

# **SYNTHESIS OF COENZYME A ANALOGUES**

A Thesis submitted by

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July 1997  
~~September 1996~~

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## **STATEMENT**

The accompanying thesis submitted for the degree of Ph.D. entitled "Synthesis of CoA analogues" is based on work conducted by the author in the Department of Chemistry of the University of Leicester mainly during the period between October 1990 and March 1995.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references. None of the work has been submitted for another degree in this or any other University.

**Signed:**

**Date:**

## **ACKNOWLEDGEMENTS**

I dedicate this thesis to my Mother and Father, Gian Kaur and Mohinder Singh (Driving Instructor) without whose constant support and encouragement this work would not have been possible. In addition I must thank my two sisters, Permjit and Ternjit who kept faith when I had lost it.

I would like to thank my supervisor professor Paul Cullis for all the help and advice he has given me during the completion of this thesis. In addition I would also like to thank my friends, Pav, Dave, Richy, Adam, Phil, Aurora and Raja who ensured life in Leicester was never boring. I must also thank Ann Lewendon and Jackie Ellis for their helpful discussions.

Finally, I wish to thank the Science and Engineering Research Council for the financial support, without which this work would not have been possible.

## ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
CAT	Chloramphenicol acetyltransferase
Cm	Chloramphenicol
CoA	Coenzyme A
DTNB	3-carboxy-4-nitrophenyl disulphide
DCC	Dicyclohexylcarbodiimide
DEAE	Diethyl aminoethyl
DTT	Dithiothreitol
IMP	Inosine 5'-monophosphate
Oxy CoA	Dethio oxy CoA
TEAB	Triethylammonium bicarbonate
Tris	Tris(hydroxy methyl) aminomethane

## Synthesis of Coenzyme A Analogues

Sarabjit S. Jhooti

### ABSTRACT

This thesis was a study of the molecular recognition process of chloramphenicol acetyltransferase (CAT). In an approach, which is complementary to site-directed mutagenesis, the substrate, acetyl Coenzyme A (AcCoA), has been modified in a variety of ways to allow quantitative evaluations of the energetics of individual enzyme-ligand interactions to be made. A series of CoA analogues have been prepared and their kinetic parameters for binding to CAT determined. These compounds had modifications in both the base and pantetheine regions of the CoA molecule.

The synthesis of CoA analogues with alternative bases involved coupling activated nucleotides with D-pantetheine-4'-phosphate which had been prepared from D-pantethine using a cyanoethyl phosphate and DCC coupling method. CoA analogues with modified pantetheine moieties were prepared by coupling pantetheine analogues with an activated adenine nucleotide.

From the kinetic studies of the CAT reaction with the modified ligands the apparent binding energies,  $\Delta\Delta G_{app}$ , of some of the substituents of AcCoA have been evaluated. Analogues in which the adenine nucleotide were substituted with alternative bases were found to be relatively poor substrates for the CAT reaction. This suggests that the adenine moiety is important in the recognition process of AcCoA binding by the enzyme. AcCoA analogues with modified pantetheine moieties were found to bind relatively well. These results suggest that the pantetheine moiety may not substantially be involved in the specificity of CoA for the enzyme, but instead contribute to the binding strength of AcCoA to CAT. Examination of analogues in which the acetyl group of AcCoA was replaced by alkyl groups suggested that they bound within the chloramphenicol binding pocket in a region eight times less hydrophobic than *n*-octanol.

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**CHAPTER 1**  
**GENERAL INTRODUCTION**

## **INTRODUCTION**

### **1.1 Molecular recognition**

Molecular interactions in biological systems are crucial to many life processes including enzyme-substrate interactions in enzyme catalysis, antigen-antibody recognition in immunity as well as DNA-protein interactions in gene regulation. Molecular recognition is the specific non-covalent interaction of two molecules which can involve two small molecules, a macromolecule binding a small ligand as in an enzyme-substrate interaction, or two macromolecules, for example DNA and protein. There are four types of weak non-covalent interaction that contribute to molecular recognition: electrostatic (ionic), hydrogen bonding, van der Waals and hydrophobic interactions. Although these interactions are well understood little is currently known about how individual interactions come together to give the high specificity required in biological systems and predicting absolute values for binding constants from a knowledge of the binding site remains problematic. One of the objectives of studies in molecular recognition is to develop an understanding of the interactions quantitatively to allow prediction of binding constants and the design of tight binding ligands simply from the knowledge of the structure and the properties of the target binding site. Success in these endeavours would have important implications for rational drug design. To control the undesirable side effects of drugs it is essential they are designed to discriminate and bind preferentially to receptor sites and enzymes involved in the disease process, while at the same time showing considerably lower affinity for other potential binding sites. This may involve discriminating between two enzymes that catalyse the same reaction but are encoded by genes either from the host or the invading organism.

#### **1.1.1 Forces involved in molecular recognition**

The four different non-covalent interactions differ in their geometric requirements, strength

and specificity as well as being affected in different ways by the local environment, be it aqueous or non-aqueous. Electrostatic interactions involve a charged group on a substrate interacting with a complementary charged group on an enzyme, the force of such an interaction is defined by Coulomb's law [1.1]:

$$F = \frac{q_1 q_2}{r^2 D} \quad \dots[1.1]$$

in which  $q_1$  and  $q_2$  are the charges on the two groups,  $r$  is the distance between them, and  $D$  is the dielectric constant of the medium. Electrostatic interactions vary in strength depending on their medium, they are strongest in vacuum where  $D$  is 1 and are weakest in medium such as water where  $D$  is 80.

Electrostatic interactions are widely observed in enzyme/ligand interactions and for example the binding of glycytyrosine to the proteolytic enzyme carboxypeptidase A<sup>1</sup> which cleaves carboxyl-terminal residues, is a good example since the electrostatic interaction is presumably part of the specificity of the exopeptidase. The negatively charged terminal carboxylate group of the substrate interacts with the positively charged guanidinium group on an arginine residue of the enzyme 2.8 Å away. In general negatively charged substrates are able to form interactions with the positively charged side chains of lysine, arginine, histidine or terminal amino groups. Positively charged substrates have potential binding sites at the negatively charged carboxylate groups of aspartate and glutamate as well as the terminal carboxylate of the polypeptide chain.

Hydrogen bonded interactions abound in biological systems, stabilising the folded forms of proteins, double helical DNA and contributing to specificity in enzyme-substrate reactions. Different types of hydrogen bonds have quite different hydrogen bond dissociation energies. Examples of those calculated *in vacuo* are: HOH...OH<sub>2</sub>, -6.4 kcal mol<sup>-1</sup>; H<sub>2</sub>O...HSCH<sub>3</sub>, -3.2 kcal mol<sup>-1</sup>; HOH...S(H)CH<sub>3</sub>, -3.1 kcal mol<sup>-1</sup>; CH<sub>3</sub>CO<sub>2</sub>-...HOH, -19.0 kcal mol<sup>-1</sup>.<sup>2</sup> They are directional and are strongest where donor hydrogen and acceptor are all linear. Charged groups have greater hydrogen bond dissociation energies because of the higher electrostatic

energies. In biological systems hydrogen bonds are particularly important because they are stable enough to provide significant binding energy, but weak enough to allow rapid dissociation.

Van der Waals or dispersion energies depend on the induction of a dipole and so polarisability is an important determinant of the strength of the interaction between atoms. Typical dispersion energies for pairs of atoms at the optimal distance of contact are for example: 0.0186 kcal mol<sup>-1</sup> for the H...H interaction; 0.1366 kcal mol<sup>-1</sup> for N...N; and 0.0679 kcal mol<sup>-1</sup> for C...C.<sup>3</sup> Although the attractive forces are weak and the van der Waals energies low, because they are additive they can make a significant contribution to binding when summed over the whole molecule. Specificity arises when there is an opportunity to make a large number of van der Waals interactions simultaneously.

Hydrophobic interactions are a major driving force in the binding of substrates to enzymes as well as protein folding and stability.<sup>4</sup> The hydrophobic bond can be described as the tendency for non-polar compounds to transfer from aqueous solution to a non polar phase. The presence of a hydrophobic compound in an aqueous environment results in the reorganization of the local hydrogen bonding network, thus resulting in the loss of entropy of solvent water. The association of hydrophobic residues is driven by the release of some of the orientated water molecules around the separate molecules or groups, which results in an increased entropy from the enhanced freedom of released water molecules. One method of measuring relative hydrophobicity of a molecule is to measure its partition between two phases, *n*-octanol and water being a commonly chosen system. It was found from partition experiments of a large number of different compounds that many substituents make constant, and additive contribution to the hydrophobicity of the parent compound. The hydrophobicity constant  $\pi$ , is defined by [1.2]:

$$\pi = \log P/P_o \quad \dots[1.2]$$

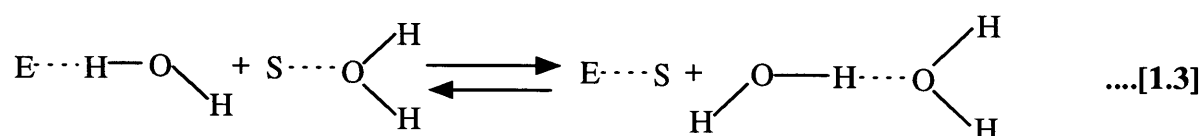
where  $P_o$  is the ratio of the solubility of the parent compound (H-S) in the organic phase to that in the aqueous phase and  $P$  is that of the substituted compound (R-S). Typical values

range from  $0.68 \text{ kcal mol}^{-1}$  ( $-\text{CH}_3$ ) to  $3.59 \text{ kcal mol}^{-1}$  ( $-\text{CH}_2\text{Ph}$ ) and negative values for polar groups  $-1.58$  ( $\text{OH}$ ).<sup>5</sup>

### 1.1.2 Binding energies of enzymes and substrates

There are problems in estimating the contribution of the different interactions to the overall binding energy of a substrate and enzyme by direct measurements outlined above because the binding process is an exchange reaction, the substrate exchanges its solvation shell of water for the binding interactions with the enzyme active site. Therefore, the problem of predicting values of binding energies in biological systems is that they represent the differences in energy between molecules interacting with water, and molecules in a complex interacting with each other.

Evaluation of the energy of electrostatic interactions is difficult because the ions involved are solvated in solution therefore their electrostatic interactions are reduced. The heterogeneity of the interior of proteins makes evaluation of the strength of the electrostatic interactions difficult since it varies markedly with the dielectric constant. The microenvironment of proteins can range from very polar near charged groups or dipoles to being non polar near hydrocarbon chains. In general solvation energies are very high, it has been estimated that a  $-\text{CO}_2^-$  ion is stabilised by about  $65 \text{ kcal mol}^{-1}$ ,<sup>3</sup> and this must be compensated for if removal from water is associated with complex formation. Similarly, it is difficult to evaluate the net energy of hydrogen bonding since the substrate (S) and the enzyme (E) are normally hydrogen bonded to water molecules in aqueous solution, and the overall energetics result from the interchange of hydrogen bonding partners [1.3].



There is a distinction between the overall energetics of the reaction and the hydrogen bond

dissociation energy. The overall energetics of the reaction depend on the relative strengths of the individual hydrogen bonds and the entropy changes that occur in both the solvent and reagents. A hydrogen bond inventory<sup>6</sup> can provide a great deal of insight into the net energetics of hydrogen bonding in water. From [1.3] it can be seen that there is no net gain in the number of hydrogen bonds. If there are no geometric constraints on bond formation and each hydrogen bond takes up its optimal conformation then to the first approximation the reaction is isoenthalpic.<sup>7</sup> The enthalpies tend to cancel out even if the bonds are of different types. Hydrogen bond formation in enzyme-substrate interactions is favoured entropically because there is a gain in entropy on the release of bound ordered water molecules into bulk water.

The evaluation of hydrophobic interactions is also difficult due to the basic differences between the hydrophobic interaction of a solute between an organic solvent and water, and the interactions which are formed by the binding of hydrophobic substrates to an enzyme. The former case involves the formation of a cavity in the organic solvent, transfer of the solute to the cavity and closure of the cavity in the aqueous phase, whereas the latter involves the occupation of a preformed cavity in the protein and the likely transfer of water from this to the aqueous phase.

### **1.1.3 Estimating binding energies from kinetics**

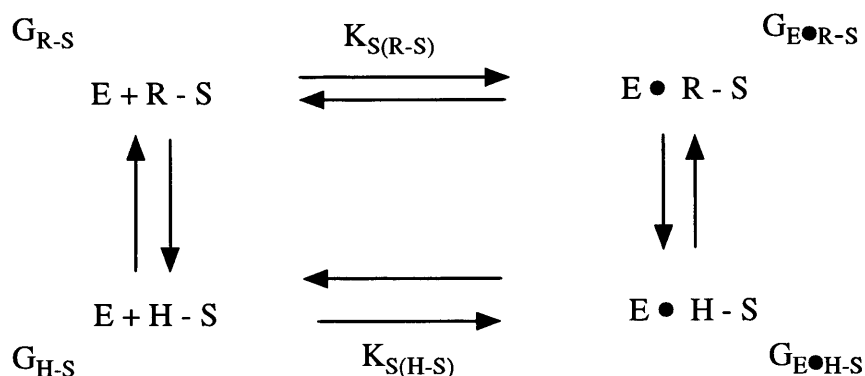
As mentioned earlier there are many difficulties in evaluating the binding energies of enzyme and substrates. Instead, the interaction energies of individual side chains within an enzyme-substrate complex can be quantified by a direct comparison of dissociation constants for the modified and unmodified complex. An apparent binding energy  $\Delta G_{app}$  is obtained.<sup>8</sup> This applies equally to modified substrates binding to a single enzyme or to single substrates binding to enzymes modified by site directed mutagenesis. In order to measure the contribution to the binding energy of a group R on a ligand R-S it is best to replace R with a



hydrogen and to compare the affinities of R-S and H-S. In the case of modified substrates the apparent binding energy of a substituent R in a substrate R-S is given by [1.4], where  $K_{s(H-S)}$  and  $K_{s(R-S)}$  are the dissociation constants from the enzyme of the modified substrate and the unmodified substrate respectively.

$$\Delta G_{app} = RT \ln (K_{s(R-S)}/K_{s(H-S)}) \quad \dots[1.4]$$

$\Delta G_{app}$  can be related in a formal manner to the energies of the specific bonds in the complex which have been altered by mutation by setting up a thermodynamic cycle (**Figure 1.1**).<sup>9</sup>



**FIGURE 1.1**

Thermodynamic cycle relating differences in dissociation constants to differences in free energies of wild type and mutant enzymes and their enzyme-substrate complexes.

If the free energy of each complex is denoted by G, then

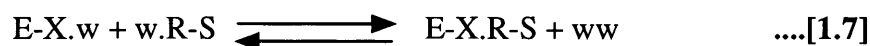
$$\Delta G_{app} = (G_{H-S} - G_{R-S}) - (G_{E\bullet H-S} - G_{E\bullet R-S}) \quad \dots[1.5]$$

It is seen from [1.5] that  $\Delta G_{app}$  depends upon the difference in free energy between E•R-S and E•H-S and those between R-S and H-S. Most energy terms cancel out, including those from the covalent modification to the substrate.  $\Delta G_{app}$  thus results from the dissociation energies of the specific bonds between enzyme and substrate that are changed and the energetic changes associated with any structural reorganization of the solvent and ligand on modification. Since the binding process is an exchange reaction between water and the

binding site  $\Delta G$  can be expressed as

$$\Delta G_{\text{app}} = (G_{\text{H-S/W}} - G_{\text{R-S/W}}) - (G_{\text{E}\bullet\text{H-S}} - G_{\text{E}\bullet\text{R-S}}) + \Delta G_{\text{reorg}} \quad \dots[1.6]$$

where  $G_{\text{H-S/W}}$  and  $G_{\text{R-S/W}}$  are the energies of interaction between modified substrate and water and that between substrate and water respectively.  $\Delta G_{\text{reorg}}$  is the reorganisational energy term which includes any perturbations to the binding of the rest of the substrate to the enzyme, and changes to the solvent arising from the modification of substrate as well as any conformational changes.  $\Delta G_{\text{app}}$  therefore results from the dissociation energies of the specific bonds between enzyme and substrate that are changed and the energetic changes associated with any structural reorganisation of the solvent and modified ligand. To conclude  $\Delta G_{\text{app}}$  is an experimental measurement of specificity of binding since it is derived from relative binding constants. On the other hand the energy term  $\Delta G_{\text{bind}}$  can be viewed as the incremental binding energy in the E-S complex.<sup>9</sup> If a group R on a substrate (S) binds to a group (X) on an enzyme then the exchange reaction in which they exchange their interactions with water to interact with each other is shown in [1.7] where w represents water.



The binding energy of X and R,  $\Delta G_{\text{bind}}$ , may be considered as a free energy of transfer of X and R from water to their binding environment. The overall energetics of the interaction, as shown in [1.8], depend on the relative individual interaction energies in [1.7] and also on any changes in entropy and energetics of solvent structure that occur.

$$\Delta G_{\text{bind}} = G_{\text{X.R}} + G_{\text{w}} - G_{\text{X.w}} - G_{\text{R.w}} \quad \dots[1.8]$$

The interaction energy of X and R is  $G_{\text{X.R}}$ , of X and w is  $G_{\text{X.w}}$ , of R and w is  $G_{\text{R.w}}$ , and the free energy of water entering bulk water and any associated changes in the free energy of the solvent is  $G_{\text{w}}$ . The value of  $\Delta G_{\text{bind}}$  depends on the fit between X and R. When there is perfect complementarity between the structures of X and R and there is no strain or undue loss of entropy on binding then the binding energy will be the maximum that can be

achieved. In this case  $\Delta G_{\text{bind}}$  tends to the intrinsic binding energy value.<sup>10</sup>  $\Delta G_{\text{bind}}$  relates the binding energy of the enzyme and R with each other compared with their binding energies to water, whereas  $\Delta G_{\text{app}}$  compares the binding energy of the enzyme and modified substrate with that of enzyme and unaltered substrate. Therefore, in general  $\Delta G_{\text{app}}$  does not equal  $\Delta G_{\text{bind}}$ . However, if the removal of a group on a substrate or enzyme allows free access of water to the cavity created by the deletion then  $\Delta G_{\text{app}}$  can be considered as a crude measure of  $\Delta G_{\text{bind}}$ .<sup>9</sup>

## **1.2 Studying molecular recognition**

In order to understand the recognition process between an enzyme and its substrate one must investigate the roles of individual amino acid residues. Classic methodologies involving the use of active site directed reagents and group specific reagents have provided a useful tool for identifying residues potentially involved in binding and catalysis, especially where the crystal structure is not available.<sup>11-14</sup> However these reagents do not address the problem of estimating non-covalent interactions between molecules. The two techniques that have been successfully employed in the study of molecular recognition are the complimentary approaches of site directed mutagenesis and the use of substrate analogues which are both discussed below.

### **1.2.1 Site directed mutagenesis**

Site directed mutagenesis particularly in conjunction with a high resolution crystal structure is a more definitive method of assigning the role of specific residues. Its advantage over covalent modification is that it can also remove a functional group and reduce the size of a residue therefore reducing ambiguity due to the introduction of steric effects. Site directed mutagenesis has the potential to allow alteration of specific amino acid side chains thus

directly probing the effects of individual residues on both binding and catalysis. The ability to substitute individual amino acid residues within a protein by any of the other naturally occurring amino acids has proved to be a powerful tool in enzymology. Site directed mutagenesis and its applications to understanding enzyme mechanisms has been reviewed extensively (Fersht, Shaw etc).<sup>8,9,15</sup> This technique has for example provided a framework for understanding the contribution of hydrogen bonding to enzyme-ligand interactions. Typically neutral hydrogen bonds fall in the range 0-1.5 kcal mol<sup>-1</sup> whereas charged hydrogen bonds tend to be larger (3-6 kcal mol<sup>-1</sup>). The extensive studies by Fersht on tRNA synthetase have been particularly important in establishing these values.<sup>6,8</sup>

Site directed mutagenesis has also been used to probe the role of catalytic groups. A particularly impressive example of this is provided by the studies of Knowles *et al* on triose phosphate isomerase.<sup>16</sup> The general base Glu 165 was replaced by an Asp residue which increases the distance between the proton on the substrate and the carboxylate by removing a methylene group from the general base. Knowles showed that although binding of the substrate is largely unaffected  $k_{cat}$  is reduced by three orders of magnitude.

However, there are limitations to the technique, at present mutagenesis routinely only allows substitution within the twenty natural amino acids specified by the genetic code. Experience in the field has shown that subtractive changes to amino acid side chains offer the most reliable mutations. Substitution of other functional groups (Asp to Asn, Glu to Gln) introduces the potential for unforeseen structural changes due to the establishment of aberrant interactions. A further disadvantage is that interactions of ligands with backbone functional groups cannot be investigated by this method. Despite the limitations, the great advantage of the technique is the ability to change the structure of a protein in a defined manner which may bring nearer the goal of redesigning enzymes with different specificities.

### 1.2.2 Substrate analogues

In contrast to site directed mutagenesis of enzymes, alteration of substrates gives a much wider range of chemically defined alterations to the interaction of enzyme and ligand and provides access to the same energetic data. It is a technique that has been widely and successfully used to study structure-activity relationships for example by Walsh in his attempt to study binding of acetyl CoA by thiolase<sup>17</sup> as well as Bartlett in his attempt to evaluate the influence of hydrogen bonding and hydrophobic effects on the inhibition of thermolysin, a zinc protease<sup>18</sup> (see **Chapter 3** for fuller discussion of substrate analogues). The use of modified ligands has several advantages over genetic manipulation, (i) variety of modifications are not limited to the side chains of the twenty naturally occurring amino acids, (ii) there is no disruption of either protein structure and stability as there can be with amino acid mutation, (iii) the ability to study interactions between ligand and amide backbone. Despite these advantages, there are limitations, often target compounds are not trivial to synthesise and paradoxically site-directed mutagenesis may be more easily accomplished.

### 1.2.3 Selecting a model system

The tools to dissect protein ligand interactions have now been identified, the next step is to select a model system to study. The enzyme chloramphenicol acetyl transferase (CAT), the enzymic effector of bacterial resistance to the antibiotic chloramphenicol in prokaryotic organisms is an attractive candidate for study for several reasons: CAT can be readily extracted and purified from *E. coli* in large amounts and can be easily stored without loss of activity. The type III variant (CAT<sub>III</sub>) has been successfully crystallized and the structures of the apoenzyme as well as the binary complexes with both substrates have been solved. The reaction catalysed by the enzyme is a relatively simple acetylation and both the substrates are amenable to changes in both functionality and stereochemistry. The enzyme is currently being exploited as a model for the development of techniques and strategies in protein

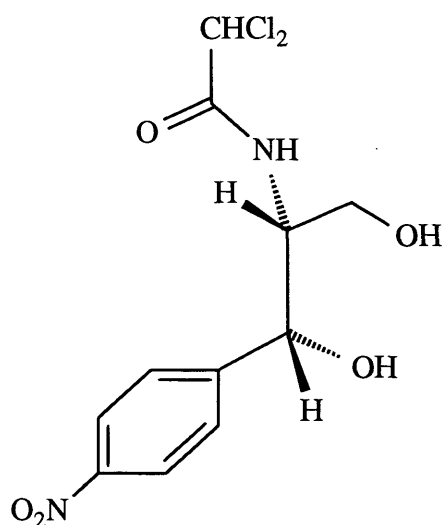
engineering (W.V. Shaw, Leicester). Much work has been done to investigate the mechanism of the catalysed reaction,<sup>19</sup> structure of the active site<sup>20</sup> as well as the interactions involved in binding the two substrates by the use of both site directed mutagenesis<sup>21,22</sup> and modified ligands.<sup>23,24</sup>

The work described in this thesis makes use of substrate analogues to investigate substrate binding by CAT in particular with the substrate acetyl CoA.

## BACKGROUND

### 1.3 Chloramphenicol: Structure and mode of action

Chloramphenicol was first isolated from *Streptomyces venezuelae* in 1947 and was identified as the antibiotic produced by this organism.<sup>25</sup> Characterisation of chloramphenicol revealed that it possessed two asymmetric carbon centres in the propanediol side chain giving rise to four possible stereoisomers, however, only the naturally occurring compound, 1R, 2R, 1-(4-nitrophenyl)-2-(N-dichloroacetamido)-1,3-propanediol has significant antimicrobial activity (**Figure 1.2**).<sup>26</sup>



**Figure 1.2**

The stereochemistry of the propanediol side chain is [1 (R), 2 (R)]

Chloramphenicol is uncharged at physiological pH, reasonably soluble in aqueous solution, and as a result of its amphiphilic nature can readily pass through biological membranes. Coupled with relatively simple chemical synthesis and a broad spectrum of sensitivity among pathogenic bacteria, chloramphenicol appeared an ideal antibiotic and received much clinical use. However, its initial wide spread use has now been severely restricted due to the identification of a number of potentially life threatening side-effects including bone marrow toxicity and adverse reactions in new born infants. The drug is currently used in veterinary practice but in man only to treat certain serious infections such as typhoid and meningitis.

The antibacterial effect of chloramphenicol is due to inhibition of protein synthesis.<sup>27</sup> In 1964 Traut and Monro demonstrated that chloramphenicol inhibits the peptide-bond forming reaction.<sup>28</sup> Chloramphenicol inhibits peptidyl transferase reactions in the absence of messenger RNA (mRNA) or 30S ribosomal units, but it does not prevent the binding of mRNA to ribosomes nor does it inhibit the non-enzymic binding of aminoacyl-tRNA into the ribosomal aminoacyl acceptor site (A site). It is thought that chloramphenicol allows the binding of the latter into the A site but prevents recognition by the peptidyl transferase of its acceptor substrate, but it is not clear how chloramphenicol achieves this. Chloramphenicol inhibits peptide chain elongation and with it the movement of ribosomes along mRNA.<sup>29</sup>

Chloramphenicol binds preferentially to the 50S subunit of ribosomes.<sup>30</sup> At bacteriostatic concentrations one molecule of chloramphenicol binds per 70S ribosome, however, at high drug concentrations another molecule can bind. Ribosomes of *E. coli* possess a high affinity site ( $K_d = 2 \mu\text{M}$ ) together with a low affinity site ( $K_d = 200 \mu\text{M}$ ) evident only at high drug concentration. The low affinity site is located on the 30S subunit but it is not known whether it is involved in the primary action of the drug. The high affinity site is located on the 50S subunit and it has been suggested that chloramphenicol binds with a slightly higher affinity to ribosomes carrying peptidyl-tRNA in the peptidyl acceptor site (P site) compared with the A site.<sup>31</sup> One of the major concerns is to establish which ribosomal proteins constitute the

peptidyl transferase and which are present in the A and P sites when the enzyme recognises its acceptor and donor substrates. Affinity labelling studies identified several ribosomal proteins in the vicinity of the peptidyl transferase centre.<sup>32</sup> In addition to ribosomal proteins, several nucleotide residues of 23S RNA have been implicated as components of the chloramphenicol binding site.<sup>33</sup> Recent evidence suggests that there is a specific interaction between rRNA and chloramphenicol within the peptidyl transferase centre of the 50S subunit.<sup>34</sup>

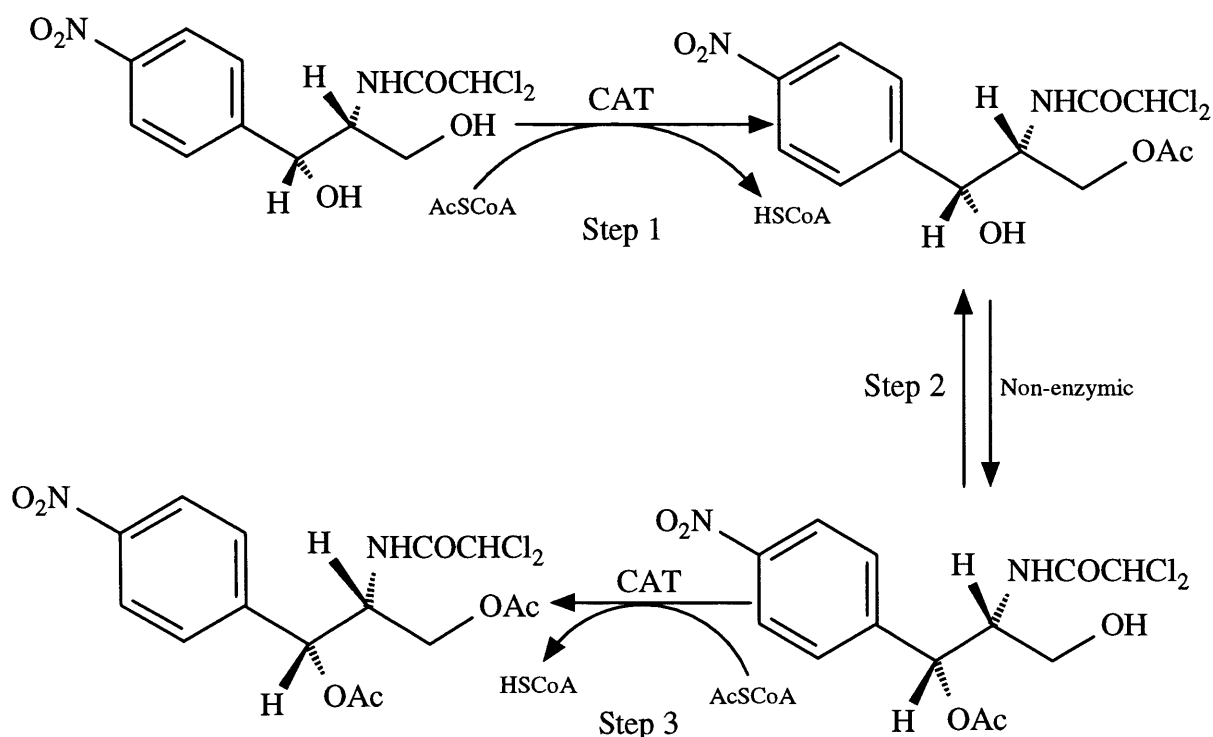
### **1.3.1 Bacterial resistance to chloramphenicol**

Soon after its introduction into clinical practice bacterial strains resistant to chloramphenicol began to emerge. Since detection in Japan in the 1950's the problem of bacterial resistance has reached huge proportions. The general trend is that when a new antibiotic is introduced there is a short period during which it is effective on a large scale, however this is followed by the appearance of a higher and higher proportion of resistant strains. This can reach a point where an organism has developed absolute resistance. Most high level resistance ( $>100 \mu\text{g ml}^{-1}$ )<sup>35</sup> to chloramphenicol is due to enzymic acetylation which is discussed in the next section. Although acetylation by CAT is the most common mode of resistance to chloramphenicol other mechanisms have been identified including exclusion from the cell,<sup>36</sup> inactivation by reduction of the nitro group,<sup>37</sup> and mutation of a ribosomal protein.<sup>38</sup>

### **1.4 Mechanism of the CAT reaction**

When bacteria containing CAT are exposed to chloramphenicol the antibiotic is converted to three acetylated products by a combination of two enzymic steps and a non enzymic isomerisation (**Scheme 1.1**).<sup>39,40</sup>





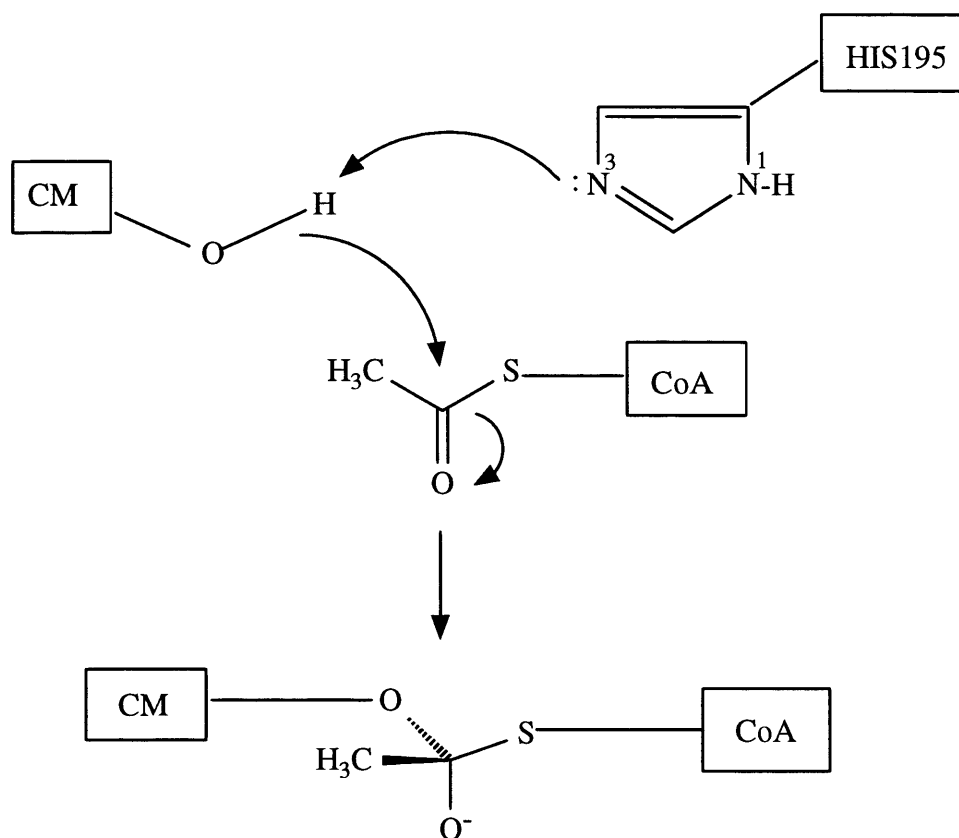
**Scheme 1.1**

#### Reactions catalysed by chloramphenicol acetyltransferase

The initial reaction catalysed by CAT (Step 1) is the acetyl CoA-dependent acetylation of the C-3 hydroxyl of chloramphenicol. The 3-*O*-acetyl chloramphenicol does not inhibit protein synthesis and neither does the product of the next step, a non enzymic and pH-dependent rearrangement (step 2) involving an intramolecular acyl transfer to the secondary (C-1) hydroxyl. The product 1-*O*-acetyl chloramphenicol can then undergo a further acetylation reaction catalysed by CAT to form 1,3-diacetyl chloramphenicol (Step 3). Of these reactions it is the first step which is of the most interest since it is sufficient to confer bacterial resistance, the second and third reactions merely result from the 1,3-diol structure of chloramphenicol and the acyl migration that occurs *in vivo* and *in vitro*. The overall rate of formation of 1,3-diacetylchloramphenicol is a function of both the rate of the slow rearrangement and the reacetylation, the latter being at least two orders of magnitude slower than the initial acetylation.<sup>41</sup> The remaining discussion on the mechanism of the CAT reaction is concerned with step 1 only. This step is freely reversible but the forward reaction

is highly favoured on energetic grounds due to the difference in free energy between acyl thioesters and oxyesters.<sup>42</sup>

The results of steady state kinetics of the forward and reverse CAT catalysed reaction suggested a rapid equilibrium mechanism leading to the formation of a ternary complex.<sup>19</sup> Although the addition of substrates in both the forward and reverse reactions is random there is a preference for AcCoA as the leading substrate in the forward reaction. Chemical modification studies were undertaken to identify which amino acid residues are important to catalysis. The affinity reagent 3-bromoacetylchloramphenicol, an analogue of the acetylated product of the forward reaction, was found to alkylate uniquely a histidine residue, His 195.<sup>43</sup> Inactivation was stoichiometric, 1 mole of inhibitor being covalently bound per mole of enzyme monomer, with the consequent loss of acetylation and hydrolytic activity associated with CAT. The modification of histidine in proteins often shows preference for one of the two tautomeric forms.<sup>44</sup> In the case of CAT the *N*<sup>3</sup>-(carboxymethyl) histidine was identified as the only alkylated amino acid, implicating the presence of a unique tautomeric form of a reactive imidazole group at the catalytic centre. As it was only the *N*-3 of the imidazole ring that was alkylated this suggests that either the imidazole ring is fixed such that only the *N*-3 nucleophilic centre is available for catalysis or that one of the two ring tautomers of His 195 is favoured on electronic grounds. X-ray crystallography studies showed that tautomeric stabilisation of the imidazole of His 195 is achieved by the presence of a novel hydrogen bond from the *N*-1 hydrogen of His 195 to the backbone carbonyl of the same residue.<sup>20</sup> The results of both kinetic and chemical modification studies led to the proposed mechanism shown in **Scheme 1.2** where the role of His 195 is one of general base.<sup>43</sup>



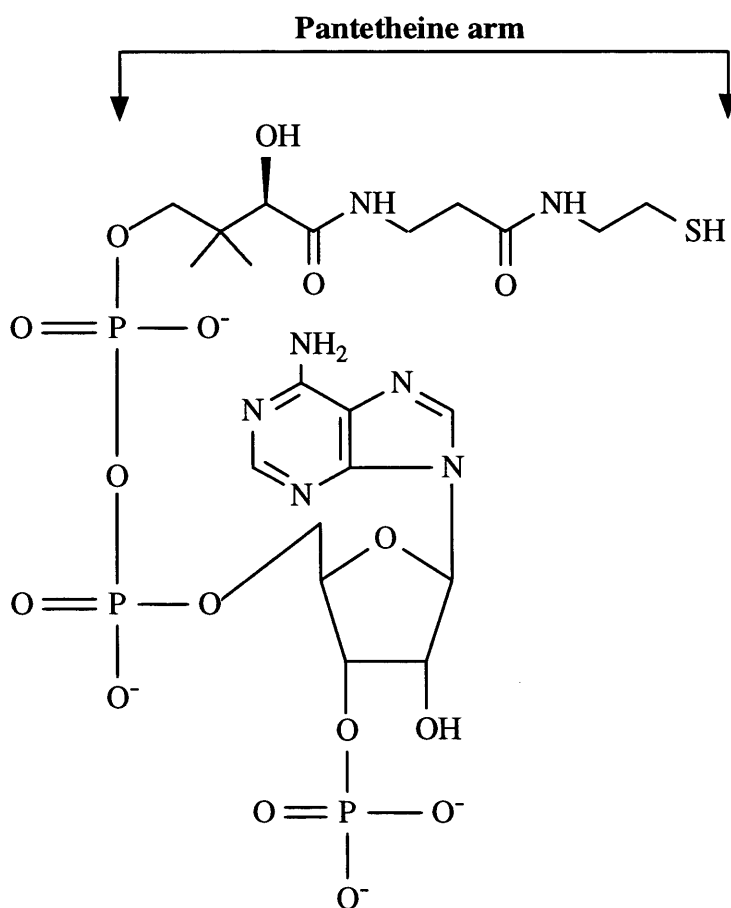
**Scheme 1.2**

Proposed reaction mechanism for CAT leading to the formation of an oxyanion tetrahedral intermediate.

His 195 increases the nucleophilicity of the primary hydroxyl group by deprotonating the C-3 hydroxyl of chloramphenicol and promoting the attack of its oxygen on the thioester carbonyl of CoA. This leads to the formation of an oxyanion tetrahedral intermediate. The availability of the structures of the two binary complexes has allowed the proposed tetrahedral intermediate to be modelled with only very small changes in either conformation of the enzyme or the position of the substrates.<sup>45</sup>

## 1.5 Coenzyme A

CoA, the cofactor in the CAT reaction is a central molecule in metabolism. It is widespread in nature and is involved in many reactions. These include, fatty acid synthesis, poly- $\beta$ -hydroxybutyrate synthesis, and the citrate synthase reaction where it condenses with oxaloacetate and water to form citrate. It was first discovered by Lipmann in 1945 as a cofactor required in many enzyme-catalysed acetylations and its structure was determined several years later (**Figure 1.3**).



Structure of coenzyme A (CoA)

**Figure 1.3**

As can be seen from the structure, CoA is composed of a highly functionalised pantetheine chain linked to an adenine nucleotide *via* a pyrophosphate bond. The terminal thiol group is

the reactive site and acyl groups are linked by a thioester bond. The  $\Delta G'$  for hydrolysis of acetyl CoA is  $-7.5 \text{ kcal mol}^{-1}$  giving it a high acetyl group transfer potential. The acetyl transfer reaction mechanism is relatively well understood in chemical terms. However little is known about the roles or significance of the other functionalities of acetyl CoA such as the adenine, ribose, phosphate or pantetheine moieties in terms of the enzyme catalysed reactions.

Over recent years both site directed mutagenesis and substrate analogues have been used to the study binding of CoA in a variety of enzyme systems (see **Chapter 3**). A particularly relevant example is that of choline acetyl transferase which catalyses the alcoholysis of acetyl CoA by choline to form the essential neurotransmitter acetyl choline.<sup>46</sup> Mautner *et al* found that the substrate analogue acetyl 3'-dephospho CoA bound with a  $K_M$  of  $820 \mu\text{M}$  compared to that of  $47 \mu\text{M}$  for acetyl CoA. This 17-fold increase in the  $K_M$  clearly suggests that the 3'-phosphate group plays an important role in the binding of acetyl CoA.

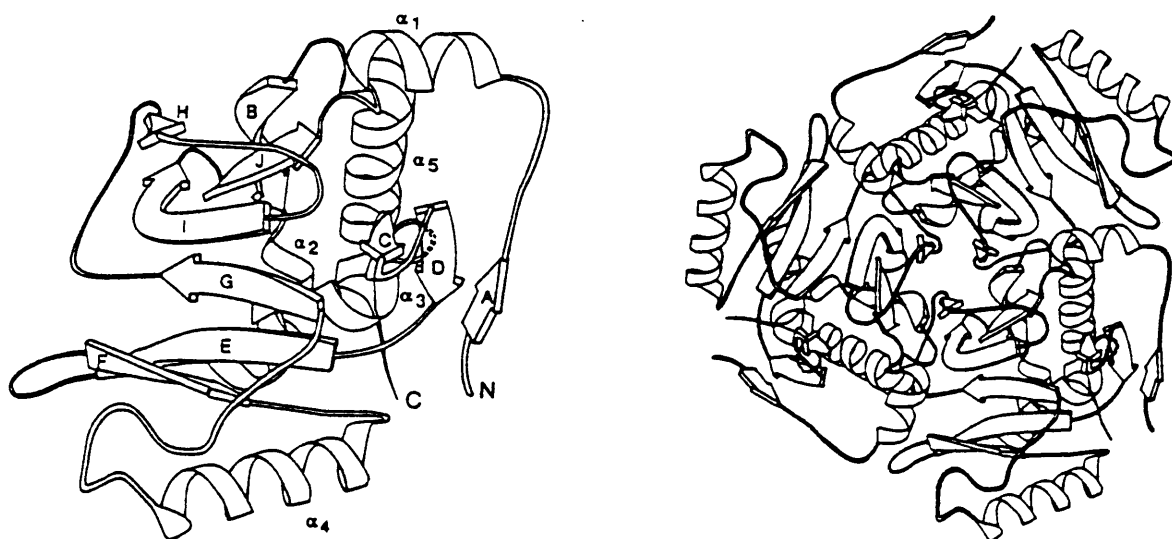
Similarly, Walsh in his studies on thiolase found that truncated CoA analogues in which the adenosine and phosphate moieties had been replaced by a trimethylacetyl group were reasonably good substrates.<sup>17</sup> As they were synthetically easier targets they were useful in probing the elements of enzymic recognition of the pantetheine portion of the substrate (see **Section 2.4**).

## 1.6 Structure of CAT

Of the eleven natural variants of CAT for which amino acid sequence data are available the type III enzyme, CAT<sub>III</sub> is the one that has been studied in greatest detail, both structurally and mechanistically.<sup>41,47</sup> CAT<sub>III</sub> is the most active of all the variants and is the variant of the enzyme discussed below and that forms the focus of this thesis.

Hydrodynamic studies showed CAT to be a trimer of identical subunits, each of mass 25,000. The active sites are located at each of the three subunit interfaces.<sup>20</sup> The dominant feature of the structure of the monomer is a six stranded predominantly antiparallel  $\beta$ -pleated sheet with

five  $\alpha$ -helices packed against one side and the end of it (**Figure 1.4**).<sup>20</sup>



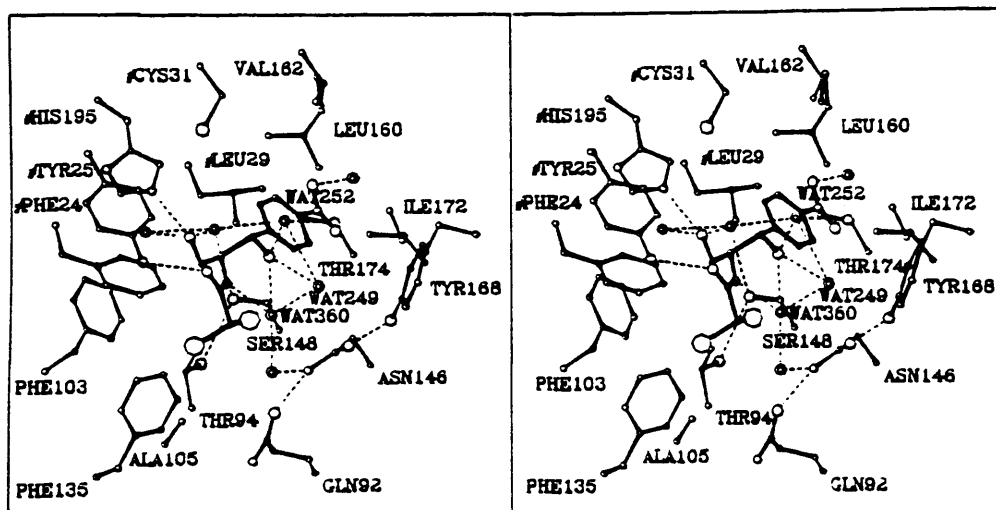
**Figure 1.4**

Structure of CAT showing the secondary structural elements of the monomer left and the trimer viewed (as the monomer) down the three fold axis.

There is an additional small three stranded  $\beta$  sheet which is formed by the N-terminus and an extended loop between helix  $\alpha_3$  and strand  $\beta_E$ . The three subunits associate so that the six stranded  $\beta$  sheet of each monomer extends across the subunit interface via the strand  $\beta_H$  of the adjacent subunit to form the trimer (**Figure 1.4**). This gives rise to eight intersubunit main chain hydrogen bonds which contribute to the stability of the trimer. The monomers associate to form a compact disc shaped trimer approximately 65 Å in diameter and 40 Å thick. On formation of the trimer about 20% of the total surface area of each monomer is buried. Hydrophobic residues make up approximately half of the buried area and there are eight charged polar side chains at the surface. Also important in stabilisation of the trimer are interactions provided by solvent molecules, five of which are buried.

### 1.6.1 The chloramphenicol binding site

The structure of the binary complex of CAT with chloramphenicol has been refined to 1.75 Å resolution.<sup>20</sup> Chloramphenicol binds in a deep pocket which is situated at the subunit interface. The high resolution structure of the binary complex shows that most of the residues forming the pocket are found on one of the two subunits forming the interface while the catalytically important His 195 is contributed by the other subunit (**Figure 1.5**). It is clear therefore, that a single monomer alone cannot be catalytically active.



**Figure 1.5**

Stereo diagram of the CM binding pocket. # indicates residues which arise from the subunit providing the His 195. Double circles represent ordered water molecules.<sup>20</sup>

Both hydrophobic and polar residues are involved in binding chloramphenicol, reflecting the amphiphilic nature of the antibiotic, and there are extensive van der Waals contacts between chloramphenicol and the side chains of the pocket. The phenyl ring of chloramphenicol packs against side chains of Leu 29, Cys 31, Leu 160, and Ile 172 and the dichloromethyl group makes van der Waals contact with Ala 105, Phe 135, and Asn 146, while the hydroxyls and acetamido groups form hydrogen bonds to ordered water molecules located in the polar side of the pocket. There are two direct hydrogen bonds between the enzyme and chloramphenicol; one between the acetamido oxygen and the side chain hydroxyl of Tyr 25,

the other between the primary (C-3) hydroxyl of chloramphenicol and the imidazole N-3 of His 195. Cullis *et al* showed that the latter contributed 2.8 kcal mol<sup>-1</sup> to binding.<sup>23</sup> This value is considerably larger than typical values for neutral hydrogen bonds in proteins which fall into the range 0.5-1.5 kcal mol<sup>-1</sup>. However, the hydrogen bond is located deep within a hydrophobic cavity which is not in contact with bulk solvent, it is therefore expected to be stronger than a hydrogen bond in a more polar environment. A third hydrogen bond exists between the C-1 hydroxyl of chloramphenicol and Thr 174 but this occurs via a bridging water molecule.

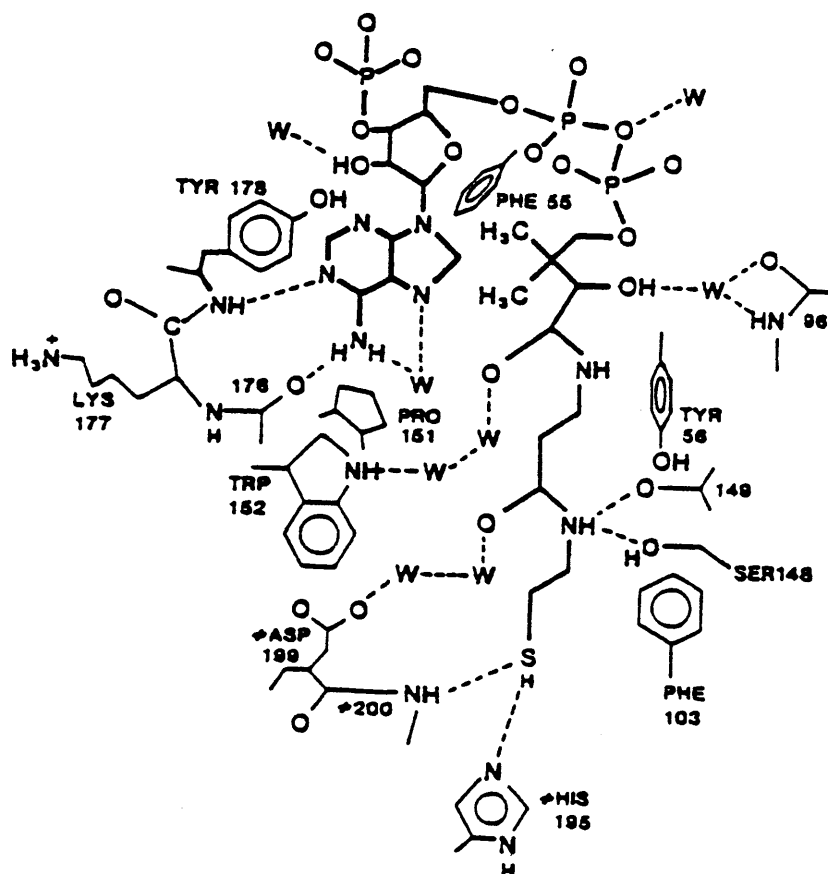
Both site directed mutagenesis and modified ligands were used to study interactions at the 1-hydroxyl substituent of the substrate within the enzyme.<sup>21</sup> The analogue 1-deoxychloramphenicol was found to be a good substrate for CAT. By a comparison with chloramphenicol the interaction between the 1-hydroxyl of chloramphenicol and the cavity that accommodates it was found to account for 0.8 kcal mol<sup>-1</sup> of binding energy. There are two water molecules associated with the primary hydroxyl group of chloramphenicol and three with the secondary hydroxyl, some or all of which are presumably displaced during the CAT reaction. The extensive hydrophobic interactions in conjunction with the hydrogen bonds reflect the amphipathic nature of chloramphenicol.

### 1.6.2 The CoA binding site

The structure of the binary complex of CAT with CoA, the product of the forward reaction, has been solved at 2.4 Å resolution.<sup>48</sup> It has been shown that binding of chloramphenicol completely blocks access to the active site from the "top" surface of the trimer. Kinetic studies showed that the substrates of CAT bind independently suggesting that CoA must gain access to the active site via a different route to that of chloramphenicol. In the refined structure of the CAT-CM complex, a tunnel leads from the active site histidine out to the lower surface of the enzyme. Apart from a short spur, this tunnel is approximately cylindrical in shape, 3-4 Å in diameter and 12 Å in length and occupied by six ordered solvent



molecules.<sup>47</sup> Modelling studies suggested that the tunnel could readily accommodate the pantetheine arm of CoA in an extended conformation placing the adenosine and phosphate moieties on the external surface of the CAT trimer. The subsequent solution of the structure of the CAT/CoA complex confirmed that this "tunnel" is the CoA binding site (**Figure 1.6**).



