Mechanistic Studies on Biotin Biosynthesis

By

Michael J. McPherson

A Thesis Submitted for the Degree of Doctor of Philosophy in

The University of Edinburgh

Department of Chemistry
University of Edinburgh
September 1995

To my Family

Acknowledgments

I would like to begin by thanking my supervisor, Dr.Robert.L.Baxter, for his advice, encouragement, enthusiasm and for proving to be an excellent guide through modern bioorganic chemistry.

I would also like to thank the members of the Baxter research group, especially Lisa McIver, Dr. Mark Bacon, Dr. Dominic Campopiano and Steve Horgan for helpful advice and sometimes relevant discussion. Many thanks also to Dr. Dima Alexeev for the work involving protein crystallography, Dr. Otto Smekal for enzyme kinetics and additional thanks to Professor Richard Ambler and Mrs Margaret Daniel for help in using the High Voltage Paper Electrophoresis apparatus.

I like to acknowledge the technical staff involved, namely Mr.John Millar, Mr. Wesley Kerr and Mr. John Parkinson for NMR spectroscopy, Mr. Alan Taylor for Mass Spectrometry and Mrs. Lorna Eades for Elemental Analysis. Thanks to the EPSRC for funding.

I must also thank my parents and family for their love and encouragement over the past three years and their generous supplements to my grant which makes me forever in their debt.

Finally, many thanks to all my friends, especially Craig, Tom, Gill, Eric and Raymond for making flat-life a sometimes arduous task and to Melanie for her love and understanding.

Abstract

Biotin is a commercially important vitamin which acts as a cofactor in enzymatic carboxylation reactions. Biotin biosynthesis occurs by similar pathways in both microorganisms and plants.

This thesis describes studies on the last two enzymes on the biotin biosynthetic pathway. The penultimate enzyme, dethiobiotin synthetase (DTBS), catalyses the *ureido* ring closure of dethiobiotin from (7*R*,8*S*)-7,8-diaminononanoate (DAPA), carbon dioxide and ATP. The final step, catalysed by biotin synthase, involves the chemically difficult insertion of sulphur into the unactivated carbon skeleton of dethiobiotin to yield biotin.

A range of analogues of DAPA differing in chain length and C-8 substitution were synthesised and found to act as slow substrates of DTBS. Steady-state kinetic and binding parameters were evaluated for these modified substrates and their relevance to the mechanism of the DTBS reaction is discussed. Crystallographic and modelling studies of one analogue bound in the active site of DTBS suggests that the enzyme may employ an alternative mechanism of *ureido* ring closure to that of the native substrate.

Biotin production was demonstrated in cell-free extracts of an *E.coli bio*B transformant and the origin of the sulphur atom in biotin was investigated. A novel cysteine desulphurase enzyme, which catalyses the desulphurisation of L-cysteine to give L-alanine and sulphur, was discovered and its possible role in the biotin synthase reaction is discussed.

[6,6-$^2H_2$]-(±)-dethiobiotin and [6,6-F_2]-(±)-dethiobiotin were identified as target compounds in intermediate trapping experiments and as selective biocides. A synthetic route to these compounds was devised and the introduction of label was explored using labelled catalysts and carbonyl transformation chemistry.

Courses Attended

Organic Research Seminars, Department of Chemistry, University of Edinburgh (3 years attendance)

Mass Spectrometry in Action, Professors J. Monaghan and J. Scrivens, ICI Ltd., (1992, 6 lectures)

Basic Course in Radiation Protection in Laboratory Work in Science and Medicine, Dr. J. D. Simpson, Radiation Protection Service, University of Edinburgh (1992, 8 lectures)

Medicinal Chemistry, Professor R. Baker and colleagues, Merck, Sharp and Dohme (1993-1995)

Recent Advances in the Synthesis and Activity of Agrochemicals, Drs. I.Boddy and P.J.Dudfield, Schering Agrochemicals Ltd. (1992, 6 lectures)

NMR and its Application to Molecules of Biological Importance, Drs. I.H.Sadler and J.Parkinson, Department of Chemistry, University of Edinburgh (1993, 6 lectures)

Chemical Development in the Pharmaceutical Industry, Dr. J.P.Clayton and colleagues, SmithKline Beecham Pharmaceuticals Ltd. (1993, 5 lectures)

Fluorine for the Organic Chemist, Professor R.Chambers, University of Durham (1994, 2 lectures)

Peroxygens in Organic Synthesis, Dr. A.Johnstone, Solvay Interox Ltd. (1994, 5 lectures)

Industrial Biocatalysis, Dr. J.T.Sime and colleagues, SmithKline Beecham Ltd. (1994, 4 lectures)

Industrial Chemistry:From Research to Development, Drs. J.D.R Vass and B.J.Box, Zeneca Ltd. (1995, 4 lectures)

Abbreviations

a.a	amino acid
AdoMet	S-adenosylmethionine
ADP	adenosine diphosphate
Amp	ampicillin
anaRNR	anaerobic ribonucleotide reductase
AOP	8-amino-7-oxopelargonic acid
aq.	aqueous
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BCP	biotin carrier protein
b.p	boiling point
br	broad
BSA	bovine serum albumin
cm	centimetere(s)
CoA	coenzyme A
d	doublet
DAPA	7,8-diaminopelargonic acid
δ_C	^{13}C NMR chemical shifts
δ_H	^{1}H NMR chemical shifts
DTB	dethiobiotin
DTBS	dethiobiotin synthetase
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
FABMS	fast atom bombardment mass spectrometry
FAD	flavin adenine dinucleotide (oxidised form)
$FADH_2$	flavin adenine dinucleotide (reduced form)
FPLC	fast protein liquid chromatography

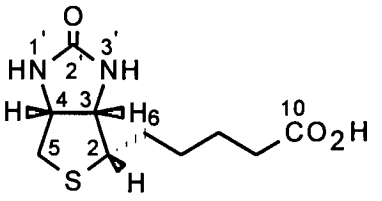
FTIR	fourier transform infrared (spectroscopy)
g	grams
hplc	high pressure liquid chromatography
HVPE	high voltage paper electrophoresis
IPTG	isopropyl β-D-thiogalactoside
J	spin-spin coupling constant
KAPA	7-keto-8-aminopelargonic acid
kDa	kilodaltons
lit.	literature
m	multiplet
M	molar
mCi	millicuries
mg	milligram
MHz	megahertz
min	minutes
mol	mole(s)
mmol	millimole(s)
m.p	melting point
MS	mass spectrometry
m/z	mass to charge ratio
NAD^+	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
nm	nanometre
NMR	nuclear magnetic resonance
$OD_{280/600}$	optical density at 280nm or 600nm

ppm	parts per million
P_i	inorganic orthophosphate
PP_i	inorganic pyrophosphate
q	quartet

Table of Contents

Chapter 1
Introduction

1.1 Biotin and its role in mammalian biochemistry



1

Our understanding of the metabolism of biotin **1** has accumulated intermittently since its discovery, at the turn of the century, as a result of the effects of nutritional deficiency. It was not until 1936 that the compound was first obtained in crystalline form from egg yolk by Kögl and Tönnis[1]. It required an additional seven years for the elucidation of its structure by du Vigneaud *et al.*[2-5] and a further two years to synthesise the vitamin[6,7]. Over a decade elapsed before Wakil *et al.*[8] discovered that biotin was an integral part of the acetyl CoA carboxylase enzyme, indicating a cofactorial role for biotin in carboxylation reactions in metabolism.

There are four biotin-dependent carboxylases important in human biochemistry[9] (**Fig.1.1**). β-Methyl crotonyl CoA carboxylase and propionyl CoA carboxylase are involved in amino acid metabolism, acetyl CoA carboxylase is a key enzyme in fatty acid biosynthesis and pyruvate carboxylase is the primary enzyme in gluconeogenesis. Because of the endogenous production of biotin by the intestinal flora, deficiency does not normally occur in man or higher animals, even when a biotin-free diet is administered. Biotin deficiency can be induced through large ingestion of raw egg white or by long-term antibiotic therapy. Symptoms suffered include dermatitis, alopecia, progressive ataxia and metabolic acidosis (see **Fig.1.2**). However, administration of supplemental biotin usually provides a quick and dramatic response to these ailments[10] (**Fig. 1.3**).

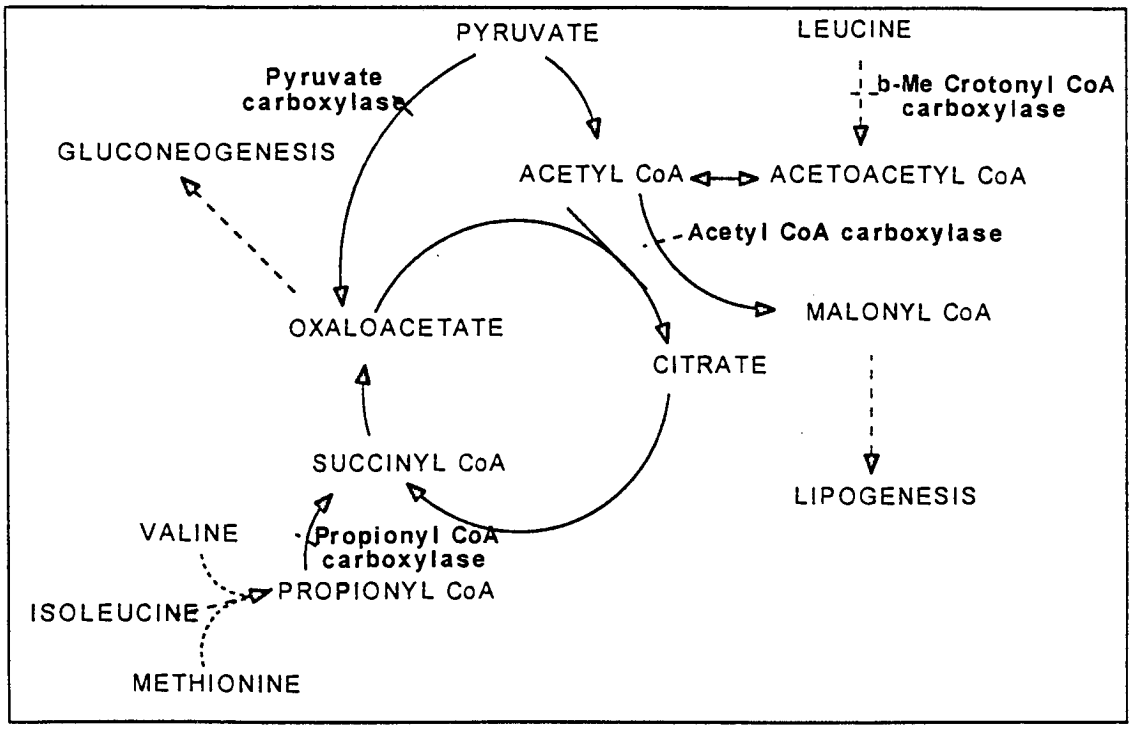


Fig.1.1 The role of biotin in cellular metabolism

Fig.1.2 Two-year old child suffering from biotin deficiency

Fig.1.3 Same child after six months of daily biotin supplements

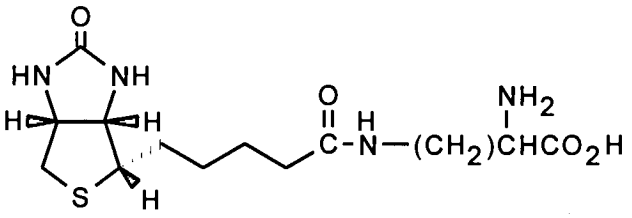
1.2 Biotin-Avidin Biotechnology

Since (+)-biotin is present in only minute amounts in biological tissues, the first total synthesis of biotin[6] provided a stepping stone towards more detailed studies on its biological role in the cell. However, despite a plethora[11] of novel enantioselective syntheses of biotin in the literature there exists only one which has been successfully employed in the commercial preparation of the vitamin[12]. The increasing demand for this vitamin has come, not only from its value in mammalian nutrition but its role as a powerful biochemical tool through the highly specific binding of biotin to the egg white protein avidin or the analogous bacterial protein streptavidin (from *Streptomyces avidinii*).

Avidin is a tetramer of four essentially identical subunits of 15 kDa each. X-ray crystal structural data[13,14] has revealed that each monomer is an eight-stranded antiparallel β-barrel, similar to that of the bacterial analogue streptavidin. The biotin binding site is located deep in the centre of the barrel where the presence of both hydrophobic and polar residues are required for its recognition. Avidin has four tryptophan residues per subunit and fluorescence studies[15] indicate that the binding of biotin shifts two or three tryptophans to an internal, hydrophobic, shielded environment and that the bound biotin is completely buried within the protein core. The high affinity ($K_d = 10^{-15}M$) for biotin is undisturbed by extremes of pH, salts or chaotrophic agents and this strong interaction, together with the facile modification of biotin's carboxylate functional group allowing the construction of a wide range of conjugates, has provided researchers with a unique tool for use in receptor studies, immunocytochemical staining and protein isolation[16].

1.3 The Biological Role of Biotin

Evidence supporting the role of biotin in the biosystem began with the isolation of biocytin **2** from yeast and the proposal that biotin existed covalently linked to a lysine residue of biotin-dependent enzymes[17]. Kasow and Lane verified this linkage of biotin to its carrier protein in the enzyme propionyl CoA carboxylase[18] and this manner of coupling has been shown in all biotin enzymes studied to date[19].



2

Biotin has now been shown to have a definitive cofactorial role for a group of carboxylases that can be subdivided into three classes.

1) In Class I (carboxylase) enzymes, biotin serves as a covalently bound "CO_2 carrier" for reactions in which CO_2 is fixed into acceptors such as pyruvate, acetyl-CoA, β-methyl-crotonyl-CoA and urea. The ATP-dependent carboxylation and subsequent transfer are summarized in the two partial reactions shown below :

$$\text{Enz-biotin} + \text{ATP} + HCO_3^- \xrightleftharpoons{Mg^{2+}} \text{Enz-biotin-}CO_2^- + \text{ADP} + P_i + H^+$$

$$\text{Enz-biotin-}CO_2^- + \text{R-H} \rightleftharpoons \text{Enz-biotin} + \text{R-}CO_2^-$$

2) The Class II (decarboxylase) enzymes catalyse the decarboxylation of β-keto acids and their thioesters and are responsible for regulation of sodium transport in anaerobes. Substrates for these enzymes include oxaloacetate, methylmalonyl-CoA and glutaconyl-CoA.

This class of enzymes serve as important energy transducers that do not require ATP and their reactions can be summarised as follows :

$$\text{Enz-biotin} + \text{R-CO}_2^- \rightleftharpoons \text{Enz-biotin-CO}_2^- + \text{R-H}$$

$$\text{Enz-biotin-CO}_2^- + 2(\text{Na}^+)_{in} \rightleftharpoons \text{Enz-biotin} + \text{HCO}_3^- + 2(\text{Na}^+)_{out}$$

3) The one Class III enzyme identified, transcarboxylase (EC 2.1.3.1), plays a key role in the fermentation process in *Propionibacterium shermanii* and involves the following transcarboxylation :

$$\text{Enz-biotin} + \text{oxalacetate} \rightleftharpoons \text{Enz-biotin-CO}_2^- + \text{pyruvate}$$

$$\text{Enz-biotin-CO}_2^- + \text{propionyl CoA} \rightleftharpoons \text{Enz-biotin} + \text{methylmalonyl CoA}$$

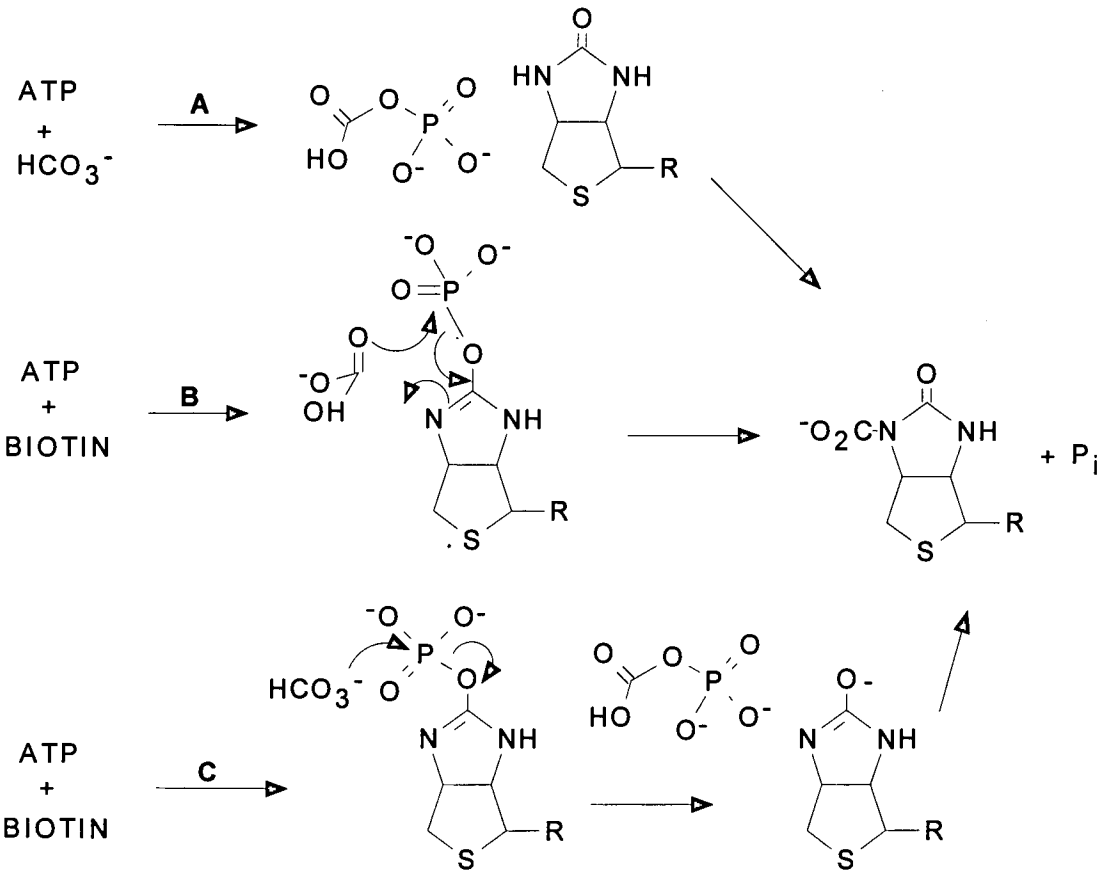
1.4 Mechanism of Carboxylation

The structural data[108] obtained from X-ray analysis of (+)-biotin provided an early insight into its mechanism of carboxylation. Since the distance between N-3' and C-6 of biotin is only 0.28nm, this steric hindrance from the valeric acid side chain suggested that carboxylation of biotin involved reversible formation of a N-1' carboxybiotin intermediate.

The isolation and characterisation of the methyl ester of N-1` carboxybiotin by Lynen *et al.*[20] suggested the enzymic intermediate in β-methyl crotonyl CoA carboxylase was N-1' carboxybiotin. Bruice *et al.*[21] suggested this structural assigment was equivocal and suggested an intermediary role for O-carboxybiotin which rearranged to the more stable N-acyl material during methylation and isolation. However, the synthesis of authentic N-1 carboxybiotinol[22] and the demonstration of its ability to act as a competent substrate for the biotin carboxylase and

carboxytransferase sub-units of *E.coli* acetyl CoA carboxylase provided convincing evidence that N-1 carboxybiotin must be a true biochemical intermediate.

Early ^{18}O tracer studies by Kaziro *et al.*[23] using [^{18}O]- bicarbonate as a substrate for propionyl CoA carboxylase demonstrated that two ^{18}O atoms from $HC^{18}O_3^-$ ended up in the carboxyl group of malonyl CoA and that the third was found in the phosphate derived from ATP. Three pathways for the mechanism of carboxylation have emerged, all consistent with the above observations (see **Scheme 1.1**)



Scheme 1.1

Pathway **A** involves activation of bicarbonate by ATP to produce carboxyphosphate. This activated bicarbonate (or CO_2 derived from it) then carboxylates the N-1 of biotin[24]. Pathway **B**[25,26] involves the

formation of O-phosphobiotin. This isoimide intermediate reacts with bicarbonate to give the carboxylated biotin and phosphate. The third pathway **C**[27] also utilises O-phosphobiotin as an intermediate, but this is then attacked by bicarbonate to carboxyphosphate and the *ureido* anion of biotin which in turn react to carboxylate biotin.

Discriminating between these proposed pathways has proved difficult since isotope exchange in partial reactions of several biotin-dependent carboxylases[24,28] and intermediate trapping[29,30] have proved unsuccessful. However, stereochemical experiments have provided evidence towards the exclusion of certain mechanisms.

Knowles *et al.*[31] synthesised the [γ-^{17}O,γ-^{18}O]-γ-phosphorothioate of ATP and utilised this chiral substrate to examine the stereochemical fate of the γ-phosphogroup of ATP in the reaction catalysed by pyruvate carboxylase. This experiment relied on the fact that enzyme-catalysed phospho group transfer involves inversion of configuration at phosphorus[32]. Therefore pathway **A** and **B** are expected to proceed with inversion of configuration whereas pathway **C** proceeds with net retention. They have argued that pseudorotatory effects (upon formation of O-phosphobiotin) were not applicable due to lack of enzymic precedent. Determination of the [^{16}O, ^{17}O, ^{18}O]-thiophosphate chirality resulted in overall inversion of configuration and this effectively rules out pathway **C**.

Concern regarding the low nuleophilicity of biotin's N-1 has been partially resolved. The proposed[33] high energy dianionic tetrahedral intermediate that would result from nucleophilic attack upon carboxyphosphate can be avoided through prior dissociation to the more electrophilic carbon dioxide and P_i. Although the failure to detect ATP/solvent oxygen exchange in positional isotope exchange experiments[30] does not discount carbon dioxide as the carboxylating

species, the inability to trap the carboxyphosphate intermediate may suggest high reactivity or subsequent dissociation at the active site.

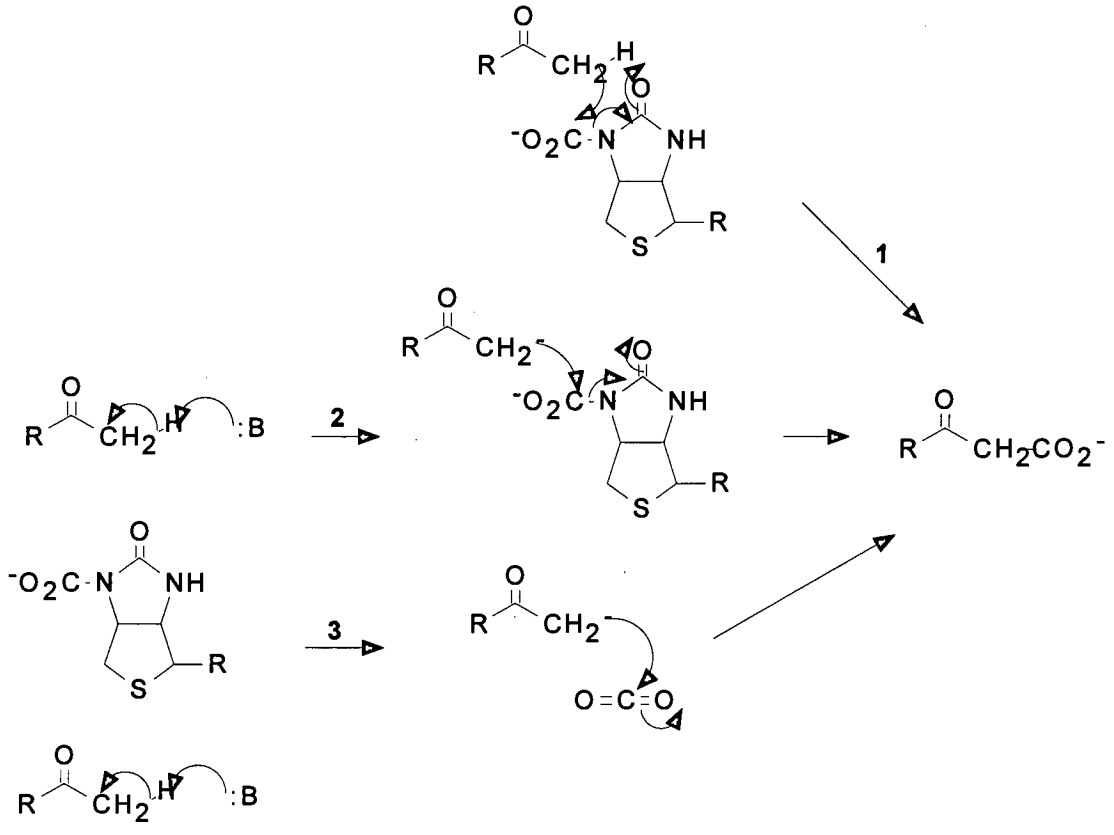
However, the ability of biotin carboxylase to synthesise ATP by utilising carbamoyl phosphate as a substrate[34] and its bicarbonate-dependent ATPase activity strengthen the view that carboxylation of biotin may proceed through pathway **A**.

1.5 Mechanism of Carboxyl Transfer

The early experiments of Retey and Lynen[35] on propionyl CoA carboxylase provided evidence on the stereochemistry and proton exchange involved in the enzymic reaction. They established that the reaction proceeds with retention of configuration at the site of carboxylation and that the exchange of protons between solvent and substrate was only accompanied with overall turnover. These early experiments led to a variety of proposals[35,36] involving either a concerted process **1**, or stepwise mechanisms such as **2** and **3** as shown in **Scheme 1.2.**

Resolution of these proposals was obtained by Stubbe *et al.*[37] who examined the action of propionyl CoA carboxylase on β-fluoropropionyl CoA. They observed a 6-fold increase in fluoride release compared to ADP formation and no evidence for the production of fluoromethylmalonyl CoA. The release of fluoride ion is indicative of α-proton abstraction from the substrate and the formation of ADP corresponds to the accumulation of biotin-CO_2. These results indicated that hydrogen abstraction could occur without concomitant CO_2 transfer from carboxylated biotin to the substrate. Thus the concerted mechanism **1** is not applicable and the authors suggested that the non-concerted

process should occur with "normal" substrates and that there must be a group at the active site of the enzyme that functions as proton acceptor.



Scheme 1.2

This mechanistic issue was settled by Knowles *et al.*[38] who employed double isotope fractionation to evaluate the rate-determining steps in transcarboxylase. Although both [^{13}C]-pyruvate and [$^{2}H_3$]-pyruvate gave primary isotope effects which didn't rule out either mechanism, the correct mechanism was distinguished by examining the consequence of substrate deuteration on the ^{13}C isotope effect. The ^{13}C effect with protio-substrate (2.3%) fell significantly on administration of deuterio-substrate (1.4%) indicating the carboxylation is a stepwise process which effectively rules out the concerted pathway **1**.

1.6 The Biotin Operon

The enzymes of biotin synthesis in *E.coli* are encoded by the *bio* operon which is located at 17 min on the genetic map[39] between the attachment site of the lambda prophage (attλ) and the *uvr*B gene. Complementation analysis by Rolfe[40] and Cleary and Campbell[41] indicated that the operon contained 5 complementation groups, namely *bio*A, *bio*B, *bio*C, *bio*D and *bio*F. An unlinked gene, *bio*H, has also been discovered[42] along with *bio*P, a permeability gene and *bio*R, a regulatory gene[43] (**Fig. 1.3**).

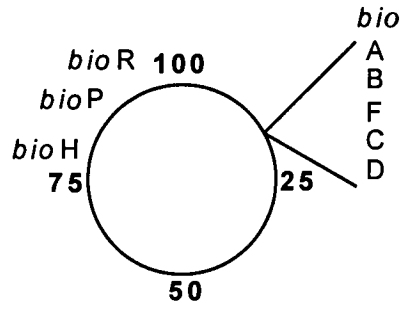
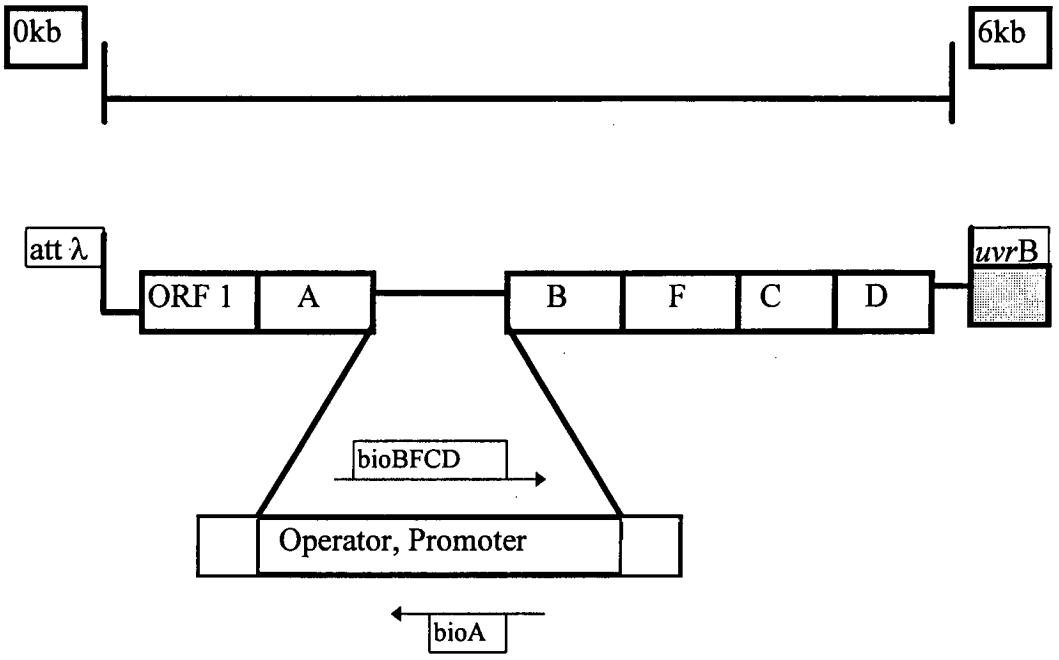


Fig.1.3. Chromosomal locations of the *E.coli bio* genes (minutes)

Cleary *et al.*[44] and Adhya *et al.*[45] established the gene order of the operon using deletion mapping and after determining the gene activity of deletion mutants concluded that the operon was divergently transcribed : *bio*B, F, C and *bio*D being transcribed as a unit from left to right and *bio*A from right to left, from a promoter/operator region located between *bio*A and *bio*B (see **Fig.1.4**).



*bio*A gene product - DAPA aminotransferase (430 a.a, 47,403 Da)

*bio*B gene product - Biotin synthase (346 a.a, 38,665 Da)

*bio*F gene product - KAPA synthetase (384 a.a, 41,599 Da)

*bio*C gene product - Unknown (prior to pimeloyl CoA, 251 a.a, 28,322 Da)

*bio*D gene product - Dethiobiotin synthetase (218 a.a, 23,917 Da)

ORF 1- Open Reading Frame (158 a.a, 17,101 Da)

Fig.1.4. The *bio* operon of *Escherichia coli*

1.7 Regulation of the *bio* operon

The earliest evidence for the control of biotin biosynthesis was discovered when Pai and Lichstein[46] noted that the total cellular biotin concentration decreased with increased biotin concentration in the growth media. The nature of this inhibition was shown to be repression rather than feedback inhibition. Eisenberg *et al.*[47] examined the interaction of the biorepressor with the operator site and concluded that the repressor protein is the product of the *bir*A (*bio*R) gene, a 35.3 kDa protein now known as biotin holoenzyme synthetase. This bifunctional protein serves as both the biotin activating enzyme and the transcriptional regulator for the *bio* operon. The *bir*A protein can activate biotin in the presence of ATP to form biotinyl-AMP, the co-repressor, which remains tightly bound to the repressor protein. This complex can either bind to the operator site and inhibit transcription or transfer the biotinyl moeity to a lysine residue on the apoenzyme of the biotin carboxyl carrier protein (BBCP) subunit of acetyl CoA carboxylase. In summary, the *bir*A gene product can catalyse each of the three reactions shown below :

Biotin + ATP + *bir*AGP $\longrightarrow$ biotinyl-5'-AMP-*bir*AGP + PP_i

Biotinyl-5'-AMP-*bir*AGP + apoenzyme $\longrightarrow$ holoenzyme + AMP + *bir*AGP

Biotinyl-5'-AMP-*bir*AGP + operator $\longrightarrow$ biotinyl-5'-AMP-*bir*AGP-operator

Thus the *bir*A gene product synthesises its own co-repressor which is unique property among DNA binding proteins. The three dimensional crystal structure of the repressor protein displays three domains including a helix-loop-helix DNA binding motif[48]. Consequently the level of expression of the biosynthetic enzymes and the amount of biotin synthesised *de novo* is dependant on the intracellular biotin concentration

and on the concentration of unbiotinylated carrier protein to which biotin must be attached to fulfill its essential metabolic role (see **Fig.1.5**)

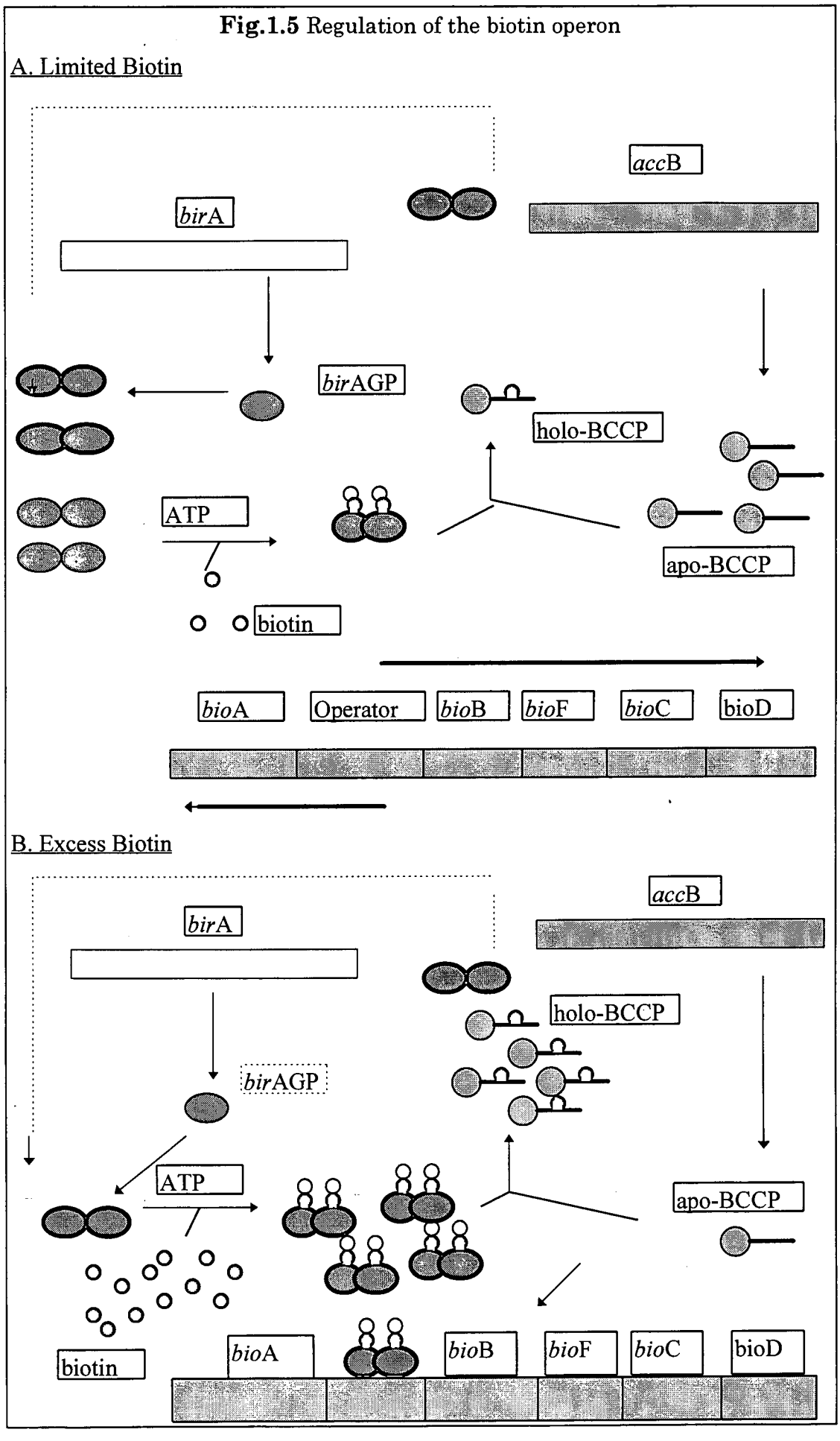
1.8 The Biosynthesis of Biotin

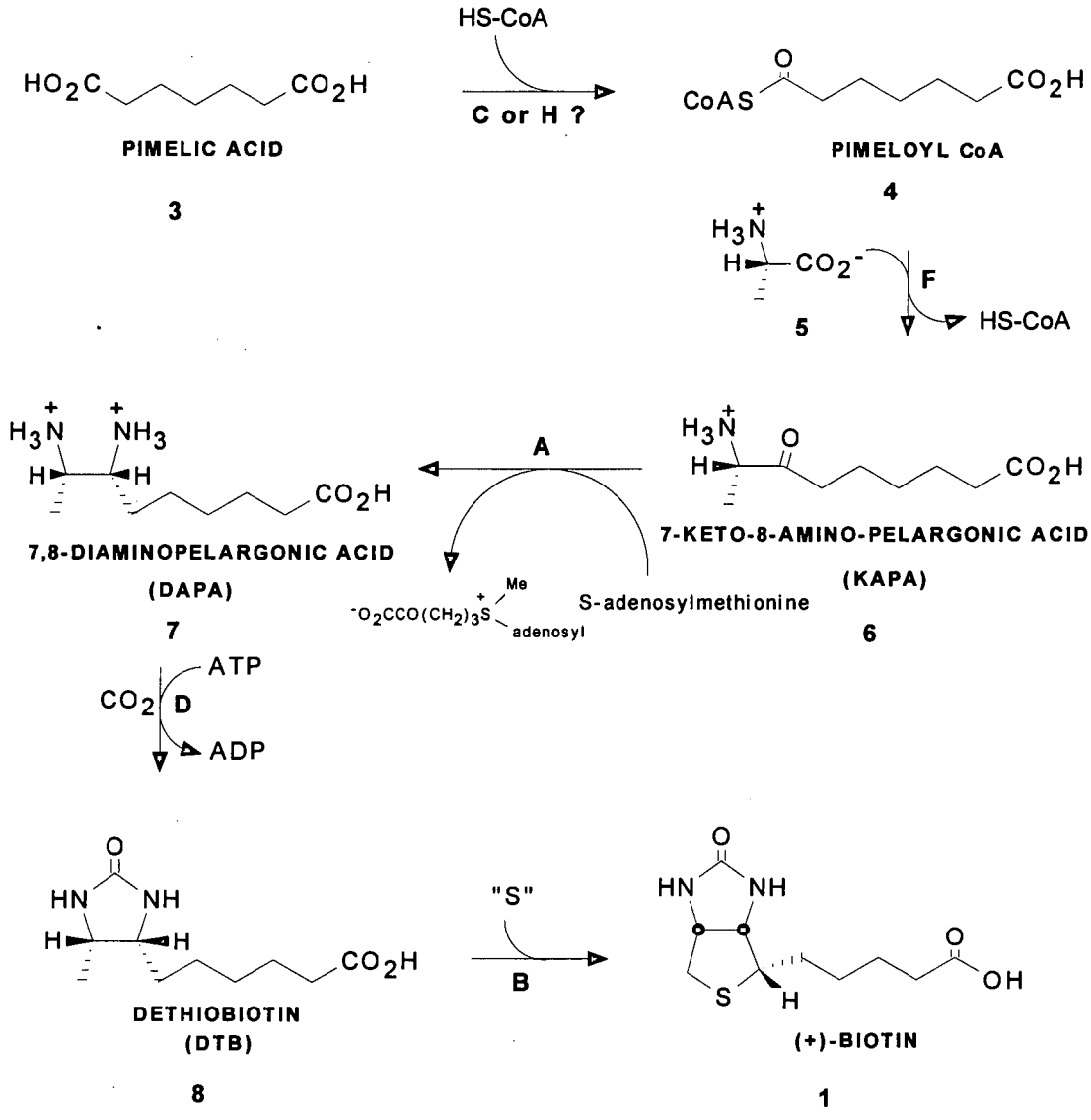
The nature of the biosynthetic and degradation pathways of biotin has preceded our knowledge of its mode of action. The miniscule quantities required to maintain normal cell function and therefore the limited amounts of possible metabolic intermediates available present a relatively unattractive target for biosynthetic studies. However, detailed investigation into the biogenesis of biotin has clarified the essentials of its biosynthetic construction.

Early bacterial studies by Mueller[49-51] and du Vigneaud[52] provided the first insights into the biosynthesis of biotin. The requirement of pimelic acid **8** and biotin **2** as a growth factor for *Corynebacterium diphtheriae* and the discovery[53] that dethiobiotin was as effective as biotin in supporting the growth of *Saccharomyces cerevisiae* led to the use of radiotracer methods, substrate analogues and genetic mutants to interpret the major steps in the biosynthesis of the coenzyme. On the basis of their results and those of other workers, studies with *Escherichia coli* mutants prompted Eisenberg *et al.*[54] to propose the pathway for biotin biosynthesis given in **Scheme 1.3**.

