The Synthesis of Intermediates and Inhibitors of Biotin Biosynthesis

By

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For Mum and Dad.

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Courses attended

Organic research seminars, Department of Chemistry, University of Edinburgh (1995-1998)

Royal Society of Chemistry, Perkin Division, Scottish Regional Meetings (1995-1998)

Basic Course on Radiation Protection in Laboratory Work in Science and Medicine, Radiation Protection Service, University of Edinburgh, 1998.

Defining, Representing and Using Precisely-defined Pharmacophores, Drs. H. Broughton and L. Castro, Merck, Sharp and Dohme Postgraduate Lecture Course, (1996).

The Synthesis of Fine Chemicals, Prof. Sandy McKillop (University of East Anglia), Postgraduate Lecture Course, (1996).

Modern NMR Spectroscopy, Prof. Peter Sadler, Drs. I Sadler, D. Reed and D. Uhrin, Postgraduate Lecture Course, (1998).

Synthons in Organic Synthesis, Prof. E. Vilsmaier (University of Kaiserslautern), Postgraduate Lecture Course, (1998).

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Abstract

The sulfur containing vitamin, *biotin* has a cofactorial role for a number of carboxylation enzymes responsible for a variety of essential metabolic processes. Biotin is produced in minute quantities by micro-organisms and plants via a similar biosynthetic route that has few parallels in mammalian biochemistry, making it an attractive target for inhibition studies.

This thesis describes the development of synthetic strategies for intermediates of the biotin biosynthetic pathway, and some structurally related compounds.

An early step in biotin biogenesis is the decarboxylative condensation of pimeloyl-CoA and L-alanine to give 8-amino-7-oxononanoic acid (AON), catalysed by the *bio*F gene product, 8-amino-7-oxononanoate synthase (AONS). A novel preparation of (±)-AON is described here, along with a description of various alternative strategies which have been employed in the synthesis of this, and related compounds.

Dethiobiotin synthetase (DTBS), the penultimate enzyme in the biotin biosynthetic pathway, catalyses the incorporation of a carbonyl moiety between the adjacent amino groups of 7,8-diaminononanoic acid (DAN), in a process utilising ATP and carbon dioxide. A phosphorus containing structural analogue of a postulated intermediate in this process has been synthesised.

The study of the interactions between these compounds and the purified enzymes will facilitate the design and development of mechanism-based enzyme-inhibitors.

Abbreviations

a.a.	Amino acid
ADP	Adenosine diphosphate
ALAS	5-Aminolaevulinic acid synthase
AMP	Adenosine monophosphate
AON	8-Amino-7-oxo nonanoic acid
AONS	8-Amino-7-oxo nonanoic acid synthase
aq.	Aqueous
Ar.	Aromatic
ATP	Adenosine triphosphate
Boc	*tertiary*-Butoxycarbonyl
b.p.	Boiling point
br.	Broad
BTEAC	Benzyl triethylammonium chloride
bzl	Benzyl
Cbz	Benzyloxycarbonyl
CoASH	Coenzyme A (reduced form)
conc.	Concentrated
δ_C	^{13}C NMR Chemical shifts
δ_H	^{1}H NMR Chemical shifts
d	Doublet
Da	Daltons
DAN	7,8-Diaminononanoic acid
DANS	7,8-Diaminononanoic acid synthase
DCC	Dicyclohexylcarbodiimide
DCM	Dichloromethane
DIC	Diisopropylcarbodiimide
DMF	N,N-Dimethyl formamide
DTB	Dethiobiotin
DTBS	Dethiobiotin synthetase

FAB-MS	Fast atom bombardment mass spectrometry
FPLC	Fast protein liquid chromatography
FTIR	Fourier transformed infrared (spectroscopy)
g	Grams
HOMO	Highest occupied molecular orbital
HMDS	1,1,1,3,3,3-Hexamethyldisilazane
HMPA	Hexamethylphosphoramide
HPLC	High performance liquid chromatography
Hz	Hertz
J	Spin-spin coupling constant (in Hertz)
LDA	Lithium diisopropylamide
m	Multiplet
M	Molar
MALDI-TOF	Mass-assisted laser desorption ionisation - time of flight
mcpba	*meta*-Chloroperbenzoic acid
min	Minute(s)
mol	Mole(s)
m.p.	Melting point
MS	Mass spectrometry
m/z	Mass to charge ratio
NBS	N-Bromosuccinimide
NMR	Nuclear magnetic resonance
P_i	Inorganic phosphate
PLP	Pyridoxal-5'-phosphate
PMP	Pyridoxamine-5'-phosphate
ppm	Parts per million
pro	Prochiral
PTC	Phase transfer catalysis
PTC/OH	Hydroxide-ion initiated phase transfer catalysis
q	Quartet
RT	Room Temperature

SAM	S-Adenosyl-L-methionine
t	Triplet
THF	Tetrahydrofuran
tlc	Thin layer chromatography
TMS-Br	Bromotrimethylsilane
TMS-I	Iodotrimethylsilane

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1.1 Biotin : A General Introduction

At the turn of the century, nutritional science was beginning to focus on the idea of micronutrients in the diet. Particular attention was concentrated on the yeast growth factor termed "bios", coenzyme R, a bacterial growth and respiration factor, and vitamin H, a rat nutrition factor. It was only realised a number of years later that these three lines of research were dealing with the same substance, biotin **(1)** which was subsequently shown to be a carboxylation cofactor.

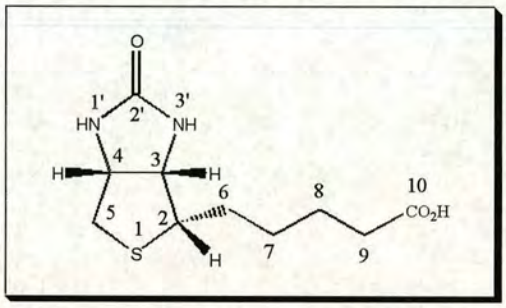


Figure 1 : D-(+)-Biotin (1)

Biotin was first isolated by Kogl and Tonnis in 1935, who obtained it as its crystalline methyl ester from 200 kg of dried egg yolk.[1] Its structure was elucidated through degradative studies by Du Vigneaud and coworkers[2] seven years later, with additional confirmation of its identity coming from the first total synthesis of (racemic) biotin by Harris *et al.* in 1944.[3] X-ray crystallographic studies[4,5] revealed biotin to have a bicyclic structure consisting of a imidazolidone ring cis-fused to a tetrahydrothiophene ring. The valeric acid side chain, attached to the C-2 of the tetrahydrothiophene ring, is *cis* with respect to the ureido ring. (It may be noted that the separation between the C-6 and 3'-N atoms is 28.6 pm, significantly less than the combined van der Waal's radii of 35 pm, resulting in a considerable steric interaction).

1.2 The Biological Role of Biotin

It was with the discovery by Wakil *et al.*[6] that biotin was an essential component in the acetyl-CoA carboxylase enzyme, that the central role of biotin in carboxylation

reactions was established. The isolation of biocytin **(2)** from yeast[7] (see figure 2), by Wright and Skeggs led to the proposition that functionally active biotin was covalently fused, *via* an amide linkage to the ε–amino group of a lysyl residue on the enzyme. Verification of this postulate came from the work of Kosow and Lane[8] who demonstrated that biotin was indeed linked in this manner to the biotin-carrier protein (BCCP) of the propionyl-CoA carboxylase enzyme (see figure 2).

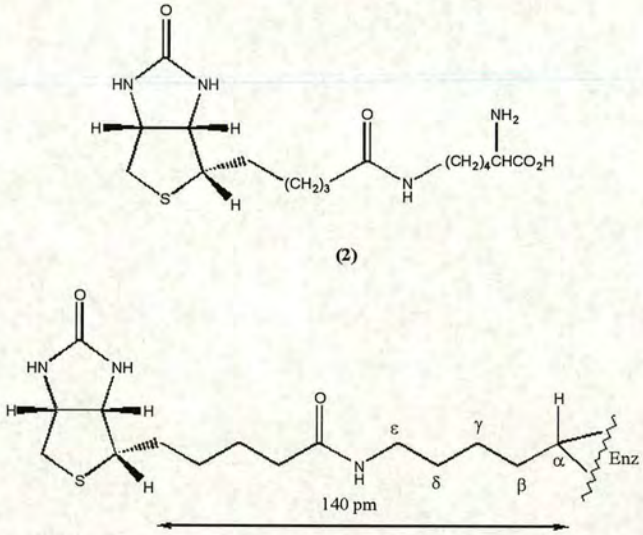


Figure 2 : Biocytin (2) and biotin attached to the BCCP

The long flexible carbon-chain linking biotin and the carrier protein-subunit enables the prosthetic group to swing between the two catalytic domains of the carboxylase enzyme, allowing the transfer of the carboxyl group from the donor to the acceptor molecules.

The carboxylases for which biotin has shown to have a definite cofactorial role, can be subdivided into three functional categories :

1) In the class 1 (carboxylation) enzymes biotin mediates the transfer of a carboxylate group from bicarbonate to acceptor molecules such as pyruvate, propionyl-CoA, acetyl-CoA, β–methylcrotonyl-CoA, geranyl-CoA and urea. The ATP-dependent

carboxylation of biotin and subsequent transfer are summarised 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 2 (decarboxylation) enzymes mediate the transport of sodium ions in anaerobes and are linked to the decarboxylation of β–ketoacids and their CoA thioesters, such as oxalacetate, (S)-methylmalonyl-CoA and glutaconyl-CoA. The processes involved can be summarised thus:

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

$$\text{enz-biotin-}CO_2^- + 2\,(Na^+)_{in} \rightleftharpoons \text{enz-biotin} + HCO_3^- + 2\,(Na^+)_{out}$$

3) The only class 3 (transcarboxylation) enzyme is methylmalonyl-CoA : pyruvate carboxytransferase (EC 2.1.3.1), found in *Propionibacterium shermanii*, which couples the two following reactions:

$$\text{enz-biotin} + \text{oxaloacetate} \rightleftharpoons \text{enz-biotin-}CO_2^- + \text{pyruvate}$$

$$\text{enz-biotin-}CO_2^- + \text{propionyl-CoA} \rightleftharpoons \text{enz-biotin} + \text{methylmalonyl-CoA}$$

There are four biotin-dependent carboxylases important in human (vertebrate) biochemistry[9] : β–methylcrotonyl-CoA carboxylase (MCC) and propionyl-CoA carboxylase (PCC) are involved in amino acid metabolism, acetyl-CoA carboxylase (ACC) is a key enzyme in fatty acid biosynthesis (lipogenesis) and pyruvate carboxylase (PC) is the primary enzyme in gluconeogenisis (see figure 3).

Due to the endogenous production of biotin by intestinal flora,[10] deficiency does not normally occur in man or higher vertebrates, even when a biotin-free diet is administered. The dietary requirement for man is not known with certainty, due to the poor understanding of the uptake mechanisms of biotin from the intestine, and of

the magnitude of biotin production in the gut. In humans, a deficiency in biotin may be induced by the consumption of a large amount of raw eggs, or egg white and by prolonged antibiotic treatment. Symptoms include alopecia, dermatitis, various metabolic disorders, nausea and behavioural abnormalities. However, remedial treatment with biotin supplements (dietary or intravenously) usually results in a full and speedy recovery.[11]

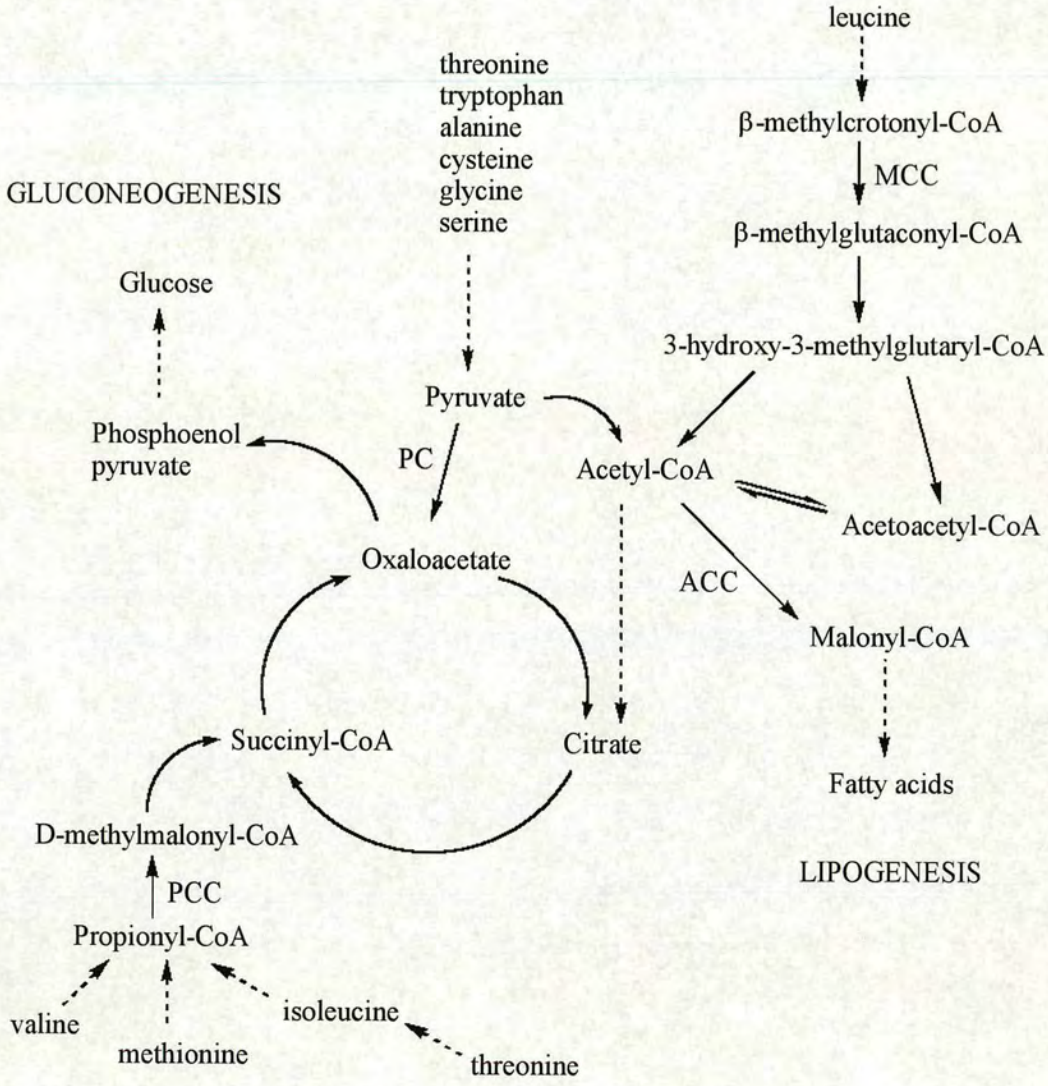


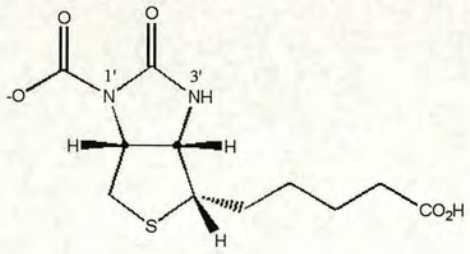
Figure 3 : Scheme showing the role of the four biotin-dependent enzymes in human biochemistry

1.3 Biotin-Avidin Technology

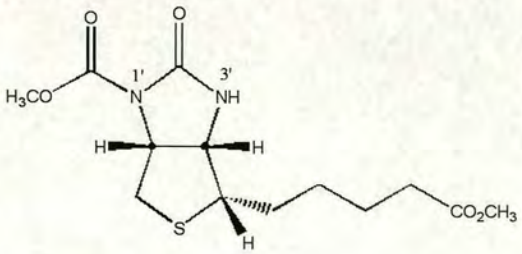
The glycoprotein avidin, found in egg whites, has an extremely high binding affinity for biotin ($K_D = 10^{-15}$ M), that is unaffected by extremes of pH, salt concentrations or chaotropic reagents such as guanidine.[12] Avidin, and the analogous bacterial proteins streptavidin and streptavin (produced by *Streptomyces* microorganisms) specifically bind molecules containing a *cis*-imidazolidin-2-one moiety. Biotin may be coupled in a facile manner to a wide range of antigens, antibodies, enzymes and fluorescent markers without adversely affecting its strong affinity to avidin. This has provided researchers with a valuable tool for the study of biochemical and immunochemical processes *in vitro*.[13]

1.4 The Mechanism of Biotin Carboxylation

The severe steric crowding between the 3'-N and C-6 of biotin[14] would suggest that the likely structure of carboxylated biotin would involve the reversible formation of a carbamate at the 1'-N position. This was shown to be the case by the isolation of the dimethyl ester of 1'-N-carboxybiotin **(4)** by Lynen *et al.*,[15] from the diazomethane trapping of the enzymatic intermediate in a model system where free (+)-biotin was carboxylated by β-methylcrotonyl-CoA carboxylase in the presence of ATP and Mg^{2+}. The x-ray analysis of the bis-*p*-bromoanilide derivative of carboxybiotin[16] confirmed the position of the carboxy group on the 1'-N position (see figure 4).



1'-N-carboxybiotin **(3)**



(4)

Figure 4 : 1'-N-Carboxybiotin (3) and its dimethyl ester (4)

The apparent chemical inertness of the N'-carboxy species led Bruice *et al.*[17] to suggest that the active enzymatic species was in fact O-carboxybiotin, which rearranged to the more stable N-acyl compound during methylation and isolation. However, this initially attractive proposal was ruled out by the demonstration that synthetically prepared 1'-N-carboxybiotinol was a chemically and kinetically competent substrate for the acetyl-CoA carboxylase enzyme.[18]

The question of whether CO_2 or bicarbonate was the substrate in the carboxylation of biotin was addressed by Kaziro *et al.*[19] in 1962, by tracer experiments using [$^{18}O_3$]-bicarbonate as a substrate for propionyl-CoA carboxylase. Their finding that two ^{18}O atoms ended up in the carboxyl group of methylmalonyl-CoA, and the third resided in P_i, strongly suggested that HCO_3^- was the true substrate (but this did not rule out a subsequent dehydration in the active site to generate CO_2). These experiments also showed that the bicarbonate ion is directly involved in the cleavage in the cleavage of the β,γ-bond of Mg-ATP.

Of the large number of pathways proposed for the mechanism of carboxylation of biotin, three of the more 'mechanistically plausible' routes are summarised in figure 5.

Pathway (A) (suggested by Kaziro[19]) involves the preactivation of bicarbonate by ATP forming carboxyphosphate **(5)**, which then directly, or after decomposition to enzyme-bound CO_2 [20] carboxylates the N'-1 of biotin.

Pathway (B) (put forward by Lynen[21]) involves the activation of biotin by phosphorylation of the urea oxygen. The resultant phosphobiotin then reacts with bicarbonate possibly in a concerted six-electron pericyclic process, to generate N-1'-carboxybiotin **(3)**.

Pathway (C)[22] also involves the formation of O-phosphobiotin as an intermediate, but this is then attacked by bicarbonate to generate carboxyphosphate **(5)** and the ureido anion of biotin, which in turn react (possibly with the pre-formation of CO_2) to yield carboxybiotin.

In an attempt to clarify the issue, Hansen and Knowles[23] synthesised [γ–^{17}O, γ–^{18}O]-γ–phosphorothioate of ATP, a chiral analogue of ATP, and used this as a substrate for the pyruvate carboxylase enzyme. The rationale behind this experiment being that enzymatic displacements at phosphorus have been universally demonstrated to proceed with an 'in-line' geometry and invert the configuraton.[24] Consequently pathways (A) and (B) are expected to proceed with inversion of configuration, with pathway (C) resulting in a net retention of configuration.

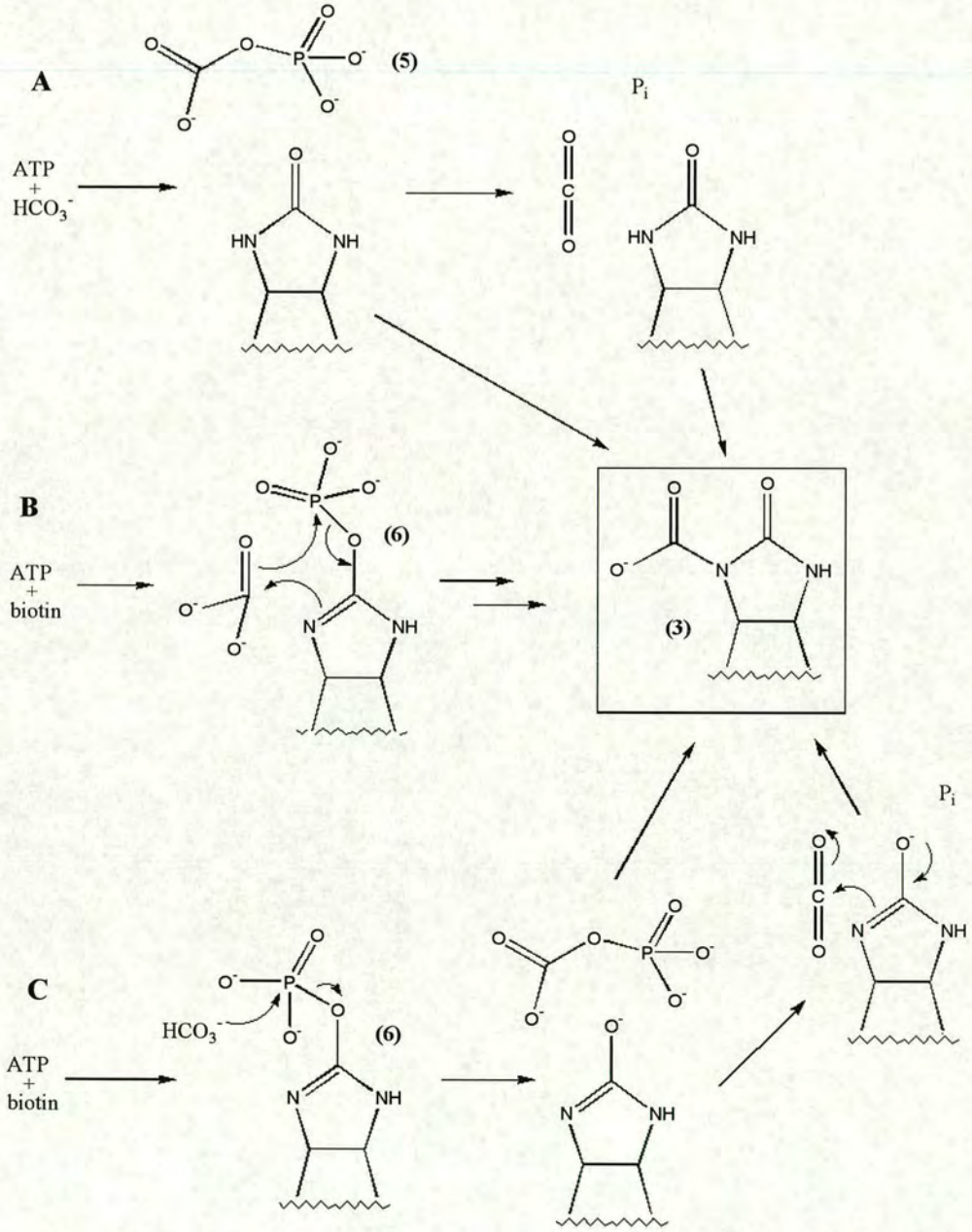


Figure 5 : Three proposed mechanisms for the carboxylation of biotin

Determination of the chirality of the [^{16}O, ^{17}O, ^{18}O]-thiophosphate product revealed that the carboxylation process had proceeded with overall inversion of stereochemistry at the phosphorus centre, effectively ruling out pathway (C). Based on the complete lack of enzymatic precedent, the authors argued that postulated pseudorotatory effects (upon the formation of the O-phosphobiotin) were unlikely to occur.

Incubation studies by Lane and co-workers[25] showed the ability of biotin carboxylase to produce ATP from Mg-ADP and carbamoyl-phosphate (an more stable, isosteric analogue of carboxyphosphate), thus supporting the intermediacy of carboxyphosphate **(5)** and hence pathway (A). This route implies that a *ureido* nitrogen of biotin functions as a nucleophile, a proposition that did not initially appear mechanistically attractive, but now appears to be the most likely path.[26]

Despite intense research, none of the suggested mechanisms above have been fully verified experimentally, principally due to the problems associated with performing reliable isotopic exchange experiments on reversible processes, and the failure to trap any of the proposed intermediate species.

1.4 The Mechanism of Carboxyl Transfer

Through experiments on propionyl-CoA carboxylase , Retey and Lynen[27] were able to demonstrate that carboxylation proceeds with retention of configuration at the carbon that accepts the carboxyl moiety. They also established that proton exchange between the solvent and substrate only occurred with enzymatic turnover. These observations led them to propose a concerted, pericyclic mechanism for the carboxyl transfer (reaction 1 in figure 6).

However, the chemically-unprecedented nature of pathway (1) prompted further investigation to determine whether or not the mechanism was concerted or stepwise.

Abeles and Stubbe[28] found that the propionyl-CoA carboxylase enzyme catalysed the elimination of HF from β-fluoropropionyl-CoA. This suggested the intermediacy of a

substrate carbanion, and hence a stepwise mechanism (2) was proposed. Knowles,[26] prompted by the results of kinetic experiments performed by Sauers and co-workers,[20] has suggested a similar process where decarboxylation preceds the attack of the substrate carbanion (pathway 3).

Further evidence supporting the operation of a stepwise mechanism was supplied by Knowles and O'Keefe[29] and Cleland *et al.*[30] who employed double isotope fractionation experiments to identify the rate determining steps in transcarboxylase and pyruvate carboxylase respectively.

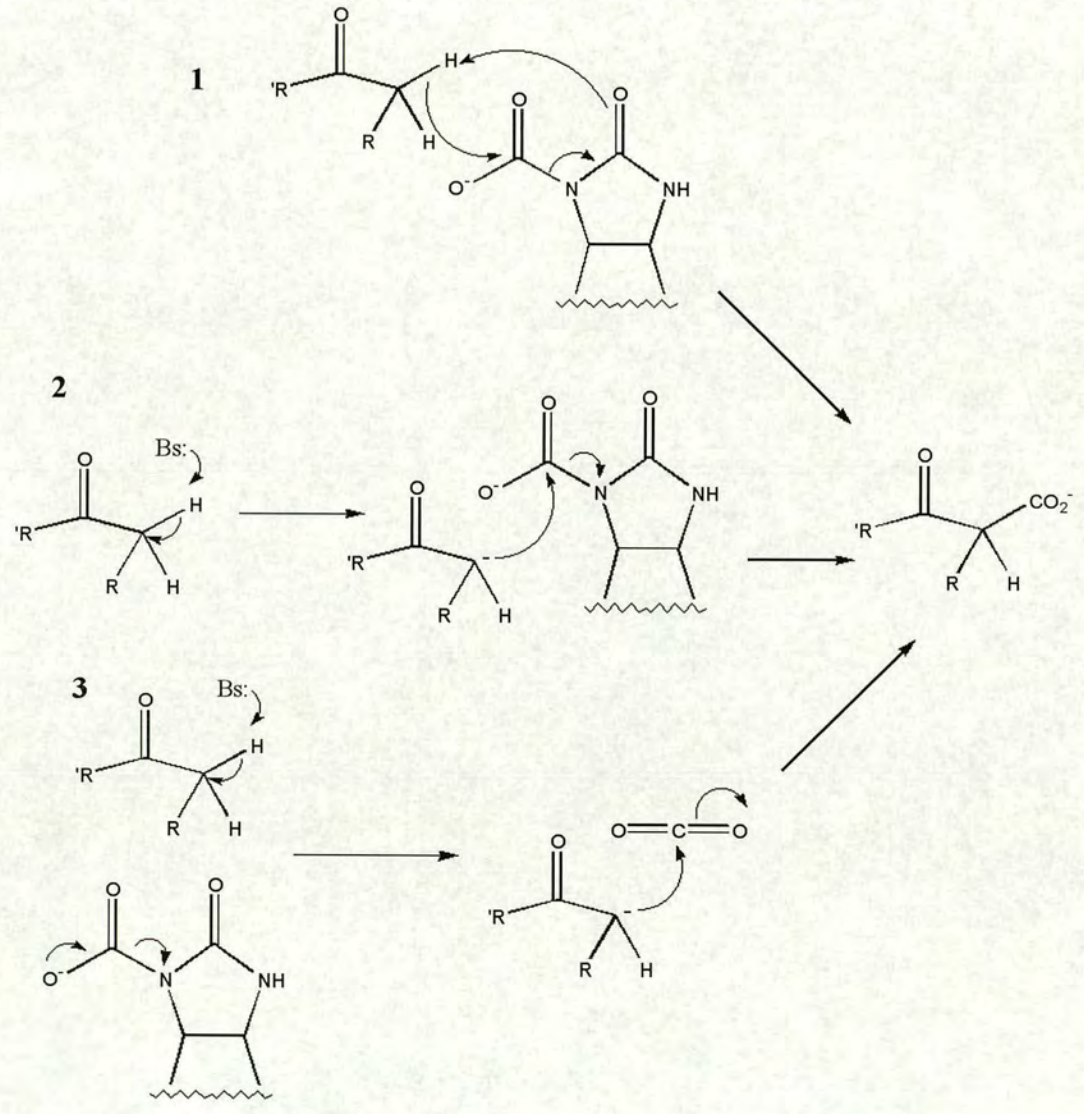


Figure 6 : Three proposed carboxyl-transfer mechanisms

Knowles and O'Keefe correlated the kinetic isotope effects measured for the substrates [$^{1}H_3$]- and [$^{2}H_3$]-pyruvate with the natural variation in ^{13}C abundance of malonyl-CoA. Detailed kinetic analysis revealed that the rate of the carboxylation reaction was dependent upon a carbon-hydrogen and a carbon-carbon bond cleavage, with both processes displaying primary isotope effects. The authors demonstrated that the kinetic measurements were consistent with a 'balanced stepwise', and not a concerted mechanism. Cleland and coworkers[30] drew parallel conclusions from the kinetic analysis of similar experiments performed on the pyruvate carboxylase catalysed process.

1.6 The Elucidation of the Microbial Biotin Biosynthetic Pathway

The microbial biotin biosynthetic pathway has been the subject of intensive study since the discovery of the true role of biotin in cell biochemistry in the early 1950's.[31] A vast array of genetic manipulations, radiolabel feeding studies and substrate analogue incubations have been used, on a variety of bacterial strains, to elucidate the sequence of enzymatic conversions required to biosynthesise this vitamin.

Early studies by Mueller *et al.*[32] demonstrated that pimelic acid **(6)** could substitute for biotin in supporting the growth of certain strains of *Cornebacterium diphtheriae*. In the same year, Eakin and Eakin[33] were able to show that the addition of pimelic acid and cysteine (or cystine) to a growing culture of *Aspergillus niger* increased the excretion of biotin vitamers into the growth medium. Dethiobiotin **(10)** was found by Du Vigneaud *et al.*[34] to be as effective as biotin in supporting the growth of the yeast *Saccharomyces cerevisiae*. Evidence for the incorporation of the intact pimelic acid moiety into both dethiobiotin and biotin, in both *Phycomyces blakesleeanus* and *Aspergillus niger*, was presented simultaneously in 1962 by Elford and Wright[35] and Eisenberg[36] using [1,7-^{14}C]-pimelic acid. However, it must be noted that studies with other organisms failed to support this finding.[37]

Later that same year Lezius, Ringelman and Lynen[38] put forward a proposal for a biotin biosynthetic pathway, based on isotopic studies with a species of *Achromobacter* grown on isovaleric acid, in the presence of either [3-^{14}C]-L-cysteine or $^{14}CO_2$. (see figure 7). The route starts with the condensation of three malonyl-CoA molecules, with the sulfur atom being incorpoated at an early stage, *via* the decarboxylative condensation of pimeloyl-CoA **(7)** with cysteine to generate 9-mercapto-8-amino-7-oxopelargonate **(11)**. However in their experiment, 40% of the label incorporated could not be localised 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. Their rationale for the formation of dethiobiotin (isolated by Dittmer[39] and Tatum[40]) was that it arose from the abortive condensation of pimeloyl-CoA with alanine.

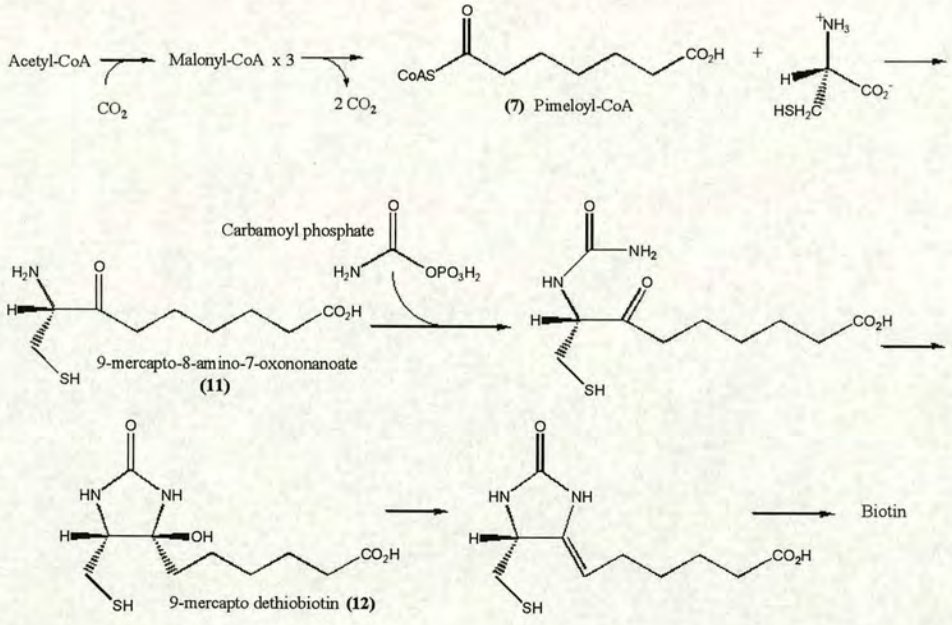


Figure 7 : Biosynthetic route to biotin proposed by Lezius *et al.*

Independent work by Eisenberg[41-43] and Iwahara,[44,45] showed that [1,7-^{14}C]-pimelic acid was incorporated into an avidin-uncombinable vitamer, which could be transformed into labelled dethiobiotin or biotin. Both groups tentatively identified this compound as 7-keto-8-aminopelargonic acid (KAPA, **8** modern name 8-amino-7-

oxononanoate, AON) and suggested an intermediary role for this vitamer in biotin biosynthesis. The observations of Dittmer and Du Vigneaud[34] and Pontecorvo,[46] that the growth of various bacterial strains could be promoted by the addition of certain pelargonic acid derivatives, led Okumura *et al.*[47,48] to perform experiments in order to increase the production of monosodium glutamate (MSG) by a *Brevibacterium* species auxotrophic for biotin. By ordering the pelargonic acid derivatives in terms of the concentrations required for maximal MSG formation, they proposed another pathway to biotin (see figure 8). These findings were in agreement with the earlier proposals of Tatum[40] who had suggested that sulfur was incorporated at a later stage in biotin biogenesis.

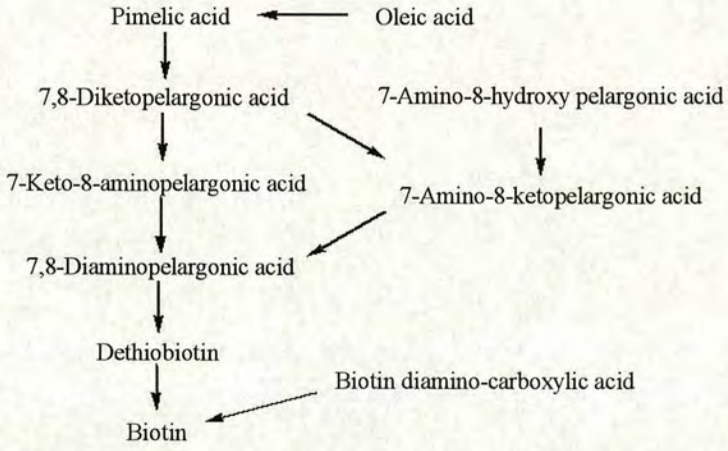


Figure 8 : Biosynthetic route to biotin proposed by Okumura

Finally Eisenberg and Rolfe[49] in 1968 proposed a biosynthetic route to biotin in *E. coli* (see figure 9). This was based upon earlier evidence but also rationalised the biotin vitamer excretion patterns of a number of mutant biotin auxotrophs of *E. coli*, in which they had identified the intermediates through their chromatographic properties. The pathway, which is now known to be ubiquitous in nature, starts with the decarboxylative condensation of L-alanine and pimeloyl-CoA **(7)** yielding 8-amino-7-oxononanoic acid (AON, **8**). Transamination leads to the formation of 7,8-diaminononanoic acid (DAN, **9**), into which a carbonyl moiety from CO_2 is

subsequently inserted to generate dethiobiotin (DTB, **10**). Finally a sulfur atom is incorporated into the molecule to give biotin **(1)**.

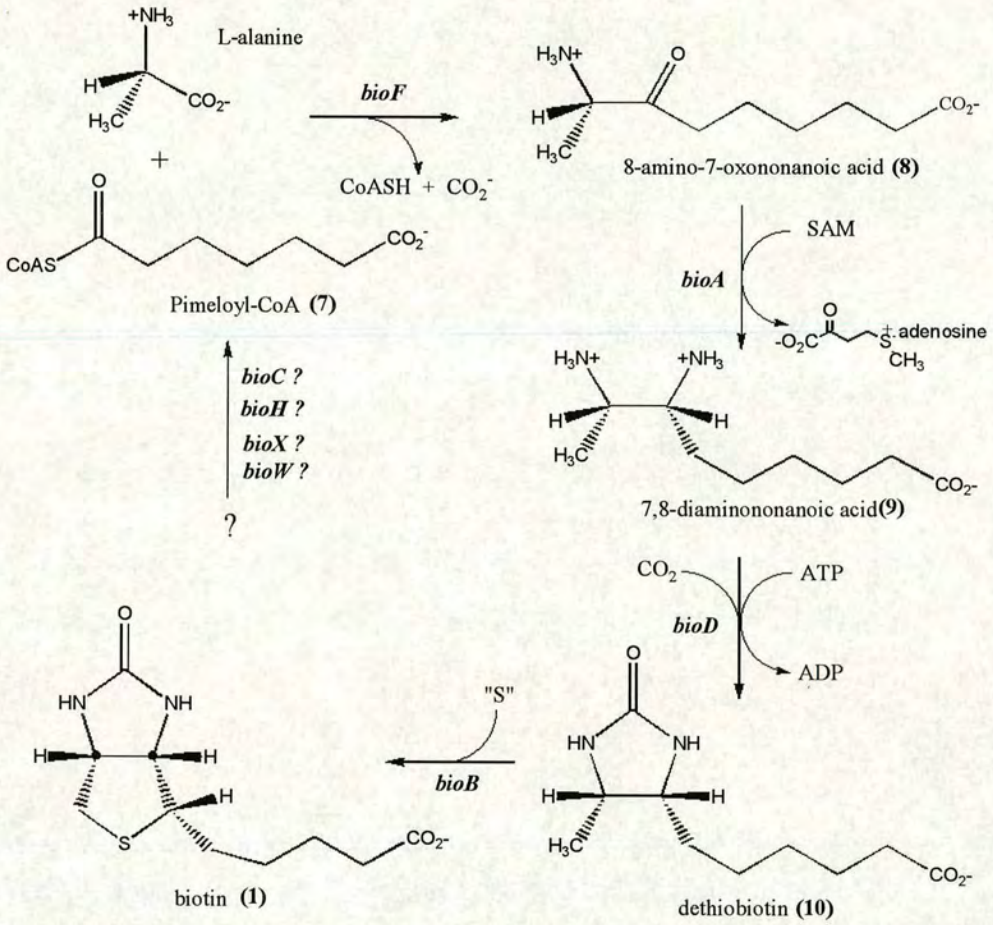


Figure 9 : The biotin biosynthetic pathway (proposed by Eisenberg)

1.7 Genetic Aspects of Biotin Biosynthesis in *Escherichia coli*

1.7.1 The Biotin Operon

Biotin biosynthesis in *E. coli* is controlled by the genes of the *bio* operon (biotin locus), which is located at 17 min. on the genetic map, between the attachment site of the lambda prophage (*attλ)* and the *uvr*B gene of *E. coli*[50] (see figure 10). Complementation analysis by Rolfe[51] and Cleary and Campbell,[52] indicated that the operon contained five complementation groups: *bio*A, *bio*B, *bio*C, *bio*D and *bio*F.

Cleary *et al*[53] and Adhya *et al.*[54] established the order of the genes using deletion mapping. They concluded that the four genes *bio*B,F,C and D were transcribed as a unit from left to right, and *bio*A from right to left, from a promoter located between *bio*A and *bio*B (see figure 11).

A further gene, *bio*H[55,56] has been found at a distant site on the map, but at this time little is known about its function except that it may encode for an enzyme necessary for the production of pimeloyl-CoA. Eisenberg[56] also identified two other genes : *bio*P, a permeability gene, and *bio*R a regulatory gene. Barker and Campbell[57] later identified a gene known as *bir*A, which was subsequently shown to be identical to the *bio*R gene discovered by Eisenberg.

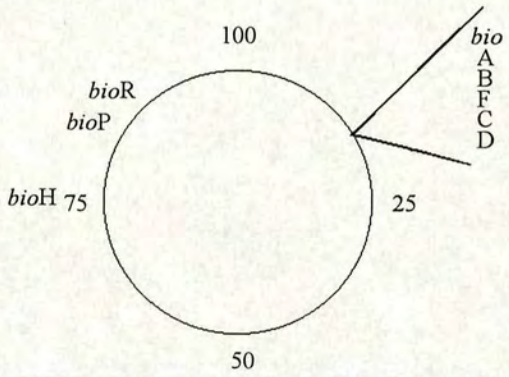


Figure 10 : The chromosomal locations of the *E. coli bio* genes (minutes)

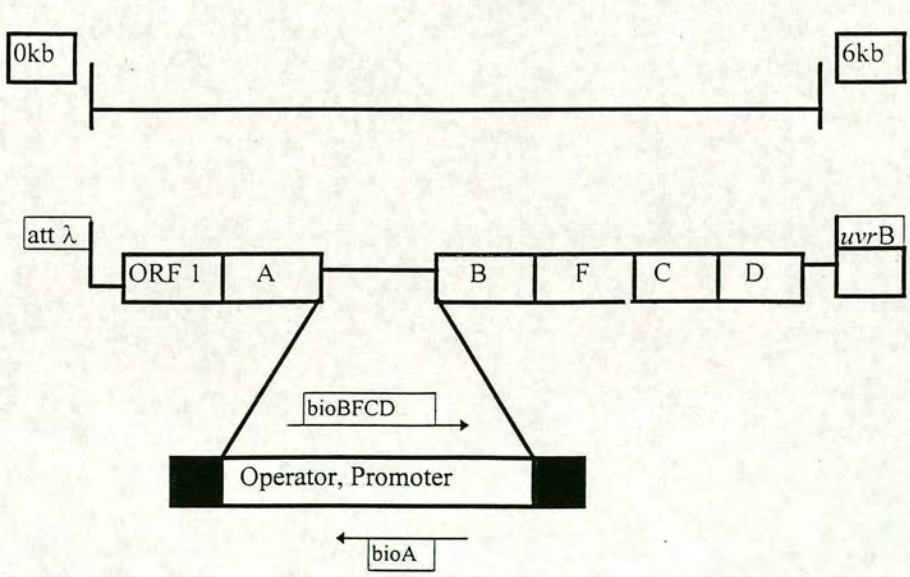


Figure 11 : The *bio* operon

The genes of the *bio* operon:

*bio*A gene product - 7,8-Diaminononanoate synthase (430 a.a., 47 403 Da)

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

*bio*F gene product - : 8-Amino-7-oxononanoate synthase (384 a.a., 41 599 Da)

*bio*C gene product - Unknown function, 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)

*bio*R gene product - regulatory protein : *Bir*A (321 a.a., 35.3 kDa)

*bio*P gene product - permeability protein

*bio*H gene product - Unknown function, prior to pimeloyl-CoA (256 a.a., 28.5 kDa)

1.7.2 Regulation of the Biotin Operon

In 1965 Pai and Lichstein first showed that biotin biosynthesis in *E. coli* was controlled by the concentration of biotin in the growth media.[58] Eisenberg later demonstrated that this was controlled by repression rather than feedback inhibition.[59] Regulation of gene expression is now known to involve *Bir*A, a bifunctional protein that serves as a biotin activating enzyme and as a transcriptional regulator.[60] *Bir*A converts biotin to biotinyl-AMP (biotinyl-5'-adenylic acid) which is the activated form required for ligation of biotin to the biotin carboxyl-carrier protein (BCCP). The intermediate in this reaction, the *bir*A-biotinyl-AMP complex functions as a corepressor, binding to the 40 base-pair *bio* operator, repressing transcription of the biotin biosynthetic genes. Thus the *Bir*A protein synthesises its own corepressor which is a unique property amongst DNA binding proteins. Recent studies have determined the structure of the biotin-holoenzyme synthetase[61] and its mechanism of binding to DNA.[62]

1.8 The enzymes of Biotin Biogenesis in *E. coli*

1.8.1 The Synthesis of Pimeloyl-Coenzyme A

Pimelic acid **(6)** has been shown to stimulate the production of biotin vitamers in several bacterial species,[63] but opinion is divided upon whether it is directly or indirectly used in the microbial biosynthetic pathway.

A gene (*bio*W) has been identified in higher plants[64] and in *Bacillus*[65] which encodes for a specific pimeloyl-CoA synthetase. The *Bacillus sphaericus* protein has been overexpressed and shown to be a homodimer, with a subunit mass of 28 kDa. It catalyses the ATP linked condensation between HSCoA and pimelic acid, yielding pimeloyl-CoA and pyrophosphate. However, an analogous gene has not thus far been identified in *E. coli.*

Ploux[65] established that the uptake of free pimelate into the cell, although shown to be far more rapid in *Bacillus*, does occur in *E. coli*. This permeability problem with pimelate was suggested as a reason for the discrepancy between the results of experiments performed with different bacterial species,[66] to determine whether or not pimelic acid was the immediate precursor to pimeloyl-CoA.