**Figure 1.6**

Schematic diagram of the CoA binding site of CAT. # indicates residues which arise from the subunit providing His 195. W represents ordered water molecules.<sup>48</sup>

The tunnel is composed of predominantly hydrophobic residues (Phe 103, Tyr 56, Trp 152, Phe 96) that provide van der Waals interactions with the apolar groups of the substrate. These interactions presumably contribute significantly to the overall binding energy. The pantetheine arm binds between  $\beta_E$  and  $\beta_G$  and forms interactions with several residues from these  $\beta$ -strands. Of the several hydrogen bonds to pantetheine only 3 do not involve bridging water molecules (Gly 200 amide  $\rightarrow$  S81, His 195 N3  $\rightarrow$  S81-H and Ser 148 hydroxyl  $\rightarrow$

N71-H).(The numbering system eg S81 refers to CoA and is the one employed by Remington *et al*<sup>49</sup>, see **Appendix 1**).

The adenosine moiety of CoA binds in a well defined pocket close to the protein surface. The binding surface is composed predominantly of hydrophobic residues (Tyr 178, Phe 55 and Pro 151) as well as the main chain atoms of residues 176→178 (**Figure 1.6**). Pro 151 is conserved in all 11 known CAT sequences and Tyr 178 is an aromatic amino acid in 10 of 11 known sequences, Phe 55 is not conserved. The *gem* dimethyl groups of the pantetheine arm form a major part of the adenine binding pocket by forming van der Waals contacts with the adenine ring. N1 and N10 of the adenine ring form hydrogen bonds with the main chain amide of residue 178 and the main chain carbonyl oxygen of residue 176 respectively. Both the ribose ring and the 3'-phosphate group are solvent accessible and form no major interactions with the enzyme although the 2'-hydroxyl group of the ribose ring forms a hydrogen bond via an ordered water molecule to the main chain amide of residue 55.

Day<sup>22</sup> in an attempt to discern the importance of the hydrophobic effect in the binding of CoA to CAT looked at the effect of substituting two highly conserved residues (Tyr 56 and Tyr 178) with less hydrophobic side chains. Deletion of the hydroxyl group of Tyr 56 (Phe 56 CAT) had only a modest effect on the steady state parameters of CAT. In fact binding of acetyl CoA to both free enzyme ( $K_d$ ) and the CAT-chloramphenicol complex ( $K_M$ ) was slightly enhanced in keeping the role of hydrophobicity in the interaction between acetyl CoA and Tyr 56, while  $k_{cat}$  was reduced by only 40%. Thus, although Tyr 56 is conserved in all known CAT variants its hydroxyl does not appear to have a major role in acetyl CoA binding.

As expected, decreasing the hydrophobicity of residue 56 by means of subtractive substitutions weakened acetyl CoA binding and reduced  $k_{cat}$ . Removal of the entire hydroxyphenyl group (Tyr 56→Ala) resulted in modest changes in both  $K_M$  and  $k_{cat}$ , with a corresponding decrease in transition-state stabilisation of 1.6 kcal mol<sup>-1</sup>. The introduction of a charged group had a profound effect on both binding and catalysis. Lys 56 bound acetyl CoA 28-fold less than the wild-type enzyme and catalysed the acetylation of chloramphenicol

over 140-fold more slowly. Transition state stabilisation was reduced by almost 5 kcal mol<sup>-1</sup> and the apparent free energy of binding acetyl CoA was reduced by 2 kcal mol<sup>-1</sup>. The data for the mutant protein Thr 56 did not fit the general trend seen for the other substitutions. Although the hydrophobicity of the side chain of residue 56 was greatly reduced in Thr 56 CAT compared to wild type enzyme, the affinity for acetyl CoA was enhanced. Modelling studies suggested that the  $\beta$ -hydroxyl of Thr 56 may form novel interactions with the bound acetyl CoA which could be sufficiently strong to compensate for the decrease in local hydrophobicity of the CoA binding site.

Subtractive substitutions at Tyr 178 resulted in large increases in both the  $K_M$  and  $K_d$  for acetyl CoA, with only a small change in the  $k_{cat}$ . This was in contrast to the effects of equivalent substitutions at Tyr 56 which decreased binding affinity and catalysis by similar extent. The hydroxyl group of Tyr 178, which is not conserved made no significant contribution to CoA binding which is to be expected from the structure of the CAT-CoA binary complex. Subtraction of the hydroxyphenyl group (Ala 178 CAT) resulted in a 27-fold decrease in affinity for acetyl CoA with only a small decrease in  $k_{cat}$  which corresponded to a decrease in transition-state energy of 2.4 kcal mol<sup>-1</sup>.

As observed for the series of substitutions at Tyr 56, the changes in the kinetic parameters for substitutions at Tyr 178 were also related to the hydrophobicity of the residue side chain. Reducing the hydrophobicity of the side chain resulted in a progressive decrease in affinity for acetyl CoA, such that Thr 178 CAT had an affinity for acetyl CoA more than 30-fold lower than wild type CAT.

Many enzymes which bind anionic substrates or cofactors generally contain critical arginine residues in their ligand binding sites.<sup>50</sup> Surprisingly there are no such ion pairs between the charged pyrophosphate of CoA and the positively charged residues on the enzyme and neither are the pyrophosphate anions positioned at the positive poles of any  $\alpha$ -helices. However, studies (Day 90)<sup>24</sup> revealed that there are long range electrostatic interactions between CoA and the side chains of the highly conserved residues Lys 54 and Lys 177. The  $\epsilon$  amino group of Lys 54 which is conserved in 11 of the 12 known CAT sequences was shown

to contribute 1.3 kcal mol<sup>-1</sup> to the binding of acetyl CoA via interactions with 3' and 5' phosphoanions of CoA. Lys 177, which is a basic residue absolutely conserved in all known CAT sequences is part of a  $\beta$ -bulge which accommodates the adenine ring of CoA. It was shown to contribute only 0.4 kcal mol<sup>-1</sup> to the productive binding of acetyl CoA via long range interactions ( $\sim 14$  Å) with the 5'  $\alpha$  and  $\beta$  phosphoanions of CoA.

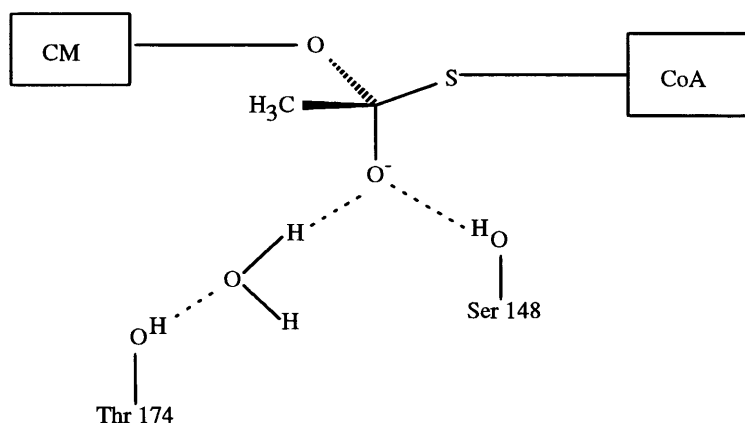
It is interesting to note that the combined energetic contribution of Lys 54 and Lys 177 to acetyl CoA binding (1.7 kcal mol<sup>-1</sup>) is less than that (2.4 kcal mol<sup>-1</sup>) for the simple hydrophobic interaction between Tyr 178 and the adenine ring of CoA.

The  $K_M$  for acetyl 3'-dephospho CoA is 671  $\mu$ m,<sup>99</sup> this is 7-fold greater than that for acetyl CoA (93  $\mu$ m) which implies that the 3'-phosphate group may be important for CoA binding. The role of the 3'-phosphate may be to favour the preferred conformation of CoA rather than serve as the anionic pole of an electrostatic effect. The latter role most probably falls either to one or both of the oxyanions of the phosphodiester backbone of CoA, this based on the differing inhibitory effects of ATP, 5'-AMP and 3'-AMP.<sup>24</sup>

Overall the contribution to transition state stabilisation of the 3'-phospho ADP moiety of CoA (compared to pantetheine) has been shown to be of the order of 6.2 kcal mol<sup>-1</sup>.<sup>24</sup> The conformation of bound CoA is similar to that observed for CoA bound to citrate synthase, the only other CoA binding enzyme to be crystallised.<sup>49</sup> The pantetheine arm binds in an extended conformation and the adenosine phosphate moiety adopts a folded conformation such that the adenine ring is in van der Waals contact with the *gem* dimethyl groups of the pantetheine arm. However, CoA bound to CAT adopts a much less folded conformation than that bound to citrate synthase and the characteristic internal hydrogen bonds between N7 and O52, O20 O51 (*via* a water molecule) are not present in CoA bound to CAT (numbering system of CoA is that used by Remington *et al*,<sup>49</sup> **Appendix 1**). The CoA binding sites of the two enzymes also differ quite significantly. In CAT there is no equivalent of the proposed "adenine recognition loop" and there are no ion pairs between the three phosphoanions of CoA and arginyl residues of the enzyme.

### 1.6.3 The catalytic centre of CAT<sub>III</sub>

The structure of the active site of CAT<sub>III</sub> shows that N-3 of His 195 is in hydrogen bonding distance from both 3'-hydroxyl of chloramphenicol (2.8 Å) and the thiol of CoASH (3.3 Å) and is thus ideally situated to deprotonate either group, either to promote the forward or reverse reactions, respectively. The N-1 of imidazole is suitably positioned to form a hydrogen bond with the main chain carbonyl oxygen of the same residue, which would provide the tautomeric stabilisation required for efficient catalysis. A search of the Brookhaven data bank revealed no other examples of this type of hydrogen bonding. Modelling of the proposed tetrahedral intermediate (**Scheme 1.2**) has shown that it is more readily accommodated than the ternary complex implying enzyme-transition state complementarity.<sup>45</sup> It is catalytically advantageous for the enzyme to be complementary to the structure of the transition state of the substrate rather than to the original structure. The increase in binding energy as the structure changes to that of the transition state lowers the activation energy of  $k_{\text{cat}}$  and so increases the rate of reaction. Modelling studies of the transition state (**Figure 1.7**) also suggested that the oxyanion of the tetrahedral intermediate could be stabilised by hydrogen bonding to a conserved serine (Ser 148).<sup>45</sup> The hydroxyl group of Ser 148 is 3.2 Å away from the putative oxyanion and so there is the possibility of hydrogen bonding between them. The same residue is 4.3 Å away from the C-3 hydroxyl of chloramphenicol and so is not likely to play a direct role in the acetyl transfer. Substitution of Ser 148 for Ala results in a 54 fold decrease in  $k_{\text{cat}}$  with only a small change in the substrate  $K_{\text{M}}$  values which is consistent with its primary role in transition state binding. Removal of the side chain completely (Ser 148→Gly) gives rise to a mutant enzyme (Gly 148 CAT) with a  $k_{\text{cat}}$  reduced only 10-fold. A plausible explanation for this is that the larger cavity in Gly 148 CAT, as compared to Ala 148 CAT, admits a water molecule which can replace the hydroxyl of Ser 148 as a hydrogen bond donor and can contribute to the stabilisation of the transition state.



**FIGURE 1.7**

The proposed tetrahedral intermediate

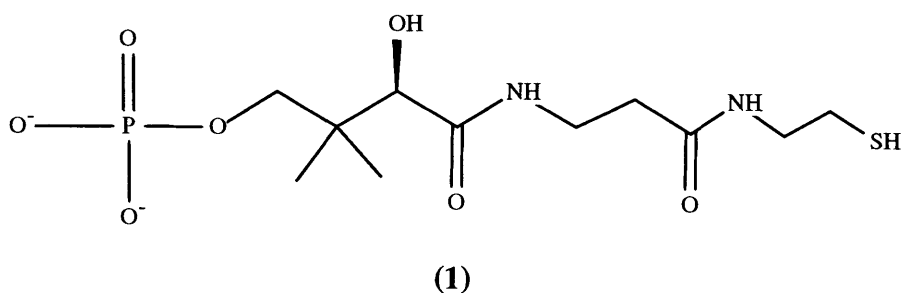
The tetrahedral oxyanion also forms a hydrogen bond, via a bridging water molecule, to a second residue, Thr 174. Although the interaction is indirect, the conservation of this chain and the results of site directed mutagenesis support the view that Thr 174 plays a role in catalysis.<sup>51</sup>

The transition state is also stabilized by hydrophobic interactions. The side chains of three highly conserved hydrophobic residues (Phe 33, Phe 158, and Leu 160) are so positioned as to make it likely that they interact not only with the methyl group of the tetrahedral intermediate, but also with the methyl of the trigonal acetyl group of acetyl CoA in its binary complex with CAT.<sup>22</sup> Whereas Phe 158 and Leu 160 are provided by the subunit to which both substrates are bound, Phe 33 arises from the facing subunit, which bears the catalytic His 195. The substitution of Phe 33 by alanine (Ala 33 CAT) results in a 2.5-fold increase in  $K_M$  for acetyl CoA and a comparable decrease in  $k_{cat}$ , yielding an estimate for the contribution of Phe 33 to transition-state stabilisation of  $1.8 \text{ kcal mol}^{-1}$  which is only slightly less than that of the hydroxyl of Ser 148.

## 1.7 Aims

The aim of the work carried out for this thesis was to gain a deeper understanding of the molecular recognition of acetyl CoA by chloramphenicol acetyltransferase, and in particular to develop a quantitative understanding of the origin of specificity. This was to be accomplished by "mutating" the substrate acetyl CoA. This approach provides a wide range of chemically defined alterations to the interaction of the enzyme and substrate and allows quantitative evaluations of the energetics of individual enzyme-ligand contacts to be made. By systematically removing or replacing a certain substituent of acetyl CoA the alternative substrates were specifically designed so that the contribution to binding and catalysis of each of the substituents of acetyl CoA could be estimated quantitatively.

The first step in the synthesis of CoA involves the synthesis of D-pantetheine-4'-phosphate (1). **Chapter 2** investigates the various syntheses of (1) and the synthesis of analogues of (1), precursors of pantetheine based CoA analogues. **Chapter 3** discusses the general synthesis of CoA which was adapted for the synthesis of pantetheine based analogues and the use of alternative bases for the synthesis of base analogues. **Chapter 4** discusses the kinetic results obtained for all the CoA analogues synthesised. **Chapter 5**, the final chapter, is the experimental section. It includes the synthesis and full characterisation of the compounds discussed above together with the kinetic methods.



**CHAPTER 2**  
**SYNTHESIS OF D-PANTETHEINE-4'-PHOSPHATE AND PANTETHEINE**  
**ANALOGUES**



## 2.1 Introduction

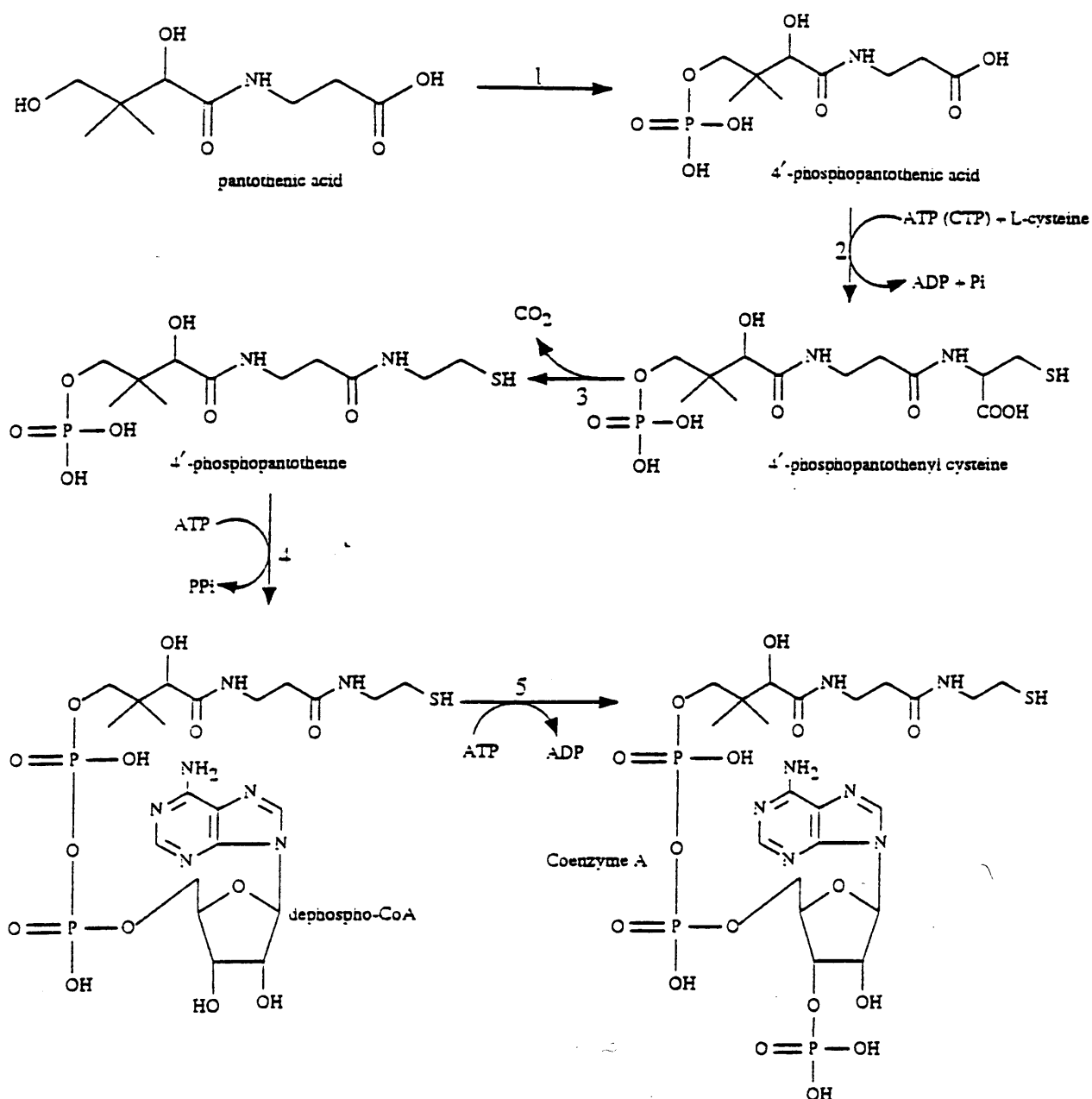
As mentioned in section 1.5 CoA was first discovered by Lipmann in 1945 as a cofactor required in many enzyme-catalysed acetylations. It is required by all organisms and plays an essential role in both carbohydrate and fat metabolism. The molecule acts as an acyl group activator by the formation of thioester linkages, and as a precursor to the pantetheine-4'-phosphate prosthetic group of the acyl carrier protein in fatty acid synthesis. The pathway of CoA biosynthesis from pantothenic acid is shown in **Scheme 2.1**. In mammals pantothenic acid is required as a vitamin but it must be synthesised from pantoic acid and  $\beta$ -alanine in micro-organisms.<sup>52</sup> Originally the pathway proposed by Hoagland and Novelli (1954)<sup>53</sup> involved the phosphorylation of pantetheine rather than pantothenic acid. However Ward et al (1955)<sup>54</sup> demonstrated pantothenate kinase activity in *P. morganii* and suggested the alternative route as shown (**Scheme 2.1**). Pantothenic acid is widely distributed in nutrition and its absence in the diet can cause human deficiencies. Other vertebrates fed on pantothenate deficient diets show diverse symptoms, including skin lesions, gastrointestinal ulcers and nervous disorders.<sup>55</sup>

The properties of the first two enzymes of the biosynthetic pathway have been studied in both *P. morganii*<sup>56</sup> and partially purified rat liver extracts.<sup>57</sup> The synthetase was substrate specific for phosphopantothenic acid in both cases, but in *P. morganii*, reaction with CTP instead of ATP was required. Phosphopantothenyl cysteine decarboxylase has been highly purified from horse liver,<sup>58</sup> but was found not to require pyridoxyl 5'-phosphate for activity as was previously reported.<sup>59</sup>

Early studies on the transferase and dephosphoCoA kinase activities were carried out by Hoagland and Novelli<sup>53</sup> who partially purified pig liver cytosol enzymes. Both activities were  $Mg^{2+}$  dependent, and the transferase was found to catalyse the only reversible step in the pathway. It was shown by Abiko<sup>57</sup> that the enzymes could only form CoA from D-pantetheine-4'-phosphate via the dephosphoCoA intermediate.

The regulation of CoA biosynthesis is generally thought to occur at the pantothenate kinase

step.<sup>60,61</sup> Abiko<sup>57</sup> found this enzyme to be inhibited by both CoA and D-pantetheine-4'-phosphate each with a  $K_i$  of 0.25 mM. Halvorsen and Skrede<sup>62</sup> later reported acetyl CoA to be a more potent inhibitor of pantothenate kinase than CoA, finding  $K_i$  values of 47  $\mu$ M for CoA and 1.4  $\mu$ M for acetyl CoA. Control of CoA biosynthesis in cardiac tissue was examined by Robishaw et al<sup>63</sup> who determined that the rate of flux through each of the reactions on perfusion of rat hearts with [<sup>14</sup>C]-pantothenic acid. They also came to the same conclusion that pantothenate kinase is the primary rate controlling step. They also found this enzyme to be inhibited by glucose, pyruvate, fatty acids and  $\beta$ -hydroxybutyrate.

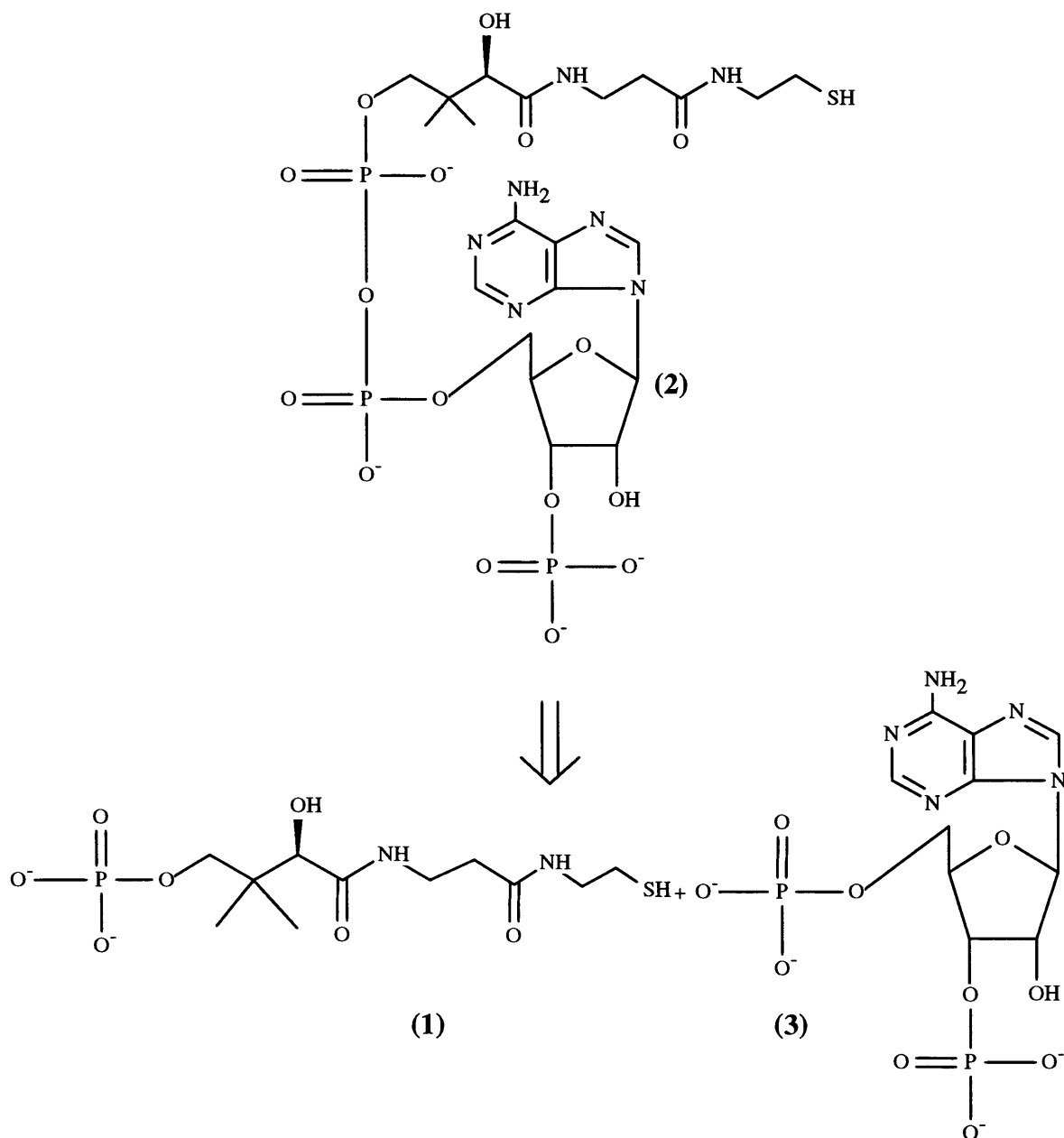


- 1 : pantothenate kinase  
 2 : 4'-phosphopantothenyl-L-cysteine synthetase  
 3 : 4'-phosphopantothenyl-L-cysteine decarboxylase  
 4 : 4'-phosphopantotheine adenyltransferase  
 5 : dephospho-CoA kinase

Scheme 2.1

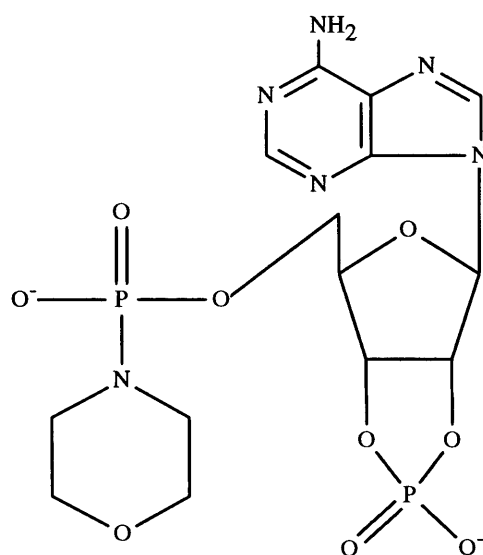
## 2.2 The synthesis of CoA

The development of methods to synthesise CoA was pioneered by Khorana in the late 1950's.<sup>64</sup> A retrosynthetic analysis of CoA (2) (Scheme 2.2) showed that it could be disconnected into two major units, D-pantetheine-4'-phosphate (1) and adenosine-3',5'-phosphate (3).



**Scheme 2.2**  
Retrosynthetic analysis of CoA

Although many alternative approaches to the synthesis of CoA are possible he identified early on the central problems of the synthesis: (a) a pyrophosphate bridge between the two major components, pantetheine and the nucleoside, and (b) a phosphomonoester group in the 3'-position of the adenosine moiety. From a general consideration of the chemistry of phosphate esters at different levels of esterification and of the behaviour of phosphomonoesters bearing vicinal hydroxyl groups, it was concluded that the most promising approach lay in the activation of the 5'-phosphomonoester group of an adenine nucleotide, which already carries the "extra" phosphate group in the form of a diester (**Figure 2.1**). The cyclic diester grouping in the structure was not expected to interfere in the pyrophosphate forming reaction, as had previously been shown in model experiments using simple diesters of phosphoric acid.<sup>65</sup> The major problems in this approach were thus (a) the synthesis of D-pantetheine-4'-phosphate, (b) the synthesis of structure in **Figure 2.1**, and (c) the formation of the pyrophosphate bond, followed by ring opening of the 2',3'-cyclic phosphate group.



Adenosine-2',3'-cyclic phosphate 5'-phosphomorpholidate (**4**)

**Figure 2.1**

## 2.3 Molecular recognition of CoA

The approach most commonly employed by synthetic chemists to probe the interactions between enzymes and substrates is to synthesise substrate analogues. Such analogues can be used in the form of alternative substrates which allow the study of both binding and catalysis, or as inhibitors which give information about binding only.

In the case of CoA, developing methods for its synthesis has provided a versatile route which has allowed the synthesis of many CoA analogues such as dephospho CoA, dethio CoA and guano CoA.<sup>64,66,67</sup> Such analogues have been instrumental in unravelling the specificity in coenzyme binding for several enzymes including choline acetyltransferase,<sup>46</sup> carbon monoxide dehydrogenase<sup>68</sup> and thiolase.<sup>69</sup> However, the synthetic routes to such analogues are often long and yields poor.

An alternative to long complicated synthesis of the coenzyme would be simpler but tight binding analogues of CoA that would be more amenable to chemical synthesis. For example pantetheine, a truncated derivative of CoA in which the adenosine 3'-phosphate-5'-diphosphate moiety (3'-phosphoADP) has been removed, and substituted pantetheines have proven to be good substrates for biosynthetic thiolase,<sup>69</sup> although D-pantetheine-4'-phosphate has been shown not to inhibit rat brain choline acetyltransferase.<sup>70</sup> Such truncated analogues of CoA have been previously synthesised by Day<sup>24</sup> in an attempt to determine their suitability as substrates for the CAT system. The results which are of relevance to this work are now discussed.

## 2.4 Truncated analogues of CoA

### 2.4.1 *S*-Acetyl D-pantetheine (Figure 2.2)

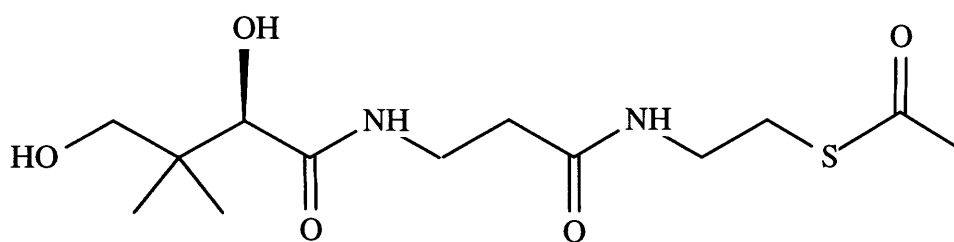


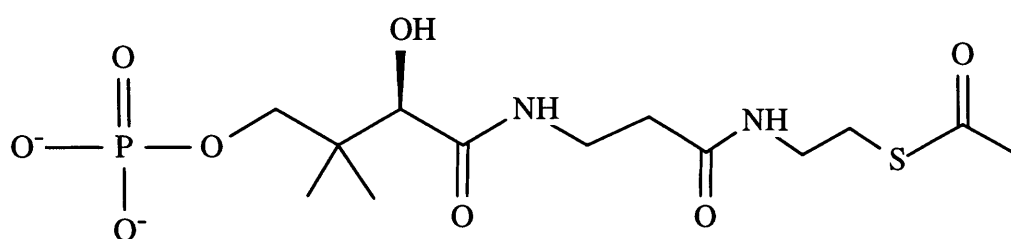
Figure 2.2

*S*-Acetyl D-pantetheine was found to be a very poor substrate for CAT. The  $K_M$  for *S*-acetyl D-pantetheine is more than 1500-fold greater than that for acetyl CoA and the  $k_{cat}$  is reduced 13-fold (**Table 2.1**). The removal of the 3'-phosphoADP moiety of CoA therefore results in a  $2 \times 10^4$ -fold decrease in the specificity ( $k_{cat}/K_M$ ) of the enzyme for the coenzyme. The 3'-phosphoADP moiety of CoA contributes  $2.9 \text{ kcal mol}^{-1}$  and  $4.3 \text{ kcal mol}^{-1}$  to the binding of CoA to the free enzyme ( $K_d$ ) and to the CAT/chloramphenicol binary complex ( $K_M$ ) respectively. Its removal also reduces transition-state stabilisation by  $6.2 \text{ kcal mol}^{-1}$ . Thus assuming that the enzyme catalysed reactions, using acetyl CoA and *S*-acetyl D-pantetheine as the acyl donor have the same rate determining step then the 3'-phosphoADP moiety of CoA appears to contribute more binding energy for binding of the transition state, than to the binding to either the free enzyme or the CAT/chloramphenicol binary complex. *S*-Acetyl D-pantetheine also resulted in a 20-fold increase in the  $K_M$  for chloramphenicol, corresponding to a weakening in the binding of the chloramphenicol to the CAT/CoA complex of  $1.8 \text{ kcal mol}^{-1}$ . For both substrates removal of the 3'-phosphoADP moiety from CoA results in greatly reduced binding to the corresponding binary complex to form a ternary complex, with much smaller effect on binding to the free enzyme to form a binary complex. Thus, a major function of the 3'-phosphoADP moiety of CoA appears to be the correct formation of a productive ternary complex and stabilisation of the transition-state, although it clearly influences affinity for acetyl CoA in binary complex formation.

### 2.4.2 Substituted pantetheines

In an attempt to improve the binding of *S*-acetyl D-pantetheine, Day synthesised several analogues substituted at the primary hydroxyl group which could potentially retain the specific enzyme/substrate interactions found for CoA (**Table 2.1**).<sup>24</sup>

### 2.4.3 *S*-Acetyl D-pantetheine-4'-phosphate (Figure 2.3)



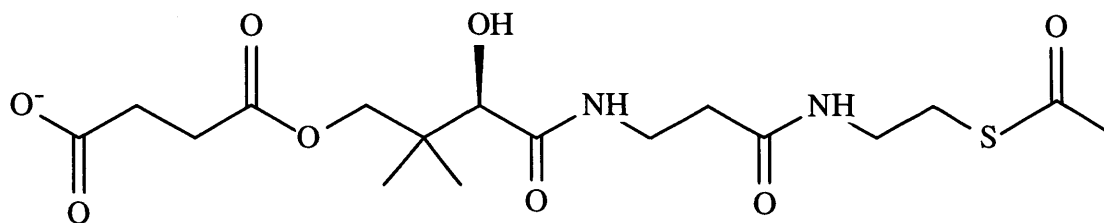
**Figure 2.3**

D-pantetheine-4'-phosphate an intermediate in the biosynthesis of CoA<sup>53</sup> might be expected to strengthen binding of pantetheine by introducing possible electrostatic interactions with Lys 54 and/or Lys 177 (**Figure 1.6**). However the presence of two negative charges, where only one would be present in CoA may perturb binding. Although the  $K_M$  is almost 5-fold lower than that for *S*-acetyl D-pantetheine, the  $K_M$  for chloramphenicol and  $k_{cat}$  are relatively unchanged (**Table 2.1**). In fact, the phosphate group contributes only 1.0 kcal mol<sup>-1</sup> in apparent binding energy, consistent with the relatively small changes in  $\Delta G_{app}$  observed for mutations which removed electrostatic interactions between the above lysyl residues and CoA.<sup>24</sup> This result suggests that the phosphate groups of *S*-acetyl D-pantetheine-4'-phosphate and CoA probably bind in a similar position.

### 2.4.4 *S*-Acetyl 4'-*O*-succinyl D-pantetheine (Figure 2.4)

The introduction of succinyl groups, which are similar in length to diphosphate moieties, to the primary hydroxyl group of pantetheine were expected to allow interactions normally associated with the 5'-phosphoanion group of CoA on binding.

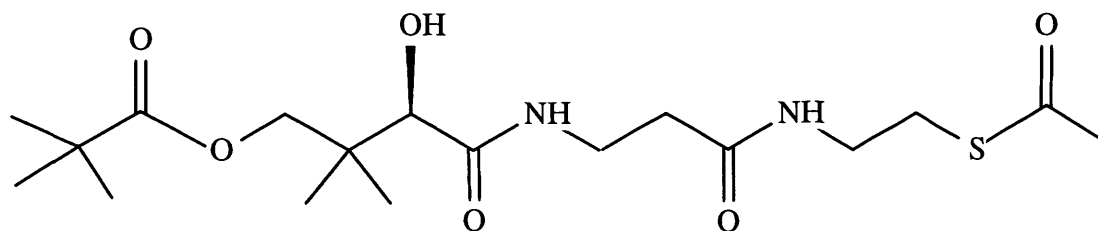




**Figure 2.4**

Although the  $K_M$  for *S*-acetyl 4'-*O*-succinyl D-pantetheine is 4.4-fold lower than that for *S*-acetyl D-pantetheine, it is still more than 300-fold greater than that for acetyl CoA (**Table 2.1**). As in the case of *S*-acetyl D-pantetheine, the  $K_M$  for chloramphenicol is greatly increased and  $k_{cat}$  is decreased. The 4-fold increase in  $k_{cat}/K_M$  for *S*-acetyl 4'-*O*-succinyl D-pantetheine, as compared with *S*-acetyl D-pantetheine, yields a calculated contribution to binding for the charged succinyl group of  $0.8 \text{ kcal mol}^{-1}$ .

#### 2.4.5 *S*-Acetyl 4'-*O*-pivaloyl D-pantetheine (Figure 2.5)



**Figure 2.5**

When CoA is bound to CAT the adenine moiety of CoA is in close proximity to the end of the pantetheine arm and is in fact in van der Waals contact with the dimethyl group. Thus, the addition of a large, relatively hydrophobic group could possibly enhance binding by exploiting the hydrophobic adenine binding surface. Such an analogue, *S*-acetyl 4'-*O*-pivaloyl D-pantetheine, in which the 3'-phosphoADP has been replaced by a trimethylacetyl (pivaloyl) group has proven to be a good substitute for acetyl CoA in the thiolase reaction.<sup>17</sup> However, Day observed that the rate in the standard assay was more than 600-fold lower than that observed with acetyl CoA as the acetyl donor and is similar to that of acetyl pantetheine suggesting that the presence of a trimethyl group does not significantly improve binding of pantetheine.

**Table 2.1** Steady state kinetic parameters for alternative acyl donors<sup>24</sup>

Acyl donor(A)	$k_{cat}(s^{-1})$	$K_M A(mM)$	$K_M B(mM)$	$K_d A(mM)$	$K_d B(\mu M)$
Acetyl CoA	600	0.093	12	0.031	4
<i>S</i> -Acetyl D-pantetheine	46	143	243	3.9	7
<i>S</i> -Acetyl D-pantetheine-4'-phosphate	49	30	175	1.1	6
<i>S</i> -Acetyl 4'- <i>O</i> -succinyl-D-pantetheine	42	32	189	1.1	7
<i>S</i> -Acetyl D-pantetheine (+5mM 3' AMP)	44	74	72	73	7

Chloramphenicol (B)

#### 2.4.6 The importance of the 3'-phosphoADP moiety of CoA

Day<sup>24</sup> has suggested that in the absence of the 3'-phosphoADP of CoA, it is possible that *S*-acetyl D-pantetheine may be able to bind in other, less catalytically favourable conformations. For example, the pantetheine arm of *S*-acetyl D-pantetheine could bind deeper into the CoA "tunnel" such that the acetyl moiety begins to interfere with chloramphenicol binding, resulting in higher than expected  $K_M$  values for both substrates, and possibly also a decrease in  $k_{cat}$ . Due to the relatively non specific nature of many of the interactions between the enzyme and the pantetheine arm of CoA, alternative binding modes for *S*-acetyl D-pantetheine are not unreasonable. Most hydrogen bonds to the pantetheine arm involve one or more water molecules and the direct interaction between the side chain of Ser 148 and N71 is rather long (**Figure 1.6, Appendix 1**). Also removal of the 3'-phosphoADP moiety of CoA removes part of the pantetheine binding site as the adenine ring is normally in

van der Waals contact with the dimethyl group. However, the changes in  $K_M$  observed on addition of charged groups to the primary hydroxyl group of pantetheine also support the view that *S*-acetyl D-pantetheine does bind in a similar manner to the pantetheine of CoA.

#### 2.4.7 Effect of 3'-AMP on *S*-acetyl D-pantetheine binding and catalysis

The possibility that adenine nucleosides might be able to substitute for the 3'-phosphoADP moiety of CoA in the catalytic reaction has been investigated by Day.<sup>24</sup> With acetyl CoA as the acetyl donor, the addition of 3'-AMP results in a 25% decrease in activity, compared with a 50% increase in activity when *S*-acetyl D-pantetheine is the acetyl donor. The  $K_M$  for both *S*-acetyl D-pantetheine and chloramphenicol is reduced 2-3 fold but the  $k_{cat}$  is virtually unchanged (**Table 2.1**). The 3'-AMP appears to increase activity slightly, however the adenosine and pantetheine moieties of CoA must be covalently linked for full activity.

#### 2.4.8 Conclusion

Truncated analogues of CoA, based on *S*-acetyl D-pantetheine, are very poor substrates for CAT. Such analogues appear to have low affinities and their use results in a large increase in the  $K_M$  value for chloramphenicol. It is likely that *S*-acetyl D-pantetheine either binds to CAT in a different manner than does the pantetheine arm of CoA, or that it fails to induce a conformation change which may normally occur on CoA binding.

Due to the high  $K_M$  values and potential problems with alternative binding modes or conformational changes, pantetheine-based analogues are unsuitable as a tool to investigate the role of specific interactions between CoA and CAT. Therefore, in order to study interactions of CoA with CAT a total synthesis of CoA is required.

As mentioned earlier the two major synthetic targets in the synthesis of CoA are D-pantetheine-4'-phosphate (1) and adenosine-2',3'-cyclic phosphate 5'-phospho-

morpholidate (4). The remainder of this chapter will concentrate on the synthesis of D-pantetheine-4'-phosphate, the first step of CoA synthesis. General phosphorylation methods were sought which could be used not only to phosphorylate pantetheine but also analogues of pantetheine for the synthesis CoA analogues.

## RESULTS AND DISCUSSION

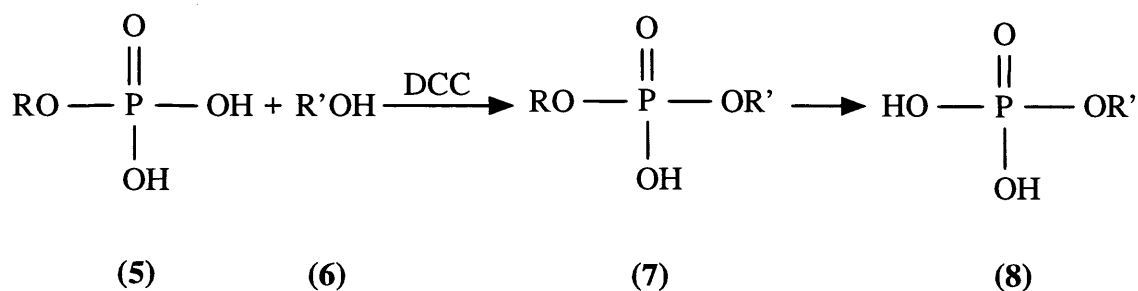
### 2.5 Synthesis of D-pantetheine-4'-phosphate (1)

There are several methods available to synthesise CoA (see **Chapter 3**) but the method developed by Khorana (**Section 2.2**) is the most versatile. The first step in the total synthesis of CoA using this method involves the synthesis of D-pantetheine-4'-phosphate. Since the early discoveries that inorganic phosphate and organic phosphate esters are fundamental to living processes much attention has been devoted to their synthesis. As a result many reagents have been developed for the conversion of alcohols to phosphate esters, each reagent unique in its selectivity and mode of deprotection.

In synthesising the monoester D-pantetheine-4'-phosphate a reagent must be chosen which can selectively phosphorylate the primary hydroxyl group and be deprotected without affecting the rest of the molecule. Several phosphorylating agents were used in the attempt to synthesise D-pantetheine-4'-phosphate, for each the background to the reagent, mechanism and suitability are discussed.

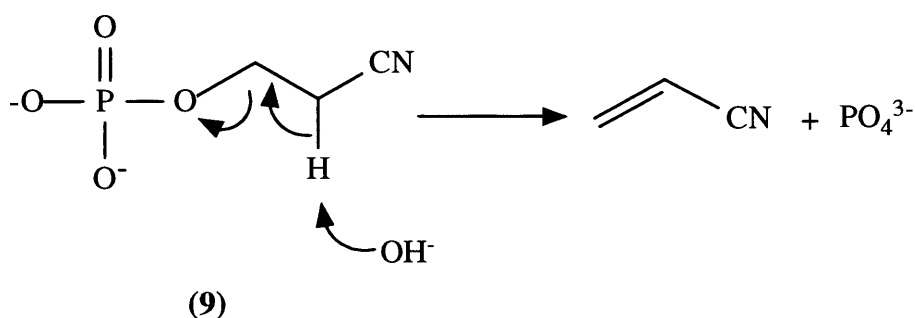
#### 2.5.1 Cyanoethyl phosphate and dicyclohexylcarbodiimide

This method was developed by Tener<sup>71</sup> in the 1950's and was based on the earlier observation<sup>72</sup> that phosphodiester (7) could be readily prepared from a monoalkyl phosphate (5) and an alcohol (6) by allowing them to react in anhydrous pyridine with DCC (**Scheme 2.3**).



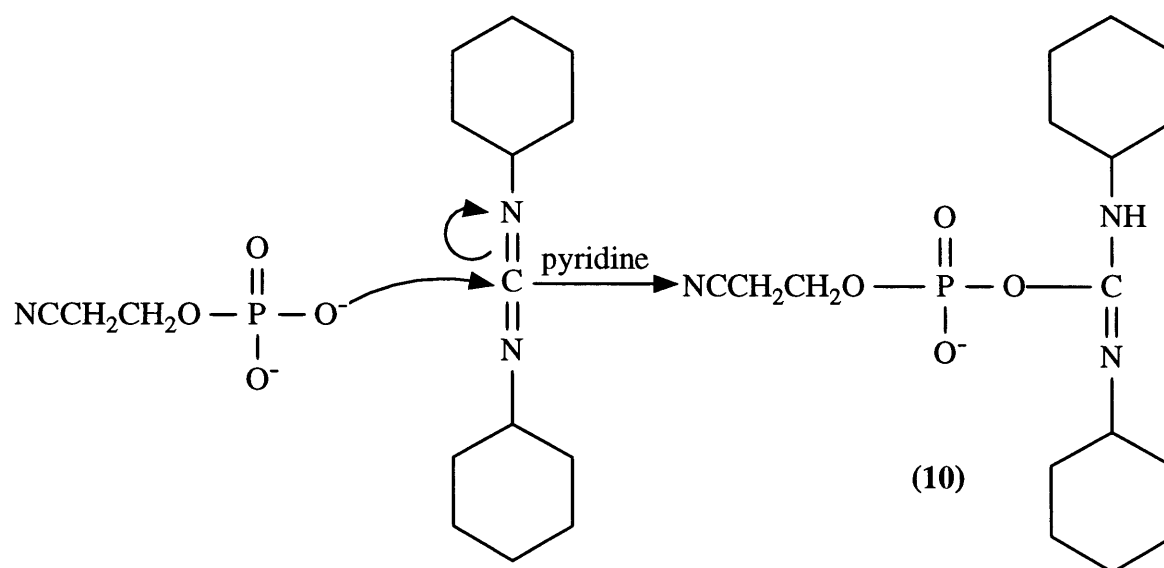
**Scheme 2.3**

In the phosphorylation procedure an alkyl phosphate (5) was chosen such that after coupling it to the alcohol, as shown in **Scheme 2.3**, its alkyl group could be selectively removed. The net result of the reaction is the conversion of the alcohol (6) to its phosphate ester (8). The reagent which satisfied this requirement best was 2-cyanoethyl phosphate (9). It was chosen because it had been shown to break down under very mild alkaline treatment and liberate orthophosphate, presumably by the reaction in **Scheme 2.4**.<sup>73</sup>



**Scheme 2.4**

The phosphorylation reaction with this commercially available reagent is carried out in anhydrous pyridine. In general, phosphorylating agents are sensitive to traces of water, however, 2-cyanoethyl phosphate offers the advantage of being completely stable and is converted into the active phosphorylating species (10) only when brought into reaction with DCC (**Scheme 2.5**).

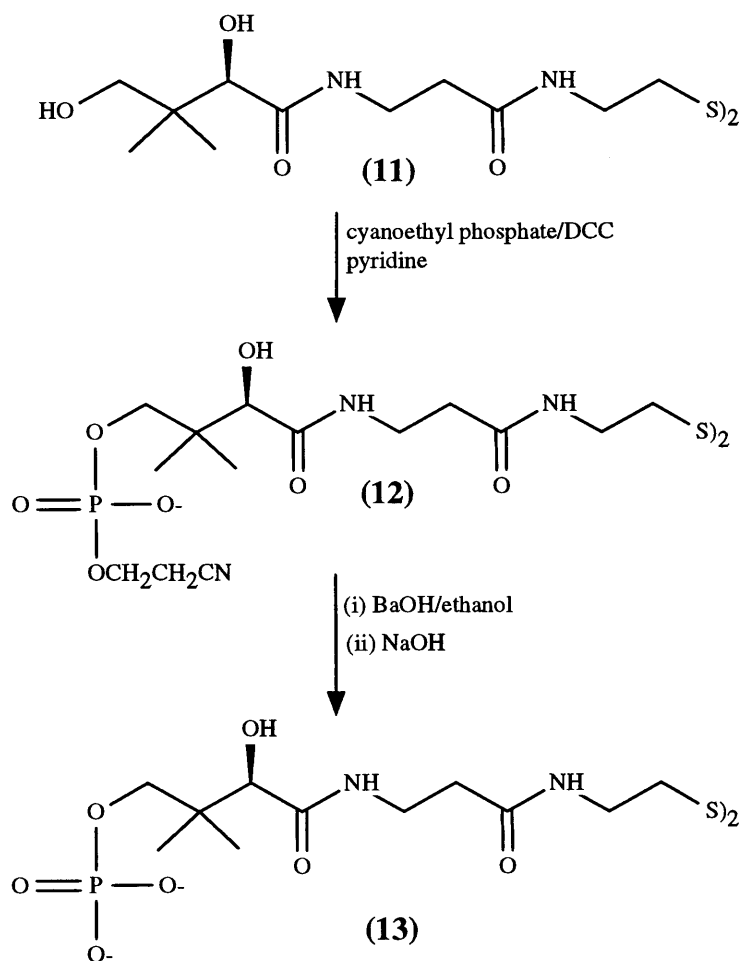


**Scheme 2.5**

Formation of activated phosphorylating species (10)

Water will decompose this activated intermediate and care is taken to remove as much water as possible from the reaction mixture. However, in practice absolutely anhydrous conditions are not required because an excess of DCC is normally added and last traces of water are removed by the acid catalysed hydration of DCC to form dicyclohexylurea. One other precaution necessary when using this reagent is to exclude the presence of strong bases, since they prevent the formation of the activated intermediate. For this reason, and because of solubility problems, barium 2-cyanoethyl phosphate is only converted to the pyridinium salt before use. This reagent has proved particularly successful in the synthesis of nucleotides, these include examples from both the ribo- and deoxyribonucleotide series as well as the dinucleotides.<sup>71</sup>

The first step in the synthesis of (1) involves the conversion of D-pantethine (11), the commercially available disulfide form of D-pantetheine, to D-pantethine-4'-phosphate (13) as shown in **Scheme 2.6**

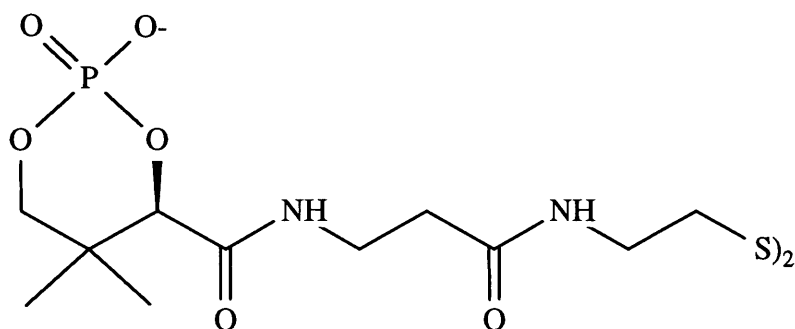


**Scheme 2.6**

D-Pantethine was left to react with the reactive intermediate (10) overnight in anhydrous pyridine. The unreacted cyanoethyl phosphate was removed by precipitating the excess reagent as the barium salt while the product was still at the diester level (12). Barium salts of phosphodiester are generally very soluble in aqueous alcohol, whereas barium 2-cyanoethyl phosphate is not. This method has also been successfully employed in the synthesis of 5-fluorodeoxyuridylic acid.<sup>71</sup>

Due to the diol nature of (11) there is competition to form a six membered cyclic phosphate, D-pantethine-2',4'-cyclic phosphate (14), (Figure 2.6) via an intramolecular transesterification. The conditions of the experiment must be carefully controlled to limit excess formation of this undesired product by in particular maintaining the temperature of the

reaction vessel below 35 °C.