This route proceeds by condensation of L-alanine **5** with pimeloyl CoA **4** to give 7-keto-8-aminopelargonic acid (7-KAPA) **6**. Transamination then leads to 7,8-diaminopelargonic acid (DAPA) **7**. DAPA subsequently reacts with carbon dioxide in the presence of ATP to generate dethiobiotin **8**. Finally sulphur is inserted into dethiobiotin to yield biotin **1**.

Fig.1.5 Regulation of the biotin operon





Scheme 1.3 . Biosynthesis of Biotin

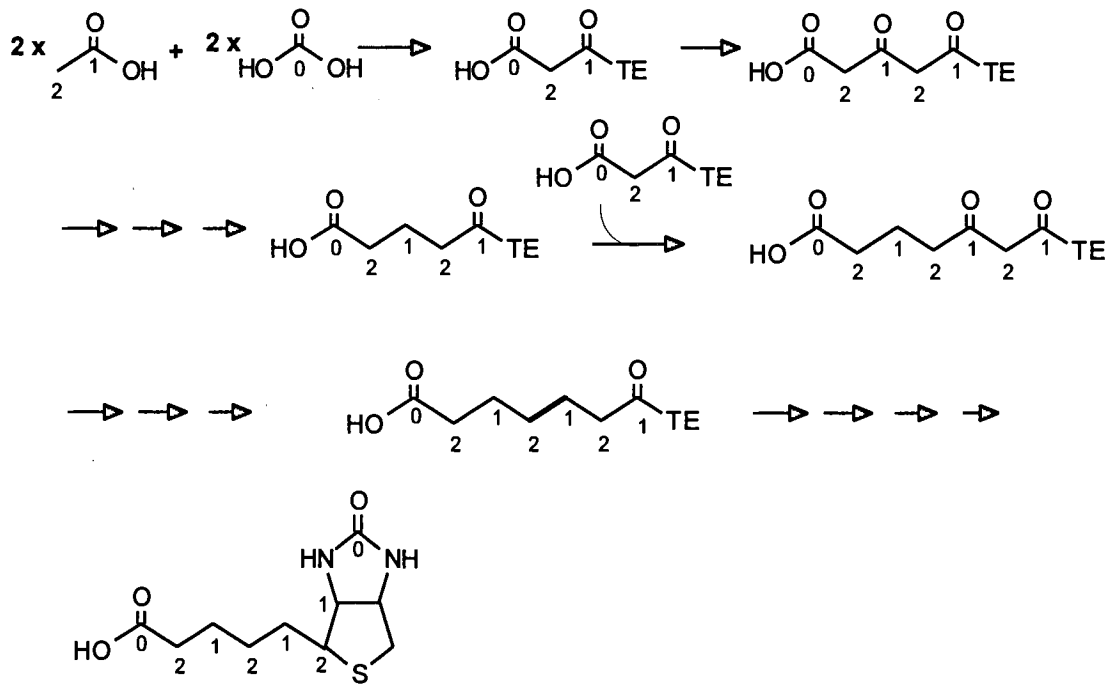
Initial tracer studies by both Eisenberg[55] and Elford and Wright[56] established the precursorial role of pimelic acid. Both performed feeding experiments with [1,7-^{14}C] pimelic acid and isolated the labelled metabolites, biotin and dethiobiotin from *Phycomyces blakesleeanus* and biotin-l-sulphoxide from *Aspergillus niger*. However the origin of pimelic acid is still unclear. Lynen *et al.*[57] proposed, based on early labelling studies with $^{14}CO_2$ in *Achromobacter*, that the biosynthetically aberrant

C-7 dicarboxylic acid derived pimelate group of biotin was constructed from three malonate units, the starter unit retaining the CO_2 derived carboxylate which became the C-9 of biotin.

Malonyl units have been shown to act as starter groups in other pathways, such as the biosynthesis of tetracycline[58]. However, in his experiment, Lynen could not locate 40% of the label incorporated and consequently the data does not exclude other biosynthetic routes including C_2 degradation of a higher homologue or C_2 addition to a lower homologue. Higher homologues of pimelic acid have been shown to stimulate biotin production but this has been attributed to β-oxidation[58a)]

Ogata[59] found that of several odd numbered chain dicarboxylic acids, only glutaric acid appeared to be essential for biotin biosynthesis in strains of *Agrobacterium radiobacter* whilst pimelic acid was inactive. Although the investigators mooted the possibility of a new biosynthetic pathway to biotin, it cannot be ruled out that the organism can activate glutaric but not pimelic acid to the CoA derivative and subsequently add a C_2 unit.

Two recent investigations[60,61] into the origin of carbon atoms from biotin have shed new light on the ambiguous of role of pimelic acid. Feeding experiments in *E.coli* by Ifuku *et al.*[61] and Sanyal *et al.*[60] with [1-^{13}C]-acetate and [2-^{13}C]-acetate suggest that the formation of pimeloyl CoA may follow a fatty acid metabolic pathway (see **Scheme 1.4**).



Scheme 1.4 . Proposed modified fatty acid synthesis pathway for formation of pimeloyl-CoA in *E.coli.* The darker bonds indicate intact acetate units. Three arrows in a row indicates reduction, dehydration, and reduction typical of fatty acid biosynthesis. Four arrows in a row indicate the subsequent steps in the bioconversion of pimeloyl-CoA into biotin.

Both groups examined the ^{13}C incorporation into biotin and ^{13}C-NMR spectroscopy of the isolated metabolites revealed labelling patterns inconsistent with the synthesis of pimeloyl-CoA from octanoate by α- and ω-oxidation, nor the formation of pimelate from tryptophan, lysine, diaminopimelate or by the α-keto acid elongation of 2-oxoglutarate.

The independent labelling data found required the two carboxyl groups of pimelate to be metabolically distinct and this effectively rules out an intermediary role for free pimelic acid. These results reinforce the early proposals of Lynen *et al.* but the authors point out that we are left with the evolutionary conundrum that biotin may be directly required for its own biosynthesis[62].

Previous work by Eisenberg and co-workers[55] showed the culture filtrate of *Phycomyces blakesleeanus* contained an unknown vitamer incapable of binding avidin and unlabelled by [^{35}S]-sulphate indicating the lack of the *ureido* and tetrahydrothiophene bicyclic ring structure. Labelling studies implicated an intermediary role in biotin synthesis and early hypotheses accounting for the vitamers properties inferred that its biosynthesis involved condensation of a three carbon unit, possibly serine or alanine, analogous to the formation of δ-amino laevulinic acid from succinyl CoA and glycine during porphyrin biosynthesis[63].

The vitamer was subsequently isolated and identified as 7-keto-8-aminopelargonic acid, 7-KAPA **6**. The ability of 7,8-diaminopelargonic acid and dethiobiotin to support the growth of yeast and *Aspergillus nidulans* corroborated evidence obtained from genetic experiments. Chemical mutagenesis provided biotin auxotrophs of *E.coli* blocked at certain enzymic stages of biotin's biosynthesis and the excretion products from the culture filtrates of these mutants were subsequently isolated and identified as 7-keto-8-aminopelargonic acid, diaminopelargonic acid and dethiobiotin.

The *bio*F gene has recently been cloned from *Bacillus sphaericus*, The protein has been overexpressed in *E.coli*, and the 7-keto-8-aminopelargonate synthase purified and partially characterised[64]. The enzyme is a monomer of 41kDa with UV absorptions characteristic of pyridoxal-5'-phosphate-dependent enzymes. K_m values for L-alanine and pimeloyl-CoA are 3mM and 1μM respectively. The protein shares high sequence similarity with 5-aminolevulinic acid synthase from *Bradyrhizobium japonicum* which catalyses the condensation of glycine with succinyl CoA in the first step of porphyrin biosynthesis.

The bioconversion of KAPA **6** to DAPA **7** has been demonstrated in resting cells of *E.coli*[65,66] and the initial results from both studies have

suggested methionine as the most effective amino donor with ATP, Mg^{2+} and pyridoxal phosphate as cofactors. This combination inferred possible *in vitro* S-adenosylmethionine synthetase (ATP:methionine S-adenosyltransferase) activity and indeed SAM was ten times more effective than ATP and methionine in DAPA formation. The *bio*A gene product has been purified and has been shown to be a dimer of approximately 47 kDa per subunit. The source of the amine for the transamination is the 2-amino group of S-adenosylmethionine. The amino group is transferred by a ping-pong mechanism to the PLP cofactor on the enzyme and then onto 7-KAPA[65].

1.9 The Mechanism of Dethiobiotin Synthetase

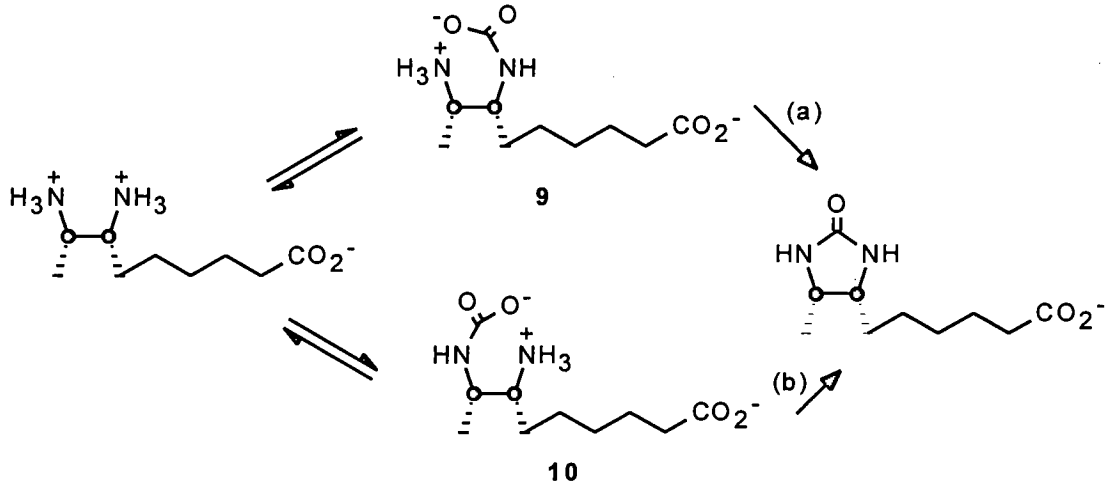
Dethiobiotin synthetase (EC 6.3.3.3) is a functionally unique, CO_2-utilising enzyme, which catalyses the formation of the ureido ring of dethiobiotin from DAPA. Initial evidence for a precursor-product relationship between DAPA and DTB was suggested from genetic and biochemical analysis of biotin auxotrophs of *E.coli*[67], and this was experimentally confirmed through demonstration of the bioconversion of DAPA to DTB, first in resting cells[68] and then in cell-free extracts[69,70] of *E.coli*.

Eisenberg and Krell[71] purified the *E.coli* enzyme, established its dimeric nature (~50kDa), and found that , in addition to DAPA, the reaction's substrate requirements included CO_2 , Mg^{2+} ions and ATP. The inability of avidin to inhibit the enzyme suggested that this is one of the rare CO_2-utilising reactions that does not involve biotin as a prosthetic group.

The dethiobiotin synthetase (*bio*D) gene has recently been cloned from both λ*bio*256 and from *E.coli* genomic DNA into a modified pBR322 derived plasmid. Expression in *E.coli* and purification of the enzyme[72] to

homogeneity have allowed a more detailed examination of the mechanism of ureido ring closure.

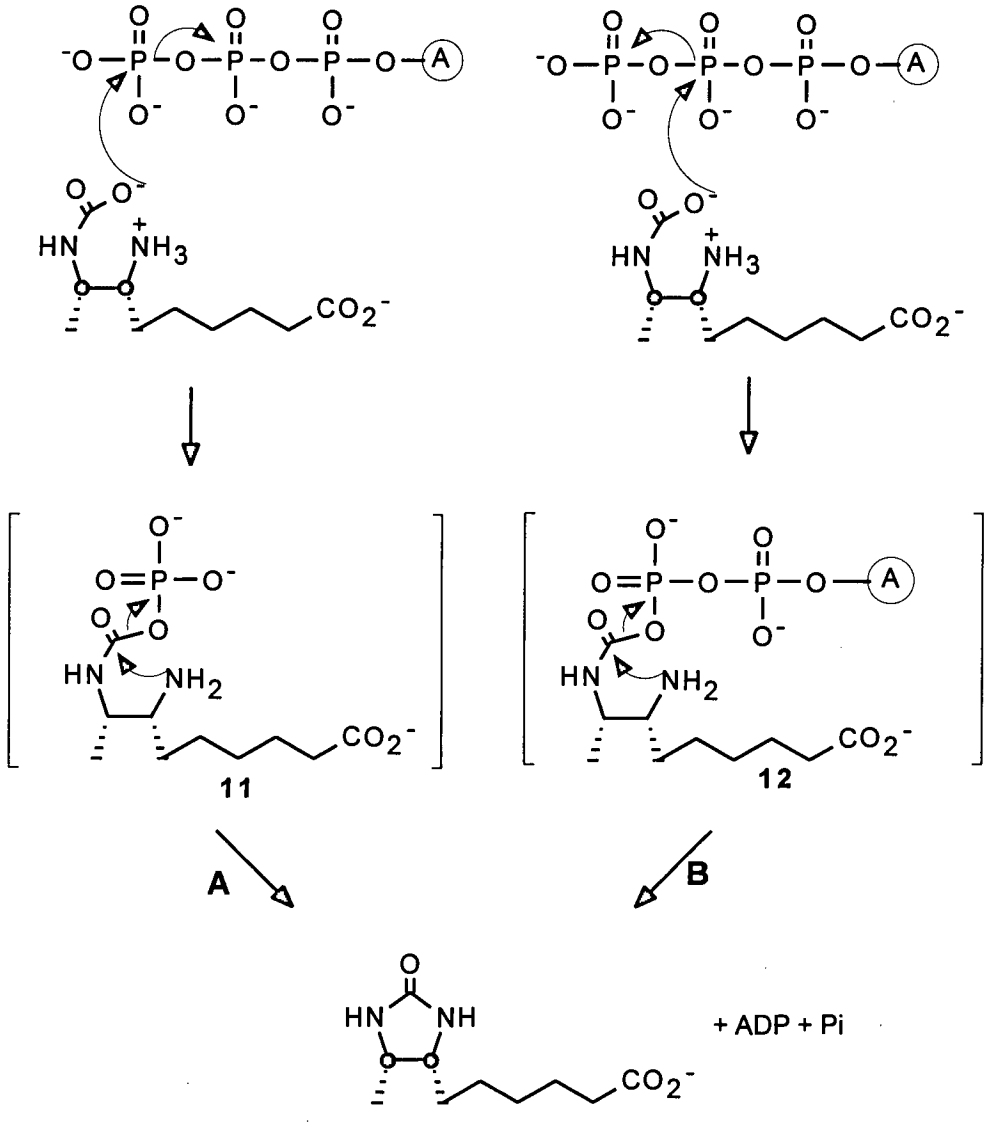
Baxter *et al.*[73] established the stoichiometry of the overall reaction involved formal hydrolysis of one molecule of ATP to ADP and phosphate per molecule of dethiobiotin synthesised. To obtain an indication of the order in which the three substrates, DAPA, CO_2 and ATP are utilised by DTBS, they performed a series of competitive experiments where the enzyme was preincubated with either [^{14}C]hydrogencarbonate or with labelled bicarbonate in combination with either DAPA or ATP. The higher incorporation of radiolabel into DTB from enzyme solutions preincubated with DAPA and [^{14}C]HCO_3^- was rationalised by a reaction sequence which implicated an intermediate carbamate (**9** or **10**) derived from DAPA and CO_2 (**Scheme 1.5**).



Scheme 1.5

Trapping experiments provided distinction between the two possible intermediates (**9** and **10**) and they proposed the first covalent bond forming step of the reaction sequence catalysed by DTBS involved formation of carbamate **10** from DAPA.

From these results, they inferred the formation of a mixed anhydride of the carbamate accompanied the hydrolysis of ATP and they synthesised γ-[$^{18}O_4$]-γ,β-[^{18}O]ATP to elucidate the nature of the activated intermediate[74]. Incubation of the labelled substrate with DAPA, HCO_3^- and Mg^{2+} ions and analysis of ^{18}O distribution in the products by ^{31}P NMR revealed a principal ion peak corresponding to [$^{18}O_3$, ^{16}O]phosphate which implicated the transient intermediate formed *via* pathway **A** in **Scheme 1.6**.



Scheme 1.6

The availability of highly purified dethiobiotin synthetase has allowed its crystallisation and subsequent structural refinement to atomic resolution. Two groups[75,76] have determined the structure of DTBS and their respective studies agree on a number of points. The DTBS monomer folds into a compact structure with the parallel β-sheet forming a core surrounded by α-helices as shown in **Figure 1.6**.

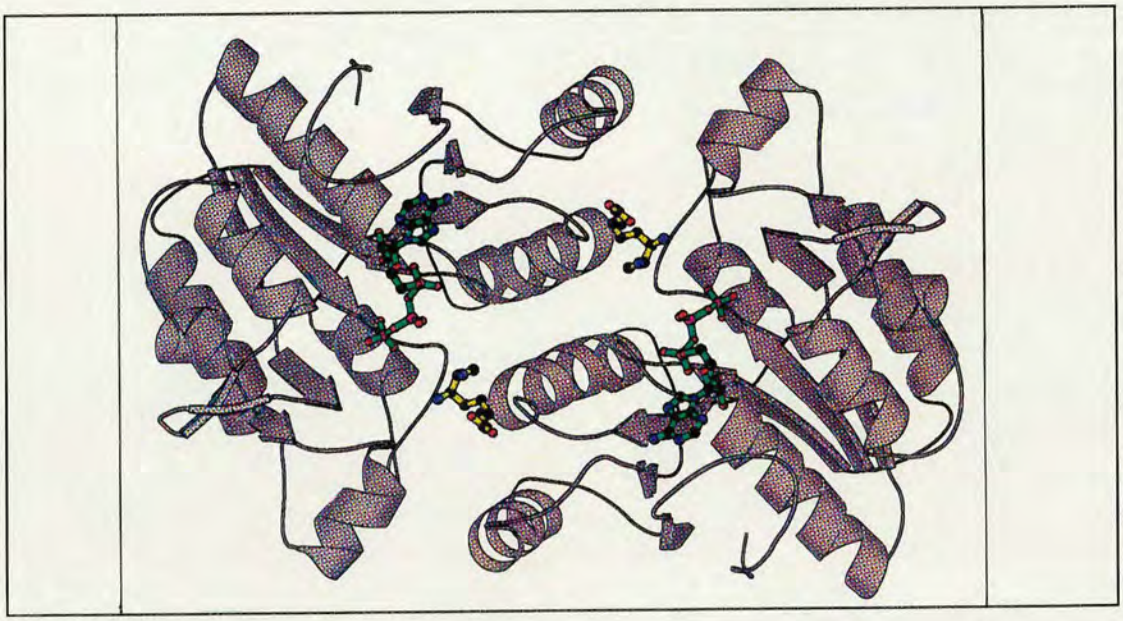


Fig.1.6 General view of the dimer of DTBS

The topology of the subunit belongs to the class of α/β domains. The two monomers have an extensive contact area and although many of the interactions are mediated by water molecules, this may facilitate monomer-monomer movement which may occur during enzyme turnover. The dimer is saucer-shaped, the concave surface housing the putative active site. Alexeev *et al.*[76] found the sulphate ions occupying sites at the bottom of the concave surface are consistent with the positions of the phosphates in the ATP-binding site. One of the sulphates (S1) is postioned exactly in the loop formed by residues 8-16 which is typical of a mononucleotide-binding motif known as the P-loop[77]. They used the position of the sulphates and the superposition of the P-loop of the Ha-*ras* oncogene product p21 onto the DTBS structure to model ATP-binding.

Corroboration of this model came from X-ray data from a crystal soaked in Mg-adenylyl-imidodiphosphate (Mg-AMP-PNP) and dethiobiotin.

Recent studies[78a] have pinpointed the binding site for the DAPA substrate on the enzyme (**Figures 1.7** and **1.8**). It binds at the interface between monomers with the carboxyl end in a pocket between Tyr187 and the main chain N atoms of loop 149-151 at the start of helix α6. The hydrophobic region of the substrate makes contacts with the conserved Ile52. The 7-amino group interacts with Ser41 and W1 while the 8-amino group makes contact with Thr11. Both groups interact with the sulphate ion S2. In solution, the significant absorption change observed at 290nm in the absence of ATP corresponds to the rotation of Tyr187 to accomodate DAPA in the binding site of DTBS. Refined structural data from DTBS crystals under an atmosphere of carbon dioxide has revealed that the 7-amino carbamate of DAPA is the true enzyme-bound intermediate formed (**Fig.1.8**) with a concomitant displacement of water W1 and sulphate S2. This contradicts the earlier labelling experiments of Baxter *et al.* but this may be due to lyophilisation of the enzyme during their work-up protocol which may have initiated carboxyl transfer from the 7-amino group to form the more thermodynamically stable 8-amino carbamate of DAPA. While there is no significant sequence homology between *E.coli* DTBS and any other protein of known sequence except the similar enzymes from *B. sphaericus*[78b], *Serratia marcescens*[78c] and *Brevibacterium flavum*[78d], the 3-D structure reveals similarities with three proteins of unrelated sequence, namely nitrogenase iron protein from *Azotobacter vinelandii* (NIP), adenylosuccinate synthetase from *E.coli* and the Ha-*ras* oncogene product p21. All four enzymes are dimers of identical monomers but there is no similarity in the dimer organisation. All contain the P-loop structural motif which suggests nucleotide binding may be the only common feature between this diverse group of enzymes.

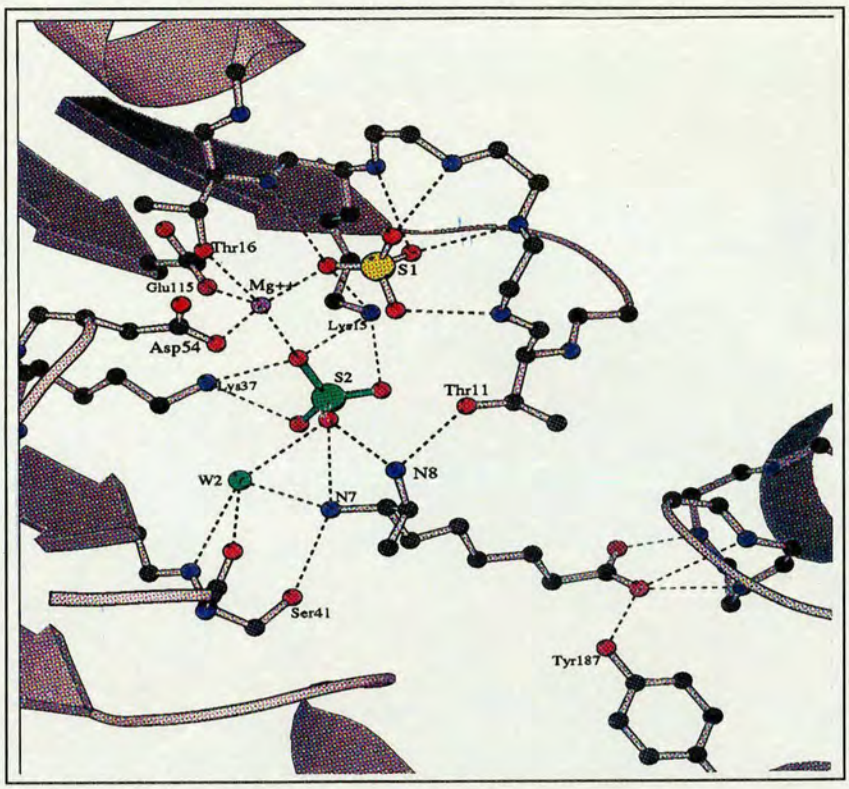


Fig. 1.7. The position of DAPA in the binding site of DTBS under an atmoshere of nitrogen. The DAPA molecule binds between the interface of the monomers. Sulphate ions, S1 and S2, correspond to the α- and γ-phosphates of ATP respectively. Broken lines denote hydrogen bonding interactions.

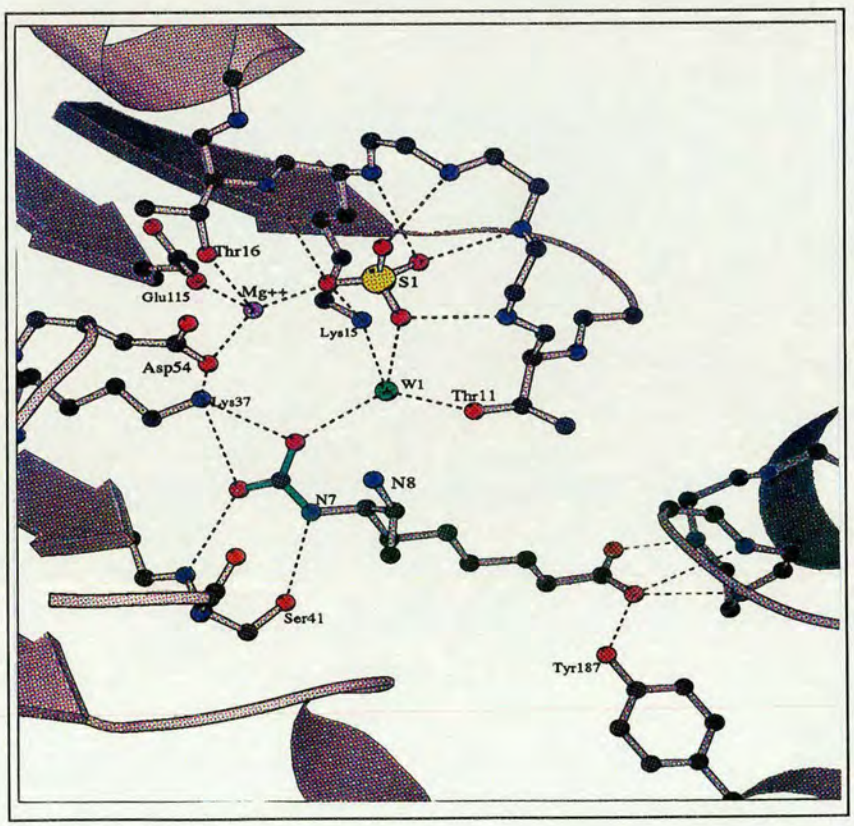


Fig.1.8 Active site of DTBS under an atmosphere of CO_2. The 7-amino carbamate of DAPA formed results in a concomitant displacement of S2 and water W2.

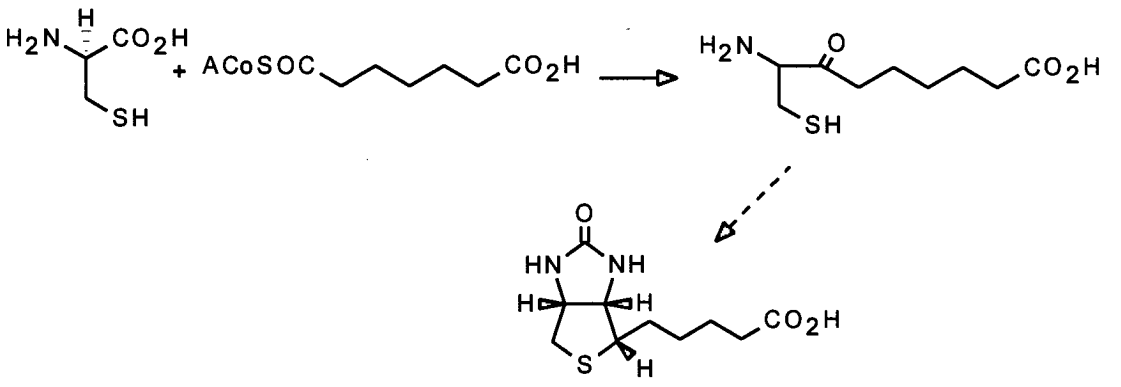
However, the similar folding of the polypeptide chains of the four proteins indicates a possible evolutionary relationship and these similarities may be features characteristic of a distinct family of ATP-dependent enzymes.

1.10 The Origin of the Sulphur Donor

The most unusual step in the biosynthesis of biotin is undoubtedly the conversion of dethiobiotin into biotin. The precise nature of the sulphur donor and the mechanism of incorporation into dethiobiotin still presents two major unsolved problems associated with this transformation.

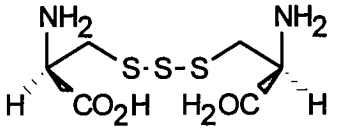
Niimura[80] utilised sulphur-starved cells of *Saccaromyces cerevisiae* to investigate the effectiveness of various compounds to act as sulphur sources in biotin biosynthesis, and found a decreasing order of efficiency : methionine sulphoxide, methionine, Na_2S, $NaHSO_4$, Na_2SO_4, homocysteine, S-adenosylmethionine and methyl mercaptan. The group found that cystine, cysteine, methionine sulphone, S-methylthioadenosine, choline sulphate and glutathione were inactive as sulphur donors. Labelling experiments[81] with [^{35}S]-methionine gave radioactive biotin. Shimada[82] corroborated this result with tracer studies in *A.niger*.

Very early tracer studies with *Achromobacter* had led to the hypothesis[79] of a precursorial role for L-cysteine in biotin biosynthesis according to **Scheme 1.7**. However reinvestigation by Marquet[85] refuted the hypothesis by incorporation experiments with [3-^{14}C,^{35}S]-L-cysteine which failed to yield radioactive biotin in the *Achromobacter* cells. Similar experiments on the same organism showed conversion of [2,3-^{3}H,-^{14}C]-(±)-dethiobiotin into biotin without change in the tritium to carbon-14 ratio and hence signify a similar pathway in *Achromobacter* as in other organisms.



Scheme 1.7

A variety of radiolabelled sulphur compounds have been utilised in dethiobiotin incorporation experiments. Although lipoic acid stimulated biotin production in *A.niger*, administration of ^{35}S-lipoic acid gave relatively low levels of incorporation in biotin suggesting the stimulating effect of lipoate is due to factors other than the donation of sulphur.[83]



13

The use of thiocystine **13** as a source of sulphur in the biological medium offers synchronous availability of both organic reduced sulphur (i.e cysteine) and inorganic sulfane sulphur to the cell. Metabolic studies using L-[^{35}S-sulfane]-thiocystine demonstrated the compound's ability to serve as a sulphur source for *E.coli* grown in a defined sulphur-free medium. The labelled sulphur is rapidly assimilated into cysteine and other sulphur-containing metabolites, including biotin. In conclusion, all the sulphur atoms of thiocystine were thought to contribute approximately equally toward biotin biosynthesis.

Alternatively, since Marquet[85] was unable to detect significant label incorporation from L-^{35}S-cysteine into biotin in *Achromobacter*, this prompted reinvestigation into the contribution of cystine, methionine and thiocystine to sulphur metabolism in *E.coli*.[86] Labelling studies indicated that *E.coli* grown in the presence of L-^{35}S-methionine gave no labelled biotin, however the addition of L-^{35}S-cystine led to approximately 80% of isolated biotin being labelled. Utilisation of L-[^{35}S-sulphane]-thiocystine by the organism produced biotin possessing approximately 29% of the label.

The equally effective sulphur metabolism of both cystine and thiocystine by *E.coli* prompted DeMoll and Shive[86] to explore the ability of these sulphur sources to metabolically complement or compete with each other as sulphur donors. When *E.coli* was grown in the presence of 50mM L-thiocystine and 75mM L-[^{35}S]-cystine, approximately 40% of the isolated biotin was determined to have incorporated ^{35}S. The theoretically possible was 50% if all sulphur was utilised equally. In accordance with this observation, addition of L-[^{35}S-sulfane]-thiocystine and unlabelled cystine resulted in a significantly lower level of incorporation of ^{35}S. The inability of *Achromobacter* to incorporate ^{35}S from cysteine might be due to the inefficiency of the organism to utilise exogeneous cysteine. Metabolic studies in *E.coli*[87] have shown that externally supplied cysteine must first be oxidised to cystine before transportation and utility by *E.coli*. The close relationship of the precursorial sulphur pool of cysteine with that of biotin has been demonstrated by DeMoll and Shive[88]. Feeding experiments with [^{34}S]SO_4^{2-} and L-[^{34}S-sulfane]-thiocystine in *E.coli* and analysis by GC-MS showed that internal cysteine and excreted biotin were labelled to the same extent.

More recently, investigation into the effect of sulphur compounds on the bioconversion of dethiobiotin to biotin by resting cells and protoplasts of a

*Bacillus sphaericus bio*B transformant[89] implicated a similar role for L-cysteine and L-cystine. In marked contrast, results[90] obtained from sulphur compounds tested in cell free extracts of the same cells found S-adenosyl-L-methionine was active while both L-cysteine and L-methionine had no significant effect. Similar results were obtained for cell free extracts of *E.coli*[91]. These authors postulated the role of SAM as a sulphur donor in biotin biosynthesis but recent labelling studies in cell-free extracts of *B.sphaericus*[92] have demonstrated the lack of incorporation of ^{35}S from ^{35}S-SAM into biotin. Low incorporation of ^{35}S-label into biotin was observed upon incubation of ^{35}S-L-cysteine in crude cell-free extracts of *B.sphaericus*[92] and *E.coli.*[93] Both D- and L-cysteine have been found to possess stimulatory effects in a recent study on the conversion of dethiobiotin to biotin in cell-free extracts of an *E.coli bio*B transformant[94]. However, until the experiments can be repeated with an active purified system the sulphur donor still remains to be characterised.

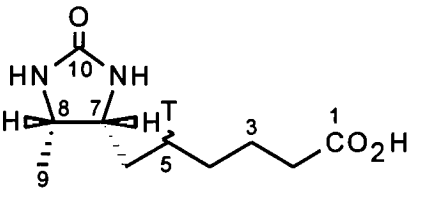
1.11 The Mechanism of Biotin Synthase

The second major problem associated with the conversion of dethiobiotin into biotin concerns the mechanism of sulphur introduction. Early investigations by Li *et al.*[96] involved administering a mixture of [1- or 10-^{14}C]-dethiobiotin and randomly tritiated dethiobiotin to cultures of *A.niger* and subsequent isolation of radioactive biotin sulfone. From their results they postulated that approximately four hydrogens might be lost in the conversion of dethiobiotin into biotin, which could be accounted for by two unsaturation steps in the overall mechanism.

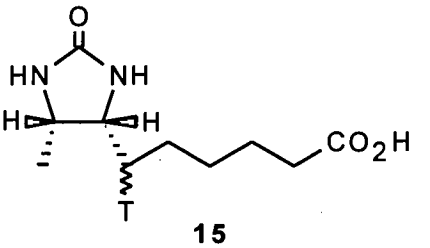
The uncertainties resulting from the use of randomly tritiated dethiobiotin in these double label experiments prompted a reinvestigation into the degree of hydrogen abstraction using specifically tritiated forms of dethiobiotin. Parry found that previously published syntheses of

dethiobiotin[97-100] proved unsatisfactory for specific labelling, due to scrambling of the label in later stages and consequently developed a novel stereospecific synthesis[101] of (±)-dethiobiotin useful for labelling purposes. The synthesis was modified to allow the introduction of tritium into the possible sites where hydrogen abstraction may occur during sulphur insertion, namely C-5 to C-9[102,103].

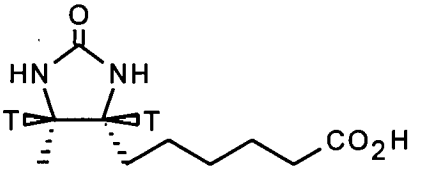
The samples of tritiated (±)-DTB were each mixed with [10-^{14}C]-(±)-DTB[104] and the doubly labelled precursors were then administered to cultures of *A.niger* (ATCC 1004) using the methods of Li *et al.*[96] The precursors were not resolved since only (+)-DTB appears to serve as biotin precursor[105]. The results indicated that sulphur introduction at C-9 and C-6 took place without loss of hydrogen from C-7, C-8 and C-5.



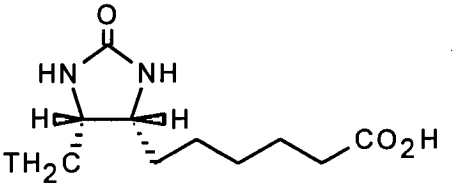
14



15



16



17

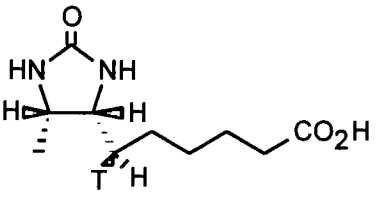
Although unsaturation at any of these sites during the (+)-dethiobiotin to (+)-biotin transformation was deemed improbable, the possibility of enzymic removal and replacement of hydrogen from these positions cannot be ruled out.

The incorporation of [9-^{3}H]-(±)-DTB **17** into biotin proceeds with about 30% tritium loss. This is consistent with the removal of one hydrogen atom from the methyl group which exhibits little or no isotope effect but

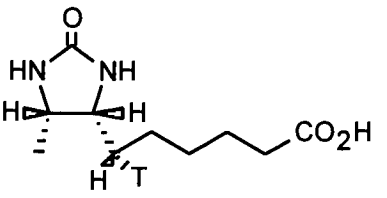
the nature of the reaction associated with the oxidation of the methyl group of dethiobiotin is still unknown. The incorporation of 6(RS)[6-^{3}H]-(±)-DTB **15** into biotin proceeded with 47% tritium loss. This figure is indicative of removal of one hydrogen atom from C-6 of dethiobiotin. Thus, Parry concluded that two hydrogens were removed from dethiobiotin on its conversion to biotin.

Parry's work with the eukaryotic organism *A.niger* was subsequently complemented by research from Marquet's group with the prokaryotic organism *E.coli*[106,107]. [7,8-^{3}H]-(±)-dethiobiotin and [5-$^{2}H_2$]-(±)-dethiobiotin were prepared using an alternative synthetic approach to that of Parry. Each of the labelled dethiobiotins were administered to an auxotrophic mutant of *E.coli* (C124) whose biosynthetic pathway to biotin is blocked before dethiobiotin. Administration of [7,8-^{3}H]-(±)-dethiobiotin in conjunction with [1-^{14}C]-(±)-dethiobiotin gave radioactive biotin which retained all of the tritium label. The possibility of hydrogen migration was dismissed by administration of [7,8-$^{2}H_2$]-(±)-DTB followed by mass spectral analysis of the isolated biotin which showed that no loss of deuterium had occurred from either position. In a similar experiment [5-$^{2}H_2$]-(±)-DTB afforded biotin with retention of both deuterium atoms. Finally, administration of [9-$^{2}H_3$]-(±)-DTB to *E.coli* (C124) gave biotin labelled with two deuterium atoms at C-9. This observation agrees with Parry's interpretation of the change in tritium to carbon-14 ratio accompanying the incorporation of [9-^{3}H, 10-^{14}C]-(±)-DTB in biotin by *A.niger*.

Parry's investigation into the stereochemical events occurring during sulphur insertion was initated by the stereospecific synthesis[104] of both (6R)[6-^{3}H]-(±)-DTB **18** and (6S)[6-^{3}H]-(±)-DTB **19** .



18

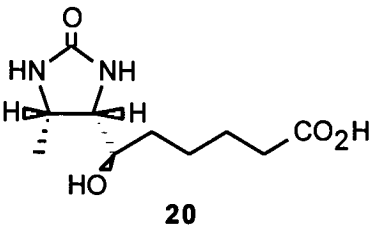


19

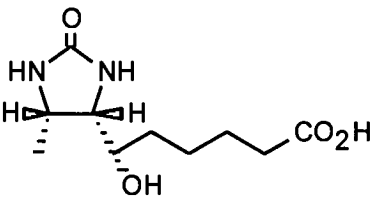
The samples of chirally tritiated dethiobiotin were each mixed with [10-^{14}C]-(±)-DTB and the doubly labelled precursors administered to *A.niger*. Isolation of (+)-biotin sulphone purified as its methyl ester to constant specific activity and constant $^{14}C/^{3}H$ ratio showed conclusively that sulphur is introduced with loss of the 6-*pro* S hydrogen atom. Since the absolute configuration of (+)-biotin at C-6 is known to be S[108], it follows that sulphur is introduced at C-6 with retention of configuration.

The stereochemistry observed in the nature of the transformation suggests that the functionalisation mechanism may be a single step process. An alternative possibility such as hydroxylation, activation of the hydroxyl group and displacement by a sulphur nucleophile (although without precedent) would presumably result in an inversion of configuration since biological hydroxylations at saturated C-atoms usually proceed with retention of configuration[109].

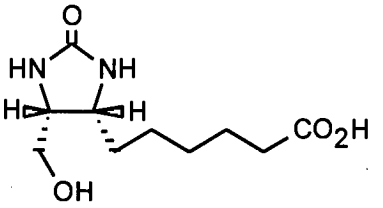
Marquet[110] provided evidence against the intermediacy of hydroxylated forms of dethiobiotin. 9-Hydroxydethiobiotin **22** and the two epimers of 6-hydroxydethiobiotin (**20** and **21**) were synthesised and precursor evaluation of the three hydroxydethiobiotin compounds in *E.coli* C124 found that none of the synthetic derivatives supported the growth of the organism.