Recent reports by Flint *et al.*[67] and Ifuku *et al*[68] have shed more light upon the origin of the carbon atoms of pimeloyl-CoA in *E. coli*, and have reinforced some of the original propositions of Lynen and coworkers.[38] Ifuku performed a number of labelling studies on a biotin-overproducing strain of *E. coli*, using [3-^{13}C]-alanine, D-[1-^{13}C]-glucose, [1-^{13}C]-acetate and [2-^{13}C]-acetate as the sole carbon sources. Labelling patterns in DTB and biotin isolated from the growth medium were consistent with acetyl-CoA acting as the precursor for pimeloyl-CoA synthesis. Experiments by Flint have mirrored these findings, with the C-3, C-6 and C-8 atoms of biotin shown to originate from the C-1 of acetate, and the C-2, C-7 and C-9 coming from the C-2 of acetate (see figure 12).

These results are in agreement with the earlier findings of Lynen *et al.*[38] who proposed that pimeloyl-CoA was constructed from the condensation of three malonyl-

CoA molecules, resulting in the two carboxyls of pimelate being metabolically distinct. Ifuku found that label was incorporated into the C-10 of biotin when [1-^{13}C] as well as [2-^{13}C]-acetate was used as the sole carbon source. This indicates that this carboxyl group may originate from the $^{13}CO_2$ produced by the tricarboxylic acid (TCA) cycle.

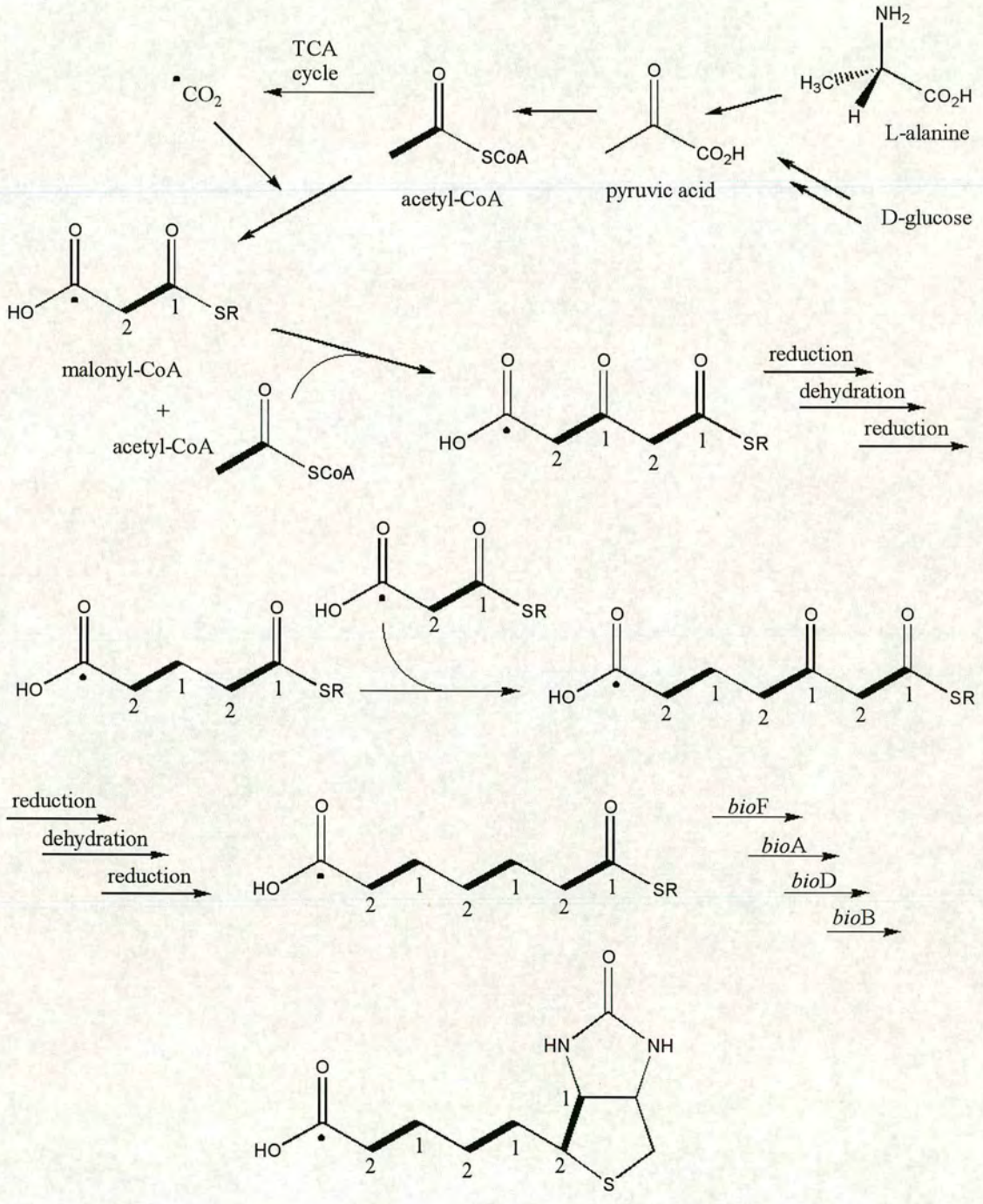


Figure 12 : Proposed mechanism of acetate incorporation into biotin

It has been proposed that the *bio*H and *bio*C genes in *E. coli* encode for two proteins involved in the biosynthesis of pimeloyl-CoA. At this time neither of the gene products have been isolated, and their nature and exact function remains unknown.[60,69] Lemoine *et al*[70] have recently published a speculative paper in which they have suggested that the *bio*C gene product may act as an acyl-carrier protein (ACP), catalysing the stepwise condensation of the malonyl-CoA starter group to form pimeloyl-ACP. They have postulated that the *bio*H protein then transfers the pimeloyl moiety from the active cysteinyl residue of *bio*C directly to coenzyme A, preventing the accumulation of free pimelate inside the cells. However, no experimental evidence has been presented to support these proposals.

1.8.2 The *bio*F Gene Product : 8-Amino-7-oxononanoate Synthase (AONS)

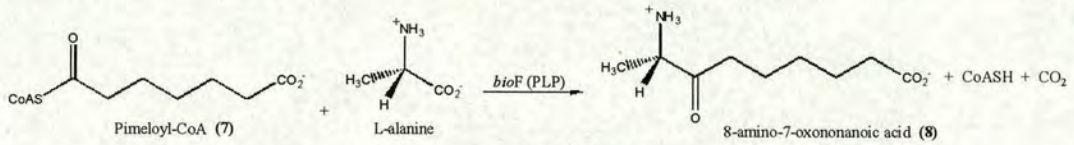


Figure 13 : The AONS catalysed process

8-Amino-7-oxononanoic acid synthase (AONS), the *bio*F gene product, catalyses the decarboxylative condensation between pimeloyl-CoenzymeA **(7)** and L-alanine in a stereospecific manner. AONS shares a common requirement for the cofactor pyridoxal-5'-phosphate (PLP, **15**) with three other α-oxoamine synthases :[71,72]
i) 5-Aminolaevulinic acid synthase (ALAS), which catalyses the condensation between glycine and succinyl-CoA, the first step in porphyrin biosynthesis,[73] ii) Glycine-acetyl transferase, which mediates the formation of 2-amino-3-oxobutanoate from acetyl-CoA and glycine[74] and iii) Serine-palmitoyl transferase, which catalyses the condensation between palmitoyl-CoA and serine.[75] Mechanistic studies on these proteins are limited; the best studied being ALAS.

In the ALAS mechanism, the first enzymatic step is the basic abstraction of the *pro*-R α-proton from the external aldimine formed between PLP and glycine. The carbanion generated then condenses with succinyl-CoA to give the Schiff-base of α-amino-β-oxoadipic acid. Subsequent decarboxylation and transimination with an active site lysine residue, yields 5-aminolaevulinate (see figure 14).

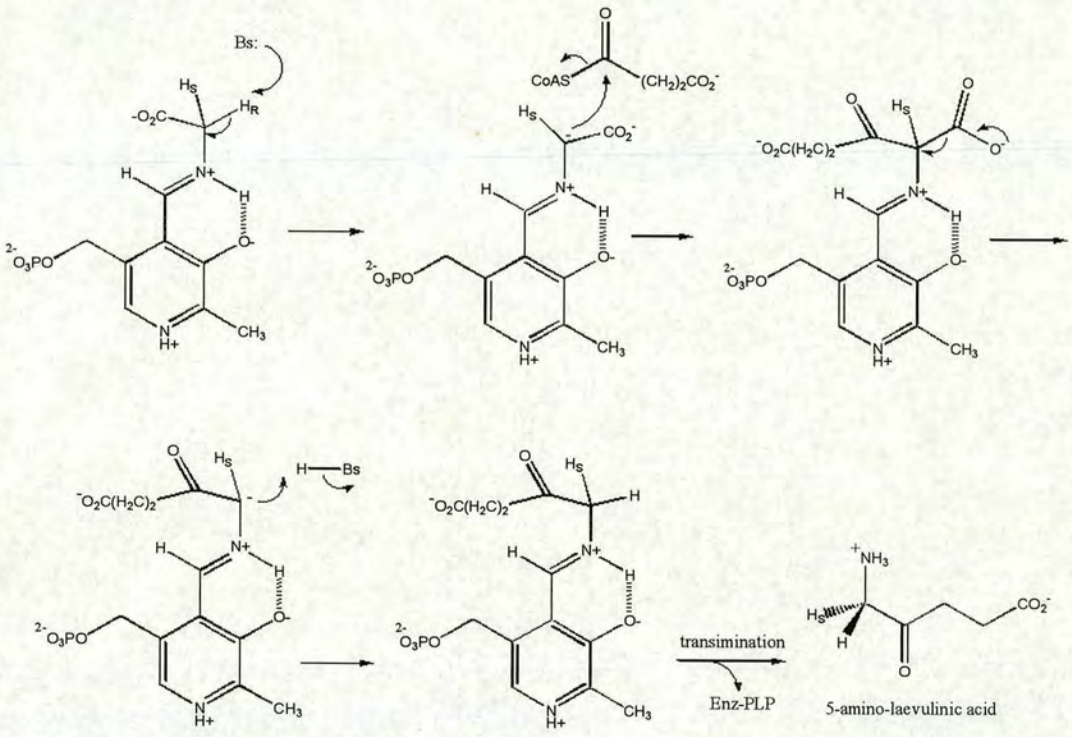


Figure 14 : Mechanism of 5-aminolaevulinic acid formation in ALAS

The *bio*F gene product has recently been cloned from *B. sphaericus*, and overexpressed in *E. coli*.[76] The purified protein is a dimer of 82 kDa, showing an absorption band at 425 nm, characteristic of a PLP-containing enzyme. Preliminary x-ray co-ordinates for the crystallised enzyme[77] have been published, but as yet no structure for the *Bacillus* enzyme has been reported.

The structure of the AONS enzyme from *E. coli* has recently been determined in our laboratory.[72] The protein is a homodimer with a tertiary structure similar to, but distinct, from the other PLP-dependent enzymes whose three-dimensional structures are known. The active site residues have been identified, and a putative diphosphate

binding site for CoA has been located. (This will be discussed in more detail in the next chapter).

Ploux and co-workers[78] observed that when L-alanine was incubated with *B. sphaericus* AONS and PLP in deuterated water buffered at pD 7.0, there was a stereospecific, time-dependent exchange of the α–proton with deuterium which followed first order kinetics. A kinetic isotope effect on the reaction velocity (V), when L-[^{2}H]- alanine was the substrate, $^{D}V = 1.3$, was taken as indicating a partially rate limiting proton abstraction at that position (combined with a postulated partially rate-limiting reprotonation step).

The above results have led Ploux *et al.* to suggest that the condensation follows an analogous mechanism to ALAS, with proton abstraction, and not decarboxylation, being the first step, and to propose that all the PLP-dependent α-oxoamine synthases share a common mode of action (see figure 15).

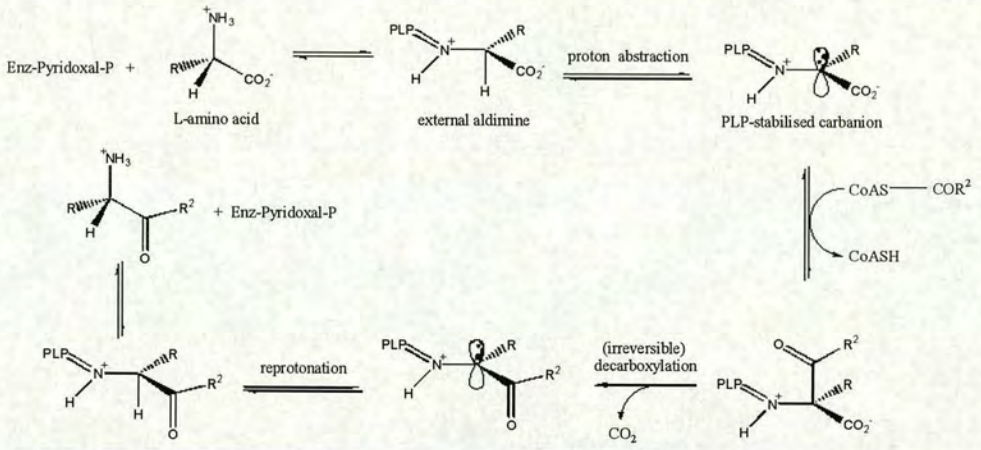


Figure 15 : Proposed mechanism shared by α-oxoamine synthases (Ploux *et al.*)

1.8.3 The *bio*A Gene Product : 7,8-Diaminononanoate Synthase (DANS)

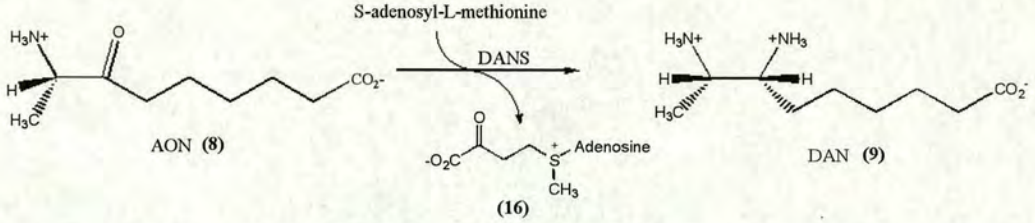


Figure 16 : The *bio*A catalysed transamination process

The *bio*A gene product, S-adenosyl-L-methionine : 8-amino-7-oxononanoic acid amino-transferase, or 7,8-diaminononanoate synthase (DANS), catalyses the stereospecific transfer of an amino group from S-adenosyl-L-methionine (SAM or Ado-met, **13**) to the *re* face of AON **(8)** forming 8(S),7(R)-diaminononanoate (DAN, **9**). The *E. coli* protein was purified to 86% homogeneity by Eisenberg and Stoner,[79] and found to be a dimer with an estimated weight of 47000 ± 3000 kDa per subunit and an isoelectric point of 4.7. Eisenberg and Stoner[80] demonstrated that the α–amino group of SAM, and not that of L-methionine as Pai[81] had originally suggested, was transferred. Pai's findings can now be attributed to a residual amount of Ado-met synthetase enzyme being present in the purified DAN-aminotransferase solution.

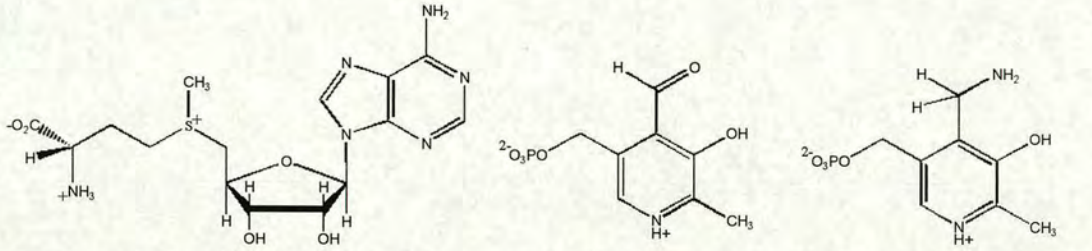


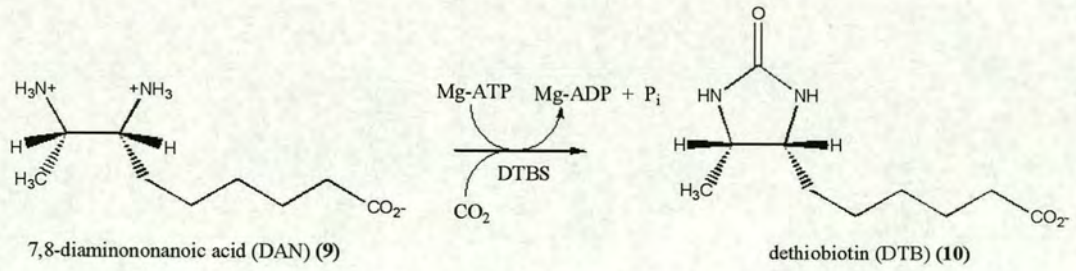
Figure 17 : The structure of SAM, PLP and PMP

The cofactor, pyridoxal-5'-phosphate (PLP, **14**) is vital for enzymatic activity, with the amino group being transferred via a ping-pong mechanism from SAM **(13)** to the PLP, forming pyridoxamine-5'-phosphate (PMP, **15**), and then to AON to yield DAN

and regenerate PLP.[82] The implied product of the reaction, S-adenosyl-2-oxo-4-methylthiobutyric acid **(16)**, has never been isolated in any enzymatic incubations, and it has been suggested[79] that it readily decomposes under non-enzymatic conditions to 5'-methylthioadenosine and 2-oxo-3-butenoic acid (although these compounds have never been detected).

Eisenberg[31] has suggested that the *bio*A and *bio*F gene products could be derived from a common ancestral gene, due to the high similarity in function, substrate specificity and cofactorial requirements of the two proteins. Subsequent work by Otsuka[83] showed that there was only 20% amino-acid homology between the *bio*A and *bio*F products, although the PLP and SAM binding site homologies were found in similar positions on the proteins. It is noteworthy that there is a higher sequence homology, (27%) between DAN-aminotransferase from *E. coli* and human ornithine aminotransferase (EC 2.6.1.13). Analogously there is a 41% sequence homology between 8-AON synthase from *E. coli* and 5-aminolaevulinic acid synthase from *Bradyrhyzobium japonicum* (EC 2.3.1.37). This suggests that even though they may share a common ancestry, the *bio*A and *bio*F genes probably diverged before the creation of vertebrates, as they now belong to two separate enzymatic families.

1.8.4 The *bio*D Gene Product : Dethiobiotin Synthetase (DTBS)



7,8-diaminononanoic acid (DAN) **(9)** dethiobiotin (DTB) **(10)**

Figure 18 : The DTBS-catalysed formation of dethiobiotin

Dethiobiotin synthetase (DTBS) catalyses the penultimate step in biotin biosynthesis (see figure 18), the formation of the *ureido* ring of biotin; converting DAN **(9)** to dethiobiotin (DTB, **10**). It is one of the few CO_2-utilising enzymes in nature that does not involve biotin as a prosthetic group, and the only one known to catalyse an N-carboxylation.[84]

Studies with resting cells of a mutant strain of *E. coli*[85] unable to grow on dethiobiotin, provided the first direct evidence of a precursor-product relationship between DAN and DTB. Using cell free extracts, Eisenberg and Krell[86] were able to demonstrate the requirement for CO_2, ATP and Mg^{2+}, and the direct incorporation of a ^{14}CO moiety from $^{14}CO_2$ into the *ureido* ring of dethiobiotin. After purification of the enzyme to near-homogeneity, Eisenberg and Krell[87] were able to show that the active form was a homodimer with a molecular weight of *ca.* 42 kDa. Enzyme-incubations with added avidin, and with carbonic anhydrase proved that CO_2 and not HCO_3^- was the true substrate, and that biotin was not involved as a prosthetic group. These observations, combined with the identification of ADP as a product, led to the proposition of a three-step mechanism (see figure 19).

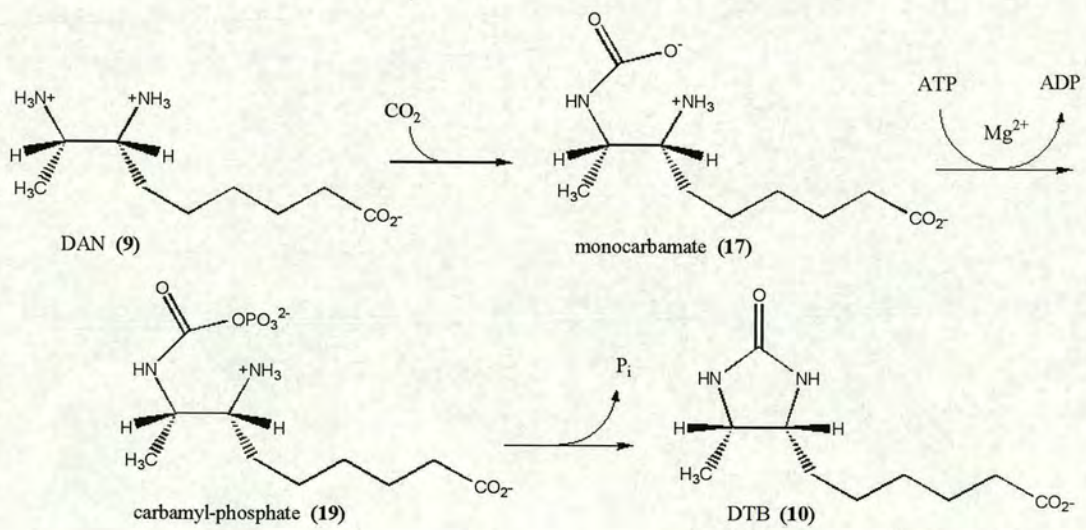


Figure 19 : Proposed mechanism of *ureido*-ring formation

The first step involves the reversible formation of a monocarbamate **17** from DAN and CO_2. The next stage is the activation of the carbamate with ATP, forming a substituted carbamyl-phosphate **19**. Subsequent nucleophilic attack from the (deprotonated) vicinal amino group at the reactive carbonyl centre generates the *ureido* ring of DTB, with phosphate being displaced.

The cloning of the *bio*D gene from both λ*bio*256 and from *E. coli* genomic DNA into a modified pBR322 derived plasmid, has allowed the DTBS enzyme to be overexpressed and purified to homogeneity.[88] This has allowed a more detailed investigation into the intimate mechanism of the *ureido* ring-forming process to be undertaken.

The stoichiometry of the overall process was confirmed by Baxter *et al.*,[89] who through a linked assay system, proved that one mole of ATP was required to produce one mole of DTB, indicating that a single activation step is involved in the formation of two amide bonds. Baxter and co-workers also performed a number of radiolabel pulse-chase experiments to indicate the order in which the three substrates: DAN, CO_2 and ATP were utilised by DTBS. These involved the pre-incubation of DTBS with i) $H^{14}CO_3^-$ alone, ii) $H^{14}CO_3^-$ and DAN or iii) $H^{14}CO_3^-$ and ATP, and after a five minute period, the addition of a 'chase' solution containing an excess of unlabelled bicarbonate and the other substrates. The finding that the largest degree of radioincorporation of ^{14}C into DTB arose from the pre-incubation of DAN with $H^{14}CO_3^-$, indicated that the initial step was the formation of the carbamate.

Whilst initial methylation trapping experiments suggested that the 8-amino-carbamate **17** was the active enzyme-bound species,[89] subsequent crystallographic information obtained *via* analysis of the enzyme-DAN-carbamate complex indicated that the 7-amino-carbamate **18** was the true intermediate[90,91] (see figure 20).

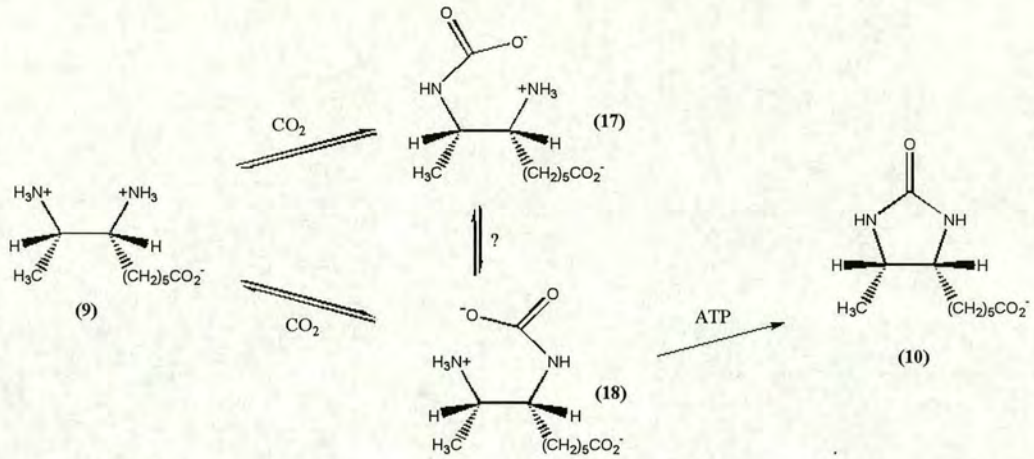


Figure 20 : Mechanism of DAN-carbamate formation

Evidence submitted by Gibson *et al.*[92] stated that the diazomethane trapping of mixtures containing DAN, [$^{14}CO_2$], and a molar excess of DTBS, led to the isolation of four times as much labelled N-7-amino carbamate, as N-8-carbamate, suggesting that the enzyme preferentially formed the N-7-regioisomer. By analogy to the earlier experiments by Baxter *et al.*,[89] pulse-chase experiments showed that the binary complex of DTBS and N-7-DAN-carbamate became kinetically committed upon the addition of MgATP. These authors also demonstrated the preference of DTBS to cyclise the DAN-N-7-carbamate mimic **20** to the corresponding lactam **21**, compared to the weakly inhibitory properties of the N-8-DAN-carbamate mimic **22** (see figure 21). It was conjectured that this showed the enzyme's specificity for carbamate formation and activation at the N-7 position.

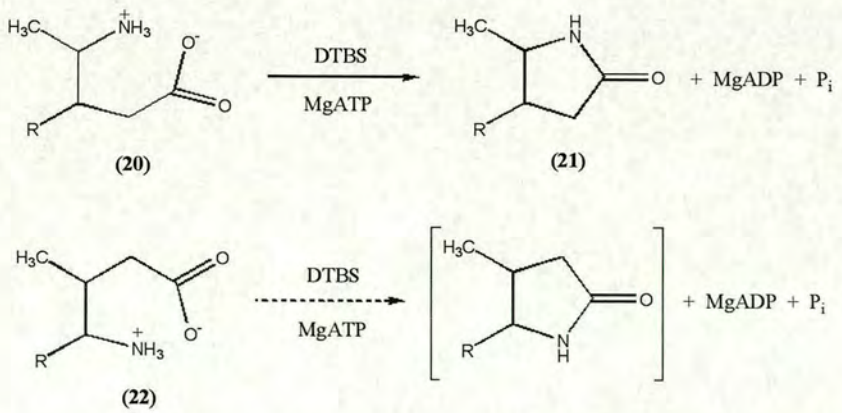


Figure 21 : DTBS-Catalysed lactam formation (Gibson *et al.*)

Trapping experiments and stopped-flow, UV spectrophotometric measurements by Baxter *et al.*[90] have suggested that the first reaction catalysed by DTBS proceeds in two stages. The initial step involves the binding of DAN to DTBS to form a substrate-enzyme complex **23**. This is followed by the reaction of this complex with CO_2 affording a DTBS-DAN-carbamate complex **24** (shown in figure 22).

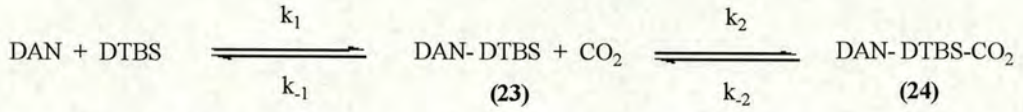


Figure 22 : Kinetic model for the enzyme-substrate-carbamate complex formation

Kinetic experiments conducted with and without Mg^{2+} ions indicated that neither the binding of CO_2 to the enzyme, nor N-carboxylation, occur to any significant extent in the absence of Mg^{2+}.

Through the incubation of the labelled substrate [$^{18}O_4$]-γ,β-[^{18}O]-ATP, plus DAN, bicarbonate and Mg^{2+} ions, with DTBS, Baxter and Baxter[93] were able to identify the reactive intermediate involved in the ring-closure process. Through the analysis of ^{18}O distribution in the products by ^{31}P NMR, a principal ion peak corresponding to [$^{18}O_3$,^{16}O]-phosphate was detected, which implied the operation of pathway (B), and the intermediacy of the carboxy-phosphate mixed anhydride species **25** (see figure 23).

Subsequently Gibson[94] through product analysis by high resolution mass spectrometry, was able to demonstrate that ^{18}O was incorporated into both the inorganic phosphate and DTB formed in the DTBS catalysed reaction between DAN, ATP and $C^{18}O_2$, in an ^{18}O-enriched aqueous buffer. She also identified a product (detectable by tlc), capable of incorporating radiolabel from either $^{14}CO_2$, [9-^{3}H]- and [1,7-^{14}C]-DAN or [γ-^{33}P]-ATP, as the carbamyl-phosphate mixed anhydride **25**. A solution containing **25** was separated from a DTBS incubation mixture quenched with ammonium acetate (pH 4), at low temperature. Subsequent kinetic experiments showed that the intermediate had a lifetime at 0 °C of *ca.* 25 minutes at pH 7.5.

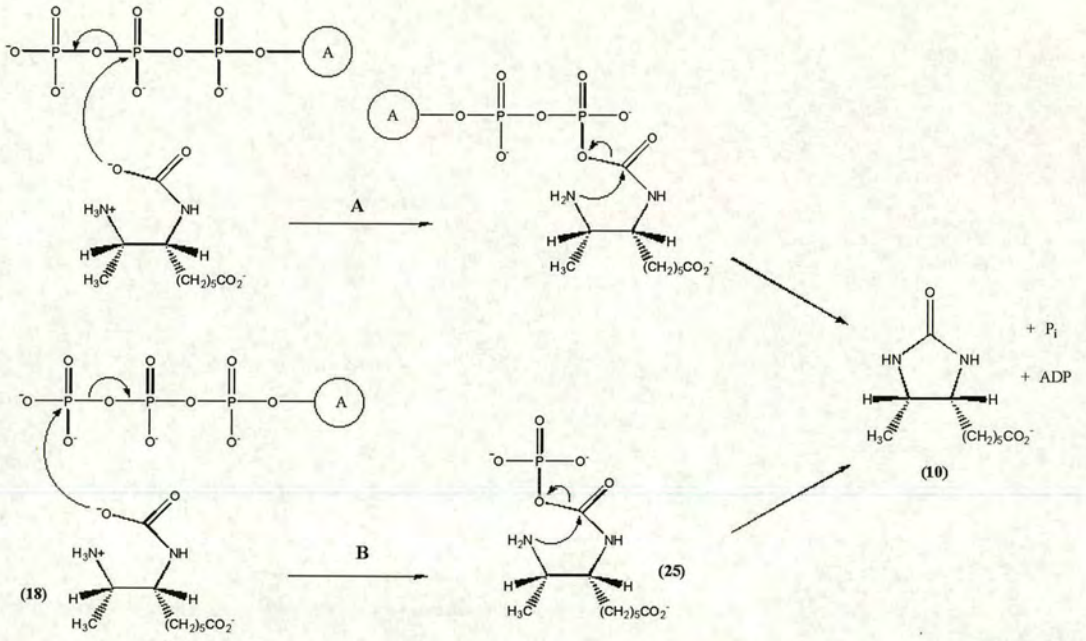


Figure 23 : Two possible ring-closure mechanisms

Gibson has also shown that the ratio of radiolabel incorporated into DAN and DTB, obtained after either acidic or basic treatment of pre-incubated mixtures of DTBS, DAN ATP and $^{14}CO_2$, is consistent with the intermediacy of a mixed anhydride species. The rationale here relies on the likelihood of the mixed anhydride being hydrolysed under acidic conditions, and ring-closing under basic conditions (as shown in scheme 24).

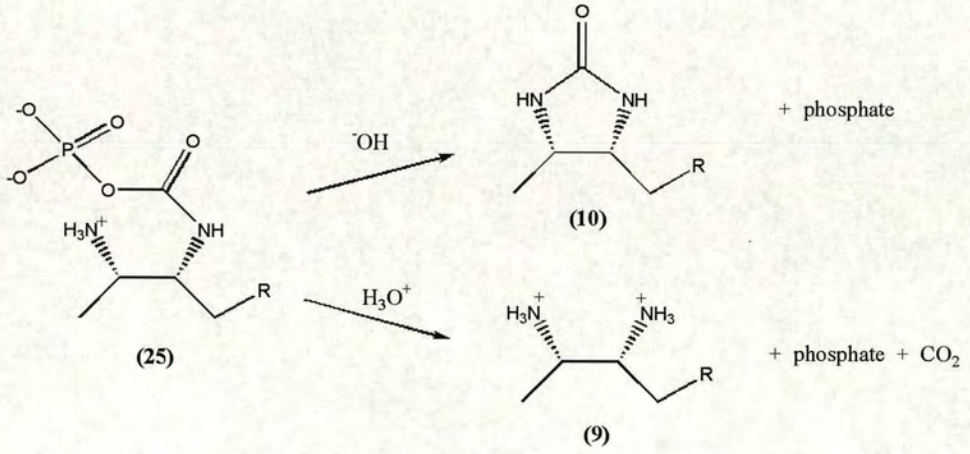


Figure 24 : Decomposition of the reactive intermediate

The availability of highly purified dethiobiotin synthetase has allowed its crystallisation and subsequent x-ray structural refinement to atomic resolution. Two groups [84, 95] have independently determined the structure of DTBS from *E. coli*, and these studies agree on the majority of issues. The DTBS monomer has been shown to consist of one subunit, belonging to the class of α/β domains. This is folded into a compact structure, with a central twisted β-sheet forming a core surrounded by α-helices (shown in figure 25).

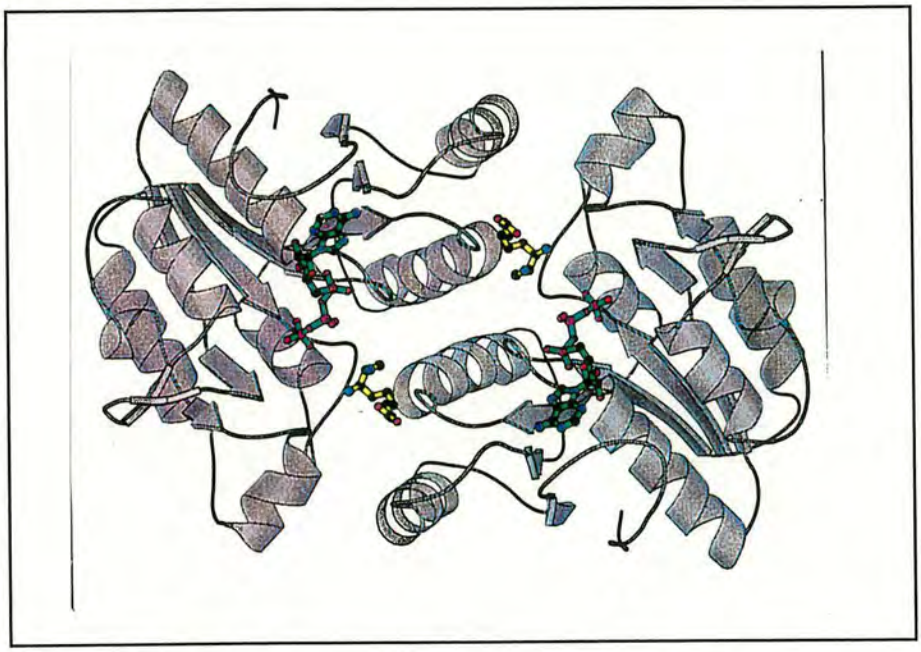


Figure 25 : General view of the DTBS dimer

The dimer is saucer-shaped, with the concave surface between the monomer units housing the putative active site. The two monomers have an extensive contact area, with many of the interactions mediated by water molecules. This may facilitate the relative movement of the monomers which is thought to occur during enzyme turnover.

Alexeev *et al.* [84] found that two sulfate ions were located at the base of the concave surface of the dimer, consistent with the positions of the phosphates in the ATP-binding site. One of the sulfates (S1 in figure 26) was positioned exactly in the loop formed by residues 8-16, indicative of a mononucleotide binding motif known as the P-loop [96].

Subsequently, x-ray analysis of DTBS which had been soaked with a solution of DAN and Mg^{2+} [90], revealed that the substrate binding site was located between the interface of the two subunits (see figure 26). One of the carboxylate oxygens was shown to bind to the main-chain nitrogen of Asn-153, with the other held between the positively polarised peptide nitrogens of Gly-150-Cys151-Ile-152 (at the start of helix α6), and the hydroxyl of Tyr187, which had moved to optimise substrate binding. In solution, this rotation was accompanied by a significant change in the absorption spectrum at 290-300 nm (in the absence of ATP). The hydrophobic alkyl chain of the bound DAN was positioned in a groove defined by the Cα of Gly188, Cγ of Thr122, Cα of Ser81 and Cδ of Pro82 on the other subunit of the native structure.

X-ray structures of the DTBS-DAN-Mg^{2+} complex in the presence of CO_2 [90], revealed the formation of a mono-carbamate at the N-7 position of the substrate. The (S2) sulfate ion (in figure 26), which was present before DAN carboxylation, is replaced by the carboxylate group of the DAN-carbamate and a water molecule (see figure 27). Thr11 becomes coordinated to water molecule W1 rather than to the N-8 amino group of the substrate, and W1 in turn forms hydrogen bonds with the oxygen of the DAN-carbamate and the ε-amino group of Lys 15.

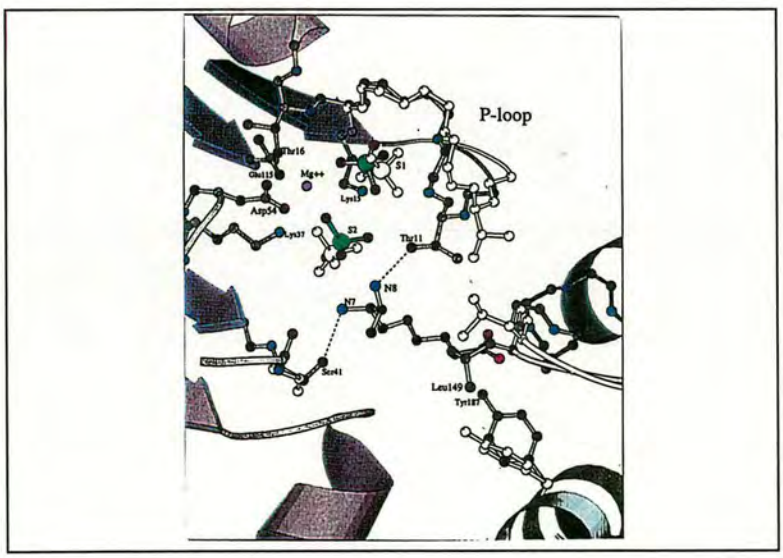


Figure 26 : The binding of DAN to DTBS under an atmosphere of N_2

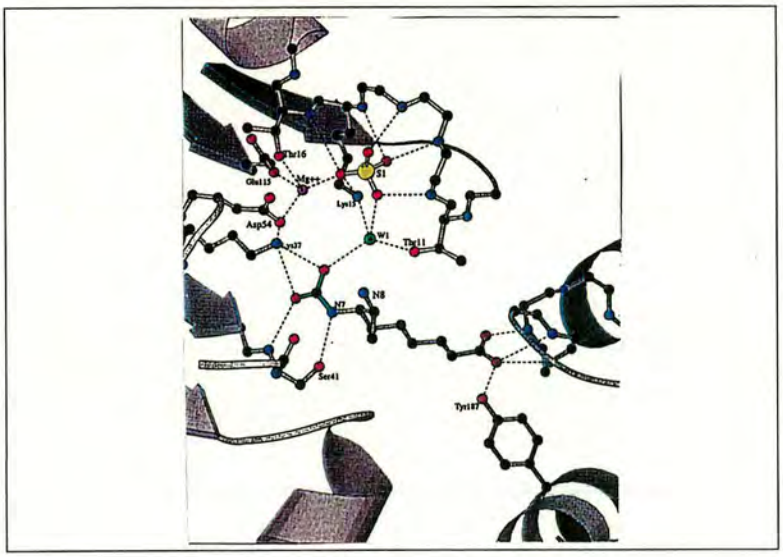


Figure 27 : The binding of DAN to DTBS under an atmosphere of CO_2

The essential roles of Lys15 and Lys37 were demonstrated by Yang *et al.* [97] through mutational studies, which showed that replacement of either one of these residues led to almost complete loss of enzymatic activity. The replacement of Thr11 with valine resulted in a 24 000-fold increase in the observed K_M(ATP), which led the authors to conjecture that this residue was essential for the steady-state affinity for ATP.

In DTBS the topology of the P-loop is identical to the classical mononucleotide-binding fold, but the sequence contains an extra residue. This forms a small bulge in the middle of the loop, occupied by Thr11, which is hydrogen-bonded to the N-8 amino group of the substrate. This interaction is essential for the correct orientation of the N-7 amine to allow carboxylation. Hence the extended P-loop simultaneously binds both the ATP and the substrate, bringing them together in order to facilitate the carbamate phosphorylation [90].

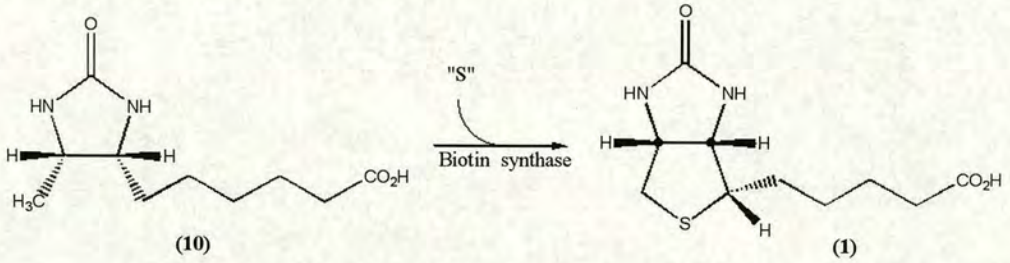
Lindqvist *et al* [98] have obtained electron density images by x-ray kinetic crystallography, showing the enzyme-MgADP-carbamic-phosphoric acid anhydride binding to the putative active-site residues. The relative position of the N-7 nitrogen atom of DAN, and the γ-phosphate of ATP, have led the authors to conjecture that the nucleophilic attack of the N-7 carbamate oxygen, on the the γ-phosphorus atom

occurs with an in-line geometry. The transferred phosphoryl group was found to have moved *ca.* 1.4 Å from its position in the ATP complex, to the one observed in the intermediate complex. This suggests that the reaction may proceed with inversion of configuration at the phosphorus atom.

The primary sequence of DTBS shows little homology with other proteins, except the analogous enzymes from *B. sphaericus*[99] *Serratia marcesens*,[100] *Brevibacterium flavum*[101] and *Mycobacterium leprae*.[102] However, the 3-D structure reveals similarities with three proteins of unrelated sequence and function; nitrogenase iron protein (NIP) from *Azotobacter vinelandii*, adenylosuccinate synthetase (purA) from *E. coli* and the Ha-ras oncogene product p21.[84] All four enzymes are homodimers, but there is no similarity in the dimer organisation. There is however a shared P-loop motif, which suggests that nucleotide binding may be the major common feature between this diverse group of enzymes.[90]

1.8.5 The *bio*B Gene Product : Biotin Synthase

Biotin synthase catalyses the final step in biotin biosynthesis, the incorporation of a sulfur atom into dethiobiotin **(10)** to give biotin **(1)**.



(10) (1)

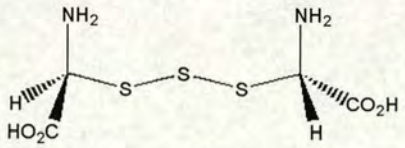
Figure 28 : The biotin synthase catalysed process

1.8.5.1 The Sulfur Donor

Lezius *et al.*[38] in their original proposal for the biotin biosynthetic pathway used tracer studies with *Achromobacter* as a basis for their suggestion of a precusorial role

for L-cysteine. However reinvestigation by Marquet *et al.*[103] using incorporation experiments with [3-^{14}C,^{35}S]-cysteine, failed to yield radioactive biotin and demonstrated that this pathway was non-operational in *Achromobacter*. Niimura *et al.*,[104] using sulfur-starved cells of *S. cerevisiae*, found that methionine, methionine sulfoxide and Na_2S were the most effective sulfur donors, and that cysteine and cystine were inactive. Labelling experiments[105] with [^{35}S]-methionine gave radioactive biotin, a result later corroborated by Shimada[106] with tracer studies in *A. Niger*. These apparently anomalous results may be due the cells being unable to incorporate exogenously supplied cysteine, as it has been demonstrated in *E. coli* that only cystine can be transported and metabolised by the cell.[107]

Although lipoic acid was shown to have a stimulatory effect upon biotin production in *A. niger*, only low levels of [^{35}S] incorporation were observed when [^{35}S]-lipoic acid was administered, suggesting that lipoate was not the sulfur donor.[108]



(26)

Figure 29 : Thiocystine (26)

Thiocystine **(26)**, a source of both organic reduced sulfur (i.e. as cysteine), and inorganic sulfane, has been used by a number of researchers to probe the mechanism of sulfur uptake into biotin. Radiolabelling feeding experiments with thiocysteine, carried out by White[109] found that both forms of sulfur were incorporated into biotin in approximately equal amounts. However, DeMoll and Shive[110,111] using competitive feeding experiments, demonstrated that cysteine was a more effective sulfur donor than thiocystine in *E. coli*, and that radiolabel from [^{35}S]-methionine was not incorporated into biotin.

Recent studies on cell-free systems[112] suggest that cysteine is the source of sulfur in biotin biosynthesis, but this remains to be proven in purified systems.