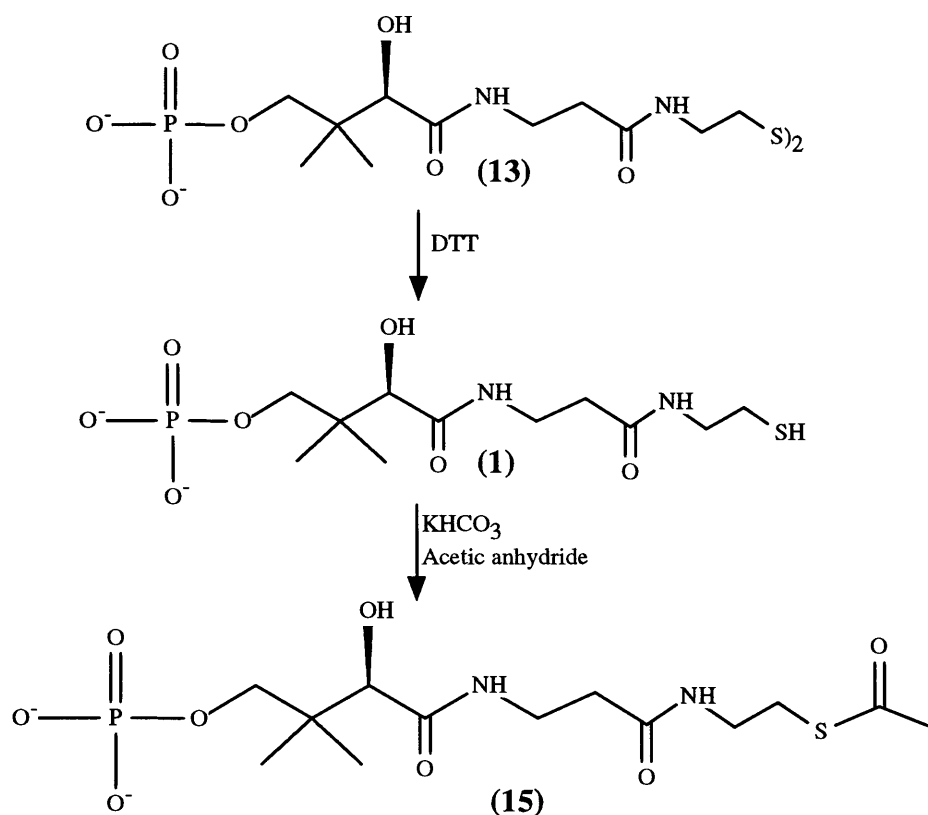


D-pantetheine-2',4'-cyclic phosphate (**14**)

**Figure 2.6**

The cyanoethyl protecting group was removed by treating the reaction mixture with dilute sodium hydroxide solution and leaving on ice for 30 minutes. The next stage involved converting the D-pantetheine-4'-phosphate (**13**) to D-pantetheine-4'-phosphate (**1**), this involved reducing the disulphide bond of (**13**) using the reducing agent dithiothreitol (DTT) to form the free thiol (**1**) (**Scheme 2.7**). Compound (**1**) was purified at this stage by DEAE Sephadex ion exchange to remove the contaminating DTT, orthophosphate and cyclic phosphate present in the reaction mixture. Compound (**1**) is not very stable as the free thiol group is easily oxidized back to the disulfide (**13**) so it must be stored as *S*-acetyl D-pantetheine-4'-phosphate (**15**) instead. The final step, the acetylation of the free thiol by acetic anhydride (**Scheme 2.7**) involved a method analogous to the acetylation of commercially available CoA to acetyl CoA (see **Section 5.3.2**).<sup>74</sup>





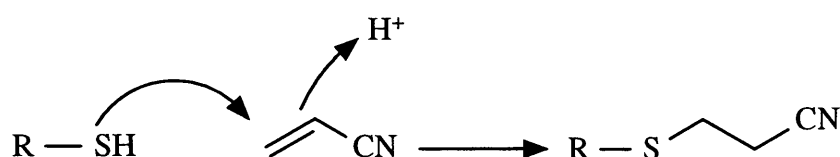
**Scheme 2.7**

This final product, *S*-acetyl D-pantetheine-4'-phosphate (15) was purified by DEAE Sephadex ion exchange to remove the bicarbonate salt as well as any disulfide (13) that may still be present.

The yields obtained for (15) using this method were consistent with reported yields,<sup>24</sup> *ie* in the region of 11%. The low yield is not a problem for the synthesis of *S*-acetyl D-pantetheine-4'-phosphate as the starting material D-pantethine is commercially available. However, the synthesis of pantetheine based analogues may involve multi step syntheses prior to phosphorylation which could limit the amount of material available.

In order to make the above method more attractive for the phosphorylation of pantetheine based analogues an attempt was made to improve the final yield. It was thought the low yields were probably due to loss of material during the barium precipitation step, *ie* some of

the product (**12**) may have remained undissolved in the ethanol and was therefore lost on filtration. In order to improve the yield it was decided to omit this step and rely on ion exchange to separate the product from the inorganic phosphate produced in the deprotection step. However a problem was encountered in that the large amount of acrylonitrile produced on deprotection was able to react with the product of the next step, ie the thiol (**1**) produced by the reduction of the disulphide as shown in **Scheme 2.8**.



The reaction of D-pantetheine-4'-phosphate with acrylonitrile

### Scheme 2.8

Two methods were developed to overcome the problem of this side reaction, (i) after deprotection of the phosphate using NaOH, the product (**13**) was purified by DEAE Sephadex ion exchange to separate it from the inorganic phosphate, cyclic phosphate and acrylonitrile present in the reaction mixture. The purified product was then reduced and acetylated as before. (ii) After deprotection the aqueous reaction mixture was washed several times with chloroform to remove all the acrylonitrile. This was followed by the reduction and acetylation steps. Both methods proved successful in eliminating the acrylonitrile side reaction.

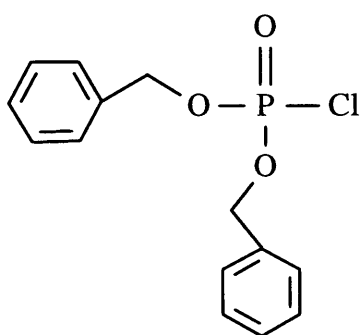
In attempting to purify the final product (**15**), it became apparent that the *S*-acetyl group was not completely stable to the conditions of the ion exchange column. <sup>1</sup>H NMR spectra showed contamination of the product with D-pantethine-4'-phosphate (**13**). Subsequent columns were unable to remove this impurity but caused only a reduction in the yield. It is possible that the triethylammonium hydrogen carbonate buffer gradient contained traces of primary amines which may have hydrolysed the *S*-acetyl group to a thiol which could have reoxidised to form the disulfide (**13**).

Several alternative methods were investigated for the purification of the final product, the

best was found to be a HPLC column (5ASAX) in conjunction with an alternative phosphoric acid gradient. Using this method there was no contamination of (15) with the disulphide (13) and the final yield was in the region of 45%, a vast improvement on the reported yields of 11%. Although the yield is much improved it is still relatively low for such a key reaction in the synthesis of CoA. Alternative phosphorylating reagents were investigated which were hoped could offer higher yields.

### 2.5.2 Dibenzyl phosphorochloridate

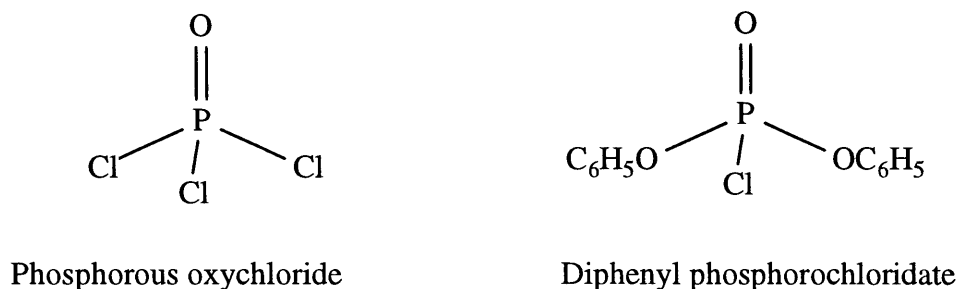
Dibenzyl phosphorochloridate (16), (Figure 2.7) has been the most successful phosphorylation reagent to be employed in the field of nucleotide chemistry.



Dibenzyl phosphorochloridate (16)

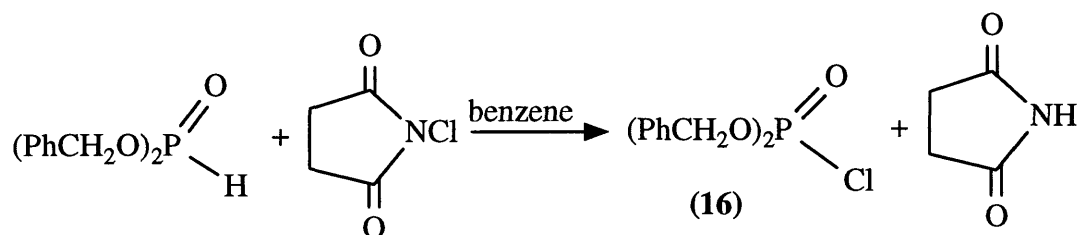
**Figure 2.7**

It was first developed by Todd<sup>75</sup> to overcome the problems of poor selectivity and difficulties of deprotection common with existing phosphorylating agents at the time, for example diphenyl phosphorochloridate and phosphorous oxychloride (Figure 2.8).



**Figure 2.8**

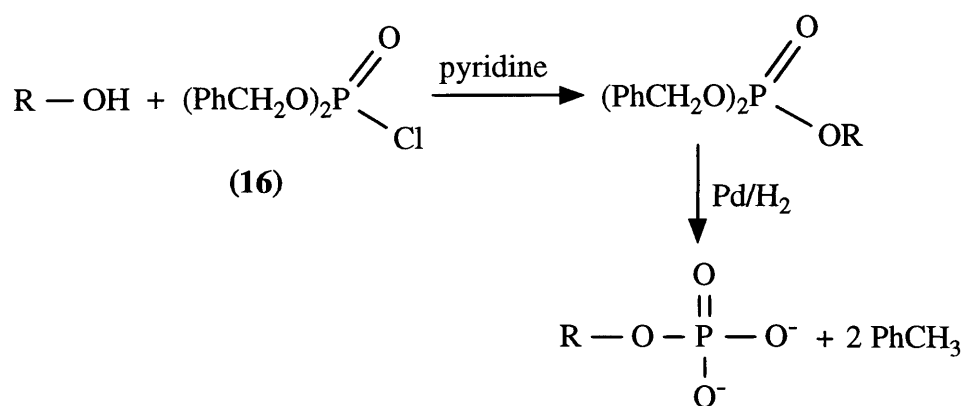
The reagent **(16)** which was employed in the synthesis of D-pantetheine-4'-phosphate was prepared from dibenzyl phosphite and *N*-chlorosuccinimide which are both commercially available as shown in **Scheme 2.9**.<sup>64</sup>



Preparation of dibenzyl phosphorochloridate

**Scheme 2.9**

As the reagent is itself unstable it could not be stored therefore it had to be prepared from dibenzyl phosphite immediately before use. The general mechanism for the phosphorylation reaction is shown in **Scheme 2.10**.



**Scheme 2.10**

General phosphorylation using dibenzyl phosphorochloridate

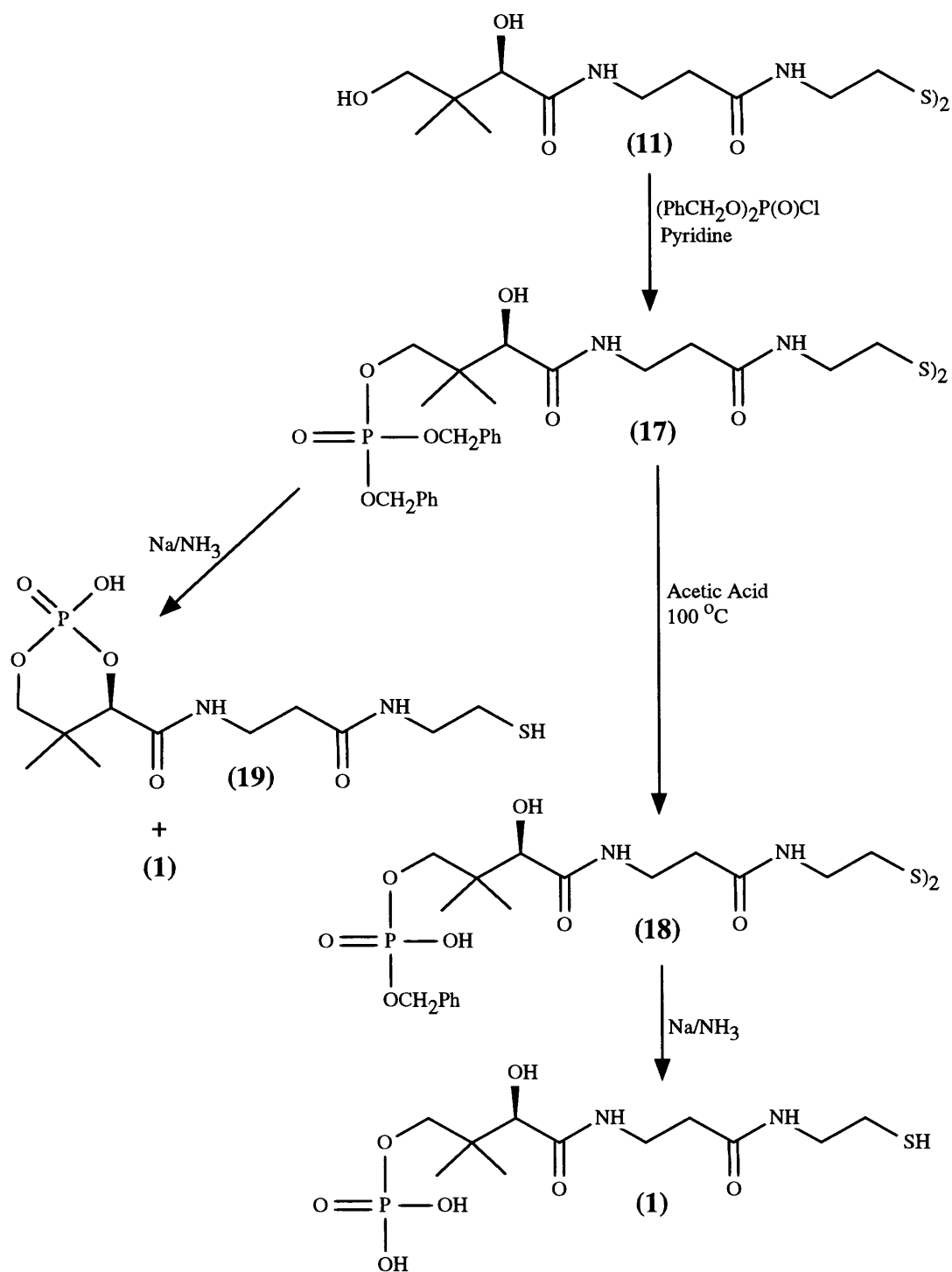
Not only does the reagent readily phosphorylate most primary alcoholic functions the dibenzyl groups can be readily removed from the intermediate phosphotriester by mild catalytic hydrogenolysis using palladium catalyst.

In some cases, in particular where the alcohol contains sulphur atoms as in the case of D-pantethine, alternative methods of deprotection must be sought to remove the benzyl groups as sulphur atoms cause the "poisoning" of palladium catalysts. A suitable alternative

is the use of sodium in liquid ammonia whereby the reaction proceeds by an alternative radical mechanism. The reagent has been used successfully in the synthesis of a variety of nucleotides and has been extensively reviewed by Khorana.<sup>76</sup>

It should be noted however that the reagent is not very powerful and is very often unsatisfactory for example for the phosphorylation of secondary hydroxyl groups in deoxyribonucleosides,<sup>77</sup> the hydroxyl group in serine<sup>78</sup> and the secondary hydroxyl group in pantolactone.<sup>79</sup> Furthermore, the reagent must be used at low temperature, and long reaction periods must be avoided due to the high susceptibility of the benzyl groups to nucleophilic attack by pyridine, the medium of the reaction. The quaternization reaction yields phosphate anions which in turn lead to pyrophosphate forming exchange reactions.

The reaction scheme for the conversion of D-pantethine (**11**) to D-pantetheine-4'-phosphate using (**16**) is shown in **Scheme 2.11**.



**Scheme 2.11**

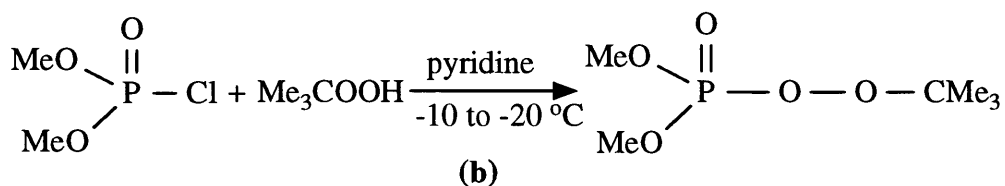
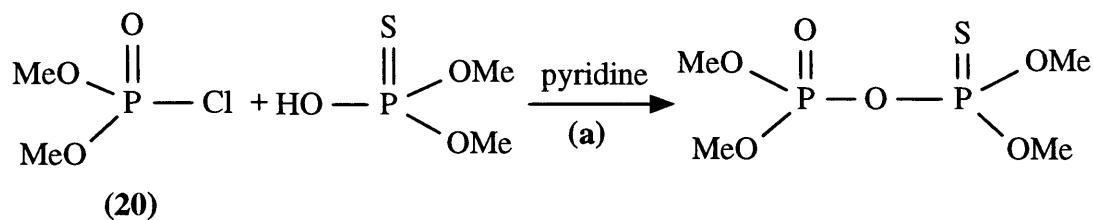
D-Pantethine was dissolved in dry pyridine and frozen in a dry ice bath and a solution of dibenzyl phosphorochloridate was added and the solution was left to stand over night. Initial experiments by Khorana<sup>64</sup> had shown that the phosphorylation followed by the simultaneous removal of the benzyl groups and reduction of the disulphide with sodium in liquid ammonia

gave invariably two major phosphorus and sulfhydryl containing compounds. The side product was identified as D-pantetheine 2',4'-cyclic phosphate (**19**) a compound which had been previously been synthesised by Baddiley and Thain.<sup>80</sup> The formation of the cyclic phosphate presumably occurred as a transesterification reaction of the phosphate triester (**17**), the initial product of phosphorylation, during treatment of sodium in liquid ammonia. Many examples of this general type of reaction are known<sup>81</sup> and more specifically, the experience of Baddiley and Thain<sup>80</sup> during the condensation of pantooyl lactone-2-diphenyl phosphate with *N*-β-alanyl-2-mercaptoethylamine provides a close analogy.

The intramolecular transesterifications are known to occur far more rapidly at the tertiary phosphate ester level than at the diester level<sup>81</sup> and in order to minimise it (**17**) was treated with 50% acetic acid at 100 °C to convert it to (**18**). Subsequent treatment with sodium and liquid ammonia gave a product which was essentially pure D-pantetheine-4'-phosphate. Traces of the cyclic diester (**19**) which were present probably arose during isolation of (**17**) after phosphorylation in pyridine, although care was taken to use as mild conditions as possible. The trace contamination was, however, not considered serious as it could be easily separated by ion exchange. After acetylation the yields obtained for (**15**) were in the range of 10% which is much lower than the reported yield of 45%.<sup>64</sup>

### **2.5.3 Dimethyl phosphorochloridate**

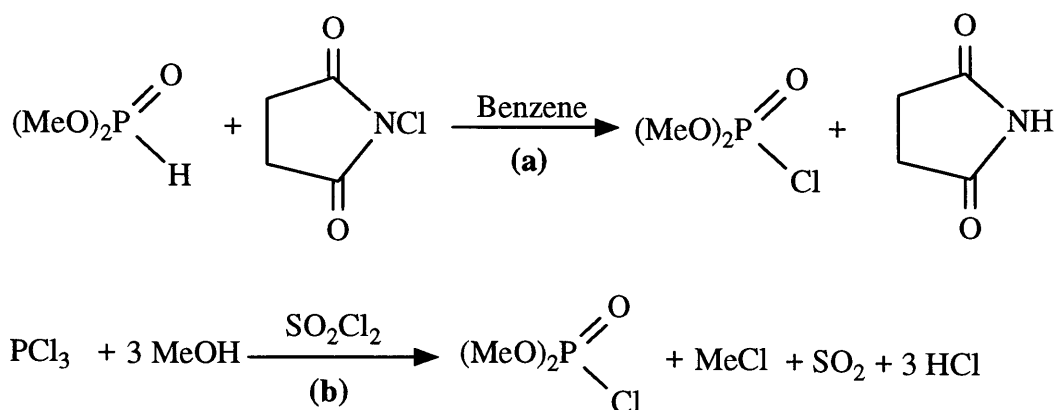
Dimethyl phosphorochloridate (**20**), **Scheme 2.12**, was first synthesised by Fiszler and Michalski in 1952.<sup>82</sup> Since then it has been employed in a variety of different synthesis including the preparation of tetramethyl thiopyrophosphate (**Scheme 2.12a**)<sup>83</sup> and dimethyl *t*-butylperoxyphosphate (**Scheme 2.12b**).<sup>84</sup>



**Scheme 2.12**

It was first applied to the field of CoA chemistry by Drueckhammer in a combined chemical and enzymatic synthesis of coenzyme A analogues.<sup>85</sup> This combined chemical and enzymatic approach is discussed in more detail in **Chapter 3**.

Compound (20) is not commercially available so had to be synthesised prior to the phosphorylation reaction. It can be prepared in two ways, either directly before use in an analogous method to that for dibenzyl phosphorochloridate (**Scheme 2.13a**) or by a general method for alkyl phosphoryl chloride developed by Fiszer which allows storage of the compound for several months (**Scheme 2.13b**).<sup>82</sup>



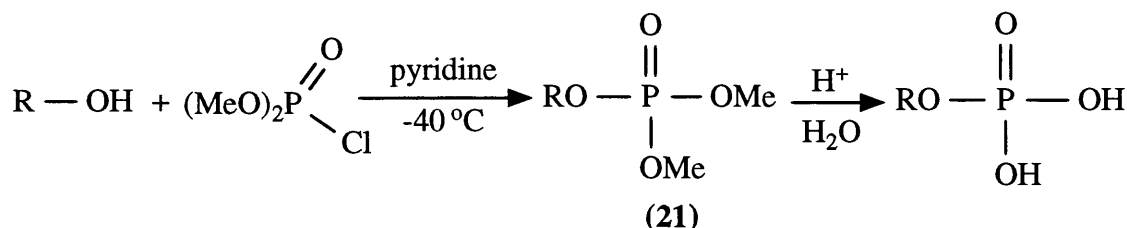
**Scheme 2.13**

Preparation of dimethyl phosphorochloridate

Method **b** was selected as the preparation had to be done only once and so reduced the time



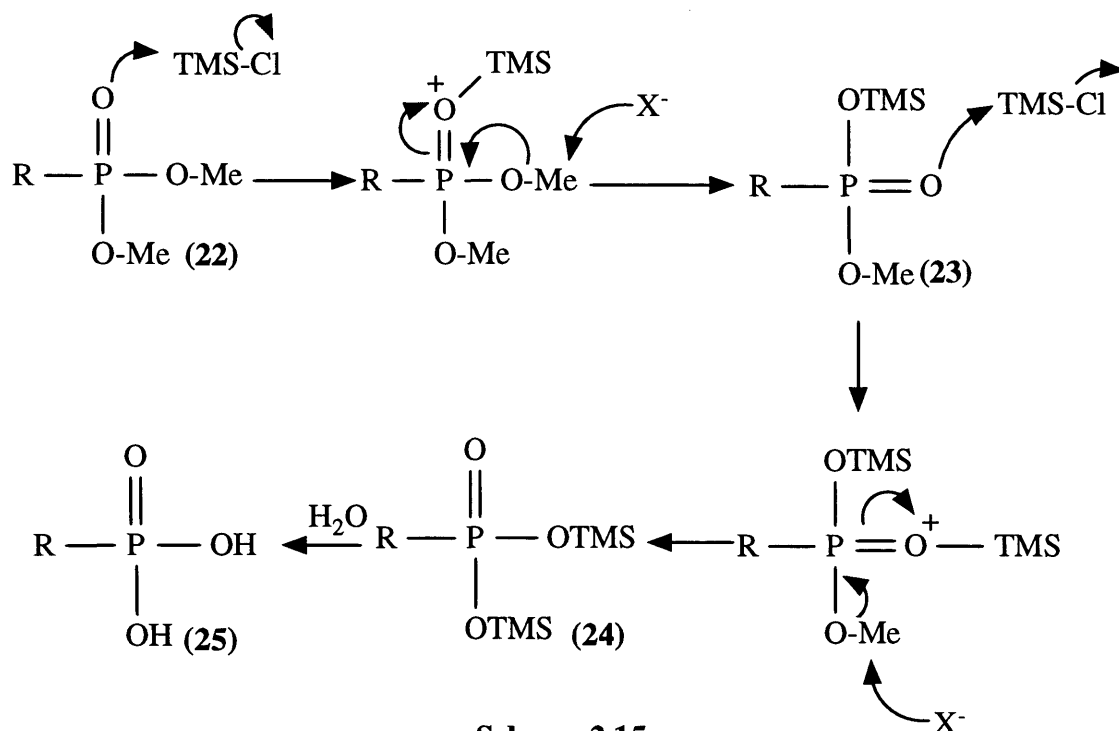
of the phosphorylation reaction. The reagent was prepared by adding phosphorous trichloride dropwise to a solution of methanol in dry benzene before adding a solution of the chlorinating agent sulphuryl chloride. The desired product (**20**) was purified by distillation under reduced pressure. The general method for phosphorylation of alcohols using (**20**) is illustrated in **Scheme 2.14**.



**Scheme 2.14**

A problem with conventional acid catalysed hydrolytic dealkylation as shown in **Scheme 2.14** is that it is often unsuitable as (**21**) may consist of, or comprise, a functional group that is too delicate to survive the harsh conditions involved. Instead an improved method for the deprotection of the dimethyl groups involves a two step process whereby (**21**) is converted to the corresponding silyl esters which are susceptible to very mild hydrolysis. This method is based on the earlier observation by Kosolopoff<sup>86</sup> that bis(trimethylsilyl)phosphonates (**24**), (**Scheme 2.15**), prepared from alkylphosphonate precursors by the action of chlorotrimethylsilane (**26**) hydrolyse to phosphonic acids (**25**) on contact with neutral H<sub>2</sub>O at room temperature.

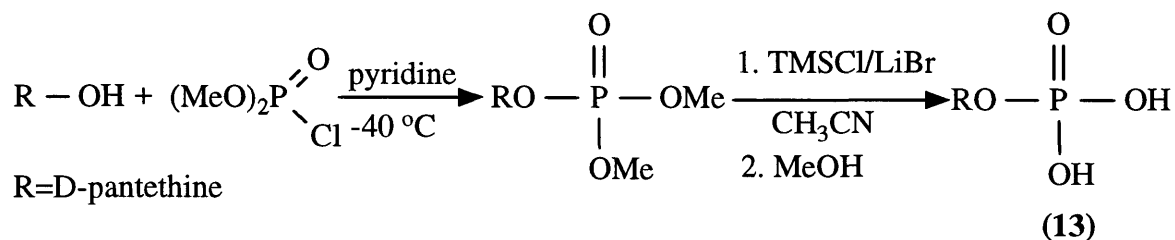
The proposed mechanism (**Scheme 2.15**) for the reaction of (**22**) with (**26**) involves attack on silicon by the phosphoryl oxygen of (**22**) followed by substitution of the displaced halide ion on a phosphonate ester alkyl group of the same molecule to give the mixed alkyl trimethylsilyl diester of the phosphonic acid (**23**); a second cycle of the same reaction sequence would then yield the bis(trimethylsilyl) phosphonate (**24**).



**Scheme 2.15**

In considering that the halide participates both as a leaving group and nucleophile it was found that replacement of (26) with bromotrimethylsilane enhanced the rate of reaction to give quantitative yields of the bis(trimethylsilyl) esters under exceedingly mild conditions in 1-2 hours at 25 °C.

The proposed synthesis of D-pantethine-4'-phosphate (13) is shown in **Scheme 2.16**.



**Scheme 2.16**

The method was analogous to that developed by Drueckhammer for the synthesis of S-(phenyl)-thiopantothenate-4'-phosphate (27) from S-(phenyl)-thiopantothenate (28) (Scheme 2.19).<sup>85</sup> D-Pantethine was dissolved in dry pyridine and cooled to -40 °C before a

solution of dimethyl phosphorochloridate in benzene was added dropwise. After 3-4 hours water was added to quench the reaction, the reaction mixture was concentrated under reduced pressure and dissolved in dichloromethane. The reaction mixture was washed with sodium hydrogen carbonate solution and then ammonium chloride, the combined organics were dried over  $\text{MgSO}_4$  and concentrated under reduced pressure. The crude reaction mixture was dissolved in acetonitrile and deprotection of the phosphate group was carried out by the method developed by Kosolopoff.<sup>86a</sup> Chlorotrimethylsilane and lithium bromide were added and the reaction mixture was stirred at 45 °C for 27 hours. After quenching the reaction with methanol the product was purified by DEAE Sephadex ion exchange. The yields for (13) were very low and in the region of 5-8%. It was thought that the greatest loss of material occurred during the work up. The work up involved several aqueous wash steps, as the product is relatively polar it is possible that much of the material was lost to the aqueous layers. This problem was not encountered with dibenzyl protected D-pantetheine-4'-phosphate as it is much more hydrophobic in character therefore less likely to dissolve in the aqueous work up. As the yields were very low it was decided not to continue with this reagent in the phosphorylation of D-pantethine as other methods offered much higher yields.

The most satisfactory phosphorylating reagent used in the synthesis of D-pantetheine-4'-phosphate was found to be the cyanoethyl barium phosphate with DCC coupling. Not only were the yields the highest, but it was also the easiest to use, required the mildest conditions and involved the least number of steps. Due to the reactive nature of the *S*-acetyl group the product was not stable to conventional Sephadex ion exchange so HPLC techniques had to be employed to purify (15).

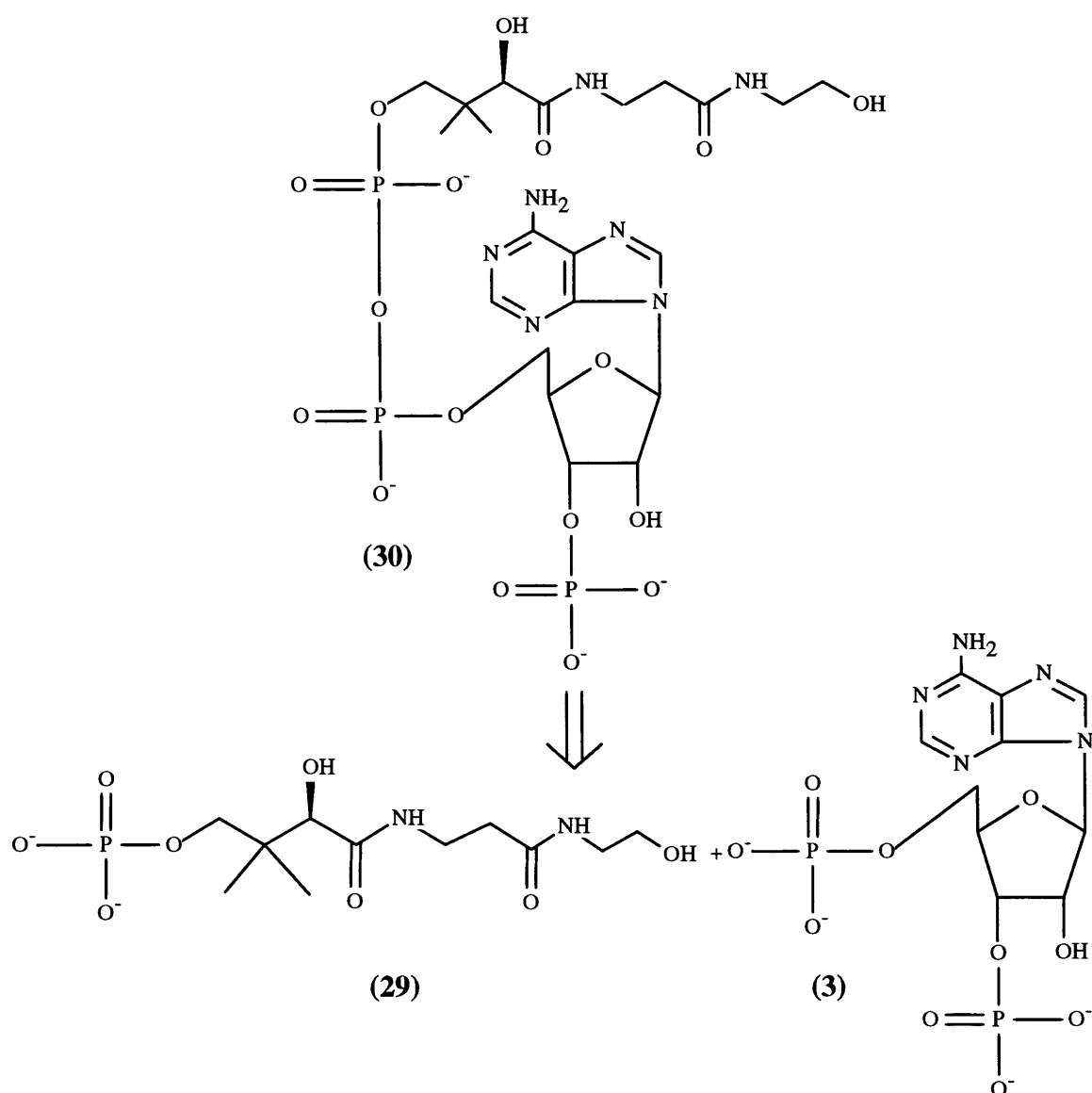
## 2.6 Synthesis of D-pantetheine-4'-phosphate analogues

In order to study the molecular recognition of CoA by CAT using substrate analogues, versatile synthetic methods are required for the synthesis of these analogues. If one first considers CoA analogues with modifications in the pantetheine portion they can be prepared in either of two ways; (i) either by synthesising analogues of D-pantetheine-4'-phosphate and reacting with (4), (**Figure 2.1**) or (ii) directly from CoA to give modifications in the thiol and thiolester regions (see **Chapter 3**). The most versatile method by far is method (i), several syntheses of pantetheine have been developed which can be modified for the synthesis of pantetheine analogues.<sup>66,87,88</sup> The key step in the synthesis of the CoA analogue, the coupling of the pantetheine analogue and the adenine nucleotide is discussed in more detail in the next chapter. The remainder of this chapter will concentrate on the synthesis of the pantetheine portion of the CoA analogues.

### 2.6.1 Synthesis of dethio D-oxypantetheine-4'-phosphate (29)

The oxygen analogue of CoA, dethio oxy CoA (oxy CoA) (**30**), has been previously prepared by a method developed by Stewart *et al*<sup>89</sup> in order to investigate the structural requirements of CoA for interaction with the enzyme phosphotransacetylase which catalyses the formation AcCoA from acetyl phosphate and CoA (see **Section 3.1**).

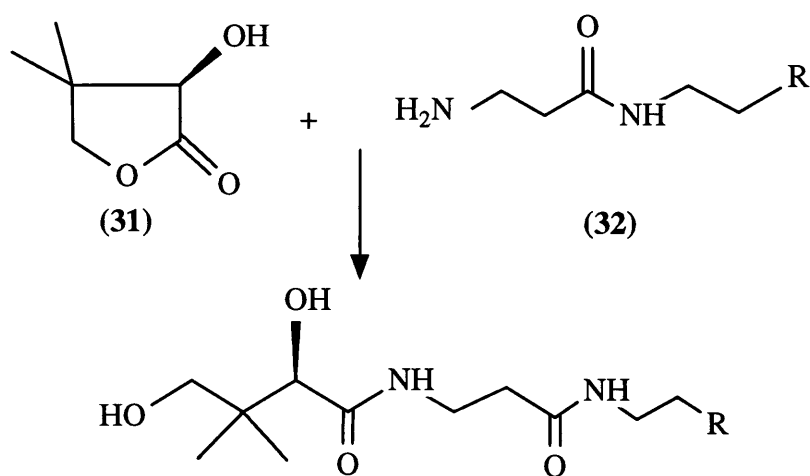
In order to extend these studies to the CAT system to examine the effect on CoA binding by replacing the thiol for an hydroxyl group a total synthesis of oxy CoA was required. A retrosynthetic analysis of oxy CoA showed that it could be disconnected into two major units, dethio D-oxypantetheine-4'-phosphate ( **29**) and adenosine-3',5'-phosphate (**Scheme 2.17**).



**Scheme 2.17**

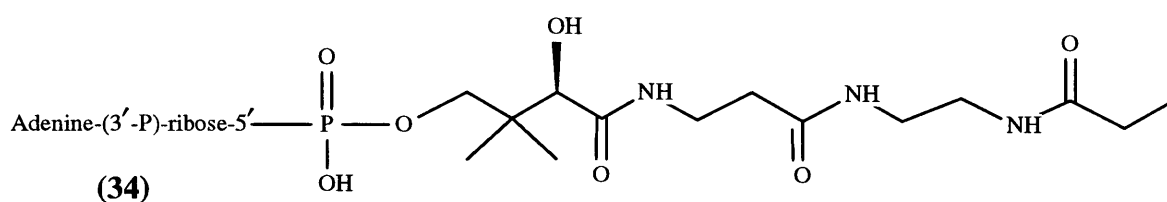
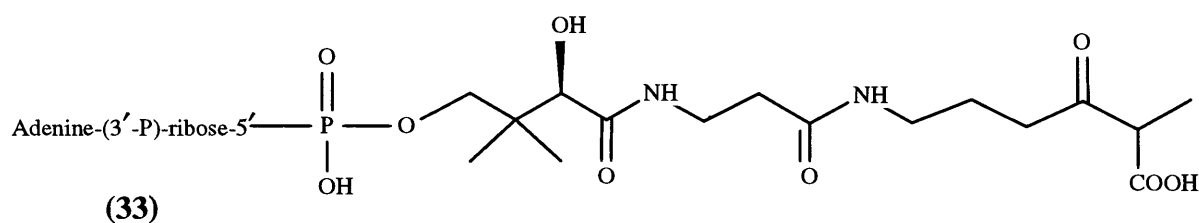
Retrosynthetic analysis of oxy CoA

The first step in the synthesis of oxy CoA involves the synthesis of dethio D-oxypantetheine-4'-phosphate. This has previously been synthesised using the successful method developed by Stewart *et al*<sup>89</sup> which involved the condensation of D-pantolactone (31) with an aletheine derivative (32) as shown in **Scheme 2.18**.



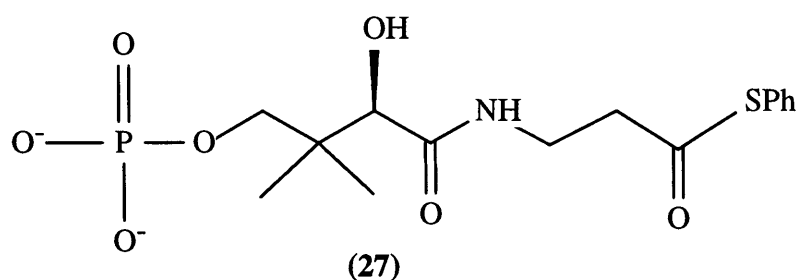
(32a) R=SH, pantetheine  
R=OH, oxypantetheine  
**Scheme 2.18** R=CH<sub>3</sub>, Dethiapantetheine

This method allows the cysteamine portion of the molecule to be replaced by highly functionalised groups. For example the method has also allowed the synthesis of more complicated analogues including methylmalonylcarba (dethia)-CoenzymeA (33) and propionyl-Aza (dethia)-CoenzymeA (34) which have been used to determine the mechanism of methylmalonyl CoA mutase<sup>90</sup> and biotin containing transcarboxylase<sup>91</sup> respectively, (Figure 2.9).



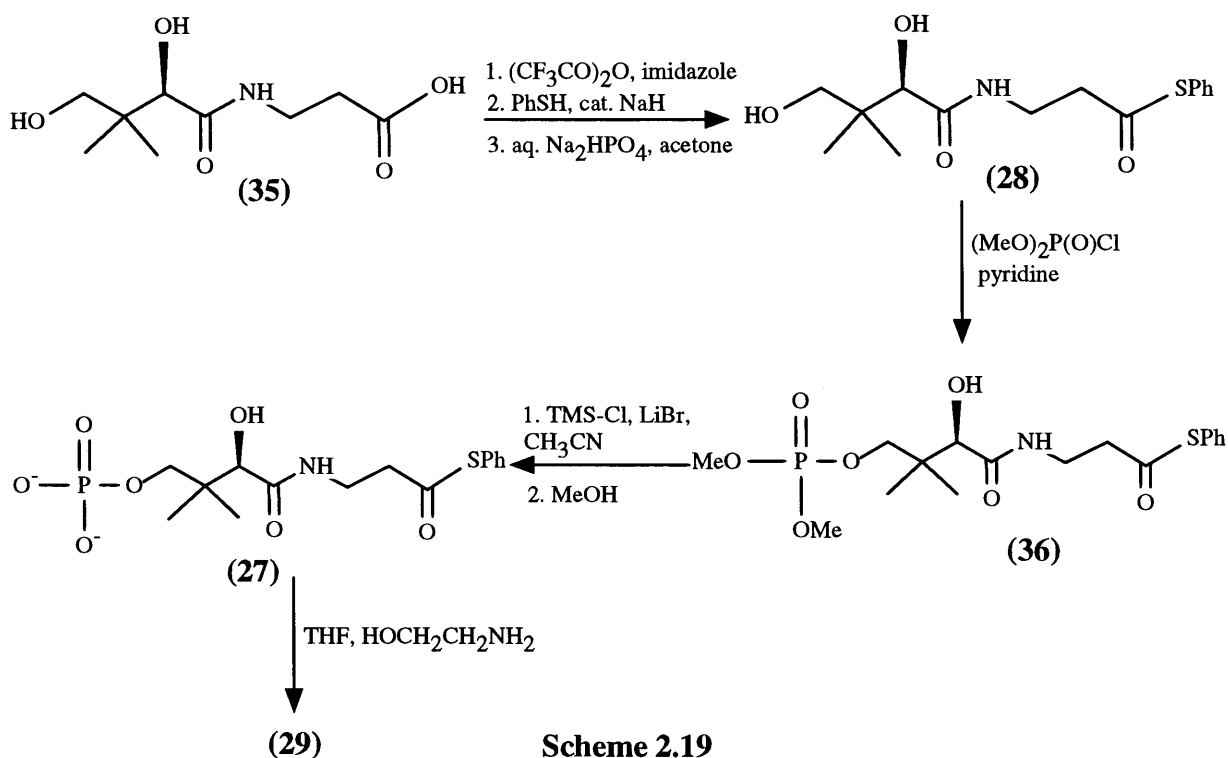
**Figure 2.9**

However during the course of this work an alternative synthesis was employed. During their work on the development of a combined chemical and enzymic approach for the synthesis of pantetheine based analogues of CoA (see **Section 3.1.2**) Drueckhammer *et al*<sup>85</sup> prepared *S*-(phenyl) thiopantothanate-4'-phosphate (**27**) (**Figure 2.10**).



**Figure 2.10**

Compound (**27**) has been used as an intermediate in the synthesis of a variety of pantetheine based CoA analogues for mechanistic studies of citrate synthase<sup>92</sup> (see **Section 3.1.2**). The thiophenol is a very good leaving group which allows (**27**) to be reacted with a variety of amines to give a range of D-pantetheine-4'-phosphate analogues. It was envisaged that reacting (**27**) with aminoethanol would yield the desired product (**29**). The route to (**29**) is shown in **Scheme 2.19**.

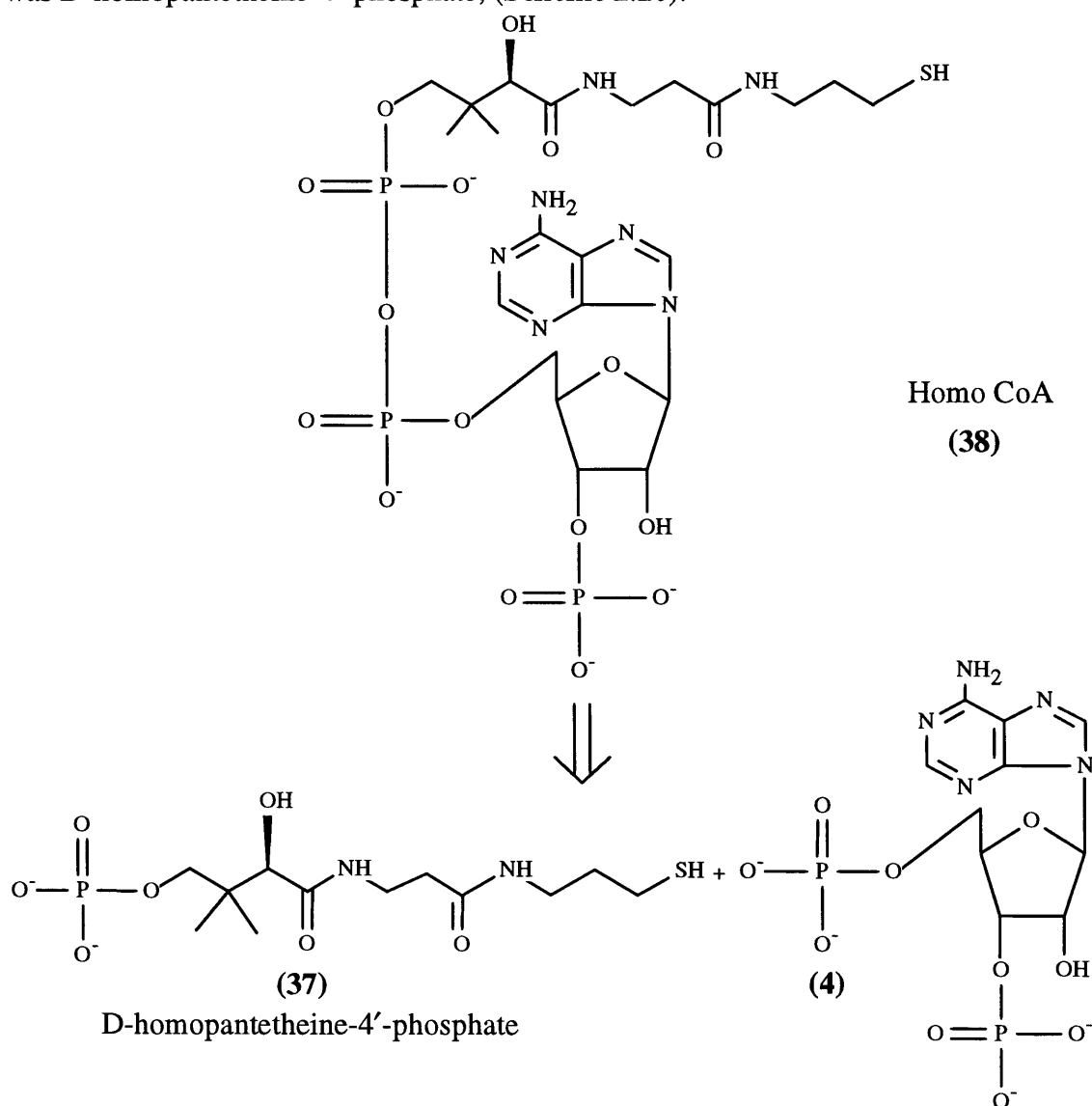


Pantothenic acid (**35**) was first converted to the acylimidazole with concurrent trifluoroacetylation of the hydroxyl groups. Reaction of the acylimidazole with thiophenol followed by selective hydrolysis of the trifluoroesters gave compound (**28**). Phosphorylation was accomplished by conversion to the dimethyl phosphate ester (**36**) followed by removal of the methyl groups as discussed in **Section 2.5.3** to give (**27**). The final step involved reacting (**27**) with 3 equivalents of aminoethanol in THF solvent. The reaction was complete after 20 minutes and the final product was purified by DEAE Sephadex ion exchange to give a yield of 52%. The next step which involves coupling (**29**) with an adenine nucleotide is discussed in more detail in the next chapter.



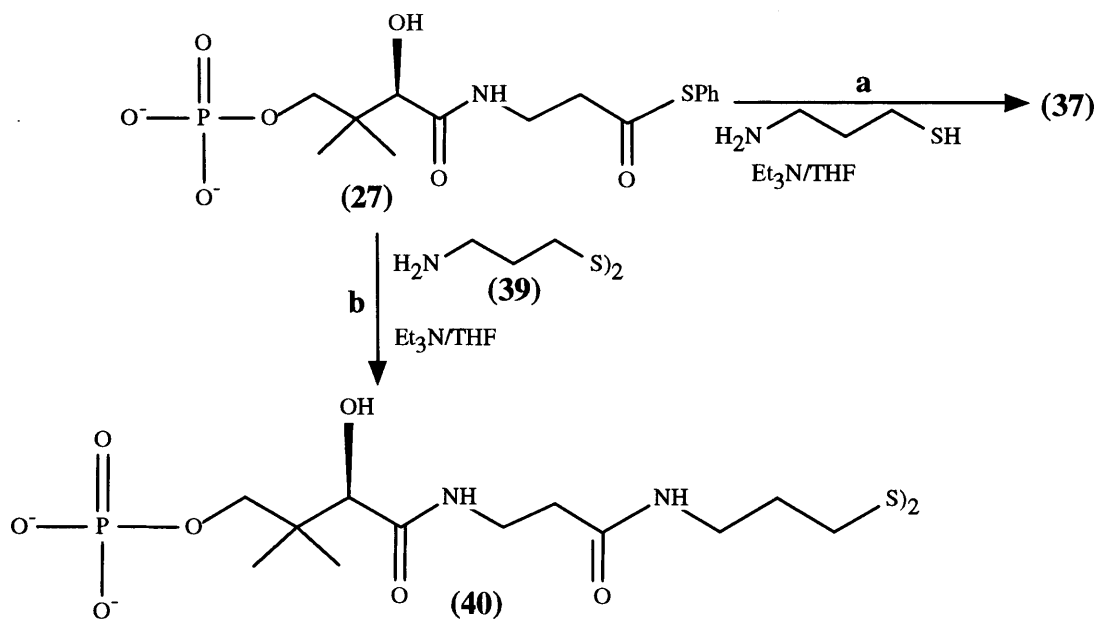
### 2.6.2 Synthesis of D-homopantetheine-4'-phosphate (37)

Homo CoA (38) has been previously synthesised by Shimizu *et al* method for the purpose of phosphotransacetylase inhibition studies.<sup>88</sup> For the purpose of this work the total synthesis of *S*-acetyl homo CoA was attempted in order to examine the effect on both binding and catalysis of CoA by increasing the pantetheine chain length by a methylene group in the cysteamine portion. A retrosynthetic analysis showed that the key pantetheine intermediate was D-homopantetheine-4'-phosphate, (Scheme 2.20).



**Scheme 2.20**  
Retrosynthetic analysis of Homo CoA

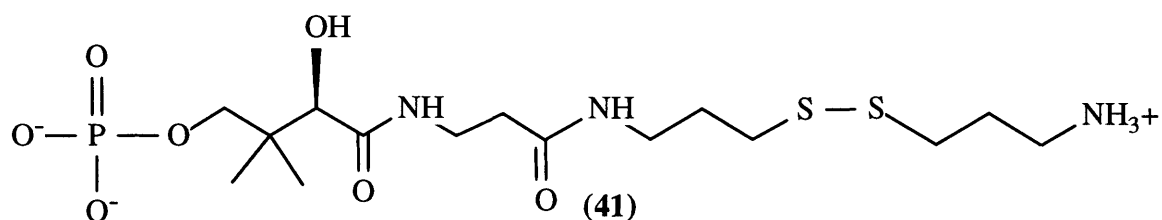
Initially the proposed synthesis of (37) involved reacting the synthon (27) with aminopropanethiol as shown in **Scheme 2.21a**. However problems were encountered in that the 3-aminopropanethiol, which had to be prepared from 1-aminopropanol, proved difficult to handle and attempts to react it with (27) were unsuccessful.



D-homopantethine-4'-phosphate

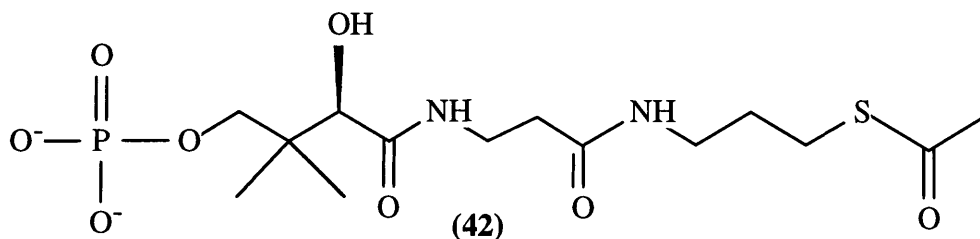
**Scheme 2.21**

Instead it was decided to react (27) with bis(3-aminopropyl) disulfide (39), (**Scheme 2.21b**), which can be easily prepared and stored as the hydrobromide salt. The product, D-homopantethine-4'-phosphate (40) is the dimeric form of (37) and can be easily converted to (37) by simple reduction of the disulphide bond. The synthesis involved reacting two equivalents of (27) with the bis(3-aminopropyl) disulfide (39) in THF. The reaction was complete within 1 hour and the product was purified by DEAE Sephadex ion exchange to give a yield of 30%. One of the side products of the reaction was (41) which was produced by the amine (39) reacting with only one molecule of (27) (**Figure 2.11**).