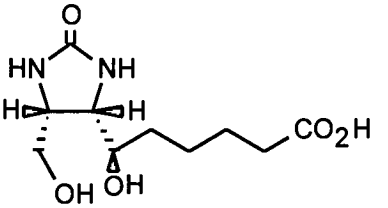
20



21



22

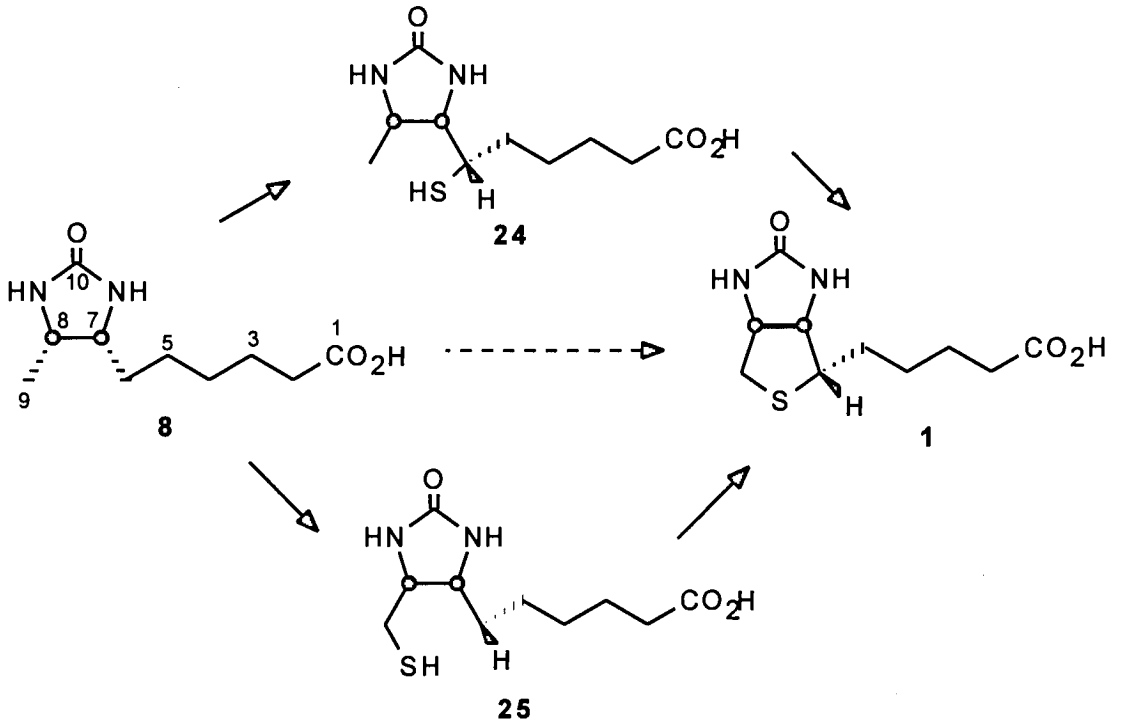


23

Transport studies showed that all three compounds can enter the cells so the absence of growth could not be attributed to permeability problems. Emoto *et al.*[111] also prepared the dihydroxydethiobiotin **23** and obtained similar results. On the conversion of dethiobiotin to biotin, it appears unlikely that hydroxylation takes place at C-9 or C-6. However, one cannot rule out the involvement of hydroxylated intermediates that are tightly bound to the enzyme(s) catalysing the introduction of sulphur.

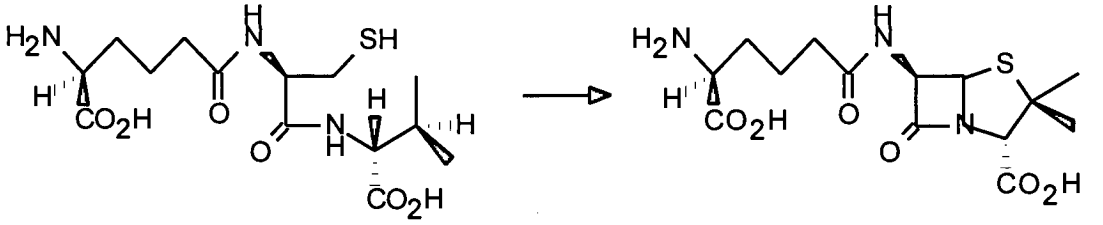
In addition to these studies, stereochemical investigation into biotin formation by Arigoni[112] demonstrated that chirally labelled [9-^{3}H,^{2}H] dethiobiotin was incorporated into biotin with complete loss of chirality at C-9. The authors postulate the involvement of a conformationally labile methylene radical at C-9.

Recent investigations have led to the proposition of an intermediary role for thiols **24** and **25** (**Scheme 1.8**) on the conversion of dethiobiotin to biotin.



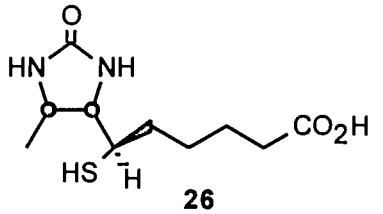
Scheme 1.8

The intermediacy of the thiol derivatives is attractive on mechanistic grounds since insertion of sulphur in a single step would involve activation at two separate, saturated sites in a single reaction. Whilst the latter reaction has no biosynthetic analogy, closure of a thiol intermediate to afford a ring structure is precedented by the ring closure of (LLD)-2-aminoadipoylcysteinylvaline to isopenicillin N[113]. (**Scheme 1.9**)

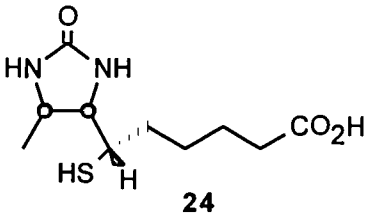


Scheme 1.9

Biosynthetic evaluation of the putative precursor **25** began with its synthesis by Marquet *et al.*[114]. Synthesis of the disulphide dimer of (±)-9-mercaptodethiobiotin has also been carried out by Baxter *et al.*[115]. The possible role of (±)-9-mercaptodethiobiotin **25** as an intermediate in biotin formation was supported by growth experiments on *bio*-mutants in *E.coli.* The thiol did not support growth, even at very high concentrations, of SA291, a *bio*ABFCD deletion mutant suggesting the compound does not substitute for biotin as a coenzyme. However, it was found to support normal growth of an *E.coli bio*A mutant (strain 6435) which requires 7,8-diaminopelargonate, dethiobiotin or biotin for growth. The evidence suggests the primary thiol may act as a biosynthetic intermediate for the conversion of dethiobiotin into biotin in *E.coli.*



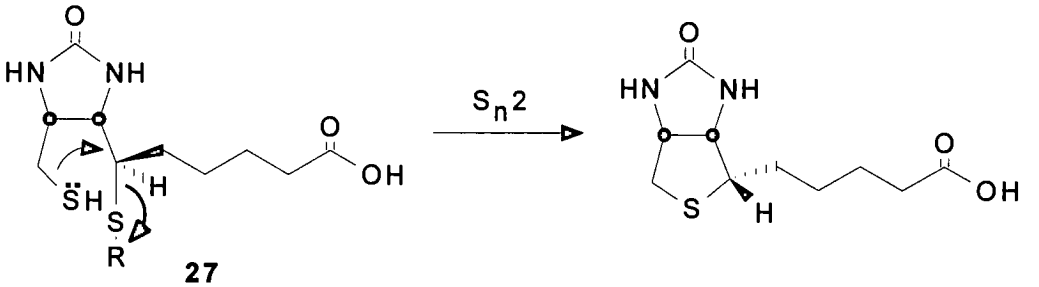
26



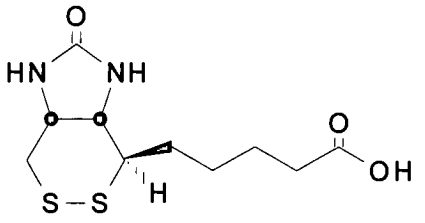
24

The synthesis and biological evaluation of the putative intermediate thiols in *B.sphaericus* was recently reported by Marquet *et al.*[116] . Three possible thiols (**24**, **25** and **26**) were synthesised and tested for growth promoting activity with the biotin requiring strain *E.coli* C124 (*bio*A-). One of the secondary thiols **24** was found to be inactive while the other **26** was almost as efficient as biotin. The primary thiol **25** also promoted growth but at significantly higher concentrations. Labelling studies using [^{35}S]-**26** produced biotin which was devoid of radioactivity.

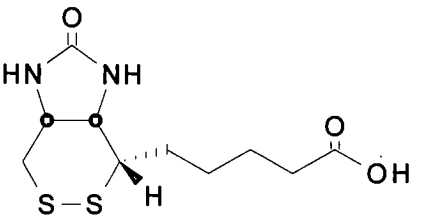
Correlation experiments with biotin and epibiotin established that the active thiol corresponded to the 6-(R)-isomer **26** with a configuration opposite to that of biotin. This result was consistent with [^{35}S] loss and they postulated the involvement of an intermediate dithiol **27** which allowed the rationalisation of the stereochemistry of sulphur insertion.



27



28



29

Introduction of sulphur with inversion at C-6, followed by S_N2 type substitution would proceed with overall retention of configuration as shown by Parry[117] but on synthesis of the postulated disulphides (**28** and **29**) , both compounds were found to be inactive in the growth assays of *E.coli* C124. Tracer studies with (+)-[^{35}S]-**25** as precursor resulted in only 1% label retention so the deuterated thiol (+)-9-[$^{2}H_2$]-**25** was utilised in incorporation experiments. Mass spectrometric analysis revealed a complex desulphurisation process.

Biological evaluation of the three thiols (**24**, **25** and **26**) in the resting cells of *Bacillus sphaericus* revealed that only the primary thiol **25** was transformed into biotin with only 10% observed conversion yield compared with dethiobiotin. However, incubation of (+)-[^{35}S]-**25** with the resting cells in the presence of DTT indicated clearly that thiol **25** was converted into biotin without degradation. This result was verified by a similar study with (+)-9- [$^{2}H_2$]- **25** which exhibited no loss of deuterium on its bioconversion.

The incongruity observed in the growing cells of *E.coli* and resting cells of *B.sphaericus* necessitated a comparison with the bioconversion by growing cells of *B.sphaericus* since resting cells of *E.coli* C124 do not produce any biotin from dethiobiotin. Experiments with (+)-9-[$^2H_2$]-**25** revealed similar results with those observed with *E.coli.* Marquet concludes that the results are qualitatively identical for growing cells of both bacteria and that a complex desulphurating pathway competes with the clean cyclisation observed with resting cells.

A recent investigation[118] into biotin biosynthesis in cells of *Lavandula vera* has shown that all bacterial intermediates from pimelic acid could be detected in higher plant cells. One of these compounds co-chromatographed with an authentic sample of 9-mercaptodethiobiotin and a labelled sample was incorporated into biotin. The compound was also as effective as biotin in supporting the growth of *E.coli* strain *bio*B105. This evidence strongly suggests the final step of biotin biosynthesis in both bacteria and in higher plants proceeds through two distinct stages : formation of the primary thiol of dethiobiotin and ring closure to give biotin.

The conversion of dethiobiotin to biotin has recently been demonstrated in cell-free extracts of *E.coli* genetically engineered to overexpress the *bio*B gene product[91a)]. Among the cofactors tested, ferrous or ferric ions, NADPH, SAM and KCl were found to stimulate the reaction. Similar cofactor requirements were found in cell-free extracts of a *B.sphaericus bio*B transformant but in addition to those reported, FAD was also found to have a stimulatory effect upon the enzymatic conversion[91b)]. Both groups agree that an unidentified enzyme(s) beside the *bio*B gene product is required for the bioconversion of DTB to biotin.

This development of overexpression systems capable of producing relatively large amounts of protein in *bio*B transformants has led to the purification of the protein encoded by the *E.coli bio*B gene[119]. The active form of the *bio*B gene product apppears to be a homodimer (82kDa) which contains one [2Fe-2S] cluster per monomer. This protein functions in the conversion of dethiobiotin to biotin in crude extracts devoid of the *bio*B gene product. Therefore the 82 kDa protein alone is not sufficient to catalyse the biotin synthase reaction since additional unidentified factors from the crude extract are required. In addition to these unidentified factors, the authors find that both iron and SAM appear to be essential for the reaction, and NADPH greatly stimulates it. The required iron is in addition to that in the cluster of the bioB gene product. The role of additional iron is unknown, although its worth noting the isopenicillin N-synthase, which catalyses a reaction similar to that catalysed by biotin synthase (sulphur added to an unactivated carbon atom), also requires loosely bound iron[120]. However, they found unlike the IPNS reaction which requires O_2, the biotin synthase reaction proceeds at the same rate in both anaerobic and aerobic conditions. The K_m for DTB in the biotin synthase reaction catalysed by the the mixture of the crude extract of KS302δ*bio* and the *bio*B gene product was found to be 2μM. The turnover number based on the amount of bioB gene product was calculated as $1h^{-1}$ which is unusually low for an enzyme. This number may increase when all proteins neccessary for the reaction are purified and the reaction conditions are optimised.

Analysis of the predicted amino acid sequences of the *bio*B gene product from *E.coli*, *B.sphaericus* and *S.cerevisiae* indicates the conservation of six cysteine residues[121]. Resonance Raman spectroscopy of the *E.coli bio*B gene product affirms the presence of four cysteinyl ligands to the [2Fe-2S] cluster of this protein. The presence of an unusual Cys-X-X-X-Cys-X-X-Cys motif in the three proteins and also in the sequence of *E.coli* lipoic

acid synthase (the *lip*A gene product) suggests the presence of similar iron-sulphur clusters[119].

EPR studies on the *E.coli bio*B gene product indicate the [2Fe-2S] cluster is unstable upon reduction with dithionite and the original spectrum is not regenerated when the reduced form is reoxidised. This instability may suggest the sulphur added to dethiobiotin possibly originates from the Fe-S cluster during catalysis. The function of the unidentified factors in the crude extract may involve cluster resynthesis after each turnover.

Flavodoxin has recently been found to be required for the biotin synthase reaction in *E.coli*.[122] The authors suggest that one of the additional unidentified protein(s) still required may therefore be ferredoxin (flavodoxin) $NADP^+$ reductase. In *E.coli*, flavodoxin is constitutively synthesised and provides electrons for the reductive activation of pyruvate formate-lyase[123] and cobalamin-dependent methionine synthase[124], both of which require SAM as a cofactor. Recent studies on lysine 2,3-aminomutase[125] and anaerobic ribonucleotide reductase[126] show reaction similarities between the two enzymes and the reaction catalysed by biotin synthase. The presence of an Fe-S cluster, the requirement of SAM and the removal of hydrogen from an unactivated carbon atom all suggest formation of a radical in the biotin synthase reaction.

The fact that (9R)[9-^{1}H,^{2}H,^{3}H]-dethiobiotin administered to resting cells of *A.niger* results in scrambling of the isotopes in the isolated biotin with its implication that a conformationally labile methylene radical may be formed at C-9 of dethiobiotin supports this observation.

The scope of this research was to investigate the mechanistic aspects of biotin biosynthesis in *E.coli* , in particular the reactions catalysed by dethiobiotin synthetase and biotin synthase.

Chapter 2

The Mechanism of Dethiobiotin Synthetase Biological Evaluation of Substrate Analogues of (7R,8S)-7,8-Diaminononanoate

2.1 Introduction

Dethiobiotin synthetase (EC 6.3.3.3) catalyses the first ring closure in the biosynthesis of biotin, resulting in the formation of the *ureido* ring of dethiobiotin (DTB) **8** from (7*R*,8*S*)-7,8-diaminononanoate (DAPA) **7** (**Fig.2.1**)

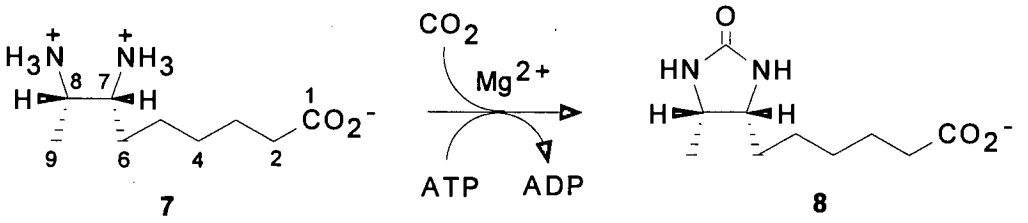


Fig.2.1. The reaction catalysed by dethiobiotin synthetase (DTBS)

This process is of particular interest in that it is one of the few "carbon dioxide" trapping reactions known to date which does not involve biotin as a prosthetic group. It shares distinction with the C-carboxylating enzymes phosphoenolpyruvate carboxykinase[127], ribulose-1,5-diphosphate carboxylase/oxidase[128] and the vitamin K dependent protein glutamyl carboxylase[129] as one of the few biocatalysts which utilise CO_2 in preference to hydrogen carbonate as a substrate. However DTBS remains unique since it is the only one to involve N-substrate carboxylation in its mechanism. These aspects have received great attention from researchers resulting in the enzyme's purification[72], characterisation of the intermediary events involved in the *ureido* ring formation[73,74] and ultimately its crystallisation[72] and structural characterisation to atomic resolution[75,76].

The kinetic aspects of this unique transformation have been investigated[130] employing both stopped-flow and steady-state kinetic techniques to characterise the nature and the sequence of the individual steps involved in the mechanism of DTBS.

2.2 Synthesis of aberrant DAPA analogues

The information obtained from both crystallographic and kinetic characterisation of this enzyme has prompted the synthesis of modified substrates as probes to explore the limitations of the DAPA binding site and examine the synthetic versatility of the reaction catalysed by DTBS. The 9-methyl group and 7-hexanoate side chain were identified as sites for synthetic modification and the analogues **30-35** were targetted for synthesis.

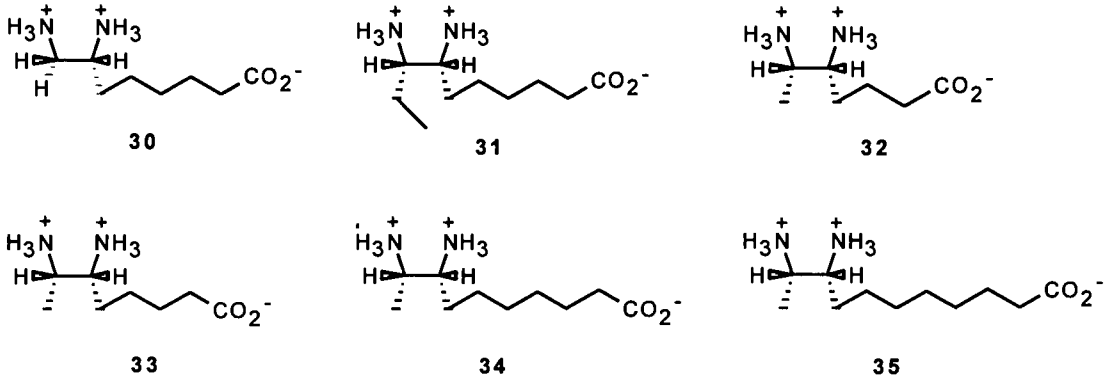


Fig.2.2 Aberrant DAPA analogues targetted for synthesis

A large number of potential synthetic routes could be employed for analogue synthesis and there are a plethora[11] of biotin syntheses reported in the chemical and patent literature. However, the synthetic approach to these analogues was limited to proven methodology successfully employed in related work (see **Chapter 4**).

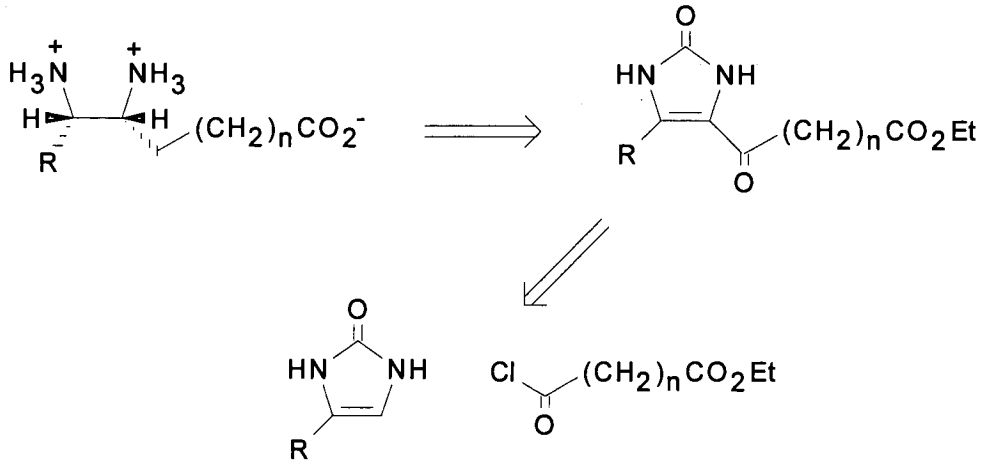
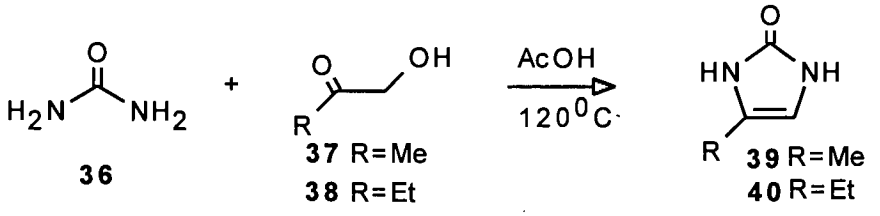
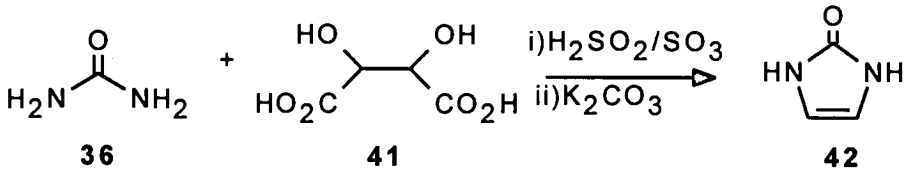


Fig2.3 Retrosynthetic analysis of DAPA

Retrosynthetic analysis of DAPA indicated that the Friedel-Crafts acylation of imidazolin-2-one could provide a convenient route to the target compounds (see **Fig.2.3**). This route can accomodate the proposed modifications of the native substrate skeleton, and the modifed tetrahydroimidazolin-2-ones are immediate precursors to both dethiobiotin and DAPA analogues so potential inhibitors for DTBS and biotin synthase could be obtained from the same synthesis.



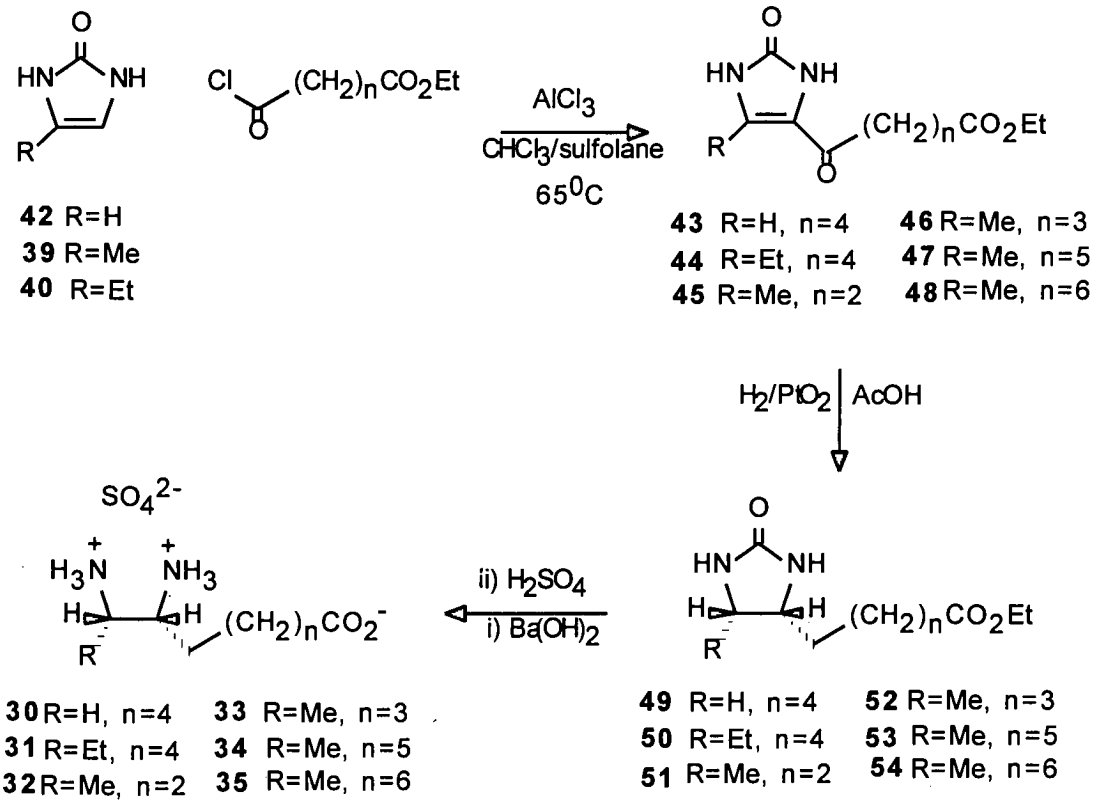
4-Methyl-4-imidazolin-2-one **39** was prepared by condensation of acetol **37** and urea **36** in 25% yield. This one-pot synthesis[131] allowed modification of the alkyl moeity and the corresponding 4-ethyl-4-imidazolin-2-one **40** was prepared by a similar condensation of urea and 1-hydroxy-2-butanone **38**.



36 **41** **42**

Imidazolin-2-one 42 was prepared by the method of Hilbert[132] which involved the condensation of urea **36** and (+-)-tartaric acid **41** in fuming sulphuric acid to give the imidazolinone-4-carboxylic acid upon which subsequent decarboxylation with potassium carbonate gave the corresponding imidazolin-2-one **42** in 75 % yield.

Friedel-Crafts acylation was performed on the substituted imidazolinones with acid chlorides of varying chain length (see **Scheme 2.1**) and the results are summarised in **Table 4.1**



42 R=H
39 R=Me
40 R=Et

43 R=H, n=4 **46** R=Me, n=3
44 R=Et, n=4 **47** R=Me, n=5
45 R=Me, n=2 **48** R=Me, n=6

30 R=H, n=4 **33** R=Me, n=3
31 R=Et, n=4 **34** R=Me, n=5
32 R=Me, n=2 **35** R=Me, n=6

49 R=H, n=4 **52** R=Me, n=3
50 R=Et, n=4 **53** R=Me, n=5
51 R=Me, n=2 **54** R=Me, n=6

Scheme 2.1 Synthetic route to racemic DAPA analogues

R	n	Product	Yield (%)	CHN calcd. (%)	CHN found (%)
H	4	**43**	35	C 55.0,H,6.7,N,11.65	C,55.2,H,6.65,N,11.4
Et	4	**44**	30	C,58.2,H,7.5,N,10.45	C,58.45,H,7.45,N,10.3
Me	2	**45**	39	C,53.1,H,6.25,N,12.4	C,53.1,H,6.3,N,12.3
Me	3	**46**	42	C,55.0,H,6.7,N,11.65	C,54.65,H,6.5,N,11.65
Me	5	**47**	40	C,58.2,H,7.5,N,10.45	C,58.0,H,7.8,N,10.15
Me	6	**48**	33	C,59.55,H,7.85,N,9.9	C,59.4,H,7.55,N,10.0

Table 4.1. Products obtained from the Friedel-Crafts acylation of substituted imidazolin-2-ones. Yields were calculated from the starting imidazolin-2-one **39**, **40** or **42**.

Compounds **43-48** were hydrogenated over Adam's catalyst (PtO_2) at room temperature and atmospheric pressure to give the corresponding racemic dethiobiotin ethyl ester analogues **49-54** (see **Table 4.2**)

R	n	Product	Yield (%)	FAB MS required	FAB MS found
H	4	**49**	85	229.1552	229.1551
Et	4	**50**	65	257.1865	257.1865
Me	2	**51**	90	215.1395	215.1403
Me	3	**52**	77	229.1552	229.1549
Me	5	**53**	88	257.1865	257.1865
Me	6	**54**	78	271.2022	271.2021

Table 4.2 Products from the catalytic hydrogenation of the substituted imidazolinones

The racemic DAPA analogues **30-35** were obtained from the corresponding aberrant (±)-dethiobiotin ethyl esters by hydrolysis with barium hydroxide in a sealed vial for three days (see **Table 4.3**). The success of the reaction was identified by the disappearance of the characteristic *ureido* carbonyl group at 164ppm in the ^{13}C nmr spectra of these DAPA analogues.

R	n	Product	Yield (%)	CHN calcd. (%)	CHN found (%)
H	4	**30**	55	C,35.3,H,7.35,N,10.0	C,35.25,H,7.1,N,10.0
Et	4	**31**	35	C,40.0,H,8.05,N,9.3	C,39.7,H,8.35,N,9.1
Me	2	**32**	45	C,32.5,H,7.0,N,10.85	C,32.2,H,6.9,N,10.9
Me	3	**33**	57	C,35.3,H,7.4,N,10.3	C,35.1,H,7.5,N,10.25
Me	5	**34**	48	C.40.0,H,8.05,N,9.3	C,40.0,H,8.35,N,9.1
Me	6	**35**	52	C,42.0,H,8.35,N,8.9	C,42.3,H,8.7,N,8.9

Table 4.3 Racemic DAPA analogues obtained from the hydrolysis of the corresponding (±)-dethiobiotin ethyl ester analogues

The isolated compounds were used in subsequent biological experiments as their racemates as it appears the enzyme is specific for the (R,S)-isomer of the substrate and the (S,R)-isomer exhibits little or no inhibitory effects.

2.3 Kinetic Studies

2.3.1 Catalytic activity of the aberrant analogues

The efficiency of the analogues to act as enzymic substrates was determined spectrophotometrically using a linked assay by which the ATP to ADP conversion was monitored by assay of ADP produced. The oxidation of NADH was coupled to the production of ADP by the addition of phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase in excess. Enzyme kinetic experiments were performed by Dr. Otto Smekal and the steady-state kinetic parameters obtained for these analogues are shown in **Table 4.4**

DAPA analogue	R	n	K_m(app) (M)	k_{cat}(app) (s^{-1})	k_{cat}/K_m(app) ($M^{-1}s^{-1}$)
(±)-DAPA **55**	Me	4	4.1×10^{-6}	8.3	2.0×10^{6}
(R-1)-(±)-DAPA **30**	H	4	1.2×10^{-2}	0.5	4.5×10^{1}
(R+1)-(±)-DAPA **31**	Et	4	9.0×10^{-3}	2.0	2.2×10^{2}
(n-2)-(±)-DAPA **32**	Me	2	4.5×10^{-3}	1.7	3.8×10^{2}
(n-1)-(±)-DAPA **33**	Me	3	2.1×10^{-3}	2.7	1.3×10^{3}
(n+1)-(±)-DAPA **34**	Me	5	3.5×10^{-3}	4.0	1.1×10^{3}
(n+2)-(±)-DAPA **35**	Me	6	5.3×10^{-3}	0.5	9.4×10^{1}

Table 4.4. Steady-state kinetic parameters obtained for aberrant DAPA analogues. Initial rate coefficients for each analogue were determined at a fixed concentration of enzyme and under conditions where all other native co-substrates (CO_2, ATP and Mg^{2+}) were held at concentrations of *ca.* $5K_m$. (app=apparent)

The above results reflect that all the analogues are substrates of DTBS but all are poorer than the racemic parent substrate. Comparison between analogues (n-1) and (n+1) indicates that increasing or decreasing the aliphatic backbone by one CH_2 group results in a similar fall in catalytic efficiency. Lengthening the native chain by two methylene groups results in a further decrease in the k_{cat}/K_m ratio but by one order of magnitude less than shortening the side chain by two carbons. It is apparent that DTBS is extremely versatile in its ability to utilise aberrant substrates of varying chain length but the enzyme functions at the optimum rate only with the native hexanoate side chain present in DAPA.

Replacing the methyl group of DAPA with H results in a dramatic fall in catalytic efficiency by five orders of magnitude. Kinetic studies on the ethyl analogue, (R+1)-(±)-DAPA, **31**, gave a similar drop in k_{cat}/K_m(app) ratio but only by four orders of magnitude. Removing the methyl group from the native substrate appears to have the most drastic effect. The conformational freedom induced by this subtle change in substrate structure may suggest the aberrant DAPA analogue cannot adopt the correct conformation for DTBS-substrate complex formation. The presence of the methyl group of DAPA may help to stabilise the enzyme-substrate complex through putative interaction with hydrophobic residues on the enzyme. If so then the large drop in catalytic efficiency observed with the ethyl DAPA analogue might be attributed to steric interactions associated with the conservative replacement of a methyl group to an ethyl group (see **Section 2.4**)

2.4 Binding studies

The range of k_{cat}/K_m ratios observed for these pseudo-subtrates necessitated a study of their interaction with DTBS to determine how alteration of the native structure affects the initial substrate binding to the enzyme.

The first reaction catalysed by DTBS proceeds in two distinct stages. The first step involves the binding of DAPA to DTBS to form a subtrate-enzyme complex. Secondly, this complex reacts with CO_2 to afford a DTBS-DAPA carbamate complex. This is shown in **Fig.2.4**

$$\text{DAPA} + \text{DTBS} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{DAPA-DTBS} + CO_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{DAPA-DTBS}.CO_2$$

Fig.2.4 Kinetic model for the first two steps of the DTBS reaction in the absence of ATP.

The results of trapping experiments[130], UV absorption changes upon substrate binding and on subsequent reaction with CO_2 lend support to this model. Absorption maxima at 290(±4) and 301(±3) nm correspond to the DTBS-DAPA complex and the DTBS-DAPA.CO_2 state respectively. Thus, stopped-flow experiments have allowed the first two steps in the reaction to be quantified spectrophotometrically.

In the absence of cooperative interactions between substrate binding sites each of the above steps can be treated as pseudo-first-order processes with the rate coefficients k_{obs1} and k_{obs2}. Measurements of the specific rates of binding of DAPA and its analogues to DTBS were determined over a DAPA (analogue) concentration range of 10-500μM in the presence of Mg^{2+} (2mM). Analysis of the spectrophotometric data using a non-linear regression approach allowed calculation of the DTBS-DAPA

(analogue) equilibrium dissociation constant. The results of these binding studies are shown in **Table 2.5**

From the results, it is apparent that the modifications in the parent substrate significantly alter the rates of DTBS-DAPA analogue complex formation. The modified DAPA analogues **33-35** which differ only in chain length exhibit slow interactions with k_1's for association one order of magnitude lower than racemic DAPA. However, the variation in R group resulted in a reduction in the rate of association by two orders of magnitude for (R-1)-(±)-DAPA **30** and (R+1)-(±)-DAPA **31** with k_1 values of 0.35 and 0.5 s^{-1} respectively. These results suggest that the stereochemical binding constraints imposed by DTBS lowers the rate of binding of these compounds to the enzyme (in the absence of ATP).

SUBSTRATE	R	n	K_{d1} (M)	k_1 ($M^{-1}s^{-1}$)	k_{-1} (s^{-1})
(±)-DAPA **55**	Me	4	3.7×10^{-3}	12.0	4.4×10^{-2}
(R-1)-(±)-DAPA **30**	Me	4	3.0×10^{-5}	0.5	1.5×10^{-5}
(R+1)-(±)-DAPA **31**	Me	4	5.0×10^{-5}	0.35	1.75×10^{-5}
(n-2)-(±)-DAPA **32**	Me	2	1.05×10^{-4}	1.7	1.8×10^{-4}
(n-1)-(±)-DAPA **33**	Me	3	2.2×10^{-4}	2.3	5.1×10^{-4}
(n+1)-(±)-DAPA **34**	Me	5	1.8×10^{-4}	4.7	8.5×10^{-4}
(n+2)-(±)-DAPA **35**	Me	6	4.3×10^{-4}	0.8	3.4×10^{-4}

Table 2.5 Binding constants for the interaction of DTBS with DAPA analogues

The association rate constants for **30** and **31** indicate that the removal or extension of the 9-methyl of DAPA significantly alters the binding of the analogue to the enzyme. Indeed, crystallographic analysis[78] of the native substrate in the active site of DTBS suggests that the 9-methyl group of DAPA may form a hydrophobic contact with the ring methylenes of Pro79. Here it should be noted that proline is one of the more hydrophobic amino acid residues with a hydrophobicity index of 10.9[133].

Upon binding, the pseudosubstrates exhibit rates of dissociation between two and three orders of magnitude less than the racemic parent substrate. These results seemed rather surprising since, on DTBS-DAPA analogue complex formation, it was thought that the modified substrates would bind less well which would lead to an increase in the rate of dissociation. It appears however that shortening or lengthening the native chain may reduce steric interactions or increase hydrophobic contact with the enzyme. This rationale may also apply to the interaction of (R-1)-(±)-DAPA **30** and (R+1)-(±)-DAPA **31** with DTBS where replacing the methyl group of DAPA with H or Et contributes to the significant fall in dissociation rate constant, k_{-1}, observed for these two analogues.

These initial observations suggest that it is binding of these analogues to the enzyme which is rate-limiting in the DTBS catalysis of *ureido* ring formation. However, since these experiments were performed in the absence of ATP it must be noted that the possible conformational changes induced by bound ATP could affect the binding of DAPA and its analogues.

We also examined the kinetics of the second step of the DTBS transformation, namely the reaction of CO_2 with the DTBS-DAPA analogue complexes to form the enzyme-bound carbamate. These results are summarised in **Table 2.6**

SUBSTRATE	R	n	K_{d2} (M)	k_2 ($M^{-1}s^{-1}$)	k_{-2} (s^{-1})
(±)-DAPA **55**	Me	4	4.7×10^{-2}	3.8	0.18
(R-1)-(±)-DAPA **30**	Me	4	1.2×10^{-2}	1.8	0.002
(R+1)-(±)-DAPA **31**	Me	4	6.3×10^{-3}	0.7	0.004
(n-2)-(±)-DAPA **32**	Me	2	2.0×10^{-2}	2.7	0.054
(n-1)-(±)-DAPA **33**	Me	3	2.7×10^{-2}	3.2	0.090
(n+1)-(±)-DAPA **34**	Me	5	1.1×10^{-2}	1.5	0.017
(n+2)-(±)-DAPA **35**	Me	6	4.5×10^{-2}	2.6	0.120

Table 2.6 Kinetic data obtained for the reaction of CO_2 with the DTBS-DAPA analogue complex

The data obtained suggests that in all cases the rate of DTBS-DAPA analogue carbamate formation is similar to that of the native substrate. All the pseudosubstrates, with the exception of **31** (k_2=0.70 $M^{-1}s^{-1}$), have k_2 values within the same order of magnitude as (±)-DAPA **55**. The increase in size of the alkyl moeity in **31** may introduce a steric constraint which forces the 7-amino group out of the optimal binding site and thus slows carbamate formation. It is noteworthy that, once formed, the aberrant carbamates do not readily dissociate from the enzyme on comparison with DAPA-CO_2. The synthetic modifications may act to stabilise the carbamate intermediate, a feature already observed in the binding of the substrate analogues to DTBS.

2.5 Modelling studies of the active site of DTBS

The results obtained from the kinetic studies clearly show that all the DAPA analogues synthesised act as slow substrates of DTBS. Although comparison of the kinetic parameters suggests differences in binding, it is difficult to recognise a clear pattern at this stage without the aid of X-ray studies. Recent crystallographic characterisation[134] of the enzyme-DAPA and enzyme-DAPA carbamate complexes in the presence and absence of Mg^{2+} ions carried out in this laboratory have revealed details of protein-substrate and protein-intermediate interactions which stabilise the binding of DAPA and the DAPA N-7 carbamate intermediate to the active site of the enzyme. (see **Figs. 1.7** and **1.8**)

The role of Mg^{2+} ion in the DTBS reaction has also been determined crystallographically and the conformational changes between the Mg^{2+}-substrate-enzyme complex (coloured atoms) and the Mg^{2+}-free complex (white atoms) are shown in **Fig.2.5**

In the absence of Mg^{2+}, the Thr11 and Ser41 hydroxyls are directed away from the substrate binding site. However, in the Mg^{2+}-complexed form, the Thr11-Ser41 Cα-Cα distance is shortened by 1.3 Å and both side chain hydroxyls are rotated towards the diamino group binding site. The substrate is bound between the two crystallographically identical monomers of the dimeric DTBS.

Earlier work[76] has suggested that the positions of the two bound sulphates, S1 and S2, could be considered to mimic the positions of the α- and γ-phosphates of the ATP molecule. Thus, binding of Mg^{2+} to the enzyme leads to a movement of the P loop which has two consequences: the phosphate binding site alters to permit better binding of the α- and γ-phosphates of ATP and the hydroxyl groups of Thr11 and Ser41 rotate towards the bound Mg^{2+}, and each other, allowing formation of H-bonds between these residues and the amine groups of the substrate.

