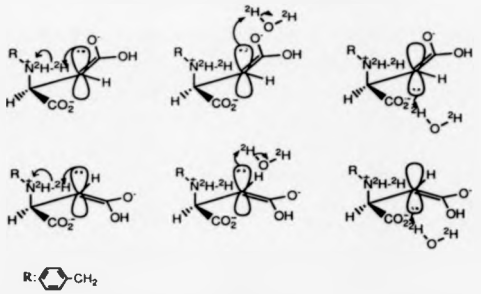
(Scheme 3:3)

Bada et al[56] investigated the addition of ammonia to fumaric acid in ^2H_2O and also obtained a non stereospecific reaction. They suggested that the carbanion intermediate (28) (assuming that no rotation took place) could give two stereochemical products depending on whether its protonation occured in a cis or trans manner to the ammonia (Scheme 3:4).



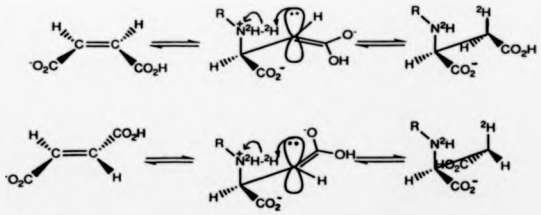
(Scheme 3:4)

This argument can also be applied to our case. In fact three possible ways of protonation could be envisaged for the carbanions in ^2H_2O (Scheme 3:5).



(Scheme 3:5)

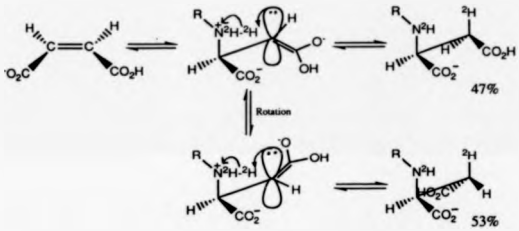
Consequently, it was decided to investigate this reaction using dioxan as solvent in the absence of water. The protonation of the carbanion could be expected to occur *via* an intramolecular reaction as shown in Scheme (3:6) leading to a stereospecifically labelled aspartate by an overall cis-addition. If no rotation took place, maleic and fumaric acids should lead to the two desired deuterated aspartates (Scheme 3:6).



(Scheme 3:6)

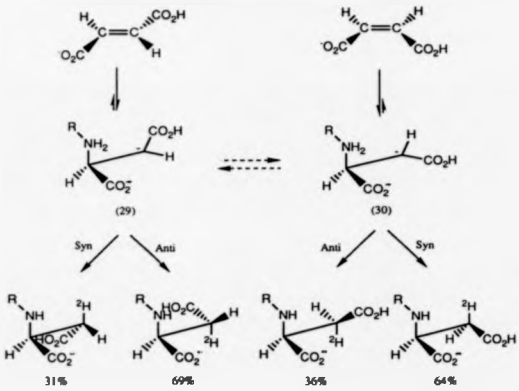
The reaction in dioxan between benzylamine and fumaric acid did not take place even after the reaction time was increased from one hour to thirteen hours. However, maleic acid reacted and a sample of aspartate was obtained. From the ^{1}H and ^{2}H NMR spectra, the deuterium label was located in the pro-R position (47%) and in the pro-S position (53%). The percentage of deuteration was high (92%). From the mass spectrum, the absence of dideuterated aspartate set aside the possibility of exchange of the prochiral protons as well as the reversibility of the reaction.

As a result, it seems that a rotation of the carbon-carbon bond may have been involved leading to this approximately 1:1 mixture of deuterated labelled aspartate at C-3. (Scheme 3:7).



(Scheme 3:7)

The results obtained in $^{2}H_{2}O$ are summarised in Scheme (3:8) and may be tentatively explained as follows:



(Scheme 3:8)

After addition of benzylamine, equilibration of the conformational isomers of the intermediate zwitterion is obtained rapidly relative to the rate of protonation, therefore the same equilibrium mixture of stereoisomeric zwitterions is formed from both maleic and fumaric acids.

Thus it can be concluded:

1. If the addition is strictly anti then species (30) reacts half as fast as species (29), or
2. If the reaction is strictly syn then species (30) reacts twice as fast as species (29).

or

3. If both syn and anti additions are possible then there are 3 possibilities
   (a) species (29) gives predominantly anti addition, species (30) gives predominantly syn addition, or

(b) Both species (29) and (30) give predominantly syn addition but protonation of species (30) is considerably faster than protonation of species (29) therefore species (30) reacts faster than (29). or

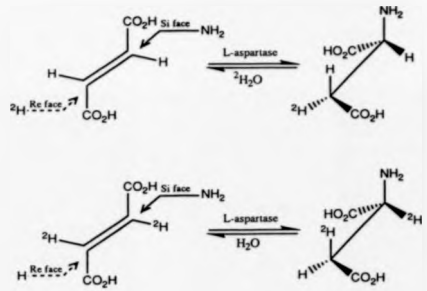
(c) Reverse of (b) when trans addition becomes predominant.

Alternatively but unlikely, after addition of benzylamine, if the protonation is rapid compared with rotational equilibration, the species (29) and (30) react preferentially anti and syn respectively but the preference is the same in both cases (i.e. 2:1).

The Michael addition with this open chain system was concluded to be non stereospecific, perhaps due to the rotation of the carbon-carbon bond of the intermediates (29) and (30). This is in contrast to the results obtained for cyclic compounds[57,58] for which nucleophilic addition has been reported to be either cis or trans. Due to the lack of stereospecificity, the addition of benzylamine to maleic and fumaric acids was not investigated further.

3.3 CIRCULAR DICHROISM STUDIES ON STEREOSPECIFICALLY DEUTERATED ASPARTATES

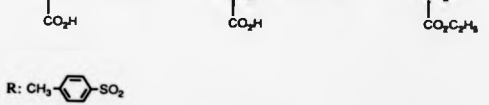
To produce deuterated aspartates, enzymatic methods seemed to be the obvious alternative. Indeed, the enzyme aspartase is known to catalyze an anti-addition of ammonia to the double bond of fumaric acid leading to L-aspartic acid. Young et al[59] used it to prepare (2S, 3R)-[3-$^2H_1$]- and (2S, 3S)-[2,3-$^2H_2$]-aspartic acids on a large scale from fumaric acid and [2,3-$^2H_2$]-fumaric acid respectively (Scheme 3:9).



(Scheme 3:9)

A sample of each of the aspartates was kindly provided by Dr. Young. At the same time, the opportunity was available to use Dr. Drake's vibrational infra red circular dichroism spectrometer[60] ; therefore it seemed possible to use this technique for the determination of the absolute configuration of the chiral centres of aspartate.

The insolubility of aspartate in carbon tetrachloride (solvent of choice for VCD measurements), led us to chemical derivatization of the former. N-Tosylation[29] followed by esterification with ethanol[61] afforded the corresponding deuterated diethyl N-p-toluenesulfonyl aspartates (Scheme 3:10)



(Scheme 3:10)

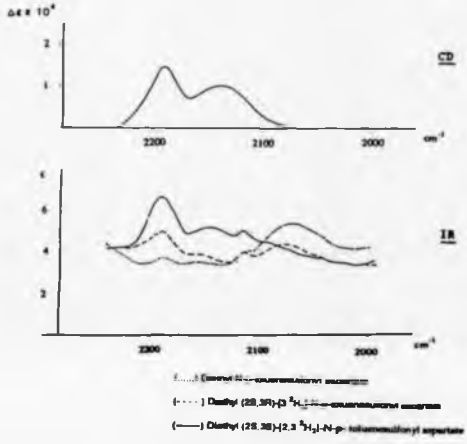
The normal infra red and vibrational infra red circular dichroism spectra (Figure 3:5) of these compounds in carbon tetrachloride were recorded in the C-D stretching region (2200 to 2000 cm^{-1}).

Diethyl (2S, 3R)-[3-$^2H_1$]-N-p-toluenesulfonyl aspartate and the non-deuterated sample exhibited no circular dichroism in the C-D stretching region. However, a strong positive circular dichroism was observed for diethyl (2S, 3S)-[2,3-$^2H_2$]-N-p-toluenesulfonyl aspartate. This could be attributed to the presence of deuterium at carbon 2. Consequently, only the assignment of the configuration of carbon 2 seemed possible. However, the signal to noise ratio being small, the validity of the method must be tested to prove that the result was not the consequence of an artefact of the instrument. To do this, the CD spectrum of one of two further compounds was also required.

One possibility was diethyl (2R)-[2$^2H_1$]-N-p-toluenesulfonyl aspartate, which should give a CD spectrum identical to that exhibited by diethyl (2S, 3S)-[2,3-$^2H_2$]-N-p-toluenesulfonylaspartate but negative in sign.

The second was racemic diethyl (2$^2H_1$)-N-p-toluenesulfonyl aspartate which should be characterized by the absence of vibrational CD.

The chemical synthesis of diethyl (2R)-[2$^2H_1$]-N-p-toluenesulfonyl aspartate was not straightforward. Indeed, it has been reported to have been achieved, after long and tedious experiments, *via* cobalt(III) chelates[62]. Enzymatically, L-2-deuterioaspartic acid can be prepared, by the use of glutamate-oxaloacetate transaminase[63] and L aspartase, but not D-2-deuterioaspartic acid. Therefore, the racemate of diethyl (2^2H)-N-p-toluenesulfonyl aspartate had to be prepared. Thanassi[64] has claimed to have prepared α-deuterated or tritiated amino acids *via* the incorporation of tritium or deuterium into the α-carbon concomitant with decarboxylation of the substituted aminomalonate precursor ($NH_2CR(COOH)_2$). Application of this method to the formation of α-deuterated aspartic acid was carried out according to the synthetic sequence indicated in Scheme (3:11).

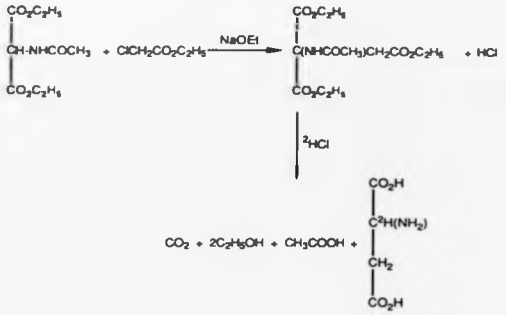


(.....) Diethyl (2S,3R)-[3 $^{2}H_{1}$]-2-isocyanosuccinate

(- - - -) Diethyl (2S,3R)-[3 $^{2}H_{1}$]-2-isocyanosuccinate enigmas

(——) Diethyl (2S,3S)-[2,3 $^{2}H_{2}$]-*N*-*p*-toluenesulfonyl aspartate

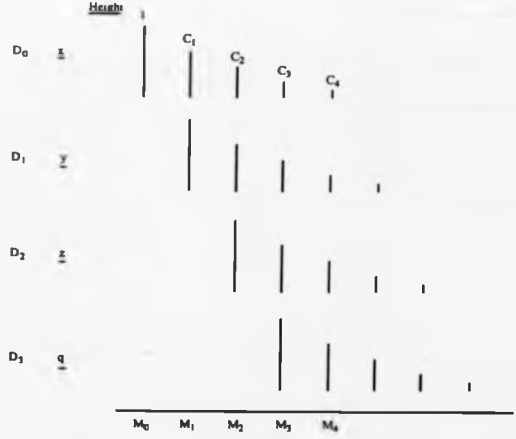
Figure(3.5)



(Scheme 3:11)

The resulting deuterated aspartate was then derivatized to diethyl N-p-toluenesulfonyl aspartate as before. Analysis by ^{1}H, ^{2}H NMR and by mass spectrometry indicated that deuterium was located at the α-position and at the β-position of the aspartate. This was not unexpected after hydrolysis, deacylation and decarboxylation of the substituted aminomalonate intermediate in boiling ^{2}HCl. Cohen et al[65] have actually reported that aspartic acid can be selectively deuterated at the β-carbon when heated with 6M ^{2}HCl.

The percentage of the non-deuterated (D_0), monodeuterated (D_1), dideuterated (D_2) and trideuterated (D_3) diethyl N-p-toluenesulfonyl aspartate were evaluated from the mass spectrum. The intensities (M_0, M_1, M_2, M_3, M_4) of the peaks due to the molecular ion were the result of the relative fractional contribution of the non-, mono-, di- and tri-deuterated species denoted as x, y, z and q respectively (Figure 3:6).



Figure(3:6)

Assuming the same relative intensities for each series, therefore the relative heights for the peaks of all four series are: 1, C_1, C_2, C_3 and C_4.

Consequently, the ratio $\frac{M_1}{M_0}$, $\frac{M_2}{M_0}$ and $\frac{M_3}{M_0}$ can be evaluated as follows:

$$R_1 = \frac{M_1}{M_0} = \frac{xC_1 + y}{x} \Rightarrow \frac{M_1}{M_0} = C_1 + \frac{y}{x} \qquad \text{Let } \frac{y}{x} = W_1$$

In the same way $R_2 = \frac{M_2}{M_0} = C_2 + W_1 C_1 + \frac{z}{x}$ $\qquad \frac{z}{x} = W_2$

$$R_3 = \frac{M_3}{M_0} = C_3 + W_1 C_2 + W_1 C_2 + \frac{q}{x} \qquad \frac{q}{x} = W_3$$

Therefore $\frac{q}{x} = W_3 \Rightarrow \frac{q + x}{x} = W_3 + 1$

$$q + x = x\,(W_3 + 1) \qquad A$$

and $\frac{y}{x} = W_1$

$\frac{z}{x} = W_2$

$$\Rightarrow y + z = x\,(W_1 + W_2) \qquad B$$

Addition of equations (A) + (B) gives

$$y + z + q + x = x\,[W_1 + W_2 + W_3 + 1]$$

Therefore the percentage of non deuterated compound is equal to:

$$\frac{x \times 100}{y + z + q + x} = \frac{100}{W_1 + W_2 + W_3 + 1}$$

In the same way:

The percentage of monodeuterated compound is:

$$\frac{y \times 100}{y + z + y + x} = \frac{W_2 \times 100}{W_1 + W_2 + W_3 + 1}$$

The percentage of dideuterated compound is:

$$\frac{z \times 100}{y + z + q + x} = \frac{W_2 \times 100}{W_1 + W_2 + W_3 + 1}$$

The percentage of trideuterated compound is:

$$\frac{q \times 100}{y + z + q + x} = \frac{W_3 \times 100}{W_1 + W_2 + W_3 + 1}$$

N.B.: Details of the calculation are given in the appendix.

Note that C_1, C_2 and C_3 were evaluated using a computer program and values 0.185, 0.073 and 0.011 respectively were assigned for isotopic distribution of non deuterated diethyl-N-p-toluenesulfonyl aspartate.

M_0, M_1, M_2 and M_3 were extracted from the mass spectrum with respective values of 1.5, 18.4, 17.9 and 6.6.

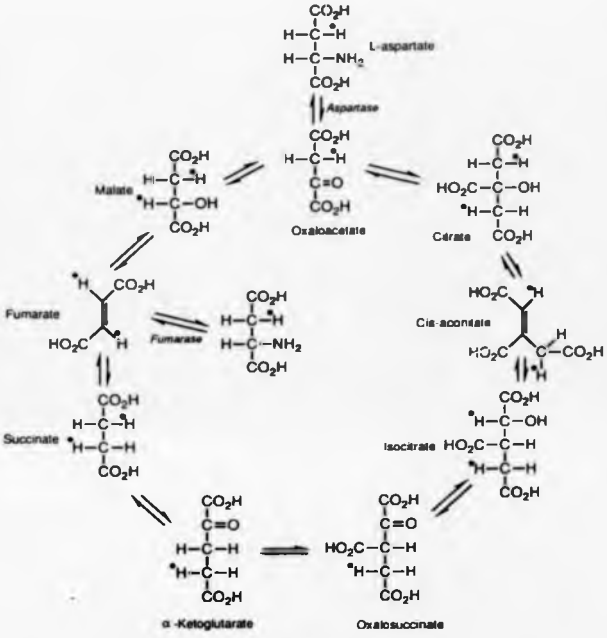
Therefore, the deuterated sample of diethyl N-p-toluenesulfonyl aspartate was a mixture of:

nondeuterated species	4.1%
monodeuterated	49.5%
dideuterated	39.4%
trideuterated	7.0%

As a result, 95.9% of the sample was concluded to be deuterated at the α-carbon. The presence of deuterium at the β-position did not affect the CD measurement (no CD spectrum for diethyl (2S, 3S)-[3-$^2H_1$]-N-p-toluenesulfonyl aspartate) which was obtained as previously in CCl_4. No CD was observed in the carbon-deuterium stretching region of this deuterated sample, thus this method of analysis was validated.

3.4 BIOLOGICAL TRACER EXPERIMENTS

The incorporation of (2S, 3R)-[3- $^2H_1$]- and (2S, 3S)-[2,3- $^2H_2$]-aspartic acids by Penicillium cyclopium into penicillic acid was investigated. Despite different feeding techniques (e.g. addition of the precursor at once or over a period of several days) significant incorporation of deuterium into penicillic acid was not observed. However, by ^2H NMR analysis of two samples of penicillic acid obtained in different experiments, the label could be located on the methoxy- or methyl-group of penicillic acid. The inconsistent results and lack of incorporation could be due to the fact that malonyl-CoA formed from aspartic acid entered into the tricarboxylic acid cycle. This would invariably lead to a dilution or loss of the label (Scheme 3:12). To verify this hypothesis, malate, an intermediate in the tricarboxylic cycle could be used as a precursor and could possibly be incorporated to a greater extent with less dilution. For this, malic acid with deuterium label at the prochiral centre is needed.


L-aspartate
Aspartase
Malate
Oxaloacetate
Citrate
Fumarate
Fumarate
Cis-aconitate
Succinate
Isocitrate
α -Ketoglutarate
Oxalosuccinate

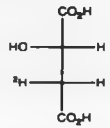
(Scheme 3:12)

CHAPTER 4

PREPARATION OF ENANTIOMERICALLY PURE ESTERS OF CIS- AND TRANS-2,3-EPOXYSUCCINATE : A PRECURSOR OF MALIC ACID

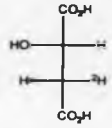
4.1 INTRODUCTION

For biosynthetic experiments, L- malic acid is required with a deuterium label at C_3 in the pro-R (31a) or pro-S (31b) position.



(2S,3R)-[3-$^2H_1$]-Malic acid

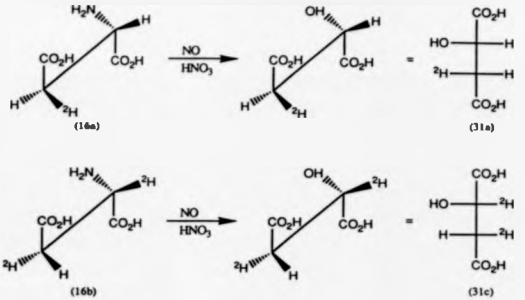
(31a)



(2S,3S)-[3-$^2H_1$]-Malic acid

(31b)

One possible straightforward method uses the stereospecifically deuterated aspartate obtained by the action of L-aspartase on fumaric acid (see Chapter 3): By treatment with nitric oxide in nitric acid[66], (2S,3R)-[3$^2H_1$]-aspartic acid (16a) and (2S,3S)-[2,3-$^2H_2$]-aspartic acid (16b) would give (2S,3R)-[3$^2H_1$]-malic acid (31a) and (2S,3S)-[2,3-$^2H_2$]-malic acid (31c) respectively (Scheme 4:1).

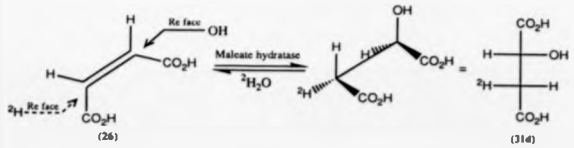


(Scheme 4:1)

Alternatively, one could use the reversible hydration of fumaric acid to malic acid. This reaction occurred non-enzymatically at elevated temperature over a wide range of pH (0-13). Different mechanisms[56,67,68,69,70] have been postulated for this reaction, although one involving a carbanion intermediate[56,67,69,70], as analogous to the one proposed for the amination of fumarate (see Chapter 3), seemed to be preferred. Nevertheless, the addition of water across the double bond was non-stereoselective, leading to D,L-malic acid with deuterium in both the pro-R and pro-S positions.

However, two hydro-lyases are known to catalyze the reversible stereospecific addition of water to the double bond of either fumaric acid or its cis-isomer, maleic acid.

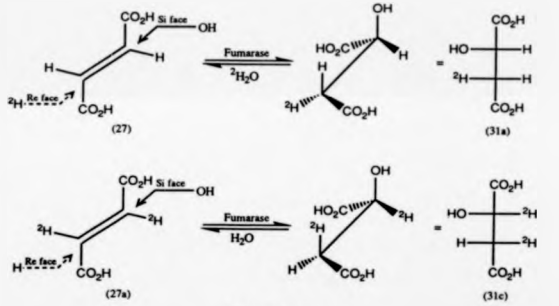
The hydration in $^{2}H_{2}O$ of maleic acid (26) was catalyzed by maleate hydratase[71] but unfortunately yielded (2R,3R)-[3-$^{2}H_{1}$]-malic acid (31d) which was the "abnormal" enantiomer with respect to tricarboxylic acid cycle. The over-all stereochemistry corresponded to an anti-addition (Scheme 4:2).



(Scheme 4:2)

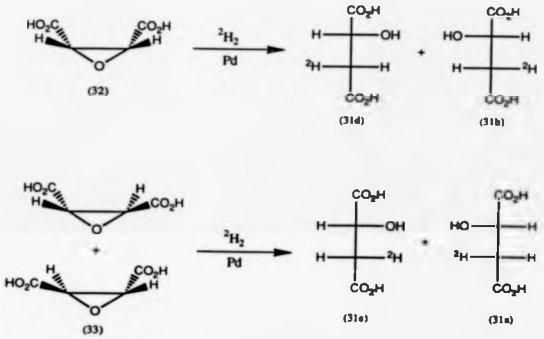
Fumarase[72,73], on the other hand, added water stereospecifically across the double bond of fumaric acid (27) also with an overall anti-addition.

England[71,73] using this enzyme prepared (2S,3R)-[3-$^2H_1$]-malic acid (31a) from fumaric acid (27) in ^2H_2O and (2S,3S)-[2,3-$^2H_2$]-malic acid (31c) from dideuterofumarate (27a) in H_2O (Scheme 4:3).



(Scheme 4:3)

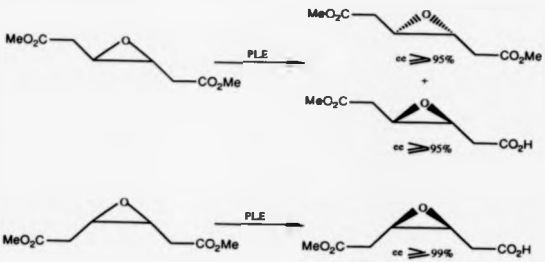
More challenging was the preparation of stereospecifically deuterated malic acid *via* the reduction of cis- and trans-2,3-epoxysuccinic acids. Hydrogenolysis of these epoxysuccinic acids had been reported[74]. At pH 7 with palladium on charcoal as catalyst, the opening of the epoxy ring took place in a trans fashion with inversion of configuration producing malic acid as a main product. Hydrogenolysis with deuterium yielded (2S,3S)-[3^2H]-malic acid (31b) and (2R,3R)-[3^2H_1]-malic acid (31d) from cis-epoxysuccinic acid (32) and (2S,3R)-[3^2H_1]-malic acid (31a) and (2R,3S)-[3^2H_1]-malic acid (31c) from racemic trans-epoxysuccinic acid (33) (Scheme 4:4).



(Scheme 4:4)

Consequently, the production of (2S,3S)-[3-$^2H_1$]-malic acid (31b) and (2S,3R)-[3-$^2H_1$]-malic acid (31a) could be achieved by the reduction of enantiomerically pure derivatives of cis- and trans-2,3-epoxysuccinic acid respectively. The use of biotransformations seemed to be the obvious approach for producing these enantiomerically pure compounds. Enzymes such as esterases or lipases might hydrolyze enantioselectively diesters of cis- and trans-2,3-epoxysuccinate. This latter

investigation was encouraged by the reported work on dimethyl 3,4-epoxyhexanedioate[75]. The geometrical isomers of this compound were hydrolyzed by pig liver esterase (PLE) with high selectivity producing enantiomerically pure compounds with an ee ≥ 95% (Scheme 4:5).

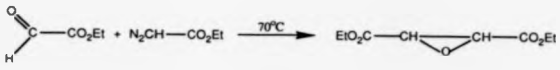


(Scheme 4:5)

Methods for preparation of the isomers of 2,3-epoxysuccinic acids reported in the literature seem to be laborious. The disodium salt of fumaric acid in water reacted with N-bromosuccinimide to yield the corresponding bromohydrin which, without separation from the reaction mixture, was apparently converted to the disodium salt of trans-2,3-epoxysuccinate in 60.6% yield by addition of aqueous sodium hydroxide[76].

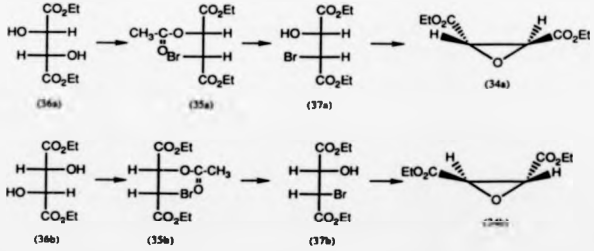
More efficient, but quite tedious was the method using hydrogen peroxide with sodium tungstate as a catalyst[77,78], in the pH range of 4-5.5, a method necessitated by the unreactivity of fumaric and maleic acids towards peroxyacetic and peroxybenzoic acids. This unreactivity was attributed to the electron withdrawing effect of the carbonyl group of these two compounds. As a result, disodium cis- and trans-epoxysuccinate were prepared by this method from maleic and fumaric acid respectively, the free acids

being obtained from the barium salts with an overall yield of 77% for the cis- and 50% for the trans-oxirane. The cis-epoxysuccinic acid was esterified with methanol to dimethyl cis-2,3-epoxysuccinate in 52% yield[79]. Therefore, the yield of the overall reaction being of only 26% this approach was considered unsatisfactory. The direct preparation of diethyl 2,3-epoxysuccinate was also reported. A mixture of isomers was obtained from the reaction between glyoxylate and ethyldiazoacetate[80], which by ^1H NMR was evaluated to be in the ratio of 2:1 (Scheme 4:6).