**Figure 2.11**

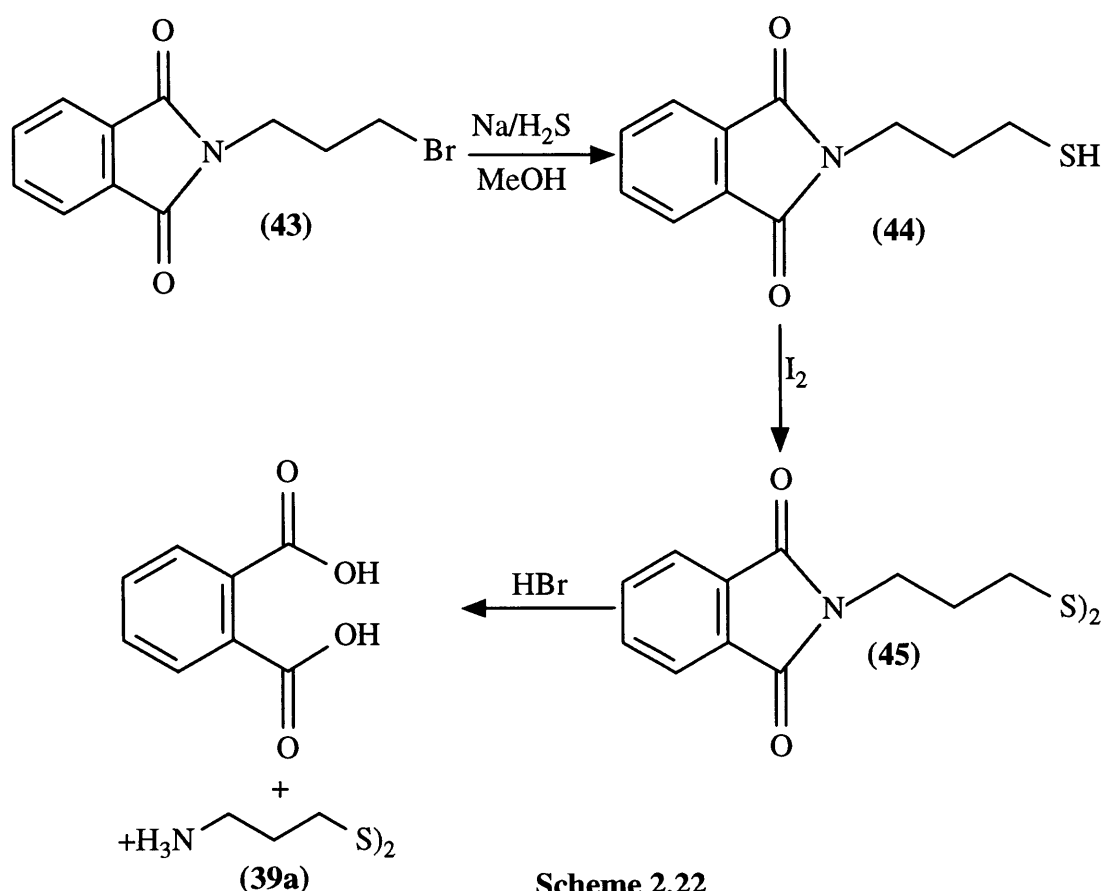
The undesired **(41)** was converted to D-homopantetheine-4'-phosphate **(37)** by reducing the disulfide with DTT in an analogous method to that of D-pantetheine-4'-phosphate synthesis. In order to prevent it from oxidizing into the dimer **(40)** to form a mixture of products **(37)** was acetylated to give S-acetyl D-homopantetheine-4'-phosphate **(42)** which can be stored without decomposition, (**Figure 2.12**).



**Figure 2.12**

D-homopantetheine-4'-phosphate **(40)** was not converted to **(37)** at this stage. Instead it was decided to couple both **(40)** and **(42)** with the adenine nucleotide as discussed in the next chapter. The former could be easily converted to the desired acetyl CoA analogue by simply reducing the disulphide bond followed by acetylation of the free thiol using acetic anhydride.

The amine **(39)** used in **Scheme 2.21b** is not commercially available and had to be synthesised from *N*-(3-bromopropyl) phthalamide **(43)** prior to reaction as shown in **Scheme 2.22**.



Scheme 2.22

Compound (43) was reacted with NaSH generated by treating a solution of sodium in methanol with  $\text{H}_2\text{S}$  to give N-(3-thiopropyl) phthalamide (44). Compound (44) was dissolved in boiling methanol and treated with powdered iodine to oxidise the thiol to give the bis(3-phthalamidopropyl) disulfide (45). The phthalamide protecting group was removed by dissolving (45) in acetic acid and treating with concentrated HBr. The phthalic acid side product was removed by evaporating the reaction mixture to dryness and then taking it up in a little water whereby the phthalic acid remained undissolved. The HBr salt of (39), compound (39a) could be stored without the risk of decomposition and could be converted to the amine (39) prior to reaction by passing it through an ion exchange column (Dowex 1<sup>-</sup>OH form).

The various syntheses of pantetheine have provided versatile routes for the synthesis of a

wide range of pantetheine based analogues. During the course of this work the versatile synthon (**27**) developed by Drueckhammer<sup>85</sup> proved particularly successful in the synthesis of dethio D-oxypantetheine-4'-phosphate and D-homopantetheine-4'-phosphate, precursors of oxy CoA and homo CoA respectively.

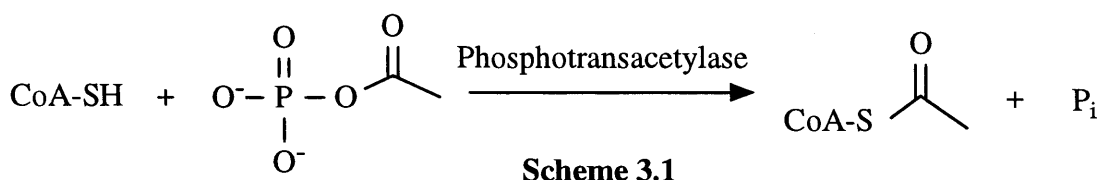
**CHAPTER 3**  
**SYNTHESIS OF CoA ANALOGUES**

### 3.1 Introduction

As mentioned previously, one method of investigating interactions between an enzyme and CoA is to analyse the effects of structural analogues of the coenzyme on the enzyme catalysed reaction. Chemical synthesis of pantetheine and CoA have provided versatile routes for the synthesis of various CoA analogues. Such analogues have found many applications in a variety of CoA-dependent enzymes. They have been used as mechanistic probes for enzyme catalysis,<sup>17</sup> provided an insight into structural specificity,<sup>93</sup> as well as helping elucidate the structure of transition state intermediates.<sup>92</sup>

#### 3.1.1 Molecular recognition of CoA by phosphotransacetylase.

Using an approach similar to that used in our work, Shimizu *et al* investigated the structural requirements of CoA to interaction with the enzyme phosphotransacetylase which catalyses the formation of AcCoA from acetyl phosphate and CoA (**Scheme 3.1**).<sup>93</sup>



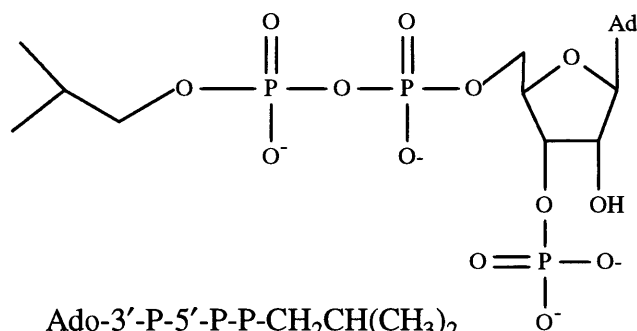
Kinetic analysis of various CoA analogues and segments (**Table 3.1**) helped identify the key elements for recognition and binding. The analogues oxy CoA and desulfo CoA which are modified in the thiol region of CoA were found to be potent competitive inhibitors of the acetyl transfer reaction, however analogues in which the cysteamine moiety was removed (de-cysteamine CoA amide, de-cysteamine CoA and decysteamine CoA nitrile) were also found to inhibit competitively but with much greater  $K_i$ 's. De- $\beta$ -alaninyl CoA, which is also a competitive inhibitor of CoA had no coenzyme activity, although it contains a thiol group. De-aletheine CoA amide, which lacks the aletheine moiety, was also a competitive inhibitor of CoA.

Nucleotide analogues such as Ado-3'-P-5'-P-P-CH<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>, Ado-3'-P-5'-P-P-CH<sub>3</sub> and Ado-3'-P-5'-P-P inhibited phosphotransacetylase competitively with CoA (**Figure 3.1**). In contrast Ado-3'-P-5'-P, 5'-ADP and 5'-ATP did not effect the enzyme. Therefore Ado-3'-P-5'-P-P was the smallest compound among the various analogues found to compete with CoA. This suggests that the nucleotide, which corresponds to the nucleotide moiety of CoA, may be essential for this coenzyme to interact with the CoA site of the enzyme. This view is supported by the finding that the enzyme gained the ability to acetylate pantetheine in the presence of Ado-3'-P-5'-P-P and Ado-3'-P-5'-P-P-CH<sub>3</sub>, in absence of the nucleotide pantetheine is inert to acetylation. These results suggest that the Ado-3'-P-5'-P-P or its methyl ester induced acetylation of pantetheine by specific interaction with a CoA site of phosphotransacetylase. Therefore it seems likely that the nucleotide moiety of CoA is essential for recognition by the enzyme.

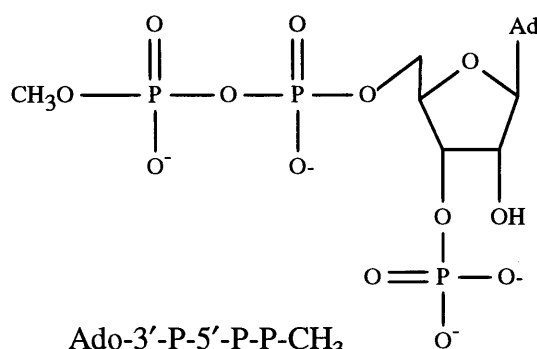
Analogues in which the D-form of the hydroxyl group in the pantoyl moiety were modified, CoA (L) and keto CoA, had inhibitory effects as well as coenzyme activity by themselves (**Table 3.1**). The dual effect may be due to their interacting with a site on the enzyme combining with CoA. De-cysteamine CoA (L) nitrile was also found to compete with CoA. These results suggest that analogues with a modified secondary hydroxyl can still combine with the CoA site of the enzyme. However, the affinity of de-cysteamine CoA (L) nitrile for the enzyme was much lower than the D-form analogue suggesting that the D-OH group of CoA contributes significantly to the strength of binding of this coenzyme with the enzyme protein.

$\alpha$ -Methyl CoA,  $\beta$ -methyl CoA and homo CoA in which the cysteamine moiety of CoA is modified all compete with CoA, however the affinities of these analogues were much less than those of desulfo CoA and oxy CoA. The diminished affinity of these analogues suggest that the cysteamine moiety may be significant in the binding strength. This is supported by

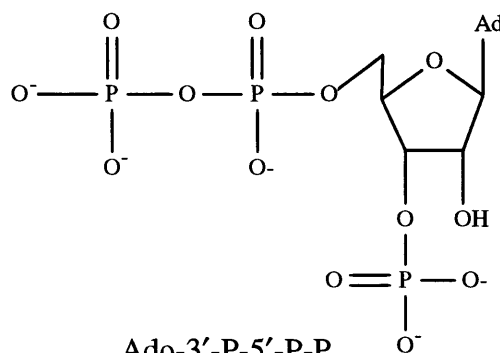




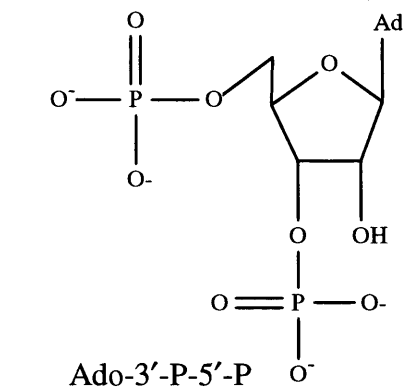
(adenosine 3'-phosphate 5'-pyrophosphate isobutyl ester)



(adenosine 3'-phosphate 5'-pyrophosphate methyl ester)



(adenosine 3'-phosphate 5'-pyrophosphate)



(adenosine 3', 5'-bisphosphate)

**Figure 3.1**

the shift in affinity between de-cysteamine CoA amide and desulfo CoA. Of these analogues only  $\alpha$ -methyl showed any enzymic activity. The lack of activity of  $\beta$ -methyl CoA and homo CoA may be due to steric hindrance near the SH group.

De-adenine inosine CoA (ino CoA), de-adenine guano CoA (guano CoA) and 3'-dephospho CoA (dephospho CoA) showed almost no inhibition of phosphotransacetylase, and only low coenzyme activity (**Table 3.1**). This indicates that modification of the nucleotide moiety resulted in considerable loss of interaction of CoA with the enzyme molecule supporting the view that the nucleotide moiety of CoA is essential for this coenzyme.

From the above results it can be concluded that the complete structure of the CoA molecule is strictly required for full function of the coenzyme. The structure of Ado-3'-P-5'-P-P, which corresponds to the nucleotide moiety has been shown to be important for recognition of CoA by the enzyme. The pantetheine moiety of CoA does not seem to contribute substantially to the specificity of this coenzyme by virtue of the fact that analogues with modified cysteamine and those lacking part of the pantetheine moiety could combine specifically with the CoA site. Moreover, neither pantetheine nor D-pantetheine-4'-phosphate themselves interacted with the enzyme. These results seem to suggest that the pantetheine moiety may not substantially be involved in the specificity of CoA for the enzyme, but instead contribute significantly to the strength of binding of CoA to phosphotransacetylase.

The enzyme CAT offers the advantage over phosphotransacetylase in that the crystal structure of the binary CAT-CoA complex has been solved. Extending the above work to the CAT system therefore allows analysis of kinetic data in conjunction with computer aided molecular modelling which allows interpretation of the data in terms of loss or introduction of specific interactions.

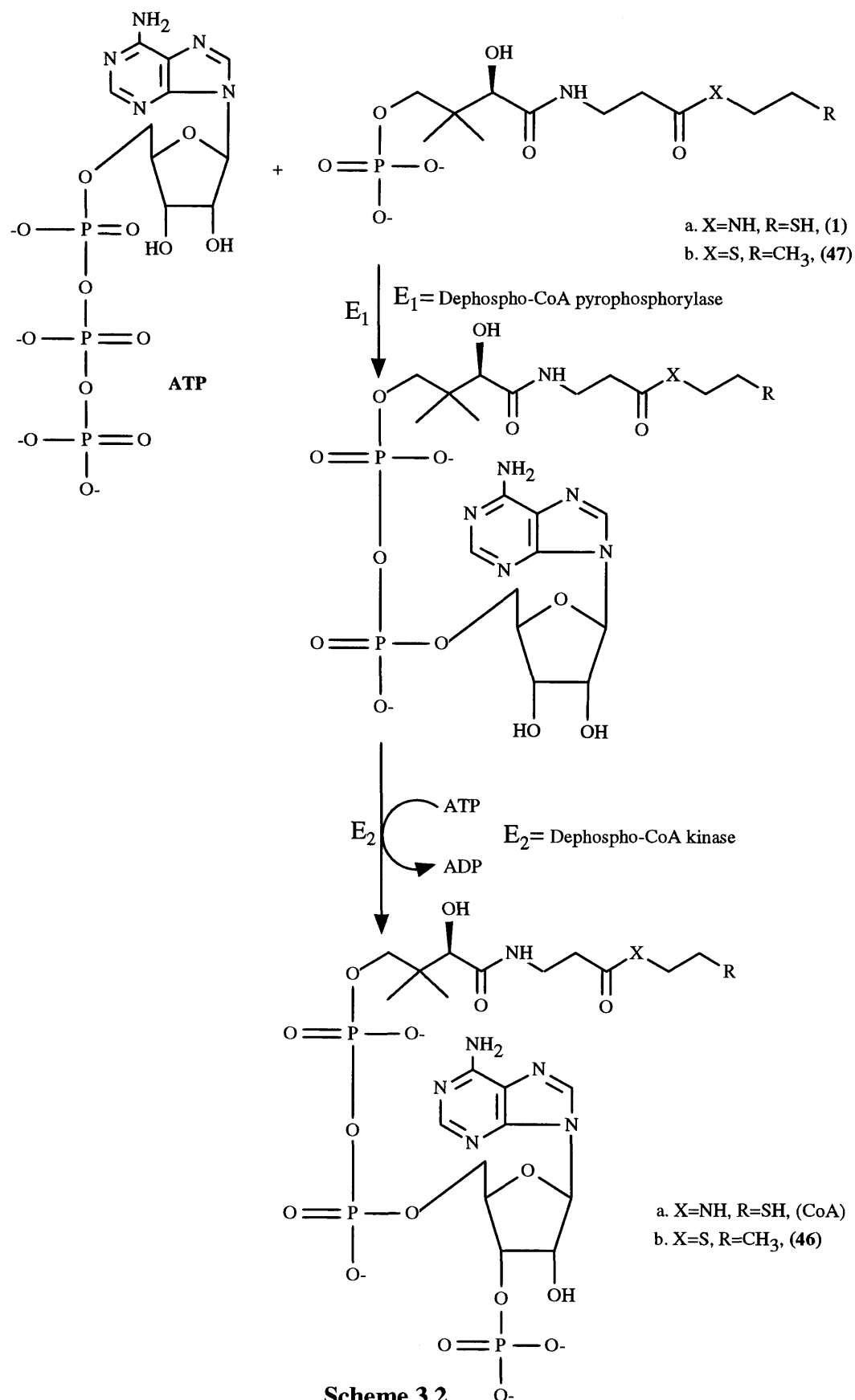
Trivial name	Chemical structure	$K_i \times 10^4$ (M)	Coenzyme activity s acetyl formation (%)
CoA	$\begin{array}{c} \text{OH}(\text{D}) \\   \\ \text{Ado-3'-P-5'-P-P-CH}_2\text{C}(\text{CH}_3)_2\text{-CH---CONH-CH}_2\text{CH}_2\text{-CONH-CH}_2\text{CH}_2\text{-SH} \end{array}$	1.2**	
Oxy-CoA	Ado-----CONH-CH <sub>2</sub> CH <sub>2</sub> -OH	0.010	
Desulfo-CoA	Ado-----CONH-CH <sub>2</sub> CH <sub>3</sub>	0.040	
De-cysteamine-CoA amide	Ado-----CONH-CH <sub>2</sub> CH <sub>2</sub> -CONH <sub>2</sub>	1.1	
De-cysteamine-CoA	Ado-----CONH-CH <sub>2</sub> CH <sub>2</sub> -COOH	2.5	
De-β-alanine-CoA	Ado-----CONH-CH <sub>2</sub> CH <sub>2</sub> -SH	1.8	
De-cysteamine-CoA nitrile	Ado-----CONH-CH <sub>2</sub> CH <sub>2</sub> -CN	2.0	
De-alethine-CoA amide	Ado-----CONH <sub>2</sub>	1.2	
	Ado-3'-P-5'-P-P-CH <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	33	
	Ado-3'-P-5'-P-P-CH <sub>3</sub>	22	
	Ado-3'-P-5'-P-P	43	
CoA(L)	$\begin{array}{c} \text{OH}(\text{L}) \\   \\ \text{Ado-----CH-----SH} \end{array}$	---	5.5
Keto-CoA	$\begin{array}{c} \text{O} \\    \\ \text{Ado-----C-----SH} \end{array}$	—	37
De-cysteamine-CoA(L) nitrile	$\begin{array}{c} \text{OH}(\text{L}) \\   \\ \text{Ado-----CH-CONH-CH}_2\text{CH}_2\text{-CN} \end{array}$	23	
β-Methyl-CoA	$\begin{array}{c} \text{CH}_3 \\   \\ \text{Ado-----CONH-CH}_2\text{CH-SH} \end{array}$	2.5	
α-Methyl-CoA	$\begin{array}{c} \text{CH}_3 \\   \\ \text{Ado-----CONH-CHCH}_2\text{-SH} \end{array}$	3.1	
Homo-CoA	Ado-----CONH-CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> -SH	10	
Inosine-CoA	Ino-----SH		0.4
Guano-CoA	Guo-----SH		0.2
Dephospho-CoA	Ado-5'-P-P-----SH		0.6

Table 3.1

### 3.1.2 Molecular recognition of CoA by citrate synthase

A variety of different methods exist for the synthesis of CoA and CoA analogues,<sup>64,66</sup> however one of the most interesting was the combined chemical and enzymatic approach developed by Drueckhammer *et al* during their work on the molecular recognition of transition state analogues of CoA for the enzyme citrate synthase.<sup>92</sup>

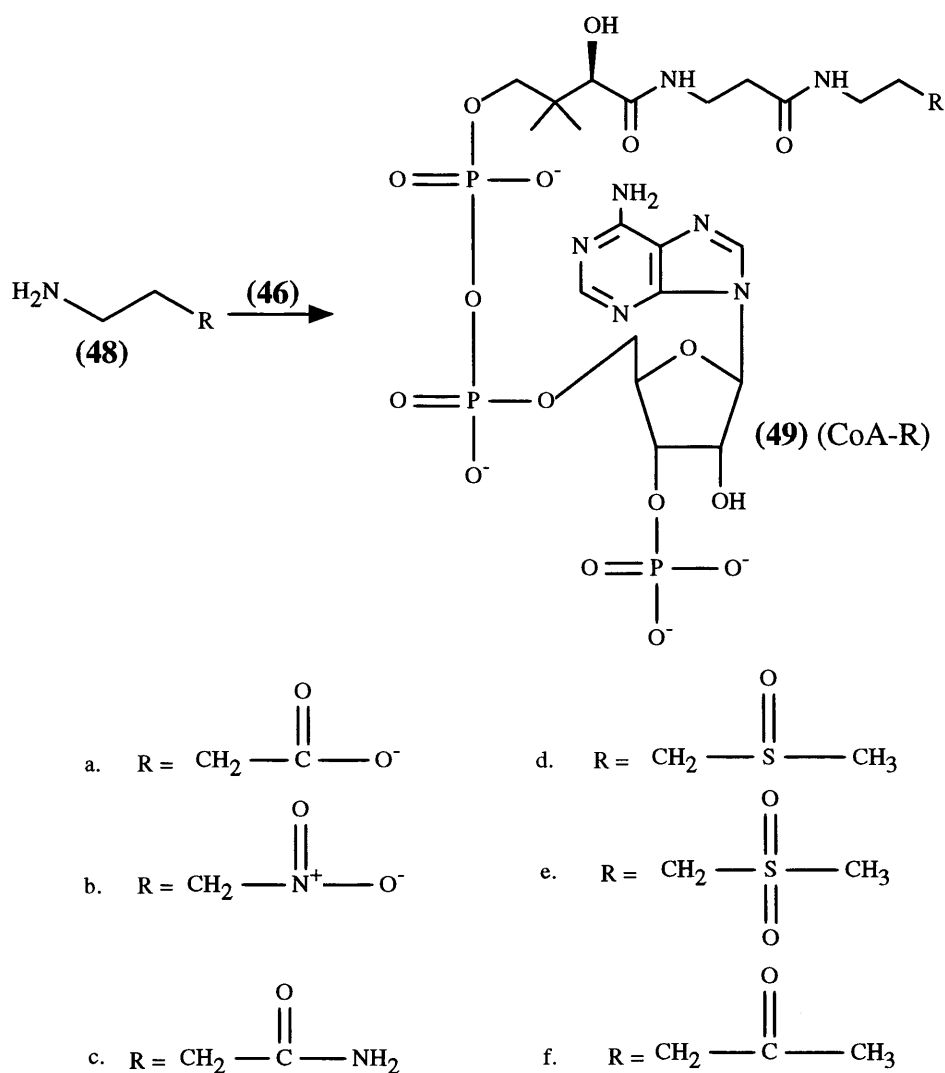
As the coupling of the adenylate and D-pantetheine-4'-phosphate moieties and regiospecific phosphorylation of the 3'-phosphate are the two primary challenges in the chemical synthesis of CoA and its analogues, the enzymes catalyzing the last two steps of CoA biosynthesis (see **Section 2.1**) were used to prepare the CoA analogue (**46**), (**Scheme 3.2**) having an amide replaced by a thioester from the pantetheine analogue (**47**).



It was expected that (47), which differs from the natural substrate by replacement of an amide bond by a thioester and the thiol by a methyl group should serve as an efficient substrate for the enzymes of CoA biosynthesis.

It was envisaged that by aminolysis with a variety of amines (46) would serve as a versatile synthon for a wide range of CoA and CoA ester analogues modified in the thiol/thiolester portion of the molecule. Since the specific functionality of interest is introduced in a final chemical step, each analogue would not be subject to substrate specificity limitations of the CoA biosynthetic enzymes.

Analogues of CoA were synthesised by the reaction of (46) with amines (48a-f) as shown in Scheme 3.3.



**Scheme 3.3**



substrate acetyl CoA suggests that **(49a)** is a good mimic of a high energy intermediate formed in the reaction. As **(49a)** is viewed as good mimic of the enolate form of acetyl CoA, this might appear to support an enolate mechanism. However **(49a)** may alternatively bind in a protonated form as an enol mimic. The  $K_i$  for **(49a)** with citrate synthase has been found to decrease with increasing pH. This is consistent with the binding of **(49a)** in a protonated form as an enol mimic but could also be explained by protonation of an amino acid residue in the enzyme active site.

The nitro analogue **(49b)** is expected to mimic the enolate and not an enol, though it is a less ideal enolate than carboxylate due to its net neutral charge and the lower basicity and hydrogen bonding ability. The lack of strong inhibition by **(49b)** provides evidence against the formation of an enolate intermediate. The amide analogue **(49c)** may be viewed as an enol mimic, with C=O in place of C=CH<sub>2</sub> and NH<sub>2</sub> in place of OH, though it is also possible that it binds as a ground state analogue with NH<sub>2</sub> in place of the methyl group of acetyl CoA. The **(49c)** is not expected to be sufficiently acidic ( $pK_a$ , 15) to form an enzyme bound anion as an enolate mimic. The potent inhibition observed by **(49c)** may be further evidence for an enol intermediate.

### **Binding studies of transition state analogues of CAT**

Drueckhammer briefly extended his studies to the CAT system, the sulfoxide **(49d)** and sulfone **(49e)** were chosen as easily accessible analogues of acetyl CoA designed to mimic the tetrahedral intermediate or transition state in the CAT reaction (**Scheme 1.2**). However the binding affinities for CAT were lower than that of acetyl CoA suggesting that **(49d)** and **(49e)** are not good mimics of the tetrahedral species. A possible explanation for the lack of enhanced activity of the sulfoxide **(49d)** relative to acetyl CoA for CAT is that the methyl group is a poor recognition element for binding. CAT may not recognise the CoA moiety, the sulfoxide oxygen, and the methyl groups as being in a pyramidal arrangement versus the planar-trigonal arrangement of acetyl CoA



With the sulfone (**49e**), potential hydrogen bonding of one oxygen atom to the base (His 195), which serves to deprotonate the hydroxyl nucleophile and the oxyanion binding site were expected to lead to enhanced binding. An explanation for the relatively low affinities of (**49d**) and (**49e**) for CAT is that the sulfoxide and sulfone oxygens are not sufficiently basic or good enough hydrogen bond acceptors to form strong interactions with the enzyme. The carboxylate analogue (**49a**) was found to be an even weaker inhibitor than the sulfoxide and sulfone indicating that a hydrogen bond accepting carboxylate group is not sufficient to mimic the tetrahedral intermediate.

Not surprisingly the ketone analogue (**49f**) had a binding affinity almost identical to that of acetyl CoA. The ketone is presumably not sufficiently electrophilic to form a stable hemiacetal with chloramphenicol in the enzyme active site.

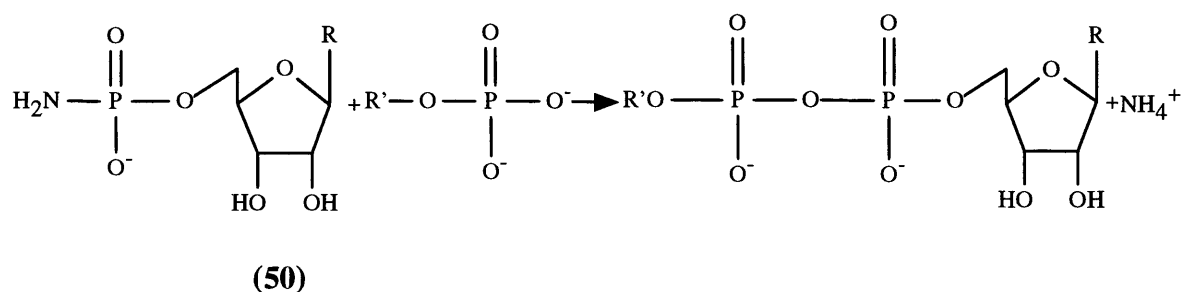
## 3.2 Synthesis of CoA: Khorana

### 3.2.1 Synthesis of activated nucleotides

As mentioned in **Chapter 2** the key targets in the synthesis of CoA were D-pantetheine-4'-phosphate (1) and the activated nucleotide, adenosine-2',3'-cyclic phosphate 5'-phosphomorpholidate (4). The synthesis of (1) was discussed in detail in chapter 2, this section will concentrate on the synthesis of the activated nucleotide (4).

The problems of the synthesis of nucleoside-5' di- and tri-phosphates and the unsymmetrical diesters of pyrophosphoric acid, to which class the nucleotide coenzymes in particular CoA belong, have long been the subject of extensive studies.<sup>94</sup>

Work pioneered by Khorana initially concentrated on nucleoside 5'-phosphoramidates (50) as the reactive intermediates in the specific synthesis of the pyrophosphate bond according to the equation in **Scheme 3.5**.<sup>95</sup>



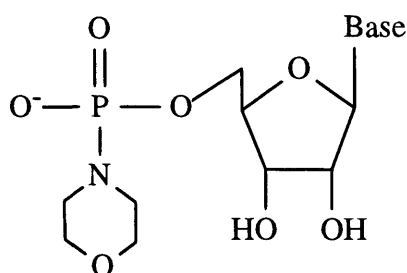
R=purine or pyrimidine

**Scheme 3.5**

The phosphoramidates (50) themselves have been prepared in high yield by the direct condensation of the unprotected ribonucleoside 5'-phosphates with ammonia in the presence of DCC. The application of the nucleoside 5'-phosphoramidates in the synthesis of nucleotide coenzymes was demonstrated by the synthesis of uridine diphosphate glucose and flavin adenine dinucleotide in satisfactory yields.<sup>94</sup> Nevertheless a serious difficulty was encountered in the choice of solvent for the pyrophosphate forming reaction. Pyridine was

found to be by far the preferred medium of reaction but of all the ribonucleoside 5'-phosphoramidates only the uridine derivative was soluble in anhydrous pyridine. Further work continued to investigate nucleoside 5'-phosphoramidates derived from alkyl amines of widely varying basicities in order to find more reactive and more conveniently soluble phosphoramidates.

Studies<sup>95</sup> showed the best compromise between the ease of preparation and reactivity of the amidate was found in the use of the base morpholine ( $pK_a$ , 8.36) which gave the morpholidate (**51**) (Figure 3.2).



Nucleoside 5'-phosphomorpholidate (**51**)

**Figure 3.2**

The general procedure for the preparation of the different nucleoside 5'-phosphomorpholidates consisted of refluxing a solution of the nucleotide, for example adenosine 5'-phosphate, with five equivalents of each of DCC and the amine in 67% aqueous tertiary butyl alcohol. This method is completely general and morpholidates of a variety of nucleotides have been prepared.<sup>95</sup>

The reaction of ribonucleoside-5'-phosphomorpholidates with a second phosphomonoester component in anhydrous pyridine has provided a general method for the synthesis of a variety of nucleotide coenzymes. Khorana has applied this method to synthesis of a variety of coenzymes including uridine diphosphate glucose and uridine diphosphate galactose and yields have been uniformly satisfactory, varying between 65 and 70%.

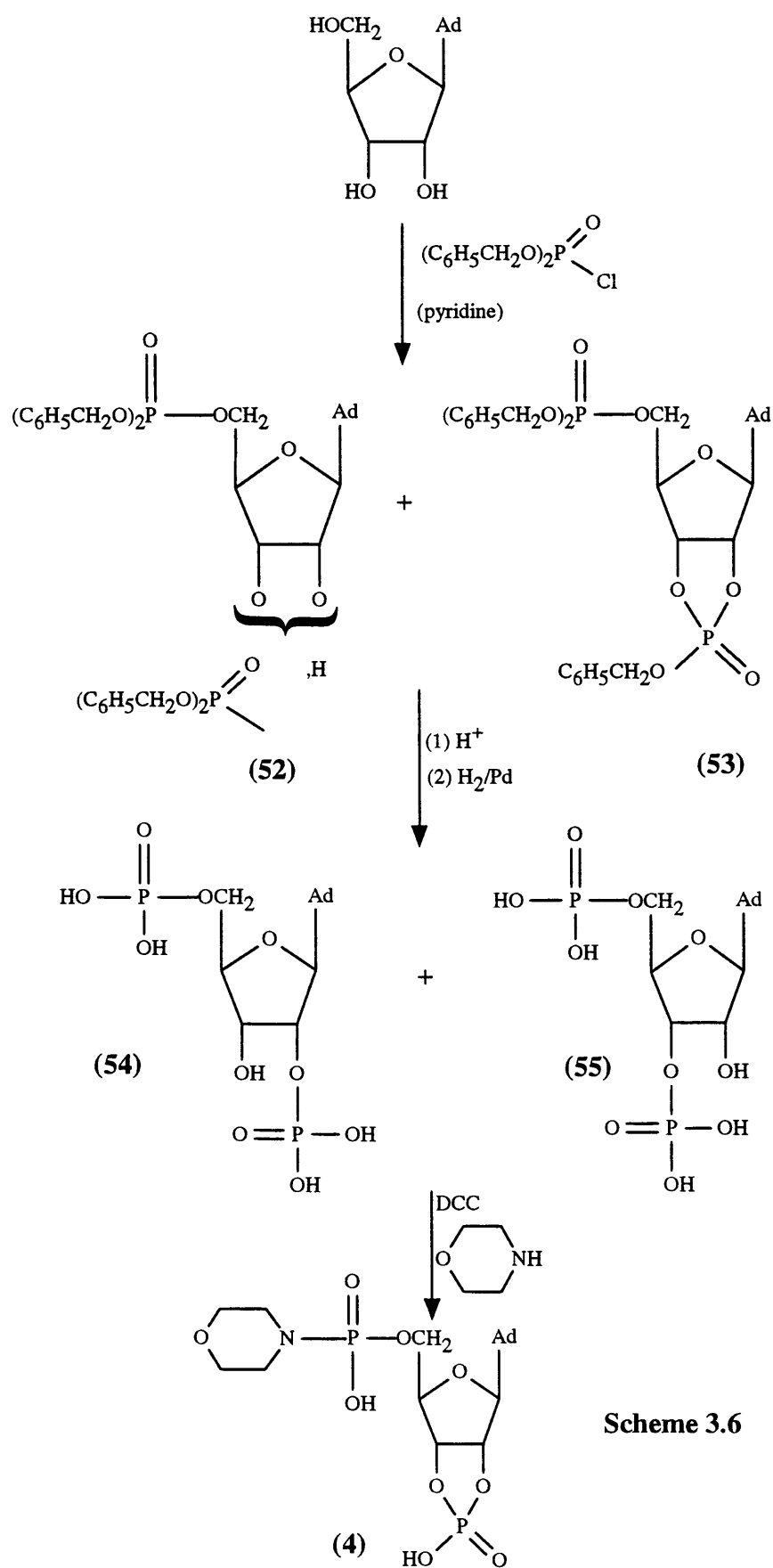
### 3.2.2 Synthesis of adenosine 2',3'-cyclic phosphate 5'-phosphomorpholidate (4)

The final step in the synthesis of the activated nucleotide involves introducing a cyclic phosphate group between the 2' and 3' positions of the ribose ring. **Scheme 3.6** shows the reactions used to prepare this key intermediate (4). Adenosine is phosphorylated with an excess of dibenzyl phosphorochloridate (16) in anhydrous pyridine and the total products (52), (53) are treated with 33% acetic acid to effect partial debenzylation. Subsequent hydrogenolysis and purification gave the diphosphates in approximately 70% yield. The diphosphates (54), (55), as expected contained the 2',5' and 3',5'-isomers in equal amounts. Direct treatment of the diphosphates with DCC and morpholine gave the desired (4) in 91-98% yield.<sup>64</sup>

### 3.2.3 Synthesis of CoA: The problem of the 3'-phosphate

In the original work by Khorana<sup>64</sup> the final step in the synthesis of CoA involved reaction of the bis(4-morpholine *N,N'*-dicyclohexylcarboxamidinium) salt of (4) with three equivalents of D-pantetheine-4'-phosphate in anhydrous pyridine (**Scheme 3.7**). The initial product was anhydro Coenzyme A (56) which was not isolated, instead the total reaction mixture was treated with acid to open the cyclic phosphate ring. Ion exchange of the reaction mixture gave an elution profile which consisted of two major peaks which were identified as iso-Coenzyme A (57) and Coenzyme A. The two products could be resolved quite well by chromatography on an ECTEOLA cellulose anion exchanger. The combined yield of CoA and iso CoA (57) in the synthesis was 65% from (1); of this at least one half was the desired CoA.

The alkaline hydrolysis of adenosine-2',3'-cyclic phosphate had previously been found<sup>96</sup> to give 65-70% of the 3'-phosphate, and it was, therefore, of interest to attempt alkaline ring opening of (56) so as to increase the proportion of the desired CoA. However, the alkaline conditions that were found necessary for the completion of the ring opening in (56) caused

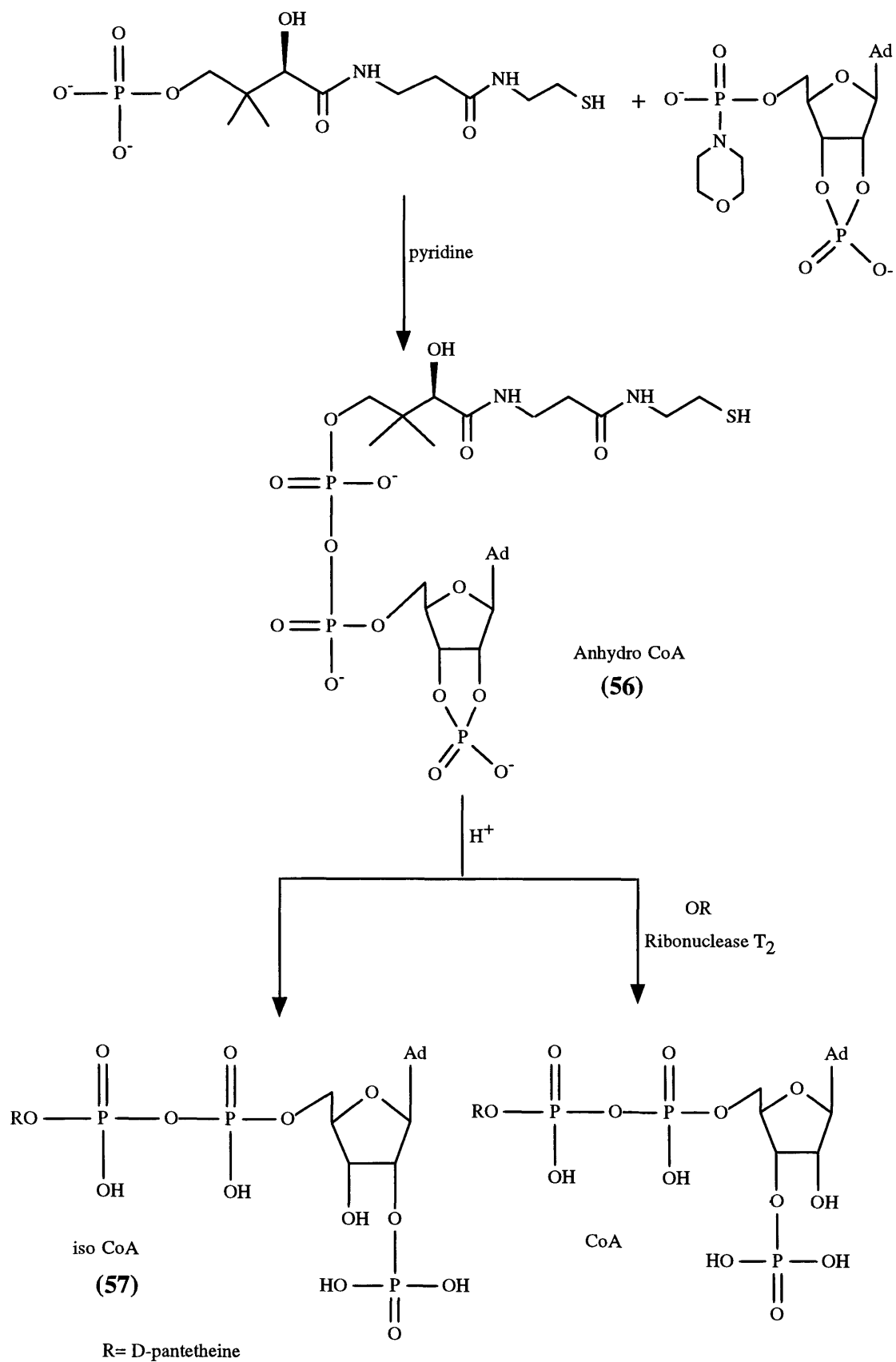


**Scheme 3.6**

Synthesis of adenosine-2',3' cyclic phosphate-5'-phosphomorpholidate (**4**)

considerable breakdown of the pyrophosphate linkage.

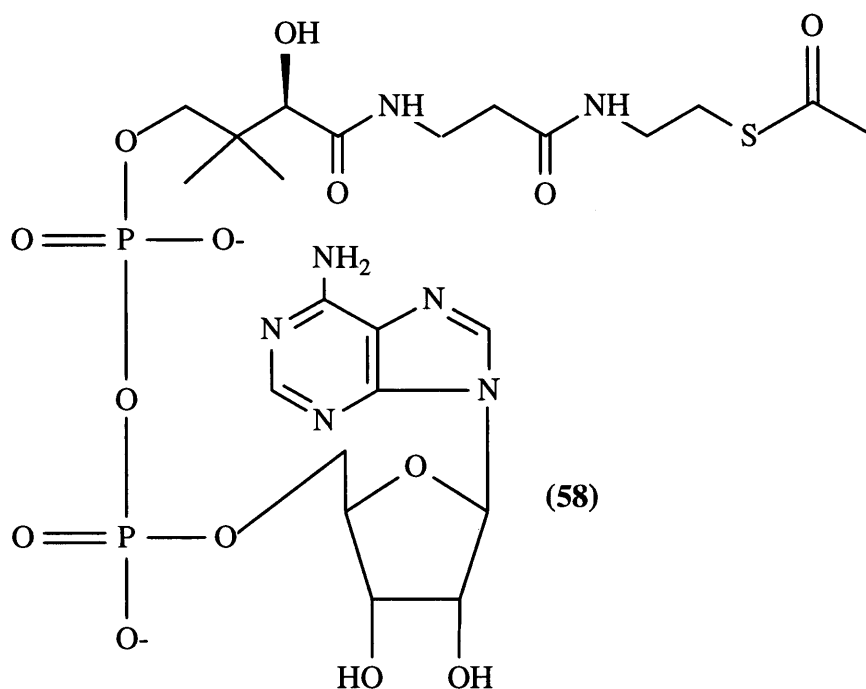
Since chemical hydrolysis of the 2',3'-cyclic phosphate moiety in such compounds gave rise to a mixture of the 2' and 3'-phosphates, methods involving specific enzymic cleavage were sought in an attempt to improve the yield of the desired 3'-phosphate (Michelson).<sup>97</sup> Digestion of adenosine-(2',3'-cyclic phosphate)-5'-phosphate with crude rattlesnake venom had been shown to yield the 3',5'-diphosphate exclusively. A more convenient enzyme, *takadiatase* ribonuclease T<sub>2</sub> is known to split adenosine 2',3'-cyclic phosphate to the 3'-phosphate,<sup>98</sup> and it can be readily obtained free of phosphatase and pyrophosphatase activity. Hydrolysis of adenosine-(2',3'-cyclic phosphate)-5'-pyrophosphate with ribonuclease T<sub>2</sub> yielded adenosine-(3'-phosphate)-5'-pyrophosphate uncontaminated by the 2' isomer and without significant cleavage of the pyrophosphate linkage. Replacing the acid catalysed hydrolysis of the cyclic phosphate employed by Khorana by enzymic cleavage with ribonuclease T<sub>2</sub> considerably improved the yield of the desired CoA (63%).



**Scheme 3.7**

### 3.2.4 Synthesis of S-acetyl 3'-dephospho CoA (58)

As the synthesis and selective enzymic cleavage of the cyclic phosphate is not trivial it was decided to concentrate our efforts on synthesising the synthetically easier target of *S*-acetyl 3'-dephospho CoA (58), (Figure 3.3). The reaction with the commercially available activated nucleotide, adenosine 5'-monophosphomorpholidate (AMP morpholidate) which lacks the 2',3'-cyclic phosphate moiety is analogous to that of CoA synthesis and avoids the problems associated with the 3'-phosphate group. The  $K_M$  for acetyl dephospho CoA is 671  $\mu$ M which is only approximately 7-fold greater than for acetyl CoA.<sup>99</sup> This compromise offers synthetically easier targets which can still give meaningful kinetic results.



Structure of *S*-acetyl 3'-dephospho CoA

**Figure 3.3**

For all subsequent work (except analogues modified to give alkyl groups in place of the acetyl group) the structures of all the analogues synthesised will be based on that of dephospho CoA, ie they will be lacking a phosphate group on the 3'-position of the ribose ring.



### 3.3 Results and discussion

During the course of this work a variety of CoA analogues were prepared for the purpose of molecular recognition studies of CoA by the enzyme CAT. The analogues were divided into three categories; (i) pantetheine based analogues, (ii) alkylCoA analogues, and (iii) nucleotide base analogues.

#### 3.3.1 Pantetheine based analogues of CoA.

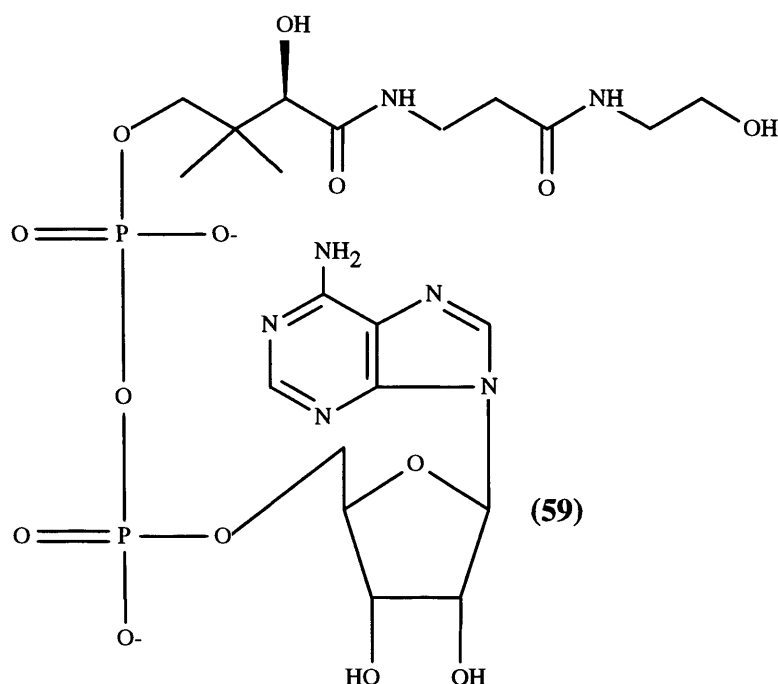
##### *S*-Acetyl dephospho CoA (**58**)

Compound (**58**) is not a pantetheine based analogue but represents the first analogue to be synthesised and the structure that all pantetheine and base analogues are based on.

As mentioned previously (Section 3.2.4) (**58**) lacks a phosphate from the 3'-OH position of the ribose ring. It has been previously prepared from commercially available dephospho CoA isolated from biosynthetic sources and has been shown to be relatively good substrate for the CAT reaction ( $K_M=671\ \mu\text{M}$ ).

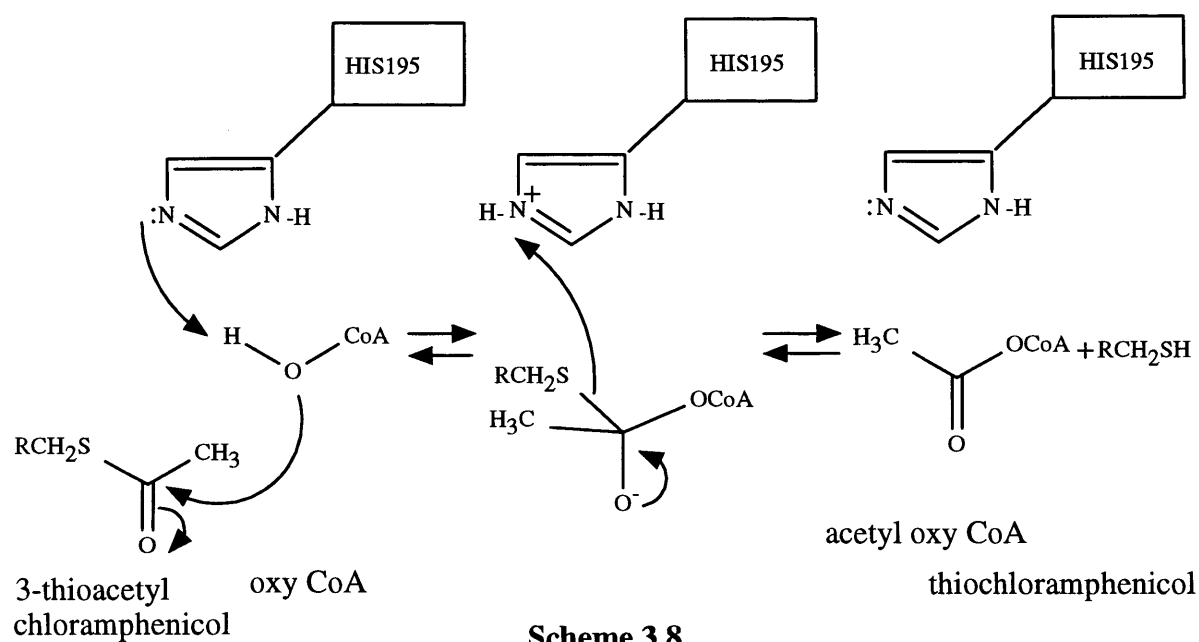
During the course of this work (**58**) was prepared by chemical synthesis to confirm the results obtained by the biosynthetic (**58**). *S*-Acetyl D-pantetheine-4'-phosphate (**15**) prepared by the cyanoethyl phosphate and DCC coupling method was reacted with three equivalents of AMP morpholidate in anhydrous pyridine. The reaction was left to proceed overnight and after removing all traces of pyridine the product (**58**) was purified by HPLC to give a yield of 96%.

