

Molecular Recognition Studies Of Chloramphenicol Acetyltransferase By Substrate Modification

A Thesis submitted by

Kerensa J. Paul

for the degree of Doctor of Philosophy
in the Faculty of Science of the
University of Leicester

Department of Chemistry
University of Leicester
Leicester LE1 7RH

September 1993

UMI Number: U554434

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U554434

Published by ProQuest LLC 2015. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346



750192785X

STATEMENT

The accompanying thesis submitted for the degree of Ph.D. entitled "Molecular Recognition Studies of Chloramphenicol Acetyltransferase by Substrate Modification" is based on work conducted by the author in the Department of Chemistry of the University of Leicester mainly during the period between October 1989 and October 1992.

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: Kerensa J. Paul Date: 29th Sep 1993

ACKNOWLEDGEMENTS

I would like to thank my supervisor Professor Paul Cullis for all the help and advice he has given me during the past four years.

Special thanks go to Ann Crane for all the time and assistance she has given me, especially with the drawing of diagrams both for this thesis and for the presentation of posters. My thanks and appreciation also go to Lewis Paul for the loan of his computer which enabled me to type up this thesis, and to Helen Johnson and Adam Chappell for their assistance with the typing.

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

Molecular Recognition Studies of Chloramphenicol Acetyltransferase by Substrate Modification

Kerensa J. Paul

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, chloramphenicol (CM), has been modified in a variety of ways to allow quantitative evaluations of the energetics of individual enzyme-ligand interactions to be made. Nineteen diastereo- and enantiopure chloramphenicol analogues have been prepared and their kinetic parameters as substrates for CAT determined. These compounds were derived from L- and D-amino acids and chloramphenicol base, (1R, 2R)-2-amino-1-(*p*-nitrophenyl)-propan-1,3-diol.

The asymmetric synthesis of a series of 2-amino 1,3-diols via alternative routes was investigated. The first started with the N-BOC-protected amino acid D-serine and involved conversion of the acid to a ketone with organolithium reagents followed by asymmetric reduction to give the diol. For the second route N,O-diprotected D-serinal was converted to the amino diols via reaction with organometallic reagents. Addition of $[RCu] \cdot MgX_2$ ($X=Br$ or Cl) to the aldehyde led to essentially enantiopure single diastereomers with the *threo*-configuration.

From kinetic studies of the CAT reaction with the modified ligands the apparent binding energies, $\Delta\Delta G_{app}$, of each of the substituents of chloramphenicol has been evaluated. Examination of analogues in which the *p*-nitrophenyl group of chloramphenicol was replaced by alkyl groups suggested that the aryl binding pocket is a local hydrophobic region approximately 1.5 times more hydrophobic than *n*-octanol. There is a linear free energy relationship between the specificity constant, k_{cat}/K_m , of these ligands and the hydrophobicity of the alkyl substituent. The incremental apparent binding energy for each methylene group bound in this pocket is estimated to be $1.0 \text{ kcal mol}^{-1}$ and it is proposed that this binding site can only enclose an alkyl chain up to C_4 . A large number of chloramphenicol analogues were found to be substrates for CAT. This is believed to be due to the dominant hydrophobic interactions in the CAT:CM complex and the lack of many direct hydrogen bonds, which for some ligands apparently leads to alternative binding modes.

CONTENTS

CHAPTER 1 GENERAL INTRODUCTION

INTRODUCTION

1.1	MOLECULAR RECOGNITION	1
1.1i	Interactions Between Nonbonded Atoms	3
1.1ii	Binding Energies of Enzymes and Substrates	4
1.1iii	Examples of the Calculation of Binding Energies for Different Systems	8

BACKGROUND

1.2	CHLORAMPHENICOL	17
1.3	ANTIBIOTICS	19
1.3i	Mode of Action of Chloramphenicol and its Binding to Ribosomes	19
1.3ii	Bacterial Resistance to Antibiotics	21
1.4	MECHANISM OF THE CAT REACTION	23
1.5	STRUCTURE OF CAT	26
1.5i	The Binding Site of Chloramphenicol	27
1.5ii	The Binding Site of Coenzyme A	28
1.5iii	The Active Site	30
1.6	STUDIES OF THE CHLORAMPHENICOL BINDING SITE OF CAT BY SITE-DIRECTED MUTAGENESIS AND SUBSTRATE MODIFICATION	32
1.7	THIS THESIS	39

CHAPTER 2 SYNTHESIS OF CHLORAMPHENICOL DERIVATIVES VIA THE AMINO ACIDS SERINE AND THREONINE

2.1	INTRODUCTION	41
-----	--------------	----

RESULTS AND DISCUSSION

2.2	CONVERSION OF CARBOXYLIC ACIDS TO KETONES WITH ORGANOMETALLIC REAGENTS	46
2.2i	Tertiary Alcohol Formation	48
2.2ii	Application to the Synthesis of Chloramphenicol Analogues	50
2.2iii	Synthesis of the Methyl and Butyl Ketone from N-BOC-D-Serine	51

2.3	CONVERSION OF KETONES TO SECONDARY ALCOHOLS	55
2.3i	Synthesis of the Methyl and Butyl Alcohol	58
2.4	SYNTHESIS OF 2-N-DICHLOROACETAMIDO-BUTAN-1,3-DIOL	60
2.5	ALTERNATIVE SYNTHESIS OF 2-N-DICHLOROACETAMIDO-BUTAN-1,3-DIOL	62
2.5i	Synthesis of N-Dichloroacetamido-L-Threoninol	62
2.5ii	Synthesis of N-Dichloroacetamido-D-Threoninol	65
2.5iii	Verification of Relative Configuration and Optical Purity	66
2.6	COMPARISON AND CONCLUSION	69

CHAPTER 3 ASYMMETRIC SYNTHESIS OF CHLORAMPHENICOL DERIVATIVES VIA A PROTECTED α -AMINO ALDEHYDE

3.1	INTRODUCTION	71
3.1i	Preparation of N-Protected α -Amino Aldehydes	72
3.1ii	Reactions of N-Protected α -Amino Aldehydes	73
3.1iii	Aldehyde Stability	77
RESULTS AND DISCUSSION		
3.2	SYNTHESIS OF THE GARNER ALDEHYDE	80
3.2i	Stability of the Garner Aldehyde	83
3.3	REACTION OF THE GARNER ALDEHYDE WITH MeMgBr	84
3.4	REACTION OF THE GARNER ALDEHYDE WITH VARIOUS ORGANOMETALLIC REAGENTS	86
3.4i	Conformation of the <i>Threo</i> and <i>Erythro</i> Assignment	92
3.4ii	Proposed Mechanisms	94
3.5	APPLICATION TO THE SYNTHESIS OF CHLORAMPHENICOL ANALOGUES	97
3.5i	Verification of Relative Configurational and Optical Purity	100
3.6	CONCLUSION	105

CHAPTER 4 KINETIC STUDIES AND THE HYDROPHOBIC EFFECT

4.1	INTRODUCTION	106
4.2	KINETIC STUDIES	108
4.3	KINETIC RESULTS	110
4.3i	Partition Coefficients and the Hansch Equation	111
4.3ii	Analysis of Kinetic Results and Estimation of Incremental Binding Energies	114
4.4	THE HYDROPHOBIC EFFECT	120
4.5	1-DEOXYCHLORAMPHENICOL AND ANALOGUES	126
4.5i	Kinetic Results	131

CHAPTER 5 ADDITIONAL KINETIC STUDIES AND OVERALL CONCLUSIONS

5.1	INTRODUCTION	135
5.2	ANALOGUES OF CHLORAMPHENICOL IN WHICH THE DICHLOROACETYL SUBSTITUENT IS VARIED	136
5.2i	Kinetic Results	137
5.3	ALTERNATIVE SUBSTRATES FOR CAT	140
5.3i	Kinetic Results	143
5.4	ENANTIODISCRIMINATION	147
5.5	OVERALL CONCLUSIONS	156

CHAPTER 6 EXPERIMENTAL

6.1	GENERAL EXPERIMENTAL DETAILS	
6.1i	Materials	159
6.1ii	Solvents	159
6.1iii	Instrumentation and Methods	159
6.2	SYNTHESIS RELATING TO CHAPTER 2	161
6.3	SYNTHESIS RELATING TO CHAPTER 3	173
6.4	SYNTHESIS RELATING TO CHAPTER 4	205

6.5	SYNTHESIS RELATING TO CHAPTER 5	214
6.6	KINETIC STUDIES	
6.6i	Preparation of TSE Buffer	225
6.6ii	Preparation of Acetyl CoA	225
6.6iii	Assay of CAT Activity	225
6.6iv	Kinetic Analysis	226
REFERENCES		227

CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

1.1 MOLECULAR RECOGNITION

Molecular recognition processes control all chemical and biological reactions vital to life. Essentially, molecular recognition is the interaction of two molecules, be they two simple molecules whose interaction involves mutual recognition, or two macromolecules such as DNA and protein. Of relevance to this work is the molecular recognition involved in the interaction between a large molecule and a small one such as that between an enzyme and its substrate.

The ability of individual molecules to recognise and discriminate between closely related partners is a key determinant of many fundamental processes such as enzymic catalysis, gene regulation and biological fidelity in DNA and protein synthesis. The goal of molecular recognition studies is to understand in a quantitative manner the origin of this specificity. Understanding molecular recognition processes, particularly those which influence the binding of small molecules to complex proteins, is a vital component of rational drug design. Drugs must be highly discriminating at the molecular level so that diseases can be controlled without any undesirable side effects. It is therefore essential that drug molecules are designed to recognise and bind selectively to target receptors or enzymes identified as being involved in the disease process, and to show considerably lower affinities for other potential binding sites. At the limit, this may involve discriminating between two enzymes that catalyse the same reaction but are encoded respectively by host and invading agent (virus or bacteria).

Recent developments in structural and molecular biology have greatly enhanced the ability to examine the structure-function relationships of biological macromolecules and to obtain new insights into the molecular recognition processes which form the basis of

biological specificity. It is the goal of much recent research to make quantitative estimates of the thermodynamic contributions of individual interactions to the overall molecular recognition process. This has been facilitated by high resolution structural studies based on NMR spectroscopy or X-ray crystallography to identify key interactions and to define structural changes in both the receptor and/or ligand made by genetic or chemical means. The increasing number of enzymes and enzyme-substrate complexes whose structures are available at high resolution provides an important framework within which to determine the basic rules that govern biological molecular recognition.

Site-directed mutagenesis involving the systematic changing of one amino acid residue to another in a protein, and substrate modification are two valuable means of quantitatively evaluating the energetic contributions to binding and catalysis of individual enzyme-ligand interactions. Such a use of site-directed mutagenesis is well established, as is the general principle that amino acid substitutions should not disrupt the protein structure.¹ This approach, however, is limited in the comparatively small number of possible changes that can be made, being restricted at present to the side chains of the twenty naturally occurring amino acids found in proteins and specified by the genetic code.² A further disadvantage is that interactions of ligands with backbone functional groups cannot be investigated by this method. "Mutation" of the substrate, however, affords a considerably wider range of chemically defined alterations to the enzyme-ligand interaction and provides access to the same energetic data.

To achieve in-depth studies in the area of molecular recognition requires the collaboration of biochemists, molecular biologists and chemists. Use of substrate modification alongside genetic manipulation provides a powerful tool in analysing the enzyme-substrate interaction. Once the various interactions are understood in sufficient detail it should be possible, from a knowledge of the structure and properties of the binding site, to design *de novo* tight-binding ligands. Such an approach would define

target molecules for the synthetic chemist which would be further refined by the usual structure-activity analysis.

1.1i Interactions Between Nonbonded Atoms

Although chemists have mastered the control of covalent bonds, in order to be predictive it is necessary to understand the weak forces involved in noncovalent interactions. The four types of interactions that contribute to such molecular recognition are **electrostatic, van der Waals, hydrogen bonding and hydrophobic**. The quantitative importance of electrostatic interactions, those between charged or dipolar atoms and molecules, is difficult to calculate because the energies depend on the dielectric constant of the surrounding medium. It is particularly difficult to calculate electrostatic effects in proteins because the dielectric constant is not uniform throughout the protein but depends on the microenvironment. 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 any atoms. Typical dispersion energies for pairs of atoms at the optimal distance of contact are for example: 0.0186 kcal mol⁻¹ for the H...H interaction; 0.1366 kcal mol⁻¹ for N...N; and 0.0679 kcal mol⁻¹ for C...C.³ 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. Different types of hydrogen bonds have quite different hydrogen bond dissociation energies. Examples of calculated values *in vacuo* are: HOH...OH₂, -6.4 kcal mol⁻¹; H₂O...HSCH₃, -3.2 kcal mol⁻¹; HOH...S(H)CH₃, -3.1 kcal mol⁻¹; CH₃CO₂⁻...HOH, -19.0 kcal mol⁻¹.⁴ Charged groups have greater hydrogen bond dissociation energies because of the higher electrostatic energies. Hydrogen bonds in biological systems are particularly important because they are stable enough to provide significant binding energy, but sufficiently weak to allow rapid dissociation. The hydrophobic "bond" can be considered as the tendency of

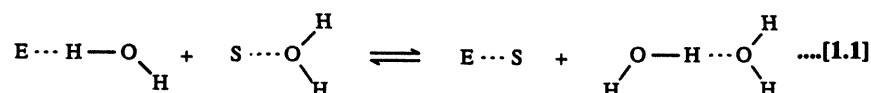
nonpolar compounds to transfer from an aqueous solution to an organic phase. The classic theory of the hydrophobic effect is that it arises primarily from the strong attractive forces between water molecules which must be disrupted or distorted when a nonpolar solute is dissolved in the water.⁵ To date, however, many conflicting explanations have been offered for the behaviour of hydrophobic molecules in water and these are discussed in detail in **Chapter 4**. One particular method for measuring the hydrophobicity of a molecule is to measure its partition between two phases, *n*-octanol and water being a commonly chosen system. The distribution of the solute depends on the competing tendencies of the hydrophobic regions to enter the organic phase and the polar regions to be solvated and hence stabilised by the aqueous phase. From such partition experiments the methylene group has an incremental Gibbs free energy of transfer from *n*-octanol to water of 0.68 kcal mol⁻¹.⁶

1.1ii Binding Energies of Enzymes and Substrates

There are several difficulties in estimating the contributions of the different types of interactions to the overall binding energy of a substrate and enzyme from the physical measurements outlined in **Section 1.1i**. The main reason for this is that the binding process is an exchange reaction, the substrate exchanges its solvation shell of water for the binding site of an enzyme. Therefore, the major 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.

It is difficult for example to evaluate the energy of salt linkages because the ions are solvated in solution and the energy of interaction in the complex critically depends on the dielectric constant of the surrounding medium. The solvation energies are very high, it is estimated that a -CO_2^- ion is stabilised by about 65 kcal mol⁻¹,³ and this must

be compensated for if removal from water is associated with complex formation. Similarly in the case of hydrogen bonds evaluation of the net energy is difficult because 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 [1.1].



A distinction must be made between the overall energetics of the exchange reaction and the hydrogen bond dissociation energy. The energetics depend on the enthalpies of formation of each of the bonds and the entropy changes in both solvent and reagents. By simply carrying out a hydrogen bond inventory⁷ a great deal of insight into the net energetics of hydrogen bonding in water can be gained. As can be seen in [1.1] 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 a first approximation the reaction is isoenthalpic.⁸ The enthalpies tend to cancel out even if the bonds are of different types. Even though there is an increase in entropy on the formation of intramolecular bonds in the enzyme-substrate complex since the substrate is probably immobilised in the complex anyway there is no further loss of entropy on the formation of the intramolecular hydrogen bonds. 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.^{5a}

The evaluation of hydrophobic interaction energies is also not easy because there are 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.

Instead of using model reactions a quantitative analysis of the energetics of individual interactions within the enzyme-substrate complex can be achieved by a direct comparison of dissociation constants for the modified and unmodified complexes. Such a comparison yields an apparent binding energy, ΔG_{app} , for the interaction.⁹ This can be applied to modified substrates binding to a single enzyme or to a single substrate binding to modified enzymes derived from site-directed mutagenesis of the wild-type enzyme. In order to measure the contribution to the binding energy of a group R on a ligand R-S it is usual to replace R by H and to compare the affinities of R-S and H-S. For the case of altered substrates, the apparent binding energy of a substituent R in a substrate R-S is given by [1.2], where $K_{s(H-S)}$ and $K_{s(R-S)}$ are the dissociation constants from the enzyme of the altered substrate and the unmodified substrate respectively.

$$\Delta G_{app} = RT \ln (K_{s(R-S)}/K_{s(H-S)}) \quad \dots[1.2]$$

ΔG_{app} can be related to the energies of the specific bonds in the complex that have been altered in the modification according to a simple thermodynamic cycle (Figure 1).¹⁰

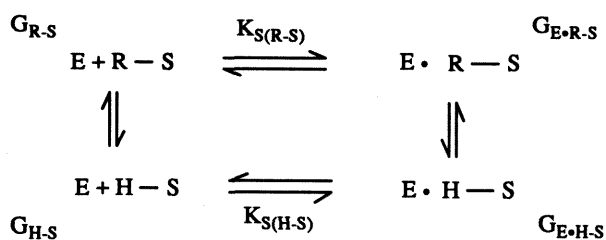


Figure 1.1

Thermodynamic cycle relating differences in dissociation constants to differences in free energies of unmodified and altered substrates and their enzyme-substrate complexes.

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

$$\Delta G_{app} = (G_{H-S} - G_{R-S}) - (G_{E-H-S} - G_{E-R-S}) \quad \dots[1.3]$$

From [1.3] it can be seen that ΔG_{app} depends upon the differences in free energy between E-R-S and E-H-S and those between R-S and H-S. Since the binding process is an exchange reaction between water and the binding site ΔG_{app} can be expressed as

$$\Delta G_{app} = (G_{H-S/w} - G_{R-S/w}) - (G_{E-H-S} - G_{E-R-S}) + \Delta G_{reorg} \quad \dots[1.4]$$

where $G_{H-S/w}$ and $G_{R-S/w}$ is the energy of interaction between modified substrate and water and that between substrate and water. ΔG_{reorg} is a reorganisational energy term that includes any conformational changes, perturbations to the binding of the rest of the substrate to the enzyme, and changes to the solvent arising from the modification of substrate. ΔG_{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. Since ΔG_{app} is derived from relative binding constants it is an experimental measure of binding. On the other hand ΔG_{bind} is equivalent to an incremental binding energy in the E-S complex.¹⁰ If a group R on a substrate binds to a group X on an enzyme then the exchange reaction is shown in [1.5] where w represents water.



The binding energy of X and R, ΔG_{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 [1.6], depend on the relative individual interaction energies in [1.5] and also on any changes in entropy and energetics of solvent structure that occur.

$$\Delta G_{\text{bind}} = G_{X \cdot R} + G_w - G_{X \cdot w} - G_{R \cdot w} \quad \dots[1.6]$$

The interaction energy of X and R is $G_{X \cdot R}$, of X and w is $G_{X \cdot w}$, of R and w is $G_{R \cdot w}$, and the free energy of water entering bulk water and any associated changes in the free energy of solvent is G_w . The value of ΔG_{bind} depends on the fit between X and R. When there is perfect complementarity between the group R and its binding cavity in the enzyme 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 ΔG_{bind} tends to the intrinsic binding energy value.¹¹ ΔG_{bind} relates the binding energy of enzyme and R with each other compared with their binding energies to water, whereas ΔG_{app} compares the binding energy of enzyme and modified substrate with that of enzyme and unaltered substrate. Therefore, in general ΔG_{app} does not equal ΔG_{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 ΔG_{app} can be considered as a crude measure of ΔG_{bind} .¹⁰

1.1iii Examples of the Calculation of Binding Energies for Different Systems

Fersht has used both site-directed mutagenesis and substrate modification to obtain binding energies for specific hydrogen bonds. Mutations of several side chains at the active site of tyrosyl-tRNA synthetase were designed to remove hydrogen-bond donating groups from the enzyme in the enzyme-substrate complex. Application of the theoretical work outlined above gave the apparent binding energies of several hydrogen bonds from the tyrosyl-tRNA synthetase (an enzyme that catalyses the formation of tyrosyl adenylate) to its substrates.^{7,9} The results showed that deletion of a hydrogen bond to an uncharged hydrogen bond donor or acceptor weakens binding by 0.5-1.8 kcal mol⁻¹.¹² These values are much smaller than the hydrogen bond dissociation energies calculated *in vacuo*. The weaker binding on removal of -XH from

an enzyme E-XH is thought to result from small changes in solvation energies and possible losses of dispersion energy in the complex because deletion potentially causes a cavity between the substrate and enzyme which the missing group once occupied. In contrast, the deletion of a bond to a charged donor or acceptor weakens binding by as much as 3-6 kcal mol⁻¹.¹³ The higher value reflects the higher bond dissociation energies of charged groups. The deletion of hydrogen-bonding groups from a substrate gave similar results when the glycogen phosphorylase-glucose complex was investigated.¹⁴ Removal of a hydrogen-bonding partner of a neutral group was shown to weaken the binding energy by about 1.5 kcal mol⁻¹ and that for a charged group by about 3 kcal mol⁻¹.

Bartlett and co-workers have used substrate modification 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 shown in Figure 1.2.¹⁵

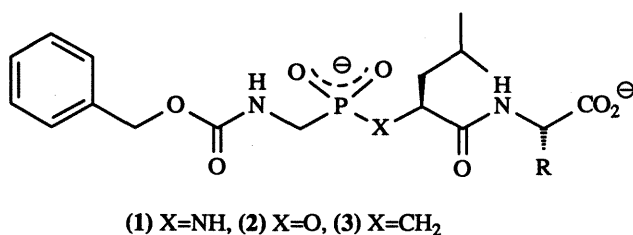


Figure 1.2

X-ray crystal structures of the thermolysin complex of (1) and (2) have shown that the two are essentially superimposable, and the specific interactions that were identified between the inhibitors and the active site are shown in Figure 1.3.¹⁶

Replacement of the phosphorus linked NH group with an oxygen atom to give a series of phosphonate esters (2) resulted in a uniform decrease in binding affinity of

4 kcal mol⁻¹ across the series.^{15b} There are two main factors which can explain the difference in binding energies of the amidates and the esters.

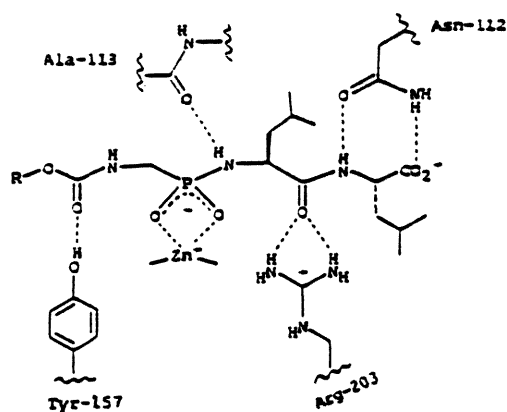


Figure 1.3

Interactions between the inhibitors and thermolysin in the vicinity of the phosphorus and amide linkages.

As can be seen from Figure 1.3 the -NH- for -O- substitution causes the loss of a hydrogen-bonding interaction because of the inability of the ester to donate a hydrogen bond to the carbonyl oxygen of Ala 113, and 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 energy of the amidate-enzyme hydrogen bond is partially offset by the greater free energy loss on desolvation of the amidates compared with the esters, due largely to the hydrogen bonding capability of the former. Using free energy perturbation calculations Bash *et al.* supported this interpretation by also obtaining a value of 4 kcal mol⁻¹ for the difference in binding energy between the amides (1) and the esters (2).¹⁷ They did this by separately calculating the differences in the energy of their interactions with the active site and with solvent. In a similar manner Kollman and Merz predicted the effect of substituting the -NH- group with a -CH₂- group.¹⁸ They accurately predicted that the

binding of the phosphinates (3) to thermolysin would be almost the same as that of the phosphonamidates (1) despite the former not being able to hydrogen-bond to the Ala 113 residue.

Bartlett then prepared a series of phosphinates (3) and demonstrated that the replacement of the -NH- group with a -CH₂- moiety alters the affinity of the inhibitors by a relatively constant amount across the series.^{15c} The phosphinates (3) were shown to bind with only a 0.1 kcal mol⁻¹ decrease in binding affinity compared with the corresponding phosphonamidates (1), which is in contrast to the phosphonates (2) which bind an average of 4 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 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 phosphinates which is offset by the decrease in hydrophilicity making the phosphinates easier to desolvate. In the case of the phosphonates (2) 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 the hydrophobic and hydrogen-bonding characteristics of the phosphorus substituents.

Williams has recently tried to develop a quantitative understanding of molecular recognition phenomena based on a combined experimental and theoretical approach.¹⁹ He presents an equation for the estimation of approximate binding constants for bimolecular associations in solution. The free energy of binding, ΔG , is separated into five factors as shown by [1.7]. This expression is only applicable if the ligand and receptor show good complementarity and there is no strain in the bound complex, that

is if the conformations of the bound components correspond closely to conformational energy minima in the separated states.

$$\Delta G = \Delta G_{t+r} + \Delta G_r + \Sigma \Delta G_i + \Delta G_{vdW} + \Delta G_H \quad \dots[1.7]$$

ΔG_{t+r} corresponds to the probability of bringing the ligand and receptor together, it is the change in translational and rotational energy upon association. The association of two molecules is always unfavourable entropically due to the loss of three degrees of translational freedom and three degrees of rotational freedom on formation of the complex. By also taking into account the small enthalpy loss on association ΔG_{t+r} can be estimated. Williams presents a plot of molecular weight of ligand against ΔG_{t+r} from which the adverse effect on the binding constant can be determined. For example, for a ligand of mass 150, ΔG_{t+r} is adverse to binding by a factor of 10^{10} which corresponds to approximately 60 kJ mol^{-1} . He has shown that ΔG_{t+r} is relatively insensitive to the shape of the ligand. A molecule of a given weight has the same value of ΔG_{t+r} to within 4 kJ mol^{-1} whatever its shape.

ΔG_r corresponds to the probability of orienting the ligand appropriately once it has formed the complex. It is the adverse free energy change, which is largely entropic, associated with the restriction of any internal rotations of either component on formation of the complex. In agreement with Page and Jencks,²⁰ Williams estimates the value of ΔG_r to be approximately $5\text{-}6 \text{ kJ mol}^{-1}$ per rotor frozen out upon complexation.

$\Sigma \Delta G_i$ is the sum of the free energy of binding that results from the interaction between polar functional groups in the complex. This value requires a knowledge of the intrinsic binding energies for every portion of a component involved in the binding interaction. These values can be obtained experimentally by the method of comparison of dissociation constants or taken from previously calculated values. It should be noted

that by definition intrinsic binding energies are essentially the same value for a similar environment and the same solvent. However, until a detailed quantitative understanding of the molecular recognition process has been gained it is not known whether intrinsic binding energies have the potential to be fundamental and useful constants.

ΔG_{vdw} is a favourable term that arises because the molecular packing in a complex, whether it is intramolecular or intermolecular, may be more efficient than that of the individual components in the solvent. Only hydrocarbon-to-hydrocarbon interactions in the complex need be considered because the polar interactions are included in the $\Sigma \Delta G_i$ term.

ΔG_H is due to the hydrophobic effect and so is only relevant to binding in aqueous solution. It corresponds to the favourable release of water from the hydrocarbon surfaces as they are removed from exposure to water upon binding. Williams has calculated a favourable effect on binding of $0.125 \text{ kJ mol}^{-1}$ per \AA^2 of hydrocarbon removed from exposure to water by the binding process.²¹ This value was obtained from considerations of thermodynamic data on the solubilities of hydrocarbons in water. Williams later adjusted this value to be 0.19 kJ mol^{-1} per \AA^2 by correcting for the favourable entropy of mixing.²²

As previously mentioned, [1.7] is only applicable to systems which show good complementarity and no strain, for example binding sites which evolved under the pressure of natural selection to maximise the binding strength. For a more general case an additional factor, $\Delta H_{\text{conform}}$ must be included in the equation. $\Delta H_{\text{conform}}$ represents the total conformational strain energy produced upon binding due, for example, to the introduction of unfavourable bond lengths and bond angles. Before association there is essentially no strain present in either component.

Williams has applied his expression [1.7] for estimating binding constants to the interaction in aqueous solution of the antibiotics vanomycin and ristocetin A with the peptide cell wall analogue N-Ac-D-Ala-D-Ala and related ligands.¹⁹ He assumes for these systems that $\Delta H_{\text{conform}}$ and ΔG_{vdW} are zero. The complex formed upon the binding of ristocetin A to N-Ac-D-Ala-D-Ala is shown in Figure 1.4.

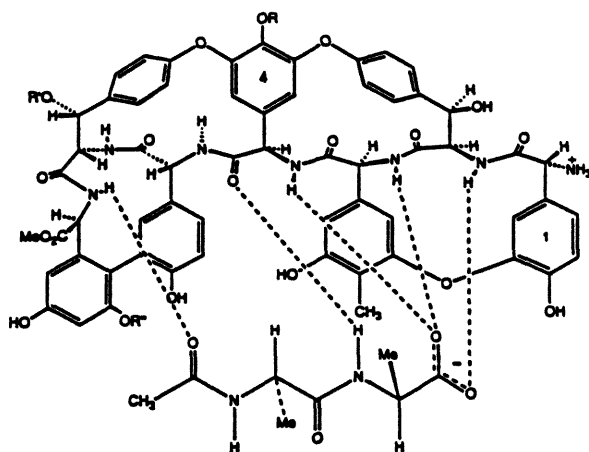


Figure 1.4

Interactions between the cell wall peptide analogue N-Ac-D-Ala-D-Ala and the antibiotic ristocetin A. The broken lines indicate intermolecular hydrogen bonds.

By comparing the binding free energies of two ligands which differ in their binding to a common receptor by removal of an amide-amide bond, the intrinsic binding energy of that bond can be measured. In order to obtain the intrinsic binding free energy for the amide-amide interaction from the acetyl carbonyl group of N-Ac-D-Ala-D-Ala to the antibiotics (the left most hydrogen bond in Figure 1.4) the thermodynamic parameters for N-Ac-Gly-D-Ala (4) and N-Ac-D-Ala (5) were compared (Figure 1.5).

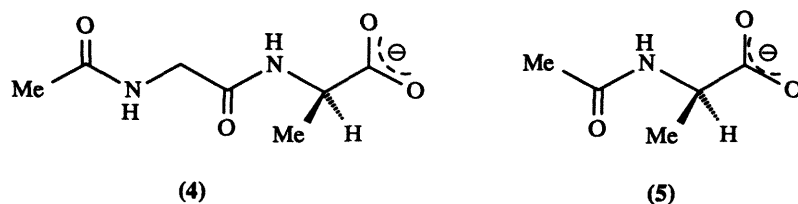


Figure 1.5

In the case of the latter ligand (5) the hydrogen bond being considered is deleted. To calculate the required intrinsic binding energy the differences in enthalpy [$\Delta(\Delta H)$] and entropy [$\Delta(T\Delta S)$] of binding the two ligands are required. Calorimetric data on the binding of the two ligands to vancomycin and ristocetin A gave a mean value of -1 kJ mol^{-1} for $\Delta(\Delta H)$, this is the enthalpy of the hydrogen bond, $\Delta H_{\text{H-bond}}$. The data also show that the entropy change, $\Delta(T\Delta S)$, has a mean value of 10 kJ mol^{-1} . This change in the entropy of binding arises from three sources: i) the difference in rotational and translational free energies of the two ligands which is estimated to be 3 kJ mol^{-1} . This value has been calculated on a semi-empirical basis using gas-phase rotational and translational free energies which are then corrected to allow for the fact that the binding occurs in aqueous solution; ii) the freezing out of two rotors of glycine on formation of the hydrogen bond which has an unfavourable entropy of 10 kJ mol^{-1} ; iii) the entropy change, $T\Delta S_{\text{H-bond}}$ associated with the hydrogen bond formation. Thus

$$\begin{aligned}\Delta(T\Delta S) &= 10 = T\Delta S_{\text{H-bond}} - 3 - 10 \\ \therefore T\Delta S_{\text{H-bond}} &= 23 \text{ kJ mol}^{-1}.\end{aligned}$$

The intrinsic binding energy for the amide-amide hydrogen bond is therefore

$$\begin{aligned}\Delta G_i &= \Delta H_{\text{H-bond}} - T\Delta S_{\text{H-bond}} = -1 - 23 \\ \therefore \Delta G_i &= -24 \text{ kJ mol}^{-1} \text{ (5.7 kcal mol}^{-1}\text{)}.\end{aligned}$$

This value is significantly higher than those previously reported by Fersht for individual uncharged hydrogen bonds.¹²

It should be noted that Williams has subsequently re-evaluated the strength of this amide-amide hydrogen bond.²³ The above estimate of the value of ΔG_{tr} assumed that there was no residual overall relative translation and rotation of the associating components (A and B) in the complex (A·B), however, it is now thought that this situation is unlikely in practice.²⁴ Williams now regards residual relative motions, including new soft vibrations, remaining in the complex as constituting translational and rotational entropy of A and B that was not lost. Also, the initial value of ΔG_r of 5-6 kJ mol⁻¹ for each rotation removed upon association corresponded to severe restriction or complete loss of a rotation, but Williams now argues that smaller values of 3.5-5 kJ mol⁻¹ may be appropriate for associations involving noncovalent bonds.²⁴ Furthermore, initially no increase in the binding of (4) over (5) was attributed to the hydrophobic effect but this has been reassessed and it is now estimated that this contributes to a difference of -8 kJ mol⁻¹. Therefore, by removing credit for residual motions and allowing for a larger hydrophobic effect than originally envisaged, the "new" estimated value for the amide-amide hydrogen bond is -(4-7) kJ mol⁻¹ which corresponds closely to the existing view of these bonds of -2 to -8 kJ mol⁻¹.^{12,25}

The work carried out for the purpose of this thesis was the synthesis of a wide range of modified ligands as a means of making quantitative evaluations of the energetics of individual enzyme-ligand contacts. The enzyme being investigated is chloramphenicol acetyltransferase (CAT), the enzymic effector of bacterial resistance to the antibiotic chloramphenicol (CM).²⁶ There are several reasons why CAT is an attractive candidate for detailed analysis of its molecular recognition process: i) the reaction it catalyses is a relatively simple one; ii) the functionality and stereochemistry of chloramphenicol, one of its substrates, can be altered in a variety of significant ways; iii) the system is being used extensively in protein engineering studies (W.V.Shaw, Leicester University); iv) high resolution crystal structures of the binary complexes of the type III CAT are available.²⁷

BACKGROUND

1.2 CHLORAMPHENICOL

Chloramphenicol was the first broad-spectrum antibiotic brought into medicinal use and also the first to be produced by organic synthesis rather than by fermentation. It is a fermentation product of a soil bacterium (*Streptomyces venezuelae*) and was first isolated in 1947.²⁸ Subsequent characterisation revealed that although its structure was relatively simple it was novel in two respects and interesting stereochemically.²⁹ Chloramphenicol has a N-dichloroacetyl substituent and it was the first natural product found to contain a nitro group. It possesses two asymmetric carbon atoms in the propanediol side chain which gives rise to four possible stereoisomers. It is, however, only the naturally occurring D-(-)-*threo*-2-N-dichloroacetamido-1-*p*-nitrophenylpropan-1,3-diol (**6**) which has significant antimicrobial activity (Figure 1.6).³⁰

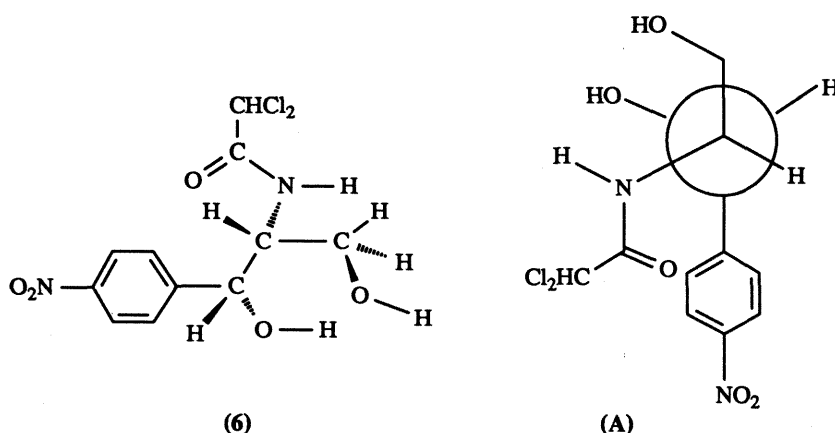


Figure 1.6

In 1952, Dunitz carried out an X-ray diffraction study of the dibromoacetyl analogue of chloramphenicol which showed that the antibiotic exists in a preferred conformation (A) where the two hydroxyl groups are within 2.7 Å of one another.³¹

Due to the close proximity of these two groups he proposed that an intramolecular hydrogen bond was the force responsible for stabilising the (A)-conformer. Independent investigations have supported this hypothesis by concluding that this conformation also exists in solution.³² However, Bustard *et al.* re-examined the conformation of chloramphenicol in solution by NMR and IR spectroscopy and these studies did not reveal the presence of intramolecular hydrogen bonds.³³ This led them to believe that the (A)-conformer might be stabilised through minimal polar and/or solvent interactions. After recent work using high dilution FT-IR Fitzhugh has proposed that (A) is stabilised entirely by noncovalent means through the complementary action of both intramolecular hydrogen bonding and dipolar attractive forces between the carbonyl oxygen and the *p*-nitrophenyl group (Figure 1.7).³⁴

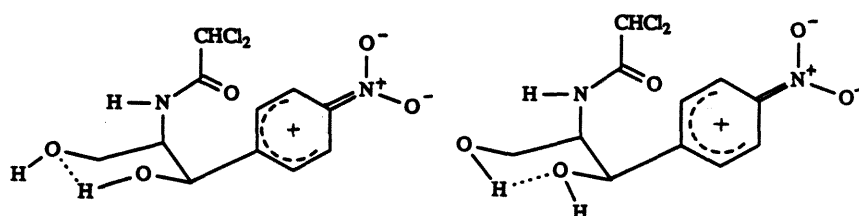


Figure 1.7

The preferred conformer of chloramphenicol showing two pairs of intramolecularly hydrogen-bonded O-H groups.

There are two important molecular recognition events that involve chloramphenicol. i) The binding of CM to the bacterial ribosome which is crucial to its antibiotic action,³⁵ and ii) the recognition of CM by CAT which is the molecular basis of bacterial resistance to the antibiotic.²⁶ Although the former recognition process is the most biologically interesting little is known about it (see Section 1.3i). In contrast, the molecular recognition of chloramphenicol by CAT is known at atomic resolution and provides an excellent test-bed for developing a quantitative understanding. The latter binding site may be viewed as a surrogate for the ribosome site allowing the exploration of the important recognition features of chloramphenicol.

1.3 ANTIBIOTICS

Although all antibiotics are able to prevent the growth and multiplication of susceptible bacteria, microbial inhibition is not brought about by a common mechanism. After many years of extensive study of the mechanisms by which specific antibiotics interact with components of bacterial cells it was possible to divide antibiotic action into five groups according to the general biochemical site upon which they acted.³⁶ These five targets for antibiotic action are: 1) energy metabolism; 2) the function of bacterial membranes; 3) the synthesis of protein; 4) the metabolism of nucleic acid and 5) the synthesis of peptidoglycan. Each of these sites however is complex and different antibiotics within a particular group may have different modes of action.

Chloramphenicol prevents peptide bond formation and so belongs to group 3. Among the antibiotics which inhibit protein synthesis many show selective toxicity in that they are active against prokaryotes but not against eukaryotes (or vice versa) e.g. chloramphenicol, whereas others can inhibit growth of both prokaryotic and eukaryotic cells e.g. puromycin. The majority of antibiotics whose action is targeted at protein synthesis do so by inhibiting functions of ribosomes, the multi-macromolecular complexes on or in which decoding of the genetic message occurs.

1.3i Mode of Action of Chloramphenicol and its Binding to Ribosomes

After it was discovered that chloramphenicol inhibits protein synthesis in bacteria³⁷ it was soon shown that at bacteriostatic concentrations the antibiotic does not inhibit the synthesis of cell-wall peptidoglycan,³⁸ nucleic acids³⁹ or polysaccharides.⁴⁰ As well as inhibiting protein synthesis in prokaryotes chloramphenicol can also achieve this in intact mitochondria⁴¹ and chloroplasts,⁴² but not in mammalian or plant cells.^{35a} In 1964 Traut and Monro demonstrated that chloramphenicol inhibits the peptide

bond-forming reaction.⁴³ Chloramphenicol inhibits peptidyl transferase reactions in the absence of messenger RNA (mRNA) or 30S ribosomal subunits, 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 widely supposed 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.³⁵

Studies by Vazquez on the binding of ¹⁴C-labelled chloramphenicol to ribosomes showed that the antibiotic bound preferentially with the 50S subunit.⁴⁴ At bacteriostatic concentrations one molecule of chloramphenicol binds per 70S ribosome, however, another molecule can bind at high drug concentrations.⁴⁵ Work by Lessard and Pestka confirmed the existence of a second ribosomal binding site for chloramphenicol.⁴⁶ Ribosomes of *E. coli* possess a high affinity site ($K_d = 2 \times 10^{-6}$ M) together with a low affinity site ($K_d = 2 \times 10^{-4}$ M) evident only at high drug input. This second 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 larger 50S subunit and it has been suggested that chloramphenicol binds with slightly higher affinity to ribosomes carrying peptidyl-tRNA in the peptidyl donor site (P site) compared with the A site.⁴⁷ One of the main concerns is to establish which ribosomal proteins constitute the peptidyl transferase and which are present in the A and P sites where the enzyme recognises its acceptor and donor substrates. Affinity labelling studies have identified several ribosomal proteins in the vicinity of the peptidyl transferase centre.⁴⁸ In addition to ribosomal proteins, several nucleotide residues of 23S RNA have been implicated as components of the chloramphenicol binding site.⁴⁹ Recent evidence suggests that there is a specific interaction between rRNA and chloramphenicol within the peptidyl transferase centre of the 50S subunit.⁵⁰

By 1950 chloramphenicol was being produced synthetically and was being widely used as a promising broad-spectrum antibiotic (i.e. it was effective against both Gram-positive and Gram-negative bacteria). However, its clinical applications were severely curtailed after reports of toxic side-effects being associated with its use, which included bone marrow toxicity and adverse reactions in newborn infants. Some of the undesirable effects could possibly be related to the action of chloramphenicol upon mitochondrial metabolism in the spleen and bone marrow. The drug is currently used in veterinary practice, but is used only on a very limited scale in man. Soon after its introduction as an antibiotic it was discovered that many bacteria had become resistant to chloramphenicol and in the vast majority of cases this was due to the presence of the enzyme chloramphenicol acetyltransferase.

1.3ii Bacterial Resistance to Antibiotics

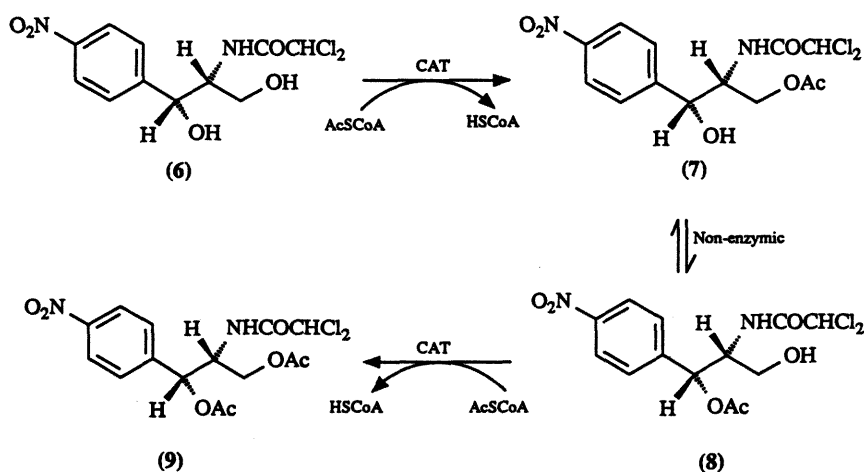
The sudden and epidemic spread of resistance to multiple antibiotics was first detected in Japan in the 1950s. Since then the problem of bacterial resistance has reached huge proportions. The general trend is that when a new antibiotic is introduced there is a short period during which it is effective on a large scale, but this is followed by the appearance of a higher and higher proportion of resistant strains, even up to the point where an organism has developed an absolute resistance. For example, the penicillin dose presently used to control Gram-positive bacteria is several thousand times that used in the forties. For more than thirty years penicillin was the drug used for the treatment of gonorrhoea but its continued prolonged therapeutic use world-wide has led to the development of gonococci that are completely resistant to penicillin.⁵¹

Microorganisms can achieve resistance to antibiotics through biochemical, physiological and morphological modifications. There are five main ways in which this can be achieved:⁵¹

1. Modification of the bacterial site at which the antibiotic acts so that it is insensitive to the drug yet still able to carry out its normal physiological function - this type of resistance mechanism accounts for sulphonamide resistance among pneumococci.
2. Prevention of access of the antibiotic to its active site - loss of cell wall permeability to antimicrobial agents has been detected in ampicillin-resistant mutants of *Salmonella typhimurium* and *E.coli* which have been shown to possess modified cell wall polysaccharides.
3. Reduction in the physiological importance of the target site - certain penicillin and cephalosporin resistant bacteria make use of this mechanism.
4. Duplication of the target enzyme, the second version being the one resistant to inhibition - this type of resistance is effective against the antimicrobial agent trimethoprim.
5. Synthesis by the bacteria of an enzyme capable of inactivating the antibiotic - many clinically important bacteria confer resistance in this way, the enzymes either destroy the antibiotics by cleaving one or more covalent bonds or by chemical modification of key functionalities rendering them inactive. β -lactamases are an example of the first type, they effect the hydrolysis of the β -lactam ring of penicillins and cephalosporins. Enzymes that inactivate antibiotics by substitution are generally of three distinct types, adenylating, phosphorylating and acetylating enzymes. CAT belongs to the class of enzyme that catalyses the acetylation of the antibiotic, and is central to this thesis.

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 nonenzymic isomerisation (Scheme 1.1).^{26,52}



Scheme 1.1

Reactions catalysed by chloramphenicol acetyltransferase.

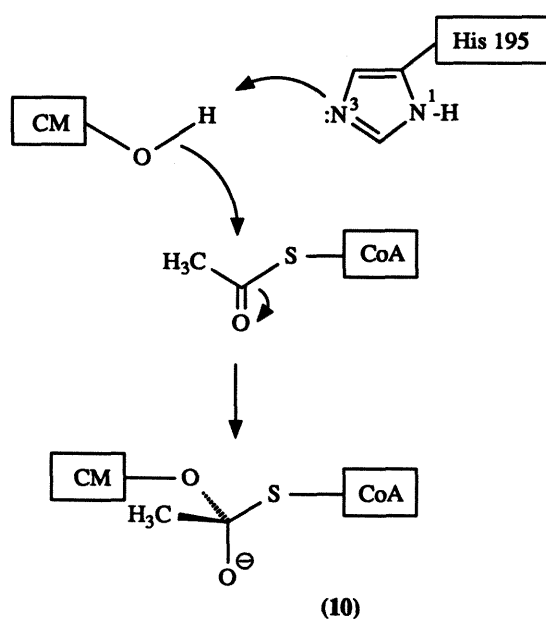
The initial acetylation reaction (Step 1) is catalysed by CAT and is dependent on acetyl coenzyme A (AcSCoA) as the acyl donor. The primary (C-3) hydroxyl group of chloramphenicol is acetylated to give 3-O-acetylchloramphenicol (7) which fails to bind tightly to bacterial ribosomes and so is inactive as an antibiotic.⁵³ (7) then undergoes a nonenzymic, pH-dependent rearrangement (Step 2) involving an intramolecular acyl transfer to the secondary (C-1) hydroxyl group to give 1-O-acetylchloramphenicol (8) which exposes the primary hydroxyl to reacetylation.⁵⁴ (8) is then converted to 1,3-diacetylchloramphenicol (9) by the second CAT catalysed acetylation (Step 3).

Of these reactions it is the first step which is of most interest since it leads to 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 the rate of the slow rearrangement step and that of the reacetylation, the latter being at least two orders of magnitude slower than the initial acetylation.⁵⁵ 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 due to the difference in free energy between acetyl thioesters and acetyl oxyesters.⁵⁶

The results of steady-state kinetic studies of the forward and reverse CAT catalysed reaction suggest a rapid-equilibrium mechanism leading to the formation of a ternary complex.⁵⁷ Although the addition of substrates in both the forward and reverse reactions is random, a preference for AcSCoA as the leading substrate has been detected in the forward reaction. Chemical modification studies were undertaken to find out which amino acid residues play a role in catalysis. 3-Bromoacetylchloramphenicol, an affinity reagent that is an analogue of the acetylated product of the forward reaction catalysed by CAT, was found to uniquely alkylate a histidine residue, His 195.⁵⁸ Inactivation was stoichiometric, 1 mole of the inhibitor being covalently bound per mole of enzyme monomer, with the consequent loss of acetylation and hydrolytic activity associated with CAT. Histidine modification in proteins often shows preference for one of the two possible tautomeric forms.⁵⁹ In this case N³-(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. Since 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 has shown 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.^{27a}

The results of the 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.⁵⁸



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 carbonyl of the thioester of CoA. This leads to the formation of an oxyanion tetrahedral intermediate (10). The availability of the structures of the two binary complexes has allowed the proposed intermediate to be modelled with only very small changes in either the conformation of the enzyme or the position of the substrates.⁶⁰

1.5 STRUCTURE OF CAT

There are eleven natural variants of CAT for which amino acid sequence data are available, but it is the type III enzyme, CAT_{III}, which has been studied in the greatest detail, both structurally and mechanistically.^{55,61} CAT_{III} is the most active of known CAT variants and is the enzyme that is the subject of this work. When CAT is referred to it will be concerning CAT_{III}.

CAT is a trimer of identical subunits, each with a mass of 25,000, and the active sites are located at each of the three subunit interfaces (see Figure 1.9).²⁷ The secondary structure of the monomer consists of a six-stranded mixed parallel and antiparallel β -pleated sheet with five α -helices packed against one side and the end of it (Figure 1.8).

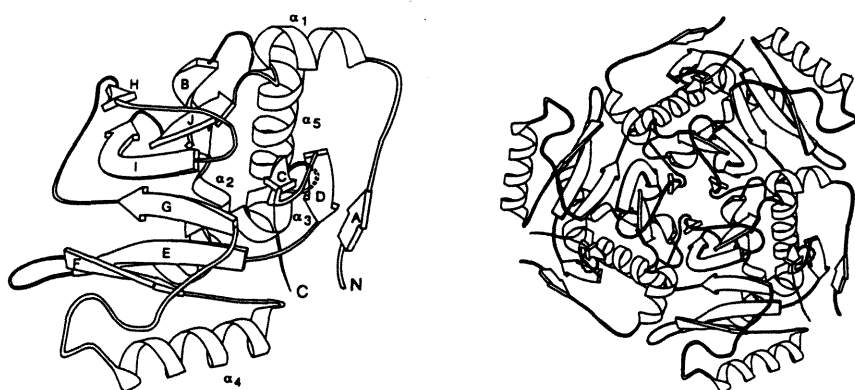


Figure 1.8

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

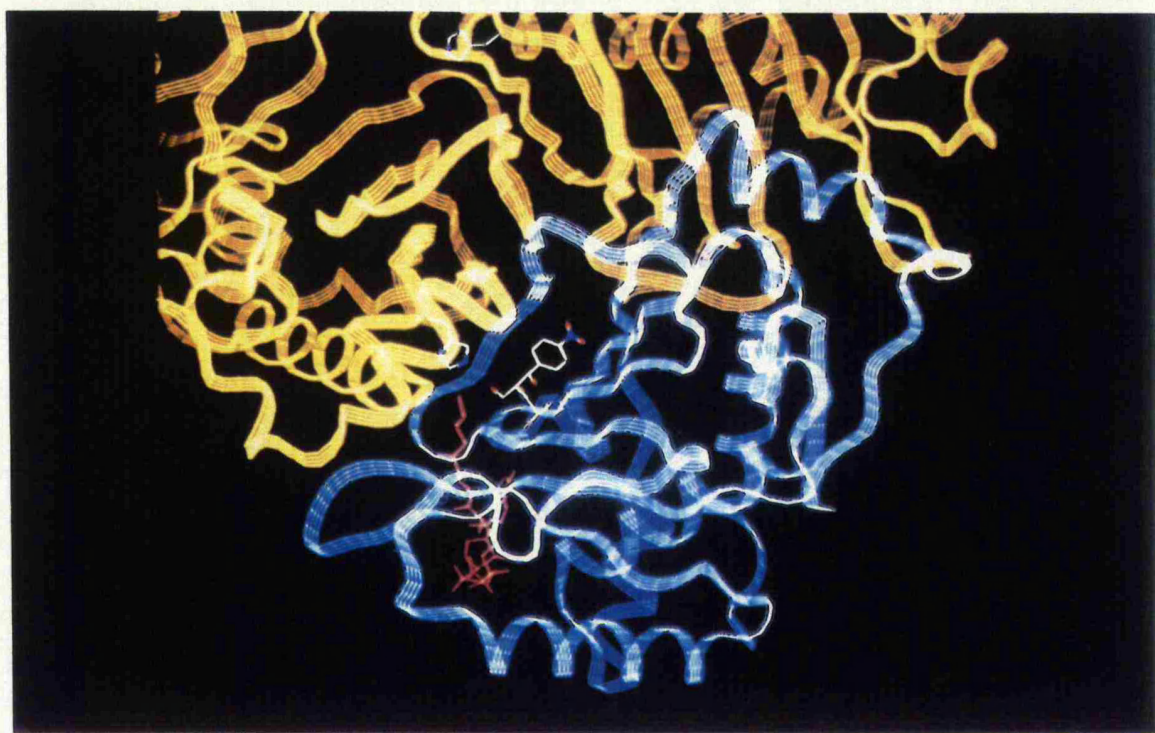
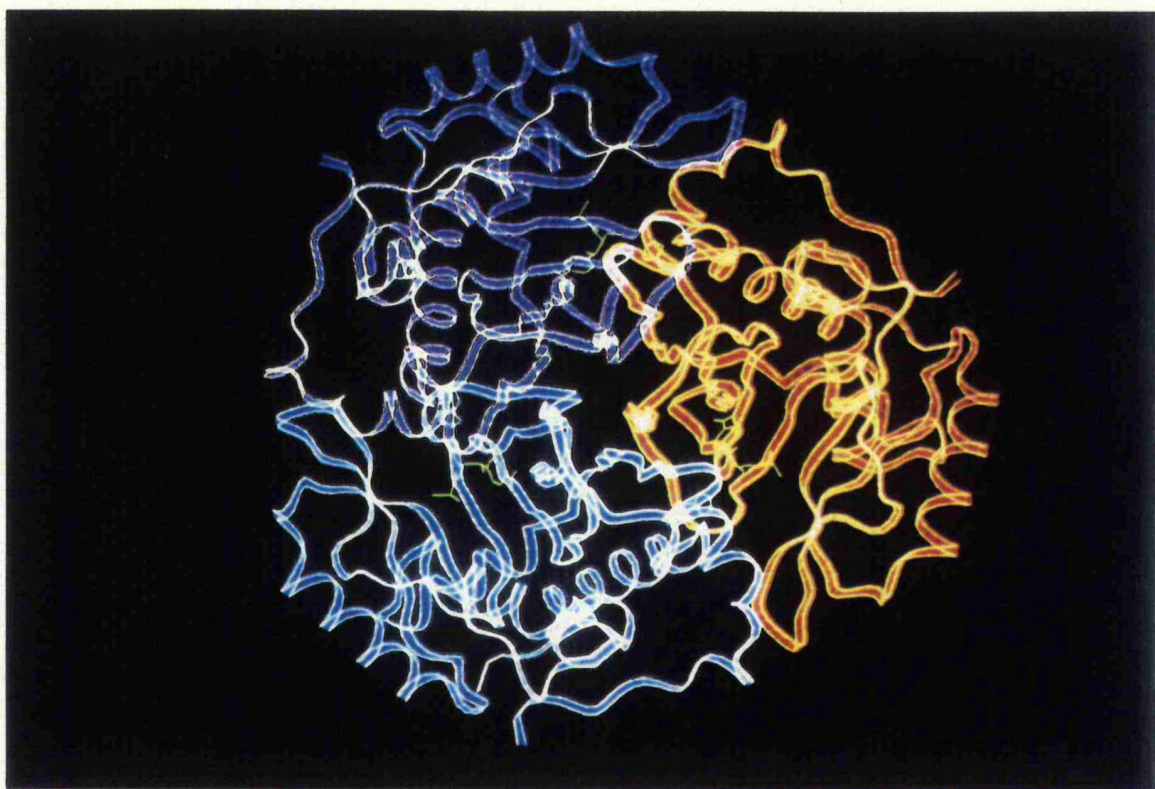
There is an additional small three-stranded β sheet which is formed by the N-terminus and an extended loop between helix α_3 and strand β_E . The three subunits associate so that the six-stranded β sheet of each monomer extends across the subunit interface via

Figure 1.9 (opposite)

(Top) Model of CAT showing the trimer of identical subunits. Chloramphenicol (shown in green) is bound at each of the three subunit interfaces.

(Bottom) Model of both chloramphenicol and CoA (red) bound at one of the three active sites of CAT.