(Scheme 4:6)

The method elaborated by Mori et al[81] led to the preparation of optically pure diethyl trans-2,3-epoxysuccinate (34). The production of the acetoxybromide (35) from one enantiomer of diethyl tartrate (36) was the key step, as it occurred stereospecifically. Its deprotection to the bromohydrin (37) followed by ring closure with sodium ethoxide yielded optically pure epoxide (34). Two Walden inversions having taken place during this series of reactions resulted in retention of the configuration of carbons 2 and 3 of diethyl tartrate (36) in the diethyl epoxysuccinate (34) (Scheme 4:7).

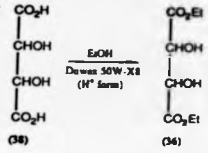


(Scheme 4:7)

4.2 PREPARATION OF DIETHYL 2,3-EPOXYSUCCINATE *VIA* DIETHYL 2-HYDROXY-3-BROMOSUCCINATE

4.2.1 Preparation of diethyl trans-(2S,3S)-epoxysuccinate (34a)

To obtain only (2S,3R)-[3-$^{2}H_{1}$]-malic acid (31a) by catalytic hydrogenation of diethyl trans-2,3-epoxysuccinate (Scheme 4:4), diethyl (2S,3S)-epoxysuccinate (34a) must be synthesized. This could be done as described in the introduction using Mori's method[81] and therefore by starting from diethyl (2S,3S)-tartrate (36a) (Scheme 4:7a). Esterification of tartaric acid (38) was achieved in benzene by reflux with ethanol using as a catalyst the ionic exchange resin, Dowex 50W-X8(H^+ form), with yields of up to 91% (Scheme 4:8). The water formed was displaced by azeotropic removal using a Dean-Stark apparatus. When the reaction was complete, filtration to remove the catalyst and evaporation of the solvent under reduced pressure gave the desired ester. Thus no treatment of the reaction mixture with bicarbonate to eliminate the catalyst was involved which would have led to loss of ester.



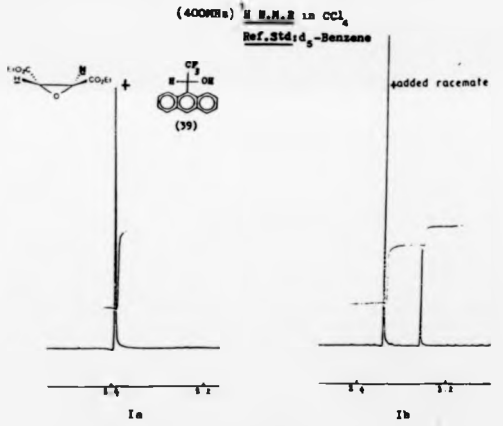
(38) (34)

(Scheme 4:8)

Reaction of diethyl (2S,3S)-tartrate (36a) with hydrogen bromide (30% w/v) in acetic acid gave the crude diethyl (2R)-acetoxy-(3S)-bromosuccinate (35a) in a yield of 91%. Deprotection of the latter by heating in ethanol with a small amount of hydrogen bromide (30% w/v) gave the bromohydrin (37a). As reported[81] the yield was of 73% before silica gel column purification and distillation. Further purification, which was necessary for the following experiment caused it to drop to 55%. Furthermore, it must be noted that this reaction could lead back to the starting material (36a). This might be dependent upon the quality of the supplied hydrogen bromide and particularly its water content. The (2S,3S)-epoxide (34a) was obtained from the

hydroxybromide (37a) by closure with NaOEt in ethanol in 84% yield. The enantiomeric purity of (34a) was checked by the measurement of the optical rotation. The measured specific rotation, $[\alpha]_D^{27}$ = +108.4° (C = 0.289, ether) was in good agreement with the literature value[81] $[\alpha]_D^{23}$ = +105.5° (C = 1.413, ether). To confirm this unambiguously, a 400 MHz ^{1}H NMR spectrum was run in carbon tetrachloride/d_6-benzene in the presence of 3 equivalents of the chiral solvating agent (S)-(+)-2,2,2-trifluoro-1-(9-anthryl)-ethanol (39) (Figure 4:1a). The spectrum was re-recorded in the presence of added racemate made by the above, but starting from diethyl D,L-tartrate. The splitting of the singlet corresponding to the epoxy hydrogens occurred only in this latter case (Figure 4:1b).

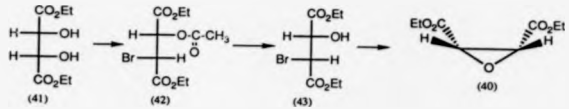
These experiments showed that the diethyl (2S,3S)-epoxysuccinate (34a) was enantiomerically pure (ee ≥97%), and that the epoxy hydrogens of this enantiomer corresponded to the downfield singlet obtained from the chiral solvating agent ^{1}H NMR experiment. Finally, the overall yield could be estimated to be at the best of about 40%



Figure(4:1)

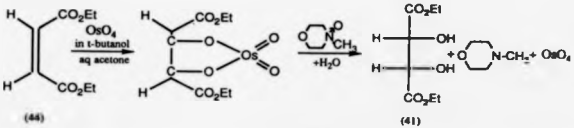
4.2.2 Preparation of diethyl cis-2,3-epoxysuccinate (40)

In the same way, it should be possible to prepare the meso compound diethyl cis-2,3-epoxysuccinate (40) from diethyl meso-tartrate (41) (Scheme 4:9).



(Scheme 4:9)

As meso tartaric acid is not commercially available, diethyl meso-tartrate (41) was synthesized from diethyl maleate (44) by an improved catalytic osmium tetroxide oxidation using N-methyl-morpholine-N-oxide[82] to regenerate the osmium tetroxide (Scheme 4:10).



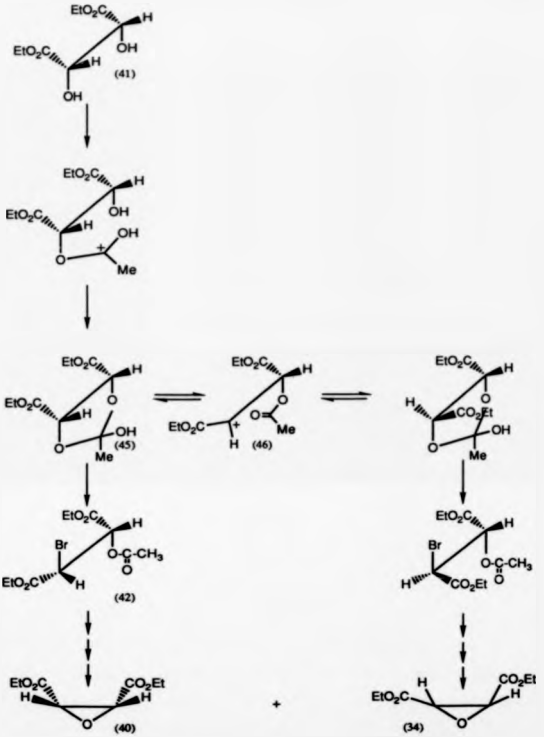
(Scheme 4:10)

Safe and easily carried out at room temperature, this process provided diethyl meso-tartrate (41) in high yield (79%) compared to the 41% yield obtained using hydrogen peroxide[83] instead of tertiary amine-N-oxide. This loss of yield could be attributed to the further oxidation of the vicinal diol to the α-ketol. Treatment of diethyl meso-tartrate (41) at 0°C with a solution of hydrogen bromide in glacial acetic acid (30% w/v) gave the acetoxy bromide (42) in poor yield (30%). Diethyl 2,3-diacetoxysuccinate was certainly one of the by-products of the reaction. However, the crude acetoxy bromide (42) was then refluxed in ethanol containing a catalytic amount of hydrogen bromide to give, after distillation and purification by flash chromatography, a colourless oil identified as the bromohydrin (43) in 6% yield.

Diethyl meso-tartrate (41) was identified by ^{13}C NMR as the by-product of this reaction.

Crude diethyl 2,3-epoxysuccinate was subsequently obtained by the action of NaOEt on the bromohydrin (43) in a yield of 54%. By ^{1}H NMR, the crude diethyl 2,3-epoxysuccinate was determined to be a mixture of cis- and trans-isomers in a ratio of 54/46 respectively. Two singlets, each corresponding to the epoxy hydrogens of each of the isomers, at 3.71 and 3.69 ppm, were assigned to diethyl cis- and trans-2,3-epoxysuccinate respectively. This assignment which was performed by re-running the ^{1}H NMR spectrum in the presence of added diethyl trans-2,3-epoxysuccinate (34) (see Section 4.2.1).

The formation of diethyl 2,3-diacetoxysuccinate as a by-product as well as the obtention of the cis- and trans-epoxides could eventually be rationalized by looking at the mechanism of the formation of diethyl 2-acetoxy-3-bromosuccinate (42). The mechanism[84] of the reaction as shown in Scheme (4.11) involved monoacetylation of the diol (41), followed by its cyclization to the 1,3-dioxolan-2-ylium ion (45) and then capture of this intermediate by bromide ion. The cyclisation to the "ylium ion" intermediate (45) implied that the hydroxy group and protonated acetoxy group were in proximity to form this planar five member ring. For the formation of this ring, diethyl meso-tartrate (41) must adopt the less stable of the eclipsed conformation in which the two ethyl ester groups were closed to each other, otherwise the formation of the diacetoxy compound would be favoured. In addition, the formation of cis-and trans-oxiranes might be the result of the formation of this acyclic cation (46) which causes racemization of (45).



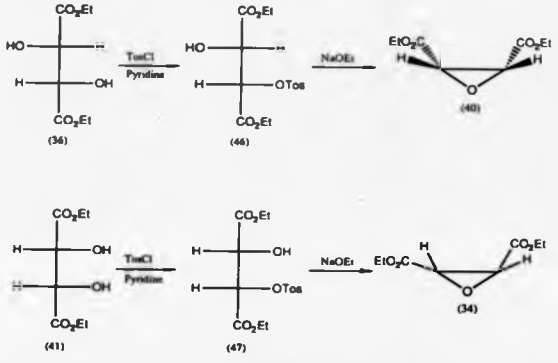
(Scheme 4:11)

4.3 PREPARATION OF DIETHYL 2,3-EPOXYSUCCINATE *VIA* DIETHYL 2-TOSYL TARTRATE

4.3.1 Preparation of diethyl 2-tosyl tartrate by conventional method

The formation of these epoxides was attempted *via* the monotosylate of diethyl tartrate (46,47) (Scheme 4.12).

Using a similar approach, Nelson and Burke[85] reported the production of (2R)-3-(O-allyloxyphenoxy)-1,2-epoxypropane in 42% yield *via* the monotosylation of (2R)-3-tosyloxy-1,2-propanediol acetonide. The tosylate was prepared in a crude yield of 88% using an equimolar quantity of tosyl chloride in pyridine-benzene.



(Scheme 4:12)

Diethyl meso-tartrate (41) was treated with an equimolar quantity of tosyl chloride in pyridine-benzene. The crude yellow oil obtained, tested by Tlc, was a mixture of three compounds. These compounds were identified by ^1H NMR as diethyl 2-tosyl butenedioate, diethyl 2,3-ditosyl-meso-tartrate and in 40% yield, diethyl 2-tosyl-meso-tartrate (47). Lowering the temperature of the reaction or adding the reactants more slowly failed to improve the yield of the monotosylate, and a tedious

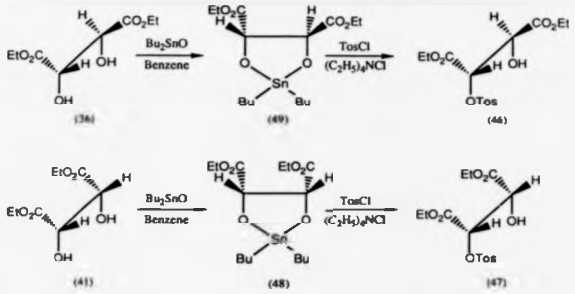
purification by flash chromatography was therefore needed.

The monotosylate of diethyl D,L-tartrate (46) was obtained in 22% yield from the reaction mixture, which also contained a mixture of ditosylate and elimination products.

4.3.2 Mono-O-p-toluenesulfonylation of diethyl tartrate *via* a cyclic stannylene derivative

At the time that the monotosylation of diethyl tartrate was under investigation, a relevant paper was published by Grouiller et al[86]. The regioselective O-p-toluenesulfonylation of nucleosides was described using dibutyltin oxide as a base under a solid/liquid phase-transfer conditions, a variant of the liquid-liquid phase transfer elaborated by Garegg et al[87] for the synthesis of partially substituted carbohydrates. Previously, Moffat and co-workers[88] used the reaction between vicinal diols and dibutyltin oxide to form cyclic dibutylstannylene derivatives of nucleosides. Subsequent reaction with tosyl chloride and triethylamine resulted in regioselective monotosylation in high yield.

As a result, it was decided to attempt the synthetic sequence outlined in Scheme (4.13). Dibutylstannylenes of diethyl meso-(48) and D,L-(49)tartrate, obtained as white crystalline solids, were prepared in quantitative yield by reaction of equimolar quantities of the diols and dibutyltin oxide in boiling benzene, with azeotropic water removal using a Dean-Stark apparatus. The cyclic stannylene from diethyl (2S,3S) tartrate has previously been obtained by Schanzer and his co-workers[89], who used it to form stereospecifically a single macrocyclic product with pimeloyl dichloride. Treatment of (48) and (49) with one equivalent of tosyl chloride and tetraethylammonium chloride in acetonitrile gave, again in quantitative yield, diethyl 2-tosyl-meso-tartrate (47) as a colourless oil and diethyl 2-tosyl-D,L-tartrate (46) as a white solid respectively.



(Scheme 4:13)

4.3.3 Diethyl trans-2,3-epoxysuccinate (34) from diethyl 2-tosyl-meso-tartrate (47)

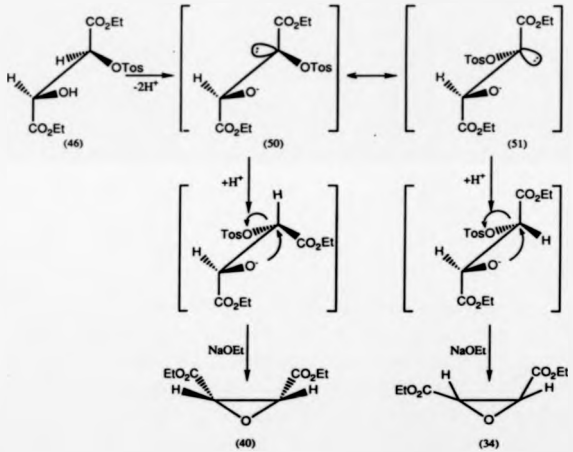
As with the bromohydrin of diethyl (2S,3S)-tartrate (37a), treatment of diethyl 2-tosyl-meso-tartrate (47) with a slight excess of NaOEt in ethanol afforded, in 84% yield, the racemate of diethyl trans-2,3-epoxysuccinate (34) as a colourless liquid (Scheme 4:12)

4.3.4 Diethyl meso-2,3-epoxysuccinate (40) from diethyl 2-tosyl-D,L-tartrate (46)

When sodium ethoxide solution was added to an ethanolic solution of the diethyl 2-tosyl-D,L-tartrate (46) at $0^{o}C$ and then left to warm up at room temperature, a mixture of diethyl cis-(40) and trans-(34) epoxysuccinate was obtained, in a low yield, in a ratio of 81 to 19 respectively. From the reaction mixture a precipitate was always produced, and the reaction never went to completion. An attempt using another base, 1,8-diazabicyclo[5.4.0] undec-7-ene (DBU) in ether, also gave a mixture of isomers, but due to the extent of the impurities in the crude mixture and the low yield, this method was not investigated further. Much better yield was obtained by the addition of the ethanolic solution of the tosylate (46) to the solution of NaOEt over a period of 8 hours at $0^{o}C$, followed by a further 30 hours at $4^{o}C$. A ratio of 74 of cis-(40) to 26 of trans-(34) isomers was obtained. These two

epoxides were readily separated by flash chromatography and diethyl cis-2,3-epoxysuccinate (40) was obtained in 23.5 % with a purity ≥ 97% by ^{1}H NMR and HPLC.

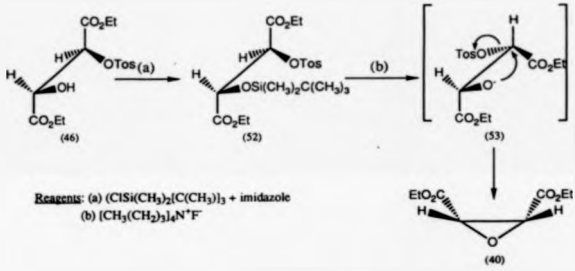
One possible mechanism to explain the mixture of diastereomers is indicated in Scheme (4.14). It involved the abstraction of the proton on the carbon bearing the tosylate group to form the carbanion intermediate (50). At this stage, if the reaction was kinetically controlled, the formation of the cis-isomer (40) would be favoured. On the other hand, if the reaction was thermodynamically controlled, this carbanion would isomerise to the more stable intermediate (51) and as a result trans-isomer (34) would be formed preferably.



(Scheme 4:14)

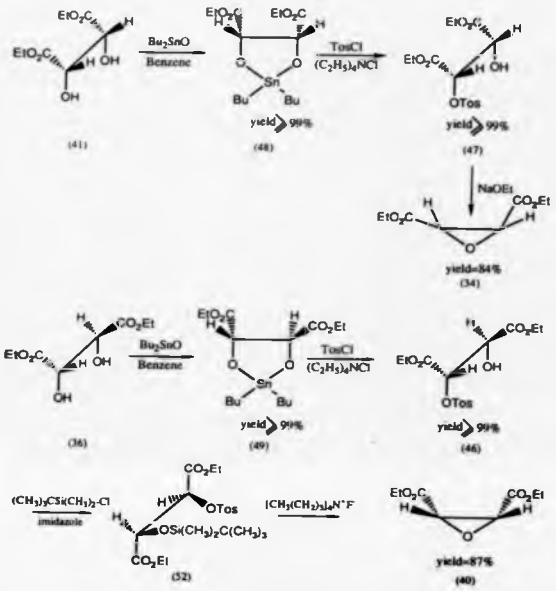
4.3.5 Preparation of diethyl cis-2,3-epoxysuccinate *via* the silylation of diethyl 2-tosyl-D,L-tartrate

The difficulties encountered in the preceeding method probably arose from the use of strong bases. However, it is known that alkoxide anion can be generated under essentially mild conditions by treatment of silyl ethers with fluoride ion[90]. It was anticipated that the use of the method would lead to a stereochemically clean reaction. Consequently, diethyl 2-tosyl-D,L-tartrate (46) was treated with tert-butyldimethylsilyl chloride to give the silyl ether (52) quantitatively. Treatment of (52) with tetra-n-butylammonium fluoride in tetrahydrofuran gave (40) in 87% yield, presumably *via* (53). The absence of strong base can account for the improvement in yield compared to the previous method.



(Scheme 4:15)

In summary, the synthesis of diethyl cis-(40) and trans-(34) 2,3-epoxysuccinate were realized by new routes (Scheme 4:16). These new simple approaches afforded the two monomers in high yield and high diastereoisomeric excess, and from this point of view were superior to the methods previously reported in the literature (see Section 4.1).



(Scheme 4:16)

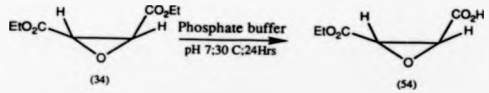
As racemic diethyl trans-2,3-epoxysuccinate (34) was being produced in contrast to Mori's method[81], a resolution using enzymes was needed. For the meso compound, diethyl cis-2,3-epoxysuccinate (40), the use of biotransformations was also investigated in an attempt to produce the corresponding monoacid in enantiomerically pure form.

4.4 PREPARATION OF ENANTIOMERICALLY PURE DERIVATIVES OF CIS- AND TRANS-2,3-EPOXYSUCCINATE

4.4.1 Enzymatic resolution of diethyl trans-2,3-epoxysuccinate (34)

4.4.1.1. Enzymatic hydrolysis

Initially, it was hoped to use an enzyme - catalyzed hydrolysis to achieve a kinetic resolution. Ester hydrolysis using enzymes such as PLE, α-chymotrypsin and lipases from different origins were first investigated. However, in aqueous solution, diethyl trans-2,3-epoxysuccinate (34) underwent a spontaneous hydrolysis (Scheme 4:17). At 30°C, in phosphate buffer at pH 7, the decomposition was followed by maintaining the pH at 7 using an automatic titrator to add a solution of sodium hydroxide. The velocity at which the epoxide decomposed varied from experiment to experiment, but generally about 25% of the ester groups were hydrolyzed in 4 hours at 30°C. The extent of hydrolysis reached 50% in 24 hours, then slowed down considerably. The reaction seemed to be dependent on temperature and the volume of the aqueous medium (tris-buffer, phosphate buffer) but these phenomena were not investigated further. However, the product of hydrolysis, ethyl(2,3)-epoxysuccinic acid (54) was identified by ^{1}H NMR and mass spectroscopy.

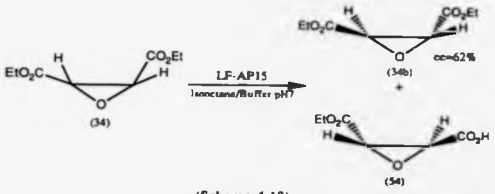


(34) (54)

(Scheme 4:17)

Nevertheless, the hydrolysis in a non-miscible two-phase system (water : isooctane) was investigated with the lipase from Rhizopus javanicus (LF-AP15), following the excellent results obtained with it previously in transesterification reactions (described below in section (4.4.1.2)). With stirring at room temperature, racemic diethyl trans-2,3-epoxysuccinate (34) in a mixture of isooctane : pH 7 buffer (ratio 4:1 respectively) was hydrolyzed with lipase from Rhizopus javanicus (LF-AP15). By the

use of an automatic titrator, the reaction was stopped after 25.6% hydrolysis of the ethyl groups and from the organic phase diethyl (2R,3R)-epoxysuccinate (34b) was obtained in 36% yield with ee = 62% (optical purity determined by the chiral solvating agent experiment described in Section (4.2.1) (Scheme 4:18).



(Scheme 4:18)

Reactions carried out without enzyme (Table 4:1) showed that the partition of substrate between organic and aqueous phases was a crucial factor. The isooctane must be in reasonable quantity to "protect" the substrate against spontaneous hydrolysis.

Mass of diethyl trans-2,3-epoxysuccinate (mg)	25.4	25.4
Volume of pH 7 phosphate buffer (1 M) (ml)	2	8
Volume of isooctane (ml)	8	2
Mass of epoxide (34) collected in the organic phase after 24 hours stirring at room temperature (mg)	21.6	9.1

Partition of diethyl trans-2,3-epoxysuccinate in a biphasic system.

(Table 4:1)

Since Klibanov[91] reported the use of lipases to catalyze transesterification between tributyrin and various alcohols in wholly organic media containing as little as 0.015% of water, this versatile potential of enzymes to work in organic solvents has received more attention[92,93,94]. Therefore it was decided that enzymatic transesterification in pure organic solvents was a better approach, removing completely the problem of spontaneous hydrolysis, although this had been partially overcome by the use of the two-phase system described above.

4.4.1.2 Enzymatic transesterifications

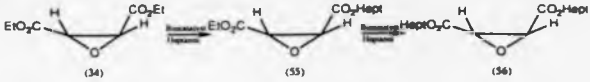
Accordingly, the lipases mentioned in Table (4:2) were tested as catalysts of the transesterification reaction between the racemic diethyl trans-2,3-epoxysuccinate (34) and heptanol in isooctane. The rate of the reaction was significantly enhanced when the optimal temperature of these enzymes in aqueous media was chosen and generally a temperature between 40-50°C was selected.

Origin of the lipases	Name	Optimal temperature*
Candida cyclindracea	CCL	-
Pig pancreas	PPL	-
Pseudomonas fluorescens	LP	50°C
Aspergillus niger	LAP6	45°C
Rhizopus javanicus	LF-AP15	40°C

* Optimal temperatures were provided by the suppliers and referred to aqueous media. No data in organic media has so far been determined.

(Table 4:2)

The progress of the reaction was monitored simply by gas chromatography using a SE 30 3% column. A temperature program made it possible to show the production of two new compounds which corresponded to two consecutive transesterifications on racemic diethyl trans-2,3-epoxysuccinate (34). These compounds were identified as monoethyl monoheptyl trans-2,3-epoxysuccinate (55) and diheptyl trans-2,3-epoxysuccinate (56) (Scheme 4:19).



(Scheme 4:19)

Since on the chromatogram, the area of each peak was approximately proportional to the number of carbon atoms in the product formed (characteristic of flame ionization detector system), it was possible to estimate at which time the reaction should be stopped. Each epoxide could be obtained in good yield after purification by flash chromatography.

The enantiomeric purity of each sample was determined by an ^1H NMR experiment using 3 equivalents of (S)-(+)-2,2,2-trifluoro-1-(9-anthryl)-ethanol (39). Like racemic diethyl trans-2,3-epoxysuccinate (34), the epoxy hydrogens of each enantiomer of the enantiomeric mixture of diheptyl trans-2,3-epoxysuccinate (56) appeared as a singlet (Figure 4:2b). For compound (55), each enantiomer was characterized by an AB system for the two non-equivalent epoxy hydrogens (Figure 4:2a). The ee values for each experiment were calculated and summarised in (Table 4:3).