### Synthesis of dethio dephospho oxy CoA (59) (Figure 3.4)



**Figure 3.4**

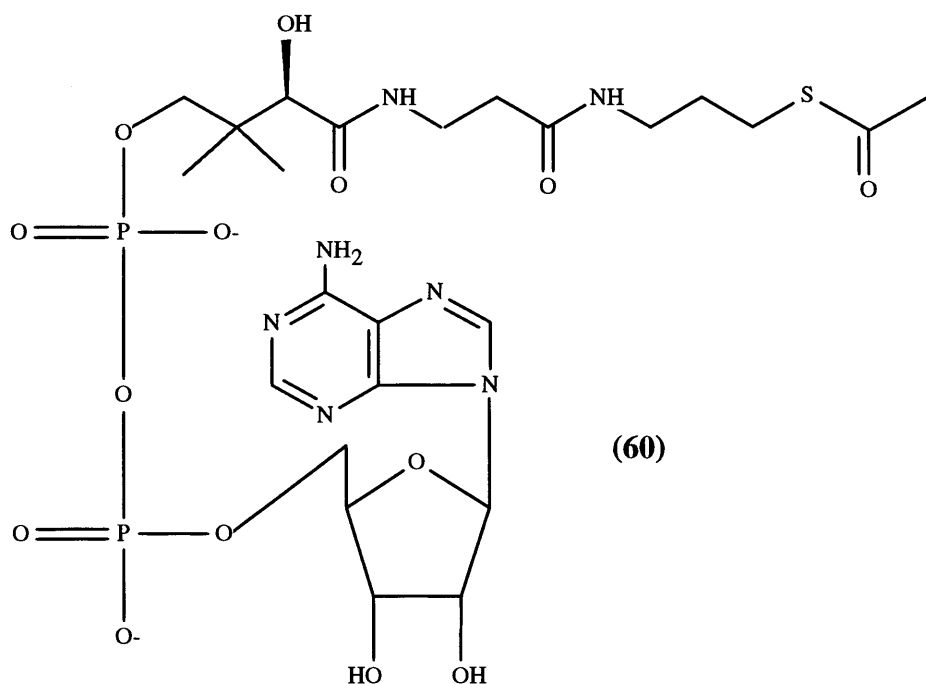
The oxy analogue (59) was prepared to examine its effect on binding to the CoA binding site of CoA. However, there is an interesting application of oxy CoA that as yet remains unexplored. In the normal CAT reaction both substrates are ideally positioned for the forward and reverse reactions. Reversing the functionality of the two substrates (*ie* acetyl CoA to oxy CoA, and chloramphenicol to 3-thioacetylchloramphenicol) inverts the chemistry and potentially moves the hydroxyl group further away from the catalytic histidine (**Scheme 3.8**). It would be interesting to test to what extent the His 195 can catalyse the reaction as catalysis may require movement of the catalytic residue. The chloramphenicol analogue 3-thioacetyl chloramphenicol has been previously synthesised at Leicester and these reverse functionality experiments offer an interesting extension of the current work.



The first step, the synthesis of dethio D-oxypantetheine-4'-phosphate (**29**) was completed as discussed in **Section 2.6.1**. The next step, coupling to the adenosine nucleotide was carried out in an analogous method to dephospho CoA synthesis. Three equivalents of (**29**) were reacted with AMP morpholidate in anhydrous pyridine. The reaction was left to proceed overnight and after all traces of pyridine had been removed the product was purified by DEAE Sephadex ion exchange chromatography and dethio dephospho oxy CoA was obtained in 60% yield as the triethylammonium salt.

### Synthesis of *S*-acetyl dephospho homo CoA (**60**) (Figure 3.5)

For the purpose of this work *S*-acetyl dephospho homo CoA was synthesised to see the effect on binding and catalysis of CoA by increasing the pantetheine chain length by a methylene group in the cysteamine portion.



**Figure 3.5**

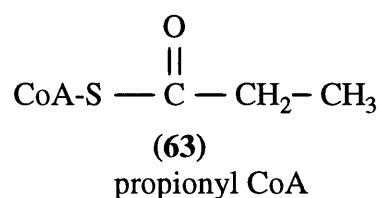
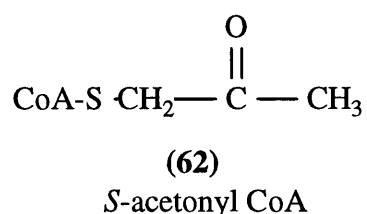
The key intermediate D-homopantethine-4'-phosphate (**40**) was synthesised as discussed in **Section 2.6.2**. As the synthesis of (**40**) involved a multi step synthesis there was a limiting amount of material of available for the next step, coupling to the adenosine nucleotide. In order to make maximum use of the limited amount of material, (**40**) was reacted with six equivalents of AMP morpholidate. The reaction conditions were analogous to that of CoA synthesis, however the reaction which was monitored by  $^{31}\text{P}$  NMR by following the disappearance of the mono-phosphate of (**40**) and appearance of the pyrophosphate of dephospho homopantethine CoA (**61**) took one week to reach completion. After removing the pyridine solvent the product, (**61**), the disulfide form of dephospho homo CoA, was purified by DEAE Sephadex ion exchange chromatography to give a yield of 55% as the triethylammonium salt.

The final step involved reducing the disulfide bond using DDT and acetylating the free thiol using acetic anhydride in an analogous method to acetylation of CoA. Acetylation was complete when the reaction mixture gave a negative DTNB test for free thiols. The final product *S*-acetyl dephospho homo CoA (**60**) was purified by HPLC to give a yield of 10% as

the ammonium salt.

### 3.3.2 Thioester analogues of CoA

Thioether-containing structural analogues of acetyl CoA have been shown to be competitive inhibitors of CoA dependent enzymes. For example *S*-acetonyl CoA (**62**) is a potent inhibitor with respect to acetyl CoA, of citrate synthase, phosphotransacetylase, and carnitine acetyltransferase.<sup>100</sup>

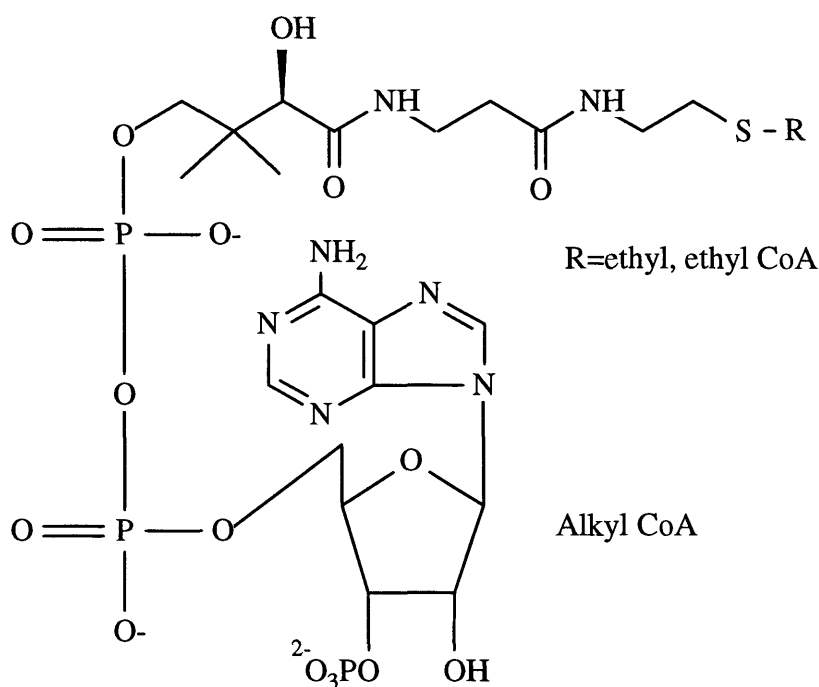


Work by Kleanthous and Shaw<sup>19</sup> showed that propionyl CoA (**63**) is a good substrate for CAT ( $K_M = 167 \text{ M}$ ,  $k_{\text{cat}} = 709 \text{ s}^{-1}$ ) suggesting that the active site can accommodate an extra methylene group without greatly reducing binding. Acetonyl CoA has been shown to be a competitive inhibitor of CAT with respect to acetyl CoA. However the  $K_i$  is more than 8-fold greater than the  $K_d$  for acetyl CoA determined from steady state kinetic analysis suggesting that the position of the substrate carbonyl group has a significant effect on binding.

Reduction of the acetonyl CoA with sodium borohydride produced a mixture of epimers of 2-hydroxypropyl CoA which could perhaps act as a transition state analogue. A mixture of the epimers gave a  $K_i$  which is 25-fold lower than that of the trigonal parent compound (**62**), suggesting that the tetrahedral geometry may allow formation of transition state-like interactions. Enhanced binding of the 2-hydroxypropyl CoA might be expected if the catalytic reaction proceeds via a tetrahedral intermediate, however due to the presence of an extra methylene group in the compound the introduction of novel interactions cannot be ruled out.

*S*-Alkyl CoA compounds (**Figure 3.6**), in particular *S*-ethyl CoA can fully replace AcCoA in

a variety of CoA dependent enzymes as shown by Wieland *et al.*<sup>101</sup> For example ethyl CoA could fully replace AcCoA in avian liver pyruvate carboxylate, an enzyme which is totally dependant on AcCoA for catalytic activity. Similarly methyl CoA and butyl CoA were active but showed markedly diminished effectiveness which is consistent with studies that suggest that the acyl binding part of the regulatory site of the avian liver enzyme optimally accommodates a chain length of two carbon atoms.<sup>102</sup>



**Figure 3.6**

On the other hand, phosphotransacetylase (from *C. kluyveri*) was as expected inhibited by *S*-alkyl CoA analogues (**Table 3.2**). The analogues acted as competitive inhibitors with respect to CoA. From this and work with several AcCoA dependent enzymes it was evident that replacement of the carbonyl group does not significantly alter the specific binding properties of CoA.<sup>101</sup>

Compound	$K_i$ (mM)
Methyl CoA	1.7
Ethyl CoA	1.0
Propyl CoA	1.8
Butyl CoA	2.8
Acetyl CoA	$K_M$ 0.32

Competitive inhibition of phosphotransacetylase by acetyl CoA analogues.<sup>101</sup>

**Table 3.2**

In addition ethyl CoA, a non hydrolyzable thioether analogue of AcCoA has been found to be a competitive inhibitor with respect to AcCoA ( $K_i=100\ \mu\text{M}$ , Day, unpublished results). As it can produce a stable (non productive) ternary complex with chloramphenicol it has proven invaluable in mechanistic studies of CAT.<sup>103,104</sup> As part of this present work a series of analogues of alkyl CoA were prepared with increasing chain length (Methyl-Hexyl). Inhibition studies were carried out to see to what extent the CoA binding pocket, which is remarkably tolerant to increased chain length, could accommodate these analogues.

This work is similar to studies by Paul<sup>105</sup> to study the binding of chloramphenicol to CAT. A series of analogues were prepared in which the *p*-nitrophenyl group of chloramphenicol was replaced by alkyl groups of increasing chain length (Methyl-Hexyl) to probe the incremental binding energy derived from this part of the molecule. Results showed that there was a linear free energy relationship between the specificity constant,  $k_{cat}/K_M$ , of the ligands and the hydrophobicity of the alkyl substituent. The incremental apparent binding energy for each methylene group bound in the aryl binding pocket was estimated to be  $1.0\ \text{kcal mol}^{-1}$  and it was proposed that this binding site could only enclose an alkyl chain up to  $C_4$ .

The alkyl CoA analogues were prepared by the general method used by Wlassics and Anderson<sup>106</sup> for ethyl CoA synthesis (**Table 3.3**). The alkyl iodide (3.5 mol equiv) in ethanol was added to a solution of CoA and  $\beta$ -mercaptoethanol (2.5 mol equiv) in  $\text{KHCO}_3$  (pH 10). The reaction was complete when a negative test for unreacted thiol was obtained. The

unreacted alkyl iodide was extracted into ethyl acetate and the CoA thioether was purified by reverse phase HPLC. The concentration of alkyl CoA was determined spectrophotometrically by assuming that the extinction coefficient,  $\epsilon=15\,000\text{ cm}^{-1}\text{ M}^{-1}$ .

**Table 3.3**

Alkyl Group	Yield (%)
Me ( <b>64</b> )	81
Et ( <b>65</b> )	85
Pr ( <b>66</b> )	75
Bu ( <b>67</b> )	69
Pentyl ( <b>68</b> )	34
Hexyl ( <b>69</b> )	19
Isobutyl ( <b>70</b> )	16
Benzyl ( <b>71</b> )	62

### 3.3.3 Base Analogues

Studies of metabolic pathways and biosynthetic mechanisms have lead to the discoveries of a large number of unsymmetrical pyrophosphates derived from each of the four commonly occurring ribonucleoside 5'-phosphates. The type of biochemical functions that the different compounds perform appears to be determined by the nature of the ribonucleoside component; for example, uridine and guanosine nucleotides are largely involved in carbohydrate metabolism and cytidine compounds in phospholipid biosynthesis. Adenosine in the form of CoA is largely involved in acetyl transfer reactions. No examples of CoA with an alternative base exist in nature, guano CoA and ino CoA the only two to be synthesised to date were used by Shimizu *et al* to show the importance of base recognition of CoA binding to

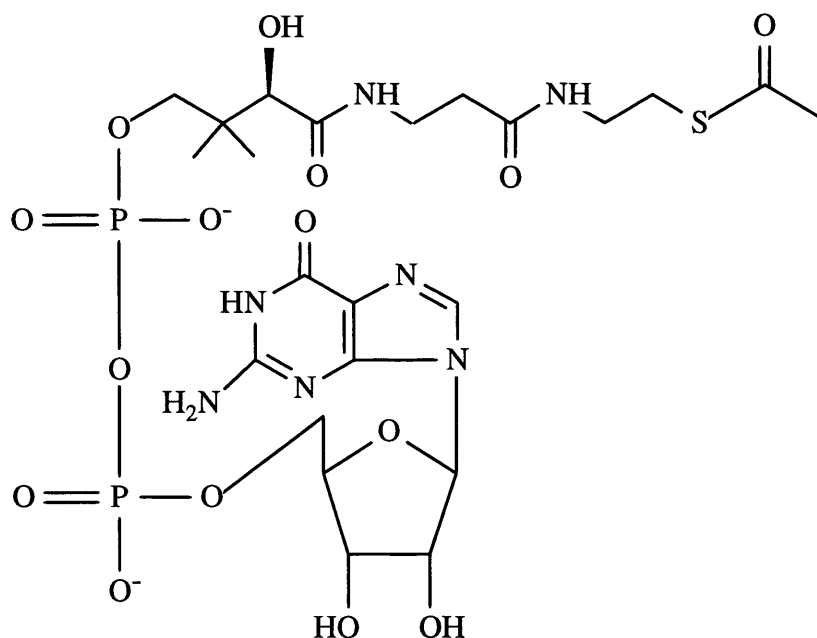


phosphotransacetylase.<sup>93</sup>

A series of base analogues of dephospho CoA were synthesised to see if CAT unlike phosphotransacetylase could tolerate modification in the base region of CoA.

### ***S*-Acetyl dephospho guano CoA (72) (Figure 3.7)**

Compound (72) differs from AcCoA in that the adenosine moiety has been replaced by guanosine. The main differences between the two molecules is that the amino group at C<sub>6</sub> of adenosine which can act as a two hydrogen bond donator is replaced by a one hydrogen bond accepting carbonyl group. Also guanosine introduces an amino group at C<sub>2</sub>, this has the potential to introduce novel interactions, however, unfavourable steric interactions cannot be ruled out. N<sub>1</sub> of the ring has also gained a hydrogen atom which has the potential to form a hydrogen bond.



*S*-Acetyl dephospho guano CoA (72)

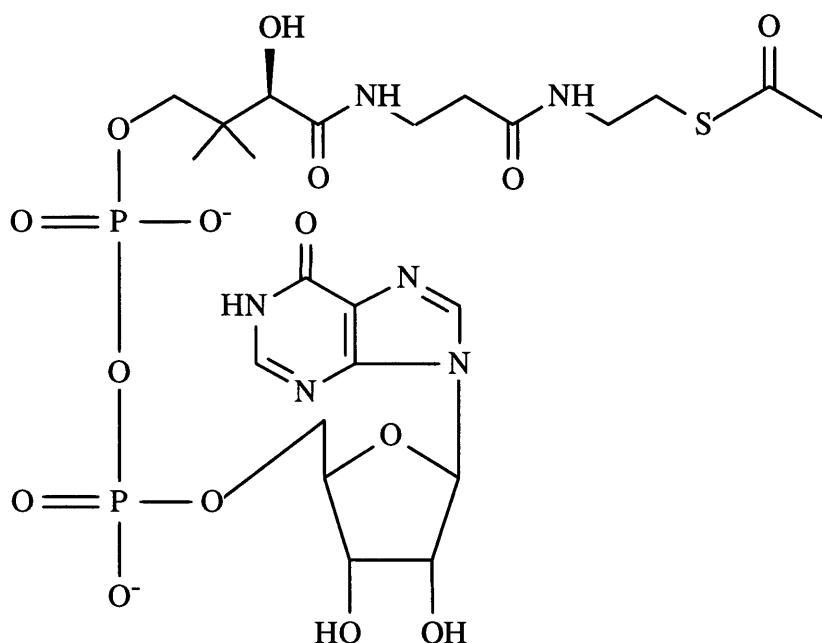
**Figure 3.7**

Compound (72) was synthesised in an analogous method to *S*-acetyl dephospho CoA: three

equivalents of *S*-acetyl D-pantetheine-4'-phosphate were coupled to the commercially available guanosine 5'-monophosphomorpholidate in anhydrous pyridine. The reaction was left to proceed overnight and after all traces of pyridine had been removed the product was purified by DEAE Sephadex ion exchange to give a yield of 17.5% as the triethylammonium salt.

***S*-Acetyl dephospho ino CoA (73) (Figure 3.8).**

The main differences between the inosine analogue of CoA (73) and CoA are the introduction of a carbonyl group at C<sub>6</sub> and a hydrogen atom at N<sub>1</sub>. As with guanine the introduction of the carbonyl removes a group which can potentially act as a two hydrogen bond donor and replaces it with a group capable of acting as a one hydrogen acceptor.



*S*-Acetyl dephospho ino CoA (73)

**Figure 3.8**

The final step in the synthesis of (73) involves coupling (15) with inosine 5'-monophosphomorpholidate (IMP morpholidate) in anhydrous pyridine. The morpholidate is not commercially available so had to be prepared prior to the coupling step. It was

Chemical reaction scheme showing the synthesis of a phosphoramidite derivative. The reactant is a nucleoside with a phosphate group (O=P(O<sup>-</sup>)(O<sup>-</sup>)-O-), a ribose sugar, and an inosine base. It reacts with DCC and morpholine to form a phosphoramidite intermediate where the phosphate is linked to a morpholine ring.

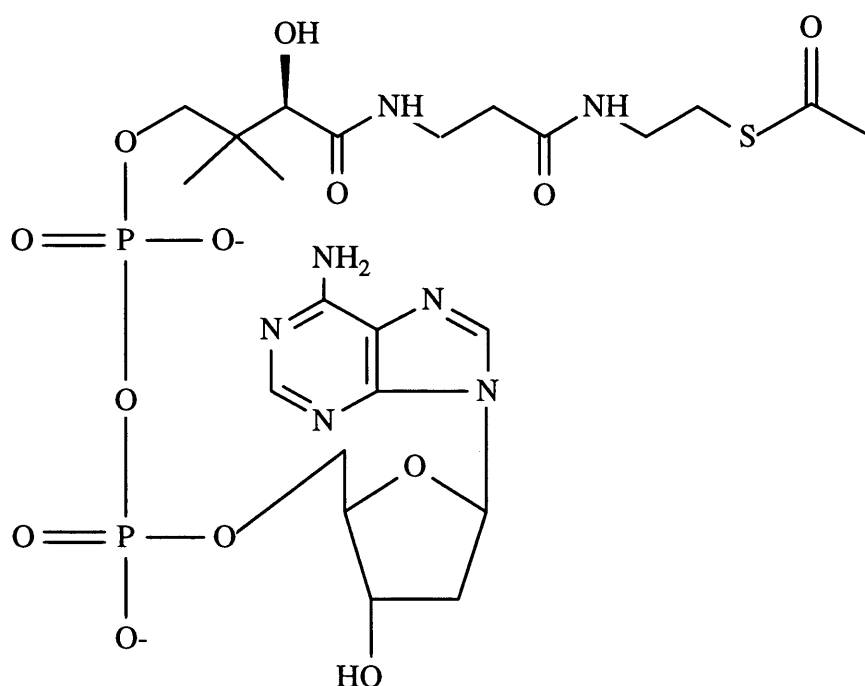
After removal of the <sup>t</sup>butyl alcohol the product was purified by DEAE sephadex ion exchange to give a yield of 97% for the morpholidate as the triethylammonium salt. Coupling to **(15)** was carried out in an analogous method to the other base analogues and the final yield of **(73)** after purification by HPLC was 80% as the ammonium salt.

CC(=O)SCCCNC(=O)CCNC(=O)[C@H](O)C(C)(C)OP(=O)([O-])OP(=O)([O-])O[C@@H]1O[C@H](c2c(C)nc(=O)[nH]c2=O)[C@H](O)[C@H]1O

94

The base analogues prepared by Shimizu *et al*<sup>93</sup> for the phosphotransacetylase studies contained the purine bases guanosine and inosine. No examples of pyrimidine CoA compounds exist in nature and no such compounds have been previously synthesised for the purpose of inhibition studies of CoA dependent systems. As the adenine ring forms part of the pantetheine binding site it was expected that (74) which contains thymidine in place of adenosine would bind with a much lower affinity than the guano and ino CoA analogues both of which contain purine bases. Compound (74) was prepared by an analogous method to the other base analogues of CoA. Three equivalents of (15) were coupled with the commercially available thymidine 5'-monophosphomorpholidate in anhydrous pyridine. The product was purified by HPLC to give (74) in 75% yield as the ammonium salt.

**S-Acetyl 2'-deoxy 3'-dephospho CoA (75) (Figure 3.10)**



*S*-Acetyl 2'-deoxy 3'-dephospho CoA (75)

**Figure 3.10**

The ribose moiety, in particular the 2'-OH has proven to be important in the binding of adenosine 5'-monophosphate to both the enzymes threonine deaminase and glycogen

phosphorylase.<sup>107</sup> In the CAT system however the 2'-OH group forms no direct interaction with the enzyme but does form a hydrogen bond via an ordered water molecule to the main chain amide of residue of residue 55 (see **Section 1.6.2**). The analogue *S*-acetyl 2'-deoxy 3'-dephospho CoA in which the 2'-OH has been replaced by a hydrogen atom was prepared in order to study the contribution to binding made by the 2'-hydroxyl of the ribose ring of the CoA molecule.

The synthesis was analogous with the other base analogues, 2'-deoxyadenosine 5'-monophosphomorpholidate which is commercially available was coupled with three equivalents of **(15)** in anhydrous pyridine. The product was purified by HPLC to give **(75)** in 69% yield as the ammonium salt.

### 3.4 Conclusions.

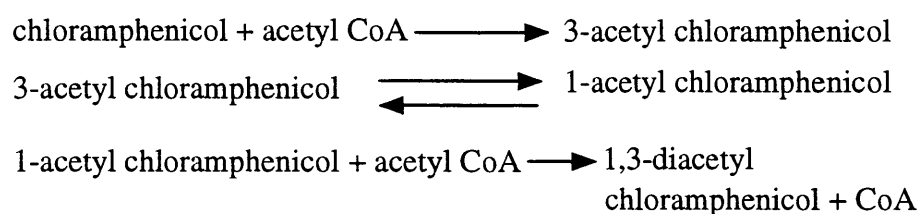
The versatile synthesis of CoA developed by Khorana<sup>64</sup> has allowed the synthesis of a variety of CoA analogues. However due to the problems associated with introducing the 3'-phosphate to the ribose ring synthetically easier targets based on dephospho CoA were prepared instead. A combination of CoA syntheses developed by Drueckhammer<sup>85</sup> and Khorana<sup>64</sup> were used to synthesise both dephospho oxy CoA and *S*-acetyl dephospho homo CoA. The general method for alkyl CoA synthesis developed by Wlassics and Anderson<sup>101</sup> allowed the synthesis of a series of alkyl CoA analogues while the general method for nucleoside 5'-phosphomorpholidate synthesis developed by Khorana<sup>64</sup> provided a route for the synthesis of base analogues of CoA.

**CHAPTER 4**  
**KINETIC STUDIES**

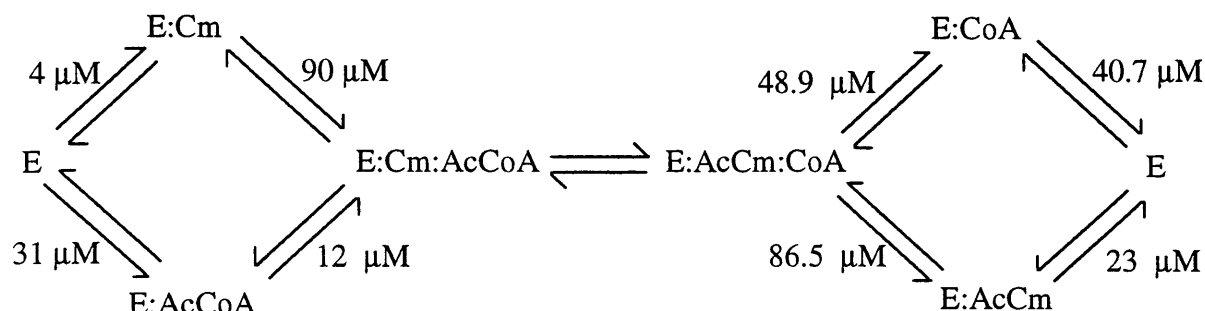
## RESULTS AND DISCUSSION

### 4.1 Introduction

As mentioned in chapter 1, CAT catalyses the conversion of the antibiotic chloramphenicol to several acetylated products through a combination of enzymic and non-enzymic steps. The initial reaction is that of the acetyl CoA-dependant acetylation of the C-3 hydroxyl of chloramphenicol. A non enzymic rearrangement involving an intramolecular acyl transfer converts the product to 1-*O*-acetyl chloramphenicol which can serve as a substrate for the second round of acetylation at the C-3 hydroxyl to yield 1,3-diacetyl chloramphenicol.



In a sense, the second and third reactions above are unnecessary complications arising from the 1,3-diol structure of chloramphenicol and the acyl migration that takes place *in vivo* and *in vitro*. The first step, the acetyl CoA dependant acetylation of the 3-hydroxyl of chloramphenicol has been analysed by steady-state kinetics.<sup>19,104</sup> The use of alternative acyl donors and acceptors, as well as natural substrates, has yielded data that favour the view that the reaction proceeds to the formation of a ternary complex by a rapid equilibrium mechanism wherein the addition of substrates may be random but a preference for AcCoA as the leading substrate can be detected. The scheme showing the possible order of binding of substrates to CAT is shown in **Figure 4.1**, the binding constants for each of the binary and ternary complexes have been previously determined by Ellis *et al*<sup>104</sup> and Lewendon (unpublished results).

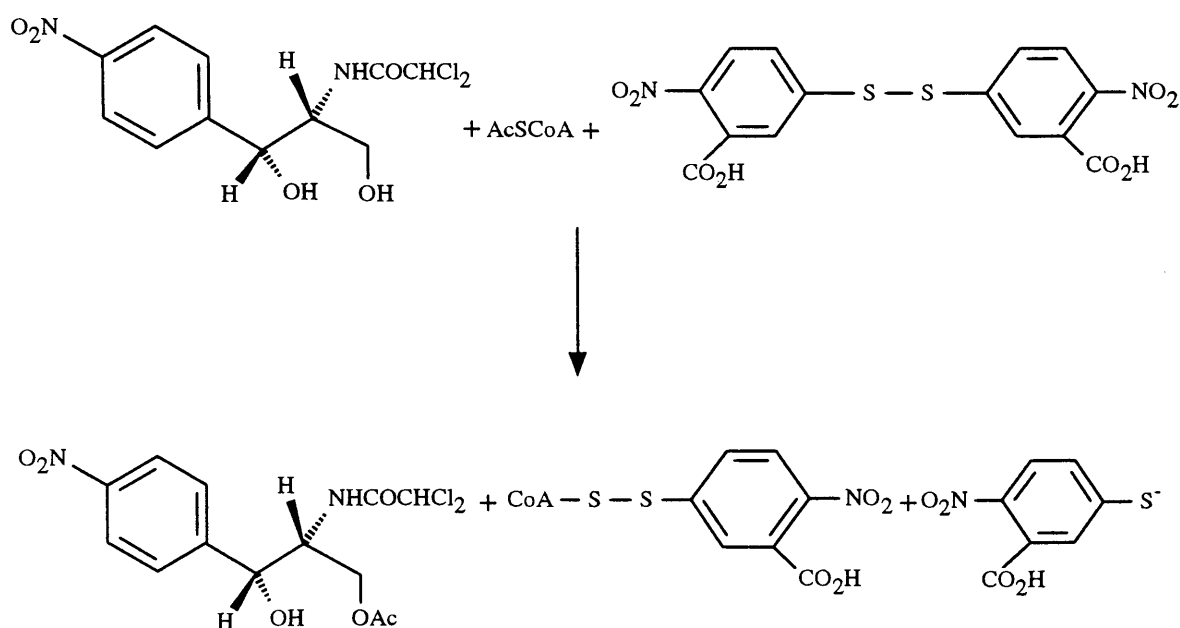


**Figure 4.1**

An examination of the reaction catalysed by the type III CAT variant by Ellis<sup>104</sup> which included the measurement of individual rate constants by stopped-flow fluorimetry found that both product release and ternary complex interconversion are likely to be involved in rate determination of the CAT<sub>III</sub> reaction.

## 4.2 Kinetic studies

The rate of 3-*O*-acetylation of chloramphenicol by the CAT catalysed reaction and assay of CAT activity was measured by UV spectrophotometry by measuring the release of CoA by its reaction with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), as shown in **Scheme 4.1**.<sup>108</sup>



**Scheme 4.1**



As shown above, reaction of CoASH with DTNB yields 1 equiv. of 3-carboxy-4-nitrophenyl sulfide, this has a molar extinction coefficient of 13,600 at 412 nm, and the rate of reaction is measured by monitoring the increase in absorbance at this wavelength.

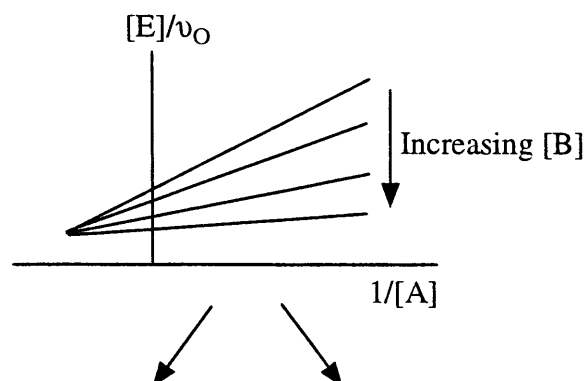
Kinetic coefficients in the initial rate equation [4.1]<sup>19</sup> were calculated from linear intercept and slope replots from computer generated (GrafIt) double-reciprocal plots.

$$\frac{[E]}{v_o} = \phi_o + \frac{\phi_A}{[A]} + \frac{\phi_B}{[B]} + \frac{\phi_{AB}}{[A][B]} \quad \dots[4.1]$$

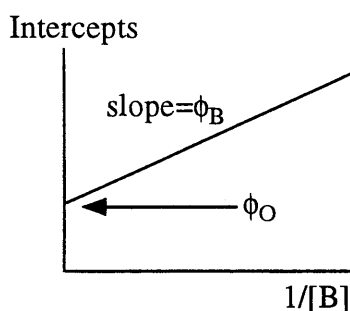
In equation [4.1], [E] is the monomer concentration of active enzyme, calculated from the CAT standard assay (see **Section 5.3.3**);  $v_o$  is the linear initial rate determined from each assay; [A] is the chloramphenicol concentration; and [B] is the AcCoA analogue concentration. Each double-reciprocal plot was constructed from a matrix of four chloramphenicol and four coenzyme analogue concentrations, and all reactions were performed in triplicate resulting in a total of 48 assays per substrate.

In order to determine the  $K_M$  and  $k_{cat}$  values for each substrate three graphs were plotted (**Figure 4.2**).

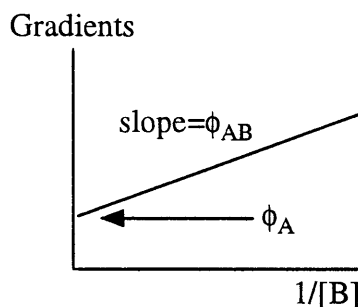
Graph 1 **Primary double-recipricol plot**



Graph 2 **Intercept replot**



Graph 3 **Gradient replot**

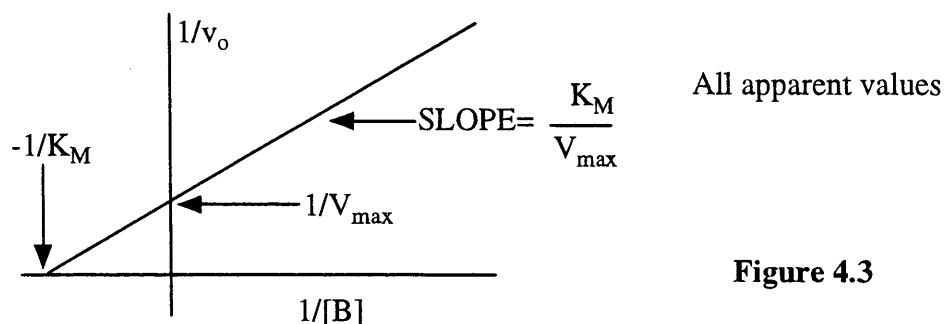


**Figure 4.2**

Graph 1 was a plot of  $[E]/v_o$  against  $1/[A]$ ; Graph 2 was a plot of the intercepts from Graph 1 against  $1/[B]$ ; and Graph 3 was a plot of the gradients from Graph 1 against  $1/[B]$ . The kinetic coefficients  $\phi_o$  and  $\phi_A$  are equal to the intercept of the plot from Graph 2 and Graph 3 respectively. From these values the kinetic parameters  $K_M$  and  $k_{cat}$  can be calculated, as shown by [4.2].

$$\text{For substrate A, } k_{cat} = \frac{1}{\phi_o} \quad \& \quad K_M = \frac{\phi_A}{\phi_o} \quad \dots[4.2]$$

In certain cases, where there was only a limited amount of CoA analogue available it was not possible to perform a full 4×4 kinetic analysis so apparent  $K_M$  and  $k_{cat}$  values were determined. This was achieved by plotting one graph (**Figure 4.3**) of  $1/[B]$  against  $1/v_o$  at constant chloramphenicol concentration (100  $\mu\text{M}$ ).

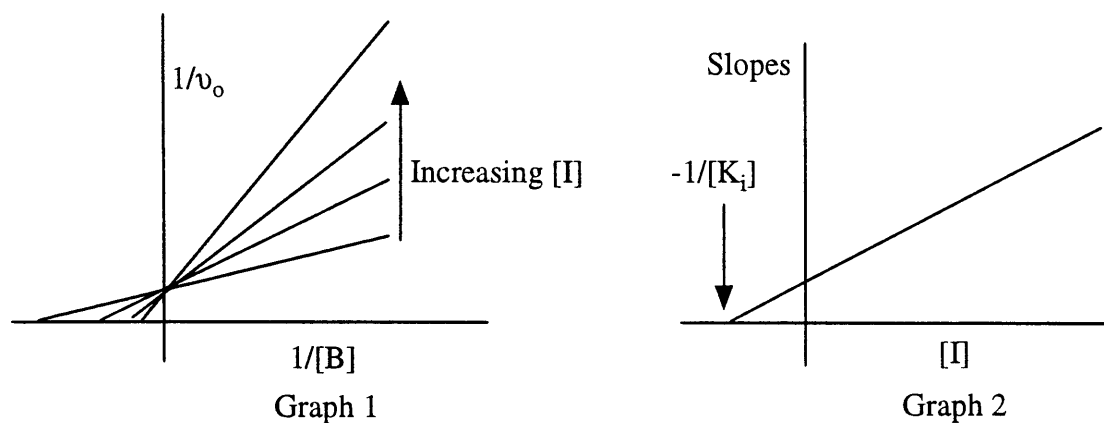


**Figure 4.3**

The apparent  $K_M$  was easily determined from the graph whereas the apparent  $k_{cat}$  was determined from equation [4.3].

$$\frac{[E]}{V_{max}} = \frac{1}{k_{cat}} \quad \dots[4.3]$$

In order to determine the binding constants of inhibitors ( $K_i$ ), two graphs were plotted (**Figure 4.4**): Graph 1 was a plot of  $1/v_o$  against  $1/[B]$  at constant chloramphenicol concentration ( $100 \mu M$ ); Graph 2 was a plot of the gradients from Graph 1 against  $[I]$ .



**Figure 4.4**

### 4.3 Analysis of kinetic results and estimation of incremental binding energies

$k_{\text{cat}}/K_M$  (also referred to as the "specificity constant") is the apparent second-order rate constant for the conversion of free enzyme and substrate to enzyme and products. The contribution to binding,  $\Delta\Delta G_{\text{app}}$ , of a substituent R can be measured by comparing values of  $k_{\text{cat}}/K_M$  for the two substrates R-S and H-S. In **Section 1.1.3** it was shown that  $\Delta\Delta G_{\text{app}}$  could be calculated by a comparison of the dissociation constants,  $K_s$ , of the two substrates, [1.4]. It is however, usually more reliable to use the alternative equation [4.4] because the quantity  $k_{\text{cat}}/K_M$  includes both the activation energies and the binding energies, and avoids the underestimates that may result from using  $K_s$  values only.<sup>6a,109</sup>

$$\Delta\Delta G_{\text{app}} = -RT \ln \left[ \frac{(k_{\text{cat}}/K_M)_{(\text{R-S})}}{(k_{\text{cat}}/K_M)_{(\text{H-S})}} \right] \quad \dots[4.4]$$

In order to make a quantitative analysis of an individual protein/ligand interaction the group being investigated is usually replaced by a proton. If the group is replaced by another substituent then the apparent binding energy could also include possible favourable and/or unfavourable interactions according to the precise substitution. In [4.4],  $-\Delta\Delta G_{\text{app}}$  is the incremental Gibbs free energy of transfer of R from enzyme to water, relative to H.

## 4.4 Kinetic results

### 4.4.1 Base analogues

One of the major questions in the study of CoA recognition is why does CoA require such a complicated structure to perform simple acetylation reactions. An examination of its structure shows that it is composed of a highly functionalised chain connected to an adenine nucleotide via a pyrophosphate bond. Walsh<sup>17</sup> during his studies on thiolase found that substituting the nucleotide with the hydrophobic pivaloyl group only had modest effects on binding and catalysis. Although crystallographic data is not available, the most likely explanation is that the nucleotide moiety is located in a local hydrophobic environment which allows the pivaloyl group to form hydrophobic interactions with the enzyme. Such pivaloyl analogues have been shown to be poor substrates for CAT.<sup>24</sup> Crystallographic studies have shown that not only does the adenine nucleotide form hydrogen bonds with the enzyme but it is also forms part of the pantetheine binding pocket. Work on another CoA utilising enzyme, phosphotransacetylase,<sup>93</sup> has shown that in this enzyme the adenine nucleotide is also essential for recognition by the enzyme. Results of kinetic studies (**Chapter 3**) showed that adenosine-3'-phosphate-5'-pyrophosphate inhibited phosphotransacetylase competitively with CoA while the nucleotide analogues of ino CoA, guano CoA, and dephospho CoA showed almost no inhibition and only low coenzyme activity. In the present study the molecular recognition of the adenine nucleotide moiety during the binding process of CoA by CAT was investigated. In analogous work to that performed by Shimizu on phosphotransacetylase,<sup>93</sup> CoA analogues containing alternative nucleotide bases were prepared (**Section 3.3.3**) and their kinetic parameters as substrates were determined, (**Table 4.1**).

**Table 4.1**

	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$K_{\text{M}}$ ( $\mu\text{M}$ )	$k_{\text{cat}}/K_{\text{M}}$
AcCoA	600	93	6.45
(58)	1370	606	2.26
(58)*	550	671	0.83
(73)	105	87900	0.0012
(72)	76.9	6109	0.01
(74)	1149	2111	0.54
(75)	1042	381	5.25

\* values obtained by Day *et al* using enzymically prepared (58)<sup>99</sup>

It must be noted that the relatively low amounts of CoA analogues available for kinetic analysis allowed only a limited set of kinetic experiments to be conducted. As a result it was not possible to estimate both slope and ordinate intercepts of the Lineweaver-Burk plots with reasonable precision. Because kinetic experiments could not be determined in triplicate as usual there are likely to be larger errors on these measurements as compared to the  $K_{\text{M}}$  and  $k_{\text{cat}}$  for the natural substrates.

#### 4.4.2 S-Acetyl dephospho CoA (58)

If one first considers (58) the CoA analogue in which the 3'-phosphate group was replaced by a hydroxyl group it is evident that unlike with phosphotransacetylase it is a relatively good substrate for CAT (**Table 4.1**). Compared to AcCoA the  $K_{\text{M}}$  of chemically synthesised (58) was found to increase by a factor of 6.5 while the  $k_{\text{cat}}$  was approximately doubled. The specificity constant ( $k_{\text{cat}}/K_{\text{M}}$ ) for (58) compared to AcCoA is reduced by a factor of 3 which corresponds to a  $-\Delta\Delta G_{\text{app}}$  of  $0.62 \text{ kcal mol}^{-1}$  for the 3'-phosphate group. This value is rather small but is consistent with previous findings (see **Section 1.6.2**) that the 3'-phosphate group

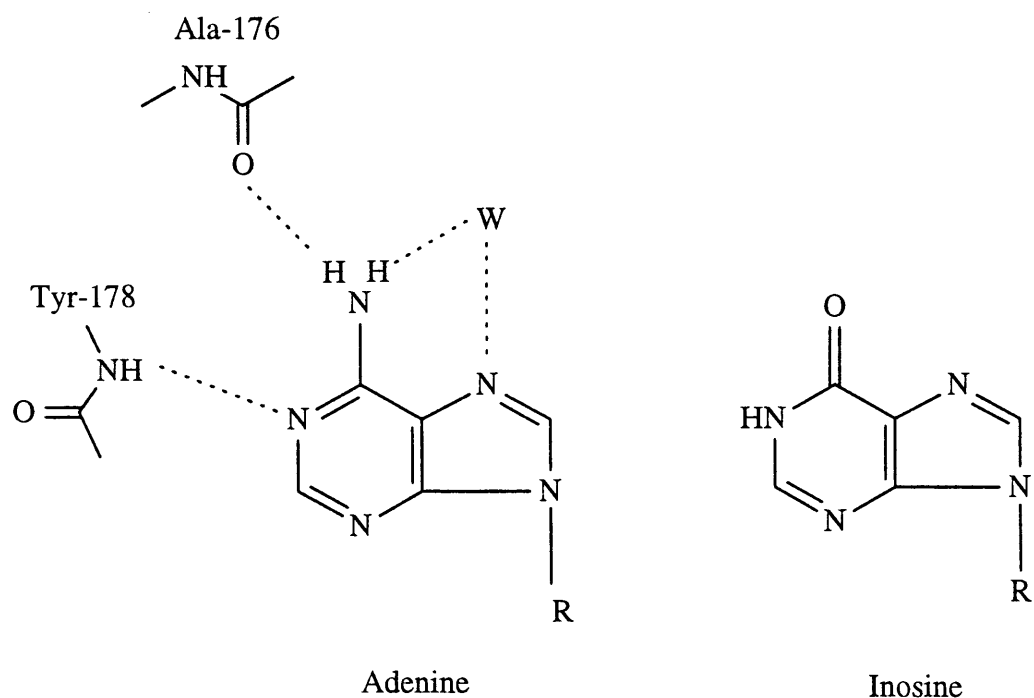
is not directly involved in any ion pairs but instead makes only long range electrostatic interactions with the  $\epsilon$ -amino group of Lys 54. The results tend to suggest that the conformation of **(58)** is similar to that of bound AcCoA. A change in conformation could perturb other interactions between the enzyme and substrate which would result in a greater loss of binding energy.

Day *et al* also determined the kinetic parameters of **(58)** using 3'-dephospho CoA which had been prepared enzymically. While the  $K_M$  value is similar to my findings his  $k_{cat}$  differs somewhat, as a result they report a value of 1.22 kcal mol<sup>-1</sup> of binding energy for the phosphate group. The reasons for the discrepancy are unclear as one cannot comment on the purity of the commercially available 3'-dephospho CoA used by Day and the accuracy of his kinetic data. The *S*-acetyl 3'-dephospho CoA used during the course of this work was fully characterised and showed no traces of impurity, for this reason it was decided to use my data as the comparison for the other base analogues.

#### 4.4.3 *S*-Acetyl 3'-dephospho ino CoA (**73**)

The inosine analogue of AcCoA was also shown to be a substrate for CAT (**Table 4.1**) unlike in the case for phosphotransacetylase. The effect of replacing the adenine base by inosine was to increase the  $K_M$  by a factor of 150 compared to acetyl dephospho CoA **(58)** and reduce  $k_{cat}$  by a factor of 13. The specificity constant of **(73)** compared to **(58)** is reduced by a factor of 2000 which corresponds to a  $-\Delta\Delta G$  of 4.5 kcal mol<sup>-1</sup>. In the present case the loss of binding energy cannot be attributed to the loss of a single interaction as a single functional group has not been replaced by a hydrogen atom.

An examination of the structure of inosine shows that the hydrogen bonding potential of the functional groups is opposite to that of adenine (**Figure 4.5**). The hydrogen bond donating amino group at C<sub>6</sub> has been replaced by a hydrogen bond accepting carbonyl group, while the N<sub>1</sub> of adenine has gained a hydrogen atom giving it hydrogen bond donating potential.



**Figure 4.5**

An examination of the CAT:CoA binary complex (**Figure 1.6**) shows that the substitution with inosine has the potential to remove a total of 4 hydrogen bonds. In adenine the amino group at C<sub>6</sub> forms two hydrogen bonds, one directly to the amide backbone of residue of 176 and the other to N<sub>7</sub> of the purine ring *via* an ordered water molecule while N<sub>1</sub> forms a hydrogen bond with the amide backbone of residue 178 (**Figure 4.5**).

Fersht<sup>6b</sup> in his work on tyrosyl-tRNA synthetase and Withers *et al*<sup>110</sup> on glycogen phosphorylase-glucose complex showed that deletion of a hydrogen bonding partner of a neutral group weakens binding energy by a value in the range of 0.8-1.8 kcal mol<sup>-1</sup>.

In the present case, substituting with inosine potentially removes four neutral hydrogen bonding partners between CAT and CoA, this would be expected to weaken binding by a value in range of 3.2-7.2 kcal mol<sup>-1</sup>.

It is possible that the inosine may form alternative hydrogen bonds with the enzyme. Preliminary molecular modelling studies which assumes that (73) binds in a similar conformation to AcCoA showed that the inosine group did not form any new hydrogen bonds with CAT, however there was potential to form a hydrogen bond between the carbonyl at C<sub>6</sub>



and N<sub>7</sub> *via* a bridging water molecule as there is in the CAT:CoA binary complex.

As there are no hydrogen bond donors or acceptors within hydrogen bonding distance of the new groups in inosine the results of both kinetic studies and molecular modelling suggest that the difference in specificity for (73) and (58) is due mainly to the loss of hydrogen bonding interactions to the enzyme. The experimental value of 4.5 kcal mol<sup>-1</sup> of binding energy is consistent for the loss of 3-4 neutral hydrogen bonds as calculated by Fersht.<sup>6b</sup>

The results show the importance of the precise nature of hydrogen bond donors and acceptors in molecular recognition. It is interesting to note that even though CAT has not faced evolutionary pressure to discriminate between adenosine and inosine nucleotides, the hydrogen bond network fortuitously in place effects a substantial discrimination which is expressed in both K<sub>M</sub> and k<sub>cat</sub>.

#### 4.4.4 S-Acetyl 3'-dephospho guano CoA (72)

The guanine analogue (72) was shown to be a substrate for CAT (Table 4.1). Compared to (58) substituting the adenine by guanine weakened binding (K<sub>M</sub>) by a factor of 10 and reduced k<sub>cat</sub> by a factor of 18. Guanine is similar in structure to inosine except there is an additional amino group at C<sub>2</sub> (Figure 3.7). The specificity constant for (72) compared to (73) is 8-fold higher suggesting that (72) is a better substrate. Comparing the kinetic parameters of (72) and (73) the -ΔΔG<sub>app</sub> for the NH<sub>2</sub> at C<sub>2</sub> was calculated to be 1.26 kcal mol<sup>-1</sup>.

This suggests that it is possible that the new NH<sub>2</sub> group is forming favourable interactions with the enzyme possibly a hydrogen bond with the enzyme. Inspection of the CAT:dephospho guano CoA structure did not support this view and showed no suitable hydrogen bond donors or acceptors within hydrogen bonding distance, however this does not discount the possibility of a hydrogen bond to the protein *via* ordered water molecules. It must be noted that there are limitations to the molecular modelling studies. Compound (72) is modelled into the CoA binding pocket using the co-ordinates of CoA in the CAT:CoA binary complex, the adenine base is then substituted by guanine using the Biosym software. It is

possible that (72) may bind in a different conformation to that of CoA which would allow the NH<sub>2</sub> or indeed other groups to form interactions both favourable and unfavourable with CAT. Until the binary complex of the CAT:dephospho guano CoA complex is solved the results of current molecular modelling studies should only be used as rough guides.

#### 4.4.5 S-Acetyl dephospho thymo CoA (74)

Due to the conformation of the coenzyme when bound to CAT, the adenine moiety of CoA is in close proximity to the pantetheine arm and is in fact in van der Waals contact with the *gem* dimethyl groups (Section 1.6.2). This interaction forms a major part of the adenine binding pocket, therefore, for the substitution of adenine by thymine, a pyrimidine base, one would expect (74) to be a poor substrate for CAT. In fact (74) was shown to be a relatively good substrate for CAT (Table 4.1). Compared to (58) binding was reduced by a factor of 3.5 while the  $k_{cat}$  remained relatively unchanged. The specificity constant of (74) compared to (58) was reduced by a factor of 4 which corresponds to a  $-\Delta\Delta G_{app}$  of 0.84 kcal mol<sup>-1</sup> for the substitution.

One would have predicted that replacing the adenine base by thymine would have reduced binding by a greater extent than that for inosine and guanine: not only is there a loss of hydrogen bonds to the enzyme but also hydrophobic and van der Waals interactions are also lost. The lower than expected value can be understood if one considers the factors which can compensate for the loss of the above interactions. Compound (74) may be able to form a network of novel interactions to the enzyme, although these are not readily identified by molecular modelling. They cannot however be discounted since the conformation of the bound (74) maybe different to that of CoA in the binary CAT:CoA complex. Molecular modelling studies do however show that the methyl group at C<sub>5</sub> is in close proximity to a bound water molecule. It is possible that the binding of (74) releases the bound water molecule to bulk solvent which would increase the entropy of the system and thus reduce the loss of binding energy.

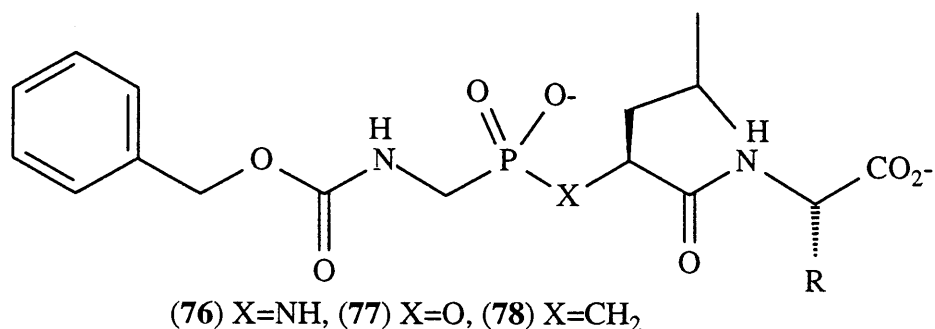
Many of the hydrogen bonds between CoA and CAT are relatively long or involve one or more water molecules so alternative binding modes cannot be ruled out. For example in the binding of chloramphenicol in which there are also relatively few direct hydrogen bonds, the 1-*O*-acetyl chloramphenicol, a product of the non-enzymic rearrangement of 3-*O*-acetyl chloramphenicol was found to have a specificity constant 150-fold lower than chloramphenicol. Results of both site directed mutagenesis and substrate analogues studies suggested that the 1-*O*-acetyl chloramphenicol is unlikely to bind in the cavity next to the 1-OH group in the CAT:CM crystal structure. Instead it has been shown that there are alternative binding modes, both non-productive and productive, for the binding of 1-*O*-acetyl chloramphenicol arising from the considerable flexibility in the active site.<sup>21</sup>

Desolvation energies are also an important factor, if one considers the structures of both adenine and thymine one would expect thymine to have a lower energy of desolvation. The lower energy of desolvation could compensate for the loss of some of the interactions lost on the substitution (see next section for a fuller discussion of the effect of desolvation energies).

#### 4.4.6 *S*-Acetyl 2'-deoxy 3'-dephospho CoA (75)

From **Table 4.1** we can see that (75) is a relatively good substrate for CAT. The apparent  $K_M$  of (75) is 3-fold higher than AcCoA but is 2-fold lower than that of (58). The binding process of (75) to CAT can be viewed as an exchange reaction, the substrate exchanges its solvation shell of water for the binding interactions with the enzyme active site. Such solvation energies can be very high (**Section 1.1.2**) and must be compensated for if removal from water is associated with complex formation.