The molecular modelling was carried out with the Biosym software using the coordinates of the two binary complexes taken from the X-ray structures.²⁷



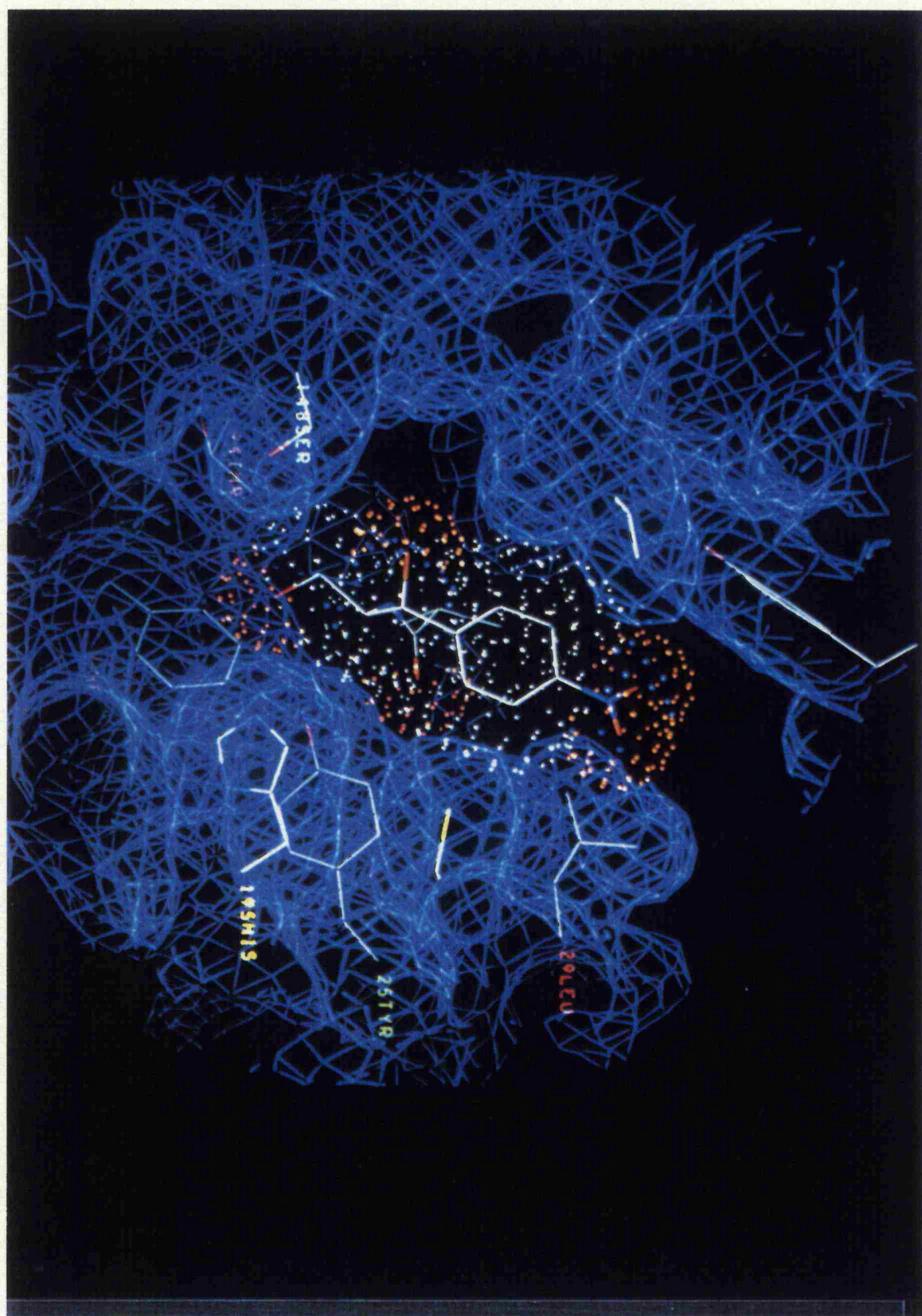
1.5i The Binding Site of Chloramphenicol

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

Figure 1.11 (opposite)

Model of the CAT:CM binary complex. Chloramphenicol is shown bound in the deep binding pocket situated at the subunit interface.

The molecular modelling was carried out with the Biosym software using the coordinates of the binary complex taken from the X-ray structure.^{27a}



Both hydrophobic and polar residues are involved in binding chloramphenicol, and there are extensive van der Waals contacts between chloramphenicol and the side chains of the pocket (see Figure 1.11). The phenyl ring of chloramphenicol lies next to the side chains of Leu 29, Cys 31, Leu 160 and Ile 172, and the dichloro group makes van der Waals contact with Ala 105, Phe 135 and Asn 146. The interaction of chloramphenicol with CAT involves only two direct hydrogen bonds, one between the carbonyl oxygen of the dichloroacetamido group of chloramphenicol and the hydroxyl of Tyr 25 and the other between the C-3 hydroxyl of chloramphenicol and the imidazole N-3 of His 195. A third hydrogen bond exists between the C-1 hydroxyl and Thr 174 but this occurs via a bridging water molecule. 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 together with the hydrogen bonds reflect the amphipathic nature of chloramphenicol.

1.5ii The Binding Site of Coenzyme A

The structure of the binary complex of CAT with CoA, the product of the forward reaction, has been solved at 2.4 Å resolution.^{27b} When CoA binds the pantetheine arm is in an extended conformation with the polar phosphate groups in contact with solvent on the enzyme surface and the thiol group completely buried (Figure 1.12). The pantetheine binding pocket appears as a tunnel which leads from the surface of the protein into the catalytic site. It is lined with hydrophobic residues which make van der Waals contact with the apolar groups of CoA. Although the adenine ring binds in a hydrophobic pocket it makes two hydrogen bonds to main chain atoms and one to an ordered water molecule. The majority of the polar groups of CoA are hydrogen bonded to ordered water molecules rather than to specific residues.

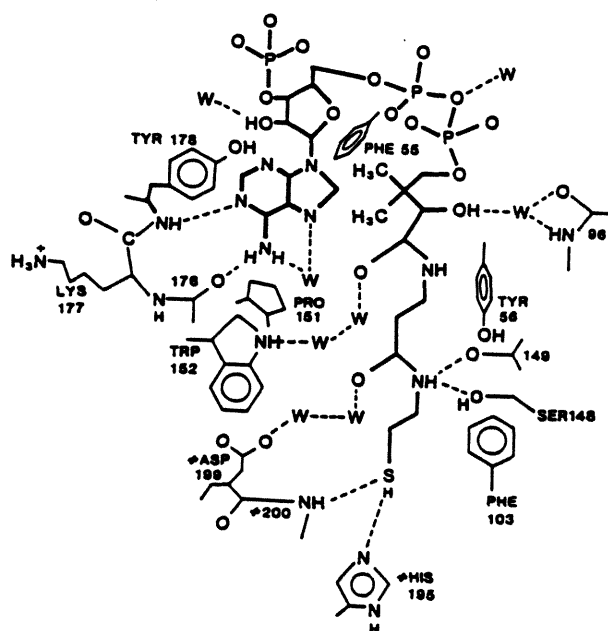


Figure 1.12

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

Chloramphenicol and acetyl CoA 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, positioned in the upper surface of the enzyme (as viewed in **Figure 1.8**), is linked to the CoA binding tunnel which emerges from the lower surface. Therefore, when no substrates are bound to CAT, this provides a solvent-accessible channel which runs from one side of the molecule to the other.

1.5iii The Active Site

As discussed in Section 1.4, the N-3 of the imidazole ring of His 195 was identified as the nucleophilic/basic centre responsible for catalysis. In the binary complex of CAT and chloramphenicol this nitrogen is 2.8 Å away from the C-3 hydroxyl of chloramphenicol which is consistent with its role as a general base.^{27a} It has been shown that there is no obvious need for a conformational change in the ternary complex to bring the reactive groups of substrates and enzyme into close proximity since in the binary complex of CAT and CoA the sulphur of CoA is only 3.3 Å from the N-3 imidazole nitrogen of the catalytic histidine residue. Modelling of the proposed tetrahedral intermediate (10) has shown that it is more readily accommodated than the ternary complex implying enzyme-transition state complementarity.⁶⁰ 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_{cat} and so increases the rate of reaction. A further result of the modelling studies is that a conserved residue, Ser 148, may play a role in the stabilisation of the transition state (Figure 1.13).

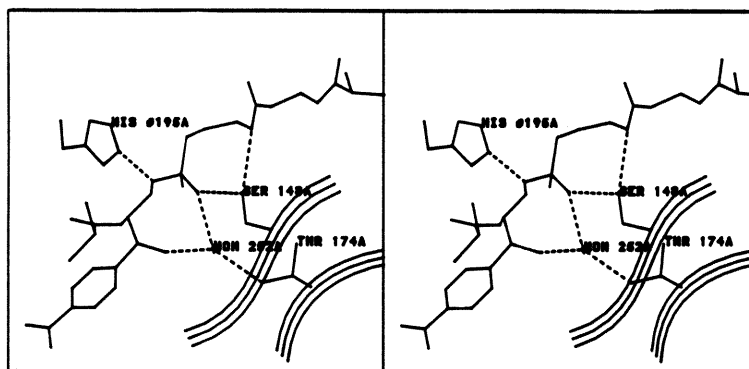


Figure 1.13

Stereo diagram of the proposed tetrahedral intermediate. Parallel lines indicate the position of the C_{α} backbone.

Ser 148 is 3.2 Å from the putative oxyanion and so there is the possibility of hydrogen bonding between them, whereas the same residue lies 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. A second residue, Thr 174, is also involved in the transition-state stabilisation, by forming a hydrogen bond to the tetrahedral oxyanion via an ordered water molecule.

1.6 STUDIES OF THE CHLORAMPHENICOL BINDING SITE OF CAT BY SITE-DIRECTED MUTAGENESIS AND SUBSTRATE MODIFICATION

A large number of site-directed mutants of CAT have been prepared to assess substrate recognition, catalysis and general aspects of protein structure and stability. For the specific purpose of this thesis it is modifications to the chloramphenicol binding site that are of interest. Both site-directed mutagenesis and substrate modification have been employed to investigate enzyme-ligand interactions at the C(1)-hydroxyl substituent of chloramphenicol.

In the binary complex of CAT and chloramphenicol the substrate lies in a deep pocket where it makes extensive van der Waals contacts with the side chains lining the pocket, but there is a small cavity situated at the base of the pocket which in part accommodates the 1-hydroxyl group of chloramphenicol.^{27a} The cavity is lined by the side chains of Ser 148, Leu 160, Ile 172 and Thr 174, and is occupied by three water molecules, Wat 249, Wat 252 and Wat 360 (Figure 1.14). Wat 252 forms a bridging hydrogen bond between the 1-hydroxyl of chloramphenicol and the hydroxyl group of Thr 174.

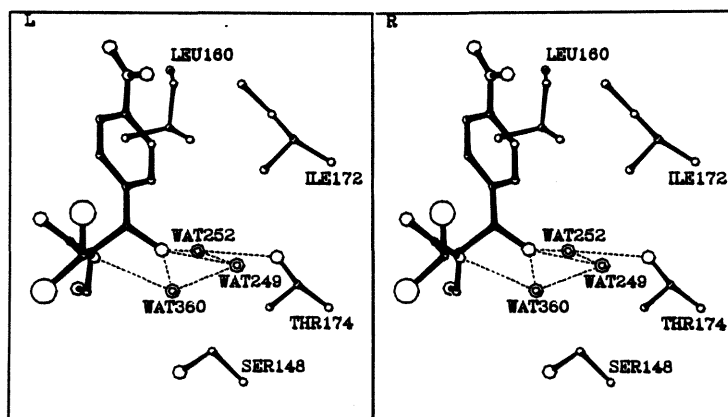
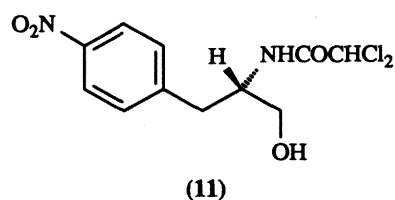


Figure 1.14

Stereo diagram of the cavity inpart occupied by the 1-hydroxyl group of CM.

In Section 1.4 it was noted that the acetylation of 1-O-acetylchloramphenicol (8) to give 1,3-diacetylchloramphenicol (9), Step 3 Scheme 1.1, was slow compared to Step 1 which gave 3-O-acetylchloramphenicol (7). Modelling suggested that the acetyl substituent of (8) could be accommodated in the 1-hydroxyl cavity but this would probably cause the displacement of bound water and a minor rearrangement of either the substrate or enzyme to alleviate unfavourable van der Waals contacts. In order to examine the binding of 1-O-acetylchloramphenicol, Leu 160 was substituted by glutamine to give Q160CAT and phenylalanine to give F160CAT.⁶² Glutamine is approximately isosteric with leucine but less hydrophobic and so could affect the organisation of the water molecules, whereas phenylalanine was expected to impair the binding due to steric effects.

Initially the kinetics were determined for the reaction of wild-type CAT with chloramphenicol (6), 1-O-acetylchloramphenicol (8) and 1-deoxychloramphenicol (11) wherein the 1-hydroxyl is replaced by a hydrogen atom (Figure 1.15).⁶²



1-Deoxychloramphenicol

Figure 1.15

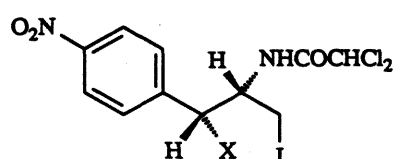
The results showed that for wild-type CAT the specificity constant, k_{cat}/K_m , for 1-O-acetylchloramphenicol was 150-fold lower than for chloramphenicol which confirmed the relative inefficiency of Step 3 to give the diacetyl product. The difference arises mainly from the large decrease in k_{cat} suggesting that either the side chain of Ser 148 is displaced and so disrupts coordination of the oxyanion of the transition-state, or that 1-O-acetylchloramphenicol is bound in an alternative mode to

that observed in the binary complex of CAT and chloramphenicol. The 1-deoxy analogue (11) was found to be a good substrate for CAT, and by comparison with chloramphenicol the interaction between the 1-hydroxyl of chloramphenicol and the cavity was calculated to account for only 0.8 kcal mol⁻¹ of binding energy.

The kinetics of the above substrates, (6), (8) and (11) were then determined with the mutant enzymes. The results show that the L160Q substitution caused an increase in K_m values which is as expected if hydrophobic considerations are dominant in the binding of chloramphenicol with CAT because the amide substitution causes the binding to become more hydrophilic. The specificity constants however, indicated that the substitution did not enhance or impair discrimination against the alternative substrates.

In the case of the L160F substitution it was shown that the binding affinities of all three substrates were only very slightly altered in comparison with wild-type CAT, whereas the k_{cat} values were significantly reduced. There was a slightly enhanced discrimination against the analogues but this was not thought to be due to the steric hindrance of the aryl group of Phe 160. The structure of the binary complex of chloramphenicol and Phe 160 CAT revealed that although the introduction of the aromatic substituent did not impair the apparent binding affinity of CAT for chloramphenicol, the substrate was bound in an alternative and probably nonproductive mode. However, since Phe 160 CAT can acetylate chloramphenicol and the 1-deoxy and 1-acetyl analogues this implies that there is an additional productive mode for these substrates.

In order to examine the tolerance of wild-type CAT for increasing chain length of the C(1)-substituent, a series of potential competitive inhibitors were prepared wherein the 3-hydroxyl of chloramphenicol was replaced by iodine and the C(1)-substituent was either a hydroxyl, acetyl or propionyl group (Figure 1.16).⁶²



- (12) X=OH, 3-Iodochloramphenicol
 (13) X=OAc, 1-Acetyl-3-iodochloramphenicol
 (14) X=OCOCH₂CH₃, 3-Iodo-1-propionylchloramphenicol

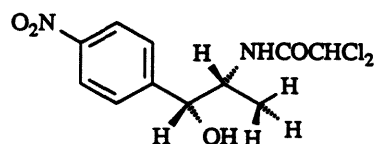
Figure 1.16

The above analogues were all found to be potent inhibitors of wild-type CAT, the K_i values were 3.7 μ M, 7.2 μ M and 29 μ M for (12), (13) and (14) respectively. Modelling studies on (14) had suggested that the binding of the propionyl moiety would cause a major displacement of the rest of the molecule. The high affinity observed for (14) suggests that the chloramphenicol binding site of CAT is extremely accommodating in its response to substitution of the 1-hydroxyl of chloramphenicol, and is further evidence that the 1-acetyl group is unlikely to bind in the cavity next to the 1-hydroxyl in the native CAT-chloramphenicol structure.

From these studies it can be concluded that the acyl substituents of these analogues do not bind in close proximity to Leu 160 as does the 1-hydroxyl of chloramphenicol, and at least two novel binding modes for the acetyl acceptor are implied by the kinetic and structural analyses. The lack of many direct hydrogen bonds together with the dominant hydrophobic interactions in the CAT-chloramphenicol complex seem to allow for such alternative binding modes.

Substrate modification studies have been carried out to examine the interaction of the 3-hydroxyl group of chloramphenicol with the N-3 of the imidazole of His 195.⁶³ In principle, site-directed mutagenesis could have been used to probe this interaction but there were several factors which disfavoured this approach: i) His 195 is central to catalysis and so substituting it with other amino acids that are not hydrogen-bond

acceptors will almost certainly lead to little or no catalytic activity, as has indeed been found, and so steady-state kinetics could not be used to evaluate the interaction; ii) enzyme mutagenesis may lead to disruptive structural changes that are difficult to detect; iii) the N-1 hydrogen of His 195 forms a novel hydrogen bond to the backbone carbonyl of the same residue which is likely to be of importance to the geometry of the active site. It was therefore decided to alter the substrate initially by replacing the hydroxyl group with a hydrogen, leading to 3-deoxychloramphenicol (15), (Figure 1.17).



(15)

3-Deoxychloramphenicol

Figure 1.17

The results of kinetic studies showed that deletion of the 3-hydroxyl of chloramphenicol caused a decrease in binding affinity. 3-Deoxychloramphenicol was found to be a competitive inhibitor with a K_i of 48 μM compared to a K_d for chloramphenicol of 3.6 μM . Comparison of these results gave an apparent binding energy, ΔG_{app} , of 1.5 kcal mol^{-1} for the hydrogen bond between the C(3)-hydroxyl of chloramphenicol and His 195. This value is at the high end of the range for neutral hydrogen bonds in enzyme-substrate complexes as reported by Fersht¹² indicating a relatively strong hydrogen bond. It was thought that the calculated value might underestimate the contribution to binding of this hydrogen bond because deletion of the hydroxyl group may also cause a loss of favourable van der Waals interactions, but this could be balanced by a favourable hydrophobic interaction, 3-deoxychloramphenicol being significantly more hydrophobic than chloramphenicol.

In order to probe more fully the environment of the 3-hydroxyl group a series of 3-halo-3-deoxychloramphenicol derivatives (16), (17), (18) and (19) were prepared (Figure 1.18).⁶³ Substitution of the 3-hydroxyl group with a halogen removes the hydrogen-bond donor to His 195 and also affects the solvent interactions of the resulting analogues.

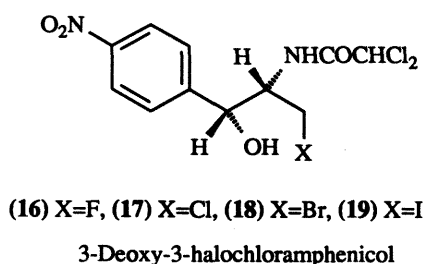


Figure 1.18

Kinetic results showed that the binding affinity of these compounds increased in the order $F < Cl < Br < I$, and it was thought that hydrophobic effects would explain the observed trend. The hydrophobicity of 3-deoxychloramphenicol and the halo derivatives was estimated on an empirical basis using an HPLC method which had previously shown that the retention time on a reverse-phase column was related to the partition coefficient between water and an organic phase.⁶⁴ A plot of $\ln(1/K_i)$ versus hydrophobicity of inhibitors showed a linear free energy relationship. This was consistent with all the ligands being bound to CAT in the same manner and that their major interactions with CAT were not disrupted, even in the case of the iodo derivative (19), the most sterically demanding substitution. Chloramphenicol was shown not to fall on the straight line, binding with a higher affinity than predicted from its hydrophobicity due to the formation of the hydrogen bond of interest. It was expected that in the absence of the hydrogen bond chloramphenicol would fall on the same regression line as the other ligands. On this basis, the extent to which chloramphenicol is displaced from the line can be considered as a direct measure of the additional stabilisation energy provided by having a hydroxyl group at the C(3)-position

in the native enzyme-chloramphenicol complex. This analysis gave a binding energy, ΔG_{bind} , of 2.7 kcal mol⁻¹. This value is larger than the ΔG_{app} value obtained by simply replacing the hydroxyl group with a hydrogen and as such is higher than the typical values reported by Fersht.

The overall results show that the binding of the C(3)-position of chloramphenicol by CAT is dominated by hydrophobic effects. This is reflected by the linear free energy relationship shown by the inhibitors and also by the larger than normal value of ΔG_{bind} . The hydrogen bond being studied is located in a comparatively hydrophobic cavity which is out of contact with bulk solvent, it is therefore expected to be stronger than a hydrogen bond in a more polar environment. The difference in ΔG_{app} and ΔG_{bind} may also in part be contributed to favourable van der Waals interactions between the hydroxyl group and CAT.

1.7 THIS THESIS

The aim of the work carried out for this thesis was to gain a deeper understanding of the molecular recognition of chloramphenicol by chloramphenicol acetyltransferase, and in particular to develop a quantitative understanding of the origin of this specificity. This was to be achieved by "mutating" the substrate chloramphenicol. 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 chloramphenicol the alternative substrates were specifically designed so that the contribution to binding and catalysis of each of the substituents of chloramphenicol could be estimated quantitatively.

Chapter 2 investigates a synthetic route to chloramphenicol analogues in which the *p*-nitrophenyl group of CM is replaced by an alkyl group R. The aim was to prepare a series of compounds which have the same relative configuration as chloramphenicol and in which the length of the R group is systematically increased. It was anticipated that this could be achieved via conversion of the amino acid D-serine to a series of ketones with organolithium reagents RLi, followed by asymmetric reduction of the ketones to give the required *threo*-1,3-diols.

An alternative approach to the above required compounds is discussed in Chapter 3. The proposed route to the chloramphenicol analogues is via a suitably protected α -amino aldehyde. It was predicted that reaction of the aldehyde with an appropriate organometallic reagent would lead to the desired *threo*-1,3-diols.

The chloramphenicol analogues in which the *p*-nitrophenyl group was replaced by alkyl groups of increasing chain length were prepared to probe the incremental binding energy derived from this part of CM. Chapter 4 discusses the kinetic results obtained

for these ligands as CAT substrates. As shown in the CAT:CM binary complex^{27a} the phenyl ring of chloramphenicol is located in a hydrophobic pocket and it was therefore predicted that these substrates would show a linear free energy relationship with respect to the hydrophobicity of the R group. In addition, by preparing a range of *p*-substituted chloramphenicol analogues, the role of the nitro group in binding of chloramphenicol by CAT could be investigated. The synthesis and kinetic studies of these compounds are also discussed in Chapter 4.

In addition to making systematic changes to chloramphenicol more radical alterations to the ligand are examined to see if there is potential for using CAT as a "reagent" in organic synthesis. The preparation and kinetic studies of these alternative substrates are discussed in Chapter 5. The role of the N-acyl group in the binding of chloramphenicol by CAT is also discussed. The origin and energetics of enantiodiscrimination shown by CAT is investigated because it was not known if CAT would be able to bind and catalyse the acetylation of a compound which has the opposite relative configuration of chloramphenicol. Chapter 5 ends with a summary of the overall conclusions that can now be made regarding the interaction of CAT with its substrates and is a culmination of the results obtained for this thesis.

Chapter 6, 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 CHLORAMPHENICOL DERIVATIVES VIA THE AMINO ACIDS SERINE AND THREONINE

2.1 INTRODUCTION

The major part of the synthetic work that was carried out involved the preparation of a series of 1,3-diols in which the *p*-nitrophenyl group of chloramphenicol (**6**) was replaced by an alkyl group R (**20**).

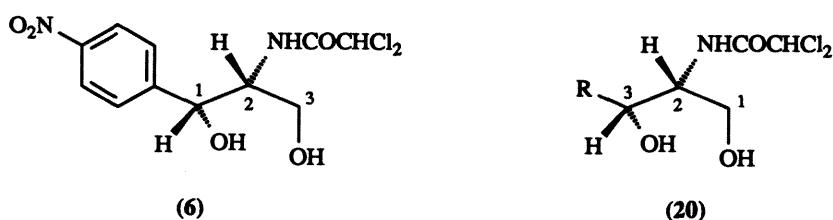


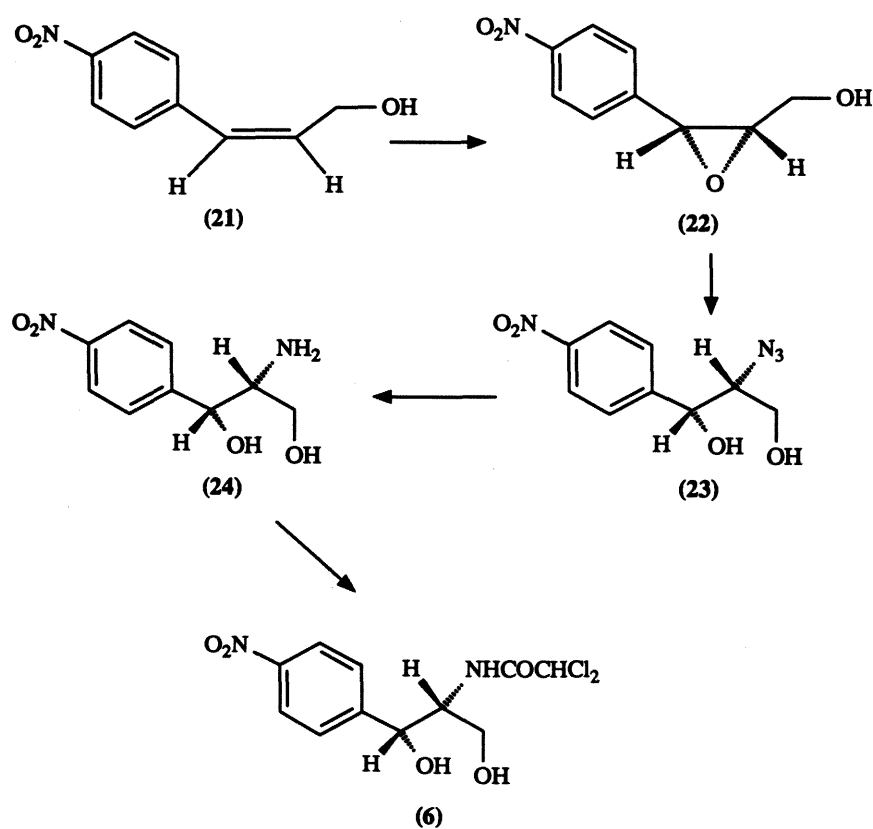
Figure 2.1

It should be noted that when using conventional numbering the secondary hydroxyl of chloramphenicol is at C-1 whereas with the proposed analogues it is at C-3 (see Figure 2.1).

In order to obtain meaningful comparisons of the interaction of chloramphenicol and CAT calculated from kinetic data with that for analogues in which the *p*-nitrophenyl group has been replaced by various alkyl groups, it was imperative that the absolute configuration of these analogues at both chiral centres was the same as in chloramphenicol. It was therefore necessary to find a general route in which R could be systematically varied and which would give enantiomerically pure compounds with the required configuration (*D-threo* in the case of chloramphenicol), see Figure 2.1.

Chloramphenicol is produced commercially by the addition of benzaldehyde to β -nitroethanol to yield 2-nitro-1-phenylpropan-1,3-diol to give mostly *threo*-racemate, followed by reduction and subsequent transformation to chloramphenicol.⁶⁵ Only two asymmetric syntheses of the antibiotic have been reported, both of which are very recent.⁶⁶

The first route started with *Z*-(*p*-nitro)cinnamyl alcohol (21) which was subjected to titanium isopropoxide catalysed asymmetric epoxidation, the reaction taking 7 days at -20°C (Scheme 2.1).^{66a}

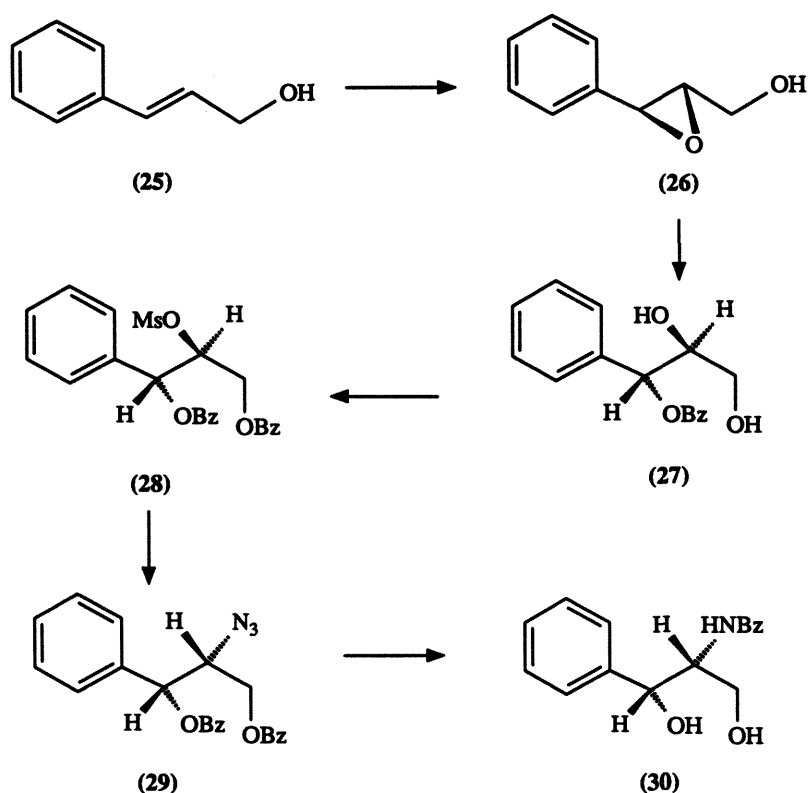


Scheme 2.1

The epoxide (22) was opened regioselectively at C-2 with *anti*-selectivity using sodium azide. C-2 was attacked preferentially on steric grounds due to the aryl group being bulkier than the hydroxymethyl functionality. By carrying out the reaction under acid catalysis the C-2 selectivity was enhanced because the carbocationic character in the transition state is stabilised more at C-2 than C-3 due to the presence of the

electronegative *p*-nitrophenyl moiety. The azide diol (23) was converted to the amino diol (24) which was then reacted with methyl dichloroacetate to give chloramphenicol.

The second route also employed an asymmetric epoxidation but started from *E*-cinnamyl alcohol (25).^{66b} The epoxidation was carried out with L-(+)-diisopropyl tartrate under Sharpless conditions (Scheme 2.2).



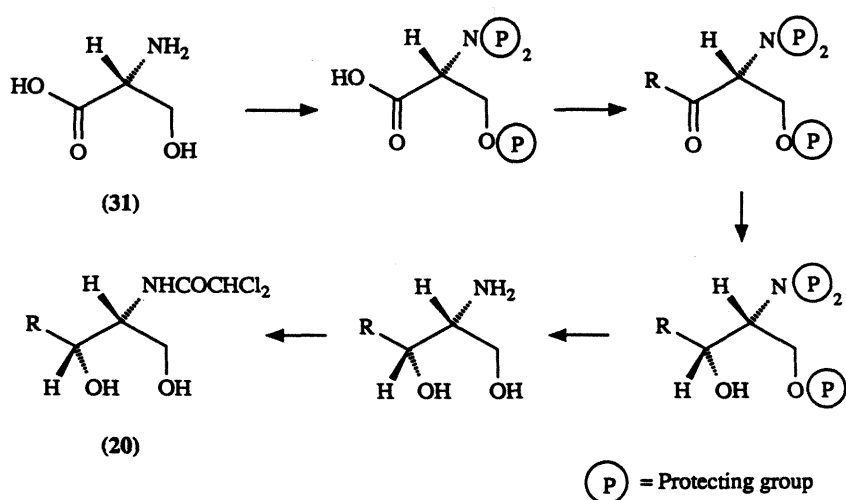
Scheme 2.2

The epoxide (26) was ringed opened regioselectively with benzoic acid in the presence of titanium isopropoxide. The benzoate ion was shown to have attacked the epoxide with inversion of configuration at C-3 to give the diol (27). Benzylation and subsequent mesylation of (27) gave (28) which was then reacted with sodium azide to afford

compound (29). N-benzoyl-amino diol (30) was obtained after reduction of (29) and subsequent hydrolysis. (30) could then be converted to chloramphenicol following the procedure described by Controulis.⁶⁵

Although it may be possible to prepare the required chloramphenicol analogues (20) using similar methodology to that shown above it is not an ideal general route, in part, because the required starting materials are not readily available. In the development of routes to optically active compounds the use of chiral starting materials is highly advantageous. For example, α -amino acids are chiral, nonracemic natural products and as such are valuable building blocks in stereocontrolled synthesis. Most of the common L-amino acids have long been utilised in this way, and with much diversity.⁶⁷ However, due now to the commercial availability of the unnatural antipode (the enantiomeric D-form) of many amino acids, the versatility of these chiral starting materials has greatly increased. The synthesis described in this and the subsequent chapters shows how the use of only a few amino acids can be exploited to make a large range of chloramphenicol analogues.

In order to prepare the series of 1,3-diols shown in Figure 2.1 it was initially thought that a synthetic route could be found which started with the amino acid D-serine (31), which provides the appropriate absolute configuration at C-2. With D-serine suitably protected the key step in the variation of R can be achieved by the direct conversion of the carboxylic acid to the ketone by the reaction of an organolithium reagent, RLi (see Scheme 2.3).



Scheme 2.3

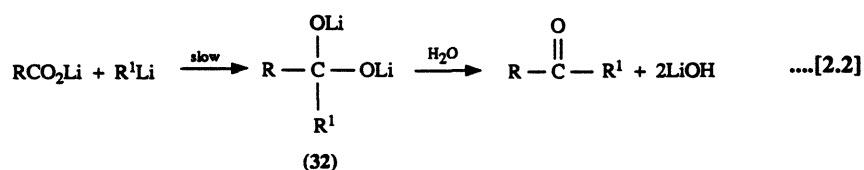
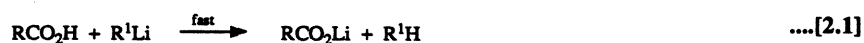
The reduction of α -amino ketones has been studied extensively with varying degrees of success.⁶⁸ In general the reported cases refer to either, racemic starting materials and so little information is available regarding possible racemisation during their synthesis and reactions, or to systems bearing groups on nitrogen that are difficult to cleave. By finding appropriate conditions to stereoselectively reduce the ketone to the required alcohol and then deprotecting and reacting the free amine with methyl dichloroacetate the proposed route shown in Scheme 2.3 should lead to the required product (20).

RESULTS AND DISCUSSION

2.2 CONVERSION OF CARBOXYLIC ACIDS TO KETONES WITH ORGANOLITHIUM REAGENTS

A general method for the synthesis of ketones is the reaction of carboxylic acids with organolithium reagents which has been reviewed by Jorgenson.⁶⁹ The synthetic potential of the reaction was first recognised in 1933 when Gilman and Van Ess discovered this transformation as a side reaction during their studies of the carbonation of organolithium reagents.⁷⁰ They made the significant observations that the reaction of benzoic acid (1 equiv.) with phenyllithium (2 equiv.) gave benzophenone (37%) and triphenylcarbinol (14%), and that refluxing lithium benzoate (1.4 equiv.) with phenyllithium (1 equiv.) for 5.5h gave only benzophenone (70%). Later work by Tegnér⁷¹ investigating the reaction of 2 equiv. of methyllithium with 1 equiv. of a series of carboxylic acids for 1h gave the corresponding methyl ketones and no tertiary alcohols.

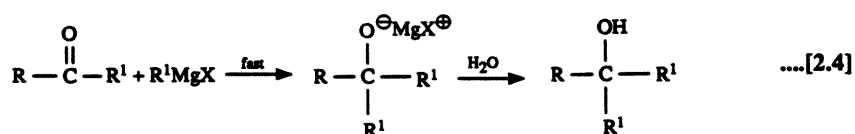
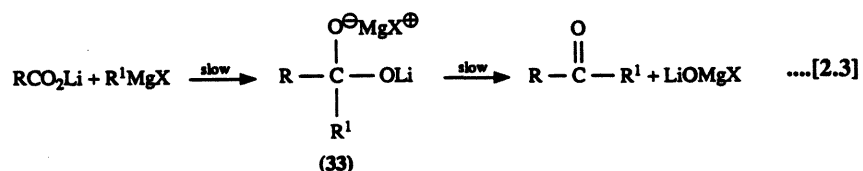
The reaction of 2 moles of organolithium reagent with 1 mole of carboxylic acid can be considered as taking place in two discrete steps.



The first step [2.1], which is very fast, produces the lithium salt of the carboxylic acid. The second step [2.2] is the relatively slow reaction of the lithium carboxylate with the second mole of organolithium reagent to form the intermediate dilithium salt (32) which only decomposes on work-up to give the ketone.

The success of the reaction in the synthesis of ketones is believed to be due to the stability of the dilithium salt which does not break down until the hydrolysis step. The significance of this stability can be seen by comparing this reaction with that of organolithium reagents with carboxylic esters, acid chlorides or anhydrides. In the latter cases the major product is generally the tertiary alcohol rather than the ketone and this is due to the facile decomposition of the organometallic intermediate to a ketone in the reaction mixture prior to hydrolysis.

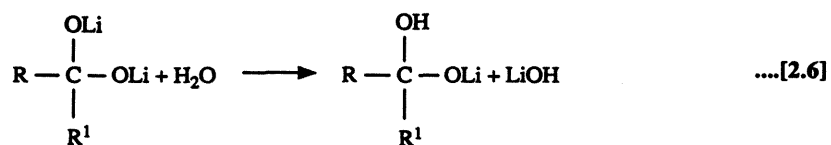
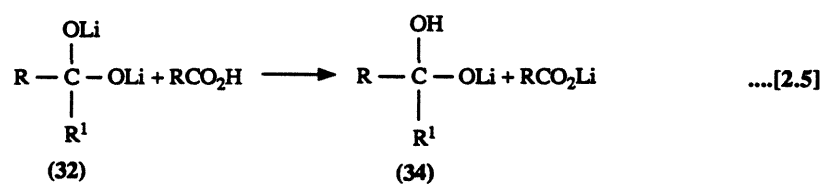
Further evidence to support the theory that it is the stability of the dilithio adduct (32) that is important is shown by the work of Levine *et al.*⁷² They conducted a series of reactions in which equimolar amounts of phenyllithium and the lithium salts of various carboxylic acids were refluxed for 24h and then quenched with water. In six out of the seven reactions they obtained high yields of ketone and no carbinol. In contrast to this they found that the reaction of equimolar amounts of various Grignard reagents with lithium *n*-butyrate and lithium benzoate gave, after refluxing for 24h, mixtures of ketone and tertiary alcohol, and in most cases it was the alcohol that was the major product. From these results they suggest that in the reactions using Grignard reagents free ketone may slowly be formed during the reaction prior to hydrolytic work-up of the intermediate adduct [2.3]. The ketone then reacts rapidly with more Grignard reagent to give the halomagnesium salt of the tertiary alcohol as shown in [2.4].



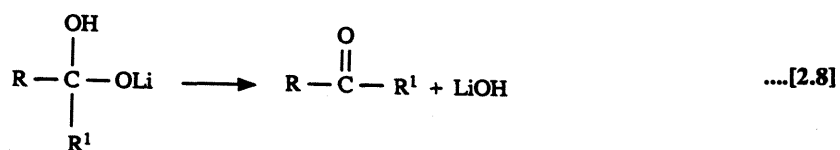
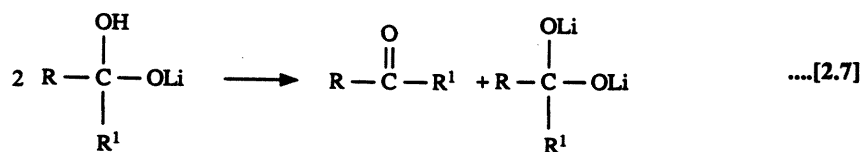
It is the better leaving-group tendency of -OMgX compared to -OLi that makes the intermediate (33) less stable than its dilithio counterpart.

2.2i Tertiary Alcohol Formation

One of the main concerns associated with the conversion of a carboxylic acid to a ketone is the prevention of the most general side reaction that can occur and that is the formation of a tertiary alcohol. The alcohol is produced via the generation of free ketone in the presence of organolithium reagent, and the ketone is formed from the monolithium salt (34) which can be produced in two ways [2.5] and [2.6].⁶⁹



It is the decomposition or disproportionation of (34) that gives rise to free ketone as shown by [2.7] and [2.8].⁷³

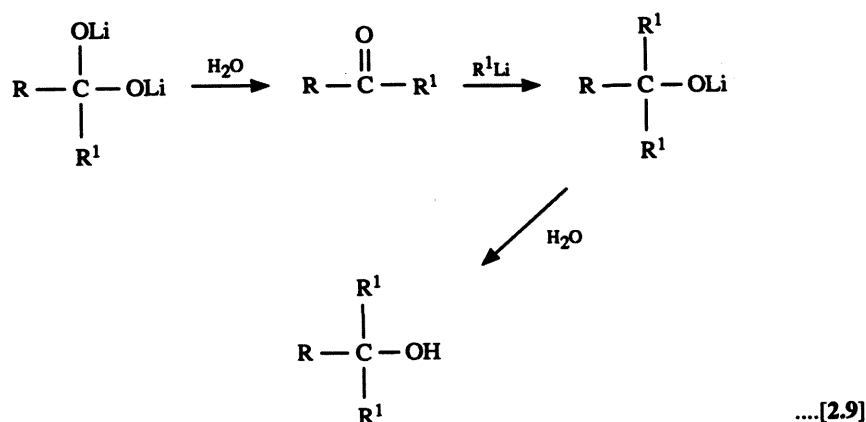


There are two ways in which the reaction shown in [2.5] can occur during the addition of the carboxylic acid and the organolithium reagent:

1. If the rate of mixing of the carboxylic acid and organolithium reagent is not very much faster than the reaction of the dilithium salt (32) with free carboxylic acid.
2. If the rate of reaction of the lithium salt of the carboxylic acid with the organolithium reagent is not significantly slower than the reaction of the carboxylic acid with the organolithium reagent.

In theory it is possible to prevent the reaction shown in [2.5] from occurring if free acid and (32) are not present together i.e. by adding the carboxylic acid to the organolithium reagent with very fast stirring. However, it is impossible to stop the reaction entirely due to the rate of mixing being crucial. Therefore, the reaction should be carried out by the slow addition of the organolithium reagent to the free acid with very efficient stirring. In this way the carboxylic acid is converted to the lithium carboxylate before any appreciable amount of (32) has been produced.

As shown in [2.6] tertiary alcohol can also be formed during the hydrolysis, particularly if a significant amount of unreacted organolithium reagent remains at the end of the reaction. Work by Levine *et al.* has shown that the amount of carbinol formed in the reaction can depend on the reaction time.⁷² By increasing the reflux time by increments, from 0.5h to 24h, for the reaction of equimolar amounts of lithium *n*-butyrate and phenyllithium they obtained increasing amounts of ketone and decreasing amounts of tertiary alcohol. A reflux time of 0.5h gave *n*-butyrophenone (39%) and diphenyl-*n*-propylcarbinol (30%) whereas after 24h only *n*-butyrophenone (85%) was obtained. They suggest that during a short reaction time tertiary alcohol results from a slow reaction between the lithium carboxylate and the organolithium reagent combined with a slow hydrolysis of the dilithium intermediate. The unreacted organolithium compound then reacts during the hydrolysis with ketone to give the carbinol [2.9].



Alcohol formation however can be minimised by taking special precautions in the work-up. By adding the reaction mixture slowly, dropwise and with rapid stirring to a large volume of the hydrolysing medium the organolithium reagent should be immediately hydrolysed before it can react with the free ketone.

2.2ii Application to the Synthesis of Chloramphenicol Analogues

As shown in the proposed route (Scheme 2.3) the amino acid D-serine was chosen as the carboxylic acid which was to be converted to a series of ketones using organolithium reagents.

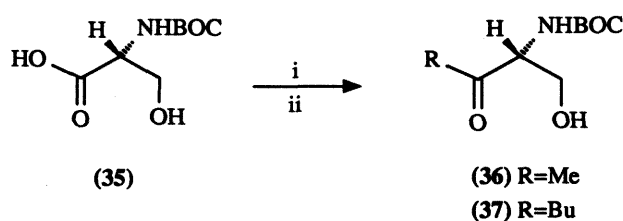
The presence of an amino group has been shown not to interfere in the conversion of a carboxylic acid to a methyl ketone.⁶⁹ However, since possible solubility difficulties had been anticipated, protection of the amino group of D-serine was considered to be advantageous. Protection with a bulky group would aid with solubility problems and it was decided to protect the amino group as the *tert*-butoxycarbonyl (BOC) amide. N-BOC-D-serine is commercially available and the BOC group can be readily removed by brief treatment with a strong acid such as trifluoroacetic acid.

It was initially thought that the hydroxyl group of D-serine would have to be protected, particularly in order to increase carboxylate solubility. However, it has been shown that the hydroxyl group generally does not interfere with the reaction if a sufficient excess of organolithium reagent is present to allow one equivalent of organolithium per hydroxyl group.⁷⁴ Protection of the hydroxyl group was therefore considered to be unnecessary, but tetrahydrofuran (THF) was chosen as the reaction solvent to overcome the possibility of the reaction being very slow due to the insolubility of the dilithium salt of the hydroxy carboxylic acid.^{74a,b,c} As well as increasing solubility THF is also a favourable solvent because it is miscible with the aqueous hydrolysing medium which aids in the work-up.

As shown in the previous section special precautions must be taken during the experimental procedure in order to obtain a good yield of the required ketone. The reactions were carried out by slow dropwise addition of the organolithium reagent to a rapidly stirred solution of N-BOC-D-serine in THF at -78°C. The reaction mixture was then stirred at 0°C for 1h and at room temperature for a further 3h. The hydrolysis step was carried out by slow, portionwise addition of the reaction mixture to a large volume of ice-water with fast stirring.

2.2iii Synthesis of the Methyl and Butyl Ketone from N-BOC-D-Serine

It was decided to initially make only the methyl and the butyl ketone. This was because it was not known if the proposed route would be a viable way of preparing a whole series of compounds, and also it was thought that the difference in size of the two alkyl groups might be consequential in this and/or subsequent reactions. Using the carefully chosen reaction conditions given in Section 2.2ii the conversion of N-BOC-D-serine to its corresponding methyl and butyl ketone was carried out as shown in Scheme 2.4.



Reagents: i) RLi (6-8 equiv.), THF, -78°C → R.T., 5h.
 ii) Water.

Scheme 2.4

The reaction was carried out under a variety of conditions, and the results are shown in Table 2.1. From this it can be seen that the crude yields were reasonable but despite the product being contaminated with only very minor impurities as shown by TLC, the overall yield after purification was disappointingly low. The main impurity was a highly non-polar compound which was thought to possibly have a polymeric nature.

When MeLi as the LiBr complex was used the reaction mixture was in solution throughout or, as in some cases, a small amount of fine precipitate formed during the addition of the organolithium reagent but this redissolved as the addition continued. However, when only MeLi was used (Entry 1) a large amount of solid formed during its addition and this did not dissolve throughout the entire reaction period. TLC indicated that after 5h no reaction had occurred and this is thought to have been due to the insolubility of the dilithium salt of the hydroxy carboxylic acid.

The results show that the use of 6 or 8 equivalents of organolithium reagent makes very little difference to the overall yield (Entries 5 and 8). However, in the former reaction a fine precipitate was observed which readily redissolved, but in the latter case a much larger amount of gelatinous solid formed approximately half-way through the addition of the BuLi which made stirring very difficult, but this did later dissolve.

Table 2.1 Experimental Results for the Reaction of N-BOC-D-Serine with Organolithium Reagents.

Entry	Quantity of N-BOC-D-serine	Organolithium Reagent	Number of Equivalents	Crude yield ^a (%)	Yield ^b after Purification (%)
1	0.5 g	1.4 M MeLi in Et ₂ O	6	No reaction	
2	0.5 g	1.5 M MeLi as LiBr complex in Et ₂ O	6	41	30
3	1.5 g	1.5 M MeLi as LiBr complex in Et ₂ O	6	59	40
4	2.5 g	1.5 M MeLi as LiBr complex in Et ₂ O	6	61	44
5	0.5 g	2.0 M BuLi in pentane	6	31	16
6	0.5 g	2.0 M BuLi in pentane	6	48 ^c	18
7	2.75 g	2.0 M BuLi in pentane	6	60	37
8	0.5 g	2.0 M BuLi in pentane	8	43	19
9	1.5 g	2.0 M BuLi in pentane	8	44	24

^a Based on weight of total organic extractable material - see text. ^b Purification was by flash column chromatography. ^c An alternative work-up procedure was used - see text.

Entry 6 shows the result of an alternative work-up procedure which was investigated by Rubottom as a means of minimising tertiary alcohol formation in the hydrolysis step.⁷⁵ Instead of quenching the reaction by addition to ice-water as carried out in all the other reactions, a large excess of chlorotrimethylsilane was added at 0°C to the reaction mixture followed by 1 M NaHCO₃ after the solution had warmed to room temperature. It should be noted that Rubottom used 1 M HCl rather than NaHCO₃ but it was thought that in this case the acidic conditions might cause the removal of the BOC group. After

standard extraction and purification the product was obtained in only 18% yield, thus showing the choice of work-up made no difference to the yield.

The factor which appeared to have the greatest influence on the overall yield was the scale of the reaction. It was found that the greater amount of N-BOC-D-serine used the higher the yield (Entries 2, 3 and 4, 5 and 7, 8 and 9). The data obtained for the methyl ketone (36) and the butyl ketone (37), including accurate mass, were consistent with their proposed structures. The ^1H NMR signals corresponding to the methyl CH_3CO -protons of (36) and the methylene $-\text{CH}_2\text{CO}-$ protons of (37) were at the expected chemical shifts, occurring at δ 2.25 ppm and δ 2.49-2.67 ppm respectively.

2.3 CONVERSION OF KETONES TO SECONDARY ALCOHOLS

Having prepared the methyl ketone (36) and the butyl ketone (37) the next stage was to reduce these compounds to their corresponding secondary alcohols. The reductions had to be carried out asymmetrically in order to achieve the required configuration at C-3, i.e. the *threo*-product (38) (see figure 2.2).

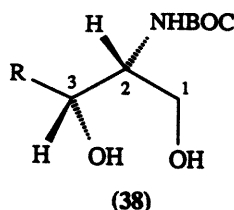
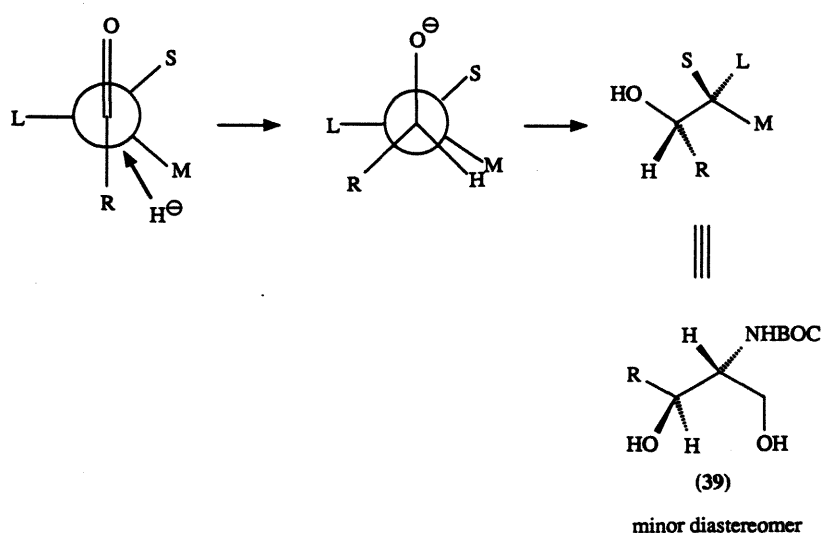
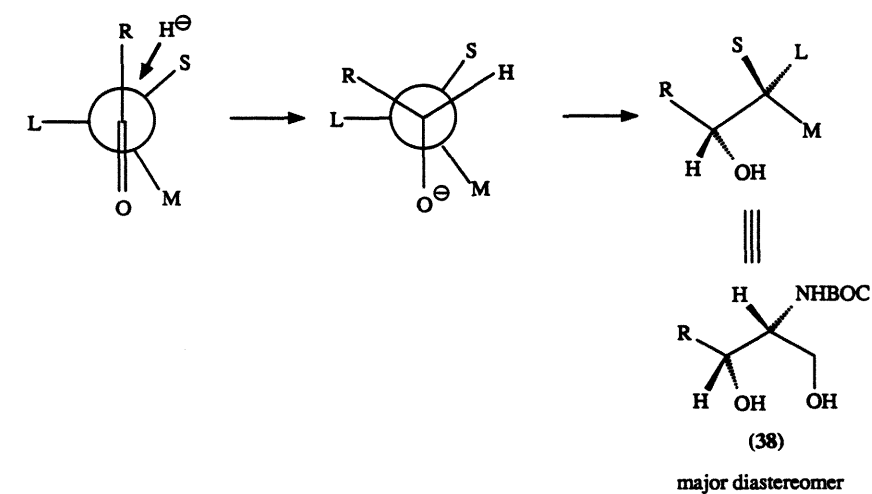


Figure 2.2

It was decided to initially use sodium borohydride to see what selectivity was achieved with a simple, nonchiral reducing agent. Since the keto group has an α -chiral centre its two faces are diastereotopic and so nucleophilic addition can lead to two possible diastereomers. However, it was difficult to predict which diastereomer would be the major product because there are several factors to consider.

If only steric interactions were important in affecting the transition state then applying Cram's rule and using Felkin-Ahn conformations⁷⁶ the predicted major product diastereomer would have the desired configuration. Work by Felkin and Ahn has shown that when the α -carbon has a small (S), medium (M), and large (L) group then the most reactive conformation, i.e. that which leads to the major product, is where the groups are staggered so that the carbonyl oxygen is between the large and medium groups. The minor product is formed when the carbonyl group lies between the large and small groups. In both conformations nucleophilic attack occurs *anti* to the large group as shown overleaf.

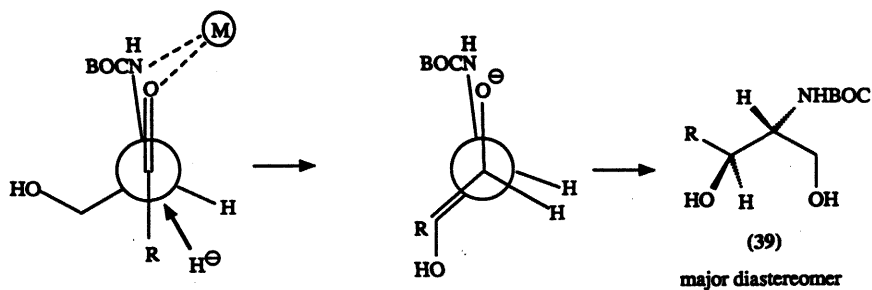
Felkin-Ahn Conformations



It can be seen that in these conformations the most important steric interactions are determined by R, and so it can be predicted that the larger the R group the greater the preference for the most reactive conformation and so the higher the selectivity.

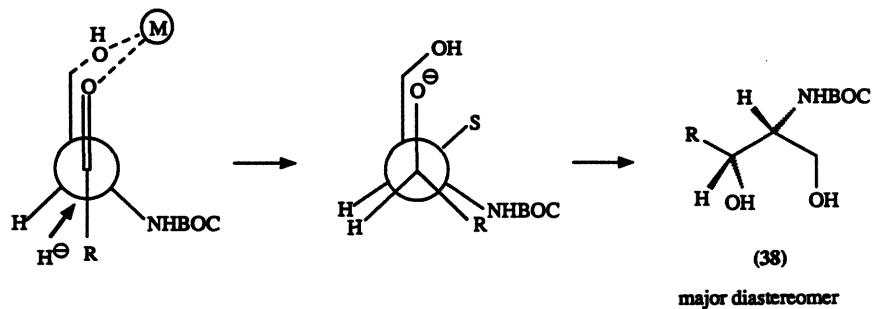
There is also the possibility of chelation control.⁷⁷ If the nitrogen lone pair coordinates to the metal then the carbonyl group and the nitrogen would be held *syn*-periplanar by a 5-membered chelate ring. In this relatively rigid conformation preferential attack occurs from the less hindered side to give the major diastereomer which in this case would be the unrequired *erythro*-product (39) as shown below.

α -Chelation control with the Urethane Nitrogen



However, if the reaction is controlled by β -chelation with the hydroxyl oxygen and the carbonyl oxygen coordinating to the metal, then the major diastereomer would be the required product (38) as shown below.

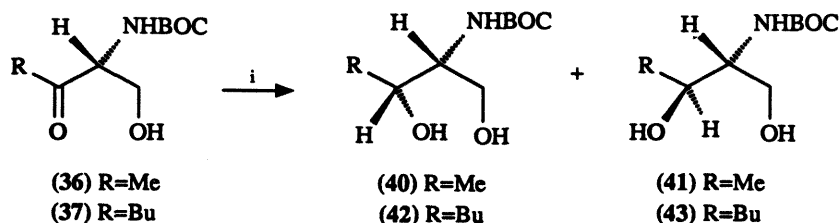
β -Chelation with the Hydroxyl Oxygen



As can be seen there are several possible ways in which the asymmetric reaction may be controlled. Because the various models discussed above lead to prediction of different diastereomers as the major product it was decided that the reduction should be studied experimentally.