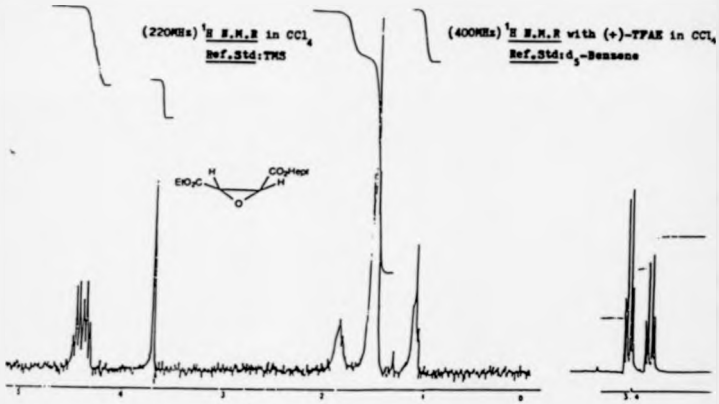
Hept	Lipase (source used)	Alcohol* used	Temperature (°C)	Time (days)	Ratio of compounds (% of mass) (34)	(55)	(56)	% ee (34)	(55)	(56)
1	CCL	A	RT	3	53	47		28	43	
	(47h/r)	A	40	22		49	51			7
2	PPL	A	40	24	60	40		0.00		
3	LP	A	50	9		47	53		21	20
4	LAPS	A	40	37	40	60		8	14	
5	CCL	B	55	2	45	55		21		
6	LPAP15	A	40	48	25	37.5	37.5	>97		>97

* A = Heptanol; B = isobutyl alcohol

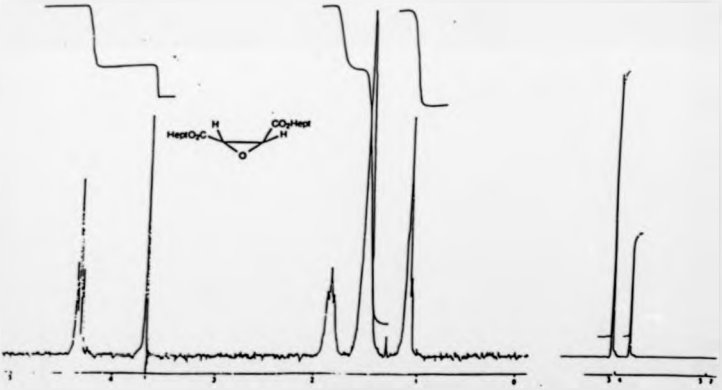
Reactions done using racemic diethyl trans-2,3-epoxysuccinate (34) (168 mg; 0.83 mmol) dissolved in a mixture of isooctane (12 ml) and heptanol (0.5 ml)

Transesterification of diethyl trans-2,3-epoxysuccinate using lipases of different origins.

(Table 4:3)



2a



2b

Figure(4:2)

From this table, one can see that the ee values were far from satisfactory for experiments 1, 2, 3, 4. An attempt to use isobutyl alcohol (experiment 5) instead of heptanol proved that lipase from Candida cyclindracea (CCL) could accept a bulkier alcohol, but no improvement in the ee value resulted. Also, to be pointed out that all the enzymes tested except lipase from Aspergillus niger (LAP 6) used preferentially the diethyl (2S,3S)-epoxysuccinate (34a).

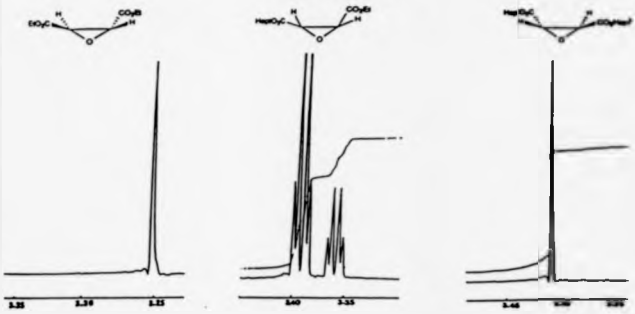
Nevertheless, the experiment 6 with lipase from Rhizopus javanicus (LF-AP15) gave very good results which deserved to be investigated in great detail.

After 15 days at room temperature, no reaction had been catalyzed by this lipase. However, its use at 40°C, after an average of 48 days gave a mixture of compounds (34), (55) and (56) in a ratio of about 25/37.5/37.5 respectively. Sometimes it was necessary, to add a small quantity of water (0.2%) to the reaction mixture to initiate the transesterification reaction.

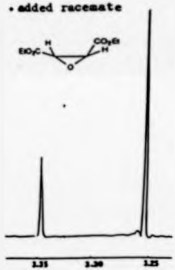
The enantiomeric purity of compounds (34) and (56) after column chromatography was determined to be ≥ 97% by the use of 400 MHz ^{1}H NMR run in presence of 2 equivalents of (S)-(+)-TFAE (39) (Figure 4:3). The same reaction using diethyl(2S,3S)-epoxysuccinate (34a) was performed using this enzyme under the same conditions as for the racemate (34). In only 20 days, no starting material was left and a mixture of (55) and (56) was obtained. After flash chromatography, each compound was tested by ^{1}H NMR with (S)-(+)-TFAE (39) and by addition of the corresponding racemate, it was deduced unambiguously that the mixed ester (2S,3S)-(55) and the diheptyl ester (2S,3S)-(56) epoxysuccinates corresponded to the downfield signal, as was also the case for diethyl(2S,3S)-epoxysuccinate (34a) (Figure 4:1).

(400MHz) ^{1}H E,H,E with (+)-TFAE in CCl_4

Ref.Std:d_5-Benzene



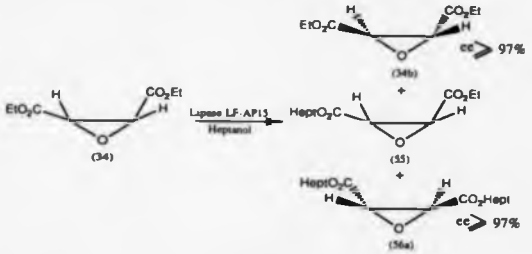
• added racemate



• added racemate

Figure(4:3)

In summary, this enzymatic reaction in pure organic solvent using a lipase from Rhizopus javanicus (LF-AP15) as a biocatalyst provided diethyl(2R,3R)-epoxysuccinate (34b) and diheptyl (2S,3S)-epoxysuccinate (56a) with an ee > 97% (Scheme 4:20).



(Scheme 4:20)

The slowness of this process (about 48 days) was the major drawback of this transesterification reaction. However, knowing that the mode of action of lipases was related to the presence of an interface[95], it was likely that enzymatic rate could be enhanced by providing an interface for the enzyme. An interface could be "created" by means of adsorption of the enzyme to hydrophobic surfaces such as siliconized glass beads[96].

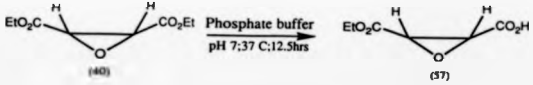
Biofix E_1, a macro-porous ceramic (kaolinite ($Al_2O_3 \cdot 2SiO_2 \cdot 2H_2O$)) designed for enzyme immobilization, was used successfully in our case. The adsorption of the enzyme on to this support, done by removal of water under reduced pressure from a slurry of lipase (10 mg) and biofix (100 mg) dramatically increased the rate of transesterification of racemic diethyl trans-2,3-epoxysuccinate (34) with heptanol (Biofix without enzyme did not catalyse the transesterification reaction). Indeed, by GC analysis after 4 days at 40°C no starting material (34) was present and the quantity of compound (55) was negligible compared to compound (56). However, the rate of

reaction varied considerably according to the percentage of water in the preparation, resulting in a lack of reproducibility of the reaction. Therefore, no ee values were determined. A more detailed study should be undertaken to optimize the conditions and to study the relationship between enhancement of the rate of transesterification and factors such as the water content of the preparation and the ratio of biofix-enzyme.

4.4.2 Preparation of enantiomerically pure esters of cis-2,3-epoxysuccinate

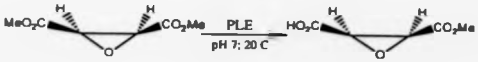
4.4.2.1 Enzymatic hydrolysis and transesterification

As for the trans epoxide (34), diethyl cis-2,3-epoxysuccinate (40) was tested for spontaneous hydrolysis in pH 7 phosphate buffer (0.1M) at 37°C. 50% of the ester groups were hydrolyzed in 12.5 hours at 37°C (Scheme 4:21). Consequently, any enzymatic hydrolysis with this substrate must be done in a much shorter time to obtain a reasonable ee. For example, if the reaction was done in 4.5 hours and stopped after 50% of ester hydrolysis, as much as 22.6% of the ester groups could have been hydrolyzed non enzymatically and consequently the worst ee expected would be of 54.8%.



(Scheme 4:21)

During the course of this investigation, a paper was published by Jones et al[97] who described the hydrolysis of dimethyl cis-2,3-epoxysuccinate by PLE at pH 7 and 20°C in 4.5 hours. The corresponding monoacid was obtained in 69% yield with the low ee value of 31%, but the problem of spontaneous hydrolysis was not mentioned (Scheme 4:22).



(Scheme 4:22)

Thus, it was decided to carry out the same reaction with diethyl cis-2,3-epoxysuccinate (40) with PLE at pH 7 and room temperature. In 4 hours, the reaction reached 50% hydrolysis and, after isolation and purification by Kugelrohr distillation, the monoethyl 2,3-epoxysuccinic acid (57) was analyzed by ^1H NMR in presence of 1 equivalent of (R)-(+)-α-methylbenzylamine for the determination of the enantiomeric purity[97]. An ee of 21% was obtained, which was much lower than the expected value of about 54.8% and because of this, the enzymatic hydrolysis of diethyl cis-2,3-epoxysuccinate (40) was not developed further.

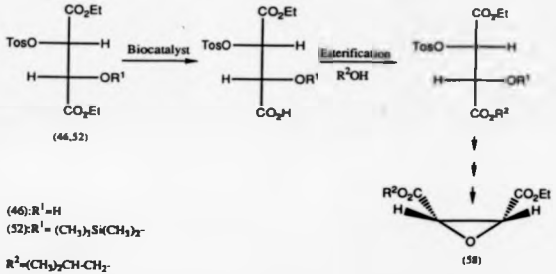
Again, a transesterification reaction seemed to be the answer and the screening of lipases of different origins was undertaken. However, no transesterification was observed, possibly due to the recognized inhibitor potential of epoxide compounds[98,99]. Indeed, racemic trans-epoxysuccinate derivatives have been reported to have an inhibitor action on some thiol proteases[100].

Consequently, the production of enantiomerically pure derivatives of cis-2,3-epoxysuccinic acid from diethyl cis-2,3-epoxysuccinate (40) by an enzymatic route seemed to be a difficult task to achieve.

4.4.2.2 Hydrolysis of diethyl 2-tosyl tartrate

Since the direct enzymatic resolution of diethyl cis-2,3-epoxysuccinate (40) seems to be difficult, a new indirect approach was investigated. This consisted of the regioselective enzymatic hydrolysis of one of the compounds preceding the formation of the cis-epoxide (40) i.e. diethyl 2-tosyl tartrate (46) or tert-butyl dimethyl silyl ether of diethyl 2-tosyl tartrate (52) which could be easily prepared enantiomerically pure from diethyl (2S,3S)-tartrate (36a) or diethyl (2R,3R)-tartrate (36b).

The monoacid obtained would then be esterified with isobutyl alcohol, leading to the production of an unsymmetrical diester of cis-epoxysuccinate in enantiomerically pure form (58) (Scheme 4:23).



(46): R^1=H
(52): R^1= $(CH_3)_2Si(CH_3)_2$-

R^2=$(CH_3)_2CH$-CH_2-

Hypothetical regioselective hydrolysis of enantiomerically pure compounds (46) or (52) for the preparation of enantiomerically pure derivatives of cis-epoxysuccinate(58).

(Scheme 4:23)

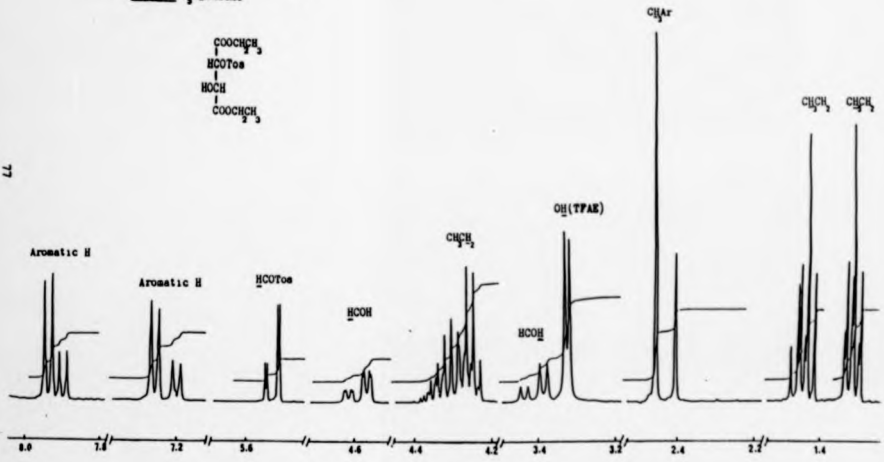
Lipase from Pseudomonas, PLE and α-chymotrypsin were assayed for hydrolysis against tert-butyldimethyl silyl ether of diethyl 2-tosyl-D,L-tartrate (52), which proved not to be a substrate for these enzymes presumably as a consequence of steric effects. Consequently we studied the hydrolysis of diethyl 2-tosyl-D,L-tartrate (46), keeping in mind that the purpose of this experiment was principally the identification of a regioselective enzyme, each enantiomer of (46) being available by chemical synthesis starting from diethyl (2S,3S)- or (2R,3R)- tartrate (36).

At 25°C, in phosphate buffer at pH 7 using α-chymotrypsin as biocatalyst, the hydrolysis of diethyl 2-tosyl-D,L-tartrate (46) was followed by the volume of NaOH added from an automatic autotitrator. The reaction was stopped at about 25% hydrolysis of the ester groups. The non-hydrolyzed starting material (46) and the product of hydrolysis were obtained by extraction of the reaction mixture with ethyl acetate at pH 7 and 2 respectively. The starting material was purified by flash column chromatography, eluting with petrol-ethyl acetate (8/2), and was obtained in 48% yield. Using 2 equivalents of the chiral solvating agent (S)-(+)-TFAE (39) in carbon tetrachloride, the 400 MHz ^{1}H NMR was recorded and showed that a mixture of enantiomers was obtained with baseline splitting of all the protons of the diethyl 2-tosyl-D,L-tartrate (46) except the ethyl ester protons, which were only partially resolved (Figure 4:4). This indicated that from a special arrangement the tosyl group interacted with (S)-(+)-TFAE (39), producing the unexpected splittings. The ee values were determined from the signal corresponding to the methyl substituent of the tosyl group because its simplicity (singlet) and intensity resulted in the greatest accuracy of the measurement (Table 4:4).

The crude monoacid, collected in 44% yield, was reesterified to diethyl 2-tosyl-tartrate (46) in 57% yield using dicyclohexylcarbodiimide (DCC) in the presence of 1-hydroxybenzotriazole hydrate (HOBT), which by formation of highly active esters increased the rate of the reaction and consequently has been used as a racemization-suppressing agent in peptide synthesis[101]. The ee could then be determined from the chiral solvating agent ^{1}H NMR experiment (Table 4:4).

(400MHz) ^{1}H N.M.R with (+)-TFAE in CCl_4

Ref.Std:d_6-Benzene



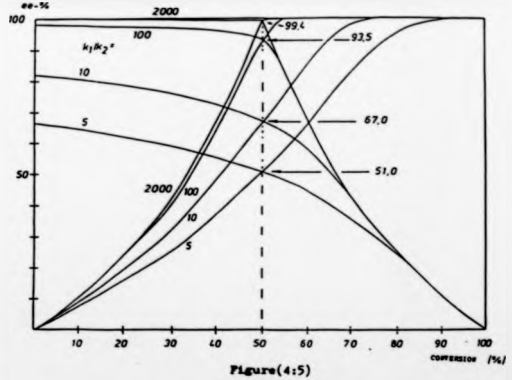
Figure(4:4)

Expt	conversion (%)	nonhydrolyzed starting material (44)		Product of hydrolysis		Calc. of E from eq. 3	
		Yield (%)	ee (%)	crude yield (%)	ee (%)	for SM(44)	for P
1	24.7	47.9	58.8	44.0	65.4	7.2	9.1
2	26.3	47.3	69.4	44.4		8.8	
3	31.2	39.9	>97			14.9	

Hydrolysis of diethyl 2-tosyl-D,L-tartrate by α-chymotrypsin at varying degrees of conversion.

(Table 4:4)

From Table (4:4) one can see that the ee of the non hydrolyzed starting material increased with the extent of the hydrolysis and consequently it could be obtained in enantiomerically pure form (experiment 3, Table (4:4)), although at the expense of the yield; while the ee of the product would continuously decrease. The variation of the ee values for product and non-hydrolyzed starting material with the degree of hydrolysis can be estimated from the enantiomeric ratio E of the reaction using the graph (Figure 4:5) which resulted from Sih et al's quantitative analysis of the kinetics of enzymatic resolutions of enantiomers[102].



Figure(4:5)

Considering a simple mechanism (equation 1) in which R was the faster reacting enantiomer, the "enantiomeric ratio" E was deduced (equation 3) from the integration of equation 2 corresponding to the steady state kinetics applied to the system (equation 1)

$$\left.\begin{array}{l} R + E \underset{k_2}{\overset{k_R}{\rightleftharpoons}} ER \xrightarrow{k_2} EP \xrightarrow{k_4} E + P \\ S + E \underset{k_2'}{\overset{k_S}{\rightleftharpoons}} ES \xrightarrow{k_3'} EP \xrightarrow{k_4'} E + Q \end{array}\right| \quad \text{equation 1}$$

$$\frac{v_R}{v_S} = \frac{V_R}{V_S} \cdot \frac{K_S}{K_R} \cdot \frac{[R]}{[S]} \qquad \text{equation 2}$$

in which v_R, v_S = partial reaction rates

V_R, V_S = maximal velocities

K_S, K_R = Michaelis constants

$$E = \frac{V_R/K_R}{V_S/K_S} = \frac{\ln[(1-c)(1-ee(S))]}{\ln[(1-c)(1+ee(S))]} \qquad \text{equation 3}$$

where "conversion ratio" $c = 1 - \dfrac{(R + S)}{(R_0 + S_0)}$

Also, the relationship between c, ee (SM) and ee (P) could then be established

By definition ee (SM) = $\dfrac{[S] - [R]}{[R] + [S]}$ ee (P) = $\dfrac{[P] - [Q]}{[P] + [Q]}$

and $[R] + [S] = 1-c$ $[P] + [Q] = c$

$$\Longrightarrow \quad \frac{ee(SM)}{ee(P)} = \frac{c}{1-c} \qquad \text{equation 4}$$

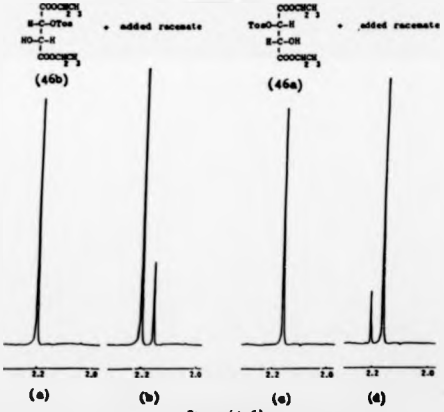
This equation (4) made it possible to check the result obtained from experiment (1) (Table 4:4); c was deduced to be equal to 47.3% when starting material (SM) and

product (P) had the ee values of 58.8 and 65.4% respectively, which was in quite reasonable agreement with the experimental value of 49.4%. Furthermore E was calculated from equation (3) to be 8.6, a value which was double-checked with the experimental data (Table 4:4).

The assignment of the two singlets obtained by ^1H NMR for the methyl substituent of the tosyl group in the case of the racemic diethyl 2-tosyl-tartrate (46) was easily achieved by preparation of each enantiomer from diethyl (2S,3S)- or (2R,3R)-tartrate by the route involving cyclic stannylene derivatives (see Section 4.3.2). Both were obtained as colourless oils in 95% yield (diethyl 2-tosyl-D,L-tartrate was a crystalline solid). From the chiral solvating agent experiment using a 400 MHz ^1H NMR instrument, the splitting of the methyl group was observed only when the racemate was added. It was concluded that the diethyl 2-tosyl-(2R,3R)-tartrate (46b)gave the downfield signal and upfield signal was assigned to the diethyl 2-tosyl-(2S,3S)-tartrate (46a) (Figure 4:6).

(400MHz) ^{1}H R,H,R with (+)-TFAE in CCl_4

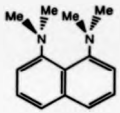
Ref.Std:d_6-Benzene



Figure(4:6)

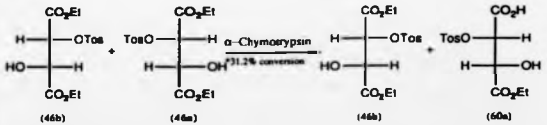
After the hydrolysis of diethyl 2-tosyl-D,L-tartrate (46) by α-chymotrypsin the analysis by ^1H NMR in presence of (S) - (+) - TFAE (39) of the non hydrolyzed starting material (if not optically pure) and product (after reesterification with ethanol) gave for the methyl signal of the tosyl group spectra identical to (b) and (d) in Figure (4:6) respectively. Consequently, it was concluded that α-chymotrypsin exhibited an enantioselective preference for the (2S,3S)-enantiomer (46a).

The determination of the regiospecificity of this reaction was also achieved by re-running the ^1H NMR spectrum of the crude monoacid obtained from the enzymatic hydrolysis in the presence of 1 equivalent of the proton sponge (59) which with its remarkably strong basic character [103] ionised the free acid group. In the ^1H NMR spectrum this ionisation resulted in an upfield shift from 5.40 to 5.20 ppm of the signal corresponding to the methine proton of the carbon bearing the tosylate group. The signal at 4.78 ppm corresponding to the other methine proton remained unchanged. Consequently, it was concluded that α-chymotrypsin cleaved regiospecifically the ester group adjacent to the tosylated hydroxyl group of the diethyl-tartrate.



(59)

The results obtained for this biocatalyzed reaction are summarized in Scheme (4:24)

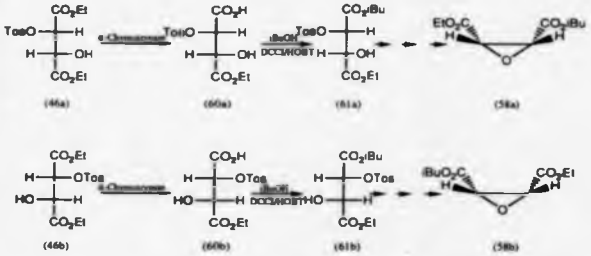


*Exp 3 (see table 4:4)

(Scheme 4:24)

4.4.2.3 Preparation of enantiomerically pure 1-ethyl 4-isobutyl cis-(2,3)-epoxysuccinate

It was expected that if diethyl 2-tosyl-(2S,3S)-tartrate (46a) and diethyl 2-tosyl-(2R,3R)-tartrate (46b) were treated separately with α-chymotrypsin, the corresponding monoacids (60a) and (60b) would be obtained in 100% yield and lead to 1-ethyl 4-isobutyl (2S,3R)-epoxysuccinate (58a) and 1-ethyl 4-isobutyl (2R,3S)-epoxysuccinate (58b) respectively (Scheme 4:25).



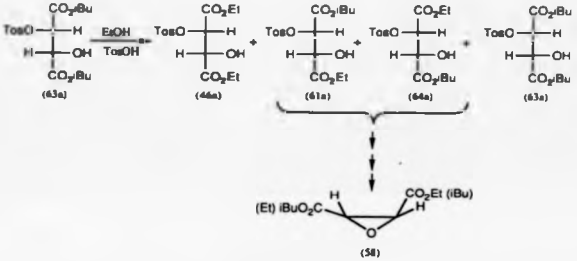
(Scheme 4:25)

Being the "faster" enantiomer to be hydrolyzed by α-chymotrypsin, diethyl 2-tosyl-(2S,3S)-tartrate (46a) was chosen for the initial experiment.

The scaling up of the hydrolysis reaction was easily done. In 21 hours at a temperature of 35°C, with α-chymotrypsin (210 mg; 10710 units), the compound (46a) (4.07 g; 11 mmol) was hydrolyzed to give in 92% crude yield the corresponding monoacid (60a). Reesterification of (60a) with isobutyl alcohol as described in Section (4.4.2.2) using DCC with HOBT afforded the mixed ester of 2-tosyl (2S,3S)-tartrate (61a) in 48% yield after column chromatography. Formation of the tert-butyldimethylsilyl ether intermediate (62a) (in 98% yield), followed by treatment with Bu_4NF (Section 4.3.5.) gave 1-ethyl 4-isobutyl (2S,3R)-epoxysuccinate (58a) as a

colourless liquid in 81% yield. Since no enantiomerically pure diesters of cis-2,3-epoxysuccinate have been reported, the specific rotation $[\alpha]_D^{24}$ = + 3.33 (c = 3.225, $CHCl_3$) did not give any indication of the enantiomeric purity, which had to be checked by another means. Again, the chiral solvating agent ^1H NMR experiment seemed the ideal tool. But first, the racemate of ethyl isobutyl cis-(2,3)-epoxysuccinate (58) was needed.

Diisobutyl 2-tosyl-(2S,3S)-tartrate (63a) was previously obtained by attempting to re-esterify with isobutyl alcohol the monoacid (60a) using as catalyst the ionic exchange resin Dowex 50W-X8(H^+ form). Thus, synthesis of the racemate seemed possible as indicated in (Scheme 4:26).



(Scheme 4:26)

Transesterification of compound (63a) with ethanol was realized in the presence of p-toluenesulfonic acid, a more powerful catalyst than the ionic exchange resin. This reaction could be followed by Tlc and was stopped when under UV detection, ethyl isobutyl 2-tosyl-tartrate was estimated in greater quantity than the corresponding diisobutyl and diethyl esters. The ethyl isobutyl 2-tosyl tartrate was isolated in 58% yield and then could be converted to the ethyl isobutyl cis-2,3-epoxysuccinate which had a specific rotation of $[\alpha]_D^{22}$ = + 2.98 (c = 1.775, $CHCl_3$).

Two conclusions could be drawn:

- Chemical transesterification of compound (63a) was to a great extent regioselective

or

- The epoxide (58a) obtained was of low enantiomeric purity leading to small specific rotation.