1.8.5.2 The Mechanism of Biotin Synthase

On the basis of radiolabel feeding studies with *A. niger,* Li *et al.*[113] conjectured that the conversion of DTB to biotin involved two desaturation steps in the overall mechanism. However, uncertainties arising from the use of randomly tritiated DTB in these double label experiments prompted Parry to reinvestigate these findings through the administration of specifically tritiated forms of dethiobiotin. These studies[114,115] revealed that the introduction of sulfur at the C-6 and C-9 positions took place without loss of hydrogen from C-7, C-8 or C-5. This suggests that a mechanism which involves desaturation at these positions is unlikely, although it does not exclude the possibility of specific enzymatic proton removal and replacement at these sites. Parry showed that the incorporation of [9-^{3}H]-(±)-DTB into biotin proceeded with ca. 30 % tritium loss, and that 6(RS)[6-^{3}H]-(±)-DTB was incorporated with 47 % tritium loss. Both of these results are consistent with the removal of one hydrogen from the C-9, and one hydrogen from the C-6 positions in the enzymatic transformation. Parry's work with the eukaryotic *A. niger* was complemented by research from Marquet's group with the prokaryotic *E. coli* organism,[116,117] who found that the administration of [7,8-^{3}H]-(±)-, [7,8-$^{2}H_2$]-(±)- and [5-$^{2}H_2$]-(±)-DTB resulted in the formation of biotin with no loss of label.

The synthesis of chirally tritiated dethiobiotin by Parry [118] has allowed the precise nature of the stereochemical events occurring at the C-6 position, during the sulfur insertion process to be elucidated. [6-(R)-^{3}H]-dethiobiotin **(27)** yielded biotin with 91 % retention of label upon administration with 10-^{14}C dethiobiotin to *A. niger*, whereas [6-(S)-^{3}H]-dethiobiotin **(28)** yielded biotin with 93 % loss of label. Thus the 6-*pro*-S-hydrogen is lost at the C-6 position with retention of configuration.

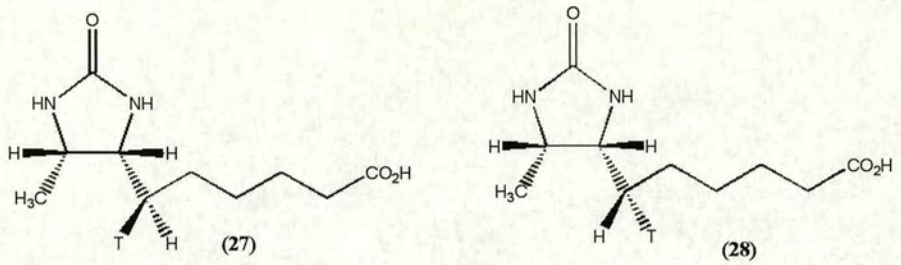


Figure 30 : Chirally tritiated DTB molecules (Parry *et al.*)

Arigoni[119] attempted to investigate the stereochemistry at the C-9 position, through feeding studies using DTB containing a chirally labelled methyl group ([9-^{3}H, ^{2}H]-dethiobiotin, **29**). The isolation of biotin configurationally scrambled at the C-5 position (i.e. the tetrahydrothiophene ring methylene), led to the proposition that functionalisation of this position involves a methylene radical intermediate (see figure31).

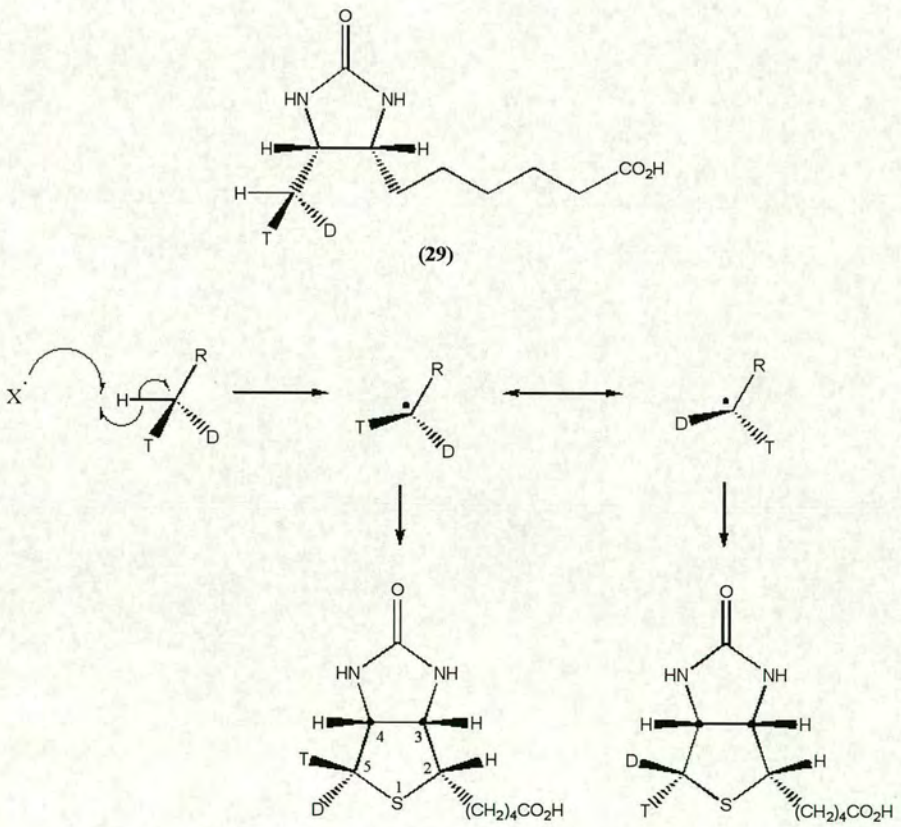


Figure 31 : Proposed mechanism of scrambling (Arigoni *et al.*)

Whilst the retention of stereochemistry at the C-6 observed in the bioconversion of DTB to biotin suggests that the functionalisation mechanism may be a single step process, both hydroxyls[120,121] and thiols[122,123] have been proposed as intermediates.

Marquet[120] has provided evidence against the intermediacy of hydroxylated forms of dethiobiotin by showing that neither 9-hydroxydethiobiotin nor 6(R)- and 6(S)-hydroxydethiobiotin could support the growth of the *E. coli* C124 mutant auxotroph. This was supported by the findings of Emoto *et al.*[121] through analogous studies with 6,9-dihydroxydethiobiotin. However, these results do not rule out the involvement of transient enzyme-bound hydroxylated intermediates.

The thiol containing compounds 6- and 9-mercaptodethiobiotin, have been proposed as intermediates in the tetrahydrothiophene ring-forming process (see figure 31).[122,123]

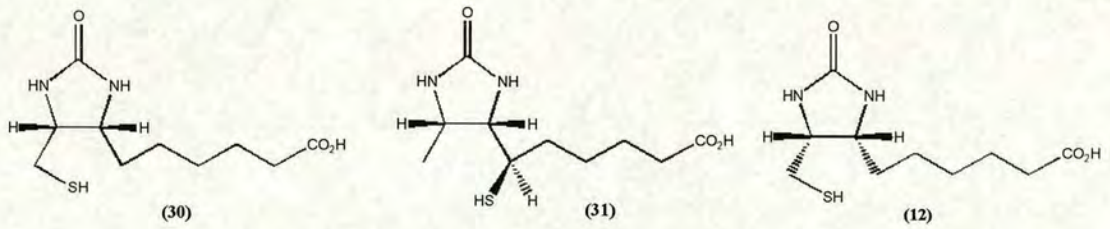


Figure 31 : Proposed thiol intermediates

Baxter[123] has shown that synthesised (±)-9-mercaptodethiobiotin **(30)** can support normal growth of an *E. coli bio*A mutant (strain 6435) which requires DAN, DTB or biotin for growth. Marquet *et al.*[122] demonstrated that the secondary thiol **31** and to a lesser extent **30** could replace DTB or biotin in a biotin-auxotrophic strain of *B. sphaericus*. However, labelling studies using [^{35}S]-**(31)** produced biotin devoid of radioactivity. Biological evaluation of the thiols **30** and **31** in resting cells of *B. sphaericus* revealed that only the primary thiol **30** was transformed into biotin with only 10 % observed conversion yield compared with dethiobiotin. However,

subsequent incubation of [^{35}S]-**(12)** with the resting cells in the presence of DTT indicated clearly that the thiol was converted into biotin without degradation.

A recent investigation by Baldet[124] has identified **12** as an intermediate in the biotin biosynthetic pathway in cells of *Lavandula vera*. The isolated thiol was subsequently shown to be as effective as biotin in supporting the growth of the *bio*B105 strain of *E. coli*. Shaw[112] has recently reported the isolation of **12** from a cell free extract containing biotin synthase from *E. coli*, providing additional evidence supporting the intermediacy of 9-mecaptodethiobiotin **(12)** in the biotin synthase process.

The development of overexpression systems capable of producing relatively large amounts of protein in *bio*B transfromants has led to the purification of the protein encoded by the *E. coli bio*B gene.[125] The active form of the *bio*B gene product appears to be a homodimer (82 kDa) which contains one [2Fe-2S] cluster per monomer. However, the *bio*B gene product alone is incapable of converting dethiobiotin to biotin, with an additional number of other proteins and low molecular weight compounds being essential for catalytic competence in cell-free extracts. The components which have thus far been suggested to be essential[112] are; flavodoxin, flavodoxin NADPH-dependent oxidoreductase, a third protein fraction that requires thiamine pyrophosphate to maintain its activity during purification, cysteine, S-adenosylmethionine, Fe^{2+}, thiamine pyrophosphate, one of the amino acids asparagine, aspartate, glutamine or serine, and NADPH.

1.9 The Aims of Research

Recent studies have suggested that the biosynthesis of biotin proceeds by an apparently identical pathway in higher plants, fungi and bacteria,[124] *via* transformations that have few parallels in mammalian biochemistry. Higher organisms such as humans, do not need to synthesise their own biotin as a sufficient amount is produced by enteric flora. These facts, allied with the lack of an active transport system for biotin in plants and micoorganisms, makes the biosynthetic pathway an attractive target for inhibition studies. Consequently, the main corpus of this research was directed towards the identification of, and the development of synthetic approaches to, possible inhibitors of biotin biosynthesis.

The first main objective was to synthesise the first two intermediates of the biotin biosynthetic pathway: namely pimeloyl-Coenzyme A and 8-amino-7-oxononanoic acid (AON), to be used in the study of the AONS and DANS enzymes from *E. coli.* The second objective was to develop general methodology applicable to the synthesis of compounds structurally related to AON.

Chapter Two

The Synthesis of Intermediates from the Biotin Biosynthetic Pathway

2.1 The (Chemical) Synthesis of Pimeloyl-CoA

Two methods for the synthesis of pimeloyl-CoA **(7)** have been reported in the literature: i) a pimeloyl-CoA synthetase (from *B. sphaericus*) catalysed condensation of HSCoA and pimelic acid,[124] and ii) a trans-thioesterification reaction involving the displacement of an aromatic thiol from pimelic acid by coenzyme A, in a mildly basic solution.[59,76] However, the reports on these procedures are extremely vague, and none of the authors give yields or proper analytical data on the product. Indeed, most have relied upon a hydroxamate,[76,126] or Ellman's-type[59,126] assay to estimate both purity and yield.

The procedure we selected for the synthesis of pimeloyl-CoA **(7)**, was that described by Ploux *et al.* [76] (see figure 32).

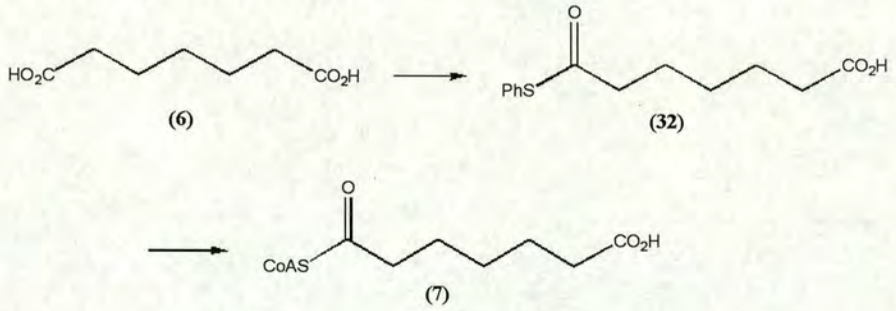


Figure 32 : Synthesis of pimeloyl-CoA

Treatment of the di-acid chloride of pimelic acid with thiophenol in DCM afforded the thiophenol mono-ester **32** in 32 % yield. The low yield is due to the formation of a significant amount of the dithioester. However, we elected not to optimise this step as **32** could easily be separated from the reaction mixture and only small amounts were required. The treatment of HSCoA with **32** in aq. $NaHCO_3$ at pH 8 gave the desired product **7**, contaminated with unreacted starting materials and hydrolysis by-products. This was extracted with DCM to remove thiophenyl pimelate, thiophenol and pimelic acid, leaving an aqueous layer containing **7** and HSCoA.

Two protocols were evaluated for the purification of the product. The first method used was anion-exchange chromatography (using a MONO-Q column on FPLC),

eluting with a linear gradient of 0 to 1.5 M triethylammonium buffer. The eluant was monitored continuously at 260 nm. Unreacted HSCoA was the main contaminant, and eluted before the pimeloyl-CoA. Lyophilisation of the product containing fractions afforded **7** as a colourless solid which was characterised by mass spectrometry (MALDI-TOF, MH^+ 910.0) and by the comparison of its chromatographic properties on paper with those reported in the literature.[59]

The second protocol employed was preparative reverse-phase HPLC, which was found to a more efficient method of purification. A 5-50 % linear acetonitrile / water gradient over 30 minutes was found to be optimal. The eluant was continuously monitored at λ = 214 nm, so that non-aromatic impurities (i.e. compounds that did not contain an adenine, or a thiophenol moiety) and salts could also be detected, giving a better indication of the product purity.

Initially the yields of pimeloyl-CoA obtained were less than 10%, although by modification of the reaction conditions and changing the method of purification, this was improved to *ca.* 60%. It was observed that maintenance of the correct pH during the reaction course was of critical importance, and the use an inert atmosphere (preventing HSCoA oxidation) was also advantageous.

2.2 The Synthesis of 8-amino-7-oxononanoic acid (AON)

2.2.1 The First Synthetic Approach to AON

Prior to this work the only reported synthesis of 8-amino-7-oxononanoate (AON) (trivial name 7-keto-8-aminopelargonic acid, KAPA) was in a Japanese patent.[127] However, as other laboratories had encountered problems in repeating this procedure it was decided that a new synthetic route would be undertaken.

Previous work in our laboratories towards the synthesis of AON-related compounds had centred on the use of cyclohexane derivatives such as **33** being key intermediates in the overall synthetic strategy (see figure 33). This type of methodology has been

used extensively in the literature in the preparation of various 1,6-difunctional compounds.[128] The Baeyer Villiger oxidaton[129] of the cyclohexane ring to afford the corresponding lactone, and the functional group interconversion (FGI) of the alcohol to the amine (shown in figure 33) were completed successfully, by a previous lab worker, for R = Et and Me, to generate 6-aminooctanoic acid and 6-aminoheptanoic acid respectively.[130]

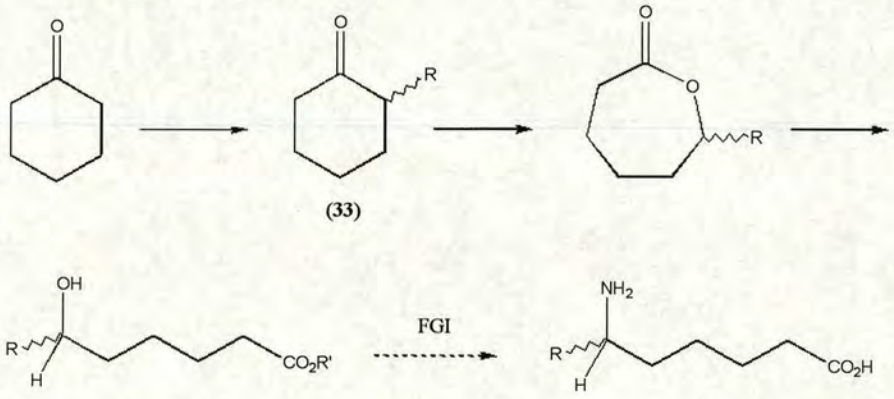


Figure 33 : The preparation of long-chain amino-acids

In the first attempted synthesis of 8-(R,S)-amino-7-oxononanoic acid **(8)**, a strategy related to the one above was formulated (see figure 34). Enamine chemistry could be used to construct a substituted cyclic ketone **36**, which could be oxidised under Baeyer-Villiger conditions to give the corresponding lactone **37**. De-methylation of the ether moiety, possibly by iodotrimethylsilane,[131] would give the hydroxyl compound **38**, which could then be selectively converted to the amine **34**, whilst the adjacent hydroxyl remained protected as a lactone (corresponding to the three dashed arrows). Subsequent methoxide-mediated lactone ring-opening, followed by oxidation and saponification would give the desired product, (±)-AON.

Cycloheptanone was converted to its morpholine enamine **35** in moderate yield as described by Stork *et al.*.[132] Lewis-acid catalysed condensation of **35** with the dimethyl acetal of acetaldehyde[133] proceeded in modest yield, with significant amounts of polymeric impurities being formed. The two expected diastereomers were formed in a 2 : 1 ratio, as estimated by the comparison of the integrals associated with

the methyl protons at the C-9 and C-10 positions. However it was not possible to assign specific resonances to either the *threo* or the *erythro* diastereomers due to the complicated, and broad nature of the NMR spectrum obtained (due to the various conformations that may be adopted by the cycloheptane ring). The multiplet at 3.59 ppm (associated with the C-8 methine proton) is poorly defined, and the signal due to the C-7 methine proton is hidden under the complex broad multiplet corresponding to the C-2 methylene protons.

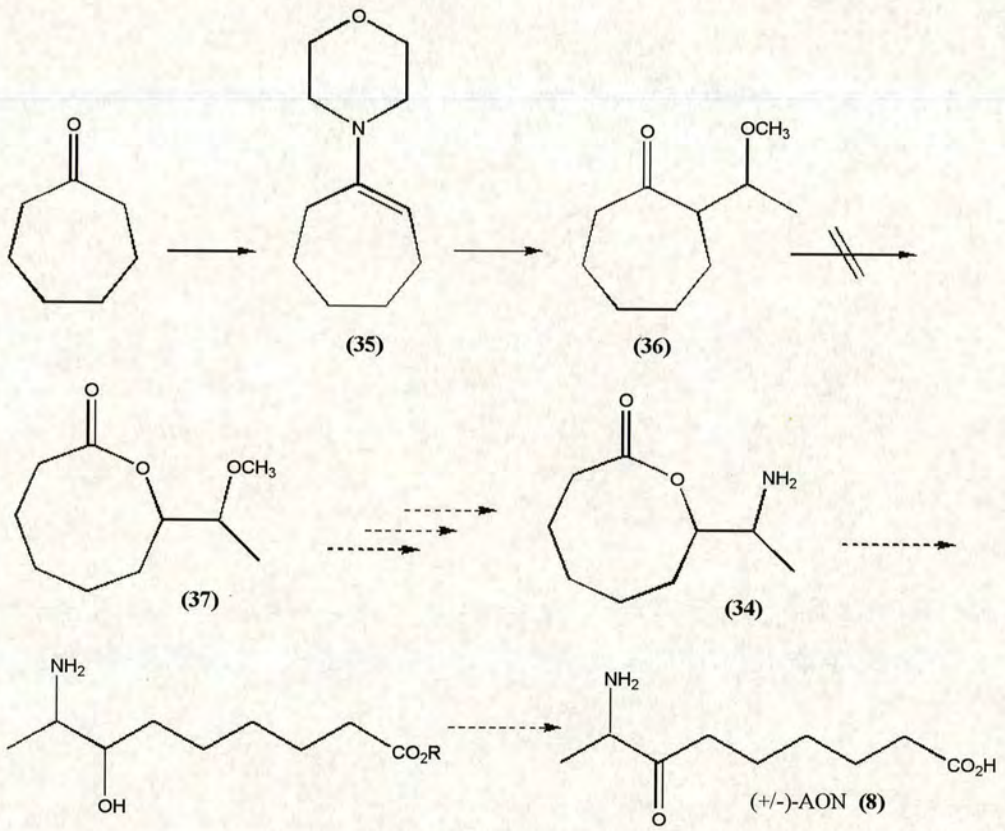


Figure 34 : First attempted route to (±)-AON

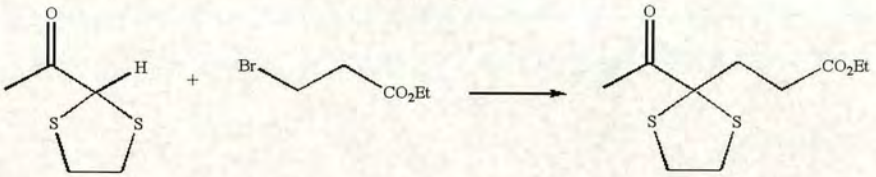
The Baeyer-Villiger lactonisation of the diastereomeric cyclic ketones **36** to afford the substituted lactones **37** proved extremely problematic, and could not be successfully performed under a variety of oxidative conditions. The reaction of **36** with *m*-chloroperbenzoic acid, trifluoro-peracetic acid (generated *in situ* from trifluoroacetic anhydride and urea-hydrogen peroxide) and urea-hydrogen peroxide[134] under various conditions did not give any detectable amounts of the desired compounds.

A number of examples where alkyl-substituted cycloheptanones have been stereospecifically oxidised to the corresponding octolactones have been reported in the literature.[135,136] Analogously, investigations performed within our laboratories [137] have shown that the Baeyer-Villiger oxidation of the α–methyl, and α-ethyl derivatives of cycloheptanone proceed cleanly and in high yield with various oxidants, to give the corresponding substituted cyclic lactones. This makes the failure of this particular Baeyer-Villiger oxidation appear quite unique.

With the considerable problems associated with the oxidation of the ketone encountered at a fairly early stage in the reaction pathway, this approach was abandoned and an alternative strategy was developed.

2.2.2 The Second Synthetic Approach to (±)-AON

Dauben and Walker[138] relied on the alkylation of the anion of ketothioacetal **38** with ethyl 3-bromopropanoate, to generate a key intermediate **39** in their total synthesis of the sesquiterpene isocomene (see figure 35).



(38) (39)

Figure 35 : Synthesis of the α-oxodithiolane in Isocomene Synthesis

If ethyl 6-bromohexanoate was used instead of ethyl 3-bromopropanoate, this would generate the desired nonanoic acid backbone with a mono-thioketal-protected α-diketone moiety at the 7 and 8 positions. The reductive amination of the free ketonic-carbonyl would yield a masked α-oxoamine, which could be deprotected to give the desired product, (±)-AON (see scheme 36).

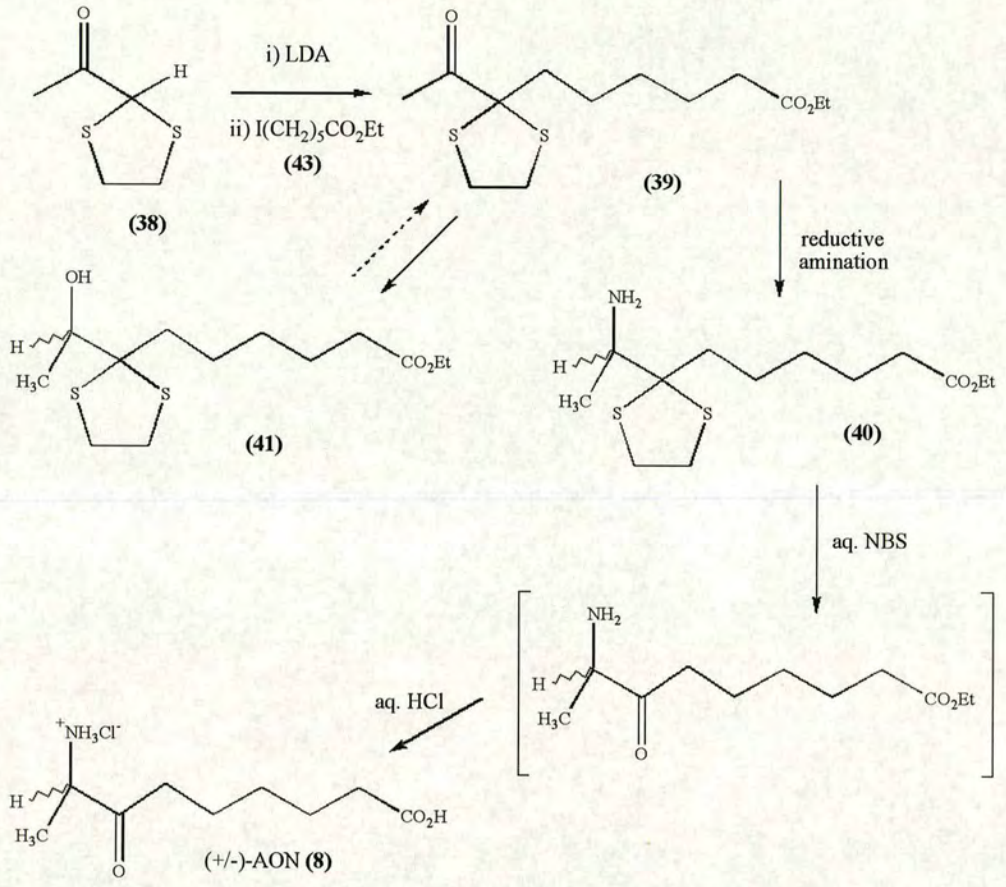


Figure 36 : Synthesis of (±)-8-amino-7-oxononanoic acid

The synthesis of acetyl-dithiolane **38** was performed as described by Leir,[139] in a two step process with an overall yield of 55 % (see figure 37). The purification of the intermediate **42** proved non essential, and the desired product could be obtained as a (pungent smelling) golden oil by distillation of the final crude reaction mixture.

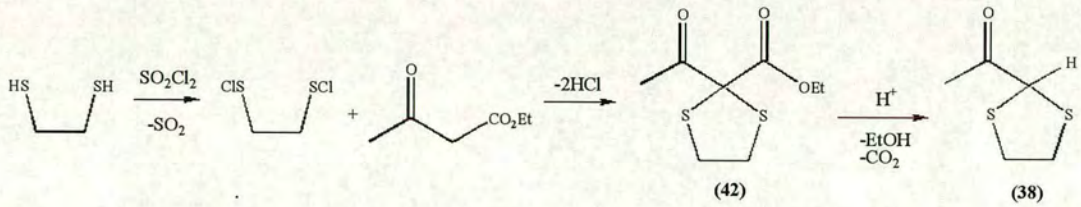


Figure 37 : Synthesis of 2-acetyl-1,3-dithioloane (38)

However, the alkylation of the lithiate of **38**, to generate the α-oxodithiolane **39** proved more difficult. Reaction of dithiolane **38** with LDA in THF at -78°C, gave a deep red-coloured anion that was unreactive towards ethyl 6-bromohexanoate, even in excess, for prolonged periods, and at elevated temperatures. This proved also to be the case when DMF, or HMPA were used as co-solvents.

The bromo-ester was quantitatively converted to the more reactive iodo-compound **43** *via* reaction with sodium iodide.[140,141] When a solution containing a slight molar excess of **43** in HMPA, was added to the lithiate of **38** in THF at -78°C and the reaction was allowed to proceed at RT for 24 hours, the desired product **39** was isolated in 96% yield.

The reductive amination of the ketone **39** was initially performed using the conditions described by Borch and *et al.*[142] using ammonium acetate. The yields of amine **40** obtained from the reaction were capricious and never above 50 %, even when the regents were scrupulously purified before use. Changing the dehydration agent from 4Å to 3Å molecular sieves, or to $CaCl_2$, and altering the molar ratio of sodium cyanoborohydride present, had little effect upon the yield of **40** obtained.

Another drawback from the use of this one-pot, imine formation and reduction process, was the significant level of alcohol **41** formed in a side reaction, from the cyanoborohydride reduction of the unreacted ketonic compound **39**.

An attempt to recycle the alcohol byproduct **41**, *via* a Jones oxidation[143] of the hydroxyl function, to give the starting ketone **39** was unsuccessful. The sole isolated products were tentatively assigned as an inseparable mixture of *cis* and *trans* ethyl 8-oxo-7-(1,3-dithiolan-1,3-dioxide)-nonanoate **(44)** (see figure 38).

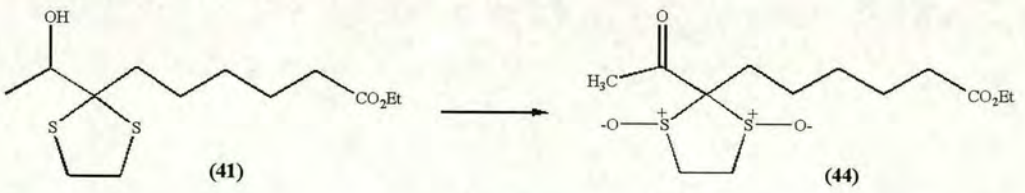


Figure 38 : Oxidation of the alcohol 41

Because of the complications with the Borch reductive amination an alternative two-step procedure was evaluated.[144] Ketone **39** was reacted with hexamethyl disilazane, then reduced with a methanolic solution of $NaCNBH_3$. Hydrolytic workup afforded the primary amine **40** (see figure 39). The use of this method eliminated the formation of the alcohol byproduct **41**, but led only to a modest improvement in the amount of **40** obtained (*ca.* 60%).

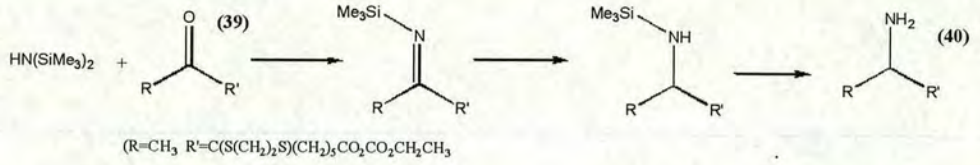


Figure 39 : Reductive amination of 39 with hexamethyl-disilazane / $NaCNBH_3$

The removal of the thio-acetal protecting group of **40** was achieved rapidly (5 minutes) and in high yield (>90 %) by the addition a solution of **40** in acetonitrile, to a stirred solution of N-bromosuccinimide in ice-cold 80 % aq. acetonitrile.[145] The crude product from this step was not purified, but was immediately added to a dilute aqueous solution of acetic acid and HCl to afford the N-protonated form. This reduced the potential for self condensation of the product. Previous work in our laboratories had demonstrated that α-oxoamines are prone to rapid pyrazine formation under neutral or basic conditions[146] (see figure 40).

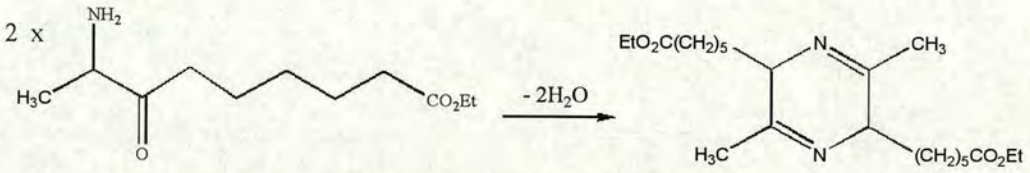


Figure 40 : Pyrazine formation via self-condensation

The AON salt formed, was separated from succinimide, and other minor impurities, by ion exchange chromatography on Dowex-50X resin (H^+ form) eluting with a linear 0 to 1.5 M HCl gradient. Lyophilisation afforded (±)-8-amino-7-oxononanoic acid as its hydrochloride salt. The colourless crystalline material is stable at room temperature, and is not extremely hydroscopic.

2.3 The Synthesis of 8(S)-amino-7-oxononanoic acid

With a satisfactory preparation of the racemic product developed, we began work on developing a stereospecific route to 8(S)-amino-7-oxononanoic acid, the biologically active isomer.

A small number of methods for the synthesis of chiral α-oxoamines exist in the literature.[147,148] Schollkopf *et al.*[147] have synthesised a selection of α-oxo-α-amino-acid esters by the reaction of lithiated *bis*-lactim ethers of 2,5-diketopiperazines with acid chlorides, followed by acidic hydrolysis. Holladay and Rich in their synthesis of a statine isostere,[148] utilised a Grignard addition to Boc-protected leucinal, followed by an oxidation, to generate a chiral α-oxoamine. Analogously, N-protected amino-acid aldehydes have been utilised extensively in the synthesis of chiral β-amino alcohols.[149,150]

Previous work in our laboratories[146] has shown that N-Boc protected alaninal **45**, was unreactive to long-chain Grignard reagents such as **50** (see figure 41). Consequently we decided to synthesise N,N-dibenzyl-alaninal **47**, as it has been demonstrated that this is reactive to a wide range of nucleophilic reagents,[151,152] including Grignard reagents, to afford a wide range of β-amino alcohols in high yield, and with high diastereomeric selectivity.

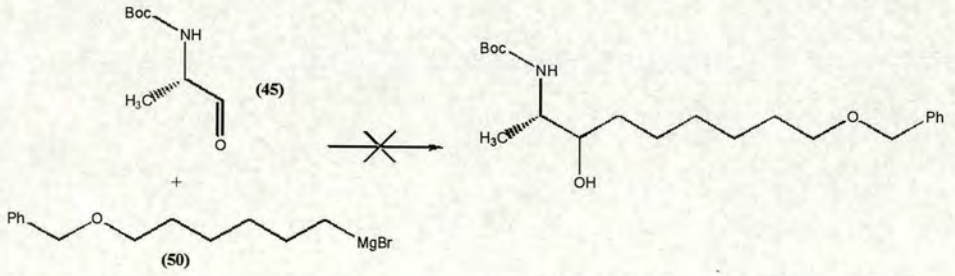


Figure 41 : Previously-attempted Grignard addition (Wang Pu)

The synthesis and subsequent reaction of N,N-dibenzyl-alaninal with long-chain carbon nucleophiles, should indicate whether the N-Boc-aldehyde is inherently less

reactive, or whether its chemical inertness is due to the nature of the nucleophile used, and the conditions employed.

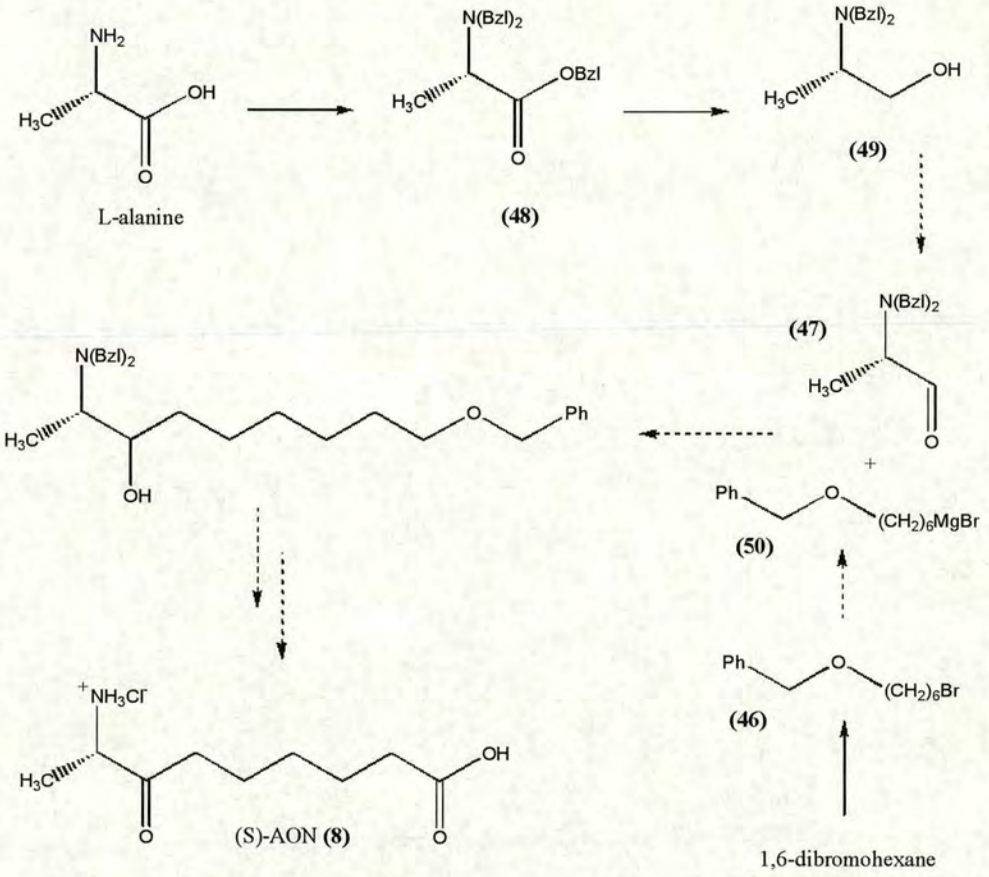


Figure 42 : Proposed route to 8-(S)-AON (hydrochloride salt)

The benzyl ester **48** was prepared in 76 % yield from L-alanine and benzyl bromide using a standard procedure.[151] The ester was smoothly reduced to the corresponding alcohol **49**, within 4 hours using a 3 molar equivalence of $LiAlH_4$ slurried in dry ether at 0°C,[153] in 84 % isolated yield. Using methodology previously developed in our laboratories,[146] the bromo-ether **46** was prepared in 36% yield from 1,6-dibromohexane and sodium benzoxide.

At this point in the synthesis of 8-(S)-amino-7-oxononanoic acid (HCl salt), Mioskowski *et al.*[154] published a stereospecific synthesis of both the (R) and (S) enantiomers of AON. This was accomplished by a similar route starting with N-Boc-

alanine as shown in figure 43. For this reason it was decided not to pursue the synthesis further.

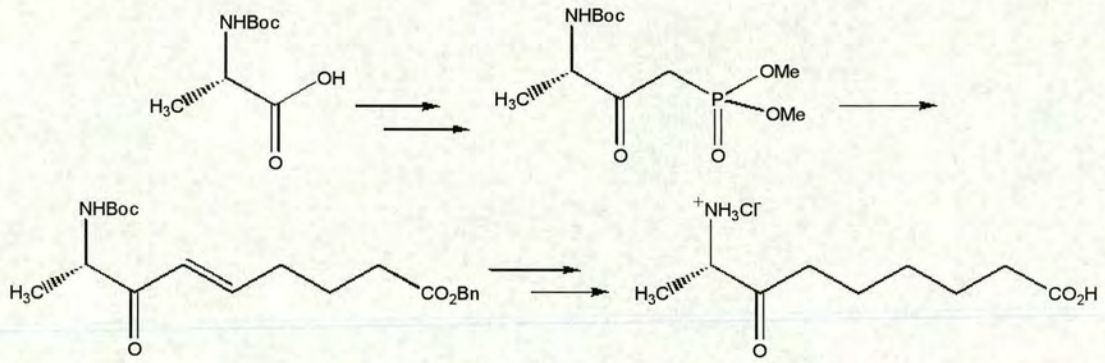


Figure 43 : Synthesis of 8(S)-AON (Mioskowski *et al.*)

2.4 The Biosynthesis of 8-amino-7-oxonoanoate in *E. coli*

2.4.1 The Cofactor Pyridoxal-5'-Phosphate

The first two enzymes of the known biotin biosynthetic pathway in *E. coli* are dependent upon the cofactor pyridoxal-5'-phosphate (PLP).[56]

PLP has a cofactorial role in a large number of enzymes, which fall into five main categories:

1) Racemases
2) Decarboxylases
3) Deaminases and transaminases
4) Decarboxylative synthases
5) Enzymes which catalyse elimination / addition reactions

The cofactorial role of PLP in these enzymes is to act as an electron sink, and stabilise carbanionic intermediates that develop during enzymatic catalysis. The conjugation between the imine and the hetero-aromatic pyridine ring results in an efficient delocalisation of negative charge.

The PLP-dependent enzymes function *via* :

1) initial imine formation,

2) chemical transformations *via* carbanionic intermediates, and

3) hydrolysis of the product imine

Pyridoxal phosphate catalyses a wide variety of chemical reactions at the α–,β–and γ–positions of the common amino-acids, and at the corresponding positions of other biologically important amino compounds. Both the *bio*F and *bio*A gene products utilise the chemistry of PLP to afford chemical transformations at the α–carbon centre.

Dunathan[155] has postulated that the residues within the active site are positioned so as to orientate the PLP-external aldimine in a certain way, to place one of the Cα sustituents perpendicular to the plane of the aldimine π-system. This 'freezing' of rotation around the Cα-N bond can be achieved through a combination of ionic and steric interactions between the substrate and the active site residues. This particular σ-bond will then be cleaved in a facilitated manner due to the large overlap between its HOMO with the LUMO of the extended π-system of the imine and the pyridinium ring (see figure 44). X-ray crystallographic studies have shown this relationship to hold true for all the systems studied.[156]

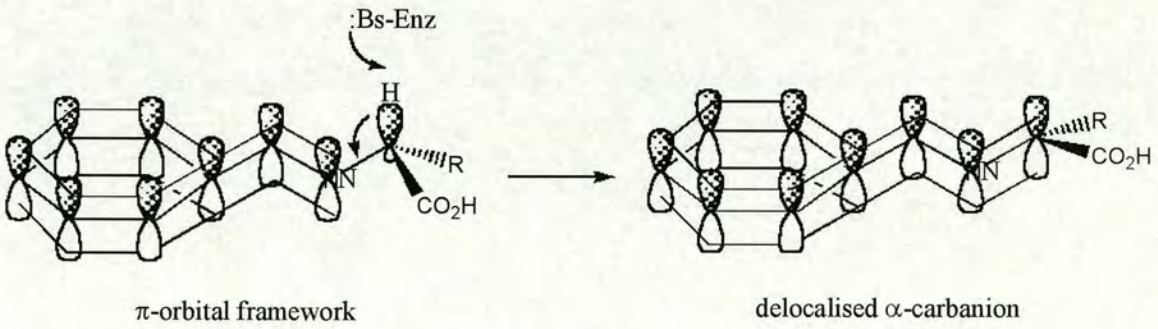


Figure 44 : Diagram showing the α-hydrogen bond orientated perpendicular to the plane of the conjugated π-system of the PLP-aldimine

Thus it is the nature of the enzyme that governs the reactivity of the substrate-PLP aldimine, leading to a specific pathway of catalysis being followed. This prevents the wide variety of reactions which PLP can catalyse from occurring simultaneously. Consequently the efficacy of the enzyme lies not only in the facilitation of certain chemical processes, but in the prevention of possible alternatives.

2.4.2 The Structure and Proposed Mechanism of AONS from *E. coli*

AONS, along with 5-aminolaevulinate synthase (ALAS), serine palmitoyl transferase and 2-amino-3-oxobutyrate CoA ligase, belongs to the small sub-group of PLP-dependent enzymes which catalyse the condensations between amino-acids and CoA thioesters, with concomitant decarboxylation.

The x-ray crystal structures for the *apo*- and PLP-bound forms of AONS from *E. coli* have recently been determined to 1.65 Å resolution by workers in our group [72].

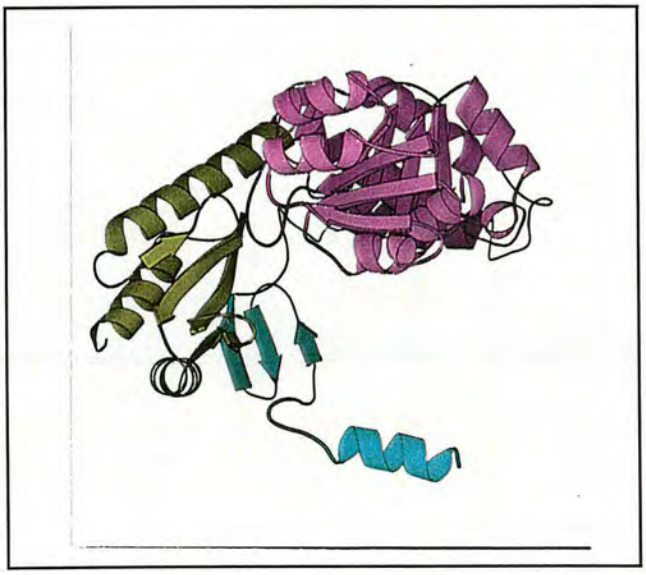


Figure 45 : Monomeric structure of AONS

The active form of the enzyme is a homodimer, with each monomer consisting of three subunits. There is a small N-terminal (blue) domain linked to a central (major)

domain (purple) which consists of a seven-stranded β-sheet. The sheet is flanked by two α-helices, and is curved around two more. This is joined to a C-terminal domain (yellow) comprising of approximately 100 residues (see figure 45).

The overall structure can be likened to an open left hand, with the thumb representing the N-terminal helix, and the fingers being the α-helices of the central domain. The rest of the N-terminal domain and the C-domain form the ball of the thumb and the heel of the hand, with the remainder of the palm and the fingers being the central domain. The N-terminal α-helical "thumb" lies on top of the α-helical "fingers" of the opposite monomer of the dimer forming an "intermolecular strap" (see figure 46).

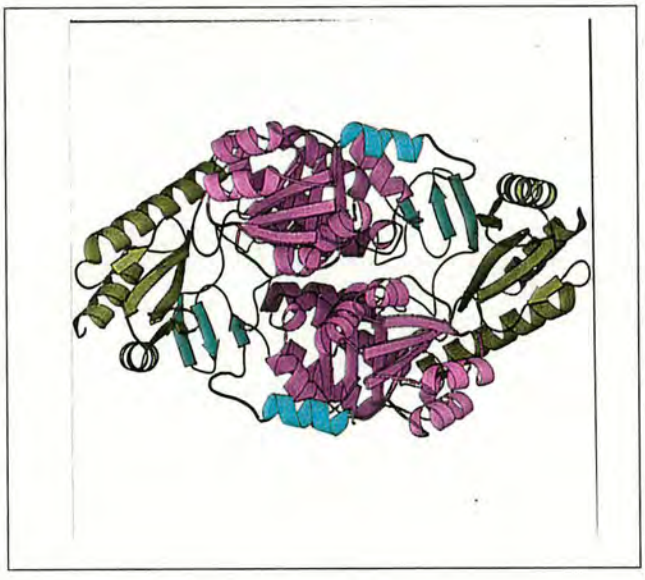


Figure 46 : The active, dimeric form of AONS

The PLP cofactor is attached to Lys236 located at the base of a deep cleft, which allows space for the pantotheinate arm of CoA to be admitted. A cluster of positively charged residues at the entrance to this cleft, near the centre of the dimer, forms the putative diphosphate binding site for CoA.