2.3i Synthesis of the Methyl and Butyl Alcohol.

Reduction of the methyl and butyl ketone (36) and (37) was carried out with sodium borohydride in ethanol at room temperature as shown in Scheme 2.5.



Reagents: i) NaBH₄ (5 equiv.), EtOH, RT., 2-3h.

Scheme 2.5

After work-up and purification by flash column chromatography the corresponding diols were obtained in good yield (65% combined yield for (40) and (41), and 71% for (42) and (43)). Satisfactory data, including accurate masses, were obtained for the products.

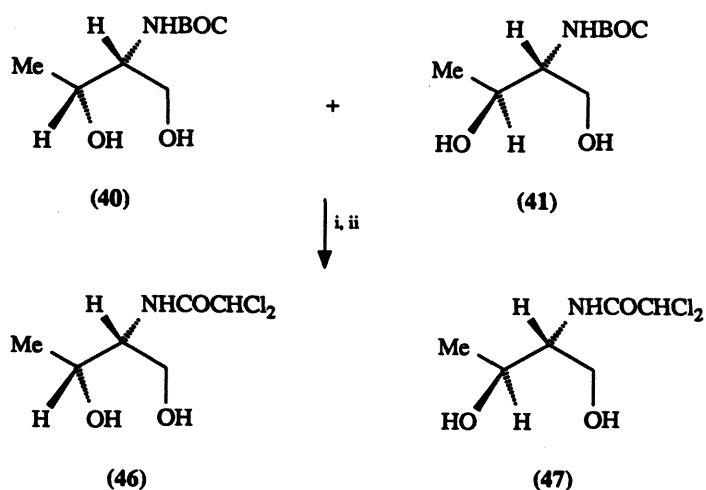
The ¹H NMR spectrum of the mixture of diastereomers (40) and (41) showed the two sets of doublets at δ 1.19 ppm and δ 1.24 ppm corresponding to the methyl protons of CH₃CH(OH) to be in a ratio of 1:2.1 respectively as calculated from the peak heights (a ratio of 1:2.3 was obtained from the integration). In the case of the ¹H NMR spectrum of the diastereomers (42) and (43) the doublets at δ 5.40 ppm and δ 5.50 ppm corresponding to the NH signal were examined and found to be in a ratio of 1:3.4 respectively as calculated from peak heights (and 1:3.0 from the integration). It should be noted that at this stage it was not possible to assign the relative configuration of the major product.

Despite the fact that the products were purified by flash column chromatography the diastereomers were not separated as shown by TLC, and so the calculated ratios were considered to be an indication of the stereoselectivity of the reduction, and therefore

show that the reductions had occurred with low diastereoselectivity. However, since greater selectivity was achieved with the butyl compound this suggests that steric interactions were important and so the reactions had possibly occurred mainly under nonchelation control.

2.4 SYNTHESIS OF 2-N-DICHLOROACETAMIDO-BUTAN-1,3-DIOL

The mixture of N-BOC diols (40) and (41) were deprotected by reaction with trifluoroacetic acid which caused the reaction mixture to become extremely dark. Extraction of the product was difficult due to its highly polar nature and (2R, 3R)-2-aminobutan-1,3-diol (44) and (2R, 3S)-2-aminobutan-1,3-diol (45) were afforded in a low combined crude yield (53%). This material was immediately reacted with methyl dichloroacetate, the reaction mixture being heated at reflux in dry ethanol (see Scheme 2.6). The product was obtained by removing remaining starting material with an acid wash. No further purification was carried out due to the small scale of this second step (<70 mg).



Reagents : i) $\text{CF}_3\text{CO}_2\text{H}$ (10 equiv.), R.T., 1h.
 ii) $\text{Cl}_2\text{CHCO}_2\text{Me}$ (2 equiv.), EtOH, Δ , 5h.

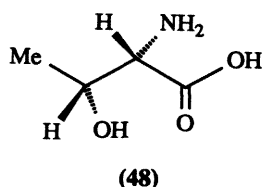
Scheme 2.6

The ^1H NMR spectrum of (46) and (47) was difficult to interpret due to the mixture of products. However, the two singlets at δ 6.33 ppm and δ 6.37 ppm corresponding to the $-\text{CHCl}_2$ proton were seen to be in a ratio of 1.8:1 respectively as calculated from the

peak heights (and 1.7:1 from the integration). Determination of which diastereomer, (46) or (47), was the major product is discussed in the remainder of this chapter.

2.5 ALTERNATIVE SYNTHESIS OF 2-N-DICHLOROACETAMIDO-BUTAN-1,3-DIOL

The naturally occurring amino acid L-threonine (48) has the required absolute configuration at both C-2 and C-3 (see Figure 2.3).



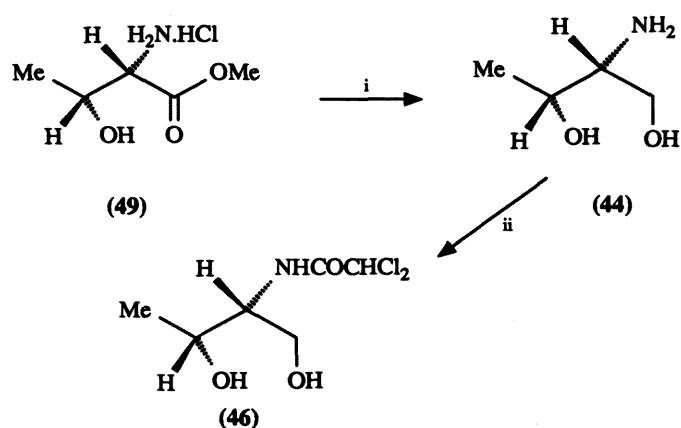
2S, 3R L-(-)-Threonine

Figure 2.3

By preparing the methyl analogue of chloramphenicol (20, R=Me) from L-threonine a compound will have been synthesised whose absolute configuration at both chiral centres is known. It can then be used to assign the absolute configuration of the methyl analogues arising from the route discussed above. By comparing this compound with the mixture of diastereomers (46) and (47) previously prepared via the methyl ketone (36) the major diastereomer formed in the sodium borohydride reduction of the ketone to the alcohol can be directly assigned.

2.5i Synthesis of N-Dichloroacetamido-L-Threoninol

Since L-threonine methyl ester hydrochloride (49) is commercially available this was the starting material used rather than L-threonine. N-Dichloroacetamido-L-threoninol (46), (2R, 3R)-2-N-dichloroacetamido-butan-1,3-diol, was prepared in two steps as shown overleaf in Scheme 2.7.



Reagents: i) NaBH₄ (7 equiv.), water, <4°C, 20h.
 ii) Cl₂CHCO₂Me (2 equiv.), EtOH, Δ, 7h.

Scheme 2.7

It is generally considered that sodium borohydride does not reduce esters. However, there are reports in the literature that in certain cases, especially in the field of sugar chemistry, reduction of the ester group to the corresponding primary alcohol has been observed.⁷⁸ Work by Seki *et al.* has shown that the reduction of α-amino acid esters (including D-threonine ethyl ester) with sodium borohydride occurs without racemisation to give optically active α-amino alcohols, and that the optimum reaction conditions were the use of 4 moles or more of sodium borohydride in either water at 0°C or 50% aqueous ethanol heated under reflux.⁷⁹

L-threonine methyl ester hydrochloride (49) was reduced with sodium borohydride in water, the reaction mixture being stirred at <2°C for 2h and then left to stand in a fridge overnight. The work-up described by Seki for the preparation of D-threoninol involved a continuous extraction with ether for over 100h and the obtained yield was only 47% showing the difficulty of extracting such a highly water soluble compound into organic solvent. Due to the impracticality of such an extraction an alternative work-up was used following a method developed by J. Williams (Leicester University).⁸⁰ This consisted of several steps:

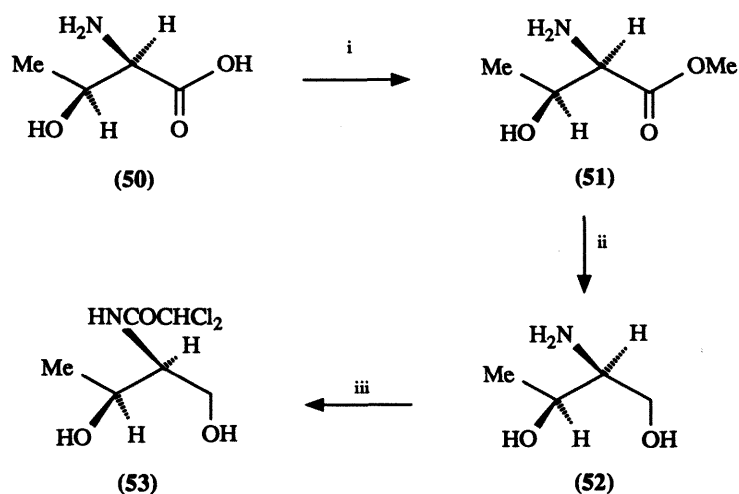
1. Formation of L-threoninol oxalate and its extraction into boiling ethanol followed by evaporation to give a solid residue.
2. Basification with aqueous barium hydroxide, filtration and evaporation to give a residue containing free amino alcohol, barium oxalate and barium hydroxide.
3. Addition of hot ethanol followed by filtration to remove barium oxalate and evaporation of the supernatant to give a residue.
4. Addition of hot acetone, filtration to remove barium hydroxide and evaporation of the final filtrate to give L-threoninol.

Although this procedure was somewhat involved L-threoninol (44) was afforded in 72% yield after final purification by Kugelrohr distillation. Due to the instability of the free base it was reacted immediately with methyl dichloroacetate to give, after purification by flash column chromatography, N-dichloroacetamido-L-threoninol (46) in 54% yield.

The data obtained for (46), including C, H and N microanalysis and an accurate mass, were consistent with its proposed structure. The low resolution mass spectrum showed the typical pattern of molecular ions containing two chlorine atoms. When recorded in D₂O both the ¹³C and ¹H NMR spectra gave very sharp signals, allowing in the latter case determination all the coupling constants, including the J_{AX}, J_{BX} and J_{gem} constants corresponding to the CH_XCH_AH_B(OH) protons. It was noted however, that when recorded in CDCl₃ the ¹³C NMR spectrum showed either two sets of signals or broad peaks. Similarly in the ¹H NMR spectrum the signals were mainly broad, but when the probe temperature was raised to 40°C and then to 50°C there was a sharpening of peaks. This phenomenon is believed to be due to the restricted rotation about the amide bond due to a resonance interaction, or to partial N-H/D exchange.

2.5ii Synthesis of N-Dichloroacetamido-D-Threoninol.

In order to examine the origin and energetics of enantiodiscrimination shown by CAT the enantiomer of (46) was also synthesised. N-Dichloroacetamido-D-threoninol (53), (2S, 3S)-2-N-dichloroacetamido-butan-1,3-diol, was prepared in three steps starting from D-threonine (50), (see Scheme 2.8).



Reagents: i) SOCl₂ 1.1 equiv.), MeOH (8 equiv.), 50°C, 4.5h.
ii) NaBH₄ (4 equiv.), water, <4°C, 20h.
iii) Cl₂CHCO₂Me (1.5 equiv.), EtOH, Δ, 7h.

Scheme 2.8

D-Threonine was reacted with thionyl chloride in dry methanol using an adaptation of a literature method.⁸¹ The resulting methyl ester was initially recovered as the hydrochloride but this material was found to be extremely hygroscopic making handling very difficult. However, conversion to the free base gave D-threonine methyl ester (51) as a non-hygroscopic solid in 47% yield.

Conversion of (51) to (53) was carried out in the same way as its corresponding enantiomer as detailed in the previous section. The reduction to give D-threoninol (52)

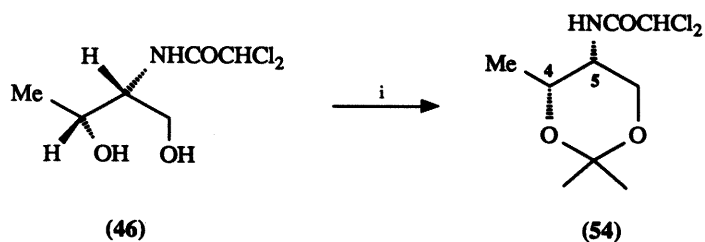
and its subsequent reaction with methyl dichloroacetate afforded N-dichloroacetamido-D-threoninol (**53**) in good yield (63% and 72% respectively for the two steps). The data obtained for (**53**) were identical to that for (**46**) consistent with it being the enantiomer. The ^1H NMR signal for H-C(3) was in this case confirmed by irradiation of the Me-C(3) signal which caused the former signal, a doublet of quartets, to collapse to a doublet.

The results of the enantiospecificity of CAT towards N-dichloroacetamido-L-threoninol (**46**) and dichloroacetamido-D-threoninol (**53**) are discussed in Chapter 5.

2.5iii Verification of Relative Configuration and Optical Purity.

Since N-dichloroacetamido-L-threoninol (**46**) derived from L-threonine was to be used as a standard it was imperative that its relative configuration and enantiomeric purity was confirmed. In order to verify its relative configuration and show that the sodium borohydride reduction of the methyl ester to its corresponding primary alcohol had occurred without epimerisation the acetonide derivative of (**46**) and the Mosher esters⁸² of (**46**) and (**53**) were prepared.

The acetonide (**54**) was afforded in high yield (86%) from the reaction of (**46**) with 2,2-dimethoxypropane (DMP) and pyridinium *p*-toluenesulphonate (PPTS) as shown in Scheme 2.9.



Reagents: i) DMP (20 equiv.), PPTS (1 equiv.), CH₂Cl₂, R.T., 20h.

Scheme 2.9

^1H NMR analysis of (54) showed that the crucial coupling constant between H-C(4) and H-C(5) was 1.6 Hz. The signal for H-C(4) was confirmed by irradiation of the Me-C(4) signal which caused the former signal, a doublet of quartets, to collapse to a doublet. For six-membered rings in the chair conformation $J_{\text{axial, equatorial}}$ is typically below 5 Hz and $J_{\text{axial, axial}}$ above 10 Hz.⁸³ This vicinal coupling constant value of 1.6 Hz is therefore consistent with an axial-equatorial relationship of the protons H-C(4) and H-C(5) of (54) in the chair conformation (Figure 2.4) and so confirms the *threo* stereochemistry. The structure of (54) was confirmed by its mass spectrum and accurate mass.

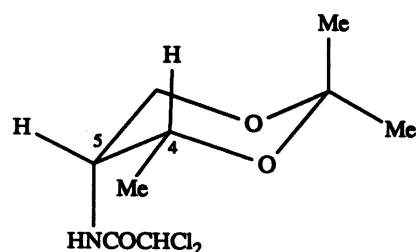
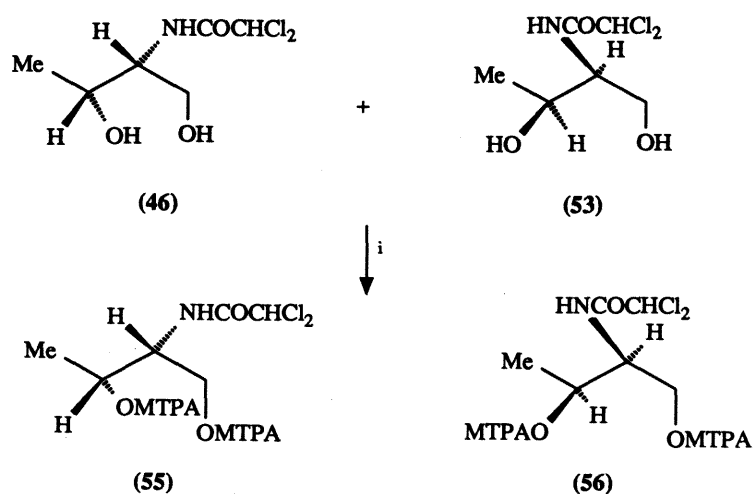


Figure 2.4

The Mosher ester of a mixture of equal quantities of (46) and (53) was prepared using (R)- α -methoxy- α -trifluoromethyl-phenylacetyl chloride (MTPA-Cl) as shown overleaf in Scheme 2.10.



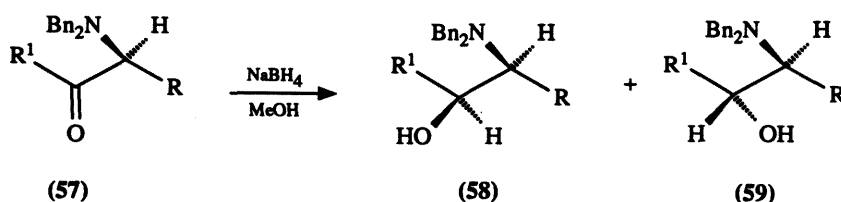
Reagents: i) MTPA-Cl (6 equiv.), pyridine, CCl₄, R.T., 20h.

Scheme 2.10

¹⁹F NMR of (55) and (56) showed four signals at -71.40 ppm, -71.46 ppm, -71.78 ppm and -71.82 ppm all of equal intensity. The Mosher ester of (53) only was also prepared and its ¹⁹F NMR showed two signals at -71.40 ppm and -71.82 ppm in a ratio of 1:1. The measured optical rotation of (46) was +8.5° and -8.4° for (53).

These results therefore confirm that both enantiomers, (46) and (53), were prepared without any detectable racemisation and that, as expected, the relative configuration of N-dichloroacetamido-L-threoninol (46) is consistent with the *threo*-structure.

A first systematic study of a non-racemising synthesis and stereoselective reduction of chiral α -amino ketones has recently been reported in the literature.⁸⁴ It was shown that reduction of the *N,N*-dibenzyl-protected α -amino ketone (57) with sodium borohydride in methanol occurred under nonchelation control to give (58) and (59) in a ratio of >9:1 (Scheme 2.11).



Scheme 2.11

Additional functionality in the *R*-substituent was found not to effect the stereoselectivity, high *threo*-selectivity being achieved in the reduction of the *L*-serine derived ketone (57), $\text{R}=\text{CH}_2\text{OBn}$.

Therefore, if *O*-benzyl-*D*-serine was protected at the nitrogen in a similar way, i.e. as the *N,N*-dibenzyl derivative rather than the *NHBOC* derivative, then the proposed route may give the required analogues (20) as the major products. Alternatively, different reducing agents could be examined for the reduction of the ketone. This particular route however was not investigated any further, in part, because of the comparatively low yields achieved in the synthesis of the intermediate ketone. The results suggest that the reduction of the ketone had occurred under chelation control, via α -chelation with the urethane nitrogen (as shown in Section 2.3) and this led to the decision to pursue a different approach which is discussed in the following chapter.

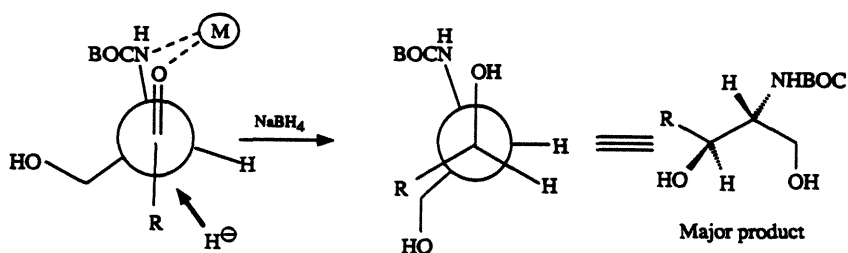
CHAPTER 3

ASYMMETRIC SYNTHESIS OF CHLORAMPHENICOL DERIVATIVES VIA A PROTECTED α -AMINO ALDEHYDE

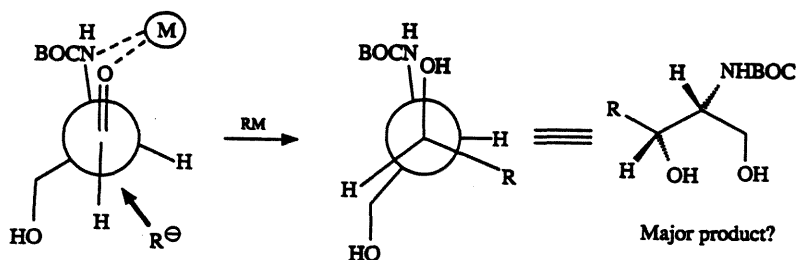
3.1 INTRODUCTION

As discussed in Chapter 2, conversion of the alcohol to the ketone gave mainly the unrequired *erythro*-product. Consideration of these results predicted that if the same model applied, i.e. α -chelation control, reaction of the corresponding aldehyde with an appropriate organometallic reagent should lead directly to the desired *threo*-1,3-diol as the major diastereomer (see below). This chapter discusses an alternative route to the required chloramphenicol analogues (20) via a suitably protected aldehyde.

Ketone to Alcohol Conversion



Aldehyde to Alcohol Conversion

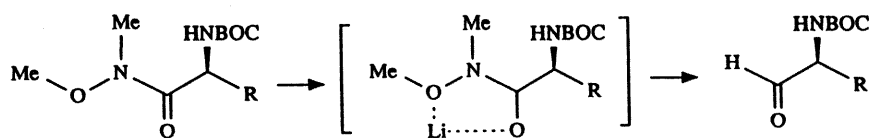


Aldehydes are an important class of compounds due to their versatility in organic synthesis, and they are especially useful if they are chiral and enantiopure. Due to the availability of both L- and D-amino acids, as discussed in Section 2.1, α -amino acids are a valuable source of chiral substrates. When the amino functionality is suitably protected they can be transformed into the corresponding α -amino aldehydes without

racemisation. Due to the increasing interest in these aldehydes and their potential applications, the preparation and reactions of optically active α -amino aldehydes have recently been reviewed by Jurczak⁸⁵ and Reetz.⁸⁶

3.1i Preparation of N-Protected α -Amino Aldehydes

α -Amino aldehydes are mainly obtained from α -amino acids. The general method of preparation is via esters or active amides of α -amino acids which are then reduced to the corresponding aldehydes. A common reducing agent for the reduction of N-BOC- or N-Cbz-protected methyl or ethyl esters is diisobutylaluminium hydride (DIBAL).⁸⁷ Reported yields of the required aldehyde are varied but generally high. A disadvantage of the reaction is that it often affords some corresponding amino alcohol produced by further reduction of the required aldehyde. This also applies to the reduction of imidazolides with lithium aluminium hydride (LiAlH_4).⁸⁸ Nonetheless, there are examples in the literature which indicate that the reaction can occur without overreduction, such as the reduction of N-BOC-protected 3,5-dimethylpyrazolides with LiAlH_4 .⁸⁹ Fehrentz and Castro have reported an efficient method which occurs without racemisation or overreduction.⁹⁰ The reduction of N-BOC-protected N-methoxy-N-methyl amides with LiAlH_4 proceeds via a stable lithium-chelated intermediate which precludes further reduction due to intramolecular complexation, and so upon hydrolysis only aldehyde is obtained (Scheme 3.1).⁹⁰



Scheme 3.1

Lubell and Rapoport achieved similar results for the reduction of N-[9-(9-phenylfluorenyl)]-L-alanine isoxazolidide.⁹¹

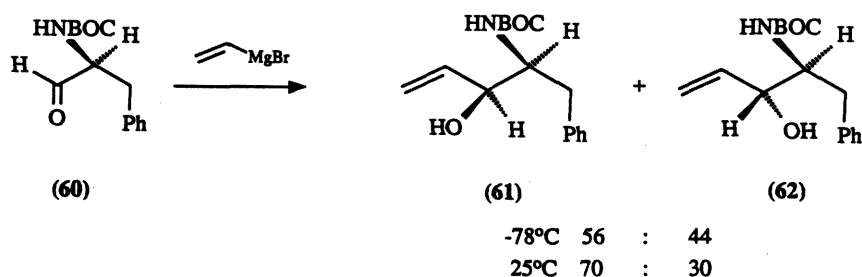
An alternative approach is the oxidation of N-protected α -amino alcohols which are generally obtained from the reduction of N-protected α -amino acids with borane-THF⁹² or sodium borohydride-lithium chloride,⁹³ or from the reduction of the corresponding methyl ester with sodium borohydride-calcium chloride.⁹⁴ Evans and co-workers have shown that the reduction of N-BOC-L-leucine with borane-THF followed by oxidation with chromium trioxide-pyridine (Collin's reagent) occurred with complete retention of chiral integrity.⁹⁵ Oxidation with a variety of activated dimethyl sulphoxide reagents is also free from racemisation.^{94,96} An alternative method suitable for α -amino alcohols with nonpolar side chains is the enzymic preparation of nonprotected α -amino aldehyde semicarbazones using horse liver alcohol dehydrogenase followed by hydrolysis to regenerate the aldehyde.⁹⁷

3.1ii Reactions of N-Protected α -Amino Aldehydes

The three main types of reactions which use the carbonyl group of N-protected α -amino aldehydes to form a new chiral centre are aldol additions, Diels-Alder reactions, and organometallic additions. It is of great importance to find highly stereoselective methods of carrying out C-C bond formations, and with the above reactions this has been achieved with varying degrees of success. Aldol addition reactions with N-protected α -amino aldehydes are generally characterised by fairly low diastereoselectivity^{87a,91,95,98} and can occur via either chelation⁹⁹ or nonchelation¹⁰⁰ control. However, Diels-Alder reactions of N-BOC-protected α -amino aldehydes with activated 1,3-dienes has been shown to occur with high diastereoselectivity in favour of the chelation-controlled product¹⁰¹ with the stereoselectivity being reversed to give almost exclusively the nonchelation-controlled product with N,N-dibenzyl protected aldehydes.^{86,102} The reaction of organometallic reagents with N-protected α -amino aldehydes is of great interest in the context of carrying out C-C bond formations stereoselectively, and it is especially important to this work. The examples outlined

below show that a number of factors can influence the selectivity, including temperature and choice of protecting groups.

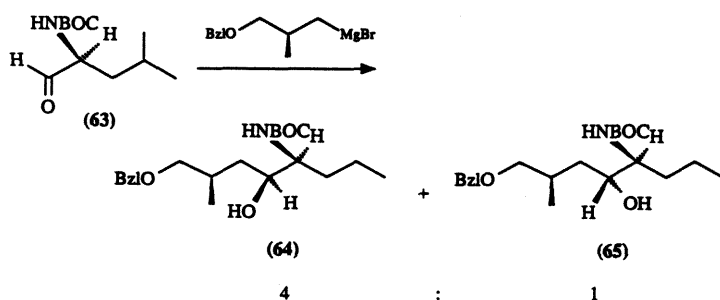
In order to prepare novel dipeptide analogues Hanson and Lindberg carried out the addition of vinylmagnesium bromide to N-BOC-L-phenylalaninal (**60**) as shown in Scheme 3.2.¹⁰³



Scheme 3.2

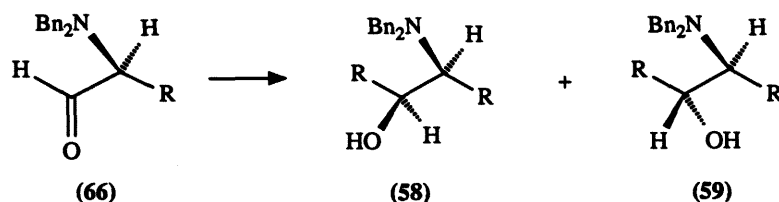
When the addition was carried out at -78°C the chelation-controlled *threo*-product was slightly favoured, (**61**) and (**62**) being afforded in a ratio of 56:44. It was found, however, that the selectivity was improved by carrying out the addition at 25°C, which gave a 70:30 ratio of (**61**) to (**62**).

The reaction of N-BOC-L-leucinal (**63**) with a chiral Grignard reagent occurred with higher diastereoselectivity (Scheme 3.3), the *threo*-product (**64**) being the major diastereomer. The two products (**64**) and (**65**) were formed in a ratio of 4:1.¹⁰⁴



Scheme 3.3

In contrast, Reetz has found that the reaction of N,N-dibenzylamino aldehydes (**66**) with Grignard and alkyl lithium reagents (Scheme 3.4) occurs with nonchelation control i.e. the *erythro*-product (**59**) is the favoured diastereomer, and the reaction is generally highly diastereoselective (>90%).^{100a}

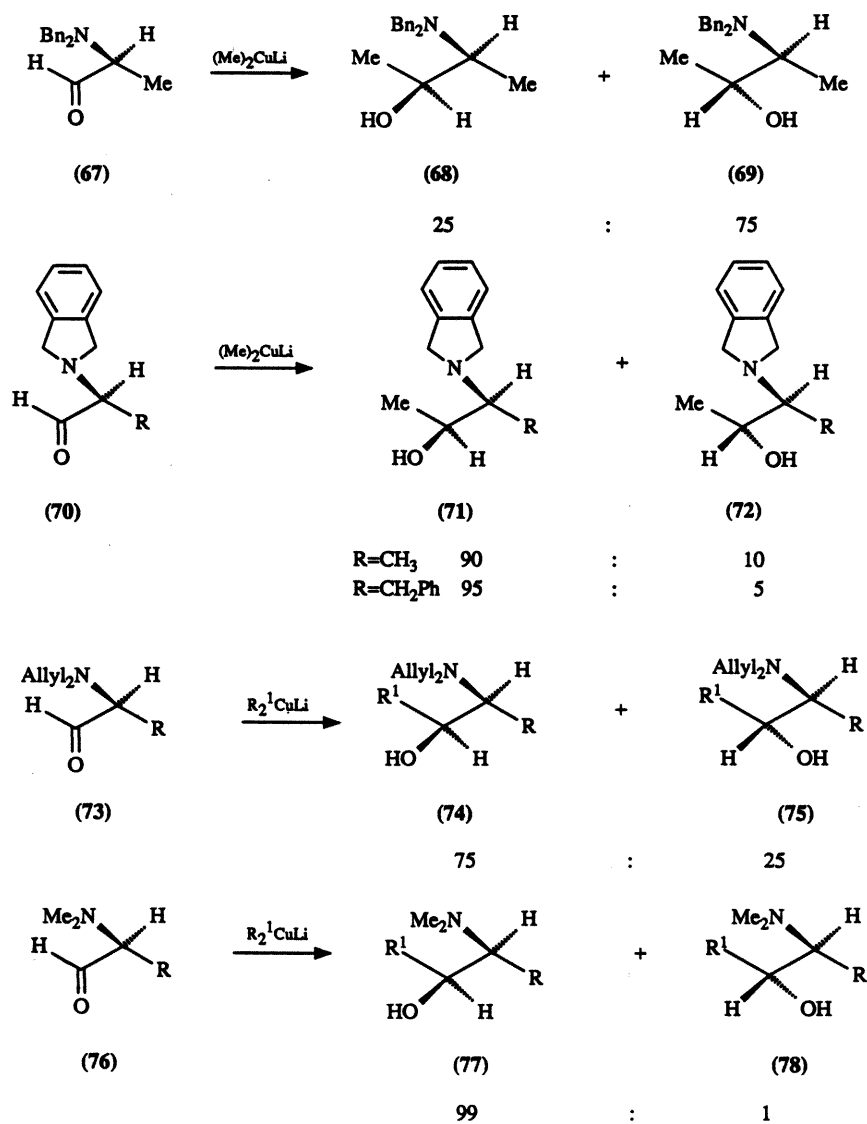


Scheme 3.4

It was only on addition of the very reactive allylmagnesium chloride (C_3H_5MgCl) that the degree of selectivity was reduced, (**58**) and (**59**) being formed in a ratio of 28:72 respectively. It was shown that the ratio could be improved by prior titration of the Grignard reagent with $ClTi(NEt_2)_3$, reaction of $C_3H_5Ti(NEt_2)_3$ with (**66**) gave (**58**) and (**59**) in a ratio of 7:93. Cuprates, however, were shown to reduce the selectivity. Reversal of selectivity was seen with Lewis acidic or chelating agents of the type CH_3TiCl_3 or allylSiMe₃/SnCl₄. The results show that with $RMgX$, RLi and R_2CuLi no chelation effects were observed, chelation only being possible when strong Lewis acidic reagents were used. It was thought that formation of the 5-membered chelate ring must be prevented on steric grounds.

To investigate the above proposal the Grignard-like addition of cuprates to aldehydes with less sterically demanding protecting groups was examined (Scheme 3.5).^{86,100a} The results show the effectiveness of protective-group "tuning". By changing from benzyl protecting groups to the smaller groups present in (**70**), (**73**) and (**76**) a complete reversal of diastereoselectivity was achieved. It should be noted that it is only the protecting group that effects the selectivity since the reaction of (**70**) and (**73**) with

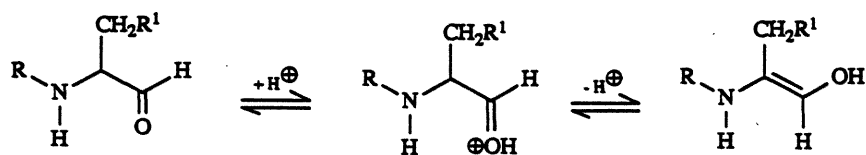
alkyllithium reagents shows no selectivity as does the reaction of (76) with Grignard reagents.



Scheme 3.5

3.1iii Aldehyde Stability

N-Protected α -amino aldehydes are relatively unstable both configurationally and chemically, particularly in solution. Ito *et al.* investigated the optical stability of various N-Cbz- α -amino aldehydes during chromatography on silica gel.^{87b} They found that the order of the degree of racemisation was N-Cbz-S-Bzl-L-cysteinyl \gg N-Cbz-L-phenylalanyl $>$ N-Cbz-L-leucinal \gg N-Cbz-N^G-nitro-L-argininal, and that the longer the contact time with the silica the much greater the extent of racemisation. The mechanism they proposed to account for the racemisation involves acid-catalysed enolisation to give the enol form of the amino aldehyde as shown in Scheme 3.6.



Scheme 3.6

Aldehydes with a R^1 group at the β -position which is enol-stabilising can racemise very quickly during exposure to silica gel. This was shown with the cysteine derivative ($\text{R}^1=\text{SH}$) which after 6h exposure underwent 99% racemisation.^{87b} Since the only structural difference between cysteine and serine is that the former has a thiol group and in the latter this is replaced with an hydroxyl group, it was anticipated that similar racemisation problems could occur with a protected serinal derivative.

As shown, the optical lability of α -amino aldehydes depends on their structure. Work by Evans *et al.* has illustrated that even crude N-BOC-L-leucinal has some configurational lability.⁹⁵ It retained its chirality reasonably well in the cold ($\geq 5\%$ racemisation after 9 days at -30°C) but after 9 days at room temperature it racemised relatively rapidly (62% racemisation), its optical rotation going from $+18.2^\circ$ to $+6.9^\circ$, thus showing the need to use the aldehyde as soon as possible after its preparation.

Garner and Park, however, have described the synthesis of N,O-diprotected L-serinal (79) and L-threoninal (80), (Figure 3.1), and they report that they have unambiguously demonstrated that these aldehydes remain configurationally stable during purification by either flash column chromatography or vacuum distillation.¹⁰⁵

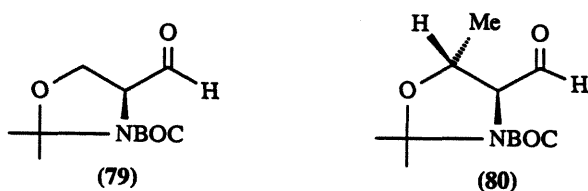
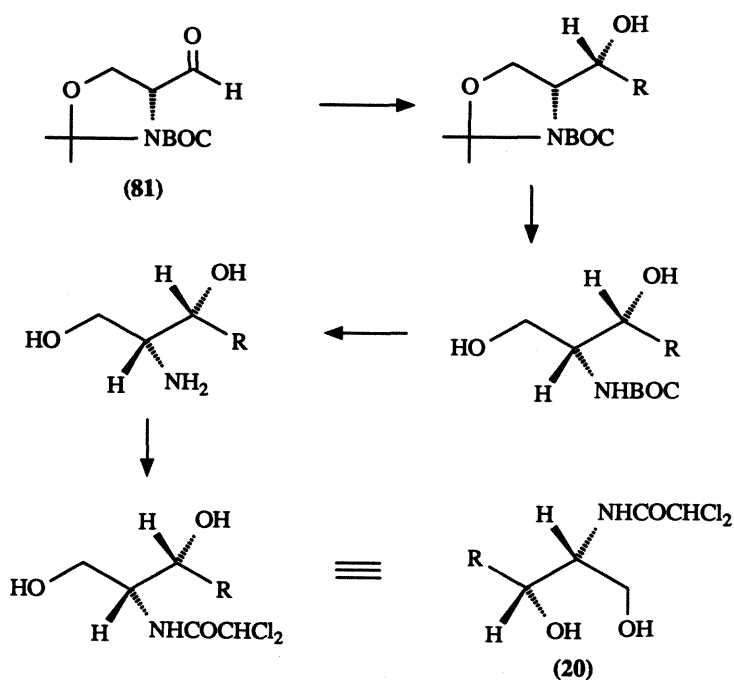


Figure 3.1

N,O-Diprotected D-serinal (81), the enantiomer of (79), was thought to be a suitably protected form of serinal ideal for the asymmetric synthesis of the required chloramphenicol analogues (20).



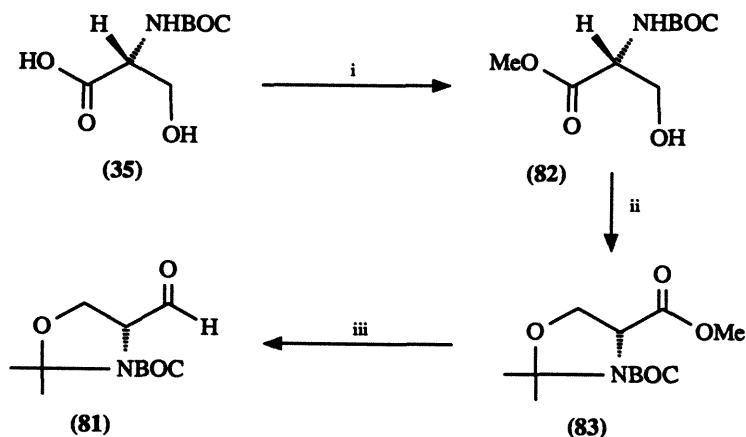
Scheme 3.7

Scheme 3.7 shows a proposed route for the required analogues, the crucial step in which is the conversion of the formyl group to the hydroxy alkyl group. In order for this to be a viable route to (20) it was necessary to be able to carry out this reaction highly stereoselectively to preferentially afford the required *threo*-product, and also to be able to systematically vary R. The aldehyde (81), referred to in the literature as the Garner aldehyde, was prepared and its chemistry investigated.

RESULTS AND DISCUSSION

3.2 SYNTHESIS OF THE GARNER ALDEHYDE

The Garner aldehyde (**81**) was prepared from N-BOC-D-serine via the reduction of an oxazolidine ester (**83**) with DIBAL. The synthesis involved three steps and the procedure used was a modified version of that reported by Garner and Park (Scheme 3.8).¹⁰⁵

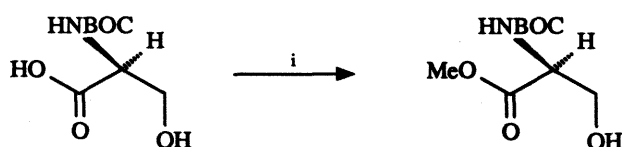


Reagents: i) CH_2N_2 (1.5 equiv.), Et_2O , 0°C , 0.5h.
ii) DMP (2 equiv.), $\text{BF}_3 \cdot \text{OEt}_2$ (0.1 equiv.), acetone, R.T. 48h.
iii) DIBAL (2 equiv.), dry toluene, -78°C , 2.5h.

Scheme 3.8

The first step was protection of the acid by forming the methyl ester (**82**) by reaction with diazomethane. The scale of the reaction was limited by the size of the Diazald apparatus and consequently a maximum of 10 g of the protected amino acid could be processed at any one time. The reaction was repeated several times and always gave quantitative yields, further purification by chromatography or distillation was not necessary. A recent report in the literature, published after the research had been carried

out for this thesis, describes an alternative procedure for the preparation of the methyl ester due to the limitations of using diazomethane. This method makes use of the Mitsunobu reaction (see Scheme 3.9) and is reported to proceed in almost 100% yield on a large scale (up to 100 g).¹⁰⁶



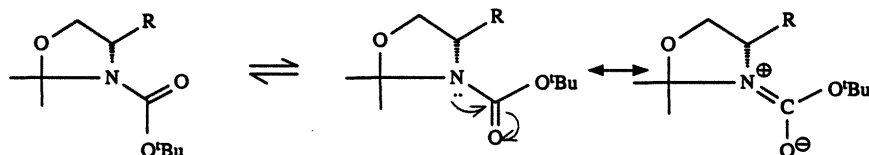
Reagents: i) $\text{P}(\text{Ph})_3$ (1.1 equiv.), dry MeOH (1.1 equiv.), DEAD (1.1 equiv.), dry Et_2O , $< 45^\circ\text{C}$, 1h.

Scheme 3.9

Protection of the O-H and N-H functionalities was achieved by forming a 2,2-dimethyloxazolidine ring. The Garner method involved the slow distillation of a solution of the methyl ester, 2,2-dimethoxypropane (DMP) and *p*-toluenesulphonic acid as the catalyst in benzene,¹⁰⁵ but it has been reported that boron trifluoride etherate is a preferable catalyst giving higher yields and simpler reaction conditions.¹⁰⁷ For these reasons the ester (**82**) was reacted with DMP and a catalytic amount (10%) of $\text{BF}_3 \cdot \text{OEt}_2$ in acetone giving the oxazolidine (**83**) in 81-86% yield after purification by flash column chromatography. The reaction was found to be fairly slow, it was generally left at room temperature for 2 days. When more $\text{BF}_3 \cdot \text{OEt}_2$ was added the reaction was quicker but a certain amount of deprotection was also observed giving D-serine methyl ester as a side product.

The aldehyde (**81**) was prepared by reduction of the methyl ester with DIBAL. By carefully keeping the reaction mixture below -68°C throughout the reaction and also during the quenching with cold methanol, the aldehyde was produced in 70-80% yield after purification by flash column chromatography. It was thought that the Garner aldehyde would not racemise on silica because enolisation would create an unfavourable interaction with the rigid N-BOC moiety.

The data obtained for compounds (82), (83) and (81) were consistent with their proposed structures and the literature data.¹⁰⁵ The important features of the IR spectra include i) the broad signal at 3420 cm^{-1} for (82) corresponding to the O–H and N–H groups which is absent for (83 and (81) and ii) the carbonyl stretching frequencies, which occur at 1750 cm^{-1} and 1710 cm^{-1} for (82) corresponding respectively to the ester and urethane groups, at 1760 cm^{-1} and 1710 cm^{-1} for (83) and at 1740 cm^{-1} and 1710 cm^{-1} for (81). The accurate mass of each compound was correct and in all three cases was obtained as the $[\text{MH}]^+$ molecular ion. An important point must be made about the NMR spectra. The ^1H and ^{13}C NMR data for (83) and (81) given in Chapter 6 show two sets of signals when the spectra were recorded at room temperature. Garner also found this but when he raised the probe temperature to 75°C for recording the ^1H NMR spectrum of (83) and to 60°C for (81) the signals merged into one set.¹⁰⁵ On raising the probe temperature to 60°C for recording the spectra of (83) we found that each pair of the ^{13}C signals became one signal with the exception of the two isopropylidene methyl carbons, but in the case of the ^1H NMR the signals were found to broaden, indicating that the probe temperature was not high enough for the coalescence point to be reached. These spectral results are a general phenomenon of the oxazolidene system and are believed to be due to the restricted rotation about the amide bond due to resonance interaction in the carbamate (see below).



The oxazolidene derivatives exist as slowly interconverting rotomers on the NMR time scale and samples therefore require heating to obtain averaged spectra.

3.2i Stability of the Garner Aldehyde

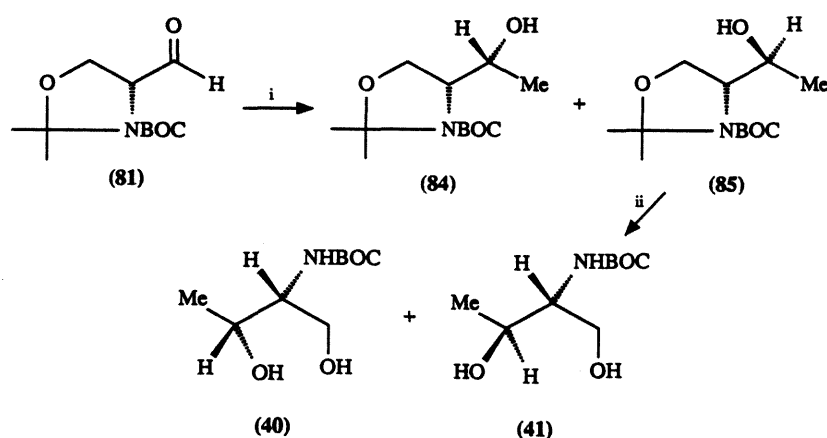
It had been incorrectly assumed that the Garner aldehyde was both chemically and configurationally stable if left for a period of time after its purification. Initially a single batch of (81) was prepared and then several reactions were carried out with it over a period of 2 weeks. It was found that if the aldehyde was not reacted immediately after its preparation then the yields of required product were very low. For this reason the DIBAL reduction of the oxazolidine methyl ester (83) was carried out many times, on each occasion only preparing a sufficient amount of aldehyde for purifying and reacting immediately.

The optical rotation of three dry samples of the aldehyde stored under nitrogen in a fridge was recorded after varying lengths of time. It was found that after only 2 days of storage the rotation had gone from $+91.8^\circ$ to $+78.4^\circ$, and after 3 weeks it was as low as $+55^\circ$. As reflected by the low yields of product, the change in optical rotation is believed to be due mainly to the chemical instability of the aldehyde rather than racemisation. If the ^1H NMR spectrum of (81) was recorded several days after its preparation it was noted that the integration of the $-\text{CHO}$ proton was lower than expected. Therefore, one possibility is that after a short period of time the aldehyde is converting to its corresponding hydrate.

3.3 REACTION OF THE GARNER ALDEHYDE WITH MeMgBr

Having prepared the aldehyde the next stage was to test the prediction given in the introduction of this chapter that the aldehyde would react with an organometallic reagent with α -chelation control as the ketones (36) and (37) had when they were reacted with sodium borohydride.

The first organometallic reagent reacted with (81) was the Grignard reagent MeMgBr (see Scheme 3.10). The reaction was carried out in THF, and after addition at -78°C the reaction mixture was stirred at room temperature for 3h followed by heating at reflux for a further 1h. Standard aqueous work-up of quenching with sat. NH_4Cl , extraction into ether and washing with 0.5 M HCl and sat. NaCl, followed by purification by flash column chromatography gave the alcohols (84) and (85) in 51% combined yield.



Reagents i) MeMgBr (1.5 equiv.), THF, $-78^{\circ}\text{C} \rightarrow \Delta$, 4h.
ii) *p*-TsOH.H₂O (0.04 equiv.), MeOH, Δ , 2h.

Scheme 3.10

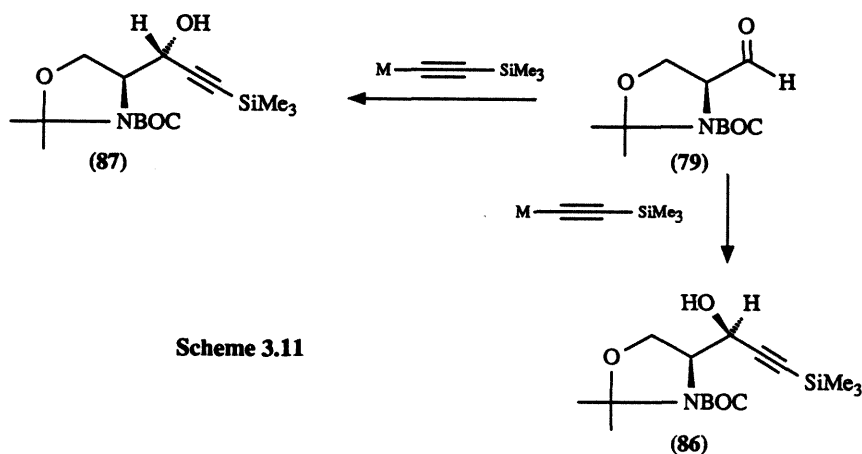
It was difficult to determine the ratio of the two possible diastereomers (84) and (85) from the ^1H NMR spectrum because apart from the presence of two products there was the additional problem of doubling up of signals as explained in Section 3.2.

A ^{13}C NMR spectrum was also not suitable for accurate d.e. determination because the *tert*-butyl peak dominated the spectrum and the other signals were weak and somewhat broadened. Therefore, because of these reasons, the ratio of products was determined from the ^1H NMR spectrum of the subsequent mixture of deprotected products (40) and (41) which were obtained by treatment of (84) and (85) with methanolic *p*-toluenesulphonic acid at reflux temperature (Scheme 3.10). The BOC group was stable to these acidic reaction conditions, but surprisingly, when deprotection of (84) and (85) was carried out in trifluoroacetic acid in an attempt to remove the BOC group and open the oxazolidine ring in one step, only removal of the BOC group was observed. Purification of the products (40) and (41) was not necessary due to the reaction having gone to completion. It should be noted that although the oxazolidines (84) and (85) were purified by flash column chromatography the two diastereomers were not separated on TLC. Therefore, the ratio of the deprotected products was considered to be the same as that obtained in the Grignard reaction.

The ^1H NMR spectrum of the mixture of N-BOC diols (40) and (41) showed the two sets of doublets at δ 1.20 ppm and δ 1.26 ppm corresponding to the methyl protons of $\text{CH}_3\text{CH}(\text{OH})$ - to be in a ratio of 1.2:1 respectively as calculated from peak heights (a ratio of 1.1:1 was obtained from the integration). The results therefore show that there was virtually no diastereoselectivity in the reaction of the aldehyde with MeMgBr . However, comparison of the ^1H NMR spectrum of the diastereomers (40) and (41) prepared via the aldehyde with the spectrum of the corresponding diols (40) and (41) formed from the reduction of the methyl ketone (36), (Section 2.3f), suggested that it was the required *threo*-isomer (40) that was the slightly favoured product i.e. it appeared that there was a slight preference for the reaction to occur via α -chelation control as opposed to nonchelation.

3.4 REACTION OF THE GARNER ALDEHYDE WITH VARIOUS ORGANOMETALLIC REAGENTS

A literature search on the Garner aldehyde¹⁰⁸ revealed the great potential for its usage as a chiral synthon in stereoselective synthesis, and in particular, work by Herold on the synthesis of *D-erythro*- and *D-threo*-sphingosine derivatives has shown encouraging results. Herold has shown that the reaction of the Garner aldehyde (79) with metallated ethynyltrimethylsilane (Scheme 3.11) selectively affords either the corresponding *threo*- or *erythro*-product, (86) and (87) respectively, depending upon the choice of metal and the reaction conditions employed.^{108a}



Scheme 3.11

Selected results of Herold's work are given overleaf in Table 3.1 which shows the high degree of diastereoselectivity (ds) that can be achieved with the Garner aldehyde. Herold has shown that using lithiated ethynyltrimethylsilane in THF considerable *erythro*-selectivity was obtained (Entry 2). When cation-complexing agents were added the *erythro*-selectivity increased even more, HMPT being the most effective agent affording (87) with 95% diastereoselectivity (Entry 1).

Table 3.1 Addition of Metallated Ethynyltrimethylsilane to the Garner Aldehyde

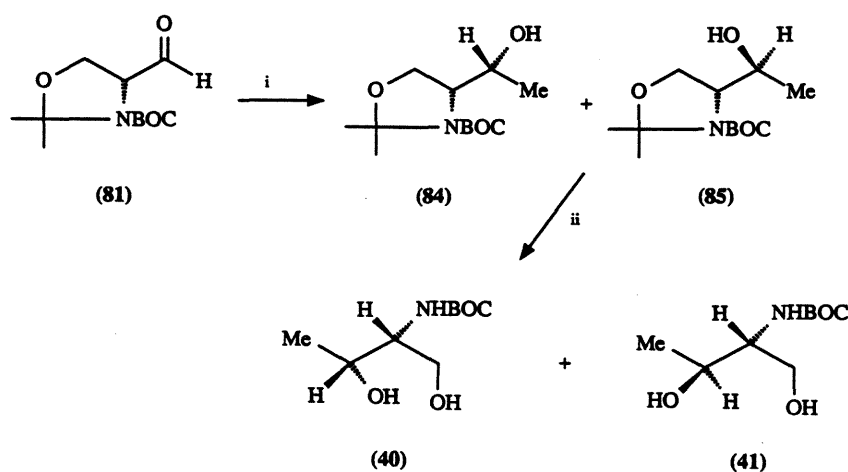
Entry	M	Additive	Solvent	86:87	ds (%)
1	Li	HMPT	THF	1:20	95
2	Li	none	THF	1:8	89
3	MgBr	none	THF	1:7	87
4	Li	ZnBr ₂	Et ₂ O	11:1	91
5	MgBr	CuI	THF/Me ₂ O	20:1	95

In contrast, high *threo*-selectivity was observed when anhydrous ZnBr₂ was added to the lithiated acetylide in Et₂O (Entry 4), but the highest *threo*-selectivity obtained was in the reaction of (79) with the Grignard reagent in the presence of CuI with THF and Me₂S as solvent. As the table shows, the latter reaction afforded (86) with 95% diastereoselectivity (Entry 5). Herold also noted that the choice of solvent can influence the selectivity. When the reaction was carried out with the Grignard reagent and ZnBr₂ as the additive in THF there was 71% diastereoselectivity in favour of the *erythro*-product, whereas a reversal of selectivity was observed when the same reaction was carried out in Et₂O, this affording a diastereoselectivity of 84% in favour of the *threo*-product.^{108a} This effect is presumably a result of competing coordination to the metal, with the solvent preferentially coordinating to the metal when the reaction was carried out in THF and the oxazolidene preferentially coordinating when in ether.

Even though direct extrapolation of Herold's work using metallated ethynyltrimethylsilane, to other organometallic reagents would be unjustified, it was decided to investigate various reaction conditions used by Herold. It was anticipated

that this would lead to a general method for the preparation of the required chloramphenicol analogues (20).

The Garner aldehyde was reacted with a metallated methyl reagent using three different reaction conditions as shown in Scheme 3.12.



Reagents: i) Method B MeLi (1.5 equiv.), THF, -78°C, 2h.
 Method C MeLi (1.5 equiv.), ZnBr₂ (2.0 equiv.), Et₂O, 0°C → RT, 20h.
 Method D MeMgBr (2 equiv.), CuI (2.9 equiv.), THF/Me₂S (5:1), -78°C → RT, 10h.
 ii) p-TsOH.H₂O (0.08 equiv.), MeOH, Δ, 2h.

Scheme 3.12

Method B followed a procedure used by Herold^{108a} and involved the addition of (81) to MeLi in THF at -78°C followed by stirring for 2h at that temperature. After the standard aqueous work-up as given in Section 3.3, the mixture of alcohols (84) and (85) was obtained in 46% combined yield.

Method C was based on a procedure reported by Asami.¹⁰⁹ MeLi and (81) were added to a suspension of ZnBr₂ in Et₂O at 0°C and the reaction mixture was left to warm to room temperature overnight. Standard work-up gave (84) and (85) in only 16% yield. Initially it was thought that this low yield was due to the ZnBr₂ which may not have

been totally dry, but when the reaction was repeated using a new batch of anhydrous ZnBr_2 a very poor yield was still obtained. The reaction was also attempted with fresh MeLi and MeLi as the LiBr complex but they too afforded little product. Therefore, the low yield that was obtained is believed to be due to the instability of the aldehyde which for these reactions had been stored for a couple of weeks prior to its use.

Method D followed Herold's procedure^{108a} and involved the addition of MeMgBr to CuI in THF/Me₂S (5:1) at -78°C. The reaction mixture was stirred at -78°C for 0.5h and at -30°C for 0.5h, followed by addition of (81) at -78°C. The reaction mixture was then left at to warm to room temperature over 9h. Standard work-up afforded the diastereomeric alcohols (84) and (85) in 63% combined yield.

As explained in Section 3.3 the ratio of the two possible diastereomers had to be determined from the product afforded in the deprotection step i.e. (40) and (41), which in all cases was obtained in high yield and purification by column chromatography was not carried out. The product ratios were calculated from the ¹H NMR spectra by examining the doublet corresponding to the methyl protons of CH₃CH(OH)–, as it had been for the product obtained via reaction of (81) with MeMgBr (Section 3.3). The ratios of the *threo:erythro* products obtained from the different methods are shown overleaf in Table 3.2 which also includes the result from the MeMgBr reaction.

Since examination of prior usage of the Garner aldehyde showed no examples of simple alkyl organometallic addition a direct comparison of these results could not be made, the majority of the literature reactions being metallated vinyl^{108b,c} or alkynyl^{108a,d-g} addition or Diels-Alder cycloaddition.^{108h,i,j} As shown in Table 3.2 low diastereoselectivity was achieved with addition of both the organolithium reagent (Entry 1) and Grignard reagent (Entry 2) to the aldehyde, and, as expected, addition of anhydrous ZnBr_2 to the MeLi reaction (Entry 3) reversed the selectivity in favour of the required *threo*-product. The degree of selectivity in the latter reaction was not as high as was hoped but this may have been due to the actual reaction conditions.