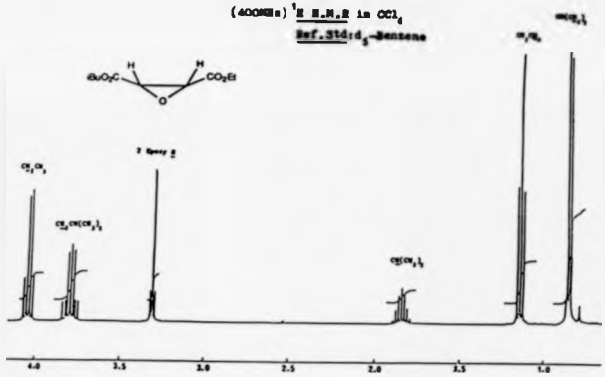
At this stage the same synthesis was carried out with the racemate of diethyl 2-tosyl tartrate (46). Ethyl isobutyl cis-(2,3)-epoxysuccinate obtained had a $[\alpha]_D^{22}$ = 0.00 (c = 4.49, $CHCl_3$). A 400 MHz ^1H NMR of the compound was recorded and the assignments were as follows (Figure 4:7a)

^1H NMR, 400 MHz (CCl_4, d_6-benzene/d_5-benzene = δ 7.20 ppm) : δ = 0.85 (d of d, 6H, $CH(CH_3)_2$; 1.15 (t, 3H, J = 7.14 Hz, CH_2CH_3); 1.79 - 1.89 (m, 1H, $CH(CH_3)_2$) 3.29-3.32 (AB system, CH-O-CH); 3.75 - 3.84 (m, 2H, CH_2CH); 4.04 ppm (q, 2H, J = 7.14 Hz, CH_2CH_3).

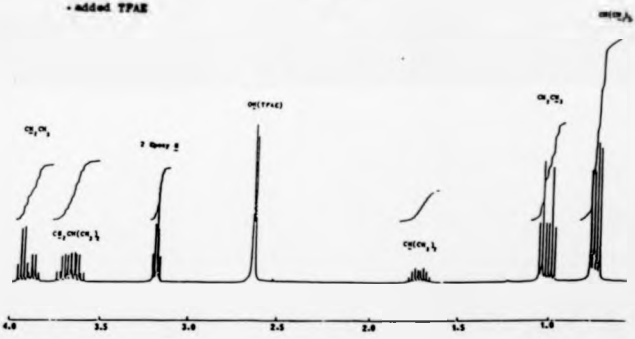
The spectrum was re-run in the presence of 4 equivalents of (S) - (+) - TFAE (39). In this case, splitting of the epoxy hydrogens was incomplete but the signals corresponding to the ethyl ester and methyl of the isobutyl group were baseline separated and consequently the ee values were deduced from them (Figure 4:7b). The 2 epoxides made previously were tested in the same conditions.

The epoxide with $[\alpha]_D^{24}$ = + 3.33 (c = 3.225, $CHCl_3$) was found to be enantiomerically pure with an ee of ≥ 97%. Indeed, the splitting of signals was observed only when some racemate was added (Figure 4:8a). This permitted assignment of the upfield triplet and quartet corresponding to the ethyl ester of 1-ethyl 4-isobutyl (2S,3R)-epoxysuccinate (58a) but the doublet of doublets corresponding to the methyl of the isobutyl group of this compound corresponded to the downfield signals (Figure 4:8b).

On the other hand, the epoxide with $[\alpha]_D^{22}$ = + 2.98 (c = 1.775, $CHCl_3$) was found to have an ee of 94% (Figure 4:9). The major enantiomer being 1-ethyl 4-isobutyl



7a

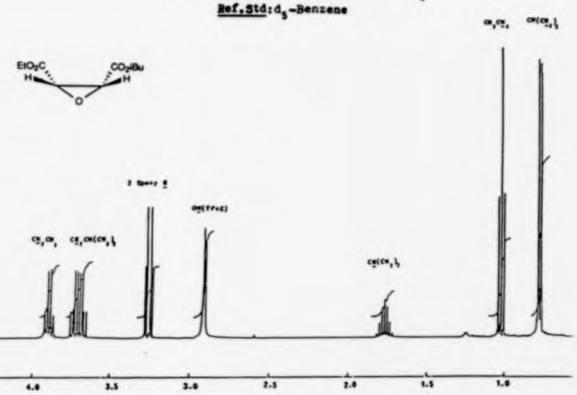


7b

Figure (4:7)

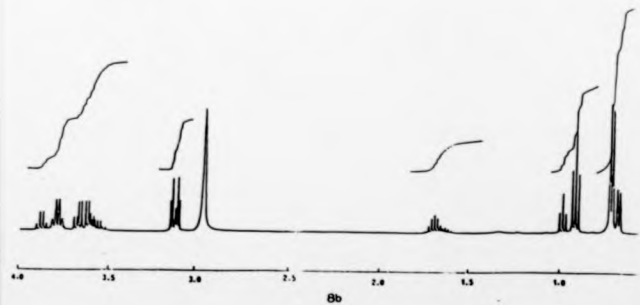
(400MHz) ^{1}H N.M.R with (+)-TFAE in CCl_4

Ref.Std:d_3-Benzene



8a

•added racemate

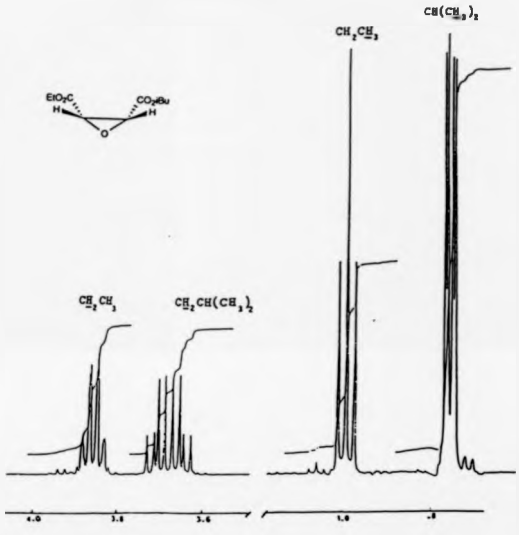


8b

Figure(4:8)

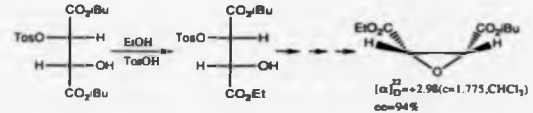
(400MHz) ^{1}H N.M.R with (+)-YPAE in CCl_4

Ref.Std: d_6-Benzene



Figure(4:9)

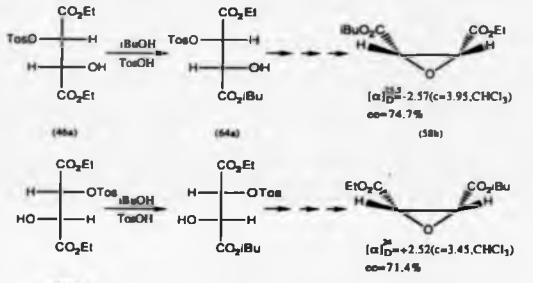
(2S, 3R)-epoxysuccinate (58a), it was consequently deduced that the transesterification with ethanol of compound (63a) occurred regiospecifically at the ester group next to the carbon bearing the hydroxyl group (Scheme 4:27)



(63a) (61a) (58a)

(Scheme 4:27)

Further experiments were carried out to check the validity of the method. Rotations and ee values were measured and reported in (Scheme 4:28).



(46a) (64a) (58b)

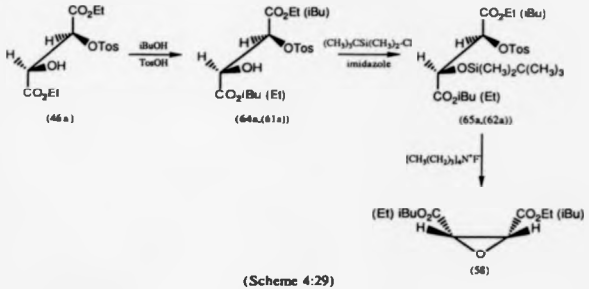
(46b) (64b) (58a)

(Scheme 4:28)

Thus, mixed esters of 2-tosyl (2S,3S)- or (2R,3R)-tartrate obtained by chemical transesterification led to epoxides with ee values ranging from 71% to 94%. This variability was certainly a direct consequence of the conditions of the transesterification reactions, which varied slightly from experiment to experiment.

It should be pointed out that one could obtain an estimate of the enantiomeric purity of the epoxide to be produced if the ^{13}C NMR of the tert-butylsilyl ether

intermediate (Scheme 4:29) was examined. When a mixture of compounds (65a, 66a) were obtained, a set of two singlets for each carbon of the ester groups appeared on the ^{13}C NMR (The height of each singlet reflects the quantity of each compound in the mixture).



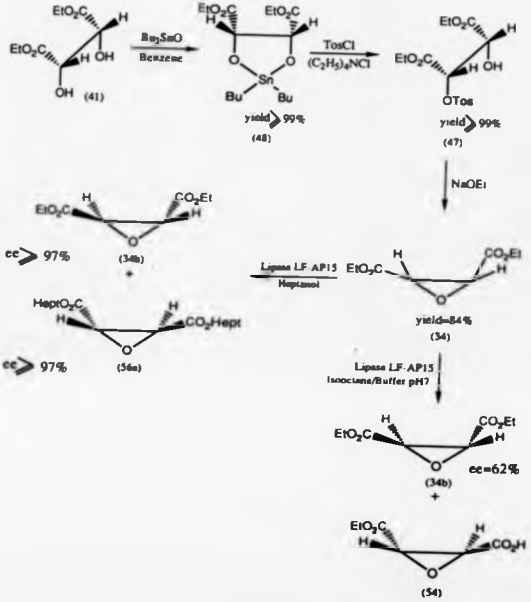
(Scheme 4:29)

4.5 CONCLUSION

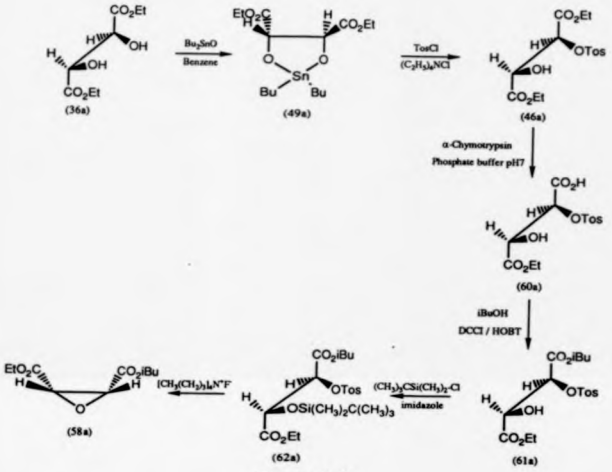
This investigation provides a more convenient method for production in high yield of diethyl cis- or trans-2,3-epoxysuccinates than those reported in the literature (see Section 2.1)

Moreover, it underlines the valuable application of enzymes in asymmetric synthesis, which in this case led to epoxides with ee ≥ 97%, compared to the chemical route which involved great difficulty or the impossibility of achievement of the same goal. The significant achievement was the production of an enantiomerically pure diester of cis-2,3-epoxysuccinate which so far had been reported to have failed[97] or proven to be very difficult[104]. This epoxide has received a lot of attention because it had been recognized as a potential chiron precursor for the gypsy moth sex pheromone disparlure[105] and as a valuable carbopenem precursor[97,99].

The syntheses accomplished successfully during this investigation are outlined in Schemes 4:30) and (4:31).


(41)
Bu2SnO
Benzene
(48)
yield > 99%
TosCl
(C2H5)4NCl
(47)
yield > 99%
NaOEt
(34b)
ee > 97%
+
(56a)
ee > 97%
Lipase LF AP15
Heptanol
yield=84%
(34)
Lipase LF AP15
Isooctane/Buffer pH7
(34b)
ee=62%
+
(54)

(Scheme 4:30)



(Scheme 4:31)

As a result, it is now undoubtedly possible to obtain from these optically pure epoxides L-malic acid specifically deuterated at C_3.

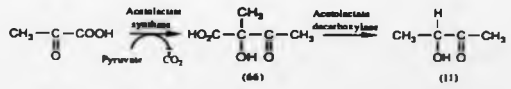
CHAPTER 5

BIOSYNTHESIS OF ACETOIN

5.1 MODE OF ACTION OF PYRUVATE DECARBOXYLASE

Acetoin (3-hydroxy-2-butanone) (11) is a four carbon chiral ketone produced by microorganisms (fungi[106,107,108] or bacteria[109,110,115]), higher plants[111] and animal tissues[112]. Its metabolism involves thiamine pyrophosphate (TPP) dependent enzymes, the α-carboxylases.

In the bacterium Aerobacter aerogenes[111,113,114], acetoin is produced from pyruvic acid (Scheme 5:1) primarily *via* decarboxylation of α-acetolactic acid (66), an intermediate in the biosynthesis of valine. At the time of the initial experiment, the acetoin produced was assumed to be enantiomerically pure based on a value for the optical rotation for (R)-acetoin of -84 ± 3°.



(Scheme 5:1)

In contrast, in yeast[107,116] or wheat germ[111,117,118], it is produced from pyruvic acid and/or acetaldehyde as a mixture of enantiomers by a pathway not involving α-acetolactic acid. This latter case is related to our work with the aim to shed light on the mechanism of action of the yeast carboxylase enzyme. This has been subject to lengthy discussion and still remains obscure.

At first, it was suggested that two enzymes were involved in the synthesis of acetoin in yeast[111].

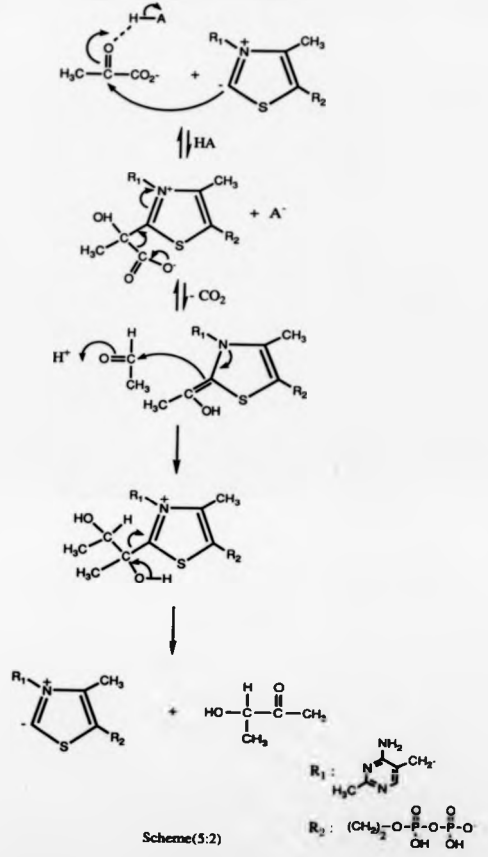
(1) A α-carboxylase which decarboxylates pyruvate to form α-hydroxyethylthiamine pyrophosphate ("active acetaldehyde") (67) and

(2) a carboligase which accomplishes the condensation of "active acetaldehyde" with acetaldehyde to form acetoin (Scheme 5:2).

However, investigations on the α-carboxylases extracted from wheat germ[111,118] and yeast[118] led to the conclusion that the α-carboxylase and the carboligase were one and the same as seen in their pH optima, requirements for cofactors such as diphosphothiamine and Mg^{2+}, purification and inactivation studies.

In addition, Singer and Pensky[111,117] produced two possible explanations for the partial asymmetric synthesis of acetoin by α-carboxylase extracted from wheat germ. The first is that it is due to the flexibility in the partial relationship during approach of the molecule of acetaldehyde to the active acetaldehyde. The second explanation requires the presence on the enzyme of several active centres which produce laevorotatory or dextrorotatory acetoin, the ratio of enantiomers being the result of the action of all these centres.

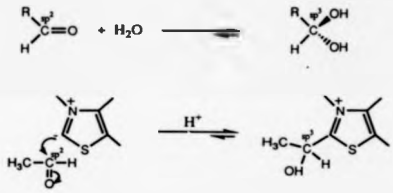
More studies[119] have supported the hypothesis of the presence of several active centres because of the dependence of the enantiomeric ratio on the relative concentrations of the substrates, acetaldehyde and pyruvate. It was suggested that one site produces a racemate and the other enantiomerically pure acetoin.



Scheme(5:2)

Previously, Juni[116] had proposed a two site- mechanism for the α-carboxylase extracted from yeast. One site permits the decarboxylation of pyruvate to form "active acetaldehyde" which can then be directly bonded to free acetaldehyde to form acetoin. Alternatively, the "active acetaldehyde" can be irreversibly transferred to the second site where it can be reversibly dissociated from the enzyme to yield free acetaldehyde. This can explain why with acetaldehyde alone as substrate, acetoin is not formed.

The lack of production of acetoin from acetaldehyde alone has also been explained by Schellenberger[120], by deduction of an analogy between TPP catalysis and hydration-dehydration equilibria. In the latter, the position of equilibrium is governed by the less stable sp^2 going to a more stable sp^3 configuration. The same idea can be applied to TPP catalysis, thus, making the release of acetaldehyde from TPP rather difficult (Scheme 5:3).



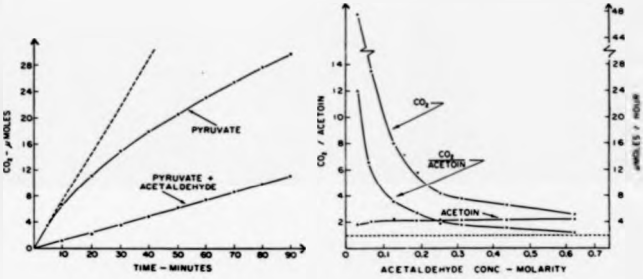
(Scheme 5:3)

Juni's conclusions have been drawn from different experiments which, although they do not provide a full description of the mechanism, reveal some interesting characteristics of the enzyme.

It has been observed that the rate of decarboxylation of pyruvate decreases with time as acetaldehyde accumulates. When pyruvate and acetaldehyde are added together

initially, the rate of decarboxylation is considerably inhibited, but remains constant with time (Figure 5:1a). If acetaldehyde is removed, the rate of decarboxylation increases again, demonstrating that acetaldehyde is inhibiting the enzyme. Moreover, the percentage of inhibiton stays constant over a wide range of pyruvate concentration indicating that acetaldehyde acts as a noncompetitive inhibitor.

Similarly, over a wide range of acetaldehyde concentration, the rate of formation of acetoin remains constant but the rate of decarboxylation falls rapidly and approaches the rate of synthesis of acetoin (Figure 5:1b).



(Figure 5:1)

However, this theory proposed by Juni does not explain why the enzyme produces a mixture of enantiomers and not an optically pure compound. This can be the consequence, as in the case of the wheat germ enzyme[119], of the relative concentration of acetaldehyde and pyruvate. To support this argument more investigations must be undertaken for which an important prerequisite is the development of an analytical method for estimating the enantiomer composition of

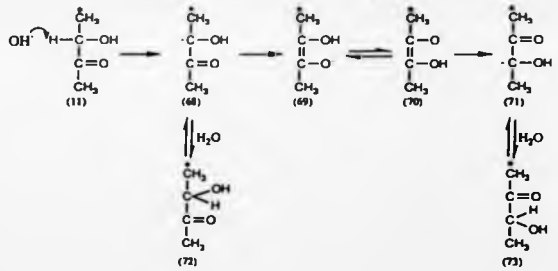
enzymatically produced samples of acetoin.

5.2 ANALYSIS OF ENANTIOMERIC MIXTURE OF ACETOIN

The analytical method to be developed must be accurate, reliable and sensitive in order to be suitable for small quantities of acetoin. Moreover, it must be mild enough to remove the possibility of racemization of this ketol. Racemization, which presumably occurs *via* the formation of an enediol or enediolate anion, is catalyzed in basic or acidic conditions.

- Base catalyzed epimerization

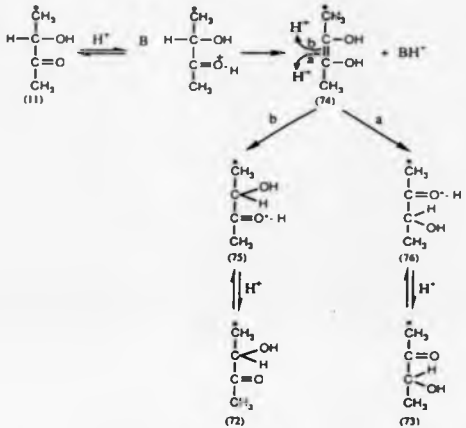
The abstraction by a base of the acidic proton on the carbinol carbon of acetoin (11) leads to an arrangement giving two carbanions (68 and 71) in equilibrium with their enediolates (69 and 70). The carbanions are different with respect to the labelled methyl and by protonation give a racemic mixture of acetoin (72 and 73) (Scheme 5:4).



(Scheme 5.4)

-Acid-catalyzed epimerisation

This reaction starts with the protonation of the ketone group of the acetoin (11) followed by abstraction of an α-proton leading to an enediol (74). The latter yields two different labelled protonated intermediates (75, 76) which by deprotonation give two different racemic mixtures (72, 73) (Scheme 5:5).

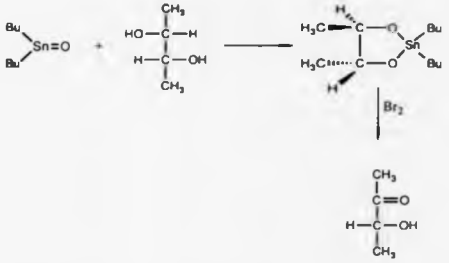


(Scheme 5:5)

This possibility of epimerisation can cause problems in the elaboration of the analytical method. However, its validity can be checked easily since the value for the specific optical rotation of the acetoin has been determined unambiguously by chemical synthesis[121] of enantiomerically pure acetoin.

The synthesis involved the formation of a cyclic stannylene from enantiomerically pure butane-2,3-diol and dibutyltin oxide. This organotin intermediate was oxidised by

bromine to give dibromodibutyltin and the corresponding enantiomerically pure acetoin (Scheme 5:6).



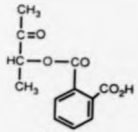
(Scheme 5:6)

(R)- and (S)-Acetoin were produced enantiomerically pure from (2R, 3R)- and (2S, 3S)-butane-2,3-diol respectively. The specific rotation, in aqueous solution, has been determined to be:

$[\alpha]_D^{2.5}$ = +82 ± 3° (c = 0.5, water) for the (S)-acetoin

and $[\alpha]_D^{27}$ = -84 ± 3° (c = 0.5, water) for the (R)-acetoin.

An isotope dilution procedure[121] has been previously developed for estimating the enantiomer composition of acetoin samples. This involved incubation of [^{14}C] pyruvate with α-carboxylases of different origins. The radiolabelled acetoin isolated was converted into the acid phthalate (77) and recrystallized to constant radioactivity as the dicyclohexylammonium salt. The latter was then subjected to a reverse isotope dilution analysis by the addition of inactive, previously resolved dicyclohexylammonium salt of the acid phthalate of (+)- or (-)- acetoin.



(77)

Although this method was accurate and reliable, it was rather time consuming. Moreover, it required the availability of different materials such as ^{14}C pyruvate and resolved (+)- or (-)- acid phthalate salts of acetoin for its application.

5.3 CONCLUSION

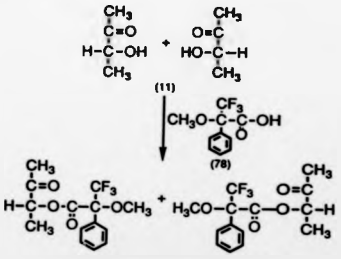
Before exploring the mode of action of pyruvate decarboxylase, it was necessary to devise a method which eliminates all the inconveniences of the isotope dilution procedure. Covalent diastereomers such as esters may be separated by chromatographic techniques or distinguished by NMR. properties which were to be exploited in the development of the analytical method.

CHAPTER 6

ENANTIOMERIC ANALYSIS OF ACETOIN

6.1 INTRODUCTION

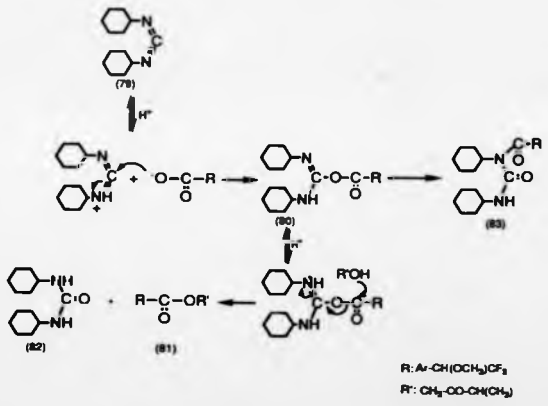
The enantiomeric composition of alcohols has often been determined indirectly *via* the formation of diastereoisomeric esters[122,123] which could then be quantified using techniques such as HPLC, GLPC and NMR. These esters have been prepared by the use of optically pure derivatising reagents such as Mosher acid ((S)-(-)-α-methoxy-α-(trifluoromethyl)phenyl acetic acid) (78), which was intended to be used to prepare the diastereoisomeric esters of acetoin (11) (Scheme 6:1). The latter were expected to be separable by HPLC for the evaluation of the enantiomeric ratio of acetoin.



(Scheme 6:1)

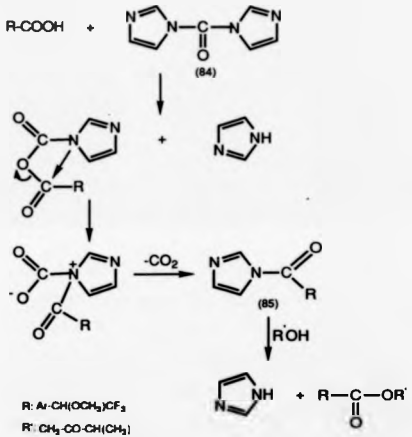
6.2 PREPARATION OF THE ESTER (S)-(-)-α-METHOXY-α-(TRIFLUOROMETHYL)PHENYL ACETATE OF ACETOIN

As mentioned in Chapter 5, these esters must be prepared cautiously in order to minimize the risk of racemization of acetoin. Therefore, it was decided to use a dehydrating agent, N,N'-dicyclohexylcarbodiimide (DCC) (79) which promotes esterification reactions under mild conditions[124]. The mechanism seems to involve the formation of the reactive intermediate O-acylisourea (80) as the result of the reaction between DCC and the carboxylic acid used ((S)-(-)-α-methoxy-α-(trifluoromethyl)phenyl acetic acid). This intermediate (80) is then converted to the ester (81) by nucleophilic attack of the alcohol producing as by-product the dicyclohexylurea (82). Alternatively, it can eventually afford, by intramolecular rearrangement, the N-acylurea (83) which lowers the yield and consequently reduces the sensitivity of the analytical method (Scheme 6.2). This side reaction has proved to be favoured at elevated



(Scheme 6.2)

temperatures and consequently the reaction was carried out at 0^{o} C. The Mosher ester of acetoin was obtained in 70% yield when reacting DCC, Mosher acid and acetoin in a ratio of 2/2/1 respectively. However, due to the difficulties encountered for its purification and with the aim of improving the yield, it was decided to use 1,1'-carbonyldiimidazole (84) as coupling agent instead of DCC. Staab[125] has showed that 1,1'-carbonyldiimidazole reacts with carboxylic acid to yield imidazolide (85) which coupled with alcoholysis affords esters under particularly mild conditions (Scheme 6:3).