Although AONS has a low overall sequence homology with the catalytic domains of the other α-oxoamine synthases, there are a number of key residues, which have been identified as active-site components in AONS, that are highly conserved (in AONS

these are His133, Glu175, Asp204, His207, Lys236 and Tyr264). This suggests that the organisation of the conserved catalytic residues within the active site is similar for all the enzymes of this sub-class of the PLP-dependent enzymes, and they share a common mechanism.

Modelling studies[72] have shown that the Cα proton of the PLP-Ala external aldimine points towards Lys236, which can move between C4' and the phosphate group of PLP, suggesting that it may be the residue that carries out the deprotonation. The acyl-CoA must then approach from the opposite *si*-face of the first quininonoid intermediate. Subsequent decarboxylation to give the second quinonoid intermediate may be assisted by PLP or the β-ketone. The final protonation must then occur from the *re*-face of the complex to achieve the observed stereochemistry (see figure 47). This scheme is in accordance with the the mechanism suggested by Ploux *et al* [78] for AONS, and with the mechanisms of other related PLP-dependent enzymes.[157]

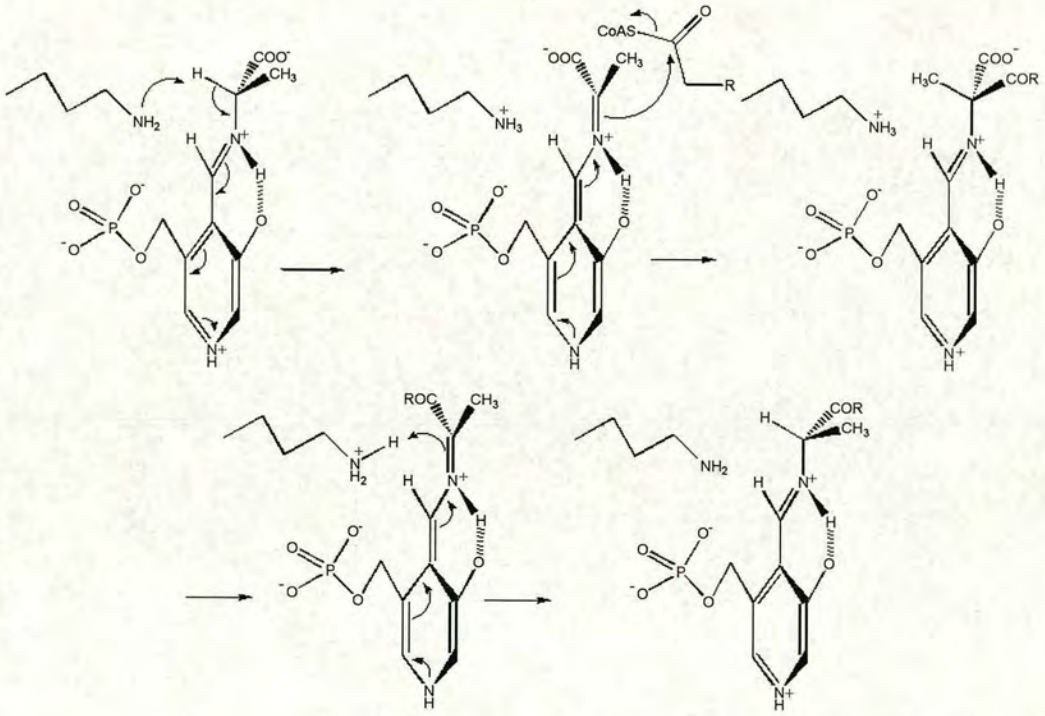


Figure 47 : Proposed AONS mechanistic scheme

At this moment the structure of the alanine-PLP (external) aldimine bound in the active site of AONS has not been resolved. However, the x-ray crystal structure of AONS with synthesised AON in the binding site has recently been solved [157] (see figure 48). As in the internal aldimine structure, His133 adopts a position parallel to the pyrimidine ring system creating an important π-interaction, as well as forming a H-bond with the ketone oxygen of AON. Arg21 anchors the carboxylate tail of AON firmly with two hydrogen bonds. Ser179 and His207 are both hydrogen bonded to the O3' of the pyridoxal phosphate, which is in turn hydrogen bonded to the iminium nitrogen.

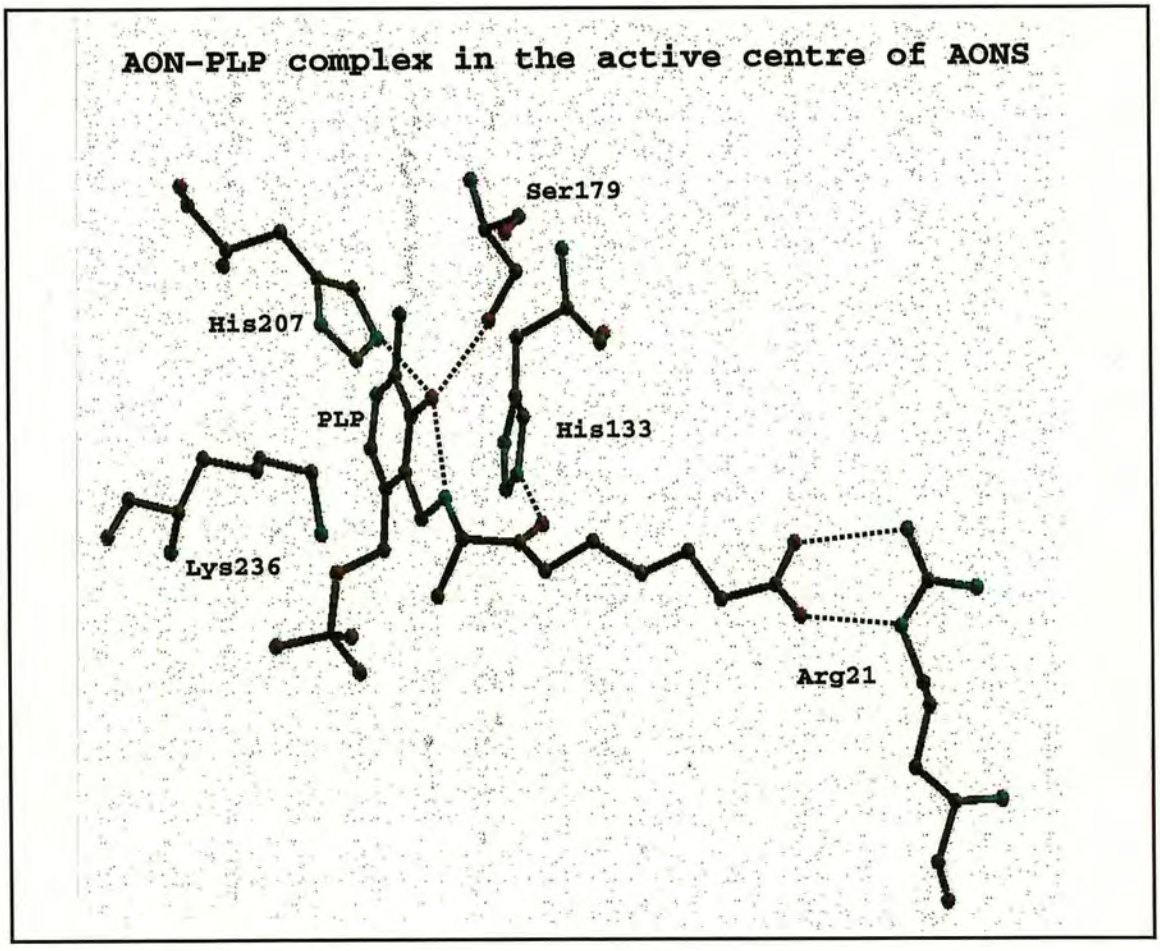


Figure 48 : AONS with AON bound in the active site

Chapter Three

The Development of Synthetic Routes to Inhibitors of AONS and DANS

3.1 Enzyme Inhibition

Generally, an enzyme inhibitor may be regarded as an agent that decreases the rate of an enzyme-catalysed reaction. Broadly speaking, enzyme inhibitors may be divided into those which are competitive (and usually, though not always reversible), and those which are non-competitive (and generally irreversible).[159]

The competitive inhibitors may be subdivided into the transition-state analogues, and the slow, tight binding inhibitors, although there is a significant amount of overlap between these two classes.

Transition-state analogues are compounds that have a structure that closely resembles the conformation of a substrate at a postulated transition state, or a transient species in the enzyme catalysed pathway. The basis for the design of such a molecule lies with the hypothesis[160,161] that an enzyme achieves its great rate enhancements by changing its conformation in such a way that the strongest interactions occur between the substrate and the active site residues at the transition state of the reaction. This implies that an analogue which has a structure similar to the transition state should bind more tightly than the substrate in the ground state.

Slow, tight binding inhibitors are compounds which either induce a conformational change in the enzyme leading to an extremely tight interaction, or compounds that form slowly reversible covalent bonds within the enzymatic active site.

The non-competitive (and predominantly irreversible) inhibitors may be broadly placed into two classes; the affinity labelling agents,[159] and the mechanism-based enzyme inacivators (suicide inhibitors).[162]

Affinity labels are compounds that contain a reactive functional group, and react with an active site residue forming a covalent bond, resulting in the irreversible inhibition of the enzyme. They may be fairly non-specific in action, undergoing reaction with more than one residue and / or protein. However, by making the structure closely resemble the natural substrate, a greater level of specificity may be obtained. These type of

compounds often contain an α-haloketone, azide or isocyanate moiety, and consequently react with nucleophilic residues such as lysine, cysteine, or serine in the active site.

A mechanism-based enzyme inactivator is a relatively unreactive compound, having a structural similarity to the substrate or product for a particular enzyme that, *via* its normal catalytic mechanism of action, converts the inactivator into a species which, without prior release from the active site, binds covalently to the enzyme.

The ability of the PLP-Schiff bases to stabilise negative charges at the α–,β–and γ–carbon atoms of the substrate has been used in the rational design of a wide range of substrate analogues that have inhibitory properties. Generally, the approach taken has been to incorporate moieties into the substrate molecule which, through the normal catalytic mechanism, generate reactive electrophilic intermediates which react with proximal, nucleophilic active site residues. This leads to the formation of a covalent bond between the substrate analogue and the enzyme causing irreversible inhibition.

The most common groups which are adventitiously incorporated into the natural substrates to create these reactive conjugated imines, ketimines, or allenes are; olefins, acetylenes, halogens, nitriles and azides amongst many others. Fluorinated compounds have been utilised extensively in the synthesis of PLP-dependent enzyme inhibitors, as the nature of the C-F bond favours elimination reactions rather than S_N2 displacements. Also the van der Waal's radius of fluorine (1.35Å) is very close to that of hydrogen (1.20Å), making steric factors relatively unimportant.[163]

A number of compounds were identified as potential inhibitors of AONS; these being either structural analogues of AON **(8)**, or the putative carboxyl intermediate in the condensation **51** (as shown in figure 49).

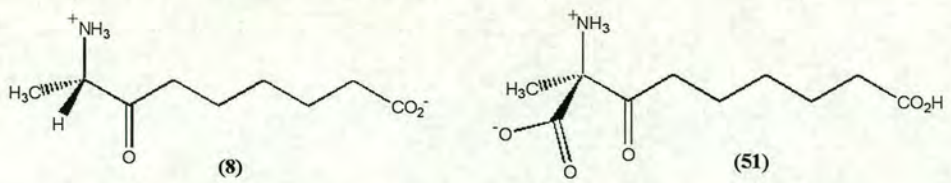


Figure 49 : AON and its putative enzymatic precursor

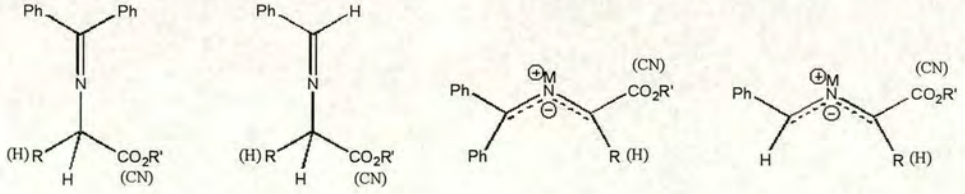
3.2 The Use of Schiff-Bases in the Synthesis of (±)-AON and Related compounds

3.2.1 The Use of Schiff-Bases in the Synthesis of Amino-Acids

Schiff-bases have been utilised extensively in the synthesis of non-proteinogenic amino-acids,[164] and consequently it was thought that this methodology could be adapted for the synthesis of analogues of **8** and **51**.

The vast majority of Schiff-base chemistry, pioneered by Phillipe Bey, Martin O'Donnell and Gilbert Stork, concerns the use of benzophenone ketimines, and benzaldehyde aldimines of amino acids and related compounds. The published chemistry in this area is almost entirely restricted to alkylations[165-167] and Michael additions,[168] and only one reference to an acylation could be located,[169] which coincidentally was in the synthesis of an inhibitor of sphingosine-1-phosphate lyase, another PLP-dependent enzyme.

Benzophenone and benzaldehyde-derived Schiff-bases such as **52** and **53** respectively may be deprotonated using a variety of organic bases, such as LDA,[165] DBU,[168] potassium t-butoxide[166] and sodium hydride,[170] at low temperature to generate the corresponding aza-allylanions **54** and **55** (see figure 50). The presence of electron withdrawing groups, such as -CN, or CO_2R adjacent to the α-proton(s), results in negligible reactivity at the imidic-carbon position,[171] when the aza-allylanions of Schiff-bases such as **54** or **55** are reacted with electrophiles.



(52) (53) (54) (55)

Figure 50 : Benzophenone and benzaldehyde imines

3.2.2 Phase Transfer Catalysis

As the aromatic Schiff-bases of amino acids (and their cyano analogues), are moderately base-stable and have α-protons with acidities between pK_a = 16 and 23,[164] they are ideally suited to reactions involving phase transfer catalysis. Consequently, a wide variety of alkylation[172,173] and Michael addition[174] reactions with Schiff-bases, under PTC conditions have been reported in the literature as routes to non-proteinogenic amino-acids and related analogues.

Phase-transfer catalysis (PTC) enables anions, or reactive species in one phase (aqueous or solid) to react efficiently with substrates soluble in a separate, organic phase. These bi-phasic processes are mediated by phase transfer catalysts, usually quaternary ammonium or phosphonium salts, or crown-ethers, which facilitate the exchange of charged species between the phases.

In hydroxide ion-initiated phase transfer catalysis[175] (PTC/OH), one of the phases is an aprotic solvent, such as toluene or dichloromethane, and the other phase is an alkali metal salt (e.g. finely ground KOH, or K_2CO_3), or a concentrated basic solution (e.g. 50 % aq. NaOH). In contrast to the standard anhydrous techniques involving organometallic reagents, PTC/OH reactions can generate the substrate anion under mild, basic conditions and do not necessarily require an inert atmosphere.

There is extensive kinetic data to suggest that aromatic Schiff-bases react via an interfacial mechanism.[175] A substrate molecule in the organic phase, located near the interface, is deprotonated by a hydroxide ion proximally positioned in the aqueous (or solid) phase, forming a metal cation-carbanion ion-pair at the interface [M^+R^-]. Exchange of the metal cation, with the cation of the quaternary (ammonium) salt, forms a lipophilic ion pair [Q^+R^-] (with concomitant release the metal-halide salt into the other phase) allowing the transfer of the carbanion into the bulk of the organic phase where it may react. A schematic for the alkylation of a Schiff's-base using PTC/OH is shown in figure 51.

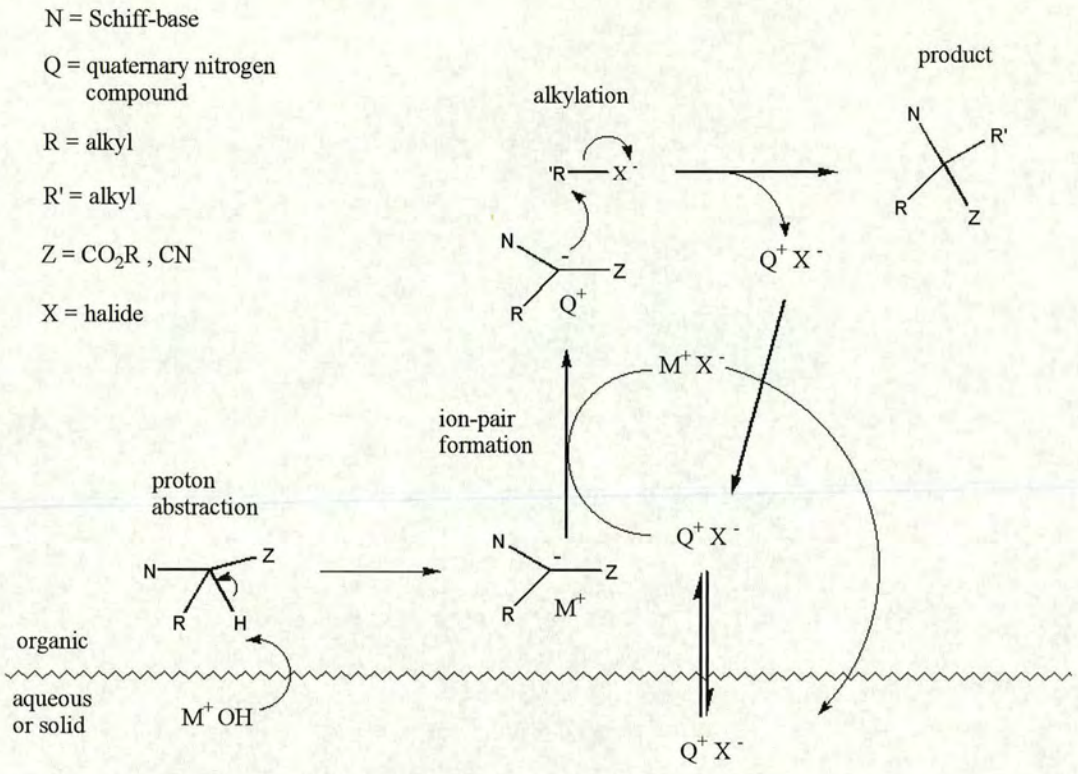


Figure 51 : Schematic diagram showing the interfacial mechanism in PTC reactions

3.2.3 An Investigation into the Acylation of Schiff-Bases

It was thought that by reacting aromatic Schiff-bases with a variety of acylating species under organic-base and / or PTC conditions, a selection of efficient, robust and general routes to α-oxoamines could be developed. The general strategy is presented in figure 52.

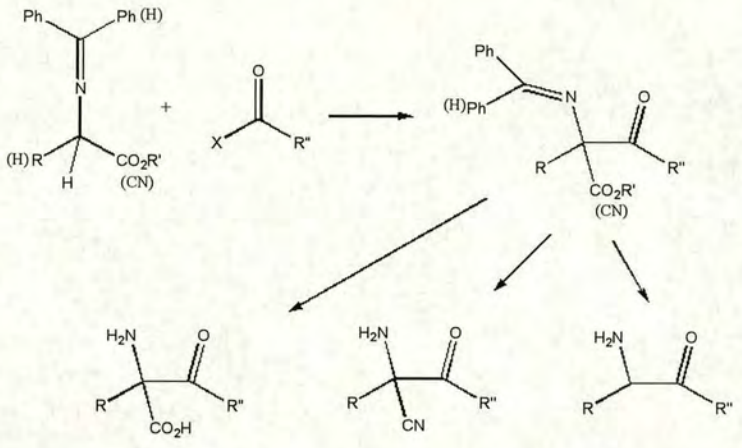


Figure 52 : General strategy for the synthesis of α-oxoamines

The benzophenone ketimines **57** and **58** were prepared in high yield *via* transimination reactions between benzophenone imine **(56)** and the hydrochloride salts of L-alanine and aminoacetonitrile respectively[165] (as shown in figure 53).

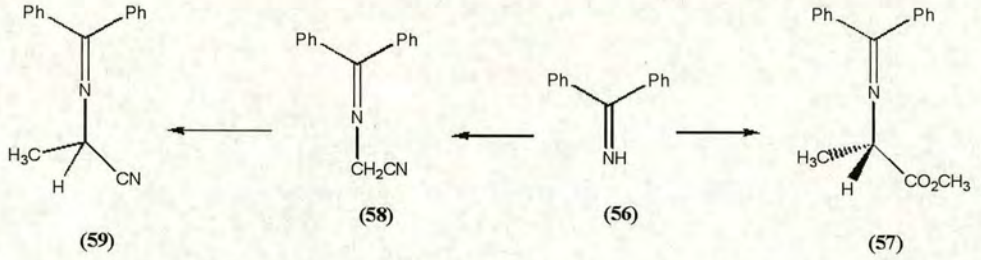


Figure 53 : Preparation of the starting imines

Imine **59** was synthesised under PTC, by the slow addition of methyl iodide to a rapidly stirring, ice-cooled mixture of **58** in toluene / 50 % aq. NaOH, using benzyl triethylammonium chloride (BTEAC) as a catalyst.[172] This general procedure has been shown to give predominantly mono-alkylated product when one equivalent of alkyl halide is used, due to the *ca.* 1000 fold reduction in acidity of the α-proton in the product compared with the starting material.[164]

To investigate whether or not acylation was feasible under PTC conditions, a number of trial reactions were performed using the benzophenone imines **58** and **59**, and a

selection of acid chlorides as shown in table 1. BTEAC was used for all the PTC reactions in a 0.1 molar equivalent, as this has been shown to be effective in both toluene / aqueous base,[172] and solid-liquid PTC mixtures.[174] The system using 50% aq. NaOH (3 molar eq.) / toluene (in equal volume to the basic solution used) and BTEAC (0.1 molar eq.) is the PTC method most widely used in the alkylation of benzophenene imines, and hence was used as the starting point for the acylation trials.

As table 1 shows, the acylation of **59** with acetyl chloride was not successful and only the starting imine was recovered upon workup. The attempted acylation of **58** with pivaloyl chloride, and with the acid chloride of mono-ethyl pimelate **(60)** was similarly unsuccessful.

Imine	Acylating agent	Phase-Transfer Conditions	Product
59	acetyl chloride 1.3 eq.	50% NaOH (3 eq.) / Toluene / BTEAC (0.1 eq.) / RT / overnight	No reaction
58	pivaloyl chloride 1 eq.	50% NaOH (3 eq.) / Toluene / BTEAC (0.1 eq.) / RT / overnight	No reaction
58	**60** 1 eq.	50% NaOH (3 eq.) / Toluene / BTEAC (0.1 eq.) / RT / overnight	No reaction

Table 1

In all of these reactions the formation of the ketimine anion was observed, as the biphasic mixture of the imine, phase-transfer catalyst, toluene and aq. NaOH was a deep red colour before the acid chloride was added. The inability of these acid chlorides to acylate the benzophenone ketimines can be ascribed to the competing hydrolysis reaction, or the unreactivity of the imine anions towards the acylating species.

To reduce acid chloride hydrolysis during the course of the reaction, non-aqueous solid-liquid PTC conditions with BTEAC and K_2CO_3 as a base were employed as shown in table 2.

Imine	Acylating agent	Phase-Transfer Conditions	Product
58	acetyl chloride 1 eq.	K_2CO_3 (0.5 eq.) / BTEAC (0.1 eq) / sonication for one hour at 45 °C	**61** (32 %)
58	pivaloyl chloride 1 eq.	K_2CO_3(0.5eq.) / BTEAC(0.1eq) / sonication for 2 hours at 45 °C	No reaction
58	pivaloyl chloride 5 eq.	K_2CO_3 (1 eq.) / BTEAC (0.1 eq) / sonication for 3 hours at 60 °C	No reaction
59	pivaloyl chloride 1 eq.	K_2CO_3 (0.5 eq.) / BTEAC (0.1 eq) / sonication for 2 hours at 45 °C	No reaction
57	**60** 2 eq.	K_2CO_3 (0.75 eq.) / BTEAC (0.1 eq) / sonication for 4 hours at 60 °C	No reaction
57	**60** 2 eq.	K_2CO_3 (0.75 eq.) / BTEAC (0.1 eq) / acetonitrile /sonication for 4 hours at 60 °C	No reaction

Table 2

When one equivalent of acetyl chloride was added to a mixture of imine **58**, finely ground anhydrous K_2CO_3 (0.5 eq.) and BTEAC (0.05eq.), and was sonicated for one hour at 45 °C, only one product, tentatively assigned as **61**, was formed (32 % yield, mixture of *cis* and *trans* forms) i.e. there had been a re-acylation of the enolised form of the mono-acylated species (see figure 54).

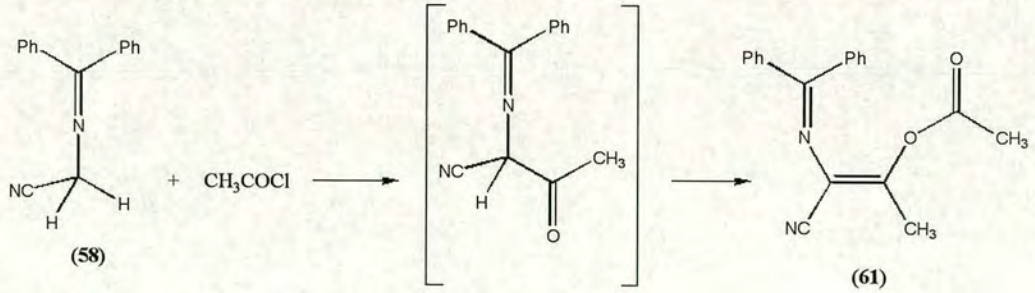


Figure 54 : The formation of 61

However, the reaction of imines **58** and **59** with pivaloyl chloride failed to generate detectable amounts of any acylated product, possibly due to steric inaccessibility. The

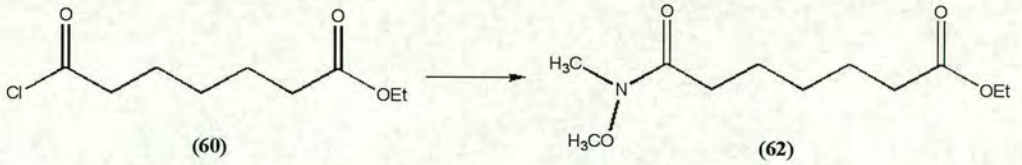
reaction of **58** with **60** (2 eq.) with, or without the presence of acetonitrile as solvent, also failed to yield any of the desired products.

Since phase transfer chemistry failed to yield any of the desired results, benzophenone imine acylations using standard anhydrous techniques were attempted (see table 3).

Imine	Acylating agent	Reaction conditions	Product
57	**62** 1.3 eq.	THF / LDA -78°C	Transesterification
59	acetyl chloride 1.5 eq.	DBU (1.2 eq)/ DMAP (0.1 eq.) / -78°C to RT	No reaction
58	**60** 2.5 eq.	THF / LDA -78°C to RT	**66** (25 %)
58	**60** 1 eq.	THF / LDA -78°C to RT	**66** (17 %)
57	**65** 1.5 eq.	THF / LDA -78°C to RT	No reaction
57	**65** 1.5 eq	THF / HMPA / LDA -78°C to RT	No reaction

Table 3

The Weinreb amide[176] of ethyl hydrogen pimelate **62** was prepared in good yield by the condensation of **60** with N,O-dimethyl hydroxylamine hydrochloride in the presence of excess pyridine (see figure 55). The thiophenol ester of methyl hydrogen pimelate **(65)** was prepared in two different ways; either by the addition of an ethereal solution of diazomethane to the thiophenol ester **32**, or by the reaction of the acid chloride of methyl hydrogen pimelate **(64)** with 1.5 eq. of thiophenol, using triethylamine to remove the HCl produced (as shown in figure 56).



(60) (62)

Figure 55 : The preparation of 62

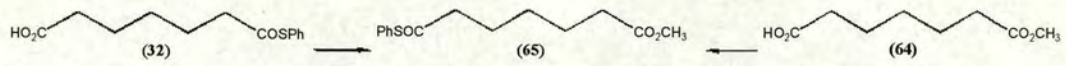


Figure 56 : Two synthetic routes to 65

When **62** was added to the lithium-anion of **57**, transesterification ensued, and the *ethyl* ester of the imine and the *methyl* ester of the Weinreb amide were isolated as the major products, and no acylation was observed. DBU and DMAP were incapable of catalysing the acetylation of **59** under the conditions employed. When 2.5 eq. of **60** was added to the LDA-derived anion of **58** at -78 °C, **66** was formed as the major product (m/z: 561 (MH^+), 391 ($M\text{-}C_9H_{14}O_3)^+$), presumably *via* an analogous mechanism to **61** as shown in figure 57. However, when the ratio of the acid chloride was reduced to 1 molar equivalent, no mono-acylated species could be isolated.

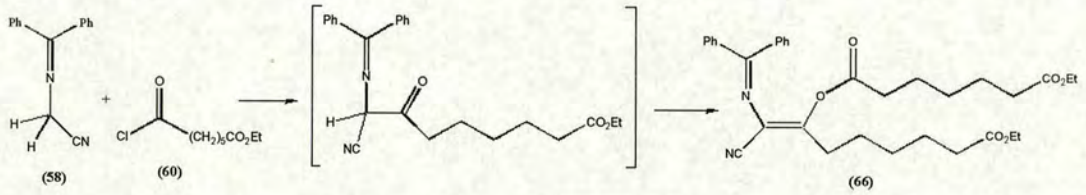


Figure 57 : The formation of 66

There was no observed reaction when the thiophenol ester **65** was added to the lithiated azaallyl anion of **57** when THF, or THF / HMPA solvent mixtures were used.

As conditions which would lead to the mono-acylation of the benzophenone imines could not be found, the chemistry of the benzaldehyde aldimines was investigated.

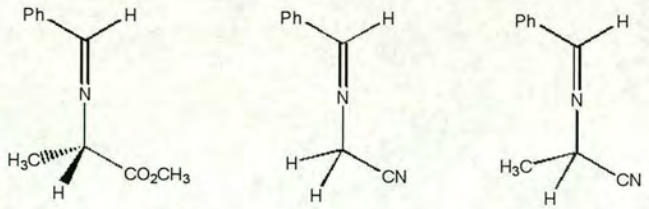


Figure 58 : Benzaldehyde aldimines

The aldimines **67** and **68** were prepared in high yield *via* the dehydrative condensation of benzaldehyde and the hydrochloride salts of alanine methyl ester and aminoacetonitrile respectively.[166] The aldimine **69** was prepared in 55 % yield by the slow addition of a molar equivalent of methyl iodide to the lithiate of **68** at -78°C. As found by O'Donnell [164] there was a significant amount of the di-methylated species formed (35%) due to **68** and **69** having roughly similar pK_a values (*ca.* 18).

The benzaldehyde aldimine of L-alanine methyl ester **(67)** was subjected to a variety of different acylation agents, using LDA and PTC to generate the azaallyl anion (as shown in table 4), to ascertain whether mono-acylation was possible.

Imine	Acylating agent	Reaction conditions	Product
67	acetyl chloride 2 eq.	K_2CO_3 (0.5 eq.) / BTEAC (0.1 eq) / sonication / 50 °C/ 5 hours	Starting materials + mixture of products
67	**60** 1.1 eq.	K_2CO_3 (0.5 eq.)/ BTEAC (0.1 eq.) / sonication / 50 °C/ 6 hours	No reaction
67	acetyl chloride 1.1 eq	LDA / THF / -78 °C to RT	No reaction
67	**70** 1.2 eq	LDA / THF / -78 °C to RT	No reaction
67	**71** 1.2 eq	LDA / THF / -78 °C to RT	No reaction
67	**60** 2.5 eq	LDA / THF / -78°C to RT / inverse addition	**72** (42 %)
67	**65** 2.5 eq	LDA / THF / -78°C to RT / inverse addition	**72** (17 %)

Table 4

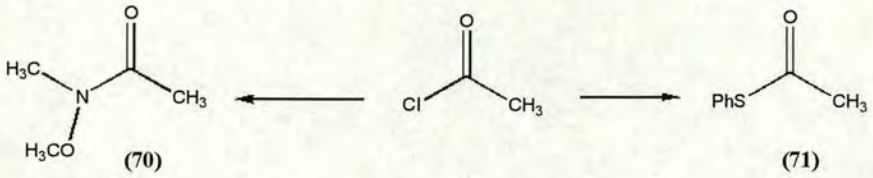


Figure 59 : Preparation of the acylating agents 70 and 71

The solid-liquid PTC reaction between acetyl chloride and **67** generated a complex mixture of products that could not be separated nor identified, as well as unreacted imine. The analogous reaction with **67** and **60** was also unsuccessful. N-methoxy-N-methyl acetamide **(70)** has been shown to be an effective acetylating agent for a number of strongly basic and nucleophilic enolate anions.[177] The amide **70** was prepared in a similar manner to **62** from acetyl chloride and N,O-dimethyl hydroxylamine hydrochloride (see figure 59). The thioester **71** was synthesised by the addition of thiophenol and excess triethylamine to a cooled solution of acetyl chloride. THF solutions of the three acetylating agents; acetyl chloride, **70** and **71** were added to the LDA-generated anion of **67** in THF at -78 °C, and the reactions were allowed to slowly warm to room temperature overnight. However none of these three reactions afforded any addition products (only starting materials were recovered).

Reverse addition is a method commonly used to prevent the over-reaction of the initially formed (reactive) product. In this method, the anion is added to a several-fold molar excess of the alkylating or acylating compound, resulting in a rapid quench of the charged species, and reducing the likelihood of the product reacting further. It was thought that this approach might be more successful than the normal-addition method (i.e. the addition of the alkylating or acylating species to the anion) which had been employed in all the previous imine acylation reactions.

Consequently, a solution of the azaallyl-anion of **67** at *ca.* -40 to -50 °C was added slowly, *via* canula to solutions of the acid chloride **60** and the thiophenol ester **65** in THF at -78 °C, which were then allowed to react whilst warming to room temperature overnight. Aqueous workup and subsequent purification of the crude organic extracts yielded the desired product **72** as a pale yellow oil, in 42 and 17 % yields respectively. The ketone **72** was slightly unstable to decomposition on silica-

gel, and was thermally unstable (having an estimated half-life of *ca.* 3 days in deuteriochloroform at room temperature). The identity of the product was confirmed by ^{1}H NMR and mass spectrometry. This was further confirmed by the acidic hydrolysis of **72**, which yielded, after purification by ion-exchange chromatography, the hydrochloride salt of (±)-AON as the main product.

The ability of the thioester **65** to successfully acylate the Schiff-base **67** means that, in effect an overall *biomimetic* synthesis of AON has been achieved (see figure 60). As in the AONS catalysed process, the first step is the formation of a Schiff-base between alanine and an aromatic aldehyde. The second process is a proton abstraction, and subsequent acylation with a thioester of pimelic acid. The final step is a decarboxylation, followed by hydrolysis of the Schiff-base to give (±)-AON.

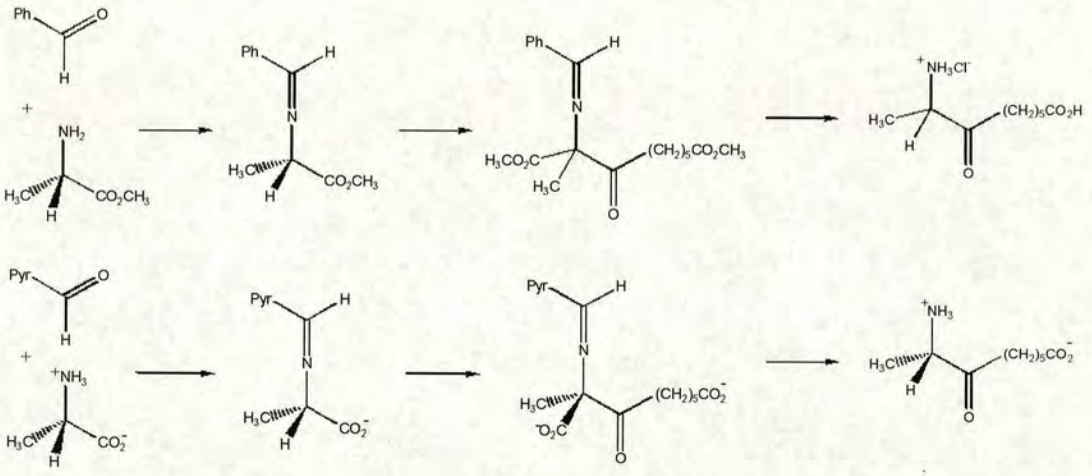


Figure 60 : Biomimetic synthesis of (±)-AON

It can be concluded from these experiments that the acid chlorides are the most effective acylation species, and that inverse addition of the azaallyl imine anions to the acylating agent is crucial for the success of the reaction. Further investigation is required to demonstrate whether or not Weinreb amides and other carboxyl activating (protecting) groups are compatible with this inverse-addition procedure. It also remains to be seen whether the benzopheneone imines, and the cyano-aldimines **68** and **69** can be selectively mono-acylated using this methodology.

3.3 The Use of Dakin-West Chemistry in the Synthesis of (±)-AON and Related Compounds

3.3.1 The Dakin-West Reaction

In 1928 Dakin and West[178] developed a method for the conversion of an α-amino acid into the corresponding α-acetylamino-alkyl methyl ketone, by the action of acetic anhydride in the presence of pyridine. Further investigations[179,180] demonstrated that carbon dioxide was evolved during the course of the reaction, and that other bases such as alkylpyridines and sodium acetate could replace pyridine. The reaction was shown to be applicable for other acid anhydrides, although in general the yields reduce significantly with increasing chain length (see figure 61).

Figure 61 : The Dakin-West reaction

Dakin and West[179] also postulated the intermediacy of an oxazol-5-one **73** in the reaction course, and demonstrated that these compounds yielded the same product as their parent amino-acid. Subsequent studies[181,182] have borne out this contention, and a general mechanism is shown in figure 62. Kinetic measurements have shown the intermediates are in equilibria and the final decarboxylative step is irreversible.

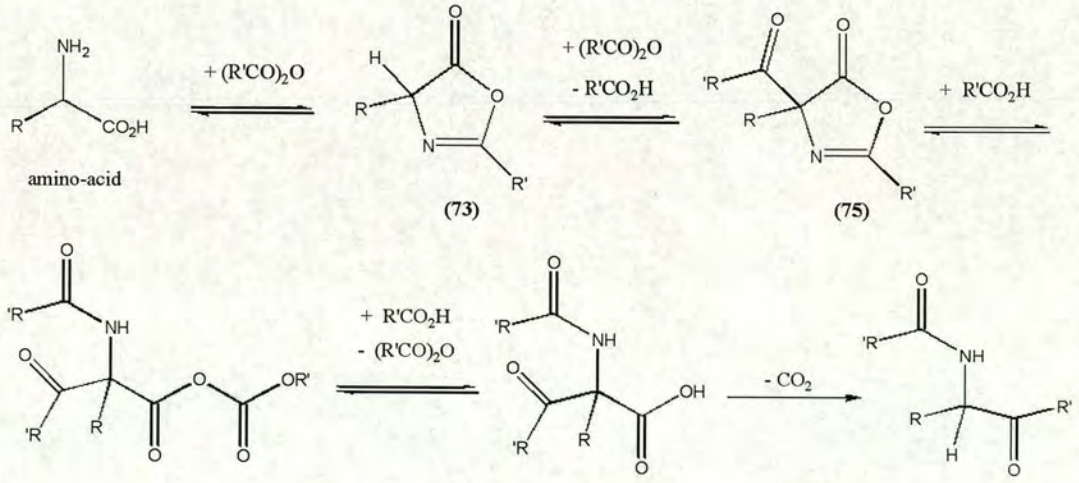


Figure 62 : Mechanism of the Dakin-West reaction

More recent research has shown that the replacement of pyridine with the acylation catalyst DMAP[183,184] can greatly enhance the reaction rate, resulting in better yields at lower reaction temperatures. Further work by Steglich and Hofle[185-188] has led to the development of a versatile 'stepwise' Dakin-West procedure, as shown in figure 63.

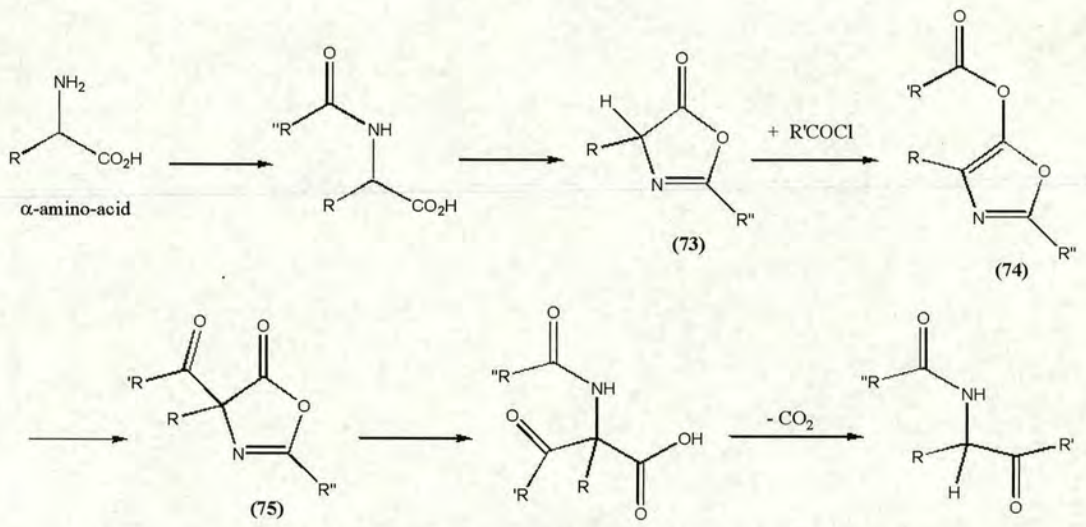


Figure 63 : The 'stepwise' Dakin - West procedure

In the 'stepwise' reaction scheme, the oxazol-5-one **73** is pre-formed, and is reacted under mild conditions (room temperature or below) with an acid chloride in the presence of triethylamine. This leads to the kinetic O-acyl product **74** being formed, which slowly isomerises to the more stable C-acyl, thermodynamic product **75**.[185] The O-acyl product can be isolated, or may be isomerised *in situ* by the addition of a catalytic amount of DMAP (which has been shown to be more efficient than pyridine and other bases).[183,184]

Using ^{1}H-NMR experiments, Steglich and Hofle[186] have demonstrated the intermediacy of a species **(76)** in the isomerisation process which disappears with the concomitant formation of the C-acylated product **75**. Consequently, the authors have proposed that the acyl migration occurs *via* the formation of the ion-pair **77**, which may equilibrate with the dihyrdopyridine-condensed species **76**, to give the thermodynamic C-acylated product **75** (see figure 64). It is noteworthy that under standard Dakin -West conditions, the O-acylated product has never been observed.

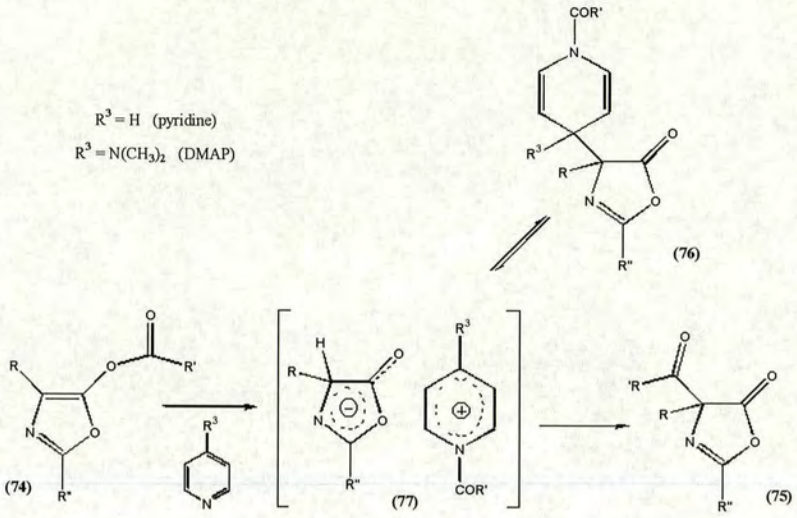


Figure 64 : Mechanism of acyl migration

Thus the 'Stepwise' Dakin-West procedure appeared to be a viable and versatile route to the synthesis of (±)-AON and related compounds. It was decided to evaluate this possibility.

3.3.2 The Synthesis of (±)-AON using Dakin-West Chemistry

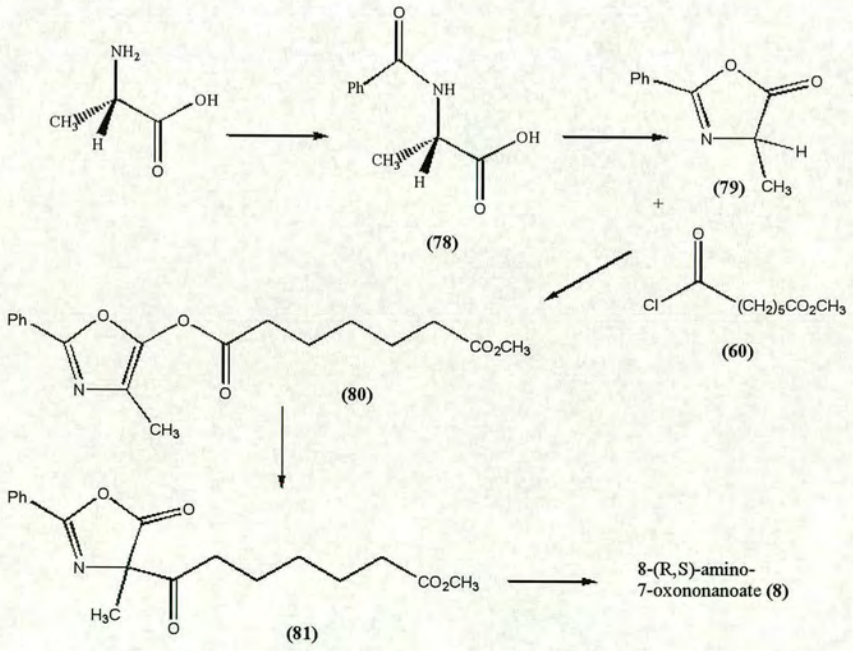


Figure 65 : A route to AON using Dakin-West chemistry

N-Benzoyl-L-alanine (**78**) was prepared from L-alanine in high yield by a standard Schotten-Bauman procedure[189] using 2M NaOH and benzoyl chloride at 0 °C. The synthesis of the oxazol-5-one **79** was achieved *via* the dehydrative self-condensation of the mixed anhydride formed between **78** and acetic anhydride[190] (see figure 66). This latter procedure was adopted, rather than a carbodiimide-activated route,[191] since it offers higher yields and a simple workup.