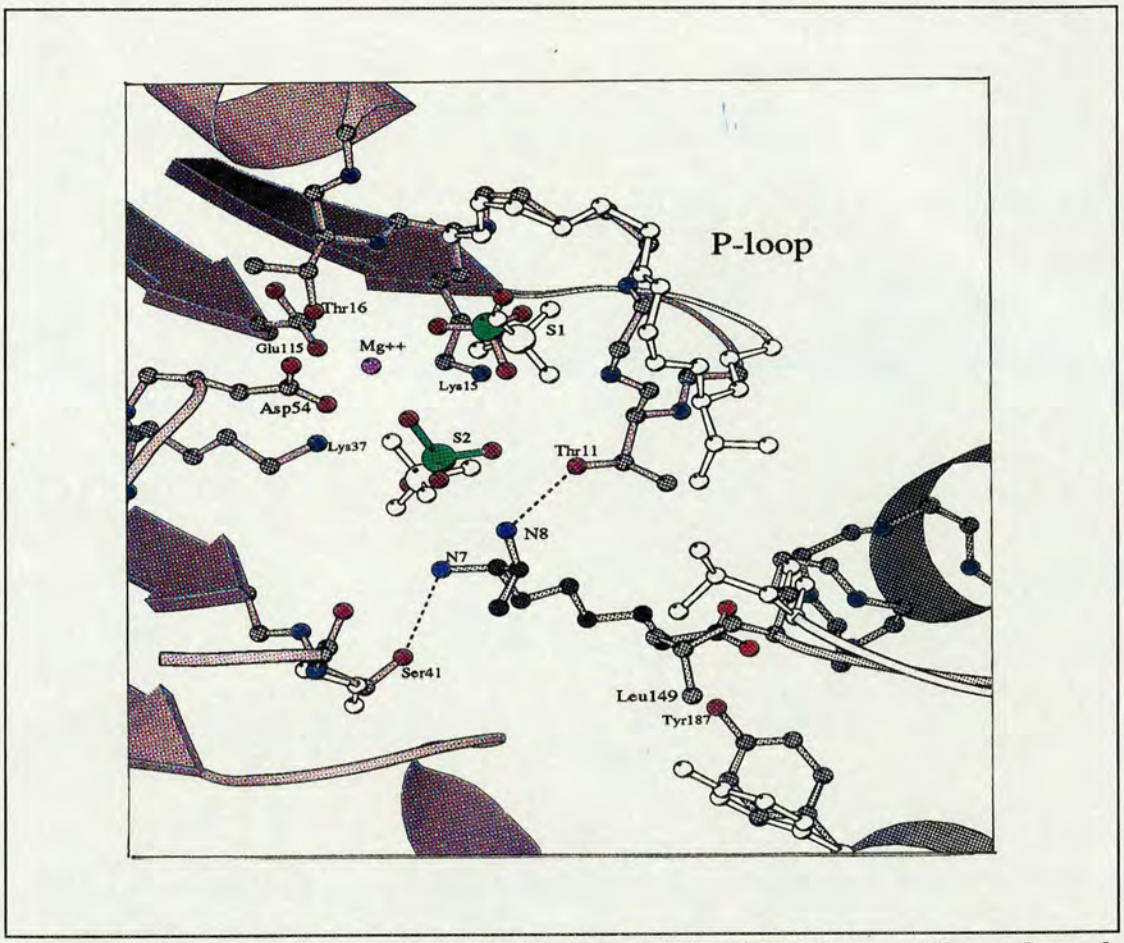


Fig.2.5 The conformational changes induced by Mg^{2+} ions. The coloured molecule represents the Mg^{2+}-complex and the open molecule corresponds to the Mg-free enzyme.(Figure drawn with MOLSCRIPT[135])

X-ray structures of the DTBS-DAPA-Mg^{2+} complex in the absence (**Fig.1.7**) and presence of CO_2 (**Fig.1.8**) were determined on a single crystal and these show in detail that carboxylation occurs at the N-7 amino group of DAPA. The sulphate S2, which is present before DAPA carboxylation and which is coordinated to the Mg^{2+} ion, the ξ-amino groups of Lys37 and Lys15, and water molecule W2, is replaced by the carboxylate group of DAPA carbamate and by a water molecule W1. Small movements (up to 0.3Å) of the Mg^{2+} ion, the sulphate anion S1 and the P-loop close the gap left by the displacement of the S2 sulphate.

In all the complexes studied, the difference electron density maps have shown unambiguously that the substrate and the intermediate bind to the same site in the enzyme. While the interactions at the diamino end of the substrate differ significantly in the two enzyme-substrate complexes (i.e in the presence and absence of CO_2), the binding of the carboxyl group of the ligand is always the same. The carboxylate of DAPA (pK_a 4.48) interacts with the positive (N-terminal) pole of the helix α6 (see **Fig.2.5**). One carboxylate oxygen binds to the main chain nitrogen of Asn153 and the other is held between the positively polarised main chain nitrogens of Gly150-Cys151-Ile152 and the hydroxyl of Tyr187, which moves to optimise substrate binding. A similar carboxylate helix dipole interaction has been observed in D-ala-D-ala ligase[137].

The hydrophobic side chain of the bound DAPA lies in a groove defined by Cα of Gly118, Cγ2 of Thr122, Cα of Ser81 and Cδ of Pro82 on the other subunit of the native structure. The side chain of Leu149 moves some 2.5Å towards the substrate providing an additional hydrophobic interaction with the polymethylene chain which helps to lock the substrate in the groove of the opposite subunit.

In contrast, the binding pattern at the amino end of the substrate is variable and subject to conformational changes of the enzyme depending on the nature of the ligand and the presence of Mg^{2+}. These changes suggest that the carboxylate end serves to anchor the substrate to the enzyme presenting the diamino functionality to the catalytic site on the other subunit.

We used this common interaction as a basis for modelling two of the aberrant analogues in the active site of DTBS. Models for (n-1)-DAPA and (n-2)-DAPA were constructed on the assumption that the carboxylate-helix dipole interaction remained the same upon binding and that H-bonding interactions between the enzyme and the amino function of the substrates would be maximised.

Figures 2.6 and **2.7** represent models for the interaction of DTBS with (n-1)-DAPA and (n-2)-DAPA respectively.

It is apparent that the constraints imposed by the carboxylate binding site in both models direct a conformational change in amino group binding such that the terminal amino group is presented for carboxylation. This is opposite to that found in DAPA where the N-7 amino group is the site of carbamate formation.

The information derived from these models suggests that DTBS may catalyse an alternative mechanism of *ureido* ring closure when utilising **32** and **33** as substrates. However, the active site models only provide a hypothetical view of analogue binding and more detailed structural analysis of the aberrant analogues in the active site was required to determine the specific enzyme-substrate interactions involved.

Fig.2.6. Hypothetical model of (n-1)-DAPA in the active site of DTBS. The reduction in chain length induces a change in amino group interaction with the enzyme.

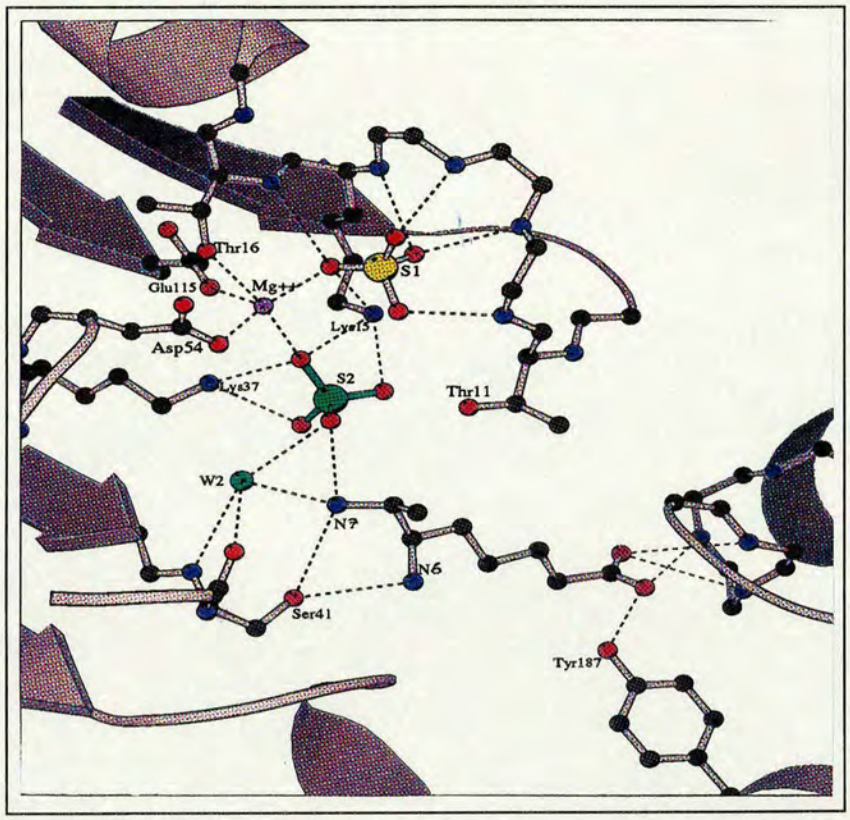
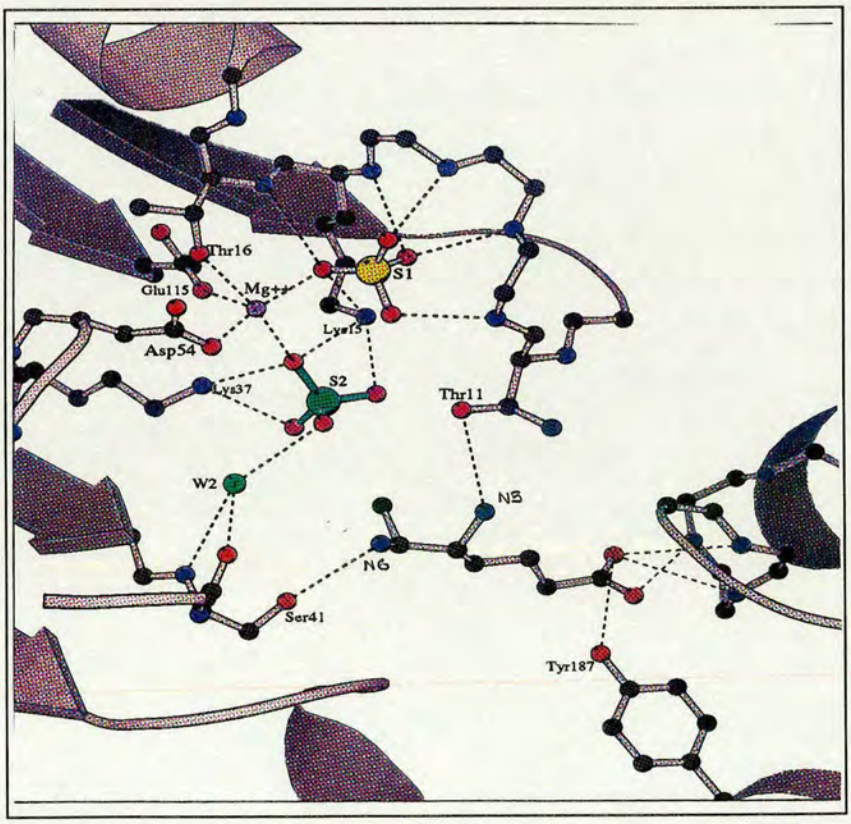


Fig.2.7 Active site of DTBS containing a model of (n-2)-DAPA. (Figures were drawn with MOLSCRIPT[135])



2.6 Crystallographic studies on the binding of (n-1)-DAPA to DTBS

Crystallographic studies on DTBS performed in our laboratory[76,134] have allowed the interaction between the enzyme and aberrant DAPA analogue **33** to be examined at atomic resolution. Structural analysis of the protein-analogue interaction was performed in collaboration with Dr. Dimitriy Alexeev and the results of this study are described below.

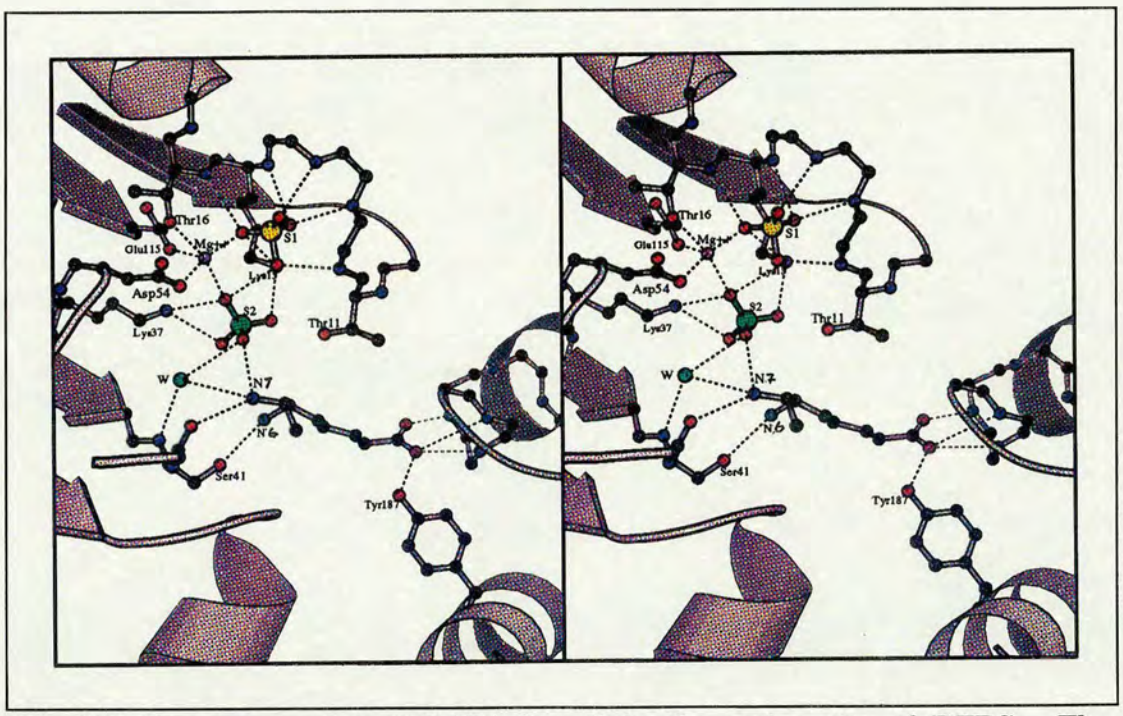


Fig.2.8 Stereoview of (n-1)-DAPA bound in the active site of DTBS. The pseudosubstrate lies between the two monomers of the enzymic homodimer. (Figure was drawn with MOLSCRIPT[135])

The (n-1)-DAPA compound binds to DTBS in a similar manner to the native substrate where analogue is straddled between the monomers of the enzyme homodimer (see **Fig.2.8**). Carboxylate binding is identical to the parent DAPA substrate and the rotation of Tyr187 on helix α7 towards the positive pole of helix α6 is clearly visible.

However, the shortened side chain directs a conformational change at the amino terminus and the site occupied by the N-7 amino group in DAPA is now replaced by the terminal amine in (n-1)-DAPA as suggested by our modelling studies. Both amines of the aberrant analogue are coordinated to Ser41 while the N-7 amino group of (n-1)-DAPA is H-bonded to the sulphate anion S2 and the water molecule W2. In addition, the monomers of the enzyme move approximately 0.4 Å closer together to accomodate the shorter substrate.

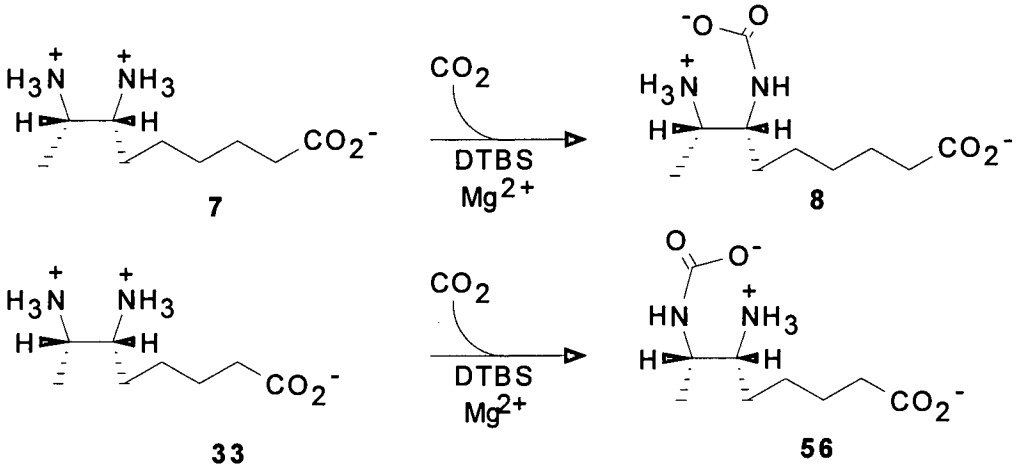


Fig.2.9 Site of DAPA carboxylation and putative site of (n-1)-DAPA carbamate formation

We were not able to detect the carboxylation of (n-1)-DAPA crystallographically which would have identified the site of carbamate formation unambiguously. However, the fact that the carboxylation active site of the enzyme is now occupied by the 7-amino group of (n-1)-DAPA suggests that this amine is carboxylated (**Fig.2.9**). If so, then it appears that DTBS exhibits remarkable versatility in dealing with small changes in substrate structure by catalysing the *ureido* ring closure of (n-1)-DAPA through an alternative mechanism.

2.7 Conclusions

The combination of kinetic, modelling and crystallographic studies have demonstrated that DTBS exhibits promiscuity in utilising aberrant DAPA analogues as substrates. Further experiments are required to determine if the enzyme employs a variable mechanism of *ureido* ring closure to achieve its catalytic goal. Fortunately, the scope of these analogues is not limited to studies on DTBS. Since the catalytic products of the DTBS reaction are modified dethiobiotin analogues, these compounds could be used as probes to study the limitations of sulphur introduction in the biotin synthase reaction. Enzymatic radiolabelling of these analogues with $H^{14}CO_3^-$ would provide a clean and efficient alternative to labelling with ^{14}C-phosgene. The information obtained from this study may prove useful in the targetted design of inhibitors of DTBS which have a potential market as selective biocides in the agrochemical industry.

Chapter 3
The Mechanism of Biotin Synthase-Enzymatic Studies on the Nature of Sulphur Donation

3.1 Establishing an *in vitro* system

Since Dittmer *et al.*[53] first demonstrated that dethiobiotin was as effective as biotin in supporting the growth of *Saccharomyces cerevisiae*, a large number of studies have appeared in the literature confirming that dethiobiotin is converted to biotin by resting and growing cells of a variety of organisms[137]. However, work on the identification of the sulphur donor and the mechanism of this unique reaction has awaited the development of overexpression systems capable of producing sufficient amounts of the enzyme involved for mechanistic investigation.

Previous studies[93] in this laboratory have involved the cloning of the *E.coli bio*B gene into a high level expression vector (pKK233-3), a plasmid with a relatively high copy number containing the *tac* promoter, to produce the recombinant plasmid pIMSB. This plasmid was used to transform *E.coli* strain DH1i (*bio*+) to give the *bio*B transformant DH1i/pIMSB. Production of biotin by induced DH1i/pIMSB cells (when excess DTB was present) was approximately 100 times that of wild type *E.coli* K-12.

During our initial investigations into establishing a functional *in vitro* system using cell-free extracts of DH1i/pIMSB, Ifuku *et al.*[91a] demonstrated the enzymatic conversion of dethiobiotin to biotin in cell-free extracts of a *bio*B transformant of *E.coli*. Their investigation into the cofactor requirements of this reaction indicated that ferrous or ferric ions, S-adenosyl methionine (AdoMet). NADPH, KCl and fructose-1,6-bisphosphate all stimulated the production of biotin from dethiobiotin.

Following this work, we also found similar cofactor requirements although in our hands fructose-1,6-bisphosphate does not significantly stimulate the reaction. (**Fig. 3.1**)

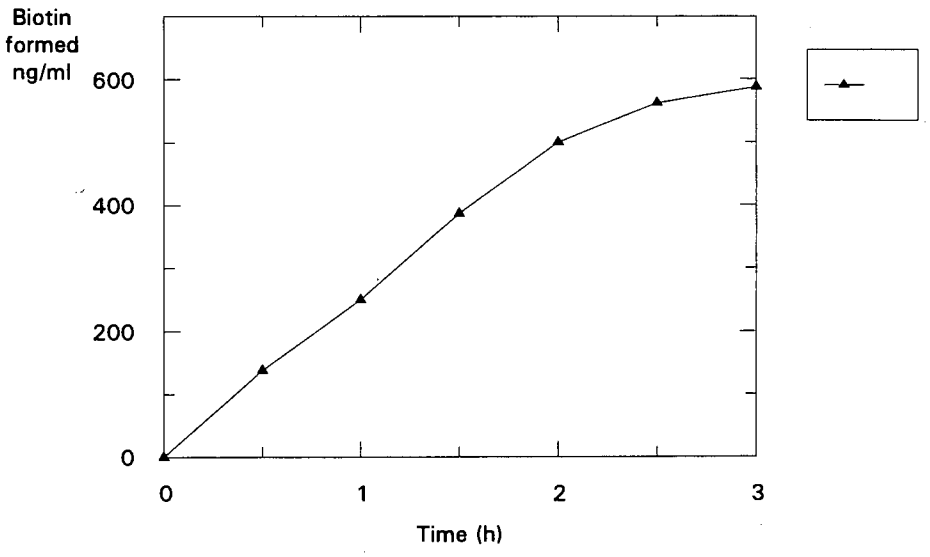


Fig 3.1. Plot of amount of biotin made with time. Biotin-forming activity was assayed under standard assay conditions using DH1i/pIMSB cell-free extract in the presence of 5mM Fe^{2+}, 0.1mM AdoMet, 1mM NADPH, 10mM KCl, 5mM L-cys and 60μM D-DTB.

In our preliminary studies on the purification of biotin synthase we also found, in agreement with Ifuku *et al.*, that an additional protein(s) was required for the enzymatic conversion of dethiobiotin to biotin. Ammonium sulphate fractionation of DH1i/pIMSB cell-free extracts showed that proteins precipitating between 20 and 50% saturation, in which the *bio*B gene product was thought to be included on the strength of SDS-PAGE, did not show any biotin-forming activity by itself. However, biotin production was detected using the *Lactobacillus plantarum* microbiological assay[138] when the reaction mixture was supplemented with cell-free extracts of PCOi (*bio*-), an *E.coli* mutant which the lacks the biotin operon.

Anion exchange chromatography of the 20-50% ammonium sulphate fraction of DH1i/pIMSB gave the elution profile shown in **Fig.3.2** Fractions were analysed by SDS-PAGE and assayed for biotin synthase activity in the presence of PCOi (bio-) cell-free extract under standard assay conditions.

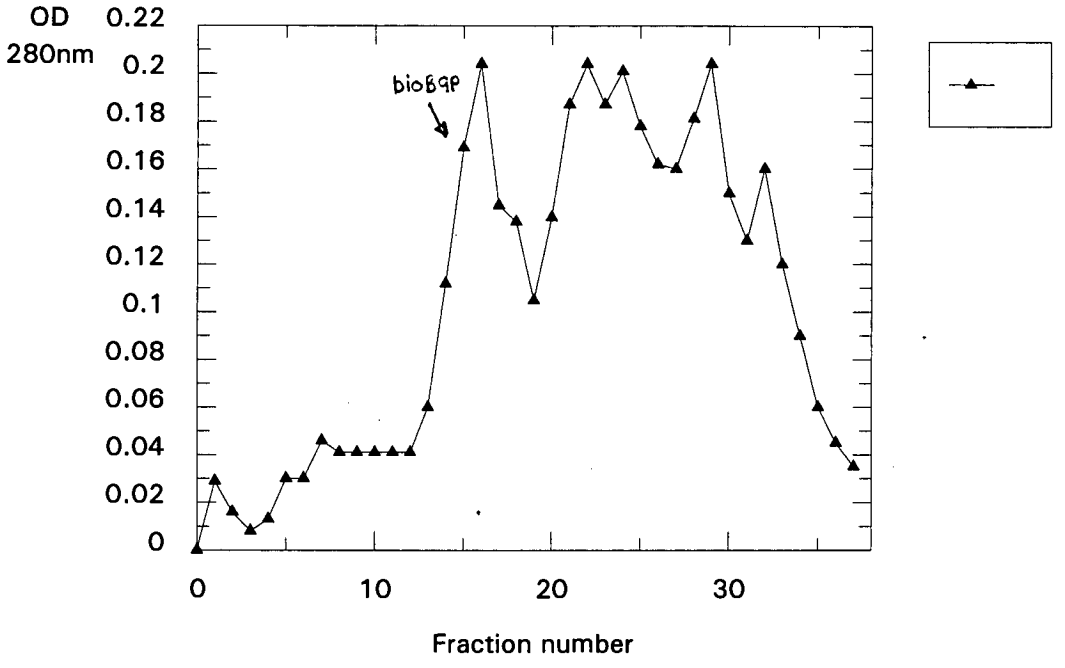


Fig.3.2 Q-sepharose anion exchange chromatography of 20-50% $(NH_4)_2SO_4$ fraction of DH1i/pIMSB.

The *bio*B gene product eluted at a salt concentration of approximately 300mM.

During the course of this work, Flint and coworkers at Du Pont reported the first purification of the *E.coli bio*B gene product[119]. The protein is a homodimer (82kDa) and contains one [2Fe-2S] cluster per monomer. In common with Ifuku's group and ourselves, they observed that the *bio*B gene product alone was insufficient in catalysing the formation of biotin from dethiobiotin and required unknown cofactors from crude extracts devoid of the *bio*B gene product. One of these unknown cofactors has been identified as flavodoxin[122]. However addition of flavodoxin alone to partially purified extracts of *bio*B gene product and other cofactors did not result in formation of biotin. The results of this study suggest that another unidentified protein(s) in addition to the *bio*B gene product and flavodoxin may be involved in the biotin-forming reaction. The authors postulate the possible involvement of ferredoxin (flavodoxin) $NADP^+$

reductase in this system but attempts at isolation have proved unsuccessful.

Although a purified system for *in vitro* biotin formation remains to be found, the information obtained from these recent investigations has been used to draw similarities between the reaction catalysed by biotin synthase and those of other enzymes. Of particular note is the requirement for Adomet in the reaction. Adomet is normally regarded as the source of methyl in methylating enzymes[139] although it has been implicated as an amino donor in DAPA synthase[65,66]. More relevant to this work is its role as a free radical initiator in the action of pyruvate formate lyase (PFL), anaerobic ribonucleotide reductase (anaRNR) and lysine 2,3-aminomutase. Since it is this action which is relevant to the biotin-forming activity of biotin synthase, some discussion of the mechanisms of these enzymes is relevant here.Their catalytic actions are shown in **Fig. 3.3**

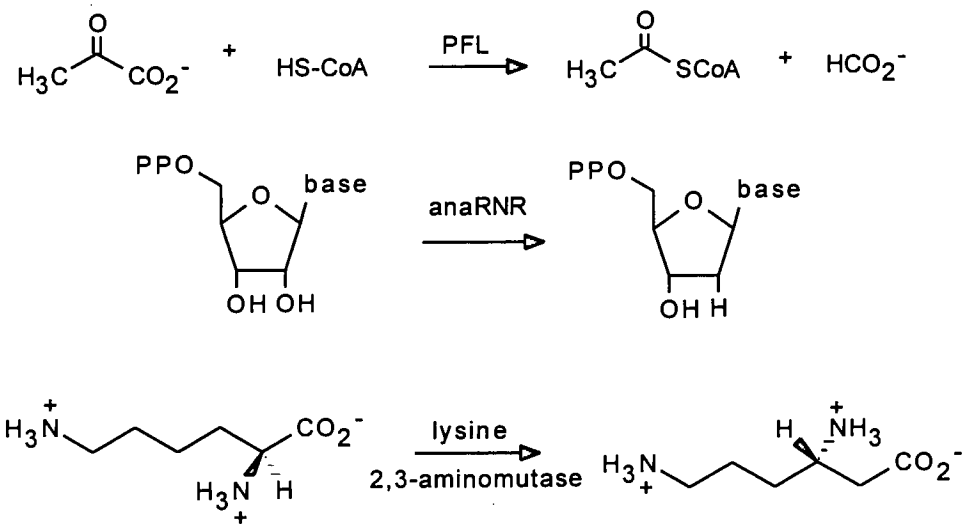


Fig.3.3 The reactions catalysed by pyruvate formate lyase (PFL), anaerobic ribonucleotide reductase (anaRNR) and lysine 2,3-aminomutase

A common dependence amongst these enzymes is the requirement of S-adenosylmethionine (AdoMet) in the reaction. The 5'-deoxyadenosyl radical produced by the one electron reduction of AdoMet is used to activate the enzyme by initiating a protein-based radical, as occurs with

PFL and anaRNR, or it may participate directly in catalysis as in the reaction catalysed by lysine 2,3-aminomutase.

Under anaerobic conditions, the activation of PFL is achieved by generating a radical at a specific glycine residue (gly734), which is predicted to lie in a β-turn of the protein[140]. The specific activating enzyme responsible for generating the glycyl radical[141], PFL activase, requires Fe(II) for activity, AdoMet and inactive PFL as substrates, with reducing power supplied by reduced flavodoxin. In addition, pyruvate is required as an allosteric activator. AdoMet is first reduced to the 5'-deoxyadenosyl radical and methionine by a single electron from flavodoxin, presumably mediated by Fe(II). The radical removes hydrogen from the C-α of PFL-gly734. This step has been demonstrated by stoichiometric incorporation of deuterium into 5'-deoxyadenosine from 2-D-glycine labelled PFL[142]. The peptide in which gly734 is replaced by L-alanine is inactive; however, the D-alanine-substituted peptide is a good substrate, which implies that the activase is stereospecific for the pro S hydrogen of PFL-gly734.

The AdoMet-dependent activation of the anaerobic ribonucleotide triphosphate reductase (anaRNR), which also requires reduced flavodoxin, most probably occurs in a manner similar to that described for PFL[143] but differs in its apparent "self-activation" i.e no auxiliary activase is required. The enzyme contains a [4Fe-4S] cluster which is presumably involved in the reduction of AdoMet to 5'-deoxyadenosyl radical and methionine. In common with PFL, this radical is used to generate a protein radical by removing hydrogen froma glycine residue which, upon sequence comparison with PFL, appears to be gly681[144].

The third AdoMet-utilising enzyme, lysine 2,3-aminomutase, contains a [4Fe-4S] cluster and Co(II), which are essential for activity[145]. However the chemistry associated with forming the 5'-deoxyadenosyl radical from AdoMet remains obscure since no external reductant is apparently

required by the enzyme. Various mechanistic hypotheses have been discussed and suggest that the iron-sulphur centre supplies an electron to reduce AdoMet[146]. Experiments have demonstrated that tritium from AdoMet labelled in the 5' position is transferred to both the substrate, lysine, and the product, β-lysine, thus implicating a direct role for the 5'-deoxyadenosyl radical in catalysis[147]. In agreement with this role, the 5'-deoxyadenosyl radical and methionine remain tighly bound to the enzyme during catalysis, although some slow release of 5'-deoxyadenosine and methionine occurs. The presence of Co(II) has led to the suggestion that (by analogy with adenosylcobalamin), the 5'-deoxyadenosyl radical could be stabilised between turnovers by coordination to the cobalt.

Radical chemistry has already been postulated in the bioconversion of dethiobiotin to biotin. Arigoni *et al.* synthesised dethiobiotin chirally labelled at the 9-position with hydrogen, deuterium and tritium to probe the mechanism of proton abstraction on its conversion to biotin in *A.niger*[112].

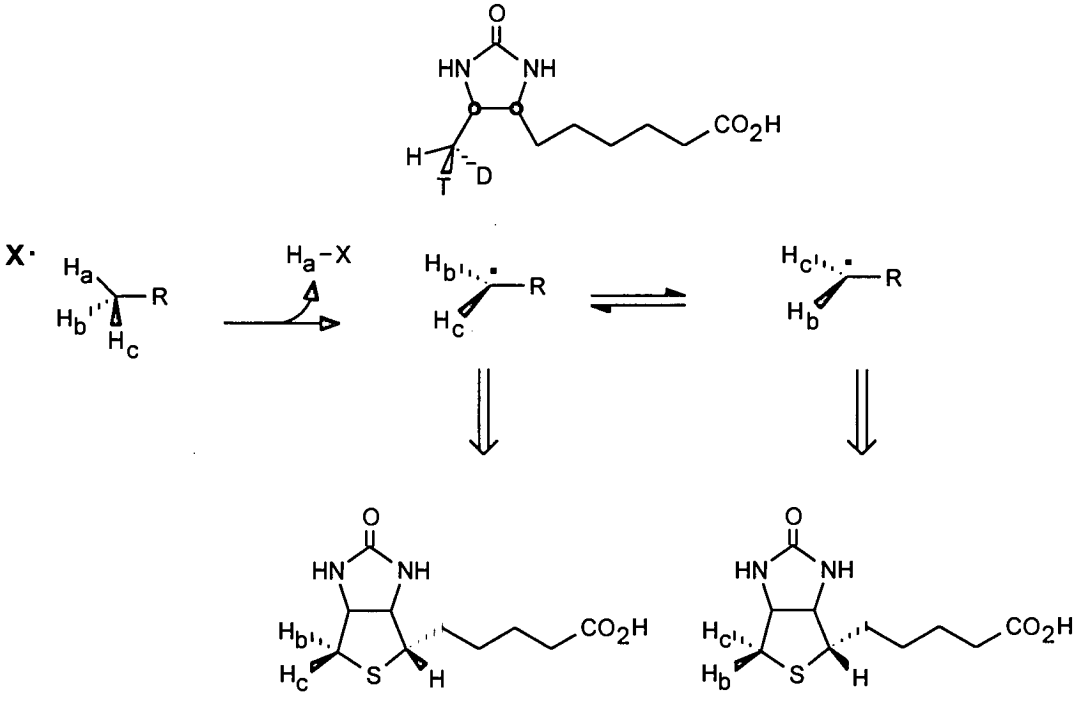


Fig.3.4 The proposed radical chemistry involved in the conversion of [9-^{2}H, ^{3}H]-(+)-dethiobiotin to biotin

The stereodistribution of the isotopes at C-1 of the isolated biotin indicated that scrambling of the isotopes had occurred. From this, the authors suggested the formation of a conformationally labile methylene at C-9 as an intermediate in the biosynthesis of biotin. (**Fig. 3.4**). The results from this experiment and analogy with other enzymes suggests a role for AdoMet in producing a radical species involved in the biotin synthase reaction.

3.2 Identification of the sulphur donor

After establishing an active *in vitro* system, we focussed our attention upon the identity of the sulphur donor in the enzymatic formation of biotin.

There is growing evidence in the literature that cysteine, if not the actual sulphur donor, is very close to the metabolic pathway whereupon sulphur is introduced into biotin[86,88]. However, incorporation experiments with intact organisms have been hampered by poor uptake of exogeneously supplied precursors and this has compelled studies at the cell-free level.

Studies in cell-free extracts of *B.sphaericus* have shown that the radiolabel from ^{35}S-AdoMet is not incorporated into biotin thus supporting the view that AdoMet acts as a cofactor and not as a sulphur source[92].

However, we observed no significant increase in biotin production on administration of L-cysteine to our active cell-free system from *E.coli* and indeed Ifuku *et al.*[91a)] have also observed that L-cysteine had no effect on the amount of biotin formed.

A major drawback in our identification of putative sulphur donors was the inability of the DH1i/pIMSB cell-free extract to withstand dialysis. Incubation of dialysed DH1i/pIMSB cell-free extract with NADPH, AdoMet, KCl, Fe^{2+}, L-cysteine and D-DTB showed no biotin-forming activity indicating that a necessary cofactor(s)/protein was lost or

inactivated upon dialysis with buffer. This phenomenon has also been observed by Ifuku *et al.*[91a]. In preliminary experiments they found that ammonium sulphate fractionation (0-90%) of their cell-free extract resulted in loss of activity. Marquet *et al.* have also observed this loss in activity upon dialysis of cell-free extracts of *B.sphaericus*[91b] and *E.coli*[94]. Despite this, radiolabel incorporation experiments have implicated a role for L-cysteine in sulphur donation in the biotin synthase reaction.
Incorporation of ^{35}S-label into biotin has been observed upon incubation of ^{35}S-L-cysteine in the presence of other cofactors in cell-free extracts of *B.sphaericus*[92] and *E.coli*[93]. However it should be noted that the specific activity of the [^{35}S]-D-biotin produced from [^{35}S]-L-cysteine is much lower than that predicted on the basis of direct incorporation of label from the substrate into biotin indicating that some dilution of the radiolabel occurs in the experiment. The most obvious factor contributing to this observation is simply that the radiolabelled cysteine is diluted by unlabelled amino acid entrained in the incubation mixtures.
Indeed quantitative amino acid analysis of the supernatant of DH1i/pIMSB after protein denaturation indicated approximately 400μM of L-cysteine was present.

In order to gain an insight into the mechanism of sulphur donation, we incubated [^{14}C]-L-cysteine with an aliquot of DH1i/pIMSB cell-free extract and monitored the fate of the radioactive amino acid. This was achieved by separation of the constituent amino acids by High Voltage Paper Electrophoresis (HVPE) at 3kV and pH 2.0. Under these conditions, the sole radioactive product was [^{14}C]-L-alanine. Similar results were obtained in control incubations with cell-free extracts lacking the *bio*B gene product, implying the desulphurisation of [^{14}C]-L-cysteine is not a consequence of *bio*B gene product activity. The results of these experiments are shown in **Fig.3.5**

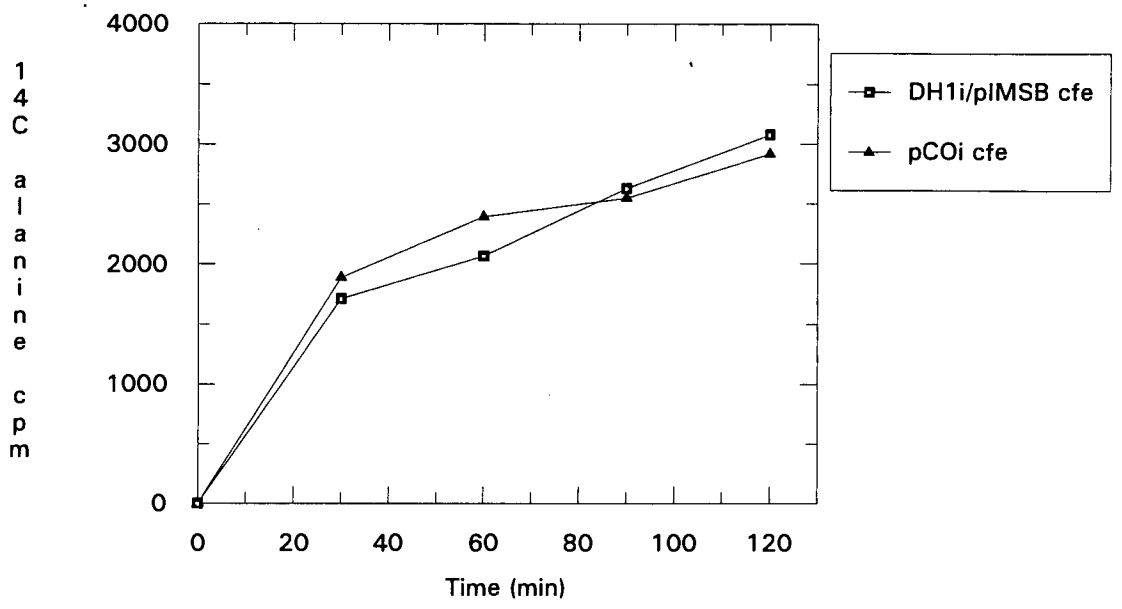
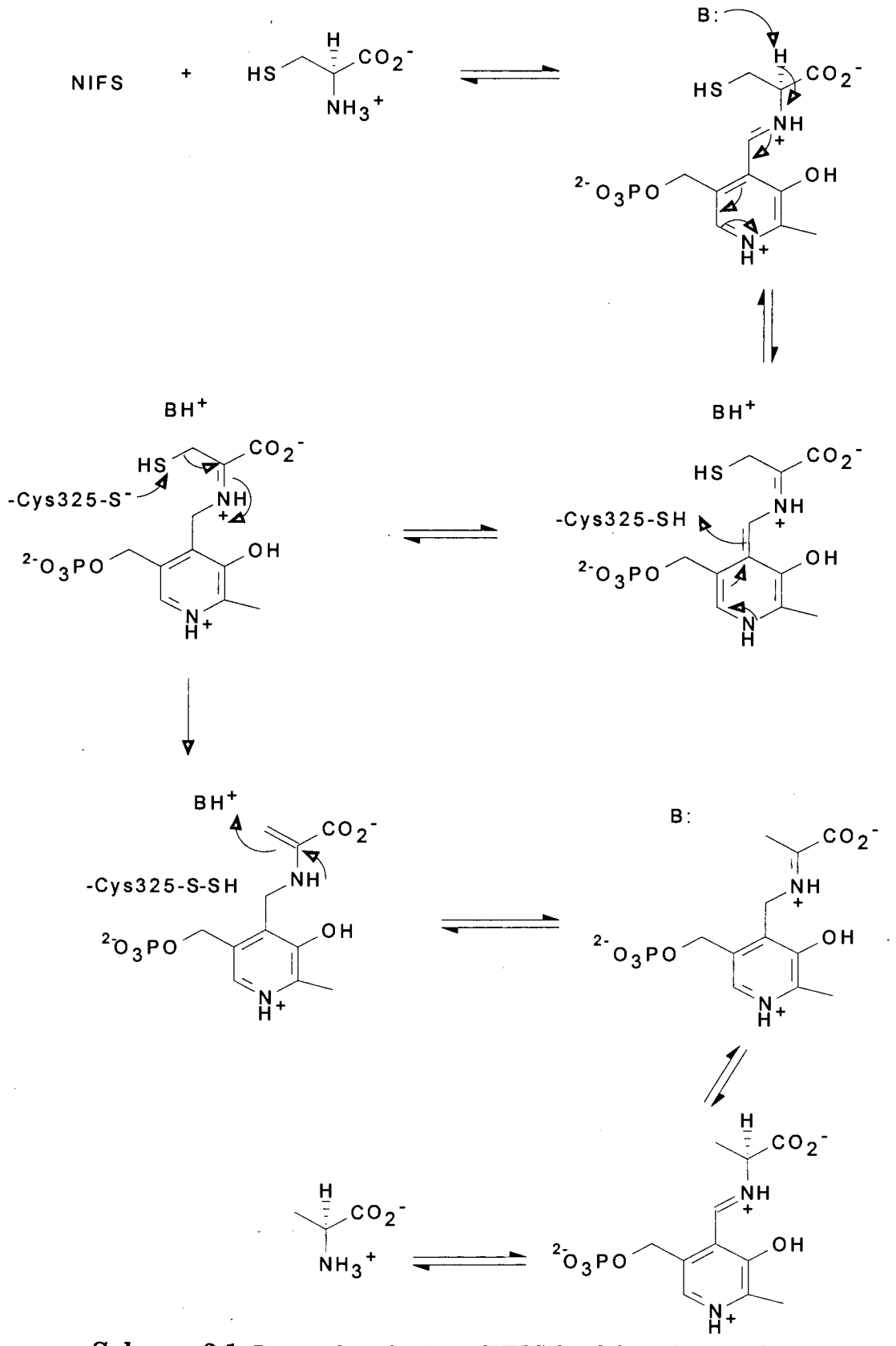


Fig.3.5 Plot of ^{14}C-alanine formation against time.