In relevant studies Bartlett and co-workers used substrate modifications to evaluate the influence of hydrogen bonding and hydrophobic effects on the inhibition of thermolysin, a zinc protease. They prepared several series of analogues which included those in **Figure 4.6**.<sup>18</sup>



**Figure 4.6**

X-ray crystal structures of the thermolysin complex of (76) and (77) have shown that the two are essentially superimposable.<sup>111</sup> Replacement of the phosphorus linked NH group with an oxygen atom to give a series of phosphonate esters (77) resulted in a uniform decrease in binding affinity of 4 kcal mol<sup>-1</sup> across the series.<sup>18b</sup> The substitution causes the loss of a hydrogen bonding interaction because of the inability of the ester to donate a hydrogen to the carbonyl oxygen of an alanine amide backbone. The loss of this interaction was judged to be the major cause of the reduced binding affinity of the esters. However, since the binding process is an exchange reaction the difference in solvation of the phosphonamidates and the phosphonates is also an important factor. The favourable amide-enzyme hydrogen bond is partially offset by the greater free energy loss of desolvation of the amidates compared with the esters, due largely to the hydrogen bonding capability of the former. Bash *et al* also supported this interpretation, by separately calculating the differences in the energy of the interactions of both amides (76) and the esters (77) with the active site and with solvent, they also obtained a value of 4 kcal mol<sup>-1</sup>.<sup>112</sup> In a similar manner Kollman and Merz accurately predicted that substituting the -NH- group with a -CH<sub>2</sub>- group the binding of the phosphinates (78) to thermolysin would be almost the same as that of the phosphonamidates (76) despite the former not being able to form a hydrogen bond to the alanine residue.<sup>113</sup>

Bartlett prepared a series of phosphinates (78) and demonstrated that the replacement of the -NH- with a -CH<sub>2</sub>- moiety alters the affinity of the inhibitors by relatively constant amounts across the series.<sup>18c</sup> Compared to the corresponding phosphonamidates (76) the phosphinates

(78) were shown to bind with only a  $0.1 \text{ kcal mol}^{-1}$  decrease in binding affinity which is in contrast to the phosphonates (77) which bind an average of  $4 \text{ kcal mol}^{-1}$  less tightly. These differences in binding affinity show that the difference in inhibitor-active site interaction must be balanced against the differences in solvation of the inhibitor. The binding affinities of the phosphinates and phosphonamidates are approximately the same which can be explained by the loss of the hydrogen bond in the enzyme-inhibitor complex for the phosphinate which is offset by the decrease in hydrophilicity making the phosphinates easier to desolvate. In the case of phosphonates (77) the loss of the hydrogen bond active site interaction was not balanced by a comparable decrease in solvation energy which accounts for the decrease in binding affinity when compared with the phosphonamidates. Bartlett concluded that the affinities can be attributed to the importance and relative magnitudes of both hydrophobic and hydrogen-bonding characteristics of the phosphorus substituents.

In the case of (75), removal of the 2'-OH moiety lowers the energy of desolvation compared to (58) by virtue of the hydrogen bonding capabilities of the hydroxyl group. Normally (75) would offset the higher desolvation energy with a favourable hydrogen bond between the 2'-OH and the enzyme. However, as can be seen from the CAT:CoA binary complex the 2'-OH makes no direct contacts with CAT therefore it cannot compensate for its higher energy of desolvation compared to (75). The net result is that (75) has a 2-fold higher specificity constant than (58) which corresponds to a  $0.11 \text{ kcal mol}^{-1}$  increase in binding affinity for the loss of the 2'-OH group.

#### 4.4.7 Conclusions

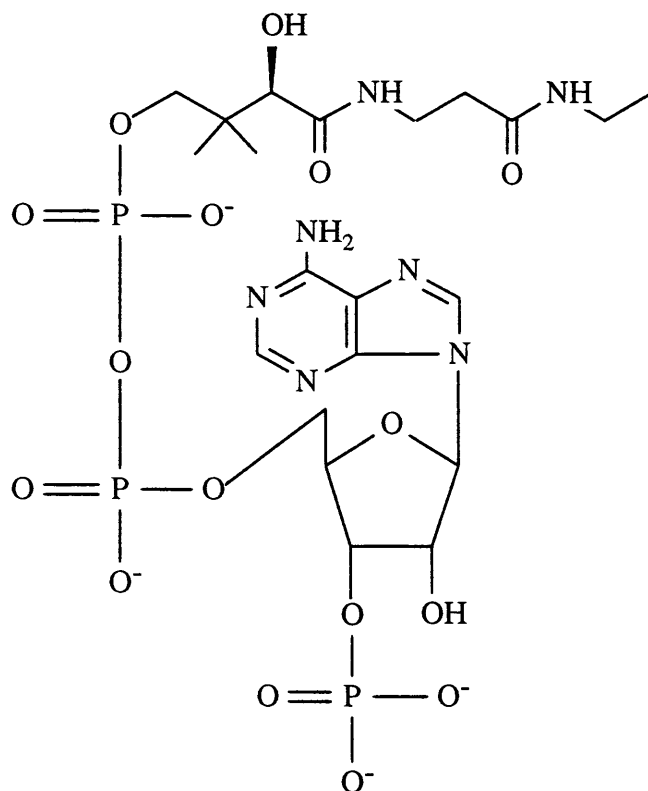
The results of both steady state kinetics and molecular modelling show that the adenine nucleotide is an important element in the recognition of CoA by CAT. By comparing the kinetic parameters of AcCoA and *S*-acetyl 3'-dephospho CoA (58) the contribution to binding of the 3'-phosphate was estimated to be  $0.62 \text{ kcal mol}^{-1}$ . Similarly comparing the kinetic

parameters of (58) with *S*-acetyl 2'-deoxy 3'-dephospho CoA (75) showed that removal of the 2'-OH group improved binding by 0.11 kcal mol<sup>-1</sup>, this being in part explained by a difference in solvation energies of the two substrates. Substituting the adenine base with another purine base, for example inosine and guanine reduced both binding and catalysis considerably, due in part to the loss of hydrogen bonding interactions to the enzyme. However, the guanine analogue lost less binding energy probably as a result of adventitious interactions between the extra amino group and the enzyme. Thymo CoA on the other hand, a pyrimidine CoA analogue was a relatively good substrate for CAT. Compared to (58) it had a lower  $k_{\text{cat}}$  but the  $K_{\text{M}}$  was very similar. Compared to both (72) and (73) it lost less binding energy which is probably in part due to the formation of novel interactions not present in the CAT:CoA binary complex and a much lower energy of desolvation of thymine compared to the purine bases. In order to gain a fuller picture of individual interactions between the analogues and CAT the binary complexes must be solved for each analogue. The results of current molecular modelling studies can only serve as a rough guide to the possible interactions within the CoA binding pocket.

#### 4.5 Coenzyme A analogues with modified pantetheine moieties

Work carried out by Shimizu on phosphotransacetylase using various CoA analogues and segments showed that the pantetheine moiety did not seem to contribute substantially to the specificity of the enzyme.<sup>93</sup> This conclusion was reached by virtue of the fact that analogues lacking part of the pantetheine chain could combine specifically with the CoA site and also the fact that D-pantetheine-4'-phosphate was unable to interact with the enzyme. During the course of this work several analogues were prepared with modifications in the cysteamine portion of the pantetheine chain. The aim was to study the effects these modifications had on the binding and catalysis of CoA by CAT.

#### 4.5.1 Dethio CoA (79) (Figure 4.7)



Structure of dethio CoA (79)

**Figure 4.7**

The commercially available CoA analogue (79) in which the terminal thiol has been replaced by a hydrogen atom was studied in order to quantify the contribution made to binding by the terminal thiol group. Kinetic studies showed it to be a competitive inhibitor with respect to AcCoA ( $K_i=360\ \mu\text{M}$ ).

By a comparison of the dissociation constant of (79) with the  $K_m$  of CoA the binding energy,  $-\Delta\Delta G_{\text{app}}$ , for the -SH group was calculated to be  $-0.84\ \text{kcal mol}^{-1}$ . From the CAT:CoA binary complex (**Figure 1.6**) it can be seen that the thiol group forms two hydrogen bonds with CAT. One via S-H to N<sup>3</sup> of His-195 (2.37 Å long) and the other between sulphur and the amide backbone hydrogen of residue 200 (2.57 Å long). The value of  $0.84\ \text{kcal mol}^{-1}$  of binding energy is a relatively low  $\Delta\Delta G_{\text{app}}$  for the loss of two hydrogen bonds as Fersht has previously calculated the free energy of binding for a single hydrogen bond between a thiol

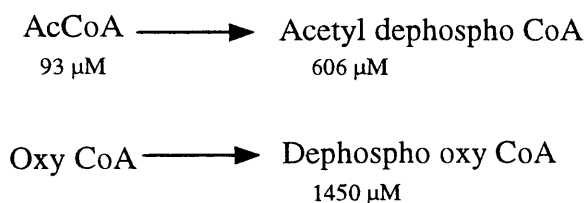
and a neutral partner to be in the region of 1 kcal mol<sup>-1</sup>.

The lower than expected binding energy for the thiol group can in part be explained by a lower energy of desolvation associated with (79) compared to CoA. In addition the pantetheine arm has become more hydrophobic in character therefore it may form favourable interactions with the largely hydrophobic binding pocket of CoA. The two hydrogen bonds via the -SH moiety to CAT are both relatively long (2.1 Å being the length of a good hydrogen bond) so are both expected to be weaker and less than the approximate value of 1.0 kcal mol<sup>-1</sup> calculated by Fersht.

#### 4.5.2 Dethio 3'-dephospho oxy CoA (59)

The CoA analogue, oxy CoA, in which the terminal thiol was replaced by a hydroxyl group has been previously shown to be very potent competitive inhibitor of the acetyl transfer reaction with phosphotransacetylase.<sup>93</sup> The dephospho analogue of oxy CoA, (59), also was shown to be a competitive inhibitor with respect to AcCoA ( $K_i=1450\ \mu\text{M}$ ) with the CAT system.

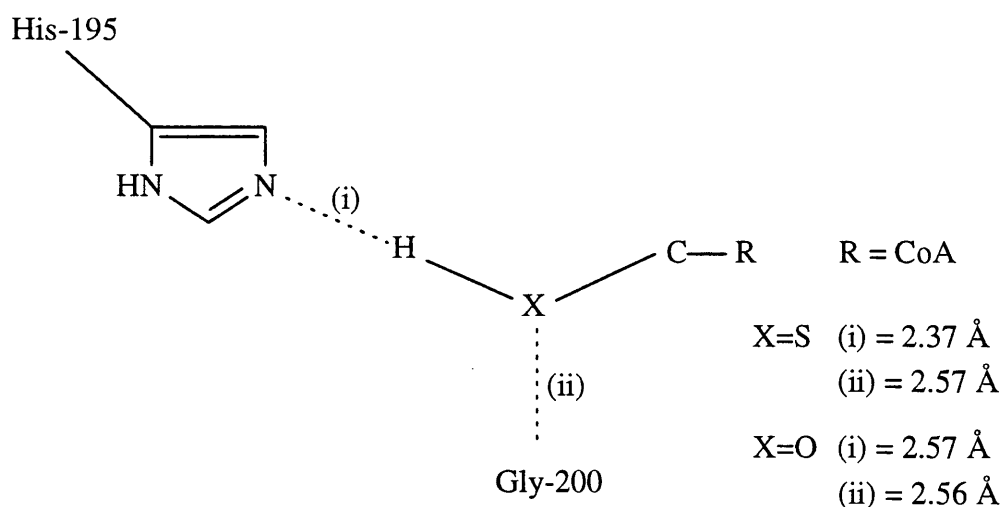
In order to obtain an apparent binding energy for the hydroxyl group a comparison of the  $K_i$  of oxy CoA with that of dethio CoA is required. However in the present case (59) lacks the 3'-phosphate group so it cannot be directly compared with dethio CoA as the binding energy would also incorporate the binding energy associated with the phosphate group. With the data available it is possible to estimate the  $K_i$  of oxy CoA as shown below.



In the case of AcCoA, the loss of the 3'-phosphate group increases the  $K_M$  by a factor of 6.5. If one assumes that the loss of the phosphate group would cause a similar reduction in



binding of oxy CoA then would also expect (59) to have a binding constant which is 6.5-fold lower than that of oxy CoA, this would give oxy CoA a binding constant in the region of 223  $\mu\text{M}$ . Comparing this value with the  $K_i$  of dethio CoA the apparent binding energy of the hydroxyl group is calculated to be  $0.28 \text{ kcal mol}^{-1}$ . This value is 3-fold lower than the apparent binding energy obtained for the thiol group. There are a number of factors which can possibly explain this difference.



**Figure 4.8**

Molecular modelling studies show that the length of the hydrogen bond to the His 195 residue of CAT would be longer in oxy CoA (**Figure 4.8**) than in CoA. This hydrogen bond is already relatively weak in CoA so would be expected to be weakened further in oxy CoA thus lowering its contribution to the binding energy. Again desolvation energies would be expected to have a significant effect, the hydroxyl group would be expected to have a higher energy of desolvation than the thiol thus reducing its apparent binding energy if this is not adequately compensated for in the enzyme ligand complex.

As the inhibition constant of (59) is relatively low it can be used in the reverse functionality experiments discussed in **Section 3.3.1**. Although a full synthesis of oxy CoA incorporating the 3'-phosphate is desirable the above compound offers the compromise of an easier synthetic target and meaningful kinetic results. Due to time limitations I was unable to

perform these studies however the work is currently being carried out by other workers.

#### 4.5.3 *S*-Acetyl 3'-dephospho homo CoA (60)

Previous studies have shown that the active site of CAT can accommodate acyl groups of greater than two carbon chain length. Propionyl CoA in particular was shown to be good substrate with a  $K_M$  of 167  $\mu\text{M}$ .<sup>19</sup> *S*-Acetonyl CoA was also shown to be a competitive inhibitor of CAT with respect to CoA with a  $K_i$  of 248  $\mu\text{M}$ .<sup>24</sup> This is approximately 2.5-fold greater than the  $K_M$  of acetyl CoA which suggests that the position of the substrate carbonyl group has a significant effect on binding.

In the present case the structure of (60) can be considered to be very similar to that of *S*-acetonyl CoA, in both cases the position of the carbonyl group has been extended by a single methylene group. However the two are quite different, *S*-acetonyl CoA is a ketone whereas (60) is a thioester. As a result there are likely to be differences in their solvation energies which could result in their kinetic parameters differing somewhat.

Firstly kinetic analysis of (60) showed it to be poor substrate for CAT, having an apparent  $K_M$  of 15.1 mmol and an apparent  $k_{\text{cat}}$  of only 9.0  $\text{s}^{-1}$ . Comparing with the kinetic parameters of (58) the extra methylene group was calculated to weaken binding by 4.4  $\text{kcal mol}^{-1}$ . The inhibition constant of (60) in contrast to the  $K_M$  determination was found to be 650  $\mu\text{M}$  suggesting it binds very well. A comparison with the kinetic parameters of (58) suggests that the extra methylene group weakens binding by only 0.04  $\text{kcal mol}^{-1}$ . The  $K_i$  of (60) did not show a 2.5-fold increase with respect to *S*-acetyl 3'-dephospho CoA as seen in the case of *S*-acetonyl CoA and AcCoA, however one must not only consider the differences in solvation energies but also that without the 3'-phosphate group (60) may have alternative binding modes which would affect the interpretation of the data.

In order to understand the difference between the  $K_M$  and the  $K_i$  values for (58) and (60) one must examine the structure of the CAT:CoA binary complex. CoA binds with its pantetheine

arm in an extended conformation (**Figure 1.6**), placing the polar phosphate groups on the surface of the enzyme in contact with the solvent while completely burying the thiol group. The adenine ring binds in a hydrophobic pocket, but makes two hydrogen bonds to main-chain atoms and a third to an ordered water molecule, satisfying all its hydrogen bonding potential. In the case of (**60**) where both the nucleotide and pantetheine moieties are fully docked it is likely that the *S*-acetyl group is not optimally positioned to serve as a substrate. In order to function as a substrate (**60**) would have to "pull back" the pantetheine a little such that the acetyl group is in the correct position to participate in the acetyl transfer reaction. The net result would be a disruption of many of the interactions in both the base and pantetheine regions responsible for the binding of (**60**), this would result in a large decrease in binding which is illustrated by the large rise in  $K_M$ . To function as an inhibitor on the other hand the substrate does not need to pull back the pantetheine arm but instead can extend it further into the acyl binding site or perhaps further into the chloramphenicol binding pocket. The relatively low  $K_i$  for (**60**) suggests that the active site can indeed readily accommodate an extra methylene group without greatly reducing binding. As with the case of *S*-acetyl CoA and propionyl CoA (see **Section 3.3.2**) results of steady state kinetic analysis suggest that the position of the substrate carbonyl has a significant effect on binding.

#### 4.5.4 Conclusions

An examination of the inhibition constants ( $K_i$ ) for the CoA analogues with modified pantetheine moieties shows that they all bind relatively well. The removal of the thiol group (**79**) and the introduction of an extra methylene group (**60**) only had modest effects on binding which is in contrast to results for base analogues which were shown to be relatively poor substrates in comparison. The findings of these initial results would seem to suggest that they are consistent with that of phosphotransacetylase in which the base region was shown to be important for recognition while the pantetheine moiety important for the strength of binding of CoA to the enzyme.

## 4.6 Alkyl CoA analogues

As discussed in **Chapter 1**, one of the primary aims of research in the field of molecular recognition is to make quantitative estimates of the thermodynamic contributions of individual non-covalent interactions to the overall binding energy of a ligand to an enzyme. Studies, in particular by Fersht, have established typical values for hydrogen bonds,<sup>6b</sup> however rather less has been reported on hydrophobic "bonding". The majority of amino acid residues that form both the CoA and chloramphenicol binding pockets possess hydrophobic side chains (see **Figures 1.6 and 1.5**) suggesting that this is a major source of specificity in CAT.

Paul<sup>105</sup> analysed the contribution of the *p*-nitrophenyl group of chloramphenicol to the binding and specificity exhibited by CAT. A series of chloramphenicol analogues were synthesised in which the *p*-nitrophenyl group was replaced by alkyl groups of increasing chain length. The *p*-nitrophenyl group is located in a pocket lined by hydrophobic residues and it was expected that these compounds in which the hydrophobic interactions are progressively built back into the ligand would probe the incremental binding energy derived from this part of chloramphenicol.

During work on the analysis of the mechanism of CAT, Kleanthous and Shaw showed that it was able to transfer acyl groups of increased chain length. In fact both propionyl CoA ( $K_M = 167 \mu\text{M}$ ) and butyryl CoA ( $K_M = 271 \mu\text{M}$ ) were shown to be relatively good substrates for CAT.<sup>19</sup> As an extension of this work a series of analogous experiments to those performed by Paul were carried out. The acetyl group of AcCoA was replaced by a series of alkyl groups of increasing chain length. It was expected that these groups which have been shown to be readily accommodated by the enzyme would probe the incremental binding energy derived from interactions with the hydrophobic residues in both the CoA and chloramphenicol binding sites. The results may also help clarify the intriguing "docking" process of the

pantetheine arm that takes place during CoA binding. In the normal CAT reaction the two substrates approach the active site from opposite faces of the enzyme and approach is facilitated via the tunnel which extends through the protein and accommodates the pantetheine arm. The chloramphenicol binding pocket is linked to the CoA binding tunnel, therefore, when no substrate is bound to CAT there is a solvent-accessible channel which runs from one side of the molecule to the other. Previous unpublished studies have shown that a multisubstrate analogue linking CoA to chloramphenicol does not bind to CAT. This suggests that the chloramphenicol moiety is too bulky to pass through the tunnel that accommodates the pantetheine arm. The benzyl analogue of CoA (**71**) in particular was synthesised to probe the binding of a structure which resembles a less bulky version of the multisubstrate analogue.

The CoA analogues in which the acetyl group was replaced by an alkyl group were all shown to be good inhibitors of CAT as shown in **Table 4.2**.

**Table 4.2**

	$K_i$ ( $\mu\text{M}$ )	$K_i/K_M$
CoA*	86.5	-
Me ( <b>64</b> )	350	4.05
Et ( <b>65</b> )	105	1.21
Pr ( <b>66</b> )	115	1.33
Bu ( <b>67</b> )	101	1.17
Pentyl ( <b>68</b> )	85	0.98
Hexyl ( <b>69</b> )	72.5	0.84
Isobutyl ( <b>70</b> )	47.5	0.55
Benzyl ( <b>71</b> )	70	0.81

\*  $K_m$  of CoA<sup>104</sup>

Both the  $K_i$  and  $K_d$  for ethyl CoA (**65**) have previously been determined and the values are 103  $\mu\text{M}$  (kinetic studies, Day unpublished results) and 12  $\mu\text{M}$  (by fluorescence) respectively.<sup>114</sup> As the  $K_i$  of (**65**) most closely resembles the  $K_M$  of AcCoA it is likely that  $K_i$  is a measure of binding to the ternary complex of CAT and not the binary complex.

For the substrates R=Methyl–Hexyl there is a clear trend in the series. Increasing the size of the alkyl group improves binding to the ternary complex, ethyl CoA being the only exception. Both the isobutyl and benzyl CoA analogues which after first consideration may have been thought to bind weakly due to steric effects are infact found to bind tightly.

The interaction of a substrate with its active site involves either complete or partial desolvation of both ligand and active site. Only when hydrogen bonds to water are replaced by appropriate hydrogen bonds between enzyme and substrate do the energetics of this balance out. In addition, the displacement of water molecules from the active site has a favourable entropy, which is considered to be part of the hydrophobic effect. To this extent biological specificity can be seen to arise at least in part from the ease of removal of the ligand from solvent water. As the binding of a substrate by an enzyme involves the removal of the substrate from an aqueous environment to the hydrophobic regions of the enzyme this can be considered as analogous to partitioning between aqueous and organic phases.

If the trend seen in the data in **Table 4.2** arises from the differences in the hydrophilic/hydrophobic character of the ligands there should be a linear free energy relationship between the binding or specificity of CAT and the hydrophobicities of the ligand.

#### 4.6.1 Partition coefficients and the Hansch Equation

The distribution of solute between two phases in which it is soluble has long been the subject of study. Investigation over a century ago showed that the ratio of concentrations of solute

distributed between immiscible solvents did not depend on relative volumes of solution used.<sup>115</sup> However this is only the case if a single molecular species is being considered as partitioned between two phases.<sup>116</sup> Partitioning was then treated as an equilibrium process where the tendency of any single molecular species of solute to leave one solvent and enter another would be a measure of its activity in that solvent. It is however, only the relatively recent use of partition coefficients as extra thermodynamic reference parameters for "hydrophobic bonding" in biochemical and pharmacological systems that has generated renewed interest in their measurement.<sup>5</sup>

A variety of work has been carried out to examine the relative roles of the various binding forces which determine the ways a solute distributes itself between two phases.<sup>4b,4c</sup> A study of hydrocarbons in water showed that although there is a favourable entropy change the compounds are highly insoluble. The reluctance to mix with water is the result of a large entropy change for the process, it is the large energy of reordering the hydrocarbon solute and the water solvent molecules which keeps them in separate phases when placed together. It is this phenomenon that regulates the distribution of non polar solute molecules in an apolar solvent-water system. The origin of the large negative entropy change that is involved in partitioning between aqueous and non aqueous phases was first clearly appreciated by Frank and Evans.<sup>4c</sup> They reached the conclusion that when organic compounds are placed in water, the water molecules arrange themselves around the apolar parts in what were later referred to as "flickering clusters", and it is the removal of these water molecules from the apolar part of the solute that results in a large entropy change in the randomization of the water molecules.

Hydrogen bonding within the organic phase is a factor that must be considered in evaluating the energy requirements for phase transfer, moreover, one must also address the fact that rather large amounts of water (in molar terms) may be present in certain solvents during the partitioning process. For example, octanol dissolves in water only to the extent of 4.5 mM, however the molar concentration of water in octanol is 2.3 M, and that in butanol is 9 M.

Therefore, when a solute molecule transfers from the aqueous phase to the organic phase it is most likely that in octanol the polar functions would be more or less solvated by water and/or the hydroxyl group of the alcohol. Certain solvents such as alcohols and amines can act as both donors and acceptors in hydrogen bonding which increases their versatility as solvents. Since there are many -NH- and OH groups present in enzymes and membranes the alcohol-water system, in particular *n*-octanol water, has become widely used as an extrathermodynamic reference system for evaluating partitioning of active compounds in biological systems.

As introduced in **Section 1.1.1**, Hansch developed a comparative substituent constant  $\pi$ , which was found to be of great use in evaluating the lipo-hydrophilic character of a molecule upon which biological activity is highly dependent.<sup>117</sup>

$$\pi = \log (P/P_o)$$

Hansch determined the partition coefficients between *n*-octanol and water for a large number of mono- and disubstituted benzenes from which he was able to calculate  $\pi$  values for 67 functional groups.<sup>118</sup> He found that  $\pi$  values varied for a given substituent from system to system depending on its electronic environment, however the range over which it varied is not great. In addition, when strong electronic interaction is absent  $\pi$  is constant and additive in character. This additivity is of value when making gross modifications in the structure of biologically active compounds in order to make more active or selective drugs. Hansch has shown that  $\pi$  can be used to account for the hydrophobic forces involved in the binding of organic compounds by proteins<sup>119</sup> and enzymes.<sup>120</sup>

In **Section 4.3** it was mentioned that the contribution to binding of a substituent R can be measured by comparing the values of  $k_{cat}/K_M$  for two substrates. In the present case the  $K_i$ 's are a measure of the dissociation constant for the enzyme-inhibitor complex and they can be used to define  $\Delta G_{app}$  as discussed in **Section 1.1.3**.

$$\Delta G_{app} = RT \ln (K_S(R-S)/K'_S(H-S)) \quad \dots[1.4]$$



In the above equation  $\Delta G_{\text{app}}$  is the incremental Gibbs free energy of transfer of R from the enzyme to water relative to H. It is only in certain cases that the result of [1.4] can be interpreted as incremental binding energies. It is valid in the present case as the increasing length of the side chain sequentially fills up an extending binding site. The calculated values of  $\Delta G$  (obtained from the data in **Table 4.2** and equation [1.4]) are shown in **Table 4.3** together with the Gibbs energy of transfer of the alkyl groups from *n*-octanol to water.

**Table 4.3**

	$-\Delta G_{\text{app}}^{\text{a}}$ (kcal mol <sup>-1</sup> )	$\pi$	$\Delta G_{\text{t}}^{\text{b}}$ (kcal mol <sup>-1</sup> )
Me (64)	0.83	0.5	0.68
Et (65)	0.11	1.0	1.36
Pr (66)	0.17	1.5	2.04
Bu (67)	0.09	2.0	2.72
Pentyl (68)	-0.01	2.5	3.41
Hexyl (69)	-0.10	3.0	4.09
Isobutyl (70)	-0.35	-	-
Benzyl (71)	-0.12	-	-

a The Gibbs free energy of transfer from CAT to water

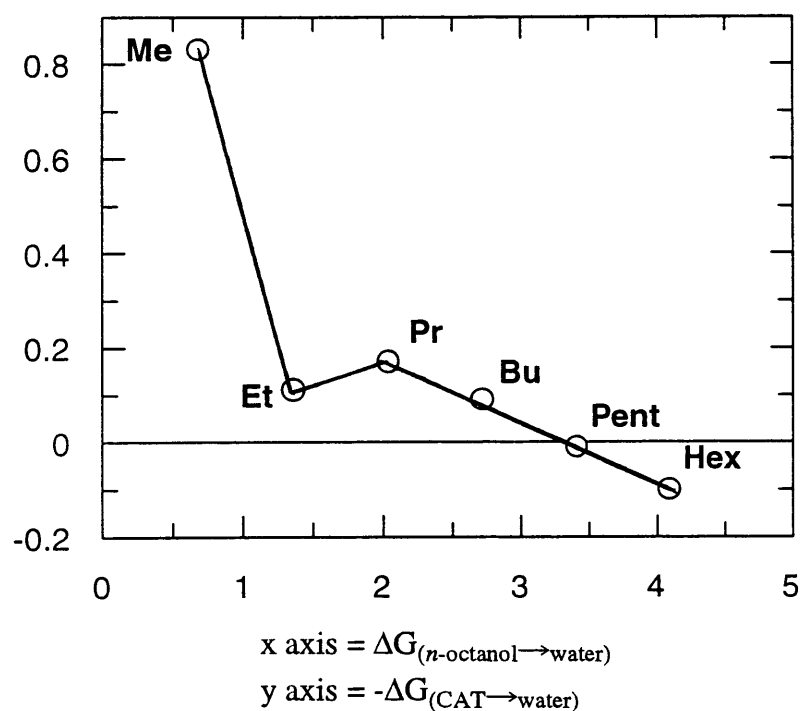
b The Gibbs free energy of transfer from *n*-octanol to water

The  $\Delta G_{\text{t}}$  values were calculated from the Hansch equation [1.2],  $RT \ln (P/P_0)$ , ie  $2.303 RT\pi$  being the incremental Gibbs energy of transfer of the group R from *n*-octanol to water (relative to H). For a range of model systems the incremental Gibbs energy of transfer from *n*-octanol to water for a methylene group is 0.68 kcal mol<sup>-1</sup>.<sup>5</sup> The observed additivity breaks down for long alkyl chains where the chains have the opportunity to adopt more compact

structures where presumably the alkyl chain associates with itself either intra or inter-molecularly.

A plot of  $-\Delta G_{\text{app}}$  for the substrates R=Methyl-Hexyl ((64)→(69)) against  $\Delta G(n\text{-octanol-water})$  is shown in **Figure 4.9**, the gradient of the linear portion of the plot ( $C_3$ - $C_6$ ) is -0.13. Since the slope is smaller than unity this suggests that the region accommodating these alkyl groups is less hydrophobic than *n*-octanol.

**Figure 4.9 Relationship between the Gibbs free energy of transfer from CAT to water and the Gibbs free energy of transfer from *n*-octanol to water for a series of alkyl groups**



Energies are in  $\text{kcal mol}^{-1}$  and are relative to the hydrogen atom.

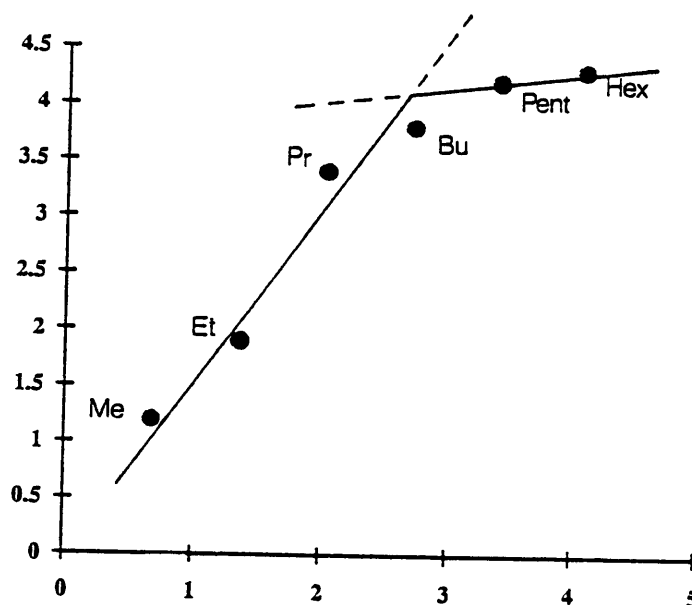
**Figure 4.9**

An analogous plot of  $\Delta \Delta G_{\text{app}}$  for a range of chloramphenicol analogues in which the *p*-nitrophenyl group of chloramphenicol was replaced by alkyl groups of increasing chain length against  $\Delta G_t$  was performed by Paul (**Figure 4.10**).<sup>105</sup>

As shown in the CAT:CM binary complex (**Figure 1.5**) the phenyl ring of chloramphenicol is located in a hydrophobic pocket and it was expected that compounds in which the

opportunities for hydrophobic interactions are progressively built back into the ligand would show a linear free energy relationship with respect to the hydrophobicity of the group.

**Figure 4.10 Relationship between the Gibbs free energy of transfer from CAT to water and the Gibbs free energy of transfer from *n*-octanol to water for a series of alkyl groups**



x axis =  $\Delta G(\text{n-octanol} \rightarrow \text{water})$

y axis =  $-\Delta\Delta G(\text{CAT} \rightarrow \text{water})$

**Energies are in kcal mol<sup>-1</sup> and are relative to the hydrogen atom**

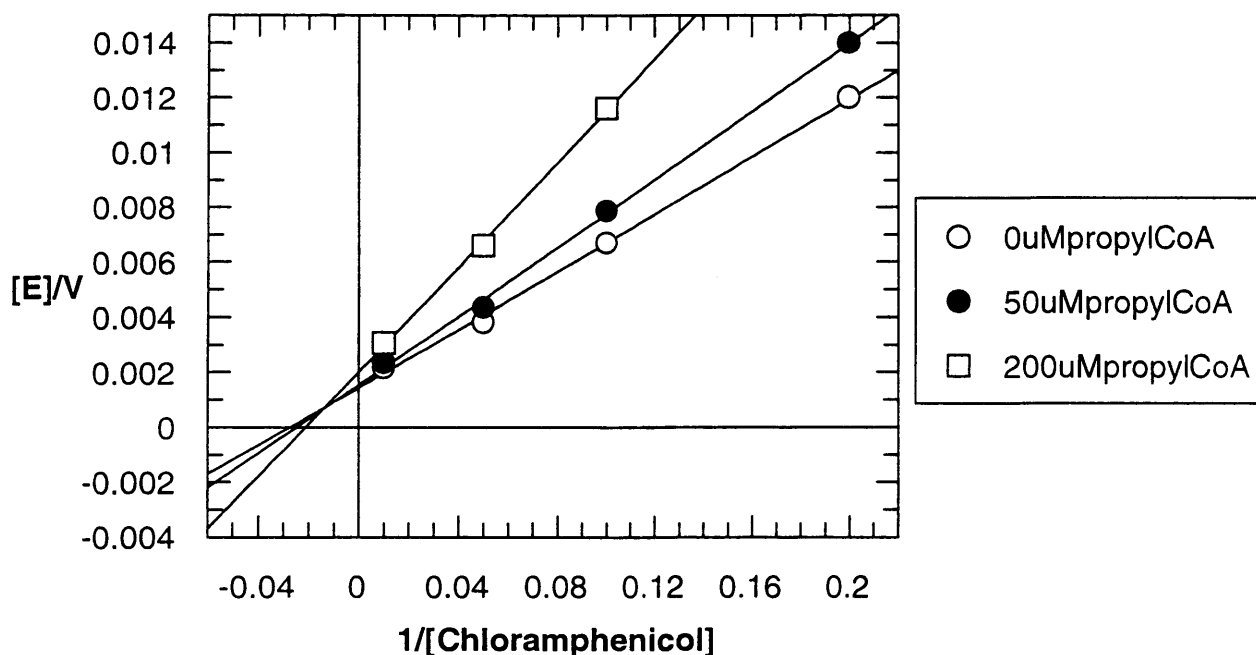
As can be seen from **Figure 4.10** the plot is linear up to C<sub>4</sub> (R=Butyl) at which point there appears to be a break and the plot begins to level off. This is readily explained by the fact that C<sub>4</sub> represents the point at which additional methylene groups are no longer located within the active site cleft but protrude in to the solvent. This being the case the terminal two methylene groups of for example the hexyl analogue are solvated to similar extents in both the free state and within the binary complex, and furthermore, they do not make significant adventitious contacts with the enzyme. The gradient of the linear portion of the plot (C<sub>1</sub>-C<sub>4</sub>) is 1.5 suggesting that the aryl binding pocket is 1.5 times more hydrophobic than *n*-octanol. This is similar to the case for chymotrypsin where the hydrophobic pocket appears to be 2.2 times more hydrophobic than *n*-octanol.<sup>121</sup>

In the case of the alkyl CoA analogues it is evident from the graph (**Figure 4.9**) that both the methyl and ethyl analogues do not fit the linear relationship exhibited by the other straight chain analogues. Firstly the ethyl analogue, the one which has a structure most resembling AcCoA was shown to be good inhibitor and had a  $K_i$  (105  $\mu\text{M}$ ) very similar to the  $K_M$  of AcCoA. Methyl CoA on the other hand has a much higher  $K_i$  (350  $\mu\text{M}$ ) than the other analogues. The most likely explanation for the weaker binding of the methyl analogue is that in the active site of CAT three highly conserved hydrophobic residues (Phe 33, Phe 158, and Leu 160) are so positioned as to make it likely that they interact with the methyl (C-2) of the trigonal acetyl group of AcCoA in its binary complex with CAT. Site directed mutagenesis studies by Day<sup>22</sup> showed that the subtractive substitution of Phe 33 by alanine removed an interaction between the aromatic ring and the C-2 methyl of AcCoA which resulted in a 2.5 fold increase in the  $K_M$  of AcCoA (252  $\mu\text{M}$ ). Methyl CoA which lacks the interaction to the Phe 33 residue and also the other hydrophobic residues in the active site has a binding constant of 350  $\mu\text{M}$  which is 3.5-fold higher than the  $K_M$  of AcCoA. As there are similarities in the two cases it is of no surprise they have similar binding constants.

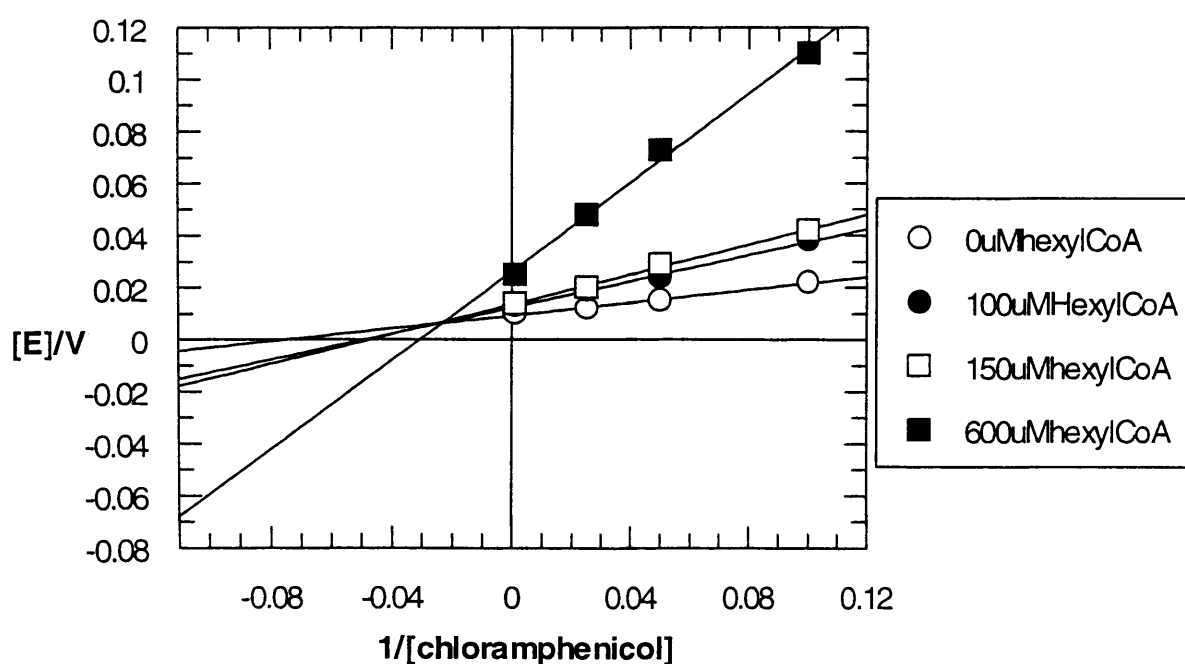
Alternatively it is possible that the binding of methyl CoA results in "burying" of a water molecule within the cavity which normally accommodates the  $\text{CH}_3$  moiety of the ethyl analogue. This would have an adverse effect on the entropy of the system and account for the higher inhibition constant compared to the others. Extending the length of the methyl chain to give ethyl CoA would expel the buried water molecule from the acyl binding site which would increase the entropy of the system. This would improve the binding of ethyl CoA to the enzyme, which is confirmed by its much lower inhibition constant. Extending the chain further as in the case of the propyl CoA analogue could either result in the alkyl chain binding in a different mode within the acyl binding site or being forced into the chloramphenicol binding site. The results of preliminary kinetic studies (**Figure 4.11**) showed that the alkyl CoA analogues are also competitive inhibitors with respect to chloramphenicol. These results support the view that the alkyl groups with a chain length of

greater than two carbon atoms are located within the chloramphenicol binding pocket. This is supported by molecular modelling studies which have shown that the extending alkyl chain can be modelled into the chloramphenicol binding site with little steric hindrance (Figure 4.12 A).

**The effect of propylCoA on the  $K_M$  of chloramphenicol**



**The effect of HexylCoA on the  $K_M$  of chloramphenicol**



**Figure 4.11**

The linear portion of the graph which corresponds to the straight chain analogues Propyl-Hexyl ((66)→(69)) was found to have a gradient of -0.13. Since the slope is smaller than unity it suggests that the region within the chloramphenicol binding pocket accommodating the alkyl groups is less hydrophobic than *n*-octanol. From the linear portion of the graph it was calculated that for each additional CH<sub>2</sub> moiety there is a decrease in  $-\Delta G_{\text{app}}$  by a factor of 0.09 kcal mol<sup>-1</sup>. This is in contrast to the binding site of *p*-nitrophenyl group of chloramphenicol where the incremental binding energy for each methylene group was estimated to be 1.0 kcal mol<sup>-1</sup>.

If one considers the residues forming the pocket accommodating the alkyl chain there is a mixture of hydrophobic and polar side chains (Thr 88, Phe 97, Asn 140, Ser 140 Leu 154 and Thr 166). This is far less hydrophobic than the pocket accommodating the phenyl ring of chloramphenicol which lies next to the side chains of Leu 29, Cys 31, Leu 160 and Ile 172). The amphipathic nature of the alkyl CoA binding pocket explains why it is 8-fold less hydrophobic than *n*-octanol.

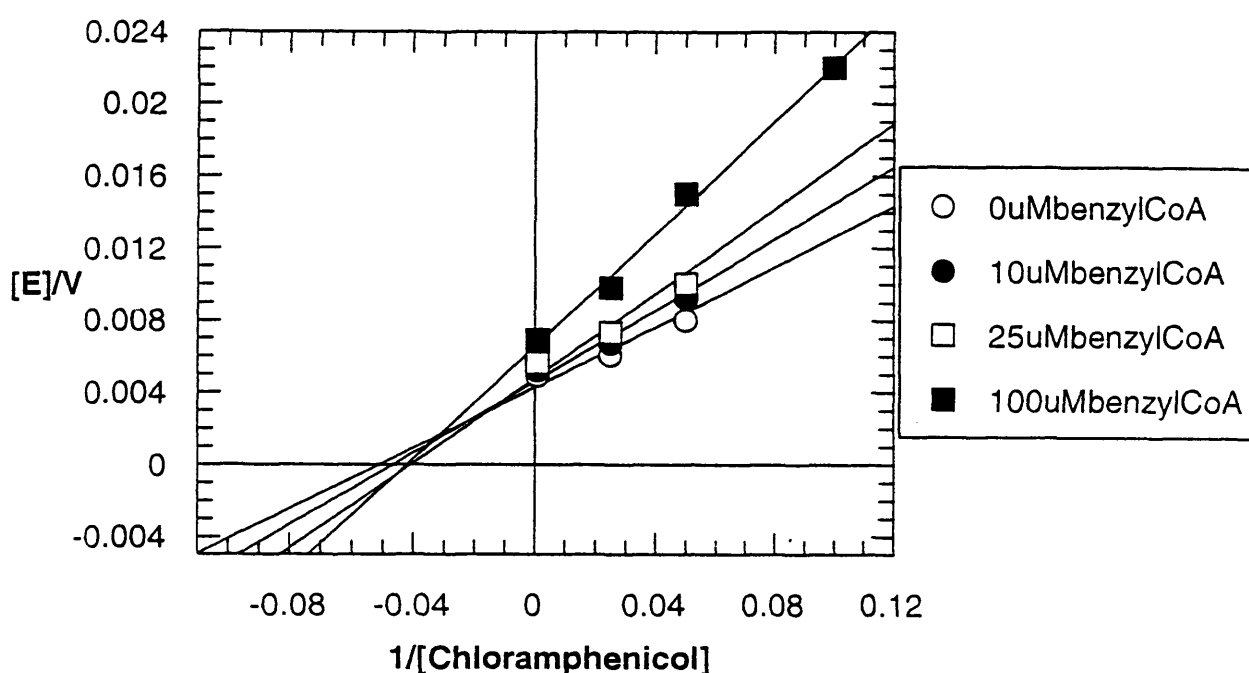
It is possible that extending the alkyl chain further may force it into the *p*-nitrophenyl binding pocket. It is possible these analogues would show similar kinetic results to those obtained by Paul. However not only is there difficulty in synthesising these analogues but they may adopt more folded conformations which would make interpretation of the data difficult. Still this would offer an interesting extension to the current work.

The results of kinetic analysis have shown that the multisubstrate analogue cannot bind to the enzyme. However, the benzyl CoA analogue which has some similarity in structure to the multisubstrate analogue was found to be a relatively good inhibitor of CAT with the benzyl group contributing 0.12 kcal mol<sup>-1</sup>. Molecular modelling studies have shown that the benzyl group can be modelled into the active site with little steric hindrance (**Figure 4.12 B**). However due to the size of the benzyl group it is possible that it is unable to pass through the pantetheine binding site and it may have alternative binding modes. Results of preliminary kinetic studies showed that benzyl CoA is a competitive inhibitor with respect to

chloramphenicol (**Figure 4.13**). This suggests that the benzyl group does indeed bind in the chloramphenicol binding pocket and may possibly bind as shown in **Figure 4.12 B**.

The benzyl group is located in a region close to the hydrophobic side chains of Thr 88, Phe 97, 152 and Leu 154 which would be expected to be the major factor for the improved binding of benzyl CoA.

#### The effect of benzyl CoA on the $K_M$ of chloramphenicol



**Figure 4.13**

Unlike benzyl CoA the multisubstrate analogue has an additional *N*-dichloroacetamido side chain in the chloramphenicol moiety. This additional group which is rather bulky may prohibit passage of the multisubstrate analogue along the binding pocket. A possible extension to the current work would be to synthesise the multisubstrate analogue lacking the *N*-dichloroacetamido group and to see if it is able to bind to the enzyme.

The isobutyl analogue of CoA was synthesised in order to examine effect of branched side chains on binding to CAT. The isobutyl CoA was shown to be an excellent inhibitor of CAT with a  $K_i$  of 47.5  $\mu\text{M}$ . Molecular modelling studies showed that the isobutyl group of the analogue could be modelled into the chloramphenicol binding pocket such that it is in close

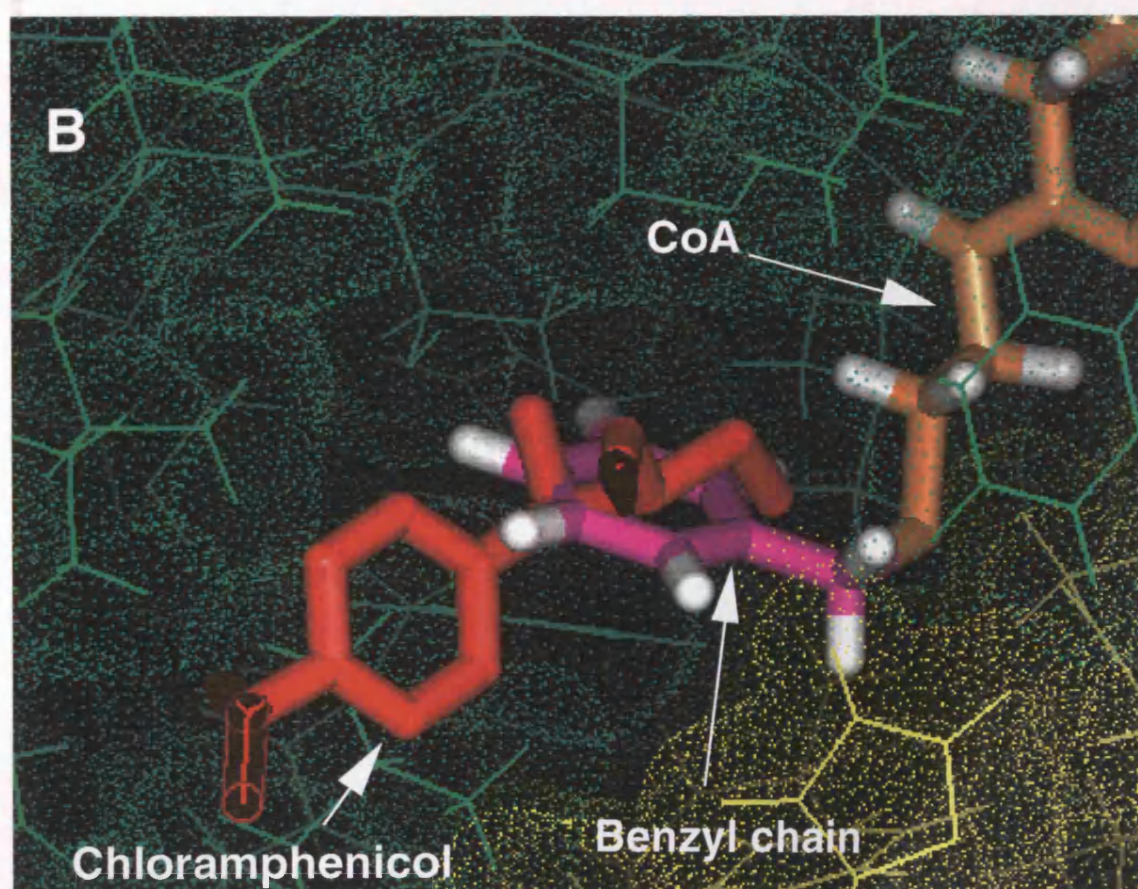
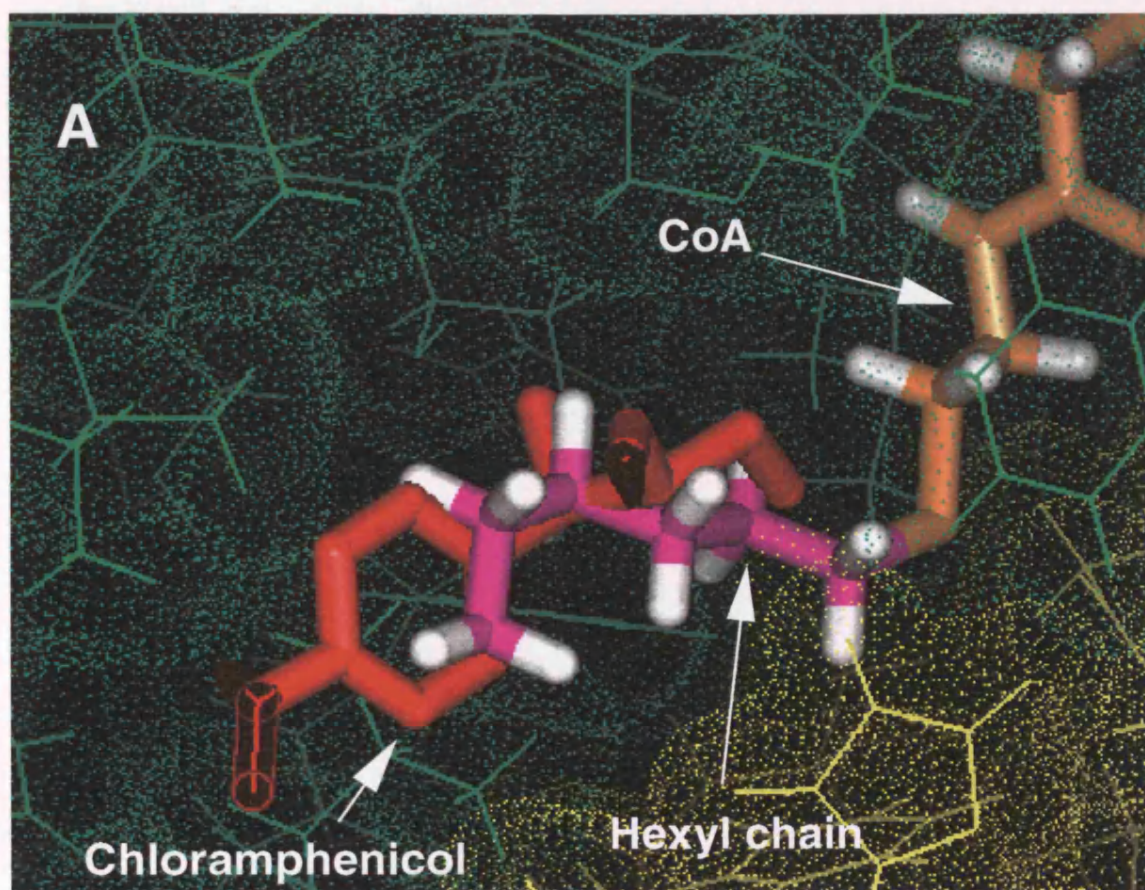
**FIGURE 4.12 (opposite)**

**A** The Molecular modelling of the hexyl group of hexylCoA into the chloramphenicol binding pocket of CAT.

**B** The Molecular modelling of the benzyl group of benzylCoA into the chloramphenicol binding pocket of CAT.

The molecular modelling was carried out with the Biosym software using the coordinates of the binary complexes taken from the X-ray structures.<sup>20,48</sup>





chloramphenicol (**Figure 4.13**). This suggests that the benzyl group does indeed bind in the chloramphenicol binding pocket and may possibly bind as shown in **Figure 4.12 B**.