Table 3.2 Addition of Methyl Organometallic Reagents to the Garner Aldehyde

Entry	Method	Organometallic Reagent	Additive	Solvent	<i>Threo</i> : <i>Erythro</i> (84):(85) ^a	ds (%)
1	B	MeLi	none	THF	1:2.5 (1:3)	71
2	A	MeMgBr	none	THF	1.2:1 (1.1:1)	55
3	C	MeLi	ZnBr ₂	Et ₂ O	1.6:1 (2.8:1)	62
4	D	MeMgBr	CuI	THF/ Me ₂ S	20 - >100:1 ^b (25 - >100:1)	95 - >99

^a The ratios were calculated from peak heights and the ratios in brackets were calculated from the integration.

^b The results were obtained from several experiments.

Recent work by Coleman on the addition of vinyl organometallic reagents to the Garner aldehyde has revealed some interesting results, the most significant of which are shown overleaf in Table 3.3.^{108b}

The reaction corresponding to Entry 2 (Table 3.3) involved precomplexing the aldehyde with ZnCl₂ followed by addition of vinyl lithium. Unexpectedly, this had little effect on the diastereoselectivity of the reaction compared to Entry 1, the slight increase in the proportion of *threo*-product being possibly a result of the change of solvent rather than the addition of the Lewis acid. For Entry 3 vinyl zinc chloride was prepared from vinyl lithium and ZnCl₂ and then added to the aldehyde, this resulted in a reversal of diastereoselectivity affording the *threo*-adduct highly stereoselectively. As shown by Entry 4, addition of vinyl zinc chloride to the aldehyde precomplexed with ZnCl₂ had no effect on the selectivity.

Table 3.3 Addition of Vinyl Organometallic Reagents to the Garner Aldehyde

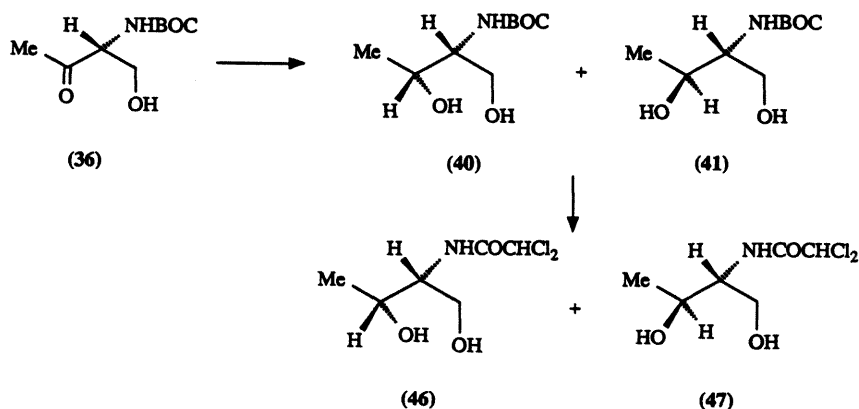
Entry	Vinyl Nucleophile	Solvent, Temperature	Equiv.	Additive	Solvent	<i>Threo:Erythro</i>
1	C ₂ H ₃ Li	THF, -78°C	1.6	none	THF	1:5
2	C ₂ H ₃ Li	pent/Et ₂ O, -78°C	3.0	1.0 equiv. ZnCl ₂	Et ₂ O	1:3
3	C ₂ H ₃ ZnCl	pent/Et ₂ O, -78°C	1.5-3.0	none	Et ₂ O	6:1
4	C ₂ H ₃ ZnCl	pent/Et ₂ O, -78°C	3.0	1.0 equiv. ZnCl ₂	Et ₂ O	6:1

Coleman's results suggest that to favour the *threo*-product the important factor is the vinyl nucleophile rather than the addition of a chelating reagent to the aldehyde.

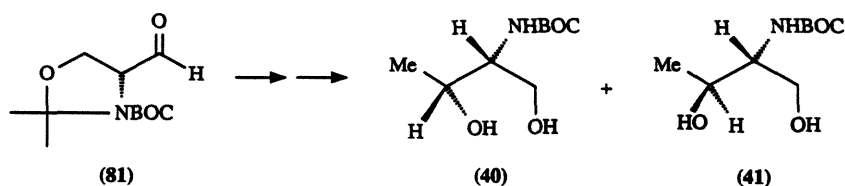
Since Method C (Table 3.2) involved the immediate addition of MeLi to a mixture of the aldehyde and ZnBr₂, rather than addition of the aldehyde to methylzinc bromide, the modest selectivity observed in the reaction is in accord with Coleman's work. In contrast, excellent diastereoselectivity in favour of the required *threo*-adduct was achieved by the reaction of the Garner aldehyde with [MeCu].MgBr (prepared from MeMgBr and CuI in THF/Me₂S, Method D, Table 3.2). This reaction was repeated several times and routinely afforded the *threo*-product almost exclusively. It is not known why such high selectivity is observed with organocopper compounds, but Macdonald suggests that it is the depressed reactivity of the carbon nucleophile compared to the Grignard reagent that is responsible for this behaviour.¹¹⁰

3.4i Confirmation of the *Threo* and *Erythro* Assignment

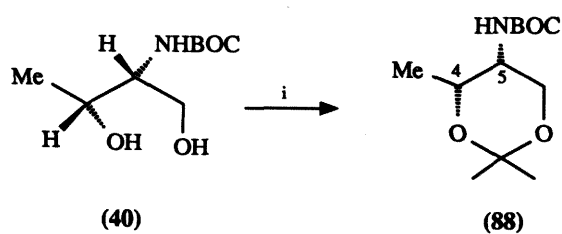
As discussed in Section 2.5 the minor diastereomer formed from the reduction of the methyl ketone (36) was the required *threo*-product (40) as verified by comparison of the mixture of diastereomers (46) and (47) with authentic N-dichloroacetamido-L-threoninol (46) prepared via L-threonine.



Therefore, by comparing the ¹H NMR spectra of (40) and (41) formed via organometallic addition to the Garner aldehyde with the spectrum of the corresponding diols formed from the methyl ketone the *threo*- and *erythro*-product could be tentatively assigned.



Confirmation of these assignments was obtained by preparing the acetonide derivative of (40) which had been prepared via Method D and so was essentially diastereomerically pure. The acetonide (88) was afforded in good yield from DMP and PPTS as shown in Scheme 3.13.



Reagents: i) DMP (20 equiv.), PPTS (1 equiv.), CH_2Cl_2 , RT, 20h.

Scheme 3.13

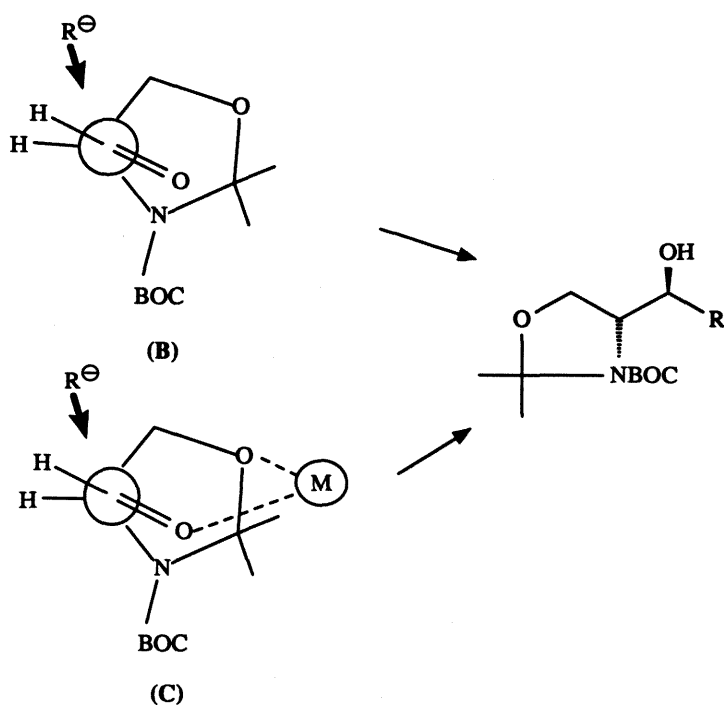
The ^1H NMR spectrum of (88) showed that the coupling constant between H-C(4) and H-C(5) was 1.9 Hz. This value is consistent with an axial-equatorial relationship of these two protons,⁸³ and so confirms that the major diastereomer formed via Method D was the required *threo*-product.

3.4iii Proposed Mechanisms

In proposing mechanisms that account for the stereoselectivity in the systems discussed above it should be noted that the many functional groups present offer several alternative chelate structures, and the relative contribution of these possible chelates, as well as relevant nonchelate models, will influence the stereochemical outcome of addition.

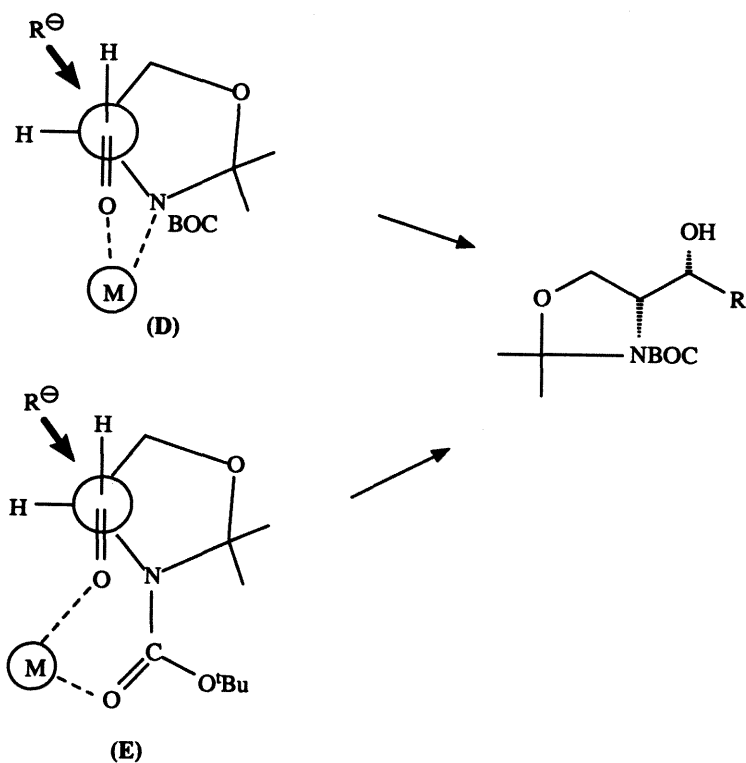
Formation of the *erythro*-product is thought to proceed via either a nonchelated Felkin-Ahn type transition state (B)⁷⁶ or a β -chelation-controlled model (C)^{108,1} in which the ring oxygen and the carbonyl form a 6-membered chelate with the metal ion, with nucleophilic attack occurring from the least hindered face.

Proposed transition states for *erythro* formation

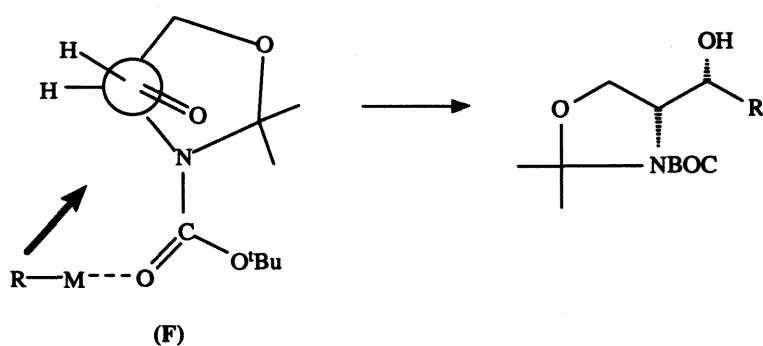


Predominant *threo*-selectivity can be ascribed to several possible models. It is generally considered that the *threo*-product is formed via either α -chelation control with the urethane nitrogen (D) or δ -chelation control with the urethane carbonyl (E).^{108f,g,j}

Proposed transition states for *threo* formation



The results of Coleman's work however are not consistent with the proposed chelation models (D) and (E). He offers an alternative model^{108b} which occurs with a coordinated delivery of nucleophile via a transition state such as (F) to account for the observed *threo*-selectivity (see overleaf).

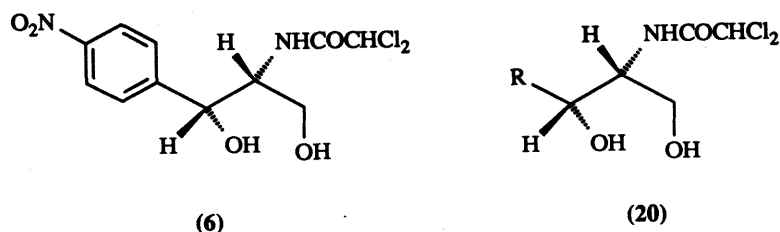


The above model could explain the results of the reaction of the Garner aldehyde with $[\text{MeCu}]\cdot\text{MgBr}$, the reagent precomplexing with the urethane carbonyl and thereby adding from the *re*-face.

It should be noted that the above transition states are merely proposed mechanisms to rationalise the formation of either the *threo*- or *erythro*-product. It is speculated that the cases which proceed with low selectivity are the result of competition between the different models of stereochemical control. In conclusion, it is clear that many factors can influence the stereochemical course of the reaction and these include temperature and solvent effects, choice of metal and addition of chelating reagents.

3.5 APPLICATION TO THE SYNTHESIS OF CHLORAMPHENICOL ANALOGUES

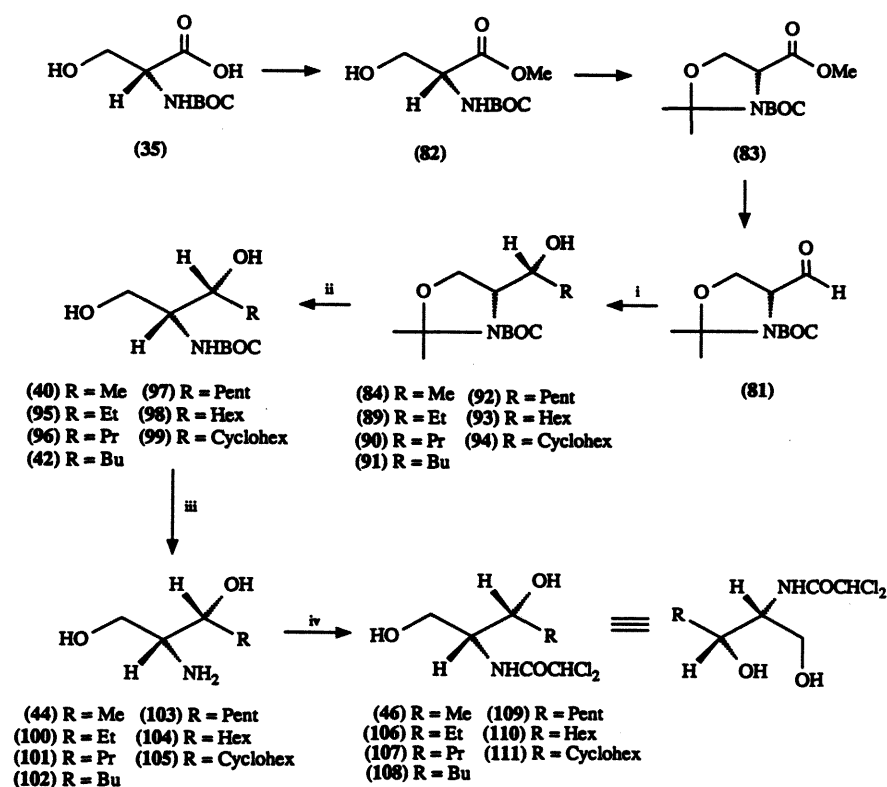
Since such exceptionally high *threo*-selectivity was achieved with the reaction of the Garner aldehyde (81) with MeMgBr in the presence of CuI in THF/Me₂S this was thought to be a suitable general route for the preparation of the series of chloramphenicol analogues (20) in which the *p*-nitrophenyl group of chloramphenicol (6) was replaced by various alkyl groups.



A further advantage to this route is that the aldehyde could be prepared in 3 steps in a high overall yield (60-70%) and also, through experience of using the aldehyde, its limitations with regard to stability were known. The synthetic route for the preparation of the required analogues is shown overleaf in Scheme 3.14, and this was used to prepare a total of seven compounds, six of which had straight chain alkyl groups (R=Me, Et, Pr, Bu, Pent and Hex) and the seventh an alicyclic group (R=Cyclohex).

The Garner aldehyde (81) was prepared in several batches as described in Section 3.2 and was used immediately after its purification. The hydroxy oxazolidines (84) and (89-94) were all prepared in a similar manner following Method D (Section 3.4), and generally afforded in good yield after purification by flash column chromatography, with the exception of the cyclohexyl analogue (94) which was afforded in a very low yield (18%). The IR spectra of (84) and (89-94) showed peaks at 3600-3400 cm⁻¹ inferring the presence of an O-H group, but the room temperature NMR data for these compounds were difficult to fully interpret due to the broadness of the signals as

previously discussed. It was also observed that if the NMR samples were left in solution for any length of time then some deprotection occurred to give the corresponding N-BOC diols. Correct accurate masses were obtained for all the analogues and the low resolution mass spectra showed fragmentations consistent with their proposed structures, including the characteristic loss of $\cdot\text{RCHOH}$.



Reagents: i) RMgX (2 equiv.), CuI (2.9 equiv.), THF-DMS (5:1), $-78^\circ\text{C} \rightarrow \text{R.T.}$, 10h.
 ii) $p\text{-TsOH}\cdot\text{H}_2\text{O}$ (0.08 equiv.), MeOH, Δ , 2h.
 iii) 1 M HCl, dioxane, Δ , 0.5h.
 iv) $\text{Cl}_2\text{CHCO}_2\text{Me}$ (2.5 equiv.), EtOH, Δ , 6h or $\text{Cl}_2\text{CHCO}_2\text{Me}$ (5 equiv.), Et_3N (1 equiv.), MeOH, R.T., 24h.

Scheme 3.14

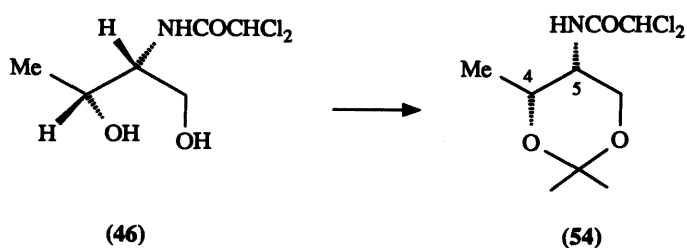
The first deprotection step, which was carried out as previously described with *p*-toluenesulphonic acid in methanol, gave the corresponding N-BOC diols (40), (95-96), (42), and (97-99) in high yield (62-95%) after purification by flash column chromatography. The data obtained for all the products were consistent with their structures. The ^1H NMR spectra showed an N-H and two O-H D_2O exchangeable protons, and also confirmed that in all cases the *threo*-product was obtained with >97% diastereoselectivity. The low resolution mass spectra of all the analogues routinely showed fragments corresponding to the loss of $\cdot\text{CH}_2\text{OH}$, $\cdot\text{RCHOH}$ and $\cdot\text{O}^t\text{Bu}$, and the accurate mass of each compound was correct. The observed $[\alpha]_{\text{D}}$ values of the N-BOC diols showed a reversal of rotation compared with those of the hydroxy oxazolidines and the final N-dichloroacetylated products, they were also found to be very low, and in the case of (99) it was too low to read.

Removal of the BOC group had previously been carried out by treatment with trifluoroacetic acid (Section 2.3) but the reaction mixture showed a tendency to darken considerably. It was therefore decided for the second deprotection step, which involved removing the BOC group of (40), (95-96), (42) and (97-99), to use the procedure described by Herold.^{108a} This involved heating at reflux a solution of the N-BOC diols and 1 M HCl in dioxane for 0.5h. This method afforded the amino alcohols (44) and (100-105) in a high crude yield (68-100%). The free amine bases are unstable and difficult to handle and so they were reacted immediately with methyl dichloroacetate to afford, after purification by flash column chromatography, the final products (46) and (106-111) in varying yields (42-81%). The propyl analogue (107) was prepared by the reaction of (101) with methyl dichloroacetate in ethanol at reflux temperature, this was the method previously used to prepare N-dichloroacetamido-L-threoninol (46), N-dichloroacetamido-D-threoninol (53) and the mixture of diastereomers (46) and (47) prepared via the methyl ketone. As shown by TLC a large amount of starting material remained unreacted using this method and so an alternative procedure was used for the preparation of the remaining analogues. This method followed a procedure described

by Schumacher ¹¹¹ and involved the addition of 1 equiv. of Et₃N to a solution of the crude amino alcohols and methyl dichloroacetate in methanol at room temperature. Even though the yields of product were varied they tended to be higher than those obtained using the previous method. CD₃OD and/or D₂O solutions were used for recording the NMR spectra of (46) and (106-111) which gave very sharp signals and confirmed that all the compounds were >97% diastereomerically pure. In all cases the ¹H NMR spectra showed a distinct ABX pattern corresponding to the -CH_XCH₂(OH) protons which allowed the J_{AX}, J_{BX} and J_{gem} coupling constants to be calculated. The spectrum of the methyl analogue (46) was particularly well defined enabling calculation of all the possible coupling constants. Correct accurate masses were obtained for all the analogues and the low resolution mass spectra showed the typical pattern of molecular ions containing two chlorine atoms. With the exception of the cyclohexyl analogue (111) all the compounds were highly crystalline and correct C, H and N analysis was obtained for (46) and (106-110).

3.5i Verification of Relative Configuration and Optical Purity

The *threo*-assignment of the N-BOC diol (40) prepared via the aldehyde was confirmed by the preparation of its acetonide derivative (88) and analysis of its ¹H NMR spectrum as discussed in Section 3.4i. Verification of the *threo*-configuration of the final product (46) was obtained in an analogous manner by preparing its acetonide derivative. The acetonide (54) was afforded in high yield (91%) from the reaction of (46) with DMP and PPTS as shown overleaf in Scheme 3.15. The ¹H NMR spectrum of (54) showed that the coupling constant between H-C(4) and H-C(5) was 1.7 Hz, which as previously discussed is consistent with an axial-equatorial relationship of these two protons. The data obtained for (54) prepared via the aldehyde were identical to that of the acetonide derivative of authentic N-dichloroacetamido-L-threoninol, consistent with it being the same diastereomer.



Reagents: i) DMP (20 equiv.), PPTS (1 equiv.), CH_2Cl_2 , RT, 20h.

Scheme 3.15

Further evidence of the relative configuration of the final products derived from the Garner aldehyde was obtained from the X-ray structure of the propyl analogue (107) which is shown in Figure 3.2.

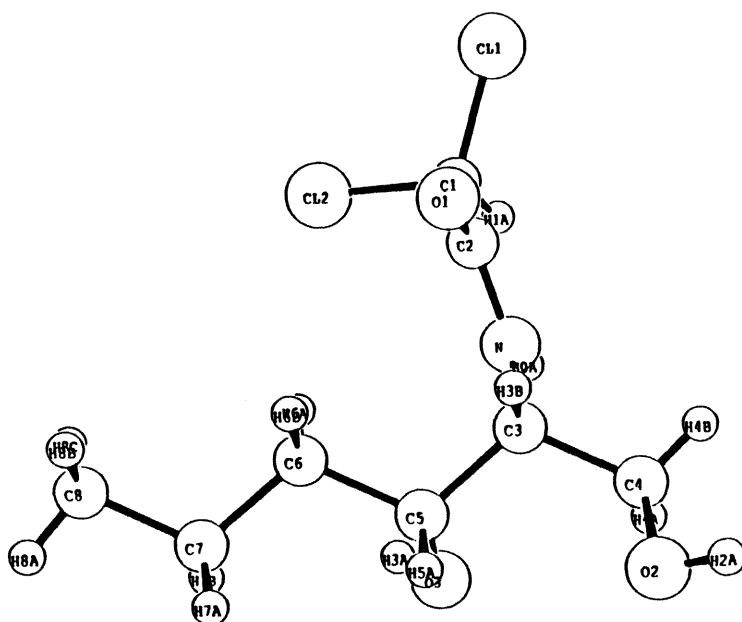


Figure 3.2

X-ray crystal structure of (2R, 3R)-2-N-dichloroacetamidoheptan-1,3-diol.

The X-ray structure shows that (107) exists in a preferred conformation (G), Figure 3.3. Comparison of (G) with the preferred conformation (A) of chloramphenicol (see Section 1.2) shows that the intramolecular hydrogen bonds proposed to exist between the two hydroxyl groups of CM are clearly absent in the (G)-conformer.

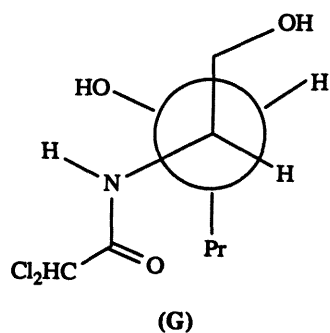


Figure 3.3

The X-ray structure of (107) does however show the presence of intermolecular hydrogen bonds (Figure 3.4).

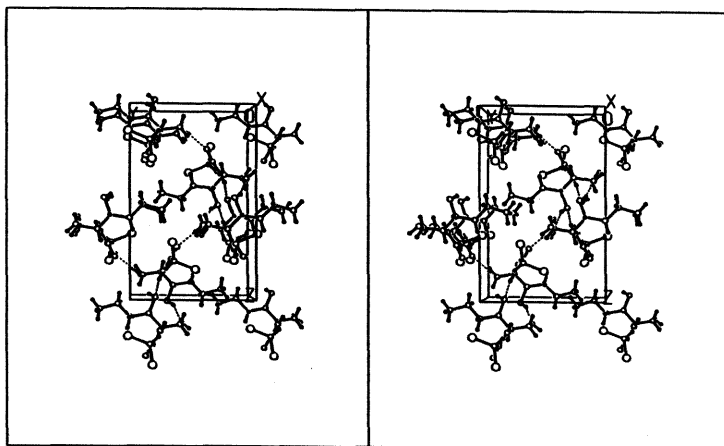


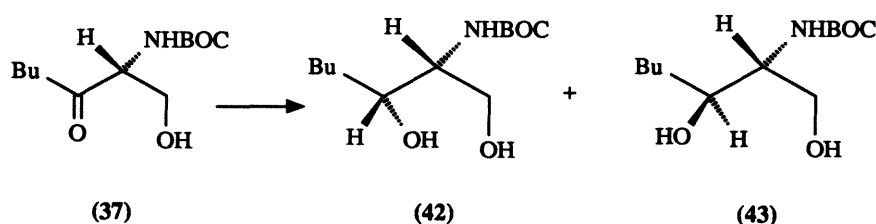
Figure 3.4

Stereo diagram of a unit cell of (2R, 3R)-2-N-dichloroacetamido-1,3-hexanediol.

As shown in Figure 3.4 there are hydrogen bonds between H3A and O2 and between H2A and O1. These are strong hydrogen bonds, the distances between them being 1.943 Å and 1.913 Å respectively.

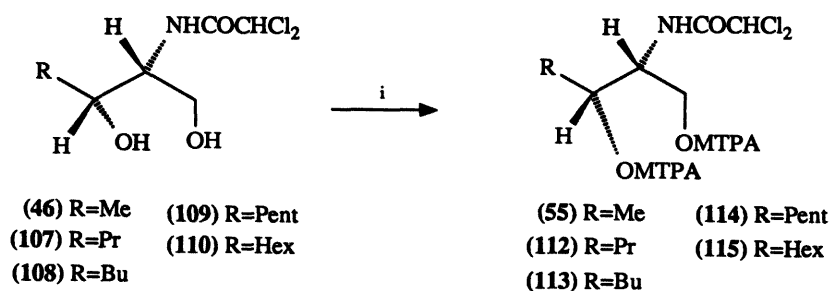
Comparison of the data obtained for (46), derived from the aldehyde, and N-dichloroacetamido-L-threoninol (46), derived from L-threonine, showed that the two products were identical. This was also verified by preparing an NMR sample of a mixture of the two products prepared via both routes, the resulting ^1H NMR spectrum showing that the two compounds were the same diastereomer.

As discussed in Section 2.5 the minor diastereomer formed via the sodium borohydride reduction of the methyl ketone (36) was shown to be *threo*-product. In a similar way the configuration of the minor product from the reduction of the butyl ketone (37) was confirmed.



A sample of the butyl N-BOC diol (42) prepared from the aldehyde was added to an NMR sample of the mixture of diastereomers (42) and (43) derived from the reduction of the butyl ketone. The resulting ^1H NMR spectrum confirmed that the minor diastereomer had the same configuration as (42) i.e. it was the *threo*-product. For comparative purposes the doublet corresponding to the $-\text{NH}$ proton was the simplest to examine. The spectrum of the mixture of diastereomers (42) and (43) showed the two doublets at δ 5.49 ppm and δ 5.31 ppm to be in a ratio of 3.4:1 respectively, and after addition of (42) prepared via the aldehyde the spectrum showed an increase in intensity of the signal at δ 5.31 ppm.

The Mosher esters of (46) and (107-110) were prepared using MTPA-Cl (Scheme 3.16). and their ^{19}F NMF spectra indicated that all the compounds had been prepared without any detectable racemisation. (The Mosher ester of the ethyl analogue (106) and the cyclohexyl analogue (111) could not be prepared due to an insufficient amount of material being available).



Reagents: i) MTPA-Cl (6 equiv.), pyridine, CCl_4 , R.T., 20h.

Scheme 3.16

Comparison of the ^{19}F NMR spectrum of the Mosher ester (55) of the methyl analogue with the spectrum of the Mosher esters prepared from the racemic mixture of N-dichloroacetamido-D-threoninol (53) and N-dichloroacetamido-L-threoninol (46) and that of the Mosher ester of (53) only (see Section 2.5iii), confirmed that (46) prepared via the aldehyde was the same enantiomer as authentic N-dichloroacetamido-L-threoninol. This was also verified by the measured optical rotation which was $+8.7^\circ$ for (46) prepared via the aldehyde and $+8.5^\circ$ for N-dichloroacetamido-L-threoninol.

3.6 CONCLUSION

The work carried out for this thesis together with that reported in the literature¹⁰⁸ shows great potential for use of the Garner aldehyde in the stereoselective synthesis of 2-amino-1,3-diols. Use of varying reaction conditions together with the availability of both D-serine and L-serine gives a versatile enantio- and diastereospecific route leading to all four possible diastereomers. The results discussed in this Chapter show that the required chloramphenicol analogues (20) were successfully prepared via addition of $[RCu].MgX_2$ ($X=Br$ or Cl), to the D-serine derivative of the Garner aldehyde, and that all final products were essentially single diastereomers formed without racemisation.

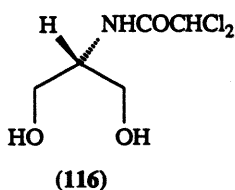
CHAPTER 4

KINETIC STUDIES AND THE HYDROPHOBIC EFFECT

4.1 INTRODUCTION

As discussed in Chapter 1, one of the main aims of much recent research in the field of molecular recognition is to make quantitative estimates of the thermodynamic contributions of individual noncovalent interactions to the overall binding energy of a ligand to an enzyme. Although different groups have established typical values for hydrogen bonds rather less work has been reported on hydrophobic "bonding". The majority of the amino acid residues that form the chloramphenicol binding pocket possess hydrophobic side chains (see Figure 1.10) suggesting that this is a major source of specificity in CAT. Section 1.6 discussed an analysis of the contribution of the 3-hydroxyl group of chloramphenicol to the binding of CAT which demonstrated a linear free energy relationship between the dissociation constants for binding to CAT and the hydrophobicity of the inhibitors. The work discussed in this chapter is an analysis of the contribution of the *p*-nitrophenyl group of chloramphenicol to the binding and specificity exhibited by CAT.

Previous work by J. Williams (Leicester Univ.) has shown that N-dichloroacetyl serinol (116), an analogue of chloramphenicol in which the *p*-nitrophenyl group is replaced by a hydrogen, is a substrate for CAT.⁸⁰



N-Dichloroacetyl serinol

Figure 4.1

Although the structure differs significantly from chloramphenicol having destroyed the asymmetry in the molecule (C-2 is now a prochiral centre) it is believed that CAT binds this analogously to CM because of the enantiotopic discrimination which was shown in

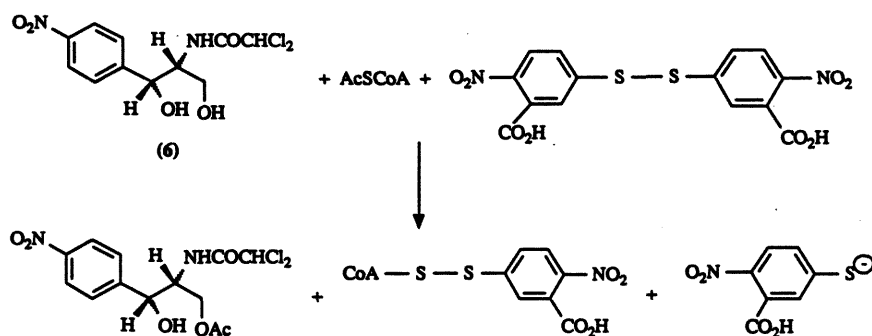
the first acetylation step.⁸⁰ This deletion dramatically increases K_m and decreases k_{cat} , however, because CAT_{III} is such a catalytically efficient enzyme it was possible to determine reliable kinetic parameters – $K_m=15$ mM and $k_{cat}=20$ s⁻¹ cf 11.6 μ M and 599 s⁻¹ respectively for chloramphenicol.⁵⁵ The change in k_{cat}/K_m corresponds to a free energy difference of 6.3 kcal mol⁻¹, which corresponds to the ΔG_{app} for the *p*-nitrophenyl group.

Chapter 3 described the synthesis and characterisation of a series of chloramphenicol analogues in which the *p*-nitrophenyl group was replaced by alkyl groups of increasing chain length. These compounds, in which the opportunities for hydrophobic interactions are progressively built back into the ligand, were prepared to probe the incremental binding energy derived from this part of chloramphenicol and so extend the above approach.

RESULTS AND DISCUSSION

4.2 KINETIC STUDIES

The rate of 1-O-acetylation of the chloramphenicol analogues by the CAT catalysed reaction and assay of CAT activity was measured by UV spectrophotometry by monitoring the release of CoA by its reaction with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), see Scheme 4.1.¹¹²



Scheme 4.1

As shown above, reaction of CoASH with DTNB yields 1 equiv. of 5-thio-2-nitrobenzoic acid, 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] were calculated from linear intercept and slope replots from manually drawn double-reciprocal plots.⁵⁷

$$\frac{[E]}{v_0} = \phi_0 + \frac{\phi_A}{[A]} + \frac{\phi_B}{[B]} + \frac{\phi_{AB}}{[A][B]} \quad \dots[4.1]$$

In equation [4.1], [E] is the monomer concentration of active enzyme, calculated from the CAT standard assay (see Section 6.6); v_0 is the linear initial rate determined from each assay; [A] is the substrate concentration; and [B] is the AcCoA concentration. Each double-reciprocal plot was constructed from a matrix of four substrate and four coenzyme concentrations, and all reactions were performed in triplicate resulting in a total of 48 assays per substrate.

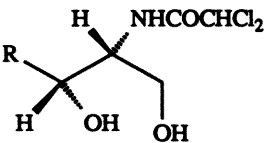
In order to determine the K_m and k_{cat} values for each substrate three graphs had to be plotted: Graph 1 was a plot of $[E]/v_0$ against $1/[A]$; Graph 2 a plot of the gradients from Graph 1 against $1/[B]$; and Graph 3 a plot of the intercepts from Graph 1 against $1/[B]$. The kinetic coefficients ϕ_0 and ϕ_B 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].

$$\text{For substrate A, } K_m = \frac{\phi_B}{\phi_0} \text{ and } k_{cat} = \frac{1}{\phi_0} \quad \dots[4.2]$$

4.3 KINETIC RESULTS

The chloramphenicol analogues in which the *p*-nitrophenyl group was replaced by an alkyl group were all shown to be substrates for CAT. The kinetic parameters for these ligands as CAT substrates are shown in Table 4.1.

Table 4.1 Kinetic parameters for a series of CAT substrates

			
R	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m
H (116) ^a	15.00	20	1.3
Me (46)	8.70	90	10
Et (106)	2.80	103	37
Pr (107)	0.77	300	390
Bu (108)	0.35	303	867
Pent (109)	0.16	263	1644
Hex (110)	0.13	263	2023
C.Hex (111)	0.30	500	1667

^a The preparation and determination of kinetic parameters for (116) was carried out by J. Williams (Leicester University).

As expected, for the substrates where R = H→Hex, there is a clearly identifiable trend in the series. Increasing size of the alkyl group improves both the binding (as approximated by K_m) and the turnover number (k_{cat}), and this trend continues as far as

the butyl derivative (108) where the maximum rate is achieved. For the pentyl and hexyl derivatives (109) and (110) the decrease in K_m is noticeably less pronounced and the catalytic rate has decreased.

The interaction of a substrate with its active site involves either the complete or partial desolvation of both ligand and active site. The energetics of this balance out only if hydrogen bonds to water are replaced by appropriate hydrogen bonds between enzyme and substrate. In addition, displacement of water molecules from the active site has an attendant 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. If the binding of a substrate by an enzyme involves the removal of the substrate from an aqueous environment to the hydrophobic region of the enzyme this can be considered as analogous to a partitioning between aqueous and organic phases. Since the aryl ring of chloramphenicol is in a hydrophobic pocket it was predicted that the above substrates would show a relationship between the hydrophobicity of the R group and the energies involved in their binding. If the trend seen in the data in Table 4.1 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 ligands.

4.3i Partition Coefficients and the Hansch Equation

The distribution of a solute between two phases in which it is soluble is a subject that has been studied for many years. Over a century ago investigations showed that the ratio of the concentrations of solute distributed between two immiscible solvents was a constant and did not depend on the relative volumes of solutions used.¹¹³ In 1891, Nernst stated that the partition coefficient would only be a constant if a single molecular species was being considered as partitioned between the two phases.¹¹⁴ 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 extrathermodynamic reference parameters for "hydrophobic bonding" in biochemical and pharmacological systems that has generated renewed interest in their measurement.⁶

A variety of work has been carried out to examine the relative roles of the various binding forces which determine the way a solute distributes itself between two phases.^{5b,c} A study of hydrocarbons in water showed that although the enthalpy change is favourable the compounds are highly insoluble. This is the result of a large entropy change for the process, the large energy cost of reordering the hydrocarbon solute and the water molecules is what keeps the two in separate phases. It is this phenomenon that regulates the distribution of nonpolar solute molecules in an apolar solvent-water system. It was Frank and Evans who first clearly appreciated the origin of the large negative entropy change that is associated with partitioning between aqueous and nonaqueous phases.^{5b} They concluded 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 that it is the removal of these water molecules that results in a large entropy change due to the randomisation of the water molecules.

In evaluating data from solvent-water distribution studies potential hydrogen bonding within the organic phase must be considered, as must the fact that quite large amounts of water may be present in certain solvents when saturated during the partitioning process. *n*-Octanol, for example, dissolves in water only to the extent of 4.5 mM, but the 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 seems likely that in octanol the polar functions would be more or less solvated by the dissolved water

and/or the hydroxyl group of the alcohol. Solvent versatility however, increases when it can act as both a hydrogen bond donor and acceptor, as with alcohols and amines. Since there are many -NH- and -OH groups present in enzymes and membranes the alcohol-water system, and in particular *n*-octanol-water, is most widely used as an extrathermodynamic reference system for evaluating partitioning of active compounds in biological systems. Increasing the hydrocarbon chain length in the alcohol increases the dissimilarity of the alcohol-water phases and increases the sensitivity to solute changes.

In 1963 Hansch demonstrated the advantage of using partition coefficients in connection with the Hammett constant σ to correlate the effect of a given substituent on the growth-promoting activity of phenoxyacetic acids and the bactericidal action of chloramphenicol analogues on various bacteria.¹¹⁵ He developed a comparative substituent constant π , which was found to be of great use in evaluating the lipo-hydrophilic character of a molecule upon which biological activity is highly dependent.¹¹⁶ π is defined as the difference in the logarithms of the partition coefficients of the substituted and unsubstituted compounds, as shown by [4.3].

$$\pi = \log (P_X/P_H) \quad \dots[4.3]$$

In the above equation P_H is the partition coefficient of a parent compound between two solvents and P_X is that of a derivative. The reference system is *n*-octanol-water. π , like σ , is a free energy related constant, it is a measure of the relative free energy change resulting from moving a derivative from one phase to another.

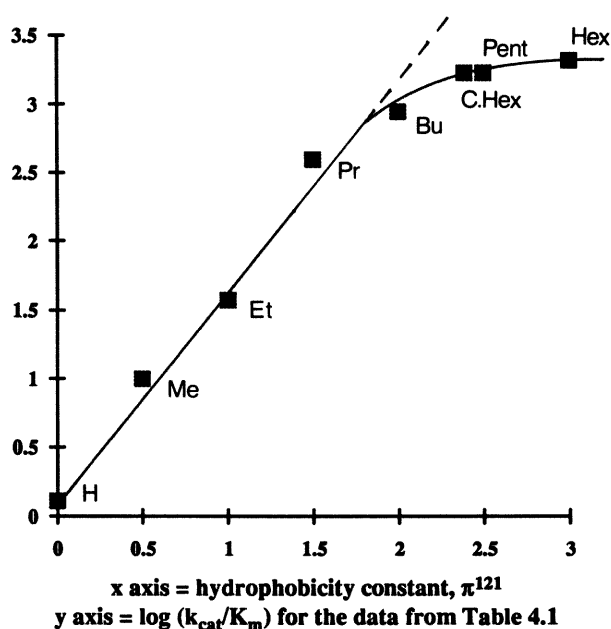
Hansch went on to determine the partition coefficients between *n*-octanol and water for 203 mono- and disubstituted benzenes from which he calculated π values for 67 functional groups.¹¹⁷ It was found that although π varies for a given substituent from system to system depending on its electronic environment, the range over which it varies is not great. When strong electronic interaction is absent π is constant and

additive in character. The additive nature of π 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 π can be used to account for the hydrophobic forces involved in the binding of organic compounds by proteins¹¹⁸ and enzymes.¹¹⁹

4.3ii Analysis of Kinetic Results and Estimation of Incremental Binding Energies

k_{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, and according to the transition state theory the $\log(k_{cat}/K_m)$ is proportional to the transition-state binding energy.¹²⁰ Using the data from Table 4.1, a plot of the $\log(k_{cat}/K_m)$ against the hydrophobicity constant π shows a good linear free energy relationship, see Figure 4.2. Although in considering the solvation of polyfunctional organic molecules the assumption of group additivity may not always be a good one, it is valid in this case because the series of substrates used in the study involves extending a straight saturated hydrocarbon chain while keeping the other functional groups in the molecule constant. The good fit for this data provides evidence that the alkyl group is located in the choramphenicol *p*-nitrophenyl binding pocket and that this is a local hydrophobic region. It shows that the relative differences between transition-state binding energies can be accounted for by differences in the hydrophobicity of the side chain R.

Figure 4.2 Hydrophobic Interaction at the *p*-Nitrophenyl Binding Site



As can be seen from Figure 4.2, the plot is linear up to C_4 ($R = Bu$) at which point there appears to be a break and the plot begins to level off. This is readily explained by the expectation that from this point additional methylene groups are no longer located within the active site cleft but protrude into 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.

In the CAT:CM binary complex the phenyl ring is in the mouth of the cleft that forms the chloramphenicol binding pocket and the nitro group is in contact with solvent as shown in Figure 4.3.

Figure 4.3 (opposite)

Model of the CAT:CM binary complex showing that the nitro group and a chlorine of chloramphenicol (dark blue) are not located in the active site cleft but are in contact with solvent.

The molecular modelling was carried out with the Biosym software using the coordinates of the binary complex from the X-ray structure.^{27a}

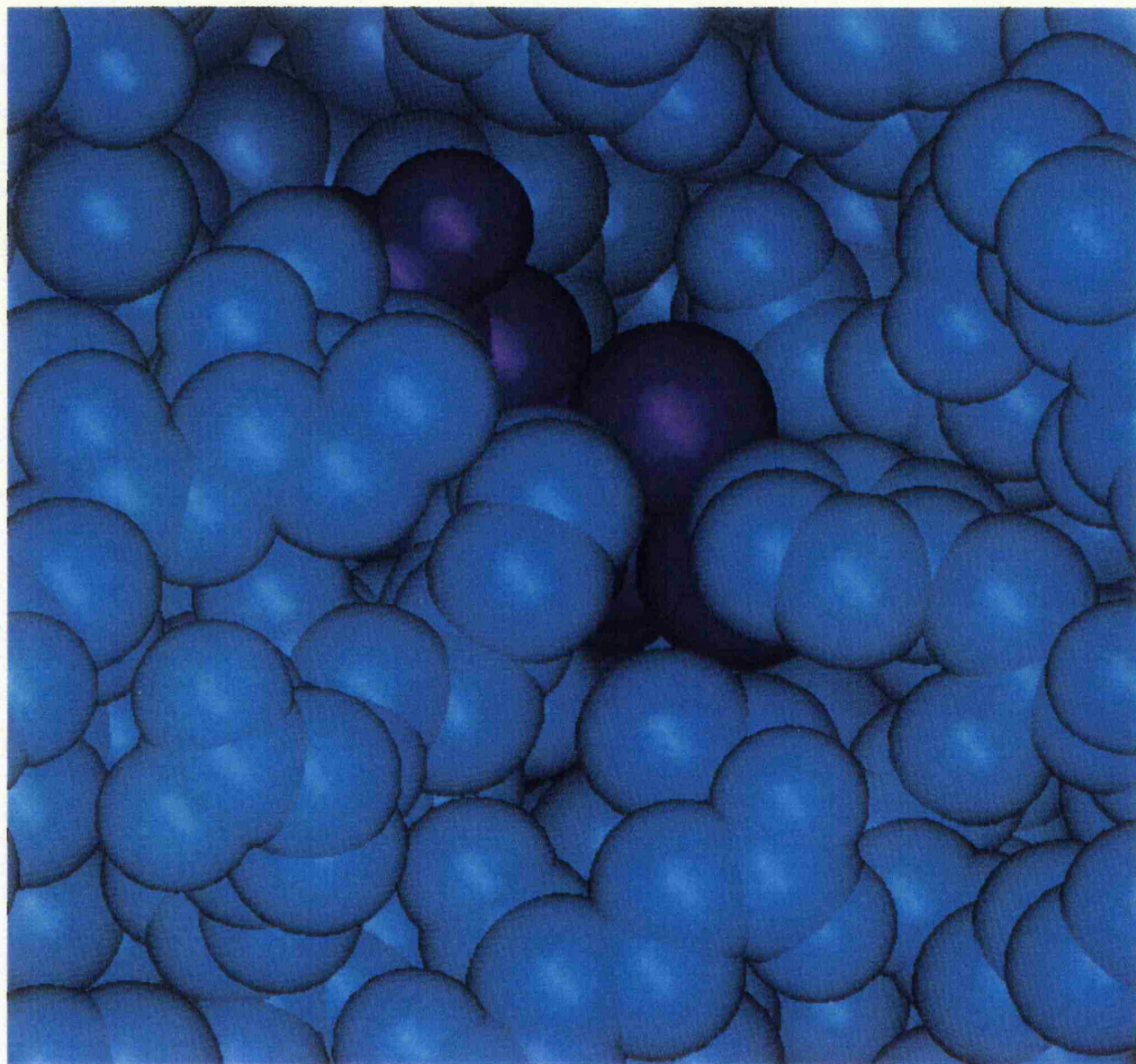


Figure 4.4 is a comparison of the lengths of the various alkyl chains in extended conformation with the corresponding atomic separations in the aryl ring and the *p*-nitrophenyl group of chloramphenicol. This suggests that the upper limit for alkyl chains to be fully accommodated in the hydrophobic cleft is reached at C₄, i.e. with the butyl analogue (108), and this is in agreement with the above experimental results.

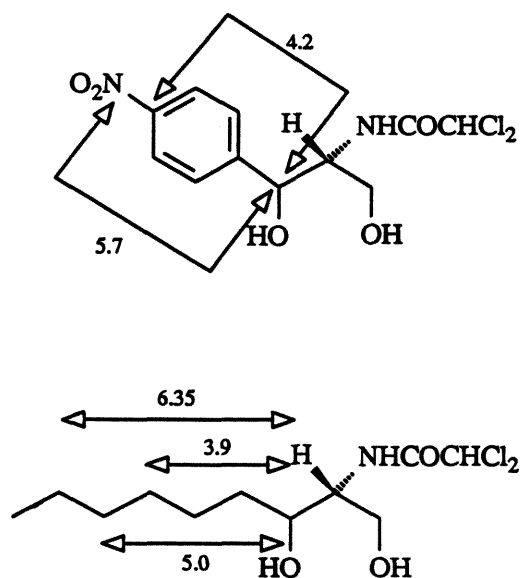


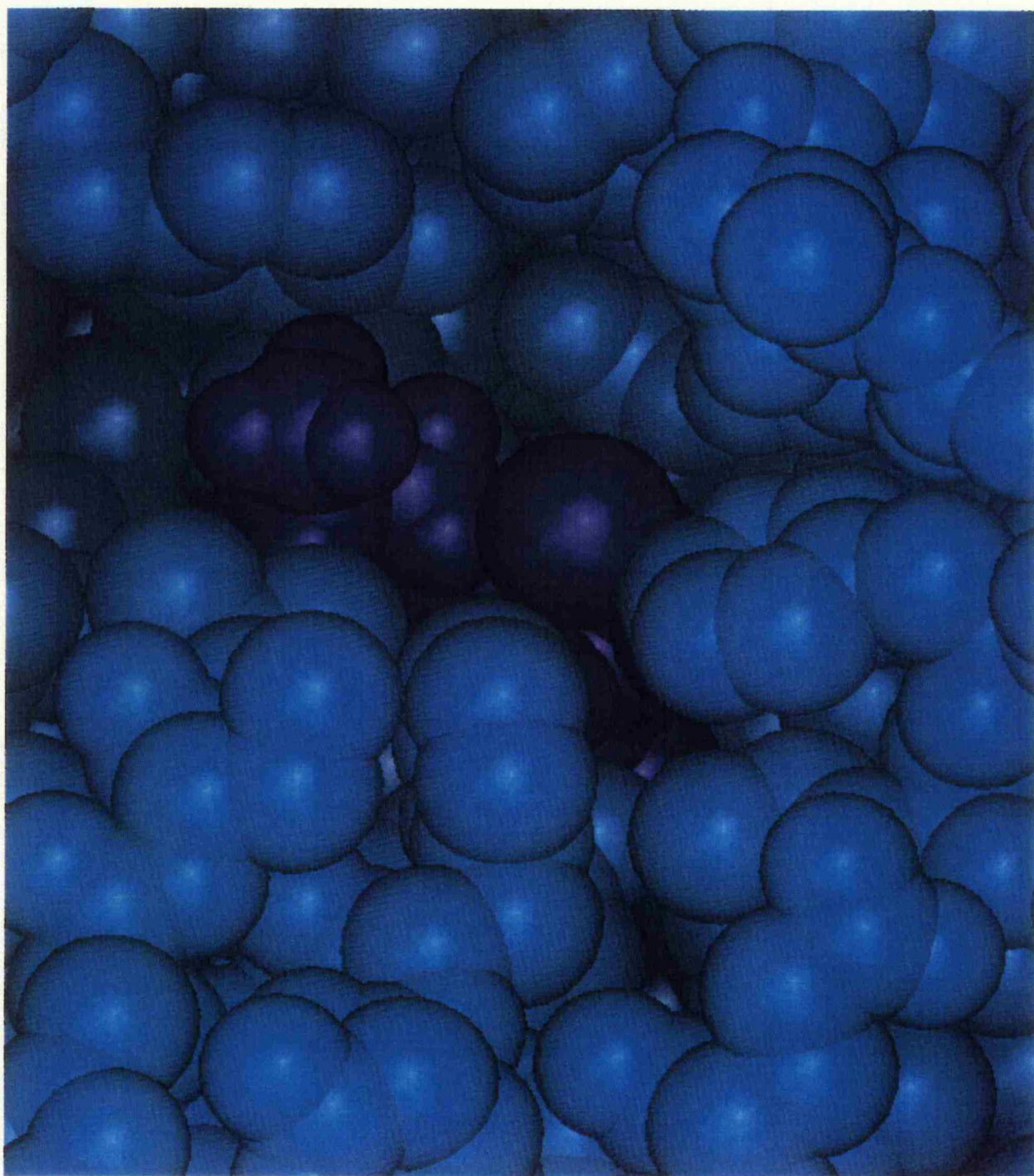
Figure 4.4

Further evidence to support this proposal is shown in Figure 4.5. Taking the coordinates of the CAT:CM binary complex the hexyl analogue (110) has been modelled into the chloramphenicol binding site using the Biosym software, and as the model shows, the terminal two methylene groups of the hexyl chain are in contact with solvent, as is found with the $-\text{NO}_2$ group of chloramphenicol.

Figure 4.5 (opposite)

Model of the binary complex of CAT and the hexyl analogue, (2R,3R)-2-N-dichloroacetamidononan-1,3-diol (**110**), showing that the terminal methylene groups of (**110**) (dark blue) are not accommodated in the hydrophobic cleft but protrude into solvent.

The molecular modelling was carried out using the Biosym software by taking the coordinates of the CAT:CM complex from the X-ray structure^{27a} and replacing the *p*-nitrophenyl group of chloramphenicol with a hexyl chain.



The contribution to binding, $\Delta\Delta G_{app}$, of the substituent R can be measured by comparing the values of k_{cat}/K_m for the two substrates R-S and H-S. In Section 1.1iii it was shown that $\Delta\Delta G_{app}$ could be calculated by comparing the dissociation constants, K_s , of the substrates, [1.2]. It is however, usually more reliable to use an alternative equation [4.4] because the quantity k_{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.^{7a,120}

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

When making a quantitative analysis of the energetics of an individual interaction the group being investigated is usually replaced by a proton. If the group was replaced by another substituent then the apparent binding energy could also include possible favourable and/or unfavourable interactions according to the precise substitution. In the above equation $-\Delta\Delta G_{app}$ 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 results of [4.4] can be interpreted as incremental binding energies. In the present case it is valid for substrates $R = Me \rightarrow Bu$ because the increasing length of the side chain sequentially fills up an extended binding site.

The calculated values of $\Delta\Delta G_{app}$ (obtained from the data in Table 4.1 and [4.4]) are shown in Table 4.2 together with the Gibbs energy of transfer of the alkyl groups from *n*-octanol to water.

Table 4.2

R	$-\Delta\Delta G_{\text{app}}^{\text{a}}$ (kcal mol ⁻¹)	$-\Delta\Delta G_{\text{app}}$ per CH ₂	π	$\Delta G_{\text{t}}^{\text{b}}$ (kcal mol ⁻¹)
Me	1.2	1.20	0.5	0.68
Et	1.9	0.95	1.0	1.36
Pr	3.4	1.13	1.5	2.04
Bu	3.8	0.95	2.0	2.72
Pent	4.2	0.84	2.5	3.41
Hex	4.3	0.72	3.0	4.09

^a The Gibbs free energy of transfer from CAT to water

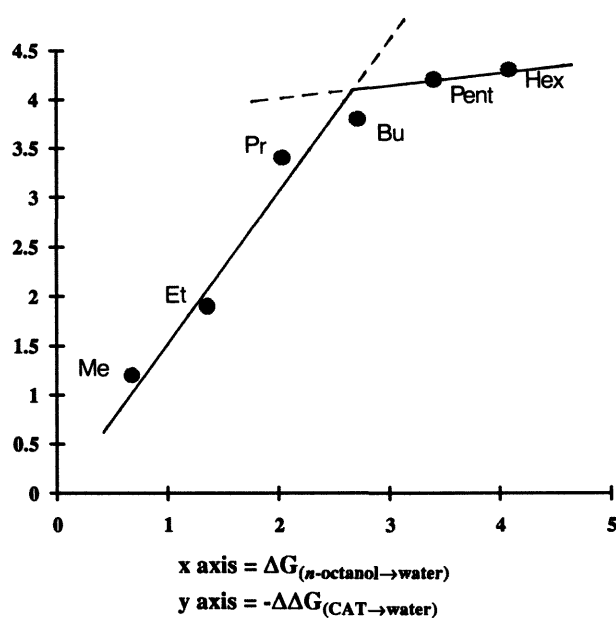
^b The Gibbs free energy of transfer from *n*-octanol to water

The ΔG_{t} values were calculated from the Hansch equation [4.3], $RT\ln(P_X/P_H)$, i.e. $2.303 RT\pi$, being the incremental Gibbs energy of transfer of the group R from *n*-octanol to water (relative to H). The substitution of, for example, a methyl group for a hydrogen is the same as the addition of a methylene group since it is equivalent to the interposing of a -CH₂- group between the hydrogen and the rest of the molecule. 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⁻¹.⁶ The observed additivity only 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 intermolecularly.

A plot of $-\Delta\Delta G_{\text{app}}$ for the substrates R = Me→Hex against $\Delta G_{(n\text{-octanol} \rightarrow \text{water})}$ is shown in Figure 4.6. The gradient of the linear portion of the plot (C₁→C₄) is 1.5. Since the slope is greater than unity this suggests that the chloramphenicol aryl binding pocket is more hydrophobic than *n*-octanol, indeed 1.5 times more hydrophobic. This is also the

case for chymotrypsin, where the hydrophobic binding pocket appears to be 2.2 times more hydrophobic than *n*-octanol.¹²² (A similar analysis of subtilisin showed it to be 1.2 times more hydrophobic than ethanol or dioxane).¹²³

Figure 4.6 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 kcal mol^{-1} and are relative to the hydrogen atom.