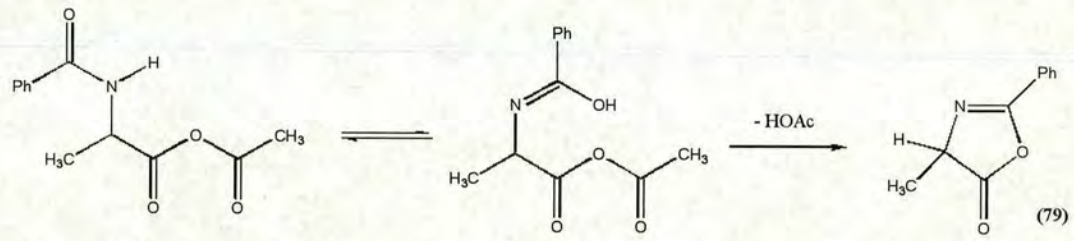


Figure 66 : Oxazol-5-one formation

Optical rotation measurement confirmed that complete racemisation had occurred during the oxazolone ring formation step (the melting point of N-benzoyl-L-alanine **(78)** corresponded closely to the literature value[192] of 150-151°C, and the melting point of oxazolone **79** (35-37 °C) was in agreement with the literature value of 37-39°C[193] for the racemate).

The triethylamine-catalysed condensation of oxazolone **79** and acid chloride **60** was performed analogously to the procedure described for the acetylation of **79** by Steglich and Hofle,[185] except that in this case the reaction was maintained at 0 °C for 3 hours, then stirred at ambient temperature for 18 hours. After aqueous workup and purification by silica-gel chromatography, the desired product **80** was obtained in 84% yield as a colourless oil.

Isomerisation of the 5-acyloxazole **80** to the 4-acyloxazolone **81** was achieved in moderate yield by stirring a solution of **80** in THF with a 3% molar equivalent of DMAP overnight at room temperature. Longer reaction times led to a greater degree of conversion from the O- to the C-Acyl product, but this was accompanied by slow thermal decomposition of the desired product, leading to lower overall yields being

obtained. The thermodynamic product **81** appears to be less thermally stable than the kinetic product **80** (with an estimated half-life in deuteriochloroform of *ca.* 1 week at room temperature). For this reason it was decided to examine a one-pot, hydrolytic oxazolone ring-opening, de-esterification, decarboxylation and de-benzoylation procedure under acidic conditions, as an alternative.

The desired product (±)-AON (hydrochloride salt) was obtained in moderate yield (36% after purification), by vigorously stirring the oxazolone **81** in 4M aqueous HCl at 80 °C overnight. The main by-products formed (identified by a combination of tlc and ^{1}H-NMR) were alanine, N-benzoyl alanine, pimelic acid, and mono-methyl pimelate. All of these could be removed by repeated extraction of the acidic product solution with chloroform, except alanine, which could be separated in the final ion-exchange purification.

The formation of these products is consistent with hydrolytic attack at the ketonic carbonyl (pathway **1** in figure 67) competing with the desired hydrolysis at C-5 of the oxazolone ring (pathway **2**).

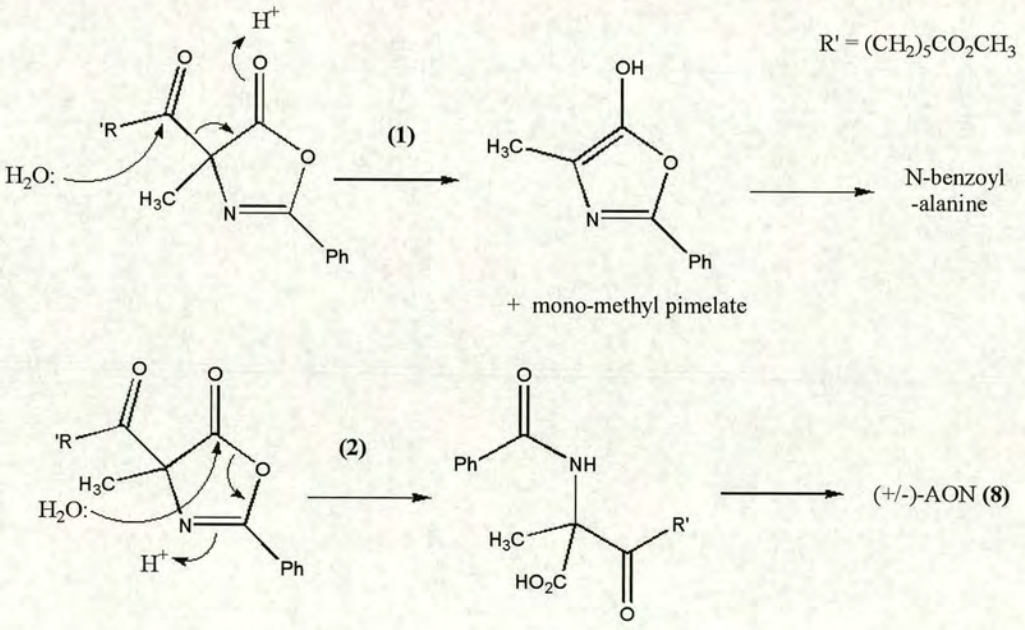


Figure 67 : Acidic hydrolysis of 4-acyl-oxazol-5-one (81)

Similar findings have been reported for the hydrolytic ring opening of more simple 4-acyloxazol-5-ones to the corresponding N-benzoyl-amino ketones, with analogous results also obtained for aminolysis and methanolysis.[187]

In brief conclusion, this route has been shown to be successful for the synthesis of (±)-AON, but more work will be required to develop this pathway for the synthesis of AON analogues.

3.3.3 Considerations for Future Development of the Strategy

It has been noted previously[187,191] that heating the 4-acyloxazol-5-one with pyridine and acetic anhydride, or oxalic acid can effect a mild and non-hydrolytic, decarboxylative ring opening with minimal reaction at the ketone carbonyl. However, the application of this method for the ring-opening of 4-acyloxazol-5-one **81** remains to be investigated.

The potential use of this route for the synthesis of the putative carboxylated intermediate in the *bio*F catalysed process **(51)** was considered.
There is a possibility that the oxazolone **81** could be hydrolysed under mild conditions to the α-acyl-α-(N-benzoyl)-amino-acid **82**. However the harsh (acidic) conditions required to remove the N-benzoyl group would probably lead to an (acid catalysed) β-decarboxylation, resulting in the formation of (±)-AON **(8)**. This might be avoided if the ketone functionality was protected as a dithiolane, or dithiane,[145] prior to the acidic benzoyl removal step. The dithioacetal protection could then be removed in a mild manner, either with NBS or a mercuric salt, to generate (±)-**51** (see figure 68).

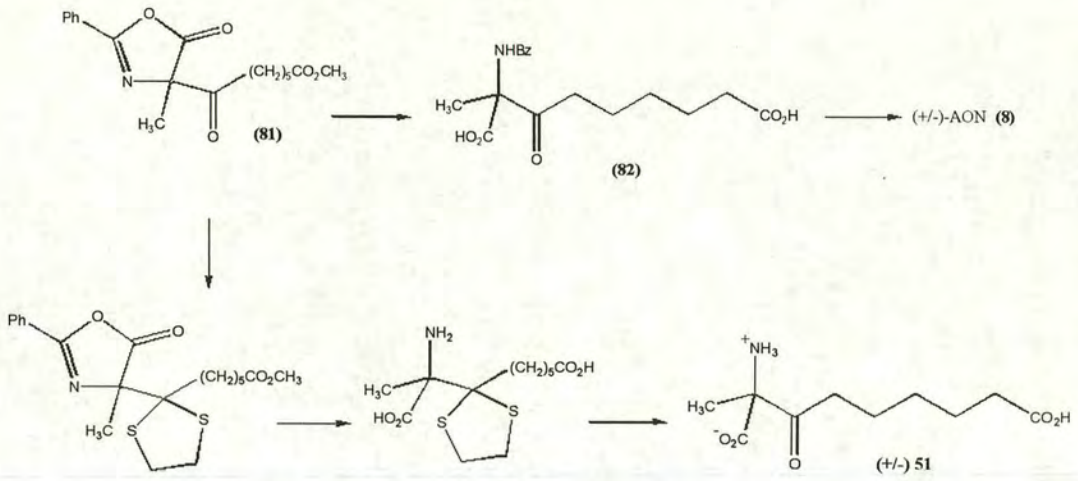


Figure 68 : Proposed route to (±)-51

3.4 A route to an Affinity Labelling Agent for DANS (AONS)

The synthesis of an affinity label for the *bio*A or *bio*F enzymes was considered.

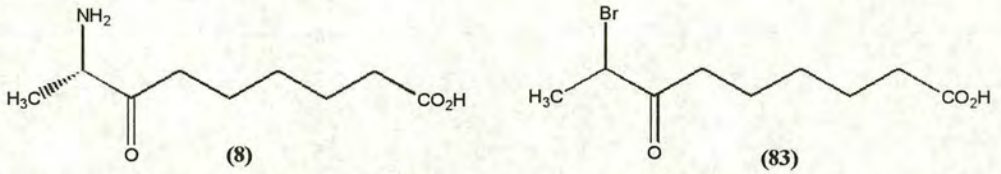


Figure 69 : (S)-AON and affinity label analogue (83)

Species **83** can be proposed as a plausible candidate for an affinity reagent, which may inactivate DANS (and / or possibly AONS). As with the natural substrate, AON **(8)**, the target compound **83** contains a nonanoic acid carbon backbone and has a ketone functionality at the C-7 position, and is therefore capable of forming an external aldimine with the PMP-cofactor in the enzyme active site.

The α-bromoketone **83** has an analogous structure to bromopyruvate, which Morino and Okamoto[195,196] have shown to be a slow inactivator of pig heart aspartate aminotransferase. This affinity reagent may react with an active site nucleophile, e.g. the lysine residue involved in forming the internal aldimine with the PLP cofactor,

resulting in enzyme alkylation as shown in pathway **(a)**. Alternatively there is also the possibility that **83** may form a Schiff-base with the PMP (present in DANS) leading to enzyme alkylation *via* a Michael type attack from an active site nucleophile following the elimination of a bromide ion, as shown in pathway **(b)**.

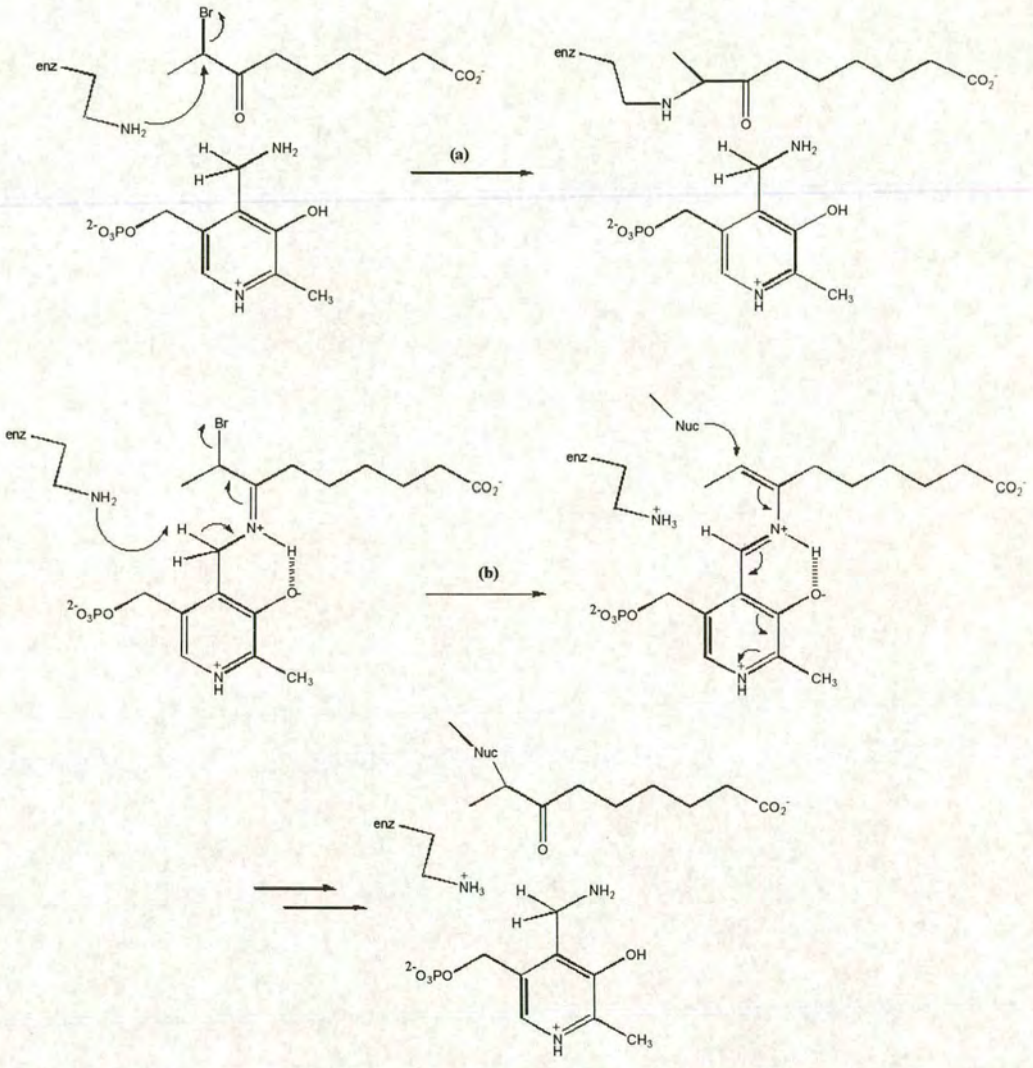


Figure 70 : Possible mechanism of DANS inhibition

The alcohol **41,** the by-product from the reductive amination of ketone **39** in the dithiolane synthetic pathway (see figure 36), appeared to be a good starting material for the synthesis of the bromo-compound **83**, as shown in figure 71.

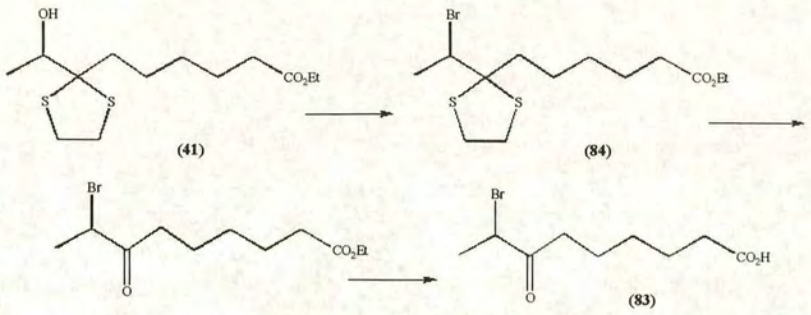


Figure 71 : Proposed route to the affinity label 83

However when the bromination of **41** was attempted using triphenylphosphine / bromine[197] in DCM at 0 °C, the major product obtained (75 %) was not the desired product **84**, but a rearranged product **85**. Possible mechanisms for this rearrangement are shown in figure 72.

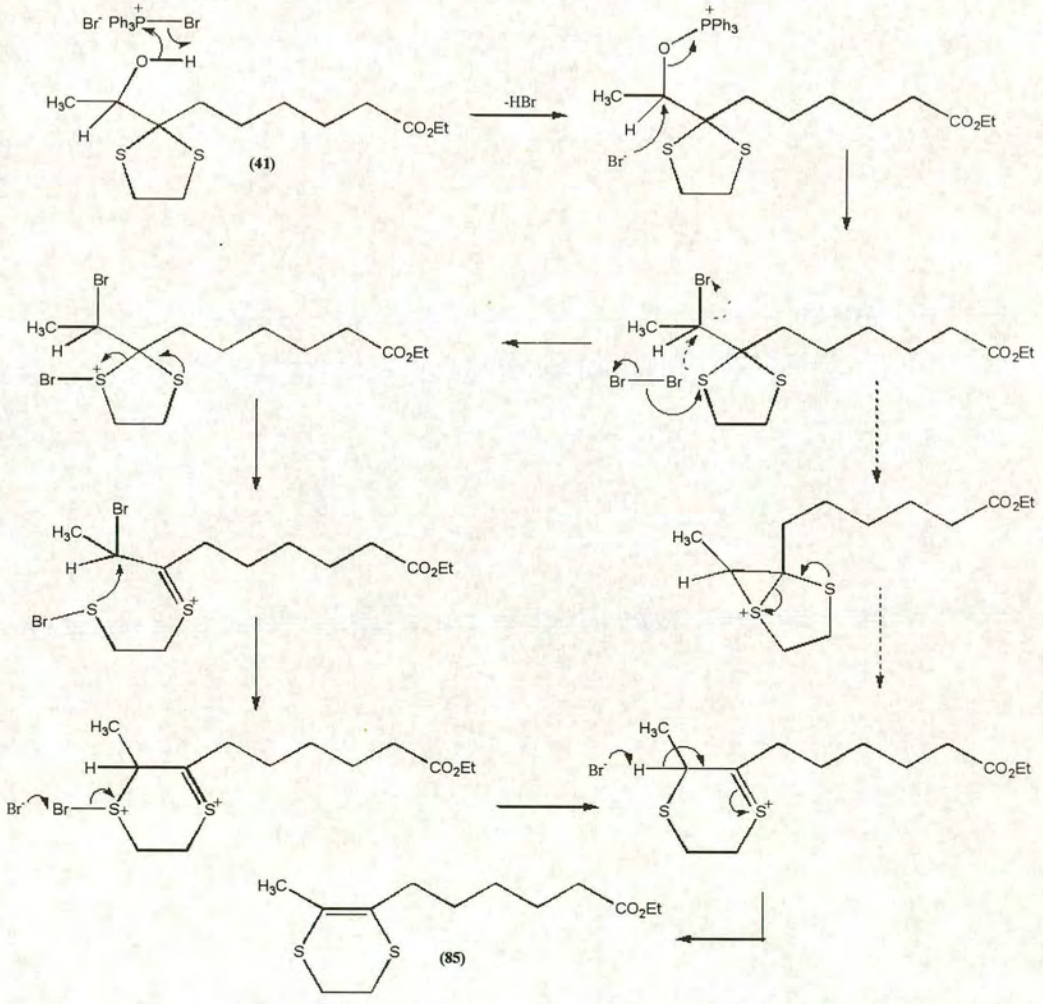


Figure 72 : Possible mechanisms for the formation of 85

Donatelli and Chen[214] and Lee *et al.*[215] have reported that 2,2-disubstituted-1,3-dithiolane mono-sulfoxides can undergo an analogous rearrangement under acidic conditions to give the corresponding 2,3-disubstituted-5,6-dihydrodithiines. (see figure 73).

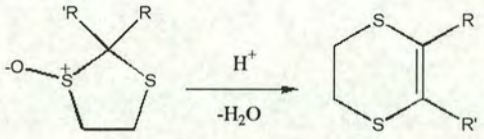


Figure 73 : Formation of 2,3-disubstituted dithiines

Rubinstein and Wuerthele[216] have demonstrated that 2,3-disubstituted-5,6-dihydrodithiines are also formed when α-bromoketones are reacted with ethane-1,2-dithiol under acid conditions with the azeotropic removal of water.

An alternative three step route to **83** would be to remove the dithiolane moiety prior to the bromination process, and saponification. However, when the hydroxy compound **41** was treated with NBS at -5 °C in 80 % aqueous acetonitrile; conditions similar to those employed in the dithiolane deprotection previously used in the synthesis of (±)-AON, the main product obtained was the disulfoxide **87** (68 %), and the desired α-hydroxy ketone **86** was only isolated in 27 % yield (see figure 73). It may be noted that the dithiolane protecting group was removed in 94 % yield from the analogous amine **40** using an identical procedure.

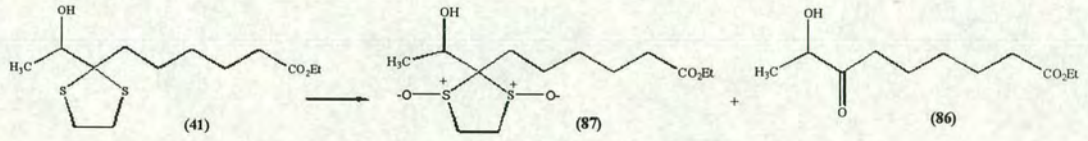


Figure 73 : Disulfoxide formation

A number of authors have reported that stoichiometric amounts of Cl^+ (generated *in situ* from iodobenzene dichloride[217] or 1-chlorobenzotriazole[218,219] in either aqueous acetone or acetonitrile) can selectively oxidise 1,3-dithiolanes to the corresponding

mono- or *di*-sulfoxides within minutes at 0°C. Prolonged reaction times and higher temperatures were found to result in further oxidation to give di-sulfones.

With this anomalous sulfur oxidation occurring in competition with the dithiolane deprotection of **41**, it was conjectured that this might be minimised if the hydroxyl was derivatised. The protecting group could then be removed afterwards to allow bromination of the hydroxylated compound, which could be saponified to give the desired product **83** (see figure 74).

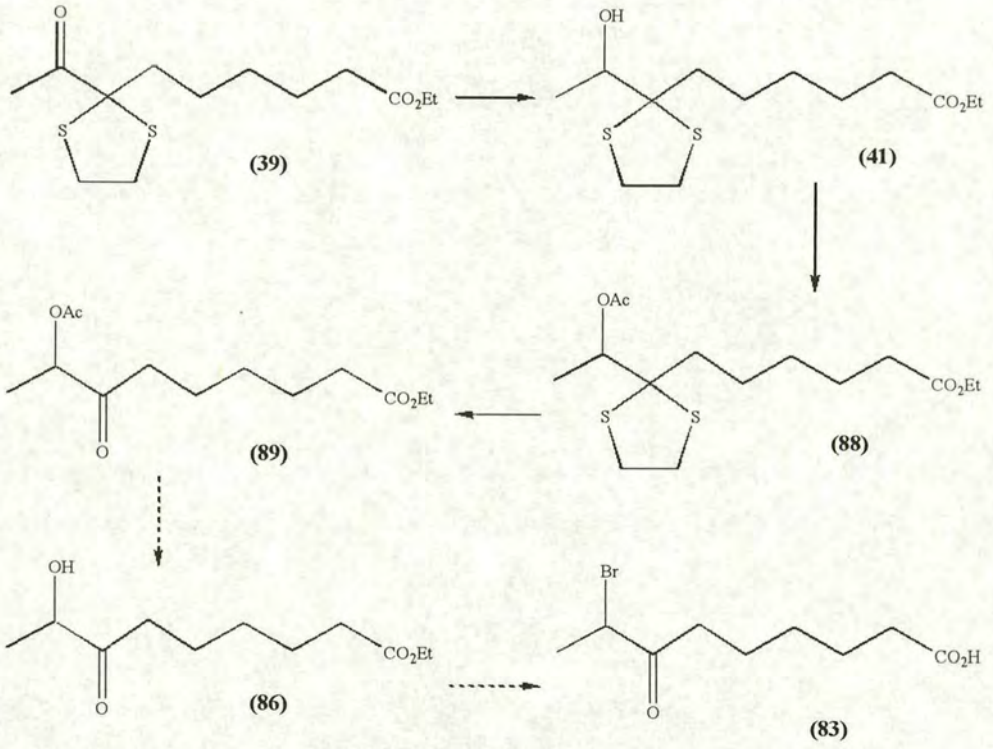


Figure 75 : Second proposed route to 83

Since an insufficient amount of the alcohol **41** was available (as a by-product of the reductive amination of **39** using the one-pot, ammonium acetate procedure) it was synthesised in 98 % yield by the reduction of ketone **39** with $NaBH_4$ in ethanol / DCM at room temperature. This compound had identical NMR characteristics, and co-eluted on tlc with the material previously obtained. The alcohol **41** was converted in high yield (94 %) to its acetate derivative **88** by reaction with excess acetic anhydride

in pyridine. Unlike compound **41**, the dithiolane group of **88** was efficiently removed by NBS in aq. acetonitrile (as performed previously), to give the ketone **89** in 89 % yield. This implies that the Br^+-mediated removal of the dithiolane moiety from **88** occurs more quickly than for **41**, and consequently there is less time for the slower sulfur oxidation process to take place.

Regrettably, lack of material and the preferential pursuit of other synthetic targets has meant that this route has not been taken any further.

Chapter Four

The Inhibition of Dethiobiotin Synthetase

4.1 The Formation of the *Ureido* ring of Dethiobiotin

Recent work in our laboratory[198] has shown that the binding of the terminal methyl group plays an important role in the DTBS-catalysed reaction. Comparison of the crystal structures of the amino-carbamate **18**, and DTB bound to DTBS has revealed that the enzyme affects a change in conformation of the bound DAN-carboxyphosphate intermediate **25** to set-up the correct geometry for the *ureido* ring closure. There is a *ca.* 60 ° rotation around the C_7-C_8 bond which places the methyl moiety in a new hydrophobic binding pocket (see figure 76). The nature of the protein architecture within the active site is such that this rotation is optimised for the methyl group.

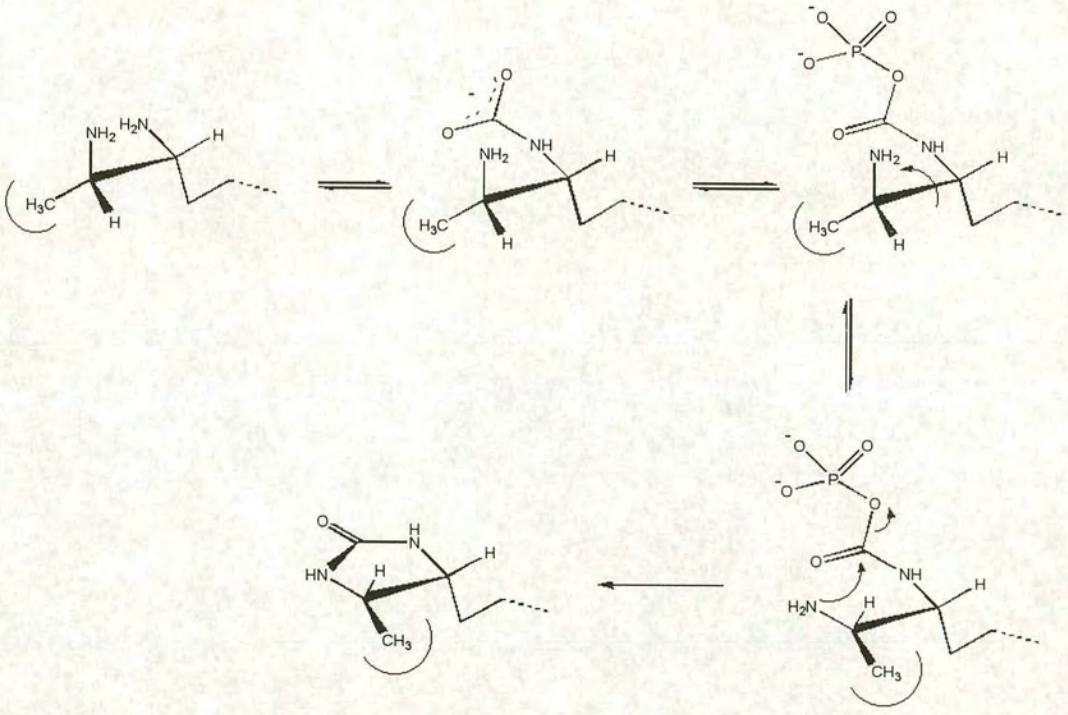
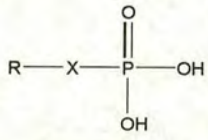


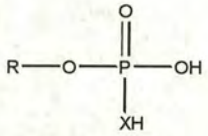
Figure 76 : The role of the methyl group in the *ureido* ring closure reaction

4.2 The Synthesis of a Potential Inhibitor of Dethiobiotin Synthetase

In the past, phosphate esters have been identified as prototypes for the design and synthesis of analogues that would posses modified chemical, and hence biological properties.[199] In general, most attention has been placed upon the isosteric replacement of either an internal, or an external oxygen atom of the phosphate, with nitrogen (X = NR'), sulfur (X = S) or methylene (X = CH_2) functions (see figure 77). Internal oxygen substitution leads to the phosphoramidate **90**, thiolphosphate **91** and phosphonate **92** analogues. These substitutions preserve the general ionisation pattern of the parent phosphate monoester, without leading to a large difference in the geometrical arrangement around the phosphorus centre.



Internal oxygen replacement



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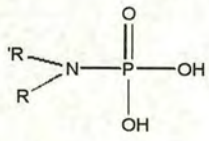
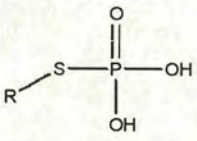


(90)



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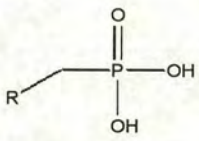
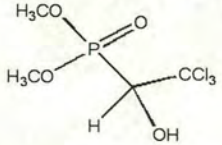


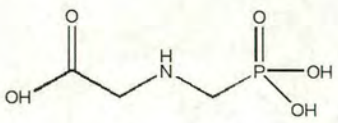
(92)

Figure 77 : Phosphate ester analogues

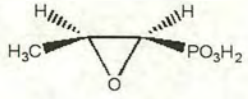
Amongst examples of phosphonate analogues of phosphate esters that have found practical applications are i) the insecticide trichlorfon **(93)**, ii) the weedkiller glyphosate **(94)** and iii) the antibiotic phosphonomycin **(95)** (see figure 78).



(93)



(94)

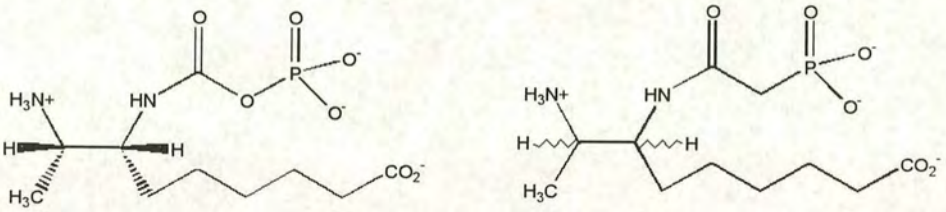


(95)

Figure 78 : Commercially used phosphonate compounds

The phosphonate isostere **96** of the mixed anhydride species **25**, the proposed intermediate in the penultimate step of biotin biosynthesis,[93,94] was identified as a target compound (see figure 79). **96** should adopt a conformation similar to **25** in the binding site of DTBS, but due to the methylene incorporation, would be unable to undergo an internal phosphate displacement. If there was a strong non-covalent interaction between **96** and the enzyme, as may be postulated for a short-lived enzymatic intermediate, then the compound would be expected to reversibly inhibit the enzyme.

If this was shown to be the case, then **96** could be catagorised as a transition state analogue.[200]



(25) (96)

Figure 79 : The postulated DTBS enzymatic intermediate and isosteric analogue

The amine **40**, an intermediate from the dithiolane-based AON synthesis, should be a good starting point for the synthesis of **96**, as it contains a mono-protected 7,8-difunctional moiety. The general strategy here was to protect the primary amine at the C-8 position, and then convert the 7,7-(ethylenedithio)-group to an amine, which could be coupled to a protected phosphonoacetate compound. The formed phosphono-amide could then be fully deprotected to give the desired product (see figure 80).

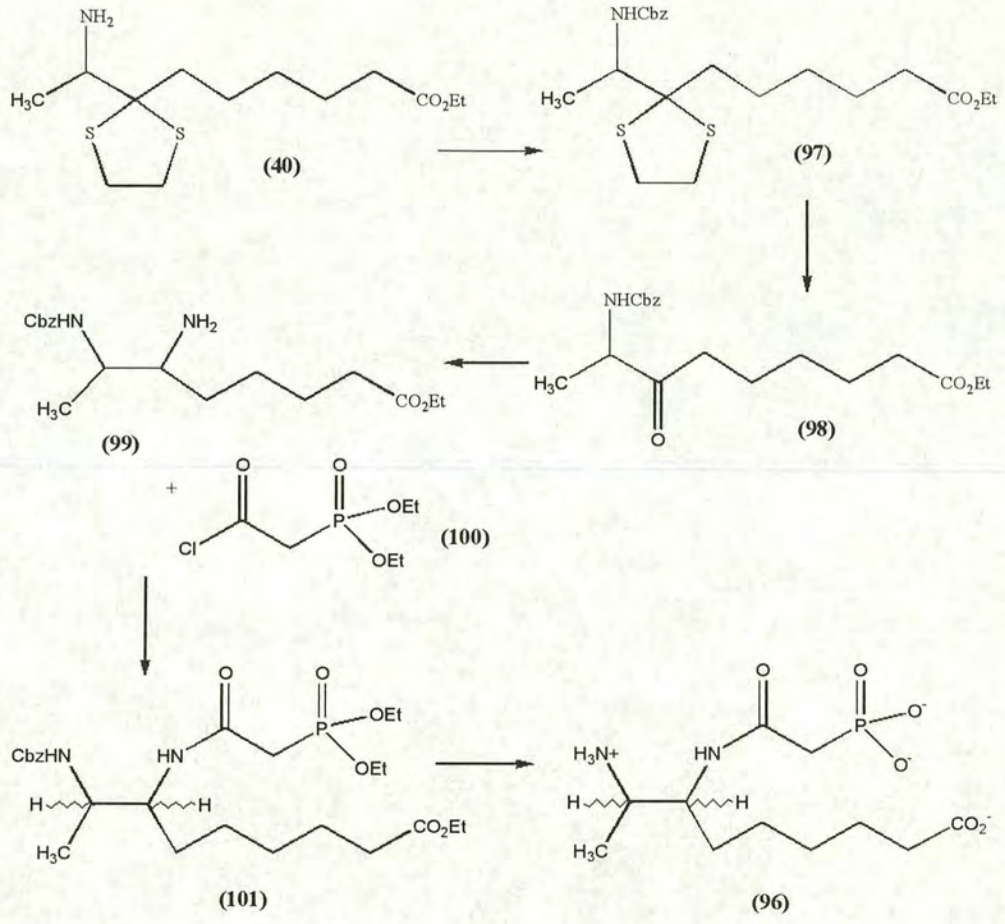
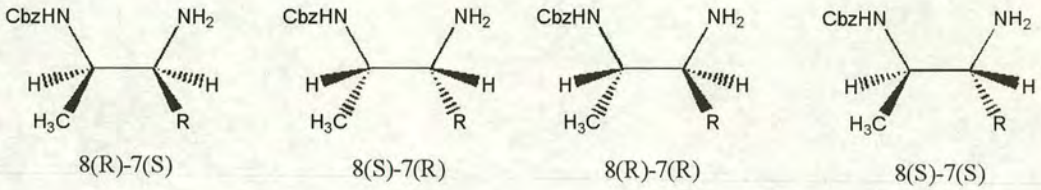


Figure 80 : Proposed synthesis of the intermediate-isosteric analogue (96)

The amine **40** was converted to its N-benzyloxycarbonyl derivative **97** in 88 % yield, using benzyl chloroformate. Deprotection of the dithiolane compound **97** was performed as before, to give the ketone **98** in high yield. The reductive amination of **98** was initially performed using the one-pot, ammonium acetate / $NaCNBH_3$ procedure used previously. However as found in the synthesis of **40**, the yields of this reaction were both modest and variable, and competing ketone reduction occurred. When the HMDS / $TiCl_4$ two-step reductive amination procedure was used, **99** was formed in 51 % yield and no alcohol formation was observed.

Whilst the reductive amination of ketone **39** generates a racemic product **40**, the analogous reaction with the prochiral ketone **98** results in the formation of two diastereomers. Examination of the ^1H NMR spectrum of the product indicated that it

contained both diastereomers in a 1:1 ratio. This was clear from comparison of the integrals for the doublets at 1.04 and 1.14 ppm, corresponding to the two C-9 methyl protons of the diastereomeric mixture. This ratio was constant for both of the reductive amination protocols.



erythro diastereomeric pair **(104)** ***threo*** diastereomeric pair **(105)**

Figure 81 : The diastereomeric amines formed

Although both of the diastereomeric amines **104** and **105** (see figure 81) were identical on tlc, they proved separable by adsorption column chromatography on silica-gel, eluting with a gradual DCM / methanol gradient. Diastereomer (a) eluted first (C-9 methyl doublet at 1.14 ppm, amido NH resonance at 5.18 ppm, C-8 at 54.7, urethane C=O at 156.1) followed by diastereomer (b) (methyl doublet at 1.04, NH at 5.27, C-8 at 54.8, urethane C=O at 155.8). At 250 MHz, the signals at 2.7 and 3.8 ppm on the ^{1}H NMR spectrum, corresponding to the C-7 and C-8 methyne protons respectively, were unresolved for both diastereomers (a) and (b). Selective decoupling experiments at 600 MHz also failed to resolve these resonances clearly enough to obtain any of the coupling constants for these protons.

However, separation of the diastereomers was far from quantitative and only small amounts of pure material could be separated. For this reason it was decided to continue with the next step of the synthesis using a mixture of the diastereomeric amines - with the hope that subsequent diastereomeric mixtures would prove more tractable to separation

The peptide coupling was initially performed using dicyclohexylcarbodiimide (DCC) as the activating agent for diethyl phosphonoacetate. This afforded the desired product **101** in modest yield (34 %). The main problem with this procedure was the

separation of the product from the dicycloheyl urea formed as a byproduct. The DCU also displayed similar solubility and chromatographic properties to **101** necessitating repeat chromatography of the initially obtained product.

A diisopropylcarbodiimide (DIC) / 1-hydroxy-4-carbethoxytriazole (HOCt) coupling was then attempted in the presence of Hunig's base (diisopropylethylamine). This afforded **101** in higher yield (54 %), but again the urea biproduct proved difficult to remove from the product mixture.

The use of the acid chloride of diethyl phosphonoacetate **(100)**, in the synthesis of **101** was tested by conducting a trial reaction with isopropylamine (see figure 82). Phosphono-amide **106** was formed cleanly, in good yield by the addition of isopropylamine to a solution of **100** in DCM at room temperature.

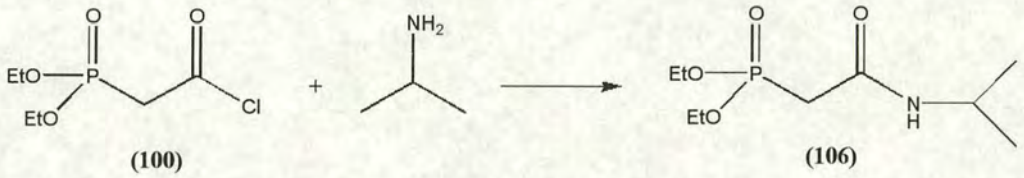


Figure 82 : Trial coupling reaction

The purity of **100** was determined by ^{1}H NMR spectroscopy; observing the disappearance of the methylene signal for the acid at 2.97 ppm (2H, doublet, J_{PH} 22 Hz), and the appearance of the methylene signal for the acid chloride **100** at 3.49 ppm (2H, doublet, J_{PH} 21.2).

Development of this synthesis showed that the best results were obtained by the dropwise addition of excess triethylamine to a solution of amine **99** and a 2 molar equivalent of **100** (freshly prepared by reacting diethyl phosphonoacetate with 2 molar equivalents of oxalyl chloride), in DCM at ambient temperature. Using this procedure, **101** was formed in 70% isolated yield, without the previously encountered problems of urea contamination.

The *erythro* and *threo* diastereomers of **101** were again only partially separable by silica-gel chromatography, so the use of reverse phase HPLC was investigated as a

means of purification. Although the compound could be cleanly separated from starting materials and by-products on a C_8 column, the diastereomers proved inseparable.

A large number of methods known for the removal of esters, ethers and urethanes, which are commonly used as protecting groups, involve the use of halide ions under non-hydrolytic conditions.[131,201-203] It was thought that this would be the most effective method for the full deprotection of **101** under mild conditions. To avoid the unnecessary wastage of material, a trial reaction was first performed on the previously synthesised compound **106** (see figure 83)

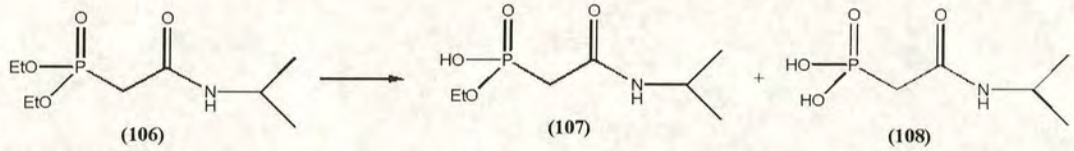
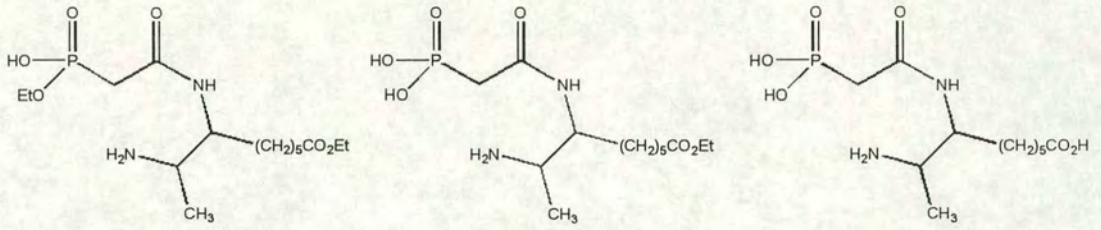


Figure 83 : Trial deprotection of 106

Treating **106** with a 10 molar equivalent of bromotrimethylsilane (TMS-Br)[201] in DCM at room temperature for 18 hours, resulted in formation of the deprotected phosphonate **108** in around 65-70 % yield, with the remainder of material being the mono-phosphonate ester **107** (by comparison of the integrals for the doublets at 2.70 and 2.72 corresponding to the methylene protons of **107** and **108** respectively in the ^{1}H NMR spectrum).

Treatment of a solution of **101** in DCM, with a 20 molar equivalent of TMS-Br at 35°C for 18 hours led to a mixture of three species being formed; the desired product **96**, its ethyl ester **110**, and the mono-phosphonate ester **109** (see figure 84). The products were identified by a combination of ^{1}H NMR and mass spectroscopy [m/z 310 **(96)**, 338 **(110)** and 362 **(109)**]. The relative amounts present could not be accurately determined due to the superimposition of the signals corresponding to the diastereomeric phosphonate methylene protons at 2.70 ppm, and the phosphonate and alkyl ethyl ester methylene, and methyl protons at 3.95 and 1.10 ppm respectively.



(109) (110) (96)

Figure 84 : The products from the TMS-Br deprotection of 101

From these two experiments, it can be clearly seen that the ease of removal of the protecting groups is : NH-Cbz > P(O)OEt > C(O)OEt, which is in agreement with the findings of Lott *et al.*[131] and McKenna and Schmidhauser.[201] Consequently, the more reactive iodotrimethylsilane (TMS-I)[131,202] was used in the same molar ratio (20 eq.), under analogous conditions. This yielded a mixture of two species; the desired product **96** and its ethyl ester **110** in roughly 80 % and 20 % amounts respectively. However, when a 25 molar excess of TMS-I was used, and the reaction was stirred for 48 hours at 45 °C, **96** was isolated in 95 % yield, as a mixture of diastereomers. Once again however, the diastereomeric mixture proved intractable to chromatographic separation.

The biological properties of the *erythro* and *threo* diastereomers of the transition-state mimic **96** are currently under investigation.

Chapter Five

Conclusions and Suggestions for Future Work

The first two intermediates of the known biotin biosynthetic pathway in *E. coli* have been successfully synthesised. Pimeloyl-CoA has been synthesised in 21 % yield by the modification of a two-step literature procedure.[76] Three alternative approaches have been taken in the synthesis of the second known biosynthetic intermediate, 8-amino-7-oxononanoate (AON).

A five-step pathway using Umpolung chemistry, has been used to synthesise (±)-AON in 10 % overall yield. (±)-AON synthesised by this pathway has been soaked into AONS crystals, and the x-ray structural elucidation of the external PLP-AON aldimine bound in the enzyme active site has been achieved [158].

Methodology for the acylation of various aromatic Schiff-bases has been developed, and using this approach a three-step synthesis of (±)-AON has been accomplished in 20 % overall yield. This route should prove applicable to the construction of structurally related α-oxoamines. In a modification of this strategy, a three step biomimetic synthesis of (±)-AON has been achieved, involving the acylation of a deprotonated aromatic aldimine with a thioester, mirroring the *bio*F-PLP mediated process.

The use of both solid-liquid and liquid-liquid phase transfer catalysis (PTC) in the acylation of aromatic Schiff-bases has been investigated. However, further work is required to determine whether or not PTC is a feasible alternative to standard anhydrous techniques for these systems.

(±)-AON has also been synthesised by a five step route utilising Dakin-West chemistry in an overall yield of 10%. The versatile nature of this pathway, combined with the mild conditions employed in the oxazol-5-one acylation step, should make it applicable for the synthesis of other biologically important metabolites such as 5-aminolaevulinic acid and dehydrosphinganine. This route may be also practicable for the synthesis of the putative carboxylated AON biosynthetic intermediate **51**, and similar α-oxo-α-amino acids.

The complimentary nature of the three synthetic approaches to AON developed should facilitate the future construction of a wide variety of structurally related compounds.

Dethiobiotin synthetase (DTBS) catalyses the penultimate step in biotin biosynthesis, the formation of the *ureido* ring moiety of dethiobiotin. A phosphonate isostere of the putative carbamoyl-phosphate intermediate in this process, identified as a possible DTBS inhibitor, has been synthesised in 4.5 % overall yield by a nine step procedure. Unfortunately it was not possible to separate the diastereomeric intermediates in the later stages of this pathway. Future work would be directed towards choosing an alternative N-protection strategy which would facilitate diastereomer separation during the synthesis. However the mixture should enable us to demonstrate whether or not this transition state analogue affects the postulated inhibitory properties towards DTBS.