The benzyl group is located in a region close to the hydrophobic side chains of Thr 88, Phe 97, 152 and Leu 154 which would be expected to be the major factor for the improved binding of benzyl CoA.

Unlike benzyl CoA the multisubstrate analogue has an additional *N*-dichloroacetamido side chain in the chloramphenicol moiety. This additional group which is rather bulky may prohibit passage of the multisubstrate analogue along the binding pocket. A possible extension to the current work would be to synthesise the multisubstrate analogue lacking the *N*-dichloroacetamido group and to see if it is able to bind to the enzyme.

The isobutyl analogue of CoA was synthesised in order to examine effect of branched side chains on binding to CAT. The isobutyl CoA was shown to be an excellent inhibitor of CAT with a  $K_i$  of 47.5  $\mu\text{M}$ . Molecular modelling studies showed that the isobutyl group of the analogue could be modelled into the chloramphenicol binding pocket such that it is in close

proximity to the hydrophobic side chains of Thr 87, Phe 97, 152 and Leu 154. The additional binding energy associated with these interactions would explain the incremental binding energy of 0.25 kcal mol<sup>-1</sup> for the isobutyl group.

#### 4.7 Conclusions

In the binding of alkyl CoA analogues molecular modelling studies showed that alkyl groups with a chain length greater than two carbons could be modelled into the chloramphenicol binding pocket. As with chloramphenicol there was also a linear relationship between the hydrophobicity of the ligand and the apparent binding energy. The gradient suggested that the binding pocket accommodating the alkyl groups was 8 times less hydrophobic than *n*-octanol. This is consistent with molecular modelling studies which showed the region accommodating the chain to be much less hydrophobic than the *p*-nitrophenyl binding pocket of chloramphenicol, and in fact to be quite amphipathic in nature. The binding pocket can also accommodate benzyl and isobutyl groups which suggests that it can also bind bulky hydrophobic side chains very tightly.

For the pocket accommodating the alkyl CoA groups of CoA a plot of  $-\Delta G_{\text{app}}$  for the substrates against  $\Delta G(\text{n-octanol-water})$  was found to be lower than unity. This is not unexpected because there are basic differences between the hydrophobic interaction of a solute between an organic solvent and water and the binding of hydrophobic substrates to an enzyme. The former involves the formation of a cavity in the organic solvent, transfer of the solute to the cavity and closure of the cavity in the aqueous phase, whereas the latter case involves transfer of the substrate to a preformed cavity in the enzyme and the likely transfer of water from this to the aqueous phase.

**CHAPTER 5**  
**EXPERIMENTAL**

## **5.1 GENERAL EXPERIMENTAL DETAILS**

### **5.1.1 Reagents and Solvents**

Unless otherwise stated all solvents and chemicals were obtained from Aldrich Chemical Company Ltd or Sigma Chemical Ltd. Solvents were purified following the methods of Perrin and Armarego<sup>122</sup>, as follows:- benzene, diethyl ether, and tetrahydrofuran were distilled immediately before use, under dry nitrogen from sodium and benzophenone. Dichloromethane, ethyl acetate and petroleum ether were distilled from powdered calcium hydride under nitrogen. Pyridine was distilled from potassium hydroxide and triethylamine was distilled prior to use. Methanol and ethanol were distilled from magnesium and iodine under dry nitrogen. The distilled water used throughout these experiments was obtained from a milli-Q-purification system.

### **5.1.2 Apparatus, Instrumentation and Techniques**

All 90MHz <sup>1</sup>H NMR spectra were recorded on a varian EM-390 spectrometer. High field <sup>1</sup>H NMR (300MHz), <sup>13</sup>C NMR (75MHz) and <sup>31</sup>P NMR (121MHz) spectra were recorded on a Bruker AM-300 spectrometer. Spectra were recorded in CDCl<sub>3</sub>, using tetramethylsilane (TMS) as the internal standard ( $\delta=0.0\text{ppm}$ ) or D<sub>2</sub>O using 3-(trimethylsilyl) propionic acid sodium salt as the internal standard ( $\delta=0.0\text{ppm}$ ) unless otherwise stated. The following descriptions are used to describe the <sup>1</sup>H NMR spectra: br=broad, d=doublet, m=multiplet, q=quartet, quin=quintet. Coupling constants (J) are reported in Hertz (Hz). For the <sup>13</sup>C NMR peaks the following descriptions were used: q=methyl (CH<sub>3</sub>), t=methylene (CH<sub>2</sub>), d=methine (CH), s=carbon with no hydrogens attached (C). The <sup>31</sup>P peaks were all completely decoupled and chemical shifts are reported relative to the tetrahydroxyphosphonium ion (85% H<sub>3</sub>PO<sub>4</sub>)<sup>123</sup> standard ( $\delta=0.0\text{ ppm}$ ) in which downfield shifts are reported as positive. High resolution mass spectra were performed on a Kratos Concept 1H double-focusing mass



spectrometer.

Flash chromatography was carried out according to the method of Still *et al.* using silica (Merck and Co., Kiesel 60, 230-400 mesh).<sup>124</sup> Thin layer chromatography (t.l.c) was conducted on precoated aluminium sheets (60F-254) with a 0.2 mm layer thickness manufactured by Merck and Co.

### 5.1.3 Ion exchange chromatography

Ion exchange chromatography was performed using DEAE Sephadex A-25 resin from Pharmacia. Before use it was left to swell for 24 hours in 1 M ammonium bicarbonate solution. It was purified and contaminants were removed by subsequent washings with excess volumes of 1 M  $\text{NH}_4\text{HCO}_3$  solution and distilled water. Triethylammonium bicarbonate (TEAB) buffer, the eluent, was prepared as a 1M stock solution. Initially the triethylamine (101 g, 139 ml) was dissolved in distilled water and made to a 1M standard solution in 1 litre. Gaseous carbon dioxide was then bubbled through the mixture at 25 °C until the pH of the solution approached pH 7.0. The necessary concentrations of buffer were prepared as required by the appropriate dilutions of volumes of the stock solution with distilled water. Fractions were collected in test tubes. UV absorbing materials were directly observed as they eluted by use of a Cecil UV-detector and recorded on a chart recorder. Evaporation of fractions gave solid TEAB which was broken down and removed by co-evaporation with methanol several times.

### 5.1.4 HPLC

HPLC was carried out using a Gilson 712 system fitted with 306 pumps, dynamax mixer and a Rainin Dynamax UV-1 detector. Preparative HPLC of S-acetyl D-pantetheine-4'-phosphate (1) was carried out using a Phasesep S5SAX column while monitoring at 220 nm. Eluent A consisted of 0.5 M  $\text{H}_3\text{PO}_4/\text{KOH}$ ; pH3, and eluent B consisted of 0.5 M  $\text{H}_3\text{PO}_4/\text{KOH}$ ;

pH3-CH<sub>3</sub>CN (1:1 v/v). The compound was eluted at 1 ml per minute with a linear gradient of 10% B (0 min) increasing to 45% (20 min) before returning to 10% (25 min). The tubes containing (1) (ret. time 9.5 minutes) were pooled and desalted using a Phasesep ODS2 reverse phase column with detection at 220 nm. Eluent A consisted of H<sub>2</sub>O and eluent B consisted of H<sub>2</sub>O-CH<sub>3</sub>CN (1:1 v/v). The compound was eluted at 5 ml per minute with a linear gradient of 0% B (0 min) increasing to 80% (50 min) before returning to 0% (60 min). The potassium salt of (1), (ret. time 30 minutes) was freeze dried and stored at -18 °C.

CoA analogues were purified using a Phasesep ODS2 reverse phase column with detection at 260 nm. Eluent A consisted of 0.2 M ammonium acetate pH6 and eluent B consisted of methanol. Each analogue was purified by using one of the following mobile phases:

Mobile phase A; compounds were eluted at 5 ml per minute with a linear gradient of 0% B (0 min) increasing to 80% (45 min), maintained at 80% (52 min) before returning to 0% (60 min).

Mobile phase B; compounds were eluted at 5 ml per minute with a linear gradient of 30% B (0 min) increasing to 50% (50 min) before decreasing to 0% (57 min).

Mobile phase C; compounds were eluted at 5 ml per minute with a linear gradient of 20% B (0 min) increasing to 50% (50 min) before returning to 20% (60 min).

The compounds were freeze dried and stored at -18 °C as the ammonium salts.

### 5.1.5 NMR data nomenclature

For pantetheine and all pantetheine based analogues the nomenclature is based on D-pantetheine as shown in **Appendix 2**. For all analogues of CoA the nomenclature used by Lee and Sarma has been employed, where A refers to adenosine protons and P refers to pantetheine protons, (see **Appendix 3**).<sup>125</sup> Not all <sup>13</sup>C signals were visible on certain spectra due to poor noise to signal ratio.

## 5.2 Synthesis

### Preparation of D-panethine-4'-phosphate (13)

#### Cyanoethyl phosphate and dicyclohexylcarbodiimide method

To cyanoethyl phosphate, barium salt (0.75 g, 2.33 mmol) was added Dowex 50W (H<sup>+</sup>) resin and the mixture stirred until the cyanoethyl phosphate dissolved. The supernatant was collected and dry pyridine (4 ml) was added. After evaporating to dryness and resuspending in dry pyridine several times to produce the pyridine salt, the product was finally resuspended in dry pyridine (7.5 ml). To this was added dicyclohexylcarbodiimide (0.48 g, 2.30 mmol) and D-pantethine (0.5 g, 0.90 mmol) and the reaction mixture was stirred for 24 hours at room temperature. The reaction mixture was filtered and water (6.5 ml) was added to the filtrate. After 1 hour the mixture was filtered again and the filtrate evaporated to dryness. The reaction mixture was resuspended in NaOH (7.50 ml, 2 M) and after incubation on ice for 30 minutes Dowex 50W(H<sup>+</sup>) resin was added until the pH ≤ 2. After filtering again the filtrate was brought to pH 7.5 by the addition of KOH (3 M).

The reaction mixture was diluted with water (150 ml) and applied to an ion exchange column (130 ml) of DEAE-Sephadex resin. The column was eluted with a gradient of TEAB (50-400 mM), pH 7.5 over 18 hours (total volume of eluent used 1600 ml). The eluent from the column was continuously monitored by a UV detector at  $\lambda$  220 nm. The appropriate fractions were combined, concentrated and the TEAB removed as before. The product (13) eluted at approximate buffer concentration 180-220 mM, and was isolated as a clear gum (0.525 g, 52%).

$\delta_P$ (121MHz:CD<sub>3</sub>OD) 3.79ppm

$\delta_H$ (300MHz:CD<sub>3</sub>OD) 4.0 (1 H, s, 2'-H), 3.9 (1 H, dd,  $J^3_{P-H}$  7.2,  $J_{Gem}$  9.88, 4'-H), 3.5 (5 H, m, 4'-H, 1-H, 4-H), 3.2 (q, Et<sub>3</sub>NH, CH<sub>2</sub>), 2.8 (2 H, t,  $J$  6.8, 5-H), 2.5 (2 H, t,  $J$  6.61, 2-H), 1.3 (t, Et<sub>3</sub>NH, CH<sub>3</sub>), 1.0, (3 H, s, 5'-H), 0.83, (3 H, s, 6'-H).



See appendix 2 for nomenclature.

$\delta_C$ (75MHz:CD<sub>3</sub>OD) 175.44(s), 173.71(s), 75.40(d), 72.40(dt,  $^2J_{P-C}$  5.03), 46.98(t, Et<sub>3</sub>NH), 40.22 (ds,  $J$   $^3J_{P-C}$  6.6), 39.65(t), 38.43(t), 36.53(t), 36.31(t), 22.36(q), 19.66(q), 9.19(q, Et<sub>3</sub>NH).

m/z (FAB) 816 (MEt<sub>3</sub>NH<sup>+</sup>, 77%), 715 (MH<sup>+</sup>, 60), 617 (31), 255 (100).

### Preparation of *S*-acetyl-D-pantetheine-4'-phosphate (15)

Compound (13) (0.5 g, 0.45 mmol) was dissolved in H<sub>2</sub>O (6.5 ml) and DTT (137 mg, 0.89 mmol) was added and the reaction mixture was stirred for 20 minutes. KHCO<sub>3</sub> (1 ml, 1 M) and NaOH (0.1 ml, 1 M) were added and the total thiol concentration was measured by finding the absorbance at 412 nm at 25 °C for 10  $\mu$ l of solution in TSE buffer (990  $\mu$ l) containing 1 mmol DTNB. Acetic acid (2 equiv) was added and the solution was vortexed and left of ice. If after 20 minutes a negative DTNB test for free thiols was not obtained more acetic anhydride was added. (15) was purified by HPLC as the potassium salt (277 mg, 65%).

$\delta_P$ (121MHz:D<sub>2</sub>O) 1.34 ppm

$\delta_H$ (300MHz:D<sub>2</sub>O) 3.96 (1 H, s, 2'-H), 3.7 (1 H, s,  $J$   $^3J_{P-H}$  4.95,  $J_{Gem}$  9.7, 4'-H), 3.5 (3 H, m, 1-H, 4'-H), 3.3 (2 H, t,  $J$  6.4, 4-H), 2.95 (2 H, t,  $J$  6.4, 5-H), 2.4 (2 H, t,  $J$  6.5, 2-H), 2.3 (3 H, s, SCH<sub>3</sub>), 0.95, (3 H, s, 5'-H), 0.9, (3 H, s, 6'-H).

$\delta_C$ (75MHz:D<sub>2</sub>O) 201.30(s), 175.21(s), 174.357(s), 74.85(d), 71.22(dt,  $^2J_{P-C}$  5.5), 38.90(t), 38.65(ds,  $^3J_{P-C}$  7.7), 35.76(t), 35.62(t), 30.33(q), 28.65(t), 21.22(q), 18.81(q).

m/z (FAB) 401.2 (MH<sup>+</sup>, 44%), 255.5 (36).

### Preparation of *S*-acetyl-D-pantetheine-4'-phosphate (15)

#### Dibenzyl phosphorochloridate method

D-pantethine (0.831 g, 1.5 mmol) was dried by three evaporation of its solutions in

anhydrous pyridine (10 ml each), dissolved in pyridine (30 ml) and frozen in a dry ice bath. A solution of dibenzyl phosphorochloridate (from 1.33 ml, 6 mmol of dibenzyl phosphite and 810 mg of N-chlorosuccinimide) in benzene (10 ml) was then added and the solution was thawed and rapidly refrozen. It was then allowed to stand overnight in a deep freeze (-18 °C). Water (5 ml) was added and after 15 minutes the pale yellow solution was carefully evaporated *in vacuo* with a bath temperature less than 35 °C. The residue was dissolved in ethyl acetate and extracted three times each with sulphuric acid (2 M), sodium bicarbonate (10%) and saturated sodium sulphate. The organic phase was then dried over sodium sulphate and the solvent evaporated *in vacuo* giving a thick syrup which was dissolved in glacial acetic acid (10 ml) and heated on a steam bath for 1 hour after the gradual addition of water (10 ml) over the first 5 minutes. The acetic acid was then evaporated under reduced pressure and the residue repeatedly evaporated with methanol until the odour of acetic acid was no longer noticeable. The residue was dissolved in liquid ammonia (25 ml) and a solution of sodium in ammonia was added until a blue colour persisted. Dry methanol was then added to destroy the blue colour after which the ammonia was evaporated by gentle warming leaving a dry white powder. An aqueous slurry of Dowex 50 (H<sup>+</sup>) resin was quickly added to this residue until the solution was acidic. The pH was adjusted to pH 7.5 with NaOH and the product was purified by ion exchange. The tubes that gave a positive thiol test were pooled and reduced to approx. 7 ml. KHCO<sub>3</sub> (1 M, 1 ml) and NaOH (1 M, 0.1 ml) were added and the total thiol concentration was measured by finding the absorbance at 412 nm at 25 °C for 10 µl of solution in TSE buffer (990 µl) containing 1 mmol DTNB. Acetic acid (2 equiv) was added and the solution was vortexed immediately. The solution was left on ice for 10 minutes and (15) was purified by HPLC as the potassium salt (129 mg, 9%).

#### **Preparation of S-acetyl 3'-dephospho CoA (58)**

The potassium salt of (15) (36 mg, 75.6 µmol) in water (10 ml) was passed through a column of Dowex 50 (pyridinium form) (3 ml), and the solution was evaporated to dryness *in vacuo*.

The residue was dried three times by evaporation with anhydrous pyridine (each 10 ml), dissolved in dry pyridine (5 ml) and added to a solution in anhydrous pyridine (5 ml) of the salt of adenosine 5'-phosphomorpholidate (18 mgs, 25.1  $\mu$ mol) which was dried beforehand in the same way as above. The reaction mixture was further dehydrated by evaporation three times with anhydrous pyridine, dissolved in pyridine (3 ml), and kept at room temperature overnight. The solvent was evaporated *in vacuo*, and the traces of remaining pyridine were thoroughly removed by several evaporations with water (each 10 ml).

(58) was purified by HPLC (method A, ret. time 24 minutes) as the ammonium salt (24.15  $\mu$ mol, 96%)

$\delta_P$ (121MHz:D<sub>2</sub>O) -11.31 ppm (br)

$\delta_H$ (300MHz:D<sub>2</sub>O) 8.5 (1 H, s, AH-8), 8.24 (1 H, s, AH-2), 6.11 (1 H, d, *J* 5.70, AH-1'), 4.8 (1 H, m, AH-2', HOD), 4.53 (1 H, m, AH-3'), 4.39 (1 H br m, AH-4'), 4.23 (2 H, br m, AH-5'), 4.02 (1 H, s, PH-3'), 3.85 (1 H, m, PH-1'), 3.57 (1 H, m, PH-1'), 3.45 (2 H, t, *J* 6.43, PH-5'), 3.32 (2 H, t, *J* 6.40, PH-8'), 2.96 (2 H, t, *J* 6.39, PH-9'), 2.43 (2 H, t, *J* 6.49, PH-6'), 2.34 (3 H, s, SC(O)CH<sub>3</sub>), 0.92 (3 H, s, PCH<sub>3</sub>-2''), 0.78 (3 H, s, PCH<sub>3</sub>-2').

**See appendix 3 for nomenclature.**

$\delta_C$ (75MHz:D<sub>2</sub>O) 203.50(s), 177.31(s), 176.59(s), 157.93(s), 155.06(d), 142.51(s), 121.29(s), 89.68(d), 86.58(d), 86.47(d), 76.98(d), 76.80(d), 74.51(t), 72.97(d), 67.83(t), 41.20(t), 41.04(s), 38.05(t), 37.96(t), 32.61(q), 30.96(t), 23.53(q), 20.83(q).

*m/z* (FAB) 730 (MH<sup>+</sup>, 0.1%), 255 (20), 136 (100).

### **Preparation of S-acetyl 3'-dephospho guano CoA (72)**

The triethylammonium salt of (15) (442 mg, 0.73 mmol) was dissolved in pyridine and the solution was evaporated to dryness *in vacuo*. The residue was dried three times by evaporation with anhydrous pyridine (each 10 ml), dissolved in dry pyridine (5 ml) and added to a solution in anhydrous pyridine (10 ml) of the salt of guanosine 5'-phosphomorpholidate (177 mg, 0.24 mmol) which was dried beforehand in the same way as above. The reaction

mixture was further dehydrated by evaporation three times with anhydrous pyridine, dissolved in pyridine (3 ml), and kept at room temperature overnight. The solvent was evaporated *in vacuo*, and the traces of remaining pyridine were thoroughly removed by several evaporations with water (each 10 ml).

The reaction mixture was diluted with water (100 ml) and applied to an ion exchange column (100 ml) of DEAE Sephadex resin. The column was eluted with a gradient of TEAB (20-250 mM), pH 7.5 over 17 hours (total volume of elutant used 1600 ml). The eluent from the column was continuously monitored by a UV detector at  $\lambda$  260 nm. The appropriate fractions were combined, concentrated and the TEAB removed as before. The product (**72**) eluted at approximate buffer concentration 140-160 mM, and was isolated as a clear gum (42.8  $\mu$ mol, 17.5%).

Note this experiment was carried out before the HPLC purification method had been developed.

$\delta_P$ (121MHz:D<sub>2</sub>O) -9.36 (d, *J* 21.71), -9.86 (d, *J* 21.87).

$\delta_H$ (300MHz:D<sub>2</sub>O) 8.0 (1 H, s, GH-8), 5.80 (1 H, d, *J* 5.96, GH-1'), 4.8 (1 H, m, GH-2', HOD), 4.5 (1 H, br m, GH-3'), 4.2 (3 H, br m, GH-4',5'), 4.0 (1 H, s, PH-3'), 4.0 (1 H, m, PH-1'), 3.45 (5 H, m, PH-1',5',8'), 3.10 (br q, Et<sub>3</sub>NH), 2.97 (2 H, t, *J* 6.76, PH-9'), 2.43 (2 H, t, *J* 6.73, PH-6'), 2.31 (3 H, s, SC(O)CH<sub>3</sub>), 1.30 (t, Et<sub>3</sub>NH), 1.08 (3 H, s, PCH<sub>3</sub>-2''), 0.79 (3 H, s, PCH<sub>3</sub>-2').

*m/z* (FAB) 847 (MEt<sub>3</sub>NH<sup>+</sup>, 0.1%), 746 (MH<sup>+</sup>, 0.3), 154 (100).

### Preparation of *S*-acetyl 2'-deoxy 3'-dephospho CoA (**75**)

The potassium salt of (**15**) (100 mg, 0.21 mmol) in water (5 ml) was passed through a column of Dowex 50 (pyridinium form) (3 ml), and the solution was evaporated to dryness *in vacuo*. The residue was dried three times by evaporation with anhydrous pyridine (each 10 ml), dissolved in dry pyridine (5 ml) and added to a solution in anhydrous pyridine (10 ml) of the salt of 2'-deoxyadenosine 5'-phosphomorpholidate (45 mg, 69  $\mu$ mol) which was dried

beforehand in the same way as above. The reaction mixture was further dehydrated by evaporation three times with anhydrous pyridine, dissolved in pyridine (3 ml), and kept at room temperature overnight. The solvent was evaporated *in vacuo*, and the traces of remaining pyridine were thoroughly removed by several evaporations with water (each 10 ml). (**75**) was purified by HPLC (method B, ret. time 11 minutes) as the ammonium salt (48.3  $\mu$ mol, 69%).

$\delta_P$ (121MHz:D<sub>2</sub>O) -11.0 (d, *J* 21.79), -11.57 (d, *J* 21.97).

$\delta_H$ (300MHz:D<sub>2</sub>O) 8.50 (1 H, s, AH-8), 8.2 (1 H, s, AH-2), 4.75 (1 H, m, AH-3', HOD), 6.49 (1 H, t, *J* 7.8, AH-1'), 4.3 (1 H, m, AH-4'), 4.2 (2 H, m, AH-5'), 4.0 (1 H, s, PH-3'), 3.85 (1 H, m, PH-1'), 3.6 (1 H, m, PH-1'), 3.45 (2 H, t, *J* 6.34, PH-5') 3.3 (2 H, t, *J* 6.39, PH-8'), 2.95 (2 H, t, *J* 6.4, PH-9'), 2.80 (1 H, m, AH-2'), 2.6 (1 H, m, AH-2'), 2.4 (2 H, t, *J* 6.50, PH-6'), 2.33 (3 H, s, SC(O)CH<sub>3</sub>), 0.91 (3 H, s, PCH<sub>3</sub>-2''), 0.79 (3 H, s, PCH<sub>3</sub>-2').

$\delta_C$ (75MHz:D<sub>2</sub>O) 203.49(s), 177.30(s), 176.60(s), 155.86(s), 152.11(d), 150.98(s), 121.29(s), 88.62(d), 88.50(d), 86.47(d), 76.80(d), 74.51(t), 72.97(d), 67.83(t), 41.93(t) 41.20(t), 41.04(s), 38.05(t), 37.96(t), 32.61(q), 30.96(t), 23.53(q), 20.83(q).

*m/z* (FAB) 916 (M(Et<sub>3</sub>N)<sub>2</sub><sup>+</sup>, 1%), 815 (MEt<sub>3</sub>NH<sup>+</sup>, 42), 714 (MH<sup>+</sup>, 25), 255 (80).

#### Preparation of *S*-acetyl dephospho thymo CoA (**74**)

The potassium salt of (**15**) (43 mg, 0.9  $\mu$ mol) in water (5 ml) was passed through a column of Dowex 50 (pyridinium form) (3 ml), and the solution was evaporated to dryness *in vacuo*. The residue was dried three times by evaporation with anhydrous pyridine (each 10 ml), dissolved in dry pyridine (5 ml) and added to a solution in anhydrous pyridine (10 ml) of the salt of thymidine 5'-phosphomorpholidate (19 mg, 0.3  $\mu$ mol) which was dried beforehand in the same way as above. The reaction mixture was further dehydrated by evaporation three times with anhydrous pyridine, dissolved in pyridine (3 ml), and kept at room temperature overnight. The solvent was evaporated *in vacuo*, and the traces of remaining pyridine were thoroughly removed by several evaporations with water (each 10 ml).

Compound (**74**) was purified by HPLC (method A, ret. time 24 minutes) as the ammonium salt (22.5  $\mu$ mol, 75%).

$\delta_P$ (121MHz:D<sub>2</sub>O) -11.00 (d, *J* 20.76), -11.80 (d, *J* 20.62).

$\delta_H$ (300MHz:D<sub>2</sub>O) 7.7 (1 H, s, TH-1), 6.4 (1 H, d, *J* 6.89, TH-1'), 4.60 (1 H, br m, TH-3'), 4.16 (3 H, m, TH-4',5'), 4.03 (1 H, s, PH-3'), 3.90 (1 H, m, PH-1'), 3.6 (1 H, m, PH-1'), 3.5 (2 H, m, PH-5'), 3.35 (2 H, t, *J* 6.38, PH-8'), 3.02 (2 H, t, *J* 6.40, PH-9'), 2.90 (2 H, m, TH-2'), 2.5 (2 H, m, PH-6'), 2.36 (3 H, s, SC(O)CH<sub>3</sub>), 1.91 (3 H, s, TCH<sub>3</sub>-2), 0.97 (3 H, s, PCH<sub>3</sub>-2''), 0.83 (3 H, s, PCH<sub>3</sub>-2').

$\delta_C$ (75MHz:D<sub>2</sub>O) 203.54(s), 181.49(s), 177.33(s), 176.62(s), 169.11(s), 154.29(s), 140.02(d), 114.34(s), 88.032(dd, <sup>3</sup>*J*<sub>P-C</sub> 9.15), 87.620(d), 76.87(d), 74.57 (dt, <sup>2</sup>*J*<sub>P-C</sub> 6), 73.50(d), 66.10(dt, <sup>2</sup>*J*<sub>P-H</sub> 5.25), 61.89(s), 41.31(t), 41.04(s), 38.09(t), 37.90(t), 32.65(q), 30.99(t), 24.39(q), 23.59(q), 20.91(q).

### Preparation of inosine 5'-monophosphomorpholidate (**80**)

A solution of DCC (0.824 g, 4.0 mmol) in <sup>t</sup>butyl alcohol (15 ml) was added dropwise to a refluxing solution of inosine 5'-monophosphate (0.348 g, 1 mmol of free acid) in a mixture of water (10 ml) and purified morpholine (0.34 ml, 4.0 mmol). The addition was completed in 3-4 hours and the mixture was further refluxed for several hours until only one peak in <sup>31</sup>P nmr. The mixture was cooled to room temperature and any crystalline material present was removed by filtration. The filtrate was evaporated *in vacuo* until the t-butyl alcohol was largely removed and the remaining aqueous phase was diluted with water (100 ml) and applied to an ion exchange column (130 ml) of DEAE Sephadex resin. The column was eluted with a gradient of TEAB (20-200 mM), pH 7.5 for 17 hours (total volume of elutant used 1600 ml). The eluent from the column was continuously monitored by UV detection at  $\lambda$  260 nm. The appropriate fractions were combined, concentrated and the TEAB removed as before. The product (**80**) eluted at approximately 60-80 mM and was isolated as a clear gum (0.69 g, 97%).

$\delta_P$ (121MHz:D<sub>2</sub>O) 7.86(s)

$\delta_H$ (300MHz:D<sub>2</sub>O) 8.44 (1 H, s, 8-H), 8.09 (1 H, s, 2-H), 6.06 (1 H, d, *J* 5.58, 1'-H), 4.71 (1 H, t, *J* 5.31, 2'-H), 4.39 (1 H, m, 3'-H), 4.22 (1 H, m, 4'-H), 4.04, (2 H, m, 5'-H), 3.58 (4 H, m, NCH<sub>2</sub>CH<sub>2</sub>O), 3.20 (q, Et<sub>3</sub>NH), 3.08 (4 H, m, NCH<sub>2</sub>CH<sub>2</sub>O), 1.30 (t, Et<sub>3</sub>NH).

$\delta_C$ (75MHz:D<sub>2</sub>O) 158.86(s), 146.69 (d), 140.62(s), 125.50(s), 89.48(d), 85.87 (d), 85.76(d), 75.98(d), 72.16(d), 68.46(t), 68.37(t), 65.46(t), 65.39(t), 46.56(t).

### Preparation of S-acetyl 3'-dephospho ino CoA (73)

The potassium salt of (15) (61 mg, 127  $\mu$ mol) in water (5 ml) was passed through a column of Dowex 50 (pyridinium form) (3 ml), and the solution was evaporated to dryness *in vacuo*. The residue was dried three times by evaporation with anhydrous pyridine (each 10 ml), dissolved in dry pyridine (5 ml) and added to a solution in anhydrous pyridine (5 ml) of the salt of inosine 5'-phosphomorpholidate (30 mg, 42  $\mu$ mol) which was dried beforehand in the same way as above. The reaction mixture was further dehydrated by evaporation three times with anhydrous pyridine, dissolved in pyridine (3 ml), and kept at room temperature overnight. The solvent was evaporated *in vacuo*, and the traces of remaining pyridine were thoroughly removed by several evaporations with water (each 10 ml).

Compound (73) was purified by HPLC (method C, ret. time 21 minutes) as the ammonium salt (33.9  $\mu$ mol, 80%).

$\delta_P$ (121MHz:D<sub>2</sub>O) -10.90 (d, *J* 20.70), -11.28 (d, *J* 20.75).

$\delta_H$ (300MHz:D<sub>2</sub>O) 8.45 (1 H, s, IH-8), 8.20 (1 H, s, IH-2), 6.11 (d, 1 H, *J* 5.5, IH-1'), 4.8 (1 H, m, IH-2', HOD), 4.55 (1 H, m, IH-3'), 4.37 (1 H, br m, IH-4'), 4.22 (2 H, br m, IH-5'), 4.02 (1 H, s, PH-3'), 3.85 (1 H, PH-1'), 3.6 (1 H, m, PH-1'), 3.48, (2 H, br m, PH-5'), 3.35 (2 H, t, *J* 6.39, PH-8'), 2.92 (2 H, t, *J* 6.39, PH-9'), 2.43 (2 H, t, *J* 6.49, PH-7'), 2.33 (3 H, s, SC(O)CH<sub>3</sub>), 0.93 (3 H, s, PCH<sub>3</sub>-2''), 0.79 (3 H, s, PCH<sub>3</sub>-2').

$\delta_C$ (75MHz:D<sub>2</sub>O) 203.51(s), 177.34(s), 176.62(s), 161.117(s), 148.822(d), 142.33(s), 126.42(s), 90.150(d), 86.69(d), 86.57(d), 77.09(t), 76.82(t), 74.58(t), 72.938(d), 67.77(t),

41.21(t), 38.07(t), 32.64(q), 30.98(t), 23.56(q), 20.84(q).

### **Preparation of S-(Phenyl)-thiopantothenate (28)**

To a stirred suspension of pantothenic acid sodium salt (5 g, 20.7 mmol) in dry THF (40 ml) under a nitrogen atmosphere at 0 °C was added trifluoroacetic acid anhydride (9.1 ml, 64.2 mmol) dropwise over 5 minutes. The reaction mixture was stirred at 0 °C until all of the salt dissolved. Imidazole (4.95 g 72.5 mmol) was added in one portion and the mixture was stirred for 30 minutes at 30 °C, then an additional hour at room temperature. The reaction mixture was vacuum filtered to remove the white crystalline precipitate, which was rinsed with dry THF. The filtrate was then concentrated to ~30 ml by rotary evaporation. A solution of thiophenol (2.76 ml, 27 mmol) in dry THF (20 ml), with a catalytic amount of sodium hydride (~1 mg), was then added in one portion to the reaction mixture which was then stirred at room temperature overnight. The reaction mixture was filtered and the precipitate was rinsed with ether. The combined filtrate and washing were concentrated under reduced pressure, dissolved in acetone (40 ml), and cooled in an ice bath. Sodium bicarbonate (20 ml saturated aqueous solution) was added and the mixture was stirred for 15 minutes. Brine (20 ml) and ethyl acetate (40 ml) were added and the layers separated. The aqueous phase was further extracted with ethyl acetate (2×40 ml). The combined organic fractions were washed with saturated aqueous ammonium chloride, dried over  $\text{MgSO}_4$  and concentrated to yield a clear, slightly yellow liquid. TLC analysis of this material in ethyl acetate/petrol 5:3 showed residual trifluoroacetic esters at  $R_f$  0.69 and 0.79, so the material was dissolved in acetone and stirred with phosphate buffer pH 7 until TLC analysis showed little remaining esters (left overnight). Compound (28) has  $R_f$  0.19 in this solvent. Ethyl acetate (EtOAc, 50 ml) and brine (15 ml) were added, and the organic phase was separated. The aqueous phase was extracted with EtOAc and the combined organics were partially dried over  $\text{MgSO}_4$ , concentrated, and evaporated with toluene under reduced pressure to remove residual water. This material was purified by flash chromatography on silica gel (3×20 cm) eluting first with



EtOAc/petrol 2:1 (200 ml) then with 100% EtOAc to yield **(28)**, (3.18 g, 49%).

$\delta_{\text{H}}$ (300MHz:CDCl<sub>3</sub>) 7.41 (5 H, m, SC<sub>6</sub>H<sub>5</sub>), 4.90 (1 H, br s, N-H D<sub>2</sub>O exchangeable), 4.1 (1 H, s, 2'-H), 3.50 (2 H, m, 1-H), 3.45 (2 H, s, 4'-H), 2.89 (2 H, t, *J* 6.1, 2-H), 1.01 (3 H, s, 5'-H), 0.88 (3 H, s, 6'-H).

$\delta_{\text{C}}$ (75MHz:CDCl<sub>3</sub>) 196.57(s), 173.82(s), 134.37(d), 129.57(d), 129.21(d), 126.97(s), 77.09(d), 70.87(t), 42.77(t), 39.14(s), 34.87(t), 20.88(q), 20.39(q).

### Preparation of dimethyl phosphorochloridate (20)

To a solution of methanol (41.72 ml, 1.04 mol) in dry benzene (90 ml) was added dropwise at 8-10 °C a solution of PCl<sub>3</sub> (30 ml, 0.345 mol) in dry benzene (90 ml). The mixture was stirred for 1 hour and then a solution of sulphuryl chloride (72.8 ml, 0.34 mol) in dry benzene (90 ml) was added dropwise at 8-10 °C. The mixture was warmed to RT, concentrated and distilled under reduced pressure 74 C, 25 mm Hg (lit.,<sup>82</sup> 80 C, 25 mm Hg), to yield the title compound **(20)** (34.8 g, 70%).

$\delta_{\text{P}}$ (121MHz:Benzene) 6.12 ppm

### Preparation of *S*-(phenyl)-thiopantothenate-4'-dimethyl phosphate (36)

Compound **(28)** (2 g, 6.43 mmol ) was dissolved in dry pyridine (10 ml) and evaporated to dryness twice under reduced pressure to remove residual water. Compound **(28)** was dissolved in a final portion of dry pyridine and cooled in a -40 °C bath (acetonitrile-dry ice). Dimethyl phosphorochloridate in benzene (10 ml) was added dropwise into the reaction vessel over two minutes and stirring was continued at -40 °C for 3-4 hours. Water (3 ml) was added and the mixture was concentrated under reduced pressure to yield a yellow oil, which was dissolved in dichloromethane (30 ml) and washed with potassium bicarbonate (2×20 ml, 1M), then ammonium chloride (20 ml, saturated). The combined aqueous phases were extracted with dichloromethane and the combined organics were dried over MgSO<sub>4</sub> and

concentrated under reduced pressure. The resulting oil was purified by flash chromatography on silica gel using EtOAc as eluent ( $R_f=0.20$ ) yielding **(36)**, (0.85 g, 32%).

$\delta_P(121\text{MHz};\text{CDCl}_3)$  2.56 ppm

$\delta_H(300\text{MHz};\text{CDCl}_3)$  7.39 (6 H, m,  $\text{SC}_6\text{H}_5$ , N-H  $\text{D}_2\text{O}$  exchangeable), 4.88 (1 H, d,  $J$  5.90, OH  $\text{D}_2\text{O}$  exchangeable), 4.10 (1 H, m, 4'-H), 3.95 (1 H, d,  $J$  5.68, 2'-H, s on addition of  $\text{D}_2\text{O}$ ), 3.73 (7 H, m, 4'-H,  $(\text{MeO})_2$ ), 3.55 (2 H, m, 1-H), 2.90 (2 H, t,  $J$  6.11, 2-H), 1.09 (3 H, s, 5'-H), 0.90 (3 H, s, 6'-H)

$\delta_C(75\text{MHz};\text{CDCl}_3)$  196.15(s), 172.54(s), 134.40(d), 129.48(d), 129.17(d), 127.19(s), 73.75(dt,  $^2J_{P-C}$  5.97), 73.50(d), 54.50(dq,  $^2J_{P-C}$  5.59), 42.97(t), 39.36(ds,  $^3J_{P-C}$  6.1), 34.85(t), 20.83(q), 18.94(q).

$m/z$  (FAB) 420 ( $\text{MH}^+$ , 74%), 338 (41), 310 (18), 294 (100), 211 (35), 142 (13).

#### **Preparation of *S*-(phenyl)-thiopantothenate-4'-phosphate (27)**

Compound **(36)** (0.98 g, 2.34 mmol) was dissolved in dry acetonitrile (5 ml) then evaporated twice under reduced pressure to remove residual water, and finally dissolved in dry MeCN (5 ml). Trimethylsilylchloride (TMSCl, 3ml, 23 mmol) and lithium bromide (0.43 g, 4.91 mmol) were added. The reaction mixture was heated with stirring at 45 °C under nitrogen for 27 hours. White crystals pervaded the solution.  $^1\text{H}$  NMR analysis showed complete disappearance of the starting material. The reaction mixture was evaporated, treated with methanol, evaporated and finally treated with MeCN and evaporated to yield a tan-white foam.

The crude material was not purified but used directly in the next stage.

#### **Preparation of dethio D-oxypantetheine-4'-phosphate (29)**

Compound **(27)** (0.87 g, 2.2 mmol) was dissolved in THF (10 ml) and ethanolamine (0.67 ml, 11 mmol) was added in one portion and the reaction mixture was stirred at room temperature

for 30 minutes. The THF was removed under reduced pressure to yield a white solid which was dissolved in water (200 ml). Any undissolved material was removed by filtration and the product applied to an ion exchange column (130 ml) of DEAE Sephadex resin. The column was eluted with a gradient of TEAB (20-250 mM), pH 7.5 for 18 hours, (total volume of eluent used 1600 ml). The eluent from the column was continuously monitored by a UV detector at  $\lambda$  260 nm. The appropriate fractions were combined, concentrated and the TEAB removed as before. The product (**29**) eluted at approximate buffer concentration 120-160 mM and was isolated as a clear gum (0.64 g, 52%).

$\delta_P(121\text{MHz:D}_2\text{O})$  0.42 ppm

$\delta_H(300\text{MHz:D}_2\text{O})$  4.01 (1 H, s, 2'-H), 3.75 (1 H, dd,  $^3J_{P-H}$  4.79,  $J_{\text{Gem}}$  9.75, 4'-H), 3.65 (2 H, t,  $J$  5.59, 5-H), 3.55 (1 H, dd,  $^3J_{P-H}$  4.91,  $J_{\text{Gem}}$  10.06, 4'-H), 3.46 (2 H, t,  $J$  6.55, 1-H), 3.27 (2 H, t,  $J$  5.54, 4-H), 3.15 (q, Et<sub>3</sub>NH, CH<sub>2</sub>), 2.46 (2 H, t,  $J$  6.55, 2-H), 1.25 (t, Et<sub>3</sub>NH, CH<sub>3</sub>), 0.94 (3 H, s, 5'-H), 0.87 (3 H, s, 6'-H).

$\delta_C(75\text{MHz:D}_2\text{O})$  177.71(s), 176.69(s), 77.15(d), 73.52(dt,  $^2J_{P-C}$  5.18), 62.48(t), 49.29(t, Et<sub>3</sub>NH), 44.11(t), 40.85(ds,  $^3J_{P-C}$  7.87), 38.10(t), 37.97(t), 23.42(q), 21.28(q).

$m/z$  (FAB) 444 (EtNH<sup>+</sup>, 10%), 343 (MH<sup>+</sup>, 2), 255 (22), 154 (100), 136 (80).

### Preparation of 3'-dephospho oxy CoA (**59**)

The triethylammonium salt of (**29**) (0.698 g, 1.26 mmol) was dissolved in pyridine and the solution was evaporated to dryness *in vacuo*. The residue was dried three times by evaporation with anhydrous pyridine (each 10 ml), dissolved in dry pyridine (5 ml) and added to a solution in anhydrous pyridine (10 ml) of the salt of adenosine 5'-phosphomorpholidate (0.30 g, 4.2 mmol) which was dried beforehand in the same way as above. The reaction mixture was further dehydrated by evaporation three times with anhydrous pyridine, dissolved in pyridine (10 ml), and kept at room temperature overnight. The solvent was evaporated *in vacuo*, and the traces of remaining pyridine were thoroughly removed by several evaporations with water (each 10 ml).

The reaction mixture was diluted with water (200 ml) and applied to an ion exchange column (130 ml) of DEAE Sephadex resin. The column was eluted with a gradient of TEAB (20-250 mM), pH 7.5 for 18 hours, (total volume of eluent used 1600 ml). The eluent from the column was continuously monitored by UV detection at  $\lambda$  260 nm. The appropriate fractions were combined, concentrated and the TEAB removed as before. The product (**59**) eluted at approximate buffer concentration 120-160 mM and was isolated as a clear gum (0.25 mmol, 20%).

$\delta_P(121\text{MHz:D}_2\text{O})$  -11.40(br)

$\delta_H(300\text{MHz:D}_2\text{O})$  8.48 (1 H, s, AH-8), 8.19 (1 H, s, AH-2), 6.08 (1H, d,  $J$  5.73, AH-1'), 4.8 (1 H, m, AH-2', HOD), 4.49 (1 H, m, AH-3'), 4.35 (1 H, br m, AH-4'), 4.20 (2 H, m, AH-5'), 3.99 (1 H, s, PH-3'), 3.85 (1 H br dd, PH-1'), 3.60 (3 H, m, PH-1', PH-9'), 3.45 (2 H, t,  $J$  6.59, PH-5'), 3.31 (2 H, t,  $J$  5.66, PH-8'), 3.14 (q, Et<sub>3</sub>NH, CH<sub>2</sub>), 2.45 (2 H, t,  $J$  6.59, PH-6'), 1.23 (t, Et<sub>3</sub>NH, CH<sub>3</sub>), 0.89 (3 H, s, PCH<sub>3</sub>-2''), 0.76 (3 H, PCH<sub>3</sub>-2').

$\delta_C(75\text{MHz:D}_2\text{O})$  177.29(s), 176.71(s), 157.47(s), 154.53(s), 89.63(d), 86.50(d), 76.93(d), 76.60(d), 74.52(t), 72.97(d), 67.76(t), 62.51(t), 49.28(t, Et<sub>3</sub>NH), 44.10(t), 41.04(s), 38.09(t), 38.02(t), 23.51(q), 20.80(q), 10.89(q, Et<sub>3</sub>NH).

$m/z$  (FAB) 874 (MEt<sub>3</sub>NH<sup>+</sup>, 11%), 672 (MH<sup>+</sup>, 41), 154 (100)

### Preparation of bis(3-phthalamidopropyl) disulphide (**45**)

In a 500 ml three neck round bottomed flask with a reflux condenser a solution of sodium (2.7 g, 0.12 mol) in dry methanol (260 ml) was treated with H<sub>2</sub>S (dried over CaCl<sub>2</sub> and P<sub>2</sub>O<sub>5</sub>) until it went yellow. *N*-(3-bromopropyl) phthalamide (15 g, 0.06 mol) was added and the mixture was heated on a water bath for 2 hours. The reaction mixture was then diluted with water and made weakly acidic with HCl. The product was extracted into CHCl<sub>3</sub> and the layer washed with 10% potassium bicarbonate solution to neutrality. The organic layer was dried over MgSO<sub>4</sub> and then removed *in vacuo* to leave a thick brown oil. For further purification, the oil was dissolved in methanol (30 ml) and to it was added a solution of lead tetra-acetate

(13.3 g, 0.03 mol) in methanol (30 ml). The mixture was clarified with acetic acid and the precipitated yellow lead mercaptide was filtered off washed with ether, suspended in hot methanol and treated with  $\text{H}_2\text{S}$ . The lead disulphide was filtered off, washed with ether and the  $\text{H}_2\text{S}$  and methanol were removed *in vacuo*.

The mercaptan was dissolved in boiling methanol and treated with powdered iodine until the appearance of a weak brown colour.  $\text{CHCl}_3$  (50 ml) was added to the reaction mixture and the excess iodine was removed by washing with 10% sodium bisulphite solution. The organic layer was dried over  $\text{MgSO}_4$  and concentrated under reduced pressure. The disulphide appeared first oily and then crystallised to a white mass, (10.02 g, 81%).

$\delta_{\text{H}}$ (300MHz: $\text{CDCl}_3$ ) 7.75 (4 H, AA'BB'), 3.80 (2 H, t,  $J$  6.91, H-3), 2.74 (2 H, t,  $J$  7.22, H-1), 2.1 (2 H, quin,  $J$  7.03, H-2).

$\delta_{\text{C}}$ (75MHz: $\text{CDCl}_3$ ) 168.61(s), 134.32(d), 132.41(s), 123.60(d), 37.43(t), 37.14(t), 28.5(t).

#### **Preparation of bis(3-aminopropyl) sulphide (HBr salt) (39a)**

Compound (**45**) (6 g, 0.014 mol) was taken up in acetic acid (8 ml) and HBr (48%, conc) was added until the floating oil dissolved. The reaction mixture was then refluxed for 12 hours. The reaction mixture was poured into cold water and the white plates of phthalic acid were filtered off. The filtrate was evaporated to dryness and the brown residue was taken up in a little water whereby some phthalic acid remained undissolved. After several times evaporating to dryness the residue was taken up into ethanol and precipitated with diethyl ether (3.6 g, 76%).

$\delta_{\text{H}}$ (300MHz: $\text{D}_2\text{O}$ ) 2.81 (2 H, t,  $J$  7.53, H-3), 2.45 (2 H, t,  $J$  7.04, H-1), 1.78 (2 H, quin,  $J$  7.29, H-2).

$\delta_{\text{C}}$ (75MHz: $\text{D}_2\text{O}$ ) 38.44(t), 34.05(t), 26.32(t).

### Preparation of bis(3-aminopropyl) disulphide (39)

A solution of Dowex 1 (OH form) was added to (39a) (133 mg, 0.38 mmol). After 20 minutes the Dowex was filtered off and the solution evaporated to dryness to yield the title compound (39) as a clear gum (66 mg, 97%).

$\delta_{\text{H}}$ (300MHz:D<sub>2</sub>O) 2.81 (2 H, t, *J* 7.26, H-3), 2.74 (2 H, t, *J* 7.02, H-1), 1.80 (2 H, quin, *J* 7.13, H-2).

$\delta_{\text{C}}$ (75MHz:D<sub>2</sub>O) 41.97(t), 38.17(t), 33.70(t).

### Preparation of D-homopantethine-4'-phosphate (40)

Compound (27) (0.824 mmol) was dissolved in THF (5 ml) and to it was added a mixture of (39) (66 mg, 0.37 mmol) and triethylamine (0.49 ml, 3.48 mmol) in H<sub>2</sub>O. The reaction mixture was stirred overnight. The THF was concentrated under reduced pressure and the residue was taken up in H<sub>2</sub>O (200 ml) and any undissolved material was removed by filtration. The reaction mixture was applied to an ion exchange column (130 ml) of DEAE Sephadex resin. The column was eluted with a gradient of TEAB (50-400 mM), pH 7.5 for 18 hours, (total volume of eluent used 1600 ml). The eluent from the column was continuously monitored by UV detection at  $\lambda$  220 nm. The appropriate fractions were combined, concentrated and the TEAB removed as before. The product (40) eluted at approximate buffer concentration 190-210 mM and was isolated as a clear gum (126 mg, 30%).

$\delta_{\text{P}}$ (121MHz:CD<sub>3</sub>OD) 2.99 ppm

$\delta_{\text{H}}$ (300MHz:CD<sub>3</sub>OD) 3.80 (1 H, s, 2'-H), 3.65 (1 H, dd, <sup>3</sup>*J*<sub>P-H</sub> 6.32, *J*<sub>Gem</sub> 9.93, 4'-H), 3.26 (3 H, m, 1-H, 4'-H), 3.05 (2 H, m, 4-H), 2.95 (q, Et<sub>3</sub>NH, CH<sub>2</sub>), 2.55 (2 H, t, *J* 7.16, 6-H), 2.25 (2 H, t, *J* 6.70, 2-H) 1.65 (2 H, quin, *J* 7.02, 5-H), 1.1 (t, Et<sub>3</sub>NH, CH<sub>3</sub>), 0.85 (3 H, s, 5'-H), 0.66 (3 H, s, 6'-H).

$\delta_C$ (75MHz:CD<sub>3</sub>OD) 175.46(s), 173.65(s), 75.30(d), 72.40(dt, <sup>2</sup>J<sub>P-C</sub> 5.25), 47.47 (t, Et<sub>3</sub>NH), 40.16 (ds, <sup>3</sup>J<sub>P-C</sub> 7.13), 39.02(t), 36.76(t), 36.50(t), 36.35(t), 29.91(t), 21.97(q), 19.61(q), 9.13(q, Et<sub>3</sub>NH).

m/z (FAB) 844 (MEt<sub>3</sub>NH<sup>+</sup>, 13%), 742 (MH<sup>+</sup>, 16).

### Preparation of 3'-dephospho homopantethine CoA (61)

The triethylammonium salt of (40) (180 mg, 0.157 mmol) was dissolved in pyridine and the solution was evaporated to dryness *in vacuo*. The residue was dried three times by evaporation with anhydrous pyridine (each 5 ml), dissolved in dry pyridine (5 ml) and added to a solution in anhydrous pyridine (10 ml) of the salt of adenosine 5'-phosphomorpholidate (667 mg, 0.94 mmol) which was dried beforehand in the same way as above. The reaction mixture was further dehydrated by evaporation three times with anhydrous pyridine, dissolved in pyridine (5 ml), and kept at room temperature for 1 week. The solvent was evaporated *in vacuo*, and the traces of remaining pyridine were thoroughly removed by several evaporations with water (each 10 ml).

The reaction mixture was diluted with water (100 ml) and applied to an ion exchange column (130 ml) of DEAE Sephadex resin. The column was eluted with a gradient of TEAB (50-450 mM), pH 7.5 for 18 hours, (total volume of eluent used 1600 ml). The eluent from the column was continuously monitored by UV detection at  $\lambda$  260 nm. The appropriate fractions were combined, concentrated and the TEAB removed as before. The product (61) eluted at approximate buffer concentration 200-235 mM and was isolated as a clear gum (152 mg, 55%).

$\delta_P$ (121MHz:CD<sub>3</sub>OD) -9.38 (br).