The incremental binding energy for a methylene group in the aryl binding pocket of CAT is therefore estimated to be $1.0 \text{ kcal mol}^{-1}$. This value is similar to those of the incremental hydrophobic free energy of a $-\text{CH}_2-$ group calculated from a range of model systems. These values together with various explanations of the hydrophobic effect are discussed in the following section.

4.4 THE HYDROPHOBIC EFFECT

In aqueous solution one of the most important enzyme-ligand interactions is that between lipophilic regions in the substrate and the protein. The variety of conflicting explanations that have been offered (see below) for the behaviour of lipophilic particles in water are a clear reflection of the current state of uncertainty with respect to the physical principles that govern molecular recognition. Indeed, even from model systems a range of values have been obtained for, for example, the incremental hydrophobic free energy of a $-\text{CH}_2-$ group.

The classic theory of the hydrophobic effect originates from the anomalously large loss of entropy which accompanies dissolution of nonpolar substances in water.⁵ The "classical" hydrophobic effect arises primarily from the reorganisation of the normal hydrogen-bonding network in water by the presence of a hydrophobic compound. A highly polar or ionic solute forms strong bonds to water molecules which more than compensates for the disruption or distortion of the hydrogen bonds existing in pure water, and so tends to be readily soluble. However, with nonpolar groups no such compensation occurs. In order to preserve the number of hydrogen bonds the water molecules arrange themselves around the nonpolar groups, and so it is a loss of entropy rather than bond energy that leads to an unfavourable free energy change for the process. Not all authors however, regard the entropy factor as the basis for the hydrophobic effect. In particular, it has been argued that dispersive interactions are entirely¹²⁴ or partially¹²⁵ sufficient to explain thermodynamic data obtained with noble gases or alkanes in water. Various explanations which have been offered to explain the effect together with estimated values of the free energy change for the process are discussed below.

Tanford^{5a,126} defines hydrophobic free energy in terms of the unitary free energy of transfer of a nonpolar solute from a nonpolar reference solvent, such as a hydrocarbon, to an aqueous medium. The hydrophobic free energy (i.e. the difference between the free energy of the interaction of the solute with the hydrocarbon and that with water) is given by [4.5] and can be determined from solubility measurements.

$$\mu_{\text{W}}^0 - \mu_{\text{HC}}^0 = RT \ln (X_{\text{HC}}/X_{\text{W}}) \quad \dots[4.5]$$

X_{HC} is the concentration of solute in the organic phase at equilibrium in mole fraction units, X_{W} is that in water, and μ_{W}^0 and μ_{HC}^0 are the standard chemical potentials of water and hydrocarbon respectively. This equation is only valid for very dilute solutions. If it is the pure liquid hydrocarbon that is being considered (i.e. the solute is the same as the solvent) then [4.6] applies, where X_{S} is the solubility of the hydrocarbon in water in mole fraction units.

$$\mu_{\text{W}}^0 - \mu_{\text{HC}}^0 = -RT \ln X_{\text{S}} \quad \dots[4.6]$$

Using solubility measurements made by McAuliffe,¹²⁷ Tanford has shown that a plot of the hydrophobic free energy, as defined by [4.6], against the number of carbon atoms in the solutes alkyl chain is linear for normal saturated aliphatic hydrocarbons.^{5a} From this he obtained a free energy value of 0.884 kcal mol⁻¹ per -CH₂- increment. It was found that for branched or cyclic hydrocarbons the free energy of transfer is less than for an unbranched chain of the same number of carbon atoms. This is in agreement with the idea that the hydrophobic free energy arises from contacts between hydrocarbon and water at the solute-solvent interface and so would be expected to be a function of the surface area of the hydrocarbon molecule rather than chain length.

By re-examining reported data Tanford went on to confirm that the hydrophobic free energy for linear, branched and cyclic hydrocarbons was proportional to the surface area of the cavity created by the solute in the aqueous solution.¹²⁶ Previous work by Hermann had shown this to be so, and he calculated the free energy per unit area, at the

distance of closest approach of water molecules to the cavity surface, to be $33 \text{ cal mol}^{-1} \text{ per } \text{\AA}^2$ at 25°C .¹²⁸ This value corresponds to a hydrophobic free energy of $1.049 \text{ kcal mol}^{-1}$ per $-\text{CH}_2-$ increment. The plot drawn by Hermann indicated that solutes of similar size to He or Ne would show no hydrophobic effect, which is contrary to experimental observations. Tanford, using the same solubility data and computed cavity surface area as Hermann, replotted the results including some extra data points. He obtained a straight line which passed through the origin and corresponded to a hydrophobic free energy of $25 \text{ cal mol}^{-1} \text{ per } \text{\AA}^2$. A further plot by Tanford, this time using relative areas obtained by manual packing of models,¹²⁹ gave a value of $20 \text{ cal mol}^{-1} \text{ per } \text{\AA}^2$.

From his empirical results Tanford concluded that the hydrophobic repulsive free energy between water and hydrocarbon chains (straight, cyclic or branched) is proportional to the area of contact measured at the distance of closest approach of water molecules. This value of $20\text{-}25 \text{ cal mol}^{-1} \text{ per } \text{\AA}^2$, which corresponds to $630\text{-}795 \text{ cal mol}^{-1}$ per $-\text{CH}_2-$ increment, is significantly less than that calculated by Hermann. The uncertainty of these figures is mainly due to the uncertainty of the measurement of contact area, but also depends on the accuracy of the best straight line plot of the data points.

Tanford also remarks that although the origin of the hydrophobic effect is a result of a large entropy change, the enthalpies and entropies of transfer are not necessarily regular functions of contact area. The very large change in heat capacity for the process of transfer to water is believed to be due to the water molecules at the solute-water interface arranging themselves in two or more ways.⁵⁰ It is probably the shape of the cavity that leads to these different states, but even though these arrangements must differ in enthalpy and entropy, they must do so in a mutually compensating way since it has been shown that the free energy change is linearly dependent on the cavity surface area.

Cramer, on the other hand, regards dispersive interactions as sufficient to explain thermodynamic data obtained with noble gases and alkanes in water. As presented by several authors including Hansch¹³⁰ and Tanford,¹²⁶ Cramer shows that there is a linear relationship between the bulk of the solute and its free energy of partitioning between water and *n*-octanol.¹²⁴ From the slope of the least-square lines he calculates the transfer energy of each incremental $-\text{CH}_2-$ to be $-0.72 \text{ kcal mol}^{-1}$. Previous descriptions of the hydrophobic effect have focused on the unique solvation properties of water. In contrast, Cramer has separated the partitioning equilibrium into two components: solvation by water and solvation by *n*-octanol.¹²⁴ He argues that if the prevailing theories of the hydrophobic effect are correct and thus result almost exclusively from bulk-dependent repulsions between water and solute, then i) a plot of solvation free energy (ΔG_s) by water versus molecular volume should resemble the similar plot of free energy of transfer from water to *n*-octanol (ΔG_t), and ii) a plot of ΔG_s by *n*-octanol should be an almost horizontal line. This, however, was not the case. For both plots of ΔG_s^0 against solute molecular volume Cramer constructs a straight line for the rare gases, and another straight line for the C_2 - C_5 alkanes. The resulting V-shape line for *n*-octanol is quite similar to the V-shape obtained for water. Cramer concludes that the two lines of different slope obtained for *n*-octanol require two different modes of solute-solvent interaction, one for the rare gas-solvent interaction and one for the *n*-alkane-solvent interaction. He also suggests that because both solvents give rise to V-shaped lines the mechanism of solvation by *n*-octanol and water must have many common features, the linear relationship between partition coefficient and molecular volume resulting from mutual compensation between relatively irregular solvation energies in water and *n*-octanol.

Abraham wanted to establish whether or not water is unique with respect to the solvation of hydrocarbons. He observed that plots of ΔG^0_s for a series of solutes against solute radius gave good straight lines with every solvent studied with the exception of water.¹³¹ For water, a straight line was obtained for solution of the rare gases but not for the solution of alkanes, and he suggests the latter gives rise to anomalous free energies. It should be noted that any analysis of ΔG^0_s values and solute size will not give unambiguous results because of the inherent difficulty of assigning solute radii.

Following Cramer,¹²⁴ Abraham constructed plots of ΔG^0_s against solute molecular volume for *n*-alkanes and rare gases. Taking *n*-octanol and benzene he shows that, instead of drawing two straight lines resulting in a V-shape, a smooth curve can be drawn through all the points. From this he implies, in contrast to Cramer, that the mechanism of solvation of the rare gases and the *n*-alkanes could be similar. Cramer's analysis in terms of ΔG^0_s and molecular volume is largely a personal judgement as to whether the plot for *n*-octanol is treated as two straight lines, or whether it is actually a smooth continuous curve.

Abraham went on to show that ΔG^0_s values for both rare gases and alkanes in over sixteen nonaqueous solvents are well correlated through two linear equations, [4.7] and [4.8], where *R* is a solute parameter related to solute radius.

$$\Delta G^0_{s(\text{solvent})} = m \Delta G^0_{s(\text{benzene})} + c \quad \text{....[4.7]}$$

$$\Delta G^0_{s(\text{solvent})} = l R + d \quad \text{....[4.8]}$$

When applied to ΔG^0_s values for solution in water, the equations showed conclusively that the solution of alkanes (but not rare gases) in water is quite anomalous. Once again, this implies that in any nonaqueous solvent the nature of the solution process of the rare gases and *n*-alkanes is basically the same, as is the solution of rare gases in water and in organic solvents, but the solution of *n*-alkanes in water is fundamentally different from solution in the nonaqueous solvents. The ΔG^0_s values for the *n*-alkanes in water were

more positive than expected confirming the existence of the hydrophobic effect. Further reasons why Cramer and Abraham have made different conclusions are that Abraham used considerably more data with regard to both the number of solutes and solvents, and Cramer's analysis relies very much on a given set of molecular radii or volumes.

From the standard free energies of solution of gaseous solutes, Abraham obtained a value of $0.92 \text{ kcal mol}^{-1}$ per $-\text{CH}_2-$ increment for transfer of *n*-alkanes from hexane to water.¹³¹ This was separated into a favourable gas \rightarrow hexane contribution of $0.74 \text{ kcal mol}^{-1}$ and an unfavourable (hydrophobic) gas \rightarrow water contribution of $0.18 \text{ kcal mol}^{-1}$. From this it appears that the hydrophobic effect is very small but this value for the gas \rightarrow water transfer includes a negative effect due to a normal solution process as well as the positive hydrophobic effect. The increment for the gas \rightarrow water transfer of $0.18 \text{ kcal mol}^{-1}$ was further separated into a true (unfavourable) hydrophobic effect of $0.54 \text{ kcal mol}^{-1}$ and a favourable normal solvent effect of $0.36 \text{ kcal mol}^{-1}$. Therefore it can be seen that the actual hydrophobic effect per $-\text{CH}_2-$ increment estimated at $0.54 \text{ kcal mol}^{-1}$ is much larger than the overall effect in the gas \rightarrow water transfer of $0.18 \text{ kcal mol}^{-1}$. Abraham's conclusions are in terms of free energy but whether or not the solution of hydrocarbons in water is anomalous in terms of enthalpy or entropy can not be determined on the basis of this free energy data.

Comparison of the above values of the hydrophobic effect per $-\text{CH}_2-$ increment with the estimated incremental binding energy for a methylene group in the aryl binding pocket of CAT ($1.0 \text{ kcal mol}^{-1}$) shows the latter value to be slightly higher. This is as expected 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 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 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.

4.5 1-DEOXYCHLORAMPHENICOL AND ANALOGUES

In order to explore the role of the nitro group in binding of chloramphenicol by CAT a range of *p*-substituted 1-deoxychloramphenicol analogues were prepared (Figure 4.7), and their kinetic parameters as substrates for CAT were determined. In the X-ray structure of the CAT:CM binary complex the nitro group is largely solvent accessible although some protein contact with one of the oxygens appears to exist.^{27a} The strong electron withdrawing properties of a nitro group on a benzene ring could in principle give rise to an electronic contribution to binding through, for example, donor-acceptor π stacking. To test this it was necessary to compare the binding of ligands with *p*-substituents that differed significantly in electronic effect, ranging from strongly electron withdrawing to strongly electron donating.

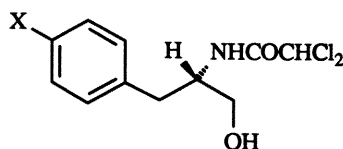
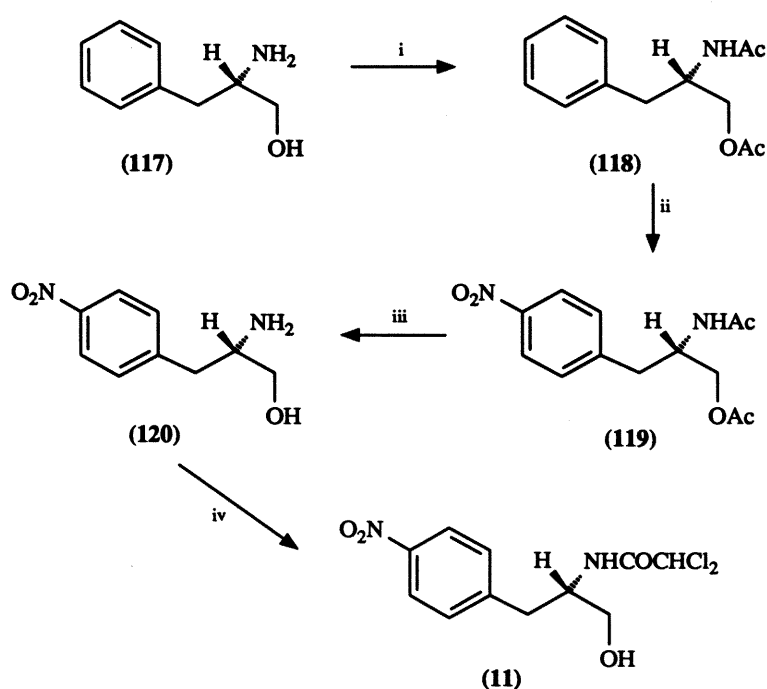


Figure 4.7

In principle, the synthesis of such compounds could be achieved by the general route described in Chapter 3. However, the easy access to the corresponding 1-deoxy compounds (Figure 4.7) with $X = \text{NO}_2$, H, OH and OMe from the amino acids L-phenylalanine and L-tyrosine provided a quick way to access the influence of the *p*-substituent on binding. Although the X-ray structure^{27a} shows a hydrogen bond to the 1-hydroxyl of chloramphenicol from Thr 174 it is via a bridging water molecule and the energetic contribution to binding is small ($0.8 \text{ kcal mol}^{-1}$).⁶² Indeed, 1-deoxy-chloramphenicol (11) is a good substrate for CAT, particularly because of the removal of complications from acyl migration and a second round of acetylation leading to 1,3-diacetylchloramphenicol (9) seen with the natural substrate.^{26,52}

1-Deoxychloramphenicol (11), $X=\text{NO}_2$, was prepared in four steps from the amino alcohol L-phenylalaninol (117) which has the desired absolute configuration at C-2 (see Scheme 4.2).



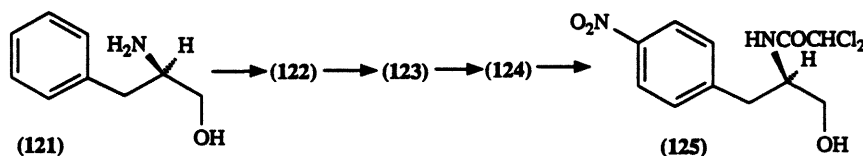
Reagents: i) $(\text{MeCO})_2\text{O}$ (2.1 equiv.), dry pyridine, $<4^\circ\text{C}$, 20h.
 ii) Fuming HNO_3 , $0^\circ\text{C} \rightarrow \text{R.T.}$, 2h.
 iii) 5% aq. HCl , Δ , 2.5h.
 iv) $\text{Cl}_2\text{CHCO}_2\text{Me}$ (2.5 equiv.), EtOH , Δ , 3h.

Scheme 4.2

L-Phenylalaninol was protected as the diacetyl derivative by reaction with acetic anhydride and dry pyridine at $<4^\circ\text{C}$ for 20h. After work-up N,O-diacetyl-L-phenylalaninol (118) was afforded in high yield (91%) and further purification was not necessary. The next step was nitration of the protected amino alcohol by treatment with fuming nitric acid, addition was carried out at 0°C and the reaction mixture was then left to warm to room temperature. Work-up afforded N,O-diacetyl-

L-*p*-nitrophenylalaninol (119) in high yield (91%). The ^1H NMR spectrum of the product showed that the nitration had also produced 17% of the corresponding *ortho*-product (as calculated from the peak height of the doublets corresponding to the -NH-proton), but this was not removed at this stage. Deprotection was carried out by heating (119) in 5% aqueous HCl at reflux for 2.5h, and L-*p*-nitrophenylalaninol (120) was obtained in a high crude yield (90%). Due to the instability of the free amine, (120) was reacted immediately with methyl dichloroacetate in dry ethanol to afford, after purification by flash column chromatography, 1-deoxychloramphenicol (11) in 82% yield. Despite achieving reasonable separation of the *para*- and *ortho*- products on TLC, flash column chromatography did not completely separate the two products even after a number of attempts. Repeated recrystallisations were then carried out to remove the small amount of *ortho*-material that had not been separated by chromatography. The data obtained for (11) were consistent with its proposed structure including a correct accurate mass and C, H and N analysis.

The enantiomer of (11), N-dichloroacetamido-D-*p*-nitrophenylalaninol (125), was also synthesised. It was prepared via the same route as (11) but starting from D-phenylalaninol (121) as shown in Scheme 4.3.

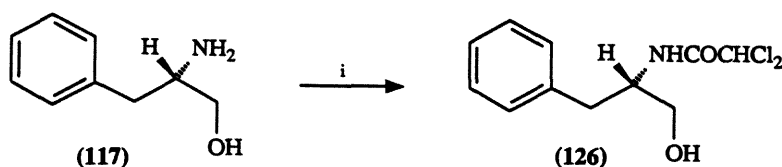


Scheme 4.3

Protection of the amino alcohol to give the N,O-diacetylated product was again afforded in high yield (86%) as was the subsequent nitration (94%) to give N,O-diacetyl-D-*p*-nitrophenylalaninol (123). Deprotection of (123) to give D-*p*-nitrophenylalaninol (124) followed by reaction with methyl dichloroacetate afforded, after purification by flash column chromatography, N-dichloroacetamido-D-*p*-nitrophenylalaninol (125) in

82% yield. Similar purification difficulties as encountered with (11) were also found with (125), but again, repeated recrystallisations successfully removed the small proportion of remaining *ortho*-product. The data obtained for (125) were identical to that for (11), consistent with it being the enantiomer. Results of the kinetic study of (125) with CAT are discussed in Chapter 5.

N-Dichloroacetamido-L-phenylalaninol (126), X=H, was prepared in one step from L-phenylalaninol as shown in Scheme 4.4.

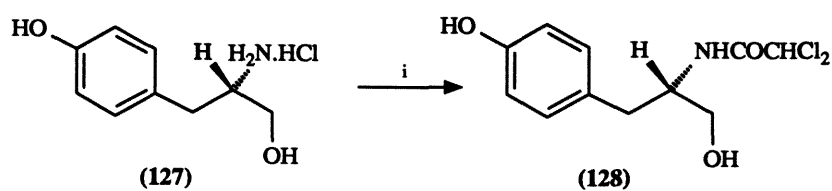


Reagents: i) $\text{Cl}_2\text{CHCO}_2\text{Me}$ (2 equiv.), EtOH, Δ , 5h.

Scheme 4.4

Reaction of L-phenylalaninol (117) with methyl dichloroacetate in ethanol at reflux temperature afforded N-dichloroacetamido-L-phenylalaninol in 57% yield after purification by flash column chromatography. The data obtained for (126) were consistent with its proposed structure including a correct C, H and N analysis.

N-Dichloroacetamido-L-tyrosinol (128), X=OH, was also prepared in only one step, this time starting from the amino alcohol L-tyrosinol which has the desired absolute configuration at C-2 (Scheme 4.5). L-Tyrosinol hydrochloride (127) was reacted with methyl dichloroacetate in methanol with 1.2 equiv. of dry triethylamine at room temperature. After purification by flash column chromatography, dichloroacetamido-L-tyrosinol was afforded in 72% yield. The data obtained for (128) were consistent with its proposed structure including a correct accurate mass and C, H and N analysis.



Reagents: i) $\text{Cl}_2\text{CHCO}_2\text{Me}$ (5.1 equiv.), Et_3N (1.2 equiv.), MeOH, R.T., 48h.

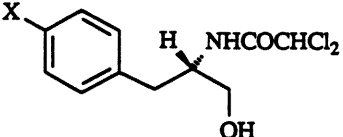
Scheme 4.5

Attempts to methylate the *p*-hydroxyl group of (128) to give the corresponding *p*-methoxy compound (129), $\text{X}=\text{OMe}$, were problematic and led to mixtures that were difficult to separate. Unfortunately, due to time restraints, a pure sample of (129) was not obtained and so its kinetic parameters as a substrate for CAT could not be determined.

4.5i Kinetic Results

1-Deoxychloramphenicol (11), N-dichloroacetamido-L-phenylalaninol (126) and N-dichloroacetamido-L-tyrosinol (128) were all shown to be substrates for CAT. The kinetic parameters for the ligands as CAT substrates are shown in Table 4.3.

Table 4.3 Kinetic parameters for a series of CAT substrates

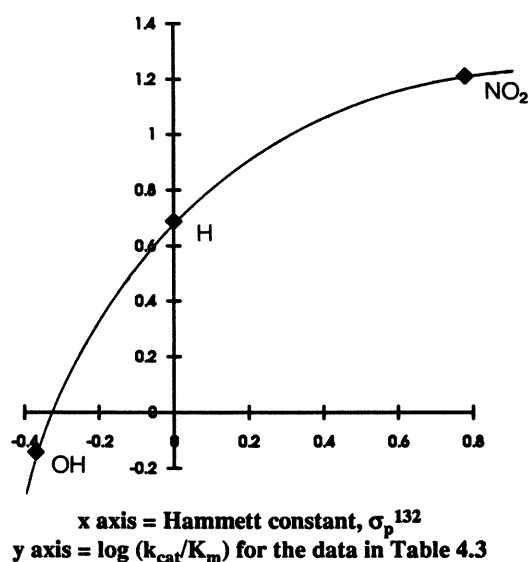
			
X	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m
NO_2 (11)	20 (23) ^a	323 (312) ^a	16.15
H (126)	47	230	4.89
OH (128)	215	154	0.72

^a Literature value ⁶²

As reflected by the plot of $\log k_{\text{cat}}/K_m$ against hydrophobicity (Figure 4.2), the results obtained for the chloramphenicol analogues in which the *p*-nitrophenyl substituent was replaced by an alkyl group suggested that hydrophobic interactions were not involved in the binding and specificity of the nitro group of chloramphenicol by CAT. If, however, using the data from Table 4.3, a plot of $\log k_{\text{cat}}/K_m$ versus the Hammett constant σ_p shows a linear free energy relationship then this would be evidence that there is an electronic contribution to the binding of the *p*-substituents (see Figure 4.8). Even though it is appreciated that it may not be considered fully reliable to use only three

points to construct a graph, it is thought to be valid in this case because it is only a general trend in the plot that is being sought.

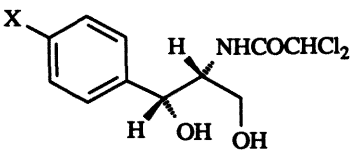
Figure 4.8



As shown in Table 4.3 there is a clear trend in the series, going from a strongly electron donating group (128) to a strongly electron withdrawing group (11) improves both the binding and the catalytic rate. However, the above plot suggests that this is not a linear free energy relationship.

Further work carried out at Leicester University has examined a range of *p*-substituted chloramphenicol analogues to assess their binding by CAT.¹³³ These compounds, all of which were obtained from the Research Laboratories of Parke Davis & Co., possessed *p*-substituents which were electron withdrawing. The kinetic data for these ligands as CAT substrates is however limited. The K_m values of some, but not all, of the substrates are available and are given overleaf in Table 4.4.

Table 4.4 Binding constants for a series of CAT substrates

		
X	K_m (μM)	σ_p
H (130)	85	0
I (131)	15	0.18
Br (132)	20	0.23
CH ₃ CO (133)	good substrate	0.50
CN (134)	12	0.66
SO ₂ CH ₃ (135)	poor substrate	0.72
NO ₂ (6)	11.6	0.78

As found with the 1-deoxychloramphenicol analogues, the binding of the above substrates generally improves with the strength of the electron withdrawing group. The main exception to this, however, was found with the chloramphenicol analogue with the *p*-SO₂CH₃ group (135). If the binding of the *p*-substituent was dominated by electronic effects then it would be predicted that (135) would have a similar K_m to chloramphenicol, which it clearly does not. Although there is a tendency for the binding and specificity of the substrates to improve with the electron withdrawing capabilities of the *p*-substituents, the overall results imply that there is not a strong electronic contribution. This result is not unexpected since the nitro group in the CAT:CM complex is largely in contact with solvent.

The kinetic parameters obtained for 1-deoxychloramphenicol and N-dichloroacetamido-L-phenylalaninol can be used to determine the contribution to binding, $\Delta\Delta G_{app}$, of the $-NO_2$ group and the secondary hydroxyl group of chloramphenicol. Using equation [4.4] and the kinetic parameters for 1-deoxychloramphenicol and N-dichloroacetamido-L-phenylalaninol an apparent binding energy, $-\Delta\Delta G_{app}$, of $0.7 \text{ kcal mol}^{-1}$ is obtained for the $-NO_2$ group. Similarly by comparing the kinetic parameters of chloramphenicol and 1-deoxychloramphenicol a binding energy of $0.7 \text{ kcal mol}^{-1}$ (lit. value $0.8 \text{ kcal mol}^{-1}$)⁶² is calculated for the secondary hydroxyl group of chloramphenicol. This value is fairly low for a hydrogen bond¹² and shows that this hydroxyl contributes little to binding.

CHAPTER 5

ADDITIONAL KINETIC STUDIES AND OVERALL CONCLUSIONS

5.1 INTRODUCTION

The previous chapter described a systematic changing of the chloramphenicol ligand in order to probe the hydrophobic component of the binding of the *p*-nitrophenyl ring. In this chapter more radical changes to the ligand have been made. Some preliminary exploration of the possible range of structures that may serve as substrates for CAT has been undertaken as a prelude to exploiting the CAT-catalysed reaction to access enantiopure chiral molecules of potential application in organic synthesis. In addition, the role of the N-acyl group in binding has been investigated to evaluate the importance of the hydrogen bond to the carbonyl oxygen located in the X-ray structure.^{27a} With these final results the energetics of almost all the individual CAT:CM interactions will now have been evaluated quantitatively. The enantiodiscrimination shown by CAT has been investigated, in particular the interaction of CAT with N-dichloroacetamido-D-threoninol has been studied extensively. This chapter ends with a summary of the overall conclusions that can now be made regarding the interaction of CAT with its substrates, and in particular with chloramphenicol.

5.2 ANALOGUES OF CHLORAMPHENICOL IN WHICH THE DICHLOROACETYL SUBSTITUENT IS VARIED

As discussed in Section 1.5i there are only two direct hydrogen bonds between CAT and chloramphenicol, one between the C-3 hydroxyl of CM and the imidazole N-3 of His 195 and the other between the carbonyl oxygen of the dichloroacetyl group of CM and the hydroxyl of Tyr 25. It is the latter hydrogen bond that is of interest to the work described in this section, the aim was to investigate the effect of varying the electronegative groups of the acetyl substituent.

A series of chloramphenicol analogues were prepared in which the dichloroacetyl group was varied (Figure 5.1).

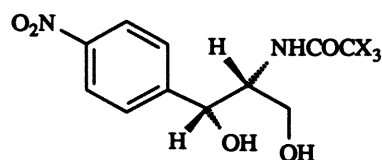
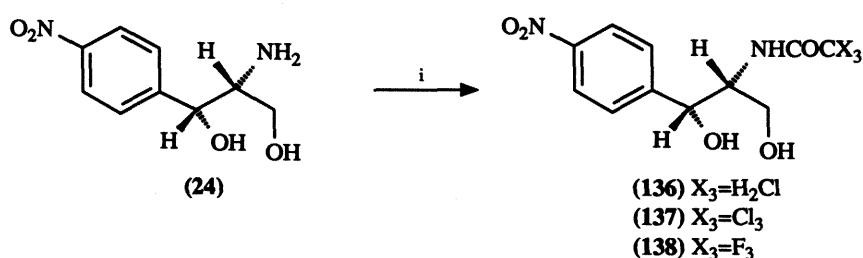


Figure 5.1

Three compounds were synthesised, (1R, 2R)-2-N-chloroacetamido-1-(*p*-nitrophenyl)-propan-1,3-diol (**136**), $X_3 = H_2Cl$, (1R, 2R)-2-N-trichloroacetamido-1-(*p*-nitrophenyl)-propan-1,3-diol (**137**), $X_3 = Cl_3$ and (1R, 2R)-2-N-trifluoroacetamido-1-(*p*-nitrophenyl)-propan-1,3-diol (**138**), $X_3 = F_3$. They were all prepared in one step by reaction of chloramphenicol free base (**24**), (1R, 2R)-2-amino-1-(*p*-nitrophenyl)-propan-1,3-diol, with the appropriate methyl acetate (see Scheme 5.1).



Reagents: i) $\text{CX}_3\text{CO}_2\text{Me}$ (5 equiv.), Et_3N (1 equiv.), MeOH , Δ , 1h.

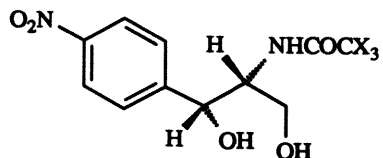
Scheme 5.1

Aminolysis of methyl chloroacetate occurred slower and in a lower yield than that of methyl trichloroacetate and methyl trifluoroacetate. After 10h at reflux temperature (136), $X_3 = \text{H}_2\text{Cl}$, was afforded in 54% yield whereas (137), $X_3 = \text{Cl}_3$, and (138), $X_3 = \text{F}_3$, were obtained in >80% yield after 1h. The data obtained for all three compounds were consistent with their proposed structures. A correct accurate mass was obtained for (136) and the low resolution mass spectrum showed the typical pattern of molecular ions containing one chlorine atom. The material was obtained as a white foam which solidified on standing, attempted recrystallisations were unsuccessful and so C, H and N analysis was not carried out. A correct C, H and N analysis however was obtained for (137) and (138). It should be noted that in the ^{13}C NMR spectra of (137) and (138) the quaternary carbon of CCl_3 and CF_3 was not seen, but the low resolution mass spectrum of (137) indicated the presence of molecular ions containing three chlorine atoms.

5.2i Kinetic Results

As shown in Table 5.1, the analogues of chloramphenicol in which the dichloroacetyl substituent was varied were all shown to be good substrates for CAT. The table includes the kinetic parameters for chloramphenicol and also for (1R, 2R)-2-N-acetamido-1-(*p*-nitrophenyl)-propan-1,3-diol (139), $X_3 = \text{H}_3$, which had previously been prepared by an Undergraduate at Leicester University.

Table 5.1 Kinetic parameters for a series of CAT substrates

			
X_3	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m
H_3 (139)	52	286	5.5
H_2Cl (136)	36	606	16.8
HCl_2 (6)	12	599	49.9
Cl_3 (137)	6	185	30.8
F_3 (138)	20	200	10.0

The carbonyl oxygen of the dichloroacetyl group of chloramphenicol is directly hydrogen-bonded to CAT, and so theoretically the greater the electronegativity of the acetyl substituents the greater the strength of the hydrogen bond. However, since the binding process is an exchange reaction, if the substrate could potentially bind tighter to the enzyme due to an increase in the strength of the hydrogen bond it would also be harder to desolvate and so there would be a greater free energy loss on transfer of the substrate from water to enzyme. The aim was to determine the optimum balance, and as expected this was obtained with the natural substrate chloramphenicol as reflected by the specificity constant, k_{cat}/K_m .

As shown in Table 5.1, there is a clear trend in the series. Increasing the number of chlorine substituents from 0→3 improves the binding (as approximated by K_m), with the trichloro analogue (137) actually binding tighter than chloramphenicol. The crystal structure of the binary complex of CAT:CM^{27a} suggests that the two chlorines of

chloramphenicol make van der Waals contact with the enzyme, and so the tighter binding of (137) could be due to an increase in van der Waals contact with the additional chlorine and/or to an increase in the hydrogen bond strength.

By comparing the kinetic parameters of (139), $X_3=H_3$, and chloramphenicol, an apparent binding energy, $\Delta\Delta G_{app}$, of 1.3 kcal mol⁻¹ for the dichloro group of chloramphenicol is obtained. This contribution to binding is presumably due to the increase in the strength of the hydrogen bond and also to the extra van der Waals contacts on going from CH₃ to CHCl₂. The effect of removing the dichloroacetyl group altogether has also been investigated.¹³⁴ Chloramphenicol base (24) was found to be a poor substrate for CAT with a greatly increased K_m of 4300 μ M and a k_{cat} of 123 s⁻¹. The standard assays are carried out at pH 7.5, at this pH (24) is protonated therefore making desolvation difficult, as reflected by the high K_m . Evidence to support this was obtained when the pH was raised to 8.5. Under these conditions it was found that the binding of chloramphenicol base was dramatically improved, its kinetic parameters being $K_m=64.5 \mu$ M and $k_{cat}=161$ s⁻¹. Comparison of these parameters with those of chloramphenicol itself give an apparent binding energy, $\Delta\Delta G_{app}$, for the COCHCl₂ group of 1.8 kcal mol⁻¹. This result together with the $\Delta\Delta G_{app}$ of 1.3 kcal mol⁻¹ for the dichloro group implies that the strength of the hydrogen bond between the carbonyl oxygen of chloramphenicol and Tyr 25 is greatly influenced by the presence of the electronegative chlorine atoms.

5.3 ALTERNATIVE SUBSTRATES FOR CAT

There has been considerable interest in the use of enzymes as "reagents" in organic synthesis, particularly to give access to enantiopure chiral molecules.¹³⁵ In principle, if CAT is able to discriminate between a pair of enantiomers it could be used in such a way. For example, if a racemic mixture of β -amino alcohols were subjected to CAT and acetyl CoA, and CAT catalysed the acetylation of one enantiomer in preference to the other, this would be a means of separating the two enantiomers. The acetylated product and unreacted alcohol could be extracted from the catalyst and coenzyme and then be separated by, for example, flash column chromatography.

This would be particularly useful because β -amino alcohols are biologically and pharmaceutically interesting compounds. There are many cases where these structures are found as components of complex active substances or natural products.¹³⁶ Examples of which are statine (140) and sphingosine (141), Figure 5.2. Statine is a constituent of a number of protease inhibitors, notably pepstatin which is a strong inhibitor of aspartic proteases such as pepsin and renin,¹³⁷ and sphingosine is the backbone component of the biologically important sphingolipids and is usually present as the D-(+)-*erythro* isomer.¹³⁸

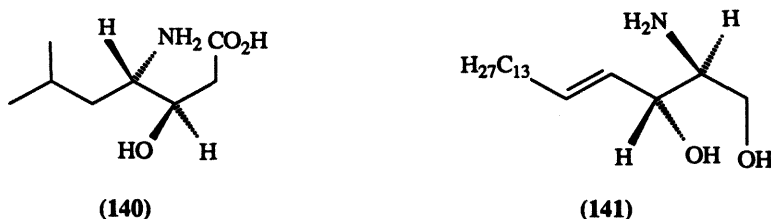


Figure 5.2

A series of mono alcohols were prepared (Figure 5.3) in order to make a preliminary investigation of the range of structures that may be substrates for CAT.

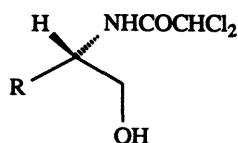
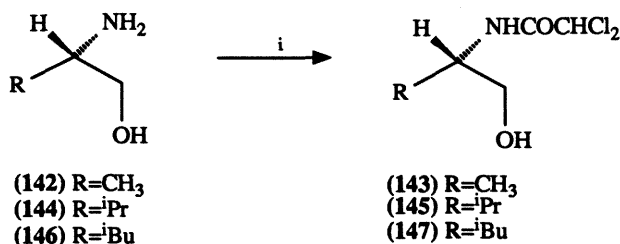


Figure 5.3

The only similarities between these compounds and chloramphenicol are the primary hydroxyl and N-dichloroacetyl group, and also the same relative configuration as CM was retained at C-2. As discussed in Chapter 4, even though chloramphenicol has the 1,3-diol structure the presence of a secondary hydroxyl group was not considered to be a prerequisite for possible CAT substrates because it has previously been shown that the C-1 hydroxyl of CM only contributes 0.7-0.8 kcal mol⁻¹ to binding. The four compounds that were prepared were all derived from naturally occurring amino acids.

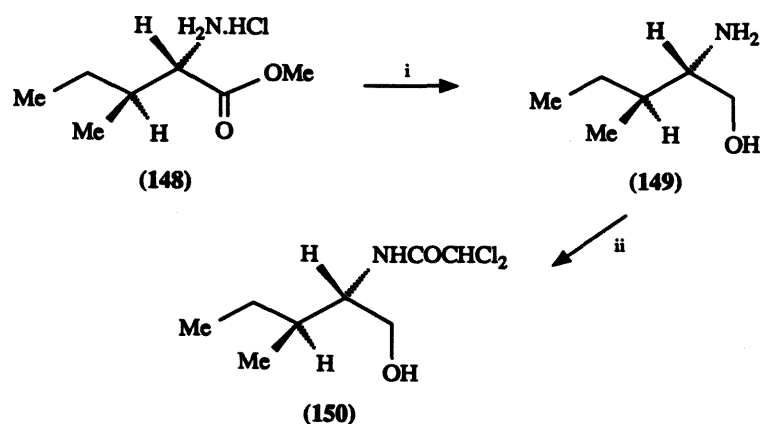
(2S)-2-N-Dichloroacetamido-1-propanol (143), R=CH₃, (2S)-2-N-dichloroacetamido-3-methyl-1-butanol (145), R=ⁱPr, and (2S)-2-N-dichloroacetamido-4-methyl-1-pentanol (147), R=ⁱBu, were obtained in high yield (87-94%) by aminolysis of methyl dichloroacetate with L-alaninol (142), R=CH₃, L-valinol (144), R=ⁱPr, and L-leucinol (146), R=ⁱBu, respectively (see Scheme 5.2).



Reagents: i) Cl₂CHCO₂Me (2 equiv.), EtOH, Δ, 6h.

Scheme 5.2

(2S,3S)-2-N-Dichloroacetamido-3-methyl-1-pentanol (**150**), $R=CH_3CH_2(CH_3)CH$, was prepared in two steps by reduction of L-isoleucine methyl ester hydrochloride followed by reaction with methyl dichloroacetate (Scheme 5.3).



Reagents: i) $NaBH_4$ (4 equiv.), water, $<4^\circ C$, 20h.
 ii) Cl_2CHCO_2Me (2.5 equiv.), EtOH, Δ , 7h.

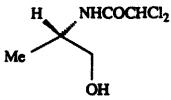
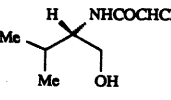
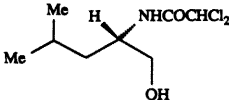
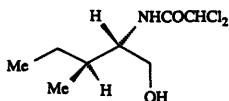
Scheme 5.3

L-Isoleucine methyl ester hydrochloride (**148**) was reduced with sodium borohydride in water to afford L-isoleucinol (**149**) in moderate yield (58%) after purification by Kugelrohr distillation. The amino alcohol (**149**) was then reacted immediately with methyl dichloroacetate in ethanol to give N-dichloroacetamido-L-isoleucinol (**150**) in 99% yield. The data obtained for (**143**), (**145**), (**147**) and (**150**), including C, H and N analysis and accurate mass, were consistent with their proposed structures.

5.3i Kinetic Results

All four mono alcohols were shown to be CAT substrates, the kinetic parameters for which are shown in Table 5.2.

Table 5.2 Kinetic parameters for alternative CAT substrates

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m
	4.1	4.4	1.07
	1.1	3.4	3.09
	1.2	5.2	4.33
	1.7	2.0	1.18

When carrying out assays to determine the kinetic parameters of a substrate for CAT, the rate of acetylation is measured by monitoring the release of CoA by its reaction with DTNB (see Section 4.2). It has been shown that simple hydrolysis of AcCoA does occur and this is another source of CoA in the assay mixture,¹³⁴ the rate of this, however, is very slow, approximately 10^4 times slower than the rate of acetylation of chloramphenicol which is $600 s^{-1}$. Even though the turnover number (k_{cat}) of each of the

mono alcohols is very low the possibility that the measured rate is due to hydrolysis of AcCoA or even to contaminating chloramphenicol can be excluded. For each set of assays where the AcCoA concentration was constant and the substrate concentration was varied it was shown that the rate of release of CoA increased with the concentration of substrate, and was therefore due to acetylation of the substrate.

This series of compounds were prepared to find out whether there is potential for using CAT in organic synthesis. These alcohols lack many of the features of chloramphenicol and the initial aim was to see if they would in fact be substrates for CAT. Future work could examine the extent of the range of alcohols that CAT could recognise and catalyse the acetylation of, leading possibly to the kinetic resolution of racemic amino alcohols. It should be noted that these were only preliminary investigations to see how specific CAT is, but as the following discussion shows the above results have led to a greater understanding of the binding process and the interactions between CAT and its substrates.

The results obtained for N-dichloroacetamido-L-alaninol (143) can be compared with those for N-dichloroacetyl serinol (116) since the only difference in their structure is that in the former there is a proton at C-3 and in the latter a hydroxyl group. As shown in Table 4.1 the kinetic parameters for (116) are: $K_m=15$ mM, $k_{cat}=20$ s⁻¹ and $k_{cat}/K_m=1.3$. The change in k_{cat}/K_m gives an apparent binding energy, $\Delta\Delta G_{app}$, of only 0.12 kcal mol⁻¹ for the secondary hydroxyl group. This value is considerably lower than the 0.7 kcal mol⁻¹ obtained from the kinetic parameters of chloramphenicol and 1-deoxychloramphenicol (Section 4.5i). A possible explanation for this difference is that for the serinol derivative (116) the lack of a substituent in the *p*-nitrophenyl binding site may allow rotation about the bond as shown overleaf in Figure 5.4. Therefore, on formation of the hydrogen bond to the hydroxyl of (116) a rotational degree of freedom would be frozen out and this would have an adverse entropy change.

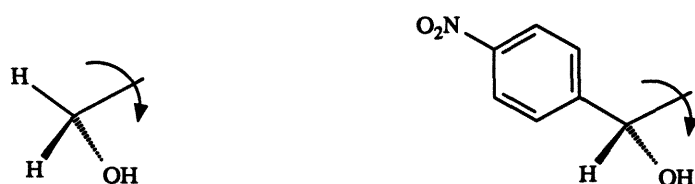


Figure 5.4

In the case of chloramphenicol, rotation about the bond shown in Figure 5.4 is prevented due to the binding of the large *p*-nitrophenyl group, and so formation of the hydrogen bond does not have to freeze out this rotation therefore the hydrogen bond appears stronger.

Comparison of the kinetic parameters of N-dichloroacetamido-L-alaninol with 1-deoxychloramphenicol (Table 4.3) allow an apparent binding energy for the *p*-nitrophenyl group to be calculated. The change in k_{cat}/K_m corresponds to a free energy difference of $5.7 \text{ kcal mol}^{-1}$ which is similar to the value of $6.3 \text{ kcal mol}^{-1}$ obtained from the kinetic parameters of N-dichloroacetyl serinol and chloramphenicol. It was previously estimated that the aryl binding pocket of chloramphenicol is approximately 1.5 times more hydrophobic than *n*-octanol (Section 4.3iii). The free energy of transfer, ΔG_t , of a phenyl ring from *n*-octanol to water is $2.91 \text{ kcal mol}^{-1}$, therefore if the above proposal is correct it could be predicted that the aryl ring of chloramphenicol would contribute approximately $4.4 \text{ kcal mol}^{-1}$ to binding. Examination of the kinetic parameters of N-dichloroacetamido-L-alaninol and N-dichloroacetamido-L-phenylalaninol (126) gives an apparent binding energy for the aryl ring of $5.0 \text{ kcal mol}^{-1}$ which is in good agreement given the errors in measurement.

N-Dichloroacetamido-L-valinol (145) and N-dichloroacetamido-L-threoninol (46) ($K_m=8.7 \text{ mM}$, $k_{cat}=90 \text{ s}^{-1}$, Table 4.1) are isosteric, the former having a methyl group at C-3 and the latter an hydroxyl group. Comparison of their kinetic parameters shows that the binding of (145) (as approximated by K_m) is greater than that of (46). However,

N-dichloroacetamido-L-threoninol is a better substrate for CAT as reflected by the specificity constant k_{cat}/K_m . As with chloramphenicol, the secondary hydroxyl is probably hydrogen bonded to Thr 174 via a bridging water molecule and the additional stabilisation provided by this lowers the activation energy of k_{cat} and so improves catalysis.

As shown in Table 5.2 N-dichloroacetamido-L-leucinol (147) is the best substrate for CAT out of the mono alcohol series. The additional binding energy provided by the $i\text{Pr}$ group, which is presumably bound in the hydrophobic pocket which accommodates the *p*-nitrophenyl group of chloramphenicol, also contributes to the catalytic rate. Even though the binding of N-dichloroacetamido-L-isoleucinol (150) is similar to that of (147) it is a poorer substrate for CAT as shown by the specificity constant. This is probably due to the fact that it has a second chiral centre at C-3. The ethyl group is expected to be positioned in the *p*-nitrophenyl binding pocket and so consequently the methyl group is forced into an area normally occupied by a proton, the result of which is an increase in the energy of activation and a reduction in k_{cat} .

5.4 ENANTIODISCRIMINATION

N-Dichloroacetamido-D-threoninol (**53**), (Figure 5.5), was prepared in order to investigate enantiodiscrimination shown by CAT, its synthesis was previously described in Section 2.5ii.

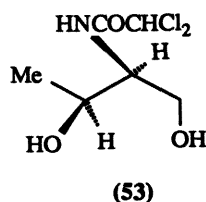


Figure 5.5

Since (**53**) has the opposite relative configuration of chloramphenicol it was not known if it would be a substrate for CAT. Enzymes show varying degrees of stereospecificity, however, as a class the transferases are generally much more substrate- and stereo-specific.

Kinetic studies on the reaction of CAT with (**53**) revealed that it was, indeed, a substrate, with a smaller K_m compared to N-dichloroacetamido-L-threoninol (**46**) but the rate of acetylation, k_{cat} , was considerably reduced. These results were rather surprising since the inversion of the two chiral centres was expected to disrupt the major polar interactions with the protein.

Table 5.3 Kinetic parameters for a pair of enantiomers

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m
N-Dichloroacetamido-L-threoninol (derived from L-threonine)	8.7	91	10.5
N-Dichloroacetamido-D-threoninol (derived from D-threonine)	5.0	0.8	0.16

For the analogous CAT-catalysed reaction to occur i.e. acetylation of the primary hydroxyl, the C-1 hydroxyl of (53) would have to be positioned next to His 195, the amino acid directly involved in the catalysis (Section 1.4).

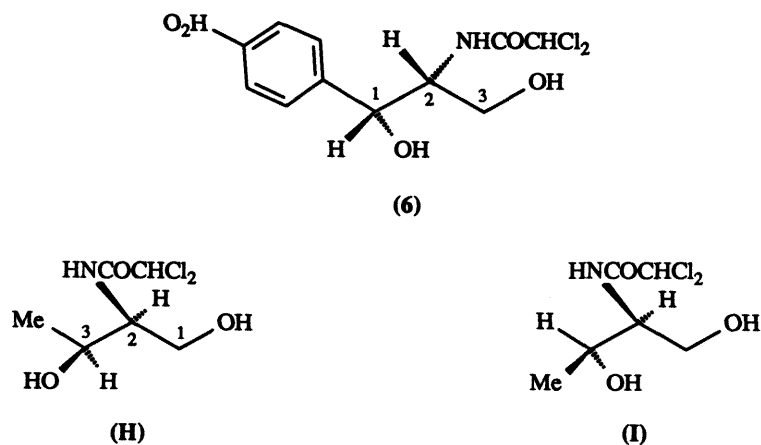


Figure 5.6

As shown by Figure 5.6, in this orientation, (H), the comparatively large N-dichloroacetyl group is displaced from the normal position in chloramphenicol (6), although there may be a conformation of (53) that can compensate for this. In the

absence of such a compensating conformational change there would be a loss of the direct hydrogen bond between Tyr 25 and the dichloroacetyl carbonyl group of chloramphenicol (Section 1.5i). Furthermore, for N-dichloroacetamido-D-threoninol to retain the hydrogen bond which occurs between the secondary hydroxyl of chloramphenicol and Thr 174 via a bridging water molecule, then it is likely that a further conformational change is required e.g. the methyl group would have to be accommodated in the chloramphenicol H-C(1) pocket as shown in conformation (I), (Figure 5.6).

In contrast, by changing the orientation of (53) in the active site, (J) in Figure 5.7, the N-dichloroacetyl group of (53) is positioned correctly in the chloramphenicol dichloroacetyl pocket but the secondary (C-3) hydroxyl lies next to His 195.

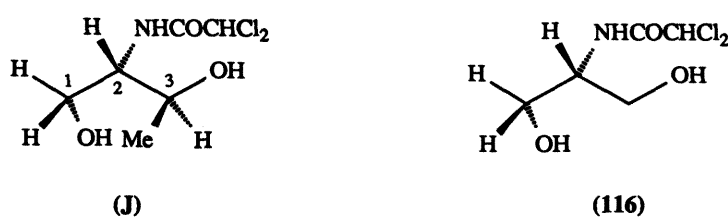
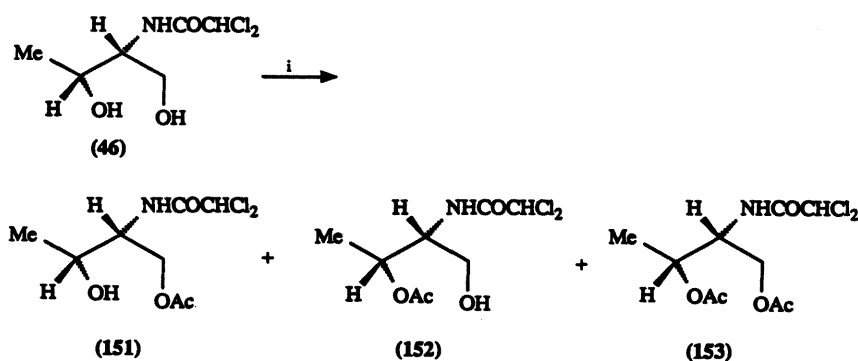


Figure 5.7

Positioned in this way the only difference between N-dichloroacetamido-D-threoninol and N-dichloroacetyl serinol (116) is a methyl group at C-3 rather than a hydrogen. If (53) bound to CAT in this orientation the normal hydrogen bonds would be retained and the only difficulty would be accommodating the methyl group in the site that normally accommodates a hydrogen.

The possibility that the very slow reaction of (53) could occur via (J) could not be excluded. That is to say, CAT could in principle process a pair of enantiomers in two different ways i.e. by catalysing the acetylation of the primary (C-1) hydroxyl of N-dichloroacetamido-L-threoninol (46) and the secondary (C-3) hydroxyl of N-dichloroacetamido-D-threoninol (53).

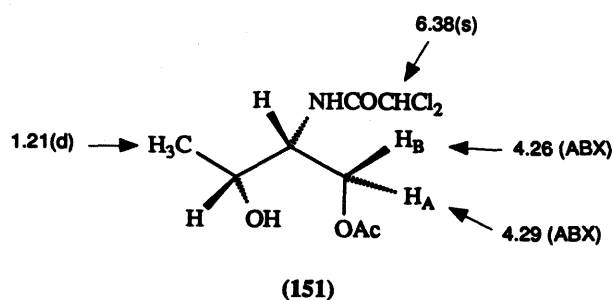
In order to investigate this proposal the CAT catalysed reactions of (46) and (53) were monitored by ^1H NMR over a period of approximately 1h. To assist with the analysis of the enzymic reaction 1-O-acetyl- (151), 3-O-acetyl- (152) and 1,3-diacetyl-N-dichloroacetamido-L-threoninol (153) were prepared in advance in order to determine ^1H NMR data for each compound. They were synthesised by the reaction of N-dichloroacetamido-L-threoninol and acetic anhydride in pyridine (Scheme 5.4).



Reagents: i) Acetic anhydride (1 equiv.), pyridine, $<4^\circ\text{C}$, 3 days.

Scheme 5.4

The mixture of products was separated by flash column chromatography to afford 1,3-diacetyl-N-dichloroacetamido-L-threoninol (20%), 1-O-acetyl- (22%) and 3-O-acetyl-N-dichloroacetamido-L-threoninol (7%). The polarity of the products on TLC was shown to be in the order 3-acetyl- > 1-acetyl- > 1,3-diacetyl-, and their identification was confirmed by the ^1H NMR spectrum of each compound. The crucial chemical shifts with respect to identification were those corresponding to the H-C(3) proton and the prochiral methylene protons at C(1). Selected chemical shifts in ppm observed for (151), (152) and (153) are shown in Figure 5.8, the spectra of (149) and (150) were recorded in D_2O and that of (151) in CDCl_3 .



The signals for H-C(3) and H-C(2) slightly overlapped at 4.01 - 4.16 (m).

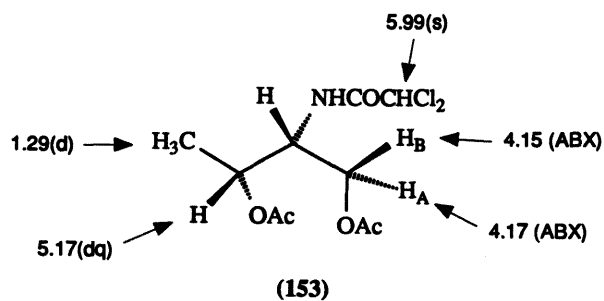
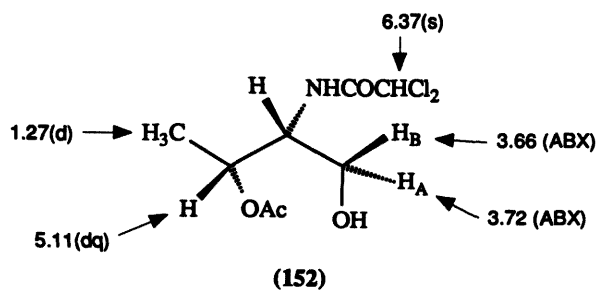


Figure 5.8

Typical δ values for methylene and methine protons attached to carbons bearing an -OH and an -OCOR group are given in Table 5.4. The observed values are consistent with those given in the table confirming the identification of each compound.

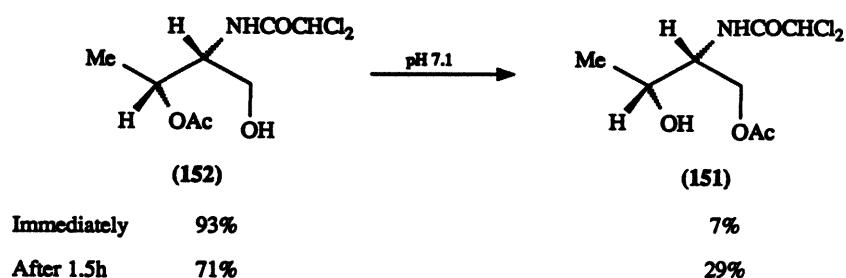
Table 5.4

Protons	Typical Value ¹³⁹ δ (ppm)
-CH ₂ OH	3.6
-CH(OH)-	3.9
-CH ₂ OCOR	4.1
-CHOCOR	4.8

The CAT catalysed reaction of both enantiomers, (46) and (53), was continuously monitored by ¹H NMR and shown to produce the same product throughout the reaction period, and this was the primary acetylated product, 1-O-acetyl-N-dichloroacetyl threoninol, the secondary acetylated product was not detected. After each reaction had been monitored for 1h the reaction mixture was acidified to pH 5-6 (in an attempt to inhibit the isomerisation reaction, see Scheme 1.1), extracted into ethyl acetate, and evaporated to give a mixture of product and unreacted N-dichloroacetamido-threoninol. The residue was then examined by ¹H NMR in D₂O solvent, and in both cases the presence of 3-O-acetyl-N-dichloroacetyl threoninol could now be detected. The extracted product from the reaction of the L-enantiomer (46) was shown to consist of 79% 1-O-acetylated product and 21% 3-O-acetylated product, and that from the reaction of the D-enantiomer (53), 74% and 26% respectively. The percentages were calculated from the peak height of the singlet corresponding to the -CHCl₂ proton.

The results imply that it was the primary acetylated product that formed in the CAT catalysed reaction of N-dichloroacetamido-D-threoninol (53), with 3-O-acetyl-N-dichloroacetamido-D-threoninol being produced during the work-up and/or while in the solvent. To confirm that 1-O-acetyl-N-dichloroacetamido-D-threoninol was the initial product it was necessary to determine whether the secondary acetylated product could

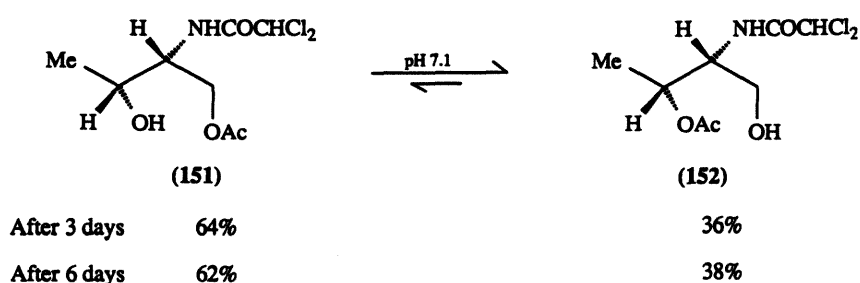
isomerise immediately to the corresponding primary acetylated product under the NMR reaction conditions. A sample of (152) was dissolved in the D₂O buffer (pH 7.1) and its ¹H NMR spectrum was recorded immediately and then after a further 1.5h (Scheme 5.5). The ratios were calculated from the peak height of the doublets corresponding to the Me-C(3) protons.