The desulphurisation of L-cysteine to L-alanine is not without enzymic precedent. The *nif*s gene product (NIFS) of *Azotobacter vinelandii* is a pyridoxal phosphate-dependent homodimer (88kDa) that catalyses the desulphurisation of L-cysteine to yield L-alanine and sulphur[148]. This activity is required for the full activation of the two metalloproteins that are the components of nitrogenase. (The role of nitrogenase in biological nitrogen fixation has been extensively reviewed[149].)

These components, Fe protein and MoFe protein, both contain metalloclusters which are required for their respective activities and it is suggested that NIFS participates in the biosynthesis of the nitrogenase metalloclusters by providing the inorganic sulphur required for Fe-S core formation. Dean *et al.*[150] have suggested NIFS catalyses the formation of an enzyme-bound persulphide which is the active species for providing the inorganic sulphur necessary for Fe-S cluster biosynthesis and experimental evidence has led to the proposed mechanism for the desulphurisation of L-cysteine catalysed by NIFS shown in **Scheme 3.1**

The salient and novel feature of this mechanism is nucleophilic attack by an active site cysteinyl thiolate anion on the sulphur of a cysteine-PLP



Scheme 3.1 Proposed mechanism of NIFS desulphuration reaction

adduct which results in formation of a cysteinyl persulphide and an enamine derivavtive of L-alanine. Site-directed mutagenesis experiments, which show that cys325 is required for cysteine desulphurase activity, support this mechanism.

Olefinic amino acids are often utilised as mechanism-based suicide inhibitors of PLP-dependent enzymes[151] so we used L-allylglycine to investigate the mechanism of cysteine desulphurisation in *E.coli*.
Aliquots of DH1i/pIMSB cell-free extract were incubated with various concentrations of L-allylglycine (0, 5, 10, 20 amd 50mM). After dialysis to remove excess L-allylglycine, each aliquot was incubated with [^{14}C]-L-cysteine and the formation of [^{14}C]-L-alanine was monitored. (**Fig.3.6**)

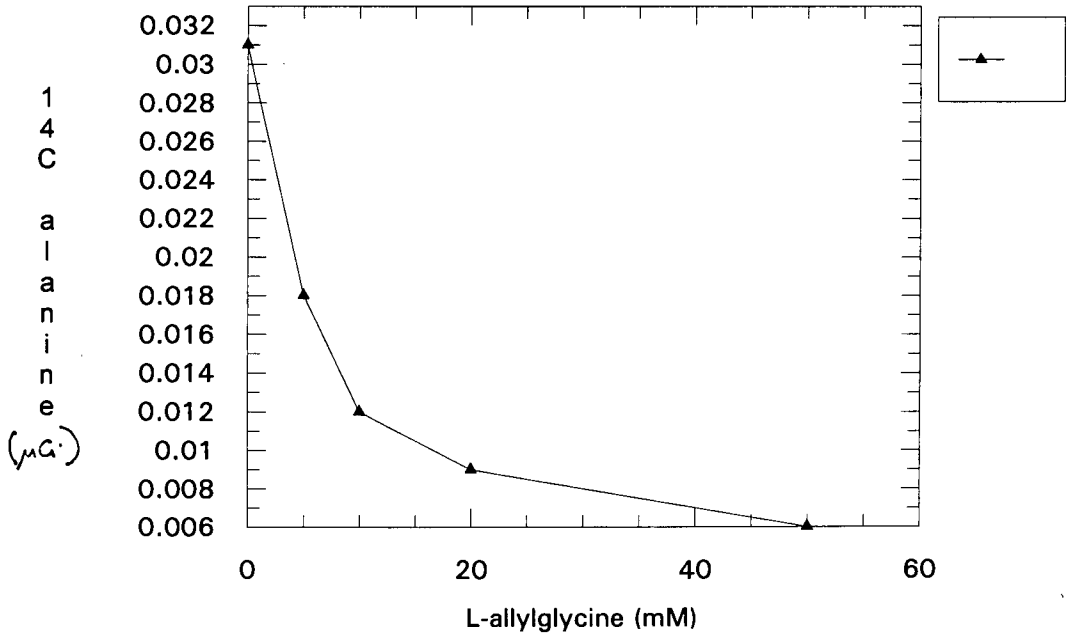
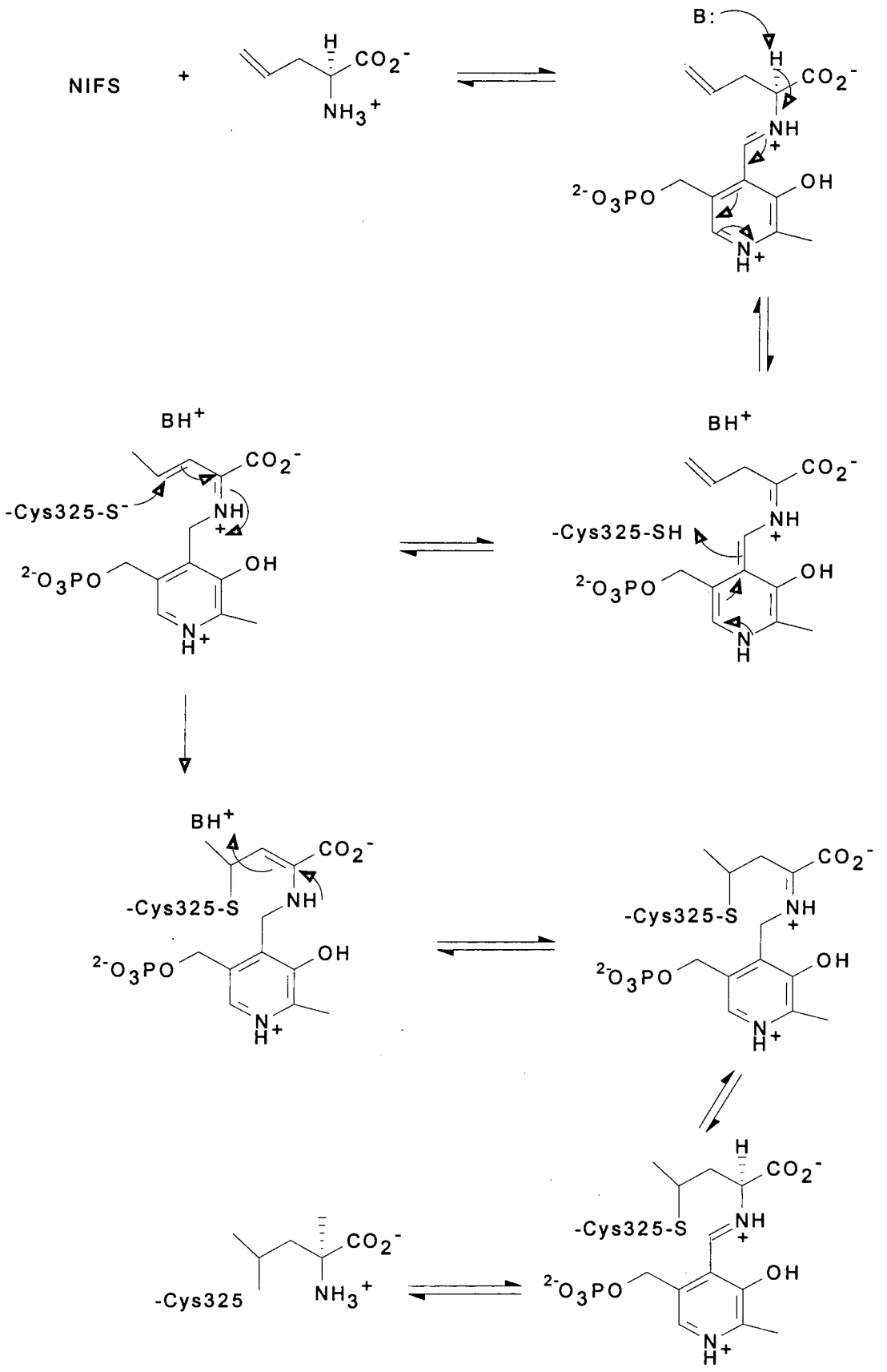


Fig.3.6 Inhibition of *E.coli* cysteine desulphurase activity by L-allylglycine

The observed loss in cysteine desulphurase activity was not reversed by the addition of L-cysteine and PLP suggesting L-allylglycine inactivates the enzyme irreversibly.

These results imply the *E.coli* enzyme catalysing the desulphurisation of L-cysteine to L-alanine and sulphur is pyridoxal phosphate-dependent. Dean *et al.* have examined the mechanism-based suicide inhibition of NIFS by L-allylglycine in detail and their results[150] have led to the proposed mechanism shown in **Scheme 3.2.** This route involves a mechanism-based suicide inactivation in which the enzyme tautomerises the terminal double bond of L-allylglycine followed by nucleophilic attack by the proposed active cysteinyl residue at the γ-carbon. This mechanism has precedence in cystathionine γ-synthase which catalyses the formation γ-methylcystathionine by condensation of PLP-allyglycine adduct and L-cysteine[152].



Scheme 3.2 Proposed mechanism of inhibition of NIFS by L-allylglycine

3.3 The Purification of *E.coli* cysteine desulphurase

After demonstrating cysteine desulphurase activity in cell-free extracts of both DH1i/pIMSB and pCOi we hoped to proceed by purifying the cysteine desulphurase to homogeneity and establish if this enzyme was involved in the biotin synthase reaction. The use of radiolabelled substrate would ensure the detection of small amounts of protein from the various chromatographic purification procedures employed.

Ammonium sulphate fractionation of DH1i/pIMSB cell-free extract was performed and the proteins precipitating between 20 and 50% saturation exhibited cysteine deulphurase activity. The 20-50% ammonium sulphate fraction was dialysed to remove the salt and applied to a DEAE-cellulose anion exchange column. The column was washed with Tris buffer (50mM, pH7.5) and the proteins eluted with a linear salt gradient (0-800mM NaCl). The elution profile is shown in **Fig.3.7**

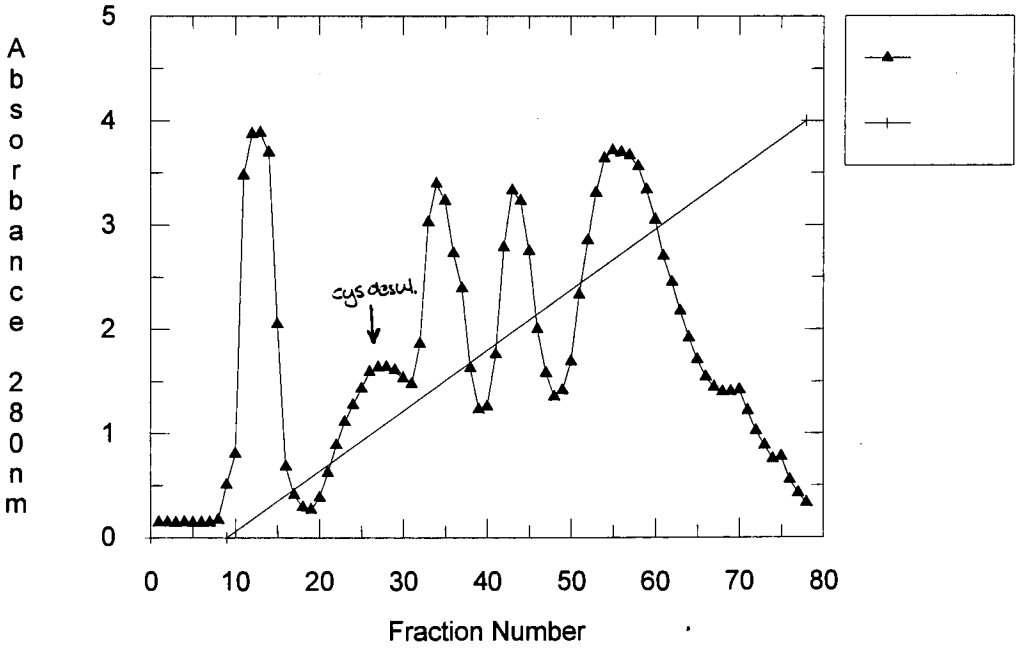


Fig.3.7 DEAE-cellulose anion exchange chromatography of 20-50% ammonium sulphate fraction of DH1i/pIMSB cell-free extract. Fractions were assayed for cysteine desulphurase activity under standard [^{14}C]-L-cysteine assay conditions.

E.coli cysteine desulphurase eluted between 200 and 300mM salt concentration. Active fractions were pooled, concentrated and dialysed against Tris buffer (50mM, pH 7.5) to remove salt.

The concentrated sample was subjected to further anion exchange chromatography on Mono Q resin using FPLC. Bound proteins were eluted with a linear salt gradient (0-500mM) to yield the chromatographic trace shown in **Fig.3.8**

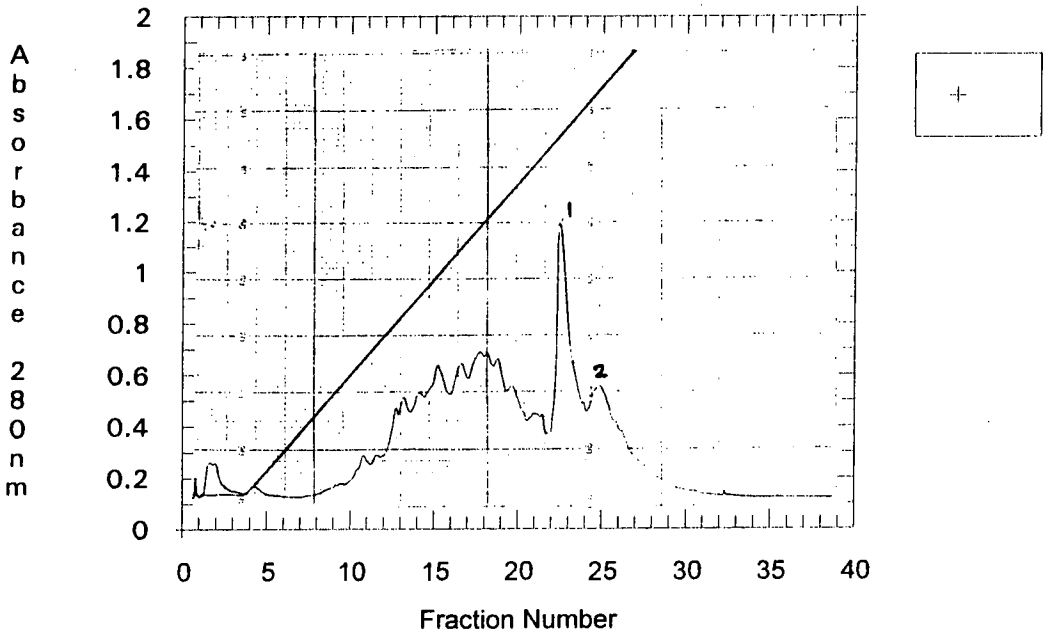


Fig.3.8 MonoQ-sepharose anion exchange chromatography of pooled active fractions from DEAE column. Fractions were analysed for cysteine desulphurase activity under standard [^{14}C]-L-cysteine assay conditions.

Active cysteine desulphurase eluted between 380 and 400mM NaCl concentration.

The specific activity of *E.coli* cysteine desulphurase was monitored at each stage of the purification (**Fig.3.9**) and protein content was analysed by Bradford assay and SDS gel electrophoresis (**Fig.3.10**).

Procedure	Total Protein (mg)	Total Activity (nmol[^{14}C]-ala h^{-1})	Specific Activity (nmol[^{14}C]-ala h^{-1} mg protein^{-1})
crude DH1i/pIMSB cell-free extract	306	161.3	0.53
20-50% $(NH_4)_2SO_4$	120	96	0.80
DEAE-cellulose	7.5	9.7	1.28
Mono Q	1	1.67	1.67

Fig.3.9 The purification of *E.coli* cysteine desulphurase. Incubations were carried under standard assay conditions with [^{14}C]-L-cysteine (0.2μCi, 291.3mCi/mmol), L-cysteine (200μM) and DTT (2mM).

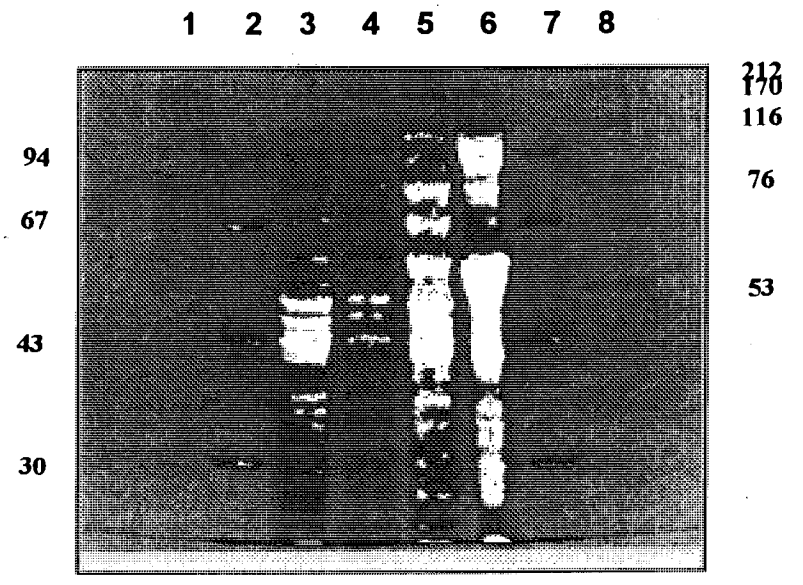


Fig.3.10 7.5% SDS-PAGE analysis of each stage in the purification of *E.coli* cysteine desulphurase. Lanes **3-6** show crude DH1i/pIMSB cell-free extract, 20-50% ammonium sulphate fraction, DEAE-cellulose fraction and Mono Q fraction respectively. Low and High molecular weight standards are shown in kilodaltons.

This analysis showed the acculmulation of two major soluble polypeptides **A** and **B** of approximate molecular weight 55 and 90 kDa respectively. SDS-PAGE of peak **2** from the chromatographic separation of proteins on the Mono Q anion exchange column showed that the major band was the 55kDa protein. This fraction displayed significantly lower cysteine desulphurase activity on comparison with the fractions from peak 1. From this, the 90kDa protein was tentatively assigned as *E.coli* cysteine desulphurase.

Incubation of DH1i/pIMSB cell-free extract with the MonoQ protein concentrate and the necessary cofactors did not result in an increase in biotin-forming activity. This suggests that either cysteine desulphurase is not intimately involved in the biotin synthase reaction or that other unidentified factors in the reaction are rate-limiting. It is clear that an active purified system is neccesary before the individual stages in this enzymic transformation can be elucidated.

However, a role for cysteine desulphurase in the biotin synthase reaction can be justified by considering a number of observations already encountered.

Incorporation experiments with *bio*B transformants of both *E.coli*[93] and *B.sphaericus*[92] have shown the production of labelled biotin upon incubation with [^{35}S]-L-cysteine. Previous studies have indicated that the sulphide present in iron-sulphur proteins from *E.coli* likely originates from cysteine sulphur[153]. Also, Takahashi *et al.* have observed incorporation of radiolabel from [^{35}S]-L-cysteine into the Fe-S cluster of [2Fe-2S] ferredoxin from spinach chloroplasts[154].

EPR studies on purified *bio*B gene product indicates that the [2Fe-2S] cluster is highly unstable in the reduced state[119]. The possibility that the sulphur added to dethiobiotin comes from the Fe-S cluster may suggest that its instability is associated with the loss of sulphur from the cluster

during catalysis. If the sulphur does come from the cluster, then an integral part in enzyme turnover would be cluster resynthesis.

By analogy with the *nif*S gene product, it is plausible that *E.coli* cysteine desulphurase is involved in the synthesis of *bio*B gene product Fe-S cluster.

Important progress has been made over recent years in the field of iron-sulphur proteins[155] but little is still known about the assembly of iron-sulphur clusters within the cell and their insertion into the various apoproteins. Attempts to reconstitute several iron-sulphur proteins by chemical methods have been more or less successful[156] but because of the toxic nature of the reagents employed[157] and the non-specific chemical reactions[158] involved, the physiological relevance of such models is doubtful.

Evidence has been reported on the possible involvement of sulphurtransferases in the biosynthesis of iron-sulphur clusters. 3-Mercaptopyruvate sulphurtransferase activity was observed in both mitochondria and cytosol, but its involvement in the *in vitro* formation of the iron-sulphur cluster of adrenodoxin requires cysteine transaminase activity which is only present in the cytosol[159]. Rhodanese (thiosulphate-cyanide sulphurtransferase) activity has been detected in mitochondrial fractions and has been implicated in metallocluster biosynthesis by its ability to reconstitute the chromophore of apoferredoxin *in vitro*[160]. However, the role of rhodanese in Fe-S cluster formation has been argued against because rhodanese has not been detected in appreciable levels in certain bacteria known to contain nonheme iron-sulphur proteins. Moreover, any enzyme that catalyses the formation of sulphide could be expected to assist reconstitution of apoproteins *in vitro* because such reconstitution occurs spontaneously, provided that sufficient sulphide and iron are available[161].

Genes present in nonnitrogen fixing organisms such as *Lactobacillus delbrueckii*[162], *Bacillus subtilis*[163], *Saccharomyces cerevisiae*[162] and *Candida maltosa*[162] have been shown to have extraordinary sequence identity when compared to *nif*S. Although the specific functions of the *nif*S-like genes from these organisms are not yet established, the results shown here support the view that cysteine desulphurase activity may represent a universal mechanism for the activation of sulphur required for metallocluster biosynthesis.

3.4 Conclusion

Upon establishing an active *in vitro* system for the conversion of dethiobiotin to biotin, the mechanism of sulphur donation was investigated. [^{14}C]-L-cysteine was found to be converted to [^{14}C]-L-alanine upon incubation with DH1i/pIMSB cell-free extract. Enzymatic inhibition of [^{14}C]-alanine formation with L-allylglycine suggests the enzyme responsible for this transformation uses PLP chemistry in its action.

Partial purification of the *E.coli* cysteine desulphurase and analysis by SDS-PAGE showed the accumulation of two soluble polypeptides of molecular weight 55 and 90kDa . The latter protein was thought to be the cysteine desulphurase on the basis that purified extracts containly mostly 55kDa protein exhibited significantly less desulphurase activity.

This previously unreported enzyme has precedent in cysteine desulphurase from *Azotobacter vinelandii* which is thought to be involved in nitrogenase metallocluster biosynthesis.

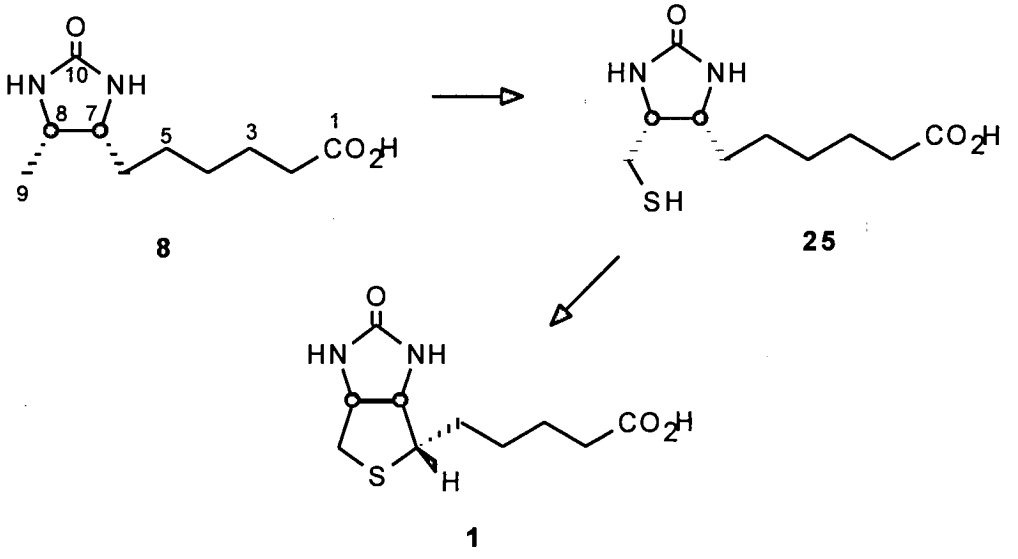
Although it is unknown whether *E.coli* cysteine desulphurase is involved in the bioconversion of dethiobiotin to biotin, analogy with the *nif*S gene product and the discovery of *nif*S-like genes from other nonnitrogen fixing organisms suggest that this enzyme may be involved in the sequestering of sulphur required for Fe-S cluster formation in metalloproteins.

Chapter 4

The Mechanism of Biotin Synthase-Synthetic Approaches to Labelled Analogues of Dethiobiotin

4.1 Introduction

The final step in the biosynthesis of biotin, which involves the insertion of sulphur into the saturated C-H bonds at C-6 and C-9 of dethiobiotin, is perhaps the most interesting and unusual stage in the synthesis of the coenzyme. The lack of chemical precedent and hence the mechanistic novelty of this transformation has stimulated an active area of research. Recent investigations in *E.coli*[115] and *B.sphaericus*[116] have implicated an intermediary role for 9-mercaptodethiobiotin **25** in the biosynthesis of biotin (**Scheme 4.1**) and feeding studies[118] with [^{3}H]-pimelic acid in *Lavandula vera* resulted in the formation of a compound which co-chromatographed with an authentic sample of 9-mercaptodethiobiotin. However, this putative precursor has yet to be isolated and characterised in bacterial cell extracts.

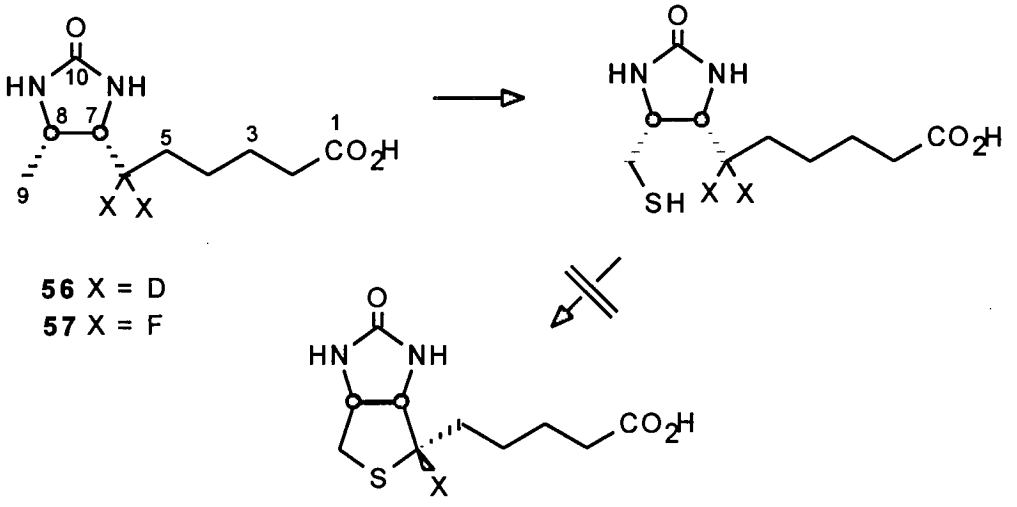


Scheme 4.1

One of the aims of this thesis was to investigate the mechanistic aspects of biotin biosynthesis *in vitro* through the synthesis of substrate analogues of dethiobiotin specifically labelled at the C-6 position.

Administration of these aberrant analogues to *bio*B transformants should retard abstration at C-6 and inhibit ring closure (**Scheme 4.2**).

Identification of the enzymic products of these slow arrest/trapping experiments would hopefully provide an insight into the mechanism associated with this transformation.

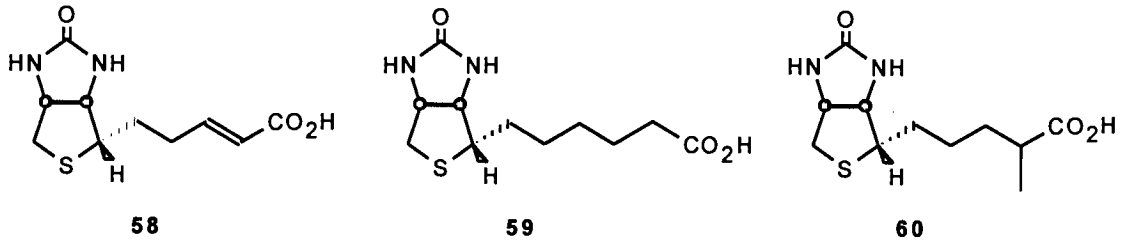


56 X = D
57 X = F

Scheme 4.2

The scope of these analogues is not only restricted to mechanistic studies. Biotin is essential for the production of cell membrane lipids in all organisms and is synthesised in limited amounts by plants and microorganisms but not animals. Thus analogues of biotin biosynthetic precursors which inhibit the enzymes of its biosynthesis should have potential value as microbial and agrochemical biocides. Some derivatives of biotin precursors have already been shown to exhibit anti-microbial activity (**Fig.4.1**).

α -Dehydrodethiobiotin **58** represses transcription of both l and r strands of the biotin operon[164,165] while homobiotin **59** and α-methylbiotin **60** also exhibit weak co-ordinate repression of transcription[165]. Their mode of action appears to be as pseudo-substrates of *bir*A where they function as corepressors of *bio* gene expression.

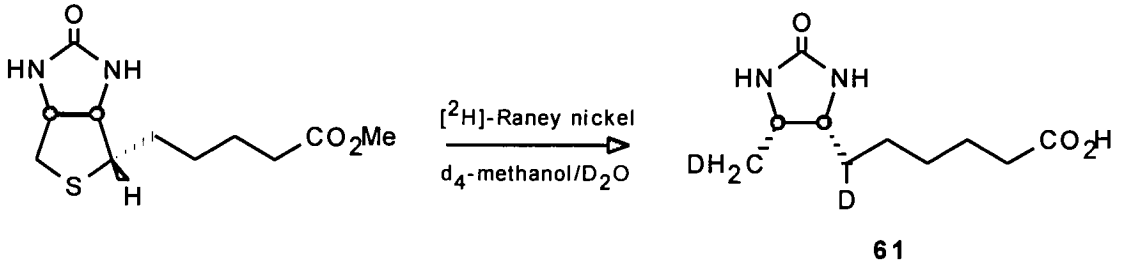


58 59 60

Fig. 4.1 Anti-metabolites of biotin biosynthesis

4.1 The Synthesis of [6,9-$^2H_2$]- (+)-dethiobiotin

The use of Raney nickel in the hydrodesulphurisation of sulphur compounds is well documented[166] and was employed in the structure elucidation of biotin by du Vigneaud *et al.*[2-5] The desulphurisation process proceeds with retention of configuration at both chiral centres in dethiobiotin. Despite extensive research[167], the mechanism of desulphurisation reactions at metal surfaces still remains poorly understood.



61

Fig. 4.2 The deuterodesulphurisation of (+)-biotin methyl ester

Initial studies began with the synthesis of [6,9-$^2H_2$]-dethiobiotin **61** from biotin using [^{2}H]-Raney nickel (**Fig.4.2**). Preparation of the deuterated catalyst was achieved by the method of Mozingo[168] using nickel-

aluminium alloy and sodium deuteroxide in D_2O. Analysis of the labelled product by ^1H nmr (360 MHz) revealed, on comparison with dethiobiotin, a change in the C-9 methyl, C-6 methylene and bridgehead H-7 and H-8 multiplicity. On insertion of deuterium at C-9, the two hydrogen atoms of the methylene group become stereochemically non-equivalent. A change in the complexity and integral was also observed in the unresolved multiplet at δ 1.46-1.54 indicating the introduction of deuterium at C-6. The deuterodesulphurisation of biotin also resulted in changes in the multiplicities of the H-7 and H-8 methine resonances..

Spin decoupling experiments augmented these initial observations. Irradiation of the H-8 methine resonance resulted in collapse of the H-7 methine signal at δ 3.77 to a doublet. This was accompanied by collapse of the C-9 methylene signal to a singlet. The presence of an additional resonance observed as a shoulder on the C-9 CH_2 signal indicated however that the methylene group might be diastereotopic. Irradiation of the H-7 methine signal led to collapse of the H-8 methine multiplet at δ 3.90 to an unresolved multiplet and small changes in the multiplet of the C-3 and C-6 methylene resonances at δ 1.46-1.60. The ^{13}C DEPT spectrum provided additional evidence for the introduction of two deuterium atoms at C-6 and C-9 of dethiobiotin (**Fig.4.3**). Spectral comparison with dethiobiotin (**a**) revealed the disappearance of the positive C-9 methyl singlet at δ 14.16 and the appearance of a negative triplet at δ 13.89 (**b**).

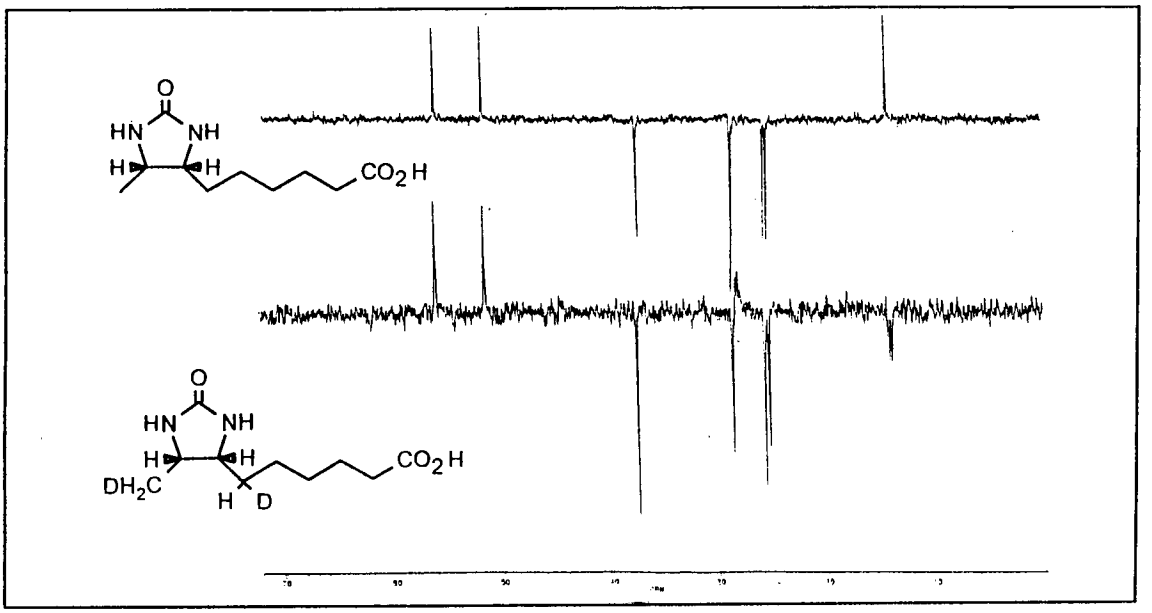


Fig.4.3 ^{13}C DEPT (3Π/4) nmr spectra of **a**) dethiobiotin and **b**) [6,9-$^{2}H_2$]-dethiobiotin

The shift to lower frequency in the C-6 signal of [6,9-$^{2}H^{2}$]-dethiobiotin is the result of a deuterium isotope effect. As observed, the α-shift is in the order of 0.27ppm per deuterium and 0.1 ppm for the β-shift. The appearance of a positive triplet resonance at 27.86 ppm verified the introduction of deuterium at C-6.

Analysis of the ^{2}H-nmr spectrum of **61** gave supporting evidence for the regiospecific incorporation of the label. Strong resonances at δ 1.04 and δ 1.50 indicated the direct introduction of deuterium atoms into C-9 and C-6 respectively. The smaller signals at δ 3.89 and δ 3.78 can be attributed to bridgehead proton exchange. The latter signal was of lower intensity to that of the H-8 resonance and the lower incorporation of ^{2}H at C-7 is probably due to steric hindrance at the H-7 position.

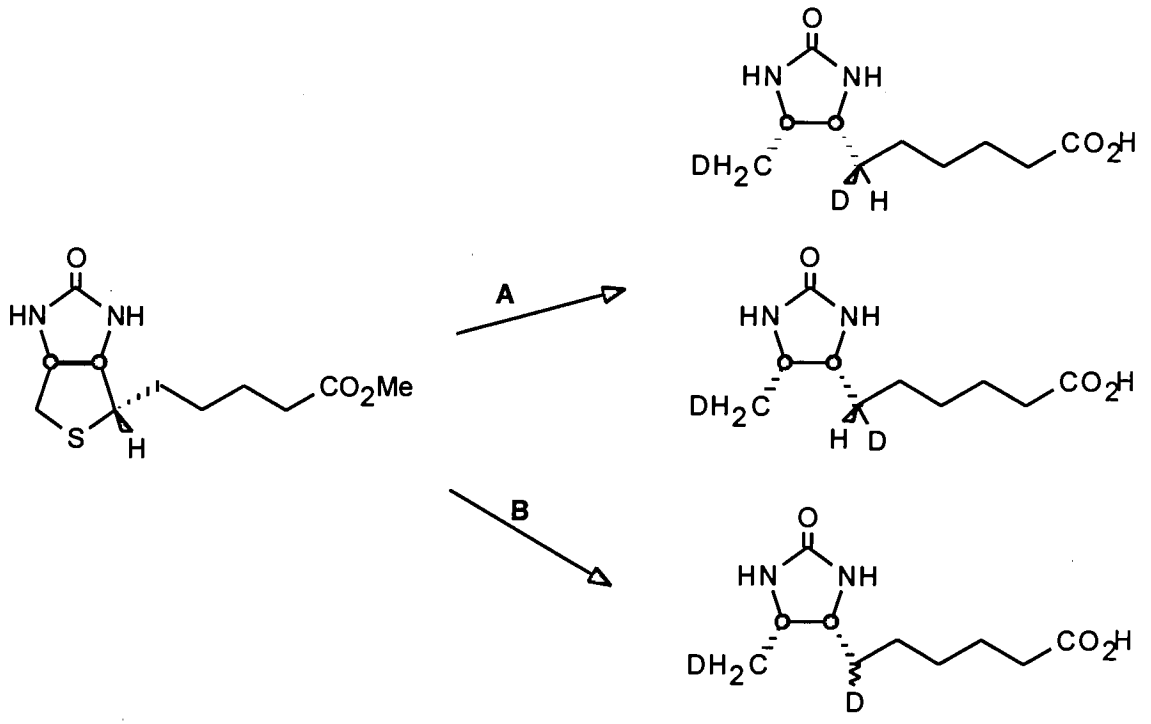


Fig 4.4. Deuterodesulphurisation of (+)-biotin methyl ester with **A** retention/inversion or **B** loss of chirality

Further ^{1}H nmr analysis was required to establish whether chirality was retained at C-6 after deuterodesulphurisation of biotin.