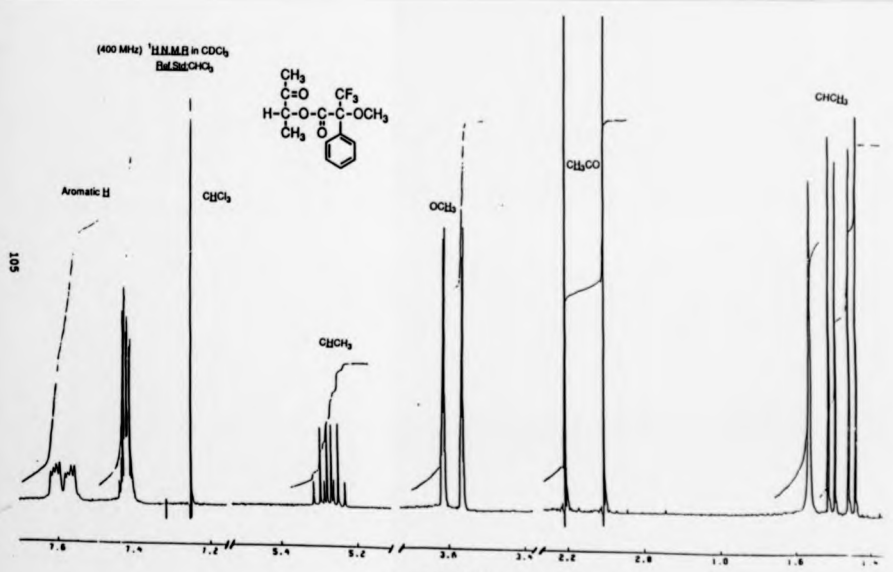


(Scheme 6:3)

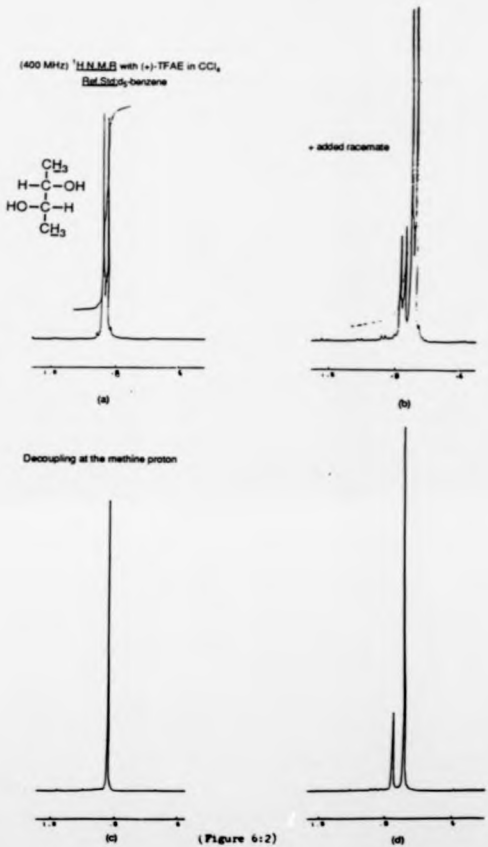
Despite no real improvement of the yield, the purification by flash chromatography led to purer Mosher ester than previously with the DCC method. The separation of the diastereoisomers was then achieved by HPLC using a reverse phase column. However, for accurate determination of the diastereomeric ratio, purification of the sample on silica gel was necessary, thus leading to the possibility of fractionation. This purification was also required for the analysis using the ^{1}H NMR spectrum which showed splitting of methyl, methoxy and acetyl signals in the case of a diastereomeric mixture (Figure 6:1). Consequently, ^{19}F NMR was used instead as it permitted the analysis on the crude ester directly. The diastereomeric ratio could be determined from the fluorine resonances which are reliable since the signals are simple and in an uncongested region.

Racemic acetoin was used for the elaboration of the method and enantiomerically pure (R)-(-)-acetoin was prepared from (2R,3R)-butanediol *via* the organotin intermediate (see Scheme 5:6), with the aim of checking if racemization occurs during esterification.

It is noteworthy to mention that the isomeric purity of (2R,3R)-butanediol was checked before use. The presence of the meso-compound could be easily detected by recording a ^{13}C NMR spectrum. On the other hand, the enantiomeric ratio could be determined directly by ^{1}H NMR in presence of 1.5 equivalents of the chiral solvating agent (S)-(+)-2,2,2-trifluoro-1-(9-anthryl)-ethanol (39) (Figure 6:2a). The spectrum was re-recorded in the presence of added racemic (2,3)-butanediol, which showed a splitting of the methyl signal (Figure 6:2b). Decoupling at the methine signal led to a singlet for the methyl groups (Figure 6:2c, d) and consequently an accurate direct method for the measurement of the enantiomeric ratio of (2,3)-butanediol.


(400 MHz) ¹H N.M.R in CDCl₃
Ref.Std:CHCl₃
Aromatic H
CHCl₃
CHCH₃
OCH₃
CH₃CO
CHCH₃
105
7.6
7.4
7.2
5.4
5.2
3.6
3.4
2.2
2.0
1.8
1.6

Figure(6:1)


(400 MHz) ¹H.N.M.R with (+)-TFAE in CCl_4
Ref.Std:d_6-benzene
CH_3
H–C–OH
HO–C–H
CH_3
+ added racemate
(a)
(b)
Decoupling at the methine proton
(c)
(d)

(Figure 6:2)

Results of the different experiments carried out with DCC and carbonyldiimidazole, as well as the ^{19}F spectra are reported in Table (6:1) and Figure (6:3) respectively.

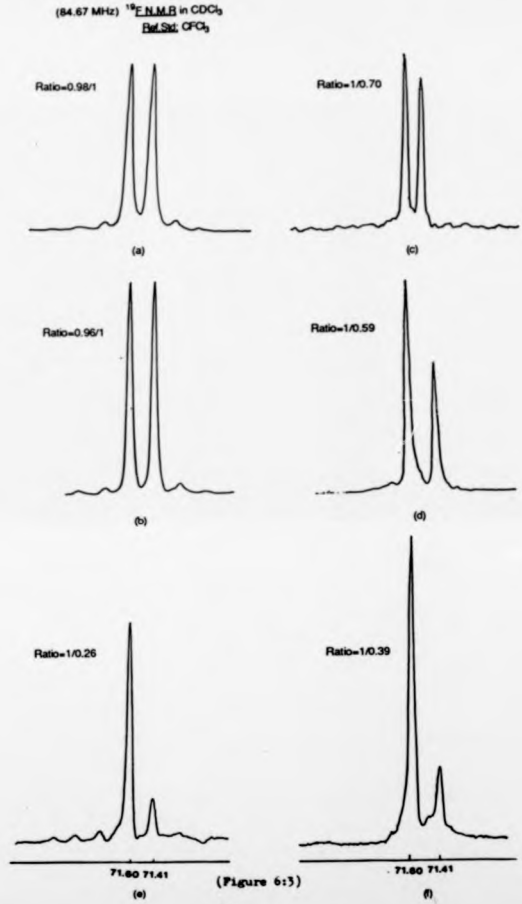
	Reagents		Ratio	Diastereoisomeric ratio		Spectrum reported in (Figure 6:3)
	acid	Coupling agent	acetoin/reagent	Experimental	Theoretical	
Racemic acetoin	Mosher Acid	1,1'-carbonyl diimidazole	2/1	0.96:1	1:1	a
			1/2	0.98:1	1:1	e
		DCC	2/1	1.02:1	1:1	d
			1/2	1.03:1	1:1	c
R-(-)-acetoin		1,1'-carbonyl diimidazole	1/2	1.02:1	0:1	e
		DCC	1/2	1.62:1	0:1	f

Determination by ^{19}F NMR of the diastereoisomeric ratio of samples of (S)-(-)-α-methoxy-α-(trifluoromethyl)phenyl acetate of acetoin.

Table (6:1)

Using racemic acetoin, the diastereomeric ratio should have been 1:1. With 1,1'-carbonyldiimidazole as the coupling agent, and considering the experimental error, the ratio obtained was in good agreement. However, with DCC the ratio changed significantly when acetoin was used in excess. This result could be explained in terms of asymmetric induction making the method using DCC unreliable.

Moreover, when using enantiomerically pure acetoin, a racemization with both coupling agents took place (up to 28%) as the two diastereoisomers of the Mosher esters were detectable by ^{19}F NMR (Figure 6:3 e,f). This evidence ruled out the possibility of use of these indirect methods for the analysis of the enantiomeric ratio of acetoin.


(84.67 MHz) ^{19}F N.M.R in $CDCl_3$
Ref.Std: $CFCl_3$
Ratio=0.98/1
(a)
Ratio=1/0.70
(c)
Ratio=0.96/1
(b)
Ratio=1/0.59
(d)
Ratio=1/0.26
71.60 71.41
(e)
Ratio=1/0.39
71.60 71.41
(f)

(Figure 6:3)

CHAPTER 7

EXPERIMENTAL

7.1 GENERAL NOTES

Nuclear magnetic resonance spectra were recorded using the instruments listed below operating at the frequencies given in the table:

Spectrometer	Frequency/MHz		
	^{1}H	^{13}C	^{19}F
Bruker WH400	400.13	100.62	
Bruker WH90		22.63	84.67
Perkin-Elmer R34	220		

Chemical shifts, unless otherwise stated, are quoted in ppm downfield from a tetramethylsilane internal reference.

Mass spectra were recorded using a Kratos MS 80 spectrometer.

Infra red spectra were recorded using a Perkin-Elmer 580-B spectrophotometer as either liquid films or NaCl discs.

Optical rotations wered recorded using an "Optical Activity Ltd." AA-1000 polarimeter at 589 nm in a 2 dm path length cell.

Vibrational infra red circular dichroism spectra wered recorded by Dr. A. F. Drake,

Birkbeck College, London, using an own-built spectrometer[60].

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

Gas-liquid chromatographic analysis was performed using a Pye 204 gas chromatograph. The columns used were 1.8 m length. Nitrogen was used as carrier gas at a flow rate of 30 ml/minute.

High pressure liquid chromatography was performed using a Gilson system comprising the following components: a model 302 piston pump, a model 802 C manometric module and a HM holochrome UV/Vis detector.

pH-stat experiments were performed using a Radiometer Copenhagen RTS 822 recording titration system.

Radioactivity measurements were made with an LKB RackBeta 1215 liquid scintillation counter using Packard 229 scintillation cocktail.

Thin-layer chromatography was performed on Merk Kieselgel F_{254} 0.2 mm precoated plates. Spot detection was by UV fluorescence quenching, iodine vapour or methyl red (0.02 g) in ethanol-water (60/40). Mobilities are quoted as R_F values where R_F = distance moved by substance/distance moved by solvent front.

Flash chromatography was performed on Merk Kieselgel 60 silica gel (230-400 mesh).

All solvents were distilled before being used. Ether refers to diethyl ether and petrol to the petroleum fraction boiling in the range 40-60° C .

The enzymes used were obtained from the sources given below and had the listed activities:

Enzyme	Source	Activity (eu/mg protein)
PLE (EC 3.1.1.1)	Boehringer	130
PPL (EC 3.1.1.3)	Sigma, Type 2	35-70
CCL (EC 3.1.1.3)	Amano	1,40-2,800
LAP 6	Amano	60
LF-AP 15	Amano	150
LP	Amano	30

7.2 EXPERIMENTAL SECTION TO CHAPTER 3

7.2.1 Penicillic acid fermentation

7.2.1.1 Preparation of medium for fermentation

Raulin Thom medium was used for these fermentations. Its composition was as follows:

Glucose	50 g
Tartaric acid	2.67 g
Ammonium tartrate	2.67 g
Diammonium hydrogen phosphate	0.40 g
Ammonium sulphate	0.17 g
Potassium carbonate	0.40 g
Magnesium carbonate	0.27 g
Ferrous sulphate heptahydrate	0.047 g
Zinc sulphate heptahydrate	0.047 g
Distilled water to 1 litre	

The medium was dispersed into cotton wool-plugged conical flasks for sterilisation by autoclaving at 121°C for 20 minutes (20 ml into 50 ml flasks and 40 ml into 100 ml flasks).

7.2.1.2 Preparation of inoculum

Penicillium cyclopium was grown on potato dextrose agar plate for long period storage (several months) at 4°C. Spores from one plate was used to inoculate Raulin-Thom media (2 x 20 ml) which was left to grow for 24 hours at 24°C.

7.2.1.3 Preparation of fermentation broth and isolation of penicillic acid (7)

Flasks containing Raulin-Thom media (40 ml/flask) were inoculated with the inoculum (0.8 ml) prepared as described above and were incubated at 24°C. The flasks used for the experiments were selected on the basis of maximum mycelial growth. After 13 days of growth, penicillic acid could be isolated as follows. The

fermentation broth was filtered to remove the mycelium and the filtrate (pH = 3) was extracted with chloroform. The chloroform phase was dried over sodium sulphate and after filtration and evaporation afforded a yellow oil which crystallized on long standing. Penicillic acid was then crystallized from chloroform-cyclohexane (1/3) giving a white cottony solid (mp: 83-84°C) lit[36] mp = 84°C.

^{1}H NMR, 220 MHz ($CDCl_3$/TMS): δ = 1.78 (3H, s, CH_3); 3.93(3H, s, OCH_3); 5.17 (1H, s, C = C(H)H); 5.25 (1H, s, HC-$COCH_3$); 5.52 ppm (1H, s, C = C(H)H).

7.2.1.4 Tracer experiment using radiolabelled aspartic acid

L-(3R)-[3^3H, 3^{14}C]-aspartic acid (0.644μC ^{14}C, 6.5 μC ^{3}H) dissolved in sterile water (6 ml) was distributed on the 5th day of growth between 6 flasks of fermentation broth. On the 13th day, crude penicillic acid (0.286g) was recovered which was then recrystallized from chloroform-cyclohexane (1/3) (0.19 g; mp = 83-84°C). Using the liquid scintillation counting technique, fractions of this sample were measured for radioactivity content. After a second recrystallisation, the radioactivity in penicillic acid being estimated to be constant, ^{14}C incorporation and ^{3}H retention could be deduced (see section 3.1).

7.2.2 Preparation of D,L-aspartic acid (16) *via* the formation of the benzylamine salt of N-benzyl-D,L-aspartic acid (25)

7.2.2.1 Preparation of unlabelled N-benzyl-D,L-aspartic acid

Preparation of benzylamine salt of N-benzyl-D,L-aspartic acid (25)

To a solution of maleic acid (2.00 g; 17.26 mmol) in water (3.5 ml) was added dropwise to avoid excessive heating a solution of benzylamine (3.70 g; 34.52 mmol) in water (2 ml). The mixture was gradually brought to reflux, held at reflux for one hour and allowed to cool down. On addition of acetone (200 ml) a heavy white precipitate appeared instantaneously. The mixture was left overnight and then the product, crude benzylamine salt of N-benzyl-D,L-aspartic acid, was filtered off and

dried under vacuum (3.70 g; 99%). mp = 163.5-164.5°C. This compound was not purified further.

Preparation of N-benzyl-D,L-aspartic acid

The crude benzylamine salt of N-benzyl-D,L-aspartic acid (2.18 g; 6.61 mmol) was dissolved in sodium hydroxide solution (15%; 30 ml). The benzylamine was extracted with diethyl ether (3 x 10 ml). The remaining alkaline solution was acidified with HCl to pH 1 and left at 2°C for a few days to crystallize (Crystallisation was actually difficult to obtain). White crystals were obtained (0.64 g; 43%) mp = 181-185°C. These were then recrystallized as described above, washed with water and dried over phosphorus pentoxide (0.03 mm Hg) at 80°C for several hours, to give pure N-benzyl-D,L-aspartic acid (0.53 g; 36%), mp = 184-186°C (lit[50,51] 194°C up to 209°C).

^{1}H NMR, 400 MHz(^2H_2O + K_2CO_3 45%/HO^2H = δ 4.75 ppm) : δ = 2.41 (1H, AB part of ABX syst, J_{AB} = 16.03 Hz, J_{BX} = 8.53 Hz; C_3-H_{proS}); 2.55 (1H, AB part of ABX, J_{AB} = 16.03 Hz, J_{AX} = 4.88 Hz, C_3-H_{proR}); 3.50 (1H, X of ABX syst, J_{AX} = 4.88 Hz; J_{BX} = 8.53 Hz; HCNH), 3.80 and 3.91 (2H, AB syst, J_{AB} = 12.82 Hz, CH_2Ar); 7.30-7.40 ppm (5H, m, Aromatic H).

IR (Nujol mull) : ν = 2940 brs (NH_2^+, OH); 2860 br s; 1740 m (CO), 1575 m 1595 cm^{-1} sh (CO).

MS (CI) : m/z = 224(MH)$^+$, 260, 178, 134, 108, 91.

Elemental analysis : Found C, 59.38; H, 5.93; N, 6.44. $C_{11}H_{13}NO_4$ requires C, 59.18; H, 5.87; N, 6.27.

For hydrogenolysis of this compound and NMR data of aspartic acid see sections (7.2.2.2) and (3.2) respectively.

7.2.2.2 Preparation of labelled aspartic acid (16)

From maleic acid (26)

. Preparation of benzylamine salt of N-benzyl-D,L-3[$^2H_1$] aspartic acid in ^2H_2O

For a maximum deuteration, the reactants were deuterated as follows:

A solution of freshly distilled benzylamine (10 g; 93.30 mmol) in benzene (25 ml) was shaken with deuterium oxide (99.8 atom%, 2 x 8 ml). The dideuteriobenzylamine was recovered by the separation of the deuterium oxide and removal of the benzene on a rotary evaporator.

The maleic acid (2.37 g; 20.40 mmol) was dissolved in deuterium oxide (99.8 atom%, 5 ml) and the solution was evaporated until dryness.

The dideuteriobenzylamine (4.59 g; 42.1 mmol) in ^2H_2O (3 ml) was added dropwise to deuterated maleic acid (2.41 g; 20.4 mmol) in ^2H_2O (2.5 cm^3).

The reaction was then carried out as indicated in section (7.2..1.1) to afford white crystals of crude benzylamine salt of N-benzyl-D,L-3[$^2H_1$]-aspartic acid (6.58 g; 97%) mp = 160-166^oC.

. Preparation of ammonium -D,L-3[$^2H_1$]-aspartate

Due to the difficulties encountered for the preparation of N-benzyl-D,L-aspartic acid, it was decided to use its benzylamine salt for the preparation of aspartate.

A solution of the benzylamine salt of N-benzyl-D,L-3[$^2H_1$]aspartic acid (3.29 g; 9.90 mmol) and palladium on charcoal (10%; 0.5 g) in glacial acetic acid was stirred at 60^oC under H_2 (atmospheric pressure) for 10 hours. The catalyst was filtered off, washed with formic acid and the combined filtrate and washings were evaporated under vacuum until dry. Crude D,L-3[$^2H_1$]-aspartic acid (1.99 g) was obtained. This was dissolved in water (100 ml) and was placed on a Dowex 50W-X8 (H^+ form) ionic exchange resin column (35 g). The column was eluted with an ammonia solution (4M). The fractions which gave a positive reaction with ninhydrin were evaporated under vacuum and finally freeze-dried. Ammonium-D,L-3[$^2H_1$]-aspartate

(1.24 g; 80.4%) was obtained.

Preparation of benzylamine salt of N-benzyl-D,L-3[$^2H_1$]-aspartic acid in dioxan

The reactants were prepared as follows:

A solution of freshly distilled benzylamine (4.51 g; 42.00 mmol) in benzene (10 ml) was shaken with deuterium oxide (99.8 atom%; 2 x 4 ml). The benzene solution was separated and dried by azeotropic distillation of benzene-water. Dioxan (20 ml) was added and distilled from the mixture until the distillate was pure dioxan. The dideuteriobenzylamine solution was then used immediately.

Maleic acid (2.37 g; 20.4 mmol) was dissolved in deuterium oxide (99.8 atom%; 5 ml). The solution was evaporated until dry and kept overnight under vacuum over phosphorus pentoxide.

The reaction was then carried out as indicated below:

The dideuteriobenzylamine in dioxan (20 ml) was added dropwise to deuterated maleic acid (2.41 g; 20.40 mmol) as a solid under dioxan (20 ml) under dry conditions. The mixture, under nitrogen, was gradually brought to reflux and held at reflux for 1 hour. The reaction was then carried out as in section (7.2.2.1) affording benzylamine salt of N-benzyl-D,L-3[$^2H_1$]-aspartic acid in 87% yield.

From fumaric acid (27)

The reactions were performed in deuterium oxide and in dioxan as indicated for maleic acid. However, due to the insolubility of fumaric acid in deuterium oxide, this latter was used as such for the reaction resulting in a lower percentage deuteration in aspartic acid.

The benzylamine salt of N-benzyl-D,L-aspartic acid was obtained in quantitative yield.

The ammonium salt of aspartate was obtained in 70% yield with a percentage deuteration of 85%.

Furthermore, the reaction of fumaric acid with benzylamine in dioxan did not take place despite a longer period of reflux (up to 13 hours).

7.2.3 Preparation of diethyl-N-p-toluenesulfonyl aspartate

7.2.3.1 Preparation of N-p-toluenesulfonyl aspartic acid

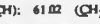
Sodium hydroxide (0.30 g; 7.58 mmol) was dissolved in distilled water (5 ml) and aspartic acid (0.23 g; 1.73 mmol) was added. N-p-toluenesulfonylchloride (0.67 g; 3.50 mmol) was added to the solution which was brought rapidly to a temperature of 80°C and maintained at the same temperature for 5 $\frac{1}{2}$ hours. After cooling, the pH of the solution was brought to 1 using concentrated HCl and was extracted with ether (2 x 30 ml). The combined ether extracts were dried over Na_2SO_4 and the ether was removed by evaporation under reduced pressure, and finally lyophilised to give a sticky white solid (0.30 g; 60%; mp = 85-87°C) which was used without further purification.

^1H NMR, 220 MHz (d_6-acetone/d_5-acetone = δ 2.10ppm) : δ = 2.45 (3H, s, $ArCH_3$); 2.90(2H, d, CH_2); 4.30(1H, m, H-C-NH-); 6.70(1H, d, H-C-NH-); 7.45(2H, d, aromatic H); 7.85 ppm (2H, d, aromatic H).

7.2.3.2 Preparation of diethyl-N-p-toluenesulfonyl aspartate

To ethanol (5.3 ml) was added, at 0°C and under nitrogen, acetylchloride (0.16 ml, 2.25 mmol). Then at room temperature was added N-p-toluenesulfonyl aspartic acid (0.30 g; 1.04 mmol) in ethanol (4.3 ml). The mixture was brought to 90°C and this temperature was maintained for 3 hours and then left to cool down overnight. The following day, the solution was evaporated to dryness to give a pale yellow oil which was crystallized from diethyl ether-petrol to give white needles (0.30 g; 84%, mp = 75-76°C) Lit[29] mp = 77°C.

^1H NMR, 400 MHz ($CDCl_3/CHCl_3$ = δ 7.27) : δ = 1.13 (3H, t, J = 7.15 Hz, CH_2CH_3); 1.24 (3H, t, J = 7.15 Hz, CH_2CH_3); 2.42 (3H, s, $ArCH_3$); 2.83 (1H, AB of ABXY, J_{AB} = 16.94 Hz, J_{AX} = 4.33 Hz, HC-CH_2); 2.95 (1H, AB of ABXY, J_{AB} = 16.94 Hz, J_{BX} = 4.92 Hz, H-C-CH_2); 4.00-4.09 (2H, m, CH_2CH_3); 4.12 (2H, q, J = 7.15 Hz, CH_2CH_3); 4.13 (1H, X of ABXY, J_{AX} = 4.33 Hz, J_{BX} = 4.92 Hz, J_{XY}*

8.07 Hz, H-C-CH_2): 5.64 (1H, broad d, Y of ABXY, J_{XY} = 8.04 Hz, -NH-); 7.27-7.36 (2H, m, ); 7.73-7.77 ppm (2H, m, ).

^{13}C NMR, 100.62 MHz ($CDCl_3/CDCl_3$ = δ 76.90): δ = 13.72 (CH_2CH_3), 13.95 (CH_2CH_3); 37.92 (CH_2CH); 52.17 (CH_2CH); 61.02 (CH_2CH_3); 62.04 (CH_2CH_3), 127.16 (); 129.53(); 137.00(); 143.53 (); 169.80 (COO); 169.99 ppm (COO).

IR (Nujol mull) : ν = 3280 (NH), 1730 cm^{-1}(CO).

MS(CI) : m/z = 361(M + NH_4)$^+$; 344(MH)$^+$; 270 (M-$CO_2C_2H_5$)$^+$; 188 (M- )$^+$, 155 ( )$^+$.

(2S, 3R)-[3-$^2H_1$]- and (2S, 3S)-[2,3-$^2H_2$]-aspartic acids were converted into the corresponding deuterated diethyl-N-p-toluenesulfonyl aspartate by the same procedure given above.

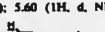
- Diethyl-(2S, 3S)-[2,3-$^2H_2$]-N-p-toluenesulfonyl aspartate

^1H NMR, 220 MHz ($CDCl_3$/TMS) : δ = 1.15 (3H, t, CH_2CH_3); 1.25 (3H, t, CH_2CH_3); 2.45 (3H, s, $ArCH_3$); 2.85 (1H, s, CH^2H); 4.05 (2H, q, CH_2CH_3); 4.15 (2H, q, CH_2CH_3); 5.65 (1H, s, NH); 7.35 (2H, d, ); 7.75 ppm (2H, d, ).

^2H NMR, 61.41 MHz ($CHCl_3/CDCl_3$ = δ 7.24) : δ = 2.91 (1^2H, s, CH^2H); 4.11 ppm (1^2H, s, ^2HCNH).

MS (CI) : m/z = 363 (M + NH_4)$^+$, 346 (MH)$^+$; 272 (M-CO_2H_5)$^+$; 190 (M- )$^+$, 155 ()$^+$.