Scheme 5.5

As shown in Scheme 5.5 the initial NMR sample contained 7% of the primary acetylated product but it was not possible to determine whether this was a result of immediate isomerisation, or whether the sample of (152) which had been purified by flash column chromatography contained a small amount of (151). After being in solution for 1.5h the amount of (151) present had increased to only 29% thus confirming that the 1-O-acetyl-N-dichloroacetamido-D-threoninol observed during the monitoring of the CAT reaction was the product obtained in the CAT catalysed acetylation of N-dichloroacetamido-D-threoninol.

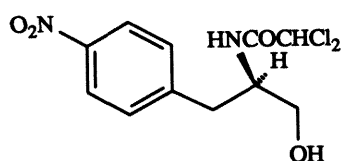
The equilibrium of the isomerisation reaction was also studied. A sample of 1-O-acetyl-N-dichloroacetamido-L-threoninol (151) was dissolved in the D₂O buffer (pH 7.1) and its ¹H NMR spectrum recorded after 3 days and after 6 days (Scheme 5.6 overleaf). The above results imply that the equilibrium position lies in favour of the primary acetylated compound, (151) and (152) existing in a ratio of approximately 2:1 respectively.



Scheme 5.6

Having shown that the enzymic acetylation of N-dichloroacetamido-D-threoninol produces the unexpected primary acetylated product the next consideration is how is the substrate bound at the active site? There is clearly no direct evidence to suggest that it is bound at the active site with the same conformation as its enantiomer. One possibility is that there is more than one mode of binding of this substrate, such that it may bind in an alternative unreactive mode at the active site in competition with a weaker productive mode of binding. Work on 1-substituted chloramphenicol analogues in conjunction with site directed mutagenesis and X-ray structure determination has previously suggested alternative binding modes for CAT substrates⁶² (see Section 1.6). At present work is being carried out at York University to obtain a X-ray structure of the binary complex of CAT and N-dichloroacetamido-D-threoninol which will assist greatly in the interpretation of the observed results.

Having determined that CAT could bind and acetylate N-dichloroacetamido-D-threoninol (53), we decided to examine another analogue which has the opposite relative configuration of chloramphenicol. The compound investigated was N-dichloroacetamido D-*p*-nitrophenylalaninol (125), the enantiomer of 1-deoxychloramphenicol, its synthesis was previously described in Section 4.5.



(125)

Since (53) was shown to have been acetylated at the primary hydroxyl, implying that the N-dichloroacetyl substituent could be accommodated in the active site of CAT, it was thought that (125) would be a potential substrate. Kinetic studies, however, implied that this was not the case. Further investigations showed that (125) was in fact a potent competitive inhibitor of CAT with a K_i of 15.0 μM . Comparison with its enantiomer, 1-deoxychloramphenicol, which has a K_m of 20 μM (Table 4.3) shows that the inhibitor is bound very tightly by CAT, however, since (125) is not acetylated by CAT this suggests that it too is bound in an alternative nonproductive mode.

5.5 OVERALL CONCLUSIONS

As shown in Chapter 2.5 a wide range of diastereo- and enantiopure chloramphenicol analogues have been successfully synthesised, the majority of which were derived from L- and D-amino acids. By studying the kinetics of the CAT reaction with these modified ligands the contribution to binding and catalysis, $\Delta\Delta G_{app}$, of each of the substituents of chloramphenicol has been evaluated quantitatively. When examining binding energies of specific interactions it is important to define how they were derived. In each case $\Delta\Delta G_{app}$ was calculated using equation [4.4] and as such is the difference between the binding energies of the functional groups involved in the comparison. It would be incorrect to interpret these values as the actual binding energies of the specific groups, ΔG_{bind} , because as discussed in Section 1.1iii there is a fundamental difference between ΔG_{app} and ΔG_{bind} . However, if the removal of a group on the substrate allows access of water to the region where the deletion occurs then ΔG_{app} can be considered as a crude approximation of ΔG_{bind} . The apparent binding energies for the different substituents of chloramphenicol are shown in Figure 5.9.

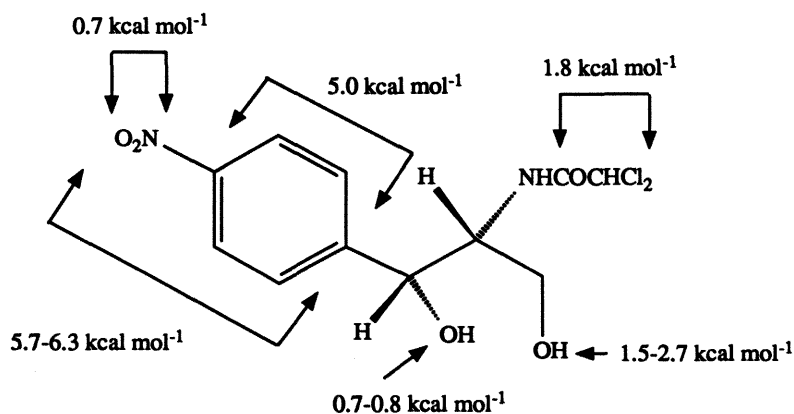


Figure 5.9

The necessity of acknowledging which two substrates were used for obtaining the above values can be seen by the following examples. By comparing the kinetic parameters of 1-deoxychloramphenicol (11) and N-dichloroacetamido-L-alaninol (143), (Section 5.3i), the contribution to binding of the *p*-nitrophenyl group of chloramphenicol was estimated to be 5.7 kcal mol⁻¹, whereas comparing chloramphenicol and N-dichloroacetyl serinol (116), (Section 5.1), a value of 6.3 kcal mol⁻¹ was obtained. The apparent binding energy of the secondary hydroxyl group of chloramphenicol was calculated as 0.7-0.8 Kcalmol⁻¹ by comparing chloramphenicol with 1-deoxychloramphenicol, however a considerably lower value, 0.12 Kcalmol⁻¹, was obtained when N-dichloroacetyl serinol was compared with N-dichloroacetamido-L-alaninol (Section 5.3i). The above differences in binding energy clearly depend on the basis of the comparison.

Examination of the aryl binding pocket of chloramphenicol using a series of analogues in which the *p*-nitrophenyl group of CM was replaced by alkyl groups suggests that this region of the active site is approximately 1.5 times more hydrophobic than *n*-octanol (Chapter 4). The linear free energy relationship shown by these substrates is evidence that the alkyl chain is located in this pocket and that it is a local hydrophobic region. The incremental apparent binding energy for each methylene group bound in the hydrophobic cleft is estimated to be 1.0 kcal mol⁻¹. The binding site can only enclose an alkyl chain up to C₄, if the chain is longer than this it is proposed that the terminal methylene groups will be in contact with solvent. This is demonstrated by the break in the linear free energy plot and confirmed by molecular modelling of these ligands in the CAT X-ray structure.

As a result of the work carried out for the purpose of this thesis together with previous studies^{62,63} it can be concluded that hydrophobic effects are dominant in chloramphenicol recognition by CAT. A large number of chloramphenicol analogues have been shown to be substrates for CAT. It is believed that this is due to a lack of many direct hydrogen bonds together with the dominant hydrophobic interactions in the CAT:CM complex,

which in some cases apparently leads to alternative binding modes. However, the apparent lack of specificity may be a reflection of the fact that CAT is such a catalytically efficient enzyme, the k_{cat} for its natural substrate being 600 s^{-1} . If, k_{cat}/K_m of the altered substrates is compared with that of chloramphenicol it can be seen just how specific CAT actually is towards chloramphenicol.

CHAPTER 6

EXPERIMENTAL

6.1 GENERAL EXPERIMENTAL DETAILS

6.1i Materials

All chemicals were obtained from:- Aldrich Chemical Company Ltd. (Gillingham, Dorset), Sigma Chemical Company Ltd. (Poole, Dorset), or Lancaster Synthesis (Morecambe, Lancs.), with the exception of Coenzyme A which was obtained from Pharmacia LKB Biotechnology (Milton Keynes, Bucks.).

6.1ii Solvents

Dichloromethane was distilled from calcium hydride.

Diethyl ether (ether) was distilled from LiAlH_4 under a nitrogen atmosphere.

Ethyl acetate was distilled.

Methanol and ethanol were distilled from magnesium and iodine under a nitrogen atmosphere and stored over 3 Å and 4 Å molecular sieves respectively.

Tetrahydrofuran (THF) was passed through an alumina column and distilled from sodium in the presence of benzophenone under a nitrogen atmosphere.

Toluene was dried over calcium chloride, distilled and stored over 5 Å molecular sieves.

Triethylamine was distilled from calcium hydride and stored over 4 Å molecular sieves.

Water was obtained from the Elgastat Option 4 water purifier.

6.1iii Instrumentation and Methods

Thin layer chromatography (TLC) was performed on Merck & Co. silica gel 60 F₂₅₄ plates and aqueous KMnO_4 was used for staining. Flash column chromatography was carried out using silica (Merck & Co., Kiesel 60, 230-400 mesh).

Melting points were determined using a Kofler hotstage and are uncorrected.

Optical rotations were determined at ambient temperature with a Perkin-Elmer 141 polarimeter and are the average of at least six measurements.

Microanalysis (C, H & N) was performed by Butterworth Laboratories, Teddington, Middlesex.

Infra red (IR) spectra were recorded with a Perkin-Elmer 298 spectrometer.

^1H , ^{13}C and ^{19}F NMR spectra were recorded with a Bruker AM 300 spectrometer. For ^1H and ^{13}C spectra, trimethylsilane (TMS) or 2,2-dimethyl-2-silapentane-5-sulphonic acid sodium salt (DSS) was used as an internal standard, and for ^{19}F spectra trichlorofluoromethane (CFCl_3) was used.

Low resolution mass spectra [Electron Ionisation (EI)] were recorded with a Kratos Concept H mass spectrometer. High resolution accurate mass spectra [EI or Chemical Ionisation (CI)] were also recorded with the above spectrometer or carried out by the SERC Mass Spectrometry Centre, University College of Swansea.

Ultra violet (UV) spectra were recorded on a Shimadzu UV-visible UV-240 spectrophotometer.

6.2 SYNTHESIS RELATING TO CHAPTER 2

(3R)-3-Amino-3-N-*tert*-butoxycarbonyl-4-hydroxybutan-2-one (36)

1.5 M MeLi (as the LiBr complex) in ether (50 ml, 75.00 mmol) was added dropwise over 45 min. to a rapidly stirred solution of N-*tert*-butoxycarbonyl-D-serine (2.50 g, 12.20 mmol) in THF (100 ml) at -78°C, under a nitrogen atmosphere. The reaction mixture was stirred at 0°C for 1h and at room temperature for 3h.

The reaction mixture was added slowly portionwise to a rapidly stirred mixture of ice-water (150 ml) and ether (100 ml). The phases were separated and the aqueous phase extracted with ether (2×100 ml). The combined organics were dried (Na₂SO₄) and evaporated to residue *in vacuo* to give an orange oil which was purified by flash column chromatography (3:1 dichloromethane-ethyl acetate) to give the title compound (1.081 g, 44%) as a colourless oil.

R_f 0.42 (ethyl acetate);

[α]_D reading too low to record;

ν_{max} (CH₂Cl₂)/cm⁻¹ 3670w, 3600w, 3420m, 3050w, 2970m, 2930w, 1705s, 1495s, 1390w, 1365s, 1315w, 1230m, 1165s, 1060m, 1025w, 955w, 900w, 885w, 865w;

δ_H (300 MHz; D₂O+CD₃OD) 1.41 (9H, s, C(CH₃)₃), 2.25 (3H, s, CH₃CO), 3.83 (1H, ABX system, J_{BX}=4.2 Hz, J_{gem}=11.8 Hz, CH₂OH), 3.93 (1H, ABX system, J_{AX}=4.3 Hz, J_{gem}=11.8 Hz, CH₂OH), 4.25-4.27 (1H, m, CHCH₂OH);

δ_C (75 MHz; D₂O+CD₃OD) 29.35 (CH₃), 30.30 (CH₃), 63.35 (CH₂), 64.89 (CH), 83.94 (C), 159.97 (C), 212.95 (C);

m/z 203 (M⁺, <1%), 161 (12), 160 (100), 133 (38), 130 (38), 119 (17), 117 (22), 104 (58), 89 (15), 73 (15);

$C_9H_{18}NO_4$ [MH]⁺ requires 204.1236
found 204.1235.

(2R)-2 Amino-2-N-*tert*-butoxycarbonyl-1-hydroxyheptan-3-one (37)

Method A 6 equivalents of BuLi

2 M BuLi in pentane (40 ml, 80.00 mmol) was added dropwise over 45 min. to a rapidly stirred solution of N-*tert*-butoxycarbonyl-D-serine (2.75 g, 13.41 mmol) in THF (100 ml) at -78°C, under a nitrogen atmosphere. The reaction mixture was stirred at 0°C for 1h and at room temperature for 3h.

The reaction mixture was added slowly portionwise to a rapidly stirred mixture of ice-water (150 ml) and ether (100 ml). The phases were separated and the aqueous phase extracted with ether (2×100 ml). The combined organics were dried (Na₂SO₄) and evaporated to residue *in vacuo* to give an orange oil which was purified by flash column chromatography (3:1 dichloromethane-ethyl acetate) to give the title compound (1.219 g, 37%) as a colourless oil.

Method B 6 equivalents of BuLi with an alternative work up

2 M BuLi in pentane (7.25 ml, 14.50 mmol) was added dropwise over 45 min. to a rapidly stirred solution of N-*tert*-butoxycarbonyl-D-serine (0.495 g, 2.42 mmol) in THF (20 ml) at -78°C, under a nitrogen atmosphere. The reaction mixture was stirred at 0°C for 1h and at room temperature for 3h.

Chlorotrimethylsilane 7.0 ml, 55.00 mmol) was added rapidly to the stirred reaction mixture at 0°C, and the resulting colourless solution was left to warm to room temperature. 1 M NaHCO₃ (15 ml) was added slowly and the mixture was stirred for 0.5h. The phases were separated and the aqueous extracted with ether (2×25 ml). The combined organics were washed with water (15 ml), dried (Na₂SO₄) and evaporated to residue *in vacuo* to give a brown oil which was purified by flash column chromatography

(3:1 dichloromethane-ethyl acetate) to give the title compound (0.107 g, 18%) as a colourless oil.

Method C 8 equivalents of BuLi

Following the same procedure as Method A except using 8 equiv. of BuLi rather than 6 equiv., *N*-*tert*-butoxycarbonyl-D-serine (1.50 g, 7.32 mmol) and 2 M BuLi in pentane (30 ml, 60.00 mmol) gave, after purification by flash column chromatography (3:1 dichloromethane ethyl acetate), the title compound (0.432 g, 24%) as a colourless oil.

R_f 0.53 (ethyl acetate);

$[\alpha]_D -18.3^\circ$ ($c=1.0$ in CH_2Cl_2);

ν_{max} (CH_2Cl_2)/ cm^{-1} 3670w, 3600w, 3420m, 3060w, 2960m, 2930m, 2870m, 1705s, 1490s, 1390w, 1370m, 1230w, 1165m, 1050m, 1025w, 980w, 900w, 860w;

δ_H (300 MHz; CDCl_3) 0.91 (3H, t, $J=7.3$ Hz, CH_3CH_2), 1.19-1.66 (13H, s and m, $\text{C}(\text{CH}_3)_3$ and $\text{CH}_3(\text{CH}_2)_2$), 2.49-2.67 (2H, m, CH_2CO), 2.83 (1H, br s, OH, exchanged with D_2O), 3.87 (1H, ABX system, $J_{\text{BX}}=3.8$ Hz, $J_{\text{gem}}=11.5$ Hz, CH_2OH), 3.95 (1H, ABX system, $J_{\text{AX}}=3.9$ Hz, $J_{\text{gem}}=11.5$ Hz, CH_2OH), 4.24-4.40 (1H, m, CHCH_2OH), 5.67 (1H, d, $J=5.7$ Hz, NH, slowly exchanged with D_2O);

δ_C (75 MHz; CDCl_3) 13.81 (CH_3), 22.27 (CH_2), 25.50 (CH_2), 28.33 (CH_3), 39.70 (CH_2), 61.61 (CH), 63.01 (CH_2), 80.21 (C), 155.96 (C), 208.25 (C);

m/z 246 ($[\text{MH}]^+$, 2%), 190 (11), 172 (29), 160 (94), 159 (10), 104 (100), 87 (21), 86 (11), 85 (80);

$\text{C}_{12}\text{H}_{24}\text{NO}_4$ $[\text{MH}]^+$ requires 246.1705
found 246.1707.

**(2R, 3R)-2-Amino-2-N-*tert*-butoxycarbonylbutan-1,3-diol (40) and
(2R, 3S)-2-Amino-2-N-*tert*-butoxycarbonylbutan-1,3-diol (41)**

A solution of the methyl ketone (36) (0.449 g, 2.21 mmol) in ethanol (10 ml) was added dropwise over 5 min. to a stirred solution of sodium borohydride (0.452 g, 11.90 mmol) in ethanol (10 ml) at room temperature. The reaction mixture was stirred for 3h at room temperature.

The reaction mixture was evaporated to residue *in vacuo*, taken up in 1.19 M acetic acid (10 ml), and when effervescence had ceased ethylene glycol (0.66 ml, 11.85 mmol) was added. The reaction mixture was basified with NaOH and extracted with dichloromethane (5×10 ml). The combined organics were dried (Na₂SO₄) and evaporated to residue *in vacuo* to give a colourless oil which was purified by flash column chromatography (ethyl acetate) to give a 1:2.1 mixture of the title compounds (40) and (41) respectively (0.294 g, 65%) as a white wax-like solid.

R_f 0.28 (ethyl acetate);

m.p. 57-69°C (without further purification);

ν_{max} (CH₂Cl₂)/cm⁻¹ 3610w, 3500br, 3430w, 2970w, 2930w, 1700s, 1495m, 1365m, 1220w, 1165m, 1050br, 860w;

δ_H (300 MHz; CDCl₃) 1.24 [1.19] (3H, d, J=6.5 Hz [J=6.4 Hz], CH₃CH), 1.45 (9H, s, C(CH₃)₃), 3.30-3.51 (3H, br m, CHCH₂OH and 2×OH, two of which exchanged with D₂O), 3.63-4.12 (3H, m, CH₂OH and CH₃CH), 5.59 [5.47], (1H, J=7.5 Hz [J=7.8 Hz], NH, exchanged with D₂O); N.B. For the sets of signals which were clearly due to the two different diastereomers those corresponding to the minor diastereomer are shown in square brackets, otherwise only one value or range is given;

δ_C (75 MHz; D_2O) 20.95 [21.55] (CH_3), 30.43 (CH_3), 60.22 [59.83] (CH), 63.65 [64.28] (CH_2), 69.82 [69.10] (CH), 83.71 (C), 160.87 (C); N.B. Signals corresponding to the minor diastereomer are shown in square brackets;

m/z 206 ($[MH]^+$, 1%), 174 (22), 160 (50), 143 (73), 132 (47), 118 (33), 104 (60), 88 (15), 87 (63), 75 (10), 74 (100), 70 (13);

$C_9H_{20}NO_4$ $[MH]^+$ requires 206.1392
found 206.1392.

**(2R, 3R)-2-Amino-2-N-*tert*-Butoxycarbonylheptan-1,3-diol (42) and
(2R, 3S)-2-Amino-2-N-*tert*-Butoxycarbonylheptan-1,3-diol (43)**

Following the method used for the preparation of (40) and (41), the butyl ketone (37) (0.314 g, 1.28 mmol) and sodium borohydride (0.243 g, 6.40 mmol) gave, after purification by flash column chromatography (ethyl acetate), a 1:3.4 mixture of the title compounds (42) and (43) respectively (0.245 g, 78%) as a white wax-like solid.

R_f 0.39 (ethyl acetate);

m.p. 54-67°C (without further purification);

ν_{max} (CH_2Cl_2)/ cm^{-1} 3610w, 3500br, 3430m, 3050w, 2960m, 2930m, 2870w, 1700s, 1495s, 1400m, 1365m, 1330w, 1240w, 1165s, 1050m, 870w, 750br;

δ_H (300 MHz; $CDCl_3$) 0.88-0.93 (3H, m, CH_2CH_3), 1.20-1.65 (15H, m, $C(CH_3)_3$ and $(CH_2)_3CH_3$), 3.45-4.13 (6H, m, $CH(OH)CHCH_2OH$, two of which exchanged with D_2O), 5.50 [5.40] (1H, d, $J=8.1$ Hz [$J=9.1$ Hz], NH, slowly exchanged with D_2O); N.B. For the set of signals which was clearly due to the two different diastereomers that corresponding to the minor diastereomer is shown in square brackets, otherwise a range is given;

δ_C (75 MHz; $CDCl_3$) 13.88 (CH_3), 22.54 [22.44] (CH_2), 27.99 [27.69] (CH_2), 28.29 (CH_3), 33.88 [33.64] (CH_2), 54.96 [54.39] (CH), 62.24 (CH_2), 73.60 (CH), 79.55 (C),

156.09 [156.51] (C); N.B. Signals corresponding to the minor diastereomer are shown in square brackets;

m/z 248 ([MH]⁺, 2%), 216 (18), 174 (28), 160 (66), 144 (19), 143 (100), 116 (99), 115 (12), 104 (65), 95 (11), 88 (12), 87 (96);

C₁₂H₂₆NO₄[MH]⁺ requires 248.1862
found 248.1862.

(2R, 3R)-2-N-Dichloroacetamidobutan-1,3-diol (46)
and (2R, 3S)-2-N-Dichloroacetamidobutan-1,3-diol (47)

Trifluoroacetic acid (1 ml, 12.98 mmol) was added to the 1:2.1 mixture of the protected amino diols (40) and (41) prepared via the methyl ketone (36) (0.251 g, 1.22 mmol). The reaction mixture was stirred at room temperature for 1h.

The reaction mixture was evaporated to residue *in vacuo* and extracted with ethyl acetate (10×5 ml). The combined organics were dried (Na₂SO₄) and evaporated to residue *in vacuo* to give a crude mixture of (2R, 3R)-2-aminobutan-1,3-diol (44) and (2R, 3S)-2-aminobutan-1,3-diol (45) (0.068 g, 53%) as a yellow oil which was reacted immediately.

Methyl dichloroacetate (0.13 ml, 1.26 mmol) was added to a solution of (44) and (45) (0.068 g, mmol) in dry ethanol (5 ml). The reaction mixture was heated to reflux and aged at that temperature for 5h.

The reaction mixture was evaporated to residue *in vacuo* to give an orange oil which was taken up in ethyl acetate (5 ml). The solution was washed with 2 M HCl (2×1 ml), dried (Na₂SO₄) and evaporated to residue *in vacuo* to give a 1:1.8 mixture of the title compounds (46) and (47) respectively (0.071 g, 51%) as a yellow oil.

δ_H (300 MHz; D₂O) 1.18 [1.17] (3H, d, J=5.1 Hz [J=6.5 Hz], CH₃CH), 3.61-4.05 (4H, m, CHCH₂OH and CH₃CH), 6.33 [6.37] (1H, s, CHCl₂); N.B. For the sets of signals which were clearly due to the two different diastereomers, those corresponding to the minor isomer are shown in square brackets, otherwise a range is given.

(2R, 3R)-2-N-Dichloroacetamidobutan-1,3-diol (46)

(N-Dichloroacetamido-L-threoninol)

A solution of L-threonine methyl ester hydrochloride (3.940 g, 23.24 mmol) in water (40 ml) was added dropwise over 10 min. to a stirred solution of sodium borohydride (4.00 g, 0.11 mmol) in water (40 ml) at <2°C. The reaction mixture was stirred for 2h at <2°C and then left to stand in a fridge overnight.

The reaction mixture was acidified to pH 4 with aqueous oxalic acid and evaporated to residue *in vacuo*. The residue was extracted with boiling ethanol (3×50 ml) and the combined organics were evaporated to residue *in vacuo*. The residue was dissolved in water (60 ml), basified to pH 12 with barium hydroxide and filtered. The aqueous solution was evaporated to residue *in vacuo*, hot ethanol (40 ml) was added and the mixture filtered. The ethanolic solution was evaporated to residue *in vacuo*, hot acetone (40 ml) was added and the mixture filtered. The filtrate was evaporated to residue *in vacuo*, dissolved in dry ethanol, dried (Na₂SO₄) and evaporated to residue *in vacuo* to give a yellow oil which was purified by Kugelrohr distillation (80°C, 2×10⁻³ mbar) to give (2R, 3R)-2-aminobutan-1,3-diol (L-threoninol) (44) (1.750 g, 72%) as a colourless oil which was reacted immediately.

Methyl dichloroacetate (3.45 ml, 33.32 mmol) was added to a solution of L-threoninol (1.750 g, 16.67 mmol) in dry ethanol (20 ml). The reaction mixture was heated to reflux and aged at that temperature for 7h.

The reaction mixture was evaporated to residue *in vacuo*, triturated with petroleum ether (2×5 ml) and purified by flash column chromatography (ethyl acetate) to give the title compound (1.942 g, 54%) as a white solid.

R_f 0.36 (ethyl acetate);

m.p. 82.5-84°C (from ethyl acetate/hexane);

[α]_D +8.5° (c1.0 in EtOH);

C₆H₁₁NO₃Cl₂ requires C, 33.35; H, 5.1; N, 6.5%

found C, 33.3; H, 5.0; N, 6.4%;

ν_{max} (nujol/cm⁻¹ 3350m, 3240m, 3070w, 1685s, 1570m, 1335w, 1305w, 1235w, 1215w, 1200w, 1150w, 1100w, 1065m, 1020m, 1010w, 960w, 930w, 900w, 845w, 805m, 780w, 720w, 670w;

δ_H (300 MHz; D₂O buffer, pH 7.1) 1.17 (3H, d, J=6.5 Hz, CH₃CH), 3.66 (1H, ABX system, J_{BX}=7.6 Hz, J_{gem}=11.6 Hz, CH₂OH), 3.75 (1H, ABX system, J_{AX}=4.9 Hz, J_{gem}=11.6 Hz, CH₂OH), 3.88 (1H, ddd, J=3.8, 4.9, 7.6 Hz, CHCH₂OH), 4.02 (1H, dq, J=3.8, 6.5 Hz, CH₃CH), 6.38 (1H, s, CHCl₂);

δ_C (75 MHz; D₂O) 21.63 (CH₃), 59.87 (CH), 63.62 (CH₂), 68.70 (CH), 68.93 (CH), 169.95 (C);

m/z 216 ([MH]⁺, 2%), 186 (24), 184 (38), 170 (14), 168 (23), 166 (35), 155 (53), 153 (82), 120 (12), 118 (37), 106 (8), 85 (13), 83 (23), 76 (12), 74 (30), 71 (9), 70 (100);

C₆H₁₂NO₃Cl₂ [MH]⁺ requires 216.0194

found 216.0193.

Methyl (2R, 3S)-2-amino-3-hydroxybutanoate (51)
(D-Threonine methyl ester)

Thionyl chloride (9.40 ml, 12.88 mmol) was added dropwise over 10 min. to a stirred suspension of D-threonine (13.94 g, 11.71 mmol) in dry methanol (38.0 ml, 93.93 mmol) at <5°C. The reaction mixture was left to warm to room temperature and then stirred for 4.5h in a water-bath maintained at 50°C.

The reaction mixture was evaporated to residue *in vacuo* and basified to pH 9 with 10% NaHCO₃ solution. The reaction mixture was evaporated to residue *in vacuo* and extracted with dichloromethane (10×40 ml). The combined organics were washed with saturated brine (20 ml), dried (Na₂SO₄) and evaporated to residue *in vacuo* to give the title compound (7.35 g, 47%), as a white solid).

R_f not recorded-baseline with ethyl acetate;

m.p. 61-63°C (without further purification);

ν_{max} (CH₂Cl₂)/cm⁻¹ 3610w, 3400m, 3230w, 2980m, 2960m, 2880w, 1735s, 1605w, 1590w, 1435m, 1400m, 1265br, 1205s, 1190m, 1120m, 1055m, 1040w, 1015w, 990m, 930w, 910w, 850br;

δ_H (300 MHz; CDCl₃) 1.22 (3H, d, J=6.4 Hz, CH₃CH), 2.46 (3H, br s, OH and NH₂, exchanged with D₂O), 3.28 (1H, d, J=4.9 Hz, CHCO₂CH₃), 3.75 (3H, s, CH₃O), 3.93 (1H, dq, J=5.0, 6.4 Hz, CH₃CH);

δ_C (75 MHz; CDCl₃) 19.90 (CH₃), 52.10 (CH₃), 60.07 (CH), 68.26 (CH), 174.66 (C).

(2S, 3S)-2-N-Dichloroacetamidobutan-1,3-diol (53)
(N-Dichloroacetamido-D-threoninol)

Following the method used for the preparation of N-dichloroacetamido-L-threoninol (46), D-threonine methyl ester (51), (1.138 g, 8.56 mmol) and sodium borohydride

(1.30 g, 34.21 mmol) gave after purification by Kugelrohr distillation (80°C, 2×10^{-3} mbar) D-threoninol (**52**) (0.570 g, 63%) as a colourless oil which was reacted immediately.

Methyl dichloroacetate (0.77 ml, 7.44 mmol) was added to a solution of D-threoninol (0.520 g, 4.95 mmol) in dry ethanol (10 ml). The reaction mixture was heated to reflux and aged at that temperature for 7h.

The reaction mixture was evaporated to residue *in vacuo*, triturated with petroleum ether (2 \times 2.5 ml) and purified by flash column chromatography (ethyl acetate) to give the title compound (0.769 g, 72%) as a white solid.

R_f 0.36 (ethyl acetate);

m.p. 82-83.5°C (from ethyl acetate/hexane);

[α]_D -8.4° (c1.0 in EtOH);

ν_{\max} (nujol)/cm⁻¹ 3350m, 3240br, 3070w, 1685s, 1570m, 1335w, 1300w, 1235w, 1215w, 1200w, 1150m, 1100w, 1065m, 1020m, 1010w, 960w, 930w, 900m, 845w, 805m, 780w, 720w, 670w;

δ_{H} (300 MHz; D₂O buffer, pH 7.1) 1.17 (3H, d, J=6.5 Hz, CH₃CH), 3.66 (1H, ABX system, J_{BX}=7.6 Hz, J_{gem}=11.7 Hz, CH₂OH), 3.75 (1H, ABX system, J_{AX}=4.9 Hz, J_{gem}=11.7 Hz, CH₂OH), 3.88 (1H, ddd, J=3.9, 4.9, 7.6 Hz, CHCH₂OH), 4.02 (1H, dq, J=3.9, 6.5 Hz, CH₃CH), 6.38 (1H, s, CHCl₂); N.B Signal for CH₃CH confirmed by irradiation at δ 1.17;

δ_{C} (75 MHz; CDCl₃) 20.02 (CH₃), 56.03 and 56.12 (CH), 62.55 (CH₂), 66.56 (CH), 66.76 (CH), 165.66 and 165.73 (C);

m/z 216 ([MH]⁺, 1%), 186 (25), 184 (40), 170 (17), 168 (29), 166 (46), 155 (64), 154 (11), 153 (100), 120 (16), 118 (50), 106 (8), 85 (11), 84 (10), 83 (19), 76 (9), 74 (41), 71 (9), 70 (98);

$C_6H_{12}NO_3Cl_2$ [MH]⁺ requires 216.0194
found 216.0199.

(4R, 5R)-5-N-Dichloroacetamido-2,2-dimethyl-4-methyl-1,3-dioxan (54)

Pyridinium *p*-toluenesulphonate (0.156 g, 0.62 mmol) was added to a solution of N-dichloroacetamido-L-threoninol (prepared via L-threonine) (0.134 g, 0.62 mmol) and 2,2-dimethoxypropane (1.50 ml, 12.22 mmol) in dichloromethane (20 ml) under a nitrogen atmosphere. The reaction mixture was stirred at room temperature overnight.

Ether (15 ml) was added and the organics were washed with half-saturated brine (10 ml), dried (Na₂SO₄) and evaporated *in vacuo* to give a white solid which was purified by flash column chromatography (ethyl acetate) to give the title compound (0.137 g, 86%) as a white solid.

R_f 0.60 (ethyl acetate);

m.p. 92.5-95°C (without further purification);

δ_H (300 MHz; CDCl₃) 1.14 (3H, d, J=6.3 Hz, CHCH₃), 1.43 (3H, s, CCH₃), 1.50 (3H, s, CCH₃), 3.74-3.82 (2H, m, CH₂CH and one of OCH₂), 4.11-4.16 (1H, m, other of OCH₂), 4.22 (1H, dq, J=1.7, 6.3 Hz, CHCH₃), 6.04 (1H, s, CHCl₂), 7.25 (1H, d, J=8.4 Hz, NH); N.B. Signal for CHCH₃ confirmed by irradiation at δ1.12;

δ_C (75 MHz; CDCl₃) 17.69 (CH₃), 18.48 (CH₃), 29.70 (CH₃), 47.57 (CH), 64.14 (CH₂), 66.45 (CH), 66.95 (CH), 99.26 (C), 164.10 (C);

m/z No M⁺, 242 (14), 240 (21), 211 (11), 198 (13), 182 (10), 180 (15), 157 (11), 155 (65), 153 (100), 132 (11), 118 (25), 114 (11), 71 (14), 70 (73);

$C_9H_{16}NO_3Cl_2$ [MH]⁺ requires 256.0507
found 256.0507.

Mosher esters of a racemic mixture of N-dichloroacetamido-L-threoninol and N-dichloroacetamido-D-threoninol (55) and (56)

(R)- α -methoxy- α -trifluoromethyl-phenylacetyl chloride (MTPA-Cl) (50 μ l, 242 μ mol) was added rapidly to a mixture of N-dichloroacetamido-L-threoninol (4.3 mg, 19.9 μ mol) and N-dichloroacetamido-D-threoninol (4.3 mg, 19.9 μ mol) in dry pyridine (8 drops) and carbon tetrachloride (5 drops). The reaction mixture was left to stand at room temperature for 16h.

Water (2 ml) was added and the mixture extracted with ether (3 \times 15 ml). The combined organics were washed with 0.5 M HCl (4 ml), water (4 ml) and saturated Na₂CO₃ (7 ml), dried (Na₂SO₄) and evaporated to residue *in vacuo* to give the title compounds (0.026 g) as a colourless oil/solid mixture.

δ_F (282.36 MHz; CDCl₃) -71.40, -71.46, -71.78, -71.82, (1:1:1:1).

Mosher ester of N-dichloroacetamido-D-threoninol (56)

Following the method used for the preparation of (55) and (56), N-dichloroacetamido-D-threoninol (8.6 mg, 39.8 μ mol) and MTPACl (50 μ l, 242 μ mol) gave the title compound (0.033 g) as a colourless oil/solid mixture.

δ_F (282.36 MHz; CDCl₃) -71.40, -71.82, (1:1).

6.3 SYNTHESIS RELATING TO CHAPTER 3

Methyl (2R)-2-Amino-2-N-*tert*-butoxycarbonyl-3-hydroxypropanoate (82) (N-*tert*-Butoxycarbonyl-D-serine methyl ester)

Diazomethane was prepared using the Aldrich Diazald Apparatus and following the Aldrich procedure.¹⁴⁰ Diazald (22.5 g, 0.105 mol) in ether (200 ml) was added dropwise to a solution of 2-(2-ethoxyethoxy)ethanol (65 ml), ether (35 ml) and potassium hydroxide (11.25 g) in water (18 ml) at 65°C. The ethereal distillate should have contained approximately 3.1 g (0.074 mol) of diazomethane.

The ethereal diazomethane was added dropwise over 15 min. to a stirred solution of N-BOC-D-serine (10.0 g, 0.049 mol) in ether (300 ml) at 0°C. Addition continued until the reaction mixture became slightly yellow in colour. After standing at 0°C for 30 min. the excess diazomethane was destroyed by dropwise addition of acetic acid. The resulting solution was washed with half-saturated NaHCO₃ solution (150 ml) and saturated brine (100 ml), dried (Na₂SO₄) and evaporated to residue *in vacuo* to give the title compound (10.68 g, 100%) as a pale yellow oil.

R_f 0.37 (1:1 dichloromethane-ethyl acetate);

[α]_D -10.0° (c1.2 in CH₂Cl₂);

ν_{max} (film)/cm⁻¹ 3420br, 2980m, 2960m, 2940m, 2890w, 1750s, 1710s, 1510m, 1460m, 1440m, 1390m, 1370m, 1250m, 1210m, 1165m, 1090m, 1055m, 870w, 850w, 810w, 770w;

δ_H (300 MHz; CDCl₃) 1.45 (9H, s, C(CH₃)₃), 3.24 (1H, br s, OH, exchanged with D₂O), 3.78 (3H, s, OCH₃), 3.86 (1H, ABX system, J_{BX}=3.6 Hz, J_{gem}=11.2Hz, CH₂OH), 3.97 (1H, ABX system, J_{AX}=3.7 Hz, J_{gem}=11.2 Hz, CH₂OH), 4.37 (1H, m, CHCH₂OH), 5.64 (1H, d, J=8.0 Hz, NH, exchanged with D₂O);

δ_C (75 MHz; $CDCl_3$) 28.29 (CH_3), 52.55 (CH_3), 55.74 (CH), 63.12 (CH_2), 80.22 (C), 155.82 (C), 171.51 (C);

m/z No M^+ , 164 (12%), 133 (13), 120 (11), 91 (15), 57 (100);

$C_9H_{18}NO_5$ $[MH]^+$ requires 220.1185
found 220.1185.

Methyl (4R)-3-*tert*-butoxycarbonyl-2,2-dimethyl-4-oxazolidinecarboxylate (83)

Boron trifluoride etherate (0.6 ml, 4.9 mmol) was added to solution of N-BOC-D-serine methyl ester (10.68 g, 0.049 mol) and 2,2-dimethoxypropane (12 ml, 0.098 mol) in acetone (100 ml) at room temperature, under a nitrogen atmosphere. The reaction mixture was stirred at room temperature for two days after which time TLC indicated only a trace of starting material remaining.

The reaction mixture was evaporated to residue *in vacuo* to give a pale orange oil which was taken up in dichloromethane (100 ml). The solution was washed with saturated $NaHCO_3$ solution (60 ml) and saturated brine (40 ml), dried (Na_2SO_4) and evaporated to residue *in vacuo* to give a pale yellow liquid which was purified by flash column chromatography (5:1 dichloromethane-ethyl acetate) to give the title compound (10.91 g, 86%) as a colourless liquid.

R_f 0.65 (1:1 dichloromethane-ethyl acetate);

$[\alpha]_D +53^\circ$ (c 1.30 in $CHCl_3$) (lit., $+53^\circ$);¹⁰⁵

ν_{max} (film)/ cm^{-1} 2980m, 2960m, 2940m, 2890w, 1760s, 1710s, 1480m, 1460m, 1440m, 1385s, 1365s, 1290w, 1270w, 1250m, 1205m, 1170m, 1095m, 1070m, 1055m, 1005w, 945w, 870w, 850m, 810w, 770m;

δ_{H} (300 MHz; CDCl_3) 1.42 and 1.50 (9H, s, $\text{C}(\text{CH}_3)_3$), 1.54 (3H, s, CH_3), 1.64 and 1.67 (3H, s, CH_3), 3.76 (3H, s, OCH_3), 4.01-4.08 and 4.11-4.18 (2H, m, OCH_2), 4.37-4.40 and 4.47-4.50 (1H, m, CHCO_2Me);

δ_{C} (75 MHz; CDCl_3) 24.79 and 25.17 (CH_3), 24.95 and 26.03 (CH_3), 28.27 and 28.34 (CH_3), 52.26 and 52.37 (CH_3), 59.21 and 59.25 (CH), 65.99 and 66.24 (CH_2), 80.27 and 80.84 (C), 94.38 and 95.01 (C), 151.15 and 152.05 (C), 171.22 and 171.63 (C);

δ_{C} (75 MHz; C_6D_6 ; 65°C) 24.71 and 25.65 (CH_3), 25.29 and 26.43 (CH_3), 28.40 (CH_3), 51.63 (CH_3), 59.77 (CH), 66.38 (CH_2), 79.86 (C), 95.14 (C), 151.50 (C), 171.38 (C);

m/z 260 ($[\text{MH}]^+$, 8%), 244 (15), 204 (36), 186 (17), 160 (22), 144 (100), 120 (13), 100 (20), 84 (20), 57 (100);

$\text{C}_{12}\text{H}_{22}\text{NO}_5$ $[\text{MH}]^+$ requires 260.150
found 260.150.

(4R)-3-*tert*-Butoxycarbonyl-2,2-dimethyl-4-formyloxazolidine (81)

1.0 M DIBAL in toluene (54 ml, 0.054 mol) was added dropwise over 1h to a stirred solution of the oxazolidine methyl ester (83) (7.00 g, 0.027 mol) in dry toluene (45 ml) at -78°C , under a nitrogen atmosphere. The rate of addition was adjusted so as to keep the internal temperature below -68°C . The reaction mixture was stirred at -78°C for an additional 2.5h.

The reaction was quenched by slow dropwise addition of cold methanol (13 ml), again keeping the internal temperature below -68°C . The resulting white mixture was added over 15 min to a stirred solution of ice-cold 1 M HCl (150 ml) and the aqueous mixture was extracted with ethyl acetate (3×150 ml). The combined organics were washed with brine (150 ml), dried (Na_2SO_4) and evaporated to residue *in vacuo* to give a very pale

yellow oil which was purified by flash column chromatography (9:1 dichloromethane-ethyl acetate) to give the title compound (4.81 g, 78%) as a colourless liquid.

R_f 0.62 (9:1 dichloromethane-ethyl acetate);

$[\alpha]_D^{+91.8^\circ}$ (c1.34 in CHCl_3) (lit., $+95^\circ$);¹⁰⁵

ν_{max} (film)/ cm^{-1} 2980m, 2940m, 2890w, 2805w, 2720w, 1740s, 1710s, 1480m, 1460m, 1380br, 1265m, 1250m, 1210w, 1170m, 1095m, 1060m, 950w, 850m, 825w, 810w, 770m;

δ_H (300 MHz; CDCl_3) 1.44 and 1.52, 1.50 and 1.56 (12H, s, $\text{C}(\text{CH}_3)_3$ and CH_3), 1.61 and 1.65 (3H, s, CH_3), 4.03-4.13 (2H, m, OCH_2), 4.19-4.23 and 4.31-4.37 (1H, m, CHCOH), 9.55 and 9.60 (1H, s, COH);

δ_C (75 MHz; CDCl_3) 23.80 and 24.70 (CH_3), 25.78 and 26.70 (CH_3), 28.27 (CH_3), 63.44 and 63.89 (CH_2), 64.72 (CH), 80.99 and 81.30 (C), 94.32 and 95.04 (C), 151.28 (C), 199.24 and 199.32 (C);

m/z 230 ($[\text{MH}]^+$, <1%), 201 (13), 200 (60), 156 (48), 144 (50), 114 (34), 100 (100), 84 (17), 83 (22);

$\text{C}_{11}\text{H}_{20}\text{NO}_4$ $[\text{MH}]^+$ requires 230.1398

found 230.1395.

(4R, 1'R)-3-*tert*-Butoxycarbonyl-2,2-dimethyl-4-(1'-hydroxyethyl)oxazolidine (84)
and (4R, 1'S)-3-*tert*-Butoxycarbonyl-2,2-dimethyl-4-(1'-hydroxyethyl)oxazolidine (85)

Method A

3.0 M MeMgBr in ether (2.5 ml, 7.50 mmol) was added to a stirred solution of the aldehyde (81) (1.130 g, 4.93 mmol) in THF (15 ml) at -78°C , under a nitrogen

atmosphere. The reaction mixture was stirred at room temperature for 3h and then heated to reflux and aged at that temperature for 1h.

Saturated NH_4Cl (40 ml) was added and the mixture extracted with ether (3×60 ml). The combined organics were washed with 0.5 M HCl (40 ml) and saturated brine (40 ml), dried (Na_2SO_4) and evaporated to residue *in vacuo* to give an orange oil which was purified by flash column chromatography (3:1 dichloromethane-ethylacetate) to give a 1.2:1 mixture of the title compounds (84) and (85) respectively (0.614 g, 51%) as a colourless oil.

Method B

A solution of the aldehyde (81) (0.390 g, 1.70 mmol) in THF (2 ml) was added to a stirred solution of 1.5 M MeLi (as the LiBr complex in ether) (1.7 ml, 2.55 mmol) in THF (10 ml), at -78°C , under a nitrogen atmosphere. The reaction mixture was stirred at -78°C for 2h.

Saturated NH_4Cl (20 ml) was added and the mixture was left to warm to room temperature. After dilution with water (10 ml) the mixture was extracted with ether (2×40 ml). The combined organics were washed with 0.5 M HCl (20 ml) and saturated brine (20 ml), dried (Na_2SO_4) and evaporated to residue *in vacuo* to give a yellow oil which was purified by flash column chromatography (2:1 dichloromethane-ethyl acetate) to give a 1:2.5 mixture of the title compounds (84) and (85) respectively (0.192 g, 46%) as a pale yellow oil.

Method C

1.4 M MeLi in ether (2.6 ml, 3.64 mmol) was added to a stirred mixture of the aldehyde (81) (0.575 g, 2.51 mmol) and anhydrous ZnBr_2 (1.131 g, 5.03 mmol) in ether (20 ml) at 0°C , under a nitrogen atmosphere. Stirring was continued and the reaction mixture was left to warm to room temperature over 20h.

Saturated NH_4Cl (20 ml) was added to the mixture and after dilution with water (10 ml) the aqueous layer was extracted with ether (2×40 ml). The combined organics were washed with 0.5 M HCl (20 ml) and saturated brine (20 ml), dried (Na_2SO_4) and evaporated to residue *in vacuo* to give a colourless oil which was purified by flash column chromatography (5:1 dichloromethane-ethyl acetate) to give a 1.6:1 mixture of the title compounds (84) and (85) respectively (0.061 g, 16%) as a colourless oil.

Method D

3.0 M MeMgBr in ether (5.8 ml, 17.40 mmol) was added to a stirred mixture of copper (I) iodide (4.85 g, 25.53 mmol) in THF (50 ml) and dimethyl sulphide (10 ml) at -78°C , under a nitrogen atmosphere. The reaction mixture was stirred at -78°C for 0.5h and at -30°C for a further 0.5h. A solution of the aldehyde (81) (2.00 g, 8.73 mmol) in THF (8 ml) was then added at -78°C . Stirring was continued and the reaction mixture was left to warm to room temperature over 9h.

Saturated NH_4Cl (80 ml) was added to the mixture and after dilution with water (30 ml) the aqueous layer was extracted with ether (2×120 ml, 1×80 ml). The combined organics were washed with 0.5 M HCl (80 ml) and saturated brine (80 ml), dried (Na_2SO_4) and evaporated to residue *in vacuo* to give a mixture of a yellow oil and a white solid. Ethyl acetate (50 ml) was added to the mixture and the undissolved solid removed by filtration. The ethyl acetate solution was evaporated to residue *in vacuo* to give a yellow oil which was purified by flash column chromatography (4:1 dichloromethane-ethyl acetate) to give a >100:1 mixture of the title compounds (84) and (85) respectively (1.34 g, 63%) as a colourless oil which partially solidified on standing.

R_f 0.24 (5:1 dichloromethane-ethyl acetate);

$[\alpha]_D +8.4^\circ$ (c1.0 in CH_2Cl_2);

ν_{max} (CH_2Cl_2)/ cm^{-1} 3610w, 3440w, 2990w, 2940w, 2890w, 1700s, 1660m, 1500m, 1460w, 1390m, 1370s, 1230w, 1170s, 1110w, 1080w, 1060w, 860br;

δ_{H} (300 MHz; CDCl_3) 1.17 (3H, d, $J=6.2$ Hz, CHCH_3), 1.49 (12H, s, $\text{C}(\text{CH}_3)_3$ and CH_3), 1.58 (3H, s, CH_3), 3.65-4.12 (5H, m, one of which exchanged with D_2O);

δ_{C} (75 MHz; CDCl_3) 18.58 (CH_3), 27.02 (CH_3), 28.37 (CH_3), 29.72 (CH_3), 63.28 (CH), 64.50 (CH_2), 69.73 (CH), 81.15 (C), 94.07 (C), C=O not seen;

m/z No M^+ , 200 (5%), 190 (6), 144 (8), 130 (8), 100 (22), 84 (12), 57 (100);

$\text{C}_{12}\text{H}_{24}\text{NO}_4$ $[\text{MH}]^+$ requires 246.1705
found 246.1705.

(4R, 1'R)-3-*tert*-Butoxycarbonyl-2,2-dimethyl-4-(1'-hydroxypropyl)oxazolidine (89)

Following Method D used for the preparation of (84), 1.0 M EtMgBr in THF (18.3 ml, 18.30 mmol) and the aldehyde (81) (2.09 g, 9.13 mmol) gave, after purification by flash column chromatography (5:1 dichloromethane-ethyl acetate), the title compound (1.56 g, 66%) as a colourless oil.

R_f 0.40 (5:1 dichloromethane-ethyl acetate);

$[\alpha]_{\text{D}} +16.1^\circ$ (c 1.0 in CH_2Cl_2);

ν_{max} (CH_2Cl_2)/ cm^{-1} 3670w, 3440w, 3060w, 2970m, 2940m, 2880w, 1710s, 1500m, 1365s, 1220s, 1170m, 1090m, 1060w, 850w, 700br;

δ_{H} (300 MHz; CDCl_3) 0.98 (3H, t, $J=7.3$ Hz, CH_2CH_3), 1.25-1.67 (17H, m), 3.47-4.07 (5H, m, one of which exchanged with D_2O);

δ_{C} (75 MHz; CDCl_3) 18.60 (CH_3), 24.79 (CH_2), 27.10 (CH_3), 28.37 (CH_3), 29.71 (CH_3), 62.06 (CH), 64.82 (CH_2), 72.93 (CH), 81.20 (c), 93.95 (C), 155.75 (C);

m/z No M^+ , 201 (10%), 200 (12), 144 (40), 100 (45), 84 (8), 57 (100);

$\text{C}_{13}\text{H}_{26}\text{NO}_4$ $[\text{MH}]^+$ requires 260.186
found 260.186.

(4R, 1'R)-3-*tert*-Butoxycarbonyl-2,2-dimethyl-4-[1'-hydroxybutyl]oxazolidine (90)

Following Method D used for the preparation of (84), 2.0 M PrMgCl in ether (8.7 ml, 17.40 mmol) and the aldehyde (81) (2.00g, 8.73mmol) gave, after purification by flash column chromatography (4:1 dichloromethane-ethyl acetate), the title compound (1.51 g, 63%) as a colourless oil.

R_f 0.48 (4:1 dichloromethane-ethyl acetate);

[α]_D +23.0° (c1.0 in CH₂Cl₂);

ν_{max} (CH₂Cl₂)/cm⁻¹ 3440w, 3060w, 3000m, 2960m, 2940w, 2870w, 1715s, 1500m, 1400br, 1365s, 1220s, 1170m, 1090m, 1060w, 850w, 700br;

δ_H (300 MHz; CDCl₃) 0.94 (3H, t, J=6.9 Hz, CH₂CH₃), 1.33-1.70 (19H, m), 3.47-4.08 (5H, m, one of which exchanged with D₂O);

δ_C (75 MHz; CDCl₃) 14.03 (CH₃), 18.57 (CH₃), 18.83 (CH₂), 28.37 (CH₃), 29.71 (CH₃), 33.83 (CH₂), 62.32 (CH), 64.78 (CH₂), 72.21 (CH), 81.20 (C), 94.10 (C), 155.81 (C);

m/z 274 ([MH]⁺, <1%), 202 (42), 201 (21), 200 (21), 160 (14), 158 (21), 144 (53), 143 (77), 116 (11), 115 (16), 102 (40), 100 (100), 87 (61), 84 (30), 73 (17), 69 (15);

C₁₄H₂₇NO₄ M⁺ requires 273.194
found 273.194.

(4R, 1'R)-3-*tert*-Butoxycarbonyl-2,2-dimethyl-4-(1'-hydroxypentyl)oxazolidine (91)

Following Method D used for the preparation of (84), 2.0 M BuMgCl in ether (8.7 ml, 17.40 mmol) and the aldehyde (81) (2.00 g, 8.73 mmol) gave, after purification by flash column chromatography (5:1 dichloromethane-ethyl acetate), the title compound (1.11 g, 45%) as a colourless oil.

R_f 0.48 (5:1 dichloromethane-ethyl acetate);

$[\alpha]_D +38.0^\circ$ (c1.0 in CH_2Cl_2);

$\nu_{\max}(\text{CH}_2\text{Cl}_2)/\text{cm}^{-1}$ 3410br, 3060w, 2960w, 2940w, 2870w, 1710s, 1660w, 1460w, 1400m, 1380m, 1370m, 1225m, 1170m, 1110w, 1085w, 1060w, 850br, 700br;

δ_H (300 MHz; CDCl_3) 0.91 (3H, t, $J=7.0$ Hz, CH_2CH_3), 1.25-1.70 (21H, m), 3.62-4.05 (5H, one of which exchanged with D_2O);

δ_C (75 MHz, CDCl_3) 14.05 (CH_3), 22.73 (CH_2), 24.35 (CH_3), 27.13 (CH_3), 27.37 (CH_3), 27.51 (CH_2), 34.01 (CH_2), 62.28 (CH), 64.84 (CH_2), 73.78 (CH), 81.10 (C),
C=O not seen;

m/z No M^+ , 201(18%), 200(13), 172(14), 144(42), 100(100), 83(9), 69(17);

$\text{C}_{15}\text{H}_{29}\text{NO}_4$ M^+ requires 287.210
found 287.210.

(4R, 1'R)-3-*tert*-Butoxycarbonyl-2,2-dimethyl-4-(1'-hydroxyhexyl)oxazolidine (92)

Following Method D used for the preparation of (84), 2.0 M PentMgBr in ether (9.6 ml, 19.20 mmol) and the aldehyde (81) (2.20 g, 9.61 mmol) gave, after purification by flash column chromatography (4:1 dichloromethane-ethyl acetate), the title compound (2.44 g, 84%) as a pale yellow oil.

R_f 0.60 (5:1 dichloromethane - ethyl acetate);

$[\alpha]_D +41.1^\circ$ (c 1.0 in CH_2Cl_2);

$\nu_{\max}(\text{CH}_2\text{Cl}_2)/\text{cm}^{-1}$ 3400br, 2930m, 2860w, 1705s, 1660w, 1470br, 1400s, 1380s, 1370s, 1240w, 1170m, 1110w, 1085w, 1060w, 850br, 700br;

δ_H (300 MHz; CDCl_3) 0.89 (3H, t, $J=6.6$ Hz, CH_2CH_3), 1.23-1.70 (23H, m), 3.62-4.25 (5H, m, one of which exchanged with D_2O);

δ_C (75 MHz; $CDCl_3$) 14.05 (CH_3), 22.64 (CH_2), 25.07 (CH_2), 27.07 (CH_3), 28.38 (CH_3), 29.73 (CH_3), 31.70 (CH_2), 31.89 (CH_2), 62.28 (CH), 64.83 (CH_2), 73.84 (CH), 81.04 (C), 94.01 (C), C=O not seen;

m/z 301(M^+ , <1%), 230 (22), 201 (21), 200 (22), 186 (27), 170 (12), 156 (11), 144 (56), 143 (60), 130 (27), 115 (18), 114 (11), 101 (16), 100 (100), 87 (60), 84 (33), 83 (51), 71 (19), 69 (27);

$C_{16}H_{31}NO_4$ M^+ requires 301.225
found 301.225.

(4R, 1'R)-3-*tert*-Butoxycarbonyl-2,2-dimethyl-4-[1'-hydroxyheptyl]oxazolidine (93)

Following Method D used for the preparation of (84), 2.0 M HexMgBr in ether (9.6 ml, 19.20 mmol) and the aldehyde (81) (2.20 g, 9.61 mmol) gave, after purification by flash column chromatography (4:1 dichloromethane-ethyl acetate), the title compound (2.83 g, 94%) as a pale yellow oil.

R_f 0.64 (5:1 dichloromethane-ethyl acetate);

$[\alpha]_D^{+42.1^\circ}$ (c1.0 in CH_2Cl_2);

ν_{max} (CH_2Cl_2)/ cm^{-1} 3680w, 3610w, 3400br, 2930s, 2860m, 1700s, 1660w, 1470br, 1400s, 1380s, 1370s, 1240w, 1205w, 1170m, 1085w, 1060m, 850m, 700br;

δ_H (300 MHz; $CDCl_3$) 0.88 (3H, t, $J=6.6$ Hz, CH_2CH_3), 1.20-1.70 (25H, m), 3.32-4.20 (5H, m, one of which exchanged with D_2O);

δ_C (75 MHz; $CDCl_3$) 14.09 (CH_3), 22.63 (CH_2), 24.65 (CH_2), 27.05 (CH_3), 28.38 (CH_3), 29.38 (CH_2), 29.67 (CH_2), 29.72 (CH_3), 31.84 (CH_2), 62.31 (CH), 64.87 (CH_2), 73.75 (CH), 81.15(C), 94.05 (C), C=O not seen;

m/z No M^+ , 244 (30%), 201 (22), 200 (39), 184 (120), 156 (13), 144 (58), 143(97), 115(21), 100 (100), 87 (47), 84 (17), 83(15), 71 (26), 69 (11);

$C_{17}H_{33}NO_4$ M^+ requires 315.241
found 315.241.