Since the methylene protons at C-6 in dethiobiotin are diasterotopic, we would expect the individual proton resonances to occur at different chemical shifts. Analysis of proton dispersion, signal and integral intensity of the labelled compound, in conjunction with spin decoupling experiments, should indicate whether retention of chirality, inversion or racemisation has occurred at that site (**Fig.4.4**).

Initial ^{1}H nmr studies on dethiobiotin methyl ester at 200MHz showed no clear dispersion of the C-6 methylene protons. The methylene signals of interest were hidden under the unresolved multiplet at δ 1.44-1.60 so a variety of lanthanide shift reagent (LSR) titration experiments were performed.

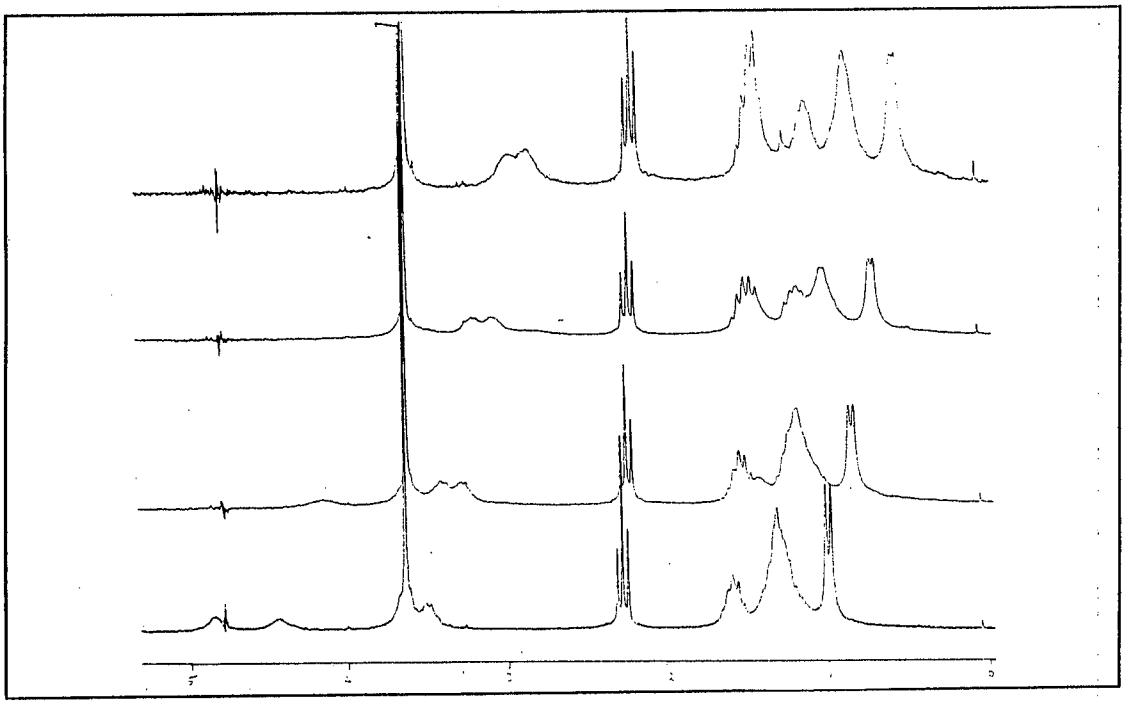


Fig.4.5 ^1H nmr spectrum of titration of $Pr(FOD)_3$ with dethiobiotin methyl ester

Titration of $Pr(FOD)_3$ with dethiobiotin methyl ester did produce a shift effect in the correct region of the spectrum however the accompanying line broadening prevented the use of spin-decoupling for proton assignment (**Fig.4.5**).

Distinction between the 6-*proS* and 6-*proR* signals in the spectrum of **61** was however apparent at 600MHz. Low resolution COSY of the aliphatic region (δ 0-4.3) displayed a cross peak at δ 1.49 which corresponded to a coupling assignment between the H-7 bridgehead proton (δ 3.70) and the C-6 methine proton. Examination of unlabelled dethiobiotin methyl ester revealed resolution in the region δ 1.42-1.54 (**Fig.4.6**) with two proton integration and this multiplet was attributed to the C-6 methylene group.

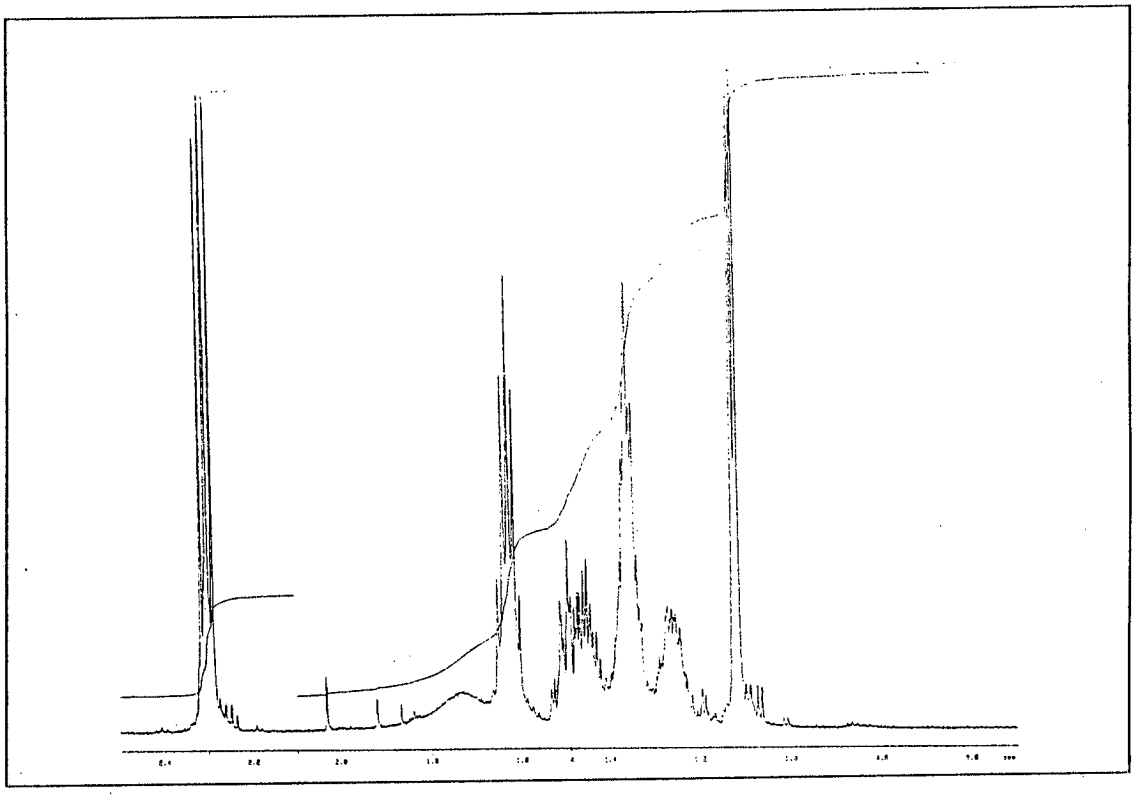


Fig. 4.6 ^{1}H nmr spectrum of (+)-dethiobiotin methyl ester at 600MHz

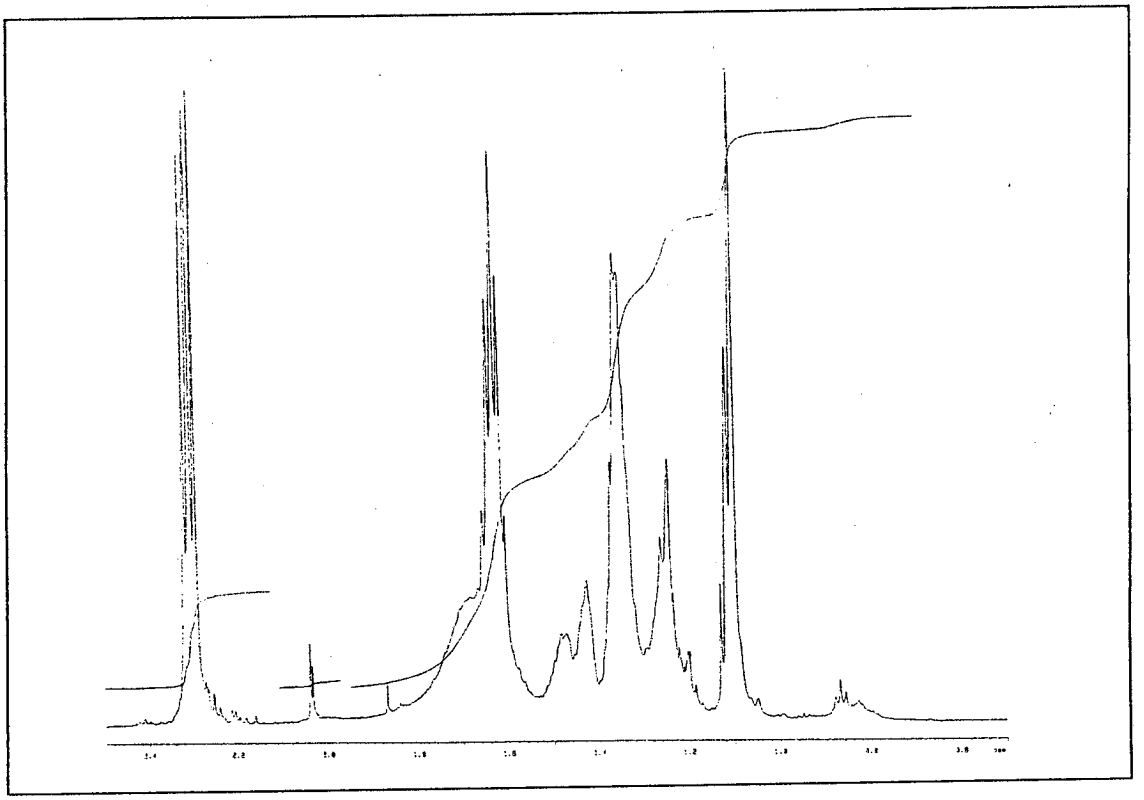


Fig. 4.7 ^{1}H nmr spectrum of [6,9-$^{2}H_2$]-(+)-dethiobiotin methyl ester at 600 MHz

On examination of the ^1H nmr spectrum of [6,9-$^2H_2$]-dethiobiotin methyl ester (**Fig.4.7**) the multiplet at δ 1.42-1.52 was found to be of reduced intensity and both regions of this multiplet integrated for approximately 0.5H. This observation indicated racemisation at C-6 had occurred and so excluded the use of this compound in future biological experiments.

4.2 Approaches towards the synthesis of [6,6-$^2H_2$]- and [6,6-F_2]-(±)-dethiobiotins

The lack of stereospecificity in the introduction of deuterium at C-6 of dethiobiotin using ^2H-Raney nickel suggested that a better approach would be to construct dethiobiotin precursors geminally labelled at the 6 position. Introduction of two ^2H's at C-6 would circumvent any problems of racemisation in the synthesis. Additionally, the 6,6-dideutero- and 6,6-difluorodethiobiotins were targetted for synthesis.

The latter analogue is of particular relevance since fluorinated derivatives of biological molecules frequently have dramatic effects on enzymatic reactions. The van der Waals radius of the fluorine atom (1.35Å) is closest to that of the hydrogen atom (1.20Å) while the C-F and C-H bond lengths (1.26-1.41Å compared to 1.08-1.11Å) are approximately the same. The substitution of fluorine for hydrogen does not dramatically alter the steric environment in the molecule thus allowing fluorinated precursors to mimic the metabolic fate of the parent hydrogen compounds[169]. The increase in bond strength associated with the substitution of hydrogen for fluorine creates a substrate resistant to metabolic transformations which require manipulation of the C-F bond[170]. The large difference in electronegativity between fluorine (4.0) and hydrogen (2.1) translates into a substantial elctronic effect on nearby carbon centres. In addition, C-F bonds substantially increase the

lipophilicities of fluorinated precursor analogues. The combination of these effects has firmly established the use of fluorine-containing analogues of biological intermediates in the pharmaceutical and agrochemical industry.

Biotin and its derivatives have provided an attractive target for total synthesis. The first total synthesis of biotin was acheived by Harris *et al.*[6] and since then a large number of syntheses have been reported by numerous research groups[11].

Novel synthetic approaches have included the use of carbohydrate[171], aromatic[172] and aliphatic[173] precursors while photochemical[174] and cycloaddition[175] methodology has been utilised to maintain enantioselectivity at biotin's 3 chiral centres. However , few of these approaches are applicable to synthesis requiring label incorporation at C-6 of dethiobiotin.

Retrosynthetic analysis of the proposed compounds suggested they should be available through manipulation of the 6-keto moeity of **62**.(**Fig.4.8**) This product is available from a Friedel-Crafts acylation of 4-methyl-4-imidazolin-2-one **39**.

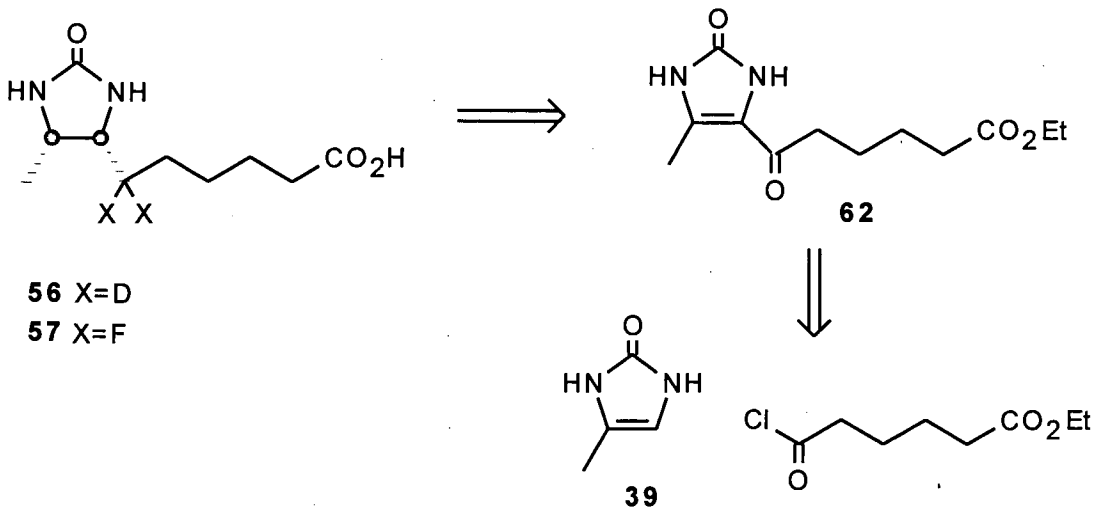
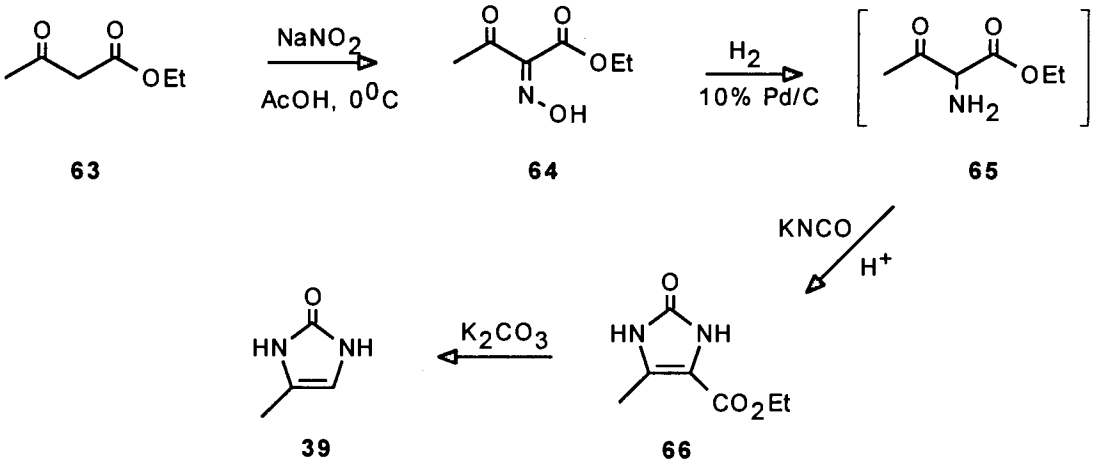


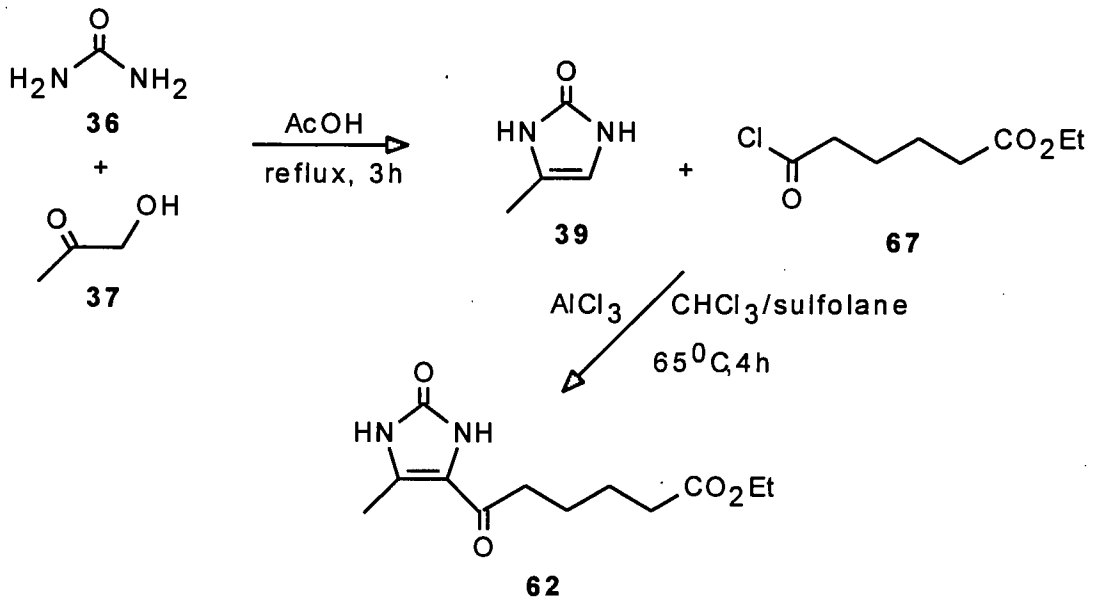
Fig.4.8 Retrosynthetic analysis of target compounds

Previous routes[176] to 4-methyl-4-imidazolin-2-one have utilised ethyl acetoacetate **63** as the starting material (**Scheme** 4.3). The route proceeds through fomation of the α-oxime of ethyl acetoacetate **64** by the addition of sodium nitrite in glacial acetic acid. Catalytic hydrogenation to the intermediate amine **65**, and treatment with an acidic solution of potassium isocyanate affords the cyclised product 4-methyl-5-ethoxycarbonyl-2-imidazolinone **66**. Subsequent saponification and decarboxylation gives the desired compound **39** in approximately 20 % overall yield.



Scheme 4.3

The construction of the ureido ring skeleton by this procedure appeared time-consuming and necessitated refinement. Interest[177] in the cardiotonic properties of 4-methyl-(5-substituted benzoyl)-imidazolin-2-one derivatives has resulted in a recent one-step synthesis of 4-methyl-4-imidazolin-2-one by Andrews[131] and this procedure was employed here.



Scheme 4.4

The condensation of acetol **37** and urea **36** in glacial acetic acid gave a dark coloured residue which, on extraction and crystallisation from water, afforded the cyclised product 4-methyl-4-imidazolin-2-one **39** in 25% yield.

Friedel-Crafts acylation of **39** with ethyl 5-chloroformyl pentanoate **67** using the method of Duschinsky and Dolan[178] gave 6-keto-dehydrodethiobiotin ethyl ester **62** in 52% from the starting imidazolinone.

Duschinsky and Dolan[178] have investigated the hydrogenation of **62** to dethiobiotin ethyl ester and found two distinct steps in the reduction of the α,β-unsaturated ketone. Hydrogenation of 6-keto-dehydrodethiobiotin ethyl ester with Adams catalyst (PtO_2) resulted in rapid uptake of 2 moles of hydrogen and reduction of the 6-keto moeity to a methylene group. This was confirmed through the isolation of dehydrodethiobiotin ethyl ester. A third mole of hydrogen was consumed more slowly to give dethiobiotin ethyl ester in good yield.

We reasoned that the reduction of the 6-keto group could be performed with deuterium in place of hydrogen and this would provide a rapid and effective method for introduction of a geminal label into the 6-position of dethiobiotin.

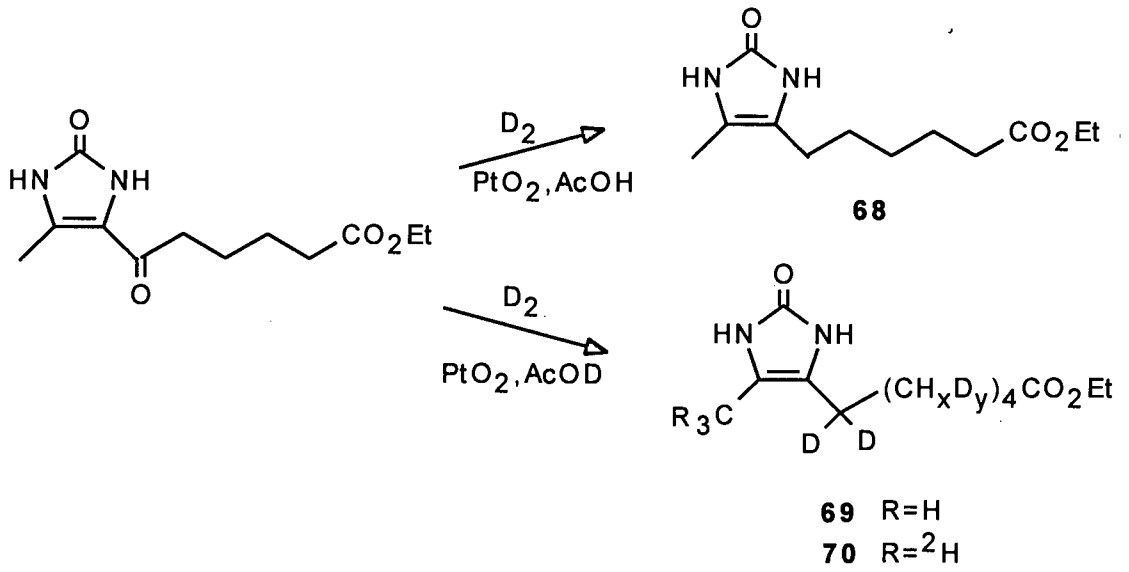


Fig. 4.9 Deuterogenolysis of 6-keto-dehydrodethiobiotin ethyl ester

The reduction of 6-keto-dehydrodethiobiotin ethyl ester was accomplished over pre-reduced Adams catalyst in glacial acetic acid under an atmosphere of deuterium at 25°C. After 3 hours, two molar equivalents of deuterium had been consumed and crystallisation from ethanol afforded a white microcrystalline solid which co-chromatographed with authentic **68**.

The ^{13}C DEPT nmr spectrum of this product displayed signals at 116.92 and 112.59 ppm corresponding to the vinyl carbons at C-8 and C-7 respectively. This indicated the imidazolinone double bond remained unreduced. However the disappearance of the α,β-unsaturated carbonyl resonance at 188.4 ppm, which was associated with reduction of the 6-keto group, resulted in the appearance of a methylene signal at 33.11 ppm. This indicated the formation of undeuterated dehydrodethiobiotin

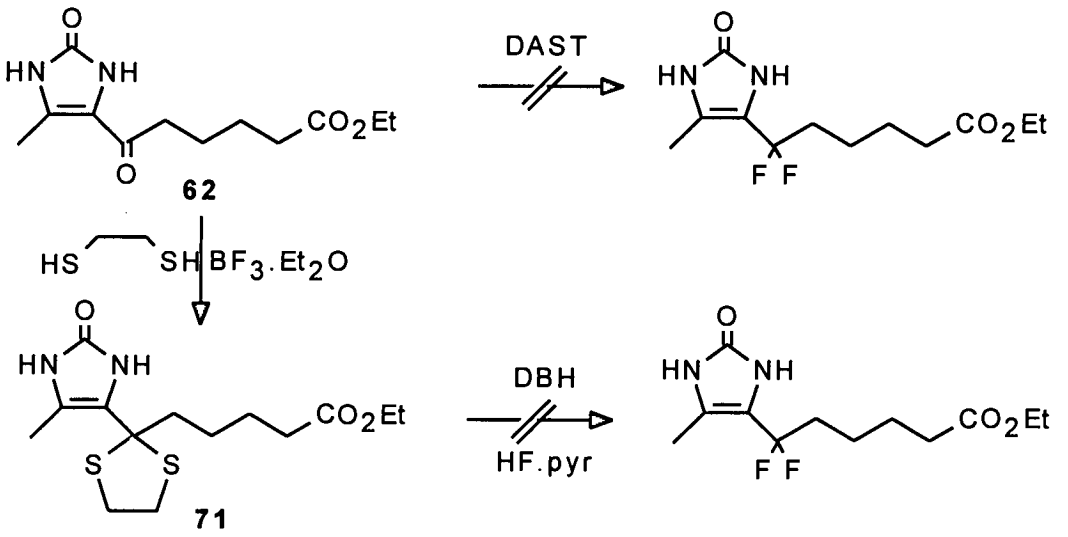
ethyl ester **68** and this was attributed to the use of non-deuterated solvent in the reaction (**Fig.4.9**).

The reaction was repeated in d-acetic acid under similar conditions and the product isolated upon the uptake of moles of two moles of deuterium. However, the spectral properties of the reduced material proved anomalous. ^{1}H nmr of the product revealed the disappearance of the singlet at δ 2.02 associated with the allylic methyl at C-9. Moreover, the multiplet at δ 1.67, attributed to the resonance of 4 methylene protons from the aliphatic side chain, only integrated for 2.5 protons. The presence of the imidazolinone double bond and reduction of the ketone was confimed by ^{13}C DEPT nmr but of the 5 methylene signals in the spectrum, the resonances at 27.85 ppm and 27.96 ppm were of reduced intensity relative to those of dehydrodethiobiotin ethyl ester. These preliminary observations suggested deuterogenolysis of the 6-keto group was successful but involved complete exchange of the C-9 methyl protons and partial exchange of the side chain protons as a consequence (**Fig.2.9**). ^{2}H nmr and FAB mass spectrometry supported this non-specific distribution of deuterium and this method of label incorporation was abandoned in the hope a more regiospecific alternative could be found.

Attention was turned to the preparation of 6,6-difluorodethiobiotin. The *gem*-difluoro group is prepared, most frequently, from the corresponding ketone or aldehyde. However, this approach is often limited by the inconvenience of obtaining and using the reagents required for this conversion. Sulphur tetrafluoride with hydrogen fluoride as a catalyst, the classical method for achieving this transformation[179], generally provides good yields of the desired product, but its use requires extreme caution because of its toxicity, reactivity and volatility. Pressure equipment constructed from fluorine-resistant material such as stainless steel bombs must be used[180], and high temperatures are frequently

necessary. Diethylaminosulphur trifluoride (DAST) has provided a milder alternative to SF_4 as a fluorinating agent[181].

Attempted conversion of 6-keto-dehydrodethiobiotin ethyl ester into the corresponding difluoromethylene compound with DAST was unsuccessful under a variety of conditions (up to 48 hours in neat DAST at reflux) affording only unreacted starting material (**Scheme 4.5**).



Scheme 4.5

Sondej and Katzenellenbogen[182] have reported that *gem*-difluoro compounds can be prepared from ketones by formation of the corresponding 1,3-dithiolane, followed by reaction with 1,3-dibromo-5,5-dimethylhydantoin and pyridinium poly(hydrogen fluoride).

Reaction of **62** with 1,2-ethanedithiol and boron trifluoride etherate as Lewis acid gave 6-dithioketal-dehydrodethiobiotin ethyl ester **71** in 85% yield. Reaction of this compound with pyridinium poly(hydrogen fluoride) and a variety of N-halo oxidants resulted in consumption of starting

material but analysis of the reaction products by ^{19}F nmr revealed no incorporation of fluorine into the molecule. Most fluorinations in the literature utilising carbonyl transformation chemistry involve isolated ketones which may be more electrophilic towards fluoride attack than the conjugated systems in **62** and **71**.

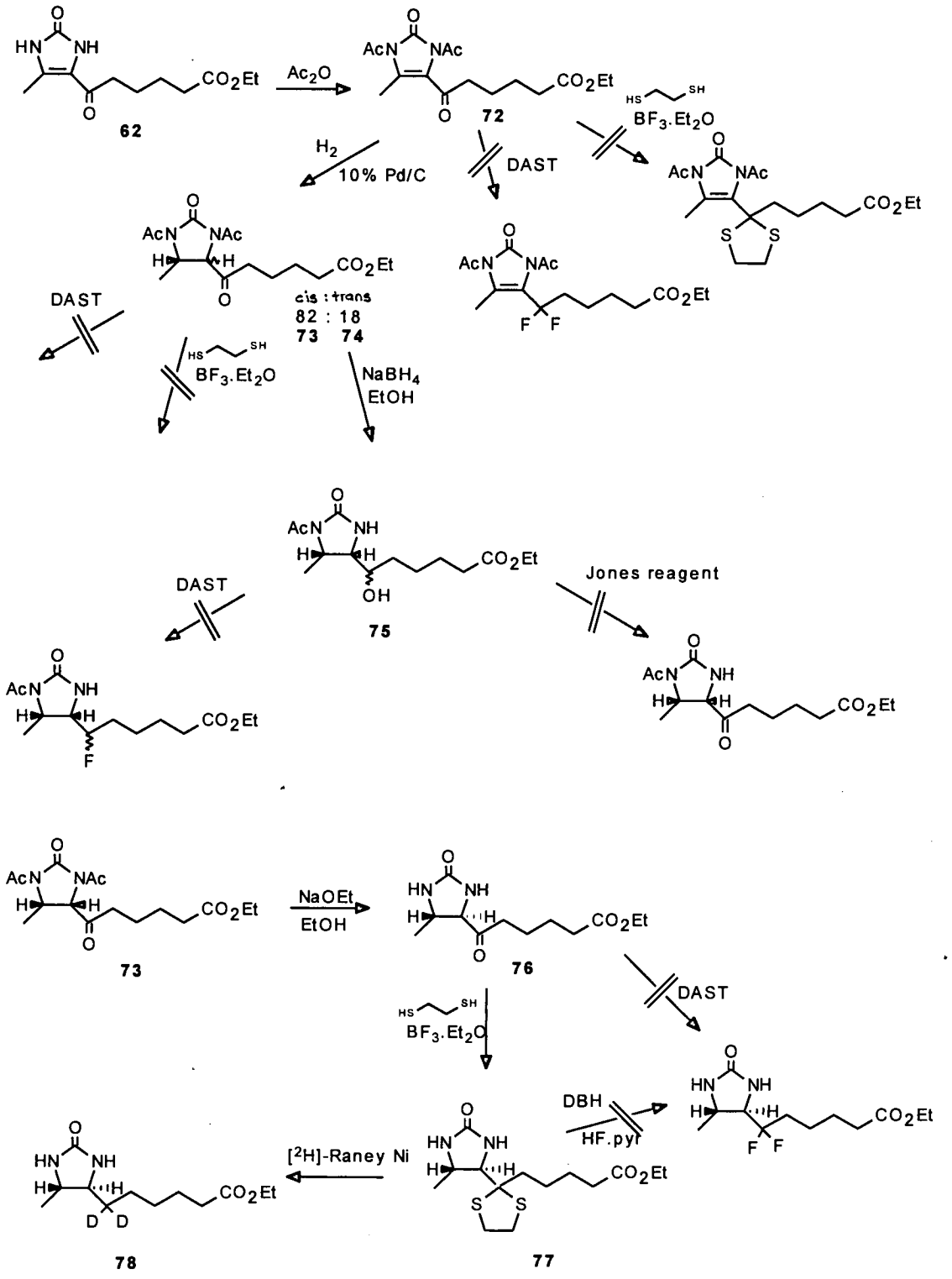
It has been shown that derivatisation of the imidazolinone nitrogens can render the double bond susceptible to selective hydrogenation with respect to the 6-keto functionality[110]. Acetylation of **62** with acetic anhydride and crystallisation from aqueous ethanol yielded 6-keto-N,N'-diacetyl-dehydrodethiobiotin ethyl ester **72** in 82% yield. Catalytic hydrogenation of **72** at room temperature and pressure over 10% palladium on charcoal in 1,4-dioxan produced a mixture of *cis* and *trans* 6-keto-N,N'-diacetyl-(±)-dethiobiotin ethyl esters (82:18) **73** and **74** which were separated by dry flash chromatography.(**Scheme 4.6**)

^{1}H nmr analysis of the *cis* ketone **73** showed the doublet of the H-7 proton was shifted to higher frequency by 0.5 ppm relative to that of the *trans* isomer and displayed the expected larger *cis* coupling constant of 9.8 Hz. The coupling constant between the 9-methyl group and the H-8 methine proton ($J_{9,8}$=6.5Hz) is similar in both **73** and **74**.

Perversely 6-keto-N,N'-diacetyl-(±)-dethiobiotin ethyl ester was also resistant to fluorination with DAST and attempts at dithioketalisation proved abortive. The reluctance of **73** to form the corresponding 6-dithioketal compound can be attributed to the N'-acetyl group which can sterically hinder the 6-keto moeity and prevent reaction. However, removal of the acetyl groups to decrease this interaction was not practical since deacetylation with acid or base resulted in epimerisation at C-7 of **73** to give the more thermodynamically stable *trans* isomer **76**. However, Marquet[110] had noted that the reduction of the ketone **73** with $NaBH_4$ to afford the N,N'-diacetyl alcohol also gave the N-acetyl alcohol **75** as a side

product so we decided to use this procedure as a method of selective deacetylation. Optimisation of the reaction conditions gave only the N-acetyl alcohol, 6-(*R,S*)-hydroxy-N-acetyl-(±)-dethiobiotin ethyl ester **75**, as the principal product (**Scheme 4.6**).

Surprisingly, attempts to convert the secondary alcohol into the corresponding ketone even with strong oxidising agents such as Jones reagent, $KMnO_4$ and MnO_2 proved futile. The compound was equally resistant to conversion to the monofluoro derivative with DAST. Model studies[183] indicate that strong NH-OH intramolecular hydrogen bonding can occur in **75**. The broad absorptions at 3460 and 3240 cm^{-1} in the IR spectrum of this compound and its resistance to oxidation imply that such may be the case. To test the rationale behind these observations we removed the acetyl groups from **73** with sodium ethoxide and this resulted in the expected epimerisation at C-7 to yield 6-keto-*trans*-(±)-dethiobiotin ethyl ester **76**. This was easily converted to its dithioketal derivative **77** by treatment with 1,2-ethanedithiol and $BF_3.Et_2O$. Although unreactive to oxidative fluorodesulphurisation using the conditions described earlier, this compound underwent deuterodesulphurisation using [^{2}H]-Raney nickel resulting in formation of [6,6-$^2H_2$]-*trans*-(±)-dethiobiotin ethyl ester **78**. The structure of **78** was confirmed by ^{1}H and ^{13}C DEPT nmr in conjunction with FAB mass spectrometry.


62
72
DAST
H_2
10% Pd/C
$BF_3.Et_2O$
cis : trans
82 : 18
73 74
$NaBH_4$
EtOH
75
Jones reagent
73
NaOEt
EtOH
76
DBH
HF.pyr
[2H]-Raney Ni
77
78

Scheme 4.6

4.3 Conclusion

Both [6,6-$^2H_2$]-(±)-dethiobiotin and [6,6-F_2]-(±)-dethiobiotin have proved difficult targets for synthesis using the methodology adopted here. The synthesis of labelled dethiobiotin using deuterated Raney nickel and the observation that the introduction of deuterium involves racemisation at C-6 of dethiobiotin supports the current hypothesis that Raney nickel reactions, although still in doubt, probably occur by a free-radical mechanism[184].

It is clear from these studies that carbonyl transformation chemistry is not suited for the introduction of fluorine into these molecules while non-specific distribution of label or epimerisation has impeded formation of labelled dethiobiotin with the correct sterochemistry for biological evaluation. Alternative strategies which can circumvent these problems will be required for the eventual synthesis of these specifically labelled probes.

Chapter 5

Addendum and Suggestions for Future Work

5.0 Addendum and Suggestions for Future Work

After completion of this work, Birch and her co-workers at Lonza AG reported their investigation into the low molecular weight and protein components required for *in vitro* activity of the biotin synthase complex from *E.coli*[185]. They found that cofactor requirements included NADPH, Fe^{2+}, AdoMet, L-cysteine and high concentrations of one of the amino acids asparagine, aspartate, glutamine or serine. They also found that an unidentified thiamine pyrophosphate (TPP)-dependent protein is required in addition to the *bio*B gene product, flavodoxin and ferredoxin (flavodoxin) $NADP^+$ reductase for activity. The role of TPP and the amino acids other than cysteine is unknown. The role of TPP is noteworthy since TPP is normally associated with decarboxylation of α-ketoacids (e.g pyruvate in pyruvate dehydrogenase, pyruvate decarboxylase and acetolactate synthase). TPP seems to be required to maintain the activity of one of the proteins involved in the biotin synthase reaction although the proteins function is unknown. Here, it may be that TPP acts as an allosteric agent for one of the enzymes involved in the reaction. For optimal activity in the assays *in vitro*, the amino acids are required at concentrations above those naturally found in *E.coli* cells, and Birch *et al.* suggest that they may be directly involved in the reaction *in vitro*, or may have a regulator role.

An alternative argument could be that high concentrations of an amino acid help to protect the protein complex either specifically (through an allosteric effect) or merely as a buffer component.

Future work must involve the purification of *E.coli* cysteine desulphurase and determining its role (if any) in the biotin synthase reaction. Location and sequencing of the gene encoding this protein may provide an insight into its function in *E.coli* and perhaps its existence in other non-nitrogen fixing organisms. It is interesting to speculate whether TPP acts

allosterically and it should be relatively easy to determine if *E.coli* cysteine desulphurase and the TPP-dependant protein described above are one and the same.

The synthesis and characterisation of the modified DAPA analogues has now allowed the enzymatic synthesis of radiolabelled dethiobiotin analogues with DTBS and $H^{14}CO_3^-$ to be performed. This should ascertain whether sulphur introduction is affected by chain length in the biotin synthase reaction.

The dideutero- and difluorodethiobiotins have strong applications as selective biocides and further research into this area may lead to their eventual synthesis.

The current evidence suggests that biotin synthase can be included in a growing section of enzymes catalysing reactions by a radical process. However, it is clear we still in the early stages of understanding this unique transformation and only through the complete purification and characterisation of all the components of the biotin synthase system can the mechanism of sulphur insertion be elucidated.

Chapter 6

Experimental

EXPERIMENTAL

All starting materials were purchased from commercial sources (Sigma-Aldrich Inc., Fluka and Lancaster Synthesis unless otherwise stated) and used without further purification. All solvents used in chemical reactions were dried and distilled before use. Diazomethane was prepared as an ethereal solution fron Diazald by the procedure of De Boer and Backer[196] Merck silica 60 (70-230 mesh) was used for column chromatography and thin layer chromatography (tlc) was carried out on 200 x 200 x 0.2mm layers of Merck $60F_{254}$ silica. Components were visualised by UV, phosphomolybdic acid spray reagent or by exposure to iodine vapour. Biotin and its derivatives were identified on tlc by colour reaction with 0.2% N,N-dimethylaminocinnamaldehyde in 2% conc. sulphuric acid/ethanol[192]. Melting points were determined on a Reichert 7905 hot-stage apparatus and are uncorrected. IR spectra were recorded on a Biorad SP3200 FTIR spectrophotometer. Samples were prepared as liquid films or as nujol mulls unless otherwise stated. NMR spectra were recorded on Jeol PMX60, Bruker WP80, WP200, AX250, WM360 and Varian VXR600S spectrometers using tetramethylsilane (δ=0.0) as an external standard. Coupling constants (J) are given in Hz. Fast Atom Bombardment (FAB) mass spectra were measured using a Kratos MS 50 TC spectrometer and were carried out using either glycerol or thioglycerol as sample matrices.. Elemental analyses were determined using a Perkin Elmer 2400 elemental analyser. Radioactivity was determined as counts per minute (cpm) on a LKB 1212 RackBeta liquid scintillation counter using Optiphase Hi-Safe 3 liquid scintillant and ^{14}C-hexadecane (306.1dpm mg^{-1}) as an external standard. Sonication was performed using an M.S.E Soniprep 150 ultrasonic disintegrator. Protein gel electrophoresis was performed using a Bio-Rad Protein II apparatus (Hercules, CF, USA)

Biotin Methyl Ester **1a**

D-Biotin 1 (1.51g, 5.1mmol) was slurried in methanol (20ml). An ethereal solution of diazomethane was added until a green/yellow colour persisted. The ether was removed *in vacuo* to afford a white solid. Analysis by tlc (silica, methanol/chloroform (1:20)) indicated the reaction had gone to completion . Recrystallisation from methanol yielded biotin methyl ester (1.33g, 83%) as a white needles, m.p. 165-167°C (lit.[5] 165-167°C);

^{2}H-*Raney Nickel*

A solution of sodium deuteroxide (4g, 0.098mol) in D_2O (8ml) was placed in a beaker, stirring magnetically and cooled in an ice-bath to 10°C. Nickel-aluminium alloy (2g) was added in small portions with stirring so that the temperature did not rise above 20°C. The mixture was removed from ice and allowed to attain room temperature. The contents were then stirred until evolution of gas became slow (~5 hours). The supernatant liquid was then decanted and the nickel washed with D_2O until neutral pH. The nickel was transferred to a dry sample bottle and stored under 99.8 atom% D_2O.