- Diethyl-(2S, 3R)-[3-$^2H_1$]-N-p-toluenesulfonyl aspartate

^1H NMR, 220 MHz ($CDCl_3$/TMS) : δ = 1.15 (3H, t, CH_2CH_3); 1.25 (3H, t, CH_2CH_3); 2.45 (3H, s, $ArCH_3$); 2.95 (1H, brs, CH^2H); 4.00-4.20 (5H, m, 2 x CH_2CH_3, H-C-NH); 5.60 (1H, d, NH); 7.35 (2H, d, ); 7.75 ppm (2H, d, ).

^2H NMR, 61.41 MHz ($CHCl_3/CDCl_3$ = δ 7.25) : δ = 2.82 ppm (1^2H, s, CH^2H).

MS (CI) : m/z = 362 ($M + NH_4$)$^+$, 345 (MH^+), 271 ($M-CO_2C_2H_5$)$^+$; 189 (M-)$^+$, 155 ( )$^+$.

* CD measurements are reported in section (3.3)

7.2.3.3 Attempted preparation of

diethyl-D,L-[2-$^2H_1$]-N-p-toluenesulfonyl aspartate

A solution of sodium ethoxide [generated by the addition of sodium (0.14 g; 5.98 mmol) to dry ethanol (9 ml)] was added to diethylacetamidomalonate (1.14 g, 5.23 mmol) at room temperature under nitrogen. To the mixture was added dry ethyl chloroacetate (0.57 ml, 5.49 mmol) and a few crystals of sodium iodide. The mixture was left at room temperature with stirring for 4 days and heated for 2 $\frac{1}{2}$ hours using a steam bath. The sodium chloride was removed by filtration and the filtrate was evaporated to dryness to give a pale yellow oil. This was treated with concentrated ^2HCl (Aldrich, 37% w/w solution in ^2H_2O, 99 atom%^2H) at reflux for 3 hours. The solution was evaporated under reduced pressure, the residue was dissolved in distilled water (1 ml) and the pH of the solution was adjusted to pH 3 with concentrated ammonia. The mixture was concentrated slightly *in vacuo* and left at 0°C to crystallize. Crude deuterated aspartic acid (0.50 g) was collected and recrystalized from water to give a white crystalline solid (0.44 g, 63%). This gave a positive reaction with ninhydrin.

The deuterated aspartic acid obtained was derivatized to diethyl-N-p-toluenesulfonyl aspartate following the above procedure (section 7.2.3.1 and 7.2.3.2).

^1H NMR, 220 MHz ($CDCl_3$/TMS) : δ = 1.15 (3H, t, $CH_2\underline{CH_3}$); 1.25 (3H, t, $CH_2\underline{CH_3}$); 2.45 (3H, s, $Ar\underline{CH_3}$); 2.80-3.00 (xH, m, $\underline{CH_2}$, $\underline{CH}^2H$); 4.05 (2H, q, $\underline{CH_2}$-CH_3); 4.15 (2H, q, $\underline{CH_2}CH_3$); 5.65 (1H, s,-NH-); 7.35 (2H, d, ); 7.80 ppm (2H, d, ).

^2H NMR, 61.41 MHz ($CHCl_3$/$CDCl_3$ = δ 7.25): δ = 2.79 (1^2H, m, $CH^2\underline{H}$); 2.91 (1^2H, m, -$CH^2\underline{H}$-); 4.11 ppm (1^2H, s, $^2\underline{H}CNH$).

MS (CI): m/z (%) = 344 (M + 1; 1.5); 345 (M + 2; 18.4), 346 (M + 3, 17.9) 347

(M + 4; 6.6); 348 (M + 5; 1.8)

% of nondeuterated compound	4.09 %
monodeuterated compound	49.46%
dideuterated compound	39.38%
trideuterated compound	7.06%

* CD measurements are reported in section (3.3).

7.2.4 Tracer experiments using deuterated aspartic acid

These experiments were carried out as indicated in section (7.2.1.4). However, the precursor was added on the 5th day, at once or over a period of several days. On the 13th day, the penicillic acid was harvested and after recrystallisation analyzed by ^2H NMR.

^2H NMR, 61.41 MHz ($CHCl_3/CDCl_3$ = δ 7.25) : δ = 3.39 ppm (α^2H, s, O-C^2H_3).
^2H NMR, 61.41 MHz ($CHCl_3/CDCl_3$ = δ 7.25) : δ = 1.64 ppm (α^2H, s, C^2H_3).

7.3 EXPERIMENTAL SECTION TO CHAPTER 4

7.3.1 Synthesis of diethyl trans-2,3-epoxysuccinate (34) via diethyl 2-hydroxy-3-bromosuccinate (37)

7.3.1.1 Preparation of diethyl tartrate (36)

To (2S, 3S)-tartaric acid (19.84 g; 132 mmol) suspended in benzene (82 ml) was added ethanol (40 ml) and dowex 50W-X8 (H^+ form) ionic exchange resin (5.37 g). The mixture was brought to reflux using a Dean and Stark apparatus to collect the water formed during the esterification. The reaction could take up to 24 hours which corresponded to the total disappearance of tartaric acid into solution. The resin was filtered off, washed several times with fresh benzene and the solvent was removed under reduced pressure. The crude oil was purified by distillation in a Kugelrohr apparatus (135°C/0.1 mm Hg) to give diethyl (2S, 3S)-tartrate (36a) as a colourless oil (24.92 g; 91%).

Following the same procedure:

- Diethyl (2R, 3R)-tartrate (36b) was obtained in 89% yield. Distillation at 135°C/0.1 mm Hg.

- Racemic of diethyl tartrate was similarly obtained in 82% yield (not taking into account that D,L-tartaric acid contained an unknown number x of water molecules). Distillation at 122.5°C/0.05 mm Hg.

^1H NMR, 220 MHz ($CDCl_3$/TMS) : δ = 1.35 (t, 6H, CH_2CH_3); 2.70 - 2.40 (broad s, 2H, 2OH); 4.37(q, 4H, CH_2CH_3); 4.60 ppm (s, 2H, CHCH).

^{13}C NMR, 100.62 MHz ($CDCl_3$/$CDCl_3$ = δ 76.90 ppm): δ = 13.95 ($CH_2\underline{C}H_3$); 62.25 ($\underline{C}H_2CH_3$); 71.95 (HCOH); 171.42 ppm (COO).

IR (film): ν = 3480 brs(OH); 1750 cm^{-1} brs(CO).

7.3.1.2 Preparation of diethyl 2-acetoxy-3-bromosuccinate (35)

To stirred and ice-cooled diethyl D,L-tartrate (23.19 g; 112 mmol) was added dropwise a solution of HBr (30%) in glacial acetic acid (68.1 ml). When the addition was complete, the mixture was stirred for 15 minutes at 0°C, then for 4 hours in the dark at room temperature. The mixture was poured into ice-water (75 ml) and the aqueous solution was extracted several times with ether. The combined ethereal extracts were washed with saturated aqueous NaCl solution, dried with $MgSO_4$ overnight, filtered and evaporated under reduced pressure to give a pale yellow oil (32.00 g; 91%) which was used without purification for experiment (7.3.1.3.).

^1H NMR, 220 MHz ($CDCl_3$/TMS): δ = 1.31 (t, 6H, $CH_2\underline{CH_3}$); 2.11 (s, 3H, $\underline{CH_3}COOH$); 2.19 (s, 3H, $CO(O\underline{CH_3})$); 4.20 - 4.40 (m, 4H, $\underline{CH_2}CH_3$); 4.82 (d, 1H, $\underline{CH}Br$); 5.63 ppm (d, 1H, $\underline{CH}OAc$).
IR (film): ν = 1752 cm^{-1} brs (CO).

Following the same procedure,

Diethyl (2S)-acetoxy-(3R)-bromosuccinate (35a) was prepared in 81% yield.

7.3.1.3 Preparation of diethyl 2-hydroxy-3-bromosuccinate (37)

To a solution of racemic diethyl 2-acetoxy-3-bromosuccinate (30.50 g; 98 mmol) in dry ethanol (259 ml) was added a solution of HBr (30%) in glacial acetic acid (8.5 ml). The mixture was brought to reflux for 4 hours under nitrogen and then the solution was evaporated under reduced pressure. The residue was distilled (bp 88-90°C/0.02 mm Hg) to afford a colourless oil (19.32 g; 73%) which was further purified by flash chromatography eluting with ethyl acetate-petrol (1/1). The bromohydrin (37) was obtained as a colourless oil R_F 0.44 (14.40 g; 55%) after a distillation in the Kugelrohr apparatus (130°C/0.08 mm Hg).

^1H NMR, 220 MHz ($CDCl_3$/TMS): δ = 1.32 (t, 6H, $CH_2\underline{CH_3}$); 3.49 (d, 1H, OH); 4.24 - 4.42 (m, 4H, $\underline{CH_2}CH_3$); 4.64 - 4.80 ppm (m, 2H, $\underline{CH}\underline{CH}$).
IR (film): ν = 1740 brs (CO); 3465 cm^{-1} brs(OH).

Prepared in the same way:

Diethyl (2S)-hydroxy-(3R)-bromosuccinate (37a) was obtained in 62% yield after flash chromatography.

7.3.1.4 Preparation of diethyl trans-2,3-epoxysuccinate (34)

A solution of sodium ethoxide was prepared by addition of sodium (0.28 g; 12.30 mmol) to dried ethanol (6.6 ml), to which was added dropwise at 0^0C a solution of racemic diethyl 2-hydroxy-3-bromosuccinate (2.63 g; 9.77 mmol) in dried ethanol (2.3 ml). After the addition was completed, the mixture was left to stir at room temperature for 1 hour, neutralized with glacial acetic acid and finally the solvent removed under reduced pressure at low temperature. The residue was taken up into ice-water and extracted with ether (3 x 35 ml). The combined ethereal fraction was washed with saturated aqueous NaCl solution, dried overnight with $MgSO_4$ and after filtration, concentrated *in vacuo* to give a slightly yellow oil (1.66 g; 90%). Then a distillation in a Kugelrohr apparatus (130^0C/0.1 mm Hg) afforded a colourless liquid (1.54 g; 84%) which could be further purified by flash chromatography eluting with ethyl acetate-petrol (1/4) although this led to partial decomposition of diethyl trans-2,3-epoxysuccinate; R_F 0.50 in petrol-ethyl acetate (3/1) (1.09 g; 59%).

^1H NMR, 220 MHz ($CDCl_3$/TMS): δ = 1.32 (t, 6H, $CH_2\underline{CH_3}$); 3.68 (s, 2H, trans epoxy $\underline{H}$); 4.29 ppm (q, 4H, $\underline{CH_2}CH_3$).
IR (film): ν = 1745 cm^{-1} brs (CO).

Following the same procedure:

· Diethyl (2S, 3S)-epoxysuccinate (34a) was obtained after distillation in 81% yield.

Specific rotation measurement:

$[\alpha]_D^{27}$ = +93.9 (c = 0.360, $CHCl_3$).

$[\alpha]_D^{27}$ = + 108.4° (c = 0.289, ether) Lit[81] $[\alpha]_D^{23}$ = +105.5° (c = 1.413, ether).

Enantiomeric purity was checked by the ^1H NMR spectrum at 400 MHz in the presence of 3 equivalents of (S)-(+)-2,2,2-trifluoro-1-(9-anthryl) ethanol (39).

^1H NMR, 400 MHz (CCl_4, d_6-benzene/$d_5$benzene = δ 7.20 ppm): δ = 1.08 (t, 6H, J = 7.14 Hz, $CH_2\underline{CH_3}$); 3.38 (s, 2H, trans epoxy H); 3.95 ppm (q, 4H, J = 7.14 Hz, $\underline{CH_2}CH_3$).

Splitting of the singlet at 3.38 ppm was only observed in the presence of racemic compound indicating an ee ≥ 97%. Another singlet appeared at higher field 3.30 ppm which corresponded to diethyl (2R, 3R)-epoxysuccinate (34b)

7.3.2 Attempt at the synthesis of diethyl cis-2,3-epoxysuccinate (40) via the corresponding diethyl 2-hydroxy-3-bromosuccinate (43).

7.3.2.1 Preparation of diethyl meso-tartrate (41)

Diethyl maleate 97% pure (21.75 g; 122.60 mmol) was added slowly to a mixture of N-methylmorpholine-N-oxide (22.29 g; 184.60 mmol), water (61 ml), acetone (25 ml) and osmium tetroxide (80 mg) dissolved previously in tert-butanol (10 ml). The reaction was maintained at room temperature with a water bath and then left to stir overnight under nitrogen. The mixture was filtered after the addition of a slurry composed of sodium hydrosulfite (1 g) and magnesium silicate (12 g) in water (80 ml). The filtrate was neutralized to pH 7 with H_2SO_4(0.5 M) and after evaporation of acetone under reduced pressure, the pH was adjusted to 2. The solution was saturated with NaCl and extracted with ethyl acetate. The aqueous phase was concentrated by azeotroping with n-butanol and extracted again with ethyl acetate. The combined ethyl acetate layers were dried and evaporated under reduced pressure to give a yellow solid (24.44 g; 97%) which upon recrystallisation from ether-petrol furnished white needles (19.98 g; 79%) mp = 56 - 57.5° C. Lit[83] mp = 55°C.

^{1}H NMR, 220 MHz ($CDCl_3$/TMS): δ = 1.30 (t, 6H, $CH_2\underline{CH_3}$); 3.35 (br, s, 1H, OH); 4.20 - 4.42 (m, 4H, $\underline{CH_2}CH_3$); 4.59 ppm (s, 2H, $\underline{H}COH$).

^{13}C NMR, 100.62 MHz ($CDCl_3$/$CDCl_3$ = δ 76.90 ppm): δ = 13.91 ($\underline{CH_3}CH_2$); 62.09 ($\underline{CH_2}CH_3$); 72.80 (HCOH); 170.89 ppm (COO).

IR (Nujol mull): ν = 3420 brs (OH), 1755 and 1740 cm^{-1} s (CO).

MS (EI): m/z = 207 $(MH)^+$, 133 $(M\text{-}CO_2Et)^+$, 104 $(M\text{-}(CO_2Et) - (Et))^+$, 29 $(CH_2CH_3)^+$.

High resolution MS: $C_8H_{15}O_6$ (M+H); cal. 207.0868, found. 207.0874.

Elemental analysis: Found: C, 46.65; H, 6.98. $C_8H_{10}O_6$ requires: C, 46.60; H, 6.85%.

7.3.2.2 Preparation of diethyl 2-acetoxy-3-bromosuccinate (42)

The reaction was carried out as indicated in section (7.3.1.2). The ^{1}H NMR of the crude mixture showed the presence of acetic acid and diethyl 2,3-diacetoxysuccinate. The yield for the production of diethyl 2-acetoxy-3-bromosuccinate was estimated by ^{1}H NMR at 30% with a ratio of 44.5/55.5 respectively for the acetoxybromide and diacetoxy compound.

^{1}H NMR, 220 MHz ($CDCl_3$/TMS): δ = 1.23 - 1.44 (m, $CH_2\underline{CH_3}$); 2.11 (s, 3H, $\underline{CH_3}$ COOH); 2.20(s, $CO(CH_3)$); 4.17 - 4.47 (m, $\underline{CH_2}CH_3$); 4.65 (d, 2H, CHBr); 5.58 (d, 2H, $\underline{CH}OAc$); 5.67 ppm (s, 2H, AcOC$\underline{H}$ - C$\underline{H}$OAc).

The deprotection of the acetoxybromide was carried out on this crude mixture without further purification.

7.3.2.3 Preparation of diethyl 2-hydroxy-3-bromosuccinate (43)

As previously described in section (7.3.1.3), a colourless oil (7.94 g; 66%) was obtained after distillation in a Kugelrohr apparatus (bp 160^{0}C/0.05 mm Hg). Flash chromatography eluting with ethyl acetate-petrol (1/1) was performed and the bromohydrin was collected as a colourless oil R_F 0.45 (0.73 g; 6%) with diethyl

tartrate obtained as a by-product of the reaction.

^{1}H NMR, 220 MHz ($CDCl_3$/TMS): δ = 1.33 (t, 6H, $CH_2\underline{CH_3}$); 3.38 (d, 1H, OH); 3.22 - 4.45 (m, 4H, $\underline{CH_2}CH_3$), 4.79 - 4.88 ppm (m, 2H, $C\underline{H}C\underline{H}$).

7.3.2.4 Preparation of diethyl cis-2,3-epoxysuccinate (40)

The procedure followed was the same as in section (7.3.1.4). The bromohydrin (0.53 g; 1.97 mmol) made previously was treated with NaOEt in dried ethanol. By ^{1}H NMR, the crude yellow oil (0.20 g; 54%) was a mixture of diethyl cis- and trans- epoxysuccinate in a ratio of 54.3/45.7 respectively.

^{1}H NMR, 220 MHz ($CDCl_3$/TMS): δ = 1.20 - 1.48 (m, 6H, $CH_2\underline{CH_3}$); 3.69 (s, 2H, trans epoxy $\underline{H}$); 3.71 (s, 2H, cis epoxy $\underline{H}$); 4.20 - 4.45 ppm (m, 4H, $C\underline{H_2}CH_3$).

N.B. For these experiments, no mass spectra could be reported due to the lack of mass spectra service at the time in the department.

7.3.3 Preparation of diethyl 2,3-epoxysuccinate *via* diethyl 2-tosyl tartrate (46,47)

7.3.3.1 Preparation of diethyl 2-tosyl tartrate (46,47) by the conventional method

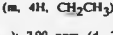
p-Toluenesulfonyl chloride (1.27 g; 6.70 mmol) in anhydrous benzene (20.6 ml) was added slowly over a period of 8 hours to an ice-cold solution of diethyl meso-tartrate (1.37 g; 6.70 mmol) dissolved in dry pyridine (5.50 ml), and left to stir for 4 days at room temperature. The reaction mixture was diluted with ether (55 ml) and after filtration, washed successively with 1M HCl (4 x 28 ml) and water (5 x 28 ml). The ethereal solution was dried over Na_2SO_4 and evaporated under reduced pressure to furnish a yellow oil (1.65 g). On Tlc, 4 compounds were detected R_F 0.58, 0.50, 0.43 and 0.36 using petrol-ethyl acetate (1/1). After flash chromatography eluting with ethyl acetate-petrol (1/5) these compounds were identified as being respectively: p-toluenesulfonyl chloride (0.14 g); diethyl 2-tosyl butenedioate (0.21 g).

diethyl 2,3-ditosyl-meso-tartrate (0.33 g) and diethyl 2-tosyl-meso-tartrate (0.97 g; 40%).

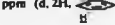
· (Cis and trans)-1,4-diethyl 2-tosyl butenedioate:

^{1}H NMR, 220 MHz ($CDCl_3$/TMS): δ = 1.15 - 1.38 (m, 6H, $CH_2\underline{CH_3}$); 2.49 (s, 3H, $Ar\underline{CH_3}$); 4.10 - 4.32 (m, 4H, $\underline{CH_2}CH_3$); 6.22 (s, H, $\underline{H}_{cis}$); 6.77 (s, H, $\underline{H}_{trans}$); 7.40 (d, 2H, ); 7.90 ppm (d, 2H,).

· Diethyl 2,3-ditosyl-meso-tartrate:

^{1}H NMR, 220 MHz ($CDCl_3$/TMS): δ = 1.23 (t, 6H, $CH_2\underline{CH_3}$); 2.47 (s, 3H, $Ar\underline{CH_3}$); 4.12 - 4.32 (m, 4H, $\underline{CH_2}CH_3$); 5.37 (s, 2H, $\underline{CH}\underline{CH}$); 7.35 (d, 4H, 2 x ); 7.81 ppm (d, 4H, 2 x ).

· Diethyl 2-tosyl-meso-tartrate (47):

^{1}H NMR, 220 MHz ($CDCl_3$/TMS): δ = 1.18 - 1.40 (m, 6H, $CH_2\underline{CH_3}$); 2.47 (s, 3H, $Ar\underline{CH_3}$); 3.17 (d, 1H, OH); 4.07 - 4.41 (m, 4H, $\underline{CH_2}CH_3$); 4.60 - 4.70 (d of d, 1H, $\underline{H}COH$); 5.35 (d, 1H, $\underline{H}COTos$); 7.40 (d, 2H, ); 7.9 ppm (d, 2H, ).

· Diethyl 2-tosyl-D,L-tartrate (46):

Following the same procedure this compound was prepared from diethyl D,L-tartrate in 22% yield as a mixture with diethyl 2-tosyl butenedioate and diethyl 2,3-ditosyl-D,L-tartrate.

^{1}H NMR, 220 MHz ($CDCl_3$/TMS): δ = 1.20 - 1.40 (m, 6H, $CH_2\underline{CH_3}$); 2.47 (s, 3H, $Ar\underline{CH_3}$); 3.15 (d, 1H, OH); 4.04 - 4.40 (m, 4H, $\underline{CH_2}CH_3$); 4.75 (d of d, 1H, $\underline{H}COH$); 5.38 (d, 1H, $\underline{H}COTos$); 7.40 (d, 2H,); 7.88 ppm (d, 2H,).

7.3.3.2 Preparation of diethyl 2-tosyl tartrate (46,77) *via* cyclic stannylene derivatives of diethyl tartrate (48,49)

Preparation of dibutylstannylene of diethyl tartrate (48,49)

A mixture of diethyl meso-tartrate (14.38 g; 69.70 mmol) and

dibutyltin oxide (17.39 g; 69.80 mmol) in benzene (115 ml) was brought to reflux using a Dean and Stark apparatus. After 1 hour, the water formed (1.25 ml) was collected but the mixture was left to reflux for a further 5 hours. After distillation of the benzene (80 ml), the reaction mixture was left to cool down. A white solid precipitated which was filtered, dried in a dessicator over freshly cut shavings of paraffin wax and used as such for the following experiment (29.45 g, 97%). A fraction was crystallized several times from benzene and sent for analysis (mp=decomposed at 158°C).

IR (Nujol mull): ν = 1745, 1760 cm^{-1} sh (CO).

MS(EI): m/z = 439 $(MH)^+$, 438 M^+, 381 $(M\text{-}Bu)^+$, 336, 223, 177 $(SnBu)^+$.

Elemental analysis : Found : C, 44.09; H, 7.19. $C_{16}H_{30}O_6Sn$ requires C, 43.97; H, 6.92.

· Dibutylstannylene of diethyl D,L-tartrate (49)

Following the same procedure this compound was obtained as a white crystalline solid in 95% yield (mp = 138 - 139°C).

IR(Nujol mull): ν = 1755, 1730 cm^{-1} (CO).

MS(EI): m/z = 439$(MH)^+$, 438 M^+, 381 $(M\text{-}Bu)^+$, 336, 223, 177 $(SnBu)^+$.

Elemental analysis : Found: C, 44.13; H, 7.14. $C_{16}H_{30}O_6Sn$ requires C, 43.97; H, 6.92.

Preparation of diethyl 2-tosyl tartrate (46,47)

A mixture of dibutyltin oxide stannylene of diethyl meso-tartrate (4.14 g; 9.47 mmol), p-toluenesulfonyl chloride (1.82 g; 9.54 mmol) and tetraethylammonium chloride (1.75 g; 9.52 mmol) in acetonitrile (200 ml) was stirred at room temperature for 24 hours. The solution was evaporated under reduced pressure to give an oily mixture which was purified by flash chromatography petrol-ethyl acetate (7/3) to afford diethyl 2-tosyl-meso-tartrate (47) (3.23g; 95%) as a colourless oil (R_F 0.46 in petrol-ethyl

acetate (1/1).

^{1}H NMR, 220 MHz ($CDCl_3$/TMS): δ = 1.15 - 1.40 (m, 6H, $CH_2\underline{CH_3}$); 2.47 (s, 3H, $ArCH_3$); 3.17 (d, 1H, OH); 4.10 - 4.44 (m, 4H, $\underline{CH_2}CH_3$), 4.63 - 4.72 (d of d, 1H, HCOH); 5.37 (d, 1H, HCOTos); 7.42 (d, 2H,);7.92 ppm(d, 2H,).
^{13}C NMR, 100.62 MHz ($CDCl_3$/$CDCl_3$ = δ 76.90 ppm): δ = 13.80 ($CH_2\underline{CH_3}$); 13.84 ($\underline{CH_2}CH_3$) 21.56 ($ArCH_3$); 62.20 ($\underline{CH_2}CH_3$); 62.75 ($\underline{CH_2}CH_3$); 71.06 (HOCH); 78.37 (HCOTos); 128.00( ); 129.63 ( ); 133.14( );
145.13( ); 165.22(COO). 169.64 ppm (COO).
IR (sol in $CHCl_3$) : ν = 3540 br(OH), 1755 sh(CO), 1750 cm^{-1} (CO).
MS(EI) : m/z = 361(MH)$^+$, 360M$^+$, 287(M-$COOCH_2CH_3$)$^+$, 155(),
91($ArCH_3$)$^+$.
High resolution MS : $C_{15}H_{21}O_8S$ (MH)$^+$, cal. 361.0957, found. 361.0954.
Elemental analysis : Found : C, 50.31, H, 5.60. $C_{15}H_{20}O_8S$ requires C, 49.99, H, 5.59.

· Diethyl 2-tosyl-D,L-tartrate (46)

The reaction was carried out as above and after flash chromatography with petrol-ethyl acetate (1/1) (R_F 0.43) the compound was obtained as a white solid after crystallisation with ether-petrol (91%; mp = 62.5 - 64.0°C).

^{1}H NMR, 220 MHz ($CDCl_3$/TMS) : δ = 1.22 - 1.35 (m, 6H, $CH_2\underline{CH_3}$); 2.49 (s, 3H, $ArCH_3$); 3.17 (d, 1H, OH); 4.07 - 4.42 (m, 4H, $\underline{CH_2}CH_3$); 4.79 (d of d, 1H, HCOH); 5.41 (d, 1H, HCOTos); 7.44 (d, 2H, );7.93 ppm (d, 2H, ).
^{13}C NMR, 100.62 MHz ($CDCl_3$/$CDCl_3$ = δ 76.90 ppm): δ = 13.81 ($CH_2\underline{CH_3}$); 21.53 ($ArCH_3$); 62.37 ($\underline{CH_2}CH_3$); 62.92 ($\underline{CH_2}CH_3$); 71.15 (HOCH); 77.14 (HCOTos), 128.07 (); 129.56 ( ); 133.09 ( ); 145.14
( ). 165.65 (COO); 169.72 ppm (COO).

IR(Nujol mull): ν = 3470 br s(OH), 1775 s (CO), 1750 cm^{-1} s (CO).

MS(EI): m/z = 361$(MH)^+$, 360M^+, 91 $(ArCH_3)^+$.

High resolution MS : $C_{15}H_{21}O_8S(MH)^+$, cal 361.0957; found 361.0961.