**(4R, 1'R)-3-tert-Butoxycarbonyl-2,2-dimethyl-4-[1'-cyclohexyl-1'-hydroxymethyl]
oxazolidine (94)**

Following Method D used for the preparation of (84), 2.0 M CyclohexMgCl in ether (9.0 ml, 18.00 mmol) and the aldehyde (81) gave, after purification by flash column chromatography (4:1 dichloromethane-ethyl acetate), the title compound (0.510 g, 18%) as a colourless oil.

R_f 0.59 (5:1 dichloromethane-ethyl acetate);

$[\alpha]_D +35.8^\circ$ (c1.0 in CH_2Cl_2);

ν_{max} (CH_2Cl_2)/ cm^{-1} 3440br, 2980w, 2930m, 2860w, 1705m, 1660m, 1480w, 1450w, 1400s, 1380m, 1370s, 1240w, 1205w, 1170m, 1110w, 1060w, 860w, 845w, 700br;

δ_H (300 MHz; $CDCl_3$) 1.10-1.79 (26H, m), 3.41-4.15 (5H, m, one of which exchanged with D_2O);

δ_C (75 MHz; $CDCl_3$) 24.18 (CH_3), 24.87 (CH_2), 26.03 (CH_2), 26.31 (CH_2), 26.58 (CH_2), 27.12 (CH_3), 28.27 (CH_3), 30.38 (CH_2), 41.51 (CH), 59.85 (CH), 65.20 (CH_2), 78.32 (CH), 81.18 (C), 94.81 (C), C=O not seen;

m/z 313 (M^+ , 1%), 201 (20), 200 (16), 198 (10), 145 (13), 144 (53), 143 (18), 100 (100), 95 (25), 84 (20), 83 (24);

$C_{17}H_{31}NO_4$ M^+ requires 313.225
found 313.225.

**(2R, 3R)-2-Amino-2-N-*tert*-butoxycarbonylbutan-1,3-diol (40) and
(2R, 3S)-2-Amino-2-N-*tert*-butoxycarbonylbutan-1,3-diol (41)**

p-Toluenesulphonic acid (0.003 g, 0.015 mmol) was added to a solution of the mixture of oxazolidines (84) and (85) prepared via Method A (0.114 g, 0.465 mmol) in dry methanol (3 ml). The reaction mixture was heated to reflux and aged at that temperature for 2h.

The reaction mixture was evaporated to residue *in vacuo*, basified with 10% NaHCO₃ and extracted with dichloromethane (4×5 ml). The combined organics were washed with saturated brine (5 ml), dried (Na₂SO₄) and evaporated to residue *in vacuo* to give a 1.2:1 mixture of the title compounds (40) and (41) respectively (0.089 g, 93%) as a colourless oil.

δ_{H} (300 MHz; CDCl₃) 1.20 [1.26] (3H, d, *J*=6.4 Hz [*J*=6.4 Hz], CH₃CH), 1.45 (9H, s, C(CH₃)₃), 3.42-3.56 (1H, br m, CHCH₂OH), 3.60-4.17 (5H, m, CH₂OH and CH₃CH(OH), two of which exchanged with D₂O), 5.41 [5.51] (1H, d, *J*=9.0 Hz [*J*=8.3 Hz], NH, exchanged with D₂O); N.B. For the sets of signals which were clearly due to the two different diastereomers those corresponding to the minor isomer are shown in square brackets, otherwise only one value or range is given.

p-Toluenesulphonic acid (0.011 g, 0.058 mmol) was added to a solution of the mixture of oxazolidines (84) and (85) prepared via Method B (0.167 g, 0.682 mmol) in dry methanol (5 ml). The reaction mixture was heated to reflux and aged at that temperature for 2.5h.

The reaction mixture was evaporated to residue *in vacuo*, basified with 10% NaHCO₃ and extracted with dichloromethane (5×5 ml). The combined organics were washed with saturated brine (5 ml), dried (Na₂SO₄) and evaporated to residue *in vacuo* to give a 1:2.5

mixture of the title compounds (40) and (41) respectively (0.098 g, 71%) as a colourless oil.

δ_H (300 MHz; $CDCl_3$) 1.26 [1.20] (3H, d, $J=6.5$ Hz [$J=6.4$ Hz], CH_3CH), 1.45 (9H, s, $C(CH_3)_3$), 3.38-3.55 (1H, br m, $CHCH_2OH$), 3.67-4.16 (5H, m, CH_2OH and $CH_3CH(OH)$, two of which exchanged with D_2O), 5.52 [5.41] (1H, d, $J=8.4$ Hz [$J=9.0$ Hz], NH, exchanged with D_2O); N.B. For the sets of signals which were clearly due to the two different diastereomers those corresponding to the minor diastereomer are shown in square brackets, otherwise only one value or range is given.

p-Toluenesulphonic acid (0.004 g, 0.021 mmol) was added to a solution of the mixture of oxazolidines (84) and (85) prepared via Method C (0.055 g, 0.224 mmol) in dry methanol (5 ml). The reaction mixture was heated to reflux and aged at that temperature for 4h.

The reaction mixture was evaporated to residue *in vacuo*, basified with 10% $NaHCO_3$ and extracted with dichloromethane (5×5 ml). The combined organics were washed with saturated brine (5 ml), dried (Na_2SO_4) and evaporated to residue *in vacuo* to give a 1.6:1 mixture of the title compounds (40) and (41) respectively (0.032 g, 70%) as a colourless oil.

δ_H (300 MHz; $CDCl_3$) 1.22 [1.28] (3H, d, $J=6.4$ Hz [$J=6.5$ Hz], CH_3CH), 1.46 (9H, s, $C(CH_3)_3$), 2.51 (2H, br s, 2×OH, exchanged with D_2O), 3.44-3.58 (1H, br m, $CHCH_2OH$), 3.71-4.17 (3H, m, CH_2OH and CH_3CH), 5.15-5.45 (1H, br, NH, exchanged with D_2O); N.B. For the set of signals which was clearly due to the two different diastereomers that corresponding to the minor diastereomer is shown in square brackets, otherwise only one value or range is given.

(2R, 3R)-2-Amino-2-N-*tert*-butoxycarbonylbutan-1,3-diol (40)

p-Toluenesulphonic acid (0.076 g, 0.40 mmol) was added to a solution of the oxazolidine (84) prepared via Method D (1.233 g, 5.03 mmol) in dry methanol (50 ml). The reaction mixture was heated to reflux and aged at that temperature for 2h.

The reaction mixture was evaporated to residue *in vacuo*, basified with 10% NaHCO₃ solution (20 ml) and extracted with dichloromethane (5×40 ml). The combined organics were washed with saturated brine (40 ml), dried (Na₂SO₄) and evaporated to residue *in vacuo* to give a viscous oil which was purified by flash column chromatography (2:1 ethyl acetate-dichloromethane) to give the title compound (0.750 g, 73%) as a colourless oil.

R_f 0.28 (ethyl acetate);

[α]_D -8.5° (c2.0 in CH₂Cl₂);

ν_{max} (CH₂Cl₂)/cm⁻¹ 3620w, 3510br, 3440w, 3060w, 2980w, 2940w, 2890w, 1710s, 1500m, 1390w, 1365m, 1220m, 1165m, 1090w, 860w;

δ_H (300 MHz; CDCl₃) 1.22 (3H, d, J=6.4 Hz, CH₃CH), 1.45 (9H, s, C(CH₃)₃), 3.00 (2H, br s, 2×OH, exchanged with D₂O), 3.44-3.57 (1H, br m, CHCH₂OH), 3.79 (2H, d, J=4.3 Hz, CH₂OH), 4.08-4.16 (1H, m, CH₃CH), 5.31 (1H, d, J=6.4 Hz, NH, exchanged with D₂O);

δ_C (75M Hz; CDCl₃) 20.22 (CH₃), 28.37 (CH₃), 55.77 (CH), 64.42 (CH₂), 68.22 (CH), 79.68 (C), 156.70 (C);

m/z No M⁺, 174 (<5%), 160 (<5), 143 (<5), 132 (<5), 118 (6), 104 (7), 87 (10), 74 (27), 57 (100);

C₉H₂₀NO₄ [MH]⁺ requires 206.139
found 206.139.

(2R, 3R)-2-Amino-2-N-*tert*-butoxycarbonylpentan-1,3-diol (95)

Following the method used for the preparation of (40), the oxazolidine (89) (1.360 g, 5.25 mmol) and *p*-toluenesulphonic acid (0.080 g, 0.42 mmol) gave, after purification by flash column chromatography (1:1 ethyl acetate-dichloromethane), the title compound (0.837 g, 73%) as a colourless oil.

R_f 0.33 (ethyl acetate);

[α]_D -5.0° (c2.5 in CH₂Cl₂);

ν_{max} (CH₂Cl₂)/cm⁻¹ 3620w, 3510br, 3440w, 3060w, 2940w, 2880w, 1705s, 1500m, 1390w, 1370m, 1235br, 1165m, 1050br, 860br;

δ_H (300 MHz; CDCl₃) 0.96 (3H, t, J=7.4 Hz, CH₃CH₂), 1.45 (9H, s, C(CH₃)₃), 1.48-1.58 (2H, m, CH₃CH₂), 3.13 (2H, br s, 2×OH, exchanged with D₂O), 3.55-3.65 (1H, br m, CHCH₂OH), 3.77 (2H, d, J=4.6 Hz, CH₂OH), 3.81-3.85 (1H, m, C₂H₅CH), 5.30 (1H, br s, NH, exchanged with D₂O);

δ_C (75 MHz; CDCl₃) 10.15 (CH₃), 26.98 (CH₂), 28.37 (CH₃), 54.12 (CH), 64.24 (CH₂), 73.30 (CH), 79.59 (C), 156.63 (C);

m/z No M⁺, 188 (25%), 160 (18), 146 (24), 143 (43), 132 (42), 104 (34), 88 (100), 87 (44), 71 (15), 70 (10);

C₁₀H₂₂NO₄ [MH]⁺ requires 220.155
found 220.155.

(2R, 3R)-2-Amino-2-N-*tert*-butoxycarbonylhexan-1,3-diol (96)

Following the method used for the preparation of (40), the oxazolidine (90) (1.025 g, 3.75 mmol) and *p*-toluenesulphonic acid (0.057 g, 0.30 mmol) gave, after purification by flash column chromatography (1:1 ethyl acetate-dichloromethane), the title compound (0.835 g, 95%) as a colourless oil.

R_f 0.35 (ethyl acetate);

[α]_D -5.7° (c1.0 in CH₂Cl₂);

ν_{max} (CH₂Cl₂)/cm⁻¹ 3690w, 3620w, 3500br, 3440w, 3050m, 2980m, 2930w, 2880w, 2300w, 1710s, 1500m, 1420m, 1360s, 1255s, 1220s, 1165m, 1090w, 890m, 720br;

δ_H (300 MHz; CDCl₃) 0.93 (3H, t, J=7.0 Hz, CH₃CH₂), 1.32-1.66 (13H, m, C(CH₃) and CH₃(CH₂)₂), 2.98 (2H, br s, 2×OH, exchanged with D₂O), 3.47-3.64 (1H, m, CHCH₂OH), 3.78 (2H, d, J=4.6 Hz, CH₂OH), 3.86-3.98 (1H, m, C₃H₇CH), 5.29 (1H, d, J=5.8 Hz, NH, slowly exchanged with D₂O);

δ_C (75 MHz; CDCl₃) 14.01 (CH₃), 18.85 (CH₂), 28.36 (CH₃), 36.18 (CH₂), 54.53 (CH), 64.28 (CH₂), 71.53 (CH), 79.59 (C), 156.61 (C);

m/z 234 ([MH]⁺, <1%), 202 (24), 160 (35), 146 (36), 143 (43), 104 (34), 102 (100), 87 (43), 85 (11), 73 (12);

C₁₁H₂₄NO₄ [MH]⁺ requires 234.1705

found 234.1705.

(2R, 3R)-2-Amino-2-N-*tert*-butoxycarbonylheptan-1,3-diol (42)

Following the method used for the preparation of (40), the oxazolidine (91) (1.008 g, 3.51 mmol) and *p*-toluenesulphonic acid (0.053 g, 0.28 mmol) gave, after purification by flash column chromatography (2:1 ethyl acetate-dichloromethane), the title compound (0.787 g, 91%) as a colourless oil.

R_f 0.39 (ethyl acetate);

[α]_D -6.5° (c1.0 in CH₂Cl₂);

ν_{max} (CH₂Cl₂)/cm⁻¹ 3690w, 3620w, 3500br, 3440w, 3050m, 2980m, 2940w, 2870w, 2300w, 1710s, 1500m, 1420m, 1365m, 1255s, 1220m, 1165m, 1070br, 890m, 720br;

δ_{H} (300 MHz; CDCl_3) 0.90 (3H, t, $J=6.9$ Hz, CH_3CH_2), 1.20-1.65 (15H, m, $\text{C}(\text{CH}_3)_3$ and $\text{CH}_3(\text{CH}_2)_3$), 3.50-3.62 (1H, br m, CHCH_2OH), 3.72 (2H, d, $J=5.2$ Hz, CH_2OH), 3.76-3.97 (3H, br m, $\text{C}_4\text{H}_9\text{CH}$ and $2\times\text{OH}$, 2 of which exchanged with D_2O), 5.40 (1H, d, $J=9.1$ Hz, NH, slowly exchanged with D_2O);

δ_{C} (75 MHz; CDCl_3) 14.01 (CH_3), 22.64 (CH_2), 27.84 (CH_2), 28.37 (CH_3), 33.73 (CH_2), 54.57 (CH), 64.05 (CH_2), 71.56 (CH), 79.58 (C), 156.65 (C);

m/z No M^+ , 216 (8%), 174 (9), 160 (25), 143 (32), 142 (19), 116 (72), 104 (23), 87 (52), 57 (100);

$\text{C}_{12}\text{H}_{26}\text{NO}_4$ $[\text{MH}]^+$ requires 248.186
found 248.186.

(2R, 3R)-2-Amino-2-N-*tert*-butoxycarbonyloctan-1,3-diol (97)

Following the method used for the preparation of (40), the oxazolidine (92) (2.291 g, 7.61 mmol) and *p*-toluenesulphonic acid (0.115 g, 0.61 mmol) gave, after purification by flash column chromatography (1:1 ethyl acetate-dichloromethane), the title compound (1.393 g, 70%) as a colourless oil.

R_f 0.44 (1:1 ethyl acetate-dichloromethane);

$[\alpha]_{\text{D}} -8.3^\circ$ (c1.0 in CH_2Cl_2);

ν_{max} (CH_2Cl_2)/ cm^{-1} 3690w, 3610w, 3500br, 3440w, 3050m, 2980m, 2940w, 2870w, 2300w, 1710m, 1495m, 1420m, 1365s, 1260s, 1165m, 1060br, 890m, 725br;

δ_{H} (300 MHz; CDCl_3) 0.88 (3H, t, $J=6.5$ Hz, CH_3CH_2), 1.24-1.60 (17H, m, $\text{C}(\text{CH}_3)_3$ and $\text{CH}_3(\text{CH}_2)_4$), 3.50-3.62 (1H, br m, CHCH_2OH), 3.72 (2H, d, $J=4.9$ Hz, CH_2OH), 3.62-4.00 (3H, br m, $\text{C}_5\text{H}_{11}\text{CH}$ and $2\times\text{OH}$, 2 of which exchanged with D_2O), 5.38 (1H, d, $J=9.0$ Hz, NH, slowly exchanged with D_2O);

δ_C (75 MHz; $CDCl_3$) 14.04 (CH_3), 22.57 (CH_2), 25.35 (CH_2), 28.38 (CH_3), 31.80 (CH_2), 34.05 (CH_2), 54.52 (CH), 64.28 (CH_2), 71.83 (CH), 79.58 (C), 156.62 (C);

m/z No M^+ , 230 (15%), 188 (15), 174 (28), 160 (17), 144 (13), 143 (46), 130(100), 104 (33), 87 (50), 83 (14), 75 (27);

$C_{13}H_{28}NO_4$ $[MH]^+$ requires 262.2018

found 262.2018.

(2R, 3R)-2-Amino-2-N-*tert*-butoxycarbonylnonan-1,3-diol (98)

Following the method used for the preparation of (40), the oxazolidine (93) (2.644 g, 8.39 mmol) and *p*-toluenesulphonic acid (0.128 g, 0.67 mmol) gave, after purification by flash column chromatography (1:1 ethyl acetate-dichloromethane), the title compound (1.425 g, 62%) as a colourless oil.

R_f 0.46 (1:1 ethyl acetate-dichloromethane);

$[\alpha]_D -8.4^\circ$ (c 1.0 in CH_2Cl_2);

ν_{max} (CH_2Cl_2)/ cm^{-1} 3690w, 3620w, 3500br, 3400w, 3050m, 2980m, 2930m, 2860w, 2300w, 1710m, 1500m, 1420m, 1370w, 1255s, 1165m, 1060br, 890m, 725br;

δ_H (300 MHz; $CDCl_3$) 0.88 (3H, t, $J=6.8$ Hz, CH_3CH_2), 1.23-1.54 (19H, m, $C(CH_3)_3$ and $CH_3(CH_2)_5$), 3.07 (2H, br, 2 \times OH, exchanged with D_2O), 3.52-3.64 (1H, br m, $CHCH_2OH$), 3.77 (2H, d, $J=4.5$ Hz, CH_2OH), 3.84-3.96 (1H, br m, $C_6H_{13}CH$), 5.27 (1H, d, $J=8.6$ Hz, NH, slowly exchanged with D_2O);

δ_C (75 MHz; $CDCl_3$) 14.07 (CH_3), 22.61 (CH_2), 25.62 (CH_2), 28.37 (CH_3), 29.27 (CH_2), 31.76 (CH_2), 34.10 (CH_2), 54.49 (CH), 64.38 (CH_2), 71.94 (CH), 79.57 (C), 156.60 (C);

m/z No M^+ , 244 (1%), 202 (2), 188 (4), 170 (5), 160 (4), 144 (15), 143 (18), 130 (13), 104 (10), 87 (16), 75 (100);

$C_{14}H_{30}NO_4$ [MH]⁺ requires 276.2175
found 276.2175.

(1R, 2R)-2-Amino-2-N-*tert*-butoxycarbonyl-1-cyclohexylpropan-1,3-diol (99)

Following the method used for the preparation of (40), the oxazolidine (94) (0.510 g, 1.63 mmol) and *p*-toluenesulphonic acid (0.025 g, 0.13 mmol) gave, after purification by flash column chromatography (1:1 ethyl acetate-dichloromethane), the title compound (0.310 g, 73%) as a colourless oil.

R_f 0.39 (1:1 ethyl acetate-dichloromethane);

[α] reading too low to record;

ν_{max} (CH₂Cl₂)/cm⁻¹ 3630w, 3440w, 3050m, 2990m, 2930m, 2860w, 2300w, 1710m, 1500m, 1420m, 1370w, 1255s, 1165m, 1060w, 1045w, 890m, 725br;

δ_H (300 MHz; CDCl₃) 0.89-1.01 (2H, m, C₆H₁₁), 1.18-1.50 (13H, m, C₆H₁₁ and C(CH₃)₃), 1.60-1.82 (4H, m, C₆H₁₁), 1.97-2.04 (1H, m, C₆H₁₁), 3.38-3.90 (6H, m, CH(OH)CHCH₂OH, two of which exchanged with D₂O), 5.41 (1H, d, *J*=8.6 Hz, NH, slowly exchanged with D₂O);

δ_C (75 MHz; CDCl₃) 25.94 (CH₂), 26.02 (CH₂), 26.39 (CH₂), 28.36 (CH₃), 28.94 (CH₂), 29.28 (CH₂), 40.35 (CH), 51.91 (CH), 64.06 (CH₂), 75.62 (CH), 79.52 (C), 156.47 (C);

m/z 274 ([MH]⁺, <1%), 242 (16), 200 (8), 186 (36), 160 (11), 144 (19), 143 (66), 142 (100), 134 (13), 115 (12), 104 (29), 95 (31), 87 (49), 82 (100), 75 (61);

$C_{14}H_{28}NO_4$ [MH]⁺ requires 274.2018
found 274.2017.

(2R, 3R)-2-N-Dichloroacetamidobutan-1,3-diol (46)

A solution of the protected diol (40) (0.470 g, 2.29 mmol) in dioxane (20 ml) and 1 M HCl (10 ml) was heated to reflux and aged at that temperature for 0.5h.

The reaction mixture was evaporated to residue *in vacuo*, basified with 2 M NaOH (10 ml) and extracted with ethyl acetate (6×50 ml). The combined organics were dried (Na_2SO_4) and evaporated to residue *in vacuo* to give crude (2R, 3R)-2-aminobutan-1,3-diol (44) (0.163 g, 68%) as an orange oil which was reacted immediately.

Methyl dichloroacetate (0.80 ml, 7.73 mmol) and triethylamine (0.22 ml, 1.57 mmol) were added to a solution of the amino diol (44) (0.163 g, 1.55 mmol) in dry methanol (4 ml). The reaction mixture was stirred at room temperature for 2 days.

The reaction mixture was evaporated to residue *in vacuo* to give a pale orange oil which was taken up in ethyl acetate (10 ml). The solution was washed with 2 M HCl (2×1 ml), dried (Na_2SO_4) and evaporated to residue *in vacuo*. The residue was triturated with petroleum ether (2×2 ml) and purified by flash column chromatography (ethyl acetate) to give the title compound (0.260 g, 78%) as a white solid.

R_f 0.36 (ethyl acetate);

m.p. 82-84°C (ethyl acetate);

$[\alpha]_D +8.7^\circ$ (from ethyl acetate/hexane);

$\text{C}_6\text{H}_{11}\text{NO}_3\text{Cl}_2$ requires C, 33.35; H, 5.1; N, 6.5%

found C, 33.3; H, 5.1; N, 6.25%;

ν_{max} (nujol)/ cm^{-1} 3350m, 3250m, 3070w, 1690s, 1575m, 1340w, 1305w, 1235w, 1220w, 1205w, 1150w, 1100w, 1070m, 1025w, 1010w, 960w, 900w, 805m, 780w, 715w, 670w;

δ_H (300 MHz; D_2O) 1.19 (3H, d, $J=6.5$ Hz, $CHCH_3$), 3.67 (1H, ABX system, $J_{BX}=7.6$ Hz, $J_{gem}=11.7$ Hz, CH_2OH), 3.77 (1H, ABX system, $J_{AX}=4.9$ Hz, $J_{gem}=11.7$ Hz, CH_2OH), 3.87-3.92 (1H, m, $CHCH_2OH$), 4.04 (1H, dq, $J=3.9, 6.5$ Hz, $CHCH_3$), 6.39 (1H, s, $CHCl_2$);

δ_C (75 MHz; CD_3OD) 21.65 (CH_3), 59.83 (CH), 63.60 (CH_2), 68.60 (CH), 68.95 (CH), 169.81 (C);

m/z 216 ($[MH]^+$, 1%), 188 (5), 186 (28), 184 (40), 172 (9), 170 (19), 168 (30), 166 (43), 157 (12), 155 (58), 154 (13), 153 (67), 152 (11), 120 (17), 118 (50), 106 (10), 85 (16), 84 (12), 83 (26), 76 (15), 74 (38), 71 (14), 70 (100);

$C_6H_{12}NO_3Cl_2$ $[MH]^+$ requires 216.0194
found 216.0200.

(2R, 3R)-2-N-Dichloroacetamidopentan-1,3-diol (106)

Following the method used for the deprotection of (40), the protected diol (95) (0.807 g, 3.68 mmol) in dioxane (40 ml) and 1 M HCl (20 ml) gave crude (2R, 3R)-2-aminopentan-1,3-diol (100) (0.304 g, 69%) as an orange oil which was reacted immediately.

Methyl dichloroacetate (1.30 ml, 12.55 mmol) and triethylamine (0.36 ml, 2.59 mmol) were added to a solution of the amino diol (100) (0.304 g, 2.55 mmol) in dry methanol (7 ml). The reaction mixture was stirred at room temperature for 24h.

The reaction mixture was evaporated to residue *in vacuo* to give a pale orange oil which was taken up in ethyl acetate (10 ml). The solution was washed with 2 M HCl (2× 2.5 ml), dried (Na_2SO_4) and evaporated to residue *in vacuo*. The residue was triturated with petroleum ether (2× 2.5 ml) and purified by flash column chromatography (2:1 ethyl acetate-dichloromethane) to give the title compound (0.252 g, 43%) as a white solid.

R_f 0.37 (ethyl acetate);

m.p. 83-84°C (from ethyl acetate/hexane);

$[\alpha]_D$ +9.5° (c1.0 in EtOH);

$C_7H_{13}NO_3Cl_2$ requires C, 36.5; H, 5.7; N, 6.1%

found C, 36.7; H, 5.5; N, 6.1%;

ν_{max} (nujol)/ cm^{-1} 3350m, 3260w, 1690s, 1560m, 1330w, 1230w, 1210w, 1140w, 1105w, 1050w, 1030w, 960w, 900w, 920w, 805m, 770w, 675m;

δ_H (300 MHz; CD_3OD) 0.95 (3H, t, $J=7.4$ Hz, CH_2CH_3), 1.43-1.52 (2H, m, CH_2CH_3), 3.61 (1H, ABX system, $J_{BX}=6.3$ Hz, $J_{gem}=10.9$ Hz, CH_2OH), 3.68 (1H, ABX system, $J_{AX}=6.4$ Hz, $J_{gem}=10.9$ Hz, CH_2OH), 3.73-3.78 (1H, m, $CHCH_2OH$), 3.89-3.97 (1H, m, $CHCH_2OH$), 6.35 (1H, s, $CHCl_2$), 8.03 (1H, d, $J=8.7$ Hz, NH);

δ_C (75 MHz; CD_3OD) 10.58 (CH_3), 27.97 (CH_2), 56.28 (CH), 62.60 (CH_2), 67.71 (CH), 72.22 (CH), 166.96 (C);

m/z No M^+ , 202 (4%), 200 (18), 198 (27), 182 (18), 180(23), 157 (10), 155 (57), 154 (12), 153 (87), 149 (11), 130 (10), 128 (10), 120 (16), 118 (48), 88 (12), 86 (14), 85 (18), 83 (25), 76 (13), 72 (11), 71 (47), 70 (100), 69 (17);

$C_6H_{10}NO_2Cl_2$ M^+-CH_2OH requires 198.0089

found 198.0075.

(2R, 3R)-2-N-Dichloroacetamidohexan-1,3-diol (107)

Following the method for the deprotection of (40), the protected diol (96) (0.736 g, 3.16 mmol) in dioxane (30 ml) and 1 M HCl (15 ml) gave crude (2R, 3R)-2-aminohexan-1,3-diol (101) (0.420 g, 100%) as a pale orange oil which was reacted immediately.

Methyl dichloroacetate (0.80 ml, 7.73 mmol) was added to a solution of the amino diol (34), (0.420 g, 3.16 mmol) in dry ethanol (10 ml). The reaction mixture was heated to reflux and aged at that temperature for 6h.

The reaction mixture was evaporated to residue *in vacuo*, triturated with petroleum ether (2×2.5 ml) and purified by flash column chromatography (ethyl acetate) to give the title compound (0.435 g, 56%) as a slightly off-white solid.

R_f 0.40 (ethyl acetate);

m.p. 109.5-111°C (from ethyl acetate/hexane);

[α]_D +13.5° (c1.0 in EtOH);

C₈H₁₅NO₃Cl₂ requires C, 39.4; H, 6.2, N, 5.7%

found C, 39.1; H, 6.1; N, 5.5%;

ν_{max} (nujol)/cm⁻¹ 3405m, 3360m, 3270m, 3060w, 1690s, 1555m, 1350w, 1335w, 1295w, 1225m, 1205m, 1140w, 1125w, 1110m, 1065w, 1035m, 955m, 870m, 810m, 770w, 725m, 645m;

δ_H (300 MHz; D₂O+CD₃OD) 0.86 (3H, t, J=6.8 Hz, CH₂CH₃), 1.21-1.51 (4H, m, (CH₂)₂CH₃), 3.62 (1H, ABX system, J_{BX}=7.5 Hz, J_{gem}=11.5 Hz, CH₂OH), 3.70 (1H, ABX system, J_{AX}=5.5 Hz, J_{gem}=11.5 Hz, CH₂OH), 3.77-3.84 (1H, m, CHCH₂OH), 3.88-3.93 (1H, m, CHPr), 6.33 (1H, s, CHCl₂);

δ_C (75 MHz; CDCl₃) 13.93 (CH₃), 18.81 (CH₂), 36.08 (CH₂), 54.29 (CH), 63.15 (CH₂), 66.42 (CH), 70.92 (CH), 165.34 (C);

m/z No M⁺, 214 (14%), 212 (27), 194 (17), 157 (11), 155 (65), 154 (15), 153 (100), 120 (14), 118 (39), 85 (18), 70 (65), 60 (13), 55 (10);

C₈H₁₆NO₃Cl₂ [MH]⁺ requires 244.0507

found 244.057.

(2R, 3R)-2-N-Dichloroacetamidoheptan-1,3-diol (108)

Following the method for the deprotection of (40), the protected diol (42) (0.300 g, 1.21 mmol) in dioxane (10 ml) and 1 M HCl (5 ml) gave crude (2R, 3R)-2-aminoheptan-1,3-diol (102) (0.178 g, 100%) as a light brown solid which was reacted immediately.

Methyl dichloroacetate (0.63 ml, 6.08 mmol) and triethylamine (0.17 ml, 1.22 mmol) were added to a solution of the amino diol (102) (0.178 g, 1.21 mmol) in dry methanol (3 ml). The reaction mixture was stirred at room temperature for 24h.

The reaction mixture was evaporated to residue *in vacuo* to give a pale orange oil which was taken up in ethyl acetate (5 ml). The solution was washed with 2 M HCl (2×1.5 ml), dried (Na₂SO₄) and evaporated to residue *in vacuo*. The residue was triturated with petroleum ether (2×1.5 ml) and purified by flash column chromatography (ethyl acetate) to give the title compound (0.164 g, 53%) as a white solid.

R_f 0.44 (ethyl acetate);

m.p. 79-81°C (from ethyl acetate/hexane);

[α]_D +15.5°(c1.0 in EtOH);

C₉H₁₇NO₃Cl₂ requires C, 41.9; H, 6.6; N, 5.4%

found C, 41.8; H, 6.5; N, 5.5%;

ν_{max} (nujol)/cm⁻¹ 3420br, 3305m, 1670s, 1550m, 1314w, 1220w, 1200w, 1145br, 1100w, 1070w, 1050w, 970m, 910w, 860w, 810m, 765w, 720m, 665m;

δ_H (300 MHz; D₂O+CD₃OD) 0.82 (3H, t, J=7.3 Hz, CH₂CH₃), 1.23-1.41 (6H, m, (CH₂)₃CH₃), 3.59 (1H, ABX system, J_{BX}=7.6 Hz, J_{gem}=11.5 Hz, CH₂OH), 3.67 (1H, ABX system, J_{AX}=5.5 Hz, J_{gem}=11.5 Hz, CH₂OH), 3.75-3.80 (1H, m, CHCH₂OH), 3.87-3.92 (1H, m, CHBu), 6.31 (1H, s, CHCl₂);

δ_C (75 MHz; CD₃OD) 14.30 (CH₃), 23.63 (CH₂), 28.94 (CH₂), 34.74 (CH₂), 56.52 (CH), 62.50 (CH₂), 67.65 (CH), 70.53 (CH), 166.84 (C);

m/z 258 ([MH]⁺, 2%), 230 (9), 229 (17), 228 (43), 227 (27), 226 (50), 210 (12), 208 (18), 157 (31), 156 (47), 155 (71), 154 (61), 153 (79), 128 (12), 121 (8), 120 (28), 119 (27), 118 (62), 99 (15), 95 (11), 86 (11), 85 (11), 83 (17), 81 (43), 71 (28), 70 (100), 69 (22), 61 (12);

C₉H₁₈NO₃Cl₂ [MH]⁺ requires 258.0644
found 258.0664.

(2R, 3R)-2-N-Dichloroacetamidooctan-1,3-diol (109)

Following the method used for the deprotection of (40), the protected diol (97) (1.263 g, 4.84 mmol) in dioxane (50 ml) and 1 M HCl (25 ml) gave crude (2R, 3R)-2-aminooctan-1,3-diol (103) (0.697 g, 89%) as a light brown solid which was reacted immediately.

Methyl dichloroacetate (2.20 ml, 21.25 mmol) and triethylamine (0.60 ml, 4.31 mmol) were added to a solution of the amino diol (103) (0.697 g, 4.33 mmol) in dry methanol (11 ml). The reaction mixture was stirred at room temperature for 24h.

The reaction mixture was evaporated to residue *in vacuo* to give a pale orange oil which was taken up in ethyl acetate (15 ml). The solution was washed with 2 M HCl (2×3 ml) and water (3 ml), dried (Na₂SO₄) and evaporated to residue *in vacuo*. The residue was triturated with petroleum ether (2×3 ml) and purified by flash column chromatography (1:1 ethyl acetate-dichloromethane) to give the title compound (0.956 g, 81%) as a white solid.

R_f 0.49 (ethyl acetate);

m.p. 89.5-91°C (from ethyl acetate/hexane);

[α]_D +16.3° (c1.0 in EtOH);

$C_{10}H_{19}NO_3Cl_2$ requires C, 44.1; H, 7.0; N, 5.15%

found C, 44.4; H, 6.9; N, 5.0%;

ν_{max} (nujol)/ cm^{-1} 3420br, 3340w, 3300m, 3100w, 1670s, 1550m, 1315w, 1220w, 1205m, 1140w, 1070w, 1045w, 1035w, 980w, 930m, 905w, 855w, 815m, 760w, 720m, 665m;

δ_H (300 MHz; D_2O+CD_3OD) 1.09 (3H, t, $J=6.7$ Hz, CH_2CH_3), 1.40-1.75 (8H, m, $(CH_2)_4CH_3$), 3.79 (1H, ABX system, $J_{BX}=6.3$ Hz, $J_{gem}=10.9$ Hz, CH_2OH), 3.87 (1H, ABX system $J_{AX}=6.4$ Hz, $J_{gem}=10.9$ Hz, CH_2OH), 3.97-4.16 (2H, m, $CHCH_2OH$ and CH_{pent}), 6.55 (1H, s, $CHCl_2$);

δ_C (75 MHz; D_2O+CD_3OD) 14.35 (CH_3), 23.57 (CH_2), 26.40 (CH_2), 32.89 (CH_2), 35.01 (CH_2), 56.51 (CH), 62.51 (CH_2), 67.65 (CH), 70.55 (CH), 166.84 (C);

m/z No M^+ , 242 (14%), 240 (23), 157 (11), 155 (66), 153 (100), 118 (27), 95 (20), 84 (11), 70 (47);

$C_{10}H_{20}NO_3Cl_2$ $[MH]^+$ requires 272.0820

found 272.0820.

(2R, 3R)-2-N-Dichloroacetamidononan-1,3-diol (110)

Following the method used for the deprotection of (40), the protected diol (98) (1.289 g, 4.69 mmol) in dioxane (50 ml) and 1 M HCl (25 ml) gave crude (2R, 3R)-2-aminononan-1,3-diol (104) (0.765 g, 93%) as a light brown solid which was reacted immediately.

Methyl dichloroacetate (2.30 ml, 22.21 mmol) and triethylamine (0.61 ml, 4.38 mmol) were added to a solution of the amino diol (104) (0.765 g, 4.37 mmol) in dry methanol (11 ml). The reaction mixture was stirred at room temperature for 24h.

The reaction mixture was evaporated to residue *in vacuo* to give an orange oil which was taken up in ethyl acetate (15 ml). The solution was washed with 2 M HCl (2×3 ml) and water (3 ml), dried (Na₂SO₄) and evaporated to residue *in vacuo*. The residue was triturated with petroleum ether (2×3 ml) and purified by flash column chromatography (1:1 ethyl acetate-dichloromethane) to give the title compound (0.937 g, 75%) as a white solid.

R_f 0.50 (ethyl acetate);

m.p. 85-87°C (from ethyl acetate/hexane);

[α]_D +17.0° (c1.0 in EtOH);

C₁₁H₂₁NO₃Cl₂ requires C, 46.2; H, 7.4; N, 4.9%

found C, 45.9; H, 7.1; N, 4.9%;

ν_{max} (nujol)/cm⁻¹ 3400br, 3340m, 1685s, 1550m, 1320w, 1200w, 1150w, 1100w, 1070m, 1025br, 970w, 860w, 820m, 810m, 775w, 720m;

δ_H (300 MHz; D₂O+CD₃OD) 0.89 (3H, t, J=6.9 Hz, CH₂CH₃), 1.16-1.60 (8H, m, (CH₂)₄CH₃), 3.61 (1H, ABX system, J_{BX}=6.3 Hz, J_{gem}=10.9 Hz, CH₂OH), 3.69 (1H, ABX system, J_{AX}=6.4 Hz, J_{gem}=10.9 Hz, CH₂OH), 3.82-3.97 (2H, m, CHCH₂OH and CHhex), 6.35 (1H, s, CHCl₂);

δ_C (75 MHz; D₂O+CD₃OD) 14.41 (CH₃), 23.57 (CH₂), 26.63 (CH₂), 30.28 (CH₂), 32.82 (CH₂), 35.00 (CH₂), 56.45 (CH₂), 56.45 (CH), 65.45 (CH), 65.52 (CH₂), 67.62 (CH), 70.58 (CH), 166.83 (C);

m/z No M⁺, 256 (10%), 254 (17), 238 (14), 236 (22), 202 (19), 157 (10), 155 (66), 154 (17), 153 (100), 120 (11), 119 (22), 118 (33), 117 (21), 113 (11), 109 (43), 86 (21), 85 (12), 84 (25), 83 (23), 81 (12), 71 (16), 70 (82), 69 (16), 67 (22);

C₁₁H₂₂NO₃Cl₂ [MH]⁺ requires 286.0977

found 286.0977.

(1R, 2R)-1-Cyclohexyl-2-N-dichloroacetamidopropan-1,3-diol (111)

Following the method used for the deprotection of (40), the protected diol (99) (0.200 g, 0.73 mmol) in dioxane (10 ml) and 1 M HCl (5 ml) gave crude (1R, 2R)-2-amino-1-cyclohexylpropan-1,3-diol (105) (0.120 g, 94%) as a white solid which was reacted immediately.

Methyl dichloroacetate (0.36 ml, 0.35 mmol) and triethylamine (0.10 ml, 0.72 mmol) were added to a solution of the amino diol (105) (0.120 g, 0.69 mmol) in dry methanol (2 ml). The reaction mixture was stirred at room temperature for 24h.

The reaction mixture was evaporated to residue *in vacuo* to give an orange oil which was taken up in ethyl acetate (5 ml). The solution was washed with 2 M HCl (2×1 ml) and water (1 ml), dried (Na₂SO₄) and evaporated to residue *in vacuo*. The residue was triturated with petroleum ether (2×1 ml) and purified by flash column chromatography (2:1 ethyl acetate-dichloromethane) to give the title compound (0.083 g, 42%) as a white solid.

R_f 0.44 (ethyl acetate);

m.p. 38-40°C (without further purification);

[α]_D +14.0° (c1.0 in EtOH);

ν_{max} (CH₂Cl₂)/cm⁻¹ 3620m, 3420m, 2930s, 2860m, 1690s, 1520m, 1450w, 1320w, 1210w, 1105w, 1060m, 1045m, 970w, 920w, 890w, 865s, 835w, 810m, 700br;

δ_H (300 MHz; CD₃OD) 0.88-1.11 (2H, m, C₆H₁₁), 1.14-1.45 (4H, m, C₆H₁₁), 1.60-1.85 (4H, m, C₆H₁₁), 1.96-2.06 (1H, m, C₆H₁₁), 3.52 (1H, dd, J=1.9, 8.5 Hz, C₆H₁₁CH), 3.59 (1H, ABX system, J_{BX}=6.1 Hz, J_{gem}=10.8 Hz, CH₂OH), 3.66 (1H, ABX system, J_{AX}=7.0, J_{gem}=10.8 Hz, CH₂OH), 4.06-4.11 (1H, m, CHCH₂OH), 6.37 (1H, s, CHCl₂);

δ_C (75 MHz; CD_3OD) 27.11 (CH_2), 27.14 (CH_2), 27.49 (CH_2), 30.09 (CH_2), 30.43 (CH_2), 42.03 (CH), 53.86 (CH), 62.93 (CH_2), 67.70 (CH), 74.65 (CH), 166.53 (C);

m/z No M^+ , 254 (8), 252 (13), 156 (10), 155 (65), 154 (24), 153 (100), 152 (12), 118 (22), 111 (11), 107 (24), 83 (13), 70 (19);

$C_{11}H_{20}NO_3Cl_2$ $[MH]^+$ requires 284.0820
found 284.0819.

(4R, 5R)-5-Amino-5-N-*tert*-butoxycarbonyl-2,2-dimethyl-4-methyl-1,3-dioxan (88)

Pyridinium *p*-toluenesulphonate (0.218 g, 0.87 mmol) was added to a solution of (2R, 3R)-2-amino-2-N-*tert*-butoxycarbonylbutan-1,3-diol (40) (0.178 g, 0.87 mmol) and 2,2-dimethoxypropane (2.10 ml, 17.10 mmol) in dichloromethane (10 ml), under a nitrogen atmosphere. The reaction mixture was stirred at room temperature overnight. N.B. The N-BOC protected amino diol (40) had been prepared via the aldehyde (81).

Ether (20 ml) was added and the organics were washed with half-saturated brine (15 ml), dried (Na_2SO_4) and evaporated to residue *in vacuo* to give a yellow oil which was purified by flash column chromatography (ethyl acetate) to give the title compound (0.139 g, 65%) as a colourless oil.

R_f 0.57 (ethyl acetate);

δ_H (300 MHz; $CDCl_3$) 1.12 (3H, d, $J=6.3$ Hz, $CHCH_3$), 1.40 (3H, s, CCH_3), 1.46 (12H, s, $C(CH_3)_3$ and CCH_3), 3.42-3.48 (1H, m, CH_2CH), 3.74 (1H, ABX system, $J_{BX}=1.8$ Hz, $J_{gem}=11.9$ Hz, OCH_2), 4.08 (1H, ABX system, $J_{AX}=2.0$ Hz, $J_{gem}=11.9$ Hz, OCH_2), 4.13 (1H, dq, $J=1.9, 6.3$ Hz, $CHCH_3$), 5.32 (1H, d, $J=9.7$ Hz, NH); N.B. Signal for $CHCH_3$ confirmed by irradiation at δ 1.12;

δ_C (75 MHz; $CDCl_3$) 17.71 (CH_3), 18.58 (CH_3), 28.37 (CH_3), 29.70 (CH_3), 47.90 (CH), 65.11 (CH_2), 67.25 (CH), 79.23 (C), 98.86 (C), 155.86 (C).

(4R, 5R)-5-N-Dichloroacetamido-2,2-dimethyl-4-methyl-1,3-dioxan (54)

Pyridinium *p*-toluenesulphonate (0.033 g, 0.13 mmol) was added to a solution of (2R, 3R)-2-N-dichloroacetamidobutan-1,3-diol (**46**) (0.028 g, 0.13 mmol) and 2,2-dimethoxypropane (0.32 ml, 2.61 mmol) in dichloromethane (7 ml), under a nitrogen atmosphere. The reaction mixture was stirred at room temperature overnight. N.B. (**46**) had been prepared via the aldehyde (**81**).

Ether (5 ml) was added and the organics were washed with half-saturated brine (3 ml), dried (Na₂SO₄) and evaporated to residue *in vacuo* to give a white solid which was purified by flash column chromatography (ethyl acetate) to give the title compound (0.030 g, 91%) as a white solid.

R_f 0.60 (ethyl acetate);

m.p. 92-94°C (without further purification);

δ_H (300 MHz; CDCl₃) 1.14 (3H, d, J=6.3 Hz, CHCH₃), 1.44 (3H, s, CCH₃), 1.50 (3H, s, CCH₃), 3.74 3.81 (2H, m, CH₂CH and one of OCH₂), 4.11-4.16 (1H, m, other of OCH₂), 4.22 (1H, dq, J=1.7, 6.3 Hz, CHCH₃), 5.99 (1H, s, CHCl₂), 7.19 (1H, br, NH);

δ_C (75 MHz; CDCl₃) 17.58 (CH₃), 18.35 (CH₃), 29.59 (CH₃), 47.47 (CH), 64.04 (CH₂), 66.33 (CH), 66.86 (CH), 99.18 (C), 164.00 (C).

Mosher ester of (2R, 3R)-2-N-dichloroacetamidobutan-1,3-diol (55)

(R)-α-methoxy-α-trifluoromethyl-phenylacetyl chloride (MTPA-Cl) (50 μl, 242 μmol) was added rapidly to a mixture of (2R, 3R)-2-N-dichloroacetamidobutan-1,3-diol (0.0086 g, 39.8 μmol) in dry pyridine (8 drops) and carbon tetrachloride (5 drops). The reaction mixture was left to stand at room temperature for 16h.

Water (2 ml) was added and the mixture extracted with ether (3×15 ml). The combined organics were washed with 0.5 M HCl (4 ml), water (4 ml) and saturated Na₂CO₃ (7 ml), dried (Na₂SO₄) and evaporated to residue *in vacuo* to give the title compound (0.024 g) as a colourless oil/solid mixture.

δ_F (282.36 MHz; CDCl₃) -71.45, -71.77 (1:1).

Mosher ester of (2R, 3R)-2-N-dichloroacetamidohexan-1,3-diol (112)

Following the method used for the preparation of (55), (2R, 3R)-2-N-dichloroacetamidohexan-1,3-diol (0.0097 g, 39.8 μ mol) and MTPA-Cl (50 μ l, 242 μ mol) gave the title compound (0.028 g) as a colourless oil/solid mixture.

δ_F (282.36 MHz; CDCl₃) -71.21, -71.80 (1:1).

Mosher ester of (2R, 3R)-2-N-dichloroacetamidoheptan-1,3-diol (113)

Following the method used for the preparation of (55), (2R, 3R)-2-N-dichloroacetamidoheptan-1,3-diol (0.0104 g, 40.3 μ mol) and MTPA-Cl (50 μ l, 242 μ mol) gave the title compound (0.029 g) as a colourless oil/solid mixture.

δ_F (282.36 MHz; CDCl₃) -71.13, -71.80 (1:1).

Mosher ester of (2R, 3R)-2-N-dichloroacetamidoctan-1,3-diol (114)

Following the method used for the preparation of (55), (2R, 3R)-2-N-dichloroacetamidoctan-1,3-diol (0.0108 g, 39.7 μ mol) and MTPA-Cl (50 μ l, 242 μ mol) gave the title compound (0.031 g) as a colourless oil/solid mixture.

δ_F (282.36 MHz; CDCl₃) -71.13, -71.78 (1:0.8).

Mosher ester of (2R, 3R)-2-N-dichloroacetamidononan-1,3-diol (115)

Following the method used for the preparation of (55), (2R, 3R)-2-N-dichloroacetamidononan-1,3-diol (0.0114 g, 39.9 μ mol) and MTPA-Cl (50 μ l, 242 μ mol) gave the title compound (0.024 g) as a colourless oil/solid mixture.

δ_F (282.36 MHz; $CDCl_3$) -71.13, -71.78 (1:0.4).

6.4 SYNTHESIS RELATING TO CHAPTER 4

(2S)-1-O-Acetyl-2-N-acetyl-2-amino-3-phenyl-1-propanol (118)

(N,O-Diacetyl-L-phenylalaninol)

Acetic anhydride (2.65 ml, 28.11 mmol) was added to a solution of L-phenylalaninol (2.018 g, 13.36 mmol) in dry pyridine (20 ml) at 0°C. The solution was stirred at 0°C for 5h and then left to stand in a fridge overnight.

After warming to room temperature the reaction mixture was poured into water (50 ml), stirred for 5 min and extracted with ethyl acetate (3×75 ml). The combined organics were washed with 2 M HCl (50 ml), 10% NaHCO₃ solution (50 ml) and water (50 ml), dried (Na₂SO₄) and evaporated to residue *in vacuo* to give the title compound (2.858 g, 91%) as a white solid.

R_f 0.34 (ethyl acetate);

m.p. 127-128.5°C (without further purification);

[α]_D -18.5° (c 1.0 in CH₂Cl₂);

ν_{max} (nujol)/cm⁻¹ 3300m, 3100w, 1730s, 1645s, 1550m, 1310w, 1275m, 1260w, 1195w, 1130w, 1105w, 1040w, 1030w, 975w, 920w, 895w, 860w, 755m, 720w, 700m;

δ_H (300 MHz; CDCl₃) 1.92 (3H, s, COCH₃), 2.07 (3H, s, COCH₃), 2.81 (1H, ABX system, J_{BX}=7.5 Hz, J_{gem}=13.8 Hz, C₆H₅CH₂), 2.87 (1H, ABX system, J_{AX}=6.6 Hz, J_{gem}=13.8 Hz, C₆H₅CH₂), 4.04 (2H, d, J=5.1 Hz, CH₂OAc), 4.37-4.47 (1H, m, CHCH₂OAc), 6.01 (1H, d, J=8.3 Hz, NH), 7.17-7.31 (5H, m, C₆H₅);

δ_C (75 MHz; CDCl₃) 20.80 (CH₃), 23.26 (CH₃), 37.47 (CH₂), 49.49 (CH), 64.83 (CH₂), 126.71 (CH), 128.56 (CH), 129.13 (CH), 137.03 (C), 169.74 (C), 170.95 (C).

(2R)-1-O-Acetyl-2-N-acetyl-2-amino-3-phenyl-1-propanol (122)

(N,O-Diacetyl-D-phenylalaninol)

Following the method used for the preparation of (118), D-phenylalaninol (3.036 g, 20.11 mmol) and acetic anhydride (4.0 ml, 42.43 mmol) gave the title compound (4.076 g, 86%) as a white solid.

R_f 0.34 (ethyl acetate);

m.p. 126-127.5°C (without further purification);

[α]_D +17.0° (c1.0 in CH₂Cl₂);

ν_{max} (nujol/cm⁻¹ 3300m, 3100w, 1730s, 1645s, 1550m, 1310w, 1275m, 1260m, 1195m, 1130w, 1105w, 1040m, 1030m, 980w, 920w, 895w, 860w, 750m, 735w, 720w, 700m;

δ_H (300 MHz; CDCl₃) 1.92 (3H, s, COCH₃), 2.06 (3H, s, COCH₃), 2.78 (1H, ABX system, J_{BX}=7.5 Hz, J_{gem}=13.8 Hz, C₆H₅CH₂), 2.87 (1H, ABX system, J_{AX}=6.7 Hz, J_{gem}=13.8 Hz, C₆H₅CH₂), 4.04 (2H, d, J=5.0 Hz, CH₂OAc), 4.36-4.47 (1H, m, CHCH₂OAc), 6.11 (1H, d, J=8.3 Hz, NH), 7.16-7.31 (5H, m, C₆H₅);

δ_C (75 MHz; CDCl₃) 20.80 (CH₃), 23.24 (CH₃), 37.43(CH₂), 49.45 (CH), 64.82 (CH₂), 126.67 (CH), 128.53 (CH), 129.11 (CH), 137.05 (C), 169.76 (C), 170.93 (C).

(2S)-1-O-Acetyl-2-N-acetyl-2-amino-3-(p-nitrophenyl)-1-propanol (119)

(N,O-Diacetyl-L-p-nitrophenylalaninol)

N,O-Diacetyl-L-phenylalaninol (2.348 g, 9.99 mmol) was added portionwise over 5 min. to stirred fuming nitric acid (10 ml) at 0°C. The reaction mixture was left to warm to room temperature and stirring was continued at this temperature for a further 50 min.

The reaction mixture was poured onto ice (20 g), basified with NaHCO₃ and extracted with ethyl acetate (4×50 ml). The combined organics were washed with

water (2×50 ml), dried (Na₂SO₄) and evaporated to residue *in vacuo* to give the title compound (2.552 g, 91%) as a white solid.

R_f 0.30 (ethyl acetate);

m.p. 173-180°C (without further purification);

[α]_D -25.0° (c1.0 in CH₂Cl₂);

ν_{max} (nujol)/cm⁻¹ 3280m, 3100w, 2715w, 1725s, 1650s, 1605w, 1555m, 1515s, 1350s, 1315w, 1305w, 1270s, 1195w, 1150w, 1125w, 1105w, 1050m, 980w, 970w, 925w, 895w, 860w, 850w, 805w, 750w, 720m, 700m;

δ_H (300 MHz; CDCl₃) 1.95 (3H, s, COCH₃), 2.11 (3H, s, COCH₃), 2.93 (1H, ABX system, J_{BX}=7.4 Hz, J_{gem}=13.8 Hz, (C₆H₄NO₂)CH₂), 3.00 (1H, ABX system, J_{AX}=7.0 Hz, J_{gem}=13.8 Hz, (C₆H₄NO₂)CH₂), 4.03-4.15 (2H, m, CH₂OAc), 4.42-4.55 (1H, m, CHCH₂OAc), 5.88 (1H, d, J=8.2 Hz, NH), 7.39 (2H, d, J=8.7 Hz, C₆H₄), 8.16 (2H, d, J=8.7 Hz, C₆H₄);

δ_C (75 MHz; (CD₃)₂SO) 20.50 (CH₃), 22.40 (CH₃), 36.30 (CH₂), 48.60 (CH), 64.88 (CH₂), 123.06 (CH), 130.18 (CH), 145.96 (C), 146.64 (C), 168.89 (C), 170.10 (C).

(2R)-1-O-Acetyl-2-N-acetyl-2-amino-3-(p-nitrophenyl)-1-propanol (123)
(N,O-Diacetyl-D-p-nitrophenylalaninol)

Following the method used for the preparation (119), N,O-diacetylphenylalaninol (3.460 g, 14.72 mmol) and fuming nitric acid (15 ml) gave the title compound (3.857 g, 94%) as a white solid.

R_f 0.30 (ethyl acetate);

m.p. 171-178°C (without further purification);

[α]_D +22.0° (c1.0 in CH₂Cl₂);

ν_{\max} (nujol)/cm⁻¹ 3280m, 3100w, 2715w, 1725s, 1650s, 1605w, 1555m, 1515s, 1350s, 1315w, 1305w, 1270s, 1195w, 1150w, 1125w, 1105w, 1045m, 980w, 965w, 925w, 895w, 860w, 850w, 805w, 750w, 720m, 700m;

δ_{H} (300 MHz; CDCl₃) 1.96 (3H, s, COCH₃), 2.11 (3H, s, COCH₃), 2.93 (1H, ABX system, $J_{\text{BX}}=7.4$ Hz, $J_{\text{gem}}=13.8$ Hz, (C₆H₄NO₂)CH₂), 3.00 (1H, ABX system, $J_{\text{AX}}=7.0$ Hz, $J_{\text{gem}}=13.8$ Hz, (C₆H₄NO₂)CH₂), 4.03-4.15 (2H, m, CH₂OAc), 4.42-4.55 (1H, m, CHCH₂OAc), 5.83 (1H, d, $J=8.3$ Hz, NH), 7.39 (2H, d, $J=8.7$ Hz, C₆H₄), 8.16 (2H, d, $J=8.7$ Hz, C₆H₄);

δ_{C} (75 MHz; (CD₃)₂SO) 20.52 (CH₃), 22.41 (CH₃), 36.30 (CH₂), 48.60 (CH), 64.88 (CH₂), 123.08 (CH), 130.20 (CH), 145.97 (C), 146.65 (C), 168.87 (C), 170.10 (C).

(2S)-2-N-Dichloroacetamido-3-(*p*-nitrophenyl)-1-propanol (11)

(1-Deoxychloramphenicol)

N,O-Diacetyl-L-*p*-nitrophenylalaninol (2.309 g, 8.25 mmol) in 5% HCl (80 ml) was heated to reflux and aged at that temperature for 2.5h.

After cooling to room temperature the reaction mixture was extracted with ether (2× 50 ml). The aqueous layer was basified to pH 11 with 1 M NaOH and extracted with ethyl acetate (5×75 ml). The combined organics were dried (Na₂SO₄) and evaporated to residue *in vacuo* to give crude (2S)-2-amino-3-(*p*-nitrophenyl)-1-propanol, (L-*p*-nitrophenylalaninol) (120) (1.454 g, 90%) as a pale yellow solid which was reacted immediately.

Methyl dichloroacetate (1.90 ml, 18.35 mmol) was added to a solution of L-*p*-nitrophenylalaninol (1.454 g, 7.42 mmol) in dry ethanol (20 ml). The reaction mixture was heated to reflux and aged at that temperature for 3h.

The reaction mixture was evaporated to residue *in vacuo* to give a yellow solid which was taken up in ethyl acetate (20 ml). The solution was washed with 2 M HCl (2× 10 ml), dried (Na₂SO₄) and evaporated to residue *in vacuo*. The residue was triturated with petroleum ether (2×10 ml) and purified by flash column chromatography (4:1 dichloromethane-ethyl acetate) to give the title compound (1.868 g, 82%) as a pale yellow solid. Repeated recrystallisations (ethyl acetate/hexane) were then carried out to remove the small proportion of *ortho*-material that had not been separated by chromatography.