Chapter Six

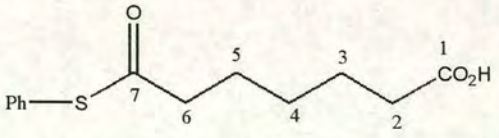
Experimental

General Experimental

All starting materials were purchased from commercial sources (generally Sigma-Aldrich, Fluka, Fisons and Acros), and were routinely purified by distillation or crystallisation before use. Merck (BDH) silica gel 60 (particle size 35 - 60 μm) was used for (wet) flash column chromatography. Thin layer chromatography (tlc) was carried out on aluminium sheets pre-coated with Merck silica gel 60 F_{254}. Solvent systems for tlc were either diethyl ether / hexane (1:1-4), ethyl acetate / hexane (1:2-6), dichloromethane / methanol (9:1) or butanol / acetic acid / water (3:1:1). Organic extracts were routinely dried over anhydrous sodium sulfate, or magnesium sulfate prior to evaporation *in vacuo*. Melting points were measured on an electrically heated Griffin melting point apparatus and are uncorrected. Infra-red spectra were recorded on a Biorad FTS7 Fourier Transform infra-red spectrophotometer. Samples were prepared as liquid films or as nujol mulls unless otherwise stated. ^{1}H-NMR spectra were recorded on a Varian Gemini 2000 (200 MHz), or a Brucker AX 250 (250 MHz), a Brucker GSX-1 (270 MHz) or a Varian Unity Inova 600 MHz instrument. The units of chemical shift (δ) are given in ppm, and coupling constants (J) are in Hz. Mass spectra were obtained by fast atom bombardment (FAB-MS) (with either glycerol or thioglycerol as matrices), or by electron impact (E.I.-MS), using a Kratos MS 50-TC spectrometer.

Preparations

6-(Phenylthiocarbonyl) hexanoic acid (32) [76]



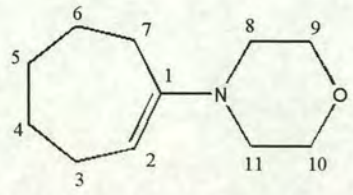
Pimelic acid **(6)** (3.00 g, 18.8 mmol) was stirred with an excess of thionyl chloride (4.0 cm^3, 56 mmol) at 60°C for 1 hour with the exclusion of moisture ($CaCl_2$ drying tube). After evaporation *in vacuo* the resultant oily material was dissolved in dry DCM (5 cm^3) and thiophenol (2.0 cm^3, 18 mmol) was added dropwise, over 5 mins.

with stirring at ambient temperature. The reaction was stirred at room temperature for 4 hours, then was extracted with a solution of $NaHCO_3$ [3 x 7 cm^3, 2 % (w/v)] and with water (7 cm^3). The aqueous phases were combined, acidified (pH 1, conc. HCl) and extracted with DCM (3 x 10 cm^3). The combined organic layers were dried (Na_2SO_4) then evaporated *in vacuo* to yield a pale yellow oil which was purified by silica-gel chromatography (cyclohexane / ethyl acetate / acetic acid: 15:4:1 by vol.) to give **32** as a colourless solid (1.48g, 5.87 mmol, 32%); m.p. 45-46 °C (lit. [76] 45-46°C), δ_H (200 MHz, $CDCl_3$) 1.30-1.45 (2H, m, 4-CH_2), 1.52-1.75 (4H, m, 3- and 5-CH_2), 2.30 (2H, t, J 7.2, 2-CH_2), 2.60 (2H, t, J 7.3, 6-CH_2), 7.35 (5H, s, Ar-*H*), 10.6 (1H, br s, CO_2H), δ_C (62.9 MHz, $CDCl_3$) 22.8, 23.7 and 26.9 (C-3, -4 and -5), 32.4 (C-2), 41.9 (C-6), 126.5, 127.9, 128.0 and 133.2 (5 x aromatic-C), 178.8 (C-1), 196.1 (C-7), ν_{max} (nujol, cm^{-1}) 1710 (CO_2 and COS). Found M^+, 252.08222 (EI-MS), $C_{13}H_{16}O_3S$ requires 252.08202.

Pimeloyl-Coenzyme A (7) [76]

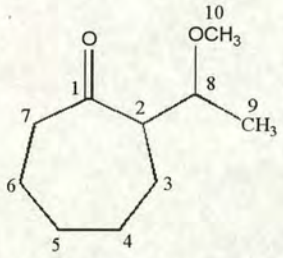
CoASH, (11 mg, 14 μmol) was dissolved in a degassed solution of $NaHCO_3$ [1 cm^3, 2% (w/v)], and **32** (28 mg, 29 μmol) in methanol (0.4 cm^3) was added. The pH was adjusted to 8 (pH paper) by the addition of $NaHCO_3$ [2% (w/v)], and the reaction mixture was stirred for 5 hours at ambient temperature, under an atmosphere of nitrogen. The pH was then adjusted to 2, by the careful addition of conc. HCl. The reaction mixture was washed with DCM (3 x 4 cm^3), and concentrated by lyophilisation before being purified by HPLC on an Aquapore OD-300 C_8 reverse phase column, eluting (5 ml/min) with a linear 5%-50% acetonitrile / water gradient (0.1% TFA), monitoring at λ = 214 nm. Thus **7** (8.5 mg, 9.3 μmol, 65 %) was obtained as a colourless solid ; m/z (MALDI-TOF) 910.0 (MH^+).

1-Morpholino-1-cycloheptene (35) [132]



Cycloheptanone (40.0 g, 350 mmol), morpholine (36.6 g, 420 mmol) and *p*-toluene sulfonic acid (0.40 g) were dissolved in toluene (100 cm^3), and heated under reflux (6 hours), with the water generated being removed by Dean-Stark apparatus. Evaporation of the toluene *in vacuo* gave an yellow oil which was purified by distillation under reduced pressure to give **35** as a clear, colourless oil (44.0 g, 238 mmol, 68%) ; b.p.140-150 °C/ 20 mm Hg (lit. [132] 133-135 °C/ 17 mm Hg), δ_H (250 MHz, $CDCl_3$) 1.34-1.46 (4H, m, 4- and 5-CH_2), 1.63-1.71 (2H, m,6-CH_2), 1.99-2.06 (2H, m, 3-CH_2), 2.16-2.20 (2H, m, 7-CH_2), 2.65 (4H, t, J 4.8, 8- and 11-CH_2), 3.66 (4H, t, J 4.8, 9- and 10-CH_2), 4.82 (1H, t, J 6.9, 2-CH).

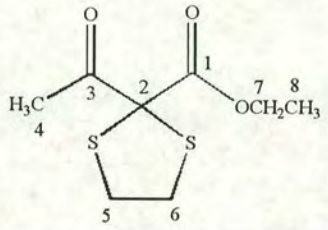
2-(1-Methoxyethyl)-1-cycloheptanone (36)



Enamine **35** (8.00g, 44.1 mmol) was added dropwise, with stirring, to a solution of acetaldehyde dimethyl acetal (4.50 g, 50.0 mmol) and boron trifluoride etherate (7.48 g, 52.7 mmol) in dry DCM (50 cm^3) at -42°C (dry ice / acetonitrile) under argon. After stirring for 3 hours, the reaction mixture was carefully quenched by the addition of aq. HCl (50 cm^3, 10 mM), and was stirred for a further hour. The organic layer was separated and washed with water (50 cm^3) and brine (30 cm^3) before being dried (Na_2SO_4). Evaporation of the solvent *in vacuo* yielded a dark-brown oil (6.55 g) which was purified by silica gel chromatography (hexane / ethyl acetate) to give a clear, colourless oil **36** (1.64 g, 9.7 mmol, 22 %) as a mixture of diastereomers (formed in a 2:1 ratio); b.p. 80-90 °C / 0.5 mm Hg, ν_{max} (neat, cm^{-1}) 1690 (C=O), δ_H ($CDCl_3$, 250 MHz) 1.03 (1H, d, J 6.2, 9-CH_3), 1.06 (2H, d, J 6.2, 9-CH_3), 1.25-1.50 (4H, m, 4- and 5-CH_2), 1.60-1.95 (4H, m, 3- and 6-CH_2), 2.30-2.60 (3H, m, 7-CH_2 and 2-CH), 3.21 (2H, s, 10-CH_3), 3.22 (1H, s, 10-CH_3), 3.59 (1H, m, 8-CH), δ_C (62.9 MHz, $CDCl_3$) 15.9 and 16.9 (C-9), 24.3, 24.6, 24.7, 25.5, 25.8, 29.6, 29.7 and 30.4 (C-3, -4, -5 and 6), 43.8 and 44.1 (C-7), 56.3 and 56.7 (C-10), 57.3 and 58.6 (C-2),

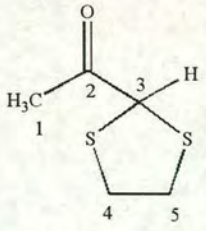
77.6 and 77.7 (C-8), 215.4 and 216.2 (C-1), Found : M^+, 170.13104 (EI-MS), $C_{10}H_{18}O_2$ requires 170.13069.

2-Carbethoxy-2-acetyl-1,3-dithiolane (42) [139]



Sulfuryl chloride (14.33 g,106 mmol) was added dropwise over 30 mins., with stirring, to a solution of ethane-1,2-dithiol (5.00 g, 53.1 mmol) in dry DCM (50 cm^3) at -10 °C under an inert atmosphere. The resultant brown solution was stirred at 0°C for a further 30 mins. before ethyl acetoacetate (6.91 g, 53.1 mmol) in DCM (5 cm^3) was added dropwise with stirring at 0-5 °C, over a 1 hour period. The reaction mixture was then stirred at room temperature for 5 hours, before being filtered to remove a small amount of white polymeric material. After removal of the solvent *in vacuo* , the resultant oil was distilled under reduced pressure yielding a yellow oil **42** (7.83 g, 35.6 mmol, 67 %); b.p. 145°C / 0.6 mm Hg (lit. [139] 125°C / 0.1 mm Hg), ν_{max} (neat, cm^{-1}) 1708 (CO), 1740 (CO_2), δ_H (200 MHz, $CDCl_3$) 1.30 (3H, t, J 7.2, 8-CH_3), 2.41 (3H, s, 4-CH_3), 3.41 (4H, m, 5- and 6-CH_2), 4.28 (2H, q, J 7.2, 7-CH_2), δ_C (62.9 MHz,$CDCl_3$) 13.7 (C-8), 25.6 (C-4), 40.1 (C-5 and -6), 63.0 (C-7), 76.5 (C-2), 168.4 (C-1), 197.0 (C-3). Found M^+, 220.02287 (EI-MS), $C_8H_{12}O_3S_2$ requires 220.02279.

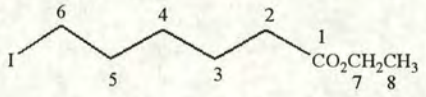
2-Acetyl-1,3-dithiolane (38) [139]



A mixture of **42** (3.24 g, 147 mmol), conc. H_2SO_4 (1.4 cm^3), water (5.5 cm^3) and glacial acetic acid (0.7 cm^3) was stirred and heated under reflux for 24 hours. The

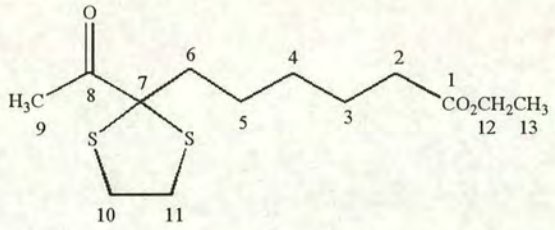
mixture was allowed to cool to room temperature before being extracted with DCM (3 x 10 cm^3). The combined organic extracts were washed with brine (20 cm^3) before being dried (Na_2SO_4), and evaporated *in vacuo* to give a brown oil. Distillation under reduced pressure afforded **38** as a clear yellow oil (1.78 g, 120 mmol, 82 %); b.p. 135°C / 0.5 mm Hg (lit. [139] 73°C / 0.05 mm Hg), ν_{max} (neat, cm^{-1}) 1717 (CO), δ_H (200 MHz, $CDCl_3$) 2.32 (3H, s, 1-CH_3), 3.32 (4H, s, 4- and 5-CH_2), 4.84 (1H, s, 3-CH), δ_C (50.28 MHz, $CDCl_3$) 24.1 (C-1), 37.6 (C-4 and -5), 56.4 (C-3), 201.2 (C-2). Found : M^+, 148.00189 (EI-MS), $C_5H_8OS_2$ requires 148.00166.

Ethyl 6-iodo hexanoate (43) [140]



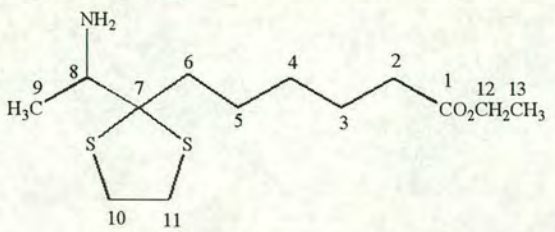
Ethyl 6-bromohexanoate (2.10 g, 1.68 cm^3, 9.41 mmol) was added dropwise, with stirring, to a solution of anhydrous NaI (2.86g, 19.1 mmol) in acetone (35cm^3, freshly distilled from $CaSO_4$), with the exclusion of light. The solution was stirred for 1 hour then heated under reflux for one further hour. After allowing it to cool, the solution was poured into hexane (150 cm^3), then filtered through a glass sinter, before the solvent was evaporated *in vacuo*. Removal of the residual solid (filtration) yielded a golden oil, which was purified by distillation to give **43** as a clear colourless oil (2.28 g, 8.75 mmol, 93 %); b.p. 100°C / 5 mm Hg (lit. [140] b.p. 135-137 °C / 16 mm Hg) ; δ_H (200 MHz, $CDCl_3$) 1.25 (3H, t, J 7.2, 8-CH_3), 1.45 (2H, m, 4-CH_2), 1.65 (2H, m, 3-CH_2), 1.84 (2H, m, 5-CH_2), 2.30 (2H, t, J 7.4, 2-CH_2), 3.18 (2H, t, J 7, 6-CH_2), 4.11 (2H, q, J 7.2, 7-CH_2), δ_C (50.28 MHz, $CDCl_3$) 5.2 (C-6), 12.8 (C-8), 22.4, 28.5, 31.7 and 32.7 (C-2, -3, -4 and -5), 58.9 (C-7), 172.1 (C-1). Found : M^+, 270.01168 (EI-MS), $C_8H_{15}IO_2$ requires 270.01169.

Ethyl 7,7-(ethylenedithio)-8-oxo-nonanoate (39)



Acetyl dithiolane **38** (0.350 g, 2.36 mmol) was added dropwise to a stirred solution of LDA [2.71 mmol; prepared from diisopropylamine (0.38 cm^3, 2.71 mmol) and n-butyllithium (1.25 cm^3, 1.94 M in pentane)] in THF (5cm^3) at -78°C under argon. The red solution was stirred for 1 hour then ethyl 6-iodohexanoate **43** (1.50 g, 5.56 mmol) in HMPA (freshly distilled from CaH_2) (3.5cm^3) was added dropwise. The reaction was stirred for 4 hours at -78°C, then for 24 hours at room temperature before being poured into a stirred solution of saturated ammonium chloride (10 cm^3), and extracted with ethyl acetate (4 x 10 cm^3). The combined organic layers were washed with a saturated aqueous lithium chloride solution (20 cm^3) (to remove HMPA), then brine (20 cm^3), before being dried (Na_2SO_4). Evaporation of the solvent on a rotary evaporator yielded an orange oil (0.70 g), which was purified by silica-gel chromatography (ethyl acetate / hexane) to give **39** as a pale yellow oil (0.666 g, 2.30 mmol, 96 %); δ_H ($CDCl_3$, 200 MHz) 1.24 (3H, t, J 7.2, 13-CH_3), 1.37 (4H, m, 4- and 5-CH_2), 1.62 (2H, m, 3-CH_2), 2.11 (2H, m, 6-CH_2), 2.27 (2H, t, J 7.4, 2-CH_2), 2.39 (3H, s, 9-CH_3), 3.36 (4H, s, 10- and 11-CH_2), 4.09 (2H, q, J 7.2, 12-CH_2), δ_C (62.9 MHz, $CDCl_3$) 14.0 (C-13), 25.1 (C-9), 24.4, 26.7 and 28.9 (C-3, -4 and -5), 33.9 (C-2), 39.2 (C-6), 40.1 (C-10 and -11), 60.0 (C-12), 75.2 (C-7), 173.4 (C-1), 202.7 (C-8). Found : MH^+, 291.10887 (EI-MS), $C_{13}H_{23}O_3S_2$ requires 291.10811.

Ethyl-7,7-(ethylenedithio)-8-(R,S)-amino-nonanoate (40)



Method 1

Sodium cyanoborohydride (22 mg, 345 μmol), **39** (94 mg, 325 μmol) and ammonium acetate (0.265g, 3.45 mmol, recrystallised from methanol, then vacuum dried) in methanol (3 cm^3) was stirred over 4Å molecular sieves for 36 hours under an atmosphere of nitrogen. After filtration, the excess methanol was removed *in vacuo*, and HCl (1 M, 10 cm^3) was added. The mixture was extracted with ether (2 x 10 cm^3), then solid NaOH was added to bring the aqueous solution to pH >10. After saturation with NaCl, the solution was extracted with ethyl acetate (4 x 10 cm^3). The combined ethyl acetate extracts were dried (Na_2SO_4), and evaporated *in vacuo* to give pure amine **40** as a clear colourless oil (49 mg, 168 μmol, 52 %).

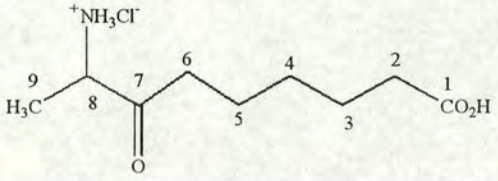
Method 2

A solution of titanium tetrachloride (0.6 eq., 5.57 mmol, 5.57 cm^3, 1M in DCM) was added dropwise to a stirred solution of triethylamine (3 eq., 2.81 g, 27.8 mmol), **39** (2.69 g, 9.28 mmol) and 1,1,1,3,3,3-hexamethyldisilazane (1.5 eq., 2.25 g, 13.9 mmol) in dry DCM (70 cm^3) under an inert atmosphere at room temperature. The dark-brown reaction was left overnight, then quenched by the careful addition of a solution of $NaCNBH_3$ (3 eq., 1.75 g, 27.8 mmol) in dry methanol (25 cm^3). After allowing to react for 30 mins. at room temperature, aq. NaOH was added (50 cm^3, 1M), then the phases were separated. The aqueous phase was extracted with a further portion of DCM (50 cm^3), and the combined DCM layers were evaporated *in vacuo*. The oily residue was then partitioned between ether (30 cm^3) and aq. HCl (50 cm^3, 1M), and after separation of the phases, the ethereal layer was extracted with aq. HCl (1M, 2 x 25 cm^3). The aqueous layers were combined, the pH was raised to 12 (NaOH), and NaCl was added until saturation, before it was extracted with ethyl acetate (3 x 50 cm^3). The combined organic extracts were dried ($MgSO_4$), and solvent was removed on a rotary evaporator to yield amine **40** as a clear, colourless oil (1.59 g, 5.47 mmol, 59 %)

δ_H ($CDCl_3$, 200 MHz) 1.16 (3H, d, J 6.6, 9-CH_2), 1.25 (3H, t, J 7.2, 13-CH_3), 1.26-1.42 (2H, m, 4-CH_2), 1.56-1.69 (4H, m, 5- and 3-CH_2), 1.86-1.94 (2H, m, 6-CH_2), 2.30 (2H, t, J 7.5, 2-CH_2), 3.06 (1H, q, J 6.6, 8-CH), 3.21-3.25 (4H, m, 10- and 11-CH_2), 4.12 (2H, q, J 7.2, 12-CH_2), δ_C (62.9 MHz, $CDCl_3$) 14.2 (C-13),19.5 (C-9),

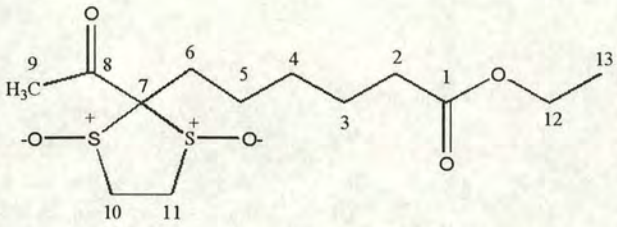
24.8, 25.8 and 29.2 (C-3, -4 and -5), 34.2 (C-2), 40.9 (C-6), 40.2 (C-10 and -11), 54.7 (C-8), 60.1 (C-12), 80.2 (C-7), 173.6 (C-1). Found : M^+, 291.13267 (EI-MS), $C_{13}H_{25}NO_2S_2$ requires 291.13161.

8-(R,S)-Amino-7-oxononanoic acid, (±)-8-AON (Hydrochloride salt) (8)



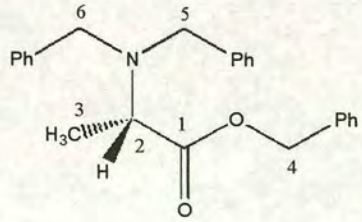
A solution of amine **40** (56 mg, 192 mmol) in acetonitrile (0.5 cm^3) was added dropwise to a rapidly stirring solution of N-bromosuccinimide (0.153 g, 859 mmol) in 80 % aq. acetonitrile (2 cm^3) at -5 °C. The orange solution was stirred for 5 minutes at -5°C (salt / ice), then stirred whilst slowly allowed to warm to room temperature for a further 5 minutes. After quenching with saturated sodium sulfite solution (2 cm^3), water was added (5 cm^3), and the aqueous reaction mixture was extracted with ether (2 x 10 cm^3). Evaporation of the ethereal layer *in vacuo* yielded a yellow oil which was immediately added to a solution of acetic acid (2 cm^3) and conc. HCl (0.5 cm^3) in water (4 cm^3). The reaction mixture was heated under reflux for 18 hours, and then after allowing to cool, was extracted with chloroform (3 x 5 cm^3). The water was removed by lyophilisation to yield a colourless solid which was purified on Dowex 50-X cation-exchange resin (H^+ form), eluting with a linear 0-1.5 M HCl gradient. Thus **34** was obtained as its colourless hydrochloride salt (21 mg, 93.9 mmol, 49 %) ; ν_{max} (nujol, cm^{-1}) 3134 and 3040 (NH_3^+ and CO*OH*), 1754 (*CO*OH), 1727 (CO, ketone), δ_H (D_2O, 200 MHz), 1.13-1.24 (2H, m, 4-CH_2), 1.39 (3H, d, J 7.5, 9-CH_3), 1.35-1.53 (4H, m, 3- and 5-CH_2), 2.22 (2H, t, J 7.3, 2-CH_2), 2.52 (2H, m, 6-CH_2), 4.10 (1H, q, J 7.5, 8-CH), δ_c (D_2O, 62.9 MHz) 14.6 (C-9), 22.2, 23.9 and 27.5 (C-3, -4 and -5), 33.7 (C-2), 37.8 (C-6), 54.8 (C-8), 179.0 (C-1), 209.3 (C-7). Found : MH^+, 188.12882 (FAB-MS), $C_9H_{18}NO_3$ requires 188.12867.

Oxidation of 41



To a solution of **41** (108 mg, 370 μmol) in acetone (20 cm^3) was added Jones' reagent (0.45 cm^3, *ca.* 4 mmol) over 1 minute whilst stirring at 0°C. After 10 mins. stirring at 0°C, the reaction mixture was partitioned between water (20 cm^3) and ether (20 cm^3). After separation of the phases, the aqueous phase was extracted with a further portion of ether (20 cm^3). The combined ethereal extracts were washed with Na_2CO_3 solution (20 cm^3, 2% w/v), water (20 cm^3) and brine (20 cm^3) before being dried over $MgSO_4$. Evaporation of the solvent *in vacuo* yielded a brown oil which was purified by silica gel chromatography (DCM / ethyl acetate) to give a pale yellow oil (60 mg, 186 μmol, 50%) tentatively assigned as a mixture of *cis* and *trans* diastereomers of ethyl 8-oxo-7-(1,3-dithilan-1,3-dioxide)-nonanoate **(44)**; δ_H ($CDCl_3$, 200 MHz) 1.25 (3H, t, J 7.2, 13-CH_3), 1.30-1.50 (2H, m, 4-CH_2), 1.50-1.75 (4H, m, 3- and 5-CH_2), 2.00-2.30 (2H, 2H, m, 6-CH_2), 2.28 (2H, t, J 7.5, 2-CH_2), 2.32 (3H, s, 9-CH_3), 3.20-3.40 (3H, m, 10- and 11-CH_2), 3.60-3.75 (1H, m, 10- and 11-CH_2), 4.11 (2H, q, J 7.2, 12-CH_2). Found M^+, 32.09176 (EI-MS), $C_{13}H_{22}O_5S_2$ requires 322.09087.

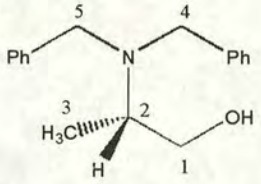
Benzyl N,N-dibenzyl-L-alaninate (48) [151]



Benzyl bromide (5.99 g, 35.3 mmol) was added to a stirred mixture of L-alanine (1.00 g, 11.2 mmol) and sodium carbonate (4.74 g, 44.8 mmol) in ethanol (12 cm^3) and water (12 cm^3). The mixture was heated under reflux (90 °C) for 3 hours, then allowed to cool to room temperature. Water (20 cm^3) was added, then the reaction mixture was extracted with DCM (3 x 20 cm^3). The combined organic extracts were

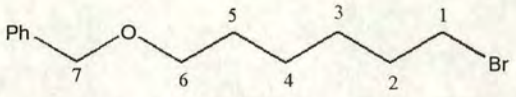
dried (Na_2SO_4) and solvent was removed *in vacuo* to give a yellow oil which was purified using silica-gel chromatography (hexane / ether) yielding **48** as a clear, colourless oil (3.20 g, 8.91 mmol, 80 %); ν_{max} (neat, cm^{-1}) 1730 (CO), δ_H (200 MHz, $CDCl_3$) 1.39 (3H, d, J 7.1, 3-CH_2), 3.61 (1H, q, J 7.1, 2-CH), 3.69 and 3.89 (4H, AB system, J_{AB} 14.0, 5- and 6-CH_2), 5.20 and 5.29 (2H, AB system, J 12.3, 4-CH_2), 7.24-7.48 (15H, m, Ar-H), δ_C ($CDCl_3$, 62.9 MHz) 14.8 (C-3), 54.2(C-5 and -6), 56.0 (C-2), 65.8 (C-4), 126.7,127.5, 127.6, 128.0, 128.1, 128.2, 128.4, 135.9 and 139.6 (18 x Ar-C), 173.3 (C-1), Found : M^+, 359.18985 (EI-MS), $C_{24}H_{25}NO_2$ requires 359.18853.

N,N-Dibenzyl-2-(S)-amino-1-propanol (49) [151]



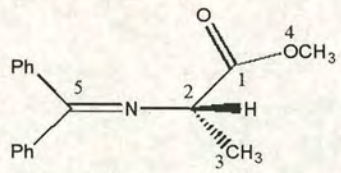
N,N-dibenzyl-L-alanine benzyl ester **48** (0.470 g, 1.31 mmol) in dry ether (3 cm^3) was added dropwise to a slurry of $LiAlH_4$ (0.158 g, 4.16 mmol), with cooling (ice bath). The reaction was slowly allowed to warm to room temperature, and was stirred for a further 4 hours before being carefully quenched by the addition of NaOH (2.5 cm^3, 0.5 M). The aluminium salts were removed by filtration and washed with ether, before the liquid phases were separated. The organic extract was washed with water (5 cm^3) and brine (5 cm^3), before being dried (Na_2SO_4). Solvent was removed *in vacuo* to give a pale yellow oil, which was purified by silica gel chromatography (ether / hexane) to yield **49** as a clear oil (0.280 g, 1.10 mmol, 84%); ν_{max} (neat, cm^{-1}) 3432 (OH), δ_H (200 MHz, $CDCl_3$) 0.98 (3H, d, J 6.6, 3-CH_2), 2.99 (1H, m, 2-CH), 3.12 (1H, br.s, OH), 3.35 and 3.82 (4H, AB system, J 13.2, 4- and 5-CH_2), 3.41 (2H, m, 1-CH_2), 7.2-7.4 (10H, m, Ar-H), δ_C ($CDCl_3$, 62.9 MHz) 8.5 (C-3), 52.7 (C-4 and -5), 54.0 (C-2), 62.5 (C-1), 127.1 (2 x *p*-C) 128.3 and 128.8 (8 x *o,m*-C), 139.1 (2 x *ipso*-C), Found : M^+, 255.16231 (EI-MS), $C_{17}H_{21}NO$ requires 255.16231.

6-Benzyloxybromohexane (46) [204]



A solution of 1,6-dibromohexane (5.12 g, 20.3 mmol) and NaOBz (2.64 g, 20.3 mmol) in dry THF (100 cm^3) was stirred at ambient temperature under an inert atmosphere for three days. After evaporation of the solvent, the residue was taken up in ether (30 cm^3), and washed with aq. HCl (20 cm^3, 1M), aq. Na_2CO_3 (20 cm^3, 10 % w/v) and brine (20 cm^3) before being dried ($MgSO_4$). Evaporation of the solvent *in vacuo* yielded a yellow oil which was purified by silica-gel chromatography (hexane / ether) to give **46** as a clear, colourless oil (2.01 g, 7.40 mmol, 36 %); δ_H (200 MHz, $CDCl_3$) 1.39-1.44 (4H, m, 3- and 4-CH_2), 1.60-1.67 (2H, m, 5-CH_2), 1.83-1.90 (2H, m, 2-CH_2), 3.40 (2H, q, J 7.2, 6-CH_2), 4.47 (2H, q, J 7.0, 1-CH_2), 4.50 (2H, s, 7-CH_2), 7.31-7.34 (5H, m, Ar-H), Found : M^+, 270.06183 (EI-MS), $C_{13}H_{19}BrO$ (^{79}Br) requires 270.06193.

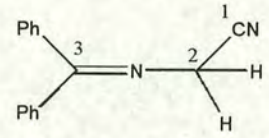
Methyl 2-[N-(diphenylmethylene)]-L-alaninate (57) [205]



Benzophenone imine (1.00 g, 5.52 mmol) and L-alanine-methyl ester hydrochloride (0.770 g, 5.52 mmol) in DCM (20 cm^3) was stirred at room temperature for 24 hours with the exclusion of moisture ($CaCl_2$) tube. The reaction mixture was filtered to remove NH_4Cl and evaporated to dryness on a rotary evaporator. The residue was taken up in ether (30 cm^3) and washed with water (2 x 20 cm^3) then brine (20 cm^3), before being dried ($MgSO_4$). Removal of the solvent *in vacuo* yielded a clear colourless oil which slowly crystallised to give the title compound as a colourless solid (1.43 g, 96 %); m.p. 70-71 °C (lit. [205] m.p. 69-72 °C), δ_H (200 MHz, $CDCl_3$) 1.41 (3H, d, J 6.8 Hz, 3-CH_3), 3.72 (3H, s, 4-CH_3), 4.17 (1H, q, J 6.8 Hz, 2-CH), 7.15-7.65 (10H, m, Ar-H), δ_C (50.28 MHz, $CDCl_3$) 19.4 (C-3), 52.3 (C-2), 60.8 (C-4), 127.9, 128.1, 128.2, 128.5, 128.5, 128.8, 128.9, 129.0 and 130.5 (10 x Ar-C), 136.4

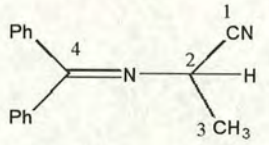
and 139.7 (2 x *ipso*-C), 169.9 (C-5), 173.6 (C-1), m/z (CI) 268 (MH^+), 208 [(M-CO_2CH_3)$^+$], 77 ($C_6H_5^+$).

[(Diphenylmethylene)-amino]-acetonitrile (58) [165]



Prepared analogously to **57** from benzophenone imine (1.00g, 5.52 mmol) and aminoacetonitrile hydrochloride (0.510 g, 5.52 mmol) in DCM (20 cm^3), to give **58** as a colourless solid (1.11 g, 93%); m.p. 82-83°C (lit. [165] m.p. 81-82°C), δ_H (200 MHz, $CDCl_3$) 4.22 (2H, s, 2-CH_2), 7.1-7.7 (10H, m, Ar-H), δ_C (50.28 MHz, $CDCl_3$) 41.5 (C-2), 117.8 (C-1), 127.4, 128.5, 128.7, 129.1, 129.4 and 129.6 (8 x *o,m*-C), 129.7 and 131.4 (2 x *p*-C), 135.0 and 138.4 (2 x *ipso*-C), 174.5 (C-3), m/z (EI) 221 (M^+), 193 [(M-HCN)$^+$], 180 [(M-CH_2CN)$^+$], 77 ($C_6H_5^+$).

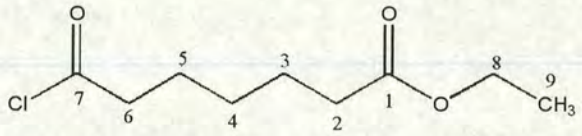
2-(Diphenylmethylene-amino)-propionitrile (59) [172]



58 (3.00 g, 13.6 mmol), Benzyl triethylammonium chloride (BTEAC) (0.310 g, 1.36 mmol), 50% aqueous sodium hydroxide (3.8 cm^3, 47.6 mmol) and toluene (3 cm^3) were placed in a round-bottomed flask, stoppered and stirred at 0°C for 1 minute. Methyl iodide (2.33 g, 16.3 mmol) was added to the reaction mixture via syringe over 2 hours at 0°C. The reaction was stirred for 2 hours at 0°C, then allowed to warm to room temperature and stirred for a further 18 hours. The reaction mixture was poured into a separatory funnel containing DCM (40 cm^3) and water (40 cm^3). After the separation of the layers, the aqueous phase was extracted with a portion of DCM (40 cm^3), and the combined organic extracts were washed with water (40 cm^3), then brine (40 cm^3), before being dried over $MgSO_4$. Evaporation of the solvent *in vacuo* yielded an orange oil (3.60 g), which was further purified by column chromatography to give the title compound as a yellow oil (2.16 g, 9.23 mmol, 68%); δ_H (200 MHz,

$CDCl_3$) 1.55 (3H, d, J 7.0, 3-CH_3), 4.32 (1H, q, J 7.0 Hz, 2-CH), 7.20-7.70 (10H, m, Ar-H), δ_C (62.9 MHz, $CDCl_3$) 20.8 (C-3), 48.0 (C-2), 120.3 (C-1), 127.1, 128.1, 128.8, 128.8 and 128.9 (8 x *o,m*-C) 129.2 and 131.0 (2 x *p*-C), 134.9 and 138.2 (2 x *ipso*-C), 172.2 (C-4), m/z (EI) 234 (M^+), 207 [$(M\text{-}HCN)^+$], 77 ($C_6H_5^+$). Found: M^+, 234.11599, $C_{16}H_{14}N_2$ requires 234.115699.

Ethyl pimeloyl chloride (60) [206]



Oxalyl chloroide (1.35 g,10.6 mmol) was added to ethyl hydrogen pimelate (1.00 g, 5.32 mmol) in a flask fitted with a reflux condenser under nitrogen. The mixture was stirred for 45 mins. at room temperature, then for 1 hour at 60°C. Evaporation of volatile components *in vacuo* gave an essentially quantitative yield of **60** as a clear oil, which was purified by distillation; b.p. 100-105 °C / 4 mm Hg (lit. [206] b.p. 126-129 °C / 8.5 mm Hg), δ_H (200 MHz, $CDCl_3$) 1.25 (3H, t, J 7.2, 9-CH_3), 1.32-1.45 (2H, m, 4-CH_2), 1.57-1.82 (4H, m, 3- and 5-CH_2), 2.30 (2H, t, J 7.2, 2-CH_2), 2.89 (2H, t, J 7.3, 6-CH_2), 4.12 (2H, q, J 7.2, 8-CH_2).

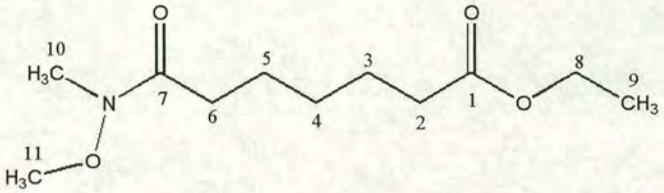
Acetylation of 58 with acetyl chloride

(All solids were dried and finely ground prior to use)

Acetyl chloride (75 mg, 955 μmol) was added to a dry flask containing **58** (210 mg, 955 μmol), potassium carbonate (63 mg, 456 μmol) and BTEAC (12 mg, 53 μmol). The flask was stoppered then the mixture was sonicated for one hour at 45 °C, before being partitioned between water (10 cm^3) and DCM (10 cm^3). After separation of the phases, the aqueous phase was extracted with DCM (5 cm^5), then the organic layers were combined and washed with water (10 cm^3), brine (10 cm^3) and dried ($MgSO_4$). Evaporation of the solvent *in vacuo* yielded an orange oil which was purified by silica gel chromatography (hexane / ethyl acetate) to give a pale yellow oil, tentatively assigned as 2-(Diphenylmethylene-amino)-3-acetoxy-but-2-enonitrile **(61)**, (92 mg, 306 μmol, 32 %); ν_{max} (neat, cm^{-1}) 2252 and 2219 (C=N), 1771 (C=O), δ_H (68.8

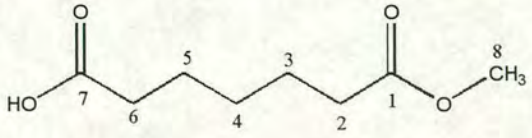
MHz, $CDCl_3$) 1.90 (1H, br.s,), 2.13 (1.5H, s), 2.18 (1.5H, s) and 2.23 (2H,s), 7.3-7.6 (10H, m, Ar-H), m/z (EI) : 305 (MH^+), 245 ($M-CH_3CO_2^+$), 219 ($M-C_4H_6O_2^+$).

Ethyl 7-[N-methoxy-N-methylamido]-7-oxo-heptanoate (62) [207]



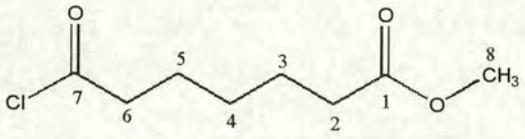
A solution of **60** (1.65 g, 7.99 mmol) in dry chloroform (10 cm^3) was added to a slurry of N,O-dimethyl hydroxylamine hydrochloride (0.790 g, 8.10 mmol) in dry chloroform (90 cm^3) stirring at room temperature under an atmosphere of nitrogen. After being cooled to ice/water temperature, neat pyridine (0.13 g, 1.65 mmol) was carefully added to the stirring reaction mixture over 10 mins. After the reaction had been left stirring for 30 mins at 0°C, it was allowed to warm to room temperature, where it was stirred for 12 hours, after a $CaCl_2$ drying tube had been fitted. The reaction mixture was then partitioned between ether (30 cm^3), and a mixture of saturated brine (30 cm^3) and water (50 cm^3) in a separatng funnel. After the layers were separated, the organic phase was washed with brine (50 cm^3) and dried ($MgSO_4$), before the solvent was removed by rotary evaporation. The resultant pale yellow oil was purified by silica gel chromatography to give **62** as a clear, colourless oil (1.64 g, 7.12 mmol, 89 %); δ_H (200 MHz, $CDCl_3$) 1.24 (3H, t, J 7.2, 9-CH_3), 1.33-1.44 (2H, m, 4-CH_2), 1.60-1.73 (4H, m, 3- and 5-CH_2), 2.30 (2H, t, J 7.2, 2-CH_2), 2.43 (2H, t, J 7.1, 6-CH_2), 3.19 (3H, s, 10-CH_3), 3.68 (3H, s, 11-CH_3), 4.12 (2H, q, J 7.2, 8-CH_2), δ_C (62.9 MHz, $CDCl_3$) 14.0 (C-9), 24.0, 24.5 and 28.6 (C-3, -4 and -5), 31.4 (C-6), 32.0 (C-10), 34.0 (C-2), 60.0 (C-8), 61.0 (C-11), 173.5 (C-1), 174.2 (C-7). Found: M^+, 231.14706 (EI-MS), $C_{11}H_{21}NO_4$ requires 231.1470583.

Methyl hydrogen pimelate (63) [208]



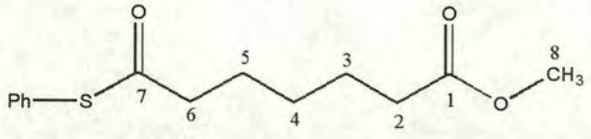
Freshly distilled thionyl chloride (22.28 g, 190 mmol) was added dropwise, with stirring to a flask containing pimelic acid **6** (10g, 62.4 mmol), which was subsequently heated to 50°C and stirred for one hour. The volatile components were removed by evaporation *in vacuo*, then the oily residue was added to a dry flask containing DCM (20 cm^3). Methanol (1.60 g, 50 mmol) was carefully added over 15 minutes (with ice/water cooling). A $CaCl_2$ drying tube was fitted, and the reaction was stirred for 18 hours at room temperature. After careful quenching by the addition of aq. $NaHCO_3$ (20 cm^3, 2% w/v), DCM (20 cm^3) was added and the aqueous and organic phases were separated. The organic extract was washed with aq. $NaHCO_3$ (20 cm^3, 2% w/v), then brine (10 cm^3) before being dried over $MgSO_4$. Evaporation of the solvent yielded a pale yellow oil, which was purified by distillation under vacuum to give **63** as a clear, colourless oil (13.55 g, 77.9 mmol, 41%); b.p. 95 °C / 0.5 mm Hg (lit. [208] b.p. 123 °C / 0.3 mm Hg), δ_H (200 MHz, $CDCl_3$) 1.34-1.46 (2H, m, 4-CH_2), 1.61-1.73 (4H, m, 3- and 5-CH_2), 2.29 (2H, t, J 7.1, 2-CH_2), 2.33 (2H, t, J 7.2, 6-CH_2) 3.64 (3H, s, 8-CH_3), δ_C (62.9 MHz, $CDCl_3$) 24.4, 24.7 and 28.6 (C-3, -4 and -5), 34.0 (C-2 and -6), 51.7 (C-8), 174.3 (C-1), 179.9 (C-7).

Methyl pimeloyl chloride (64) [209]



Prepared as described for **60**, from **63** (1.50 g, 8.62 mmol) and oxalyl chloride (2.10 g, 17.2 mmol) to give **64** as a clear, colourless oil in quantitative yield; b.p. 85 °C / 1 mmHg (lit. [209] b.p. 76-78 °C / 0.3 mmHg), δ_H (200 MHz, $CDCl_3$) 1.31-1.46 (2H, m, 4-CH_2), 1.55-1.79 (4H, m, 3- and 5-CH_2), 2.31 (2H, t, J 7.5, 2-CH_2), 2.88 (2H, t, J 7.3, 6-CH_2), 3.65 (3H, s, 8-CH_3).

Methyl-6-(phenylthiocarbonyl)-hexanoate (65)



Method 1

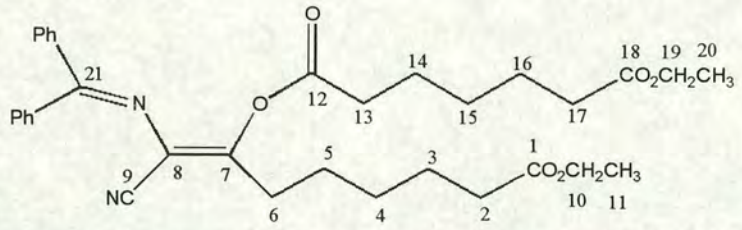
Prepared analogously to **32**, from methyl ester **63** (5.00 g, 28.7 mmol), and thiophenol (4.82 g, 43.1 mmol) to give **65** as a clear colourless oil (5.57 g, 21.0 mmol, 73 %), after purification by silica-gel chromatography (hexane / ether).

Method 2

Freshly prepared diazomethane (25 mmol in 40 cm^3 of ether) was added over 15 minutes to a rapidly stirring solution of thioester **32** (1.05 g, 4.17 mmol) in ethanol (45 cm^3) at 0 °C. The reaction was stirred for 30 minutes at 0°C, then for 18 hours at ambient temperature. Evaporation of the solvent *in vacuo* gave a quantitative yield of **65** as a clear, colourless oil.

δ_H (200 MHz, $CDCl_3$) 1.32-1.47 (2H, m, 4-CH_2), 1.56-1.80 (4H, m, 3- and 5-CH_2), 3.32 (2H, t, J 7.3, 2-CH_2), 2.66 (2H, t, J 7.4, 6-CH_2), 3.67 (3H, s, 8-CH_3), 7.40 (5H, s, Ar-H), δ_C (200 MHz, $CDCl_3$) 23.1, 23.7 and 27.0 (C-3, -4 and -5) 32.4 (C-2), 42.0 (C-6), 50.1 (C-8), 126.5, 127.8, 128.0 and 133.2 (6 x Ar-C), 172.7 (C-1), 196.0 (C-7) Found: M^+, 266.09770 (EI-MS), $C_{14}H_{18}O_3S$ requires 266.09767.