Elemental analysis : Found : C, 49.84; H 5.52. $C_{15}H_{20}O_8S$ requires C, 49.99; H, 5.59.

7.3.3.3 Diethyl 2,3-epoxysuccinate (34,40) from diethyl 2-tosyl tartrate (46,47)

Diethyl-trans-2,3-epoxysuccinate (34)

To a solution of sodium ethoxide prepared by addition of sodium (0.23 g;10.0 mmol) to dry ethanol (5.7 ml) was added dropwise at 0^oC a solution of diethyl 2-tosyl meso tartrate (2.88 g; 8.00 mmol) in dry ethanol (2 ml). After the addition was completed, the mixture was left to stir at room temperature for 1 hour, neutralized with glacial acetic acid and the solvent removed under reduced pressure at low temperature. The residue was taken up into ice-water and extracted with ether (3 x 35 ml). The combined ethereal fractions were washed with saturated aqueous NaCl solution, dried overnight with $MgSO_4$ and after filtration, concentrated *in vacuo* to give a slightly yellow oil (1.38 g; 92%). A distillation in a Kugelrohr apparatus (90^oC/0.07 mm Hg) afforded diethyl trans-2,3-epoxysuccinate as a colourless oil (1.26 g; 84%).

^1H NMR, 220 MHz ($CDCl_3$/TMS): δ = 1.33 (t, 6H, $CH_2\underline{CH_3}$); 3.71 (s, 2H, Trans epoxy H); 4.14 - 4.44 (app q, 4H, $\underline{CH_2}CH_3$).

^{13}C NMR, 100.62 MHz ($CDCl_3$/$CDCl_3$ = δ 76.90 ppm): δ = 13.91 ($CH_2\underline{CH_3}$); 51.91 (C-O-C); 62.11 ($\underline{CH_2}CH_3$); 166.65 ppm (COO).

IR (film): ν = 1750 cm^{-1} br s (CO).

MS(EI): m/z = 189$(MH)^+$, 161, 143 $(M - OCH_2CH_3)^+$, 115 $(M - CO_2CH_2CH_3)^+$, 87, 71, 43, 29 $(CH_2CH_3)^+$.

High resolution MS : $C_8H_{13}O_5(MH)^+$, cal : 189.0763; found: 189.0808.

Elemental analysis : Found : C, 50.99; H, 6.71. $C_8H_{12}O_5$ requires: C, 51.06, H, 6.43.

Preparation of diethyl cis-2,3-epoxysuccinate (40)

The addition of sodium ethoxide in ethanol to a solution of diethyl 2-tosyl-D,L-tartrate resulted invariably in the solidification of the reaction mixture, even if the volume of ethanol or the temperature was increased. The result was systematically a mixture of cis- and trans-epoxides in a ratio of about 81 : 19% respectively with the reaction never going to completion (yield of ± 53% after Kugelrohr distillation of the crude mixture). The best compromise was the addition of the tosylate to the NaOEt solution and the reaction was consequently carried out as follows:

A solution of diethyl 2-tosyl-D,L-tartrate (8.23 g; 22.80 mmol) in dry ethanol (38 ml) was added at 0°C over a period of 8 hours to a solution of sodium ethoxide (0.70 g; 30.4 mmol in 26 ml of ethanol). The reaction was left at 4°C for 40 hours in an attempt to bring the reaction to completion. The reaction was brought to pH 7 by addition of glacial acetic acid and the solution was evaporated under reduced pressure at low temperature. The white precipitate obtained was dissolved in ice-water and extracted several times with ether. The ethereal solution was dried overnight with Na_2SO_4. After filtration and evaporation, a yellowish oil (4.46 g) was obtained which by ^1H NMR was identified as a mixture of cis- and trans-epoxides in a ratio of 74/27% respectively. By Tlc, trans-epoxide migrated faster (R_F 0.50) than cis epoxide (R_F 0.38) petrol-ethyl acetate (3/1). A distillation in a Kugelrohr apparatus (95°C, 0.08 mm Hg) afforded a colourless oil (2.35 g; 55%). After flash chromatography with petrol-ethyl acetate (85/15), pure diethyl cis-2,3-epoxysuccinate was obtained as a colourless oil (1.01 g; 23.5%). Purity of the epoxide could be easily checked by HPLC using a Spherisorb C_{18} reverse phase column. Acetonitrile-water (1/1) mixture was used with UV detection at λ = 214 nm.

R_T of cis epoxide = 5.25' R_T of trans epoxide = 6.30'.

^1H NMR, 220 MHz ($CDCl_3$/TMS) : δ = 1.31 (t, 6H, J = 7.2 Hz, $CH_2\underline{CH_3}$); 3.73

(s, 2H, cis epoxy H); 4.31 ppm (q, 4H, J = 7.2 Hz; CH_2CH_3).

^{13}C NMR, 100.62 MHz ($CDCl_3/CDCl_3$ = δ 76.90 ppm): δ = 13.91 ($CH_2\underline{C}H_3$); 52.45 ($\underline{C}$-O-$\underline{C}$); 61.90 ($\underline{C}H_2CH_3$), 165.57 ppm (COO).

IR (film) : ν = 1750 cm^{-1} br s (CO).

MS(EI) : m/z = 188 $M^{\bullet}$, 161, 143 (M - OCH_2CH_3)$^{\bullet}$, 115 (M - $CO_2CH_2CH_3$)$^{\bullet}$, 87, 71, 43, 29 (CH_2CH_3)$^{\bullet}$.

Elemental analysis : Found : C, 51.35, H, 6.14. $C_8H_{12}O_5$ requires C, 51.06; H, 6.43.

7.3.3.4 Formation of diethyl cis-2,3-epoxysuccinate (40) *via* the tert-butyldimethyl silyl ether of diethyl 2-tosyl-D,L-tartrate (52)

Formation of the tert-butyldimethyl silyl ether of diethyl 2-tosyl-D,L-tartrate (52)

Diethyl 2-tosyl-D,L-tartrate (7.08 g; 19.60 mmol) and imidazole (4.60 g; 67.60 mmol) was dissolved in DMF (17.4 ml). To this mixture, under nitrogen, was added tert-butyl dimethyl silyl chloride (5.12 g; 34.0 mmol). The reaction was warmed to 25°C and stirred for 2½ hours. The reaction mixture was taken up in ethyl acetate (300 ml) and washed twice with water (2 x 50 ml). The ethyl acetate phase was dried overnight with $MgSO_4$. Filtration and evaporation under reduced pressure afforded a yellow oil which was purified by a flash chromatography with petrol-ethyl acetate (8/2). The tert-butyl dimethylsilyl ether of diethyl 2-tosyl-D,L-tartrate (52) was obtained as a viscous colourless oil (9.30 g; 100%), R_F 0.55 in petrol-ethyl acetate (3/1).

^1H NMR, 220 MHz ($CDCl_3/CHCl_3$ = δ 7.30 ppm): δ = 0.00 (s, 3H, $SiCH_3$); 0.09 (s, 3H, $SiCH_3$); 0.84 (s, 9H, $C(CH_3)_3$); 1.13 - 1.31 (m, 6H, CH_2CH_3); 2.43 (s, 3H, $ArCH_3$); 3.91 - 4.29 (m, 4H, CH_2CH_3); 4.74 (d, 1H, HCOSi); 5.33 (d, 1H, HCOTos); 7.37 (d, 2H, ); 7.85 ppm (d, 2H, ).

^{13}C NMR 100.62 MHz ($CDCl_3$/$CDCl_3$ = δ 76.90 ppm) : δ = -5.81 ($SiCH_3$); -5.02 ($SiCH_3$); 13.71 ($CH_2\underline{C}H_3$); 13.84 ($CH_2\underline{C}H_3$); 18.09 ($\underline{C}(CH_3)_3$); 21.52 ($Ar\underline{C}H_3$); 25.38 ($C(\underline{C}H_3)_3$); 61.69 ($\underline{C}H_2CH_3$); 62.09 ($\underline{C}H_2CH_3$); 72.72 (SiOCH); 78.51 (HCOTos); 128.01 (); 129.44 ( ); 133.60 ( ); 144.73 ( ); 166.15 (COO); 168.81 ppm (COO).

IR (film) : ν = 1750, 1740 cm^{-1} br s (CO).

MS(CI): m/z = 492 ($M + NH_4$)$^+$; 475 ($M + H$)$^+$; 459 ($M - CH_3$)$^+$, 417 ($M - C(CH_3)_3$), 229, 189, 155 (SO_2-)$^+$; 91 ($ArCH_3$)$^+$.

Elemental analysis : Found : C, 53.26; H, 6.95. $C_{21}H_{34}O_8SSi$ requires: C, 53.14; H, 7.22.

Formation of diethyl cis-2,3-epoxysuccinate (4) from the tert-butyldimethyl silyl ether of diethyl 2-tosyl-D,L-tartrate (52)

The tert-butyldimethylsilyl ether of diethyl 2-tosyl-D,L-tartrate (0.31 g; 0.65 mmol) was dissolved in dry THF (7.6 ml) under nitrogen and tetrabutylammonium fluoride 1M (0.8ml) in THF was added. After 2 hours, additional tetrabutylammonium fluoride (0.4 ml) was added and the reaction was left a further 3 hours. The reaction mixture was then evaporated under reduced pressure and the residue obtained dissolved in ice-water (10 ml) and extracted several times with ether (3 x 60 ml). The ethereal phase was dried with Na_2SO_4 overnight. Filtration and evaporation of the solvent under reduced pressure afforded a crude viscous oil (289.2 mg) which was purified by flash chromatography with petrol-ethyl acetate (85/15) to give diethyl cis-2,3-epoxysuccinate (R_F 0.38 petrol- ethyl acetate (3/1) (108.5mg; 87%). Purification of this epoxide by flash chromatography led sometimes to partial decomposition and a Kugelrohr distillation (117.5°C at 0.3 mm Hg) was generally a good enough alternative for purification of this epoxide.

^1H NMR, 220 MHz ($CDCl_3$/TMS): δ = 1.31 (t, 6H, J = 7.2 Hz, $CH_2\underline{C}H_3$); 3.72 (s, 2H, cis epoxy H); 4.29 ppm (q, 4H, J = 7.2 Hz, $\underline{C}H_2CH_3$).

^{13}C NMR, 100.62 Hz ($CDCl_3/CDCl_3$ = δ 76.90 ppm): δ = 13.75 ($CH_2\underline{C}H_3$); 52.30 (C-O-C); 61.67 ($\underline{C}H_2CH_3$), 165.45 ppm (COO).

IR (film): ν = 1750 cm^{-1} br s (CO).

MS(EI): m/z = 188 $M^{\bullet}$, 161, 143 $(M - OCH_2CH_3)^+$, 115 $(M - CO_2CH_2CH_3)^+$, 87, 71, 43, 29 $(CH_2CH_3)^+$.

High resolution MS : $C_8H_{12}O_5$: cal. 188.0684; found : 188.0699.

Elemental analysis : Found : C, 50.87; H, 6.47. $C_8H_{12}O_5$ requires C, 51.06; H, 6.43.

7.3.4 Preparation of enantiomerically pure diesters of cis- and trans-2,3-epoxysuccinate

7.3.4.1 Enzymatic resolution of diethyl trans-2,3-epoxysuccinate (34)

Decomposition of diethyl trans-2,3-epoxysuccinate (34) in phosphate buffer at pH 7

Diethyl trans-2,3-epoxysuccinate (0.20 g; 1.04 mmol) was suspended in pH 7 phosphate buffer (0.1 M, 6 ml) and stirred vigorously at 30°C. The addition of NaOH (0.306 M) from an autotitrator maintained the pH of the solution at 7 while also monitoring the reaction. When the volume of NaOH added was equal to 3.75 ml, the reaction mixture was extracted with ethyl acetate (4 x 20 ml). The pH of the aqueous phase was then lowered to 1 with HCl (5 M), re-extracted with ethyl acetate (4 x 20 ml) and the organic phase was dried over Na_2SO_4. After filtration, the ethyl acetate was removed by rotary evaporation to give a crude colourless liquid (0.12 g; 69%) identified as being the corresponding monoethyl 2,3-epoxysuccinic acid (54).

^1H NMR, 220 MHz ($CDCl_3$/TMS): δ = 1.33 (t, 3H, $CH_2\underline{CH_3}$); 3.73 (app s, 2H, trans epoxy H); 4.31 (q, 2H, $\underline{CH_2}CH_3$); 6.80- 7.60ppm (br s, 1H, COOH).

MS(+ve CI, ammonia) : m/z = 178 $(M + 18)^+$, 161 $(M + 1)^+$.

Hydrolysis of diethyl trans-2,3-epoxysuccinate (34) by lipase from Rhizopus javanicus (LF-AP15) using a two phase system

Racemic diethyl trans-2,3-epoxysuccinate (135.00 mg; 0.72 mmol) was dissolved in isooctane (38 ml). To this stirred mixture was added pH 7 phosphate buffer (1M, 9.5 ml) containing lipase from Rhizopus javanicus (9600 units, 64 mg). NaOH (0.099 M) was added from an autotitrator to maintain the pH of the aqueous phase at 7. When the hydrolysis reached 25.6% of the ester groups (NaOH added = 3.69 ml), the organic phase was collected and evaporated under reduced pressure. After drying over silica gel in a dessicator under vacuum, a crude colourless liquid (48.5 mg; 36%) was obtained.

The enantiomeric purity of this compound was determined by the ^1H NMR spectrum at 400 MHz in the presence of 3-equivalents of (S)-(+)-2,2,2- trifluoro-1-(9-anthryl)-ethanol (39).

^1H NMR, 400 MHz (CCl_4, d_6-benzene/d_5-benzene = 7.20 ppm) : δ = 1.00 (t, 6H, J = 7.1 Hz, $CH_2\underline{CH_3}$); 3.31 (s, (2R, 3R)-epoxy H); 3.37 (s,(2S, 3S)-epoxyH); 3.87 ppm (q, 4H, J = 7.14 Hz; $\underline{CH_2}CH_3$).

Peak intensities:	major peak	3.31 ppm	14.12
	minor peak	3.37 ppm	3.34

Enantiomeric excess = 62%

Transesterification of diethyl trans-2,3-epoxysuccinate (34) with heptanol

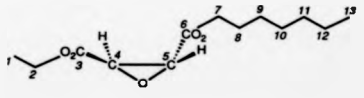
A typical transesterification reaction was carried out as follows:

To diethyl trans-2,3-epoxysuccinate (0.10 g, 0.54 mmol) in isooctane (12 ml) was added heptanol (0.49 ml), LP(750 units, 25 mg) and the mixture was left to stir at 50°C. Samples were periodically withdrawn and analyzed by gas chromatography. R_T of compounds (34), (55) and (56) were 5.4', 20.7' and 28.15' respectively by the use

of a temperature program (130°C for 10 minutes then an increment of 6°C/min was applied up to 250°C). On the 9th day, a GC was run in slightly different conditions than previously described to optimize the measurement of the relative quantities of each compound (130°C for compound (34), 170°C for compound (55) and 200°C for (56)). No starting material was detectable and compounds (55) and (56) were in a proportion of 47/53 respectively. The enzyme was filtered off through cotton wool and washed with fresh isooctane. The combined filtrate was evaporated under reduced pressure and the heptanol distilled off using a Kugelrohr apparatus. A crude colourless liquid (0.15 g) was obtained which by Tlc gave, after exposure to iodine vapour, two distinct spots. Compounds (55) and (56) corresponded respectively to R_F of 0.39 and 0.49 (petrol-ethyl acetate) (3/1). Flash chromatography using petrol-ethyl acetate (95/5) gave 1-ethyl 4-heptyl trans-2,3-epoxysuccinate (55) (51.4 mg; 81%) and diheptyl epoxide (56) (78.3 mg; 81%) both as colourless oils. According to the mass of each compound, the ratio was 45.5/54.5 for (55/56) which was in good agreement with the GC results.

The determination of the enantiomeric purity of each sample was done using ^1H NMR and 3 equivalents of (S)-(+)-2,2,2-trifluoro-1-(9-anthryl)-ethanol (220 MHz) or 2 equivalents (400 HMz instrument).

· 1-ethyl 4-heptyl trans-2,3-epoxysuccinate (55)



^1H NMR, 220 MHz ($CDCl_3$/TMS) : δ = 0.83 - 0.99 (m, 3H, H of C_{13}); 1.19 - 1.53 (m, 11H, H of $C_{12,11,10,9,1}$); 1.58 - 1.79 (m, 2H, H of C_8); 3.69 (app s, 2H, trans epoxy H); 4.11 - 4.43 ppm (m, 4H, H of $C_{7,6}$).

^{13}C NMR, 100.62 MHz ($CDCl_3$/$CDCl_3$ = δ 76.90 ppm) : δ = 13.89 ($C_{1,13}$); 22.40 (C_{12}); 25.57 (C_{11}); 28.28 (C_{10}) 28.67 (C_9); 31.51 (C_8); 51.92 ($C_{4,5}$); 62.08 (C_2); 66.23 (C_7); 166.66 (COO); 166.73 ppm (COO)

IR (film) : ν = 1750 cm^{-1} br, s (CO).

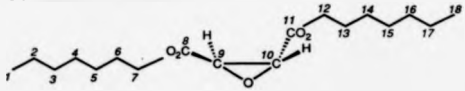
MS(EI) :m/z = 259 $(MH)^+$, 161, 133, 115 (M - $CO_2C_7H_{15}$); 98, 87, 70, 57$(C_4H_9)^+$, 41, 29 $(C_2H_5)^+$.

High resolution MS : $C_{13}H_{23}O_5(M + H)^+$, cal: 259.1545; found : 259.1576.

Elemental analysis : Found : C, 60.40; H, 8.80; $C_{13}H_{22}O_5$ requires C, 60.45; H, 8.58.

The chiral solvating agent ^1H NMR experiment showed that the mixed ester (2S, 3S)-epoxysuccinate obtained had an ee of 21.3%

· Diheptyl trans-2,3-epoxysuccinate (56)



^1H NMR, 220 MHz ($CDCl_3$/TMS) : δ = 0.74 - 1.03 (m, 6H, H of $C_{18,1}$); 1.15 - 1.47 (m, 16H, H of $C_{2,3,4,5,13,14,15,16}$); 1.55 - 1.80 (m, 4H, H of $C_{6,13}$); 3.68 (s, 2H, trans epoxy H) ; 4.09 - 4.33 ppm (m, 4H, H of $C_{7,12}$).

^{13}C NMR, 100.62 MHz ($CDCl_3$/$CDCl_3$ = δ 76.90 ppm) : δ = 13.85 ($C_{1,18}$); 22.39 ($C_{2,17}$); 25.56 ($C_{3,16}$); 28.27 ($C_{4,15}$); 28.66 ($C_{5,14}$); 31.50 ($C_{6,13}$); 51.91 ($C_{9,10}$); 66.20 ($C_{7,12}$); 166.73 ppm ($C_{11,8}$).

IR (film) : ν = 1760 cm^{-1} brs (CO).

MS(EI) : m/z = 329 $(MH)^+$, 231, 143 $(CO_2C_7H_{15})^+$, 99$(C_7H_{15})^+$, 57$(C_4H_9)^+$.

High resolution MS: $C_{19}H_{32}O_5$ $(M + H)^+$ cal: 329.2327; found : 329.2315.

Elemental analysis : Found : C, 65.82; H, 9.91; $C_{18}H_{32}O_5$ requires C, 65.82; H, 9.82.

The chiral solvating agent ^1H NMR experiment showed that the diheptyl (2S, 3S)-epoxysuccinate obtained had an ee of 20.5%.

The transesterification reaction using Rhizopus javanicus (FL-AP15) was carried out at 40°C with diethyl (2S, 3S)-epoxysuccinate (34a) made enantiomerically pure (see section 7.3.1.4). After 20 days, compounds (55a) and (56a) were obtained and analyzed by 400 MHz ^1H NMR in presence of (S)-(+)-2,2,2-trifluoro-1-(9-anthryl)-ethanol in CCl_4.

· 1-ethyl 4-heptyl trans-(2S, 3S)-epoxysuccinate (34a)

In the 400 MHz ^1H NMR spectrum, an AB system at 3.39 ppm was obtained for the epoxy hydrogens. Splitting of the AB system at 3.36 ppm was only observed on addition of the racemate (ee ≥ 97%). The downfield signal was thus assigned to the (2S, 3S)-enantiomer.

Specific rotation measurement. $[\alpha]_D^{2.5} = +73.38^o$ (c = 0.539, $CHCl_3$).

· Diheptyl trans-(2S, 3S)-epoxysuccinate (56a)

In the 400 ^1H NMR spectrum, a singlet at 3.37 ppm was obtained for the epoxy hydrogens. Splitting of the singlet at 3.37 ppm was only observed on addition of the racemate. The downfield signal was assigned to the (2S, 3S)-enantiomer.

Specific rotation measurement. $[\alpha]_D^{2.7} = +59.34^o$ (c = 0.439, $CHCl_3$)

In addition, the reaction with Rhizopus javanicus (LF-AP15) using the racemate of diethyl trans-(2,3)-epoxysuccinate (34) led after 48 days to compounds (34), (55) and (56). ^1H NMR (as above) showed that diethyl (2R, 3R)-epoxysuccinate and diheptyl (2S, 3S)-epoxysuccinate (56a) were both obtained with ee ≥ 97% (see section 4.4.1.2).

7.3.4.2 Preparation of enantiomerically pure diesters of cis-2,3-epoxysuccinate

Spontaneous hydrolysis of diethyl cis-2,3-epoxysuccinate (40) in phosphate buffer at pH 7.

The reaction was done as indicated for the trans epoxide (see section 7.3.4.1).

The crude compound, obtained in 98% yield, was purified by Kugelrohr distillation (150°C at 0.01 mm Hg) to give a colourless liquid (77%) which was fully characterized as 1-ethyl 2,3-epoxysuccinic acid (57).

^{1}H NMR, 220 MHz ($CDCl_3$/TMS) : δ = 1.33 (t, 3H, J = 7.3 Hz, CH_3); 3.83 (AB system, 2H, cis epoxy H); 4.34 (q, 2H, J = 7.3 Hz, CH_2); 7.87 ppm (br s, 1H OH).
^{13}C NMR, 100.62 MHz ($CDCl_3$/$CDCl_3$ = δ 76.90): δ = 13.77 ($CH_2\underline{C}H_3$); 52.71 (C-O-C); 62.70 ($\underline{C}H_2CH_3$); 166.28 ($\underline{C}OOCH_2$); 168.56 ppm (COOH)
At low temperature, 2 peaks corresponding to the epoxy carbons can be resolved.
IR (film) : ν = 1750 cm^{-1} br s (CO).
MS (EI): m/z = 161(MH)$^+$, 143 (M-OCH_2CH_3)$^+$, 115 (M-$CO_2CH_2CH_3$)$^+$.
High resolution MS : $C_6H_9O_5$, cal : 161.0450 found : 161.0433.

Hydrolysis of diethyl cis-2,3-epoxysuccinate (40) using PLE as biocatalyst

To diethyl cis-2,3-epoxysuccinate (0.19 g; 1.00 mmol), suspended in pH 7 phosphate buffer (0.1 M, 6 ml) stirred vigorously at 37°C, was added, dissolved in phosphate buffer (0.3 ml), PLE (260 units) obtained from the centrifugation of 200 µl of the PLE preparation supplied by Boehringer. The progress of the hydrolysis was monitored by addition of NaOH (0.99 M) from an autotitrator maintaining the pH at 7. After 4 hours, the reaction stopped when 1 equivalent of NaOH (0.99 ml) had been added. The reaction mixture was brought to pH 2 by addition of HCl (5 M), extracted with ethyl acetate (4 x 20 ml) and the organic phase was dried over Na_2SO_4. After filtration and evaporation under reduced pressure a crude colourless liquid (0.15 g; 92%) was obtained, which was further purified by Kugelrohr distillation (87.5°C at 0.07 mm Hg).

^{1}H NMR, 220 MHz ($CDCl_3$/TMS) : δ = 1.33 (t, 3H, CH_3); 3.83 (AB system, 2H, cis epoxy H); 4.34 (q, 2H, CH_2); 7.87 ppm (br s, 1H, OH).

A 400 MHz ^{1}H NMR of this sample was run in presence of 1 equivalent of (R)-(+)-α-methylbenzylamine. A splitting of the AB system at 3.83 ppm was observed producing two AX systems at (3.46 and 3.30 ppm) and (3.46 and 3.26 ppm) corresponding to each enantiomer. The ee was determined to be 25%. Partial splitting was also obtained for the quartet and triplet of the ethyl group.

Enzymatic hydrolysis of diethyl 2-tosyl-D,L-tartrate (46)

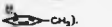
To racemic diethyl 2-tosyl tartrate (2.07 g; 5.70 mmol) dissolved in dichloromethane (2.5 ml) was added pH 7 phosphate buffer (0.1 M, 35 ml). To the mixture stirred at 25°C was added α-chymotrypsin (210 mg; 10710 units), and the pH was maintained at 7 by titration with aqueous sodium hydroxide (0.307 M) from an autotitrator. After 22 hours, the reaction was terminated by acidification of the reaction mixture to pH 2 with HCl (5 M). The volume of sodium hydroxide added (9.25 ml) corresponded to the hydrolysis of 24.7% of all ester groups. After neutralisation to pH 7 with sodium hydroxide (5 M), the aqueous mixture was extracted with ethyl acetate for the recovery of the non-hydrolyzed diethyl 2-tosyl tartrate. The product of hydrolysis was obtained by ethyl acetate extraction of the aqueous phase after reacidification to pH 2. After drying over Na_2SO_4, filtration and evaporation of the ethyl acetate under reduced pressure, monoethyl 2-tosyl tartaric acid (60) was collected as a yellowish oil.

Diethyl 2-tosyl tartrate was subjected to flash chromatography eluting with petrol-ethyl acetate (8/2) and collected as a colourless oil (0.99 g; 47.8%) which sometimes crystallized on long standing depending upon the enantiomeric purity of the sample. Following the recording of the ^{1}H NMR spectrum of the compound in carbon tetrachloride in the presence of 2 equivalents of (S)-(+)-2,2,2-trifluoro-1-(9-anthryl)-ethanol with d_5-benzene as a reference, baseline splitting of all the hydrogens was observed except for the ethyl hydrogens (practically resolved). The methyl of the tosylate group appeared in the 2.20 ppm region as 2 singlets in a ratio of 79/21 for

the downfield and upfield singlets respectively. The ee was determined (58.8%) and as a result of the assignment of each singlet using enantiomerically pure enantiomers prepared by chemical methods, diethyl 2-tosyl-(2R, 3R)-tartrate (46a) corresponded to the predominant enantiomer of the mixture.