[6,9-$^2H_2$]-(+)-*dethiobiotin* **61**

Biotin methyl ester **1a** (100mg, 0.39mmol) was dissolved in d_4-methanol (4ml) and heated under reflux with [^{2}H]-Raney nickel (1ml, ~0.6g W-2 suspension in D_2O, pH12) for 1 hour. Tlc (silica, 20% methanol/chloroform) of the reaction mixture indicated the reaction had gone to completion. The reaction mixture was filtered and the nickel washed with water (15ml). The filtrate and combined washings were acidified to pH4 with acetic acid and passed through an AG50 H^+ (x8) cation exchange column. Ethanol (12ml) was added to the eluate and the solvent removed *in vacuo* to give [6,9-$^2H_2$]-(±)-dethiobiotin **61** (43mg, 51%), δ_H (D_2O, 360MHz) 3.88 (1 H, m, H-8), 3.77 (1 H, m, H-7), 2.16 (2 H,

t, *J*7.4, -CH_2-CO_2H), 1.46-1.60 (3 H, m), 1.28-1.40 (4 H,m), 1.09 (2 H, m, *J*6.0, DCH_2-) ppm; δ_C (D_2O, DEPT) 55.82, 51.31, 37.33, 28.38, 27.85, 25.52, 25.10 and 13.89 ppm; δ_D (H_2O) 3.89, 3.78, 2.16,1.50, 1.34, 1.27 and 1.10 ppm

[6,9-$^2H_2$]-(+)-dethiobiotin methyl ester **61a**

This compound was prepared using a similar procedure to that described above using [^{2}H]-Raney nickel at pH7,

m/z (FAB , thioglycerol) 231.1677 [(M+1)$^+$, $C_{11}H_{19}N_2O_3{}^2H_2$ requires 231.1677])

4-*Methyl*-4-*imidazolin*-2-*one* **39**

To a solution of urea **36** (48g) in acetic acid (70ml) at 80°C was added acetol **37** (20.8g) and solid phosphomolybdic acid (10mg). The resulting reaction mixture was heated at reflux for 1.5 hour, then evaporated *in vacuo* until the weight was 95g, the residue taken up in water (95ml) and the pH adjusted to 8 with solid sodium carbonate. The basic solution was extracted with n-butanol (3 x 200ml) and the combined butanol extracts washed with sat.aq.NaCl solution (1x50ml). The organic extract was evaporated to 300ml and placed on ice overnight whereupon a colourless solid separated and was removed by filtration. The filtrate was evaporated to dryness, the residue taken up in hot water (10ml) and cooled to 0°C which resulted in crystallisation of the imidazolinone **39** (5.5g, 20%), m.p 204-208°C (lit.[131], 202-204°C); δ_H ([$^2H_6$]DMSO, 60MHz) 9.48 (2 H, br d), 1.83 (3 H, s), 5.90 (1 H, s) ppm

Ethyl 5-*chloroformylpentanoate* **67**

Adipic acid monoethyl ester (12.34g, 0.071mol) was heated at reflux in thionyl chloride (35ml) for 30 minutes. Excess thionyl chloride was removed by gentle heating (<40°C) of the mixture under reduced

pressure. The resulting product was purified by distillation under vacuum (0.3mm Hg) to afford ethyl 5-chloroformylpentanoate **67** as a clear yellow liquid (15.2g, 90%); b.p. 58°C (0.3mm Hg), δ_H ($CDCl_3$, 60 MHz) 4.20 (2 H, q, *J* 7.1), 2.98 (2 H, m), 2.38 (2 H, m), 1.75 (4 H, m), 1.30 (3 H, t, *J* 7.2) ppm; υ_{max} (film)/cm^{-1} 1797 and 1728.

6-*Keto*-7,8-*dehydrodethiobiotin ethyl ester* **62**

To a stirred solution of 4-methyl-4-imidazolin-2-one **39** (5g, 0.052mol) in dry $CHCl_3$ (30ml) and sulfolane (15ml) anhydrous aluminium trichloride (21g, 3xeq.) was added in aliquots held at ice temperature and under N_2. Ethyl 5-chloroformylpentanoate **67** (11.5g, 0.06mol, 1.15xeq.) was then added slowly. The reaction mixture was heated at 65°C under N_2 for 5 hours (until HCl evolution had ceased). Tlc analysis (silica, 20% methanol/ethyl acetate) indicated the reaction had gone to completion. The reaction mixture was cooled to room temperature and poured onto a ice/water mixture (100ml) containing sodium carbonate (8g). Ether (100ml) was then added, the reaction mixture stirred at ice temperature for 30 mins and the resulting tan coloured precipitate isolated by filtration. The crude product was recrystallised from ethanol/water (1:1) to yield 6-keto-7,8-dehydrodethiobiotin ethyl ester **62** (5.4g, 42%) as yellow needles, m.p 171-173°C (lit.[178], 174-176°C); d_H ([$^2H_6$]DMSO, 360MHz) 10.73 (1 H, br s), 10.18 (1 H, br s), 4.03 (2 H, q, *J* 7.1), 2.56 (2 H, t, *J* 6.7), 2.29 (2 H, t, *J* 7.0), 2.25 (3 H, s), 1.52 (4 H, m), 1.16 (3 H, t, *J* 7.1) ppm; ν_{max}(Nujol)/cm^{-1} 1733, 1697, 1640 and 1605.

6-*Dithioketal*-7,8-*dehydrodethiobiotin ethyl ester* **71**

A solution of 6-keto-7,8-dehydrodethiobiotin ethyl ester **62** (0.4g, 1.6mmol) in $CHCl_3$ (5ml) and 1,2-ethanedithiol (0.14ml, 1eq) was stirred under N_2 and boron trifluoride etherate (0.4ml, 2eq) added. After stirring for 3 hours the mixture was washed with water (2x5ml), 1M KOH

solution (3x5ml) and again with water (2x5ml). The solvents were removed *in vacuo* to yield 6-dithioketal-7,8-dehydrodethiobiotin ethyl ester **71** (0.44g, 85%) as a yellow gum; δ_H ($CDCl_3$, 200MHz) 10.72 (1H, br s), 9.70 (1 H, br s), 4.02 (2 H, q, *J* 7.1), 3.30 (4 H, m), 2.26 (4 H, m), 2.08 (3 H, s), 1.52 (2 H, m), 1.29 (2 H, m), 1.15 (3 H, t, *J* 7.0) ppm; δ_C 11.09, 13.91, 24.46, 27.02, 33.72, 39.02, 39.97, 42.81, 59.89, 66.39, 114.69, 153.51, 173.01 ppm; *m/z* (FAB, thioglycerol) 331.1149 [$(M+1)^+$, requires $C_{14}H_{23}N_2O_3S_2$ 331.1149]

6-*Keto*-N,N'-*diacetyl-7,8-dehydrodethiobiotin ethyl ester* **72**

6-Keto-7,8-dehydrodethiobiotin ethyl ester **62** (0.70g, 2.7mmol) was heated at reflux in acetic anhydride for 3 hours. The solvent was removed *in vacuo* to afford 6-keto-N,N'-diacetyldehydrodethiobiotin ethyl ester (0.80g) as a light brown residue, which dissolved ether (10ml) and the solvent evaporated *in vacuo* to yield the crude diacetyl derivative as a solid. Recrystallisation from ethanol/water (2:1) afforded 6-keto-7,8-dehydrodethiobiotin ethyl ester **72** (0.76g, 82%) as white needles, m.p 71-73°C (lit.[110], 71-71.5°C); δ_H ($CDCl_3$, 200MHz) 4.11 (2 H, q, *J* 7.1), 2.61 (3H,s), 2.58 (s, 3H), 2.35 (4 H,m), 2.09 (3 H, s), 1.76 (4 H, m), 1.23 (3 H, t, *J* 7.1) ppm; ν_{max}/cm^{-1} 1750, 1725, 1685

6-*Keto*-N,N'-*diacetyl*-(±)-*dethiobiotin ethyl ester* **73**

6-Keto-N,N'-diacetyl-7,8-dehydrodethiobiotin ethyl ester **72** (100mg, 0.3mmol) in 1,4-dioxan (5ml) was hydrogenated at room temperature and pressure over 10% Palladium on charcoal (100mg). The theoretical uptake was 6.72cm^3 of hydrogen for 1 mole equivalent, the actual uptake was 9cm^3, completed after twelve hours. The catalyst was filtered off through celite and the filtrate evaporated *in vacuo* to leave a clear oil (92mg, 90%); a mixture of *cis* **73** and *trans* **74** isomeric products (82:18) by ^1H nmr analysis. The isomers were separated by dry flash

chromatography using ethyl acetate/chloroform (1:4) as the eluting solvent to yield the title compound **73** (76mg,75%) as a clear oil; δ_H ($CDCl_3$, 360MHz) 4.78 (1 H, d, *J* 9.8), 4.55 (1 H, m), 4.04 (2 H, q, *J* 7.1), 2.46 (3H, s), 2.44 (3H, s), 2.50 (2 H, m), 2.25 (2 H, m), 1.59 (4 H, m), 1.17 (3 H, t, *J* 7.1), 1.12 (3 H, d, *J* 6.5) ppm.

6-Hydroxy-N-acetyl-(±)-dethiobiotin ethyl ester **75**

6-Keto-N,N'-diacetyl-(±)-dethiobiotin ethyl ester **73** (314mg, 0.92mmol) in ethanol (8ml) was cooled in an ice bath to 0°C. To the stirring solution was added sodium borohydride (38mg, 1mmol) in small aliquots. The reaction mixture was allowed to return to room temperature and left to stir for 1.5 hours. Acetic acid was added dropwise to destroy any excess sodium borohydride and the reaction mixture was poured onto ice/water (20ml). The aqueous mixture was then extracted with ethyl acetate (4x25ml), then washed with water (20ml). The organic extracts were dried (Na_2SO_4) and the solvent removed *in vacuo* to afford a clear oil. Crystallisation from ethanol gave 6-hydroxy-N-acetyl-(±)-dethiobiotin ethyl ester **75** (179mg, 65%) as a white microcrystalline solid, m.p. 128-130°C (lit.[110] 129-130°C); δ_H ($CDCl_3$, 200MHz) 6.74 (1H, br s), 4.55 (1H, m), 4.12 (2H, q, *J* 7.0), 3.63 (1H, br s), 3.54 (1H, m), 2.91 (1H, br m), 2.40 (3H, s), 2.28 (2H, t, J 7.1), 1.60-1.27 (6H, m), 1.26 (3H, d, *J* 6.5), 1.19 (3H, t, *J* 7.0) ppm; ν_{max}/cm^{-1} 3460, 3240, 1735, 1685, 1665

6-Keto-trans-(±)-dethiobiotin ethyl ester **76**

To a stirring solution of 6-keto-N,N'-diacetyl-(±)-dethiobiotin ethyl ester **73** (1.00g, 2.94mmol) in dry ethanol (5ml) under a N_2 atmosphere was added freshly prepared sodium ethoxide (240mg, 3.5mmol) and the reaction mixture left to stir for one hour. AGW 50 (H^+) cation exchange resin was added to the reaction mixture and left to stir for thirty minutes. The resin was filtered off and the solvent evaporated *in vacuo* to give a

yellow oil. Dry flash chromatography (6% methanol/dichloromethane) gave the title compound **76** as a clear oil (320mg, 43%); δ_H ($CDCl_3$, 200MHz) 6.50 (1H, br s), 6.00 (1H, br s), 4.03 (2H, q, *J* 7.1), 3.91 (1H, d, J 4.8), 3.65 (1H,m), 2.53 (2H, m), 2.25 (2H, m), 1.55 (4H, m), 1.29 (3H, d, *J* 6.3), 1.18 (3H, t) ppm; δ_C ($CDCl_3$, 50mhz) 202.3 173.20, 162.85, 67.42, 60.11, 51.01, 37.70, 33.76, 24.06, 22.19, 13.99 ppm

[6,6-$^2H_2$]-trans-(±)-dethiobiotin ethyl ester **78**

6-Keto-*trans*-(±)-dethiobiotin ethyl ester **76** (50mg, 0.20mmol) in CH_2Cl_2 (2ml) and 1,2-ethanedithiol (33μL, 2eq) were stirred under N_2 and boron trifluoride etherate (25μL, 1eq) added. The reaction mixture was stirred rapidly for two hours and tlc (silica, 15% methanol/dichloromethane) indicated the reaction had gone to completion. The mixture was diluted with CH_2Cl_2 (10ml) and washed with saturated $NaHCO_3$ solution (2x5ml), 15% NaOH solution (2x5ml) and brine (2x5ml). The organic extract was dried (Na_2SO_4) and the solvent removed *in vacuo* to give 6-thioketal-trans-(+-)-dethiobiotin ethyl ester **77** (55.3mg, 83%) as a white solid. This was not isolated but dissolved in d_4-methanol (2ml) and [^{2}H]-Raney Nickel (~0.5g, 1ml W-2 suspension, 50% slurry in D_2O) was added. The reaction mixture was heated under reflux for 4 hours and tlc (silica, 15% methanol/dichloromethane) indicated the reaction had gone to completion. The catalyst was filtered off through a celite plug and the solvent evaporated *in vacuo* to leave [6,6-$^2H_2$]-trans-(±)-dethiobiotin ethyl ester **78** as a clear oil (34mg, 84%), δ_H ($CDCl_3$, 200MHz) 5.21 (1H, br s), 5.04 (1H, br s), 4.08 (2H, q, *J* 6.9), 3.42 (1H, m), 3.19 (1H, d, *J* 6.1), 2.27 (2H, m), 1.64-1.54 (2H, m), 1.31 (4H, m), 1.23 (3H, t, *J* 7.0), 1.19 (3H, d, *J* 6.0) ppm; δ_C ($CDCl_3$, 50MHz) 173.54, 162.80, 60.13, 54.06, 51.01, 34.01, 28.73, 24.99, 24.55, 21.05, 14.10 ppm; *m/z* (FAB, thioglycerol) 245.1838 [$(M+1)^+$, $C_{12}H_{21}N_2O_3{}^2H_2$ requires 245.1834]

7,8-Dehydrodethiobiotin ethyl ester **68**

A solution of 6-keto-7,8-dehydrodethiobiotin ethyl ester **62** (1.50g, 5.9mmol) in glacial acetic acid (15ml) was added to pre-reduced Adams catalyst (PtO_2) (500mgs) in glacial acetic acid (5ml) and the reaction mixture was stirred under an atmospheric pressure of deuterium for 6h . Calculated uptake for 2 moles = 264 cm^3; uptake after 6 hours = 270cm^3. The catalyst was filtered off through a celite plug and the solvent evaporated *in vacuo* to afford a light yellow oil. The residue was treated with ethanol and , on cooling (ice), a white precipitate appeared. This was filtered off to give 7,8-dehydrodethiobiotin ethyl ester **68** as colourless needles (0.80g, 56%), m.p. 190-192°C (lit.[178] 194-196°C); δ_H ($CDCl_3$, 200MHz) 10.15 (1H, br s), 10.01 (1H, br s), 4.18 (2H, q, *J* 7.1), 2.38 (2H, t, *J* 7.3), 2.25 (2H, m), 2.02 (3H, s), 1.76-1.58 (6H, m), 1.46-1.38 (2H, m), 1.28 (3H, t, *J* 7.1) ppm; δ_C 173.53, 154.28, 116.92, 112.59, 59.45, 33.11, 27.70, 27.43, 23.80, 22.45, 12.62, 7.03 ppm; *m/z* (FAB, thioglycerol) 241.1663, $(M+1)^+$, $C_{12}H_{21}N_2O_3$ requires 241.1662

Polydeuterated-7,8-dehydrodethiobiotin ethyl ester **70**

A stirring solution of Adams catalyst (PtO_2) (250mg) in d_1-acetic acid (3ml) was pre-reduced under an atmosphere of D_2 for 30 minutes. 6-Keto-7,8-dehydrodethiobiotin ethyl ester **62** (0.55g, 2.18mmol) in AcOD (1ml) was added and the reaction mixture was stirred under an atmospheric pressure of D_2 until tlc (silica, $CHCl_3$/MeOH/AcOH, 90:5:5) indicated the reaction had gone to completion (calculated for moles of deuterium: 108cm^3; Found : 123cm^3) The catalyst was filtered off through a celite plug and the solvent evaporated *in vacuo* to give a white solid. Recrystallisation from ethanol gave the title compound **70** as white needles. m.p. 186-187°C; δ_H ($CDCl_3$, 250MHz) 10.15 (1H, br s), 10.00 (1H, br s), 4.05 (2H, q, *J* 7.1), 2.23 (2H, t, *J* 7.5), 1.50-1.32 (3H, m), 1.23 (2H, m), 1.16 (3H, t, *J* 7.1) ppm; δ_C ($CDCl_3$, 50MHz) 173.62, 155.46, 117.07,

112.71, 60.05, 34.02, 27.96, 27.85, 24.32, 14.02; δ_D (H_2O, 55MHz) 2.39, 2.22, 1.99, 1.55, 1.33 ppm; *m/z* (FAB, thioglycerol) 242 (+^2H, 2.9%), 243 (+$^2H_2$, 13.5%), 244 (+$^2H_3$, 13.5%), 245 (+$^2H_4$, 30.9%), 246 (+$^2H_5$, 58.5%), 247 (+$^2H_6$, 77.8%), 248 (+$^2H_7$, 83.7%), 249 (+$^2H_8$, 77.4%), 250 (+$^2H_9$, 51.4%), 251 (+$^2H_{10}$, 23.7%)

Imidazolin-2-one **42**

A ground mixture of (±)-tartaric acid **41** (21g, 0.14mol) and urea **36** (7.5g, 0.125mol) was slowly added to a stirring solution of fuming sulphuric acid (50ml) such that the temperature did not exceed 65^{0}C without external cooling. The resultant mixture was heated at 80^{0}C for 1 hour until the evolution of gas had ceased, cooled to 20^{0}C and poured onto crushed ice (500g). The resulting brown coloured precipitate was then washed with water (250ml) and acetone (50ml) to afford 4-carboxyimidazolin-2-one (9.5g, 53%), m.p. 226-230^{0}C (lit.[132] 232-235^{0}C). A solution of this compound (9g, 0.07mol) in aq.K_2CO_3 (0.25M, 100ml) was heated at reflux for 4 hours, charcoal (1g) added, and the solution heated at reflux for a further 0.5 hours. The solution was hot filtered and the solvent evaporated under reduced pressure to *ca.* 50ml and the resulting precipitate washed with acetone (20ml) and ether (20ml) to give imidazolin-2-one **42** (4.7g, 75%), m.p. 238-240^{0}C (lit.[132] 240-241^{0}C); δ_H ([$^2H_6$]-DMSO, 60MHz) 9.70 (2H, br d), 6.23 (2H, br s) ppm

4-Ethyl-4-imidazolin-2-one **40**

A mixture of urea **36** (48g, 0.8mol) and glacial acetic acid (70ml) was heated until a clear solution was obtained whereupon 1-hydroxy-2-butanone **38** (24.7g, 0.28mol) and solid phosphomolybdic acid (10mg) was added. The reaction mixture was heated under reflux for 3 hours. The acetic acid was removed *in vacuo* then 1,4-dioxan (100ml) added and evaporated to remove any residual acetic acid azeotropically. The brown

coloured residue was dissolved in water (150ml) and solid sodium carbonate was added until the pH of the solution was *ca.* 9. The resulting mixture was extracted with CH_2Cl_2 (2x400ml) and then n-butanol (3x300ml). The combined n-butanol extracts were evaporated *in vacuo*, the resulting orange oil taken up in water (25ml) and cooling at ice temperature resulted in the formation of a yellow solid . The solid was collected by filtration and recrystallisation from water gave 4-ethyl-4-imidazolin-2-one **40** as a yellow microcrystalline solid (8g, 25%),m.p .174-175^{0}C (lit.[187],179-180^{0}C); δ_H ([$^2H_6$]-DMSO, 200MHz) 10.03 (1H, br s), 9.65 (1H, br s), 5.92 (1H, s), 2.20 (2H, q, *J* 7.5), 1.03 (3H, t, *J* 7.5) ppm; δ_C ([$^2H_6$]-DMSO, 50MHz) 172.30, 124.11, 103.30, 18.69, 12.62 ppm; *m/z* (FAB, thioglycerol) 113.0713 $(M+1)^+$, $C_5H_9N_2O$ requires 113.0714

Friedel-Crafts acylation of imidazolin-2-ones

A slurry of imidazolinone (30mmol) in sulfolane (12.4ml) and dry chloroform (30ml) was cooled to ice bath temperature and anhydrous aluminium trichloride (20.1g, 150mmol, 5eq) was added portionwise. After the addition was complete, the temperature was raised to 65^{0}C and the acid chloride monoethyl ester (45mmol, 1.5eq) was added dropwise over a period of an hour. On heating an additional 3 hours, tlc (silica, 20% methanol/ethyl acetate, phosphomolybdic acid visualisation) showed the reaction had gone to completion. The reaction mixture was poured onto ice (200g) containing Na_2CO_3 (4.4g) in water (25ml). The hydrolysis mixture was stirred rapidly and diethyl ether (150ml) was added. The resulting precipitate was isolated by filtration and dried under vacuum to give the crude product. Recrystallisation from ethanol/water (1:1) gave the corresponding substituted imidazolin-2-ones :

4-(5-Ethoxycarbonylpentanoyl)-imidazolin-2-one **43**

(2.35g, 35%), m.p.242°C (lit.[186] 239-242°C); (Found: C,55.2; H,6.65; N,11.4. $C_{11}H_{16}N_2O_4$ requires C, 55.0; H, 6.7; N, 11.65%); δ_H ([$^2H_6$]-DMSO, 200MHz) 10.77 (1H, br s), 10.48 (1H, br s), 7.62 (1H, s), 4.00 (2H, q, *J* 7.1), 2.56 (2H, t, *J* 7.0), 2.28 (2H, t, *J* 7.0), 1.52 (4H, m), 1.15 (3H, t, *J* 7.1) ppm; δ_C ([$^2H_6$]-DMSO) 187.35, 172.16, 153.50, 122.82, 120.32, 59.07, 32.97, 32.64, 23.60, 23.46, 13.47 ppm

4-Ethyl-(5-ethoxycarbonylpentanoyl)-imidazolin-2-one **44**

(2.41g, 30%), m.p. 104-105°C (lit.[187] 108-109°C); (Found : C, 58.45; H, 7.45; N, 10.25. $C_{13}H_{20}N_2O_4$ requires C, 58.2; H, 7.5; N, 10.45%); δ_H ([$^2H_6$]-DMSO, 200MHz)) 10.81 (1H, br s), 10.24 (1H, br s), 4.01 (2H, q, *J* 7.1), 2.65 (2H, q, *J* 7.5), 2.56 (2H, m), 2.28 (2H, m), 1.51 (4H, m), 1.15 (3H, t, *J* 7.1), 1.10 (3H, t, *J* 7.5) ppm; δ_C ([$^2H_6$]-DMSO, 50MHz) 188.90, 172.92, 152.89, 135.44, 118.03, 59.82, 38.65, 33.48, 24.21, 23.19, 18.79, 14.24, 13.09 ppm

4-Methyl-5-(3-ethoxycarbonylpropanoyl)-imidazolin-2-one **45**

(2.64g, 39%), m.p. 227-228°C ; (Found : C, 53.05; H, 6.3; N, 12.3. $C_{10}H_{14}N_2O_4$ requires C, 53.1; H, 6.25; N, 12.4%); δ_H ([$^2H_6$]-DMSO, 200MHz) 10.78 (1H, br s), 10.26 (1H, br s), 4.01 (2H, q, *J* 7.1), 2.84 (2H, t, *J* 5.8), 2.52 (2H, t, *J* 5.8), 2.27 (3H, s), 1.16 (3H, t, *J* 7.1) ppm; δ_C ([$^2H_6$]-DMSO, 50MHz) 186.76, 172.50, 152.71, 130.28, 118.91, 59.97, 34.01, 27.55, 14.20, 11.88 ppm

4-Methyl-5-(4-ethoxycarbonylbutanoyl)-imidazolin-2-one **46**

(3.02g, 42%), m.p. 186°C; (Found : C, 54.65; H, 6.5; N, 12.0. $C_{11}H_{16}N_2O_4$ requires C, 55.0; H, 6.7; N, 11.65%); δ_H ([$^2H_6$]-DMSO, 80MHz) 10.72 (1H, br s), 10.13 (1H, br s), 4.00 (2H, q, *J* 7.1), 2.59 (2H, t, *J* 6.1), 2.30 (2H, t, *J* 6.2), 2.25 (3H, s), 1.76 (2H, m), 1.16 (3H, t, *J* 7.1) ppm; δ_C ([$^2H_6$]-DMSO,

50MHz) 188.19, 172.86, 152.72, 130.18, 119.18, 59.95, 38.05, 32.97, 19.22, 14.26, 11.89 ppm

4-Methyl-5-(6-ethoxycarbonylhexanoyl)-imidazolin-2-one **47**
(3.21g, 40%), m.p. 152-154°C; (Found : C, 58.0; H,7.8; N, 10.15. $C_{13}H_{20}N_2O_4$ requires C, 58.2; H, 7.5; N, 10.45%); δ_H ([$^2H_6$]-DMSO, 250MHz) 10.74 (1H, br s), 10.19 (1H, brs), 4.01 (2H, q, *J* 7.1), 2.54 (2H, t, *J* 7.2), 2.25 (3H, s), 2.22 (2H, t, *J* 6.75), 1.51 (4H, m), 1.26 (2H, m), 1.15 (3H, t, *J* 7.1) ppm; δ_C ([$^2H_6$]-DMSO, 50mhz) 188.73, 172.98, 152.68, 129.80, 119.12, 59.79, 39.26, 33.55, 28.26, 24.47, 23.42, 14.22, 11.81 ppm

4-Methyl-5-(7-ethoxycarbonylheptanoyl)-imidazolin-2-one **48**
(2.79g, 33%), m.p. 154°C; (Found : C, 59.4; H, 7.55; N, 10.0. $C_{14}H_{22}N_2O_4$ requires C, 59.55; H, 7.85; N, 9.95%); δ_H ([$^2H_6$]-DMSO, 250 MHz) 10.74 (1H, br s), 10.19 (1H, br s), 4.01 (2H, q, J 7.1), 2.53 (2H, t, *J* 7.2), 2.25 (3H, s), 2.21 (2H, t, *J* 7.2), 1.49 (4H, m), 1.25 (4H, m), 1.15 (3H, t, *J* 7.1) ppm; δ_C ([$^2H_6$]-DMSO, 50MHz) 188.80, 172.99, 152.67, 129.74, 119.13, 59.76, 39.60, 33.57, 28.42, 24.46, 23.57, 14.22, 11.81 ppm

Catalytic hydrogenation of substituted imidazolinones
The substituted imidazolinone (1.5mmol) was dissolved in glacial acetic acid (2ml) and added to a stirring solution of pre-reduced Adams catalyst (PtO_2, 150mg) in glacial acetic acid (2ml). The reaction mixture was hydrogenated at room temperature and atmospheric pressure until the uptake of hydrogen had ceased. The catalyst was filtered off through a celite plug and the solvent evaporated *in vacuo* to afford the corresponding modified dethiobiotin ethyl esters as clear oils which were of sufficient purity for the next step:

4-(5-Ethoxycarbonylpentyl)-imidazolidin-2-one **49**

From **43** (291mg, 85%); δ_H ($CDCl_3$, 250MHz) 6.24 (1H, br s), 5.88 (1H, br s), 4.06 (2H, q, *J* 7.1), 3.72 (1H, m), 3.53 (1H, m), 3.06 (1H, m), 2.23 (2H, t, *J* 7.5), 1.60-1.47 (4H, m), 1.31-1.25 (4H, m), 1.18 (3H, t, *J* 7.1) ppm; δ_C ($CDCl_3$, 50MHz) 173.51, 165.11, 60.04, 52.86, 46.45, 35.36, 33.92, 28.65, 24.88, 24.46, 14.00 ppm; m/z (FAB, thioglycerol) 229.1551. $(M+1)^+$, $C_{11}H_{21}N_2O_3$ requires 229.1552

4-Ethyl-5-(5-ethoxycarbonylpentyl)-imidazolidin-2-one **50**

From 44 (250mg, 65%); δ_H($CDCl_3$, 250MHz) 7.89 (1H, br s), 6.18 (1H, br s), 4.08 (2H, q, *J* 7.1), 3.69-3.58 (2H, m), 2.29 (2H, t, *J* 7.2), 1.67-1.24 (10H, m), 1.21 (3H, t, *J* 7.1), 0.91 (3H, t, *J* 7.2) ppm; δ_C($CDCl_3$, 50MHz) 173.55, 164.85, 60.08, 57.72, 56.05, 34.00, 29.20, 28.83, 25.87, 24.51, 22.44, 14.04, 10.55 ppm; *m/z* (FAB, thioglycerol) 257.1865. $(M+1)^+$, $C_{13}H_{25}N_2O_3$ requires 257.1865.

4-Methyl-5-(3-ethoxycarbonylpropyl)-imidazolidin-2-one **51**

From **45** (289mg, 90%); δ_H ($CDCl_3$, 250MHz) 6.22 (1H, br s), 5.86 (1H, br s), 4.07 (2H, q, *J* 7.1), 3.78 (1H, m), 3.69 (1H, m), 2.30 (2H, t, *J* 7.2), 1.49 (4H, m), 1.20 (3H, t, *J* 7.1), 1.07 (3H, d, *J* 6.3) ppm; δ_C ($CDCl_3$, 50MHz) 173.07, 164.57, 60.26, 55.70, 51.25, 33.72, 28.97, 21.51, 15.28, 13.97 ppm; *m/z* (FAB, thioglycerol) 215.1403. $(M+1)^+$, $C_{10}H_{18}N_2O_3$ requires 215.1395

4-Methyl-5-(4-ethoxycarbonylbutyl)-imidazolidin-2-one **52**

From **46** (263mg, 77%); δ_H ($CDCl_3$, 250MHz) 6.35 (1H, br s), 6.01 (1H, br s), 4.03 (2H, q, *J* 7.1), 3.75 (1H, m), 3.63 (1H, m), 2.25 (2H, t, *J* 7.6), 1.55 (2H, m), 1.39-1.24 (4H, m), 1.17 (3H, t, *J* 7.1), 1.03 (3H, d, *J* 6.4) ppm; δ_C ($CDCl_3$, 50MHz) 173.32, 164.70, 60.03, 55.76, 51.18, 33.71, 29.10, 25.67, 24.52, 15.24, 13.94 ppm; *m/z* (FAB, thioglycerol) 229.1549. $(M+1)^+$, $C_{11}H_{20}N_2O_3$ requires 229.1552

4-Methyl-5-(6-ethoxycarbonylhexyl)-imidazolidin-2-one **53**

From **47** (338mg, 88%); δ_H ($CDCl_3$, 250MHz) 6.06 (1H, br s), 5.76 (1H, br s), 4.04 (2H, q, *J* 7.1), 3.79 (1H, m), 3.63 (1H, m), 2.22 (2H, t, *J* 7.3), 1.77-1.21 (10H, m), 1.19 (3H, t, *J* 7.1), 1.06 (3H, d, *J* 6.3) ppm; δ_C ($CDCl_3$, 50MHz) 173.61, 164.58, 60.00, 56.01, 51.27, 34.02, 29.34, 28.98, 28.65, 26.03, 24.59, 15.32 ppm; 14.00; *m/z* (FAB, thioglycerol) 257.1865. $(M+1)^+$, $C_{13}H_{25}N_2O_3$ requires 256.1865

4-Methyl-5-(7-ethoxycarbonylheptyl)-imidazolidin-2-one **54**

From **48** (316mg, 78%); δ_H ($CDCl_3$, 250MHz) 6.12 (1H, br s), 5.89 (1H, br s), 4.06 (2H, q, *J* 7.1), 3.76 (1H, m), 3.63 (1H, m), 2.21 (2H, t, *J* 7.2), 1.56-1.24 (12H, m), 1.17 (3H, t, *J* 7.1), 1.03 (3H, d, *J* 6.4) ppm; δ_C ($CDCl_3$, 50MHz) 173.59, 164.65, 59.91, 55.96, 51.20, 34.01, 29.32, 29.09, 28.72, 26.09, 24.58, 15.24, 13.94 ppm; *m/z* (FAB, thioglycerol) 271.2022. $(M+1)^+$, $C_{14}H_{27}N_2O_3$ requires 271.2021.

Preparation of aberrant DAPA analogues

Substituted imidazolidin-2-one (0.7mmol) and barium hydroxide octahydrate (500mg) in water (2ml) were heated in a sealed vial at 130°C for 3 days. After cooling, the reaction mixture was filtered to remove any excess barium hydroxide and the filtrate and washings (total volume 5ml) were acidified to pH1.5 with concentrated sulphuric acid. The resulting barium sulphate precipitate was removed by centrifugation, the supernatants combined and the solvent evaporated *in vacuo* to *ca.* 1 ml. On addition of methanol (3ml), a white solid precipitated out which was collected by filtration. Recrystallisation from water/methanol afforded the corresponding DAPA analogues isolated as their sulphate salts:

7,8-(±)-Diaminooctanoic acid sulphate **30**

From **49** (104mg, 55%), m.p. 238-239°C; (Found:C, 35.25; H, 7.1; N, 10.0. $C_8H_{20}N_2O_6S$ requires C, 35.3; H, 7.35; H, 10.0%); δ_H (D_2O, 250MHz) 3.70(1H, m), 3.51 (1H, m), 3.02 (1H, m), 2.25 (2H, t, *J* 7.3), 1.62-1.43 (4H, m), 1.32-1.25 (4H, m) ppm; δ_C (D_2O, 50MHz) 178.15, 51.12, 46.23, 34.17, 29.1, 26.1, 25.92, 24.91 ppm

7,8-(±)-Diaminodecanoic acid sulphate **31**

From **50** (74mg, 35%), m.p 249-250°C; (Found:C, 39.7; H, 8.35; N, 9.1. $C_{10}H_{24}N_2O_6S$ requires C, 40.0; H, 8.05; N, 9.3%); δ_H (D_2O, 250MHz) 3.64-3.51 (2H, m), 2.38 (2H, t, *J* 7.3), 1.87-1.38 (10H, m), 1.05 (3H, t, *J* 7.4) ppm; δ_C (D_2O, 50MHz) 179.03, 54.20,52.49, 33.64, 27.82, 27.65, 24.32, 23.85, 21.42, 9.22 ppm

5,6-(±)-Diaminoheptanoic acid sulphate **32**

From **51** (71mg, 45%), m.p. 236-238°C; (Found:C, 32.2; H, 6.9; N, 10.9. $C_7H_{18}N_2O_4S$ requires C, 32.5; H, 7.0; N, 10.85%); δ_H (D_2O, 250MHz) 3.88-3.73 (2H, m), 2.36 (2H, t, *J* 7.3), 1.77-1.48 (4H, m), 1.03 (3H, d, *J* 6.4) ppm; δ_C (D_2O, 50MHz) 178.29, 55.73, 51.51, 33.55, 28.32, 21.18, 14.33 ppm

6,7-(±)-Diaminooctanoic acid sulphate **33**

From **52** (108mg, 57%), m.p. 244-245°C; (Found : C, 35.1; H, 7.5; N, 10.25. $C_8H_{20}N_2O_6S$ requires C, 35.3; H, 7.4; N, 10.3%); δ_H (D_2O, 250MHz) 3.72 (1H, m), 3.57 (1H, m), 2.39 (2H, t, *J* 7.3), 1.78-1.56 (6H, m), 1.48-1.41 (2H, m), 1.36 (3H, d, *J* 7.0) ppm; δ_C (D_2O, 50MHz) 178.61, 52.28, 48.20, 33.29, 28.56, 23.92, 23.66, 12.85 ppm

8,9-(±)-Diaminodecanoic acid sulphate **34**

From **53** (100mg, 48%), m.p. 257-258°C; (Found:C, 40.0; H, 8.35; N, 9.1. $C_{10}H_{24}N_2O_6S$ requires C, 40.0; H, 8.05; N, 9.3%); δ_H (D_2O, 250MHz) 3.74 (1H, m), 3.61 (1H, m), 2.43 (2H, t, *J* 7.3), 1.81-1.59 (4H, m), 1.45 (3H, d, *J* 7.0), 1.39 (6H, m) ppm; δ_C (D_2O, 50MHz) 177.79, 53.57, 48.38, 33.76, 33.64, 28.85, 27.84, 24.31, 24.12, 13.01 ppm

9,10-(±)-Diaminoundecanoic acid sulphate **35**

From **54** (115mg, 52%), m.p. 274°C; (Found:C, 42.3; H, 8.7; N, 8.9. $C_{11}H_{26}N_2O_6S$ requires C, 42.0; H, 8.35; N, 8.9%); δ_H (D_2O, 250MHz) 3.75 (1H, m), 3.58 (1H, m), 2.30 (2H, t, *J* 7.3), 1.78-1.55 (4H, m), 1.41 (3H, d, *J* 7.0), 1.34 (8H, m) ppm; δ_C (D_2O, 50MHz) 181.36, 53.55, 48.34, 35.41, 28.15, 28.05, 27.99, 27.91, 24.84, 24.35, 13.02 ppm

Purification of E.coli Dethiobiotin Synthetase

The *bio*D gene from λ*bio*256 DNA was cloned into a modified pUC8 vector into which a 91bp *tac* promoter block has previously been inserted. The recombinant plasmid, pBD8, was used to transform *E.coli* JM83[130]. *E.coli* JM83/pBD8 was grown in LB medium (500ml) containing 50μg/ml ampicillin for 12h at 37°C in shake flasks (250rpm). Aliquots of the culture (50ml) were diluted to 500ml with fresh ampicillin containing medium and shaken for 6h at 37°C. The cells were harvested by centrifugation (5000g, 10min, 4°C) and the cell pellet resuspended in 50mM Tris buffer (pH 7.5) containing $MgSO_4$ (5mM) and DTT (300mM)[Buffer A] (typically 5ml/L of culture suspension). The concentrated cell suspension was sonicated for 14min on ice (14 bursts of 30s with 30s cooling periods) and centrifuged (15,000g, 20min, 4°C). The cell debris were resuspended in 10ml Buffer A and the sonication and centrifugation procedure repeated. The supernatants were combined and 2% protamine sulphate in Buffer A was slowly added. The solution was

stored at 4°C overnight and then centrifuged (15,000g, 20min, 4°C). The supernatant was adjusted to 45% in $(NH_4)_2SO_4$ by slow addition of $(NH_4)_2SO_4$, stirred for 5h at 4°C and centrifuged (15,000g, 30min, 4°C). The protein pellet was solubilised in Buffer A and loaded on a Sephacryl S-200 (3 x 40cm) equilibriated with Buffer A and the proteins eluted at a flow rate of 1ml/min. Fractions containing DTB synthetase were pooled and applied to a Sepharose Q fast flow anion exchange column (2 x 30cm) equilibriated with 50mM bis-tris-propane-HCl (pH7.2) containing $MgSO_4$ (5mM) and DTT (300mM)

[buffer B]. The column was washed with Buffer B (30ml) and the proteins eluted with a linear salt gradient (0-250mM NaCl in Buffer B). Active fractions were combined and dialysed against 50% (NH_4) 4 dissolved in Buffer A (final pH 7.2) for 15h. The precipitate was removed by centrifugation (10,00g, 20 min, 4°C) and resuspended in Buffer A (5ml). Desalting was effected by FPLC on a Pharmacia desalting column (1 x 10cm) using Buffer A as eluant affording homogeneous protein (1.2mg/ml; typically 3 mg/L of the original culture).

Determination of Dethiobiotin Synthetase Activity

The assay was based on the procedure described by Rosing *et al.*[188]. Solutions were prepared immediately prior to the assay and stored on ice during mixing:

Solution A- Tricyclohexylammonium phosphoenol pyruvate (10mg/ml, 20mM), disodium ATP (10mg/ml, 17mM), $MgSO_4$ (0.33mM) and KCl (1.33mM); Solution B- disodium NADH (10mg/ml, 14mM); Solution C- pyruvate kinase (2U/μl) and L-lactate dehydrogenase (1U/μl) suspended in ammonium sulphate (3.2M, pH6.0); Solution D- DTT (3mM) in tris-HCl (50mM, pH 8.0) was kept at ambient temperature before mixing. A 2.34 ml aliquot of a solution of $NaHCO_3$ (0.1M) and DTT (3mM) in tris-HCl (50mM, pH 7.7) (having a final pH of 8.0 at 25°C) was stirred in a

1cm pathlength plastic cuvette at 25°C and aliquots of solution A (300µl), solution B (50µl), solution C (10µl) and solution D (200µl) added. After 2 min the enzyme solution was added (50µl, 2µM) and after a further 10 min 10-50µl of an aqueous solution of DAPA sulphate (1mg/ml) was added. The NADH absorbance at 340nm was monitored prior to addition of substrate and for a further 10 min. The activity of DTB synthetase, corrected for non-specific ATP'ase activity, was calculated as (ΔA_{340} min^{-1}) x 0.482 units, where one unit is defined as the consumption of 1µmol NADH (or ATP) min^{-1}.