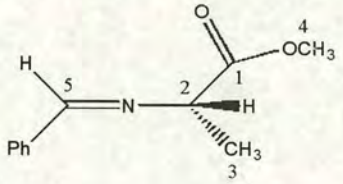
7-[O-1'-oxo-6'-(ethoxycarbonyl)]-8-(benzhydrylidene-amino)-8-cyano-oct-7-enoic acid ethyl ester (66)



A solution of **58** (0.395 g, 1.79 mmol) in THF (3 cm^3) was added dropwise to a solution of LDA [2.05 mmol; prepared from diisopropylamine (0.29 cm^3, 2.05 mmol) and n-butyllithium (1.40 cm^3, 1.57 M in hexane)] in THF (4 cm^3) at -78°C under argon. The deep-red solution was stirred for 1 hour then **60** (0.920 g, 4.78 mmol) in THF (2 cm^3) was added over 15 minutes. The reaction was stirred for 2 hours at -78°C, then allowed to warm to ambient temperature over 2-3 hours, where it was stirred for a further 18 hours under argon. It was then quenched by the addition of a saturated ammonium chloride solution (10 cm^3), and after 5 mins. was partitioned between water (10 cm^3) and ether (20 cm^3). After separation, the aqueous phase was

extracted with ether (10 cm^3), and the combined ethereal layers were washed with water (20 cm^3) and brine (20 cm^3) before being dried ($MgSO_4$). Evaporation of the solvent gave an orange oil which was purified by silica-gel chromatography to yield **66** as a pale yellow oil (0.250 g, 447 μmol, 25 %); ν_{max} (neat, cm^{-1}) 2192 (CN), 1764 (C=O), 1732 (OC=O), 1658 (C=N and C=C), δ_H (250 MHz, $CDCl_3$) 1.25 (3H, t, J 7.2, 11-CH_3), 1.26 (3H, t, J 7.2, 20-CH_3), 1.30-1.45 (4H, m, 4- and 15-CH_2), 1.50-1.75 (8H, m, 3-, 5-, 14- and 16-CH_2), 2.28 (4H, t, J 7.2, 2- and 17-CH_2), 2.48 (2H, t, J 7.5, 13-CH_2), 2.80 (2H, t, J 7.2, 6-CH_2), 4.12 (2H, q, J 7.2, 10-CH_2), 4.12 (2H, q, J 7.2, 19-CH_2), 7.25-7.75 (10H, m, Ar-H), δ_C (62.9 MHz, $CDCl_3$) 14.4 (C-11 and 20), 24.4, 24.7, 24.8, 25.9, 28.6, 28.8, 30.1, 34.0, 34.2, 34.3 and 34.3 (C-2, -3, -4, -5, -6, -13, -14, -15, -16 and -17), 60.4 (C-10 and -19), 112.0 (C-8), 115.2 (C-9), 128.4, 128.7, 128.7, 129.4 and 129.7 (8 x *o,m*-C), 130.5 and 131.6 (2 x C-*p*), 135.6 and 139.0 (2 x C-*ipso*), 161.7 (C-21), 170.7 (C-7), 171.0 (C-12), 173.7 (C-1 and -18), m/z 561 (MH^+), 391 [$(M-C_9H_{14}O_3)^+$]. Found: MH^+, 561.29723 (CI-MS), $C_{33}H_{41}N_2O_6$ requires 561.29646.

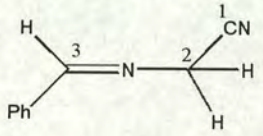
2-(S)-(Benzylidene-amino)-propanoic acid methyl ester (67) [210]



A mixture of L-alanine-methyl ester (HCl salt) (5.00 g, 35.8 mmol), benzaldehyde (3.80 g, 35.8 mmol), triethylamine (7.25 g, 71.6 mmol) and magnesium sulfate (4.50 g, anhydrous, oven dried for 24 hours) were stirred at ambient temperature for 18 hours with the exclusion of moisture ($CaCl_2$ drying tube). After filtration, the solution was partitioned between ether (100 cm^3) and water (150 cm^3). The organic layer was separated, then washed with water (100 cm^3) and brine (100 cm^3) before being dried ($MgSO_4$). Evaporation of solvent *in vacuo* gave a yellow oil which was purified by silica-gel chromatography (hexane / ethyl acetate) to yield **67** as a pale yellow oil (5.65 g, 29.6 mmol, 83 %); δ_H (200 MHz, $CDCl_3$) 1.53 (3H, d, J 6.9, 3-CH_3), 3.75 (3H, s, 4-CH_3), 4.16 (1H, q, J 6.9, 2-CH), 7.41-7.80 (5H, m, Ar-H), 8.32 (1H, s, 5-

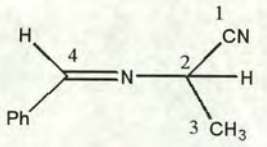
CH), δ_C (50.28 MHz, $CDCl_3$) 18.1 (C-3), 50.8 (C-2), 66.6 (C-4), 127.2 127.3 (4 x *o,m*-C), 129.8 (*p*-C), 134.4 (*ipso*-C), 161.7 (C-5), 171.7 (C-1), m/z (CI) 192 (MH^+), 132 [$(M\text{-}HCO_2CH_3)^+$], 77 (C_6H_5).

(Benzylidene-amino)-acetonitrile (68) [211]



Prepared analogously to **67** from aminoacetonitrile (hydrochloride salt) (2.62 g, 28.0 mmol), benzaldehyde (3.29 g, 28.0 mmol), triethylamine (5.74 g, 56.6 mmol), magnesium sulfate (3.5 g), DCM (50 cm^3) to give **68** as a colourless solid (3.61 g, 25.1 mmol, 89 %); m.p. (22-24 °C), δ_H (200 MHz, $CDCl_3$) (mixture of rotamers) 4.65 (1H 2-CH_2), 4.65 (1H, 2-CH_2), 7.41-7.80 (5H, m, Ar-H), 8.51 (0.5H, 4-CH), 8.51 (0.5H, 4-CH), δ_C (50.28 MHz, $CDCl_3$) 44.2 (C-2), 114.2 (C-1), 127.3 and 127.5 (4 x *o,m*-C), 130.5 (*p*-C), 133.5 (*ipso*-C), 163.4 (C-3). Found: M^+, 144.06878 (EI-MS), $C_9H_8N_2$ requires 144.06875.

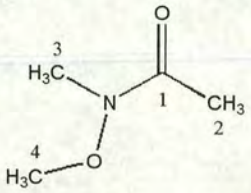
2-(R,S)-(Benzylidene-amino)-propionitrile (69) [168]



A solution of **68** (2.40 g, 16.8 mmol) in THF (2.5 cm^3) was added dropwise to a stirred solution of LDA [17.9 mmol; prepared from diisopropylamine (2.50 cm^3, 17.9 mmol) and n-butyllithium (12.8 cm^3, 1.46 M in hexane)] in THF (17 cm^3) at -78°C under argon. The deep-red solution was stirred for 1 hour then methyl iodide (2.38 g, 16.8 mmol) was added dropwise over 10 minutes. The reaction was stirred for 1 hour at -78°C, then for 18 hours at room temperature before being quenched by the addition of water (25 cm^3). The mixture was extracted with ether (2 x 40 cm^3), then the combined ethereal layers washed with water (40 cm^3) and brine (40 cm^3), before being dried ($MgSO_4$). Evaporation of the solvent *in vacuo* gave an orange oil (2.5 g), which was purified by silica-gel chromatography (ethyl acetate / hexane) to yield **69** as

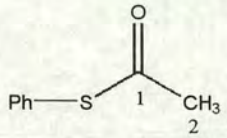
a pale yellow oil (1.46 g, 9.24 mmol, 55 %); δ_H (200 MHz, $CDCl_3$) 1.67 (3H, d, J 7.0, 3-CH_3), 4.69 (1H, dq, J 7.0 and 1.6, 2-CH), 7.41-7.91 (5H, m, Ar-H), 8.50 (1H, d, J 1.6, 4-CH), δ_C (50.28 MHz, $CDCl_3$) 19.7 (C-3), 51.8 (C-2), 117.4 (C-1), 127.4 and 127.7 (4 x *o,m*-C), 130.4 (*p*-C), 133.6 (*ipso*-C), 161.2 (C-4), Found: M^+, 158.08398 (EI-MS), $C_{10}H_{10}N_2$ requires 158.08440.

N-methoxy-N-methyl acetamide (70) [177]



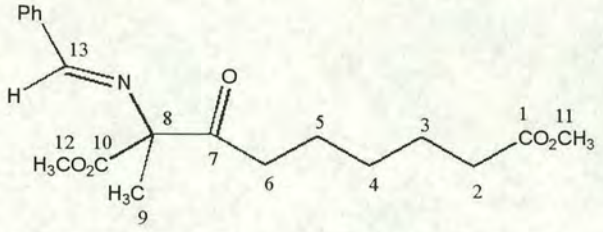
Prepared analogously to **62** from acetyl chloride (3.29 g, 41.9 mmol), N,O-dimethyl-hydroxylamine hydrochloride (4.50 g, 46.1 mmol) and pyridine (7.30 g, 92.3 mmol) in chloroform (50 cm^3), yielding a yellow oil which was purified by distillation *in vacuo* to give **70** as a clear, colourless oil (3.05 g, 29.6 mmol, 71 %); b.p.35-40 °C / 9 mm Hg (lit. [177] b.p. 40-44 °C / 20 mm Hg), ν_{max} (neat, cm^{-1}) 1664 (CON), δ_H (200 MHz, $CDCl_3$) 2.11 (3H, s, 2-CH_3), 3.16 (3H, s, 3-CH_3), 3.66 (3H, s, 4-CH_3), δ_C (62.9 MHz, $CDCl_3$) 18.4 (C-2), 30.6 (C-3), 59.7 (C-4), 171.0 (C-1).

Acetic acid thiophenol ester (71) [212]



Prepared analogously to **64** from thiophenol (1.07 g, 9.74 mmol), acetyl chloride (0.76 g, 9.7 mmol) and triethylamine (1.18 g11.7 mmol) in DCM (30 cm^3), yielding a yellow oil which was purified by distillation *in vacuo* to give **71** as a clear, colourless oil (1.08 g, 7.11 mmol, 73 %); b.p.85 °C / 5 mm Hg (lit. [212] b.p. 70 °C / 0.9 mm Hg), δ_H (200 MHz, $CDCl_3$) 2.43 (3H, s, 2-CH_3), 7.42 (5H, s, Ar-H), δ_C (62.9 MHz, $CDCl_3$) 29.9 (C-2), 127.7, 128.9, 129.2 and 134.2 (6 x Ar-C), 193.7 (C-1). Found: M^+, 152.02985 (EI-MS), C_8H_8SO requires 152.02959.

Ethyl 8-(R,S)-(benzylidene-amino)-8-(methoxycarbonyl)-7-oxononanoate (72)



From the acid chloride

A solution of the lithium-anion of **67** (prepared by the addition of **67** (0.2.40 g, 1.25 mmol) to LDA (1.35 mmol) at -78 °C, and stirring for one hour) in THF (10 cm^3) at *ca.* -50 to -70 °C, was added dropwise to a solution of acid chloride **64** (0.600 g, 3.12 mmol) in THF (20 cm^3) at -78 °C, under an inert atmosphere. After 4 hours of stirring at -78 °C, the reaction was allowed to slowly warm up to ambient temperature over 3 hours, and was stirred for a further 18 hours. Water (30 cm^3) was added to quench the reaction, and after 5 minutes the mixture was extracted with ether (2 x 30 cm^3). The combined ethereal layers were washed with sodium carbonate solution (50 cm^3, 5 % w/v), water (50 cm^3) and brine (30 cm^3) before being dried ($MgSO_4$). Evaporation of the solvent *in vacuo* gave an orange oil, which was purified by silica-gel chromatography (hexane / ether) to yield **72** as a pale yellow oil (0.182g, 525 μmol, 42 %).

From the thiophenol ester

Prepared analogously to above from **67** (0.660 g, 2.48 mmol), **65** (200 mg, 1.04 mmol) to give **72** as a pale yellow oil (62 mg, 177 μmol, 17 %).

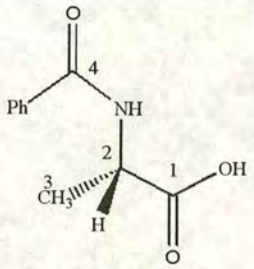
δ_H (200 MHz; $CDCl_3$) 1.24-1.41 (2H, m, 4-CH_2) 1.56 (4H, m, 3- and 5-CH_2), 1.60 (3H, s, 9-CH_3), 2.30 (2H, t, J 7.6, 2-CH_2), 2.74-2.84 (2H, m, 6-CH_2), 3.65 (3H, s, 11-CH_3), 3.76 (3H, s, 12-CH_3), 7.28-7.87 (5H, m, Ar-H), 8.23 (1H, s, 13-CH). Found: M^+, 347.17344 (EI-MS), $C_{19}H_{25}O_5N$ requires 347.17327.

8-(R,S)-Amino-7-oxononanoic acid (Hydrochloride salt) (8)

A mixture of **72** (50 mg, 144 μmol), acetic acid (1 cm^3) and 4M HCl (5 cm^3) was stirred at 80 °C for 24 hours. After allowing to cool, water (5 cm^3) was added, and the reaction mixture was extracted with chloroform (3 x 10 cm^3). Evaporation of the

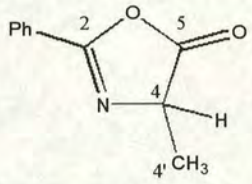
water *in vacuo* yielded a crystalline material which was purified by ion-exchange chromatography (Dowex 50-X (H^+-form), 0-1.5 M HCl linear gradient) to afford **34** as a colourless solid (18 mg, 80 μmol, 55 %), which showed identical chromatographic properties on tlc (R_f = 0.45 (butanol, acetic acid, water, 3:1:1, visualisation by ninhydrin), and had identical ^1H NMR characteristics as a sample previously synthesised via an alternative route.

N-Benzoyl-L-alanine (78) [192]



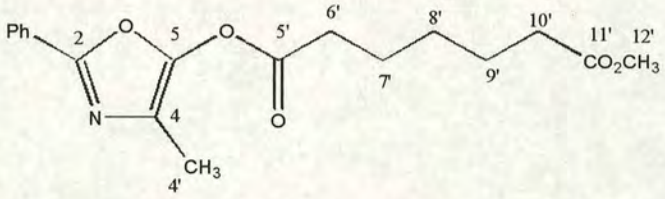
Alanine (6.00 g, 67.3 mmol) was dissolved in a solution of 0.6 M NaOH (20 cm^3) and stirred with ice / water cooling. Benzoyl chloride was added until the pH was *ca.* 8, then 2M NaOH and the remainder of the benzoyl chloride (10.22 g, 72.7 mmol, in total) were added in tandem over 1.5 hours, maintaining the pH between 7.8 and 8.5, and the temperature between 0 and 5 °C. The reaction mixture was then left for 18 hours, without stirring at 4 °C for the reaction to proceed to completion. The pH was then raised to *ca.* 10, and the solution was extracted with ethyl acetate (3 x 50 cm^3). After lowering the pH to *ca.* 2, the cloudy aqueous layer was extracted with ethyl acetate (4 x 50 cm^3), and the organic extracts were combined, washed with brine (1 x 100 cm^3) and dried ($MgSO_4$) before the solvent was removed *in vacuo*. The solid product was recrystallised (ethanol / water) to give **78** as a colourless solid (10.65 g, 55.2 mmol, 82 %) ; m.p. 149-151 °C (lit. [192] 150-151 °C), δ_H (200 MHz, $CDCl_3$) 1.60 (3H, d, J 7.2, 3-CH_3), 4.85 (1H, dq, J 7.2 and 5.8, 2-CH), 6.81 (1H, br.d, J 5.8, NH), 7.4-8.1 (5H, m, Ar-H), δ_C (50.28 MHz, $CDCl_3$) 17.0 (C-3), 51.1 (C-2), 126.7, 128.3, 131.7 and 132.9 (6 x Ar-C), 169.5 (C-4), 179.9 (C-1). Found : M^+, 193.07425 (EI-MS), $C_{10}H_{11}NO_3$ requires 193.07389.

2-Phenyl-4-(R,S)-methyl-oxazolin-5-one (79) [193,194]



78 (2.55 g, 13.2 mmol) was added to a stirred mixture of dioxane (30 cm^3) and acetic anhydride (30 cm^3) at 50 °C with the exclusion of water ($CaCl_2$ drying tube). The solution was stirred for one hour at 50-60 °C, removed from the heat, then stirred for a further 2 hours. Evaporation *in vacuo* (4 hours) yielded a pale yellow oil which was filtered through a plug of silica to give a colourless oil which solidified upon standing, yielding **79** as colourless solid (2.19 g, 12.5 mmol, 95%) ; m.p.35-37 °C (lit. [193] 37-39 °C), ν_{max} (neat, cm^{-1}) 1823 (CO_2), 1651 (C=N), δ_H (200 MHz, $CDCl_3$) 1.58 (3H, d, J 7.6, 4'-CH_3), 4.45 (1H, q, J 7.6, 4-CH), 7.4-7.8 (5H, m, Ar-H), δ_C (62.9 MHz, $CDCl_3$) 16.7 (C-4'), 60.8 (C-4), 125.6 (C-*ipso*), 127.6 and 128.6 (4 x *o,m*-C), 132.6 (C-*p*), 161.3 (C-2), 178.8 (C-5).

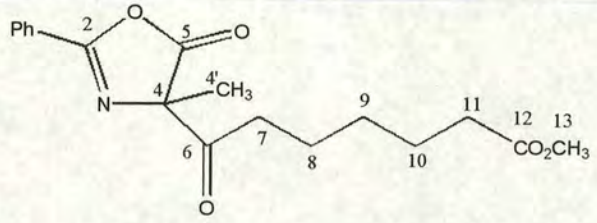
2-phenyl-4-methyl-5-[6-(methoxycarbonyl)-hexyloxy]-oxazole (80)



Acid chloride **64** (0.568 g, 2.95 mmol) was added dropwise, over 5 minutes, to a solution of oxazolone **79** (0.490 g, 2.80 mmol) and triethylamine (0.283 g, 2.80 mmol) in THF (7 cm^3) whilst stirring at 0-5°C under an inert atmosphere. The reaction temperature was maintained at ice / water temperature for 3 hours, then was allowed to rise to room temperature overnight. After removal of the THF by rotary evaporation, the crude product was taken up in ether (20 cm^3), and extracted with 0.3M HCl (20 cm^3) and water (20 cm^3), before being dried ($MgSO_4$). Evaporation of the solvent *in vacuo* yielded a yellow oil, which was purified by silica gel chromatography (ether / hexane) to give **80** as a clear, colourless oil (0.720 g, 2.17 mmol, 77 %); δ_H (200 MHz, $CDCl_3$) 1.41-1.51 (2H, m, 8'-CH_2), 1.60-1.83 (4H, m,

7'- and 9'-CH_2), 2.10 (3H, s, 4'-CH_3), 2.36 (2H, t, J 7.3, 10'-CH_2), 2.64 (2H, t, J 7.3, 6'-CH_2), 3.68 (3H, s, 12'-CH_3), 7.4-7.9 (5H, m, Ar-H), δ_C (62.9 MHz, $CDCl_3$) 10.2 (C-4'), 24.0, 24.2 and 28.2 (C-7', -8' and -9'), 33.0 and 33.5 (C-6'and 10'), 51.3 (C-12'), 120.3 (C-*ipso*), 125.6 and 128.5 (4 x C-*o,m*) 127.0 (C-4), 129.9 (C-*p*), 145.5 (C-5), 154.7 (C-2), 169.7 (C-5'), 173.7 (C-11'), Found : M^+, 331.14189 (EI-MS), $C_{18}H_{21}NO_5$ requires 331.14197.

2-phenyl-4-(R,S)-methyl-4[-6-(methoxycarbonyl)-hexyloxy]-oxazol-5-one (81)



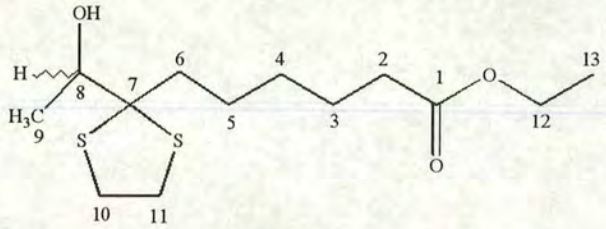
A solution of oxazole **80** (0.194 g, 586 μmol) and N,N-dimethyl-4-pyridinamine (DMAP) (9 mg, 73 μmol) in THF (3 cm^3) was stirred at room temperature under an inert atmosphere for 10 hours. After removal of the THF *in vacuo* the crude product was taken up in a mixture of ether (10 cm^3) and 0.1M HCl (10 cm^3). The ethereal phase was separated and washed with water (10 cm^3) and brine (10 cm^3), then dried ($MgSO_4$). Evaporation of the solvent *in vacuo* yielded a yellow oil which was purified by silica gel chromatography (hexane / ether) to give **81** as a pale yellow oil (89 mg, 270 μmol, 46 %); δ_H (200 MHz, $CDCl_3$) 1.20-1.36 (2H, m, 9-CH_2), 1.52-1.67 (4H, m, 8- and 10-CH_2), 1.71 (3H, s, 4'-CH_3), 2.27 (2H, t, J 7.5, 11-CH_2), 2.39-2.77 (2H, m, 7-CH_2), 3.64 (3H, s, 13-CH_3), 7.44-8.05 (5H, m, Ar-H), Found : M^+, 331.14177 (EI-MS), $C_{18}H_{21}NO_5$ requires 331.14197

8-Amino-7-oxononanoic acid (Hydrochloride salt) (8)

A mixture of oxazolone **81** (20 mg, 60 μmol) and 4M HCl (3 cm^3) was stirred at 80 °C for 24 hours. After allowing to cool, water (3 cm^3) was added, and the reaction mixture was extracted with chloroform (5 x 6 cm^3). Evaporation of the water *in vacuo* yielded a crystalline material which was purified by ion-exchange chromatography (Dowex 50-X (H^+-form), 0-1.5 M HCl linear gradient) to afford **34**

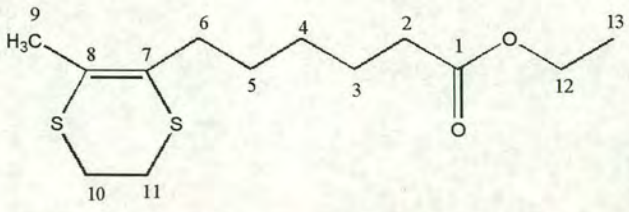
as a colourless solid (5 mg, 22 μmol, 36 %), which showed identical chromatographic properties on tlc (R_f = 0.45 (butanol, acetic acid, water, 3:1:1, visualisation by ninhydrin), and had identical ^1H NMR characteristics as a sample previously synthesised via an alternative route.

Ethyl 8-(R,S)-hydroxy-7,7-(ethylenedithio)-nonanoate (41)



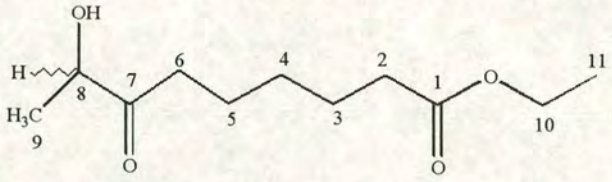
Sodium borohydride (24 mg, 634 μmol, freshly purified) was added to a rapidly stirring solution of ketone **39** (156 mg, 538 μmol) in DCM (4 cm^3) and ethanol (4 cm^3) at 0 °C. After one hour the reaction was quenched by the addition of acetone (2 cm^3), then the reaction mixture was partitioned between water (25 cm^3) and ether (25 cm^3). The phases were separated and the aqueous layer was extracted with a portion of ether (25 cm^3). The combined ethereal extracts were washed with brine (20 cm^3), and dried ($MgSO_4$) before being evaporated *in vacuo* to yield alcohol **41** as a clear colourless oil (155 mg, 529 μmol, 98 %); δ_H (200 MHz, $CDCl_3$) 1.24 (3H, t, J 7.2, 13-CH_3), 1.29 (3H, d, J 6.1, 9-CH_3), 1.20-1.42 (2H, m, 4-CH_2), 1.55-1.73 (4H, m, 3- and 5-CH_2), 1.70-1.90 (2H, m, 6-CH_2), 2.29 (2H, t, J 7.5, 2-CH_2), 3.26 (4H, s, 10- and 11-CH_2), 3.87 (1H, q, J 6.1, 8-CH), 4.11 (2H, q, J 7.2, 12-CH_2), δ_C (50.28 MHz, $CDCl_3$) 12.8 (C-13), 17.1 (C-9), 23.4, 24.5 and 27.9 (C-3, -4 and -5), 32.9 (C-2), 36.5 (C-6), 38.4 and 38.8 (C-10 and -11), 58.8 (C-12), 72.3 (C-8), 77.3 (C-7), 173.2 (C-1). Found : MH^+, 293.12492 (FAB-MS), $C_{13}H_{25}O_3S_2$ requires 293.12451.

Attempted Bromination of 41



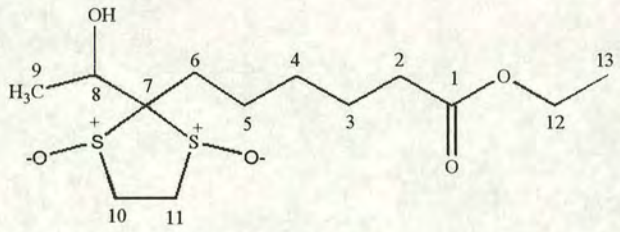
Bromine (82 mg, 513 μmol) was added over 15 mins to a solution of triphenyl phosphine (135 mg, 513 μmol) in DCM (2 cm^3) stirring at -5 °C under nitrogen. After allowing to react for 10 mins., a solution of **41** (150 mg, 513 μmol) and triethylamine (52 mg, 513 μmol) in DCM (3 cm^3) was added over 30 mins., maintaining the temperature between -5 and 0 °C. The reaction was stirred for 1.5 hours at 0 °C, then for a further 18 hours at room temperature. Ether (4 cm^3) was added, and the resultant precipitate was removed *via* filtration, with the solid being washed with a further portion of ether (4 cm^3). The combined ethereal filtrates were evaporated *in vacuo* to give a brown/green oil which was purified using silica gel chromatography to yield a pale yellow oil assigned 7,8-(ethylenedithio)-non-7-enoic acid ethyl ester **(85)** (106 mg, 387 μmol, 75 %); ν_{max} (neat) 1731 (C=O), 1604 (C=C), δ_H ($CDCl_3$, 270MHz) 1.25 (3H, t, J 7.2,13-CH_3), 1.30-1.42 (2H, m, 4-CH_2), 1.45-1.55 (2H, m, 5-CH_2), 1.55-1.70 (2H, m, 3-CH_2), 1.91 (3H, s, 9-CH_3), 2.21 (2H, t, J 7.0, 6-CH_2), 2.30 (2H, t, J 7.4, 2-CH_2), 3.13 (4H, s, 10 and 11-CH_2), 4.12 (2H, q, J 7.2, 12-CH_2), δ_C ($CDCl_3$, 68.8 MHz) 14.4 (C-12), 21.0 (C-9), 25.0, 28.6, 28.7, 29.0 and 29.7 (C-2, -3, -4, -5 and -6), 34.4 and 35.1 (C-10 and -11), 60.4 (C-12), 117.7 (C-8), 122.6 (C-7), 173.9 (C-1), m/z (EI) : 274 (M^+), 229 [$(M-C_2H_5O)^+$], 145 [$(M-C_7H_{13}O_2)^+$].

Ethyl 8-(R,S)-hydroxy-7-oxononanoate (86)



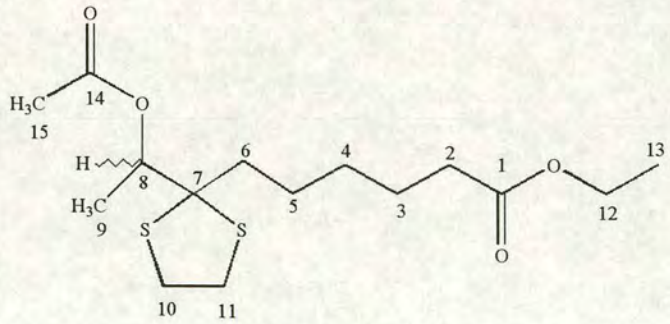
Prepared analogously to **40** from **41** (0.135g, 462 μmol) and NBS (0.410 g, 2.31 mmoles), to give **86** (the minor product) as a clear, colourless oil (27 mg, 125 μmol, 27 %); δ_H (270 MHz, $CDCl_3$) 1.24 (3H, t, J 7.2, 11-CH_3),1.25-1.4 (2H, m, 4-CH_2), 1.38 (3H, d, J 7.1, 9-CH_3), 1.60-1.72 (4H, m, 3- and 5-CH_2), 2.30 (2H, t, J 7.4, 2-CH_2), 2.36-2.60 (2H, m, 6-CH_2), 4.12 (2H, q, J 7.2, 10-CH_2), 4.24 (1H, J 7.1, 8-CH), δ_C (62.9 MHz, $CDCl_3$) 14.4 (C-11), 20.0 (C-9), 23.3, 24.8 and 28.8 (C-3, -4 and -5),

34.2 (C-2), 37.4 (C-6), 60.5 (C-10), 72.8 (C-8), 173.8 (C-1), 212.2 (C-7). Found : M^+, 216.13611 (EI-MS), $C_{11}H_{20}O_4$ requires M, 216.1361593.



The major product was a clear colourless oil assigned as 8-hydroxy-7-(1,3-dithiolan-1,2-dioxide)-nonanoic acid ethyl ester (mixture of *cis* and *trans* diastereomers) **(87)** (101 mg, 314 μmol, 68 %); ν_{max} (neat) 3312 (br., OH), 1726 (C=O), 1036 (S-O), δ_H ($CDCl_3$, 270MHz) 1.26 (3H, t, J 7.2,13-CH_2), 1.35-1.55 (2H, m, 4-CH_2), 1.49 (3H, d, J 6.2, 9-CH_3), 1.55-1.75 (5H, m, 3-, 5- and 6-CH_2), 2.00-2.10 (1H, m, 6-CH_2), 2.30 (2H, t, J 7.3, 2-CH_2), 3.05 (1H, br.s, 8-OH), 3.35-3.45 (2H, m, 11-CH_2), 3.70-3.80 (1H, m, 10-CH_2), 3.95-4.02 (1H, m, 10-CH_2), 4.12 (2H, q, 12-CH_2), 4.50-4.60 (1H, m), δ_C ($CDCl_3$, 68.8 MHz) 14.4 (C-13), 21.0 (C-9), 24.6, 24.7, 24.9, 29.8 and 34.3 (C-2, -3, -4, -5 and -6), 48.4 (C-11), 51.7 (C-10), 60.5 (C-12), 68.0 (C-8), 90.8 (C-7), 173.9 (C-1). Found MH^+, 325.11488 (FAB-MS), $C_{13}H_{25}O_5S_2$ requires 325.11434.

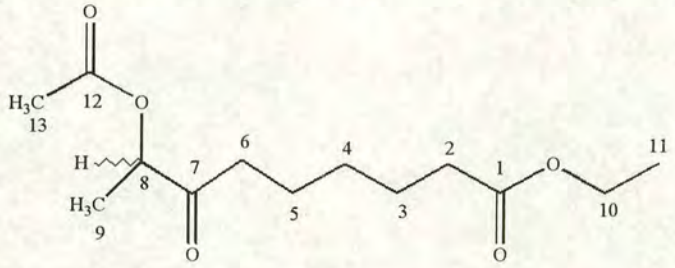
Ethyl 8-(R,S)-(acetoxy)-7,7-(ethylenedithio)-nonanoate (88)



Alcohol **41** (500 mg, 1.71 mmol), pyridine (3 cm^3) and acetic anhydride (3 cm^3) in DCM (10 cm^3) were stirred for 18 hours with the exclusion of moisture ($CaCl_2$ drying tube). Evaporation of the volatile components *in vacuo* (3 hours) yielded fairly pure acetylated product **88**, which was further purified by silica gel chromatography (hexane / ether) to give **88** as a clear colourless oil (535 mg,1.60 mmol, 94 %); δ_H

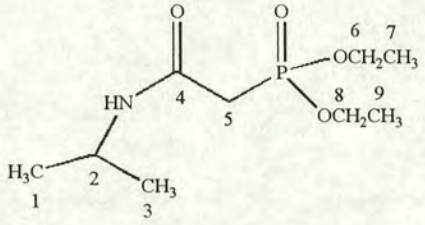
(200 MHz, $CDCl_3$) 1.25 (3H, t, J 7.2, 13-CH_3), 1.29 (3H, d, J 6.3, 9-CH_3), 1.20-1.45 (2H, m, 4-CH_2), 1.55-1.73 (4H, m, 3- and 5-CH_2), 1.80-1.95 (2H, m, 6-CH_2), 2.06 (3H, s, 15-CH_3), 2.30 (2H, t, J 7.5, 2-CH_2), 3.22-3.26 (4H, m, 10- and 11-CH_2), 4.11 (2H, q, J 7.2, 12-CH_2), 5.13 (1H, q, J 6.3, 8-CH), δ_C (62.9 MHz, $CDCl_3$) 14.1 (C-13), 16.5 (C-9), 21.1 (C-15), 24.7, 25.6 and 29.2 (C-3, -4 and-5), 34.2 (C-2), 38.1 (C-6), 39.8 and 40.2 (C-10 and -11), 60.1 (C-12), 74.1 (C-8), 77.1 (C-7),170.1 (C-14), 173.6 (C-1). Found : M^+, 334.12632 (EI-MS), $C_{15}H_{26}O_4S_2$ requires 334.12725.

Ethyl 8-(R,S)-(acetoxy)-7-oxononanoate (89)



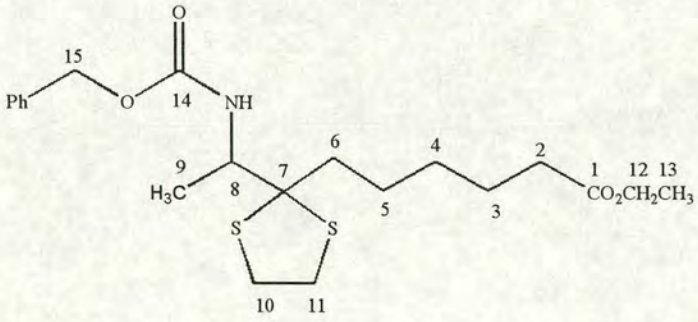
Acetylated compound **88** (140 mg, 419 μmol) in acetonitrile (1 cm^3) was added over 30 seconds, to a rapidly stirring solution of NBS (450 mg, 2.52 mmol) in 80 % aq. acetonitrile (3 cm^3) at *ca.* -2°C. The bright orange solution was stirred for 10 mins. at *ca.* -2 to 0 °C, then for a further 10 mins. at 0 °C to 10 °C, before being quenched by the addition of a saturated solution of sodium sulfite (5 cm^3), and partitioned between a mixture of ether (20 cm^3) and water (20 cm^3). After the separation of the phases, the aqueous layer was extracted with ether (20 cm^3), then the combined ethereal layers were washed with water (2 x 20 cm^3) and brine (20 cm^3) before being dried ($MgSO_4$). Evaporation of the solvent *in vacuo* gave an orange oil which was purified by silica-gel chromatography (ether / hexane) to yield **89** as a clear colourless oil (96 mg, 372 μmol, 89 %); δ_H (250 MHz, $CDCl_3$) 1.23 (3H, t, J 7.2, 11-CH_3), 1.36 (3H, d, J 7.1, 9-CH_3), 1.27-1.37 (2H, m, 4-CH_2), 1.53-1.67 (4H, m, 3- and 5-CH_2), 2.11 (3H, s, 13-CH_3), 2.27 (2H, t, J 7.5, 2-CH_2), 2.36-2.55 (2H, m, 6-CH_2), 4.11 (2H, q, J 7.2, 10-CH_2), 5.05 (1H, q, J 7.1, 8-CH), δ_C (62.9 MHz, $CDCl_3$) 14.1 (C-11), 16.0 (C-9), 20.6 (C-13), 22.6, 24.5 and 28.4 (C-3, -4 and -5), 34.0 (C-2), 37.8 (C-6), 60.1 (C-10), 74.5 (C-8), 170.3 (C-12), 173.5 (C-1), 207.5 (C-7). Found : M^+, 258.14609 (EI-MS), $C_{13}H_{22}O_5$ requires 258.14673

(Isopropylcarbamoyl-methyl)-phosphonic acid diethyl ester (106) [213]



Isopropylamine (0.100 g, 1.72 mmol) was added, dropwise over 2 mins., to a solution of freshly prepared diethyl phosphonoacetyl-chloride (0.178 g, 832 μmol) in dry DCM (2 cm^3), under nitrogen at room temperature. The reaction was stirred for 5 hours, then the volatile components were removed *in vacuo*, to yield a yellow oil. Purification using silica-gel chromatography (methanol / chloroform) yielded **106** as a clear, colourless oil (155 mg, 653 μmol, 78 %); δ_H (200 MHz , $CDCl_3$) 1.10 (6H, d, J 6.6, 1- and 3-CH_3), 1.28 (6H, t, J 7.1, 7- and 9-CH_3), 2.74 (2H, d, J 20.6, 5-CH_2), 4.03 (1H, heptet, J 6.6, 2-CH), 4.07 (4H, A of ABX, $^3J_{PH}$ 8.1 and $^3J_{HH}$ 7.1, 6- and 8-CH_2), 6.50 (1H, br.s, NH), δ_C (62.9 MHz, $CDCl_3$) 16.2 and 16.2 (C-7 and -9), 22.4 (C-1 and -3), 35.0 (C-5, d, $^1J_{PC}$ 130.0), 41.7 (C-2), 62.5 (C-6 and -8, d, $^2J_{PC}$ 6.5), 162.8 (C-4, d, $^2J_{PC}$ 3.2), Found: M^+, 237.11315 (EI-MS), $C_9H_{20}NO_4P$ requires 237.11300

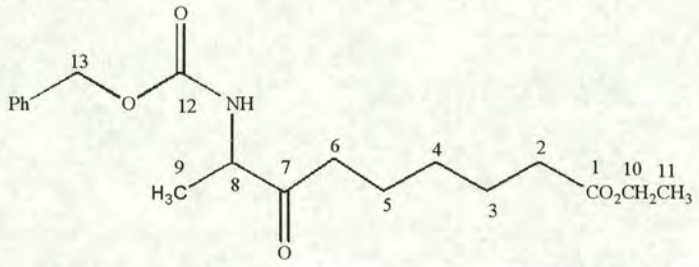
Ethyl [(7,7)-ethylenedithio]-8-(R,S)-[(N-benzyloxycarbonyl)-amino]-nonanoate (97)



Triethylamine (30 mg, 296 μmol) was added dropwise to a solution of **40** (46 mg, 158 μmol) and benzyl chloroformate (35 mg, 205 μmol) in DCM (2 cm^3) and was stirred for 12 hours at room temperature. Ether (10 cm^3) was added, then the mixture was separated from 1M HCl (10 cm^3), water (10 cm^3) and brine (10 cm^3), before being dried (Na_2SO_4). Evaporation of the solvent *in vacuo* left a pale yellow oil

which was purified by silica gel chromatography (hexane / ethyl acetate) to give **97** as a clear colourless oil (59 mg, 138 mmol, 88%); ν_{max} (neat,cm^{-1}) 3411 and 3344 (NH), 1735 (CO_2R), 1700 (CON), δ_H (200 MHz, $CDCl_3$) 1.19 (3H, t, J 7.2, 11-CH_3), 1.21 (3H, d, J 7.0, 9-CH_3), 1.32 (2H, m, 4-CH_2), 1.63 (4H, m, 5- and 3-CH_2), 1.92 (2H, t, J 8, 6-CH_2), 2.29 (2H, t, J 7.4, 2-CH_2), 3.31 (4H, s, 10- and 11-CH_2), 4.12 (1H, m, 8-CH_2), 4.12 (2H, q, J 7.2, 10-CH_2), 5.11 (2H, s, 15-CH_2), 5.13 (1H, m, NH), 7.35 (5H, s, Ar-H), δ_C (62.9 MHz, $CDCl_3$) 14.2 (C-13), 19.1 (C-9), 24.7, 25.8 and 29.1 (C-3, -4 and -5), 34.1 (C-2), 40.0 (C-10), 40.1 (C-11), 42.1 (C-6), 53.8 (C-8), 60.1 (C-12), 66.6 (C-15), 77.1 (C-7), 129.0 and 128.4 (5 x aromatic C), 136.5 (*ipso*-C), 155.7 (C-14), 173.6 (C-1), Found: M^+, 425.16903 (EI-MS), $C_{21}H_{31}NO_4S_2$ requires 425.16945.

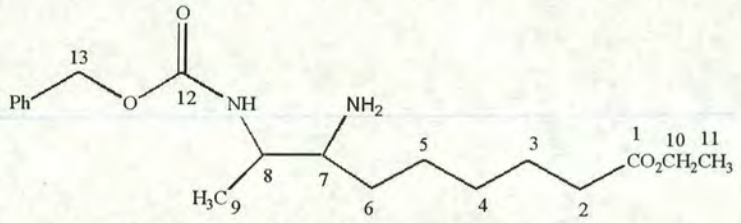
Ethyl 7-oxo-8-(R,S)-[(N-benzyloxycarbonyl)-amino]-nonanoate (98)



Dithiolane **97** (16 mg, 38 mmol) in acetonitrile (0.4 cm^3), was added dropwise, with stirring, to a solution of N-bromosuccinimide (36 mg, 202 mmol) in 80% aq. acetonitrile (0.7 cm^3) at -4°C. The red solution was stirred for 7 mins at -4°C, then allowed to warm to room temperature over 5 minutes, before being quenched by the addition of saturated sodium sulfite solution (1 cm^3). The mixture was extracted with ether (3 x 3 cm^3), and the combined organic extracts were washed with water (6 cm^3) and brine (6 cm^3) before being dried (Na_2SO_4). Evaporation of solvent *in vacuo* yielded a pale yellow oil (14 mg) which was purified by silica gel chromatography (hexane / ethyl acetate), to give **98** as a clear, colourless oil (12 mg, 35 mmol, 94%); δ_H (200 MHz, $CDCl_3$) 1.18 (3H, t, J 7.2, 11-CH_3), 1.15-1.30 (2H, m, 4-CH_2), 1.28 (3H, d, J 7.4, 9-CH_3), 1.45-1.65 (4H, m, 5- and 3-CH_2), 2.22 (2H, t, J 7.5, 2-CH_2), 2.43 (2H, m, 6-CH_2), 4.05 (2H, q, J 7.2, 10-CH_2), 4.30 (1H, m, 8-CH), 5.03 (2H, s, 13-CH_2), 5.48 (1H, br.s, NH), 7.28 (5H, s, Ar-H), δ_C (62.9 MHz, $CDCl_3$) 14.1 (C-

11), 17.8 (C-9), 23.0, 24.5 and 28.4 (C-3, -4 and -5), 33.9 (C-2), 38.7 (C-6), 55.3 (C-8), 60.1 (C-10), 66.7 (C-13), 127.9, 128.0 and 128.4 (5 x aromatic-C), 136.1 (*ipso*-C), 155.4 (C-12), 173.4 (C-1), 208.8 (C-7), Found : M^+, 349.18865 (EI-MS), $C_{19}H_{27}NO_5$ requires 349.18892.

Ethyl 7-amino-8-(R,S)-[(N-benzyloxycarbonyl)-amino]-nonanoate (99)



The title compound was prepared via the two methods used to synthesise the analogous amine **40** and obtained as a mixture of 2-diastereomers, each were separable by silica-gel chromatography (DCM / methanol, (a) eluting first) (Yield quoted is for total mass of both diastereomers, formed in a 1:1 ratio by both methods).

98 (0.171 g, 490 μmol), ammonium acetate (0.460 g, 6.0 mmol), $NaCNBH_3$ (37 mg, 0.6 mmol) in methanol (6 cm^3), stirred for 48 hours at room temperature, yielded (96 mg, 275 μmol, 56 %) of **99** as a clear, colourless oil.

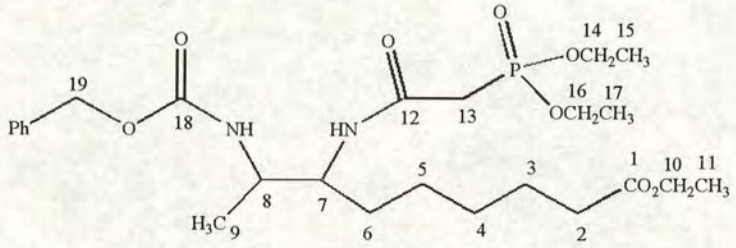
98 (0.157g, 450 μmol), $TiCl_4$ (239 μmol, 0.24 cm^3 of a 1M solution in DCM), hexamethyl disilazane (75 mg, 477 μmol) and triethylamine (144 mg, 1.43 mmol) in DCM (5 cm^3) were stirred for 18 hours, then quenched with a solution of $NaCNBH_3$ (1.43 mmol, 90 mg in 1.5 cm^3 of dry methanol). Upon workup as described previously, (79 mg, 227 μmol, 51%) of **99** was obtained as a clear, colourless oil.

Diastereomer (a) : δ_H ($CDCl_3$, 200 MHz) 1.14 (3H, d, J 6.6, 9-CH_3), 1.25 (3H, t, J 7.2, 11-CH_3), 1.20-1.50 (6H, m, 3-, 4- and 5-CH_2), 1.50-1.70 (2H, m, 6-CH_2), 2.27 (2H, t, J 7.5, 2-CH_2), 2.72 (1H, m, 7-CH_2), 3.66 (1H, m, 8-CH_2), 4.11 (2H, q, J 7.2, 10-CH_2), 5.09 (2H, s, 13-CH_2), 5.18 (1H, br.d, J 8.0, NH), 7.33-7.38 (5H, m, Ar-H), δ_C (62.9 MHz, $CDCl_3$) 14.1 (C-11), 18.7 (C-9), 24.7, 25.8 and 29.0 (C-3, -4 and -5), 33.9 (C-6), 34.1 (C-2), 50.0 (C-7), 54.7 (C-8), 60.1 (C-10), 66.4 (C-13), 127.9 and 128.4 (5 x Ar-C), 136.6 (C-*ipso*), 156.1 (C-12), 173.6 (C-1).