N.B. All data corresponding to this compound (46a) have been reported in section (7.3.3.2).

^{1}H NMR of the crude monoacid (0.84 g; 44%) was consistent with the proposed structure.

^{1}H NMR, 220 MHz ($CDCl_3$/TMS) : δ = 1.30 (t, $CH_2\underline{CH_3}$); 2.07 (s, 3H, $\underline{CH_3}$-CO-OCH_2CH_3); 2.45 (s, 2H, $\underline{CH_3}$Ar); 4.00 - 4.40 (m, $CH_2\underline{CH_3}$); 4.78 (d, 1H, $\underline{H}$COOH); 5.40 (d, 1H, $\underline{H}$COTos); 5.70 - 6.30 (br s, 1H OH); 7.39(d,2H, ); 7.85 ppm (d,2H, ).

After addition of 1 equivalent of proton sponge (59), the ^{1}H NMR spectrum showed an upfield shift from 5.40 ppm to 5.20 ppm of the doublet corresponding to the hydrogen of the carbon bearing the tosylate group. Consequently, the product of hydrolysis corresponded to 4-ethyl 2-tosyl tartaric acid (60).

The latter was then reesterified with ethanol using the following procedure:

To a mixture of 4-ethyl 2-tosyl tartaric acid (0.80 g; 2.4 mmol), HOBT (0.32 g; 2.4 mmol) and ethanol (1.1 ml) in dichloromethane (4.2 ml) stirred under nitrogen at 0^{0}C was added dicyclohexylcarbodiimide (0.61 g; 2.9 mmol). The reaction was left at 0^{0}C for a further hour and then left to warm up to room temperature overnight. The mixture was filtered and the precipitate washed several times with dichloromethane (3 x 4 ml). The combined dichloromethane fractions were washed with HCl 5M (2 ml) and with water. After drying for $\frac{1}{4}$ hour over Na_2SO_4, filtration and evaporation of the solvent under reduced pressure, the crude diethyl 2-tosyl tartrate obtained was purified by flash chromatography to give a viscous liquid (0.49 g; 56.5%).

From the ^{1}H NMR experiment with chiral solvating agent (as above) , the ee was estimated to be 65.4% and corresponded to a sample enriched with diethyl 2-tosyl-(2S,

3S) tartrate as judged by the ratio of 17.3/82.7 for downfield and upfield signals of the methyl of tosylate group respectively.

Large scale α-chymotrypsin hydrolysis of diethyl 2-tosyl-D-tartrate (46a)

To increase the rate of hydrolysis and decrease the quantity of enzyme used, the reaction was carried out under the same conditions as indicated previously, but at a temperature of 35°C.

Consequently, hydrolysis by α-chymotrypsin (210 mg; 10710 units) of diethyl 2-tosyl-D-tartrate (4.07 g; 11 mmol) was completed in 21 hours. After extraction with ethyl acetate of the acidified reaction mixture (pH 2 by addition of HCl (5 M)), monoethyl 2-tosyl-D-tartaric acid (3.44 g, 92%) was collected as a crude colourless oil used as such for the next experiment.

Re-esterification of 4-ethyl 2-tosyltartaric acid (60a)

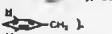
In the same way as indicated in section (7.3.4.2), reesterification with isobutylalcohol was achieved using DCCI in the presence of HOBT. The quantities of reagents was as follows:

4-ethyl 2-tosyltartaric acid	1.02 g ; 3.08 mmol	in dichloromethane (5.4 ml)
HOBT	417 mg ; 3.08 mmol	
isobutyl alcohol	2.2 ml	
DCCI	783 mg ; 3.79 mmol	

Identical work up of the reaction afforded a crude sample of 4-ethyl 1-isobutyl 2-tosyltartrate (1.64 g) which was purified by flash chromatography with petrol-ethyl acetate (8/2) to yield a colourless oil (R_F 0.26 petrol-ethylacetate (3/1)) (0.58 g; 48%).

^1H NMR, 220 MHz ($CDCl_3$/TMS) : δ = 0.93 (d, 6H, $CH(CH_3)_2$); 1.30 (t, 3H, CH_2CH_3); 1.83 - 2.09 (m, 1H, $CH(CH_3)_2$); 2.46 (s, 3H, $ArCH_3$); 3.13 (d, 1H, OH);

3.99 (d, 2H, CH_2CH); 4.03 - 4.20 (m, 2H, CH_2CH_3); 4.75 (d of d, HCOH); 5.39 (d, 1H, HCOTos); 7.39 (d, 2H,); 7.88 ppm (d, 2H,).

^{13}C. 100.65 Hz ($CDCl_3$/$CDCl_3$ = δ 76.90 ppm) = 13.75 (CH_2CH_3); 18.70 ($CH(CH_3)_2$); 21.47 ($ArCH_3$); 27.52 ($CH(CH_3)_2$); 62.83 (CH_2CH_3); 71.17 (HCOH); 72.15 ($CH_2CH(CH_3)_2$); 77.12 (HCOTos); 128.00 (); 129.54 (); 133.14 ( ); 145.07 ( ); 165.69 (COO); 169.68 ppm (COO).

IR (film) : ν = 3500 br s (OH), 1770 sh, 1745 cm^{-1} s (CO).

MS(EI) : m/z = 389 $(MH)^+$; 155 (SO_2- ); 91 ($ArCH_3$).

High resolution MS : $C_{17}H_{25}O_6S$ (M + H), cal 389.1270; found 389.1215.

Elemental analysis: Found C, 52.72; H, 6.27 $C_{17}H_{24}O_6S$ requires C, 52.57; H, 6.23.

Transesterification of diethyl 2-tosyl tartrate (46) using p-toluenesulfonic acid as a catalyst

Transesterification of diethyl 2-tosyl tartrate or diisobutyl 2-tosyl tartrate was done according to the following procedure using the appropriate alcohol.

A mixture of diethyl 2-tosyl tartrate (1.98 g; 5.49 mmol), isobutyl alcohol (0.8 ml), p-toluenesulfonic acid (0.07 g; 0.37 mmol) in benzene (15 ml) was brought to reflux under nitrogen. The reaction was monitored by Tlc in petrol-ethyl acetate (3/2) at regular intervals. When under UV detection, the ethyl isobutyl 2-tosyl tartrate was estimated to be in greater quantity than the corresponding diesters, the reaction was stopped. After evaporation under reduced pressure, the residue was dissolved in ether and washed successively with sodium hydrogen carbonate (5%), then water and the ether phase was dried over Na_2SO_4. After filtration and evaporation of the solvent under reduced pressure, flash chromatography (petrol-ethyl acetate (8/2)) afforded a colourless oil, 1-ethyl 4-isobutyl 2-tosyl tartrate as the major product (1.24 g; 58%). For more details concerning this reaction, refer to section (4.4.2.3).

Formation of the tert-butyldimethylsilyl ether of 4-ethyl 1-isobutyl 2-tosyl tartrate (62a)

The procedure followed was the same as indicated in section (7.3.3.4). The ether was obtained in 97.5% yield after purification by flash chromatography (petrol-ethyl acetate (9/1) (R_f 0.30) as a viscous oil which solidified after long standing.

^{1}H NMR, 220 MHz ($CDCl_3/CHCl_3$ = δ 7.30 ppm): δ = 0.00(s, 3H, $SiCH_3$); 0.09 (s, 3H, $SiCH_3$); 0.85 (s, 9H, $SiC(CH_3)_3$); 0.93 (d, 6H, $CH(CH_3)_2$); 1.23 (t, 3H, CH_2CH_3), 1.84 - 2.03 (m, 1H, $CH(CH_3)_2$); 2.46 (s, 3H, $ArCH_3$); 3.30 - 4.19 (m, 4H, CH_2); 4.75 (d, 1H, HCOSi); 5.35 (d, 1H, HCOTos); 7.37 (d, 2H, ); 7.86 ppm (d, 2H, ).

^{13}C NMR, 100.62 MHz ($CDCl_3/CDCl_3$ = δ 76.86 ppm) : δ = -5.80 ($SiCH_3$); -5.00 ($SiCH_3$); 13.76 (CH_2CH_3); 18.02 ($SiC(CH_3)_3$); 18.80 ($CH(CH_3)_2$); 21.41 ($ArCH_3$); 25.35 ($C(CH_3)_3$); 27.40 ($CH(CH_3)_2$); 61.57 (CH_2CH_3); 72.06 ($CH_2CH(CH_3)_2$); 72.71 (SiOCH); 78. 46 (HCOTos); 127.88 (); 129.40 (); 133.80 (); 144.64 (); 166.22 (COO); 168.77 ppm (COO).

IR (film) : ν = 1750, 1740 cm^{-1} brs.

MS(CI) : m/z = 520 ($M + NH_4$)$^+$, 503 ($M + H$)$^+$, 445 ($M - iBu$)$^+$, 155 ()$^+$, 91 ()$^+$.

Elemental analysis : Found C, 54.90; H, 7.76. $C_{23}H_{38}O_8SiS$ requires C, 54.95; H, 7.62.

For 1-ethyl 4-isobutyl 2-tosyltartrate (61), obtained as a mixture with 4-ethyl 1-isobutyl 2 tosyl tartrate (64) (see previous section)

^{13}C NMR, 100.62 MHz ($CDCl_3/CDCl_3$ = δ 76.90 ppm) : δ = -5.85 ($SiCH_3$); -5.05 ($SiCH_3$); 13.59 (CH_2CH_3); 17.94 ($SiC(CH_3)_3$); 18.74 ($CH(CH_3)_2$); 21.34 ($ArCH_3$); 25.27 ($C(CH_3)_3$); 27.26 ($CH(CH_3)_2$); 61.96 (CH_2CH_3); 71.55 ($CH_2CH(CH_3)_2$); 72.61 (SiOCH); 78.44 (HCOTos); 127.86 (); 129.36 (); 133.57 (); 144.64 (); 166.01 (COO); 168.74 ppm(COO).

Preparation of 1-ethyl 4-isobutyl (2S, 3R) epoxysuccinate (58a)

The procedure followed was the one indicated in section (7.3.3.4), using the mixed esters of 2-tosyl tartrate (42a) obtained as indicated in section (8.4.2.5). After purification by flash chromatography (petrol-ethyl acetate (85/15), distillation using a Kugelrohr apparatus (90°C at 0.01 mm of Hg) afforded as a colourless liquid 1-ethyl 4-isobutyl (2S, 3R)-epoxysuccinate in 81% yield.

^1H NMR, 400 MHz (CCl_4; d_6-benzene/$d_5$benzene = δ 7.20 ppm) : δ = 0.84 (d, 3H, $CH(\underline{CH_3})_2$); 0.86 (d, 3H, $CH(\underline{CH_3})_2$); 1.15 (t, 3H, J = 7.1 Hz, $CH_2\underline{CH_3}$); 1.79 - 1.89 (m, 1H, $C\underline{H}(CH_3)_2$); 3.30 - 3.32 (AB system, 2H, cis epoxy H); 3.75 - 3.84 (m, 2H, $OC\underline{H_2}CH$); 4.04 ppm (q, 2H, J = 7.1 Hz, $C\underline{H_2}CH_3$).

^{13}C NMR, 100.62 MHz ($CDCl_3/CDCl_3$ = δ 76.89 ppm) : δ = 13.84 ($CH_2\underline{CH_3}$); 18.73 ($CH(\underline{CH_3})_2$); 27.46 ($\underline{C}H(CH_3)_2$); 52.42 ($\underline{C}$-O-$\underline{C}$); 61.76 ($\underline{C}H_2CH_3$); 71.76 ($\underline{C}H_2CH$); 165.49 (COO); 165.56 ppm (COO).

IR (film) : ν = 1750 cm^{-1} brs (CO).

M(EI) : m/z = 217 $(M + H)^+$; 161, 143 $(M - CO_2CH_2CH_3)^+$ 115 $(M - CO_2CH_2CH(CH_3)_2)^+$ 71, 57 $(CH_2CH(CH_3)_2)^+$; 29 $(CH_2CH_3)^+$.

High resolution MS : $C_{10}H_{17}O_5(M + H)^+$: cal. 217.1076; found 217.1089.

Elemental analysis : Found : C, 55.30; H, 7.50 $C_{10}H_{16}O_5$ requires C, 55.55; H, 7.46.

Specific rotation measurement:

$[\alpha]_D^{24}$ = +3.33 (c = 3.225, $CHCl_3$).

The ^1H NMR spectrum at 400 MHz in the presence of 4 equivalents of (S)-(+)-2,2,2-trifluoro-1-(9-anthryl)ethanol using CCl_4 as solvent and d_5-benzene as reference was run.; splittings were observed only in the presence of racemic compound (see section 4.4.2.3). Thus the ee ≥ 97%.

7.4 EXPERIMENTAL SECTION TO CHAPTER 6

7.4.1 Preparation of monomeric acetoin

Acetoin (crystalline dimers, 5 g) was dissolved in water (20 ml). The aqueous phase was then continuously extracted with ether for 18 hours. After drying the ethereal extract over $MgSO_4$, most of the ether was distilled off under nitrogen. The concentration of the acetoin solution could then be deduced by ^1H NMR using as internal standard a solution of 4-methylbenzophenone of known concentration.

^1H NMR, 220 MHz ($CDCl_3$/TMS) : δ = 1.40 (d, 3H, $CHC\underline{H}_3$); 2.22 (s, 3H, $COC\underline{H}_3$); 4.29 ppm(q, 1H, $C\underline{H}$-CH_3).

7.4.2 Preparation of the ester (-)-α-methoxy-α-(trifluoromethyl)-phenyl acetate of acetoin

7.4.2.1 Using N,N' dicyclohexylcarbodiimide

(-)-α-Methoxy-α-(trifluoromethyl)phenyl acetic acid (0.53 g; 2.26 mmol) and N,N'-dicyclohexylcarbodiimide (0.47 g; 2.27 mmol) were mixed in THF (10 cm^3) at 4° C. A freshly prepared solution of the monomeric of racemic acetoin (0.10 g; 1.13 mmol) was immediately added together with pyridine (0.5 ml). After stirring the mixture for four days at 4° C, the dicyclohexylurea was filtered off. The filtrate was treated with acetic acid (0.5 ml) to decompose any excess N,N'-dicyclohexyl carbodiimide. After 15 minutes, dicyclohexylurea was removed by filtration. The filtrate was taken to dryness *in vacuo* and the residue was dissolved in ether (150 ml). After washing successively with HCl , sodium bicarbonate (5%) and water, the ether solution was dried over Na_2SO_4 and evaporated to give a yellow liquid. The crude material was purified by flash chromatography, eluting with petrol-ether (3/1), to afford a viscous colourless oil (0.24 g, 70%). For data see section (7.4.2.2).

7.4.2.2 Using 1,1'-carbonyldiimidazole

(-)-α-Methoxy-α-(trifluoromethyl) phenylacetic acid (0.46 g; 1.96 mmol) and 1, 1'-carbonyldiimidazole (0.32 g, 197 mmol) were stirred in THF (1 cm^3) under an

atmosphere of dry nitrogen for 1 hour. A solution of monomeric racemic acetoin (0.084 g, 0.95 mmol) freshly prepared in THF (0.5 cm^3) was added and the mixture stirred for 12 hours. THF was evaporated and the residue was dissolved in ether (50 ml) and washed successively with HCl (5 ml, 5M), sodium bicarbonate (10 ml, 5%) and water. The ether solution was dried overnight with Na_2SO_4 and evaporated to give a yellow liquid (0.21 g). After purification by flash chromatography (petrol-ether (3/1)), a colourless oil was obtained (0.22 g, 76%).

^1H NMR, 400 MHz ($CDCl_3/CDCl_3$ = δ : 7.25 ppm) : δ = 1.45 (d, $CHCH_3$); 1.50 (d, $CHCH_3$); 2.10 (s, CH_3CO); 2.20 (s, CH_3CO); 3.56 - 3.57 (m, OCH_3); 3.61 - 3.62 (m, $O\text{-}CH_3$); 5.26 (q, J = 7.1 Hz, $CHCH_3$); 5.29 (q, J = 7.0 Hz, $CHCH_3$); 7.40 - 7.44 (m, 3H, Aromatic H); 7.55 - 7.62 ppm (m, 2H, Aromatic H).

MS(CI) : m/z = 322 $(M + NH_4)^+$.

Elemental analysis : Found C, 55.32; H, 5.18, $C_{14}H_{15}F_3O_4$ requires C, 55.27; H, 4.97.

7.4.2.3 Analysis

The ester (-)-α-methoxy-α-(trifluoromethyl)phenyl acetate of acetoin was purified by high pressure liquid chromatography using a S5 OD52 C-18 reverse phase column. The diastereoisomers could be sucessfully separated using this column and consequently, the diasteresisomeric ratio deduced.

The high pressure liquid chromatography was performed using as solvent a mixture of methanol-water (40/60).

Detection was by UV at λ = 214

Flow rate 0.75 ml/min

Retention times 120 minutes

136 minutes.

However, the ratio of the diastereoisomers was deduced on a routine basis using ^{19}F NMR. The sample was dissolved in $CDCl_3$ and $CFCl_3$ was used as internal standard.

^{19}F NMR, 84.67 MHz ($CDCl_3/CFCl_3$) : 71.41, 71.60 ppm.

For the results of the different experiments, see section 6.2.

7.4.3 Preparation of optically pure (R)-(-)-Acetoin

Dibutyltin oxide (1.40 g; 5.60 mmol) and (R,R)-2,3-butanediol (0.50 g; 5.60 mmol) were refluxed in benzene (20 ml) with azeotropic removal of water by using a Dean and Stark apparatus. The mixture was heated at reflux overnight. The solvent was evaporated and the product was recrystallised twice from benzene to give white crystals of 2,2-dibutyl-4,5-dimethyl-2-stanna-1,3-dioxolane (1.40 g; 76%) mp 132° C Lit[121] mp = 134 - 136° C.

The stanna-dioxolane (1.40 g, 4.40 mmol) was dissolved in dichloromethane (20 cm^3) and a solution of bromine (227 µl, 698 mg; 4.4 mmol) in dichloromethane (6.5 ml) was added dropwise with stirring. The mixture was left overnight and then the solution was washed twice with water (50 ml) and the combined washings filtered. The aqueous solution was subjected to continuous extraction with ether. The ether solution was then dried with Na_2SO_4, filtered and distilled over nitrogen to afford the monomer of (R)-(-)-acetoin ready for the preparation of the ester (see section 6.2).

^1H NMR, 220 MHz ($CDCl_3$/TMS) : δ = 1.40 (d, 3H, $CHCH_3$); 2.22 (s, 3H, $COCH_3$); 4.29 ppm(q, 1H, $CHCH_3$).

Specific rotation measurement: $[\alpha]_D^{22.5}$ = -84° (c = 0.6, water).

7.4.4 2,3-Butanediol : NMR Experiments

To detect the presence of meso 2,3-butanediol in a racemic sample a ^{13}C NMR spectrum was obtained. A mixture of meso- and racemic 2,3-butanediol gave the following ^{13}C spectrum.

^{13}C NMR, 22.63 MHz ($CDCl_3/CDCl_3$ = δ 77.00 ppm) : δ = 16.55 (meso CH_3); 18.70 (racemate CH_3); 70.50 (meso CH); 71.87 ppm (racemate CH).

Evaluation of the enantiomeric composition of 2,3-butanediol

^1H NMR (220 MHz $CDCl_3$/TMS) : δ = 1.10 (d, 6H, $2CH_3$); 3.30 - 3.50 (m, 2H, CHCH); 3.60 ppm (brs, OH).

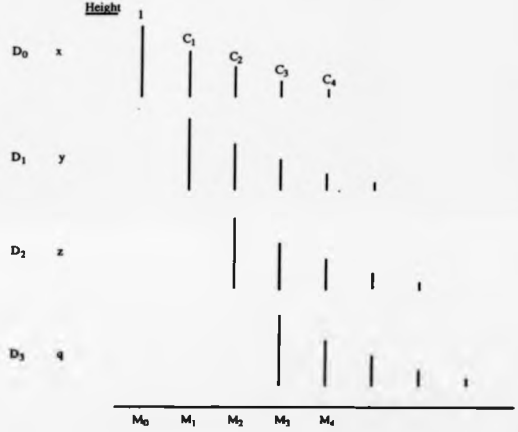
A 400 MHz ^1H NMR was run after addition of 1.5 equivalents of the chiral solvating agent (S)-(+)-2,2,2-trifluoro-1-(9-anthryl)ethanol in CCl_4 with d_5-benzene as reference.

Splitting of the doublet at 1.10 ppm was observed only in the presence of a racemic compound. (2R, 3R)-(-)-2,3-butanediol gave one doublet only. After addition of racemate, it was deduced that (2R, 3R)-(-)-2,3-butanediol corresponded to the upfield doublet.

APPENDIX

Composition of the mixture of deuterated diethyl N-p-toluenesulfonyl aspartates by mass spectral analysis

The percentage of the non-deuterated (D_0), monodeuterated (D_1), dideuterated (D_2) and trideuterated (D_3) diethyl N-p-toluenesulfonyl aspartate were evaluated from the mass spectrum. The intensities (M_0, M_1, M_2, M_3, M_4) of the peaks due to the molecular ion were the result of the relative fractional contribution of the non-, mono-, di- and trideuterated species donated as x, y, z and q respectively.



Figure(3:6)

Assuming the same relative intensities for each series, therefore the relative heights for the peaks of all four series are: 1, C_1, C_2, C_3 and C_4.

Consequently, the ratio $\frac{M_1}{M_0}$. $\frac{M_2}{M_0}$ and $\frac{M_3}{M_0}$ can be evaluated as follows:

$$R_1 = \frac{M_1}{M_0} = \frac{x\,C_1 + y}{x} \Rightarrow \frac{M_1}{M_0} = C_1 + \frac{y}{x} \qquad \text{Let } \boxed{\frac{y}{x} = W_1}$$

$$R_2 = \frac{M_2}{M_0} = \frac{x\,C_2 + y\,C_1 + z}{x} = \frac{\frac{y}{W_1}\,C_2 + y\,C_1 + z}{\frac{y}{W_1}}$$

$$= C_2 + W_1\,C_1 + \frac{W_1\,z}{y}$$

$$\frac{M_2}{M_0} \cdot (C_2 + W_1\,C_1) = \frac{W_1 z}{y} = \frac{W_1 z}{x\,W_1} = \boxed{\frac{z}{x} = W_2}$$

$$R_3 = \frac{M_3}{M_0} = \frac{x\,C_3 + y\,C_2 + z\,C_1 + q}{x}$$

$$= \frac{x\,C_3 + x\,W_1\,C_2 + x\,W_2\,C_1 + q}{x}$$

$$\frac{M_3}{M_0} \cdot (C_3 + W_1\,C_2 + W_2\,C_1) = \boxed{\frac{q}{x} = W_3}$$

Therefore $\frac{q}{x} = W_3 \Rightarrow \frac{q + x}{x} = W_3 + 1$

$$q + x = x\,(W_3 + 1) \qquad \text{(a)}$$

and $\frac{y}{x} = W_1$, $\frac{z}{x} = W_2$ $\Rightarrow$ $y + z = x\,(W_1 + W_2)$ (b)

Addition of equations (a) + (b) gives

$$y + z + q + x = x\,[W_1 + W_2 + W_3 + 1]$$

Therefore:

The percentage of non deuterated compound is equal to:

$$\frac{x \times 100}{y + z + q + x} = \frac{100}{W_1 + W_2 + W_3 + 1} \qquad (c)$$

The percentage of monodeuterated compound is:

$$\frac{y \times 100}{y + z + q + x} = \frac{W_1 \times 100}{W_1 + W_2 + W_3 + 1} \qquad (d)$$

The percentage of dideuterated compound is:

$$\frac{z \times 100}{y + z + q + x} = \frac{W_2 \times 100}{W_1 + W_2 + W_3 + 1} \qquad (e)$$

The percentage of trideuterated compound is:

$$\frac{q \times 100}{y + z + q + x} = \frac{W_3 \times 100}{W_1 + W_2 + W_3 + 1} \qquad (f)$$

Determinations of W_1, W_2, W_3

$$R_1 = \frac{M_1}{M_0} = C_1 + W_1 \Rightarrow W_1 = R_1 - C_1$$

$$R_2 = \frac{M_2}{M_0} = W_2 + (C_2 + W_1 C_1) \Rightarrow W_2 = R_2 - (C_2 + W_1 C_1)$$

$$W_2 = R_2 - [C_2 + C_1 (R_1 - C_1)]$$

$$\Rightarrow W_2 = R_2 - C_2 - C_1 (R_1 - C_1)$$

$$R_3 = \frac{M_3}{M_0} = W_3 + C_3 + W_1 C_2 + W_2 C_1$$

$$R_3 = \frac{M_3}{M_0} = W_3 + C_3 + C_2 (R_1 - C_1) + C_1 [R_2 - C_2 - C_1(R_1 - C_1)]$$

$$\Rightarrow \quad W_3 = R_3 - [C_3 + C_2 (R_1 - C_1) + C_1 (R_2 - C_2 - C_1(R_1 - C_1))]$$

Calculations

C_1, C_2 and C_3 were evaluated using a computer program and values 0.185, 0.073 and 0.011 respectively were assigned for isotopic distribution of nondeuterated diethyl N-p-toluenesulfonyl aspartate. M_0, M_1, M_2, M_3 were extracted from the mass spectrum with respective values of 1.5, 18.4, 17.9 and 6.6.

$$R_1 = \frac{M_1}{M_0} = \frac{18.4}{1.5} = 12.27$$

$$R_2 = \frac{M_2}{M_0} = \frac{17.9}{1.5} = 11.93$$

$$R_3 = \frac{M_3}{M_0} = \frac{6.6}{1.5} = 4.40$$

$$\Rightarrow \quad W_1 = R_1 - C_1 = 12.08$$

$$W_2 = R_2 - (C_2 + W_1 C_1) = 9.62$$

$$W_3 = R_3 - (C_3 + W_1 C_2 + W_2 C_1) = 1.72$$

Replacing these values in equations (c), (d), (e) and (f) led to:

% of nondeuterated species	4.1%
monodeuterated	49.5%
dideuterated	39.4%
trideuterated	7.0%

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TITLE

SYNTHETIC AND ENZYMATIC STUDIES RELATED TO THE BIOSYNTHESIS OF PENICILLIC ACID AND ACETOIN

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