Kinetic measurements

UV measurements were carried out using a Hewlett Packard 8452A Diode Array Spectrophotometer with a SFA-20 Rapid Kinetics Stopped-Flow Accessory (Hi-Tech Scientific Ltd., Salisbury, UK). Reaction temperatures were maintained at 25°C (+-1°C) on 0.1 M tris buffer containing 50mM KCl, 0.2mM EDTA, 0.5mM 2-mercaptoethanol (and 2mM $MgCl_2$) adjusted to pH7.8 at 25°C with enzyme concentrations of 0.5-1.0 µM. Data were collected using a HP Vectra VL2 4/50 computer and analysed on line using various software products (HP 89532A General Scanning Software, HP 89532K UV-Visible Kinetics Software and SPECFIT v2.0, Spectrum Software Associates, Chapel Hill, NC, USA). The coefficients k_{obs}, k_{obs1} and k_{obs2} were determined using non-linear regression by a combination of the Levenberg-Marquardt algorithmn and the simplex method. Values of k_1, k_{-1} and k_2 were obtained from k_{obs}/[substrate] plots. k_{-2} was estimated by fitting the kinetic model to time dependent absorption changes in the 200-400 nm range using experimental values for k_1, k_{-1} and k_2 and initial conditions [DAPA analogue]=4mM, [DTBS]=60µM, [CO_2]=2mM and [DTBS-Mg^{2+}-DAPA analogue]=[DTBS-Mg^{2+}-DAPA analogue.CO_2]=0

Protein Assays

Accurate protein concentrations for kinetic measurements were determined by UV spectrophotometry using corrections to allow for interfering substances[189]. For three independent experiments reproducibility was +- 2%. The Bradford method[190] (standardised against BSA) was used for routine protein concentration determinations.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using the discontinuous buffer system of Laemmli[191]. Molecular weight determinations were carried using SDS-PAGE and phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme as low molecular weight standards. Myosin, α_2-macroglobulin, β-galactosidase, transferrin and glutamic dehydrogenase were used as high molecular weight standards.

Crystallisation of Dethiobiotin Synthetase

Crystallisation of the protein was effected from 50mM cacodylate-HCl buffer (pH 6.0) by dialysis against a 0.5M $(NH_4)_2SO_4$ solution in the same buffer as reported earlier[72].

The substrate soaking experiments were carried out as follows. The native enzyme crystals were grown in the absence of Mg^{2+} and were of typical dimension 0.2mm x 0.2mm x 0.2mm. Crystals were added to a well solution containing $MgSO_4$ (20mM) and (n-1)-(+-)-DAPA (100mM) and left to soak overnight. Details of the X-ray data sets collected from crystals of DTBS soaked under various conditions are shown in **Table 6.1**

Substrate	Res. (Å)	Complete (%)	I/sigI	Rmerge (%)	Mult.	a	b	c
(n-1)-DAPA	2.3	99.9 (100.0)	21.1 (5.8)	9.8 (33.9)	4.9	72.71	49.48	61.19
AA-(n-1)-DAPA-Mg^{2+}	2.0	91.6 (92.5)	16.2 (7.0)	4.4 (9.5)	2.4	72.15	48.62	60.57
CO_2-(n-1)-DAPA-Mg^{2+}	1.9	99.5 (97.1)	17.0 (4.1)	5.7 (21.5)	3.1	71.78	48.25	60.62

Table 6.1 Crystallographic details of data collection and processing. Data was recorded for (n-1)-DAPA (in the absence of Mg^{2+}) at room temperature and under an ambient atmosphere (AA). The other two data sets were collected at a temperature of 100K and under the atmosphere stated.

40% glycerol was used as cryoprotectant for cryocooling experiments. For the carboxylation experiment the crystals were equilibriated in an atmosphere of CO_2 for 3 hours before cryocooling.

X-ray data for the substrate soaked crystals were collected on station 7.2 at the SRS, Daresbury Laboratory at a wavelength of 1.488A using an 18cm MAR image plate system. Data were processed with DENZO-SCALEPACK[193].

Preliminary binding studies at room temperature of crystals soaked in (n-1)-(±)-DAPA (100mM) in the absence of Mg^{2+} showed no substrate binding. These crystals of DTBS were isomorphous with the native crystals without the substrate and the difference Fourier map with coefficients (F_{soak}-F_{nat}), Φ_{nat} was featureless indicating that no binding occurred without Mg^{2+}. The cryocooled crystals of DTBS, previously soaked in the (n-1)-(±)-DAPA (100mM) and $MgSO_4$ (20mM), were not isomorphous with the native crystals but to crystals containing the parent DAPA at 100K, the structure of which was already determined to 1.6Å resolution.

The structure factors F_{DAPA}, Φ_{DAPA} from the refined model of the native substrate bound to DTBS were used in the Fourier analysis. The Fourier maps with coefficients ($3F_{soak}$-$2F_{DAPA}$), Φ_{DAPA} for the soaks in the presence of Mg^{2+} showed the substrate clearly in the ambient atmosphere and under CO_2. The density corresponding to the bound substrate was the major feature of both maps and the substrate could be localised unambiguously. The shortened substrate was easily recognised in the map that used phases from the bound native substrate.

The starting model was built with the program 'O'[194] and refined with the program XPLOR[195] independently for both cases. The difference Fourier analysis based on the phases from each model indicated that the bound structures are indistinguishable and no carboxylation was detected. The R-factor between the two models was 15.4%.

BIOTIN SYNTHASE

Bacterial Strains and Plasmids

E.coli DH1i (*bio*+, *recA*, *hsc*1R, *thi*1, placi) was used as a host strain. The *bio*ABFCD mutant pCOi (*bio*-, Δ(*attλ bio uvr*B), *thr*, *leu*, *thi*, placi) lacks the biotin operon and therefore has an absolute requirement of biotin for growth. The *E.coli bio*B gene was cloned from λ*bio*256 DNA. The recombinant plasmid pIMSB was obtained from the insertion of *bio*B into a pKK223-3 (Pharmacia) derived vector which was under the control of the *tac* promoter.

Preparation of cell-free extracts of DH1i/pIMSB

E.coli DH1i/pIMSB was grown in LB medium (10ml) supplemented with ampicillin (50μg/ml) and tetracycline (12.5μg/ml) overnight at 37°C and this was used to innoculate 500ml of identical broth. The cells were grown in shake flasks (250rpm) at 37°C until the OD_{600} was 1.0 (*ca.*3h). Aliquots of this culture (50ml) were diluted to 500ml with fresh

supplemented LB medium and shaken at 37°C until OD_{600} was 0.4 (*ca.*2h). The cells were then induced with IPTG at a final concentration of 10μM and grown for a further 3h (final OD_{600} *ca.*1.8). The cells were collected by centrifugation (5,000rpm, 5min, 4°C) and the cell pellet resuspended in Tris buffer (50mM, pH 7.5) (1g wet weight cells/ml). The concentrated cell suspension was sonicated for 10 minutes on ice (10 bursts of 30s with 30s cooling periods) and centrifuged (15,000rpm, 15min, 4°C). The supernatants were collected and stored as 5ml aliquots at -20°C.

pCOi cell-free extract was prepared in a similar manner.

Partial Purification of bioB gene product

DH1i/pIMSB cell-free extract (5ml, 34mgml^{-1}) was fractionated at ice temperature by the addition of solid ammonium sulphate to 20% saturation, left to stir for 2h at 4°C and then centrifuged (15,000g, 30min, 4°C). The supernatant was brought to final concentration of 50% with solid ammonium sulphate at ice temperature, left to stir for 2h at 4°C and then centrifuged (15,000g, 30min, 4°C). the pellet was resuspended in Tris buffer (50mM, pH 7.5) (5ml) and dialysed against the same buffer overnight. The dialysed fraction (1ml) was loaded onto Q-sepharose anion exchange column (30cm × 3cm) equilibriated with Tris buffer (50mM, pH 7.5). The column was washed with Tris buffer (50ml) and the proteins eluted (1.2mlmin^{-1}) witha linear salt gradient (0-1M NaCl in the same buffer). The *bio*B gene product eluted between 300mM and 400mM salt.

^{14}C CAP-SEP Biotin Assay

A.Incubation

A standard reaction mixture contained p*bio*B extract (100μl), pCOi (*bio*-) (100μl) and the necessary cofactors in a final volume of 500μl. The

cofactors and their final concentrations are as follows: S-adenosyl-L-methionine (0.1mM), KCl (10mM), NADPH (1mM), $Fe(NH_4)_2(SO_4)_2$ (5mM), L-Glutamic acid (20mM), Thiamine pyrophospate (0.5mM), L-cysteine (5mM) and 20µl ^{14}C-DTB (0.2µCi/20µL) (0.75µg/0.2µCi). All cofactor solutions were made up in Tris buffer (50mM, pH 7.5). The reaction mixture was incubated at 37°C for two hours. The reaction was stopped by the addition of trichloroacetic acid (200µl, 10%w/v). DTB (20ml, 10mg/ml) and biotin (20ml, 10mg/ml) were added as carriers , the eppendorf tilted and the precipitated protein spun down in a microcentrifuge (15,000g, 30sec).

B. Supernatant work-up

A CAP-SEP C18 reverse phase column was washed with methanol (2mls) then distilled water (5mls). The supernatant from the reaction mixture was loaded onto the column and washed with acetic acid (2ml, 1%v/v) and distilled water (2ml). The radioactive dethiobiotin and biotin were then eluted with methanol (1ml) into an eppendorf and the solvent blown off with nitrogen. The dry sample was resuspended MeOH/0.1M NaOH (100µl, 1:1). An aliquot (10µl) was applied to a tlc plate (silica, 10 x 10cm) and run using a chloroform: 15% methanol: 0.1%formic acid (17:3:0.2) solvent system. The tlc plate was allowed to dry and was exposed overnight to a hyperfilm-ßmax autoradiography film (Amersham). After exposure, the film was developed (5min), fixed (10min) and rinsed in cold water (5min). Radioactive biotin (Rf 0.47) was identified from the developed film and the amount of biotin synthesised was calculated by scintillation counting of the radioactive zones cut from the tlc plate.

Microbiological Biotin Assay

The incubation procedure was as described above except (+)-DTB (50μM) was used in place of ^{14}C-DTB and the reaction was stopped by boiling for 5min. After centrifugation, the supernatant was assayed for biotin using the paper disc plate method with *Lactobacillus plantarum*[138]. Several standards of authentic biotin were used on each assay plate (0.1, 0.2, 0.5, 1.0, 5.0, 10.0 μg/ml) and data obtained was the average of at least duplicate experiments.

Cysteine Desulphurase Radiochemical Assay

A typical incubation contained an aliquot of protein extract (50μl), ^{14}C-L-cystine (Amersham, (10μl, 0.2μCi, 291.3mCi/mmol) and L-cysteine (200μM) in Tris buffer (50mM, pH 7.5) containing DTT (2mM) . The reaction mixture was made up to a final volume of 70μl and incubated at 37^{0}C for 3h. The reaction was stopped by boiling for 5 min and the denatured protein centrifuged (15,000g, 30s). An aliquot (5μl) of the supernatant was removed for scintillation counting. The remaining supernatant (40μl) was transferred to a fresh eppendorf and 10μl L-cysteine (1mg/ml) and 10μl L-alanine (1mg/ml) were added as carriers. The sample was then applied to Whatman No.3MM paper (30 x 90cm) and 10μl L-cysteine (1mg/ml) and 10μl L-alanine (1mg/ml) applied *ca.* 4 cm either side of the radioactive sample. The amino acids were separated using High Voltage Paper Electrophoresis (3000V, pH2, 30min). After drying, the flanking marker strips were cut from the electrophoretogram, wet with ninhydrin (2%w/v) in acetone, dried at 40^{0}C and L-cysteine (R_f 0.25) and L-alanine (R_f 0.45) indentified.. The band corresponding to L-alanine was cut from the unstained section of the electrophoretogram and eluted with 0.1M ammonia solution. Scintillation fluid (5ml) was added to the eluate (*ca.*1ml) and the radioactivity measured.

Inactivation of E.coli Cysteine Desulphurase

DH1i/pIMSB cell-free extract (1ml, 34 mg/ml) was dialysed against 100ml Tris buffer (50mM, pH 7.5) overnight. Aliquots (200µl) were transferred to five eppendorfs and L-allylglycine added to a final concentration of 0, 5, 10, 20, and 50mM (Final volume=400µl). The five reaction mixtures were incubated at 37°C for 2h. The excess L-allylglycine was removed by dialysis with Tris buffer (50mM, pH7.5, 100ml)) for 2h and 100µl aliquots of each were transferred to five new eppendorfs. ^{14}C-L-cystine (Amersham, 10µl, 0.2µCi,), L-cysteine.HCl (200µM) and Tris buffer (50mM, pH7.5) containing DTT (2mM) were added to a final volume of 150µl and the reaction mixtures incubated at 37°C for 4h. The reaction was terminated by boiling for 5min and the precipitated proteins were centrifuged (15,000g, 30s). The supernatants were analysed by HVPE as described above.

Purification of E.coli Cysteine Desulphurase

DH1i/pIMSB cell-free extract (10ml, 30mg/ml) was fractionated at ice temperature by the addition of solid ammonium sulphate to 20% saturation, left to stir for 2h at 4°C and then centrifuged (15,000g, 30min, 4°C). The supernatant was brought to a final concentration of 50% with solid $(NH_4)_2SO_4$ at ice temperature, left to stir for 2h at 4°C and then centrifuged (15,000g, 30min, 40C). The pellet was resuspended in Tris buffer (50mM, pH7.5) (8ml) and dialysed against Tris buffer (50mM, pH7.5, 250ml) at 4°C overnight. The dialysed fraction (3ml, 70mg/ml) was loaded onto a DEAE-cellulose anion exchange column (3.5cm x 21cm) equilibrated with Tris buffer (50mM, pH7.5). The column was washed with Tris buffer (50mM, pH 7.5, 100ml) and the proteins eluted (1.5 ml/min) with a linear salt gradient (0-750mM NaCl in the same buffer). Active fractions were combined and dialysed against Tris buffer (50mM, pH 7.5) at 4°C overnight. The dialysed sample (1ml) was loaded onto a

Mono Q Sepharose column (FPLC) and the proteins eluted (1ml/min) with a linear salt gradient (0-600mM NaCl in the same buffer). Cysteine desulphurase eluted between 380mM and 400mM NaCl.

References

REFERENCES

1. F.Kögl and B.Tönnis, *Z. Physiol. Chem.*, **242**, 43 (1936)

2. K. Hofmann, D.B. Melville and V.du Vigneaud, *J. Biol. Chem.*, **141**, 207 (1941)

3. K.Hofmann, G.W.Kilmer, D.B.Melville and V.du Vigneaud, *J. Biol. Chem.*, **145**, 403 (1942)

4. V.du Vigneaud, K.Hofmann and D.B.Melville, *J. Am. Chem. Soc.*, **64**, 188 (1942)

5. D.B.Melville, A.W.Moyer, K.Hofmann and V.du Vigneaud, *J. Biol. Chem.*, **146**, 487 (1942)

6. S.A.Harris, D.E.Wolf, R.Mozingo and K.Folkers, *Science*, **97**, 447 (1943)

7. S.A.Harris, D.E.Wolf, R.Mozingo, R.C.Anderson, G.E.Arth, N.R.Easton, D.Heyl, A.N.Wilson and K.Folkers, *J. Am. Chem. Soc.*, **66**, 1756 (1944)

8. S.J.Wakil, E.B.Titchener and D.M.Gibson, *Biochem.Biophys.Acta*, **29**, 225 (1958)

9. J.P Bonjour, *Ann.N.Y.Acad.Sci.*, **447**, 97 (1985)

10. H.M. Swick and C.L.Klien, *Ann.N.Y. Acad.Sci.*, **447**, 430 (1985)

11. E.G.Baggiolini, H.L.Lee, G.Pizzolato, and M.R.Uskovic, *J.Am.Chem.Soc.*, **104**, 6460 (1982) and references cited therein

12. M.W.Goldberg and L.H.Sternbach, U.S. Patents 2,489,232; 2,489,235 and 2,489,238 (1949)

13. O.Livnah, E.A.Bayer, M.Wilchek, and J.L.Sussman, *Proc.Natl.Acad.Sci.*USA, **90**, 5076 (1993)

14. L.Pugliese, A.Coda, M.Malcovati, and M.Bolognesi, *J..Mol.Biol.*, 698 (1993)

15. G.P.Kurzban, G.Gitlin, E.A.Bayer, M.Wilchek, and P.M.Horowitz, *Biochemistry*, **28**, 8537 (1989)

16. "Avidin-Biotin Technology", *Methods in Enzymol.*, **184**, 1 (1992)

17. L.D. Wright and H.R.Skeggs, *Proc.Soc.Exp.Biol.Med.*, **56**, 95 (1944)

18. D.P.Kasow and M.D.Lane, *Biochem.Biophys.Res.Comm.*, **5**, 191 (1961)

19. D.B.McCormick and L.D.Wright, *Compr.Biochem.*, **21**, 81 (1971)

20. S.A.Harris, R.Mozingo, D.E.Wolf, A.N.Wilson, and K.Folkeus, *J.Am.Chem.Soc.*, **67**, 2096 (1945)

21. F.Lynen, J.Knappe, E.Lorch, G.Jutting, and E.Ringelmann, *Angew. Chem.*, **71**, 481 (1959)

22. T.C.Bruice, A.F.Hegarty, *Proc.Natl.Acad.Sci.*USA, **65**, 805 (1970)

23. R.B.Guchait, S.E.Polakis, D.Hollis, C.Fenselau, and M.D.Lane, *J.Biol.Chem.*, **247**, 1349 (1974)

24. Y.Kaziro, L.F.Hass, P.D.Boyer, and S.Ochoa, *J.Biol.Chem.*, **237**, 1460 (1960)

25. F.Lynen, *Biochem. J.*, **102**, 381 (1967)

26. R.Kluger and P.D.Adawadker, *J.Am.Chem.Soc.*, **98**, 3741 (1976)

27. R.Kluger, P.P.Davis, and P.D.Adawadker, *J.Am.Chem.Soc.*, **101**, 5995 (1979)

28. R.E.Barden, C.H.Fung, M.F.Utter, and M.C.Scrutton, *J.Biol.Chem.*, **247**, 1323 (1972)

29. J.C.Wallace, N.B.Phillips, M.A.Snoswell, G.J.Goodall, P.V.Attwood, and D.B.Keech, *Ann.N.Y.Acad.Sci.*, **447**, 169 (1985)

30. i) T.Ogita and J.R.Knowles, *Biochemistry*, **27**, 8028 (1988)
ii) P.A.Tipton and W.W.Cleland, *Biochemistry*, 27, 4325 (1988)

31. D.E.Hansen and J.R.Knowles, *J.Am.Chem.Soc.*, **107**, 8304 (1985)

32. J.R.Knowles, *Ann.Rev.Biochem.*, **49**, 877 (1980)

33. S.E.Polakis, R.B.Guchait, and M.D.Lane, *J.Biol.Chem.*, **247**, 1335 (1972)

34. I.Climent and V.Rubio, *Arch.Biochem.Biophys.*, 251, 465 (1986)

35. J.Retey and F.Lynen, *Biochem.Z.*, **342**, 256 (1965)

36. D.J.Prescott and J.L.Rabinowitz, *J.Biol.Chem.*, **243**, 1551 (1968)

37. J.Stubbe and R.H.Abeles, *J.Biol.Chem.*, **252**, 8338 (1979)

38. S.J.O'Keefe and J.R.Knowles, *Biochemistry*, **25**, 6077 (1986)

39. A.L.Taylor, *Bacteriol.Rev.*, **34**, 155 (1970)

40. B.Rolfe, *Virology*, **42**, 643 (1969)

41. P.P.Cleary and A.Campbell, *J.Bacteriol.*, **112**, 830 (1972)

42. M.Schwartz, *J.Bacteriol.*, **92**,, 1083 (1966)

43. M.A.Eisenberg, *Metabolic Pathways*, Academic Press, New York, 27 (1975)

44. P.P.Cleary, A.Campbell, and R.Chang, *Proc.Natl.Acad.Sci.*USA, **69**, 2219 (1972)

45. S.Adhya, P.P.Cleary, and A.Campbell, *Proc.Natl.Acad.Sci.*USA, **61**, 956 (1968)

46. C.H.Pai and H.C.Lichstein, *Biochim.Biophys..Acta*, **100**, 28 (1965)

47. M.A.Eisenberg and C.Starr, *J.Bacteriol.*, **96**, 1291 (1968)

48. P.K.Howard, J.Shaw, and A.Otsuka, *Gene*, **35**, 321 (1985)

49. J.H.Mueller, *J.Biol.Chem.*, **119**, 121 (1937)

50. J.H.Mueller, *J.Bacteriol.*, **34**, 163 (1937)

51. J.H.Mueller, *Proc.Soc.Exp.Biol.Med.*, **36**, 706 (1937)

52. V.du Vigneaud, K.Dittmer, E.Hague and B.Long, *Science*, **96**, 186 (1942)

53. K.Dittmer, D.B.Melville and V.du Vigneaud, *Science*, **99**, 203 (1944)

54. M.A.Eisenberg, *Adv.Enzymology*, **38**, 317 (1973)

55. M.A.Eisenberg, *Biochem.Biophys.Res.Comm.*, **38**, 437 (1962)

56. H.L.Elford and L.D.Wright, *Biochem.Biophys.Res.Comm.*, **10**, 373 (1963)

57. H.Lezius, E.Ringelmann and F.Lynen, *Biochem.Z.*, **336**, 510 (1963)

58. i) J.R.D.McCormick, E.R.Jensen, N.H.Arnold, H.S.Corey, U.H.Joachim and S.Johnson, *J.Am.Chem.Soc.*, **90**, 7127 (1968)
and ii) R.Thomas et al., *J.Chem.Soc.Chem.Commun.*, 667 (1983)

59. K.Ogata, Y.Izumi and K.Tam, *Agric.Biol.Chem.*, **37**, 1087 (1975)

60. I.Sanyal, S.Lee, and D.H.Flint, *J.Am.Chem.Soc.*, **116**, 2637 (1994)

61. O.Ifuku, H.Miyaoka, N.Koga, Jiro, S.Haze, Y.Wachi, and M.Kajiwa, *Eur.J.Biochem.*, **220**, 585 (1994)

62. K.Magnuson, S.Jackowski, C.O.Rock, and J.E.Cronan Jr., *Microbiol.Rev.*, **57**, 522 (1993)

63. M.Akhtar, H.M.Aboud, G.Barnard, P.M.Jordan and Z.Zaman, *Phils.Trans.R..Soc.London.Ser.B.*, **13**, 273, 99 (1976)

64. O.Ploux and A.Marquet, *Biochem.J*, **283**, 327 (1992)

65. M.A.Eisenberg and G.LStoner, *J.Bacteriol.* **108**, 1135 (1975)

66. C.H.Pai, *J.Bacteriol.*, **105**, 793 (1971)

67. B.Rolfe and M.A.Eisenberg, *J. Bacteriol.*, **96**, 515 (1968)

68. M.A.Eisenberg and K.Krell, *J.Bacteriol.*, **98**, 1227 (1969)

69. M.A.Eisenberg and K.Krell, *J.Biol.Chem.*, **244**, 5503 (1969)

70. C.H.Pai, *J.Bacteriol.*, **99**, 696 (1969)

71. K.Krell and M.A.Eisenberg, *J.Biol.Chem.*, **245**, 6558 (1970)

72. D.Alexeev, S.M.Bury, C.W.G.Boys, M.A.Turner, L.Sawyer, A.J.Ramsey, H.C.Baxter and R.L.Baxter, *J.Mol.Biol.*, **235**, 774 (1994)

73. R.L.Baxter, A.J.Ramsey, L.A.McIver and H.C.Baxter, *J.Chem.Soc., Chem. Commun.*, 559 (1994)

74. R.L.Baxter and H.C.Baxter, *J.Chem.Soc., Chem. Commun.*, 759 (1994)

75. W.Huang, Y.Lindqvist, G.Schneider, K.J.Gibson, D.Flint, and G.Lorimer, *Structure*, **2**, 407 (1994)

76. D.Alexeev, R.L.Baxter, and L.Sawyer, *Structure*, **2**, 1061 (1994)

77. G.E. Schulz, *Curr.Opin.Struct.Biol.*, **2**, 61 (1992)

78. a)D.Aleexev, R.L.Baxter *et al.*, unpublished results

b) R.Gloeckler.,et al., and Y.Lemoines, *Gene*, **87**, 63 (1990)

c) N.Sakurai, Y.Imai, H.Akatsuka, E.Kawai, S.Komatsubara,and T.abd Tosa, SWISSPROT data bank, accession P36572 (1993)

d) K.Hatakeyama, K.Hohama, A.A.Vertes, M.Kobayashi, Y.Kurusu, and H.Yukawa, *DNA sequence*, **4**, 177 (1994)

79. A.Lezius,E.Ringelman and F.Lynen, *Biochem.Z.*, **336**, 510 (1963)

80. T.Niimura, T.Suzuki and Y.Sahashi, *J.Vitaminol.*, **10**, 218 (1964)

81. i) T.Niimura, T.Suzuki and Y.Sahashi, *J.Vitaminol.*, **10**, 224 (1964)

ii) T.Niimura, T.Suzuki and Y.Sahashi, *J.Vitaminol.*(Japan), **29**, 86 (1964)

82. K.Shimada, *Yakugahu Zaoshi* (Jap), **87**, 351 (1967)

83. H.L.Elford and L.D.Wright, *Arch.Biochem.Biophys.*, **123**, 1456 (1968)

84. R.H.White, *Biochem.*, **21**, 4271 (1982)

85. F.Frappier and A.Marquet, *Biochem. Biophys. Res. Comm.*, **103**, 1288 (1981)

86. E.DeMoll and W.Shive, *Biochem.Biophys.Res.Comm.*, **110**, 243 (1983)

87. R.B.Roberts, P.H.Abelson, D.B.Cowie, E.T.Bolton and R.J.Britten, *Studies of Biosynthesis in E.coli.*, 318-405 (1955)

88 . E.DeMoll, R.H.White and W.Shive, *Biochem.*, **23**, 558 (1984)

89. A.Fujisawa, T.Abe, I.Ohsawa, S.Shiozaki, K.Kamogawa, and Y.Izumi, Biosci.Biotech.Biochem., **57**, 740 (1993)

90. A.Fujisawa, T.Abe, I.Ohsawa, K.Kamogawa, and Y.Izumi, *FEMS Microbiol.Lett.*, **110**, 1 (1993)

91a) O.Ifuku, J.Kishimoto, S.Haze, M.Yanagi, and S.Fukushima, *Biosci.Biotech.Biochem.*, **56**, 1780, (1992)

b) T.Oshiro, M.Yamamoto, Y.Izumi, B.T.SBui, D.Florentin, and A.Marquet, *Biosci.Biotech.Biochem.*, **58**, 1739

92. D.Florentin, B.T.S.Bui, A.Marquet, T.Oshiro, and Y.Izumi, *Comptes Rendus de l'Academie des Sciences*, **317**, 485 (1994)

93. D.Camp, Ph.D thesis, University of Edinburgh (1993)

94. T.Oshiro, M.Yamamoto, B.T.S.Bui, D.Florentin, A.Marquet, and Y.Izumi, *Biosci.Biotech.Biochem.*, **59**, 943 (1995)

96. H.Li, D.B.McCormick and L.D.Wright, *J.Biol.Chem.*, **243**, 6442 (1968)

97. J.B.Bourguin, O.Schnider and A.Grüssner, *Helv.Chim.Acta*, **28**, 528 (1945)

98. J.L.Wood and V.du Vigneaud, *J.Am.Chem.Soc.*, **67**, 210 (1945)

99. R.Duschinsky and L.A.Dolan, *J.Am.Chem.Soc.*, **67**, 2079 (1945)

100. G.Swain, *J.Chem.Soc.*, 1552 (1948)

101. R.J.Parry, M.G.Kunitani and O.Viele, *J.Chem.Soc.Chem.Commun.*, 321 (1975)

102. R.J.Parry and M.G.Kunitina, *J.Am.Chem.Soc.*, **98**, 4024 (1971)

103. R.J.Parry and M.V.Naidu, *Tet.Letts.*, 4783 (1980)

104. R.J.Parry and M.G.Kunitina, *Methods in Enzymol.*, **62D**, 353 (1979)

105. K.Ogata, *Methods in Enzymol..*, **18A**, 390 (1970)

106. G.Guillerm, F.Frappier, M.Gaudry and A.Marquet, *Biochimie,* **59**, 119 (1977)

107. F.Frappier, M.Jouany, A.Marquet ,A.Olesker and J.C.Tabet, *J.Org.Chem.*, **47**, 2252 (1982)

108. J.Trotter and J.A.Hamilton, *Biochem.*, **5**, 713 (1966)

109. R.J.Parry, *Bioorganic Chem.*, Vol.II, 247-272, Academic Press, N.Y. (1978)

110. F.Frappier, G.Guillern, A.G.Salib and A.Marquet, *Biochem.Biophys.Res.Comm.*, **91**, 521 (1979)

111. H.Ohuri, N.Sueda and S.Emoto, *Agric.Biol.Chem.*, **42**, 865 (1978)

112. F.B.Marti, Ph.D Dissertation (ETH 7236), Eidgenossischen Technischen Hochschule Zurich, Zurich (1983)

113. L.D.Hatfield, J.W.Fisher, F.C.Jose and R.D.G.Cooper, *Tet.Letts*, 4897 (1970)

114. L.Even. D.Florentin, and A.Marquet, *Bull.Soc.Chim.Fr.*, **127**, 758 (1990)

115. R.L.Baxter, D.J.Camp, A.Coutts and N.Shaw, *J.Chem.Soc.Perkin.Trans.*1, 255 (1992)

116. A.Marquet, F.Frappier, G.Guillerm, M.Azoulay, D.Florentin and J-C.Tabet, *J.Am.Chem.Soc.*, **115**, 2139 (1993)

117. R.J.Parry, *Tetrahedron*, **39**, 1215 (1983)

118. P.Baldet, H.Gerbling, S.Axiotis, and R.Douce, *Eur.J.Biochem.*, **217**, 479 (1993)

119. I.Sanyal, G.Cohen, and D.H.Flint, *Biochemistry*, **33**, 3625 (1994)

120. V.J.Chen, A.M.Orville, M.R.Harpel, C.A.Frolik, K.K.Surerus, E.Munck, and J.D.Limpscomb, *J.Biol.Chem.*, **264**, 21677 (1989)

121. S.Zhang, I.Sanyal, G.H.Bulboaca, A.Rich, and D.H.Flint, *Arch.Biochem.Biophys.*, **309**, 29 (1994)

122. O.Ifuku, N.Koga, S.Haze, J.Kishimoto, and Y.Wachi, *Eur.J.Biochem.*, **224**, 173 (1994)

123. J.Knappe, F.A.Neugebauer, H.P.Blaschkowski, and M.Ganzler, *Proc.Natl.Acad.Sci.USA*, **81**, 1332 (1984)

124. R.V.Banerjee, S.R.Harder, S.W.Ragsdale and R.G.Matthews, *Biochemistry*, **29**, 1129 (1990)

125. P.A.Frey and G.H.Reed, *Adv.Enzymol.Relat.Areas Mol.Biol.*, **66**, 1 (1993)

126. E.Mulliez, M.Fontecave, J.Gaillard and P.Reichard, *J.Biol.Chem.*, **268**, 2296 (1993)

127. A.M.Jabalquinto and E.Cardemil, *Biochim.Biophys.Acta*, **1161**, 85 (1993)

128. F.C.Hartman and M.R Harpel, *Ann.Rev.Biochem.*, **63**, 197 (1994)

129. D.P.Morris, B.A.M.Soute, C.Vermeer and D.W.Stafford, *J.Biol.Chem.*, **268**, 8735 (1993)

130. R.L.Baxter, H.C.Baxter, M.Fuhrmann, L.A.McIver, A.J.Ramsey and O.Smekal, *submitted for publication*

131. G.C.Andrews (Pfizer Inc.), WO patent WO 86.02070 (1986)

132. G.E.Hilbert, *J.Chem.Soc.*, **54**, 3413 (1932)

133. Hydrophobicity index

134. D.Alexeev, R.L.Baxter, O.Smekal and L.Sawyer, *Structure*, in press (1995)

135. P.J.Kraulis, *J.Appl.Crystallogr.*, **24**, 946 (1991)

136. C.Fan, P.C.Moews, C.T.Walsh and J.R.Knox, *Science*, **266**, 439 (1994)

137. M.A.Eisenberg, *Adv.Enzymol.Relat.Areas Mol.Biol.*, **38**, 317 (1973)

138. L.D.Wright and H.R.Skeggs, *Proc.Soc.Exp.Biol.Med.*, **56**, 95 (1944)

139. L.Stryer in "Biochemistry", 493-494, 2nd Ed., W.H.Freeman and Co., San Francisco (1981)

140. A.F.Wagner, M.Frey, F.A.Neugebauer, W.Schafer and J.Knappe, *Proc.Natl.Acad.Sci.USA*, **89**, 996 (1992)

141. K.K.Wong, B.W.Murray, S.A.Lewisch, M.K.Baxter, T.W.Ridky, L.Ulissi-DeMario and J.W.Kozarich, *Biochemistry*, **32**, 14102 (1993)

142. M.Frey, M.Rothe, A.F.V.Wagner and J.Knappe, *J.Biol.Chem.*, **269**, 12432 (1994)

143. V.Bianchi *et al.*, *Biochem.Biophys.Res.Comm.*, **197**, 792 (1993)

144. X.Y.Sun, J.Harder, M.Krook, H.Jornvall, B.M.Sjornberg and P.Reichard, *Proc.Natl.Acad.Sci.USA*, **90**, 577 (1993)

145. R.M.Petrovich, F.J.Ruzicka, G.H.Reed and P.A.Frey, *Biochemistry*, **31**, 10774 (1993)

146. P.A.Frey and G.H.Reed, *Adv.Enzymol.Relat.Areas Mol.Biol.*, **66**, 1 (1993)

147. J.Basaniak, M.L.Moss and P.A.Frey, *J.Biol.Chem.*, **264**, 1357 (1989)

148. L.Zheng, R.H.White, V.L.Cash, R.F.Jack and D.R.Dean, *Proc.Natl.Acad.Sci.USA*, **90**, 2754 (1993)

149. J.Kim and D.C.Rees, *Biochemistry*, **33**, 389 (1994) and references cited therein

150. L.Zheng, R.H.White, V.L.Cash and D.R.Dean, *Biochemistry*, **33**, 4714 (1994)

151. A.J.L.Cooper, *Ann.Rev.Biochem.*, **52**, 187 (1983)

152. P.Brzovic, E.L.Holbrook, R.C.Greene and M.F.Dunn, *Biochemistry*, **29**, 442 (1990)

153. R.H.White, *Biochem.Biophys.Res.Comm.*, **112**, 66 (1983)

154. Y.Takahashi, A.Mitsui, Y.Fujita and H.Matsubara, *Plant Physiol.*, **95**, 104 (1991)

155. R.Cammack, *Adv.Inorg.Chem.*, **38**, 281 (1992)

156. J.A.Fee and G.Palmer, *Biochim.Biophys. Acta*, **245**, 175 (1971)

157. J.W.Bodrick and J.Rabinowitz in Iron-Sulfur Proteins (W.Lovenberg ed.), vol.III, 100, Academic Press, NY and London (1977)

158. K.McCarthy and W.Lovenberg, *J.Biol.Chem.*, **243**, 6436 (1968)

159. T.Taniguchi and T.Kimura, *Biochim.Biophys.Acta*, **364**, 284 (1974)

160. A.Koj, J.Frendo and L.Woitczak, *FEBS Lett.*, **57**, 42 (1975)

161. W.Sanberg, M.C.Graves and J.C.Rabinowitz, *Trends.Biochem.Sci.*, **12**,56 (1987)

162. P.Leong-Morgenthaler, C.Kolman, S.G.Oliver, H.Hottinger and D.Soll, *Biochimie*, 76, 45 (1994)

163. D.Sun and P.Setlow, *J.Bacteriol.*, **175**, 1423 (1993)

164. A.Vrancic and A.Guha, *Nature New Biol.*, **245**, 105 (1974)

165. M.A.Eisenberg, *J.Bacteriol.*, **123**, 248 (1975)

166. Belen'kii, in Belen'kii, "Chemistry of Organosulfur Compounds", Ellis Horwood, Chichester, 193 (1990)

167. B.C.Wiegand and C.M.Friend, *Chem.Rev.*, **92**, 491 (1992)

168. R.Mozingo, *Org.Syn.Coll.*, **3**, 181 (1955)

169.a) G.Resnati, *Tetrahedron*, **49**, 9385 (1993)

b) S.Brandage, O.Dahlman, A.Mahieu and L.Morah, *Acta Chem.Scand.*, **36**, 67 (1982)

c) P.H.Buist, J.M.Findlay, G.Leger and R.A.Pan, *Tet.Letts.*, **28**, 3891 (1987)

170.a) M.B.Chenoweth and L.P.McCarty, *Pharmacol.Rev.*, **15**, 673 (1963)

b) M.Richardson and M.S.Mariott, *Ann.Rep.Med.Chem.*, **22**,159 (1987)

171. T.Ravindranathan, V.Shivajat, D.Hiremath, R.Rajagopala and A.V.Roe, *Carbohydr.Res.*, **134**, 332 (1984)

172. P.N.Confalone, G.Pizzolato and M.R.Uskovic, *Helv.Chim.Acta*, **59**, 1005 (1976)

173. R.A.Volkmann, J.T.Davis and C.N.Meltz, *J.Am.Chem.Soc.*, **105**, 5946 (1983)

174. R.A.Whitney, *Can.J.Chem.*, **59**, 2650 (1981)

175. V.Alcazar, I.Tapia and J.R.Moran, *Tetrahedron*, **46**, 1057 (1990)

176. see ref.178 and R.Duschinsky and L.A.Dolan, *J.Am.Chem.Soc.*, **70**, 657 (1948)

177. Schnettler et al., *J.Med.Chem.*, **25**, 1477 (1982)

178. R.Duschinsky and L.A.Dolan, *J.Am.Chem.Soc.*, **67**, 2079 (1945)

179. G.A.Boswell, W.C.Ripka, R.M.Scribner and C.W.Tullock, *Org.React.* (NY), **21**, 20 (1974)

180. C.W.Tullock, F.S.Fawcett, W.C.Smith and D.D.Coffman, *J.Am.Chem.Soc.*, **22**, 539 (1960)

181. W.J.Middleton, *J.Org.Chem.*, **40**, 574 (1975)

182. S.C.Sondej and J.A.Katzenellenbogen, *J.Org.Chem.*, **51**, 3508 (1986)

183. A.Coutts, PhD thesis, University of Edinburgh, 1988

184. Bonner and Grim in Kharasch; Meyers "The Chemistry of Organic Sulphur Compunds" vol.2, Perganon, NY, 35-71 and 410-413 (1966)

185. O.M.Birch, M.Fuhrmann and N.M.Shaw, *J.Biol.Chem.*, **270**, 19158 (1995)

186. S.I.Zavylov, *Iv.Akad.Nauk.SSSR., Ser.Khim.*, **10**, 2335 (1972)

187. R.Duschinsky and L.A.Dolan, *J.Am.Chem.Soc.*, **68**, 2350 (1946)

188. J.Rosing, D.A.Harris, A.Kemp.Jr and E.C.Slater, *Biochim.Biophys.Acta*, **376**, 13 (1975)

189.a) V.F.Kalb and R.W.Berlow, *Anal.Biochem.*, **82**, 362 (1977)

b) J.R.Whitaker and P.E.Granum, *Anal.Biochem.*, **109**, 156 (1980)

c) W.J.Waddell, *J.Lab.Clin.Med.*, **48**, 311 (1956)

190. M.M.Bradford, *Anal.Biochem.*, **116**, 53 (1976)

191. U.K.Laemmli, *Nature*, **227**, 680 (1970)

192. H.Falter, K.Jagasimhalu and D.A.Day, *Anal.Biochem.*, **67**, 359 (1975)

193. Z.Otwinowski, DENZO. A program for Automatic Evaluation of Film Densities, Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT

194. T.A.Jones, J.Y.Zou, S.W.Cowan and M.Kjeldgaard, *Acta Crystallogr.*, **A47,** 110 (1991)

195. A.T.Brunger and A.Krukowski, *Acta Crystallogr.*, **A46**, 585 (1990)

196. De Boer and Backer, *Org.Synth.Coll.*, Vol. **IV**, 250 (1963)