Diastereomer (b) : δ_H ($CDCl_3$, 200 MHz), 1.04 (3H, d, J 6.6, 9-CH_3), 1.25 (3H, t, J 7.2, 11-CH_3), 1.20-1.50 (4H, m, 3- and 4-CH_2), 1.50-1.70 (4H, m, 5-and 6-CH_2), 2.29 (2H, t, J 7.5, 2-CH_2), 2.75 (1H, m, 7-CH_2), 3.72 (1H, m, 8-CH_2), 4.12 (2H, q, J 7.2, 10-CH_2), 5.09 (2H, s, 13-CH_2), 5.27 (1H, br.d, J 8.4, NH), 7.33-7.38 (5H, m, Ar-H), δ_C (62.9 MHz, $CDCl_3$) 14.1 (C-11), 18.7 (C-9), 24.7, 26.0 and 29.6 (C-3, -4 and -5), 33.9 (C-6) 34.1 (C-2), 50.0 (C-7), 54.8 (C-8), 60.1 (C-10), 66.4 (C-13), 127.9 and 128.4 (5 x Ar-C), 136.6 (C-*ipso*), 155.8 (C-12), 173.6 (C-1).

Found : M^+, 350.22013 (EI-MS), $C_{19}H_{30}N_2O_4$ requires 350.22056.

Ethyl 7-[N-(diethylphosphonoacetyl)-amido]-8(R,S)-benzyloxycarbonylamino nonanoate (101)



Method 1

A solution of amine **99** (18 mg, 50 mmol) in dry DCM (1 cm^3), was added to a solution of DCC (12 mg, 59 mmol) and diethyl phosphonoacetate (11 mg, 55 mmol) in dry DCM (1 cm^3) at ambient temperature under an inert atmosphere. The reaction mixture was stirred for 12 hours and then filtered, with the solid washed with cold DCM (2 x 2 cm^3). The filtrate and combined washings were separated from aqueous NaOH (0.5 M, 2 cm^3), water (2 cm^3) and brine (2 cm^3) then dried (Na_2SO_4). Evaporation of the solvent *in vacuo* yielded a cloudy yellow oil which was purified by silica gel chromatography (DCM / methanol) to give **101** as a colourless oil (9 mg, 17 mmol, 34 %).

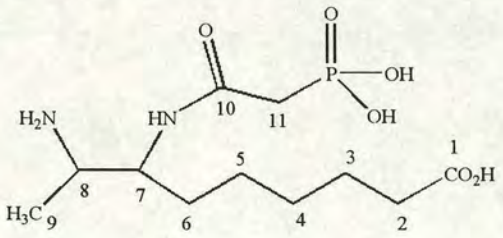
Method 2

A solution of diethyl phosphonoacetyl chloride **(100)** (32 mg, 150 μmol), [freshly prepared by the reaction of diethyl phophonoacetate (30 mg, 153 μmol) with oxalyl chloride (38 mg, 299 μmol)] in DCM (2 cm^3), was added to a flask containing a solution of amine **99** (20 mg, 57 μmol) in dry DCM (2 cm^3), whilst stirring under an

atmosphere of nitrogen at room temperature. Triethylamine (20 mg, 200 μmol) was added over 2 mins., then the reaction was stirred for 24 hours. DCM (5 cm^3) and NaOH (0.5 M, 7 cm^3) were added, and after separation of the phases, the organic extract was dried ($MgSO_4$). Evaporation of the solvent in vacuo yielded a pale yellow oil, which was purified using silica-gel chromatography (DCM / methanol) to give **101** (mixture of diastereomers) as a colourless oil (21 mg, 40 μmol, 70 %).

δ_H (200 MHz, $CDCl_3$) 1.09 (1.5H, d, J 6.9, 9-CH_3), 1.15 (1.5H, d, J 6.9, 9-CH_3), 1.21 (3H, t, J 7.2, 11-CH_3), 1.25 (3H, t, J 7.2, 15-CH_3), 1.26 (3H, t, J 7.2, 17-CH_3),1.10-1.40 (4H, m, 4- and 5-CH_2), 1.40-1.60 (4H, m, 6- and 3-CH_2), 2.20 (2H, t, J 7.5, 2-CH_2), 2.70 (1H, d, J 20.6, 13-CH_2), 2.78 (1H, d, J 20.6, 13-CH_2),3.70-3.90 (2H, m, 7- and 8-CH_2), 4.03 (2H, q, J 7.2, 14-CH_3), 4.03 (2H, q, J 7.2, 16-CH_3), 4.04 (2H, q, J 7.2, 11-CH_3), 5.01 (2H, s, 19-CH_2), 5.22 (0.5H, br.d, J 8.4, 8-NH), 5.46 (0.5H, br.d, J 8.6, 8-NH), 6.51 (0.5H, br.d, J 9.6, 7-NH), 6.60 (0.5H, br.d, J 9.8, 7-NH), 7.27-7.30 (5H, m, Ar-H), δ_C (250 MHz, $CDCl_3$) 14.1 (C-11), 16.1, 16.2 and 16.2 (C-15 and -17), 14.1 and 18.9 (C-9), 24.7, 25.4, 25.7, 28.7, 29.6 and 31.9 (C-3, -4, -5 and -6), 34.1 (C-2), 35.0 (C-13, d, $^1J_{PC}$ 137), 35.0 (C-13, d, 125.8), 50.3 and 50.5 (C-7), 53.4 and 54.1 (C-8), 60.1 (C-10), 62.6 (C-14 and -16, d, $^2J_{POC}$ 6.7), 62.7 (C-14 and -16, d, $^2J_{POC}$ 6.5), 66.4 (C-19), 127.8, 127.9, 128.0, 128.3 and 128.3 (5 x Ar-C), 136.5 (C-*ipso*), 155.8 and 156.2 (C-18), 164.1 (C-12, d, $^2J_{PC}$ 6.5), 173.6 (C-1). Found: MH^+, 529.26729 (FAB-MS), $C_{25}H_{42}N_2O_8P$ requires 529.26788.

8-amino-7-[phosphonoacetylamido]-nonanoic acid (96)



Iodo-trimethylsilane (140 mg, 700 μmol) was added dropwise to a solution of **101** (15 mg, 28 μmol) in dry DCM (3 cm^3) at room temperature under argon. The reaction was stirred at 45 °C for 48 hours before being quenched by the addition of water (10 cm^3) and chloroform (5 cm^3). After the separation of the layers, the aqueous phase was extracted repeatedly with chloroform (7 x 15 cm^3). Lyophilisation

afforded **96** (mixture of diastereomers a and b) as a pale brown solid (8.5 mg, 26 μmol, 95 %); δ_H (250 MHz, D_2O) 1.20 (1.5H, d, J 6.8, 9_b-CH_3), 1.29 (1.5H, d, J 6.7, 9_a-CH_3), 1.20-1.50 (4H, m, 4- and 5-CH_2), 1.50-1.70 (4H, m, 3- and 6-CH_2), 2.34 (2H, t, J 7.3, 2-CH_2), 2.78, 2.79, 2.80, 2.81, 2.87, 2.88, 2.88 and 2.89 (2H, AB of ABX, 11-CH_2), 3.32 (0.5H, br. dq 6.6 and 3.2, 8_a-CH_2), 3.49 (0.5H, br. dq J 6.6 and 3.7, 8_b-CH_2), 3.88 (0.5H, dt J 6.6 and 3.7, 7_a-CH), 4.11 (0.5H, br. dt J 3.5 and 6.5, 7_b-CH), δ_C (62.9 MHz, D_2O) 11.8 and 15.0 (C-9), 24.0, 24.3, 24.8, 27.6 (C-3, -4 and -5), 29.1 and 29.7 (C-6), 33.6 (C-2), 37.2 (C-11, d, $^1J_{PC}$ 125), 37.4 (C-11, d, $^1J_{PC}$ 124), 51.2 (C-7), 51.6 and 52.2 (C-8), 170.1 (C-10, d, $^2J_{PC}$ 4), 179.1 (C-1), δ_P (D_2O, 101.26 MHz) 17.74 (br. s). Found : M^+, 310.12967 (FAB-MS), $C_{11}H_{23}N_2O_6P$ requires 310.12938.

Chapter Seven

References

1 F. Kogl and B. Tonnis, *Z. Physiol. Chem.*, 1936, **242**, 43.

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

3 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.*, 1944, **66**, 1756.

4 W. Traub, *Nature*, 1956, **178**, 649.

5 J. Trotter and J. A. Hamilton, *Biochemistry*, 1966, **5**, 713.

6 S. J. Wakil, E. B. Titchener, and D. M. Gibson, *Biochim. Biophys. Acta*, 1958, **29**, 225.

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

8 D. P. Kosow and M. D. Lane, *Biochem. Biophys. Res. Commun.*, 1962, **7**, 439.

9 J. P. Bonjour, Handbook of Vitamins : Nutritional, Biochemical and Clinical Aspects, ed. L. J. Machlin, Marcel Dekker, 1984, 403.

10 M. Ohsugi, Y. Imanishi, T. Teraoka, K. Nishimura, and S. Nakao, *J. Nutr. Sci. Vitaminol.*, 1990, **36**, 447.

11 L. Sweetman and W. L. Nyham, *Ann. Rev. Nutr.*, 1986, **6**, 317.

12 C. C. Whitehead, Biotin in animal Nutrition, *F-Hoffmann-La Roche & Co.Ltd Animal Nutrition and Health*, 1988, 5.

13 "Avidin-Biotin Technology", *Methods Enzymol.* 1992, **184**, 1.

14 C. M. Visser and R. M. Kellogg, *J. Mol. Evol.*, 1978, **11**, 171.

15 F. Lynen, J. Knappe, E. Lorch, G. Jutting, and E. Ringelman, *Angew. Chem.*, 1959, **71**, 481.

16 C. Bonnemere, J. A. Hamilton, L. K. Steinrauf, and J. Knappe, *Biochemistry*, 1965, **4**, 240.

17 T. C. Bruice and A. F. Hegarty, *Proc. Natl. Acad. Sci. USA*, 1970, **65**, 805.

18 R. B. Guchait, S. E. Polakis, D. Hollis, C. Fenselau, and M. D. Lane, *J. Biol. Chem.*, 1974, **237**, 1460.

19 Y. Kaziro, L. F. Hass, P. D. Boyer, and S. Ochoa, *J. Biol. Chem.*, 1962, **237**, 1460.

20 C. K. Sauers, W. P. Jencks, and S. Groh, *J. Am. Chem. Soc.*, 1975, **97**, 5546.

21 F. Lynen, *Biochem. J.*, 1967, **102**, 381.

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

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

24 J. R. Knowles, *Annu. Rev. Biochem.*, 1980, **49**, 877.

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

26 J. R. Knowles, *Annu. Rev. Biochem*, 1989, **58**, 195.

27 J. Retey and F. Lynen, *Biochem. Z.*, 1965, **342**, 256.

28 J. Stubbe and R. H. Abeles, *J. Biol. Chem.*, 1979, **252**, 8338.

29 S. J. O'Keefe and J. R. Knowles, *Biochemistry*, 1986, **25**, 6077.

30 P. V. Attwood, P. A. Tipton, and W. W. Cleland, *Biochemistry*, 1986, **25**, 8197.

31 M. A. Eisenberg, *Adv. Enzymol. Relat. Areas. Mol. Biol.*, 1973, **36**, 317.

32 J. H. Mueller, *J. Biol. Chem.*, 1937, **119**, 121.

33 R. E. Eakin and E. A. Eakin, *Science*, 1942, **96**, 187.

34 K. Dittmer and V. du Vigneaud, *Science*, 1944, **100**, 129.

35 H. L. Elford and L. D. Wright, *Biochem. Biophys. Res. Commun.*, 1963, **10**, 373.

36 M. A. Eisenberg, *Biochem. Biophys. Res. Commun.*, 1962, **8**, 437.

37 S. Okumura, R. Tsugawa, T. Tsunoda, and S. Matozoki, *Agric. Chem. Soc.*, 1962, **36**, 605.

38 H. Lezius, E. Ringelman, and F. Lynen, *Biochem. Z.*, 1963, **336**, 510.

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

40 E. L. Tatum, *J. Biol. Chem.*, 1945, **160**, 455.

41 M. A. Eisenberg and R. Maseda, *Biochem. J.*, 1966, **101**, 598.

42 M. A. Eisenberg, *Biochem. J.*, 1966, **101**, 601.

43 M. A. Eisenberg and R. Maseda, *Biochemistry*, 1969, **9**, 108.

44 S. Iwahara, M. Kikuchi, T. Tochikura, and K. Ogata, *Agr. Biol. Chem.*, 1966, **30**, 304.

45 S. Iwahara, M. Kikuchi, T. Tochikura, and K. Ogata, *Agr. Biol. Chem.*, 1967, **31**, 694.

46 G. Pontecorvo, *Adv. Genet.*, 1953, **5**, 141.

47 S. Okumura, R. Tsugawa, T. Tsunoda, and S. Matozaki, *J. Agric. Chem. Soc. (Jap.)*, 1962, **35**, 599.

48 S. Okumura, R. Tsugawa, T. Tsunoda, and S. Matozaki, *J. Agric. Chem. Soc. (Jap.)*, 1962, **36**, 204.

49 B. Rolfe and M. A. Eisenberg, *J. Bacteriol.*, 1968, **96**, 515.

50 A. L. Taylor, *Bacteriol. Rev.*, 1970, **34**, 155.

51 B. Rolfe, *Virology*, 1969, **42**, 643.

52 P. P. Cleary and A. Campbell, *J. Bacteriol.*, 1972, **112**, 830.

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

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

55 M. Schwartz, *J. Bacteriol.*, 1966, **92**, 1083.

56 M. A. Eisenberg, "Metabolic Pathways", ed. D. M. Greenberg, Academic Press, New York, 1975, 27.

57 D. F. Barker and A. M. Campbell, *J. Mol. Biol.*, 1981, **146**, 469.

58 C. H. Pai and H. C. Lichstein, *Biochim. Biophys. Acta.*, 1965, **100**, 28.

59 M. A. Eisenberg and C. Star, *J. Bacteriol.*, 1968, **96**, 1291.

60 M. A. Eisenberg, *Ann. N.Y. Acad. Sci.*, 1984, **447**, 335.

61 K. P. Wilson, L. M. Shewchuk, R. G. Brennan, A. J. Otsuka, and B. W. Matthews, *Proc. Natl. Acad. Sci. USA*, 1992, **89**, 9257.

62 D. Beckett and B. W. Mattews, *Methods Enzymol.*, 1997, **279**, 362.

63 Y. Izumi, Y. Tani, and H. Yamada, *Bull. Instr. Chem. Res. Kyoto Univ.*, 1980, **58**, 434.

64 H. Gerbling, S. Axiotis, and R. Douce, *J. Plant Physiol.*, 1994, **143**, 561.

65 O. Ploux, P. Soularue, A. Marquet, R. Gloekler, and Y. Lemoines, *Biochem. J.*, 1992, **287**, 685.

66 M. A. Eisenberg, 'Biosynthesis of Biotin and Lipoic acid', *Escherichia coli* and *Salmonella Typhimurium*, ed. F. C. Neidhardt *et al.*, American. Society for Microbiology, Washington D.C., 1987, 544.

67 I. Sanyal, S. L. Lee, and D. H. Flint, *J. Am. Chem. Soc.*, 1994, **116**, 2637.

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

69 N. Koga, J. Kishimoto, S. I. Haze, and O. Ifuku, *J. Ferment. Biooeng.*, 1996, **81**, 482.

70 Y. Lemoine, A. Wach, and J. M. Jeltsch, *Mol. Microbiol.*, 1996, **19**, 645.

71 F. W. Alexander, E. Sandmeier, P. K. Mehta, and P. Christen, *Eur. J. Biochem.*, 1994, **219**, 953.

72 D. Alexeev, M. Alexeeva, R. L. Baxter, D. J. Campopiano, S. P. Webster, and L. Sawyer, *J. Mol. Biol.*, 1998, 1.

73 Z. Zaman, P. M. Jordan, and M. Akhtar, *Biochem. J.*, 1973, **135**, 257.

74 J. J. Mukherjee and E. E. Dekker, *J. Biol. Chem.*, 1987, **262**, 14441.

75 A. H. Merrill and D. D. Jones, *Biochim. Biophys. Acta.*, 1990, **1037**, 24.

76 O. Ploux and A. Marquet, *Biochem. J.*, 1992, **283**, 327.

77 S. Spinelli, O. Ploux, A. Marquet, C. Anguille, C. Jelsch, C. Cambillau, and C. Martinez, *Acta Cryst.*, 1996, **D52**, 866.

78 O. Ploux and A. Marquet, *Eur. J. Biochem.*, 1996, **236**, 301.

79 G. L. Stoner and M. A. Eisenberg, *J. Biol. Chem.*, 1975, **250**, 4029.

80 M. A. Eisenberg and G. L. Stoner, *J. Bacteriol.*, 1971, **108**, 1135.

81 C. H. Pai, *J. Bacteriol.*, 1971, **105**, 793.

82 G. L. Stoner and M. A. Eisenberg, *J. Biol. Chem.*, 1975, **250**, 4037.

83 A. J. Otsuka, M. R. Buoncristiani, P. K. Howard, J. Flamm, C. Johnson, R. Yamamoto, K. Uchida, C. Cook, J. Ruppert, and J. Matsuki, *J. Biol. Chem.*, 1988, **263**, 19577.

84 D. Alexeev, R. L. Baxter, and L. Sawyer, *Structure*, 1994, **2**, 1061.

85 M. A. Eisenberg and K. Krell, *J. Bacteriol.*, 1969, **98**, 1227.

86 M. A. Eisenberg and K. Krell, *J. Biol. Chem.*, 1969, **244**, 5503.

87 K. Krell and M. A. Eisenberg, *J. Biol. Chem.*, 1970, **245**, 6558.

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

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

90 D. Alexeev, R. L. Baxter, O. Smekal, and L. Sawyer, *Structure*, 1995, **3**, 1207.

91 W. Huang, J. Jia, K. J. Gibson, W. S. Taylor, A. R. Rendina, G. Schneider, and Y. Lindqvist, *Biochemistry*, 1995, **34**, 10985.

92 K. J. Gibson *et al.*, *Biochemistry*, 1995, **34**, 10976.

93 R. L. Baxter and H. C. Baxter, *J. Chem. Soc., Chem. Commun.*, 1994, 759.

94 K. J. Gibson, *Biochemistry*, 1997, **36**, 8474.

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

96 G. E. Schulz, *Curr. Opin. Struct. Biol.*, 1992, **2**, 61.

97 G. Yang, T. Sandalova, K. Lohman, Y. Lindqvist, and A. R. Rendina, *Biochemistry*, 1997, **36**, 4751.

98 H. Kack, K. J. Gibson, Y. Lindqvist, and G. Schneider, *Proc. Natl. Acad. Sci. USA*, 1998, **95**, 5495.

99 R. Gloeckler, I. Ohsawa, D. Speck, C. Ledoux, S. Bernard, M. Zinsius, D. Villeval, T. Kisou, K. Kamogawa, and Y. Lemoines, *Gene*, 1990, **87**, 63.

100 N. Sakurai, Y. Imai, H. Akatsuka, E. Kawai, and S. Komatsubara, *SWISSPROT data bank*, 1993, accession code P36572.

101 K. Hatakeyama, K. Hohama, A. A. Vertes, M. Kobayashi, Y. Kurusu, and H. Yukawa, *DNA sequence*, 1994, **4**.

102 *SWISSPROT data bank*, accession code P36572.

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

104 T. Niimura, T. Suzuki, and Y. Sahashi, *J. Vitaminol.*, 1964, **10**, 218.

105 T. Niimura, T. Suzuki, and Y. Sahashi, *J. Vitaminol.*, 1964, **10**, 224.

106 K. Shimada, *Yakugahu Zashi (Jap.)*, 1964, **87**, 351.

107 R. B. Roberts, D. B. Cowie, P. H. Abelson, E. T. Bolton, and R. J. Britten, *Studies of Biosynthesis in E. coli*, 1955, **607**, 318.

108 H. L. Elford and L. D. Wright, *Arch. Biochem. Biophys.*, 1968, **123**, 1456.

109 R. H. White, *Biochemistry*, 1982, **21**, 4271.

110 E. DeMoll and W. Shive, *Biochem. Biophys. Res. Commun.*, 1983, **110**, 243.

111 E. DeMoll, R. H. White, and W. Shive, *Biochemistry*, 1984, **23**, 558.

112 N. M. Shaw, O. M. Birch, A. Tinschert, V. Venetz, R. Dietrich, and L. A. Savoy, *Biochem. J.*, 1998, **330**, 1079.

113 H. C. Li, D. B. McCormick, and L. D. Wright, *J. Biol. Chem.*, 1968, **243**, 6442.

114 R. J. Parry and M. G. Kunitani, *Methods Enzymol.*, 1979, **62D**, 353.

115 R. J. Parry and V. Naidu, *Tetrahedron Lett.*, 1980, **21**, 4783.

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

117 F. Frappier, M. Jouany, A. Marquet, A. Olesker, and J. C. Tabet, *J. Org. Chem.*, 1982, **47**, 2257.

118 D. A. Trainor, R. J. Parry, and A. Gitterman, *J. Am. Chem. Soc.*, 1980, **102**, 1467.

119 F. B. Marti, *PhD Thesis, Eidgenossischen Technischen Hochschule (ETH), Zurich*, 1983.

120 F. Frappier, G. Guillerm, A. G. Salib, and A. Marquet, *Biochem. Biophys. Res. Commun.*, 1979, **91**, 521.

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

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

123 R. L. Baxter, D. J. Campopiano, A. Coutts, and N. Shaw, *J. Chem. Soc., Perkin Trans. 1*, 1992, 255.

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

125 I. Sanyal, G. Cohen, and D. H. Flint, *Biochemistry*, 1994, **33**, 3625.

126 Y. Izumi, H. Morita, Y. Tani, and K. Ogata, *Agr. Biol. Chem.*, 1972, **36**, 519.

127 T. Suyama and S. Kaneo, *Chem. Abstr.*, 1964, **60**, 4013 g.

128 S. G. Warren, 'Organic Synthesis : The Disconnection Approach ', John Wiley and Sons. Ltd., 1982.

129 A. Baeyer and V. Villiger, *Chem. Ber.*, 1899, **32**, 3625.

130 S. G. Horgan, *PhD. Thesis, University of Edinburgh, Edinburgh*, 1995.

131 R. S. Lott, V. S. Chauhan, and C. M. Stammer, *J. Chem. Soc., Chem. Commun.*, 1979, 1979.

132 G. Stork, A. Brizzolara, H. Landesman, J. Szmuszkovicz, and R. Terrell, *J. Am. Chem. Soc.*, 1963, **85**, 207.

133 O. Takazawa, K. Kogami, and K. Hayashi, *Bull. Chem. Soc. Jpn.*, 1984, **57**, 1876.

134 M. S. Cooper, H. Heaney, A. J. Newbold, and W. R. Sanderson, *Synlett.*, 1990, 533.

135 J. S. Clark and A. B. Holmes, *Tetrahedron Lett.*, 1988, **29**, 4333.

136 J. C. McWilliams and J. Clardy, *J. Am. Chem. Soc.*, 1994, **116**, 8378.

137 R. S. McAlpine, *Personal Communication.*

138 W. B. Dauben and D. M. Walker, *J. Org. Chem.*, 1981, **46**, 1103.

139 C. M. Leir, *J. Org. Chem.*, 1972, **37**, 887.

140 N. J. Leonard and W. E. Goode, *J. Am. Chem. Soc.*, 1950, **72**, 5404.

141 G. Rosenkranz, O. Mancera, J. Gatica, and C. Djerassi, *J. Am. Chem. Soc.*, 1950, **72**, 4077.

142 R. F. Borch, M. D. Bernstein, and H. D. Durst, *J. Am. Chem. Soc.*, 1971, **93**, 2897.

143 K. Bowden, I. M. Heilbron, E. R. H. Jones, and B. C. L. Weedon, *J. Chem. Soc.*, 1946, 39.

144 C. L. Barney, E. W. Huber, and J. R. McCarthy, *Tetrahedron Lett.*, 1990, **31**, 5547.

145 E. J. Corey and B. W. Erickson, *J. Org. Chem.*, 1971, **36**, 3553.

146 P. Wang, *PhD. Thesis, University of Edinburgh, Edinburgh*, 1995.

147 U. Schollkopf, K. O. Westphalen, J. Schroder, and K. Horn, *Liebigs. Ann. Chem.*, 1988, 781.

148 M. W. Holladay and D. H. Rich, *Tetrahedron Lett.*, 1983, **24**, 4401.

149 M. T. Reetz, M. W. Drewes, and A. Schmitz, *Angew. Chem. Int. Ed. Engl.*, 1987, **26**, 1141.

150 C. W. Alexander and D. C. Liotta, *Tetrahedron Lett.*, 1996, **37**, 1961.

151 P. L. Beaulieu and D. Wernic, *J. Org. Chem.*, 1996, **61**, 3635.

152 M. T. Reetz, M. W. Drewes, B. R. Matthews, and K. Lennick, *J. Chem. Soc., Chem. Commun.*, 1989, 1474.

153 J. W. B. Cooke, S. G. Davies, and A. Naylor, *Tetrahedron*, 1993, **49**, 7955.

154 D. Lucet, T. L. Gall, C. Mioskowski, O. Ploux, and A. Marquet, *Tetrahedron Asymm.*, 1996, **7**, 985.

155 H. C. Dunathan, *Proc. Natl. Acad. Sci. USA*, 1966, **55**, 712.

156 H. Hayashi, *J. Biochem.*, 1995, **118**, 463.

157 R. A. John, *Biochim. Biophys. Acta.*, 1995, **1248**, 81.

158 D. Alexeev, *Personal communication.*

159 M. I. Page, *Chem. Ind. (London)*, 1981, 144.

160 A. G. Ogston, *Discuss. Faraday. Soc.*, 1955, **20**, 161.

161 L. Pauling, *Chem. Eng. News*, 1946, **24**, 1375.

162 R. R. Rando, *Acc. Chem. Res.*, 1975, **8**, 281.

163 J. T. Welch, *Tetrahedron*, 1987, **43**, 3123.

164 M. J. O`Donnell, W. D. Bennett, W. A. Bruder, W. N. Jacobsen, K. Knuth, B. LeClef, R. L. Polt, F. G. Bordwell, S. R. Mrozack, and T. A. Cripe, *J. Am. Chem. Soc.*, 1988, **110**, 8520.

165 M. J. O`Donnell and R. L. Polt, *J. Org. Chem.*, 1982, **47**, 2663.

166 G. Stork, A. Y. W. Leong, and A. M. Touzin, *J. Org. Chem.*, 1976, **41**, 3491.

167 P. Bey and J. P. Vevert, *Tetrahedron Lett.*, 1977, **17**, 1455.

168 O. Tsuge, K. Ueno, S. Kanemasa, and K. Yorozu, *Bull. Chem. Soc. Jpn.*, 1987, **60**, 3347.

169 A. Boumendjel and S. P. F. Miller, *Tetrahedron Lett.*, 1994, **35**, 819.

170 D. Ferroud, J. P. Genet, and R. Kiolle, *Tetrahedron Lett.*, 1986, **27**, 23.

171 P. Sulmon, N. D. Kimpe, and N. Schamp, *Synthesis*, 1989, 8.

172 M. J. O`Donnell and T. M. Eckrich, *Tetrahedron Lett.*, 1978, **47**, 4625.

173 M. J. O`Donnell, S. Wu, and J. C. Huffman, *Tetrahedron*, 1994, **50**, 4507.

174 J. O. Opio, S. Labidalle, H. Galons, and M. Miocque, *Synth. Commun.*, 1991, **21**, 1743.

175 M. Rabinovitz, Y. Cohen, and M. Halpern, *Angew. Chem. Int. Ed. Engl.*, 1986, **25**, 960.

176 S. Nahm and S. M. Weinreb, *Tetrahedron Lett.*, 1981, **22**, 3815.

177 T. A. Ostler and T. M. Harris, *Tetrahedron Lett.*, 1983, **24**, 1851.

178 H. D. Dakin and R. West, *J. Biol. Chem.*, 1928, **78**, 745.

179 H. D. Dakin and R. West, *J. Biol. Chem.*, 1928, **78**, 757.

180 J. Attenburrow, D. F. Elliott, and G. F. Penny, *J. Chem. Soc.*, 1948, 310.

181 Y. Iwakura, F. Toda, and H. Suzuki, *J. Org. Chem.*, 1967, **32**, 440.

182 N. L. Allinger, G. L. Wang, and B. B. Dewhurst, *J. Org. Chem.*, 1974, **39**, 1731.

183 W. Steglich and G. Hofle, *Angew. Chem. Int. Ed. Engl.*, 1969, **8**, 981.

184 W. Steglich and G. Hofle, *Tetrahedron Lett.*, 1970, **54**, 4727.

185 W. Steglich and G. Hofle, *Angew. Chem. Int. Ed. Engl.*, 1968, **7**, 61.

186 W. Steglich and G. Hofle, *Tetrahedron Lett.*, 1968, **13**, 1619.

187 W. Steglich and G. Hofle, *Chem. Ber.*, 1969, **102**, 883.

188 W. Steglich and G. Hofle, *Chem. Ber.*, 1969, **102**, 899.

189 J. March, 'Advanced Organic Chemistry : Reactions, Mechanisms and Structure', Fourth edn., Wiley-Interscience, 1992, 392.

190 H. E. Carter, *Org. React.*, 1946, **3**, 198.

191 A. M. M. Mjalli, K. T. Chapman, and M. MacCoss, *Bioorg. Med. Chem. Lett.*, 1993, **3**, 2693.

192 V. Daffe and J. Fastrez, *J. Chem. Soc., Perkin. Trans. 2.*, 1983, 783.

193 F. M. F. Chen, K. Kuroda, and N. L. Benoiton, *Synthesis*, 1979, 230.

194 V. Daffe and J. Fastrez, *J. Chem. Soc., Perkin. Trans. 2.*, 1983, 789.

195 Y. Morino and M. Okamoto, *Biochem. Biophys. Res. Commun.*, 1970, **40**, 600.

196 M. Okamoto and Y. Morino, *J. Biol. Chem.*, 1972, **248**, 82.

197 G. A. Wiley, R. L. Hershkowitz, B. M. Rein, and B. C. Chung, *J. Am. Chem. Soc.*, 1964, **86**, 964.

198 M. McPherson, *PhD Thesis, University of Edinburgh, Edinburgh,* 1995.

199 G. M. Blackburn, *Chem. Ind. (London)*, 1981, 134.

200 R. B. Silverman, 'Mechanism-based Based Enzyme Inactivation: Chemistry and Enzymology', vol. 1, CRC Press, 1988, 3.

201 C. E. McKenna and J. Schmidhauser, *J. Chem. Soc., Chem. Commun.*, 1979, 739.

202 M. E. Jung and M. A. Lyster, *J. Org. Chem.*, 1977, **42**, 3761.

203 D. Ben-Ishai and A. Berger, *J. Org. Chem.*, 1952, **17**, 1564.

204 K. Mori and Z. Qian, *Liebigs. Ann. Chem.*, 1994, 291.

205 R. A. Polt, M. A. Peterson, and L. DeYoung, *J. Org. Chem.*, 1992, **57**, 5469.

206 W. F. Huber, *J. Am. Chem. Soc.*, 1951, **73**, 2730.

207 M. P. Sibi, J. W. Christensen, S. G. Kim, and M. Eggen, *Tetrahedron Lett.*, 1995, **36**, 6209.

208 F. C. Pennington, W. D. Celmer, W. M. McLamore, V. V. Bogert, and I. A. Solomons, *J. Am. Chem. Soc.*, 1953, **75**, 109.

209 T. Tanaka, S. Kurozumi, T. Toru, M. Kobayashi, S. Miura, and S. Ishimoto, *Tetrahedron*, 1977, **33**, 1105.

210 P. Bey, J. P. Vevert, V. V. Dorsellaer, and M. Kolb, *J. Org. Chem.*, 1979, **44**, 2732.

211 O. Tsuge, K. Ueno, S. Kanemasa, and K. Yorozu, *Bull. Chem. Soc. Jpn.*, 1986, **59**, 1809.

212 J. V. Paukstelis and M. Kim, *J. Org. Chem.*, 1974, **39**, 1503.

213 V. I. Lomakina and e. al., *J. Gen. Chem. USSR (Eng. Transl.)*, 1966, **36**, 465.

214 C.H. Chen and B. A. Donatelli, *J. Org. Chem.*,1976, **41**, 18, 3053.

215 W. S. Lee, K. Lee, K. D. Nam and Y. J. Kim, *Tetrahedron*, 1991, **47**, 38, 8091.

216 H. Rubinstein and M. Wuerthele, *J. Org. Chem.*, 1969, **34**, 9, 2762.

217 G. Barbieri, M. Cinquini and F. Montanari, *J. Chem. Soc. (C)*, 1968, 659.

218 W. D. Kingsbury and C. R. Johnson, *J. Chem. Soc., Chem. Commun.*,1969, 365.

219 P. R. Heaton, J. M. Midgley and W. B. Whalley, *J. Chem. Soc., Perkin 1,* 1978, 1011.

Appendix

Publication

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3
Amino Acids Aren't Only for Peptide Synthesis - Enzymatic and Biomimetic Syntheses of a Vitamin from L-Alanine

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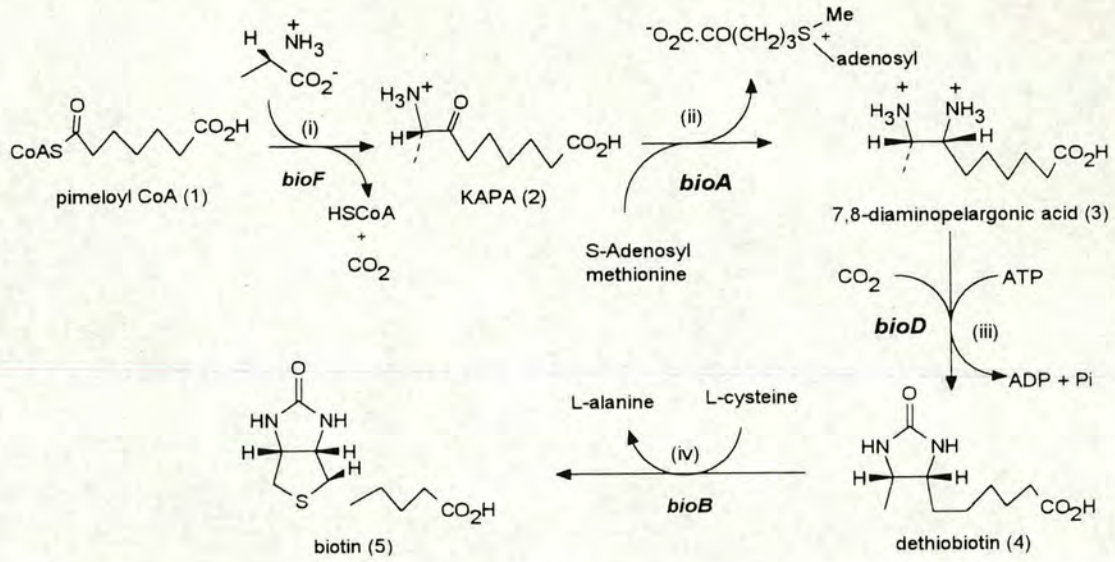
Introduction

In Nature, the α-amino acids serve not only as building blocks for the synthesis of proteins but also as starting materials for syntheses of a bewildering number of other important products. Dissection of these pathways has been rewarded not only by an intimate understanding of the mechanisms of biosynthetic processes but also by discovery of new chemistry. In this context, it has been recognised for many years that the biosynthesis of almost all the B vitamins start from amino acid precursors and involve quite unique chemistry. However, despite their importance and their ubiquitous presence in Nature, the fact that vitamins are produced in minute quantities, by enzymes present in barely detectable amounts, has seriously limited the study of these reactions. Over the past five years, however, the application of the techniques of molecular biology to this field has led to rapid progress. Biotin biosynthesis is a typical case.

Biotin **5**, the cofactor of almost all carboxylase and transcarboxylase enzymes, is a commercially important compound used not only as a vitamin in food supplements but also, in view of its ability to make tight complexes with avidin and streptavidin, as a probe in molecular and cell biology. Because biotin is found in only miniscule amounts in producing organisms, all commercially used biotin is currently obtained by total synthesis. More than fifteen syntheses of biotin have been published in the last four decades. Although the commercial chemical synthesis has been reduced now to only twelve steps - biotin producing organisms can effect the efficient synthesis of biotin from L-alanine and pimelic acid in only five reactions (Scheme 1) - suggesting that we might still have a little chemistry to learn from Nature.

The route shown in Scheme 1 was determined by early work by Campbell and by Eisenberg and appears to be ubiquitous in Nature [1]. Each of the reactions shown are enzymatically unusual: Step (i) has a few parallels - one notably being the first step in vitamin B_{12} synthesis catalysed by 5-aminolevulinic acid synthase; Step (ii) is a trans-amination - but is unique in requiring S-adenosylmethionine as the nitrogen donor; Step

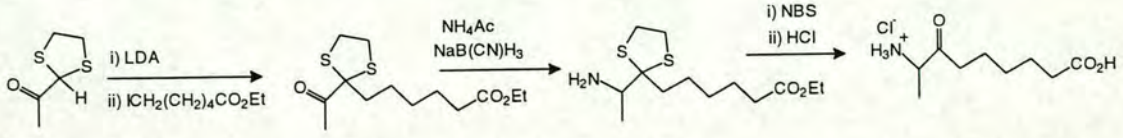


Scheme 1. *The biotin pathway.*

(iii) is unique, here CO_2, and not HCO_3^-, is involved and the ring closure involves only one activation step for formation of two C-N bonds. The final step (iv) is chemically the most intriguing; involving the introduction of sulfur at two apparently chemically unreactive sites to form a tetrahydrothiophene ring. Recent work on the enzymes of biotin biosynthesis in Edinburgh, and elsewhere, has led to not only to insight into the details of the reactions involved but also to the development of enzymatic and biomimetic chemical routes to the immediate precursor of biotin.

Results and Discussion

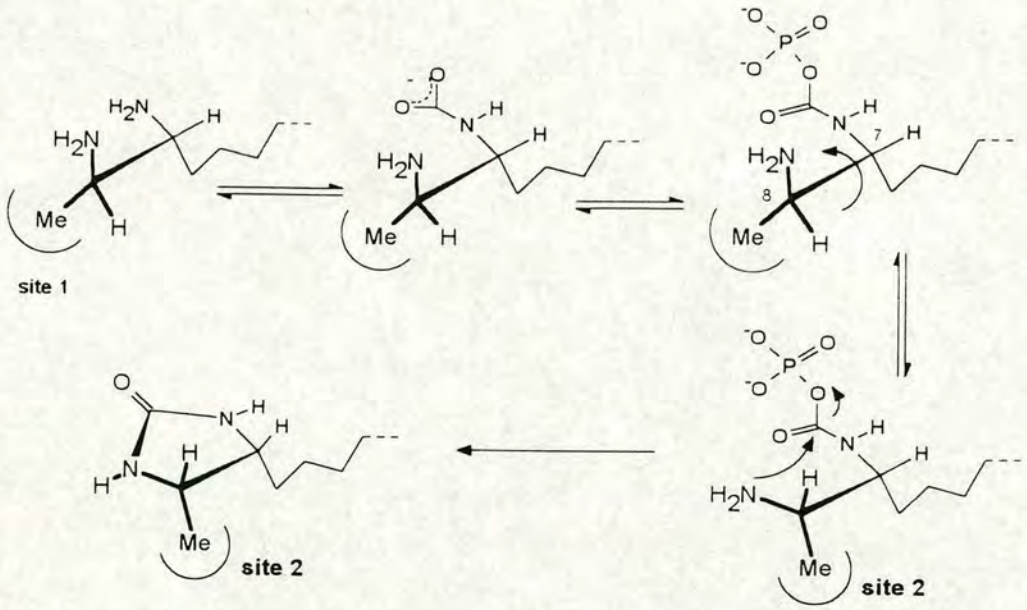
At the start this work, we were confronted by a problem - the production of the *E. coli* enzymes is controlled at the genetic level by feedback inhibition mediated by binding of the biotin holoenzyme/biotin-AMP anhydride complex to the operator region of the biotin operon [2]. Thus we had first to subclone all of the genes in the operon and express each of these individually. This was non-trivial, complicated by the lack of convenient restriction sites and the overlap of some of the gene reading frames. Two of the individual genes (*bio*B and *bio*F) proved extremely difficult to overexpress - but now all of these enzymes are routinely available in 20mg/L amounts. The second problem was the non-availability of intermediates. Syntheses of diaminopelargonate **3** (from dethiobiotin) and pimeloyl CoA **1** proved straightforward - but that of novel ketoamino acid **2** was more challenging (Scheme 2).



Scheme 2. ***Synthesis of (±)ketoaminopelargonic acid 2.***

A combination of chemical and biological techniques has allowed us to examine each step in the pathway in detail. Exemplative of this work is the study of the penultimate

enzyme, dethiobiotin synthetase. This catalyses the third step in the pathway - the formation of the *ureido* ring of **4** by dethiobiotin synthetase. Superficially, this reaction seems quite straightforward but the fact that the enzyme uses CO_2 and only a single ATP $\rightarrow$ ADP + P_i turnover poses a problem. How can two N-C bonds be formed with only a single activation step? The answer has emerged from a combination of chemical, kinetic and crystallographic studies from the group in Edinburgh, and also from work in Upsala and Delaware [3]. The first problem was to determine the order of substrate addition and we found that **3** and $^{14}CO_2$ were combined enzymatically, in the absence of ATP, to give an unstable DAPA carbamate. The next step was shown by ^{18}O labelling to involve formation of a phosphoric acid anhydride species which undergoes attack by the neighbouring amino group to give the product **4** [3a-c] (Scheme 3). The structural criteria which are important in the series of reactions catalysed by the enzyme has now been revealed by crystallographic and kinetic studies using synthetic substrates and intermediate mimics. Substitution of the terminal methyl group of **3** with either H or Et results a 10^3 fold lowering of enzymatic efficiency indicating that methyl group binding is an important element in the reaction.

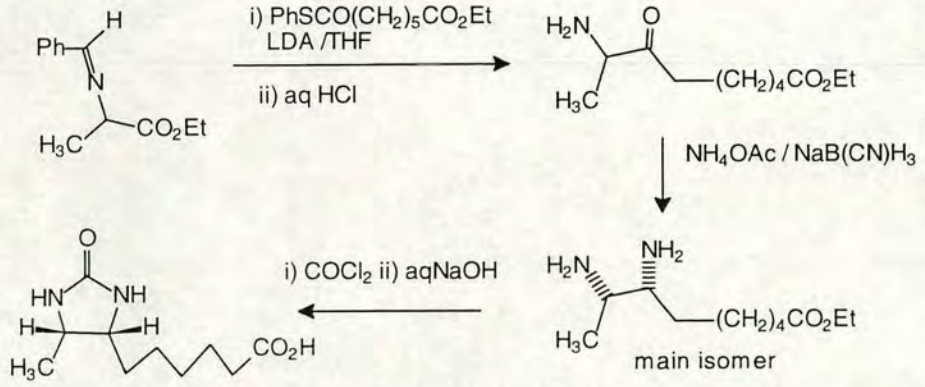


Scheme 3. ***The role of methyl group binding in the ureido ring closure reaction.***

Crystallography has also revealed that the conversion of DAPA carbamate **3a** to DTB **4** by the enzyme involves a change in conformation of the Me group. This can be rationalised in that the *ureido* ring closure step requires a *ca* 60° rotation round the C_7-C_8 bond of the phosphate-carbamate mixed anhydride to effect an effective angle of attack of the 8-amino group for ring closure which places the Me group in a new binding pocket in the protein architecture. This rotation is optimised for methyl group interaction with the hydrophobic regions of the reaction sites in the protein skeleton.

Studies on the mechanisms of the earlier enzymes of the pathway in Edinburgh and Paris [4] have augmented our knowledge and with adequate amounts of the enzymes in hand we have now been able to complete a catalytic *ex vivo* synthesis of dethiobiotin

from L-alanine. For most biochemical pathways just adding starting materials to an enzyme mixture can result in an equilibrium state with no significant final product formation. However here, as in the B_{12} studies of Scott and his coworkers [5], the enzymatic machinery works in our favour. Since the K_M's of each of the enzymes are extremely low, dethiobiotin can be produced in virtually quantitative yield from L-alanine and pimeloyl CoA by enzymic catalysis *in vitro* [Steps (i) to (iii), Scheme 1].



Scheme 4. ***A biomimetic synthesis of (±) dethiobiotin 4.***

What we have learned from the biosynthesis has also been applied to designing a biomimetic synthesis of (±)dethiobiotin (Scheme 4). However completion of the pathway requires *in vitro* biochemical or chemical realisation of the final step - the introduction of a sulfur atom between C-6 and C-9 of the dethiobiotin skeleton to form the final tetrathiophene ring (Scheme 1). We know now that 9-mercaptodethiobiotin is a probable intermediate and that this conversion is a complex S-adenosylmethionine initiated radical process, requiring not only the dimeric [2Fe-2S] biotin synthase protein, flavodoxin and flavodoxin reductase, but also a *nifS*-type cysteine desulfurase [6]. The current biological chemistry challenge is to determine the mechanism of the enzyme system - and the future chemical challenge will be to design a synthetic reaction which will be able to mimic the efficiency of the biological process.

References

1. For a review see Eisenberg M.A. in *Escherichia coli and Salmonella typhimurium* (Neidhardt, F.C. ed), vol 2, pp 544-550, American Society for Microbiology, Washington, USA (1987).
2. Buoncristiani, M.R., Howard, P.K., and Otsuka, A.J., *Gene,* 44 (1986) 255.
3. (a) Baxter R.L., Ramsey A.J., *et al, J. Chem.Soc., Chem.Commun.* (1994) 559; (b) Baxter, R.L. and Baxter H.C., *ibid*, (1994) 759; (c) Gibson, K.J., Lorimer, J.H., *et al, Biochemistry*, 34 (1995) 10976; (d) Alexeev, D., Baxter, R.L. and Sawyer, L.., *Structure*, 2 (1994) 1061; (e) Alexeev, D., Baxter, R.L., *et al, ibid*, 3 (1995) 1207; (f) Huang, W.J., Linquist, Y., *et al, Structure*, 2 (1994) 407; (g) Huang, W., Jia, J., *et al, Biochemistry*, 34 (1995) 10985.
4. Ploux, O. and Marquet, A., *Biochem. J.*, 283 (1992) 327.
5. Roesner, C.A. and Scott, A.I., *Chemistry and Biology*, 3 (1996) 325.
6. Zheng, L., White, R.H., *et al, Biochemistry*, 33 (1994) 4714.