R_f 0.40 (ethyl acetate);

m.p. 147.5–148.5°C (from ethyl acetate/hexane);

[α]_D -12.0° (c1.0 in EtOH);

C₁₁H₁₂N₂O₄Cl₂ requires C, 43.0; H, 3.9; N, 9.1%

found C, 43.3; H, 4.0; N, 9.15%;

ν_{max} (nujol/cm⁻¹) 3440br, 3260m, 3095w, 1675s, 1610w, 1600w, 1560m, 1515s, 1345s, 1235w, 1210w, 1180w, 1110w, 1080w, 1055w, 1020w, 960w, 905w, 865s, 855s, 810s, 750w, 720w, 700m;

δ_H (300 MHz; CD₃OD) 2.90 (1H, ABX system, J_{BX}=9.3 Hz, J_{gem}=13.8 Hz, (C₆H₄NO₂)CH₂), 3.13 (1H, ABX system, J_{AX}=5.3 Hz, J_{gem}=13.8 Hz, (C₆H₄NO₂)CH₂), 3.60 (2H, d, J=5.3 Hz, CH₂OH), 4.13–4.21 (1H, m, CHCH₂OH), 6.14 (1H, s, CHCl₂), 7.48 (2H, d, J=8.8 Hz, C₆H₄), 8.14 (2H, d, J=8.8 Hz, C₆H₄);

δ_C (75 MHz; CD₃OD) 37.51 (CH₂), 54.52 (CH), 63.82 (CH₂), 67.54 (CH), 124.32 (CH), 131.43 (CH), 147.46 (C), 147.99 (C), 166.33 (C);

m/z 306 (M⁺, 2%), 277 (33), 275 (48), 223 (18), 180 (10), 179 (32), 174 (26), 173 (11), 172 (90), 171 (12), 170 (100), 165 (52), 154 (34), 152 (54), 137 (46), 136 (20), 120 (10), 119 (11), 107 (11), 106 (19), 91 (24), 90 (23), 89 (18), 85 (13), 83 (21), 78 (14), 77 (13);

$C_{11}H_{12}N_2O_4Cl_2 \cdot M^+$ requires 306.0174
found 306.0174.

(2R)-2-N-Dichloroacetamido-3-(*p*-nitrophenyl)-1-propanol (125)
(N-Dichloroacetamido-D-*p*-nitrophenylalaninol)

Following the method used for the preparation of (11), N,O-diacetyl-D-*p*-nitrophenylalaninol (2.059 g, 7.35 mmol) in 5% HCl (80 ml) gave crude D-*p*-nitrophenylalaninol (124) (1.192 g, 83%) as a pale yellow solid which was reacted immediately.

Methyl dichloroacetate (1.50 ml, 14.49 mmol) was added to a solution of D-*p*-nitrophenylalaninol (1.140 g, 5.82 mmol) in dry ethanol (15 ml). The reaction mixture was heated to reflux and aged at that temperature for 5h.

The reaction mixture was evaporated to residue *in vacuo* to give a pale yellow solid which was taken up in ethyl acetate (15 ml). The solution was washed with 2 M HCl (2× 5 ml), dried (Na_2SO_4) and evaporated to residue *in vacuo*. The residue was triturated with petroleum ether (2×5 ml) and purified by flash column chromatography (4:1 dichloromethane-ethyl acetate) to give the title compound (1.473 g, 82%) as a pale yellow solid. Repeated recrystallisations (ethyl acetate/hexane) were carried out to remove the small proportion of *ortho*-material that had not been separated by chromatography.

R_f 0.40 (ethyl acetate);

m.p. 147-148.5°C (from ethyl acetate/hexane);

$[\alpha]_D +11.0^\circ$ (c1.0 in EtOH);

ν_{\max} (nujol)/ cm^{-1} 3440br, 3280m, 3100w, 2400w, 1675s, 1600w, 1560w, 1515s, 1345s, 1235w, 1150w, 1110w, 1070w, 1055w, 1020w, 960w, 865m, 855m, 850m, 810m, 720m, 700m;

δ_{H} (300 MHz; $\text{D}_2\text{O}+\text{CD}_3\text{OD}$) 2.90 (1H, ABX system, $J_{\text{BX}}=9.3$ Hz, $J_{\text{gem}}=13.7$ Hz, $(\text{C}_6\text{H}_4\text{NO}_2)\text{CH}_2$), 3.12 (1H, ABX system, $J_{\text{AX}}=5.1$ Hz, $J_{\text{gem}}=13.7$ Hz, $(\text{C}_6\text{H}_4\text{NO}_2)\text{CH}_2$), 3.61 (2H, d, $J=5.0$ Hz, CH_2OH), 4.15-4.22 (1H, m, CHCH_2OH). 6.15 (1H, s, CHCl_2), 7.47 (2H, d, $J=8.6$ Hz, C_6H_4), 8.11 (2H, d, $J=8.6$ Hz, C_6H_4);

δ_{C} (75 MHz; $\text{D}_2\text{O}+\text{CD}_3\text{OD}$) 37.50 (CH_2), 54.50 (CH), 63.80 (CH_2), 67.52 (CH), 124.34 (CH), 131.42 (CH), 147.46 (C), 148.00 (C), 166.32 (C);

m/z No M^+ , 277 (12%), 275 (19), 223 (6), 179 (13), 174 (10), 172 (64), 170 (100), 165 (23), 154 (17), 152 (27), 137 (22), 106 (12), 90 (10);

$\text{C}_{11}\text{H}_{12}\text{N}_2\text{O}_4\text{Cl}_2$ M^+ requires 306.0174
found 306.0174.

(2S)-2-N-Dichloroacetamido-3-phenyl-1-propanol (126)

(N-Dichloroacetamido-L-phenylalaninol)

Methyl dichloroacetate (1.45 ml, 14.00 mmol) was added to a solution of L-phenylalaninol (1.058 g, 7.00 mmol) in dry ethanol (10 ml). The reaction mixture was evaporated to residue *in vacuo*, triturated with petroleum ether (2×10 ml) and purified by flash column chromatography (ethyl acetate) to give the title compound (1.055 g, 57%) as a white solid.

R_f 0.56 (ethyl acetate);

m.p. 90-91°C (from ethyl acetate/hexane);

$[\alpha]_{\text{D}}$ -15.8° (c 1.0 in EtOH);

$C_{11}H_{13}NO_2Cl_2$ requires C, 50.4; H, 5.0; N, 5.3%

found C, 50.8; H, 5.1; N, 5.3%;

ν_{max} (nujol)/ cm^{-1} 3270m, 3100w, 1675s, 1570m, 1310w, 1260w, 1235w, 1210w, 1150w, 1075w, 1060w, 1050w, 1030w, 960w, 910w, 875w, 855w, 810m, 755m, 720m, 700m, 660m;

δ_H (300 MHz; $CDCl_3$) 2.90 (1H, ABX system, $J_{BX}=7.1$ Hz, $J_{gem}=13.8$ Hz, $C_6H_5CH_2$), 2.93 (1H, ABX system, $J_{AX}=7.2$ Hz, $J_{gem}=13.8$ Hz, $C_6H_5CH_2$), 3.11 (1H, br s, OH, exchanged with D_2O), 3.61 (1H, ABX system, $J_{BX}=4.8$ Hz, $J_{gem}=11.1$ Hz, CH_2OH), 3.68 (1H, ABX system, $J_{AX}=3.9$ Hz, $J_{gem}=11.1$ Hz, CH_2OH), 4.11-4.21 (1H, m, $CHCH_2OH$), 5.91 (1H, s, $CHCl_2$), 7.05 (1H, d, $J=8.1$ Hz, NH, exchanged with D_2O), 7.20-7.32 (5H, m, C_6H_5);

δ_C (75 MHz; $CDCl_3$) 36.61 (CH_2), 53.33 (CH), 62.67 (CH_2), 66.44 (CH), 126.78 (CH), 128.61 (CH), 129.24 (CH), 139.87 (C), 164.40 (C).

m/z 261 (M^+ , 3%), 230 (11), 172 (25), 170 (38), 154 (13), 152 (20), 134 (82), 120 (19), 117 (11), 105 (14), 103 (13), 92 (57), 91 (100), 85 (10), 83 (15), 78 (14), 77 (16).

(2S)-2-N-Dichloroacetamido-3-(p-hydroxyphenyl)-1-propanol (128)

(N-Dichloroacetamido-L-tyrosinol)

Methyl dichloroacetate (2.30 ml, 22.21 mmol) and triethylamine (0.73 ml, 5.25 mmol) were added to a solution of L-tyrosinol hydrochloride (0.886 g, 4.35 mmol) in dry methanol (10 ml). The reaction mixture was stirred at room temperature for 2 days.

The reaction mixture was evaporated to residue *in vacuo* to give a pale orange oil which was taken up in ethyl acetate (10 ml). The solution was washed with 2 M HCl (2×2.5 ml) and water (2.5 ml), dried (Na_2SO_4) and evaporated to residue *in vacuo*. The residue was triturated with petroleum ether (2×10 ml) and purified by flash column

chromatography (ethyl acetate) to give the title compound (0.876 g, 72%) as a white solid.

R_f 0.54 (ethyl acetate);

m.p. 151-151.5°C (from ethyl acetate/hexane);

$[\alpha]_D$ -10.5° (c1.0 in EtOH);

$C_{11}H_{13}NO_3Cl_2$ requires C, 47.5; H, 4.7; N, 5.0%

found C, 47.6; H, 4.8; N, 4.9%;

ν_{max} (nujol)/ cm^{-1} 3470w, 3370br, 3270m, 3100w, 1675s, 1600w, 1570m, 1515m, 1320w, 1250w, 1240w, 1220m, 1205w, 1185w, 1115w, 1080w, 1055m, 1025m, 955w, 915w, 880w, 830w, 815w, 805m, 760w, 720m, 635m;

δ_H (300 MHz; CD_3OD) 2.68 (1H, ABX system, $J_{BX}=7.9$ Hz, $J_{gem}=13.9$ Hz, $(C_6H_4OH)CH_2$), 2.84 (1H, ABX system, $J_{AX}=6.3$ Hz, $J_{gem}=13.9$ Hz, $(C_6H_4OH)CH_2$), 3.53 (1H, ABX system, $J_{BX}=5.6$ Hz, $J_{gem}=11.1$ Hz, CH_2OH), 3.75 (1H, ABX system, $J_{AX}=5.0$ Hz, $J_{gem}=11.1$ Hz, CH_2OH), 3.97-4.06 (1H, m, $CHCH_2OH$), 6.18 (1H, s, $CHCl_2$), 6.69 (2H, d, $J=8.5$ Hz, C_6H_4), 7.04 (2H, d, $J=8.5$ Hz, C_6H_4), 8.31 (1H, d, $J=8.2$ Hz, NH);

δ_C (75 MHz; $CDCl_3+CD_3OD$) 35.10 (CH_2), 53.29 (CH), 61.65 (CH_2), 65.91 (CH), 114.72 (CH), 127.95 (C), 129.75 (CH), 154.91 (C), 164.44 (C);

m/z No M^+ , 170 (2%), 150 (21), 107 (70), 94 (8), 77 (9), 60 (10);

$C_{11}H_{14}NO_3Cl_2$ $[MH]^+$ requires 278.0351

found 278.0351.

6.5 SYNTHESIS RELATING TO CHAPTER 5

(1R, 2R)-2-N-Chloroacetamido-1-(*p*-nitrophenyl)-propan-1,3-diol (136)

Methyl chloroacetate (2.0 ml, 22.82 mmol) and triethylamine (0.64 ml, 4.60 mmol) were added to a solution of (1R, 2R)-2-amino-1-(*p*-nitrophenyl)-propan-1,3-diol (0.976 g, 4.60 mmol) in dry methanol (15 ml). The reaction mixture was heated to reflux and aged at that temperature for 10h.

The reaction mixture was evaporated to residue *in vacuo* to give a yellow oil which was taken up in ethyl acetate (20 ml). The solution was washed with 2 M HCl (2×5 ml) and saturated brine (5 ml), dried (Na₂SO₄) and evaporated to residue *in vacuo*. The residue was triturated with petroleum ether (2×10 ml) and purified by flash column chromatography (1:1 ethyl acetate-dichloromethane) to give the title compound (0.717 g, 54%) as a white foam which solidified on standing.

R_f 0.39 (ethyl acetate);

m.p. 87-88°C (from ethyl acetate/hexane);

ν_{\max} (nujol)/cm⁻¹ 3450br, 3260m, 1660s, 1560m, 1520m, 1340s, 1290w, 12445m, 1190w, 1150w, 1105w, 1060s, 970w, 940w, 880w, 860w, 840m, 820w, 750w, 725br, 705w, 680w;

δ_{H} (300 MHz; CD₃OH) 3.61 (1H, ABX system, J_{AB} =6.0 Hz, J_{gem} =10.9 Hz, CH₂OH), 3.78 (1H, ABX system, J_{AX} =6.9 Hz, J_{gem} =10.7 Hz, CH₂OH), 3.94 (1H, J =13.7 Hz, CH₂Cl), 4.01 (1H, d, J =13.7 Hz, CH₂Cl), 4.14-4.20 (1H, m, CHCH₂OH), 5.14 (1H, d, J =2.8 Hz, ArCHOH), 7.64 (2H, d, J =8.7 Hz, Ph), 8.18 (2H, d, J =8.8 Hz, Ph);

δ_{C} (75 MHz; CD₃OD) 43.12 (CH₂), 58.15 (CH), 62.35 (CH₂), 71.41 (CH), 124.11 (CH), 128.23 (CH), 148.47 (C), 151.63 (C), 169.02 (C);

m/z No M^+ , 153 (38%), 152 (12), 151 (94), 150 (76), 138 (22), 137 (10), 136 (86), 135 (24), 121 (44), 120 (28), 119 (100), 118 (24), 106 (27), 105 (28), 104 (19), 93 (12), 92 (12), 84 (25), 83 (11), 79 (10), 78 (12), 77 (76), 76 (20);

$C_{11}H_{14}N_2O_5Cl$ $[MH]^+$ requires 289.059
found 289.059.

(1R, 2R)-1-(p-nitrophenyl)-2-N-trichloroacetamido-propan-1,3-diol (137)

Methyl trichloroacetate (2.80 ml, 23.54 mmol) and triethylamine (0.66 ml, 4.74 mmol) were added to a solution of (1R, 2R)-2-amino-1-(p-nitrophenyl)-propan-1,3-diol (1.002 g, 4.73 mmol) in dry methanol (15 ml). The reaction mixture was heated to reflux and aged at that temperature for 1h.

The reaction mixture was evaporated to residue *in vacuo* to give a yellow oil which was taken up in ethyl acetate (15 ml). The solution was washed with 2 M HCl (2×3.5 ml) and saturated brine (3.5 ml), dried (Na_2SO_4) and evaporated to residue *in vacuo*. The residue was triturated with petroleum ether (2×10 ml) and purified by flash column chromatography (2:1 ethyl acetate-dichloromethane) to give the title compound (1.385 g, 82%) as a pale yellow solid.

R_f 0.52 (ethyl acetate);

m.p. 95.5-97°C (from ethyl acetate/hexane);

$C_{11}H_{11}N_2O_5Cl_3$ requires C, 36.9; H, 3.1; N, 7.8%

found C, 37.05; H, 2.8; N, 7.7%;

ν_{max} (nujol)/ cm^{-1} 3540w, 3420m, 1680s, 1510s, 1360s, 1340w, 1315w, 1305w, 1220w, 1150w, 1115w, 1050m, 1040w, 975w, 905w, 850m, 815m, 760w, 740w, 720w, 700w, 680m;

δ_H (300 MHz; CD_3OD) 3.69 (1H, ABX system, $J_{BX}=6.2$ Hz, $J_{gem}=11.0$ Hz, CH_2OH), 3.85 (1H, ABX system, $J_{AX}=6.9$ Hz, $J_{gem}=11.0$ Hz, CH_2OH), 4.13-4.21 (1H, m, $CHCH_2OH$), 4.85 (2H, s, $2\times OH$), 5.19 (1H, d, $J=2.8$ Hz, $ArCHOH$), 7.65 (2H, d, $J=8.7$ Hz, Ph), 8.02 (1H, d, $J=9.0$ Hz, NH), 8.19 (2H, d, $J=8.7$ Hz, Ph);

δ_C (75 MHz; CD_3OD) 59.98 (CH), 61.99 (CH_2), 71.31 (CH), 124.19 (CH), 128.21 (CH), 148.57 (C), 151.26 (C), 163.78 (C), CCl_3 not seen;

m/z No M^+ , 189 (17%), 187 (38), 153 (30), 152 (11), 151 (46), 150 (56), 135 (14), 120 (10), 119 (12), 118 (10), 117 (12), 106 (17), 105 (12), 104 (25), 93 (14), 87 (23), 85 (82), 84 (10), 83 (100), 77 (21), 70 (12), 69 (10);

$C_{11}H_{15}N_3O_5Cl_3$ $[M+NH_4]^+$ requires 374.0077
found 374.0077.

(1R, 2R)-1-(*p*-nitrophenyl)-2-N-trifluoroacetamidopropan-1,3-diol (138)

Methyl trifluoroacetate (2.40 ml, 23.87 mmol) and triethylamine (0.66 ml, 4.74 mmol) were added to a solution of (1R, 2R)-2-amino-1-(*p*-nitrophenyl)-propan-1,3-diol (1.002 g, 4.73 mmol) in dry methanol (15 ml). The reaction mixture was heated to reflux and aged at that temperature for 1h.

The reaction mixture was evaporated to residue *in vacuo* to give a yellow oil which was taken up in ethyl acetate (15 ml). The solution was washed with 2 M HCl (2×3.5 ml) and saturated brine (3.5 ml), dried (Na_2SO_4) and evaporated to residue *in vacuo*. The residue was triturated with petroleum ether (2×10 ml) and purified by flash column chromatography (2:1 ethyl acetate-dichloromethane) to give the title compound (1.243 g, 85%) as a pale yellow solid.

R_f 0.55 (ethyl acetate);

m.p. 127-128°C (from ethyl acetate/hexane);

$[\alpha]_D +14.5^\circ$ (c1.0 in EtOH);

$C_{11}H_{11}N_2O_5F_3$ requires C, 42.9; H, 3.6; N, 9.1%

found C, 43.2; H, 3.4; N, 9.2%;

ν_{\max} (nujol)/ cm^{-1} 3530w, 3380m, 3220m, 3080m, 1700s, 1580w, 1520m, 1370m, 1350s, 1290w, 1250w, 1210m, 1190s, 1150s, 1100w, 1060m, 970w, 905w, 880w, 840w, 820w, 740w, 710m, 680w;

δ_H (300 MHz; CD_3OD) 3.62 (1H, ABX system, $J_{BX}=6.7$ Hz, $J_{gem}=11.2$ Hz, CH_2OH), 3.82 (1H, ABX system, $J_{AX}=6.1$ Hz, $J_{gem}=11.2$ Hz, CH_2OH), 4.21-4.27 (1H, m, $CHCH_2OH$), 5.12 (1H, d, $J=3.7$ Hz, $ArCHOH$), 7.63 (2H, d, $J=8.4$ Hz, Ph), 8.19 (2H, d, $J=8.8$ Hz, Ph);

δ_C (75 MHz; CD_3OD) 58.69 (CH), 61.99 (CH_2), 71.68 (CH), 124.17 (CH), 128.30 (CH), 148.60 (C), 151.16 (C), 159.22 (C), CF_3 not seen;

m/z No M^+ , 290 (2%), 153 (17), 151 (14), 150 (18), 139 (100), 138 (11), 136 (11), 106 (11), 77 (13), 70 (16), 69 (10);

$C_{11}H_{15}N_3O_5F_3$ [$M+NH_4$] $^+$ requires 326.0964

found 326.0964.

(2S)-2-N-Dichloroacetamido-1-propanol (143)

(N-Dichloroacetamido-L-alaninol)

Methyl dichloroacetate (4.00 ml, 38.63 mmol) was added to a solution of L-alaninol (1.50 ml, 19.20 mmol) in dry ethanol (15 ml). The reaction mixture was heated to reflux and aged at that temperature for 6h.

The reaction mixture was evaporated to residue *in vacuo*, triturated with petroleum ether (2×10 ml) and purified by flash column chromatography (7:3 dichloromethane-ethyl acetate) to give the title compound (3.196 g, 89%) as a white solid.

R_f 0.51 (ethyl acetate);

m.p. 84-85°C (from ethyl acetate/hexane);

$[\alpha]_D$ -11.3° (c1.0 in EtOH);

$C_5H_9NO_2Cl_2$ requires C, 32.3; H, 4.9; N, 7.5%

found C, 32.65; H, 4.8; N, 7.4%;

ν_{max} (nujol)/ cm^{-1} 3330m, 3250m, 3080m, 1675s, 1675s, 1575m, 1360w, 1310w, 1245m, 1220w, 1210w, 1150m, 1115m, 1060s, 985w, 975w, 930w, 910w, 875m, 815m, 770w, 750w, 720m, 670m;

δ_H (300 MHz; D_2O) 1.18 (3H, d, $J=6.8$ Hz, CH_3CH), 3.56 (1H, ABX system, $J_{BX}=6.5$ Hz, $J_{gem}=11.6$ Hz, CH_2OH), 3.64 (1H, ABX system, $J_{AX}=4.8$ Hz, $J_{gem}=11.6$ Hz, CH_2OH), 3.95-4.05 (1H, m, $CHCH_2OH$), 6.29 (1H, s, $CHCl_2$);

δ_C (75 MHz; D_2O) 17.99 (CH_3), 50.89 (CH), 66.59 (CH_2), 68.92 (CH), 168.89 (C);

m/z No M^+ , 156 (30%), 154 (46), 102 (7), 85 (7), 83 (10), 73 (13), 59 (30);

$C_5H_{10}NO_2Cl_2$ $[MH]^+$ requires 186.009

found 186.009.

(2S)-2-N-Dichloroacetamido-3-methyl-1-butanol (145)

(N-Dichloroacetamido-L-valinol)

Methyl dichloroacetate (2.50 ml, 24.14 mmol) was added to a solution of L-valinol (1.255 g, 12.18 mmol) in dry ethanol (10 ml). The reaction mixture was heated to reflux and aged at that temperature for 5h.

The reaction mixture was evaporated to residue *in vacuo* to give a white solid which was triturated with petroleum ether (2x5 ml) and then taken up in ethyl acetate (15 ml). The solution was washed with 2 M HCl (2x2 ml), dried (Na_2SO_4) and evaporated to residue *in vacuo* to give the title compound (2.260 g, 87%) as a white solid.

R_f 0.55 (ethyl acetate);

m.p. 92.5-94°C (from ethyl acetate/hexane);

$[\alpha]_D$ -15.0° (c1.0 in EtOH);

$C_7H_{13}NO_2Cl_2$ requires C, 39.3; H, 6.1; N, 6.5%

found C, 39.2; H, 6.1; N, 6.6%;

ν_{max} (nujol)/ cm^{-1} 3285m, 3090w, 1670w, 1670s, 1565m, 1270w, 1220w, 1210w, 1150w, 1080w, 1025w, 1025w, 970w, 935w, 895w, 855w, 805m, 770w, 720w;

δ_H (300 MHz; D_2O) 0.89 (3H, d, $J=6.8$ Hz, CH_3CH), 0.94 (3H, d, $J=6.8$ Hz, CH_3CH), 1.81-1.92 (1H, m, $(CH_3)_2CH$), 3.57-3.76 (3H, m, $CHCH_2OH$), 6.33 (1H, s, $CHCl_2$);

δ_C (75 MHz; $CDCl_3$) 18.57 (CH_3), 19.44 (CH_3), 29.01 (CH), 57.65 (CH), 62.40 (CH_2), 66.66 (CH), 164.96 (C);

m/z No M^+ , 189 (9%), 185 (7), 184 (63), 182 (100), 172 (18), 170 (30), 154 (11), 152 (170), 130 (11), 128 (13), 112 (6), 85 (15), 83 (22), 76 (12), 72 (66), 70 (15), 69 (29), 60 (42), 57 (8), 56 (21), 55 (66);

$C_7H_{14}NO_2Cl_2$ $[MH]^+$ requires 214.0402

found 214.0402.

(2S)-2-N-Dichloroacetamido-4-methyl-1-pentanol (147)

(N-Dichloroacetamido-L-leucinol)

Methyl dichloroacetate (2.50 ml, 24.14 mmol) was added to a solution of L-leucinol (1.50 ml, 11.79 mmol) in dry ethanol (10 ml). The reaction mixture was heated to reflux and aged at that temperature for 6h.

The reaction mixture was evaporated to residue *in vacuo*, triturated with petroleum ether (2×10 ml) and purified by flash column chromatography (7:3 dichloromethane-ethyl acetate) to give the title compound (2.526 g, 94%) as a white solid.

R_f 0.60 (ethyl acetate);

m.p. 73.5-75°C (from ethyl acetate/hexane);

[α]_D -12.8° (c1.0 in EtOH);

C₈H₁₅NO₂Cl₂ requires C, 42.1; H, 6.6; N, 6.1%

found C, 42.2; H, 6.5; N, 6.0%;

ν_{max} (nujol)/cm⁻¹ 3320br, 3230m, 3080m, 1685s, 1560m, 1320w, 1295w, 1270w, 1245w, 1215m, 1205m, 1165w, 1155w, 1110w, 1080m, 1060m, 1030m, 1015m, 975w, 955w, 930w, 890w, 860w, 850w, 810m, 750w, 720w, 670m, 640w;

δ_H (300 MHz; D₂O) 0.88 (3H, d, J=6.6 Hz, CH₃CH), 0.90 (3H, d, J=6.7 Hz, CH₃CH), 1.30-1.50 (2H, m, (CH₃)₂CHCH₂), 1.54-1.65 (1H, m, (CH₃)₂CH), 3.52 (1H, ABX system, J_{BX}=6.7 Hz, J_{gem}=11.6 Hz, CH₂OH), 3.62 (1H, ABX system, J_{AX}=4.6 Hz, J_{gem}=11.6 Hz, CH₂OH), 3.96-4.04 (1H, m, CHCH₂OH), 6.29 (1H, s, CHCl₂);

δ_C (75 MHz; CDCl₃) 22.13 (CH₃), 22.97 (CH₃), 24.87 (CH), 39.88 (CH₂), 50.58 (CH), 64.73 (CH₂), 66.56 (CH), 164.51 (C);

m/z No M⁺, 198 (36%), 196 (57), 172 (6), 170 (13), 162 (7), 156 (18), 154 (32), 142 (14), 140 (21), 130 (22), 128 (36), 86 (10), 85 (15), 83 (49), 76 (11), 70 (22), 69 (100), 60 (23), 57 (28), 55 (42);

C₈H₁₆NO₂Cl₂ [MH]⁺ requires 228.056

found 228.056

(2S, 3S)-2-N-Dichloroacetamido-3-methyl-1-pentanol (150)
(N-Dichloroacetamido-L-isoleucinol)

A solution of L-isoleucine methyl ester hydrochloride (2.30 g, 12.67 mmol) in water (25 ml) was added dropwise over 10 min. to a stirred solution of sodium borohydride (1.90 g, 50.00 mmol) in water (20 ml) at $<2^{\circ}\text{C}$. The reaction mixture was stirred for 5h at $<2^{\circ}\text{C}$ and then left to stand in a fridge overnight.

The aqueous mixture was extracted with ethyl acetate (6 \times 50 ml) and the combined organics were washed with saturated brine (50 ml), dried (Na_2SO_4) and evaporated to residue *in vacuo* to give a pale yellow oil. The crude product was purified by Kugelrohr distillation (40°C , 3×10^{-4} mbar) to give (2S, 3S)-2-amino-3-methyl-1-pentanol, (L-isoleucinol) (149) (0.857 g, 58%) as a colourless oil which was reacted immediately.

Methyl dichloroacetate (1.90 ml, 18.35 mmol) was added to a solution of L-isoleucinol (0.847 g, 7.24 mmol) in dry ethanol (10 ml). The reaction mixture was heated to reflux and aged at that temperature for 7h.

The reaction mixture was evaporated to residue *in vacuo* to give a white solid which was triturated with pentane (2 \times 5 ml) and then taken up in ethyl acetate (15 ml). The solution was washed with 2 M HCl (2 \times 2 ml), dried (Na_2SO_4) and evaporated to residue *in vacuo* to give the title compound (1.631 g, 99%) as a white solid.

R_f 0.58 (ethyl acetate);

m.p. $88-90^{\circ}\text{C}$ (from ethyl acetate/hexane);

$[\alpha]_D -11.0^{\circ}$ (c1.0 in EtOH);

$\text{C}_8\text{H}_{15}\text{NO}_2\text{Cl}_2$ requires C, 42.1; H, 6.6; N, 6.1%

found C, 42.0; H, 6.8; N, 6.1%;

ν_{\max} (nujol)/ cm^{-1} 3280m, 3250m, 3090m, 1670m, 1670s, 1565m, 1320w, 1275w, 1255w, 1220m, 1210m, 1150m, 1080m, 1035w, 1020w, 970w, 915w, 895m, 855w, 810m, 765m, 720m, 640m;

δ_{H} (300 MHz; CDCl_3) 0.92 (3H, t, $J=7.4$ Hz, CH_3CH_2), 0.97 (3H, d, $J=6.8$ Hz, CH_3CH), 1.11-1.25 (1H, m, CH_3CH_2), 1.46-1.59 (1H, m, CH_3CH_2), 1.68-1.82 (1H, m, $\text{CH}_3\text{CH}_2\text{CH}$), 2.98 (1H, br s, OH, exchanged with D_2O), 3.74-3.84 (3H, m, CHCH_2OH), 6.02 (1H, s, CHCl_2), 6.96 (1H, d, $J=7.9$ Hz, NH, exchanged with D_2O);

δ_{C} (75 MHz; CDCl_3) 11.14 (CH_3), 15.48 (CH_3), 25.30 (CH_2), 35.38 (CH), 56.43 (CH), 62.34 (CH_2), 66.66 (CH), 164.73 (C);

m/z No M^+ , 198 (34%), 196 (55), 172 (18), 170 (31), 162 (6), 154 (11), 152 (14), 142 (13), 140 (22), 130 (14), 128(24), 86 (15), 85 (14), 83 (39), 76 (11), 71 (14), 70 (22), 69 (100), 63 (36), 57 (30), 55 (25);

$\text{C}_8\text{H}_{16}\text{NO}_2\text{Cl}_2$ $[\text{MH}]^+$ requires 228.056
found 228.056.

(2R, 3R)-1,3-O-Diacetyl, 1-O-Acetyl and 3-O-Acetyl-2-N-dichloroacetamidobutan-1,3-diol (153), (151), and (152)

Acetic anhydride (0.22 ml, 2.33 mmol) was added to a solution of N-dichloroacetamido-L-threoninol (46) (0.501 g, 2.32 mmol) in dry pyridine (5 ml) at 0°C . The reaction mixture was left to stand in a fridge for three days.

After warming to room temperature the reaction mixture was poured into water (20 ml), stirred for 5 min. and extracted with ethyl acetate (3×50 ml). The combined organics were washed with 2 M HCl (20 ml) and saturated NaHCO_3 (20 ml), dried (Na_2SO_4) and evaporated to residue *in vacuo* to give a pale orange oil. The mixture of products was purified by flash column chromatography (7:3 dichloromethane-ethyl acetate) to give 1,3-O-diacetyl-2-N-dichloroacetamido-L-threoninol (0.139 g, 20%) as a white solid,

1-O-acetyl-2-N-dichloroacetamido-L-threoninol (0.133 g, 22%) as a white solid and 3-O-acetyl-2-N-dichloroacetamido-L-threoninol (0.042 g, 7%) as a colourless oil.

(2R, 3R)-1-O-Acetyl-3-O-acetyl-2-N-dichloroacetamidobutan-1,3-diol (**153**):

R_f 0.73 (7:3 dichloromethane-ethyl acetate);

m.p. 93.5-95.5°C (without further purification);

δ_H (300 MHz; CDCl₃) 1.29 (3H, d, J=6.4 Hz, CH₃OH), 2.07 (3H, s, CH₃CO), 2.09 (3H, s, CH₃CO), 4.15 (1H, ABX system, J_{BX}=6.2 Hz, J_{gem}=11.5 Hz, CH₂OAc), 4.17 (1H, ABX system, J_{AX}=6.0 Hz, J_{gem}=11.5 Hz, CH₂OAc), 4.26-4.34 (1H, m, CHCH₂OAc), 5.17 (1H, dq, J=4.3, 6.4 Hz, CH₃CH), 5.99 (1H, s, CHCl₂), 6.79 (1H, d, J=9.0 Hz, NH);

δ_C (75 MHz; CDCl₃) 17.05 (CH₃), 20.54 (CH₃), 20.90 (CH₃), 52.55 (CH), 62.51 (CH₂), 66.14 (CH), 68.56 (CH), 164.41 (C), 169.99 (C), 170.49 (C).

(2R, 3R)-1-O-Acetyl-2-N-dichloroacetamidobutan-1,3-diol (**151**):

R_f 0.47 (7:3 dichloromethane-ethyl acetate);

m.p. 88-89°C (without further purification);

δ_H (300 MHz; D₂O) 1.21 (3H, d, J=6.4 Hz, CH₃CH), 2.09 (3H, s, CH₃CO), 4.01-4.16 (2H, m, CHCH₂OAc and CH₃CH), 4.26 (1H, ABX system, J_{BX}=7.3 Hz, J_{gem}=11.4 Hz, CH₂OAc), 4.29 (1H, ABX system, J_{AX}=5.6 Hz, J_{gem}=11.4 Hz, CH₂OAc), 6.38 (1H, s, CHCl₂);

δ_C (75 MHz; D₂O) 21.50 (CH₃), 22.82 (CH₃), 57.12 (CH), 66.45 (CH₂), 68.45 (CH), 68.76 (CH), 170.04 (C), 176.55 (C).

(2R, 3R)-3-O-Acetyl-2-N-dichloroacetamidobutan-1,3-diol (**152**):

R_f 0.35 (7:3 dichloromethane-ethyl acetate);

δ_{H} (300 MHz; D₂O) 1.27 (3H, d, $J=6.4$ Hz, CH₃CH), 2.09 (3H, s, CH₃CO), 3.66 (1H, ABX system, $J_{\text{BX}}=7.1$ Hz, $J_{\text{gem}}=11.8$ Hz, CH₂OH), 3.72 (1H, ABX system, $J_{\text{AX}}=5.1$ Hz, $J_{\text{gem}}=11.8$ Hz, CH₂OH), 4.04-4.15 (1H, m, CHCH₂OH), 5.11 (1H, dq, $J=5.4$ Hz, 6.4 Hz, CH₃CH), 6.37 (1H, s, CHCl₂).

6.6 KINETIC STUDIES

6.6i Preparation of TSE Buffer

TSE buffer was made 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.

6.6ii Preparation of Acetyl CoA

Coenzyme A (100 mg) was added to distilled water (7 ml) and KHCO_3 (1 M, 1 ml) cooled in ice. Acetic anhydride (25 μl) 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 μl of the AcCoA solution in TSE buffer (990 μl) containing 1 mmol 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 μl), 0.25 mM chloramphenicol (50 μl of 5 mM), the AcCoA solution (5 μl) and CAT (10 μl). The concentration of acetyl CoA was determined by finding the absorbance at 260 nm at 25°C ($\epsilon_{260}=15.4 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$), and it was diluted to 10 mM. Acetyl CoA was stored at -20°C as 500 μl aliquots.

6.6iii 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 μmol of chloramphenicol to product per minute. The standard assay mixture contained TSE buffer (930 μl containing 1 mM DTNB), 0.4 mM acetyl CoA

(40 μ l of 10 mM) and 0.1 mM chloramphenicol (20 μ l of 5 mM). The reaction was initiated by the addition of CAT (10 μ l) and was monitored at 412 nm.

6.6iv Kinetic Analysis

Measurement of the linear initial rates of acetylation of the chloramphenicol 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 ranged from $\approx 0.5 \times K_m$ – $8 \times K_m$) and four AcCoA concentrations (25 μ M, 50 μ M, 100 μ M and 400 μ 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 from manually drawn double-reciprocal plots.⁵⁷

REFERENCES

REFERENCES

- 1 J. R. Knowles, *Science*, 1987, **236**, 1252;
W. V. Shaw, *Biochem. J.*, 1987, **236**, 1
- 2 J. R. Roesser, M. S. Chorghade & S. M. Hecht, *Biochemistry*, 1986, **25**, 6361
- 3 A. Warshel & M. Levitt, *J. Mol. Biol.*, 1976, **103**, 227
- 4 S. J. Weiner, P. A. Kollman, D. A. Case, U. C. Singh, C. Ghio, G. Alagona, S. Profeta & P. Weiner, *J. Am. Chem. Soc.*, 1984, **108**, 765
- 5 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
- 6 A. Leo, C. Hansch & D. Elkins, *Chem. Rev.*, 1971, **71**, 525
- 7 A. R. Fersht, *Trends Biochem. Sci.*, 1984, **9**, 145;
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
- 8 W. P. Jencks, *Catalysis and Enzymology*, McGraw-Hill, 1969;
J. Hine, *J. Am. Chem. Soc.*, 1972, **94**, 5766
- 9 T. N. C. Wells & A. R. Fersht, *Biochemistry*, 1986, **25**, 1881
- 10 A. R. Fersht, *Biochemistry*, 1988, **27**, 1577
- 11 W. P. Jencks, *Proc. Natl. Acad. Sci. U.S.A.*, 1981, **78**, 4046
- 12 A. R. Fersht, *Trends Biochem. Sci.*, 1987, **12**, 301
- 13 D. M. Lowe, G. Winter & A. R. Fersht, *Biochemistry*, 1987, **26**, 6038
- 14 I. P. Street, C. R. Armstrong & S. G. Withers, *Biochemistry*, 1986, **25**, 6021
- 15 a) P. A. Bartlett, & C. K. Marlowe, *Biochemistry*, 1983, **22**, 4618;
b) P. A. Bartlett, & C. K. Marlowe, *Science*, 1987, **235**, 568;
c) B. P. Morgan, J. M. Scholtz, M. D. Ballinger, I. D. Zipkin & P. A. Bartlett, *J. Am. Chem. Soc.*, 1991, **113**, 297
- 16 D. E. Tronrud, H. M. Holden & B. W. Matthews, *Science*, 1987, **235**, 571

- 17 P. A. Bash, P. A. Kollman, U. C. Singh, F. K. Brown & R. Langridge, *Science*, 1987, 235, 574
- 18 K. M. Merz & P. K. Kollman, *J. Am. Chem. Soc.*, 1989, 111, 5649
- 19 D. H. Williams, J. P. H. Cox, A. J. Doig, M. gardner, U. Gerhard, P. T. Kaye, A. R. Lal, I. A. Nicholls, C. J. Salter & R. C. Mitchell, *J. Am. Chem. Soc.*, 1991, 113, 7020
- 20 M. I. Page & W. P. Jencks, *Proc. Natl. Acad. Sci. U.S.A.*, 1971, 68, 1678
- 21 A. J. Doig & D. H. Williams, *J. Mol. Biol.*, 1991, 217, 389
- 22 D. H. Williams, *Aldrichim. Acta*, 1991, 24, 71;
K. A. Sharp, I. A. Nicholls, R. F. Fine & B. Honig, *Science*, 1991, 252, 106
- 23 M. S. Searle, D. H. Williams & U. Gerhard, *J. Am. Chem. Soc.*, 1992, 114, 10697
- 24 M. S. Seale & D. H. Williams, *J. Am. Chem. Soc.*, 1992, 114, 10690
- 25 B. A. Shirley, P. Stanssens, U. Hahn & C. N. Pace, *Biochemistry*, 1992, 31, 725
- 26 W. V. Shaw, *J. Biol. Chem.*, 1967, 242, 687
- 27 a) A. G. W. Leslie, *J. Mol. Biol.*, 1990, 213, 167;
b) A. G. W. Leslie, P. C. E. Moody & W. V. Shaw, *Proc. Natl. Acad. Sci. U.S.A.*, 1988, 85, 4133
- 28 J. Ehrlich, Q. R. Bartz, R. M. Smith, D. A. Joslyn & P. R. Burkholder, *Science*, 1947, 106, 417
- 29 M. C. Rebstock, H. M. Crooks, J. Controulis & Q. R. Bartz, *J. Am. Chem. Soc.*, 1949, 71, 2458
- 30 R. E. Maxwell & V. S. Nickel, *Antibiot. Chemother.*, 1954, 4, 289
- 31 J. D. Dunitz, *J. Am. Chem. Soc.*, 1952, 74, 995
- 32 O. Jardetzky, *J. Biol. Chem.*, 1963, 238, 2498
- 33 T. M. Bustard, R. S. Egan & T. J. Perun, *Tetrahedron*, 1973, 29, 1961
- 34 A. L. Fitzhugh, *Bioorg. Med. Chem. Lett.*, 1991, 1, 253
- 35 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

- 36 E. F. Gale, *Pharmacol. Rev.*, 1963, 15, 481
- 37 E. F. Gale & T. F. Paine, *Biochem. J.*, 1951, 48, 298
- 38 R. Hancock & J. T. Park, *Nature*, 1958, 181, 1050
- 39 E. F. Gale & J. P. Folkes, *Biochem. J.*, 1953, 53, 493
- 40 H. E. Hopps, C. L. Wisseman & F. E. Hahn, *Antibiot. Chemother.*, 1954, 4, 857
- 41 L. W. Wheeldon & A. L. Lehninger, *Biochemistry*, 1966, 5, 3533
- 42 R. J. Ellis, *Science*, 1969, 163, 477
- 43 R. R. Traut & R. E. Monro, *J. Mol. Chem.*, 1964, 10, 63
- 44 D. Vazquez, *Nature*, 1964, 203, 257
- 45 A. D. Wolfe & F. E. Hahn, *Biochim. Biophys. Acta*, 1965, 95, 146
- 46 J. L. Lessard & S. Pestka, *J. Biol. Chem.*, 1972, 247, 6909
- 47 A. Contreras & D. Vazquez, *Eur. J. Biochem.*, 1977, 74, 539
- 48 O. Pongs, R. Bald & V. A. Erdmann, *Proc. Natl. Acad. Sci. U.S.A.*, 1973, 70, 2229; N. Sonnenberg, M. Wilchek & A. Zamir, *Proc. Natl. Acad. Sci. U.S.A.*, 1973, 70, 1423
- 49 R. Skinner, E. Cundliffe & J. Schmidt, *J. Biol. Chem.*, 1983, 258, 12702
- 50 R. T. Marconi, J. S. Lodmell & W. E. Hill, *J. Biol. Chem.*, 1990, 265, 7894
- 51 S. M. Hammond & P. A. Lambert, *Antibiotics & Antimicrobial Action*, The Camelot, Press Ltd., Southampton, 1978
- 52 Y. Suzuki & S. Okamoto, *J. Biol. Chem.*, 1967, 242, 4722
- 53 W. V. Shaw & J. Unowsky, *J. Bacteriol.*, 1968, 95, 1976
- 54 Y. Nakagawa, Y. Nitahara & S. Miyamura, *Antimicrob. Agents Chemother.*, 1979, 16, 719
- 55 W. V. Shaw & A. G. W. Leslie, *Annu. Rev. Biophys. Chem.*, 1991, 20, 363
- 56 W. P. Jencks, S. Cordes & J. Carriolo, *J. Biol. Chem.*, 1960, 235, 3608
- 57 C. Kleanthous & W. V. Shaw, *Biochem. J.*, 1984, 223, 211
- 58 C. Kleanthous, P. M. Cullis & W. V. Shaw, *Biochemistry*, 1985, 24, 5307
- 59 H. J. Vogel & W. A. Bridger, *Biochem. Soc. Trans.*, 1983, 11, 315

- 60 A. Lewendon, I. A. Murray, W. V. Shaw, M. R. Gibbs & A. G. W. Leslie, *Biochemistry*, 1990, 29, 2075
- 61 W. V. Shaw, *CRC Crit. Rev. Biochem.*, 1983, 14, 1
- 62 I. A. Murray, A. Lewendon, J. A. Williams, P. M. Cullis, & W. V. Shaw, *Biochemistry*, 1991, 30, 3763
- 63 P. M. Cullis, A. Lewendon, W. V. Shaw & J. A. Williams, *Biochemistry*, 1991, 30, 3758
- 64 D. J. Minick, J. H. Frenz, M. A. Patrick & D. A. Brent, *J. Med. Chem.*, 1988, 31, 1923; H. Terada, *Quant. Struc.-Act. Relat.*, 1986, 5, 81
- 65 J. Controulis, M. C. Rebstock & H. M. Crocks, *J. Am. Chem. Soc.*, 1949, 71, 2463
- 66 a) A. V. R. Rao, S. P. Rao & M. N. Bhanu, *J. Chem. Soc., Chem. Commun.*, 1992, 859; b) B. Lou, Y. Zhang & L. Dai, *Chem. Ind.*, 1993, 7, 249
- 67 G. M. Coppola & H. M. Schuster, *Asymmetric Synthesis*, Wiley, New York, 1987; J. Martens, *Top. Curr. Chem.*, 1984, 124, 165; K. Drauz, A. Kleemann & J. Martens, *Angew. Chem. Int. Ed. Engl.*, 1982, 21, 584
- 68 M. Tramontini, *Synthesis*, 1982, 605
- 69 M. J. Jorgenson, *Org. React (N. Y.)*, 1970, 18, 1-97
- 70 H. Gilman & P. R. Van Ess, *J. Am. Chem. Soc.*, 1933, 55, 1258
- 71 C. Tegnér, *Acta Chem. Scand.*, 1952, 6, 782
- 72 R. Levine, M. J. Karten & W. M. Kadunce, *J. Org. Chem.*, 1975, 40, 1770
- 73 P. H. Ogden & G. C. Nicholson, *Tetrahedron Lett.*, 1968, 3553
- 74 a) R. A. Schneider & J. Meinwald, *J. Am. Chem. Soc.*, 1967, 89, 2023
b) N. Danieli, Y. Mazur & F. Sondheimer, *J. Am. Chem. Soc.*, 1962, 84, 875; *Tetrahedron*, 1966, 22, 3189
c) J. A. Marshall & M. T. Pike, *Tetrahedron Lett.*, 1966, 4989
d) C. H. Heathcock & T. R. Kelly, *Tetrahedron*, 1968, 24, 3753
e) D. J. Cram & K. R. Kopecky, *J. Am. Chem. Soc.*, 1959, 81, 2748
- 75 G. M. Rubottom & C. Kim, *J. Org. Chem.*, 1983, 48, 1550

- 76 M. Chérest, H. Felkin & N. Prudent, *Tetrahedron Lett.*, 1968, 2199; N. T. Anh, *Top. Curr. Chem.*, 1980, 88, 145
- 77 M. T. Reetz, *Angew. Chem., Int. Ed. Engl.*, 1984, 23, 556
- 78 E. Schenker, *Angew. Chem.*, 1961, 73, 81
- 79 H. Seki, K. Koga, H. Matsuo, S. Ohki, I. Matsuo & S. Yamada, *Chem. Pharm. Bull.*, 1965, 13, 995
- 80 J. Williams, unpublished work
- 81 M. Brenner & W. Huber, *Helv. Chim. Acta*, 1953, 36, 1109
- 82 J. A. Dale, D. L. Dull & H. S. Mosher, *J. Org. Chem.*, 1969, 34, 2543
J. A. Dale & H. S. Mosher, *J. Org. Chem.*, 1970, 11, 4002
- 83 L. M. Jackman & S. Sternhell, *Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry*, Pergamon Press, Oxford, 1969, 2nd Edition, 280-300
- 84 M. T. Reetz, M. W. Drewes, K. Lennick, A. Schmitz & X. Holdgrün, *Tetrahedron Asymmetry*, 1990, 1, 375
- 85 J. Jurczak & A. Golebiowski, *Chem. Rev.*, 1989, 89, 149
- 86 M. T. Reetz, *Angew. Chem. Int. Ed. Engl.*, 1991, 30, 1531
- 87 a) D. H. Rich, E. T. Sun, & A. S. Boparai, *J. Org. Chem.*, 1978, 43, 3624
b) A. Ito, R. Takahashi & Y. Baba, *Chem. Pharm. Bull.*, 1975, 23, 3081
c) A. Golebiowski, U. Jacobsson & J. Jurczak, *Tetrahedron*, 1987, 43, 3036
d) M. M. Hann, P. G. Sammes, P. D. Kennewell & J. B. Taylor, *J. Chem. Soc., Perkin Trans. 1*, 1982, 307
- 88 H. Zemlicka & M. Murata, *J. Org. Chem.*, 1976, 41, 3317
- 89 M. Narita, S. Otsuka, S. Kobayashi, M. Ohno, Y. Umezawa, H. Morishima, S. Saito, T. Takita & H. Umezawa, *Tetrahedron Lett.*, 1982, 23, 525
- 90 J. A. Fehrentz & B. Castro, *Synthesis*, 1983, 676
- 91 W. D. Lubell & H. Rapoport, *J. Am. Chem. Soc.*, 1987, 109, 236
- 92 C. F. Stanfield, J. E. Parker & P. Kanellis, *J. Org. Chem.*, 1981, 46, 4799
- 93 Y. Hamada & T. Shioiri, *Tetrahedron Lett.*, 1982, 23, 1193

- 94 J. R. Luly, J. F. Dellaria, J. J. Plattner, J. L. Soderquist & N. Yi, *J. Org. Chem.*, 1987, 52, 1487
- 95 K. E. Rittle, C. F. Homnick, G. S. Ponticello & B. E. Evans, *J. Org. Chem.*, 1982, 47, 3016
- 96 Y. Hamada & T. Shioiri, *Chem. Pharm. Bull.*, 1982, 30, 1921
- 97 L. Andesson & R. Wolfenden, *Anal. Biochem.*, 1982, 124, 150
- 98 S. H. Rosenberg, J. J. Plattner, K. W. Woods, H. H. Stein, P. A. Marcotte, J. Cohen & T. Perun, *J. Med. Chem.*, 1987, 30, 1224
- 99 Y. Ohfuné & H. Nishio, *Tetrahedron Lett.*, 1984, 25, 4133
- 100 a) M. T. Reetz, M. W. Drewes & A. Schmitz, *Angew. Chem. Int. Ed. Engl.*, 1987, 26, 1141; b) J. F. Dellaria & R. G. Maki, *Tetrahedron Lett.*, 1986, 27, 2337
- 101 S. Danishefsky, S. Kobayashi & J. F. Kerwin, *J. Org. Chem.*, 1982, 47, 1981; J. Jurczak, A. Golebiowski & T. Bauer, *Synthesis*, 1985, 928
- 102 J. Jurczak, A. Golebiowski & J. Raczko, *J. Org. Chem.*, 1989, 54, 2495; M. M. Midland & M. M. Alfonso, *J. Am. Chem. Soc.*, 1989, 111, 4368
- 103 G. J. Hanson & T. Lindberg, *J. Org. Chem.*, 1985, 50, 5399
- 104 M. W. Holladay & D. H. Rich, *Tetrahedron Lett.*, 1983, 24, 4401
- 105 P. Garner & J. M. Park, *J. Org. Chem.*, 1987, 52, 2361
- 106 E. Branquet, P. Durand, L. Vo-Quang & F. Le Goffic, *Synthetic Communications*, 1993, 23, 153
- 107 T. Moriwake, S. Hamano, S. Saito & S. Torii, *Chemistry Letters*, 1987, 2085
- 108 a) P. Herold, *Helv. Chim. Acta.*, 1988, 71, 354; b) R. S. Coleman, A. J. Carpenter, *Tetrahedron Lett.*, 1992, 33, 1697; c) P. Garner & J. M. Park, *J. Org. Chem.*, 1988, 53, 2979; d) P. Garner & J. M. Park, *J. Org. Chem.*, 1990, 55, 3772; e) M. Nakagawa, A. Tsuruoka, J. Yoshida & T. Hino, *J. Chem. Soc., Chem. Commun.*, 1990, 603; f) P. Garner, J. M. Park & E. Malecki, *J. Org. Chem.*, 1988, 53, 4395; g) S. Nimkar, D. Menaldino, A. Merrill & D. Liotta, *Tetrahedron Lett.*, 1988, 29, 3037; h) D. Kahne, D. Yang & M. Lee, *Tetrahedron Lett.*, 1990, 31, 21; i) P. Garner & S. Ramakanth, *J. Org. Chem.*, 1986, 51, 2609; j) P. Garner,

- Tetrahedron Lett.*, 1984, 25, 5855; k) G. Casiraghi, L. Colombo, G. Rassu & P. Spanu, *J. Chem. Soc., Chem. Commun.*, 1991, 603; l) A. Dondoni, G. Fantin, M. Fogagnola & P. Merino, *J. Chem. Soc., Chem. Commun.*, 1990, 854; m) A. Dondoni, G. Fantin, M. Fogagnola & P. Pedrini, *J. Org. Chem.*, 1990, 55, 1439; o) G. Casiraghi, L. Colombo, G. Rassu & P. Spanu, *Tetrahedron Lett.*, 1989, 30, 5325; p) G. Casiraghi, M. Cornia & G. Rassu, *J. Org. Chem.*, 1988, 53, 4919; q) A. Dondoni, G. Fantin, M. Fogagnola & A. Medici, *J. Chem. Soc., Chem. Commun.*, 1988, 10
- 109 M. Asami & R. Kimura, *Chemistry Letters*, 1985, 1221
- 110 K. Mead & L. Macdonald, *J. Org. Chem.*, 1985, 50, 422
- 111 D. P. Schumacher, J. E. Clark, B. L. Murphy & P. A. Fischer, *J. Org. Chem.*, 1990, 55, 5294
- 112 W. V. Shaw, *Methods Enzymol.*, 1975, 43, 737
- 113 Berthelot & Jungfleisch, *Ann. Chim. Phys.*, 1872, 4, 26
- 114 W. Nernst, *Z. Phys. Chem.*, 1891, 8, 110
- 115 C. Hansch, R. M. Muir, T. Fujita, P. P. Maloney, F. Geiger & M. Streich, *J. Am. Chem. Soc.*, 1963, 85, 2817
- 116 C. Hansch & T. Fujita, *J. Am. Chem. Soc.*, 1964, 86, 1616
- 117 T. Fujita, J. Iwasa & C. Hansch, *J. Am. Chem. Soc.*, 1964, 86, 5175
- 118 K. Kiehs, C. Hansch & L. Moore, *Biochemistry*, 1966, 5, 2602
- 119 C. Hansch, E. W. Deutsch & R. N. Smith, *J. Am. Chem. Soc.*, 1965, 87, 2738
- 120 A. Fersht, *Enzyme Structure and Mechanism*, Freeman, New York, 2nd edition
- 121 C. Hansch & E. Coats, *J. Pharm. Sci.*, 1970, 59, 731
- 122 V. N. Dorovskaya, S. D. Varfolomeyev, N. F. Kazanskaya, A. A. Klyosov & K. Martinek, *FEBS Lett.*, 1972, 23, 122
- 123 D. A. Estell, T. P. Graycar, J. V. Miller, D. B. Powers, J. P. Burnier, P. G. Ng & J. A. Wells, *Science*, 1986, 233, 659
- 124 R. D. Cramer III, *J. Am. Chem. Soc.*, 1977, 99, 5408
- 125 M. H. Abraham, *J. Am. Chem. Soc.*, 1982, 104, 2085

- 126 J. A. Reynolds, D. B. Gilbert & C. Tanford, *Proc. Nat. Acad. Sci. USA*, 1974, 71, 2925
- 127 C. McAuliffe, *J. Phys. Chem.*, 1966, 70, 1267
- 128 R. B. Hermann, *J. Phys. Chem.*, 1972, 76, 2754
- 129 M. J. Harris, T. Higuchi & J. H. Rytting, *J. Phys. Chem.*, 1973, 77, 2694
- 130 A. Leo, C. Hansch & P. Y. C. Jow, *J. Med. Chem.*, 1976, 19, 611
- 131 M. H. Abraham, *J. Am. Chem. Soc.*, 1979, 101, 5477
- 132 C. Hansch & A. Leo, *Substituent Constants for Correlation Analysis in Chemistry and Biology*, John Wiley & Sons, London, 1979
- 133 J. Ellis, unpublished work
- 134 A. Lewendon, unpublished work
- 135 A. Fischli, *Modern Synthetic Methods*, 1980, 2, 269
- 136 G. W. Fleet & P. W. Smith, *Tetrahedron Lett.*, 1985, 26, 1469; D. H. Rich, *J. Med. Chem.*, 1985, 28, 264; J. F. Kennedy & C. A. White, *Bioactive Carbohydrates*, Ellis Horwood, Chichester, 1983; D. H. Rich, B. J. Moon & A. S. Boparai, *J. Org. Chem.*, 1980, 45, 2283; R. Nishizawa & T. Saino, *J. Med. Chem.*, 1977, 20, 510
- 137 H. Umezawa, T. Aoyagi, H. Morishima, H. Matsuzaki, M. Hamada & T. Takeuchi, *J. Antibiot.*, 1970, 23, 259
- 138 N. K. Kochetkov & G. P. Smirnova, *Adv. Carbohydr. Chem. Biochem.*, 1986, 44, 387; F. Sarmientos, G. Schwarzmann & K. Sandhoff, *Eur. J. Biochem.*, 1985, 146, 59; S. Hakomori, *Ann. Rev. Biochem.*, 1981, 50, 733
- 139 D. H. Williams & I. Fleming, *Spectroscopic Methods in Organic Chemistry*, McGraw-Hill Book Co. Ltd., Maidenhead, 1980, 3rd Edition, 136-137
- 140 Aldrich, *Technical Information Bulletin*, 1982, AL-121