$\delta_H$ (300MHz:CD<sub>3</sub>OD) 8.60 (1 H, br s, AH-8), 8.22 (1 H, br s, AH-2), 6.10 (1 H, d, *J* 5.64, AH-1'), 4.75 (1 H, br m, AH-2'), 4.48 (1 H, br m, AH-3'), 4.25 (3 H, br m, AH-4', AH-5'), 4.10 (2 H, br m, PH-1',3'), 3.50 (3 H br m, PH-1', PH-5'), 3.15 (q, Et<sub>3</sub>NH, CH<sub>2</sub>, PH-8'), 2.62 (2 H, t, *J* 7.11, PH-10'), 2.40 (2 H, br t, PH-6'), 1.85 (2 H, br m, PH-9'), 1.30 (t, Et<sub>3</sub>NH, CH<sub>3</sub>),

0.97 (3 H, s, PCH<sub>3</sub>-2''), 0.81 (3 H, s, PCH<sub>3</sub>-2').

$\delta_C$ (75MHz:CD<sub>3</sub>OD) 175.46(s), 173.67(s), 153.71(d), 88.98(d), 85.52(d), 76.60(d), 74.89(d), 73.30(t), 71.98(d), 66.50(t), 47.40(t, Et<sub>3</sub>NH), 40.20(s), 39.02(t), 36.75(t), 36.57(t), 36.35(t), 29.89(t), 22.29(q), 19.35(q), 9.18(q, Et<sub>3</sub>NH).

### Preparation of *S*-acetyl 3'-dephospho homo CoA (60)

Compound (61) (112 mg, 98  $\mu$ mol) was dissolved in water (3 ml) and DTT (30 mg, 195  $\mu$ mol) was added and the reaction mixture was stirred for 20 minutes. KHCO<sub>3</sub> (1 ml, 1 M) and NaOH (0.1 ml, 1 M) were added and the concentration of free thio was measured. Acetic anhydride (2 equiv) was added and the solution was vortexed and left on ice. If after 20 minutes a negative DTNB test for free thiols was not obtained more acetic anhydride was added. Compound (60) was purified by HPLC (method A, ret. time 26.5 minutes) as the ammonium salt (21.0  $\mu$ mol, 10%).

$\delta_P$ (121MHz:D<sub>2</sub>O) -11.30 (br).

$\delta_H$ (300MHz:D<sub>2</sub>O) 8.54 (1 H, s, AH-8), 8.30 (1 H, s, AH-2), 6.12 (1 H, d,  $J$  5.68, AH-1'), 4.8 (1 H, m, AH-2', HOD), 4.53 (1 H, m, AH-3'), 4.39 (1 H, br m, AH-4'), 4.22 (2 H, br m, AH-5'), 4.01 (1 H, s, PH-3'), 3.84 (1 H, dd,  $^3J_{P-H}$  4.24,  $J_{Gem}$  9.80, PH-1'), 3.57 (1 H, dd,  $^3J_{P-H}$  4.30,  $J_{Gem}$  9.35, PH-1'), 3.45 (2 H, t,  $J$  6.42, PH-5'), 3.17 (2 H, t,  $J$  6.72, PH-8'), 2.82 (2 H, t,  $J$  7.14, PH-10'), 2.44 (2 H, t,  $J$  6.48, PH-6'), 2.32 (3 H, s, SC(O)CH<sub>3</sub>), 1.71 (2 H, quin,  $J$  6.92, PH-9'), 0.91 (3 H, s, PCH<sub>3</sub>-2''), 0.78 (3 H, s, PCH<sub>3</sub>-2').

$\delta_C$ (75MHz:D<sub>2</sub>O) 201.22(s), 177.32(s), 176.43(s), 156.39(s), 152.85(d), 143.26(s), 89.92(d), 86.71(d), 86.60(d), 77.10(d), 76.61(d), 74.50(dt,  $^2J_{P-C}$  5.93), 72.95(d), 67.73(t), 41.51(ds,  $^3J_{P-C}$  7.80), 40.68(t), 38.13(t), 38.02(t), 32.62(q), 30.76(t), 28.77(t), 23.86(t), 23.52(q), 20.83(q).



### Preparation of methyl CoA (64)

Methyl iodide (20  $\mu$ l, 0.33 mmol) in ethanol (2.5 ml) was added to a solution of CoA (100 mg, 0.13 mmol) in  $\text{KHCO}_3$  (2.5 ml, pH 10). The reaction mixture was maintained at pH 10 by the addition of KOH (3 M). After 2 hour, a negative DTNB test for unreacted thiols was obtained. The unreacted methyl iodide was extracted into ethyl acetate and the product was diluted with water (100 ml) and applied to an ion exchange column (100 ml) of DEAE Sephadex resin. The column was eluted with a gradient of TEAB (20-200 mM), pH 7.5 for 18 hours, (total volume of eluent used 1600 ml). The eluent from the column was continuously monitored by UV detection at  $\lambda$  260 nm. The appropriate fractions were combined, concentrated and the TEAB removed as before. The product (**64**) eluted at approximate buffer concentration 120-140 mM and was isolated as a clear gum (89.6  $\mu$ mol, 81%).

$\delta_{\text{P}}(121\text{MHz};\text{D}_2\text{O})$  2.06 (s), -9.51 (d,  $J$  20.93), -10.06 (d,  $J$  20.51).

$\delta_{\text{H}}(300\text{MHz};\text{D}_2\text{O})$  8.60 (1 H, s, AH-8), 8.20 (1 H, s, AH-2), 6.15 (1 H, d,  $J$  5.85, AH-1'), 4.85 (1 H, m, AH-2', AH-3', HOD), 4.50 (1 H, m, AH-4'), 4.30 (2 H, m, AH-5'), 4.10 (1 H, s, PH-3'), 4.01 (1 H, dd,  $^3J_{\text{P-H}}$  4.60,  $J_{\text{Gem}}$  9.50, PH-1'), 3.50 (1 H, dd,  $^3J_{\text{P-H}}$  4.80,  $J_{\text{Gem}}$  9.37, PH-1'), 3.45 (2 H, t,  $J$  6.71, PH-5'), 3.35 (2 H, s,  $J$  6.82, PH-8'), 3.2 (q,  $\text{Et}_3\text{NH}$ ,  $\text{CH}_2$ ), 2.55 (2 H, t,  $J$  7.06, PH-9'), 2.45 (2 H, t,  $J$  6.69, PH-6'), 2.10 (3 H, s,  $\text{SCH}_3$ ), 1.30 (t,  $\text{Et}_3\text{NH}$ ,  $\text{CH}_3$ ), 1.10 (3 H, s,  $\text{PCH}_3$ -2''), 0.80 (3 H, s,  $\text{PCH}_3$ -2').

$\delta_{\text{C}}(75\text{MHz};\text{D}_2\text{O})$  175.54(s), 173.06(s), 157.04(s), 153.67(d), 150.97(s), 141.04(d), 120.5(s), 88.58(d), 84.59(d), 75.64(d), 74.84(d), 73.37(t), 66.43(t), 47.32 ( $\text{Et}_3\text{NH}$ ), 40.18(s), 39.40(t), 36.61(t), 36.32(t), 34.07(t), 22.44(q), 19.06(q), 15.09(q), 9.09( $\text{Et}_3\text{NH}$ ).

$m/z$  (FAB) 984 ( $\text{M}(\text{Et}_3\text{N})_2^+$ , 5%), 883 ( $\text{MEt}_3\text{NH}^+$ , 20), 782 ( $\text{MH}^+$ , 68), 255 (100), 227 (28).

### Preparation of ethyl CoA (65)

Ethyl iodide (26  $\mu$ l, 0.33 mmol) in ethanol (2.5 ml) was added to a solution of CoA (100 mg,

0.13 mmol) in  $\text{KHCO}_3$  (2.5 ml, pH 10). The reaction mixture was maintained at pH 10 by the addition of KOH (3 M). After 2 hours, a negative DTNB test for unreacted thiols was obtained. The unreacted ethyl iodide was extracted into ethyl acetate and the product was diluted with water (100 ml) and applied to an ion exchange column (100 ml) of DEAE Sephadex resin. The column was eluted with a gradient of TEAB (20-200 mM), pH 7.5 for 18 hours, (total volume of eluent used 1600 ml). The eluent from the column was continuously monitored by UV detection at  $\lambda$  260 nm. The appropriate fractions were combined, concentrated and the TEAB removed as before. The product (**65**) eluted at approximate buffer concentration 110-130 mM and was isolated as a clear gum (94  $\mu\text{mol}$ , 85%).

$\delta_{\text{P}}$ (121MHz:D<sub>2</sub>O) 1.98 (s), -9.58 (d,  $J$  20.93), -9.96 (d,  $J$  20.51).

$\delta_{\text{H}}$ (300MHz:D<sub>2</sub>O) 8.60 (1 H, s, AH-8), 8.20 (1 H, s, AH-2), 6.30 (1 H, d,  $J$  5.98, AH-1'), 4.80 (2 H, m, AH-2', AH-3', HOD), 4.55 (1 H, m, AH-4'), 4.30 (2 H, m, AH-5'), 4.1 (1 H, s, PH-3'), 3.95 (1 H, dd,  $^3J_{\text{P-H}}$  5.18,  $J_{\text{Gem}}$  9.69, PH-1'), 3.45 (1 H, dd,  $^3J_{\text{P-H}}$  5.25,  $J_{\text{Gem}}$  9.72, PH-1'), 3.35 (2 H, t,  $J$  6.68, PH-5'), 3.29 (2 H, t,  $J$  6.98, PH-8'), 3.10 (q, Et<sub>3</sub>NH, CH<sub>2</sub>), 2.59 (2 H, t,  $J$  6.90, PH-9') 2.50 (2 H, q,  $J$  7.38, CH<sub>2</sub>CH<sub>3</sub>). 2.41 (2 H, t,  $J$  6.69, PH-6'), 1.20 (t, Et<sub>3</sub>NH, CH<sub>3</sub>), 1.15 (3 H, t,  $J$  7.39, CH<sub>2</sub>CH<sub>3</sub>), 1.10 (3H, s, PCH<sub>3</sub>-2''), 0.79 (3H, s, PCH<sub>3</sub>-2').

$\delta_{\text{C}}$ (75MHz:D<sub>2</sub>O) 175.44(s), 173.58(s), 156.94(s), 153.54(d), 150.96(d), 141.06(s), 120.07(s), 88.66(d), 84.48(d), 75.64(d), 74.90(d), 73.40(t), 66.41(t), 47.29(Et<sub>3</sub>NH), 40.18(t), 40.08(s), 36.59(t), 36.33(t), 31.53(t), 26.35(t), 22.45(q), 19.15(q), 15.22(q), 9.13(Et<sub>3</sub>NH).

$m/z$  (FAB) 998 ( $\text{M}(\text{Et}_3\text{N})_2^+$ , 3%), 897 ( $\text{MEt}_3\text{NH}^+$ , 20), 796 ( $\text{MH}^+$ , 79), 255 (100), 227 (33).

### Preparation of propyl CoA (**66**)

Propyl iodide (31  $\mu\text{l}$ , 0.33 mmol) in ethanol (2.5 ml) was added to a solution of CoA (100 mg, 0.13 mmol) in  $\text{KHCO}_3$  (2.5 ml, pH 10). The reaction mixture was maintained at pH 10 by the addition of KOH (3 M). After 2 hours, a negative DTNB test for unreacted thiols was obtained. The unreacted propyl iodide was extracted into ethyl acetate and the product (**66**)

was purified by HPLC (method A, ret. time 30.0 minutes) as the ammonium salt (97.5  $\mu$ mol, 75%).

$\delta_P$ (121MHz:D<sub>2</sub>O) 1.98 (s), -9.51 (d, *J* 20.76), -10.01 (d, *J* 20.76).

$\delta_H$ (300MHz:D<sub>2</sub>O) 8.61 (1 H, s, AH-8), 8.20 (1 H, s, AH-2), 6.12 (1 H, br d, AH-1'), 4.85 (br m, AH-2', AH-3'), 4.45 (1 H, br m, AH-4'), 4.35 (2 H, br m, AH-5'), 4.08 (1 H, s, PH-3'), 3.85 (1 H, br dd, PH-1'), 3.45 (1 H, br dd, PH-1'), 3.35 (2 H, t, *J* 6.69, PH-5'), 3.2 (2 H, t, *J* 7.16, PH-8'), 2.59 (2 H, t, *J* 7.20, PH-9'), 2.49 (2 H, t, *J* 7.23, SCH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 2.43 (2 H, t, *J* 6.70, PH-6'), 1.57 (2 H, sextet, *J* 7.30, SCH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 1.09 (3 H, s, PCH<sub>3</sub>-2''), 0.97 (3 H, t, *J* 7.33, SCH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 0.79 (3 H, s, PCH<sub>3</sub>-2').

$\delta_C$ (75MHz:D<sub>2</sub>O) 177.35(s), 176.47(s), 153.86(s), 149.41(s), 89.85(d), 86.21(d), 76.70(d), 76.64(d), 74.57(t), 67.62(t), 41.37(t), 41.01(s), 36.12(t), 36.35(t), 32.85(t), 24.93(t), 23.56(q), 20.69(q), 15.31(q).

*m/z* (FAB) 810 (MH<sup>+</sup>, 25%), 255 (90), 227 (43).

### Preparation of butyl CoA (67)

Butyl iodide (37  $\mu$ l, 0.33 mmol) in ethanol (2.5 ml) was added to a solution of CoA (100 mg, 0.13 mmol) in KHCO<sub>3</sub> (2.5 ml, pH 10). The reaction mixture was maintained at pH 10 by the addition of KOH (3 M). After 4 hours, a negative DTNB test for unreacted thiols was obtained. The unreacted butyl iodide was extracted into ethyl acetate and the product (67) was purified by HPLC (method A, ret. time 33.5 minutes) as the ammonium salt (89.7  $\mu$ mol, 69%).

$\delta_P$ (121MHz:D<sub>2</sub>O) 1.98 (s), -9.53 (d, *J* 20.74), -10.01 (d, *J* 20.75).

$\delta_H$ (300MHz:D<sub>2</sub>O) 8.60 (1 H, s, AH-8), 8.41 (1 H, s, AH-2), 6.20 (1 H, br d, AH-1'), 4.8 (br m, AH-2', AH-3' HOD), 4.53 (1 H, br m, AH-4'), 4.30 (2 H, br m, AH-5'), 3.95 (1 H, s, PH-3'), 3.75 (1 H, br d, PH-1'), 3.55 (1 H, br d, PH-1'), 3.35 (2 H, t, *J* 5.96, PH-5'), 3.20 (2 H, t, *J* 6.58, PH-8'), 2.49 (2 H, t, *J* 6.55, PH-9'), 2.41 (2 H, t, *J* 7.22, SCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 2.31 (2 H, t, *J* 5.96, PH-6'), 1.35 (2 H, quin, *J* 7.17, SCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 1.20 (2 H sextet, *J* 7.17,

SCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 0.85 (3 H, s, PCH<sub>3</sub>-2''), 0.70 (6 H, m, PCH<sub>3</sub>-2', SCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>).

δ<sub>C</sub>(75MHz:D<sub>2</sub>O) 177.36(s), 176.78(s), 153.04(s), 148.30(s), 89.95(d), 86.22(d), 76.72(d), 76.66(d), 74.57(t), 87.80(t), 41.38(t), 41.01(s), 38.13(t), 38.03(t), 33.57(t), 33.34(t), 32.89(t), 23.94(t), 23.58(t), 20.92(t), 15.56(t).

m/z (FAB) 824 (MH<sup>+</sup>, 84%)

### Preparation of isobutyl CoA (70)

Isobutyl iodide (22 μl, 0.19 mmol) in ethanol (2.5 ml) was added to a solution of CoA (60 mg, 0.13 mmol) in KHCO<sub>3</sub> (2.5 ml, pH 10). The reaction mixture was maintained at pH 10 by the addition of KOH (3 M). After 18 hours, a negative DTNB test for unreacted thiols was obtained. The unreacted isobutyl iodide was extracted into ethyl acetate and the product (70) was purified by HPLC (method A, ret. time 36 minutes) as the ammonium salt (12.89 μmol, 16%).

δ<sub>P</sub>(121MHz:D<sub>2</sub>O) 1.96 (s), -9.53 (d, *J* 20.76), -10.06 (d, *J* 20.75).

δ<sub>H</sub>(300MHz:D<sub>2</sub>O) 8.65 (1 H, s, AH-8), 8.45 (1 H, s, AH-2), 6.30 (1 H, br d, AH-1'), 4.90 (br m, AH-2', AH-3', HOD), 4.65 (1 H, br m, AH-4'), 4.35 (2 H, br m, AH-5'), 4.10 (1 H, s, PH-3'), 3.95 (1 H, br d, PH-1'), 3.65 (1 H, br d, PH-1'), 3.51 (2 H, t, *J* 5.96, PH-5'), 3.35 (2 H, t, *J* 6.58, PH-8'), 2.66 (2 H, t, *J* 6.23, PH-9'), 2.45 (4 H, m, PH-6', CH<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>), 1.79 (1 H, nonet, *J* 6.64, CH<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>), 0.95 (9 H, m, PCH<sub>3</sub>-2'', CH<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>), 0.85 (3 H, s, PCH<sub>3</sub>-2').

δ<sub>C</sub>(75MHz:D<sub>2</sub>O) 177.38(s), 176.48(s), 154.59(s), 150.20(s), 89.80(d), 86.23(d), 76.83(d), 76.63(d), 74.56(t), 67.85(t), 42.94(t), 41.41(t), 41.02(s), 38.13(t), 38.04(t), 33.53(t), 30.61(d), 23.82(q), 23.57(q), 20.89(q).

m/z (FAB) 824 (MH<sup>+</sup>, 31%).

### Preparation of pentyl CoA (68)

Pentyl iodide (26  $\mu$ l, 0.19 mmol) in ethanol (2.5 ml) was added to a solution of CoA (60 mg, 0.13 mmol) in  $\text{KHCO}_3$  (2.5 ml, pH 10). The reaction mixture was maintained at pH 10 by the addition of KOH (3 M). After 4 hours, a negative DTNB test for unreacted thiols was obtained. The unreacted pentyl iodide was extracted into ethyl acetate and the product (**68**) was purified by HPLC (method A, ret. time 36.0 minutes) as the ammonium salt (26.7  $\mu$ mol, 34%).

$\delta_{\text{P}}(121\text{MHz:D}_2\text{O})$  1.99 (s), -9.53 (d,  $J$  20.76), -10.01 (d,  $J$  20.75).

$\delta_{\text{H}}(300\text{MHz:D}_2\text{O})$  8.70 (1 H, s, AH-8), 8.42 (1 H, s, AH-2), 6.20 (1 H, d,  $J$  5.14, AH-1'), 4.8 (br m, AH-2', AH-3', HOD), 4.55 (1 H, br m, AH-4'), 4.28 (2 H, br m, AH-5'), 4.02 (1 H, s, PH-3'), 3.85 (1 H, br d, PH-1'), 3.60 (1 H, br d, PH-1'), 3.45 (2 H, t,  $J$  6.35, PH-5'), 3.29 (2 H, t,  $J$  6.68, PH-8'), 2.60 (2 H, t,  $J$  6.68, PH-9'), 2.50 (2 H, t,  $J$  7.29,  $\text{SCH}_2$ ), 2.40 (2 H, t,  $J$  6.30, PH-6'), 1.50 (2 H, quin,  $J$  7.03,  $\text{SCH}_2\text{CH}_2$ ), 1.25 (4 H, m,  $\text{SCH}_2\text{CH}_2\text{CH}_2\text{CH}_2$ ), 0.95 (3 H, s,  $\text{PCH}_3$ -2''), 0.80 (6 H,  $\text{PCH}_3$ -2',  $\text{SCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ ).

$\delta_{\text{C}}(75\text{MHz:D}_2\text{O})$  177.36(s), 176.49(s), 152.48(s), 151.08(d), 147.45(s), 145.05(d), 121.08(s), 90.06(d), 86.27(d), 76.85(t), 76.75(d), 76.64(d), 74.59(t), 67.77(t), 41.38(t), 41.06(s), 38.14(t), 38.04(t), 33.64(t), 32.88(t), 31.08(t), 24.23(t), 23.57(q), 20.93(q), 15.92(q)

### Preparation of hexyl CoA (69)

Hexyl iodide (29  $\mu$ l, 0.19 mmol) in ethanol (2.5 ml) was added to a solution of CoA (60 mg, 0.13 mmol) in  $\text{KHCO}_3$  (2.5 ml, pH 10). The reaction mixture was maintained at pH 10 by the addition of KOH (3 M). After 8 hours, a negative DTNB test for unreacted thiols was obtained. The unreacted hexyl iodide was extracted into ethyl acetate and the product (**69**) was purified by HPLC (method A, ret. time 40 minutes) as the ammonium salt (14.5  $\mu$ mol, 19%).

$\delta_{\text{P}}(121\text{MHz:D}_2\text{O})$  1.94 (s), -9.58 (d,  $J$  20.79), -10.01 (d,  $J$  20.79).

$\delta_{\text{H}}$ (300MHz:D<sub>2</sub>O) 8.60 (1 H, s, AH-8), 8.31 (1 H, s, AH-2), 6.10 (1 H, br d, AH-1'), 4.70 (br m, AH-2', AH-3', HOD), 4.50 (1 H, br m, AH-4'), 4.20 (2 H, br m, AH-5'), 3.95 (1 H, s, PH-3'), 3.80 (1 H, br d, PH-1'), 3.51 (1 H, br d, PH-1'), 3.35 (2 H, t, *J* 6.08, PH-5'), 3.20 (2 H, t, *J* 6.62, PH-8'), 2.55 (2 H, t, *J* 6.62, PH-9'), 2.45 (2 H, t, *J* 7.23, SCH<sub>2</sub>), 2.35 (2 H, t, *J* 6.13, PH-6'), 1.45 (2 H, quin, *J* 7.06, SCH<sub>2</sub>CH<sub>2</sub>), 1.15 (6 H, m, SCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 0.85 (3 H, s, PCH<sub>3</sub>-2''), 0.70 (6 H, m, PCH<sub>3</sub>-2', SCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>).

$\delta_{\text{C}}$ (75MHz:D<sub>2</sub>O) 177.30(s), 176.49(s), 152.62(s), 151.10(d), 147.57(s), 133.30(s), 90.08(d), 86.31(d), 76.75(d), 74.61(t), 67.79(t), 41.39(t), 41.06(s), 38.14(t), 38.49(t), 33.68(t), 33.23(t), 32.90(t), 31.44(t), 30.28(t), 24.55(t), 23.59(q), 20.94(q), 16.00(q).

### Preparation of benzyl CoA (71)

Benzyl bromide (23  $\mu$ l, 0.20 mM) in ethanol (2.5 ml) was added to a solution of CoA (62 mg, 0.009 mM) in KHCO<sub>3</sub> (2.5 ml, pH 10). The reaction mixture was maintained at pH 10 by the addition of KOH (3 M). After 7 hours, a negative DTNB test for unreacted thiols was obtained. The unreacted benzyl bromide was extracted into ethyl acetate and the product was diluted with water (100 ml) and applied to an ion exchange column (100 ml) of DEAE Sephadex resin. The column was eluted with a gradient of TEAB (20-200 mM), pH 7.5 for 18 hours, (total volume of eluent used 1600 ml). The eluent from the column was continuously monitored by UV detection at  $\lambda$  260 nm. The appropriate fractions were combined, concentrated and the TEAB removed as before. The product (**71**) eluted at approximate buffer concentration 230-250 mM and was isolated as a clear gum (41.9  $\mu$ mol, 62%).

$\delta_{\text{P}}$ (121MHz:D<sub>2</sub>O) 2.08 (s), -9.56 (d, *J* 20.71), -9.92 (d, *J* 20.76).

$\delta_{\text{H}}$ (300MHz:D<sub>2</sub>O) 8.60 (1 H, s, AH-8), 8.20 (1 H, s, AH-2), 7.30 (5 H, m, C<sub>6</sub>H<sub>5</sub>), 6.12 (1 H, d, *J* 6.03, AH-1'), 4.8 (br m, AH-2', AH-3' HOD), 4.50 (1 H, m, AH-4'), 4.30 (2 H, m, AH-5'), 4.15 (2 H, m, PH-3', PH-1') 3.70 (2 H, s, CH<sub>2</sub>C<sub>6</sub>H<sub>5</sub>), 3.50 (3 H, PH-1', PH-5'), 3.30 (2 H, m, PH-8'), 3.15 (m, Et<sub>3</sub>NH, CH<sub>2</sub>), 2.49 (2 H, t, *J* 7.11, PH-9'), 2.41 (2 H, t, *J* 6.68,

PH-6'), 1.30 (t, Et<sub>3</sub>NH, CH<sub>3</sub>), 1.09 (3 H, s, PCH<sub>3</sub>-2''), 0.80 (3 H, s, PCH<sub>3</sub>-2').

$\delta_C$ (75MHz:D<sub>2</sub>O) 175.52(s), 173.62(s), 153.73(d), 139.80(s), 129.93(d), 129.36(d), 127.87(d), 88.64(d), 75.62(d), 74.87(d), 73.40(t), 66.37(s), 47.33(Et<sub>3</sub>NH), 40.18(s), 39.69(t), 36.59(t), 36.45(t), 36.33(t), 31.35(t), 31.35(t), 22.45(q), 19.09(q), 9.10(Et<sub>3</sub>NH).

### 5.3 Kinetic studies

#### 5.3.1 Preparation of TSE buffer

TSE buffer was prepared from 50 mM tris(hydroxymethyl)aminomethane (tris), 100 mM sodium chloride and 0.1 mM ethylenediaminetetra-acetic acid disodium salt (EDTA) in distilled water. The pH was adjusted to 7.5 with conc. HCl.

#### 5.3.2 Preparation of acetyl CoA

Coenzyme A (100 mg) was added to distilled water (7 ml), KHCO<sub>3</sub> (1 M, 1 ml) and NaOH (1 M, 0.1 ml) cooled in ice. Acetic anhydride (25  $\mu$ l) was added and the solution was vortexed immediately. The solution was left to stand on ice for 10 min. The concentration of remaining CoA was checked by finding the absorbance at 412 nm at 25 °C for 10  $\mu$ l of the AcCoA solution in TSE buffer (990  $\mu$ l) containing 1 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). The absorbance was <0.02 and so the solution was assayed for AcCoA. The assay mixture was made up from TSE and 1 mM DTNB (935  $\mu$ l), 0.25 mM chloramphenicol (50  $\mu$ l of 5 mM), the AcCoA solution (5  $\mu$ l) and CAT (10  $\mu$ l). The concentration of acetyl CoA was determined by finding the absorbance at 260 nm at 25 °C ( $\epsilon_{260}$ =15.4 $\times$ 10<sup>3</sup> M<sup>-1</sup>cm<sup>-1</sup>), and it was diluted to 10 mM. Acetyl CoA was stored at -20 °C as 500  $\mu$ l aliquots.

### 5.3.3 Assay of CAT activity

CAT activity was assayed spectrophotometrically at 25 °C by monitoring the release of CoASH by reaction with DTNB. One unit of enzyme activity is defined as the amount that will convert 1  $\mu\text{mol}$  of chloramphenicol to product per minute. The standard assay mixture contained TSE buffer (930  $\mu\text{l}$  containing 1 mM DTNB), 0.4 mM acetyl CoA (40  $\mu\text{l}$  of 10 mM) and 0.1 mM chloramphenicol (20  $\mu\text{l}$  of 5 mM). The reaction was initiated by the addition of CAT (10  $\mu\text{l}$ ) and was monitored at 412 nm.

### 5.3.4 Kinetic analysis

Measurement of the linear rates of acetylation of chloramphenicol by acetyl CoA analogues employed the same conditions used in the CAT standard assay but over various concentrations. The assay mixtures were made up from four substrate concentrations (which range ideally from  $\sim 0.3 \times K_M$ – $3 \times K_M$ ) and four chloramphenicol concentrations (5  $\mu\text{M}$ , 10  $\mu\text{M}$ , 20  $\mu\text{M}$  and 100  $\mu\text{M}$ ). Each rate was measured in triplicate giving a total of 48 assay mixtures per substrate. Kinetic parameters were determined from linear slope and intercept replots fitted from computer (GrafFit) double reciprocal plots.

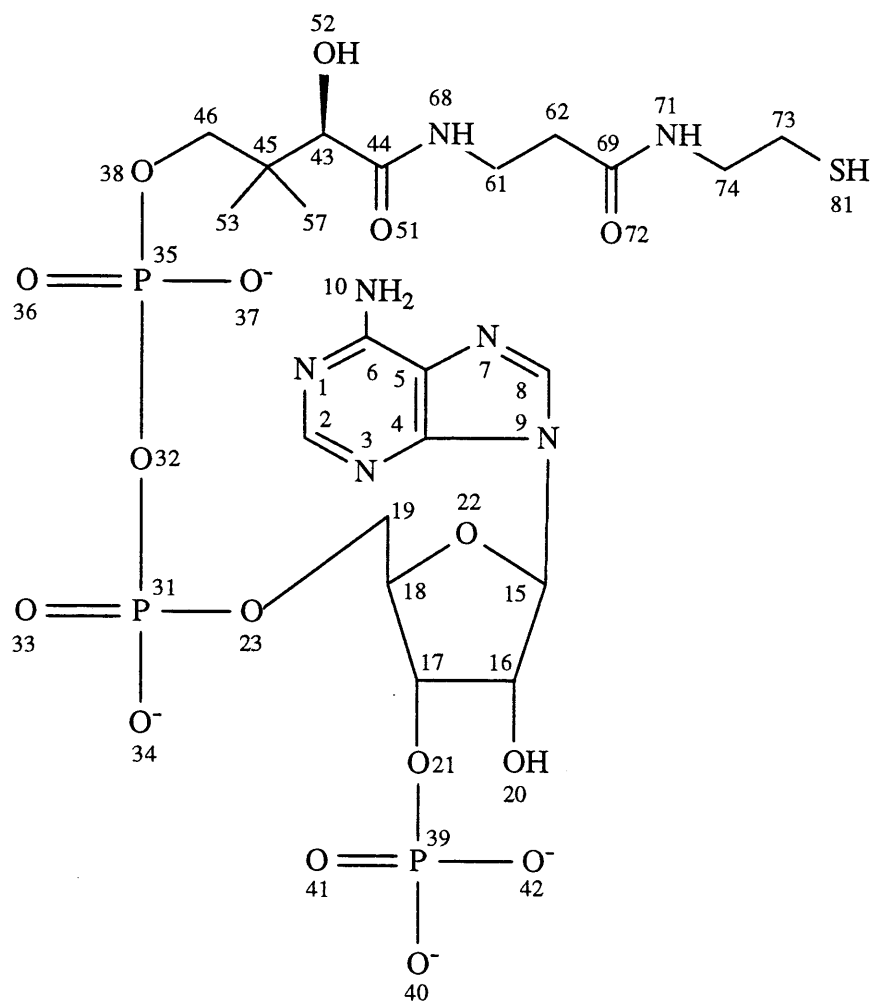
Concentrations of inhibitors and acetyl CoA were varied in the standard assay during mixed inhibition studies that routinely involved chloramphenicol concentration  $\sim 10$  times  $K_M$ . Similarly the concentration of inhibitors and chloramphenicol were varied in the standard assay that included acetyl CoA at fixed concentration  $\sim 4$  times  $K_M$ . The  $K_i$  values were determined from linear slope replots derived from double reciprocal plots as described.



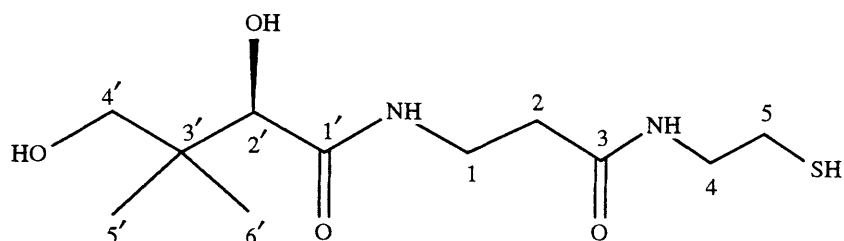
## **APPENDICES**

## APPENDIX 1

Numbering system of CoA used by Remington *et al.*<sup>49</sup>

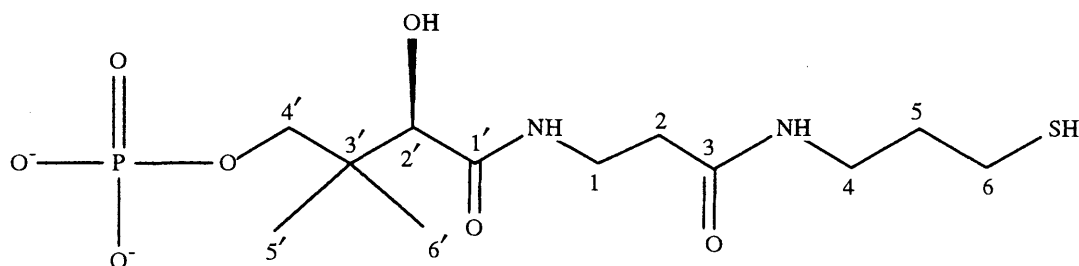


## APPENDIX 2



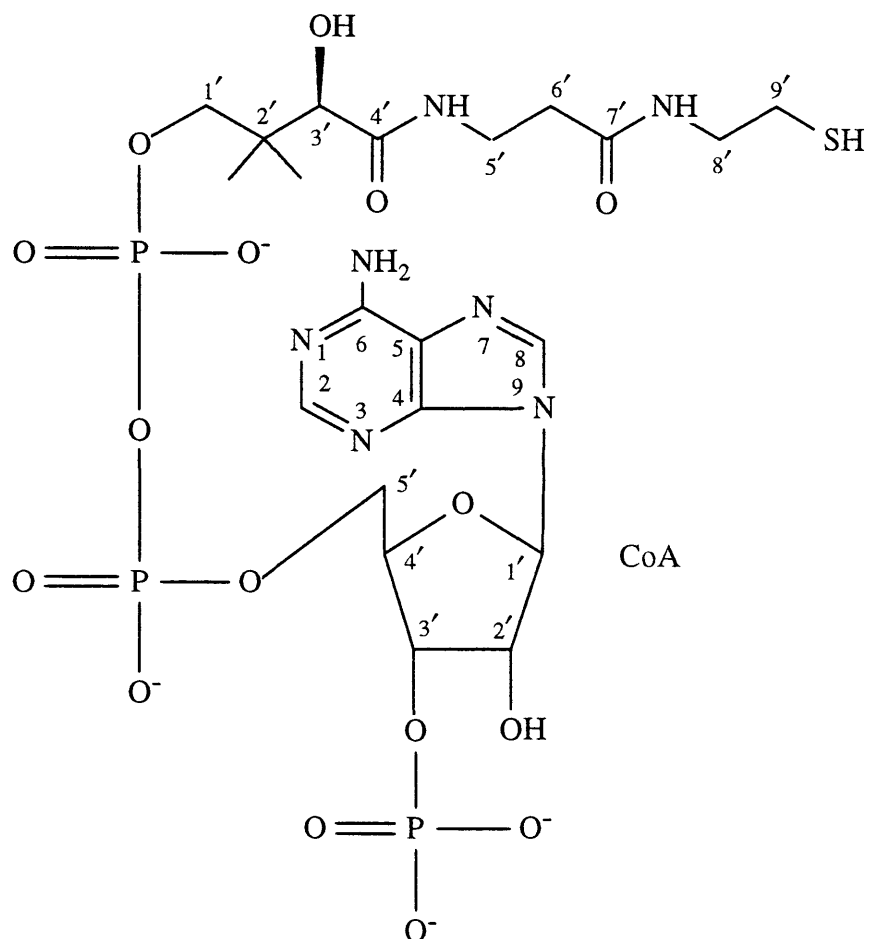
### D-pantetheine

Nomenclature of D-pantetheine and all pantetheine based analogues is based on D-pantetheine as shown above, the only exception being D-homopantetheine-4'-phosphate which has an extra methylene group in the cysteamine portion of the molecule as shown below.



### D-homopantetheine-4'-phosphate

## APPENDIX 3



Nomenclature used by Lee and Sarma<sup>125</sup>, A refers to adenosine protons and P refers to pantetheine protons (G refers to guanosine and T refers to thymine protons for guano and thymoCoA respectively).

## REFERENCES

- 1 L. Stryer, *Biochemistry*, W. H. Freeman & Company, New York, 1981
- 2 S. J. Weiner, P. A. Kollman, D. A. Chase, U. C. Singh, C. Ghio, G. Alagona, S. Profeta & P. Weiner, *J. Am. Chem. Soc.*, 1984, **106**, 765
- 3 A. Warshel & M. Levitt, *J. Mol. Biol.*, 1976, **103**, 227
- 4 a) C. Tanford, *The Hydrophobic Effect*, Wiley, New York, 1973;  
b) W. Kauzman. *Adv. Protein Chem.*, 1959, **14**, 1;  
c) H. S. Frank & M. W. Evans, *J. Chem. Phys.*, 1945, **13**, 507
- 5 A. Leo, C. Hansch & D. Elkins, *Chem. Rev.*, 1971, **71**, 525
- 6 a) A. R. Fersht, *Trends Biochem. Sci.*, 1984, **9**, 145;  
b) A. R. Fersht, J. P. Shi, J. Knill-Jones, D. M. Lowe, A. J. Wilkinson, D. M. Blow, P. Brick, P. Carter, M. M. Y. Waye & G. Winter, *Nature*, 1985, **314**, 235
- 7 W. P. Jencks, *Catalysis and Enzymology*, McGraw-Hill, 1969;  
J. Hine, *J. Am. Chem. Soc.*, 1972, **94**, 5766
- 8 a) T. N. C. Wells & A. R. Fersht, *Biochemistry*, 1986, **25**, 1881  
b) A. R. Fersht, *Trends Biochem. Sci.*, 1987, **12**, 301  
c) D. M. Lowe, G. Winter & A. R. Fersht, *Biochemistry*, 1987, **26**, 6038
- 9 A. R. Fersht, *Biochemistry*, 1988, **27**, 1577
- 10 W. P. Jencks, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 4046
- 11 F. J. M. Daemen & J. F. Riordan, *Biochemistry*, 1974, **13**, 2865
- 12 A. Chaidaroglou, D. J. Brezinski, S. A. Middleton & E. R. Kantovitz, *Biochemistry*, 1988, **27**, 8338
- 13 G. Schoellman & E. Shaw, *Biochemistry*, 1963, **2**, 252
- 14 E. Shaw, M. Mares-Guia & W. Cohen, *Biochemistry*, 1965, **4**, 2219
- 15 W. V. Shaw, *Biochem. J.*, 1987, **246**, 1
- 16 D. Straus, R. Raines, E. Kawashima, J. R. Knowles & W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.*, 1985, **82**, 2272
- 17 J. T. Davis, R. N. Moore, B. Imperiali, A. J. Prat, K. Bobayashi, S. Masamune, A. J. Sinskey & C. T. Walsh, *J. Biol. Chem.*, 1987, **262**, 82

- 18 a) P. A. Bartlett & C. K. Marlowe, *Biochemistry*, 1983, **22**, 4618  
b) P. A. Bartlett & C. K. Marlowe. *Science*, 1987, **235**, 569  
c) B. P. Morgan, J. M. Scholtz, M. D. Ballinger, I. D. Zipkin & P. A. Bartlett, *J. Am. Chem. Soc.*, 1991, **113**, 297
- 19 C. Kleanthous & W. V. Shaw, *Biochem. J.*, 1984, **223**, 211
- 20 A. G. W. Leslie, *J. Mol. Biol.*, 1990, **213**, 167
- 21 I. A. Murray, A. Lewendon, J. A. Williams, P. M. Cullis & W. V. Shaw, *Biochemistry*, 1991, **30**, 3763
- 22 P. J. Day & W. V. Shaw, *J. Biol. Chem.*, 1992, **267**, 5122
- 23 P. M. Cullis, A. Lewendon, W. V. Shaw & J. A. Williams, *Biochemistry*, 1991, **30**, 3758
- 24 P. J. Day, *The acetyl CoA binding site of chloramphenicol acetyltransferase*, 1990, PhD thesis, University of Leicester
- 25 J. Ehrlich, Q. R. Bartz, R. M. Smith, D. A. Joslyn & P. R. Burkholder, *Science*, 1947, **106**, 417
- 26 R. E. Maxwell & V. S. Nickel, *Antibiot. Chemother.*, 1954, **4**, 289
- 27 E. F. Gale & T. F. Paine, *Biochem. J.*, 1951, **48**, 298
- 28 R. R. Traut & R. E. Monroe, *J. Mol. Chem.*, 1964, **10**, 63
- 29 a) E. Cundliffe, E. F. Gale, P. E. Reynolds, M. H. Richmond & M. J. Waring, *The Molecular Basis of Antibiotic Action*, John Wiley & Sons, London, 1981, 460-468;  
b) D. Gottlieb & P. D. Shaw, *Antibiotics I, Mechanism of Action*, 1967, 308-330
- 30 D. Vazquez, *Nature*, 1964, **203**, 257
- 31 A. Contreras & D. Vazquez, *Eur. J. Biochem.*, 1977, **74**, 539
- 32 O. Pongs, R. Bald & V. A. Erdmann, *Proc. Natl. Acad. Sci. U.S.A.*, 1973, **70**, 2229; N. Sonnenberg, M. Wilcheck & A. Zamir, *Proc. Natl. Acad. Sci. U.S.A.*, 1973, **70**, 1423
- 33 R. Skinner, E. Cundliffe & J. Schmidt, *J. Biol. Chem.*, 1983, **258**, 12702
- 34 R. T. Macaroni, J. S. Lodmell & W. E. Hill, *J. Biol. Chem.*, 1990, **265**, 7894
- 35 W. V. Shaw & J. Unowsky, *J. Bacteriol.*, 1968, **95**, 1976

- 36 B. J. Backman, K. B. Low & A. L. Taylor, *Bacteriol. Rev.*, 1976, **40**, 116
- 37 G. N. Smith, & C. S. Worrel, *J. Bacteriol.*, 1953, **65**, 313
- 38 G. A. Baughman & S. R. Fahnstock, *J. Bacteriol.*, 1979, **137**, 1315
- 39 W. V. Shaw, *J. Biol. Chem.*, 1967, **242**, 687
- 40 Y. Suzuki & S. Okamoto, *J. Biol. Chem.*, 1967, **242**, 4722
- 41 W. V. Shaw & A. G. W. Leslie, *Annu. Rev. Biophys. Chem.*, 1991, **20**, 363
- 42 W. P. Jencks, S. Cordes & J. Carriuolo, *J. Biol. Chem.*, 1960, **235**, 3608
- 43 C. Kleanthous, P. M. Cullis & W. V. Shaw, *Biochemistry*, 1985, **24**, 5307
- 44 H. G. Vogel & W. A. Bridger, *Biochem. Soc. Trans.*, 1983, **11**, 315
- 45 A. Lewendon, I. A. Murray, W. V. Shaw, M. R. Gibbs & A. G. W. Leslie, *Biochemistry*, 1990, **29**, 2075
- 46 S. F. Currier & H. G. Mautner, *Biochemistry*, 1977, **16**, 1944
- 47 W. V. Shaw, *CRC Crit. Rev. Biochem.*, 1983, **14**, 1
- 48 A. G. W. Leslie, P. C. E. Moody & W. V. Shaw, *Proc. Natl. Acad. Sci. U.S.A.*, 1988, **85**, 4133
- 49 S. Remington, G. Wiegand & R. Huber, *J. Mol. Biol.*, 1982, **158**, 111
- 50 J. F. Riordan, K. D. McElvany & C. L. Borders jnr., *Science*, 1977, **195**, 884
- 51 A. Lewendon & W. V. Shaw, *J. Biol. Chem.*, 1993, **268**, 20997
- 52 G. W. E. Plaut, C. M. Smith & W. L. Alworth, *Ann. Rev. Biochem.*, 1974, **43**, 899
- 53 H. B. Hoagland & G. D. Novelli, *J. Biol. Chem.*, 1954, **207**, 767
- 54 G. B. Ward, G. M. Brown & E. E. Snell, *J. Biol. Chem.*, 1955, **213**, 869
- 55 L. E. Lloyd, B.E. McDonald & E. W. Crampton, *Fundamentals of Nutrition*, W. H. Freeman & Co., San Fransisco, 2<sup>nd</sup>edn.
- 56 G. M. Brown, *J. Biol. Chem.*, 1959, **234**, 370
- 57 Y. Abiko, *J. Biochem.*, 1967, **61**, 290
- 58 R. Scandurra, E. Barboni, F. Granata, B. Pensa & M. Costa, *Eur. J. Biochem.*, 1974, **49**, 1
- 59 R. Scandurra, M. Marcucci & G. Federica, *Acta. Vitaminol. Enzymol.*, 1970, **24**, 122



- 60 Y. Abiko, *Metabolic Pathways*, Academic Press, Inc., New York, 1975
- 61 S. Jackowski & C. D. Rock, *J. Bacteriology*, 1981, **148**, 926
- 62 O. Halvorsen & S. Skrede, *Eur. J. Biochem.*, 1982, **124**, 211
- 63 J. D. Robishaw, D. Berkich & J. R. Neely, *J. Biol. Chem.*, 1982, **257**, 10967
- 64 J. G. Moffatt & H. G. Khorana, *J. Am. Chem. Soc.*, 1961, **83**, 663
- 65 S. Roseman, J. J. Distler, J. G. Moffatt & H. G. Khorana, *J. Am. Chem. Soc.*, 1961, **83**, 659
- 66 M. Shimizu, *Methods in Enzymology*, Academic Press, New York, 1970, **18A**, 322
- 67 M. Shimizu, O. Nagase, Y. Hosokawa, H. Tagawa, Y. Abiko & T. Suzuki, *Chem. Pharm. Bull. (Tokyo)*, 1966, **14**, 683
- 68 S. A. Raybuck, N. R. Bastian, W. H. Orme-Johnson & C. T. Walsh. *Biochemistry*, 1988, **27**, 7698
- 69 J. T. Davis, Hwang-Hsing Chen, R. Moore, Y. Nishitani, S. Masamune, A. J. Sinskey & C. Walsh, *J. Biol. Chem.*, 1987, **262**, 90
- 70 J. Rossier, *Biochem. J.*, 1977, **165**, 321
- 71 G. M. Tener, *J. Am. Chem. Soc.*, 1961, **83**, 159
- 72 H. G. Khorana, W. E. Razzell, P. T. Gilham, G. M. Tener & E. H. Pol, *J. Am. Chem. Soc.*, 1957, **79**, 1002
- 73 E. Cherbuliez & J. Rabinowitz, *Helv. Chim. Acta.*, 1956, **39**, 1461
- 74 E. J. Simon & D. Shemin, *J. Am. Chem. Soc.*, 1953, **75**, 2520
- 75 J. Baddiley & A.R. Todd, *J. Chem. Soc.*, 1947, 648
- 76 H. G. Khorana, *Some recent developments in the chemistry of phosphate esters of biological interest*, Wiley, New York, 1961
- 77 D. H. Hayes, A. N. Michelson & A. R. Todd, *J. Chem. Soc.*, 1955, 808
- 78 S. Li & R. E. Eakin, *J. Am. Chem. Soc.*, 1955, **77**, 1866
- 79 J. Baddiley & E. M. Thain, *J. Chem. Soc.*, 1951, 246
- 80 J. Baddiley & E. M. Thain, *J. Chem. Soc.*, 1953, 903
- 81 H. G. Khorana, G. M. Tener, R. S. Wright & J. G. Moffatt, *J. Am. Chem. Soc.*, 1957,

79, 430

- 82 B. Fiszer & J. Michalski, *J. Rocznici Chem*, 1952, **26**, 688
- 83 R. A. McIvor, G. D. McCarthy & G. A. Grant, *Can. J. Chem.*, 1956, **34**, 1819
- 84 G. Sosnovsky & E. H. Zaret, *J. Org. Chem.*, 1969, **34**, 968
- 85 D. P. Martin & D. G. Drueckhammer, *J. Am. Chem. Soc.*, 1992, **114**, 7287
- 86 a) G. M. Kosolopoff, *Organophosphorus Compounds*, J. Wiley & Sons, Inc., New York, 1950
- b) C. E. McKenna, M. T. Higa, N. H. Cheung & M. McKenna, *Tetrahedron Letters*, 1977, 155
- 87 C. J. Stewart, *Liebigs Ann. Chem.*, 1978, 57
- 88 M. Shimizu, O. Nagase, Y. Hosokawa & H. Tagawa, *Tetrahedron*, 1968, **24**, 5241
- 89 T. L. Miller, G. L. Rowley & C. J. Stewart, *J. Am. Chem. Soc.*, 1966, **88**, 2299
- 90 M. Michenfelder & J. Reteý, *Angew. Chem. Int. Ed. Engl.*, 1986, **25**, 366
- 91 H. Martini & J. Reteý, *Angew. Chem. Int. Ed. Engl.*, 1993, **32**, 278
- 92 D. P. Martin, R. T. Bibart & D. G. Drueckhammer, *J. Am. Chem. Soc.*, 1994, **116**, 4660
- 93 M. Shimizu, T. Suzuki, Y. Hosokawa, O. Nagase & Y. Abiko, *Biochim. Biophys. Acta*, 1970, **222**, 307
- 94 J. G. Moffatt & H. G. Khorana, *J. Am. Chem. Soc.*, 1958, **80**, 3756
- 95 J. G. Moffatt & H. G. Khorana, *J. Am. Chem. Soc.*, 1961, **83**, 649
- 96 L. A. Happel, P. R. Whitfield & R. A. Markham, *Biochem. J.*, 1955, **60**, 19
- 97 A. M. Michelson, *Biochim. Biophys. Acta*, 1961, **50**, 605
- 98 M. Naoi-Tada, K. Sato-Asano & F. Egami, *J. Biochem.*, 1959, **46**, 757
- 99 P. J. Day, W. V. Shaw, M. R. Gibbs & A. G. W. Leslie, *Biochemistry*, 1992, **31**, 4198
- 100 P. Rubenstein & R. Dryer, *J. Biol. Chem*, 1980, **255**, 7858
- 101 H. P. Blaschkowski, J. Knappe & Th. Wieland, *Febs Lett.*, 1979, **98**, 81
- 102 M. F. Utter, R. E. Barden & B. L. Taylor, *Adv. Enzymol.*, 1975, **42**, 1
- 103 A. Lewendon, I. A. Murray, W. V. Shaw, M. R. Gibbs & A. G. W. Leslie, *Biochemistry*, 1994, **33**, 1944

- 104 J. Ellis, C. R. Bagshaw & W. V. Shaw, *Biochemistry*, 1995, **34**, 16852
- 105 K. J. Paul, *Molecular recognition studies of chloramphenicol acetyltransferase by substrate modification*, 1993, Ph.D thesis, University of Leicester
- 106 I. D. Wlassics & V. E Anderson, *Biochemistry*, 1989, **28**, 1627
- 107 A. Nakazawa, M. Tokushige, O. Hayaishi, M. Ikehara & Y. Mizuno, *J. Biol. Chem.*, 1967, **242**, 3868
- 108 W. V. Shaw, *Methods Enzymol.*, 1975, **43**, 737
- 109 A. Fersht, *Enzyme Structure and mechanism*, Freeman, New York, 2nd edition
- 110 I. P. Street, C. R. Armstrong & S. G. Withers, *Biochemistry*, 1986, **25**, 6021
- 111 D. E. Tronrud, H. M. Holden & B. W. Mathews, *Science*, 1987, **235**, 571
- 112 P. A. Bash, P. A. Kollman, U. C. Singh, F. K. Brown & A. R. Langridge, *Science*, 1987, **235**, 574
- 113 K. M. Merz & P. K. Kollman, *J. Am. Chem. Soc.*, 1989, **111**, 5649
- 114 J. Ellis, C. R. Bagshaw & W. V. Shaw, *Biochemistry*, 1991, **30**, 10806
- 115 C. Berthelot & M. Junfleisch, *Ann. Chem. Phys.*, 1872, **4**, 26
- 116 W. Nernst, *Z. Phys. Chem.*, 1891, **8**, 110
- 117 C. Hansch & T. Fujita, *J. Am. Chem. Soc.*, 1964, **86**, 1616
- 118 T. Fujita, J. Iwasa & C. Hansch, *J. Am. Chem. Soc.*, 1964, **86**, 5175
- 119 K. Kiehs, C. Hansch & L. Moore, *Biochemistry*, 1966, **5**, 2602
- 120 C. Hansch, E. W. Deutsch & R. N. Smith, *J. Am. Chem. Soc.*, 1965, **87**, 2738
- 121 V. N. Dorovskaya, S. D. Varfolomeyev, N. F. Kazanskaya, A. A. Klyosov & K. Martinek, *FEBS. Lett.*, 1972, **23**, 122
- 122 D. D. Perrin & W. L. F. Armarego, *Purification of Laboratory Chemicals*, Pergammon Press, New York, 1966
- 123 W. E. Morgan & J. R. Van Wazer, *J. Am. Chem. Soc.*, 1975, **97**, 6347
- 124 W. C. Still, M. Kahn & A. Mitra, *J. Org. Chem.*, 1978, **43**, 2923
- 125 C. Lee & H. Sarma, *J. Am. Chem. Soc.*, 1975, **97**, 1225